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par

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LES ONCOGENES DES VIRUS DES LEUCEMIES AIGUES AVIAIRES ET LEURS HOMOLOGUES CELLULAIRES: STRUCTURE ET FONCTION

soutenue le 10 mars 1987 devant la commission d'examen

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Les terreurs, ces ténèbres de l'esprit il faut donc, pour les dissiper, non les rayons du soleil ni les traits lumineux du jour, mais l'étude rationnelle de la nature.

Lucrèce
De Natura Rerum

Le gôut d'explorer est une pulsion fondamentale, aussi bien chez l'être humain que chez le petit chat.Sa justification première est de l'ordre du pur plaisir de découvrir.

Hubert Reeves
L'heure de s'enivrer

Ce travail a été réalisé dans l'unité d'oncologie moléculaire que dirige Dominique Stehélin. Cette thése doit beaucoup à ses avisés conseils, et je voudrais également le remercier pour sa disponibilité constante ainsi que pour la philosophie de la recherche qu'il sait communiquer.

Que Monsieur le président, Madame et Messieurs les membres du jury soient assurés de l'honneur qu'ils me font en acceptant de juger ce travail.

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Je remercie Nini -qui tout en alliant une grande dextérité au clavier à un sens aigu de la gestion-possède néanmoins un petit défaut: elle est susceptible.....

Enfin, je remercie tous les membres de l'U186, pour leur aide constante et leur sympathie.

Merci Michèle, pour tes judicieuses corrections.

PLAN

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RESUME

Les rétrovirus oncogènes responsables chez l'animal de l'appariton de néoplasmes variés sont le résultat d'une recombinaison entre le génome d'un virus non transformant, et des gènes cellulaires phylogénétiquement conservés, les oncogènes.

Nous avons analysé la structure du génome de 7 rétrovirus aviaires responsables de l'apparition de leucémies aiguës chez l'animal. Nos travaux ont permis d'isoler 4 oncogènes erbA, erbB, myc et myb.

-erbA et erbB sont portés par le virus AEV induisant des érythroblastoses;

-myc est présent dans 4 virus (dont MH2) induisant une myélocytomatose;

-myb est porté par deux virus transformant les myéloblastes.

L'analyse du génome de MH2 nous a amené à supposer l'existence d'un oncogène supplémentaire au sein de ce virus, lequel a pu être ulterieurement cloné dans le laboratoire et dénommé mil.

Le clonage moléculaire des oncogènes cellulaires montre que les gènes viraux sont des versions tronquées en 5' et en 3' des ces gènes, ce qui suggère que cette amputation peut être un des mécanismes de leur activation oncogénique.

La mesure du taux d'ARN correspondant à ces différents gènes cellulaires nous a permis de montrer que les cellules hématopoïétiques immatures des différentes lignées érythroïde, myéloïde et lymphoïde (mais pas les cellules matures), accumulent les ARN myc et myb. Cela suggére que le produit normal de ces gènes est impliqué dans la prolifération de ces cellules indifférenciées, et que la transformation des cellules hématopoïétiques par les virus étudiés n'est pas tributaire de l'expression de ces oncogènes pour se réaliser.

Afin d'aborder d'une façon plus biologique les mécanismes de la transformation cellulaire, nous avons introduit l'oncogène myc humain sous le contrôle transcriptionnel d'un promoteur viral, dans des fibroblastes embryonnaires de cailles: ces cellules se sont transformées et nous avons pu démontrer que la sur expression du produit myc normal était suffisante pour transformer ces cellules.

D'autre part, le virus MH2 portant deux oncogènes, nous avons pu montrer à l'aide de mutants amputés dans l'un ou l'autre de ces oncogènes que le gène mil possède une activité biologique décelable sur des cellules nerveuses quiescentes; le produit du gène mil fait proliférer ces cellules que le produit du gène myc parvient alors à transformer.

L'étude des rétrovirus transformants s'est avérée essentielle pour aborder les mécanismes de la transformation cellulaire, ainsi que les processus fondamentaux que sont la mitose et la différenciation.

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GLOSSAIRE

-**Activation** : modification entrainant le dérèglement d'un oncogène, aboutissant à une modification quantitative ou qualitative de son produit.

-**ADN polymérase ARN dépendante** = Rétropolymérase = Réverse transcriptase = Rétrotranscriptase : enzyme d'origine rétrovirale capable de convertir une matrice ARN en ADN.

-ALV : "Associated Leukosis Viruse", rétrovirus aviaire compétent pour la réplication et dépourvu de gène oncogène.

-BL : "Burkitt Lymphoma" , Lymphome de Burkitt.

-bp : paires de bases d'ADN ; kbp, millier de paires de bases.

-CSF : "Colony Stimulating Factor", hormone indispensable à la survie, la prolifération et la différenciation des cellules hématopoïétiques.

-**Différenciation** : Mécanismes biochimiques induisant un changement dans le génotype et/ou le phénotype cellulaire.

-DLV : "Defective Leukemia Viruses", rétrovirus aviaire induisant des leucémies aiguës et défectif pour la réplication.

-"**enhencer**": séquence activatrice de la transcription.

-**épissure** : mécanisme permettant l'élimination des introns d'un ARN.

-"**helper**": ALV auxilliaire, permettant la propagation de rétrovirus aviaires défectifs pour la réplication.

-kb : kilobases, milliers de bases d'ARN.

-kd : kilodaltons.

-LTR : "Long Terminal Repeat", séquence constituée de U3RU5 (350 bp) et présente à chaque extrémité de l' ADN proviral.

-**Oncogène** : gène dont le produit peut induire la transformation cellulaire. Par extension, gène fréquemment impliqué dans les tumeurs.

v-onc : gène oncogène viral.

c-onc : gène oncogène cellulaire; la dénomination des oncogènes doit rappeler le type de maladie induit (par exemple erb pour **é**rythroblastose), le lieu de découverte (mil pour **M**ill Hill), le nom du découvreur (abl pour Mr **A**belson) ou encore une particularité biochimique (trk pour **t**yrosine **k**inase). Les c-onc humains sont notés ONC.

-px : protéine x mature.

-ppx : phospho-protéine x.

-Px : protéine de fusion x.

-Pr : protéine précurseur.

-PK : Protéine Kinase.

-QEC : "Quail Embryo Cells", cellules d'embryons de cailles japonaises de 10 jours, essentiellement constituées de fibroblastes.

-QNR : "Quail Neuroretina Cells", cellules de neurorétines d'embryons de cailles japonaises de 7 jours.

-**Transformation cellulaire** : Evénement induisant une perturbation dans la prolifération et/ou la différenciation cellulaire, aboutissant à l'apparition d'une tumeur dans un organisme. *In vitro* la transformation se traduit par une modification de la morphologie et des conditions de croissance.

PRESENTATION DU MEMOIRE

Dans l'introduction de ce mémoire nous avons présenté les résultats permettant d'impliquer les oncogènes dans la prolifération et la différenciation cellulaire. Nous avons également envisagé les divers mécanismes capables de rendre compte de l'activation des oncogènes dans les tumeurs spontanées. Il est évident que les oncogènes cellulaires ne sont pas là pour transformer les cellules, et que ce terme recouvre en fait notre ignorance de leurs fonctions exactes. Devant l'ampleur du domaine, nous nous sommes limités la plupart du temps à ne citer que les travaux les plus récents et cette introduction est conçue comme un document de travail pour les personnes désirant se familiariser avec ces sujets. Ce domaine évoluant très vite, il n'est pas possible d'être exhaustif, et nous sollicitons l'indulgence du lecteur .

Les cancers proviennent de la prolifération anarchique de quelques cellules. Une perturbation de des mécanismes susceptibles d'amener une multiplication cellulaire (mitose/différenciation et leur régulation, surveillance immunologique...) peut être à la base de leur apparition. Dans de nombreux tissus, la mitose qui permet la croissance est notamment régulée par la différenciation qui restreint la division cellulaire en modifiant le statut de la cellule. Certaines voies biochimiques permettant mitose et différenciation sont très semblables sinon identiques, expliquant en partie pourquoi un même gène peut (selon le type cellulaire) induire l'une ou l' autre réaction. Certains des gènes cellulaires qui jouent un rôle dans la prolifération s'avèrent également être des gènes oncogènes, lorsqu'une anomalie dans leur structure ou leurs éléments de régulation aboutit à leur "activation". Les rétrovirus, qui possèdent la remarquable propriété de pouvoir recombiner avec du matériel cellulaire ont cloné et activé certains de ces oncogènes qui sont à la base de leur pouvoir transformant. Ces virus sont répandus dans le règne animal, un même oncogène pouvant être capturé par des virus d'espèces différentes. Ces virus constituent une source privilégiée d' oncogènes, une autre source étant l'ADN de certaines tumeurs spontanées.

Dans ce mémoire, nous présentons un certain nombre de résultats que nous avons obtenus en étudiant le pouvoir transformant de rétrovirus aviaires induisant chez l'animal des leucémies aiguës. Nous avons eu la chance de commencer ce travail au moment où le premier oncogène cellulaire venait d'être découvert, démasquant ainsi les perspectives de l'oncologie génétique. Ces circonstances jointes au fait que ce mémoire rapporte un travail collectif, expliquent que plusieurs rétrovirus ainsi que leurs oncogènes soient étudiés. Nous avons tout d'abord analysé la structure du génome de ces virus, ce qui nous a permis de définir leur analogie avec les rétrovirus dépourvus d'oncogènes, puis de mettre en évidence les oncogènes qu'ils contiennent. Ces derniers nous ont permis d'accéder à la structure et à l'expression de leurs équivalents cellulaires dans les cellules normales, ou transformées par différents agents. Le clonage moléculaire de ces oncogènes

cellulaires a permis d'en préciser la structure fine et de suggérer quelques mécanismes d'activation oncogénique. Nous avons pu montrer, en réintroduisant l'oncogène MYC dans des cellules saines, qu'une perturbation des signaux de contrôle de cet oncogène suffisait à transformer les cellules.

Enfin, certains de ces rétrovirus contenant deux oncogènes, nous avons étudié pour l'un de ces virus (MH2), un modèle biologique permettant de mettre en évidence une synergie entre les produits viraux dans la transformation de ces cellules.

INTRODUCTION

A - GENERALITES

De tous les grands problèmes biologiques, celui de la transformation cellulaire maligne rencontre actuellement le plus d'échos, à la fois au sein de la communauté scientifique et du grand public. Cet intérêt est motivé par la grande complexité du sujet mais également par l'aspect dramatique fréquemment revêtu par la maladie qui en découle. Le cancer doit son succès à son polymorphisme, à peu près tous les tissus peuvent être atteints, et toutes les espèces concernées. La plupart des métazoaires peuvent être affectés par des désordres touchant la croissance et la différenciation cellulaire, aboutissant à l'apparition de tumeurs localisées ou disséminées.

Ces tumeurs, induites par de nombreux agents (Hiatt et al., 1977) peuvent être limitées à l'organisme porteur; dans ce cas la tumeur dérive d'une seule cellule transformée (origine clonale). La tumeur peut se répandre dans la population par la propagation de virus oncogène (origine polyclonale). D'une façon générale, les tumeurs induites par les différents virus oncogènes sont indiscernables des tumeurs induites par les agents non biologiques (physiques ou chimiques) et sont largement répandues dans le règne animal (RNA Tumor Viruses, 1982). L'existence de tels virus a permis l'étude au niveau moléculaire de l'apparition et de la maintenance de ces tumeurs, travaux qui ont littéralement explosé depuis la fin des années 70. Il était en effet inconcevable de "pêcher" au sein d'un génome eucaryote le ou les gènes impliqués dans les processus malins. Par contre, le génome rudimentaire des virus oncogènes a pu être directement étudié, et dans le même temps, ces virus ont été utilisés pour "sonder" le génome de la cellule hôte.

De tous les virus oncogènes, les plus simples font partie de la famille des rétrovirus. Cette famille est composée de virus à ARN et se subdivise en trois sous-familles :

(1) les Oncovirinae qui regroupent tous les virus oncogènes ainsi que quelques représentants non oncogènes;

(2) les Lentivirinae, qui regroupent les virus des affections lentes dégénératives du système nerveux, tel que le virus visna du mouton, et probablement le virus humain LAV (HIV) responsable du syndrome d'immunodéficience acquise (SIDA) (Rabson et Martin, 1985);

(3) les Spumavirinae qui produisent des infections persistantes en l'absence de tout signe clinique.

Tous ces virus possèdent la particularité de dépendre, pour parasiter la cellule hôte, d'un intermédiaire ADN synthétisé par un enzyme viral : l'ADN polymérase ARN dépendante encore appelée rétropolymérase (Temin et Baltimore, 1972, Verma, 1977).

B - LES RETROVIRUS

I - PRESENTATION GENERALE

Tous les rétrovirus ont en commun d'autres caractéristiques : ils possèdent un ARN

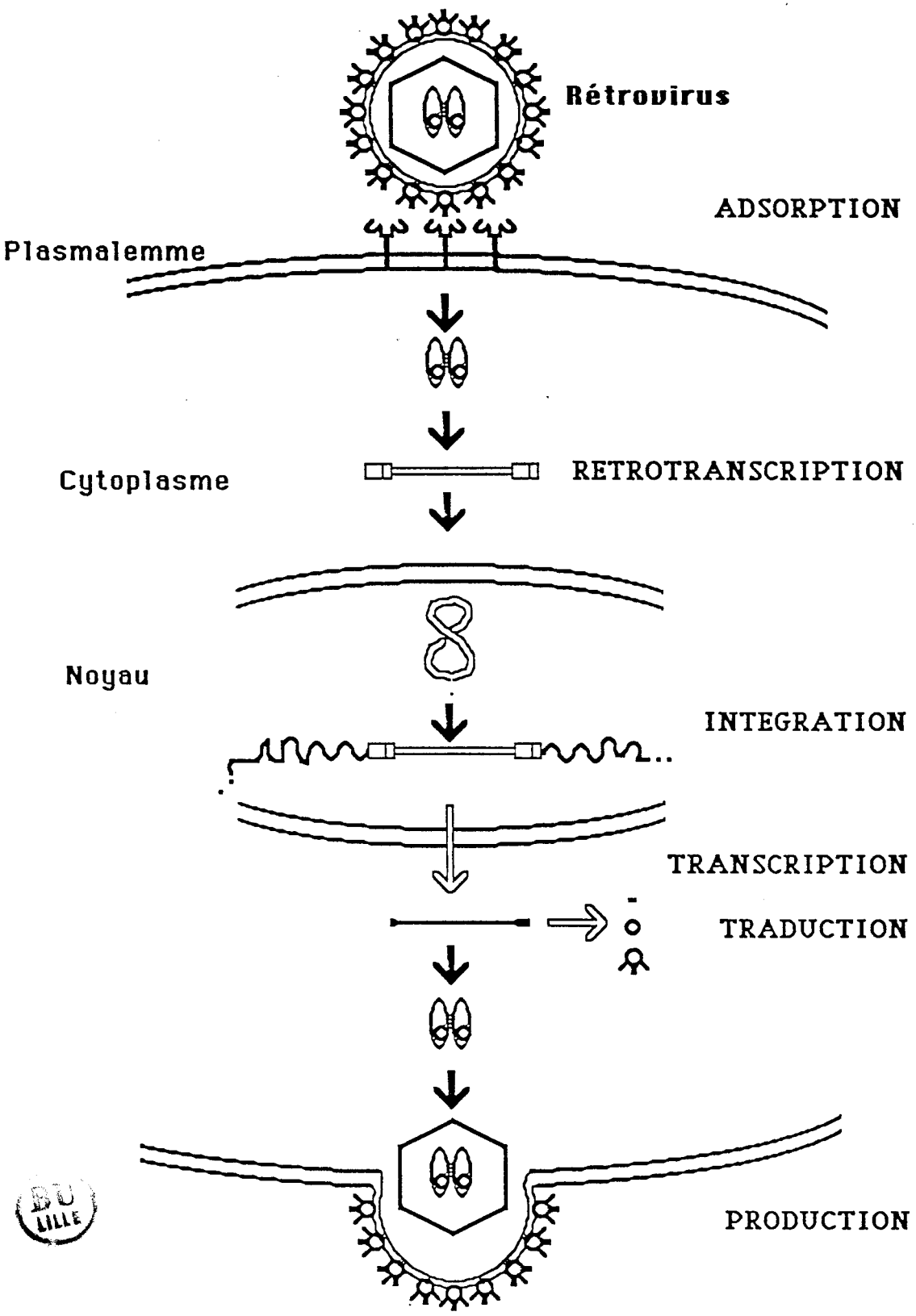


Figure 1 : Schéma général de la propagation des rétrovirus.

génomique diploïde (de coefficient de sédimentation de 60-70 S) constitué de deux sous-unités. L'extrémité 5' de l'ARN porte le classique capuchon (cap) m7 5'Gppp5' Gm (Furuichi et al., 1975) et l'extrémité 3' est polyadénylée (Bender et Davidson, 1976). De polarité positive, l'ARN peut être directement utilisé pour synthétiser les protéines virales. Cependant, cet ARN semble exister sous deux formes (circulaire et relaxée) et c'est la forme relaxée qui servirait à synthétiser les protéines, la forme circulaire étant encapsidée (Darlix, 1986). Le génome des rétrovirus compétents pour la réplication est constitué de trois gènes codant pour les protéines de structure; ce sont, de 5' en 3' les gènes gag codant pour les protéines internes, pol codant pour la réverse transcriptase et env codant pour les protéines d'enveloppe responsables de l'infectivité du virus.

- La stratégie de propagation utilisée par tous les rétrovirus est la même (Figure 1) :

- la particule virale s'adsorbe sur les récepteurs présents à la surface de la cellule hôte;

- le virus pénètre dans la cellule et son ARN, associé à la réverse transcriptase est aussitôt transcrit en ADN double brin linéaire, puis circularisé;

- cet ADN s'intègre dans le génome de la cellule où il est transcrit par la machinerie cellulaire en ARN génomique et éventuellement sous-génomique (produit par l'épissure de l'ARN génomique, afin de permettre la traduction des gènes situés en 3' du génome);

- les protéines virales sont synthétisées à partir de ces ARN le plus souvent sous la forme de précurseurs polypeptidiques, ultérieurement phosphorylés, glycosylés et clivés selon les besoins du virus. Le clivage des polypeptides précurseurs se ferait juste avant le bourgeonnement des particules virales en certains sites privilégiés de la membrane (sur lesquels seront fixées les glycoprotéines d'enveloppe du futur virus).

Il est à noter que la stratégie idéale, pour un rétrovirus parasite total, consiste à être le moins nocif possible pour la cellule et si possible même, passer inaperçu en laissant la cellule le répliquer avec son propre génome. De ce point de vue, c'est ce qu'ont réalisé avec succès les rétrovirus endogènes qui sont la plupart du temps inoffensifs pour l'animal porteur.

II - MORPHOLOGIE DES RETROVIRUS

Ces virus peuvent être classés en 4 types morpholologiques sur la base des observations de microscopie électronique (Gross, 1970). Cette classification essentiellement descriptive recouvre cependant des différences fonctionnelles.

a-Les particules de type A : ce sont des particules non infectieuses, intracellulaires, de 60 à 90 nm de diamètre, avec un centre clair entourées d'une double coque. Ces particules peuvent être intra-cytoplasmiques ou intra-cisternales; leur rôle est mal connu. Leur génome est apparenté au génome des particules de type B et D (Ono et al., 1985).

b-Les particules de type B : ce sont des particules de 125 à 130 nm de diamètre dont le nucléoïde dense est excentré dans la particule enveloppée. Le virus de la tumeur mammaire de la souris (MMTV) est un virus de type B.

c-Les particules de type C : ce type morphologique regroupe la majorité des rétrovirus. Les particules enveloppées ont un diamètre de 80 à 110 nm, un nucléoïde clair en position centrale qui mature en bourgeonnant à la surface de la cellule.

d-Les particules de type D : ces particules ne se rencontrent que chez les primates; d'un diamètre de 100 à 120 nm, elles ont un nucléoïde dense, excentré, mais différent des particules de type B par leurs images de maturation.

Enfin, sur des données de séquences nucléotidiques, un cinquième groupe, E, pourrait être créé pour le virus de la leucémie bovine (BLV) et les virus lymphotropiques T humains (HTLV) (Sagata et al., 1985).

III - STRUCTURE DU GENOME VIRAL

Les rétrovirus sont des parasites cellulaires évolués et très spécialisés. Leur génome est utilisé au mieux pour coder les différentes protéines nécessaires à la vie du virus. Une même séquence nucléotidique peut servir à coder des polypeptides non apparentés, en utilisant des cadres de lecture différents. Les éléments du génome dépourvus de capacité codante (à ce jour du moins) représentent environ 10% de l'ARN viral et contiennent les différentes séquences de régulation contrôlant la transcription, le mécanisme d' épissure, la rétrotranscription et l'encapsidation du génome viral. Certains de ces signaux peuvent avoir un impact important sur la vie de la cellule elle-même, par exemple, les séquences contrôlant la transcription du provirus sont capables d'agir sur l'expression de nombreux gènes cellulaires.

Nous allons privilégier, pour les besoins de ce mémoire, un rétrovirus aviaire transformant et compétent pour la réplication, le "Rous Sarcoma Virus" ou RSV (Coffin, 1979; RNA Tumor Viruses, 1982). Le génome de ce virus (qui est l'un des premiers virus sarcomatogènes découverts (Rous 1911)), est actuellement très bien connu, et sa séquence nucléotidique est déterminée (RNA Tumor viruses 1982; 1985; Schwartz et al., 1983). Ce virus sarcomatogène contient les trois gènes de structure des rétrovirus, mais également un gène oncogène src (Stéhelin et al., 1976a) qui lui confère sa capacité transformante (Figure 2). Son génome est composé de deux molécules d'ARN identiques de 9305 nucléotides (pour le RSV-PR-C) soit 37 S de coefficient de sédimentation, liées entre elles par leurs extrémités 5' (Bender et Davidson, 1976) et présentant de nombreuses structures secondaires et tertiaires (Darlix et al.,1985; Darlix, 1986). A chaque ARN contenant l'information virale est associée une molécule de t-ARN.

Le terme générique de RSV regroupe en fait plusieurs virus extrêmement proches : les virus B77 (Bratislava), CZ (Carr-Zilber), EH (Engelbreth-Holm), HA (Harris), PR (Prague), SR (Schmidt-Ruppin) et BH pour un autre RSV dépourvu du gène env (le virus Bryan). Les produits du gène env permettent une classification du RSV et des virus apparentés en sous classes, selon les récepteurs présents à la surface des cellules hôtes. La compatibilité entre les glycoprotéines virales d'enveloppe et les récepteurs cellulaires se traduit pour le virus par une infection possible. Les

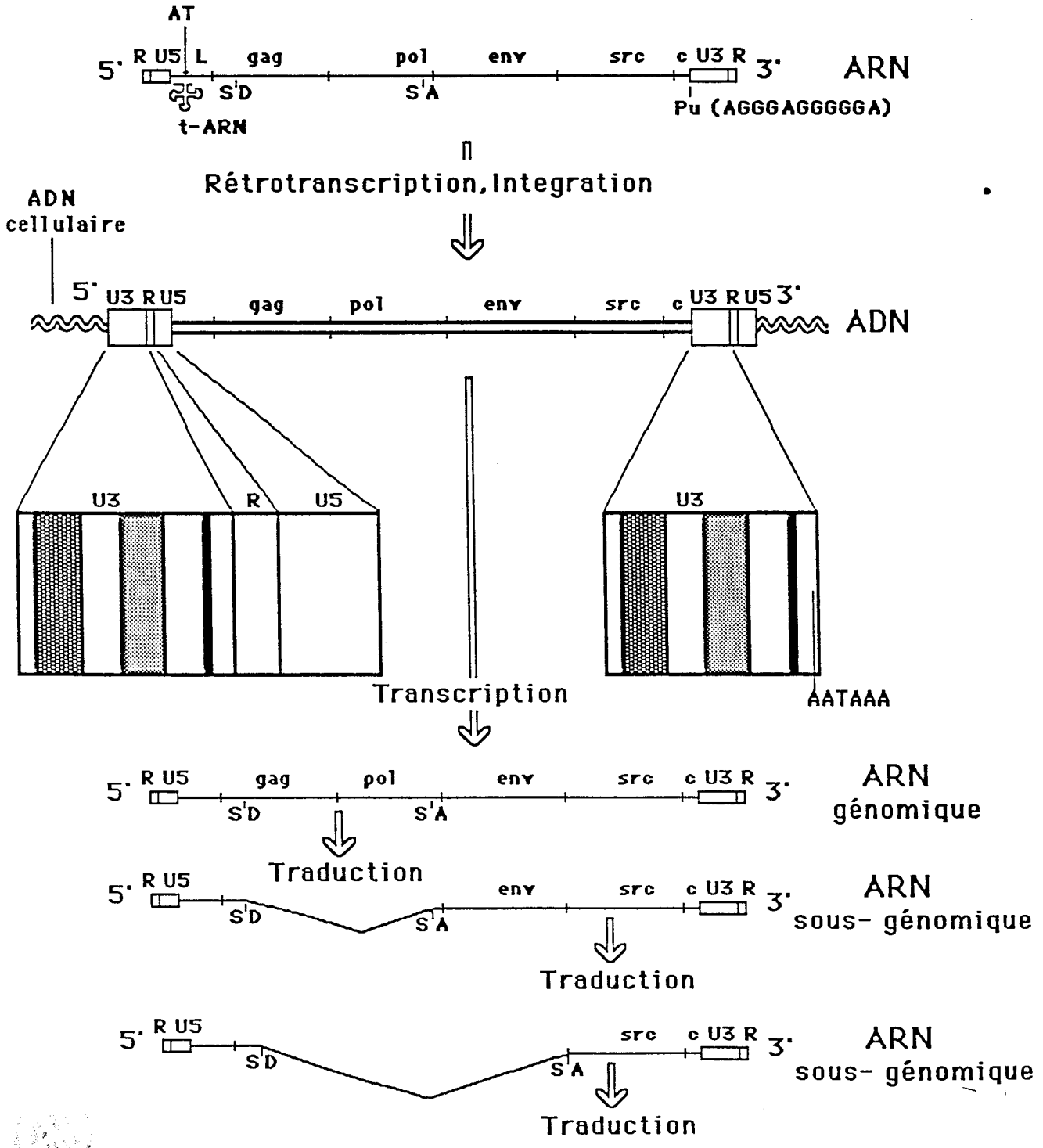


Figure 2 : Structure et expression d'un rétrovirus ASV.

(génome viral, ARN; provirus, ADN)

Activateur; Promoteur ; Initiateur .

différents sous-groupes déterminés de cette façon sont notés de A à I. Ainsi, Bratislava est de sous-groupe C, Carr-Zilber : D, Engelbreth-Holm : A, Harris : B, Prague selon les isolats : A,B,C,E, et Schmidt-Ruppin de sous-groupe A,B,D, ou E. Le virus Bryan dépourvu d'enveloppe n'a pas de sous-groupe. Les différents composants décrits plus loin (séquences de régulation et gènes) du génome viral se décomposent de la façon suivante de 5' en 3' :

5' Cap-R-U5-AT-L-gag-pol-env-src-U3-R-$A_{(200)}$ 3'

Certains des signaux présents sur la molécule d'ARN viral ne seront utilisés que dans la réplique ADN de cette molécule. La structure de l'ADN proviral intégré, de par le mode d'action de la rétropolymérase, n'est pas strictement semblable à la molécule d'ARN : les régions U3 et U5 se retrouvent répétées en 5' et 3' du génome pour aboutir à une structure connue sous le nom de LTR (Long Terminal Repeat). Cette duplication se fait évidemment au niveau des signaux présents sur la molécule d'ADN (Shank et Varmus, 1978; Coffin, 1979). Ainsi la structure du provirus est la suivante :

5' U3-R-U5-AT-L-gag-pol-env-src-U3-R-U5 3'

a-Les séquences non codantes

1) La séquence R : elle varie selon les virus entre 16 et 80 nucléotides (21 pour le RSV-PR-C) et est répétée aux extrémités 5' et 3' du génome viral (Haseltine et al., 1977; Coffin et al., 1978a). Elle semble avoir un rôle clé lors de la rétropolymérisation de l'ARN en ADN (voir plus loin).

2) La séquence U5 : elle varie selon les virus de 76 à 120 nucléotides (80 pour le RSV-PR-C) et ne semble pas avoir de fonction particulière (Dhar et al., 1980; Shimotohno et al., 1980). Cette région est très strictement conservée au sein des virus d'une même espèce. U5 a permis entre les différents virus une classification qui révèle quelques faits curieux d'un point de vue phylogénétique : par exemple, la région U5 du virus de la réticuloendothéliose aviaire (REV) est plus proche des virus endogènes de singe que des autres virus mammifères ou aviaires.

3) La séquence AT : cette séquence représente le site d'attachement du t-ARN sur l'ARN génomique et elle est complémentaire des 16 à 19 nucléotides de la région 3' du t-ARN (18 pour le RSV-PR-C). L'extrémité de ce t-ARN servira d'initiateur pour la rétropolymérase. Le t-ARN fixé sur l'ARN génomique est différent selon les virus : le t-ARN des RSV est le t-ARNtrp. Ce site de fixation de l'ARN de transfert est situé à 101 nucléotides de l'extrémité 5' de l'ARN viral du RSV-PR-C.

4) La séquence L : cette séquence est située à la fin de la région AT et au début de la région codante du gène gag. Elle varie selon les virus entre 250 (RSV-PR-C) et 300 nucléotides (MMTV) (Dhar et al., 1980; Shimotono, 1980). Cette séquence a une grande importance par les signaux qu'elle porte :

-La séquence L serait impliquée dans l'encapsidation des ARN viraux (Shank et Linial, 1980; Nishizawa et al., 1985; Darlix, 1986).

-La séquence L participe à la formation des dimères d'ARN, par l'intermédiaire

de ponts hydrogènes (Haseltine et al., 1977; Dhar et al., 1980).

- La séquence L contient les signaux nécessaires à la fixation des ribosomes sur la molécule d'ARN (Petersen et Hackett, 1985).

5) La séquence U3 : Cette séquence fait partie d'une région qui a tout d'abord été définie comme la région C, à savoir une séquence de 600 nucléotides conservée par les mutants du RSV devenus non transformants après avoir perdu le gène src (td RSV, Wang et al,.1975). Cette séquence est maintenant définie comme étant la partie 3' du génome, réitérée lors de la rétropolymérisation de l'ARN viral en ADN (voir plus loin). Cette séquence particulière varie entre 250 nucléotides (RSV-PR-C) et 1200 nucléotides (pour le virus MMTV).

U3 contient au moins deux types de signaux essentiels différents : le signal induisant la polyadénylation de l'ARN viral, AAUAAA (nucléotides 9285-9290) (Montell et al., 1983; Chen et Barker, 1984) bornant l'ARN en 3'. Le deuxième type de signal permet de contrôler la transcription du provirus et sera utilisé dans l'ADN proviral. La numérotation des nucléotides du LTR (U3-R-U5) situé en 5' se fait de façon négative à partir du 1er nucléotide de la séquence R (noté lui +1) selon le sens utilisé lors de la transcription de l'ADN. Ainsi le signal de polyadénylation AATAAA est situé en position -2 à -7 dans la séquence U3 du LTR 5'. Les séquences utilisées pour contrôler la production de l'ARN viral sont de 3 types :

a)Une séquence activant la transcription de l'ADN et qui agit en cis sur la molécule. Elle est située dans le LTR en position -219 à -139 (Luciw et al., 1983; Cullen et al., 1985a et b). Ce type de séquence (activateur) peut stimuler la transcription de gènes adjacents, à plusieurs kilobases près, indépendamment de son orientation ou de sa position (Koury et Gruss, 1983). Cette structure nucléotidique est présente dans la plupart des éléments de régulation de virus (à ADN ou à ARN) d'une façon très conservée (Koury et Gruss, 1983). Des séquences similaires existent dans le génome cellulaire (Fried et al., 1983; Schöler et Gruss, 1984) et ces éléments de régulation peuvent n'être utilisés que dans certaines conditions (Banerji et al., 1983; Gillies et al., 1983). Ces séquences fixent probablement des protéines nucléaires, différentes selon les tissus, ce qui permettrait une expression tissus-spécifique des gènes qu'elles contrôlent (Davidson et al.,1986).

b) Le second type de séquence régulatrice est constitué par le promoteur de transcription situé dans le LTR du nucléotide -135 à -45. Cette région de l'ADN, en l'absence de séquences activatrices définies plus haut, peut permettre un taux de transcription de l'ordre de 6% du taux maximum obtenu avec le LTR complet (Cullen et al., 1985a). L'élimination de cette seule séquence promotrice, par contre, réduit de 99% le taux de transcription de l'ADN (Cullen et al., 1985a). La distinction entre séquence activatrice et séquence promotrice n'est pas artificielle dans la mesure où le "pouvoir" de cette dernière séquence s'abolit très rapidement avec l'éloignement du point d'initiation de l'ARN, et devient inefficace à seulement 100 bp de ce site (Cullen et al., 1985a).

c) Enfin, une troisième séquence, appelée initiateur ou "Hogness box" de structure TAT(A ou T)$_4$G, est localisée 24 nucléotides avant le point d'initiation de la synthèse

d'ARN par l'ARN polymérase II (Yamamoto et al., 1980; Benoist et Chambon, 1981; Mathis et Chambon, 1981). Ce signal existe dans tous les rétrovirus (et la plupart des gènes eucaryotes) et est localisé du nucléotide -30 à -23 (TTTTAAG) dans la séquence du RSV-PR-C. L'initiateur contrôle l'initiation correcte de l'ARN mais n'a qu'une légère influence sur le taux de transcription (Gilmartin et Parson, 1983; Mathis et Chambon, 1981).

La structure du LTR dans l'ADN proviral autorise l'utilisation de ces signaux dans la séquence U3 située en 5' du génome pour initier la synthèse des ARN viraux. Toutefois la présence de la séquence U3 en 3' de l'ADN intégré, dans le deuxième LTR, peut permettre l'expression de l'ADN cellulaire adjacent au provirus intégré (Figure 2). Cette propriété est particulièrement intéressante car elle peut expliquer pourquoi des virus non transformants par eux-mêmes, tels les avian leukosis virus (ALV) peuvent en certains cas induire des tumeurs en activant des gènes cellulaires indésirables par l'intermédiaire de ces séquences régulatrices (Hayward et al., 1981; Fung et al., 1983; Corcoran et al., 1984).

La séquence U3 ne semble pas posséder de capacité codante en général mais ce point ne semble pas clair pour MMTV qui possède une séquence U3 particulièrement longue. Sa séquence montre un cadre de lecture ouvert permettant la synthèse d'une protéine de 36 000 daltons (Donehower et al., 1981). Une autre particularité dévolue à la région U3 de MMTV est de posséder des sites qui permettent à une hormone glucocorticoïde (la dexaméthasone) d'augmenter de 10 à 100 fois la concentration d'ARN viral dans les cellules infectées (Ringold et al., 1975).

Le rôle essentiel de ces séquences dans le contrôle de la transcription permet d'expliquer la pathogénie particulière de tel ou tel isolat viral en fonction de ses séquences U3 (Davis et al., 1985). Lenz et al. (1984) ont pu montrer que ces séquences contrôlaient le pouvoir leucémogène d'un rétrovirus murin, et Linney et al. (1984) ont impliqué ces mêmes séquences dans le blocage transcriptionnel affectant le virus MuLV dans les cellules de tératocarcinome F9. Ainsi, des expériences de biologie moléculaire ont permis de montrer que la substitution des séquences régulatrices de la transcription d'un rétrovirus murin par celles d'un virus à ADN capable de se repliquer dans ces cellules, était suffisante pour restaurer l'infectivité du rétrovirus (Linney et al., 1984). Un autre exemple de l'importance de ces séquences dans le pouvoir pathogène de ces virus est apporté par l'analyse de virus endogènes devenus leucémogènes et présentant des modifications structurales dans leurs LTR, conduisant accidentellement à la création de séquences activatrices de la transcription. Cependant, d'autres éléments du génome viral doivent aussi intervenir pour conférer aux virus dépourvus d'oncogène leur pouvoir transformant (Robinson et al., 1985; Shank et al., 1985).

b-Les signaux d'encapsidation

L'encapsidation de l'ARN viral est un événement obligatoire du cycle infectieux, et certaines séquences virales sont indispensables pour sa réalisation. Les séquences nucléotidiques impliquées ont pu être définies avec une certaine précision par l'étude soit de mutants spontanés de rétrovirus ayant perdu la capacité d'encapsider leur ARN (Shank et Linial, 1980; Nishizawa et al., 1985;

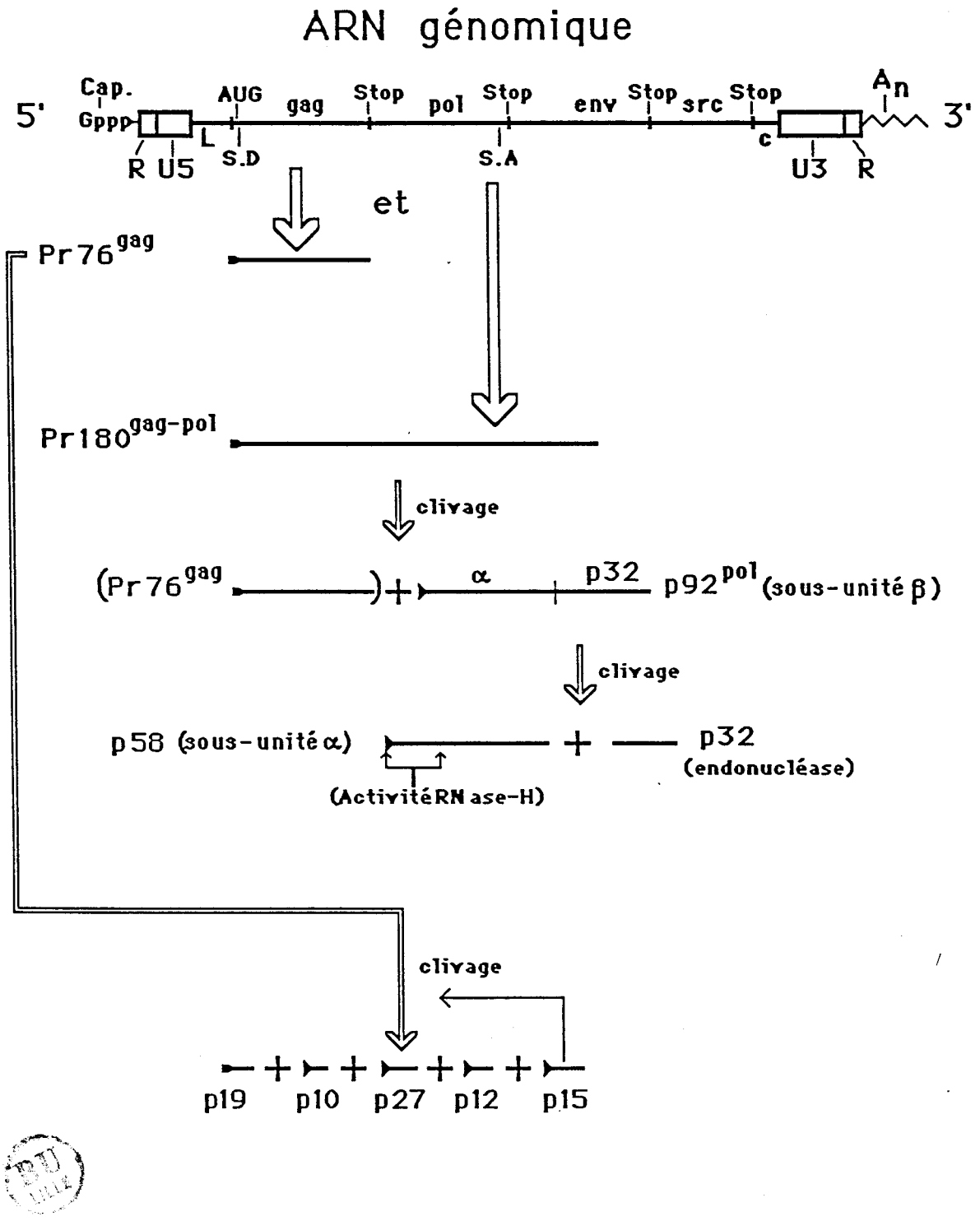


Figure 3 : Les protéines codées par le génome viral et leur maturation.

Darlix, 1986), soit de mutants construits *in vitro* à l'aide de molécules partiellement amputées de séquences nucléotidiques situées en 5' ou en 3' du génome (Watanabe et Temin, 1982; Pugatch et Stacey, 1983; Sorge et al., 1983). Ces séquences agissent en cis sur la molécule qui les porte et sont localisées (en 5') dans la séquence L, avant le début du gène gag et (en 3' de la molécule) dans les séquences directement répétées encadrant le gène src (Sorgue et al., 1983; Nishizava et al., 1985). La séquence nucléotidique minimale indispensable n'est pas connue. L'interaction de protéines virales avec ces séquences nucléotidiques parait probable.

c-Les gènes viraux

1) Le gène gag

Les gènes gag des différents rétrovirus codent pour un polypeptide précurseur de toutes les protéines de la capside interne et variant de 70 000 à 80 000 daltons de poids moléculaire. Le gène gag du RSV synthétise un précurseur de 76 000 daltons ($Pr76^{gag}$), qui est phosphorylé (mais non glycosylé) et associé à la membrane plasmique (Eisenman et Vogt, 1978). Le $Pr76^{gag}$ est synthétisé à partir d'un ARN apparemment indistinguable de l'ARN génomique qui est présent dans les particules virales (Hayaward, 1977). L'AUG utilisé pour cette synthèse est situé à 379 nucléotides de l'extrémité 5' de l'ARN et représente le $4^{ème}$ AUG présent dans cette séquence (RNA Tumor Viruses, 1982). Le gène gag contient également, 15 nucléotides après cet AUG, le signal permettant d'épisser l'ARN génomique.

La maturation du précurseur semble nécessiter l'action d'une protéase cellulaire pour libérer la protéine p15 douée d'une activité protéolytique du précurseur, car le $Pr76^{gag}$ ne possède pas de propriété autoprotéolytique (von der Helm, 1977). Deux sites majeurs de clivage, constitués de deux à quatre résidus hydrophobes suivis par une méthionine sont présents entre p27 et p12, et entre p12 et p15 (Vogt et al.,1979). L'organisation du $Pr76^{gag}$ est la suivante :

NH2 p19-p10-p27-p12-p15 COOH (Figure 3).

a) La p19

La p19 existe à la fois sous une forme phosphorylée sur une sérine (pp19) et non phosphorylée au sein du virus.Dans le virion, elle est en association avec l'ARN génomique et avec l'enveloppe lipidique (Montelaro et al., 1978). Il semblerait que la p19 soit physiquement liée à la gp37, une glycoprotéine constitutive de la membrane lipidique de l'enveloppe (Montelaro et al., 1978). La p19 semblerait également jouer un rôle dans la régulation du taux d'épissage de l'ARN génomique et dans le métabolisme des ARN messagers viraux (Leis et al, 1980).

b) La p10

Cette protéine est la seule protéine du gène gag à se trouver dans la partie externe de l'enveloppe. Sa fonction est inconnue.

c) La p27

Cette protéine hydrophobe représente le composant majeur de la capside virale. Elle pourrait constituer, de par sa propriété à former des assemblages ordonnés, les sous-unités de la capside virale (RNA Tumor Viruses, 1982).

d) La p12

C'est la deuxième protéine qui s'accroche à l'ARN viral, mais contrairement à la p19 qui s'y accroche de façon spécifique et en petit nombre, la p12 constitue l'élément essentiel des ribonucléoprotéines virales. Cette protéine (qui occupe 4 nucléotides par site) se fixe de façon non spécifique sur tous les ARN, sur la base d'une interaction ionique entre l'ARN et ses résidus lysine et arginine (RNA Tumor Viruses, 1982). Elle pourrait jouer un rôle lors de l'introduction de l'ARN viral dans la capside.

e) La p15

Cette protéine se trouverait dans le virus entre la capside interne et l'enveloppe lipidique (RNA Tumor Viruses, 1982). Elle possède une activité protéasique sur le $Pr76^{gag}$, sur ses intermédiaires (von der Helm, 1977; Vogt et al., 1979), ainsi que sur la grande sous-unité de la réverse transcriptase (Moelling et al., 1980).

Enfin, le gène gag pourrait également diriger (dans un autre cadre de lecture que celui utilisé pour la synthèse de la p19) la traduction d'un produit activant en trans la production des ARN messagers (Broome et Gilbert, 1985). Cet activateur de transcription serait traduit à partir d'un ARN utilisant un signal accepteur d'épissure cryptique (nucléotide 681) raboutant l'AUG du gène GAG avec les 401 nucléotides suivants (jusqu'au nucléotide 1080). Un peptide activateur de 123 acides aminés pourrait ainsi être produit.

2) Le gène pol

Le produit du gène pol qui constitue (peut-être) l'originalité des rétrovirus (Varmus, 1985) est synthétisé sous la forme d'un polypeptide précurseur de 180 000 daltons de poids moléculaire contenant le $Pr76^{gag}$ lié à la rétropolymérase. Le $Pr180^{gag\text{-}pol}$ est traduit à partir d'un ARN de taille génomique. Cependant, la séquence du RSV montre qu'un codon ambre (UAG) est situé à la fin de la p15 du gène gag, et la traduction de l'ARN viral en présence d'un t-ARN suppresseur n'augmente pas la quantité de $Pr180^{gag\text{-}pol}$ comme on aurait pu s'y attendre, si un mécanisme de ce type contournait ce problème, et comme c'est le cas pour le virus MLV (Weiss et al., 1978). De plus, même si la barrière UAG peut être franchie, le cadre de lecture de la polymérase étant différent de celui du gène gag, le produit synthétisé ne correspondrait pas à la rétropolymérase. Il est plus simple de proposer qu'un mécanisme d'épissure supprime 50 à 60 nucléotides entre la fin de p15 et le début de la polymérase, de façon à remettre les gènes gag et pol dans le même cadre de lecture. Toutefois, un tel messager n'a pas encore été caractérisé. Jachs et Varmus (1985) ont récemment

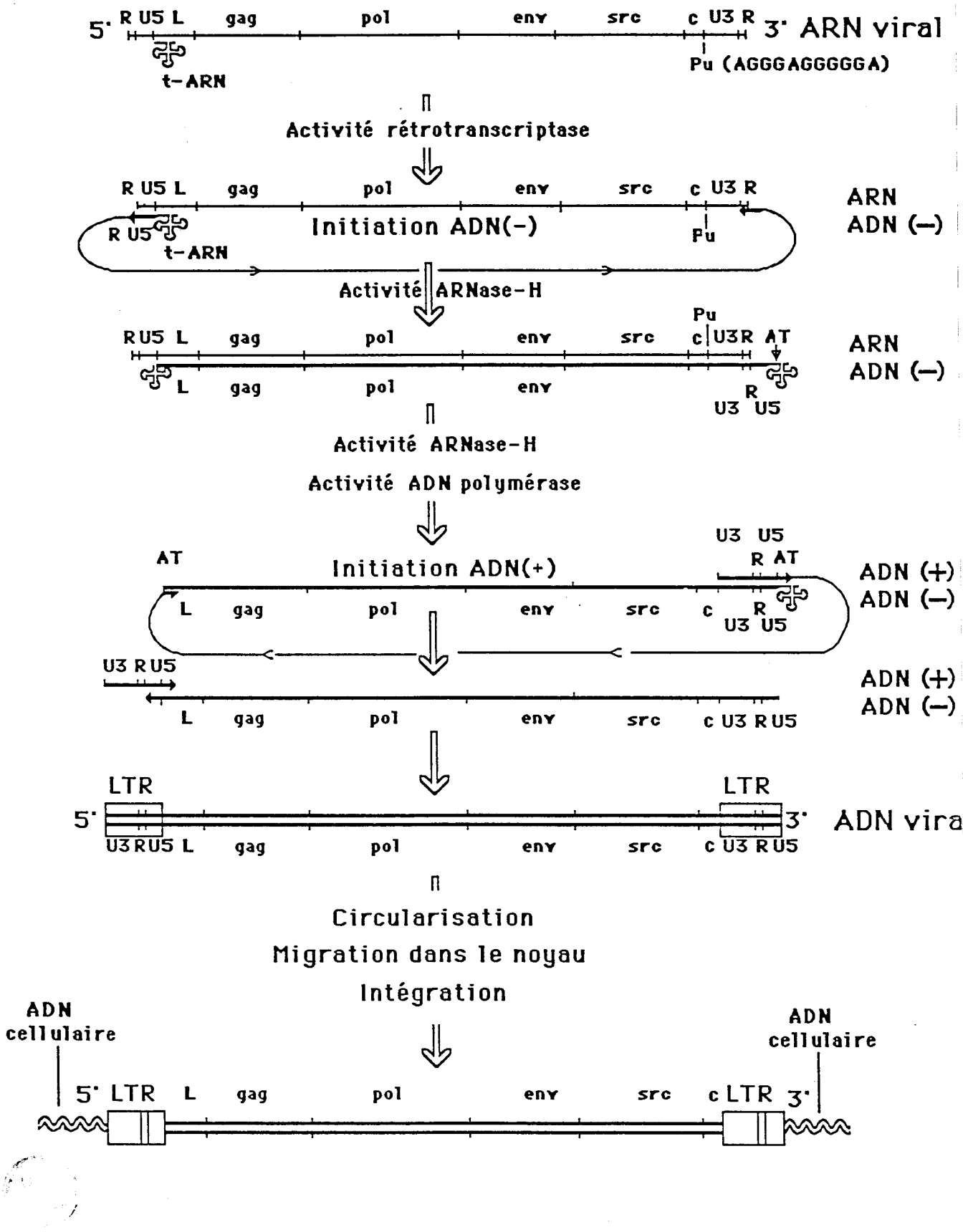


Figure 4 : Rétrotranscription de l'ARN viral.
(d'après Gilboa et al., 1979)

rapporté des expériences de traduction de $Pr180^{gag\text{-}pol}$ *in vitro* à partir de l'ARN messager génomique. Les auteurs postulent que dans ce cas, c'est le ribosome lui-même qui permet le changement de cadre de lecture aboutissant à la synthèse du polypeptide correct. Si ce mécanisme de traduction existe également i*n vivo*, l'épissage précédemment postulé n'est plus nécessaire et l'ARN génomique conventionnel coderait pour le Pr76 et le Pr180.

Contrairement au produit du gène gag présent à plusieurs milliers d'exemplaires par virus, il n'y a que 40 à 100 molécules de polymérase par virion (RNA Tumor Viruses, 1982).

a) La réverse transcriptase

La réverse transcriptase est constituée de deux sous-unités notées α et β respectivement de 58 000 et 92 000 daltons de poids moléculaire, dont l'analyse peptidique révèle que la sous-unité α est un produit de clivage de la sous-unité β et que les deux protéines ont la même extrémité NH2 terminale (RNA Tumor Viruses, 1982). Le dimère β-β initial pourrait être clivé (peut-être par la p15 du gène gag, Moelling et al., 1980) pour générer le complexe αβ, plus un fragment de 32 000 daltons de poids moléculaire doué d'une activité ADN endonucléasique Mn^{++} dépendante. Ces différents polypeptides sont tous présents dans la particule virale. Le clivage du complexe αβ par la chymotrypsine produit un fragment de 24 000 daltons de poids moléculaire, correspondant à l'extrémité NH2 terminale de la protéine et présentant une activité ribonucléasique H (RNase H) s'exerçant sur les hybrides ARN-ADN et non sur l'ARN libre ou sur l'ARN double brin (Verma, 1977).

La rétropolymérase fait partie du complexe ribonucléoprotéique viral et est probablement fixée sur le t-ARN. La présence de la rétropolymérase semble essentielle pour la pénétration du t-ARN dans la particule virale. Il existe en effet des mutants de virus aviaires, dépourvus de polymérase, qui n'encapsident pas de t-ARN (Levin et Seidman, 1979; Sawyer et Hanafusa, 1979) et des mutants de virus murins qui encapsident la rétropolymérase et le t-ARN en absence d'ARN génomique.

b) Mécanisme d'action de la rétropolymérase (Figure 4)

1°) La polymérase accrochée au t-ARN fixé sur la molécule d'ARN génomique synthétise le "strong stop DNA" comportant R-U5 de polarité négative (donc complémentaire de l'ARN, Figure 4).

2°) L'activité RNAse H de la rétropolymérase lui permettrait de libérer l'extrémité d'ADN complémentaire de la région R. Cet enzyme a également la propriété d'hydrolyser la queue polyadenylée de l'ARN viral, libérant ainsi l'extrémité 3' de la séquence R de l'ARN (Olsen et Watson, 1985). Cet ADN peut s'hybrider avec la région R présente à la fin de toutes les molécules d'ARN viral. La polymérase peut alors continuer la synthèse du brin (-) d'ADN jusqu'au site de fixation du t-ARN.

3°) La synthèse du brin d'ADN (+) se ferait grâce à la fixation d'une autre molécule de la rétropolymérase sur un site riche en purines, présentant un grand degré de

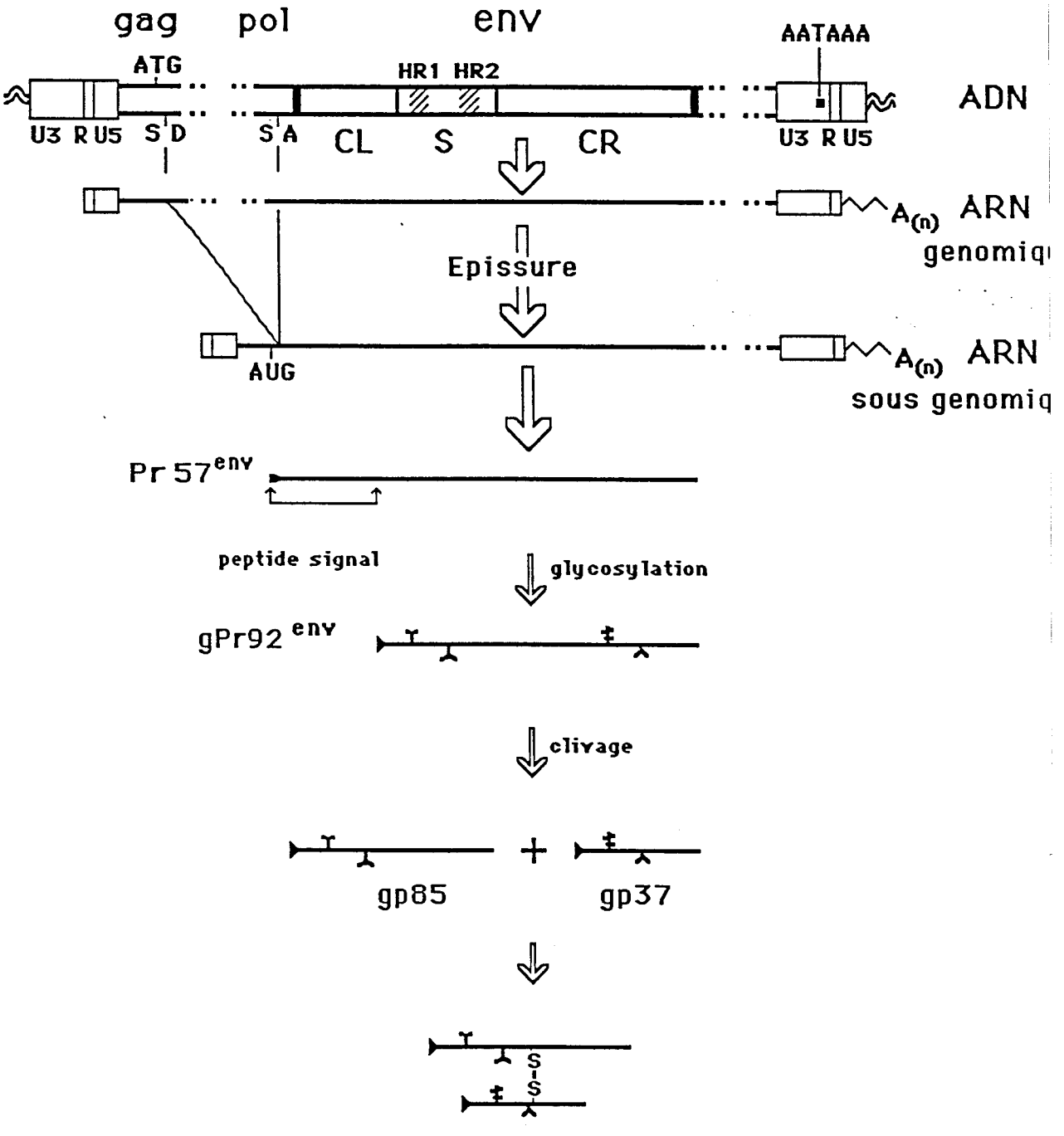


Figure 5 : Le gène env.

Structure et expression.

conservation à travers tous les rétrovirus et situé en 5' de U3 (séquence AGGGAGGGGGA pour le RSV-PR-C); les fragments d'ARN (quelques nucléotides) produits par l'activité RNase H de la rétropolymérase pourraient également servir de point d'initiation de la synthèse de l'ADN (+) en s'hybridant avec l'ADN (-) (Varmus et al., 1979). Cet ADN (+) est connu sous le nom de "(+) strong stop DNA" et contient U3-R-U5-AT.

4°) Ce fragment d'ADN pourrait être apparié à une chaîne d'ADN (-) synthétisée, une hybridation se faisant entre la région AT (+) du "(+) strong stop DNA" et la région AT (-) du brin (-) de l'ADN synthétisé initialement (le même brin d'ADN (-) peut être utilisé (comme sur la Figure 4), mais un autre brin d'ADN (-) peut également servir de matrice (Gilboa et al., 1979).

5°) Une fois le pont établi entre le "(+) strong stop DNA" et le brin (-) d'ADN, la polymérase peut achever la copie complète des deux brins d'ADN en produisant aux deux extrémités de l'ADN la structure LTR : U3-R-U5. Ce modèle d'action de la rétropolymérase semble général pour tous les rétrovirus (Figure 4). Quelques points de détail ne sont pas encore éclaircis, par exemple, la formation du pont entre le "(+) strong stop DNA" et le brin (-) nécessite l'élimination du t-ARN fixé sur la molécule en 5', ce qui demande peut-être l'action d'un facteur extérieur à la polymérase.

3) Le gène env

Les protéines d'enveloppe des rétrovirus sont traduites à partir d'un ARN sous génomique produit par l'épissage de l'ARN viral. La structure de cet ARN est :

cap-R-U5-L-env-src-U3-R-poly A pour le RSV et

cap-R-U5-L-env-U3-R-poly A pour un ALV (Hayward, 1977; Weiss et al., 1977; Lee et al,. 1979). Le site "donneur" d'épissage se trouve au tout début du gène gag, et le site accepteur se trouve dans le gène pol (nucléotide 5078), environ 110 nucléotides avant la fin de ce gène (RNA Tumor Viruses, 1982; Figure 5). La localisation membranaire (Lee et al., 1979) des polysomes contenant cet ARN est attendue puisque les protéines d'enveloppe sont des glycoprotéines et que biosynthèse et transport des produits du gène env suivent le schéma établi pour les protéines transmembranaires (Blobel et Dobberstein, 1975). Le peptide signal probablement clivé très vite *in vivo* est ici défini par les six premiers codons du gène gag, la fin du gène pol (dans un autre cadre de lecture que celui de la rétropolymérase) et le début du gène env. Le précurseur $Pr57^{env}$ semble être très rapidement glycosylé lors de son passage dans le réticulum endoplasmique pour fournir le $gPr92^{env}$, contenant les glycoprotéines d'enveloppe gp85 et gp37 dans cet ordre : NH2-gp85-gp37-COOH (Hayman, 1978). De tous les gènes des ALV ou RSV, le gène env est le plus polymorphe, ce qui rend compte des différents sous-groupes existants dans ces virus. Toutefois, le gène env ne varie pas de la même façon tout au long de sa séquence, et il est possible d'individualiser trois régions au sein de ce gène (Coffin et al., 1978b) : CL, S et CR, pour common left region, subgroup coding et common right region. C'est la région S (0.7 kb) qui est impliquée dans le type du sous-groupe défini par le gène env, et cette région S occupe le centre de la séquence dévolue à la gp85. Cette région variable a pu être structurée en deux zones, hr1

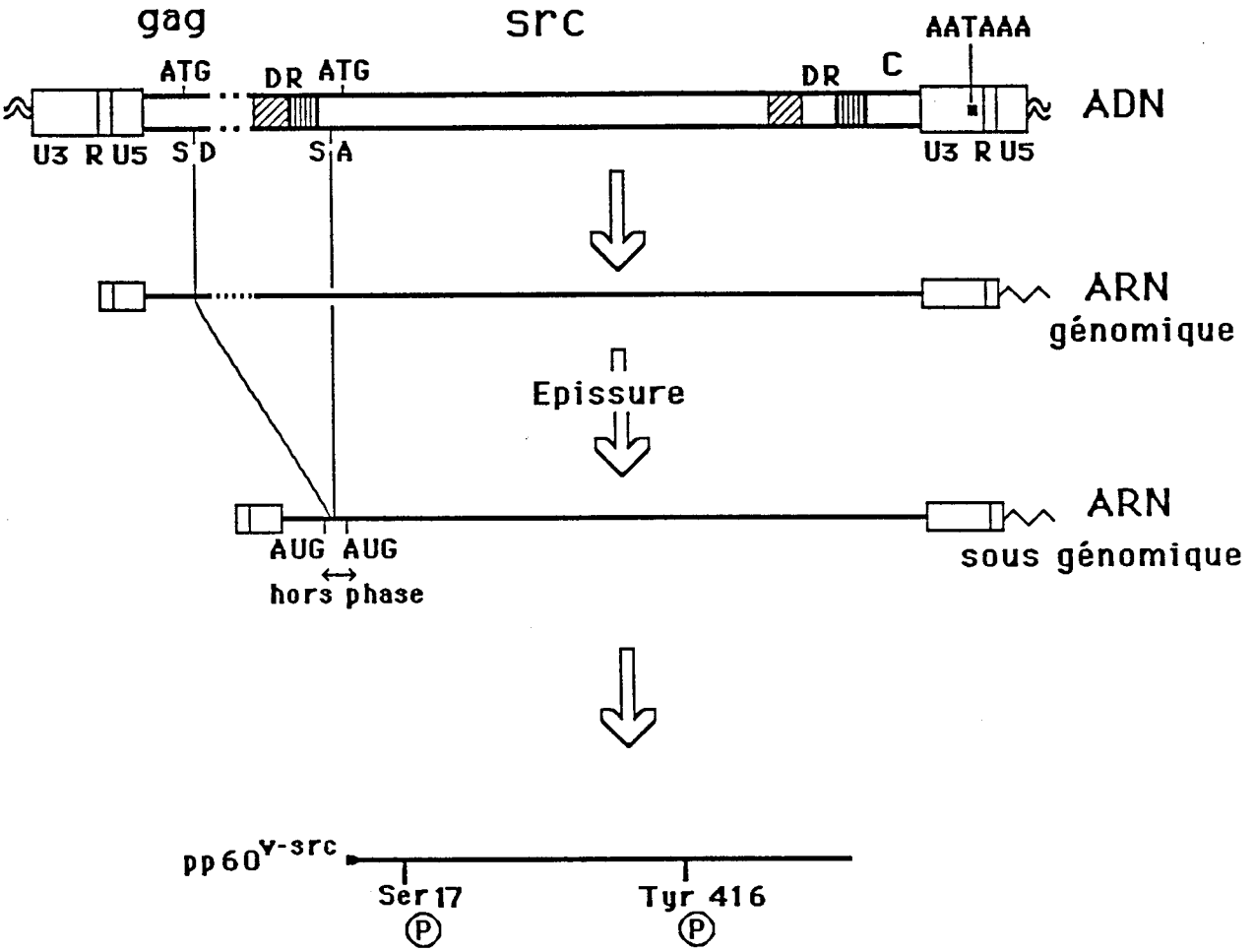


Figure 6 : Le gène src

Structure et expression.

(débutant au nucléotide 5632) et hr2 (débutant au nucléotide 5846), codant respectivement pour 32 et 27 acides aminés (hr pour "Host range", Dorner et al., 1985) (Figure 5). La portion carboxy terminale de la gp37 (codée par la région CR) pourrait être impliquée dans le transport de la protéine dans l'appareil de Golgi (Wills et al., 1984).

Les sous-groupes viraux définis par le gène env sont notés de A à I. Les sous-groupes de A à D appartiennent aux virus leucémogènes aviaires exogènes (ou aux virus endogènes ayant recombiné avec le gène env d'un virus exogène), le sous groupe E caractérise les virus endogènes du poulet. Toutefois, la grande similitude de séquence présente dans les gènes env codant pour ces différents sous-groupes laisse penser que ce gène a pu évoluer, par mutations à partir d'une séquence ancêtre, pour pouvoir coder pour les différents types d'enveloppe existants (Fujita et al., 1978).

Les sous-groupes F et G appartiennent aux gènes d'enveloppe de virus endogènes de deux espèces différentes de faisan (virus RPV et GPV) et ces gènes env n'ont que peu ou pas d'homologies avec les gènes env de sous-groupes A à E (Fujita et al., 1978; Hu et al., 1978a).

Le sous-groupe H appartient à un virus endogène de perdrix Hongroise et le sous-groupe I vient d'être attribué à un virus endogène de caille californienne (Troesch et Vogt, 1985).

C'est contre les produits du gène env que se fait la réponse immunitaire majeure de l'animal infecté, par l'intermédiaire d'anticorps neutralisants (Rubin et al., 1962), ce qui diminue considérablement l'efficacité d'infection d'un animal adulte.

4) Le gène src

La dénomination des gènes oncogènes doit se faire par trois lettres, et rappelera de préférence le type de tumeur induit, ou le nom de l'auteur ou encore le lieu de découverte (Coffin et al., 1981). Dans le cas précis, src fait référence aux sarcomes induits par le RSV. Comme pour le gène env, le gène src est traduit à partir d'un ARN de taille sous-génomique (Figure 6), produit d'épissure de l'ARN 37 S, et de structure cap-R-U5-L-src-U3-R-poly A (Hayward et al., 1977; Lee et al., 1979). Le site "donneur" d'épissure est celui du gène gag, et le site "receveur" semble être situé, pour le RSV-PR-C, au tout début du gène src, 70 nucléotides en 5' du codon d'initiation, nucléotide 7053 (RNA Tumor Viruses, 1982). Il est à noter que la traduction du gène src contrairement à celle du gène env, utilise l'AUG interne du gène et non celui du gène gag .

La séquence du gène src présente un cadre de lecture ouvert de 1590 nucléotides, codant pour 530 acides aminés, ce qui conduit à prédire un polypeptide de 58 500 daltons de poids moléculaire, tout à fait compatible avec celui de la $p60^{src}$ mise en évidence dans les cellules transformées par le RSV (Brugge et Erikson, 1977). Entre le codon d'arrêt UAG de la fin du gène env et l'AUG du début du gène src il existe 265 nucléotides non traduits pour le RSV-PR-C. De même, entre la fin de src et le début de la séquence U3, il existe 350 nucléotides non traduits pour le RSV-PR-C. Ces régions non codantes sont intéressantes, car elles possèdent une séquence nucléotidique répétée qui joue un rôle important dans l'encapsidation du génome viral. Cette séquence répétée est de 120 nucléotides mais son arrangement est un peu particulier dans la mesure où les 120 nucléotides présents en 5' sont en 3' séparés en deux régions (80 et 40 nucléotides) par l'insertion d'une

séquence unique de 135 nucléotides (Figure 6). La présence de ces séquences répétées pourrait être due à la capture du gène src cellulaire par le virus, qui aurait conduit à la duplication des séquences virales adjacentes. Une conséquence de la présence de ces séquences au sein du virus pourrait être la fréquente excision de src obtenue par une recombinaison entre ces séquences répétées, et la génération de mutants non transformants du RSV.

Le produit du gène src a pu être mis en évidence à la fois *in vivo* (Brugge et Erikson, 1977) et *in vitro* après traduction de l'ARN sous-génomique du gène viral (Bishop et al., 1980). Il s'agit d'un produit légèrement différent en taille selon les souches virales, et suffisamment polymorphe pour que des immun sérums anti $pp60^{src}$ ne reconnaissent pas obligatoirement les protéines src de tous les RSV. Toutefois, il s'agit là de différences mineures n'affectant pas la fonction de la $pp60^{src}$. La $pp60^{src}$ est une phosphoprotéine kinase phosphorylant les protéines sur les tyrosines (Colett et al., 1980). La $pp60^{src}$ serait douée d'une activité autophosphorylante sur sa tyrosine 416. L'autre acide aminé dont la phosphorylation pourrait réguler l'activité de l'enzyme serait la sérine 17 phosphorylée par une protéine kinase AMP-c dépendante d'origine cellulaire (Collett et al., 1979). Le RSV n'est pas le seul virus à porter un gène oncogène, et le gène src sera traité plus en détail dans le chapitre consacré aux oncogènes.

IV-INTEGRATION DES RETROVIRUS ET VIRUS ONCOGENES

a-Intégration des rétrovirus

L'intégration de l'ADN synthétisé après la pénétration de l'ARN viral dans le cytoplasme cellulaire est nécessaire pour la fabrication de nouvelles particules virales et la maintenance de l'infection. Cependant, cette étape cruciale dans le cycle parasitaire du rétrovirus n'est pas encore très clairement comprise. Cette intégration requiert :

1) une molécule d'ADN proviral dont la structure (linéaire, circulaire, avec un seul ou deux LTR) n'est pas très bien définie (ces différents ADN sont en effet infectieux), mais quelques nucléotides présents sous forme de séquences inversées répétées en 3' de U5 et 5' de U3 jouent probablement un rôle essentiel (Chen et Barker, 1984).

2) des facteurs enzymatiques cellulaires et viraux pour effectuer les différentes opérations d'ouverture de l'ADN et ligature des brins cellulaires et viraux (Duyk et al., 1983).

3) des sites d'intégration dans l'ADN cellulaire, dont le type et la nature ne sont pas définis et qui peuvent être extrêmement discrets (Chen et Barker, 1984).

Cependant, la détermination de la séquence nucléotidique de la jonction ADN cellulaire-ADN viral de plusieurs provirus intégrés a permis de faire une observation importante pour la compréhension du mécanisme d'intégration. Tous les provirus analysés à ce jour présentent une duplication de 4 à 6 nucléotides d'ADN cellulaire de part et d'autre du site d'intégration, le provirus intégré perdant deux nucléotides par rapport à l'ADN non intégré (Hughes et al., 1981; Van Beveren et al., 1982; Chen et Barker, 1984). Cette particularité a amené les auteurs à comparer les rétrovirus à des éléments transposables bactériens et à leur supposer un mode d'intégration comparable (Temin, 1980; Baltimore, 1985).

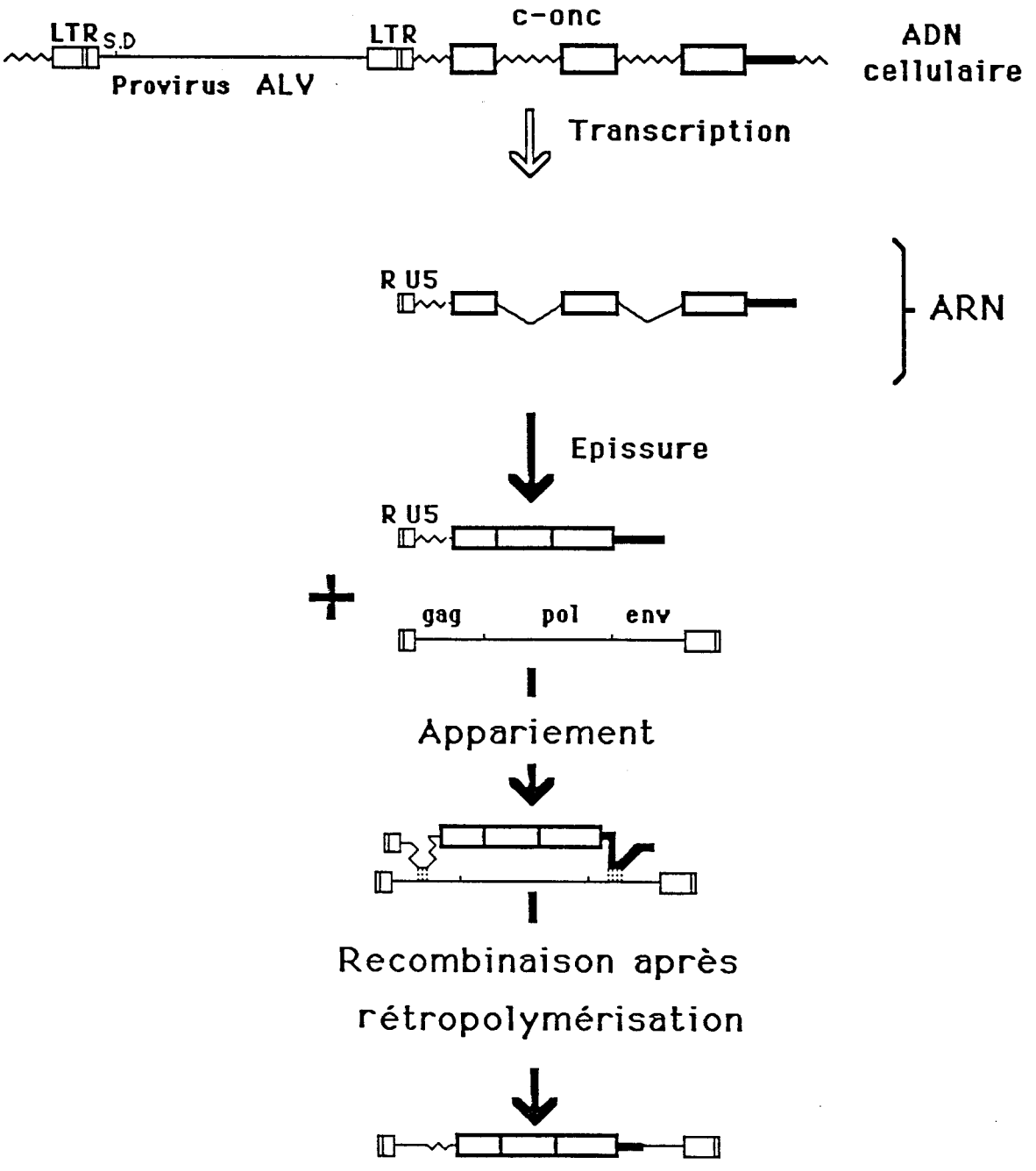


Figure 7 : Un mécanisme possible pour la formation d'un rétrovirus oncogène.

L'origine des rétrovirus n'est pas connue, mais il existe dans le matériel génétique des eucaryotes des séquences d'ADN répétées ou moyennement répétées qui ont une structure proche de celle des rétrovirus (Sun et al., 1984; Baltimore, 1985; Wichman et al., 1985). Ces séquences possèdent en effet un promoteur de transcription (pour l'ARN polymérase III) (Adeniyi-Jones et Zasloff, 1985), sont encadrées par des séquences directement répétées (Jagadeeswaran et al., 1981; Wichman et al., 1985) et sont capables d'une réplication autonome de façon extra-chromosomique.Ces séquences pourraient peut-être représenter des ancêtres de rétrovirus.

L'intégration des rétrovirus dans le génome de la cellule hôte est sous la dépendance de facteurs cellulaires gouvernant l'insertion du matériel génétique viral dans l'ADN cellulaire. La nature de ces facteurs n'est pas connue, mais dépend notamment du stade de différenciation de la cellule infectée. Ce point a pu être montré à l'aide de lignées cellulaires de tératocarcinomes murins: les cellules les moins différenciées ne permettent pas l'intégration du provirus infectant, alors que cette intégration est rendue possible une fois la différenciation induite (Niwa et al., 1983). Des facteurs cellulaires doivent probablement interagir avec des séquences virales pour réguler l'intégration des rétrovirus infectants.

b-Les virus endogènes

Des rétrovirus endogènes ont été répertoriés dans de très nombreuses espèces animales, l'homme compris (Jaenisch, 1983; Horn et al., 1986). Ils se comportent comme du matériel cellulaire génomique. Ces virus peuvent être d'acquisition ancienne et servir dans ce cas de marqueur phylogénique, comme par exemple entre les chats et les singes (Benveniste et Todaro, 1974) soit être d'acquisition plus récente comme chez le poulet (Frisby et al., 1979). Dans l'espèce de poulet white-Leghorn, 16 formes différentes d'un virus endogène Rav-0 ont été répertoriées, la plupart d'entre elles étant défectives pour la réplication (Astrin et al., 1980). Chez l'homme des rétrovirus endogènes (essentiellement incomplets) ont été localisés sur les chromosomes 1, 5, 7, 8, 11, 14, 17 et 18 (Horn et al., 1986). La présence ou l'absence de virus endogène ne semble pas affecter les poulets, contrairement à la souris où les virus endogènes peuvent devenir leucémogènes dans certaines conditions (Chattopadhyay et al., 1982).

c-Recombinaison des rétrovirus avec des gènes cellulaires

L'infection d'un animal à l'aide d'un rétrovirus non transformant peut permettre dans certains cas (RNA Tumor Viruses, 1982) l'émergence de virus oncogènes. Ces derniers sont le produit d'une recombinaison entre les séquences nucléotidiques des gènes de structure du rétrovirus non transformant et une séquence d'origine cellulaire provenant d'un oncogène (c-onc). Cette recombinaison a probablement lieu en deux temps, dans l'ADN tout d'abord, puis dans l'ARN. Une suite d'événements aboutissant à la création d'un rétrovirus oncogène est fort bien décrite dans la génération du virus aviaire érythroblastosant AEV-H. Dans ce cas, un ALV s'intègre dans le locus c-erbB; l'un de ses LTR permet l'expression d'une quantité importante d'ARN de ce gène. Un deuxième événement intervenant au niveau de l'ARN suppose un appariement entre des séquences nucléotidiques de l'ALV et des séquences nucléotidiques du gène erbB. La

onc	virus	maladie	hôte	protéine v-onc	activité	localis.	prot. c-onc	activité	localis.	Références
sis	SSV	Sarcome	Singe	P28 $^{env-sis}$	PDGF (chaine β)	Excrétée (1)	p30	PDGF (chaine β)	Excrétée	1) Huang et al., 1984
	Pi-FeSV	Sarcome	Chat	P76 $^{gag-sis}$						
erbB	AEV-ES4	Sarcome Erythro.	Poulet	gp74 $^{v-erbB}$	Tyr. Kin.	Membrane plasmique	gp170 (Homme)	Tyr. Kin. Réc. EGF	Tran. Mbr.	
fms	SM-FeSV	Sarcome	Chat	gP 180 $^{gag-fms}$	Tyr. Kin.	Membrane plasmique	(2) gp165	Tyr. Kin. Réc. CSF-1	Tran. Mbr.	2) Sherr et al., 1985
ros	UR2	Sarcome	Poulet	P68 $^{gag-ros}$	Tyr. Kin.	Membranes				
src	RSV S1 S2	Sarcome	Poulet	pp60 $^{v-src}$ (3) pp62 $^{s-src}$	Tyr. Kin.	Face. int. du Plasmaleme	pp60	Tyr. Kin.	Mbr. et Cytosquel.	3) Ikawa et al., 1986
fps/fes	Fu-ASV	Sarcome	Poulet	P130 $^{gag-fps}$	Tyr. Kin.	Membrane plasmique	p98	Tyr. Kin.		
	ST-FeSV	Sarcome	Chat	P85 $^{gag-fes}$	Tyr. Kin.	Membrane plasmique	p92			
yes	Y73-ASV	Sarcome	Poulet	P90 $^{gag-yes}$	Tyr. Kin.	Membrane plasmique	(4) p59-62	Tyr. Kin.		4) Sudol et Hanafusa 1986
fgr	GR-FeSV	Sarcome	Chat	P70 $^{gag-actin-fgr}$	Tyr. Kin.					
abl	Ab-MLV	Leuc. préB	Souris	P160 $^{gag-abl}$	Tyr. Kin.	Membrane plasmique	p150		(5) Cytoplasme	5) Hunter et Cooper, 1985
	HZ2-FeSV	Sarcome	Chat	P98 $^{gag-abl}$	Tyr. Kin.	Membrane plasmique				
kit	HZ4-FeSV	Sarcome	Chat	P80 $^{gag-kit}$	(6) Tyr. Kin.					6) Besmer et al., 1986
sea	S13	Sarcome Erythro.	Poulet	gP155 $^{env-sea}$ → gP85+gP70	Tyr. Kin. (gP70) (7)	(7) Cytoplasme				7) Hayman et al., 1985
——	TP_1-FeSV	Sarcome	Chat	gP 83 $^{gag-onc}$	Tyr. Kin.					

TABLE I: Oncogènes viraux et leurs homologues cellulaires
(RNA Tumor Viruses, 1985).

onc	virus	maladie	hôte	protéine v-onc	activité	localis.	prot. c-onc	activité	localis.	Références
Ha-ras	Ha-MSV	Sarcome Erythro.	Rat	p21 $^{v\text{-}Ha\text{-}ras}$	Thr. Kin. fixe GTP GTPase	Mbr. face int.	Ha-ras p21	Thr. Kin. fixe GTP GTPase	Mbr. face int.	
	BalB-MSV	Hémangio-sarcome	Souris							
Ki-ras	Ki-MSV	Sarcome Erythro.	Rat	p21 $^{v\text{-}Ki\text{-}ras}$			Ki-ras p21			
mos	Mo-MSV	Sarcome	Souris	P37 $^{env\text{-}mos}$	Thr/Ser. Kin.	Cytoplasme				
mil /raf	MH2	Carcinome	Poulet	P100 $^{gag\text{-}mil}$	Thr/Ser. Kin.	Cytoplasme	p71 (8)			8)Patshinsky et al., 1986
	MSV-3611	Sarcome	Souris	gP90 $^{gag\text{-}raf}$ P75 $^{gag\text{-}raf}$	Thr/Ser. Kin.					
fos	FBj-MSV	Ostéo-sarcome	Souris	p55 $^{v\text{-}fos}$		Noyau	p55 (9)		Noyau	9)Curran et al., 1984
myc	MC29	Myélocyto. Carcinome	Poulet	P110 $^{gag\text{-}myc}$	Affinité pour l'ADN	Noyau	p58	Affinité pour l'ADN	Noyau	
	MH2	Carcinome	Poulet	p61/63 $^{v\text{-}myc}$						
myb	AMV	Myélobl.	Poulet	p45 $^{v\text{-}myb}$	Affinité pour l'ADN	Noyau	p75 (10)	Affinité pour l'ADN	Noyau	10)Klempnauer et al., 1984
	E26	Myélobl. Erythro.	Poulet	P135 $^{gag\text{-}myb\text{-}ets}$						
ski	SKV-ASV	Carcinome	Poulet	P110 $^{gag\text{-}ski\text{-}pol}$		Noyau				
ets	E26	Erythro.	Poulet	P135 $^{gag\text{-}myb\text{-}ets}$		Noyau	p54 (11)		Cytoplasme	11) Ghysdael et al., 1986
erbA	AEV-ES4	Sarcome Erythro.	Poulet	P75 $^{gag\text{-}erbA}$		Noyau (12)	p40/46	Réc. Hormones Thyr.		12) Sap et al., 1986
rel	REV-T	Réticulo-endothél.	Dinde	P59 $^{env\text{-}rel}$		Noyau (13) Cytoplasme				13)Gilmore et Temin, 1986

TABLE I (suite)

rétropolymérase "sauterait" ensuite de la matrice virale sur l'ARN cellulaire, puis repasserait sur la matrice virale pour créer une molécule d'ADN hybride capable de s'intégrer (Figure 7).

La recombinaison entre un rétrovirus et un oncogène ne se fait pas tout à fait n'importe où dans le génome rétroviral. Certaines séquences tel le gène gag, semblent privilégiées dans ces

événements de recombinaison. Le mécanisme précis de ce processus n'est pas connu, mais la séquence GAGG semble fréquemment impliquée au point de recombinaison (Besmer et al., 1986). Or cette séquence a déjà été retrouvée lors de recombinaisons chromosomiques dans le génome cellulaire (Piccoli et al., 1984). GAGG pourrait représenter un signal reconnu par des enzymes cellulaires chargés des remaniements de l'ADN.

C-LES ONCOGENES

Le terme d'oncogène sera utilisé ici au sens large. Cette dénomination sous-entend en fait que les produits de ces gènes sont impliqués dans la transformation cellulaire. Cependant, la preuve directe de l'activité oncogène n'existe pas pour tous les gènes qui sont pourtant regroupés sous ce vocable. Parfois, ce terme sera appliqué à des séquences nucléotidiques dont on sait seulement qu'elles sont fréquemment remaniées dans certaines tumeurs. Il faut noter que l'application de cette règle amènerait à considérer comme oncogènes les gènes d'immunoglobulines transloqués dans les tumeurs lymphoïdes. Accessoirement, les gènes d'immunoglobulines peuvent jouer un rôle indirect essentiel dans ces types de transformation, en permettant peut être translocation et expression de certains c-onc.

Le premier oncogène a été isolé à partir d'un virus aviaire sarcomatogène, le RSV-PR-C, en 1976, (Stéhelin et al., 1976b). Depuis, environ 45 oncogènes distincts ont pu être caractérisés notamment par l'intermédiaire de rétrovirus (Bishop, 1985). Ces oncogènes viraux (v-onc) dérivent de gènes cellulaires (c-onc) (Table I) qui se trouvent plus ou moins modifiés après leur capture et leur propagation par le virus. L'interférence du produit d'un v-onc avec le déroulement de la mitose et/ou de la différenciation cellulaire conduisant à la transformation, suppose une action (directe ou indirecte) du produit de ces gènes dans l'exécution de ces programmes. L'étude du mode d'action de ces produits dans la cellule nécessite tout d'abord de définir les critères de la transformation cellulaire *in vitro*.

I-LES CRITERES DE LA TRANSFORMATION CELLULAIRE

a-Transformation des cellules non hématopoïétiques

In vivo, la détection du pouvoir transformant d'un virus est simple : l'inoculation de quelques particules de virus sarcomatogène (le RSV-PR-C par exemple), dans un animal susceptible, provoque en une semaine une tumeur au point d'inoculation (Rous, 1911). *In vitro*, les fibroblastes aviaires infectés par ce même virus vont présenter, après quelques jours de culture, une série de modifications évidentes. Les cellules deviennent rondes et réfringentes, traduisant ainsi les

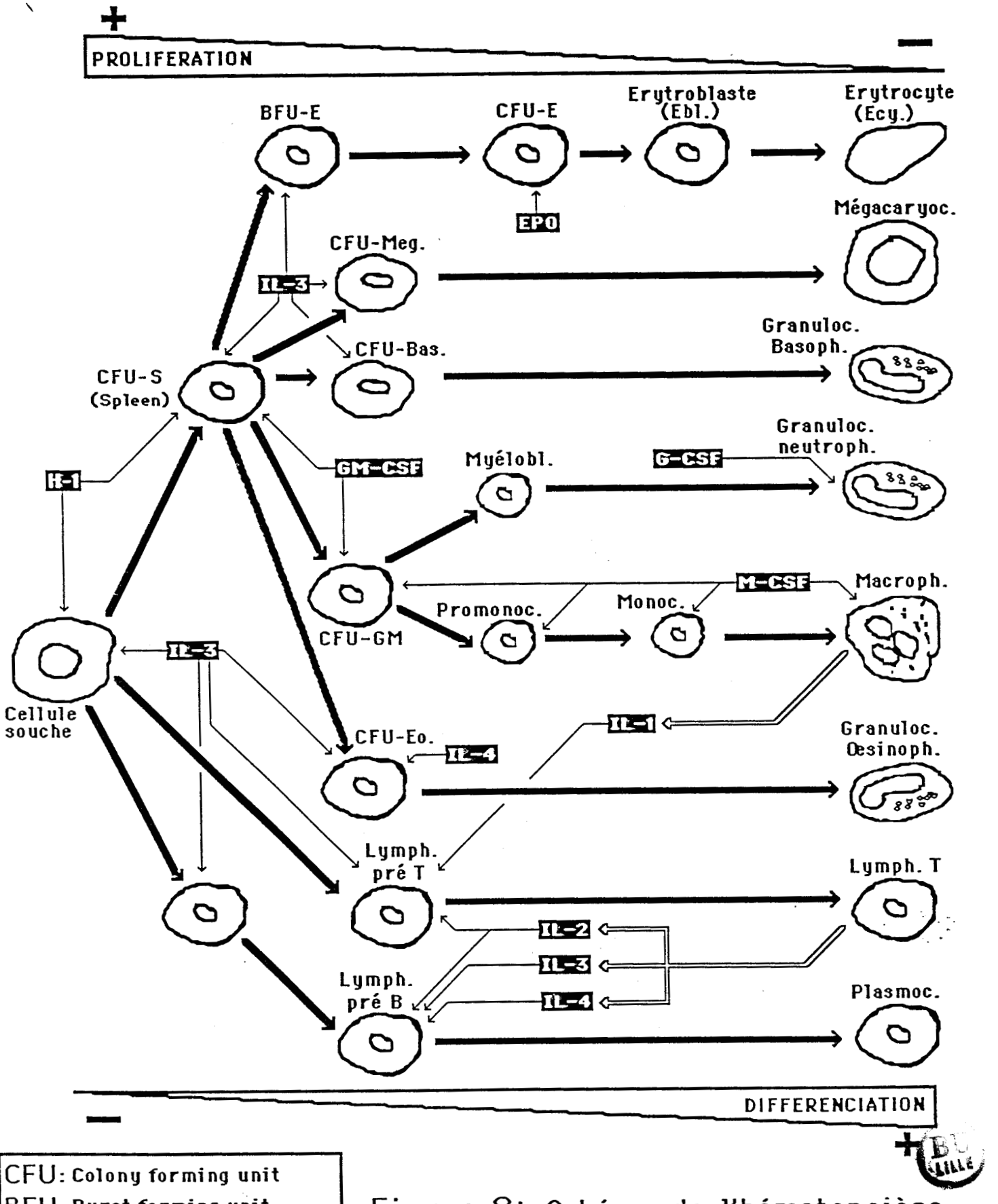
changements que subit le cytosquelette (Edelman et Yahara, 1976). De nouvelles caractéristiques de croissance apparaissent. Sur substrat solide, les fibroblastes transformés ont besoin de concentrations de sérum moins élevées que les cellules saines pour se multiplier, perdent l'inhibition de contact caractérisant les cellules normales (Dulbecco, 1970) et peuvent également donner naissance à des colonies en milieu semi-solide (agarose ou méthylcellulose (Stoker et al., 1968)). Des modifications de la membrane plasmique interviennent : des néo-antigènes de surface surgissent (Kurth et Bauer, 1975) associés à des modifications des glycoconjugués, alors que d'autres protéines disparaissent, tel la LETS protéine (fibronectine) (Olden et Yamada, 1977). Des modifications du métabolisme apparaissent : le transport des sucres est considérablement augmenté dans les cellules transformées (Hatanaka, 1974), des protéases sont excrétées, tel l'activateur du plasminogène (Reich, 1976). Enfin, au niveau génique, on estime qu'un millier de transcrits nouveaux sont induits dans les cellules transformées par le RSV (Groudine et Weintraub, 1980).

La plupart de ces observations sont valables quelque soit l'agent transformant (Kahn et Shin, 1979) mais apparemment, seul la capacité à former des colonies en milieux semi-solides semble être liée (mais pas à 100%) avec la tumorigénicité de ces cellules dans un animal syngénique (ou à défaut dans les souris nude, qui ne présentent que très peu de phénomènes de rejets) (Kahn et Shin, 1979). Posséder un marqueur fiable de la tumorigénicité est utile, car le degré de "transformation" conféré *in vitro* aux cellules par un même agent peut varier considérablement d'un système biologique à l'autre, et il existe certains virus oncogènes (ou certains mutants) ainsi que de nombreuses substances chimiques qui peuvent ne conférer aux cellules qu'un phénotype transformé partiel (Kahn et al., 1982).

La part revenant à chacune des modifications décrites plus haut dans l'acquisition de la capacité tumorigène est variable d'un système cellulaire à l'autre. C'est le produit de l'oncogène qui induit le phénotype transformé, comme l'ont bien montré les mutants de virus oncogènes ainsi que les réversions phénotypiques obtenues après disparition ou suppression transitoire du produit du gène oncogène impliqué (Quade et al., 1981; Drebin et al., 1985). Le mode d'action du produit de ces gènes est différent selon le type d'oncogène, mais il se dégage actuellement quelques grands groupes fonctionnels incluant la plupart des oncogènes connus. Toutefois, si des mécanismes initiateurs de la transformation sont établis, on ne comprend pas encore comment toute la cascade des événements aboutissant au phénotype "transformé" se met en place.

b-Transformation des cellules hématopoïétiques

La séparation qui est faite ici, entre les cellules sanguines et les autres, vient de ce que les problèmes de différenciation vont dominer ceux de la transformation des cellules hématopoïétiques. Ces cellules, qui se multiplient en suspension et poussent de ce fait naturellement dans un milieu semi-solide, privent l'expérimentateur d'un moyen simple de caractériser *in vitro* leur transformation. Les cellules hématopoïétiques transformées proviennent souvent de la prolifération d'une cellule immature morphologiquement indistinguable de son équivalent normal. Les cellules hématopoïétiques dérivent d'une cellule souche indifférenciée (Figure 8), douée d'un grand pouvoir de multiplication afin de pouvoir répondre aux exigences de l'organisme. Cette cellule souche va se



CFU: Colony forming unit
BFU: Burst forming unit
CSF: Colony stimulating factor

Figure 8: Schéma de l'hématopoièse

Facteur de croissance	Poids Moléculaire	Cellules cibles	Poids Moléculaire du récepteur	Références
IL-3 (Interleukine 3)	23-26 kd	Cellule souche CFU-S BFU-E CFU-GM CFU-Eo CFU-Meg Pré. des Granulocytes Pré. des Macrophages	50-70 kd	Metcalf, 1985
IL-4 (Interleukine 4)	20 kd	Lymphocytes B et T Mastocytes		Lee et al., 1986
GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor)	23 kd	CFU-GM CFU-Eo CFU-Meg		Walker et Burgers, 1985
G-CSF (Granulocyte-Colony Stimulating Factor)	25 kd	Sous-popul. des CFU-GM Pré. des Granulocytes	150 kd	Nicola et al., 1985
M-CSF (Macrophage-Colony Stimulating Factor)	76 kd	Sous-popul. des CFU-GM Pré. des Macrophages	150 kd	Kawasaki et al., 1985
EPO (Erythropoïétine)	20 kd	CFU-E		Krantz et Goldwasser, 1984
H-1 (Hémopoïétine-1) (Origine humaine)	39 kd	Cellule souche CFU-S (en synergie avec IL-3 et M-CSF)		Stanley et al., 1986

TABLE II : Facteurs de croissance des cellules hématopoïétiques (espèce murine).

différencier, perdre graduellement son pouvoir prolifératif et sa capacité à donner plusieurs types cellulaires distincts (Clarkson et al.,1978). Cette différenciation ne s'effectue pas au hasard, mais en réponse à des inducteurs précis émis par différents types de cellules (Cline et Golde, 1979; Whetton et Dexter, 1986). Toutes les substances intervenant dans le contrôle de l'hématopoïèse ne sont pas connues, mais un certain nombre d'entre elles ont pu être caractérisées (Table II).

La différenciation de la cellule souche produit essentiellement trois classes de cellules : des cellules multipotentes, capables de donner naissance aux différentes lignées sanguines, des cellules engagées dans une voie de différenciation définie, mais encore immatures, et enfin des cellules matures spécialisées qui ne se multiplient plus. La prolifération, l'engagement dans une lignée particulière et la différenciation cellulaire sont sous le contrôle d'une famille d'hormones, des glycoprotéines, connues sous le nom de "colony stimulating Factors" (CSF) (Table II). Chaque CSF est défini par sa capacité à stimuler la croissance de colonies de cellules d'une lignée précise, reconnaissable par sa descendance différenciée dans un milieu de croissance semi-solide (Metcalf, 1977; Cline et Golde, 1979). La complexité de cette régulation est accrue par le rôle que joue le micro-environnement, les cellules du stroma produisant également leurs substances régulatrices (Cline et Golde, 1979). Parmi les différents CSF, on distingue le M-CSF (ou CSF-1) qui stimule spécifiquement la croissance des macrophages et de leurs précurseurs directs, le G-CSF qui stimule la prolifération des précurseurs de granulocytes, le GM-CSF (ou CSF-2), stimulant la croissance des cellules à l'origine des macrophages et granulocytes et enfin le Multi-CSF (ou interleukine 3 (IL3), produit par des lymphocytes T activés) qui permet la formation de colonies de précurseurs érythroïde, éosinophile, mégacaryocytaire et mastocytaire ainsi que la stimulation de la cellule souche elle-même (Metcalf, 1985; Whetton et al., 1986; Figure 8). Ces CSF sont nécessaires pour la survie, la multiplication et la différenciation des cellules hématopoïétiques, montrant par là leur effet pléïotropique. Les CSF pourraient provoquer la différenciation de leurs cellules cibles en induisant l'expression de protéines (MGI-2, macrophage granulocyte inducing proteins 2 pour la lignée myéloïde, peut être similaires au facteur DIF produit par des lymphocytes activés, Leung et Chiao, 1985) qui permettraient le bon déroulement de la différenciation. Il semblerait exister une anomalie de couplage entre l'action des CSF et l'expression des protéines MGI-2 dans les cellules leucémiques (Loten et Sach, 1982; Symonds et Sach, 1982).

L'intervention des oncogènes dans la transformation des cellules hématopoïétiques ne fait aucun doute, puisque la plupart des rétrovirus peuvent être leucémogènes (Figure 21) (Clarkson et al.,1978). Il existe une spécificité entre le type de cellule transformée et le type d'oncogène actif (Graf et al., 1980), bien que plusieurs oncogènes différents puissent interférer avec des cellules de la même lignée sanguine (c'est par exemple le cas de la lignée érythroïde qui peut être perturbée par les oncogènes myb-ets, erb, ras, abl, et fps (Graf et al., 1986). Quand leur différenciation peut s'effectuer, les cellules hématopoïétiques transformées perdent leur caractéristiques tumorales et participent au fonctionnement de l'organisme (Gootwine et al., 1982) imitant ainsi d'autres types cellulaires (cellules de tératocarcinomes) où une perturbation de la différenciation est responsable du phénotype transformé (Mintz et Illemensee, 1975). De nombreuses études montrent que les cellules hématopoïétiques infectées par des rétrovirus sans oncogènes se transforment de façon

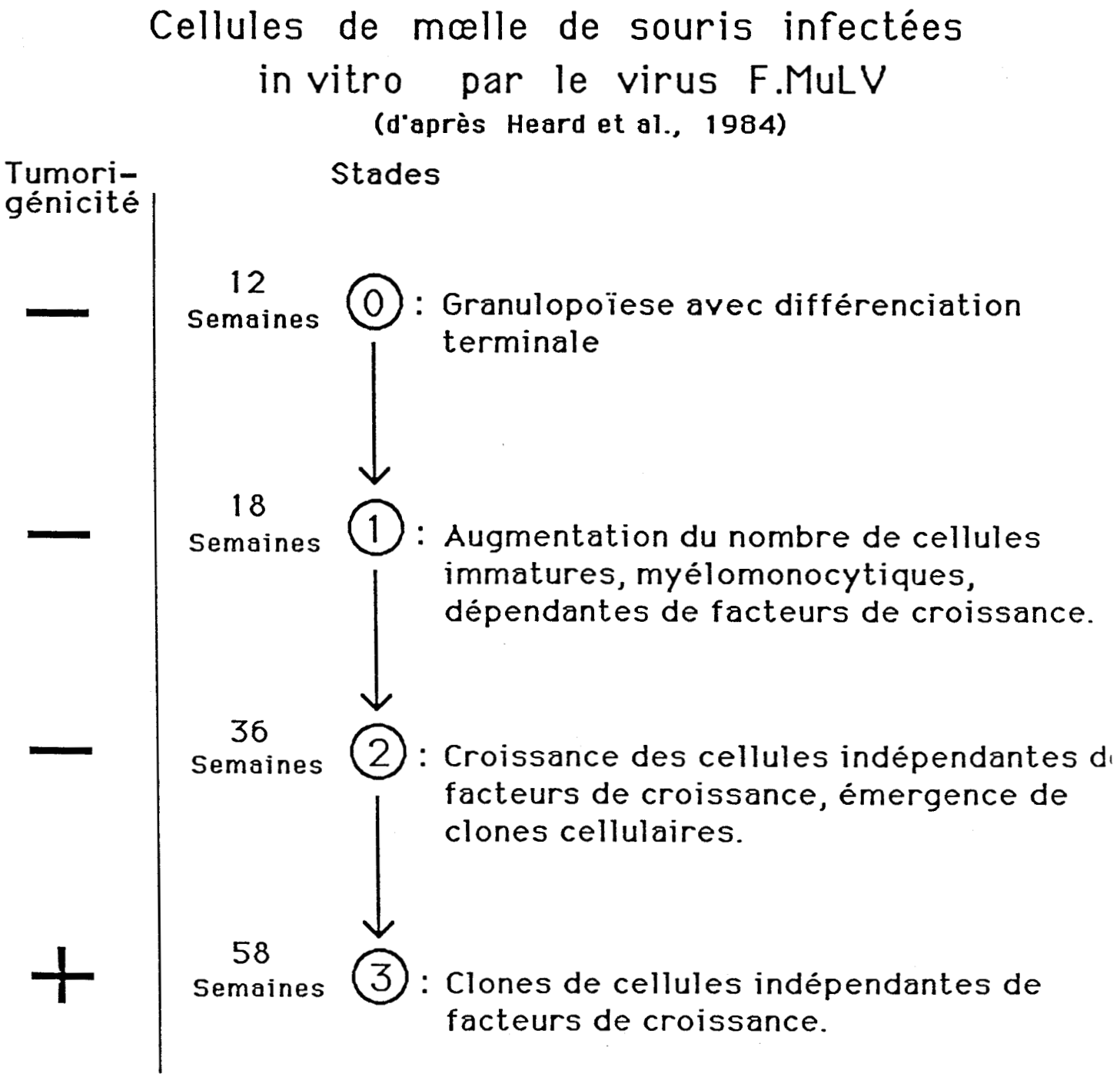


Figure 9 : Modèle de transformation progressive par un rétrovirus.

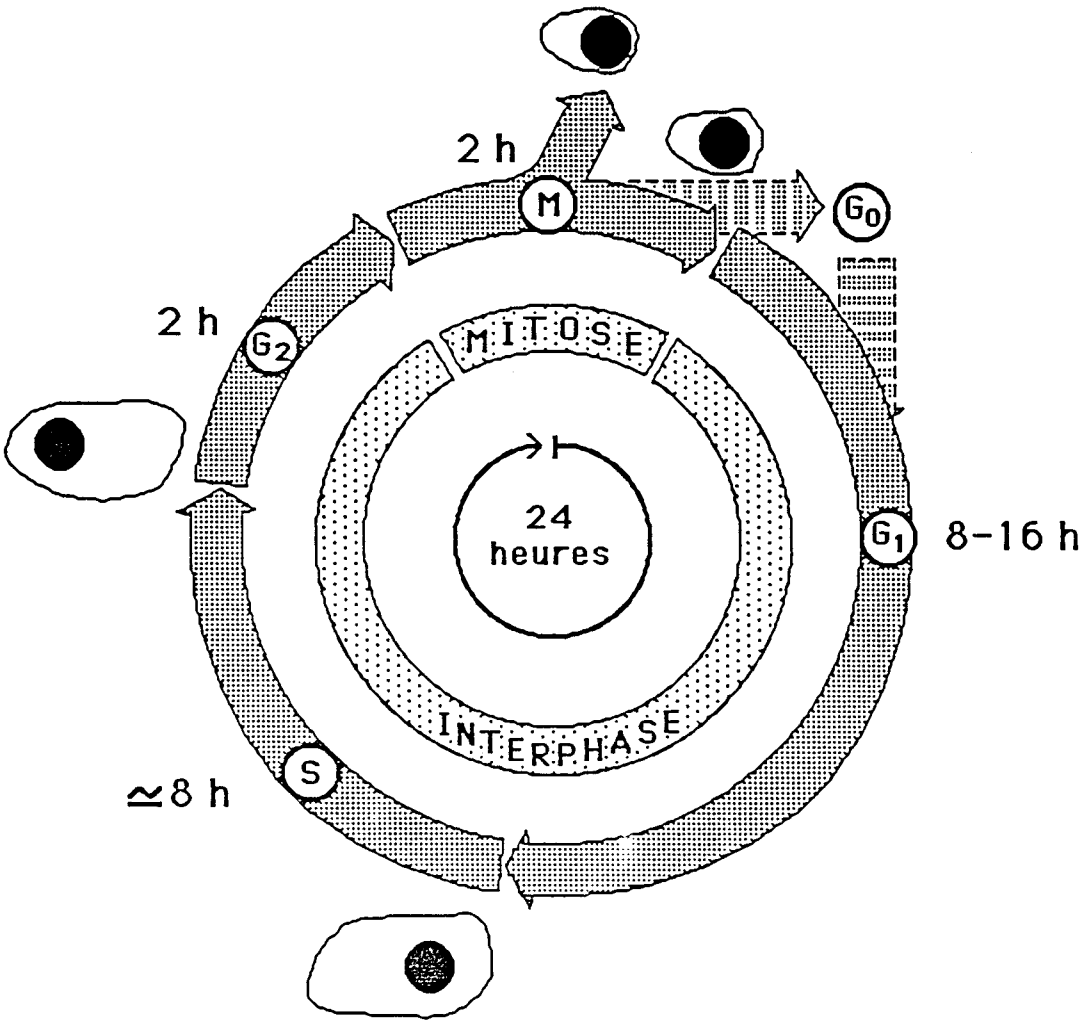


Figure 10 : Le cycle cellulaire.

(La phase G_0 est facultative.)

progressive : elles deviennent, par paliers, insensibles aux différents CSF, puis franchement tumorigènes (Figure 9; Tambourin et al., 1981; Heard et al., 1984; O'Donnell et al., 1985) suggérant ainsi l'implication de plusieurs gènes cellulaires dans ce processus.

II-MODE D'ACTION DES PRODUITS D'ONCOGENES

On doit distinguer ici le mode d'action (largement inconnu) des produits des c-onc du mode d'action des produits des v-onc. Les rôles physiologiques que remplissent les c-onc sont indirectement suggérés par le profil d'expression de ces gènes selon les tissus, ou selon les stades de différenciation cellulaire pendant lesquels ils sont transcrits, ainsi qu'à partir de la fonction attribuée aux v-onc correspondants. Ainsi, de très nombreuses études ont permis de montrer que la transcription des c-onc n'était pas uniforme selon les tissus, selon les stades de différenciation et selon l'oncogène considéré, suggérant un rôle spécifique pour nombre d'entre eux (Gonda et al., 1982; Muller et al., 1982; Shibuya et al., 1982; Coll et al., 1983a; Muller, 1983; Kelly et al., 1983; Sheiness et Gardinier, 1984; Mitchell et al., 1985; Pfeifer-Ohlsson et al., 1985). Toutefois, d'après le type même de la "maladie cancéreuse", il parait évident que les oncogènes viraux et cellulaires interviennent au moins dans le contrôle de la différenciation et dans celui de la mitose.

a-Les oncogènes et la mitose

L'étude de la division cellulaire permet de découper la mitose en quatre étapes (G1, S, G2, M) (Figure 10) qui doivent être étroitement régulées. Un certain nombre de polypeptides facteurs de croissance sont connus pour induire les cellules à se diviser (Goustin et al., 1986). Ces polypeptides extra-cellulaires doivent signaler à la cellule que l'heure de la mitose est arrivée; pour cela, une interaction avec des protéines membranaires est indispensable. Une fois fixé sur son récepteur membranaire, le facteur de croissance délivre un signal à la cellule par l'intermédiaire de modifications touchant la structure du récepteur. Ce signal est probablement véhiculé jusqu'au cytoplasme par des protéines membranaires. Ensuite, il doit être relayé jusqu'au noyau, par l'intermédiaire des seconds messagers chargés d'acheminer tous les messages de l'environement extra-cellulaire. Dans le noyau, s'activeront les gènes effecteurs responsables de la progression cellulaire à travers toutes les autres phases du cycle (Figure 10).

Le produit des oncogènes peut agir à tous les points de la propagation du signal:

-en étant le facteur de croissance;

-en étant le récepteur (ou en mimant son action);

-en transmettant le signal mitogène du récepteur activé;

-en contrôlant la production des seconds messagers intra-cytoplasmiques;

-en agissant directement dans le noyau.

1) Les facteurs de croissance et leurs récepteurs

Les facteurs de croissance diffèrent des hormones dans la mesure où ils agissent pour la plupart sur leur lieu de production. Ils peuvent soit stimuler la croissance de la cellule qui les

produit (stimulation autocrine) soit stimuler la croissance d'une cellule ne les produisant pas (stimulation paracrine). Certaines cellules réagissent à un seul facteur de croissance, d'autres types cellulaires demanderont plusieurs facteurs de croissance administrés selon un ordre précis, pour que la synthèse d'ADN puisse se faire (Pledger et al., 1978; Stiles et al., 1979; Leof et al., 1982). Cette cascade d'événements à été particulièrement bien étudiée dans une lignée de fibroblastes de souris (BALB-c 3T3). Ces cellules rendues quiescentes (phase Go) par la privation du milieu de culture en sérum, peuvent être amenées à se multiplier si elles sont exposées à du PDGF (platelet derived growth factor, facteur de croissance produit en grande quantité dans les plaquettes), mais cette induction (encore appelée compétence) ne dépassera le stade G1 situé entre la fin de la mitose précédente et le début de la synthèse d'ADN (d'une durée de 12 heures, Leof et al, 1982) que si d'autres facteurs de croissance sont fournis à la cellule (EGF: epidermal growth factor, somatomédine C ou insuline). Dans ce cas la synthèse d'ADN peut avoir lieu (phase S) et la cellule progresse à travers la phase G2 jusqu'à la mitose (M) (Pledger et al., 1978; Leof et al., 1982). La cellule exposée au PDGF est compétente, et pendant les 12h de la phase G1 doivent s'exprimer les gènes qui lui permettront d'accéder à la phase S (Smith et Stiles, 1981; Cochran et al., 1983).

