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Titre

La phosphorylation de NS1 est un événement clef durant la propagtion du virus minute de la souris (MVMp).

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Abbreviations

AAV	Adeno Associated Virus
ADN	DesoxyriboNucleic Acid
ARN	Ribonucleic Acid
ATP	Adenosine triphosphate
CDK	Cyclin-Dependent Kinase
CKII	Casein Kinase II
Fig.	Figure
h	hour(s)
kb	kilobase
kDa	kilo Dalton
LTAg	Large T Antigen
Mda	Mega Dalton
min	minute(s)
MVMi	immunosuppressive strain of Minute virus of Mice, lymphotropic
MVMp	prototype strain of Minute Virus of Mice, fibroblastic
NES	Nuclear Export Signal
NLS	Nuclear Localisation Signal
NPC	Nuclear Pore Complex
NS (1 or 2)	Non Structural protein (1 or 2)
$NS1^0$	un(der)phosphorylated NS1
$NS1^p$	native NS1
NTP	Nucleotide triphosphate
PKC	Protein Kinase C
RPA	Replication Protein A
SAPK	Stress Activated Protein Kinases
SV40	Simian Virus 40
TPA	12-O-tetradecanoyl-phorbol-13-acetate
VP (1, 2 or 3)	Viral Protein (1, 2 or 3)

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Résumé

Les Parvovirus sont de petits virus non enveloppés ayant un génome constitué d'un ADN simple brin, d'environ 5kb. Ils infectent une variété d'espèces animales dont le spectre d'étend de l'embranchement des arthropodes jusqu'à l'homme. Le genre des parvovirus autonomes est caractérisé par sa capacité à produire des virions dans des cellules hôtes en l'absence de virus aidant. Les parvovirus autonomes, dont le virus prototype, le virus minute de la souris (MVMp), sujet de notre travail, se répliquent de manière préférentielle dans des cellules transformées. Ils possèdent, du fait de cet oncotropisme et de leur activitè cytolytique, des capacités oncosuppressives. La principale thématique de notre département réside dans l'investigation des mécanismes de ces effets oncolytiques sur les cellules transformées, une propriété attribuée à la protéine non-structurale, NS1. Les parvovirus sont uniques parmi les virus animaux puisqu'ils ne possèdent au sein de leur famille aucun membre induisant ou promouvant la transformation cellulaire. Ils font ainsi, l'objet d'investigations dont le but est d'utiliser ces virus comme vecteurs en thérapie génique. Cet axe de recherche également développé au sein de notre département.

Notre groupe, travaille plus particulierement sur la régulation de la réplication de l'ADN des parvovirus autonomes. C'est dans ce contexte, que j'ai été impliqué dans la caractérisation de la protéine non-structurale NS1, en particulier dans l'étude du mode de régulation des nombreuses fonctions attribuées à ce polypeptide. Il est connu depuis longtemps que la "régulation de fonctions" peut être attribué à des événements de phosphorylation, étant donné que le simple ajout d'un groupement phosphate à une protéine lui confère de nouvelles propriétés, principalement en modifiant la conformation du polypeptide. Ces altérations structurelles permettent à la protéine de passer d'une conformation dite "active" ˆ une forme "inactive" ou inversement. Ces changements structurels et fonctionnels ont été déjà bien étudiés, comme le révèle les exemples des divers facteurs de transcription, des kinases dépendantes de cyclines, ou encore de la protéine large T de SV40. Dans le cas de NS1, il a été établi depuis une dizaine d'années que cette protéine devient phosphorylée lors d'une infection. Toutefois, la signification de cette modificationest resté obscur jusqu'à ce jour. NS1 possède un arsenal d'activités biochimiques intervenant lors du cycle réplicatif du virus. Cette protéine est ainsi capable de lier et d'hydrolyser l'ATP, et possède des activités de coupure de l'ADN opérant de manière site spécifique et induisant une liaison covalente entre NS1 et l'extrémité 5' ainsi coupée. NS1 est également capable de séparer les brins d'ADN (activité hélicase), de moduler en trans des promoteurs viraux et cellulaires, et d'induire des changements conduisant à la mort de la là cellule hôte. D'autres protéines virales, tel que l'antigène Large T de SV40 (LTAg), qui posséde des

homologies de séquence avec NS1, exercent des rôles-clefs similaires lors de la progression du cycle viral. Pour LTAg, il a été montré que les fonctions de réplication sont régulées par phosphorylation. Ceci nous a conduits à postuler que les fonctions de NS1 pouraient être elles aussi sous le contrôle de la phosphorylation.

A l'aide d'essais *in vitro* développés récemment et permettant d'analyser les fonctions réplicatives de NS1, nous avons testé notre hypothèse de travail en comparant les activités de la protéine à l'état natif, $NS1^P$, par rapport à son état dephosphorylé, $NS1^0$. Nous avons ainsi mis en évidence qu'en comparaison de $NS1^P$, $NS1^0$ est sévèrement altérée dans sa fonction hélicase, et à un moindre degrés, dans ses activités d'hydrolyse de l'ATP (activité ATPasique) et de coupure de l'ADN, alors que son affinité pour la séquence cible (ACCA)2-3 est augmentée. En présence des protéines kinases contenues dans les extraits cellulaires de réplication, $NS1^0$ posséde toutes les fonctions nécessaires ˆ la résolution et la réplication du "dimer-bridge" (jonction entre les unités génomiques composant la forme réplicative dimérique). Ceci a put être imputè à la réactivation de la fonction de réplication de $NS1^0$ suite à la rephosphorylation du polypeptide *in vitro*. Encouragé par ces résultats, nous avons ètendu l'étude de la phosphorylation de NS1 à l'infection naturelle de cellules permissives de souris (lignée A9) par MVMp. NS1 est phosphorylée sur des résidus sérine et thréonine, majoritairement au sein d'une r_gion de 18kDa, situé entre les acides aminés 315 et 482; cette région comprend le domaine hélicase de NS1. La phosphorylation de la protèine NS1 *in vivo* precéde sa liaison covalente à l'ADN, marqueur de l'implication du polypeptide viral dans la réplication. Nos résultats montrent ainsi pour la première fois une séquence temporelle *in vivo* entre la phosphorylation de NS1 et son activité de réplication. Par ailleurs, nous avons observé que le taux de modification post-traductionnelle de NS1, en particulier de phosphorylation, augmente lors d'une infection virale. Cette évolution s'accompagne de changements qualitatifs dans le profil de phosphorylation du polypeptide viral.

Afin de tenter d'identifier les protéine-kinases impliquées dans la phosphorylation et la régulation de NS1, nous avons établi un système de réplication *in vitro* dépouvu de protéine-kinase endogènes. Dans ce conditions, NS1 à l'état natif ($NS1^P$) permet la réplication d'un plasmide contenant l'origine gauche de réplication du parvovirus selon le mode dit de "rolling circle", tandis que NS1 à l'état déphosphorylé ($NS1^0$), en est incapable. Des fractionnements d'extraits de cellules Hela ont ensuite été réalisés et ont conduit ˆ l'identification de deux composants protéiques essentiels, capable en combaison de phosphoryler $NS1^0$ et de rètablir au moins en partie sa compétence pour la réplication dans le système sus-mentionné. Ces fractions sont enrichies en protéine kinase C (PKC), ce qui peut être rapproché du fait que la réactivation de $NS1^0$ dépend de l'ajout de cofacteurs caractéristiques de cette famille de kinases. Un de ces composants est capable, enrichi en protéine-kinases C atypiques, est capable à lui seul de réactiver $NS1^0$ pour son activité hélicase. La capacité de PKC atypiques recombinantes à réactiver la fonction hélicase de $NS1^0$ a récemment été confirmée (Detwiller-S communication personnelle).

Finalement, l'implication de la famille des protéines kinases C dans la régulation de fonctions distinctes de NS1 a été étayé par des études réalisés au moyen de mutants de NS1. Ces derniers ont été construits par mutagenèse dirigée et concernent des sites consensus de phosphorylation par la protéine kinase C, situés dans une région de NS1 mentionnée ci-dessus comme étant une cible majeure de phosphorylation (le fragment de 18kDa). Ces mutants ont permis l'identification de deux sites fonctionnels de phosphorylation *in vivo* de NS1, les résidus thréonine 403 et 435. L'analyse biochimique de ces mutants a révélé leur incapacité à répliquer l'ADN viral. Des analyses plus fines ont montré que le mutant T435A est altéré dans ses activités hélicase et de coupure de l'ADN, alors que le mutant T403A n'est seulemetn déficient pour la coupure de l'ADN (dans des conditions physiologiques, c'est-à-dire lorsqu'une protéine cellulaire accessoire est nécessaire au clivage). La thréonine 435 est un cible de phospohrylation in vitro par la classe des protèine-kinases C atypiques, en accord avec le rôle proposé ci-dessus pour ces kinases dans la régulation des fonctions réplicatives (en particulier hélicase) de NS1. L'analyse des propriétés biochimiques du mutant T435A a formé une première indication en faveur d'une régulation différentielle des fonctions de NS1 par la phosphorylation. Bien qu'inactif pour la réplication, ce mutant est en effet capable de trans-activer le promoteur viral p38 (qui permet la synthèse des protéines de la capside). En conclusion, l'ensemble de nos résultat indiquent que la phosphorylation de NS1 par des membres de la famille des protéines kinases C représente un événement majeur impliquè dans la régulation des fonctions de cette protéine virale lors d'un cycle réplicatif.

Abstract

Parvovirus are small, non-enveloped viruses with a 5kb single-strand linear DNA as a genome that infect a variety of species ranging from arthropod to men. The genus autonomous parvovirus is characterised by their independence of helper viruses for progeny virion production. Minute Virus of Mice (MVMp), the prototype strain of the autonomous parvoviruses, under investigation here, preferentially replicates in transformed cells, performing a lytic cycle. A main task of our department consists in investigation of the oncotropism and the oncolytic effects on transformed cells, a property that has been attributed to the major non-structural protein, NS1. In addition, since they do not promote or induce cell transformation but exert oncosuppressive properties, a unique feature among animal viruses, they are prime candidates for gene therapy vectors. This important parvoviral application is investigated in our department as well. Within this departmental structure, our group investigates the regulation of parvoviral DNA replication and my thesis project in this context consisted in the characterisation of the non-structural protein NS1, in particular the modes of regulation of this multifunctional polypeptide. "Regulation of functions" is well-known to be a major task, and often it is attributed to phosphorylation events, since this simple addition of a phosphate group to a protein is able to change its properties, mostly by conformational changes within the structure of the polypeptide chain. These alterations enable the protein to switch from an active to an inactive conformation, which has been studied for a variety of proteins and is exemplified by transcription factors such as the AP1 family, by the cyclin dependent kinases, or by the nuclear translocation of SV40 large T antigen. In the case of NS1, it has been known for a decade that the polypeptide becomes phosphorylated during infection, no indication, however, was obtained for a functional relevance of this modification. NS1 carries out a variety of biochemical activities in the course of a viral infection. Thus, it binds and hydrolyses ATP, has site-specific endonuclease activity leaving the polypeptide covalently attached to the 5' end of the nicked DNA, unwinds DNA, trans-modulates cellular and viral promoters, and induces changes to the host cell leading to cell death. Other viral proteins, such as SV40 Large T antigen (LTAg), to which NS1 has many similarities, including a striking sequence homology within the helicase domain, exert a similar key-role during the course of a viral infection. For the well studied LTAg, it has been shown that replicative functions are regulated by phosphorylation. Due to the many similarities between NS1 and this viral protein, we extended the known similarities and hypothesised that NS1 functions might be regulated by phosphorylation as well. Taking advantage of recently developed *in vitro* assays that

have been used to analyse NS1 replicative functions and to determine its domain structure, we investigated the relevance of this proposal, comparing the properties of native and dephosphorylated NS1. Thus, by analysing the biochemical functions of native $NS1^P$ derived from recombinant vaccinia viruses with its dephosphorylated counterpart $NS1^O$ we revealed that $NS1^O$ was severely impaired for intrinsic helicase and to a minor extent for ATPase and nickase activities, whereas the affinity to the target DNA sequence $[ACCA]_{2-3}$ was enhanced. Nevertheless, in the presence of endogenous protein kinases in replication extracts, $NS1^O$ produced all functions necessary for resolution and replication of the left-end dimer-bridge, indicating a reactivation of $NS1^O$ by rephosporylation. Confident from these results, we further characterised phosphorylation of NS1 *in vivo* during the course of a natural MVMp infection in A9 cells. NS1 is phosphorylated on serine and threonine residues, located mainly on an 18 kDa fragment, between amino acids 315 and 482, encompassing the NS1 helicase domain. Moreover, phosphorylation of NS1 take place before it exerts its replicative functions, manifested by covalent attachment to the viral DNA. In addition, we were also able to demonstrate that the NS1 phosphorylation pattern alters during the virus cycle. These time course experiments confirmed that there is an early synthesis of non-structural proteins before the capsid proteins become apparent. Moreover, we showed an increase in the post-translational modification of NS1, due to the accumulation of slower migrating NS1 species in by Western blot. We were also able to demonstrate that the rate of NS1 phosphorylation increases during the course of an infection. In order to identify some of the protein kinases involved in phosphorylation and regulation of NS1, we established an *in vitro* replication system that is devoid of endogenous protein kinases. While native $NS1^P$ supports rolling circle replication of plasmids containing the left-end origin of replication under these conditions, dephosphorylated $NS1^O$ is impaired. Consecutive fractionation of HeLa cell extracts, lead to the identification of two essential protein components which are able to phosphorylate $NS1^O$. These protein fractions are enriched in protein kinase C (PKC) and reactivation of $NS1^O$ is dependent upon addition of characteristic PKC co-factor. One of these "activating" components, was able to restore $NS1^O$ helicase activity due to atypical PKCs enriched in this purified fraction. The regulation of NS1 unwinding functions by atypical PKC has been recently confirmed using recombinant PKCλ (Detwiller-S personal communication). Finally, the involvement of the protein kinase C family in the regulation of distinct NS1 function was further substantiated, by site directed mutagenesis of conserved PKC consensus phosphorylation sequences, which are located within the main region of NS1 targeted for phosphorylation during MVM infection. These mutants allowed to identify two phosphorylation sites in NS1, namely the threonine 403 and 435. Analysing these mutants for biochemical activities, we showed that the mutagenesis impaired replicative functions of NS1. More refined analyses revealed that T435A mutant was impaired for nicking and helicase activity, whereas, T403A mutant was impaired in nicking assay in the context where an accessory protein

is required. The first indication for differential regulation of NS1 functions by phosphorylation derived from the biochemical properties of T435A. While inactive for replication, this mutant was clearly able to trans-activate the p38 promoter driving the capsid gene. Interestingly, together with the finding that Thr435 is impaired for DNA unwinding and the identification of a target phosphorylation site in vivo we were also able to demonstrate that it consists of a phosphorylation site for atypical PKC in vitro. Since these kinases have been shown to regulate NS1 helicase function in vitro it consist of a candidate regulatory element for NS1 functions. In conclusion, phosphorylation of NS1 by members of the protein kinase C family is of major importance for a proper regulation of the viral protein functions during virus propagation.

Introduction

I) Parvovirus

I-1) General properties of parvoviruses

a) Physicochemical characteristics

Parvoviruses are small non-enveloped spherical particles which are composed by association of proteins and a single stranded linear DNA serving as a genome (83). Neither RNA, nor lipids have been detected in mature viruses. Members of the parvovirus genus are of the smallest viruses in vertebrates with a particle diameter between 20 and 25 nm and a genome of approximately 5.1kb (25). Three different forms of viral capsids are found in parvovirus-infected cells or tissues, namely full particles containing DNA, banding between 1.41 and 1.46 g/ml in CsCl gradient, empty capsids which band at 1.32 g/ml, and heterogeneous intermediate species which consists of defective particles containing deleted forms of the viral genome (70). The entire infectivity of such a fractionated virus preparation resides within the DNA-containing full virions (70). The viral capsid is assembled into an isometric structure comprising a total of about 60 polypeptides, a combination of the structural proteins VP1, VP2 and VP3 (7). The three-dimensional structures for full and empty particles of a variety of parvoviruses (CPV, FPV, MVM and B19) has been determined to atomic resolution using X-ray crystallography (5-7, 63, 66, 173). These analyses showed that one third of the VP polypeptide folds into a β-strand structure composed of eight anti-parallel β-strands, a motif that has been found in most other spherical capsids determined so far (8). The rest of the VP-structure consists of large insertions between the β-strands (8). VP1, VP2 and VP3 assemble tightly to sub-units sedimenting characteristically at 12S in sucrose gradients (160, 216, 257). 32 of these capsomers are arranged in an icosahedric shell of both full infectious viruses as well as empty capsids (83). The assembled particles are very stable, comprising high resistance to desiccation, heating, freezing and thawing, lipid solvents and even moderate levels of chaotropic agents, such as urea or SDS (18, 38).

b) History and classification

Rat virus, the first parvovirus isolate, was discovered in the late 1950s as a contaminant in a transplantable neoplasm (158). A decade later a similar agent which differed antigenetically was found in a human tumour cell line (Hep1) that was passaged in laboratory rats. This virus was termed H-1 virus (263, 264). Minute virus of mice (MVM), the main parvovirus under investigation in this study, was isolated in 1966 from a contaminated stock of mouse adenovirus (87). The linear, single stranded DNA genomes of parvovirus makes these agents unique among

the virus superfamily (198, 273). Today the family parvoviridae regroups a in large still increasing number of physically similar viruses, which replicate in the nuclei of multiple species ranging from insects to men. Just recently three new members have been characterised, the mouse parvovirus, rat parvovirus and hamster parvovirus (32, 40, 139, 184). The last international committee for parvovirus classification divided the family of parvoviridae into two sub-families on the basis of their natural hosts (198). Parvovirinae infect vertebrates, whereas Densovirinae affect invertebrates. These sub-families are further divided in genera which are based on structural and biological characteristics. This classification discriminates between the requirements for helper viruses, the preferential encapsidation of minus or plus strand DNA, and on the presence of identical or different palindromic sequences at both ends of the viral genome (Table 1). The characteristics of the individual genera of the two parvovirus subfamilies are summarised as follows:

-The genus Autonomous Parvovirus, such as the prototype strain of Minute Virus of Mice (MVMp), causes characteristic cytopatic effects to cultured cells during replication. Most strains also have the capacity to hemagglutinate red cells from at least one species. Their viral genome encodes proteins expressed from two distinct promoters.

-The genus Erythrovirus consists of two members, so far, B19 (human parvovirus) and SPV (simian parvovirus). These viruses exclusively replicate in erythrocyte progenitors cells. Transcription of the mRNA of these viruses is controlled by a single promoter.

-The genus Dependovirus is characterised best by AAV2 (Adeno-Associated Virus 2). These viruses have three promoters. In addition, for efficient propagation, they depend on a helper virus, like adenovirus or herpes virus. Under some special conditions, using carcinogenic agents, viral replication is observed in absence of a helper virus.

The subfamily Densovirinae consists also of three genera, Densovirus, Iteravirus, and Contravirus, which infect arthropods. These viruses replicate efficiently in the majority of the tissues in their host species ranging from larvae, nymph to imago. There is no need for helper viruses. They are able to induce cellular alterations, such as the hypertrophy of the nucleus during virion accumulation, manifested by formation of an intranuclear mass which is voluminous and dense.

The reminder of the introduction will be focused on autonomous parvoviruses, since the studies presented here concern the autonomous parvovirus MVMp.

Table1: Family of Parvoviridae

Parvovirinae(sub-family) Natural host: vertebrate

Autonomous parvovirus (genus)

Acronym	Full name (species)	Host	Characteristics
RV	Rat virus	Rat	
H-1	H-1 virus	Rat	
RT	RT virus	Rat	
RPV	Rat parvovirus	Rat	
MPV	Mouse parvovirus	Mouse	
MVM	Minute Virus of Mouse	Mouse	-independent of a helper virus for replication
LuIII	LuIII virus	unknown	
TVX	Tumour virus	unknown	
HaPV	Hamster parvovirus	Hamster	
PPV	Porcine parvovirus	Pig	
BPV	Bovine parvovirus	Bovine	-different palindromic sequences
FPV	Feline parvovirus	Feline	
MEV	Mink enteritis virus	Mink	
CPV	Canine parvovirus	Dog	
RPV	Racoon parvovirus	Racoon	
LPV	Lapine parvovirus	Lapin	
ADV	Aleutian Disease virus	Mink	
GPV	Goose parvovirus	Goose	

Erythrovirus

Acronym	Full name	Host	Characteristics
B19	B19 virus	Human	-Independent of a helper virus for replication
SPV	Simian parvovirus	Simian	-Identical palindromic sequences

Dependovirus or AAV

Acronym	Full name	Host	Characteristics
AAV-1	adeno-associated virus type1	singe	-dependent on helper viruses for replication
AAV-2	adeno-associated virus type2	human	
AAV-3	adeno-associated virus type3	human	
AAV-4	adeno-associated virus type4	singe	
AAV-5	adeno-associated virus type5	human	-palindromic sequences are identical
AAV-X7	bovine adeno-associated virus	bovine	
AAAV	avian adeno-associated virus	avian	
CAAV	canine adeno-associated virus	canine	
OAAV	ovine adeno-associated virus	ovine	

Densovirinae natural host: invertebrates

Densovirus

Acronym	Full name	Host	Characteristics
GmDNV	Galleria mellonela Densovirus	Galleria mellonella	-independent of a helper virus for replication
Jc DNV	Junonia coenia Densovirus	Junonia coenia	

Iteravirus

Acronym	Full name	Host	Characteristics
BmDNV	Bombix mori Densovirus	Bombix mori	-independent of helper viruses for replication

Contravirus

Acronym	Full name	Host	Characteristics
AaDNV	Aedes aegypti Densovirus	Aedes aegypti	-independent of helper viruses for replication
AaPV	Aedes albopictus parvovirus	Aedes albopictus	

I-2) Parvovirus characteristics

a) Virus/Host relationship

The impact of autonomous parvovirus infections extends from lethal disease to clinically inapparent manifestations (12, 39, 138, 169, 192). In this respect, it is of interest that the clinical symptoms observed after MVM infections are usually inapparent in adults and infants, whereas neonatal mice of some inbred strains are highly susceptible to lethal renal and/or intestinal haemorrhage, when infected experimentally with MVMi (55). The three main biological responses observed upon autonomous parvovirus infection are their teratogenic and abortive effects and their oncosupresssiv properties.

Autonomous parvovirus have been shown to be teratogenic agents, since they cause foetal and neonatal abnormalities (146). These manifestation are due to the destruction of specific cell populations characteristically proliferating rapidly during the normal course of the development, and therefore, are susceptible to parvovirus infection. In the mature animal, however, these cell populations are either resistant or replaced. Consequently, few of the viruses cause clinical disease in adults. For instance, intracerebral inoculation of neonatal animals with some parvoviruses cause runting and a characteristic « mongoloid-like » deformation (156, 265). The cranofacial and periodontal alterations appear to be due to selective viral attacks on developing skeletal and dental tissues (117). In addition, foetal infections lead, when occurring early in gestation, to a devastating attack of the mesodermal tissue of the embryo and hence result in resumption or mummification (182). At later stages of gestations intracerebral parvovirus infection cause cerebellar hypoplasia, often leading to chronic ataxia (157). This is also explained though viral depletion of a rapidly proliferating cell population, in this case cells of the cerebellar granular cortex (157, 182). Indeed, the involvement of cell populations with high mitotic activity at or subsequent to the time of infection is a consistent feature of the reported diseases (146). Several factors could influence the diseases observed after infection of foetus and explain the resistance of adult animals. The most intriguing of them is the absolute requirement of cells to enter S-phase (178, 213, 278).

Autonomous parvovirus infections have also been shown to suppress tumour formation caused by various viruses and carcinogens *in vivo*. For instance, RV was able to suppress leukaemia induction by Moloney leukaemia virus in rats (37). In addition, hamsters which had been infected with parvoviruses prior to adenovirus (266) or dimethylbenzanthracene (267) were found to become remarkably resistant to tumour induction by the latter treatments. Moreover in *nude* and *skin* mice, tumour formation from transplanted human neoplastic cells was impaired, and the size of pre-existing tumours was reduced upon parvovirus infection (108, 114).

All of these experiments indicate that parvovirus depend on rapidly dividing cells, including tumour tissues. However, although cell cycling is essential for parvovirus propagation, it is not sufficient to allow a lytic parvovirus infection. This is best exemplified by the resistance of murine embryonal carcinoma cells to MVMp infection, which become susceptible only when differentiation is induced *in vitro* (256), indicating that the differentiated state of the host cell is of paramount importance. The mechanism of parvoviruses to interfere with tumour formation has been studied extensively in cell culture and is most likely due to the cytostatic and cytotoxic functions of the viral proteins in neoplastically transformed cells.

The first evidence that parvoviruses are lytic, preferentially for transformed cells compared with their normal counterparts (termed oncolytic properties) was presented in the work of Mousset and Rommelaere (197), describing the isolation of a BALB/c 3T3 mouse fibroblaste variant, which was resistant to MVMp cell killing. These cells were able to support a productive lytic infection

with MVMp after transformation by SV40. Several investigations thereafter using different transforming agents (275) confirmed the oncolytic properties of parvoviruses. The main viral component governing this oncolytic properties was attributed to the major non-structural protein NS1 (16, 58, 196, 244), but it is increased in the presence of the small non-structural proteins NS2. The molecular mechanisms of NS1 cytotoxicity are still obscure, despite extensive research using virus infections and inducible cell lines expressing parvoviral non-structural proteins. One property leading to cell killing consists in the capability of NS1 to trans-regulate heterologous viral and cellular promoters (16, 167, 274), and it seems that cytotoxicity and trans-regulation of NS1 are contained within the same domains of the polypeptide (167). Another report presents the capacity of the non-structural protein to induce nicks into the chromosomal DNA (212) and, just recently it has been shown that parvoviruses are able to induce apoptosis (227).

A first clear indication that parvoviruses are able to induce apoptosis was obtained with B19 infection, causing morphologic changes such as nucleolar degeneration, extreme margination of the nuclear heterochromatin, and cytoplasmic vacuolisation. These features are typical of cells undergoing programmed cell death or apoptosis (193). Recently, it has been reported that B19 NS1 causes DNA fragmentation (190). Further analyses have indicated that initiation of apoptosis by NS1 occurs through induction of caspase3, but not caspase1 activation. There is no evidence that NS1-induced apoptosis is mediated by the Fas-Fas ligand pathway. Bcl2 is able to block NS1-induced apoptosis. Interestingly, there was an early report about intracellular changes caused by the autonomous parvovirus MVMp, which could be interpreted as induction of apoptosis (232), even before the recognition of apoptosis as a general phenomenon of cell death. Electron microscopic examination of synchronised RT7 cells infected with MVM revealed that with the onset of capsid assembly (around 20H after mitotic arrest, corresponding to the end of the end phase), condensation of the granular and fibrous elements of the nucleolus gave this organelle a more compact and dense appearance. In addition to the changes within the nucleolus, the nucleus became spotted with patches of condensed chromatin, and the band of heterochromatin at the periphery of the nucleus appeared denser and began to thicken. As the infection progressed, the nuclear changes became more pronounced. Within further 4h, condensation of the euchromatin became apparent and at 38h after mitotic arrest, the cells begun to lyse (232). Similar ultrastructural condensation of the nucleus were seen for BPV-infected cells as well. Recently it has been shown directly that autonomous parvovirus H-1 is able to induce apoptosis through its major non-structural protein NS1. H-1 NS1-mediated induction of apoptosis acts through the activation of caspase 3 and is accompanied with a drastic drop in the concentration of myc protein. In connection to this mechanism, cleavage of the PARP enzyme has been observed (227). In mouse fibroblasts, induction of MVMp NS1, however, did not reveal any criteria for apoptosis. Instead different mechanisms might be a major cause of cell death, as discussed above (212).

b) Parvovirus life cycle

As illustrated in Fig.1, productive infection starts with binding of the infectious particle to a specific cell surface receptor(s) of which mouse A9 cells (L-cell derivatives) have around 5.10^5 binding sites per cell(171). The virus enters the cells by a two step process. In a first and rapid phase which is sensitive to EDTA and lasts less than 30 min, the virus interacts with its receptor(172). In the second phase, which is slower (2h) and which is characterised by the resistance of infectious particle to neutralising antibody, the virus is internalised(172). For B19, an erythrovirus, globoside (the blood group P antigen) has been identified as the receptor, which is found to be present on the surface of only a few cell types (such as erythroid progenitor cells) (53). Uptake of the virus is achieved by endocytosis, targeted to late endosomes through a microtubule-dependent delivery and further transported to the nucleus(276). In MVM, two amino acid residues within the capsid appear to be of crucial importance for this process, and to determine the host range of the fibrotropic MVMp versus lymphotropic MVMi viruses(71). Since transfection of infectious clone DNA leads to a productive infection in the restrictive host cell, and virus entry into the cell seems not to be affected, it has been suggested that a host range determinant plays a role in nuclear translocation or decapsidation of the viral genome. In the nucleus, the single-strand genome is converted to a double-strand template. It has been proposed that the single strand DNA could interact with nucleolin at the so-called NUBE site, which is located within the non-structural transcription unit(34). This interaction could play a role in the control of the conversion step of MVMp DNA replication (27, 34). Unlike infection and nuclear translocation of the infectious particles, conversion of the single-strand genome to a double-strand replication intermediate requires the cell to enter S-phase (35). Using an *in vitro* system, this conversion reaction which is readily achieved in cell extracts from S-phase cells, does not occur in extracts from G1 cells, unless cyclin A is supplemented. After viral conversion, transcription and translation results in the production of NS proteins, which are then able to up-regulate the amplification of replicative forms and the synthesis of capsid proteins with a temporal co-ordination (191, 242). The production of viral genomes occurs a short time after the burst of capsid protein synthesis. The capsid proteins VP1 and VP2 are able to self-associate in the cytoplasm before entering the nucleus and assembling into capsid-like structures(175). The viral DNA is packaged into capsids concomitantly to the replication. Therein, an interaction of VP1 with the left-end terminus of the viral DNA could play a role during encapsidation of the genome (277). At this time, the 1,46g/cm^3 virus particles mature to a 1,42g/cm^3 species by proteolytic cleavage of a variable amount of VP2 into the third structural polypeptide, VP3(232, 257). Interestingly, while only VP2 is essential for encapsidation of the viral genome, VP1 seems to play a crucial role to produce infectious progeny particles(271). At the end of the virus cycle, the cells (which are blocked in late S-phase), are lysed, and the mature virions are released to start a new cycle.

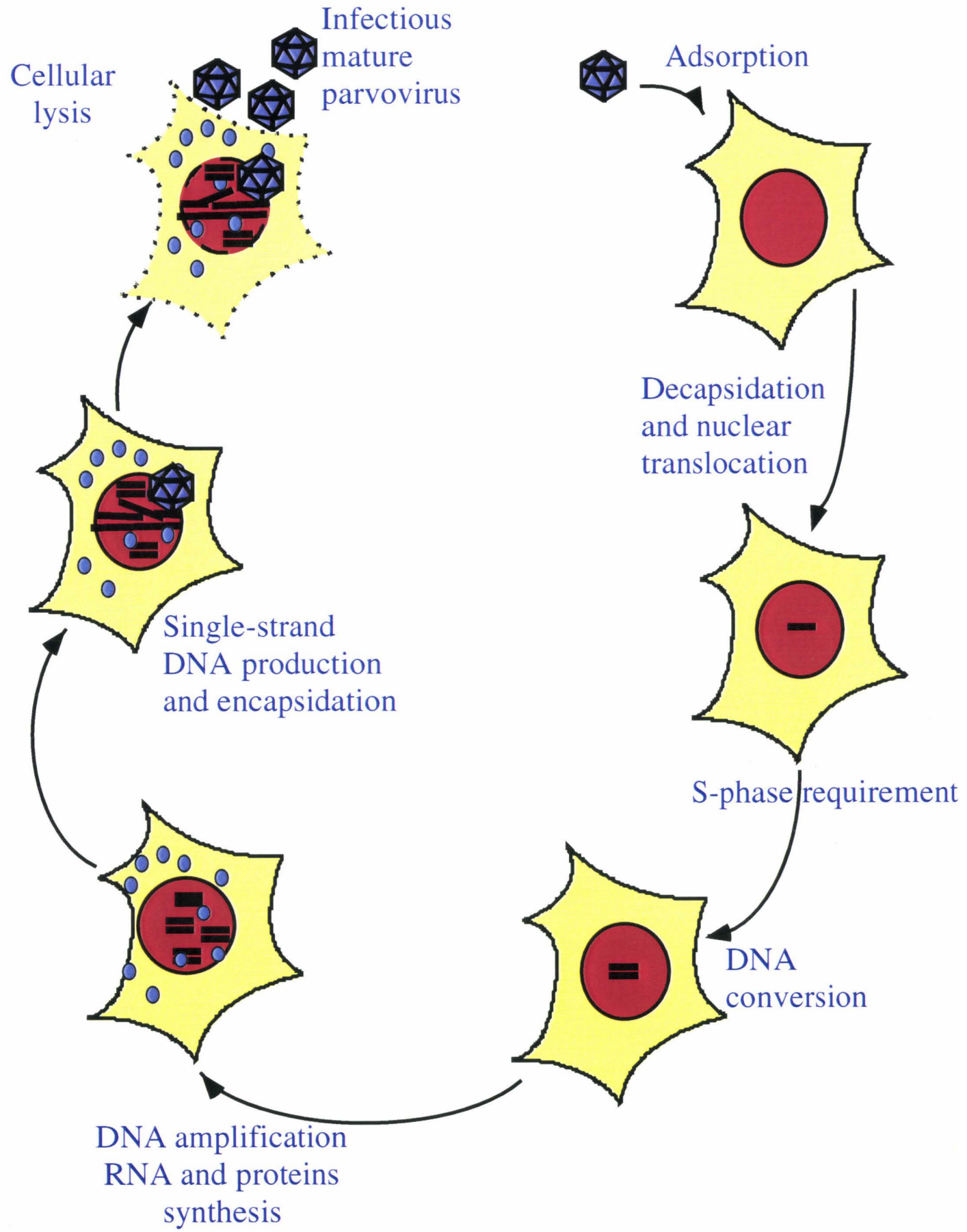

Fig. 1: Parvovirus life cycle.

—, ═ :viral DNA ● :viral proteins ⬢ :capsid

c) Genome organisation

The rodent parvovirus genome is a single-strand DNA molecule of approximately 5 kb. At either end of the viral DNA their are complementary structures which are able to form hairpin duplexes (see Fig.2) (50)A. The terminal palindromes of MVM contain the viral origins of replication. The left-end hairpin structure of virion DNA comprises 115 nucleotides that can base-pair to form a stable Y-shaped structure (23), while the right-end hairpin contains 207 nucleotides (24). The remaining part of the DNA between these two terminal structures consists of coding sequence. The right-end terminus of MVM DNA has been found in two alternative sequence orientations, termed "flip" and "flop", according to the sequence of a mismatched bubble (Fig.2). Both orientations occur with equal frequency in the populations of virion single-strand DNA (20, 25). In contrast, the left-end terminus occurs only in one orientation in virion DNA. Within the duplex stem, there is a mismatched bubble between nucleotides 25-26 and 88-91, which determines whether an origin of replication is recognised by NS1. Rodent parvoviruses generally package strands of one sense only, in a way that at least 99% of virions contain a molecule of the opposite sense with regard to RNA transcripts(198). This selective packaging makes rodent parvoviruses negative-strand DNA viruses(38).

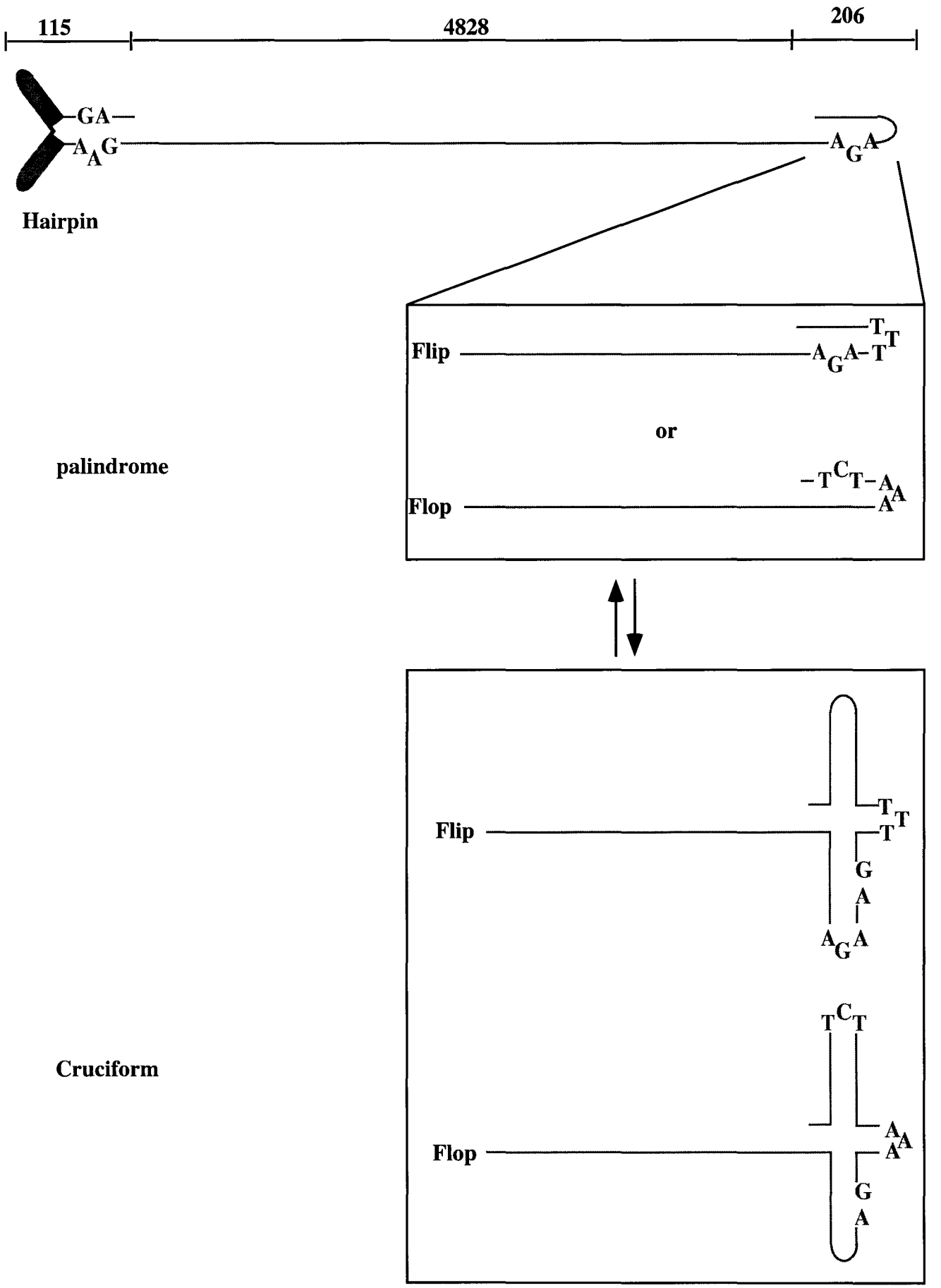

Fig.2 : Structure of the viral termini. (adapted from Cotmore and Tattersall, 1987)

By convention the genome is depicted with the 3' end of the negative-sense DNA molecule at the left (left-end) and the 5' end at the right (right-end) (Fig.3) (83). The entire genome contains two large blocks of open reading frames, separated by translational stop signals in all reading frames, located between map units 45 and 46(25). The main open reading frame in the right half of the genome encodes the capsid proteins, VP1 (83kDa) and VP2 (64kDa), with the entire sequence of VP2 located within the C-terminal three quarters of the VP1 sequence(74). These polypeptides are translated from the most abundant viral mRNA species R3, which is controlled by a promoter located at map unit 38 (P38). The third capsid protein, VP3 (62kDa) is a proteolytic product of VP2 produced during maturation (257). The block of open reading frame in the left half of the genome encodes for the non-structural proteins NS1 (83kDa) and NS2 (25kDa), which are translated from R1 and R2 mRNAs, respectively(144, 281). Both mRNAs, R1 and R2 are transcribed from a promoter at map unit 4 (P4). R2 is a spliced product of R1 lacking 30% of the genome sequence, located between map units 10 and 39(221). The excision of this large intron from pre-mRNA seems to be regulated in a virus-strain-specific manner (71). All three transcripts are spliced in the region between map units 44-46. For R2 this alternative splicing accounts for the production of three similar NS2 proteins, called NS2 major, NS2 minor and NS2 rare, divergent in their carboxy terminal part only (Fig. 3). All transcripts are processed and polyadenylated at the far right side of the genome using predominantly the most distal of four AATAAA signals (Clemens virology1987) (69).

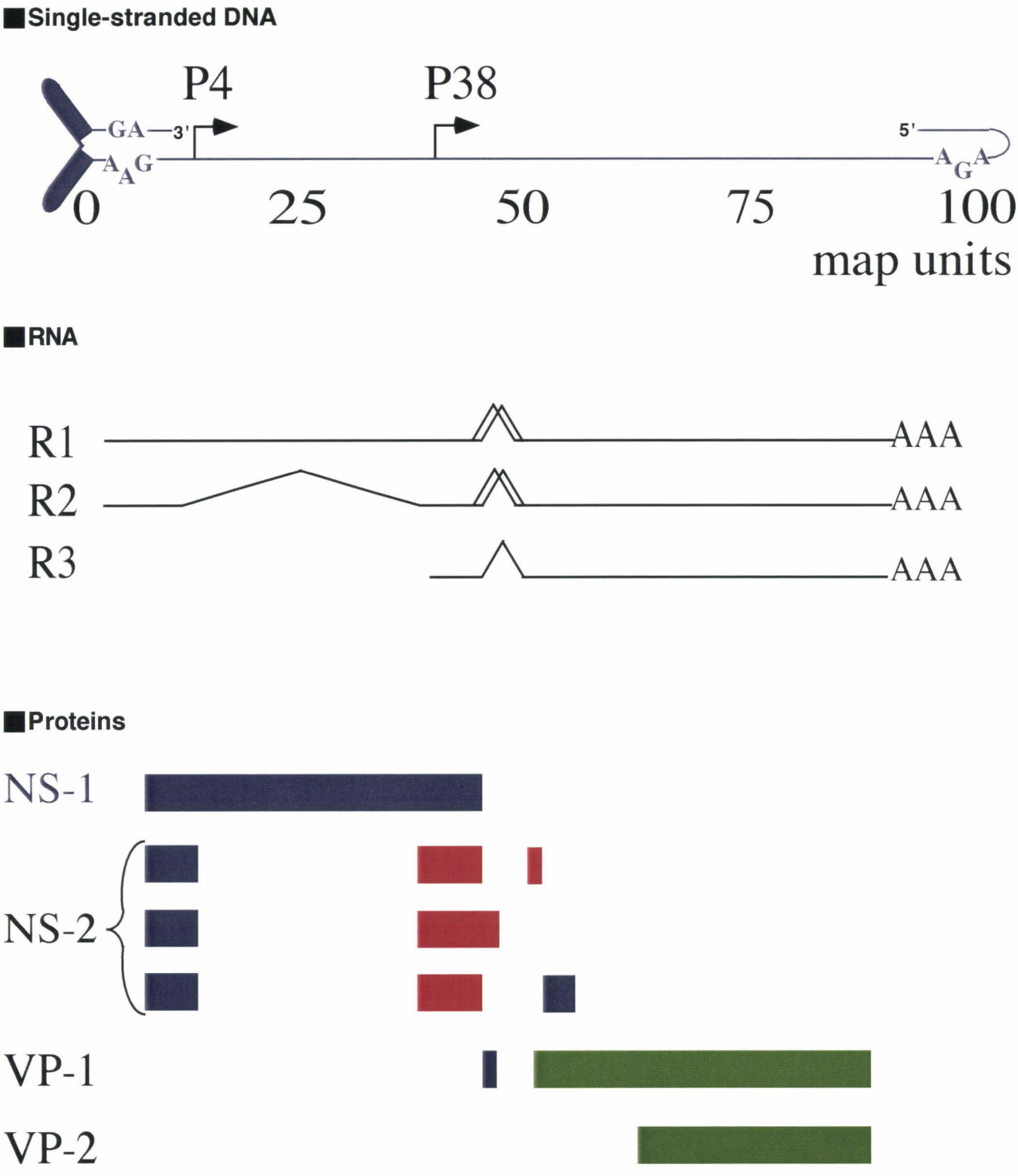

Fig.3:Genome organization of MVM. The different colors in the protein represent the alternative reading frames.

The P4 promoter

P4-driven gene expression is subjected to multiple forms of regulation(9, 98, 115, 126, 129). On one hand, the abundance of mRNAs programmed by this promoter is regulated at the level of their elongation. Indeed, the analysis of the primary and potential secondary structures of MVM DNA downstream from P4 led the prediction of the occurrence of a region of premature termination of nascent RNA chains located approximately 143 nucleotides from the transcription initiation site. The involvement of this region in the so-called attenuation of transcription was demonstrated both *in vitro* (36) and *in vivo* (228). On the other hand, positive and/or negative control of the P4 promoter is achieved at the level of transcription initiation. Therein several different transcription factors have been shown to influence the expression of the genes driven by this promoter (Fig. 4). A series of deletion and linker-scanning mutants within the MVM P4 promoter identified two region: a GC box and a TATA box, which are essential for transcription. They are localised between nucleotides 150 and 180 (-55 to -25 relative to the primary RNA start site) (9). The GC box was found to bind the transcription factor Sp1 with high affinity (222) but also SP3 (120). Additional upstream sequences localised between -139 and -55 were shown to be required for maximal transcription activity *in vivo*, and at least four distinct elements, interacting with cellular proteins, were identified (115). Transcription factors USF and NF-Y were shown to bind the enhancer E box CACATG and the CCAAC sequence, respectively. While binding of NF-Y up-modulates the promoter activity, USF binding does not appear to have a significant effect on P4 activity *in vivo* (126). Interestingly, the P4 promoter activity is stimulated in ras-transformed FREJ4 cells compared with the normal parental FR3T3 cell line. This higher transcriptional activity of the promoter in response to c-Ha-ras is mediated at least in part by two upstream CRE elements which are able to bind proteins from the ATF/CREB family. Regulation of this protein/DNA interaction involves the PKA pathway (219). In addition, another element, the Ets-binding-site (EBS) located upstream of the GC box, is involved in the response of promoter P4 to c-Ha-ras, and acts in synergism with the sp1 transcription factors (120). Finally the activity of the P4 promoter is also regulated in a cell cycle-dependent manner, showing a strong stimulation in S phase compared to the G0/G1 phase of the cell cycle. At the G1/S-phase transition, the promoter is activated through a cis-acting DNA element which interacts with cell cycle specific protein complexes involving transcription factors of the E2F family. Inhibition of the P4 promoter in cells arrested in G1 by contact inhibition, is mediated through cyclic AMP response elements (CREs) (98).

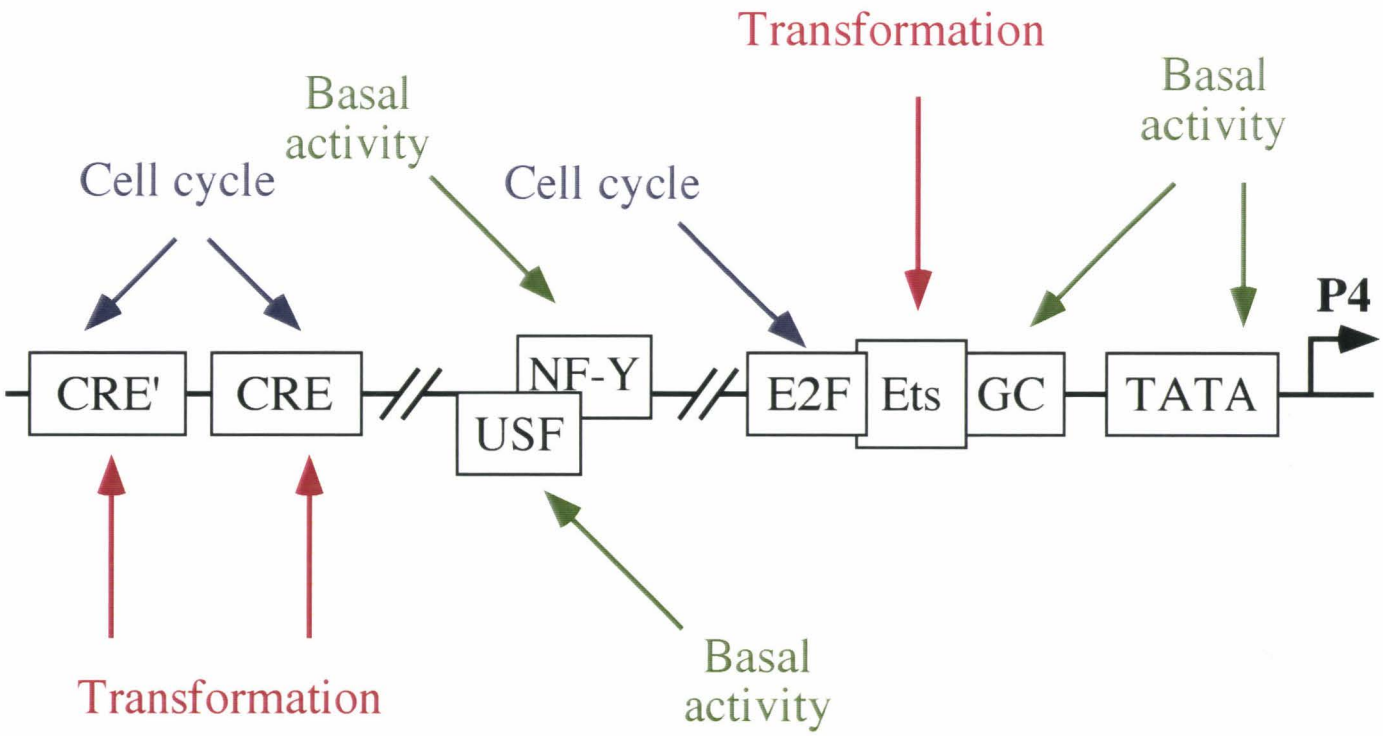

Fig.4: Regulation of the P4 promoter

P38 promoter

The P38 promoter is subject to regulation by transcription factor binding, mediated through a TATA element and a GC box which are essential to its basal activity (121). In absence of the activator parvoviral protein NS1, the P38 promoter activity is weak (230). Upon the presence of NS1, P38 is strongly induced, showing a biphasic response, depending on the NS1 concentration. Maximal trans-activation by NS1 requires a small 5' cis-acting element (tar), located between nucleotides -137 and -116 (10, 231). A comparison between the tar sequences of MVMp, CPV, ADV and H-1 virus gave a consensus AACCAA motif as the responsive cis-acting element for NS1 trans-activation (252). NS1 is able to bind to the tar region in an ATP-dependent manner. In analogy to binding studies using a similar motif located in the left-end origin, this interaction of NS1 with ATP, allows the polypeptide to oligomerize and to bind with high affinity to the $(ACCA)_{2-3}$ element. In the P38 promoter, NS1 binds a 43-bp sequence extending asymmetrically from the $(ACCA)_2$ sequence toward the TATA box of the (67). The trans-activation function of NS1 is assumed to act within its binding with SP1 and also with the general transcription factors TBP and TFIIA (alpha, beta) (176). A downstream promoter element (DPE), necessary for efficient transcription initiation from the P38 promoter was also identified. This element resides 282 to 647 base pairs down-stream of the transcription initiation site and was shown to bind NS1 either directly or indirectly (161). The same group described an additional downstream promoter element (between +95 and +129 from the P38 initiation start site), which inhibits, transcription from the P38 promoter *in vitro*, in the absence of the DPE.

