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REPLICATION ET ASSEMBLAGE DE L'ADENOVIRUS HUMAIN: ANALYSE BIOCHIMIQUE ET GENETIQUE

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Messieurs, P. BOULANGER, M. GIRARD, J. GUILLAUME,

J. KREMBEL, G. MARTIN, J. WEBER,

vous me faîtes l'honneur de bien vouloir juger cette thèse

je vous en remercie vivement

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INTRODUCTION

L'Adénovirus a présenté pour nous un double intérêt. Celui d'être une sonde métabolique, et celui d'être un modèle structural .

1) Les virus des cellules eucaryotes comme les bactériophages ne peuvent se multiplier qu'après avoir infecté une cellule. Ils deviennent alors une véritable sonde d'étude de la cellule, puisqu'ils utilisent, tout ou partie de la machinerie cellulaire. Ainsi l'adénovirus utilise les systèmes de transcription et de traduction, et les DNA polymérases de la cellule hôte pour sa reproduction ; l'étude du fonctionnement de ces systèmes enzymatiques est rendu plus facile par le fait qu'un petit nombre de gènes sont exprimés. Dans cette optique l'adénovirus est sans doute un meilleur outil que le SV_{40}, car le premier inhibe les synthèses de la cellule hôte et pas le second.

2) Les virus qui sont des structures relativement simples, provenant de l'assemblage de sous-unités répétitives sont des moyens d'approche des intéractions protéine-protéine et protéine-acide nucléique. Ces intéractions sont évolutives au cours de la morphogénèse du virion. Ilest donc possible de suivre, la formation des particules virales pour les virus de cellules de mammifères, ce qui n'est pas le cas des virus des plantes.

Notre travail avait pour but l'étude des étapes de l'assemblage de l'adénovirus aboutissant à la formation de la capside contenant un génome infectieux et du rôle du DNA dans la morphogénèse virale. Nous avons utilisé plusieurs voies d'approche : l'analyse génétique, biochimique et immunologique, en essayant de combiner les informations obtenues par les différentes méthodes. Il serait peut être souhaitable de faire appel à d'autres techniques complémentaires, telles les études de Biophysique des diverses particules.

Avant d'exposer nos résultats, nous avons fait un bref rappel des connaissances actuelles concernant l'adénovirus et en particulier le cycle lytique.

Nos résultats sont présentés sous forme d'un résumé, résultats et discussion. Les résultats détaillés sont rassemblés en annexe puisqu'ils ont fait l'objet des publications suivantes :

1) Isolation and phenotypic characterization of human adenovirus type 2 temperature-sensitive mutants
Martin, G.R., Warocquier, R., Cousin, C., D'Halluin, J.C., and Boulanger, P.A.
J. Gen. Virol. (1978) 41, 303-314

2) Adenovirus early function required for protection of viral and cellular DNA
D'Halluin J.C., Allart, C., Cousin, C., Boulanger, P.A. and Martin, G.R.
J. Virol. (1979) 32, 61-71

3) Température-sensitive mutant of adenovirus type 2 blocked in virion assembly : accumulation of light intermediate particles
D'Halluin, J.C., Milleville, M., Boulanger, P.A. and Martin, G.R.
J. Virol. (1978) 26, 344-356

4) Adenovirus type 2 assembly analyzed by reversible cross-linking of labile intermediates
D'Halluin, J.C., Martin, G.R., Torpier, G., and Boulanger, P.A.
J. Virol. (1978) 26, 357-363

5) Morphogenesis of human adenovirus type 2 studied with fiber and fiber and penton base-defective temperature-sensitive mutants
D'Halluin, J.C., Milleville, M., Martin, G.R., and Boulanger, P.A.
J. Virol. (1980) 33, sous presse

6) Human adenovirus type 2 protein IIIa. II Maturation and encapsidation
Boudin, M.L., D'Halluin, J.C., Cousin, C., and Boulanger, P.A.
Virology (1980) 101, sous presse

7) Novobiocin blocked adenovirus DNA replication and encapsidation
D'Halluin, J.C., Milleville, M., and Boulanger, P.A.
Nucleic. Acad. Res. (soumis à publication)

Ce travail a en outre fait l'objet des communications suivantes :

1) D'Halluin J.C., Warocquier R., et Martin G.R.
Un nouvel intermédiaire dans la maturation de l'adénovirus type 2
Société Française de microbiologie (section de Virologie)
Réunion de Nice, 7 Mai 1977

2) D'Halluin J.C. et Martin G.R.
Mutants d'adénovirus défectifs dans la synthèse du DNA viral
VIéme Réunion francophone des Onco-DNA virologistes
Les Embiez 3-5 Octobre 1977

3) D'Halluin JC, Allart C., Devaux C., and Martin G.R.
Studies on DNA temperature sensitive mutants of Adénovirus type 2
XIth Meeting of European Tumor Virus Group
Balatonfured, 8-12 Mai 1978

4) D'Halluin,J.C., Martin G.R. and Boulanger PA
Adenovirus type 2 assembly analyzed by reversible cross-linking of wild type and ts mutants labile intermediates
EMBO Workshop on the Molecular Biology of Adenoviruses
Örenäs, Suède, 11-16 Juin 1978

5) D'Halluin J.C. and Milleville, M.
Assemblage de l'adénovirus de type 2 : analyse des intermédiaires labiles après fixation reversible
Société de Chimie Biologique
Forum des Jeunes, Villeneuve d'Ascq, Juin 1978

6) D'Halluin J.C., Cousin C., Milleville M., and Boulanger P.A.
Temperature-sensitive mutant of adenovirus type 2 defective in DNA synthesis initiation and DNA protection
Tumour Virus Meeting on SV 40, Polyoma and adenoviruses
Cambridge, England 16-21 Juillet 1979

7) D'Halluin J.C.
Une protéine précoce de l'adénovirus nécessaire à la protection du DNA et à la réplication
RCP : Mécanismes et régulation de la réplication chez les eucaryotes
Villeurbanne, 1-2 Décembre 1979

GENERALITES

I Introduction

Les adénovirus furent découverts en 1953 par Rowe et al. (1953) dans des cultures de tissus d'amygdale humaine, d'où leur nom. Ils sont responsables de certaines affections respiratoires, de conjonctivites et de certaines affections digestives. C'est pour l'adénovirus que fut mis en évidence pour la première fois, le pouvoir oncogène, chez le hamster nouveau né, d'un virus animal (Trentin et al., 1962 ; Huebner et al., 1962).

Les adénovirus peuvent avoir trois types d'interaction avec une cellule

a) entraîner une transformation maligne de la cellule

b) la tuer sans produire de particule virale : c'est le cycle abortif

c) la tuer en produisant une grande quantité de particules : c'est le cycle productif ou lytique. Le cycle lytique se divise en deux périodes :

la phase précoce qui précéde la réplication du DNA ; la phase tardive qui suit la synthèse du DNA et se poursuit jusqu'à la lyse de la cellule avec libération d'une quantité importante de particules infectieuses.

L'adénovirus a fait l'objet de nombreuses revues générales, par exemple, en ce qui concerne :

- l'absorption sur les cellules. Lonberg-Holm and Philipson, 1974
- le cycle lytique. Philipson and Lindberg, 1974
- la réplication du DNA. Winnacker, 1978
- l'intégration du DNA viral dans le DNA cellulaire. Doerfler, 1977
- la transcription. P. Lemay 1978
- la génétique. Ginsberg and Young, 1977

Nous ne ferons qu'évoquer les grandes lignes du cycle lytique et rappeler l'existence des cycles abortif et transformant.

II Structure de la particule virale

II - 1 Architecture de la particule

Les adénovirus sont des virus non-enveloppés de 65-80 nm de diamètre Au microscope électronique leur capside se présente sous forme icosaédrale, et composée de sous-unités, les capsomères (Horne et al., 1959). Chaque capsomère des faces triangulaires (20 faces - 240 capsomères) est entouré de six voisins : leur symétrie hexagonale leur a fait donner le nom d'hexon. A chacun des 12 sommets se trouve un capsomère possédant cinq voisins ; appelé "penton" (Ginsberg et al., 1966). Le penton est composé d'une base, insérée dans la capside et d'une projection : la fibre (Valentine and Pereira, 1965 ; Norrby, 1966). Un nucléoide ("core"), contenant le DNA ainsi que d'autres protéines, occupe le centre de la capside (Epstein, 1959 Epstein et al., 1960 ; Bernhard et al., 1961).

Les méthodes de désintégration séquentielle du virion et d'analyse des relations de voisinage par pontage réversible des protéines de la capside permettent de reconstruire un modèle détaillé de la topographie des protéines dans la particule virale (fig 1a).

Une dialyse contre de l'eau (Laver et al, 1969), ou contre un tampon 5mM tris-Maléate pH 6,0-6,5 (Prage et al, 1970) provoque la perte préférentielle des pentons. Les particules virales dépourvues de penton sont stables, mais leur DNA est accessible aux désoxyribonucléases.

En présence de SDS, d'urée ou de pyridine, la dissociation de la particule permet d'isoler les hexons des faces triangulaires (Smith et al, 1965 ; Maizel et al, 1968 ; Prage et al, 1970). D'autres conditions expérimentales aboutissent à la formation de "groupes de neuf hexons" (Prage et al, 1970 ; Russel et al, 1971 ; Pereira and Wrigley, 1974 ; Crowther and Franklin, 1972).

Plusieurs méthodes sont décrites pour obtenir "les cores" : le chauffage de la particule (Russell et al, 1967 ; 1971) ; l'action de l'acétone (Laver et al, 1968) ; de l'urée 5 M (Maizel et al, 1968) ; de la formamide (Stasny et al, 1968). Il est également possible d'utiliser un milieu 10% pyridine, ou le procédé des congélations-décongélations répétées (Prage et al, 1968; 1970).

La composition en polypeptides a été étudiée par electrophorèse en gel de polyacrylamide en milieu dissociant.

Comme le montre la figure 1b, la particule virale contient 14 polypeptides (Everitt et al, 1973 ; Anderson et al, 1973).

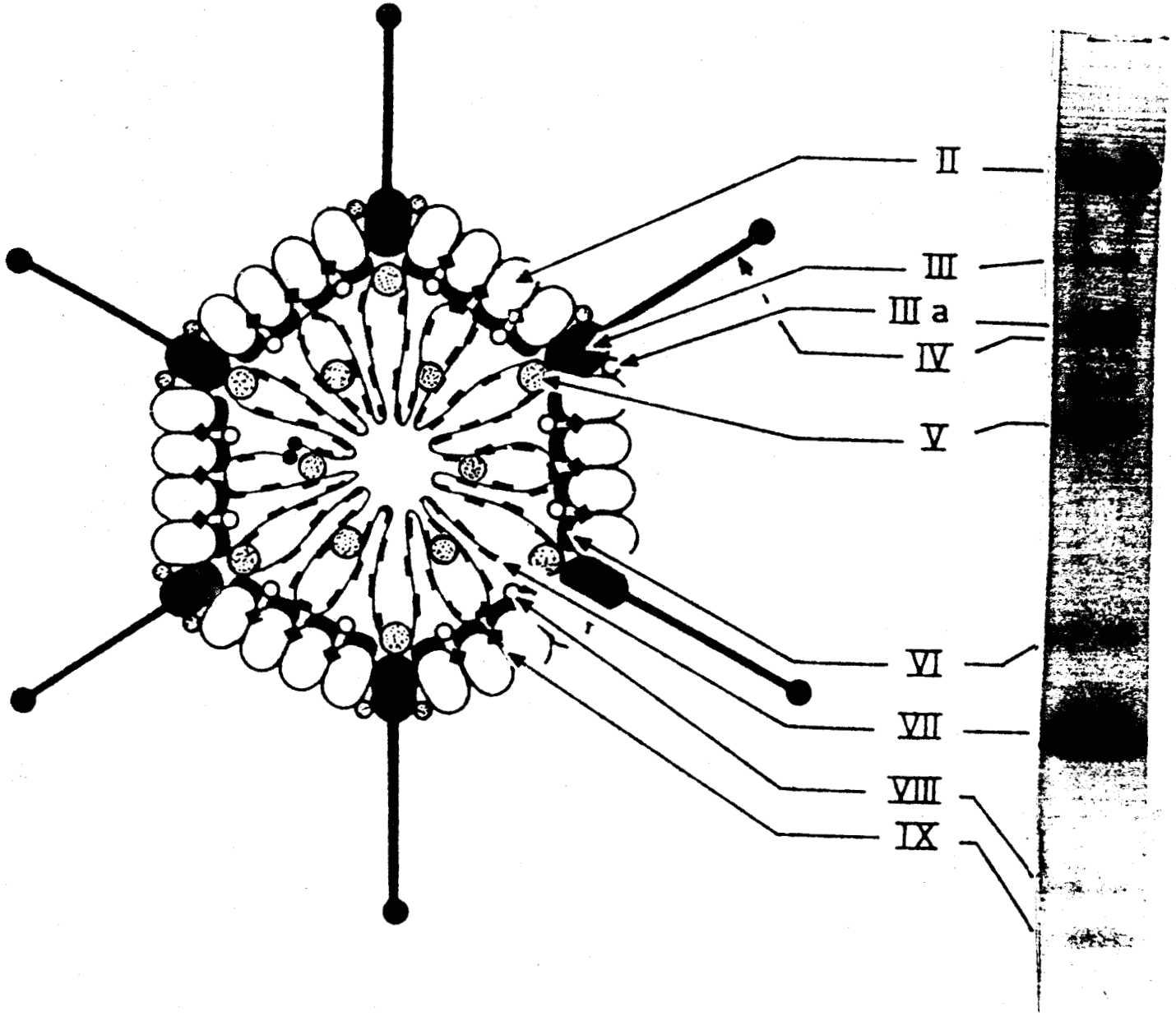

Fig 1 - Structure et composition polypeptidique de la particule virale

Le polypeptide II est le constituant de l'hexon, le polypeptide III celui de la base du penton, le polypeptide IV celui de la fibre. Les protéines V et VII sont associées au DNA dans le nucléoide. La localisation des polypeptides dans la particule est montrée sur la figure 1.

Les polypeptides VI et VIII sont liés à l'hexon après dissociation de la particule par congélation-décongélation. Le polypeptide VIII est dissocié des groupes de neuf hexons par traitement à la pyridine (Maizel et al., 1968 ; Everitt et al., 1973). Le polypeptide IX est présent dans les groupes de neuf hexons (Maizel et al., 1968, Everitt et al., 1973 ; Boulanger et al., 1979).

Après dissociation de l'apex, le polypeptide IIIa est relaché, il est localisé dans la région péripentonale (Everitt et al., 1973).

II - 2 Les Protéines structurales

Lors de l'infection d'une cellule les polypeptides viraux de structures sont synthétisés en grand excès ; seulement 1 à 5% de la fibre et de la base du penton et 20 à 30% de l'hexon sont assemblés en virus (White et al., 1969 ; Everitt et al., 1971). Les polypeptides produits en excès, sont, le plus souvent, présents sous forme de protéines multimériques (Horwitz et al., 1969). C'est à partir de cet excès de production que les protéines de structures sont purifiées pour leur étude biochimique et biophysique et leurs propriétés biologiques (par exemple pour l'hexon Boulanger et al., 1969 ; Boulanger and Puvion, 1973).

L'hexon est un tronc de cône de 11 nm de hauteur et de 8 à 9,6 nm de diamètre. Ces mesures sont déterminées par la microscopie électronique (Wilcox et al., 1963 ; Nermut, 1975) et par la diffraction des Rayons X (Tejg-Jensen et al., 1972 ; Berger et al., 1978). La protéine purifiée a une masse moléculaire de 310.000 à 360.000 (Franklin et al., 1971) et le polypeptide II migre en gel de polyacrylamide-SDS avec un poids moléculaire apparent de 120.000, soit 3 chaines polypeptidiques par hexon. La structure trimèrique de l'hexon a été confirmée par pontage inter sous-unités (Boulanger and Puvion, 1974). La sous-unité contient 7 cystéines pour 850 acides aminés. L'acide aminé en position N-terminale est bloqué par acétylation (Jörnvall et al., 1974). L'hexon posséde des déterminants antigéniques de groupe (α), de sous-groupe et de type (ε) (Norrby, 1969a). Le déterminant de type est résistant à la trypsine, la chymotrypsine, la papaine ou la subtilisine (Pettersson 1971).

La fibre a la forme d'une antenne de 2 nm de diamètre terminée par une sphérule de 4 nm de diamètre. La longueur varie avec le sérotype,

30 nm pour le type 2 (Norrby, 1969b). La fibre est glycosylée. Elle est composée de 3 polypeptides de masse moléculaire apparente 62 000 pour le type 2 (Ishibashi and Maizel, 1974). Un déterminant antigénique de type est porté par la sphérule, et un déterminant de sous-groupe par la tige (Norrby, 1969b ; Pettersson et al., 1968).

La base du penton et la fibre sont associés de manière non covalente car ils sont dissociables par le chlorhydrate de guanidine 2,5M, la pyridine à 8% (Norrby and Shaaret, 1967) ou le desoxycholate de sodium à 0,5% (Boudin et al., 1979). Le penton complet a une masse moléculaire d'environ 500.000. La base du penton est composée de 5 polypeptides de 85.000 (III) (Anderson et al., 1973). Celle-ci possède 3 déterminants antigéniques (type, sous-groupe et groupe) (Wadell and Norrby, 1969 ; Pettersson and Höglund, 1969).

Les deux protéines du core V et VII ont une masse moléculaire respective de 48.500 et 18.500. Le VII est une protéine analogue aux histones, riche en arginine (21%) et en alanine (18%) (Laver, 1970 ; Prage and Pettersson, 1971 ; Russell et al., 1971 ; Sung et al., 1977) Le nombre de polypeptides VII et V par particule est estimé à 1080 et 180 respectivement (Maizel et al., 1968 ; Everitt et al., 1973). Le polypeptide VII dérive d'un précurseur, P VII de masse moléculaire 22.500 (Anderson et al., 1973), par clivage à son extrémité N-terminale (Rekosh and Russell, 1977).

Les deux polypeptides VI et VIII, associés à l'hexon dans la particule virale, de masse moléculaire 24.000 et 13.000 dérivent de deux précurseurs, P VI et P VIII ayant respectivement 27.000 et 26.000 de masse moléculaire apparente en gel de polyacrylamide-SDS (Anderson et al., 1973, Edvardsson et al., 1976).

Le polypeptide IX qui est associé aux hexons des faces de l'icosaèdre est une protéine basique de masse moléculaire 12.000 (Boulanger et al., 1979). Ce polypeptide est indispensable au maintien de la stabilité de la capside (Jones et al., 1978).

La protéine IIIa est un monomère de 66.000, produit de clivage, à l'extrémité N-terminale d'un précurseur P IIIa de 67.000 (Lemay et al., 1980 ; Boudin et al., 1980). C'est la protéine phosphorylée majeure de la particule virale (Russell and Blair, 1977). Le composant IIIa n'est pas responsable de l'induction d'anticorps neutralisant l'adénovirus (Lemay et al., 1980).

Les polypeptides X, XI, XII apparaissent au cours de la maturation du virus (voir ci-dessous assemblage) et, du moins pour le XI et le XII,

sont considérés comme des produits de clivage des précurseurs P VII, P VI et/ou P VIII (Rekosh et al., 1977).

II - 3 Le DNA

Le chromosome de l'adénovirus est un DNA bicaténaire de masse moléculaire de 20-25 10^6 (Green et al., 1967 ; Van der Eb et al., 1969).

La composition en G + C varie d'un sous-groupe à l'autre (voir ci-dessous : II-5 classification). Le DNA du type 2 contient 59% de G + C (Pina and Green, 1965). Les deux brins peuvent être séparés :

- en centrifugation isopycnique en CsCl en présence de copolymères (poly (I, G) ou poly (U.G) (Kubinski and Rose, 1967 ; Landgraf-Leurs and Green, 1971).

- par électrophorèse en gel d'agarose alcalin

Par dénaturation puis renaturation à faible concentration de DNA des molécules circulaires monocaténaires s'observent. Ces molécules possèdent une projection bicaténaire. Elle est de 103 paires de bases pour le type 2. Cette structure en forme de "manche de poële" est le résultat de la présence d'une séquence terminale répétitive, inversée aux deux extrémités du génome (Garon et al., 1972 ; Wolfson and Dressler, 1972 ; Roberts et al., 1974). Une séquence terminale répétitive inversée est aussi présente dans le génome du virus associé à l'adénovirus (AAV) (Koczot et al., 1973). L'AAV est un virus défectif appartenant au groupe des parvovirus. Sa multiplication nécessite la présence d'un virus auxiliaire : l'adénovirus ou l'herpès simplex.

Le DNA viral extrait des particules par traitement à la pronase est une molécule bicaténaire linéaire, non permutée. Quand la particule est dissociée par le chlorhydrate de guanidine 4M, le DNA viral est circulaire Il sédimente plus rapidement que sous forme linéaire. Ces molécules circulaires deviennent linéaires si on les traite par la pronase. Une protéine est donc responsable de la circularisation du génome dans la particule virale (Robinson et al., 1973 ; Robinson and Bellet, 1974). Elle a une masse moléculaire de 55.000 et est liée à chaque extrémité 5' du DNA par un résidu deoxycytidine (Rekosh et al., 1977). Une structure semblable existe dans le phage ϕ 29 de Bacillus Subtilus (Ortin et al., 1971). C'est le cas aussi d'un virus à RNA monocaténaire : le poliovirus (Flanegan et al., 1977 ; Lee et al., 1977).

Dans la particule virale deux protéines (V et VII) sont associées au DNA dans le nucleoide. Après extraction de celui-ci par chauffage en

présence de deoxycholate de sodium ou par la pyridine ; le DNA est clivé en fragments de 200 paires de bases par la nucléase de Staphylococcus Aureus Le DNA viral est donc organisé d'une manière semblable à la chromatine des cellules eucaryotes. Chaque nucléosome contient six polypeptides VII et un polypeptide V (Corden et al., 1976). Le DNA de SV40 est aussi "assemblé" en nucléosome mais ce virus utilise les histones de la cellule hôte (Griffith, 1975 ; Germand et al., 1975).

II - 4 Enzymes présents dans la particule

Aucune activité DNA ou RNA polymérase n'est associée à la particule virale, contrairement au virus de la Vaccine.

Une endonucléase est mise en évidence après rupture de la particule par dialyse contre un tampon tris-maleate 5 mM pH 6,2 (Burlingham et al., 1971, Burlingham and Doerfler, 1972 ; Marusyk et al., 1975). Elle a les mêmes propriétés qu'une endonuclease de cellules KB non-infectées. Elle est vraisemblablement d'origine cellulaire (Reif et al., 1977 ; Tsuruo et al., 1978).

Dans les mêmes conditions de rupture de la particule, une protéine kinase cosédimente avec la particule ayant perdu le penton. Cet enzyme phosphoryle préférentiellement la protéine IIIa, mais aussi les protéines V, VI, VII et X, en utilisant le phosphate en γ de l'ATP (Blair and Russell, 1978 ; Akusjarvi et al., 1978).

II - 5 Classification

90 sérotypes différents ont été isolés dans 11 espèces animales (Tableau I, retranscrit à partir de Norrby et al., 1976). Les adénovirus aviaires n'ont pas d'antigène commun avec les adénovirus isolés de mammifères. Tous les sérotypes portent un déterminant antigénique spécifique de groupe, porté par l'hexon, à l'exception des adénovirus aviaires (Pereira et al., 1963).

Plusieurs classifications des sérotypes isolés chez l'homme ont été proposées, en utilisant des propriétés différentes :

a) la capacité à agglutiner des érythrocytes de rat ou de singe (Rosen 1960)
b) le pouvoir oncogène chez le hamster nouveau-né (Huebner et al., 1965)
c) l'homologie de séquences des DNA (Green et al., 1979a)
d) l'homologie de masse moléculaire de certains polypeptides de structure : V, VI et VII (Wadell, 1979).

Le tableau II présente la classification des types basée sur

Tableau I

Nombre de sérotypes de l'Adénovirus isolés chez différentes espèces animales

Embranchement	Hôte Naturel	Nombre de sérotypes
Mammifères	Homme	33
	Singe	24
	Equidé	1
	Bovin	8
	Ovin	3
	Canin	2
	Porcin	4
	Murin	2
Oiseaux		14

Tableau II : Classification des Adénovirus Humains

sous-groupe	sérotypes	% d'homologie du DNA dans le sous groupe (a)	% d'homologie du DNA avec les autres types (a)	% G + C (b)	Pouvoir Oncogène (c)	sous groupe hémagglutination (d)	sous groupe antigène T (e)	% d'homologie de la région transformante (f) dans un même sous-groupe	% d'homologie de la région transformante (f) avec les autres types	Masse Moléculaire des Polypeptides (g) V	VI	VII
A	12-18-31	48-69	8-20	47-49	hautement	3 B	A	35-71	2-7	46-48,5 51-51-5	25,5	18
B	3-7-11 14-16-21	89-94	9-20	49-52	faiblement	1 A, B	B	85-99	6-21	53,5-54,5	24	18
C	1-2-5-6	99-100	10-16	57-59	pas	3 A	C	98-100	1-15	48,5	24	18,5
D	8-9-10-13 15-19-20 22-23-24 25-26-27 28-29-30	94-99	4-17	57-59	pas sauf Ad 9	2 A - F	D	-	-	50,5	23,5	18
E	4	-	4-23	57	pas	3 A	-	-	-	48	24,5	18

Les différents sous-groupes sont établis par l'homologie des séquences de DNA. Il y a aussi homologie de séquences de la région du DNA indispensable à la transformation maligne. Une même classification des sérotypes est obtenu par les différences de masse moléculaire de certains polypeptides. Les propriétés d'hémagglutination sont testées avec des erythrocytes de singe Rhesus (1), humaine et de rat (2), ou de Rat (3). A représente une hémagglutination à une dilution au 1/28, B au 1/64 etc... F au 1/4.

a) d'après Green et al. 1979
b) d'après Piña and Green 1965
c) d'après Huebner et al. 1965
d) d'après Hierholzer 1973
e) d'après Huebner 1967
f) d'après Mackey et al. 1979 ; Fujinaga et al., 1979
g) d'après Wadell, 1979

l'homologie des séquences de DNA. Les résultats des autres modes de classification y ont été incorporés.

La séquence des 120 premiers nucléotides de l'extrémité gauche du génome est relativement bien conservée à travers les sous-groupes A, B, C et même pour un adénovirus simien (le SA 7) et le SV 40 (Tolun et al., 1979).

III Génétique

III-1 Organisation des gènes

Le chromosome de l'adénovirus a une capacité de codage pour 40 à 50 polypeptides. Pendant le cycle lytique 6 à 13 protéines précoces et 18 à 20 protéines tardives sont mises en évidence en gel de polyacrylamide-SDS. La localisation des gènes codant pour ces protéines est effectuée par diverses méthodes.

III-1-1 Traduction in vitro de mRNA spécifiques

La localisation de la zone codante pour une protéine est réalisée par la traduction in vitro de mRNA polyadénylés sélectionnés par hybridation sur des fragments du génome viral. Les premiers résultats furent acquis en utilisant des fragments de DNA obtenus à l'aide de l'endonuclease de restriction Eco R I (Lewis et al., 1975). Des résultats plus précis ont ensuite été obtenus par l'utilisation de fragments de DNA produits par diverses endonucléases de restriction (Lewis et al., 1976, 1977 ; Pettersson and Mathews, 1977).

L'addition de cycloheximide au début du cycle augmente la concentration de certains mRNA précoces (Buttner et al., 1976 ; Craig and Raskas, 1974 ; Halbert et al., 1979).

III-1-2 Visualisation des hybrides DNA-RNA

Les mRNA spécifiques de certaines protéines tardives sont visualisés le long du génome en microscopie électronique (Thomas et al., 1976). Les mRNA sont incubés en présence de DNA dans des conditions de fusion partielle de celui-ci. Il se forme alors un hybride DNA-RNA plus stable que le DNA double brin. La région où un mRNA s'hybride avec une séquence complémentaire de DNA apparait sous forme d'une boucle de déplacement ("R-loop"). Les premiers résultats ont été obtenus avec le mRNA de l'hexon (Westphal et al., 1976). Cette technique fut appliquée ensuite aux mRNA de la fibre et d'une protéine virale non-structurale de masse moléculaire 100 K. (Meyer et al., 1977, Chow et al., 1977a, b). La figure 2 résume les

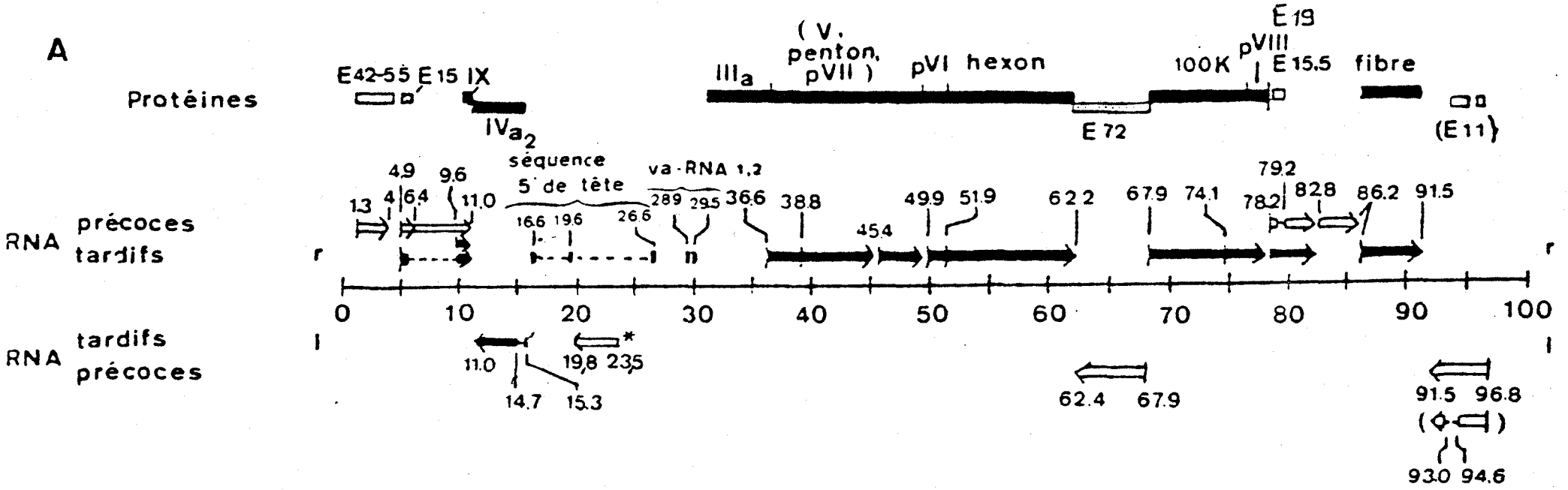

Fig 3 A - Carte génétique de l'Adénovirus 2

Les pointes des flèches représentent les extrémités 3'. Les lignes pointillées indiquent les séquences absentes dans le mRNA polysomal. Les flèches claires correspondent à l'expression des gènes précoces ; les flèches noires correspondent à l'expression des gènes tardifs.
D'après Anderson, C.W., Atkins, J.F., Broker, T.R., Chow, L.T., Gelinas, R.E., Lewis, J.B., Mathews, M.B., Pettersson, U., Roberts, J.M., and Roberts, R.J., Cell 1977, 11 et 12 ; Galos et al., 1979 (E 5) ; Persson et al., 1979c (E 3 19 K).

B

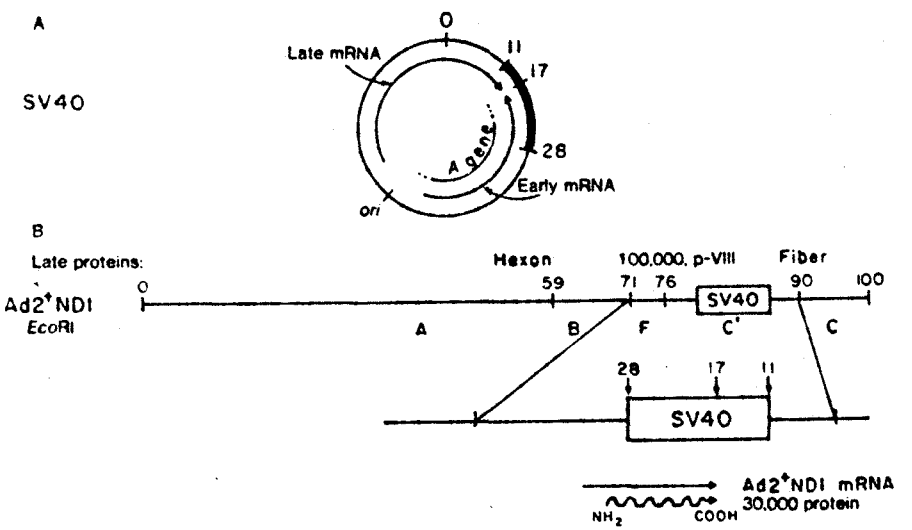

Fig 3 B - Carte génétique de l'Ad 2 ND 1

reprise de Gesteland et al., 1977

résultats obtenus par ces deux techniques. La plupart des gènes codant pour les protéines tardives à l'exception du IVa_2 sont situés sur le brin r, ainsi dénomé parce que la transcription se fait vers la droite (right). Les gènes codant pour les protéines précoces sont répartis en quatre zones majeures dénomées E_1, E_2, E_3 et E_4. Récemment une 5ème zone précoce a été mise en évidence sur le brin l (left) entre 19,8 et 23,5, le produit de ce gène n'est pas connu. Ce mRNA est présent en un petit nombre de copies : 30 copies par cellule au lieu de 500 à 1 000 pour les autres mRNA précoces (Galos et al., 1979).

III-1-3 analyse de recombinants intertypiques

La localisation de certains gènes a aussi été effectuée par l'analyse de recombinants intertypiques entre des mutants thermosensibles (ts) d'Ad 5 et des mutants ts d'Ad 2 ou d'Ad 2 ND1. L'Ad 2 ND1 est un virus hybride de l'Ad 2 qui contient un fragment de génome du SV 40. Cet hybride est non défectif car il se multiplie efficacement sur cellules de singe alors que les adénovirus humains n'y produisent qu'un cycle abortif.

La structure du DNA des recombinants est étudiée par clivage à l'aide de différentes endonucléases de restriction. Les sites de coupure sont spécifiques du sérotype étudié. La figure 3a montre, les sites de clivage par l'endonucléase de restriction Eco R I de divers adénovirus. Tandis que la figure 3b rassemble les sites de clivage de plusieurs endonucléases de restriction pour les sérotypes 2 et 5. Comme certaines protéines diffèrent par leur masse moléculaire (hexon et fibre) ou par leurspropriétés immunologiques (fibre) il est possible de localiser les gènes codant pour ces protéines (Mautner et al., 1975 ; Grodzicker et al., 1977 ; Weber and Hassell, 1979)

Le tableau III compare les résultats obtenus par ces trois techniques pour quatre polypeptides.

La figure 2 montre aussi la localisation des gènes codant pour des RNA de petite taille (5,5 S) les VA RNA (Viral Associated RNA) (Mathews, 1975 ; Soderlund et al., 1976). Ce résultat a été obtenu par hybridation sur filtre de nitrate de cellulose des VA-RNA radioactifs avec des fragments de DNA obtenus par clivage avec des endonucléases de restriction. Les fragments de DNA sont d'abord séparés en gel d'agarose ou d'acrylamide puis transférés, après dénaturation sur une membrane de nitrate de cellulose méthode de transfert ou "blotting" de Southern, 1975).

Tableau III

Localisation des gènes codant pour les polypeptides II, 100 K, IV et E 72 K

polypeptides	par traduction in vitro des mRNA a	par cartographie en microscopie électronique b	analyse de Recombinants * c
II	40,9 - 70,7	51,9 - 62,2	44 - 59
100 K^{+}	59 - 83,4	68 - 78,6 68 - 83	69 - 71
IV	85 - 100	86,3 - 91,5	85 - 98
E 72 K	58,5 - 70,7 d	62,4 - 67,9	59 - 71

* La région codante peut être plus grande que cette zone (en particulier pour la 100 K). La valeur donnéereprésente la zone codante pour le fragment type spécifique de la protéine. Ainsi pour l'hexon cette partie type-spécifique (Ad 2 / Ad 5) est localisée à la position 55-57 du génome (Weber and Hassell, 1979)

a) d'après Lewis et al. 1977

b) d'après Chow et al. 1977 b

c) Mautner et al., 1975 ; Grodzicker et al., 1977 ; Weber and Hassell, 1979)

d) d'après Lewis et al. 1976

+ 100 K : c'est une protéine non structurale de masse moléculaire 100 000.

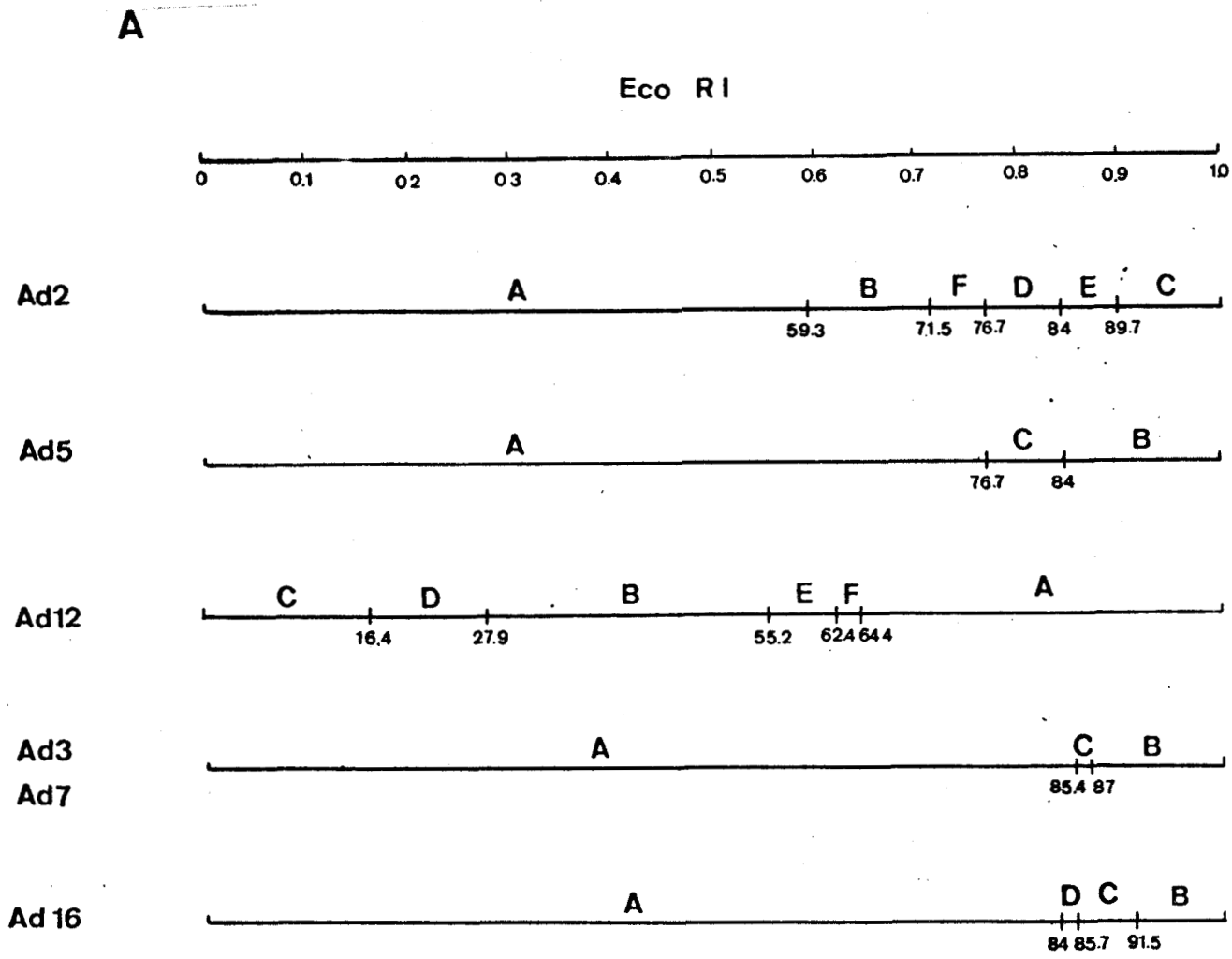

Fig 3 - Carte physique de l'adénovirus

A) de plusieurs sérotypes (2, 5, 3, 7, 12, 16) par l'endonuclease de restriction Eco RI.

B) des sérotypes 2 et 5 par diverses endonucleases de restriction. Notons qu'il n'y a pas de site différent de coupure à la gauche du site Bam H1 de 29,3 pour le type 2

B

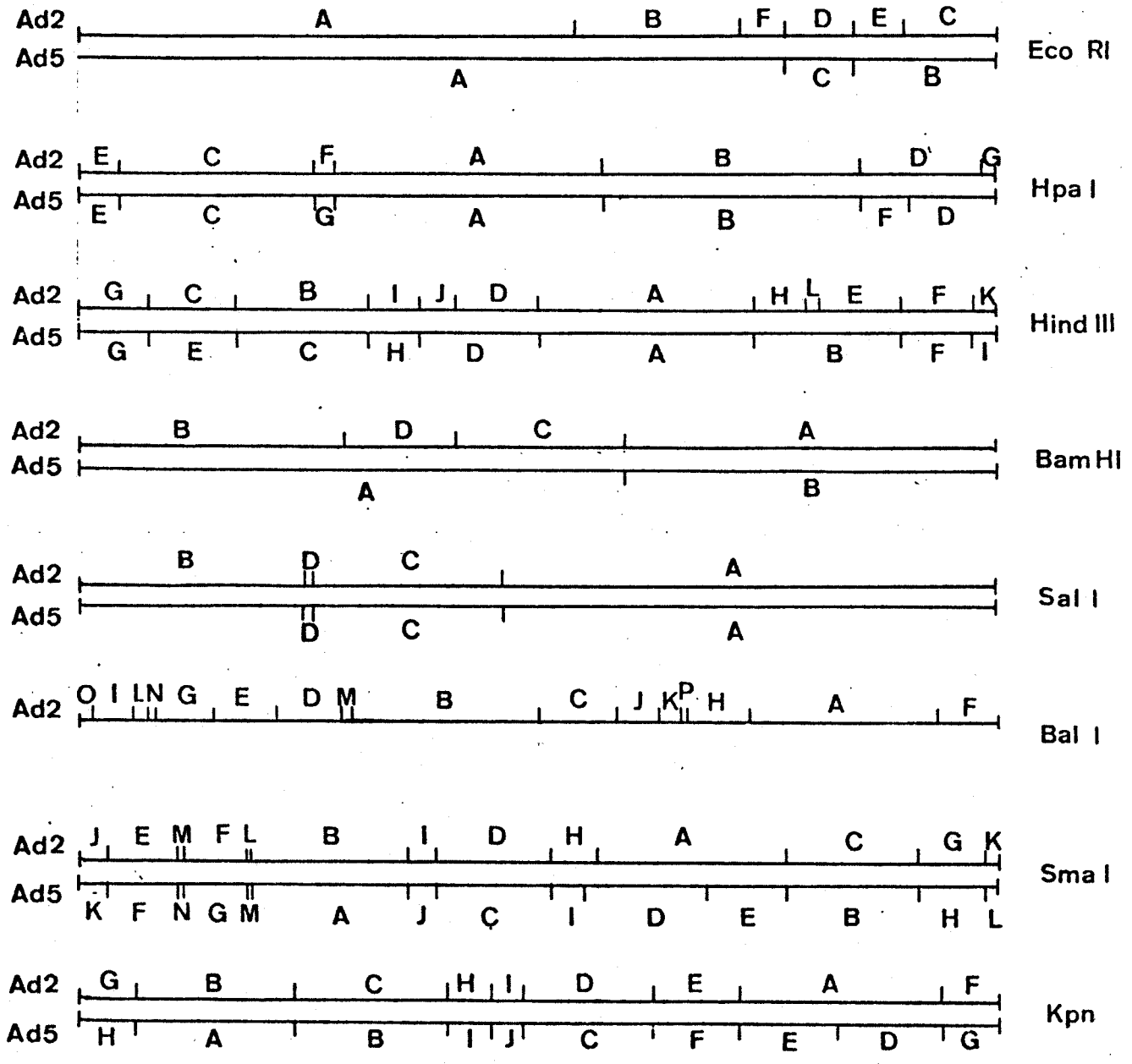
Ad2
A B F D E C
Ad5
A C B
Eco RI
Ad2
E C F A B D G
Ad5
E C G A B F D
Hpa I
Ad2
G C B I J D A H L E F K
Ad5
G E C H D A B F I
Hind III
Ad2
B D C A
Ad5
A B
Bam HI
Ad2
B D C A
Ad5
D C A
Sal I
Ad2
O I LN G E D M B C J K P H A F
Bal I
Ad2
J E M F L B I D H A C G K
Ad5
K F N G M A J Ç I D E B H L
Sma I
Ad2
G B C H I D E A F
Ad5
H A B I J C F E D G
Kpn

Tableau IV : Mutants de l'Adén.

Sérotype et type de mutant (a)	agent mutagène (b)	Fréquence d'isolement	Références
H 2 ts	NNG HNO_2	7/172 5/170	Begin and Weber 1975
H 2 ts	HNO_2	39/400	Martin et al., 1978
H 2 ts	EMS HNO_2 NH_2OH		Kathmann et al., 1976
H 5 ts	NH_2OH HNO_2 BudR	14/146 8/95 2/355	Williams et al., 1971
H 5 ts	NH_2OH HNO_2 NNG	0,01 à 0,1%	Ensinger and Ginsberg, 1972
H 5 ts	NH_2OH NNG	8/372 2/317	Takahashi 1972
H 7 ts	spontanné	-	Estes and Butel 1977
H 12 ts	UV NH_2OH BudR NNG	45/700 24/370 2/260 17/250	Shiroki et al., 1972
H 12 ts	HNO_2 NNG	5/679	Ledinko 1974
H 31 ts	UV	17/506	Suzuki et al., 1972
celo ts	NH_2OH	27/238	Ishibashi 1971
H 12 cyt	spontané UV		Takemori et al., 1968
Ad 2 ND 1 (c)			Lewis et al., 1969
Ad 2 ND 1 hr ts	NH_2OH HNO_2	3/250	Grodzicker et al., 1974
Ad 2 hr	HNO_2	4	Klessig 1977
5 hr	spontané	1	Klessig and Groczicker 1979
H 5 hs	spontané	1 mutant	Young and Williams 1975
H 5 hr	NH_2OH	7/372	Takahashi 1972
H 5 hr	HNO_2 UV	0,05%	Harrisson et al., 1977
H 5 dl in.	clivage du DNA par endonuclease de restriction reconstruction du génome, infection et recherche de mutants ayant perdu un site de coupure par une endonuclease		Jones and Shenk 1978, 1979

a) La nomenclature utiliséeest celle proposéepar Ginsberg, Williams, Doerfler and Shimojo, 1973.

Par exemple H 2 pour adénovirus humain de type 2 suivi

ts	thermosensible
hr	hôte dépendant
hs	thermo-stable
cyt	cytocidal
dl	deletions
sub	substitution
in	insersion

b) Abréviation utilisée

EMS	ethane methane sulfonate
HNO_2	acide nitreux
NH_2OH	hydroxylamine
NNG	N-méthyl-N'-nitro-N-nitrosoguanidine
BUdR	bromodeoxyuridine
UV	radiations ultra violettes

c) Ad 2 ND hybride Ad 2 SV_{40} qui se multiplie efficacement sur cellules de singe et qui contient une séquence de SV 40 .

III - 2 Mutants de l'Adénovirus

Dans l'étude du cycle lytique, abortif ou dans la transformation cellulaire, les mutants sont de précieux outils. Ils permettent de déterminer in vivo le rôle des protéines virales pour la réplication du DNA, la régulation de l'expression des gènes précoces et tardifs et la formation de la particule virale. Ils sont aussi particulièrement utiles pour mettre en évidence des étapes "fugaces". du cycle lytique.

III-2-1 Type des mutants isolés

De tous les mutants ce sont les thermosensibles qui sont le plus étudiés dans le sys tème adénovirus-cellule-permissive. Ils offrent l'avantage de pouvoir passer d'un stade permissif à un stade restrictif ou vice-versa par simple changement de température. Mais par contre la localisation du gène muté ainsi que le produit de celui-ci sont plus difficiles à déterminer que pour les mutants de délétion. Un grand nombre de mutants thermosensibles ont été isolés pour plusieurs sérotypes (2, 5, 7, 12, 31 et virus Celo). Le tableau IV résume la liste des publications décrivant l'isolement de mutants. Ce tableau précise :

- l'agent mutagène utilisé ; acide nitreux, N-Méthyl-N'- nitro-N-Nitrosoguanidine, les radiations ultraviolettes, ainsi que la bromo_deoxyuridine.
- la fréquence d'isolement de mutants

Les mutants thermosensibles sont obtenus principalement par traitement d'un stock de virus avec un agent mutagène, de manière à diminuer le titre du stock d'un facteur 10^2 à 10^5. Les clones sont isolés à la température choisie comme température permissive puis testés aux deux températures, permissive (32° - 33°C) et non permissive (38,5°-39,5°C). La fréquence des clones thermosensibles varie d'environ 1 à 10%.

Quand la bromodeoxyuridine est utilisée comme agent mutagène, l'isolement se fait après un cycle lytique en présence de BudR. Les mutants hôte-dépendants (hr) sont aussi obtenus par traitement d'un stock de virus avec un agent mutagène. Des clones sont sélectionnés sur des cellules choisies comme permissives et essayés sur cellules que l'on voudrait non permissives. Ainsi Harrisson, Graham et Willians ont isolé en 1977 des mutants qui ne se multiplient plus sur KB ou HeLa mais seulement sur HEK-clone 293. Les HEK-293 sont des cellules de rein embryonnaire humain (HEK) en lignée continue, transformées par du DNA d'adénovirus 5 fragmenté (Graham et al., 1974)

Elles contiennent 12% de la partie gauche du génome qui est exprimée et 9% de la partie droite qui n'est pas exprimée sous forme de mRNA (Graham et al., 1977 ; Aiello et al., 1979).

Ces cellules HEK 293 sont aussi les cellules permissives utilisées par Jones et Schenk (1979a)pour isoler des mutants de délétion, de substitution ou d'insertion. Ils recherchent des mutants ayant perdu un ou plusieurs sites de clivage d'une endonucléase de restriction : après clivage du DNA circulaire avec l'enzyme de restriction choisi, ils relient les fragments obtenus avec la polydeoxynucléotide ligase du phage T_4, et infectent des cellules HEK 293 ; ils ont ainsi isolé des mutants ne possèdant plus de site de coupure de l'endonucléase de restriction Xba I (Jones and Shenk, 1978, 1979)

D'autres systèmes cellulaires ont aussi été utilisés, Takahashi (1972) a isolé des mutants d'Adénovirus 5 ne poussant pas sur cellules de rein de hamster (Ham.K), cellules permissives à l'adénovirus 2 et 5 mais pas à l'adénovirus 12.

Les adénovirus humains induisent un cycle abortif dans des cellules de singe (CV_1), mais Lewis et al. 1969) ont isolé chez des hybrides Ad2 SV_{40} (Ad2-ND 1 à 4) qui sont capables de se multiplier sur CV_1. Des mutants thermosensibles ont été isolés à partir de l'Ad2 ND_1. Ces mutants ne se multiplient pas à 38,5°C sur CV_1, mais ils se comportent comme l'Ad 2 ND_1 à 33°C. A 38,5°C ils se comportent comme des mutants hôte-dépendants car ils se multiplient normalement sur HeLa (Grodzicker et al., 1974). Tout récemment Klessig a isolé plusieurs mutants ponctuels de l'adénovirus qui se multiplient aussi efficacement sur CV_1, que sur HeLa (Klessig, 1977 ; Klessig and Grodzicker, 1979).

Il est impossible de cultiver des mutants non-sens de virus eucaryotes, car il n'existe pas de système t-RNA suppresseurs comme dans le système phage-bactérie. Cependant, des mutants de l'Ad 2 ND_1 sont des mutants non-sens, pour la fonction nécessaire à la multiplication sur cellule de singe (Gesteland et al., 1977 ; Grodzicker et al., 1976).

III-2-2 complémentation

Le test de complémentation a pour but de déterminer si deux mutants ont une mutation dans le même gène ou dans deux gènes différents. Pour les mutants ts, des cellules sont infectées par deux

mutants à température non permissive, et à la fin du cycle viral la production de virus est mesurée à température permissive, et est comparée à la production de chaque mutant seul à la température non-permissive. Il est possible de calculer un indice de complémentation (CI) qui est donné par la formule suivante :

$$CI = \frac{\text{Production de la double infection}}{\text{Production la plus élevée des deux infections simples}}$$

(Russel et al., 1972 ; Suzuki et al., 1972 ; Ledinko, 1974 ; Begin and Weber, 1975). Beaucoup d'indices de complémentation sont élevés, dans certains cas ils atteignent 10^6 (Williams and Ustacelebi, 1971). La production de la double infection se rapproche quelque fois de celle du type sauvage à la même température (Ledinko, 1974).

Williams et al. (1974) estiment que deux mutants appartiennent à deux groupes de complémentation quand le CI est supérieur à 10. Avec cette limite ils obtiennent 17 groupes de complémentation pour 51 mutants dont cinq groupes concernent l'hexon (synthèse, assemblage, transport), 3 la synthèse de la fibre et 2 la synthèse du DNA. Ensinger et Ginsberg (1972) ne trouvent que trois groupes de complémentation pour les mutants ayant le phénotype hexon défectif.

D'autres auteurs trouvent trois groupes de complémentation concernant la synthèse du DNA (Ad 12 : Ledinko 1974 ; Suzuki et al., 1972 ; Ad 2 et Ad 5 Kathmann et al., 1976), ce qui semble faible par rapport au nombre de protéines précoces mises en évidence (au moins six) au cours du cycle lytique. Ce résultat apparemment paradoxal peut s'expliquer si l'on considère que les mutants hr isolés par Harrisson et al.,(1977) complémentent tous les mutants ts appartenant aux 17 groupes de Williams. Ces mutants hr concernent des protéines précoces de la région E_1 (Frost and Williams, 1978).

Quelques mutants ts appartiennent à plusieurs groupes de complémentation, ce sont vraisemblablement des doubles mutants (Suzuki et al., 1972 ; Begin and Weber, 1975) Un seul mutant ne complémente aucun autre, et il a un effet dominant sur le type sauvage, c'est le H 2 ts 48 (Carstens and Weber, 1977).

Le tableau V indique le nombre de groupes de complémentation des mutants isolés dans divers laboratoires, il indique aussi le nombre de groupes de complémentation pour quelques phénotypes (Ginsberg and Young, 1977).

III-2-3. Recombinaison et localisation des gènes

- Recombinaison de mutants d'un même sérotype

L'étude de la fréquence de recombinaison entre plusieurs mutants permet de situer chaque mutation par rapport aux autres. Un "test 3 points" a été utilisé avec un mutant thermo-résistant (hs = heat-stable) mais la nécessité d'analyser la progéniture vis à vis de sa résistance à la température rend ce test fastidieux. De plus ce mutant est localisé dans la partie gauche du génome, ce qui rend l'interprétation difficile pour les mutants de la partie droite du génome (Young and Williams, 1975). Le système le plus utilisé est la fréquence de recombinaison entre deux mutants. Pour ce faire, des cellules infectées par chaque mutant ou par le mélange des deux mutants sont cultivées à température permissive. Puis la production virale est testé à température non permissive, pour mesurer la production de ts^+, et à température permissive pour mesurer la production totale. La fréquence de recombinaison (rf) est exprimée en fonction de la formule suivante :

$$\frac{\text{titre à la température non permissive}}{\text{titre à la température permissive}} \times 2 \times 100$$

Il est le plus souvent possible de ne pas faire la correction de la fréquence de réversion des mutants seuls (Ensinger and Ginsberg, 1972 ; Williams et al., 1974). Begin and Weber (1975) soustraient la fréquence de réversion du rapport précédent pour obtenir la fréquence de recombinaison. A partir des mesures de fréquence de recombinaison il est possible de construire une carte génétique. La figure 4 montre une telle construction. Elle comprend 2 "familles" de mutants de l'Ad 5 (Williams et al., 1974 ; Ginsberg and Young, 1977).

- Analyse de recombinants entre deux sérotypes d'un même sous-groupe

L'homologie des DNA des adénovirus d'un même sous-groupe permet d'envisager une recombinaison entre des mutants de deux sérotypes. Une telle analyse a d'abord été utilisée pour des mutants thermosensibles d'Ad 5 et d'Ad 2 ND_1 (Sambrook et al., 1975).

Les plages obtenues à température non permissive (ts^+) sont amplifiées et le DNA viral est analysé par diverses endonucleases de restriction. Les sérotypes 2 et 5 présentent de nombreux sites différents entre les coordonnées 30 et 100 du génome. Il est donc possible, par l'analyse d'un grand nombre de recombinants de déterminer le fragment de génome, spécifique de type, toujours absent des recombinants et donc porteur de la mutation. Cette technique a été aussi utilisée pour la cartographie des mutants isolés par Begin et Weber (Hassell and Weber, 1978). La figure 5a montre les

Mutants thermosensibles de l'adénovirus : phénotype et groupe de complémentation

Sérotype	nb de mutants	nb de groupes de complémentation	PHENOTYPE						Références
			nb de mutant défectif dans la synthèse du DNA viral	Hexon	Transport de l'hexon	Fibre	Base du penton	Assemblage	
H 2	36	13	0	2 (1,1)$^{+}$			3(1,1,1)$^{=}$	8 (6X1,2X2)	Begin and Weber, 1975 ; Weber et al., 1975 ; Carstens and Weber, 1977
	39	14	2 (2, 1)	4 (4,3,1 1)		2 (2,1)	3(1,1,1)	3 (1,1,1)	Martin et al., 1978
	14	7	3 (1, 1, 1)						Kathman et al., 1976
H 5	51	17	1 (3)	2 (1,1)	4 (20, 7. 1, 1)	3 (3,2,1)	0	4 (3,2,1,1)	Russell et al., 1972 ; 1974 ; Williams et al. 1974 ; Wilkie et al., 1973
	15	6	2 (1, 1)	1 (6)	1 (1)	1 (1)	0	1 (5)	Ensinger and Ginsberg, 1972 ; Ginsberg et al. 1974
	10	7	2 (2, 2)	2 (1,1)					Minekava et al., 1976 : Takahashi, 1972
H 7	1	1		1					Estes and Butel, 1977
H 12	34	13	3 (5, 3, 3)	0	1 (2)	1 (2)	I (2)	3 (6 1,1)	Shiroki et al., 1972

									Shiroki and Shimojo, 1974
	10	6	2 (1, 1)	1 (1)				1 (1)	Ledinko, 1974
H 31	12	8	3 (1, 1, 1)	0	0	1 (1)	0	2 (2, 2)	Suzuki et al., 1972
Celo	49	*	1		(16)			(22)	Ishibashi, 1971

= Le premier chiffre indique le nombre de groupes de complémentation, le chiffre entre parenthèses indique le nombre de mutants pour chaque groupe de complémentation

\+ H 2 ts 3 aucune synthèse d'antigène de structure n'est décelé
H 2 ts 48 pas de synthèse d'hexon antigénique, et la fibre est anormale

* Pas de test de complémentation effectué, mais il distingue 5 groupes de changement morphologique

Tableau V (b)

Mutants "host-range" (hr) et de délétion de l'adénovirus : phénotype et groupe de complémentation

Sérotype	nb de mutants	nb de groupes de complémentation	cellules restrictives	cellules permissives	Ref
H 2			de singe	humaine	
ND 1				singe + humaine	Lewis et al., 1969
H 2 hr 400	4			singe + humaine	Klessig, 1977 ; Klessig and Grodzicker, 1979
H 2 ND 1ts		1	singe (température non permissive)		
H 5 hr	7	2 (5, 2)	HeLa ou KB	293	Harrison et al., 1977
H 5 dl	7		HeLa	293	Jones and Shenk 1979
H 5 hr	7	6	NIL	HEK	Takahashi, 1972, Minekawa et al., 1976

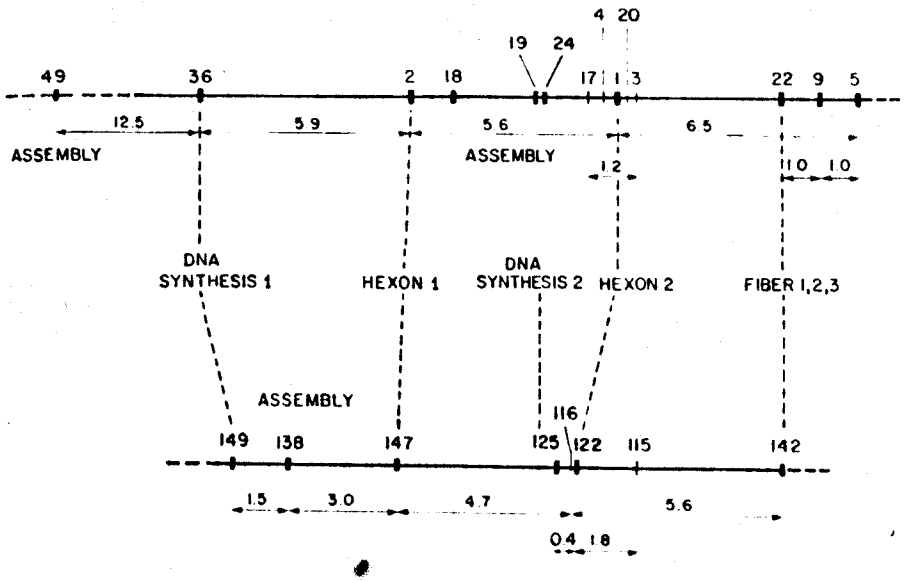

Fig 4 - Carte génétique, basée sur la fréquence de recombinaison

Construction d'une carte génétique a partir de la fréquence de recombinaison entre des mutants ts d'Ad 5.