Or la plupart des cellules transformées sont devenues autonomes vis à vis des facteurs extérieurs. Deux mécanismes de cette autonomie sont maintenant bien connus :

-les cellules transformées relachent dans leur surnageant de culture différents types de facteurs de croissance auxquels elles répondent en se divisant de façon continue (Sporn et Roberts, 1985; Ymer et al., 1985);

-les oncogènes peuvent coder directement pour les facteurs de croissance ou leurs récepteurs.

a)Les cellules transformées par l'oncogène v-mos ou v-Ki-ras produisent des facteurs de type PDGF (Bowen-Pope et al., 1984). L'oncogène v-src pour sa part est capable de rendre des cellules aviaires de la lignée myéloïde indépendantes de leur facteur de croissance, le cMGF (myelomonocytic growth factor, Adkins et al., 1984). Ce même oncogène, introduit dans un virus murin, perturbe durablement la différenciation de la cellule souche hématopoïétique de souris, peut-être par un mécanisme de même nature (Boettiger et al., 1984). L'action des oncogènes sur la mitose s'exerce également en contrôlant la production de peptides qui sont depuis longtemps associés à la transformation , les TGF (tumor growth factor). Le TGFα, un peptide apparenté à l'EGF et utilisant le même récepteur, est produit par les cellules de rongeurs transformées par v-mos ou v-Ki-ras (DeLarco et Todaro, 1978; Kaplan et al., 1982). Le TGFβ est paradoxalement doué de propriétés inhibitrices de la multiplication cellulaire (Sporn et al., 1986, voir les contrôles de l'oncogénèse) mais son mode d'action n'est pas univoque, et dépend du type cellulaire : dans certaines conditions (associé au TGFα) il peut permettre la croissance en milieu semi-solide de cellules normales (Assoian et al., 1984). Il semble que dans ce cas le TGFβ agisse spécifiquement sur la synthèse des récepteurs de l'EGF sur lesquels se fixe le TGFα (Assoian et al., 1984). Le rôle physiologique du TGFß produit par les plaquettes sanguines pourrait être d'induire la différenciation des cellules épithéliales (Masui et al.,1986). Le contrôle qu'exercent les produits des

oncogènes sur la production des différents TGF est indirect, et probablement situé au niveau transcriptionnel ou traductionnel (Sporn et Roberts, 1985).

b)L'oncogène d'un virus de sarcome simien, v-sis, présente une très grande analogie avec la chaîne β du PDGF (Waterfield et al., 1983) facteur actif sur de nombreux types cellulaires sous forme d'un dimère αβ. Les gènes codant pour chaque sous-unité, qui possèdent entre elles 40% d'homologie, sont localisés sur des chromosomes différents: α sur le chromosome 7 (bande q22) chez l'homme (Betsholtz et al.,1986), β sur le chromosome 22 (Table VI) . Chacun des homodimères est également actif sur les cellules.

Les oncogènes codant pour des récepteurs sont plus nombreux que ceux codants pour les facteurs de croissance; il existe une certaine homologie entre le récepteur du PDGF et l'oncogène v-kit isolé à partir d'un virus sarcomatogène félin (Table I; Yarden et al., 1986). L'oncogène v-erbB, isolé d'un virus d'érythroblastose aviaire (AEV), est très semblable à la portion transmembranaire et cytoplasmique du récepteur de l'EGF (Downward et al., 1984). Il existe une grande analogie entre c-fms, représentant l'équivalent cellulaire de l'oncogène trouvé dans l'isolat McDonough d'un virus de sarcome félin (v-fms), et le récepteur du CSF-1 (ou M-CSF), indispensable pour le développement des colonies de macrophages sanguins (Sherr et al., 1985). Un grand nombre d'oncogènes est rattaché au groupe des récepteurs de facteurs de croissance, à cause de l'activité tyrosine kinase présentée par leur produit (Table I). Or cette activité, rare dans la cellule, est elle-même caractéristique de nombreux récepteurs de facteurs de croissance, et il est possible qu'elle soit essentielle pour la transmission du signal mitogène par le récepteur membranaire activé. Certaines protéines phosphorylées *de novo* sur la tyrosine se retrouvent également présentes après l'action du RSV ou de l'EGF sur les cellules (Cooper et al., 1982).

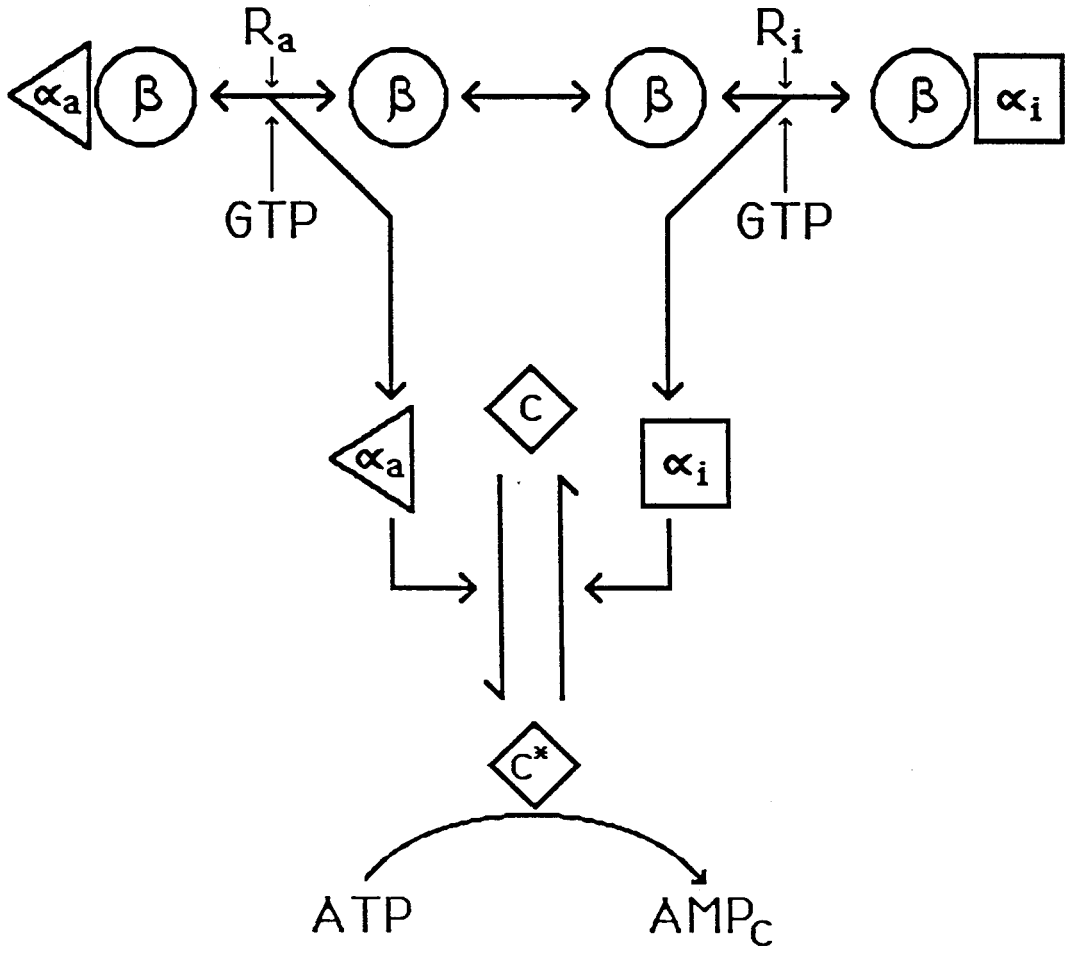
2) La transmission du signal mitotique

La transmission du signal mitotique va mettre en jeu des protéines membranaires (phospholipases, protéines kinases..) ainsi que les deuxièmes messagers cytoplasmiques, tels des ions (Na^+, K^+, H^+, Ca^{++}..) et les phospholipides de l'inositol.

Nous avons vu que certains oncogènes peuvent coder pour les facteurs de croissance ou leurs récepteurs, mais une troisième classe d'oncogènes pourrait coder pour les protéines de relais transmembranaires du signal mitotique (Sporn et Roberts, 1985). Il se trouve en effet qu'une enzyme membranaire, douée de la capacité de fixer le GTP (Cockcroft et Gomperts, 1985), pourrait contrôler l'activité de la phospholipase-C . Cet enzyme possède un rôle clé dans la régulation de la réponse cellulaire à des stimuli externes impliquant une liaison ligand-récepteur. La phospholipase-C module la production de diacyl-glycérol et d'inositol triphosphate qui sont parmi les deuxièmes messagers cellulaires. Ces produits vont à leur tour activer (entre autre) la protéine kinase-C ce qui induira finalement l'expression des gènes effecteurs. Trois enzymes membranaires au moins sont donc impliqués dans la transmission du signal.

-Régulation de la phospholipase-C :

Le produit de l'oncogène qui pourrait agir à ce niveau de propagation du signal doit :



R: Récepteur; R_a: activateur; R_i: inhibiteur

Sous-unité $G_{s\alpha}$ activée

Sous-unité $G_{s\alpha}$ inhibée

Sous-unité $G_{s\beta}$

Protéine cible, adénylate-cyclase

Figure 11 : Régulation de l'adénylate-cyclase par les protéines G.

-être membranaire;

-fixer le GTP;

-inter-agir avec des récepteurs de facteurs de croissance.

L'oncogène candidat à ce poste de contrôle, Ha-ras, a été trouvé au sein d'un virus murin sarcomatogène (Harvey Sarcoma Virus). Il code pour une protéine de 21 kd, fixant du GTP et localisée sur la face interne de la membrane plasmique (Furth et al., 1982). Le produit du gène c-ras est doué d'une activité GTPasique qui est fortement réduite dans sa forme activée par des mutations ponctuelles (voir le chapitre c-onc et tumeurs, p.30; Sweet et al., 1984), et l'ancrage de la protéine dans la membrane est requis pour son activité transformante (Willumsen et al., 1984). Cette propriété GTPasique du produit normal, jointe à une localisation membranaire de la $p21^{ras}$ suggère que l'oncogène ras pourrait être la protéine contrôlant la phospholipase-C et intervenir par ce biais dans la propagation du message mitogène.

L'action de la $p21^{ras}$ pourrait s'exercer selon le schéma proposé dans le contrôle de l'adénylate-cyclase (Gilman, 1984; Figure 11). L'adénylate-cyclase qui régule la production de l'AMPc est contrôlée par deux enzymes membranaires, les protéines Gi (inhibitrice) et Gs (stimulatrice). Ces deux protéines sont en contact avec des récepteurs de substances activant ou déprimant l'activité de l'adénylate-cyclase (tels des hormones, des neurotransmetteurs, etc..). Gi et Gs fixent du GTP dans leur sous-unité α ce qui permet la dissociation du complexe $\alpha\beta$ constitutif de ces enzymes, et l'interaction de la sous-unité α activée avec l'adénylate-cyclase. La sous-unité α est par ailleurs douée d'une activité GTPasique ce qui permet après hydrolyse du GTP, un retour de l'enzyme aux conditions initiales. La famille des protéines G fixatrices du GTP comprend, en plus de Gi et Gs une autre protéine, la transducine, qui active par l'intermédiaire d'un photorécepteur membranaire (la rhodopsine) la GMPc phosphodiestérase rétinienne (Gilman, 1984). Or, une certaine homologie existe entre la séquence nucléotidique de la transducine et celle de l'oncogène ras (Locherie et al., 1985). L'analogie entre ras et protéine G est encore renforcée par le fait que dans la levure, le produit de ce gène activé (de façon similaire à l'activation du c-ras chez les mammifères) est capable de stimuler l'activité adénylate-cyclase de ces cellules (Toda et al., 1985). Toutefois, chez les mammifères, la p21 ne participe pas directement au contrôle de cet enzyme (Bekner et al., 1985).

D'autre part, le fait que l'EGF augmente l'affinité de la $p21^{ras}$ pour le GTP (Kamata et Feramisco, 1984) suggère que cette protéine est capable d'interagir avec des récepteurs de facteurs de croissance. Enfin, un rôle direct de la $p21^{c\text{-}ras}$ dans l'initiation de la phase S a pu être mis en évidence par l'injection d'anticorps monoclonaux anti $p21^{c\text{-}ras}$ dans des NIH_{3T3} (Mulcahy et al., 1985).

-Régulation de la protéine kinase-C :

La phospholipase-C (peut-être contrôlée par ras) est responsable de l'hydrolyse du phosphatidyl inositol (4,5) diphosphate (un composant des membranes plasmiques) en diacylglycérol et inositol triphosphate (Berridge et Irvine, 1984; Nishizuka, 1984). Le diacylglycérol permet l'activation de la protéine kinase-C (PK-C, Nishizuka, 1984).

La PK-C fait partie d'une famille de protéines kinases étroitement apparentées, dont les gènes

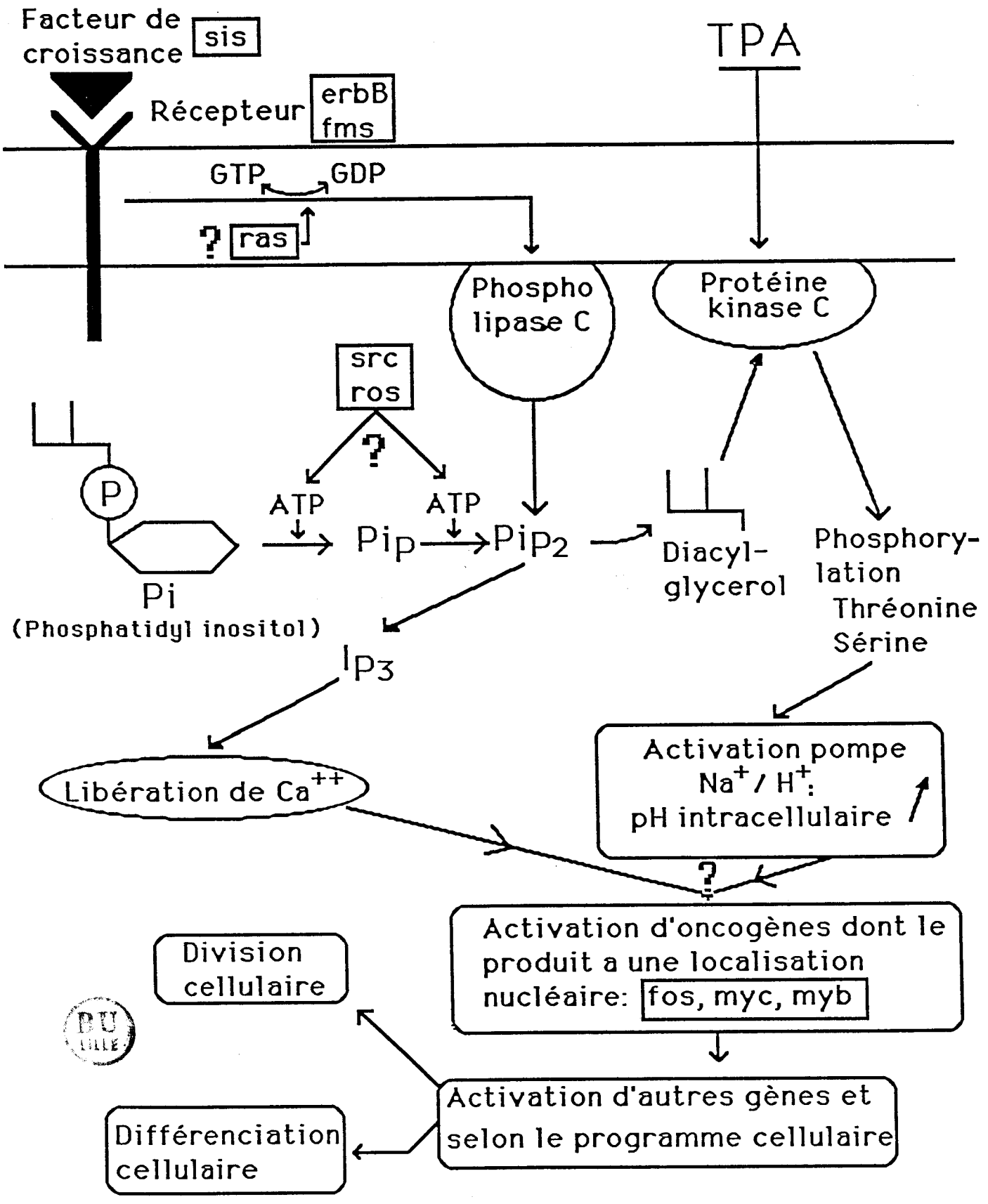


Figure 12 : La "cascade" des oncogènes.

sont localisés chez l'homme sur les chromosomes 17 (pour PK-Cα), 16 (pour la PK-Cβ) et 19 (pour la PK-Cδ) (Coussens et al., 1986a). Ces PK-C fixent le Ca^{++} et possèdent une activité sérine et thréonine-kinase. L'existence d'une famille de gènes codant pour ces enzymes reflète probablement la multiplicité des effecteurs extracellulaires auxquels ces enzymes auront à répondre.

PK-Cα est essentiellement cytoplasmique sous sa forme inactive et étroitement liée à la membrane plasmique sous sa forme activée (Nishizuka ,1984; Wolf et al., 1985). Elle semble avoir un rôle biologique important (dans l'activité métabolique des plaquettes sanguines par exemple) mais son action dans la prolifération cellulaire est suggérée par sa capacité à phosphoryler (et réguler l'activité) des récepteurs de facteurs de croissance (Fearn et King, 1985; Jetten et al., 1985). De plus, la PK-Cα est elle-même le récepteur de substances chimiques, connues sous le nom de "tumor promoters" tel le TPA (12-O-tétradécanoylphorbol-13-acétate).Le TPA contient dans sa molécule la structure du diacylglycérol activateur de PK-C (Castagna et al., 1982; Nishizuka, 1984).

Le TPA est capable de promouvoir la transformation morphologique de cellules "induites" (voir le chapitre oncogénèse multiétape et Weinstein et al., 1983). Son rôle très pléïotropique lui permettrait également d'agir sur la différenciation des cellules hématopoïétiques soit en la stimulant, soit en l'inhibant (Fibach et al., 1980). Certaines de ces activités seraient dues à l'action de PK-Cα activée par le TPA. Les mécanismes d'action précis des esters de phorbol (et de la PK-C) ne sont pas connus mais pourraient modifier le pH intra-cellulaire en agissant sur l'activité de l'échangeur membranaire Na^+/H^+ à l'aide de phosphorylations. Une rapide augmentation du pH intra-cellulaire, atteignant son maximum d'élevation 5 minutes après le contact de la cellule avec l'agent mitogène, est observée en réponse à une stimulation mitogène (Pouyssegur et al., 1985; Swann et Whitaker, 1985). La rapidité de cette alcalinisation pourrait en faire l'événement métabolique initial de la mitose (Rozengurt 1986). PK-Cα pourrait également phosphoryler et activer l'ATPase de la pompe à Ca^{++} (Nihizuka, 1986). Or, le Ca^{++} (activateur de PK-C) est également impliqué dans les mécanismes prolifératifs (Nishizuka, 1984; Villereal et al.,1985; Rozengurt,1986).

L'autre produit du clivage du phosphatidyl inositol (4,5) diphosphate par la phospholipase-C est l'inositol triphosphate qui permettrait la mobilisation du Ca^{++} intra-cellulaire essentiellement à partir du réticulum endoplasmique (Streb et al., 1983; Berridge et Irvine, 1984; Nishizuka, 1984).

De tels mecanismes contrôlant la prolifération cellulaire doivent être étroitement rétro-régulés . Ainsi, l'EGF active le métabolisme du phosphatidyl inositol, ce qui accroit l'activité de PK-C, qui en retour induirait une phosphorylation sur les acides aminés sérines et thréonines, notamment la thréonine 654 du récepteur de l'EGF. Cette phosphorylation induirait l'internalisation du récepteur et diminuerait son activité phospho-tyrosine kinase (Lin et al., 1986) permettant une rétroaction négative de l'EGF sur son récepteur. Le produit de <u>v-erbB</u> exprimant de façon constitutive son activité tyrosine kinase (Gilmore et al., 1985) échapperait à cette régulation.

-<u>Interaction entre le produit des v-onc et les deuxièmes messagers de la mitose</u> :

Les produits de certains oncogènes sont également capables d'interférer avec le métabolisme des phospholipides : le produit de <u>v-sis</u> (analogue du PDGFß) est connu pour stimuler le

métabolisme des lipides de l'inositol (Berridge et Irvine, 1984). Les protéines kinases codées par les oncogènes v-src et v-ros interagissent avec le métabolisme du phosphatidyl inositol (4,5) diphosphate (Macara et al., 1984; Sugimoto et al., 1984). Cette régulation pourrait être due à un contrôle des phospholipases par le produit de ces gènes. Une des cibles bien connues de la $pp60^{src}$, la p36 vient en effet d'être identifiée comme étant la lipocortine II calcium dépendante, qui inhibe l'activité de la phospholipase A2 (Brugge, 1986). Cet enzyme libère l'acide arachidonique à partir des phosphatidylcholine et d'autres phospholipides. L'acide arachidonique va notamment être métabolisé en prostaglandine de type E qui une fois fixée sur son récepteur stimulera l'activité adénylate-cyclase de la cellule (Rozengurt, 1986). Ce système contrôle l'activité de la PK-A AMPc dépendante, et cet enzyme (qui phosphoryle également sérine et thréonine) peut s'associer à l'activité de PK-C d'une façon positive ou négative selon le type cellulaire (Nishizuka, 1986).

Enfin, l'oncogène ras pourrait être impliqué, nous l'avons vu, dans la régulation de la phospholipase-C, et cette liste n'est probablement pas définitive.

3) La réaction nucléaire

Comment le signal mitogène parvient-il au noyau ? Le problème se pose sans doute différemment selon que le produit de l'oncogène inducteur de la transformation est nucléaire ou non (Table I). En effet, un certain nombre d'oncogènes code pour des protéines qui se localisent dans le noyau et pour certaines, se fixent à l'ADN. C'est le cas des produits oncogènes v- ou c-myc (Donner et al., 1982; Bunte et al., 1983); v- ou c-myb et du produit de fusion, v-myb-ets (Klempnauer et al., 1984; Moelling et al., 1985); c'est également vrai pour le produit de l'oncogène v-ski (qui présente la remarquable propriété d'avoir recombiné *in vitro* avec un ALV non transformant, Stavnezer et al., 1981); enfin, c'est aussi le cas des produits de v- ou c-fos (Curran et al., 1984). Le produit de l'oncogène myc peut activer en trans la région de promotion d'un gène de choc thermique de drosophile (hsp70) (Kingston et al., 1984). L'oncogène v-fos est capable d'agir en trans sur la transcription de certains gènes (Setoyama et al., 1986) et cela est également vrai pour le produit des gènes transformants de virus à ADN présentant une localisation nucléaire (Kingston et al., 1985). On peut supposer que c'est par cette capacité à accroître (ou induire) la transcription d'autres gènes que les produits d'oncogènes à localisation nucléaire agissent dans la mitose et dans la transformation cellulaire. C'est probablement dans cette catégorie qu'il faut ranger l'oncogène v-erbA. Il semble que cet oncogène code pour le récepteur des hormones thyroïdiennes T3 et T4 , bien qu'il ait perdu le site de fixation de l'hormone. La localisation de la $p75^{gag\ erbA}$ codée par le virus AEV est nucléaire (Sap et al.,1986). Les récepteurs d'hormones agissent de façon intra nucléaire en activant ou en réprimant l'expression de certains gènes (Yamamoto, 1985).

L'action du produit de certains oncogènes "non nucléaires" sur la structure même de l'ADN est suggérée par l'activité de type topoisomérasique (capable de relaxer, en présence d'ATP, l'ADN super-enroulé) présentée par la $pp60^{src}$ ainsi que par une autre protéine, présentant une activité tyrosine-kinase, tel le récepteur de l'EGF. D'après les travaux de Basu et al. (1985), il semblerait que la protéine oncogène interagisse plutôt avec la topoisomérase elle-même. Une telle régulation pourrait se révéler importante lors des mécanismes de transcription et de réplication.

L'action indirecte du produit de certains onc sur l'ADN est suggérée par l'accumulation des ARN messagers des oncogènes c-myc, c-fos et c-myb très rapidement (de l'ordre de quelques minutes) après un signal mitogène faisant passer la cellule du stade Go à G1 (Kelly et al., 1983; Greenberg et Ziff, 1984; Reed et al., 1985; Torelli et al., 1985). En fait, c-myc semble efficacement transcrit dans des fibroblastes pulmonaires de hamster chinois arrêtés au stade Go, mais les ARN messagers de ce gène sont extrêmement instables. La présence de facteurs de croissance dans le milieu de culture provoque leur stabilisation (Blanchard et al., 1985).

Des expériences de micro-injection de protéine c-myc dans le noyau de cellules 3T3 quiescentes ont permis de montrer que cette protéine remplaçait l'action du PDGF dans l'établissement de la compétence pour ces cellules (Kaczmarek et al., 1985). Cela suggère que le pic d'activation de cet oncogène suivant le contact du PDGF sur son récepteur (Kelly et al., 1983) est directement responsable de l'activation des gènes rendant ces cellules sensibles aux produits des autres facteurs de croissance permettant la synthèse d'ADN. Cependant, il a pu être montré à l'aide de mutants cellulaires que l'activation de c-myc et c-fos (suivant la fixation de l'EGF sur son récepteur) ne requérait pas la mise en route de la mitose pour s'effectuer (Bravo et al., 1985). Il s'agirait d'une réponse spécifique à l'activation du récepteur par son ligand suivant toute la cascade des événements biochimiques décrits dans la Figure 12; l'accumulation d'ARN c-myc en réponse à un signal mitogène étant notamment sous la dépendance de PK-Cα.

Cette activation en cascade de plusieurs oncogènes dont les produits agissent sur plusieurs voies métaboliques complémentaires dans le déroulement de la mitose, laisse la porte ouverte à la possibilité d'une action synergique de ceux-ci dans la transformation.

Ainsi, oncogènes cellulaires et viraux sont étroitement intriqués dans les voies métaboliques conduisant à la prolifération, mais le nombre des oncogènes impliqués dans la propagation du signal se resserre d'autant plus que le signal transformant initial est plus près du noyau. Par exemple, v-sis utilisera en tant qu'analogue du PDGF, toute la cascade des événements cellulaires qu'utilisent les facteurs de croissance pour délivrer leur signal. v-src, v-fms ou v-fes (v-onc dont les produits présentent des similitudes avec les récepteurs de facteurs de croissance) sont dépendants du produit de c-ras pour propager leur signal mitotique, alors que les produits de v-mos et v-raf supposés situés plus en amont de la cascade (du fait de leur activité sérine/thréonine-kinase pouvant peut être mimer celle de PK-C) ne le sont plus (Smith et al., 1986 et Figure 12).

b-Les oncogènes et la différenciation cellulaire

La mitose est étroitement liée à la différenciation cellulaire dans la mesure où les cellules qui se différencient arrêtent très souvent de se diviser (Le Douarin, 1979). De façon concomitante avec la réduction du potentiel mitotique, s'expriment à la surface de la cellule différenciée certains antigènes caractéristiques de son nouvel état (Feizi, 1985), ainsi que s'activent les gènes spécifiques codant pour les substances que cette cellule spécialisée aura à produire. Plusieurs systèmes cellulaires permettent d'étudier *in vitro* les mécanismes de la différenciation et l'interaction du produit des oncogènes avec celle-ci (Graf et Beug, 1978; Alema et al., 1985a; Falcone et al., 1985; Jaye et al., 1985; Mitchell et al., 1985; Muller et Wagner, 1985; Weissman et Aaronson, 1985).

L'introduction du produit d'un oncogène (viral ou cellulaire) dans une cellule immature peut soit empêcher sa différenciation ultérieure (Graf et Beug, 1978 ; Falcone et al., 1985 ; Fusco et al., 1985 ; Coppola et Cole, 1986) soit au contraire induire sa différenciation dans des conditions de culture où ce phénotype n'apparait pas (Muller et Wagner, 1984; Alema et al., 1985a). Cet effet n'est pas lié au produit d'un oncogène déterminé mais dépend du programme de différenciation de la cellule considérée.

Le produit de v-src peut inhiber la différenciation de myoblastes (Fiszman et Fuch, 1975; Falcone et al., 1985) ou de préadipocytes (Langer-Safer et al., 1985) mais peut par ailleurs induire la différenciation de cellules de phéochromocytome de rat PC12, d'origine nerveuse (Alema et al., 1985a). Contrairement à son rôle dans la plupart des cellules, le produit du gène src serait impliqué dans la différenciation plutôt que la prolifération des cellules nerveuses. Il faut noter que le produit de l'oncogène src est particulièrement exprimé dans les cellules nerveuses différenciées qui ne se divisent plus (Cottan et Brugge, 1983; Sorge et al., 1984). Les cellules de phéochromocytome PC12, qui sont capables de se différencier sous l'action du facteur de croissance NGF (Nervous growth factor) se différencient sans NGF, sous l'action de la p21 codée par des virus portant l'oncogène ras (Noda et al., 1985). La participation de la $p21^{c\text{-}ras}$ dans le processus normal de différenciation de ces cellules a également été rapportée par Hagag et al. (1986). Ces auteurs ont pu montrer que la micro-injection d'anticorps anti $p21^{c\text{-}ras}$ dans ces cellules bloque leur différenciation en réponse au NGF. D'autres oncogènes peuvent n'avoir soit aucun effet sur la différenciation, soit un effet modéré (Graf et al., 1980; Alema et al., 1985b).

Les cellules hématopoïétiques constituent un modèle privilégié pour étudier l'interférence entre les oncogènes et les phénomènes de différenciation. De nombreux virus oncogènes animaux ainsi que l'existence d'un certain nombre de marqueurs bien connus ont permis de montrer que le produit des v-onc influe sur la dépendance des cellules hématopoïétiques vis à vis des CSF et sur leur processus de différenciation. Trois situations peuvent se présenter :

1) la cellule infectée (dont la prolifération est stimulée) reste dépendante de CSF pour se différencier;

2) la cellule infectée est indépendante de CSF pour se différencier;

3) la cellule infectée est bloquée dans sa différenciation en présence ou en absence de CSF.

Par exemple, le produit de v-ras est capable de stimuler la croissance de précurseurs de cellules érythroïdes murines. Ces cellules, comme leur équivalent normal, sont dépendantes d'une glycoprotéine (l'érythropoïétine) pour se différencier (Figure 8). Par contre, cette hormone n'est plus nécessaire pour induire la différenciation des cellules transformées par le produit de v-abl. La surinfection par le virus Abelson des précurseurs érythroïdes transformés par le virus Ha-MSV (donc par v-ras) permet à la différenciation érythroïde de se produire en l'absence d'hormone. L'érythropoïétine est devenue superflue sous l'action du produit de v-abl (Waneck et al., 1986). L'effet du produit de l'oncogène sur la dépendance cellulaire en CSF peut s'exercer selon deux voies distinctes : dans le cas des cellules infectées par le virus Abelson, l'indépendance vis à vis de l'érythropoïétine s'obtient sans production de l'hormone (Wanek et al., 1986); dans le cas des cellules myéloïdes infectées par le virus MH2, le produit de l'oncogène v-mil rend ces cellules

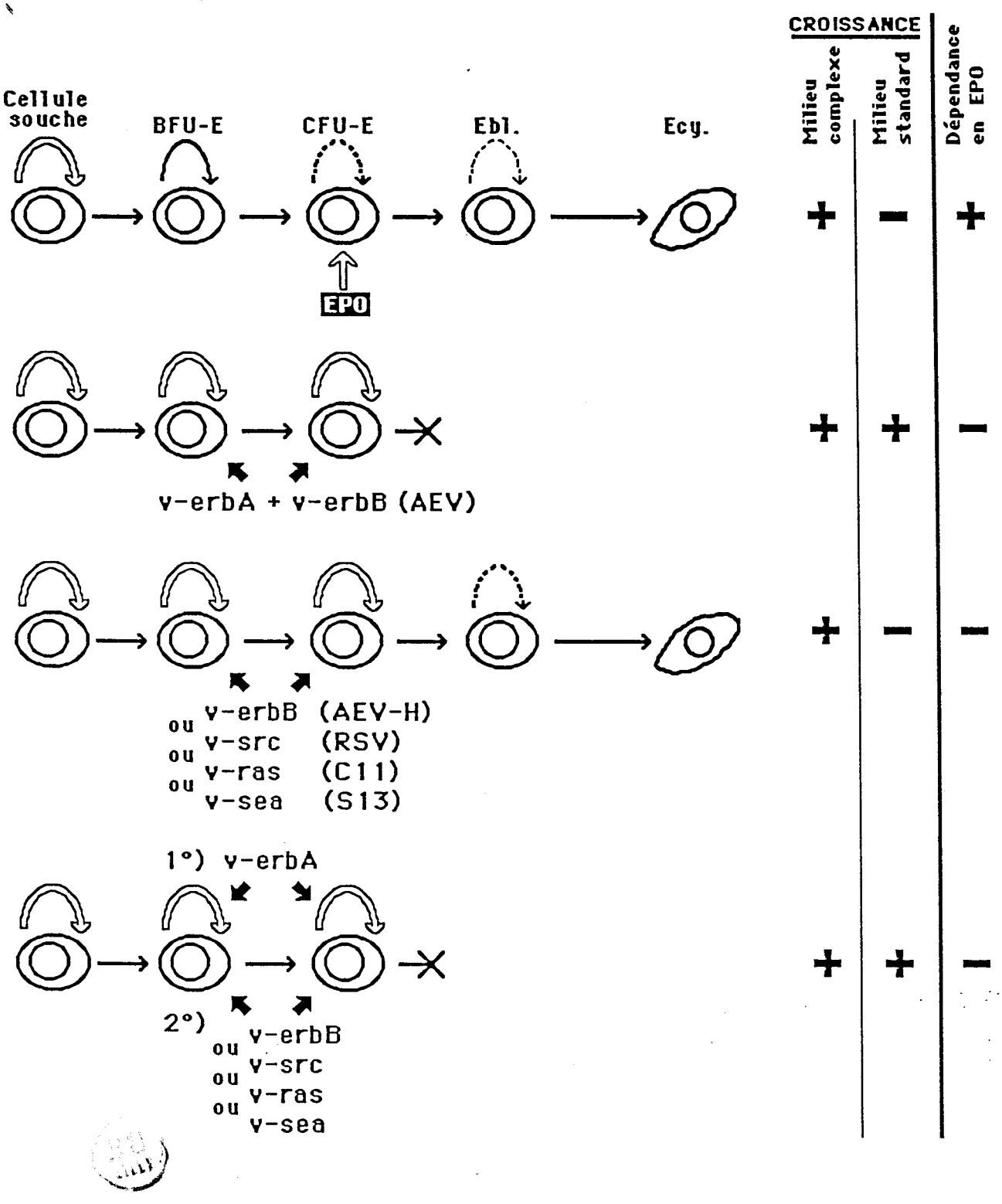


Figure 13 : Coopération entre le produit de v-onc dans la transformation de cellules hématopoïétiques.

(v-erbA seul sans effets apparents)

indépendantes de cMGF (indispensable pour leur croissance) d'une façon autocrine (les cellules infectées produisent leur propre facteur de croissance, Graf et al., 1986).

Le troisième type d'interaction entre v-onc et système hématopoïétique est fourni par le virus AEV qui contient deux oncogènes v-erbA et v-erbB; l'étude de mutants isolés *in vivo* ou construits *in vitro* a permis de montrer une action complémentaire entre v-erbA et v-erbB dans le blocage de différenciation des cellules érythroïdes transformées par AEV. Une étude récente réalisée par Kahn et al. (1986) et portant sur la coopération entre le produit v-erbA et le produit d'autres oncogènes dans la transformation des érythroblastes a permis de définir deux étapes dans cette transformation. Les produits des oncogènes v-src, v-erbB, v-ras, v-sea permettent la prolifération des érythroblastes d'une façon indépendante de l'érythropoïétine (Figure 13) (il faut noter ici que ces cellules restent dépendantes de l'hormone pour se différencier). Toutefois, ces cellules exigent un milieu de culture complexe (fenêtres de pH et de force ionique très définies, T.Graf et H.Beug, communication personnelle) pour proliférer. La surinfection de ces cellules par un virus portant l'oncogène v-erbA seul (Frikberg et al., 1983) permet leur prolifération en milieu standard et bloque leur maturation en érythrocyte même en présence d'hormone. Ces résultats suggèrent que v-erbA agirait sur le contrôle de la différenciation des érythroblastes transformés (en contrôlant peut êre le pH et/ou la force ionique), et v-erbB et les autres oncogènes cités agiraient sur le contrôle de la mitose (Graf et Beug, 1983; Frykberg et al., 1983; Yamamoto et al., 1983; Kahn et al., 1986). Ce modèle montre que plusieurs événements génétiques (fournis par le produit des v-onc) peuvent être requis pour que la cellule acquière son phénotype transformé maximal : le premier stade dissocie l'effet des hormones de croissance sur la prolifération et le second bloque la différenciation.

Une autre voie d'approche pour obtenir des informations sur le rôle du produit des oncogènes cellulaires dans les programmes de différenciation consiste à mesurer les variations du taux d'expression d'un c-onc donné après induction de la différenciation. Ainsi, c-fos est rapidement exprimé durant la différenciation de monomyélocytes ou de monocytes en macrophages, mais ne montre aucune induction lors de la différenciation des monomyélocytes en granulocytes suggérant ainsi un rôle spécifique de cet oncogène dans la différenciation monocytaire (Mitchell et al., 1985).

L'induction de c-fos est également observée lors de la différenciation des cellules de phéochromocytome PC12 (Curran et Morgan, 1985). De façon opposée, l'effondrement du taux d'ARN des oncogènes c-myc et c-myb est observé lors de la différenciation i*n vitro* de cellules myéloïdes (Gonda et Metcalf, 1984) alors que le taux d'ARN de l'oncogène c-ras lui ne varie pas. L'oncogène c-myc, introduit sous le contrôle d'un promoteur viral dans des pro-érythroblastes murins transformés par le SFFV (virus dépourvu d'oncogène), empêche la différenciation de ces cellules sous l'action du DMSO (Coppola et Cole, 1986). Cette observation va tout à fait dans le sens de l'activité mitogène attribuée au produit de c-myc. Dans un autre système, une chute du taux des ARN de l'oncogène c-sis est observée au cours de la différenciation *in vitro* de cellules endothéliales des parois de vaisseaux sanguins (Jaye et al., 1985).

Les voies métaboliques qui sont utilisées pour mener à bien la mitose fonctionnent également dans le cas de la différenciation cellulaire. Le fait qu'une cellule choisisse de se diviser ou de se

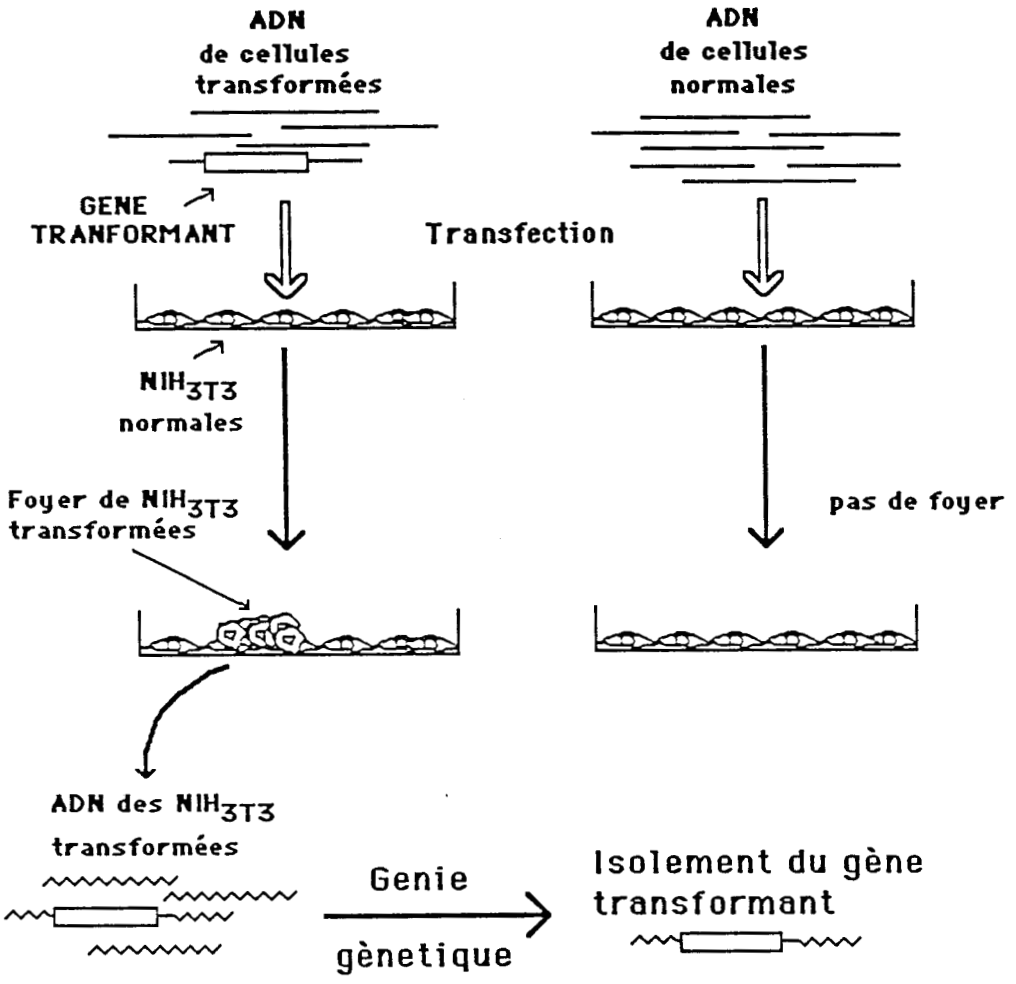


Figure 14 : Isolement par le test NIH_{3T3} de c-onc activés.

différencier en réponse au même inducteur dépend probablement du programme génétique de cette cellule au moment où elle reçoit le signal. Les oncogènes qui interagissent avec la mitose seraient donc également capables, en intervenant par les mêmes mécanismes biochimiques, d'induire la différenciation cellulaire (Figure 12).

III-ONCOGENESE MULTI-ETAPE

Un oncogène c'est bien, mais deux oncogènes, c'est mieux ! Ou encore, Attention ! Un oncogène peut en cacher un autre ! Cette formule s'est révélée particulièrement vraie dans les études tendant à isoler les gènes transformants actifs dans les tumeurs spontanées (ou induites par différents agents (Figure 14) (Cooper et Neiman, 1981; Lane et al ., 1982; De Lapeyriere et al., 1984). Toutefois, cette notion d'étapes multiples dans la transformation était déjà connue en cancérologie clinique, ou en cancérogénèse chimique (Cairns , 1975; Henning et al., 1983). Cette association de gènes est également connue dans la transformation induite par les virus à ADN (Rassoulzadegan et al., 1982; Yang et al., 1985). L'étude de l'interaction de plusieurs oncogènes dans la transformation cellulaire s'est trouvée facilitée par l'utilisation de systèmes cellulaires "induits", ne produisant pas de tumeurs chez la souris nude, tout en ayant acquis une capacité de multiplication infinie, ce que ne peuvent pas faire des cellules normales (Macceira-Coelho et al.,1977). De telles lignées cellulaires ont permis de dégager deux groupes d'oncogènes, les premiers sans action transformante sur ces cellules, les seconds au contraire doués de ce pouvoir. Par contre, les oncogènes du premier groupe sont capables d'induire sans changement morphologique l'immortalisation des cellules saines, liée à une réduction de leurs exigences vis à vis des facteurs mitogènes du sérum, alors que les oncogènes du deuxième groupe sont sans action sur ces cellules. Il semble en fait que l'immortalisation ou la transformation cellulaire dépende (dans un système donné) plus d'un problème quantitatif que d'un effet spécifique de l'oncogène.

En effet, le produit de myc semble capable, quand il est exprimé par l'intermédiaire de LTR murin, de transformer des fibroblastes et des macrophages normaux de souris (Vennström et al., 1984) alors qu'il est immortalisant sur ces cellules quand il s'exprime par l'intermédiaire d'autres types de séquences activatrices de la transcription (Mougneau et al., 1984). Cette capacité à transformer les fibroblastes ou les cellules sanguines était déjà connue depuis fort longtemps pour l'oncogène v-myc présent dans les virus aviaires (Langlois et al ., 1967). D'autre part, le produit de l'oncogène RAS activé semble immortalisant quand il est faiblement transcrit et transformant quand il est placé sous le contrôle transcriptionnel de séquences virales (Spandidos et Wilkie, 1984). Cette observation est également valable pour le produit d'un autre oncogène (p53) immortalisant quand il est faiblement transcrit, et transformant s'il est fortement transcrit sous le contrôle d'un promoteur viral (Jenkins et al., 1984; Eliyahu et al., 1985).

Un autre exemple des effets multiples du produit d' un oncogène dans la transformation a été apporté par l'oncogène ras activé dans les tumeurs induites par les cancérogènes chimiques. Nous avons vu que deux stades cellulaires peuvent être individualisés dans la transformation induite par

les carcinogènes chimiques: un stade d'initiation où les cellules non tumorigènes présentent une propension élevée à dégénérer sous l'action des promoteurs chimiques (TPA), représentant ainsi le deuxième stade tumorigène. c-Ha-ras peut être activé par mutation ponctuelle (sur l'acide aminé 61) par le DMBA (7,12-diméthylbenz(a)anthracène) dans les kératinocytes murins "initiés" formant des papillomes bénins susceptibles de dégénérer en carcinomes squameux sous l'action des esters de phorbol (Balmain et al., 1984; Brown et al., 1986; Harper et al., 1986). Ainsi la $p21^{ras}$ activée parait capable de prendre en charge l'étape d'initiation où la cellule est suffisamment "anormale" pour se transformer au contact des substances promotrices, et présenter *in vitro* une anomalie des phases tardives de la différenciation (Harper et al., 1986). C'est probablement par le biais de cette perturbation que les cellules induites sont capables de former les papillomes bénins; un deuxième événement stimulé par le TPA interviendrait alors pour transformer ces cellules. Bien que le traitement de la peau par le DMBA conduise à l'apparition de papillomes dont 85% possèdent une anomalie du c-Ha-ras, et sont sensibles aux esters de phorbol, un petit pourcentage de ceux-ci parait initié sans que ce gène soit activé; ces papillomes progresseront vers la transformation d'une façon indépendante du TPA (Harper et al., 1986).

Or, l'introduction du gène v-ras dans ces cellules "initiées" établit la phase de progression en les rendant tumorigènes (Harper et al., 1986). Ces résultats montrent que le produit d'un même gène peut être impliqué dans l'initiation des cellules (les cellules non tumorigènes ne montrent qu'une perturbation des phases terminales de la différenciation) mais également dans l'étape de progression à l'issue de laquelle les cellules sont franchement transformées. Les voies métaboliques permettant d'atteindre ces différents stades sont multiples : l'initiation peut se faire par l'intermédiaire de la voie métabolique détaillée dans le chapitre oncogènes et mitose, incluant l'oncogène c-ras et la PK-Cα (ce qui expliquerait pourquoi les papillomes où le c-ras est activé sont sensibles aux esters de phorbol, la PK-C étant, nous l'avons vu, un des récepteurs de ces substances); ce stade peut également être atteint par un autre mécanisme mal connu, mais qui permet au produit de ras de se révéler transformant.

Des études menées *in vitro* suggèrent que l'oncogène myc serait également capable d'induire l'étape "d'initiation" dans des fibroblastes d'embryons de rats, le TPA permettant ensuite leur transformation morphologique (Connan et al., 1985). Ainsi, il est clair que les carcinogènes chimiques utilisent l'arsenal des c-onc pour transformer les cellules mais que les produits de plusieurs gènes sont dans tous les cas nécessaires.

La démonstration de l'action synergique du produit d'oncogènes a été apportée par Land et al., (1983) dans la transformation de fibroblastes embryonnaires de rat. Dans ce système, l'oncogène v-myc n'induit pas sur les cultures cellulaires de modifications décelables; de son côté, le produit de l'oncogène Ha-RAS activé, qui est transformant sur différents types de cellules immortalisées (Newbold et Overell, 1983), s'avère incapable de rendre ces cellules tumorigènes; l'action simultanée du produit des deux oncogènes y parvient. Les actions synergiques ont été activement étudiées depuis en associant plusieurs produits d'oncogènes. Ainsi, une action synergique a pu être observée entre la $p21^{RAS}$ et la p53 (Parada et al., 1984). Le gène codant pour cette protéine n'est pas isolé d'un rétrovirus; la p53 est présente à des taux fréquemment élevés dans différentes

Source	Nom	Cellules dont la croissance est inhibée	Poids Moléculaire
Cellules de souris en culture	TGF β (Transforming Growth Factor)	Cellules fibroblastiques de souris (NRK ou AKR-2B)	25 kd (2 sous-unités de 12.5 kd)
Cellules épithéliales de rein (BSC-1)	BSC-1 Inhibiteur (Probablement TGF β)	BSC-1	25 kd (2 sous-unités de 12.5 kd)
Fibroblastes de souris (3T3)	FGR-s Fibroblast Growth Regulator	3T3	13 kd
Fibroblastes embryonnaires de souris		Cellules Productrices	10 à 15 kd
Foie de rat	HPI Hepatic Proliferation Inhibitor	Hépatocytes (mais pas cellules d'hépatomes)	26 kd
Glande mammaire bovine		Cellules d' Ehrlich (Ascite de carcinome mammaire)	13 kd
Cortex cérébral bovin	BSC-G (Bovine Glycoprotéine inhibitor)	Fibroblastes murins	45 kd (Glycoprotéine)

TABLE III : Facteurs inhibant la croissance cellulaire.

(Wang et Hsu, 1986)

cellules transformées mais également dans des cellules normales (Rotter et Wolf, 1985). Observation très interessante, sa localisation de cytoplasmique dans les cellules normales devient nucléaire dans les cellules transformées (Rotter et Wolf, 1985). Le clonage moléculaire de l'ARN codant pour cette protéine (Oren et Levine, 1983) a permis d'étudier directement son potentiel oncogène et de montrer que la p53 est capable d'immortaliser les cellules normales (Jenkins et al., 1984). Cette protéine pourrait intervenir i*n vivo* dans l'établissement des tumeurs (De Lapeyriere et al ., 1984). L'existence d'une synergie du même type a également été rapportée pour les oncogènes N-MYC et RAS (Schwab et al., 1985). N-MYC également dépourvu d'équivalent parmi les oncogènes de rétrovirus a été isolé sur la base de son homologie limitée avec myc et de son amplification importante dans les cellules de neuroblastomes (Schwab et al., 1983a). Ce gène code, comme MYC, pour un doublet protéique (p62-64) à localisation nucléaire (Slamon et al., 1986).

Un autre moyen de mettre en évidence une synergie entre oncogènes est fourni par des rétrovirus contenant naturellement plusieurs oncogènes. Nous avons vu dans le chapitre oncogènes et différenciation cellulaire, le cas du virus AEV au sein duquel v-erbA et v-erbB coopèrent pour transformer les cellules érythroïdes aviaires immatures (Figure 13). Un autre exemple de coopération peut être fourni par l'action des oncogènes v-mil et v-myc (tous deux portés par le rétrovirus aviaire MH2), sur des cellules de neurorétines aviaires (Béchade et al., 1985). Les cellules de neurorétines d'embryons de poule de 7 jours ne se multiplient pas *in vitro* tout en survivant. Nous avons pu montrer, en utilisant des mutants du virus MH2, isolés biologiquement ou construits *in vitro*, que le produit du gène v-mil était capable d'induire la multiplication de ces cellules. Les virus n'exprimant que le produit de l'oncogène myc sont sans effet décelable sur ces cellules. Après multiplication de ces cellules sous l'action du produit de v-mil, le produit de l'oncogène v-myc est capable de les transformer.

Enfin, une action synergique ou complémentaire peut être supposée pour certains oncogènes trouvés simultanément activés au sein d'une même cellule transformée. C'est ainsi le cas des oncogènes MYC et N-RAS identifiés dans des cellules promyélocytiques humaines (cellules HL60) et dans au moins une lignée cellulaire de lymphome de Burkitt (Murray et al ., 1983). L'oncogène N-RAS pour sa part a été isolé selon la technique de transfection de l'ADN de cellules transformées sur les cellules NIH_{3T3} (Figure 14, Table IV) et moléculairement cloné à partir de cellules de neuroblastomes; il fait partie de la famille des oncogènes RAS, mais contrairement à Ha-RAS et Ki-RAS, N-RAS n'a pas d'équivalent connu dans les rétrovirus (Shimizu et al ., 1983a).

IV- LES CONTROLES DE L'ONCOGENESE

La constitution d'un organisme nécessite de façon harmonieuse, croissance et différenciation cellulaires ce qui suppose un contrôle étroit de ces mécanismes. Ainsi, des facteurs de croissance) sont responsables de la multiplication cellulaire, d'autres subtances sont capables de prévenir celle-ci. De tels facteurs ont été obtenus à partir de tissus normaux et à partir du surnageant de

culture de cellules (Table III). En règle générale, leur action s'exerce sur les cellules saines mais pas sur les cellules transformées. Ces facteurs sont constitués par des polypeptides (souvent reliés par des ponts disulfures) et certains sont des glycoprotéines (Table III). Le mode d'action précis de tous ces facteurs n'est pas connu, mais il pourrait, comme le TGFβ par exemple, moduler l'activité des facteurs de croissance en agissant sur leurs récepteurs, ou encore induire la différenciation de certains types cellulaires.

Un mécanisme de balancier activant des gènes induisant la mitose, puis activant des gènes la réprimant, peut même être supposé. En effet, le PDGF semble induire très rapidement l'expression d'une famille de gènes (dont c-fos et c-myc) dans des fibroblastes de souris, mais cette première vague d'activation (de gènes supposés être mitogènes) est suivie d'une deuxième vague où figurent le gène du β-interféron fibroblastique et le gène de la 2'-5' oligoadénylate-synthétase qui sont anti-mitotiques (Zullo et al., 1985). Le ß-interféron contrôlant le gène c-myc semble être également capable d'induire la différenciation de cellules hématopoïétiques (Resnitzky et al.,1986); un tel produit pourra ainsi réguler la prolifération de ces cellules en les induisant à se différencier. Un mécanisme de balancier semble également exister dans la fonction de PK-C, qui tout en induisant une série d'événements aboutissant à la mitose dirige également (dans certains types cellulaires) un certain nombre de mécanismes régulateurs aboutissant à la dégradation de récepteurs membranaires et/ou à l'inactivation du système adénylate cyclase (voir le chapitre sur la mitose). PK-C est également capable d'activer la 5'-phosphomonoesterase, responsable de la dégradation de l'inositol triphosphate déprimant ainsi la cellule en second messager (Figure 12, Connolly et al., 1986). On peut supposer que la rupture de cet équilibre entre "activation" et "répression" puisse induire la transformation cellulaire.

D'autre part, l'existence de séquences nucléotidiques capables de contrôler l'expression de phénotypes transformés a clairement été montrée dans le cas de certaines tumeurs particulières, les rétinoblastomes. Dans ces tumeurs, un gène RB1, présent sur le chromosome 13 (bande q14) doit être fonctionnellement perdu sur ses deux allèles (Cavenee et al., 1983), pour que la tumeur se développe. RB1 pourrait (par exemple) être essentiel dans l'induction de la différenciation des cellules visées. Le contrôle exercé par le gène RB1 sur la prolifération cellulaire déborde le cadre des cellules de la rétine, car il a été montré récemment que la présence des deux allèles RB1 non fonctionnels était responsable de la fréquente survenue d'ostéosarcomes chez les individus atteints de la forme héréditaire du rétinoblastome (Hansen et al., 1985). D'autres tumeurs (tumeur de Wilms, carcinome pulmonaire à petites cellules, carcinome rénal à transmission familiale et les neuroblastomes) semblent également devoir perdre pour se développer les deux allèles d'un gène unique (Klein et Klein, 1985). L'existence de tels gènes "anti-oncogènes" est également suggérée par les études menées sur des hybrides somatiques non transformés, résultants de la fusion de cellules transformées et de cellules normales (Graig et Sager, 1985).

Par exemple, un contrôle négatif s'exerçe sur la capacité de v-src à induire des tumeurs *in vivo* dans des embryons de poule (Dolberg et Bissell, 1984). Les cellules embryonnaires infectées se transforment après culture *in vitro* ce qui suggère que les facteurs contrôlant la différenciation pendant la vie embryonnaire sont également capables dans ce cas de dominer le phénotype

transformé. Les mécanismes régulateurs ne sont pas tous connus, mais on peut supposer, sur la base des résultats actuels, au moins quatre types de contrôles différents pouvant affecter un oncogène : un contrôle transcriptionnel, un contrôle post-transcriptionnel, un contrôle régulant la traduction et un contrôle régulant l'accumulation du produit cible de l'oncogène.

Ainsi, il est clair que l'expression transcriptionnelle de certains oncogènes peut être régulée de façon tissu-spécifique par les séquences nucléotidiques adjacentes en 5' ou en 3' du gène. L'oncogène MOS (qui présente par ailleurs la particularité, unique chez les oncogènes, d'être dépourvu d'introns, Watson et al., 1982) possède en 5' une séquence nucléotidique qui empêche son expression transcriptionnelle après transfection dans des fibroblastes (Wood et al., 1984). Une séquence analogue est également retrouvée dans le gène c-myc de souris (Remmers et al., 1986) et le gène codant pour la p53 (Bienz-Tadmor et al., 1985) suggérant ainsi un rôle général de ce type de contrôle dans la régulation des c-onc. Ces séquences pourraient être la cible de protéines se fixant sur l'ADN (Siebenlist et al., 1984) et on peut imaginer une expression définie selon les tissus ou les stades de différenciation.

Un contrôle post-transcriptionnel est suggéré dans le cas de l'oncogène c-myc, la stabilité de l'ARN synthétisé étant sous la dépendance de facteurs cellulaires (Dani et al., 1984). Ce type de régulation existe également dans le cas du gène c-fos, qui doit être amputé de 67 nucléotides riches en adenines et thymidines (-189 à -123 nucléotides du signal de polyadénylation du gène, Meijlink et al., 1985) pour être traduit dans des fibroblastes. Or, en réponse à un signal de différenciation (esters de phorbol) des monocytes expriment d'importantes quantités d'ARN FOS pendant une centaine d'heures, alors que la protéine correspondante devient indécelable dans les deux heures qui suivent l'induction (Mitchell et al., 1985). Un facteur cellulaire intervient donc de façon transitoire pour permettre l'expression de la protéine.

Enfin, on peut supposer d'après les expériences de Noda et al. (1983) qu'un contrôle peut s'exercer sur l'accumulation du produit cible pour un oncogène donné. Ces auteurs ont pu montrer que des révertants morphologiques de cellules de souris transformées par l'oncogène v-ras ne pouvaient plus être transformés par certains autres (v-fes et v-src) alors que la transformation par v-mos ou v-sis était toujours possible. On peut supposer que le produit cible du gène v-ras a disparu de ces cellules.

Intervenant probablement par un autre biais, les gènes du complexe majeur d'histocompatibilité (essentiels dans les mécanismes immunologiques de rejets modulés par les lymphocytes cytotoxiques) peuvent empêcher une cellule transformée d'être tumorigène (Wallich et al., 1985). L'importance de leur rôle dans la transformation est attestée par l'inhibition de ces gènes sous l'effet du produit E1A de l'adénovirus type 12, ce qui permet à la cellule de devenir tumorigène en échappant au contrôle de ces lymphocytes cytotoxiques (Bernards et al., 1983). Situé sur la même voie de régulation, il faut signaler le rôle du "Tumor Necrosis Factor" (TNF) synthétisé par les macrophages activés. Ce produit de 156 amino acides est lytique et induit une nécrose tumorale *in vivo,* et une mort cellulaire préférentielle des cellules transformées *in vitro* (Fransen et al., 1985). Ce produit pourrait également agir par l'intermédiaire de mécanismes de différenciation : une complète homologie vient d'être observée entre DIF responsable de la différenciation des cellules

Oncogènes	Produits	Cellules d'origine	Références
Ha-RAS*	p21 (Fixe le GTP et GTPase)	EJ: carcinome de la vessie	Santos et al., 1982
Ki-RAS*		Carcinome pulmonaire	Der et al., 1982
N-RAS		Sk-N-SH: Neuroblastome	Taparowsky et al., 1983
B-lym	p8 apparentée aux transférines	RP9: lymphome B aviaire.	Goubin et al., 1983
T-lym-1		S49T: Lymphome murin de type T	Lane et al., 1984
RAF *		Carcinome pulmonaire	Shimizu et al., 1985
neu	p185 Tyrosine kinase	Neuroblastome.	Bargmann et al., 1986
TRK	p64 Tyrosine kinase	Carcinome du colon ascendant.	Martin-Zanca et al., 1986
MET	Tyrosine kinase?	MNNG-HOS; Lignée cellulaire humaine transformée par un carcinogène chimique.	Cooper et al., 1984
mel		NK14: mélanome.	Padua et al., 1984
dbl		Lymphome B diffus.	Eva et Aaronson, 1985
ret		Lymphome de type T.	Takahashi et al., 1985
mcf-2 ROS *	Tyrosine kinase?	MCF-7: Carcinome mammaire.	Fasano et al., 1984b Birchmeier et al., 1986
MAS	p33 Transmembrane?	Carcinome épidermoïde.	Young et al., 1986
hst		Carcinome Gastrique.	Sakamoto et al., 1986

* Oncogène déja trouvé au sein d'un rétrovirus.

TABLE IV : Oncogènes isolés par transfection de l'ADN de cellules transformées

Tumeurs ou lignées	Oncogène amplifié	Produit	Amplification	Références
HL60 (Lignée promyélocytique humaine)	MYC	p64/67 Nucléaire	20	Nowell et al., 1983
COLO 320 (Carcinome colique humain)	MYC		50	Alitalo et al., 1983
Adénocarcinome gastrique humain	MYC		15 à 30	Shibuya et al., 1985
SCLC (Carcinome pulmonaire humain à petites cellules)	MYC		20 à 76	Little et al., 1983
SCLC	L-MYC		10 à 20	Nau et al., 1985
Neuroblastomes (Lignées ou tumeurs humaines)	N-MYC	p62/64 Nucléaire	140	Schwab et al., 1983a
Y79 (Lignée de rétinoblastome humain)	N-MYC		20	Schwab et al., 1984a
MCF7 (Carcinome mammaire humain)	N-RAS	p21 Fixe GTP	10	Fasano et al., 1984
Y1 (Tumeur adénocorticale de souris)	c-Ki-ras	p21 GTPase	50	Schwab et al., 1983b
K562 (Lignée de leucémie myéloïde chronique humaine)	ABL	p145	10	Collins et Groudine, 1983
COLO 201/205 (Carcinome colique humain)	MYB	p 83 Nucléaire	10	Alitalo et al., 1984
A431 (Carcinome épidermoïde humain)	ERB B	p70 Tyr. k.	10	Linn et al., 1984
Glioblastomes humains	ERB B		10 à 60	Libermann et al., 1985
MAC117 (Carcinome mammaire humain)	NEU	p185 Tyr. k. ?	10	King et al., 1985

TABLE V : Amplification des c-onc.

Localisation		Oncogène	Références
1	Cen.-p21	N-RAS	Davis et al., 1983
	p 32	B-LYM	Morton et al., 1984
	p 32	L-MYC	Nau et al., 1985
	p 36.1-p 36.2	FGR	Tronic et al.. 1985
	q 12 - q ter.	SKI	Balazs et al., 1984
2	p 23 - p ter.	N-MYC	Schwab et al., 1984b
3	p 25	MIL1/RAF1	Bonner et al., 1984
4		MIL2/RAF2	
5	q 34	FMS	Groffen et al., 1984b
6	p 23 - q 12	Ki-RAS1	Sakaguchi et al., 1984
	q 16 - q 22	ROS	Nagarajan et al., 1986
	q 22 - q 24	MYB	Harper et al., 1983
7	p ter - q 22	ERB B	Spurr et al., 1984
	p 11 -q ter	MET	Cooper et al., 1984
8	q 11	MOS	Caubet et al., 1985
	q 24	MYC	Neel et al., 1982
9	q 34	ABL	Goff et al., 1982
11	p 15	Ha-RAS1	O'Brien et al., 1983
	q 13	INT2*	Casey et al., 1986
	q 13	BCL1*	Tsujimoto et al., 1984
	q 23 - q 24	ETS1*	DeTaisne et al., 1984
12	p 12 - p ter	Ki-RAS2	O'Brien et al., 1983
	q 14 - q ter	INT 1*	Nusse et al., 1984
14	q 21 - q 31	FOS	Barker et al., 1984
15	q 25 - q 26	FES/FPS	Harper et al., 1983
17	p 13	p53	Isobe et al., 1986
	q 11 - q 12	ERB A*	Sheer et al., 1985
	q 11 - q 22	NEU	Schechter et al., 1985
18	q 21	BCL2*	Tsujimoto et al., 1985
20	q 12 - q 13	SRC	Le Beau et al., 1984
21	q 22	ETS2*	Watson et al., 1986
22	q 11	BCR *	Heisterkamp et al., 1985
	q 12 - q 13	SIS	Bartram et al., 1984
X	p21 - q 11	A-RAF1	Huebner et al., 1986
	q 12 - q 13	Ha-RAS2	O'Brien et al., 1983

* : pas d'évidence directe d'une activité transformante

TABLE VI : Localisation chromosomique des "oncogènes".

Translocations ou remaniements	Cancer associé	c-onc "impliqué"	Références
5→5q-	Leucémie myéloïde aigüe. Anémies.	FMS	Nienhuis et al., 1985
t (9 ; 22) (q34 ; q11) (Chromosome Philadelphie)	Leucémie myéloïde chronique.	ABL BCR	Shtivelman et al., 1985
t (7; 9) (q36 ; q34)	Lymphome T	ABL	Westbrook et al.,1987
t (9 ; 11) (p22 ; q23)	Leucémie monocytique aigüe.	ETS1	Diaz et al., 1986
t (8 ; 14) (q24 ; q32) t (8 ; 22) (q24 ; q11) t (2 ; 8) (p11 à p13 ; q24)	Lymphome de Burkitt.	MYC	Berger et Bernheim, 1985
t (4 ; 11) (q21 ; q23) t (8 ; 21) (q22 ; q22)	Leucémies aigües.	ETS1 ETS2	Sacchi et al., 1986
t (14 ; 18) (q32 ; q21)	Lymphome folliculaire.	BCL2	Tsujimoto et al., 1985
t (11 ; 14) (q13 ; q32)	Lymphome B diffus. Leucémie lymphoïde chronique.	BCL1	Tsujimoto et al., 1985
t (15 ; 17) (q22 ; q21)	Leucémie promyélocytique aigüe.	p53	Le Beau et al., 1985
t (3 ; 8) (p14 ; q24)	Carcinome rénal héréditaire.	MYC	Drabkin et al., 1985

TABLE VII : Translocations chromosomiques et c-onc.

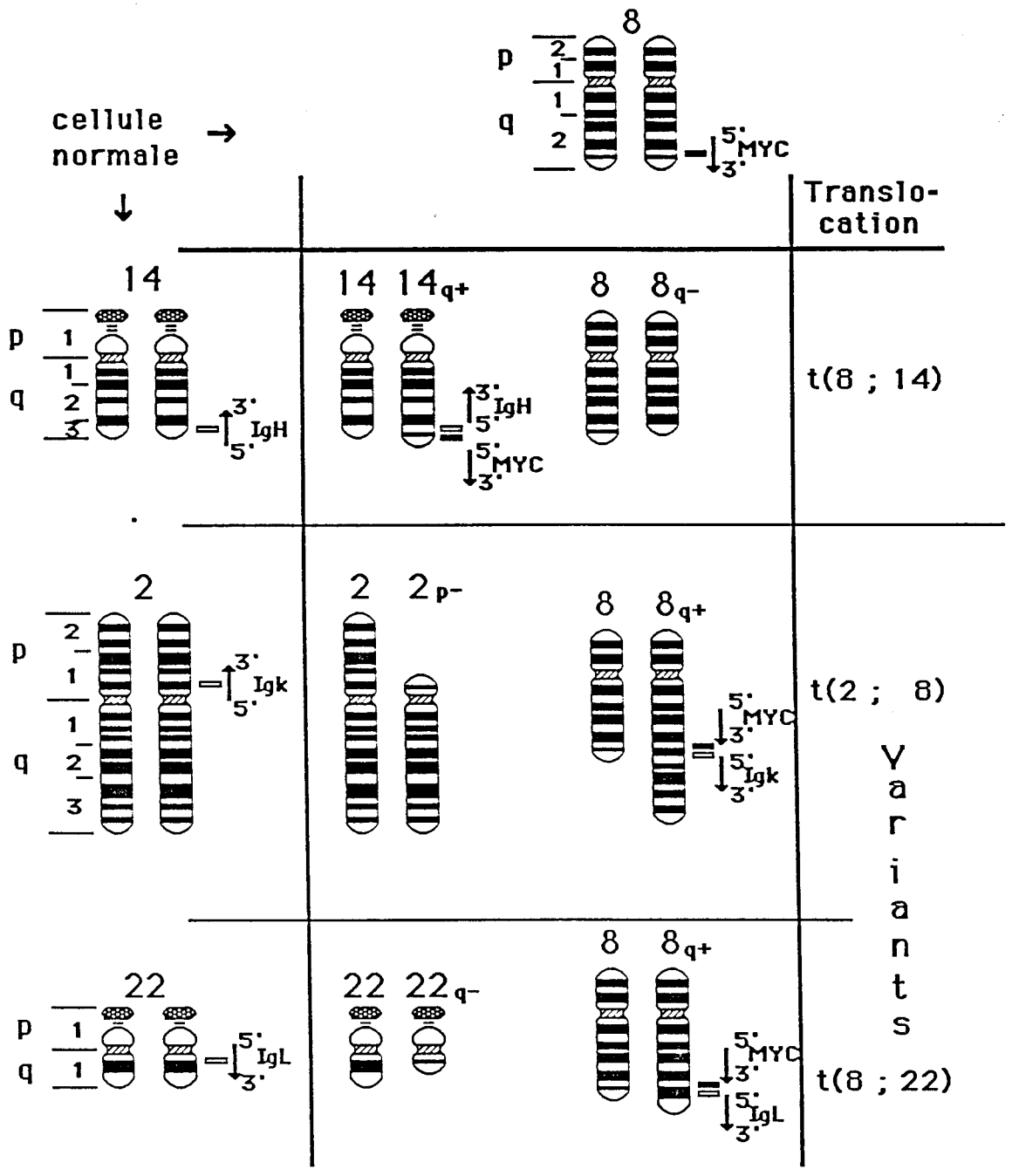


Figure 15 : Chromosomes dont la translocation est impliquée dans les Lymphomes de Burkitt.

— Localisation de l'allèle transloqué du gène MYC

= Localisation des allèles transloqués des gènes d'immunoglobulines

5'↓3' Orientation transcriptionnelle des gènes

myéloïdes humaines et le TNF (Takeda et al.,1986). Quel que soit le type de contrôle utilisé, l'expression des oncogènes est étroitement surveillée dans l'ontogénèse et au cours de la division cellulaire. Au même titre que l'activation d'oncogènes, la suppression "d'anti-oncogènes" doit être prise en compte pour expliquer l'installation d'une tumeur.

IV-C-ONC ET TUMEURS

Depuis la caractérisation des équivalents cellulaires d'oncogènes trouvés au sein de rétrovirus, de nombreuses études ont été entreprises pour déterminer si les c-onc pouvaient être impliqués d'une façon quelconque dans l'apparition de tumeurs spontanées. Et de fait, bien que le rôle exact du produit de ces gènes dans la transformation ne soit pas clairement établi, leur implication dans ce processus est maintenant certaine. L'activation d'un c-onc, c'est à dire l'événement génétique détournant le produit de ce gène de son rôle physiologique et aboutissant à la transformation cellulaire, peut se faire selon des voies différentes. Trois grands types de modifications (non mutuellement exclusifs) peuvent induire l'activation de c-onc :

1) des modifications touchant le nombre de copies (amplification) ou la localisation (translocation) d'un oncogène dans le génome;

2) des altérations ponctuelles des séquences nucléotidiques régulant l'activité transcriptionnelle d'un oncogène;

3) des altérations nucléotidiques (mutations) affectant la structure et l'activité du produit codé par cet oncogène.

Les deux premiers points impliquent l'expression accrue d'un produit cellulaire normal, alors que le dernier point suggère l'expression normale d'un produit modifié. La démonstration de l'existence de gènes activés dans certaines tumeurs a été apportée par les expériences de transfection de l'ADN de tumeurs sur des cellules réceptrices non transformées (Figure14, Table IV). La transformation de ces dernières, et le clonage moléculaire du gène impliqué a permis d'identifier un certain nombre de nouveaux oncogènes dépourvus d'équivalent viraux. Certaines tumeurs ont permis de redécouvrir des oncogènes déjà connus (Table IV) et elles ont également permis de préciser les mécanismes d'activation de telles séquences.

a-Amplification des c-onc

L'amplification d'un gène est un moyen utilisé par la cellule pour augmenter la production d'un enzyme pour faire face à un produit toxique par exemple : amplification du gène codant pour la dihydrofolate réductase lors du traitement des cellules par le méthotrexate et amplification du gène codant pour l'aspartyl transcarbamylase dans les cellules devenues résistantes à des inhibiteurs de cette fonction enzymatique (Schimke, 1982). Cette amplification induit deux types de modifications caryotypiques décelables. La première, appelée HSR (homogeneously staining regions), est visualisée après coloration par l'apparation sur les chromosomes de bandes anormalement larges là où l'amplification intervient (ce type d'amplification est stable). La seconde se fait par l'intermédiaire de "Double Minute Chromosomes" (DMC) et s'accomplit de façon

extra-chromosomique (ce type d'amplification est instable).

De tels types d'anomalies ne sont pas rares dans les cellules transformées, et récemment il a pu être montré que certains gènes oncogènes pouvaient être amplifiés par ce biais. Différents oncogènes dans différentes cellules sont susceptibles d'être amplifiés de cette façon (voir Tableau V). Dans tous les cas examinés, l'augmentation génique induit une surexpression de l'oncogène concerné qui pourrait être liée au pouvoir tumorigène de la cellule (Schwab et al., 1984a). L'amplification de N-MYC dans des neuroblastomes se voit dans les tumeurs ayant le plus mauvais pronostic, ainsi que l'amplification de MYC dans les carcinomes pulmonaires (Little et al., 1983). L'amplification de N-MYC dans les neuroblastomes est responsable du pouvoir métastatique élevé de ces cellules. Or, le gène N-MYC amplifié est responsable dans ces cellules de la répression de l'antigène de classe I du complexe majeur d'histocompatibilité (Bernards et al.,1986). Cela permettrait à ces cellules d'échapper à la survillance des lymphocytes cytotoxiques. Un modèle d'étude *in vitro* permet de correler amplification et transformation : une lignée fibroblastique de souris transfectée avec de l'ADN d'un plasmide dans lequel est inséré le gène v-src, voit cet ADN s'amplifier (x50) et les cellules devenir tumorigènes quand elles sont cultivées en basse densité de population. Si la densité de la culture est maintenue à un niveau élevé, le phénotype transformé est perdu ainsi que l'amplification du v-src dans le génome cellulaire (Glanville, 1985). L'amplification des oncogènes pourrait se faire tout d'abord sous forme de DMC, lesquels pourraient ensuite regagner l'ADN chromosomique par recombinaison homologue créant ainsi les structures HSR sur les chromosomes (Schwab et al., 1984a).

b-Translocations chromosomiques et activation d'oncogènes

A côté des anomalies caryotypiques représentées par les amplifications d'ADN, le génome des cellules transformées contient souvent des translocations ou des remaniements (amputations, inversions de fragments) affectant les chromosomes. Ces anomalies sont souvent observées sur des lignées cellulaires et il peut être difficile de déterminer celles qui sont la cause ou la conséquence de la transformation (Yunis, 1983). Cependant, l'association constante de certaines translocations avec certains types de cancers, et plus récemment la découverte d'oncogènes aux points de translocations suggèrent que les remaniements chromosomiques, comme les amplifications, sont à la base de l'expression inopportune d'oncogènes. La localisation des oncogènes sur les chromosomes (humains et murins essentiellement) a grandement facilité les études menées sur la relation oncogènes/translocation (voir Tableaux VI et VII).

Les chromosomes sont caractérisés dans les cellules en métaphase ou prométaphase (les études menées sur les translocations sont donc tributaires de la division des cellules cancéreuses, ce qui explique la plus grande fréquence des leucémies par rapport aux tumeurs solides dans les expériences rapportées). Après traitement par la trypsine, les préparations chromosomiques sont colorées au giemsa, ce qui permet d'obtenir le long du chromosome, une alternance de bandes sombres et claires caractéristiques (Harnden et al., 1981). Ce type de coloration permet de repérer précisément les chromosomes impliqués dans la translocation et, par la bande concernée, le niveau où l'anomalie intervient. Peu d'oncogènes connus ont pu être impliqués dans ce type de processus,

mais il n'est pas douteux que cette liste va s'allonger dans un futur proche. L'étude des translocations a d'ailleurs permis de découvrir de nouveaux oncogènes potentiels (Table VII) , et il est à supposer que leur nombre va croître. Les translocations les plus étudiées et les mieux connues concernent l'oncogène MYC dans les lymphomes de Burkitt, et l'oncogène ABL dans la leucémie myéloide chronique.

1) Le lymphome de Burkitt

En 1958, Burkitt publiait la première description d'un lymphosarcome de la mâchoire frappant l'enfant africain avec une fréquence de 4/100 000 (Burkitt, 1958). Ce lymphome prédomine dans les régions à paludisme endémique, et une association avec un virus à ADN (Herpès-virus de Epstein et Barr) était fréquemment trouvée (Kaplan et Szajnert, 1985). Il semblerait actuellement que l'infection de ces cellules par le virus d'Epstein-Barr permette aux cellules lymphoïdes proliférantes d'échapper au contrôle des lymphocytes T cytotoxiques ce qui faciliterait l'émergence d'un clone tumoral (Rooney et al., 1985). Le lymphome de Burkitt représente une prolifération monoclonale maligne de lymphocytes B présentant une translocation (8 ; 14) ou plus rarement (8 ; 22) ou (2 ; 8) (Kaplan et Szafnert, 1985, Figure 15). Les cellules transformées produisent une chaîne d'immunoglobuline différente selon le type de translocation:

-t (8 ; 14), les cellules produisent les chaînes lourdes d'immunoglobuline (dont les gènes ont pu être localisés sur le chromosome 14, bande q32) et l'un ou l'autre type des chaînes légères;

-t (8 ; 22) les cellules produisent des immunoglobulines dont les chaînes légères sont exclusivement de type lambda (dont les gènes sont localisés sur le chromosome 22, bande q11);

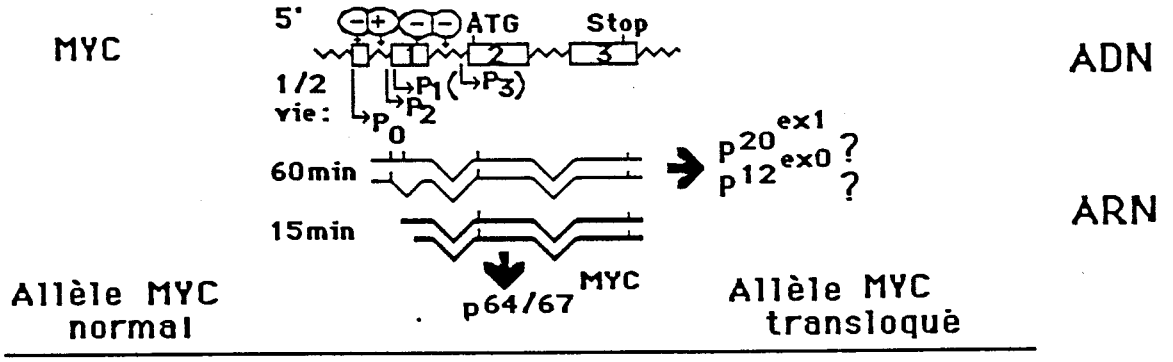
- t (2 ; 8), les cellules produisent des immunoglobulines dont les chaînes légères sont de type kappa (leurs gènes sont localisés sur le chromosome 2, bande p13).

Le point commun de toutes ces translocations vient du chromosome 8 invariablement clivé à la bande q24. La découverte de l'oncogène MYC au point de fracture à permis de l'impliquer dans la translocation (Taub et al., 1982).Le rôle de l'oncogène MYC dans l'étiologie du lymphome de Burkitt n'est pas évidente, mais semble essentiel sur la base de plusieurs observations. En effet, ce lymphome chez l'homme a son équivalent dans les autres espèces animales, et l'oncogène c-myc se retrouve impliqué à chaque fois (Klein, 1983):

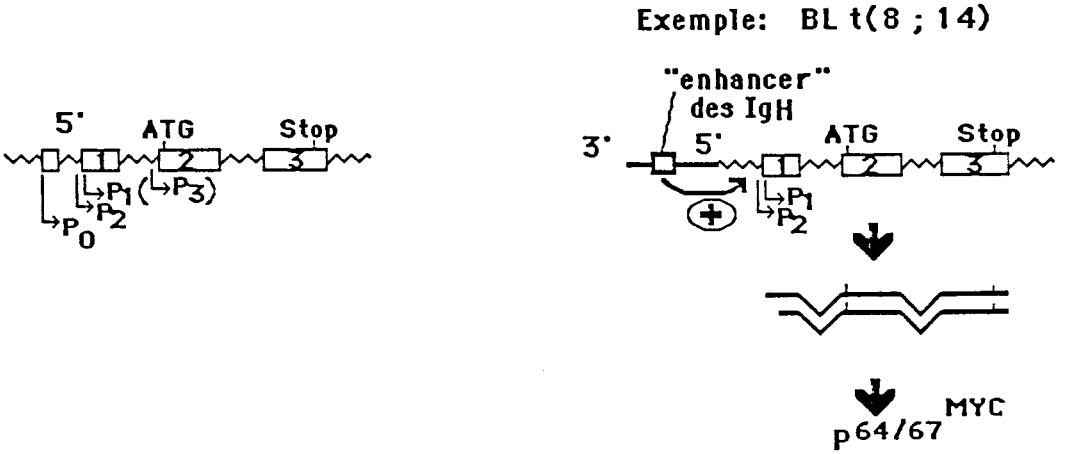
-dans les plasmocytomes murins (Shen-Ong et al., 1982);

-dans des immunocytomes spontanés de rat (translocation (6 ; 7); le gène myc étant porté dans cette espèce par le chromosome 7) (Sümegi et al., 1983).

-chez le poulet, de nombreuses études ont montré une activation du c-myc dans des lymphomes de type B induits par toute une série de rétrovirus (Hayward et al., 1981; Noari-Daloii et al., 1981; Payne et al., 1982). Enfin des lymphocytes bursiques, infectés *in vitro* par le virus HB1 portant le gène v-myc, réimplantés dans un animal chimiquement bursectomisé, induisent des lésions prénéoplasiques dans l'organe reconstitué (Neiman et al., 1985). Cette observation suggère un rôle pour le produit de myc, au moins dans les stades précoces de la transformation lymphoïde, car il est probable que l'activation d'autres oncogènes est nécessaire pour développer ce type de



1) Rôle de l'activateur transcriptionnel des gènes des immunoglobulines



2) Augmentation de la transcription par modification de signaux de régulation

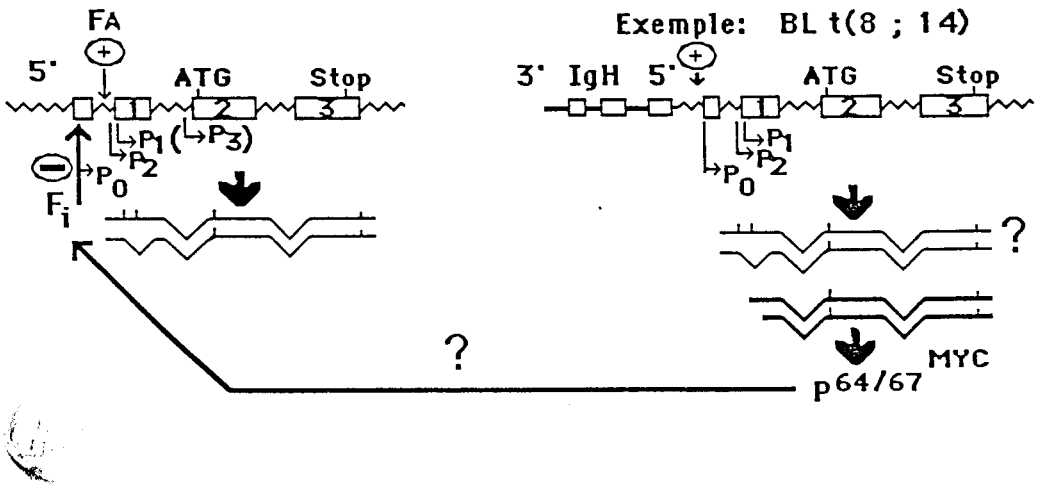


Figure 16 A : Différents modes d'activations du gène MYC dans les lymphomes de Burkitt.

□ : Exon

lymphome (Cooper et Neiman, 1981; voir le chapitre oncogénèse multi-étape.

Actuellement deux autres oncogènes activés dans des lymphomes B ont pu être caractérisés; il s'agit du gène B-LYM, identifié dans certains lymphomes de Burkitt (Diamond et al., 1983) ainsi que du gène N-RAS, également trouvé dans un lymphome de Burkitt (Murray et al., 1983).

L'étude des lymphomes de Burkitt a permis de montrer la complexité du mode de régulation d'un gène tel que MYC et font de cet oncogène l'un des mieux connus actuellement. Nous avons déjà présenté quelques uns des mécanismes régulateurs de l'expression des c-onc (voir les contrôles de l'oncogénèse. Ce gène pouvant être activé par la perturbation de l'un quelconque de ses éléments de contrôle, nous détaillerons ceux-ci.

La régulation de son expression semble pouvoir s'exercer au cours de la transcription du gène, au niveau de l'ARN synthétisé, et enfin sur les protéines traduites (Figure 16).

-Contrôles transcriptionnels :

La séquence nucléotidique complète du gène MYC est connue (Colby et al., 1983; Gazin et al., 1984); deux promoteurs de transcription situés au début de l'exon 1 (P1 et P2) permettent la synthèse de deux ARN différant de 160 bp (Taub et al., 1984) qui dirigent tous les deux l'initiation de la traduction sur l'AUG présent au tout début de l'exon 2 (nucléotides 4521-4523, Gazin et al., 1984). Ces deux promoteurs pourraient peut-être (comme cela a déjà été vu pour d'autres gènes, Shaw et al., 1985) être utilisés différemment au cours de l'ontogénèse et/ou de la différenciation (ce qui pourrait constituer un premier mode de contrôle transcriptionnel sur l'expression de ce gène). Un autre groupe de promoteurs (P0) situé 600 bp en amont de P1 et P2 semble coder pour au moins deux types d'ARN minoritaires dont la fonction n'est pas connue, mais qui pourraient traduire le cadre de lecture ouvert de l'exon1 (Bentley et Groudine, 1986). Les ARN initiés sur P0 sont plus stables que ceux initiés sur P1 et P2 (Bentley et Groudine, 1986).

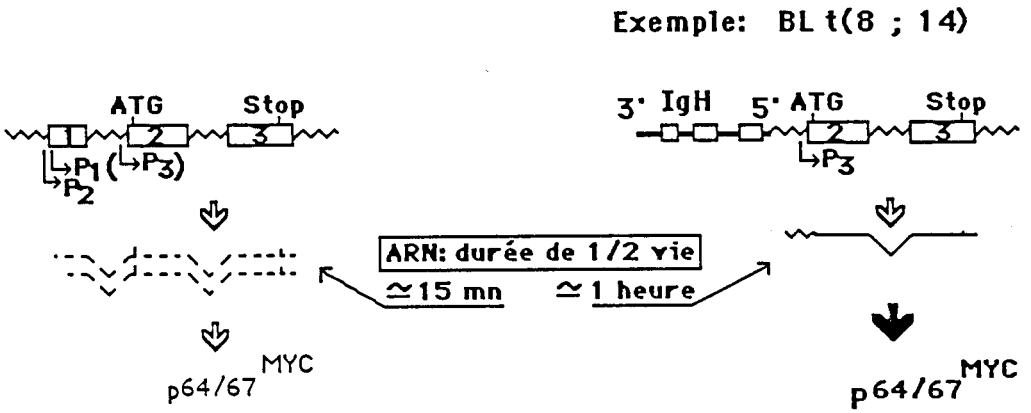
Deux régions particulières situées en 5' de ce gène ont pu être identifiées en utilisant des expériences d'hypersensibilité à la DNAse I (qui permettent de mettre en évidence les sites actifs de la chromatine). Une première région sensible semble se confondre avec les deux promoteurs du gène (ce qui refléterait leur utilisation dans l'ADN génomique), alors qu'une deuxième région située plus en 5' du gène serait activée lorsque cet oncogène n'est pas transcrit. Ce site contiendrait une séquence de contrôle négatif, agissant peut-être par l'intermédiaire de la fixation d'une protéine régulatrice (Siebenlist et al., 1984; Figure 16A). Une séquence nucléotidique désactivatrice de la transcription a récemment été mise en évidence en 5' du 1er exon de ce gène chez la souris (Remmers et al., 1986); une régulation par arrêt de l'élongation a été trouvée dans le premier intron du gène MYC (Bentley et Groudine, 1986). Co-existant avec ces différentes sequences de contrôle négatif, deux sites de contrôle positif ont pu être localisés devant le promoteur P1(Chung et al.,1986). Ainsi, la présence d'éléments activateurs de la transcription des gènes codant pour les chaînes lourdes des immunoglobulines, en perturbant ce type de régulation après translocation, activerait MYC dans cerains lymphomes de Burkitt (Hayday et al., 1984).

-Contrôles post-transcriptionnels :

-la séquence nucléotidique de l'exon 1 pourrait permettre l'élaboration d'une structure tridimensionnelle au sein de l'ARN, ce qui pourrait influer en retour sur l'efficacité de sa traduction

Allèle MYC normal | Allèle MYC transloqué

3) Stabilisation des ARN de l'allèle MYC transloqué



4) Rôle des mécanismes traductionnels dans l'accumulation des protéines MYC

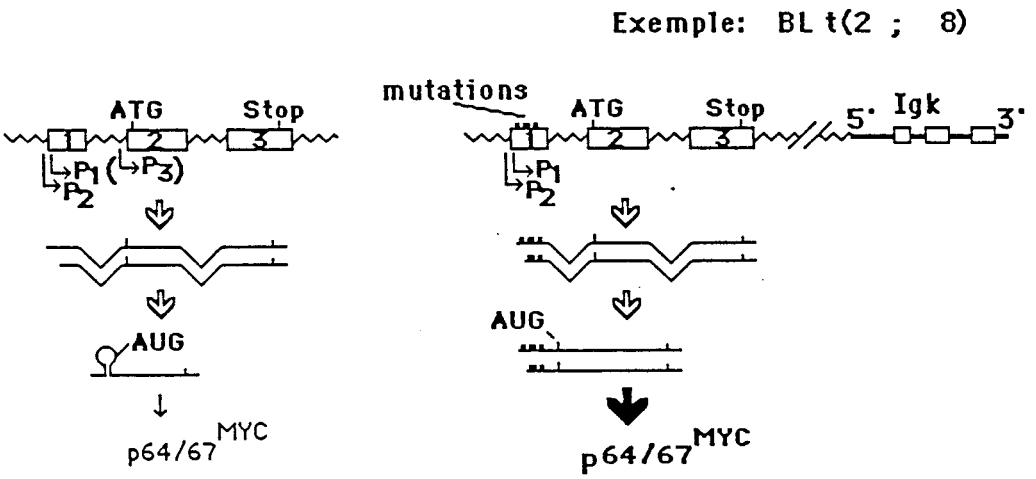


Figure 16 B : Différents modes d'activations du gène MYC dans les lymphomes de Burkitt. (suite)

(Saito et al., 1983);

-une modulation de la stabilité des ARN a également été proposée (Dani et al., 1984; Piechaczyk et al., 1985). Ce type de mécanisme, en stabilisant l'ARN, serait responsable de l'accumulation accrue des messagers provenant des loci remaniés après translocations, (Adams et al., 1983; Perry, 1983) sans modification du taux de transcription (Piechazyck et al., 1985; Figure 16B).

Le mécanisme inverse (déstabilisation) serait responsable de la réduction du taux d'ARN observé dans les cellules en réponse à l'administration de β–interféron (Knight et al., 1985). L'interféron agirait en activant la synthèse du gène de la 2'-5' oligoadénylate synthétase qui participerait avec la RNAse L à la création d'une activité endonucléasique qui dégraderait sélectivement les ARN possédant une structure partiellement double brin (Knight et al., 1985). Cette régulation, contrôlant l'activité d'un gène par une stabilisation modulable de son messager, pourrait être assez générale et a déjà été observée pour d'autres gènes dont le taux d'ARN messagers varie avec la différenciation (Mangiarotti et al., 1985).

-Contrôles à la traduction du gène :

-Le gène MYC induit la synthèse d'au moins quatre protéines légèrement différentes par leur poids moléculaire (64 à 68 kd) dont le degré de phosphorylation varie. Les phospho-protéines pp64 et pp67 ont une durée de vie courte. Les protéines p65 et p68 ne sont pas phosphorylées, p65 a une demie-vie courte, et pourrait être le précurseur de la p68 à durée de vie longue (Persson et al.,1986). Toutefois, le rôle biologique de ces différentes protéines n'est pas encore connu (Ramsay et al., 1984).

Les différents éléments de contrôle de l'oncogène MYC, remanié ou non sont tributaires du degré de différenciation de la cellule lymphoïde pour pouvoir s'exprimer. Ce point a pu être démontré grâce à la construction d'hybrides somatiques résultant de la fusion de cellules de lymphome de Burkitt avec différents types de cellules murines : lymphoblastes, plasmocytes ou fibroblastes (Croce et al., 1984; Nishikura et al., 1984; Feo et al., 1985; Nishikura et al., 1985). Ainsi, dans ces hybrides somatiques, l'oncogène remanié après translocation (8 ; 14) peut s'exprimer si les cellules de lymphome sont fusionnées avec des cellules de plasmocytome murin, mais il reste silencieux si la fusion est réalisée avec une cellule lymphoblastoïde humaine (Croce et al., 1984; Nishikura et al., 1985). Ainsi, la diversité des mécanismes mis en cause dans l'activation de MYC pourrait être notamment due au fait que les cellules transformées ne sont pas toutes au même stade de différenciation .

Le mécanisme de translocation conduit toujours MYC à la place qu'occuperaient les séquences codant pour les régions variables des gènes d'immunoglobulines (à des distances très variables selon le type de la translocation). Or, ces régions variables sont le siège de nombreuses mutations somatiques destinées à augmenter la diversité des anticorps synthétisés; les séquences MYC placées dans cet environnement pourraient muter de façon importante ce qui pourrait perturber la régulation ou la fonction de ce gène (Rabitts et al., 1983).

D'autres types de lymphomes n'impliquant pas l'oncogène MYC, mais les gènes des chaînes

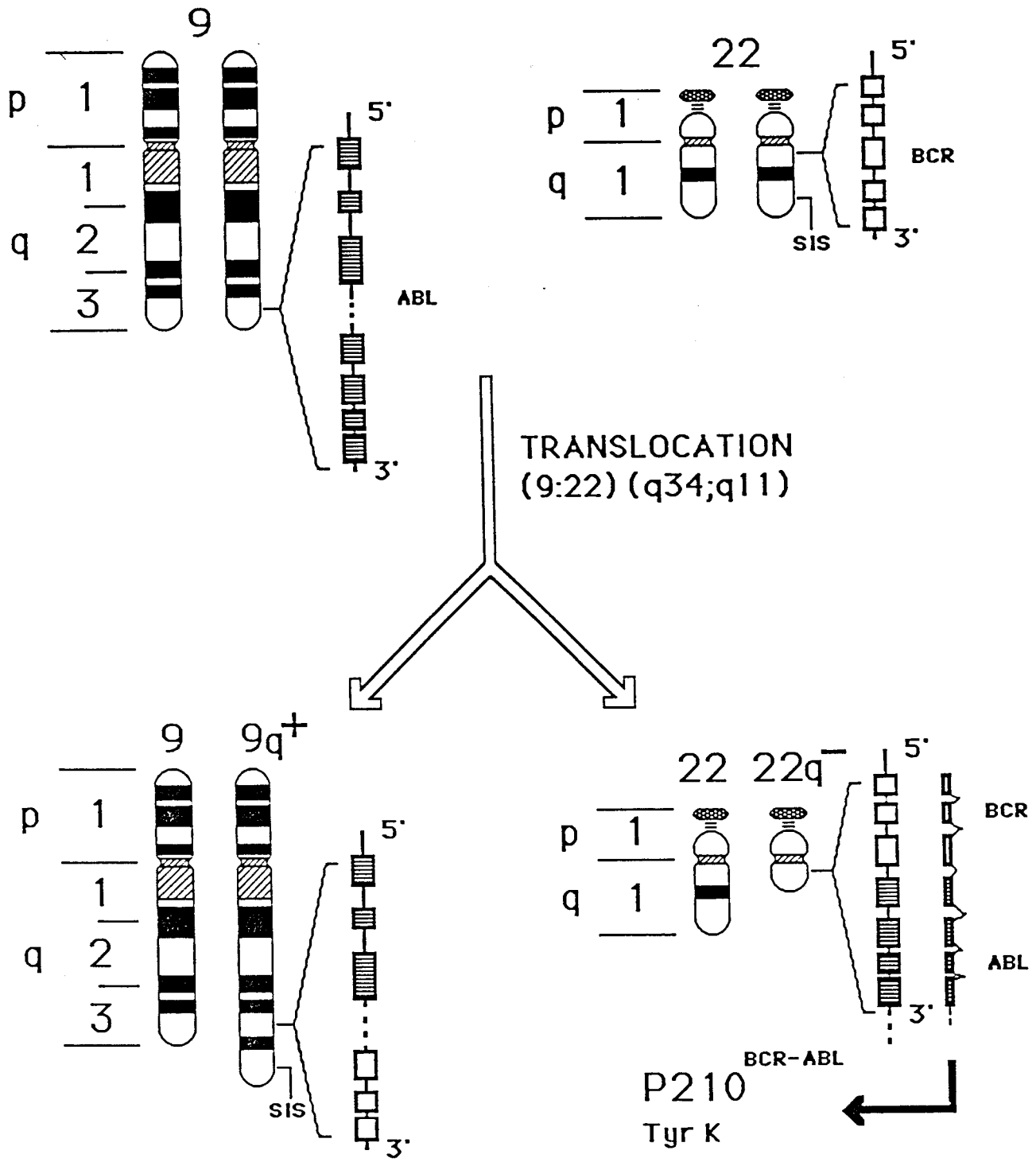


Figure 17 : Translocation 9:22 et activation de l'oncogène ABL.

lourdes d'immunoglobulines ont été décrits. Il s'agit des lymphomes B diffus ainsi que de certaines leucémies lymphoïdes chroniques de type B dans lesquelles une translocation chromosomique caractéristique a pu être mise en évidence, (11 ; 14) (q13 ; q32). Un autre type de lymphome (lymphome folliculaire) présente lui une translocation (14 ; 18) (q32 ; q21). Dans ces deux cas, la translocation permet l'expression d'une séquence cellulaire dont l'implication dans le processus oncogène est fortement suggérée par la fréquence de ce type de remaniement dans les leucémies citées. Ces séquences ont été appelées BCL1 (B cell lymphoma) pour la translocation (11 ; 14) et BCL2 pour la translocation (14 ; 18) (Tsujimoto et al., 1985). L'implication des gènes codant pour les chaînes (lourdes et légères) d'immunoglobulines dans les translocations chromosomiques est peut-être la conséquence des remaniements spectaculaires qui affectent ces gènes lors de la différenciation des lymphocytes en cellules productrices d'immunoglobulines (Milstein, 1985).

2) Le chromosome philadelphie

La leucémie myéloïde chronique est caractérisée par la prolifération et l'accumulation de cellules myéloïdes et de leurs précurseurs. Cette maladie est cliniquement divisée en deux phases. La première étape, d'une durée de 3 à 4 ans voit augmenter le compartiment cellulaire des précurseurs myéloïdes. Cette étape de leucémie chronique est suivie d'une crise blastique de 3 à 6 mois caractérisée par la diminution du potentiel de différenciation de ces cellules, ainsi que par leur incapacité à répondre aux CSF. 90 à 95 % des patients possèdent dans leurs cellules leucémiques une anomalie chromosomique spécifique représentée par un raccourcissement du chromosome 22 qui est alors appelé chromosome Philadelphie (Yunis, 1983). Ce chromosome 22 particulier ($22q^-$) est le produit d'une translocation (9 ; 22) (q34 ; q11) (Figure 17). Deux oncogènes sont proches du point de translocation, l'oncogène ABL (9q34) et SIS (22q12) (Table VI).

Une étude attentive des taux d'expression et de la structure de ces oncogènes dans les leucémies myéloïdes chroniques a permis d'impliquer l'oncogène ABL, dans la translocation (Heisterkamp et al., 1983). Sur le chromosome 22, la translocation affecte toujours une région d'ADN de 5.8 kb, qui a été appelée BCR (pour break point cluster region) (Groffen et al., 1984a). L'implication de cette séquence nucléotidique, en plus de l'oncogène ABL dans l'étiologie de la leucémie myéloïde chronique parait probable du fait de son constant réarrangement même en l'absence du marqueur chromosomique (Bartram et al., 1985). L'association des séquences ABL et BCR en une seule unité transcriptionnelle est la conséquence de cette translocation (Shtivelman et al., 1985, Figure 17). Le rôle exact de l'un et de l'autre de ces deux gènes dans la transformation myéloïde n'est pas connu, mais il est possible que le produit de la séquence BCR puisse servir à activer l'oncogène ABL dans la protéine de fusion (Heiskerkamp et al., 1985). En effet, le produit de l'oncogène ABL remanié possède une activité tyrosine kinase (mimant en cela le produit de l'oncogène v-abl), alors que le produit de l'oncogène ABL normal en est dépourvu (Konopka et al, 1984).

Ainsi, les translocations peuvent affecter l'intégrité (comme dans le cas d'ABL) ou la régulation (comme dans le cas de MYC) d'un oncogène. D'autres modes d'activation moins

Gène activé	Espèce	Tumeur ou lignée	Origine du promoteur viral	Références
c-myc	poulet	Lymphome B	ALV (RAV-1, RAV-2, td-SRA)	Hayward et al., 1981
	souris	Lymphome T	C.P.A (Intra Cisterral Particule A)	Greenberg et al., 1985
		Plasmocytome	MCF	Corcoran et al., 1984
	rat		MLV	Tsichlis et al., 1985
	chat	Lymphome T	FeLV	Neil et al., 1984
c-erb B	poulet	Erythroblastose	ALV (RAV-1)	Raisnes et al., 1985
c-myb	souris	Tumeurs myéloïdes	MLV	Shen-Ong, 1986
c-mos	souris	Plasmocytome	C.P.A	Canaani et al., 1983
pim 1	souris	Lymphome T	MCF	Cuypers et al., 1984
int 1	souris	Carcinome mammaire	MMTV	Peters et al., 1984
int 2				
int 41		Carcinome mammaire et rénal	MMTV	Garcia et al., 1986
Mlvi 1	rat	Lymphome T	MLV	Tsichlis et al., 1985
Mlvi 2				
Mlvi 3				
Mis 1	rat	Lymphome T	MLV	Jolicœur et al., 1985
IL-3	souris	Promyélocytique (WEHI 3B)	C.P.A	Ymer et al., 1985
IL-2	singe	Lymphome T (MLA-144)	GLV	Chen et al., 1985
fim 1	souris	Leucémies Myéloblastiques	F-MLV	Sola et al., 1986
fim 2				

TABLE VIII : Activation de gènes par des promoteurs viraux.

spectaculaires peuvent aboutir à des résultats semblables.

c-Activation transcriptionelle des c-onc

La translocation d'un oncogène à proximité d'une séquence activatrice de la transcription peut être responsable de sa surexpression dans la cellule. Ce mode d'activation a déjà été observé pour l'oncogène c-myc (Hayday et al., 1984; Corcoran et al., 1985). Un autre mode d'activation transcriptionnelle des oncogènes est indépendant des translocations chromosomiques. Il s'agit dans ce cas de l'insertion dans l'environnement du c-onc d'un promoteur fort, d'origine virale, et plus particulièrement d'un LTR rétroviral. L'oncogène peut être intact ou interrompu par l'insertion du LTR. Le LTR peut fournir son propre promoteur, ou uniquement ses séquences activatrices de la transcription. Dans ce cas, l'insertion du LTR peut se faire en 3' du c-onc, l'activation transcriptionnelle étant possible sur des distances de plusieurs kilobases. La LTR activation est essentiellement produite par des rétrovirus dépourvus d'oncogènes . Plusieurs oncogènes connus (c-erbB, c-mos, c-myc...) ont ainsi pu "bénéficier" de ce mode d'activation (Tableau VIII). L'étude des séquences nucléotidiques sans équivalent viral connu, activées par les LTR de rétrovirus à permis d'impliquer dans la transformation un certain nombre d'autres gènes dont l'activité oncogène directe ou indirecte est suspectée.

1) Activation des c-onc par des LTR de rétrovirus endogènes

L'activation de gènes cellulaires par les LTR de virus endogènes a pu être observée essentiellement dans l'espèce murine où ces virus sont très abondants et peut-être capables de se transposer dans le génome cellulaire (Kuff et al., 1983). Deux gènes oncogènes "authentiques", c-myc et c-mos ont ainsi pu être impliqués dans des plasmocytomes murins (Tableau VIII). Un autre gène cellulaire, codant pour un facteur de croissance de cellules hématopoïétiques (facteur IL3) est également activé par un LTR de particule A intra-cisternale dans une cellule de souris promyélocytique produisant une grande quantité de ce facteur (Ymer et al., 1985). L'activation transcriptionnelle de gènes codant pour des facteurs de croissance peut être l'un des mécanismes amenant une cellule à devenir tumorigène. En effet, l'émergence de cellules hématopoïétiques dont la croissance est indépendante de ces facteurs est un événement fréquemment observé dans la progression de ces cellules vers la tumorigénicité (Heard et al., 1984; Sporn et Roberts, 1985 Figure 9). Récemment, Lang et al. (1985) ont pu montrer que l'infection de cellules hématopoïétiques non tumorigènes, dépendantes de facteur de croissance GM-CSF pour proliférer, avec un rétrovirus construit *in vitro* et possédant le gène codant pour ce facteur, rendait ces cellules tumorigènes.

2)Activation de c-onc par les LTR de rétrovirus exogènes

De nombreuses espèces animales sont susceptibles de présenter une activation transcriptionnelle de gènes cellulaires dans certains types de néoplasmes et ce, pour plusieurs virus différents (Tableau VIII). L'oncogène le plus fréquemment activé se trouve être c-myc qui est

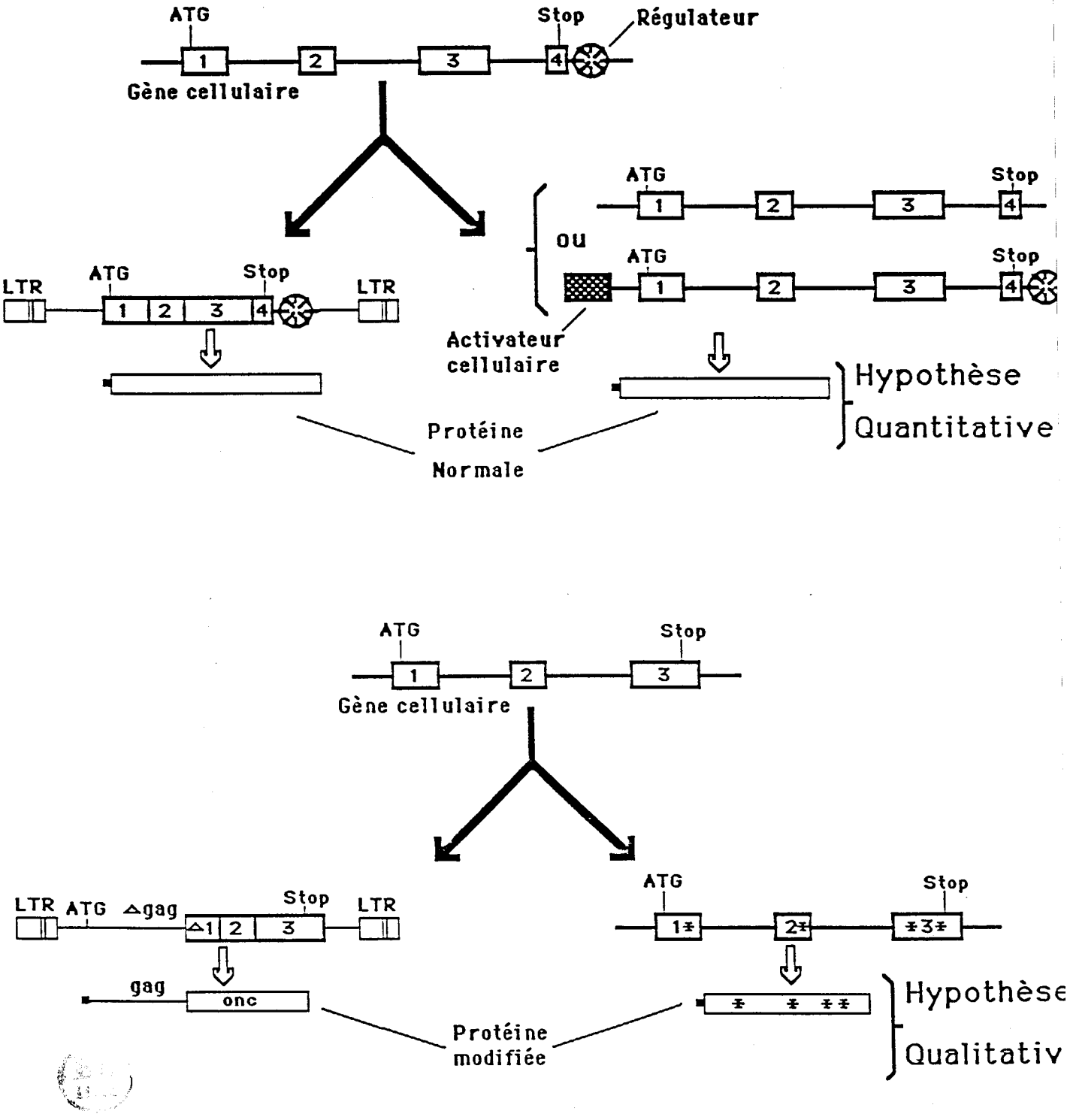


Figure 18 : Activation de c-onc par modification de la séquence nucléotidique du gène.