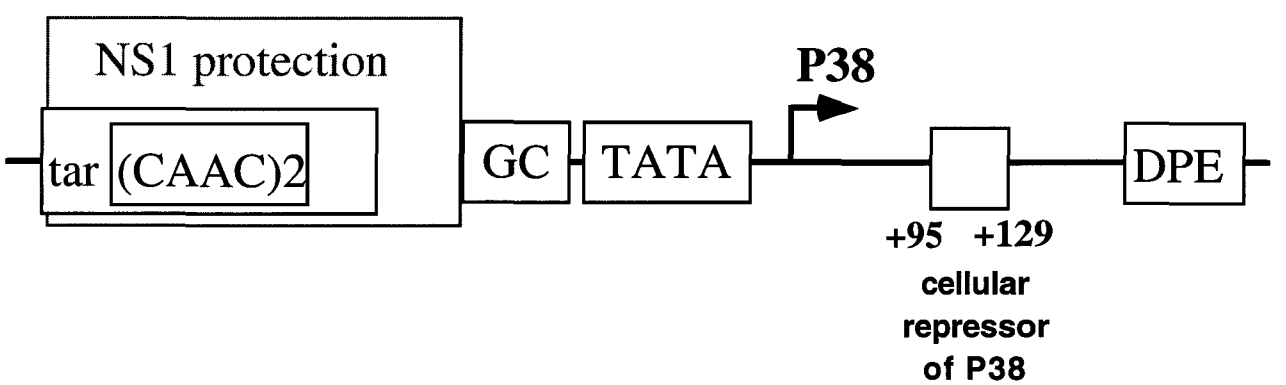

Fig.5: P38 promoter regulatory elements

d) Model of parvovirus DNA replication

In order to replicate their genome, parvoviruses have to convert their single-strand DNA genome into a double-strand transcription template. This double-strand DNA is then amplified and progeny virion DNA, i.e. the single strand genome produced and encapsidated into a protein shell, in order to form infectious particles. In a first step, the incoming particles deliver the single strand virion DNA into the cell and target it to the nucleus, where DNA replication takes place. Parvoviral DNA replication is unique among the animal viruses, since it resembles the unidirectional rolling circle replication mechanism described for bacteriophages or single-strand plasmids, rather than the bi-directional eucaryotic DNA (38). Replication of MVM DNA occurs in three phases (Fig. 6), namely the conversion of the single-strand genome to a covalently closed circle which can serve as a transcription template (steps 1 and 2), amplification of duplex replicative intermediates (steps 3-14), and the production and concomitant packaging of the progeny single-strand DNA genomes (steps 15 and 16). Figure 6 presents the rolling hairpin model that has been modified for MVM replication (84), based on previous proposals (24, 258). Synthesis of the complementary DNA strand to the covalently closed circular form (steps 1 and 2) is achieved solely by cellular components. The base-paired 3' hydroxyl of the left-end hairpin serves as a primer for elongation driven by cellular polymerases. At the right-end, meeting the 5' end of the palindrome, the 3' end of the nascent DNA strand is ligated with the 5' end of the virion DNA to form the covalently closed circular DNA form which serves as a template for transcription. These first steps (1 and 2) occur independently of NS1 proteins and are highly dependent on the cell to enter the S-phase (31, 83, 270). Recent developments using an *in vitro* replication system have shown that the presence of cyclin A could play a crucial role, since conversion of naturally incompetent G0/G1-extracts was achieved upon addition of recombinant cyclin A (35). For all further steps during viral DNA replication the viral non-structural NS1 is of crucial importance. This viral protein serves as the initiator protein for DNA replication through its site- and strand-specific nickase activity (steps 3,9 and 10), generating free 3' OH, which serve as primers for DNA polymerases. During these nicking reactions, NS1 remains covalently attached to the 5' ends of replicated DNA. In addition to initiation, NS1 is thought to unwind double-strand DNA templates in front of the replication machinery through its helicase function allowing the replication fork to proceed. NS1 is produced from transcription controlled by the viral P4 promoter using presumably the circular form DNA as an initial template.

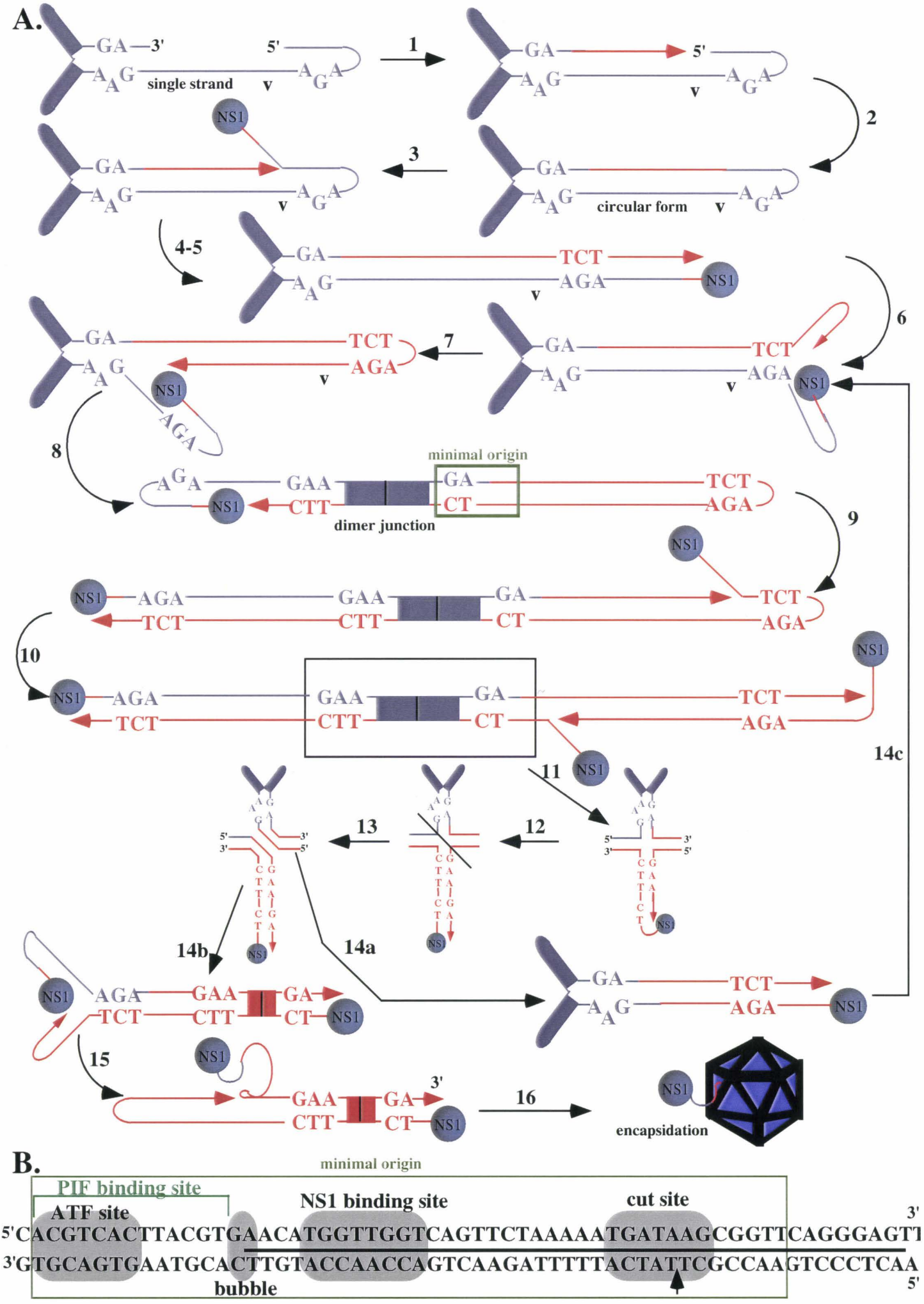

Fig.6. A: Replication model (adapted from Cotmore and Tattersall 1995).
B: Minimal left-end origin of replication.

Upon the presence of NS1, the covalently closed circular DNA form is nicked (step 3), leaving NS1 covalently attached to the 5' end of the nicked strand. The free 3' OH generated by this nicking process serves again as a primer for DNA synthesis producing a replication intermediate with a turnaround left-end and an extended right-end (step 4/5). For further synthesis, a rabbit-ear structure is formed (step 6) and strand-displacement synthesis (step 7) occurs in an unidirectional single-strand copy mechanism as seen for rolling circle replication of bacteriophage ΦX174 to generate concatemeric replication intermediates (steps 7,8). This formation of head-to-head dimers is important to conserve the virion orientation in the left-end telomere (24). The requirement of DNA polymerases for a primer and the 5' to 3' orientation of strand elongation during DNA-synthesis cause problems to reproduce the tail ends of a linear double-strand DNA such as the chromosomal ends or in this case the replication intermediates. The copy-mechanism of the right-end telomere (steps 3-5), which is found in both flip and flop orientations (see Fig.5), can be explained by a simple hairpin-transfer mechanism as proposed by Cavalier-Smith (59)for replication of chromosomal DNA. For this reaction NS1 nicks the right-end hairpin (step 3) within the origin of replication and the free 3' OH is elongated, copying the palindromic sequence across the axis of symmetry (steps 4-5). Recently it has been shown that for this right-end nicking reaction performed by NS1, an additional accessory protein, belonging to the high mobility group proteins (HMG) is required (75). Alternatively to this hairpin transfer mechanism, it is also possible that replication of the right-end telomere starts from a tail-to-tail concatemeric intermediate. This allows the formation of a Holliday structure which has been found to be nicked *in vitro* and *in vivo* by NS1, across the axis of symmetry with similar efficiencies at both origins of replication (80, 85). These naturally occurring concatemers are resolved in this way generating monomeric replicative intermediates. Unlike the right-end telomere, the left-end of the virion has been found in a single orientation only (termed flip). In order to preserve this orientation, Astell and co-workers(20) proposed that replication has to occur through a dimer-intermediate, which unlike the right-end tail-to-tail telomere-bridge, is resolved asymmetrically in order to use the complementary strand as sole replication template (steps 11-14). Detailed *in vivo* and *in vitro* studies using plasmids containing the left-end bridge or fragments thereof have confirmed the asymmetry of the resolution reaction (79, 85). The reason for this asymmetry during resolution and replication reactions at the left-end bridge was found through analyses of the minimal origin(s) of replication located within the left-end junction. These analyses revealed that some 40 nucleotides, within the stem region of the palindrome between an ATF binding site and the cut-site (see Fig.6B), serve as an origin for rolling circle replication through NS1 when cloned into circular plasmids (82). Interestingly, only the origin containing the 5'-GA-3' " bubble sequence " in the junction serves as a substrate for nicking by NS1, the 5'-GAA-3' counterpart across the axis of symmetry is silent (see products of the steps 9-10) (68). The nicking reaction driven by NS1 at the left-end origin is achieved in the presence of

a novel cellular protein called PIF (Parvovirus Initiation Factor), which binds to an ACGT-repeat located at the ATF-binding site. Since the number of nucleotides in the "bubble " region (Fig.6B) rather than the sequence content decides over activity of the origins, it is likely that the "bubble " reflects an important spacing element between PIF and NS1 binding sites. Since initiation at the active (GA) origin in the linear configuration of the left-end bridge would lead to replication of the viral palindrome rather than the complementary one, Cotmore and Tattersall(84) proposed the formation of cruciform-structure of the palindrome which allows to use the complementary strand to be copied into virion DNA and consecutive resolution of the concatemer (steps 11-14). This resolution reaction (step 14) allows both the production of single strand virion DNA (steps 14b-16) and the production of a replication intermediate that re-enters the replication cycle (steps 14a and 14c). The mechanisms for production of single-strand progeny virion DNA and the concomitant packaging (steps 15 and 16) of the genomes remains obscure. During this reaction, the terminal 24 bases to which NS1 is attached covalently at the 5' end extend outside of the capsid and can be subsequently cleaved off (78). These terminal nucleotides as well as the attached NS1 polypeptide are not essential for infectivity of the virus particles.

e) Properties of the non-structural proteins

The left part of the viral genome encodes for the non-structural proteins of MVM, the 83 kDa nuclear phosphoprotein NS1, and in addition, the three minor 25kDa non-structural proteins NS2 which differ from each other in their unique C-terminus only. Whereas the small non-structural proteins are dispensable in certain cell culture systems, the multifunctional NS1 plays a key-role during virus propagation. MVMp NS1 is a single polypeptide chain of 672 amino acids that regulates numerous aspects of the viral life cycle (Fig.7).

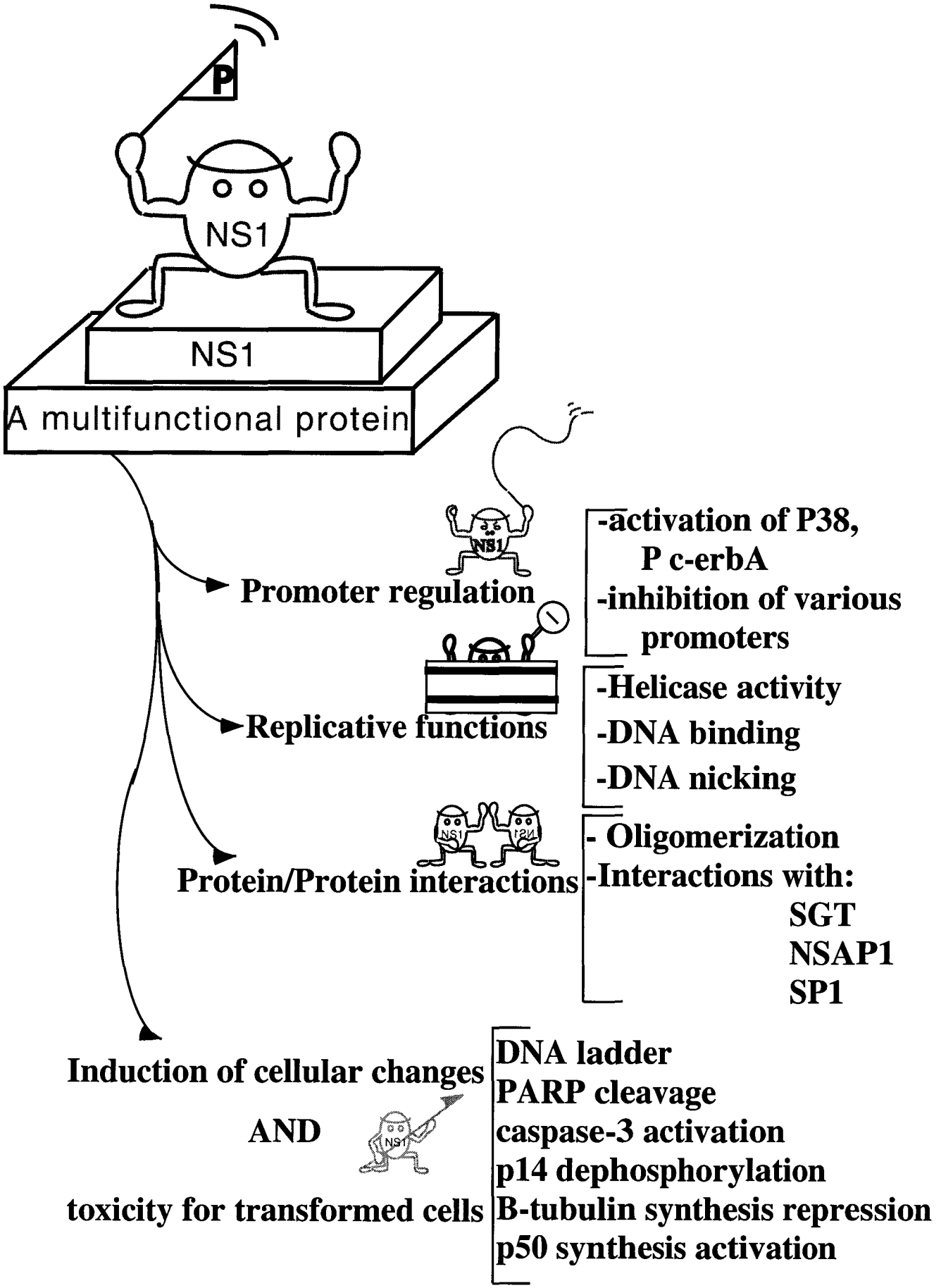

Fig.7: NS1 is a multifunctional protein.

NS1 is required for viral DNA replication, modulation of a variety of promoters including trans-activation of the p38 promoter driving capsid gene expression, and MVM-induced cytotoxicity (229). For all these activities, NS1 exerts a variety of biochemical functions, including the binding and hydrolysis of ATP. This co-factor interaction is of crucial importance since many, if not all of the NS1 functions are controlled by its ATP metabolism. This has been substantiated by mutagenesis of the NTP binding site located around lysine 405 within the NS1 polypeptide, which lead to a loss of NS1 functions ranging from DNA replication to promoter regulation and toxic activity. Most of the NS1 biochemical activities identified so far are involved in viral DNA replication. NS1 is able to bind site-specifically to (ACCA) repeats that are found through the whole viral genome, including the origins of replication (76, 165). This DNA binding is enhanced when ATP is bound to NS1, and decreases under conditions that favour hydrolysis of the nucleotide (67). Analyses of NS1 interaction with the left-end origin of replication, reveal NS1 as protecting a stretch of 43 nucleotides on both DNA strands, ranging from the middle of the bubble sequence to a position some 14 bp beyond the nick site (Fig. 6B). The size of the footprint and the requirement for ATP binding lead to the assumption that NS1 interacts with its cognate DNA recognition motif as a multimer. This ability of NS1 to oligomerize has been shown through the co-transport of a cytoplasmic mutant NS1 derivative by nuclear NS1 (209). For this self-assembly, an intact NTP binding domain of NS1 is essential. Using series of peptides corresponding to the NS1 sequence, the interaction domain of NS1 was located between amino acid 261 and 280 (Fig. 8). This domain is composed of a bipartite interaction motif which has a putative double β-strand structure (224). The functional relevance of this domain has been shown by inhibition of NS1 helicase function upon block of this interaction domain with the corresponding peptide.

In order to produce the free 3' OH which serves as primer for DNA replication, NS1 has site and strand specific endonuclease activity. For this activity, besides an intact NTP binding domain, a putative metal co-ordination site (H129) and an active site tyrosine (Y210, to which the 5' end of the nicked DNA strand becomes covalently attached) are essential (Fig.8) (208). The majority of the NS1 forms found in infected cell are not covalently attached to replicated viral DNA (86). Replication of parvoviral DNA takes place in the nuclear compartment of the cell. NS1 nuclear targeting is mediated by a bipartite lysine-rich nuclear localisation signal located around amino acid 200 ($K^{194}K(X)_{18}KKK^{216}$) (209). Mutation of the triple lysine motif within this NLS, produced a cytoplasmic NS1 mutant. The ability of nuclear NS1 to transport NLS^- mutants into the nucleus suggests that NS1 is able to oligomerize prior to nuclear translocation (209). Besides its replicative functions, NS1 also possesses the capacity to modulate promoters. Most importantly, it is able to trans-activate the late parvoviral P38 promoter driving the capsid genes (231).

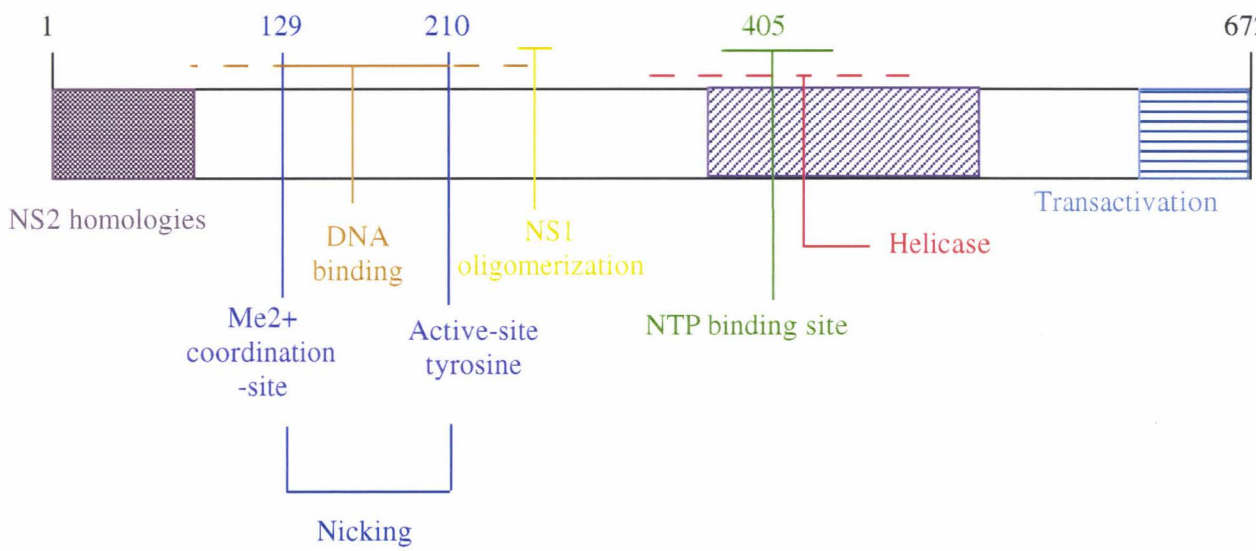

Fig. 8: NS1 domains

This trans-activation requires the binding of NS1 to the tar region upstream of the GC box within the P38 promoter and an interaction with SP1. A direct interaction with the general transcription factors TBP and TFIIA (alpha, beta) might also be of importance (176). An intact NTP-binding site is also essential for this function most likely for positioning the polypeptide correctly to serve as a transcription factor. The main domain of NS1 involved in this trans-activation process is localised within the carboxy-terminal part of NS-1 (166). Positioned correctly by an artificial lexA peptide/DNA interaction, this C-terminal domain is sufficient for transactivation. Apparently through the same domain, NS1 also trans-activates the cellular c-erbA1 promoter, for which however the mechanism is different. This trans-activation does not involve binding of NS1 to the promoter region, but instead requires the presence of a sequence motif TCAAGGTCA, that constitutes a putative nuclear receptor-binding site (274). Moreover, this sequence is necessary and sufficient for this second NS1 trans-activation mechanism.

Among the various cytotoxic and cytostatic effects of NS1 to the host cell, this activation of the c-erbA1 promoter deserves certainly further attention. It has to be mentioned in this respect that NS1 is also able to trans-inhibit a variety of cellular and heterologous viral promoters which could have dramatic consequences to the cell. A variety of other cytotoxic functions have been found for NS1, which will most likely be studied in further investigations. Among these functions, NS1 is able to trans-inhibit the replication controlled by heterologous replicons through a 157-bp sequence (262), it interferes with the cell cycle, arresting the cells in different phases (211). After infection, NS1 mostly blocks the cell in late S-phase and induces nicks into the cellular chromatin. This finding could explain the ability of NS1 to interfere with cellular DNA replication (212). Interestingly, cells having a high DNA double stand repair capacity may be protected against the parvovirus lytic effect (259). NS1 also induces cellular changes characteristic for cell death by apoptosis, as mentioned previously.

Little is known about the functions of NS2. NS2 is able to enhance the toxicity exerted by NS1 in transformed cells. The molecular mechanism for this function, is not defined yet. Experiments with truncated or mutant forms of NS2 suggested a role of NS2 in a host cell dependent manner. In human cell lines MVMp mutants defective for NS2 achieve a full lytic cycle. Yet, in certain cells, in particular of mouse origins, NS2 is essential for efficient DNA replication and completion of a full lytic cycle (200). In this respect, NS2 was reported to be required for efficient translation of viral mRNA and, in particular, for capsid formation (201). A role for NS2 was also observed in animals. MVMi infection of DBA/2 mice leads to intestinal haemorrhages and accelerates involution of hepatic hematopoiesis. In contrast, the NS2-defective virus is apathogen for animals (56), which was assigned to the requirements of NS2 for effective capsid production (77). It should also be noted that NS2 associates with specific isoforms of the 14-3-3 class of cellular proteins (52)as well as with the nuclear export protein crm1 (45). The function of these interactions however, are not determined yet.

I-3) Phosphorylation of the viral proteins.

a) The capsid proteins.

PPV capsid proteins VP-1 and VP-2 contain substantial amounts of ^{32}P after metabolic labelling using orthophosphate. All phosphorylation events occur on serine residues and to low level on threonine residues to all capsid proteins (191). The phosphoproteins can be resolved by two-dimensional gel electrophoresis into two to four distinct species, which differ in their isoelectric point by 0.05 pH units. In MVMp-infected A9 cells, VP1 and VP2 proteins are post-translationally modified before assembly. Phosphorylation occurs mainly on serine but also on threonine residues (236). When analysed by two-dimensional gel electrophoresis VP2 is migrating as 12 subtypes; assembly of the capsid does not involve (a) major VP2 subtype(s). A rearrangement in VP2 composition is imposed by DNA encapsidation, where 6 VP2 subtypes act as precursors for VP3. Five to seven VP3 isoforms are present in the mature virion (236). By tryptic peptides analysis, the main phospho-peptide in VP2 was identified as containing phosphoserine. This peptide is absent from VP3. This finding explains the decrease of ^{32}P labelling during virus maturation (183). Moreover, during capsid formation, VP proteins appear to become dephosphorylated (191).

b) The non-structural proteins NS1 and NS2.

NS1 detected by Western blots resolves in multiple species. NS1 is synthesised as one single species, that is gradually converted into a variety of slower migrating forms, most likely as a result of post-translational modifications (191). PPV NS1 is a nuclear phosphoprotein that is phosphorylated on serine and threonine residues (191). H-1 virus NS1 is also present in at least two species that can be resolved in SDS gels as faster migrating (hypophosphorylated) and slower migrating (hyperphosphorylated) species of 84kDa and 92kDa apparent molecular weight, respectively. The phosphorylation of the 84kDa species appeared to be uniformly distributed, while the hyperphosphosphorylation of the 92kDa species occurs on serine and threonine residues from a defined region of the polypeptide (215). Chemical inhibition (aphidicolin or cytosine arabinoside) of DNA replication *in vivo* was found to inhibit the conversion of the hypo- to the hyper-phosphorylated form of NS1, leading to the proposal of the implication of NS1 phosphorylation in viral replication (215). This hypothesis is attenuated due to a temperature sensitive mutant of H-1, ts2 (defective in progeny DNA synthesis at the restrictive temperature), which produce similar NS1 as wild-type does (Paradiso J.virol1984). Underphosphorylated or non-phosphorylated MVMp NS1 is migrating in one dimensional electrophoresis as a sharp band with an apparent molecular weight of 83kDa, whereas phosphorylated NS1 is migrating as a more diffuse 84-85 kDa species (86). Accordingly, ^{35}S

labelled NS1 is resolved by two-dimensional electrophoresis in the form of a continuous spot extending from pI values of 7.2 to 7.8. This heterogeneity in the electrofocusing pattern can probably be assigned to one or more other kinds of substitutents besides phosphorylation. Indeed, the corresponding ^{32}P-labelling shows that phosphorylated NS1 is present as a major heterogeneous component at neutral pI (236). NS1 is more extensively phosphorylated than NS2, VP1 or VP2 (86). MVMp NS1 phosphorylation has been reported to occur on serine residues in MVM-infected mouse LA9 cells, and to resolves in more than 18 phosphotrypic peptides (21). Baculovirus produced NS1 can be phosphorylated by various kinases *in vitro,* including casein kinase II, cdc2 kinase, MAP kinase, protein kinase C, and cAMP-dependent protein kinase

NS2 proteins are found in the cytoplasm and in the nucleus; the phosphorylated species of NS2 are predominantly cytoplasmic, whereas the non-phosphorylated species are distributed in both the nucleus and the cytoplasm (81). The three isoforms of NS2 are phosphorylated with NS2-L being the major NS2 phosphoprotein (236).

II) Protein phosphorylation.

II-1) Role of phosphorylation in the cell.

The protein kinase family comprises hundreds of diverse but related enzymes that are involved in regulation of nearly all cellular processes in eucaryotic cells. (128). The importance of protein phosphorylation in the regulation of cellular processes is demonstrated by the finding that 30% of intracellular proteins are phosphoproteins (136). In the 1950s, the first specific protein kinase to be discovered and purified was the phosphorylase kinase (162). Since then a variety of protein kinases have been detected, and the number of new members of this protein family is still growing. It is speculated, that there are more than two thousand different protein kinases in an eucaryotic cell. Currently the total number of protein kinase sequences available is approaching 400 (130). Multiple sequence alignments have indicated that all protein kinases have similar structures. Conserved features have been identified in subdomain regions of all protein kinases, and essential roles in enzyme structure and function have been assigned to residues from these subdomains.

Phosphorylation is a reversible process (Fig. 9) (143).The forward and the backward reactions are catalysed by different enzymes that are regulated in response to different stimuli. Phosphorylation of a protein can alter its properties upon addition of a phosphate to the naturally occurring amino acids, since the phosphate group, which has four oxygen atoms attached is able to participate extensively in hydrogen-bond interactions which can link different parts of the polypeptide chain(62, 73, 132). In addition, phosphorylation also participates in destabilising a defined structure, and hence allows the structure to adopt different conformations, which determine the biochemical activity(ies) of the polypeptide(253). Protein phosphorylation and dephosphorylation control many processes triggered in response to selected stimuli. The disparate conformational changes of the protein resulting from phosphorylation allow the modulation of a multitude of biological events including transcriptional regulation, cell cycle control, protein trafficking and protein-protein interactions.

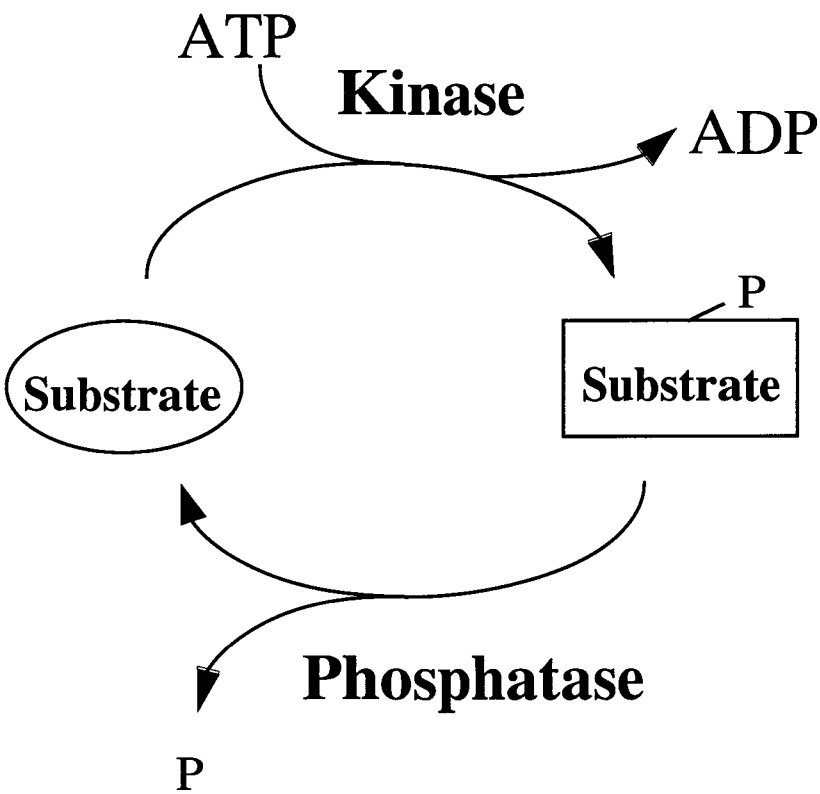

Fig. 9: Regulation by phosphorylation.

a) Transcriptional regulation by phosphorylation: The c-Jun paradigm.

The reversible phosphorylation of amino acids appears to be the most ubiquitous intracellular control mechanism to convey information from the cell surface to the nucleus where gene expression takes place. In the case of mitogen-stimulated genes, those with a DNA recognition sequences containing the heptameric motif TGA C/G TCA, are regulated through the AP1 complex. This complex consists of c-Jun homo-dimers, or alternatively hetero-dimers between c-jun and c-fos proto-oncogene products (88). c-Fos and c-Jun polypeptides are composed of several functionally independent domains (Fig. 10). The dimerization domain of c-Jun/c-Fos consists of a leucine zipper motif near the protein carboxy-terminus (14, 47). Leucine zippers are dimerization interfaces that consists of two parallel, α-helix structures. The heptad repeat of leucines participate in hydrophobic interactions among the parallel α-helices to mediate dimerization (1, 122, 226). The carboxy-terminal half of the protein encodes a basically charged DNA binding domain. This domain is able to make contact with the major groove of the pseudo-palindromic half of the DNA binding site. Upon dimerization of the polypeptides, this region is brought into close juxtaposition with the DNA (122). This binding of the heterodimer induces a DNA bending (153, 154). The amino-terminal part of the two proteins contains a trans-activation domain (15, 48, 248). For Fos, this domain encompasses two sub-domains, consisting of an acidic region and a proline rich region, whereas the c-Jun A1 region is strongly acidic (89).

For c-Jun, the DNA binding and trans-activation functions are each regulated by phosphorylation in a co-ordinated but independent manner (Fig 10). Regulation of c-Jun by phosphorylation occurs on four sites (Thr 231, Thr 239, Ser 243, Ser 249) localised in a segment preceding the DNA binding domain, and phosphorylation within this region inhibits DNA binding (51). In fact, the decrease of DNA binding after phosphorylation has been shown to be linked to a structural destabilisation of the leucine zipper α-helices (254). Moreover, regulation of c-Jun/Fos activity is regulated through a cascade of signals, including activation of PKC. This signalling pathway leads

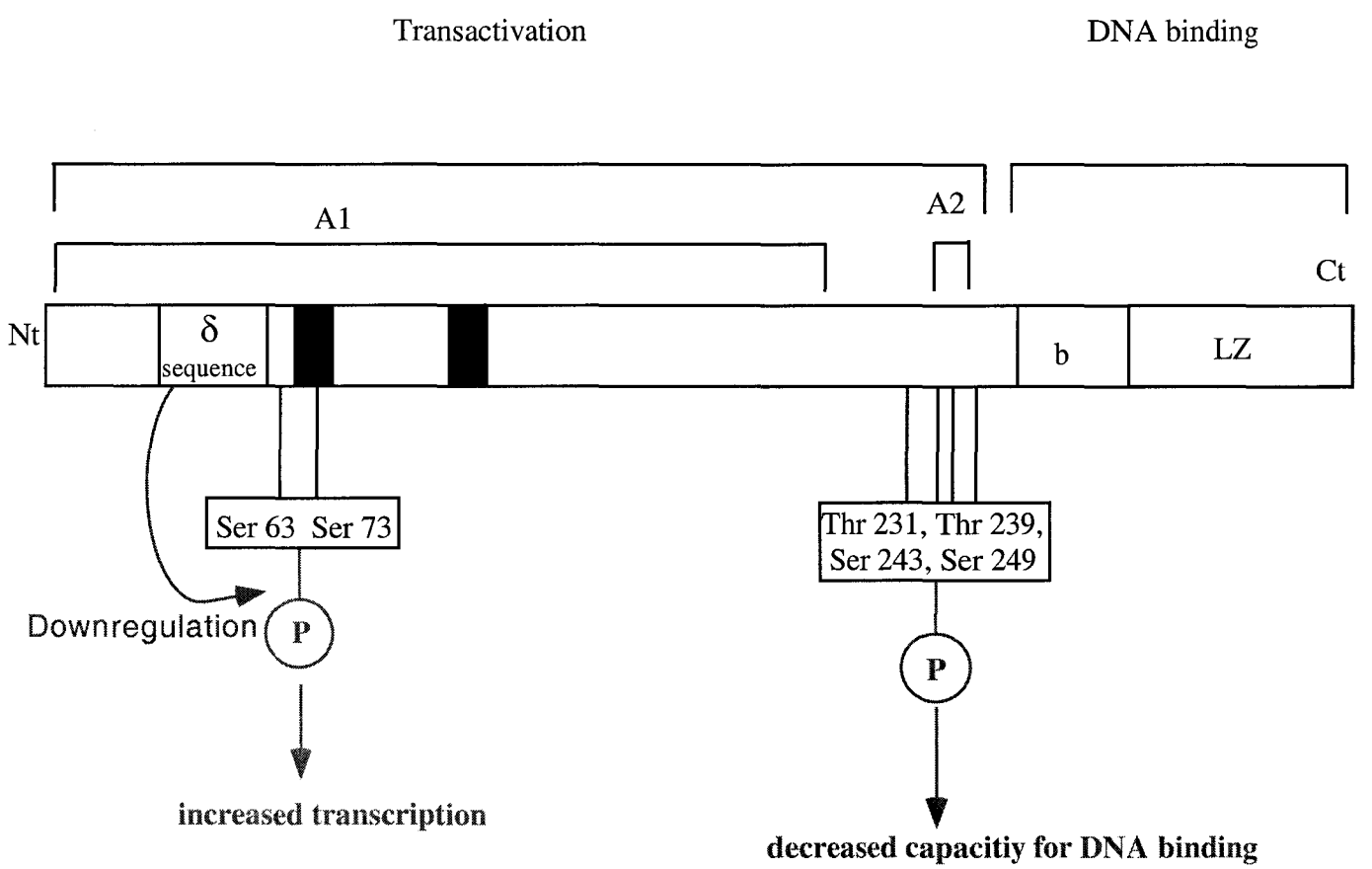

Fig. 10: Activation domains in c-Jun. The c-jun stucture is depicted with the related HOB1 and HOB2 sequences highlighted. The activation domains A1 and A2, as well as the basic domain (b) and the leucine zipper (LZ) are indicated

to dephosphorylation of Jun in this region (through protein phosphatase 2A (pp2A (43)) which in turn stimulates DNA-binding (51). Furthermore, in certain cell types, phorbol esters, activated Ras and polypeptide growth factors are able to stimulate phosphorylation of the c-Jun trans-activation domain at Ser 63 and 73, which results in increased transcription (41). The importance of these residues to c-Jun trans-activation has been assessed by mutagenesis, demonstrating that these residues are necessary for Jun interaction with the transcriptional initiator adaptator complex CBP (19).

Thus, mitogen regulation of c-Jun function is mediated through co-ordinated changes in phosphorylation of functionally distinct domains. Dephosphorylation of multiple sites near the DNA binding domain enhances DNA binding activity, whereas phosphorylation at Ser 63 and 73 within the amino-terminal region, catalysed by a family of related kinases, is necessary for transcriptional activation. These latter phosphorylation events are catalysed by pp54 (30), Jun-Nterminal kinase, (JATPK (4) or JNK (100)) or stress activated protein kinases (SAPK (163)). An interesting feature of Jun regulation is that the level of phosphorylation at Ser63 and Ser73 is dependent upon the presence of a δ sequence (amino acids 31-60), that is deleted in v-jun. The lack of this δ-motif leads to a hyperphosphorylation of v-Jun at residues 63 and 73, which causes an increase of the activation potential of v-jun (42). The δ sequence has been proposed to bind a cell-type specific repressor, probably the SAPK (90), which inhibit activation by the A1 domain (amino-acids 5-196) in certain cells (28, 29).

b) Cell cycle regulation: Structural regulation of the cyclin A-cdk2 kinase activity by phosphorylation.

The eukaryotic cell cycle is co-ordinated by several related Ser/Thr protein kinases, each consisting of a catalytic cyclin-dependent kinase (CDK) subunit and a regulatory cyclin subunit. CDK are activated by interaction with their appropriate cyclin. The transient

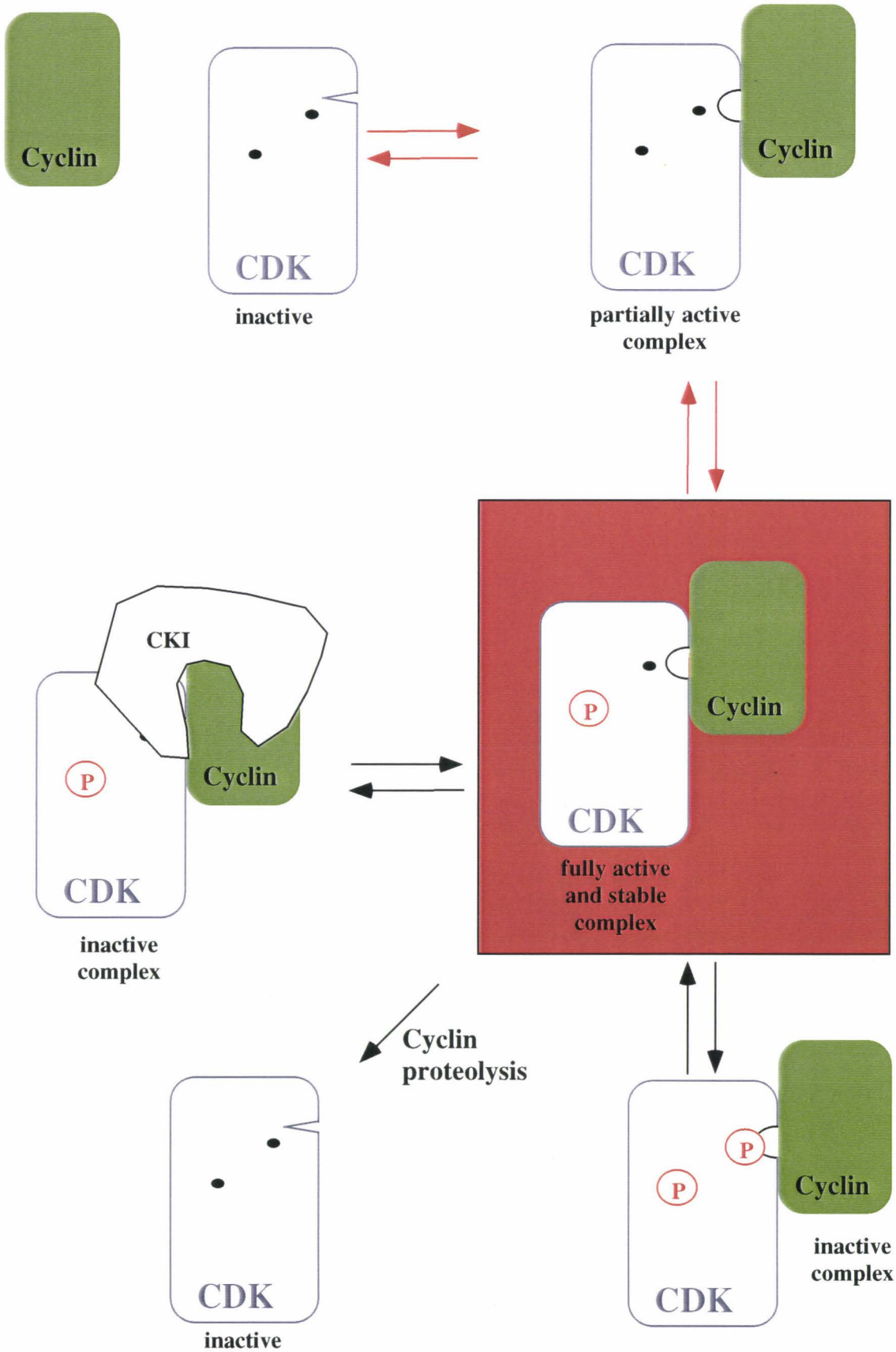

Fig. 11: Activation-inactivation process of cdk (from Martin-Castellanos and Moreno 1997).

appearance of these cyclin-CDK complexes drives cell cycle events such as cell growth (cyclinD-CDK4 and cyclinD-CDK6 in G1), DNA replication (cyclin E-CDK2 in G1/S and cyclin A-CDK2 in S) and cell division (cyclin A-CDK1 and cyclin B-CDK1 in G2 and M). The catalytic CDK subunit is inactive as a protein kinase in absence of the regulatory cyclin subunit (Fig. 11). The binding of the cyclin subunit to the catalytic kinase confers basal activity to the complex (72), whereas phosphorylation by CDK-activating kinase (CAK) at a distinct threonine residue of the CDK subunit results in full activity (118) (Fig. 11). Cyclins are a diverse family of proteins that have only limited sequence similarity, but share a homologous region of about 100 amino acids, called the cyclin box. This domain is a generalised adapter motif that recognises diverse classes of proteins involved in cell cycle control and regulation of transcription. (54). CDKs in contrast are highly homologous (220)and contain a conserved catalytic core of approximately 300 amino acids, which is common to all known eukaryotic proteins kinase (260).

The structure of cyclin A has been determined. It contains 12 α-helices, arranged in two lobes linked by two short spacers (54). There is no significant structural alteration of cyclin A upon association with CDK2 (54).

The structure of CDK2 is bilobal; it consists of an amino-terminal lobe (residues 1-85) which is rich in β-sheets and a larger carboxy-terminal lobe of mostly α-helical structure. In its inactive state, i.e. in absence of binding of a cyclin, this bilobal structure is in a closed conformation (93). The activation segment (red in Fig. 11) is folded in a way that the substrate recognition site is blocked. This segment is a common regulatory motif found in all kinases and contains a phosphorylable threonine, Thr-160 that activates the kinase. The α-helix of CDK2 contains the PSTAIRE sequence, a motif that is characteristic of the cyclin-dependent kinase family. This motif, central to the interface of the two lobes, is important for the binding of the cyclins (220).