D'après Williams et al., 1974 ; Ginsberg and Young, 1977.

résultats obtenus pour divers mutants d'Ad 2, d'Ad 2 ND_1 et d'Ad 5. Notons que les mutations de l'Ad 2 ND_1 ne sont pas localisées dans le fragment SV_{40} mais entre les positions 59-72 du DNA de l'adénovirus.

- Technique "Marker Rescue"

Le DNA viral est infectieux (Graham and Van der Eb, 1973a) et l'efficacité d'infection est augmentée en utilisant le complexe DNA-55 K. Par l'infection à température restrictive de cellules avec du DNA d'un mutant et l'un des fragments de DNA du type sauvage, des virus ts^+ sont obtenus. Cette production est le reflet d'une recombinaison dans la cellule. Puisque l'un des fragments de DNA de type sauvage permet de sauver la mutation, celle-ci est localisée dans ce fragment de génome. Le DNA déprotéinisé a d'abord été utilisé (Arrand, 1978 ; Frost and Williams, 1978), puis le complexe DNA-55 K : dans ce cas la fréquence d'apparition de plages est multipliée par un facteur 10^3 (Galos et al., 1979).

La recombinaison ayant lieu, au hasard, dans la cellule, la nature de celle-ci joue un grand rôle : l'efficacité d'obtention de plages est beaucoup plus élevée sur HEK 293 que sur HeLa ou KB (Williams, 1979).

Les résultats obtenus sont représentés dans la figure 5 b.

Les mutations des virus hr d'Ad 5 ont été localisées dans la région transformante (l'extrémité gauche du génome) par une technique similaire (Frost and Williams, 1978).

III-2-4 Phénotypes

- Mutants thermosensibles

Suivant le phénotype, nous pouvons déterminer 3 classes de mutants thermosensibles :

. défectifs dans la synthèse du DNA viral
. défectifs dans la synthèse d'une ou plusieurs protéines structurales du virus
. défectifs dans l'assemblage, bien que synthétisant normalement les protéines de structure de la particule.

La synthèse du DNA viral est mesurée par l'une de ces trois méthodes :

. hybridation DNA-DNA
. séparation à l'équilibre en gradient isopycnique de CsCl, basé

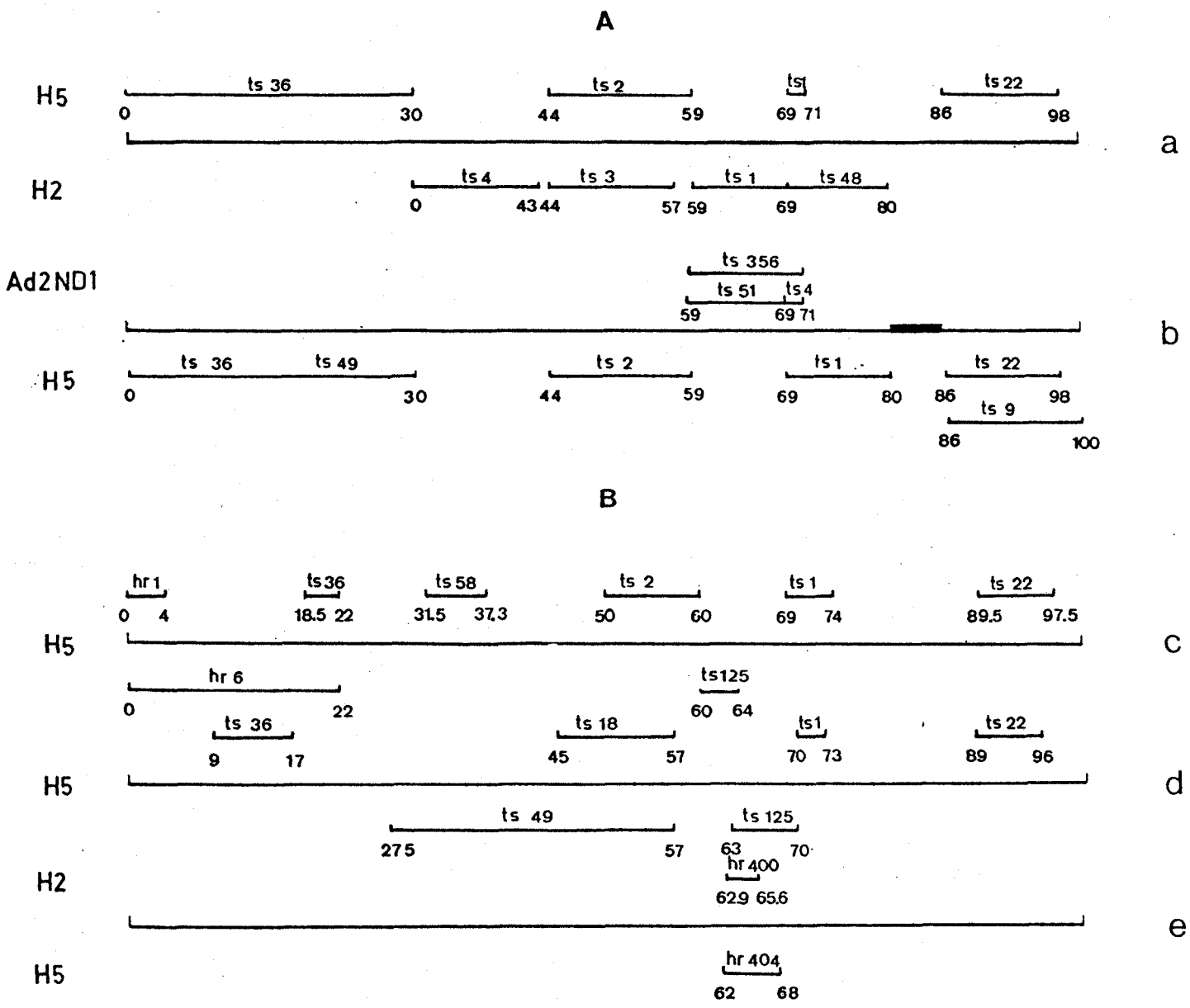

Fig 5 - Cartographie des mutations

La localisation des mutations a été effectuée par l'une des techniques suivantes :

A) Analyse des recombinants intertypiques

B) "Marqueur Rescue"

Ces résultats ont été obtenus par

a) Hassell and Weber, 1978
b) Sambrook et al., 1975
c) Frost and Williams, 1978 ; Galos et al., 1979
d) Arrand, 1978
e) Klessig and Grodzicker, 1979

sur la différence de composition en base du DNA viral et cellulaire

. sédimentation en gradient linéaire de saccharose alcalin, après lyse des cellules au sommet du gradient

De nombreux mutants ont ainsi été caractérisés pour l'adénovirus de type 2 (Kathman et al., 1976), de type 5 (Ensinger and Ginsberg, 1972 ; Wilkie et al., 1973), de type 12 (Ledinko, 1974 ; Shiroki and Shimojo, 1974), de type 31 (Suzuki et al., 1972) et pour le Celo virus (Ishibashi, 1971). L'étude de ces mutants indique que trois gènes, au moins, sont nécessaires à l'initiation de la synthèse du DNA viral. Le produit de l'un de ces gènes a été identifié : c'est la E 72 K, une protéine à affinité pour le DNA simple brin (DNA binding protein = DBP) ; cette protéine est thermosensible dans le H5 ts 125 (Van der Vliet et al., 1975).

L'antigénicité des protéines majeures de structure est visualisée, le plus souvent par immunodiffusion radiale d'un immunsérum de lapin, anti particule virale, contre les antigènes d'un lysat de cellules infectées par le mutant à température restrictive (Russell et al., 1972 ; Weber et al., 1975 ; Ensinger and Ginsberg, 1972). Martin et al. (1975) utilisent l'immunoélectrophorèse à deux dimensions (Laurell, 1965).

22 des 51 mutants testés par Williams et al. (1974) sont défectifs dans la synthèse de l'hexon. Ils appartiennent à 5 groupes de complémentation. L'un de ces groupes correspond à un défaut de transport de l'hexon du cytoplasme au noyau (Kauffman and Ginsberg, 1976). Un autre groupe de complémentation concerne les mutants d'une protéine non-structurale : la 100 K ; dans ce cas il n'y a pas formation d'hexon trimérique (Leibowitz and Horwitz , 1975 ; Mautner et al., 1975 ; Cartens and Weber, 1977).

Les mutants défectifs dans la synthèse de la fibre appartiennent à 3 groupes de complémentation (Russell et al., 1972 ; Ensinger and Ginsberg, 1972). L'un de ces mutants possède un polypeptide IV (fibre) non glycosylé (H5 ts 142)(Ginsberg and Young, 1977).

Très peu de mutants défectifs dans la synthèse de la base du penton ont été isolés à l'exception de ceux de Begin et Weber (Weber et al., 1975).

Les mutants de la classe III forment jusqu'à 8 groupes de complémentation pour l'Ad 2 (Weber et al., 1975) et 5 groupes pour l'Ad 5 (Russell et al., 1972). L'un de ces mutants (H2 ts 1)

accumule à température non permissive des particules non infectieuses contenant du DNA (Weber, 1976). Deux mutants d'Ad 5 (H5 ts 18, H5 ts 19) n'induisent pas la synthèse d'interféron dans les cellules fibroblastiques embryonnaires de poulet (CEF) à température restrictive (Ustacelbi and Williams, 1972 ; Tarodi et al., 1977).

Le tableau 5 indique le nombre de groupes de complémentation, avec le nombre de mutants dans chaque groupe. Le tableau 6 précise pour divers mutants représentatifs d'un groupe : le phénotype, la région du DNA muté, ainsi que la ou les protéines susceptibles d'être modifiées.

- Mutants "hôte-dépendants"

Les mutants hr de l'adénovirus 5 isolés par Takahashi, incapables de se multiplier sur cellules de hamster, peuvent transformer ces cellules. Il en est de même pour la souche sauvage de l'adénovirus 12. Une protéine modifiée dans ces mutants hr, et peut-être absente de l'Ad 12 est indispensable à la multiplication sur cellules de hamster (Takahashi et al., 1974).

Les cellules HeLa ou KB infectées par le mutant hr 1 (cultivé sur HEK 293) ne synthétisent pas de DNA viral mais il y a synthèse de DNA avec les mutants "hôtes dépendants" de l'autre groupe de complémentation (hr 6) (Harrisson et al., 1977). Ces deux mutants ne transforment pas les cellules embryonnaires de rat (Graham et al., 1978).

- Mutants de délétion

Les cellules embryonnaires de rat ne sont pas transformées par les mutants H5 dl 311, 312, 313, 314. La délétion de ces mutants est située autour de la position 4 du génome (Jones and Schenk, 1979).

IV Le cycle lytique

Le cycle lytique commence par l'absorption de la particule infectieuse sur la membrane cellulaire et aboutit à la lyse de la cellule permissive avec libération d'un grand nombre de particules virales (10^4 à 10^5 particules/cellule).

Le cycle infectieux est divisé en deux phases, la phase précoce qui suit la pénétration du virus et la phase tardive qui précède la lyse cellulaire. Le passage de la phase précoce à la phase tardive est ponctué par le démarrage de la synthèse du DNA viral.

Tableau VI

Corrélation entre le phénotype, et la protéine mutée

	Phénotype (a)	Localisation de la mutation (b)	Protéine mutée (c)
H 5 ts 125	DNA$^-$	63 - 64	E 72 K
H 5 ts 36	DNA$^-$	18,5 - 22	?
H 5 ts 22	fibre$^-$	89 - 96	IV
H 5 ts 2	hexon$^-$	50 - 59	II
H 2 ts 3		44 - 57	
H 5 ts 1	hexon$^-$	69 - 71	100 K
H 2 ts 48		69 - 80	100 + pVIII
H 5 ts 58	Assemblage$^-$	31,5 - 37,3	IIIa
H 2 ts 4	Assemblage$^-$	30 - 43	IIIa, V, III
H 2 ts 1	Assemblage$^-$	59 - 69	II, 100 K (E 72 K)
H 5 ts 18	Assemblage$^-$ Interferon$^-$	45 - 57	pVI, II

(a) phénotype d'après Russell et al., 1972 ; Ensinger and Ginsberg 1972 ; Weber et al., 1975

DNA$^-$ mutant défectif dans la synthèse du DNA viral

Hexon$^-$ mutant défectif dans la synthèse de l'hexon

Fibre$^-$ mutant défectif dans la synthèse de la fibre

Assemblage$^-$ mutant ne produisant pas de virus infectieux

interferon$^-$ mutant n'induisant pas la production d'interféron dans des cultures de cellules embyronnaires de poulet (CEK)

(b) repris de la figure 5a et 5 B

(c) protéine correspondante d'après la fig. 3

IV - 1 Pénétration du virus dans la cellule

La particule virale se fixe à un récepteur cellulaire par la fibre (Philipson et al., 1968). Le récepteur cellulaire est composé de trois espèces polypeptidiques majeures de poids moléculaire 78 000, 60 000 et 42 000 (Hennache and Boulanger, 1978). Cette étape de fixation est suivie de la pénétration dans le cytoplasme par remaniement de la membrane (Hennache et al;, 1979). Au cours du passage à travers la membrane cellulaire, ou dans le cytoplasme, la particule virale perd 5% de sa masse protéique (le penton) (Sussenbach, 1967). La particule migre rapidement vers la membrane nucléaire, où le DNA, sans doute sous forme de nucleoide("core")est injecté dans le noyau, laissant dans le cytoplasme une capside vide (Morgan et al., 1969).

Sergeant et al. (1979) ont montré qu'au stade précoce le DNA viral était organisé dans les noyaux en nucléosomes comme la chromatine cellulaire. Après extraction, même dans des conditions douces, le DNA viral des noyaux de cellules infectées , n'est pas lié à des protéines. Cependant un complexe nucléoprotéinique a été mis en évidence après l'infection de cellules avec des particules immatures produites par le mutant H2 ts 1 à température non-permissive. Ce complexe nucleoprotéinique sédimente comme les "cores" extraits de virus et contient, lié au DNA, les polypeptides V et P VII (précurseur du VII (Mirza and Weber, 1979). Cette étape de nucléoide dans les noyaux de cellules serait trop brève dans l'infection avec le virus sauvage et/ou il y aurait substitution des protéines du nucléoide par les histones de la cellule hôte. Ce changement serait rendu impossible dans le cas des particules de mutant H2 ts 1, par la présence du P VII. Au cours de la maturation du virus le polypeptide VII n'apparait que dans la particule virale. Le mutant H2 ts 1 est bloqué sur l'étape de maturation P VII VII. Les protéines terminales (55 K) restent attachées au DNA après l'infection (Straus et al., 1979 ; Van Wielink et al., 1979). Bien que l'infection d'une cellule par la particule virale intacte soit le processus le plus efficace, il est possible d'infecter une cellule par du DNA, en le précipitant sur les cellules avec du phosphate de calcium (Graham and Van der Eb, 1973a).Le pouvoir infectieux peut être multiplié par un facteur 10^3 si cette transfection se fait avec du complexe-DNA-protéines terminales (Talas and Butel, 1974 ; Sharp et al., 1976).

La faible infectivité du DNA déprotéinisé n'est pas due à l'absence de pénétration, mais à la dégradation de ce DNA par les endonucléases et/ou les exonucléases de la cellule hôte (Groneberg et al., 1975).

IV - 2 La phase précoce du cycle lytique

IV-2-1 Transcription des gènes précoces

Les mRNA viraux précoces représentent 1% des mRNA néosynthétisés (Warocquier, 1969). Les mRNA cytoplasmiques sont complémentaires de 20-25% du génome viral (Craig and Raskas, 1974 ; Sharp et al., 1974). Les RNA nucléaires au stade précoce, en saturent une plus grande partie (Wall et al., 1972 ; Sharp et al., 1974).

Les mRNA précoces sont codés par 5 régions du génome. Les cinq sites d'initiation sont répartis de la manière suivante : deux sur le brin r aux positions 2 (E_1) et 81 (E_3) et trois sur le brin l aux positions 97 (E_4), 73 (E_2) et 23,5 (E_5) (Craig et al., 1975 ; Tal et al., 1974 ; Galos et al., 1979).

Les chaînes de RNA produites dans des conditions où seule l'élongation est possible ne proviennent pas de long transcrit. Aucune molécule supérieure à 26S n'a été mise en évidence (Berk and Sharp, 1977 ; Evans et al., 1977 ; Craig and Raskas, 1976; Sehgal et al., 1979) Les RNA précoces après synthèse sont "coiffés" comme tous les mRNA des cellules eucaryotes (Hashimoto and Green, 1976). Et après addition de poly A, les RNA subissent un épissage ("splicing" des anglo saxons). Ce remaniement est mis en évidence par des boucles de déplacement de DNA simple chaîne hybridé avec des mRNA cytoplasmiques (Kitchingman et al., 1977 ; Neuwald et al., 1977 ; Westphal and Lai, 1977).

Six à huit mRNA précoces sont identifiés dans le cytoplasme des cellules infectées (Philipson et al., 1974 ; Tal et al., 1974 ; Craig et al., 1975 ; Flint et al., 1975 ; Buttner et al., 1976). Sept "coiffes" (cap) différentes sont obtenues après digestion des mRNA cytoplasmiques par la RNAse T_2. Ce qui suggère l'existence d'au moins 7 mRNA précoces (Hashimoto and Green, 1976, 1979).

IV-2-2 Protéines précoces

Jusqu'à 13 polypeptides précoces, de taille variable de 72 000 (E 72 K) à 10 500 (E 10,5 K) sont visualisés, dans des extraits de cellules infectées, en gel de polyacrylamide-SDS.

9 de ces polypeptides sont précipités avec un immunsérum anti protéines précoces (Saborio and Oberg, 1976 ; Russell and Shehel, 1972 ; Anderson et al., 1973 ; Walter and Maizel, 1974 ; Harter et al., 1976 ; Chin and Maizel, 1976). Certaines protéines précoces du cycle lytique précipitent avec des immunsérums anti cellules de rat transformées par l'adénovirus 2 ou 5. Les protéines E 15 K et E 53 K sont toujours obtenues quelque soit l'immunsérum utilisé. Elles sont codées

par la région E_1 et pourraient jouer un rôle dans la transformation (Wold and Green, 1979).

Au moins deux des protéines précoces subissent une modification post traductionnelle. La E 72 K est phosphorylée in vivo et in vitro (Levinson et al., 1977 ; Jeng et al., 1977 ; Russell and Blair, 1977 ; Axelrod, 1978). Elle comporte 17 résidus phosphates sur un fragment de 25 K (Linné and Philipson, 1979). La E 19 K est glycosylée (Ishibashi and Maizel, 1974a; Persson et al., 1979c).Avant glycosylation elle apparait comme un polypeptide de 14,5 K avec un intermédiaire de glycosylation de 17,5 K ; et elle est codée par la région E_3 (fig 3) (Persson et al., 1979a).

Le rôle des protéines précoces n'est pas connu à l'exception de la E 72 K qui est une "DNA-binding-protein" (DBP). Elle a une affinité pour le DNA simple brin (Van der Vliet and Levine, 1973 ; Van der Vliet et al., 1975). Elle est requise pour la réplication du DNA viral. C'est la protéine thermosensible du mutant H5 ts 125, défectif dans la synthèse du DNA viral (Ensinger and Ginsberg, 1972 ; Van der Vliet et al., 1975). La protéine équivalente pour l'Ad 12 a un poids moléculaire de 60 000, elle est thermosensible pour le mutant H2 ts 275 (Rosenwirth et al., 1975). Elles ont donc un rôle très semblable à la protéine du gène 32 du bactériophage T_4 (Alberts and Frey, 1970).

La protéine de circularisation du DNA (55 K), qui n'est pas détectée dans les extraits de cellules infectées, en gel de polyacrylamide-SDS, est synthétisée au stade précoce (Yamashita et al., 1979). Elle n'a pas de peptide commun avec les protéines précoces E_1 (53 K) E_1 (50 K), E_2 (72 K) (Green et al., 1979b) ni avec la protéine mineure de structure IVa_2 (56 K) (Harter et al., 1979) après digestion à la trypsine.

IV-2-3 Régulation de l'expression des gènes précoces

Certaines protéines précoces (E 18 K, E 11 K) sont enrichies par traitement des cellules infectées à la cycloheximide (Harter et al., 1976). Ce traitement change aussi la quantité de mRNA synthétisé, les RNA de taille 9-11 S sont augmentés (Eggerding and Raskas, 1978).

L'étude cinétique de l'apparition des mRNA précoces montre que

le mRNA 13 S codé par la région E 1 A est synthétisé ayant les autres mRNA précoces, y compris les espèces 22 S et 13 S de la zone E 1 B (Spector et al., 1978). De plus les mutants hr de l'adénovirus 5 du groupe 1, et le mutant de délétion dl 312 (délétions de 1,5 4,5) ne synthétisent pas de mRNA précoces des régions E_1 B, E_2, E_3 et E_4. Donc un ou plusieurs des produits des gènes de la région E_1 A sont nécessaires à la transcription des autres régions précoces du génome viral (Berk et al., 1979 ; Jones and Shenk, 1979 b)

Il a été aussi rapporté que la E 72 K (la DNA-binding-protéine) a un rôle d'autorégulation de la synthèse des mRNA précoces. Dans les cellules infectées par l'ad 5 de type sauvage, la synthèse de mRNA précoces diminue à partir de la 6éme heure, ce qui n'est pas le cas pour les cellules infectées avec le mutant H5 ts 125 (Carter and Blanton, 1978 ; Blanton and Carter, 1979).

IV - 3 Réplication du DNA viral

La réplication du DNA viral a lieu dans le noyau de la cellule (Shiroki et al., 1974 ; Vlak et al., 1975a; Simmons et al., 1974). La réplication débute 6 à 8 h après l'infection le taux maximal de synthèse de DNA viral est atteint à la 16éme heure du cycle lytique, il est très réduit après la 24éme heure. Par hybridation, on a pu montrer que 500 000 équivalents génome sont présents dans la cellule à la fin du cycle lytique (Fanning and Doerfler, 1977).

Plusieurs formes de DNA viral ont été détectées dans les cellules permissives. Pendant la phase précoce , une part du DNA parental, ainsi que le DNA nouvellement synthétisé sédimente entre 50 et 100 S en saccharose alcalin. Ce DNA viral est lié de manière covalente à du DNA cellulaire (Schick et al., 1976). Cette forme de DNA viral est aussi retrouvée dans les cellules infectées avec des mutants défectifs dans la synthèse du DNA viral "mature" (Tyndall et al., 1978 ; Schick and Doerfler, 1979 ; Allart et D'Halluin, résultats non publiés). Cette forme de DNA viral, qui n'est pas précurseur de la forme mature, correspond à 1 000 à 2 000 copies de génome par cellule (Fanning and Doerfler, 1977). A la 16éme heure du cycle lytique, la grande majorité du DNA nouvellement synthétisé sédimente à 31 S en saccharose neutre ou à 34 S en saccharose alcalin. Sans action de protéase, après lyse des cellules avec du sarcosinate de sodium ou du chlorhydrate de guanidine, le DNA sédimente comme le DNA circulaire extrait des virions. Cette forme est sensible aux protéases (Girard et al., 1977). Par des marquages brefs, suivi ou non de chasse, il est possible de distinguer la forme réplicative de la forme "mature" : le DNA en cours de réplication

sédimente plus vite en saccharose neutre et a une densité supérieure en chlorure de césium (Pearson and Hanawalt, 1971 ; Van der Vliet and Sussenbach, 1972 ; Van der Eb, 1973).

Par fractionnement sur BND-cellulose, la forme réplicative contient des zones monocaténaires (Robin et al., 1973), qui sont sensibles à la nucléase S_1, spécifique des acides nucléiques monocaténaires (Pettersson, 1973 ; Robin et al., 1973). Les régions monocaténaires, qui peuvent correspondre à 15-30% du génome viral sont complémentaires de toutes les zones du génome, avec une légère surreprésentation de la partie droite du DNA viral (Flint et al., 1976).

Dans l'état actuel de nos connaissances, il n'est pas établi que la synthèse du DNA soit continue ou discontinue. Certes, en saccharose alcalin des fragments 10-12 S sont mis en évidence après des marquages très brefs (60 s) (Horwitz, 1971 ; Pearson, 1975) ou en présence d'hydroxyurée (Vlak et al., 1975b ; Winnaker, 1975). La taille de ces fragments dépend des concentrations relatives en ribo et déoxyribonucléoside-triphosphate (Winnacker, 1975). Ces fragments de type Okazaki pourraient être le résultat de l'incorporation dans le DNA de deoxyuridine puis de la réparation de l'erreur commise (une N-glycosydase enlèverait la base et une endonucléase type "endonucléase apurinique" provoquerait la coupure de la chaîne et l'excision du reste de la zone lésée). Il a été montré récemment que de l'uracile est incorporée dans le DNA d'adénovirus au cours de la réplication (Ariga and Shimojo, 1979). Un tel mécanisme a déjà été mis en évidence chez les procaryotes en particulier dans des mutants E. Coli SOF ("Small Okazaki-Fragments" (Tye and Lehman, 1977).

Ces résultats et l'absence de RNA amorce ou "primer", sont en faveur d'une synthèse continue du DNA viral. L'hypothèse de la synthèse continue est corroborée, par le fait que l'initiation de la synthèse a lieu aux deux extrémités de la molécule de DNA. Quand on transfère à température permissive des mutants défectifs dans la synthèse du DNA viral et qu'on effectue un marquage bref et une hybridation avec des fragments de DNA obtenus après action d'endonucléases de restriction, la radioactivité hybridée est plus importante aux extrémités du génome (Ariga and Shimojo, 1977 ; Sussenbach and Kuijk, 1978 ; Sussenbach et al., 1979). Cette initiation n'a lieu qu'à l'une des extrémités à la fois, par déplacement de l'autre chaîne parentale, celle-ci étant ensuite répliquée. En microscopie électronique, seules des molécules "Y" sont observées (Bourgaux et al., 1974). Ces résultats sur l'initiation sont confirmés par le fait que lors de marquage de durée inférieure à celle du cycle de réplication, la radioactivité est plus importante aux sites de terminaison. Avec cette méthode, deux sites de terminaisons sont mis en évidence, l'un

complémentaire du brin l à l'extrémité gauche, l'autre complémentaire du brin r à l'extrémité droite (Tolun and Pettersson, 1975 ; Schilling et al., 1975 ; Bourgaux et al., 1976 ; Sussenbach and Kuijk, 1977 ; Weingartner et al., 1976)

- initiation de la réplication

Les DNA polymérases ne peuvent initier la synthèse du DNA que s'il existe un 3'-OH libre. Aucun RNA "primer" n'ayant été mis en évidence, l'initiation de la synthèse fait appel à un autre mécanisme. Il n'existe pas non plus comme pour certains bactériophages de concaténaires avec un site spécifique de clivage. De plus, récemment, il a été prouvé que l'initiation de la synthèse du DNA ne fait pas appel à un mécanisme dit d'épingle à cheveux (" hairpin ") (Stillman et al., 1977). Un modèle proposé par Rekosh et al. (1977) fait intervenir la protéine terminale pour l'initiation de la synthèse du DNA (Fig 6). La protéine 55 K est liée de manière covalente à l'extrémité 5'P de chaque brin du DNA parental, par l'intermédiaire d'un résidu deoxycytidine (dCMP) qui offre donc un 3'-OH libre nécessaire à l'initiation.

Ce modèle est confirmé par quelques résultats supplémentaires :

la protéine terminale est toujours retrouvée à l'extrémité de chaînes de DNA nouvellement synthétisées, quelque soit la longueur de ces chaînes (Robinson et al., 1979 ; Stillman and Bellett, 1979). Cette protéine permettrait même l'initiation de la réplication dans un système in vitro (Challberg and Kelly, 1979). Un même modèle a été proposé pour la réplication du DNA du phage ϕ 29 (Ito et al., 1978).

- enzymes et protéines impliqués dans la réplication

Trois protéines virales, au moins, sont impliquées dans la réplication. Seule l'une d'elles est connue : la E 72 K, une DNA-binding Protein (D B P). Elle est indispensable à l'initiation de la synthèse (Van der Vliet et al., 1975) et à l'élongation des chaînes de DNA (Van der Vliet and Sussenbach, 1975 ; Van der Vliet et al., 1977 ; Sussenbach et al., 1979).

L'étude des DNA polymérases impliquées dans la réplication est faite en isolant un complexe capable de synthétiser du DNA in vitro (Brison et al., 1977 ; Kaplan et al., 1977 ; Arens et al., 1977) et par l'action d'inhibiteurs, tel le dd TTP ou l'aphidicoline, spécifiques de certaines DNA polymérases soit in vitro, soit in vivo (Longiaru et al., 1979 ; Van der Vliet and Kwant, 1978 ; Krokan et

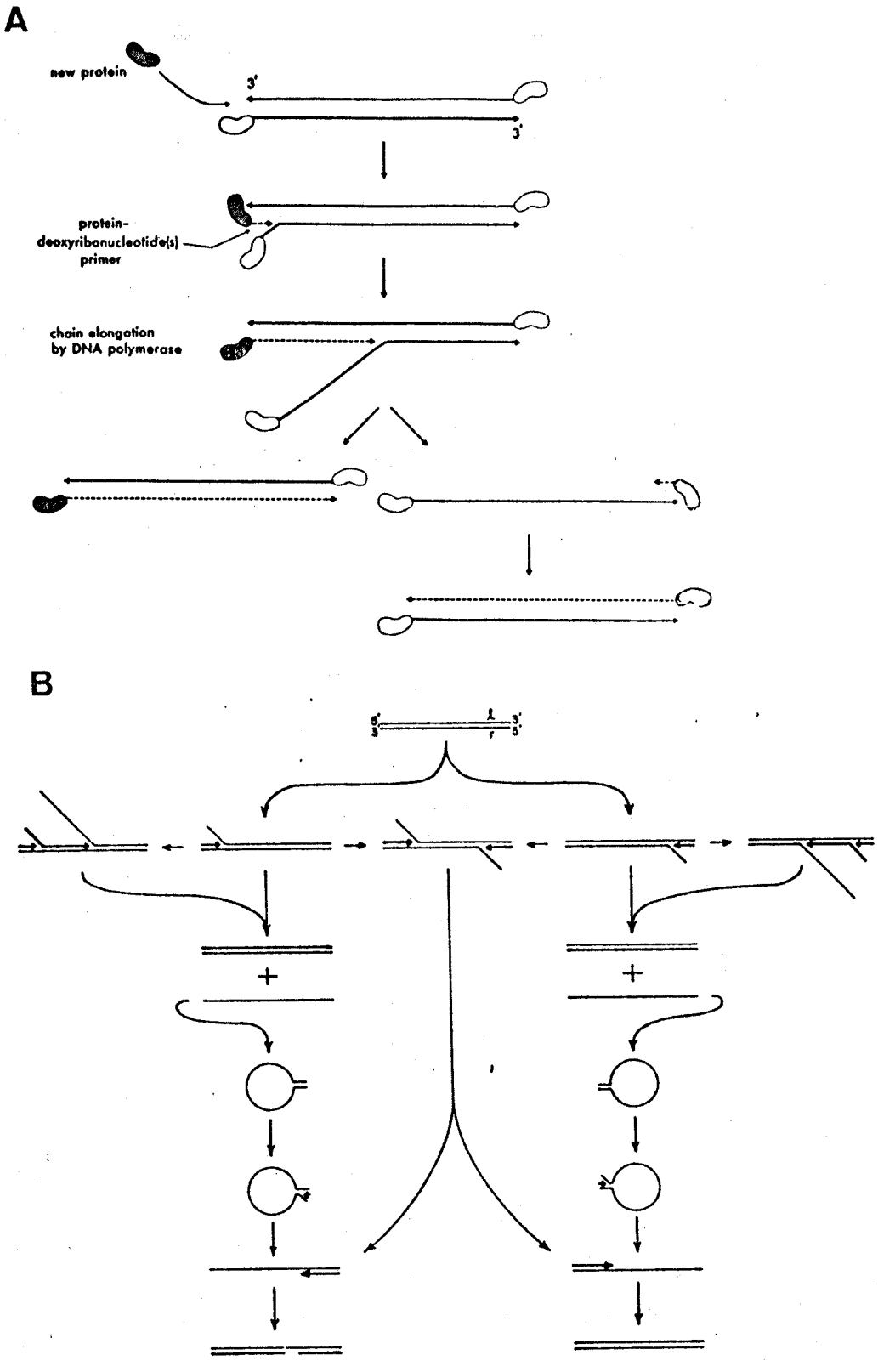

Fig.6 Réplication du DNA viral.

A) Initiation de la synthése. Ce modèle a été proposé par REKOSH et al(I977).

B) Synthése par déplacement de brin suivie de la synthése du brin déplacé. (LECHNER and KELLY,I977)

,al., 1979). Ces DNA polymérases sont d'origine cellulaire. Il semble que la DNA polymérase γ soit responsable de la réplication par déplacement de l'une des chaînes et que la DNA polymérase α réplique la chaîne de DNA déplacée (Longiaru et al., 1979 ; Krokan et al., 1979).

IV-4 Expression des gènes tardifs

La transcription puis la traduction des gènes tardifs débutent après le début de la réplication de DNA viral. La plupart des protéines tardives sont codées par le brin r à partir d'un promoteur unique situé à 16,6. Seules 2 protéines sont codées par la partie gauche du génome par rapport à ce promoteur ; la protéine IX par le brin r et la protéine IVa_2 par le brin l (voir fig. 3).

IV-4-1 Transcription des mRNA tardifs

Durant la phase tardive 40% des RNA totaux nucléaires néosynthétisés sont d'origine virale. Ils saturent 85% du brin r et 10 à 15% du brin l, alors que les mRNA saturent 65 à 100% du brin r et 25% du brin l (Pettersson and Philipson, 1974). A ce stade du cycle lytique la synthèse des rRNA cellulaires est fortement réduite (10 à 20%) (Raskas et al., 1970). Des hn RNA cellulaires sont retrouvés dans le noyau ($\simeq$ 40%) mais pas de mRNA cellulaire dans le cytoplasme, bien que la polyadénylation soit normale (Beltz and Flint, 1979).

Les précurseurs des mRNA tardifs sont transcrits par la RNA polymérase II comme le montre la sensibilité de cette transcription à l'α-amanitine dans les noyaux de cellules infectées (Price and Penman, 1972a). Des précurseurs des mRNA ont pu être mis en évidence. Ces RNA peuvent avoir une constante de sédimentation supérieure à 45 S (Parsons et al., 1971 ; Mc Guire et al., 1972). Par des marquages très brefs, sur les cellules entières ou avec des noyaux de cellules infectées des chaînes de 1 000 à 25 000 nucléotides sont marquées (Bachenheimer and Darnell, 1975). Les précurseurs les plus longs (80% du génome) s'hybrident préférentiellement avec l'extrémité droite du génome. Par blocage de l'élongation (irradiation au U.V, ou addition de D.R.B.) un seul promoteur pour le long transcrit est mis en évidence ; il est situé à 16,3 (Evans et al., 1977 ; Goldberg et al., 1977).

Les hn RNA subissent trois modifications avant d'être transportés au cytoplasme sous forme de mRNA. Une séquence polyadénylée (150 à 200 résidus d'acide adénylique) est additionnée à l'extrémité 3'-OH (Philipson et al., 1971).

L'extrémité 5' est coiffée ("cap" ou coiffe), ce qui la rend résistante aux exo-ribonucléases. Cette extrémité 5' est pour 80% des mRNA

$^{m7}G(5')^{m6}ApN^{m}p.N'$ et pour les 20% restant, $^{m7}G(5')A^{m}pN^{m}pN'$ (Mc Guire et al., 1976 ; Moss and Koczot, 1976 ; Sommer et al., 1976).

La troisième modification, a été mise en évidence par l'observation des hybrides entre un mRNA et la chaine r du DNA : trois boucles de DNA sont visualisées. Les zones hybridées correspondent aux coordonnées 16,6 - 19, 6 26,6 et la zone codante du mRNA. Ces trois premières séquences, dites "de tête", font respectivement 20 à 50, 80 et 110 nucleotides (Chow et al., 1977). Les messagers codant pour la fibre possèdent, au moins, une quatrième séquence de tête situé à la position 78,6 - 79,1 (Chow and Broker, 1978). Les RNA qui proviennent d'un long transcrit sont donc "épissés". Cette modification a été décrite d'abord pour les mRNA tardifs de l'adénovirus avant de l'être pour la plupart des mRNA des cellules eucaryotes.

Le promoteur des mRNA tardifs, à l'exception de ceux codant pour le IX et le IVa_2 est identique et situé à 16,6. Un oligonucléotide (11 nucléotides) qui contient la coiffe a été sélectionné par chromatographie sur dihydroxyboryl cellulose après digestion des mRNA à la ribonucléase T_1 (Gelinas and Roberts, 1977).

Les 2 mRNA qui codent pour les protéines IX et IVa_2, possèdent aussi une séquence "de tête" ne faisant pas partie de la zone codante.

IV-4-2 Les VA- RNA

Les RNA viraux associés sont des RNA de petite taille5,2 et 5,5 S. Lorsque la synthèse du DNA démarre, la synthèse du VA-RNA 5,2 S diminue alors que celle du VA-RNA 5,5 S augmente. Le VA-RNA 5,5 S est formé d'une chaine de 156 nucléotides dont la séquence a été déterminée (Ohe and Weissman, 1970 ; 1971). Ces deux VA-RNA sont dépourvus de séquence polyadénylée. Ils sont synthétisés par la RNA polymérase III (Price and Pennman, 1972 b). Le rôle de ces RNA reste inconnu. De plus il existe 2 types de VA-RNA 5,5 S, qui différent par 3 bases à l'extrémité 5', l'un débute par A et l'autre par G. L'un de ces deux RNA n'est pas utile au cycle infectieux sur cellule HeLa, puisque le mutant dl 309, qui n'a que la forme commencant par G, est viable (Thimmappaya et al., 1979)

IV-4-3 Synthèses des protéines tardives

Les mRNA matures après transport au cytoplasme sont traduits en protéines virales. Les séquences "de tête" pourraient être reconnues par la petite sous-unité ribosomale (Ziff and Evans, 1978).

Un grand nombre de polypeptides sont synthétisés au stade tardif. En plus des polypeptides de structure il a été mis en évidence une protéine

non structutale majeure la 100 K (masse moléculaire : 100 000).

La plupart des polypeptides de structure , Hexon, fibre, pVI, pVII sont N-acétylés(Jörnvall et al., 1974 ; Sung et al., 1977 ; Boulanger et al., 1978).

Quelques uns de ces polypeptides subissent des modifications post-traductionnelles.

a) la fibre (IV) est glycosylée (Ishibashi and Maizel , 1974a
b) la protéine IIIa, la 100 K, sont phosphorylées (Axelrod, 1978 ; Russell and Blair, 1977).
c) les protéines VI, VII, VIII proviennent de précurseurs par clivages endopeptidiques (Anderson et al., 1973). Ce clivage se produit lors de la maturation du virus (voir l'assemblage). La transformation du pVII en VII se fait par perte d'un fragment de l'extrémité N-terminale (Rekosh and Russell, 1977 ; Sung et al., 1977)

Les polypeptides de l'hexon s'associent en trimère, ceux de la fibre aussi, ceux du penton en pentamère (Boudin et al., 1978). Ils sont alors antigéniquement actifs. La 100 K semble jouer un rôle important dans la trimérisation de l'hexon. En effet les mutants thermosensibles qui sont modifiés sur la 100 K ne forment pas d'hexon antigénique (Carstens and Weber, 1977 ; Mautner et al., 1975 ; Leibovitz and Horwitz 1975).

IV-5 Morphogénèse des virions

La morphogénèse des particules virales est la construction de la particule infectieuse à partir de ces éléments constitutifs, protéines de structures, DNA. Le processus d'assemblage peut être étudié de multiples façons :

"in vivo"
- la formation de particules défectives et leur relation temporelle avec les particules infectieuses
- l'évolution des structures au cours d'une chasse après un marquage bref
- l'étude des particules produites par les mutants de la classe III, et l'évolution de ces particules après passage à la température permissive

"in vitro"
- étude des interactions protéine -protéine et protéine DNA
- reconstitution de groupes de neuf hexons et de capside
- encapsidation du DNA

IV-5-1 Les particules défectives

Lors de la purification des adénovirus en gradient de densité (CsCl), le virus s'équilibre à une densité de 1,34. Mais des particules de densité plus faible sont visibles dans le gradient. Ces particules sont défectives, et leur quantité varie d'un sérotype à l'autre.

Ce sont les sérotypes du sous-groupe II (3, 7, 16) qui en synthétisent le plus 20 à 30% de particules défectives par rapport à l'ensemble des particules formées (Daniell, 1976 ; Wadell et al., 1973 ; Tibbetts, 1977 ; Prage et al., 1972).

Leur composition en polypeptides et en DNA a été étudiée. Ces particules ne contiennent pas les deux polypeptides associés au DNA (V et VII) ni les polypeptides VI et VIII mais leurs précurseurs pVI et pVIII. Ces résultats ont été obtenus pour l'Ad 2 par Rosenwith et al., 1974 ; l'Ad 3 : Prage et al., 1972 ; l'Ad 7 : Tibbetts, 1977 ; l'Ad 12 par Burlingham et al., 1974 ; l'Ad 16 par Winberg and Wadell, 1977.

Cependant les résultats divergent en ce qui concerne le contenu en DNA de ces particules. Pour les particules de l'adénovirus 3 (Daniell, 1976) et de l'Ad 7 (Tibbetts, 1977), ces auteurs ont montré que l'extrémité gauche du génome est toujours présente. Le fragment de DNA s'agrandit avec l'alourdissement de la particule.

Il a été aussi rapporté, notamment pour l'Ad 2 et l'Ad 16 que ces particules défectives contiennent un fragment de DNA viral lié de manière covalente à du DNA d'origine cellulaire et dans ce cas le fragment de DNA viral est un morceau de n'importe quelle partie du génome (Tjia et al., 1977 ; Hammarskjöld et al., 1977).

Un troisième résultat a été obtenu avec les particules incomplètes de l'adénovirus 12. Le génome ainsi encapsidé présente une délétion de 4,5 à 8,8%, délétion qui se situe toujours dans la partie gauche du génome, à environ 8% de l'extrémité gauche (Mak et al., 1979).

Les particules défectives sont assemblées sensiblement en même temps que les particules infectieuses, mais peut-être un peu plus précocement (Rosenwirth et al., 1974 ; Sundquist et al., 1973).

Au moins une partie de ces particules incomplètes sont capables de se transformer en virions matures, comme l'ont montré

Sundquist et al (1973) par un marquage bref suivi d'une cinétique d'apparition de la radioactivité dans les différentes classes de particules.

IV-5-2 Cinétique d'apparition des différentes classes de particules

En utilisant ce même procédé Ishibashi et Maizel (1974b) ont mis en évidence une étape importante dans la formation des virus infectieux :le stade "jeunes virions". Ces particules contiennent du DNA, et les précurseurs des polypeptides VI, VII et VIII (pVI, pVII et pVIII). Ces particules s'équilibrent à une densité de 1,34 en gradient de chlorure de césium, comme les virus matures.

Mais les techniques utilisées : congélation + décongélation, extraction au Fréon, centrifugation en CsCl, si elles laissent intacts les virions matures, peuvent altérer des structures labiles. Aussi en utilisant une technique de purification plus douce ; la centrifugation en gradient de Ficoll, Edwardsson et al (1976) mettent en évidence une population d'intermédiaires d'assemblage qui sédimente à 550 S, au lieu de 750 S pour le virus infectieux. Cette population contient moins de DNA que celle qui sédimente à 750 S. Les particules qui sédimente à 550 S possèdent tous les polypeptides présents dans les jeunes virions mais aussi deux protéines non-structurales de masse moléculaire 50 K et 40 K. Après fixation des structures labiles au glutaraldehyde, 3 populations de particules de densité 1,30-1,33-1,39 sont obtenues en CsCl. Ces auteurs suggèrent que les particules de densité 1,39 en CsCl sont des "nucléoides".Les nucléoides ne sont pas mis en évidence quand on utilise une autre technique douce : la sédimentation à l'équilibre de densité en gradient de métrizamide d'un lysat cellulaire. Par cette méthode, les polypeptides pVII ou le VII, et V ne sont pas retrouvés associés à du DNA en dehors de la particule. Le DNA n'est présent qu'à l'état de complexe qu'avec la protéine terminale, ou encapsidé en virus. Ce résultat suggère que le DNA pénétre nu dans la capside, en même temps ou avant les protéines du nucléoide (Everitt et al., 1978)

Presques toutes les particules défectives décrites (Ad 3, Ad 16, Ad 7) renferment l'extrémité gauche du génome : une séquence nucléotidique de cette extrémité pourrait être reconnue pour l'encapsidation du DNA. De plus un mutant d'Ad 16 qui possède une séquence de 450 nculéotides de l'extrémité gauche du DNA de type sauvage à ses deux extrémités , produit des particules défectives ayant alternativement chacune des extrémités (Hammarskjöld, 1979).

IV-5-3 Mutant défectif dans la maturation de la particule virale

Le premier mutant ainsi étudié fut l'H2 ts 1 (Weber, 1976). Ce mutant accumule à 39,5°C des particules virales de densité 1,34 en chlorure de césium. Elles contiennent un DNA intact mais les précurseurs pVI, pVIII et pVII ne sont pas clivés. Ce sont donc des jeunes virions. Ce blocage confirme les résultats d'Ishibashi et Maizel (1974b)et apporte la preuve que les précurseurs des polypeptides VI, VII et VIII sont clivées uniquement lors de la maturation jeunes-virions ⟶ virions matures. Les particules virales de H2 ts 1 produites à 39°C ne sont pas infectieuses. Les "cores" de ces particules extraits avec du sarcosinate de sodium perdent la plus grande partie des polypeptides (V et pVII (ou VII), contrairement au virus mature (WT) (Mirza and Weber, 1977). La mutation peut être localisée dans le gène de l'hexon ou celui de la 100 K (Hassell and Weber, 1978).

Un autre mutant isolé dans le même laboratoire H2 ts 4, forme des capsides sans DNA à température non permissive. Ces particules ne contiennent pas les polypeptides VII ni pVII et ni V (Khittoo and Weber, 1977). Les polypeptides qui peuvent être mutés sont le IIIa, le V ou le III (Hassell and Weber, 1978).

En utilisant la technique de centrifugation en Ficcoll Edvardsson et al. (1978) ont étudié l'assemblage de mutants d'ad 5 (ts 5, 19, 58 et 24). Le ts 24 n'accumule aucune particule. Le ts 58 qui est muté dans le IIIa (et qui complémente l'H2 ts 4) forme des particules qui ressemblent à celles de l'Ad 2 ts 4. Les mutants H5 ts 5 et 19 défectifs dans la synthèse de fibre produisent une petite quantité de virus mature . Le H5 ts 19 accumule un peu de jeunes virions à température non permissive. Pour d'autres auteurs, le H5 ts 19 ainsi que le H5 ts 18 (tous les deux défectifs dans la production d'interféron) ne produisent pas de particule (Tarodi et al., 1979).

IV-5-4 Enzymes impliqués dans la maturation

Au stade tardif de l'infection, une endopeptidase spécifique du clivage pVII ⟶ VII a été mise en évidence dans les extraits de cellules infectées (Bhatti and Weber, 1978). Elle est capable de cliver le polypeptide pVII in vitro.

Elle est inactive ou inexistante dans tous les extraits de cellules infectées par des mutants thermosensibles et elle serait sous le contrôle d'un gène qui intervient dans l'étape ultime de la

maturation, passage du stade jeunes virions ⟶ virions matures (Bhatti and Weber, 1979). Cet enzyme peut être une protéase d'origine cellulaire mais viro-modifiée.

IV-5-5 Schéma d'assemblage

Une capside se forme par association des protéines de structure , peut-être autour d'un noyau constitué par une protéine d'échaffaudage ("scaffolding protein"). Le DNA, soit sous forme de nucléoide, soit seul est ensuite encapsidé. C'est alors le stade jeune virion. La particule devient infectieuse après le clivage des précurseurs des polypeptides VI, VIII et VII. Ce schéma est représenté fig 7 (Philipson, IV Congrès International de Virologie, La Haye, 1978).

V Cycle abortif et transformation cellulaire maligne

Dans les cellules en culture de même origine que l'hôte naturel, les adénovirus se multiplient en lysant la cellule pour être libérés dans le milieu (cycle lytique ou productif). Dans d'autres types de cellules ils peuvent induire un cycle abortif, sans production de progéniture virale, ou ils peuvent provoquer une transformation maligne. Ainsi les adénovirus humains ont un cycle abortif sur cellules de singe (AGMK, CV_1), et transforment in vitro les cellules embryonnaires de rat.

V-1 Cycle abortif

Le système le plus étudié est l'Ad 2 ou 5 et les cellules de singe. Lors de l'infection de cellules CV_1, par l'adénovirus 2, il y a synthèse des protéines précoces, du DNA viral et des protéines de structure. Mais la quantité de fibre synthétisée est beaucoup plus faible que dans le cycle lytique (Friedman et al., 1970 ; Baun et al., 1972 ; Klessig and Anderson, 1975).

En coinfection avec le SV_{40}, la production virale devient identique à celle obtenue sur des cellules permissives (O'Conor et al., 1963 ; Rabson et al., 1964). Les hybrides Ad2 SV_{40} (Ad 2 ND_1-) qui contiennent différentes séquences du SV_{40} et une délétion de l'Ad2 (fig 2) se multiplient efficacement sur CV_1 (Lewis et al., 1969). Le clone Ad 2 ND_1 synthétise une protéine de masse moléculaire 30 000 qui permet la multiplication sur cellules de singe (Lopez-Revilla and Walter, 1973 ; Grotzicker et al., 1974). Elle permet aussi la multiplication de l'Ad 5 dans les coinfections. Récemment,

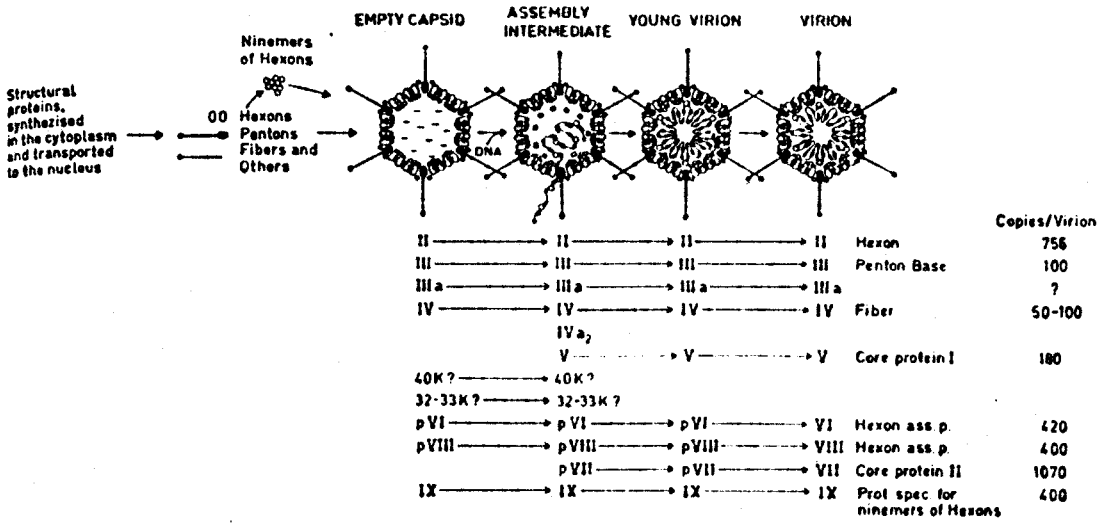

Fig 7 - Schéma d'assemblage de l'adénovirus

Ce schéma a été proposé par Philipson, au 4éme Congrès International de Virologie, La Haye, Hollande, 1978

Klessig (1977) a isolé un mutant ponctuel de l'adénovirus 2 qui se multiplie efficacement sur cellules de singe ; puis 4 autres (H2 hr 400 — 404 et H5 hr 405). La mutation est localisée entre les coordonnées 62,9-65,6, c'est à dire dans le gène codant pour la "DNA binding protein" (E 72 K) (Klessig and Grodzicker, 1979). Ces mutants hr 400-404 permettent la multiplication de l'Ad 5 ; ils agissent donc "en trans", par l'intermédiaire d'un produit diffusible : leur activité porterait sur la maturation des hnRNA en mRNA. L'Ad 2 de type sauvage produit dans les cellules de singe des mRNA codant pour le polypeptide de la fibre anormaux dans leur épissage, ce qui n'est plus le cas avec les mutants hr 400 - 404 (Klessig et al., 1979).

L'adénovirus induit un cycle abortif de même type dans des cellules de lapin en lignée continue (sirc : cellule de cornée de lapin) (Devaux et D'Halluin, résultats non publiés).

Dans les cellules de hamster (BHK 21), l'Ad 2 et l'Ad 5 se multiplient faiblement, mais l'adénovirus 12 ne s'y multiplie pas et transforme même ces cellules (Doerfler, 1968, 1969, 1970 ; Stroshl et al., 1970).

V-2 Transformation maligne cellulaire

Certains adénovirus provoquent des tumeurs chez le hamster nouveau né à haute fréquence pour les sérotypes 12, 18, 31 à faible fréquence pour les sérotypes 3, 7, 16. Enfin les sérotypes 1, 2, 4, 5, 6 ne provoquent pas de tumeurs chez cet animal (Trentin et al., 1962 ; Huebner et al., 1962). Mais tous les sérotypes sont capables de transformer des cellules de rat embryonnaire "in vitro" (Freeman et al., 1967). Les sérotypes 2 et 5 ne transforment pas, même in vitro, des cellules de hamsters ; au contraire ils s'y multiplient. Il semble donc que la transformation n'est possible que s'il n'y a pas multiplication. Ceci est confirmé par le fait suivant : des cellules de hamster peuvent être transformées par des mutants thermosensibles d'Ad 5 (Williams, 1973) et des cellules de rat par du DNA fragmenté d'Ad 5 (Graham and Van der Eb, 1973b).

Le fragment G (0-7,5) de DNA d'Ad 5 produit par l'endonucléase de restriction Hind III est suffisant pour l'initiation et le maintien de la transformation (Graham et al., 1974).

Les cellules transformées synthétisent l'antigène T révélé par un immunsérum provenant de l'animal porteur de la tumeur (Huebner et al., 1964). En utilisant divers clones de cellules de rat transformées, Green et coll. ont préparé des immunsérums. Ces immunsérums peuvent précipiter certaines protéines précoces du cycle lytique. Deux de ces protéines E 15 K et E 53 K pourraient donc jouer un rôle dans le maintien de la transformation et la synthèse d'antigène T (Wold and Green, 1979).

Les mutants H5 dl 312, dl 313, et sub 315 (délétions entre 1,5 et 10,5 ne transforment pas les cellules embryonnaires de rat (Jones and Shenk, 1979a). De même les mutants ponctuels H5 hr (2 à 7) sont incapables de transformer les cellules embryonnaires de rat. Le mutant H5 hr 1 est capable d'initier cette transformation, mais incapable de maintenir l'état transformé (Graham et al., 1978).

RESULTATS - DISCUSSION

I Isolement et caractérisation de mutants thermosensibles

I-1 Isolement de mutants thermosensibles

Par traitement, à l'acide nitreux, d'un stock d'adénovirus de type 2, préalablement cloné à 39,5°C, Martin G.R. et Cousin C. ont isolé 400 clones à 33°C. Pour chaque clone, l'efficacité de formation de plages a été mesurée aux deux températures 33°C (permissive) et 39°C (restrictive). Les clones ayant au moins un rapport du nombre de plages de 10^2 entre les deux températures ont été reclonés, deux fois.

Ces deux auteurs ont ainsi obtenu 39 mutants de l'adénovirus 2. La fréquence d'obtention de mutant est donc de 10%, sensiblement la même que celle obtenue pour l'adénovirus de type 5 par Williams et al., (1971). Les caractéristiques phénotypiques d'une trentaine de ces mutants ont été déterminées.

I-2 Phénotype

I-2-1 Synthèse du DNA viral

Le cycle lytique est ralenti à 33°C : il faut 2,5 à 3 fois plus de temps, pour obtenir la même production virale qu'à 37°C ou à 39,5°C (Ensinger and Ginsberg, 1972). A 39,5°C, comme à 37°C la synthèse du DNA est maximale entre 16 et 18 h, à 33°C cette synthèse est maximale vers la $40^{éme}$ h.

Nous avons mesuré la synthèse du DNA viral dans les cellules KB infectées par le type sauvage et de chacun des mutants, aux deux températures 33° et 39,5°C. L'analyse du DNA se fait par sédimentation en gradient de saccharose alcalin. Le DNA viral est extrait à la période de synthèse maximale : 40-$48^{éme}$ heure du cycle à 33°C, 16-$24^{éme}$ heure à 39,5°C. Trois mutants apparaissent ainsi défectifs dans la synthèse de DNA viral H2 ts 105, H2 ts 111 et H2 ts 114.

I-2-2 Synthèse des antigènes "solubles"

Nous appellons antigènes "solubles" les composants majeurs de la capside non incorporés dans les virions. La présence de ces antigènes est mise en évidence par immunoélectrophorèse bidimensionnelle (Laurell, 1965).

Les mutants peuvent être classés dans l'une des trois catégories

suivantes :

1) absence de tous les antigènes majeurs de structure, pour les mutants défectifs dans la synthèse du DNA viral

2) absence ou diminution d'un ou plusieurs antigènes

3) présence en quantité normale de tous les antigènes (H2 ts 107)

Les mutants de la classe 2 peuvent se diviser en 4 sous-classes :

a) défectifs dans la synthèse de l'hexon (H2 ts 118, H2 ts 121)

b) défectifs dans la synthèse de la fibre (H2 ts 115, H2 ts 125)

c) défectifs dans la synthèse de la fibre et de la base du penton (H2 ts 103, H2 ts 104, H2 ts 136)

d) défectifs dans la synthèse de la protéine IIIa (H2 ts 101, H2 ts 112)

I-2-3 Assemblage en particules virales

L'assemblage des particules virales, à température non-permissive, a été étudié en microscopie électronique sur des coupes cellulaires. Beaucoup des mutants, ainsi étudiés, forment des particules, mais ells sont, pour la plupart, de morphologie anormale. C'est le cas notamment pour les mutants H2 ts 104 et H2 ts 112. Les cellules infectées par le mutant H2 ts 118, accumulent du matériel dense à la périphérie du noyau comme pour le mutant défectif dans le transport de l'hexon H5 ts 147 (Kauffman and Ginsberg, 1976). Mais contrairement à ce mutant les cellules infectées par le H2 ts 118 ne produisent pas d'hexon antigéniquement actif.

I-3 Groupes de complémentation

Nous pouvons distinguer 13 ou 14 groupes de complémentation. Le tableau VII donne pour chaque groupe de complémentation, les mutants correspondants, la classe phénotypique et quelques cas de mutants d'autre laboratoire ayant des propriétés semblables.