⇨ : Transcription

essentiellement impliqué dans les lymphomes T du chat, du rat et de la souris, induits respectivement par les virus FeLV, MuLV et MCF . L'activation transcriptionnelle de c-myc par les LTR de rétrovirus exogènes à également été signalée dans des lymphomes de type B, chez la souris et le poulet. L'activation de ce gène est en effet, ainsi que nous l'avons vu, un événement très précoce de la stimulation des cellules lymphoïdes B et T par différents agents mitogènes. L'activation transcriptionnelle d'un autre oncogène (c-erbB déjà identifié au sein d'un rétrovirus aviaire) a été vue dans le cas d'érythroblastoses induites par le virus Rav1 dans la souche de poulets L15 (Raines et al., 1985). L'insertion du LTR rétroviral se faisant à l'intérieur du gène, seule la partie 3' de celui-ci se trouve exprimée.

D'autres gènes candidats au statut d'oncogène ont pu être identifiés dans des lymphomes thymiques de rat (Tableau VIII). Ces oncogènes n'ont pas d'homologies avec des oncogènes connus et sont situés sur des chromosomes distincts (Jolicoeur et al., 1985; Tsichlis et al., 1985). Toutefois ils n'ont pas encore pu être impliqués de façon causale dans l'apparition de ces lymphomes. Dans les tumeurs solides, trois régions de l'ADN sont les sites d'insertion privilégiés du virus de la tumeur mammaire de la souris (MMTV) dans les carcinomes induits par ce virus (Peters et al., 1984; Garcia et al., 1986). La synthèse d'un ARN correspondant à l'activation transcriptionnelle de la région int-1 et int-41 est observée (Fung et al., 1985; Garcia et al., 1986) quand le virus s'insère à cet endroit . Une intégration du virus MMTV avec activation transcriptionnelle du site int-41 a également été observée dans le cas d'un carcinome rénal. Cela suggère l'implication d'un produit codé par ce gène dans l'apparition de la tumeur.

Dans certaines tumeurs, une activation simultanée des gènes int-1 et int-2 a même été vue, ces deux oncogènes pouvant peut-être coopérer dans la transformation cellulaire (Peters et al., 1986). En effet, le produit du gène int-1, artificiellement exprimé dans des cellules épithéliales, semble capable d'induire une transformation partielle de ces cellules (Brown et al., 1986).

Enfin, un gène cellulaire codant pour un facteur de croissance (IL2) produit par des lymphocytes T activés se trouve sous le contrôle transcriptionnel d'un LTR de virus leucémogène de gibbon dans les cellules d'un lymphome de type T (MLA-144, Chen et al.,1985).

Ainsi, aucune spécificité particulière de l'oncogène activé ou du type cellulaire impliqué n'est mise en évidence dans la transformation induite par les LTR de rétrovirus exogènes ou endogènes. Cela est probablement dû au fait que les rétrovirus s'insèrent en de très nombreux endroits dans la cellule et que les mécanismes de transformation, pour une cellule donnée, sont multiples.

d-Activation de c-onc par modification de la séquence nucléotidique

L'augmentation ou l'acquisition d'une activité transformante pour un oncogène donné peut être tributaire de modifications (minimes ou étendues) affectant cet oncogène. Trois types de modifications ont pu être recensés :

1) des modifications résultant de la fusion de la séquence codante de l'oncogène avec la portion codante d'un autre gène cellulaire (ou plus fréquemment un gène de structure rétroviral) aboutissant à la synthèse d'un produit hybride (Figures 17 et 18);

2) des modifications plus subtiles résultant de mutations ponctuelles affectant la séquence

codante de cet oncogène (Figure 18);

3) des modifications affectant les séquences non codantes du gène oncogène. Dans ce cas, c'est le produit normal qui est actif une fois que son ARN est amputé des séquences qui permettent à un contrôle post transcriptionnel de s'exercer (Figure 18).

L'importance des modifications structurales, induites grâce à la fusion de séquences exogènes, dans l'acquisition des propriétés transformantes du produit des oncogènes a été particulièrement bien étudiée grâce aux rétrovirus oncogènes. Ces derniers ayant transduit des c-onc synthétisent dans de nombreux cas un produit oncogène hybride contenant une portion (plus ou moins importante) du produit des gènes de structure (essentiellement gag ou env). L'importance de ces fragments rétroviraux au sein du produit oncogène est variable selon le type d'oncogène et le type cellulaire dans lequel ils s'expriment. Le clonage moléculaire du génome de ces virus et les manipulations menées *in vitro* à l'aide des techniques du génie génétique ont permis de préciser le rôle de ces séquences exogènes dans l'activité transformante.

1) Rôle des séquences exogènes dans l'activité transformante des c-onc

Un des exemples les plus frappants de l'importance des séquences rétrovirales dans l'expression d'une activité transformante pour le produit d'un oncogène, est apporté par l'oncogène v-abl. Le virus A-MuLV est un virus murin défectif pour la réplication qui a recombiné avec le gène cellulaire c-abl (Table I). Ce virus code pour une protéine de fusion gag-abl de poids moléculaire variable selon les isolats de ce virus mais contenant 30 kd amino terminaux codés par le gène gag. Les poids moléculaires de la protéine varient entre 90 et 160 kd, mais toutes ces protéines possèdent une activité tyrosine kinase. Ces virus induisent *in vivo* des sarcomes ainsi que des leucémies lymphoïdes de type pré B et sont capables de transformer *in vitro* des cellules lymphoïdes et fibroblastiques. Le clonage moléculaire du provirus a permis, entre autre, de construire des mutants amputés de portions variables des séquences gag et abl du virus. L'introduction du virus résultant dans des fibroblastes ou des cellules lymphoïdes montre que les séquences virales, et plus précisément l'extrémité NH2 de la séquence gag est indispensable pour l'expression du pouvoir transformant de la protéine de fusion dans les cellules lymphoïdes mais pas dans les cellules fibroblastiques (Prywes et al., 1983). Une amputation partielle du gène gag en aval de ses 34 premiers acides aminés est sans effet sur la transformation fibroblastique qui est maintenue, mais abolit toute transformation lymphoïde. Cette différence est dûe à une déstabilisation de la protéine amputée dans les cellules lymphoïdes (Prywes et al., 1985). L'importance de la portion amino terminale d'origine exogène dans l'activation du pouvoir transformant du produit abl peut s'exercer à deux niveaux :

1)En conférant à la protéine une activité tyrosine kinase. En effet, la protéine c-abl normale ne présente pas d'activité tyrosine kinase décelable (Konopka et al., 1984). Cette même protéine remaniée après la recombinaison du gène ABL avec le locus BCR dans la leucémie myéloïde chronique (Figure 17) (Hiesterkamp et al., 1985) possède cette activité (Konopka et al., 1984). Dans ce cas la portion NH2 terminale codée par les séquences du gène BCR pourrait jouer le même rôle que les séquences gag dans le cas de la protéine virale. L'activité tyrosine kinase (essentielle

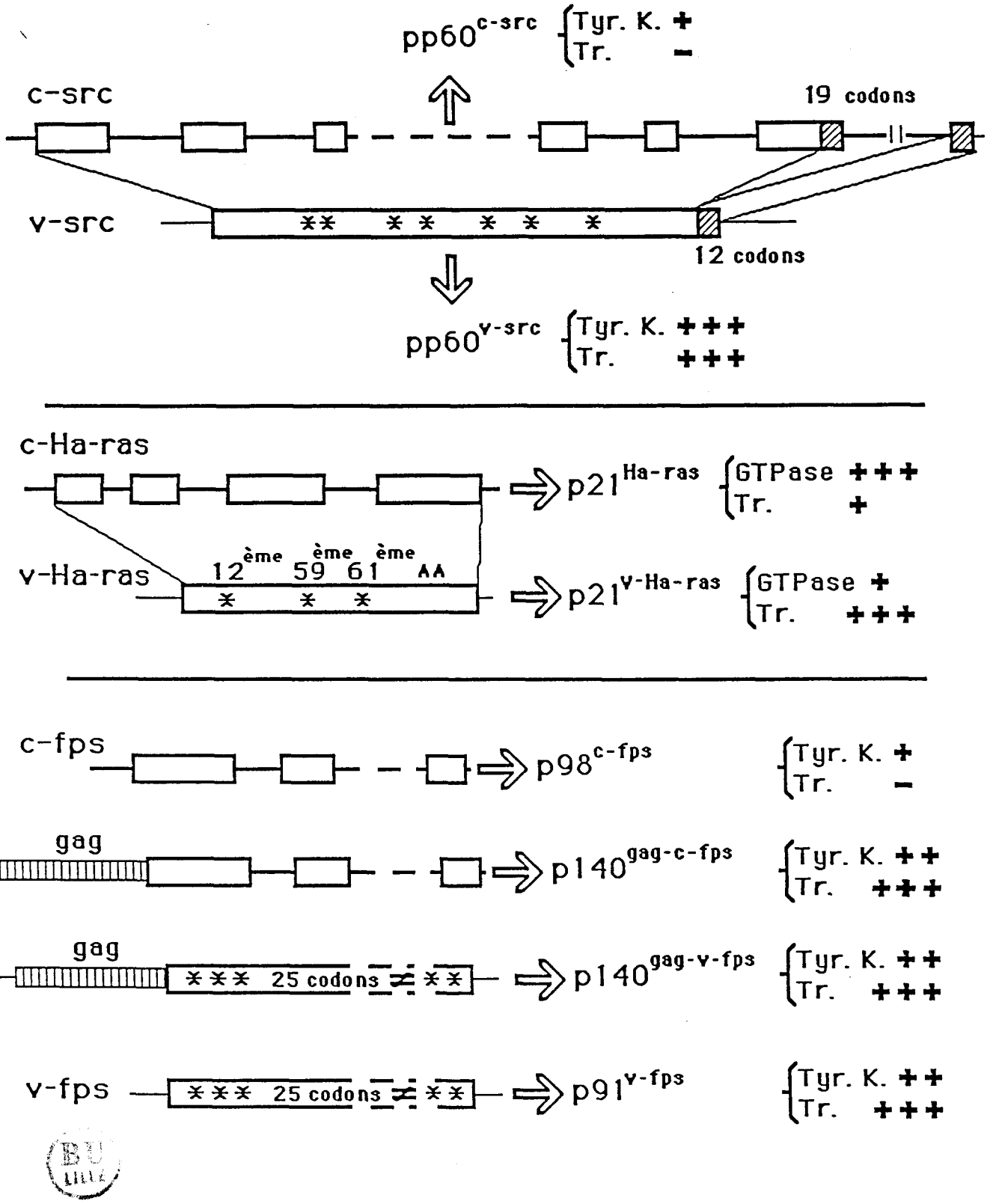


Figure 19 : Trois exemples d'activation de c-onc.

* : Mutation ponctuelle

Tyr. K. : activité tyrosine kinase

Tr. : transformation

pour l'expression du pouvoir transformant de ce produit oncogène) pourrait être tributaire d'une structure particulière de la protéine abl.

2) Cette portion amino terminale pourrait également être importante pour obtenir une localisation de la protéine dans le compartiment cellulaire adéquat. Les acides aminés de la portion codée par le gène gag sont déterminants dans la localisation de la protéine de fusion gag-abl du virus A-MuLV dans la membrane cytoplasmique, cette localisation conditionnant peut-être à son tour la transformation cellulaire.

Un autre exemple de l'importance de la portion protéique codée par le gène gag dans l'activité transformante d'une protéine oncogène s'observe dans le cas de l'activation du produit du gène fps. Le gène fps ayant recombiné avec un virus aviaire (Table I) (virus sarcomatogène de Fujinami ou FSV) est exprimé sous la forme d'une protéine de fusion de 140 kd de poids moléculaire (Feldman et al., 1980). Cette protéine est également douée d'une activité tyrosine kinase (Feldman et al., 1980). La suppression des séquences du gène gag dans le provirus cloné est sans effet sur l'activité transformante du virus modifié (Foster et Hanafusa, 1983, Figure 19).

Cependant l'insertion du gène c-fps (qui code dans l'espèce aviaire pour une protéine de 98 kd de poids moléculaire douée d'une activité tyrosine kinase, Table I; Mattey-Prevot et al., 1982), à la place du gène v-fps au sein du virus FSV dépourvu des séquences gag, aboutit à la création d'un virus non transformant. La réinsertion de gag dans ce virus inactif recrée une protéine de fusion gag-c-fps transformante (Foster et al., 1985). A côté de ces exemples dans lesquels la séquence virale exogène a pu être montrée importante, il existe d'autres cas de gènes oncogènes hybrides pour lesquels le rôle des séquences exogènes n'est pas encore connu. Ainsi, l'oncogène fgr, isolé à partir d'un virus sarcomatogène félin (Gr-FeSV), code pour une protéine de 70 kd de poids moléculaire contenant les déterminants amino terminaux (p15) codés par le gène gag, puis 128 acides aminés codés par une portion du gène d'actine et enfin le reste de la protéine codé par le gène fgr (Table I; Naharro et al., 1984). Un oncogène récemment mis en évidence grâce à la technique de transfection de l'ADN de cellules transformées sur cellules NIH_{3T3}, TRK, (provenant d'un carcinome du colon ascendant, Pulciani et al., 1982; Table IV) se révèle également être une fusion de deux gènes cellulaires. Une portion de TRK (221 acides aminés) serait synthétisée à partir d'un fragment de gène codant pour une tropomyosine non musculaire, le restant (420 acides aminés) étant synthétisé par une séquence nucléotidique codant probablement pour une protéine de la famille des tyrosine kinases (Martin-Zanca et al., 1986). Dans ce cas la portion tropomyosine pourrait activer la portion tyrosine kinase de la molécule en modifiant sa structure ou sa localisation subcellulaire.

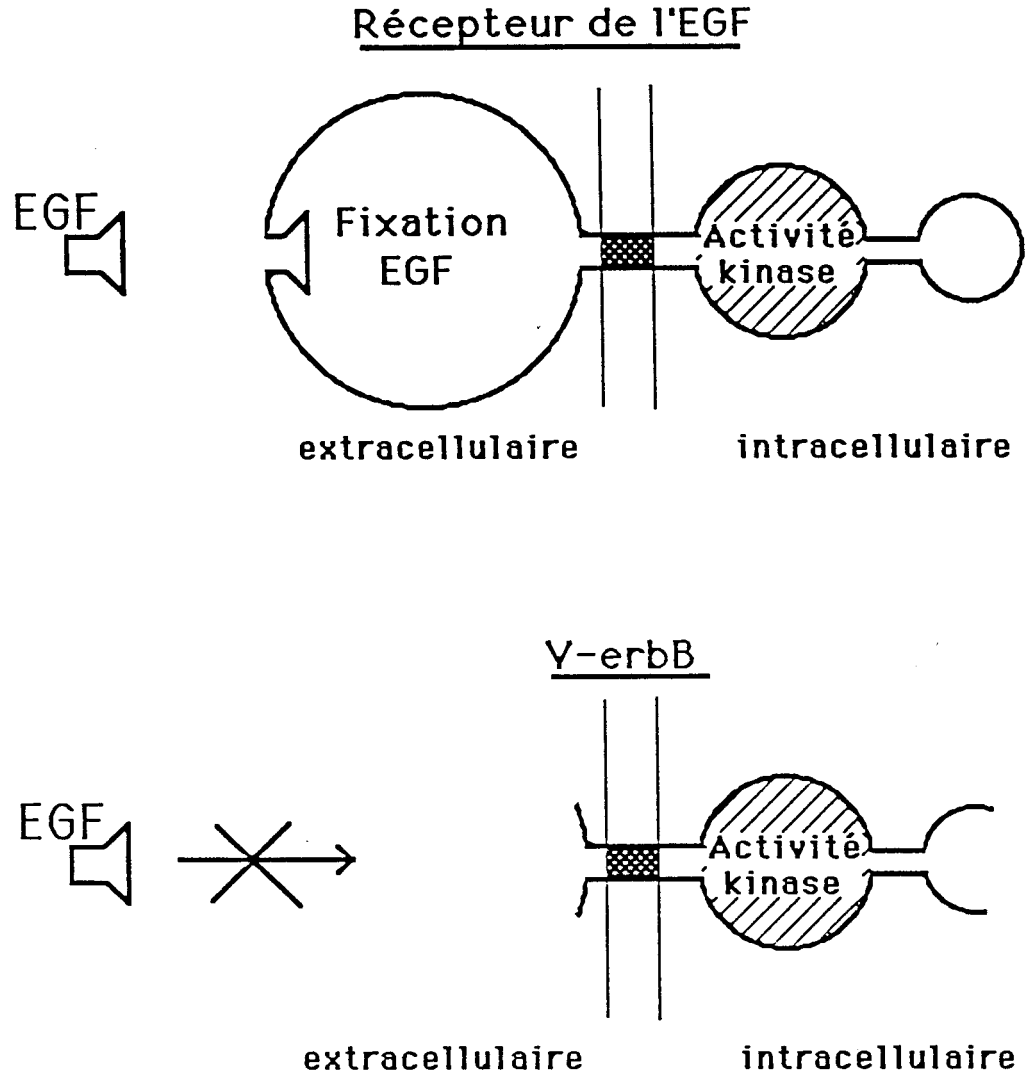
Un autre exemple de gène cellulaire hybride est apporté par l'oncogène ret. Cet oncogène obtenu par transfection de l'ADN d'un lymphome du type T sur NIH_{3T3} semble s'être créé au cours du processus de transfection lui-même (Takahashi et al., 1985) à partir de deux séquences d'ADN distinctes (Table IV). Enfin, nous mentionnerons un autre produit oncogène hybride, la $P135^{gag\text{-}myb\text{-}ets}$ du virus aviaire E26. Ce virus induit une érythroblastose accompagnée de la prolifération de cellules myéloïdes *in vivo*. Son produit oncogène contient à son extrémité NH2 272 acides aminés codés par le gène gag (la p19 et une partie de la p27) 283 acides aminés codés par le

gène myb tronqué et enfin 491 acides aminés ets (Leprince et al., 1983; Nunn et al., 1983; Nunn et al., 1984). La participation des séquences ets et/ou gag dans l'activité transformante de la P135 n'est pas encore démontrée mais semble probable dans la mesure ou le virus AMV (Avian Myeloblastosis Virus) qui ne contient que l'oncogène myb n'induit pas d'érythroblastose mais une myéloblastose *in vivo* (Raddke et al., 1982; voir la section résultats).

2) Rôle des mutations dans l'activation des c-onc

L'importance des mutations ponctuelles dans l'activation d'un oncogène a initialement été mise en évidence pour le produit codé par les gènes ras. La famille des oncogènes ras code pour des protéines de 21 kd de poids moléculaire très étroitement apparentées. Ces protéines ont la propriété de fixer le GTP, et sont présentes très tôt dans l'évolution puisque, nous l'avons vu, la levure possède deux gènes extrêmement proches des gènes ras mammifères (Powers et al., 1984). Les $p21^{v\text{-}Ha\text{-}}$ et v-Ki-ras possèdent en plus de cette activité fixatrice du GTP la capacité de s'autophosphoryler (sur la thréonine, résidu 59) (Shih et al., 1982; Taparowsky et al., 1983). La protéine cellulaire c-Ha-ras ne possède pas cette propriété, le codon 59 codant pour une alanine (Taprowski et al., 1983). En plus de la mutation sur le $59^{ème}$ acide aminé, les gènes v-ras portent une autre mutation sur le $12^{ème}$ acide aminé (Gly dans le c-ras, Arg dans v-Ha-ras et Ser dans v-Ki-ras) (Taparowski et al., 1983). Une surexpression du produit du gène c-Ha-ras normal dans des cellules de souris NIH_{3T3} est suffisante pour transformer ces cellules (cette surexpression est obtenue après activation transcriptionnelle du gène Chang et al., 1982). Toutefois, l'efficacité transformante de cette construction, comparée à celle d'une construction similaire contenant le v-Ha-ras est fortement réduite, et le produit du gène normal ne transforme pas des cellules primaires non immortalisées (Spandidos et Wilkier, 1984).

L'importance des mutations dans l'activation du pouvoir transformant du produit des gènes ras a pu être mise en évidence directement de deux façons. Tous les oncogènes ras activés dans des tumeurs ou dans certaines lignées cellulaires possèdent une de ces mutations caractéristiques (la plus fréquente étant la mutation sur le codon du $12^{ème}$ acide aminé, Reddy et al., 1982; Tabin et al., 1982; Capon et al., 1983; Taparowski et al., 1983). Enfin, la mutagénèse chimique à l'aide de bisulfite de sodium, réalisée sur le gène Ha-RAS1 normal cloné dans un plasmide, a permis de montrer que les mutations affectant les codons des $12^{ème}$, $13^{ème}$, $59^{ème}$ et $63^{ème}$ acides aminés étaient chacune capable d'activer le pouvoir transformant de ce gène (Fasano et al., 1984a). Des expériences de mutagénèse dirigée, réalisées *in vitro* sur le codon du $12^{ème}$ acide aminé ont permis de montrer que la substitution du physiologique Gly par n'importe quel autre acide aminé (à l'exception de la proline) permettait d'activer le pouvoir transformant de Ha-RAS1 (Seeburg et al., 1984). Cette observation permet d'expliquer pourquoi une substitution du $12^{ème}$ acide aminé de la p21 est la plupart du temps retrouvée dans les tumeurs où cet oncogène est impliqué. D'autre part, Gly où Pro brise la structure hélicoïdale de l'extrémité NH2 de la p21, et ce sont les deux seuls acides aminés qui n'activent pas le pouvoir transformant de cette protéine (Seeburg et al., 1984). Ainsi cette minime modification structurale, apportée par la modification d'un seul nucléotide au niveau de l'ADN, pourrait expliquer l'activation du gène ras. Cette activation semble dépendre



Conséquences de ces clivages

- Message mitogène délivré en continu
- Plus d'internalisation du récepteur lié à l'EGF
- Perte des mécanismes régulateurs

Figure 20 : Activation d'un gène oncogène par amputations dans sa séquence nucléotidique.

notamment d'une réduction du pouvoir GTPasique de la protéine (Mc Grath et al., 1984). La diminution de ce pouvoir GTPasique pourrait à son tour induire toute la cascade des événements aboutissant à la mitose et/ou à la différenciation cellulaire (Figure 12). D'autres oncogènes sont également susceptibles d'être activés par des mutations affectant leur séquence codante.

C'est le cas de l'oncogène neu, isolé par le test de transfection de l'ADN de cellules transformées (neuroblastomes) sur les cellules indicatrices NIH_{3T3} (Figure 14 et Table IV). Cet oncogène code pour une protéine de 185 kd transmembranaire, supposée jouer le rôle de récepteur pour un facteur de croissance non identifié et présente une homologie avec le produit du gène erbB (ce qui l'a fait initialement appeler erbB2). Des expériences de construction de gène chimérique réalisées *in vitro* entre l'allèle du gène neu normal (non transformant) et l'allèle transformant isolé de cellules tumorales de rats montrent qu'une mutation ponctuelle dans la région transmembranaire est suffisante pour que le produit de ce gène soit transformant (Bargmann et al.,1986).

L'oncogène c-src doit lui-aussi être modifié pour que son produit devienne transformant. Le gène c-src aviaire (Takeya et Hanafusa., 1983) code pour une pp60 ayant également une activité tyrosine kinase (Colett et al., 1979b). Toutefois, une sur-expression de la $pp60^{c\text{-}src}$ dans différents types de cellules, à l'aide d'un rétrovirus artificiel, ne permet pas la transformation de ces cellules (Iba et al., 1984; Parker et al., 1984). Une analyse attentive des propriétés biochimiques des deux types de pp60 montre que la protéine cellulaire possède une activité tyrosine kinase restreinte par rapport à la protéine virale (Coussens et al., 1985). Cette restriction est à la fois quantitative (portant sur la quantité des protéines phosphorylées dans la cellule infectée) et qualitative (concernant les cibles spécifiques de phosphorylations par ces protéines, Coussens et al., 1985). Les séquences nucléotidiques des gènes v-src et c-src présentent quelques différences qui pourraient être responsables du comportement différent de ces deux protéines. Le changement le plus important porte sur les acides aminés carboxy terminaux qui sont différents dans les deux produits. Les nucléotides codant pour les 12 derniers acides aminés de la $pp60^{v\text{-}src}$ proviennent de l'ADN situé en 3' de la fin du c-src. Ainsi le gène cellulaire code pour une pp60 avec 19 acides aminés carboxy terminaux absents de la $pp60^{v\text{-}src}$ du virus RSV-SRA (Takeya et Hanafusa, 1983; voir page 5 pour la nomenclature de ces virus) et 8 autres acides aminés diffèrent entre la $pp60^{c\text{-}src}$ et la $pp60^{v\text{-}src}$ (Figure 19). L'étude de nouveaux virus ayant recombiné avec l'oncogène c-src (Ikawa et al., 1986), ainsi que celle de variants spontanés ou artificiels du RSV construits *in vitro* et contenant le c-src (Levy et al., 1986; Yaciuk et Shalloway, 1986) montrent que deux types d'altérations sont susceptibles de rendre la $pp60^{c\text{-}src}$ transformante :

- une mutation ponctuelle dans la portion "tyrosine kinase",
- une modification de son extrémité carboxy terminale. Dans tous ces cas, l'activité kinase de la protéine modifiée est comparable à celle de la $pp60^{v\text{-}src}$.

Un autre exemple d'activation d'un produit d'oncogène par remaniements de la protéine codée est fourni par l'oncogène c-erbB. Cet oncogène qui code en fait pour le récepteur de l'EGF, semble activé après clivage de son extrémité amino terminale, qui est également la portion de la protéine qui fixe l'EGF (Figure 20; Downward et al., 1984; Ullrich et al., 1984; Henry et al., 1985). Le pouvoir transformant du gène c-erbB n'a pu être établi *in vitro* sur des fibroblastes (Lin et al.,

1986) et d'importantes informations concernant son activation sont fournies par l'étude d'érythroblastoses induites *in vivo* par le virus aviaire Rav1, dans la souche de poulets L15 (Fung et al., 1983; Raisnes et al., 1985; Beug et al., 1986). Dans 100% de ces érythroblastoses survenant chez 80% des poulets infectés, le gène c-erbB est activé par le LTR du provirus intégré dans l'intron situé en 5' du premier exon cellulaire retrouvé au sein du virus AEV (Henry et al., 1985; Raines et al., 1985; Beug et al., 1986). Cette LTR activation permet fréquemment la propagation d'un rétrovirus ayant recombiné avec l'oncogène (Yamamoto et al., 1983; Beug et al., 1986). Le clivage de la portion fixatrice de l'EGF semble crucial dans l'activation du pouvoir transformant du récepteur (tout au moins en ce qui concerne la transformation des cellules érythroïdes). Ce clivage permet peut-être au récepteur tronqué de délivrer son signal mitogène par l'intermédiaire de son activité tyrosine kinase constitutive (Gilmore et al., 1985; Kris et al., 1985). De plus, cette amputation permet au récepteur d'échapper à l'internalisation puis à la dégradation lysosomiale suivant la fixation de l'EGF (Lin et al., 1986). Le gène v-erbB du virus AEV possède également une amputation de son extrémité carboxy terminale (acides aminés 1154 à 1186 ,Hunter, 1984). Cette deuxième amputation semble indispensable pour que le récepteur tronqué soit capable de transformer les fibroblastes (Beug et al., 1986). Cette portion absente dans l'oncogène viral contient un site d'autophosphorylation (tyrosine 1173). Une fois phosphorylé en réponse à la fixation de l'EGF, le fragment porteur de cette tyrosine pourrait interagir avec le site catalytique du récepteur (Hunter, 1984). Le mécanisme par lequel son amputation permettrait la transformation des fibroblastes reste obscur.

Une anomalie de la portion 3' des c-onc codant (probablement) pour des protéines réceptrices de facteurs de croissance et douées d'une activité tyrosine kinase, semble la règle dans l'activation de ces gènes ayant recombiné avec un rétrovirus. C'est le cas, nous l'avons vu, pour les gènes c-src et c-erbB, mais cela semble également être vrai pour les gènes c-fms et c-ros (ce dernier possède une certaine homologie avec le gène codant pour le récepteur de l'insuline). Ainsi, la perte constante de la tyrosine carboxy terminale semble, seule ou avec d'autres anomalies associées, un événement clé de l'activation de ce type d'oncogènes (Coussens et al., 1986b).

Enfin, le pouvoir transformant de l'oncogène cellulaire codant pour la p53 peut être indifféremment activé à l'aide d'un promoteur fort de transcription (tel le LTR du RSV) ou à l'aide d'une amputation de 157 bp entre les nucléotides 163 et 320. Cette amputation permet la synthèse d'une protéine stable qui peut exercer son effet biologique même si le gène est exprimé avec un promoteur faible de transcription (Jenkins et al., 1985).

3)Activation des c-onc après amputation de séquences nucléotidiques non codantes

Ce mode d'activation particulier ne porte pas sur l'intégrité du produit codé, mais sur celle des séquences régulatrices dont l'action s'exercera à un niveau transcriptionnel ou post-transcriptionnel.

L'exemple le plus significatif du mode d'activation transcriptionnel est apporté pour l'oncogène c-mos. Cet oncogène, identifié nous l'avons vu, grâce au virus sarcomatogène murin Mo-MSV (Table I), semble régulé d'une façon particulièrement stricte, puisque son expression n'a

pu être décelée, et ce à des taux très faibles, que dans les organes génitaux (testicules et ovaires) de souris (Propst et van de Woode, 1985). Cet oncogène semble contrôlé par la présence d'une séquence inhibitrice de la transcription située en 5' de la séquence codante du gène. Une activation du c-mos nécessite deux événements : la ligature du gène avec les séquences d'un LTR murin, ainsi que l'amputation de la séquence inhibitrice (Wood et al., 1984).

Le modèle le plus démonstratif d'une activation par amputation de séquences impliquées dans une régulation post-transcriptionnelle est apporté pour l'oncogène c-fos. Le produit de c-fos, pour transformer des fibroblastes de souris NIH_{3T3} doit être sous le contrôle transcriptionnel d'un promoteur rétroviral. Il doit également être amputé d'une séquence de 67 nucléotides riche en adénines et thymidines située en 3' du signal de polyadénylation du gène, donc en dehors de sa séquence codante (Miller et al., 1984; Meijlink et al., 1985). Il faut noter que ces deux conditions (LTR activation et amputation des 67 bp) ne sont pas requises pour qu'un gène c-fos exogène puisse s'exprimer dans des cellules de tératocarcinome de souris (Muller et Wagner, 1984; nous avons vu dans le chapitre les contrôles de l'oncogénèse quelques exemples de ce type).

Ainsi, deux classes d'oncogènes peuvent être individualisées :

- les oncogènes du premier groupe, dont le produit normal est transformant (par exemple c-fos, c-mos, c-myc, p53, c-ras...), seront activés par des perturbations affectant leur régulation donc plus probablement des modifications importantes dans leur environnement génique (translocations, amplifications, amputations...);

- les oncogènes du deuxième groupe, exigeant une modification de leur séquence codante pour être transformants (c-erbB, neu, c-src...) seront essentiellement activés par des perturbations affectant de façon ponctuelle leur séquence codante, donc notamment par les carcinogènes chimiques.

Il est intéressant de noter que certains c-onc (c-ras, p53 ..) peuvent indifféremment être activés par les deux mécanismes; on peut s'attendre à les trouver plus souvent impliqués dans les tumeurs spontanées que les autres. Curiosité supplémentaire, les gènes dont le produit est à localisation nucléaire figurent tous (pour autant qu'ils aient été étudiés sous cet angle) dans le premier groupe de notre classification. Bien que l'on en soit réduit aux hypothèses, on peut supposer que ces protéines oncogènes sont dépendantes d'une structure très strictement définie, pour être actives au niveau de l'ADN (ou de l'ARN). D'autre part, ces protéines ne paraissent pas régulées à partir d'un contrôle de leur structure moléculaire, comme c'est le cas pour les protéines tyrosine kinases, ce qui limite le rôle des modifications structurales dans leur activation.

SYSTEME EXPERIMENTAL ET RESUME DES RESULTATS

D-SYSTEME EXPERIMENTAL

Notre travail a porté sur l'étude des virus défectifs des leucémies aiguës aviaires (DLV). La première partie des résultats présente la structure du génome de ces virus, ainsi que la caractérisation des oncogènes qu'ils contiennent. La deuxième partie du travail présente la structure de trois c-onc : c-erbB, c-myb et c-erbA isolés chez le poulet (animal d'origine) et chez l'homme à partir des v-onc correspondants. Enfin, la troisième partie présente une approche plus biologique du rôle des oncogènes identifiés grâce à l'étude des DLV.

Les DLV regroupent (Table IX) une dizaine de virus possédant en commun la propriété de s'attaquer notamment au système hématopoïétique de l'animal hôte (Figure 21). Ces virus sont défectifs pour la réplication et nécessitent la présence d'un virus auxiliaire (helper) pour se propager. Ils sont extrêmement agressifs, puisque la mort intervient pour 100 % des animaux de 5 à 30 jours après l'infection (Graf et Beug, 1978; RNA Tumor viruses, 1985). Le virus REV-T (lui-même prototype d'une famille plus vaste) se distingue des autres DLV par sa grande analogie avec les virus de mammifères. REV-T requiert la présence d'un virus auxiliaire particulier qui ne peut jouer le rôle de helper pour les autres DLV. Le virus S13 bien qu'isolé en 1935 n'est étudié que depuis peu. La biologie de ces virus est complexe et il faut pouvoir discerner dans la pathologie qu'ils induisent la part que prend le virus "helper". Ce travail a été effectué essentiellement durant la dernière décennie et s'est développé grâce à des systèmes de culture de cellules hématopoïétiques *in vitro* qui ont grandement contribués à éclaircir le pouvoir transformant de chacun des DLV (Graf et Beug, 1978).

Ainsi, le virus AEV qui induit *in vivo* une érythroblastose ainsi que des sarcomes (surtout en injection intra-musculaire) est capable de transformer *in vitro* des fibroblastes ainsi que des érythroblastes. Ces derniers prolifèrent et sont incapables de se différencier en érythrocytes (Gazzolo et al.,1980). L'obtention de virus AEV thermosensibles pour la maintenance de cet état indifférencié (la différenciation peut s'effectuer à 42°) a permis de montrer qu'au moins une protéine virale était impliquée dans ce blocage (Graf et al.,1978; Beug et al., 1982a; Samarut et Gazzolo, 1982).

Les virus MC29, CMII, MH2 et OK10 sont capables de transformer des cellules myéloïdes *in vitro* (Graf et Beug, 1978 ; Gazzolo et al., 1979), les virus MC29 et CMII étant capables d'induire une myélocytomatose *in vivo*. L'isolement de virus de ce type, thermosensibles pour la transformation des cellules hématopoïétiques n'a pu être possible que très récemment et pour MH2 seulement (Palmieri, 1986; von Weisäcker et al.,1986). Ce type de virus semble également interférer avec la différenciation ultérieure de la cellule myéloïde transformée, (Graf et Beug, 1978; Gazzolo et al., 1979). Cette différenciation peut s'achever à température non permissive dans les cellules myéloïdes transformées par le virus MH2 ts 41 (Palmieri 1986). Le virus MH2 permet la

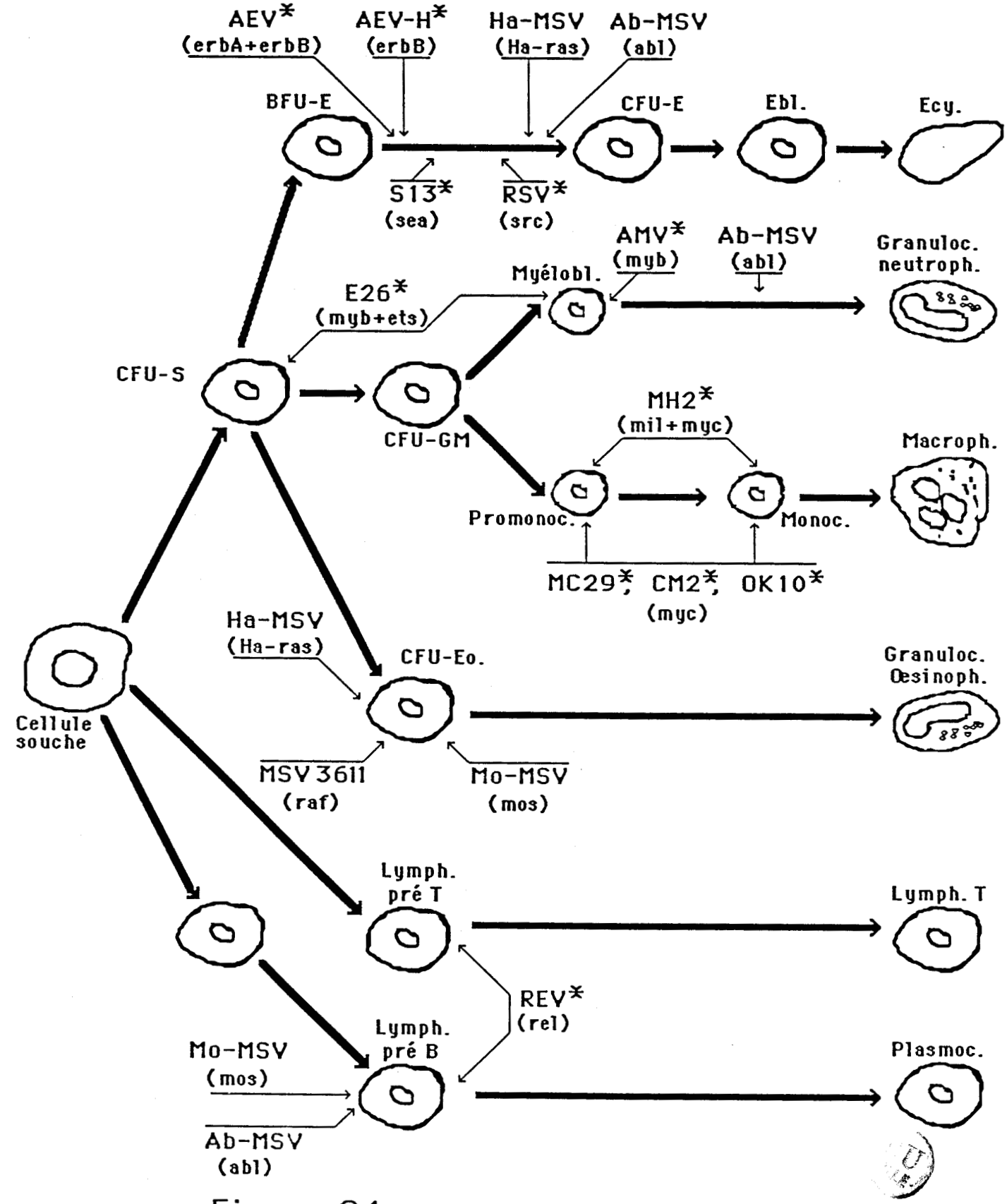


Figure 21 : Rétrovirus transformants et système hématopoïétique.

* rétrovirus aviaire

Virus	Maladies induites	Oncogènes	Année d'isolement
AEV-ES4	Erythroblastose, sarcome.	erb B, erb A	1933
AEV-H		erb B	1983
MC29	Myélocytomatose, carcinome.	myc	1964
CMII	Myélocytomatose.	myc	1964
OK10	Endothéliome.	myc	1979
MH2	Endothéliome, carcinome.	myc, mil	1927
AMV	Myéloblastose.	myb	1941
E26	Myéloblastose, érythroblastose.	myb, ets	1962
S13	Erythroblastose, sarcome.	sea	1935
REV-T	Réticuloendothéliose.	ret	1964

TABLE IX : Les virus défectifs des leucémies aviaires.

croissance de cellules myéloïdes transformées en absence de cMGF, ce que ne permettent pas MC29, CMII, et OK10. En effet, ces virus transforment les cellules myéloïdes, mais celles-ci restent tributaires du cMGF pour leur croissance (Adkins et al., 1984).

Les virus AMV et E26 sont également capables de transformer *in vitro* des cellules de la lignée myéloïde, mais celles ci paraissent plus immatures que les cellules cibles de MC29 (Graf et Beug,1978; Gazzolo et al.,1979). Un virus AMV thermosensible pour le blocage de différenciation a été isolé en 1983 par Moscovici (Moscovici et Moscovici, 1983) suggérant que là encore un produit viral empêche la maturation des cellules transformées. Le virus E26 présente quelques particularités additionnelles par rapport à AMV, puisque ce virus est capable de transformer *in vitro* des cellules érythroïdes, ce qui reflète sa capacité à induire une érythroblastose *in vivo* (Moscovici et al.,1981; Radke et al.,1982; Moscovici et al.,1983). Ce virus code pour une protéine de fusion de 135 kd de poids moléculaire (Beug et al.,1982b) dans laquelle le déterminant qui implique la protéine dans le noyau cellulaire serait responsable de l'activité myéloblastosante du virus. Un mutant de E26 thermosensible pour la transformation des myéloblastes a pu être isolé par le groupe de T.Graf et ce virus possède une protéine qui se fixe sur l'ADN d'une façon considérablement réduite à température non permissive, par rapport à la P135 d'un virus E26 sauvage (Moelling et al., 1985).

Au moment où ce travail a été initié, nous ne connaissions rien de la structure de ces virus, mais la caractérisation des protéines virales isolées dans des cellules transformées par MC29 et MH2 laissait penser que ces virus devaient être apparentés aux ALV tout en contenant des séquences nucléotidiques étrangères à ceux-ci (Barlati et de Giuli-Morghen, 1978). Le système biologique une fois clarifié, et certains virus clairement associés à la transformation (ou au blocage de différenciation) d'une cellule du système hématopoïéitique, les virus défectifs des leucémies aiguës sont apparus comme particulièrement intéressants pour approcher, au niveau moléculaire, les problèmes de prolifération et de différenciation cellulaire. Ces virus étant défectifs pour la réplication, il a été possible d'obtenir des cellules transformées (non productrices, ne contenant que le génome du DLV transformant). L'existence ou l'obtention de telles cellules nous a permis de réaliser le travail de comparaison entre le génome des DLV et celui de l'ALV "helper".

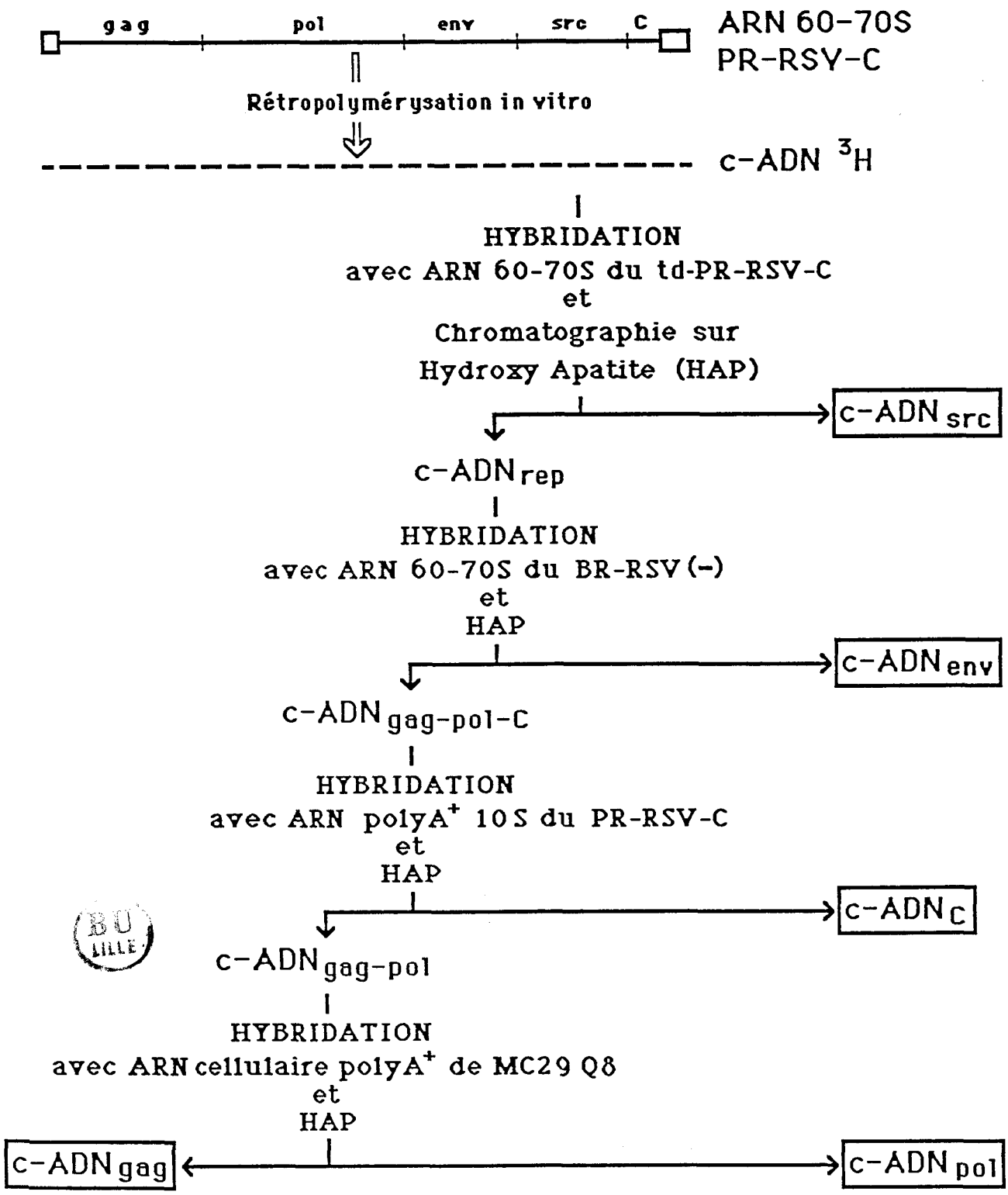


Figure 22 : Préparation des réactifs utilisés pour définir la structure du génome des DLV.

E-RESUME DES RESULTATS

I- PREMIERE PARTIE: STRUCTURE DU GENOME DES DLV

Les publications 1 et 2 rapportent l'isolement et la caractérisation de trois séquences nucléotidiques exogènes au sein des DLV. Ces résultats ont pu être obtenus, grâce aux techniques d'hybridation des acides nucléiques en phase liquide, en soustrayant de la séquence totale d'un DLV prototype (AEV, MC29 et AMV) les séquences apparentées à son virus auxiliaire, l'homologie entre chacun des DLV et l'ALV helper étant par ailleurs déterminée à l'aide des sondes complémentaires des gènes de structure et présentées dans la Figure 22).

Les séquences spécifiques (v-onc) d'abord appelées v-erb, v-mac et v-myb puis v-erb, v-myc et v-myb (pour rappeler la maladie induite par les virus qui les portent), sont d'origine cellulaire, et phylogénétiquement conservées. Cela est attesté par l'existence d'une hybridation significative entre nos différentes sondes et de l'ADN d'origine aviaire (caille, canard, faisan), de l'ADN de saumon ainsi que de l'ADN humain. Les pourcentages d'hybridation décroissent avec la divergence évolutive par rapport au poulet pris comme référence. Ces séquences cellulaires sont présentes à raison de 1 à 3 copies par génome (ainsi que le montre la valeur du cot 1/2 obtenu pour 50% d'hybridation). Les ARN correspondants sont accumulés dans des fibroblastes aviaires à un taux de quelques copies par cellule. Ainsi, ces c-onc représentent des séquences "uniques", faiblement exprimées dans les cellules fibroblastiques.

Dans le génome des DLV, le contenu en séquences nucléotidiques dérivées du génome du helper est variable d'un virus à l'autre (de 28 à 77 %) et d'un gène structural à l'autre. Le virus OK10 possède un gène gag complet (publication N°3) alors que le virus AMV est totalement dépourvu de séquence env. Enfin, le fait que tous les virus induisant le même type de transformation contiennent la même séquence spécifique milite fortement en faveur d'un rôle causal du produit de ces v-onc dans les phénomènes de transformation/différenciation de cellules hématopoïétiques bien définies.

La publication N°2 montre que le contenu en séquences apparentées aux gènes de structure (gag, pol, env) additionné aux séquences v-onc est insuffisant pour expliquer la taille de l'ARN viral produit par les virus MH2 et E26.

-Les différents ARN poly A+ des cellules transformées sont séparés selon leur poids moléculaire après migration dans un gel d'agarose. Une fois ces ARN transférés puis fixés sur un support solide, les ARN viraux sont révélés à l'aide d'une sonde complémentaire du v-onc étudié: technique du northern blot.

Pour pouvoir prédire la taille du génome des DLV nous avons dû déterminer la longueur de la séquence oncogène ainsi que celle des régions apparentées au génome du virus helper. Pour toutes ces séquences nous avons procédé de la façon indiquée dans l'article N°6 pour déterminer la longueur de v-erb. Les résultats obtenus indiquaient une valeur de 3700 bp pour AEV, 1800 bp pour MC29 et 1600 bp pour AMV (nous savons actuellement que ces résultats sont sur-estimés de 20% pour AEV et MC29, et de 50% dans le cas d'AMV). L'estimation que nous avons faite sur la

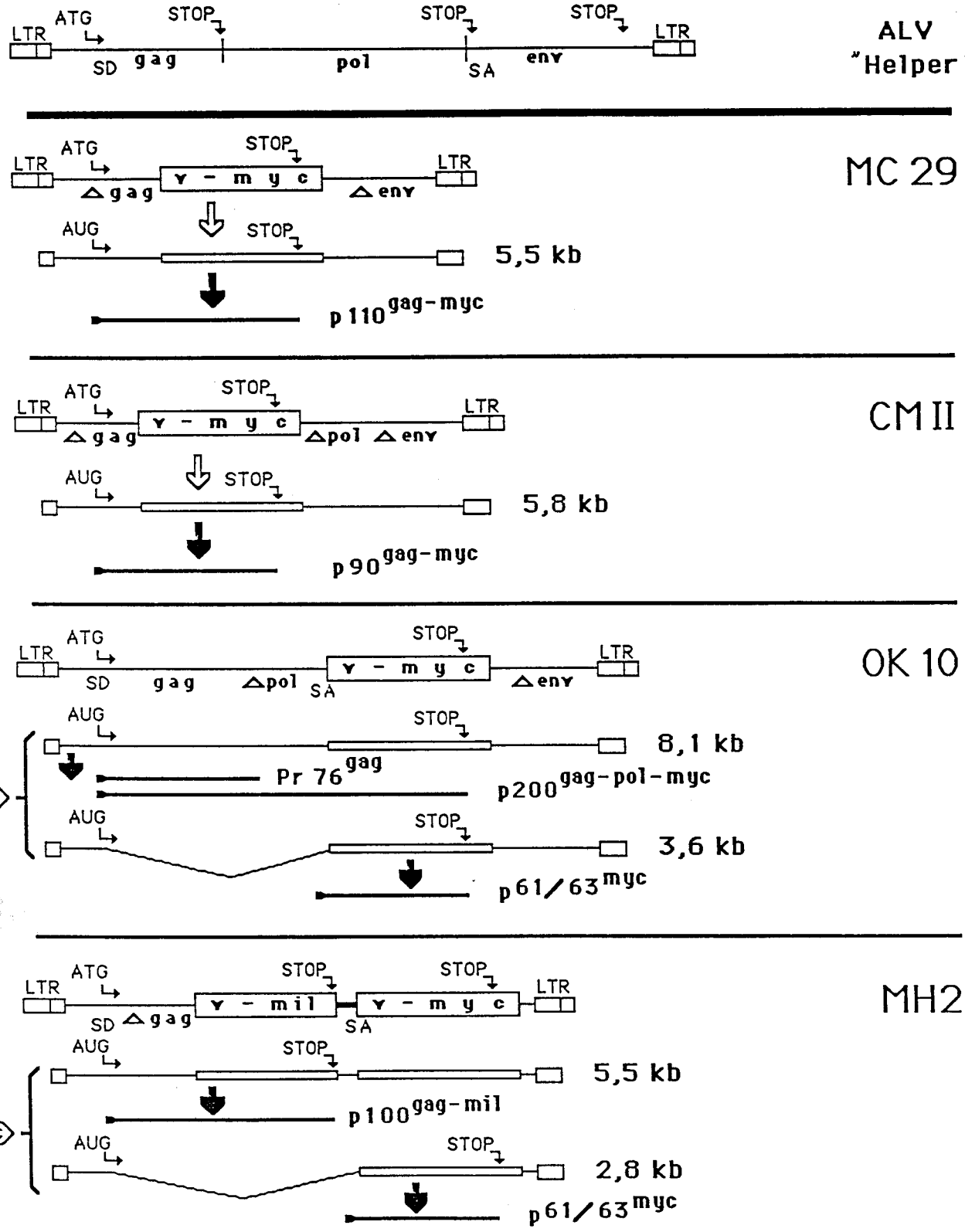


Figure 23 : Structure et modes d'expression des virus contenant l'oncogène myc.

⇨ Transcription ; ➔ Traduction

taille en nucléotides des séquences apparentées au virus helper, bien que très approximative (car nous ignorions la longueur exacte des différents gènes viraux) nous a conduit à supposer la présence d'une séquence exogène additionnelle au sein de MH2 et E26. Le clonage moléculaire du génome du virus E26 a permis de caractériser pour ce virus une deuxième séquence également d'origine cellulaire et phylogénétiquement conservée. Cette séquence a été appelée ets (Leprince et al., 1983 ; Nunn et al., 1983).

Dans les articles N° 3, 4 et 5 nous reportons des expériences menées afin de définir le mode d'expression du v-onc selon l'isolat viral. La publication N° 3 affine notre connaissance du génome du virus OK10 (Figure 23). Ce virus a souffert d'une amputation partielle des gènes pol et env mais possède un gène gag complet, permettant la production de particules rétrovirales non infectieuses dépourvues d'activité transcriptase réverse. A l'aide des techniques de microscopie électronique, nous avons pu visualiser les structures d'hétéroduplexes réalisées entre des molécules d'ARN du virus OK10 et l'ADN du génome de son helper. Nous avons pu montrer que le gène myc s'était inséré entre les gènes pol et env (ce qui explique que ces gènes soient amputés, après cet événement de recombinaison).

Le produit de l'oncogène v-myc est exprimé dans ces cellules par l'intermédiaire d'un ARN sous-génomique produit par un mécanisme d'épissage de l'ARN viral. Cet ARN permet la traduction de protéine myc dépourvue de déterminants codés par les gènes de structure, situation inattendue au sein des virus de ce type qui dirigent la synthèse de protéines gag-onc (Barlati et de Giuli-Morghen, 1978). Ces résultats suggéraient pour la première fois que les acides aminés codés par le gène gag présents au sein de la protéine de fusion n'étaient pas essentiels dans l'expression du pouvoir oncogène de myc.

La publication N°4 nous a permis d'étendre cette observation au virus MH2 qui exprime également les séquences v-myc par l'intermédiaire d'un ARN sous-génomique. Cet article montre en outre que la $p100^{gag\text{-}onc}$ mise en évidence dans les cellules transformées par ce virus (Hu et al., 1978b) ne peut être une $p100^{gag\text{-}myc}$ contrairement à l'idée généralement admise à cette époque (RNA Tumor virus, 1982). En effet, le gène v-myc se trouve en 3' du génome viral. Ainsi, la séquence exogène supplémentaire, dont l'existence avait pu être soupçonnée d'après les expériences d'hybridation en phase liquide, est adjacente au résidu du gène gag de MH2. Le clonage moléculaire ultérieur de ce provirus a permis de la caractériser en détail (Coll et al., 1983b ; Jensen et al., 1983). Comme pour E26, cette séquence additionnelle, v-mil , est d'origine cellulaire et est phylogénétiquement conservée. Elle représente le gène oncogène (v-raf) d'un isolat de virus sarcomatogène murin (Kan et al., 1984). Ainsi, MC29 et CMII codent pour une protéine de fusion (P110 et P90) $^{gag\text{-}myc}$ alors que OK10 et MH2 peuvent synthétiser un produit uniquement codé par le gène v-myc.

Toutefois, cette conclusion doit être tempérée par les résultats présentés dans l'article N°5 qui rapporte le clonage moléculaire et la caractérisation d'un provirus MH2 correspondant uniquement à la propagation de l'ARN sous-génomique de ce virus. En effet, la séquence nucléotidique de l'extrémité 5' de ce provirus (Cl25) montre que l'AUG du gène gag est fusionné dans le même cadre de lecture que le reste des séquences du gène myc, fusion résultant du mécanisme d'épissage.

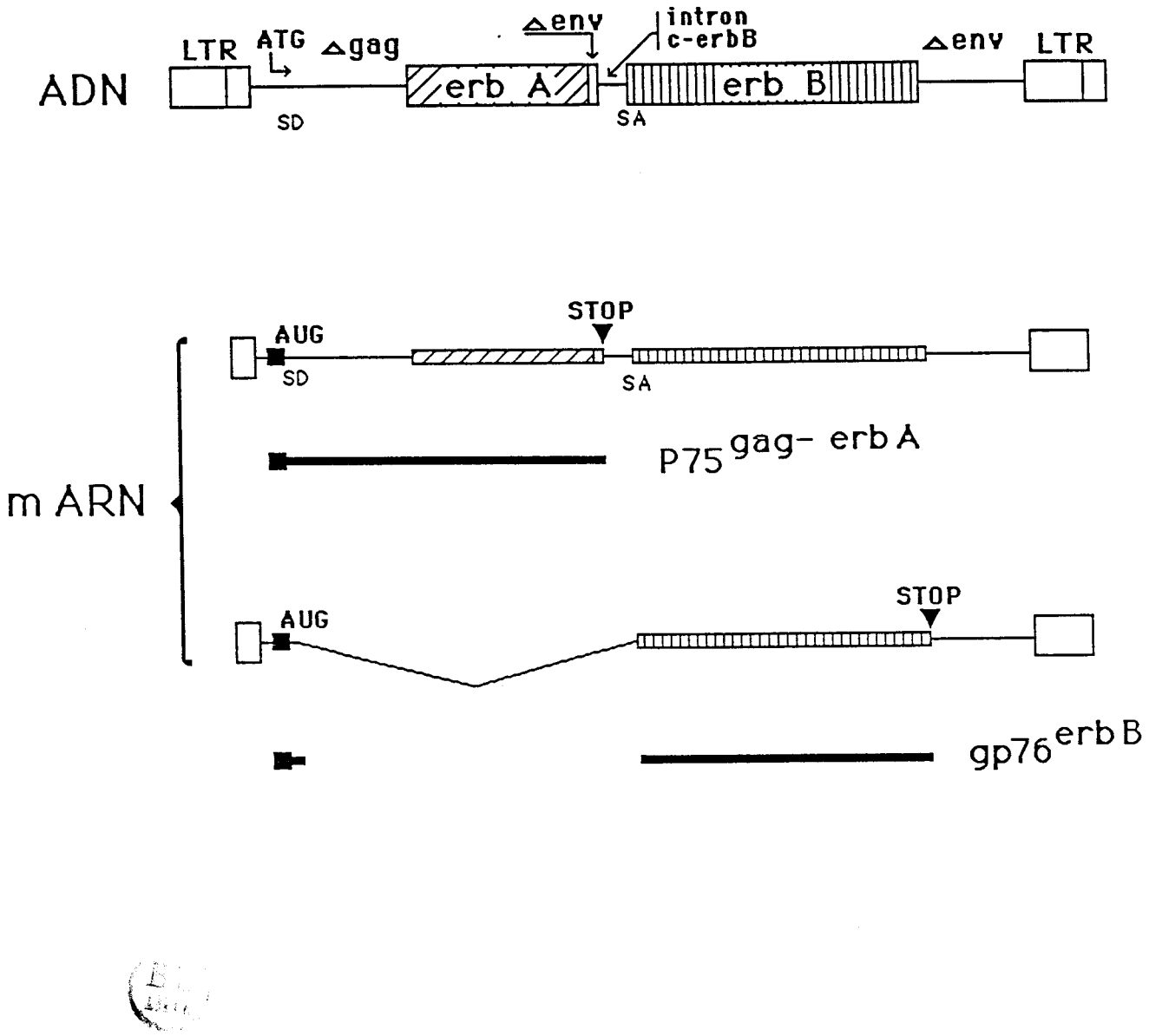


Figure 24 : Structure et mode d'expression du virus de l'érythroblastose aviaire, AEV.

Ainsi, ces cellules produisent le même doublet de protéines v-myc que le virus complet de départ, les 6 premiers acides aminés du gène gag (de l'AUG au site d'épissage) sont probablement présents à l'extrémité NH2 de l'une des deux protéines (p61-63) codées par MH2. Ce doublet pourrait être dû à l'utilisation des deux AUG dans le même cadre de lecture (le premier dans le gène gag, le second dans le gène myc), conséquence du "balayage" ribosomal lors de la traduction (Kozak, 1986). Cependant, ainsi que nous le montrons dans l'article N°14 présenté dans la troisième partie des résultats, ces séquences gag n'apportent aucune contribution décelable dans le pouvoir transformant de la protéine v-myc. Le virus Cl25 constitue probablement l'unité réplicative minimale trouvée dans un rétrovirus. Son étude permet de mieux cerner les séquences nucléotidiques impliquées dans l'encapsidation et la propagation des ARN viraux. Nous avons montré, en transfectant l'ADN de ce clone moléculaire sur des fibroblastes embryonnaires de caille que cette molécule était biologiquement active et capable de transformer ces cellules. La surinfection de ces dernières à l'aide d'un virus helper permet de récupérer des particules transformantes se propageant avec la même efficacité que l'isolat biologique. Ainsi, le site d'encapsidation décrit par Pugatsch et Stacey (1983) au début du gène gag (545ème nucléotide) ne semble pas indispensable à la propagation de ce virus. Celui-ci utilise probablement les sites d'encapsidation localisés dans les regions L (Nishizawa et al., 1985) et U3 (Sorge et al., 1983) en dehors des séquences codantes (Figure 2).

Les articles 6, 7 et 8 caractérisent la structure et la stratégie d'expression du virus de l'érythroblastose aviaire AEV (Figure 24). La publication N°6 montre que ce virus exprime dans les cellules transformées deux ARN ce qui suggère l'existence de deux v-onc au sein d'AEV, l'ARN génomique codant pour une protéine de fusion gag-onc de 75 kd (Hayman et al., 1979). La complexité de la séquence spécifique du virus AEV est grande (ca 3700 bp) et pourrait effectivement représenter deux gènes (ce qui sera confirmé, voir publications 7, 8 et 10 et Vennström et Bishop, 1982). Ces gènes seront appelés erbA et erbB. La stratégie d'expression de ce virus est la même, que les cellules transformées soient des cellules d'origine aviaire (érythroblastes ou fibroblastes) ou d'origine mammifère (cellules fibroblastiques de rat, 208F).

D'autre part, les articles 3, 4 et 6 montrent que l'arrêt de transcription de l'ARN des rétrovirus intégrés dans des clones cellulaires (la culture ne dérive que d'une seule cellule transformée) peut se faire en dehors du site normal d'arrêt, situé dans la séquence U3 du LTR 3' du provirus intégré. En effet, dans des cellules transformées par OK10, MC29, CMII, MH2 et AEV nous avons pu mettre en évidence des ARN viraux d'une taille supérieure à celle du génome. Cela suggère que certaines molécules d'ARN se terminent dans les séquences nucléotidiques adjacentes au site d'intégration viral. Une telle observation a déjà été faite dans des clones de cellules mammifères transformées par le RSV (Quintrell et al., 1980). De même, certaines séquences cellulaires peuvent être transcrites sans être liées à d'autres séquences virales que les séquences U5 (dans les cellules Q8, fibroblastes de caille non productrices de virus, transformées par le virus MC29, article N°4). Nous savons que cette activation transcriptionnelle peut être déterminante dans le cas de cellules infectées par des virus non transformants ; dans le cas des virus portant un oncogène, le rôle éventuel de tels gènes parait limité. Il n'est pas impossible que certains de ces gènes soient

importants pour conférer un avantage aux cellules transformées (l'immortalisation par exemple). Toutefois il ne s'agit là que d'hypothèses.

Les articles 7 et 8 précisent la structure du virus AEV grâce à la détermination (par l'équipe de B.Debuire) de la séquence nucléotidique d'un clone moléculaire du virus AEV (Vennström et al.,1980). Les limites 3' du gène v-erbA et 5' du gène v-erbB sont précisément fixées grâce à la séquence nucléotidique du début du gène c-erbB ayant recombiné avec le virus (le clonage de ce gène cellulaire est présenté dans la publication N°9).

L'article N°7 montre que la protéine codée par le gène v-erbA peut être séparée en deux domaines. Le domaine 1, codé par la moitié 5' du gène est riche en cystéines et en lysines, et montre une analogie importante avec la portion fixatrice de l'ADN des récepteurs d'hormones glucocorticoïdes et œstrogènes (Weinberger et al., 1985; Green et al., 1986). Le domaine 2, codé par la moitié 3' du gène viral, présente une analogie avec la famille des anhydrases carboniques de type II. Nous savons actuellement que la portion erbA de la $p75^{gag\text{-}erbA}$ correspond au récepteur des hormones thyroïdiennes T3 et T4, mais que le virus a perdu la possibilité de fixer l'hormone. D1 correspond au domaine qui se fixe sur l'ADN, et D2 au domaine qui fixera l'hormone dans le produit c-erbA (Sap et al.,1986).

La séquence nucléotidique du début de c-erbB révèle que deux virus ayant indépendamment recombiné avec ce gène (AEV-ES4 et AEV-H) débutent la protéine v-erbB au même endroit, ce qui suggère que l'activation oncogénique du récepteur de l'EGF nécessite notamment la perte de son domaine extra-cellulaire (celui-ci étant perdu dans les deux virus). L'ARN sous-génomique est produit en utilisant le signal accepteur d'épissure présent au tout début du premier exon cellulaire c-erbB capturé par le virus; la phase de lecture étant identique dans le gène gag et le gène cellulaire, on peut supposer que l'AUG utilisé pour synthétiser la protéine transformante dérive du gène viral et non du c-erbB.

ARTICLES

1 à 8

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Three new types of viral oncogene of cellular origin specific for haematopoietic cell transformation

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The RNAs of seven replication-defective leukaemia virus (DLV) strains contain three types of unique sequences, which correlate with the capacity of a given virus strain to transform erythroblasts, macrophage-like cells and myeloblasts, respectively. These sequences, termed erb, mac *and* myb, *have their counterparts in the normal DNA of avian and mammalian species. Our results indicate that DLVs represent recombinants between a common 'vector' related to a chicken endogenous virus and one of three types of cellular gene possibly involved in haematopoietic differentiation.*

On the basis of their oncogenic properties, avian retroviruses are assigned to either of two major classes. Of many characterised natural field isolates, most (non-defective avian leukaemia viruses, or ALVs) are 'weakly oncogenic' and cause predominantly lymphatic leukaemia after a latency period of months to years following inoculation into susceptible strains of chickens. The ALVs most frequently occur as independent agents containing all the genetic information required for viral replication. In contrast, several avian retroviruses are highly oncogenic, causing disease after a latency period of days to weeks. These include the replication-competent avian sarcoma viruses (ASVs, or Rous sarcoma viruses) which readily induce sarcomas in susceptible birds and transform fibroblasts *in vitro*, and also the replication-defective leukaemia viruses (DLVs) which require helper ALVs for propagation in cultured cells (for review, see ref. 1).

The seven available independent isolates of DLVs have been assigned to three subgroups based on the types of neoplasm they induce and on the differentiation phenotypes of haematopoietic cells they transform *in vitro*[1,2]. (1) The avian erythroblastosis-type viruses (AEV strains R and ES4 are probably identical[3]) induce erythroblastosis *in vivo* and transform erythroblasts *in vitro*. (2) The avian myelocytomatosis-type viruses (MC29, CMII, OK10 and MH2) transform macrophage-like cells *in vitro* and some strains induce myelocytomatosis *in vivo*. (3) The avian myeloblastosis-type viruses (AMV and E26) induce myeloblastosis *in vivo* and transform myeloblasts *in vitro*. AEV can also induce sarcomas and transforms fibroblasts *in vitro*, and MC29-type viruses can induce carcinomas and occasional sarcomas and transform epithelial cells and fibroblasts *in vitro*[1,2]. In this report we have investigated whether the oncogenicity of these viruses could be related to the presence of specific nucleotide sequences in their genome. Such a relationship would imply that part or all of these specific sequences represent new viral oncogenes.

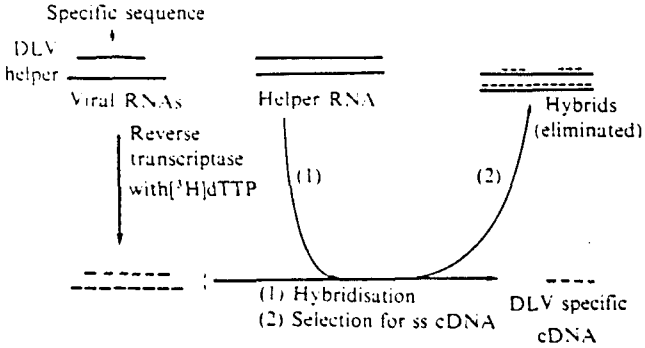

Fig. 1 Scheme for the preparation of DLV-specific cDNAs. Cloned AEV (RAV-2) and MC29 (RAV-2) pseudotypes, obtained by rescuing these viruses with RAV-2 from cloned non-producer fibroblasts, were propagated in chicken fibroblasts. Viral RNAs were extracted from the pelleted viruses, then transcribed into labelled cDNA using *in vitro* reverse transcription with purified AMV reverse transcriptase, ^{3}H-dTTP and unlabelled dCTP, dGTP, dATP and calf thymus priming. In a negative selection step each cDNA mixture was hybridised to an excess of RNA extracted from RAV-2 grown separately. Single-stranded DLV-specific cDNAs were then isolated after fractionation on hydroxyapatite. AMV-specific cDNA was prepared in a similar way, but using RNA of AMV (helper) pseudotype obtained from the plasma of infected chickens, and a mixture of ALV helper RNAs extracted from RAV-1, RAV-2 and MAV-2 (0) viruses for the selection. Non-hybridisable radioactivity was eliminated from the cDNAs by a positive selection with homologous RNAs[9] (not shown). Specific activities of the ^{3}H-cDNAs were $\sim 50 \times 10^6$ c.p.m. per μg. Details of these experiments will be described elsewhere.

DLVs are related to non-defective avian leukaemia viruses

DLVs were originally isolated from chickens in association with their natural replication-competent helper viruses belonging to the ALV group. Attempts to complement DLVs with helpers unrelated to ALVs have failed[4]. A possible homology between certain genomic sequences of the DLVs and ALV genes[5-7] was investigated by testing clones of avian cells containing the DLV provirus without its helper. Such cells, usually unable to produce virus[8], still contain several RNA transcripts of the DLV provirus. We tested whole RNA of such nonproducer cells, using molecular hybridisation with labelled probes representing a homogeneous transcript of an ALV, td Pr-B (cDNArep), or of the *src* gene of ASVs (cDNAsarc)[9]. Table 1 indicates that all the DLV genomes tested contain ALV-related nucleotide sequences in different amounts (28–77%), but lack the *src* gene of ASV[9,10]. However, the detected homology to ALV cannot account for the entire genetic content of the DLVs, which all contain 6,000–8,000 nucleotides in their 28–34S genome[11,12]. Thus, these viruses may contain sets of nucleotide sequences unrelated to ALVs.

DLV-specific sequences

We looked for specific sequences by analysing the prototype strain of each of the three DLV subgroups, namely AEV, MC29 and AMV. Clone viral strains were obtained for AEV and MC29 by superinfection of nonproducer cells with the plaque-purified helper virus RAV-2. The rescued viruses were then

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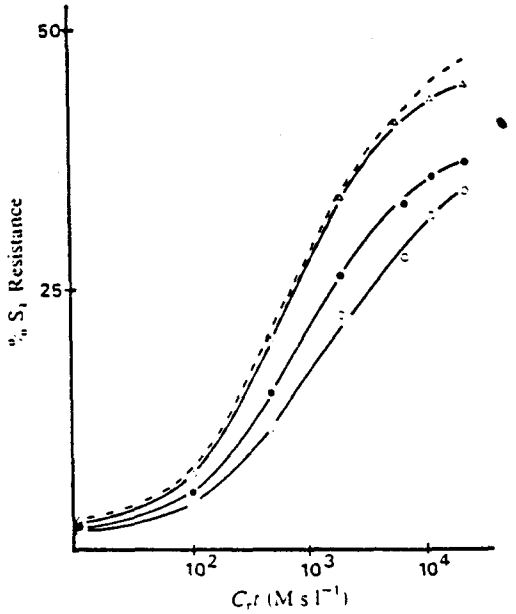

Fig. 2 DLV-specific sequences have their couterparts in normal chicken DNA. Aliquots of 11-day chicken embryo DNA (200 μg per point, sheared to 4–6S) were hybridised with the different specific cDNAs (1,000 c.p.m. per point = 0.02 ng) in stringent conditions (0.6 M NaCl, 68 °C) at increasing C_0t values. Hybrids formed were tested for their resistance to S_1 endonuclease. ●—●, cDNAaev; △—△, cDNAmc29; ○—○, cDNAamv; ---- cDNAsarc. Similar results were obtained with normal quail embryo DNA (not shown).

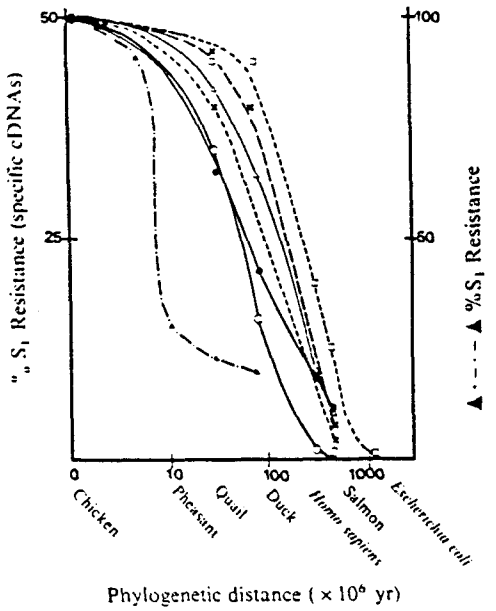

Fig. 3 Stability of cellular DLV-related specific sequences during evolution. Aliquots of DNA extracted from different species having diverged increasingly long ago with speciation were hybridised in the specific conditions described in Fig. 2. The values obtained were standardised arbitrarily, for comparison purposes, to 50% for the chicken, which was also taken as the origin of the scale indicating phylogenetic distance. ●—●, cDNAaev; △—△, cDNAmc29; ○—○, cDNAamv; □- - -□, cDNAsarc. Also shown (▲ · — · —▲) are results obtained with ^{3}H-labelled chicken unique sequence DNA[14] (1,000 c.p.m. per point = 20 ng) in similar conditions (standardised to 100% for homologous reassociation) and with cDNAs representing genes known to be conserved throughout the evolution of vertebrates; × — —×, cDNAlgo (ref. 16) for globin; × - - - -×, cDNAova (ref. 16) for ovalbumin.

tested for their oncogenic potential *in vitro* (using the bone marrow transformation assay[2]) and propagated in chicken erythroblasts (for AEV) or chicken fibroblasts (for MC29). AMV was obtained in high yields from the plasma of infected chickens.

The preparation of specific cDNAs of these viruses (Fig. 1) was similar to the selection of cDNA specific for the *src* gene of ASVs[3]. cDNAs of AEV, MC29 and AMV were selected so that they could still hybridise to their RNAs of origin, but not to the RNA of the helper viruses used to grow the viral stocks or any of the other ALVs tested. These specific cDNAs were denoted cDNAaev for AEV, cDNAmc29 for MC29 (ref. 13) and cDNAamv for AMV.

The distribution of viral nucleotide sequences related to these specific cDNAs was explored by hybridising them at plateau C_rt values to several viral RNAs. The results (Table 2) allow several conclusions to be drawn. First, the cDNAs tested are specific for the DLVs used for their preparation. Second, they represent distinct sets of sequences, unrelated to each other by cross-hybridisation experiments, and third, cDNAmc29 shows extensive homology to RNA of CMII, OK10 and MH2, as does cDNAamv to E26 RNA. As will be described elsewhere, the three cDNAs exhibited exactly the same specificity when tested with cellular RNAs extracted from nonproducer cells, further demonstrating that the probes indeed detect sequences specific for the three types of DLV.

To investigate the origin of the DLV-specific sequences, DNA extracted from normal avian cells was tested for its ability

Table 1 DLV genomes in nonproducer cells are related to ALVs

DLV strain	Type of nonproducer cell	Annealing (% S_1 resistance) with cDNArep	cDNAsarc
AEV	chicken fibroblast	28	<3
MC29	quail fibroblast	45	<3
CMII	quail fibroblast	41	<3
OK10	quail fibroblast	77	<3
MH2	quail fibroblast	32	<3
AMV	chicken myeloblast	50	<3
E26	chicken myeloblast	36	<3

Total RNA was extracted from the different avian nonproducer cells and hybridised in stringent conditions (25-μl aliquot containing 1–10 mg ml^{-1} RNA, 0.6 M NaCl, 68 °C) with cDNAs (1,000 c.p.m. per point = 0.02 ng) to plateau C_rt values (defined as 10× the $C_rt_{1/2}$). cDNArep and cDNAsarc were prepared as described earlier[3], but using calf thymus-primed polymerase reactions[14]. Hybrids were assayed by S_1 nuclease. No correction for the expression of endogenous RAV-O or cellular sarc was made as they gave values below 3% in each case.

to hybridise with cDNAaev, cDNAmc29 and cDNAamv. Figure 2 shows that all three types of sequences have their counterparts in normal cellular chicken DNA and, as judged from the $C_0t_{1/2}$ values, that they are present in the non-repetitive DNA at one or two copies each per haploid genome. The low plateau values observed in Fig. 2 are due to the competition of DNA reassociation with the DNA–cDNA hybridisation reaction; transcription analyses (see below) show that most or all of the specific sequences are present in normal chicken DNA. Results obtained with molecular hybridisation in liquid phase in stringent conditions show that the bulk of the non-repetitive chicken DNA sequences have been lost relatively rapidly during evolution: labelled chicken unique sequence DNA barely anneals (Fig. 3) to the DNA of species from another genus. For example, quail and chicken, which probably evolved from a common ancestor some 20–40 Myr ago, have DNAs that share less than 25% homology when cross-hybridised (Fig. 3). Similarly, ALV-related 'virogene' sequences have recently been shown to be absent in some species of the Galliformes[14]. In contrast, cellular sequences related to cDNAaev, cDNAmc29

Table 2 DLVs contain unique nucleotide sequences

RNA derived from	Annealing (% S_1 resistance) with cDNAaev	cDNAmc29	cDNAamv
AEV	100	<3	<3
MC29	<3	100	<3
CMII	<3	96	<3
OK10	<3	91	<3
MH2	<3	66	<3
AMV	<3	<3	100
E26	<3	<3	68
ALV helpers (control)	<3	<3	<3

Viral 50–70S RNAs were extracted and hybridised to plateau C_0t values (>10 M s l^{-1}) in stringent conditions (0.6 M NaCl, 68 °C) with the specific cDNAs (1,000 c.p.m. per point = 0.02 ng). The hybrids formed were tested for S_1 nuclease resistance. Results obtained were standardised to the values of the homologous reaction. Maximum hybridisation of cDNAaev with AEV RNA was 95%; that of cDNAmc29 with MC29 RNA 90% and that of cDNAamv with AMV RNA 95%. The DLVs tested were cloned pseudotypes with RAV-1 or RAV-2 helper ALV, with the exception of AMV and E26, which were not cloned. The ALV helper RNAs tested as controls were obtained from RAV-1, RAV-2, RAV-49, RAV-50, MAV-2 (0). The numbers boxed indicate the important homologies.

and cDNAamv have been highly conserved throughout the phylogeny of the higher vertebrates. As also shown in Fig. 3, their 'evolutionary half lives' are comparable to those of known stable genes such as globin or ovalbumin. The latter values resemble thoses obtained earlier for the transforming gene *src* of ASVs[15–17] and agree with the results recently described for MC29 by Sheiness *et al.*[18].

As normal cells contained nucleotide sequences related to highly transforming viruses, we studied the rate of transcription of such sequences. Total RNA extracted from normal chicken (or quail) fibroblasts was hybridised to the different specific cDNAs. Figure 4 shows that these sequences are all transcribed at a low level (a few copies per cell), as previously found for the expression of "cellular *src*" (refs 16, 19). Cellular sequences related to cDNAaev are transcribed at two or three copies per cell; cellular sequences related to cDNAmc29 are reproducibly expressed at a higher level (5–10 copies per cell), as Sheiness *et al.*[18] found. In contrast, cellular sequences related to cDNAamv are transcribed at lower levels in fibroblasts (one copy per cell). As can be seen in Fig. 4, all these cellular sequences reach plateau hybridisation values close to 100%, and must therefore be essentially fully represented in normal cellular nucleic acids.

Three new oncogenes

Our results show that all seven defective leukaemia viruses studied contain part or all of one out of three different sets of specific nucleotide sequences complementary to cDNAaev, cDNAmc29 or cDNAamv, which have their counterpart in normal cells of higher vertebrates. None of these sequences is related to the transforming gene *src* of ASVs. Although it is not understood how recombinants between ALV sequences and cellularly derived sequences can acquire oncogenic capacity, several indications favour the hypothesis that three specific transforming genes, termed *erb*, *mac* and *myb*, are detected by cDNAaev, cDNAmc29 and cDNAamv, respectively. (1) There is a strict correlation between the presence of *erb*, *mac* or *myb* sequences in a given virus and its selective capacity to generate transformed erythroblasts, macrophage-like cells or myeloblasts[1,2,20]. (2) The properties of these sequences and their origin are reminiscent of results obtained earlier for the transforming gene *src* of ASVs[15]: size, cellular origin and phylogenetic stability[16,17]. (3) As indicated by heteroduplex mapping studies with MC29 (ref. 21), these sequences do not appear as bits of sequences randomly distributed throughout the viral RNA, but rather as continuous stretches located inside the viral genome. (4) Temperature-sensitive mutants have recently been isolated from AEV, indicating that a virus-coded protein must be synthesised to maintain the undifferentiated state of transformed erythroblasts[22]. (5) All DLVs tested contain the 5' region of the ALV genome (partial *gag* gene[12]). In the strains studied so far (MC29 and MH2) this region was located adjacent to the DLV-specific sequence[21,23]. In addition, cells nonproductively transformed by AEV, MC29, CMII and MH2 express novel polyproteins of molecular weight 75,000 (ref. 3), 110,000 (refs 6, 7), 90,000 (ref. 7) and 100,000 (ref. 4), respectively, with the structure: partial *gag*-*x* or *y*, where *x* or *y* represent polypeptide moieties which are antigenically unrelated to known ALV proteins, and presumably correspond to part or all of the specific *erb* or *mac* nucleotide sequences. That these polyproteins could be involved in the transformation process is indicated by the finding that the type of tryptic peptide pattern exhibited by the non-*gag* portion of the polyprotein of a given DLV closely correlates with the transformation specificity of the virus (M. J. Hayman, personal communication, and ref. 24). In their general structure and ability to code for *gag*-related polyproteins, avian DLVs resemble replication-defective mammalian retroviruses capable of inducing leukaemias or sarcomas with a short period of latency, such as the Abelson[25] murine virus and feline sarcoma virus[26,27].

Avian DLVs behave like recombinants between an ALV-related vector virus of low oncogenicity and unknown origin[14] and an evolutionarily stable set of nucleotide sequences of cellular origin. In this regard they also closely resemble ASVs (for review, see ref. 11). The mechanism of such a recombination remains to be elucidated, but could be related to the jumping polymerase hypothesis proposed recently by Coffin[28]. How frequently such a recombination occurs is not known, although about 30 DLV-like and 1–3 ASV-like independent isolates have been reported[2]. However, a transduction of cellular sequences would not necessarily lead to such severe effects as the generation of a highly oncogenic virus and may therefore be much more

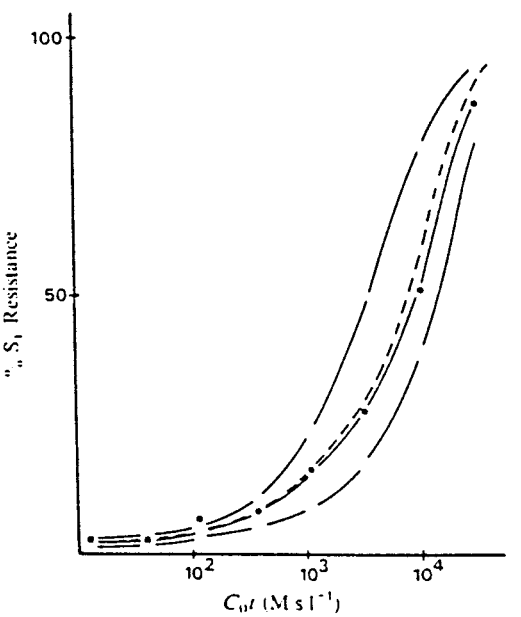

Fig. 4 Transcription of the cellular DLV-related specific sequences in normal fibroblasts. Total RNA was extracted from normal chicken embryo fibroblasts and hybridised at increasing C_0t values (varying RNA concentration up to 10 mg ml^{-1}) in stringent conditions (0.6 M NaCl, 68 °C), to the specific cDNAs (1,000 c.p.m. per point = 0.02 ng). Hybrids were scored for their S_1 endonuclease resistance. Symbols are as in Fig. 2.

frequent than anticipated. Indeed, we have recently found in an ASV stock a new virus of unknown oncogenicity containing apparently unknown cellular genetic material (N. Pluquet and D.S., unpublished).

That only a partial homology was observed between the specific sequences of MC29 and MH2 as well as between the specific sequences of AMV and E26 (Table 2) can be explained in three ways—these viruses acquired cellular genes which already differed in their sequences; they acquired the same gene and the observed differences reflect a divergence introduced by passage of the virus; a second recombination event occurred in MH2 and E26. The finding that there are only one or two copies per haploid genome of these genes present in cellular DNA is consistent with the second possibility. The observation that there is a somewhat lower degree of homology of cDNAamv to cellular DNA than that of the other types of specific cDNA tested (Fig. 2) might reflect the long passage history of AMV and would also agree with the second of the above interpretations. But hybridisation experiments between cDNAmc29 and MH2 RNA carried out in non-stringent conditions do not show the significant increase of annealing which the second interpretation would predict (M.R., S.S. and D.S., unpublished) and thus leave that question unanswered.

The role of DLVs in leukaemic transformation could be explained by a generalisation of the 'differentiation block' hypothesis[22,24]: the oncogenes of DLVs are homologous to normal cellular genes ('cellular *erb*, *mac* or *myb*') coding for lineage-specific haematopoietic differentiation proteins; the transforming proteins act by competitively inhibiting the corresponding cellular proteins[2,17], thus leading to a block of differentiation and subsequent leukaemogenesis. This hypothesis would predict that RNA sequences corresponding to the cellular *erb*, *mac* and *myb* genes are preferentially expressed in normal erythroblasts, macrophages and myeloblasts, respectively. Spontaneous leukaemogenesis may then simply result from a malfunction of such genes. Indeed, several different genes may be able to induce the same type of leukaemia. In this respect, it would be interesting to know whether there are viruses causing the same type of leukaemia but containing different cell-related oncogenes. Such viruses would also be useful for elucidating why the DLVs also induce sarcomas or carcinomas, an additional specificity for non-haematopoietic tissues difficult to explain by the differentiation block theory.

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Three New Types of Viral Oncogenes in Defective Avian Leukemia Viruses. I. Specific Nucleotide Sequences of Cellular Origin Correlate with Specific Transformation

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On the basis of their oncogenic properties, avian retroviruses can be assigned to either of two major classes. Of many characterized natural field isolates, the vast majority (nondefective avian leukemia viruses [ALVs]) are weakly oncogenic and cause predominantly lymphatic leukemia after a prolonged latency period of months to years following inoculation of susceptible strains of chickens. ALVs most frequently occur as independent agents and contain all the genetic information required for viral replication. In contrast, several other avian retroviruses are highly oncogenic and produce disease after a short latency period of days to weeks. These highly oncogenic viruses include the replication-competent avian sarcoma viruses (ASVs) (or Rous viruses), which readily induce sarcomas in susceptible birds and transform fibroblasts in vitro, and the replication-defective leukemia viruses (DLVs), which require helper ALVs for their propagation in cultured cells (for review, see Graf and Beug 1978).

The seven available independent isolates of DLVs have been recently assigned to three subgroups (Table 1) based on the types of neoplasms they induce and on the differentiation phenotypes of hematopoietic cells transformed in vitro by these viruses (Beug et al. 1979). These subgroups are: (1) Avian erythroblastosis virus (AEV) (strains R and ES4 have been shown to be probably identical [Hayman et al. 1979b]) induces erythroblastosis in vivo and transforms erythroblasts in vitro. (2) Avian myelocytomatosis-type viruses (MC29, CMII, OK10, and MH2) transform macrophagelike cells in vitro and some strains induce myelocytomatosis in vivo. (3) Avian myeloblastosis-type viruses (AMV and E26) induce myeloblastosis in vivo and transform myeloblasts in vitro.

In addition, AEV is capable of inducing sarcomas and transforming fibroblasts in vitro; MC29-type viruses are capable of inducing carcinomas and occasionally sarcomas and of transforming epithelial cells and fibroblasts in vitro (Graf and Beug 1978; Beug et al. 1979), although AEV and MC29 have been shown to lack the *src* gene of ASVs (Stéhelin and Graf 1978). In most cases, these viruses induce the same type of neoplasm as in the chicken of origin and thus are not likely to represent laboratory artifacts.

The aim of our work was to determine if the oncogenicity of these viruses could be related to the presence of specific nucleotide sequences in their genome. Such a relationship would then imply that part or all of these specific sequences represent new viral oncogenes.

DLVs Are Related to Nondefective ALVs

DLVs were originally isolated from chickens in association with their natural replication-competent helper viruses belonging to the ALV group. Attempts to complement DLVs with helper viruses unrelated to ALVs have failed thus far (Hu et al. 1978) and indicate a possible homology between certain genomic sequences of the DLVs and ALV genes (Bister et al. 1977; Hu and Vogt 1979; Hayman et al. 1979b). Such a homology was verified by testing avian cell clones infected by pseudotypes of the DLVs, thus containing in their DNAs a given DLV provirus, but no ALV helper provirus (AEV, Graf et al. 1976; MC29, Bister et al. 1977; CMII, Graf et al. 1977; MH2, S. Saule, unpubl.; OK10 and E26, Graf et al. 1979b; AMV, T. Graf, unpubl.). These cells, usually unable to produce virus (nonproducer or NP cells), still contain numerous RNA transcripts of the DLV provirus. Whole RNA of these NP cells was analyzed, using molecular hybridization with labeled probes representing homogeneous transcripts of an ALV ($cDNA_{rep}$), of parts of an ALV genome ($cDNA_{gag\text{-}pol}$; $cDNA_{env}$), or of the *src* gene of ASVs ($cDNA_{src}$) (Tal et al. 1977; Frisby et al. 1979). Table 2 indicates that all the DLV genomes tested contain ALV-related nucleotide sequences in variable amounts, but lack the *src* gene of ASV. However, the detected homology to ALVs cannot account for the entire genetic content of the DLVs, which all contain about 6000–8000 nucleotides in their genomic RNAs

Table 1. Defective Avian Leukemia Viruses

Virus type	Virus strain[a]	Country and year of origin	Type of neoplasm in chicken of origin	Predominant types of neoplasms induced[a]
AEV	ES4	Denmark, 1933	sarcoma and myelocytomatosis	erythroblastosis; sarcomas
	R	Denmark, 1931	erythroblastosis	erythroblastosis; sarcomas
MC29	MC29	Bulgaria, 1964	myelocytomatosis	myelocytomatosis; liver and kidney carcinomas
	CMII	Federal Republic of Germany, 1964	myelocytomatosis	myelocytomatosis
	MH2	England, 1927	endothelioma (?)	liver and kidney carcinomas; sarcomas; monocytic leukemia (?)
	OK10	Finland, 1975	endothelioma (?)	endothelioma (?)
AMV	AMV BAI/A	United States, 1941	neurolymphomatosis	myeloblastosis
	E26	Bulgaria, 1962	leukemia	erythroblastosis

Modified from Graf and Beug (1978).

[a]Other neoplasms such as lymphatic leukemia, osteopetrosis, and nephroblastoma are omitted from this list, since they are attributed to helper viruses present in stocks of DLVs.

(see below). Thus, these viruses must contain nucleotide sequences unrelated to ALVs.

DLVs Contain Nucleotide Sequences Not Related to ALVs

The search for specific sequences was conducted by analyzing the prototype strain of each of the three DLV subgroups, namely, AEV, MC29, and AMV. Cloned viral strains were obtained for AEV and MC29 by superinfection of nonproducer cells with the plaque-purified helper-virus RAV-2. The rescued viruses were then tested for their oncogenic potential in vitro using the bone marrow transformation assay (Graf et al. 1979a) and then propagated in chicken erythroblasts (for AEV) or chicken fibroblasts (for MC29). AMV was obtained in high yields from the plasma of infected chickens.

The strategy used to prepare specific cDNAs of these viruses was similar to that previously used to select cDNA specific for the *src* gene of ASVs (Stéhelin et al. 1976a) and is outlined in Figure 1. Briefly, cDNAs of AEV, MC29, and AMV were selected so that they were still capable of hybridizing to their RNAs of origin, but could not hybridize to the RNAs of the helper viruses used to grow the virus stocks or of any of the other ALVs tested. These specific cDNAs were denoted $cDNA_{aev}$ for AEV, $cDNA_{mc29}$ for MC29 (Sheiness et al. 1978), and $cDNA_{amv}$ for AMV.

The distribution of viral nucleotide sequences related to these specific cDNAs was explored by hybridizing them at plateau C_rt values to several viral RNAs. The results shown in Table 3 allowed us to draw several conclusions: (1) The cDNAs tested are specific for the DLVs used for their preparation. (2) They represent distinct sets of sequences, unrelated to each other by cross-hybridization experiments. (3) $cDNA_{mc29}$ shows extensive homology to CMII, OK10, and MH2 RNAs, as does $cDNA_{amv}$ to E26 RNA. As will be described elsewhere, the three cDNAs exhibited exactly the same specificity when tested with cellular RNAs extracted from NP cells, further demonstrating that the probes indeed detect sequences specific for the three types of DLVs.

The complexities of the specific cDNAs were shown to be 3700 nucleotides for $cDNA_{aev}$ and 2100 nucleotides for $cDNA_{amv}$ (S. Saule et al., in prep.); $cDNA_{mc29}$ was shown to comprise 1800 nucloetides (Sheiness et al. 1978).

Table 2. DLV Genomes in NP Cells Are Related to ALVs

DLV strain	Type of NP cell	Annealing (% nuclease-S1 resistance) with $cDNA_{rep}$	$cDNA_{gag\text{-}pol}$	$cDNA_{env}$	$cDNA_{src}$
AEV	chicken fibroblast	28	15	16	<3
MC29	quail fibroblast	45	30	66	<3
CMII	quail fibroblast	41	24	79	<3
OK10	quail fibroblast	77	87	45	<3
MH2	quail fibroblast	32	25	10	<3
AMV	chicken myeloblast	50	59	< 3	<3
E26	chicken myeloblast	36	13	60	<3

Total RNA was extracted from the different avian NP cells and hybridized under stringent conditions (25-μl aliquot containing 1–10 mg/ml of RNA, 0.6 M NaCl at 68°C) with cDNAs (1000 cpm/point = 0.02 ng) to plateau C_rt values (defined as 10 times the $C_rt_{1/2}$). cDNAs were prepared as described earlier, but using calf-thymus-primed polymerase reactions (Stéhelin et al. 1976a; Tai et al. 1977). Hybrids were assayed by nuclease S1. No correction for the expression of endogenous RAV-0 or cellular *sarc* was made since they gave values below 3% in each case.

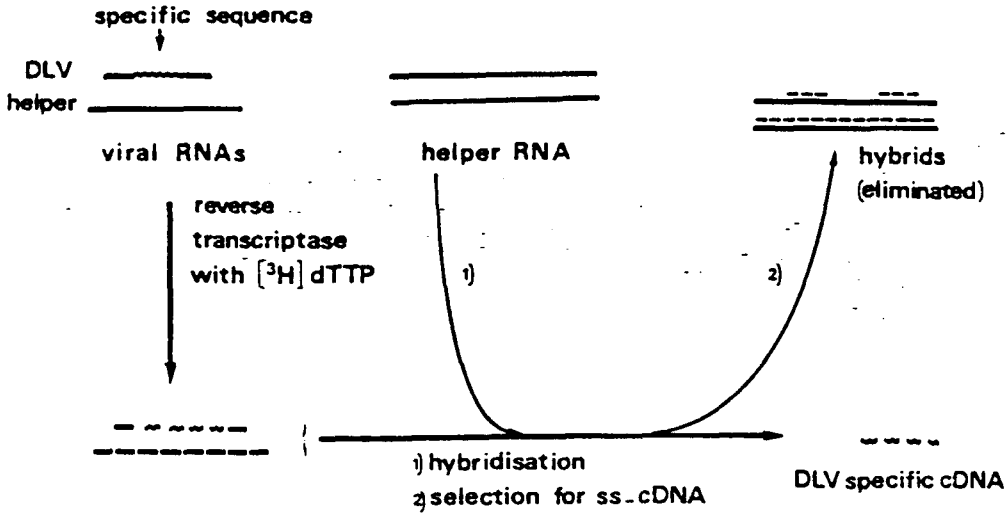

Figure 1. Scheme for the preparation of DLV-specific cDNAs. Cloned AEV (RAV-2) and MC29 (RAV-2) pseudotypes, obtained by rescuing these viruses with RAV-2 from cloned NP fibroblasts, were propagated in chicken erythroblasts (line 6C2) and fibroblasts, respectively. Viral RNAs were extracted from the pelleted viruses and then transcribed into labeled cDNA using in vitro reverse transcription with purified AMV reverse transcriptase, [^{3}H]dTTP and unlabeled dCTP, dGTP, and dATP, and calf-thymus-priming. In a negative selection step, each cDNA mixture was hybridized to an excess of RNA extracted from RAV-2 grown separately. Single-stranded DLV-specific cDNAs were then isolated after fractionation on hydroxyapatite. AMV-specific cDNA was prepared in a similar way, but using RNA of AMV (helper) pseudotype obtained from the plasma of infected chickens and a mixture of ALV helper-virus RNAs extracted from RAV-1, RAV-2, and MAV-2 (0) viruses for the selection. Specific activities of the [^{3}H]cDNAs were about 50×10^6 cpm/μg. Details of these experiments will be described elsewhere.

Size of the Genomic RNAs of DLVs

Among the DLVs, the genomic sizes of only MC29 and MH2 were determined recently by heteroduplex analysis to be about 5700 nucleotides long (Hu et al. 1979).

We used a different approach to study the sizes of the remaining DLVs. Medium was harvested (4-hr harvests) from cells infected with cloned pseudotypes of each DLV. The viral RNAs were extracted and separated by vertical agarose gel electrophoresis with rRNA markers of *Escherichia coli* (16S and 23S), quail (18S and 27S), or CV-1 cells (18S and 28S). RNA blots were performed according to the method of Alwine et al. (1977) and hybridized to [^{32}P]cDNA$_{rep}$ and, when appropriate, to [^{32}P]cDNA specific for AEV, MC29, or AMV. The results of these experiments, shown in Figure 2, led to the following conclusions: (1) All strains tested contain helper 34S RNAs detected by [^{32}P]cDNA$_{rep}$, and (2) specific bands corresponding to the DLV genomes are revealed by the specific cDNAs (30S–31S); the presence of additional minor bands seen occasionally has not been explained thus far. A notable exception is OK10 RNA which shows a much larger genome about

Table 3. DLVs Contain Unique Nucleotide Sequences

RNA derived from	Annealing (% nuclease-S1 resistance) with		
	cDNA$_{aev}$	cDNA$_{mc29}$	cDNA$_{amv}$
AEV	100	<3	<3
MC29	<3	100	<3
CMII	<3	96	<3
OK10	<3	91	<3
MH2	<3	66	<3
AMV	<3	<3	100
E26	<3	<3	68
ALV helper viruses (control)	<3	<3	<3

Viral 50S–70S RNAs were extracted and hybridized to plateau C_rt values (over 10 M·sec/liter) under stringent conditions (0.6 M NaCl at 68°C) with the specific cDNAs (1000 cpm/point = 0.02 ng). The hybrids formed were tested for nuclease-S1 resistance. Results obtained were standardized to the values of the homologous reaction. Maximum hybridization of cDNA$_{aev}$ with AEV RNA was 95%, cDNA$_{mc29}$ with MC29 RNA 90%, and cDNA$_{amv}$ with AMV RNA 95%. The DLVs tested were cloned pseudotypes with RAV-1 or RAV-2 helper ALV, with the exception of AMV and E26 which were not cloned. The ALV helper-virus RNAs tested as controls were obtained from RAV-1, RAV-2, RAV-49, RAV-50, and MAV-2 (0).

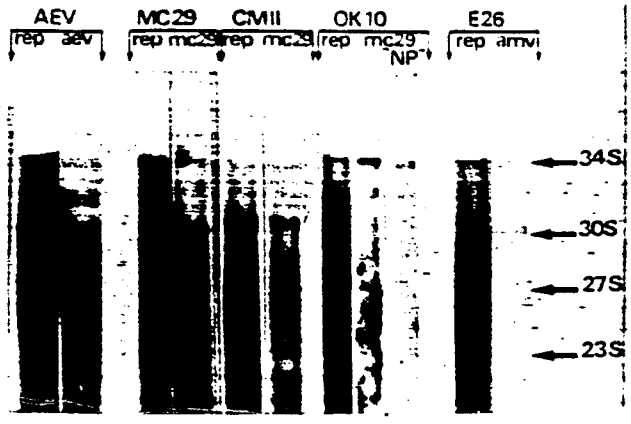

Figure 2. Sizes of the DLV genomes. DLV (helper) RNAs were extracted from 4-hr-harvested supernatants, fractionated by electrophoresis on vertical agarose gels, and then blotted according to the method of Alwine et al. (1977). The blots were hybridized to ^{32}P-labeled $cDNA_{rep}$ (*left*) or specific $cDNA_{aev}$, $cDNA_{mc29}$, $cDNA_{amv}$ (*right*). The slot marked "NP" contained RNA of OK10 harvested from "nonproducer" cells without helper virus. Autoradiography was performed for 1–10 days as described by Frisby et al. (1979). In general, the ratio of DLV to helper RNAs was ≤1, with the exception of CMII where it was about 4 to 1.

34S, in agreement with the high annealing seen for this virus with ALV cDNAs in Table 2. With such a genome, OK10 is obviously less defective than the other DLVs and is likely to be produced in the medium of NP cells containing only the OK10 provirus. This was indeed the case, as shown in Figure 2 where the slot marked "NP" contains RNAs extracted from the medium of "nonproducer" cells. Although OK10 seems to have most of the *pol* sequences, no active polymerase could be demonstrated (M. B. Raes and D. Stéhelin, unpubl.), which agrees with the fact that this virus is not infectious (C. Lagrou et al., in prep.).

Genetic Content of the DLVs

From our results, a provisional map was drawn (Fig. 3) of the genetic content of the DLVs studied (the size of MH2 was taken from previously described observations of Duesberg et al. 1978). Several remarks can be made. (1) All the genomes contain at least 650 nucleotides in the *gag* gene; this could correspond to the N-terminal part (p19) of the *gag* gene found to be present in the *gag*-gene-related proteins of DLVs. It may also be essential for the growth of these viruses or may contain an initiation site for the translation of a *gag-x* polyprotein (see Discussion). (2) The order of the different sets of nucleotide sequences is not known and has been chosen according to the known order of ALV structural genes and the putative location of sequences responsible for the *gag*-gene related proteins. There is good agreement between the size of these polyproteins and our nucleotide sequence data. No non-*gag*-gene-related polyproteins corresponding to comprising *env* sequences in these viruses have been detected. (3) There is no requirement for the presence in these viruses of nucleotide sequences related to the *env* gene of ALVs, since some lack most or all of such sequences (see Table 2). (4) MH2 and E26 contain nucleotide sequences unaccounted for by our hybridization studies. (5) Hybridization studies performed with $cDNA_{gag\text{-}pol}$ and with $cDNA_{gag\text{-}pol\text{-}C}$ (S. Saule et al., unpubl.) suggest that DLVs contain at least part of the *C* region detected in all avian viruses studied thus far.

DLV-specific Sequences Represent Transduced Normal Cellular Genes

To investigate the origin of DLV-specific sequences, DNA extracted from normal avian cells was tested for its ability to hybridize with $cDNA_{aev}$, $cDNA_{mc29}$, and

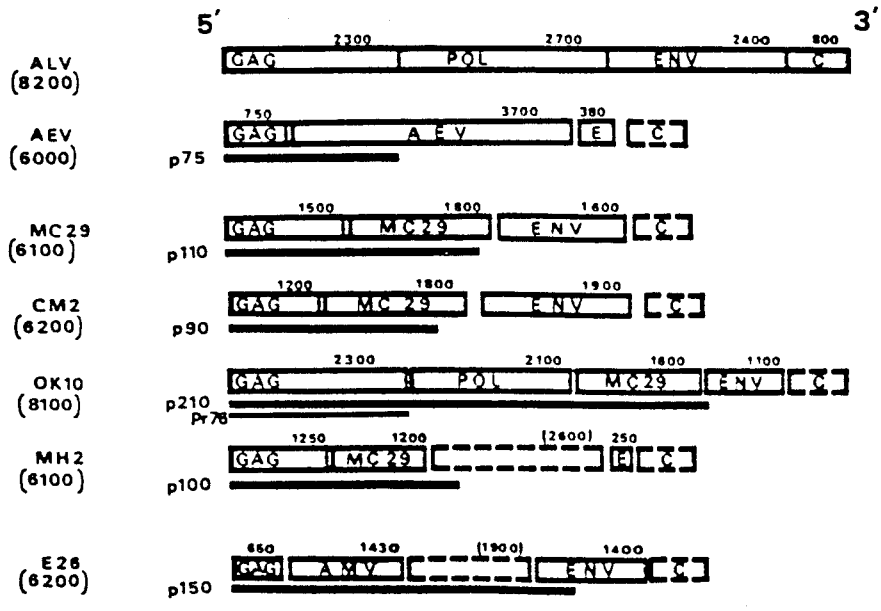

Figure 3. Genetic maps of the DLV RNAs (see text). Standard deviation for the annealing values was ±5% (for the polyproteins related to OK10 and E26 viruses, see Graf et al. [this volume]).

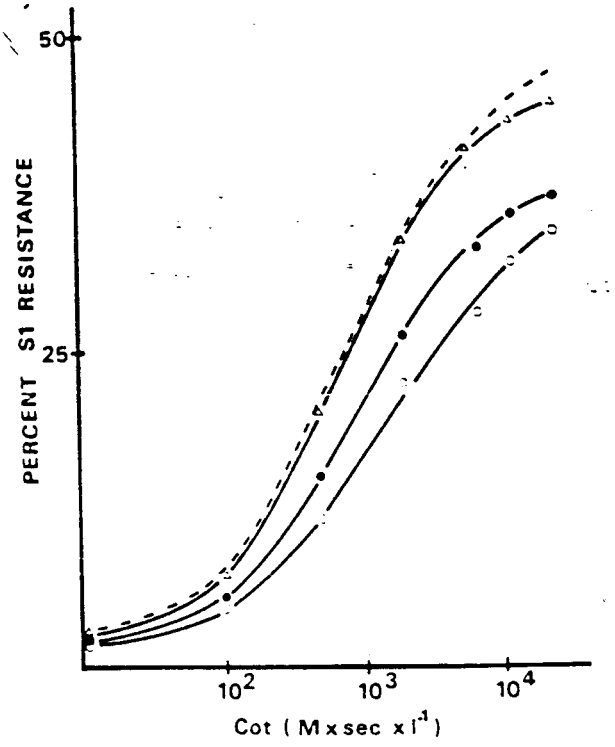

Figure 4. DLV-specific sequences have their counterparts in normal chicken DNA. Aliquots of 11-day-old chicken embryo DNAs (200 μg/point, sheared to 4S–6S) were hybridized with the different specific cDNAs (1000 cpm/point = 0.02 ng) under stringent conditions (0.6 M NaCl at 68°C) at increasing C_0t values. Hybrids formed were tested for their resistance to endonuclease S1. (●) $cDNA_{aev}$; (△) $cDNA_{mc29}$; (○) $cDNA_{amv}$; (--------) $cDNA_{sarc}$. Similar results were obtained with normal quail embryo DNA.