Upon interaction of cyclin A with both lobe of CDK2, there is a conformational change in the CDK2 activation segment and the PSTAIRE helix (Fig.11). This leads to a partial activation of the kinase complex through realigning the active-site residues and relieving the steric blockage

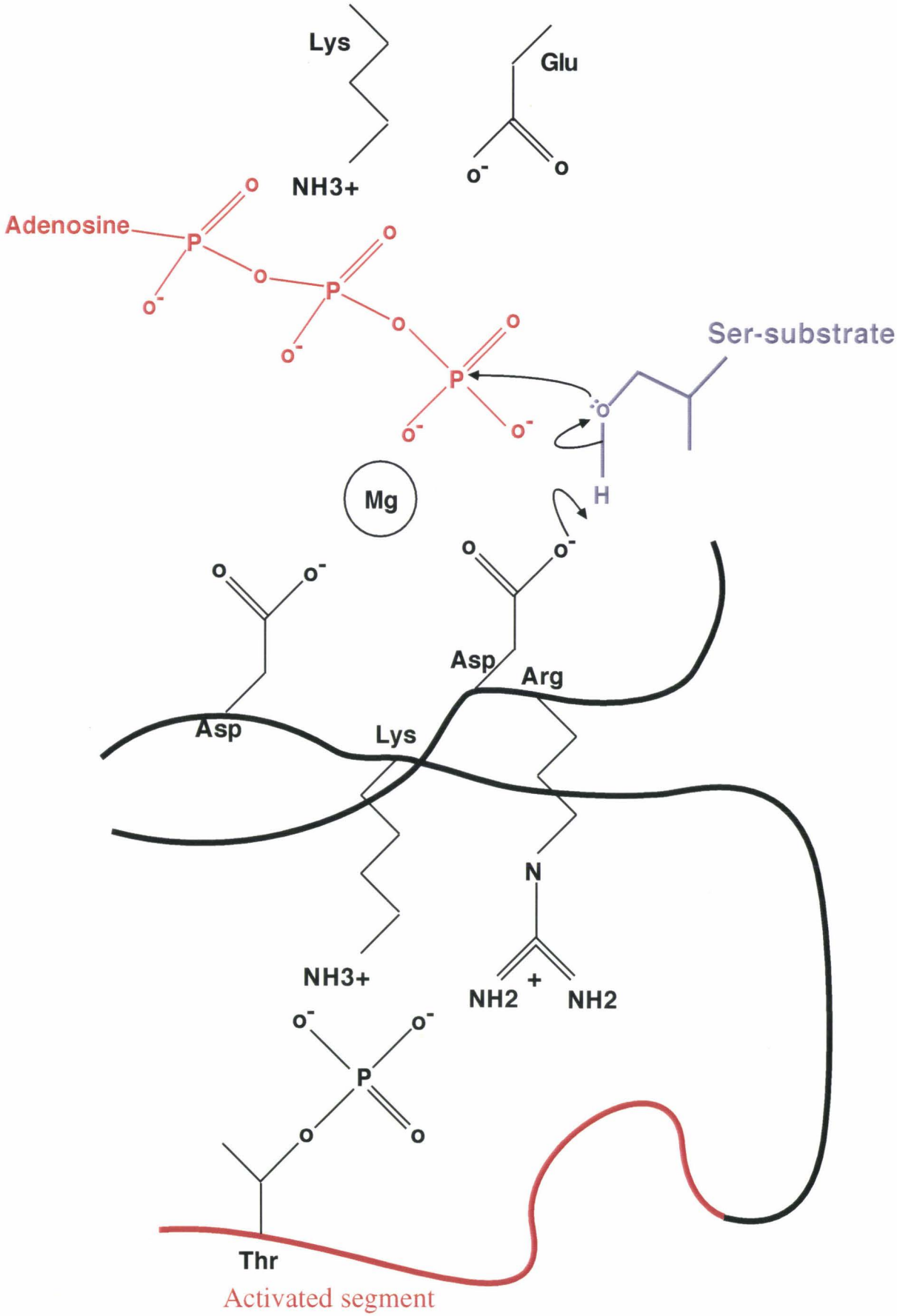

Fig. 12: Schematic representation of activation of a protein kinase.

at the entrance of the catalytic site. This complex has basal intrinsic kinase activity and exposes the Thr160 within the activating segment for phosphorylation. The correct alignment of the ATP with respect to the catalytic loop (which contain the active Asp) and the activating segment appears crucial for activity (194, 210). The kinase reaction is performed through a base-catalysed attack by the active Asp through its COO^- (Fig. 12). This Asp has to be aligned with the Ser-substrate and the ATP for maximal kinase activity. An Arg residue precedes the catalytic Asp. Upon phosphorylation of the Thr160 on the activating segment, the phosphate stabilises the positively charged Arg in a way that it aligns correctly whit the substrate and ATP. The phosphorylated CDK2-cyclin show a 17-fold increase in activity compared with the non-phosphorylated complex (142). Note also in Fig. 11, that there are four ways to inactivate the CDK-cyclin complex: by phosphorylation leading to a completely inactive complex, by dephosphorylation, leading to a partially active complex, by proteolysis of the cyclin or by binding of a Cyclin Kinase Inhibitor (CKI) to the complex.

c) Regulation of protein trafficking

The nucleus is separated from the cytoplasm by a double membrane structure, the nuclear envelope (Fig.13) (103). Nuclear protein import and export are mediated by receptor proteins that recognise nuclear localisation sequences (NLSs) or nuclear export sequences (NESs) and target the NLS- or NES-bearing proteins to the nuclear pore complex (NPC). NPCs are very large structures of about 125 MDa and consist of 100-200 different proteins (214). They are localised in the nuclear envelope and control the entire transport in/out of the compartment. Although there are exceptions, proteins larger than 45kDa require an NLS in order to be efficiently targeted to the nucleus. An NLS is defined as the sequences necessary and sufficient for nuclear localisation (92). They function through recognition/ligand-receptor-like interactions and constitute an entry signal rather than a signal for retention within the nucleus (245). The archetype of NLS is the motif (Pro-Lys-Lys-Lys-Arg-Lys-Val) of SV40 large T antigen. Recently, a second nuclear import pathway that allows the rapid re-entry into the nucleus of proteins that also participate in nuclear export of mature mRNAs, has been identified, involving the M9 NLS and the Nab2p NLS, which are independent of the classical pathway (113, 269). Both pathway involve two step in transferring the NLS containing protein in the nucleus. In a first step, the substrate binds to an adaptator protein (karyopherin or penetratin) that mediates the docking of NLS proteins to the periphery of the nuclear-pore complex (205). This step is independent of energy. The second step involves the binding of the adaptator protein to transporter proteins and requires energy. This step translocates and releases the nuclear protein through the central channel of the pore complex in the nucleus. Both pathways differ in the nature of the adaptator proteins and the nature of the NLS. This implies that the cells has the possibility to regulate the entry of at least two type of proteins in a different ways. The first step, which involves protein-protein

interaction of the NLS protein with the karyopherin, is phosphorylation-dependent. Similar to the SH2 domain recognising a tyrosine phosphate or 14-3-3 protein recognising a phosphoserine in the target polypeptide (199), the interaction between karyopherin and the substrate is dependent upon phosphorylation of a residue near the NLS (255). An excellent example of this regulation by phosphorylation consists in the nuclear translocation of LTAg (Fig. 13). Nuclear localisation of LTAg is dependent on the NLS located between amino acid 126-132. Three phosphorylation sites present near the NLS are able to regulate the kinetics of nuclear transport of LTAg. The CKII sites (serines at position 111 and 112) increase the rate of NLS-dependent nuclear import so that maximal accumulation of LTAg in the nucleus occurs within 15-20 min, whereas in absence of CKII phosphorylation the same accumulation takes more than 10h (141, 233). The phosphorylation event enhances the recognition of the LTAg NLS by the importin 58/97 heterodimer (134). In contrast to the enhancing effects of this phosphorylation at Ser111 and Ser112, phosphorylation at Thr124 by cdc2 inhibits nuclear transport (140). This complex regulatory system for SV40 LTAg nuclear localisation demonstrates the existence of specific mechanisms regulating nuclear entry, and shows that the NLS is not the sole determinant for nuclear translocation.

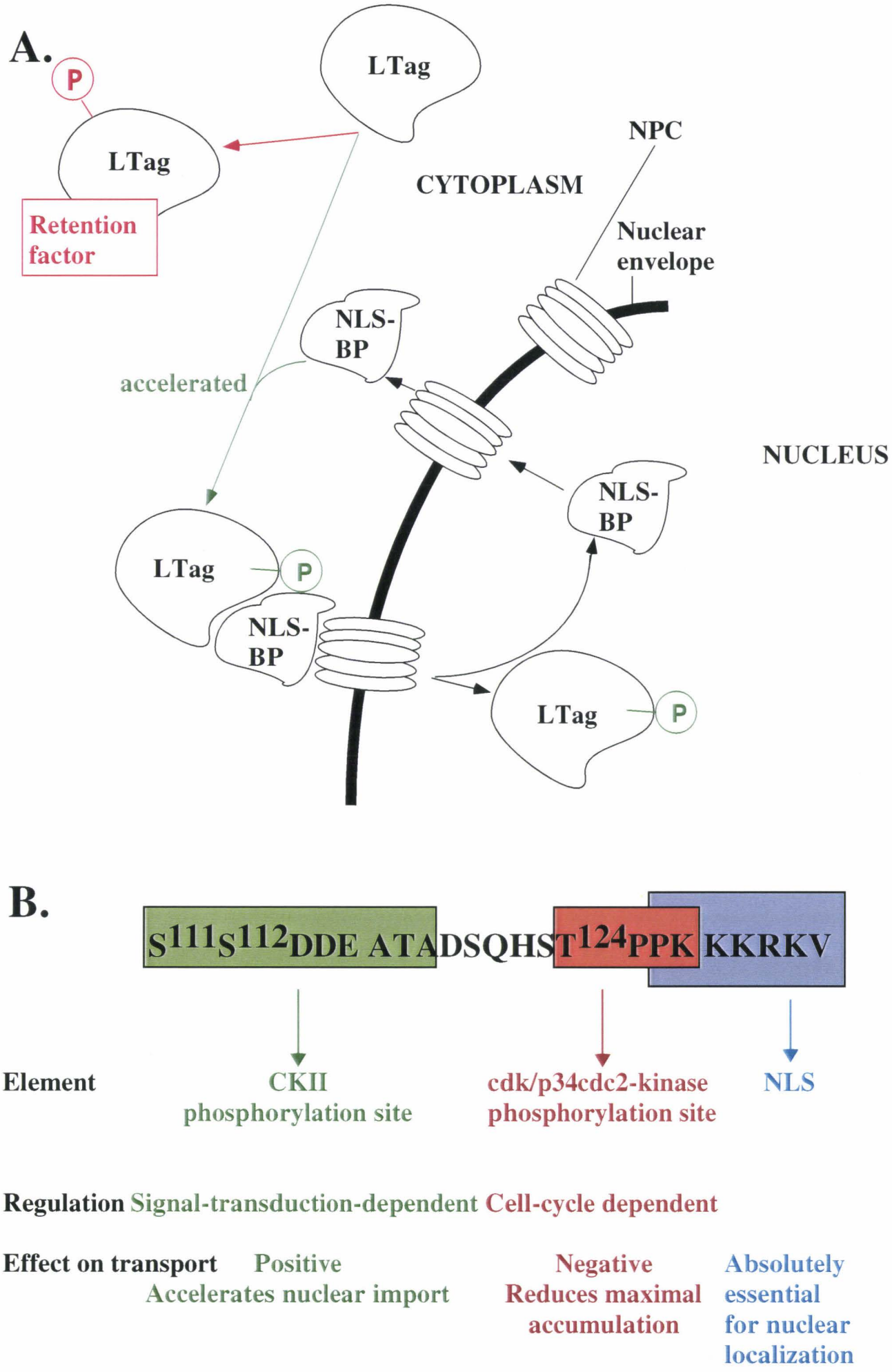

Fig. 13: A. Regulation of LTAg nulcear localization by phospohrylation. B. Phosphorylation sites involved in LTAg nuclear localization

II-2) Protein Kinase C (PKC)

a) Structure

The protein kinase C (PKC) was first identified as a protease-activated serine/threonine kinase and subsequently shown to be activated reversibly by neutral lipids, such as diacylglycerol (DAG) (225). In fact, this is a growing heterogeneous family of phospholipid-dependent kinases that can be divided into three categories on the basis of their cofactor requirements and structure. In the new classification of protein kinases, PKC can be found in the class of "AGC group" protein kinases encompassing serine and threonine kinase diacylglycerol-activated and phospholipid dependent (127). Protein Kinases C, like the other kinases, transfer the γ-phosphate group of ATP to a substrate, and liberate ADP and phosphorylated substrate (see Fig. 9). The modular structure of PKC is composed by the junction of a regulatory N-terminal domain, and a catalytic C-terminal domain (Fig. 14) (2). These domains form a succession of variable and constant (from C1 to C4) regions(96). The constant regions are modules with specific binding properties and correspond to functional parts(147). The catalytic domain is composed of the C3 and C4 regions that are characteristic for all PKCs and represent the ATP-binding site (C3) and the region responsible for binding of the substrate and for the catalytic activity (C4) (204). While the catalytic domains of different PKCs show a high degree of homology (see Fig. 14), the regulatory domains are diverse. This diversity in the regulatory domain provides the basis for the subdivision of the PKC family in three subgroups: the classical, novel and atypical PKC. As shown in Fig. 14 the regulatory domains of the classical and novel PKCs contain the C1 conserved region(203). This region includes just over 120 residues, combining two tandem repeats of a cysteine-rich domain (46, 155, 203). In contrast, atypical PKCs do only contain one of these repeats. Functionally, the typical C1 region (from the classical and novel PKC) has been identified as the region where diacylglycerol or phorbol esters are able to bind. The truncated C1 domain of atypical PKCs does not bind diacylglacerol and phorbol esters (148), but is implicated in protein-protein interactions (101). It has recently become evident that the fundamental structure of this domain consists of a single, rather than the double, cysteine-rich region (137). The regulatory domain of classical PKCs do contain the conserved C2 region. The structure of C2 reveals a β sheet-rich domain with a Ca^{2+}-binding pocket. This pocket is made by two loops comprising sequences at the amino and carboxyl termini of the C2 core, which come together to form an "aspartate-lined mouth" composed of five aspartate residues that co-ordinate the interaction with divalent cations (202, 243, 251).

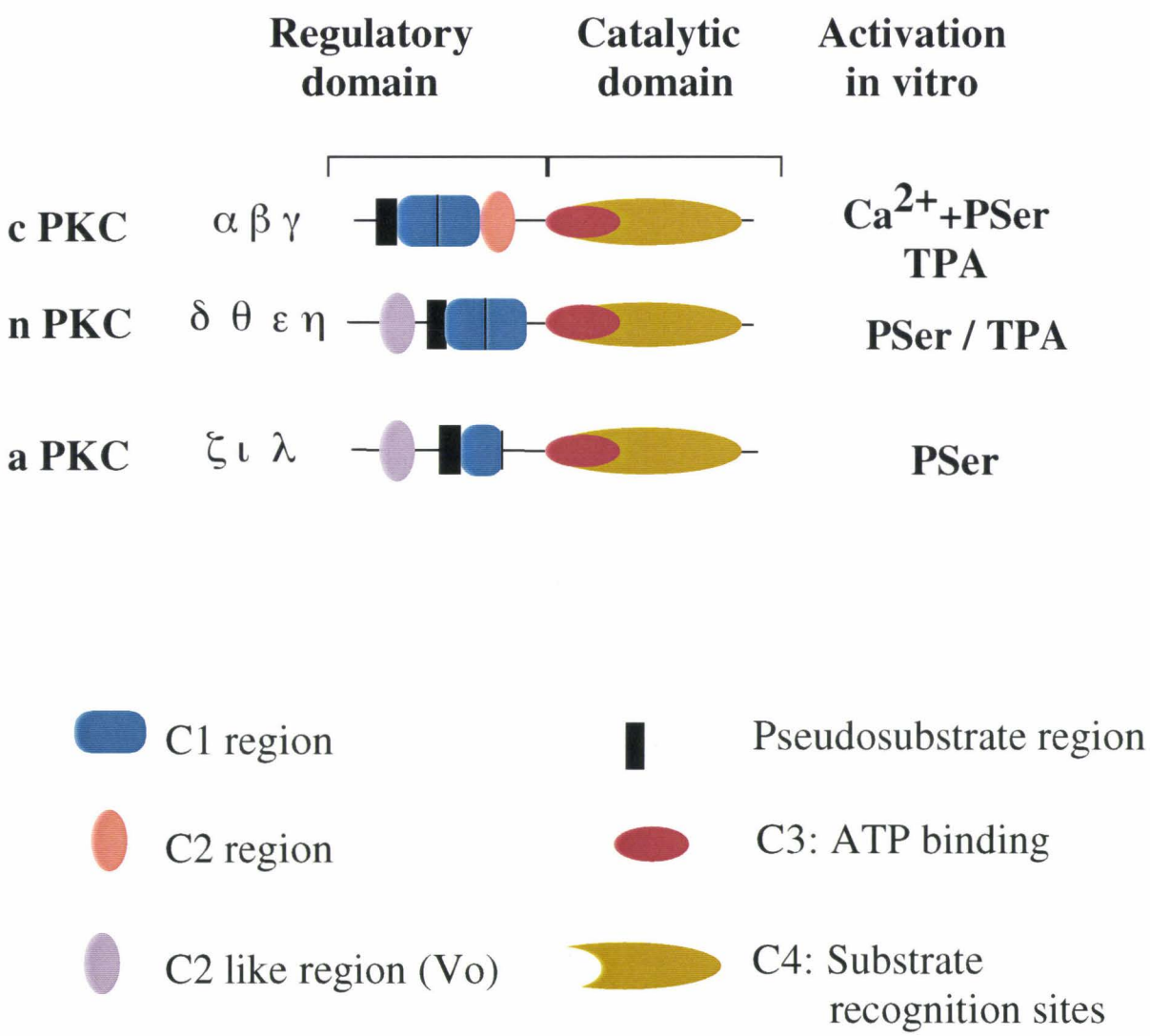

Fig. 14: Characteristics of protein kinase C isoforms

Ca^{2+} binding induces a significant conformational change within the C2 domain, exposing residues that serve as membrane anchors (203, 243), and thereby leading to the activation of classical PKCs(112). A variety of independent structural and biochemical approaches have suggested that the fully saturated C2 domain binds at least two metal ions rather than just one (202). Initially, the regulatory domains of the novel and atypical PKCs were considered to lack the C2 region, but recently it became evident that a region previously defined as a V0 extension is structurally related to the C2 region of the classical PKCs. Due to the lack of aspartates that bind Ca^{2+}, Vo is now called a C2-like region (250). This C2 like region does not require Ca^{2+} in order to bind phosphatidylserine or membranes (203). It is worth noting that the various C2-like regions show considerable differences in amino acid composition and therefore also differ considerably in their functions.

An additional functional region identified in PKC consists of the pseudosubstrate region (133) (Figs. 14 and 15). The amino acid sequence of the pseudosubstrate region resembles the recognised substrate site, while the target Ser/Thr present in the genuine substrate is replaced by an inert alanine. This pseudosubstrate region is also involved in the regulation of PKC activity(11, 179, 195).

The consensus phosphorylation site

K/R	X	X	S/T				
	K/R	X	S/T				
			S/T		X	K/R	

Pseudosubstrate

α	A	R	K	G	A	L	R	Q	
β	C	R	K	G	A	L	R	Q	K
γ	A	R	K	G	A	L	R	Q	K
δ	N	R	R	G	A	I	K	Q	A
θ	Q	R	R	G	A	I	K	Q	A
ε	K	R	Q	G	A	V	R	R	R
η	K	R	Q	R	A	M	R	R	R
ζ	Y	R	R	G	A	R	R	W	R
λ	Y	R	R	G	A	R	R	W	R

Fig. 15: Sequences of PKC pseudosubstrate regions and comparaison with the PKC consensus sequences

b) PKC regulation

PKC is synthesised in the cell as an immature inactive polypeptide (Fig. 16) (109, 111). Three phosphorylation steps are performed to transform this polypeptide into a mature PKC that can be activated by cofactors. In the processing of protein kinase C, the first site to be phosphorylated is located within the activation loop (152). Following this phosphorylation, PKC becomes additionally modified on Thr 641 (for PKC βII) (152). This phosphorylation appears to lock the protein in a catalytically competent conformation. The third phosphorylation step occurs at Ser 660 and correlates with the release of protein kinase C into the cytosol, suggesting that it directs the subcellular localisation of the kinase (49, 111, 152). This mature protein kinase C can be activated by at least three ligands such as phosphatidylserine, diacylglycerol, and Ca^{2+} (207). Deduced from their respective structure, as described above, the classical PKCs are regulated by all three of these ligands, whereas the novel PKC are regulated by phosphatidylserine and diacylglycerol, and the atypical PKC by phosphatidylserine only. Protein kinase C is maintained in an inactive conformation by an auto-inhibitory pseudosubstrate region which occupies the substrate binding cavity (C4 domain) (149, 151, 217). The pseudosubstrate region was identified using synthetic peptides derived from this sequence, which are able to inhibit PKC (133). Upon interaction with cofactors, a conformational change is induced in the polypeptide chain displacing the pseudosubstrate region from the C4 domain, rendering the active-site accessible for the substrate. This regulation is similar to the displacement of the vivo of other allosterically regulated kinases by corresponding second messengers (149).
PKC can also be activated by other pathways, in addition to the ones mentioned above(225). For example, arginine-rich polypeptides or polyamines like protamine, are able to bind protein kinase C, activate the enzyme and become phosphorylated in the absence of lipids or Ca^{2+} (168). There are also reports about regulation of distinct PKC isoforms in a cell type-specific manner. In addition to the triple phosphorylation involved in the maturation of PKC, further phosphorylation events have been shown to modulate PKC activity. Thus, in salivary gland, PKCδ is phosphorylated on tyrosine residues in response to activation of fluid secretion by neurotransmitters binding to phospholipase C-linked receptors (249), and there is evidence that PKCδ tyrosine phosphorylation is able to increase (170) or decrease (99) PKCδ activity. There are also reports showing a dual effect of ceramide. It inactivates PKCα by inducing its dephosphorylation and on the contrary, activates PKCζ (177). Interestingly, PKC is also known to be regulated in a spacial manner(188). Upon activation by diacylglycerol, PKC translocates to different subcellular sites, where it is able to phosphorylate target proteins(188). In the case of PKCα, the inactive isoform is found in the cytoplasm of fibroblasts. Upon activation with TPA, PKCα migrates to the nucleus by a new, energy and cytoskeleton-dependent mechanism, and exerts its kinase activity in nuclear component (57, 135, 240).

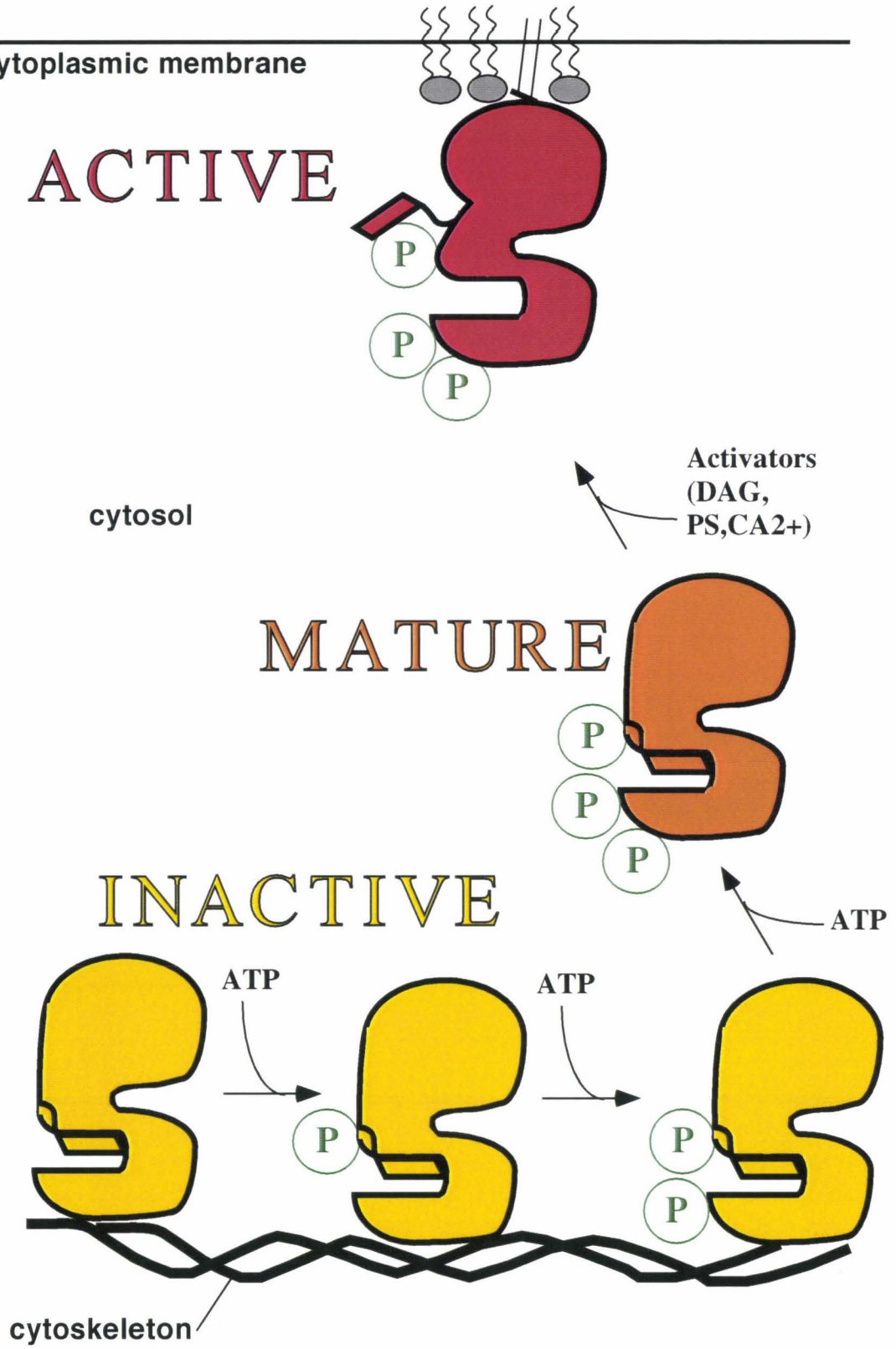

Fig. 16: Activation of PKC. Newly synthesized PKC (yellow) is phosphorylated on three residues to become mature (orange). This mature form is activated (red) by cofactors .

Cytosolic PKC also seems to be regulated in a different manner than the membrane bound PKC, in a way that the cytosolic PKC is activated by free fatty acids, instead of phosphatidyl serine (44).

c) PKC activity

The protein kinase C subfamily belongs to a group of kinases that phosphorylate their substrates on serine and/or threonine residues (127). Spacing and accommodation of phospho-acceptor residues are important determinants of whether a residue will be phosphorylated or not. Thus the varying susceptibility of target residues is related to the tightness of the juxtaposition of the hydroxyl-group on the phospho-acceptor residue with the phosphate-donating ATP molecule (247). In order to achieve a correct juxtaposition, a specific pocket is required for serines/threonines, since their OH groups are in close proximity to the C-backbone of the substrate protein. Tyrosines, the OH group of which is further out of the C-backbone of the protein, need alternative alignment (261). Amino acids surrounding the phospho-acceptor residue also contribute to its positioning, rationalising the often high degree of conservation of such residues over the various substrates for a particular kinase. This high degree of conservation of the environment of the phospho-acceptor residue determines the consensus phosphorylation site of a given kinase (150, 218). In the case of PKC, the consensus phosphorylation site resembles that of PKA and calmodulin-dependent kinase I (CaMK I), since basic residues are in the vicinity of the phospho-acceptor residue (150, 218). The actual defined consensus sequence is composed as follows (150, 218):

K/R X X S/T
or K/R X S/T
or S/T X K/R

This consensus sequence is a common target of all the PKCs, but does not reflect the specificity of each individual PKC isoform (97). Indeed, albeit targeted on the same consensus, each PKC has distinct substrates, thereby exhibiting specificity in their capacity for substrate recognition (131, 268). The amino acid composition around the phosphorylation consensus sequence seems to play a role. The determinants for this specificity at the level of the PKC structure are not clear (206). Alignments of catalytic domain amino acid sequences of distinct PKC isoforms reveals a high degree of conservation of residues which are involved in positioning the phospho-acceptor residue and are thought to be essential for the catalytic processes or for the stability of the catalytic loop structure (96). However, several variable amino acid clusters exist within this structure, which suggests, at least theoretically, that differential interactions with substrates may take place in the catalytic domain (96). In addition, the cofactors also seem to influence the specificity of PKC for their substrate (234, 280).

III) Large T Antigen (LTAg) of Simian Virus 40: The regulation of the viral initiator protein by phosphorylation, an example.

A variety of functional similarities and a striking sequence homology within the putative helicase domain (22) between parvovirus NS1 and SV40 LTAg led parvovirologist to use the well studied SV40 system as a model for parvovirus replication. Therefore, I would like to introduce the main features of SV40 and in particular LTAg.

The genome of the papovavirus simian virus 40 (SV40) is a circular, double-strand DNA composed of 5243 bp (33). The genome is divided into two functionally distinct genetic regions of about equal size, the early region encoding the regulatory proteins, and the late region encoding the structural polypeptides forming the virus shell. Transcripts from the early promoter produce two proteins, the small (17 kDa) and the large (90 kDa) tumour antigen (33). The large tumour antigen (LTAg) is one of the best studied eucaryotic proteins. LTAg is a phosphoprotein of 708 amino acids which exerts a variety of functions (181). The domain structure of LTAg is presented in Fig. 17. The DNA binding domain located between amino acid 131-259 allows LTAg to bind with high affinity to target DNA sequences containing the pentanucleotide GAGGC in a defined arrangement (64, 159, 185). Specific DNA binding of LTAg to two major sites in the regulatory region of the viral genome is essential (235, 246). Binding of LTAg to site I mainly represses the early transcription, whereas binding to site II is a crucial step during initiation of SV40 DNA replication (145, 235, 246). Just outside of this domain, there is a region that is in close contact to the target DNA in LTAg-DNA complexes. This region consists of a zinc finger loop (amino acids 302-318) (174, 282). A second domain of LTAg that harbours the site for ATP-hydrolysis is located between amino acid 418 and 627 (116). This function is essential for LTAg to unwind double-strand DNA. Bound to the origin of replication, LTAg is able to initiate unwinding of this region of the viral genome in order to allows the initiation of replication and the establishment of replication forks (94, 279). Further on, LTAg works as a 3' to 5' helicase in front of the replication machinery to allow the replication fork to proceed. In addition, LTAg also regulates promoters (13, 91, 189)and induces cellular DNA replication (17, 102)in infected cells. Also well known is the ability of LTAg to transform cells *in vitro* (60, 65)and to induces tumour formation in new-born animals (33, 107). The DNA binding, ATPase and helicase activities of LTAg are not essential for LTAg-mediated transformation (26, 180). In order to achieve many of its functions, LTAg is able to self-assemble to higher order of oligomers(235). It is also able to interact with a variety of cellular proteins, including p53 (164), pRb (95) and p107(110), which leads to transformation of the host cell.

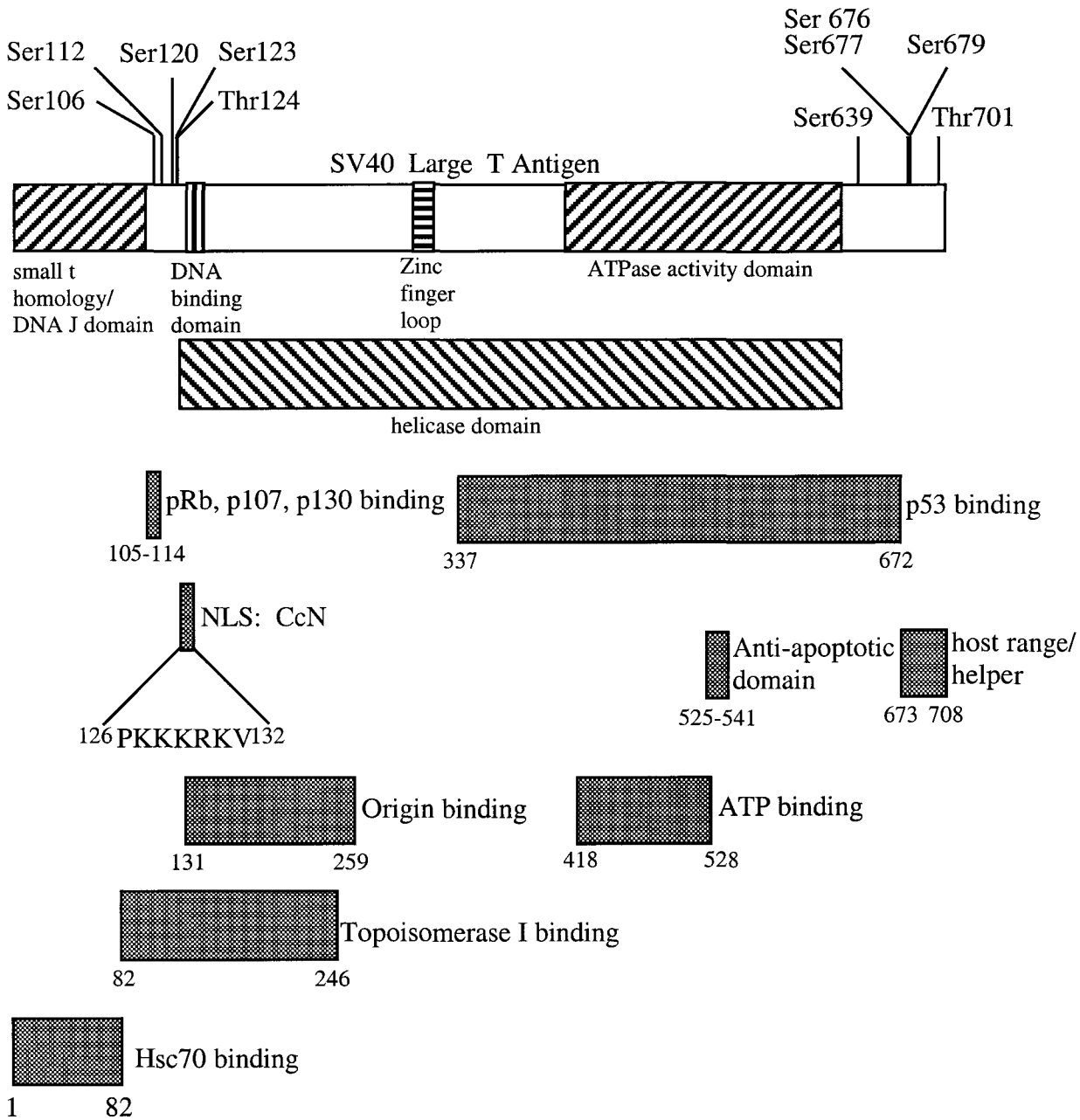

Fig. 17: Phosphorylation sites and domains of LTAg. (Adapted from Prives-C. Cell 1990.vol.61 p.735-738)

In addition, LTAg interacts with key replication enzymes, such as DNA polymerase α , RPA and topoisomerases (104-106), as well as with the transcription factor AP2 (187), the heat shock protein p73 (237), the TATA box binding protein TBP (125), cyclinA and p33cdk2 (3).
In order to co-ordinate its various tasks, the multi-functional protein LTAg is a target for multiple phosphorylation and dephosphorylation events that are able to regulate its activities during the virus cycle (223). LTAg is phosphorylated at serine/threonine residues. Many of the serine residues that are phosphorylated *in vivo* have been reported to be phosphorylated *in vitro* specifically by casein kinases I and II (124). As shown in Fig. 17 target phosphorylation sites of LTAg *in vivo* are mainly located within the N- and C-terminal parts of the polypeptide. They lead to the activation of LTAg for specific tasks, as determined by site directed mutagenesis. The replacement of serine and threonine residues to inert alanines in target phosphorylation sites of LTAg have determined Ser 120, Ser123 and Thr124 to be important for viral DNA replication (241). Indeed, LTAg need to be phosphorylated at Thr124, and requires dephosphorylation at Ser120 and Ser123 in order to be fully active for replication (see Fig. 18). Interestingly, when LTAg is produced in mammalian COS-1 cells, the viral protein becomes hyperphosphorylated at Thr124, Ser120 and Ser123 leading to a replication incompetent polypeptide *in vitro*, while expression in insect cells phosphorylates the protein at Thr124 only. This species is highly competent for replication, while the unphosphorylated, bacterially derived species is unable to achieve replication (Fig. 18). This production of differentially phosphorylated LTAg *in vivo* allowed to study its functional regulation by phosphorylation *in vitro* by purified kinases and phosphatases in great detail. In order to become activated for replication, LTAg is phosphorylated at Thr124 by cyclin/CDK complexes. Thr124 is phosphorylated in the cytoplasm soon after synthesis of LTAg. This Thr phosphate group has a slow turn over rate (61). The requirements for phosphorylation is thought to co-ordinate SV40 DNA replication with the S-phase of the cell in order to achieve maximal virus production during the lytic cycle of the virus. Analyzing specific biochemical activities of LTAg involved in replication, it has been determined that the initiation of replication at the viral origin is impaired by the lack of phosphorylation of Thr124.

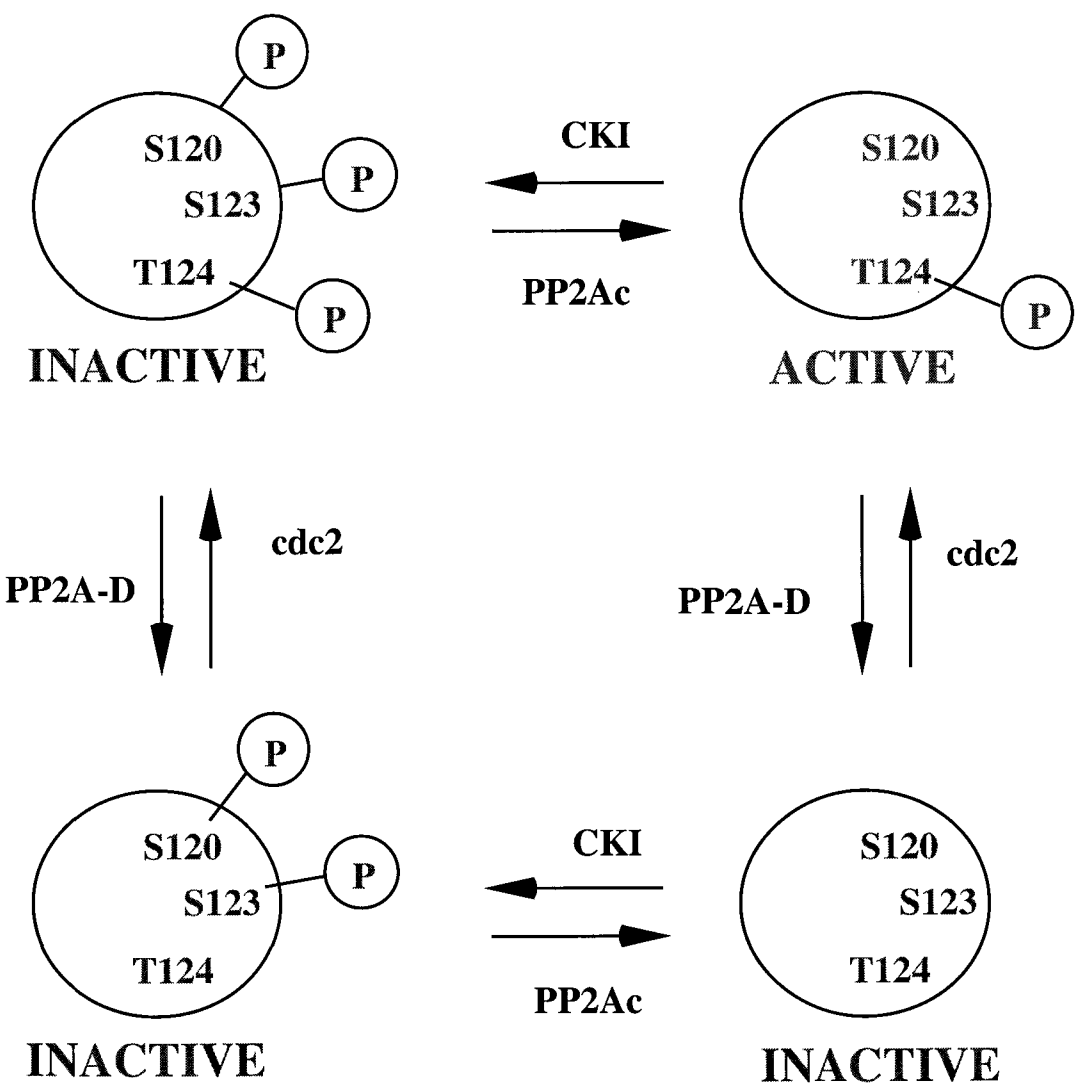

Fig. 18: Regulation of LTAg replicative functions by phosphorylation.

Underphosphorylated LTAg is impaired with regard to DNA-binding to site II, which is essential to allow local unwinding of the double-strand replication origin. In contrast binding to site I (located in close vicinity to the origin), intrinsic helicase function (which allows the replication to proceed after initiation), ATPase activity, and oligomer formation are not affected by phosphorylation.

Binding of LTAg to the SV40 origin occurs in two discrete steps, consisting in assembly of a first LTAg hexamer on one half-site of the origin, followed by the assembly of a second hexamer on the other half-site. Phosphorylation of LTAg at Thr124 induces conformational changes for these steps (185). Most likely phosphorylated Thr124 interacts with charged amino acids within the minimal DNA binding domain (186). After assembly of the two hexamers on the DNA, dephosphorylation of LTAg at Ser120 and Ser123 causes a change in the interactions between the hexamers and permits contacts, which would otherwise be prevented (123). These alterations of protein-protein interactions by dephosphorylation and the consecutive conformational changes of the double hexamer complex result in the initial opening of the duplex to form the replication bubble. Within the open duplex, the two LTAg hexamers serve as helicases unwinding the duplex DNA in front of the replication fork, after initiation of DNA replication by pol-α primase. Phosphorylation of Ser120 and 123 which inhibits LTAg replication activity, take place in the nuclei of infected cells and is characterised by a rapid phosphate turnover ($T_{1/2}$, 1,7H for Ser 123, (238)). Phosphorylation of Ser123 depends upon prior phosphorylation at Ser120 to generate a recognition determinant for a novel form of CKI which recognises structural rather than sequence determinants of the target substrate (119, 239, 272). It is worth noting that LTAg is also regulated at the level of nuclear entry. The rate of nuclear import is regulated by the CKII phosphorylation site (Ser 111/112 (141)); furthermore phosphorylation at Thr 124 adjacent to the NLS (amino acid 126-132) determines the maximal extent of nuclear accumulation (140). The regulation of LTAg phosphorylation is also complicated by the fact that phosphorylation at certain sites is controlled by the differential phosphorylation of other sites. For example, phosphorylation of Ser120 and Ser123 is reduced when Ser677 is dephosphorylated (239). All phosphorylation sites at the C-terminus (Ser676, 676, 679 and Thr701 but not Ser639) are located within a region that is not essential for DNA replication, but involves host range functions. Finally, the phosphorylation of Ser677, 679 and 639 are important for the transforming capacity of LTAg by an unknown mechanism (239, 241). While phosphorylation of Ser677 and Ser697 promotes transformation, Ser639 has an inhibitory effect on this LTAg function. Last but not least, as shown in Fig. 17, Ser106 and Ser112 are within the region of LTAg required for its binding to the product of the retinoblastoma tumour susceptibility gene (Rb) (124), and phosphorylation of these sites is most likely involved in transformation as well.

Comparison of MVM NS1 with SV40 LTAg.

For the virus.

	MVMp	**SV40**
virion	single strand ~5Kb	double strand ~5Kb
early promoter	+	+
late promoter	transactivated by NS1	transactivated by LTAg
tumorigenicyty	oncotropism and oncolytic activity	transforming capacity

For NS1/LTAg

	NS1	**LTAg**
Phosphorylation on:	Ser and Thr	Ser and Thr
subcellular localisation	mainly nuclear	mainly nuclear
self-association	+	(higher orders oligomers up to double hexamers)
association with cellular proteins	SP1, SGT, TBP,TFIIA (α,β)	p53, pRB, p107, p130, topoI, DNA-polα, RPA, p300, p400, cycA, TBP, hsc70
ATP binding/ATPase	+	+
DNA binding	high affinity for ACCA	high affinity for GAGGC
helicase activity	+	+
trans-modulation of promoters	+	+
activation of kinase(s)	increases p14 phosphorylation	increases CK2, cdk2 and cdc2 activities
apoptosis	induction of apoptosis	anti-apoptotic and apoptotic domains
Site and strand specific DNA nicking	+	-

Objectives

Autonomous parvoviruses such as Minute Virus of Mice (MVMp) have a very small genome, consisting of a single-strand linear DNA molecule of approximately 5.1kb. More than half of the coding capacity of this genome is needed to produce the capsid shell, protecting the genetic information during infection cycles and allowing its efficient transfer from cell to cell. The remaining part allows of the genome has a regulatory function and allows the virus to efficiently multiply. For this purpose, the following strategy is used by parvoviruses. Viral DNA amplification and production of progeny virus particles require host cells to enter S-phase, when the cellular replication machinery is available to the virus for its own use. This implies that the synthesis of viral cytotoxic proteins and capsid constituents is delayed until cells reach S phase, which results first from the lack of formation of a double-strand transcription template in quiescent cells, and second from the S-phase dependence of the early promoter controlling the expression of the regulatory non-structural proteins. To compensate for the very limited coding capacity of parvoviruses, a number of viral activities are carried out by a single multifonctional regulatory protein designated NS1. The NS1 polypeptide combines a range of functions including promoters trans-regulation, various replicative activities and toxic effects on host cells. The various functions of NS1 have be temporally co-ordinated in the course of the virus cycle, so that viral DNA become amplified prior to its packaging and progeny viruses are produced before cell die. My thesis represents a first attempt at unravelling the regulation of NS1 functioning, of which nothing was known at the beginning of my work. In analogy with SV40 LTAg, which shows functional similarities and sequence homology with NS1, we made the hypothesis that NS1 functions are controlled at least in part by phosphorylation at specific residues, either through activation or repression of distinct functions. This hypothesis is in agreement with the fact that NS1 is phosphorylated in infected cells. The multifunctional NS1 protein may become differentially phosphorylated in the course of infection in order to achieve its various tasks in a well-ordered way. Our first goal was to determine the relevance, if any of phosphorylation to the control of NS1 activity(ies). To this end, in vitro assays were used, allowing distinct NS1 function to be monitored, and the influence of phosphorylation on these functions to be assessed. In case phosphorylation indeed turned out to influence NS1 functioning, it was then of interest to identify both, (the) kinase(s) controlling NS1 activity(ies), and the NS1 target sites involved in such regulation. This was attempted, on the one hand, by using fractionated cell extracts and recombinant proteins in reaction performed under defined conditions, and on the other hand, by modifying NS1 phosphorylation sites by directed mutagenesis. Finally, it is important to evaluate

the physiological meaning of the NS1 modification pinpointed by mean of the in vitro assays. A first hint of the functional role of NS1 phosphorylation was sought by investigating whether the pattern of NS1 modification varies during the course of a natural virus infection.

Results

I) *In vitro* Activities of Minute Virus of Mice of the Non-structural Protein NS1 are Regulated by Phosphorylation.

Submitted, January 12, 1997

In vitro Replication Activities of Minute Virus of Mice Non-structural Protein NS1 are Regulated by Phosphorylation.

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Key words: Parvovirus MVM, Non-structural protein NS1, Phosphorylation, Biochemical Activities, Replication

Abstract

NS1, the 83 kDa major non-structural protein of minute virus of mice (MVM), is a multifunctional nuclear phosphoprotein which is required in a variety of steps during progeny virus production, early as well as late during infection. NS1 is the initiator protein for viral DNA replication, binds specifically to target DNA motifs, has site-specific single-strand nickase, intrinsic ATPase, and helicase activities, trans-regulates viral and cellular promoters and exerts cytotoxic stress on the host cell. In order to investigate whether these multiple activities of NS1 might be regulated by post-translational modifications, in particular phosphorylation, we expressed His-tagged NS1 by recombinant vaccinia viruses in HeLa cells, dephosphorylated it at

serine and threonine residues with calf intestine alkaline phosphatase, and compared biochemical activities of the purified un(der)phosphorylated (NS1O) with the "native" (NS1P) polypeptide. Biochemical analyses of replicative functions of NS1O, revealed a severe reduction of intrinsic helicase and to a minor extent of ATPase and nickase activities, whereas affinity to the target DNA sequence [ACCA]2-3 was enhanced compared to NS1P. In the presence of endogenous protein kinases found in replication extracts, NS1O showed all functions necessary for resolution and replication of the 3'dimer-bridge, indicating reactivation of NS1O by re-phosporylation. Partial reactivation for helicase activity was found as well when NS1O was incubated with protein kinase C.

Introduction

Replication of the single-stranded, linear DNA genome of parvoviruses involves the formation of a series of monomeric and concatemeric duplex DNA intermediates produced by an unidirectional, single-strand copy mechanism (19, 66). This mode of replication resembles the rolling circle DNA replication (RCR) mechanism described for bacteriophages, single-strand plasmids, and geminiviruses (3, 35). The first step of parvovirus DNA replication, the conversion of the single-stranded genome to a monomeric duplex, is executed by cellular components only, and is primed directly from the 3'hydroxyl-group of the nucleotide that is base-paired through folding back of a terminal palindromic structure (4, 18). For later stages in the infectious cycle, replication initiates at site-specific, single-strand nicks introduced by a virally coded initiator protein into origin sequences which are located at either end of the genome (17, 23, 64). The minimal origin sequence at the 3'end of parvovirus minute virus of mice (MVM) DNA has been determined (16). The DNA motif responsible for specific interaction with the initiator protein (25), as well as the target site for the nicking activity, and therefore covalent attachment, of this protein have been mapped (16).

The viral initiator protein involved in MVM DNA replication is a pleiotropic 83 kDa nuclear phosphoprotein called NS1 (for non-structural protein 1) (18, 24). A number of studies carried out in vivo (22, 39, 48) or in vitro (4, 17, 21, 56) with wild type NS1 or derivatives modified by site-directed mutagenesis, have clearly demonstrated the key role of this protein during distinct steps of parvoviral DNA replication. Indeed, NS1 proved to be the only viral protein necessary for viral DNA replication in all cell types. In particular, NS1 is required for the hairpin transfer of the right-end telomere of monomeric replicative forms (4) and the resolution of concatemeric replication intermediates (17, 21) as determined with recombinant NS1 proteins produced by vaccinia (53) or baculoviruses (1). One-step partial purification of NS1 has been achieved by immunoaffinity chromatography (70), conventional chromatography (13), or Ni2+-NTA agarose

columns taking advantage of a [His]6-TAG engineered to the N-terminus of the viral product (56), and has allowed a variety of biochemical activities to be assigned to the NS1 protein, relative to its replicative functions. In addition, some of these activities could be mapped to distinct domains of the multifunctional protein by the use of site-directed NS1 mutants (see Fig. 1A). Thus, NS1 forms oligomers (54, 60), exhibits intrinsic ATP-binding, ATPase and helicase activities (13, 70), binds site-specifically to an [ACCA]2-3-element present at multiple positions in the viral genome (11, 25), mediates the site-specific single-strand nicking of replication origins located in the left- (12, 56) and right-end (27) terminal sequences, and becomes covalently attached to the 5'end of replicated viral DNA (4, 16, 20, 23). Besides its multiple functions during viral DNA replication, NS1 possesses a C-terminal acidic transcription-activating domain (37) that is able to trans-regulate the parvoviral and various heterologous cellular and viral promoters (29, 38, 61, 67, 68). Furthermore, NS1 can induce cytotoxic and/or cytostatic stress in sensitive host cells (6, 7, 52), for which the N- and C-terminal part of the polypeptide appears to be important (38).

The different biochemical activities of the multifunctional NS1 protein can be assumed to be regulated in a temporal order, at least in part through post-translational modifications. Indeed, NS1 was found to be phosphorylated in vivo (1, 14, 24, 50). Precedents for regulatory pathways involving post-translational modifications, in particular phosphorylation, can be found in the control of cell cycle (33), and neoplastic transformations (44). The dependency of parvoviruses on host cell entry into S-phase (18), as well as their preferential replication and toxicity in neoplastic cells (15, 52), raise the possibility that NS1 may also be regulated through phosphorylation. It is worth noting that there is a striking functional, structural, and even sequence homology between NS1 and the simian virus 40 large T antigen (SV40 LT) (2), which both initiate viral DNA replication, trans-regulate homologous and heterologous promoters and disturb host cells. Interestingly, the replicative functions of SV40 LT have been reported to be modulated through phosphorylation (31, 59), in order to assure DNA replication to occur during S-phase of the cell cycle. Analysis of distinct replicative functions of LT revealed that this coordination is achieved specifically through its origin unwinding function (8, 46, 49). Little is known about the pattern or timing of phosphorylation of the parvovirus NS1 protein, but there is evidence to support that MVM NS1 phosphorylation occurs already early in infection and persists throughout the infection cycle (14, 18, 24). Phosphorylated amino acids in porcine parvovirus NS1 were found to be for the most part serine and to a lower extent threonine residues (14, 50). No tyrosine phosphorylation of NS1 has been reported so far (1, 14, 50). The implications of phosphorylation in NS1 functions, however, remained elusive to date.