Deux de nos mutants, défectifs dans la synthèse du DNA, ne complémentent pas l'H5 ts 36 ; ils sont complémentés tous les trois par le mutant H5 ts 125 ; le mutant H2 ts 111 représente donc un unique groupe de complémentation ; ses propriétés (voir ci-dessous) sont très différentes de l'H2 ts 206, autre

Tableau VII

Caractéristiques de mutants thermosensibles : complémentation et phénotype

Groupe de complémentation	Mutants	Classe (a)	Mutant même groupe (b)	Mutant ayant les même propriétés
D	105-114	I	H 5 ts 36	
G	111	I		
A	121	II Hex$^-$		
B	102-110-117	II Hex$^-$		
	122			
H	118	II Hex$^-$		
K	106-108-119	II Hex$^-$		
E	115	II Fibre$^-$	H 5 ts 22	
L	116-125	II Fibre$^-$		
C	104	II bP et F$^-$		H 2 ts 1
F	103	II bP et F$^-$		
M	136	II bP et F$^-$		
N	101	II IIIa$^-$		
I	112	II IIIa$^-$	H 2 ts 4	
J	107	III		

b Mutant isolé dans un autre laboratoire appartenant au même groupe de complémentation

H5 ts 36, H5 ts 22 isolé par Williams et al., 1971
H2 ts 1, H2 ts 4 isolé par Begin and Weber, 1975

a Classe phénotypique :

I Mutant défectif dans la synthèse du DNA viral
II Mutant défectif dans la synthèse d'un antigène de structure
Hex$^-$ Défectif dans la synthèse de l'hexon
Fibre$^-$ Défectif dans la synthèse de la fibre
bP et Fibre$^-$ Défectif dans la synthèse de la base du penton et de la fibre
IIIa$^-$ Défectif dans la synthèse de la protéine IIIa
III Mutant défectif dans l'assemblage, bien que les protéines de structures soient synthétisées

mutant défectif dans la synthèse du DNA (Kathmann et al., 1976). Il semble donc qu'il y ait au moins quatre fonctions précoces qui participent avec les enzymes cellulaires (DNA polymérase α et γ, etc) à la réplication du DNA viral.

Les mutants défectifs dans la synthèse de l'hexon appartiennent à 4 groupes.

Nous trouvons 2 groupes défectifs dans la synthèse de la fibre ; Williams et al., (1974) en trouvent 3 ayant ce phénotype.

Trois groupes de complémentation ont le phénotype : défectif dans la synthèse de la base du penton et de la fibre.

Les mutants défectifs dans la synthèse de la protéine IIIa appartiennent à deux groupes, auxquels il faut adjoindre un groupe représenté par le mutant H5 ts 58. L'H2 ts 112 et l'H2 ts 4 sont dans le même groupe.

II Mutants défectifs dans la synthèse du DNA viral

A température restrictive, ces mutants ne synthétisent pas de DNA viral sous la forme qui est encapsidable. Ils inhibent cependant la synthèse du DNA cellulaire, bien que cette inhibition soit plus faible dans les cellules infectées par les mutants H2 ts 105 et H2 ts 114.

L'inhibition de la synthèse du DNA cellulaire n'est donc pas liée à la réplication du DNA viral, ni à la synthèse des protéines tardives. Cependant, des séquences virales sont intégrées au génome cellulaire et sont corépliquées avec le DNA cellulaire au cours de la phase précoce de l'infection (résultats non exposés). Les mêmes résultats ont été trouvés par Schick and Doerfler (1979), Tyndal et al., (1978).

II-1 Synthèse de DNA après passage à la température permissive

A la 16ème heure du cycle lytique, après passage à la température permissive la synthèse du DNA viral est restaurée immédiatement. Cette synthèse a lieu même en présence d'un inhibiteur de la synthèse protéique (la cycloheximide par exemple). Les protéines nécessaires à la réplication sont donc présentes, mais non fonctionnelles à 39,5°C. Notons que la faible synthèse du DNA cellulaire dans les cellules infectées par les mutants H2 ts 105 et H2 ts 114 est totalement inhibée après passage à 33°C.

II-2 Arrêt de la synthèse du DNA viral après passage à la température restrictive

Les résultats diffèrent pour les deux groupes de complémentation. L'arrêt de la synthèse du DNA viral est immédiat pour le mutant H2 ts 111, bien qu'il semble y avoir un peu d'élongation des chaînes initiées à 33°C ; mais il n'y a pas de nouvelle initiation de chaînes à 39,5°C. Au contraire, dans le cas des mutants H2 ts 105 et H2 ts 114, la synthèse du DNA viral se ralentit progressivement : 60% d'inhibition après 2 h à 39,5°C, et par marquage en présence de BUdR à température non permissive, des molécules HH (lourdes-lourdes) sont obtenues. L'initiation se ralentit, mais reste possible pendant au moins un cycle de réplication du DNA.

Il s'agit de résultats préliminaires, concernant les mutants H2 ts 105 et H2 ts 114 ; le reste de cette étude ne concerne que le mutant H2 ts 111.

II-3 Devenir du DNA viral synthétisé à 33°C après transfert à 39,5°C

Le DNA viral, synthétisé à 33°C, sédimente en un pic hétérogène après passage à 39,5°C. A température restrictive, la taille des fragments diminue avec le temps d'incubation. Une étude cinétique du comportement de ces fragments de DNA en gradient de saccharose neutre et alcalin, montre que les coupures sont d'abord monocaténaires avant de devenir bicaténaires.

Cette dégradation du DNA viral n'est pas liée à la réplication. Le blocage de la synthèse du DNA par l'hydroxyurée ou l'ara C ne diminue pas cette fragmentation. Ce phénomène ne peut pas être lié a un mécanisme de réparation du DNA, car la fragmentation est indépendante de la radioactivité spécifique du DNA, ainsi que de l'isotope radioactif utilisé, ^{3}H ou ^{14}C.

Le DNA viral n'est pas protégé par une préincubation de 16 heures à 33°C avant le passage à 39,5°C, sauf pour une très faible fraction de DNA qui est vraisemblablement encapsidée

II-4 Devenir du DNA parental

A 39,5°C, le DNA parental du mutant H2 ts 111 est faiblement dégradé. Par contre, si les cellules sont incubées à 33°C avant d'être transférées à 39,5°C, le DNA parental est totalement fragmenté. Cette dégradation intervient de même manière si les cellules sont maintenues artificiellement en phase

précoce (par addition d'hydroxyurée). Ceci suggère un changement important dans la structure du DNA viral au cours de la phase précoce (peut être dans sa structure en nucléosomes), ainsi que l'existence d'une fonction précoce, nécessaire à la protection du DNA contre les endonucléases cellulaires dont certaines sont "viro-stimulées" (Burlingham and Doerfler, 1972).

II-5 Devenir du DNA cellulaire après infection avec le mutant H2 ts 111

Le DNA cellulaire préalablement marqué à la thymidine ^{14}C, subit une dégradation à 39,5°C après infection des cellules avec le mutant H2 ts 111. Cette fragmentation est fortement stimulée si les cellules infectées sont cultivées à 33°C avant leur passage à 39,5°C. Dans ces conditions 70 à 85% du DNA cellulaire a une constante de sédimentation inférieure à 30S en saccharose alcalin. Cette dégradation est aussi fonction de la multiplicité d'infection sans passage préalable à 33°C.

II-6 La mutation du H2 ts 111 n'est pas dominante

Ce mutant est complémenté très efficacement par un grand nombre de mutants. De plus, la dégradation du DNA de la cellule hôte est totalement inhibée par la co-infection avec un autre mutant (H2 ts 114). Ce n'est donc pas une mutation dominante.

II-7 La liaison DNA-55 K est altérée juste après passage à 39,5°C

En 1978, Coombs et Pearson, ont montré que le DNA viral, lié de manière covalente avec la protéine 55 K est retenu spécifiquement sur filtre de fibre de verre à pH 7,4 0,3M NaCl. Cette fixation dépend de la conformation de la protéine. Le DNA viral de cellules infectées par le mutant H2 ts 111 après une période brève à 39,5°C, a été isolé en gradient de saccharose (5-20%) en présence de chlorhydrate de guanidine (4M). Le DNA, qui sédimente à 31 S, après dialyse, n'est pas retenu sur filtre alors que le DNA viral des cellules infectées maintenues à 33°C, l'est. Le complexe DNA-55 K pourrait donc subir une modification importante à 39,5°C : coupure de la liaison 55 K-DNA par exemple, ou protéolyse partielle de la protéine ou encore une modification conformationelle de ce complexe.

II-8 Nature de la progéniture de la complémentation H2 ts 111 x H5 ts 36

A partir de la production à 39,5°C de la coinfection H2 ts 111, H5

ts 36, 40 clones ont été isolés à 33°C. Après amplification, les polypeptides viraux de cellules HeLa infectées avec chaque clône à 33°C, marqués à la ^{35}S-méthionine, sont analysés en gel de polyacrylamide SDS. 39 de ces clones sont de l'adénovirus de type 5. Le 40ème est un mélange d'Ad2 et d'Ad 5.

L'adénovirus 2 s'est donc faiblement répliqué, lors de la coinfection avec l'Ad 5. La protéine terminale "thermosensible pour le H2 ts 111", n'a pu servir à l'initiation de nouvelles molécules de DNA, et seul le mutant possédant une "protéine normale" a pu répliquer son DNA.

Ce résultat est un argument en faveur du rôle de la 55 K dans l'initiation de la synthèse du DNA viral, rôle suggéré par Rekosh et al., (1977). Un résultat similaire a été obtenu pour les mutants thermosensibles de ϕ 29 en ce qui concerne la protéine terminale liée au DNA (produit du gène 3) (Salas et al., 1978).

II-9 La fonction mutée dans l'H2 ts 111 pourrait être une double fonction

L'H2 ts 111 présente donc deux phénotypes :

- absence d'initiation et d'élongation des "chaînes" de DNA
- absence de protection du DNA viral et cellulaire.

Ces deux fonctions semblent ne faire intervenir qu'une seule mutation. En effet, les quatre révertants isolés ont pour ces deux phénotypes les mêmes propriétés que le type sauvage.

II-10 Complémentation et recombinaison dans les cellules HEK-293

La production virale, à 39,5°C, n'est pas augmentée si nous utilisons des cellules HEK-293, à la place de cellules HeLa. Les HEK-293, sont des cellules transformées par l'adénovirus 5 qui expriment des fonctions virales. Ces fonctions exprimées ne complémentent pas la fonction lésée du H2 ts 111, contrairement aux fonctions absentes des mutants H5 hr 1-7, et H5 dl 311-315 (Harrisson et al., 1977, Jones and Schenk, 1979).

Mais lors de l'infection de cellules HEK-293 à 33°C par le mutant H2 ts 111, nous décellons dans la progéniture virale, une quantité importante de recombinants. La fréquence de recombinants (ts^+), atteint 5% si cette fréquence est mesurée sur HeLa, et même 7% si elle l'est sur HEK-293. La fréquence de réversion sur HeLa est inférieure à 10^{-5}.

Il y a donc recombinaison entre le DNA viral et le DNA cellulaire. De plus la mutation de l'H2 ts 111 est porté par une partie du génome qui est intégrée dans les cellules HEK-293 {(12% de l'extrémité gauche et 9% de l'extrémité droite (Graham et al., 1977, Aeillo et al., 1979)}. Mais nous ne pouvons exclure qu'il s'agisse d'une plus grande fréquence de réversion sur ces cellules par rapport aux cellules HeLa.

II-11 Origine de la fragmentation du DNA viral

L'introduction de coupures dans les molécules de DNA cellulaire ou viral fait appel à divers mécanismes et pour chacun d'eux, il existe un moyen de protection ou une possibilité de réparation :

- restriction-modification ; (cf revue Arber, 1974)
- endonucléases, exonucléases et leurs inhibiteurs
- lésions du DNA et systèmes de réparations (cf Grossman et al., 1975)

Quel est le système défaillant dans les cellules infectées par le mutant H2 ts 111 ?

Ce mutant n'étant pas dominant, il ne s'agit donc pas d'une activité accrue d'introduction de coupures dans le DNA, mais plutôt d'une absence de protection ou de réparation. La quantité de fragments de DNA n'est pas augmentée, la taille de ceux-ci n'est pas réduite, par un accroissement de la radioactivité spécifique du DNA, ce qui n'est pas en faveur d'une mutation dans les systèmes de réparation.

Une autre hypothèse serait la coupure par des endonucléases et/ou l'absence de ligature du DNA survenant au cours de la réplication. De nombreuses protéines ont été isolées ayant un rôle de protection du DNA monocaténaire et joueraient le rôle d'inhibiteurs des nucléases pour ce même type de DNA in vitro telle la protéine E 72 K (Nass and Frenkel, 1978 ; 1979).

Il n'a pas été décrit pour les cellules eucaryotes d'endonucléase de type restriction, bien que le DNA cellulaire soit modifié par méthylation. Le DNA d'adénovirus contient moins de bases modifiées que le DNA de la cellule hôte (0,04% de 5 méthylcytosine par cytosine pour le DNA viral et 3,75% pour le DNA de cellules KB) (Gunthert et al., 1976).

D'autres protéines introduisent des coupures transitoires dans le DNA, ce sont les enzymes de type "nicking-closing" qui relaxent les supertours positifs ou négatifs pour permettre la réplication (cf revue Wang and Liu, 1979)

Un tel système pourrait être lésé dans le mutant H2 ts 111,

D'autres études sont donc nécessaires pour :

- confirmer que la mutation affecte la protéine terminale par exemple comparaison des peptides produits par clivages avec des endopeptidases, avec le type sauvage ("finger-printing").
- connaitre le rôle de cette protéine dans la protection du DNA ; c'est à dire :
  - . la stabilité des nucléosomes dans les cellules infectées
  - . son éventuel participation aux complexes multienzymatiques de relaxation du DNA (Topoisomérases)
- situer la mutation sur le génome

III Assemblage de la particule virale

III-1 Méthodes d'extraction et de purification des virus

L'étude des étapes de la formation de la particule virale infectieuse nécessite d'isoler les intermédiaires d'assemblage dans leur état natif. Les méthodes d'extraction et de purification ne doivent pas dégrader ces intermédiaires qui peuvent être très labiles puisque ce sont des structures incomplétes, non stabilisées. Edvardsson et al., (1976) utilisent la centrifugation en gradient linéaire de ficoll, pour séparer les diverses classes de particules. Ils les obtiennent en traitant aux ultrasons les noyaux de cellules infectées. Dans nos mains, cette technique s'est révélée très aléatoire, une grande partie des particules s'agglomeraient et n'étaient donc plus séparées dans le gradient de ficoll. De plus, le traitement aux ultrasons, ne permet pas de maintenir intact le DNA viral.

Nous avons donc utilisé la technique décrite par Wallace et Kates (1972) qui consiste à lyser les noyaux au $(NH_4)_2\ SO_4$ 0,3M pH 8, et à diluer ce lysat immédiatement. Les particules virales sont ensuite séparées par centrifugation dans un gradient linéaire de saccharose. Les particules ainsi isolées ne peuvent être repurifiées par centrifugation isopycnique en gradient de CsCl, solution très hypertonique (2,7M en moyenne), sans "consolidation" préalable. La fixation la plus utilisée est le pontage au glutaraldehyde, mais après action de ce fixateur, irréversible, il n'est plus possible d'étudier la composition polypeptidique de ces particules. C'est pourquoi nous utilisons un autre agent de pontage, un diimidoester clivable ; le chlorhydrate de diméthyl 4, 4'dithiobisbutyrimidate (DMTB) pour maintenir la structure des intermédiaires labiles pendant leur

purification.

III-2 Intermédiaires mis en évidence dans les cellules infectées par l'adénovirus de type sauvage

En gradient linéaire de saccharose, deux populations qui sédimentent à 750 et 600S sont mises en évidence. La population 750S cosédimente avec le virus mature. La population 600S apparait 30 minutes après la fin d'un marquage bref. La quantité maximale est atteinte après 1h30 de "chasse".

La purification des particules 600S par centrifugation en gradient de CsCl après fixation au DMTB donne 2 classes de particules de densité respectives 1,315 et 1,37. Les particules de densité 1,315 ne contiennent ni de DNA, ni les polypeptides du nucléoide (V et VII). Elles possèdent les précurseurs des polypeptides VI et VIII et deux protéines non structurales, de masse moléculaire 50 000 et 39 000 (50 K et 39 K). Les intermédiaires lourds (1,37 de densité) possèdent du DNA viral 31S. Ils diffèrent des virus matures par leur composition en polypeptides. Comme les intermédiaires légers 1,315, ils ne contiennent pas les protéines V et VII, mais ils n'ont pas les protéines 50 K et 39 K.

Les particules qui sédimentent à 750S s'équilibrent à une densité de 1,345 en chlorure de césium. Elles correspondent à un mélange de jeunes virions et de virions matures.

Ces trois classes de particules sont différentes en microscopie électronique. En coloration négative, la partie centrale des particules 1,315 est moins dense aux électrons que celle des deux autres types de particules (1,37 et 1,345). L'intermédiaire lourd (1,37 de densité) présente une structure plus arrondie et moins nettement icosaèdrique que le virus de densité 1,345.

Une étude cinétique montre que les intermédiaires légers apparaissent avant les intermédiaires lourds. Ceux-ci évoluent en jeunes virions qui deviennent des virions infectieux. Les protéines de structure sont rapidement utilisées pour la formation des intermédiaires légers, 30 minutes après leur synthèse. Le DNA viral est encapsidé à partir de 2h30 après sa synthèse. Un même délai, a été trouvé par Everitt et al., (1977) pour l'encapsidation du DNA.

Les intermédiaires lourds, de densité 1,37 apparaissent avec la même cinétique pour les sérotypes 2 et 3. Ils représentent donc une étape

dans la formation de virus infectieux, et non pas une "voie de garage" qui aboutirait à des particules défectives. En effet, dans cette hypothèse, le type 3 qui produit une grande quantité de particules défectives, devrait synthétiser plus d'intermédiaires lourds que le type 2.

III-3 Accumulation d'intermédiaires légers dans les cellules infectées par des mutants thermosensibles

Les mutants H2 ts 101, H2 ts 112 et H5 ts 58, défectifs dans la synthèse de la protéine IIIa, accumulent des intermédiaires légers à température restrictive. Ces particules sédimentent à 600S en gradient de saccharose, et après fixation au DMTB, elles s'équilibrent en une population homogène de densité 1,315 en CsCl. Ces particules ne contiennent pas les protéines du nucléoide (V et VII) mais elles possèdent deux protéines non-structurales de masse moléculaire 50 K et 39 K. Elles ressemblent aux intermédiaires légers présents dans les cellules infectées par le type sauvage. Certaines protéines sont phosphorylées, c'est le cas en particulier des protéines 100 K, 68 K, 50 K et 39 K.

Les intermédiaires légers accumulés par le mutant H2 ts 112 renferment un fragment de DNA. Ce fragment a une taille d'environ 7S en gradient de saccharose alcalin. C'est du DNA bicaténaire. Il est principalement constitué par des séquences virales, mais il s'hybride, à un taux faible, avec du DNA cellulaire. Ce fragment n'est pas spécifique : toutes les séquences du génome viral sont statistiquement représentées. Le DNA n'est pas retenu à 0,3M NaCl sur filtre de fibre de verre (GF/C) ; il est donc démuni de protéines liées de manière covalente aux extrémités du DNA de virus mature. Ce fragment de DNA peut être le résultat d'une incorporation non-spécifique au cours de l'extraction.

Réversibilité et maturation des intermédiaires légers

Par passage à température permissive, il est possible de restaurer la production virale. Cette restauration est totale si les cellules sont transférées avant la 16ème h, et elle devient très faible à partir de la 20ème heure du cycle lytique. La maturation des intermédiaires d'assemblage doit donc être étudiée le plus prés possible de la 16ème heure.

De plus, il est nécessaire de bloquer l'utilisation du "pool" des protéines synthétisées pour former de nouveaux intermédiaires à la température permissive ; ce blocage est obtenu par addition d'un inhibiteur de la

synthèse protéique (cycloheximide) (Sundquist et al., 1973).

Dans ces conditions, les intermédiaires peuvent évoluer jusqu'au stade de virus infectieux. Les intermédiaires, accumulés par les trois mutants, désignés ci-dessus, se maturent par passage à 33°C en présence ou en absence de cycloheximide. L'analyse en gel de polyacrylamide-SDS des polypeptides présents dans les intermédiaires non maturés révéle la présence d'une protéine supplémentaire, de masse moléculaire variant de 28 à 32 K en fonction de la période d'incubation à 33°C. Cette protéine est en quantité plus importante dans le cas du mutant H5 ts 58. Cette protéine est phosphorylée.

Les particules accumulées par le mutant H2 ts 112 sont semblables à celles accumulées par le H2 ts 4 (Khittoo and Weber, 1977).

Après dialyse contre un tampon à basse force ionique (5mM Tris-maleate pH 6,2), ces intermédiaires possèdent une endonucléase active à pH 4,5, comme la particule infectieuse.

III-4 Mutants défectifs dans la synthèse de la base du penton et de la fibre

Deux de ces mutants (H2 ts 103 et H2 ts 136) ne forment pas de particule à 39,5°C. Au contraire, un grand nombre de particules sont trouvées dans les cellules infectées par l'H2 ts 104. Ces particules ressemblent aux jeunes virions décrits par Ishibashi et Maizel (1974b) et aux virions accumulés par l'H2 ts 1 (Weber, 1976).

Les précurseurs des polypeptides VI, VII et VIII ne sont pas clivés dans la cellule à 39,5°C, ils le sont après passage à 33°C. En bloquant la formation de nouvelles particules (par l'addition de cycloheximide), il est possible de suivre l'évolution de celles déjà formées. Ainsi après passage à 33°C, l'analyse des polypeptides de ces particules en gel de polyacrylamide-SDS révèle la présence des protéines VI, VII et VIII. Les jeunes virions accumulés à 39,5°C se maturent à 33°C.

Cette modification concerne aussi la structure interne de la particule. Après dissociation à 56°C, en présence de 0,5% de déoxycholate de sodium, des particules formées à 39,5°C, le DNA sédimente à 40S. Après le même traitement des particules extraites de cellules transférées à 33°C, le DNA sédimente à 180-200S et se trouve lié aux protéines V et VII. La structure en nucléoide n'est stable qu'au stade de virion mature. La stabilité plus

faible pour le nucléoide extrait des jeunes virions a été aussi décrite pour les particules accumulées par l'H2 ts 1 (Mirza and Weber, 1977).

III-5 Mutants défectifs dans la synthèse de la fibre

L'H2 ts 115, étant probablement un double mutant, muté sur la protéine IV (fibre) et PVI, comme le suggère l'analyse en gel de polyacrylamide-SDS, a été moins étudié.

Les cellules infectées par les mutants H2 ts 125 ou H2 ts 116 présentent une quantité très réduite de protéines VI, VII et VIII, ce qui indique la formation d'un petit nombre de virus matures. Dans le lysat cellulaire, analysé par sédimentation en gradient linéaire de saccharose, nous trouvons un peu de virus mais beaucoup de particules qui sédimentent à 600S. La transformation intermédiaire ⟶ virus n'est pas amplifiée par passage à la température permissive. Les virus formés à 39,5°C ont toutes les propriétés des virus infectieux. Ce résultat est semblable a celui obtenu lors de l'infection de cellules de singe par l'adénovirus 2 (type sauvage), cellules dans lesquelles une diminution de la quantité de fibre synthétisée entraine une réduction drastique de la formation de virus. La fibre pourrait jouer un rôle important dans la quantité de particules virales formées, bien que dans le cycle lytique elle soit produite en grand excès (2 à 5% seulement est encapsidé).

Ces deux mutants (H2 ts 116 et H2 ts 125) ont une fibre glycosylée mais le polypeptide IV a une masse moléculaire de 60 K au lieu de 62 K pour le polypeptide de la fibre de l'Ad 2. Cette protéine bien qu'ayant la même masse moléculaire que la fibre de l'Ad 5 ne réagit pas avec un immunsérum antifibre de type 5 . La fibre des mutants H2 ts 116 et 125 synthétisée à 33°C, bien qu'ayant une masse moléculaire réduite a une conformation très semblable à la fibre de l'adénovirus 2 de type sauvage, comme le révèlent les réactions immunologiques.

La réduction de la masse moléculaire de la fibre de 62 à 60 K, et son unicité indiquent que le trimère est formé de 3 polypeptides identiques. L'existence de trois groupes de complémentation pour les mutants fibre (-) doit être le reflet :

- soit d'une complémentation intracistronique
- soit de facteurs intervenant dans la maturation de la fibre ou l'assemblage des trois sous-unités

. soit des deux phénomènes à la fois

Les résultats de la fréquence de recombinaison entre les mutants fibre (-) suggèrent qu'il y a vraisemblablement complémentation intracistronique (Williams et al., 1974).

III-6 Evolution des antigènes dans les particules

Après dissociation du virus infectieux à 56°C, en présence de déoxycholate de sodium, 4 pics de précipitation antigènes-anticorps sont obtenus en immunoelectrophorèse bidimensionnelle avec un immunsérum anti-protéines de structure . Les quatre protéines ainsi précipitées sont de l'anode à la cathode l'hexon, la base du penton, le IIIa et la fibre.

L'analyse immunologique des particules accumulées par les divers mutants thermosensibles révèle que :

- les intermédiaires légers contiennent seulement de l'hexon antigéniquement actif et de la fibre (sauf pour les mutants défectifs dans la synthèse de la fibre). Le IIIa et la base du penton ne sont pas à l'état antigéniquement réactif

- les jeunes virions, accumulés par l'H2 ts 104 ne possèdent pas de base de penton antigéniquement active

- dans les particules maturées à 33°C, les 4 protéines majeures réagissent avec un immunsérum anti-protéines de structure .

La protéine IIIa et la base du penton subissent au cours de l'assemblage des modifications qui leur font perdre leurs propriétés immunologiques au stade d'intermédiaire léger. Ce changement pourrait être dû au masquage du site de reconnaissance de l'anticorps par une protéine. Un polypeptide de masse moléculaire 56 K est coprécipité avec le IIIa à l'aide d'un immunsérum anti IIIa et de la protéine A de Staphylococcus Aureus. Ce polypeptide 56 K migre comme un constituant mineur du virus infectieux : le IVa_2, mais ce polypeptide est absent des intermédiaires légers (1,315). Il pourrait donc s'agir d'un produit de dégradation de la protéine IIIa. La différence d'antigénécité peut refleter un changement conformationel dû par exemple au clivage du polypeptide IIIa, présent dans les intermédiaires pour donner le polypeptide IIIa présent dans le virus infectieux (voir ci-dessous).

III-7 Clivage du PIIIa en IIIa

Weber et al. (1977) ont rapporté l'existence d'une hétérogénéité dans la protéine IIIa, avec 2 espèces protéiques migrant avec des masses moléculaires apparentes de 68 000 à 66 000. Dans le virus infectieux le polypeptide IIIa a une masse moléculaire de 66 000. Lors d'un marquage bref, ce polypeptide a une masse moléculaire de 67 000 (67 K).

Dans les intermédiaires d'assemblage légers et lourds, le IIIa a une masse moléculaire de 67 K. La même taille est retrouvée pour ce polypeptide dans les jeunes virions. Ces deux formes (66 K et 67 K) sont phosphorylées. La différence de migration ne s'explique donc pas par une différence de phosphorylation mais sans doute par un clivage protéolytique. Le clivage du polypeptide 67 K (P IIIa) en IIIa, intervient au cours de la maturation de la particule virale. Ce clivage est contemporain des trois autres clivages (PVI, PVIII, PVII) bien qu'il semble être un peu plus rapide. Le clivage se produit à l'extrémité N terminale par enlévement de 10 à 15 acides aminés. Le N terminal du IIIa est la glycine, et n'est pas bloqué (Lemay et al., 1980).

IV La novobiocine inhibe la synthèse du DNA viral et son encapsidation

L'encapsidation du DNA est-elle un phénomène passif ou actif ? Cette encapsidation se fait-elle avec des changements de structure ? Pour répondre à ces questions, il est utile de pouvoir agir sur la conformation du DNA ou sur des enzymes agissant sur cette conformation. Et comme le DNA est, dans le virus organisé en nucléosome, il est possible qu'un enzyme du type "nicking-closing" joue un rôle lors de l'empaquetage de l'acide nucléique. C'est pourquoi nous avons étudié l'effet de la novobiocine sur la synthèse et l'encapsidation du DNA. La novobiocine est un antibiotique qui inhibe l'activité DNA gyrase des procaryotes (cf. Champous, 1978).

IV-1 La novobiocine inhibe l'initiation de la synthèse du DNA viral

Ajoutée au milieu de culture à 200 µg/ml, cette drogue inhibe 95% de la synthèse du DNA viral, comme celle du DNA cellulaire. A 100 µg/ml, cette inhibition est de l'ordre de 60%.

L'inhibition est très rapide : 15 min après son addition, le plateau de synthèse résiduel est atteint. Ce blocage dans la synthèse du DNA est réversible : après enlévement de la drogue la synthèse du DNA reprend,

même en l'absence de synthèse de protéines. Le DNA synthétisé en présence de novobiocine sédimente en gradient de saccharose alcalin à 34 S. L'élongation n'est donc pas affectée par cette drogue, seule l'initiation de nouvelles chaînes de DNA est altérée.

Si la cible de cet antibiotique est la même dans les cellules eucaryotes que dans les cellules procaryotes ce qui semble le cas (Mattern and Painter, 1979), Cette activité DNA gyrase, créant des supertours négatifs serait donc nécessaire à la réplication du DNA viral.

IV-2 La novobiocine inhibe l'encapsidation du DNA viral

Ajoutée après un marquage bref des protéines et du DNA, la novobiocine empêche la formation de virus et même d'intermédiaires d'assemblage sédimentant à 600 S. Cet effet pourrait être le résultat de l'inhibition de la synthèse de protéines. On sait en effet qu'une synthèse continue de protéines est nécessaire à la formation des intermédiaires d'assemblage. L'addition d'hydroxyurée n'empêche pas la formation de virus mature. Une néosynthèse de DNA n'est pas requise pour la formation de virus, le DNA préexistant pouvant être encapsidé.

Si dans des cellules infectées par l'H2 ts 112, on laisse s'accumuler à 39,5°C des intermédiaires légers et qu'on ajoute la novobiocine au moment du transfert à 33°C, il n'y a pas, dans ce cas, de maturation des intermédiaires en virus. Une conformation adéquate du DNA ou la possibilité de modifier cette conformation est donc nécessaire à son encapsidation. Notre préférence va à la deuxième hypothèse, plus probable, car la novobiocine n'agit pas directement sur la conformation du DNA, mais plutôt par l'intermédiaire d'un blocage d'activité enzymatique.

IV-3 Assemblage des mutants défectifs dans la synthèse du DNA

Les protéines virales qui jouent un rôle dans l'initiation de la synthèse du DNA viral peuvent avoir aussi un rôle dans son encapsidation. Des cellules infectées par l'H5 ts 36 ou l'H2 ts 111, cultivées à température permissive, assemblent une quantité réduite de virus si elles sont transférées à 39,5°C juste avant la période de marquage. Le taux d'assemblage devient pratiquement nul lorsque les cellules sont transférées à 39,5°C 30 min avant le marquage.

Dans ces conditions, il n'y a pas de réduction du taux de synthèse des protéines. Le complexe enzymatique, nécessaire à la réplication du DNA servirait donc au moins en partie à son encapsidation.

V Schéma de l'assemblage de l'adénovirus de type 2

Nous pouvons diviser le processus d'assemblage en deux étapes importantes :

1) formation d'une capside dépourvue de DNA

2) encapsidation du DNA et maturation de la particule

V-1 Formation d'une capside sans DNA

L'élaboration d'une capside, dans le noyau des cellules infectées nécessite la synthèse de protéines de structure, leur assemblage en protéines multimériques fonctionnelles et leur transport du cytoplasme au noyau.

C'est la protéine majeure de la capside qui joue un rôle clef dans cet assemblage ; aucun mutant défectif dans la synthèse de l'hexon n'assemble de telles particules. Les mutants hexon (-) appartiennent à quatre groupes de complémentation et nous pouvons distinguer au moins trois types d'altération :

- modification du polypeptide II
- absence de trimérisation : cette trimérisation est effectuée avec l'intervention d'au moins une protéine, la 100 K qui est défective pour les mutants H5 ts 1, H5 ts 17, H5 ts 20, H2 ts 48 (Mautner et al., 1975 ; Leibowitz and Horwitz, 1975 ; Carstens and Weber, 1977 ; Carstens et al., 1979)
- absence de transport du trimère hexon du cytoplasme au noyau. Ce transport est altéré pour le mutant H5 ts 147 et semble faire intervenir le polypeptide PVI (Kauffman and Ginsberg, 1976).

Les mutants défectifs dans la synthèse de la fibre assemblent, en quantité réduite certes, des particules, bien qu'il n'y ait pas de penton complet formé ; ou alors, cette formation est instable dans la cellule et elle est stabilisée dans la particule virale, puisque de la fibre est retrouvée dans ces particules, notamment pour le mutant H2 ts 125. La base du penton change de conformation suivant qu'elle se trouve à l'état libre ou à l'état associé à la fibre (Lemay and Boulanger, 1980).

Les mutants sus de Φ 29 qui ne synthétisent pas de fibre, assemblent des particules sans fibre qui sont infectieuses (Reilly et al., 1977).

Pour deux mutants (H2 ts 103, H2 ts 136) défectifs dans la synthèse de la base du penton et de la fibre, aucune particule n'est formée. Le penton co l'hexon jouent un rôle important dans la formation de la capside dépourvue de DNA. Le troisième mutant (H2 ts 104) assemble des particules virales.

Les mutants des trois groupes de complémentation défectifs dans la synthèse de la protéine IIIa assemblent des capsides sans DNA.

D'autres facteurs que les protéines majeures de structure interviennent dans cet assemblage. Des mutants qui synthétisent en quantité normale ces protéines peuvent être défectifs dans l'assemblage, c'est le cas du mutant H2 ts 107.

Cet assemblage nécessite la synthèse continue de protéines, puisqu'il est rapidement bloqué par l'addition de cycloheximide ou d'émitine (Sundquist et al., 1974) alors que les protéines de structure sont en très large excès.

La protéine limitante pourrait être une protéine d'échaffaudage ("scaffolding protein"). La 50 K présente dans les IM (1,315) mais pas visible dans les extraits de cellules infectées, peut avoir cet fonction. Elle ne serait pas recyclée, ce qui nécessiterait sa synthèse en continu. Cette protéine pourrait jouer un rôle en collaboration avec d'autres protéines.

Les protéines d'échaffaudage jouent un rôle important dans la formation des "pré-têtes" des bactériophages à DNA bicaténaire, comme λ, T_4, T_7, P_{22} (cf. revue générale de Casjens and King, 1975 ; Showe and Kellenberger, 1976).

Une autre hypothèse est que ces particules sans DNA, ou possèdant un très petit fragment, sont le résultat de la dégradation, au cours de l'extraction, de structures plus complexes, comme il a été rapporté récemment pour l'assemblage du SV_{40} (Garber et al., 1978 ; Coca Prados and Hsu, 1979 ; Baumgartner et al., 1979). Dans le cas de ce virus, la capside se forme autour du minochromosome (DNA organisé comme la chromatine

avec des histones cellulaires). La maturation de la particule consiste en la consolidation de la nucléocapside et au départ de l'histone H1.

Nous pouvons exclure cette possibilité, dans l'assemblage de l'adénovirus pour deux raisons :

- les particules de densité 1,315 ne sont pas vides ; elles contiennent en leur centre du matériel dense aux électrons
- le génome viral n'existe pas lié aux polypeptides du nucléoide dans la cellule ; il se retrouve seulement lié aux protéines V et VII (PVII) à partir du stade de jeunes virions.

V-2 Encapsidation du DNA et maturation de la particule

Pour les bactériophages à DNA bicaténaire, l'encapsidation du DNA viral suit la disparition de la protéine d'échauffaudage de la pré-tête. Cette étape est aussi l'étape ultime de la réplication du DNA, l'obtention du génome mature, par exemple la formation des extrémités cohésives du DNA de λ (5' monocaténaire) (Emmons, 1974).

Deux protéines (50 K, 39 K) présentes dans les intermédiaires d'assemblage légers sont absentes des particules contenant du DNA 31 S. Comme elles ne sont pas retrouvées en quantité importante dans les extraits de cellules infectées, elles sont probablement dégradées pour permettre l'entrée du DNA. Persson et al.,(1979b) suggèrent que la 50 K joue un rôle dans la maturation de la particule virale. Il nous semble que c'est son départ qui permettrait le début de la maturation de la particule virale par l'encapsidation du DNA. Son rôle serait alors très voisin des protéines d'échaffaudage des bactériophages, telle la protéine codée par le gène 8 de P 22 (Casjens and King, 1974 ; Lenk et al., 1975 ; Earnshaw et al., 1976).

Le DNA n'est pas encapsidé dans les particules accumulées par les mutants des groupes de complémentation défectifs dans la synthèse de la protéine IIIa. Ces particules contiennent les protéines 50 K et 39 K, l'étape bloquée peut être le départ, ou la protéolyse de ces protéines. L'encapsidation du DNA est aussi réduite dans les mutants défectifs dans la synthèse de la fibre (H2 ts 125) ce qui suggère que la conformation de l'apex joue un rôle dans l'entrée du DNA. La protéine IIIa est localisée dans la zone péripentonale (Everitt et al., 1975).

Cette encapsidation semble se faire par l'une des faces de l'icosaèdre (vestige ou précurseur de la fixation de la queue des bactériophages ?). Le DNA pénètre par son extrémité gauche, il est donc linéaire à ce stade ; il est peut-être circularisé dans la particule.

Le DNA, qui est encapsidé, doit avoir une conformation adéquate, ou il doit pouvoir changer de conformation au cours de l'assemblage, puisque cette étape est inhibée par la novobiocine. Ce changement de conformation est certainement le passage d'une structure en nucléosome du DNA associée à des protéines non encore identifiées, en la structure du nucléoide avec les protéines V et PVII dans une première étape et avec les protéines V et VII, au stade de virus infectieux. Avant la formation du nucléoide, le DNA peut être, dans une étape transitoire, non lié à des protéines (stade IM 1,37).

Après la formation du nucléoide, quatres polypeptides subissent un clivage par une endopeptidase(PIIIa, PVI, PVIII, PVII). Le PIIIa serait le premier peptide clivé. Cette endopeptidase n'est active qu'à ce stade du cycle infectieux ; son origine est inconnue mais elle est sous le contrôle de gène (s) viral (aux) (Bhatti and Weber, 1979). Après ces clivages, la particule devient infectieuse. Cette étape n'est pas réalisée pour les mutants H2 ts 1 (Weber, 1976) et H2 ts 104 (cf. résultats ci-dessus). Les clivages endopeptidasiques sont très importants dans la morphogénèse des bactériophages (cf. Laemmli, 1970, pour T_4 et revue générale de Casjens and King, 1975). Un modèle d'assemblage de l'adénovirus est proposé sur la fig 8.

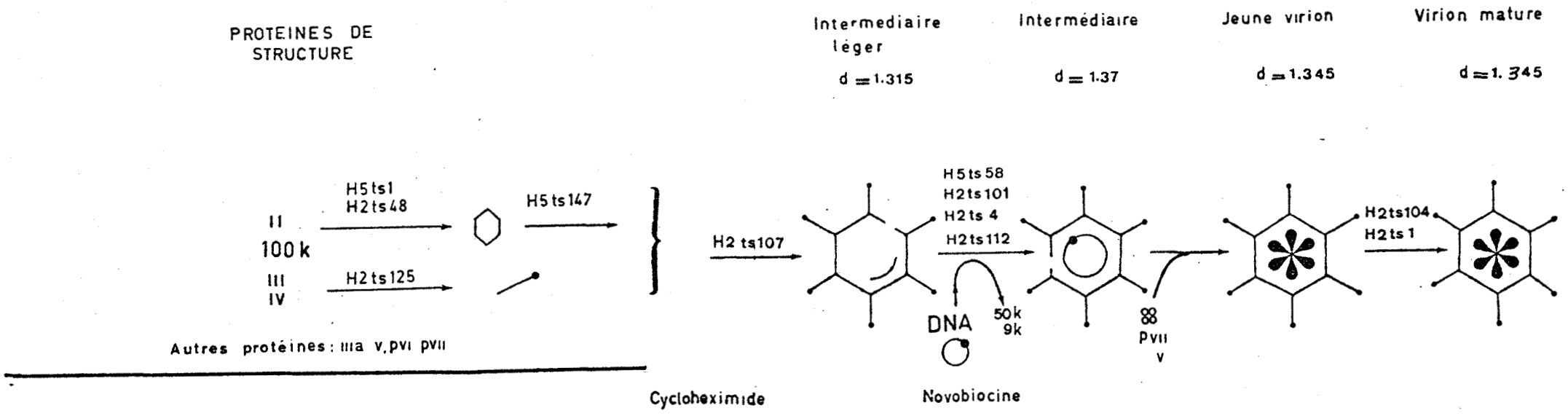

Fig 8 - Schéma d'assemblage de l'adénovirus

La composition en polypeptides est indiquée en dessous des diverses particules. Les mutants inscritsau dessus des flèches d'évolution des particules indiquent les étapes non réalisées dans les cellules infectées par ces divers mutants. Les particules isolées de ces cellules sont donc du type de celles situées à gauche du mutant mentionné

CONCLUSION

Parmi les 40 mutants ts isolés dans notre laboratoire, l'un deux, défectif dans la synthèse du DNA viral représente un unique groupe de complémentation. La fonction mutée est nécessaire à l'initiation de la synthèse du DNA viral, mais aussi au maintien de l'intégrité du génome viral. La protéine mutée pourrait être la 55 K, protéine de circularisation du DNA. Ce mutant nous a permis de confirmer qu'il y a recombinaison entre le DNA viral et le génome de la cellule-hôte, en dehors de la transformation cellulaire.

L'utilisation de l'immunoelectrophorèse bidimensionnelle comme technique de caractérisation sérologique de mutants d'adénovirus, nous a permis de mettre en évidence, l'absence d'un seul composant, la protéine IIIa, dans quatre mutants thermosensibles (H2 ts 4, H2 ts 101, H2 ts 112, H5 ts 58), défectifs dans la morphogénèse du virion. Ces mutants appartiennent à 3 groupes de complémentation.

Nous avons montré que, dans la formation de la particule virale infectieuse il existe deux classesd'intermédiaire d'assemblage de densité 1,315 et 1,37. Les particules de densité 1,37 proviennent des intermédiaires 1,315 après élimination des protéines 50 K et 39 K et encapsidation du DNA ; elles évoluent en "jeunes virions" après la pénétration des protéines du nucléoide (V et VII).

Les mutants thermosensibles défectifs dans la synthèse de la protéine IIIa, accumulent des particules de densité 1,315. Celles-ci sont capables d'évoluer en virus infectieux après passage à température permissive. L'étude de cette évolution est rendue possible par le blocage de la formation de nouvelles structures pré-capsidales (addition de cycloheximide).

De même les jeunes virions formés dans les cellules infectées par H2 ts 104 se transforment en virus infectieux après passage à 33°C.

Une modification de la fibre, telle que celle observée dans les mutants H2 ts 103, 104, 115, 116, 125, entraine une réduction de la quantité de virus formés, ce qui suggère que l'intégrité de la protéine fibre est indispensable à la morphogénèse ou à la stabilité du virion.

Lors de la formation de la particule infectieuse, deux protéines de structure : la base du penton et le IIIa, subissent des modifications conformationnelles comme le révèle la perte transitoire de leurs propriétés antigéniques au

stade intermédiaire d'assemblage.

La protéine IIIa (66 K) provient d'un précurseur : le P IIIa (67 K) par clivage endopeptidasique ; cette modification intervient au cours de la maturation des jeunes virions en virions infectieux.

La synthèse du DNA viral est inhibée par l'addition de novobiocine. Un enzyme, de type "DNA gyrase", serait donc nécessaire à la réplication.

Cet antibiotique empêche, également l'encapsidation du DNA. L'encapsidation nécessiterait donc la participation d'un enzyme de "relaxation" transitoire du DNA, bien que le DNA d'adénovirus ne soit pas circulaire de façon covalente, ou "supertorsadé", qui pourrait être le même enzyme que la gyrase évoquée plus haut.

ANNEXES

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Isolation and Phenotypic Characterization of Human Adenovirus Type 2 Temperature-Sensitive Mutants

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SUMMARY

Thirty-nine temperature-sensitive (*ts*) mutants that fail to grow at 39·5 °C but develop normally at 33 °C have been isolated from a nitrous-acid-treated stock of a wild-type strain of type 2 human adenovirus. The frequency of *ts* mutants among the surviving viruses was about 10%. Complementation tests in doubly infected cell cultures at restrictive temperature permitted the assignment of 19 of these mutants to 11 complementation groups. They were characterized phenotypically according to their soluble capsid antigen production quantified by two-dimensional immunoelectrophoresis, virus DNA synthesis, as measured by alkaline sucrose gradient sedimentation of 34S DNA, and virion morphogenesis, as analysed by electron microscopy of cell sections. Two complementation groups were defective for DNA synthesis, four for soluble hexon production and two groups for total penton (penton base + fibre), while one group revealed no fibre production. Two complementation groups presented a normal antigen pattern, but the particles exhibited altered morphology as observed in cell sections.

INTRODUCTION

Animal viruses and particularly adenoviruses are considered, with reason, to be invaluable tools in investigating the regulation of replication and transcription of DNA, the translation of m-RNAs, and malignant transformation in eukaryotic systems. Essential to these investigations are viruses deficient in different properties. Host-range, conditionally lethal temperature-sensitive and deletion mutants have therefore been isolated.

Temperature-sensitive mutants of human adenovirus type 2 (Begin & Weber, 1975; Kathmann *et al.* 1976), type 5 (Williams *et al.* 1971; Ensinger & Ginsberg, 1972), type 7 (Estes & Butel, 1977), type 12 (Shiroki *et al.* 1972; Ledinko, 1974) and type 31 (Suzuki *et al.* 1972) have been selected in several laboratories. Their properties have been reviewed recently (Ginsberg & Young, 1976, 1977).

Thirty-nine temperature-sensitive (*ts*) mutants of human adenovirus type 2 (H 2) have been isolated in our laboratory. Twenty-five have been characterized and 19 of them have been assigned to 11 groups of complementation. Some biochemical and immunological characteristics are reported in the present study. Certain of these *ts* mutants, such as penton-defective mutants, or a DNA-negative mutant which maintains a tight inhibition of host DNA synthesis at restrictive temperature, have not been reported so far for adenovirus type 2.

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METHODS

Virus and cells. Human adenovirus type 2 (H 2) wild type (WT) originally obtained from Dr J. F. Williams (Carnegie-Mellon Institute, Pittsburgh, U.S.A.) was grown on KB cells cultured in suspension (2 to $2{\cdot}5 \times 10^5$ cells/ml) in Eagle's minimal essential medium supplemented with 5% horse serum.

Virus was titrated by the plaque assay or by the fluorescent focus unit assay (Philipson *et al.* 1968) on HeLa cells at 37 °C. The WT virus to be used for mutagenesis underwent two cycles of plaque purification at 37 °C.

Human adenovirus type 5 (H 5) *ts* mutant 36 was kindly supplied by Dr J. F. Williams. H 5 *ts*-125, originally isolated by Dr H. S. Ginsberg was obtained from Dr W. C. Russell.

Mutagenesis of adenovirus 2. Nitrous acid was used as the mutagen (Williams *et al.* 1971). Plaque-purified WT stock (0·1 ml) with a titre of 6×10^9 plaque-forming units p.f.u./ml was diluted with 1·9 ml of 0·7 M-$NaNO_2$ in 1 M-sodium acetate buffer, pH 4·6, at room temperature for 8 min. The reaction was stopped by addition of four volumes of cold 1 M-tris-HCl buffer, pH 7·9, and the final mixture diluted a further 50 times with Eagle's medium. Samples thus diluted were frozen and stored at −70 °C until used.

Isolation of ts mutants. The mutagenized stock was diluted to give 30 to 40 plaques/plate and plated on HeLa cell monolayers at 33 °C. After 14 days of incubation at 33 °C, the plaques which appeared were removed with a Pasteur pipette and resuspended in 1 ml tris-saline (0·15 M-NaCl, 0·01 M-tris-HCl, pH 7·5). Virus was released by three cycles of freezing and thawing and the resulting virus isolate was tested for plaque formation on HeLa cell monolayers at 33 °C and 39·5 °C. Those virus isolates which gave a 33/39·5 °C p.f.u. ratio > 100 were plaque-purified twice more and tested again for plaquing ratio before a final working mutant stock was prepared at 33 °C. According to the proposed nomenclature for adenovirus mutants (Ginsberg *et al.* 1973) the block numbers 101 to 200 have been allotted to these *ts* mutants.

Complementation tests. HeLa cell monolayers were doubly infected at an input multiplicity of 5 p.f.u. of the two *ts* mutants/cell. After 2 h of adsorption at 33 °C, unadsorbed virus was rinsed off and the cells treated for 30 min with an antiserum against whole type 2 adenovirus, at a dilution of 1 : 100/1 : 200. The cells were rinsed again and further incubated at 39·5 °C for an additional 40 h. Control cultures were singly infected in parallel with 10 p.f.u. of each mutant/cell. At the end of the incubation period, the cells were scraped off the dishes into the culture medium, disrupted by three cycles of freezing and thawing and virus titrated using the fluorescent focus assay at 33 °C. Complementation index was given as the ratio of yield of the double infection to that of the higher of the two single infections at 39·5 °C, expressed as p.f.u. or f.f.u./ml. Complementation was considered as positive when this complementation index was at least 10 (Williams *et al.* 1971).

Antisera. Whole adenovirus type 2 (Ad 2) virion antiserum was obtained as follows: 0·5 ml adenovirus particles suspension purified by two CsCl bandings (Green & Piña, 1963), was mixed with 0·5 ml complete Freund's adjuvant (Difco Lab., Mich.) and injected intradermally into a rabbit in twenty 0·05 ml portions. Three weeks later, 0·25 ml adenovirus suspension in 0·25 ml complete Freund's adjuvant was injected intramuscularly, and the animal was bled 10 days after this last injection.

Antisera against purified hexon, penton, fibre and virion polypeptide IX were also prepared by immunizing rabbits in a similar way. Hexon, penton and fibre antigens were purified by a four-step procedure previously described (Boulanger & Puvion, 1973). Ad 2 polypeptide IX was purified according to the procedure of Everitt *et al.* (1973).

Crossed immunoelectrophoresis. An adaptation of the procedure described by Weeke (1973) was employed. First dimension: 5 to 20 μl samples were applied in wells punched in agarose gel (four wells on a 90 × 110 × 1·5 mm glass plate, corresponding to 15 ml 1% agarose). Electrophoresis was carried out at 10 V/cm for 70 min in agarose gel buffered with barbitone, pH 8·6, ionic strength 0·02. The electrophoresis tank was refrigerated at 15 °C by tap-water circulation. After the run, the agarose gel was divided into four slabs, each of them corresponding to one well. Each of the four first-dimension gel slabs was then transferred to a glass plate (100 × 100 × 1·5 mm) along one edge, and the remaining part of the plate was filled with antibody-containing agarose (50 to 200 μl antiserum mixed with 12 ml 1% agarose solution maintained in a 52 °C water bath). Second dimension: 16 h, 3 V/cm, 15 °C, in antibody-containing agarose buffered at pH 8·6. After electrophoresis in the second dimension, the gel was washed in saline, pressed, dried, stained with Coomassie brilliant blue R-250 (0·5% in 50% ethanol–10% acetic acid) and destained in 10% acetic acid – 50% ethanol.

Quantitative estimation of the soluble adenovirus antigens was made either by measuring the area enclosed in the precipitate (expressed as height × width at half-height) or by drawing the precipitate outline on transfer paper, cutting out the drawing, and weighing it. The antiserum was calibrated for each adenovirus antigen by running purified hexon, penton and fibre solutions separately (Martin *et al.* 1975). Their protein contents were determined by the method of Lowry *et al.* (1951).

DNA analysis. KB cells in suspension culture were infected with WT or *ts* mutants and labelled with ^{3}H-thymidine (2 μCi/ml, 25 Ci/mmol) at 16 to 24 h post infection (p.i.) at 39·5 °C. Control cultures at 33 °C were labelled from 40 to 48 h p.i. Cells were harvested, resuspended in tris-saline and loaded on top of a 5 to 20% alkaline sucrose gradient made in 0·3 M-NaOH, 0·7 M-NaCl, 1 mM-Na EDTA overlayered with 0·2 ml of 0·5 M-NaOH, 0·05 M-Na EDTA. After standing for 16 h at 4 °C, the gradients were centrifuged for 5 h at 35000 rev/min and 4 °C in a SW 41 rotor. Fractions were collected dropwise from the bottom and assayed for acid-precipitable radioactivity.

Electron microscopy. Cell samples were harvested 36 h after infection at 39·5 °C, fixed with 2·5% glutaraldehyde in 0·1 M-cacodylate buffer, pH 7·2 and post-fixed in 1% osmium tetroxide. After dehydration in an alcohol gradient, the samples were embedded in Araldite and sectioned. Sections were stained with lead citrate and examined in an Hitachi HU-12 electron microscope.

RESULTS

Isolation of ts mutants

The stock of type 2 adenovirus mutagenized by 0·7 M-nitrous acid was screened for *ts* mutants. From 400 plaques isolated at random at 33 °C, 39 mutants were isolated without selection pressure, i.e. a frequency of 10%. The 33/39·5 °C plaque-forming ratio ranged from 10^2 to 10^5, whereas the ratio for the WT varied from 1·5 to 2. The tightness of the mutants was estimated by comparing the yields from single growth cycles at 33 and 39·5 °C in cells infected at an m.o.i. of 10 p.f.u./cell. Mutants with a minimal 33/39·5 °C yield ratio lower than 10^2 were considered as leaky. As summarized in Table 4, only a few mutants were found to be leaky (H 2 *ts* 101 and 112).

Synthesis of virus structural antigens

Two-dimensional immunoelectrophoretic analysis of WT-infected HeLa cell extracts using an antiserum against whole virion reveals five main precipitate peaks which have been

previously identified and quantified (Martin *et al.* 1975). Peak 1 corresponds to hexon antigen, peak 2 to free penton base, peak 3 corresponds to the fibre antigenic determinants of the complete penton, and peak 4 to the penton base antigenic determinants of the complete penton; peak 5 has been identified as free fibre antigen (Fig. 1*a*).

The capacity of the *ts* mutants to induce the synthesis of immunologically active capsid antigens was tested at both permissive and restrictive temperatures by the two-dimensional immunoelectrophoretic technique. Three principal types of two-dimensional antigenic patterns were reproducibly observed: (i) a drastic reduction or a disappearance of one or several structural antigens; (ii) a homogeneous reduction of all the virus antigens; and (iii) an antigenic pattern similar to that of the WT. Among the *ts* mutants of the first serological class, some were defective in fibre production at 39·5 °C and showed no free fibres and no complete penton (*ts* 115, Fig. 1*b*), while others were reduced in penton (penton base+fibre) production (*ts* 104, Fig. 1*c*) and still others in hexon synthesis (Fig. 1*d* to *f*). Significant differences were visible in the antigenic pattern of the hexon-defective *ts* mutants, and three subclasses of patterns were distinguishable: (i) in hexon-defective mutants of subclass I (H 2 *ts* 118 and *ts* 121) hexon was produced in minute amounts, non-quantifiable (Fig. 1*d*); (ii) in subclass II (H 2 *ts* 102, *ts* 110, *ts* 117 and *ts* 122) the hexon production was 15 to 20% of the normal rate (Fig. 1*e*); (iii) in subclass III (H 2 *ts* 106, *ts* 108 and *ts* 119) the hexon production was 30 to 40% of the normal synthesis, but still attained the level of complete penton antigen (Fig. 1*f*). These hexon-defective mutants appear to belong to four different complementation groups designated as A, B, H and K (see Tables 3 and 4).

Virus DNA synthesis

The analysis of virus DNA synthesis in cells infected (m.o.i. = 25) by the *ts* mutants at both permissive (33 °C) and restrictive (39·5 °C) temperatures was carried out by centrifugation of ^{3}H-thymidine-labelled infected cell DNA in alkaline sucrose gradients. The velocity sedimentation pattern obtained with *ts* mutants (Fig. 2*b*) was compared with the WT pattern obtained at the same temperature (Fig. 2*a*): the synthesis of 34S virus DNA was drastically reduced at 39·5 °C for three *ts* mutants which appeared, therefore, to be DNA-negative: H 2 *ts* 105, 111 and 114. During infection with *ts* 105 and 114, host DNA synthesis was only slightly depressed at the non-permissive temperature, whereas this synthesis was strongly altered during infection with *ts* 111 at 39·5 °C. Experiments, to be published elsewhere, have shown that these mutants are not decapsidation mutants.

Virus capsid morphogenesis

The assembly of virus was studied by electron microscopy of cells infected with *ts* mutants at the restrictive temperature under identical m.o.i. conditions (25 p.f.u./cell). Virus particles were analysed with respect to their number and morphology in cell sections. Four different types of intranuclear particle patterns were observed: (i) a total absence of detectable virus particles; (ii) less than 50 particles per nucleus; (iii) the number of intranuclear particles ranging from 100 to 500; (iv) more than 1000 particles per section. Fig. 3*b* shows part of a section of HeLa cell infected with *ts* 104, exhibiting more than 1000 particles per section. Most of these particles presented an irregular contour but seemed to contain a densely-stained internal core. As already shown by immunoelectrophoresis, *ts* 104 presented an abnormal pattern of antigens, containing no visible penton and fibre cross-reactivity. In contrast, most of the intranuclear virus particles of *ts* 112, which showed a normal pattern of synthesis of soluble antigens, exhibited an altered structure: they appeared

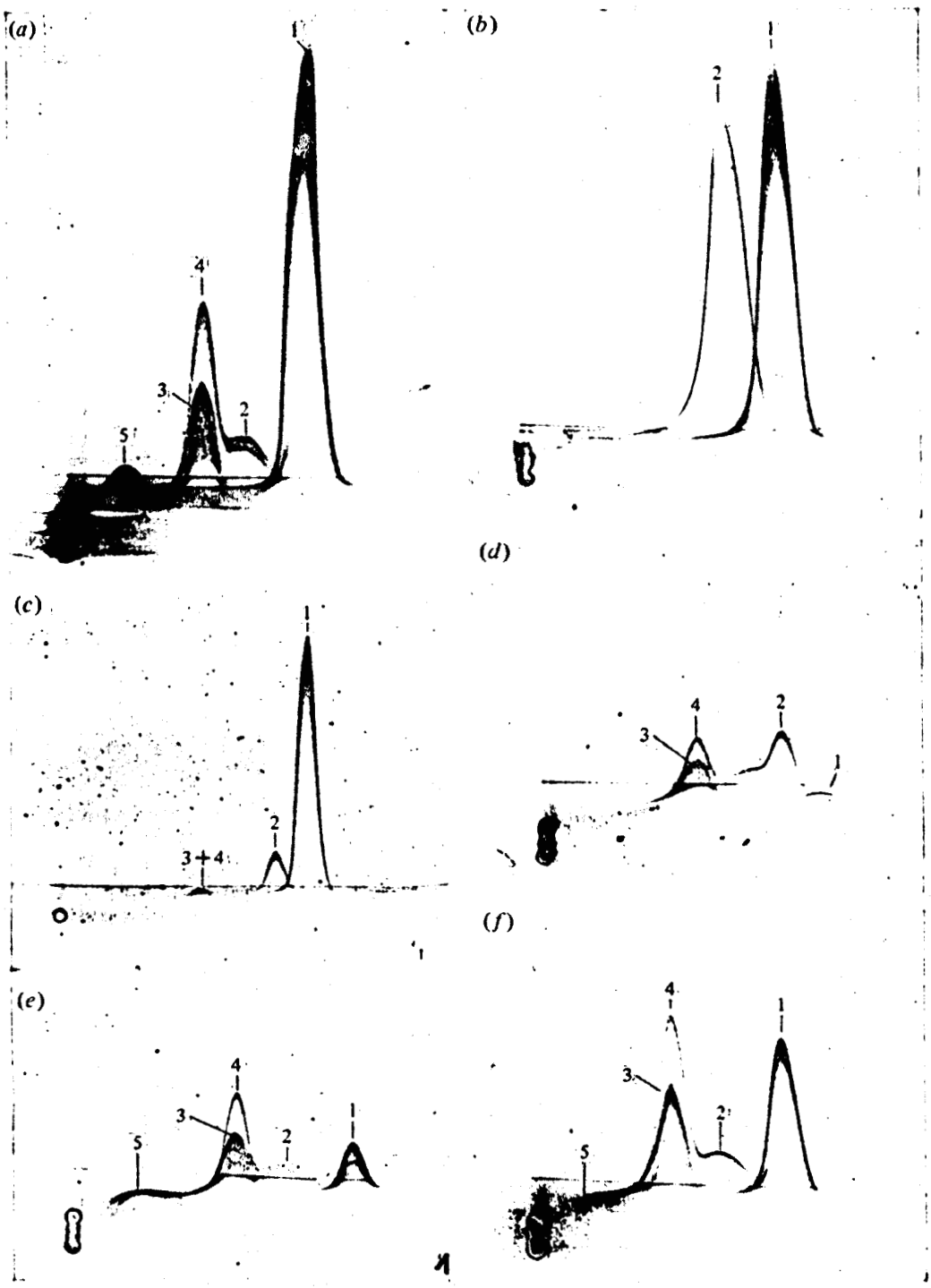

Fig. 1. Serological analysis of lysates of cells infected with adenovirus 2 WT or *ts* mutants at 39·5 °C. Six × 10^6 HeLa cells were disrupted in 100 μl of hypotonic buffer and 10 μl of cell lysate loaded in the well; 100 μl of antiserum against whole Ad 2 virion was added to agarose gel in the second dimension (plates *a*, *b*, *d* to *f*). On plate (*c*), 5 μl of cell extract was reacted against 50 μl of antiserum. (*a*) WT; (*b*) fibre-defective *ts* 115; (*c*) penton-defective *ts* 104; (*d*) hexon-negative *ts* 121; (*e*) hexon-defective *ts* 102; (*f*) hexon-defective *ts* 106.