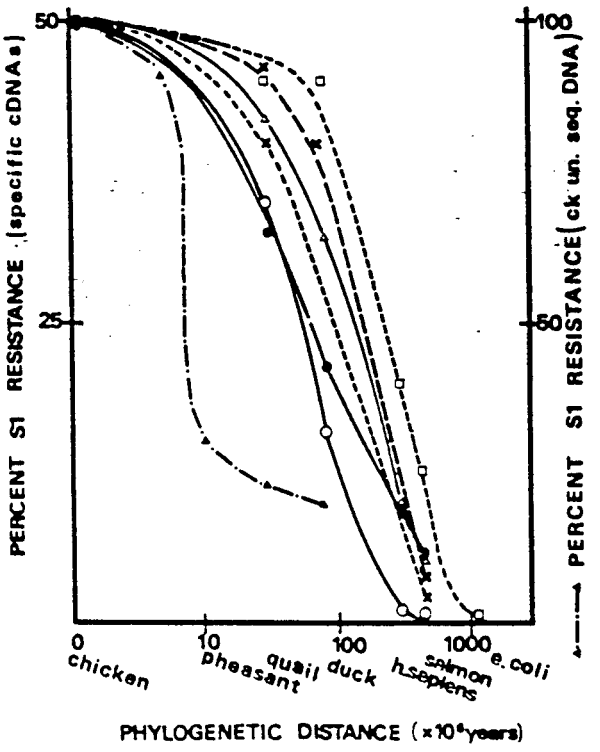

Figure 5. Stability of cellular DLV-related specific sequences during evolution. Aliquots of DNA extracted from different species that diverged increasingly long ago with speciation were hybridized under the specific conditions as described in Fig. 2. For comparison, the values obtained were standardized arbitrarily to 50% for the chicken; this was also taken as the origin of the scale indicating phylogenetic distance. (●) $cDNA_{aev}$; (△) $cDNA_{mc29}$; (○) $cDNA_{amv}$; (--------) $cDNA_{sarc}$; (▲) results obtained with the [^{3}H]-labeled chicken DNA unique sequence (Frisby et al. 1979) (1000 cpm/point = 20 ng) in similar conditions (standardized to 100% for homologous reassociation) and with cDNAs representing genes known to be conserved throughout the evolution of vertebrates. (x-----x) $cDNA_{glo}$ for globin; (x--------x) $cDNA_{ova}$ for ovalbumin (Stéhelin et al. 1978).

$cDNA_{amv}$. Figure 4 shows that all three types of sequences have their counterparts in normal cellular chicken DNA and, as judged from the $C_0t_{1/2}$ values, that they are present in the nonrepetitive DNA at one to two copies each per haploid genome. The low plateau values observed in Figure 4 are due to the competition of DNA reassociation with the DNA-cDNA hybridization reaction; transcription analyses (see below) show that most or all of the specific sequences are present in normal chicken DNA. Results obtained with molecular hybridization in liquid phase under stringent conditions show (Fig. 5) that the bulk of the nonrepetitive chicken DNA sequences are rapidly lost during evolution (~75% of the nuclease-S1-resistant hybrids formed are lost in the 30 million years of evolution from chicken to quail). Similarly, ALV-related virogene sequences, which have recently been shown to be absent in some species of the *Gallus* (Frisby et al. 1979), are also phylogenetically unstable. In contrast, cellular sequences related to $cDNA_{aev}$, $cDNA_{mc29}$, and $cDNA_{amv}$ are highly conserved throughout the phylogeny of higher vertebrates. As also shown in Figure 5, their evolutionary half-lives are comparable to that of known stable genes such as globin or ovalbumin. The latter values resemble those obtained earlier for the transforming *src* gene of ASVs (Stéhelin et al. 1976b, 1978; Spector et al. 1978a) and agree with the result recently described for MC29 by Sheiness and Bishop (1979).

Cellular DLV-related Specific Sequences Are Transcribed at Low Levels in Normal Cells

Since normal cells contain nucleotide sequences related to highly transforming viruses, it was of interest to study the rate of transcription of such sequences. Total RNA extracted from normal chicken (or quail) fibroblasts was hybridized to the different specific cDNAs. Figure 6 shows that these sequences are all transcribed at a low level (a few copies per cell). Again, similarity with the previously described cellular *sarc* expression is found (Stéhelin et al. 1978; Spector et al. 1978b), with cellular sequences related to $cDNA_{aev}$ transcribed at two to three copies per cell; cellular sequences related to $cDNA_{mc29}$ are reproducibly expressed at a higher level (five to ten copies/cell), as also found by Sheiness and Bishop (1979). In contrast, cellular sequences related to $cDNA_{amv}$ are slightly less

transcribed in fibroblasts (one copy/cell). As shown in Figure 6, all these cellular sequences reached plateau hybridization values close to 100% and therefore must be essentially fully represented in normal cellular nucleic acids (see also Roussel et al. 1979).

DISCUSSION

Three New Types of Viral Oncogenes Specific for Leukemic Transformation

Our results have shown that all seven defective leukemia viruses studied contain part or all of one of three different sets of specific nucleotide sequences complementary to $cDNA_{aev}$, $cDNA_{mc29}$, or $cDNA_{amv}$. These specific sequences have their counterparts in normal cells of higher vertebrates, where their divergence correlates with the phylogeny of speciation. None of these sequences are related to the transforming *src* gene of ASVs. Although it is not yet understood how recombinants between ALV sequences and cell-derived sequences can acquire oncogenic capacity, several indications favor the hypothesis that three specific transforming genes, which we have termed *erb*, *mac*, and *myb*, in agreement with Graf et al. (this volume), are detected by $cDNA_{aev}$, $cDNA_{mc29}$, and $cDNA_{amv}$, respectively. (1) There is a strict correlation between the presence of *erb*, *mac*, or *myb* sequences in a given virus and its selective capacity to generate transformed erythroblasts, macrophagelike cells, or myeloblasts (Graf et al., this volume; Gazzolo et al. 1979; Beug et al. 1979). (2) The properties of these sequences and their origin are reminiscent of results obtained earlier for the transforming *src* gene of ASVs, i.e. size, cellular origin, and phylogenetical stability (Stéhelin et al. 1978; Spector et al. 1978a,b). (3) As indicated by heteroduplex mapping studies with MC29 (Hu et al. 1979), these sequences do not appear as bits of sequences randomly distributed throughout the viral RNA, but rather as continuous stretches located inside the viral genome. (4) Temperature-sensitive mutants have recently been isolated from AEV, indicating that a virus-coded protein must be synthesized to maintain the undifferentiated state of transformed erythroblasts (Graf et al. 1978). (5) All DLVs tested contain the 5′ region of the ALV genome (partial *gag* gene).

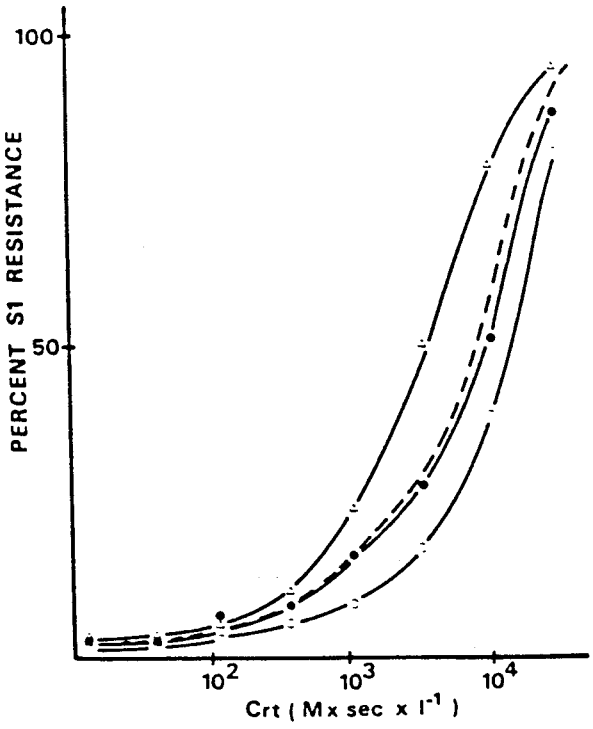

Figure 6. Transcription of the cellular DLV-related specific sequences in normal fibroblasts. Total RNA was extracted from normal chicken embryo fibroblasts and hybridized at increasing C_0t values (varying RNA concentration up to 10 mg/ml) under stringent conditions (0.6 N NaCl at 68°C) to the specific cDNAs (1000 cpm/point = 0.02 ng). Hybrids were scored for their endonuclease S1 resistance. (●) $cDNA_{aev}$; (△) $cDNA_{mc29}$; (○) $cDNA_{amv}$; (--------) $cDNA_{src}$.

In the strains studied so far (MC29 and MH2), this region was located adjacent to the DLV-specific sequence (Mellon et al. 1978; Hu et al. 1979). In addition, cells nonproductively transformed by AEV, MC29, CMII, and MH2 express novel polyproteins with molecular weights of 75,000 (Hayman et al. 1979), 110,000 (Bister et al. 1977; Hayman et al. 1979a), 90,000 (Hayman et al. 1979a), and 100,000 (Hu et al. 1978), respectively, with the structure: partial *gag-x* or *gag-y*, where *x* or *y* represents polypeptide moieties that are antigenically unrelated to known ALV proteins and presumably correspond to part or all of the specific *erb* or *mac* nucleotide sequences. That these polyproteins could be involved in the transformation process is indicated by the finding that the type of tryptic peptide pattern exhibited by the non-*gag*-gene portion of the polyprotein of a given DLV closely correlates with the transformation specificity of the virus (Graf et al., this volume; M. J. Hayman, pers. comm.) In their general structure and in their ability to code for *gag*-gene-related polyproteins, avian DLVs resemble replication-defective mammalian retroviruses capable of inducing leukemias or sarcomas with a short latency period, such as the Abelson (Witte et al. 1978) murine leukemia virus and feline sarcoma virus (Stephenson et al. 1977; Sherr et al. 1978).

Avian DLVs behave like recombinants (Fig. 3) between an ALV-related vector virus of low oncogenicity and unknown origin (Frisby et al. 1979) and an evolutionary stable set of nucleotide sequences of cellular origin; in this regard they also closely resemble ASVs (for review, see Bishop 1978). The mechanism of such a recombination remains unclear, but it could be related to the jumping polymerase hypothesis proposed recently by Coffin (1979). How frequently such a recombination occurs is not known. Since the beginning of this century, about 30 DLV-like and one to three ASV-like independent isolates have been reported (Graf and Beug 1978). Recent studies by Hanafusa and coworkers (1977) have shown that

cellular *sarc* sequences can be rescued at high frequencies by infection of chicks with mutants of ASV partially deleted in the *src* gene. However, a transduction of cellular sequences may not necessarily lead to such dramatic effects as the generation of a highly oncogenic virus and therefore may be much more frequent than anticipated. In fact, we have recently found in one ASV stock a new virus containing apparently unknown cellular genetic material (N. Pluquet and D. Stéhelin, unpubl.).

That only a partial homology was observed between the specific sequences of MC29 and MH2, as well as the specific sequences of AMV and E26 (Table 3), can be explained in three ways: (1) These viruses acquired cellular genes that already differed in their sequence, or (2) they acquired the same gene and the observed differences reflect a divergence introduced by passage of the virus, or (3) a second recombination event happened in MH2 and E26. The finding that there are only one to two copies per haploid genome of these genes present in cellular DNA is consistent with the second possibility. The observation that there is a somewhat lower degree of homology of $cDNA_{amv}$ to cellular DNA than for other types of specific cDNAs tested (Fig. 4) might reflect the long passage history of AMV and also would be in agreement with the second of the above interpretations. However, hybridization experiments performed between $cDNA_{mc29}$ and MH2 RNA in nonstringent conditions do not show a significant increase of annealing as the second interpretation would predict (M. Roussel et al., unpubl.) and thus leave that question open.

Possible Role in Leukemic Transformation of the Transforming Genes of DLVs and Their Cellular Counterparts

Recent studies with a temperature-sensitive mutant of AEV indicate that a viral gene product required for maintenance of transformation causes a block of differentiation in its hematopoietic target cells (Graf et al. 1978). This observation, together with the findings that DLVs can infect and are expressed in nontarget hematopoietic cells (Graf et al. 1979a and this volume) and that phylogenetically stable cellular sequences exist that are related to the oncogenes of DLVs, leads to the following hypothesis: The transforming genes of DLVs are homologous to normal cellular genes coding for lineage-specific hematopoietic differentiation proteins; the transforming proteins act by competitively inhibiting the corresponding cellular proteins (Graf and Beug 1978), thus leading to a block of differentiation and subsequent leukemogenesis. This hypothesis also provides an explanation for the target-cell specificity of DLVs and predicts that RNA sequences homologous to *erb*, *mac*, and *myb* are preferentially expressed in normal erythroblasts, macrophages, and myeloblasts, respectively. We are currently trying to purify hematopoeitic target cells for DLVs in order to test this prediction. In this regard, it would also be interesting to determine whether or not erythroblastosis occasionally induced after injection of chicken embryos with ALVs (Fredrickson et al. 1965) expresses increased amounts of *erb* RNA.

Another assumption of the hypothesis is that the oncogenes of DLVs represent modified versions of their cellular counterparts, as also proposed in general terms in Temin's protovirus hypothesis (Temin 1971). Such a modification may have occurred by a fusion of the cellular oncogenes with an endogenous ALV or by an aberrant splicing of the transduced sequences, thus leading to oncogenicity. Preliminary experiments indicate that the cellular *erb*, *mac*, and *myb* genes occur in pieces and therefore are likely to be subject to splicing mechanisms (S. Saule et al., unpubl.).

The capacity of AEV-type viruses to induce sarcomas and of MC29-type viruses to induce carcinomas suggests an additional specificity of *erb* and *mac* genes for nonhematopoietic tissues. This intriguing specificity is difficult to explain by the differentiation block hypothesis, although it is interesting to note that normal fibroblasts, which can be transformed by AEV, MC29, and ASV but not by AMV, express higher levels of cellular *erb*, *mac*, and *sarc* sequences than of cellular *myb* sequences (see Fig. 6). It is still unknown whether or not DLVs code for only one gene responsible for transformation that could exert a pleiotropic effect on different types of target cells, as suggested by the experiments of Calothy et al. (1978) with ASV.

The complexity of the DLV-specific probes is between 1800 and 3700 nucleotides, depending on the viral strain, thus allowing in some cases for enough space to code for more than one gene. In fact, a mutant of AEV has now been isolated that has lost its capacity to transform erythroblasts but is still transforming fibroblasts (Graf et al. 1979a; Royer-Pokora et al. 1979). On the other hand, results obtained with the *ts*34 mutant of AEV (Graf et al. 1978) suggest that a single mutation affects the transforming capacity of the virus for both erythroblasts and fibroblasts (Graf et al. 1979a). Clearly, more work is needed along these lines to resolve these observations.

If indeed DLV-induced leukemogenesis is the result of a competitive inhibition of a cellular differentiation protein by the viral transforming protein, spontaneous leukemogenesis may simply result from a malfunction of the latter. Our newly developed probes should be useful for determining whether or not cells from spontaneous leukemias express differentiation-specific sequences related to avian DLVs.

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Subgenomic mRNA in OK10 Defective Leukemia Virus-Transformed Cells

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OK10, a defective leukemia virus, is produced as a defective particle by so-called nonproducer transformed quail fibroblasts. OK10 defective viral particles contain an 8-kilobases (kb)-long genomic RNA, lack any detectable reverse transcriptase activity, and are not infectious. We studied the genetic content of OK10 RNA extracted from both virions and infected cells. As shown by RNA-cDNA hybridizations in stringent conditions, about 77% (6.4 kb) of the OK10 8.0-kb RNA was related to avian leukosis viruses in the three structural genes *gag*, *pol*, and *env*, as well as in the *c* region. The remainder of the OK10 genome-encoding capacity (≤1.6 kb) was homologous to the MC29-specific transforming sequence *myc*(m) and therefore has been named *myc*(o). *Eco*RI restriction analysis of the OK10 integrated proviral DNA with different probes indicated the presence of only one provirus in the OK10 QB5 clone, which agreed with the gene order: 5′-*gag*-Δ*pol*-*myc*(o)-Δ*env*-*c*-3′. Heteroduplex molecules formed between the viral OK10 8.0-kb RNA and the 6.8-kb *Sac*I DNA fragment of the Prague A strain of Rous sarcoma virus confirmed that structure and indicated that the *myc*(o) sequence formed a continuous RNA stretch of 1.4 to 1.6 kb long between Δ*pol* and Δ*env*. We also examined the *myc*(o)-containing mRNA's transcribed in OK10-transformed cells. OK10-transformed quail fibroblasts (OK10 QB5) transcribed two mRNA species of 8.0 and 3.6 kb containing the *myc*(o) sequence. The genetic content of the 3.6-kb species made it a possible maturation product of the genome size 8-kb species by splicing out the *gag* and *pol* sequences. In OK10-transformed bone marrow cells (OK10 BM), a stable bone marrow-derived cell line producing OK10, the *myc*(o) sequence was found in four RNA species of 11.0, 8.0, 7.0, and 3.6 kb. Again, the genetic content of these mRNA's indicated that (i) the 3.6-kb species could be spliced out of the 8.0-kb-genome size mRNA and (ii) the 11.0-kb-long mRNA could represent a read-through of the OK10 provirus, the corresponding maturation product being, then, a 7.0-kb mRNA. The 7.0- and 3.6-kb mRNA's both contained the *myc*(o) sequence, but no sequences related to the *gag* or *pol* gene. In conclusion, whereas the *myc* sequences have been generally thought to be expressed through a *gag-onc* fusion protein, as for MC29 and CMII viruses, our experiments indicate that they could also be expressed as a non-*gag*-related product made from a subgenomic mRNA in the OK10-transformed cells.

OK10 has been classified as a defective leukemia virus of the MC29 subgroup based on the differentiation phenotype of the hematopoietic cells it transforms in vitro (9). Bone marrow cells infected by these viruses (MC29, MH2, CMII, and OK10) resemble transformed macrophages and express differentiation markers of the myeloid lineage (2). These findings have been confirmed by biochemical studies. The transforming potential of MC29-type viruses is associated with the presence in their genome of a specific nucleotide sequence called *myc* (22, 26). The OK10 allele of the *myc* sequence has been called *myc*(o). The *myc* sequence has no homology with the *src* gene of Rous sarcoma viruses (RSVs) or with the structural genes (*gag*, *pol*, and *env*) of avian leukosis viruses (ALVs) (22). The *myc* sequence has been proposed, therefore, to be an oncogene (26). As for the *src* gene, *myc* is closely related to conserved nucleotide sequences (*c-myc*) found in the DNA of uninfected vertebrate cells (22).

The genetic structure of all defective leukemia viruses characterized so far conforms to the same basic model. They all contain ALV-related sequences and a unique nucleotide sequence of cellular origin (30). However, they are all deleted in ALV-related genes, which are essential

for replication, and therefore need a helper virus (ALV) for their propagation in cultured cells (9). Cells transformed by MC29 contain a novel 110,000-dalton (110K) fusion protein comprising part of the *gag* polyprotein linked to the translation product of the *myc* sequence (4). Such a product is made in the cell from a genome-size viral mRNA. A similar structure has been proposed for OK10 (5), with a 200,000-dalton (200K) fusion protein (20). Our results suggest the alternate possibility that in OK10 virus the transforming gene product could also be a non-*gag* protein translated from a subgenomic viral mRNA.

MATERIALS AND METHODS

Cells and viruses. Defective leukemia virus nonproducer quail fibroblast clones (OK10 QB5 [10, 20], MC29 Q8 [4]) were obtained through the courtesy of T. Graf and K. Bister. OK10-transformed bone marrow cells (OK10 BM) (19) were kindly provided by L. Hortling. The transformation-defective Prague C strain of RSV (PR-RSV-C) was obtained originally from R. Junghans. All viruses were grown on C/E chicken fibroblasts as described elsewhere (29).

Preparation of virus-specific cDNA's. Radiolabeled cDNA transcripts were synthesized in exogenous reactions, using 50 to 70S viral RNA templates, calf thymus primer, and purified avian myeloblastosis virus polymerase, as previously described (23, 32). The steps for selection of cDNArep (representing the transformation-defective PR-RSV-C genome), cDNAsrc, cDNAgag, cDNApol, cDNAenv, and cDNAc are outlined in Fig. 1A. cDNArep, cDNAsrc, and cDNAenv were selected as described previously (29, 31). cDNAc was prepared by hybridizing the remaining cDNA containing the *gag-pol-c* sequences to plateau value to 10S (ca. 800 nucleotides) polyadenylated [poly(A^+)] RNA of PR-RSV-C and then recovered after selection (29, 31). The 10S RNA was prepared from spontaneously nicked 70S RNA (100 μg) fractionated by sucrose gradient centrifugation followed by polyadenylate [poly(A)] selection over polydeoxythymidylate-cellulose (these two steps being repeated sequentially twice). Such 10S RNA should contain the entire *c* sequences and may contain some *src* or *env* sequences (already eliminated from the cDNA by the previous steps) but no *pol* or *gag* sequences. cDNAgag was finally separated from the remaining cDNA by annealing to MC29 Q8 cellular poly(A^+) RNA at a plateau C_0t value of 2,000 mol·s liter^{-1}. That RNA, extracted from a cell containing the MC29 genome without helper sequences, was used, since MC29 virus has been reported to have most of the *gag* sequences but no *pol* sequences (27). Specificities of these cDNA's are validated in Table 1. cDNA-myc complementary to the specific region of MC29 virus [*myc*(m)] was made as described previously (26).

cDNA5′ was prepared according to the published procedure (8). Alternatively, probes for *gag*, *pol*, *env*5′, or *env*3′ were prepared by nick translation (21) of DNA fragments B, A, C, and D (Fig. 1B) originating from a λ phage clone containing the *Sac*I 6.8-kilobase (kb) fragment of PR-RSV-A DNA, a gift of J. Taylor. The fragments were purified twice by electrophoresis

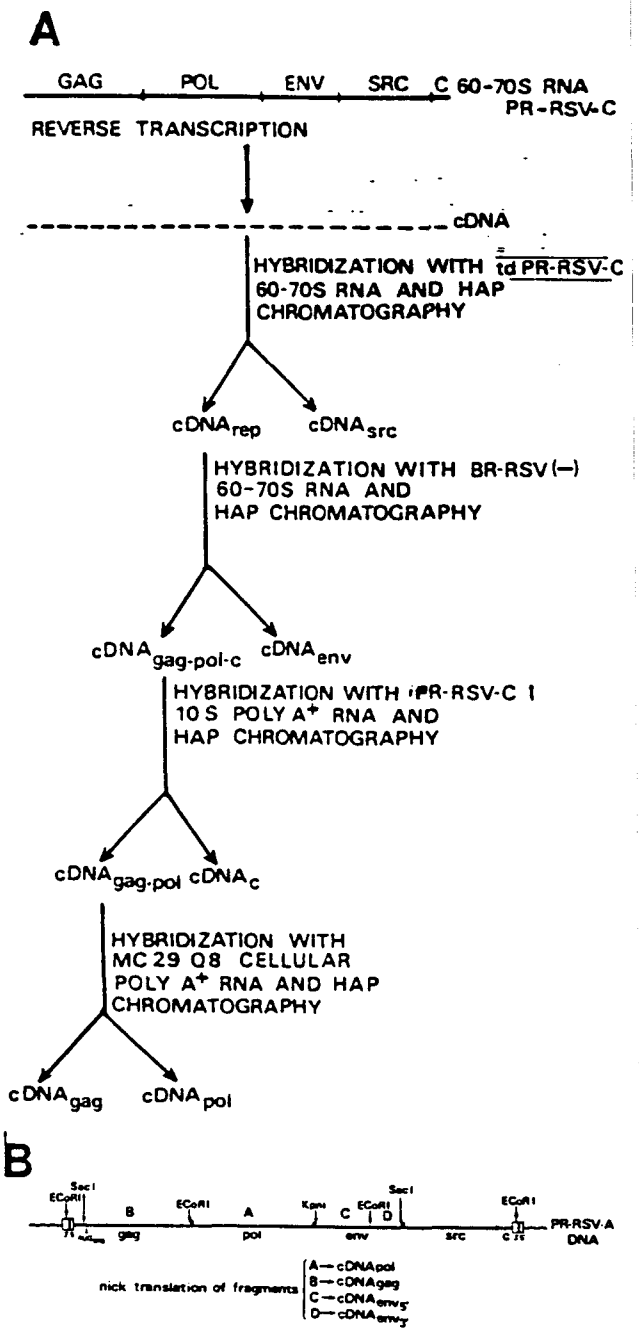
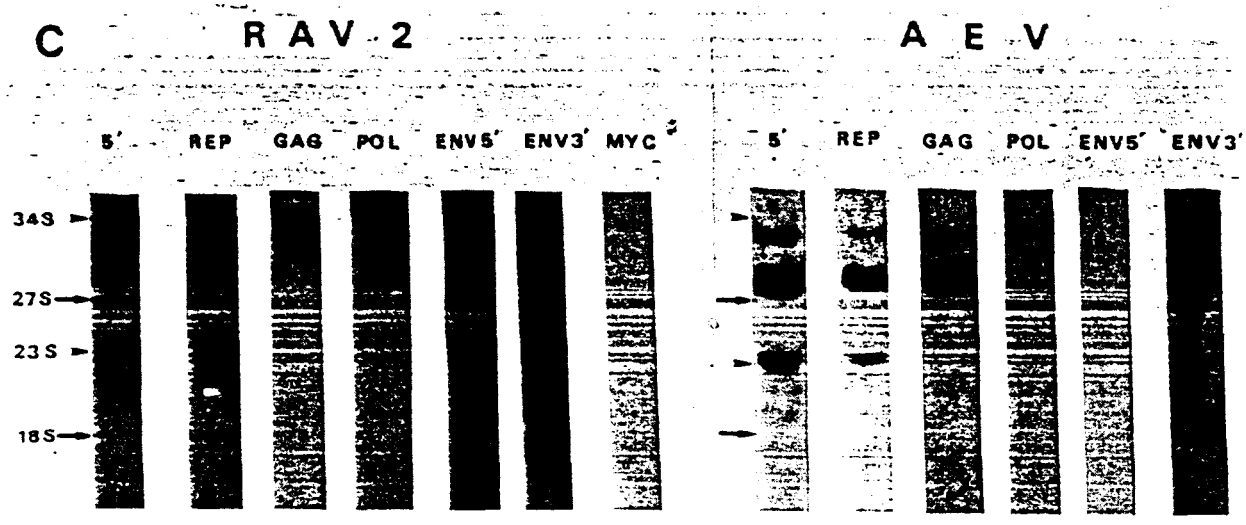


FIG. 1. Preparation of cDNA's. (A) cDNArep, cDNAgag-pol-c, cDNAc, and cDNAgag were recovered as RNA-cDNA hybrids after hydroxyapatite (HAP) chromatography. The hybrids were alkali treated, neutralized, and ethanol precipitated before the next step of selection. (B) The *Sac*I DNA fragment of PR-RSV-A was in phage Charon 16A. The insert was excised by endo·R-*Sst*I, which is an isozyme of endo·R-*Sac*I. Nick-translated cDNA's were prepared from fragments A, B, C, and D isolated by agarose gel electrophoresis after the suitable restriction cleavages. (C) Hybridization of cellular RAV-2- and AEV (nonproducer erythroblasts, clone HD3)-infected cells with nick-translated ^{32}P-labeled DNA. Total poly(A^+) RNAs were denatured by glyoxal treatment, separated on 1% agarose gels, transferred to diazobenzyloxymethyl paper, and hybridized with ^{32}P nick-translated fragments A, B, C, D, and cDNAmyc. The RNA content of HD3 cells was previously described (23). rRNA's from chicken fibroblasts were used as standard length markers.



in 0.8% agarose gels before the nick translation reaction, which was performed by using a New England Nuclear nick translation kit.

Reverse transcriptase assay. OK10 defective viral particles were purified from 1 liter of OK10 QB5 cell culture medium as described previously (15). The exogenous reverse transcriptase assay was then carried out as follows: purified virions were incubated at 38°C for 2 h with various Nonidet P-40 concentrations in 0.1 M Tris-hydrochloride (pH 8.1)–8 × 10^{-3} M $MgCl_2$–1% β-mercaptoethanol–5 × 10^{-5} M each dATP, dGTP, and dCTP–10^{-5} M dTTP–0.02 mCi of [^{3}H]dTTP.

Nucleic acid hybridization. Viral and cellular RNAs for liquid hybridization studies were extracted and purified as described previously (23). Standard liquid hybridization reaction mixtures contained 0.6 M NaCl, 2 × 10^{-3} M EDTA, 2 × 10^{-2} M Tris-hydrochloride (pH 7.4), 500 μg of calf thymus DNA per ml as carrier, 2,000 cpm (0.04) ng) of ^{3}H-labeled cDNA's, and appropriate RNAs in large excess. Hybridizations were conducted in glass capillaries at 68°C, and the extent of annealing was monitored by digestion with S1 nuclease.

Agarose gel electrophoresis of poly(A)-containing cellular and viral RNAs. Poly(A)-containing cellular and viral RNAs were prepared as described elsewhere (34). RNA samples were denatured by glyoxal treatment (18) and submitted to electrophoresis in 1% agarose horizontal gels.

Transfer hybridization of RNAs. Size-separated RNAs were transferred to diazobenzyloxymethyl paper prepared as described previously (1). The northern blots were hybridized with ^{32}P-labeled cDNA's as described previously (17), except the washing procedure which was performed as follows. After a 10-min wash in 1× Denhardt solution–2× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate), the blots were submitted to mild sonication in 0.1× SSC–0.1% sodium dodecyl sulfate for 1 to 3 min at room temperature and dried (Pons sonicator, 3 liters, 100 W, 40 kHz; Bioblock, Strasbourg, France). The blots were then subjected to autoradiography (Kodak X-ray films, Dupont Lightning Plus X-ray intensifying screens, −70°C).

Heteroduplex mapping. The 6.8-kb *Sac*I DNA fragment of PR-RSV-A cloned in lambda (Charon 16A) was excised by *Sst*I, an isozyme of *Sac*I, and purified by agarose gel electrophoresis. The gene content of this DNA fragment is shown in Fig. 1B (25). The OK10 QB5 viral 8.0-kb RNA purified by sucrose gradient centrifugation (16) was hybridized to the *Sst*I PR-RSV-A fragment by using the high formamide conditions developed by Casey and Davidson (6). The heteroduplexes were dialyzed for 1 h at 37°C against 1 M glyoxal and then dialyzed for 4 h at 4°C against 0.01 M Tris (pH 7.4)–0.001 M EDTA.

The heteroduplexes were spread from a 41% formamide hyperphase containing 0.1 M Tris (pH 8.5)–0.01 M EDTA–50 μg of cytochrome *c* per ml onto a hypophase of water. pMB9 plasmid DNA was included as an internal-length standard. Molecules were picked up on 300-mesh copper grids covered with a thin carbon film and were colored with uranyl acetate. Dried molecules were shadowed with a platinum-carbon mixture (95:5%, wt/wt), using a Balzer electron bombardment gun and a quartz crystal film thickness monitor. A 7° shadow angle and a mean shadow thickness of 0.3 mm, with a specimen rotation speed of ca. 80 rpm, were used for low rotary shadowing.

High-molecular-weight DNA isolation. Cells were gently resuspended (10^7 cells per ml) in an extraction buffer containing 0.05 M Tris-hydrochloride (pH 7.4)–0.1 M NaCl–0.01 M EDTA–100 μg of proteinase K per ml and lysed by adding slowly, under gentle rocking, Sarkosyl to a 1% final concentration. After overnight digestion, CsCl was added (2.54 g per 2 ml of lysate; ρ = 1.7 g/ml), and the DNA was centrifuged to equilibrium. The band of viscous DNA was collected through a large hole made in the bottom of the tube and dialyzed extensively against 0.001 M Tris-hydrochloride (pH 7)–0.0001 M EDTA.

Restriction endonuclease mapping. Restriction endonucleases were obtained from Boehringer Mannheim (Mannheim, West Germany) and Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). The DNA digestions were performed according to the supplier's instructions. DNA fragments were separated by electrophoresis in 0.8% agarose horizontal gels and transferred to nitrocellulose as previously described (28).

TABLE 1. Specificities of cDNA's used and results[a]

RNA SPECIES	% S_1 RESISTANCE with cDNA							
	rep	gag-pol-c	gag	pol	env	src	c⁻	myc
E. COLI	3	3	2	2	3	1	2	1
10 S Pr C POLY A+	9	14	2	2	4	nd	100 [70]	nd
Pr C	100	100 [85]	100	100 [80]	100 [75]	100 [90]	100	1
rd Br Q16	68	nd	nd	nd	3	98	97	nd
td Pr C	100 [87]	100	100	100	100	3	100	2
MC 29 Q8	45	38	100 [65]	5	66	13	38	100 [97]
OK 10 QB5	77	87	84	96	45	13	77	90
OK 10 BM	81	nd	nd	nd	77	2	nd	90
[1] COMPLEXITIES [Kb]	8.0	nd	2.0[a]	3.0[b]	2.2	1.8[2]	0.33	1.6[3]

[a] RNA extracted from different viruses (PrC, transformation-defective PrC, replication-defective Br, OK10 BM) or cells were hybridized under stringent conditions (0.6 M NaCl, 68°C) with cDNA's (2,000 cpm per point = 0.04 ng) to plateau C_rt values (10 times the $C_rt_{1/2}$ and C_rt: 4×10^4 mol·s liter^{-1} for *E. coli*). Results obtained were standardized to the values of the homologous reaction (numbers in brackets). The cDNA's were prepared as described in the legend to Fig. 1A. cDNAmyc was prepared as described by Sheiness et al. (26). [1] The complexity of the cDNAs were calculated according to Saule et al. (23); [2] according to Sheiness et al. (26); [3] according to Stehelin et al. (29). [a] cDNAgag contains only the *gag* sequences conserved in MC29; [b] cDNApol contains, in addition to the *pol* sequence of PR-RSV-C, the *gag* sequences deleted in MC29. ND, Not done.

The Southern blots were hybridized to specific ^{32}P-labeled cDNA's, washed, dried, and submitted to autoradiography as previously described (24).

Biological and physical containment. All experiments performed with the EK2-certified phage Charon A16 containing the *Sac*I DNA fragment of PR-RSV-A and its certified host *Escherichia coli* DP50 *supF* were carried out according to L2 B2 conditions in the nomenclature adopted by the French Committee (7) (equivalent to P2 EK2 in the National Institutes of Health nomenclature).

RESULTS

OK10 RNA of a nonproductively transformed quail fibroblast clone. The OK10 QB5 quail fibroblast clone has been reported to be nonproducing (10, 20, 30). It does, in fact, produce particles that accumulate in the culture fluid, but these particles are noninfectious (20, 30), suggesting the absence of a competent helper virus in these cells. The total RNA of these cells was analyzed to study the structure of the 8- to 8.6-kb OK10 RNA (5, 30). We used labeled cDNA probes related to all or parts of an ALV genome prepared as described in the legend to Fig. 1A, as well as cDNA probes corresponding to the *myc*(m) sequences of MC29 (cDNAmyc) and to the *src* gene (cDNAsrc) of PR-RSV-C. These probes were characterized by plateau hybridization experiments (Table 1). As shown, all probes exhibited the expected specificities. We thus used these probes to analyze the viral RNA of OK10 virus (Fig. 2; Table 1). Table 1 showed that the viral RNA of QB5 cells was closely related (77%) to an ALV cDNArep probe and contained homology in the *gag*, *pol*, *env*, and *c* regions. It hybridized extensively with cDNAmyc and did not contain sequences related to cDNAsrc. The following observations also resulted from Table 1 data: (i) the 84% hybridization with cDNAgag probably reflects some divergence in this gene, since it has been shown that the entire Pr76 *gag* precursor polypeptide

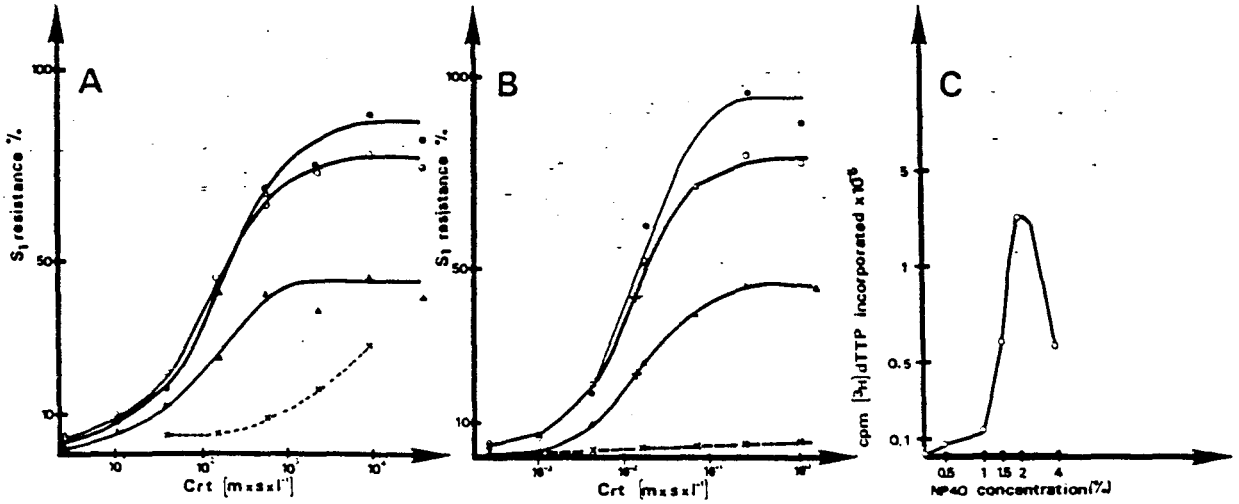


FIG. 2. Hybridization of (A) total cellular OK10 QB5 RNA and (B) 50 to 70S RNA from OK10 QB5-defective viral particles to cDNA's. (A) Hybridizations were performed in 22 μl with 0.3 mg of RNA at the highest C_rt and different ^3H-labeled cDNA's at the C_rt values indicated. ^{32}P-labeled cDNArep was included as a standard. Symbols: cDNAsrc, ×; cDNArep, ○, △; cDNAenv, ▲; cDNAmyc, ●. (B) Hybridizations were performed as in (A) with ^3H-labeled cDNA's and 20 ng of viral RNA at the highest C_rt. ^{32}P-labeled cDNArep was included as a standard. Symbols: cDNAsrc, ×; cDNArep, ○, △; cDNAenv, ▲; cDNAmyc, ●. $C_rt_{1/2}$ values on curves for *env* and *rep* have been marked by (+) symbols to indicate their similar experimental value. (C) Endogenous reverse transcriptase activity of OK10 QB5-defective particles. Virions from 1 liter each of OK10 QB5 and SR-RSV-E culture supernatants were purified separately as described in reference 15. Each point contained 0.053 μg of OK10 QB5 and 0.017 μg of SR-RSV-E virion proteins. Incubation were performed for 2 h at 37°C in the presence of increasing concentrations of Nonidet P-40. Symbols: SR-RSV-E, ○; OK10 QB5, ●.

was made in these cells (20); (ii) the 96% annealing with cDNApol indicated the presence of most or all of the *pol* gene, although this number may be an overestimation due to some *gag* sequences being present in this probe (see Table 1); (iii) cDNAenv annealed only to 45%, indicating that the *env* gene of OK10 was partially missing, or quite diverged (12), from its ALV homolog; (iv) the homology of OK10 RNA in the *c* region was much higher than for MC29, (and also for MH2; unpublished data), 35% as tested by this method.

We next tested the amount of OK10 RNA produced within the OK10 QB5 cells as well as in the culture fluid harvested from them. Figure 2A indicated about 330 copies of intracellular viral RNA ($C_rt_{1/2}$, 120 mol·s liter^{-1}), and the colinearity of the curves obtained with cDNAmyc and cDNAenv showed that these sequences were present in stoichiometric amounts. Figure 2B showed a similar pattern for the virus produced in the culture fluid of these cells. From the yield of RNA, we deduced a production of ca. 5×10^7 virions per ml. As reported previously (20), this virus was noninfectious and had no polymerase activity (Fig. 2C).

Proviral DNA of OK10 in the QB5 cells. According to the ALV relatedness determined above for OK10 viral RNA, we made the assumption that some of the *Eco*RI sites present in the DNA of several strains of RSVs and ALVs (25) (Fig. 1B) could be conserved in OK10. Our assumption was correct (Fig. 3). The *Eco*RI pattern of digestion of OK10 QB5 cellular DNA yielded three fragments of 2.7, 1.6, and 0.8 megadaltons (Md) hybridizing with cDNArep. These fragments were further analyzed by using DNA probes to the *gag*, *pol*, and *env* (3′ or 5′ part) genes, prepared as described in the legend to Fig. 1B, as well as cDNA5′ and cDNAmyc, prepared as described by Friedrich et al. (8) and Sheiness et al. (26), respectively. These probes exhibited the expected specificities as documented by their hybridization with northern blots containing polyadenylated RNA extracted from chicken fibroblasts infected by Rous-associated virus-2 (RAV-2) and chicken erythroblasts (HD3) transformed by avian erythroblastosis virus (AEV) (Fig. 1C; 23). The *gag* probe did not hybridize to the RAV-2 or AEV subgenomic RNAs. The *pol* probe contained probably about 160 bases related to *gag* (sequence data from D. Schwartz, R. Tizard, and W. Gilbert [personal communication], referred to throughout this paper), but did not hybridize with AEV RNA, which lacks these sequences (16, 27, 33), or to viral *env* sequences. The *env* 5′ probe contained about 190 bases into the *pol* gene (sequence data cited) and did not hybridize with AEV RNA, unlike the *env*3′ probe, which scored a different set of sequences. Finally,

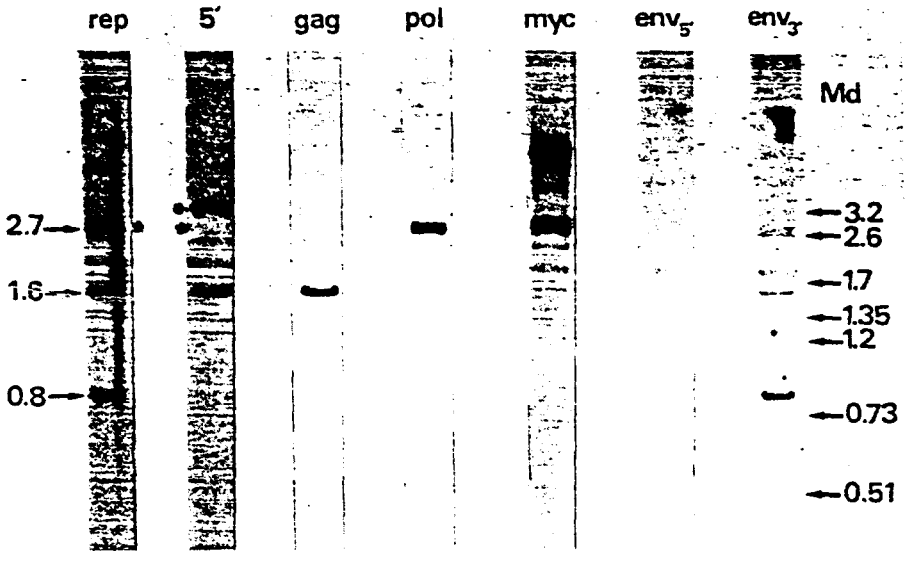


FIG. 3. *Eco*RI mapping of OK10 QB5 proviral DNA. DNA from OK10 QB5 cells was isolated and digested by *Eco*RI as described in the text. DNA fragments separated by electrophoresis in a 0.8% horizontal agarose gel were hybridized to different ^{32}P probes. The DNA fragments used as size markers were obtained by digestion with *Kpn*I, *Sst*I, and *Eco*RI of the clone containing the *Sac*I DNA fragment of PR-RSV-A. The probes used are listed across the top. Note that the band at 2.7 Md with the rep probe is different from the one seen at 3.2 Md with the 5' probe (*).

cDNAmyc did not hybridize with the RNA of RAV-2-infected fibroblasts (Fig. 1C).

Using these probes with Southern blots containing *Eco*RI digests of OK10 QB5 cell DNA, we reached the following conclusions (Fig. 3). (i) As expected, the 1.6-Md fragment hybridized with cDNA5' and with the *gag* probe only, thus representing probably the 5' part of the OK10 genome. (ii) The 2.7-Md DNA fragment hybridized with the *pol* probe and DNAmyc but not with cDNA5' or to the *gag* or the two *env* probes. This fragment thus appeared to represent the internal part of the OK10 genome. This indicated that the *myc*(o) sequences, if contiguous, had to be located next to the 3' part of *pol* or within *pol*. Furthermore, that this fragment showed no hybridization with the two *env* probes indicated that it did not contain a significant part of the *env* sequences at the left of the *Eco*RI site in the *env* gene of ALV. This is compatible with the liquid hybridization value of 45% observed with OK10 RNA and ^3H-labeled cDNAenv (Fig. 2A and B; Table 1) (unless these sequences were so diverged that they were no longer detected under Southern blot conditions). (iii) The 0.8-Md DNA fragment hybridized only with the *env*3' probe and thus represented the 3' part of the viral DNA. The size of this fragment coincided with the 3' *Eco*RI fragment observed for an ALV (25) and indicated that this *Eco*RI site probably also was conserved in OK10 (unless by coincidence the site was lost and a new site appeared in its vicinity). We concluded from these experiments that the usual *Eco*RI sites of RSVs and ALVs (i.e., Pr-C or Schmidt-Ruppin strain [SR-A]) were conserved in OK10, including the one in the large terminal repeats (LTR). Of the two bands seen with cDNA5', one has been shown to represent the 5'-*gag* fragment of 1.6 Md. The other band at 3.2 Md, larger than 2.7 Md, did not hybridize to any other viral probes and thus represented the 3' LTR fragment extending into the cellular DNA. This indicated that the QB5 cells contained only one provirus. The *myc*-containing band at high molecular weights represented the cellular equivalent (*c-myc*) DNA since it did not hybridize to any of the viral probes.

Heteroduplex mapping of OK10 RNA. The observations described above favored the following structure of OK10 viral RNA: 5'-*gag*-(Δ*pol-myc*(o))-*env*3'-*c*-3'. Further investigations were undertaken. We built heteroduplexes between the OK10 viral 8.0-kb RNA and the 6.8-kb *Sst*I DNA fragment of PR-RSV-A and analyzed the hybrids by electron microscopy. From sequence data, the *Sst*I (or *Sac*I, isoenzyme) DNA fragment of PR-RSV-A lacks ca. 250 bases at the 5' end of the genome, left of the *gag* gene, and contains the whole *pol* and *env* genes, but the *c* region is totally missing, as are the 5' and 3' large terminal repeats (Fig. 1B). We detected in the electron microscope only one type of heteroduplex molecule. All hybrids contained reproducibly a single large substitution loop flanked by two double-stranded regions of 4.33 ± 0.065

and 0.98 ± 0.06 kb (Fig. 4). The two strands of the loop were of similar sizes, 1.43 ± 0.11 and 1.55 ± 0.07 kb, indicating that the inserted sequences were comparable in length to the deletion of ALV-related sequences (we could not determine which of the strands was RNA). These observations agreed with the results of the Southern blots and with the size of OK10 RNA as well as with the 90% annealing of OK10 mRNA with cDNAmyc, representing a com-

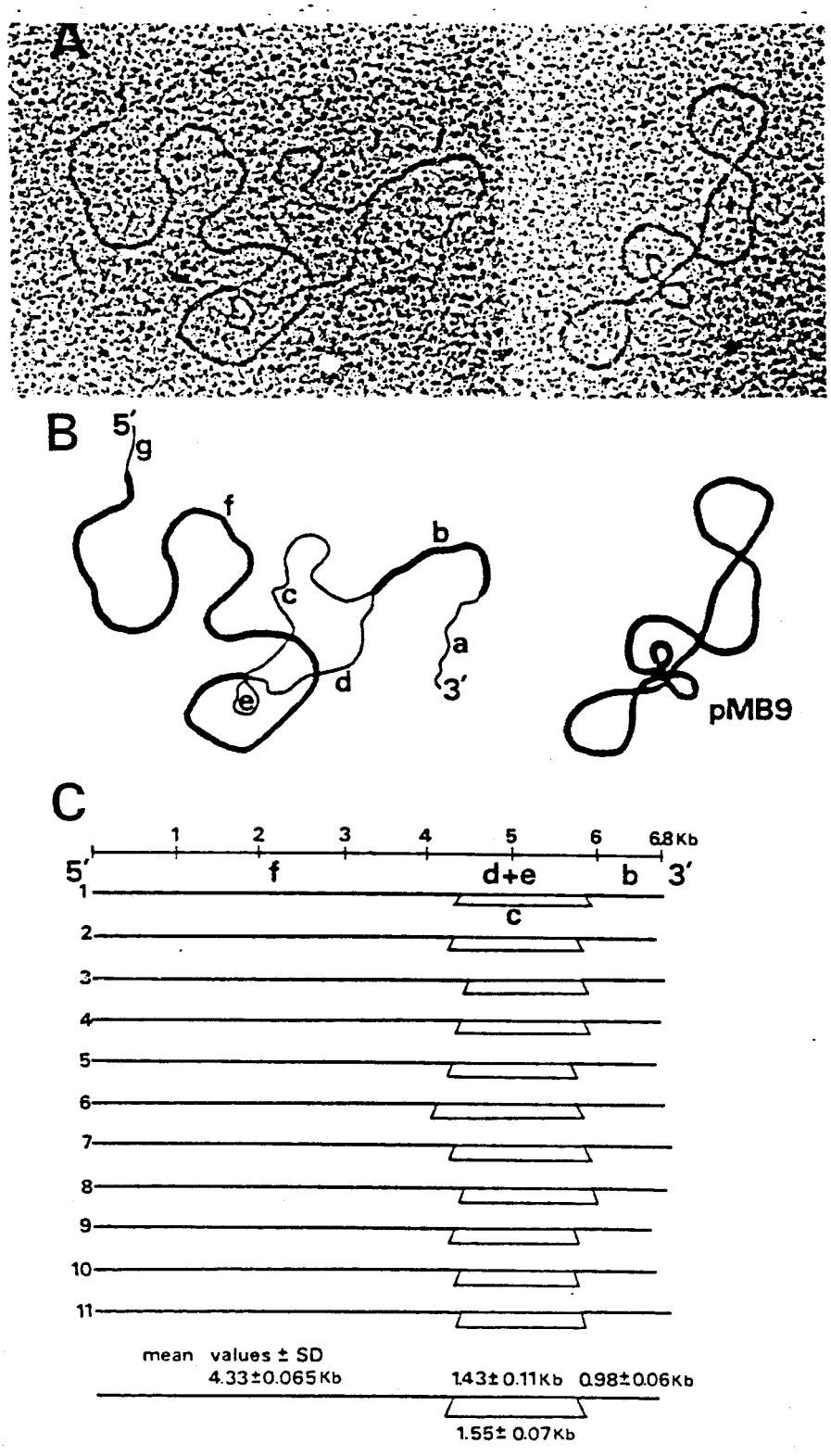


FIG. 4. Electron micrograph of OK10 RNA PR-RSV-A DNA heteroduplexes. One typical hybrid molecule (A) observed in the electron microscope was drawn on a transparency (B). In (C), we present the measurements made on 11 molecules (n), using pMB9 as a length standard. Standard deviation = Σ (variations from mean value)/n.

plexity of 1.6 kb (26). Thus, the *myc*(o) sequence in OK10 appeared to be very similar to the corresponding *myc*(m) sequence in MC29 (13). We next had to order the two double-stranded stretches of 4.33 and 0.98 kb, deciding between the structures *gag*-Δ*pol*-*myc*(o)-Δ*env* and Δ*gag*-*myc*(o)-Δ*pol*-Δ*env*. Since OK10 virus makes the Pr76 polyprotein (20), its *gag* gene must be complete and is expected from sequence data of RSV to amount to 2.1 kb (sequence data cited above) minus the ca. 0.25 kb of missing sequences of the *Sac*I fragment in the *gag* region, a total of ca. 1.85 kb. Such a size is incompatible with the size of the small fragment. Thus, it had to be included with the *pol* sequences in the 4.3-kb double-stranded stretch. It followed that the 0.98-kb segment had to correspond to the *env* sequences present in OK10, confirming the hybridization data that the 5′ half of the *env* gene was missing, rather than the whole *env* gene being present but considerably mismatched. Consistent with this interpretation was the fact that most molecules observed by electron microscopy had a single-stranded tail of ca. 0.6 kb following the 0.98-kb region, which would be expected for the *c*-poly(A) stretch of the OK10 RNA. On the other end, the molecules also had single-stranded tails up to 0.25 kb that were likely to represent the viral RNA sequences at the 5′ end of the OK10 genome and missing in the 6.8-kb *Sst* DNA fragment of Pr-RSV-A. The OK10 genome was thus consistent with the structure 5′-*gag*-*pol*-*myc*(o)-Δ*env*-*c*-poly(A).

We always observed a looplike structure of 0.37 to 0.1 kb at the 5′ end of the insertion-substitution loop. This observation was not studied further, but could indicate that the OK10 genome had a small deletion somewhere at the end of the *pol* gene.

Subgenomic viral mRNA in OK10 QB5-transformed cells. Having determined the organization of the OK10 genome, we next analyzed the strategy of viral expression in OK10-transformed cells.

Poly(A$^+$) QB5 cell RNA was fractionated by agarose gel electrophoresis and transferred to activated diazobenzyloxymethyl paper. The blots were then hybridized with different labeled probes. Two viral RNA species accumulated in these cells (Fig. 5A). The genomic OK10 RNA of the expected 8.0-kb size (30) hybridized to the different ^{32}P-labeled probes tested, cDNArep, cDNA5′, and the *gag* and *pol* nick-translated fragments, as well as to cDNAmyc.

Surprisingly, a second *myc*-containing viral mRNA species was observed at 3.6 kb that hybridized with cDNArep and cDNA5′ (not with the *gag* or the *pol* probe) and strongly with cDNAmyc and the *env* probe (Fig. 5A). Since we have shown that these cloned cells contained only one OK10 proviral DNA (Fig. 3), the 3.6-kb species had the characteristics of a spliced subgenomic RNA of the possible structure 5′ leader-*myc*(o)-*env*3′-*c*-poly(A). Indeed, our finding could correspond to an abnormal OK10 provirus in this clone that would have retained the property of transforming only fibroblasts. We thus performed similar experiments with a chicken

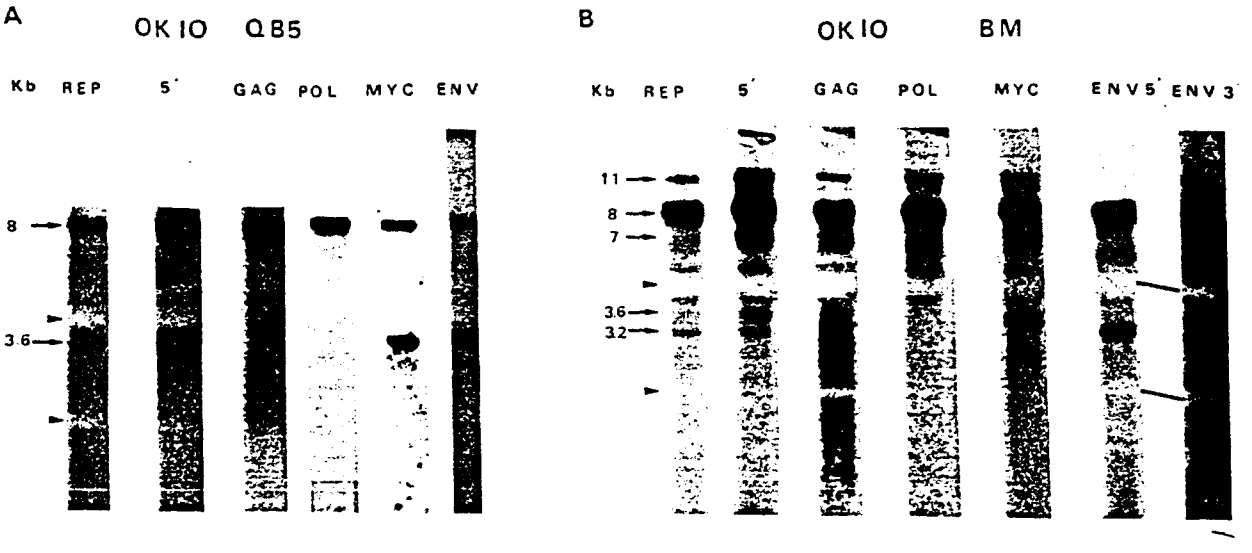


FIG. 5. Size and genetic content of *myc*(o)-containing mRNA's in OK10-transformed cells. Poly(A$^+$) RNAs were denatured by glyoxal treatment, separated on agarose gels, transferred to diazobenzyloxymethyl paper, and hybridized with the ^{32}P probes DNAgag, DNApol, DNAenv3′, and DNAenv5′ were nick translated and are described in the legend to Fig. 1. rRNA's from chicken fibroblasts and from *E. coli* were used as standard length markers. (A) OK10 QB5 intracellular poly(A$^+$) RNA. Probes are listed across the top. (B) OK10 BM intracellular poly(A$^+$) RNA.

bone marrow cell line (OK10 BM) obtained by in vivo tranformation with the original OK10 virus (kindly provided by N. Oker Blom and L. Hortling). Although this cell line produced infectious virus (19), we observed again, with the 8.0-kb band and among other species that will be analyzed further below, the other OK10-derived mRNA species of 3.6 kb (Fig. 5B) that hybridized as expected to the different probes used: to cDNArep and cDNA5', not to the *gag* or the *pol* probe, to cDNAmyc, not to *env*5', and to *env*3'.

Supergenomic mRNA in OK10 BM cells. Although quite complicated, the pattern of viral transcripts (Fig. 5B) present in the OK10 BM cells presented other interesting features besides the 8.0- and 3.6-RNAs just described.

Two mRNA's indicated the presence of a helper virus in these cells. One species at 8.0 kb coincided with the OK10 genomic RNA and could be shown with the ^{32}P-labeled *env*5' probe, although these sequences are absent from the OK10 genome. This helper produced, as expected, a subgenomic mRNA at 3.2 kb that reacted with cDNArep, cDNA5', and not with the probes to the *gag*, *pol*, and *myc* sequences, but again with *env*5' and *env*3', thus representing probably the spliced *env*-mRNA (17).

Two other viral species that contained *myc*(o)-related sequences were also observed in these cells. An 11.0-kb band reacted to the same probes as the 8.0-kb OK10 RNA. Similarly, a 7.0-kb species reacted with the same probes as the OK10 subgenomic 3.6-kb mRNA. Thus, these two bands were OK10 related but of abnormally large size. We thus hypothesized that they represented species that extend into the cellular DNA where they were modulated (at termination or initiation or both) by cellular signals. Despite this, the 7.0-kb species was apparently spliced, possibly from the 11.0-kb mRNA. These data and hypotheses are summarized in Fig. 6. Alternatively, these cells could contain an additional OK10-like provirus of larger size.

DISCUSSION

Genetic structure of OK10. Although OK10 does not contain nucleotide sequences related to the *src* gene of RSVs (22), this virus, like all known MC29-type viruses, can transform specific hematopoietic target cells in vitro (2) as well as avian fibroblasts (9). Its ability to produce a transformed phenotype in these cells has been associated with the presence in its genome of a unique nucleotide sequence termed *myc*(o) (22). This sequence has its counterpart in normal

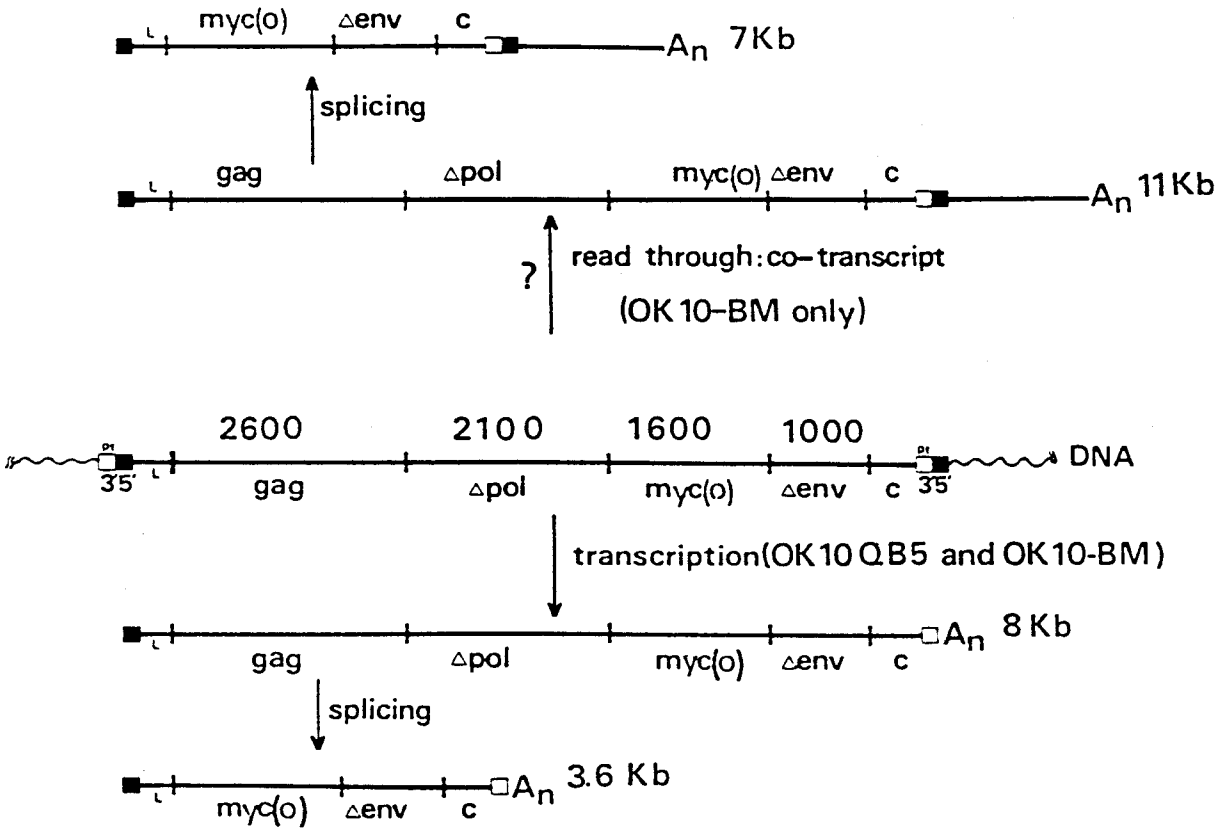


FIG. 6. Transcription and processing of OK10 mRNA's. This model has been built to explain the results obtained and presented in Fig. 5, favoring the 3' cellular-extended 11.0-kb RNA species in OK10 cells. Pt, Putative transcription promoter and termination signals. The 11-kb supertranscript could as well extend upstream or at both ends of the OK10 provirus.

cell DNA (22) and is unrelated to any known RSV/ALV genes (30). Our studies showed that the OK10 *myc*(o) allele of the putative *myc*-transforming gene formed a continuous stretch of 1.43 to 1.55 ± 0.07 kb in the 8.0-kb-long OK10 genomic RNA extracted from defective viral particles produced by OK10 QB5 cells. The *myc*(o) sequence is flanked by sequences that are isogenic to part or all of the *gag*, *pol*, and *env* genes of ALVs.

The *gag* gene is fully represented in OK10 genomic RNA. This finding fits with the fact that OK10-defective viral particles containing the *gag* gene products p27, p19, and p15/p12 (20) are produced by OK10 QB5 cells. The *pol* gene is deleted by about 0.5 kb at the 3′ end, and that could explain why no polymerase activity could be detected in OK10 QB5-defective viral particles (Fig. 2C). The *env* gene is deleted by ca. 0.9 kb at the 5′ end, and again this finding has been corroborated by the absence of any detectable *env* gene-related proteins in OK10 QB5-defective particles (20). Moreover, in OK10 QB5 cells, two proteins were identified, the OK10 76K, antigenically related to the *gag* gene, and the OK10 200K, antigenically related to the *gag* and *pol* genes but not to the *env* gene product (20). Tryptic peptide mapping showed that the OK10 76K protein was almost indistinguishable from the *gag* gene product $Pr76^{gag}$ of an ALV and that the OK10 200K polyprotein contained all but one methionine tryptic peptide of $Pr180^{gag\text{-}pol}$, the missing peptide being a *pol* peptide (20). In short, the genetic structure of OK10 as probed by hybridization and heteroduplex mapping, i.e. 5′-*gag*-Δ*pol*-*myc*(o)-Δ*env*-*c*-poly (A), is compatible with determinations by T1 fingerprinting (5) and with immunological analyses of the OK10-coded proteins present in OK10 QB5 cells and the OK10 QB5-defective particles. We have no clear explanation for the presence of the 0.37-kb looplike structure at the 5′ end of the deletion-substitution loop observed reproducibly in the heteroduplexes studied; it could represent an additional deletion in the OK10 genome, probably near the end of the *pol* gene. The OK10 200K polyprotein covers the total OK10 *gag*-Δ*pol*-*myc*(o) coding capacity (ca. 6.2 kb) and has been described as a fusion product between the OK10 *gag* and *pol* gene-coded polypeptides and the *myc*(o) cellular information. Similarly nonproducer quail fibroblasts transformed by the other MC29-related defective leukemia viruses MH2 and CMII also contain *gag*-related polyproteins which were shown to be fusion products of the *gag* gene-coded polypeptides and possibly the *myc* cellular derived information (4, 11, 14). Therefore, the oncogenic potential of MC29-related viruses might be expressed through *gag-myc* or *gag*-Δ*pol-myc* proteins translated from genome-size mRNA's.

Subgenomic mRNA of OK10. It must be pointed out that, in the case of OK10, the amount of 200K polyprotein observed is very low and similar to the precursor of the polymerase $180^{gag\ pol}$ polyprotein observed in ALV-infected cells. This is about 20 to 50 times less abundant than the *gag-onc* fusion protein in MC29- or CMII-infected cells, although the OK10 virus is, like MC29 or CMII, acutely transforming and similar to that of MC29. We raise here the possibility that the OK10-transforming protein could be made in another way. The 3.6-kb subgenomic mRNA observed in abundance in the OK10-transformed cells studied represents a candidate of choice for such a function with its structure 5′-*myc*(o)-Δ*env*-*c*-poly(A) where the ALV-related sequences represent, at most, ca. 2.2 kb (see Results). Thus, this RNA is likely to contain 3.6 minus 2.2 = 1.4 kb of *myc*(o), which represents most or all of the *myc*(o) of OK10. The interesting implication of these experiments is that they allow us to think that a given single transforming gene information could be translated in a *gag-onc* fusion protein (in MC29) as well as in a non-*gag* protein (in OK10), the important feature for transformation being, then, the efficient translation of the active gene in the correct phase no matter by which strategy of mRNA expression. Alternatively, the 3.6-kb RNA only would code for a competent transforming protein in OK10 virus, the P200 polypeptide being inactive because of an early termination or an incorrect frame. The latter possibility is less likely, since one methionine-containing tryptic peptide related to the MC29 P110 polyprotein in the *myc* region has been found for OK10 P200 (20). The structure of the 3.6-kb spliced mRNA will have to be determined more precisely to determine how the splice occurs. If indeed the *myc*(o) sequence is next to the 5′ leader, it must contain a splice acceptor site compatible with the donor site at the 5′ end of the viral RNA. Another possibility is that the splice signal of the *env* gene is still conserved and used and that the insertion-deletion occurred all within the *env* gene. The structure of this mRNA would then be 5′ leader-5′-*env* splice acceptor site-*myc*(o)-*env*3′-*c*-poly(A). Such a possibility would then also explain the small loop observed at the *pol-env* junction in the heteroduplex shown in Fig. 4. The loop would represent the DNA corresponding to the 3′-end *pol* deletion of OK10 and would be followed by a small double-stranded stretch corresponding to the 5′ end of *env*, presumably then conserved in OK10 and located at the 5′ side of the *myc* insertion but too small to anneal with our *env*5′ probe. This hypothesis remains to be documented further.

In conclusion, OK10 virus presents at least two interesting features compared with MC29. (i) It is produced as a noninfectious form without helper in the culture fluid of nonproducer cells. (ii) It produces in fibroblasts, but also in in vivo transformed myeloid cells (19), a subgenomic spliced mRNA containing most or all of the *myc*(o) sequences. Its putative transforming protein could thus be a non-*gag* polypeptide rather than a *gag-myc* fusion protein as in MC29. Preliminary findings (S. Saule, M. B. Raes, C. Lagrou, and D. Stehelin, unpublished data) indicate that MH2-transformed cells also contain a subgenomic mRNA species that hybridizes with cDNAmyc.

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Two different types of transcription for the myelocytomatosis viruses MH2 and CMII

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The four avian defective leukemia retroviruses (DLVs) MC29, CMII, MH2 and OK10 all transform primarily macrophages in an *in vitro* bone marrow transformation assay, and contain specific nucleotide sequences closely related to the *myc* gene of MC29. These viruses were thought to express their oncogenic potential through a *gag-myc* fusion polyprotein, since fusion polyproteins were found in all tested cells transformed by MC29. We show here that MH2 virus does not conform to this model. Whereas MC29 produces only one mRNA detectable by RNA blotting in productively transformed cells, we reported recently that OK10 induced the synthesis of two *myc*-containing mRNAs, the smaller species being a spliced mRNA and a possible candidate for a transforming protein lacking *gag* determinants. However, the studies with OK10 were ambiguous because this virus produced also, in infected cells, a fusion protein containing *gag*, *pol* and *myc* determinants. We have therefore investigated the transcription pattern of the two other members of this group of viruses, namely CMII and MH2. Our results show that CMII resembles MC29 whereas MH2 produces, as OK10, two mRNAs containing *myc*-related sequences. However, unlike OK10, the MH2 fusion protein of 100 kd described previously cannot contain *myc* determinants and thus is likely to produce from its subgenomic mRNA a *v-myc* protein-lacking *gag* determinants. We thus conclude that the product of the *v-myc* oncogene is transforming with (MC29) or without (MH2) its fusion to *gag* determinants and that the multiple oncogenic spectrum is not basically affected since MH2 and MC29 both transform macrophages, fibroblasts and epithelial cells.

Key words: hybridizations/myelocytomatosis viruses/Northern/transcription/oncogene

Introduction

The group of myelocytomatosis viruses belongs to the avian defective leukemia retroviruses (DLVs) (Graf and Beug, 1978). Four independent isolates, called MC29, CMII, OK10 and MH2 contain a similar transforming gene, *v-myc*, of cellular origin (*c-myc*), most likely derived from the chicken (Roussel *et al.*, 1979; Sheiness and Bishop, 1979; Stehelin *et al.*, 1980) and transduced by these viruses as a result of insertion-deletion events at the expense of viral structural genes. Due to their genetic rearrangements these viruses are defective for replication and need, in order to propagate, the presence of an avian leukosis helper virus (ALV) containing the genes for the three replicative functions: *gag* (capsid proteins), *pol* (reverse transcriptase) and *env* (required for virion infectivity).

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The *in vitro* transforming properties of the myelocytomatosis viruses are similar, as expected, since they carry a similar oncogene (Graf and Stehelin, 1982): all of them transform fresh chicken bone marrow to yield macrophage-like transformed cells and also transform fibroblasts in culture (Graf and Beug, 1978). *In vivo*, the pathogenicity of these viruses is complex. OK10 and MH2 seem to have a slightly different oncogenic spectrum from that of MC29 and CMII; MC29 and CMII induce myelocytomatosis (Loliger and Shubert, 1966; Alexander *et al.*, 1979) whereas OK10 and MH2 have not been shown to do so (Graf and Beug, 1978; Linial, 1982). In addition, the induction of sarcomas and carcinomas has been reported for MC29, MH2 and OK10 (Moscovici *et al.*, 1977; Graf and Beug, 1978).

MC29 expresses its oncogenic potential through a fusion protein (p110) containing both *gag* and *myc* determinants (Bister *et al.*, 1977; Hayman *et al.*, 1979; Ramsay and Hayman, 1980) which has DNA-binding properties (Donner *et al.*, 1982; Abrams *et al.*, 1982). That this protein is associated with transformation was indicated by the isolation of MC29 mutants with altered transforming abilities and fusion proteins altered in their *myc* determinants (Ramsay *et al.*, 1980). An MH2 fusion protein of 100 kd with *gag* determinants was also described by Hu *et al.* (1978) although, importantly, no *myc* determinants were demonstrated. Since CMII and OK10 virus were subsequently shown to express *gag-myc* fusion proteins (of 90 kd, Hayman *et al.*, 1979 and 200 kd, Ramsay and Hayman, 1980, respectively), it became generally accepted that the viral *myc* oncogene was always expressed as a *gag-myc* fusion protein (Bister and Duesberg 1980; Coffin *et al.*, 1981). Some authors even proposed that transformation was due to the fusion of *v-myc* with *gag* determinants (Bister and Duesberg, 1980). As a result of these data, MH2 has been said to code for such a protein (Bister and Duesberg, 1980; Coffin *et al.*, 1981; Bishop and Varmus 1982) although no single direct experiment supported this statement and despite some indirect evidence against it (Neiman *et al.*, 1981; Linial, 1982; Ramsay *et al.*, 1982). Recently Chiswell *et al.* (1981) and Saule *et al.* (1982) showed that OK10 produced, in infected cells, a spliced subgenomic mRNA containing *myc*, but no *gag* sequences. This suggested that a *myc* protein lacking *gag* determinants, could be involved in the process of transformation as an alternative agent to the already decribed p200. Unfortunately, the OK10 system proved to be too complex to provide a clear answer. We thus decided to re-examine the virus MH2 for which no *myc* determinants had been demonstrated in the p100 fusion protein since it was possible that the *myc* product was made from a spliced mRNA with no *gag* determinants. The results presented here indeed support this hypothesis.

The availability of four independent isolates of *myc*-containing viruses provides a unique opportunity to explore this controversial subject. This paper is the first to conclude unambiguously that one of these viruses, MH2, makes a subgenomic mRNA containing all the *myc* information, that the corresponding product cannot be a fusion protein and that this different pattern of expression does not drastically

modify the transformation spectrum of MH2 virus compared with the other members of this family.

Results

Genetic content and transcription pattern of MH2 and CMII

Using ^{32}P-labelled probes, we investigated the genome structure and the transcription pattern of MH2. We also studied CMII, for which a possible subgenomic RNA has not been formally ruled out so far. Total polyadenylated cellular RNAs from non-producer quail cells transformed by MH2 (clone MH2 QB2) or CMII (clone CMII QA1, Graf *et al.*, 1977), as well as MC29 (clone MC29 Q8, Bister *et al.*, 1977) for comparison, was analyzed by gel electrophoresis and blotting onto diazobenzyloxymethyl (DBM) paper. The blots were hybridized using nick-translated ^{32}P-labelled probes (Rigby *et al.*, 1977) from the genes *gag, pol* and *env* (3′ or 5′ region), prepared as described (Saule *et al.*, 1982) (Figure 1), or utilizing: *cDNA5′* (Friedrich *et al.*, 1977) *cDNAmyc* (Sheiness *et al.*, 1978) and *cDNArep* (representative of ALV RNA, Saule *et al.*, 1982).

The results are shown in Figure 2. Using *cDNA5′*, three mRNAs were detected in the MC29 non-producer (NP) cells (A). The smallest species (0.9 kb) hybridized only with *cDNA5′* and thus may be of cellular origin [long terminal repeat (LTR) promotion of an unknown cellular sequence]. Similar data were previously reported for lymphoid cells transformed *in vivo* by an ALV (Payne *et al.*, 1981; Neel *et al.*, 1981), as well as for mammalian cells transformed by B77 ASV (Bishop *et al.*, 1980). In agreement with the data reported in Stehelin *et al.*, 1980, no *pol* or *env5′* sequences were detected, while the *rep, gag, myc* and *env3′* probes recognized two main large RNA species of ~5.5 kb, closely related in size (a third band was seen, for which we have no explanation). As shown in Figure 2B, hybridization with the *rep* and *myc* probes of polyadenylated RNAs found in uncloned chicken fibroblasts transformed by MC29 (RAV2) revealed the normal genomic RNA of MC29 to be the smaller viral species of the doublet observed in Q8 NP cells. The MC29 related supergenomic RNA may be, therefore, interpreted as a read-through product of viral transcription into the host DNA, or, alternatively, as an upstream initiation of the viral transcript in the adjacent cellular DNA. Confirming previous reports (Sheiness *et al.*, 1981), no subgenomic viral RNA was found in MC29 transformed cells.

CMII displayed the same basic pattern of transcription, with minor differences. The *cDNA5′* probe was able to reveal two RNAs (Figure 2C), which again may be interpreted as a genomic RNA species of 5.8 kb, also found in CMII (RAV2) mass-transformed chicken fibroblasts with a *myc* probe (Figure 2D), and a read-through transcript of supergenomic size. The two RNAs hybridized not only with both *rep* and *myc* probes, but also with a *pol* probe (Figure 2C) confirming previous data (Bister and Duesberg, 1980) obtained by T1 fingerprinting. As can be calculated from the nucleotide sequence, our *pol* probe contained 160 bp related to the 3′ part of the *gag* gene but, as previously reported by Hayman *et al.* (1979), the CMII virus does not contain this region and therefore the hybridization seen in Figure 2C was not due to a trivial contamination of our *pol* probe. CMII also hybridized to both *env5′* and *env3′* probes, confirming the presence of extensive *env*-related sequences, in comparison with MC29 (Stehelin *et al.*, 1980).

MH2 produces a subgenomic mRNA

In contrast to CMII, the MH2 QB2 non-producer clone showed a subgenomic mRNA transcript. The *cDNA5′* revealed three mRNA species, the smallest of which (2.8 kb) hybridized with a *myc* probe but not with *gag* (Figure 2E) or with *pol* or *env5′* probes (Figure 3). This new species must thus represent a subgenomic RNA, spliced from the 5.5-kb

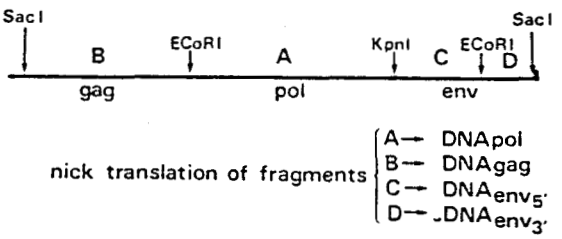

Fig. 1. Preparation of nick-translated probes. Nick-translated probes were prepared from fragments A, B, C and D isolated by agarose gel electrophoresis after suitable restriction cleavages of the *Sac*I DNA fragment of PR-RSV-A.

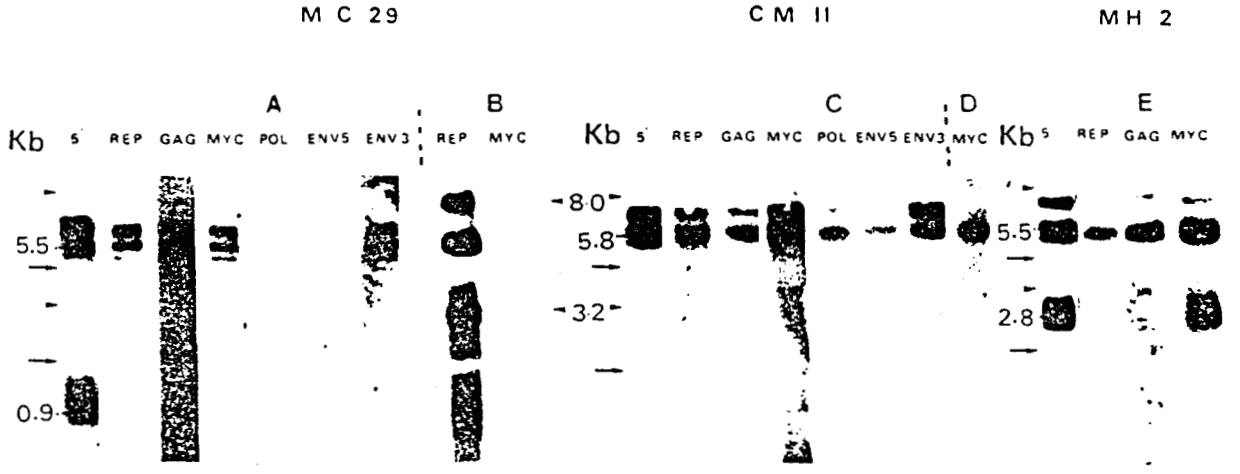

Fig. 2. Size and genetic content of *myc*-containing mRNA in MC29, CMII and MH2-transformed cells. Polyadenylated RNAs were denatured by glyoxal treatment, separated on agarose gels, transferred to DBM paper and hybridized with the ^{32}P probes listed across the top. The different cell RNAs tested were from: (A) a non-producer quail fibroblast clone, MC29 Q8; (B) MC29 (RAV2) mass infected chicken fibroblasts; (C) a non-producer quail fibroblast clone, CMII QA1; (D) CMII (RAV2) mass infected chicken fibroblasts; (E) A non-producer quail fibroblast clone, MH2 QB2. Symbols are: ▶ sizes of ALV related viral products; →ribosomal 28S and 18S markers; — sizes of DLV-related viral and cellular products.

MH2 genomic transcript. Again, the presence of a supergenomic RNA hybridizing like the genomic RNA with all the probes in Figure 2E could be interpreted as a product of read-through of the MH2 provirus.

The MH2 (RAV3) culture contain several viruses

Since the QB2 clone was derived from a single MH2-transformed quail fibroblast, we considered the possibility that a peculiar transcription process, yielding a subgenomic RNA, occurred in this particular clone. We, therefore, prepared polyadenylated RNAs from MH2 (RAV3) mass infected chicken fibroblasts (the supernatant of which had been used to clone the MH2 virus in the QB2 cells) and analysed it as described above. Figure 3 shows the quite complex hybridization pattern obtained with *cDNA5'* and *cDNAmyc*, on agarose-fractionated polyadenylated RNA from MH2 (RAV3) chicken fibroblasts as compared with that from MH2 QB2 quail cell clone. MH2 (RAV3) cells contained as many as five mRNA species hybridizing with *cDNA5'*. The 8-kb and 3.2-kb RNAs, which did not hybridize with *cDNAmyc*, represented the RAV3 helper transcription products (Lee *et al.*, 1979). The largest RNA found (9.0 kb) was unlikely to represent a supergenomic species resulting from read-through since such a species would not appear in a mass culture infection, the proviral integration occurring probably at many sites (Varmus *et al.*, 1981). Therefore, we favoured the hypothesis that this 9.0-kb RNA hybridizing with *cDNA5'*, *rep*, *pol* and *env5'* probes, but not with the *myc* probe, nor with *cDNAsrc* (S. Saule, unpublished data) represented a new recombinant viral RNA, the characterization of which is now in progress. Two other bands were resolved with *cDNA5'* at 5.8 kb and 2.8 kb. The 2.8-kb RNA also hybridized with *cDNAmyc* and thus probably represented the spliced subgenomic mRNA found in the non-producer MH2 QB2 cells. The 5.8-kb RNA hybridized weakly with *cDNAmyc* and was larger than the genomic MH2 RNA (5.5 kb) expressed in the non-producer clone. Surprisingly this RNA hybridized with *pol* and *env5'* probes (Figure 3) and so it may represent an abundant MH2 genome RNA variant different from the one cloned in the non-producer cells MH2 QB2. The fact that the MH2 subgenomic RNA had the same size (2.8 kb) in both cases, and that no hybridization was found with an *env5'* probe for the MH2 (RAV3) subgenomic RNA, allowed us to locate the *myc* sequence within the 3'-terminal region of the MH2 virus. The size of this RNA suggested that it contained probably all of the MH2 *myc* sequence.

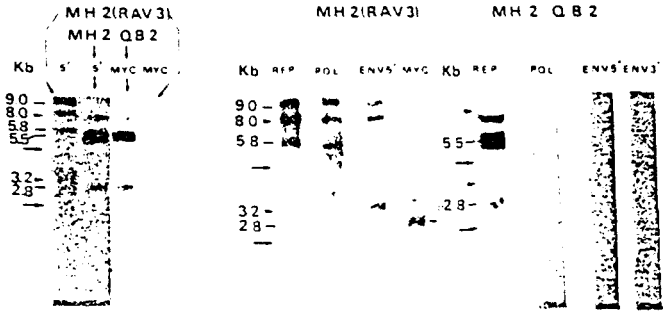

Fig. 3. Size and genetic content of virus-coded mRNAs in MH2-transformed cells. Poly(A)$^+$ RNA treated as in Figure 2 were hybridized with the probes listed across the top. Cellular RNAs used were from: MH2 (RAV3) transformed chicken fibroblasts and MH2 QB2 non-producer quail fibroblasts. Symbols are: ► sizes of ALV-related viral products; → ribosomal 28S and 18S markers. The numbers indicated size markers in kb; — sizes of DLV-related viral products.

Molecular cloning of MH2 QB2 provirus confirms the 3' location of v-myc

The structure proposed for MH2 from RNA analyses was confirmed at the DNA level, by the molecular cloning of the proviral DNA from MH2 QB2 cells. A gene library was constructed by inserting a partial *Mbo*I digest of MH2 QB2 DNA into the *Bam*HI site of λ phage L47. The library was screened for plaques hybridizing with both *myc* and *rep* probes. A typical phage, λ MH2 Q1 was shown to contain the complete MH2 QB2 provirus, and restriction analyses by Southern blotting with different labelled probes allowed the construction of the restriction map presented in Figure 4. The *myc* gene in phage λ MH2 Q1 mapped, as expected from the RNA studies, at the 3' end of the genome, to the right of the ~1.6 kbp of the as yet uncharacterized sequences. Details of the molecular cloning and properties of this phage will be described elsewhere (J .Coll *et al.*, in preparation).

Discussion

Our results indicate that CMII resembles closely MC29, producing only one genome size RNA in infected cells. These two viruses have very similar transforming properties.

In contrast to CMII and MC29, MH2 presents an interesting feature. Mass cultured MH2 infected cells produce a subgenomic spliced RNA species of 2.8 kb containing 5' and *myc* nucleotide sequences. A transformed, single quail cell clone (QB2) that contains a variant of MH2 lacking *pol* and *env* sequences replaced by as yet unknown sequences also produces the 2.8-kb, spliced *myc*-containing RNA. The molecular cloning of MH2 proviral DNA from this quail cell clone confirms the location of the *myc* gene in the 3' region of the genome (Figure 4), to the right of the yet unidentified X sequences found in this variant. Thus, the *myc* gene in MH2 can be located at the 3' end of the viral genome after *env* sequences in the case of MH2 RAV3 (Figure 5) or after the X sequences in the variant (Figures 4 and 5). This localization is not compatible with the production by MH2 of a p100 fusion protein (Hu *et al.*, 1978) containing *gag* and *myc* determinants that has been proposed in the literature (Bister and

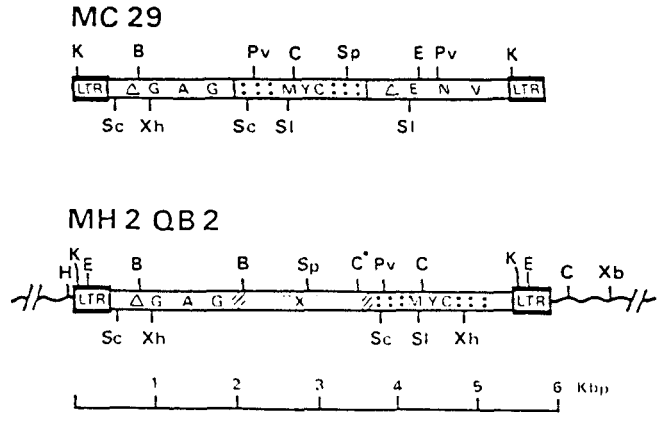

Fig. 4. Restriction map of MH2 QB2 proviral DNA in λ phage MH2 Q1. The restriction map of λ phage MH2 Q1 was established using several endonucleases. The *myc* and X sequences were located by Southern blotting analyses with specific labelled probes. For comparison, the linearized viral DNA of MC29 is indicated (from Vennstrom *et al.*, 1981). Restriction endonuclease indicated are: H: *Hind*III; K: *Kpn*I; E:*Eco*RI; Sc: *Sac*I; B: *Bam*HI; Xh: *Xho*I; Sp: *Sph*I; C: *Cla*I (*: site methylated in dam$^+$ host bacteria); Pv: *Pvu*II; Sl: *Sal*I; Xb: *Xba*I. Note that the *v-myc* sequences are not identical in the two viruses, since the restriction sites *Sph*I in MC29 and *Xho*I in MH2 Q1 DNA are not present in the DNA of the other virus.

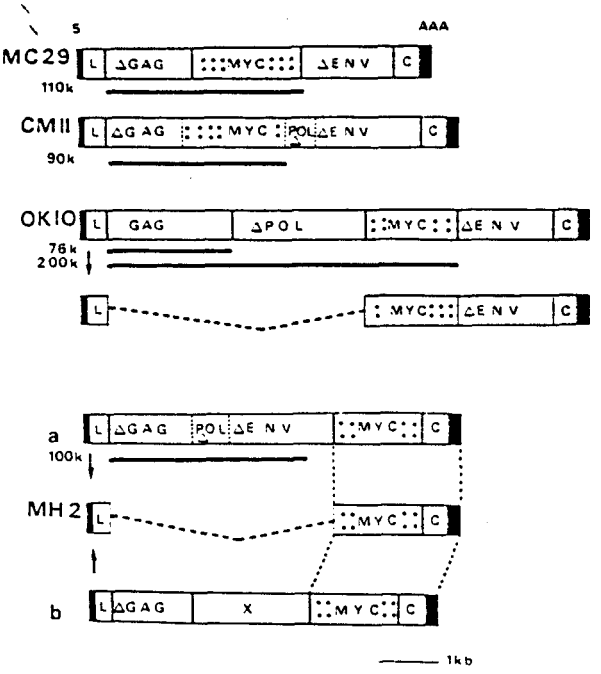

Fig. 5. Transcription and processing of the four *myc*-containing viruses. This model has been drawn to account for the results obtained for MH2 (RAV3) (a) and MH2 QB2 or its molecular clone (b) and presented in Figures 2, 3 and 4. For comparison, the genomes of MC29, CMII and OK10 viruses are also indicated.

Duesberg, 1980; Bishop and Varmus, 1982) but never demonstrated. In fact, we suggest that the p100 protein of MH2 lacks *myc* determinants since the size of p100 (~3 kb of coding RNA) cannot extend into the *myc* region located at the 3′ end of the ~6 kb long viral genome (see Figures 4 and 5). Our finding explains the following recent observations: p100 was shown to contain both *gag* and *pol* determinants (Neil *et al.*, 1981); the extent to which MC29 p100 and MH2 p100 differed was unexpected when they were analyzed by peptide mapping (Neil *et al.*, 1981); *myc*-specific phosphothreonine amino acids, essential for transformation, as shown for MC29, have not been reported in the MH2 p100 (Ramsay *et al.*, 1982); the isolation of a MH2 mutant still transforming that lacked the p100 protein (Linial, 1982). We conclude, therefore, that p100 cannot be a *myc*-transforming protein. Rather, the *myc* gene may yield a non-*gag* protein that has so far not been detected because of the lack of *myc*-specific antisera.

In this respect, MH2 resembles OK10 which also produces a *myc*-containing subgenomic RNA in infected cells (Chiswell *et al.*, 1981; Saule *et al.*, 1982), that could code for a non-*gag*, *myc*-containing transforming protein. However, in such cells, OK10 also produces a small amount of a 200-kd protein that is a fusion polypeptide with *gag*, *pol* and *myc* determinants (Ramsay and Hayman, 1980). The possibility that this large fusion protein is involved in transformation by OK10 has not been ruled out. In contrast, this possibilty does not exist for MH2. It follows that two distinct patterns in the processing of *myc* sequences are found among the *myc*-containing retroviruses: a *gag-myc* fusion protein in MC29 and CMII; a putative non-*gag*-transforming protein in MH2 and probably also in OK10. How the two different processing patterns relate to the different biological properties of these viruses is now of prime interest. Whereas all four viruses transform macrophages and fibroblasts in culture, MH2 and OK10 appear, *in vivo*, to exhibit a slightly different oncogenic spectrum to those of MC29 and CMII [i.e., the latter two viruses but not the former pair induce myelocytomatosis (Graf and Beug, 1978)]. Such pathology studies were often performed with uncharacterized virus stocks that hampered rigorous comparisons and should now be repeated using molecularly cloned isolates.

Finally, our experiments have shown that the MH2 viral stock contained a variant virus present in the MH2 QB2 cell clone. On the basis of the hybridization results, it is not possible to explain the size of the genome RNA (5.5 kb) without admitting the existence of an as yet unidentified, helper-virus unrelated, nucleotide sequence (X) of ~1.6 kbp, the presence of which is confirmed by molecular cloning (λ phage MH2 Q1) of this variant. This sequence could have originated from cellular DNA and could represent an independent second insertion of genetic material within the viral genome. The possible contribution of this sequence to the transforming properties and oncogenic spectrum of this new viral isolate remains to be examined and will require site mutagenesis in the X or *myc* domains.

Materials and methods

Cells, viruses and cloned DNA fragments

The cell lines, CMII QA1, and the viruses MC29 RAV2 and CMII RAV2 were obtained through the courtesy of T. Graf and H. Beug. The cell line MC29 Q8 was obtained from K. Bister and the MH2 RAV3 fibroblasts were kindly provided by C. Moscovici through L. Gazzolo. The non-producer MH2 QB2 quail fibroblast clone was established in our laboratory according to Hu *et al.* (1978) after infection of quail fibroblasts by MH2 (RAV3) virus at low multiplicity and subsequent selection in agar. All non-producer clones described released infectious, chicken embryo fibroblasts transforming virus upon rescue with a helper virus.

The *Sac*I 6.8-kb fragment of PR-RSV-A DNA inserted in a Charon 16A phage was a gift of J. Taylor. This fragment excised by *Sst*I, an isozyme of *Sac*I, was purified by agarose gel electrophoresis and cut by *Eco*RI and *Kpn*I in order to prepare specific fragments for *gag5′* (*Sac*I-*Eco*RI); *pol* (*Eco*RI-*Kpn*I); *env5′* (*Kpn*I-*Eco*RI) and *env3′* (*Eco*RI-*Sac*I) (see Figure 1). These fragments were purified twice by electrophoresis in 0.8% agarose gels before labelling by nick-translation (New-England Nuclear).

Preparation of ^{32}P-specific cDNA probes

The preparation and characterization of all probes was extensively described in Saule *et al.* (1982). The *cDNAmyc* synthesized in the exogenous reaction (Taylor *et al.*, 1976), using 50–70S RNA viral templates (MC29 RAV2) and purified avian myeloblastosis virus polymerase, was selected as described by Sheiness *et al.* (1978). Representative cDNA (*cDNArep*) from RAV2 was prepared according to Saule *et al.* (1982). Strong stop cDNA (*cDNA5′*) was prepared according to Friedrich *et al.* (1977).

Agarose gel electrophoresis of poly(A)-containing cellular RNAs

Poly(A)-containing cellular RNAs were prepared as described (Verma, 1978). RNA samples were subsequently denatured by glyoxal/dimethyl sulfoxide treatment (McMaster and Carmichael, 1977) and submitted to electrophoresis in 1% agarose horizontal gels, submerged in phosphate 0.01 M buffer, pH 6.8.

Transfer-hybridization of RNAs

Size separated RNAs were transferred to DBM paper, prepared as described by Alwine *et al.* (1977). The blots were hybridized with ^{32}P-labelled DNA (or cDNA) probes as described (Saule *et al.*, 1982) washed, and submitted to autoradiography. When rehybridization of the same blot was performed, the DBM paper was dehybridized with 97% formamide in EDTA 10^{-2} M for 1 h at 80°C, washed and processed as previously described.

Cloning of MH2 (QB2) proviral DNA

DNA was extracted from MH2 (QB2) cells and subjected to a partial cleavage with the restriction endonuclease *Mbo*I. Fragments of 15–20 kbp in size were selected by agarose gel electrophoresis and inserted into the *Bam*HI site of λ phage L47 by ligation to agarose gel selected phage arms. The chimeric DNA was packaged *in vitro* into λ phage capsids. The resulting gene library (10^6 plaque forming units) was screened with a *rep* and *myc* probe as described by Benton and Davis (1977) and double-positive plaques were purified and amplified. A typical phage, λ MH2 Q1, contained the complete

MH2 DNA within ~14 kbp of cell DNA, and was used for restriction mapping analyses and Southern blotting (Southern, 1975).

Acknowledgements

We thank T. Graf and H. Beug for having provided us with the cell lines OK10 QB5, CMII QA1, K. Bister for the MC29 Q8 cells, J.M. Bishop for the MC29 plasmid, A. Gegonne for help in making the DNA gene library, A. Begue for excellent technical assistance, N. Devassine for patient typing and C. Dissous for helpful discussion. This work was suported by the INSERM (CRL 79.5.054.2 and 802.036), CNRS (AI, ERA 225) and the Institut Pasteur de Lille.

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Characterization of a *myc*-Containing Retrovirus Generated by Propagation of an MH2 Viral Subgenomic RNA

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We have previously isolated, from wild-type MH2 virus that contains the two oncogenes *mil* and *myc*, mutants defective in one or the other oncogene product. We report here the molecular cloning and extensive characterization of MH2 CL25 provirus lacking the v-*mil* oncogene. Our results indicate that this virus corresponds to the propagation of the 2.8-kilobase subgenomic RNA of MH2l.

The avian retrovirus Mill-Hill 2 (MH2) is a replication-defective retrovirus that induces liver and kidney carcinomas in fowl and transforms fibroblasts, macrophages, chondroblasts, and neuroretina cells in culture (1, 2, 7). The genome of MH2 contains two cell-derived oncogenes: v-*myc*, also found in three other retroviruses (MC29, CMII, OK10) (19, 21), and v-*mil* (4, 12), related to the *src* gene of Rous sarcoma virus (RSV) (6, 13). In MH2-transformed cells v-*mil* is expressed as a 100-kilodalton polyprotein resulting from the fusion of *gag* and *mil* sequences (11, 17, 19). v-*myc* is expressed as a 61- to 63-kilodalton nuclear protein encoded by a 2.8-kilobase subgenomic mRNA (9, 17, 22).

We defined distinct effects of the *mil* and *myc* oncogenes by studying the properties of MH2-infected neuroretina cells. Chicken embryo neuroretina cells which normally do not multiply in vitro are induced to proliferate upon infection with MH2 but not with MC29, CMII, and OK10 (2, 3). From two distinct viral stocks of wild-type (wt) MH2 we isolated two mutants, MH2 CL16 and MH2 CL25, lacking the v-*mil* oncogene. These mutants failed to transform neuroretina cells or to induce their proliferation but retained the ability to transform avian embryo cells. To precisely define the genetic organization of these v-*mil*-defective mutants, we molecularly cloned a v-*myc*-containing provirus from MH2 CL25-transformed quail embryo cells. We present here data indicating that this mutant corresponds to the propagation of a retroviral particle containing subgenomic MH2 RNA species.

Molecular cloning of MH2 CL25 provirus. A transformed quail embryo cell clone containing MH2 CL25 provirus was isolated in soft agar and used as a source of DNA to construct a gene library. High-molecular-weight DNA was partially digested with *Eco*RI restriction endonuclease and ligated with purified lambda Charon 4A arms, packaged, and amplified as reported previously (22). Several probes were prepared: a long terminal repeat (LTR) probe was derived from a provirus of RSV, strain Schmidt-Ruppin A (RSV-SRA) (5). Fragments of the retroviral *gag* gene were derived from Prague strain (RSV-PrA) as previously described (22). Two *myc* probes were used: a 5′ v-*myc* probe (*Hpa*I-*Pst*I fragment derived from pMH2Hd [4]) and a 3′ c-*myc* probe representing the 3′ half of chicken exon 3 and described in reference 23. Agarose gel-purified fragments were labeled through nick translation reactions (20) (Amersham nick translation kit) in the presence of [^{32}P]dCTP according to the supplier's instructions. By use of LTR and *myc* probes, two lambda phages were isolated, purified, and amplified. From one of these phages, lambda MH2 CL25, we subcloned a 10-kilobase-pair (kbp) *Eco*RI fragment containing the complete provirus into the *Eco*RI site of pKH47 plasmid DNA (10), yielding plasmid pMH2-CL25. A detailed restriction map of the provirus is shown in Fig. 1.

The hybridization pattern obtained allowed the following observations. (i) The subcloned MH2 CL25 provirus contained a *Kpn*I restriction site within its LTRs as attested by the 2.2-kbp band hybridizing with LTR, *gag*, and *myc* probes. Such a band was also observed with the DNA of quail embryo cells transformed by the original isolate of MH2 CL25 pseudotyped by Rous-associated virus 1 (RAV-1) (Fig. 2). (ii) By *Sac*I digestion of the provirus, an internal 0.30-kbp restriction fragment was found that hybridized with both *gag* and 5′ *myc* probes (Fig. 1). This 0.30-kbp fragment replaced a 3.2-kbp fragment observed previously in the provirus of wt MH2 analyzed similarly, which in addition hybridized to a *mil* probe (4), indicating that in the MH2 CL25 mutant the *gag* and *myc* sequences had become proximal. Such a *gag-myc* junction could be explained by an extensive deletion of the *mil* sequences, or it could have resulted from the integration of a reverse-transcribed spliced subgenomic viral RNA. We thus analyzed the precise boundary between the *gag* and *myc* portions within the 0.30-kbp *Sac*I fragment. The fragment was purified on agarose gel, cleaved by *Hin*fI restriction endonuclease, and subjected to nucleotide sequencing by the Maxam and Gilbert procedure (15). Figure 1 presents our results, compared with the nucleotide sequence of the *gag* gene of RSV-SRA (24) (since the similar region in wt MH2 has not been sequenced before) and with the nucleotide sequence of the *myc* gene of wt MH2 (6, 13).

It appears that the *gag-myc* junction in MH2 CL25 occurred precisely between the splice donor site of the *gag* sequence and the splice acceptor site of the *myc* gene, bringing the open reading frames together in the correct phase. Therefore, the MH2 CL25 *myc* protein may initiate at the AUG of *gag* located 18 nucleotides upstream from the splice donor site (Fig. 1).

The *myc* gene of molecularly cloned MH2 CL25 is biologically active. We next examined whether our pMH2-CL25 was biologically active. Supercoiled pMH2-CL25 was used

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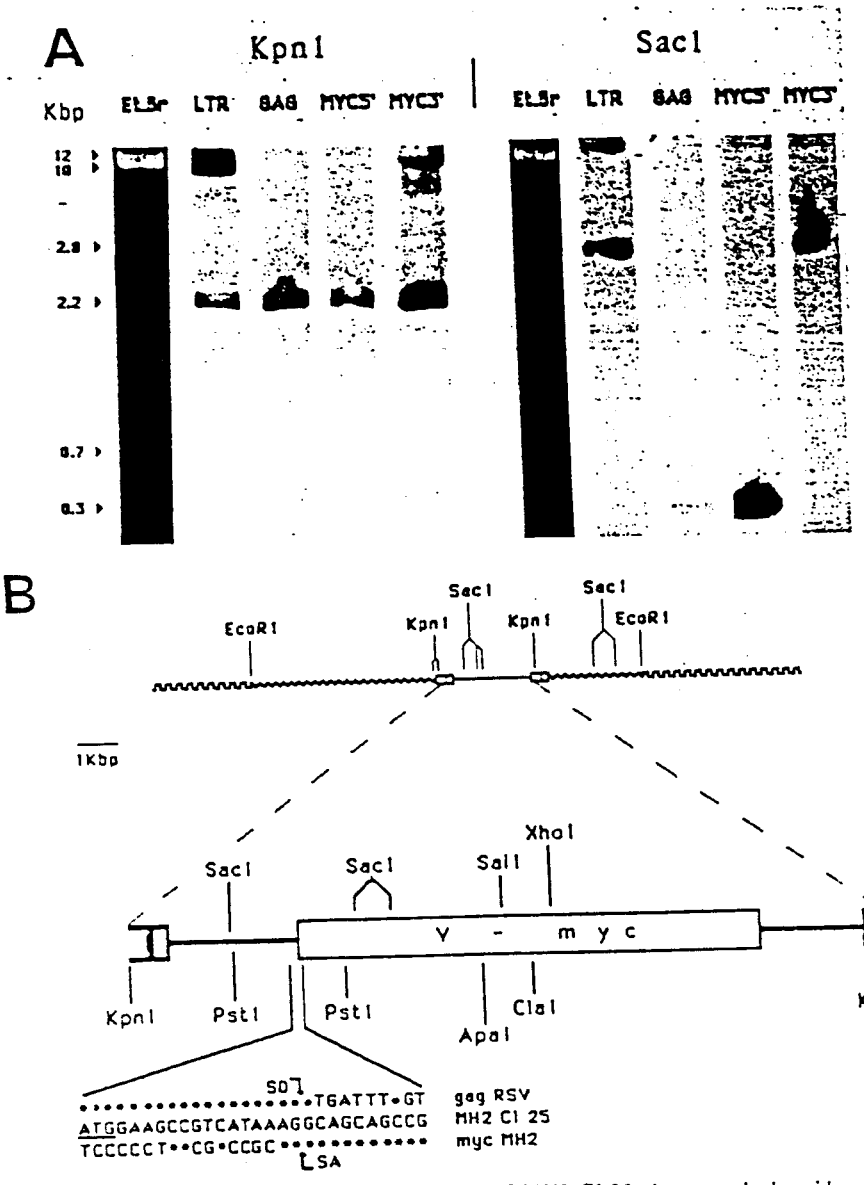

FIG. 1. Restriction map of pMH2-CL25 DNA. The restriction map of MH2 CL25 phages and plasmids was established with several endonucleases. The viral domains were located by Southern blot analyses with specific ^{32}P-labeled probes corresponding to LTR sequences from RSV-SRA, the *gag* gene from Pr-RSV-A, a 5' v-*myc* probe derived from wt MH2, and a 3' c-*myc* probe. (A) *Kpn*I or *Sac*I digestions were performed on pMH2-CL25 DNA. Fragments were separated on 1% agarose gels, visualized by ethidium bromide (EtBr) staining, and then transferred to nitrocellulose and hybridized with the probes quoted above. (B) Organization deduced from Southern blot analysis of MH2 CL25 DNA and nucleotide sequence of the *gag-myc* junction. A 162-nucleotide *Hin*fI fragment was sequenced by the Maxam and Gilbert procedure (15). The determined sequence was compared with the *gag* sequence of RSV (24) and with the v-*myc* sequence of wt MH2 (6, 13). Asterisks denote common nucleotides; SD, splice donor site of the *gag* gene; SA, splice acceptor site of the *myc* gene. Symbols: ——, MH2 CL25 proviral DNA; ∿∿, plasmid DNA; ▬, cellular DNA; ▭, LTR.

to transfect quail embryo cells. DNA (30 μg) was precipitated by the calcium phosphate method on 10^6 quail embryo cells (8). After overnight exposure, cultures were reseeded in 100-mm dishes and maintained in low-serum medium (Dulbecco modified Eagle medium supplemented with 5% fetal calf serum). Ten days later distinct foci had appeared (about 1 focus per μg of DNA). Groups of morphologically transformed cells were picked, pooled, and grown up in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. These cells were checked for the presence of MH2 CL25 proviral DNA and v-*myc* protein. Figure 2 shows the Southern blot analyses of DNA from quail embryo cells transformed by wt MH2 (RAV-1), MH2 CL16 (RAV-1), and MH2 CL25 (RAV-1) and cells transfected with pMH2-CL25. After *Kpn*I restriction endonuclease digestion, known to cut within the proviral LTRs, a 2.2-kbp *myc*-hybridizing band was detected in MH2 CL16- and MH2 CL25-transformed cell DNAs (in addition to the 5.5-kbp band corresponding to the endogenous c-*myc* gene seen in normal quail embryo cells [data not shown]). After *Eco*RI digestion of the same DNAs, a similar 2.2-kbp *myc*-hybridizing band appeared in MH2 CL16 DNA but not in MH2 CL25 DNA (in addition to the 16-kbp endogenous c-*myc* gene band [23]). This was expected for MH2 CL16 since this clone was derived from wt MH2 QB2 cells in which both restriction sites were found previously in the proviral LTRs (4). The

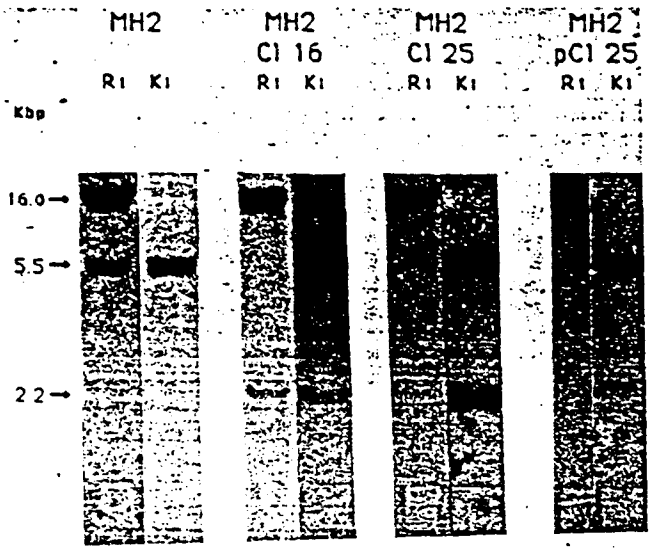

FIG. 2. Southern blot analysis of DNA from quail embryo cells transformed by wt MH2 and mutants. High-molecular-weight DNA from infected cells was digested with *Eco*RI (RI) and *Kpn*I (K). Size-separated cellular DNA fragments were transferred to nitrocellulose and hybridized with the 3′ c-*myc* probe. The transforming viruses used (RAV-1 helper pseudotypes) are indicated on top of the figure. pMH2-CL25 corresponds to quail cells transformed by the plasmid containing the molecularly cloned MH2 CL25.

progenitor of MH2 CL25 would be in this respect more related to the one described by Jansen et al. (12) that similarly lacks the LTR *Eco*RI site.