In order to investigate the impact of phosphorylation on NS1 activities, NS1 was purified from recombinant vaccinia virus-infected HeLa cells (53, 56), and tested for a variety of biochemical activities, either in its "native" form or after dephosphorylation with calf intestine alkaline phosphatase. The native protein, as well as its dephosphorylated derivative incubated with endogenous protein kinases present in fully competent replication extracts, were both able to carry out all functions necessary for resolution and replication of the 3'dimer-bridge plasmid containing the MVM left-end replication origin (17). In contrast, in absence of any added protein kinases, the ATPase and especially the intrinsic helicase activities of the dephosphorylated polypeptide were markedly reduced compared to native NS1, while the specific binding to the 3'origin was enhanced. Re-phosphorylation by a commercially available protein kinase C preparation led to a partial reactivation of dephosphorylated NS1 for its helicase activity.

Material and Methods

Viruses and cells

Recombinant vaccinia viruses were propagated in monolayer cultures of BSC-40 or HeLa cells and purified over a sucrose cushion as previously described (41), except for the release of virus from infected cells by three cycles of freezing and thawing instead of sonification. MVMp was propagated in A9 cells. HeLa, BSC-40, and A9 cells were grown as monolayers in Dulbecco's Modified Eagle Medium (DMEM) containing 5% fetal calf serum. HeLa-S3 were grown in suspension using Spinner-bottles in Joklik's medium containing 5% fetal calf serum.

Production and purification of native and dephosphorylated NS1

NS1 was produced from recombinant vaccinia viruses in suspension cultures of HeLa-S3 cells. Cells were collected by low speed centrifugation and resuspended in DMEM without serum containing 15 pfu/cell each of vTF7-3 (32) together with the appropriate recombinant vaccinia viruses containing the NS1 gene under control of the bacteriophage T7 promoter (51, 53). The cell/virus suspension (5 ml = 107 cells) was transferred into a 150 mm2 tissue culture dish, incubated for 2 hr and supplemented to 20 ml with DMEM containing 10% fetal calf serum and 190 mM NaCl to favor cap-independent translation from the encephalomyocarditis virus leader sequence present in the pTM-1 based constructs (51, 53, 54, 56). Cultures of 2x108 cells were harvested 18 h post infection, nuclear extracts were prepared (21) and His-NS1 was purified using Ni2+-NTA agarose (Qiagen) columns (56). For dephosphorylation, nuclear extracts containing His-NS1 were adjusted to the appropriate buffer conditions (100 mM Tris-HCl, pH 8.5; 1 mM ZnCl), supplemented with the protease inhibitors PMSF (phenylmethylsulfonylfluoride) (174 mg/ml), leupeptin (1 mg/ml), aprotinin (1 mg/ml), and pepstatin (1 mg/ml), and incubated for 15 min at 37oC in the presence of 150 mg/ml of calf intestine alkaline phosphatase (Boehringer Mannheim). Dephosphorylated NS1 was immediately purified from other proteins by affinity chromatography using Ni2+-NTA agarose columns. NS1 preparations were analyzed by discontinous SDS-PAGE, and proteins were detected by Coomassie-blue staining. All mutant NS1 proteins used as controls were tested for their described biochemical properties.

Production and purification of bacterially expressed GST-NS1

pBacGST-EK-NS1 was constructed by inserting the BamHI fragment of the cloning intermediate pT-GST-NS1 into pGEX-5X-2 (Pharmacia). pT-GST-NS1 was obtained by polymerase chain

reaction (PCR) amplification of a fragment encompassing the glutathione transferase (GST)-part of pGEX-5X-2 with the primer pair 5'-CAGTATCCATGGCCCCTATAC-3' and 5'-TCACGCCATGGCCGCTCGA-3', digested with the restriction endonuclease NcoI and inserted into pTHis-NS1 (56). For expression of the GST-NS1 fusion protein, Sure bacteria were transformed with pBacGST-EK-NS1 and grown overnight at 37oC. Cultures were diluted 1:4 and further amplified for 3 h at 32oC before production of GST-NS1 was induced with 1 mM IPTG (Isopropyl-b-D-thiogalactopyranoside) for 2.5 h at 32oC. Bacteria were then collected by centrifugation, suspended in sonication buffer (20 mM HEPES-KOH, pH 8.0, 300 mM KCl, 0.05% Nonidet-P40, 0.1 mM DTT), and digested with 1 mg/ml of lysozyme for 10 min at 37oC. The suspension was supplemented with 25 mM EDTA together with the protease inhibitors PMSF, leupeptin, and aprotinin, and the bacteria were disrupted by sonification. The soluble proteins were cleared from insoluble material by centrifugation at 100,000 g for 1 h, and soluble NS1 was purified by addition of 300 ml of glutathione-sepharose (Pharmacia) per liter culture. After being allowed to attach for 2 h at 4oC and extensive rinsing with sonication buffer, bound NS1 was eluted with 10 mM glutathione in sonication buffer at pH 7.5. Since GST is able to self-associate, GST-NS1 is present as a dimer as determined by Fast Performance Liquid Chromatography on Superose 6 columns (57). To avoid this artefact, the 27.5 kDa GST polypeptide was cleaved off by treatment with 0.25 mg enterokinase (Boehringer Mannheim) per mg NS1 for 15 min at 30oC, and GST-free NS1 was further purified on a 5'AMP column (Pharmacia).

In vivo 32P-labeling and tryptic phosphopeptide analysis

After infection with MVMp (10 pfu/cell) in serum-free DMEM for 30 min, A9 cultures were incubated for 18 h in medium containing 5% fetal calf serum. Cultures (107 cells) were then labeled with [32P]-orthophosphate (ICN, 10-10 Ci/cell) in complete medium without phosphate (Gibco/BRL) for an additional 4 h period, and harvested directly in 1 ml of RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Na-deoxycholate, 1% Triton X-100), containing the protease inhibitors PMSF, leupeptin and pepstatin as well as phosphatase inhibitors (20 mM NaF, 5 mM b-glycophosphate, 5 mM p-nitrophenyle phosphate, 5 mM sodium molybdate, 1 mM sodium orthovanadate, 5 mM sodium phosphate). Immunoprecipitations were carried out using 5ml of aNSN, an antiserum raised against the N-terminal 91 amino acids of MVM NS proteins (26), for 2 h at room temperature. Immune complexes were precipitated with protein A-sepharose (Pharmacia), washed three times with RIPA buffer and further purified by 10% SDS-PAGE. [32P]-labeled proteins were revealed by autoradiography after blotting on polyvinylidene fluoride (PVDF) membranes (Millipore) (42) , and the band corresponding to NS1 was excised. Digestion of membrane bound NS1 was performed with 50 units of trypsin (Promega) for 18 h at 37oC. Tryptic peptides contained in the supernatant were recovered by lyophilization and analyzed on thin layer cellulose plates (MERCK) in two dimensions, by electrophoresis using pH 1.9 buffer and chromatography in phosphochromatography buffer (5).

Site-specific binding of NS1 to the MVM 3'origin of replication

Site-specific binding assays using NS1 and the MVM 3'origin were performed as described (25). Briefly, plasmid pL1-2TC, which contains the minimal active 3'replication origin (16), was digested with restriction enzymes Sau3A and NarI, and the DNA fragments were labeled at the 3'end by filling in with sequenase (Amersham), [a-32P]dGTP and unlabeled dATP, dCTP, and dTTP. Binding assays were carried out in 100 ml of 20 mM Tris-HCl, pH 8.0, 10% glycerol, 1% NP-40, 5 mM DTT, and 100 mM NaCl, supplemented with labeled, fractionated pL1-2TC DNA, 500 ng oligo d[I,C], 0.5 mM g-S-ATP, and 50 ng of purified NS1. After allowing interactions to take place for 30 min on ice, 2 ml of antiserum aNSN was added and the incubation was continued for another hour. Immune complexes were precipitated with protein A-sepharose, deproteinized, and analyzed by non-denaturing 7% PAGE in the presence of 0.1% SDS.

Site-specific nicking of the 3'origin of replication
NS1-mediated site-specific nicking and the consequent covalent attachment of NS1 to the 5'end of the nicked product, were analyzed according to Nüesch et al., (56). The substrate containing the 3'origin of replication was obtained as a 95 bp EcoRI fragment of pL1-2TC. This fragment was end-labeled by fill-in reaction using sequenase, 32P-dATP and unlabeled dTTP. Approximately 1 ng of substrate was incubated with 20 ng of NS1 in the presence of 3 mM ATP for 1 hr at 37oC. The reaction was stopped by adding 0.1% SDS and 2.5 mM EDTA, and immunoprecipitations were performed using aNSN. The immune-complexes were freed from proteins by proteinase K digestion and phenol/chloroform extraction and analyzed by electrophoresis on 8% sequencing gels.

Helicase assay
Helicase assays were carried out as described (56). M13-VAR used as substrate was prepared by annealing the reverse primer (Amersham) to M13 single-strand DNA (Amersham) followed by extension for 5 min at room temperature in the presence of sequenase, dNTPs, including [a-32P]dATP. 32P-labeled fragments of varying length were obtained by addition of dideoxy-GTP and further incubation for 20 min. Purified NS1 (2 to 200 ng as indicated) was incubated with 20 ng of substrate for 30 min to 1 h in the presence of 3 mM ATP, and when indicated, an ATP regeneration system that consisted of 25 mM phosphocreatine and 25 ng of phosphocreatine kinase. The reactions were stopped by addition of SDS and EDTA, and the products were analyzed by 7% non-denaturing PAGE in the presence of 0.1% SDS.

ATPase assay
NS1 used for ATPase assays was further purified by centrifugation through a 1.5 ml 15 to 40% glycerol gradient at 50,000 rpm using a TLS55 rotor (Beckman) for 18 h at 4oC. 20 fractions (75 ml) were collected from the top, with peak amounts of NS1 present around fraction 9. The ATPase activity was measured of individual fractions which had been adjusted to their respective NS1 contents, as determined by Coomassie-blue staining after SDS-PAGE. ATPase assays were performed according to Wilson et al., (70), using 2 to 20 ng of NS1 protein in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 5 mM DTT, 0.01% NP-40, 30 mM ATP supplemented with 0.5 mCi [g-32P]ATP (Amersham, 3000 Ci/mmol) and 0.1 mg single-stranded DNA, for 20 min at room temperature. The reaction was terminated by addition of 100 ml of 7.5% (wt/vol) acid-washed charcoal in 50 mM HCl, 5 mM H3PO4, and free phosphate was separated from unreacted charcoal-bound ATP by centrifugation. A 50 ml sample of the supernatant containing [32P]i was analyzed by scintillation counting.

In vitro resolution and replication reactions with the left-end bridge fragment.
Resolution of the 3'dimer-bridge of MVM DNA was investigated in vitro as previously described, using pLEB711 as a substrate (17). Approximately 100 ng of purified NS1 expressed from HeLa cells or dephosphorylated NS1 was supplied to HeLa replication extracts. The reaction mixture was incubated for 2 h at 37oC in the presence of dNTPs including 32P-dATP, ATP and an ATP regeneration system. The NS1-attached labeled products were recovered by immunoprecipitation with aNSN, digested with the restriction endonuclease ScaI, and the resulting fragments were further separated for NS1-bound and NS1-free fractions by centrifugation. These products were analyzed independently by 1% agarose gel electrophoresis.

In vitro phosphorylation of NS1
Purified dephosphorylated NS1 (100 ng) was treated with either one of the following protein kinases for 30 min at 37oC in the presence of 10 mCi [g-32P]ATP, in 20 mM HEPES-KOH, pH 7.5, 5 mM MgCl2, 5 mM KCl, 0.1 mM DTT: 0.1 U of protein kinase C (Sigma), 5 mU of casein kinase II (Boehringer Mannheim), 20 mg of the catalytic subunit of cAMP/GMP dependent kinase (Sigma), or 10 ng of cdc2 complex (UBI). In some experiments, 500 ng of total proteins from HeLa cell extracts were used to phosphorylate NS1 instead of defined protein kinases. The reaction was terminated by addition of 0.1% SDS and 2.5 mM EDTA, and heating for 30 min at 70oC. 32P-labeled NS1 was purified by immunoprecipitation with aNSN, and analyzed by SDS-PAGE.

Results

Production of native and dephosphorylated NS1

In order to evaluate the complexity of NS1 phosphorylation, potential phosphorylation sites were predicted by computer analyses, and in vivo phosphorylation experiments were carried out with MVM infected cells (Fig. 1 B,C). Computer searches limited to three main protein kinases (protein kinase C, PKC; casein kinase II, CKII; and cyclic AMP/GMP dependent kinase, PKA) revealed over 35 consensus phosphorylation sequences within the NS1 polypeptide (Fig. 1B). Their complexity was confirmed by tryptic peptide mapping of in vivo labeled NS1 (Fig. 1C). Depending on the cell line tested and the time of analysis during infection, eight to twelve distinct phosphorylated peptides could be detected, as illustrated in Fig. 1C for NS1 produced in A9 cells 18 h post infection. At least four of these phosphopeptides were consistently present in NS1 preparations from all MVM infected cell lines tested so far, and constitute potential candidates for phosphorylation-dependent regulation sites. In order to determine the relevance of NS1 phosphorylation for MVM propagation, the present study was undertaken to test whether phosphorylation indeed has an impact on NS1 replication functions. To this end, NS1 was produced by recombinant vaccinia viruses in HeLa cells (53), dephosphorylated, or not, on serine and threonine residues using calf intestine alkaline phosphatase, and purified on Ni2+-NTA agarose through a N-terminal [His]6-TAG (56). The efficiency of the dephosphorylation procedure was determined by addition of vaccinia virus-produced NS1 that was 32P-labeled either in vivo using orthophosphate or in vitro by treatment with protein kinase C in the presence of [g-32P]ATP. As shown in Fig. 2, the dephosphorylation procedure removed the 32P-label efficiently from NS1 (Fig. 2B) under conditions which caused relatively little NS1 degradation (Fig. 2A), as determined after SDS-PAGE by autoradiography and Coomassie-blue staining, respectively. In addition to using full length NS1, we performed many of the subsequent biochemical analyses in parallel with a mutant NS1 polypeptide (54) that lacks the C-terminal 67 amino acids (NS1:dlC67) corresponding to the transcription-activating domain (38).

Biochemical characterization of dephosphorylated versus native NS1

Purification of functionally active NS1, development of a variety of distinct biochemical assays, and generation of specific mutants, obtained by site-directed mutagenesis, allowed a number of activities to be assigned to the NS1 protein, such as site-specific interaction with a consensus DNA motif (25), single-strand site-specific nicking and covalent attachment to the left-end origin (13, 56), intrinsic helicase function, and ATPase activity (70). These developments prompted us to determine whether phosphorylation had any impact on these properties of NS1, by comparing native and dephosphorylated NS1 purified from recombinant vaccinia virus-infected HeLa cells.

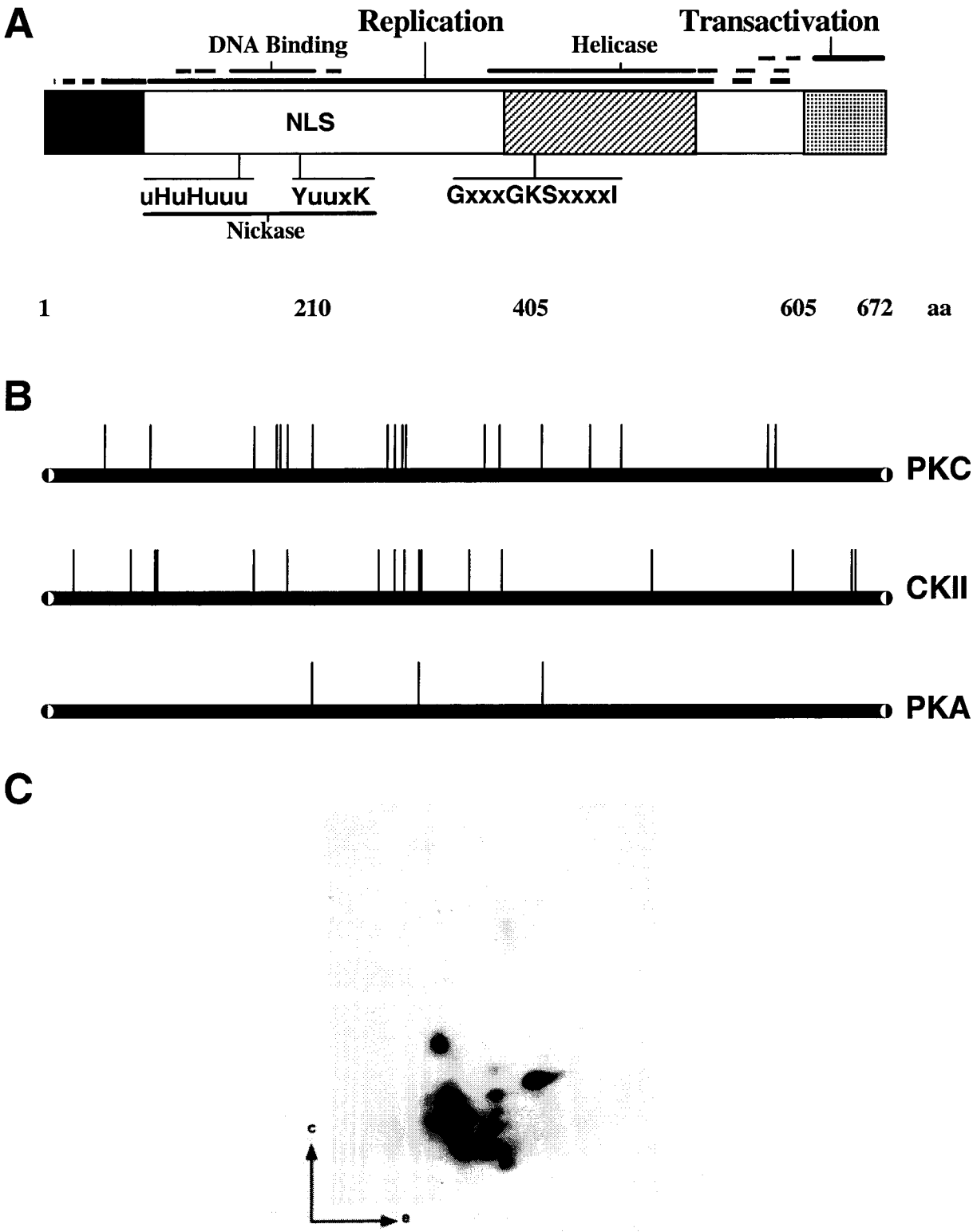


Figure 1. NS1 phosphorylation.

(A) Schematic representation of NS1 and its functional domains as determined by mutational analyses (for references see main text). The black, hatched, and stippled boxes correspond to the common N-terminus of NS1 and NS2, the homology region between NS1 and SV40 large T antigen, and the region deleted in the mutant NS1:*dl*C67. NLS, nuclear localization signal. The consensus sequences for the Metal coordination site, the linking tyrosine, and the nucleotide binding site are indicated. aa, amino acids.
(B) NS1 consensus sites of phosphorylation by protein kinase C (PKC), casein kinase II (CKII), and cAMP/GMP dependent kinase (PKA) as determined by computer alignments using HUSAR.
(C) Tryptic phosphopeptide map of *in vivo* labeled NS1 isolated from A9 cells incubated with 32P-orthophosphate after MVMp infection. NS1 was immunoprecipitated using antiserum αNSN, digested with trypsin and analyzed by two dimensional electrophoresis (e) and chromatography (c).

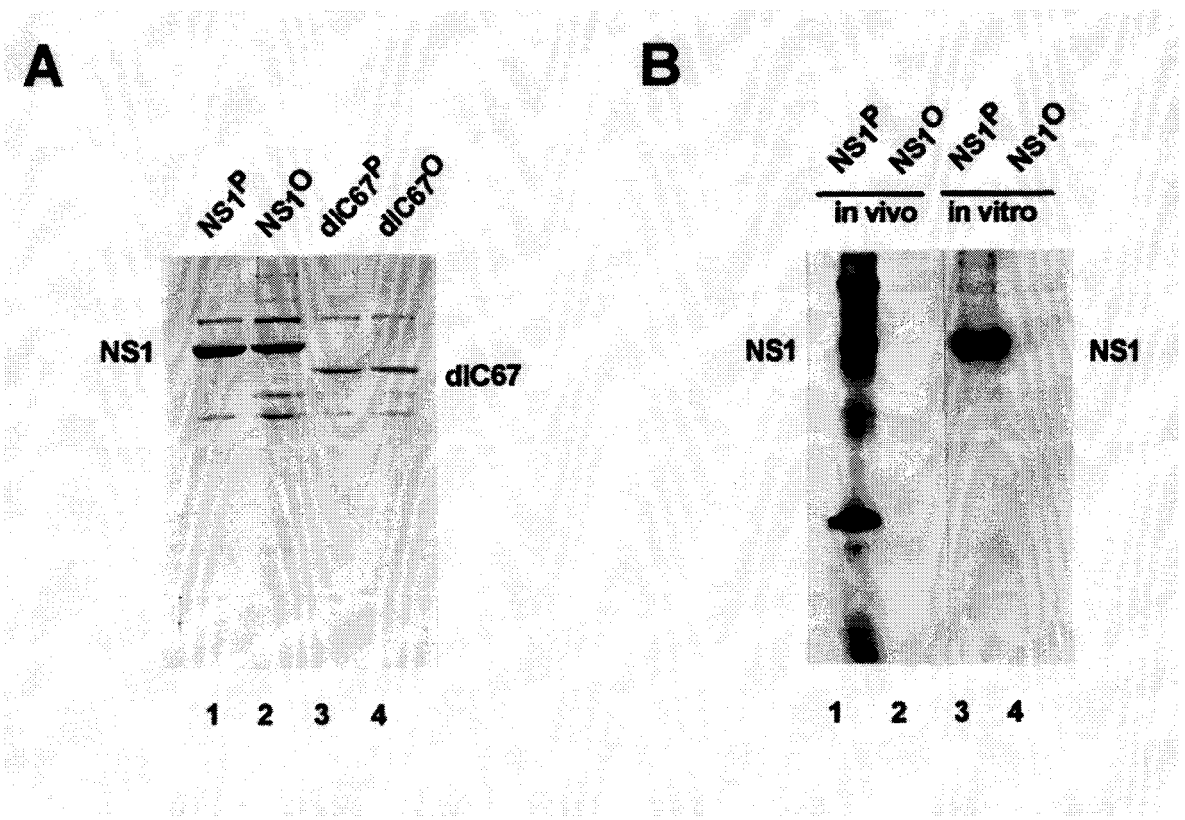


Figure 2. Production, dephosphorylation, and purification of NS1.

(A) Full length (wt) and C-terminally truncated (*dl*C67) NS1 were produced in HeLa cells from recombinant vaccinia viruses. Nuclear extracts containing NS1 were treated (dephosphorylated samples, "O") or not (native samples, "P") with calf intestine alkaline phosphatase, and the NS1 polypeptides were purified by affinity chromatography through their N-terminal [His]6-TAG. The purified samples were analyzed by SDS-PAGE and Coomassie-blue staining. Lanes 1, 2: wild type NS1; 3, 4: NS1*dl*C67; 1, 3: native NS1; 2, 4: dephosphorylated NS1.

(B) Wild type NS1, produced by recombinant vaccinia viruses was metabolically 32P-labeled *in vivo* using 106 HeLa cells, and extracted by repeated freezing and thawing. Alternatively dephosphorylated NS1 was 32P-labeled *in vitro* using PKC. The 32P-labeled proteins were then mixed with the same amount of nuclear extracts containing NS1 as used in (A). Phosphatase treated or untreated samples were then purified on Ni2+-NTA agarose and analyzed by SDS-PAGE and autoradiography. Lanes 1, 2: NS1 labeled *in vivo* in HeLa cells; 3, 4: NS1 labeled *in vitro* using recombinant PKC. 1, 3: mock treatment; 2, 4: treatment with calf intestine alkaline phosphatase; The low degree of purification of *in vivo* 32P-labeled NS1 in lane 1 is probably due to the formation of aggregates caused by the freezing and thawing procedure used to release the 32P-labeled proteins from cells.

n order to measure the site-specific binding of NS1 to the [ACCA]2-3-element located within the 3'origin of replication, plasmid pL1-2TC was used as a substrate, after digestion with Sau3A/NarI and end-labeling of the restriction fragments by fill-in reaction using sequenase (25). The 32P-labeled plasmid fragments were incubated with purified NS1 in the presence of non-specific competitor DNA (oligo d[I,C]) and non-hydrolysable g-S-ATP. Specific NS1:DNA complexes were immunoprecipitated using aNSN, an antiserum raised against the N-terminal 91 amino acids of MVM NS proteins (26), digested with proteinase K and analyzed by non-denaturing PAGE in the presence of SDS. Mutant NS1 Y210, which contains a substitution of the linkage tyrosine for covalent attachment to replicated viral DNA (56) and which has been shown to be severely impaired for site-specific binding to the origin, served as a negative control in these assays. As illustrated in Fig. 3, full length NS1 and C-terminally deleted NS1:dlC67 were both able to immunoprecipitate the plasmid fragment containing the 3'origin, when supplied in either native or dephosphorylated form, indicating that NS1 phosphorylation is not required for the specific interaction with the [ACCA]2-3 element. On the contrary, dephosphorylated NS1 proteins exhibited consistently a more than 3-fold higher affinity for the origin-containing fragment than did the native polypeptides.

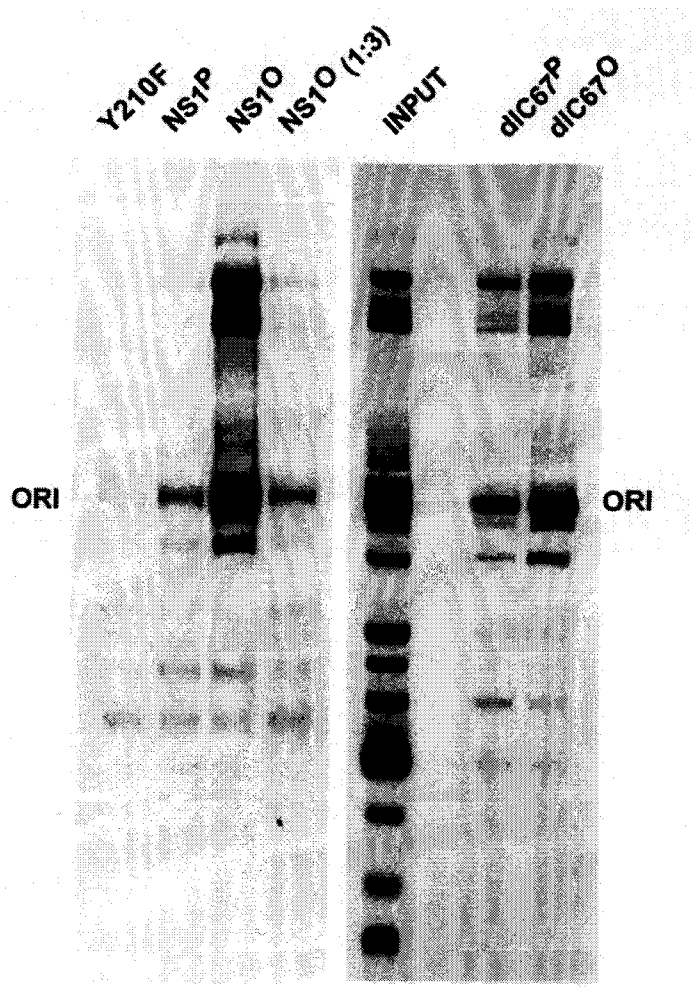


Figure 3. Site-specific binding of native (P) versus dephosphorylated (O) NS1 wt and *dl*C67.

Purified NS1 was incubated with 32P-labeled, Sau3A/NarI digested pL1-2TC plasmid containing the MVM active 3'origin of replication, in the presence of γ-S-ATP. NS1:DNA complexes were immunoprecipitated using αNSN, and their DNA constituents were revealed by autoradiography after 7% non-denaturing PAGE in the presence of 0.1% SDS. The fragment containing the MVM origin is denoted "ori". The NS1 mutant Y210F served as a control.

The NS1-mediated nicking of the 3'origin and the consequent covalent attachment of the polypeptide to the 5'end of the nicked strand are essential to initiate resolution and replication of MVM DNA replication intermediates (17, 21, 22). This initial nicking reaction can be reproduced in vitro using purified NS1 and a cloned 3'origin (3'end-labeled 95 bp EcoRI fragment of pL1-2TC) under low salt conditions without any additional cellular components (56). Site-specific nicking is dependent on the supply of hydrolysable ATP and of NS1 that contains intact ATP-binding, metal coordination, and linking tyrosine consensus domains (56). These requirements were confirmed by the present study showing that significant nicking was achieved by native full-length and dlC67 NS1 in the presence of ATP, but not by the NTP-binding site (K405R) and active-site tyrosine (Y210F) mutants or in the presence of g-S-ATP instead of hydrolysable ATP (Fig. 4). When dephosphorylated NS1 was tested in this assay, nicking was also found to occur in an ATP-dependent way, albeit to a significantly lower extent than with native NS1. Considering the efficiency of dephosphorylation (Fig. 2B), these results suggest that phosphorylation is not a prerequisite for nicking activity of NS1. Yet the significantly lower capacity of dephosphorylated versus native NS1 for 3'ori nicking in the present assay, points to this NS1 function as a potential target for phosphorylation-mediated up-regulation. Current investigations, in which 3' origin nicking reactions are performed in the presence of a purified cellular co-factor, the parvovirus initiation factor PIF, support these findings (10).

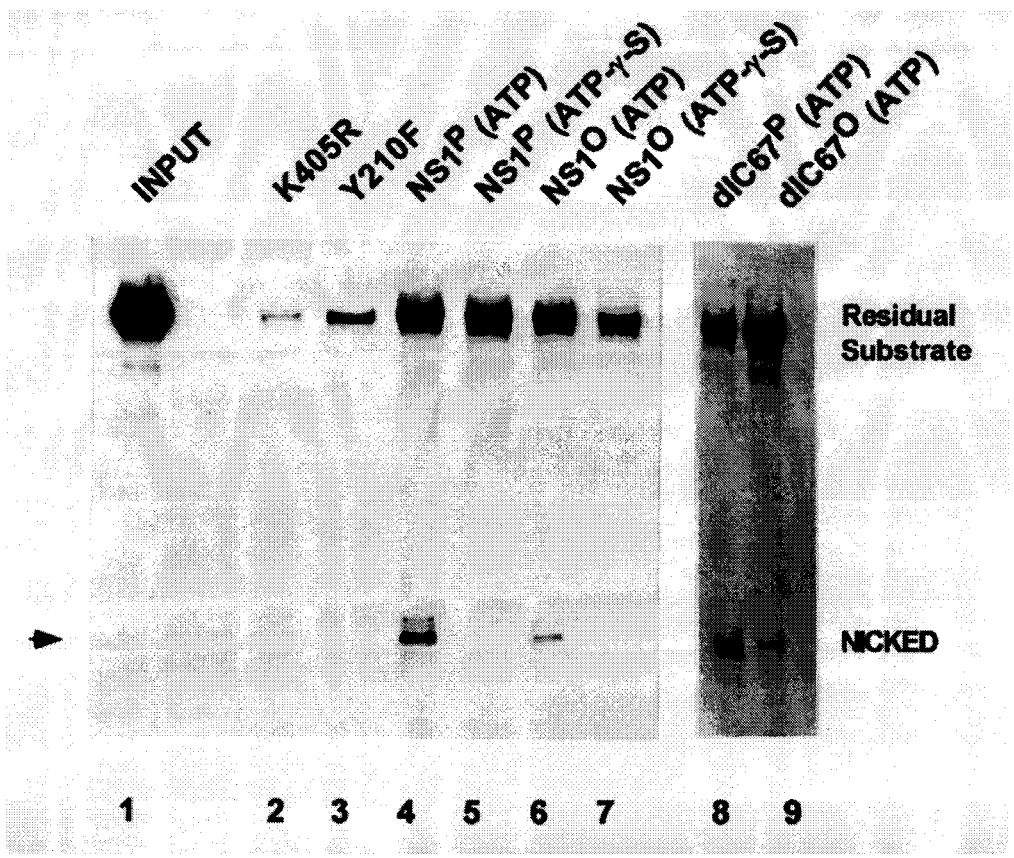


Figure 4. Site-specific nicking of native (P) versus dephosphorylated (O) NS1 wt and *dl*C67. The MVM active replication origin was isolated as a 95 bp EcoRI fragment from plasmid pL1-2TC, 3'end-labeled and used as a substrate for nicking by NS1 in the presence of ATP or γ-S-ATP as indicated. Site-specific nicking generates a labeled DNA fragment of 53 nt in length that remains covalently attached with NS1. Nicked DNA fragments were recovered by immunoprecipitation using αNSN, deproteinized, and analyzed for their size on 8% sequencing gels. The size of nicked products is indicated by arrow heads. NS1 mutants K405R and Y210F served as negative controls. Lane 1: input substrate (1:20 dilution); 2, 3: negative controls using mutant NS1; 4 to 7: wild type NS1; 8, 9: NS1*dl*C67; 2-4, 6, 8, 9: reactions in the presence of 2 mM ATP; 5, 7: reactions in the presence of ATP-γ-S

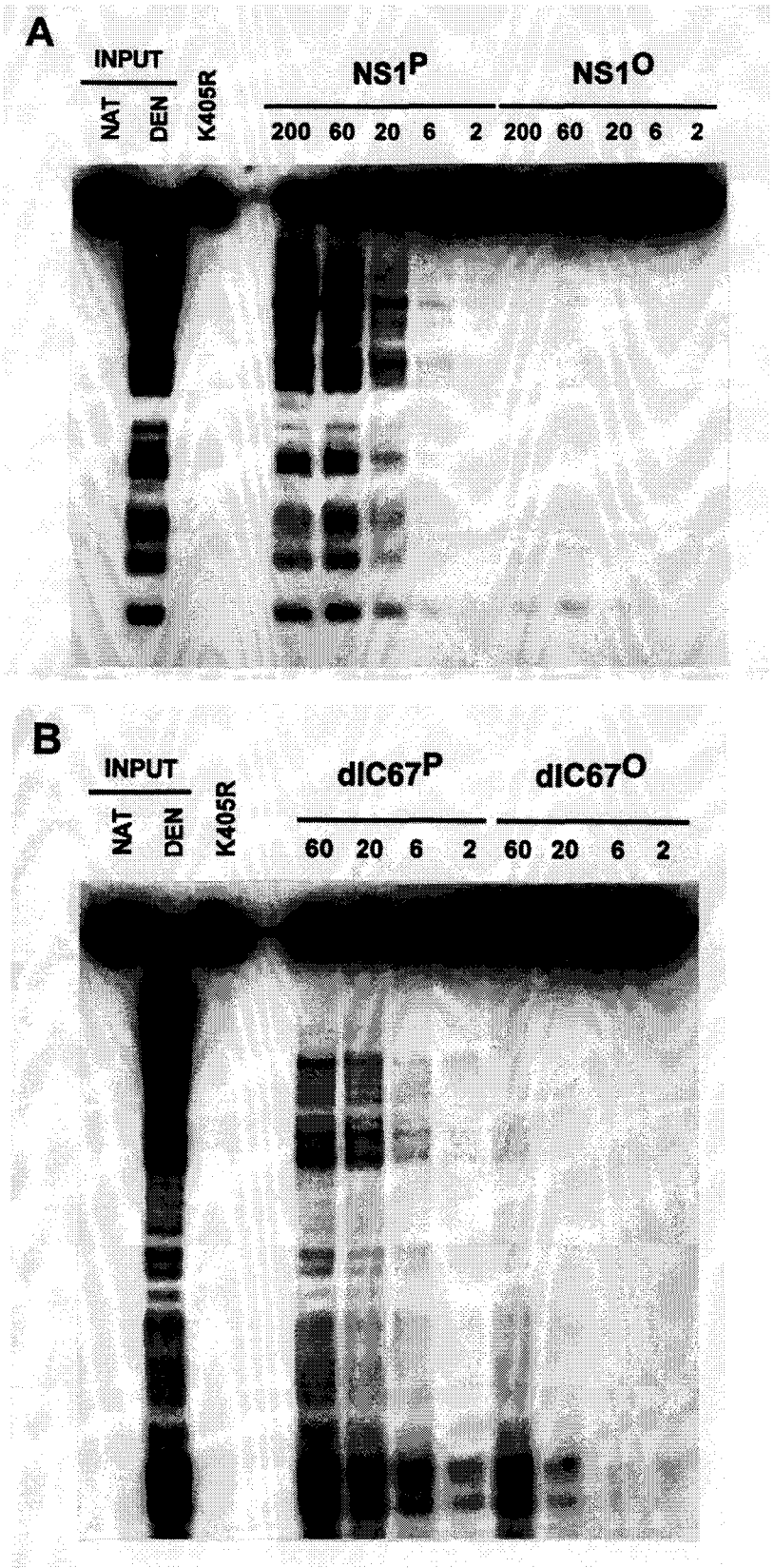


Figure 5. Helicase activity of purified native (P) and dephosphorylated (O) NS1 wt (panel A) and *dl*C67 (panel B).

Helicase assays were carried out by incubating 20 ng of M13-VAR (various length 32P-labeled fragments annealed to circular M13 DNA) with the indicated amounts (ng) of NS1 in the presence of ATP. The reaction products were analyzed by 7% non-denaturing PAGE in the presence of 0.1% SDS. NS1 mutant K405R served as a negative control. Native and heat-denatured input DNA are shown on the left as references.

NS1 is thought to facilitate strand-displacement synthesis during MVM DNA replication through its intrinsic helicase activity. In the presence of hydrolysable ATP, NS1 proved able to unwind DNA fragments of a size of up to 600 nucleotides from circular M13 DNA templates (56). Fig. 5 presents a titration experiment in which the unwinding activities of HeLa cell-derived native and dephosphorylated NS1 were compared, using the M13-VAR substrate that consists of various length 32P-labeled fragments annealed to circular M13 DNA (56). The NS1 mutant K405R, which is helicase-deficient due to an amino acid substitution at the conserved lysine 405 position in the NTP-binding domain (53, 56), served as a negative control to ascertain the NS1 dependence of the unwinding reactions measured. Wild type and C-terminally deleted NS1:dlC67 both exhibited helicase activity, the extent of which was reduced more than 30-fold as a result of dephosphorylation. Therefore, the NS1 helicase function appears to be under a tight control mediated by phosphorylation.

Furthermore, we investigated a possible effect of NS1 phosphorylation on the ATPase activity that is involved in a variety of biochemical activities described for NS1. Purified NS1 was supplied with [g-32P]ATP in the presence of single-stranded DNA as a co-factor (13), and the release of labeled phosphate was measured by scintillation counting. In order to minimize contamination with endogenous ATPases present in HeLa cell extracts, and due to fluctuations inherent in the assay, NS1 preparations were subjected to a further purification step by centrifugation through a 15 to 40% glycerol gradient. Fraction 7, 8, and 9 (of which fraction 9 corresponded to the peak amount of NS1) were analyzed individually, matched for their NS1 content as determined by Coomassie-blue staining after SDS-PAGE. The average values from multiple experiments were calculated with their standard deviation for two independent dephosphorylated NS1 preparations (#1, #2) and expressed as relative ATPase activities compared to native NS1 samples. As presented in Fig. 6, dephosphorylation was associated with a marked impairment (4 to 8 fold) of the ATPase activity of native NS1 protein, although a significant residual activity could still be detected. In order to confirm the measured ATPase activity to be derived from NS1, the mutant K405R was used as a negative control. Similar mutations within the NTP-binding domains of ADV NS1 (13) and other ATPases (65) have been shown to completely abolish the ATPase activity of the respective polypeptides.

NS1 replication activity

All of the above presented analyses of native and dephosphorylated NS1 have taken advantage of assays developed to analyze distinct replicative functions of this protein (36, 56, 63). In order to investigate whether dephosphorylated NS1, deficient for nickase and helicase function could regain these activities when properly re-phosphorylated, we compared native and dephosphorylated NS1 for their ability to resolve and replicate a plasmid containing the 3' head-to-head dimer-bridge in crude cellular replication extracts that can be assumed to contain the appropriate protein kinases.

NS1 activity was tested in vitro using HeLa cell extracts supplemented with pLEB711 as a substrate, in the presence of dNTPs including 32P-dATP, ATP and an ATP-regenerating system. The 32P-labeled products were analyzed by agarose gel electrophoresis after immunoprecipitation and ScaI restriction digestion (17). Figure 7 shows that, in the presence of replication-competent native NS1, the dimer-bridge is nicked asymmetrically leading predominantly to the extension of the GAA arm by NS1-mediated strand-displacement synthesis, and to the production of a covalently closed turnaround form of the TC arm. This pattern is due to resolution of the asymmetric palindromic structure as described previously (19), whith the palindrome arms named after the unpaired sequence making up a "bubble" in the 3'terminal hairpin of viral DNA (17). No replication was detected using the mutant NS1:Y210F, which contains a mutation in the active-site tyrosine. Dephosphorylated NS1 also proved competent for dimer-bridge resolution and replication, although the efficiency was somewhat lower than with the native NS1 (Fig. 7). This result clearly demonstrates that the phosphatase treatment did not cause an irreversible inactivation of NS1. The significant replication capacity of the dephosphorylated NS1 in cell extracts contrasts with the almost complete inactivity of this protein in the helicase assay (Fig. 5). This difference might be ascribed either to NS1 helicase function being dispensable for replication or by the presence of protein kinases in HeLa replication extracts which are able to re-phosphorylate the phophatase treated NS1. The failure to isolate so far NS1 helicase mutants that are still active in replication assays (36, 56, 57), rather argues against the former possibility. Furthermore, the replication extracts used for in vitro replication assays were subsequently found to be able to re-phosphorylate phosphatase treated NS1 (see below and Fig. 8). Similar results have been obtained with the truncated NS1:dlC67 that lacks the C-terminal 67 amino acids, or when A9 cell extract were used in replication experiments instead of HeLa cells (data not shown). These results confirm that the C-terminal domain of NS1 is not essential for replication activity, as previously indicated (38) and suggest that the presumed up-regulation of NS1 resolution and replication activity by phosphorylation can take place to a significant extent in the absence of this domain.

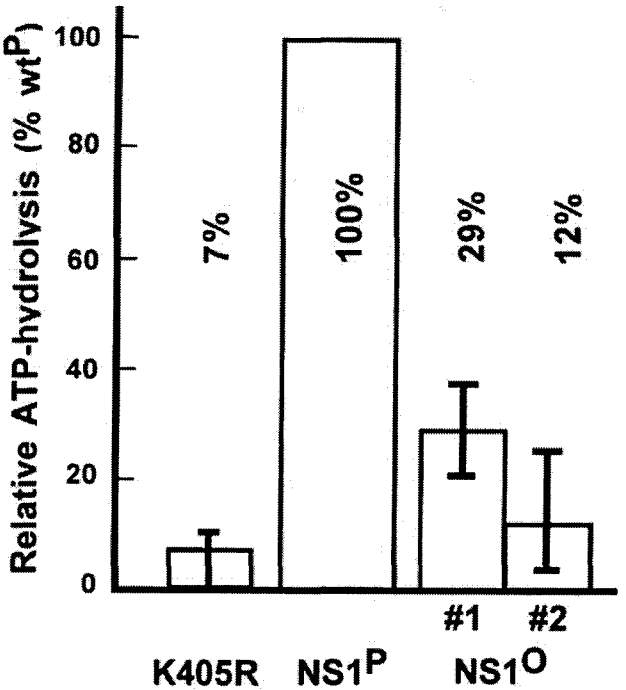


Figure 6. Effect of dephosphorylation on ATPase activity of NS1.

Release of labeled Pi was determined by scintillation counting after incubation of [γ-32P]ATP with native (P) or dephosphorylated (O) NS1. Average values from multiple assays using different sucrose gradient fractions are shown with their standard deviation bars for two independent NS1O preparations (#1,#2). The NS1 mutant K405R served as a negative control. Data are expressed relative to the ATPase activity of native wild type NS1.

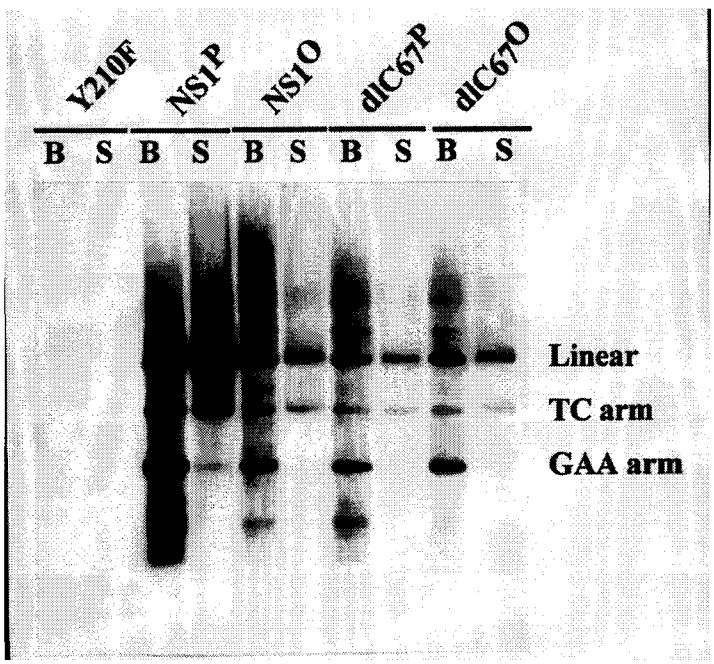


Figure 7. *In vitro* resolution of the 3'dimer-bridge by native (P) and dephosphorylated (O) NS1 wt and *dl*C67.

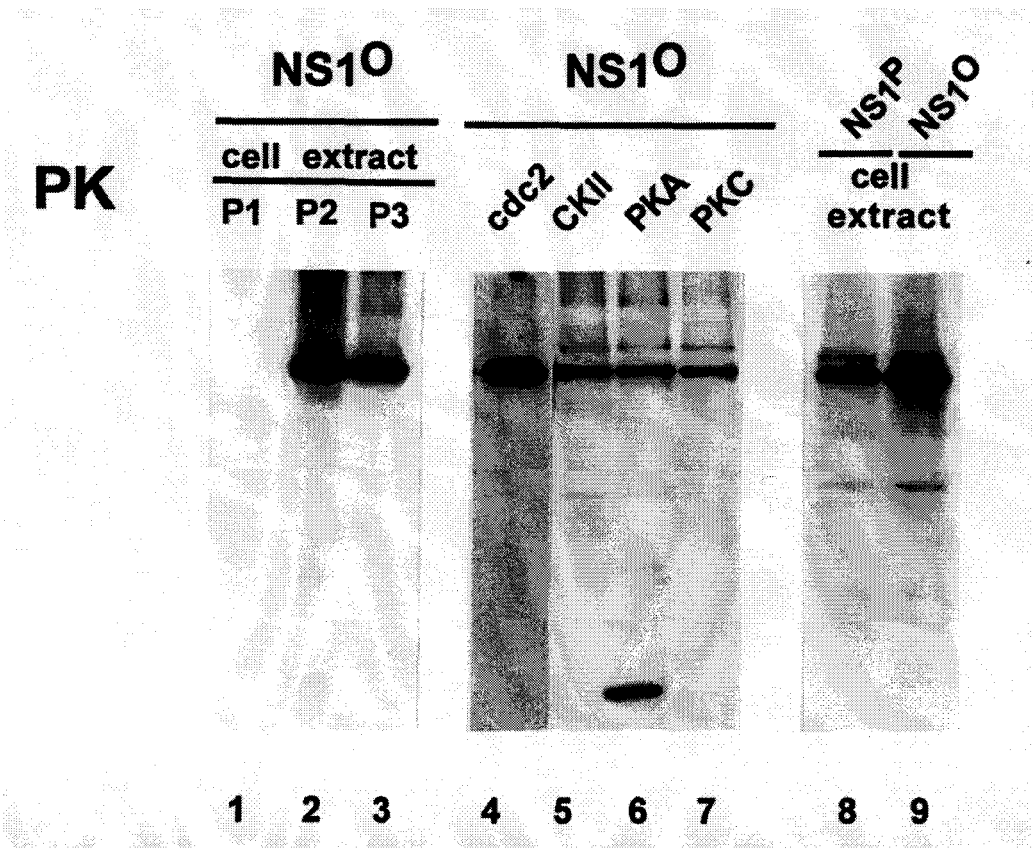
The 3' dimer-bridge containing plasmid pLEB711 was subjected to resolution and replication in fully competent HeLa replication extracts in the presence of dNTP including [α-32P]dATP, ATP and an ATP regeneration system, using native or dephosphorylated wild type NS1 and *dl*C67, respectively. The reactions were stopped by adding 0.2% SDS and heating at 70oC for 30 min to disrupt non-covalent binding of NS1 to the replicated DNA. The newly synthesized 32P-labeled DNA was then immunoprecipitated using αNSN, digested by ScaI, allowing the resolved NS1-attached (B) products and NS1-free (S) products to be isolated and analyzed separately by 1% agarose gel electrophoresis and autoradiography. The linearized unresolved plasmid, labeled either by non-specific nick-translation (S), or by rolling circle replication and incomplete resolution (B) of the circular plasmid, as well as the complete resolution products, the TC, and GAA arms of the resolved plasmid are indicated. As reported previously, *in vitro* resolution of pLEB711 by NS1 is asymmetric, producing predominantly the extended form of the GAA arm (NS1-bound) and the turnaround form of the TC arm (NS1-free).

Reactivation of NS1 helicase activity by in vitro phosphorylation

In order to test the assumption that phosphatase-treated NS1 can be reactivated by in vitro phosphorylation, we analyzed for an NS1 function that can be measured in the absence of cellular components (i.e. without any endogenous protein kinases). The helicase activity of NS1 was studied in this respect, given its above-mentioned dramatic suppression as a result of NS1 dephosphorylation. First, we determined whether phosphatase-treated NS1 could indeed be re-phosphorylated in vitro by incubation with [g-32P]ATP in the presence of either crude cell extracts as a supplier of protein kinases, or commercially available cAMP/GMP dependent kinase, protein kinase C, casein kinase II, or cdc2 complex. As shown in Fig. 8, dephosphorylated NS1 was indeed a target for these various kinases under in vitro conditions. As expected from previous studies showing that a variety of phosphoproteins are underphosphorylated in vivo compared with their possible phosphorylation in vitro (43), native NS1 could also be further phosphorylated in vitro, yet to a lower extent than phosphatase-treated NS1 (Fig. 8C).