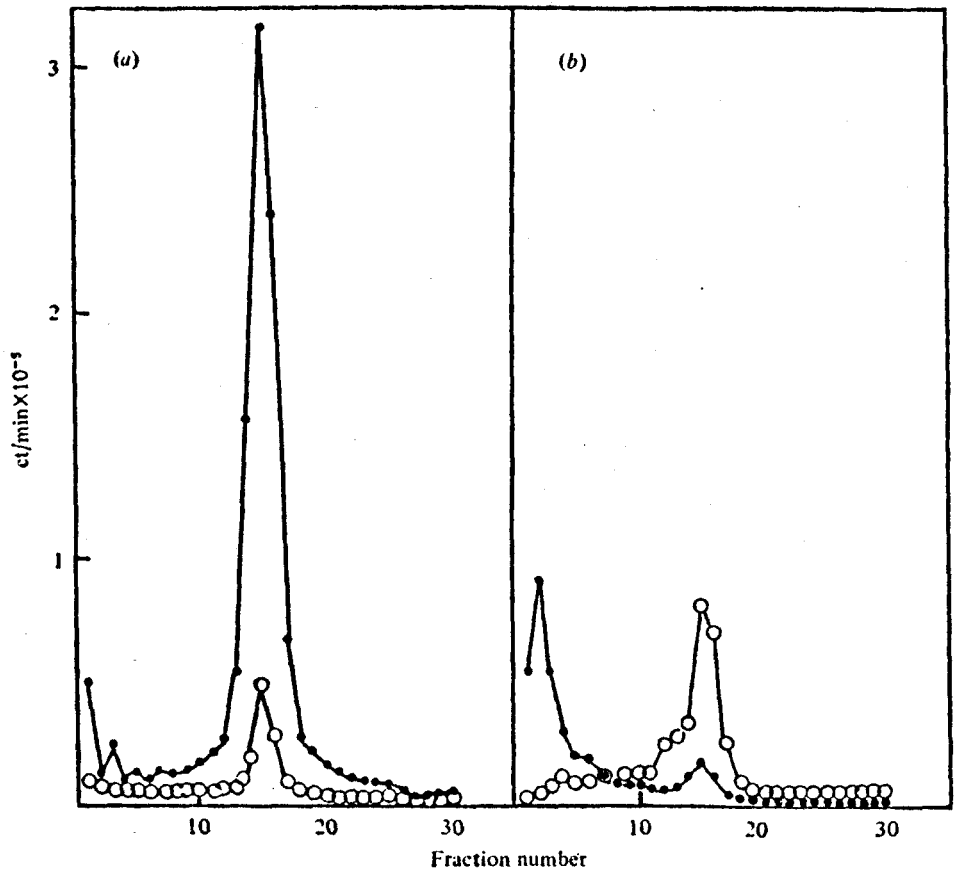

Fig. 2. Synthesis of virus DNA analysed by velocity sedimentation in alkaline sucrose gradient. HeLa cells infected with WT or *ts* mutants were labelled with ^{3}H-thymidine. (*a*) WT; (*b*) profile representative of *ts* 105 and 114. Fraction 15 corresponds to the position of 34S virion marker DNA. ○—○, 33 °C; ●—●, 39·5 °C.

devoid of densely-stained core, suggesting a thermosensitive lesion in a late function required for virus maturation (Fig. 3*c*).

In some cases dense amorphous material was visible in the cytoplasm surrounding the nucleus. These dense perinuclear inclusions were observed in infection with hexon-defective mutants showing no (or only a few) intranuclear particles, such as *ts* 110, 118, 121 and 123. Fig. 3*d* presents the dense inclusions induced by H 2 *ts* 118, which was reminiscent of the intracytoplasmic lesion reported for two hexon-defective adenovirus mutants H 2 *ts* 3 (Weber *et al.* 1977) and H 7 *ts* 19 (Estes & Butel, 1977).

Complementation

Complementation tests were carried out to group the *ts* mutants, and particularly those which presented the same phenotype, such as the DNA-negative mutants, the hexon-defective, penton- and fibre-defective mutants. The 19 mutants studied here fell into 11 groups of complementation. The complementation between the DNA-negative mutants was determined by two different methods: (i) comparison of the virus yields in single and

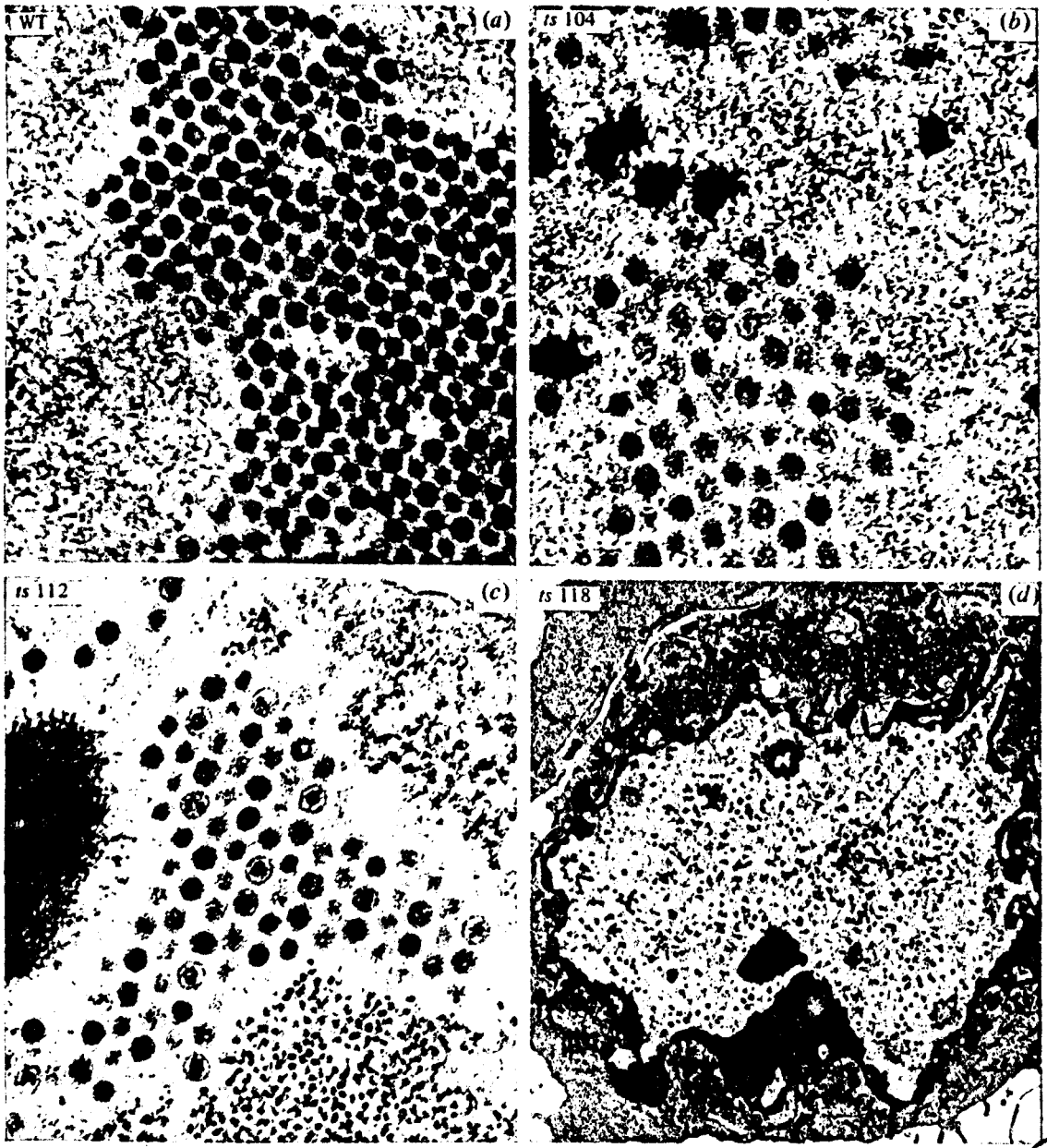

Fig. 3. Virion morphogenesis – electron microscopy of cells infected at 39·5 °C with (*a*) WT, (*b*) *ts* 104, (*c*) *ts* 112 and (*d*) *ts* 118. Many particles in (*b*) and (*c*) appear abnormal in morphology, lacking their densely stained inner core. In (*d*) electron-dense material is visible surrounding the nucleus. Magnifications: (*a*)–(*c*) ×80000; (*d*) ×10500.

Table 1. *Complementation between DNA-negative* ts *mutants of type 2 adenovirus (H 2)**

Mutants	*ts* 105	*ts* 111	*ts* 114
		Virus yield†	
ts 105	(9×10^6)	83 ($7{\cdot}5\times10^8$)	1·05 ($9{\cdot}6\times10^6$)
ts 111	—	(6×10^6)	65 ($3{\cdot}9\times10^8$)
ts 114	—	—	($3{\cdot}3\times10^6$)
		Virus DNA synthesis‡	
ts 105	(21650)	7·1 (153840)	0·7 (15550)
ts 111	—	(4220)	14·1 (105500)
ts 114	—	—	(7460)

* Complementation tests were performed as described in Methods.

† Values in table are complementation indices (CI), calculated from the virus yield, as explained in Methods. Complementation was considered as positive when the CI was $\geqslant$ 10. Values in parentheses are yields of infection expressed as p.f.u./ml.

‡ KB cells were infected at an input multiplicity of 25 p.f.u./cell with each mutant and labelled with 10 μCi/ml of ^{3}H-thymidine from 16 to 24 h p.i. The virus DNA was analysed as in Fig. 2. Values in the table are ratios of label in 34S DNA peak of the double infection to that of the higher of the two single infections at 39·5 °C, expressed as total ct/min. The leakiness of *ts* 105 explains the value of 7·1 for the CI calculated from the rates of DNA synthesis.

Table 2. *Complementation indices between DNA negative* ts *mutants of human type 5 (H 5) and type 2 (H 2) adenovirus**

Mutants	H 5 *ts* 36	H 5 *ts* 125	H 2 *ts* 111	H 2 *ts* 114	H 2 *ts* 105
H 5 *ts* 36	.	300	400	4·2	5·4
H 5 *ts* 125	.	.	50	600	120
H 2 *ts* 111	.	.	.	300	400
H 2 *ts* 114	.	.	.	.	4·0
H 2 *ts* 105	.	.	.	.	.

* Values in table are complementation indices calculated from the virus yields, as described in Methods.

Table 3. *Complementation between hexon-defective* ts *mutants of adenovirus 2**

Mutants	102	106	108	110	117	118	121	122	123
102	.	14	10	1·6	0·4	5200	46	1·2	0·4
106	.	.	4	12	10	56	27	11	NT†
108	.	.	.	19	18	1500	58	10	NT
110	.	.	.	.	5·3	600	20	1·0	1·0
117	.	.	.	.	.	1000	320	4·5	1·3
118	.	.	.	.	.	.	410	75	1700
121	.	.	.	.	.	.	.	30	1
122	.	.	.	.	.	.	.	.	NT
123	.	.	.	.	.	.	.	.	.‡

* Values in table are complementation indices, calculated from the virus yields, as described in Methods.

† NT = not tested.

‡ *Ts* 123 which did not complement both *ts* 102 and *ts* 121 appears as a possible double mutant.

double infections at 39·5 °C as indicated in Methods; (ii) comparison of the amount of label in the peak of 34S DNA in single and double infections at restrictive temperature. In the second method the complementation index was the ratio of virus DNA synthesis of the double infection to virus DNA synthesis of the higher of the two infections at 39·5 °C. Complementation was considered as positive with respect to DNA synthesis when the ratio was higher than 2. The results were concordant for both kinds of analysis. As shown in

Table 4. *Properties of* ts *mutants of human adenovirus type 2 at the restrictive temperature* (39·5 °C)

Mutant	Complementation group	Virus DNA synthesis	Inhibition of host DNA synthesis	Soluble antigens* Hexon	Penton base	Fibre	Virion morphogenesis (Intranuclear particles) Average no. per nucleus section	Morphology of particles†	Ratio of yields 33/39·5 °C‡
Class I									
ts 105	D	−	−	+	+	+	100	N+A	$3{\cdot}2\times10^{2}$
ts 114	D	−	−	+	+	+	50	N+A	$4{\cdot}0\times10^{2}$
ts 111	G	−	+	+	+	+	50	N+A	$1{\cdot}5\times10^{2}$
Class II§									
H (−)									
ts 118	H	+	+	− (0·0)	+	+	0	.	$5{\cdot}9\times10^{4}$
ts 121	A	+	+	− (0·0)	+	+	50	N	$1{\cdot}1\times10^{3}$
ts 102	B	+	+	± (0·18)	+	+	0	.	$8{\cdot}4\times10^{3}$
ts 110	B	+	+	± (0·19)	+	+	0	.	$1{\cdot}6\times10^{5}$
ts 117	B	+	+	± (0·19)	+	+	ND‖	ND	$4{\cdot}1\times10^{5}$
ts 122	B	+	+	± (0·15)	+	+	0	.	ND
ts 106	K	+	+	+ (0·33)	++	++	50	A	$5{\cdot}0\times10^{2}$
ts 108	K	+	+	+ (0·38)	++	+	500	N+A	$2{\cdot}1\times10^{3}$
ts 119	K	+	+	+ (0·31)	++	+	500	N	$9{\cdot}0\times10^{2}$
ts 109	ND	+	+	±	+	+	ND	ND	ND
ts 120	ND	+	+	±	+	+	ND	ND	$1{\cdot}3\times10^{2}$
ts 123	ND	+	+	±	+	+	0	.	$3{\cdot}0\times10^{6}$
ts 124	ND	+	+	−	+	+	ND	ND	ND
ts 126	ND	+	+	±	+	+	ND	ND	$4{\cdot}0\times10^{3}$
P (−)									
ts 103	F	+	+	++	±	±	ND	ND	$1{\cdot}2\times10^{4}$
ts 104	C	+	+	++	±	±	1000	N+A	$1{\cdot}3\times10^{5}$
ts 116	C	+	+	++	+	+	500	A	$1{\cdot}0\times10^{3}$
F (−)									
ts 115	E	+	+	++	++	−	1000	A	$2{\cdot}0\times10^{3}$
ts 125	E	+	+	++	++	−	1000	A	$2{\cdot}0\times10^{2}$
Class III									
ts 101	ND	+	+	++	++	++	1000	N+A	$2{\cdot}2\times10^{1}$
ts 107	J	+	+	++	++	++	500	N+A	$1{\cdot}7\times10^{2}$
ts 112	I	+	+	++	++	++	1000	N+A	$2{\cdot}7\times10^{1}$

* Soluble antigens: ++, amount of antigen identical to wild-type (WT) production; +, amount of antigen reduced by comparison with WT; ±, minute amount of antigen; −, no antigen detectable or measurable. Values in brackets are percents of WT hexon production at 39·5 °C, quantified by two-dimensional immunoelectrophoresis.

† Morphology of intranuclear particles visible on cell section: N, normal aspect; A, altered morphology.

‡ Minimal ratio of yields from cultures infected with a m.o.i. of 10 f.f.u./cell at both temperatures and harvested at 36 h (39·5 °C) and 92 h (33 °C) p.i.

§ Symbols: H (−), hexon-minus; P (−), penton-minus; F (−), fibre-minus mutants.

‖ ND = not determined.

Table 1, two of our three DNA negative *ts* mutants of adenovirus 2 belonged to the same complementation groups, whereas *ts* 111 was in a different group. These three DNA-negative *ts* mutants were tested with the adenovirus type 5 *ts* mutants 36 and 125. *Ts* 111 was complemented by both H 5 *ts* mutants and was therefore different from them. In contrast, there was a very low complementation efficacy between H 5 *ts* 36 and H 2 *ts* 105 or 114, suggesting that the same function was mutated for these three mutants (Table 2).

Nine hexon-minus mutants were analysed by complementation and four complementation groups emerged from the crosses (Table 3). *Ts* 102, 110, 117 and 122 which produced minute amounts of hexon antigen, belonged to the same group (B), whereas *ts* 106 and 108, producing appreciable, although reduced, quantities of hexon, were in another group (K). The *ts* 118 (H) and *ts* 121 (A) which complemented each other and the other hexon-defective *ts* mutants appeared in two different groups: both of them failed to synthesize hexon antigen (Fig. 1*d*).

DISCUSSION

We have presented here the phenotypic characterization of 25 *ts* mutants of human adenovirus type 2, among which 19 could be assigned to 11 complementation groups. They were analysed with respect to their synthesis of virus DNA, soluble antigens and capsid morphogenesis. Their biological properties are summarized in Table 4. The interest of the serological characterization is obviously limited: most of the *ts* mutants appeared abnormal in production of soluble capsid antigen(s), but some of them exhibited a normal two-dimensional immunoelectrophoretic pattern. However, these antigens were normally produced in excess by cells infected with adenoviruses and some change in the over-production of the capsid antigens did not necessarily signify that the thermosensitive lesion was located on this particular antigen. Similarly a normal pattern of antigens does not imply a functional integrity of these antigens. In addition, since labile capsid intermediates could fall apart during extraction (Edvardsson *et al.* 1976), virion morphogenesis was analysed by electron microscopy of virus particles in cell sections. According to the soluble antigen pattern in two-dimensional immunoelectrophoresis, three serological classes could be discerned. In class I, there was a global and homogeneous reduction of all the virus antigens. In class II, there was a drastic reduction of one (or several) soluble antigen(s). In class III, the antigenic pattern was similar to that of WT.

The first class contained three DNA-negative mutants, whose minor antigen production represented either a certain degree of leakiness of the system, or the result of transcription and translation of the parental genomes. Biochemical analysis of these three early mutants, H 2 *ts* 105, 111 and 114, arranged in two complementation groups (Table 1), confirmed previous findings that the onset of adenovirus DNA replication was not required to shut-off of host DNA synthesis (Ensinger & Ginsberg, 1972). H 2 *ts* 105 and H 2 *ts* 114, partially depressed host DNA synthesis at restrictive temperature, while the H 2 *ts* 111 maintained this host DNA synthesis shut-off at 39·5 °C to the same extent as WT (to be published). This suggested that virus DNA replication and cellular DNA synthesis inhibition were independent and dissociable functions. However, it must be considered that host DNA synthesis inhibition may simply be due to the leakiness of all the DNA-negative *ts* mutants examined to date.

Our three mutants H 2 *ts* 105, 111 and 114 were complemented by H 5 *ts* 125 efficiently, suggesting that their mutated functions were different from that involved in H 5 *ts* 125 (Ginsberg & Young, 1977). By contrast, H 5 *ts* 36 complemented poorly both *ts* 105 and *ts* 114 (Table 2), suggesting a lesion of a similar function. However, since H 5 *ts* 36 inhibits almost totally the host DNA synthesis at 39·5 °C (Wilkie *et al.* 1973), which was not the case for either H 2 *ts* 105 or H 2 *ts* 114, it is possible that their lesions are in fact different. Other DNA-negative mutants of Ad 2 have been isolated and characterized recently (Kathmann *et al.* 1976): H 2 *ts* 206 is complemented by H 5 *ts* 125 and H 5 *ts* 36 with a high efficiency, and does not inhibit the host DNA synthesis. Maintenance of H 2 *ts* 206 for 4·5 h at 32·5 °C results in normal synthesis of virus DNA during the subsequent

incubation phase at 39·5 °C, whereas the synthesis of H 2 *ts* 105 and H 2 *ts* 114 DNA is inhibited upon shift-up, whatever the time of incubation at permissive temperature. H 2 *ts* 206 appears therefore as an earlier mutant than H 2 *ts* 105 and H 2 *ts* 114.

In the serological class II, the hexon-defective mutants could be arranged in four complementation groups, and divided into three serological subclasses, according to their immunological patterns. In the light of recent observations (Ginsberg & Young, 1977), it was scarcely surprising that, using a complementation index of greater than or equal to 10, four complementation groups were found for the hexon-defective *ts* mutants. Hexon being a multimeric protein, intracistronic complementation are possible and have been suggested (Williams *et al.* 1974). The low complementation efficacy between mutants of groups B and K appears to support this hypothesis. Preliminary biochemical data suggested that *ts* 121 (A) was mutated on the hexon polypeptide gene, whereas *ts* 118 (H) was altered on the hexon assembly (unpublished results). The other hexon-defective mutants of both groups B and K, which complemented poorly (*ts* 102, 110, 117 and 122, and *ts* 106, 108 and 119) might be tentatively gathered in a unique group. However, the properties of the mutants of groups B and K were significantly different (Table 4), which justifies the arrangement in two separate groups for the moment.

Among the other late *ts* mutants of the serological class II, H 2 *ts* 104 and H 2 *ts* 115, which were respectively penton-defective and fibre-defective, assembled their capsids at restrictive temperature (Table 4). This phenomenon was reminiscent of fibre-minus mutants of phage-ϕ 29, which can assemble without their head-fibres (Reilly *et al.* 1977), and suggested that the penton-fibre structure was not indispensable for adenovirus assembly. Fibre-defective *ts* mutants of adenovirus 5 also assemble capsids (Edvardsson *et al.* 1978).

The serological class III mutants contained three mutants with normal patterns of antigens, and abundant intranuclear particles of both normal and abnormal features (Table 4). These mutants, H 2 *ts* 101, *ts* 107 and *ts* 112, appeared to be altered in a late function required for capsid assembly and/or virus maturation. This was the case for H 2 *ts* 112 which has been found to be blocked at a stage of assembly preceding the encapsidation of virus DNA; this mutant accumulates light intermediate particles of density 1·315 (D'Halluin *et al.* 1978).

As already mentioned (Russell *et al.* 1972), one of the main interests in these *ts* mutants of adenovirus type 2, a representative of Rosen's subgroup III (Rosen, 1960), is the possibility of preparing major or minor virus components not normally accessible to investigation: the fibre-defective mutant H 2 *ts* 115 is thus used in our laboratory for isolation of penton base which accumulates in the absence of fibre and of antigen IIIa (Everitt *et al.* 1973) which is usually masked by complete penton in two-dimensional immunoelectrophoretic patterns.

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Adenovirus Early Function Required for Protection of Viral and Cellular DNA

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Studies were done to characterize a DNA-negative temperature-sensitive (*ts*) mutant of human adenovirus type 2, H2 *ts*111. The temperature-sensitive defect, which was reversible on shift-down in the absence of protein synthesis, was expressed as early as 2 h postinfection, and the results of density-labeling experiments are in agreement with at least a DNA replication initiation block. On shift-up, after allowing viral DNA synthesis at permissive temperatures, the newly synthesized viral DNA and the mature viral DNA were cleaved into fragments which sedimented as a broad peak with a mean coefficient of 10–12S. This cleavage was more marked in the presence of hydroxyurea as the DNA synthesis inhibitor. Parental DNA in infected cells was degraded to a much lesser extent regardless of the incubation temperature. In contrast, the parental DNA was strongly degraded when early gene expression was permitted at 33°C before shift-up to 39.5°C. Furthermore, cellular DNA was also degraded at 39.5°C in *ts*111-infected cells, the rate of cleavage being related to the multiplicity of infection. This cleavage effect, which did not seem to be related to penton base-associated endonuclease activity, was also enhanced when early gene expression was allowed at 33°C before shift-up. The *ts*111 defect, which was related to an initiation block and endonucleolytic cleavage of viral and cellular DNA, seemed to correspond to a single mutation. The implication of the *ts*111 gene product in protection of viral and cellular DNA by way of a DNase-inhibitory function is discussed.

Thermosensitive (*ts*) mutants which cannot replicate DNA at nonpermissive temperatures have been isolated from human adenovirus type 2 (Ad2) (10, 12), Ad5 (5, 30), Ad12 (11, 23), and Ad31 (25, 26). Partial characterization of these mutants suggests that adenovirus replication needs at least three virus-coded early gene products (8, 10, 23), each probably being required for a step in chain initiation. Moreover, a very early function has been detected which does not seem to be directly involved in DNA synthesis (10). To our knowledge, no other DNA synthesis-related early function associated with adenovirus DNA-negative phenotypes has been previously reported.

In the present paper, an early function which is altered in cells infected at the nonpermissive temperature with the H2 *ts*111 DNA-negative mutant (12) is reported. Data presented show that viral as well as cellular DNAs are degraded throughout the replicative cycle. The results suggest that an early viral function prevents both viral and cellular DNAs from endonuclease-like cleavage.

MATERIALS AND METHODS

Cells and virus. KB cells were cultured in suspension in Joklik-modified F13 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% horse serum. HeLa cells were grown as monolayers in Eagle minimum essential medium containing 10% calf serum.

The wild-type (WT) Ad2 was originally supplied by J. F. Williams (Carnegie-Mellon Institute, Pittsburgh, Pa.). The Ad2 thermosensitive mutant *ts*111 has undergone preliminary characterization (12). Virus particles were produced and purified as previously described (4). Infectivity was measured by the fluorescent focus assay technique (16) or by the plaque method on HeLa cells. The infectivity titers were expressed in fluorescent focus units or PFU. Cells were infected at a multiplicity of infection (MOI) of 10 to 100 PFU/cell as indicated in each experiment.

Radioisotopes and counting method. [^{3}H]thymidine (27 Ci/mmol) and [^{14}C]thymidine (53.6 mCi/mmol) were purchased from the Commissariat à l'Energie Atomique (Saclay, France). The radioactive samples were precipitated with cold 10% trichloroacetic acid and filtered on Whatman GF-C filters. The filters were washed successively with 2% trichloroacetic acid and cold ethanol, air dried, and counted in a

toluene-based scintillation fluid in an Intertechnique liquid spectrometer.

Labeling conditions. Infected cells were labeled with [^{3}H]thymidine (5 μCi/ml). For pulse-chase experiments, 5×10^{-5} M nonradioactive thymidine (Sigma Chemical Co., St. Louis, Mo.) was added. Density labeling of DNA was accomplished by adding 10^{-4} M 5-bromo-2'-deoxyuridine (Sigma). Noninfected HeLa cells were labeled with [^{14}C]thymidine (1 μCi/ml). WT and *ts*111-purified, DNA-labeled virus particles were prepared from infected cells maintained at 33°C and labeled with [^{3}H]thymidine (1 μCi/ml) from 24 to 96 h. Temperature shifts were made on HeLa cell monolayers by replacing the medium with a medium prewarmed at the required temperature.

Analysis of DNA by zonal sedimentation. (i) Alkaline sucrose gradient centrifugation. The method used for alkaline sucrose gradient centrifugation was that described by Doerfler (5). A total of 5×10^5 to 1×10^6 cells suspended in 100 μl of Tris-saline (0.15 M NaCl + 0.01 M Tris-hydrochloride, pH 7.5) were loaded on top of a 5 to 20% alkaline sucrose gradient (0.3 M NaOH + 0.7 M NaCl + 0.01 M EDTA) and overlaid with 0.4 ml of lysis buffer (1 M NaOH + 0.05 M EDTA). After standing for 16 h at 4°C, the gradients were centrifuged at 4°C in an SW 41 rotor at 35,000 rpm for 5 (viral DNA) or 3 (cellular DNA) h.

(ii) Centrifugation in neutral condition. A total of 10^6 cells were suspended in 0.01 M Tris + 0.001 M EDTA + 0.3 M NaCl + 0.03 M trisodium citrate (pH 7.4) containing 0.5% Sarkosyl (Ciba-Geigy) and 1 mg of pronase (Boehringer; predigested at 37°C for 3 h and heated for 2 min at 85°C before use) per ml and lysed for 3 h at 37°C. Lysates were gently pipetted on top of a 5 to 20% neutral sucrose gradient (1 M NaCl + 0.01 M EDTA + 0.05 M Tris + 0.1% Sarkosyl, pH 7.4) and centrifuged for 5 h at 35,000 rpm at 4°C in an SW 41 rotor. 5-Bromo-2'-deoxyuridine density-labeled DNA was analyzed by centrifugation on a self-generating CsCl gradient (1.7 g/ml) for 72 h at 35,000 rpm and 20°C in an R 50Ti rotor. The gradients were collected dropwise from the bottom of the tubes, and the fractions were assayed for acid-precipitable radioactivity.

Assay for penton base-associated endonuclease activity. The assay for penton base-associated endonuclease activity was carried out as described by R. G. Marusyk (personal communication).

Virus particles (WT and *ts*111) were purified by two cycles of equilibrium centrifugation in CsCl. The virion preparations were diluted to the same optical density (at 260 nm) per milliliter. Virion-derived pentons were obtained by dialysis of virions versus distilled water for 2 h, followed by dialysis against 0.005 M Tris-maleate buffer, pH 6.2, for 16 h at 20°C. The dialysate was centrifuged at 35,000 rpm for 2 h at 20°C in an SW 50.1 rotor. The supernatant, dialyzed for 2 h against 0.01 M citrate, pH 4.5, was used as a source of virion-derived pentons. Adenovirus DNA was extracted from purified WT adenovirus. The incubation mixture consisted of 0.01 M NaCl, 0.01 M citrate, 0.002 M $MgCl_2$, pH 4.5, and 1.5 μg of Ad2 DNA plus an aliquot of the virion-derived penton in a total volume of 20 μl. The reaction was carried out at 39.5°C for 10 min and was stopped by adding 0.01 M EDTA. The size of the DNA was determined by electrophoresis in agarose gel.

Inhibitors. Cycloheximide (Boehringer-Mannheim) was used at a concentration of 20 μg/ml, and hydroxyurea (Serva) was used at a final concentration of 0.01 M.

RESULTS

Viral DNA synthesis after temperature shift-up and shift-down. The efficiency of viral DNA synthesis shutoff after shift-up to the nonpermissive temperature was studied. KB cells infected with *ts*111 were incubated at 33°C for 40 h and subsequently shifted to 39.5°C or kept at 33°C. The infected cells were then labeled with [^{3}H]thymidine from 40 to 42 h post-infection (p.i.). Analysis of labeled DNA at 42 h p.i. on alkaline sucrose gradients showed that there was almost no viral DNA synthesis after shift-up (Fig. 1). Shift-down experiments were performed to determine if the *ts*111 DNA block was reversible. KB cells infected with *ts*111 were maintained at 39.5°C for 17 h and shifted to 33°C or kept at 39.5°C. [^{3}H]thymidine was then added from 17 to 19 h p.i. Viral DNA synthesis immediately resumed after the shift-down even when protein synthesis was inhibited by cycloheximide (Fig. 2).

These data indicate that after shift-up to 39.5°C the capacity to synthesize *ts*111 DNA slows down rapidly and that inactivation of the thermosensitive *ts*111 function is readily reversible, even in the absence of protein synthesis. The existence of fragments lighter than 34S, noticed particularly in cycloheximide-treated cells, suggested the possibility of viral DNA degradation or lack of ligation of Okazaki-like fragments.

Reversibility of the *ts*111 defect by temperature shift-down: infectious virus and viral DNA. To establish the time at which the *ts*111 altered function was expressed in the infectious cycle, *ts*111-infected cells were incubated for varying periods at 39.5°C and shifted down to 33°C. The reversibility of the mutation was determined by titrating the infectious *ts*111 virus produced and by quantitating the synthesized 34S DNA.

HeLa cells infected with *ts*111 were incubated for varying periods of time at 39.5°C and shifted down to 33°C until 98 h p.i. The infectious virus present in crude cell lysates was titrated by the fluorescent focus assay. Figure 3b shows the curve obtained by plotting the values of final virus yields versus time. As early as 2 h p.i., a strong effect was observed at the nonpermissive temperature. It can be concluded, therefore, that the function altered in *ts*111 infection is essential very early in the productive cycle. Analysis of

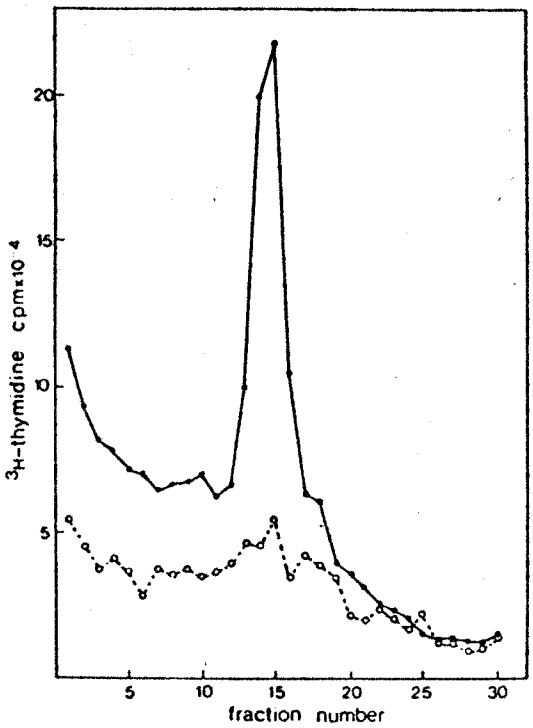

FIG. 1. *Analysis in alkaline sucrose gradients of the DNA synthesized on shift-up to 39.5°C in KB cells infected with mutant ts111. KB cell suspensions inoculated with H2 ts111 at an MOI of 25 PFU/cell were incubated at 33°C for 40 h and subsequently shifted to 39.5°C (○) or kept at 33°C (●). The infected cells were labeled with [^{3}H]thymidine (10 μCi/ml) from 40 to 42 h p.i. At the end of the labeling period, the cells were washed, and aliquots of 5 × 10^5 cells were lysed in alkali on top of alkaline sucrose gradients. The conditions of centrifugation have been described. The direction of sedimentation is to the left.*

the viral DNA synthesis capacity was carried out by infecting KB cells with *ts*111 and incubating the infected cells at 39.5°C for 10, 16, 20, 24, and 32 h before shift-down to 33°C until 44 h p.i. The infected cells were then labeled with [^{3}H]thymidine between 40 and 44 h p.i. Alkaline sucrose gradient analysis revealed that extracted DNA sedimented almost exclusively at 34S. The amount of viral DNA synthesized was determined from the radioactivity in the 34S peak, and the results were expressed as a percentage of the amount of labeled 34S viral DNA synthesized in *ts*111-infected cells at the permissive temperature (Fig. 3a). It was noticed that DNA synthesis was much less rapidly depressed than expected from the previous results. This suggests that a fraction of the viral DNA which appeared on shift-down was somehow unable to evolve into infectious virions.

Viral DNA replication and *ts*111 function. The possible implication of the altered *ts*111 function in the viral DNA replication process was determined as follows. KB cells infected with *ts*111 at 33°C and labeled with [^{3}H]thymidine from 40 to 42 h p.i. were chased for 4 h at 33 or 39.5°C in the presence of 5-bromo-2′-deoxyuridine (10^{-4} M). After Sarkosyl-pronase treatment, the infected cell lysates were subjected to equilibrium centrifugation in cesium chloride. Controls were performed with WT virus (not shown). 5-Bromo-2′-deoxyuridine was not incorporated into viral DNA in the shift-up experiment, whereas a peak was observed at the heavy-

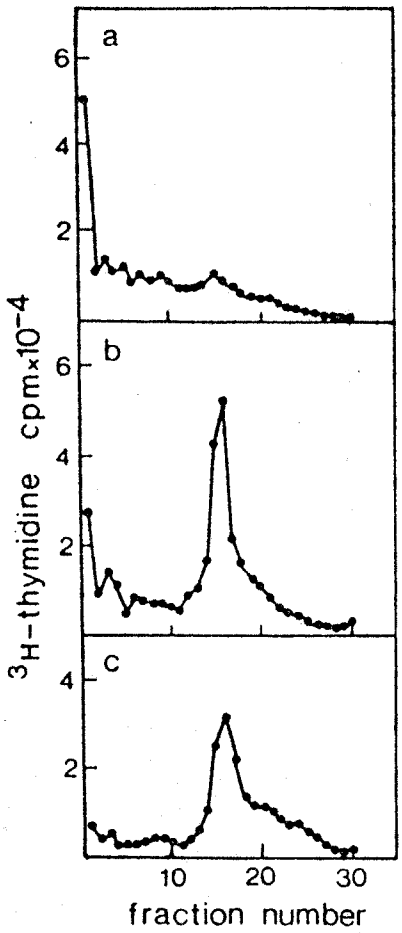

FIG. 2. *Analysis in alkaline sucrose gradients of the DNA synthesized on shift-down to 33°C in KB cells infected with ts111. KB cells inoculated with H2 ts111 at an MOI of 25 PFU/cell were incubated at 39.5°C for 17 h and kept at 39.5°C (a) or shifted to 33°C (b) in the presence of 20 μg of cycloheximide per ml (c). The cells were labeled from 17 to 19 h p.i. with [^{3}H]thymidine (10 μCi/ml). At the end of the labeling period, the cells were treated as described in the legend to Fig. 1.*

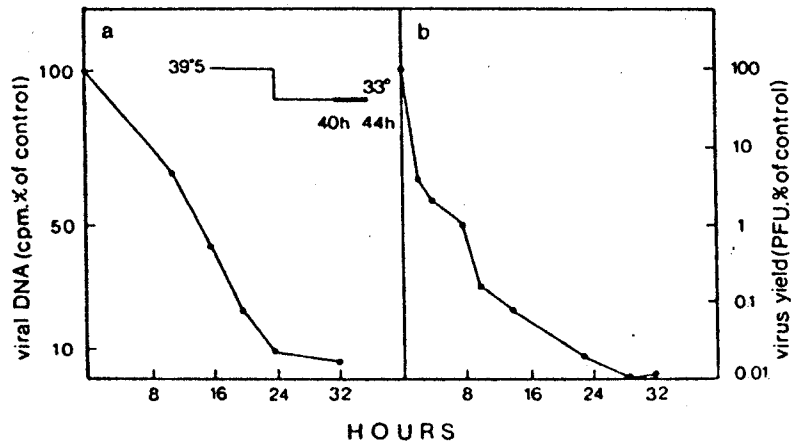

FIG. 3. *Time dependence of the ts111 defect reversibility. (a) Viral DNA synthesis: 5 × 10⁵ KB cells infected with ts111 (MOI = 25) were incubated at 39.5°C for 0, 10, 16, 20, 24, and 32 h before shift-down to 33°C until 44 h p.i. The cells were labeled from 40 to 44 h p.i. with [³H]thymidine (10 μCi/ml). At the end of the labeling period, the cells were treated as described in the legend to Fig. 1. The amounts of synthesized viral size DNA were determined from the radioactivity in the 34S peak. The results are expressed as a percentage of the quantity of labeled viral DNA in ts111-infected cells incubated at 33°C until 44 h p.i. (b) Virus yields: 5 × 10⁶ HeLa cells infected with ts111 (MOI = 25) were incubated at 39.5°C for different lengths of time before shift-down to 33°C until 98 h p.i. Infectious virus production was monitored by the fluorescent focus method, and the results are expressed as a percentage of the virus yield at 98 h p.i. at 33°C. The x axis indicates the time p.i. at which cultures were shifted down to 33°C.*

light density position with extracts from cells maintained at 33°C (Fig. 4). These results indicate that observable reinitiation and elongation do not take place at the nonpermissive temperature.

Fate of newly synthesized viral DNA. To determine whether the viral DNA synthesized at the permissive temperature was affected when the mutation was expressed, the following experiment was carried out. KB cells infected with *ts*111 were incubated at 33°C and labeled with [³H]thymidine between 40 and 42 h p.i. The infected cells were separated into three aliquots; one was stored at +4°C before analysis, and cold thymidine was added to the two others before incubation at 39.5 or 33°C until 46 h p.i. Labeled DNA was analyzed in alkaline sucrose gradients (Fig. 5). A predominant 34S peak was observed just after labeling, together with 40–100S intermediate-size DNA and heavy cellular DNA (Fig. 5a) as described previously (2, 7, 20). After a 4-h chase at 33°C, the 34S peak diminished and slower-sedimenting fragments appeared (Fig. 5b). When the 4-h chase was performed at 39.5°C, the radioactivity was found at sedimentation rates between 7 and 34S (Fig. 5c). Trichloroacetic acid-precipitable counts remained constant during the chases. The *ts*111 lesion, which appeared fully expressed at 39.5°C, was therefore also observed at 33°C, although to a much lesser degree. Under the same conditions, the WT DNA appeared stable (12). These data indicate that the function modified by the *ts*111 mutation is essential to the maintenance of normal-length viral DNA.

The low-molecular-weight DNA material was of viral origin since it hybridized at a rate of >90% with Ad2 WT DNA and at <2% with control KB cell DNA. When analyzed in agarose gel electrophoresis, the cleaved DNA showed a polydisperse pattern, with a band smearing between the position of *Eco*RI fragments A and C of Ad2 DNA used as markers, suggesting a low degree, if any, of cleavage specificity (not shown).

Effect of DNA synthesis inhibition on viral DNA degradation. To determine whether the *ts*111 lesion affects the DNA molecules during their replication cycle or their stability independently of the replication process, hydroxyurea was used as a DNA synthesis inhibitor (24, 28). *ts*111-infected HeLa cells were incubated at 33°C for 42 h and labeled with [³H]thymidine between 40 and 42 h p.i. The infected cells were divided into three equal parts; one was removed immediately after labeling and stored at +4°C until analyzed, and cold thymidine was added to the two others, which were immediately shifted to 39.5°C and maintained at this temperature for 4 h. Hydroxyurea was added to one aliquot at the time of shift-up. DNA was analyzed in alkaline and neutral su-

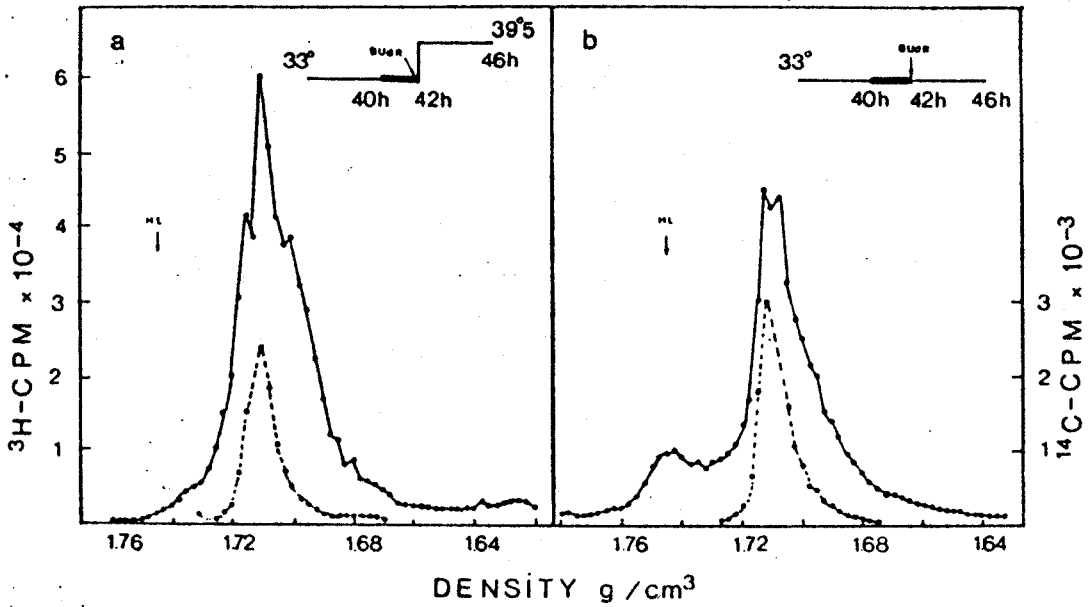

FIG. 4. *CsCl density gradient centrifugation of H2 ts111 DNA labeled with 5-bromo-2'-deoxyuridine (BUdR) at 39.5°C. KB cells infected with H2 ts111 (MOI = 15) were incubated for 42 h at 33°C and labeled with [^{3}H]thymidine (10 μCi/ml) from 40 to 42 h. BUdR (10^{-4} M) and nonradioactive thymidine (5×10^{-5} M) were added at 42 h p.i. Cells were harvested after a further 4 h of incubation at (a) 39.5 or (b) 33°C and treated with Sarkosyl and pronase as described in the text. Viral DNA was analyzed by centrifugation on a self-generating CsCl gradient (1.7 g/ml) in the presence of ^{14}C-labeled Ad2 marker DNA. HL indicates the heavy-light density position. Symbols: ●——●, ^{3}H; ●---●, ^{14}C.*

crose gradients (Fig. 6). Immediately after labeling, single-stranded DNA was found at 34S and at lower S values, confirming the occurrence of partial degradation even at the permissive temperature (Fig. 6a). After the chase at 39.5°C, the DNA peak was observed at a sedimentation rate always lighter than 34S (Fig. 6b). When hydroxyurea was added at the time of shift-up, the amount of light DNA fragments strongly increased (Fig. 6c). These results suggest that the main altered function in *ts*111-infected cells is not related to the viral DNA replication process, since DNA breakdown still occurred at a higher rate in the absence of DNA replication.

Analysis on neutral sucrose gradients showed that viral DNA sedimented between 31S and 10S (Fig. 6d), or in some experiments only at 31S. Radiolysis did not account for these results since the sedimentation pattern was not modified regardless of the total incorporated radioactivity. Therefore, single-strand "nicks," starting points of the degradation process, may explain these data.

Pulse-chase of viral DNA. It was shown above that newly synthesized viral DNA was cleaved into small fragments at 39.5°C. Since mature viral DNA might be protected against degradation, it was of interest to study the fate of labeled viral DNA at 39.5°C after different periods of chase at 33°C. KB cells infected with *ts*111 at 33°C and labeled with [^{3}H]thymidine from 40 to 42 h p.i. were shifted up to 39.5°C for 4 h at 42, 46, 50, and 64 h p.i. Hydroxyurea was added at the time of shift-up to prevent viral DNA from being repaired during the chase period at 39.5°C.

The amount of label observed in the alkaline sucrose gradient 34S peak after the 2-h pulse decreased slightly after an 8-h chase at 33°C, and only a few lighter fragments appeared. In contrast, whenever the chase period at 33°C was followed by a shift-up to 39.5°C, marked DNA fragmentation occurred (not shown). These data suggest that the function altered in *ts*111 infection is essential to the integrity of viral DNA regardless of its time of synthesis.

Fate of parental DNA. Since synthesized viral DNA was broken down at 39.5°C, an experiment on the fate of parental DNA at the nonpermissive temperature was performed. KB cells were infected with [^{3}H]thymidine-labeled *ts*111 virions, and the parental viral DNA was examined in alkaline sucrose gradients under the following conditions of incubation: (i) 39.5°C for 16 h (Fig. 7a); (ii) 33°C for 48 h (Fig. 7b); (iii) 33°C for 24 h and shift-up for 16 h (Fig. 7c); (iv)

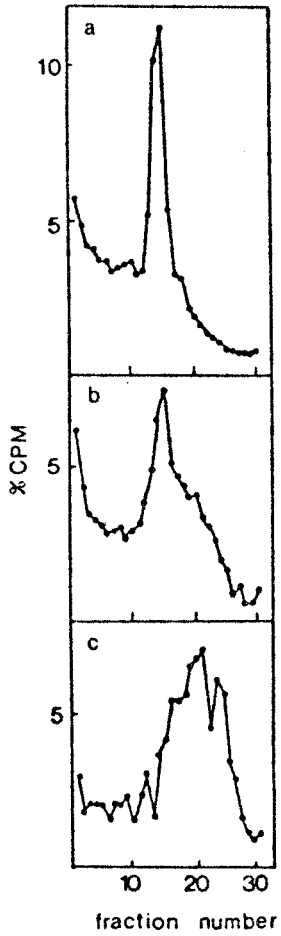

FIG. 5. *Analysis of newly synthesized DNA in KB cells infected with ts111. KB cell suspensions were infected with ts111 (MOI = 25), incubated at 33°C for 42 h, and labeled from 40 to 42 h p.i. with [^{3}H]-thymidine (10 μCi/ml). (a) Cells harvested at 42 h p.i. (b) Cells incubated and radioactivity chased at 33°C until 46 h p.i. (c) Cells incubated at 39.5°C and chase performed until 46 h p.i. Analysis was performed on alkaline sucrose gradients as indicated in the legend to Fig. 1. The results are expressed as a percentage of counts per minute (CPM) in the gradient.*

33°C for 48 h and shift-up for 16 h (Fig. 7d); (v) addition of hydroxyurea and then incubation at 33°C for 48 h and shift-up for 16 h (Fig. 7e). A control experiment was carried out by coinfecting KB cells with DNA-labeled *ts*111 and unlabeled WT for 18 h at 39.5°C (Fig. 7f). The *ts*111 parental DNA was slightly degraded at 39.5°C (Fig. 7a) and 33°C (Fig. 7b). In contrast, incubation at 33°C for 24 (Fig. 7c) and 48 (Fig. 7d) h followed by a shift-up at 39.5°C for 16 h provoked a strong degradation of the parental DNA. This degradation was much more marked when hydroxyurea was added (Fig. 7e). The data presented indicate that parental DNA is only slightly degraded when infection takes place at the nonpermissive temperature, whereas it is almost completely broken down when early genes are expressed for 24 and 48 h at 33°C before incubation at 39.5°C.

Fate of prelabeled cellular DNA. Since the above results suggested that the *ts*111 altered function might be implicated in the protection of viral DNA, it was of interest to establish whether this function was specific for protection of viral DNA or could also affect the fate of cellular DNA. HeLa cell monolayers were labeled at 37°C for 24 h with [^{14}C]thymidine before infection with *ts*111 at 39.5°C. Similar experiments were performed with WT-infected cells or cells doubly infected with *ts*111 and *ts*114. *ts*114 is a DNA-negative mutant which complements *ts*111 (12). The cellular DNA was analyzed in alkaline sucrose gradients at 40 h p.i. When cells were infected with *ts*111, a large fraction (46%) of the cellular DNA sedimented between 20 and 50S (Fig. 8a), whereas single infection with WT (not shown) or double infection with *ts*111 and *ts*114 (Fig. 8b) resulted in reduced degradation (20 and 18%). These data show that the normal function of the product modified in *ts*111 infection may be inhibition of nonspecific cellular nucleases.

Influence of MOI on cellular DNA breakdown. During previous experiments, it was noticed that the *ts*111 mutant could not be used at a high MOI as high levels of late cytotoxicity were produced (unpublished data). It was of interest, therefore, to look at the fate of cellular DNA when cells were infected at several different MOIs. HeLa cells in monolayer were labeled at 37°C for 48 h with [^{14}C]thymidine and chased for 24 h. The cells were infected at 39.5°C with *ts*111 at MOIs of 5, 25, 50, 100, and 200 PFU/cell. The following controls were carried out at the same temperature: mock-infected cells, WT-infected cells (MOI = 100), and WT-*ts*111 double-infected cells (MOI = 5 or 50 each). DNA was analyzed on alkaline sucrose gradients at 24 h p.i. Results are summarized in Table 1. Whereas DNA breakdown in control experiments was 24 and 27%, it increased with *ts*111 infection from 34% at an MOI of 5 to 63% at an MOI of 200. The MOI does, then, affect the rate of DNA degradation. These data may be explained in at least two ways: (i) *ts*111 infectious virus may contain a modified structural protein with enhanced endonucleolytic activity; (ii) the amount of the putative altered protein could be a function of the number of expressed genomes. The data presented above do not allow a clear choice between the two explanations.

Penton base-associated endonuclease

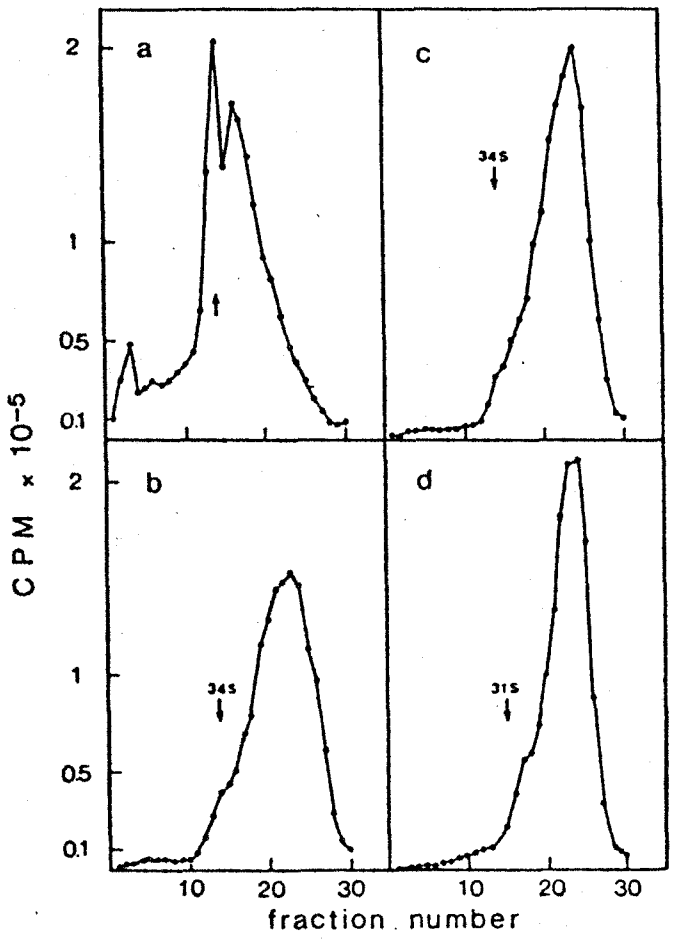

FIG. 6. *Fate of the newly synthesized viral DNA on shift-up to 39.5°C in the presence of hydroxyurea. KB cells infected with ts111 (MOI = 25) were incubated at 33°C until 42 h p.i. and labeled with [³H]thymidine (10 μCi/ml) from 40 to 42 h. The added radioactivity was chased with 5 × 10⁻⁵ M nonradioactive thymidine. Analysis in alkaline sucrose gradients was performed as indicated in the legend to Fig. 1. (a) Cells at 42 h p.i. (b) Cells after 4-h chase at 39.5°C. (c) Cells after 4-h chase at 39.5°C in the presence of hydroxyurea (10 mM). Analysis in neutral sucrose gradients was performed as described in the text. (d) Cells after 4-h chase at 39.5°C in the presence of hydroxyurea (10 mM). The arrow indicates the position of the Ad2 marker DNA.*

activity. Endonuclease activity has been found in association with the penton subunit of adenovirus (3, 13). This activity has also been described and characterized in extracts from uninfected and adenovirus-infected cells (17, 18, 29).

An experiment was carried out to establish if the penton base-associated endonuclease activity was modified in the *ts*111 virion. Virion-derived pentons (WT and *ts*111) and Ad2 DNA were prepared, and the assays were performed as described in Materials and Methods. Three concentrations (1×, 5×, 10×) of WT and *ts*111 virion-derived pentons were studied. The degree of cleavage was almost identical for the WT and *ts*111 penton preparations at each concentration (not shown). This result shows that the *ts*111 mutation apparently does not affect the penton-associated endonuclease activity. This is in agreement with the finding that this activity is of cellular origin (L. Tsang and R. G. Marusyk, personal communication).

Relationship between synthesis of early proteins and cellular DNA breakdown. The possibility that an increase in cellular DNA breakdown was related to the enhanced synthesis of *ts*111-induced early proteins was studied by infecting cells with *ts*111 at the permissive temperature for a convenient period before shift-up to 39.5°C, thus allowing expression of the mutation. HeLa cells were labeled for 72 h with [¹⁴C]thymidine. The cells were infected with

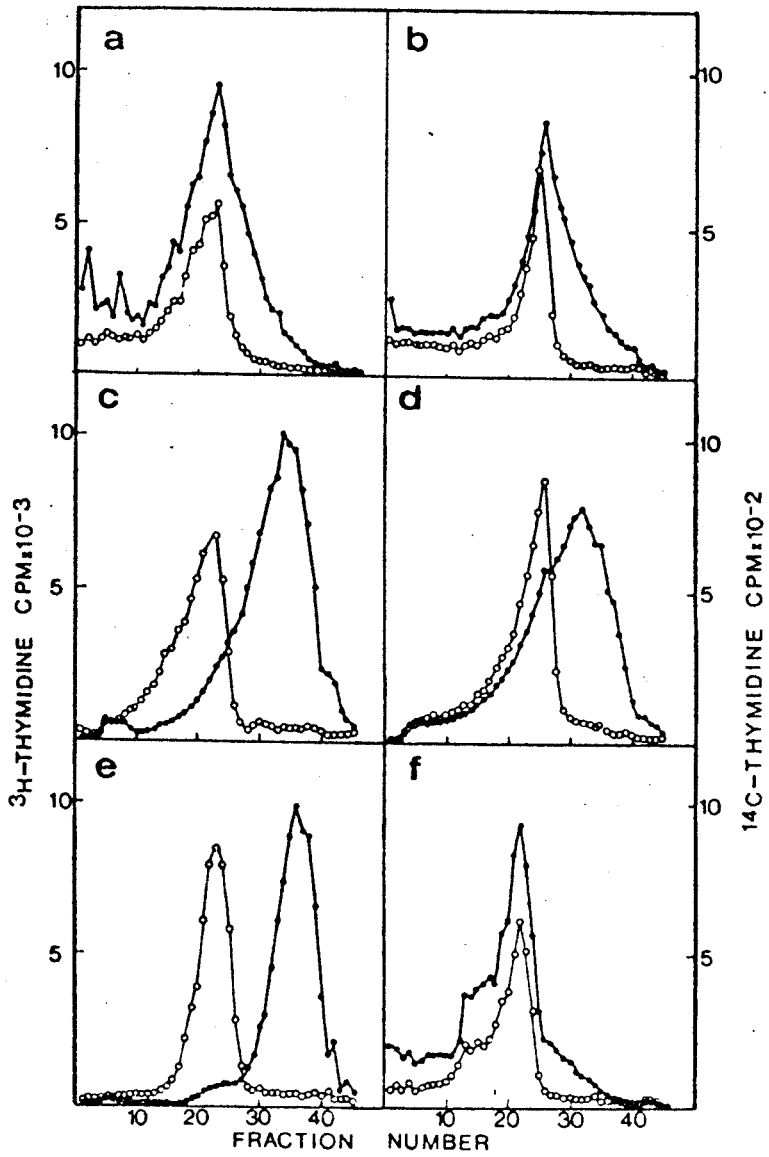

FIG. 7. *Size distribution of ^{3}H-labeled Ad2 DNA after various conditions of incubation of KB cells infected with ^{3}H-labeled ts111 virus. KB cell suspensions were infected with ^{3}H-labeled ts111 virus (2.5 × 10^{-4} cpm/PFU) at an MOI of 100. After incubation under several conditions, the DNA was analyzed by zonal sedimentation in alkaline sucrose density gradients as described (●). ^{14}C-labeled Ad2 marker DNA was added on top of the gradient (○). (a) Incubation at 39.5°C for 16 h. (b) Incubation at 33°C for 48 h. (c) Incubation at 33°C for 24 h followed by 16 h of incubation at 39.5°C. (d) Incubation at 33°C for 48 h followed by 16 h of incubation at 39.5°C. (e) Incubation at 33°C for 48 h followed by 16 h of incubation at 39.5°C in the presence of hydroxyurea (10 mM). (f) Incubation at 39.5°C for 16 h after coinfection with WT (MOI = 100) and ^{3}H-labeled ts111 (MOI = 100).*

*ts*111 at 33°C for 24 h in the presence of hydroxyurea to maintain them in the early phase of the virus cycle. Infected cells were then shifted up to 39.5°C for 24 h with or without hydroxyurea. Controls were performed with WT. The results summarized in Table 2 show that preincubation at 33°C before shift-up greatly increased cellular DNA breakdown. These data suggest that a modified *ts*111 early protein, functional at 33°C, must lose its DNA-protective function at 39.5°C.