Finally, to determine whether the transformed cells produced the expected p61-63 *myc* doublet protein (9), [^{35}S]methionine-labeled total cellular extracts were challenged with rabbit anti-*myc* serum prepared by immunization with a bacterially expressed polypeptide corresponding to the product of the 5′ part of exon 3 from the human *myc* gene (F. Ferre, manuscript in preparation). Immunoprecipitation was performed as previously described (2). Immunoprecipitated proteins, characterized by their apparent molecular weight in polyacrylamide gel, are shown in Fig. 3. As can be seen, the p61-63 doublet was detected in cells transformed by wt MH2 or the mutants studied (lanes 1); such bands were not seen with preabsorbed serum (lanes 2).

We concluded from these experiments that the molecularly cloned provirus pMH2 CL25 was biologically active and apparently indistinguishable from the starting mutant isolate.

We then addressed whether pMH2-CL25 could produce recovered infectious virus. Transformed quail embryo cells obtained upon transfection with pMH2-CL25 DNA were superinfected with RAV-1 helper virus. Supernatant medium collected 2 weeks later was titrated for transforming activity by a focus assay on quail embryo cells (29) and contained 0.6 $\times$ 10^3 focus-forming units per ml. This titer was roughly similar to those obtained in similar conditions with cultures infected with wt MH2 (1.5 $\times$ 10^3 focus-forming units per ml), MH2 CL16 (0.7 $\times$ 10^3 focus-forming units per ml), and MH2 CL25 (0.9 $\times$ 10^3 focus-forming units per ml), all pseudotyped with RAV-1 helper virus. We concluded from these results that the virus rescued from pMH2-CL25 provirus was not defective in packaging.

Our results show that the MH2 CL25 and MH2 CL16 mutants derive from wt MH2 and appear to correspond to the encapsidation of subgenomic RNA in viral particles that can propagate as RAV pseudotypes.

The conserved infectious titers show that encapsidation signals are present in these types of molecules. Three kinds of encapsidation signals have been characterized in avian retroviruses. (i) Nishizawa et al. (16) reported packaging sequences (Ni-PS) in the 5′ leader of a temperature-sensitive mutant of RSV. These sequences reside between nucleotides 109 and 356, i.e., between the primer-binding site of the tRNA and the initiation codon of the *gag* gene. A mutant virus, TK15, deleted in this region is defective in packaging. Previous work by Shank and Linial (25) reported sequences that may serve similar functions in the first 600 nucleotides at the 5′ end of the RSV-Pr genome. (ii) Pugatsch and Stacey (18) mapped packaging sequences (Pu-PS) at the *Sst*II restriction site (545 nucleotides from the 5′ end) in the *gag* gene of RSV-SRA. These sequences map 3′ of the splice donor signal and are lost in *env* (and *src*) subgenomic mRNAs that splice out the intronic *gag-pol* (*env*) sequences from the viral genome. Sequences serving similar functions have also been reported for spleen necrosis virus (30). (iii) Sorge et al. (26) reported packaging sequences (So-PS) in the direct repeat unit 3′ to the *src* gene of RSV, slightly upstream from the U3 sequences.

Although no direct evidence is yet available, wt MH2 is likely to contain all three packaging signals described, since the leader and *gag* sequences are well conserved among most avian retroviruses and since the direct repeat unit that may encompass the So-PS signal was found in wt MH2 by Kan et al. (13), although the U3 sequences appeared quite distantly related to RSV. In contrast, the mutant MH2 CL25 (and MH2 CL16) corresponds to subgenomic RNA that should lack the putative Pu-PS signal lost during the splicing process. This is also true for the *env* subgenomic mRNA described by Stacey (27). Since the titers of our mutants MH2 CL25 and MH2 CL16 are still about half of the wt MH2 titer, we conclude that the Pu-PS signal, if present, does not

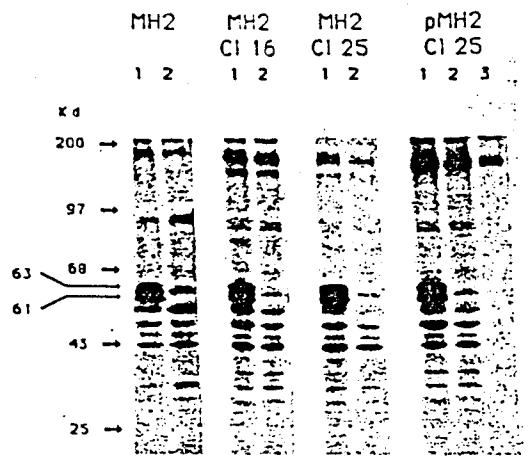

FIG. 3. Immunoprecipitation of v-*myc* proteins in quail embryo cells transformed by wt MH2 and mutants. Cells were labeled for 45 min with [^{35}S]methionine, lysed, and incubated with rabbit anti-human c-*myc* serum prepared with a bacterially expressed polypeptide (lane 1) or the same antiserum preincubated with the corresponding polypeptide (lane 2). Lane 3 corresponds to rabbit preimmune serum. Standard molecular size markers from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) are listed on the left of the figure (Kd, kilodaltons).

play a decisive role in the packaging process of wt MH2 or the mutants.

The titers measured separately for wt MH2 and the mutants in similar conditions would predict a ratio of ca. 60 to 40% of the corresponding particles present in routinely passaged stocks of MH2 virus. Instead, we never detected more than 10% mutant particles in such stocks. Clearly, when promiscuously mixed in such stocks, wt MH2 appears to exhibit a selective advantage over the mutant particles, an observation for which we have as yet no clear explanation.

The isolation and characterization of the MH2 CL25 mutant represents the first demonstration of transmissible pseudotype particles containing an oncogene embedded in a subgenomic mRNA. An analogous situation may have been encountered with *src*-containing deleted proviruses in Syrian hamster tumor cell lines such as H-19 (28) or deletion mutants described by Koyama et al. (14). Such experiments might help in finding the minimum structural genetic elements required to allow the propagation of a retrovirus, and our results may be used for the construction of vectors for the introduction of foreign genes into host cells. Finally, the tumorigenic potential of our mutants is currently being investigated.

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Characterization of the Oncogene (*erb*) of Avian Erythroblastosis Virus and Its Cellular Progenitor

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Avian erythroblastosis virus (AEV) induces primarily erythroblastosis when injected intravenously into susceptible chickens. In vitro, the hematopoietic target cells for transformation are the erythroblasts. Occasional sarcomas are also induced by intramuscular injection, and chicken or quail fibroblasts can be transformed in vitro. The transforming capacity of AEV was shown to be associated with the presence of a unique nucleotide sequence denoted *erb* in its genomic RNA. Using a simplified procedure, we prepared radioactive complementary DNA ($cDNA_{aev}$) representative of the *erb* sequence at a high yield. Using a $cDNA_{aev}$ excess liquid hybridization technique adapted to defective retroviruses, we determined the complexity of the *erb* sequence to be 3,700 ± 370 nucleotides. AEV-transformed erythroblasts, as well as fibroblasts, contained two polyadenylated viral mRNA species of 30 and 23S in similar high abundance (50 to 500 copies per cell). Both species were efficiently packaged into the virions. AEV-transformed erythroblasts contained additional high-molecular-weight mRNA species hybridizing with $cDNA_{aev}$ and $cDNA_{5'}$ but not with cDNA made to the helper leukosis virus used ($cDNA_{rep}$). The nature and the role, if any, of these bands remain unclear. The *erb* sequence had its counterpart in normal cellular DNA of all higher vertebrate species tested, including humans and fish (1 to 2 copies per haploid genome in the nonrepetitive fraction of the DNA). These cellular sequences (c-*erb*) were transcribed at low levels (1 to 2 RNA copies per cell) in chicken and quail fibroblasts, in which the two alleged domains of AEV-specific sequences corresponding to the 75,000- and 40,000-molecular-weight proteins seemed to be conserved phylogenetically and transcribed at similar low rates.

Among avian retroviruses, avian defective leukemia viruses (DLVs) form a group of highly oncogenic RNA viruses inducing acute leukemias with a short period of latency in susceptible birds and also occasional sarcomas or carcinomas (8).

Recently, the seven available independent isolates of DLVs have been assigned to three subgroups based on the types of malignant hemopathy that they induce in vivo and on the differentiation phenotype of hematopoietic cells that they transform in infected bone marrow cells in vitro (3, 8). (i) Avian erythroblastosis virus (AEV) causes erythroblastosis in vivo and induces the proliferation of erythroblast-like cells. It also induces sarcomas at the site of injection (24). (ii) The four avian myelocytomatosis-type viruses (MC29, CMII, OK10, and MH_2) induce the proliferation of macrophage-like transformed cells, and some of them induce carcinomas in vivo (10, 19, 20). (iii) The avian myeloblastosis viruses (AMV and E26) induce myeloblastosis in vivo and transform myeloblast-like cells in vitro (6). Studies on the genetic content of DLVs have documented the following common properties. All DLVs contain avian leukemia virus (ALV)-related nucleotide sequences in different amounts (28 to 77%) (25) but are deleted in genes necessary for viral replication (31) and therefore are replication defective and require a helper ALV for their propagation in cultured cells (8). None of these viruses contain sequences homologous to the transforming gene *src* of avian sarcoma virus (ASV) (4, 25, 27, 28). Instead, they contain one of three different sequences inserted within the ALV-related sequences, which correlate with the capacity of a given virus strain to transform erythroblasts, macrophage-like cells, or myeloblasts (25, 31). These sequences have been termed *erb*, *mac*, and *myb*, respectively. As published previously (25, 26, 31), these unique sequences have their counterpart in the normal cellular DNA. Thus, DLVs seem to be recombinants between an

ALV-related vector and normal chicken cellular DNA sequences which confer to these viruses their oncogenicity.

In this paper we describe extensively the selection and properties of a radioactive DNA complementary to the specific sequences (*erb*) of AEV ($cDNA_{aev}$). We established the complexity of the $cDNA_{aev}$ to be 3.7 ± 0.37 kilobases, using a method adapted from Young et al. (37).

This cDNA enabled us to determine unambiguously the size of the AEV genomic RNA and its products of transcription in some AEV-infected cells. Our results are consistent with the hypothesis that the viral *erb* sequences contain two different genes, one coding for the 75,000-molecular-weight (75K) *gag-erb* polyprotein (12, 15, 23; H. Beug, G. Ramsay, S. Saule, D. Stehelin, M. J. Hayman, and T. Graf, *in* B. Fields, R. Jaenisch, and F. Fox, ed., *Animal Virus Genetics, ICN-UCLA Symposium on Cell and Molecular Biology*, vol. 18, in press) and the other coding for a 40K protein as shown previously by in vitro transcription of AEV RNA (15, 21, 36). AEV-transformed erythroblasts contain additional mRNA species hybridizing with $cDNA_{aev}$ and related to ALV sequences that are packaged into the virions. These species are not seen in AEV-transformed uncloned fibroblasts or erythroblasts and may represent occasional read-through molecules extending into cellular sequences.

Normal avian DNA contains sequences (c-*erb*) related to $cDNA_{aev}$ (25). The moieties in $cDNA_{aev}$ corresponding to both the 75K and the 40K proteins are conserved in quail DNA and transcribed at similar levels. It is unclear that this is the case in more distantly related species. Nevertheless, $cDNA_{aev}$-related sequences are at least partially present in the DNAs of humans and fish at a similar frequency of 1 to 2 copies per haploid genome.

MATERIALS AND METHODS

Cells and viruses. Viruses were grown on C/E chicken fibroblasts prepared from 11-day fertile eggs (Brown Leghorn; Institut Gustave Roussy, Villejuif, France). AEV nonproducer NP 75 chicken fibroblasts (11) and AEV (Rous-associated virus type 2 [RAV-2])-producing 6C2 and 9D4 erythroblasts and nonproducing NP A6C1 erythroblasts, prepared with *ts*34 AEV (7), were obtained from T. Graf and H. Beug, AEV nonproducer NP AT1a rat fibroblasts (22) were obtained from K. Quade. The transformation-defective Prague B strain (*td* PrB) of ASV was obtained originally from R. Junghans. All other viruses used in these studies have been described elsewhere (29).

RNA extraction. Viral 50 to 70S RNA was extracted from viruses harvested from culture medium clarified from cell debris. Viruses were suspended in STE buffer (0.1 M NaCl, 0.001 M EDTA, 0.02 M Tris) containing 200 μg of proteinase K per ml and 1% sodium dodecyl sulfate (SDS). After 5 min of digestion at 37°C, the RNA was extracted twice with an equal volume of STE buffer-saturated phenol. RNA was precipitated from the aqueous phase by the addition of 2 volumes of 95% ethanol at −20°C, collected by centrifugation (10,000 × *g*, 10 min, 4°C), and dissolved in STE buffer before preparative sucrose gradient centrifugation. The 50 to 70S RNA was separated from rRNA by sedimentation through a 15 to 30% sucrose gradient made in STE buffer in an SW41 rotor at 40,000 rpm for 3.5 h at 4°C. The 50 to 70S peak was detected by UV absorption at 260 nm, pooled, and precipitated by the addition of 2 volumes of cold ethanol.

Cellular RNAs were extracted as previously described (28).

DNA extraction. Chicken embryos were homogenized in 10 ml of TE buffer (0.02 M Tris-hydrochloride [pH 7.5], 0.01 M EDTA) per embryo at 0°C for 60 s with an Ultra-Turrax homogenizer (Scientific Instruments Ltd., London, England). The mixture was then adjusted to 1% with SDS and 50 μg/ml with proteinase K. Tissue culture cells (10^7 cells per ml) were suspended in TE buffer containing 50 μg of proteinase K per ml and 1% SDS. After incubation overnight at 37°C, the DNA was extracted twice with STE buffer-saturated phenol. The final aqueous phase was made 0.2 M in sodium acetate, and the DNA was precipitated by 2 volumes of 95% ethanol. The DNA was spooled out, washed with 95% ethanol, and air dried. This DNA was dissolved overnight in TE buffer at a concentration of 1 to 2 mg/ml and then treated with pancreatic RNase (100 μg/ml; Sigma Chemical Co., St. Louis, Mo.). After 1 h at 28°C, the DNA was sonicated and incubated overnight in 0.3 M NaOH at 37°C. The mixture was adjusted to neutral pH by the addition of 3 M HCl in the presence of phenol red indicator and extracted by STE buffer-saturated phenol as described above. The DNA was then ethanol precipitated and dissolved in TE buffer (5 to 10 mg/ml) before UV spectral analysis. The ratio of absorbance at 260 nm to that at 280 nm was more than 1.9 for all DNAs tested.

Preparation of virus-specific cDNA's. Representative radioactive cDNA's were synthesized on a 70S viral RNA template with fragmented calf thymus DNA primer (34), using the exogenous AMV reverse transcriptase (J. Beard, Life Science Inc., through the auspices of the Office of Program Resources and Logistics, National Cancer Institute). The reaction mixture (for ^3H, 1 ml; for ^{32}P, 100 μl) contained the following: 20 μg of viral RNA per ml, 0.05 M Tris (pH 8.1), 0.02 M dithiothreitol, 8 mM $MgCl_2$, 0.05 M KCl, 100 μg of actinomycin D (Rhône-Poulenc) per ml, 2.5 mg of DNA primer per ml, 280 U of AMV polymerase per ml, 0.4 mM concentration each of dGTP, dATP, and dCTP, and 0.15 mM [^3H]TTP (60 Ci/mmol, 10 mCi; International Chemical Nuclear, Irvine, Calif.) or 0.002 mM [^{32}P]TTP (350 Ci/mmol, 0.5 to 1 mCi; New England Nuclear Corp., Boston, Mass.). The reaction was allowed to proceed for 45 min at 41°C and was terminated by the addition of 1% SDS (final concentration). After the reaction mixture had been digested with 100 μg of proteinase K per ml for 15 min at 37°C,

200 μg of yeast RNA per ml was added. The nucleic acids were then extracted once with phenol-STE buffer and precipitated with 2 volumes of ethanol at −20°C. The specific activities of the probes complementary to the *td* PrB genome ($cDNA_{rep}$) were as follows: [^{32}P]cDNA, 5×10^4 cpm/μg; [^{3}H]cDNA, 4.8×10^7 cpm/μg. The cDNA's were sonicated and sedimented through 5 to 20% (wt/vol) sucrose gradients made in 0.9 M NaOH–1 M NaCl–0.01 M EDTA for 24 h at 37,000 rpm in an SW41 rotor at 24°C. The cDNA's ranged in size from 3 to 10S, with a peak at 5S. A size pool of 4 to 7S was used throughout the studies described below. $cDNA_{src}$ was prepared as described previously (29), $cDNA_{5'}$ (strong stop) was prepared by the method of Friedrich et al. (5), and $cDNA_{gpc}$ was prepared by the method of Tal et al. (33).

Nucleic acid hybridization. Standard hybridization reaction mixtures (stringent conditions) contained 0.6 M NaCl, 2 mM EDTA, 0.02 M Tris (pH 7.4), 500 μg of calf thymus DNA per ml as carrier, 2,000 cpm (0.04 ng) of [^{3}H]cDNA or 2,000 cpm (0.005 ng) of [^{32}P]cDNA, and appropriate RNAs or DNAs in large excess. Hybridizations were conducted in glass capillaries at 68°C, and the extent of annealing was analyzed by digestion with S1 nuclease (17), prepared by the method of Sutton (32).

Determination of T_m. For the determination of thermal denaturation (T_m), erythroblast 6C2 DNA or normal chicken DNA (final DNA concentration of 10 mg/ml) was hybridized with AEV-specific [^{3}H]cDNA to reach a C_0t of 2×10^4 mol·s liter^{-1}. The samples were diluted to a final concentration of 0.3 M NaCl, and 20,000 cpm of [^{32}P]$cDNA_{rep}$, preannealed with normal chicken DNA, was added as an internal control. Samples were then divided into 10 aliquots and incubated in a water bath at different temperatures for 10 min. The percentage of cDNA remaining annealed to the particular cellular DNA at each defined temperature was determined by S1 nuclease digestion. The self-annealing of the cDNA was not subtracted (1 to 3%), and all hybridization values were normalized by using a correction factor corresponding to 100% of plateau hybridization of the cDNA with its homologous RNA.

Complexity of $cDNA_{aev}$. We determined the complexity of $cDNA_{aev}$ by using a method adapted from Young et al. (37), based on the fact that the complexity of a given radioactive cDNA can be defined experimentally by the $C_0t_{1/2}$ of the hybridization kinetics established between this cDNA in excess and its homologous nonradioactive RNA. Under these conditions, the $C_0t_{1/2}$ depends only on the complexity of the cDNA and is independent of any RNA heterogeneity in the hybridization reaction. One essential condition is to be in cDNA excess. To control this absolute requirement, we set the experimental procedure as follows. The hybridization kinetics were established by varying the time of hybridization of samples containing each, in addition to [^{3}H]cDNA in excess and cold RNA homologous to [^{3}H]cDNA, [^{32}P]cDNA representative of the RNA genome 100 times less concentrated than the [^{3}H]cDNA.

Thus, with [^{3}H]cDNA, we constructed a C_0t curve, and with [^{32}P]cDNA as an internal control, we constructed a C_rt curve which allowed us to estimate accurately the RNA concentration in the hybridization reaction.

We standardized our system by determining the complexity of a known [^{3}H]cDNA ($cDNA_{gpc}$) prepared and purified by the method of Tal et al. (33), except rd Bryan ASV 60 to 70S RNA was used to select the single-stranded cDNA (ss-cDNA). We similarly used [^{3}H]$cDNA_{rep}$.

Hybridizations were conducted in capillaries at 68°C in 0.6 M NaCl. See the legend to Fig. 2 for details.

Gel electrophoresis of RNA. Polyadenylated RNA was prepared from AEV-transformed cells as described elsewhere (35). The RNA was treated with glyoxal and submitted to electrophoresis on agarose gels as previously described (16, 18, 31). RNAs separated by size were then transferred to activated diazobenzyloxymethyl paper by the method of Alwine et al. (1), and the northern blots were hybridized with different [^{32}P]cDNA's as described previously (1, 16, 18, 31).

We used an original washing procedure of ours (unpublished data). After a 10-min wash in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhardt buffer, the blots were cleaned of cDNA's by mild sonication in 0.1× SSC–0.1% SDS during 1 to 3 min (Pons sonicator, 100 W, 40 kHz; Bioblock, Strasbourg, France).

The hybrids were scored by autoradiography, using Kodak XR-1 X-ray film with Du Pont Lightning-Plus screens at −70°C.

RESULTS

Preparation of $cDNA_{aev}$. We adopted the following substractive hybridization strategy to purify $cDNA_{aev}$. [^{3}H]DNA complementary to the 50 to 70S RNA of AEV (RAV-2)-producing 6C2 cloned chicken erythroblasts was synthesized as described above. The 4 to 7S cDNA size pool (30% of the total cDNA, 9.6 μg) obtained after centrifugation through an alkaline sucrose gradient was hybridized to 78 μg of RAV-2 70S RNA under nonstringent conditions (1.2 M NaCl, 60°C) to reach a C_rt of 25 mol·s liter^{-1} and a C_0t of 4 mol·s liter^{-1} (Table 1). The ss-cDNA was separated from hybrids by chromatography on hydroxyapatite. The ss-cDNA eluted from the column (7 μg) was completely sensitive to S1 nuclease as tested on an aliquot, did not hybridize to helper 70S RAV-2 RNA ($C_rt \geq 10$ mol·s liter^{-1}), but hybridized to AEV (RAV-2) RNA ($C_rt \geq 11$ mol·s liter^{-1}) with a plateau at 50% hybridization, indicating that 50% of the ss-cDNA was not virus-hybridizable cDNA (Table 1).

The ss-cDNA was then hybridized to AEV (RAV-2) 50 to 70S RNA under stringent conditions (0.6 M NaCl, 68°C; final C_rt = 4.5 mol·s liter^{-1}), and the hybrids were treated preparatively with S1 nuclease (15 min, 50°C) to digest the ss-cDNA which was not AEV specific. Proteinase K (100 μg/ml), EDTA (0.01 M), and SDS

TABLE 1. *Purification of cDNA$_{aev}$*[a]

cDNA	g × 10^{-4}	cpm × 10^{-9}	% of total cDNA	S1 resistance (%) after hybridization with:	
				AEV (RAV-2)	RAV-2
Total	32	1.6	100		
4 to 7S	9.6	0.48	30	67	37
ss after hydroxyapatite[b]	7	0.35	21.8	50	<3
ss after S1 digestion[c]	3.5	0.176	11	88	<3

[a] The data represent recoveries during the preparation of the [^{3}H]cDNA$_{aev}$ that was used in our experiments. Similar results were obtained for the preparation of [^{32}P]cDNA$_{aev}$ that was used in experiments shown in Fig. 3.

[b] A total of 9.6 μg of cDNA was hybridized to 78 μg of RAV-2 70S RNA in 1.2 M NaCl at 60°C at a final C$_r$t of 25 mol·s liter^{-1}, and ss-DNA was separated from hybrids by chromatography on hydroxyapatite (see text).

[c] A total of 7 μg of cDNA was hybridized to 33 μg of AEV (RAV-2) 50 to 70S RNA in 0.6 M NaCl at 68°C at a final C$_r$t of 4.5 mol·s liter^{-1}. ss-DNA was digested with S1 nuclease (see text), and the cDNA was recovered from hybrids by treatment with 0.3 N NaOH (12 h at 37°C to hydrolyze RNA) and ethanol precipitation after neutralization with HCl. During the procedure, the selected cDNA's were tested for their hybridization capacities at plateau C$_r$t values to AEV (RAV-2) RNA or RAV-2 RNA, as scored by the percentage of radioactivity rendered resistant to S1 nuclease.

(1%) were then added, and the mixture was incubated at 37°C for 15 min. The hybrids were phenol extracted once, treated with alkali (0.3 N NaOH for 12 h at 37°C) to eliminate the viral RNA, neutralized with HCl, and ethanol precipitated (−20°C, 12 h). After this step, 11% of the starting cDNA was recovered as ss-cDNA (Table 1) and was denoted cDNA$_{aev}$.

Specificity of cDNA$_{aev}$. The specificity of cDNA$_{aev}$ was documented more precisely by hybridizing cDNA$_{aev}$ and cDNA$_{rep}$ to viral 50 to 70S RNAs. No residual hybridization of cDNA$_{aev}$ with RAV-2 RNA could be detected (Fig. 1a), indicating the efficiency of the selection procedure. On the contrary, the kinetics with AEV (RAV-2) RNA from the 6C2 erythroblasts was as expected (Fig. 1b), showing that cDNA$_{aev}$ had maintained its hybridization qualities, with a maximum plateau value of 88%. A similar curve was obtained with cDNA$_{rep}$, indicating a favorable ratio of AEV RNA to RAV-2 RNA of near 1:1 for the viruses produced by these cells.

A possible drawback to our strategy for preparing cDNA$_{aev}$ was that we might coselect for cellular sequences specific to erythroblasts that could be encapsidated with the virus or unidentified viral sequences unrelated to AEV and present in this clone. This possibility would predict that some sequences in cDNA$_{aev}$ would not hybridize with the viral RNA in another nonerythroblastic AEV-transformed cell clone. Thus, we tested the cellular RNA from an independently prepared clone (NP 75) of chicken fibroblasts nonproductively transformed by AEV. Figure 1c shows that, indeed, the same plateau value was reached with cDNA$_{aev}$, confirming the purity of this probe, whereas the curve obtained with cDNA$_{rep}$, with similar kinetics, indicated less than 30% homology of the AEV genome with cDNA$_{rep}$.

We further analyzed the distribution of cDNA$_{aev}$-related sequences among a variety of avian retroviruses, using stringent hybridization conditions at high C$_r$t values (≥4 mol·s liter^{-1}). None of the viruses tested, except AEV (RAV-2), showed any annealing above background (Table 2). Thus, we concluded that cDNA$_{aev}$ was specific for viral nucleotide sequences found so far only in AEV.

Complexity of cDNA$_{aev}$. Complexities of cDNA probes are usually determined by measuring the percentage of labeled viral RNA rendered resistant to RNase by the cDNA present in excess. The precision of this method becomes unsatisfactory when the complexity of the cDNA represents only a small part of the viral RNA, which is often the case for a defective retrovirus obtained with sometimes a severalfold excess of helper virus. We thus used an alternative method to determine the complexity of cDNA$_{aev}$. The validity of this method is first documented here by using cDNA probes of known complexities.

Following the experimental procedure of using C$_0$t curves in cDNA excess as described by Young et al. (37), modified for better accuracy as we described above, we determined the complexities of well-characterized probes such as cDNA$_{rep}$ and cDNA$_{gpC}$ as a test for the precision of this method. Figure 2a shows the kinetics between [^{3}H]cDNA$_{rep}$ in slight excess and *td* PrB ASV RNA, on the one hand (C$_0$t curve), and [^{32}P]cDNA$_{rep}$ in a negligible amount with the same RNA, on the other hand (control C$_r$t curve). Figure 2 clearly shows that these conditions yielded a C$_0$t$_{1/2}$ value of 1.6×10^{-2} mol·s liter^{-1}, corresponding to the expected complexity of 8,000 ± 800 nucleotides of the viral RNA and matching the C$_r$t$_{1/2}$ value. The almost equimolar ratio of viral RNA and [^{3}H]cDNA$_{rep}$ would predict a plateau value of the C$_0$t curve of near 90%. In fact, the observed value was lower (60%). This was probably due to the fact that the RNA might have been slightly degraded upon long incubation times; it also could mean that [^{3}H]cDNA$_{rep}$ was not absolutely representative or not able to hybridize all along the RNA molecules. Nevertheless, the C$_0$t$_{1/2}$ value does not seem to be affected detectably by these

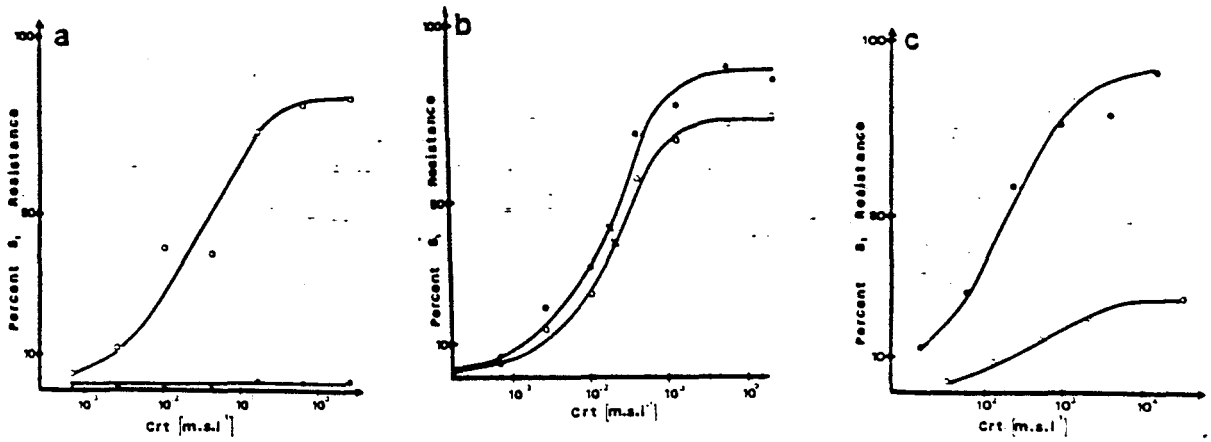

FIG. 1. *Hybridization of $cDNA_{aev}$ after the S1 nuclease selection step. [^{3}H]$cDNA_{aev}$ (●) and [^{32}P]$cDNA_{rep}$ (○) were hybridized under stringent (0.6 M NaCl) conditions to dilutions of viral RNAs in a volume of 8 μl for 15 h at 68°C. The extent of hybridization was measured by digestion with S1 nuclease. (a) Hybridization with RAV-2 RNA; (b) hybridization with AEV (RAV-2) RNA; (c) dilutions of total cellular RNA extracted from a clone (NP 75) of chicken fibroblasts nonproductively infected with AEV were hybridized to both probes in 0.6 M NaCl at 68°C for 25 h in 7 μl (0.075 mg of RNA at the highest C$_r$t). Hybridization was measured by resistance of the cDNA's to hydrolysis with S1 nuclease.*

TABLE 2. *Hybridization of viral 50 to 70S RNAs to $cDNA_{rep}$ and $cDNA_{aev}$*[a]

	Viral RNAs	Hybridization of cDNA (%) cDNA rep	cDNA aev
ALV	RAV 0	91	3
	RAV 1	79	4
	RAV 2	80	3
	RAV 49	80	3
	RAV 50	86	3
	td B77	83	2
	td Pr B	100	3
ASV	nd. B 77	94	4
	nd. Pr B	100	3
	C Z	86	4
	rd Bry	70	2
DLV	AEV (RAV 2)	86	100 (88)
	MC 29 (RAV 2)	89	5
	MH 2 (RAV 3)	89	6
	OK 10 (RAV 2)	100	3
	CM II (CM-AV)	76	3
	AMV	84	3
	E 26 (RAV 2)	89	4

[a] Hybridization reactions were performed in solution under conditions of RNA excess as described in the text. [^{32}P]$cDNA_{rep}$ was included in each reaction mixture as a standard (final $C_rt \geq 4$ mol·s liter^{-1}). The values for the percent cDNA's in hybrids were normalized to 100% for the final extent of reaction with a homologous RNA: 80% for $cDNA_{rep}$ with *td* PrB and 88% for $cDNA_{aev}$ with AEV (RAV-2).

parameters and proves the validity of this method, provided that the ^{3}H probes have been selected to exclude small pieces (<4S) that would affect the kinetics. Similar experiments are shown for [^{3}H]$cDNA_{gpC}$ in excess over *td* PrB RNA in Fig. 2b. The $C_0t_{1/2}$ value found was 4.5×10^{-3} mol·s liter^{-1} and thus corresponded to a complexity of 2,250 ± 200 nucleotides, a value which agrees with other determinations (13, 33). As expected, the $C_0t_{1/2}$ value did not vary when the ratio of cDNA to RNA was increased (Fig. 2c), but the plateau value was then lower and the precision was somehow affected.

Using this procedure for [^{3}H]$cDNA_{aev}$, we found (Fig. 2d) a $C_0t_{1/2}$ value of 7.5×10^{-3} mol·s liter^{-1}, which corresponded to a complexity of 3,700 ± 370 nucleotides. If one adds the 2,200 ± 200 nucleotides of ALV-related sequences detected in the AEV genomic RNA (25) to the 3,700 ± 370 nucleotides of the specific sequence, the AEV genomic RNA should be 5,900 ± 600 nucleotides long. These numbers are in good agreement with the size of the AEV genomic RNA as determined by northern blotting (30S) and described below and with the results obtained by analysis of heteroduplex molecules formed between a long cDNA (cDNA PrC) and the AEV genomic RNA (14).

Sizes of AEV-specific RNAs in infected cells. The strategy of transcription of the AEV provirus was analyzed to determine whether we could detect a difference in the relative level of transcription or in the sizes of viral transcripts in erythroid versus fibroblastic transformed cells.

We thus performed northern blots with polyadenylic acid-containing total cell RNA from the following AEV-transformed cells: a chicken erythroblast clone (9D4) producing AEV (RAV-2) (the virus produced here was also tested); a chicken erythroblast nonproducer clone (NP

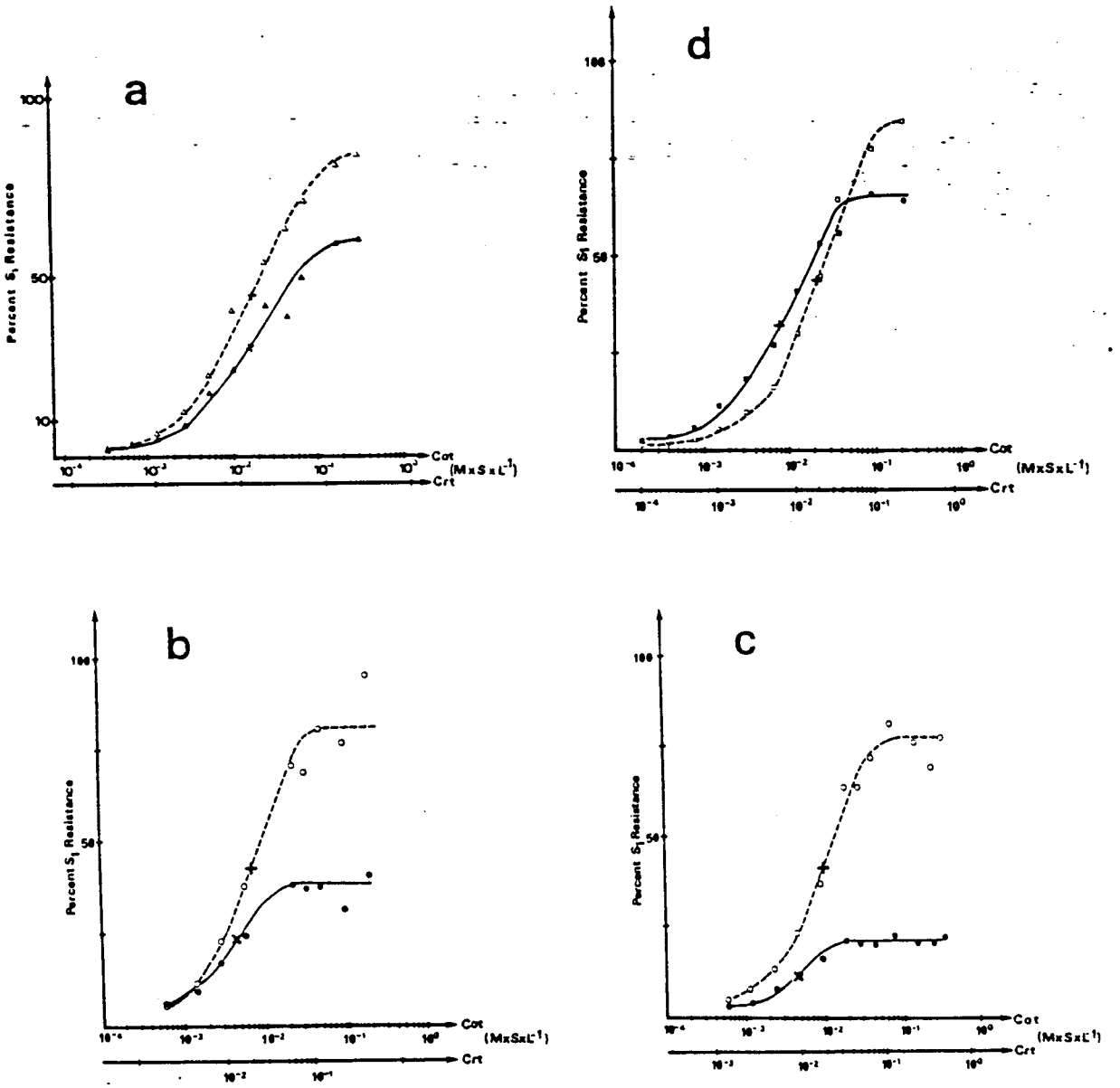

FIG. 2. *Determination of cDNA complexities. (a) Complexity of $cDNA_{rep}$. A total of 39,000 cpm (0.82 ng) of [^{3}H]$cDNA_{rep}$ (▲) and 2,000 cpm (0.005 ng) of [^{32}P]$cDNA_{rep}$ (△) were hybridized to 0.73 ng of td PrB RNA in each sample (10 μl). The times of incubation ranged from 210 s to 64 h. Hybridization was measured by resistance to S1 nuclease. (b and c) Complexity of $cDNA_{gpC}$ at two different values of cDNA excess. (b) A total of 24,000 cpm (0.37 ng) of [^{3}H]$cDNA_{gpC}$ (●) and 2,000 cpm (0.005 ng) of [^{32}P]$cDNA_{rep}$ (○) were hybridized as in (c) to 1 ng of td PrB RNA in each sample (2 μl) (gpC = 25% of viral RNA sequences). (c) A total of 17,000 cpm (0.31 ng) of [^{3}H]$cDNA_{gpC}$ (●) and 2,000 cpm (0.005 ng) of [^{32}P]$cDNA_{rep}$ (○) were hybridized to 0.29 ng of td PrB RNA in each sample. The times of incubation ranged from 225 s to 32 h for each curve. Hybridization was measured by resistance to S1 nuclease. All values were normalized to a final extent of hybridization of 75% for both ^{3}H and ^{32}P probes (factor, ×1.33). (d) Complexity of $cDNA_{aev}$. The C_0t curve was constructed as follows. Samples contained 44,000 cpm (0.92 ng; ■) of [^{3}H]$cDNA_{aev}$, 2,000 cpm (0.005 ng; □) of [^{32}P]$cDNA_{rep}$, and 0.8 ng of AEV (RAV-2) RNA in 10 μl (0.6 M NaCl, 68°C). The C_0t values were obtained by varying the time of incubation from 225 s to 37 h. Percent values were corrected by the factors ×1.14 for [^{3}H]$cDNA_{aev}$ and ×1.18 for [^{32}P]$cDNA_{rep}$.*

A6C1); and two nonproducer fibroblast clones of chicken (NP 75) and rat (NP AT1a). The results are shown in Fig. 3. For clone 9D4, three mRNA species of 34, 30, and 23S were detected with [^{32}P]$cDNA_{rep}$ (Fig. 3, lane a). The 30 and 23S species also hybridized with [^{32}P]$cDNA_{aev}$ (Fig. 3, lane b), but the 34S species that corresponded to the RAV-2 helper RNA did not. In addition, two other RNA species of high molecular weight (31 and 32S) were detected wtih [^{32}P]$cDNA_{aev}$.

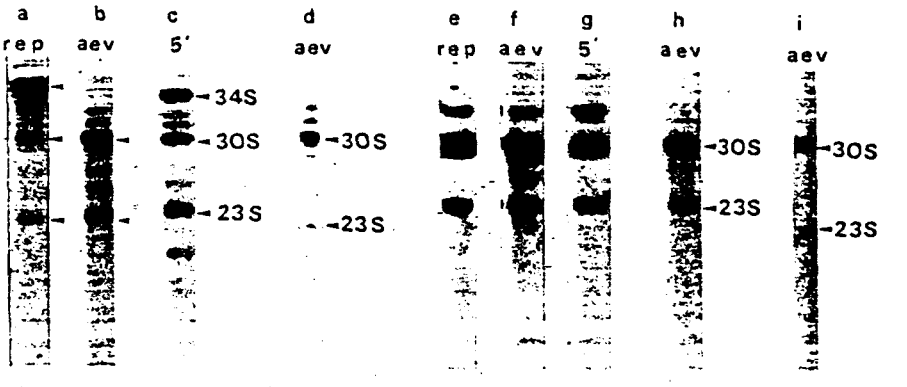

FIG. 3. *Sizes of AEV-coded RNAs. Total cellular polyadenylic acid-containing RNAs were run on 1% agarose gels, transferred to activated diazobenzyloxymethyl paper, and hybridized with [^{32}P]cDNA$_{rep}$ (lanes a and e), cDNA$_{aev}$ (lanes b, f, h, and i), or cDNA$_{5'}$ (lanes c and g). The following AEV-transformed cells were tested: chicken erythroblast clone 9D4 (lanes a, b, and c) and the AEV (RAV-2) that it produces in the culture fluid (lane d, with cDNA$_{aev}$); chicken erythroblast nonproducer clone NP A6C1 (lanes e, f, and g); chicken fibroblast clone NP 75 (lane h); and rat fibroblast clone NP AT1a (lane i).*

These bands also hybridized to cDNA$_{5'}$ (Fig. 3, lane c), indicating their viral origin or viral induction. Interestingly, all of these sequences were found in the viral RNA from the culture fluid (Fig. 3, lane d), indicating that they were probably efficiently packaged into the virions. This matter was not analyzed further.

The complicated pattern of cDNA$_{aev}$-related mRNA species in clone 9D4 raised the question of their relevance to erythroblast transformation by AEV. We thus analyzed another clone of similar origin but a nonproducer, NP A6C1. Only three mRNA species (23, 30, and 33S) were seen with cDNA$_{rep}$ (Fig. 3, lane e), cDNA$_{aev}$ (Fig. 3, lane f), or cDNA$_{5'}$ (Fig. 3, lane g). The band at 27S (Fig. 3, lane f) could not be reproduced in subsequent blots and thus represented an artifact. Thus, only the 30 and 23S species are shared between the two erythroblast clones tested, and it is unclear whether the other species observed play any role in the transformation of erythroblasts by AEV. Certainly, the cDNA$_{aev}$-related 30 and 23S species seemed to be sufficient to transform chicken or rat fibroblasts (Fig. 3, lanes h and i, respectively), and no obvious variation in the relative abundance of these species was observed in the four AEV-transformed clones tested, although the rat cells contained about 10-fold-fewer AEV-related RNA copies per cell (unpublished data of K. Quade and us).

Cellular origin of the nucleotide sequences related to cDNA$_{aev}$. We have shown previously that cDNA$_{aev}$ annealed under stringent reaction conditions (0.6 M NaCl, 68°C) to DNA from normal chickens, as well as to DNA from other birds phylogenetically diverged from chickens and to DNA from mammalian species and fish (25, 31). For the latter species, the percent annealing was low, and the copy number per cell was not known. We thus decided to analyze more closely the kinetics of these annealings. The C_0t curves constructed (Fig. 4) with the DNAs of human placenta (Fig. 4a) and salmon sperm (Fig. 4b) indicated that in both cases 1 to 2 copies of cDNA$_{aev}$-related sequences were present per haploid genome ($C_0t_{1/2}$ = 3.5 × 10^{-3} mol·s liter^{-1}). The hybridization kinetics allowed us to exclude the possibility of nonspecific hybridization, since in the same experiment, the cDNA$_{rep}$ used as a negative control did not hybridize (Fig. 4). The plateau value observed (7%) reflected the phylogenetic distance of mammals and fish from chickens, but significant annealings were detected even under our stringent conditions (0.6 M NaCl, 68°C, S1 nuclease treatment).

***T_m* studies.** We previously reported that AEV-specific sequences (v-*erb*) are transduced from normal cell DNA (c-*erb*) and might represent a cellular gene involved in the normal hematopoietic cell differentiation process (9, 25, 31).

To establish the relatedness between cellular AEV-related sequences and viral AEV-specific sequences, we determined the T_m curve of the cDNA$_{aev}$ hybridized with the normal c-*erb* sequences in chicken DNA and the T_m curve of the cDNA$_{aev}$ hybridized with the viral AEV sequences present in the DNA of 6C2 chicken erythroblasts. In each experiment, [^{32}P]cDNA$_{rep}$ preannealed with normal chicken DNA was added as an internal standard. As shown in Fig. 5, the v-*erb* sequences and their alleged progenitor c-*erb* sequences are not totally homologous. These results are very similar to those found previously for the *src* gene of ASV (30) and the *mac*-specific sequences of MC29 and their cellular equivalents (26).

Transcription of cellular AEV-related sequences in normal cells. The RNA of unin-

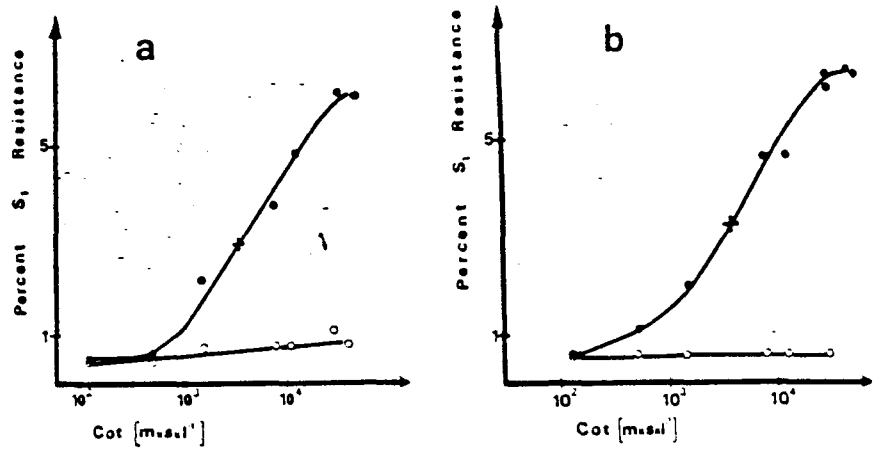

FIG. 4. *AEV-specific sequences present in the DNAs of humans (a) and salmon (b). A total of 200 μg (4 to 6S) of denatured DNA was hybridized (25 μl) with 2,000 cpm of cDNA's per point (^3H, 0.04 ng; ^{32}P, 0.005 ng) under stringent conditions (0.6 M NaCl, 68°C, S1 nuclease treatment) at increasing C₀t values to a final C₀t of 40,000 mol·s liter^{-1}. Symbols: ●, [^3H]cDNA$_{aev}$; ○, [^{32}P]cDNA$_{rep}$.*

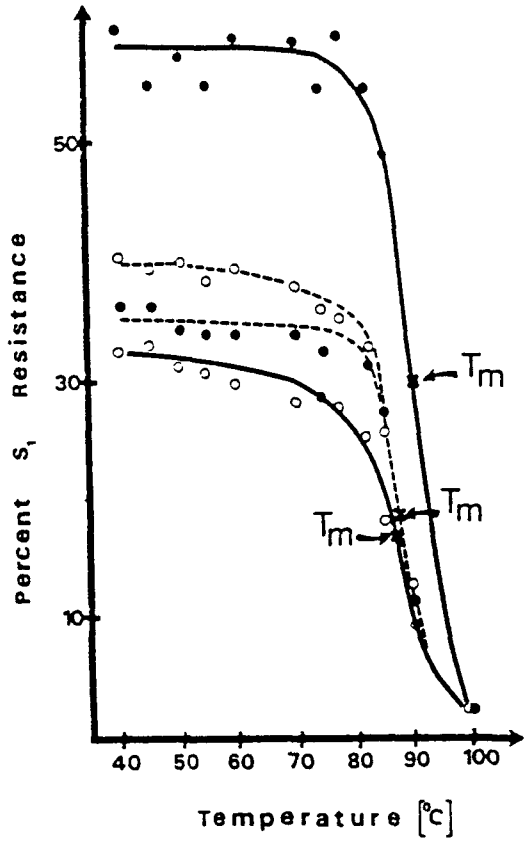

FIG. 5. *T_m profiles. The DNAs were annealed to [^3H]cDNA$_{aev}$, the T_m values were determined as described in the text, and the values were corrected for the final extent of reaction for each probe with a homologous RNA. A duplex preformed between [^{32}P]cDNA$_{rep}$ and normal chicken DNA was included in each T_m series as an internal control. Symbols: ○——○, cDNA$_{aev}$ to normal chicken DNA; ○- - -○, control; ●——●, cDNA$_{aev}$ to AEV-infected erythroblast DNA; ●- - -●, control.*

fected chicken fibroblasts contains 1 to 2 copies per cell of cDNA$_{aev}$-related sequences (25). In these experiments, the plateau value of the C$_r$t curves was near 100%, indicating that most or all of these sequences were present in the chicken DNA and transcribed. We now know that cDNA$_{aev}$ detects probably two different moieties of the specific AEV sequences corresponding to the 75 and 40K virus-coded proteins (12, 21, 36). This raised the questions of whether both domains were conserved phylogenetically and whether they were transcribed at similar frequencies. We thus conducted a similar experiment in quail.

RNA extracted from normal quail embryo fibroblasts was hybridized to different cDNA's under stringent reaction conditions. The kinetics of hybridization are shown in Fig. 6. cDNA$_{aev}$ showed extensive homology (over 90%) to the quail fibroblast RNA, with kinetics similar to those presented here for a comparison with cDNA$_{src}$. In contrast, cDNA$_{rep}$ did not hybridize to more than 5%. We thus concluded that both domains represented in cDNA$_{aev}$ are conserved in quail DNA and that both are transcribed at a frequency of 1 to 2 copies per cell in quail, as well as in chicken, fibroblasts.

DISCUSSION

Viral *erb* sequence. We have been able to prepare a cDNA (cDNA$_{aev}$) representing the specific, ALV-unrelated nucleotide sequences contained in the AEV genome. An efficient and simplified purification procedure that produces cDNA$_{aev}$ in only two selection steps, allowing an optimized yield (11% of the starting radioactivity) with no detectable residual hybridization

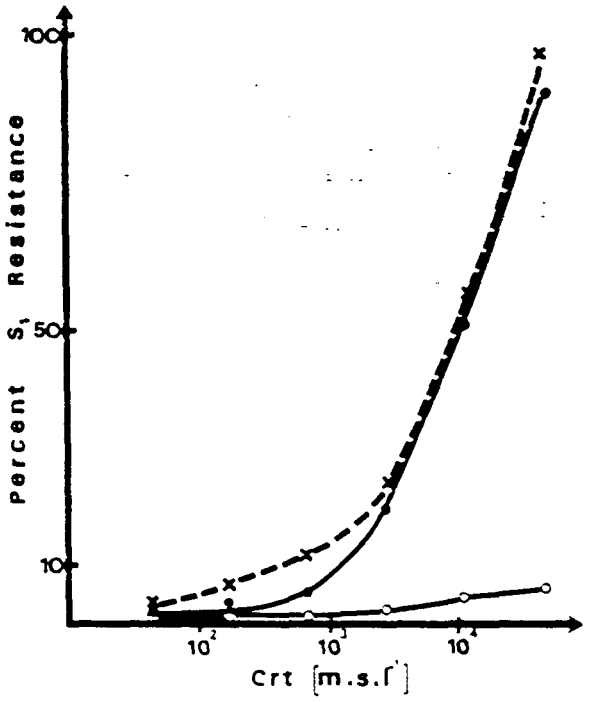

FIG. 6. *Transcription of the cellular AEV-related specific sequences in normal quail fibroblasts. Total RNA was extracted from normal quail embryo fibroblasts and hybridized (30 μl) at increasing C$_r$t values (varying the RNA concentration up to 10 mg/ml) under stringent conditions (0.6 M NaCl, 68°C) with different cDNA's (2,000 cpm each per point). The extent of hybridization was measured by S1 nuclease digestion. Symbols: ●, [^{3}H]cDNA$_{aev}$; ○, [^{32}P]-cDNA$_{rep}$; ×, [^{3}H]cDNA$_{src}$.*

with ALV RNA, has been designed.

Complexity measurements of cDNA's representing parts of viral genomes have used the protection of labeled viral RNA by cDNA excess liquid hybridization (27). Such determinations are not accurate for defective viral genomes. Indeed, the specific RNA annealed represents only a small fraction of the labeled RNA, which consists mostly of helper RNA usually in severalfold excess over the defective genomic RNA. We thus adapted the method of Young et al. (37) to deduce the complexity of the specific [^{3}H]cDNA directly from the C$_0$t$_{1/2}$ value determined in cDNA excess liquid hybridization. Under these conditions, the measure is then independent of any helper RNA excess, provided that the cDNA is driving the reaction kinetics. Such conditions were conveniently monitored by including in the reaction mixture a control cDNA ([^{32}P]cDNA$_{rep}$) made to the helper RNA by using a different radioactive label. The concentration range of viral RNA can then be carefully determined to ensure hybridization kinetics in which C$_0$t$_{1/2}$ < C$_r$t$_{1/2}$ and C$_0$t$_{1/2}$ is indicated by the ^{3}H counts and C$_r$t$_{1/2}$ is indicated by the ^{32}P counts. If such conditions are fulfilled, the plateau value of the C$_0$t curve is lower than the maximum expected under RNA excess conditions, an additional internal control for the cDNA excess requirement in such studies.

The validity of our modification was tested by determining the complexities of [^{3}H]cDNA's prepared to whole ALV RNA and also to the well-characterized *env* gene of an ALV. Measured complexities were 8.0 ± 0.8 and 2.25 ± 0.2 kilobases, respectively, values that are in agreement with published data (13, 33). The complexity of 3.7 ± 0.37 kilobases that we determined for cDNA$_{aev}$ agrees with the value of 3.25 ± 0.33 kilobases for the continuous stretch seen by Lai et al. (14) with heteroduplex mapping. Thus, the adapted method indicated here, although somehow complicated to describe but in fact easy to use, has proven to be a method of choice for determining more accurately the complexities of cDNA probes made to specific parts of defective viral genomes. It remains to be documented whether a highly unusual guanine-plus-cytosine content in such sequences would significantly modify the precision of such determinations.

Among all of the avian retroviruses tested (ALVs, ASVs, and DLVs), only AEV contains the *erb* sequences. Other viruses have been reported to induce occasionally erythroblastosis (8); thus, they must contain other transforming genes or act by a different mechanism. If other viruses that cause acute erythroid leukemias are isolated in the future, it will be of interest to determine whether their genomes show relatedness with cDNA$_{aev}$.

Two sizes of AEV-coded RNAs. Total polyadenylic acid-containing cellular RNA was tested in different types of cells by northern blotting for AEV-related sequences. RNAs from AEV nonproducer chicken fibroblasts show two distinct *erb*-related species: a 30S genomic RNA and a 23S subgenomic RNA. Such species are also found in mammalian fibroblastic cells nonproductively transformed by AEV, as well as in the RNA of chicken erythroblast clones productively or nonproductively transformed by AEV. Both species were also detected by cDNA$_{rep}$ and cDNA$_{5'}$, indicating that the 23S species probably represents a spliced viral mRNA. Surprisingly, both species are packaged efficiently into mature virions.

These results have several important implications. (i) There are two AEV-coded mRNA's in all tested AEV-transformed cells. (ii) Their sizes are identical as tested by northern blottings, indicating a similar, if not identical, processing in mammalian versus avian fibroblasts on the one hand and in hematopoietic target cells

versus nonhematopoietic cells on the other hand. (iii) The 23S species could correspond to a spliced mRNA containing the 5′ leader linked to the 3′ half of the *erb* sequence next to the Δ*env*-c-polyadenylic acid-containing ALV-related sequences seen by heteroduplex mapping (14) and in our studies (31; unpublished data). This virion-packaged mRNA is a good candidate for the AEV-specific 40K polyprotein reported by other investigators (15, 21, 36) by in vitro translation of AEV RNA, in addition to the *gag* fusion 75K polyprotein.

Additional virus-coded bands are seen with $cDNA_{aev}$ and $cDNA_{5'}$ in some infected cells. They may represent read-through transcripts into cellular sequences due to a leaky termination signal at the 3′ right-end long terminal redundancy, as recently suggested by Benz et al. (2). Such additional bands seem to be more abundant in clones producing virus (this study and our unpublished data) and could be explained by new postcloning intergrations of complete or partial viral DNA copies. The role, if any, of such bands in the transformation process of erythroblasts has not been investigated further in our studies. We also cannot exclude the alternate possibility of viral promoters inducing the transcription of some c-*erb* sequences.

c-*erb* sequence. We previously showed that normal cellular DNAs of avian and mammalian species contain nucleotide sequences (c-*erb*) related to $cDNA_{aev}$ (25, 31). A cellular origin of the c-*erb* sequences was indicated by a hybridization plateau decreasing with the phylogenetic distance from chickens to the species tested, as was found also for the cellular equivalents of the specific sequences of other transforming viruses (25, 26).

The small number of hybrids observed with nonavian DNA, though, demanded closer studies. In this paper, we showed that indeed there was a significant slope in the C_0t curve obtained with $cDNA_{aev}$ and the DNA of humans or fish (Fig. 4), indicating the presence of 1 to 2 copies of these sequences per haploid genome, as shown previously in chicken DNA (25). The important implication of these results is that it should now be possible, using $cDNA_{aev}$, to isolate human molecular clones from a human DNA library inserted in a λ phage.

It is most likely that AEV was formed by recombination of an ALV-related vector virus with the c-*erb* sequence from chickens or a species close to chickens. This is indicated by the small value of mismatching observed between the v-*erb* and the chicken c-*erb* sequences (3 to 4°C in our experiments) compared with similar experiments performed with other avian DNAs. These results are similar to those obtained previously for the *src* gene of ASV (30) and the *mac* sequences of MC29 (26) and their cellular equivalents. Such a divergence of a low percentage of bases probably reflects the increased capacity of the viruses to accumulate mutations because nonlethal mutations are efficiently selected in the viral progeny. Repeated cycles of infection and transformation could lead to the selection of mutations that would enhance the transformation activity of the *erb* gene or could extend its target cell specificity. In such a way, the structural comparison of normal cellular genes and their equivalents acquired by retroviruses could represent a suitable experimental approach to the problem of eucaryotic gene evolution.

Transcription experiments performed with total cellular RNA extracted from normal quail embryo fibroblasts and hybridized with $cDNA_{aev}$ show that most or all of the sequences of $cDNA_{aev}$ are found in the cellular RNA, although at a very low level (1 or 2 copies per cell). Similar results were obtained previously for chicken fibroblasts (25) and indicate that: (i) the c-*erb* sequences corresponding to the specific genetic information coding for both the p75 and the p40 viral polyproteins are transcribed in normal cells; (ii) similar mechanisms of transcription are operating in chicken and quail fibroblasts; (iii) these sequences are tightly controlled by the cellular machinery and kept to a low level of expression. Whether the c-*erb* sequences have any role to play in fibroblasts or in any other non-hematopoietic or hematopoietic normal cell functions remains to be shown.

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SCIENCE

Sequencing the *erb*A Gene of Avian Erythroblastosis Virus Reveals a New Type of Oncogene

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Sequencing the *erb*A Gene of Avian Erythroblastosis Virus Reveals a New Type of Oncogene

Abstract. *Avian erythroblastosis virus (AEV) contains two distinct oncogenes,* erbA *and* erbB. *The* erbB *oncogene, which is homologous to a portion of the epidermal growth factor receptor, is related to the* src *family of oncogenes and efficiently transforms erythroblasts, whereas* erbA *potentiates the effects of* erbB *by blocking the differentiation of erythroblasts at an immature stage. This "potentiator" was sequenced: the amino acid sequence deduced from it was clearly different from the sequences of other known oncogene products and was related to carbonic anhydrases. These enzymes participate in the transport of carbon dioxide by erythrocytes, the precursors of which are main targets of avian erythroblastosis virus. A* src*-related oncogene such as* erbB *in synergy with an activated specific cell-derived gene such as* erbA *can profoundly affect early erythroid differentiation.*

Two distinct oncogenes can act in synergy or complementation to transform normal cells (*1–3*). Some retroviruses contain two specific cell-derived sequences in their genome (*4–6*), probably leading to their selection as highly transforming viruses.

Avian erythroblastosis virus (AEV), a defective leukemia virus that transforms mainly erythroblasts (*7, 8*), contains two independent oncogenes, *erb*A and *erb*B (*9–14*), expressed in transformed cells from two distinct messenger RNA's (mRNA's) (*13, 15, 16*). A 5.4-kilobase (kb) mRNA codes for a cytoplasmic $P75^{gag\text{-}erbA}$ fusion protein (*17, 18*) while a subgenomic spliced 3.5-kb mRNA produces a 65 to 68-kilodalton (kD) membrane-associated glycoprotein (*19–21*). Both *erb*A and *erb*B oncogenes are ho-

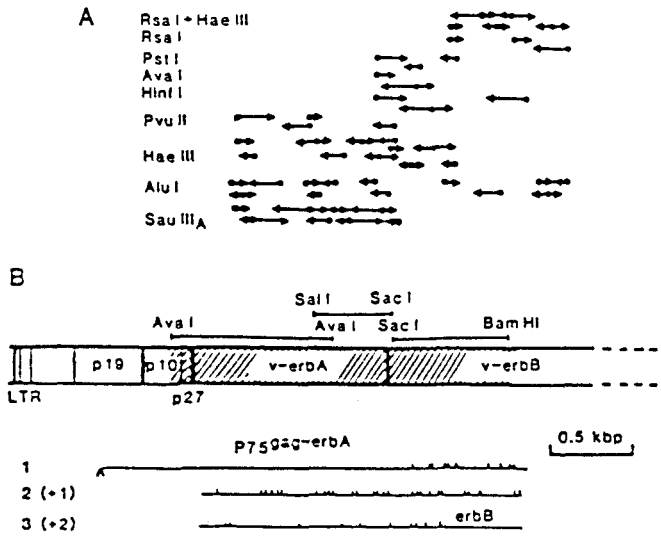

Fig. 1. (A) Sequencing strategy. The complete AEV DNA is represented linearized by Eco RI (*12*). The *erb*A sequence was obtained from three subclones, and solid bars indicate the *gag-erb* DNA inserts: 1-kbp Ava I–Ava I, 0.5-kbp Sal I–Sac I, and 0.7-kbp Sac I–Bam HI. Gel-purified restriction fragments used for DNA sequencing (restriction enzymes in the column at left) were labeled at their 5′ termini with [γ-^{32}P]adenosine triphosphate and polynucleotide kinase and sequenced by the method of Maxam and Gilbert (*31*). Closed circles indicate the cleavage points inside of each insert and horizontal arrows the direction and length of the sequenced DNA strands. (B) Reading frames in the genome of AEV. The DNA sequence was determined (hatched). The recombination of *erb*A with the viral genome occurred at the sites shown as thick vertical bars in the diagram of AEV DNA. p27 denotes the remaining coding domain for the p27 protein of the *gag* gene, which was truncated during the recombination events leading to capture of the *erb* insert. The open reading frame for $P75^{gag\text{-}erbA}$ and the two other reading frames in *erb*A are shown below the AEV DNA. A different open reading frame was detected for *erb*B. Vertical bars denote stop codons. LTR indicates a long terminal repeat.

mologous to avian and mammalian chromosomal DNA sequences (c-*erb*A and c-*erb*B) (*4*, *13*, *22*) that may play a role in the metabolism of normal cells, possibly in the differentiation of immature red blood cells (*23*).

Wild-type AEV-transformed erythroblasts are tightly blocked in their maturation (at the colony-forming unit stage) (*24*, *25*). Studies involving deletion mutants in *erb*A and *erb*B indicate that *erb*B, in vitro as well as in vivo, yields transformed erythroblast-like cells at different stages of maturation, whereas *erb*A alone induces no transformation. Thus *erb*A potentiates the transforming activity of *erb*B and appears to be responsible for the early blockage of cell differentiation within the erythroid lineage (*26–29*).

Although several oncogenes have been extensively studied, "potentiators" such as *erb*A have not been studied. Therefore we analyzed the nucleotide sequence of *erb*A. Computer analysis showed that the deduced amino acid sequence of the *erb*A stretch of $P75^{gag\text{-}erbA}$ is clearly different from the sequences of other reported transforming proteins. Thus *erb*A appears to represent a distinct new member of the oncogene families.

A molecular clone of AEV (p-AEV 11) (*30*) with biological activity was used to sequence the *erb*A oncogene by the Maxam and Gilbert procedure (*31*). Figure 1A shows the strategy used to produce the nucleotide sequence (Fig. 2) defining the *erb*A boundaries (Fig. 1) between the structural *gag* gene upstream and the second oncogene, *erb*B, downstream. The left boundary of *erb*A was assessed by comparison to the nucleotide sequence of the Prague-C strain (Pr-C) of Rous sarcoma virus (*32*). The

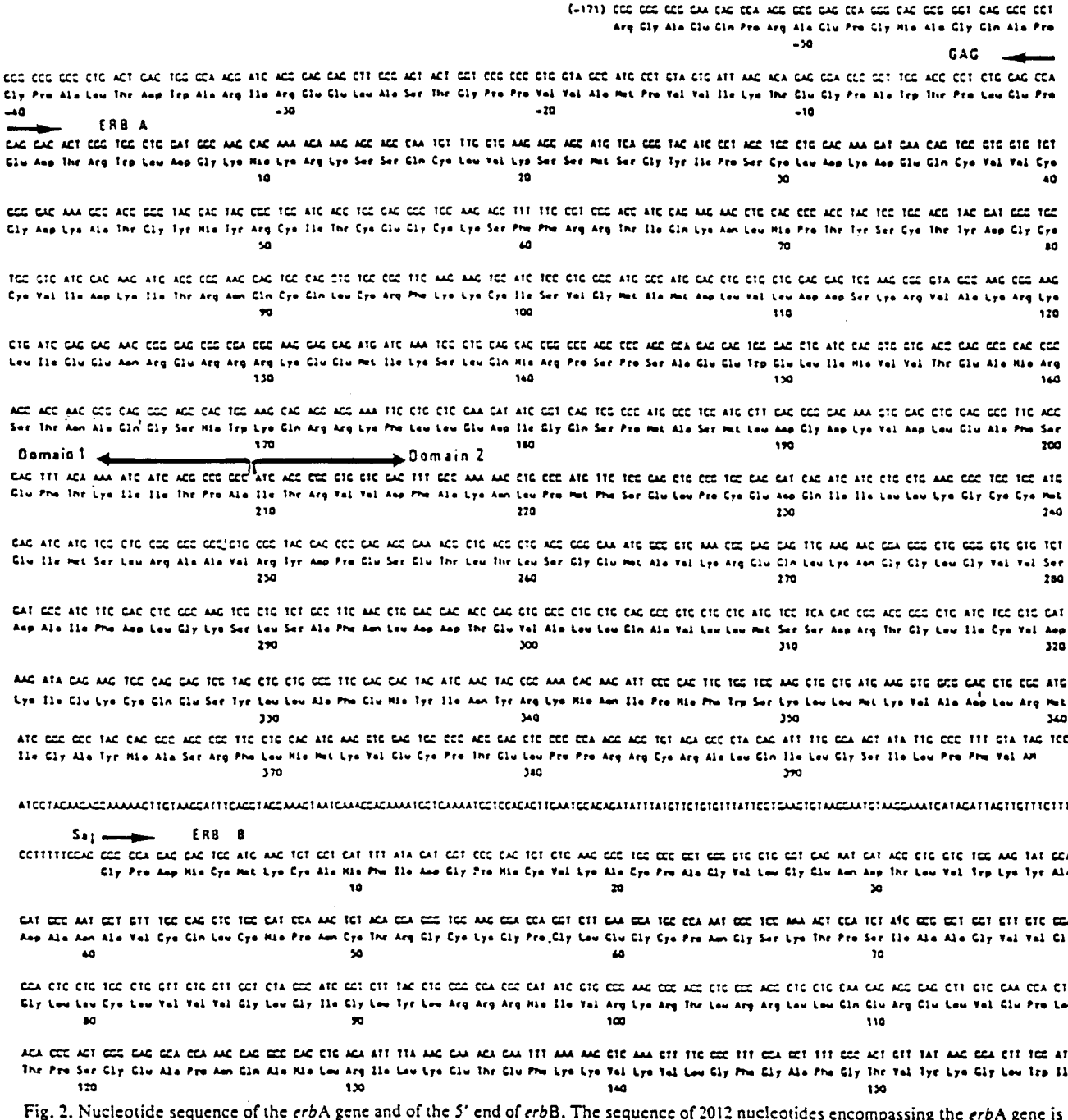

Fig. 2. Nucleotide sequence of the *erb*A gene and of the 5′ end of *erb*B. The sequence of 2012 nucleotides encompassing the *erb*A gene is shown. The deduced amino acid sequence of the *erb*A domain of $P75^{gag\text{-}erbA}$ is indicated from nucleotides 1 to 1194 and the deduced amino acid sequence of the 5′ end of *erb*B is indicated from the putative splice acceptor site (Sa, nucleotide 1370) to nucleotide 1841. Nucleotides are numbered in the right column and every tenth amino acid is numbered.

Ava I site at the extreme left of our sequence (Fig. 1) is within the coding domain for the virion protein $p10^{gag}$. The nucleotide sequences of Pr-C and AEV are almost identical from this position rightward to residue 1 in the AEV sequence, where complete divergence of the two sequences marks the point of insertion of *erb*A. This insertion is located within the coding domain for the virion protein $p27^{gag}$ (*32*).

The reading frame of *erb*A continues uninterrupted from the $p27^{gag}$ reading frame at position +1 until it is terminated by an amber codon at position 1195; this is followed by a stretch of noncoding sequences up to a putative splice acceptor (Sa in Fig. 2) site (*33*). TTTCC-TTTTTGCAG.G (T, thymine; C, cytosine; G, guanine; A, adenine) for the *erb*B gene at nucleotide 1370. It is unlikely that the other two *erb*A reading frames are used because they are frequently closed by termination codons (vertical bars in Fig. 1B). The consensus splice acceptor site at position 1370 could generate the subgenomic *erb*B mRNA in an open reading frame different from that of *erb*A, and we present the deduced partial amino acid sequence of this reading frame (Fig. 2). Thus the *erb*B product may start at the AUG (U, uracil) codon of *gag* used to produce $P75^{gag\text{-}erbA}$ (assuming that AEV and Pr-C use the same splice donor site in *gag*); the two proteins then share a few common amino acids at their NH_2 terminus. Alternatively, the *erb*B product could initiate at the in-frame AUG codon at position 1386 in our sequence (Fig. 2). The structure of *erb*B is virtually identical, in the region we studied, to the one recently described for another independent isolate of AEV, namely AEV-H, which lacks the *erb*A oncogene (*34*). Importantly, *erb*B is closely related to the *src* gene of avian sarcoma virus [(*34*) and our sequence data] and to a portion of the epidermal growth factor receptor (*35*).

The deduced amino acid sequence of *erb*A (398 amino acid residues) is shown under the nucleotide sequence in Fig. 2. The calculated molecular size of the *erb*A polypeptide is 45.4 kD, yielding for $P75^{gag\text{-}erbA}$ (*36*) a deduced molecular size of 72 kD. Cellular adenosine 3′,5′-monophosphate–dependent protein kinases phosphorylate serine or threonine residues within sequences *x*-*y*-*z*-(Ser or Thr) (*37*), where *x* and *y* are basic residues; two such serine residues are found in the *erb*A stretch at positions 14 and 15. There are no potential glycosylation sites Asn-*x*-(Thr or Ser) (*38*) in this polypeptide. Thus *erb*A may be phosphorylated but not glycosylated. Two domains can be defined within the *erb*A protein; notable features of the amino acid composition are found in the first domain of *erb*A (amino acids 1 to 209 in Fig. 2) with elevated levels of cysteine and basic residues (10 percent and 21 percent, respectively, within amino acids 1 to 131), in strong contrast with the rest of the molecule. Hydrophilicity studies with the Hopp-Woods procedure (*39*) indicate that *erb*A encodes a relatively hydrophilic product (the value for the *erb*A polypeptide is 0.1, whereas the average protein has a net hydrophilicity of 0.07), as expected for a cytoplasmic protein (*21*).

A search for similarities between the *erb*A protein and the other proteins contained in the protein data bases (*40*, *41*) revealed no obvious relationships with other oncogenes. Thus *erb*A may be a genuinely new member of this class of proteins. Domain 1 showed no salient homology with other known proteins, but a relatedness was found between the carboxyl terminal half of *erb*A (domain 2 in Fig. 2) and the carbonic anhydrase family (Fig. 3). The homology begins with *erb*A amino acid residue 219 (amino acid residue 10 for the carbonic anhydrases) and extends across 180 residues up to the carboxyl terminus of the molecule (residue 398 of *erb*A or residue 195 of the carbonic anhydrases, which continue for another 64 residues). When compared with the four mammalian carbonic anhydrases II available in the data banks we used, the overall homology was 27 percent for 180 amino acids, clustered in some portions of the molecule. For example, close to one of the known active sites of carbonic anhydrases (*42*), the homology increases to more than 51 percent (*erb*A residues 248 to 274), although the two residues reported to be important in the active site (His^{63} and Asn^{66}) (*42*) are not found in the *erb*A protein. This may imply a structural, but not a functional relationship. Another example is *erb*A residues 310 to 330, with more than 40 percent homology. This homology between the

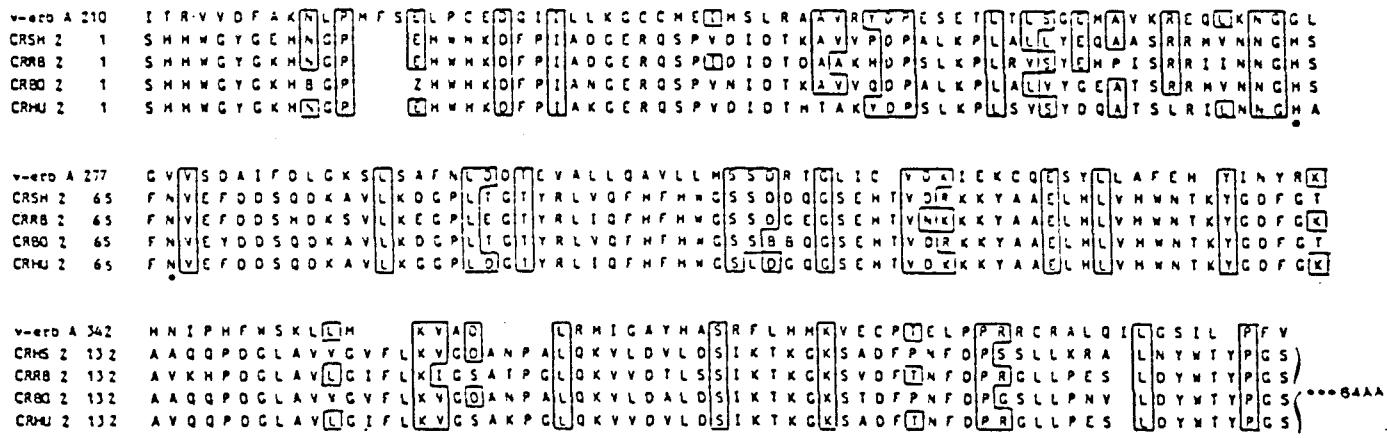

Fig. 3. Relatedness of *erb*A domain 2 to the carbonic anhydrase family. Using the sequence of the putative *erb*A polypeptide, we performed exhaustive homology searches on the NBRF (*41*) and NEWAT (*40*) protein data banks with our computer system (*47*). These searches revealed a homology encompassing 180 amino acid residues between *erb*A domain 2 and the carbonic anhydrase family. The significance of this homology was further assessed by an alignment program (*48*) and adapted by one of us (J.M.C.). The amino acid sequences of the predicted *erb*A polypeptide and of sheep (CRSH2) (*49*), rabbit (CRRB2) (*50*), bovine (CRBO2) (*51*), and human (CRHU2) (*42*) carbonic anhydrases II were compared. The optimal alignment was found when we used a deletion weight of 1 for each gap plus 0.2 times the number of residues in each gap. In this case, the Needleman-Wunsch similarity value (*52*) was found 3.8 standard deviation units above the mean of 30 shuffled sequences. Scores that are 3 or more standard deviation units above the mean can reasonably be expected to represent authentic relationships (*53*). Common residues are boxed. Closed circles indicate amino acids known to be important in the active site of carbonic anhydrases. The one-letter symbols for the amino acids are A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine; B, asparagine or aspartic acid; and Z, glutamine or glutamic acid.

*erb*A product and, for example, human carbonic anhydrase II (CRHU 2 in Fig. 3) is 3.8 standard deviation units above the mean of 30 shuffled sequences, considerably higher than the cutoff value of 3, which indicates, with statistical significance, an evolutionary relatedness (*40*) (for details of the computer program see Fig. 3). Moreover, in human carbonic anhydrase I, which has a 61 percent homology with human carbonic anhydrase II, most of the homologies depicted in Fig. 3 are conserved (not shown). Although *erb*A domain 2 is related to carbonic anhydrases, we showed that it is not the enzyme itself. Using a viral *erb*A DNA probe corresponding to domain 2, we screened both chicken and human genomic DNA libraries and found that normal DNA in both species contained a similar locus. The human cellular *erb*A domain 2 was cloned, and a nucleotide sequence was determined (corresponding to nucleotides 675 to 936 in the *erb*A domain 2 of Fig. 2). Homology at the nucleotide level was 83 percent, and the deduced amino acid sequence in the corresponding open reading frame showed 96 percent homology (the two other reading frames were closed by stop codons) (*43*). The homologies of the human *erb*A domain 2 with human carbonic anhydrase remained but were not augmented. Thus the *erb*A gene is related to but not identical to known carbonic anhydrases.

In conclusion, AEV is an unusual retrovirus in that it can specifically block the maturation of erythroid cells at an immature stage of differentiation. This is achieved by the synergistic action of two distinct oncogenes, *erb*A and *erb*B. The *erb*B product is a membrane glycoprotein (*20*, *21*) that is homologous to a portion of the epidermal growth factor receptor (*35*) and shares extensive homology with the *src* oncogene family [(*34*) and our results]. This latter homology is also functional, since both *erb*B in the absence of *erb*A and other *src* family viral genes such as *src* or *fps* (*44*) can transform erythroblasts (*28*). Such cells require complex growth conditions and partially differentiate into mature erythrocytes in vitro (*28*). In contrast, *erb*A in combination with *erb*B is capable of arresting erythroid leukemic cells at an early stage of differentiation where they are able to grow in simple tissue culture media unsuitable for normal erythroid precursors (*45*, *46*).

The finding that the deduced *erb*A protein bears statistically significant relatedness, but not identity, with carbonic anhydrases is interesting because such enzymes play a fundamental role in the CO_2 transport by erythrocytes, the precursors of which are precisely the main targets of AEV.

Our finding of this new type of oncogene leads to the idea that some *src*-related oncogenes found—with the exception of AEV—to be single transforming genes in retroviruses and to transform mainly fibroblasts can affect early hematopoietic differentiation when acting in synergy with a specific activated cell-derived gene such as *erb*A.

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The Four C-Terminal Amino Acids of the v-*erbA* Polypeptide Are Encoded by an Intronic Sequence of the v-*erbB* Oncogene

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The genome of avian erythroblastosis virus (AEV), a defective acute leukemia retrovirus, carries two distinct cell-derived oncogenes in the structure 5'Δgag-*erbA*-*erbB*-Δenv3'. The nucleotide sequence of the v-*erbA* gene was recently reported. In order to determine the boundary between the two adjacent oncogenes, the sequence of the v-*erbA*/v-*erbB* junction of AEV was compared to that of a recombinant lambda phage containing a chicken cellular sequence representing the 5' part of c-*erbB*. The four C-terminal amino acids of v-*erbA* are in fact encoded by a c-*erbB* intron-derived sequence thus demonstrating that the virus acquired a truncated c-*erbA* gene. Furthermore the 7 to 10 amino acid residues upstream from the 4 C-terminal amino acids mentioned above appeared to be derived from *env*-related sequences. The splice acceptor site at the beginning of the only open reading frame for v-*erbB* is also present and functional in c-*erbB* when expressed to generate a truncated EGF (epidermal growth factor) receptor. Thus AEV joins a truncated *erbA* gene to a truncated *erbB* gene through *env*-derived sequences and intronic sequences from c-*erbB*.

Avian erythroblastosis virus is a defective leukemia virus (DLV) which induces both erythroblastosis and fibrosarcomas in chickens (*1*). The genome of this virus contains two independent cell-derived oncogenes v-*erbA* and v-*erbB* (*2, 3*) expressed in transformed cells as two distinct mRNAs (*4–6*). A genomic 5.4-kilobase (kb) mRNA codes for a cytoplasmic 75,000-molecular weight (MW) fusion protein ($P75^{gag\text{-}erbA}$) (*7*) while a subgenomic spliced 3.5-kb mRNA produces a v-*erbB* membrane glycoprotein of 74,000 MW (*8, 9*). Recently, the v-*erbB* sequence (*10*) was identified as a portion of the EGF receptor (*11*). We have sequenced the v-*erbA* oncogene (*12*) and shown that the carboxy-terminal half of its product exhibited a significant homology with the carbonic anhydrase family. This homology begins with v-*erbA* amino acid residue 219 (amino acid residue 10 for the carbonic anhydrases) and spans ca. 180 residues toward the carboxyterminus of the molecule (residue 398 of v-*erbA* corresponding to residue 195 of the carbonic anhydrases, which continue for another 64 residues (*12*)). We also compared the nucleotide sequence of v-*erbA* to the sequences of avian viral structural genes. We found a significant homology (82%) between the end of v-*erbA* (nucleotides 1151 to 1183) and a portion of the *env* gene of Rous sarcoma virus (nucleotides 5804 to 5836) (Ref. (*13*) and Figs. 1A and B). However, the stop codon for the $P75^{gag\text{-}erbA}$ fusion protein (TAG at position 1195) did not belong to this stretch of homology.

In order to determine the exact boundary between the two oncogenes, we sequenced part of the 2.5-kbp *Eco*RI fragment of a previously described recombinant lambda phage (NO4 c-*erbB* in Ref. (*2*)) containing the cellular chicken sequences corresponding to the upstream region of v-*erbB* and we compared this sequence to the homologous region of v-*erbB*. The relevant nucleotide sequences are shown in Fig. 1A and allow the fol-

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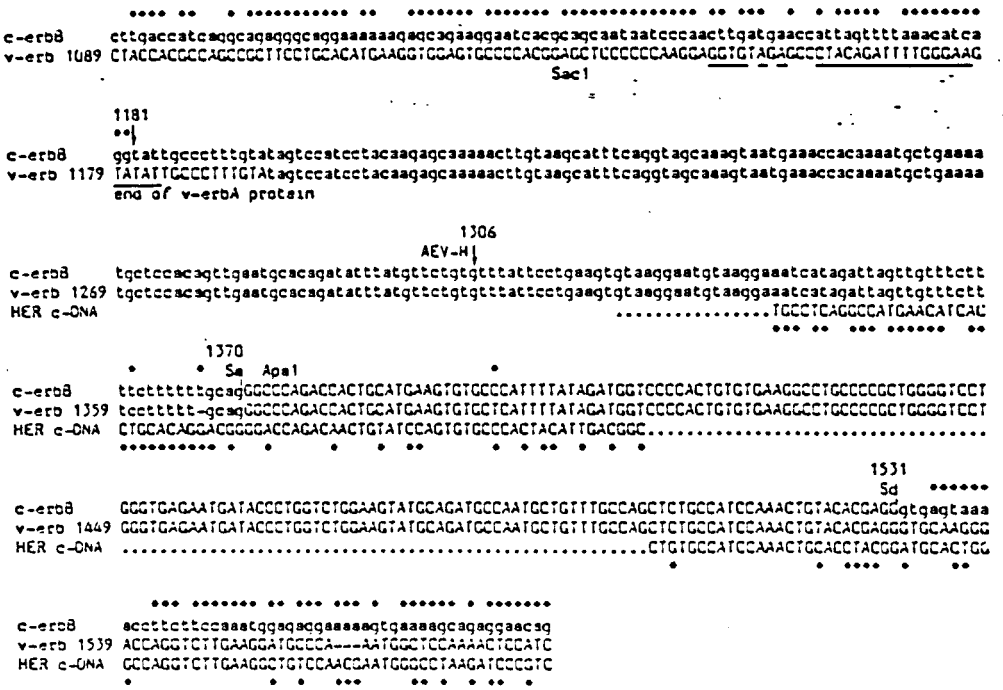
A



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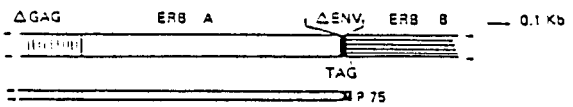

FIG. 1. (A) Nucleotide sequence of the 5' part of the chicken c-*erbB* locus: comparison to the v-*erb* (*12*) and human EGF receptor (HER) cDNA sequences (*11*). The c-*erbB* sequence was determined by the Maxam and Gilbert procedure (*17*). Coding sequences are written in capital letters and noncoding sequences in small letters. Numbers refer to v-*erb* nucleotides as in Ref. (*12*). Sa and Sd demarcate the splice acceptor and splice donor sites at the extremities of the c-*erbB* exon. Asterisks mark the differences between the cellular and the viral *erb* sequences (upper line) and between the v-*erb* and human EGF receptor cDNA nucleotide sequences (*11*) (lower line). AEV-H indicates the recombination site between c-*erbB* and an erythroblastosis virus described in Ref (*10*). The arrow shows the recombination point between *erbA* and *erbB* in AEV. Endonuclease restriction sites used to produce the probes described in Fig. 2 are indicated. Underlined nucleotides are homologous to *env* nucleotides of RSV (*13*). (B) Schematic representation showing the presence of *env*-related sequences between v-*erbA* and v-*erbB*.

lowing observations to be made. First, the homology between v-*erbB* and c-*erbB* begins at nucleotide 1181 in the viral sequence (Ref. (*12*) and Fig. 1A); this defines the upstream boundary of the c-*erbB*-related sequences in AEV. Surprisingly the four C-terminal amino acids of $P75^{gag\text{-}erbA}$ are encoded by c-*erbB*-derived nucleotides which also provide the termination codon of this protein (nucleotides 1195-1197) indicating that AEV acquired a truncated c-*erbA* locus. Second, the viral

sequences between nucleotides 1181 and 1370 correspond to part of an intron of c-*erbB*, closed in all three reading frames by numerous stop codons and ending with the conserved splice acceptor site TTTTCTTTTTTGCAG/G (Sa in Fig. 1A). This splice acceptor site is used to generate the subgenomic v-*erbB* mRNA since the latter did not hybridize on Northern blots (Fig. 2, lane 1) with a *Sac*I-*Apa*I probe (restriction sites indicated in Fig. 1A) derived from AEV DNA (nucleotides 1139 to 1374 of v-*erbB*) (*12*). As shown as a control in Fig. 2 lane 2, this mRNA hybridized with a *Sac*I-*Bam*HI probe (nucleotides 1139 to 1841 of v-*erbB*) (*12*). The splice acceptor site described above is also likely to be used during the maturation of the c-*erbB* transcript. Nucleotide 1371 of v-*erbB* corresponds to the beginning of a strong homology with the human EGF receptor cDNA (nucleotide 1918 in Ref. (11)). The c-*erbB* exon beginning with the splice acceptor site at position 1370 ends with the splice donor site G/GTGAG (*14*) at position 1528, since downstream from this site the c-*erbB* sequence diverges from that of both v-*erbB* (downstream from position 1531 in Fig. 1A) and the EGF receptor cDNA (downstream from nucleotide 2067 in Ref. *11*)). We concluded that the v-*erbB* gene is a truncated version of c-*erbB*, starting within an intron at the 5' end. These observations favor the idea that the recombination events involved to generate v-*erbB* occurred at the DNA level. How and when AEV acquired the *erbA* gene remains to be determined: *erbB* before *erbA*, *erbA* before *erbB*, or both together from a cell having undergone a translocation joining the two genes prior to their transduction by the virus? The homology detected between the end of v-*erbA* and the *env* gene of RSV suggests that two successive recombination events led to the capture of the *erb* genes by AEV. In fact, a possible mechanism leading finally to the c-*erbB* capture by an ALV (avian leukosis virus) could result from the upstream insertion of a LTR (long terminal repeat) as reported by Fung *et al.* (*15*). Such an activation would have generated an *erbB*-containing retrovirus as recently described by Yamamoto *et al.* (*10*) for AEV-H. A second recombination event at the DNA or RNA level would occur leading to the capture of *erbA*.

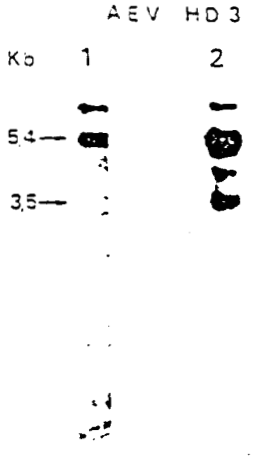

FIG. 2. Sizes of AEV-coded RNAs in AEV-transformed chicken erythroblastes HD3 (described in Ref. (*13*)). Poly(A)-containing RNA was denatured (*19*), separated on agarose gels, and transferred to nitrocellulose. Blots were hybridized with a ^{32}P-nick-translated fragment isolated from AEV proviral DNA (*20*). Lane 1, *Sac*I-*Apa*I fragment; lane 2, *Sac*I-*Bam*H1 fragment, extending largely into v-*erbB*.

Finally a salient feature of c-*erbB* activation linked to transformation appears to involve the c-*erbB* exon described here, that encodes the carboxyterminal half of the EGF receptor (*16*). Indeed the three independent *erbB*-activated oncogenes in AEV (this paper), in AEV H (*10*), and in an LTR activated c-*erbB* chicken leukemia (*15*) all concern insertions (although at different sites, see Fig. 1) clustered in the intron preceding the c-*erbB* exon sequences presented in this paper. Ullrich *et al.* (*11*) proposed that the truncated EGF receptor may escape cell regulation by loosing its EGF binding site and thus lead to cell transformation. Whether such recombinations are facilitated by a privileged domain contained within this intron remains to be documented. In any case, our observations together with the ones just quoted here, allow to predict that most c-*erbB* activations (by viral genomes, translocations, etc.) might involve recom-

bination events in the intron mentioned above.

ACKNOWLEDGMENTS

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II- DEUXIEME PARTIE: STRUCTURE DES C-ONC

Les résultats présentés dans la 1ère partie nous ont permis de caractériser 4 oncogènes au sein des DLV. Ces oncogènes dérivent de c-onc phylogénétiquement stables. Dans cette deuxième partie des résultats, nous présentons des travaux réalisés dans le but de définir la structure de ces gènes cellulaires afin de construire des expériences permettant d'accéder à leur fonction .

Les articles N°9 et N°10 rapportent le clonage moléculaire et l'analyse de la structure des deux gènes cellulaires c-erbA et c-erbB. L'oncogène c-erbB (article N°9) a été cloné à partir d'une banque d'ADN génomique de poulet (Dodgson et al.,1979) à l'aide de sondes moléculaires spécifiques du gène v-erbB. 6 phages contenant des séquences d'ADN se recouvrant, nous ont permis de reconstituer la structure du c-erbB de poulet homologue au gène viral. Nous avons vérifié à l'aide de la technique deSouthern (l'ADN digéré par des enzymes de restriction est séparé selon sa taille après migration dans un gel d'agarose, transféré sur un support solide et hybridé avec une sonde radioactive), que la structure de ce gène se retrouvait dans l'ADN cellulaire, et n'était pas un artéfact de clonage. La réalisation d'hétéroduplex entre des molécules d'ARN du virus AEV et l'ADN phagique ont permis de définir les structures introniques (formant des boucles de non appariement entre ADN et ARN) et exoniques (structures double brin). Toutefois, cette définition est faite par référence à l'ARN viral, et certains exons peuvent être différents dans l'ARN cellulaire. Par exemple, la séquence nucléotidique (article N°8) du premier exon de c-erbB défini par cette technique montre que l'ARN viral contient 180 nucléotides d'intron et que le premier "exon" de 280 nucléotides ne contient en fait que 100 nucléotides codants. Ainsi, comme v-myb (cf article 12), v-erbB ne correspond qu'à une portion (les douze derniers exons) du gène codant pour le récepteur de l'EGF.

La structure du gène c-erbA est beaucoup plus complexe, et nous l'avons analysée chez le poulet et chez l'homme. L'article N°10 montre que l'ADN génomique contient en fait deux gènes extrêmement proches de v-erbA. Un locus contenant tout v-erbA a pu être identifié dans les deux espèces (domaine 1, erbA5' + domaine 2, erbA3') ainsi qu'un locus ou nous n'avons pu isoler que le domaine 2. La séquence nucléotidique du fragment d'ADN cellulaire humain et aviaire contenant le domaine 2 révèle une classique structure exon-intron .

Ces deux domaines pourraient représenter deux domaines fonctionnels de la protéine, le domaine 1 permettant sa fixation sur l'ADN, le domaine2 permettant l'interaction avec les hormones thyroïdiennes (Sap et al.1986 ; Weinberger et al.1986). Le locus c-erbA humain complet (D1+D2) présente la particularité de posséder une inversion de l'ADN homologue au D2 viral (Figure 25). Cette inversion est étonnante car la séquence nucléotidique du fragment D2 du locus humain révèle que le seul cadre de lecture possible dans cette séquence est le même que celui utilisé par le virus. Or, ce locus (à cause de son inversion) est incapable de diriger la synthèse d'une protéine erbA du type de celle présente dans le virus et responsable du blocage de différenciation des érythroblastes infectés (Graf et Beug, 1983, Figure 13). Ainsi, si la protéine cellulaire erbA (D1 + D2) a la même fonction *in vivo* (notamment faire proliférer les cellules érythroïdes immatures) que la protéine virale, un mécanisme d'inversion de l'ADN doit se produire dans la cellule adéquate pour placer D1 et D2 dans le même sens de lecture. Une étude attentive des

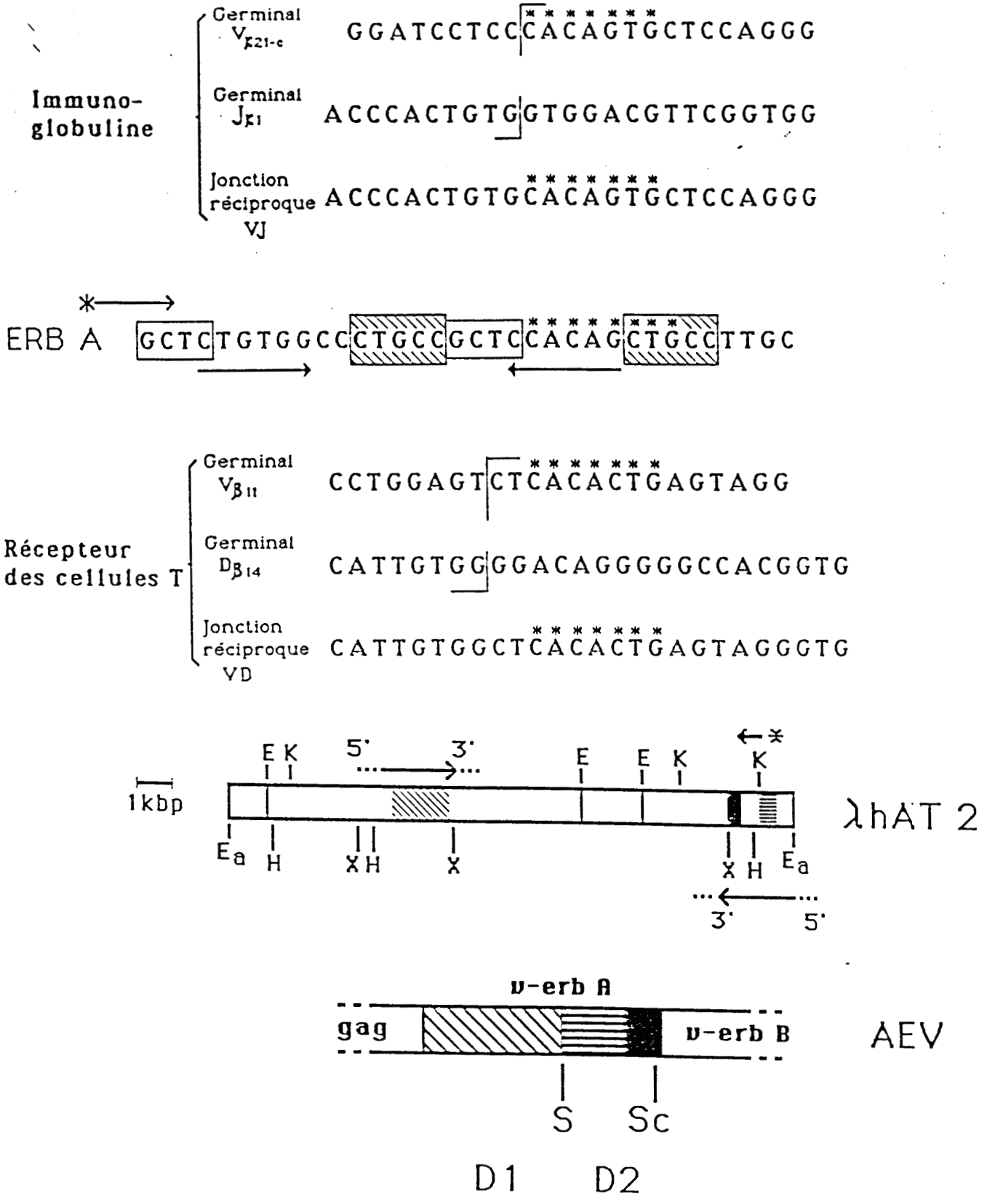

Figure 25 : Structure du gène ERB A.

nucléotides situés au point de retournement supposé de D2 (premiers nucléotides non codants) révèle le motif CACACGTG. Or, les motifs CACAGTG et CACACTG sont trouvés respectivement au point de réarrangement des fragments V et J (Jλ1) des gènes d'immunoglobulines (Hope et al., 1986) et au point d'inversion du fragment Vß14 codant pour une région variable de la chaine ß du récepteur des lymphocytes T (Malissen et al., 1986). Ces données suggèrent qu'un enzyme cellulaire pourrait prendre en charge le clivage de l'ADN à ce site, et un mécanisme d'inversion du type de celui décrit dans le retournement de la région V du gène codant pour les récepteurs de lymphocytes T pourrait replacer D1 et D2 dans une configuration de type v-erbA (Figure 25). L'étude de la structure de l'ADN apparenté à ce locus erbA dans l'ADN humain par la technique de Southern montre que tous les types cellulaires que nous avons étudiés (placenta, lymphomes de Burkitt, cellules érythroïdes K562 provenant d'une leucémie myéloïde chronique, leucémies érythroblastiques très immatures, cellules d'une lignée de tératocarcinome humain) contiennent la forme germinale du c-erbA (D1+D2) telle que nous l'avons clonée.

Nous ne connaissons pas la situation du gène c-erbA dans l'ADN de poulet car cette région a toujours résisté aux tentatives de clonage moléculaire. Si l'hypothèse que nous faisons (le produit du c-erbA (D1 + D2) est important pour la prolifération et/ou la différenciation des cellules immatures normales) est correcte, c-erbA serait un gène de différenciation et l'inversion de fragment d'ADN, l'un des mécanismes utilisés par la cellule pour contrôler l'activation de ce type de gènes.

L'article N°11 rapporte le clonage moléculaire et la caractérisation de l'oncogène c-myb dans l'ADN de poulet et l'ADN humain. Les résultats obtenus montrent que cet oncogène possède également la structure éclatée caractéristique des gènes eucaryotes. La différence de taille considérable entre la séquence v-myb (800 nucléotides) et l'ARN épissé du gène c-myb (3.8 kb chez l'homme et 4.0 kb chez le poulet) suggère que le virus n'a recombiné qu'avec une partie seulement de ce gène. Cette amputation est peut-être essentielle dans l'activation du pouvoir transformant de ce gène, car E26 (qui a également recombiné, de façon indépendante avec c-myb) contient la même région de ce gène (Leprince et al., 1983).

ARTICLES

9 à 11

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Molecular cloning and characterization of the chicken DNA locus related to the oncogene *erb*$_B$ of avian erythroblastosis virus

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Chicken cell DNA contains sequences which are homologous to the avian erythroblastosis virus oncogene *v-erb*. These cellular sequences *(c-erb)* have been isolated from a library of chicken cell DNA fragments generated by partial digestion with *Alu*I and *Hae*III and shown to be shared by at least two loci in the chicken DNA. One of them, denoted *c-erb*$_B$, contains ~1.8 kilobase pairs of chicken DNA homologous to the 3' part of the *v-erb* oncogene (*v-erb*$_B$). Restriction mapping studies show that the *c-erb*$_B$ DNA sequences homologous to *v-erb*$_B$ are distributed among six *Eco*RI fragments located in a single genomic region. Heteroduplexes between *v-erb*$_B$ viral RNA and cloned *c-erb*$_B$ DNA show that the chicken DNA sequences homologous to *v-erb*$_B$ are interrupted by 11 DNA sequences not present in the *v-erb* oncogene. We conclude from our data that the *c-erb*$_B$ locus might represent the cellular progenitor for the *v-erb*$_B$ domain of the *v-erb* oncogene.

Key words: viral oncogene/cellular oncogene/DNA cloning/restriction analysis/heteroduplex mapping

Introduction

Recent progress in retroviral oncology supports the theory that some cancers could be due to DNA rearrangements that bring cellular genes under the control of highly active transcription promoters (Klein, 1981). Moreover, it has been shown that the retroviral oncogenes, *v-onc*, are homologous to "normal" genes of their host cells, *c-onc*, which may or may not be interrupted by intervening sequences (Stéhelin *et al.*, 1976, Roussel *et al.*, 1979, Goff *et al.*, 1980, Jones *et al.*, 1980, Francini *et al.*, 1981, Shalloway *et al.*, 1981). For example, the transforming gene of the avian sarcoma viruses *v-src*, which contains an uninterrupted coding sequence of 1590 bp (Czernilofsky *et al.*, 1980b), is homologous to chicken DNA sequences interrupted by five or six intervening sequences (Shalloway *et al.*, 1981), whereas the cellular gene *c-mos*, homologous to the transforming gene of Moloney sarcoma virus *v-mos*, is colinear with the viral sequences (Jones *et al.*, 1980). Experimental data suggest that the retroviral oncogenes represent cellular genes that play essential roles in cell growth and/or differentiation (Beug *et al.*, 1979, Stéhelin *et al.*, 1980). If such genes evade cell regulation and are continuously expressed at a high level their products might change the growth and/or affect the differentiation programme of the cell and lead directly or indirectly to malignant transformation. This occurs in retrovirus-transformed cells where the cell-derived viral oncogenes, stably integrated in the host genome, are continuously transcribed under the control of a viral promoter, at a level 30–100 times higher than their cellular progenitors (Shank *et al.*, 1978, Czerlinofsky *et al.*, 1980a). This also occurs in some avian leukosis virus (ALV) induced lymphomas where the "non-transforming" ALV integrates immediately upstream from the cellular counterpart *c-myc* of the viral oncogene *v-myc*, providing this cellular gene with a promoter (present in the viral large terminal repeat) and increasing its transcription by a factor of 30 to 100 (Hayward *et al.*, 1981). Finally, a constructed vector consisting of the *c-mos* gene flanked by viral sequences necessary for replication, integration, and transcription induced a transformed phenotype in target cells into which it was transfected (Oskarsson *et al.*, 1980).

That the cellular progenitors of viral oncogenes are important for cell growth regulation and differentiation is particularly well documented by biological and biochemical studies on defective avian leukaemia viruses (DLV). These viruses contain oncogenes called *v-erb*, *v-myc*, and *v-myb* (Roussel *et al.*, 1979) which transform and block the differentiation of specific haematopoietic target cells *in vitro* (Graf and Beug, 1978; Graf *et al.*, 1978; Beug *et al.*, 1979). Again these viral oncogenes seem to be derived from normal cellular genes that could code for specific proteins involved in the control of haematopoietic differentiation (Graf and Beug, 1978).

Avian erythroblastosis virus (AEV) is a DLV that induces erythroleukaemia with a short period of latency and transforms both avian fibroblasts and avian erythroblasts in culture (Graf *et al.*, 1976). The AEV viral oncogene *(v-erb)*, is homologous to avian and mammalian chromosomal DNA sequences *(c-erb)* (Saule *et al.*, 1981). In the AEV RNA, *v-erb* is flanked by sequences isogenic to the ALV *gag* and *env* genes (Lai *et al.*, 1979; Saule *et al.*, 1981; Vennström *et al.*, 1980) (Figure 1) and, in all AEV-transformed cells tested, it is transcribed into two mRNAs with lengths of 6 and 3.2 kb. The 6-kb RNA species is a genomic-size mRNA whereas the 3.2-kb species, which contains ~2 kb from the 3' half of *v-erb*, might be generated by splicing of the 6-kb RNA (Figure 1) (Sheiness *et al.*, 1981; Saule *et al.*, 1981). These two mRNAs probably encode two different proteins. The 6-kb RNA codes for a p75 gag-erb fusion polyprotein (Hayman *et al.*, 1979), while *in vitro* translation studies suggest that the 3.2-kb mRNA could code for a p40 erb protein, unrelated to the p75 protein (Figure 1) (Lai *et al.*, 1980; Pawson and Martin, 1980). Accordingly, it has been proposed that the AEV oncogene may contain two functional domains, *v-erb*$_A$ and *v-erb*$_B$, acquired from two different cellular progenitors (Sheiness *et al.*, 1981; Saule *et al.*, 1981). Thus, *v-erb*$_A$ can be defined as the domain of *v-erb* coding for the erb part of p75, i.e., ~1 kb of the 5' part of *v-erb*. The *v-erb*$_B$ domain is defined by the sequences present in the 3.2-kb mRNA species, i.e., ~2 kb of the 3' half of *v-erb* (Figure 1).

This paper describes our attempts to characterize the chicken DNA counterpart of *v-erb*. In chicken DNA frac-

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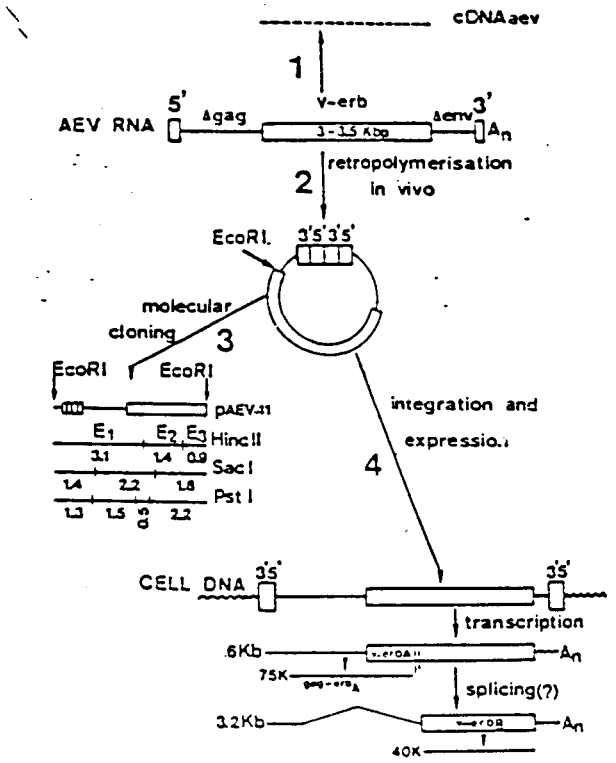

Fig. 1. Schematic representation of AEV replication cycle and molecular cloning. 1: The preparation of $cDNA_{aev}$, specific for *v-erb* has been extensively described (Saule *et al.*, 1981). 3: The circular AEV proviral DNA has been cloned in λgt.WES.B. by Vennstrom *et al.* (1980), and then recloned in the *Eco*RI site of PBR 313 (pAEV-11). The 1.8-kbp *Sac*I and the 2.2-kbp *Pst*I AEV DNA fragments are respectively 7-kbp and 8-kbp long when the total plasmid DNA is digested by *Sac*I or *Pst*I.