It was then investigated whether the lost helicase activity after phosphatase treatment could be recovered at least in part, by re-phosphorylation of the viral protein in vitro. To this end, phophatase-treated NS1 was incubated with protein kinases that were either commercially available in a purified enough state, i.e. did not present significant helicase and/or DNase activity (casein kinase II and protein kinase C), or were present in crude extracts which could be used at dilutions exhibiting no measurable endogenous helicase activity. Casein kinase II failed to reactivate the helicase function of phosphatase-treated wild type and NS1:dlC67, and did not affect the helicase activity of the native protein (Fig. 9A). In contrast, protein kinase C proved able to restore substantially the helicase activity of dephosphorylated NS1 wild type as well as dlC67 (Fig. 9B). Since the extent of NS1 phosphorylation achieved by both protein kinases was similar (Fig. 8), this result suggests that the up-regulation of NS1 depends on its phosphorylation at specific site(s). Furthermore, the helicase activity of phosphatase-treated NS1 could also be rescued by incubation of the viral product with phosphocellulose fraction P2 (150 mM to 400 mM NaCl elution) derived from HeLa replication extracts (Fig. 9B), but not with fraction P3 (400 mM to 1000 mM NaCl elution) (data not shown). It is worth noting in this respect that protein kinase C and casein kinase II segregate into fraction P2 and P3, respectively (57, 71).

In order to ascertain that the above-mentioned modulations in helicase activity were due to the phosphorylation state of NS1 and not to side-effects caused by the treatments used, NS1 was also produced in bacteria. NS1 was expressed as a GST-NS1 fusion protein, purified on glutathione sepharose, and further analyzed after cleavage of the 27.5 kDa GST N-terminus with enterokinase. Despite possible protein phosphorylation in bacteria (43), figure 9 shows that little helicase activity could be detected using up to 100 ng of bacterially produced NS1



Eigure 8. *In vitro* phosphorylation of NS1.

Phosphatase treated (O) or native (P) wild type NS1 was incubated with fractionated cell extract or commercially available protein kinases in the presence of [γ-32P]ATP, immunoprecipitated with αNSN, and revealed by autoradiography after SDS-PAGE. HeLa S100 cell extract was fractionated by chromatography on a phosphocellulose column P11 (Whatman) into P1 (150 mM NaCl flow-through), P2 (150 mM to 400 mM NaCl elution), and P3 (400 mM to 1000 mM NaCl elution) fractions. cdc2, cdc2 complex; CKII, casein kinase II; PKA, catalytic subunit of cAMP/GMP dependent kinase; PKC, protein kinase C. Lanes 1-7, 9: dephosphorylated NS1 used as substrate; 8: native NS1 used as substrate; 1: protein kinases from fraction P1; 2, protein kinases from fraction P2; 3: protein kinases from fraction P3; 4-7: commercially available protein kinases, cdc2 complex, casein kinase 2, cAMP/GMP dependent kinase (catalytic subunit), protein kinase C, respectively; 8, 9: protein kinases from HeLa replication extracts. The heavily labeled 32 kDa protein in lane 6 probably corresponds to the autophosphorylated catalytic subunit of PKA present in high amounts in the reaction.

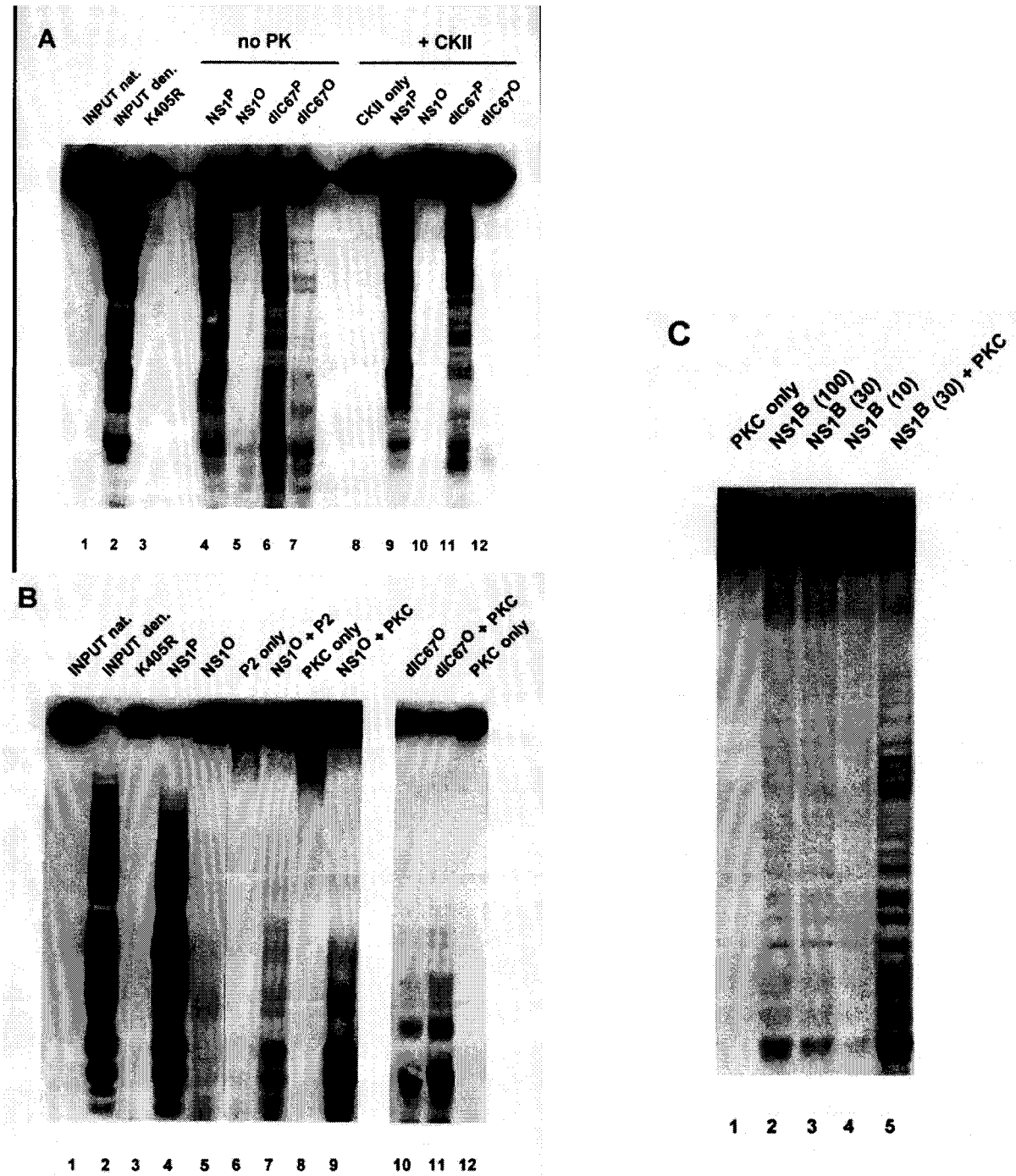


Figure 9. *In vitro* reactivation of NS1 helicase function with protein kinases.

Helicase assays were carried out as in Fig.5, using 10 ng of native (P) or 30 ng of phosphatase treated (O) wild type NS1 or *dl*C67 from recombinant vaccinia virus-infected HeLa cells, or indicated amounts (ng) of bacterially produced NS1. The NS1 mutant K405R served as a negative control. Native and denatured input DNAs are shown as references.
(A) Vaccinia virus-produced NS1 was incubated with 5 mU casein kinase II (CKII) or without protein kinase (no PK). Lane 1: native input substrate, 2: heat-denatured input substrate; 3: mutant NS1 used as negative control; 4, 5, 9, 10: wild type NS1; 6, 7, 11, 12: NS1*dl*C67; 4, 6, 9, 11: native NS1; 5, 7, 10, 12: dephosphorylated NS1; 4 to 7: no protein kinases added; 8 to 12: casein kinase 2 added to the reactions.
(B) Dephosphorylated vaccinia virus-produced NS1 was incubated with P2 fraction derived from HeLa cells (as in Fig. 8), or protein kinase C (PKC, 0.1U). Lane 1: native input substrate; 2: heat-denatured input substrate; 3: mutant NS1 used as negative control; 4: native wild type NS1 used as positive control; 4, 5, 7, 9: wild type NS1; 10, 11: NS1*dl*C67; 5, 7, 9, 10, 11: dephosphorylated NS1; 6, 7: fraction P2 added to the reaction; 8, 9, 11, 12: protein kinase C added to the reaction.
(C) Bacterially produced NS1 (NS1B) was tested either in absence (lanes 2 to 4), or in the presence of 0.1 unit of protein kinase C (lane 5). The amount of NS1 used in the assays is indicated (ng). Lane 1: helicase assay using 0.1 unit of PKC in the absence of NS1.

per reaction in absence of added protein kinases. However, when the reaction was supplied with protein kinase C, a striking increase was observed in the helicase activity shown by the bacterially-produced NS1 preparation, which was now activated to more than 10% of the level determined for an equivalent amount of native, HeLa-derived NS1. Therefore, these results substantiated the dependence of NS1 helicase function on a phosphorylation pattern that cannot be achieved effectively in a bacterial context, but is provided to a significant extent by incubation with PKC.

Discussion

The NS1 protein of MVM, a parvovirus, exhibits a number of functional analogies with the large T antigen (LT) of simian virus 40 (SV40), a papovavirus. NS1 is able to form oligomers, bind and hydrolyze NTPs, and shows DNA helicase activity, site-specific interaction with target DNA motifs, and transcriptional regulation, in addition to serving as the essential initiator protein for viral DNA replication, all properties shared with SV40 LT (reviewed in (31, 59)). Furthermore, NS1 shows striking amino acid homology with SV40 LT within the putative helicase domain (2). The replicative activity of SV40 LT is regulated positively and negatively by phosphorylation. The cyclin-dependent kinase cdc2 activates LT by phosphorylation of threonine 124 (46, 49), leading to the formation of double-hexamers competent for bi-directional unwinding of the SV40 origin in vitro. Interestingly, mutation of T124 to alanine, while blocking this activation of the unwinding function has no effect on the intrinsic DNA helicase activity of the molecule (49). Recently, it has been shown that phosphorylation of T124 activates the minimal DNA-binding domain of LT for specific binding to the central pentanucleotide repeats in the SV40 core origin, even in the absence of the LT amino acid sequences required for hexamer formation (47). This suggests that, although T124 is located N-terminal to the minimal DNA-binding domain, phosphorylation of this residue may induce conformational changes within the DNA-binding domain itself. Negative regulation of LT occurs predominantly through phosphorylation of serines 120 and/or 123, which is catalyzed in vitro by a novel form of casein kinase I (CKI), in a reaction which appears to be highly dependent upon the tertiary structure of LT (8), and independent of the usual amino acid context characteristic of other members of the casein kinase I family (9). The exact step of the interaction between LT and the SV40 origin at which this control operates is yet to be determined. Presumably these opposing regulatory phosphorylations, in addition to others, operate in the infected cell to modulate the different activities of LT in an optimal temporal scheme.

The present study comparing the replicative properties of dephosphorylated NS1 (NS1O) with native NS1 (NS1P), both produced in HeLa cells, provides preliminary evidence for a regulation of NS1 by phosphorylation. Like SV40 LT, un- or under-phosphorylated NS1 is still able to bind

to its cognate origin sequence, and does so with substantially higher affinity than the native, phosphorylated polypeptide. This gain of function clearly demonstrates that the dephophorylation procedure used here does not affect the integrity of the polypeptide. It also suggests that the loss of nickase, helicase and ATPase activities observed upon dephosphorylation are part of an NS1 regulation by protein kinases in a way that newly synthesized NS1 has to become phosphorylated first before it is active for viral DNA replication. This hypothesis is substantiated by the finding that both, dephosphorylated NS1 as well as un(der)-phosphorylated NS1 produced in bacteria, became activated for helicase function upon phosphorylation by PKC preparations. Interestingly, all functions of NS1 investigated here are dependent upon an intact ATP-binding domain (13, 25, 56), and thus most likely controlled by ATP-binding and hydrolysis. Consequently, regulation of the NS1 ATPase by phosphorylation might be of central importance for the regulation of other activities of NS1.

ATP-dependent interaction of proteins with their target DNA has been described for a variety of processes (34, 40, 45, 58) in addition to NS1-driven initiation of viral DNA replication (25). A reason for this co-factor dependency could be that a constitutive high affinity association of these polypeptides with target DNA, as seen for many transcription factors, would restrict their multiple activities. Initiator proteins for DNA replication, such as bovine papilloma virus E1 or SV40 LT, bind site-specifically with high affinity to the origin at a distinct location within the viral genome in order to organize a pre-initiation complex. In addition, these proteins exert the origin-unwinding that is required for polymerase a primase to initiate DNA replication, and at a later stage they facilitate the copying of the parental DNA through their processive helicase activity to unwind the double-strand DNA in front of the replication fork. An alternative example is given by the pentapeptide replication factor RF-C. This protein uses ATP-dependent DNA interaction to assemble a complex with PCNA on the DNA, which is followed by scanning on the parental duplex, and positioning of this RF-C/PCNA complex at the free 3' OH of the initiator DNA. ATP-hydrolysis induces a conformational shift which stabilizes the protein complex on the DNA and allows it to target polymerase d to the DNA (34).

For NS1, site-specific binding to the origin has been shown to be dependent on the presence of ATP or a non-hydrolysable ATP-analogue such as ATP-g-S, and conditions leading to reduced ATP-hydrolysis increase the affinity of NS1 for its cognate element (25), possibly due to the involvement of bound ATP in formation of higher order oligomers (54, 69). Up-regulation of the affinity for the [ACCA]-element by dephosphorylation could favor organization of pre-initiation complexes with other replication factors known to be required for MVM replication, such as RP-A, PCNA, DNA-polymerase, or the newly described initiation factor PIF (12). In addition, NS1 has been shown to be able to interact with multiple recognition-sites distributed over the entire

genome (25), which have been proposed to serve in association with NS1 for assembly of the replicative-form DNA into nucleosome-like structures (30). This kind of high affinity interaction of NS1 with its cognate element might involve preferentially newly synthesized and therefore un(der)-phosphorylated NS1. The reduced ATPase activity seen for NS1O might contribute additionally to this phonotype.

On the other hand, a constitutive tight association of NS1 with its origin-recognition sequence might have considerable negative consequences for other replicative functions of this protein. In particular, a tight binding of NS1 to the origin would interfere with helicase activity necessary for unwinding the double-stranded template as the replication fork proceeds. The observation that both NS1 functions, nickase and helicase activity are dependent upon ATP hydrolysis, and are negatively affected by the lack of phosphorylation, indicates that they may be also controlled in part by regulation of the NS1 ATPase. However, a more complex regulation by phosphorylation, similar to that seen for SV40 LT, is probably necessary to enable the transition of NS1 from a site-specific double-strand DNA-binding protein to a helicase which has single-strand DNA-binding properties. How this transition occurs has not yet been established, but it is possible that a conformational change(s) within the NS1-multimer which occurs during ATP-hydrolysis, directly changes relative affinity of NS1 for duplex versus single-strand DNA.

NS1 is a pleiotropic protein that contributes to additional aspects of the parvovirus life-cycle besides replication, such as trans-activation of the P38-promoter which directs the expression of the capsid genes (61). NS1 is also a cytotoxic effector molecule, the expression of which can lead to the eventual death of the target cell (6). These NS1-dependent events necessary for virus propagation take place in a temporal order (62). Thus, expression of the structural genes peaks after the burst of viral DNA replication and prior to the appearance of visible cytotoxic effects (18). We have shown that NS1 dephosphorylation suppresses functions associated with initiation of viral DNA replication, while its site-specific affinity to the [ACCA]-DNA motif was enhanced. NS1 acts as a trans-activator of the P38 promoter by binding to the repeated consensus NS1 recognition motifs located within the tar element, the cis-acting element responsible for trans-activation of this promoter. The enhanced affinity of hypophosphorylated NS1 for this particular binding-site might lead to an increase in P38 promoter activity, thus tipping the balance between parvovirus DNA replication and structural gene expression. Such a regulation could favor capsid protein production at later stages of infection, and thereby promote the assembly of progeny virus particles. It remains to be determined whether pre-formed NS1 undergoes dephosphorylation, and/or newly synthesized NS1 fails to become phosphorylated, as the virus cycle progresses.

Though demonstrated here under in vitro conditions for a few distinct NS1 activities, the phosphorylation-dependent up-modulation of NS1 replicative functions may take place in infected cells. This suggestion is supported by the finding that cell extracts can fully reconstitute the replicative activities of dephosphorylated NS1, resulting in resolution and replication of the cloned dimer-bridge fragment. Moreover, helicase activity, the NS1 function most affected by dephosphorylation, could be restored by re-phosphorylation due to kinases present in the replication extracts or purified recombinant protein kinase C (28). This in vitro specificity encourages us to search for distinct protein kinases involved in the regulation of NS1 functions. During MVM infection, NS1 is phosphorylated at multiple positions, and it is presently unknown which of these phosphorylation site(s) is relevant for regulation of NS1. It is interesting to note that helicase activity of NS1O is rescued specifically by protein kinase C preparations, knowing that this protein kinase family has also been implicated in neoplastic transformation at the cellular level (44). This correlates with the longstanding observation that parvoviruses preferentially replicate in transformed cells, compared to the non-transformed parental cells (15).

Recently we have established an in vitro replication system devoid of endogenous protein kinases (28, 55), which supports initiation of replication by NS1P but not NS1O, providing further evidence that dephosphorylation of NS1 indeed down-regulates initiation of replication, and that the protein kinases are responsible for restoring the NS1 activity. This tool should allow us to screen for distinct protein kinases capable of phosphorylating NS1 and thus activating the protein for replicative functions. In addition, on-going research is aimed at identifying the phophorylation sites responsible for the regulation of distinct NS1 functions in infected cells.

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II) Phosphorylation of the Viral Non-structural Protein NS1 during MVMp infection in A9 cells.

Manuscript in preparation

Phosphorylation of the Viral Non-structural Protein NS1 during MVMp Infection in A9 cells.

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Running Titel: NS1 phosphorylation during MVM infection.
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Abstract

The major non-structural protein of parvovirus MVMp, NS1 is 83 kDa nuclear phosphoprotein which exerts a variety of different functions during the course of a viral infection. These multiple tasks range from its major involvement in viral DNA amplification, promoter regulation to the cytotoxic action to the host cell. Since these most deviant functions require different properties of a protein, it has been proposed that NS1 is regulated by post-translational modifications in particular phosphorylation. So far it has been shown that NS1 is regulated in vitro for initiation of replication by phosphorylation through members of the protein kinase C family, most likely by a control of its DNA unwinding activity. To substantiate these in vitro findings in vivo we investigated NS1 phosphorylation during the course of an MVMp infection in a natural host cell, A9 fibroblasts in comparison to characteristic features of the virus cycle. By these means we observed an altered phosphorylation pattern during the course of infection indicating that indeed different tasks of NS1 might be achieved by differential phosphorylation of the polypeptide. In addition, we present in vivo evidence that phosphorylated NS1 initiates viral DNA replication becoming covalently attached to replicated DNA. Moreover, the localization of the major phosphorylation events within the putative helicase domain and the alignment with of a phosphopeptide with in vitro phosphorylated NS1 by an "activating" kinase suggests a possible

regulation similar to the observed regulation for replicative functions in vitro during the course of a viral infection.

Introduction

Parvovirus minute virus of mice (MVM) are small icosahedral particles with a single-strand linear DNA of approximately 5.1 kb as a genome. MVM consist of two strains according to their host range requirements, the lymphotropic MVMi, and the fibrotropic prototype MVMp (4). Unlike small tumor viruses, such as simian virus 40, parvoviruses are unable to drive quiescent cells into S-phase. In contrast, they are highly dependent on the host cell to enter S-phase to achieve a productive infection. The small DNA genome of MVM encodes for two structural (VP) and at least four non-structural (NS) proteins. NS1 the major non-structural gene product is an 83 kDa, mainly nuclear phosphoprotein, which exerts a variety of functions during virus propagation (20, 21). NS1 is the only viral non-structural protein necessary in all cell types. The small non-structural proteins, NS2 consist of three similar proteins that differ in their unique C-termini only (18). These viral polypeptides are dispensable in transformed human cell lines (28, 48, 49).

In a productive parvovirus infection the single-strand genome is converted into a covalently closed monomer circle in the nucleus. This reaction is exerted in the nucleus by cellular polymerases and is able to serve as a template for transcription. Analyses performed in vitro have shown that this reaction requires S-phase components and/or components from GO/G1-phase that were stimulated by cyclin A (5). For further amplification of the viral DNA the major non-structural protein NS1 is required, which is synthesized from the "early" P4 promoter. Viral DNA amplification occurs by a single-strand strand-displacement mechanism, similar to the rolling circle replication described for bacteriophages and single-strand plasmids, initiated by NS1 through site- and strand-specific nicking within the origin sequences located at either termini of the genome (21). This mode of DNA amplification leads to the formation of concatemeric form DNA (68), which allows the reproduction of the viral telomers during resolution by NS1 (19, 23, 24). During these nicking reactions, which serve for initiation of viral DNA replication, NS1 becomes covalently attached and remains at the 5'ends of newly synthesized viral DNA (22, 25, 68). Progeny single-strand DNA are encapsidated late in infection concomitantly with replication (20) into virus particles composed of the structural proteins VP1 and VP2. These proteins are derived from transcripts controlled by the late P38 promoter. After encapsidation the NS1 protein attached to the 5' end of the virion DNA remains at the outside of the infectious particle (22).

A number of studies carried out in vivo (24, 41, 45, 66) or in vitro (3, 19, 23, 36, 50, 52) with wild type NS1 or derivatives modified by site-directed mutagenesis, have clearly demonstrated the

key role of this protein during distinct steps of parvoviral replication. In particular, NS1 is required for the hairpin transfer of the right-end telomere of monomeric replicative forms (3, 16) and resolution of concatemeric replication intermediates (19, 23). Moreover, partial purification of NS1 has allowed a variety of biochemical activities to be assigned to the NS1 protein and some of these activities could be mapped to distinct domains of the multifunctional protein by the use of these site-directed mutants (cf. Fig. 6A). Thus, NS1 forms oligomers (51, 60), exhibits intrinsic ATP-binding, ATPase and helicase activities (14, 72), binds site-specifically to an [ACCA]2-3-element present at multiple positions in the viral genome (12, 27), mediates the site- and strand-specific nicking of replication origins located in the left- and right-end terminal sequences, and becomes covalently attached to the 5' end of replicated viral DNA (13, 16, 52). Besides these multiple functions during viral DNA replication, NS1 possesses a C-terminal acidic transcription activation domain (39) that is able to transregulate the parvoviral and various heterologous cellular and viral promoters (32, 40, 62, 69, 70). Furthermore, NS1 is able to induces alterations to the host cell, such as cell cycle in S/G2-phase (58), induction of chromosome breaks (57), and apoptosis (61), which account at least in part together with the modulation of cellular promoters for the cytotoxic effects of NS1 to the host cell (9, 11, 47). By deletion mutagenesis it was shown that the N- and C-terminal part of the polypeptide appear to be import for cytotoxic action of NS1 (40). For complex NS1 functions, such as the initiation of DNA replication by NS1 or P38 promoter activation, multiple NS1 functions are required, localized in different parts of the polypeptide (12, 43, 52), whereas NS1 interaction with cellular protein components (29, 38, 43) might play an important regulatory function as well.

The multitude of NS1 activities during virus propagation, the coordinated action of biochemical activities to perform these tasks, and the temporal regulation during the course of a viral infection (65) lead to the assumption that the different NS1 functions might be regulated by post-translation modification. Since NS1 has been shown to be phosphorylated during infection (26) and the analogy to the functional and structural similar SV40 large T (LT) antigen (1) suggested a possible regulation of NS1 by phosphorylation. Indeed, comparing biochemical activities of dephosphorylated NS1O with native NS1P produced by vaccinia virus expression in HeLa cells, there were significant differences in the functional properties of these preparation in regard to replication activity. While NS1P was able to support rolling circle replication of plasmids containing an active left-end origin in absence of endogenous protein kinases, NS1O was impaired for this function unless stimulated by selected protein kinases (54). This defect could be attributed significant reduction of NS1O in ATPase, site-specific endonuclease, and in particular helicase activity, while its affinity to the cognate DNA-motif was enhanced (53).

In order to investigate whether a regulation of NS1 by phosphorylation as observed in vitro would occur during a natural infection, we analyzed the phosphorylation of NS1 during the course of an MVM infection in A9 cells. These analyses revealed that already early in infection, NS1 becomes increasingly phosphorylated on serine and threonine residues. In addition, there is a significant modulation of the NS1 phosphorylation pattern according to the different functions that are achieved by the polypeptide during the course of infection, indicating a possible modulation of NS1 activities by phosphorylation. Moreover, the involvement of phosphorylated NS1 in viral DNA replication, the localization of major phosphorylation events preceding onset of DNA amplification within the putative helicase domain, and the alignment of peptides with phosphopeptides derived from an "activating" kinase in vitro, suggest that a similar regulation of NS1 could take place in vivo.

Material and Methods

Mammalian cell lines, viruses and synchronization. A9 cells were cultured in minimal essential medium (MEM, Gibco) supplemented with 5% fetal calf serum. Hela and BSC-40 cells were grown in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 5% fetal calf serum. Synchronization was achieved by serum deprivation. The cells grown in monolayers were washed three times with PBS and incubated for three days in MEM containing 0.5% serum. Before infection of the arrested cells, the efficiency of synchronization was determined by FACS analysis. Cell population with higher than 85% GO/G1-content were then infected with 10 pfu/cell CsCl-purified MVMp and incubated for 15 h or the indicated period before release into S-phase by addition of 20% of serum. The release of the arrested cells into S-phase was again monitored by FACS analysis. Recombinant vaccinia viruses were propagated in monolayer cultures of BSC-40 cells and purified over a sucrose cushion (44).

Metabolic labeling of NS1, purification, and phosphopeptide analyses. After infection with MVMp (10 pfu/cell), A9 cultures were incubated for the indicated time in medium containing 5% fetal calf serum. Cultures (107 cells) were then labeled with [32P]-orthophosphate (ICN, 10-10 Ci/cell) or [35S]-translabel (ICN, 2x10-11/cell) in complete medium without phosphate (Gibco/BRL) for an additional 4 h period for phosphate-labeling, 2 h in complete medium lacking methionine and cysteine for 35S-labeling, respectively, and harvested directly in 1 ml of RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Na-deoxycholate, 1% Triton X-100), containing the protease inhibitors PMSF, leupeptin and pepstatin as well as phosphatase inhibitors (20 mM NaF, 5 mM b-glycophosphate, 5 mM p-nitrophenyle phosphate, 5 mM sodium molybdate, 1 mM sodium orthovanadate, 5 mM sodium phosphate). Immunoprecipitations were carried out using 10ml of aSP8 (10)[Cotmore, 1986b #91] for 2 h at room temperature. Immune complexes were precipitated with protein A-sepharose (Pharmacia), washed three times with RIPA buffer and further analyzed or purified by 10% SDS-PAGE. [32P]-labeled proteins were revealed by autoradiography after blotting on polyvinylidene fluoride (PVDF) membranes (Millipore) [Mansfield, 1994 #114] , and the band corresponding to NS1 was excised. Digestion of membrane bound NS1 was performed with 50 units of trypsin (Promega) for 18 h at 37oC. Tryptic peptides contained in the supernatant were recovered by lyophilization and analyzed on thin layer cellulose plates (MERCK) in two dimensions, by electrophoresis using pH 1.9 buffer and chromatography in phosphochromatography buffer [Boyle, 1991 #79]. Phospho amino acid analysis was performed either from the whole protein blotted on PVDF or alternatively from individual tryptic phosphopeptides recovered after two-dimensional analysis on thin-layer chromatography plates. The 32P-labeled protein/peptide was hydrolyzed in 6 M HCl at 110OC for 1 h, and the products were analyzed on thinlayer

chromatography plates by electrophoresis in two dimensions using pH 1.9 and pH 3.5 electrophoresis buffer (8) by autoradiography. The positions of phosphoserine, phosphothreonine, and phosphotyrosines were monitored by a ninhydrin reaction of 1 mg each of the unlabeled purified amino acids loaded together with the 32P-labeled digests. For CNBr-cleavage, 32P-labeled proteins were blotted on nitrocellulose excised and treated with 50 mg/ml CNBr in 70% formic acid for 1 h at room temperature (33) and analyzed by three part SDS gel (64) using 16.5%T:6%C as separation gel. For further peptide analysis the products were transferred to PVDF membranes and treated the same as the whole proteins.

Immunoprecipitated of 32P-labeled NS1/DNA complexes. NS1/DNA complexes were labeled metabolically labeled for the indicated time with [32P]-orthophosphate as described 20 h post infection in asynchronuously growing cells (53). The chase period was performed for the indicated time replacing the labeling medium with complete medium containg cold phosphate. The complexes were isolated by immunoprecipitations using aSP8 and analyzed by 10% SDS-PAGE and autoradiography. DNase-treatment of the complexes was performed after three washed in PBS by incubation (or not) with 125 units of the endonuclease Benzonase (Merck) for 30' at 37°C in a digestion buffer (Tris-HCl pH8.0, 1mM MgCl2, 100mg/ml BSA).

Western blotting and ECL detection. Proteins of 107 cells (infected or not) where harvested directly in RIPA-buffer as described for metabolically labeled proteins.. 40 mg of protein extracts where separated on 10% SDS-PAGE, blotted on nitrocellulose and the identification of the individual proteins was performed using a 1:2000 dilution of rabbit aSP8 for NS1 and aSP6 for NS2 (10), 1:1000 dilution of rabbit adenatured-capsid antibody (17) to reveal VP1 and VP2, and a 1:1000 dilution of the mouse monoclonal antibodies aPKCa, aPKCe, and aPKCi to the individual protein kinase C isoforms, respectively. Detection of the protein-antibody complex was the performed with a 1:5000 dilution of horseradish peroxidase conjugated arabbit/mouse IgG and ECL (Amersham). To analyze NS1 for phosphorylation in western blots, cell extracts were performed in water containing protease inhibitors, subjected to three cycles of freezing thawing and passed through a syringe. The resulting extract was subjected to phosphatase treatment (or not) with 20U of potato acid phosphatase for 30min at 30°C.

DNA extraction and Southern blot. Whole cell DNA extracts where performed as previously described (25) by proteinase K digestion and purification by phenol and chloroform extractions. 20 mg of DNA was separated by 0.8% agarose gel electrophoresis and transferred on a nitrocellulose membrane. Hybridization was performed with a random primed 32P-labeled DNA probe corresponding to the entire coding sequence of MVMp (42).

Flow cytometry (FACS). Two dimensional FACS analyses were performed as previously described (55), using aSP8 antiserum in order to detect NS1.

NS1 production and in vitro phosphorylation with HA-1 fraction. Dephosphorylated purified NS1O was prepared as described (53) by recombinant vaccinia virus expression, phosphatase treatment using calf intestine alkaline phosphatase, and purification on Ni2+-NTA agarose columns through an N-terminal [His]6-TAG. The protein kinase fraction HA-1 was purified from crude HeLa extracts by conventional chromatography and FPLC (phosphocellulose, DE-52, protamine, hydroxyl apatite) as described (54) and tested for its potential to reactivate dephosphorylated NS1 in helicase assays. 32P-labeled NS1 was obtained by in vitro kinase assays, subjecting 500 ng of purified NS1O to phosphorylation by HA-1 in the presence of g[32P]ATP and L-a-phosphatidyl-L-serine (54) and purified on SDS-PAGE.

Results

Phosphorylation of NS1 occurs on serine and threonine residues during MVMp infection

In vitro analyses of native NS1P derived from HeLa cell expression and its dephosphorylated counterpart have shown that replicative functions are dependent upon phosphorylation of the polypeptide (53, 54). In order to substantiate these findings under natural conditions, NS1 phosphorylation was investigated in vivo after MVMp infection in its host cell line A9 fibroblasts. Metabolically labeled NS1 was obtained 20 h post-infection, subjecting infected cells for 4 h to [32P]-orthophosphate or [35S]-methionine, respectively. After labeling, the cells were harvested, and NS1 was isolated by immunoprecipitation with aSP8 antisera (10) and analyzed by 10% SDS-PAGE (Fig. 1A). As expected from previously reported results (2, 26), a polypeptide of 83 kDa was revealed after immunoprecipitation that was heavily labeled in the presence of [32P]-orthophosphate. As indicated, by 35S-labeling, the polypeptides corresponding to the 83 kDa NS1 were resolved into several subspecies, indicating that the polypeptide consists of a heterogeneous species that is modified/phosphorylated in more than one way (63). In addition, the 65 kDa species which is recognized by NS1-antisera, the NS1* (26), revealed after 35S-labeling is only poorly if at all labeled by [32P]-orthophosphate as previously reported.

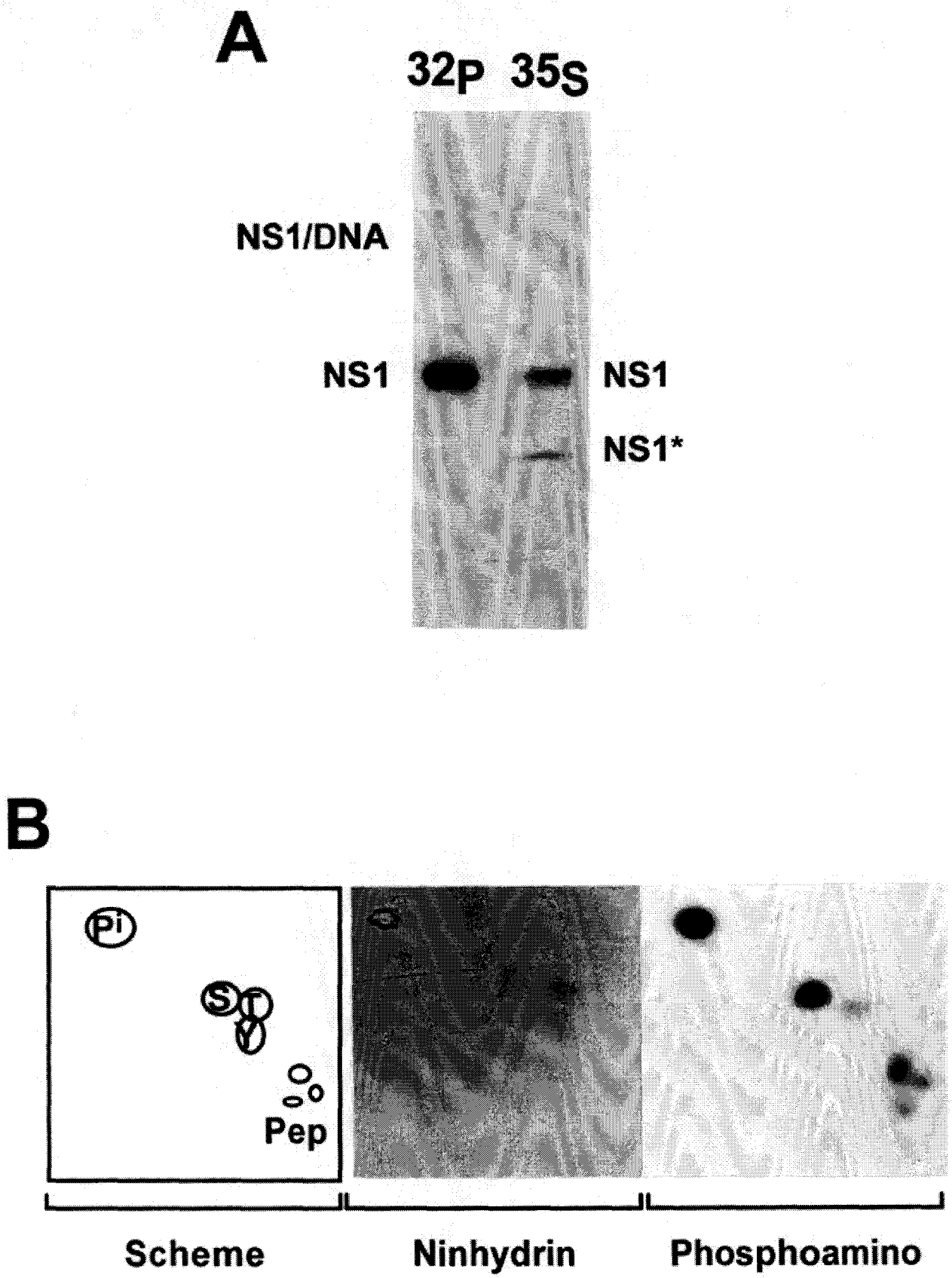

Figure 1: Analysis of NS1 phosphorylation in MVMp infected A9 cells. (A) NS1 was metabolically labeled 20 h post infection in asynchronously growing cells with [32P]-orthophosphate or [35S]-methionine, respectively, as indicated, isolated by immunoprecipitation with αSP8 (10), analyzed by 10% SDS-PAGE and revealed by autoradiography. The migration of NS1, NS1/DNA-complex (as analyzed in Fig.2) and the C-terminally truncated NS1* are indicated. (B) Phospho amino acid analysis of metabolically 32P-labeled NS1. The labeled NS1 protein was subjected the HCl treatment and the resulting hydrolysate was analyzed in two-dimensional electrophoresis using pH 3.5 buffer (8). The left panel presents a scheme interpreting the possible products of this two-dimensional analysis, the middle part shows the ninhydrin stained purified phospho amino acids added to the 32P-labeled hydrolysate serving as markers, the right panel presents the autoradiography of the phospho amino acid analysis of metabolically 32P-labeled NS1.

For further analyses NS1 was gel-purified and hydrolyzed with HCl to determine the amino acid(s) used as target phosphorylation residues. Fig. 1B presents a phosphoamino acid analyses of metabolically 32P-labeled NS1 derived from MVMp infection in A9 cells. Besides the previously reported phospho-serines (2), a significant amount of phospho-threonine was detected in MVM NS1 produced (see also Fig. 5B), similar to NS1 of porcine parvovirus (46). Consistently with all previous reports (2, 46) no phosphorylation on tyrosine residues could be detected. These results clearly demonstrates that phosphorylation of NS1 takes place during natural infections on serine and threonine residues and therefore consists of possible mechanism for regulation of NS1 functions by post-translational modifications.

Phosphorylated NS1 is replication active.

So far NS1 phosphorylation has been reported to occur during productive parvoviral infections (26, 46) and there is some evidence from in vitro assays that this kind of modification is indeed necessary for certain NS1 functions (53, 54). Therefore, it was of interest to determine whether there is a functional relevance for phosphorylated NS1 in infected cells as well. During viral DNA replication, NS1 nicks the origin through its site- and strand-specific endonuclease activity and gets covalently attached to the replicated DNA during this initiation reactions (13, 16, 25, 52). To investigate the involvement of phosphorylated NS1 during this initiation reactions for viral DNA replication we performed pulse-chase experiments.Cellular and viral components were subjected to [32P]-labeling 20 h post MVMp infection in A9 cells for one hour and before replacing the medium containing cold phosphate. Cells were harvested during the labeling and after selected times of the chase-period. 32P-labeled NS1 was isolated by immunoprecipitations with aSP8 and protein/DNA complexes were analyzed by 10% SDS-PAGE. Fig. 2A illustrates that with increasing time in the chase period, unbound [32P]-NS1 decreases with a half-life of approximately 5 h as determined by phosphoimager, while a distinct, slower migrating species, identified as a covalent NS1/DNA complex increases. Moreover, when NS1-immunoprecipitates were treated with Benzonase, an endonuclease with single- and double-strand specificity, the NS1/DNA-complex disappeared in order to convert proportionally to the "free" NS1-species. These nuclease treatments, together with the specific recognition of the complex by aSP8, clearly confirmed that the slower migrating product consists of an NS1/DNA complex. In addition, these pulse-chase analyses also demonstrate that previously phosphorylated NS1 becomes covalently bound to DNA and remains phosphorylated after the reaction, suggesting the involvement of phosphorylated NS1 during viral DNA replication. So far it has not been determined in vivo unequivocally, whether NS1 indeed needs to be phosphorylated to become covalently attached to the viral DNA as shown in vitro.

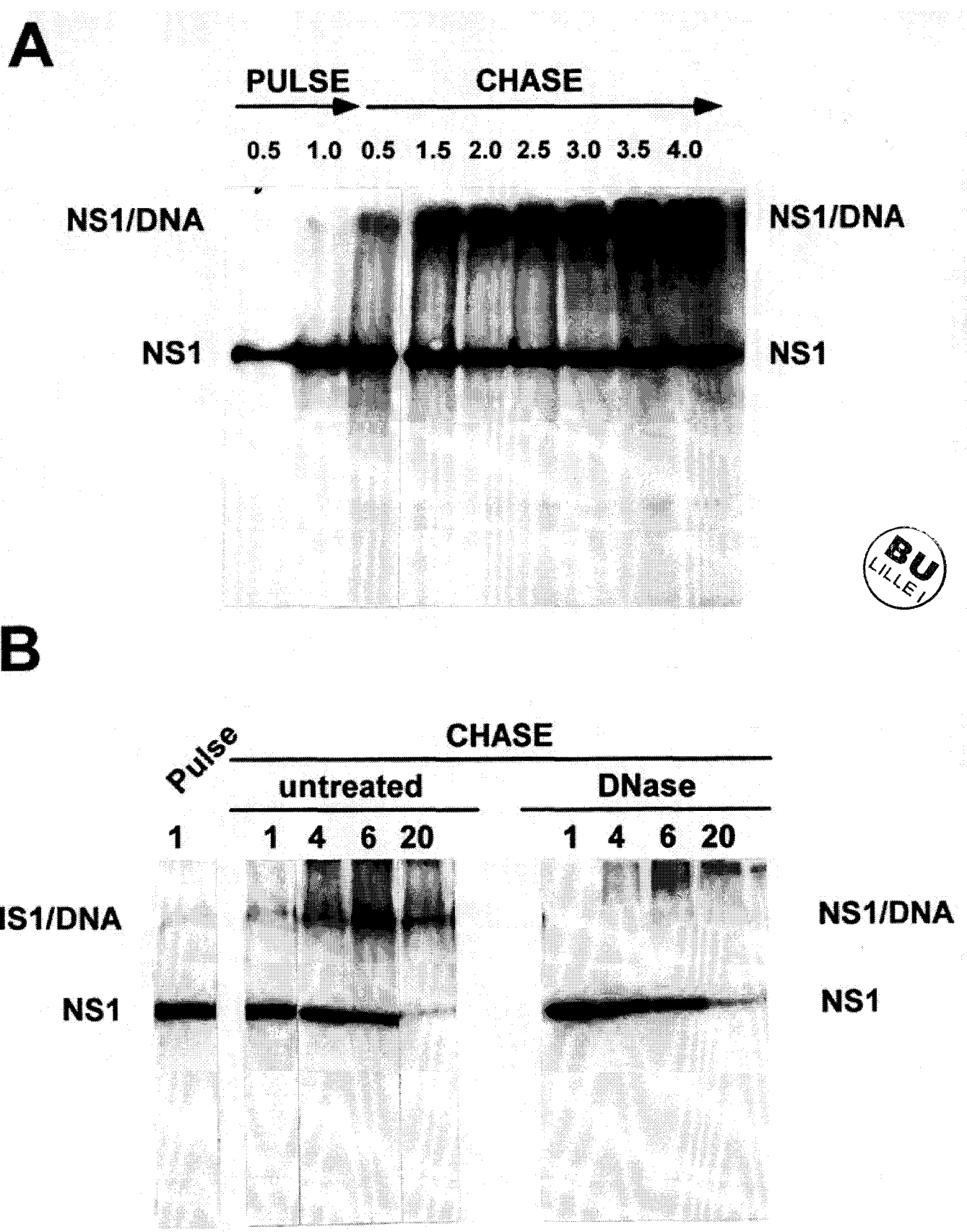

Figure 2: Analysis of metabolically labeled NS1/DNA complexes. Pulse-chase experiments of NS1-attached 32P-labeled were performed 20 h post MVMp infection in asynchronously growing A9 cells. NS1-bound products were isolated at the indicated time by immunoprecipitation with αSP8 antiserum and analyzed by 10% SDS-PAGE. (A) Pulse-chase experiment. The time of labeling during the "pulse"- as well as the "chase"-period after replacement in cold medium is indicated on the top. Migration of free NS1 as well as NS1/DNA complexes are indicated. (B) Pulse-chase comparison of untreated and phosphatase treated isolates.

NS1 phosphorylation increases during virus propagation

NS1 phosphorylation has been suggested to be a mechanism to regulate the various functions of the multifunctional protein. Therefore, it was of interest to determine first the overall phosphorylation rate of NS1during an infectious MVM cycle. A9 cells were synchronized by serum deprivation and infected with 10 pfu/cell MVMp 72 h post starvation in GO/G1-phase as determined by FACS-analysis. 12 h post infections cells were released into S-phase upon addition of medium containing 20% serum, and metabolic labeling was performed using [32P]-orthophosphate in 4 h periods between 0 and 24 h post release, together with two late time points at 36 and 52 h post release. NS1 was isolated by immunoprecipitation with aSP8 and analyzed by 10% SDS-PAGE. Fig 3A demonstrates that NS1 becomes phosphorylated immediately after onset of synthesis, as early as 4 h post release of arrested cells. However, the rate of phosphorylation remains rather weak until the majority of cells enter S-phase (12 h post release), before a massive increase occurs concomitantly with accumulation of the NS1 protein in the cell. During late time points in infection a slight decrease of de novo phosphorylation is observed which could be due to the cytotoxic effects of NS1 during MVM infections. Western blot analyses performed in parallel, confirmed that a consistent accumulation of NS1 occurs during the first 20 h post release before a slight reduction late in infection is observed (Fig. 3B). Interestingly, these western blot analyses revealed NS1 as a highly heterogeneous species with progression of the virus cycle resolving into multiple, up to five discrete bands under high resolution. Such a heterogeneity of NS1 has been reported previously analyzing metabolically labeled NS1 proteins by two-dimensional analysis which resolved the polypeptide produced after natural infection in multiple discrete isoforms (63). Phosphatase treatment of this heterogeneous NS1 population (Fig. 3B, right panel) revealed that most of the slower migrating NS1 isoforms collapsed into a residual, "fastest" migrating form. This species most likely represents the un(der)phosphorylated NS1. The extensive conversion of slower migrating species to "dephosphorylated NS1" indicates that a majority of the NS1 population is phosphorylated, and that this modification causes an altered migration in SDS-PAGE. Despite extensive phosphatase treatment there was still some residual heterogeneity present within the dephosphorylated products. This heterogeneity could reflect either a partial digest, or additional alternative post-translational modifications of NS1 that have not been described so far. It has to be mentioned that NS1 proteins detected up to 4 h post release from starvation are not derived from arrested cells. By FACS analysis it could be demonstrated that all NS1 detected during this period is associated to the reminiscent cell population that escaped cell cycle arrest and is therefore able support a productive MVM infection. In addition to NS1, we also performed western blot analyses for the minor non-structural proteins NS2 and the structural gene products VP1 and VP2 (Fig. 3C). NS2 accumulated predominantly between 12 and 20 post release, significantly after the onset of NS1

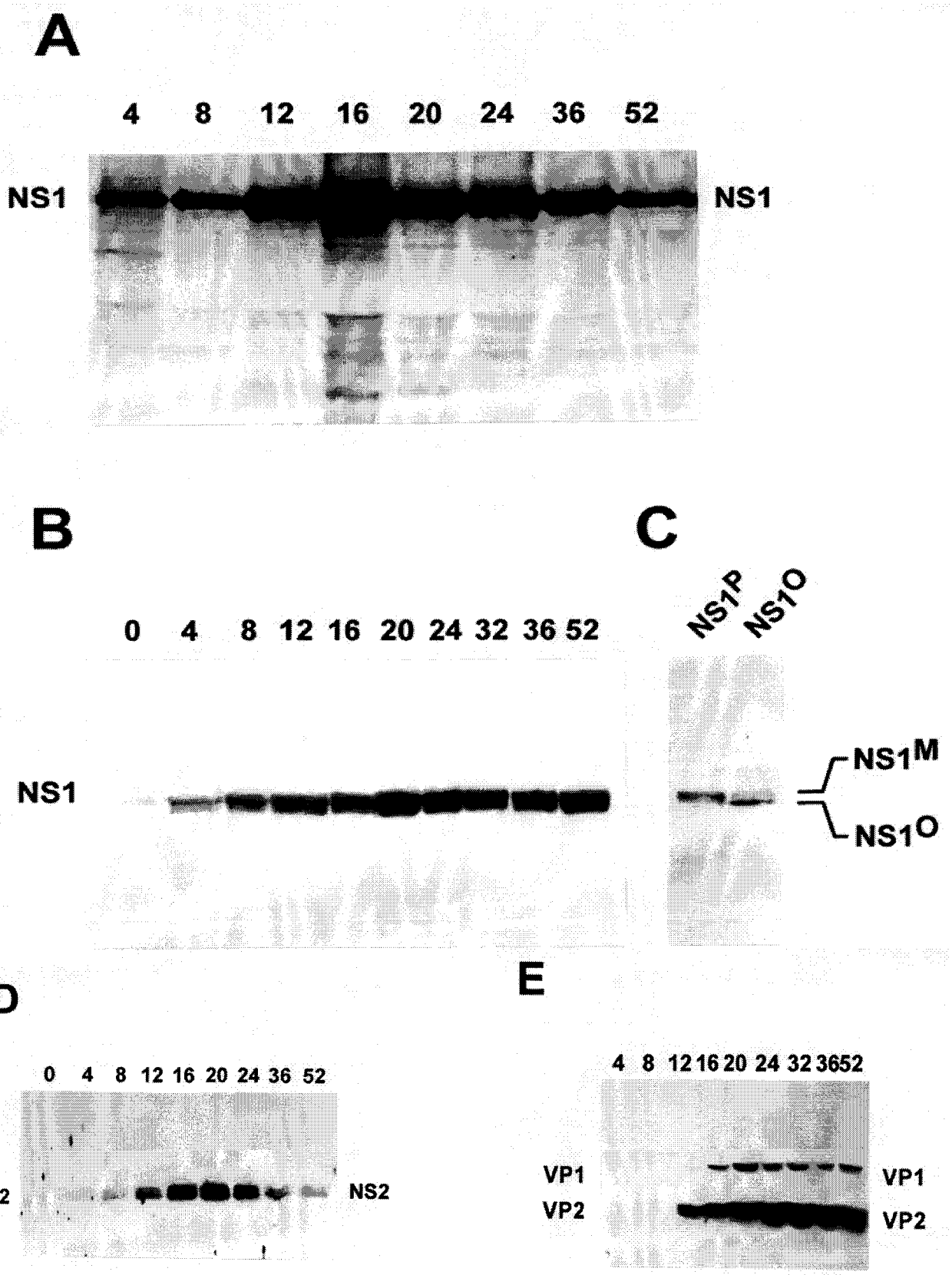

Figure 3: Characterization of MVM non-structural and capsid proteins during the course of a viral infection. Synchronized virus propagation was achieved by synchronization of A9 cells by serum starvation. The rate of overall phosphorylation of NS1 (A), the accumulation of NS1 (B), NS2 (D) and the capsid proteins (E) were analyzed at the indicated time post release of the cells into S-phase. (A) Rate of NS1 phosphorylation was measured by metabolic 32P-labeling for 4 h. 32P-labeled NS1 was isolated by immunoprecipitation with αSP8 and analyzed by 10% SDS-PAGE and autoradiography. The migration of NS1 is indicated. (B) Accumulation of NS1 protein as determined by western blot analysis. 40 μg of infected cells were analyzed by SDS-PAGE and NS1 was revealed by αSP8. The migration of NS1 is indicated. (C) Determination of NS1 phosphorylation in western blots. In order to determine whether the variability of migration of NS1 revealed in western blots is due to phosphorylation protein extracts derived 20 h post infection were analyzed untreated (NS1P) or after phosphatase digest (NS1O) on 10% SDS-PAGE by western blot. The migration of the modified NS1M and the un(der)phosphorylated NS1O are indicated. (D) Western blot analysis of NS2 during the course of a viral infection. The detection of NS2 was performed with the same antiserum used for NS1 detection, αSP8, raised against the common N-terminus of NS1 and NS2. (E) Western blot analysis of the capsid proteins during the course of a viral infection. Detection of VP1 and VP2 was performed with an antiserum raised against denatured MVM empty capsids (Cotmore personal communications).

production. The bulk synthesis of VP1 and VP2, up-regulated by NS1 P38 promoter trans-activation (62) started 12 h post release and continued until late during infection. These analyses of the overall phosphorylation of NS1 during virus propagation demonstrate that the modification of this polypeptide occurs already early in infection and therefore is able to modulate all functions of NS1 including replication. Moreover, since there is no significant decrease in the rate of phosphorylation indicating possible regulation of the polypeptide during the whole virus cycle. Due to the modifications observed in western blots, which continuously increase during the course of infection, it is not likely that late functions of NS1 such as cytotoxicity are induced by massive dephosphorylation of the polypeptide.