DISCUSSION

The temperature-sensitive mutant *ts*111 of human Ad2 is a phenotypical DNA-negative

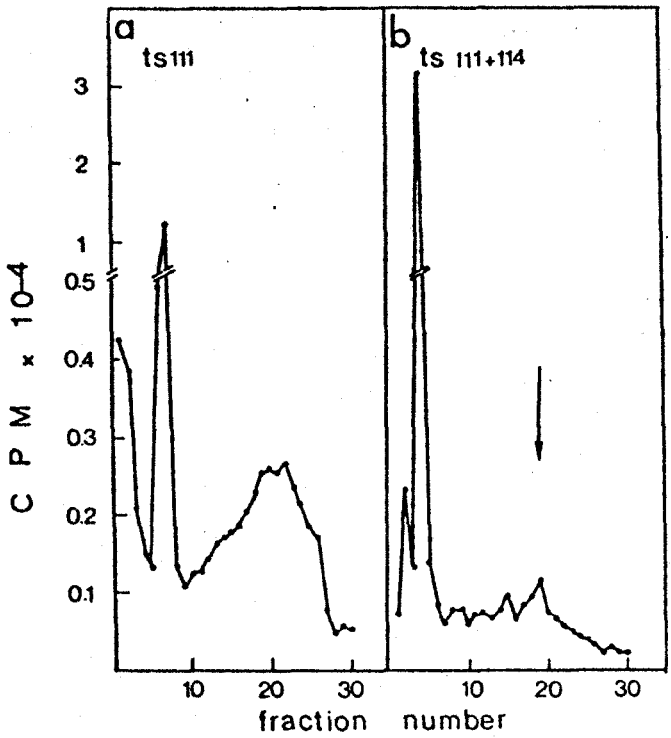

FIG. 8. *Size distribution of ^{14}C-labeled cellular DNA after ts111 infection at 39.5°C. HeLa cell monolayers were labeled with [^{14}C]thymidine (1 μCi/ml) for 24 h at 37°C. After being washed, cells were infected with (a) ts111 (MOI = 25) or (b) ts111 and ts114 (MOI = 25 each) and incubated at 39.5°C for 40 h. Labeled cellular DNA was analyzed in alkaline sucrose gradients as described in the text. The arrow indicates the position of the Ad2 marker DNA.*

mutant belonging to a unique complementation group differing from the H5 *ts*125, which is altered in the DNA-binding 72K protein function. It also complements the H5 *ts*36 by a factor of about 400 (12). H2 *ts*111 elicits a defect which provokes viral DNA synthesis shutoff on shift-up to nonpermissive temperature (Fig. 1). On shift-down, viral DNA synthesis immediately resumes, even in the presence of protein synthesis inhibitors (Fig. 2). Therefore, the altered gene product which is not degraded at 39.5°C becomes functional on shift-down to 33°C. The implicated function is essential from the beginning of the infectious cycle. However, the effect of the *ts*111 mutation is much more pronounced on the virus yield than on the amount of viral DNA synthesized (Fig. 3). These data may be explained by the fact that the production of infectious virions is the result of multiple steps, only one of which is viral DNA synthesis. Like all adenovirus DNA-negative mutants described thus far, H2 *ts*111 appears to be defective in DNA initiation at 39.5°C, as revealed by density labeling and temperature shift-up experiments. The elongation process is also affected (Fig. 4). The most important feature of the H2 *ts*111 alteration is seen when viral DNA synthesis is allowed at 33°C before shift-up to 39.5°C. The 34S DNA synthesized at the permissive temperature is rapidly degraded into small fragments at 39.5°C, with the cleavage being strongly enhanced in the presence of hydroxyurea, a DNA synthesis inhibitor (Fig. 5). These results indi-

TABLE 1. *Influence of MOI on cellular DNA degradation*[a]

Virus	MOI (PFU/cell)	% Cellular DNA at S values < 30
H2 *ts*111	5	34
H2 *ts*111	25	44
H2 *ts*111	50	59
H2 *ts*111	100	63
H2 *ts*111	200	63
WT	100	27
H2 *ts*111 + WT	2 × 5	24
H2 *ts*111 + WT	2 × 50	24
Mock-infected		25

[a] HeLa cell monolayers were labeled at 37°C for 48 h with [^{14}C]thymidine (1 μCi/ml) and chased for 24 h. After infection, cells were incubated at 39.5°C for 24 h. Cellular DNA was analyzed in alkaline sucrose gradients as described in the text.

TABLE 2. *Cellular DNA degradation after infection with WT and H2 ts111 under various conditions of incubation*[a]

Expt	Conditions of incubation	% Cellular DNA at S values < 30	
		WT	H2 *ts*111
I	33°C/48 h	6.7	36.4
	33°C/24 h + 39.5°C/24 h	11.6	86.7
	33°C/24 h + 39.5°C/24 h(+HU[b])	11.6	87.3
	39.5°C/24 h	3.3	12.0
II	33°C/24 h(+HU)	ND[c]	11.8
	33°C/48 h(+HU)	8.3	20.1
	33°C/24 h(+HU) + 33°C/24 h 33°C/24 h	7.6	23.3
	33°C/24 h(+HU) + 39.5°C/24 h	11.9	69.1
	33°C/24 h(+HU) + 39.5°C/24 h(+HU)	7.0	71.5
	39.5°C/24 h	8.0	ND
	39.5°C/24 h(+HU)	10.7	19.9

[a] HeLa cell monolayers were labeled at 37°C for 72 h with [^{14}C]thymidine (1 μCi/ml). After being washed, cells were infected with WT or *ts*111 (MOI = 10) under different conditions. Cellular DNA was analyzed in alkaline sucrose gradients as described in the text.

[b] Hydroxyurea (HU; 10 mM) was added at the beginning of the indicated incubation period.

[c] ND, Not done.

cate that protection of newly synthesized viral DNA requires an active *ts*111 gene product and suggest that the main altered function is related to an endonucleolytic-like degradation which likely starts by single-strand nicks.

When parental DNA is examined late in *ts*111 infection at 39.5 or 33°C, a fraction of the label sediments at a rate lower than 34S (Fig. 7a and b), whereas the bulk of the label cosediments with intact WT DNA marker (Fig. 7f), as previously observed with DNA-labeled WT virions (9). In contrast, extensive parental DNA degradation is observed on shift-up to 39.5°C for 16 h after 24 or 48 h of incubation at 33°C (Fig. 7c and d), particularly under conditions of DNA synthesis inhibition (Fig. 7e). According to these data, the parental DNA appears to be partially protected even in the absence of active *ts*111 gene product, which is consistent with the finding that stretches of parental DNA are found in nucleosome-like structure (22). At permissive temperature, the parental DNA enters into replication intermediates and protection may be assumed to occur by binding of the 72K protein (14) and the involvement of the *ts*111 gene product. Inactivation of this gene product on shift-up to 39.5°C would allow endonuclease cleavage.

Inhibitory activity towards cellular DNases has been demonstrated in phage-bacteria systems (1, 15, 19, 27) and during infection of KB cells by Ad5 (14). Similarly, H2 *ts*111 might be altered genetically in a DNase-inhibitory function. This hypothesis is supported by the fact that cellular DNA is markedly degraded on infection with *ts*111 at 39.5°C. This cleavage is related to MOI (Table 1) but does not seem to be due to a modification of the penton base-associated endonuclease activity (3, 13). The functional role of the observed endonucleolytic DNA cleavage is unknown. Available data do not suggest a restriction-like process. However, integration of viral DNA fragments into cellular DNA (2, 7, 20) might be one of the consequences of this activity. In this hypothesis, the enhancement of cell DNA cleavage observed when *ts*111 expression is allowed at 33°C before shift-up (Table 2) might be the result of integration of viral DNA units into cell DNA, a phenomenon which could have occurred at a normal rate at the permissive temperature. Viral DNA sequences have been found in high-molecular-weight, newly synthesized DNA from cells infected at 39.5 or 33°C with *ts*111 (data not shown) or with other Ad2 temperature-sensitive mutants (21).

From the results reported here, it appears that H2 *ts*111 presents two phenotypical characteristics: (i) absence of DNA initiation and elongation; (ii) endonucleolytic cleavage of viral and cellular DNA. As *ts*111 might be a double mutant, four revertants were isolated by culture at nonpermissive temperature and cloned. They were tested for virus yield at permissive and nonpermissive temperatures, soluble antigen production, and cellular DNA degradation. The four revertants had a WT serological phenotype and the same effect on cell DNA as WT, strongly suggesting that *ts*111 is a single mutant. Attempts to detect the implicated virus-coded protein failed: the sodium dodecyl sulfate-polyacrylamide gel pattern of the *ts*111 early proteins was similar to that of WT (not shown). Further experiments are required to determine how the *ts*111 gene product is implicated in DNA initiation and elongation and in cellular DNase inhibition.

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Temperature-Sensitive Mutant of Adenovirus Type 2 Blocked in Virion Assembly: Accumulation of Light Intermediate Particles

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A temperature-sensitive mutant of human adenovirus type 2, *ts*112, was isolated and characterized. *ts*112 was blocked in a late function required for virus maturation. At restrictive temperature, it accumulated light precursor particles that were able to mature into infectious virions upon temperature shift-down. Use of a mild extraction procedure and a reversible fixation by a cleavable diimido ester permitted the isolation and analysis of these labile intermediates in the adenovirus assembly. These accumulated particles had a sedimentation coefficient of about 600S and a buoyant density of 1.315 g/cm^3 in CsCl. They contained a DNA fragment of 7-11S and two nonvirion proteins having molecular weights of 50,000 (50K) and 39,000 (39K), respectively. They resembled in composition and morphology the light intermediate particles found in wild-type adenovirus 2, which were identified as precursors of heavy intermediates, preceding the young virions. The *ts*112 lesion was apparently located at the exit of either the 50K and/or 39K proteins and at the entry of viral DNA.

The assembly of a virus constitutes a model system for studies on protein-protein and protein-nucleic acid interactions. It also offers a possible clue for the therapy of viral diseases (6, 12). Adenovirion assembly has been studied by different approaches: in vitro reconstruction (4, 31, 32); analysis of incomplete particles, precursors of mature virions (5, 11, 14, 19, 20, 23, 24, 30); and analysis of temperature-sensitive (*ts*) mutants blocked in different steps of viral morphogenesis (28).

In our work, the assembly of human type 2 adenovirus (Ad2) was studied by biochemical and electron microscopic analysis of different classes of particles obtained with a *ts* mutant, *ts*112. These particles were isolated by centrifugation in sucrose and in CsCl gradients after reversible fixation by a cleavable diimido ester (9). *ts*112 was defective in a late stage required for virus maturation. At the nonpermissive temperature, an accumulation of precursor particles, able to mature into infectious virions upon temperature shift-down, was observed.

These light intermediate (IM) particles were reminiscent of the prohead structure described in certain double-stranded DNA bacteriophages (2, 15), and they were also found in wild-type (WT) Ad2, provided that a mild procedure of isolation and fixation was used (9).

MATERIALS AND METHODS

Cells. KB cells were grown in suspension in Joklik-modified medium F 13 (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 5% horse serum. HeLa cells were grown as monolayers in Eagle minimum essential medium, containing 10% calf serum.

Virus. The Ad2 WT was originally supplied by J. F. Williams (Carnegie-Mellon Institute, Pittsburgh, Pa.). The Ad2 *ts* mutant, *ts*112, was isolated after nitrous acid treatment of the WT stock. This was done according to the method described by Williams et al. (29), and preliminary characterization has also been performed (G. R. Martin, R. Warocquier, J. C. D'Halluin, and P. A. Boulanger, manuscript in preparation).

Virus production and purification. Stocks of WT and of *ts*112 were grown by infecting cells at a low multiplicity of infection and incubating them at 37°C for 40 h and at 33°C for 96 h, respectively. Virus particles were purified as previously described (3, 9). Infectivity was assayed by the fluorescent focus assay technique (18) or by the plaque method on HeLa cells. The titers were expressed in fluorescent focus units or PFU.

Infection and labeling conditions. Cells were infected at a multiplicity of infection of 25 PFU per cell. KB cells in suspension were centrifuged at low speed at different times postinfection (p.i.) and resuspended at a density of 10^6 cells per ml in culture medium. For pulse-labeling, the cells were labeled with

1 μCi of [^{14}C]valine per ml and 2 μCi of [^{3}H]thymidine per ml in a valine-deprived medium. For chase experiments, the cells were diluted in normal medium at 3 × 10^5 cells per ml. HeLa-cell monolayers were pulse-labeled with 6 μCi of [^{35}S]methionine per ml in a medium containing 2.5% of the normal methionine concentration. For *ts*112, the permissive temperature was 33°C and the restrictive temperature was 39.5°C.

Radioisotopes. [^{35}S]methionine (600 to 700 Ci/mmol) and [^{14}C]valine (250 to 300 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, England), and [^{3}H]thymidine (30 Ci/mmol) was purchased from the Commissariat à l'Energie Atomique (Saclay, France).

Cell fractionation and isolation of virus and IM particles. Cell fractionation was performed according to a modification of the technique of Edvardsson et al. (11). Infected cells were washed with cold phosphate-buffered saline, suspended at 4 × 10^7 cells per ml in reticulocyte standard buffer (10 mM Tris-hydrochloride, pH 7.4–10 mM NaCl–1.5 mM $MgCl_2$), and left for 10 min at 0°C. Triton X-100 was added up to a final concentration of 0.5%, and the cells were disrupted by 10 strokes in a tight-fitting Dounce homogenizer. NaCl was added up to 100 mM, and the cell lysate was centrifuged at 1,000 × *g* for 5 min. The supernatant, referred to as the cytoplasmic fraction, was adjusted to 20 mM sodium EDTA, centrifuged at 16,000 × *g* for 10 min, and analyzed on a sucrose or Ficoll gradient, as described below. The nuclei in the pellet was lysed by the method of Wallace and Kates (26). They were suspended in 50 mM Tris-hydrochloride, pH 8.0–10 mM sodium EDTA (TE buffer) adjusted to 0.3 M $(NH_4)_2SO_4$, homogenized in a tight-fitting Dounce homogenizer (three strokes), and immediately diluted with 2 volumes of TE buffer. The nuclear lysate was then centrifuged on a 30% (wt/vol) sucrose cushion at 16,000 × *g* for 10 min. The supernatant was further analyzed on sucrose (E. Merck A. G., Darmstadt, West Germany) or Ficoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden) gradients. The gradients, containing 5 to 12.5% Ficoll in 50 mM Tris-hydrochloride, pH 8.0–150 mM NaCl–10 mM sodium EDTA or 25 to 40% sucrose in 50 mM Tris-hydrochloride, pH 8.0–200 mM NaCl–10 mM sodium EDTA, were centrifuged at 85,000 × *g* and 4°C for 105 min in an SW 27 rotor over a cushion of CsCl (1.43 g/cm^3). The gradients were collected dropwise from the bottoms of the tubes, and the fractions were assayed for acid-precipitable radioactivity on Whatman GF-C filters.

Fixation of virus particles. For electron microscopic analysis, a nonreversible fixation of samples was carried out with 5% glutaraldehyde (in 0.1 M sodium phosphate buffer, pH 7.0) at room temperature for 30 min. Excess fixation by glutaraldehyde was prevented by adding 0.1 M lysine, pH 7.4, for an extra 2 h at room temperature, followed by dialysis against phosphate-buffered saline.

For biochemical analysis, a reversible fixation by a cleavable diimido ester was used (25). This second procedure was performed on virus particles obtained from sucrose or Ficoll gradients made in 20 mM sodium borate, pH 8.0, instead of 50 mM Tris-hydrochloride, pH 8.0. Samples were first reacted with 3 mg of methyl-4-mercaptobutyrimidate hydrochloride (Pierce Chemicals Co., Rockford, Ill.) per ml for 30 min at 4°C. Excess reagent was removed by dialysis against phosphate-buffered saline, containing 100 mM H_2O_2 to provoke disulfide bridge formation. The induced disulfide cross-bridges were cleaved by 2-mercaptoethanol to perform polypeptide analysis on sodium dodecyl sulfate (SDS)-acrylamide gels.

Analytical SDS-polyacrylamide gel electrophoresis. The samples were dissolved in SDS denaturing mix (62.5 mM Tris-hydrochloride, pH 6.8–4% SDS–10% 2-mercaptoethanol–6 M urea) and heated for 2 min at 100°C. The polypeptides were analyzed on a 17.5% acrylamide–0.08% bisacrylamide slab gel overlayered by a 5% acrylamide–0.13% bisacrylamide stacking gel in the discontinuous buffer system of Laemmli (16). Electrophoresis was carried out for 16 h at 30 V (constant voltage) in a Bio-Rad model 220 apparatus. The gels were stained with Coomassie brillant blue R 250, vacuum-dried, and autoradiographed with Kodak Kodirex film.

Electron microscopy. The different classes of virus particles obtained from sucrose or Ficoll gradients were examined in a Hitachi HU-12 electron microscope after staining with 1% potassium phosphotungstate, pH 7.2. They were examined nonfixed or fixed with glutaraldehyde (as above).

Extraction of DNA from virus particles. DNA present in mature virions or in IM particles was analyzed according to the procedure described by Doerfler (10), with the following modification: 10% 2-mercaptoethanol was added to the lysis buffer (0.5 M NaOH–50 mM sodium EDTA) to cleave the disulfide cross-links.

RESULTS

Comparison of virus-coded proteins in *ts*112- and WT-infected cells by pulse-chase experiments. Preliminary phenotypic characterization of the *ts* mutants of Ad2 has shown that *ts*112 induced normal synthesis of viral DNA and the major soluble antigens at the restrictive temperature. Moreover, electron microscopic controls have revealed that the *ts*112-infected cells maintained at 39.5°C contained a great number of particles with cores less dense than those of mature virions. It seemed, therefore, likely that the *ts*112 lesion was in a late function, such as precursor protein cleavage. It is known that several adenovirus proteins are derived from precursor polypeptides by specific processing (1, 11, 14, 17).

HeLa cells infected with *ts*112 and WT and maintained at 39.5 or 33°C were pulse-labeled with [^{35}S]methionine. Chase was performed in a fresh medium containing a fivefold excess of cold methionine, and incubation was continued for various lengths of time at 39.5 or 33°C. The cells were harvested, and labeled polypeptides were analyzed on SDS-polyacrylamide gels. The patterns of pulse-labeled polypeptides in *ts*112- and

WT-infected cell extracts were almost identical at 39.5°C (Fig. 1). However, significant differences could be observed in the chase experiments. The precursor polypeptide P VI (27,000 daltons [27K]) seemed to be processed, and a weak band of VI (24K) appeared. In contrast, the precursor P VII scarcely diminished, and its cleavage product, virion polypeptide VII, failed to appear during the chase in *ts*112-infected cells. The absence of cleavage P VII → VII, which has been shown to be a late event (1, 27, 28), therefore suggested that *ts*112 was defective in virus particle maturation. No structural or nonstructural viral polypeptides appeared to be thermosensitive at 39.5°C.

Isolation of *ts*112 particles by density centrifugation in CsCl. Since it appeared that *ts*112 was blocked at a late step in viral assembly, it was interesting to determine which classes of virus particles were produced in infected cells incubated at either the restrictive (39.5°C) or the permissive (33°C) temperature. *ts*112 was

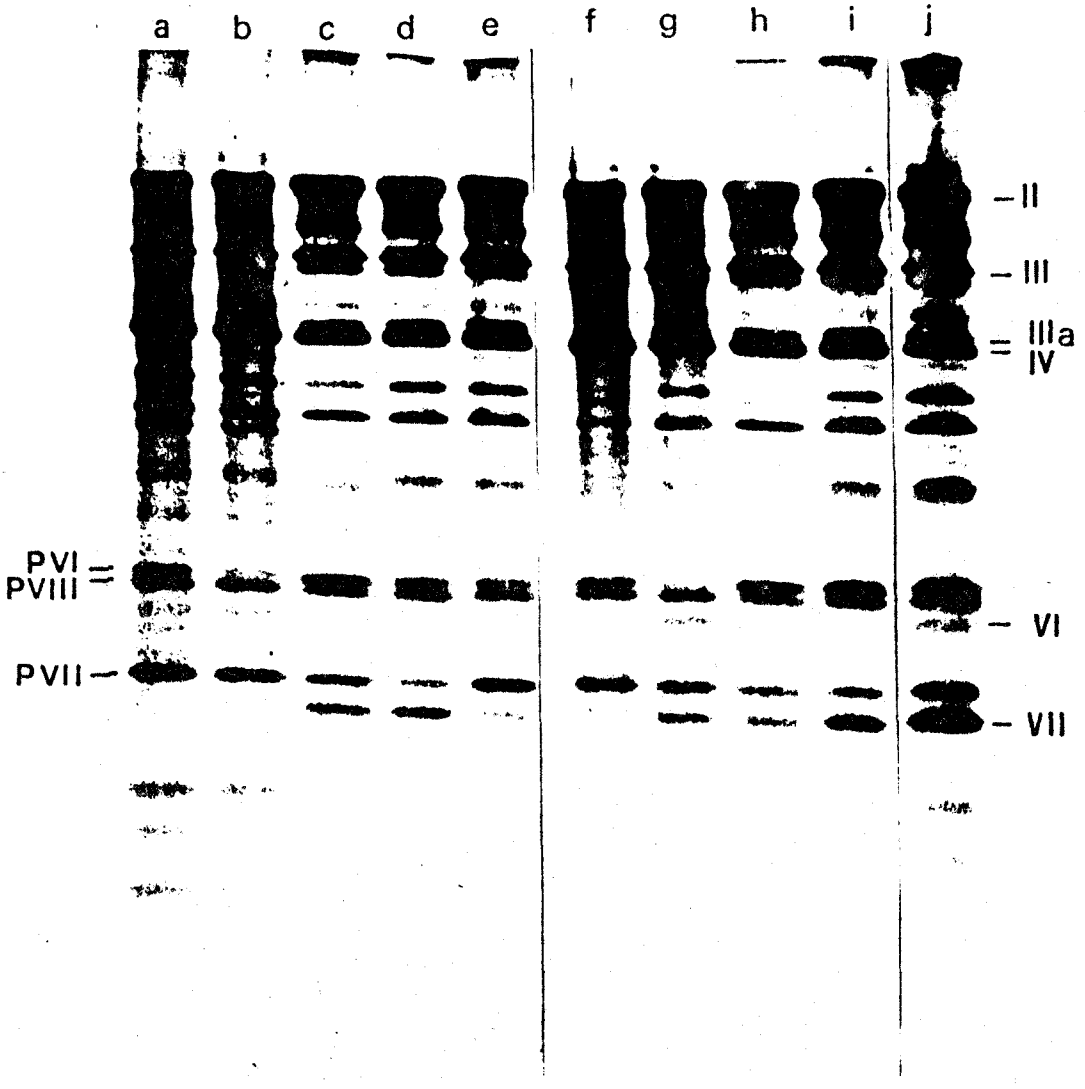

FIG. 1. *Autoradiogram of SDS-polyacrylamide gel of WT- and ts112-induced polypeptides in HeLa cells. HeLa cell monolayers infected with ts112 (a–e) and WT (f–j) were pulse-labeled with [^{35}S]methionine for 1 h at 23 h p.i. and 39.5°C and for 3 h at 69 h p.i. and 33°C and chased for different lengths of time at either 39.5 or 33°C. (a, f) Pulse at 39.5°C; (b, g) pulse and chase at 39.5°C for 16 h; (c, h) pulse at 33°C; (d, i) pulse and chase at 33°C for 24 h; (e, j) pulse at 33°C, shift-up after pulse, and chase at 39.5°C for 24 h. The cleavage of P VII into VII appears altered in ts112-infected cells at 39.5°C. Anode is at the bottom.*

grown at 39.5 and 33°C in KB cells for 36 and 80 h, respectively. The particles were extracted as described in Materials and Methods and analyzed on a self-generating CsCl gradient with an initial density of 1.34 g/cm^3. The gradient patterns showed: (i) at 33°C, a major band of virions with a buoyant density of 1.345 g/cm^3 and a minor band in the 1.29- to 1.30-g/cm^3 density zone; (ii) at 39.5°C, a minor band of mature virions and two major bands at densities close to each other, at 1.29 and 1.30 g/cm^3 (not shown). These two latter bands were collected separately and further purified from each other on preformed 1.23- to 1.34-g/cm^3 CsCl gradients.

The different bands were collected, dialyzed against 10 mM Tris-hydrochloride, pH 8.0–1 mM sodium EDTA, and heated in SDS denaturing mix, and then the polypeptides of these particles were analyzed on SDS-polyacrylamide slab gels. The light particles at 33°C (Fig. 2c) and the 1.30-g/cm^3 particles at 39.5°C (Fig. 2f) contained the same species of polypeptides as the empty particles described in the Ad3 system (11). They lacked almost totally the core proteins V and VII and contained instead the polypeptides 27K and 26K, probable precursors of virion polypeptides VI and VIII. There was a relatively greater proportion of 26K. The cleavage products VI and VIII were also present in small amounts. The 1.29-g/cm^3 particles at 39.5°C (Fig. 2g) were similar in polypeptide composition to WT incomplete particles (11, 20), except that these *ts*112 particles contained a small amount of P VII and almost no VIII and that the respective proportions of 27K and 26K were changed in favor of the 27K. The light 1.30-g/cm^3 particles produced at 39.5°C were analyzed with regard to their DNA content and were found to contain a fragment of DNA sedimenting in an alkaline sucrose gradient as a broad peak between 7 and 11S (Fig. 3). No detectable DNA was found in 1.29-g/cm^3 particles at 39.5°C. The presence of significant amounts of precursor polypeptide P VII, besides the major band of core protein VII, in the 1.345-g/cm^3 particles produced at 33°C (Fig. 2b) suggested that these particles were in some way an intermediate state between young (14) and mature virions. These results suggested that *ts*112 was blocked in a step preceding the formation of young virions. The occurrence of a large amount of light particles, even at 33°C, implied that the mutation was still expressed at the permissive temperature.

Isolation of the different classes of *ts*112 particles on sucrose or Ficoll gradients. The preceding results established that *ts*112 grown at 39.5°C produced light particles banding at a density of 1.29 to 1.30 g/cm^3 in a CsCl gradient.

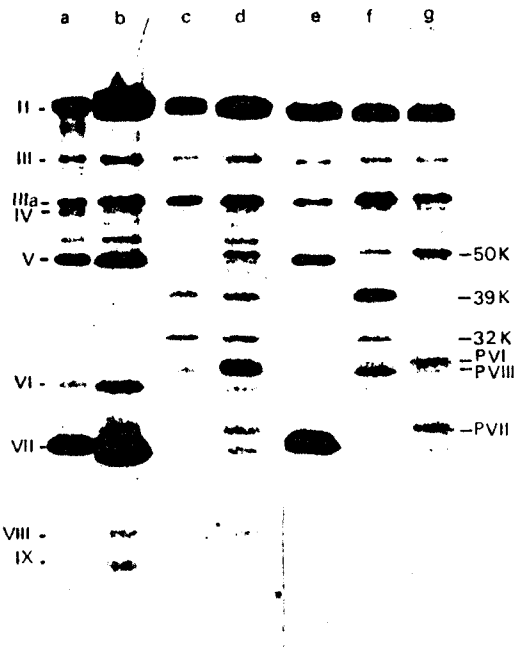

FIG. 2. *Polypeptide composition of Ad2 ts112 particles. Particles produced by ts112 at the permissive or the restrictive temperature were extracted from infected cells at 36 h p.i. at 39.5°C and at 80 h p.i. at 33°C and isolated on CsCl gradients without prior fixation. (a) Ad2 WT; (b) ts112 particles produced at 33°C and banding at 1.345 g/cm^3 in CsCl; (c) light ts112 particles produced at 33°C and found at 1.29 to 1.30 g/cm^3; (d) light ts112 particles produced at 39.5°C (1.29 to 1.30 g/cm^3). The light ts112 particles produced at 39.5°C consisted of two populations, which were further repurified on preformed CsCl gradients. (e) Control WT; (f) 1.30-g/cm^3 ts112 particles; (g) 1.29-g/cm^3 ts112 particles. (a–d) and (e–g) were two separate runs. Staining: Coomassie brilliant blue. Anode is at the bottom.*

Since it has been recently shown that adenovirus assembly IM are fragile structures, easily disrupted by purification in a CsCl gradient without fixation, the accumulated particles of *ts*112 were isolated by a mild procedure recommended by Edvardsson et al. (11). Suspension cultures of KB cells infected with *ts*112 were incubated at 39.5°C and double-labeled with [^{3}H]thymidine and [^{14}C]valine from 12 to 20 h p.i. The cells were fractionated into cytoplasm and nuclei. Extracts from each fraction (2 × 10^8 cells) were layered on top of sucrose or Ficoll gradients. Although Ficoll has been shown to be less damaging to adenovirus IM than is sucrose (11), our results showed that sucrose and Ficoll have the same effect on *ts*112 light particles. Double-labeled, mock-infected cell extracts and WT-in-

fected cell extracts were analyzed in the same way and used as controls. In mock-infected cell extracts, the DNA label was found in gradient zones corresponding to values lower than 100S and larger than 1,000S (not shown).

The sucrose gradient pattern of *ts*112-infected

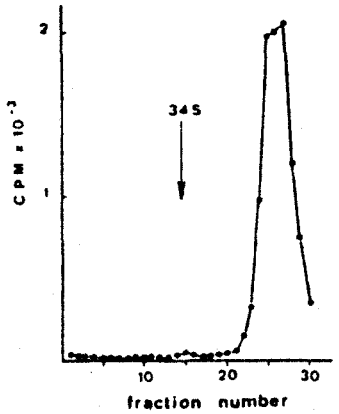

FIG. 3. *DNA analysis of Ad2 ts112 light particles. The DNA of light, 1.30-g/cm³ ts112 particles produced at 39.5°C was analyzed on a 5 to 20% alkaline sucrose gradient. The position of WT DNA (34S) is indicated by the arrow. The DNA of light ts112 particles behaves as a heterogeneous population of molecules with sedimentation coefficients ranging from 7 to 11S. Bottom is at the left.*

nucleus extract showed a slow-sedimenting, sharp peak at about 600S, referred to as *ts*112 IM; these IM contained little DNA label (Fig. 4b). The extracts from WT-infected nuclei contained two peaks. One sharp peak sedimented as intact virions at 750S and was labeled in both the DNA and the protein moieties. Another broad peak, slower than 750S, contained little DNA and had a heterogeneous sedimentation rate (Fig. 4a). The analysis of these extracts on Ficoll gradients showed the same patterns (not shown). The fractions corresponding to each peak were pooled, and the virus particles were fixed with glutaraldehyde for electron microscopic analysis and with cleavable diimido ester for further biochemical analysis, i.e., density determination in a CsCl gradient and polypeptide analysis on an SDS-polyacrylamide gel.

Density analysis of the *ts*112 IM particles. Fixed *ts*112 IM particles were analyzed in a CsCl gradient. A single sharp peak was obtained at a density of 1.315 g/cm³ (Fig. 5). The sharpness of this peak suggested a strong homogeneity in the population of these IM particles. The value of 1.315 g/cm³ and, thus, the ratio of DNA to protein content were higher than those previously determined for empty and incomplete particles (8, 11, 20). This suggested that the mild extraction procedure followed by fixation preserved the structure of the *ts*112 IM.

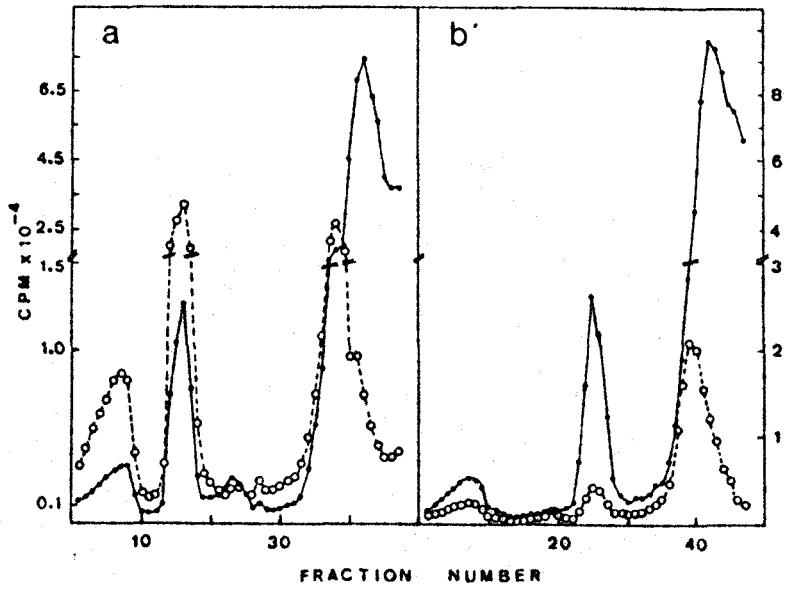

FIG. 4. *Sucrose gradient centrifugation analysis of nuclear extracts from WT- and ts112-infected KB cells. Ad2 WT- or ts112-infected KB cells were labeled with [³H]thymidine and [¹⁴C]valine from 12 to 20 h p.i., and nuclear extracts were analyzed on 25 to 40% sucrose gradients. (a) WT; (b) ts112. The peak at fraction 16 in (a) corresponds to adenovirus particles with an apparent sedimentation coefficient of 750S. The peak at fraction 25 in (b) corresponds to ts112 accumulated IM sedimenting at 600S. Symbols: ●, ¹⁴C; ○, ³H. Bottom is at the left.*

Electron microscopy of the *ts*112 IM particles. The *ts*112 material, isolated on CsCl gradient without prior fixation and banding at 1.29 to 1.30 g/cm^3, was found to contain empty particles, penetrated by stain, along with disrupted capsids, confirming the lability to adenovirus intermediates in CsCl (not shown).

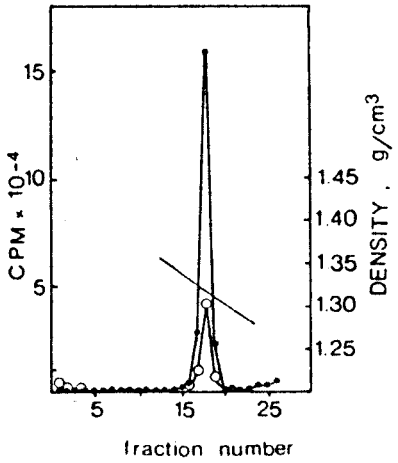

FIG. 5. *Isopycnic gradient centrifugation of ts112 IM. The ts112 IM particles, isolated on a sucrose gradient as in Fig. 4, were fixed with diimido ester and further analyzed in a CsCl gradient. A sharp band sedimenting at 1.315 g/cm^3 was obtained. Bottom is at the left.*

In the *ts*112 material isolated on a sucrose gradient and nonfixed, a unique class of particles was observed (Fig. 6a). These particles appeared similar in size and contour to mature virions, but gaps were visible in the outer capsid and the icosahedral shape was not discernible. The same particles, after fixation with glutaraldehyde (Fig. 6b), appeared rounder than mature virions and the stain penetrated irregularly into the capsids.

Polypeptide pattern of the *ts*112 IM particles. Diimido ester-fixed *ts*112 IM particles, obtained from Ficoll or sucrose gradients and further purified in CsCl gradients, were dissolved in SDS denaturing mix, and their polypeptide components were analyzed on SDS-polyacrylamide gels. No significant difference was observed between the polypeptide patterns of nonfixed IM particles obtained from Ficoll or sucrose gradients and those of fixed IM particles obtained by either method after subsequent purification in a CsCl gradient, except for the disappearance in the fixed particles of a barely visible band of P VII (Fig. 7). This suggested an effective fixation by the cleavable diimido ester. The P VII present in the IM peak of the sucrose gradient was also found in all the gradient fractions, and, most likely, it represented contaminating material that was subsequently elimi-

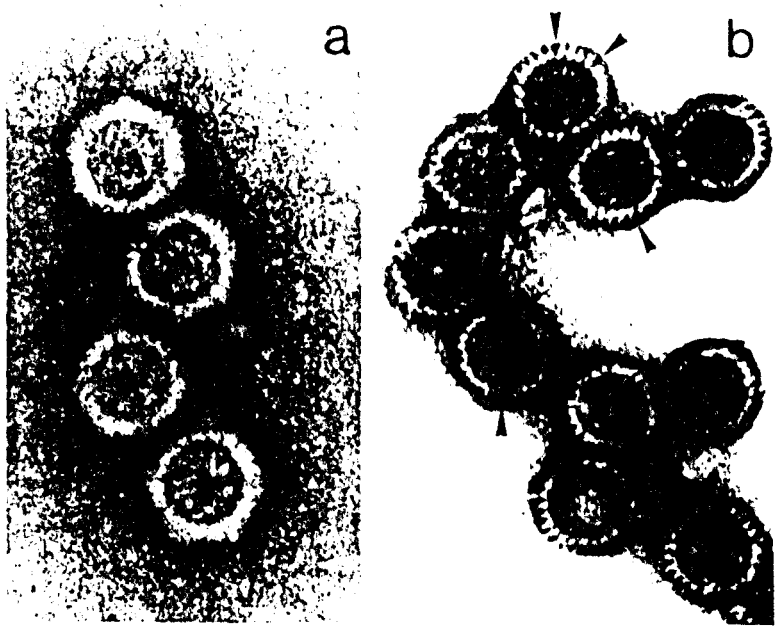

FIG. 6. *Electron microscopy of ts112 IM particles. The ts112 600S particles, obtained from a sucrose gradient (as on Fig. 4), were examined nonfixed (a). The same ts112 particles were fixed with glutaraldehyde and further purified in a CsCl gradient, and the population banding at 1.315-g/cm^3 was examined in the same conditions (b). Staining: potassium phosphotungstate. The arrows indicate gaps in the virus capsids. ×240,000.*

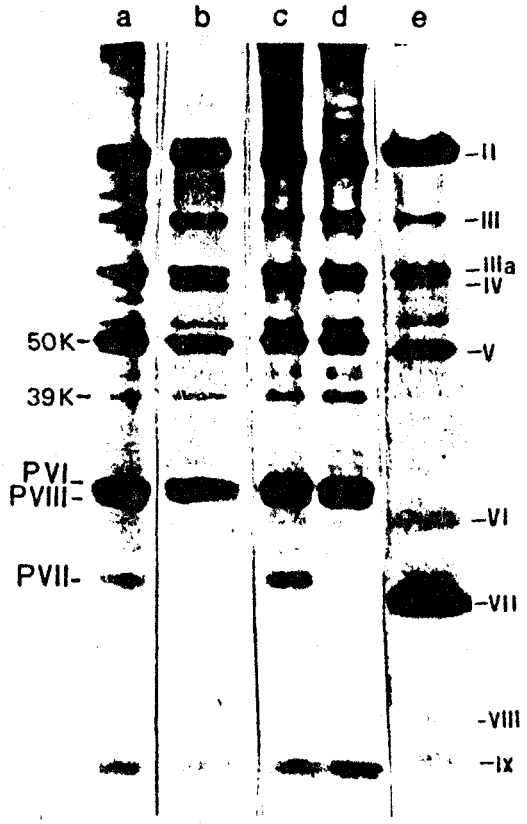

FIG. 7. *Polypeptide composition of ts112 IM particles extracted and isolated with different procedures. (a) ts112 IM extracted by sonic treatment of nuclei and isolated on Ficoll gradient before fixation, as described by Edvardsson et al. (11); (b) the same material after diimido ester fixation and further purification in a CsCl gradient; (c) ts112 IM particles extracted by ammonium sulfate treatment of nuclei and isolated on a sucrose gradient before fixation; (d) the same material after diimido ester fixation and banding in a CsCl gradient; (e) control WT. Traces of precursor P VII present in material cosedimenting with IM in a Ficoll (a) or sucrose (c) gradient disappeared upon repurification of the fixed material by banding in CsCl (b, d). The fixed purified particles in (b) and (d) show similar polypeptide patterns. Anode is at the bottom.*

nated by fixation and purification in CsCl (9).

The virion structural polypeptides II, III, IIIa, IV, IVa_2, and IX were present in the IM particles. Polypeptide V was barely visible on a stained gel and on the corresponding autoradiogram. Polypeptides VI and VII were completely missing. In contrast, the precursor polypeptides P VI and P VIII were strongly labeled. Two extra polypeptides, with apparent molecular weights of 50,000 and 39,000, were found in great amounts. This polypeptide pattern differed significantly from that obtained with CsCl-isolated light particles (Fig. 2), confirming the lability of nonfixed adenovirus IM in high-ionic-strength medium (11). The light particles obtained in CsCl gradients without prior fixation probably corresponded to breakdown products of assembly IM.

Reversibility of the mutation by temperature shift-down. The reversibility time of the mutation was determined by titrating the infectious *ts*112 obtained after shift-down at different times p.i. Temperature-shifted cultures were harvested at 87 h p.i., and infectious virus was titrated by fluorescent focus assay on crude cell lysate. Figure 8 compares the yields of infectious *ts*112 obtained after shift-down with the growth curves at each temperature. Until 18 h p.i., there was little or no effect of the nonpermissive temperature on the final virus yield. From 30 to 50 h, the effect of the mutation could not be reversed by a shift-down to a permissive temperature. Therefore, the critical period for the expression of the lesion seemed to extend from 18 to 30 h p.i. at 39.5°C. These data were used in the next experiment to choose the proper periods of labeling and temperature change.

Evolution of *ts*112 IM particles upon tem-

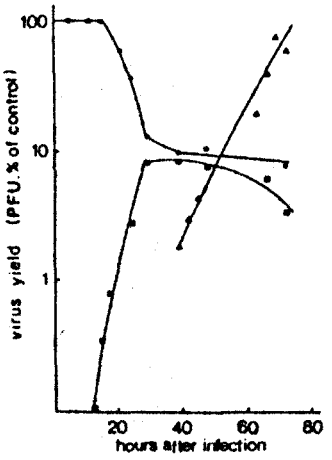

FIG. 8. *Temperature dependence of ts112 infectious particle maturation. Duplicate sets of ts112-infected KB-cell cultures were incubated at 33 or 39.5°C for different lengths of time and then harvested for determination of the virus yield at each temperature. One 39.5°C culture was shifted down to 33°C and incubated at this temperature until 87 h p.i. The virus production was titrated by the fluorescent focus method, and the results are expressed as a percentage of the virus yield at 87 h p.i. at 33°C. The x axis indicates the time p.i. at which cultures were harvested or shifted down. Symbols: ▲, ts112 growth curve at 33°C; ■, ts112 growth curve at 39.5°C; ●, ts112 yield after the shift-down to 33°C and further incubation until 87 h p.i. at 33°C.*

perature shift-down. Temperature shift-down experiments after pulse-labeling were performed to determine whether the *ts*112 IM particles could evolve into mature virions or other types of precursors. The length of the chase at 39.5°C, preceding the temperature shift-down, was first determined to get a maximum entry of the label into the IM particles and to test the efficiency of maturation of the virions. KB cells infected with *ts*112 at 39.5°C were labeled with [^{14}C]valine from 15 to 17 h p.i. and then shifted down to 33°C at 17, 20, and 24 h p.i., i.e., after 0-, 3-, and 7-h chase periods. The incubation at 33°C was continued until 88, 80, and 72 h p.i., respectively. The particles produced at the ends of these incubation periods were extracted with Freon and analyzed on CsCl gradients. A 7-h chase period at 39.5°C, preceding the shift-down, resulted in low recovery of label in mature virions (Fig. 9). In contrast, a 3-h chase at 39.5°C, preceding the shift-down, did not affect the entry of label into virions. Since it has been shown that in WT 60 to 90 min is required for appearance of maximum radioactivity in intermediates (14), a chase period of 2 h at 39.5°C, before shift-down, was chosen for the next experiment.

KB cells infected with *ts*112 at 39.5°C and labeled with [^{14}C]valine from 15 to 17 h p.i. were chased for 2 h at 39.5°C and then shifted down to 33°C. Samples of 2×10^6 cells were withdrawn at different times. The cells were fractionated, and nuclear and cytoplasmic extracts were analyzed on sucrose gradients. The total radioactivity was constant throughout the chase. Figure 10 shows the distribution of nuclear label in the sucrose gradients at 17 (a), 19 (b), 20 (c), 21 (d),

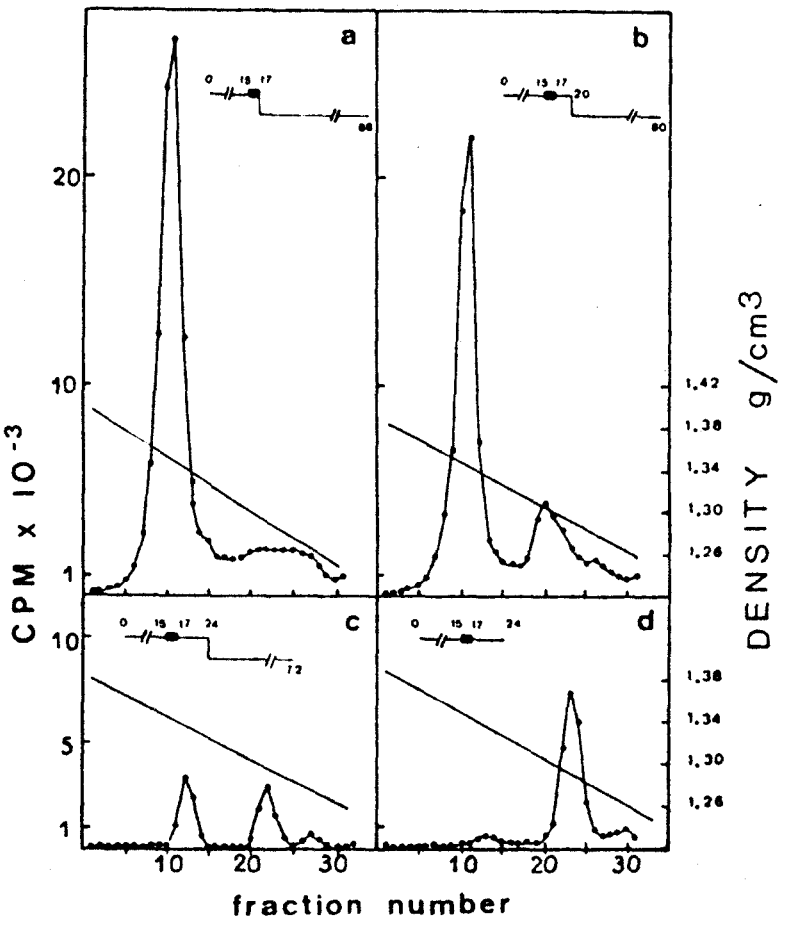

FIG. 9. *Isopycnic centrifugation of ts112 IM matured upon temperature shift-down. ts112-infected KB-cell cultures maintained at 39.5°C were pulse-labeled with [^{14}C]valine for 2 h at 15 h p.i., chased at 39.5°C for various lengths of time, and then shifted down to 33°C. The particles were extracted with Freon at the end of the incubation period and analyzed on CsCl gradients. (a) 0-h chase at 39.5°C; (b) 3-h chase at 39.5°C; (c) 7-h chase at 39.5°C; (d) control 7-h chase at 39.5°C without shift-down. Bottom is at the left.*

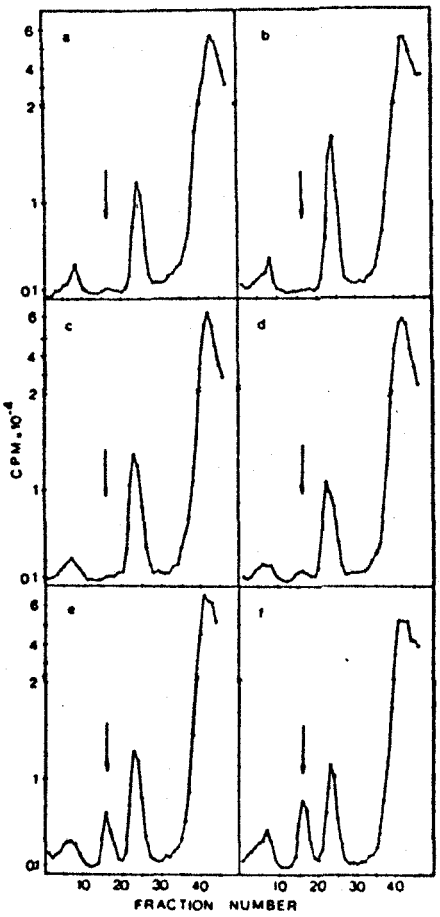

FIG. 10. *Sucrose gradient centrifugation analysis of nuclear ts112 IM matured after shift-down. ts112-infected KB-cell cultures maintained at 39.5°C were labeled with [^{14}C]valine for 2 h at 15 h p.i. (a), chased for 2 h at 39.5°C (b), and then shifted down to 33°C (c–f). Samples were withdrawn at different times, and nuclear extracts were analyzed on 25 to 40% sucrose gradients. (a) Control 2-h pulse at 39.5°C; (b) 2-h pulse at 39.5°C followed by 2-h chase at 39.5°C; (c) 1-h chase at 33°C; (d) 2-h chase at 33°C; (e) 4-h chase at 33°C; (f) 7-h chase at 33°C. Bottom is at the left.*

23 (e), and 26 (f) h p.i. The label in the IM-particle peak increased during a 2-h chase at 39.5°C and then decreased during a chase at 33°C. A peak of nuclear virus appeared at 21 h (2 h after shift-down) and increased at 23 and 26 h p.i. A peak of cytoplasmic virus was detected at 23 h p.i., i.e., 4 h after temperature shift-down (not shown). The ratios of counts per minute in the IM peaks to the total label loaded on the gradients were (in percentages): (a) 6.64; (b) 8.99; (c) 8.74; (d) 6.94; (e) 6.58; and (f) 6.53. Thus, the label in the IM peaks remained almost constant from 4 to 7 h of chase after the shift-down to 33°C.

The fact that the label in the IM peaks remained constant throughout the chase at permissive temperature, whereas the label increased in the virus peak, suggested either a de novo assembly of virions after shift-down or both a maturation of IM into virions and a formation of IM from soluble components at 33°C. However, it has to be considered that the rates of viral maturation are different at 33 and 39.5°C. This rate has been found to be approximately threefold higher at 39.5 than at 33°C (unpublished data). If the time at 33°C is corrected by a factor of 3, the peak of nuclear virus appeared first at 40 min after shift-down, in corrected time. Since it has been shown that in WT the nuclear virus peak is labeled at 1.5 h after pulse (11), it seemed unlikely that the nuclear virus label observed in Fig. 10d originated from a de novo assembly.

Effect of cycloheximide on the evolution of *ts*112 IM particles. Since the appearance of label in the mature virion peak might be due to a de novo assembly from either soluble labeled material or IM-breakdown products, it was of interest to study the fate of the IM and the appearance of mature virions in the presence of cycloheximide (20 μg/ml) as a protein synthesis inhibitor.

A KB-cell culture infected with *ts*112 at 39.5°C was pulse-labeled with [^{35}S]methionine for 15 min at 14.45 h p.i. and divided into 15 aliquots treated as indicated in Fig. 11. Nuclear and cytoplasmic extracts were pooled, to compensate possible nuclear leaks, and analyzed on sucrose gradients. The same amounts of label were loaded on the gradients. The radioactivity increased in the IM fraction until 2 h of chase at 39.5°C and then decreased (Table 1). The amount of label was lower in the IM peak and higher in the virion fraction when the shift-down to 33°C was performed at 17 rather than at 19 h p.i.

Addition of cycloheximide just after the pulse reduced drastically the assembly of IM particles. The same effect has been described for emetine (23). In contrast, the radioactivity in the IM and virus fractions was more important when cycloheximide was added at the time of shift-down, i.e., 2 h after the pulse. Thus, the cycloheximide treatment, performed just after the pulse or 2 h after the pulse, did not prevent the appearance of label in the virions when the culture was shifted down to 33°C.

These results suggested that most of this virus label derived from maturation of the IM particles accumulated at 39.5°C, and not from a de novo assembly of soluble material occurring upon the shift-down to 33°C. In addition, if IM-breakdown products were utilized in virion for-

mation, the efficiencies of reassembly upon shift-down would be similar at 17 and 19 h p.i. This was not the case, as shown in Table 1.

Polypeptide pattern of *ts*112 particles obtained in pulse-chase experiments. The fractions corresponding to the different peaks of the sucrose gradients of Fig. 10 were pooled and treated with the cleavable cross-linking reagent. The different classes of particles were further purified by centrifugation in CsCl gradients and analyzed on SDS-polyacrylamide gels. Figure 12 shows the polypeptide patterns of *ts*112 nuclear IM and virus particles obtained after temperature shift-down. The polypeptide patterns of the *ts*112 IM remained essentially unchanged throughout the chase: a 28K polypeptide visible just after the pulse disappeared during the chase, whereas a 32K polypeptide appeared. Virion polypeptide VII was absent, and traces of precursor P VII were scarcely visible. Polypeptides VI and VIII were present in slight amounts as well as polypeptide V. The nuclear virus consisted mainly of young virions, as suggested by the incomplete processing of P VI and 26K into VI and VIII and of P VII into VII (11, 14). The chase revealed no intermediate pattern between *ts*112 IM (containing the 50K and 39K) and the *ts*112 young virions. However, careful analysis of CsCl gradients of fixed nuclear particles, obtained after 4 and 7 h of shift-down, showed a minor but constant particle population banding at 1.37 g/cm^3. These short-lived 1.37-g/cm^3 intermediates were more easily detected in Ad2 WT (9).

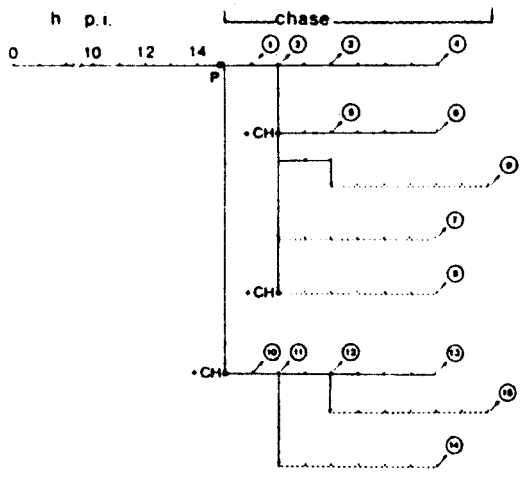

FIG. 11. *Pulse-chase and shift-down experiments in the presence or absence of cycloheximide. KB cells were infected with ts112 at 39.5°C and pulse-labeled (P) with [^{35}S]methionine (20 μCi/ml) for 15 min at 14.45 h p.i. The culture was then divided into 15 aliquots. Solid lines: subculture maintained at 39.5°C; dotted lines: subcultures shifted down to 33°C; CH: cycloheximide. Figures in circles indicate the samples withdrawn after a certain period of chase to analyze the different classes of particles, as in Fig. 10. Quantitative data are given in Table 1.*

DISCUSSION

ts mutant *ts*112 of human Ad2 induced normal synthesis of viral DNA and of viral structural and nonstructural proteins. It also produced more than 1,000 physical particles per cell, for an input multiplicity of 20 to 25 PFU per cell. However, it did not process the precursor protein of major core protein VII, although it cleaved the other precursor proteins, P VI and P VIII. In that respect it seems different from Ad2 *ts*1, which is blocked in the processing of viral protein precursors P VII, P VI, and P VIII (28). Two classes of light particles, banding at 1.29

TABLE 1. *Evolution of label in ts112 intermediates and virions in pulse-chase and shift-down experiments*[a]

Cycloheximide added	Peak[b]	^{35}S incorporation (cpm)					
		Chase period at 39.5°C				Chase periods at 39.5 and 33°C	
		1 h	2 h	4 h	8 h	2 h/39.5 + 6 h/33	4 h/39.5 + 6 h/33
No	IM	16,644 (1)	29,805 (2)	27,991 (3)	22,389 (4)	19,388 (7)	25,910 (9)
	V	0	385	824	854	6,765	4,508
2 h after pulse	IM	16,644 (1)	29,805 (2)	25,189 (5)	24,972 (6)	23,290 (8)	ND[c]
	V	0	385	408	210	9,492	ND
Just after pulse	IM	7,851 (10)	5,890 (11)	5,527 (12)	2,654 (13)	6,560 (14)	4,926 (15)
	V	0	182	53	32	1,572	343

[a] KB cells in suspension were infected with *ts*112 and pulse-labeled at 39.5°C with [^{35}S]methionine (20 μCi/ml) for 15 min at 14.45 h p.i. The culture was divided into 15 aliquots treated as indicated in Fig. 11, and particles were analyzed on sucrose gradients. Values in table are counts per minute found in each particle peak after correction for background. Figures in parentheses refer to the experiment numbers in Fig. 11.

[b] V, Virion peak (750S); IM, IM particle peak (600S).

[c] ND, Not determined.

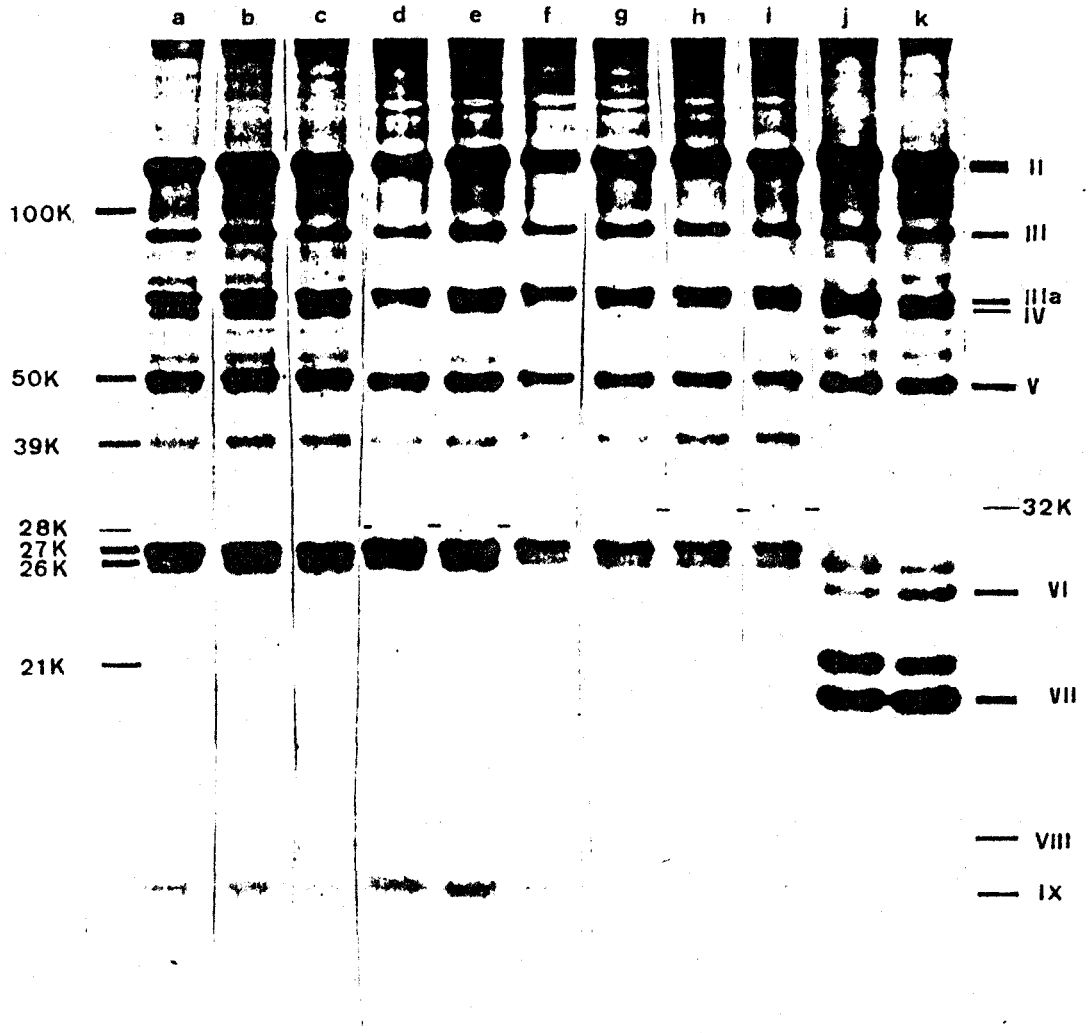

FIG. 12. *Polypeptide pattern of ts112 nuclear IM pulsed and chased at 39.5°C and shifted down to 33°C at various times. The nuclear IM, isolated on sucrose gradients as in Fig. 10, were analyzed before (a–c) or after (d–k) diimido ester fixation and further purification on a CsCl gradient. (a) Nonfixed IM peak (600S) of Fig. 10a; (b) nonfixed IM of Fig. 10b; (c) nonfixed IM of Fig. 10c; (d) fixed IM of Fig. 10a; (e) fixed IM of Fig. 10b; (f) fixed IM of Fig. 10c; (g) fixed IM of Fig. 10d; (h) fixed IM of Fig. 10e; (i) fixed IM of Fig. 10f; (j) fixed virion peak (750S) of the gradient of Fig. 10e; (k) fixed virions of the gradient of Fig. 10f. The polypeptide patterns in (j) and (k) are typical of young virions. As observed in Fig. 7, traces of precursor l' VII, present in nonfixed material cosedimenting with IM particles (a–c), disappeared in fixed and repurified material (d–f). Anode is at the bottom.*

and 1.30 g/cm^3 in a CsCl gradient, were produced by *ts*112 at the restrictive temperature. Their polypeptide patterns resembled those of incomplete particles (11, 20, 30) and those of empty particles, or top components (11, 14), found in productive infection with Ad2 WT.