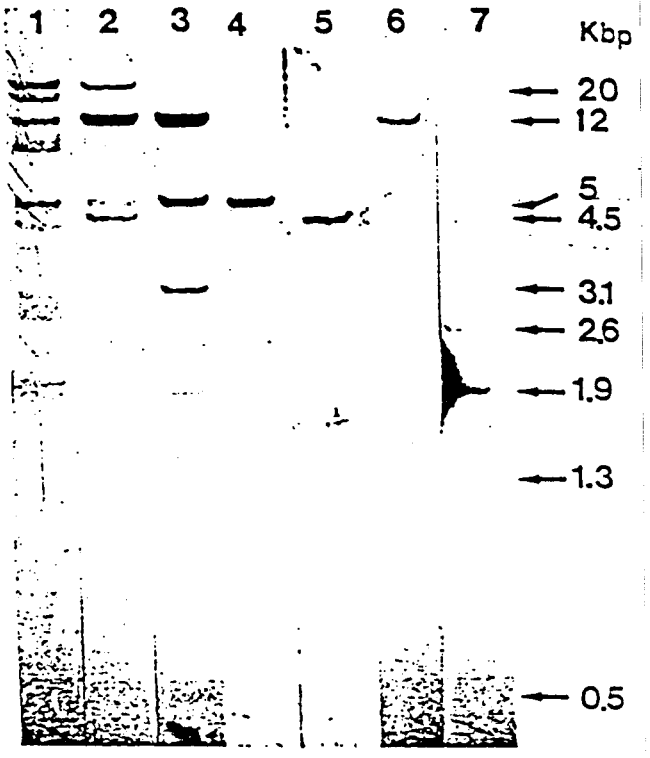

Fig. 2. Hybridization of chicken fibroblast *Eco*RI DNA fragments with radiolabelled *v-erb* and *c-erb* probes. Chicken fibroblast HMW DNA wa digested with *Eco*RI. The size-separated *Eco*RI DNA fragments were tra ferred to nitrocellulose and hybridized to the following probes: lane 1. E probe; lane 2. E_2 probe; lane 3. E_3 probe; lane 4. $cDNA_{rep}$; lane 5. NO 2.3-kbp DNA fragment as probe (NO4); lane 6. 13i 4.3-kbp DNA fragment as probe; lane 7 . 13i 1.9-kbp DNA fragment as probe.

tionated after *Eco*RI digestion, several distinct fragments could be detected with radioactive probes specific to *v-erb*. Six of these *Eco*RI fragments are located in one locus, $c\text{-}erb_B$, spanning >21 kilobase pairs (kbp). Heteroduplex studies showed that the $c\text{-}erb_B$ locus contained 1.8 kbp of DNA homologous to $v\text{-}erb_B$, interrupted by 11 regions not homologous to *v-erb*. The 20-kbp fragment containing sequences homologous to $v\text{-}erb_A$ is not reported here.

Results

c-erb is discontinuous in chicken DNA

As shown previously, $cDNA_{AEV}$ anneals to DNA isolated from normal chicken cells with a Cot ½ value indicating that there is 1 or 2 full complements of the *v-erb* oncogene per haploid genome (Saule *et al.*, 1981). This agrees with the presence of most eukaryotic genes in non-repetitive DNA. We first determined the restriction pattern of the *c-erb* sequences homologous to *v-erb* by hybridizing an *Eco*RI digest of chicken chromosomal DNA with different ^{32}P-labelled proviral DNA probes. These probes, described in Figure 1, were called E_1, E_2, and E_3 according to their polarity on the AEV proviral DNA. As shown in Figure 2, the E_3 probe, containing 0.9 kbp from the 3' part of $v\text{-}erb_B$, hybridized to *Eco*RI fragments of 0.5, 1.3, 1.9, 3.1, 5, and 12 kbp. The E_2 probe containing 1.4 kbp in the middle of *v-erb* (end of erb_A + start of erb_B) detected only *Eco*RI fragments of 4.5, 5.0, 12, and 20 kbp. The several *Eco*RI fragments detected by the E_1 probe cannot be explained by the presence of sequences homologous to the *gag*, *env*, and LTR sequences of ALV hybridizing to *Eco*RI fragments of the endogenous chick virus RAV-O. Indeed, a cDNA of the *gag*, *pol*, and *env* ge of an ALV ($cDNA_{rep}$), (Saule *et al.*, 1982) only detected 5-kbp *Eco*RI DNA fragment. Thus, the numerous ban revealed by the E_1 probe probably reflect the presence, in t distal 5' part of *v-erb*, of sequences repeated in the chick genome. Therefore, from the results described above, it a peared that ~3 kbp of the *v-erb* sequences span >44 kbp the cell DNA, indicating a split structure for *c-erb*.

Isolation of recombinant phages containing c-erb

To probe the organization of the *c-erb* sequences screened a library of chicken cell DNA generated by par digestion with *Alu*I and *Hae*III, using a $cDNA_{AEV}$ pr (Dodgson *et al.*, 1979). We selected several phages hybrid ing with $cDNA_{AEV}$, and screened them for the presence *Eco*RI DNA fragments identical in size and genetic conten those found in the *Eco*RI digest of chicken chromoso DNA and described above. After digestion of the clo DNAs with *Eco*RI, the DNA fragments were separated agarose gel electrophoresis, transferred to nitrocellulose hybridized with different radiolabelled probes. As show Figures 3A and 3B, we isolated six different overlapp clones (NO, 3CB, 3CA, JK, 13i, 10A) containing only si the chromosomal *Eco*RI fragments detected by the E_2 an probes. The order of the *Eco*RI DNA fragments in each c is presented in Figure 3C, and part of the strategy emplo to establish such maps is shown in Figure 3B and Figur None of the DNA fragments hybridized with $cDNA_{rep}$ shown). Probes E_2 and E_3 hybridized to a 12-kbp fragme

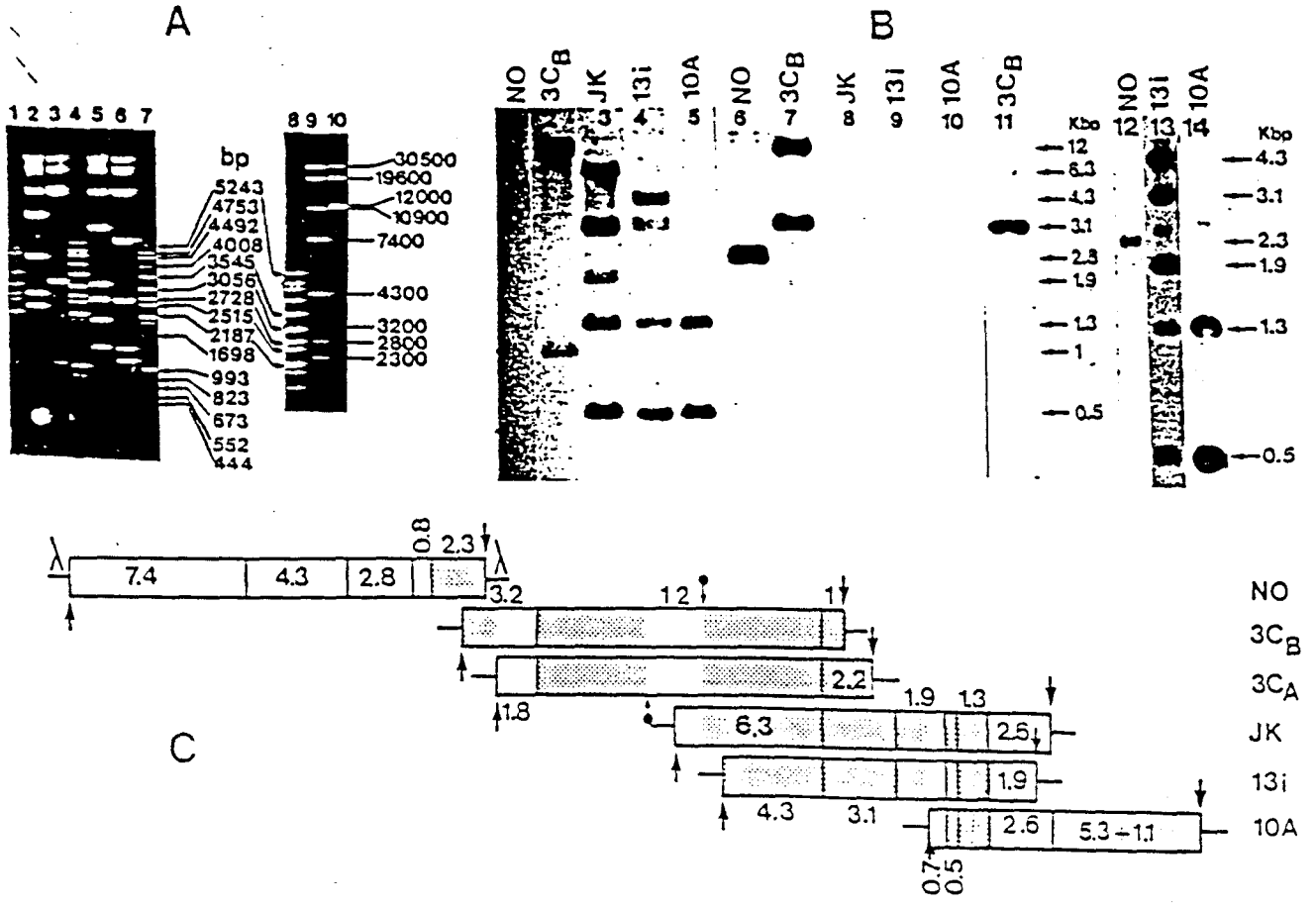

Fig. 3. Map of the chicken $c\text{-}erb_B$ locus. DNA from recombinant phages was isolated, digested with *Eco*R1, subjected to agarose gel electrophoresis, stained with ethidium bromide, blotted to nitrocellulose filters, and hybridized to specific radiolabelled probes. (A) U.v. visualization of *Eco*RI DNA fragments. Lanes 1,4,7 and 8, size marker DNA fragments obtained after digestion of SV40 DNA with different restriction enzymes; Lanes 2 and 9, clone NO DNA. Lanes 3 and 10, clone 3CB DNA. Lane 5, clone JK DNA. Lane 6, clone 10A DNA. Lanes 8,9, and 10 show a better separation between the 10.9-kbp λ-Charon 4A DNA fragment and the 12-kbp DNA fragment of clone 3CB. (B) The *Eco*RI DNA fragments separated as in (A) were hybridized to the following probes: E_1 probe (lanes 1,2,3,4,5); E_2 probe (lanes 6,7,8,9,10); NO4 probe (lane 11); $cDNA_{AEV}$ (lanes 12,13,14). (C) Restriction maps of the chicken DNA sequences cloned in the six λ-Charon 4A recombinant phages. Restriction sites are identified as follows: | *Eco*RI site in the *Eco*RI DNA linker; ⊐ *Eco*RI DNA fragments; ▲ *Kpn*I site. The shaded regions on the maps indicate that the corresponding sequences hybridized with *v-erb* specific probes. Lengths of DNA fragments are indicated in kbp.

cell DNA, showing that some sequences in these probes were adjacent in the clones. Clones NO and 3CB overlap within the 4.5-kbp *Eco*RI fragment of chicken DNA. This was shown by the fact that a radiolabelled probe made with the 2.3-kbp fragment of NO (NO4) hybridized only with the 3.2-kbp *Eco*RI fragment of clone 3CB (Figure 3B) and the 4.5-kbp *Eco*RI fragment of chicken DNA (Figure 2). Similarly, the link between clones 3CB and 13i was shown by hybridization of the ^{32}P-labelled 4.3-kbp DNA fragment of clone 13i to the 12-kbp *Eco*RI fragment of the chicken DNA (Figure 2) which is also present in clone 3CB. Lastly, the link between clones JK, 13i, and 10A was shown as follows. The two comigrating 1.9-kbp DNA fragments of clone 13i labelled with ^{32}P revealed two *Eco*RI DNA fragments of 1.9 kbp and 2.6 kbp in the chicken DNA (Figure 2) which are also present in clones JK and 10A. It is clear from the maps that the six genomic *Eco*RI fragments containing *c-erb* sequences are located in one locus, flanked by cellular sequences at least 15.3 kbp upstream and 9 kbp downstream which do not hybridize with $cDNA_{AEV}$ (Figure 3B, lanes 12,13,14) or $cDNA_{rep}$ (not shown). As will be discussed later, the 5-kbp band detected by both E_2 and E_3 probes had no counterpart in the cloned DNA fragments covering the *c-erb* locus studied. Also, the 20-kbp *Eco*RI fragment detected by the E_1

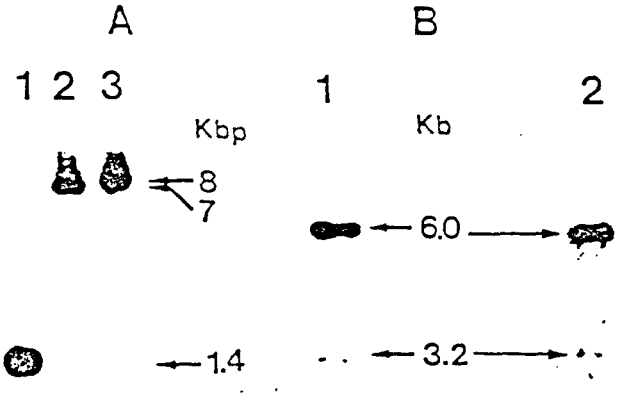

Fig. 4. Localization of the 5′ end of the $c\text{-}erb_B$ locus on $v\text{-}erb_B$. (A) pAEV-11 DNA was digested with *Hin*dI (lane 1), *Sac*I (lane 2), and *Pst*I (lane 3). The DNA fragments were separated by agarose gel electrophoresis, blotted to nitrocellulose, and hybridized with the radiolabelled NO 2.3-kbp DNA fragment as probe (NO4 probe). (B) Polyadenylated RNAs extracted from chicken erythroblasts transformed by AEV (RAV-2) were denatured with glyoxal, separated by agarose gel electrophoresis, bound to DBM paper, and hybridized with $cDNA_{AEV}$ (lane 1) and the NO4 probe (lane 2).

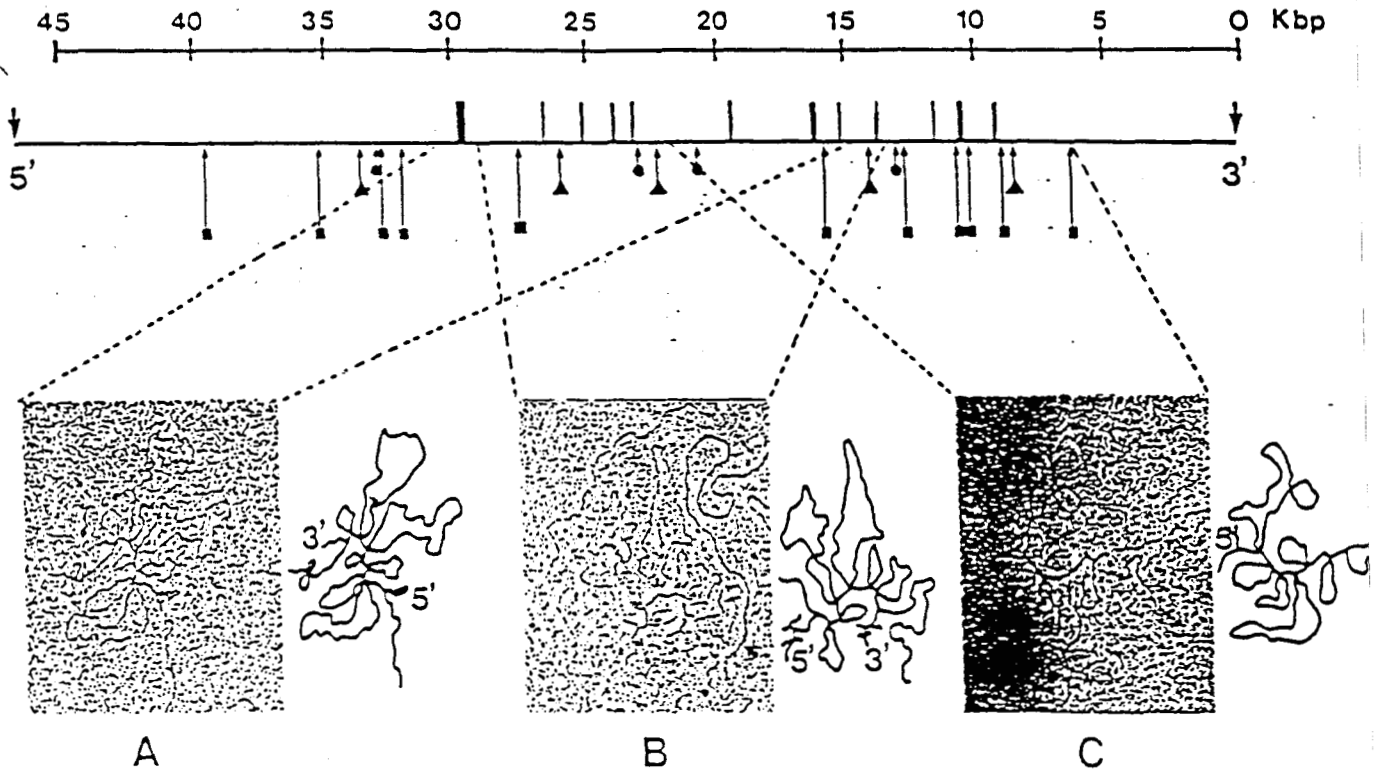

Fig. 5. Heteroduplex between viral *erb* and chicken *c-erb* cloned DNA. The entire restriction map of the *c-erb*$_B$ chicken locus has been established from results presented in Figure 3 and Figure 2. Heteroduplexes were formed between the clones 3CB (A), 3CA (B) and JK (C) covering the entire *c-erb*$_B$ locus and AEV viral RNA. The 12 DNA segments forming heteroduplexes are indicated as the above vertical bars on the restriction map where the restriction s are identified by the arrows below as follows: ■ *Eco*RI sites; ● *Kpn*I sites; ▲ *Bam*HI sites. The 5' and 3' termini of the AEV RNA are indicated c the schematic representation of heteroduplexes.

and E_2 probes on the chicken DNA blot could not be found in any of the phages isolated from the library.

To locate where the 5' part of *c-erb* maps on *v-erb*, the DNA of clone AEV-11 cut by different restriction enzymes (*Hin*cII, *Sac*I, *Pst*I, see Figure 1) was hybridized with the [^{32}P]NO4 DNA fragment (Figure 4A). The NO4 probe hybridized with the DNA fragments *Hin*cII, 1.4 kbp, *Sac*I, 7 kbp, and *Pst*I, 8 kbp. Thus, the NO4 fragment containing the 5' part of *c-erb* maps at ~2 kbp from the 3' end of the *v-erb*$_B$ domain. We then hybridized the NO4 probe to cellular polyadenylated RNA extracted from erythroblasts transformed by AEV (RAV-2). As shown in Figure 4B, the NO4 sequences were present in the 3.2-kb subgenomic AEV RNA. Accordingly, the *c-erb* sequences homologous to *v-erb*$_B$ were called *c-erb*$_B$.

The c-erb$_B$ *sequences are split in 12 "exon-like" regions*

To examine further the organization of the *c-erb*$_B$ sequences homologous to *v-erb*$_B$ in the 21-kbp long locus in chicken chromosomal DNA, we allowed heteroduplexes to form between the DNA of genomic clones and the RNA isolated from virions released by AEV-transformed erythroblasts (cell clone 6C2). As shown by the hybrid regions formed between the viral RNA and the cloned DNAs 3CA, 3CB, and JK covering the entire *c-erb*$_B$ locus (Figure 5), the *v-erb*$_B$ sequences homologous to *c-erb*$_B$ formed 12 small double-stranded regions separated by 11 non-homologous regions that appeared as single-stranded loops on the micrographs (Figure 6). Length measurements indicate that the duplex regions comprise ~1.8 kbp and the non-homologous sequences covered ~19 kbp (Table I). By comparing the heteroduplex measurements and the restriction enzyme coordinates (Figure 3C), we generated the map shown in Fig where the 12 DNA fragments forming heteroduplexes placed relative to the restriction enzymes sites.

Table I. Length of heteroduplex segments between λ-Charon 4A *c-e* clones and AEV RNA

	Double stranded segments	*n*	Internal loops		
a	280 ± 30	9	1	2900 ± 100	1
b	50 ± 10	10	2	1500 ± 100	1
c	140 ± 30	21	3	1100 ± 120	2
d	120 ± 20	20	4	620 ± 50	2
e	100 ± 15	20	5	3600 ± 200	2
f	170 ± 30	33	6	3100 ± 260	3
g	150 ± 30	34	7	800 ± 200	2
h	75 ± 16	33	8	1200 ± 110	2
i	170 ± 25	24	9	2200 ± 200	1
j	90 ± 25	15	10	800 ± 200	1
k	250 ± 45	15	11	1100 ± 100	1
l	160 ± 20	12			
Total	1755 ± 100			18920 ± 200	

The double stranded segments and internal loops notation refer to Fig 6. The lengths in kilobases ± s.d. represent the averages of measurem of *n* heteroduplex molecules. Single- and double-stranded φX174 or f DNA were used as internal standards.

Discussion

We have isolated recombinant phages containing six o several *Eco*RI DNA fragments detected in the chi

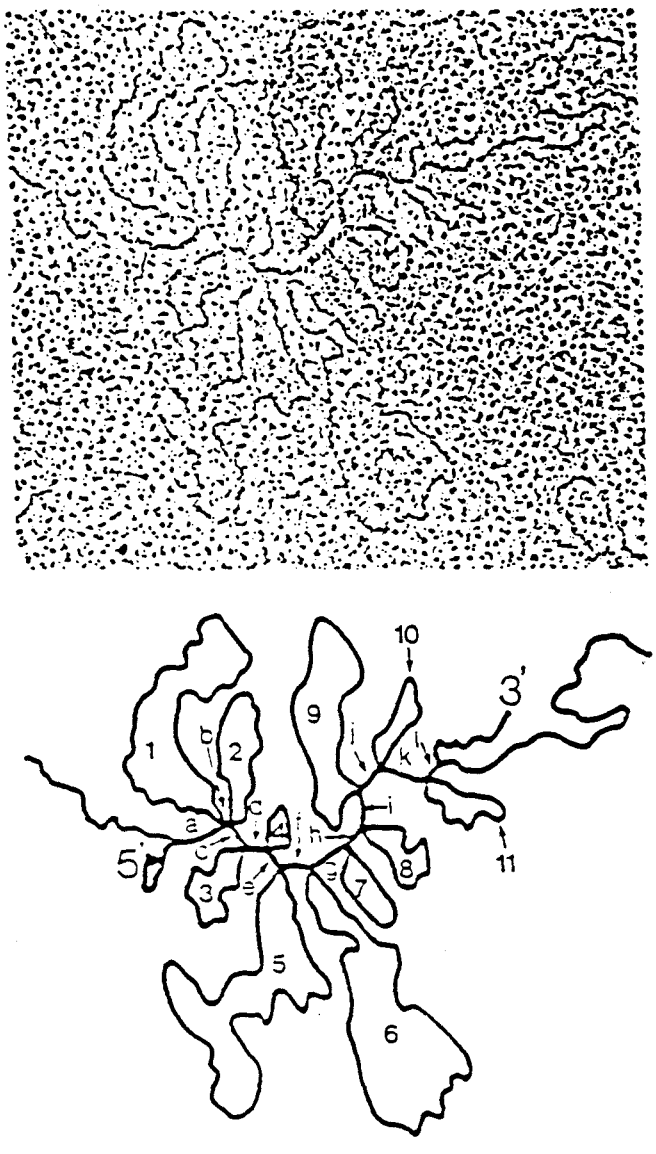

Fig. 6. Summary of the heteroduplex studies. This electron micrograph was artificially constructed from the heteroduplex-micrographs presented in Figure 5 to allow a clear visualization of homologous (letters a to l) and non-homologous (numbers 1 to 11) regions whose lengths are presented in Table 1.

chromosomal DNA by *v-erb*-specific probes. These six *Eco*RI fragments of 0.5, 1.3, 1.9, 3.1, 4.5, and 12 kbp are contiguous in one genomic locus and are flanked by cellular DNA sequences (15.3 kbp at the 5′ end and 9 kbp at the 3′ end) which do not hybridize to any of the *v-erb* probes used or to $cDNA_{rep}$. Restriction mapping studies and heteroduplex analysis with viral RNA and cloned DNA showed that ~1.8 kbp of chicken DNA in the $c\text{-}erb_B$ locus are homologous to the 3′ half of *v-erb* ($v\text{-}erb_B$) and are interrupted by 11 non-homologous regions ranging in size from 600 bp to 3600 bp. The organization of the $c\text{-}erb_B$ locus appears to be similar to many eukaryotic genes which contain coding sequences (exons) interrupted by non-coding sequences (introns). However, it would be imprudent to use the term exon to describe the $c\text{-}erb_B$ sequences homologous to $v\text{-}erb_B$, because the homologies between $v\text{-}erb_B$ and $c\text{-}erb_B$ have only been defined by heteroduplex studies. However, no substitution loops were visible on the electron micrographs, suggesting that there was no large region of non-homology between $v\text{-}erb_B$ and $c\text{-}erb_B$. That does not rule out the possibility of a small insertion in $v\text{-}erb_B$, non-homologous to $c\text{-}erb_B$, which would appear as a small single-stranded loop in the heteroduplexes.

It may indeed be possible that some of the $v\text{-}erb_B$ sequences overlapping in the E_2 and E_3 probes are non-homologous to the $c\text{-}erb_B$ sequences. These probes detect a 5-kbp *Eco*RI DNA fragment in the chicken DNA that was not found in the $c\text{-}erb_B$ locus. This raises the following interesting hypothesis: the 5-kbp DNA fragment could contain sequences (in one or several pieces) that have been inserted independently into the AEV $v\text{-}erb_B$ domain, at a site overlapping the boundary between E_2 and E_3. It follows that in the DNA-RNA heteroduplex mapping studies, such an insert would have appeared as a single-stranded loop of viral RNA instead of chicken DNA. An argument in favour of our hypothesis is that such a loop of 0.6 kb is present in the heteroduplex micrograph of clone 3CB (and 3CA) (Figure 5) that maps precisely around the *Hin*cII site between the fragments E_2 and E_3 of viral DNA. The 5-kbp fragment detected by both E_2 and E_3 probes could also be due to a deletion in the 12-kbp *Eco*RI fragment in one of the alleles of the chicken DNA tested. It could also be that this fragment belongs to a pseudogene (Vanin *et al.*, 1980). Work is in progress to isolate from the chicken library the phage containing the *Eco*RI 5-kbp fragment, in order to be able to explore these hypotheses further. Part of the $c\text{-}erb_A$ locus situated in the 20-kbp *Eco*RI fragment of chicken DNA and containing sequences homologous to $v\text{-}erb_A$ has been cloned by Vennström and Bishop (1982). Again, in this locus, 750 bp of chicken DNA homologous to $v\text{-}erb_A$ are interrupted by three non-homologous regions.

Our results, together with those obtained by others, suggest that the $c\text{-}erb_B$ locus might be a cellular gene, the progenitor of $v\text{-}erb_B$: (a) it has been shown that $cDNA_{AEV}$ detects homologous sequences in the DNA of all vertebrate cells including human cells (Roussel *et al.*, 1979; Saule *et al.*, 1981), with a hybridization plateau decreasing with the phylogenetic distance from chickens to the species tested, as was shown also for the cellular equivalents of the "c-onc" of other avian transforming viruses (Roussel *et al.*, 1979); (b) the absence of complete or defective proviruses of an endogenous avian virus in the vicinity of the $c\text{-}erb_B$ locus rules out the possibility that $c\text{-}erb_B$ was introduced into the chicken genome by infection by a related avian ancestor; (c) the presence of intervening sequences that split $c\text{-}erb_B$ into 12 pieces (see also Vennström and Bishop, 1982) is analogous to the organization of many eukaryotic split genes. A similar organization has been demonstrated for the cellular *src* gene (Shalloway *et al.*, 1981) and for one of the two Harvey sarcoma virus oncogene *v-ras* cellular homologues (Defeo *et al.*, 1981); and (d) $c\text{-}erb_B$ is transcribed in chicken cells as mRNAs of sizes 12.0 and 9.0 kb (Vennström and Bishop, 1982).

It seems that AEV originated by recombination of an ALV-related virus with cellular sequences (genes) from different loci in the chicken genome. It remains to be determined if the two domains, $v\text{-}erb_A$ and $v\text{-}erb_B$, of the oncogene generated are required for transformation of avian cells by AEV.

Materials and methods

Cells, viruses, and cloned DNA fragments

AEV-transformed erythroblasts (6C2) were obtained through the courtesy of T.Graf and H.Beug. The AEV-11 clone was kindly provided by J.B. Bishop and the chicken DNA fragment library by J.Dodgson.

Isolation of chicken erb-DNA-containing recombinant phages

The library of random chicken cell DNA fragments prepared in λ-Charon 4A (Dogdson *et al.*, 1979) was screened with a [^{32}P]cDNA specific for the *v-erb* sequence of AEV ($cDNA_{AEV}$) which was synthesized and selected as published recently (Saule *et al.*, 1981). Several individual overlapping clones were isolated as representative of one chicken locus.

Preparation of DNAs

Normal chicken fibroblast high molecular weight DNA (HMW DNA) was prepared by incubating chicken primary fibroblasts overnight at 37°C in Tris 0.01 M, pH 7.4, NaCl 0.15 M, EDTA 0.001 M, SDS 0.1% and proteinase K 100 μg/ml (10^7 cells/ml). The digest was then brought to a density of 1.7 by adding CsCl (2.545 g/2 ml) and centrifuged for 70 h at 15°C in a Beckman 50Ti rotor. HMW DNA was recovered through a large hole in the tube wall, and dialysed extensively against Tris 0.005 M, pH 7.4 and EDTA 0.001 M.

Recombinant phage DNAs were prepared as described above from phage particles grown and purified as published (Saule *et al.*, 1982).

Preparation of ^{32}P-specific DNA probe

Representative specific radiolabelled $cDNA_{AEV}$ and $cDNA_{erb}$ were synthesized in exogenous reactions using 50S–70S viral RNA templates, calf thymus DNA-primer and purified avian myeloblastosis virus polymerase (Saule *et al.*, 1981). The specificity and the complexity of these probes have been extensively described (Saule *et al.*, 1981).

Probes specific for defined regions of the *v-erb* sequence were prepared by nick translation, in the presence of [^{32}P]CTP, of DNA fragments E_1, E_2 and E_3 (Figure 1) originating from the AEV-11 clone digested by *Eco*RI and *Hin*cII (Vennstrom *et al.*, 1980). The DNA fragments were purified twice by electrophoresis in 0.8% agarose gels prior to the nick translation reaction. The specificity of these probes have also been extensively described (Vennstrom *et al.*, 1980; Sheiness *et al.*, 1981).

Restriction mapping

Restriction endonucleases were obtained from Boehringer (Mannheim, FRG) and BRL Inc. (USA). The DNA digestions were performed according to the supplier's instructions. DNA fragments obtained after restriction endonuclease digestion were separated by electrophoresis in 0.8% agarose horizontal gels and transferred to nitrocellulose as previously described (Saule *et al.*, 1982). The Southern blots were hybridized to specific [^{32}P]cDNAs, washed, dried, and submitted to autoradiography.

Isolation of AEV genomic RNA

AEV-transformed producer erythroblasts (cell line 6C2) were grown and AEV virions were isolated together with the helper RAV-2 virions, from 11 of culture medium and resuspended in 2 ml of Tris-HCl, pH 8, NaCl 0.1 M, EDTA 0.001 M (buffer A) containing 0.5% SDS and 100 μg/ml of proteinase K. After 15 min of incubation at 37°C, the digest was extracted twice with an equal volume of phenol saturated with buffer A. The RNA in the aqueous phase was precipitated overnight at −20°C by 2 volumes of ethanol. The pellet, recovered after centrifugation for 30 min at 15 000 r.p.m. (Sorvall SS34 rotor), was dissolved in Tris-HCl 0.02 M pH 7.4, 0.5 M NaCl, 0.01 M EDTA, and 0.2% SDS (buffer B) at 50°C. Poly(A)-containing RNAs were hybridized to 0.2 ml of oligo-dT cellulose (T3, Collaborative Research) equilibrated in buffer B at 50°C. The oligo-dT cellulose was washed extensively with buffer B at the same temperature and the poly(A)-containing RNA was eluted with 0.2 ml volumes of Tris-HCl 0.001 M pH 7.4 and EDTA 0.001 M (3 times) at room temperature. After addition of a one tenth the volume of sodium acetate 2 M, the RNAs were precipitated overnight at −70°C by adding three volumes of ethanol. The RNA pellet obtained after centrifugation for 30 min at 15 000 r.p.m. was resuspended in 10 μl of water. The RNAs were routinely analysed by agarose gel electrophoresis after glyoxal treatment (McMaster and Carmichael, 1977). Intact RNA (30S) preparations were kept at −70°C in 2 μl samples.

Agarose gel electrophoresis of poly(A)-containing cellular RNAs

Poly(A)-containing cellular RNAs were prepared as described (Verma, 1978). RNA samples were denatured by glyoxal treatment (McMaster and Carmichael, 1977) and submitted to electrophoresis in 1% agarose horizontal gels submerged in phosphate 0.02 M, pH 6.8.

Transfer-hybridization of RNAs

Size-separated RNAs were transferred to diazobenzyloxymethyl (DBM) paper prepared as described by Alwine, 1977. The Northern blots were hybridized with ^{32}P-labelled DNA probes, washed, and submitted to autoradiography as already described by Saule *et al.* (1982).

Heteroduplex analysis

The AEV viral RNA prepared as described above was hybridized to intact recombinant phage DNA under the high formamide conditions (Casey and Davidson, 1977). AEV RNA and λ recombinant DNAs (1 μg/ml, 1:1 molar ratio) were denatured at 75°C for 10 min in 70% formamide, Tris-HCl 0.02 M pH 8.5, NaCl 0.3 M, and EDTA 0.001 M. Samples of 10 μl were immediately transferred to a 54°C water bath and further incubated for 3 h. After incubation, 5 μl samples were immediately prepared for spreading onto a hypophase of water. The sample was either brought to 70% formamide or 50% formamide and 4 M urea, in 0.1 M Tris-HCl, pH 7.5, EDTA 0.01 M, and 50 μg/ml of cytochrome c. φX174 DNA (5386 bp) or fd DNA (6389 bp) was mixed with the samples before spreading and used as internal length markers. Molecules were picked up on 300 mesh copper grids covered with a thin carbon film or with parlodion and stained for 30 s maximum with uranyl acetate. The grids were then shadowed and observed as published (Saule *et al.*, 1982).

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The 3' half part of the cellular erbA oncogene is inverted in the human genome.

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ABSTRACT

We have previously determined the complete nucleotide sequence of the v-erbA oncogene of Avian Erythroblastosis Virus (AEV). Two domains can be defined within this gene and one of them, the 3' half of the gene (domain 2) is related to the carbonic anhydrase gene family.

DNA sequences complementary to the v-erbA oncogene have been identified in human and chicken DNA libraries. The isolated human clones were divided into two distinct groups: one, λhAT, contained the total v-erbA related sequences, but exhibited an inversion of the 3' region relative to the viral polarity, while the other only contained the 3' half part of the v-erbA sequences and was designated λhAP. Only the partial c-erbA locus (λcAP), which reresents a c-erbA related locus, was cloned in the chicken library. Hybridization analysis and sequencing data revealed that the human c-erbA inverted segment corresponds to the v-erbA-domain 2 sequence.

INTRODUCTION

Some avian retroviruses contain one or two oncogenes homologous to avian and mammalian chromosomal DNA sequences which may play an important role in the metabolism of normal cells (Roussel et al., 1979; Coll et al., 1983a; Leprince et al., 1983; Vennström and Bishop, 1982). AEV which induces erythroblastosis and sarcomas in chickens (Graf and Beug, 1978) is interesting in that respect since it carries two host cell-derived genes denoted v-erbA and v-erbB (Vennström and Bishop, 1982; Sergeant et al., 1982). In the human genome these genes are on two separate chromosomes ; c-erbA has been assigned to chromosome 17 and c-erbB to chromosome 7 (Spurr et al., 1984). In infected cells the provirus is transcribed into two distinct mRNAs. A genomic-length mRNA directs the synthesis of a 75,000 molecular weight (MW) cytoplasmic fusion protein containing both retroviral gag and erbA encoded sequences (Hayman et al., 1979). Sequencing of the v-erbA oncogene revealed that two domains can be defined within the erbA polypeptide (Debuire et al., 1984). The first domain (N-terminal half) is homologous to the human glucocorticoid receptor (Weinberger et al., 1985) and to the human estrogen receptor (Green et al., 1986; Greene et al., 1986). The second domain (C-terminal half) exhibits a relatedness with the carbonic anhydrase family (Debuire et al., 1984). A subgenomic mRNA encompassing the v-erbB gene codes for a membrane associated glycoprotein of 74,000 MW (Schmidt et al., 1985).

Erythroblasts infected by AEV are blocked in their maturation at the CFU-E stage (Samarut and Gazzolo, 1982). The molecular cloning of AEV (Vennström et al., 1980) and the construction of deletion mutants in v-erbA or v-erbB made possible the identification of the gene involved in the blockage of erythroid differentiation (Frykberg et al., 1983). v-erbA^{+}B^{-} mutants lack detectable transforming activity while v-erbA^{-}B^{+} mutants still transform both erythroblasts and fibroblasts. However erythroid cells infected by v-erbA^{-}B^{+} mutants, in contrast to AEV transformed CFU-E, differentiate spontaneously and grow only under culture conditions similar to those that promote the differentiation of normal erythroid cells *in vitro* (Graf and beug, 1983). From these data it appears that the v-erbA product is involved in the control of erythroid differentiation. This prompted us to study the structure of the c-erbA genes assuming that they could also be involved in the control of normal erythroid differentiation. We have isolated several molecular clones containing chicken and human DNA sequences complementary to the v-erbA gene. Hybridization and sequencing analysis of these clones showed that the chicken and human genomes contain a complete c-erbA locus (λhAT in human) as well as an independent locus (λcAP and λhAP respectively) corresponding to the 3' half part of the gene (domain 2 of v-erbA). Surprisingly, in λhAT, the phage containing the complete human c-erbA locus, the 3' half part of the gene (domain 2) was found in an inverted orientation compared to the v-erbA polarity.

Southern blot analysis of human genomic DNA indicated that this peculiar structure was found in all the human cells tested. Therefore the inversion of v-erbA-domain 2 related sequences in the human genome leads us to speculate on the possible rearrangement of this gene (according to a mechanism observed in human DNA for immunoglobulins (Lewis et al., 1984) and T-cell receptors (Baltimore, 1986)) to generate a c-erbA product, similar to the one found in AEV transformed cells,

and this inversion may be involved in the control of erythroid differentiation.

MATERIALS AND METHODS

Isolation of chicken and human erbA DNA containing recombinant phages

The library of random chicken DNA AluI-HaeIII fragments prepared in Charon 4A (Dogdson et al., 1979) was screened with ^{32}P DNA specific probes for the v-erb sequence of AEV (probes 1, 2 and 3 depicted in figure 1) (Sergeant et al., 1982). Two human DNA libraries (one DNA digested by EcoRI, the other digested by AluI-HaeIII) prepared in Charon 4A vector (Lawn et al., 1978) were screened with the same probes. Several individual overlapping clones were isolated as representative of the chicken λcAP and human λhAT and λhAP loci.

Preparation of DNAs

DNA was prepared from K562, a human transformed erythroid cell line (Lozzio and lozzio, 1975) and LY 47 a human lymphoid cell line (Bernheim et al., 1981). Placental DNA was obtained from healthy donors, (Sergeant et al., 1982). DNA prepared from chicken cells RP9 and MSB1, cells of lymphoid origin, and 0K10 BM cells of myeloid origin were described in Coll et al., (1983b). AEV transformed erythroblasts and normal erythrocytes from the same chicken were a generous gift of Dr T.Graf.

Cloned DNA probes

DNA fragments for the preparation of ^{32}P probes were obtained from suitable recombinant plasmids by enzyme digestions and agarose gel electrophoresis. The 2.6 kbp BamHI fragment represented the entire v-erbA gene and part of the v-erbB gene was isolated from AEV provirus cloned in pKH47 (Vennström et al., 1980). The 0.8 kbp AvaI fragment corresponds to v-erbA-domain 1 (Debuire et al., 1984); the 0.49 kbp SalI-SAcI fragment to v-erbA-domain 2 (probe 3), the 0.25 kbp SalI-PstI fragment to the 5' part of v-erbA-domain 2 (probe 4) and the 0.24 kbp PstI-SacI to the 3' part of this domain (probe 5) (9). The location of these fragments in the AEV genome have been described previously (Vennström et al., 1980; Debuire et al., 1984). Chicken and human genomic probes were as follows: probe A, a 2.8 kbp XhoI fragment corresponding to v-erbA-domain 1 isolated from the 2.8 kbp EcoRI fragment of λhAT2 subcloned in pKH47; probe B, a 2.5 kbp EcoRI-XhoI fragment corresponding to the 3' part of v-erbA-domain 2 was isolated from a 4.5 kbp EcoRI fragment of λhAT2 also subcloned in pKH47; probe C, a 2.2 kbp KpnI fragment from the latter insert corresponds to the complete v-erbA-domain 2; probe D, a 1.8 kbp EcoRI fragment of λhAP3 corresponds to the 5' part of v-erbA-domain 2; probe E, a 4.3 kbp EcoRI-HindIII fragment isolated from the 4.8 kbp EcoRI fragment of λhAP3 was relevant to the 3' part of v-erbA-domain 2; probe F, a 5.5 kbp EcoRI fragment was isolated from λcAP1 and was relevant to the complete v-erbA-domain 2.

DNA sequencing

DNA sequencing was carried out by the base-specific chemical method described by Maxam and Gilbert (1980). Fragments with 5' protruding ends were dephosphorylated with bacterial alkaline phosphatase and were subsequently 5' end-labelled with ^{32}P ATP using T4 polynucleotide kinase. The labelled fragments resulting from chemical modification and cleavage were fractionated on 25, 16 and 6% polyacrylamide gels and autoradiographed.

RESULTS

Isolation of human and chicken c-erbA genomic clones

Human and chicken DNA libraries established with lambda phage Charon 4A were screened with a complete v-erbA probe, probe 1 (described in Figure 1A). From two distinct human DNA libraries five different clones (λhAT1 and λhAT2 ; λhAP1 to λhAP3) were isolated and analyzed by restriction enzyme digestion and filter hybridization using probes 1 and 4 (Figure 1A). The restriction map of these clones is shown in Figure 1B. In order to determine more precisely the content of these clones in erbA sequences, we used probes underlined in Figure 2 to hybridize filters carrying restriction endonucleases fragments of cloned AEV DNA. The enzymes we used allowed to distinguish between domains 1 and 2 of erbA since SalI endonuclease cleaves the erbA gene 13 nucleotides upstream of domain 2 (Debuire et al., 1984). In Figure 2 we show that probe A spans the 5' part of v-erbA, its hybridization pattern was identical to the one of probe 2 used as a control. Since probe A was unable to hybridize with the small AvaI-SacI fragment (a 0.36 kbp fragment of domain 2 revealed by probe 3 that also reveals the 0.8 kbp AvaI fragment) we concluded that the 9 kbp EcoRI fragment of λhAT2 contained essentially sequences of the 5' half of erbA. In contrast, probe D (a 1.8 kbp EcoRI fragment of λhAP3) hybridized with this 0.36 kbp AvaI-SacI fragment but failed to hybridize with the large 1.6 kbp SacI-SalI fragment encompassing domain 1 (revealed by probe 2) and the 0.24 kbp PstI-SacI fragment (3' part of domain 2, data not shown). Thus the most 5' erbA sequences contained in the partial c-erbA locus are restricted to the 5' part of domain 2 (SalI-PstI fragment); the end of this domain was found in the 4.8 kbp EcoRI fragment of λhAP3, which hybridized with the 0.24 kbp PstI-SacI fragment shown in Figure 1. In λhAP1 15 kbp of non hybridizing DNA sequences preceded the EcoRI 1.8 kbp DNA fragment containing the beginning of erbA-domain 2. We concluded that the λhAP1 to λhAP3 phages only contained the 3' part of v-erbA related sequences.

Using the same strategy as above, similar patterns were found for the chicken clones λcAP1 and λcAP2 described in Figure 1B and Figure 2. Probe F (5.5 kbp EcoRI fragment) showed an identical hybridization pattern to probe 4 (data not shown). Since this probe did not hybridize with the large 1.6 kbp SalI-SacI domain 1 fragment we concluded that the erbA content of these clones was restricted to erbA-domain 2 and thus was similar to λhAP. Furthermore a strong cross-hybridization was found in stringent conditions between the cellular inserts of λhAP3 and λcAP1 (data not shown).

Comparison of the nucleotide sequences of v-erbA and the c-erbA stretch contained in λcAP2

In order to localize more precisely, with respect to the previously defined domains 1 and 2 of v-erbA, the erbA content of λcAP, we sequenced a 0.37 kbp EcoRI-BamHI DNA fragment encompassing the most 5' v-erbA related stretch found in λcAP2. Figure 3 shows the comparison between the viral and cellular chicken erbA sequences. The homology begins at nucleotide 86 of c-erbA corresponding to nucleotide 676 of the v-erbA sequence. This homology continues for 259 nucleotides in the same open reading frame as v-erbA (the two other reading frames are closed by numerous stop codons) until terminated at nucleotide 346 (nucleotide 934 of v-erbA) where complete divergence of the sequences marks the end of the v-erbA related stretch. In c-erbA, a typical splice donor consensus sequence CAGGTGAG (nucleotides 344 to 351) (Mount, 1982) is found at the breakpoint of homology (nucleotide 346) and a suitable consensus splice acceptor sequence is located at nucleotide 88. Upstream of this position, the open reading frame is immediately closed by a stop codon TAA (nucleotides 76 to 78).

The homology between the cellular exon described above and its viral equivalent is 78% at the nucleotide level and 94% at the amino acid level, with the large majority of the mutations occurring at the third nucleotide of the codons, suggesting that λcAP2 contains a related but distinct c-erbA locus.

Inverted orientation of human c-erbA-domain 2 of λhAP when compared to v-erbA domain 2

Southern blot analysis performed on the 4.4 kbp EcoRI fragment of λhAT2 inserted in PKH47 and digested with EcoRI and HindIII restriction endonucleases showed an unexpected pattern when hybridized with probes 4 and 5 (data not shown). The small HindIII-EcoRI fragment (1.2 kbp) localized in the most 3' part of λhAT2 (Figure 2) hybridized with probe 4 whereas the larger fragment hybridized with probe 5, therefore exhibiting an inverted pattern of hybridization in contrast to the expected one. In order to confirm this inverted orientation at the nucleotide level we sequenced the 1.2 kbp HindIII-EcoRI fragment. Figure 3A shows the comparison between the v-erbA sequence and the corresponding human sequence. 166 nucleotides downstream of the EcoRI site of the human fragment we found a typical Alu sequence (Haynes et al., 1981) confirming a previous report by Jansson et al., (1983) which describes two clones he-A1 and he-A2 similar (if not identical) to λhAT and λhAP respectively. In the human sequence the homology with v-erbA-domain 2 resides in the 3'→5' orientation (fragment E_a-H) when compared to v-erbA-domain 1 related sequences (Figure 3B). This homology begins at nucleotide 656 in the human sequence which corresponds to nucleotide 674 in the v-erbA gene (Figure 3A) and continues in the same open reading frame as v-erbA until nucleotide 916 (nucleotide 934 in v-erbA). The homology is 85% at the nucleotide level and 91% at the amino acid level. Like in the chicken λcAP sequences, the v-erbA related sequence is found in an exon-like structure limited by splice donor and acceptor consensus sequences similar in both species. The homology between human and viral erbA sequences starts again at nucleotide 1626 in human sequence where a typical splice acceptor consensus sequence takes place (nucleotide 935 in v-erbA) and continues in the same open reading frame until nucleotide 1852. The homology with v-erbA sequence

disappears at nucleotide 1837 in c-erbA fragment (nucleotide 1146 in the viral counterpart) beyond which the 3' end of v-erbA streche is derived from the retroviral env sequence (Henry et al., 1985). This cellular exon exhibited 86% of homology with the viral sequence at the nucleotide and amino acid level. The end of the cellular c-erbA exon is not found in this sequence.

Southern analysis of the chicken and human c-erbA loci of genomic DNA

In order to determine whether the inverted c-erbA related sequences found in λhAT were cloning artifacts or representative of the cellular erbA structure, we analyzed the c-erbA locus by Southern blot experiments. DNA from placenta, K562 and LY 47 cells was digested with restriction endonucleases and hybridized with probes A, B, C, D and E. As shown in Figure 4A and B the structure of the cellular sequences present in λhAT was similar to that of genomic DNA sequences cleaved with KpnI, HindIII and EcoRI. The same experiments were performed on chicken cellular DNA using SacI and BamHI restriction enzymes (Figure 4C). In several chicken cell types we found in the genomic c-erbA sequences a pattern of restriction sites identical to that of the isolated λcAP1 and λcAP2 DNA. However, we detected the existence of a polymorphism for SacI in the 5.5 kbp EcoRI fragment used to prepare probe F.

DISCUSSION

In this paper we described the organization of the 3' part (domain 2) of the c-erbA gene in chicken and human DNAs. We demonstrated that this gene exists as two distinct loci in both DNAs: one contains the entire v-erbA related sequences (domains 1 + 2), the other only corresponds to v-erbA-domain 2. This latter locus exhibited a typical exon-intron structure suggesting the existence of 5' v-erbA unrelated exons. Therefore the two c-erbA loci could encode two distinct proteins strongly similar in their carboxy-terminal moities but different in their amino-terminal part. We could hypothesize that the carboxy-terminus of the molecule retains the biological activity of the c-erbA product and that the amino-terminus acts as a receptor of specific ligands. It is interesting in this respect to note that v-erbA-domain 1 is homologous to the receptors of glucocorticoids and estrogens cDNAs (Weinberger et al., 1985; Green et al., 1986; Greene et al., 1986). Therefore a single ligand could induce two different biological effects, if the amino-terminus of the c-erbA protein is involved. Alternatively two distinct ligands could induce similar biological effects if the carboxy-terminus of the c-erbA protein is implicated. Since the c-erbA products have not yet been isolated we cannot distinguish between these two possibilities.

A striking feature of the complete human c-erbA locus is the inverted orientation of the second domain compared to the viral genome. This inversion was first suggested by hybridization data, then confirmed by nucleotide sequence analysis (we do not know whether this organization is similar in the chicken genome because we did not succeed in cloning the complete chicken c-erbA locus). If the v-erbA related product plays a role in human erythroid differentiation one can expect, in appropriate cells, an inversion of the second domain of the complete c-erbA locus in order to bring domains 1 and 2 in the same reading frame as in v-erbA. We do not know wether the λhAT clones contain a functional c-erbA gene. However K562 cells express mRNAs containing both domain 1 and 2

(Jansson et al., 1983 and unpub. data) and we have not isolated genomic clones containing a complete c-erbA sequence distinct from λhAT. Therfore it appeared that λhAT probably represented the human c-erbA gene and we expected to find in its nucleotide sequence signals suggesting that an inversion of domain 2 could occur. In fact, we found three interesting stretches (Figure 5):

First, 41 nucleotides upstream from the splice acceptor site delimiting the first exon of domain 2 we noticed the sequence GAGG (asterisks in Figure 3A) also found at the recombination point of retroviruses with cellular sequences (Besmer et al., 1986), and at the recombination point of c-myc with immunoglobulin sequences in mouse plasmacytomas (Piccoli et al., 1984).

Second, an inverted repeat (CTGTGG) flanked by two direct repeats GCTC and CTGCC was detected in the stretch of the sequence expected to be involved in the inversion of domain 2.

Third, overlapping the intron-exon 5' limit of the first exon of domain 2, a CACAGCTG sequence is present (in the black box in Figure 3A, asterisks in Figure 5). A similar sequence CACACTG was found in the V_β germline fragment of T-cell receptor genes at the point of inversion leading to the V-D-J rearrangement (Malissen et al., 1986). Another similar sequence CACAGTG was found in the vicinity of the recombination site of immunoglobulin joining gene segments (Hope et al., 1986; Lewis et al., 1984). This type of sequence could be a privileged site for nucleolytic enzymes and, in fact, an endonucleolytic activity that introduces a double-strand cut at the dinucleotide TG of the CACAGTGCTG↓GGTG sequence (Hope et al., 1986) has been identified in nuclear extracts of chick embryo bursa and mouse fetal liver cells. Another particularity of our sequence is the presence of a typical Alu family element, 168 nucleotides upstream from the splice acceptor site indicated in Figure 3A. This sequence might also play a role in the inversion of c-erbA-domain 2. Indeed, the structural features mentioned above do not prove that an inversion of c-erbA-domain 2 occurs in the course of erythroid cell life but the accumulation of these elements cannot be fortuitous. Moreover DNA breaking and rejoining regulated by ADP-ribosyltransferase has been shown to be involved in the differentiation of various cells (Johnstone and Williams, 1982) suggesting a link between DNA rearrangement and cellular differentiation. Thus it is tempting to speculate that c-erbA is involved in erythroid differentiation and that DNA inversion within this gene is a key mechanism in the control of this irreversible process.

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LEGENDS

Figure 1 : Restriction maps of lambda clones containing human and chicken c-erbA sequences.

The resulting maps were constructed from Southern blots of phage DNAs hybridized with probes depicted in panel A.

Panel B : restriction endonuclease cleavage map of the inserts in the lambda phages. Hatched areas represents hybridizing v-erbA fragments. E: EcoRI, E_a: EcoRI sites generated by the addition of synthetic linkers used for establishing the library, Xb: XbaI, H: HindIII, K: KpnI, B: BamHI, X: XhoI, A: AvaI, P1: PstI, Pv: PvuII, S: SalI, Sc: SacI. S* and B* indicate the allelic restriction sites in the chicken and human cellular DNA. ↓ ⊤ Sequenced fragments.

Figure 2 : Hybridization of human erbA sequences to AEV DNA.

AEV DNA cloned in the EcoRI site of lambda Charon 4A was double digested with AvaI and SacI (A-Sc), SalI and SacI (S-Sc), PvuII and SacI (Pv-Sc) restriction endonucleases. The fragments were separated in agarose gel, transferred to nitrocellulose filters and hybridized with nick-translated probes (2, A, 3, and D described in Figure 1) listed across the top of the figure.

Figure 3 : A - Nucleotide sequence of v-erbA-domain 2-related fragments in chicken and human cellular DNAs and comparison to v-erbA.

In order to align viral and human sequences we have indicated the sequence of the complementary strands of the human fragments in their 5'→3' orientation. Brackets delineate an Alu family sequence with both ends underlined and a direct repeat of 13 nucleotides (the poly A rich stretch at the left end of the Alu sequence) is written in italics. In the cellular sequences, nucleotides different from the viral sequence are indicated. Sa: splice acceptor site; Sd: splice donor site. The black box contains the sequence depicted in Figure 5. Coding triplets of the cellular sequences modifying the encoded amino acids found in the v-erbA polypeptide are boxed. The fifteen 3' nucleotides of v-erbA are in fact of env origin.

B - Representation of human c-erbA organisation compared to v-erbA (not to scale).

Domain 1

5' Domain 2 exon 1

3' Domain 2 exon 2

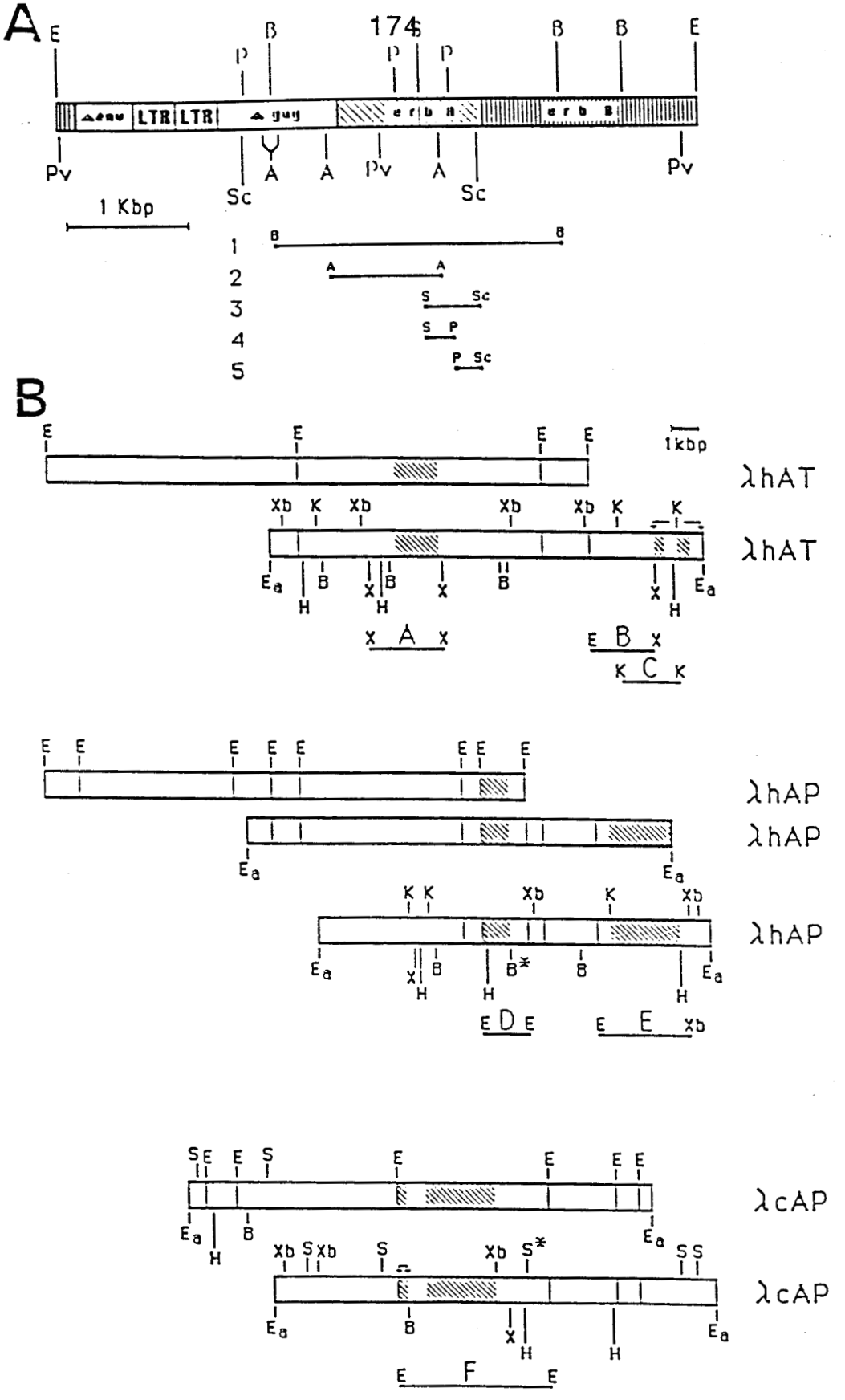
Figure 4 : Analysis of erbA related sequences in chicken and human chromosomal DNA.

Panels A and B represent human DNA digested with enzymes listed across the top. Th resulting fragments were separated in an agarose gel, transferred to a nitrocellulose filter and hybridize with the probes indicated in the figure (A, B, C, D, E, F and 3 described in Figure 1). Lane 1: K56 DNA, lane 2: Ly 47 DNA, lane 3: placental DNA.

Panel C represents chicken DNA digested with enzymes listed across the top and treated a described above. Lane 1: RP9 DNA, lane 2: OK10 BM DNA, lane 3: MSB1 DNA, lane 4: norm erythrocyte DNA, lane 5: AEV transformed erythroblast DNA (these cells were obtained from th same chicken which providied, before infection, the normal erythrocytes used in lane 4). indicate v-erbA fragments.

Figure 5 : Salient features of the human c-erbA sequence.

Direct repeats are boxed, the inverted repeat is underlined. Sequences of immunoglobul (Lewis et al., 1984) and T-lymphocyte receptor (Malissen et al., 1986) are shown for comparison.


A
E
B
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1745
LTR
LTR
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erb B
erb B
Pv
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A
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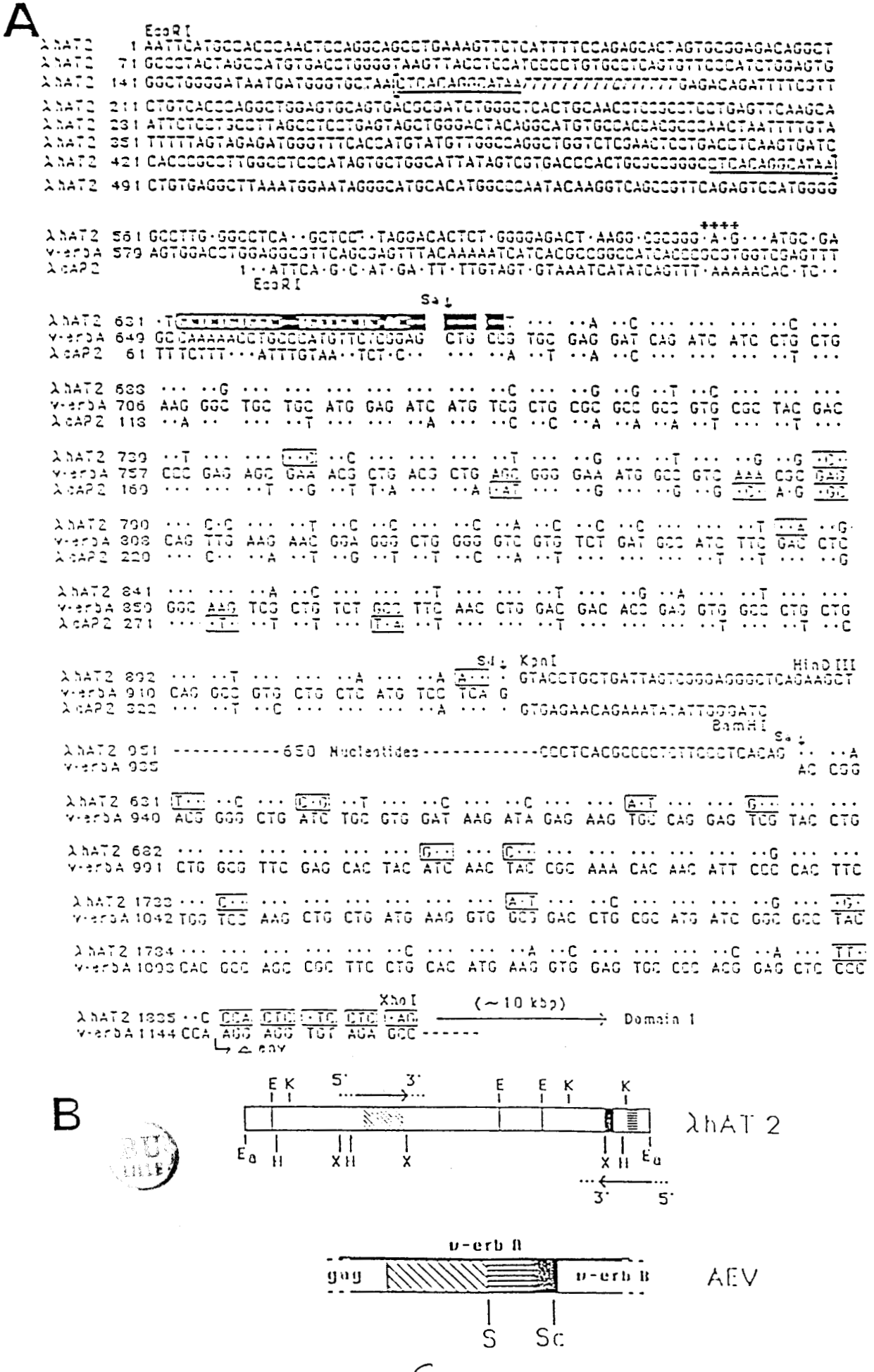
1
2
3
4
5
B
1kbp
λhAT
λhAT
λhAP
λhAP
λhAP
λcAP
λcAP
X A X
E B X
K C K
E D E
E E Xb
E F E

Figure 1


A
EcoR I
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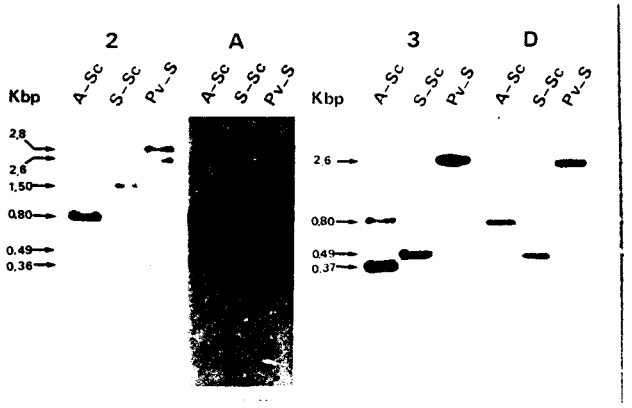
λhAT2 141 GGCTGGGGATAATGATGGGTGCTAAICTCACAGGCATAA
λhAT2 211 CTGTCACCCAGGCTGGAGTGCAGTGACGCGATCTGGGCTCACTGCAACCTCCGCCTCCTGAGTTCAAGCA
λhAT2 281 ATTCTCCTGCCTTAGCCTCCTGAGTAGCTGGGACTACAGGCATGTGCCACCACGCCCAACTAATTTTTGTA
λhAT2 351 TTTTTAGTAGAGATGGGTTTCACCATGTATGTTGGCCAGGCTGGTCTCGAACTCCTGACCTCAAGTGATC
λhAT2 421 CACCCGCCTTGGCCTCCCATAGTGCTGGCATTATAGTCGTGACCCACTGCGCCGGGCCTCACAGGCATAA
λhAT2 491 CTGTGAGGCTTAAATGGAATAGGGCATGCACATGGCCCAATACAAGGTCAGCCGTTCAGAGTCCATGGGG
λhAT2 561 GCCTTG·GGCCTCA··GCTCC·TAGGACACTCT·GGGGAGACT·AAGG·CGCGGG·A·G···ATGC·GA
v-erbA 579 AGTGGACCTGGAGGCGTTCAGCGAGTTTACAAAAATCATCACGCCGGCCATCACCCGCGTGGTCGAGTTT
λcAP2 1···ATTCA·G·C·AT·GA·TT·TTGTAGT·GTAAATCATATCAGTTT·AAAAACAC·TC··
EcoR I
Sa I
λhAT2 631 ·T··· ··· ··A ··C ··· ··· ··· ··C ···
v-erbA 649 GCCAAAAACCTGCCCATGTTCTCGGAG CTG CGG TGC GAG GAT CAG ATC ATC CTG CTG
λcAP2 61 TTTCTTT···ATTTGTAA··TCT·C·· ··· ··A ··T ··A ··C ··· ··· ··· ··T ···
λhAT2 683 ··· ··G ··· ··· ··· ··· ··· ··· ··C ··· ··G ··G ··T ··C ··· ··· ···
v-erbA 706 AAG GGC TGC TGC ATG GAG ATC ATG TCG CTG CGC GCC GCC GTG CGC TAC GAC
λcAP2 118 ··A ·· ··· ··T ··· ··· ··A ··· ··C ··C ··A ··A ··A ··T ··· ··T ···
λhAT2 739 ··T ··· ··· ··· ··C ··· ··· ··· ··T ··· ··G ··· ··T ··· ··G ··G ···
v-erbA 757 CCC GAG AGC GAA ACG CTG ACG CTG AGC GGG GAA ATG GCC GTC AAA CGC GAG
λcAP2 169 ··· ··· ··T ··G ··T T·A ··· ··A ··· ··· ··G ··· ··G ··G ··· A·G ···
λhAT2 790 ··· C·C ··· ··T ··C ··C ··· ··C ··A ··C ··C ··C ··· ··· ··T ··· ··G
v-erbA 808 CAG TTG AAG AAC GGA GGG CTG GGG GTC GTG TCT GAT GCC ATC TTC GAC CTC
λcAP2 220 ··· C·· ··A ··T ··G ··T ··T ··C ··A ··T ··· ··· ··· ··T ··T ··· ··G
λhAT2 841 ··· ··· ··A ··C ··· ··· ··T ··· ··· ··T ··· ··G ··A ··· ··T ··· ···
v-erbA 859 GGC AAG TCG CTG TCT GCC TTC AAC CTG GAC GAC ACC GAG GTG GCC CTG CTG
λcAP2 271 ··· ··T· ··T ··T ··· T·A ··T ··· ··· ··T ··· ··· ··· ··T ··· ··T ··C
Sal I KpnI HinD III
λhAT2 892 ··· ··T ··· ··· ··A ··· ··A ··· · GTACCTGCTGATTAGTCGGGAGGGCTCAGAAGCT
v-erbA 910 CAG GCC GTG CTG CTC ATG TCC TCA G
λcAP2 322 ··· ··T ··C ··· ··· ··· ··A ··· · GTGAGAACAGAAATATATTGGGATC
BamHI
λhAT2 951 ----------650 Nucleotides----------CCCTCACGCCCCTCTTCCCTCACAG ·· ··A
v-erbA 935 AC CGG
λhAT2 631 ··· ··C ··· ··· ··T ··· ··C ··· ··C ··· ··· ··· ··· ··· ···
v-erbA 940 ACG GGG CTG ATC TGC GTG GAT AAG ATA GAG AAG TGC CAG GAG TCG TAC CTG
λhAT2 682 ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··G ··· ···
v-erbA 991 CTG GCG TTC GAG CAC TAC ATC AAC TAC CGC AAA CAC AAC ATT CCC CAC TTC
λhAT2 1733 ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··C ··· ··· ··· ··G ··· ···
v-erbA 1042 TGG TCC AAG CTG CTG ATG AAG GTG GCG GAC CTG CGC ATG ATC GGC GCC TAC
λhAT2 1784 ··· ··· ··· ··· ··· ··· ··C ··· ··· ··A ··C ··· ··· ··· ··C ··A ··· ···
v-erbA 1093 CAC GCC AGC CGC TTC CTG CAC ATG AAG GTG GAG TGC CCC ACG GAG CTC CCC
XhoI (~10 kbp)
λhAT2 1835 ··C CCA CTC ··TC CTC ··AC ——————→ Domain 1
v-erbA 1144 CCA AGG AGG TGT AGA GCC ------
↳ ← env
B
E K 5' ···→··· 3' E E K K
Eo H XH X X H Eo
3' ···←··· 5'
λhAT 2
v-erb B
gag v-erb B
AEV
S Sc

Figure 3

FIGURE 2



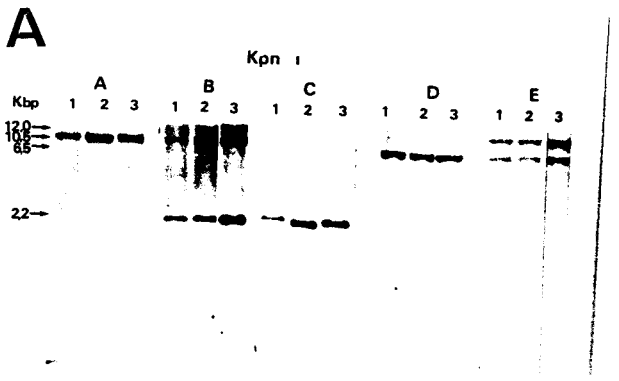
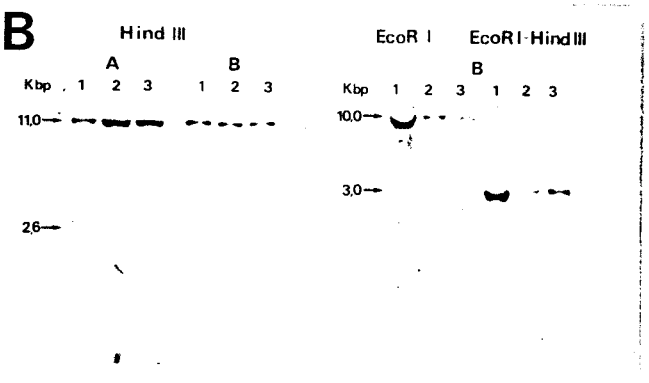
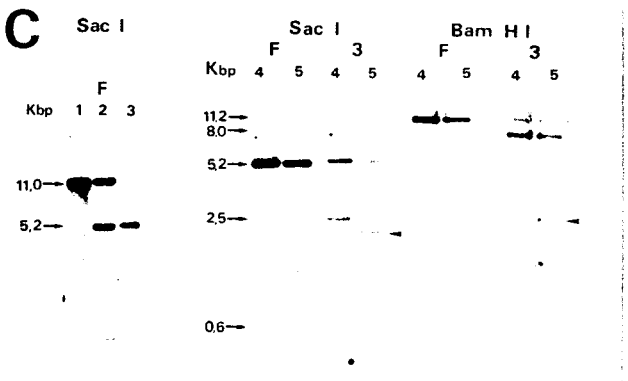


FIGURE 4





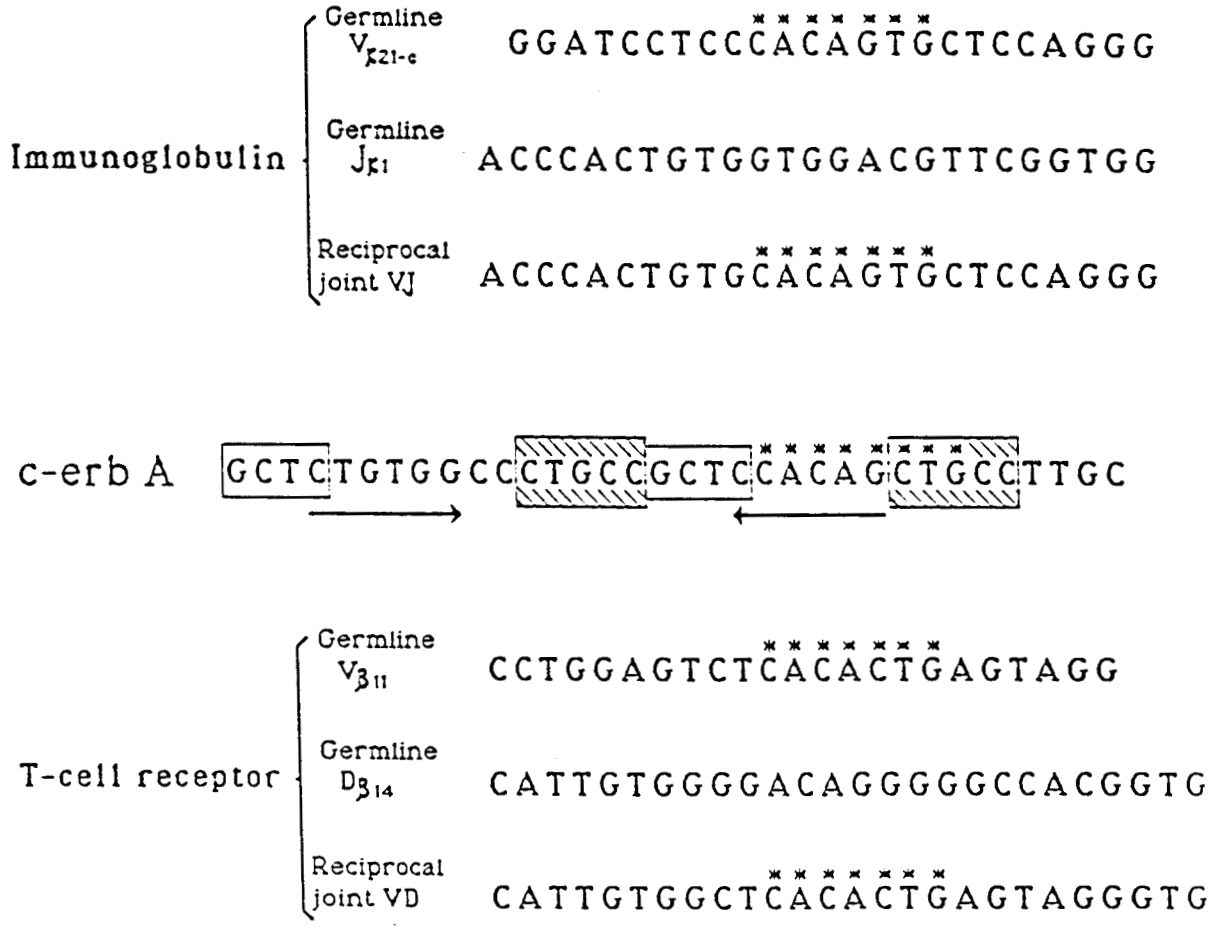

Immunoglobulin
Germline $V_{\kappa 21\text{-}e}$
GGATCCTCCCACAGTGCTCCAGGG
Germline $J_{\kappa 1}$
ACCCACTGTGGTGGACGTTCGGTGG
Reciprocal joint VJ
ACCCACTGTGCACAGTGCTCCAGGG
c-erb A
GCTCTGTGGCCCTGCCGCTCCACAGCTGCCTTGC
T-cell receptor
Germline $V_{\beta 11}$
CCTGGAGTCTCACACTGAGTAGG
Germline $D_{\beta 14}$
CATTGTGGGGACAGGGGGCCACGGTG
Reciprocal joint VD
CATTGTGGCTCACACTGAGTAGGGTG

Figure 5

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The human DNA locus related to the oncogene *myb* of avian myeloblastosis virus (AMV): molecular cloning and structural characterization

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Chicken and human cell DNA contains sequences homologous to the avian myeloblastosis virus oncogene, *v-myb*. These cellular sequences, *c-myb* (human) and *c-myb* (chicken), were isolated from libraries of human or chicken cell DNA fragments, generated by partial digestion with the restriction enzymes *Alu*I and *Hae*III, and compared. The chicken *c-myb* locus isolated from two distinct overlapping recombinant phages, contained five contiguous *Eco*RI fragments of 5.4, 1.1, 2.1, 2.2 and 9 kbp, accounting for all the bands seen with a *v-myb* probe in a complete *Eco*RI digest of chicken cellular DNA. Likewise, the screening of the human library yielded a recombinant phage hybridizing with the *v-myb* specific probe, that contained five *Eco*RI fragments of 2.8, 2.6, 2.0, 1.2 and 5.0 kbp (the last ending with an artificial *Eco*RI site, due to the construction of the library) belonging to the *c-myb* (human) locus. Probes using the *Eco*RI chicken DNA cloned fragments revealed corresponding contiguous *Eco*RI fragments in the human clone. Subsequent analyses of cellular polyadenylated mRNA extracted from human and chicken cells allowed the identification of single RNA species of 3.8 and 4.0 kb, respectively, as the representative transcripts of the *c-myb* locus in the two species. Thus, *c-myb* appears as a single locus in man and chicken, conserved with a similar structure in the two distantly related species. Our preparation of a specific human *c-myb* probe with an increased sensitivity on DNA/RNA blots should facilitate analyses concerning this gene in human normal or tumour cells or tissues.

Key words: avian myeloblastosis virus/viral oncogene/cellular oncogene/DNA cloning/RNA blotting

Introduction

Acute transforming retroviruses are responsible for the onset of different types of cancers in many vertebrate species. The avian defective leukemia viruses (DLV), whose cellular targets are primarily hematopoietic cells, is a particularly well studied group. When propagated *in vivo*, these viruses induce different types of cancers within a few weeks of inoculation and they transform hematopoietic cells *in vitro*; some strains also transform fibroblasts and epithelial cells (Graf and Beug, 1978; Graf and Stehelin, 1982). They are all defective for replication and need, for their propagation, helper virus containing the normal viral structural genes (*gag*, *pol* and *env*) (Graf and Stehelin, 1982). Their genomic RNA contains both helper virus-related sequences and specific sequences called *v-onc*, implicated in the transformation process. These *v-onc* sequences have normal cellular counterparts, *(c-onc)*, in the DNA of vertebrates and possibly invertebrates (Shilo and Weinberg, 1981). These highly transforming retroviruses are thought to have arisen from genetic recombination between poorly oncogenic helper viruses and phylogenetically stable cellular genes. How such recombination occurred is not yet well understood (Graf and Stehelin, 1982; Bishop, 1982).

The eight available DLV isolates have been divided into several groups according to their transforming potentials and *v-onc* genes. The avian myeloblastosis group includes avian myeloblastosis virus (AMV) and E26, two myeloblastosis-inducing virus strains which have been obtained as independent isolates (Graf and Beug, 1978). AMV (Beard, 1963; Moscovici, 1975) incudes myeloblastosis in chickens and transforms immature myeloid cells (Gazzolo *et al.*, 1979) as well as mature macrophages (Durban and Boettiger, 1981) from chick embryo yolk sac cultures; AMV is so far the only DLV strain which has a transforming capacity restricted to the hematopoietic lineage (Graf and Stehelin, 1982).

The 7.2-kb AMV genomic RNA contains a complete functional *gag* gene, most of the *pol* gene and a transforming sequence, *v-myb*, in place of *env* sequences (Souza *et al.*, 1980). The expression of *v-myb* is achieved through the presence of a subgenomic spliced RNA of 2.1 kb found in all AMV-transformed cells (Gonda *et al.*, 1981). This spliced RNA contains *v-myb* as well as regulatory sequences derived from the 5′ (U5) and 3′ (U3) part of the genomic RNA (Graf and Stehelin, 1982). Sequencing of *v-myb* revealed a single open reading frame, which might code for a protein of 30 000 daltons (265 amino acids) (Rushlow *et al.*, 1982) or, alternatively, of 45 000 daltons, if an upstream initiation codon is used (Klempnauer *et al.*, 1982).

E26 is another myeloblastosis virus independently isolated from a field case of 'erythroblastosis' (Ivanov *et al.*, 1962), that also harbours part of the *v-myb* sequences (Roussel *et al.*, 1979). E26 induces the proliferation of both erythroid and myeloid cells *in vivo* (Sotirov, 1981). A similar situation is observed with bone marrow cells transformed *in vitro* by E26 and seeded in erythroid- or myeloid-stimulating growth medium (Beug *et al.*, 1982; Radke *et al.*, 1982). Furthermore, E26 also transforms quail but not chicken fibroblasts in culture (Graf *et al.*, 1979), therefore showing an oncogenic spectrum different from that of AMV. In addition to *v-myb*, E26 contains a so far unidentified sequence (X) which could play a role in erythroid (and/or) fibroblastic transformation (Roussel *et al.*, 1979; Stehelin *et al.*, 1980). In contrast to AMV, the putative transforming protein of E26, designated p135, is translated from the 5.7-kb genomic length RNA (Bister *et al.*, 1982), as a fusion protein containing *gag* and *myb* determinants, as well as probably X-determinants (D.Leprince in preparation). E26 and AMV illustrate the two distinct types of expression used by retroviruses, they specify either a fusion protein (generally *gag-onc*) translated from a genomic length RNA, or an *onc* protein translated from a subgenomic RNA. The *v-myb* sequences have a cellular counterpart in chicken and mammalian DNA, called *c-myb* (Roussel *et al.*, 1979; Bergmann *et al.*, 1981). Using DNA from mouse-human somatic cell hybrids, this sequence was recently assigned to human chromosome 6 (Dalla Favera *et al.*, 1982).

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We describe our attempts to investigate the structure of the human *c-myb* DNA as compared with its chicken counterpart. In chicken DNA, digested by *Eco*RI, the *c-myb* locus was represented by five *Eco*RI fragments spanning ~20 kbp of cellular DNA. From a chicken random DNA library, we cloned two overlapping phages carrying all these *v-myb*-positive, *Eco*RI fragments and representing a single chicken DNA locus. From a human random DNA library, we found one recombinant DNA phage with a structure highly homologous to the chicken locus. Using cellular probes, we also showed that *c-myb* was transcribed in human and chicken hematopoietic cells as a single RNA transcript with a size of 3.8 and 4.0 kb, respectively. Finally, we prepared a human *c-myb* probe yielding a high signal on blots of human DNA/RNA that should facilitate studies of this oncogene in human cells and tissues.

Results

Isolation of recombinant phages containing c-myb sequences of human or chicken DNA

*Eco*RI digests of human and chicken DNA were separated by gel electrophoresis and transferred to nitrocellulose. The blots were hybridized with a *v-myb*-labelled cDNA probe prepared as described previously (Roussel *et al.*, 1979; Gonda *et al.*, 1981). Several positive fragments were detected in both human (2.8 and 2 kbp, see Figure 2A) and chicken (9.0, 5.4 and 2.2 kbp, see Figure 1A) DNA, indicating that both species contained cellular DNA *(c-myb)* related to the *v-myb* probe. To compare the structure of *c-myb* sequences in man and chicken, we screened, with our *v-myb* probe, libraries of random *Alu*I-*Hae*III human or chicken DNA fragments inserted in the λ phage Charon 4A after the attachment of synthetic *Eco*RI linkers (Maniatis *et al.*, 1978; Dodgson *et al.*, 1979). One human DNA phage (λ HM-1) containing *c-myb* sequences was isolated, whereas two distinct chicken DNA phages (λ CM-4 and CM-5) accounted for the *c-myb* locus. These three phages were characterized and compared as described below.

Characterization of the chicken c-myb locus

Analyses performed on purified *Eco*RI-digested phage DNAs by blot-hybridization using as probes *v-myb* or *Eco*RI fragments of the isolated phages showed that the two clones λ CM-4 and λ CM-5 overlapped and accounted for the *c-myb* *Eco*RI fragments seen in chicken cellular DNA (Figure 1A). The *Eco*RI pattern of these clones is shown in Figure 1B together with their hybridization with the *v-myb* probe. These fragments were ordered by restriction mapping as shown in Figure 1D. The overlapping was confirmed by the hybridization of the ^{32}P-labelled 1.5-kbp fragment (clone λ CM-4, Figure 1D) with the 9.0-kbp fragment of clone λ CM-5 (Figure 1C). The chicken *c-myb* locus was recently described by Klempnauer *et al.* (1982), and our two phages λ CM-4 and CM-5, although slightly different from theirs, cover the same locus and confirm their results.

Structure of the c-myb locus in human DNA

The human *c-myb* DNA phage (λ HM-1) restricted with *Eco*RI (Figure 2B lane 1) contained five fragments (5.0, 2.8, 2.6, 2.0 and 1.2 kbp) positive with the *v-myb* probe (Figure 2B, lane 2). This indicated that the strong band of 2.8 kbp seen in a total human DNA digest (Figure 2A) was in fact a doublet (2.8 and 2.6 kbp) not resolved in the gel and that two

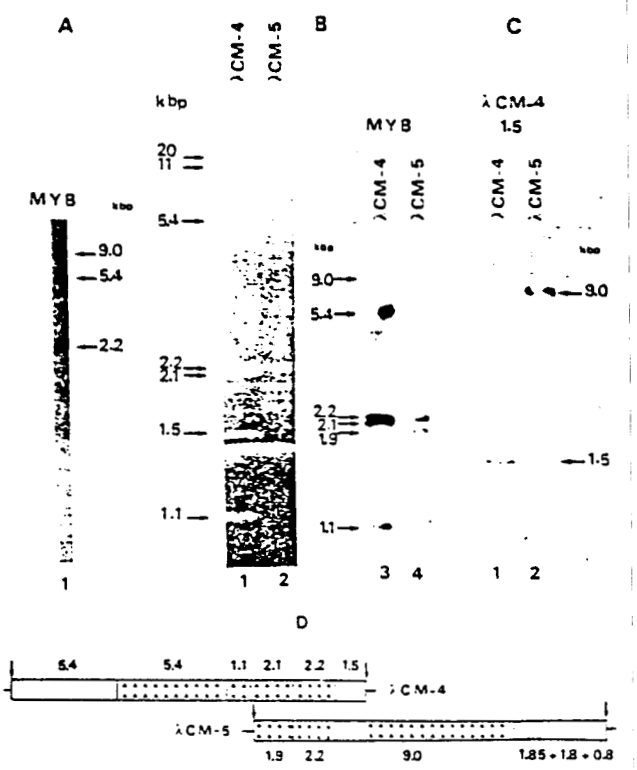

Fig. 1. Mapping of the chicken *c-myb* locus. (A) *Eco*RI-digested chicken DNA was size-separated by gel electrophoresis, transferred onto nitrocellulose and hybridized with cDNA myb. Adenovirus 2 DNA labelled *in vivo* with [^{14}C]thymidine was digested by *Eco*RI and run on the same gel as size marker (data not shown). (B) DNA from recombinant phages λ CM-4 (lane 1) and CM-5 (lane 2) was isolated, digested with *Eco*RI, subjected to 1% agarose gel electrophoresis and stained with ethidium bromide. The *Eco*RI DNA fragments separated as described above were blotted to nitrocellulose filters and hybridized to cDNA myb: λ CM-4 (lan 3) and λ CM-5 (lane 4). (C) The overlapping of the phages was demonstrated by the hybridization of the 1.5-kbp fragment of clone λ CM-4 (lane 1) with the 9.0-kbp fragment of clone λ CM-5 (lane 2). (D) Organization deduced from the above analyses of the chicken DNA sequences cloned in the recombinant phages λ CM-4 and CM-5. Boxes repr sent the *Eco*RI DNA fragments with their size indicated in kbp. ↓ Artific *Eco*RI site due to the *Eco*RI DNA linker used to construct the library. (▲▲) Fragments hybridizing with cDNA myb.

additional *c-myb* *Eco*RI DNA fragments (5.0 and 1.2 kb were not initially detected. After the determination of t structure of clone λ HM-1 (Figure 2D), we analyzed t homology between *c-myb* (human) and *c-myb* (chicken) lo and the transcriptional orientation of *c-myb* (human) by u ing size-separated *Eco*RI digests of clone λ HM-1 and d ferent ^{32}P-labelled *Eco*RI fragments of *c-myb* (chicke (Figure 1D), as probes. Figure 2C (lanes 1–5), shows th each chicken *c-myb* fragment hybridized with a sing counterpart fragment of clone λ HM-1 (see also Figures 2 and 4). Thus, the *c-myb* (human) and *c-myb* (chicke loci appeared co-linear and of similar organization. Thei fore, the orientation of *c-myb* (human) was as follow 5′-2.8-1.2-2.6-2.0-5.0-3′ by reference to the viral AMV RN defined as the 'plus' strand. The 5.0-kbp band was in fact artificial *Eco*RI fragment. It hybridized both with the *v-m* probe (Figure 2B, lane 2) and the 9.0-kbp chicken DNA fr ment containing the 3′-terminal sequences homologous to *myb* (Figure 2C, lane 5). Therefore, this *c-myb* (human) clo

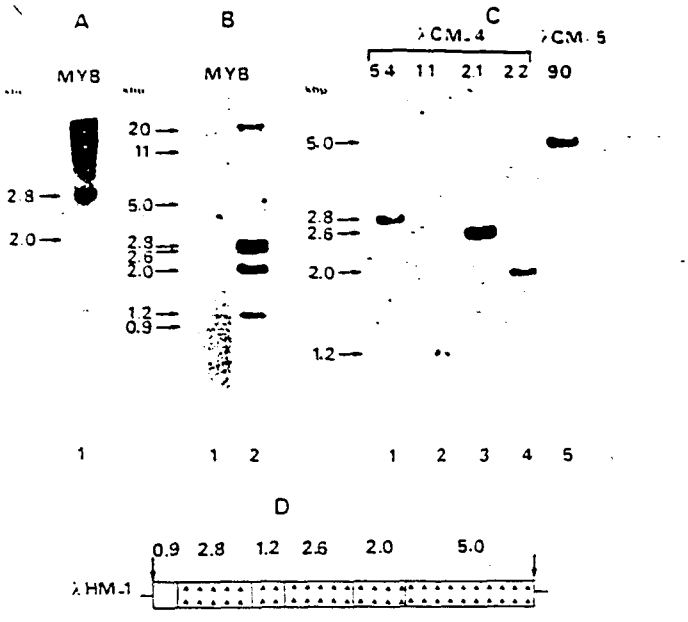

Fig. 2. The human *c-myb* locus shares structural homology with its chicken counterpart. (A) Human placenta DNA digested by *Eco*RI and blotted from agarose gel was hybridized with ^{32}P-labelled cDNA myb. (B) λ HM-1 phage DNA was digested by *Eco*RI. Fragments were separated on a 1% agarose gel visualized by ethidium bromide staining (lane 1) then transferred to nitrocellulose and hybridized with ^{32}P-labelled cDNA myb (lane 2). (C) Aliquots of clone λ HM-1 DNA digested with *Eco*RI were separated as in (B) and hybridized with the following labelled *Eco*RI fragments of the chicken clone CM-4: 5.4 kbp (lane 1); 1.1 kbp (lane 2); 2.1 kbp (lane 3); 2.2 kbp (lane 4). Lane 5 was hybridized similarly with the 9-kbp fragment of the chicken clone λ CM-5. (D) Organization deduced from the above analyses for the *c-myb* (human) locus in recombinant phage λ HM-1. Boxes represent the *Eco*RI DNA fragments with their size indicated in kbp as in Figure 1. I Artificial *Eco*RI site due to the *Eco*RI DNA linker used to construct the library. (▲▲) Fragments hybridizing with cDNA myb.

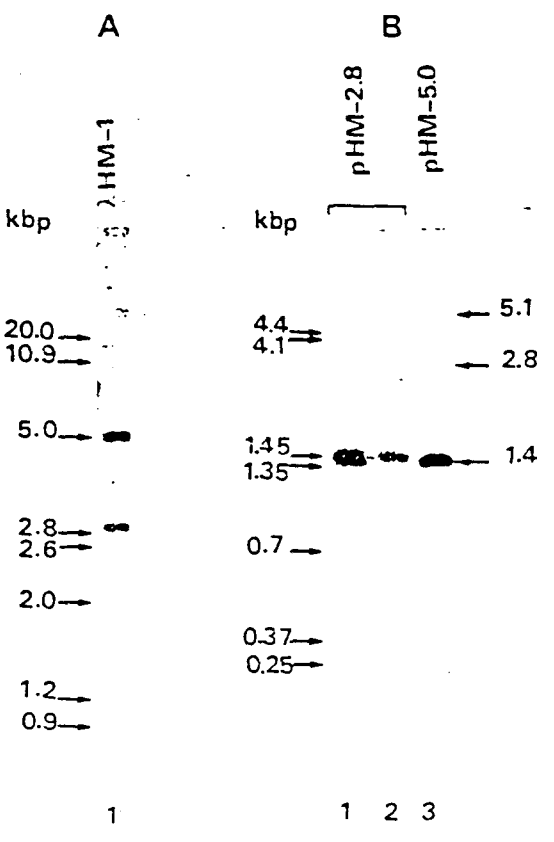

Fig. 3. Localization of human repetitive DNA sequences in clone λ HM-1. (A) λ HM-1 phage DNA was digested by *Eco*RI. Fragments were separated on a 1% agarose gel then transferred onto nitrocellulose and hybridized (lane 1) with ^{32}P-labelled rapidly reassociating total human DNA (repetitive DNA). (B) The 5.0- and 2.8-kbp *Eco*RI fragments positive with the human repetitive probe were subcloned into plasmid pKH 47 to yield pHM-5.0 and pHM-2.8, respectively. pHM-2.8 was digested either by *Eco*RI and *Xba*I (lane 1) or by *Eco*RI, *Xba*I and *Bam*HI (lane 2), submitted to gel electrophoresis, blotted onto nitrocellulose and hybridized with the [^{32}P]human repetitive DNA. The same experiment was performed with a *Xba*I digest of pHM-5.0 (lane 3). Note that the 0.7-kbp *Bam*HI-*Xba*I fragment of pHM-2.8 and the 2.8-kbp *Xba*I-*Xba*I fragment of pHM-5.0 positive with cDNA myb (see Figure 4) did not contain repetitive sequences.

probably represents all of the human DNA sequences homologous to *v-myb*.

Further analyses with the fragments of the *c-myb* (human) locus subcloned into plasmids (pKH 47, Hayashi, 1980), digested by restriction nucleases, and hybridized with viral cDNA myb allowed us to localize more precisely the regions of homology with the virus ('exon-like' fragments) in the human *c-myb* locus (Figure 4). We next examined whether the human *c-myb* locus contained repetitive DNA sequences. Human DNA was sheared, alkali-denatured, reassociated at a C_{ot} of 400 mol.s/l and fractionated on hydroxylapatite: the rapidly reassociating DNA was nick-translated and used as a ^{32}P-labelled probe to localize repetitive sequences in phage λ HM-1. The results (Figure 3A lane 1) indicated the presence of such sequences only in the 5.0- and 2.8-kbp *Eco*RI fragments. These were localized more precisely by similar experiments performed on subclones of these fragments in plasmids. They were found in the 2.8-kbp fragment within a single *Eco*RI-*Xba*I 1.45-kbp band (Figure 3B lanes 1 and 2) and in the 5.0-kbp fragment within a *Xba*I-*Xba*I 1.4-kbp band (Figure 3B lane 3). Such bands were negative with a *v-myb* probe (Figure 4).

In conclusion, *c-myb* (human) and *c-myb* (chicken) showed a highly conserved structure indicating that no extensive gene rearrangement had occurred in this locus during speciation.

Detection of human and chicken RNA transcripts with cloned human or chicken c-myb probes

The cellular analog of the AMV transforming gene, *c-myb*, is transcribed in many hematopoietic immature chicken cells as a single mRNA species of 4.0 kb (Gonda *et al.*, 1982) transcribed from the same strand as the viral RNA in AMV (Coll *et al.*, 1983). This result was confirmed in the course of the present study by analysis of the cellular RNAs from a chicken lymphoid cell line (MSB1) transformed by Marek disease-virus (MDV) (Figure 5B, lane 1). Moreover, the same hybridization pattern was obtained with the cloned 2.2-kbp *Eco*RI fragment from λ CM-4 (Figure 5B, lane 2).

We decided to prepare a human *c-myb* probe from the plasmid pHM-2.8 containing the 2.8-kbp *Eco*RI fragment of λ HM-1. When digested by *Eco*RI, *Xba*I and *Bam*HI (Figure 5A), the subclone pHM-2.8 yielded a single 0.7-kbp fragment positive with *v-myb* (Figure 5A, lane 1), and devoid of the human repetitive sequences (Figure 3B, lane 2) that, when used as a probe, allowed the detection in the human erythroid

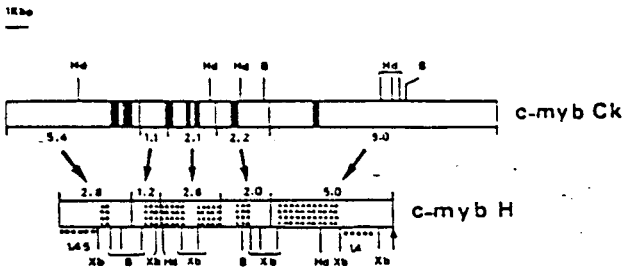

Fig. 4. *c-myb* (chicken) and *c-myb* (human) loci. Detailed restriction maps of the *c-myb* (chicken) and *c-myb* (human) loci were established as described for *Eco*RI with the following endonucleases: *Bam*HI (B), *Hind*III (Hd) and *Xba*I (Xb) (the last used only for the human clone). *c-myb* (chicken) exons (■) have been mapped according to Klempnauer *et al.*, 1982. Regions of homology with cDNA myb (▲▲) were localized in the *c-myb* (human) locus by double digestion of λ phage HM-1 or of plasmid clones containing human *c-myb* *Eco*RI fragments followed by blot hybridization. Repeated sequences (● ●) were determined similarly using a ^{32}P-labelled rapidly reassociating human DNA (see Materials and methods). Arrows indicate the human *Eco*RI fragments detected with the homologous chicken *Eco*RI fragment used as a probe.

cell line K562 of a single RNA species of ~3.8 kb (Figure 5B, lane 3). Comparative studies done on Northern blots (data not shown) or dot blots (Figure 5C) of RNA from human hematopoietic cell lines indicated that our selected partial human *myb* probe gave a much stronger signal (5- to 10-fold) than the *v-myb* probe.

Discussion

We have molecularly cloned and characterized a human *c-myb* locus in the recombinant λ phage HM-1. It spreads over at least the 13.6 kbp of DNA sequences which share homology with a *v-myb* probe. The structure of this human *c-myb* locus appears similar to its chicken equivalent which we have cloned for comparative studies; at the resolution of the techniques we used (see Figure 4), the exons are distributed similarly in the human and chicken genes. Thus, the organization of the *c-myb* locus seems to be conserved and sequence analyses will be required to evaluate more precisely this phylogenetic relatedness.

All our evidence favours a single *c-myb* locus in human as well as chicken cell DNA: our phages account for all the bands seen by *Eco*RI digestion of cell DNA and no phage with an alternate structure was found during the screening of the libraries used. Other authors have also concluded that there is a single *c-myb* locus in the chicken DNA (Klempnauer *et al.*, 1982; Perbal *et al.*, 1982b), and our results extend this conclusion to human DNA; *c-myb* differs from other *c-onc* genes which appear to belong to gene families (*c-ras*, Ellis *et al.*, 1981; *c-src*, Kitamura *et al.*, 1982) or to give rise to pseudogenes (*c-myc*, Dalla-Favera *et al.*, 1982).

The *c-myb* locus is transcribed in several immature chicken hematopoietic cells (Gonda *et al.*, 1982) as a single RNA species of 4.0 kb. Our studies indicate a similar single transcript of 3.8 kb in human cells (K562 cell line, Westin *et al.*, 1982). Thus, in both species, the size of the *c-myb* RNA is considerably larger than the 1196 nucleotides representing *v-myb* in AMV. The *v-myb*-transduced sequences lack both initiation and termination codons necessary to make the *v-myb* protein, these signals being provided by the virus (Klempnauer *et al.*, 1982). It follows that the *c-myb* locus in the two species must extend into nucleotide sequences both upstream and downstream of the homologous region to yield the 3.8-or

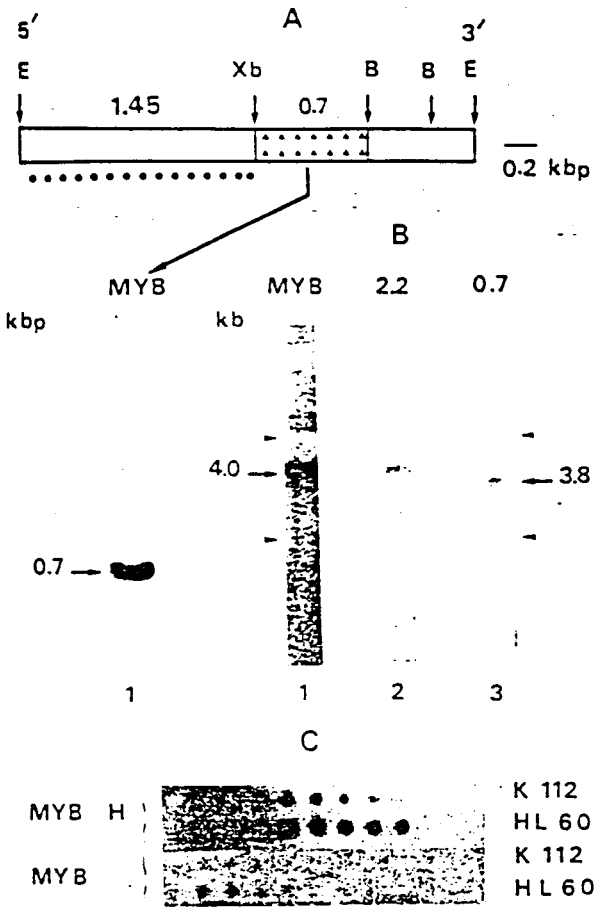

Fig. 5. Chicken and human *c-myb* mRNA transcripts. (A) The 2.8-kbp *Eco*RI fragment of clone λ HM-1 was subcloned into plasmid pKH 47: pHM-2.8. This plasmid DNA was digested simultaneously by *Eco*RI (E), *Bam*HI (B) and *Xba*I (Xb), separated by agarose gel electrophoresis and transferred to nitrocellulose. A single 0.7-kbp fragment (▲▲) hybridized with cDNA myb (lane 1) and was used as a human *c-myb*-specific probe hereafter. It contained no repetitive sequences (● ●), unlike the *Eco*RI-*Xba*I 1.45-kbp adjacent fragment. (B) Polyadenylated RNA from the chicken lymphoid cell line MSB1 (lanes 1 and 2) was denatured with glyoxal, separated by agarose gel electrophoresis, bound to activated DBM paper and hybridized with cDNA myb (lane 1) or with ^{32}P-labelled *c-myb* (chicken) 2.2-kbp *Eco*RI fragment of λ CM-5 (lane 2). Similarly, RNA from the human erythroid cell line K562 was treated as above and hybridized with ^{32}P-labelled 0.7-kbp *c-myb* (human) fragment described in (A) (lane 3). Arrow-heads indicate the 28S and 18S rRNA markers. (C) Decreasing amounts (2.5 time dilutions) of polyadenylated RNA from human hematopoietic cell lines K112 and HL60 were applied to nitrocellulose paper as described in Materials and methods. The bound RNA was hybridized with nick-translated 0.7-kbp DNA fragment (10^6 c.p.m.) from plasmid pKH HM-2.8, in stringent conditions (24 h at 41°C, hybridization buffer containing 3 x SSC, washed in 0.1 x SSC at 50°C, 4 times, 10 min) and the blot dried and exposed to X-ray film for 1 day at −70°C (MYB H). After dehybridization, the blot was rehybridized with viral cDNA myb (10^6 c.p.m.) but in non-stringent conditions (24 h 41°C, hybridization buffer containing 7.5 x SSC, washed in 2 x SSC at 41°C, 4 times, 10 min), then dried and exposed similarly (MYB).

4.0-kb RNA. We do not know if those portions of the *c-m* locus have been phylogenetically conserved. We also do n understand how a cellular gene like *c-myb* became an o cogene in the virus. Research will have to concentrate three possible mechanisms, not mutually exclusive: (1) an creased level of transcription due to the viral promoter; (2 virally truncated *c-myb* gene; (3) a *c-myb* gene mutated with the virus.

The human *c-myb* RNA was revealed using a cloned human *c-myb* probe (0.7 kbp *Bam*HI-*Xba*I fragment of the phage λ HM-1, see Figure 4) devoid of repetitive sequences, that gave at least a 5 – 10 times better signal as compared with the *v-myb* probe (see Figure 5). This human *c-myb*-specific probe should prove to be a valuable and sensitive reagent to analyse the size and accumulation of *c-myb* transcripts in many types of human cells or tumours by Northern blotting or *in situ* hybridization. It should also facilitate studies at the DNA level: for example, the *c-myb* locus has recently been mapped to human chromosome 6 q22-24 (Harper *et al.*, 1983). Translocations/deletions possibly involving this gene have been described in acute lymphoid leukemia (ALL) patients (deletions in bands 6q21-q25 or in ovarian carcinomas (translocation 6:14 with break point at 6q21) (Rowley, 1983). The availability of human specific *c-myb* probes will facilitate future studies.

Materials and methods

Cells, viruses and cloned DNA fragments

Plasma from AMV-infected birds was obtained from J.Beard (Life Science Inc.).

MDCC-MSB1 is a chicken lymphoid T-type cell line obtained by *in vitro* transformation with MDV (Nazerian and Sharma, 1979), K562 a human erythroid cell line (Lozio and Lozio, 1975), HL60, a promyelocytic cell line (Collins *et al.*, 1977) and K112, an Epstein-Barr virus-positive cell line originating from an acute granulocytic leukemia patient (A.Karpas, personal communication). The chicken and human DNA libraries were obtained through the courtesy of Dodgson *et al.* (1979) and Maniatis *et al.* (1978), respectively.

Preparation of ^{32}P virus-specific cDNAs

Representative radioactive cDNA (cDNArep) was synthesized on a 70S td PrB viral RNA template, using exogenous AMV reverse transcriptase (J.Beard, Life Science Inc., through the auspices of the Office of Program Resources and Logistics, NCI) and sonicated calf thymus DNA as primer. cDNA myb specific for AMV sequences, was selected from AMV [^{32}P]BAI-A representative cDNA by subtractive hybridization as previously described (Roussel *et al.*, 1979; Gonda *et al.*, 1981).

Isolation of chicken and human, v-myb homologous recombinant phages

Random libraries of chicken or human DNA fragments obtained by partial *Alu*I-*Hae*III digestion, and inserted with artificial *Eco*RI linkers in the *Eco*RI site of the EK2 certified vector Charon 4A, were screened with ^{32}P-labelled cDNA myb and cDNA rep probes according to Benton and Davis (1977). myb-positive, rep-negative plaques were purified twice (Maniatis *et al.*, 1978) and grown to high titer on *Escherichia coli* strain DP50.

Preparation of DNAs

Normal chicken high mol. wt. (HMW) DNA was obtained from primary cultures of chicken embryo fibroblasts prepared from 11-day-old fertile eggs (Brown Leghorn, Institut Gustave Roussy, Villejuif, France) following a classical procedure (Saule *et al.*, 1982). Human HMW DNAs was obtained by the same procedure from total human placenta. High yields of plasmid DNA were recovered from chloramphenicol (150 μg/ml) treated bacteria (12 h at 37°C) after induction of cell lysis by lysozyme (1 mg/ml) and Triton X-100 (0.1 w/v) treatment (Perbal *et al.*, 1982a). Recombinant phage DNAs were prepared as described by Saule *et al.* (1982).

Preparation of ^{32}P-labelled DNA fragments

Chicken or human *c-myb* fragments were purified twice by preparative gel electrophoresis after digestion with the appropriate enzyme(s), recovered from the gel by one cycle of freeze-thawing at −70°C and labelled by nick-translation in the presence of [^{32}P]dCTP using a New England Nuclear nick-translation kit. Specific activities of the probes were $50-100 \times 10^6$ c.p.m./μg of DNA.