Phosphorylation pattern of NS1 alters during the course of MVM infections

The heterogeneity of NS1 (Fig. 3B;(53, 63)), the phosphoamino acid analysis (Fig. 1B), and the reactivation experiments of dephosphorylated NS1 in vitro (53, 54) indicated that selected phosphorylation of NS1 rather than the overall phosphorylation state might decide upon activity of a selected function of the polypeptide. Therefore, we performed tryptic phosphopeptide analyses and compared the phosphorylation pattern of NS1 during the course of a natural MVMp infection in A9 cells. Synchronization of the virus cycle was achieved as presented under Fig. 3, synchronizing the cells by serum starvation, and the progression of the virus cycle was monitored analyzing the production of viral DNA by southern blot. As shown in Fig. 4A, the first step during viral DNA replication, the conversion from the single-strand genome to monomer duplex replicative form occurred early after release of the cells from GO/G1-phase, before NS1 was synthesized (cf. Fig. 3B). During this first phase of the virus cycle NS proteins are synthesized, with NS1 preceding the small non-structural proteins NS2 (cf. Fig. 3 B, C). Concomitant with the onset of S-phase, in a second phase, the viral DNA becomes amplified as seen with the consecutive increase of monomer and dimer replication intermediates 12 h post release, more or less at the same time as the VP-production from the viral P38 promoter becomes apparent (cf. Fig. 3C). 16 h post release, at a stage where already significant capsid proteins are present in the cell, single-strand progeny virus DNA is produced which reflects also encapsidation of the viral genomes to form infectious particles. Fig. 4B illustrates phosphorylation pattern of NS1 during these different stages of a virus cycle. Early in infection, before onset of viral DNA replication (8 h post release) four characteristic phosphopeptides (a to d) were detected, which are of low abundance. This phosphorylation might enable NS1 to establish a productive infection in the cell, i.e. functions of NS1 that are speculative and not well defined, yet. With onset of replication activities of NS1, 12 h post release five major phosphopeptides became apparent (A to E), which were observed during the remaining course of infection. While peptides "A" to "D" became more abundant phosphorylation sites with time, phosphorylation of peptide "E" remained a characteristic target phosphorylation site but did not increase with time. These five

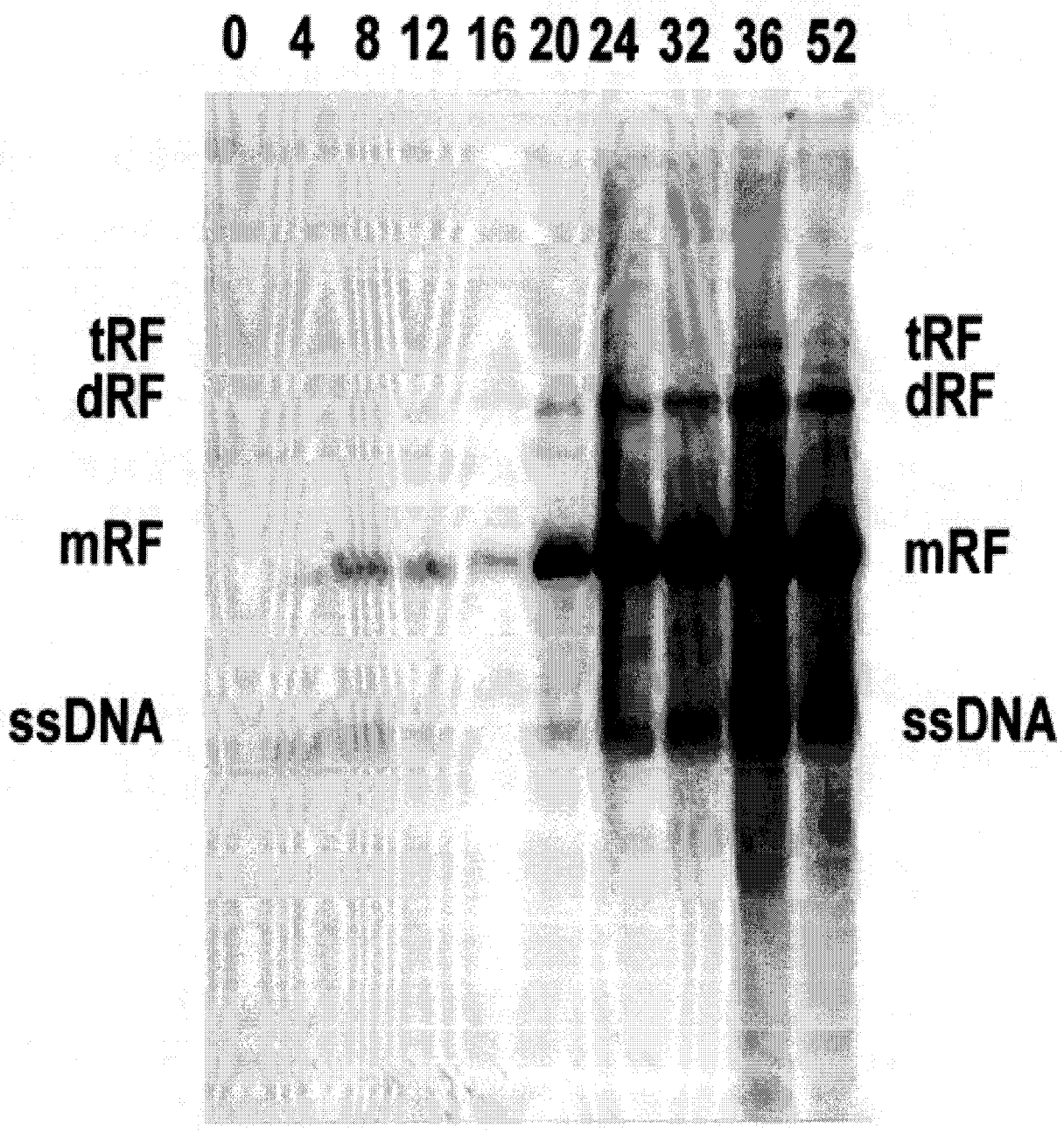

Figure 4: Production of viral DNA during the course of a synchronized MVMp infection in A9 cells. The MVM virus cycle was synchronized by cell cycle arrest and the production of different viral DNAs analyzed at the indicated time post release. Whole cell DNA was separated on 0.8% agarose gels, transferred to nitrocellulose membranes, and the viral DNA was identified by hybridization to a 32P-labeled double-strand DNA probe corresponding to the whole coding region of MVMp. The migration of the different replication intermediates and progeny single-strand DNA are indicated.

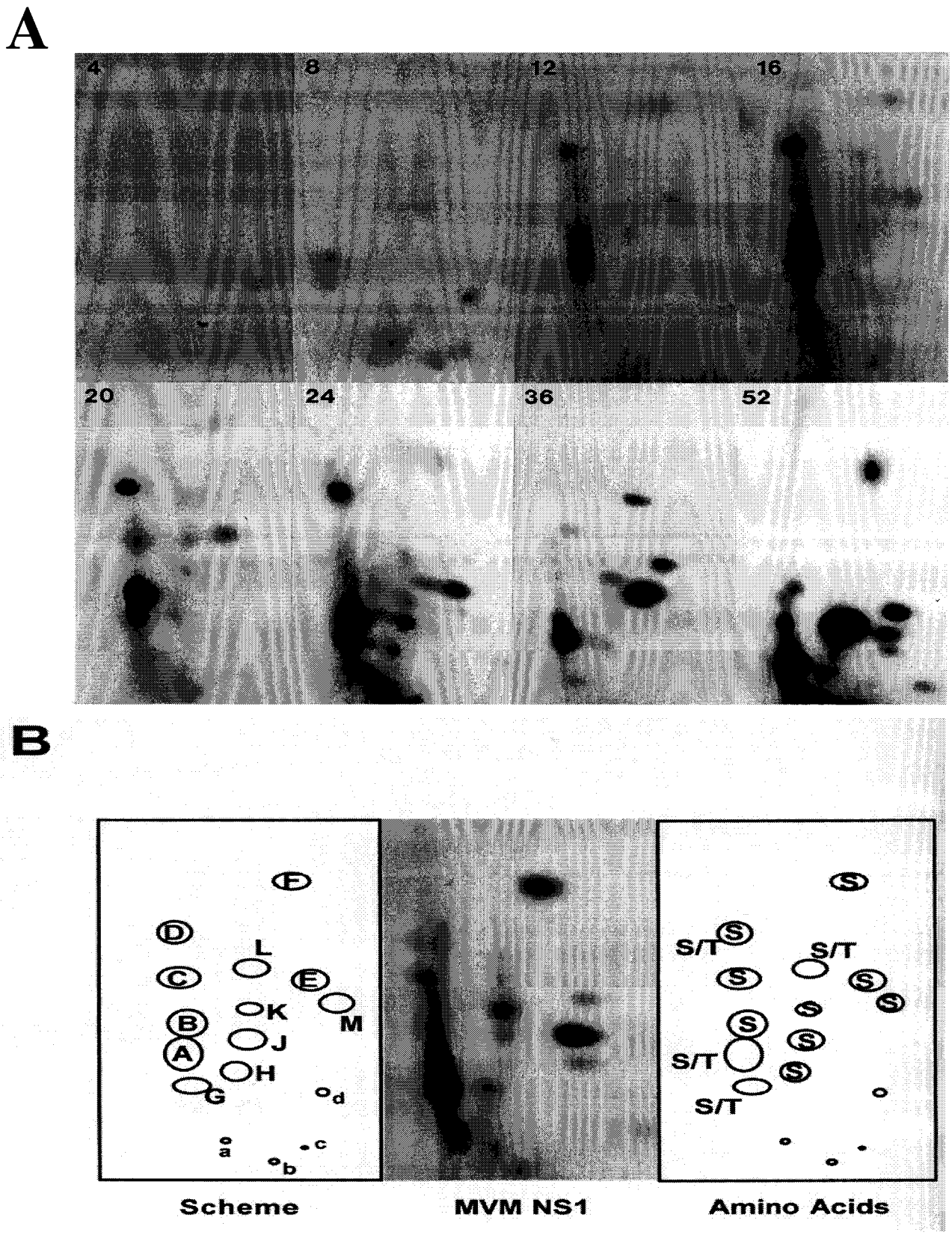

Figure 5: Phosphorylation pattern of MVM NS1 during the course of a viral infection. (A) Metabolically 32P-labeled proteins were isolated at indicated times post release from cell cycle arrest as indicated, isolated by immunoprecipitation with αSP8, gel purified, and subjected to trypsin treatments. The tryptic phosphopeptides were then analyzed by two-dimensional electrophoresis/chromatography. (B) The left panel represents a schematical representation with characteristic tryptic phosphopeptides appearing during the course of a viral infection. Small letters describe phosphopeptides detected early in infection, before onset of viral DNA amplification, Capital letters within the circle peptides appearing with or preceding DNA amplification, Capital letters outside of the circle represent peptides appearing late in infection. The middle part presents a tryptic phosphopeptide analysis of asynchronously produced NS1 24 h post infection. The right panel shows the phospho amino acid analyses of individual phosphopeptides (S stands for phosphoserine; S/T reflects the detection of both phosphoserine and phosphothreonine).

phosphorylation events might be of importance to regulate NS1 activities involved in DNA replication and P38 trans-activation, functions of NS1 that are achieved concomitantly or shortly after phophorylation at peptides A to E. In later phases of infection, reflected by capsid protein production and the appearance of single-strand viral DNA, an indication for encapsidation, a new set of phosphopeptides (F - K) became apparent, indicating the possibility for an involvement in regulation of capsid formation and/or encapsidation. Since these late phosphorylation sites became the most prominent species at times where significant cytopathic effects to the host cell are expected (36 to 52 h post release), they could also be implicated in cytotoxic functions of the NS1 protein. Altogether, the phosphopeptide analysis during the course of a viral infection clearly demonstrates that selected phosphorylation sites in NS1 are targeted together with selected activities of the multifunctional protein, indicating that differential phosphorylation could indeed consist of a mode to regulate the various tasks of NS1 during the virus cycle.

Localization of target phosphorylation sites in NS1

Regulation of NS1 functions by phosphorylation in vitro have demonstrated the necessity of distinct phosphorylation events for initiation of viral DNA replication and support of strand-displacement synthesis thereof (54). This deficiencies of un(der)phosphorylated NS1 could be explained, at least in part, by a defect in DNA unwinding functions as determined in standard helicase assays (53). A variety of biochemical activities including helicase activity have been determined for NS1 and characterized by site-directed mutagenesis (36, 39, 40, 50, 52, 60, 66, 72), leading to the identification of distinct domains in NS1 (cf. Fig. 6A). In order to obtain a further indication about the nature of a possible regulation by phosphorylation, we determined the location of in vivo phosphorylation sites in NS1. Cleavage of NS1 by CNBr at methione residues reveals a characteristic fractionation of the polypeptide (cf. Fig. 6A) that allows to localize major phosphorylation according to the domain-structure of NS1. Metabolically 32P-labeled NS1 was obtained after MVMp infection in A9 cells, isolated by immunoprecipitation with aSP8 and further purified on SDS-PAGE. The purified 32P-labeled NS1 was then subjected to CNBr-cleavage and the resulting fragments were further analyzed by three-part gel electrophoresis which reveales high resolution of small peptide fragments in one-dimension according to their molecular weight (64). As shown in Fig. 6A, most of the NS1-phosphorylation occurred on an 18 kDa CNBr-fragment corresponding to a major part of the NS1 helicase domain, whereas only minor amounts of phosphorylation were observed on an 14 kDa fragment or smaller fragments, respectively. Further fractionation of this 18 kDa peptide by trypsin digestion and the consecutive two-dimensional analysis revealed that three of the major phosphopeptides "A", "D", and "F" are derived from this part of NS1. These data indicate that this phosphorylation events, which are established prior to the amplification of viral DNA, are targeted to the NS1 helicase domain and

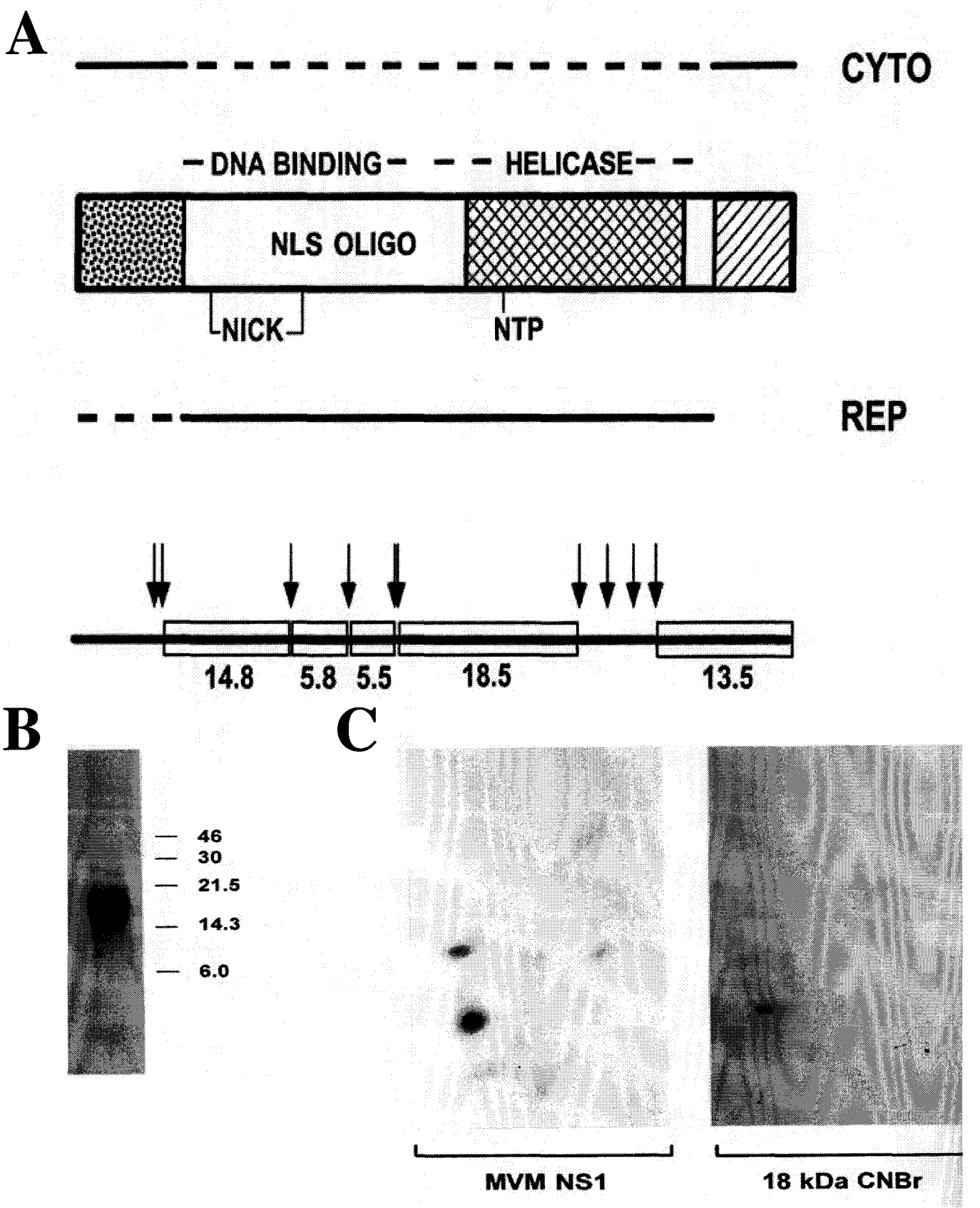

Figure 6: Localization of phosphorylation sites within the NS1 polypeptide. (A) Schematical representation of NS1. The functional domains as identified by site-directed mutagenesis are indicated. The bottom of the figure shows the cyanogen bromid cleavage pattern of NS1. (B) Cyanogen bromide cleavage of metabolically 32P-labeled purified NS1 analyzed on a three-step gel. The bulk of phosphorylation is detected on a fragment of 18.5 kDa corresponding to the main part of the putative helicase domain. Minor bands appear around 14 kDa and 6 kDa as indicated in the lower panel of Fig. 6A. (C) Tryptic phosphopeptide analysis of the 18 kDa CNBr-fragment of NS1. The left panel shows a tryptic phosphopeptide pattern of MVM NS1 derived from asynchronously growing cells (MVM NS1). The right panel presents the tryptic phosphopeptide pattern of the 18 kDa-fragment (18 kDa CNBr).

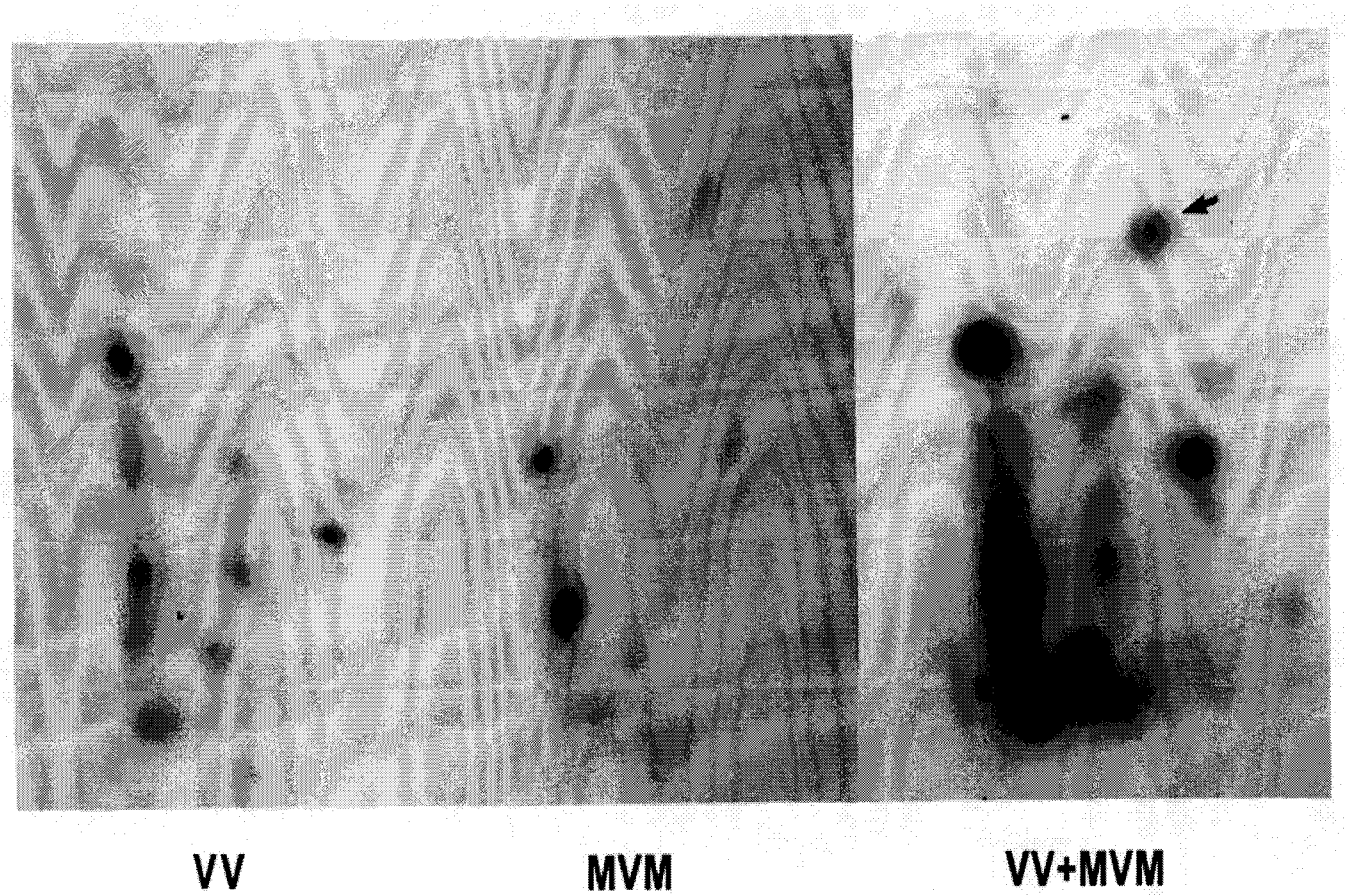

Figure 7: Comparative tryptic phosphopeptide analysis of MVM NS1 with *in vitro* phosphorylated NS1 using an "activating" protein kinase. 32P-labeled NS1 derived from metabolic labeling after MVM infection in A9 cells (MVM) was analyzed in comparison to NS1 *in vitro* phosphorylated by atypical protein kinases present in HA-1-fraction derived from HeLa cells (PK). This semi-purified fraction has been shown to reactivate dephosphorylated NS1 in helicase assays. The tryptic phosphopeptide patterns of MVM NS1 and PK NS1 were analyzed in parallel and together as an overlap to identify co-migrating phosphopeptides.

could indeed serve for activation of this biochemical activity. This possibility was further substantiated by a comparison of the tryptic phosphopeptide pattern with in vitro phosphorylated NS1 by a purified protein kinase, HA-1, which has been described to reactivate dephosphorylated NS1O for helicase function (54). NS1O was 32P-labeled in the presence of HA-1 and g[32P]ATP, gel-purified andsubjected to trypsin digest. Two-dimensional analysis was performed in parallel to [32P]-NS1 derived from MVMp infections in A9 cells, and for further alignments of the phosphopeptide analyzed as an "overlap". As shown in Fig. 7 the phosphopeptides derived from in vitro phosphorylation by HA-1 clearly aligned with the peptide(s) "A" obtained in vivo. Due to the appearance of this phosphopeptide in the course of the viral infection, the location within the putative helicase domain, and the alignment with phosphorylation site(s) that seem to be responsible for NS1 activation in vitro, it is likely that this phosphorylation event consists of a major regulatory component for NS1 replicative function.

The in vitro investigations concerning modulation of NS1 activities by phosphorylation have implicated members of the protein kinase C family to be involved in the regulation of NS1 replicative functions (31, 53, 54). In order to determine whether such a regulation is feasible in vivo as well, we determined the presence of protein kinase C during the course of an infectious cycle. Western blots were performed for characteristic members that were previously identified in A9 cells (31) and represented classical (PCKa), novel (PKCe), and most importantly atypical members (PKCl/i), which have been shown to modulate NS1 helicase activity in vitro (31). As shown in Fig. 8, all three isoforms were induced upon serum addition in both uninfected and MVM infected cells, and remained present during the course of infection, indicating that these kinases could indeed
be responsible for phosphorylation and regulation of NS1 during a natural MVMp infection in A9 cells.

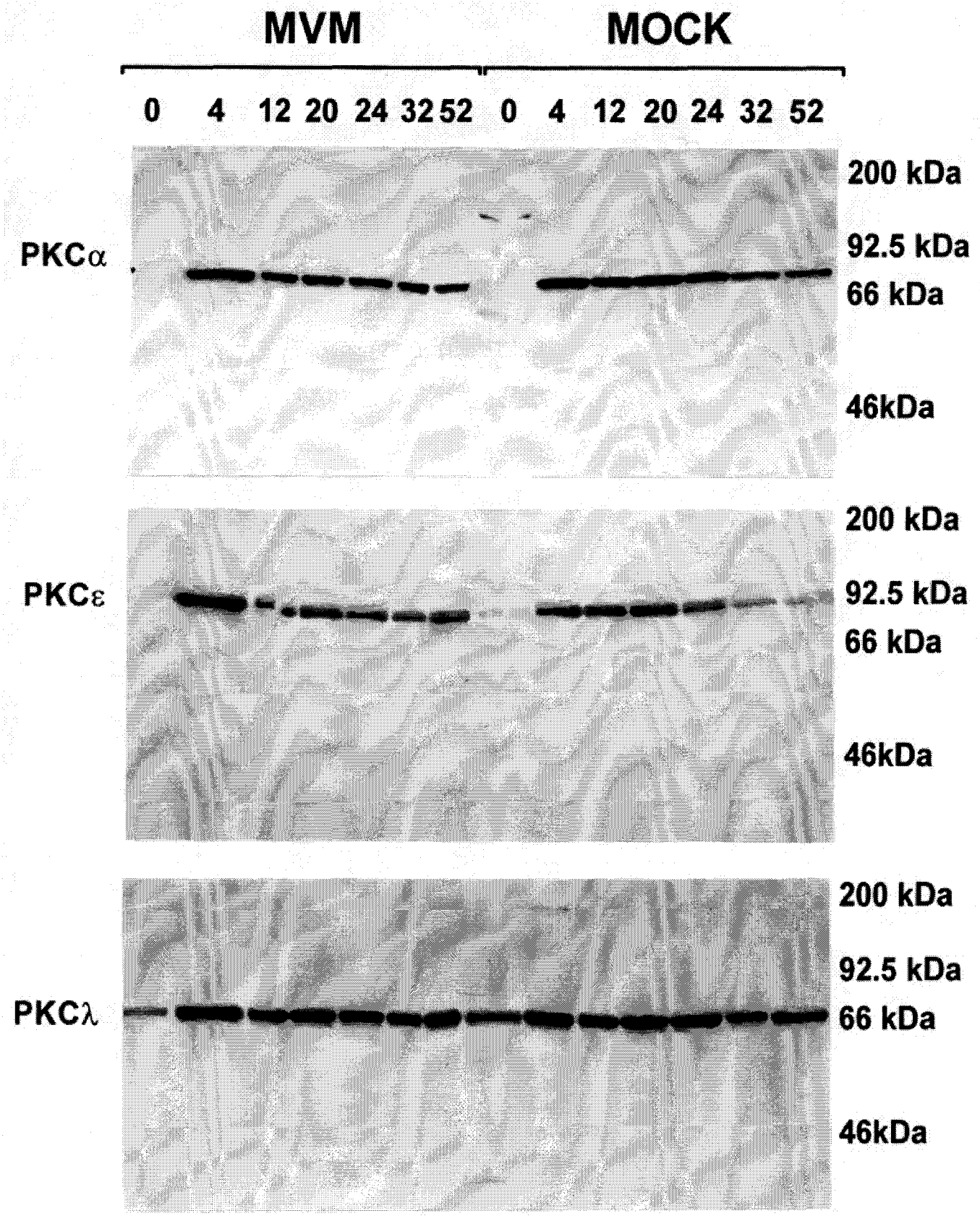

Figure 8: Detection of protein kinase C during the course of an MVM infection. Synchronized uninfected and MVM infected A9 cells were analyzed by western blot for the presence of the classical PKCα, the novel PKCε, and the atypical PKCλ/ι using isoform specific antibodies (Transduction laboratories). Times post release from cell cycle arrest are indicated.

Discussion

NS1 has a lot of similarities and even striking sequence homology to the well investigated simian virus 40 large T (LT) antigen (1). Besides their significant alteration of the cellular environment, both proteins are able to trans-regulate their viral and a variety of heterologous promoters, and most importantly they serve as the initiator proteins for viral DNA replication. In order to coordinate viral DNA replication with S-phase of the cell, SV40 LT is regulated for its replication activity by phosphorylation of T124 by cyclin/CDK2, which activates LT for origin unwinding due to a change of affinity to a target interaction site on the viral DNA (for review see (71)). MVM NS1, like SV40 LT is a phosphoprotein (26) and in vitro analyses have determined the requirements for NS1 phosphorylation in replication assays (54). The present study analyzing phosphorylation of MVM NS1, demonstrates that the phosphorylation pattern changes during the course of a viral infection in A9 cells as different NS1 activities become apparent. Main phosphorylation of NS1 occurs on serine residues and to a minor abundance on threonines located predominantly located predominantly on a CNBr-cleavage fragment (aa 315 to 482) covering a main part of the putative helicase domain of NS1. As previously reported (2, 46), no tyrosine phosphorylation could be detected in our analyses.

Despite the complexity of the NS1 phosphorylation pattern during viral infections, we attempted to correlate the appearance of distinct phosphopeptides with described functions of the polypeptide. This correlation was achieved by synchronization of the virus cycle through cell cycle arrest in GO/G1, and with analyses performed in parallel determine the occurrence of selective features of the virus infections which reflect characteristic NS1 activities. Besides monitoring the progression of the virus cycle, the appearance of the different replication intermediates (mRF, dRF, and ssDNA) also represents different replicative functions of NS1, such as the transition from viral DNA amplification to single-strand progeny virion production and encapsidation. In addition, the appearance of the capsid proteins, produced from R3 transcripts are derived through trans-activation of the "late" P38 promoter by NS1 transcriptional activities. Concerning the cytotoxic functions of NS1, there are no distinct markers identified so far. Therefore, we just considered two late time points (36 and 54 h post release) which are characterized by the occurrence of severe cytopathic effects to the host cell and the release of progeny viruses into the medium (data not shown). Selective NS1 phosphorylation as determined by tryptic phosphopeptide analyses could be characterized in three phases. Early after release of a first cell population from GO/G1 into early S-phase, at a time a first accumulation of NS1 becomes apparent and before onset of DNA amplification, NS1 is weakly but characteristically phosphorylated on four peptides which disappear later during infections. At this stage of

infection, NS1 has been shown by immunefluorescence to accumulate in little spots scattered in the nucleus which grew up to "foci-like" structures. These NS1-structures have been interpreted as replication centers (6, 20, 56). It certainly possible that NS1 is able to "organize" viral replication within the nucleus by interaction with cellular components (29) before onset of DNA amplification. Following this initial step in the virus cycle, major NS1 activities necessary for DNA replication takes place. These events occur characteristically with the appearance of four distinct phosphopeptides, three of them localized within the helicase domain of NS1, whereas a third set of peptides become apparent late(er) in infection. These latter phosphorylation events (peptides .. to ..) could regulate NS1 for its transcriptional functions and/or late activities concerned in cytotoxic/cytolytic activities of the polypeptide. It is of interest that NS1 accumulates as a major phosphorylated species and becomes increasingly phosphorylated at different sites during the course of infection, indicating that selective phosphorylation events might alter the (possible) tasks of NS1 in the virus cycle.

In analogy to SV40 LT antigen, NS1 was thought to be regulated by phosphorylation for its replication activities. This was supported by in vitro findings showing that NS1 phosphorylation is indeed required to achieve rolling circle replication of plasmids containing the minimal left-end origin of replication (54). Moreover, the detailed in vivo analyses during virus propagation presented here, clearly demonstrate that NS1 phosphorylation could serve as a mode of regulation for distinct activities, and moreover, could serve as a prerequisit for replication activities. This is strongly suggested by a the increase of NS1 phosphorylation with onset of viral DNA amplification and the findings that phosphorylated NS1 becomes covalently attached to replicated viral DNA, due to the nicking reaction which serves to initiate strand-displacement synthesis at the viral origins. Furthermore, the localization of major phosphorylation sites within the putative helicase domain and the alignment with phosphopeptides created by in vitro phosphorylation with an activating protein kinase for helicase function clearly indicated that a similar regulation/activation mechanism of NS1 occurs during MVM infection as well.

For SV40 LT phosphorylation and consecutive activation for viral DNA replication has been implicated to coordinate DNA amplification with S-phase of the cell in order to optimize production (34, 59). Unlike LT for SV40, MVM NS1 is not produced in arrested cells as demonstrated by two-dimensional FACS analysis showing that all NS1 found in serum starved cells separated with the small S-phase population (15), and there has been no evidence for an S-phase dependent activation of NS1 replicative functions. In addition, there is evidence that the dependency of parvovirus replication on cells entering S-phase is regulated on the level of NS1 production (5, 30). Therefore, it is more likely that phophorylation of NS1 serves to regulate the multifunctional protein for its different activities during virus propagation. This is supported by

the differential phosphorylation pattern of NS1 in the course of infection and the biochemical comparisons between dephosphorylated and native NS1, which indicated regulation by phosphorylation (53). Comparing the biochemical activities of NS1 which are necessary for replication with those implied to achieve P38 trans-activation, as determined so far, there are similarities but also significant differences in the properties of the two "NS1-species" required, besides (possible) interactions with cellular components (38, 43). While transcription factors usually bind constitutively with high affinity to a target DNA-sequence to coordinate the transcription machinery, DNA binding proteins involved in replication exert their binding to the DNA more flexible (7, 35, 37). Indeed, NS1 seems to achieve its trans-activation at least in part in such a matter, since it was possible to transregulate the P38 promoter at least in part by coupling the C-terminal activation domain to a heterologous DNA-binding module (39, 40). In contrast, a tight interaction with a double-strand DNA binding motif would not be favorable for NS1 during initiation of viral DNA replication, since this reaction requires flexibility of the protein during the interaction with its substrate, possibly regulated by post-translational modification of NS1. Initiation of replication is a coordinated stretch of biochemical reactions, starting with the site-specific interaction to the origin in the presence of ATP-binding (27) to allow the formation of higher order oligomers (51). Upon hydrolysis of the trinucleotide, NS1 nicks the origin and gets covalently attached to the 5' end. In analogy to other "Rep"-proteins, this endonuclease activity is achieved by local unwinding on single-strand level (37, 67), before helicase activity is required to unwind the double-strand template in front of the cellular replication machinery. Consequently, it requires the transition from a double-strand site-specific DNA-binding protein to a helicase which has affinity to single-strand DNA and is able to translocate on the template unwinding the double-strand substrate. Whether a real transition within the NS1-oligomer occurs or different NS1-species are recruited for alternative tasks is not clear yet. Either possibility, however, presents altered properties of the protein that might be achieved by differential phosphorylation. In fact, in vitro investigations have shown so far that phosphorylation is indeed required to allow this initiation process to occur (54) and that DNA unwinding functions of the polypeptide are regulated by phosphorylation through atypical members of the PKC family (31, 54). The presence of selected members of the protein kinase C family during progression of the virus cycle, and the alignment of phosphopeptide "A" with the phosphorylation pattern produced by the "activating" suggests a similar regulation during MVM infection.

So far, we obtained a variety of correlation between phosphorylation events occurring in vivo and the described regulation of NS1 replication activities in vitro, which suggests the possibility of differential regulation of NS1 during the course of infection. However, there is still a lack of information concerning the regulation of NS1 transcriptional activities and the cytotoxic function

by phosphorylation to confirm such a speculative mechanism. Currently, there are attempts under investigation to identify specific regulatory phosphorylation sites in vivo and to analyzed their impact on NS1 functions by site-directed mutagenesis. Moreover, it is also important to investigate whether the rather specific regulation of NS1 replicative functions by PKC in vitro is achieved by the same kinases during a natural infection. This could be of major interest in order to determine the host requirements for parvovirus propagation along the attempts to use these viruses for gene therapy. Last but not least, it has to be mentioned that phosphorylation is an important mode of post-translational modification to regulate the activity of a (NS1) protein. However, there are indications that alternative post-translational modifications could occur in NS1 as well (63) and might play as an important role in NS1 regulation as phosphorylation.

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III) Replicative Functions of Minute Virus of Mice NS1 Protein are Regulated by Phosphorylation through Protein Kinase C.

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Replicative Functions of Minute Virus of Mice NS1 Protein are Regulated in vitro by Phosphorylation through Protein Kinase C.

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Key words: Parvovirus MVM, Non-structural protein NS1, Rolling circle Replication, Helicase activity, Phosphorylation by PKC

Abstract

NS1, the major non-structural protein of the parvovirus minute virus of mice (MVM) is a multifunctional phosphoprotein, which is involved in cytotoxicity, transcriptional regulation, and initiation of viral DNA replication. In order to coordinate these various functions during virus propagation, NS1 has been proposed to be regulated by post-translational modifications, in particular phosphorylation. Recent in vitro studies (60) provided evidence that distinct NS1 activities, notably the intrinsic helicase function, are regulated by phosphorylation . In order to study the dependence of the initiation of viral DNA replication on NS1 phosphorylation and to identify the protein kinases involved, we established an in vitro replication system that is devoid of endogenous protein kinases and is based on plasmid substrates containing the minimal left-end origins of replication. Cellular components necessary to drive NS1 dependent rolling circle replication (RCR) were freed from endogenous serine/threonine protein kinases by affinity chromatography, and the eukaryotic DNA polymerases were replaced by the bacteriophage T4 DNA polymerase. While native NS1P supported RCR under these conditions, dephosphorylated NS1O was impaired. Using fractionated HeLa cell extracts, we identified two essential protein components which are able to phosphorylate NS1O, are enriched in protein kinase C (PKC), and

when present together, reactivate NS1O for replication. One of these components, containing atypical PKC, was sufficient to restore NS1O helicase activity. The requirement of NS1O reactivation for characteristic PKC co-factors such as Ca2+/phosphatidyl-serine or phorbol esters strongly suggests the involvement of this protein kinase family with regulation of NS1 replicative functions in vitro.

Introduction

Minute virus of mice (MVM) is the prototype of the genus parvovirus. Members of this genus consist of non-enveloped spherical particles of about 20-24 nm in diameter, comprising a linear single-strand DNA genome of approximately 5.1 kb. Parvoviral DNA encodes for the two structural (VP) and at least four non-structural (NS) polypeptides, of which the 83 kDa nuclear phosphoprotein NS1 is the only viral product necessary for viral DNA replication in all cell types (54, 22). Replication of the parvoviral genome involves the formation of monomeric and concatemeric duplex DNA intermediates. These replicative forms are produced by an uni-directional, single-strand copy mechanism (for review see (16)), which resembles the rolling circle replication (RCR) mechanism described for single-strand DNA plasmids, bacteriophages, and geminiviruses (for review see (35)). After conversion of the single-strand genome into a monomeric duplex, which is executed solely by cellular components (3), replication initiates at site-specific, single-strand nicks introduced by the viral NS1 protein into origin sequences located at either end of the genome (14, 17, 18). This cleavage reaction leaves NS1 covalently attached to the 5' end at the nick site, and generates a base-paired 3' hydroxyl which serves as a primer for DNA synthesis (8, 13, 19, 59).

The minimal origin sequences at the left-end telomere have been mapped, consisting of approximately 50 bp located within the stem of the Y-shaped terminal structure (13). This origin comprises binding-sites for the cellular component PIF (9) and for NS1 (21), and a NS1 nick-site (13). The NS1 binding- and nick-sites are separated by an A/T-rich sequence, which most likely facilitates local unwinding during the nicking reaction. In the left-end hairpin structure of the genome, between the binding-sites for PIF and NS1, there is an important mismatched "bubble", with a 5'-GAA-3' triplet on one strand opposite of a 5'-GA-3'doublet on the other. When replication through the hairpin unfolds and copies the palindrome, a double-strand intermediate is generated, in which these tri- and dinucleotide sequences are located on either side of the axis of symmetry. Although the origin sequences of both arms are nearly identical, only the arm containing the GA dinucleotide serves as an active origin for NS1 mediated rolling circle replication, while the trinucleotide counterpart remains silent (13).

Besides its keyrole as the initiator protein for viral DNA replication, NS1 is essential for several additional processes during the viral life cycle. In particular, the NS1 protein is a strong trans-activator of the parvoviral P38 promoter that controls capsid gene expression (64). Furthermore, NS1 trans-regulates non-parvoviral promoters (27, 70), and it exerts cytotoxic and/or cytostatic effects for which oncogene transformed cells appear to be preferential targets (5, 7, 53). To account for the temporal coordination of these various functions during virus multiplication, NS1 has been proposed to be regulated by post-translational modifications such as phosphorylation. NS1 was indeed found to be phosphorylated in infected cells (2, 11, 20, 51). Moreover, recent in vitro studies present differences between HeLa cell derived native NS1 from its dephosphorylated counterpart in its capacity for distinct biochemical activities involved in viral DNA replication (60).

In order to study the effect of phosphorylation on NS1-driven initiation of DNA replication, we used a previously described rolling circle replication (RCR) system, which is based on plasmid substrates containing the minimal left-end origin of replication (8, 13). This system was modified to deplete its protein components from endogenous kinases, allowing purified native NS1P to be compared with dephosphorylated NS1O in regard to their replication activities. In contrast with standard HeLa cell extracts, the "kinase-free" replication system was severely impaired to support RCR when supplied with NS1O as compared with NS1P. In reactivation experiments the combination of two distinct protein fractions from HeLa cell extracts proved able to restore at least in part the replication activity of NS1O in the kinase-free system. This reactivation was dependent upon the presence of either acid lipids and Ca2+, or the phorbol ester TPA. This dependence on co-factors together with the capacity of both fractions to phosphorylate NS1O in vitro, strongly suggest that members of the protein kinase C family are responsible for regulation of the NS1 replicative functions. Previous analyses of selected biochemical activities of native NS1P versus NS1O polypeptides have shown that the intrinsic helicase function of the viral product is strikingly dependent upon phosphorylation (60). One of the protein components, necessary to rescue the replication activity of NS1O in the kinase-free system, which was enriched in atypical PKC, was also found to reactivate the helicase function of NS1O.

Material and Methods

Viruses and cells

Recombinant vaccinia viruses were propagated in monolayer cultures of BSC-40 cells, collected and purified over a sucrose cushion (41), except for the release of virus from infected cells which was achieved by three cycles of freezing and thawing instead of sonification. Recombinant vaccinia viruses have been constructed as previously described (57). The 293 cell line was adapted to suspension and grown in spinner-bottles using Joklik's medium supplemented with 10% fetal calf serum. HeLa-S3 were grown in spinner-bottles in the presence of 5% fetal calf serum.

Production and purification of native and dephosphorylated NS1
Wild type and mutant NS1 was produced from recombinant vaccinia viruses in suspension cultures of HeLa-S3 and harvested 18 h post infection (57, 60). His-tagged NS1 present in nuclear extracts was dephosphorylated, or not, with calf intestine alkaline phosphatase, and purified immediately on Ni2+-NTA agarose columns (60). NS1 preparations were analyzed by discontinous SDS-PAGE, and proteins were detected by coomassie-blue staining. All NS1 preparations were tested for their described activities in various in vitro assays.

Plasmids
Plasmid pTMHis is a derivative of pTM-1 (52), which allows the expression of [His]6-tagged proteins. The presence of a unique NcoI-site allows the in-frame cloning of the N-terminal [His]-TAG with the initiation codon of the gene of interest. pTMHis has been constructed by insertion of the annealed oligonucleotide pair 5'-CATGCACCATCACCACCATCACGCC-ATGGAATTC-3' and 5'-GAATTCCATGGCGTGATGGTGGTGATGGTG-3' into the NcoI and SmaI cleaved pTM-1 vector. The vector used to obtain recombinant vaccinia viruses expressing His-tagged PKCa was constructed by insertion of the full length human PKCa cDNA (30) into NcoI and EcoRI cleaved pTMHis. Plasmids used as templates for in vitro replication assays, were pL1-2TC and pL1-2GAA containing the minimal active left-end MVM origin and the corresponding inactive origin, respectively (13). The bacterial expression plasmid pYT202am containing the MVM sequences nt 225 to 534, served to produce peptides for rabbit immunization and production of NS-specific antisera (23). pQE-PKCg was constructed by insertion of the BamHI to SmaI fragment (nt 1332-1956) of human PKCg cDNA (37) into pQE-30 (Qiagen), pQE-PKCz was produced by insertion of the HindII to BamHI fragment (nt 981-1403) of human PKCz cDNA (34) into pQE-32 (Qiagen).

Purification of peptides and production of antisera
Peptides from pYT202amNS, pQE-PKCg or pQE-PKCz were expressed overnight in the presence of 1 mM IPTG, extracted, and purified as described (23). Antisera were produced by multiple injections into rabbits. For western blot analyses with aPCKg or aPCKz antibodies, IgGs were affinity purified on peptide columns using the immunizing peptides (31).

Preparation of L-Threonine- and Protamine-affinity columns
Coupling of L-threonine and protamine sulfate affinity chromatography columns was performed using NIH-activated Hi-Trap columns (5 ml; Pharmacia) according to the manufacturer's instructions. L-Threonine (SIGMA) or protamine chloride (SIGMA) was dissolved in coupling buffer [0.1 M NaCO3 pH 8.3; 0.5 M NaCl] at 10 mg/ml and allowed to interact with the column material for 1 h at room temperature by recirculation.