Because CsCl gradient separation has been found to induce the formation of artifactual particles from virus IM (11), a mild method of extraction and a reversible fixation of particles by a cleavable diimido ester were performed before further analysis (9). Using this procedure, it was possible to isolate, on a Ficoll or sucrose gradient, *ts*112 particles that were accumulated at the nonpermissive temperature. These reversibly fixed *ts*112 particles constituted a homogeneous population of 600S particles banding at 1.315 g/cm^3 in a CsCl gradient. Analysis on an SDS-polyacrylamide gel after cleavage of the cross-links revealed that they lacked core protein precursor P VII and its cleavage product, VII. The other core protein, V, was also poorly

represented. In contrast, they contained two major polypeptide species, 50K and 39K. A minor band of 32,000 daltons was detected in the particles at a late stage after shift-down (Fig. 12). They also contained a small fragment of DNA, with a sedimentation coefficient ranging from 7 to 11S. Electron microscopy confirmed the homogeneity of the fixed *ts*112 particle population: it showed round particles containing an inner structure different from the core of a mature virion. It is impossible to assess, from the available data, whether the 39K protein corresponds to the nuclear phosphorylated 39K described in Ad5 (21) or to the early 39,000- to 40,000-dalton protein reported at early stages of infection by Ad2 (7, 22). However, preliminary results indicate that both 50K and 39K are phosphorylated within the *ts*112 1.315-g/cm^3 particles (not shown).

These *ts*112 particles banding at 1.315 g/cm^3 thus differed significantly in morphology and polypeptide composition from the empty particles obtained in CsCl gradients with WT (30) and with *ts*112 in the absence of fixation (Fig. 2b). These particles resembled the structures described as the prohead for bacteriophage P 22 (2, 15). They were tentatively termed "light IM." This value of 1.315 g/cm^3 (and, therefore, the ratio of DNA to protein content) was higher than that previously reported for empty and incomplete particles isolated without prior fixation (8, 20). This buoyant density was close to that of a class of top components with a buoyant density of 1.306 g/cm^3 (14) and to that of a class of IM particles found at 1.30 g/cm^3 in CsCl after glutaraldehyde fixation of nuclear intermediates extracted from nuclei by sonic treatment (11). Unfortunately, the irreversible nature of glutaraldehyde fixation does not permit any further analysis of the protein and DNA content of these 1.30-g/cm^3 particles and renders impossible comparison with the *ts*112 IM.

Because there is no ideal method available for nuclear particle extraction, there might be objections that either the 1.315- or the 1.30-g/cm^3 or both classes of particles represent fragmentation products of true intermediates existing in vivo. However, the same light 1.315-g/cm^3 particles are found in Ad2 WT as precursors of heavy IM, containing a normal viral DNA and preceding the young virions (9). In addition, the results of pulse-chase experiments in the presence of cycloheximide indicated that at least a fraction of these *ts*112 1.315-g/cm^3 particles were able to evolve into mature virions upon the shift-down to permissive temperature. All these data suggest that these light particles represent a real intermediate step in the adenovirus assembly pathway.

It has been shown that Ad2 WT IM (which probably consist of several classes of particles of different densities) contain major bands of precursor P VII, core protein V, and protein 50K. This latter protein is released upon maturation of intermediates into young virions (11). The fact that *ts*112 IM particles contain two labeled nonvirion polypeptides, 50K and 39K, further supports the hypothesis that the *ts*112 IM particles may be precursors to the Ad2 WT IM previously described (11). This hypothesis is compatible with our results showing that, upon temperature shift-down, the release of both 39K and 50K occurs simultaneously with the entry of proteins V and P VII and DNA to form young virions. This is also compatible with the results of pulse-chase labeling kinetics of Ad2 WT particles. This shows that light, 1.315-g/cm^3 particles precede "heavy IM" of 1.37 g/cm^3 of buoyant density, which, themselves, precede young virions, banding at 1.345 g/cm^3 (9).

Either 39K or 50K or both might, thus, serve as scaffolding protein(s), and the following tentative sequence of events for the adenovirus maturation process is proposed: virus capsid components → light IM (ρ = 1.315 g/cm^3) → heavy IM (ρ = 1.37 g/cm^3) → young virion (ρ = 1.345 g/cm^3) → mature virion (ρ = 1.345 g/cm^3). The *ts*112 lesion is apparently located on the exit of either 39K or 50K or both, and/or on the encapsidation of viral DNA. The exact determination of the nature and position of this *ts* lesion on the adenovirus genome requires further genetic and biochemical studies.

The occurrence of minute amounts of *ts*112 IM, similar to the heavy IM observed in WT (9), between light IM and young virions upon temperature shift-down suggests that packaging of viral DNA with the entry of V and P VII on the one hand and the exit of 39K and 50K on the other hand is a rapid process, as already observed (28). It is, thus, impossible to determine whether the entry of viral DNA precedes or follows the entry of V and P VII or if these two processes are simultaneous. Experimental data obtained with Ad2 WT (9) suggests that DNA entry occurs after the release of 39K and 50K and before the entry of core protein V and precursor P VII. This hypothesis is also supported by the recent finding of the absence of P VII-VII in the core structures preexisting outside of the virus capsid (13).

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ADDENDUM

After completion of this study, a paper was published describing the characterization of an Ad2 *ts* mutant blocked on DNA encapsidation at an early stage of empty particles at 1.30 g/cm^3, possibly preceding the 1.315-g/cm^3 IM (G. Khittoo and J. Weber, Virology 81:126–137, 1977).

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Adenovirus Type 2 Assembly Analyzed by Reversible Cross-Linking of Labile Intermediates

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Dimethyl-4,4'-dithiobisbutyrimidate dihydrochloride was used as a cleavable cross-linking reagent to maintain the structure of labile intermediates in adenovirus type 2 assembly. Analysis on sucrose gradients of nuclear adenovirus particles revealed two size classes, with sedimentation rates of 750 and 600S. After reversible fixation with diimido ester, the different classes were further separated on CsCl gradients and characterized with regard to their buoyant density, DNA content, and polypeptide composition. The 750S particles banded at 1.345 g/cm^3 in CsCl, contained a DNA with a sedimentation coefficient of 34S in alkaline sucrose gradients, and had a polypeptide composition similar to that of young virions. The 600S population consisted of two types of particles with buoyant densities of 1.315 and 1.37 g/cm^3. The 1.315-g/cm^3 particles contained a DNA fragment of 7-11S and lacked the core proteins V and VII. In their place were found precursors P VI and P VIII and two nonvirion proteins with molecular weights of 50,000 (50K) and 39,000 (39K). 34S DNA was present in the 1.37-g/cm^3 particles, which also lacked core proteins V and VII, as well as the 50K and 39K. Pulse-chase labeling kinetics suggested that the 1.315-g/cm^3 particles were anterior to the 1.37-g/cm^3 particles, themselves preceding the 1.345-g/cm^3 young virions, and that the release of both 50K and 39K, possible scaffolding proteins, was required for entry of viral DNA.

Incomplete virus particles that are coproduced with mature adenovirus during productive infection have been identified as intermediates in adenovirus assembly (4, 9, 12). They differ from mature virions in DNA content and polypeptide composition. Centrifugation in CsCl gradients has been, therefore, widely used for isolation and characterization of the different classes of particles, taking advantage of their differences in buoyant density.

However, it has been recently demonstrated that some of the adenovirus particle classes can be artifactually generated in the course of cell fractionation and isolation and that fragile intermediates can disintegrate and escape investigation (4). The necessity of using a mild isolation procedure and glutaraldehyde fixation before CsCl gradient centrifugation has been therefore clearly established (4).

The major drawback of this glutaraldehyde fixation resides in its irreversibility, which does not permit further analysis of the different classes of fixed particles isolated by CsCl gradient centrifugation. Using a mild procedure of cell extraction and a reversible cross-linking of virus particles by a cleavable diimido ester (13), it was possible to isolate and characterize some labile adenovirus type 2 (Ad2) wild-type intermediate particles in CsCl gradients and to analyze the DNA content and polypeptide composition of each class. Through the use of pulse-chase labeling kinetics, identification of some steps in the adenovirus assembly pathway became possible.

MATERIALS AND METHODS

Virus and cells. Human Ad2, originally supplied by J. F. Williams (Carnegie-Mellon Institute, Pittsburgh, Pa.), was grown on KB cells maintained in suspension culture with Joklik-modified medium F 13 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 5% horse serum.

Infection and labeling conditions. KB cells were infected at a multiplicity of infection of 25 PFU per cell. The cells were centrifuged at low speed at different times postinfection and suspended at a density of 10^7 cells per ml in culture medium containing 2.5% of the normal methionine concentration. Proteins were labeled with [^{35}S]methionine (20 μCi/ml), and DNA was labeled with [^{3}H]thymidine (20 μCi/ml).

Radioisotopes and counting method. [^{35}S]methionine (600 to 700 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, England), and [^{3}H]thymidine (30 Ci/mmol) was purchased from the Commissariat à l'Energie Atomique (Saclay, France).

The radioactive samples were precipitated with cold 10% trichloroacetic acid and filtered on Whatman GF-C filters. The filters were washed successively with cold 5% trichloroacetic acid and cold ethanol and air-dried. They were counted in a toluene scintillation fluid [5 g of 2,5-diphenyloxazole-0.3 g of 1,4-bis-(5-phenyloxazolyl)benzene in 1 liter of toluene, scintillation grade] with an Intertechnique liquid spectrometer.

Virus production and purification. Cells were concentrated 100-fold in hypotonic buffer (50 mM Tris-hydrochloride, pH 8.0–50 mM NaCl–1 mM sodium EDTA) and subjected to three cycles of freezing and thawing, and the cell lysate was extracted with an equal volume of Freon 113, as previously described (1). The aqueous phase of the Freon step was saved, layered on top of a CsCl cushion (1.43 g/cm^3), and centrifuged for 1 h at 20,000 rpm and 4°C in an SW 27 rotor. The opalescent band of virus on top of the CsCl cushion was collected and purified by equilibrium centrifugation in a self-generating CsCl gradient (1.34 g/cm^3) at 30,000 rpm and 4°C for 16 h in an SW 50 rotor. After dilution in 3 volumes of 50 mM Tris-hydrochloride, pH 8.0–1 mM sodium EDTA, the virus was layered on top of a linear gradient (9 ml; 25 to 40%, wt/vol) made in 50 mM Tris-hydrochloride, pH 8.0–200 mM NaCl–10 mM sodium EDTA–0.25% Triton X-100, placed over two cushions of 1 ml each of CsCl solutions (1.43 and 1.31 g/cm^3), and centrifuged for 2 h at 25,000 rpm and 4°C in an SW 41 rotor. The virus band concentrated between the two cushions was collected, and an equal volume of glycerol was added to this virus suspension. The virus was stored in 1-ml portions at −20°C.

Infectivity was assayed by the fluorescent focus assay technique (11) or the plaque method on HeLa cells grown as monolayers in petri dishes. The titer was expressed as fluorescent focus units or PFU.

Cell fractionation and extraction of assembly intermediates. Infected cells were washed with cold phosphate-buffered saline, suspended at 4×10^7 cells per ml in reticulocyte standard buffer (10 mM Tris-hydrochloride, pH 7.4–10 mM NaCl–1.5 mM $MgCl_2$), and left for swelling at 0°C for 10 min. Triton X-100 was added up to a final concentration of 0.5%, and the cells were disrupted by 10 strokes in a tight-fitting Dounce homogenizer. NaCl was added up to 100 mM, and the cell lysate was centrifuged at 1,000 × *g* for 5 min. The supernatant, referred to as the cytoplasmic fraction, was adjusted to 20 mM sodium EDTA to dissociate the polysomes and centrifuged at 16,000 × *g* for 10 min before analysis on sucrose or Ficoll gradients as described below.

The nuclei in the 1,000 × *g* pellet were lysed by the technique described by Wallace and Kates (15). They were suspended in 50 mM Tris-hydrochloride, pH 8.0–10 mM sodium EDTA (TE buffer) at 0°C, adjusted to 0.3 M $(NH_4)_2SO_4$, homogenized in a tight-fitting Dounce homogenizer (three strokes), and immediately diluted with 2 volumes of TE buffer. The nuclear lysate was then centrifuged over a 30% (wt/vol) sucrose cushion at 16,000 × *g* for 10 min. The nuclear supernatant was further analyzed on a sucrose or a Ficoll gradient.

Sucrose and Ficoll gradient centrifugation. Ficoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and sucrose (E. Merck A.G., Darmstadt, West Germany) gradients were used for the isolation of virus and assembly intermediate particles from cell extracts. The gradients were made of 5 to 12.5% Ficoll in 20 mM sodium borate, pH 8.0–150 mM NaCl–10 mM sodium EDTA or 25 to 40% sucrose in 20 mM sodium borate, pH 8.0–200 mM NaCl–10 mM sodium EDTA and centrifuged at 85,000 × *g* for 105 min at 4°C in an SW 27 rotor (4). A cushion of CsCl (1.43 g/ml) was placed at the bottom of each gradient. The gradients were collected dropwise from the bottoms of the tubes, and the fractions were assayed for trichloroacetic acid-precipitable radioactivity. The different populations of particles thus isolated were fixed before further electron microscopic and biochemical analyses.

Reversible fixation of virus particles. Samples from Ficoll or sucrose gradients containing complete or incomplete virus particles were reacted with 3 mg of methyl-4-mercaptobutyrimidate hydrochloride (Pierce Chemical Co., Rockford, Ill.) per ml for 30 min at 4°C in a medium devoid of primary amine (20 mM sodium borate buffer, pH 8.0–200 mM NaCl–10 mM sodium EDTA). After amidination of proteins, disulfide bridge formation was induced by dialysis against phosphate-buffered saline containing 100 mM H_2O_2. The reversibly fixed virus particles could then be further analyzed by centrifugation on a self-generating CsCl gradient (1.34 g/ml, containing 100 mM H_2O_2), by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and by electron microscopy, and the nature of their DNA could be determined. The induced disulfide cross-bridges could be cleaved by reducing agents, such as 2-mercaptoethanol or dithiothreitol, as described in the following section.

Analytical SDS-polyacrylamide gel electrophoresis. The different diimido ester-fixed virus populations separated in sucrose or Ficoll gradients and subsequently in CsCl gradients were precipitated overnight at 4°C with 10% trichloroacetic acid. The precipitate was centrifuged and washed successively with cold 5% and 0.5% trichloroacetic acid and then dissolved in SDS denaturing mix (62.5 mM Tris-hydrochloride, pH 6.8–4% SDS–10% 2-mercaptoethanol–6 M urea) and heated for 2 min at 100°C. The polypeptides were analyzed in a 17.5% acrylamide-0.08% bisacrylamide slab gel overlayered by a 5% acrylamide-0.13% bisacrylamide spacer gel in the discontinuous buffer system of Laemmli (10). Electrophoresis was carried out for 16 h at 30 V (constant voltage) with a Bio-Rad model 220 electrophoresis unit. The gels were fixed and stained with 0.3% Coomassie brilliant blue R-250 in 12% acetic acid-30% methanol, destained in the same fluid, dried under vacuum, and autoradiographed on Kodak Kodirex film.

Analysis of virus DNA. The DNA content of reversibly fixed mature virions and intermediates isolated in sucrose or Ficoll gradients and further separated on self-generating CsCl gradients was analyzed according to a modification of the procedure described by Doerfler (3). The virus suspension was adjusted to 0.5 M NaOH–50 mM sodium EDTA–10% 2-mercaptoethanol and layered on top of a 5 to 20% (wt/vol) alkaline sucrose gradient made in 0.3 M NaOH–0.7 M NaCl–1 mM sodium EDTA. After standing for 16 h at

4°C, the gradient was centrifuged for 5 h at 35,000 rpm and 4°C in an SW 41 rotor. Fractions were collected dropwise from the bottom and assayed for trichloroacetic acid-precipitable radioactivity.

Electron microscopy. The different classes of diimido ester-fixed virus particles obtained from sucrose or Ficoll gradients and further isolated in self-generating CsCl gradients were examined in a Hitachi HU-12 electron microscope after staining with 1% potassium phosphotungstate, pH 7.2.

RESULTS

Isolation and reversible fixation of Ad2 mature and intermediate particles. KB cells in suspension culture were infected with wild-type Ad2, concentrated to a density of 4×10^7 cells per ml, pulse-labeled for 15 min at 16 h postinfection with [^{35}S]methionine and [^{3}H]thymidine, and chased for 1.5 and 2.5 h. The cells were fractionated into cytoplasm and nucleus. Mock-infected cells were treated in the same way. The nuclei from Ad2-infected and mock-infected cells were lysed with 0.3 M $(NH_4)_2SO_4$ in TE buffer, and the nuclear lysate was analyzed on a sucrose gradient. Two distinct peaks of virus particles were visible on the sucrose gradient pattern of nuclear extract (Fig. 1). A first peak of virus particles sedimented in the position of mature adenovirion marker at 750S (fractions 14 to 17). This peak was scarcely visible at 1.5 h of chase and marked at 2.5 h of chase. A second peak (fractions 21 to 25), relatively homogeneous in shape, contained virus particles sedimenting at about 600S, with a ratio of DNA to protein lower than that of a normal virion. This peak was more important after a 1.5-h chase than after 2.5 h; it corresponded to Ad2 assembly intermediates (4). In mock-infected cell nucleus extracts obtained in the same way, no peak of nucleoproteinic material was found in that gradient zone (not shown). Similar results were obtained with Ficoll gradients (not shown).

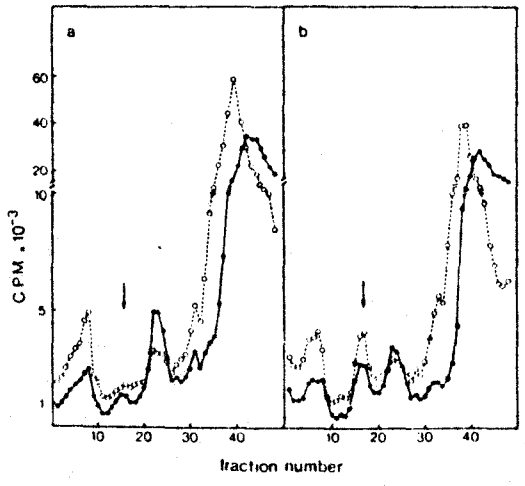

FIG. 1. *Velocity gradient analysis of wild-type Ad2 nuclear particles. Ad2-infected KB-cell cultures were pulse-labeled with [^{3}H]thymidine and [^{35}S]methionine for 15 min at 16 h p.i., and the label was chased for 1.5 h (a) and 2.5 h (b). The nuclear virus particles were extracted with $(NH_4)_2SO_4$ as in the text and analyzed on a 25 to 40% sucrose gradient with a CsCl cushion at the bottom (1.43 g/cm^3). The arrow indicates the position of mature adenovirus marker, sedimenting with an apparent sedimentation coefficient of 750S. Symbols: ●, ^{35}S; ○, ^{3}H. Bottom is at the left.*

The fractions corresponding to each peak were pooled, and the virus particles were cross-linked with the cleavable diimido ester dimethyl-4,4'-dithiobisbutyrimidate dihydrochloride, formed by oxidation of neighboring thiol groups carried by proteins previously amidinated with methyl-4-mercaptobutyrimidate (8, 13).

Buoyant density analysis of reversibly fixed Ad2 particles. The different populations of fixed virus particles were then analyzed on self-generating CsCl gradients (mean density, 1.34 g/cm^3). The virus particles sedimenting at 750S in sucrose gradients were found banding as a sharp peak at 1.345 g/cm^3, the normal buoyant density for mature and young adenovirions (Fig. 2a). The material sedimenting at 600S was found to consist of two populations of virus particles, with densities of 1.37 and 1.315 g/cm^3. The latter constituted the major class and had a lower ratio of DNA to protein label than did the 1.37-g/cm^3 species (Fig. 2b).

DNA analysis of Ad2 particles. Each population was analyzed with regard to its DNA content. DNA extracted under mild conditions was analyzed in alkaline sucrose gradients. Both 1.345- and 1.37-g/cm^3 particles contained a DNA sedimenting at 34S, the expected value for denatured complete Ad2 DNA. The 1.315-g/cm^3 particles contained a DNA fragment sedimenting as a broad peak between 7 and 11S (not shown).

Polypeptide composition of the different classes of Ad2 particles. The protein composition of the different classes of particles was analyzed on SDS-polyacrylamide slab gels after cleavage of the disulfide cross-bridges with SDS denaturing mix. Figure 3 shows the polypeptide pattern of each class of particles, compared with that of a mature adenovirion isolated at the end of a productive cycle by conventional techniques (6). Some differences could be discerned between the nuclear 1.345-g/cm^3 particles (Fig. 3e) and the mature adenovirions (Fig. 3f). The precursor proteins (P VI, P VIII, and P VII) were present, along with their cleavage products (virion proteins VI, VIII, and VII), in the 1.345-

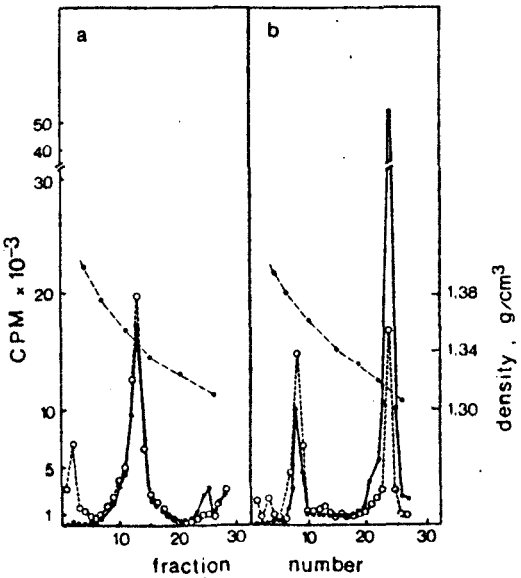

FIG. 2. *Buoyant density analysis of diimido ester-fixed adenovirus particles in CsCl gradients. The fractions corresponding to each virus particle peak obtained in sucrose gradients, as in Fig. 1b, were pooled, fixed with the bifunctional reagent dimethyl-4,4'-dithiobisbutyrimidate dihydrochloride, and further separated in a self-generating CsCl gradient (mean density: 1.34 g/cm³). (a) Lower peak of 75OS particles; (b) upper peak of 60OS particles. Symbols: ●, [³⁵S]methionine; ○, [³H]thymidine. Bottom is at the left.*

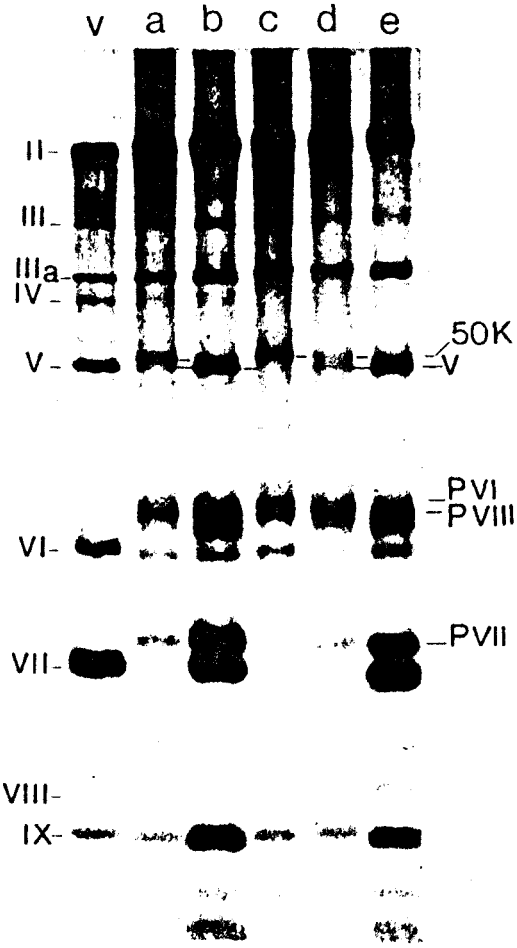

FIG. 3. *SDS-polyacrylamide gel electrophoretic analysis of Ad2 particles isolated on sucrose and CsCl gradients after reversible fixation with a cleavable diimido ester. Virus particles extracted from infected cell nuclei after a 15-min pulse with [³⁵S]-methionine at 16 h p.i., followed by a 2.5-h chase, were first isolated in sucrose gradients, reversibly fixed, and further separated in CsCl gradients. (a) 60OS intermediates isolated in a sucrose gradient, before fixation, as in Fig. 1b; (b) nonfixed 75OS virus particles from a sucrose gradient; (c) reversibly fixed intermediates of 1.315 g/cm³ of buoyant density, obtained from a CsCl gradient, as in Fig. 2b; (d) reversibly fixed intermediates banding at 1.37 g/cm³ in CsCl; (e) reversibly fixed 75OS particles banding at 1.345 g/cm³ in CsCl, as in Fig. 2a; (v) control adenovirus. Anode is at the bottom.*

g/cm³ particles, thus suggesting that they were young virions (4, 9). The 1.315-g/cm³ particles (Fig. 3c) totally lacked core proteins V and VII but possessed the precursors P VI and P VIII. In addition they contained two nonvirion proteins, with molecular weights of 50,000 (50K) and 39,000 (39K). Traces of P VII were occasionally visible. P VII were found in the intermediate peak of the sucrose gradient before fixation (Fig. 3a) and seemed to disappear almost completely from the intermediates after fixation and further purification in a CsCl gradient (Fig. 3c). P VII was, in fact, present in all sucrose gradient fractions (not shown) and, most likely, represented contaminating protein bound to particulate structures and sedimenting with a broad S range. The absence of P VII in the purified 1.315-g/cm³ particles did not result from an incomplete cleavage of cross-linked P VII, since the same reducing conditions on 1.345-g/cm³ particles released P VII monomer (Fig. 3e).

The 1.37-g/cm³ particles also contained precursors P VI and P VIII and, occasionally, traces of P VII but totally lacked core protein VII (Fig. 3d). In contrast to 1.315-g/cm³ particles the 39K and 50K were absent from the 1.37-g/cm³ species, and the V was present only in minute amounts. Both 1.315- and 1.37-g/cm³ particles lacked core structure sedimenting at 180–200S (7) after 0.5% deoxycholate treatment at 56°C (not shown). The 1.345-, 1.315-, and 1.37-g/cm³ particles occasionally contained a protein, 100K,

previously identified in Ad2 intermediates and young virions (4, 5, 9).

Pulse-chase labeling kinetic analysis of Ad2 particles. Ad2-infected KB cells were pulse-labeled at 16 h after infection for 15 min with [^{35}S]methionine and [^{3}H]thymidine and chased for 7 h. Cell samples were withdrawn at 0.5, 1, 1.5, 2.5, 4, and 7 h after the pulse and fractionated into cytoplasm and nucleus. Cytoplasmic and nuclear extracts were analyzed in sucrose gradients, and the different classes of particles thus obtained were fixed with dimethyl-4,4'-dithiobisbutyrimidate dihydrochloride and further separated on self-generating CsCl gradients. Figure 4a shows the evolution of the ^{35}S label repartition during the chase period between the different subclasses of particles, banding at 1.345, 1.315, and 1.37 g/cm^3. As early as 0.5 h after the pulse, radioactivity was present in 1.315-g/cm^3 particles and reached a maximum at 1 to 1.5 h of chase. At this time, no label was detectable in the 1.37- or in the 1.345-g/cm^3 particles. Radioactivity appeared in 1.37-g/cm^3 particles at 1.5 h of chase, increased until 2.5 h, and then progressively decreased. The nuclear virus particles of 1.345 g/cm^3 of density were only labeled at 2.5 h after the pulse, and the label reached a plateau at 4 h. Cytoplasmic virus particles (ρ = 1.345 g/cm^3) appeared labeled also at 1.5 h, with a similar labeling plateau at 4 h. The radioactivity remained constant throughout the chase period. These results suggested that the 1.315-g/cm^3 intermediate particles preceded the 1.37-g/cm^3 particles, which themselves were anterior to young virions (ρ = 1.345 g/cm^3).

The mode of entry of virus DNA into the capsid was followed by the same pulse-chase labeling kinetics. The DNA label seemed to enter the virus particle more slowly than protein label, with a lag of about 1.5 h between the two maxima of incorporation for the 1.37-g/cm^3 particles (Fig. 4b). These data suggested a relatively slow encapsidation of virus DNA.

Electron microscopic examination of Ad2 particles. Electron microscopic observations of the three classes of diimido ester-fixed particles revealed differences in their structures. The nuclear 1.315-g/cm^3 intermediates appeared similar in size to mature virions but did not present an icosahedral shape with distinct faces; the hexon capsomeres seemed less cohesively bound and less regularly arranged than those of a typical icosahedron (Fig. 5a). Their structure was reminiscent of that of Ad2 *ts*112 light particles accumulated at restrictive temperature and banding also at 1.315 g/cm^3 in CsCl (2). The

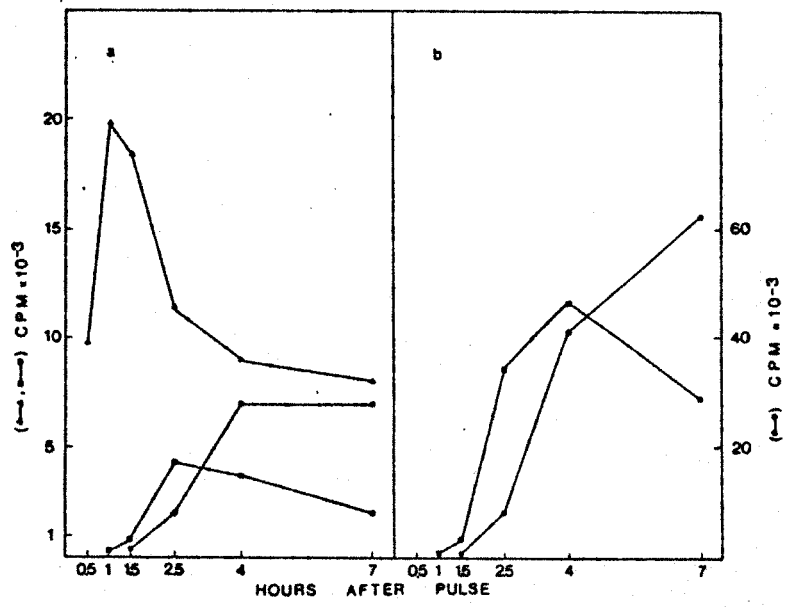

FIG. 4. *Pulse-chase labeling kinetics of Ad2 particles. Ad2-infected KB-cell cultures pulse-labeled at 16 h p.i. for 15 min with [^{35}S]methionine and [^{3}H]thymidine were chased for 7 h, and samples were withdrawn at 0.5, 1.0, 1.5, 2.5, 4, and 7 h after the pulse. Cells were fractionated into cytoplasm and nucleus, and cytoplasmic and nuclear extracts were analyzed in sucrose gradients to separate the virus particles and assembly intermediates. After reversible fixation with a diimido ester, the different size classes were further separated in CsCl gradients. The ordinate gives the value of ^{35}S (a) and ^{3}H (b) label in each particle subclass, of 1.315, 1.37, and 1.345 g/cm^3, during the chase period (abscissa). Symbols: ▲, nuclear 1.315-g/cm^3 intermediates; ■, nuclear 1.37-g/cm^3 intermediates; ●, nuclear 1.345-g/cm^3 particles.*

nuclear intermediates of 1.37 g/cm^3 of buoyant density appeared rounder and less polyhedral in shape than mature adenovirions, with vertexes hardly distinguishable (Fig. 5b). In contrast, no morphological difference could be observed between the 1.345-g/cm^3 nuclear particles and the mature virions: their icosahedral contour was clearly visible (Fig. 5c).

DISCUSSION

The question raised by the occurrence of incomplete particles in adenovirus infection is whether these particles are true preexisting intermediates in the assembly pathway or artifactual particles generated by the isolation procedure. Minimization of such artifacts is therefore the major requirement of any extraction procedure for virus assembly study. Purification and characterization of adenovirus intermediates by means of CsCl density gradient centrifugation have been shown to disrupt fragile adenovirus intermediates, and glutaraldehyde fixation has been recommended before analysis in CsCl (4). Unfortunately, neither DNA nor protein composition can be analyzed on such irreversibly fixed particles.

This major drawback has led us to use dimethyl-4,4'-dithiobisbutyrimidate dihydrochloride, a diimido ester cleavable under mild reducing conditions, which has been successfully used in neighbor analysis of proteins (13) and in the cross-linking of adenovirus with its cell receptor (8). The reversibly fixed virus particles can be isolated by density gradient centrifugation in CsCl and characterized with regard to their buoyant density, DNA content, and protein composition.

The diimido ester fixation was carried out on virus particles extracted from isolated nuclei with 0.3 M $(NH_4)_2SO_4$ and isolated on sucrose gradients. A comparison between the conditions used here and other techniques, such as sonic (4) or Freon (1) treatment of nuclei, was made with respect to their effects on the integrity of labile intermediates. The ammonium sulfate procedure appeared more reproducible and, apparently, less damaging for adenovirus intermediates, as controlled by electron microscopy and sucrose or Ficoll gradients. In addition, light intermediate particles similar to those observed in wild-type Ad2 are also found accumulated in an Ad2 temperature-sensitive mutant, *ts*112, at restrictive temperature (2), and pulse-chase labeling kinetics performed on wild-type Ad2 suggest a precursor-product relationship between the different classes of intermediate particles. All these data favor the idea that the different classes of Ad2 particles isolated here constitute true intermediates in the adenovirus assembly pathway.

As previously described (4), two size classes of Ad2 particles were separated by velocity gradient centrifugation, one sedimenting as infectious mature or young adenovirions, at 750S, and the other one sedimenting as assembly intermediates, at about 600S. After reversible fixation, the intermediate class could be further separated into two subclasses of particles, with buoyant densities of 1.315 and 1.37 g/cm^3. The 750S material consisted of a unique species of particles banding at 1.345 g/cm^3 in CsCl. Both 1.345- and 1.37-g/cm^3 particles contained a DNA with a sedimentation coefficient of 34S in alkaline sucrose gradients. The 1.315-g/cm^3 particles contained a DNA fragment of 7–11S, which might represent the beginning of the molecule entering the capsid to form the 1.37-g/cm^3 intermediate and broken during isolation. In this case, the light particles containing a piece of DNA would be at a stage intermediate in packaging, posterior to empty capsids.

After reduction of the natural and artificial disulfide bridges and SDS denaturation, the three classes of particles were analyzed on SDS-polyacrylamide gels. The 1.345-g/cm^3 particles resembled in polypeptide composition and electron microscopic aspect the young virions already characterized (4, 9), with some residue of precursor P VII of core protein VII. The 1.315-g/cm^3 particles totally lacked core proteins V and VII but contained precursors P VI and P VIII and two extra proteins (50K and 39K) not

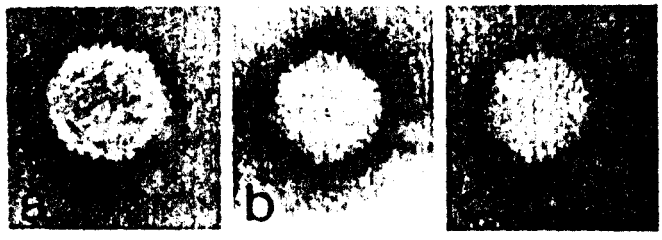

FIG. 5. *Electron microscopy of the three classes of Ad2 particles. (a) 1.315-g/cm^3 intermediates; (b) 1.37-g/cm^3 intermediates; (c) 1.345-g/cm^3 virus particles.* ×240,000.

found in mature virions. Both 50K and 39K were absent from the 1.37-g/cm^3 particles, which possessed a full length of DNA without appreciable amounts of core proteins V and P VII-VII. The 1.37-g/cm^3 particles also contained precursors P VI and P VIII. A 100K protein was visible in the three classes of particles. It is unlikely that the ammonium sulfate extraction of nuclei had removed some DNA-bound proteins, since the 72K and 45K (14) remain attached to DNA during this extraction (personal observation), and since adenovirus cores can be subjected to 2 M NaCl without detectable loss of DNA-bound protein material (7). Moreover, this extraction procedure maintains the RNA polymerases capable of elongating nascent RNA chains on DNA molecules (15).

Pulse-chase labeling kinetics revealed that 1.315-g/cm^3 particles preceded the 1.37-g/cm^3 species, which themselves preceded 1.345-g/cm^3 particles. This suggests the following sequence of events: capsid components → light intermediates (1.315 g/cm^3; 7-11S DNA) → heavy intermediates (1.37 g/cm^3; 34S DNA) → young virions (1.345 g/cm^3; 34S DNA) → mature virions (1.345 g/cm^3; 34S DNA). In this hypothetical pathway, the 50K and 39K, which are possibly scaffolding proteins, are released from the 1.315-g/cm^3 particles upon entry of 34S DNA to form 1.37-g/cm^3 particles devoid of core protein V and P VII-VII. It is possible that other intermediate steps take place between free capsid components and light intermediates.

In another study (2), we describe a temperature-sensitive mutant of Ad2 blocked at the stage of 1.315-g/cm^3 particles. Our results confirm recent findings (5) and suggest that virus DNA enters the adenovirus particle before core protein V and core precursor P VII and that DNA is not incorporated into the virion as a core structure. It remains to be elucidated whether the 7-11S DNA fragment present in the light intermediates corresponds to the same genome piece.

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Morphogenesis of Human Adenovirus Type 2 Studied with Fiber- and Fiber and Penton Base-Defective Temperature-Sensitive Mutants

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The nature, polypeptide composition, and antigenic composition of the particles formed by six human adenovirus type 2 temperature-sensitive (*ts*) mutants were studied. *ts*115, *ts*116, and *ts*125 were phenotypically fiber-defective mutants, and *ts*103, *ts*104, and *ts*136 failed to synthesize detectable amounts of fiber plus penton base at 39.5°C. The mutants belonged to five complementation groups, one group including *ts*116 and *ts*125. Except for *ts*103 and *ts*136, the other mutants were capable of producing particles at 39.5°C. *ts*116 and *ts*125 accumulated light assembly intermediate particles (or top components) at nonpermissive temperatures, with few virus particles. The sodium dodecyl sulfate polypeptide pattern of *ts*116- or *ts*125-infected cells, intermediate particles, and virus particles showed that polypeptide IV (fiber) was smaller by a molecular weight of 2,000 than that in the wild-type virion and was glycosylated. In fiber plus penton base-defective *ts*104-infected cells, equivalent quantities of top components and viruses with a buoyant density (ρ) of 1.345 g/ml (ρ = 1.345 particles) were produced at 39.5°C. These ρ = 1.345 particles corresponded to young virions, as evidenced by the presence of uncleaved precursors to proteins VI, VIII, and VII. These young virions matured upon a shift down. Virus capsid vertex antigenic components underwent a phase of eclipse during their incorporation into mature virus particles. No antigenic penton base or IIIa was detected in intermediate particles of all the *ts* mutants tested. Only hexon and traces of fiber antigens were found in *ts*104 young virions. Penton base and IIIa appeared as fully antigenically expressed capsid subunits in mature wild-type virions or *ts*104 virions after a shift down. The *ts*104 lesion is postulated to affect a regulatory function related in some way to penton base and fiber overproduction and the maturation processing of precursors PVI, PVIII, and PVII.

The adenovirus assembly pathway proceeds from soluble capsid components via multiple steps involving assembly intermediate (IM) particles separable from mature virions in CsCl gradients and identifiable by sodium dodecyl sulfate (SDS)-polyacrylamide gel patterns (5, 12). Among these IM particles, there are those devoid of 31S viral DNA, the so-called top components, banding at a buoyant density (ρ) of 1.30 to 1.31 g/ml in CsCl (4, 5, 12), heavy IM particles of ρ = 1.37 g/ml (3), and immature, or "young virions" (YV [5, 12]).

Temperature-sensitive (*ts*) mutants are invaluable tools for determining the function of human adenovirus (HAd) proteins in virion morphogenesis (11). Sets of HAd *ts* mutants have been selected and characterized in several laboratories and include HAd2 (1, 14, 21), HAd5 (7, 35), HAd7 (8), HAd12 (18, 27), and HAd31 (32).

The HAd2 penton base and fiber projection is known to be a weak point in the virus icosahedron (17). In the present study, the role of the vertex subunits in HAd2 capsid formation was studied with the aid of six *ts* mutants, three altered in a single component (fiber) and three altered in a pair of two capsid components (fiber and penton base).

MATERIALS AND METHODS

Cells and viruses. Wild-type (WT) HAd2, originally obtained from J. F. Williams (Carnegie-Mellon Institute, Pittsburg, Pa.), was grown on KB cells cultured in suspension (2.0×10^5 to 5.0×10^5 cells per ml) in Eagle Spinner medium supplemented with 5% horse serum. HeLa cells were grown in monolayers in Eagle minimal essential medium supplemented with 10% calf serum. Virus was titrated by the plaque assay or by the fluorescent focus unit (FFU) assay (25) on a HeLa cell monolayer at 37°C.

ts mutants of HAd2 (block no. 101 to 200 [21]) were isolated after nitrous acid treatment of a WT stock,

which underwent two cycles of plaque purification at 37°C. Thirty-nine *ts* mutants were thus obtained from 400 clones isolated at random (21).

Antisera. Polyspecific rabbit antiserum against HAd2 virion proteins was obtained by multiple subcutaneous injections of HAd2 particles purified by two cycles of equilibrium centrifugation in CsCl (20, 21). Monospecific antisera against hexon, penton base, fiber, or IIIa were obtained by injection of corresponding antigen-antibody precipitates freshly formed in agarose gel after two-dimensional immunoelectrophoresis (2; P. Lemay, M. L. Boudin, M. Milleville, and P. Boulanger, Virology, in press).

Complementation tests. HeLa cell monolayers were doubly infected at an input multiplicity of 5 FFU of the two *ts* mutants per cell. After 2 h of adsorption at 33°C, unadsorbed virus was rinsed off, and the cells were treated for 30 min with an anti-HAd2 virion serum at a dilution of 1:100. The cells were rinsed again and incubated at 39.5°C for an additional 40 h. Control cultures were singly infected in parallel with 10 FFU of each mutant per cell. At the end of the incubation period, the cells were scraped into the culture medium and disrupted by three cycles of freezing and thawing, and virus was titrated with the FFU assay at 33°C. The complementation index was given as the ratio of yield of the double infection to that of the higher of the two single infections at 39.5°C, expressed as FFU per milliliter. Complementation is usually considered as positive when the complementation index is at least 10 (35).

Immunological characterization of *ts* mutants. HAd2 *ts* mutants were serologically characterized by quantitation in crossed immunoelectrophoresis of the soluble antigens produced by the infected cells at 39.5°C compared with 33°C and with the WT at both temperatures (20, 21).

Quantitative and qualitative analyses of hexon, fiber, penton base, and IIIa antigens were performed in crossed immunoelectrophoresis of deoxycholate (DOC)-disrupted virions (2, 2a, 24; Lemay et al., Virology, in press). Virus particles were suspended in 0.005 M Tris-hydrochloride buffer, pH 7.8, and heated with 0.5% sodium DOC at 56°C until disappearance of the opalescence (usually 60 to 90 s). This treatment did not damage the hexon, fiber, and IIIa, but penton base was separated from the fiber projection (2; Lemay et al., Virology, in press).

Pulse-chase of infected cells. Pulse-chase labeling experiments were performed in KB cells (6×10^6 cells per ml) in methionine-deprived medium. L-[^{35}S]methionine (50 μCi/ml) was added for 20 min at different times postinfection at 39.5°C (nonpermissive temperature) or at 33°C (permissive temperature). The cells were harvested just after the pulse or chased by dilution to 3×10^5 cells per ml in medium prewarmed to the required temperature and containing 10 times the normal concentration of cold methionine. L-[^{35}S]methionine-labeled virus-induced and virion proteins were analyzed in SDS-containing polyacrylamide gels.

Analytical SDS-polyacrylamide gel electrophoresis. Samples were dissolved in an equal volume of sample buffer (0.0625 M Tris-hydrochloride, pH 6.8, containing 6 M urea, 4% SDS, and 10% 2-mercaptoethanol) and heated for 2 min at 100°C. Polypeptides were analyzed in an SDS-containing 17.5% polyacrylamide gel (acrylamide/bisacrylamide ratio of 50:0.235) overlaid by a 5% spacer gel (acrylamide/bisacrylamide ratio of 50:1.33) in the discontinuous buffer system of Laemmli (16). The gels were stained with Coomassie brilliant blue R-250, dried under vacuum, and exposed to Kodak Kodirex film.

Extraction and purification of virus particles and top components. Infected cells, harvested late in the infectious cycle (20 to 30 h after infection), were extracted with Freon 113 as previously described (3), and mature or YV (ρ = 1.345 g/ml) and light IM particles or top components (ρ = 1.30 to 1.31 g/ml) were separated in self-generating CsCl gradients (3).

Cell fractionation and extraction of assembly IM and virus particles. The method of separation of cytoplasmic and nuclear fractions and the extraction of particles from nuclei have been described in detail elsewhere (3, 4). Assembly IM and virus particles were isolated on a linear (25 to 40%) sucrose gradient: virions sediment at 750S, and assembly IM particles sediment at about 600S (3, 4).

The different classes of particles were purified further in self-generating CsCl gradients after fixation with a cleavable diimido ester (dimethyl-4,4′-dithiobisbutyrimidate [3, 4]).

Extraction and isolation of adenovirus core. Virions or IM particles were disrupted with 0.5% DOC as described above. The different subviral entities were separated in a discontinuous sucrose gradient (40 to 70%, 3-ml total volume; overlaid by a 5 to 20% gradient, 8-ml total volume; made in 0.02 M sodium borate buffer, pH 8.0, containing 1 M NaCl and 0.001 M EDTA). The gradients were centrifuged at 35,000 rpm for 2 h at 4°C in a Beckman SW41 rotor. Virus cores sediment at 180 to 200S, groups of nine hexons sediment at 50 to 60S, and isolated capsomers sediment at between 6 and 12S (2a).

Chemicals and radioisotopes. Sodium DOC was purchased from BDH, Poole, United Kingdom. A 10% solution (wt/vol) was made in water and dialyzed in the cold against distilled water. The dialyzable fraction obtained was lyophilized and used for virus disruption.

Cycloheximide (CH) was purchased from Boehringer, Mannheim, Federal Republic of Germany, and used at a concentration of 20 μg/ml of cell culture. Puromycin (Sigma Chemical Co., St. Louis, Mo.) was used at 10 μg/ml.

Methyl-4-mercaptobutyrimidate hydrochloride (Pierce Chemical Co., Rockford, Ill.) was used at a concentration of 3 mg/ml in medium devoid of primary amine. The reaction of amidination of proteins was allowed to proceed for 30 min at 4°C. Disulfide bridges were induced by dialysis against phosphate-buffered saline containing 0.1 M H_2O_2 (3). The induced disulfide cross bridges could be cleaved by an excess of reducing agent (e.g., 2-mercaptoethanol or dithiothreitol).

Bovine serum albumin (2× crystallized; Sigma Chemical Co.) was used as a standard for the protein assay (19).

L-[^{35}S]methionine (700 to 800 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, United Kingdom, and [^{3}H]thymidine (25 Ci/mmol) was from the Commissariat à l'Energie Atomique,

Saclay, France. Pulse-labeling of viral proteins and DNA was performed at 50 μCi of radioisotopes per ml for 20 min at a late time after infection (16 h postinfection). [^{3}H]glucosamine (Commissariat à l'Energie Atomique) with a specific activity of 12 Ci/mmol was used at 50 μCi/ml in prolonged labeling (16 to 24 h after infection).

RESULTS

Complementation. As shown in Table 1, *ts*103 strongly complemented *ts*104, *ts*115, *ts*116, *ts*125, and *ts*136. *ts*104 also complemented *ts*125 and *ts*136, and *ts*115 complemented *ts*116, *ts*125, and *ts*136. There was no ambiguity either for *ts*116 and *ts*136 or for *ts*125 and *ts*136, if a limit of 10 was chosen as a positive complementation index. A complementation index of 3.6 was found between *ts*104 and *ts*115, a complementation index of 6.7 was found between *ts*104 and *ts*116, and a complementation index of 7.2 was found between *ts*116 and *ts*125. This suggested that there were three complementation groups, one group including *ts*103, the second group including *ts*136, and the third group including *ts*104, *ts*115, *ts*116, and *ts*125.

However, *ts*115 and *ts*116 complemented each other, as did *ts*115 and *ts*125. In addition, the biological properties of *ts*104, *ts*115, and *ts*116 were different as shown below. In contrast, *ts*116 and *ts*125, which complemented each other poorly, could not be distinguished serologically or biochemically. Therefore, five complementation groups were suggested, two groups including the three fiber-defective mutants, *ts*115 in one group and both *ts*116 and *ts*125 in the other, and three groups corresponding to each of the three penton base and fiber-defective mutants *ts*103, *ts*104, and *ts*136.

All of the mutants, except *ts*115, were strongly complemented by HAd2 *ts*112, a mutant defective in late functions essential for a normal virus maturation (4). *ts*112 showed a serological pattern at 39.5°C similar to the WT pattern, with respect to the production of hexon, penton base, penton, and fiber (21). This result suggests that *ts*115 is a double mutant.

Serological characterization. Five of the six mutants studied have previously been characterized serologically by the two-dimensional immunoelectrophoresis pattern of cells infected with the *ts* mutants at 39.5°C (21). However, careful reexamination of the two-dimensional patterns of *ts*104 and *ts*116, previously catalogued in the same complementation group, C, indicated that the two mutants should be classified in two separate groups, C and L: *ts*116 appeared fiber defective, as did *ts*115 (group E), and presented the same polypeptide pattern as did *ts*125 (group L). *ts*104 was phenotypically penton base and fiber defective (21).

The new mutant *ts*136 failed to synthesize an excess of penton base and fiber in the infected cell and resembled the *ts*103 and *ts*104 phenotype. The biological properties of these mutants are summarized in Table 2.

***ts* mutant polypeptides in infected cells.** KB cells were infected with the WT or a *ts* mutant at 10 FFU/cell at 39.5°C and pulse-labeled for 1 h at 16 h after infection. The cell culture was divided into three portions, one being arrested just after the pulse, one being chased for 8 h at 39.5°C, and the third one being chased for 24 h at 33°C. Virus-coded polypeptides were analyzed in SDS-polyacrylamide gels.

Figure 1 shows that, except for *ts*116 and *ts*125, there was no visible cleavage of the precursors to virion proteins VI, VIII, and VII during the chase at 39.5°C. The precursor PVI of *ts*115 migrated nearer PVIII than in the WT or in the other *ts* mutants, with an apparent molecular weight of 26,500 instead of 27,000. The same difference in molecular weight was observed between polypeptide VI of *ts*115 virions produced at 33°C and polypeptide VI of the WT virus particles (see Fig. 9).

In *ts*116- and *ts*125-infected cells, the apparent

TABLE 1. *Complementation indices between fiber-defective and fiber and penton base-defective ts mutants of HAd2*

Mutant	Complementation index						
	*ts*103	*ts*104	*ts*115	*ts*116	*ts*125	*ts*136	*ts*112[a]
*ts*103 (F)	—[b]	125	67	322	360	75	75
*ts*104 (C)		—	3.6	6.7	37	56	50
*ts*115 (E)			—	20	18	26	3.9
*ts*116 (L)				—	7.2	20	52
*ts*125 (L)					—	10	135
*ts*136 (M)						—	50
*ts*112[b] (I)							—

[a] HAd2 *ts*112 is blocked in late functions essential for a normal virion maturation (4). Its serological pattern at 39.5°C resembles that of the WT (21). Letters within parentheses are complementation groups.

[b] —, Dash indicates a complementation index of 1.

TABLE 2. *Soluble capsid antigens and particles produced by fiber-defective and fiber and penton base-defective ts mutants of HAd2 at a late stage after infection at 39.5°C*[a]

Mutant	Complementation group	Soluble antigens				Particles	
		Hexon	Penton base	Fiber	IIIa	Top components	Virions
*ts*103	F	+	±	±	±	0	0
*ts*104	C	+	±	±	±	+	+ (YV)
*ts*115	E	+	+	0	±	++	± (mature)
*ts*116	L	+	+	±	±	++	± (mature)
*ts*125	L	+	+	±	±	++	± (mature)
*ts*136	M	+	0	0	0	0	0

[a] ++, Increased amount, compared with the WT; +, same amount as in the WT; ±, trace amount; 0, not detectable. Top components: light particles banding at 1.30 to 1.31 g/ml in CsCl.

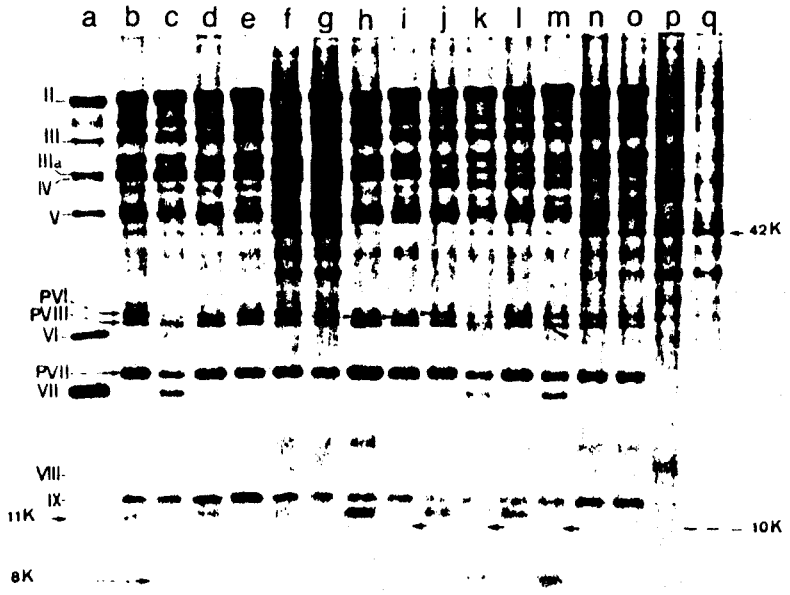

FIG. 1. *SDS-polyacrylamide gel autoradiogram of polypeptides of WT-, ts mutant-, and mock-infected KB cells at 39.5°C. The cells were pulse-labeled with [^{35}S]methionine for 1 h at 16 h after infection (b, d, f, h, j, l, n, and p) and chased for 8 h at 39.5°C (c, e, g, i, k, m, o, and q). (a) WT HAd2 virion; (b and c) WT-infected cells; (d and e) ts103; (f and g) ts104; (h and i) ts115; (j and k) ts116; (l and m) ts125; (n and o) ts136; (p and q) mock-infected cells. A 60,000-molecular-weight (60K) polypeptide is visible in place of polypeptide IV (62K) in ts116 (j)- and ts125 (l)-infected cells. Cleavage of PVII into VII occurs during the chase in WT-, ts116-, and ts125-infected cells, whereas an 11K polypeptide disappears and an 8K polypeptide appears. The label decreases in the 60 to 62K polypeptides during the chase at 39.5°C in ts115, ts116, and ts136, whereas a 10K polypeptide becomes visible. Note that actin polypeptide synthesis (42K) is not completely inhibited in ts104 and ts136 infection.*

molecular weight of polypeptide IV was 2,000 lower than that in the WT or in other *ts* mutants. In these two mutants, some cleavage of PVII to VII occurred during the chase of 39.5°C, although at a slower rate than that in the WT, a polypeptide with a molecular weight of 11,000 disappeared, and a polypeptide band with a molecular weight of 8,000 appeared. In *ts*115, *ts*116, *ts*125, and *ts*136, the fiber polypeptide (IV) decreased during the chase at 39.5°C, and an extra polypeptide band with a molecular weight of 10,000 appeared.

During a 24-h chase period at 33°C, the polypeptide patterns of the *ts* mutants were similar to that of the WT, with cleavage of the precursors PVI, PVIII, and PVII. However, the lesion of *ts*116 and *ts*125 was still apparent at 33°C, with a polypeptide with a molecular weight of 60,000 in place of the fiber polypeptide with a molecular weight of 62,000 (Fig. 2).

The possibility of a defect in the glycosylation process of the fiber protein at 39.5°C was examined by labeling infected cells with [^{3}H]glucosamine for 8 h at 16 h after infection. SDS-polyacrylamide gel analysis revealed that the *ts* mutants studied possessed a glycosylated fiber polypeptide, the same as that of the WT (13). In *ts*116 and *ts*125, the 60,000-molecular-weight polypeptide replacing polypeptide IV fiber was also glycosylated (Fig. 3).

Particles produced at the end of the infectious cycle at 39.5°C. Cells infected at 10 FFU/cell at 39.5°C were harvested 30 h after infection, and virus particles or IM particles or both were extracted with fluorocarbon and separated by buoyant density in CsCl gradients (4): virus particles (YV and mature virions) band at ρ = 1.345 g/ml, and light assembly IM particles or top components devoid of 31S viral DNA band at ρ = 1.30 to 1.31 g/ml (12).

As shown in Table 2, mutants *ts*103 and *ts*136 did not produce any detectable particles at 39.5°C, neither at ρ = 1.345 g/ml nor at ρ = 1.30 g/ml. Only soluble components were overproduced, consisting essentially of hexon capsomers. *ts*115 produced mainly top components and few virus particles of ρ = 1.345 g/ml. These ρ = 1.345 g/ml particles had a normal 31S DNA and the same polypeptide pattern as did mature WT virions (data not shown). These particles might result from a certain degree of leakiness of this mutant (21).

*ts*116 and *ts*125 also produced mainly top components at 39.5°C and a minor population of virions of ρ = 1.345 g/ml. These virions possessed a 60,000-molecular-weight polypeptide in place of the 62,000-molecular-weight (IV) polypeptide normally present in the WT (see Fig. 8f).

In contrast to the other mutants, *ts*104 produced equivalent quantities of top components and ρ = 1.345 g/ml virus particles. When analyzed in SDS-polyacrylamide gels, these ρ = 1.345 g/ml particles showed uncleaved precursors PVI, PVIII, and PVII, with only trace amounts of virion proteins VI, VIII, and VII (see Fig. 7b), thereby resembling the HAd2 *ts*1 mutant (33). These ρ = 1.345 g/ml particles might, therefore, be considered YV, according to the generally accepted nomenclature (5, 12).