Human repetitive DNA probe

DNA from human placenta was sonicated to an average size of 4 – 6S, denatured and re-annealed to a C_0t of 400 mol.s/l, then fractionated on hydroxylapatite. The double-stranded DNA recovered was dialyzed, ethanol-precipitated and used for nick-translation labelling.

Subcloning of c-myb EcoRI DNA fragments

After digestion of cloned recombinant phage DNAs by *Eco*RI, the fragments to be subcloned were purified by preparative agarose gel electrophoresis. *Eco*RI-digested plasmid pKH47 was treated with calf alkaline phosphatase. Fragment and vector pKH47 DNAs were ligated by T4 DNA ligase (Bethesda Research Laboratories Inc, USA) and used to transform calcium-treated cells of *E. coli* strain HB 101. Transformants containing the appropriate recombinant plasmids were detected by colony hybridization using different ^{32}P-labelled fragments as probes and purified twice on agar plates before large scale cultures.

Restriction mapping

Restriction endonucleases were obtained from Boehringer (Mannheim, FRG) and BRL Inc. Digested DNA fragments were size separated by electrophoresis in horizontal agarose gels and transferred to nitrocellulose according to Southern (1975).

Hybridization of blots to ^{32}P-labelled DNA, washing, and autoradiography at −70°C using Kodak X-ray films, Dupont Lightning Plus X-ray intensifying screens, were performed as previously reported (Saule *et al.*, 1982).

Agarose gel electrophoresis and transfer-hybridization of polyadenylated cellular RNAs

After extraction of total cellular RNA, polyadenylated RNAs were isolated by chromatography on oligo(dT)-cellulose (T3, Collaborative Research). RNA samples were then denatured by a glyoxal/dimethyl sulphoxide treatment and submitted to electrophoresis in 1% agarose horizontal gels in phosphate 0.02 M pH 6.8 buffer. Size-separated RNAs were transferred to diazobenzyloxymethyl (DBM) paper, hybridized with ^{32}P-labelled DNA fragments, washed and submitted to autoradiography (Saule *et al.*, 1982).

Dot blot hybridizations

These were performed essentially as described by Thomas (1980). 2.5 serial RNA dilutions were spotted onto nitrocellulose, the first spot representing −0.8 μg of polyadenylated RNA. Hybridization, washing and autoradiography were carried out as described above for RNA transferred from gels.

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III - TROISIEME PARTIE: FONCTION DES ONCOGENES

Dans cette section des résultats nous avons regroupé des articles qui portent sur l'étude de la fonction des oncogènes cellulaires et viraux que nous avons caractérisés. Nous avons utilisé deux approches complémentaires pour aborder la fonction de ces oncogènes :

1) mesurer le taux d'ARN correspondant aux c-onc dans différents types cellulaires en espérant retirer ainsi quelques indications sur le rôle de ces gènes (dans la mitose et la différenciation cellulaire);

2) introduire le gène cloné d'un c-onc dans une cellule normale afin de déterminer son potentiel transformant.

L'article N°12 présente une étude portant sur le taux d'ARN c-myc, c-myb et c-erb accumulés dans différents types de cellules hématopoïétiques aviaires. Les résultats obtenus montrent que le taux d'ARN des gènes c-myc et c-myb est élevé dans les cellules hématopoïétiques normales immatures et bas dans les cellules hématopoïétiques matures, c-erb (A + B dans cette étude) restant constant dans les deux cas.Les cellules matures et immatures ont pu être séparées à partir de la moëlle de poulets de 15 jours sur la base de leur différence de densité dans un gradient de percoll. Une étude menée sur l'accumulation de l'ARN de ces c-onc selon le type cellulaire (érythroïde, myéloïde ou lymphoïde) montre que ces trois lignées (dans ce cas nous avons utilisé des cellules transformées) sont capables d'accumuler les ARN c-myc et c-myb (Table X). La taille des ARN correspondants à ces différents c-onc, étudiée par la technique du northern blot, est la même dans tous les types cellulaires ce qui indique que le mécanisme d'épissure de ces gènes n'est pas influencé par les processus de différenciation. Nos résultats suggèrent que le mécanisme de transformation utilisé par les DLV n'est pas lié au blocage de différenciation d'une cellule exprimant le c-onc correspondant (par exemple MC29 aurait pu bloquer la différenciation des cellules hématopoïétiques exprimant l'oncogène c-myc en introduisant dans ces cellules le produit de v-myc).

Ainsi, les cellules hématopoïétiques immatures accumulent de l'ARN c-myc et c-myb à des taux 5 à 20 fois supérieurs aux taux d'accumulation décelables dans des cellules aviaires fibroblastiques ou épithéliales. L'ARN correspondant aux oncogènes erbA et erbB semble présent à un taux de base (1 à 3 copies/cellule) dans tous les types cellulaires analysés. Toutefois, en utilisant la technique d'hybridation *in situ* (cette technique, qui consiste à hybrider la sonde directement sur les cellules fixées, permet d'observer l'expression d'un gène dans une seule cellule, ce qui peut être essentiel si quelques cellules seulement expriment le gène recherché) nous avons pu mettre en évidence dans la moëlle osseuse de poulet une population restreinte (0.15% des cellules) qui fixe préférentiellement la sonde erb. Cela suggère que l'ARN des oncogènes c-erbA et/ou c-erbB est présent dans une sous-fraction des cellules hématopoïétiques que nous n'avons pu déterminer.

Si ces oncogènes sont impliqués dans la transformation cellulaire, il doit être possible de mettre en évidence dans certaines cellules transformées une accumulation d'ARN correspondant à ces c-onc. L'article N°13 rapporte une étude menée sur le taux d'expression d'ARN c-myc, c-myb

Type cellulaire	Nombre de copies par cellule des gènes: c-erb (A+B)	c-myc	c-myb	Transformation par les virus contenant: v-erb (A+B)	v-myc	v-myb
Erythroïde	ND	7 à 50	12 à 20	+	−	−
Myéloïde	0,6 à 1,2	3 à 15	0,5	−	+	+
Lymphoïde	0,8 à 1	40 à 50	1 à 30	−	+	−
Fibroblastique	0,8 à 1	1 à 3	0,5 à 1	+	+	−
Hématopoïétique immature normal	$\simeq 1$	8	10	+	+	+
Hématopoïétique mature normal	$\simeq 1$	0,5	0,5	−	+	±

TABLE X : Taux d'expression des oncogènes cellulaires c-erb (A et B), c-myc et c-myb dans certaines cellules et capacité des oncogènes viraux homologues à transformer ces cellules.

et c-erb dans différentes lignées de fibroblastes de caille transformées par le méthylcholentrène (20 MCA). Deux des cinq lignées étudiées (MCA 1-4 et MCA 3-5) contiennent des quantités d'ARN c-myc (4 et 10 fois plus que les 3 autres lignées QT4, QT5 et QT6) similaires à celles des cellules hématopoïétiques étudiées dans l'article 12. Le clonage moléculaire du gène c-myc des cellules MCA 3-5 nous a permis de montrer que l'expression de ce gène n'est pas liée à des remaniements de son locus par translocation ou amplification (contrairement à ce qui est trouvé dans la littérature pour des cellules transformées exprimant c-myc). Nous avons pu montrer que le gène c-myc des cellules MCA 3-5 et MCA 1-4 différait des autres gènes c-myc par une réduction du nombre des cytosines méthylées.

En effet, la méthylation de l'ADN eucaryote se fait essentiellement sur les cytosines (5 méthyl-cytosine) précédant les guanines (5' *CpG3') et ce type de modification de l'ADN semble jouer un rôle important dans la régulation de la transcription des gènes (Doerfler, 1981). Nous avons abordé le problème de la méthylation du gène c-myc en utilisant la couple d'enzymes HpaII-Msp1 qui reconnait le motif CCGG. L'enzyme HpaII ne clive pas l'ADN si la cytosine interne est méthylée (C*CGG). Cela nous a permis de mettre en évidence une hypométhylation spécifique du gène c-myc des cellules MCA 1-4 et MCA 3-5 sur l'un des deux allèles. L'utilisation simultanée d'enzymes de restriction (tels EcoR1, HindIII et XbaI) avec HpaII ou MspI nous à permis de localiser les sites MspI hypométhylés dans le locus c-myc. Ainsi, nous avons pu déterminer que deux sites CCGG encadrant l'exon 3' étaient hypométhylés dans les cellules MCA 3-5 et MCA 1-4. Toutefois, la technique utilisée pour étudier la méthylation de ce gène (utilisation du couple HpaII-MspI, et de sondes moléculaires définies) ne rend probablement compte que d'une toute petite fraction des méthylations affectant ce locus. Cette observation suggère que le taux d'ARN c-myc observé dans ces cellules est notamment dû à une activation transcriptionnelle du gène. Ainsi, les cellules fibroblastiques de cailles transformées par le 20 MCA ne sont pas toutes équivalentes vis à vis de l'expression d'oncogènes définis.

Cependant, la mesure du taux d'expression d'un c-onc dans une cellule normale ou transformée, ne permet pas d'accéder à la fonction de ce gène. Nous avons entrepris de déterminer si le produit du gène MYC pouvait transformer les cellules normales en cas de sur-expression, ou si des mutations somatiques étaient nécessaires pour qu'il devienne transformant. De fait, l'étude de la structure et de l'expression du gène MYC dans des cellules transformées retrouve ces deux éléments (sur-expression et/ou mutations somatiques) dans certaines cellules transformées. L'activité transformante de l'oncogène v-myc (donc ayant accumulé toutes modifications activatrices) ne se détecte pas dans le système classique de transfection sur NIH 3T3 (Figure 14), ce qui introduit une difficulté supplémentaire dans l'étude des facteurs activateurs de cet oncogène.

Nous avons vu que le pouvoir transformant du gène v-myc est révélé sans ambiguïté par des fibroblastes embryonnaires aviaires (seule espèce animale ayant produit naturellement des rétrovirus oncogènes ayant recombiné avec ce gène). Ainsi, nous avons choisi de transfecter des molécules hybrides contenant les séquences activatrices de la transcription d'un double LTR rétroviral aviaire, fusionnées au gène MYC dont la séquence nucléotidique complète a été déterminée (Gazin et al.,

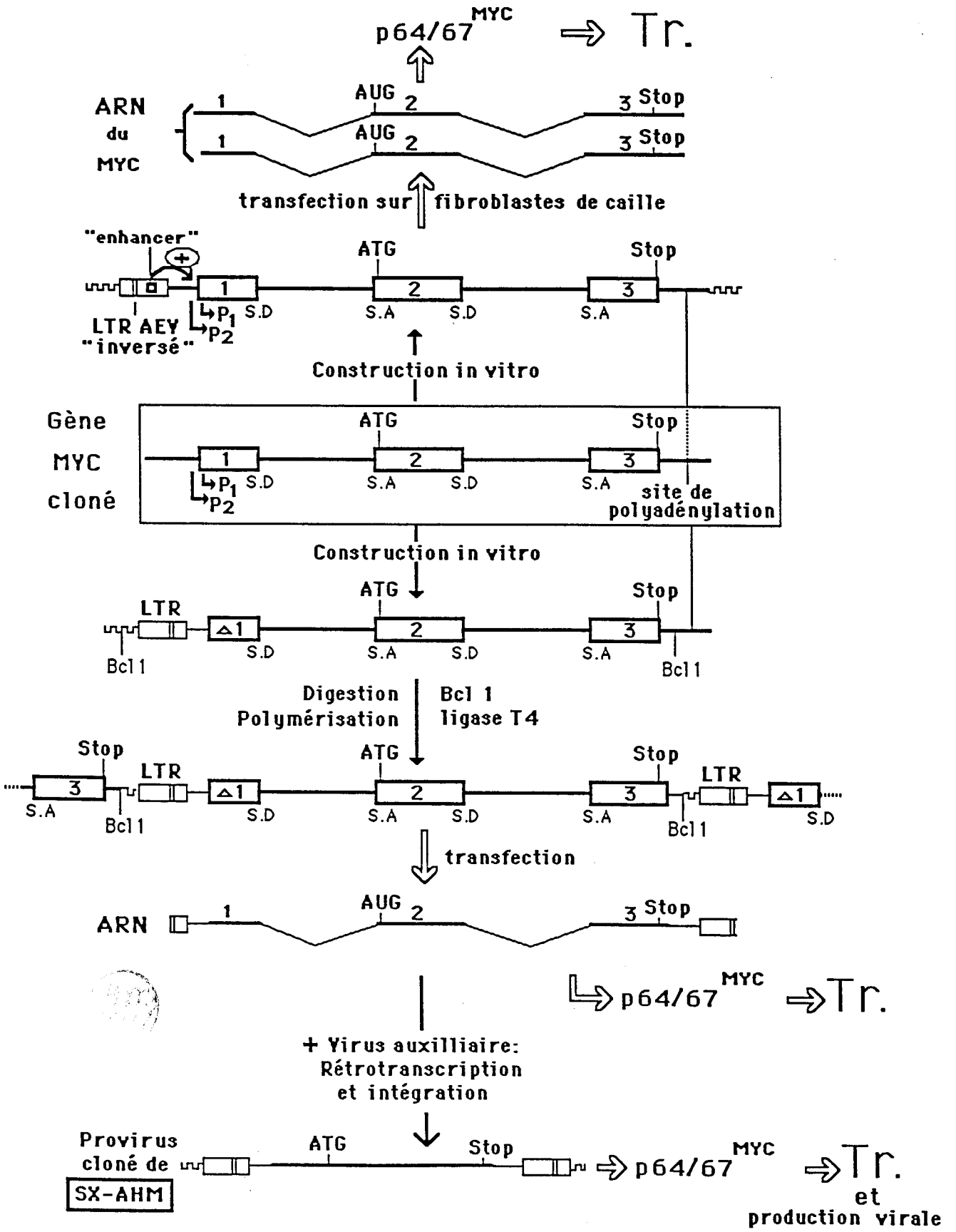

Figure 26 : Activation du gène MYC "in vitro".

1984). Si une simple sur-expression de la protéine humaine est capable de transformer les cellules, on peut s'attendre à obtenir des foyers de cellules transformées en transfectant l'ADN recombinant sur les cellules aviaires normales. Les résultats que nous avons obtenus sont réunis dans l'article N°14. Pour réaliser cette étude, nous avons construit 3 types de molécules hybrides : des molécules contenant la totalité de l'exon 1, des molécules ne contenant qu'une portion de cet exon, et des molécules dans lesquelles ces séquences sont absentes. Dans les deux derniers cas, la construction de ces recombinants nous a permis de fabriquer un rétrovirus capable de se propager. L'exon 1 a été conservé ou supprimé dans certaines constructions à cause de son influence présumée sur l'expression du produit du gène (Saito et al., 1983 ; Piechazyck et al., 1985).

La molécule recombinante contenant la totalité de l'exon 1 est construite en respectant le plus possible les signaux fonctionnels du gène. Ainsi, ce sont les propres promoteurs du gène qui sont chargés d'initier la synthèse des ARN, leur "mise en route" étant sous le contrôle des séquences activatrices contenues dans le LTR du virus AEV placé en position 3'-5' au début du gène. Une structure de type proviral est possible pour les molécules recombinantes dans lesquelles le LTR du virus AEV a été placé dans le sens 5'-3'. Grâce à l'existence d'un site unique d'enzyme de restriction Bcl1 présent dans le fragment du gène env du virus AEV (en dehors des séquences vitales du virus) et en 5' du signal de polyadénylation du gène MYC, nous avons pu polymériser à l'aide de la ligase du phage T4 l'ADN des molécules hybrides digérées par cet enzyme. Cette polymérisation a conduit à la construction de molécules du type décrit dans la Figure 26 permettant la synthèse d'ARN capables de s'intégrer dans le génome après rétro-transcription par la réverse transcriptase d'un virus auxiliaire. En l'absence d'un tel virus, ces molécules d'ADN en s'intégrant directement dans le génome permettent la synthèse d'ARN mais non de particules virales. Nous avons analysés pour chacunes des molécules recombinantes fabriquées, les ARN et protéines synthétisés dans les cellules après introduction par transfection de l'ADN chimérique.

Les résultats montrent que toutes ces molécules permettent l'expression de protéines transformantes. Les fibroblastes embryonnaires de caille se transforment et les ARN attendus d'après les signaux (promoteurs, signaux donneurs et accepteurs d'épissure, signaux de polyadénylation) présents sur les molécules recombinées sont retrouvés dans les cellules. Toutefois, l'ARN synthétisé par le recombinant vSm-AHM (v pour virus, Sm pour SmaI, site enzymatique utilisé dans l'ADN du virus AEV et du gène MYC pour réaliser le recombinant et AHM pour activated human myc) montre que des deux ARN prédits (3.2 et 2.8 kb, voir Figure 1 de l'article 14) seul le 2.8 kb est retrouvé dans les cellules transformées par la propagation de ce virus recombinant. Cela s'explique peut-être par l'existence de l'AUG du gène gag du virus AEV situé hors de la phase de lecture correcte du gène MYC dans la molécule d'ARN à 3.2 kb. Cet AUG pourrait ainsi capter les ribosomes et détourner cette molécule d'ARN des fonctions transformantes, ce qui la défavoriserait dans la culture cellulaire.

Un deuxième clone moléculaire exprime un ARN inattendu, il s'agit du clone vSX-AHM (la construction utilise le site Sac1(S) présent au début de la séquence L du virus AEV, avant l'AUG initiateur, et le site Xho1(X) présent au début de l'exon 1 du gène MYC). Dans ce cas, au lieu de

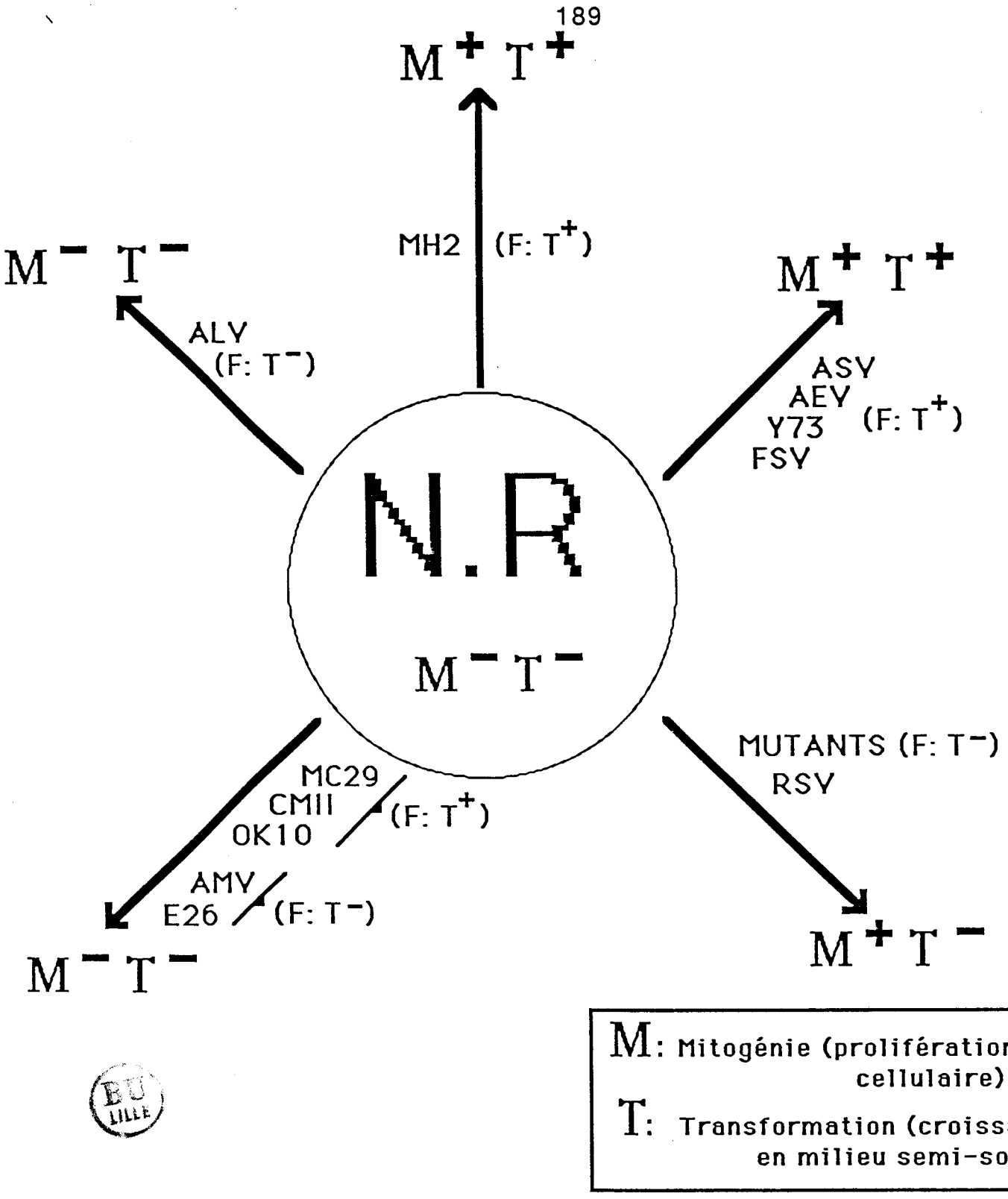

Figure 27 : Comportement des cellules de neurorétine(N.R aviaires infectées par différents virus.
F: Firoblastes.

l'ARN à 3.2 kb attendu, nous avons observé dans les cellules productrices de virus un ARN à 2.8 kb. Le clonage moléculaire réalisé pour caractériser la séquence MYC (Figure 5 de l'article 14) montre que cette molécule a été amputée, probablement au cours du processus de transfection, de 300 bp dans la séquence env résiduelle du virus AEV. Cette amputation n'affecte aucun des signaux importants de ce virus recombinant.

L'analyse des protéines exprimées dans les cellules transformées par les différents clones montre que deux types de produits peuvent être synthétisés : quand le premier AUG en phase avec la séquence MYC est l'AUG du gène, le doublet p64-67 kd décrit par Hann (Hann et Eisenman, 1984) est observé (clones pEP-AHM et vSx-AHM ; le clone pEP-AHM correspond à la construction dans laquelle les seuls promoteurs du gène humain sont utilisés, Figure 26). Quand le premier AUG en phase se trouve être l'AUG du gène gag (le cadre de lecture correct avec les séquences MYC est retrouvé après épissage de l'exon 1 et raboutage des 6 acides aminés du gène gag) une protéine de 66 kd est observée (clones vX-AHM et vSm-AHM). Ces deux types de protéines sont transformants de façon équivalente, ce qui suggère que les déterminants du gène gag ne sont pas essentiels. Ainsi, une sur-expression du produit du gène MYC suffit à transformer des fibroblastes embryonnaires de caille.

Afin de vérifier qu'aucune mutation n'était apparue dans la séquence codante de ce gène, la séquence nucléotidique de la portion codante du clone vSX-AHM a été déterminée. Cette séquence prouve l'absence de mutation . Le clone moléculaire, isolé de l'ADN des cellules transformées par le virus vSX-AHM est également transformant, de la même façon qu'un clone moléculaire du virus MC29. Ces résultats prouvent qu'une sur-expression du produit normal de ce gène suffit à transformer des cellules aviaires. Des expériences réalisées *in vivo*, en collaboration avec F.Dieterlein ont révélé que ce virus était tumorigène chez le poulet de façon similaire au virus MC29 utilisé dans les mêmes conditions.

Certains rétrovirus aviaires contiennent deux oncogènes (situation unique au sein des rétrovirus animaux). Nous avons vu précédemment que l'établissement de la transformation cellulaire, de part la multiplicité des mécanismes biochimiques permettant de l'atteindre, pouvait nécessiter l'implication de plus d'un oncogène. Ainsi, on pouvait supposer que les virus AEV, E26 et MH2 n'avaient pas fortuitement recombiné avec deux oncogènes cellulaires. Nous nous sommes intéressés à la biologie de MH2 (contenant les oncogènes mil et myc) en essayant de mettre en évidence un rôle biologique pour v-mil dans ce virus. En effet, le virus MC29 ne contenant que l'oncogène v-myc est tout à fait transformant, et *in vivo*, seul un pouvoir métastatique élevé a pu être associé aux tumeurs induites par MH2 et non un type histologique particulier (Linial, 1982).

Or, un modèle cellulaire *in vitro* développé par G.Calothy semblait singulariser MH2 au sein des virus contenant l'oncogène myc (CM2, OK10 et MC29). En effet, les cellules de neurorétines d'embryons de 7 jours de poules ou de cailles, maintenues en survie prolifèrent, et sont transformées par le virus MH2 alors que ces mêmes cellules ne réagissent pas dans les mêmes conditions à une infection par les virus MC29 ou apparentés (Figure 27). La différence de comportement observée, pour les cellules infectées par l'un ou l'autre de ces virus pouvait

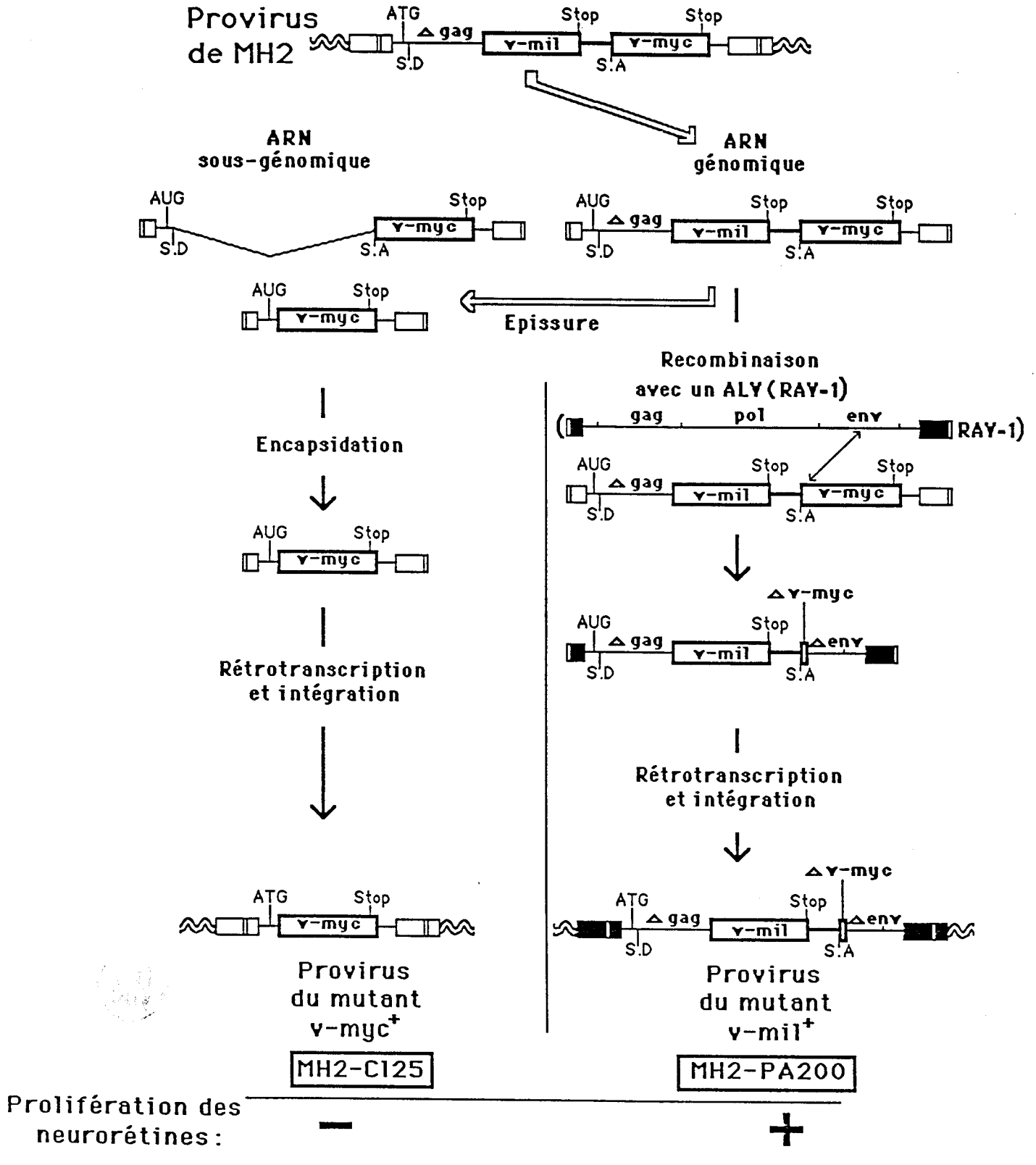

Figure 28 : Structure des ARN du virus MH2 dans une cellule infectée et caractérisation des mutants isolés biologiquement, MH2-C125 et MH2-PA200

s'expliquer soit par l'existence au sein de MH2 d'un allèle du gène v-myc particulier, soit par l'action du produit du gène v-mil, soit par une action conjointe des produits des deux oncogènes du virus MH2.

Les articles 15 et 16 présentent les résultats que nous avons obtenus dans le but de caractériser au sein du virus MH2 les rôles respectifs de mil et myc dans la prolifération/transformation des cellules de neurorétines aviaires. Le provirus MH2 étant cloné (Coll et al.,1983b), des mutants de ce virus ont été construits *in vitro*, en amputant des portions définies de ce provirus à l'aide des techniques du génie génétique. Ainsi, deux types de virus ont été créés: des virus mil+ myc-, et des virus mil- myc+. L'activité des clones moléculaires résultant de ces manipulations a été contrôlée par transfection sur des cultures de cellules de neurorétines ou de fibroblastes aviaires. Nous avons ainsi pu montrer que le virus ayant perdu myc (MH2LI-200) avait conservé la capacité d'induire la prolifération des neurorétines, alors que le virus ayant perdu mil, (MH2OB) s'avérait sans effet sur ces cellules. Ces résultats sont confirmés par l'analyse de deux mutants spontanés (isolats biologiques) du virus MH2 clonés par G. Calothy et son équipe.

Un premier lot de virus (MH2CL25, et MH2CL16) avait perdu la capacité d'induire la prolifération des neurorétines mais possédait toujours la capacité de transformer les fibroblastes. Un deuxième type de mutants (MH2PA-200) présentait le même spectre d'activité biologique que le virus LI-200. L'analyse des ARN viraux contenus dans les cellules infectées par l'un ou l'autre de ces mutants (Figure 3 de l'article 15) suggérait que les virus MH2CL25 et CL16 avaient perdu mil alors que le virus MH2PA-200 avait perdu myc (Figure 28).

Le clonage moléculaire du mutant MH2CL25 est décrit dans l'article 5. Ainsi que nous l'avons vu ce virus correspond à 1' ARN 2.8 kb du virus MH2, et codant pour les protéines myc. Ainsi, ce n'est pas un mutant à proprement parler, mais un nouveau type de virus; ce type de particule représente environ 10% d'un stock normal de MH2.

L'article N°16 présente le clonage moléculaire du virus MH2PA-200 et la caractérisation de son génome. Pour réaliser ce clonage nous avons constuit une banque d'ADN de cellules de neurorétines de caille proliférant sous l'effet de ce virus biologiquement cloné. Cette banque (l'ADN digéré partiellement par l'enzyme EcoR1 est inséré dans les sites EcoR1 de l' ADN du phage charon 4A) est criblée après étalement à l'aide d'une sonde v-mil et d'une sonde correspondant à la moitié 3' du gène env du virus auxiliaire. L'ARN viral contient en effet des séquences dérivées de ce gène. Le phage MH2PA-200 isolé de cette façon est décrit en détail dans la Figure 1 de l'article 16. La carte fine de ce virus révèle que le génome du MH2PA-200 est le fruit d'une recombinaison entre les virus RAV1 et MH2. En effet, les LTR et les séquences nucléotidiques dérivées du gène gag et du gène env proviennent du virus "helper" RAV1. Cette origine évidente pour env (totalement absent du virus MH2) et probable pour le LTR (le LTR du virus MH2PA-200 comme celui de RAV1 ne possède pas le site Kpn1 caractéristique du virus MH2 sauvage) est moins nette pour la portion du gène gag très semblable dans ces virus.

Toutefois, l'absence d'un deuxième site Pst1 dans le gène gag des virus RAV1 et MH2 PA-200 laisse penser que cette portion du gène dérive du virus auxiliaire et non de MH2. Afin de

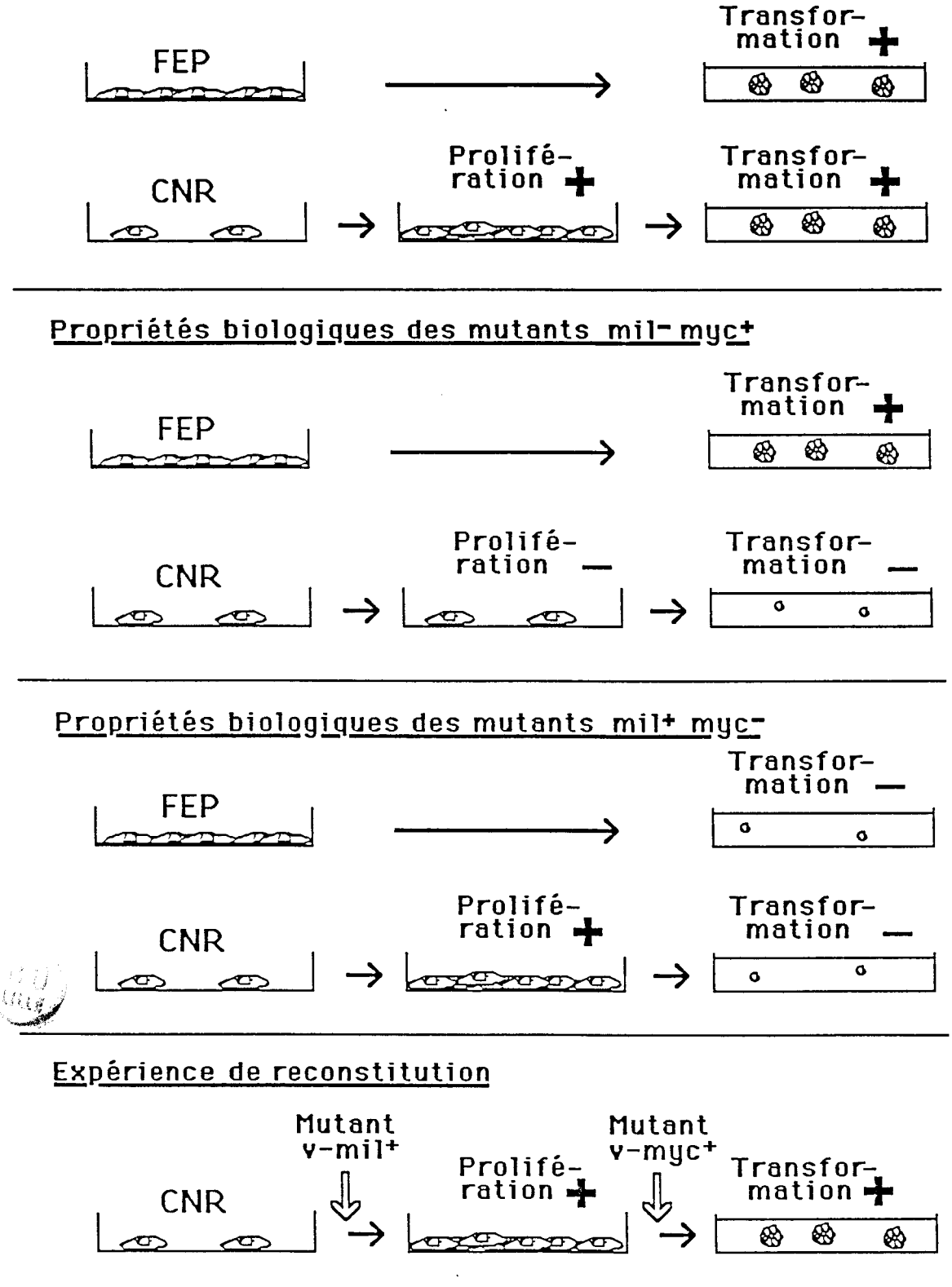

Figure 29 : Propriétés biologiques du virus MH2 et des mutants v-mil$^+$ et v-myc$^+$

préciser à quel endroit du génome la recombinaison avait eu lieu, nous avons utilisé comme sonde des fractions du gène myc. Nous avons ainsi pu montrer que le virus MH2PA-200 contenait l'extrémité 5' de ce gène et notamment le signal accepteur d'épissure, ce qui permet aux cellules infectées de produire un ARN sous-génomique contenant cette portion du gène myc, ainsi que la séquence nucléotidique résiduelle du gène env. L'isolement aisé de ce recombinant suggérait l'existence d'un facteur favorisant, soit la recombinaison, soit la sélection de ce type de virus. Cela nous a amené à étudier les virus produits par une culture de neurorétines, fraîchement infectée par le virus MH2 cloné moléculairement afin d'éliminer la possibilité que des virus de type MH2PA-200 préexistent dans nos stocks de virus.

L'analyse des acides nucléiques de ces cellules trois semaines après leur infection nous a permis de montrer l'apparition d'un nouveau virus contenant des séquences mil et env (le provirus digéré par l'enzyme EcoR1 produit un fragment contenant ces deux séquences, mais différent du fragment observé dans les cellules MH2PA-200). Ces résultats, confirmés par l'analyse de l'ARN viral, indiquaient qu'une recombinaison avait eu lieu entre le MH2 cloné moléculairement et le virus RAV1 et que le virus résultant (appelé MH2PA-201) était rapidement devenu majoritaire dans la culture de neurorétines proliférantes. Le clonage moléculaire de ce provirus nous a permis de préciser l'endroit où s'était effectuée la recombinaison en 3' de la molécule: MH2PA-201 est raccourci dans le gène myc (50 nucléotides de moins que le virus MH2PA-200) mais rallongé dans le gène env (470 nucléotides de plus).

Ces résultats ont été obtenus en réalisant la séquence nucléotidique du point de jonction myc-env de ces deux provirus. La Figure 4 de l'article 16 montre qu'il existe une certaine homologie entre ces deux séquences: 11 nucléotides identiques entre myc et env pour le virus PA-2OO et 9 pour le virus PA-201. Ces régions d'homologies pourraient être essentielles pour la génération de ces recombinants. On peut en effet supposer que cet événement se produit au cours de la rétro-transcription du génome viral (cf Figures 4 et 7). Ces séquences homologues pourraient permettre de stabiliser les hybrides ARN-ADN au cours de la rétro-transcription et permettre ainsi le "saut"de la polymérase d'une matrice (RAV1) à l'autre (MH2) puis à nouveau, en arrivant dans le gène gag retourner sur RAV1. La culture analysée n'a montré la présence que d'un seul recombinant, ce qui suggère que cet événement est rare mais que la croissance des neurorétines représente un crible positif pour le mettre en évidence. Les cellules infectées par le virus ayant perdu myc possèdent un avantage sélectif ou alors, le virus ayant recombiné se propage mieux dans la culture. Il semble en fait que ces deux éléments puissent se conjuguer pour expliquer l'émergence de ces recombinants, car lors de la surinfection de cellules de neurorétines proliférant sous l'action de v-mil par un virus contenant myc, la mortalité cellulaire devient très importante; d'autre part, le titre en particules mitogènes (virus PA-200) ou en particules transformantes (MH2 sauvage) est très différent (respectivement 10^6 et 10^3 particules /ml) ce qui suggère que le virus recombinant est bien mieux produit que le virus sauvage.

ARTICLES

12 à 16

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The Cellular Oncogenes *c-myc, c-myb* and *c-erb* Are Transcribed in Defined Types of Avian Hematopoietic Cells

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The possible role of normal chicken cellular sequences *c-erb, c-myb* and *c-myc*, together referred to as *c-onc* genes and related to the oncogenes of defective avian acute leukemia retroviruses (DLVs), was investigated by determining the accumulation of *c-onc* RNA in different avian cells an cell lines. Levels of *c-myc* and in some instances *c-myb* RNA are elevated in immature hematopoietic cells or cell lines from various lineages but more mature hematopoietic cells, as well as non-hematopoietic cells, contain only low levels. In contrast, the level of *c-erb* RNA is generally low, but high in a small number of normal bone marrow cells. The results indicate that the cellular homologues of the viral oncogenes are differentially expressed during hematopoiesis. They also indicate that the hypothesis that DLV target cells express their homologous *c-onc* genes might hold for *c-erb*, but is not valid in its simple form for *c-myc* and *c-myb*.

Defective avian leukemia viruses (DLVs) induce in susceptible chicken acute leukemias with a latency of a few weeks [1]. Three specific viral genes correlating with the type of leukemia induced and the type of cell transformed in vitro by a particular strain have been implicated in the transformation process [2, 3]. Avian erythroblastosis virus (AEV) transforms erythroblasts in vivo and in vitro and contains specific sequences termed *erb* [2, 4]. Avian myeloblastosis virus (AMV), as well as another independent viral isolate (E26) transform myeloblasts in vivo and in vitro. They contain a specific sequence termed *myb* [2, 4]. A third group of viruses, MC29, CMII, OK10 and MH2, transform macrophage-like cells in vitro and the first two strains also induce myelocytomatosis in vivo. Viruses of this group contain a specific sequence, termed *myc* (formerly denoted *mac*) [5, 2, 4]. Recent experiments conducted in quails rather than chicken, have shown a more complex oncogenic spectrum, since AEV and E26 could also induce myeloid and erythroid proliferation, respectively [6]. Although DLVs appear to transform primarily hematopoietic cells, some strains also transform fibroblasts and induce sarcomas and carcinomas (for review see [1]).

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Normal uninfected chicken cells contain nucleotide sequences closely related to all three viral oncogenes (termed *v-onc* genes). These cellular homologues, termed *c-erb*, *c-myb* and *c-myc* genes (and together referred to as *c-onc* genes) are conserved throughout the evolution of higher vertebrates [2, 4, 7, 8]. DLVs also contain sequences related to the structural genes of avian leukosis viruses (ALVs) and they thus probably arose by a recombination of cellular oncogenes with an ALV type vector [4, 9, 10, 11].

Because of the transformation specificity of DLVs in the hematopoietic system, it has been postulated that the corresponding cellular oncogenes play a role during normal hematopoiesis [2, 12]. In agreement with this, Chen reported that among several normal chicken tissues, a slightly elevated level of *c-myb* RNA was found in bone marrow, yolk sac and bursa, whereas this level was low in non-hematopoietic tissues like liver, kidney, or in normal fibroblasts [13]. It was also proposed that these genes might be expressed specifically in the hematopoietic cells corresponding to the DLV target cells ("the target cell hypothesis" [12]). The following experiments were undertaken to test these hypotheses.

MATERIALS AND METHODS

Animals and Cell Lines

Normal tissues and uninfected cells were from 2-weeks-old chicken (white Leghorn). Bone marrow cells (5×10^8 cells) of 2-weeks-old chicks were fractionated on Percoll gradients into four fractions, as described earlier [14]. Erythrocytes were from the peripheral blood, and normal macrophages were obtained as adherent cells from fresh bone-marrow in culture; fibroblasts (3rd passage) were from 11-days chicken embryo.

Unless stated otherwise, all cells described in the tables 1 and 2 originated from the chicken (white Leghorn). The quails used were *Coturnix japonica*.

Erythroid cells. AEV-transformed erythroblast cell lines HD1, HD3 and HD5 have been described by Beug et al. [3]; the erythroblast clone 4D3 originated from fresh bone-marrow cells infected with AEV and seeded in methocell semi-solid medium; this single-cell clone was picked up, amplified and used without delay. The E26 quail erythroblasts originated from the culture of spleen cells taken from an E26 (RAV1)-injected quail coming down with leukemia; these cells were of erythroid lineage since over 90% expressed the marker histone five [15]

Myeloid cells. The E26 myeloblasts were isolated from a chicken with myeloid leukemia induced by E26 (RAV-2) and cultured shortly before use. The DU65 myeloblasts were obtained similarly, but using AMV; they constitute probably an established line [16]. So do the BM2C3 and 5YS myeloblasts induced also with AMV [17] but by in vitro infection of fresh bone-marrow cells. The OK10 BM macrophage-like cells were derived by culturing the bone-marrow of a chicken diseased by the OK10 virus [18]. The cell-line HD11 was derived from fresh bone-marrow cells infected with MC29 [3].

Lymphoid cells. The MSB1 lymphoid cell line, of the T-lineage [19] originated from a bird infected by Marek's disease virus (MDV); the transplantable cell-line RP1 was obtained in a similar way [20]. The TLT and RP9 cell lines, of the B-lineage, originated from ALV-induced lymphomas [21, 22]. The TV1 lymphoid cell line was derived from a bird rendered leukemic by infection with avian reticuloendotheliosis virus (REV_T) [23]; these cells may be of pre B-pre T phenotype (H. Beug, unpublished observations). The spontaneous lymphoma in table 1 was isolated from a 2-years-old chicken by R. Friis.

Other cells. The non-producer fibroblast clones Q8NP [24] and Q5NP [25] originated from cultured quail cells transformed by MC29 and OK10 viruses, respectively. The DU 72 epithelial cell line originated from an MC29-induced hepatoma [26]. The cell line PH 310 was derived in vitro from pheasant fibroblasts transformed by AEV [27]. QT6B3 is a subclone (kindly provided by D. Spector &

J. M. Bishop) of the methyl-cholanthrene-induced quail fibroblast line QT6 [28]. This clone was superinfected by the B77 strain of ASV and cloned in soft agar to yield the clone QT6 B3-1D6 (M. Boccara & D. Stehelin, unpublished data).

Preparation of ^3H- and ^{32}P-Specific Probes

The preparation and characterization of all probes used were extensively described [2, 8]. The radiolabelled cDNA, synthesized in exogenous reactions [29] using 50–70 S-RNA template was prepared by the subtractive hybridization method [30]. The probes were specific for the four unrelated transforming genes of AEV (^{32}P or ^3H *c*DNA*erb*) AMV (^{32}P or ^3H *c*DNA*myb*) MC29 (^{32}P or ^3H *c*DNA*myc*) and ASV (^3H *c*DNA*src*). Their approximative complexities were 3.7; 2.0; 1.8 and 1.8 kb respectively. ^{32}P cDNA 5′ was prepared according to Friedrich et al. [31]. The ^{32}P probe corresponding to the *gag* region was prepared by nick translation of ASV DNA inserted into a plasmid, kindly provided by E. Stavnezer.

Nucleic Acid Hybridization

Cellular RNA for liquid hybridization studies was extracted as described previously [8]. Standard liquid hybridization reaction mixtures contained 0.6 M NaCl, 2×10^{-3} M EDTA, 2×10^{-2} M Tris hydrochloride (pH 7.4) 500 μg of calf thymus DNA per ml as carrier. Each sample contained 2000 cpm of either ^3H *c*DNA*myb*, *c*DNA*myc* or *c*DNA*src* together with 2000 cpm of ^{32}P *c*DNA*erb* as an internal control and appropriate RNAs in excess. Hybridizations were conducted in glass capillaries at 68°C and the extent of annealing was monitored by digestion with S1 nuclease. For the quantification of RNA transcripts in tables 1 and 2, one copy per cell corresponded to a Crt ½ of 4×10^4 mol. s. l^{-1}.

Numbers one were sometimes deduced from partial curves and may be accurate within a factor of two. The standard deviation on five repeated experiments with MSB1 cells was no greater than 15%.

Agarose Gel Electrophoresis of PolyA-containing Cellular RNA

PolyA-containing RNA (10 μg per lane) was denatured by glyoxal [32], separated on agarose gels and transferred to activated DBM paper [33]. Blots were hybridized as described before [8] with ca 10^6 cpm/ml of ^{32}P cDNA, washed and submitted to autoradiography.

In situ Hybridization

In situ hybridization was performed on cells cytocentrifuged onto glass slides as described by Brahic & Haase [34] with 8 μl buffer on 10^6 cells/slide and 5×10^5 cpm (10 ng) of ^3H *c*DNA*erb*. Autoradiographic exposure was 1 week; micrographs of cells counterstained with Giemsa are at final magnification of ×640.

RESULTS

c-onc Genes Are Transcribed in Immature Bone Marrow Cells and in Strains of Transformed Hematopoietic Cells

To determine whether the high level of *c-myb* transcripts observed in bone marrow cells was restricted to certain types of cells, we fractioned the cell mixture, using a Percoll density gradient, into four fractions [14]. Each fraction was then tested for its RNA content of *c-onc* genes, as shown in fig. 1. Whereas *c-erb* was found in all fractions at 1–2 copies per cell, the levels of transcripts of *c-myb* and *c-myc* were relatively high in cells of low density (blast-like cells) and decreased in the cell populations of higher density (cells at more advanced stages of differentiation).

However, the proportion of hematopoietic DLV-target cells present in normal

Table 1. *Accumulation of c-onc RNA in transformed and normal hematopoietic cells*

Cells	Transforming virus	No. of RNA copies per cell of			
		erb	*myb*	*myc*	*src*
Erythroid type					
LSCC-HD1	AEV (RAV2)	(660)	17	50	1.2
LSCC-HD5	AEV (RAV2)	(660)	13	29	1.0
4D3	AEV (RAV2)	(940)	11	40	0.9
LSCC-HD3	*ts*34 AEV (RAV2)	(400)	12	17	0.8
E26-quail erythroblasts	E26 (RAV1)	1.6	(220)	7	nd
Myeloid type					
E26 myeloblasts	E26 (RAV2)	1.2	(11)	15	1.3
LSCC-5YS	AMV	0.8	(122)	10	nd
LSCC-BM2C3	AMV	1.2	(150)	4	nd
LSCC-DU 65	AMV (MAV)	0.6	(200)	6	0.8
LSCC-OK10 BM	OK10 (OK10AV)	1.2	0.8	(400)	nd
LSCC-HD11	MC29 (RAV2)	0.8	0.5	(400)	1.2
Macrophages	None	1.0	0.5	3	0.9
Lymphoid type					
MDCC-MSB1	MDV	1.0	29	36	1.0
MDCC-RP1	MDV	0.9	1	50	nd
RECC-TV1	REV_T	1.2	1.2	36	1.0
LSCT-TLT	RPL12	0.8	2	*400*	nd
LSCC-RP9	RAV2	0.8	10	*400*	1.5
Spontaneous lymphoma (bursal, nb 4666)		1.1	0.7	4	1.0
Controls					
Chicken normal					
Spleen	None	1.5	0.5	4	1.1
Bursa	None	1.0	1	4	nd
Thymus	None	1.0	4	4	nd
Bone marrow	None	1.5	11	4	nd

For each type (see Materials and Methods), cells are listed roughly according to their known state of differentiation (from more immature to more mature). Numbers in parentheses represent transcripts corresponding to viral *onc*-genes. Numbers underlined represent cellular sequences (*c-myc*) activated by a viral promoter.

Hybridization conditions are described in Materials and Methods. Numbers represent average RNA copies per cell for each probe used, as deduced from the experimental Crt ½ values: one copy per cell taken conventionally for a Crt ½ = 4×10^{4} mol. · s · l^{-1}.
nd, Not done.

bone marrow is very small, in the order of 0.1–3% [12], and so far there are no techniques available to purify normal target cell populations in the large amount (10^7–10^8 cells) required for liquid hybridization tests. Instead, we examined the RNA levels of the *c-onc* genes in cultured transformed hematopoietic cells or cell

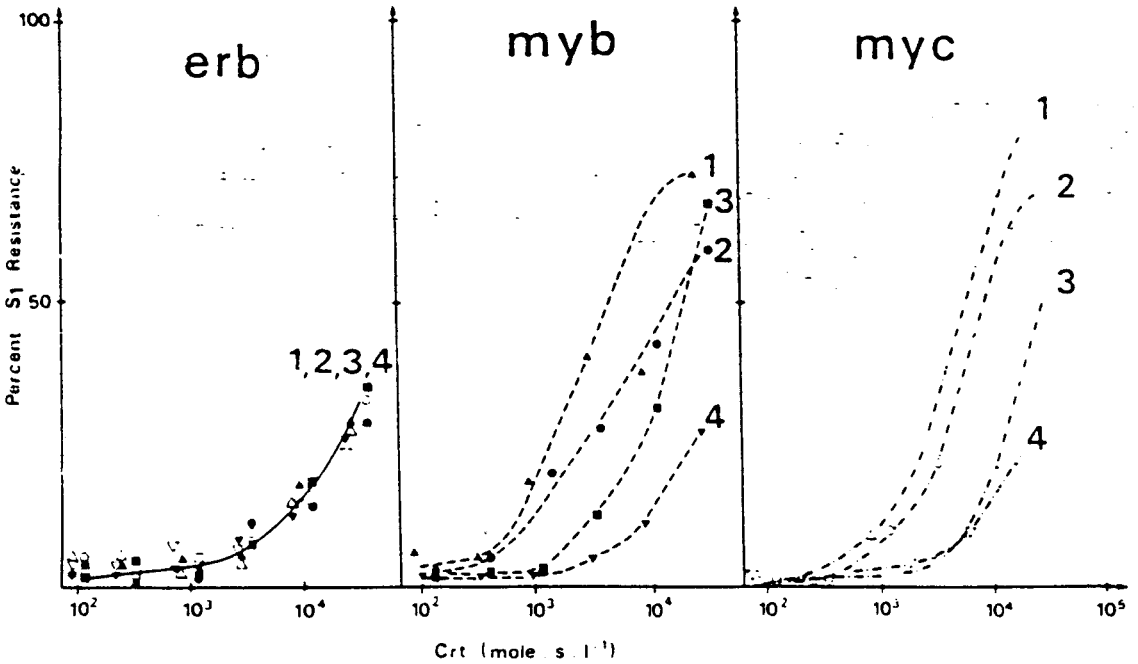

Fig. 1. Expression of *c-onc* genes in bone marrow cells fractionated according to density. Bone marrow cells (5×10^8 cells) of 2-weeks-old chicken were fractionated on Percoll gradients into five fractions, as described earlier [14]. The top fraction containing cell debris and thrombocytes was discarded. As judged from stained smears, fraction 1 contained mainly blast-like cells (▲); 2, reticulocytes (■); 3, a mixture of reticulocytes and granulocytes (●) and 4, mature erythrocytes (▼). Conditions of hybridization with cDNA *erb*, *myb*, *myc* are described in Materials and Methods. The numbers of copies per cell calculated from the curves shown are: for *c-erb* ca one in all fractions; for *c-myb* in fraction 1, 10; 2, 6; 3, 2; 4, 0.5. For *c-myc* in fr. 1, 8; 2, 6; 3.1; 4, 0.5.

lines belonging to the erythroid, myeloid or lymphoid lineages of differentiation. These cells, transformed by a variety of avian viruses, represent homogeneous populations at probably defined stages of differentiation. We also tested mature homogeneous cell populations corresponding to these lineages, where available. The results obtained after hybridization of the extracted RNAs to the various *onc*-gene-specific probes are shown in table 1.

Numbers represent average RNA copies per cell for each probe used, as deduced from the experimental Crt ½ values, with one copy per cell taken conventionally for a Crt $\frac{1}{2}=4\times10^4$ mol. s. l^{-1}. The *c-erb* sequences were found at an average level of about one copy per cell in all myeloid and lymphoid cell types tested. The fact that most of the erythroid cell clones examined were obtained by transformation of bone marrow cells with AEV precluded the measurement of the expression of the *c-erb* gene in these cells. In contrast, for erythroid proliferating cells induced with E26 in quail and exhibiting more mature phenotypes [15], as well as for mature erythrocytes, no enhanced *c-erb* transcription was seen.

For *c-myb* transcription, a strikingly different pattern was observed. Elevated levels of transcripts (11–17 copies per cell) were found in all AEV-transformed

Table 2. *Accumulation of c-onc RNA in transformed and normal non-hematopoietic cells*

Cells	Transforming virus	No. of RNA copies per cell of			
		erb	*myb*	*myc*	*src*
Transformed					
LSQC-Q8 NP	MC29	1.2	0.5	(200)	1.3
LSQC-Q5 NP	OK10	1.1	nd	(300)	1.2
LSCC-DU 72	MC29 (RAV2)	1	0.4	(1 000)	0.8
SPPC-PH 310	AEV (RAV1)	(150)	0.4	1	nd
CHQC-QT6 B3 (Methyl-cholanthrene)	None	0.5	0.4	3	1.4
QT6 B3-1D6 (Supertransformed)	B77 ASV	0.7	0.7	4	(300)
Controls					
Chicken normal					
Cultured fibroblasts	None	0.8	0.5	3	1
Muscle	None	0.9	0.6	4	0.9
Liver	None	1.2	0.6	3	1
Whole embryo	None	0.9	0.6	4	0.9

Cell types and conditions of hybridization are described in Materials and Methods. Numbers in parentheses represent transcripts from *v-onc* genes. Normal tissues were from a 2-weeks-old chicken and fibroblasts (3rd passage) from a 11-days chicken embryo (white Leghorn).
nd, Not done.

erythroid clones tested. Of interest is the fact that E26-transformed myeloblasts contained only 11 copies per cell of *myb*-related RNA sequences. This level, of an order of magnitude lower than usual retroviral transcripts, apparently suffices on one hand for the maintenance of the transformed phenotype and is comparable, on the other hand, to the levels of *c-myb* observed in AEV erythroblasts and some other cells. The two macrophage-like cell lines, transformed by the *myc*-containing viruses OK10 and MC29 did not significantly accumulate *c-myb* sequences. Finally, lymphoid cell lines transformed by different viruses contained *c-myb* sequences at variable levels ranging from 29 copies per cell in immature MSB1 lymphoblasts to ca one copy per cell in RP1 cells or a spontaneous chicken lymphoma.

The pattern observed for *c-myc* sequences presented also some interesting features (table 1). Some cells in the erythroid and lymphoid lineages accumulated elevated levels of *c-myc* (17–50 copies per cell); these are described in the literature as quite immature: AEV erythroblasts [1, 35], MSB1 T-lymphoblasts [19] and REV_T-transformed lymphoblasts [36]. Conversely, the mature cells (erythrocytes, normal macrophages) exhibited low levels (1–4 copies/cell) comparable to those published for fibroblasts [2]. A third group of cells exhibited low or intermediate levels of *c-myc*. These cells are not clearly defined in respect to

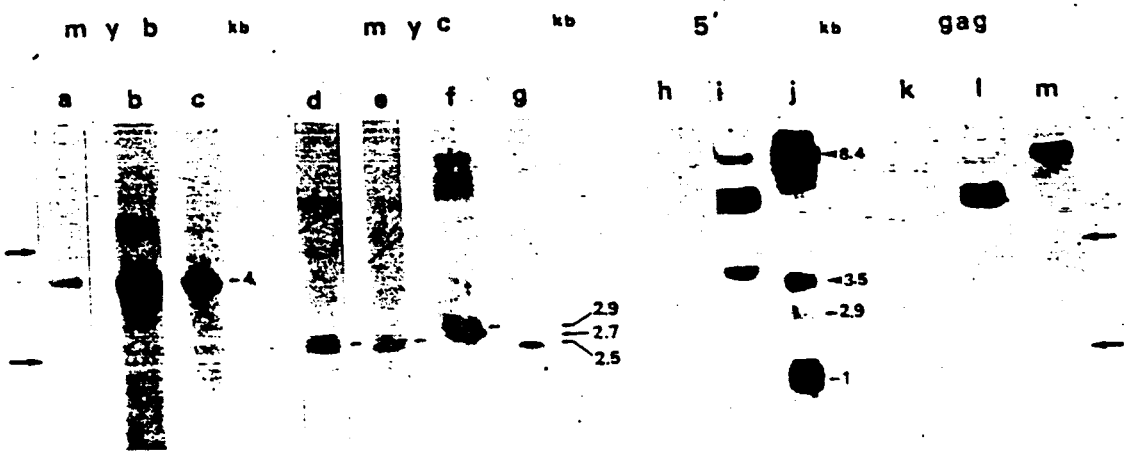

Fig. 2. Size of *c-myb* and *c-myc* transcripts in transformed chicken hematopoietic cells. Poly (A)-containing RNA was denatured, separated on agarose gels and transferred to activated DBM paper. Blots were hybridized with *a, b, c,* cDNA*myb*; or *d, e, f, g,* cDNA*myc or h, i, j,* viral cDNA5' or to *k, l, m,* a probe corresponding to the *gag* region of ASV. The different cell RNAs tested were from MSB1-cells (T-lymphoblasts, *a, d, h, k*) HD3-cells (erythroblasts, *b, e, i, l*) RP9 cells (ALV-promoted lymphoblasts, *c, f, j, m*) and TLT cells (ALV-promoted lymphoblasts, *g*). Symbols ▶, sizes of ALV-related viral products. →, ribosomal 28S and 18S markers. Numbers indicate molecular size in kb.

differentiation parameters, although E26 myeloblasts, which are probably less mature than AMV myeloblasts (H. Beug and T. Graf, unpublished data) exhibited higher levels of *c-myc*. Finally, two lympoid cell types, originating from ALV-induced bursal lymphomas (RP9 and TLT), expressed some 400 copies of the *c-myc* sequences. This is in agreement with previous reports that demonstrate that in ALV-induced bursal lymphomas *c-myc* sequences are activated by viral promoters [37, 38].

In conclusion, *c-myc* and, in some instances, *c-myb* sequences are accumulated in immature hematopoietic cells of the erythroid as well as the lymphoid lineage. Less conclusively, *c-myc* is moderately elevated in some immature myeloid cells.

Experiments performed with normal chicken (2-weeks-old) hematopoietic tissues did not in general exhibit elevated levels of *c-onc* transcripts (table 1, and [39]). This is compatible with the idea that only a minor population of cells accumulate such transcripts, which are not detectable by the liquid hybridization technique. An exception is the elevated value (11 copies per cell) observed for *c-myb* in bone marrow (and to a lesser extent in thymus). Since the fractionation done on bone marrow cells implicates a large amount of different immature cells, it remains to be documented whether in the immature bone-marrow compartment a few cells accumulate hundreds of *c-myb* transcripts, or whether a larger population of cells accumulate a moderate number of transcripts.

Sizes of c-onc Transcripts in Different Cell Types

The different pattern of expression of *c-myb* and *c-myc* observed in different hematopoietic lineages raised the question whether the corresponding mRNAs

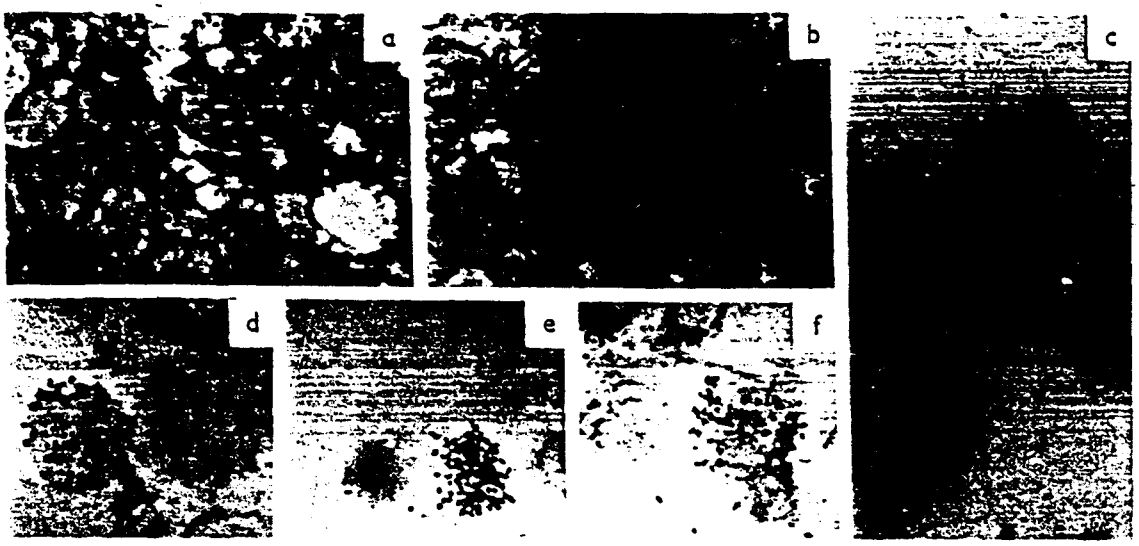

Fig. 3. In situ hybridization of chick bone marrow cells with ^{3}H *c*DNA*erb*. (*a*) AEV-transformed HD3 erythroblasts (positive control); (*b*) AMV-transformed myeloblasts (negative control); (*c, d, e, f*) uninfected bone marrow cells freshly prepared from a 2-weeks-old chick.

represented abnormally processed species. We thus examined the size of polyadenylated mRNAs extracted from lymphoid (MSB1, RP9, TLT) and erythroid (HD3) cells. The RNA transferred on DBM paper was hybridized with labelled probes representing specific cDNAs to the viral *myb* and *myc* genes, as well as to the 5′ region [31] and the structural *gag* gene of ASV RNA (Prague strain) [11]. As shown in fig. 2, with the *myb* probe, a main band of 4.0 kb was seen for MSB1, HD3 and RP9 cells (fig. 2, *a, b, c*) corresponding to the normal *c-myb* transcripts found in chicken tissues [39] and in quail fibroblasts (S. Saule and D. Stehelin, unpublished data). The *myc* probe detected a characteristic single mRNA band at 2.7 kb in both MSB1-lymphoblasts and in HD3-erythroblasts (fig. 2, *d, e*). This represented presumably the *c-myc* gene transcript, since a mRNA of similar size has been found in normal fibroblasts of chicken and quail [40]. Furthermore, for MSB1 and HD3, respectively, the *c-myb* and *c-myc* mRNAs detected were not linked to ALV-related sequences, since they did not hybridize with a 5′ cDNA (fig. 2, *h, i*) nor with a *gag*-specific probe from ASV (fig. 2, *k, l*). In contrast, ALV-induced lymphoid cells (RP9, TLT) showed abnormal sizes of the LTR-promoted *c-myc* gene (2.9 and 2.5 kb, respectively; fig. 2, *f, g*), as previously reported by Hayward and coworkers for RP9 [37], linked to the viral 5′ end (fig. 2, *j* at 2.9 kb for RP9) but not to the viral *gag* gene (fig. 2, *m* for RP9). Indeed, the *c-myb* transcripts were not initiated by a viral promoter in RP9 (no band at 4.0 in fig. 2, *j, m*) and TLT (data not shown). The additional bands, irrelevant to our discussion, seen in fig. 2, *i*, correspond to AEV-coded transcripts [8] and the ones in fig. 2, *j* to ALV-related transcripts [37]. These results show that, in the transformed hematopoietic cell lines studied, the sizes of the accumulated *c-myb* and *c-myc* mRNAs are not detectably altered. They do not indicate any accumu-

lation of abnormally processed species. It is also unlikely that these genes were activated by a viral promoter. In contrast, such modifications could easily be seen in the ALV-induced lymphoid tumor cell lines RP9 and TLT (fig. 2).

Transformation Does Not Generally Induce an Expression of c-onc Genes

The observation described above could be the result of abnormal expression of *c-onc* genes in cells, due to the transformed state. Indeed, Groudine & Weintraub [41] have shown that fibroblasts transformed by ASV, AEV and MC29 contained elevated levels of globin RNA transcripts. In RSV-transformed fibroblasts they also observed the accumulation of nuclear RNA transcripts of numerous other genes not expressed in normal fibroblasts. We thus conducted experiments to determine whether transformation had any effect on the amount of RNA transcripts of the *c-onc* genes accumulated in fibroblasts.

Table 2 shows that neither *c-erb*, *c-myb*, *c-myc* nor *c-src* sequences were induced in fibroblasts transformed by MC29, by OK10 or by AEV. Even chemically transformed quail fibroblasts, before (QT6 B3) and after (QT6 B3-1D6) supertransformation with an avian sarcoma virus, behaved normally in this respect.

In addition, the hepatoma cell line (DU 72) transformed by MC29 also showed no enhanced accumulation of the *c-onc* genes. These levels where close to those observed in normal tissues or cultured cells (table 2). We thus conclude that it is unlikely that the observation made by Groudine & Weintraub for the globin gene holds true for the *c-onc* genes studied.

The c-erb Gene is Expressed in a Minority of Normal Hematopoietic Cells

In contrast to the significant accumulation of the *c-myb* and *c-myc* genes in immature hematopoietic cells, none of the hematopoietic tissues tested contained *c-erb* transcripts above a "background" level of 1–2 copies per cell. To determine if in hematopoietic tissues a small proportion of the cell population expressed the *c-erb* gene, we used in situ hybridization, a technique allowing us to detect mRNA accumulation in individual cells. For this purpose, fresh bone marrow cells were applied to a microscope slide, fixed and annealed with cDNA*erb*. AEV-transformed myeloblasts served as negative controls. As shown in fig. 3, AEV-transformed cells (fig. 3 *a*) exhibited an average of about 15 grains per cell whereas AMV-transformed cells (fig. 3 *b*) only showed background levels of grains (no more than a maximum of 5 grains per cell in approx. 50 000 cells screened). In contrast, a few normal bone marrow cells showed (fig. 3, *d*, *e*, *f*) a strong hybridization with cDNA*erb*. Out of some 40 000 cells screened, 56 cells had over 50 grains and were considered unambiguously positive (fig. 3 *c* shows negative bone marrow cells). As estimated according to Brahic & Haase [34] these cells contained about 20–50 copies per cell of *c-erb* RNA. Because of the low proportion of such cells these transcripts were not detected by liquid hybridization experiments.

DISCUSSION

Transformed Cells Can be Used to Study the RNA Levels of c-onc Genes

To study the RNA levels of *c-onc* genes in hematopoietic cells, we have used cells transformed by different means. Such cells probably mimic stages corresponding roughly to those proceeding during normal differentiation [1]. Our results indicate that transformation as such does not generally induce the expression of *c-onc* genes (table 2). In a same given cell culture (MSB1, table 1), some *c-onc* genes are found at elevated levels (*c-myc*, *c-myb*), whereas others (*c-erb*, *c-src*) are tightly controlled. Conversely, *c-myb* and/or *c-myc* can be found at low levels in some of the transformed hematopoietic cells (table 1). Incidentally, the induction of some *c-onc* genes does not correlate with the establishment of cell lines or immortalization, since some cell lines (table 1) do not accumulate some *c-onc* transcripts (DU 65; OK10 BM), whereas fresh non-established clones show elevated levels (4D3).

A Cell Can Express a Given c-onc Without Being a Target for the v-onc

It has been proposed that the target cells for the *c-onc* genes of DLVs could be cells expressing their cellular homologues [12]. Such a proposition covers in fact two distincts aspects. (1) Is it necessary for a target cell to a given *v-onc* to express the homologous *c-onc?* (2) Is it sufficient for a cell to express a given *c-onc* to become a target for the homologous *v-onc?* Our results clearly speak against the latter proposition: some cells of the erythroid and lymphoid lineages express elevated levels of *c-myc* and/or *c-myb*. Such cells are not targets for viruses containing the homologous sequences, like MC29 or OK10 (*v-myc*) and AMV or E26 (*v-myb*), which in fact transform cells of the myeloid lineage. Thus, hematopoietic cells can express a given *c-onc* without being targets for the homologous *v-onc*. Coming back to the former proposition, our results for *c-myc* and *c-myb* do not indicate, nor do they rule out, the possibility that target cells for a given *v-onc* are cells expressing the homologous *c-onc*. Experiments along this line are hampered by the observation that usually only a small portion of a given cell population can be transformed by a given DLV. For example, chicken macrophages do not show elevated levels of *c-myc* and *c-myb* transcripts. But a few of these cells could indeed express high levels of such transcripts, undetectable by liquid hybridization experiments performed on the whole cell population, and could account for the few percent of targets readily transformed by AMV- and MC29-like viruses [42]. In situ hybridization experiments should solve the issue but were not achieved so far because of technical problems with *myc* and *myb* probes.

Turning now to *c-erb*, its pattern is quite distinct from that of the two other *c-onc* genes, since it was not seen at elevated levels in our liquid hybridization experiments. The observation of a very few positive cells (ca 0.15%) seen in normal bone marrow by in situ hybridization could implicate these sequences at a

very early stage of differentiation, perhaps in the erythroid lineage, where we could not test its level of expression. It is of interest (but at present it is not feasible) to see if such positive cells correspond to erythroid burst-forming units (BFU_E) and are targets for AEV [42, 35], in which case the target-cell hypothesis could be tenable for the *c-erb* sequences.

Finally, we examined if a given *c-onc* was expressed in a cell transformed by its viral homologue. Usually such experiments are difficult to perform, since the viral RNA is usually far more abundant than the *c-onc* corresponding RNA and thus preclude the visualization of the latter on RNA blots, despite differences in size. In one instance though (E26 myeloblasts), the viral RNA transcribed was unusually low. On the RNA blot, the *v-myb* band, at 6 kb [4], was readily seen, whereas the *c-myb* (at 4.0 kb) was undetectable in those conditions (data not shown). Thus, in this case the myeloblasts transformed by E26 did not show elevated levels of the cellular homologous oncogene.

Decreasing c-myb and c-myc Expression Could Correlate with Maturation

It is notable that in our experiments *c-myc* and *c-myb* RNA levels were found elevated in all of the immature cell lines tested, as well as in immature bone marrow cells, whereas only a few copies per cell were found in all mature or close to mature cells. In this respect, AEV has been shown to block differentiation of erythroblasts close to the colony-forming unit (CFU_E) stage [35, 44], whereas the other viruses appear to have less effect on differentiation [43, 42]. The fact, that the four AEV-transformed erythroblast cell lines tested (whether freshly transformed like 4D3 or long-term cultured like the HD1 line) exhibited similar elevated levels of *c-myc* and *c-myb*, could indicate that these *c-onc* genes are constitutively expressed at such levels in CFU_E erythroblasts. The role of these oncogenes remains to be determined. They could represent a signal involved in growth/differentiation of immature cells in the erythroid, lymphoid and perhaps myeloid hematopoietic lineages. It is compatible with this idea that, upon viral activation of *c-myc* by integration of a long terminal repeat (LTR) [37], such lymphoid cells did not mature normally and might thus be prone to subsequent transformation [45, 46].

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Increased Transcription of the c-*myc* Oncogene in Two Methylcholanthrene-induced Quail Fibroblastic Cell Lines

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The expression of three c-*onc* genes (c-*erb*, c-*myc*, c-*myb*) was investigated in five cell lines established from fibrosarcomas induced with 20-methylcholanthrene (MCA) of Japanese quails. These cell lines showed low levels of the three c-*onc* genes, with the exception of two cell lines that accumulated moderate (MCAQ 1–4) and large amounts (MCAQ3–5) of c-*myc* RNA. Molecular cloning and restriction endonuclease analyses indicated that expression of c-*myc* in these two cell lines were not associated with detectable rearrangements in the c-*myc* locus, that the size of the c-*myc* transcript (2.7 kb) in MCAQ 3–5 was similar to that of the normal c-*myc* messenger RNAs (mRNA) and that the transcriptional activation observed in MCAQ 3–5 was not mediated by the LTR (long terminal repeat) of a proximate ALV (avian leukosis virus) provirus. Finally, when analysed with the restriction enzymes Msp I and Hpa II, the c-*myc* locus of MCAQ 3–5 and MCAQ 1–4 was found hypomethylated as compared with that of the other cell lines tested that show low levels of c-*myc* transcripts. Our results suggest that one of the ways methylcholantrene could mediate transformation is by inducing an abnormal regulation of the c-*myc* gene.

The genomes of rapidly transforming retroviruses contain sequences essential to their oncogenic properties and referred to as v-*onc* genes [1, 2]. The v-*onc* genes originated from recombinational events between the genetic information of replication competent, slowly or non-transforming viruses and cellular sequences (c-*onc* genes) [3, 4]. Viruses that induce sarcomas as well as hematopoietic disorders have been described in avian as well as mammalian systems (for review, see [2]). The v-*onc* genes encode proteins probably directly responsible for the oncogenic potential of these viruses [5–7].

The c-*onc* sequences are believed to govern some unknown but important biological functions in the development and/or differentiation of the organism [8]. This assertion is mainly based on the phylogenic conservation of these sequences in a wide variety of vertebrates [9, 1, 10], as well as in some invertebrate species [11]. The intrinsic oncogenic capacities of these cellular genes have not yet been well documented, but experiments suggest that inappropriate expression of at least some c-*onc* genes might result in cell transformation [12]. For example,

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activation of the transcription of the mouse or rat c-*mos* genes by the retroviral promoter of a long terminal repeat (LTR) [13–15] induces cell transformation with high efficiency. Mechanisms others than transcriptional activation are also clearly operative [16, 17]. Indirect experimental evidence has also implicated the activation of c-*myc* in the genesis of several tumours. This gene is the progenitor of the v-*myc* oncogene, first identified in MC29, an avian acute leukaemia virus [18, 4, 19, 1]. About 80% of avian leukosis virus (ALV)-induced lymphomas show elevated expression of c-*myc* as the result of transcriptional activation by the LTR of a proximate ALV provirus [20, 21]. Activation of this gene has also recently been implicated in tumours with no known retroviral etiology, such as human Burkitt lymphomas [22] as well as murine plasmocytomas [23].

It is therefore reasonable to hypothesize that tumour induction in general, and by chemical carcinogenesis in particular, might involve alterations in the expression of known cellular oncogenes. We investigated the expression of c-*erb*, c-*myb*, c-*myc* oncogenes in five cell lines established from methylcholanthrene induced fibrosarcomas of Japanese quails [24, 25]. We chose to test the expression of these genes, because their transcripts accumulate normally only at low levels in fibroblasts [26].

We report here on two cell lines (MCAQ 1–4 and MCAQ 3–5), derived from two independent methylcholanthrene-induced tumours, that displayed transcriptional activation of c-*myc*. The c-*myc* locus in these cell lines did not show evidence of detectable rearrangements, but was found to be hypomethylated, as compared with control cells that do not accumulate elevated levels of c-*myc* mRNA.

MATERIALS AND METHODS

Cells and Viruses

MCA-induced cell lines were obtained from R. Friis for MCAQ 3–5 and MCAQ 1–4 and from C. Moscovici for QT4, QT5 and QT6. These cells were extensively described in Hayami et al. [25] and Moscovici et al. [24]. MC29 Q8, quail fibroblast non-productively transformed by MC29, were obtained from K. Bister. Q AEV (RAV1) and Q B77 represent quail fibroblasts transformed by AEV (RAV1) (a generous gift of T. Graf) and RSV B77 respectively. RP9 was a lymphoblastoid B type cell line obtained from B. Hayward through T. Graf. QEF are from Japanese quail embryos, HD3 an erythroblast cell line transformed by AEV *ts* 34 and obtained from T. Graf.

Preparation of Specific Probes

Radiolabelled cDNA transcripts were synthesized by exogenous reaction using 50S–70S appropriate RNA template (AEV (RAV2): cDNA*erb*; MC29 (RAV2): cDNA*myc*; AMV BAI: cDNA*myb*; td B77 for cDNA*rep*), calf thymus primer [^{3}H]dTTP or [^{32}P]dCTP and purified avian myeloblastosis virus polymerase as previously described [27]. Strong stop cDNA (cDNA 5′) was prepared according to Friedrich et al. [28]. Alternatively, probes for chicken 3′ exon were prepared by nick translation [29] of a DNA fragment originating from a Charon 4A clone containing the 3′ part of the c-*myc* gene (0.15 kbp after the Cla I site of the chicken c-*myc* in an artificial EcoR I site generated by the cloning strategy and extending 12 kbp in the adjacent cellular DNA). This clone was isolated with a cDNA*myc* probe from an Alu-Hae genomic library as published [30]. The 0.6 kbp EcoR I fragment (from the artificial site to the 3′ normal EcoR I site) was purified by electrophoresis in 0.8% agarose gel before the nick translation reaction, which was performed using a New England Nuclear nick translation kit.

Nucleic Acid Hybridization

Cellular RNAs for liquid hybridization were extracted and purified as described previously [27]. Standard liquid hybridization reactions contained 0.6 M NaCl, 2×10^{-3} M EDTA, 2×10^{-2} M Tris (pH 7.4), 500 μg calf thymus DNA per ml as carrier, 2000 cpm (0.04 ng), of ^{3}H-labelled cDNA, 2000 cpm (0.01 ng) of [^{32}P]cDNA*erb* as internal control and appropriate RNA in large excess. Hybridization was conducted in glass capillaries at 68°C, and the annealing extent was monitored by digestion with S1 nuclease.

Northern Blotting

PolyA-containing cellular RNAs were prepared as described [27]. RNA samples were denatured by glyoxal/DMSO treatment [31] and submitted to electrophoresis in 1% agarose gel. Size-separated RNAs were transferred to diazobenzyloxymethyl paper [32]. The northern blots were hybridized as described previously [33]. After washing, the blots were exposed at −70°C to Kodak XR5 film in the presence of an intensifying screen [27].

Southern Blotting

High molecular weight DNA was prepared as published [30], and digested by the appropriate restriction endonucleases (according to the supplier's instructions). DNA fragments were separated by electrophoresis in 0.8% agarose horizontal gels, and transferred to nitrocellulose as previously described [34]. The southern blots were hybridized to specific ^{32}P-labelled probes, washed, dried and submitted to autoradiography as previously described [30].

Cloning of MCAQ 3–5 c-myc Locus

DNA was extracted from MCAQ 3–5 cells and subjected to a partial cleavage with the restriction endonuclease EcoR I. Fragments of 15–20 kbp in size were selected by agarose gel electrophoresis and inserted into the EcoR I site of charon 4A phage by ligation to agarose gel selected phage arms. The chimeric DNA was packaged in vitro into phage capsids. The resulting gene library was screened with a chicken c-*myc* 3′ probe as described by Benton & Davis [35]. The MCAQ 1 contained the complete c-*myc* EcoR I 16 kbp fragment and was used for restriction mapping analysis and southern blotting.

RESULTS

Expression of c-myc, c-myb and c-erb Oncogenes, in Cell Lines Established from Methylcholanthrene-induced Quail Tumours

The expression of c-*myc*, c-*myb* and c-*erb* was analysed in five cell lines established from MCA-induced quail tumours, by liquid hybridization between the total cellular RNA of these cells and cDNAs specific of v-*myc*, v-*myb* and v-*erb* [26]. The cellular RNAs from several non-transformed and transformed cells were also included for comparison or as controls. As shown in fig. 1 and table 1, all of the cell lines derived from MCA-induced tumours expressed c-*myc*, c-*myb* or c-*erb* oncogenes at low levels similar to those seen in control normal quail embryo fibroblast (QEF), with two exceptions. Cell lines MCAQ 1–4 and MCAQ 3–5 accumulated respectively 3- and 8-fold higher levels of c-*myc* transcripts. No strict correlation appeared to exist between c-*myc* expression and tumorigenicity, since MCAQ 1–4 cells which express twelve copies of c-*myc* transcripts are not tumorigenic in quails, while QT6 cells which express only four copies of c-*myc* per cell are tumorigenic in quails (table 1). However, all these cell

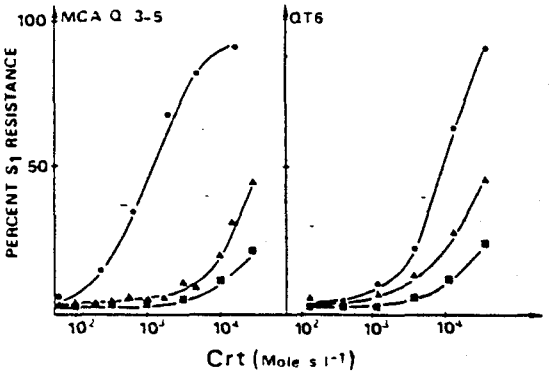

Fig. 1. Expression of c-*onc* genes in MCAQ3–5 and QT6 methylcholanthrene cell lines. Conditions of hybridization between cellular RNA and c-DNA probes specific for ▲, *erb*; ■, *myb*; ●, *myc* are described in Materials and Methods. The numbers of copies per cell are calculated from the curves with the convention of 1 copy/cell corresponding to a Crt ½ of 4×10^4 M·s·l^{-1}.

lines were derived from fibrosarcomas, showed altered morphologies and grew indefinitely in culture.

The c-myc Loci of MCAQ 3–5 and MCAQ 1–4 Do Not Appear Rearranged

Since c-*myc* transcriptional activation was shown in some instances to be linked to chromosomal translocation of the c-*myc* locus [36], we determined whether the c-*myc* locus of MCAQ 3–5 or MCAQ 1–4 was rearranged, as compared with normal QEF.

High molecular weight (HMW) DNA extracted from MCAQ 3–5 and MCAQ 1–4 cells was digested with several restriction endonucleases and southern blots of digested DNA were hybridized with a nick-translated chicken 3′ c-*myc* probe, (c-*myc* 3′ probe, see top of fig. 2). This probe was chosen because of its specificity for c-*myc*, as compared with a 5′ c-*myc* coding exon probe that may yield unspecific hybridization patterns [37]. As shown in fig. 2, the EcoR I,

Table 1. *c-onc RNA transcripts accumulated in various cells*

Cells	Transforming agent	Species	Tumor induction[a]	RNA copies[b] of c-*erb*	c-*myb*	c-*myc*
MCAQ 3–5	20 MCA	Quail	+	1	0.5	30
MCAQ 1–4	20 MCA	Quail	−	1	0.5	12
QT4	20 MCA	Quail	+	1	0.5	3
QT5	20 MCA	Quail	−	1	0.5	2
QT6	20 MCA	Quail	+	1	0.5	4
HD4EBL	AEV (NP)	Chicken	ND	(500)	17	30
RP9	RAV1	Chicken	ND	1.8	10	500
MC29 (Q8)	MC29	Quail	ND	1	0.5	(300)
QB77t	RSVB77	Quail	ND	1.2	0.6	4
QAEVRAVI	AEV(RAV1)	Quail	ND	(250)	0.4	3
QEF	NONE	Quail	−	1	0.5	4

[a] Data from Moscovici et al. [24] and Hayami et al. [25] for 20 MCA cells.
[b] Crt ½: $4\cdot10^4$ mole×s×l^{-1}=1 copy per cell.
ND, not determined. Numbers in parenthesis represent v-*onc* and not c-*onc* transcripts.

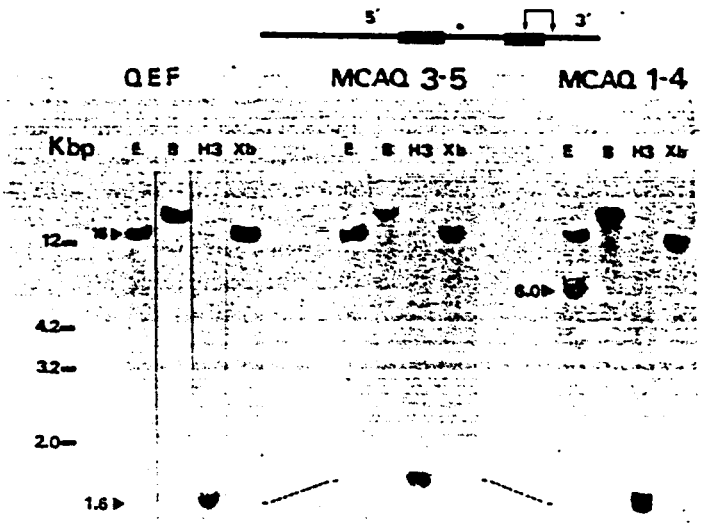

Fig. 2. Southern blot analysis of DNAs from QEF, MCAQ 3–5 and MCAQ 1–4. HMW DNA from each cell type was digested with *E*, EcoR I; *B*, BamH I; H_3, Hind III; and *Xb*, Xba I. Size-separated cellular DNA fragments were transferred to nitrocellulose and hybridized with a ^{32}P probe representing essentially chicken c-*myc* 3′ probe (shown at top; cell types are listed across the top). Numbers represent size markers (–) or indicate bands of interest (*arrowheads*), in kbp.

BamH I, Hind III and Xba I restriction patterns of the c-*myc* locus in MCAQ 3–5 and MCAQ 1–4 were found to be indistinguishable from those of control QEF. The 6 kbp c-*myc* fragment observed in the EcoR I digest of MCAQ 1–4 (fig. 2) is the result of polymorphism at this site, since it was observed in some normal quail fibroblasts (data not shown). The presence of this EcoR I site in one of the alleles of the c-*myc* gene of MCAQ 1–4 is thus probably not related to the transformed phenotype of these cells, but allows a convenient distinction between the two alleles.

We then molecularly cloned the c-*myc* locus of MCAQ 3–5 in lambdoid phage vector Charon 4A (unfortunately this work was largely engaged when we learned about the convenient MCAQ 1–4 polymorphism). The physical map of the resulting phage MCAQ 1 was constructed using several restriction endonucleases and a total v-*myc* probe or the chicken c-*myc* 3′ probe. In the map shown in fig. 3*B*, we assumed that the exon/intron organization was similar in the chicken and quail c-*myc* homologous loci. This was based on closely related restriction maps observed for the two loci (fig. 3*B*) as well as on similar results obtained with several restriction fragments of the quail c-*myc* locus used to probe c-*myc* transcripts in quail or chicken cells (data not shown). This was also indicated by the numerous Msp I sites clustered in the chicken 5′ coding exon (see sequence data in [38]) also found clustered in the corresponding quail region (see the numerous small fragments around 0.3 kbp difficult to resolve on southern blots, fig. 3*A*). From the MspI quail c-*myc* and lambda MCAQ 1 maps in fig. 3*B*, we deduce that restric-

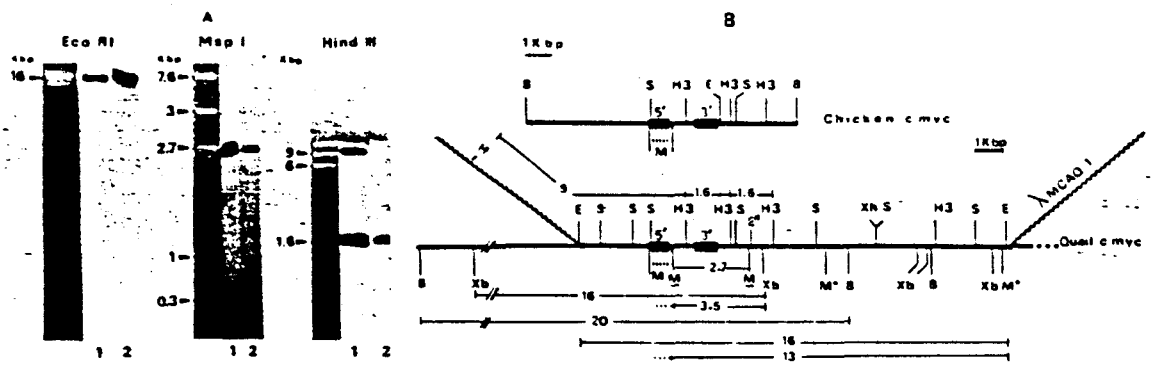

Fig. 3. Restriction map of MCAQ 1 DNA. The restriction map of phage MCAQ 1 was established using several endonucleases. The *myc* domains were located by Southern blotting analyses with specific labelled probes corresponding to total v-*myc*, or to the c-*myc* 3′ probe. (*A*) EcoR I, Msp I and Hind III digestions were performed on MCAQ 1 phage DNA. Fragments were separated on a 1% agarose gel, visualized by ethidium bromide staining then transferred to nitrocellulose and hybridized with ^{32}P-labelled cDNA*myc* (channel 1) and chicken c-*myc* 3′ probe (channel 2). (*B*) Organization deduced from southern blotting analysis of lambda MCAQ 1 digested DNA. For comparison the restriction pattern of chicken c-*myc* locus is indicated (deduced from Neel et al. [48] and Payne et al. [21]. Restriction endonucleases used were: *E*. EcoR I; *S*. Sac I; H_3, Hind III; *M*. Msp I; *Xb*, Xba I; *B*, Bamh I; *Xh*, Xho I. *M** indicates Msp I sites not precisely localized. Msp I sites of the first v-*myc*-related exon have been assigned by reference to those found in published nucleotide sequence of the chicken c-*myc* [38]. The phage arms (λ) limiting the EcoR I insert lambda MCAQ 1 were 12 kbp upstream, 19.5 downstream. Restriction sites of the quail c-*myc* locus were deduced from cell DNA bands in fig. 2. Fragment sizes are numbered in kbp. Thick bars define the v-*myc* related exons. *E** locates the polymorphic EcoR I observed in an allele of MCAQ 1–4 cells. *M* indicates sites hypomethylated in expressor cell-lines MCAQ 3–5 and 1–4 and hypermethylated in lines QT5 and QT6.

Note added in proof

Upon subcloning, the Xb site limiting the 16 and 3.5 kbp further upstream (between S and E). This does not affect any of the conclusions.

tion cleavages with BamH I and Xba I would have detected rearrangements in the c-*myc* locus up to 11 kbp upstream and 5 kbp downstream from the c-*myc* exons. We conclude that no major rearrangement occurred in the c-*myc* locus of MCAQ 3–5 and MCAQ 1–4 as compared with the normal c-*myc* quail locus (fig. 2, QEF). Furthermore, the similar relative intensities of the hybridizing bands (see figs 2 and 5) disfavour that amplification of the *myc* gene in MCAQ 3–5 and MCAQ 1–4 is responsible for the elevated transcription seen in these cells.

Characterization of the c-myc Transcript in MCAQ 3–5 Cell Line

In order to test whether the accumulation of c-*myc* transcripts in MCAQ 3–5 was due to the activation of this locus by the LTR of a nearby ALV provirus, we investigated, by northern blot hybridization experiments, the size and the genetic content of c-*myc* transcripts in these cells. Fig. 4 shows that the size of the c-*myc* transcript found in MCAQ 3–5 was undistinguishable from the normal c-*myc* mRNA found in AEV-transformed erythroblasts (these cells were chosen as control, since we know that they exhibit a normal 2.7 kb c-*myc* RNA, but accumulate about 10 times more of this RNA species than normal fibroblasts,

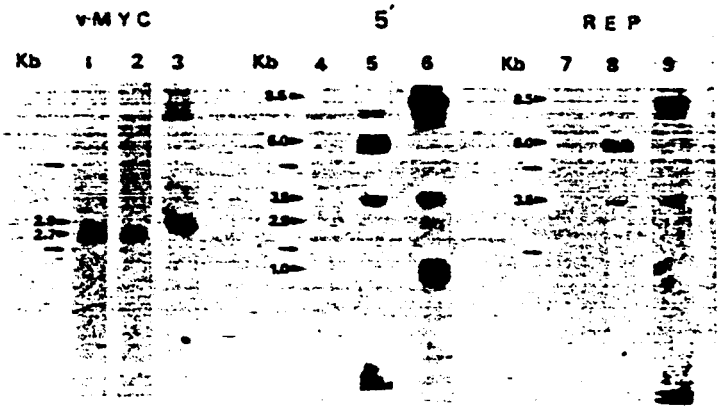

Fig. 4. Size of c-*myc* transcript in MCAQ 3–5 cell line. PolyA-containing RNA was denatured, separated on agarose gel, and transferred to activated DBM paper. Blots were hybridized with the probes listed across the top and described in Materials and Methods (v-MYC=cDNA*myc*, 5′=cDNA 5′, REP=cDNA*rep* (gag, pol, env)). MCAQ 3–5, lanes *1, 4, 7*; HD3 chicken erythroblasts, lanes *2, 5, 8*; RP9 chicken lymphoblasts, lanes *3, 6, 9*. Symbols: →, Ribosomal 28S and 18S markers; ▶, size of cellular and viral products, in kb.

providing thus a strong and well-resolved signal [26, 19] (fig. 4, *1, 2*), and distinct from the transcript of LTR-activated c-*myc* in the lymphoma line RP9 (fig. 4, *3*), clearly of a different size (2.9 kb) [20].

Using a 5′ ALV probe, no evidence of LTR-directed transcription could be found in MCAQ 3–5 cells (fig. 4, *4*); accordingly, a probe representative of an ALV genome (*rep* probe, fig. 4, *7*) detected no viral-related RNA in these cells. Such probes detected readily viral species in infected control cells (fig. 4, *5, 6, 8, 9*).

The Transcriptionally Active c-myc Locus in MCAQ 3–5 and MCAQ 1–4 is hypomethylated

Since cytosine methylation of eukaryotic DNA has been reported to modulate the regulation of transcription [39] we analysed the methylation pattern of the c-*myc* locus in MCAQ 3–5 and MCAQ 1–4 cell lines as well as in two other MCA-transformed cell lines expressing low levels of c-*myc* mRNA. We used restriction endonucleases Msp I and Hpa II that are both specific of the same restriction site CCGG. Hpa II (but not Msp I) is inhibited by internal cytosine methylation. As shown in fig. 5*A* all MCA cell DNAs cleaved with Msp I generated a 2.7 kbp band that hybridized with a c-*myc* 3′ probe. But this specific band was detected after cleavage with Hpa II only in the high c-*myc* expressor cells MCAQ 3–5 and MCAQ 1–4. This suggested that at least two Msp I sites, generating the 2.7 kbp band and flanking the 3′ c-*myc* exon, were hypomethylated (Hpa II sensitive) in MCAQ 3–5 and MCAQ 1–4 DNA (these are designated M in fig. 3*B*), but hypermethylated in the low expressor lines QT6 and QT5.

In order to localize more precisely in the c-*myc* locus the position of the methylated CCGG with respect to the c-*myc* exons, double digestions of the

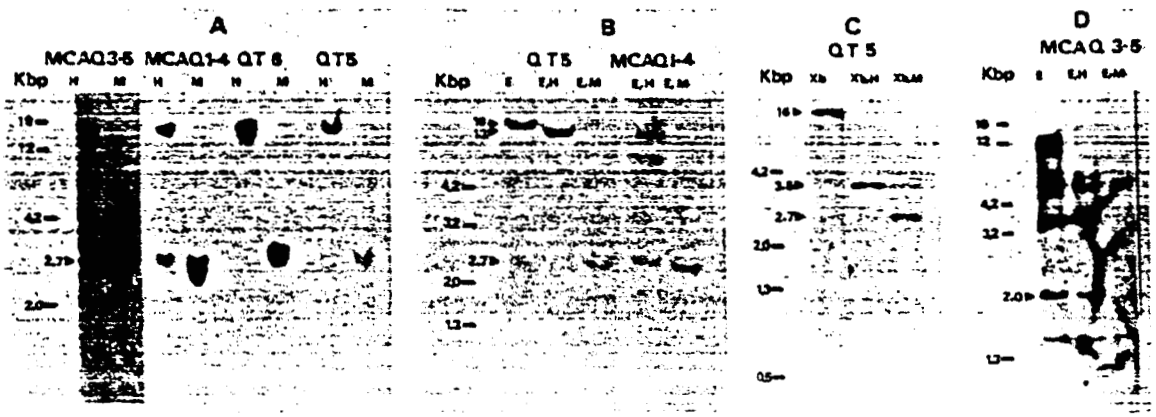

Fig. 5. Methylation pattern of the c-*myc* locus from methylcholanthrene cell lines. HMW DNA from each cell line was digested with *H*, Hpa II; *M*, Msp I; *E/H*, EcoR I–Hpa II; *E/M*, EcoR I–Msp I; *Xb/M*, Xba I–Msp I; *Xb/H*, Xba I–Hpa II and size-separated on agarose gel. DNA was then transferred to nitrocellulose and hybridized with the chicken (*A*, *B*, *C*) c-*myc* 3′ probe or (*D*) v-*myb* probe. Each cell type is listed across the top.

DNA were performed either with EcoR I and Msp I/Hpa II (fig. 5*B*) or with Xba I and Msp I/Hpa II (fig. 5*C*) and hybridized to a c-*myc* 3′ probe. The observed patterns allowed the following interpretations, when compared with the restriction map of the quail c-*myc* locus in fig. 3*B*. The EcoR I 16 kbp fragment of QT5 (fig. 5*B*) was cut to 13 kbp by Hpa II, indicating the presence of a Hpa II-sensitive site. Similarly, the Xba I 16 kbp fragment of QT5 (fig. 5*C*) was cut to 3.5 kbp by Hpa II. We thus located the methylation-sensitive site of the low expressor line QT5 within the 5′ exon of quail c-*myc* (fig. 3*B*). In both cases Msp I yielded a 2.7 kbp fragment, indicating that the Msp I site immediately upstream from the Xba I site was methylated and not cut by Hpa II (in contrast to expressor cell lines MCAQ 3–5 and 1–4, see fig. 5*A* and compare the patterns of QT5 and MCAQ 1–4 in fig. 5*B*). Similar results were obtained for QT6 (data not shown). That the EcoR I 16 kbp fragment in QT5 was only reduced to 13 kbp by Hpa II (fig. 5*B*) indicated in addition that the DNA downstream of the end of the 5′ c-*myc* exon was resistant to Hpa II, and thus methylated.

We conclude from these experiments (fig. 5, 3*B*) that (1) in the low c-*myc* expressor MCA cells (QT5, QT6), the DNA is hypermethylated downstream of the 5′ c-*myc* coding exon; (2) on the contrary, in the two high c-*myc* expressor lines the two Msp I sites limiting the 2.7 kbp fragment (designated M in fig. 3*B*) are hypomethylated (Hpa II-sensitive). Thus a good inverse correlation is found here between expression and methylation. (3) Upstream of the c-*myc* 5′ coding exon, the methylation state could unfortunately not be assessed due to unspecific hybridizations obtained with probes covering this region (not shown). It is interesting to note that in the MCAQ 1–4 cells for which the two c-*myc* alleles can be distinguished by EcoR I, it was the usual 16 kbp EcoR I fragment that was methylated, since an EcoR I/Hpa II digestion yielded the 13 kbp band characteris-

tic of a methylated allele (fig. 5*B*) and cleaved the 6 kbp allelic EcoR I band to 2.7 kbp.

Finally, we tested if the high level of c-*myc* transcripts in MCAQ 3–5 cells was due to deficient cellular methylases. This was apparently not the case, since another c-*onc* gene, c-*myb*, barely transcribed in these cells (fig. 1, table 1), was indeed found methylated. Fig. 5*D* shows the pattern of MCAQ 3–5 DNA digested with EcoR I and Hpa II (or Msp I) and hybridized with the v-*myb* probe, where the v-*myb* containing fragment of 2 kbp is observed after Hpa II, but not Msp I digestion.

DISCUSSION

The experiments described here show that two cell lines established from distinct methylcholanthrene-induced fibrosarcomas (MCAQ 3–5 and MCAQ 1–4 [25]) of adult Japanese quails accumulate elevated levels of transcripts of the c-*myc* oncogene. Three other cell lines established from young quails [24] show only a low expression of this gene, comparable to that of normal quail fibroblasts.

To explore this point further, we molecularly cloned the c-*myc* locus of line MCAQ 3–5 for which we constructed a detailed restriction map (no quail c-*myc* molecular clone has been described before). Two lines of evidences indicate that the c-*myc* locus of MCAQ 3–5 and MCAQ 1–4 cell lines is not detectably rearranged, as compared with the c-*myc* locus of normal cells. First, no differences were found between the restriction nuclease patterns of the c-*myc* locus of normal cells and that of all MCA cell lines analysed here. These analyses also showed that no c-*myc* amplification occurred in either MCAQ 3–5 or MCAQ 1–4 cell lines. Second, the size of the mRNA transcript observed in MCAQ 3–5 cell line was indistinguishable from that of normal cells [19, 40, 26]. We conclude that unlike the cases of transcriptional activation of c-*myc* in tumour cells described so far, the activation of c-*myc* transcription in cell lines MCAQ 1–4 and MCAQ 3–5 does not appear to be due to either c-*myc* translocation [36] or amplification [41]. Furthermore, we found no evidence in these cell lines for LTR-mediated activation of the c-*myc* gene by a nearby ALV provirus as described by Hayward et al. [20] for c-*myc* in chicken bursal lymphomas and by Kuff et al. [42] for the c-*mos* gene in non-virally induced mouse myelomas.

Nonetheless we demonstrated that the c-*myc* locus of MCAQ 3–5 and MCAQ 1–4 cell lines is hypomethylated when compared to that of methylcholanthrene cells that display low levels of c-*myc* transcripts. Since hypomethylation of eukaryotic DNA [39, 43] or proviral sequences [44] was shown to modulate transcription, it is tempting to link the increased expression of the c-*myc* locus in MCAQ 3–5 and MCAQ 1–4 cell to a conservative hypomethylation of this gene. This hypomethylation appears specific rather than due to a cellular defect in methyltransferases because the c-*myb* locus (which is barely expressed and thus tightly controlled in these cells) remains hypermethylated. In this respect, it is of

interest to note that inhibition of DNA methylation by chemical carcinogens has been recently proposed [45] as a possible mechanism for cellular transformation.

The unusual high level of c-*myc* expression seen in MCAQ 3–5and MCAQ 1–4 has never been found in normal (nor virally transformed) quail fibroblasts where the c-*myc* regulation appears extremely well controlled. Of course we cannot exclude that the high levels observed represent a mere coincidence unrelated to the transformation of these cells. But a more attractive and more likely hypothesis would be that the c-*myc* elevated expression detected here may play a part in the mechanism of induction of these cells by methylcholanthrene. It remains to be documented whether the c-*myc* gene product itself is directly involved in cell transformation or immortalization. The activation of a secondary transforming gene as recently shown by Goubin et al. [46] in chicken B-cell lymphomas, could be required for complete transformation.

Interestingly, the level of c-*myc* expression found in MCAQ 3–5 or MCAQ 1–4 cells is low compared with the level of transcription observed in ALV-LTR activated c-*myc* in chicken lymphomas [20] or to the virally expressed v-*myc* (by one order of magnitude, see table 1), with the exception of MC29-transformed rat cells [47]. MCAQ 3–5 cells express an average copy number (ca 30) close to that found in chicken hematopoietic immature cells [26]. This point may suggest that the transcriptional c-*myc* expression is tightly controlled in normal cells and that transformation by the MCA has removed (directly or indirectly) part of all of this control. As a consequence, the c-*myc* gene could recover its high transcription rate seen for example in immature blood cells [26].

In conclusion, we propose that in MCAQ 3–5/MCAQ 1–4 cells the c-*myc* gene regulation is altered, maybe by a slight sequence modification induced by the MCA carcinogen in the c-*myc* gene itself or in a gene involved in its regulation. A possible mutation within the coding sequence of c-*myc*, as described for the c-*ras* gene in human bladder carcinoma [17], cannot be excluded, but would not explain the increased transcription observed. Sequence data will probably be required to further explore this hypothesis.

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Transformation of quail embryo fibroblasts by a retrovirus carrying a normal human c-*myc* gene

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We have constructed avian retroviruses expressing the human c-*myc* oncogene. These viruses morphologically transformed primary quail embryo fibroblasts upon transfection and infection. Transformed cells produced viruses harboring a spliced c-*myc* gene and contained high levels of p64-67$^{c\text{-}myc}$ protein. One of these infectious viruses, vSX-AHM, was molecularly cloned and the nucleotide sequence of the spliced c-*myc* insert determined. No mutation was found within the c-*myc* coding sequence of this transforming clone when compared to the normal genomic progenitor. Thus, we concluded that no mutation within the human c-*myc* gene is required to induce primary avian embryo fibroblast transformation.

Key words: human c-*myc* oncogene/quail embryo fibroblasts

Introduction

Two types of mechanisms, not mutually exclusive, have been shown to be involved in activation of cellular oncogenes (c-*onc*). The first one refers to disorders inducing an overexpression of these genes by insertion of promotor/enhancer sequences (Hayward *et al.*, 1981; Cohen *et al.*, 1983; Fung *et al.*, 1983). The second one is characterized by the presence of mutations within the coding sequence of c-*onc* genes (Santos *et al.*, 1982; Capon *et al.*, 1983; Gambke *et al.*, 1985). These two types of activation are often found together in transforming retroviruses carrying v-*onc* (Santos *et al.*, 1982). The mechanism by which the c-*myc* oncogene is activated remains unclear since: (i) transcriptional activation of c-*myc* by viral promoters/enhancers (Hayward *et al.*, 1981; Payne *et al.*, 1982; Swift *et al.*, 1985) or cellular enhancers (Corcoran *et al.*, 1985) has been reported, but in several cases point mutations were also found within the activated c-*myc* gene (Rabbitts *et al.*, 1983, 1984; Westaway *et al.*, 1984; Showe *et al.*, 1985; Battey *et al.*, 1983); (ii) in one case the translocated c-*myc* gene was free of mutations but a *ras* oncogene was activated precluding any conclusions about the transforming role of v-*myc* (Murray *et al.*, 1983; Wiman *et al.*, 1984); and (iii) the c-*myc* sequence from transforming avian retroviruses exhibits several mutations as compared to its normal cellular counterpart (Alitalo *et al.*, 1983; Watson *et al.*, 1983; Kan *et al.*, 1984). To gain some insight on the mode of oncogenic activation of c-*myc* we constructed several recombinant plasmids in which the normal human c-*myc* gene was linked to viral promotors or enhancers. We report here that these recombinants are able to transform primary quail embryo cells as efficiently as a molecular clone of an avian v-*myc* containing provirus and that the nucleotide sequence of the activated c-*myc* insert was found free of mutations relative to the normal human c-*myc* gene. This suggests that an overexpression of the normal c-*myc* product is sufficient to induce transformation of primary avian embryo cells.

Results

Strategy of expression of the human c-myc gene in avian cells

To express the normal human c-*myc* gene in avian cells we used two distinct strategies. First we introduced human c-*myc* into the genome of an avian retrovirus so that the expression of c-*myc* was under the control of, and initiated at, a viral promotor; second, we inserted enhancer sequences (by use of an inverted LTR) in the upstream part of the c-*myc* gene. In that case c-*myc* expression was initiated at c-*myc* promotors.

Using a pKH47 vector we constructed four recombinant plasmids containing human c-*myc* sequences linked to viral sequences derived from a molecularly cloned AEV provirus (Vennström *et al.*, 1981).