Protein extraction and fractionation by column chromatography
S100 extracts from 293S and HeLa cells were prepared and fractionated on phosphocellulose columns to obtain fractions P1, P2, and P3 as described (8, 68), except that P3 was eluted at 1 M NaCl (see Fig. 2A). To remove endogenous serine/threonine kinases, P1 from a 10 liter 293 cells was further purified on a 5 ml L-Threonine affinity Hi-Trap column in buffer A (25 mM Tris pH 7.5, 1 mM EDTA, 1 mM dithiothreitol [DTT], 174mg/ml phenylmethylsulfonyl fluoride [PMSF], 10% glycerol), containing 150 mM NaCl. Individual flow-through fractions were dialyzed against buffer B (20 mM HEPES pH 7.5, 5 mM MgCl2, 5 mM KCl, 0.1 mM DTT, 17.4 mg/ml PMSF, 10% glycerol, 20% sucrose) overnight at 4oC and stored in aliquots at -80oC. All fractions were tested for their ability to phosphorylate NS1O in in vitro kinase assays to determine the extent of purification. P2-Pol was obtained by P2 fractionation on DE52 columns. P2 from a 10 liter HeLa culture was loaded on a DE52 column (5ml resin/liter original culture) in buffer A containing 50 mM NaCl. After thorough washing with the same buffer, P2-pol was eluted using buffer A containing 1 M NaCl. The eluate was dialyzed against buffer B and frozen in aliquots.
Protein kinases present in the HeLa cell derived P2 fraction were further purified by consecutive anion exchange, protamine affinity and hydroxyl apatite chromatography, as schematized in Fig. 4. (1) P2 was adjusted to 200 mM NaCl and fractionated on a DE52 column (step 2). The flow-through at 200 mM NaCl (DE-1) and the elution fractions DE-2 (200 mM to 500 mM NaCl), and

DE-3 (500 mM to 1M NaCl), were collected. All fractions were dialyzed against buffer B, frozen and stored at -80OC. The protein kinases necessary to achieve extensive reactivation of NS1O in replication assays were found to be confined to fraction DE-1. (2) Protein kinase C family members contained in DE-1 were further purified by protamine affinity chromatography (step 3), using an FPLC-system (Pharmacia). DE-1 (corresponding to a 6 liter culture) was loaded on a 5 ml protamine Hi-Trap column with a constant flow-rate of 0.5 ml/min. After collection of the flow-through (PA-1), the column was washed with buffer C (20 mM HEPES pH 7.5, 1 mM EDTA, 0.1 mM DTT, 10% glycerol) containing 50 mM NaCl. The PKC containing fraction PA-2 was then eluted using buffer C containing 1 M NaCl and the protease inhibitors PMSF (174mg/ml), leupeptin (1mg/ml), aprotinin (1mg/ml). PA-2 was dialyzed, adjusted to 50% glycerol and stored in aliquots at -80OC. (3) Protein kinase C isoforms present in fraction PA-2 were separated by FPLC on hydroxyl apatite columns (step 4). PA-2 (corresponding to 3 liter culture) was adjusted to 200 mM NaCl, loaded on 5 ml hydroxyl apatite column (MERCK) with constant flux (0.5 ml/min), and washed with 30 ml of buffer C containing 50 mM NaCl. After collection of the flow-through and wash, the HA-1 fraction was eluted from the column using buffer D (150 mM NaCl, 20 mM KPO4 pH 7.5, 10% glycerol, protease inhibitors PMSF, leupeptin, aprotinin). The protein peak was identified by UV monitoring (280 nm) and collected. HA-2 was then eluted using a linear gradient between buffer D and buffer E (150 mM NaCl, 0.5 M KPO4, 10% glycerol, protease inhibitors), and consisted of pooled fractions recovered between 120 mM and 400 mM KPO4. All fractions were dialyzed against buffer B containing 50 mM NaCl overnight at 4oC, adjusted to 50% glycerol, and frozen in aliquots at -80oC.

Replication assays.
Replication assays were carried out as described (13) in the presence of optimized amounts of the various cell fractions, using approximately 0.2 mg of His-tagged vaccinia produced NS1 (determined by coomassie-blue staining after SDS-PAGE). In the modified replication system, 3 units of T4 DNA polymerase (Boehringer Mannheim) were used instead of cellular polymerases. Each assay was carried out in a 20ml total volume of 20 mM HEPES-KOH pH 7.5, 5 mM MgCl2, 5 mM KCl, 1 mM DTT, 0.05 mM dNTP each; 4 mM ATP, 40 mM creatine phosphate, 1 mg phosphocreatine kinase, 10 mCi a[32P]dATP, and 20 ng of the appropriate DNA template. After incubation at 37oC for 2 h, the reaction was stopped by adding 60 ml of 20 mM Tris pH 7.5, 10 mM EDTA, 0.2% SDS and incubating the mixture at 70oC for at least 30 min. In order to quantify the extent of DNA replication, 3 ml of the terminated reaction mixture were spotted in duplicate on DE81 filters, washed extensively with 0.5 M Na2HPO4 and analyzed for incorporated radioactivity by scintillation counting. 32P-labeled replication products were also analyzed by agarose gel electrophoresis, either directly after proteinase K digestion and linearization of the plasmid with HindIII, or after immunoprecipitation with aNSN antisera and cleavage by the restriction endonuclease HindIII (13).

In vitro kinase reactions
In vitro kinase reactions were performed as described (60), using various amounts of protein extracts, 100 ng of dephosphorylated NS1O, and 10 mCi g[32P]ATP in 20 ml of 20 mM HEPES-KOH pH 7.5, 7 mM MgCl2, 5 mM KCl, 1 mM DTT. After incubation for 30 min at 37oC, the reactions were stopped by adding the same volume of 20 mM Tris pH 7.5, 5 mM EDTA, 0.2% SDS, and heating for 30 min at 70oC.One fifth of the reaction products were immunoprecipitated using aNSN, an antiserum raised against the common N-terminus of MVM NS-proteins (23). In vitro labeled NS1 was detected by 8% SDS-PAGE and autoradiography.

Helicase assays
Helicase assays were performed as described (59, 60) using M13-VAR as a template. Reactions using 10 - 100 ng of purified NS1 were incubated for 40 min at 37O. For reactivation experiments, titrated amounts of protein extracts were added to the reactions together with wither of the following protein kinase C co-factors: 2 mM Ca2+, 1 mg/ml phosphatidyle serine (PS), or 5 nM of 12-O-tetradecanoyl-phorbol-13-acetate (TPA). None of these PKC co-factors alone had any influence on the helicase function of native NS1P, dephosphorylated NS1O, or mutant NS1 proteins used as negative controls.

Western blot analyses
Protein extracts were fractionated by 10% discontinous SDS-PAGE, blotted on nitrocellulose membranes and revealed with rabbit antibodies directed against the most conserved domain of PKC (aPKCg, aPKCz), or with mouse antibodies specific for the atypical PKCi (Transduction Laboratories). The aPKCg or aPKCz polyclonal antibodies were affinity purified on peptide columns, used at 0.6 mg IgG per ml and revealed with 125I-protein A (ICN; 0.2mCi/ml). Mouse aPKCi antibodies were used at a 1:2500 dilution, and bound antibodies were revealed with a 1:5000 dilution of horseradish peroxidase conjugated anti-mouse IgGs using the ECL system (Amersham).

Results

NS1 phosphorylation is required for Rolling circle replication.

Previous investigations comparing biochemical activities of dephosphorylated (NS1O) versus native (NS1P) NS1, both derived from HeLa cells, revealed modulation of site-specific binding to the left-end origin, site-specific nicking, helicase and ATPase activities. In contrast, no significant difference between NS1O and NS1P could be observed in their replication

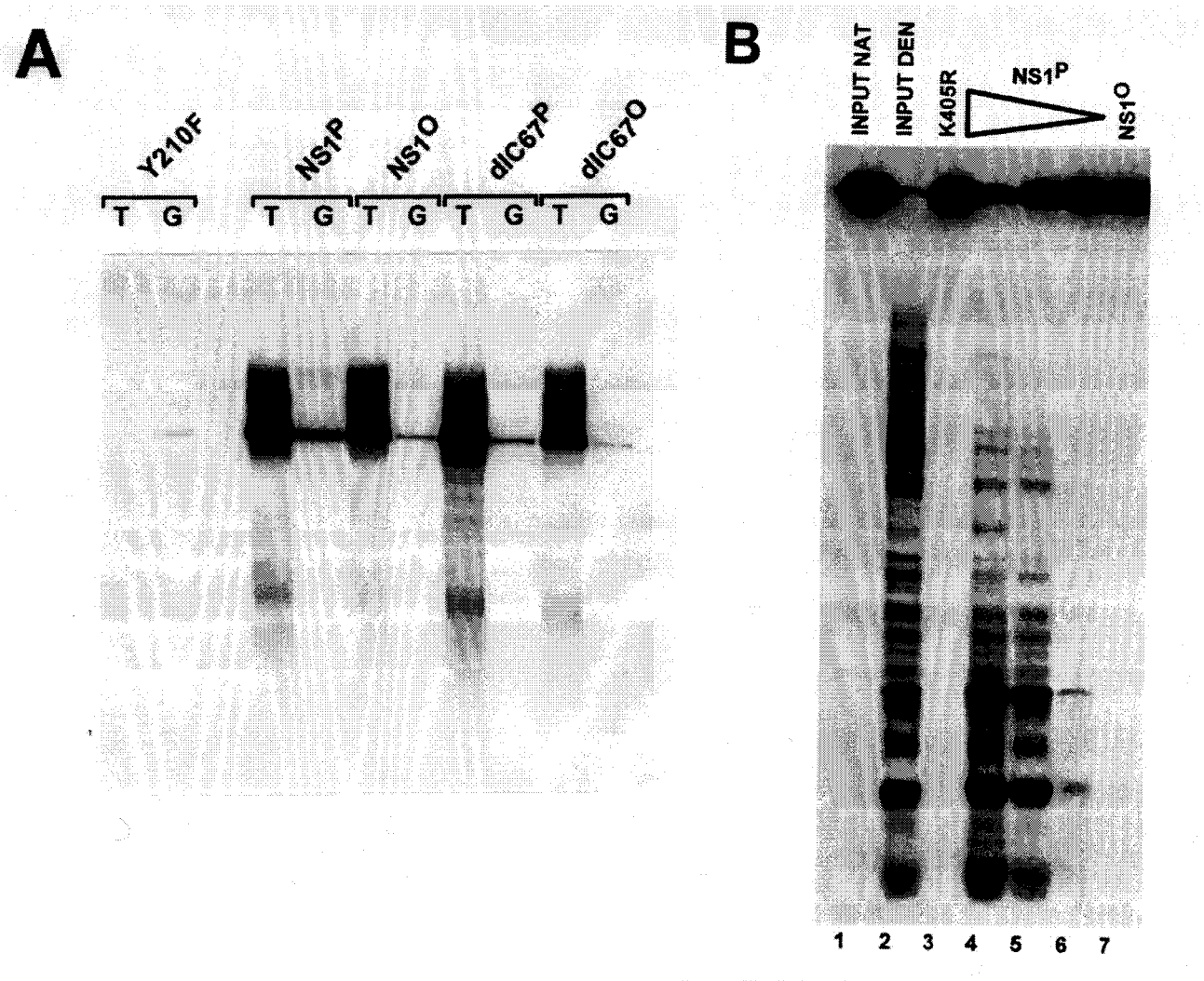


Figure 1: Comparison of native NS1P and dephosphorylated NS1O for their replicative functions . NS1 was expressed from recombinant vaccinia viruses in HeLa-S3 cells, harvested 18 h post infection, and nuclear extracts were prepared. Dephosphorylated NS1O was obtained by treatment of these nuclear extracts with calf intestine alkaline phosphatase. Native NS1P and phosphotase treated NS1O were purified from nuclear extracts Ni2+-NTA affinity chromatography, due to an Nterminal [His]6-TAG and analyzed by SDS-PAGE and coomassie-blue staining. (A) NS1P and NS1O were compared for their capacity to support rolling circle replication (RCR) in standard HeLa replication extracts, using plasmids containing the left-end active (T) or inactive (G) origin as substrates, in the presece of [32P]dATP. The reaction products were linearized with HindIII and analyzed by 0.8% agarose gel electrophoresis. The linkage tyrosine mutant Y210F (59) served as a negative control. The *dl*C67 mutant is replication competent (60) and was also analyzed in its native (P) and dephosphorylated (O) forms. (B) NS1P (100, 30, or 10 ng; lanes 4-6, respectively) and NS1O (100 ng; lane 7) were compared for their intrinsic helicase activity, using M13-VAR template, for 40 min at 37oC in the presence of 2 mM ATP. The ATP-binding site mutant K405R (57) served as a negative control. Reaction products were analyzed by 7% native PAGE in the presence of 0.1% SDS. Lanes 1 and 2, native and denatured input DNA, respectively.

capacity for dimer-bridge resolution in an in vitro assay using HeLa cell extracts (60). It was suggested that NS1O became phosphorylated by kinases present in cell extracts, leading to the development of a kinase-free replication system as presented in this study.

Resolution of the left-end dimer-bridge junction is a complicated assay, probably requiring a number of so far unknown cellular components. In addition, this assay is rather insensitive, because each initiation event is associated with synthesis of a short stretch of labeled DNA only. This prompted us to use a more simple assay, which consists in the NS1-dependent initiation of rolling circle replication (RCR) of non-palindromic substrates carrying the MVM origins of replication (13), to study the effect of phosphorylation on NS1 replicative functions. When performed with standard replication extract, wild type NS1, and an active origin of replication containing plasmid, this assay features the synthesis of several kilobases of labeled DNA, which makes it far more sensitive than dimer-bridge resolution. In addition, in absence of an active origin, or without functional NS1, only marginal replication due to repair synthesis is observed, which facilitates the analysis of the modulation of NS1 activity (13).

In a first step, NS1P was compared with NS1O regarding ability to support RCR from a parvovirus origin in standard HeLa cell extracts. Purified NS1P and NS1O were prepared as reported previously (60). Plasmids carrying the active (TC) or inactive (GAA) left-end origin of MVM DNA replication were used as substrates. The mutant NS1 derivative Y210F, which is impaired in site-specific nicking due to an amino acid substitution for the active-site tyrosine, but is proficient in helicase activity (59), served as a negative control of NS1 activity. As shown in Fig. 1A, NS1O was able to support RCR in crude HeLa cell extracts almost as efficiently as NS1P, confirming previously reported findings using cloned dimer-bridge as a substrate (60). Indeed, the replication activity of both wild type NS1 and the replication competent mutant NS1dlC67 the replication activity was reduced to only a small extent by dephophosphorylation, i.e. NS1O sustained approximately 60% of the level of [32P]-dATP incorporation into newly synthesized DNA as compared with NS1P (data not shown). To verify dephosphorylation, we also tested NS1O for its biochemical activities in absence of any additional proteins. The most striking difference between native and dephosphorylated NS1 has been described for the helicase function (60). As illustrated in Fig. 1B, NS1O helicase activity was indeed reduced more than ten fold compared to the native polypeptide, in contrast with the significant activity of NS1O in replication assays. The helicase deficient mutant NS1:K405R served as a negative control in these experiments.

One possible explanation for the replication competence of NS1O, despite the lack of helicase function, might consist in the presence of protein kinases within the cell extracts used in the former but not in the latter assay, which would lead to NS1O re-phosphorylation and consequent reactivation. To test this possibility, and eventually to establish rolling circle replication in absence of endogenous protein kinases, we first determined the presence of

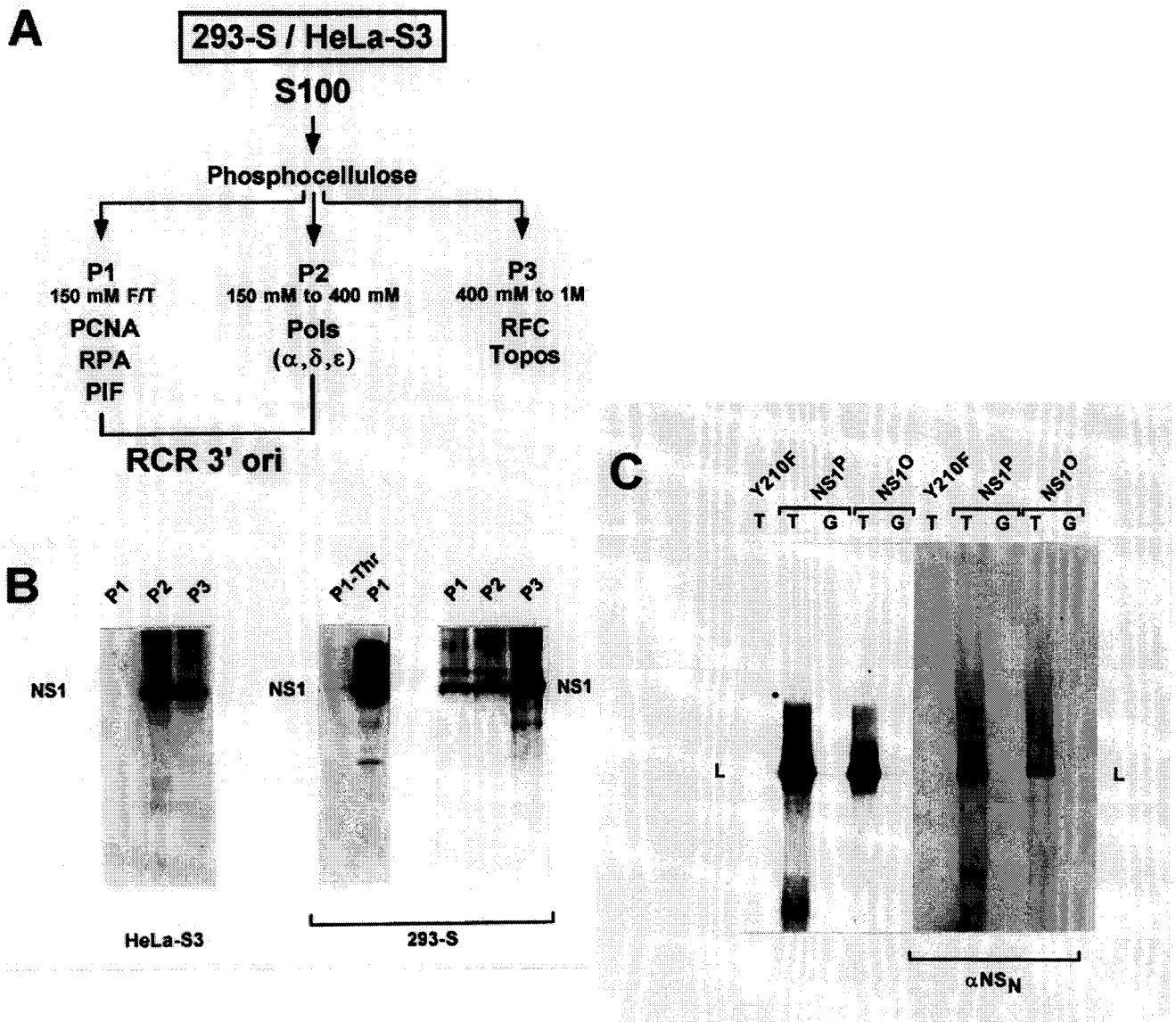


Figure 2 : Fractionation of cell extracts by phosphocellulose chromatography.
S100 Replication extracts were prepared from 293-S and HeLa-S3 cells fractionated by phosphocellulose columns according to Tsurimoto and Stillman (68). (A) Summarizing fractionation scheme of replication extracts into fractions P1, P2, and P3, and the predicted distribution of known replication factors (8, 55, 68). The NaCl concentrations to elute the components are indicated. F/T stands for flowthrough; PCNA: Proliferating Cell Nuclear Antigen; RPA: Replicator Protein A (RF-A; human ssDNA binding protein); PIF: Parvovirus Initiation Factor; Pols: Eukaryotic polymerases; RFC: Replication Factor C; Topos: Eukaryotic topoisomerases. As indicated, P1 plus P2 have been shown to contain all cellular components to support NS1 dependent rolling circle replication (RCR) at the active left-end (3') origin (8). (B) Determination of protein kinase phosphorylating NS1O which are contained within the distinct phosphocellulose fractions. NS1O was incubated with the indicated fractions in the presence of [32P]γATP for 30 min at 37oC, and analyzed by SDS-PAGE after immunoprecipitation with αNSN (23). The migration of NS1 is indicated. P1-Thr corresponds to the P1-fraction from 293-S cells after purification on L-threonine affinity columns. (C) Comparison of NS1P and NS1O in RCR assays using P1-Thr derived from 293 cells plus P2-pol derived from HeLa cells, with plasmid templates containing the minimal active (T) or inactive (G) left-end origin. Since NS1O has been shown to be activated for helicase activity by members of the PKC family (60), the reactions were carried out in the presence of the PKC co-factors Ca2+/L-α-phosphatidyl-L-serine (PS). The replication deficient NS1 mutant Y210F was used as a negative control. Linearized, labeled reaction products were analyzed by 0.8% agarose gel electrophoresis, either directly after proteinase K treatment (left panel) or after immunoprecipitation using αNSN (right panel).

NS1O phosphorylating protein kinases after fractionation of replication extracts on phosphocellulose columns, taking advantage of recent developments in the identification of host cell determinants of this reaction. As indicated in Fig. 2A, it has been shown that phosphocellulose fractions P1 and P2 derived from 293 cells are sufficient to support rolling circle replication of plasmids containing the left-end origin in the presence of wild type NS1 (8). When HeLa cell extracts were fractionated in the same way, protein kinases phosphorylating NS1 were found to by confined to fractions 2 and 3, with no detectable activity in flow-through (P1) (Fig. 2B). Yet, HeLa P1 could not be used in subsequent experiments since it failed to support significant replication in the presence of P2 and native NS1P (data not shown), leading us to prepare P1 from 293 cells. In contrast to HeLa P1, 293-P1 had a significant capacity for NS1O phosphorylation, although the bulk of kinase activity was still found in fractions P2 and P3 (Fig. 2B). To remove the residual endogenous serine/threonine kinases from 293-P1, this fraction was purified over a L-threonine-affinity column, resulting in P1-Thr that was essentially free of NS1O phosphorylating activity in comparison with the original P1 material (Fig. 2B). This "kinase-free" P1-Thr from 293 cells was then combined with fraction P2 obtained from HeLa cells, in order to supply the cellular components allowing NS1 mediated rolling circle replication. As seen in Fig. 2C, NS1P was able to trigger RCR from the active (TC) origin in the presence of P1-Thr and P2 fractions. This reaction was specific since it occurred to only little extent when the inactive (GAA) origin was used as a substrate.

Furthermore, as seen previously using standard HeLa extracts, NS1O was also able to support RCR under these conditions, i.e. in the presence of the sole protein kinases present in HeLa-P2 when Ca2+, and acid lipids, such as L-a-phosphatidyl-L-serine (PS) were supplied. Under these conditions NS1O achieved close to 50% of the RCR activity of NS1P (Fig. 2C). These results indicate that the phosphocellulose P3-fraction, as well as the protein kinases therein, are dispensable for RCR initiated by NS1(O) at the left-end origin. The inactivity of the replication deficient NS1 mutant Y210F used as a negative control, and the immunoprecipitation of labeled DNA products with NS1 antisera confirmed the specificity of the reaction on replication competent NS1 (Fig. 2C).

Fraction P2 is thought to provide the DNA polymerases necessary for NS1 dependent RCR at the left-end origin (8, 55, 68). In order to reconstitute a kinase-free system and supply DNA polymerases in absence of protein kinases, we thought to replace the whole fraction P2 by purified bacterial or bacteriophage polymerases. With native NS1P, no replication products were obtained when either P1, or P2 were omitted from the reaction (Fig. 3, lanes 1, 2). On the other hand, DNA synthesis took place when the P1-Thr fraction was supplemented with E.coli DNA polymerase I or the Klenow fragment thereof (data not shown), or with DNA polymerases from bacteriophage T7, or T4 (Fig. 3). In the presence of standard replication extracts, the active origin from pL1-2TC is recognized by NS1, allowing the establishment of an unidirectional single-

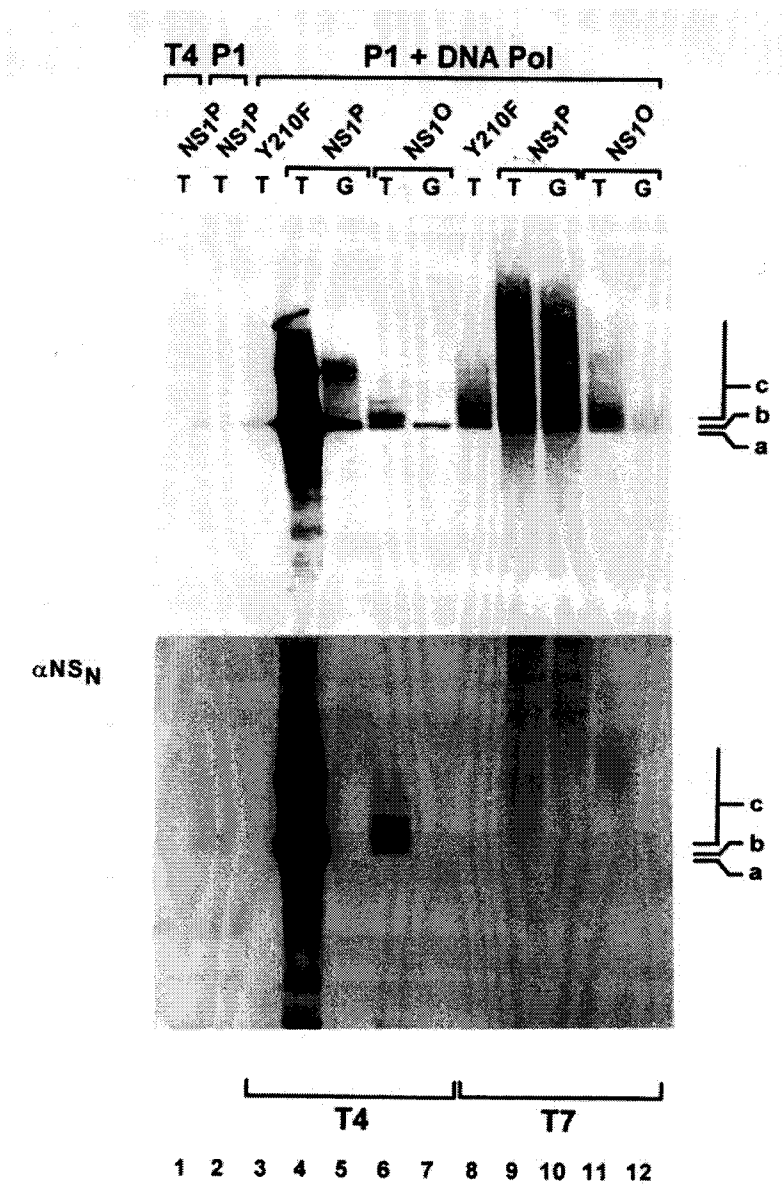


Figure 3: NS1 dependent rolling circle replication in a kinase-free *in vitro* system.
Plasmids containing the active (T) or inactive (G) left-end origin were used as substrates to measure the capacity of NS1P and NS1O to support RCR in absence of endogenous protein kinases NS1P in a system based on P1-Thr and bacteriophage DNA polymerases. T4 and T7 stand for bacteriophage T4 and T7 DNA polymerases, respectively. The replication deficient NS1 mutant Y210F was used as a negative control. Linearized 32P-labeled replication products were analyzed on 0.8% agarose gels, either directly after proteinase K digestion (top panel) or after immunoprecipitations using αNSN. (lower panel). The positions of the linearized plasmid (a) and slower migrating products (b, c) are indicated.

strand replication fork which progresses around the circular plasmid (13). Initiation of replication is achieved by site- and strand-specific nicking performed by the NS1 protein, which remains covalently attached to the 5'end of the nicked strand. In contrast to the active origin, the inactive origin in pL1-2GAA is not a substrate for nicking (8), and therefore no NS1-dependent replication occurs. We further analyzed the specificity of the RCR reactions taking place in the reconstituted, kinase-free system by using pL1-2GAA as an inactive substrate, and the mutant Y210F as replication deficient NS1 control. In addition, replication products were analyzed after immunoprecipitation with an aNS-antiserum. When E.coli Klenow fragment, DNA polymerase I (data not shown), or bacteriophage T7 polymerase (Fig. 3, lanes 8 to 12) were substituted for the eukaryotic polymerases, replication was found to occur irrespective of whether the templates contained an active (T) or inactive (G) origin. The lack of NS1 dependent initiation of the replication reactions driven by E.coli and phage T7 polymerases was also apparent from the failure of the aNS1 serum to immunoprecipitate labeled DNA products (Fig. 3 lower panel), and the significant DNA synthesis detected with the NS1 mutant Y210F (Fig. 3, lane 8). In contrast, as reported previously for SV40 DNA replication in vitro (69), T4 DNA polymerase could successfully substitute for the cellular DNA polymerases contained in P2 to give rise to a specific RCR reaction with plasmids containing the active left-end (TC) origin in the presence of P1 and NS1P (Fig. 3, lane 4). Indeed, only limited repair synthesis occurred with the substrate containing the inactive origin (Fig. 3, lane 5), while initiation was NS1 dependent, as apparent from the formation of aNS1-immunoprecipitatable replication products in the presence of wild type NS1P but not Y210F (Fig. 3, lanes 3 and 4).

Since we were able to obtain specific RCR reaction using kinase-free P1-Thr and T4 DNA polymerase in the presence of native NS1P, we further investigated the requirements of this reaction for NS1 phosphorylation. As illustrated in Fig. 3, the patterns of labeled DNA products genereated in the presence of NS1O and NS1P could be distinguished in three respects. First, the overall level of DNA synthesis was much reduced when NS1 was dephosphorylated, which strongly suggests that phosphorylation modulates the capacity of NS1 for initiation and/or promoting RCR. Second, major products of the RCR reaction taking place in the presence of NS1P migrated more slowly than linearized plasmid DNA and can be ascribed to multiple rounds of plasmid template DNA copying, yielding circular molecules with single-stranded tails of various length (13). These intermediates, marked "c" in Fig. 3, were not formed efficiently in the presence of NS1O, although labeled DNA was detected in the plasmid-length region, which argues for a role of NS1 phosphorylation in the strand-displacement synthesis during RCR (and parvovirus replication). This conclusion is in agreement with the previously reported deficiency of NS1O in helicase activity. Third, the predominantly labeled DNA product obtained with NS1O (marked "b" in Fig. 3) was slightly upshifted compared with the linearized plasmid (marked "a"). This mobility-shift is expected for DNA molecules which underwent nicking and replication

initiation, resulting in a short stretch of newly synthesized DNA in absence of extensive strand-displacement synthesis. This species "b" is not detected using replication competent NS1P, suggesting that the lack of NS1 phosphorylation is associated also with an impairment of growing strand elongation of initiated DNA molecules and not just with initiation of DNA replication. This is in agreement with recently reported in vitro assays, showing that, though proficient for site-specific nicking, NS1O is less efficient than NS1P for this function, whereas the main deficiency concerns NS1 helicase activity, which is thought to facilitate unwinding of the double-strand template to allow the replication fork to proceed (60).

Reactivation of NS1O for rolling circle replication by members of the protein kinase C family.

Since NS1O was able to support RCR in the presence of cell extracts containing endogenous protein kinases, but distinguished itself from NS1P in its low capacity to achieve this reaction in the kinase-free in vitro replication system, we further attempted to reactivate NS1O for RCR by providing exogenous cellular components. Indeed, NS1O replication activity could be stimulated to significant extent by supplying the reaction mixture with limited amounts of whole HeLa replication extracts (data not shown). This result encouraged us to fractionate HeLa cell extracts in order to characterize the NS1O-"activating" components, in particular to determine whether the rescue of NS1O replication activity co-segregated with NS1 phosphorylation by specific protein kinases as postulated.

Figure 4 gives the purification scheme of HeLa cell extracts used for these reactivation experiments. Individual fractions were tested for their ability to phosphorylate NS1O, as indicated, and their capacity to reactivate the underphosphorylated polypeptide was investigated using various concentrations either alone or in combination with other cell fractions. Native wild type NS1P and replication deficient mutant Y210F served as positive and negative controls in these assays. The specificity of the reactions was also ascertained by using active (pL1-2TC) versus inactive (pL1-2GAA) origin containing substrates, and by immunoprecipitation of NS1-bound replication products with aNSN antiserum. The chromatography steps (1) and (2) were designed to achieve a first bulk segregation of the multiple protein kinases which are able to phosphorylate NS1O. As illustrated in Fig. 2C, more than 50% of the NS1-targeted kinase activity segregated in phosphocellulose fraction P3 (step 1) and proved to be non-essential, since NS1O was almost as efficient as NS1P for RCR in the sole presence of proteins contained in P1-Thr and P2. Furthermore, neither the whole P3 fraction, nor subfractions thereof were able to activate NS1O in the kinase-free replication system (data not shown). This result indicated that specific rather than random phosphorylation of NS1 is necessary for replicative functions of the viral product. After further purification of P2 on anion exchange columns (step 2), NS1O-

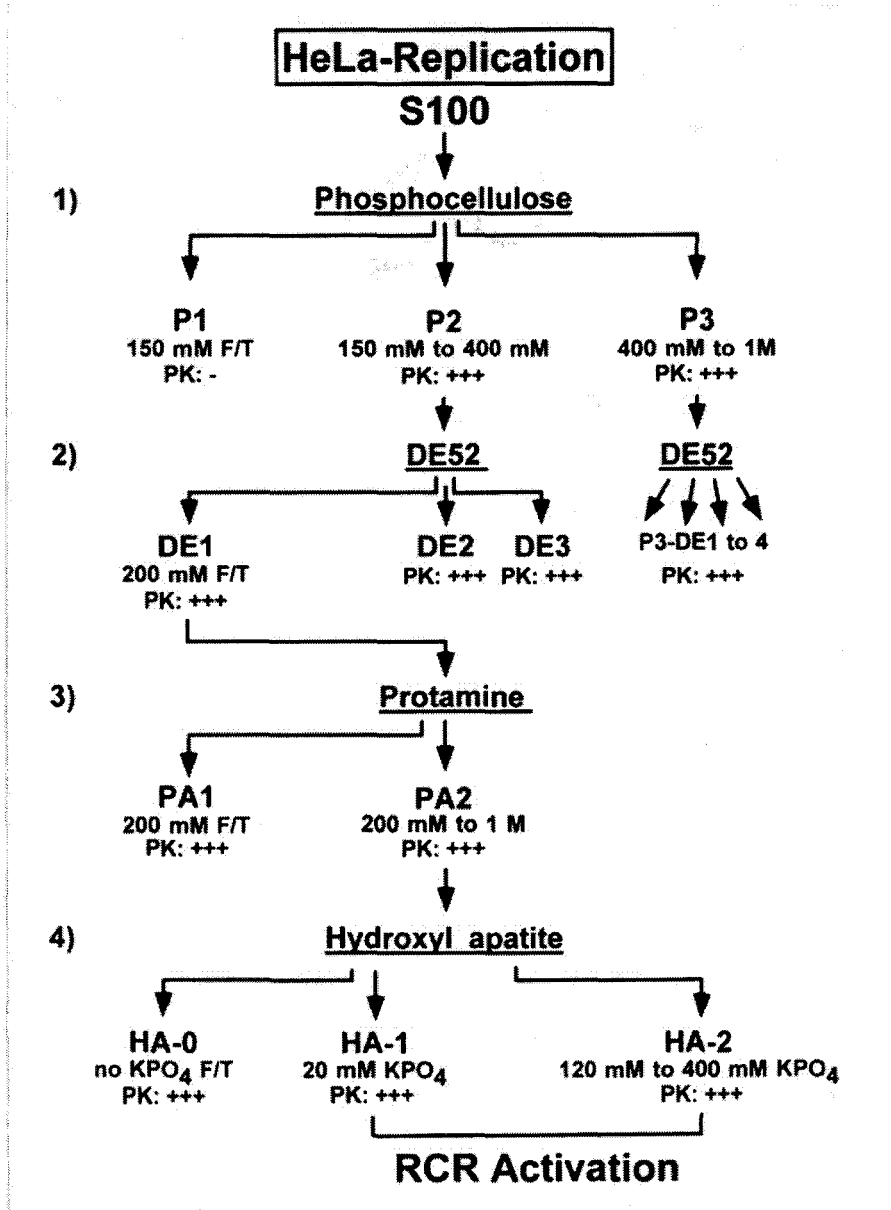


Figure 4: Purification scheme used to identify NS1O activating protein kinases.
HeLa replication extracts were analyzed for protein kinases which are able to phosphorylate and activate NS1O for RCR in a kinase-free system. Steps 1 (phosphocelluloses) and 2 (strong anion exchange) are performed for bulk separations of protein kinases, step 3 (protamine affinity) and 4 (hydroxyl apatite) to purify members of the protein kinase C family. Selective elution of the fractions under investigation from columns 1, 2 and 3, was achieved using indicated NaCl concentrations, whereas the bound components on hydroxyl apatite (4) were eluted in phosphate buffer. PK: - or PK: +++, respectively, stand for *in vitro* protein kinase activity assayed with NS1O as a substrate

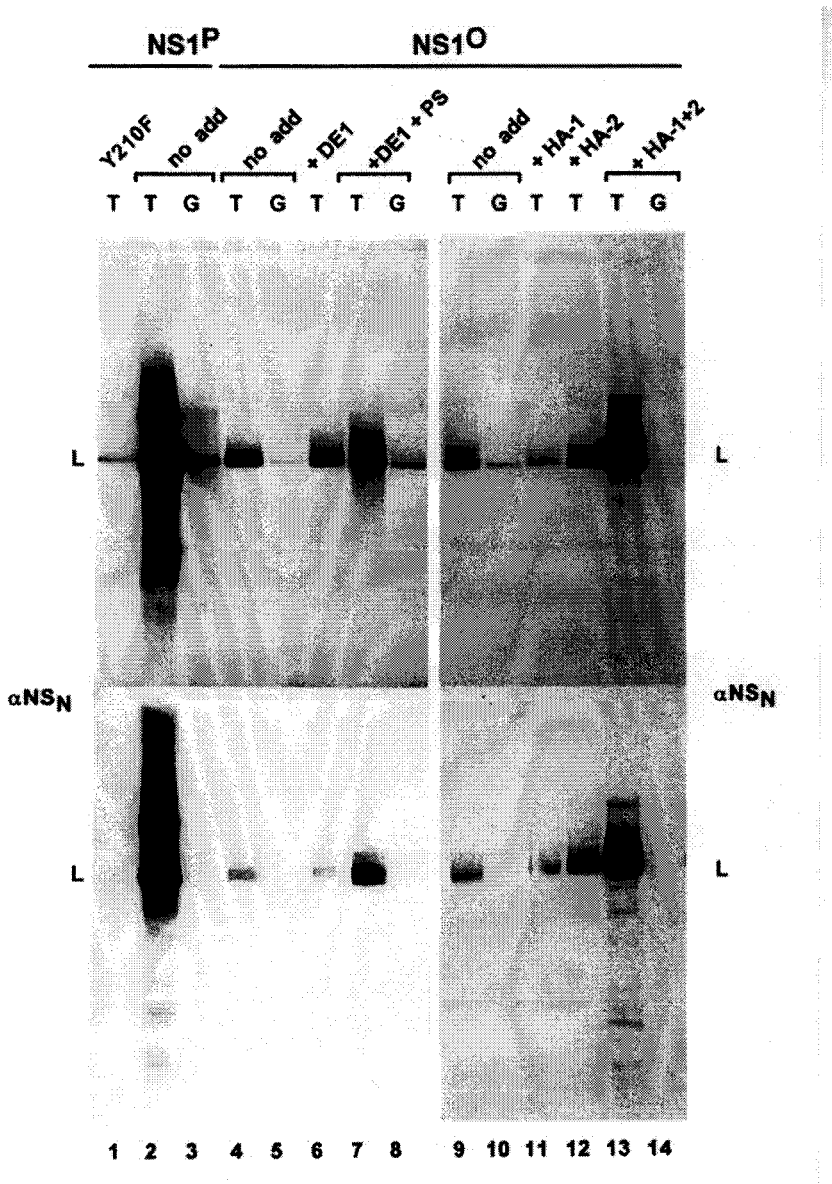


Figure 5: Activation of NS1O for RCR by fractionated HeLa extracts.

NS1 dependent rolling circle replication of plasmids containing the left-end active (T) or inactive (G) origin was determined in a kinase-free *in vitro* system based on P1-Thr and T4 DNA polymerase. Activation of NS1O (lanes 4-14) was achieved using the indicated protein components (cf. Fig. 4) and selected protein kinase C co-factors. The experimental data of the figure are a representative summary of protein/co-factor combinations, in order to activate to potential kinases. The left panel presents the dependency on PKC co-factors for NS1O activation. PS stands for Ca2+ plus L-α-phosphatidyl-L-serine (PS). The right panel shows the separation of (at least) two protein components, necessary to achieve full reactivation of the dephosphorylated polypeptide. PKC present in HA-1 have been activated by Ca2+/PS. HA-2 or HA-1+2, respectively, were activated upon addition of the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA). It has to be mentioned that HA-1 stimulation by TPA, as well as HA-2 stimulation by Ca2+/PS did not lead to activation of NS1O (data not shown) , whereas TPA can be substituted for by Ca2+/PS when both protein components are present (cf. DE-1 activation; Fig. 6). NS1 mutant Y210F (lane 1) served as a negative control. Native NS1P was used as the phosphorylated, replication competent, standard. "L" indicates the migration of the linearized plasmid. The lower panel presents reaction products after immunoprecipitation with αNSN.

stimulating activity was recovered in the low affinity DE-1 fraction, eluting between 50 mM and 200 mM NaCl (Fig. 5, lanes 4 and 7). No additional stimulation resulted from the supply of kinases present in fractions eluting at higher salt concentrations, despite their ability to phosphorylate NS1O to a significant extent in vitro (data not shown). Interestingly, DE-1 induced reactivation of NS1O for RCR in the kinase-free system was dependent upon the addition of Ca2+ and acid lipidssuch as PS (L-a-phosphatidyl-L-serine) (Fig. 5, lanes 6 and 7). Since PS is known to constitute a co-factor of protein kinase C (PKC) (44), this requirement strongly suggested that NS1O stimulation for RCR is achieved through phosphorylation by specific kinase(s), most likely members of the PKC family, rather than by providing cellular replication factors or accessory proteins. In addition, the dependency of NS1O stimulation on PKC-activators is consistent with our previous finding that the helicase activity of NS1O can be restored at least in part by commercially available protein kinase C preparations (60).

The following purification steps (3 and 4) were applied to concentrate PKCs and to separate distinct isoforms of this protein kinase family. Protamine affinity columns are commonly used to purify PKC from bulk proteins, due to the high affinity of these kinases for basic protein substrates (61). On the other hand, at least some of the structural related PKC isoforms can be separated on hydroxyl apatite columns, based on their varying requirement for Ca2+-ions and affinity to phosphate-groups (32, 62). As expected, NS1O-activating components were retained on the protamine column and recovered in fraction PA-2 after high salt elution. A further concentration of these components was achieved by hydroxyl apatite chromatography, yielding an inactive flow-through and two bound fractions of low (HA-1) and high (HA-2) affinity (eluting at 20 mM and 120-400 mM KPO4, respectively). Though inactive on their own, the HA-1 and HA-2 fractions were able to reactivate NS1O when supplied in combination to RCR assays (Fig. 5, lanes 9-14). The stimulation by HA-1/2 was a specific NS1 dependent RCR process. Indeed, no significant DNA-synthesis was observed using the inactive GAA-origin as a substrate (Fig. 5, lane 14) and the reaction products obtained with the active (TC) origin were covalently attached to NS1 as shown by immunoprecipitation (Fig. 5, bottom lane 13). As mentioned above for the DE-1 fraction, NS1O reactivation using HA-1 plus HA-2 subfractions required PKC co-factors. As examplified in Fig. 5, phorbol esters, such as TPA, which are potent activators of classical and novel protein kinase C isoforms, successfully fulfilled this requirements in replacement to Ca2+/PS, providing further evidence for the involvement of this protein kinase family in regulation of NS1 replicative functions. Alltogether, these data argue for the involvement of two or more distinct protein kinases, including at least one member of the PKC family, in the up-modulation of NS1 replicative functions.

The reactivation experiments shown above were carried out with a rather artificial replication system based on bacteriophage T4 DNA polymerase. To substantiate these data in the presence of eukaryotic polymerases, we took advantage of the requirement for this NS1O-activation for PKC co-factors. Unlike standard HeLa replication extracts, fractions P1-Thr (purified on phosphocellulose and L-Thr affinity columns) and P2-pol (purified on phosphocellulose and DE52 columns) are depleted of residual membrane structures which could serve as PKC activators (61). Assuming that the results obtained with the kinase-free system can be extrapolated to mammalian DNA polymerases, unlike NS1P, NS1O should only be active for RCR in the presence of activated PKC. In order to test this prediction,

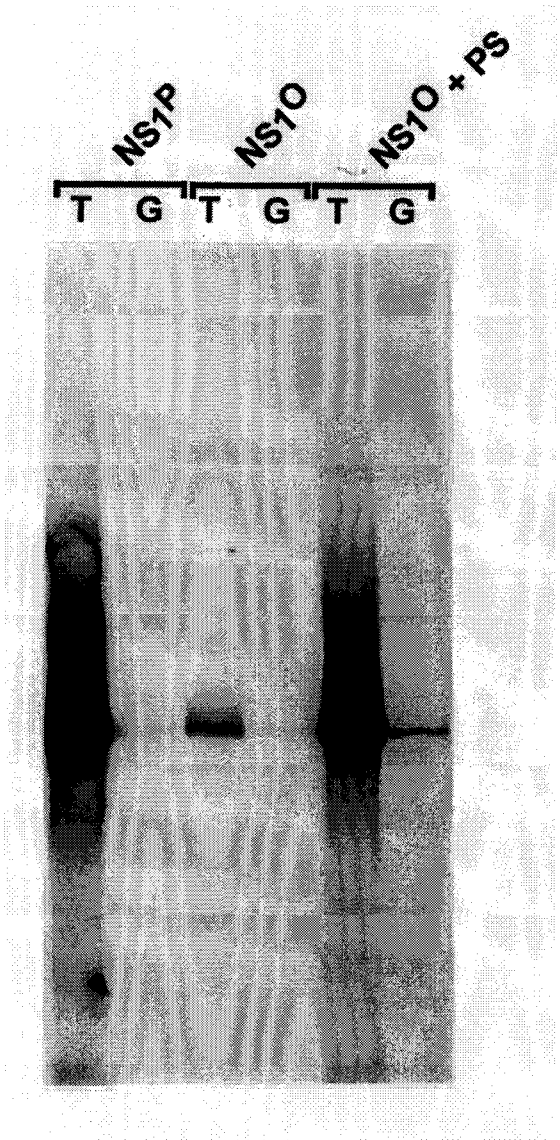
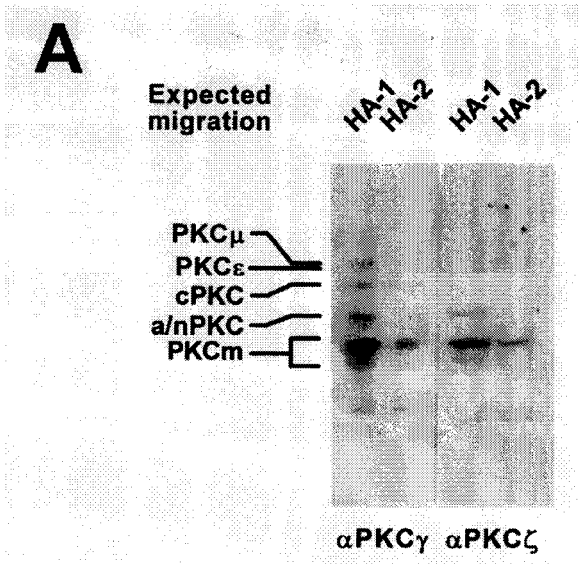


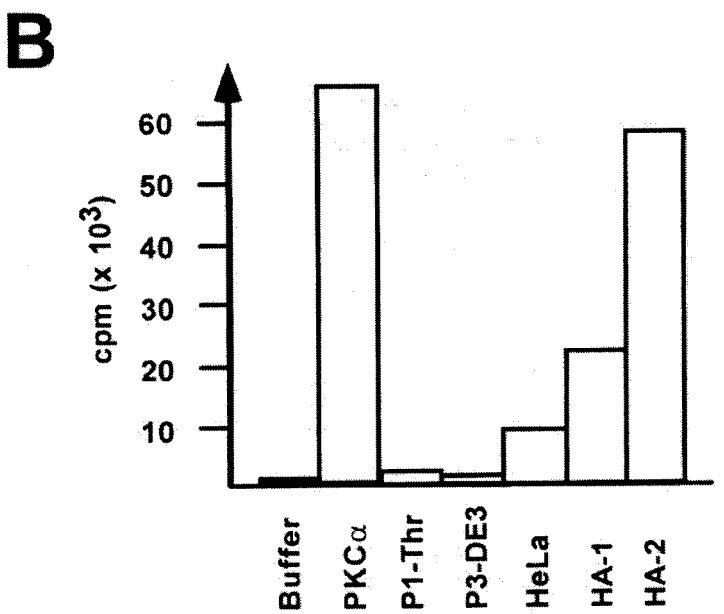
Figure 6: Effect of PKC co-factors on activation of NS1O replicative functions in an entirely eucaryotic replication system.

NS1P and NS1O were compared for RCR of plasmids containing the left-end active (T) or inactive (G) origin of replication, respectively using P1-Thr plus P2-pol (containing endogenous DNA polymerases). NS1O activity was tested either in absence or in presence (+ PS) of 1.5 μg L-α-phosphatidyl-L-serine. All reaction were performed in the presence of 2 mM CaCl2.

NS1P and NS1O were compared for RCR using P1-Thr plus P2-pol in the presence and absence of the PKC co-factors. As shown in Fig. 6, the ability of NS1O to support RCR of pL1-2TC templates under these conditions was dependent upon addition of PKC co-factors Ca2+/PS, in spite of the fact that P2-pol contains - besides DNA polymerases - multiple protein kinases which do not require additional co-factors for activation. This experiment clearly demonstrates that dephosphorylated NS1O is not irreversibly inactivated with regard to its replication function, and it extends above mentioned results to suggest that phosphorylation by distinct protein kinases is required for NS1 activity in a purely eucaryotic DNA replication system.

The protamine and hydroxyl apatite affinity of the NS1O-reactivating components, together with their co-factor requirements strongly suggest PKC to be essential to activate NS1 for replication in vitro. PKC is a family of heterogeneous isoforms which share a high degree of homology within the catalytic domain (61). We therefore produced polycolonal antibodies against most conserved parts of this region from PKCg (aa 416 to 569) or PKCz (aa 310 to 444). After affinity purification these antisera were used in Western blots to determine whether PKC proteins were present in the NS1O activating fractions HA-1 and HA-2. As illustrated in Fig. 7A, multiple proteins with the size of known PKC isoforms were indeed immunodetected within these fractions, in addition to a major product of lower molecular weight corresponding to PKCm, the proteolytically cleaved catalytic domain of PKC. To substantiate these data, we also performed PKC activity assays, using a commercially available PKC detection system (Amersham). As seen in Fig. 7B, fractions HA-1 and HA-2 were both enriched in PKC activity, compared with crude HeLa cell extracts or the "negative" controls P1-Thr (which does not contain significant protein kinase activity), and P3-DE3 (for which NS1O constitutes a target regarding in vitro phosphorylation but not replication reactivation (data not shown)). Furthermore, we evaluated whether NS1 was a target substrate for the semi-purified protein kinases within these two fractions. NS1O was subjected to in vitro kinase assays by HA-1 and HA-2 in the presence of g[32P]ATP and the labeled proteins were analyzed by SDS-PAGE. As illustrated in Fig. 7C, either of the two fractions HA-1 and HA-2 were able to phosphorylate NS1 alone or in combination. Surprisingly, under the assay conditions phosphorylation of NS1 was only slightly stimulated upon addition of PKC co-factors. However, it is presently unknown, whether additional kinases are still present within the semi-purified HA-1/2 fractions, or whether the addition of PKC co-factors could target the protein kinases for selected phosphorylation site(s) within the polypeptide. Altogether, these results clearly demonstrate the presence of highly active PKC within both fractions HA-1 and HA-2 and the ability of these kinases to phosphorylate NS1O individually or in combination (Fig. 7C).