Absence of DOC core in *ts*104 YV. The *ts*104 YV were purified as follows. The peak sedimenting at 750S in sucrose gradients was purified further in a 40 to 50% metrizamide gradient made in 0.02 M sodium borate buffer, pH 8.0 (10). Metrizamide was eliminated by exclusion chromatography on Sephadex G-50. The *ts*104 YV were subjected to DOC treatment

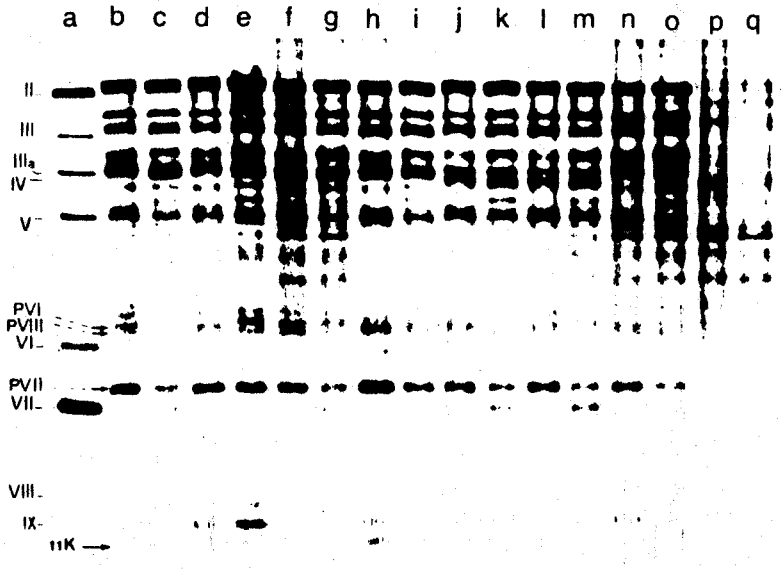

FIG. 2. *SDS-polyacrylamide gel autoradiogram of temperature-shift experiments. WT-, ts mutant-, or mock-infected KB cells were pulse-labeled for 1 h at 39.5°C at 16 h after infection (b, d, f, h, j, l, n, and p) and chased for 24 h at 33°C (c, e, g, i, k, m, o, and q). (a) WT HAd2 virion; (b and c) WT-infected cells; (d and e) ts103; (f and g) ts104; (h and i) ts115; (j and k) ts116; (l and m) ts125; (n and o) ts136; (p and q) mock-infected cells. Processing of PVII into VII and disappearance of an 11,000-molecular weight (11K) polypeptide occur upon a shift down in every infected-cell sample.*

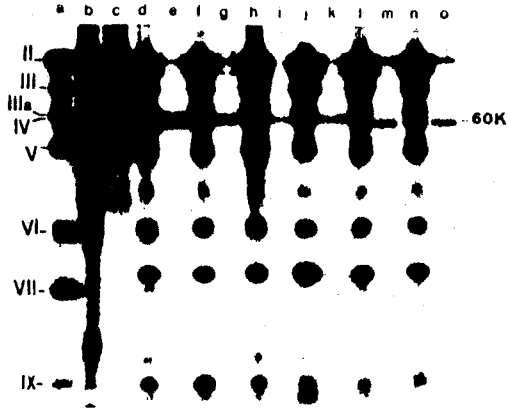

FIG. 3. *Glycosylation pattern of adenovirus-infected cell polypeptides. KB cells were mock infected (b and c) or infected with the WT (d and e), ts103 (f and g), ts104 (h and i), ts115 (j and k), ts116 (l and m), and ts125 (n and o). Labeling was performed with [^{35}S]methionine (b, d, f, h, j, l, and n) or [^{3}H]glucosamine (c, e, g, i, k, m, and o) for 8 h at 16 h after infection. (a) Control HAd2 virion. Polypeptide IV (fiber) has been found to be labeled with [^{3}H]glucosamine (13). Note the lower molecular weight of ts116 and ts125 late major glycopolypeptide: 60,000 (60K) instead of 62K. The ^{3}H label was revealed by fluorography in PPO (2,5-diphenyloxazole)-impregnated gels.*

at 56°C, and the resulting virus lysate was analyzed on a sucrose gradient as described above. Groups of nine hexons were visible at 50 to 60S, and isolated capsomers were visible at between 6 and 12S, but no viral cores were obtained from these particles. A peak of [^{3}H]thymidine-labeled viral DNA was found at 31S in the gradient (Fig. 4a). After a shift down to 33°C, the *ts*104 YV matured, as evidenced by the cleavages of PVII into VII and of PVI and PVIII into VI and VIII, respectively. DOC treatment of the temperature-shifted *ts*104 YV revealed the presence of a DNA-containing DOC core, with an apparent sedimentation coefficient of 180 to 200S (Fig. 4b).

Antigenicity of capsid proteins within the particles produced by *ts* mutants. The different classes of particles produced by mutants *ts*104, *ts*115, *ts*116, and *ts*125 were disrupted with DOC, analyzed in two-dimensional immunoelectrophoresis against antivirion serum, and compared with the DOC-disrupted WT pattern. The results are summarized in Table 3 and illustrated in Fig. 5. The top components, or light IM particles, of the mutants studied and of the WT exhibited no antigenically active penton base and IIIa: hexon was the only major detectable capsid antigen. Fiber antigen was present in a normal amount in WT top components, but was found only in trace amounts in top components of *ts*104. *ts*104 YV showed no penton base but traces of fiber antigen. However, all particles possessed the corresponding polypeptides visible in SDS-polyacrylamide gels, viz., III, IIIa, and IV (see Fig. 7 and 8).

In *ts*116- and *ts*125 top components and mature virions, no antigenic fiber was detectable, and the SDS polypeptide pattern showed the absence of the fiber polypeptide unit with a molecular weight of 62,000 and replacement by a 60,000-molecular-weight polypeptide (see Fig. 8f). As in WT top components, light particles of

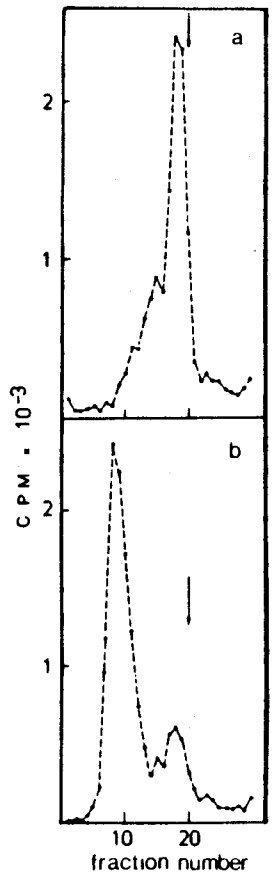

FIG. 4. *Core analysis of ts104 particles after a shift down. KB cells were infected with ts104 at 39.5°C, pulse-labeled for 20 min at 16 h postinfection with [^{3}H]thymidine (50 μCi/ml), and chased at 39.5°C for 3 h. One sample was withdrawn after the chase; virus particles were extracted, disrupted with DOC, and analyzed in a discontinuous sucrose gradient (a). The other sample (b) was chased for 16 h at 33°C in the presence of CH (20 μg/ml), and particles were treated as in (a). The arrow indicates the position of the 31S virus DNA marker.*

ρ = 1.315 g/ml accumulated by *ts*112 at restrictive temperatures (4) contained only hexon and fiber antigens. Antigenic penton base appeared only in mature virions produced normally by the WT or in minor quantities by *ts*125 at 39.5°C (Fig. 5).

Pulse-chase and temperature-shift experiments. An inhibition of protein synthesis in infected cells has been shown to block the formation of IM particles from soluble virus components (28), but does not prevent the maturation of IM particles and YV into mature virions (4). A shift down to 33°C in the presence of CH was therefore used to determine whether the IM particles or YV assembled at 39.5°C were capable of evolving into normal mature virions. Infected cells were pulse-labeled for 20 min at 39.5°C at 16 h after infection and chased with cold methionine for 2 h at 39.5°C. The cell culture was then divided into six portions. One was immediately processed for virus particle extraction (sample 1). One was further chased for 5 h at 39.5°C (sample 2). One was chased at 39.5°C for 1 h in the presence of CH (sample 3). A fourth one was chased for 1 h at 39.5°C with CH, then shifted down to 33°C, and incubated at this temperature for an additional period of 21 h with CH (sample 4). Sample 5 was chased at 33°C for 22 h in the presence of CH, and the last sample was chased at 33°C for 22 h without CH (sample 6). A scheme of this experiment is shown in Fig. 6.

The IM and virus particles were extracted from each cell sample and analyzed on sucrose density gradients. IM particles sediment as a peak of 600S, whereas virions sediment at 750S (3, 5).

Table 4 shows the results obtained with *ts*104.

TABLE 3. *Antigenicity of the major capsid proteins of DOC-treated virus particles and assembly intermediates of HAd2 ts mutants and the WT*

Virus	Type of particles analyzed	Capsid proteins[a]			
		Hexon	Penton base	Fiber	IIIa
*ts*104	Top components	+	0	±	0
*ts*104	YV	+	0	±	0
*ts*115	Top components	+	0	0	0
*ts*116	Top components	+	0	0[b]	0
*ts*125	Top components	+	0	0[b]	0
*ts*125	Mature virions	+	+	0[b]	±
*ts*112[c]	Top components	+	0	+	0
WT	Top components	+	0	+	0
WT	Mature virions	+	+	+	+

[a] +, Same amount of antigen as in the DOC-treated WT virion; ±, trace amount; 0, no detectable antigen.

[b] Presence of a 60,000-molecular-weight polypeptide in place of the fiber polypeptide of 62,000 molecular weight.

[c] HAd2 *ts* mutant defective in virion morphogenesis and accumulating light assembly IM particles (4).

In this mutant, the total label in assembly IM and virus particles decreased throughout the chase at 39.5 and 33°C, whether CH was added or not, suggesting a significant breakdown of one or both types of particles. During the chase at 39.5°C, the radioactive label seemed to enter the 600S IM particles more rapidly than from IM particles into 750S virions. However, at 33°C the label decreased at a much lesser rate in the 750S virus peak than in the 600S IM particles, especially in the presence of CH, which blocks the incorporation of soluble components into IM particles (28), suggesting either a relatively greater stability of the 750S particles at 33°C or a flow of radioactive label from 600S IM particles into 750S virions, or both.

*ts*103 and *ts*136 showed no detectable peak of labeled IM and virus particles at 39.5°C. *ts*115 showed only a peak of IM particles, with no occurrence of 750S virions, even after a shift down (data not shown). Both *ts*116 and *ts*125 assembled few 750S virions at 39.5°C, with an apparently normal maturation at either 39.5 or 33°C, as shown by the polypeptide pattern (see Fig. 8). At 33°C, the peak of 750S particles increased. The maturation process occurred also in the presence of CH at 39.5 and 33°C (data not shown).

Polypeptide pattern of particles obtained in pulse-chase and temperature-shift experiments. The 750S and 600S peaks of each of the six gradients shown in Fig. 6 were analyzed in SDS-polyacrylamide gels. The polypeptide compositions of *ts*104 and *ts*125 IM and virus particles are shown in Fig. 7 and 8. *ts*104 YV accumulated at 39.5°C were capable of maturation upon a shift down, as evidenced by the processing of precursors PVI, PVIII, and PVII and the appearance of polypeptide XI. This maturation processing also occurred when infected cells were shifted down to 33°C in the presence of CH or puromycin, suggesting that the putative virus-coded endopeptidase (33) was already synthesized but inactivated at 39.5°C (Fig. 7).

*ts*125, as well as *ts*116, showed an abnormal polypeptide pattern of assembly IM and virus particles, with a 60,000-molecular-weight polypeptide in place of the 62,000-molecular-weight fiber polypeptide. The *ts*116 and *ts*125 virions which matured at 33°C in the presence or absence of CH also contained the 60,000-molecular-weight polypeptide in place of polypeptide IV (Fig. 8 and 9).

DISCUSSION

The present study confirms previous reports on adenovirus (6, 26, 36) or bacteriophage systems (22), showing that fiber-defective mutants can assemble particles under restrictive condi-

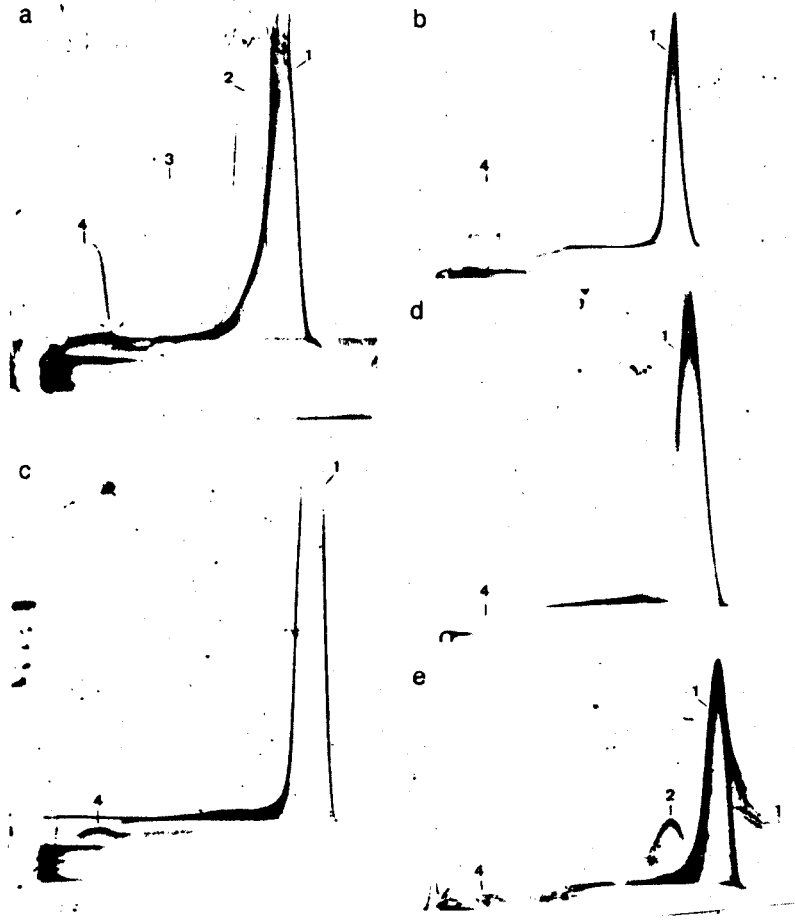

FIG. 5. *Two-dimensional immunoelectrophoretic analysis of antigenic components of WT and ts mutant particles. The particles were disrupted with 0.5% DOC before two-dimensional analysis. (a) WT mature virions; (b) WT top components (or assembly IM particles of ρ = 1.315 g/ml); (c) ts104 YV of ρ = 1.345 g/ml; (d) ts104 top components; (e) ts125 virions of ρ = 1.345 g/ml. The peaks of immune precipitates have been identified previously as follows: (1) hexon; (2) penton base; (3) protein IIIa; (4) fiber (2a, 20, 21; Lemay et al., Virology, in press). DOC dissociates complete penton into fiber and penton base (2).*

tions. The fiber-defective mutant *ts*115 underwent assembly until the stage of light IM particles (or top components), which accumulated at 39.5°C, whereas *ts*116 and *ts*125 produced few virus particles at nonpermissive temperatures. Both *ts*116 and *ts*125 synthesized fiber which was abnormal in antigenic properties and polypeptide structure (molecular weight of 60,000 instead of 62,000 for the WT fiber polypeptide unit). Despite the altered fiber structure, *ts*116 and *ts*125 IM particles were capable of maturation upon a shift down to 33°C, resulting in the production of infectious virions with a 60,000-molecular-weight protein in place of the 62,000-molecular-weight polypeptide IV (Fig. 8 and 9). The mutation of *ts*116 and *ts*125 was also expressed at 33°C, and the same 60,000-molecular-

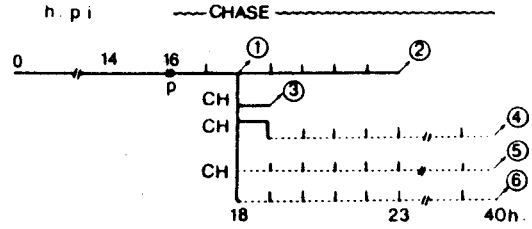

FIG. 6. *Scheme of pulse-chase and shift-down experiments in the presence or absence of CH. KB cells were infected with ts104 or ts125 at 39.5°C, pulse-labeled (P) with [^{35}S]methionine (50 μCi/ml) for 20 min at 16 h after infection (p.i.), and chased at 39.5°C (solid lines) or at 33°C (dotted lines). Samples were withdrawn at different times of the chase (numbers within circles) and analyzed in sucrose gradients to separate nuclear 750S virions and 600S IM particles.*

weight protein was synthesized in infected cells at 33°C (Fig. 2).

*ts*116 and *ts*125, as well as the other fiber- and fiber and penton base-defective *ts* mutants studied here, had an apparently normal glycosylated fiber (Fig. 3). These results suggest that (i) the site of glycosylation of fiber polypeptide corresponds to a region of the virus genome at which it is difficult to induce mutations or (ii) there are several sites of glycosylation. The occurrence in *ts*116 and *ts*125 of polypeptide IV smaller in size by a molecular weight of 2,000 than the WT polypeptide IV led to the following hypotheses: (i) the *ts*116 or *ts*125 or both mutations can generate a new initiation or termination codon on the fiber mRNA, with a polypeptide chain shortened at its N or C end; (ii) the mutations might have created a new preferential cleavage site for a cellular or virus-coded endopeptidase; (iii) whichever end of the *ts*116 or *ts*125 fiber polypeptide chain is modified, the production of infectious *ts*116 and *ts*125 at 33°C with normal infectivity and normal morphology suggests that the first 20 or the last 20 amino acids of the polypeptide IV chain were not indispensable for assembly of the normal fiber trimer structure (29) or for penton base and fiber assembly, at least at 33°C.

TABLE 4. *Pulse-chase analysis of assembly IM and virus particles of HAd2 ts104 with or without a shift down*

Sample	cpm[a]			
	600S IM (A)	750S virus (B)	Total radioactivity (A + B)	Ratio of (B − A)/(A + B)
1	35,900	22,700	58,600	−0.225
2	29,200	15,800	45,000	−0.298
3[b]	20,100	18,400	38,500	−0.044
4[b]	17,700	19,600	37,300	+0.050
5[b]	9,000	15,700	24,700	+0.271
6	12,100	20,000	32,100	+0.246

[a] The numbers correspond to the radioactivity of the samples defined in the legend to Fig. 6.
[b] CH added during the chase.

*ts*104, which is phenotypically fiber and penton base defective, accumulated equivalent quantities of top components and YV at nonpermissive temperatures. In the presence of CH, which has been shown to inhibit the de novo formation of IM particles from the pool of soluble components (28), the YV evolved into mature infectious virions after a shift down to 33°C, as evidenced by the processing of PVI, PVIII, and PVII. This mutant resembled, therefore, the HAd2 *ts*1 mutant of Weber, which is blocked at 39.5°C at a stage of immature virions with un-

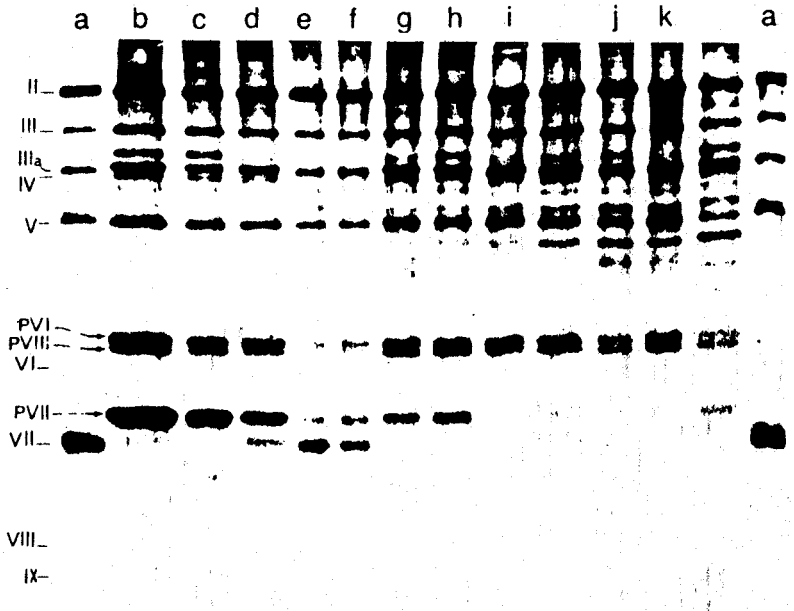

FIG. 7. *Polypeptide pattern of ts104 nuclear 750S YV (b, c, d, e, and f) and 600S IM particles (g, h, i, j, and k) produced during pulse-chase and shift-down experiments, as schematized in Fig. 6. (a) Control HAd2 virion; (b and g) sample 1 of Fig. 6; (c and h) sample 3; (d and i) sample 4; (e and j) sample 6; (f and k) sample 5. The maturation of YV is evidenced by the processing of PVII into VII upon a shift down (d, e, and f), even in the presence of CH (d and f).*

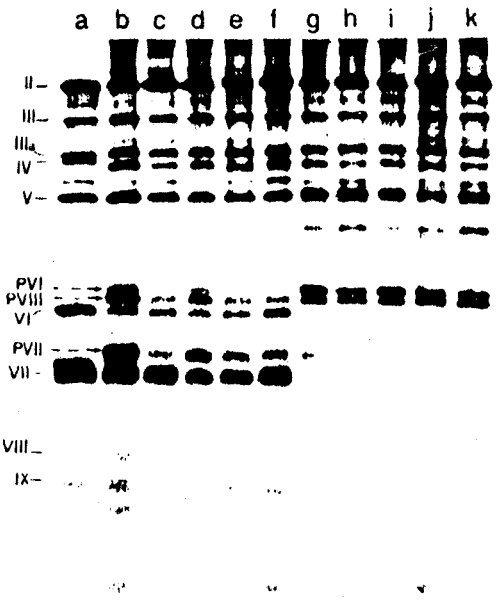

FIG. 8. *Polypeptide pattern of ts125 nuclear 750S virions (b, c, d, e, and f) and 600S IM particles (g, h, i, j, and k) produced during pulse-chase and shift-down experiments, as schematized in Fig. 6. (a) Control HAd2 virion; (b and g) sample 1 of Fig. 6; (c and h) sample 2; (d and i) sample 3; (e and j) sample 5; (f and k) sample 6. Note that after a 7-h chase at 39.5°C, the polypeptide pattern of ts125 750S virus particles resembles that of a WT, with visible maturation cleavage of PVI, PVIII, and PVII (c).*

cleaved precursors (33). These results with *ts*104 further support the hypothesis of a virus-coded or virus-induced cellular endopeptidase responsible for the cleavage maturation of precursors to virion proteins VI, VIII, and VII (30, 31; see Addendum in Proof). In another study, data which suggest a similar maturation processing of polypeptide IIIa in YV were reported (M. L. Boudin, J. C. D'Halluin, C. Cousin, and P. Boulanger, Virology, in press). *ts*104 YV were found to be devoid of a DOC core, confirming the low affinity of precursor PVII for viral DNA at low ionic strength, as previously observed (10, 23).

The antigenic properties of the major capsid subunits incorporated into the viral capsid can be studied qualitatively and quantitatively by two-dimensional immunoelectrophoresis of DOC-disrupted particles (2, 20, 21; Boudin et al., Virology, in press). This technique was applied to the virus antigens present in the different classes of particles accumulated by the *ts* mutants at 39.5°C. In light IM particles (or top components; ρ = 1.30 to 1.31 g/ml), such as those accumulated by *ts*101, *ts*104, *ts*112, and *ts*120, and the ρ = 1.315 g/ml IM particles of the WT (3), only hexon and fiber antigens formed an immune precipitate peak in two-dimensional immunoelectrophoretic analysis (Fig. 5). In DOC-disrupted YV, those produced at 39.5°C by *ts*104, only hexon and fiber antigens were detectable (Fig. 5). In contrast, hexon, penton base, fiber, and IIIa antigens were found in DOC-treated WT mature virions, as well as in *ts*104 mature virions obtained after a shift down. Penton base appeared antigenically active in the ρ = 1.345 g/ml virions produced in minute amounts by *ts*125 at 39.5°C (Fig. 5).

These data suggested a modification of the virus antigens within the capsid during virion morphogenesis, with an eclipse phase affecting the antigenicity of the vertex structures (9). Only hexon antigen seemed to remain constant in antigenicity throughout the assembly pathway (Table 5). The absence of a detectable antigen in two-dimensional immunoelectrophoretic analysis does not necessarily signify a total absence of antigenic sites. As reported elsewhere (Boudin et al., Virology, in press), modified virus

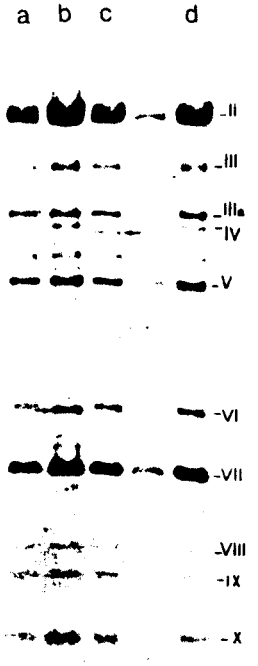

FIG. 9. *Polypeptide pattern of HAd2 infectious virus particles (ρ = 1.345 g/ml) produced at 33°C by (a) ts103, (b) ts115, (c) ts125, and (d) the WT. The molecular weights of polypeptide IV present in ts125 virion (c) and of polypeptide VI present in ts115 virion (b) are lower than those of the corresponding polypeptides of the WT (arrows). Staining: Coomassie brilliant blue R-250.*

TABLE 5. *Antigenic properties of HAd2 major capsid protein at different steps of the virion morphogenesis in WT and ts mutants*

Component	Antigenic properties[a] of following virus particles or subviral entities:								
	Soluble components			1.315-g/ml IM[b]		1.37-g/ml IM[b] (WT)	YV		Mature virions (WT)
	WT	*ts*136	*ts*107	WT	*ts*112		WT	*ts*104	
Hexon	+	+	+	+	+	+	+	+	+
Penton base	+	0	+	0	0	ND[c]	ND	0	+
Fiber	+	0	+	+	+	ND	+	±	+
IIIa[d]	+	0	+	0	0	ND	0	0	+

[a] +, Same immune precipitate peak in two-dimensional immunoelectrophoresis as with WT; ±, trace amount of antigen; 0, no antigen detectable.
[b] Data from references 3 and 4.
[c] ND, Not determined.
[d] Data from Boudin et al., Virology, in press.

antigens retained enough antigenic determinants to be selected on *Staphylococcus aureus* protein A through their specific antibody (15), but not enough to form an immune precipitate lattice within the agarose gel.

Serological and SDS-polyacrylamide gel electrophoretic analyses have shown that a number of *ts* mutants of HAd12 (27), HAd31 (32), and HAd2 (34) fail to synthesize pairs or groups of capsid components, e.g., hexon-penton base, hexon-fiber, fiber-penton base, or polypeptides IIIa-V-80K (34). Three of the *ts* mutants presented here (*ts*103, *ts*104, and *ts*136) appeared fiber and penton base defective and fell into three complementation groups (Table 1). Although double mutations cannot be totally excluded in some of these mutants, the data obtained with *ts*104 suggested a lesion of a regulatory function affecting several apparently nonlinked events, such as the antigenicity of penton base, fiber, and IIIa and the cleavage of precursors to virion proteins VI, VIII, and VII. As the accuracy of the complementation tests for grouping the *ts* mutants and determining the functions altered is obviously limited, physical mapping of the mutations appears necessary for defining the role of different genes in virus assembly.

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ADDENDUM IN PROOF

After completion of this study, a paper describing the partial characterization of an ad2-induced protease was published (A. R. Bhatti and J. Weber, Virology 96:478-485, 1979).

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VIROLOGY 101, 000–000 (1980)

Human Adenovirus Type 2 Protein IIIa

II. Maturation and Encapsidation

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Evidence is presented that a HAd2 protein IIIa precursor (PIIIa) of 67,000 daltons exists in assembly intermediates and young virions and is processed into protein IIIa (66,000 daltons). This cleavage is suggested to occur at the N terminus, and at a late stage of virus maturation, between young and mature virions. Sixty copies of IIIa were found to be present per mature virion or assembly intermediate particle, viz. one molecule per penton base subunit. The PIIIa polypeptide was antigenically different from IIIa, with a weaker antigenic reactivity toward anti-IIIa serum. This difference in antigenicity might reflect a variation in three-dimensional structure between the PIIIa polypeptide and IIIa, resulting from proteolytic cleavage. Immunoprecipitation of deoxycholate-disrupted HAd2 virions using anti-IIIa serum suggests some interaction between IIIa and the core protein VII in mature virions. Four *ts* mutants defective in virion assembly and accumulating empty particles at 39.5° were studied with regard to the occurrence of IIIa antigen in the cell pool of soluble antigens: H5 *ts* 58, H2 *ts* 4, H2 *ts* 112, and H2 *ts* 101 were shown to be phenotypically IIIa defective, and to belong to three different complementation groups. H2 *ts* 4 and H2 *ts* 112 were in the same complementation group, whereas H5 *ts* 58 and H2 *ts* 101 fell into two separate groups. Only the H5 *ts* 58 lesion has been located in the IIIa gene (E. Frost and J. F. Williams, 1978, *Virology* 91, 39–50). The IIIa antigen appears, therefore, to be a serological marker for adenovirus assembly.

INTRODUCTION

Protein IIIa from human adenovirus type 2 (HAd2) constitutes one of the major structural components of the virus capsid (Anderson *et al.*, 1973; Everitt *et al.*, 1973). In a preceding paper (Lemay *et al.*, 1980), it has been shown that this protein can be detected at a late stage after infection as one of the major soluble antigenic components in the excess pool of virus material, as are hexon, penton base, complete penton, and fiber. Some of the physical, biochemical, and immunological characteristics of IIIa have been reported (Lemay *et al.*, 1980).

The topography of IIIa and of the other virion components has been tentatively determined by sequential disintegration of the virion (Maizel *et al.*, 1968 Laver *et al.*, 1968; Prage *et al.*, 1970; Russell *et al.*, 1971; Everitt *et al.*, 1973), or by chemical crosslinking and enzymatic iodination of the virion subunits (Everitt *et al.*, 1975).

The present study deals with the stoichiometry and topology of protein IIIa within the adenovirion and its modification during the virus maturation process. This analysis was aided by the use of temperature-sensitive mutants defective in late events involved in capsid morphogenesis: H2 *ts* 112 (D'Halluin *et al.*, 1978b), H2 *ts* 4 (Khittoo and Weber, 1977), H2 *ts* 101 (Martin *et al.*, 1978), and H5 *ts* 58 (Frost and Williams, 1978; Edvardsson *et al.*, 1978).

MATERIALS AND METHODS

Viruses

Human adenovirus type 2 (HAd2), wild type (WT), originally obtained from J. F.

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Williams (Carnegie-Mellon Institute, Pittsburgh, Pa.) was propagated at 37° on KB cells maintained in suspension culture.

The HAd2 temperature-sensitive mutant H2 *ts* 112 was isolated after nitrous acid treatment of a WT stock and phenotypically characterized (Martin *et al.*, 1978; D'Halluin *et al.*, 1978b). H2 *ts* 4, a mutant defective for DNA encapsidation (Weber *et al.*, 1975; Khittoo and Weber, 1977), was a generous gift from J. Weber (Sherbrooke, Québec). The adenovirus 5 mutant H5 *ts* 58 was originally isolated by J. F. Williams (Williams *et al.*, 1971) and was obtained from W. C. Russell (Mill, London, U. K.).

Virus was titrated by the plaque assay or by the fluorescent focus unit assay (FFU; Philipson *et al.*, 1968) at 37° for the WT and 33° for the *ts* mutants. The restrictive temperature was 39.5° for the *ts* mutants.

Cells

KB cells were grown in spinner culture in Eagle's basal medium supplemented with 5% horse serum. HeLa cells were grown as monolayers in Eagle's minimum essential medium supplemented with 10% calf serum. HeLa cells were used for plaque assays. The cells were infected at a multiplicity of infection of 25 to 50 PFU per cell.

Radioactive Labeling

Adenovirus particles were labeled by adding [^{14}C]valine (250–300 mCi/mmol, Amersham, England) from 18 to 30 hr p.i. at 0.5 μCi/ml in a valine-deprived culture medium (10% of the concentration of valine in normal medium). Adenovirions used as controls were extracted with fluorocarbon and purified by conventional techniques (Green and Piña, 1963).

Pulse-chase labeling experiments were performed in KB cells (6×10^6 cells/ml) in methionine-deprived medium. L-[^{35}S]methionine (50 μCi/ml, 700–800 Ci/mmol, Amersham, U. K.) was added for 30 min at different times p.i. Cells were harvested just after the pulse or chased by dilution to 3×10^5 cells/ml in normal medium.

Infected cells were labeled with [^{32}P]orthophosphate as follows: Cells were taken at 18 hr p.i., centrifuged, and washed two times in phosphate-free culture medium. The original culture medium was saved. The cells were resuspended at 6×10^6 cells/ml in phosphate-free medium and labeled for 1 hr with 50 μCi/ml of Na-[^{32}P]orthophosphate (200 mCi/mmol, Amersham, U. K.). The chase was performed by diluting the cells in the original culture medium at 3×10^5 cells/ml. ^{32}P-Labeled virus and assembly intermediate particles were extracted and purified as described below.

Purification of Adenovirus Particles and of Assembly Intermediates (IM)

Three major species of HAd2-WT particles constituting the successive steps in virion assembly have been characterized in the following sequence: 1.315-IM, 1.370-IM, 1.345 young virions (YV), and 1.345 mature virions (MV), with reference to their buoyant densities (g/ml = ρ) in CsCl (D'Halluin *et al.*, 1978a). The method of extraction of the particles from infected cell nuclei, and of particle isolation on sucrose density gradients and CsCl gradients after reversible fixation, has been described in detail elsewhere (D'Halluin *et al.*, 1978a, b).

For rapid isolation of the bulk of IM particles or top components of ρ = 1.30–1.31 (Ishibashi and Maizel, 1974) another procedure was used. The IM and virion particles were extracted with fluorocarbon and separated on a preformed discontinuous CsCl-glycerol gradient made as follows: 0.8 ml of ρ = 1.45 CsCl in 40% glycerol, 1.4 ml of ρ = 1.33 CsCl in 26% glycerol, and 1.4 ml of ρ = 1.25 CsCl in 0.02 *M* Tris-HCl buffer, pH 7.4. The cell lysate (1.5 ml) was loaded on top of the gradient and the gradient was centrifuged for 3 hr at 35,000 rpm in a Beckman SW 50 rotor. Mature and young virions, ρ = 1.345, banded at the top of the ρ = 1.45 layer. A band of light IM particles was obtained on top of the ρ = 1.33 layer and the virus soluble components remained in the top zone at the limit of the ρ = 1.25 layer.

Immunological Analyses

Antisera. Rabbit polyspecific anti-HAd2 virion protein component serum and rabbit

monospecific anti-IIIa serum were obtained as described in the preceding paper (Lemay *et al.*, 1980).

Two-dimensional (2D) immunoelectrophoresis. Two-dimensional immunoelectrophoresis was carried out according to a modification of the basic technique of Laurell (1965), and has been described in detail elsewhere (Martin *et al.*, 1978).

Immunoprecipitation. Aliquots, 10–20 μl, of [^{35}S]methionine- or [^{14}C]valine-labeled antigen solutions were mixed with 15 μl of preimmune or immune rabbit serum and 60 μl of NET-N buffer (50 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 5 m*M* Na EDTA, 0.05% Nonidet-P40, 0.02% Na azide) supplemented with 0.02% bovine serum albumin, and incubated at 4° for 18 hr. Immune complexes were adsorbed to inactivated *Staphylococcus aureus* cells (Cowan I strain, Kessler, 1975). An aliquot (10 μl) of a 50% (w:v) washed *S. aureus* cell suspension in NET-N buffer was added to the immune precipitation mixture and incubated for 30 min at room temperature. *S. aureus* cells were pelleted by centrifugation and labeled antigens were extracted by heating the cell pellet in 25 μl of SDS–urea sample buffer for 2 min at 100° and analyzed directly on SDS–polyacrylamide gel.

Analytical SDS–Polyacrylamide Gel Electrophoresis

Samples were dissolved in an equal volume of sample buffer (0.0625 *M* Tris–HCl, pH 6.8, containing 6 *M* urea, 4% SDS, and 10% 2-mercaptoethanol) and heated for 2 min at 100°. Polypeptides were analyzed in an SDS-containing 15.5% polyacrylamide gel (acrylamide: bysacrylamide, 50:0.235) overlaid by a 5% spacer gel (acrylamide: bisacrylamide, 50:1.33) in the discontinuous buffer system of Laemmli (1970). The gels were stained with Coomassie brilliant blue R-250, dried under vacuum, and exposed to Kodak Kodirex film.

Complementation Tests

HeLa cell monolayers were doubly infected at an input multiplicity of 5 PFU of each of two *ts* mutants per cell. After 2 hr adsorption at 33°, unadsorbed virus was rinsed off and the cells were treated for 30 min with an antiserum against HAd2 virion, at a dilution of 1:100–1:200. The cells were rinsed again and further incubated at 39.5° for an additional period of 40 hr. Control cell cultures were singly infected in parallel with 10 PFU of each mutant per cell. At the end of the incubation period, the cells were scraped from the dishes into the culture medium, disrupted by three cycles of freezing and thawing, and the virus production was titrated using the fluorescent focus assay at 33°.

Complementation index (CI) was given as the ratio of yield of the double infection to that of the higher of the two single infections at 39.5° expressed as PFU/ml. Complementation was considered as positive when the CI was at least 10 (Williams *et al.*, 1971; Martin *et al.*, 1978).

Comparative Fingerprinting

Comparative fingerprinting of 67,000 (67K) precursor (PIIIa) and the 66K product (IIIa) were carried out on protein bands isolated from SDS–polyacrylamide gel and partially hydrolyzed with increasing amounts of protease (Cleveland *et al.*, 1977).

Proteases

Staphylococcus aureus protease V8 was purchased from Miles Laboratories (Slough, U. K.). Aminopeptidase M and leucine aminopeptidase were purchased from Boehringer (Mannheim, BDR). Labeled protein samples were hydrolyzed with 0.02, 0.05, 0.10, and 0.20 μg of enzyme, respectively. Some samples were hydrolyzed with a mixture of the three enzymes in the same amounts.

Protein

Protein concentration was assayed by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Cell Fractionation

It should be noted that the distinction between cytoplasmic and nuclear extracts was purely strategic, and that nuclear leaks

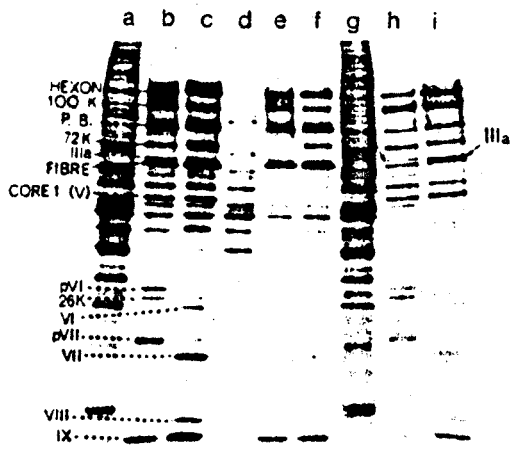

FIG. 1. Autoradiogram of SDS–polyacrylamide gel of WT-HAd2 induced polypeptides in KB cells. Cells were pulse-labeled with [^{35}S]methionine for 1 hr at 18 hr p.i. and chased for 20 hr. (a, d, g) Pulse in mock-infected cells; (b, e, h) pulse in HAd2-infected cells; (c, f, i) chase in HAd2-infected cells. (a–c) Whole cell extracts; (d–f) cytoplasmic extracts; (g–i) nuclear extracts. Anode is at the bottom. The arrows indicate the change in migration of IIIa polypeptide.

were not considered. Nuclei were prepared as follows; cells were swollen in hypotonic reticulocyte standard buffer for 10 min at 0°, and disrupted by three strokes of a tight-fitting glass Dounce homogenizer. A pellet was obtained by centrifugation at 1000 *g* for 15 min, referred to as the nuclear extract, whereas the supernatant was referred to as the cytoplasmic extract. Both nuclear and cytoplasmic extracts were dissolved, with heating, in an equal volume of SDS-sample buffer and analyzed in SDS–polyacrylamide gel.

RESULTS

The stoichiometry of protein IIIa in mature adenovirions was studied by two different methods: (i) scanning of the autoradiograms of several gel tracks of freshly prepared, SDS–urea–mercaptoethanol-dissociated, [^{14}C]valine-labeled HAd2 particles as shown in Figs. 2b and c. The amount of IIIa was determined in relation to protein IV (IV being the polypeptide subunit of the fiber). This gave an average value of 1.88 ± 0.05 for the ratio of label in bands IIIa: IV. Assuming a molecular weight of 66K for the IIIa polypeptide unit, and of 62K for the IV polypeptide unit (Anderson *et al.*, 1973), a value of 61.6 ± 1.8 copies of IIIa per virion was calculated. No correction was made for the differing valine content of IIIa (Lemay *et al.*, 1980) and the other virion components, but valine seems to be evenly distributed among the virion proteins, when comparison is made between amino acid composition of total HAd2 virion and major capsid proteins (Philipson and Pettersson, 1973).

(ii) The second method was quantitative estimation by 2D immunoelectrophoresis of IIIa and fiber polypeptides contained in adenovirions dissociated with 0.5% Na-deoxycholate (DOC) for 90 sec at 56°. The 2D immunoelectrophoresis technique has been shown to be a useful method for quantification of HAd2 soluble components (Martin *et al.*, 1975), and to quantitate antigenic components of the adenovirion, such as the penton base (Boudin *et al.*, 1979). Using different concentrations of virion-extracted, DOC-treated IIIa as standards, reacted against a polyspecific anti-HAd2 virion serum, it was possible to obtain calibration curves giving the amount of protein IIIa in a given immune precipitate peak. The protein content of the standard samples was determined by the method of Lowry *et al.* (1951) and the enclosed surface area of the precipitates, expressed as square millimeters, obtained by automatic quantitative image analysis (Boudin *et al.*, 1979). Similar calibration curves were obtained with purified, DOC-treated fiber reacted against the same antiserum as that used for IIIa calibration. This immunological method of assaying the antigenic content of HAd2 virions gave a value of 1.90 ± 0.07 for the ratio of IIIa: fiber in DOC-treated virus particles, viz. a value of 62.3 ± 2.5 copies of IIIa per virion. For immunological reasons given below, a stoichiometric study of IIIa in IM particles was only possible with the gel scanning method. This method gave a ratio of IIIa: IV of 1.78 ± 0.05 for the 1.315-IM particle, corresponding to a number of about 5 IIIa subunits per apex projection (4.85 ± 0.15).

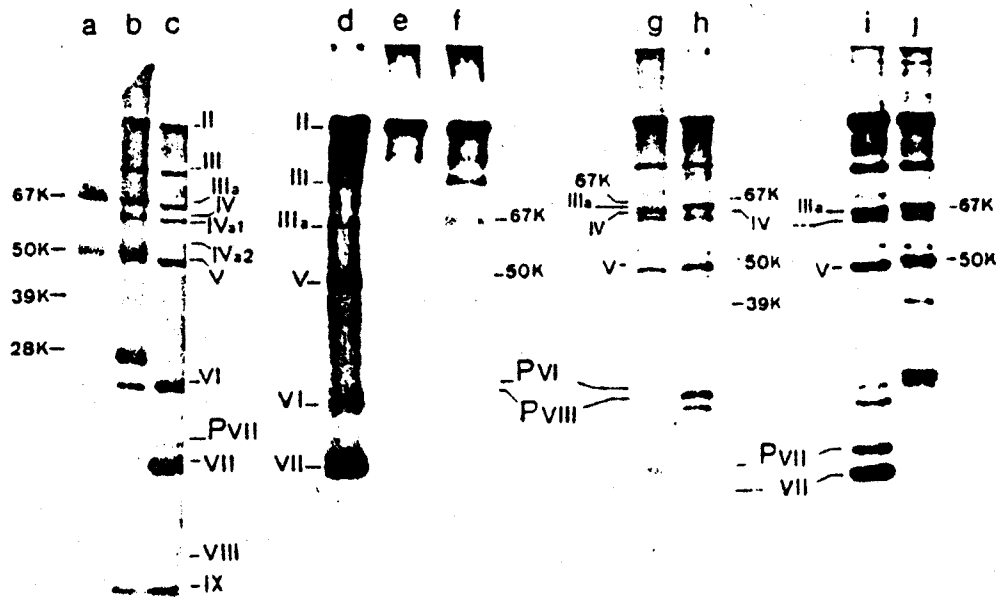

FIG. 2. Polypeptide analysis of WT-HAd2 and H2 ts 112 particles labeled with [^{32}P]orthophosphate (a) or [^{35}S]methionine (b–j). (a, b) 1.315-IM particle of H2 *ts* 112; (c, d) WT-HAd2 mature virion; (e, f) 1.37-IM particle of WT-HAd2; (g) 1.345-young virion of H2 *ts* 112 after 1-hr pulse-labeling at 39.5° and 2-hr chase at 33°; (h) H2 *ts* 112 1.315-IM; (i) 1.345-young virion of H2 *ts* 112 after 1-hr pulse at 39.5° and 8-hr chase at 33°; (j) H2 *ts* 112 1.315-IM obtained at 39.5°. Slot (f) is the same as (e) with a prolonged time of exposure on film. Slots (g) and (i) represent two different stages of young virion maturation, as shown by the different degree of processing of P-VII into VII. Slots d–f have been PPO impregnated.

Evidence for Processing of Protein IIIa during the HAd2 Infectious Cycle

As shown in Fig. 1b, SDS–gel analysis of extracts of whole KB cells infected with HAd2 and pulse-labeled with [^{35}S]methionine for 30 min at 18 hr p.i. revealed the presence of the well-defined HAd2 virion components II, III, IIIa, IV, V, IX (Everitt *et al.*, 1973), virion component precursors P-VI, P-VIII (Ishibashi and Maizel, 1974; Öberg *et al.*, 1975), and P-VII (Anderson *et al.*, 1973), and nonvirion proteins such as the 100K species and the 72K DNA-binding protein (Van der Vliet and Levine, 1970; Anderson *et al.*, 1973; Linné *et al.*, 1977; Van der Vliet *et al.*, 1978).

After a chase of 20 hr, the cleavage products appeared, viz. VI, VIII, and VII, whereas the apparent molecular weight of the 72K protein shifted to 75K (Fig. 1c). An increase in incorporation of label into the major bands II (hexon polypeptide), III (penton base), IV (fiber), V (core-1 protein), and IX was also observed.

The increase of radioactivity in band IV masked a slight change in the migration of IIIa, which superimposed with band IV in the chase pattern. This decrease in apparent molecular weight of IIIa, changing from 67K to 66K, was clearly visible in the chase pattern of cytoplasmic and, especially, nuclear extracts (Fig. 1i), in which the bands IIIa and IV were better resolved.

Molecular Weight of the IIIa Polypeptide in HAd2 Virion

The apparent molecular weight of the IIIa polypeptide unit was compared in the mature virion (MV) and IM particles. Two classes of IM particles were extracted from WT-infected cell nuclei and isolated on sucrose and CsCl gradients after re-

versible fixation: light IM banding at $\rho = 1.315$, and heavy IM at $\rho = 1.370$ (D'Halluin *et al.*, 1978a). The 1.315-IM accumulated in H2 *ts* 112-infected cells maintained at nonpermissive temperature (D'Halluin *et al.*, 1978b). Young virions (YV) were obtained by pulse-labeling cells infected with H2 *ts* 112 for 30 min at 18 hr p.i. at 39.5° and chasing at 33° for 2 hr. The peak of radioactivity at $\rho = 1.345$ in CsCl corresponds mainly to young virions containing partially cleaved precursors (Edvardsson *et al.*, 1976; D'Halluin *et al.*, 1978a).

Figure 2b shows that the 1.315-IM particles contained a polypeptide of 67K in place of polypeptide IIIa (66K) present in mature virions (Fig. 2c). The 1.370-IM particles also possessed a 67K species (Figs. 2e, f). YV particles of H2 *ts* 112 obtained after a 2-hr chase at 33° showed a partial cleavage of the precursors P-VI, P-VIII, and P-VII (30–50% of P-VII was cleaved into core protein VII), the disappearance of a 50K species, possibly a maturation protein (D'Halluin *et al.*, 1978a, b; Persson *et al.*, 1979), and the appearance of core protein V. In addition to these modifications, some 67K was still visible, whereas a 66K polypeptide corresponding to IIIa appeared (Fig. 2g).

At a later stage of evolution of the YV particles into MV particles, when 70–80% of the P-VII was cleaved into VII (8-hr chase at 33°), no trace of 67 K polypeptide remained visible (Fig. 2i). The apparent processing of 67K into 66K seemed, therefore, to occur at a faster rate than that of P-VII into VII.

Phosphorylation of IIIa in IM and MV particles

The change in apparent molecular weight of 67K into 66K might represent either proteolytic cleavage or a modification, such as phosphorylation, of IIIa between the stage of IM and MV particles. ^{32}P labeling of H2 *ts* 112 IM particles showed that the 1.315-IM particles which accumulated at 39.5°, and which evolved into mature virions upon shift-down (D'Halluin *et al.*, 1978b), contain four major phosphoproteins migrating as polypeptides of apparent molecular weights 67K, 50K, 39K, and 28K, and two minor species of 90K and 24K, respectively (Fig. 2a). This suggested that protein IIIa, a major phosphorylated protein in the mature virion (Russell and Blair, 1977) and present in phosphorylated form in 1.315-IM particles, was phosphorylated at an early stage possibly preceding its entry into IM particles. However, additional phosphorylation might not be excluded.

Precursor–Product Relationship of 67K and IIIa

The 67K polypeptide from [^{35}S]methionine-labeled H2 WT 1.315-IM particles electrophoresed in SDS–polyacrylamide gel and band IIIa from mature virions were cut off a stained gel and hydrolyzed with different concentrations of *S. aureus* protease V8, and the peptides resulting from the partial proteolysis analyzed in SDS–polyacrylamide gel (Cleveland *et al.*, 1977). Figure 3 shows that the peptide patterns were similar for 67K and IIIa with minor differences in the 20K–24K and the 48K–52K zones of the SDS–gel. These peptide fingerprints had no relationship with the other major virion proteins such as hexon, penton base (not shown), or fiber (Figs. 3k–m).

Antigenicity of IIIa in IM Particles

When 1.315-IM particles of WT, H2 *ts* 112, and H2 *ts* 101 were disrupted with DOC and analyzed in 2D immunoelectrophoresis, only two antigen peaks were found: hexon and fiber (Fig. 4). Neither IIIa, penton base, nor complete penton were visible. However, polypeptides III (penton base) and IIIa were present in these particles and were detected in SDS–polyacrylamide gels (Fig. 2b), apparently in the same stoichiometric ratio to hexon and fiber polypeptides as in the mature virion (Fig. 2c). Similarly, penton base and IIIa were not detected in DOC-treated IM particles in double diffusion tests (not shown).

The absence of antigenic reactivity might reflect a change in conformational state of 67K in the IM, or the masking of antigenic determinants by other viral component(s). [^{14}C]Valine-labeled WT 1.315-

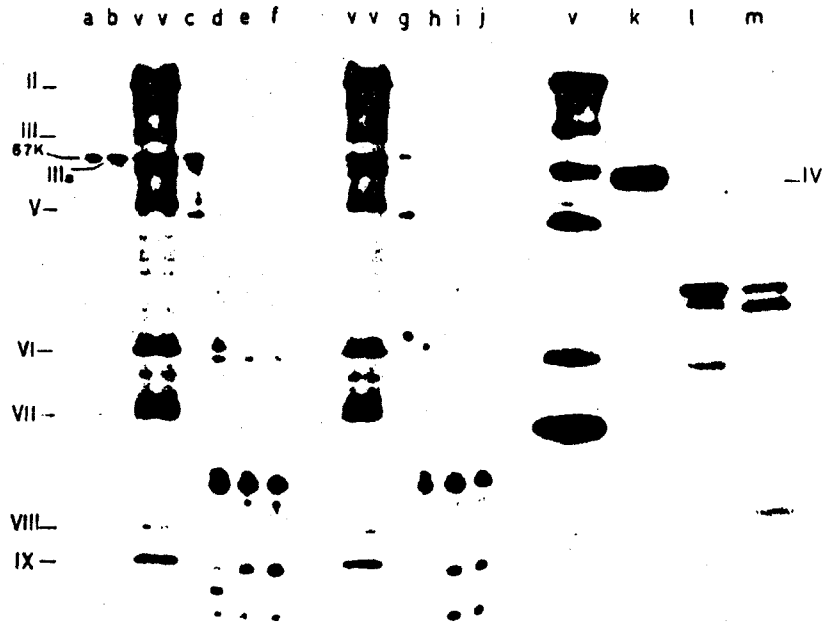

FIG. 3. Comparative fingerprints in SDS–polyacrylamide gel of 67K from 1.315-IM particle and of IIIa from HAd2 mature virion. Gel slices were hydrolyzed in gel with 0.00 (a, b), 0.02 (c, g), 0.05 (d, h), 0.10 (e, i), 0.25 μg (f, j) of *S. aureus* protease V8 for 30 min. (b–f) IIIa; (a, g–j) 67K band; (v) control HAd2 virion. For comparison, *S. aureus* protease V8 fingerprints of HAd2 fiber polypeptide are presented (k–m). Fiber was hydrolyzed with 0.00 (k), 0.05 (l), and 0.20 μg (m) of protease V8 in the same conditions as above. (a–j) and (k–m) are separate gels. Note that the cross-linking of the polyacrylamide gel (acrylamide:bisacrylamide, 50:1.33) is used to separate low molecular weight peptides but is not adequate to resolve the 67K and IIIa bands.

IM, 1.345 YV, and MV particles were therefore disrupted with DOC, then incubated with anti-IIIa serum and the antigen–antibody complexes collected by adsorption onto *S. aureus* cells. YV particles were obtained by labeling WT-infected cells for 1 hr at a late stage after infection (24 hr) and chasing for 6 hr. Particles obtained at $\rho = 1.345$ in CsCl contained mainly YV as evidenced by the incomplete processing of P-VII into VII (Fig. 5f).

As shown in Fig. 5c, a 67K polypeptide was the major antigenic component selected from the DOC-disrupted IM by the IIIa antibody, along with another component of lower molecular weight (56K) which migrated in SDS–polyacrylamide gel electrophoresis as polypeptide IVa2 of the mature adenovirion (Anderson *et al.*, 1973). Traces of hexon polypeptide were often visible, and likely corresponded to nonspecific trapping, as suggested by the precipitation of similar amounts of polypeptide II with a preimmune rabbit serum (Figs. 5d, i). In YV, IIIa also coprecipitated with a 56K protein and with discrete protein species migrating as virion proteins II, III, V, and as immature virus protein 100K, 39K, and P-VIII (Fig. 5e). Most of these polypeptides were also precipitated by a preimmune serum (Fig. 5d). Anti-IIIa antibody precipitation of components of DOC-disrupted mature virions showed three major species corresponding to IIIa, V, and VII, along with significant amounts of 56K (Fig. 5h). Stoichiometric analysis performed by gel scanning revealed that 8–10 copies of VII were precipitated per copy of IIIa.

Temperature-Sensitive Mutants of HAd2 and HAd5 Phenotypically IIIa Negative and Blocked in Virion Morphogenesis

H5 *ts* 58 has been shown to map in the region of the HAd5 genome corresponding

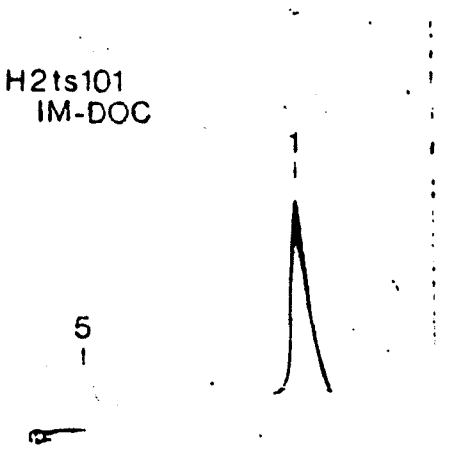

FIG. 4. Two-dimensional immunoelectrophoresis pattern of 1.315-IM of H2 *ts* 101 disrupted with 0.5% deoxycholate (DOC). Peak 1 corresponds to hexon antigen, peak 5 to fiber antigen. No penton base, complete penton, nor IIIa peak was visible. The 1.315-IM were purified without fixation. The same pattern was obtained with 1.315-IM of H2 *ts* 112.

to IIIa gene (Frost and Williams, 1978). The mutant is blocked in virion morphogenesis and accumulates empty particles at nonpermissive temperatures (Edvardsson *et al.*, 1978). Three other mutants of HAd2 accumulating the same type of empty particles were studied in complementation tests with the H5 *ts* 58: H2 *ts* 112 (D'Halluin *et al.*, 1978b), H2 *ts* 101 (Martin *et al.*, 1978), and H2 *ts* 4 (Khittoo and Weber, 1977). As shown in Table 1, these four mutants fell into three complementation groups, H2 *ts* 4 and H2 *ts* 112 belonging to the same group.

Extracts of cells infected with each of these mutants at permissive (33°) or nonpermissive temperatures (39.5°) were studied antigenically in 2D immunoelectrophoresis. Figure 6 shows that for these mutants the amount of soluble IIIa antigenic component was reduced, and was almost totally absent in extracts of H2 *ts* 4 cultured at 39.5°. The serological phenotype was therefore IIIa negative for these four *ts* mutants blocked at a late stage of virion morphogenesis.

DISCUSSION

Approximately 60 copies of component IIIa were estimated to occur in HAd2 virions, as calculated from data obtained by two different methods: (i) scanning of [^{14}C]valine-labeled virion polypeptides electrophoresed in SDS–polyacrylamide gel and estimation of the amount of IIIa relative to fiber; (ii) 2D immunoelectrophoretic quantitation of IIIa antigen in DOC-disrupted virions. Both methods gave similar results. In the topographical model of the HAd2 virion proposed by Everitt *et al.* (1975), IIIa was placed at each of the 12 vertices of the virus icosahedron, having contacts with both penton base and peripentonal hexons, and was outwardly accessible. The number of 60 IIIa copies per HAd2 virion suggests that there is one molecule of IIIa per each of the five penton base subunits (Boudin *et al.*, 1979). In the preceding paper (Lemay *et al.*, 1980) it has been shown that IIIa is a spherical molecule consisting of one polypeptide unit of 65,500 daltons. No disulfide bond was formed between IIIa and either the hexon or penton base polypeptide units. A diagonal migration of polypeptide spots was obtained in two-dimensional SDS–polyacrylamide gel when HAd2 virion was dissociated with SDS–urea sample buffer in the first dimension gel and with or without 2-mercaptoethanol in the second dimension (data not shown).

Pulse–chase experiments of KB cells infected with WT-HAd2 suggested a modifica- of the IIIa polypeptide, with a change in apparent molecular weight from 67K to 66K. This modification was difficult to see, due to the chase of label into the neighboring major fiber polypeptide (Fig. 1). The same modification was observed at a late stage in the assembly pathway of HAd2 virions when a 67K polypeptide present in IM particles of ρ = 1.315 and ρ 1.37 disappeared during the maturation of YV particles (ρ = 1.345) to MV particles. The following sequence of events has been suggested: 1.315-IM → 1.37-IM → 1.345-YV → 1.345-MV (D'Halluin *et al.*, 1978a).

The 1.315 IM particles of H2 *ts* 112 accumulating at 39.5° evolve into mature virions upon shift-down to 33° (D'Halluin *et al.*, 1978b). When H2 *ts* 112-infected cells were pulse-labeled at 39.5° and chased at 33°, label appeared in 1.345-YV, and the change in apparent molecular weight of IIIa became even more visible, with the

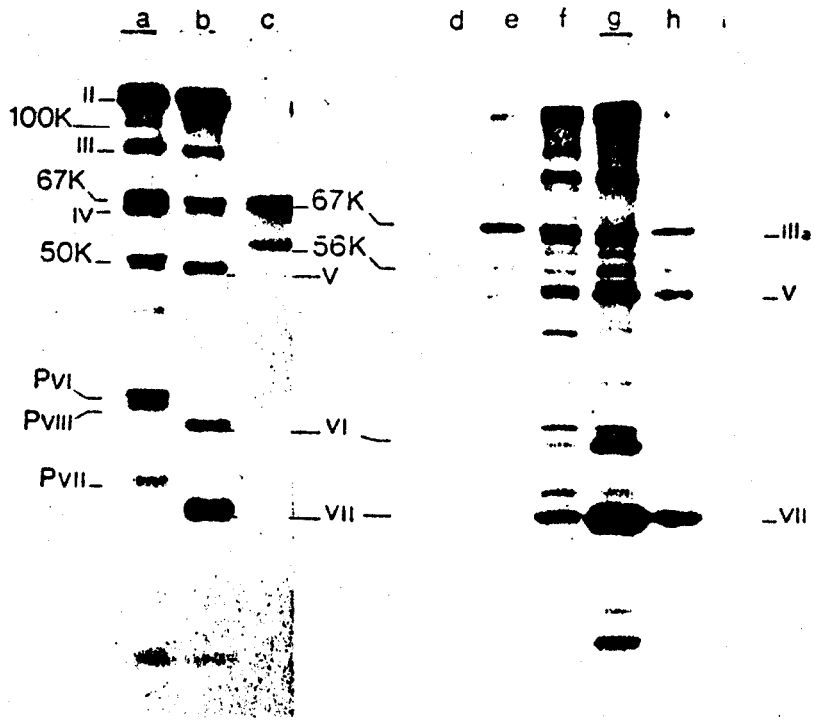

FIG. 5. Anti-IIIa immunoselection on *S. aureus* cells of capsid antigens from DOC-disrupted particles. (a) Top components of WT-HAd2; (b) control HAd2 virion; (c) DOC-treated top components of WT-HAd2 incubated with anti-IIIa serum; (d) DOC-treated young virions (YV) incubated with a preimmune serum; (e) DOC-treated YV incubated with anti-IIIa; (f) control YV; (g) control mature virions; (h) DOC-treated mature virion incubated with anti-IIIa; (i) DOC-treated mature virion incubated with preimmune serum.

simultaneous presence of the 67K precursor and 66K product in some YV particles (Fig. 2). The precursor → product relationship between the 67K and 66K (IIIa) polypeptides was demonstrated by fingerprinting in SDS–polyacrylamide gel (Fig. 3). In the HAd2 *ts* 3 mutant in which the mutation affects the group of proteins IIIa-V-80K, it has been already observed that IIIa resolved into several bands migrating between 68K and 66K (Weber *et al.*, 1977).