In three of these recombinants (pX-AHM, pSX-AHM and pSm-AHM) the human c-*myc* gene was under control of a viral promotor. Two of them (pX-AHM and pSX-AHM) contained the *Xho*I – *Eco*RI 7 kbp c-*myc* fragment (Figure 1). In pX-AHM this fragment was linked to the 2.3 kbp *Eco*RI – *Xho*I fragment of AEV proviral DNA which includes the LTRs together with the ATG and splice donor site of the *gag* gene (J.Samarut and J.H.Xhio, unpublished nucleotide sequence data). In pSX-AHM, the *gag* ATG and splice donor site were removed (see Figure 1). Therefore in this case the normal c-*myc* initiation codon was to be used. A third recombinant plasmid (pSm-AHM) was constructed by inserting the AEV-LTR in the first c-*myc* intron; this led to a complete deletion of the first c-*myc* exon (see Figure 1). Expression of the c-*myc* gene from its own promotors was obtained by construction of a fourth recombinant vector pEP-AHM which resulted from the insertion, in an inverted (3′ 5′) orientation of the AEV-LTR in the upstream part of the c-*myc* promotors (*Pvu*II site).

The transforming activity of these recombinant plasmids was assayed by transfection on quail embryo cells (QEC) using the $CaPO_4$ coprecipitation method (these cells are known to be efficiently transformed upon infection by v-*myc* containing retroviruses or transfection with the corresponding proviral DNAs).

Biological activity of the activated human c-myc gene

The four c-*myc* recombinants were found to induce transformation of QEC at levels (0.2 – 0.5 transformants/μg of DNA) similar to those observed for molecular clones of wt MH2 (pMH2-Hd) (Coll *et al.*, 1983) and wt MC29 (pMC 38) (Vennström *et al.*, 1981). No foci were found in QEC transfected with the non-activated human c-*myc* gene. When the pX-AHM, pSX-AHM or pSm-AHM c-*myc* inserts were purified after *Bcl*I endonuclease digestion [to remove the two cellular polyadenylation sites of the c-*myc* gene (Gazin *et al.*, 1984) and allow the retroviral propagation of c-*myc* RNA] and transfected together

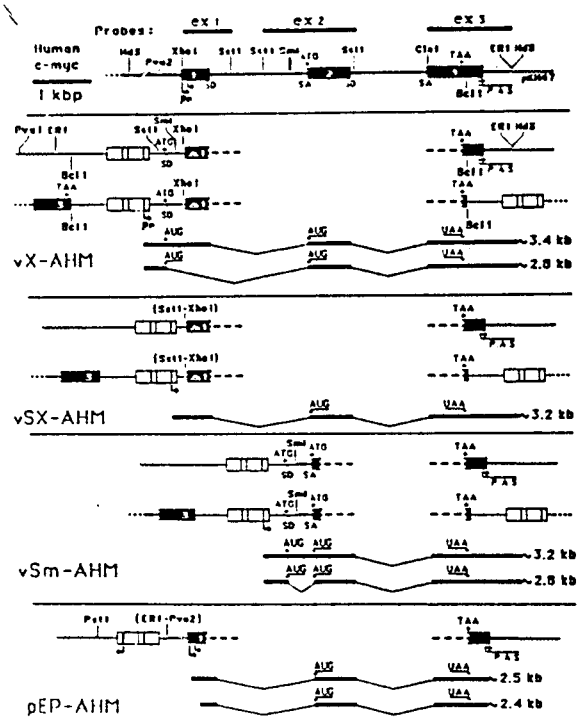


Fig. 1. Construction of human c-*myc* recombinants. The restriction map of the human c-*myc* genomic DNA used is depicted on top of the figure. For each human c-*myc* recombinant we successiveiy depicted the structure of the construction (retroviral LTR as an open box) and the structure of the ligated *Bcl*I insert (except for pEP-AHM) transfected on quail cells and the expected mRNAs. The RNAs for vX-AHM, vSX-AHM and vSm-AHM recombinants (as illustrated by the prefix v) initiate at viral promotors present in the LTR sequences and correspond to spliced RNAs able to be virally propagated; pEP-AHM mRNAs initiate at the c-*myc* promotors. Pr, promotor sequence; —, transcriptional direction; SD, splice donor site; SA, splice acceptor site; P.A.S., polyadenylation site; ■, exon; ⎯, intron; □, LTR, retroviral sequence; ~, pKH 47. ex1 delineates the extent of the probe corresponding to exon 1 (*Xho*I – *Sst*I); ex2 (*Sst*I – *Sst*I) the probe corresponding to exon 2; ex3 (*Cla*I – *Eco*RI) the probe corresponding to the third exon; *AUG*, AUG in phase with the c-*myc* sequence. (Brackets) in *Sst*I – *Xho*I and *Eco*RI – *Pvu*II means that the restriction sites were filled-in with DNA polymerase I (Klenow fragment) prior to ligation.

with pRAV1 DNA on QEC, fully transformed cell cultures producing transforming viruses were obtained (Figure 2). As shown in Figure 2C, the morphology of QEC transformed with pEP-AHM was indistinguishable from that of cells transformed with the other recombinants. We concluded from these experiments that primary quail embryo cells can be transformed when the human c-*myc* oncogene is overexpressed (see below).

Transcription pattern of the human c-myc recombinants

We next characterized the transformed cultures for c-*myc* expression at the RNA level. The QEC used were transformed upon cotransfection with pX-AHM, or pSX-AHM or pSm-AHM purified inserts and pRAV1 DNA. Each transformed cell culture produced a transforming virus named vX-AHM, vSX-AHM and vSm-AHM respectively. QEC transformed by pEP-AHM resulted from a pool of ten transformed foci, picked from transfected cultures and grown up together. Figure 3 represents the autoradiograms of Northern blots hybridized with probes specific for respectively the first, second and third c-*myc* exons. Our results clearly indicate that the spliced *myc* RNAs predicted in Figure 1 for each recombinant on the basis of nucleotide sequence data (Gazin *et al.*, 1984) are found in cells transformed by these recombinants. vX-AHM transformed cells synthesized a 3.4 kb

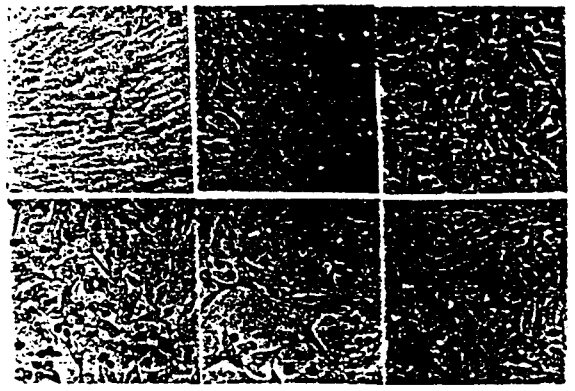


Fig. 2. Transforming ability of recombinant c-*myc* constructs. Quail embryo cell cultures were transformed as described in Materials and methods. a, normai QEC; b, QEC transformed upon cotransfection with pMC38 (*v-myc*) and pRAV1; c, d, e, QEC transformed upon cotransfection with pRAV1 and respectively X-AHM, SX-AHM and Sm-AHM Bcl1 inserts; f, QEC transformed upon transfection with pEP-AHM DNA (magnification ×200).

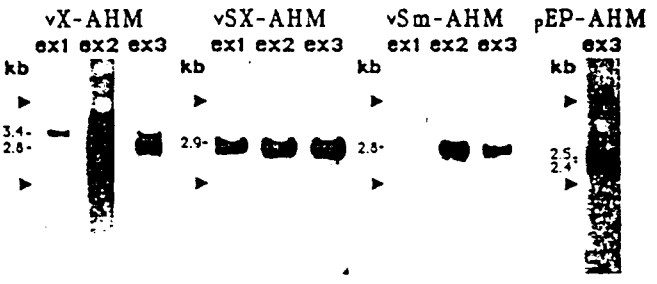
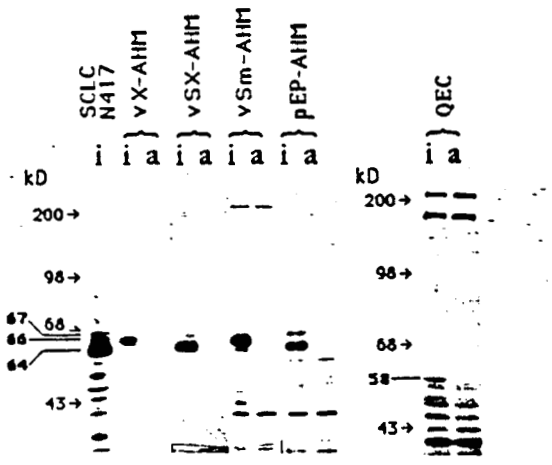


Fig. 3. Size of *myc* transcripts in quail embryo cells transformed by human c-*myc* recombinants. Poly A$^+$ containing RNA was denatured, separated on agarose gel and transferred to nitrocellulose as described (Coll *et al.*, 1983). Blots were hybridized with the ^{32}P nick translated probes described in Figure 1 and indicated on top of each lane. The transforming viruses or DNA are indicated on top of the figure. ▶, Ribosomal RNA markers.

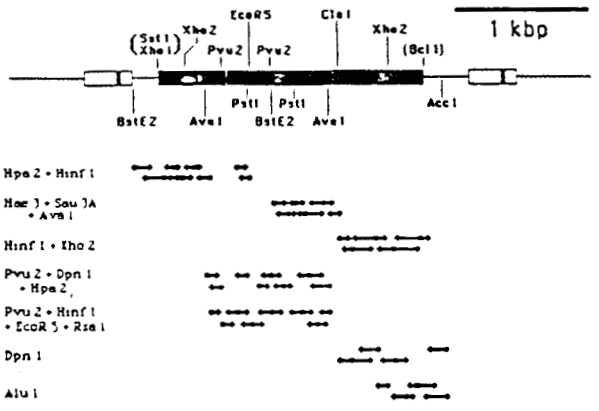
genomic RNA hybridizing with the three *myc* probes and a major subgenomic RNA (2.8 kb) lacking exon 1 and probably generated by the splicing events (described in Figure 1) joining the splice donor site of the *gag* sequence to the splice acceptor site of exon 2. In cells transformed by vSX-AHM, deletion of the splice donor site of the viral *gag* gene resulted in the synthesis of a single 2.9 kb genomic RNA hybridizing with the three *myc* probes. This RNA was smaller in size than the expected one (3.2 kb; see Figure 1). Nucleotide sequence analysis of the cloned SX-AHM proviral DNA showed that a deletion of 300 nucleotides occurred in the remaining AEV *env* sequence presumably during the transfection process (see Figure 5). In cells transformed by vSm-AHM we only detected a 2.8 kb RNA hybridizing with the *myc* exon 2 and exon 3 probes (Figure 3). This suggests that in these cells the propagated transforming RNA was the spliced transcript of the vSm-AHM predicted in Figure 1. The pool of cells transformed upon transfection with pEP-AHM DNA synthesized the expected doublet of 2.4–2.5 kb mRNAs starting at the c-*myc* promotors located in exon 1 and deduced from S1 mapping experiments (data not shown).

Proteins synthesized by the human c-myc recombinants

We determined whether cells transformed by our c-*myc* recombinants synthesized the previously described p64/67$^{c\text{-}myc}$ proteins (Hann and Eisenman, 1984). Extracts of [^{35}S]methionine labelled cells were immunoprecipitated with a rabbit anti-*myc* serum.



g. 4. Immunoprecipitation of human *myc* proteins in QEC transformed by *myc* recombinant. Labelling was performed on semi-confluent cultures eded in 100-mm Petri dishes and described in Materials and methods. ansforming viruses or DNA are indicated on top of the figure. As a ntrol the c-*myc* product in human cell line SCLC N-417 and in QEC ere immunoprecipitated in similar conditions. —, Standard molecular eight markers from BRL Inc. are listed on left of the figure.



ig. 5. Restriction map and strategy of sequencing of the c-*myc* insert of SX-AHM provirus. The restriction sites relevant in the provirus are ndicated. Gel purified restriction fragments used for DNA sequencing restriction enzymes in the left-hand column) were labelled at their 5′ termini sing [^{32}P]γ ATP and polynucleotide kinase and sequenced according to the laxam and Gilbert procedure. Closed circles indicate the cleavage points nside of each insert and horizontal arrows the direction and length of the equenced DNA strands.

As shown in Figure 4, vSX-AHM and pEP-AHM transformed ells synthesized a p64/67 doublet similar to that previously lescribed in the control SCLC N 417 human cells (Hann and Eisenman, 1984). In vX-AHM and vSm-AHM transformed cells, a 66 kd protein was observed. This difference in size can be explained by the fact that these cells synthesize a c-*myc* containing mRNA which can promote translation of a p66 protein initiated at the *gag* AUG instead of the c-*myc* AUG (Figure 1). In addition, Figure 4 shows immunoprecipitation of the endogenous QEC c-*myc* protein in similar conditions. A single faint band of 58 kd previously described by Hann *et al.* (1983) is detected.

Nucleotide sequence analysis of the c-myc insert of SX-AHM provirus

We next examined whether mutations in the c-*myc* part of the provirus had been selected for during the selection procedure. Southern blot experiments demonstrated that only one type of provirus was present in the vSX-AHM (RAV1) transformed QEC. We molecularly cloned this provirus and its transforming activity was confirmed by transfection on QEC and found similar to that of MC29 proviral DNA. Figure 5 shows the restriction map of the cloned provirus and the strategy used to determine its nucleotide sequence by the Maxam and Gilbert procedure (1980). Comparison of this sequence with that of the normal genomic c-*myc* gene (Gazin *et al.*, 1984) indicated that no mutation had occurred within the c-*myc* coding sequence during the propagation of this virus.

Discussion

The studies described above were aimed at determining whether mutations were required for the acquisition of oncogenic properties by the human c-*myc* gene. Among the *myc* alleles available, only v-*myc* genes in retroviral genomes exhibit a readily observed, single-step oncogenic effect in cell culture. This may be due either to the presence of mutations in the v-*myc* alleles (Papas and Lautenberger, 1985) or to the overexpression of this gene by retroviral promotors or to the peculiar reaction of tested biological systems towards the *myc* product. For example, expresion of v-*myc* in rat fibroblasts induces a subtle although detectable transformed phenotype measured by immortalization of these cells (Mougneau *et al.*, 1984). However, introduction of v-*myc* into a murine retrovirus leads to a recombinant able to transform rodent fibroblasts and macrophages *in vitro* (Vennström *et al.*, 1984). In another system v-*myc* induces preneoplastic transformation of avian lymphocytes in a bursal transplantation assay (Neiman *et al.*, 1985) whereas it transforms fibroblasts and macrophages of the same origin (Graf and Stehelin, 1982). Thus, it appears difficult to appreciate the respective part of transcriptional activation, somatic mutation and the biological system used in the transforming potential of the *myc* product.

A biological assay described by Land *et al.* (1983) for c-*myc* oncogene transformation requires conditions in which a second oncogene is present. This type of assay allowed Lee *et al.* (1985) to show that augmented expression of the normal human c-*myc* gene was sufficient for cotransformation of rat embryo cells with an activated *ras* gene. Our results clearly support the idea that in appropriate biological systems an overexpression of the normal c-*myc* gene is sufficient to induce cellular transformation since we did not find any mutation in the c-*myc* coding sequence isolated from avian fibroblasts transformed by the vSm-AHM recombinant retrovirus described in this paper.

Two sets of proteins are synthesized by these recombinant retroviruses: the normal *myc* doublet p64-67 found in SX-AHM and pEP-AHM transformed cells probably initiates at the c-*myc* AUG because these recombinants do not contain any *gag* sequence. The second type of *myc* protein, the p66 protein found in vX-AHM and vSm-AHM transformed cells is probably translated from the *gag* AUG, first AUG codon in phase with the *myc* gene sequence in these viruses. As a result, the p66 protein probably has six *gag* derived amino acids at its amino terminus; this could explain the difference in molecular weight between the two species, p64 and p66. Therefore, as the p66 protein and the p64–67 doublet exhibit the same oncogenic properties, the six amino acids derived from the *gag* gene in p66 presumably do not play an important role in fibroblast transformation. Similar results have been obtained by Shaw *et al.*, (1985) with deletion mutants of MC29 confirming that *gag* sequences are not required for fibroblast transformation by v- or c-*myc*.

Tumorigenesis *in vivo* is a more complex process than transformation of a cultured cell *in vitro* and it is likely that diverse alterations of c-*myc* can confer a growth advantage to cells *in vivo*. Thus we injected vSX-AHM with RAV1 helper viruses into chick embryos. Preliminary data revealed that this virus induces endotheliomas and solid tumors in the chicken (F.Dieterlein, personal communication). In summary, our results demonstrate that an overexpression of the human c-*myc* gene product is sufficient to induce avian primary embryo cell transformation and tumor formation.

Materials and methods

Construction of human c-myc recombinants

The human genomic c-*myc* clone used was isolated from a normal human DNA library as described and sequenced by Gazin *et al.* (1984). Construction of human c-*myc* recombinants was made as follows. A 2.4 kbp *PvuI* – *XhoI* fragment, including the 3' part of the *Amp* gene of pKH 47 (Hayashi, 1980) the end of the *env* gene, two functional LTRs and the beginning of the *gag* gene (with the splice donor sequence) of a cloned AEV provirus (pAEV 11, Vennström *et al.*, 1980) was purified by agarose gel electrophoresis. This fragment was introduced into p c-*myc* (pKH47 containing the 12 kbp *Eco*RI c-*myc* fragment) by ligation in the *Pvu*I (plasmidial)-*Xho*I (c-*myc*) sites of this plasmid. This generated the plasmid named pX-AHM. The nomenclature used to refer to the recombinant molecules is p for plasmid, v for virus, followed by the initial of the endonuclease used to generate the recombinant (when two enzymes are used, the first refers to the restriction site of the retroviral sequence, the second refers to the restriction sites of the c-*myc* sequence); AHM refers to activated human *myc*. The pSX-AHM DNA was derived from the pX-AHM DNA by removing the *SstI* – *XhoI* *gag* – *myc* junction fragment. The following procedure was used: the pX-AHM DNA was digested with *SstI* endonuclease and treated with DNA polymerase I, large fragment (Klenow polymerase). The same amount of this plasmid was digested with *Xho*I endonuclease and similarly treated with Klenow polymerase. These two DNAs were then digested with *Hin*dIII endonuclease (located in the plasmid) and the large *SstI* – *Hin*dIII fragment and the small *XhoI* – *Hin*dIII fragment were purified and ligated to generate pSX-AHM DNA. pSm-AHM was generated by deletion of the small *SmaI* – *SmaI* insert *gag* – *myc* exon 1 fragment of pX-AHM. pEP-AHM was obtained in four steps, and detailed strategy is available upon request. This recombinant plasmid contained an inverted (3' 5') AEV-LTR inserted in the *Pvu*II site located upstream from c-*myc* exon 1.

Transfection procedure

Primary cultures of quail embryo cells (QEC) were prepared from 10-day-old Japanese quail embryos; 2 days later the cells were passaged, and 10^6 cells were seeded onto 60-mm Petri dishes in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). Transfections were carried out as described by Graham and Van der Eb (1973) using 30 μg of purified *Bcl*I insert of c-*myc* recombinant and 5 μg of pRAV1 plasmid DNA (a gift from J.M.Bishop). In experiments without helper pRAV1 DNA, we used 30 μg of plasmid DNA. After 16 h, the transfected cells were seeded in two 100-mm Petri dishes and propagated until complete transformation. Transformed foci which appeared in transfection experiment without pRAV1 DNA were picked, pooled when necessary and grown up in DMEM 10% FCS.

Nucleic acid analysis

Total cellular RNA was extracted and fractionated on oligo (dT)-cellulose. Polyadenylated fractions were denatured by glyoxal treatment, submitted to electrophoresis in 1% agarose gels and transferred to nitrocellulose. Blots were hybridized to [^{32}P]DNA, washed and subjected to autoradiography at −70°C as previously described (Coll *et al.*, 1983).

Cloned DNA probes

DNA fragments for the preparation of ^{32}P probes were obtained from suitable recombinant plasmids by endonuclease digestion and agarose gel purification. Human c-*myc* probes corresponding to exon 1 (*XhoI* – *SstI* fragment), exon 2 (*SstI* – *SstI* fragment) and exon 3 (*ClaI* – *Eco*RI fragment) are depicted on top of Figure 1.

Cloning procedures

QEC infected with virus produced from QEC cotransfected with pSX-AHM and pRAV1 DNAs were used as source of DNA, and a partial *Eco*RI gene library was prepared in Charon 4A vector, as reported (Coll *et al.*, 1983). Subcloning of the SX-AHM provirus was performed in the *Eco*RI site of pKH 47 (Hayashi, 1980).

Protein labelling and immunoprecipitation

Labelling was performed on semi-confluent cultures seeded in 100-mm Petri dishes by incubation of the cells in 3 ml Modified Eagle Medium lacking methionine and 0.20 mCi [^{35}S]methionine for 45 min. These cells were lysed in 3 ml of 0.1% SDS, 1% Triton, 0.5% deoxycholate and 1% trasylol, Tris – HCl 10 mM pH 7.4, NaCl 150 mM, EDTA 1 mM buffer (RIPA) and clarified at 100 000 *g* for 1 h. 0.25 ml of the supernatant was then incubated for 3 h at 4°C with 5 μl rabbit antiserum prepared with a bacterially expressed polypeptide corresponding to the carboxy terminal part of the human c-*myc* protein (Martin *et al.*, 1986). 10 mg of protein A sepharose beads were added to each sample for 3 h at 4°C. Beads were washed in RIPA buffer, then in a buffer containing Tris – HCl 10 mM pH 7.4, NaCl 150 mM, EDTA 1 mM. Radioactivity was recovered from beads by boiling for 5 min in electrophoresis loading buffer (1% SDS, 5% mercaptoethanol, 10% glycerol, 50 mM Tris – HCl pH 6.8) and loaded onto 10% acrylamide gels, followed by fluorography of the dried gel (Martin *et al.*, 1986).

Nucleotide sequencing of SX-AHM c-myc insert

Plasmid DNA containing the SX-AHM molecularly cloned provirus was digested with restriction endonuclease (see Figure 5). Fragments were recovered from a 1% agarose gel and subjected to nucleotide sequencing by the Maxam and Gilbert procedure (Maxam and Gilbert, 1980). The fragments were dephosphorylated and labelled with [^{32}P]ATP and polynucleotide kinase as described (Herisse *et al.*, 1980). To separate the two labelled strands, fragments were denatured at 92°C in 30% dimethylsulfoxide and fractionated in a polyacrylamide gel. 5' labelled single-stranded fragments were recovered from the gel and subjected to chemical degradation with reagents specific for G, AG, CT, C and AC.

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Induction of proliferation or transformation of neuroretina cells by the *mil* and *myc* viral oncogenes

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The genome of the avian retrovirus MH2 contains, in addition to the v-*myc* oncogene shared with three other avian retroviruses (MC29, CMII and OK-10), a second cell-derived oncogene, v-*mil* (refs 1–3). Like the three other viruses, which contain only v-*myc*, MH2 induces mainly liver and kidney carcinomas in fowl and transforms fibroblasts and macrophages *in vitro*[4]. However, MH2 and MC29 differ in their biological properties when assayed on cultures of chicken embryo neuroretina (NR) cells. Indeed, NR cells, which normally do not multiply *in vitro*, are induced to proliferate and become transformed upon infection with MH2, whereas infection with MC29 has no apparent effect on these cells[5,6]. To analyse the functions of the two oncogenes of MH2, we isolated spontaneous and *in vitro*-constructed mutants of this virus and investigated their effects on NR cell multiplication and transformation. We report here that expression of v-*mil* is sufficient to induce NR cell proliferation, although it does not result in cell transformation. In addition, viruses expressing only the v-*myc* oncogene fail to induce any detectable change in NR cells. However, cooperation of the two oncogenes is required to achieve transformation of NR cells by MH2.

Neuroretinas, dissected from 7-day-old chicken embryos, were dissociated into essentially single-cell suspensions and plated in Eagle's basal medium containing 5% fetal bovine serum. These cultures contain only neurones and glial cells[7,8]. Infection of NR cells with MH2 resulted within 7–10 days in the appearance of actively proliferating cells which could be readily subcultured for several generations (Fig. 1*a*). These cells were morphologically transformed and displayed anchorage-independent growth capacity (results not shown). In contrast, NR cells infected with MC29, CMII or OK-10, or with the helper virus RAV-1 used as a control, retained the morphology and limited growth capacity of normal cells (Fig. 1*a*). This lack of phenotypic response was not due to a block in virus replication, because the v-*myc* products were synthesized in infected cells and supernatants of infected NR cultures contained virus that efficiently transformed chicken embryo fibroblasts (results not shown).

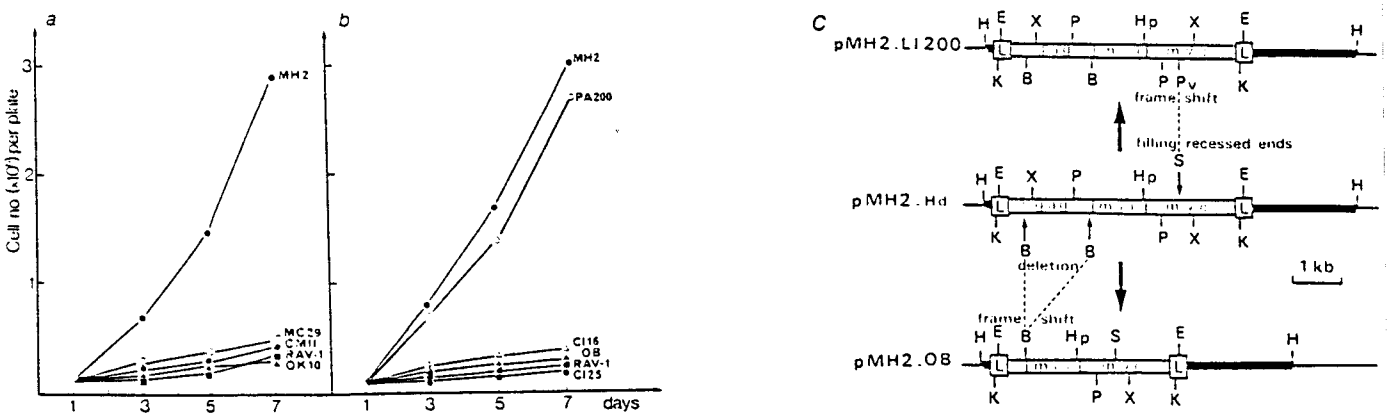

Fig. 1 Growth kinetics of virus-infected chicken neuroretina cells. NR cells were infected with MH2 (wild-type), MC29, CMII, OK-10 and RAV-1 (*a*) and MH2, MH2-PA200, MH2-cl.16, MH2-cl.25, MH2-OB and RAV-1 (*b*), then seeded at low density (~2×10^5 cells per 5-cm dish). Medium was renewed daily and cells from two aliquot dishes were counted as indicated. Viruses used were obtained by RAV-1 superinfection of non-producer clones. (*c*) Constructed mutants. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*I; X, *Xho*I; L, long terminal repeat. Open boxes represent proviral sequences and closed boxes flanking cellular sequences.

Methods. Preparation of NR cell cultures and growth assay: Neuroretinas were dissected from 7-day-old chick embryos. Cells were dissociated and resuspended in Eagle's basal medium (BME) supplemented with 5% fetal calf serum. Dishes (35 mm) containing ~2×10^6 cells were infected with the various viruses. When proliferation became evident in wild-type MH2-infected cells (7–10 days post-infection), cultures were passaged once in 5-cm dishes until MH2-infected cells reached confluency, and then seeded at low density for growth assay. Isolation of class I mutants MH2-cl.16, MH2-cl.25: Two viral stocks were used. MH2 (RAV-1) was obtained by superinfecting a non-producer quail embryo fibroblast (QEF cells) clone, MH2-QB2 (ref. 11), with RAV-1. MH2 (RAV-3) was kindly provided by Dr C. Moscovici. Both viruses exhibited comparable mitogenic and transforming properties in NR cells and directed the synthesis of $P100^{gag\text{-}mil}$ in transformed cells. QEF cells were infected at low multiplicity with MH2 (RAV-1) or MH2 (RAV-3) and seeded 6 h later at low density in soft agar-containing medium. Colonies of transformed cells developed within 2 weeks and were individually transferred to 35-mm dishes. At confluency, RAV-1 was added to rescue virus from occasional non-producer clones. Supernatants were used to infect NR cells. Media from two such clones, 16 and 25, contained virus which failed to induce NR cell growth. MH2-cl.16 originated from MH2 (RAV-1) and MH2-cl.25 from MH2 (RAV-3). The two mutant viruses were subcloned on QEF cells in soft agar, and virus stocks rescued from non-producer colonies by RAV-1 superinfection were unable to induce NR cell multiplication, but transformed chicken fibroblasts and macrophages. Isolation of class II mutant MH2-PA200: MH2-PA200 was isolated by end-point dilution of MH2 on NR cells. NR cells were infected with serial twofold dilutions of MH2 (RAV-1), to determine the highest dilution of the virus inducing cell proliferation. The reciprocal of this dilution defined the mitogenic titre of the virus in mitogenic units (MU) per ml. Several NR cultures were then infected with 0.3–0.5 MU per plate and superinfected 1 week later with RAV-1 to rescue virus from occasional non-producer cells. Virus produced by one such culture was selected for this study because it induced NR cell multiplication in the absence of morphological transformation. This variant was designated MH2-PA200 (RAV-1). Strategy used for constructed mutants (*c*): DNA of pMH2-Hd plasmid[1] was digested with *Bam*HI, ligated and transfected in *Escherichia coli* HB101 cells. A clone was selected, pMH2-OB, which lacked the 1.3-kb *Bam*HI restriction fragment derived from the *gag* gene. Sequence analysis around the recombination site indicated that translation of the pMH2-OB RNA should result in the synthesis of a product containing the amino-terminal 52 amino acids of $p19^{gag}$ linked to 5 amino acids derived from the v-*mil* sequence in a +1 frameshift (C. Henry and B. Debuire, unpublished data). To construct the MH2-LI200 mutant, DNA of pMH2-Hd plasmid was partially cleaved by *Sal*I endonuclease and cohesive termini were filled in by treatment with the Klenow fragment of DNA polymerase I. This generated a new *Pvu*I site as expected, and should result in a +1 frameshift in the translation of the v-*myc* sequence. Infectious virus (MH2-OB) was rescued from pMH2-OB DNA transfected cells using RAV-1 as helper virus.

To analyse the biological properties of the two oncogenes of MH2 further, we isolated two classes of virus mutants by their capacity to alter the phenotype of NR cells. Class I mutants were selected because they were unable to induce NR cell proliferation (see Fig. 1 legend). Two such mutants, MH2-cl.16 and MH2-cl.25, were isolated from two distinct viral stocks. MH2-cl.16 and MH2-cl.25, like wild-type MH2, transformed chicken embryo fibroblasts and macrophages (T. Graf, personal communication). In contrast to wild-type virus-infected cultures, NR cells infected with either mutant were not transformed, nor were they stimulated to grow (Fig. 1*b*). An additional mutant virus, MH2-OB, possessing similar biological properties (Fig. 1*b*), was obtained by deleting most of the *gag* sequence in plasmid pMH2-Hd (ref. 1) which contains a biologically active provirus (this yields a premature termination of the *gag-mil* protein, see Fig. 1*c*).

Two class II mutants were isolated as follows. Mutant virus MH2-PA200 was selected because it induced proliferation of NR cells, without morphological transformation (Fig. 1 legend). Such cells could be subcultured for many generations but ultimately did senesce (after ca. two month). The second mutant, MH2-LI200, with similar biological properties, was derived from plasmid pMH2-Hd (ref. 1) by generating a frameshift mutation in v-*myc*, resulting in the premature termination of its translation product (Fig. 1*c* legend).

The $P100^{gag\text{-}mil}$ translation product of v-*mil*[9,10] was readily detected by immunoprecipitation of lysates of ^{35}S-methionine-labelled cells infected with either wild-type MH2 or class II mutants (that is, MH2-PA200, Fig. 2*b*) using antisera specific for *gag* proteins (lanes 2) or for a bacterially expressed v-*mil* protein (lanes 3). In contrast, no v-*mil* specific protein was detected in fibroblasts transformed by class I mutants (Fig. 2*c–e*, respectively).

We next performed blot analyses of RNA isolated from cells infected with either wild-type or mutant viruses. As expected from previous studies[1,11], cells infected with wild-type MH2 synthesized a 5.7-kilobase (kb) genomic RNA detectable with either a v-*mil* or a v-*myc* probe (Fig. 3*c*, lanes 1 and 2, respectively) and a 2.8-kb subgenomic RNA detected with a v-*myc* probe only (Fig. 3*c*, lane 2). The 5.7-kb genomic RNA and the 2.8-kb subgenomic RNA species are known to encode respectively the $P100^{gag\text{-}mil}$ and $p60/61^{myc}$ proteins[12,13]. The 2.8-kb *myc*-specific RNA was detected in fibroblasts transformed by MH2-cl.16, MH2-cl.25 and MH2-OB at levels similar to those observed in cells transformed by wild-type MH2 (Fig. 3*a*, *b*, *d*, lanes 2). No virus-specific RNA hybridizing to the v-*mil* probe was detected in cells infected with mutant virus MH2-cl.16 and MH2-cl.25, indicating that these mutants had lost most or all of the v-*mil* sequences. In addition to the 2.8-kb RNA, cells infected with MH2-OB synthesized a 4.4-kb RNA that hybridized to both v-*mil* and v-*myc* probes (Fig. 3*d*). This, together with the size of the fragment deleted to generate pMH2-OB (1.3-kb, see Fig. 1*c*), led us to conclude that the 4.4-kb RNA was the genomic RNA of MH2-OB. The 4.0-kb species detected by the v-*mil* probe corresponds to the previously described c-*mil* endogenous transcript in fibroblasts[1] and appears on long exposures (Fig. 3*a*, *b*, lanes 1).

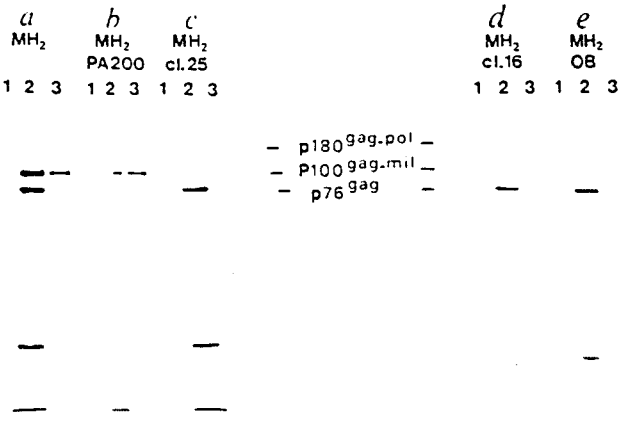

Fig. 2 Characterization of *gag* and *mil* related proteins synthesized in avian cells infected by wild-type and mutant MH2 viruses. Chicken NR cells transformed by wild-type MH2 (*a*), or infected by MH2-PA200 (*b*) and fibroblasts transformed by MH2-cl.25 (*c*), MH2-cl.16 (*d*) and MH2-OB (*e*) were incubated for 3 h in the presence of 30 $\mu Ci\ ml^{-1}$ of L-35S-methionine (specific activity 1,000 $Ci\ mmol^{-1}$), lysed and immunoprecipitates prepared as described previously[22]. Sera used were as follows: lanes 1, preimmune rabbit serum; lanes 2, rabbit anti *gag* serum; lanes 3, rabbit antiserum prepared to a bacterially expressed protein corresponding to the carboxy terminus of $P100^{gag\text{-}mil}$ (F.D. and J.G., in preparation). Immunoprecipitated proteins were analysed by SDS-polyacrylamide gel electrophoresis as described by Laemmli[23] followed by fluorography on the dried gel.

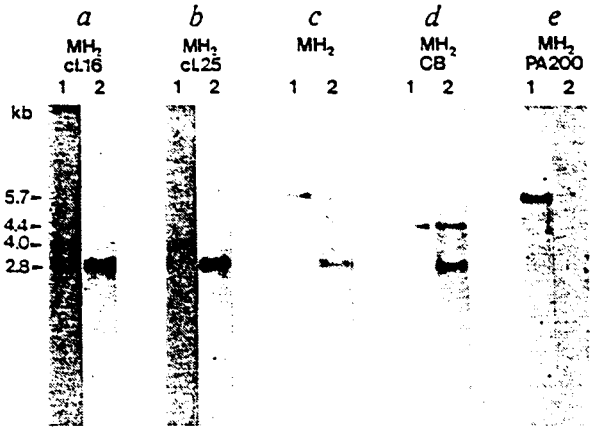

Fig. 3 Size of viral RNA in MH2 wild-type or mutant infected chicken fibroblasts and NR cells. Northern blots of polyadenylated RNA were probed in lanes 1 with a 1.1-kb *Bam*HI-*Hpa*I v-*mil*-specific fragment (see Fig. 1*c*) and in lanes 2 with a 0.6-kb *Alu*I/*Hae*III-*Eco*RI fragment derived from the last exon of the chicken c-*myc* locus[24]. The probes were labelled with ^{32}P by nick translation and used as described previously[11]. Viruses used are listed across the top of the figure. Lanes *a–d*, transformed fibroblasts; lane *e*, MH2-PA200-infected NR cells. Transformed fibroblasts in lane *c* were obtained after transfection with a molecularly cloned MH2 provirus (pMH2-Hd)[1] together with a plasmid containing a biologically active RAV-1 provirus (a generous gift from Dr J. M. Bishop).

In contrast, no RNA hybridizing to a v-*myc* probe was detected in NR cells infected with MH2-PA200 (Fig. 3*e*, lane 2). The only virus-specific RNA synthesized in these cells was a 5.7-kb RNA hybridizing to a v-*mil* probe (Fig. 3*e*, lane 1). This RNA also hybridized to an *env* probe (not shown). The MH2-PA200 provirus has been molecularly cloned and the restriction map of this provirus suggests that it has been generated by recombination between wild-type MH2 and its helper virus, resulting in the probable substitution of Δgag-pol-Δenv sequences of the helper by Δgag-mil-Δmyc sequences from wild-type MH2 (to be published elsewhere).

MH2-PA200 and MH2-LI200 were as efficient as wild-type virus in inducing proliferation of NR cells (Fig. 1*b*). However, in contrast to wild-type virus-infected cells, NR cells infected with either mutant were not morphologically transformed nor were they able to form colonies in soft agar-containing medium. Both virus mutants also failed to transform macrophages (T. Graf, personal communication) and chicken embryo fibroblasts (not shown). The latter is surprising since the mouse virus MSV3611[19] that contains v-*raf*, the mouse-derived counterpart of v-mil, readily transforms mouse fibroblasts. This may indicate a structural difference in the two transduced genes or a different response of the target cells in the chicken and mouse species. Finally, reconstitution experiments showed that proliferating NR cells infected with MH2-PA200 became rapidly transformed on superinfection with MC29 (not shown).

The results presented here show that the ability to induce proliferation and transformation of NR cells from 7-day-old chicken embryos is a characteristic property which distinguishes MH2 from the other v-*myc*-containing viruses. Characterization of mutants MH2-PA200 and MH2-LI200 demonstrates that expression of v-*mil* is both necessary and sufficient to account for the mitogenic property of MH2 in NR cells. However, infection of NR cells and fibroblasts with mutant viruses expressing only v-*mil* does not result in cell transformation. That the mutant MH2-LI200 exhibits similar biological properties indicates that the v-*mil* sequence of MH2-PA200 is similar to that of wild-type virus. The v-*mil* gene shares significant sequence homology with other oncogenes of the *src* gene family[14] which induce both proliferation and transformation of NR cells[5,15]. Interestingly, mutants in the *src* gene that, like v-*mil*, induce NR cell multiplication without transformation, have been described[5]. Several oncogenes of the *src* gene family encode plasma membrane-associated protein kinases specific for tyrosine residues, a property also shared by several growth factor receptors[16,17]. In contrast, $P100^{gag\text{-}mil}$ appears localized in the cytoplasm of transformed fibroblasts and is associated *in vitro* with a serine/threonine-specific protein kinase activity[18,19]. Such differences in intracellular localization and/or kinase specificity may explain the lack of transforming capacity of the v-*mil* product. Whether the mitogenic signals induced by the expression of v-*mil* and v-*src* in NR cells share common primary targets remains to be determined.

Mutants of MH2 expressing only the v-*myc* oncogene fail to induce transformation and proliferation of NR cells in the conditions used, although they still transform fibroblasts and macrophages. Therefore, the v-*myc* genes of MH2 and MC29 seem to be functionally indistinguishable, despite their structural differences[20,21]. However, transformation of NR cells by the v-*myc* gene can be established in cells already infected with mutants expressing only the v-*mil* gene. These results indicate that transformation of chicken NR cells by MH2 requires cooperation of the two oncogenes.

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Characterization of a MH2 Mutant Lacking the *v-myc* Oncogene

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We have previously reported that a virus, MH2-PA200, lacking the ability to transform quail embryo cells, could be isolated from wild type (wt) MH2 stocks passaged on chicken neuroretina cells. We report here the molecular cloning and extensive characterization of this MH2-PA200 provirus. Molecularly cloned MH2-PA200 DNA was found to stimulate the growth of neuroretina cells by transfection assays and our results indicate that this recombinant virus was derived from the RAV-1 helper virus, in which *v-mil* and a small part of *v-myc* of MH2 were acquired at the expense of helper (*Δgag-pol-Δenv*) sequences. In order to assess the precise boundary between the *myc* and *env* genes we determined the nucleotide sequence of the junction fragment and showed that 11 of 13 nucleotides of the *env* gene were identical to the *myc* sequence at the recombination point. The nucleotide sequence of the *myc-env* junction fragment of another similar and independently generated MH2 mutant showed similarly 9 nucleotides of homology between the *env* and *myc* sequences at the recombination point that took place at another site, suggesting that a homologous recombination occurred between MH2 and RAV-1 viruses to generate MH2-PA200 and similar mutants.

INTRODUCTION

The avian retrovirus Mill-Hill No. 2 (MH2) is a replication defective retrovirus that induces carcinomas in fowl and transforms several cell types in culture (Graf and Beug, 1978). The MH2 genome contains the *v-myc* oncogene also found in three other retroviruses (MC29, CMII, OK10) and a second cell-derived oncogene *v-mil* (Coll *et al.*, 1983; Jansen *et al.*, 1983) related to the *src* gene of RSV (Galibert *et al.*, 1984; Kan *et al.*, 1984). In MH2 transformed cells, *v-mil* is expressed as a 100-kDa polyprotein (Hu *et al.*, 1978) resulting from the fusion of *gag* and *mil* sequences (Bechade *et al.*, 1985). This cytoplasmic protein exhibits an *in vitro* phosphothreonine/phosphoserine kinase activity (Moelling *et al.*, 1984). *v-myc* is expressed as a 61/63-kDa protein doublet encoded by a 2.8-kb subgenomic mRNA (Hann *et al.*, 1983; Martin *et al.*, 1986). We recently distinguished some biological properties of the *mil* and *myc* oncogenes by studying the properties of virus-infected avian fibroblasts and embryo neuroretina cells (NR cells). wt-MH2, MC29, CMII, and OK10 which contain *v-myc* are able to transform avian fibroblasts whereas wt-MH2 which contains both *v-mil* and *v-myc* has in addition the ability to induce the proliferation of avian embryo NR cells (Bechade *et al.*, 1985). From MH2 producing chicken NR cells we isolated MH2 mutants which are unable to morphologically transform avian embryo cells and do not contain the functional *v-myc* oncogene. In order to define the genetic organization of such *v-myc* defective mutants, we molecularly cloned a *v-mil* containing provirus from MH2-PA200 proliferating quail NR cells (Bechade *et al.*, 1985). Our results suggest that MH2-PA200 was generated by homolgous recombination between the RAV-1 helper virus and MH2, resulting in the replacement of most of the helper *gag-pol-env* sequences by *v-mil* and part of *v-myc* of MH2.

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MATERIALS AND METHODS

Cells and viruses. MH2-PA200 was previously characterized (Bechade *et al.*, 1985), and was derived from a MH2 (RAV-1) pseudotype virus obtained by superinfection of a non-producer quail fibroblast clone MH2 QB2 with RAV-1. Another MH2 mutant (MH2-PA201) was isolated from 7-day-old chicken embryo neuroretina (CNR) cells infected by a virus produced by quail embryo cells (QEC) co-transfected with wt-MH2 DNA, pMH2-Hd (Coll *et al.*, 1983) and helper DNA pRAV-1 (a kind gift of J. M. Bishop).

Transfection procedure. Seven-day-old quail embryo neuroretina (QNR) cells were transfected with phage DNA according to the calcium method developed by Graham and van der Eb (1973). Precipitated DNA (35 μg) was added in 1 ml volume to 100-mm dishes containing 10^7 cells. After 1 hr incubation, cells were fed with 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Ten days later, proliferating cells were observed and were grown up in DMEM 10% FCS.

Nucleic acids analysis. High molecular weight DNA was isolated as described (Coll *et al.*, 1983). Cellular or cloned DNAs were digested with restriction endonucleases (Boehringer Mannheim, FRG, and BRL Inc., USA) according to the supplier's instructions. Fragments were size separated by agarose gel electrophoresis and transferred to nitrocellulose according to Southern (1975). Total cellular RNA was extracted and fractionated on oligo (dT)-cellulose as described (Saule *et al.*, 1983). Polyadenylated fractions were denatured by glyoxal treatment, submitted to electrophoresis in 1% agarose gels, and transferred to nitrocellulose. Blots were hybridized to ^{32}P-labeled DNA, washed and subjected to autoradiography at −70° as previously described (Saule *et al.*, 1983).

Cloned DNA probes. DNA fragments for the preparation of ^{32}P probes were obtained from suitable recombinant plasmids by endonuclease digestions and agarose gel purification.

The LTR probe was from the RSV-SRA genome and represented the 0.36-kbp *Eco*RI fragment containing the LTR sequences. The *gag* probe (*Sac*I-*Eco*RI), the *pol* probe (*Eco*RI-*Kpn*I), the 5′ *env* probe (*Kpn*I-*Eco*RI), and the 3′ *env* probe (*Eco*RI-*Sac*I) were derived from Pr-RSV-A and previously described (Saule *et al.*, 1983). *myc* probes included a 5′ *v-myc* probe (*Hpa*I-*Pst*I) derived from pMHX-Hd (Coll *et al.*, 1983) and a 3′ *c-myc* probe corresponding to the 3′ half of chicken exon 3 (Saule *et al.*, 1984). The *v-mil* probe was obtained from the 1.1-kb *Bam*HI-*Hpa*I fragment of plasmid pMH2BS (Coll *et al.*, 1983). The purified fragments were labeled through nick-translation reaction (Amersham nick-translation kit) in the presence of [^{32}P]dCTP according to the supplier's instructions.

Cloning procedures. MH2-PA200 (RAV-1) growing QNR cells were used as source of DNA and a partial *Eco*RI gene library was prepared in a Charon 4A vector, as reported (Coll *et al.*, 1983), with a minor modification in the preparation of Charon 4A arms by purification on a sucrose gradient. MH2 (RAV-1) proliferating CNR cells were used similarly as source of DNA in order to isolate *v-myc* deleted MH2 CNR provirus from a partial *Sau*3A gene library prepared in EMBL 4 vector. One such recombinant was called MH2-PA201.

Protein labeling and immunoprecipitation. Labeling was performed on semiconfluent cultures seeded in 100-mm petri dishes by incubation in 3 ml of modified Eagle's medium lacking methionine (Met) followed by the addition of 0.25 mCi [^{35}S]methionine for 60 min. Cells were lysed in 3 ml of 0.1% SDS, 1% Triton, 0.5% deoxycholate, and 1% trasylol, Tris-HCl 10 m*M*, pH 7.4, NaCl 150 m*M*, EDTA 1 m*M* buffer (RIPA), and clarified at 100,000 *g* for 1 hr. Supernatant (0.25 ml) was then incubated for 3 hr at 4° with 3 μl rabbit antiserum prepared with a bacterially expressed protein corresponding to the carboxy terminal part of P100$^{gag\text{-}mil}$ (F. Denhez and J. Ghysdael, in preparation). Protein A-Sepharose beads (10 mg) were added to each sample for 3 hr at 4°. Beads were

washed in RIPA buffer, then in a buffer containing Tris-HCl 10 m*M*, pH 7.4, NaCl 150 m*M*, EDTA 1 m*M*. Radioactivity was recovered by elution from beads by 5 min boiling in electrophoresis loading buffer (1% SDS, 5% mercaptoethanol, 10% glycerol, 50 m*M* Tris-HCl, pH 6.8) and loaded onto 10% acrylamide gels, followed by fluorography of the dried gels (Bechade *et al.*, 1985).

Preparation of the env-myc fragment and nucleotide sequencing. Subcloning of the *Eco*RI Δ*gag mil*Δ*myc*Δ*env* fragment of the MH2-PA200 provirus was performed in the *Eco*RI site of pKH47 (Hayashi, 1980). Subcloning of the *Bam*HI-*Eco*RI *mil*-Δ*myc*-Δ*env* fragment of MH2-PA201 was performed similarly in *Bam*HI-*Eco*RI sites of pKH 47. MH2-PA200 plasmid DNA was digested with *Hin*cII, digests were loaded onto a 1% agarose gel and electrophoresed overnight. The 0.4-kbp Δ*mil*-Δ*myc*-Δ*env* fragment from MH2-PA200 was then recovered and digested with *Hae*III. For MH2-PA201, plasmid DNA was digested with *Hpa*I-*Xba*I and the 0.4-kb Δ*mil*-Δ*myc*-Δ*env* fragment was recovered from a 1% agarose gel and subjected to *Hae*III enzymatic digestion. Nucleotide sequencing was performed by the Maxam and Gilbert procedure (1980). The fragments were dephosphorylated and labeled with [γ^{32}P]ATP and polynucleotide kinase as described (Herisse *et al.*, 1980). To separate the two labeled strands, fragments were denatured at 92° in 30% dimethylsulfoxide and fractionated in a polyacrylamide gel. 5′ labeled single stranded fragments were recovered from the gel and subjected to chemical degradation with reagents specific for G, AG, CT, C, and AC.

RESULTS

Molecular Cloning of MH2-PA200 Provirus

QNR cells were infected with biologically cloned MH2-PA200 (RAV-1) pseudotype virus. DNA from these cells was used as a source of DNA for the preparation of a gene library. High molecular weight DNA was partially cut with *Eco*RI restriction endonuclease, ligated with purified λ Charon 4A arms, packaged and amplified. One λ phage which hybridized with the *v-mil* and 3′ *env* probes was isolated, then purified and amplified. Phage DNA was prepared and digested with different restriction enzymes. Fragments were run on agarose gels, transferred to nitrocellulose and hybridized with different ^{32}P-nick translated probes. A detailed restriction map of the provirus is shown in Fig. 1.

The hybridization patterns obtained (Fig. 1), allowed the following observations: first, the cloned MH2-PA200 provirus contained a LTR-*gag* sequence probably derived from RAV-1 helper virus, since the *Kpn*I endonuclease site present in wt-MH2-LTR but absent in RAV-1 was not found in MH2-PA200. In addition the *Pst*I site located in the 5′ part of the *gag* sequence in both MH2-PA200 and the RAV-1 helper virus was not present in wt-MH2 (Fig. 1). Second, the MH2-PA200 provirus hybridized to the 5′ *v-myc* probe but not to the 3′ *v-myc* probe suggesting that part of the *v-myc* gene was present in this recombinant virus. Finally, both the 5′ and 3′ *env* probes hybridized to the MH2-PA200 provirus DNA. The *pol* probe did not anneal (data not shown). Therefore it appeared that the 3′ recombination point occurred between 5′ *myc* sequences of wt-MH2 and 5′ *env* sequences of RAV-1 helper.

MH2-PA200-like Mutants Are Revealed by Neuroretina Cells

The use of a specific biological assay (NR cell proliferation) reveals rapidly and efficiently MH2-PA200-like mutants. This was illustrated by analyzing proviral DNA from NR cells freshly infected with virus produced from QEC transfected with pMH2-Hd and pRAV-1 DNAs; this wt-MH2 (RAV-1) virus was passaged only once on quail embryo cells (QEC) after transfection. Chicken NR cells were passaged twice 20 days after infection with cloned wt-MH2 (RAV-1) virus in order to select proliferating cells before proviral DNA analysis. Southern blots of DNAs cleaved with *Eco*RI or other endonucleases allow-

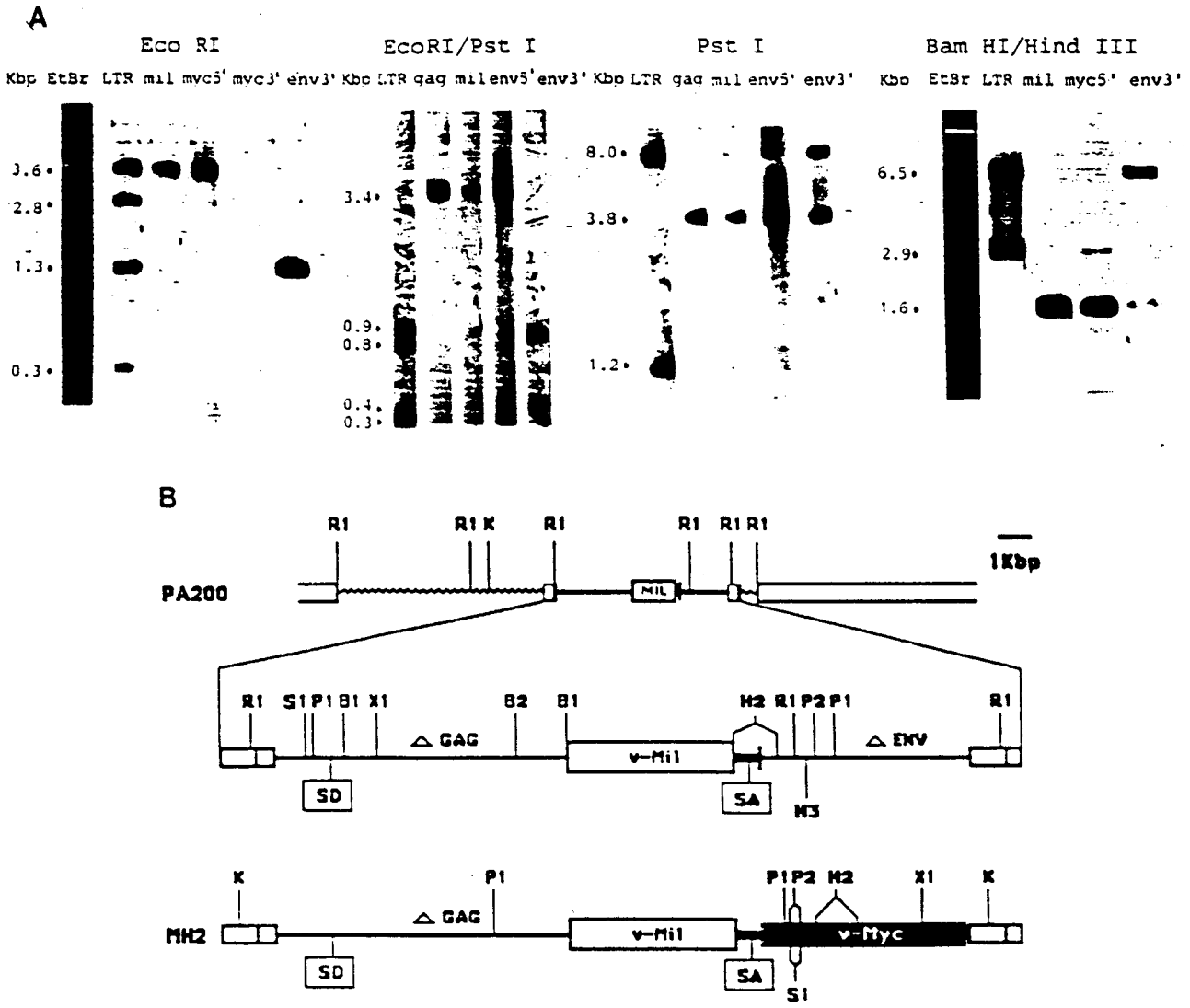

FIG. 1. Characterization of cloned MH2-PA200 proviral DNA. (A) λ MH2-PA200 phage DNA was digested with *Eco*RI, *Eco*RI-*Pst*I, *Pst*I, *Bam*HI-*Hin*dIII restriction enzymes. Digests (1 μg/lane) were separated on 1% agarose gels, transferred onto nitrocellulose, and hybridized with the nick-translated probes listed across the top of the figure. Sizes of significant hybridization positive MH2-PA200 bands are indicated (arrowheads). (B) Organization of MH2-PA200 DNA: a restriction map of the recombinant virus can be compared with that of the cloned MH2-Hd (lane 3). Restriction endonucleases used were R1, *Eco*RI; K, *Kpn*I; S1, *Sst*I; P1, *Pst*I; B1, *Bam*HI; X1, *Xho*I; B2, *Bgl*II; H2, *Hin*cII; P2, *Pvu*II; H3, *Hin*dIII. Only the restriciton sites different from those of MH2-PA200 have been indicated on wt-MH2 provirus restriction map. The first *Hin*cII site present in MH2-PA200 was also a *Hpa*I site. SD, splice donor site; SA, splice acceptor site. λ arm DNA, ▭; cellular DNA, ⁓; LTR, ▭.

ing to distinguish between parental and mutant viruses were hybridized to a *v-mil* probe (Fig. 2). For example, *Eco*RI endonuclease which cuts in the LTRs of wt-MH2 DNA revealed a provirus-sized band of 5.5 kbp in QEC transformed by wt-MH2 (first lane). No band corresponding to recombinant mutants (expected around 3.5 to 4.0 kbp if the internal *Eco*RI site in the helper *env* gene were acquired) was detected, indicating that such mutants, if present, represented a minor fraction of the proviruses. A similar experiment performed on chicken NR cells (Fig. 2, third lane) readily revealed a strong 4.0-kbp band corresponding to recombinant molecules, in addition to a faint 5.5-kbp wt-MH2 proviral band. The mutant molecules at 4.0 kbp dif-

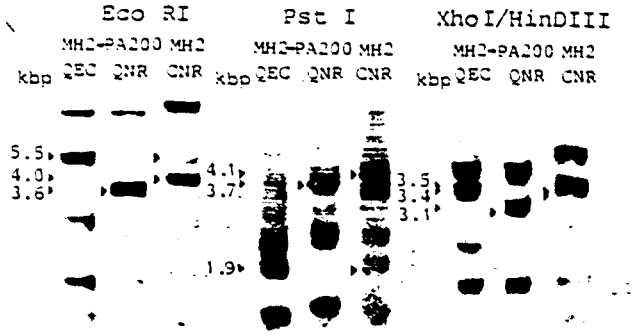

FIG. 2. Southern blot analysis of integrated proviruses from wt-MH2 and mutant infected cells. High molecular weight DNA from infected cells was digested with *Eco*RI, *Pst*I, and *Xho*I-*Hin*dIII endonucleases. Size separated cellular DNA fragments were transferred to nitrocellulose and hybridized with the *Bam*HI-*Hpa*I *v-mil* probe. Cell types and viruses are listed across the top of the figure. Arrowheads indicate *v-mil* containing DNA fragments.

fered in size from the *Eco*RI fragment of 3.6 kbp seen with MH2-PA200 mitogenized (quail) NR cells (Fig. 2 lane 2) suggesting that distinct recombination events had occurred in these two separate experiments. These results were confirmed by using other restriction endonucleases like *Pst*I or *Xho*I + *Hin*dIII (Fig. 2) or other probes like *gag* or 5′ *myc* (data not shown). Bands without arrows in Fig. 2 represent fragments of the cellular *mil* DNA. We thus conclude that molecularly cloned wt-MH2 can rapidly recombine with the RAV-1 helper to yield mutants lacking most of *v-myc* that appear to bear a selective advantage on chicken NR cells.

Next, we examined the transcription patterns of the MH2 mutants detected in Fig. 2 lanes 2 and 3. Polyadenylated mRNAs extracted from MH2-PA200 QNR and MH2 CNR cells were hybridized on Northern blots with *v-mil* and other probes. Results in Fig. 3 show that the MH2-PA200 virus exhibited a 5.6-kb genomic RNA which also hybridized with the LTR, 5′ *myc* and 3′ *env* probes. In addition, the LTR and 3′ *env* probes detected a 8.4- and a 3.2-kb viral RNA encoded by helper RAV-1 as well as a 2.4-kb band which also hybridized to the 5′ *myc* probe but not to the *v-mil* or 3′ *myc* probes. These results suggest that the splice acceptor site of the *myc* gene is conserved in this provirus and leads to the expression of a small subgenomic *myc-env* RNA. When analyzing MH2 CNR cells, a 6.0-kb MH2-RAV-1 recombinant genomic RNA was detected with a 3′ *env* probe in addition to the 8.4- and 3.2-kb helper mRNAs, confirming that this was a recombinant distinct from MH2-PA200. The existence of a putative subgenomic mRNA could not be demonstrated for this recombinant since its expected size was close to that of the RAV-1 helper *env* mRNA at 3.2 kb (Fig. 3 last lane).

Nucleotide Sequence of the myc-env Junction in the Recombinants

For further analysis of recombination, we molecularly cloned the above-described recombinant. A *Sau*3A DNA library from MH2 CNR cells in λ EMBL4 was screened with *mil* and *env* probes and a recombinant λ phage (MH2-PA201) containing a DNA insert which hybridized to both probes was isolated. The precise boundary between *myc* and *env* sequences was then determined in MH2-PA200 as well as MH2-PA201.

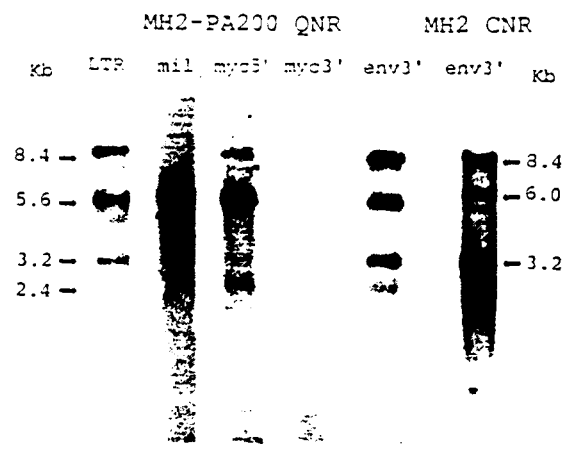

FIG. 3. Size of viral transcripts in proliferating NR cells. Poly A^+ containing RNA was denatured, separated on 1% agarose gel, and transferred to nitrocellulose. Blots were hybridized with the probes listed across the top. The size of the viral transcripts is indicated in the left- and right-hand columns of the figure. Bands at 8.4 and 3.2 kb with the 5′ *myc* probe were unexpected and probably due to a small contamination of the *myc* fragment by other MH2 related sequences.

The 0.4-kbp *Hin*cII fragment from MH2-PA200 provirus and the 0.4-kbp *Hpa*I–*Xba*I fragment from MH2-PA201 provirus were purified on agarose gels, cleaved with *Hae*III endonuclease and subjected to nucleotide sequence determination by the Maxam and Gilbert procedure. In Fig. 4 we compare the relevant nucleotide sequences to the wt-MH2 *myc* and SRA *env* sequences. In these two mutants it appeared that the recombination points were different but exhibited several similarities. In MH2-PA200, 11 homologous nucleotides divided into two stretches of five and six nucleotides were found between the *env* and *myc* sequences, whereas nine homologous nucleotides divided into two stretches of four and five nucleotides, were observed in MH2-PA201. This suggested a similar mechanism of recombination for these two viruses.

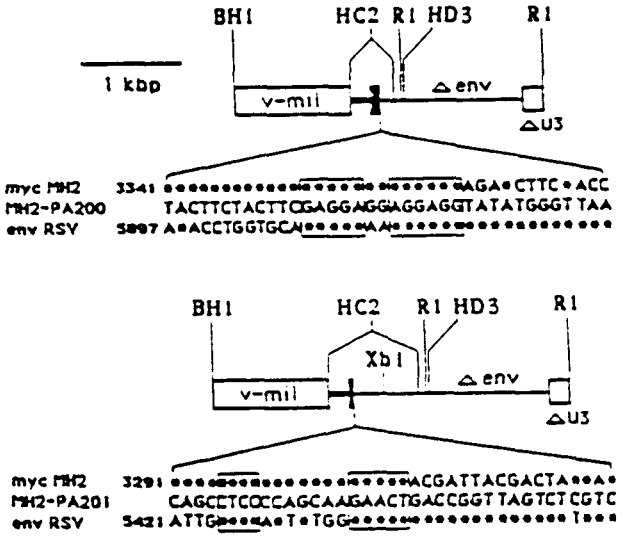

FIG. 4. Nucleotide sequence of the myc-env junction fragment. The determined nucleotide sequences of MH2-PA200 and MH2-PA201 (shown in Fig. 2 in MH2 CNR lanes) *myc-env* junction were compared to the *myc* sequence of wt-MH2 (Galibert *et al.*, 1984) and *env* sequence of RSV (Schwartz *et al.*, 1983). For both recombinants, we have indicated the structure of the *Bam*HI–*Eco*RI fragment used to determine the sequence. For MH2-PA200 the nucleotide sequence was determined by the Maxam and Gilbert procedure (1980) on both strands, whereas one strand was sequenced for MH2-PA201. ■ represents *myc;* asterisks represent nucleotides of *myc*-MH2 (upper line) and/or *env*-RSV (lower line) homologous to the nucleotides of *myc-env* junction of the mutants. Stretches of nucleotides common in the three sequences are boxed.

The mil Gene of the Molecularly Cloned MH2-PA200 Is Biologically Active

We examined whether the moleculary cloned MH2-PA200 was biologically active. λ MH2-PA200 DNA was used to perform transfection on QNR cells. DNA (30 μg) was transfected together with RAV-1 plasmid DNA (5 μg) onto 10^7 QNR cells. Ten days later, growing cells were detected and propagated. These cells were checked for the presence of MH2-PA200 by protein analysis using specific antisera derived from bacterially expressed *v-mil* polypeptide. [^{35}S]Met-labeled total cellular extracts were challenged with rabbit anti-*mil* as well as rabbit anti-*gag* sera. Immunoprecipitated proteins characterized by their apparent molecular weight in SDS-polyacrylamide gels are shown in Fig. 5. The P100$^{gag\text{-}mil}$ fusion protein was detected in these growing NR cells. Virus produced by the cultures induced the growth of fresh CNR cells (data not shown). Thus we concluded that cloned MH2-PA200 provirus was biologically active.

DISCUSSION

We reported previously that defined biological systems allowed to reveal distinct activities for the two oncogenes *v-mil* and *v-myc* of wt-MH2 (Bechade *et al.*, 1985) and to isolate mutants expressing only one or the other oncogene product. We have described mutants expressing the p61/63$^{v\text{-}myc}$ protein that transform QEC but do not induce NR cells proliferation (Martin *et al.*, 1986). We now report the characterization of mutants expressing the P100$^{gag\text{-}mil}$ gene product that induce the proliferation of NR cells, but not transformation of QEC. Two such mutants MH2-PA200 and MH2-PA201 were molecularly cloned and the 3′ recombination points were sequenced. The mutants appear to have arisen through homologous recombination between wt-MH2 and the RAV-1 helper virus used. The mutants consist of RAV-1 molecules hav-

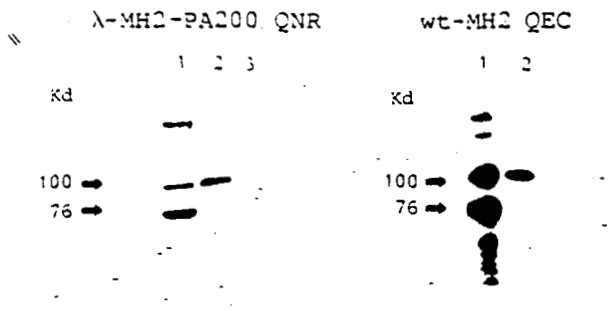

FIG. 5. Immunoprecipitation of P100$^{gag\text{-}mil}$ protein in QNR cells transfected with lambda MH2-PA200 DNA. Growing cells were labeled for 1 hr with [^{35}S]-methionine, lysed, and incubated with rabbit anti-*gag* serum prepared with the bacterially expressed *Xho*I-*Bam*HI fragment of the *gag* gene (F. Ferre, unpublished data) (lane 1); rabbit anti-*mil* serum (lane 2); and the same serum preincubated with the corresponding polypeptide (lane 3). Cell types used are indicated on top of the figure.

ing acquired *v-mil* and 200–300 nucleotides of *v-myc* from wt-MH2 at the expense of *Δgag-pol-Δenv* helper sequences. In both cases, the 3′ recombination points occurred between *myc* and *env* sequences, but at distinct location in both genes. Little information was obtained concerning the precise 5′ recombination points that occurred probably within the *gag* sequences between the *Pst*I restriction site in wt-MH2 at nucleotide 1542 (Galibert *et al.*, 1984) and the beginning of *mil* since this *Pst*I site is present in wt-MH2, absent in RAV-1 and also absent in both recombinants. Finally, both mutants are expected to still produce a subgenomic mRNA (i.e., 2.4 kb for MH2-PA200 in Fig. 3). The smallest deduced protein (for MH2-PA201) would include 6 amino acids of *gag* joined to 16 amino acids of *myc*. Although unlikely, we cannot formally exclude a participation of such a product to mitogenicity. Recombinant viruses may have occurred through reverse transcription of viral RNA, as proposed by Coffin (1979). The quite high frequency with which mutants seem to segregate in wt-MH2 transformed CNR cells could relate to two factors. First, CNR cells induced to proliferate by MH2-PA200-like viruses remain attached to cell-culture plates whereas wt-MH2 infected cells are transformed and easily released into the culture medium. They could have lost N-CAM-mediated adhesion as shown for CNR cells transformed by Rous sarcoma virus (Brackenbury *et al.*, 1984). Second, recombinant mutants appear to propagate much more efficiently than wt-MH2, since titers were found to be over 10^6 mitogenic units for MH2-PA200 (RAV-1) as compared to only 10^3 focus forming units for wt-MH2 (RAV-1). These two factors concur, separately or together to favor the selection of MH2-PA200-like mutants in infected CNR cells. Such a selection does not occur in chicken fibroblasts which may explain why this type of mutant was not observed previously.

Finally, with its *mil* oncogene MH2-PA200 resembles the mammalian retrovirus MSV 3611 that includes the mouse analog *raf* (*Jansen et al.*, 1984) of *v-mil* and has been shown to induce sarcomas in mice. We are thus currently testing the *in vivo* properties of our mutants.

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DISCUSSION

L'étude de la structure du génome ARN de certains virus défectifs des leucémies aiguës aviaires nous a permis de montrer que ces derniers dérivaient d'une recombinaison entre le génome d'un ALV peu ou pas oncogène, contenant les gènes de structure gag, pol, env et de gènes cellulaires phylogénétiquement stables responsables du pouvoir transformant de ces virus. Cette observation soulève quelques questions:

- par quels mécanismes se sont effectuées ces recombinaisons?
- quelle est la structure et le mode d'activation des c-onc correspondants?
- dans quels types cellulaires ces gènes sont-ils exprimés?
- par quels mécanismes les oncogènes viraux coopèrent-ils?

MECANISMES DE RECOMBINAISON

Les techniques que nous avons utilisées pour caractériser l'ARN viral des DLV ne permettent pas d'avoir accès à la structure fine du génome de ces virus. En effet nous avons utilisé les techniques d'hybridation moléculaire en phase liquide avec des sondes préparées de façon soustractive. Ainsi, le gène env par exemple, représente la portion du génome d'un ALV absente du virus sarcomatogène Bryan défectif dans l'enveloppe (Figure 22). La mesure du pourcentage d'hybride obtenu entre une telle sonde et l'ARN étudié permet de quantifier l'homologie existant entre les deux acides nucléiques, mais la valeur de ce résultat est tempérée par l'imprécision existant sur le contenu exact de la sonde. Cependant, cette technique d'hybridation en phase liquide fournit une bonne radiographie du génome viral. Pour obtenir davantage de précision sur la structure de ces génomes, le clonage moléculaire des virus correspondants est nécessaire et la séquence nucléotidique de ces ADN est requise. Toutefois, les résultats présentés dans la première partie montrent que le c-onc peut s'insérer dans l'un ou l'autre des trois gènes du génome viral (Figures 23 et 24).

Les séquences nucléotidiques des points de jonction virus/oncogène actuellement disponibles montrent que le motif GAGG ou CCTC (selon le brin d'ADN considéré) se retrouve fréquemment près du point de recombinaison (Figure 30). Ce motif nucléotidique se retrouve également au point de recombinaison des virus de mammifères (Besmer et al., 1986) ainsi qu'au point de recombinaison du gène c-myc avec les gènes d'immunoglobulines (Piccoli et al.,1984). Le rôle exact de ce signal n'est pas connu mais GAGG pourrait participer à une séquence reconnue par des enzymes cellulaires impliqués dans les remaniements de l'ADN. Ainsi la première partie de l'événement de recombinaison pourrait être prise en charge par ces séquences qui pourraient

AMV

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                                                                  env      myb
CCAAAAGGATGAGGTGACTAAGAAAGATGAGGCGAGCCCTCTTTTTGCAGGC|TGGAGGCACA
```

(Klempnauer et al., 1982)

E26

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   myb       ets
GATCATGG|CACCTCAGAGATGATG

              ets  env
TTTCTTTGATAGGG|CCTCCAACTCCTCGAAACCG
```

(Nunn et al., 1983 , 1984)

AEV H

```
                                                              env      erb-B
AAAAGGATGAGGTGACTAAGAAAGATGAGGCGAGCCCTCTTTTTGCAGGCAT|TTCTGACTGGAT

erb-B    env
TTACCA|ACTTCTTGCTCCTAGCTCACGGCCATGGCTGTGAGG
```

(Yamamoto et al., 1983)

AEV ES4

```
                                        gag    erb-A
ACGAGGGACCCGCTTGGACCCCTCTGGAGCCA|GAGGACACTCGGT

     erb-A    env
TCCCCCCAAGG|AGGTGTAGAGCCT
```

(Debuire et al., 1984)

MH2

```
                                    gag        mil
CGAGGGCTCTGCTACACTTGTGGATCCCC|TACAATGCCAGTAGACAGCCGGATAATTGAGG
```

(Galibert et al., 1984)

MC29

```
             gag       myc
CCCTCCCGCCCGCAG|GCAGCAGCCGCCGCGATGCCGCTCAGCGCCAGCCTCCCCAGCAA

              myc      env
TGTATTTAAGGCATT|CCTGGTGGCCCTGATAACAGCACAACCCTCACCT
```

(Reddy et al., 1983)

Figure 30 : Séquences nucléotidiques des points de recombinaison virus DLV / gène oncogène.

signaler où doit se faire, au niveau de l'ADN, la jonction entre le provirus et le c-onc. La recombinaison proprement dite pourrait se faire au niveau de l'ARN à l'aide de la réverse transcriptase d'un virus "helper" (Huang et al.,1986) au cours de la conversion de l'ARN viral en ADN. La réverse transcriptase pourrait ainsi "sauter" de la matrice virale sur l'ARN du gène oncogène. On peut supposer que l'existence de séquences nucléotidiques complémentaires entre le génome viral et le gène du c-onc stabilise de tels hybrides et favorise la création de ces chimères. De fait, de telles homologies ont été notées entre l'oncogène c-fos et le virus murin FBJ-MuLV, dans le gène gag (5 nucléotides) et en 3' du génome (10 nucléotides) (VanBeveren et al., 1983). Cela est vrai également pour l'oncogène c-raf qui présente 15 nucléotides d'homologie avec le virus auxiliaire au niveau du point de recombinaison situé en 3'(Bonner et al.,1985). Ce type de recombinaison homologue existe également dans les recombinaisons entre rétrovirus, et nous en donnons un exemple dans l'article 16. Toutefois des recombinaisons illégitimes peuvent également se produire pour créer des virus transformants, la pression de sélection introduite par l'expérimentateur permet d'identifier de tels événements, même rares.

La recombinaison se fait donc pour les DLV en différents points du génome (on notera toutefois que les points de recombinaison env-myb du virus AMV et env-erbB du virus AEV-H se font au même endroit dans le gène viral, particulièrement riche en séquences GAGG/CTCC, Figure 30) et en différents points du c-onc considéré. La jonction peut en effet se faire:

- dans un intron pour erbB (virus AEV-H et AEV-ES4),pour le gène myb du virus AMV (Klempnauer et al., 1982), pour le gène myc des virus MH2 (Galibert et al., 1984) et MC29 (Reddy et al., 1983)

-dans un exon pour le gène myb du virus E26 (Nunn et al.,1984) pour le gène mil (virus MH2, Jansen et Bister, 1985) et pour le gène erbA du virus AEV-ES4 (Sap et al., 1986).

Dans la plupart des cas, la protéine oncogène est une protéine de fusion qui s'initie dans des séquences virales. La participation virale dans la protéine de fusion est très variable d'un virus à l'autre. La part la plus importante est représentée par le gène gag pour les oncogènes qui ont fusionné dans un exon.

La présence de séquences introniques au sein du virus, permettant l'épissure du gène oncogène, restreint cette participation aux six premiers acides aminés du gène gag (c'est le cas pour les virus AMV (myb), AEV (erbB) et MH2 (myc)). Cette fusion n'est possible que si le mécanisme d'épissage conserve une phase de lecture ouverte entre le gène gag et le gène onc. Cela n'est pas vrai pour l'oncogène src qui utilise dans ce cas son propre AUG (Takeya et Hanafusa, 1983).

Les virus possédant deux oncogènes (AEV-ES4, E26 et MH2) posent le problème particulier de la capture d'un oncogène par rapport à l'autre. Il existe en effet des rétrovirus n'ayant que l'un de ces deux oncogènes, et l'on peut supposer que ce type de virus, en recombinant ensuite avec l'autre c-onc, acquiert son identité définitive. Toutefois, les deux oncogènes ne manifestent pas le même pouvoir oncogène (myc est "plus transformant" que mil, erbB est "plus transformant" que erbA et myb est actif sans ets). On peut supposer qu'un rétrovirus ayant initialement recombiné avec erbA par exemple, ne se serait pas maintenu suffisamment (par l'absence d'un avantage sélectif) pour recombiner avec un deuxième oncogène. L'inverse parait intuitivement plus facile.

D'autre part, la recombinaison simultanée avec les deux oncogènes (réunis par une translocation par exemple) parait peu vraisemblable, notamment pour les gènes v-erbA et v-erbB du fait de la présence de séquences résiduelles virales (env) entre les deux oncogènes (voir article N°8). Cependant, en l'absence de modèles expérimentaux il n'est pas possible de privilégier un mécanisme particulier.

Il est évident que pour déterminer avec précision le point d'insertion d'un c-onc dans un rétrovirus, sa séquence nucléotidique est requise. Cela nécessite bien sûr au préalable le clonage moléculaire du gène considéré à partir d'une banque d'ADN génomique inséré dans un vecteur procaryote. La sonde utilisée, complémentaire du v-onc, peut être obtenue indifféremment par la méthode d'hybridation soustractive ou par l'isolement d'un fragment de restriction du provirus cloné correspondant au gène désiré.

STRUCTURE ET ACTIVATION DES C-ONC

Le clonage moléculaire des c-onc permet de proposer des modèles d'activation d'oncogènes. Nous avons participé au clonage moléculaire de 4 c-onc: erbA, erbB, myb et myc. La structure de ces gènes est complexe, contenant des régions homologues (définies comme des exons) et non homologues (introns) à la séquence virale. L'utilisation de l'ARN viral pour définir les introns et les exons du gène cellulaire homologue peut conduire à certaines erreurs. En effet certains v-onc peuvent contenir des séquences introniques (nous avons déjà cité v-erbB (AEV-ES4 et AEV-H), myb (AMV) et myc (MH2 et MC29). De plus, l'ARN avec lequel le rétrovirus a recombiné pourrait ne correspondre qu'à un type de transcrit particulier de ce gène. En effet, le nombre de cas recensés, où un gène peut exprimer par épissage alternatif plusieurs messagers différents, augmente rapidement et les oncogènes n'échappent pas à cette tendance. Ainsi, l'oncogène Ki-RAS (Shimizu et al., 1983b) a par ce mécanisme la possibilité de diriger deux produits différents. De même, l'oncogène c-abl pourrait posséder en 5' un exon alternatif, l'épissage alternatif se produisant pour ce gène à l'endroit où s'observe la jonction BCR/ABL(Figure 17) dans la leucémie myéloïde chronique (Ben-Neriah et al., 1986). La capacité pour un gène de posséder par épissage alternatif plusieurs ARN messagers pourrait dépendre de structures secondaires dans l'ARN messager (Solnick, 1985). Ainsi, le v-onc contenu dans les rétrovirus pourrait ne pas refléter la structure du c-onc correspondant. L'analyse des ARN messagers correspondant au c-onc considéré peut aider à clarifier la situation. Cependant l'existence de plusieurs ARN pour un c-onc donné ne signe pas forcément l'existence d'un épissage alternatif pour ce gène. D'autres mécanismes peuvent être responsables de l'existence de plusieurs messagers: l'existence de plusieurs sites initiateurs de la transcription (il en existe par exemple trois fonctionnels dans le gène MYC, Bentley et Groudine, 1986) ainsi que l'existence dans l'ADN d'une famille de gènes suffisamment proches les uns des autres pour que leurs produits de transcription réagissent avec la même sonde. Le groupe des oncogènes à "famille" a en effet tendance à s'élargir, et ce d'autant plus que les conditions expérimentales permettent des appariements entre séquences nucléotidiques relativement divergentes (conditions expérimentales relachées). Ces expériences sont intéressantes car elles permettent d'isoler de nouveaux oncogènes ou des gènes dont l'étude peut aider à comprendre la

fonction de certains c-onc (Foster et al., 1986; Huleihel et al., 1986; Weinberger et al., 1986).

L'analyse des c-onc clonés moléculairement montre que la plupart des v-onc n'ont recombiné qu'avec une portion du gène cellulaire. On pouvait en effet s'attendre à ce que le virus, sélectionné par son pouvoir transformant, isole du c-onc la portion nécessaire et suffisante à la transformation cellulaire. Ainsi, v-myb est le résultat de la transduction d'une portion interne du gène cellulaire et les amputations réalisées en 5' et 3' du gène se retrouvent également dans les tumeurs ou ce gène est impliqué (Rosson et Reddy, 1986). Le clonage moléculaire du gène c-erbB et l'analyse du point de jonction virus/c-onc montre que là aussi, le clivage de ce gène ne se fait pas au hasard, et est impliqué dans son activité oncogénique. Nous avons déjà developpé les différents modèles qui peuvent rendre compte du mécanisme d'activation de c-erbB (voir chapitre activation des c-onc).

Le gène c-myc est également tronqué après sa recombinaison avec les rétrovirus aviaires, et sur les 4 isolats existants, aucun ne contient le premier exon de ce gène. La perte de cet exon 1 est également fréquente dans les translocations 8:14 impliquant ce gène (lymphomes de Burkitt). La protéine synthétisée par le v-myc ne doit différer du produit normal que par l'accumulation de mutations ponctuelles amassées au cours des cycles de réplication successifs du virus. Dans ce cas, celles-ci pourraient être importantes dans l'activation du gène. L'analyse de la séquence nucléotidique de différents c-myc (poissons, poulet, souris et humain) ainsi que différents allèles du gène v-myc montre que le triplet responsable de l'acide aminé 61 dans la protéine, code pour une thréonine alors que dans MC29 ce codon spécifie une méthionine et une alanine dans les cas de MH2 et OK10 (Papas et Lautenberger,1985). Nous avons montré que la surexpression du produit du gène MYC normal, permettait de transformer des cellules d'embryons de caille. Ce résultat montre que les mutations ne sont pas indispensables à l'activité transformante de MYC. Cependant, il n'est pas impossible qu'elles confèrent *in vivo* un avantage sélectif. Certaines de nos constructions permettant d'obtenir des virus capables de se propager, nous pourrons étudier cette possibilité directement.

Pour cet oncogène, se pose le problème du système biologique permettant de révéler son activité transformante: le produit du gène v-myc est considéré comme capable d'immortaliser les cellules mammifères mais pas de les transformer. Toutefois, le gène v-myc a déjà été décrit comme étant capable de transformer de telles cellules (Copeland et Cooper,1980; Quade et al., 1983; Vennström et al., 1984) et récemment, des cellules murines immortalisées ainsi que des cellules primaires ont été transformées à l'aide d'un rétrovirus murin portant l'oncogène c-myc de souris (Baumbach et al., 1986). Ainsi, il semble que l'action du produit de ce gène puisse dépendre de son taux d'expression dans la cellule: le gène myc (muté ou normal) transforme les cellules mammifères quand il est exprimé par l'intermédiaire d'un promoteur viral murin (Vennström et al., 1984; Baumbach et al., 1986) et les immortalise quand il s'exprime par l'intermédiaire d'un promoteur de virus à ADN (Mougneau et al., 1984).

L'étude de la structure des c-onc a également permis de révéler l'architecture inattendue du gène c-erbA; le gène viral occupe une position marginale au sein des oncogènes puisque son produit ne semble pas être directement transformant (Graf et Beug ,1983). La séquence nucléotidique d'un fragment du locus c-erbA de poulet correspondant à D2 (1erexon de D2)

présente 78% d'homologie avec son équivalent viral, mais celle-ci est de 91% au niveau de la protéine déduite; la plupart des différences observées portent sur le 3éme nucléotide des codons. Cela confirme que ce deuxième locus est très apparenté, mais différent du c-erbA complet (D1 + D2) .Ce locus pourrait correspondre chez le poulet au locus hc-erbAß décrit par Weinberger et al.,(1986) chez l'homme, et localisé sur le chromosome 3.

La séquence nucléotidique réalisée dans la même région dans le gène ERBA complet révèle 86% d'homologie avec l'équivalent viral, ce qui est considérable compte tenu de la distance phylogénétique; ce pourcentage d'homologie permet de penser que ce locus est bien celui localisé sur le chromosome 17 (Spurr et al.,1984) et non celui localisé sur le chromosome 3 qui ne présente que 74% d'homologie avec v-erbA (Weinberger et al.,1986). L'autre particularité de ce locus humain, réside dans l'inversion du D2 par rapport au D1, comparé à l'équivalent viral (Figure 25). Ce résultat est surprenant, car dans cette conformation ce locus ne peut coder pour une protéine du type de la P75 d'AEV (responsable du blocage de différenciation des érythroblastes transformés par le virus).On peut donc supposer que le D2 doit s'inverser à un moment donné pour permettre la synthèse d'un ARN portant D1 et D2 dans la même phase de lecture.

L'équipement cellulaire est pourvu en enzymes capables de provoquer des remaniements de l'ADN (dans le réarrangement des gènes d'immunoglobulines, ou dans ceux codant pour les recepteurs d'Ig des lymphocytes T) encore faut-il que l'ADN à réarranger contienne les signaux reconnus par de tels enzymes. L'examen attentif de la séquence nucléotidique à l'endroit où l'on pourrait s'attendre à ce que l'ADN bouge (à la limite de l'intron et de l'exon), révèle quelques particularités intéressantes (Figure 25). En effet, deux séquences directement répétées encadrent une séquence répétée de façon inversée. Cette accumulation parait difficilement être dûe au seul hasard; de surcroît, le motif CACAGCTG est également présent à cet endroit de la séquence ERBA. Or, CACAGTG et CACACTG, sont trouvés respectivement au point de réarrangement des gènes d'immunoglobulines (Lewis et al.,1984) et au point de réarrangement des gènes codant pour les récepteurs d'Ig (Malissen et al., 1986). Un enzyme isolé à partir de la bourse de Fabricius chez l'oiseau, ainsi que du foie de souris, clive l'ADN des gènes d'immunoglobulines après le motif CACAGTGCTG (Hope et al., 1986). Une activité "recombinase" a également été isolée à partir de cellules humaines (Hsieh et al., 1986). Enfin, l'activité ADP-ribosyltransférase augmente dans les cellules en cours de différenciation, ce qui suggère l'existence de coupures/ligatures dans l'ADN à ce moment là (Johnstone et Williams, 1982).

Toutes ces observations ne prouvent pas que le locus ERBA se remanie, et ce, au cours de la différenciation des cellules érythroïdes; la mise en évidence directe de cet événement nécessite d'obtenir une quantité suffisante et suffisamment pure de ces cellules, où l'événement s'est produit. Nous avons étudié la structure de ce gène dans l'ADN de plusieurs types cellulaires différents (placenta, lymphome de Burkitt, tératocarinome, cellule érythroïde K562 et deux leucémies érythroblastiques à cellules particulièrement immatures) sans mettre en évidence de différences. Ce réarrangement pourrait n'intervenir que très tardivement dans la lignée rouge et il faudra analyser des érythroblastes matures.

L'analyse des ADNc complémentaires des ARN ERBA isolés à partir des cellules

érythroïdes K562 suggère l'existence d'un ARN contenant les deux domaines dans la même unité transcriptionnelle. La séquence nucléotidique de certains de ces clones est en cours de détermination; celle-ci devrait permettre de déterminer si ce transcrit provient du gène ERBA qui s'est réarrangé, ou d'un locus apparenté. L'existence d'un locus très proche (capable de fixer les hormones thyroïdiennes) sur le chromosome 3 est un argument supplémentaire pour supposer que le locus inversé pourrait coder pour un récepteur particulier ne fonctionnant que dans certaines cellules. Les hormones thyroïdiennes ont un rôle pléïotropique, et on peut supposer que l'évolution a conduit à une diversification des gènes codant pour leurs récepteurs, en fonction des besoins de l'organisme. Dans ce cas, un réarrangement de l'ADN est un bon moyen pour la cellule d'éviter les retours en arrière dans les processus de différenciation, la structure de l'ADN étant irréversiblement modifiée.

Enfin, nous ne pouvons exclure la possibilité que cette structure particulière soit un "déchet" de l'évolution dépourvu d'activité. Toutefois, le fait que le cadre de lecture de D2 se soit maintenu ouvert sans pression de sélection rend cela peu probable. Il est cependant possible que le domaine1 soit dupliqué de part et d'autre du D2; le clonage moléculaire ne nous ayant pas permis de remonter à droite de D2 (Figure 25). Dans ce cas, un produit contenant D1 et D2 dans le même ordre que v-erbA pourrait être synthétisé en l'absence de tout réarrangement. L'étude des ADNc correspondants aux ARN de ce locus devrait permettre de résoudre ces questions.

EXPRESSION DES C-ONC

Les DLV interfèrent spécifiquement avec la différenciation d'une cellule cible hématopoïétique; il était dès lors logique d'essayer d'impliquer le produit des c-onc équivalents dans les mêmes processus. Pour cela, il fallait montrer au préalable que ceux-ci s'exprimaient, et que leur expression était restreinte aux populations de cellules cibles. La mesure de la quantité d'ARN correspondants à c-erb (A+B), c-myc, c-myb, et c-src a été entreprise dans plusieurs cellules à l'aide des techniques d'hydridation en phase liquide. Nous avons ainsi pu montrer que les cellules hématopoïétiques normales immatures, ainsi que des cellules transformées d'origine érythroïde et lymphoïde immatures contiennent des quantités importantes d'ARN c-myc et c-myb; c-src et c-erb sont présents à un taux de base dans toutes ces cellules. Cependant, l'expression des c-onc observée dans les cellules transformées pouvait n'être qu'une conséquence de cette transformation. Les contrôles réalisés avec d'autres types cellulaires (épithéliaux et fibroblastiques) transformés par les mêmes agents montrent que l'expression des c-onc étudiés n'est pas un mécanisme général dû à la transformation.

Ainsi, le produit de c-myb et c-myc est retrouvé dans les cellules hématopoïétiques immatures, que ces dernières soient ou non des cibles pour le v-onc correspondant. Par exemple, le gène c-myb est fortement exprimé dans des précurseurs normaux de thymocytes (Thompson et al., 1986) qui ne sont pas les cellules cibles du virus AMV. Ainsi, il apparait que l'expression d'un c-onc donné dans une cellule n'est pas un facteur suffisant pour que cette dernière soit transformée par le v-onc correspondant. D'autre part, le fait d'exprimer un c-onc particulier ne la met pas à l'abri d'une transformation par le v-onc analogue; un taux très important d'ARN c-myb est retrouvé

dans les précurseurs normaux de promyélocytes de poulets, préparés à partir de sac vitellin, et le pourcentage de cellules exprimant c-myb y est le même (5%) que celui des cellules cibles pour AMV (Duprey et Boettiger, 1985). Enfin, et cela semble être le cas le plus fréquent, une cellule peut être transformée sans exprimer le c-onc considéré; dans notre exemple, des macrophages matures n'exprimant pas c-myb peuvent être transformés par AMV (Durban et Boettiger, 1981). L'hypothèse qui voulait que le blocage de différenciation affectant les cellules hématopoïétiques transformées soit dû à une interférence entre le v-onc et le c-onc n'est probablement défendable que dans un tout petit nombre de cas.

Les résultats que nous avons obtenus sur l'expression des gènes c-erb, montrent que la plupart des cellules analysées ne contiennent que des quantités modestes de ce mélange d'oncogènes. Par hybridation *in situ* de la sonde erbA+B sur des cellules de moëlle osseuse de poulets (tissu contenant un grand nombre de cellules cibles pour le virus AEV, Graf et Beug,1978) nous avons pu observer que quelques cellules accumulaient c-erbA et/ou B. Il semblerait d'après des résultats récents (J.Samarut, communication personelle), que le sang d'embryon de poulet accumule c-erbA. On peut supposer que les cellules positives en hybridation *in situ* expriment en fait cet oncogène.

La mesure du taux d'ARN des c-onc dans les cellules aviaires non hématopoïétiques transformées a permis de montrer que certains fibroblastes de caille japonaise transformés par le 20-MCA contenaient des taux d'ARN c-myc (de 12 à 30 copies/cellules) comparables à ceux des cellules hématopoïétiques immatures. Ce phénomène parait lié à une hypométhylation d'un des allèles du gène, peut-être induite par le 20-MCA lui-même (Wilson et Jones, 1983). D'autre part, ce fibroblaste pourrait avoir été transformé, alors que physiologiquement il exprimait ce gène. La cellule (comme les cellules hématopoïétiques immatures transformées) aurait alors été "gelée" dans son développement par la transformation. On peut également supposer que la transformation a induit cette cellule à se dédiffèrencier ou à exprimer des gènes normalement silencieux (Groudine et Weintraub, 1980).

L'oncogène c-myc est souvent retrouvé exprimé dans les cellules transformées de différentes espèces. Le gène est parfois remanié (ce qui permet de supposer une implication causale de son expression dans la transformation), parfois normal, ce qui pose le problème de son rôle, car de nombreuses cellules immatures normales expriment physiologiquement cet oncogène (Pfeifer-Ohlson et al., 1985).

LES VIRUS A DEUX ONCOGENES

Trois rétrovirus aviaires (AEV, E26, et MH2) possèdent deux v-onc. Dans la mesure où existent des virus transformants ne possédant que l'un de ces deux oncogènes, le deuxième v-onc devrait favoriser le virus qui le porte; de plus, ces derniers sont stables dans le temps. Les deux oncogènes du virus E26 lui permettent de transformer plus de types cellulaires que le virus AMV pourvu du seul v-myb (Graf et Beug, 1978). Le gène erbA du virus AEV lui permet d'être beaucoup plus agressif *in vivo* que le virus ne contenant que erbB (Frykberg et al.; 1983); d'autre part, les cellules hématopoïétiques transformées *in vitro* par ce virus présentent moins d'exigences

vis à vis des conditions de culture que les cellules transformées par le mutant erbA$^-$.

Dans le cas du virus MH2, un effet propre à ce virus est mis en évidence sur les cellules de neurorétines aviaires; ce virus est capable de transformer et de faire proliférer ces cellules, alors que les virus de type MC29 ne contenant que l'oncogène v-myc (et pas l'oncogène v-mil) sont sans effet sur ces cellules dans les conditions de culture choisies. Les mutants du virus MH2 dépourvus du gène myc (virus du type PA-200) sont capables de faire proliférer les neurorétines mais pas de les transformer. Toutefois, ces cellules induites à proliférer peuvent être aisément transformées par le virus MC29 mais cela entraine rapidement une mort cellulaire très importante.

La rétine prélevée à 7 jours de développement embryologique est un tissu complexe contenant des cellules en cours de différenciation qui donneront des cellules gliales et des cellules neuronales (Okada et al.,1979). La nature de la cellule touchée par le virus n'est pas décelable morphologiquement, et il est également possible que la sensibilité de ces cellules vis à vis des produits de v-mil et v-myc varie avec le temps et leur degré de différenciation. L'effet particulier du produit du gène v-mil sur la croissance des cellules de neurorétines n'est pas restreint à ce seul tissu, mais semble s'exercer sur tous les types de cellules nerveuses que nous ayons analysés: cellules de crête et de tube neural d'embryon de poulet et de caille de 48 heures (LeDouarin, 1986), encéphale d'embryons du même âge, ainsi que de 7 jours. Des marqueurs spécifiques sont nécessaires pour déterminer la nature de la cellule cible, ce qui est indispensable pour aborder le rôle du produit du gène mil dans les cellules nerveuses.

L'utilisation d'anticorps reconnaissant les neurofilaments (filaments intermédiaires spécifiques des neurones, Prochiantz et al.,1982) permet de montrer que les cellules de cerveau et de rétine de 7 jours proliférant après une infection par le virus MH2Pa200 expriment ce marqueur (jusqu'à 50% des cellules). Donc, des neurones sont sensibles au produit de ce gène. Le virus MC29 est également capable de transformer les cellules de cerveau, mais dans ce cas, les cellules proliférantes n'expriment que peu ou pas ce marqueur (moins de 5% des cellules).

Le produit du gène v-src est également capable de faire proliférer les neurones dans les cellules de neurorétines (Pessac et Calothy, 1974; Pessac et al., 1983) mais la pp60^{src} est une tyrosine kinase, alors que le produit de v-mil possède une activité sérine/thréonine kinase (Moelling et al., 1984). La cible moléculaire de ces deux produits n'est pas forcément la même, mais il est possible que leur action utilise la même voie métabolique pour faire proliférer ces cellules. Il est particulièrement intéressant de noter dans cette optique, que nous avons réussi à faire proliférer des neurones sous l'action du produit du gène Ha-RAS activé par mutation ponctuelle. Ainsi, les différents types de protéines oncogènes impliquées dans la prolifération cellulaire (à l'exception, curieusement, des protéines à localisation nucléaire) sont capables d'induire la prolifération des neurones en culture. Le fait que v-myc soit sans effet sur la croissance de ces cellules (tout au moins chez le poulet) indique que dans certains cas, le produit de ce gène n'est pas requis pour que la division cellulaire s'effectue; or la mitose semble possible en réponse au facteur de croissance PDGF d'une façon indépendante de l'activation de c-myc (Coughlin et al., 1985). La raison pour laquelle les cellules de neurorétines n'utilisent pas la "voie" du gène myc est obscure; le problème se trouve peut-être dans le stade de différenciation de ces cellules au moment de leur

infection. Il existe en effet un certain nombre de cellules dans lesquelles le produit de l'oncogène v-myc n'induit aucune modification décelable. Ces cellules se recrutant essentiellement dans le système hématopoïétique (Graf et al.,1980); cependant, ces cellules qui dérivent toutes d'un même précurseur se spécialisent au cours de leur différenciation, certaines étant sensibles à myc, d'autres non.

Un deuxième système biologique permet de révéler l'action de v-mil; des macrophages transformés par v-myc sont dépendants du cMGF dans le milieu de culture pour assurer leur croissance, la surinfection de ces cellules par un virus de type PA-200 les rend indépendants de ce facteur. Cette indépendance est le résultat de la production autocrine de cMGF par ces cellules (Graf et al.1986). Nous ne savons pas si un mécanisme analogue est à la base de l'action de v-mil sur les cellules nerveuses.

L'absence d'effet transformant observé en réponse à v-mil est curieux dans la mesure ou le virus sarcomatogène murin MSV 3611 contenant l'oncogène v-raf (analogue murin de v-mil) est transformant *in vivo* et *in vitro* (Kan et al., 1984) et l'homologie entre les deux protéines virales est de 94%. Il faut donc supposer que, soit des mutations ponctuelles différencient les produits de ces deux oncogènes, soit qu'une différence d' espèce intervient dans la nature de la cellule cible. Il est très intéressant de savoir que le produit tronqué du gène c-mil (portion carboxyterminale) est capable d'induire la prolifération des cellules de neurorétines (ainsi que vient de le montrer C.Dozier); en effet, la partie carboxyterminale du gène c-raf artificiellement placée sous le contrôle d'un promoteur viral est capable de transformer les fibroblastes murins (Muller et Muller ,1984). Les deux équivalents cellulaires des gènes viraux se comportent donc de la même façon que ceux ci sur leurs cellules cibles respectives. Ce point est important, car le produit de c-src est sans effet transformant ou mitogène sur les cellules de rétines (Iba et al.,1985). Le mode d'activation de ces c-onc est probablement différent.

L'étude du pouvoir transformant de v-mil se heurte aux mêmes problèmes que ceux rencontrés dans l'étude de v-myc; pendant longtemps, il n'a été possible que d'immortaliser les cellules mammifères avec cet oncogène qui transformait très bien les cellules aviaires. La présence de v-mil dans MH2 renforce l'activité transformante de ce dernier (Graf et al., 1986). L'effet biologique du virus PA-200 *in vivo* n'est pas encore connu; il est possible que les cellules infectées par ce virus nécessitent des facteurs de croissance qui ne leurs sont pas fournis *in vitro* pour pouvoir être transformées mais qu'elles trouveront peut-être *in vivo*.

L'implication simultanée de plusieurs oncogènes dans la transformation cellulaire est possible et bien documentée. Les études de carcinogénèse chimique réalisées sur des cellules de peau de souris, suggèrent plusieurs événements distincts avant qu'une cellule ne devienne tumorigène: une étape d'initiation (obtenue par une seule exposition à un carcinogène chimique induisant des altérations de l'ADN), une étape de progression (impliquant plusieurs expositions à des carcinogènes n'induisant pas d'altération de l'ADN) qui permet de d'amener la tumeur du stade bénin au stade malin (Weinstein et al.,1983). Cette succession d'événements peut être prise en charge par plusieurs gènes, mais elle pourrait également être suscitée par des dérégulations successives d'un même gène. Ainsi par exemple, l'oncogène Ha-RAS activé est capable d'induire

l'immortalisation de cellules mammifères (ces cellules ne sont pas tumorigènes); placé sous le contrôle d'un promoteur fort de transcription ce gène est capable de rendre ces cellules directement tumorigènes (Spandidos et Wilkie, 1984; Pozzatti et al., 1986; Land et al., 1986) enfin, l'oncogène c-ras normal placé sous le contrôle d'un promoteur fort ne peut qu'immortaliser les cellules normales.

L'oncogène Ha-RAS activé immortalisant les cellules, associé au gène myc (viral ou cellulaire) faiblement transcrit (et donc également immortalisant) rend la cellule tumorigène (Land et al., 1983). Toutefois, les deux oncogènes associés ne rendent pas les cellules "plus" tumorigènes que le Ha-RAS activé tout seul (Pozzatti et al.,1986). Ainsi deux événements affectant le même oncogène peuvent avoir le même effet qu'un événement affectant deux oncogènes distincts.

Il est possible cependant que certaines cellules réclament impérativement le produit de deux oncogènes pour être tumorigènes. Dans le cas des oncogènes ras et myc, on peut supposer que c'est la même voie métabolique qui est sollicitée, allant de la transduction d'un signal membranaire au noyau, la sur-expression d'un seul des oncogènes activés pouvant suffire à transformer la cellule. La transformation des cellules de neurorétines par le virus MH2 pourrait nécessiter la présence des deux produits, une étape de prolifération induite par mil est peut-être nécessaire pour que le pouvoir transformant de myc puisse s'exercer. On peut supposer dans ce cas que ces cellules pourraient être transformées par myc en l'absence de mil, mais en présence d'agents mitogènes pour ces cellules.

CONCLUSION

L'étude du génome des virus aviaires responsables de leucémies aiguës chez le poulet nous a permis de montrer que ceux-ci sont le fruit d'une recombinaison d'un virus ALV peu transformant, avec des séquences cellulaires phylogénétiquement stables et responsables de leur activité transformante. L'étude de l'équivalent cellulaire des v-onc nous a permis de proposer quelques modèles d'activation de ces c-onc (par dérégulation du contrôle de l'oncogène MYC, par modifications structurales pour l'oncogène erbB). Le rôle biologique du produit de ces c-onc est toujours incertain, mais on peut supposer sur la base du profil d'expression de ces gènes, qu'ils sont impliqués dans la régulation de la mitose et/ou de la différenciation cellulaire. De ce point de vue, la stucture particulière du gène ERBA (le domaine carboxyterminal étant inversé par rapport au domaine aminoterminal tel que l'à organisé le virus AEV) laisse penser que des mécanismes impliquant des mouvements de l'ADN (du type de ceux qui président aux remaniements fonctionnels des gènes des immunoglobulines ou de leurs récepteurs) pourraient également déterminer des événements de différenciation cellulaire.

Certains de ces rétrovirus ont recombiné avec deux oncogènes, ce qui pose le problème de la coopération de leurs produits dans la transformation cellulaire. Cette coopération peut comme dans le cas d'AEV impliquer un événement bloquant la différenciation cellulaire (pris en charge par v-erbA) puis un événement transformant (dû à v-erbB) ou comme pour MH2 un événement induisant la prolifération (v-mil) puis la transformation (v-myc) des cellules cibles de ces virus.

L'étude des rétrovirus transformants de différentes espèces animales s'est avérée particulièrement féconde dans la découverte directe ou indirecte de gènes cellulaires potentiellement oncogènes. Certains de ces gènes se retrouvent dans des virus d'animaux différents, et leur action transformante se révèle sur des cellules cibles d'espèces variées ce qui indique que leur rôle biologique est fondamental dans le métabolisme cellulaire. La fonction des c-onc se précise, grâce notamment à l'étude de la mitose et de la différenciation cellulaires . L'utilisation des oncogènes est en train de déborder le domaine de la recherche pour rejoindre celui de la médecine pratique; caractériser les tumeurs vis à vis de l'expression de certains c-onc peut se révéler particulièrement utile pour définir des sous-ensembles de tumeurs qui peuvent présenter un profil évolutif différent.

D'autre part une meilleure compréhension des causes génétiques du cancer doit permettre de développer des molécules ciblées (par exemple des anticorps monoclonaux), supprimant spécifiquement les produits impliqués dans la transformation (Mulcahy et al., 1985). On doit également pouvoir induire la différenciation des cellules leucémiques grace à des facteurs clonés, ce qui doit permettre d'abolir leur pouvoir prolifératif (Leung et Chiao, 1985). Enfin, on peut espérer arriver à inhiber spécifiquement l'expression de certains oncogènes activés; il existe en effet la possibilité de bloquer l'expression d'un gène à l'aide d'ARN anti-sens capables de s'hybrider dans la cellule avec l'ARN physiologique et d'empêcher ainsi sa traduction (Izant et Weintraub, 1984).

Toutefois, l'obtention et l'utilisation de tels produits reste tributaire des efforts et des succès de la recherche fondamentale dans ce domaine.

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RESUME

Les rétrovirus oncogènes responsables chez l'animal de l'apparition de néoplasmes variés sont le résultat d'une recombinaison entre le génome d'un virus non transformant, et des gènes cellulaires phylogénétiquement conservés, les oncogènes.

Nous avons analysé la structure du génome de 7 rétrovirus aviaires responsables de l'apparition de leucémies aiguës chez l'animal. Nos travaux ont permis d'isoler 4 oncogènes erbA, erbB, myc et myb.

-erbA et erbB sont portés par le virus AEV induisant des érythroblastoses;

-myc est présent dans 4 virus (dont MH2) induisant une myélocytomatose;

-myb est porté par deux virus transformant les myéloblastes.

L'analyse du génome de MH2 nous a amené à supposer l'existence d'un oncogène supplémentaire au sein de ce virus, lequel a pu être ultérieurement cloné dans le laboratoire et dénommé mil.

Le clonage moléculaire des oncogènes cellulaires montre que les gènes viraux sont des versions tronquées en 5' et en 3' des ces gènes, ce qui suggère que cette amputation peut être un des mécanismes de leur activation oncogénique.

La mesure du taux d'ARN correspondant à ces différents gènes cellulaires nous a permis de montrer que les cellules hématopoïétiques immatures des différentes lignées érythroïde, myéloïde et lymphoïde (mais pas les cellules matures), accumulent les ARN myc et myb. Cela suggère que le produit normal de ces gènes est impliqué dans la prolifération de ces cellules indifférenciées, et que la transformation des cellules hématopoïétiques par les virus étudiés n'est pas tributaire de l'expression de ces oncogènes pour se réaliser.

Afin d'aborder d'une façon plus biologique les mécanismes de la transformation cellulaire, nous avons introduit l'oncogène myc humain sous le contrôle transcriptionnel d'un promoteur viral, dans des fibroblastes embryonnaires de cailles: ces cellules se sont transformées et nous avons pu démontrer que la sur expression du produit myc normal était suffisante pour transformer ces cellules.

D'autre part, le virus MH2 portant deux oncogènes, nous avons pu montrer à l'aide de mutants amputés dans l'un ou l'autre de ces oncogènes que le gène mil possède une activité biologique décelable sur des cellules nerveuses quiescentes; le produit du gène mil fait proliférer ces cellules que le produit du gène myc parvient alors à transformer.

L'étude des rétrovirus transformants s'est avérée essentielle pour aborder les mécanismes de la transformation cellulaire, ainsi que les processus fondamentaux que sont la mitose et la différenciation.