The possibility of a modification such as phosphorylation of 67K to explain this molecular weight change is unlikely since the 67K species was already phosphorylated in the 1.315-IM (Fig. 2a). In addition, phosphorylation of the 72K single-strand DNA-binding protein (Linné *et al.*, 1977) resulted in an apparent increase in molecular weight, from 72K to 75K. However, a difference in the degree of phosphorylation between the 67K and 66K species or some other modification might provoke the migration shift observed (Fig. 1). A proteolytic cleavage, removing 10–15 amino acid residues, might be an alternate explanation. Since glycine has been found at

TABLE 1

COMPLEMENTATION INDICES BETWEEN IIIa-DEFECTIVE *ts* MUTANTS OF H2 AND H5 ADENOVIRUSES[a]

Mutant	H5 *ts* 58	H2 *ts* 4	H2 *ts* 101	H2 *ts* 112
H5 *ts* 58	—	3235	96	30
H2 *ts* 4	—	—	382	3
H2 *ts* 101	—	—	—	35
H2 *ts* 112	—	—	—	—

[a] Complementation indices (CI) were calculated from the virus yields. As indicated under Materials and Methods, complementation was considered as positive when the CI was ≥10.

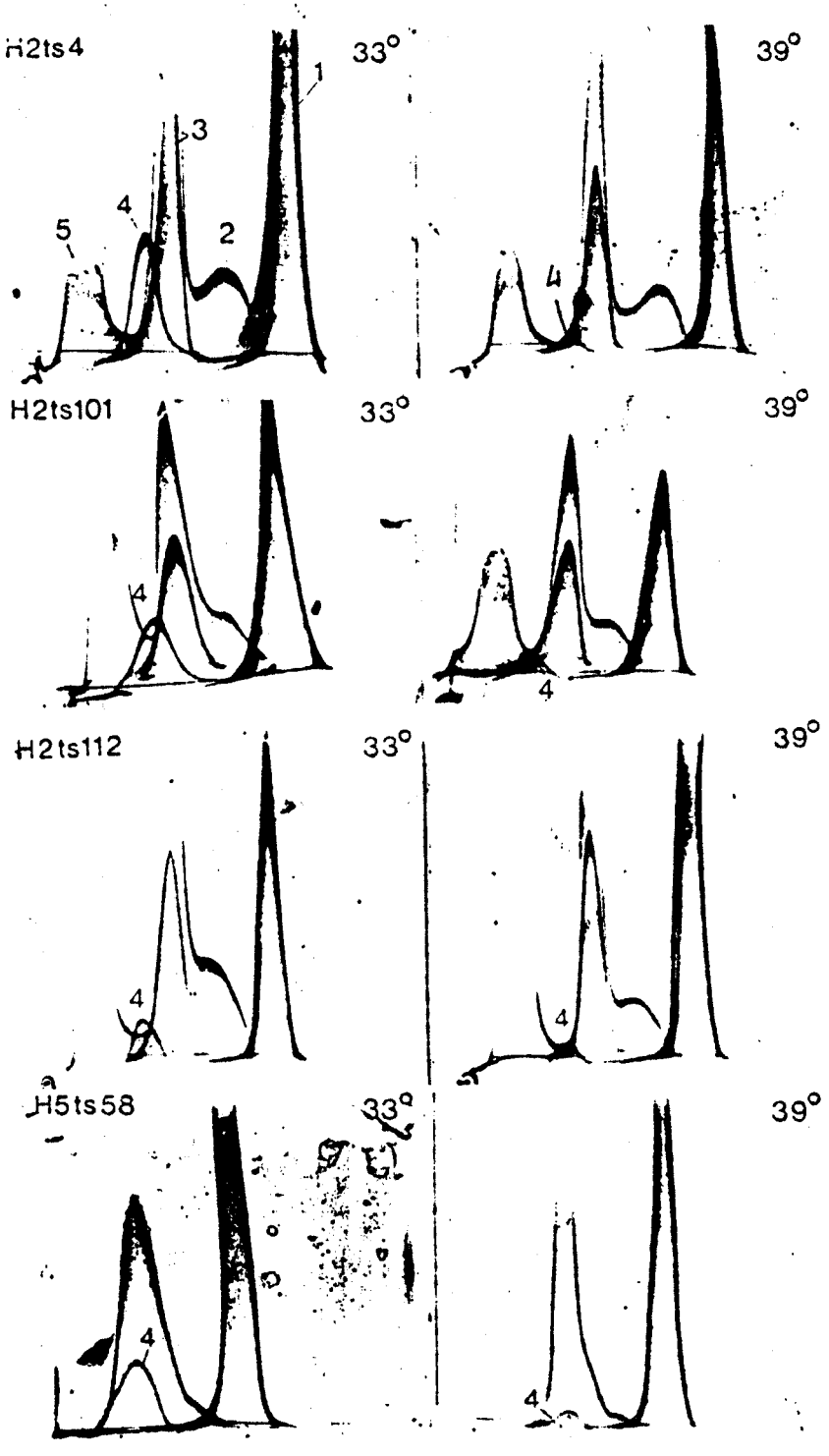

FIG. 6. Two-dimensional immunoelectrophoretic pattern of cells infected with differents *ts* mutants type 2 and 5 adenoviruses, at permissive (33°) or nonpermissive (39.5°) temperatures. Peak 1, hexon antigen; peak 2, free penton base; peak 3, complete penton with both fiber and penton base antigenic determinants; peak 4, antigen IIIa; peak 5, free fiber antigen. The decrease of IIIa peak between the 33 and 39.5° patterns is obvious. Note that the anti-HAd2 serum reveals only group-specific antigens hexon, penton base, and IIIa in HAd5 pattern.

the N terminus of virion-extracted IIIa (Lemay *et al.*, 1980) this cleavage would occur at the N-terminal end of the 67K polypeptide chain, as in the case of P-VII (Sung *et al.*, 1977) and of P-VI (Sung *et al.*, 1978). However, some proteolysis at the C-end could not be excluded. The precursor to IIIa would be processed during the maturation of YV particles at a faster rate than P-VII (Fig. 2), possibly by a virus-coded, or virus-induced protease (Weber, 1976; Bhatti and Weber, 1978).

Although polypeptide 67K was present in IM particles (Fig. 2), no IIIa antigen was detectable in 2D immunoelectrophoresis of DOC-disrupted IM particles (Fig. 4). It should be noted that these particles were not fixed prior to purification and DOC treatment and that a modification of the antigenicity due to the fixation procedure cannot be invoked. Several explanations might be proposed: (i) The 67K precursor to IIIa had an antigenicity different from that of the IIIa product; (ii) the antigenic sites carried by the 67K species did not resist the DOC treatment of the IM particles; (iii) some or all of the antigenic determinants of the IIIa precursor were masked by another virus component, the linkage of which resisted DOC; (iv) these different hypotheses are not mutually exclusive.

It was found that IIIa antigenic component present in the cell pool of HAd2 capsid components resisted DOC treatment (not shown), which excludes the second hypothesis. The possibility of another viral component binding to IIIa, with the resultant masking of IIIa antigenic sites, was explored by immunoprecipitation of DOC-disrupted virion and IM particles by anti-IIIa serum. A major protein of 56K daltons was always found coprecipitated with IIIa when immunoprecipitation was performed with DOC-treated IM particles (Fig. 5). The migration of this protein in SDS–polyacrylamide gel corresponded roughly to that of IVa2, a minor polypeptide of mature virions (Anderson *et al.*, 1973). The possible presence of contaminating anti-IVa2 antibody in the antiserum was unlikely since IVa2 was never coprecipitated with IIIa from infected cell extracts. The same 56K species was found coprecipitated with IIIa from DOC-disrupted YV and MV particles (Fig. 5). Since IIIa is antigenically fully expressed in mature virions (Lemay *et al.*, 1980), the hypothesis of antigenic determinant masking by the 56K polypeptide might be ruled out. However the possibility of proteolytic breakdown of IIIa generating a major 56K species cannot be excluded.

These results suggest that some of the antigenic determinants present in IIIa are absent from the 67K precursor. Nevertheless, sufficient antigenic sites remained available on the 67K precursor to produce soluble complexes with anti-IIIa antibody selected by adsorption onto *S. aureus* cells, although insufficient to generate an antigen–antibody lattice within the agarose gel. This change in antigenicity might reflect a difference in three-dimensional structure between 67K and IIIa relative to the proteolytic cleavage. Similar modifications of penton base antigenicity upon maturation have been observed and studied in detail elsewhere (D'Halluin *et al.*, 1980).

Coprecipitation of significant amounts of VII and V with IIIa antiserum suggests a neighbor relationship between IIIa and the core protein VII in MV particle, confirming previous results of chemical crosslinking of IIIa and VII within the virion of HAd2 (Everitt *et al.*, 1975). In addition, the data indicate a major difference in the structure of capsid vertices between YV and MV particles. Although results of immunoprecipitation have to be interpreted carefully, stoichiometric analysis by gel scanning revealed that 8–10 copies of VII were coprecipitated per copy of IIIa.

As IIIa might be implicated in capsid morphogenesis, several adenovirus *ts* mutants defective in virion morphogenesis (H5 *ts* 58, H2 *ts* 4, H2 *ts* 101, and H2 *ts* 112) were studied: all apparently phenotypically IIIa defective at 39.5° (Fig. 6). The mutants fell into three complementation groups, one containing both H2 *ts* 4 and H2 *ts* 112. Only H5 *ts* 58 has been mapped precisely in the IIIa region (Frost and Williams, 1978). The H2 *ts* 4 mutation has been located in the IIIa–V gene region, and a mutation in core protein V has been suggested (Khittoo and Weber, 1977). All these mutants accumulate empty particles

at 39.5° and, for H2 *ts* 101 and H2 *ts* 112, these particles have been shown to be assembly intermediates evolving into mature virions upon shift-down to 33° (D'Halluin *et al.*, 1978b; Martin *et al.*, 1978).

The absence or decrease of the antigenic component peak in 2D immunoelectrophoresis patterns would reflect, therefore, a block in the maturation process of adenovirus IM particles, with no maturation cleavage of the precursor (PIIIa) to IIIa, and PIIIa remaining in the cell pool and in the IM particle as a weakly antigenic 67K protein species. Thus the absence of detectable IIIa antigen in 2D immunoelectrophoresis would be equivalent to the absence of cleavage of P-VII to VII, as observed in SDS–polyacrylamide gel for assembly-defective *ts* mutants (D'Halluin *et al.*, 1978b), or for systems inhibiting the virion assembly (Warocquier and Boulanger, 1976).

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EFFECTS OF NOVOBIOCIN ON ADENOVIRUS DNA SYNTHESIS AND ENCAPSIDATION

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ABSTRACT

Novobiocin, an inhibitor of DNA gyrase implicated in bacterial, and likely mammalian, chromosome replication, inhibited the initiation, but not the elongation of human adenovirus DNA replicative synthesis. The inhibition was partially reversible, even in the presence of protein synthesis inhibitor. Novobiocin inhibited also the encapsidation of viral DNA, and this effect was independent of the block in DNA replication. It was suggested that novobiocin acted on two different functions, one involved in viral DNA replication initiation, the other in DNA encapsidation.

INTRODUCTION

Novobiocin has been found to inhibit the initiation of DNA replication in bacteria (1, 2) and in mammalian cells (3). Genetic and biochemical evidence indicates that novobiocin blocks the bacterial DNA gyrase (2, 4), an enzyme which generates negative supercoils into transiently relaxed DNA. It seemed therefore reasonable to postulate that gyrase-induced DNA surpercoiling was required for the initiation of DNA replication (5). Recent experiments using novobiocin and intercalating dye ethidium bromide favor the hypothesis that in mammalian cell DNA, an enzymatic system similar to bacterial gyrase converts the DNA template to a negatively supercoiled conformation necessary for the initiation of new DNA strands (6, 7). Comparison of novobiocin effects on UV- and X-ray-induced damage in eucaryotic DNA also supports this assumption (8).

Preliminary results of temperature shift-up with adenovirus temperature-sensitive (ts) mutants defective in viral DNA synthesis at restrictive temperature (9) have suggested that virion morphogenesis and viral DNA synthesis are related phenomena. The aim of the present study was to analyze the effects of novobiocin on the synthesis of adenovirus DNA, RNA and proteins, and to determine, with the aid of ts-mutants blocked in virion morphogenesis, and of DNA-negative ts-mutants, whether it is the initiation step of DNA replication, or a novobiocin-sensitive conformational state of the DNA molecule which is indispensable for viral DNA encapsidation.

MATERIALS AND METHODS

*Cells

KB cells were cultured in suspension at 2.5 - 5.0 x 10^5 cells/ml in spinner medium F 13 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 5 per cent horse serum. HeLa cells were grown as monolayers in Eagle's minimal essential medium, containing 10 per cent calf serum.

*Virus

Wild-type (WT) human adenovirus type 2 (H2) was propagated on KB cell spinner cultures at 37°C. The H2 temperature-sensitive mutants used in this work, H2ts111, 112, and 114 were isolated in our laboratory after nitrous acid treatment of a WT stock, and characterized (9 - 11). The H5ts36 was kindly supplied by J.F. Williams (12). The ts mutants were propagated on HeLa cell monolayers at 33°C. Infectivity was assayed by the plaque method on HeLa cells, at 37°C for the WT, at 33°C for the ts mutants. The nonpermissive temperature was 39.5°C. A virus cycle of 40 h at 37 or 39.5°C corresponds approximately to a period of 96 h at 33°C (11).

*Radioisotopes and labeling conditions

(^{35}S) methionine (600 to 700 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, U.K.). (^3H) thymidine and (^3H)-Uridine (30 Ci/mmol) were purchased from the Commissariat à l'Energie Atomique (Saclay, France). Labeling was performed in HeLa cell monolayers, infected with WT or ts mutants at a multiplicity of infection of 50 to 100.

Viral DNA or RNA was labeled with (^3H) thymidine or (^3H) uridine at 5 μCi/ml . In pulse-chase experiments, 5 X 10^{-5}M cold thymidine (Sigma Chemicals Co., St Louis, Mo.), was added to the cell culture. Mature virions and assembly intermediate particles were pulse-labeled in proteins with 5 μCi/ml of (^{35}S) methionine, in a medium containing 2.5 per cent of the concentration of methionine in normal medium.

*Isolation of mature virions (MV) and assembly intermediate particles (IM).

Infected cells were washed with cold phosphate-buffered saline, suspended at 5 X 10^7 cells/ml in reticulocyte standard buffer (0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl, 0.0015 M Mg Cl_2) and left for 10 min at 0°C. Triton X - 100 was added up to a final concentration of 0.5 per cent, and the cells were disrupted by 10 strokes of a tightfitting Dounce glass homogenizer. NaCl was added up to 0.1 M, and the cell lysate was centrifuged at 1,000 x g for 5 min. The MV and IM contained in the pellet of nuclei were extracted as follows (11). The nuclei were resuspended in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.01 M Na EDTA (TE buffer), and maintained at 0°C. The suspension was adjusted to 0.3 M $(NH_4)_2SO_4$, homogenized in a tight-fitting Dounce homoge-

nizer (3 strokes), and immediately diluted with 2 volumes of TE buffer. The nuclear lysate was then centrifuged on a 30 per cent (wt : vol) sucrose cushion at 16,000 xg for 10 min. The virus particles contained in the supernatant were analyzed on linear sucrose gradients. The gradients (25 - 40 per cent sucrose, in 0.02 M Na borate, pH 8.0, 0.2 M NaCl, 0.01 M Na EDTA) were centrifuged at 85,000 x g and 4°C for 105 min in an SW 27 rotor, over a cushion of CsCl (1.43 g/cm^3). They were collected dropwise from the bottom of the tubes, and the fractions assayed for acid-precipitable radioactivity on Whatman GF-C filters. Mature virions sediment at 700 S, and assembly intermediate particles at 600 S (11).

*Analysis of DNA by zonal sedimentation

(i) Centrifugation in alkaline sucrose gradient. 5×10^5 to 1×10^6 cells suspended in 0.1 ml of Tris-saline (0.15 M NaCl + 0.01 M Tris-HCl, pH 7.5) were loaded on top of a 5 to 20 per cent alkaline sucrose gradient (12 ml total volume) in 0.3 M NaOH, 0.7 M NaCl, 0.01 M Na EDTA, overlaid with 0.4 ml of lysis buffer (1 M NaOH, 0.05 M Na EDTA). After standing for 16 h at 4°C, the gradients were centrifuged at 4°C in an SW 41 rotor at 35,000 rpm for 5 h.

(ii) Centrifugation in neutral conditions. A total of 10^6 cells were suspended in 0.01 M Tris, 0.001 M Na EDTA, 0.3 M NaCl, 0.03 M trisodium citrate (pH 7.4) containing 0.5 per cent Sarkosyl (Ciba-Geigy) and 1 mg of pronase (Boehringer, prediges-ted at 37°C for 3 h and heated for 2 min at 85°C before use) per ml and lysed for 3 h at 37°C. Lysates were gently pipetted on top of a 5 to 20 per cent neutral sucrose gradient (1 M NaCl, 0.01 M Na EDTA, 0.05 M Tris-HCl, 0.1 per cent Sarkosyl, pH 7.4) and centrifuged for 5 h at 35,000 rpm at 4°C in an SW 41 rotor. The gradients were collected dropwise from the bottom of the tubes, and the fractions were assayed for acid-precipitable radioactivity.

*Ethidium bromide titration of supercoils in viral DNA

WT-infected cells were pulse-labeled for 2 h with (^{3}H) thymidine at 16 h post infection, and the label was chased for 1 h in the absence or the presence of novobiocin at partially inhibiting concentration (0.1 mg/ml).
The cells were harvested and fractionated into cytoplasm and nuclei as described above. The nuclei were resuspended in 8 M guanidine in 0.01 M Tris-HCl, pH 7.5, 0.01 M Na EDTA, 0.002 M 2-mercaptoethanol (TEM buffer) for 30 min at 0°C with increasing amounts of ethidium bromide (0 to 30 µg/ml). The nuclear lysates were diluted to 4 M guanidine with TEM buffer and nuclear viral DNA analyzed with adenovirus DNA marker on 5 - 20 per cent neutral sucrose gradient, preformed in TEM buffer containing 4 M guanidine and the same concentration of ethidium bromide as in the incubation mixture. The gradients were centrifuged for 16 h at 25,000 rpm and 4°C in an SW 41 rotor.

*Inhibitors

Cycloheximide (Boehringer, Mannheim, GFR) was used at a concentration of 20 µg/ml, and hydroxyurea (Serva Laboratoire, Heidelberg, GFR) at 10 mM. Novobiocin (Boehringer) was used as freshly prepared solutions. Unless otherwise stated in the text, the usual inhibiting concentration was 0.2 mg/ml.

RESULTS

1. Inhibition of cellular and viral DNA synthesis by novobiocin

WT- and mock-infected HeLa cells were pulse-labeled with (^{3}H)-thymidine for 2 h at 16 h after infection in the presence of increasing concentrations of novobiocin. Fig.1 shows that novobiocin inhibited the incorporation of (^{3}H)thymidine label into viral DNA, with 50 per cent inhibition at 0.09 mg/ml and less than 10 per cent residual DNA synthesis at 0.2 mg/ml. At this latter concentration, cellular DNA was inhibited only to 70 per cent. 0.2 mg/ml (viz. 3.2×10^{-4}M) was therefore the novobiocin concentration most frequently used in experiments where inhibition of adenovirus DNA replicative synthesis was desired.

2. Inhibition of viral DNA and protein synthesis by novobiocin

At the late times of the adenovirus cycle (24-36 h post infection) the macromolecular syntheses are essentially of viral origin. As previously observed in other systems (8), adenovirus RNA and protein synthesis was inhibited by novobiocin at concentrations three to four times higher than in the case of DNA. For RNA, 95 per cent inhibition was obtained with 0.5 mg/ml of novobiocin ; for protein, the same degree of inhibition was obtained with 1 mg/ml (Fig.2).

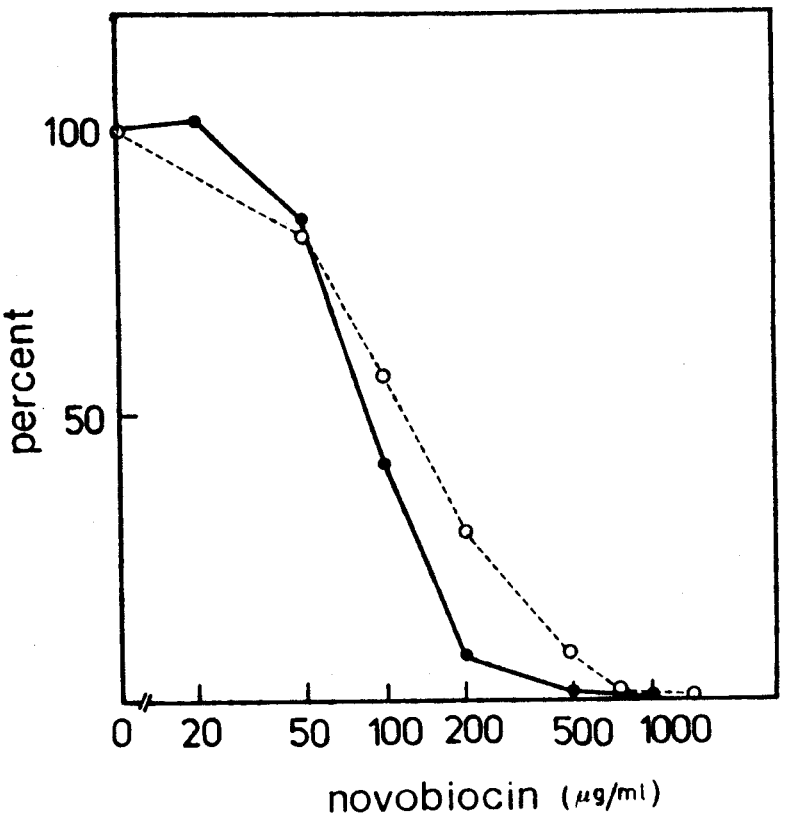

Figure 1

Dose vs response of the inhibition of cellular and viral DNA synthesis by novobiocin in HeLa cells. The data are expressed as the percentage of DNA synthesis in untreated cells after a 2 h pulse-labeling with (^{3}H)-thymidine at 16 h after infection. (O - - O) mock-infected cells ; (●-●) WT-infected cells.

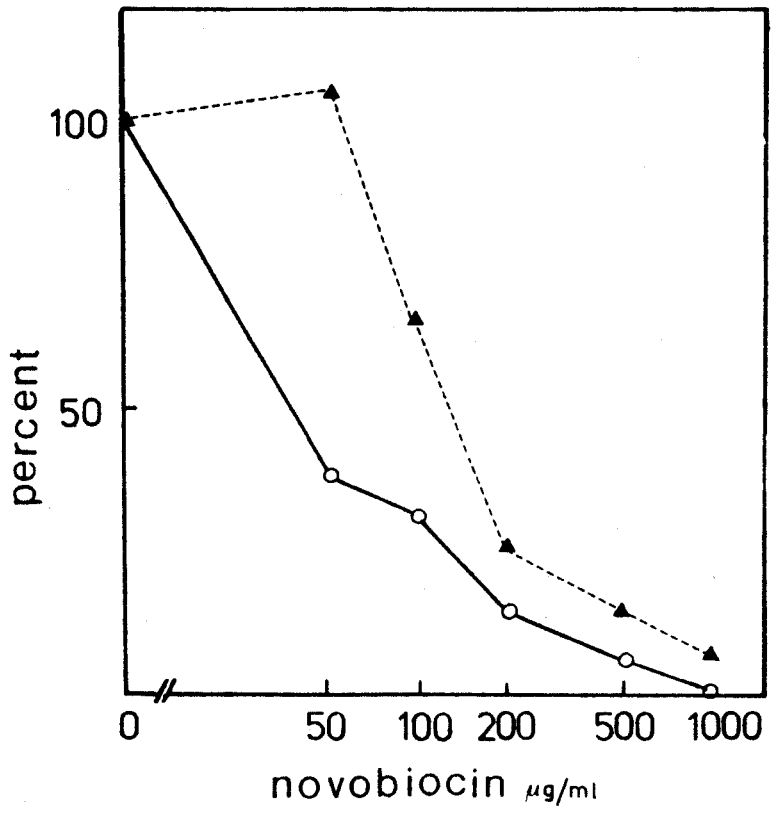

Figure 2

Dose vs response of the inhibition of adenovirus 2 RNA and protein synthesis by novobiocin in HeLa cells. The data are expressed as the percentage of synthesis in untreated infected cells after a 2 h pulse-labeling with (^{3}H)-uridine (●) or (^{35}S)-methionine (▲) at 16 h after infection.

3. Kinetics of inhibition of viral DNA and protein synthesis by novobiocin.

A kinetic study of the effect of novobiocin on viral DNA synthesis at different concentrations of inhibitor showed that the inhibition was rapid, and that a plateau was reached within 30 min after addition of the drug : 65 per cent inhibition was obtained at 0.1 mg/ml of novobiocin, and 98 per cent at 1 mg/ml (Fig.3a).

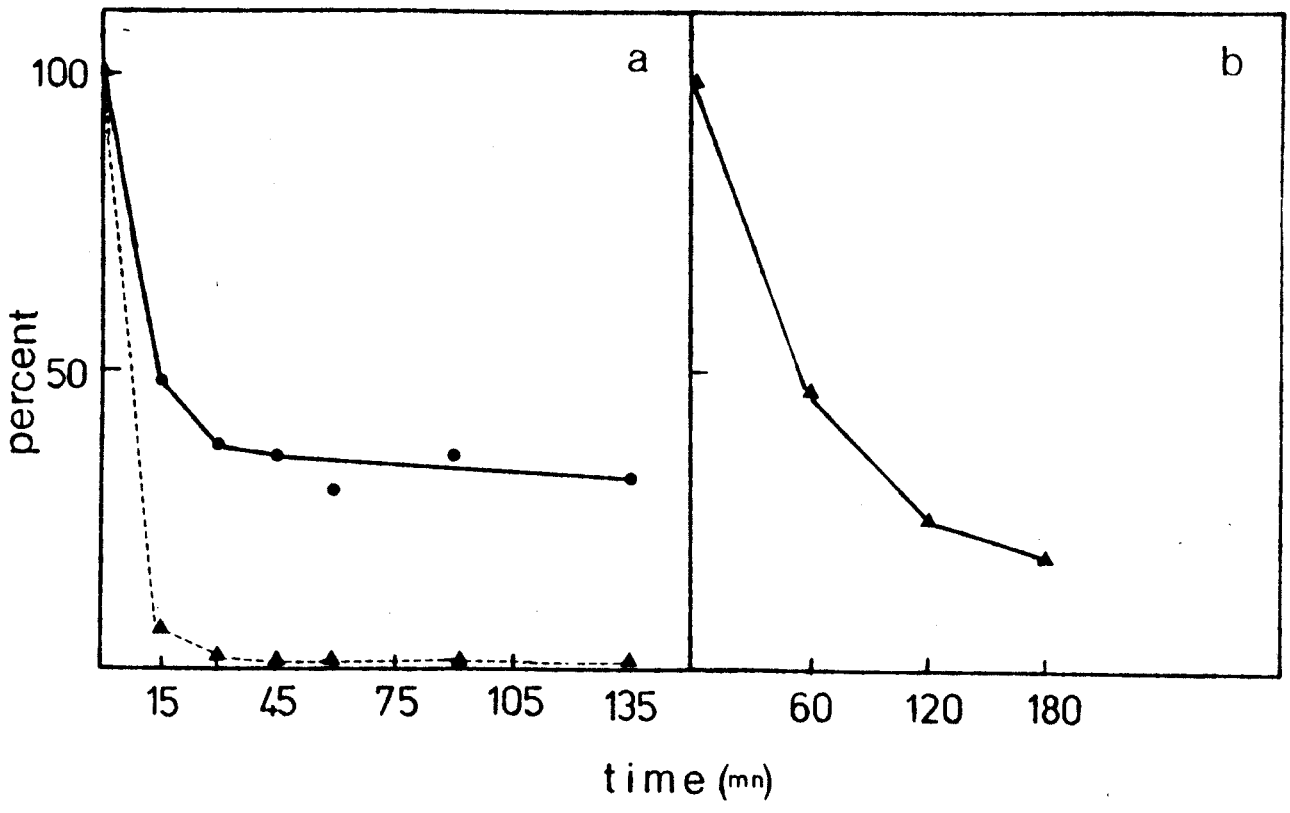

Figure 3

Kinetics of inhibition of adenovirus DNA and protein synthesis by novobiocin. The data are expressed as the percentage of synthesis in untreated infected cells after a pulse-labeling with (^{3}H)-thymidine for 15 min. (a), or (^{35}S)-methionine for 1 h (b). The X-axis indicates the time of incubation with novobiocin at 0.1 mg/ml (●) or 1 mg/ml (▲).

The inhibition of viral synthesis appeared to be slower than that of DNA synthesis : only 55 per cent inhibition was obtained after 1 h exposure to novobiocin at 1 mg/ml, and 75 per cent after 3 h (Fig.3b).

4. Reversal of the effects of novobiocin in the presence of protein synthesis inhibitor

In order to examine a possible reversal of the novobiocin inhibition, WT-infected cell monolayers were exposed to novobiocin at 1 mg/ml for 1 h at 17 h after infection, then washed and overlaid with normal prewarmed medium with or without cycloheximide as protein synthesis inhibitor. The cell were pulse-labeled with (^{3}H)-thymidine for 1 h at different times after removal of novobiocin. Each cell sample was assayed for incorporation of (^{3}H) label into DNA. Fig.4 shows that the effect of novobiocin was partially reversible : after removal of the inhibitor, the DNA synthesis was restored to 35 per cent of the level in untreated infected cells. This DNA synthesis occurred at the same level in the presence of cycloheximide, which suggested that a de novo protein synthesis was not required for the reversal of the novobiocin effect. However, it might not be excluded that the incomplete reversal of the inhibition was due to a general cytotoxic effect of the drug, as previously suggested (8).

5. Inhibition of viral DNA synthesis initiation in novobiocin-treated HeLa cells

To determine which step of viral DNA synthesis was inhibited preferentially by novobiocin (e.g. initiation, elongation, ligation, etc..), adenovirus ts mutants blocked in DNA synthesis initiation were used. H2ts114-or H5ts30-infected cell monolayers maintained at the restrictive temperature for 17 h after infection, were shifted down to the permissive temperature and pulse-labeled with (^{3}H)thymidine for 30 min. Novobiocin was added to

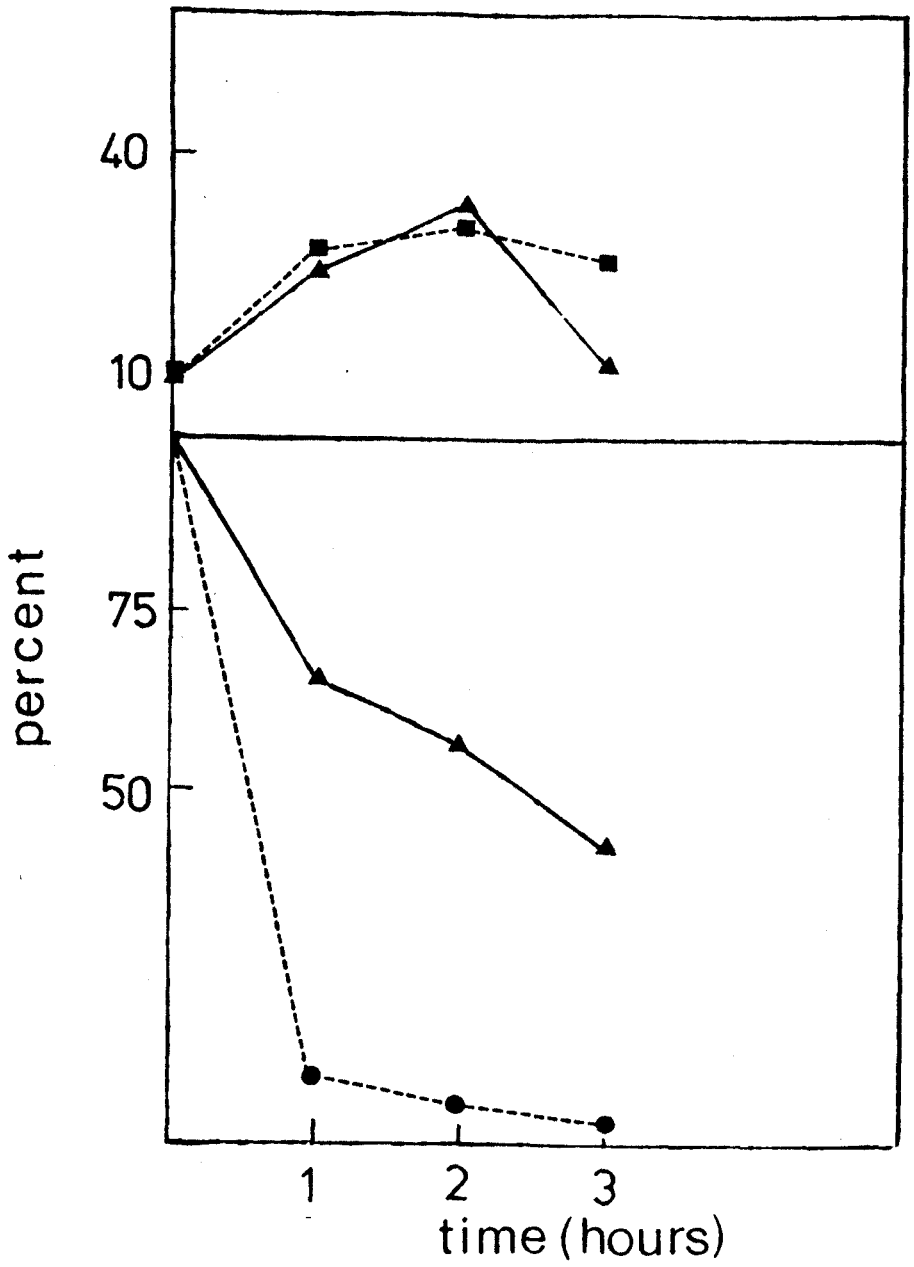

Figure 4

(a) Partial reversal of the effect of novobiocin on adenovirus DNA synthesis. WT-infected HeLa cells were exposed to novobiocin (1 mg/ml) for 1 h at 17 h after virus infection. The cell cultures were washed free of the novobiocin and pulse-labeled with (^{3}H)thymidine for 1 h at different times after removal of the drug, in the presence (▲) or the absence (■) of cycloheximide (CH).
Data are expressed as the percentage of DNA synthesis in untreated infected cells. The X-axis indicates the incubation period after removal of the novobiocin.
(b) Control WT-infected HeLa cell cultures were pulse-labeled with (^{3}H)-thymidine for 1 h at 18 h after virus infection in the presence of CH (▲) or of novobiocin (●), and incubated with the inhibitor for different periods.

the cell culture at the time of shift-down or at different times after shift-down (5, 10, 15 min) and viral DNA analyzed on sucrose gradient.

Fig.5 shows that despite an overall reduction of thymidine incorporation, whenever the time of addition of novobiocin the (^{3}H)-thymidine label always sedimented at 34 S, the coefficient for intact viral DNA, and never at lower sedimentation coefficient values. This result suggested that the elongation process remained unaltered by novobiocin, and that, once started, the synthesis of the new DNA strand pursued to its normal length.

6. Inhibition of viral DNA encapsidation by novobiocin

Another effect of novobiocin on adenovirus metabolism is shown in Fig.6. Novobiocin prevented viral DNA incorporation into mature virus particle. When novobiocin was added just after a pulse-labeling with (^{3}H)-thymidine, almost no label was found to sediment at 750 S, the sedimentation coefficient for mature virion. However, it could not be excluded that this inhibition of DNA encapsidation was due to a secondary alteration of the synthesis of some viral protein(s) indispensable for DNA maturation and encapsidation.

Although the reversal of the novobiocin effect in the presence of cycloheximide (Fig.4a) seemed to rule out this explanation, the following experiment was designed to test this hypothesis.

7. Novobiocin inhibition of viral DNA encapsidation appears as a primary event independent of protein synthesis.

Assembly intermediate particles (IM), of 1.315 in density, accumulated by H2ts112 at 39.5°C have been shown to be able to evolve into mature virions (MV, 1.345 in density) upon shift-down to 33°C (11). This maturation can occur in the presence of protein synthesis inhibitor (11, and Fig. 7d). Contrastively,

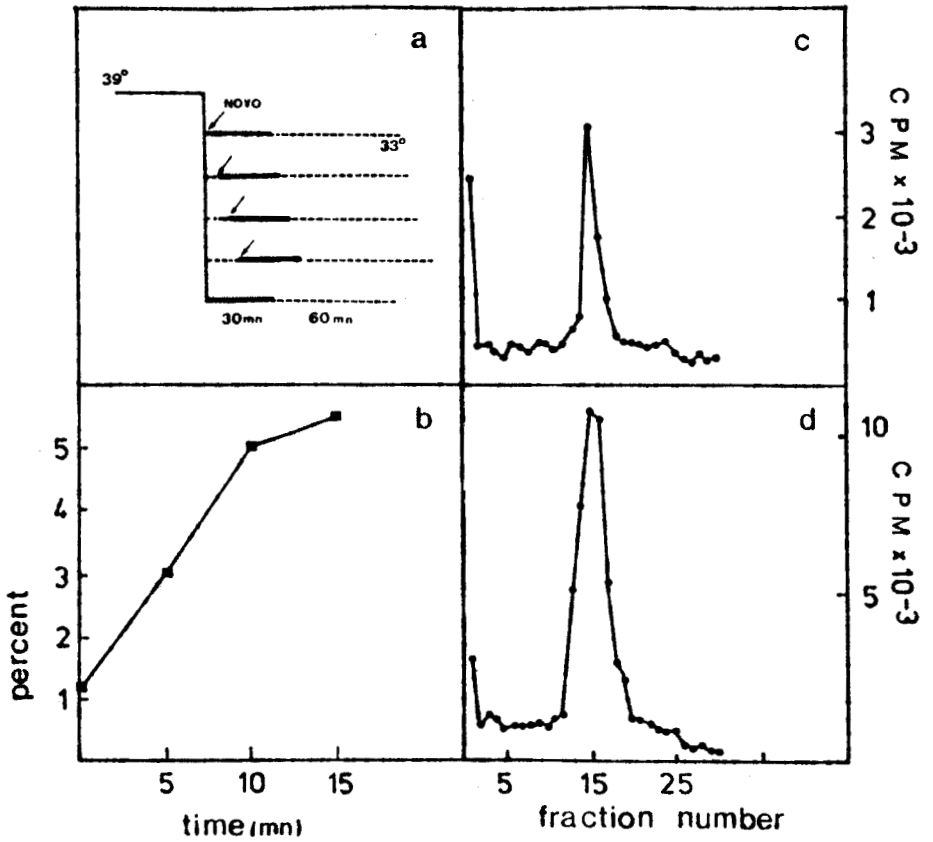

Figure 5

Effects of novobiocin on adenovirus DNA synthesis initiation and elongation. H2 ts 114-infected cell cultures maintained at 39.5°C (—) for 17 h after infection were shifted down to 33°C (---) and pulse-labeled (—▪—) with (^{3}H)-thymidine for 30 min. Novobiocin (arrows, 1 mg/ml) was added at 0, 5, 10, 15 min after the shiftdown as schematized in panel (a). Cells were harvested after a 60 min-chase period at 33°C and viral DNA analyzed on alkaline sucrose gradient. Data are expressed in (b) as the percentage of DNA synthesis in H2 ts 114-infected cells, untreated with novobiocin. Viral DNA label sedimented at 34 S, whenever the time of addition of the drug. (c) 0 min. ; (d) 10 min. Bar represents the period of labeling.

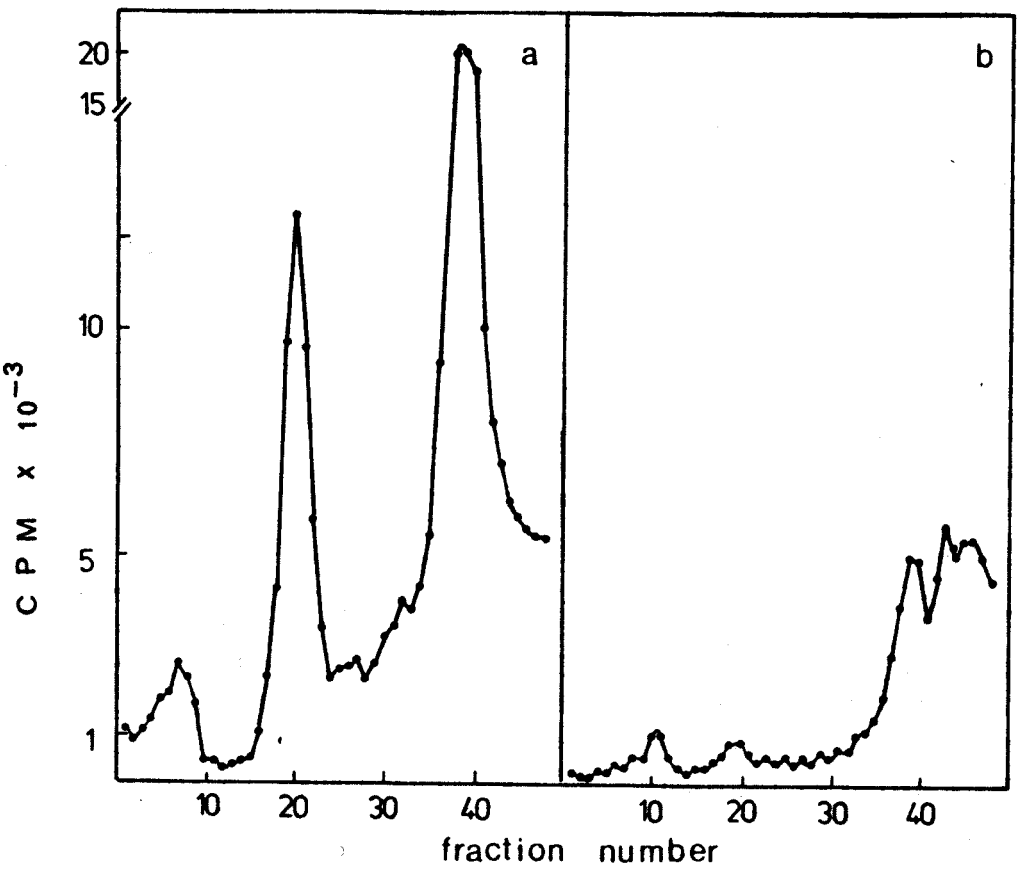

Figure 6

Novobiocin inhibition of WT adenovirus DNA encapsidation. WT-infected KB cell culture were pulse-labeled with (^{3}H)-thymidine for 20 min. at 17 h after infection. One cell culture was chased in the presence of (a) hydroxyurea (10 mM), the other in the presence of (b) novobiocin (0.2 mg/ml). The virus particles were extracted at 21 h and analyzed on sucrose gradient. The arrow indicates the position of the 750 S infectious virions (fraction 20).

when novobiocin was added at the time of shift-down, no viral DNA encapsidation occurred, and no mature virions appeared at 750 S in sucrose gradient (Fig.7c). This suggested that the inhibition of DNA encapsidation by novobiocin was a primary effect which could be expressed without protein synthesis.

8. Absence of requirement of DNA synthesis initiation for viral DNA encapsidation.

Inhibition of viral DNA encapsidation by novobiocin might be an indirect effect, secondary to an inhibition of DNA synthesis initiation. However, the following observations argued against this hypothesis. (i) WT-infected KB cells were pulse-labeled with (^{35}S)-methionine for 20 min at 16 h after infection, and hydroxyurea (HU) added just after the pulse. The cells were harvested 90 min after pulse-labeling and the virus particle populations were extracted and analyzed in sucrose gradient. As shown in Fig.8, equivalent quantites of assembly intermediates (IM, 600 S) and of mature virions (MV, 750 S) were assembled in HU-treated and in untreated cells, suggesting that virus assembly did not require a new round of DNA synthesis (see also Fig.6a). (ii) It has been shown that H2ts112 cultured at 39.5°C accumulated light IM void of DNA, which are capable of maturing upon shift-down into infectious virions containing a full length of DNA (11). When H2ts112-infected cells were pulse-labeled with (^{3}H)-thymidine before the shift-down, the population of 750 S adenovirus particles obtained at the end of the virus cycle contained labeled 31 S DNA, implying that "old" viral DNA synthesized at 39.5°C could be normally encapsidated (not shown). (iii) As previously observed (13, 14), DNA entered the virus capsid after a 3 h-lag, suggesting a relatively long maturation proccess of DNA, preceding its encapsidation. The novobiocin effect on viral DNA encapsidation appeared therefore not to involve a block in DNA synthesis initiation.

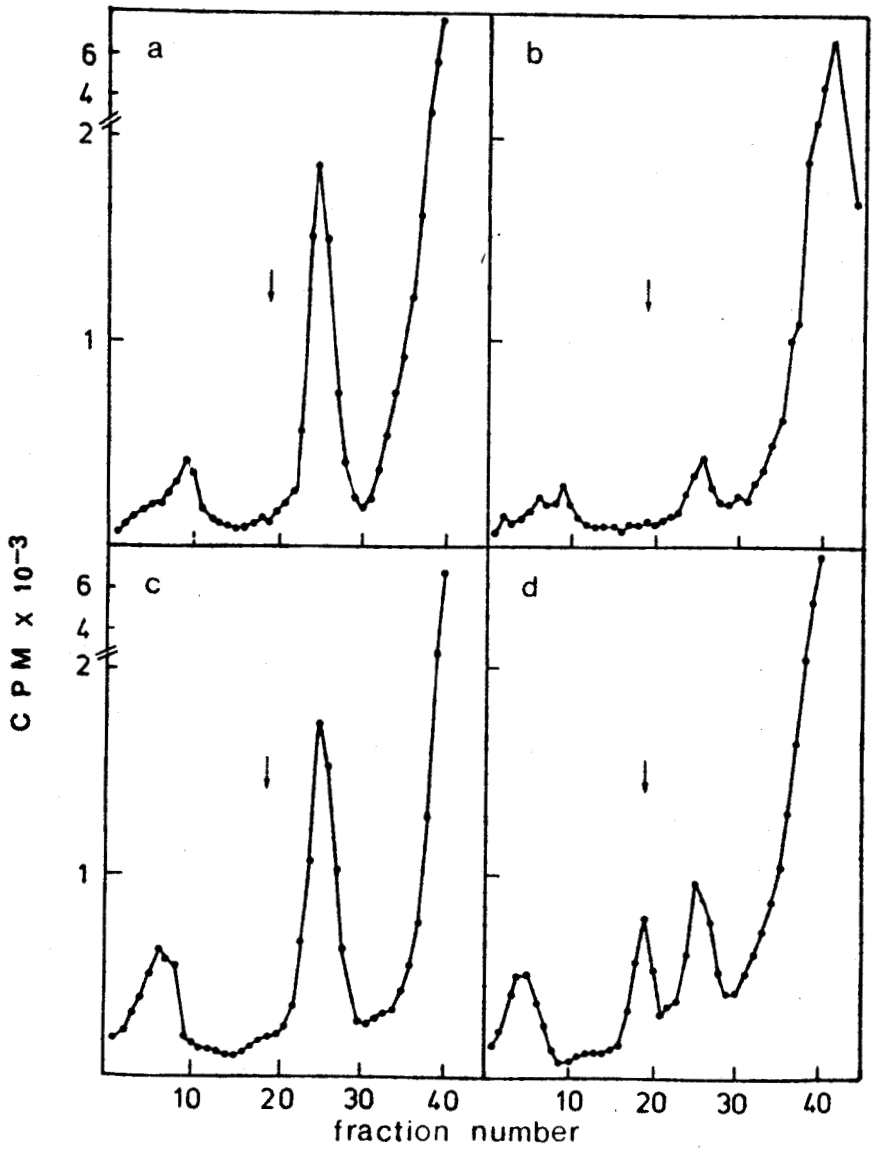

Figure 7

Novobiocin block of H2ts112 maturation. H2ts112-infected KB cell cultures maintained at 39.5°C were pulse-labeled with (^{35}S)-methionine for 20 min. at 17 h after infection, and harvested after a 2 h-chase at 39.5°C (a) in the absence, or (b) in the presence of novobiocin (0.2 mg/ml). A third cell sample (c) was harvested after a 2 h-chase at 39.5°C, followed by a 18 h chase at 33°C ; novobiocin was added at the time of shift-down. A fourth cell sample (d) served as a control of the sample (c), cycloheximide being added at the time of shift-down in place of novobiocin.

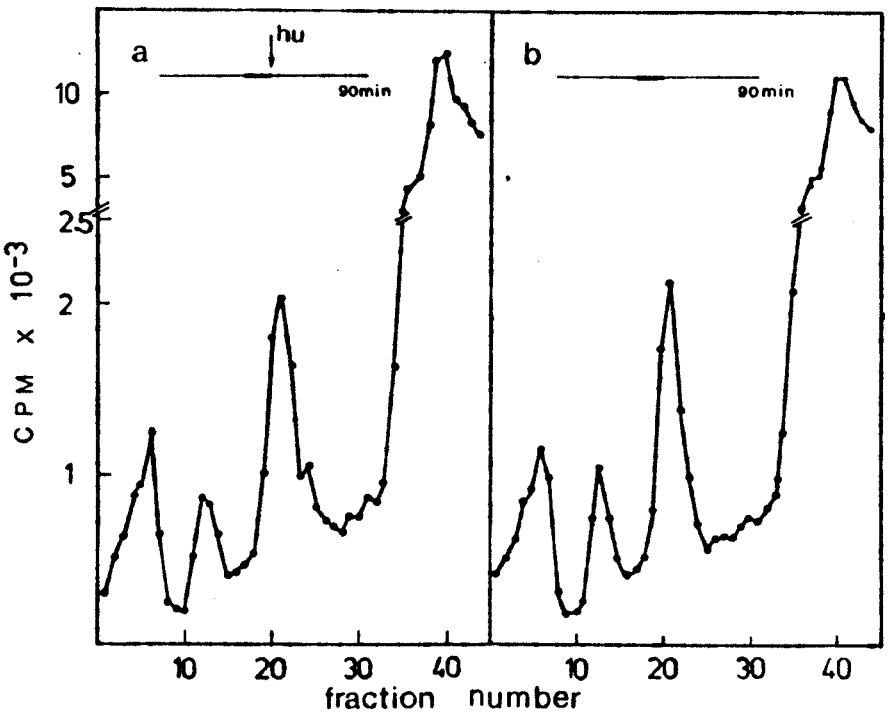

Figure 8

Adenovirus DNA encapsidation in the absence of DNA synthesis initiation. WT-infected KB cell cultures were pulse-labeled with (^{35}S)-methionine for 20 min at 16 h after infection, and chased for 90 min (a) in the presence, or (b) in the absence of hydroxyurea (HU, 10 mM). Virus particles were extracted and analyzed on sucrose gradient. Fractions 12-13 correspond to virus particles sedimenting with a sedimentation coefficient of 750 S, (mature virions) fractions 20-21 to 600 S assembly intermediates. Bar represents the period of labeling.

9. Novobiocin and adenovirus DNA conformation

It has been shown that a bimodal sedimentation profile of ethidium bromide-treated DNA reflects the relaxation of negative supercoils at low concentrations of the intercalating drug, and the introduction of positive supercoils at high concentrations (7, 15). Attempts were therefore made to determine whether novobiocin modified the sedimentation behavior of ethidium bromide-treated adenovirus DNA. Unfortunately, it was impossible to detect any difference in the sedimentation pattern of control viral DNA in the presence or the absence of ethidium bromide. This rendered impossible the evaluation of the degree of DNA relaxation with or without novobiocin. However, the absence of ethidium bromide-induced modification of viral DNA conformation suggested that the terminal protein which circularizes the adenovirus DNA molecule (16) was not capable of maintaining possible DNA supercoils in vitro.

DISCUSSION

The results presented here indicate that novobiocin inhibits adenovirus DNA replicative synthesis, and to a lesser degree, virus RNA and protein synthesis. They also strongly suggest that initiation of DNA replication and not elongation is blocked (Fig.5). This is consistent with previous reports that novobiocin affects DNA replication via its action on gyrase-induced negative supercoiling (2, 4, 7), a supercoiled DNA conformation being required for initiation of DNA replication (2, 17, 18). Inhibition of adenovirus replication is partially reversible, even in the presence of protein synthesis inhibitor (Fig.4). A striking effect of novobiocin appears to be the inhibition of viral DNA encapsidation, and therefore assembly of infectious viruses (Fig.6 and 7), which is not the result of the block in viral DNA synthesis.

Data reported here and elsewhere on adenovirus WT and ts mutants have demonstrated that initiation of DNA replication is not indispensable for DNA encapsidation and virion assembly, and that "old" DNA can be incorporated into virus capsid to form mature infectious virion (11, 13 and Fig. 6a and 8). Those data and the results obtained with novobiocin suggest that a novobiocin-sensitive, possible gyrasedependent, step of viral DNA maturation would be required for adenovirus DNA encapsidation and virion assembly : entry of viral DNA into the capsid would depend upon a negatively supercoiled conformation of the DNA molecule.

This hypothesis is favored by the observation that, in the DNA-negative ts mutants of adenovirus blocked in DNA synthesis initiation (e.g. H5ts36, H2ts111, and H2ts114) there was no DNA encapsidation and infectious virus assembly upon shift-up to the nonpermissive temperature, even after a prolonged period of incubation at the permissive temperature to permit a normal synthesis of viral DNA and proteins (Fig.9). Adenovirus-coded, or adenovirus-induced factor(s), implicated in the DNA synthesis initiation complex, would therefore also play a key role in viral DNA encapsidation. An enzyme with an activity similar to that of the bacterial or eucaryotic gyrase, responsible for the

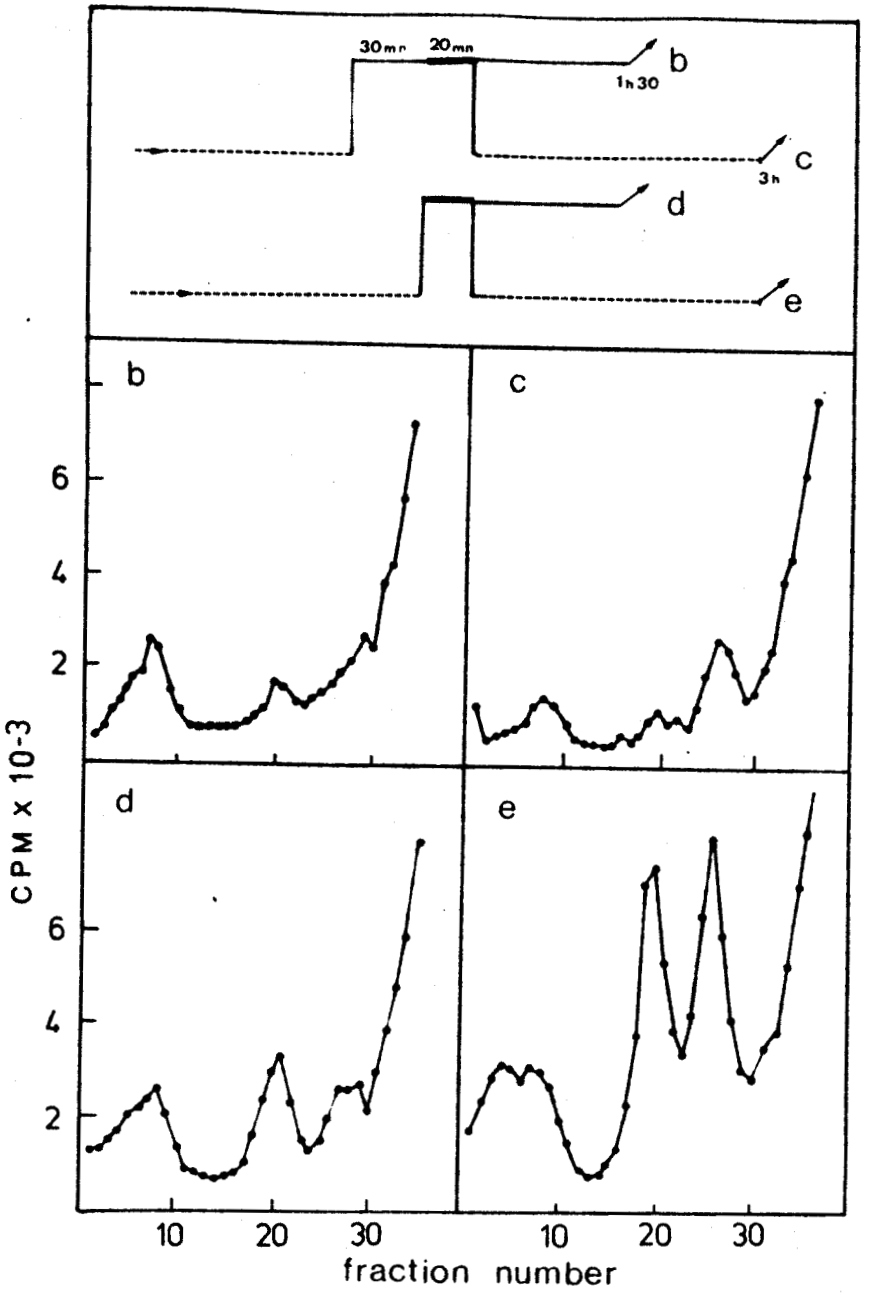

Figure 9

Block of virus particle formation in ts mutants defective in DNA synthesis initiation at nonpermissive temperature. H2ts111-infected cell cultures maintained at permissive temperature (33°C) for 40 h after infection were shifted up to 39.5°C, 30 min before or immediately before pulse-labeling with (^{35}S)-methionine for 20 min at the nonpermissive temperature. They were chased at this temperature for 90 min, or shifted down to 33°C for 3 h, as schematized in (a). Virus particles and assembly intermediates were extracted and analyzed on sucrose gradients (b-e). Fractions 19-21 correspond to 750 S virions, fractions 25-27 to 600 S assembly intermediates. Similar patterns of particles were obtained with H2ts114 and H5ts36. Bar represents the period of labeling.

introduction of negative supercoils into viral DNA, would be a good candidate for such a double function.

ACKNOWLEDGEMENTS

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LEXIQUE

1) Cellules

KB	cellule humaine en lignée continue, provenant d'un carcinome du plancher buccal
HeLa	cellule humaine en lignée continue, provenant d'un carcinome du col utérin
HEK	cellule humaine embryonnaire de rein
HEK-293	cellule HEK, transforméepar de l'adénovirus type 5
AGMK	cellule de rein de singe vert d'Afrique
CV_1	1 clone de cellules AGMK
sirc	cellule de cornée de lapin
NIL	cellule de hamster

2) Virus

SV_{40}	papovavirus, son hôte naturel est le singe
Ad 2 ND (1 à 4)	hybride Ad2-, SV_{40} possèdant un plus ou moins grand fragment de SV_{40} selon le clone. Ces hybrides se multiplient efficacement sur cellules de singe.
ϕ 29	bactériophage de Bacillus Subtilis à DNA bicaténaire circularisé par une protéine; de forme icosaèdrale

3) Nucléases

. Deoxyribonucleases

DNAse 1	(pancréas de boeuf) digère les DNA mono et bicaténaires même dans les nucléosomes

Nuclease S1 isoléed'Aspergillus oryzae, elle est spécifique des acides nucléiques simple chaine. Elle utilise le Zn^{++} comme cofacteur.

Nuclease micrococcale isoléede Staphylococcus aureus ; digère préférentiellement le DNA en dehors des nucléosomes

. Endonucleases de restriction

Eco R I endonuclease de restriction isoléede Escherichia Coli portant le plasmide de résistance R1 : (E. Coli R Y 13) site de coupure : GAATTC
CTTAAG

(pour les autres endonucleases vz : Zabeau and Roberts (1979)

4) Inhibiteurs

H.U. hydroxyurée:inhibiteur de la synthèse du DNA par réduction de la concentration des deoxynucléotides triphosphates

Ara C arabinoside-cytosine : inhibe la synthèse du DNA par compétition avec la deoxycytosine

Aphidicoline inhibe préférentiellement la DNA polymérase α (in vitro et in vivo)

dd TTP di deoxy-thymidine triphosphate : inhibe la synthèse du DNA par compétition avec la thymidine triphosphate, in vitro, ou dans les noyaux isolés

cycloheximide inhibe la synthèse protéique en empéchant la fixation du t-RNA initiateur et le relachement du t-RNA déacétylé du site A de la sous unité 60 S.

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