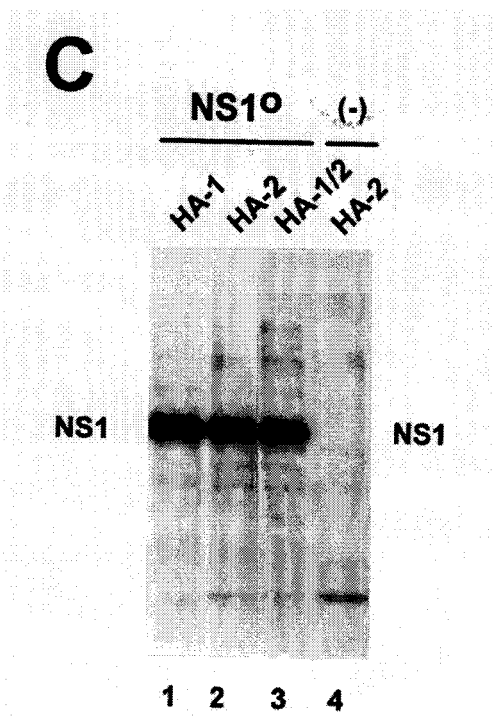


Figure 7: Detection of protein kinase C within fractions HA-1 and HA-2 from HeLa cell extracts.
(A) Western blot analysis of HA-1 or HA-2 after 10% PAGE, using peptide affinity-purified polyclonal antisera that were raised against most conserved regions of classical PKCs (PKCγ: aa 416 to 569) or atypical PKCs (PKCζ: aa 310 to 444), in order to obtain cross-reactivities between the various PKC isoforms. The left panel indicates the estimated migration positions of known PKC isoforms. cPKC stands for classical PKCα, β, and γ (approximately 80 kDa); a/nPKC for atypical PKCι/λ,ζ (70-72 kDa) and novel PKC δ, η, θ (72-78 kDa). The novel PKCε (90 kDa) and PKCμ (115 kDa) which are significantly larger than the other PKC isoforms are indicated as well. PKCm (approximately 45 kDa) denotes the catalytic domains of PKC, which are derived from proteolytic cleavage. (B) PKC activity assays (Amersham) of fractions used for reactivation of NS1O in replication assays. Buffer: negative control in absence of added protein components; PKCα: 10 ng His-TAG purified recombinant PKCα produced by recombinant vaccinia virus expression in HeLa cells; P1-Thr, fraction containing no protein kinase activity on NS1O (cf. Fig. 2B); P3-DE3: fraction proved able to phosphorylate but failed to reactivate NS1O (cf. Fig. 4); HeLa: standard HeLa replication extract; HA-1, HA-2: NS1O activating fractions in RCR assays (cf. Fig. 5). The values (except for the recombinant PKCα control) are expressed as transferred 32P-labeled substrate per 10 μg of total effector protein. (C) *In vitro* phosphorylation of 1 μg NS1O with protein fractions HA-1 and HA-2, used individually or in combination as protein kinase. Activation of PKC present within the fractions was achieved with selected co-factors. The reactions were performed in the presence of [32P]γATP for 30 min at 37oC, stopped in 0.1% SDS; 2.5 mM EDTA for 30 min at 70OC and analyzed directly by 10% SDS-PAGE. In lane 4 the NS1O substrate was omitted. The migration of NS1 is indicated.

Stimulation of NS1O helicase activity by members of the protein kinase C family.

The above mentioned analyses of NS1-driven RCR in the kinase-free in vitro replication system indicated that a major NS1 phosphorylation dependent step consisted in processive strand-displacement synthesis (Fig. 3). This step of RCR (and also parvovirus DNA replication) is thought to involve the unwinding function of NS1. Consistently, NS1O was found to be heavily impaired for this biochemical activity in standard helicase assays (Fig. 1B and (60)). In order to investigate whether the intrinsic helicase function of NS1 is regulated by the same components as those identified with the in vitro RCR system, the HA-fractions were tested for their ability to rescue NS1O in helicase assays. As illustrated in Fig. 8A, one of these fractions, namely HA-1 was able to stimulate the helicase function of NS1O to a significant extent, whereas HA-2 or the flow-through HA-0 had no detectable effect. All HA-fractions showed no helicase activity by itself, even when tested in a 100-fold excess over the amount used to stimulate the NS1O helicase activity (Fig. 8A, lanes 4 to 6), indicating that this stimulation resulted from the activation of NS1O rather then supply of (a) cellular helicase(s).

These assays were performed in the presence of Ca2+/ PS, i.e. under conditions which activate all members of the PKC family. PKC have been subdivided into three subgroups according to their co-factor requirements. Classical (a, b, and g) and novel (d, e, q, h, and m) PKC isoforms are stimulated through binding of phorbol esters such as TPA, or to a minor extent by acid lipids (PS), whereas full activation of classical PKC requires Ca2+ in addition to PS. Novel PKC do not contain a Ca2+-binding-site. Members of the last group, designated atypical PKC isoforms (i/l, andz), lack a calcium binding domain and do not respond to TPA either, but get stimulated by acid lipids. Finally, PKCm, the catalytic domain of PKC which derives from proteolytic cleavage, is constitutively active, i.e. its kinase activity is independent of co-factors (44, 61). In order to characterize the activating kinase(s) present in fraction HA-1, we further tested NS1O reactivation for helicase function in the presence of HA-1 and selected PKC co-factors. As seen in figure 8B, the HA-1 fraction had a moderate, constitutive stimulatory effect in absence of co-factors (lane 7), which may be ascribed to PKCm and/or other co-factor independent components. In contrast, this activation was significantly enhanced upon supply of PS (lane 8) while TPA failed to increase the capacity of unstimulated HA-1 to activate the helicase function of NS1O (lane 9). This PS-responsiveness and TPA-insensitivity of the HA-1 induced rescue of NS1O point to atypical PKC(s) as (one of) the effector(s) mediating the dependence of NS1-helicase activity on phosphorylation. This conclusion was substantiated by Western blot of fractions HA-1 and HA-2 with specific antibodies recognizing the atypical PKCi. HeLa replication extracts and HA-2 were used as positive and negative controls, respectively. As illustrated in Fig. 8C, PKCi was present in HeLa cell extracts, and seggregated mainly to fraction HA-1 during the purification procedure.

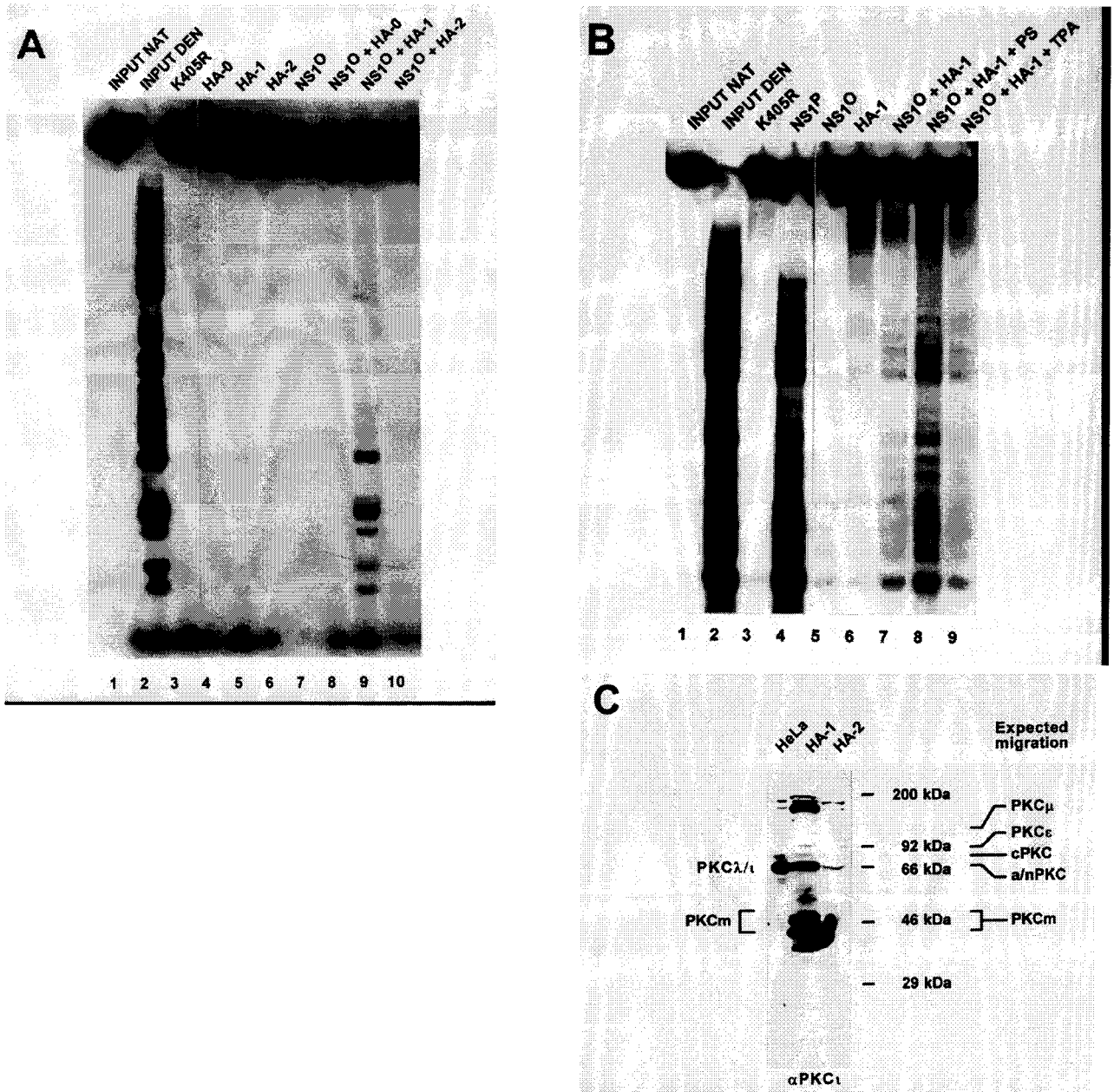


Figure 8: Reactivation of dephosphorylated NS1O for helicase activity.

Helicase assays have been performed as in Fig. 1B, using M13-VAR as a template. (A) Hydroxyl apatite column fractions HA-0, HA-1, or HA-2 were tested for their intrinsic helicase activity (lanes 4-6) and for activation of NS1O in the presence of the PKC co-factor Ca2+ /PS (lanes 8-10). Lane 7 presents helicase activity of unstimulated NS1O. The NS1 mutant K405R served as a negative control (lane 3). Lanes 1 and 2, native and denatured input DNA. The reaction products were analyzed by 7% native PAGE in the presence of 0.1% SDS. (B,C) Characterization of PKC isoforms present within the HA-1 fraction that is able to reactivate NS1O for helicase activity. (B) In order to evaluate the nature of the protein kinase(s) present in HA-1, which are able to restore NS1O helicase activity, reactivation experiments were performed in the presence of defined PKC co-factors as indicated. Atypical PKC (ι/λ, ζ) are stimulated by acid lipids (PS), but are insensitive to phorbol esters such as TPA, whereas novel and classical PKC are slightly activated by PS alone, but respond strongly to TPA. The catalytic domain of PKC, PKCm has been reported to be constitutively active. Helicase assays were performed as described above. Lane 1 and 2, native and denatured input DNA. NS1 mutant K405R served as a negative control (lane 3), native NS1P as a positive control (lane 4). Dephosphorylated NS1O was assayed either alone or in the presence of HA-1 plus the indicated PKC co-factors (lanes 5 to 9). (C) Immunodetection of atypical PKC in HA-fractions. Equal amounts of HA-1 and HA-2 were analyzed by Western blot using αPKCι antibodies (Transduction Laboratories). HeLa cell extracts served as a positive control. The size markers and the expected migration of the different PKC isoforms are indicated on the right. cPKC stands for classical PKCα, β, and γ (approximately 80 kDa); a/nPKC for atypical PKCι/λ,ζ (70-72 kDa) and novel PKC δ, η, θ (72-78 kDa). The novel PKCε (90 kDa) and PKCμ (115 kDa) which are significantly larger than the other PKC isoforms are indicated as well. PKCm (approximately 45 kDa) denotes the catalytic domains of PKC, which are derived from proteolytic cleavage. The estimated migration of PKCλ/ι as well as PKCm, which are recognized by αPCKι are indicated on the left.

In addition, this fraction contained a large proportion of polypeptides revealed by the aPKCi antibodies that were in the size range of PKCm, the proteolytic products corresponding to the constitutively active catalytic domain of PKCs. This lower molecular weight species could result from the cleavage of PKC during purification and may explain the partial HA-1 induced activation of NS1O in absence of PKC co-factors. It should also be noted that known atypical PKC isoforms are over 70% homologous at the amino acid level (44). Therefore, our data do not rule out that other (atypical) PKC isoforms than PKCi are present within the HA-1 fraction and are responsible for the activation of NS1O helicase function.

Discussion

NS1, the major non-structural protein of minute virus of mice (MVM) is involved in multiple functions necessary for progeny virus production, ranging from DNA replication to promoter regulation and toxic action on the host cell (15). Such a variety of tasks is unlikely to be achievable by a single polypeptide and usually requires the multifunctional protein to interact with heterologous proteins (42), to self-assemble into higher order oligomers (4), to associate with co-factors (61), and/or to become post-translationally modified. The latter possibility can provide an original polypeptide with functional heterogeneity through the addition of various molecule groups as catalyzed by a rather small amount of enzymes with high efficiency targeted at a large protein-pool. All of the above mentioned modes of regulation have been assigned to NS1, including interactions with cellular partners proteins (24, 36), oligomerization (58), and phosphorylation (20). Interestingly, NS1 oligomerization has been implicated in the control of replicative NS1 functions, in particular helicase activity (63). It is worth noting that NS1, self-assembly to produce higher order oligomers is dependent upon an intact NTP-binding domain (58), and might thus be regulated by association with this co-factor. Furthermore, the ATP-bound form of NS1 was found to be most competent for site-specific DNA binding (21). Indeed, ATP-binding and/or hydrolysis seems to be crucial for many NS1-functions, as apparent from the fact that mutagenesis of the ATP-binding domain abolishes all NS1 activities described so far (10, 21, 38, 39, 57-59). In its turn, it was found recently that the NS1 ATP-turnover was controlled by phosphorylation at least under in vitro conditions (60). Thus the regulation of an activity which plays a pivotal role in NS1 functions, may be traced back to the modification of NS1 through phosphorylation. NS1 dephosphorylation correlates with a reduction of NS1 ATPase activity in vitro, which is associated with an increase in the affinity of the viral product for its DNA recognition motif and with a decrease of helicase and site-specific nickase function (60). This regulation might be physiologically relevant since NS1 has been shown to be phosphorylated in

vivo (2, 11, 20). The present work was carried out to further characterize the cellular protein kinases involved in the upmodulation of NS1 replicative functions.

In this study, using an in vitro replication system devoid of endogenous protein kinases, we provide evidence that a central NS1 function necessary for progeny virus production, namely replication initiated at the left-end origin, is regulated by phosphorylation of NS1. This is reminiscent of large T antigen (LT), the initiator protein for SV40 DNA replication, which is also regulated by phosphorylation (71). LT, which is expressed in non-dividing cells and is able to drive quiescent cells into S-phase, becomes activated for replication by phosphorylation at T124 through cyclin dependent kinases. This regulation results in a coordination between SV40 and host cell DNA replication (1, 47). As in the case of SV40, parvovirus DNA replication has been shown to be dependent on the S-phase of host cells (15), yet, in contrast to LT, NS1 production is limited during G0/G1 (25, 66), and parvoviruses fail to drive quiescent cell into S-phase (15). Moreover, the extracts derived from cell arrested in G0 are able to activate the replicative functions of dephosphorylated NS1 in vitro (56). Therefore, post-translational modifications of NS1 do not account for S-phase dependency of parvovirus DNA replication. This is in agreement with the fact that, at least under in vitro conditions, members of the protein kinase C family rather than cdk2-complexes regulate NS1 replicative functions, as indicated by the failure of non-stimulated P2-and P3-fractions to reactivate NS1O for RCR. In addition, when NS1O unwinding activity was tested in the presence of semi-purified cellular components and commercially available protein kinases no activation with cyclin/cdk-complexes could be obtained despite of NS1 phosphorylation in vitro (56, 60).

Besides the S-phase dependency of parvovirus replication, and its consequent restriction to proliferating tissues (43), another feature of these small DNA viruses feature consists in their striking oncotropism (65). This tropism could be mimicked in various cell culture systems, where restrictions to parvovirus replication detected in "normal" parental cell lines were found to be at least partly overcome upon neoplastic transformation (12). In this context it is interesting to note that PKC activators, such as phorbol esters, also exert strong effects on cellular proliferation, differentiation and most intriguingly tumor promotion (44, 61). Moreover, it has been reported that overexpression of PKCe, a "novel" PKC isoform which is activated by phorbol esters and which has been detected in A9 cells the natural host cell of MVMp (26), leads to neoplastic transformation in cell culture (6, 49). These correlations of oncogenic transformation in regard to PKC activity and cell permissiveness to parvovirus replication, together with the present evidence of a role of PKC in the regulation of the pivotal viral replicator protein NS1, raise the possibility that this regulation may contribute to the oncotropism of parvoviruses.

Native NS1P was able to initiate RCR leading to extensive strand-displacement synthesis in absence of protein kinases. In contrast, NS1O had a restricted phenotype that allowed, at least to some extent, the formation of an NS1-bound replication intermediate. These intermediates migrating as a distinct species in agarose gels are thought to represent molecules in which DNA synthesis was initiated but became arrested prior to strand-displacement synthesis, what suggests that besides its role in the site-specific initiation of DNA replication, NS1 is also essential to drive the subsequent strand-displacement reaction in a phosphorylation dependent way. This is in agreement with previously reported findings that dephosphorylated NS1O is deficient in helicase activity (60), which would account for its inability to allow the replication fork to proceed during DNA-synthesis.

Therefore, regulation of the DNA unwinding activity of NS1 might be of crucial importance to turn on replication. It should be stated, however, that the helicase deficiency of NS1O could be corrected by supplementing the sole HA-1 fraction, while RCR reactivation of NS1O required both protein fractions HA-1 and HA-2. Thus, more than one protein kinase seem to be necessary to switch NS1O on for DNA replication. The additional replicative functions of NS1 which are regulated by phosphorylation, besides helicase activities are as yet undefined. One candidate is the nicking reaction which is achieved by NS1O to a detectable albeit reduced levels compared to NS1P in the RCR assays, as well as in an in vitro nicking assays in absence of additional cellular components (60). However, this reduction could also directly result from the defects of NS1O in DNA unwinding, which in analogy to other "Rep"-proteins is thought to facilitate the nickase reaction on single-strand level (35, 67), rather than an additional mode of regulation. Other potential phosphorylation dependent NS1 replicative functions might be considered by analogy to SV40 LT antigen. LT binds to the origin of replication in absence of phosphorylation (45, 46, 50) and is able to interact with components of the basic replication machinery such as RPA (48) or polymerasea primase (28, 29) to establish the replication complex by protein-protein interactions. It is tempting to speculate that NS1 may act in a similar way, knowing that LT- and NS1-driven in vitro replication share specific requirements for eukaryotic DNA polymerases or the related T4 DNA polymerase (69), and for template DNA unwinding by the helicase activity of the respective viral proteins (71). While binding to target [ACCA]-motifs on the viral DNA, NS1 fulfills functions involved not only in DNA replication but also in promoter regulation, raising the possibility that distinct NS1 phosphorylation events may regulate the interaction of the viral polypeptide with proteins from the basal replication and/or transcription machinery. NS1 was indee demonstrated to interact specifically with general transcription factors (36, 40).

NS1 has been shown to be a target for phosphorylation by many protein kinases in vitro (2, 60). Indeed, most HeLa cell fractions tested in this study were found to exhibit NS1 phosphorylation

activity. In contrast, only selected fractions of the original HeLa replication extract were able to activate NS1O for replication activity, pointing to the involvement of specific kinases and phosphorylation events in NS1 regulation. Based on the co-factor requirements of NS1O reactivation and the purification properties of effector kinases, we were able to assign, at least in part, the capacity for NS1 phosphorylation and activation in vitro to members of the protein kinase C family. In particular, the NS1 unwinding function might be regulated by atypical PKCs, given its responsiveness to acid lipids but not to phorbol esters. As stated above, regulation of the NS1 replicative functions appears to be complex and involve more than one protein kinase as indicated by the fact that rescue of NS1O for RCR required at least two protein components that could be separated by hydroxyl apatite (HA) chromatography. When combined, the active HA fractions were able to rescue the RCR functions of NS1O to a significant extent, in the presence of the phorbol ester TPA. Since TPA was unable to activate the PKC responsible for stimulating the helicase function of NS1, our results suggest that at least two PKCs may be involved in NS1 regulation. One member of the classical or novel PKC isoforms, which respond upon TPA, controlling an as yet undefined NS1 function, and another, TPA insensitive atypical PKC isoform which controlls the DNA unwinding activity of NS1. The latter PKC may be activated by the former, thereby accounting for the sole rquirement of TPA as cofactor to rescue the RCR capacity of NS1O, in agreement with the fact that PKCs are themselves regulated by phosphorylation (33). Mapping of the NS1 phosphorylation sites involved in the modulation of replicative functions, as well as further characterization of the cellular protein kinases responsible for these modifications in vitro, should contribute to unravel the posttranslational regulation of NS1 activities.

Acknowledgments

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IV) Mutagenesis of Selected Protein Kinase C Phosphorylation Sites in MVM NS1 Leads to a Loss of Phosphorylation *in vivo* and Impairs NS1 Functions.

Manuscript in preparation

Mutagenesis of Selected Protein Kinase C Phosphorylation Sites in MVM NS1 Leads to a Loss of Phosphorylation in vivo and Impairs NS1 Functions.

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Running Title: Mutagenesis of MVM NS1 PKC phosphorylation sites
Key words: Parvovirus MVM, Non-structural protein NS1, PKC phosphorylation sites, Site-directed mutagenesis.

Abstract

The multifunctional non-structural protein of parvovirus MVM, NS1 is nuclear phosphoprotein that is involved in a variety of processes during virus propagation, ranging from viral DNA replication, promoter regulation to cytotoxic action to the host cell. To coordinate all these different functions, NS1 has been proposed to be regulated by post-translational modifications, in particular phosphorylation. Indeed, NS1 is differentially phosphorylated on serine and threonine residues during the course of a viral infection. Phosphorylation residues are located mainly between amino acids 315 and 482 covering a large proportion of the putative helicase domain. Moreover, in vitro analysis have shown that replicative functions, in particular DNA unwinding activity of NS1 are regulated by members of the protein kinase C family. In order to determine possible regulatory phosphorylation sites in NS1, we performed site-directed mutagenesis of conserved target PKC phosphorylation sites in NS1, namely T363, T403, and T435 replacing the threonine substrate residues individually to inert alanines. T403 and T435 were identified as target phosphorylation sites in vivo. Both amino acids proved crucial for replication activities, whereas T435A retained the capacity to trans-activate the viral P38 promoter. Despite significant activity

for all intrinsic biochemical activities under investigation, T403A was unable to initiate viral DNA replication, while T435A was severely impaired for DNA unwinding activity as measured by helicase assays. The finding that T435 is also a target phosphorylation site in vitro for atypical PKC, which proved able to modulate NS1 helicase activity, makes T435 a target site for regulation of NS1 replicative functions.

Introduction

Parvoviruses are small non-enveloped spherical viruses with a single-strand linear DNA as a genome which encodes for two structural (VP) and at least four non-structural (NS) proteins (for review see(11)). From the non-structural proteins, only the major 83 kDa nuclear phosphoprotein NS1 is required for progeny virus production in all cell types (18, 31). This multifunctional protein is involved in many processes during the virus cycle, ranging from DNA replication and promoter regulation to cytotoxic action to the host cell. Replication of the parvoviral genome involves the formation of monomeric and concatemeric duplex DNA intermediates that are produced by an unidirectional, single-strand copy mechanism (for review see (12)), which resembles the rolling circle replication (RCR) mechanism described for single-strand plasmids, bacteriophages, and geminiviruses (for review see (23)). After conversion of the single-strand viral DNA to a covalently closed circular monomeric duplex, which is executed solely by cellular components (1), replication initiates at site-specific, single-strand nicks, which are introduced into the origin sequences by the viral non-structural protein NS1 to generate a free 3'OH to serve as a primer for DNA polymerases. This nicking reaction leaves the viral protein covalently attached at the 5'ends of replicated viral DNA (15).

Many of the replication reactions during viral DNA amplification have been reproduced at least in part using cell free systems and purified recombinant NS1. As substrates either natural MVM DNA was subjected to the reactions(1), which successfully reproduced the hairpin-transfer mechanism which occurs at the right-end of the viral genome. Alternatively, it was also possible to use cloned viral DNA-fragments, which corresponded to the right-end (tetramer) bridge (13) or the left-end (dimer-bridge) (10) to mimick resolution and replication of the concatemers found during virus propagation. Dissection of these cloned viral DNA fragments finally lead to the identification of the viral origins located at either end of the genome. These origins, when present in a circular plasmid are substrates for NS1 to initiate rolling circle replication (RCR) in vitro (9). The minimal origin sequences at the left-end telomere have been mapped, consisting of approximately 50 bp within the Y-shaped teminal structure (9). They are composed of a binding-site for the cellular component PIF (6), the NS1 binding-site (17), and the NS1 nick-site (9),

which is spaced from the NS1 binding-site by an A/T-rich sequence. This A/T-sequel most likely facilitates local unwinding of the origin by NS1 during the nicking reaction. Between the binding-sites for PIF and NS1 there is an important mismatched "bubble" where a triplet 5'-GAA-3' on one strand opposes a dinucleotide 5'-GA-3'. When replication through this hairpin unfolds and copies the palindrome to a double-strand intermediate, these tri- and dinucleotide sequences are located on either side of the axis of symmetry of the left-end bridge. Although the two origins of both arms are nearly identical, only the arm containing the GA dinucleotide serves as an active replication origin for NS1 mediated RCR, the trinucleotide counterpart remains silent (9).

Besides its keyrole during viral DNA replication, NS1 also regulates its own P4 promoter (21) and it is a strong trans-activator for the viral P38 promoter which controls the expression of the capsid proteins (40). This variety of NS1 functions during virus propagation most likely require different properties of the polypeptide, leading to selective biochemical activities of the multifunctional protein in order to achieve the required tasks. Since NS1 becomes phosphorylated in infected cells (16) it has been proposed that NS1 activities are regulated by post-translational modifications. MVM NS1 has been shown to be phosphorylated in infected cells at serine and threonine but not on tyrosine residues, whereas the phosphorylation pattern changes as the virus cycle progresses (7). Moreover, in vitro analyses using a kinase-free replication system have provided evidence for the requirement of NS1 phosphorylation to support RCR of plasmids containing the left-end origin (37). This requirement was further analyzed comparing native NS1P with dephosphorylated NS1O for selected biochemical activities required for replicative functions of the viral protein. While site-specific interaction with the cognate DNA recognition element [ACCA]2-3 was enhanced for NS1O, significant reduction of activities requiring energy exerted by ATP-hydrolysis was observed, manifested in NS1 endonuclease activity and most pronounced for DNA unwinding (36). Consecutive reactivation of the un(der)phosphorylated polypeptide determined members of the protein kinase C (PKC) family to be able to phosphorylate and activate NS1O in vitro (20, 36, 37), indicating the use of target PKC phosphorylation site(s) within NS1 to serve as regulatory elements of the various functions of the polypeptide.

In order to determine distinct phosphorylation site(s) in NS1 which are regulating the various activities of the multifunctional polypeptide, we performed site-directed mutagenesis in selected conserved PKC phosphorylation sites, changing the target amino acids T363, T403, and T435, respectively, to an inert alanine. Since the phosphorylation pattern of NS1 expressed by recombinant vaccinia viruses is very similar to the genuine MVM NS1, and since the vaccinia NS1 is a replication active polypeptide in absence of protein kinases, we examined the in vivo phosphorylation patterns of mutant NS1 proteins in comparison to wild type using this expression system. Furthermore, we investigated the impact of this mutagenesis on general NS1 functions,

such as replication activity and P38 trans-activation, as well as selected biochemical activities, e.g. site-specific binding to the left-end origin, site-specific nicking, and helicase activity. These analyses revealed, T403 and T435 as target phosphorylation sites in vivo, and the mutagenesis of these amino acid residues to alanine selectively affected distinct biochemical properties of NS1. Moreover, the coincidence that atypical PKC, which are able to regulate NS1 helicase activity in vitro (20, 37) and phosphorylate NS1 at residue T435, together with the lack of DNA unwinding activity of the NS1 mutant T435A strongly suggest the regulation of this NS1 function through phosphorylation of this PKC-site.

Material and Methods

Viruses and cells

Recombinant vaccinia viruses were constructed using pTM-1 (30) derived constructs as described (33) and the recombinant viruses were propagated in monolayer cultures of BSC-40 or HeLa cells, purified over a sucrose cushion (27), except for the release of the virus from cells by three cycles of freezing and thawing instead of sonification. MVMp was propagated in A9 cells. HeLa, BSC-40, and A9 cells were grown as monlayers in Dulbecco's Modified Eagle Medium (DMEM) containing 5% fetal calf serum. HeLa-S3 were grown in suspension using Spinner-bottles in Joklik's medium containing 5% fetal calf serum.

Plasmids and mutagenesis

The construction of the plasmid pTHis-NS1wt has been described previously (35). It consists of a modification of pTM1-NS1 in order that it expresses a [His]6-TAG followed by an enterokinase cleavage-site at the N-terminus of the MVM NS1 polypeptide. Site-directed mutagenesis in NS1 was performed by replacement of the EcoRI (nt 1085) to BstEII (nt 1885) fragment of NS1 by a PCR fragment containing the replacement of the appropriate amino acid. All PCR derived sequences have been verified for the correct coding capacity by sequencing. Site-directed substitution mutagenesis was performed by chimeric PCR as described (34) using the outside rightward primer 5'-CTAAGCGCGGCAGA-3' and leftward primer 5'-GGTAGATTGGTAACCATTC-3' together with two mutagenic overlapping primers. For replacement of T363 to alanine the mutagenic primers 5'-GCCTGACACAAGAGCCTGCAG-3' and 5'-CTGCAGGCTCTTGTGTCAGGC-3' were used, for T403A the primers 5'-GGACCAGCCAGCGCAGGCAAATC-3' and 5'-GATTTGCCTGCGCTGGCTGGTCC-3' and for T435A the primers 5'-TAATGACTGTGCCAACAAGAAC-3' and 5'-GTTCTTGTTGGGACAGTCATTA-3'. NS1-expression plasmids to perform P38 transactivation assays were constructed by transferring the mutant NS1 genes into pRSV-NS (44). EcoRV (385) to BstEII (1885) NS1-fragments carrying the mutations replaced the wild type fragment in pRSV-NS. The reporter plasmid pP38-Luc has been obtained by replacement of the cat-gene in pP38-cat (44) with the SmaI to XhoI luciferase-cassette derived from pGL-2-basic (Promega).

In vivo 32P-labeling and tryptic peptide analysis

Metabolic 32P-labeling for MVM NS1 was performed as described (36) by infection with MVMp in A9 cultures. Labeling was performed 20 h post infection for 4 h in the presence of [32P]-orthophosphate. For Vaccinia NS1 32P-labeling was performed in HeLa cells 5 h post infection with 15 pfu/cell of each vTF7-3 and the appropriate recombinant vaccinia virus containing the NS1 gene under control of the bacteriophage T7 promoter, using the same conditions as for MVM NS1. Cells were harvested directly into RIPA-buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Na-deoxycholate, 1% Triton X-100) containing protease and phosphatase inhibitors and Immunoprecipitations were carried out with aSP8 (2). Immune-complexes were further purified on SDS-PAGE, blotted on PVDF-membranes (polyvinylidene fluoride; Millipore) and the band corresponding to NS1 excised. Digestion of membrane bound NS1 was performed with 50 units of trypsin, or alternatively with 15 mg of chymotrypsin (Boehringer Mannheim) for 18 h at 37OC and the digested, purified 32P-labeled peptides were analyzed on thin layer cellulose plates (MERCK) in two dimensions, by electrophoresis using pH 1.9 buffer and chromatography in pH 3.5 phosphochromatography buffer (36).

Production and purification of wild type and mutant NS1

NS1 was produced from recombinant vaccinia viruses in suspension cultures of HeLa-S3 cells (36) using 15 pfu/cell each of vTF7-3 together with the appropriate recombinant vaccinia viruses containing the NS1 gene under control of the bacteriophage T7 promoter (33, 35). Infected cultures were harvested 18 h post infection, nuclear extracts prepared, and His-NS1 was purified using Ni2+-NTA agarose (Qiagen) columns (35). NS1 preparations were analyzed by discontinuous SDS-PAGE and proteins were detected by Coomassie-blue staining. All mutant

NS1 proteins that have been characterized previously were tested for their biochemical properties in various assays before use as controls.

In vitro resolution and replication reactions with plasmids containing MVM origins

Resolution and replication reactions were performed as previously described using pREB1412, pLEB711 (14), or the RCR plasmids pL1-2TC, pL1-2GAA, and p5'AGA (9), respectively as substrates. Approximately 100 ng of purified NS1 expressed from HeLa cells was supplied to HeLa replication extracts. The reaction mixture was incubated for 2 h at 37OC in the presence of dNTPs including a[32P]dATP, ATP and an ATP regenerating system. The NS1-attached labeled products were recovered by immunoprecipitations with aNSN (19), digested with ScaI (pREB1412; pLEB711) and the resulting fragments were further separated for NS1-bound and NS1-free fractions by centrifugation. For RCR-reactions, the reaction products were immunoprecipitated with aNSN, cleaved with HindIII, and digested by proteinase K. The 32P-labeled isolated DNA was then analyzed by 0.8% agarose electrophoresis and revealed by autroradiography.

P38-transactivation

To measure the potential of wild type NS1 and mutant derivatives to trans-activate the P38 promoter, 50 ng of pP38-Luc was co-transfected with various concentrations of the NS1 expression plasmids pRSV-NS1x into 2x105 A9 cells grown in monolayer cultures. 48 h post transfection cells were harvested into lysis-buffer (1.5 mM glyc-glycine pH 7.8; 15 mM MgSO4, 0.4 mM EGTA, 1 mM DTT, 1% Triton X-100, 10% glycerole) and measured for their luciferase activity as described (44). Luciferase activity obtained without expression vectors represents 0% activity, activity obtained with wild type NS1 was set to 100%. Average of three independent transfection experiments using various amounts of pRSV-NS1x were performed. The summarized data obtained for trans-activation with mutant NS1 are presented with standard deviations bars in percentage of activity compared to wild type NS1.

Site-specific binding of NS1 to the MVM 3' origin of replication

Site-specific binding assays using NS1 and the MVM left-end origin were performed as described (17). Briefly, plasmid pL1-2TC, which contains the minimal active left-end replication origin (9), was digested with restriction enzymes Sau3AI and NarI, and the DNA fragments were labeled at the 3'end by filling in with sequenase (Amersham), a[32P]dGTP and unlabeled dATP, dCTP, and dTTP. Binding assays were carried out in 100ml of 20 mM Tris pH 8.0, 10% glycerol, 1% NP-40, 5 mM DTT, and 100 mM NaCl, supplemented with labeled, fractionated pL1-2TC DNA, 500 ng oligo d[I,C], 0.5 mM g-S-ATP, and 50 ng of purified NS1. After allowing interactions to take place for 30 min on ice, 2 ml of antiserum aNSN was added and th incubation was continued for another hour. Immune complexes were precipitated with protein A sepharose, deproteinized, and analyzed by non-denaturing 7% PAGE in the presence of 0.1% SDS.

Site-specific nicking of the left-end origin of replication

NS1 mediated site-specific nicking and covalent attachment of NS1 to the 5' end of the nicked product were analyzed in absence of any additional protein components (35) or in the presence of the parvovirus initiation factor PIF (5). The substrate containing the left-end origin or replication was obtained as a 95 bp EcoRI fragment of pL1-2TC (9)which had been 3' end-labeled by fill-in reaction using sequenase , a[32P]dATP and unlabeled dTTP. In order to test for intrinsic site-specific endonuclease activity, approximately 1 ng of substrate was incubated with 20 ng of NS1 in the presence of 3 mM ATP for 1h at 37OC. The reaction was stopped by adding 0.1% SDS and 2.5 mM EDTA, and immunoprecipitations were performed using aNSN. The immune-complexes were freed from proteins by proteinase K digestion and phenol/chloroform extraction and analyzed on 8% sequencing gels. In the presence of the cellular protein PIF, approximately 1 ng of substrate was incubated with 20 ng of NS1, 5 ng of recombinant PIF in the presence of 3 mM ATP, 100 mM NaCl and 100 ng of non-specific competitor DNA (pUC18 cleaved with HaeIII) for 30 min at 37OC. The reaction was stopped by adding 0.1% SDS and 2.5 mM EDTA boiled for 5 min and anaylzed by non-denaturing PAGE in the presence of 0.1% SDS.

Helicase assay
Helicase assays were carried out as described (35). M13-VAR used as substrate was prepared by annealing the reverse primer (Amersham) to M13 single-strand DNA followed by extension for 5 min at room temperature in the presence of sequenase, dNTPs, including a[32P]dATP. 32P-labeled fragments of varying length were obtained by addition of dideoxy-GTP and further incubation for 20 min. Purified NS1 was incubated with 20 ng of substrate for 40 min in the presence of 3 mM ATP. The reactions were stopped by addition of SDS and EDTA and the products were analyzed by 7% non-denaturing PAGE in the presence of 0.1% SDS.

In vitro phosphorylation of NS1
Purified dephosphorylated NS1 (500 ng; (36)) was subjected to phosphorylation through activated, atypical PKC present in HA-1 fraction derived from HeLa cells (37) or 50 ng of recombinant PKCl (20) in the presence of 30 mCi g[32P]ATP for 40 min at 37OC as described (36). The reaction was stopped by addition of SDS and EDTA and heating at 70OC for 30 min. 32P-labeled NS1 was purified on SDS-PAGE and blotted on PVDF membranes for further analyses by trypsin or chymotrypsin digestion and two-dimensional phosphopeptide analyses.

Results

Site-directed mutagenesis in conserved PKC phosphorylation of NS1

Investigations of NS1 replicative functions in vitro have provided evidence for regulation of this major viral polypeptide by phosphorylation through members of the protein kinase C family (20, 36, 37). Furthermore, analyzing metabolically 32P-labeled NS1 derived from infections of the natural host of MVMp, A9 cells have presented major phosphorylation sites to be located within the putative helicase domain of NS1 (Fig. 1), segregating in a 18 kDa NS1 fragment derived from CNBr-cleavage (7). These informations lead us to perform site-directed mutagenesis of selected conserved PKC phosphorylation sites in NS1, which are located within this 18 kDa CNBr-Fragment (Fig. 1), in order to determine their impact on NS1 phosphorylation in vivo, as well as to analyze the biochemical functions of the mutant polypeptides in comparison to wild type NS1. By these means we constructed the three individual NS1 mutants T363A, T403A, and T435A, which contain a replacement of the target threonines within the PKC phosphorylation sites to an alanine.

Mutagenesis of NS1 was shown to be most deleterious for the virus, since already little alteration within the amino acid sequence often leads to a loss of the replication activities of the polypeptide (22, 33, 35, 41). Therefore, only limiting amounts of NS1 would be produced after transfection of "infectious-clone" DNA and refined studies of the recombinant protein are impossible. Previously, NS1 expression by recombinant vaccinia viruses in mammalian cells has been proven most advantages to study the effects of mutagenesis in NS1 (33, 35), and moreover, the native NS1P-protein derived from HeLa cell expression has been shown to be able to support rolling circle replication of plasmids containing the left-end origin in a kinase-free in vitro system (37). This latter finding suggested that NS1 expressed by vaccinia viruses contains the required

phosphorylation to be active for replicative functions, and that the vaccinia expression system is suitable to study the phosphorylation of the polypeptide in mammalian cells. This possibility was further investigated by comparison of the NS1 phosphorylation pattern obtained after genuine MVM infections with wild type NS1 expressed by recombinant vaccinia viruses. NS1MVM was produced by MVMp infection of A9 cells and subjected to metabolic 32P-labeling 24 h post infection. Recombinant NS1VV was produced by recombinant vaccinia virus expression in HeLa-S3 cells and subjected to metabolic 32P-labeling 4 h post infections.

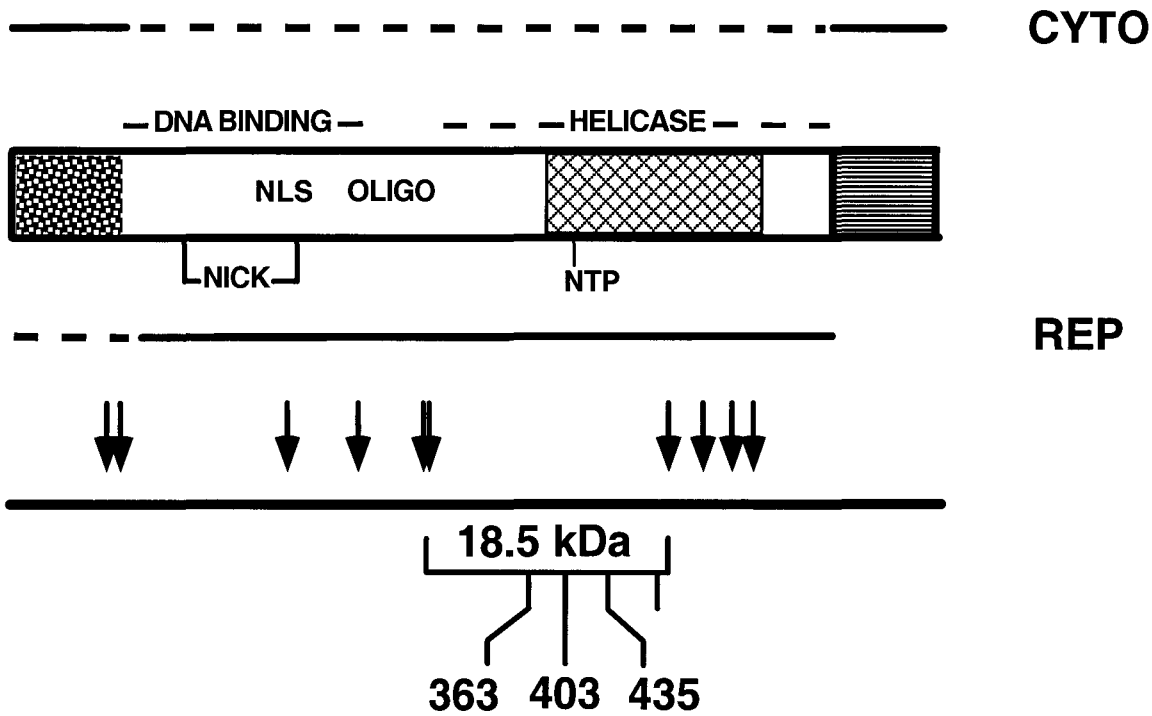

Figure 1: Determination of candidate phosphorylation sites in NS1. Schematical representation of the domain structure of NS1 as determined by site-directed mutagenesis. The bottom of the figure shows the CNBr-cleavage pattern of NS1 with the 18 kDa fragment outlined, which harbors the major residues phosphorylated during MVM infection (7). NS1 was shown to be activated *in vitro* by members of the protein kinase C family, therefore the three conserved PKC phosphorylation sites T363, T403, and T435, consist of candidate regulatory sites in NS1 and were mutagenized individually to inert alanines. The dotted area represents the common N-terminus of NS1 and NS2, the cross-hatched section the homology region to simian virus 40 large T antigen, and the hatched sequence, the C-terminal acidic transactivation domain. Defined motifs and domains in NS1 are indicated.

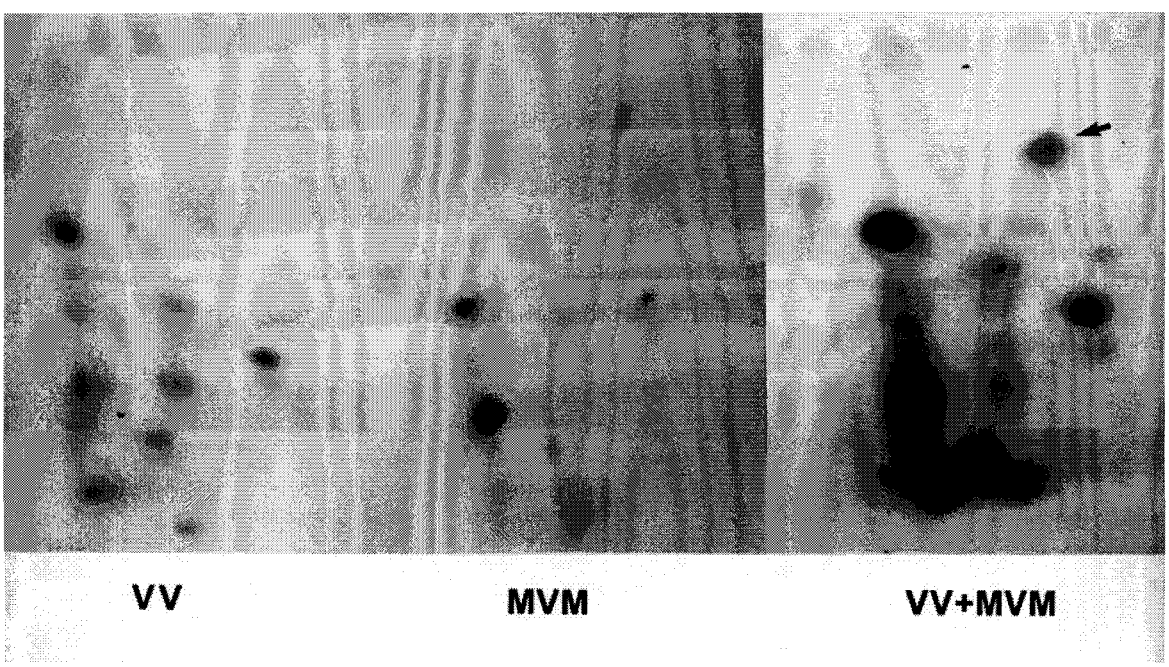

Figure 2: Comparative tryptic phosphopeptide pattern between NS1 derived from MVM infection in A9 cells and NS1 expressed by recombinant vaccinia viruses in HeLa cells. NS1 was metabolically labeled with [32P]-orthophosphate, isolated by immunoprecipitation with αSP8 antiserum (2), further purified on SDS-PAGE, and subjected to trypsin digestion. The resulting 32P-labeled tryptic peptides of NS1 derived from A9 infection and vaccinia virus production were analyzed side-by-side (VV: vaccina virus derived NS1; MVM: NS1 derived from MVM infection in A9 cells) and together (VV + MVM) by two-dimensional electrophoresis at pH 1.9 and chromatography. A characteristic phosphopeptide found in MVM-NS1 but was consistently absent in VV-NS1 is indicated in the "overlap"-experiment by an arrow.

After 4 h labeling with [32P]-orthophosphate cells were harvested and NS1 was purified by immunoprecipitations and subsequent SDS-PAGE. The purified NS1 was then subjected to trypsin digestion and analyzed in two-dimensions by electrophoresis at ph1.9 and chromatography. Fig. 2 presents a comparison between the tryptic phosphopeptide patterns of NS1MVM and NS1VV, together with an equal mixture of the two digested polypeptides. Both pattern NS1MVM and NS1VV were very similar to each other, and as evidenced from the overlap, differed only in a single major phosphopeptide. This analysis clearly demonstrates that vaccinia virus (induced) protein kinases/phosphatases have only little impact on the phosphorylation pattern of NS1, and that this expression system is suitable to analyze the major in vivo phosphorylation sites of the polypeptide.

This similarity of NS1 phosphorylated during expression by recombinant vaccinia viruses and the NS1 derived from MVM infection was encouraging to analyze the PKC phosphorylation site mutants T363A, T403A, and T435A for their phosphorylation pattern in comparison to wild type NS1 during vaccinia virus expression. Wild type and mutant NS1 proteins were expressed by recombinant vaccinia viruses in HeLa cells, metabolically labeled with orthophosphate and purified by immunoprecipitation and consecutive SDS-PAGE. The 32P-labeled NS1 proteins were digested with trypsin and analyzed by two-dimensional electrophoresis/chromatography. As shown in Fig. 3, T363A revealed the same tryptic phosphopeptide pattern as wild type NS1, indicating that the elimination of this PKC phosphorylation site did not affect a major target NS1 phosphorylation site in vivo. In contrast, when T403A and T435A were analyzed, respectively, each mutant protein lacked a distinct phosphopeptide in comparison to the wild type NS1, suggesting that these two PKC sites are target phosphorylation sites in vivo after vaccinia virus expression. Moreover, since the respective phosphopeptides were detected after a genuine MVM infection (cf. Fig. 2), it is most likely that they consist of target phosphorylation sites during MVM virus propagation as well.

Impact of mutagenesis of target phosphorylation sites on NS1 functions.

So far regulation of NS1 replicative functions was investigated with native NS1P, which is phosphorylated at least on nine different phosphorylation sites, together with polypeptides which have been "entirely" dephosphorylated on serine and threonine residues. Reactivation experiments with semi-purified protein fractions or recombinant protein kinases, however, suggested that only selected phosphorylation site(s) are responsible for regulation of NS1 replicative functions (20, 37). In order to examine, whether the phosphorylation sites identified in vivo are indeed of functional relevance or just target phosphorylation sites by chance, we performed functional assays with wild type and mutant NS1 constructs. The fact that members of the protein kinase C

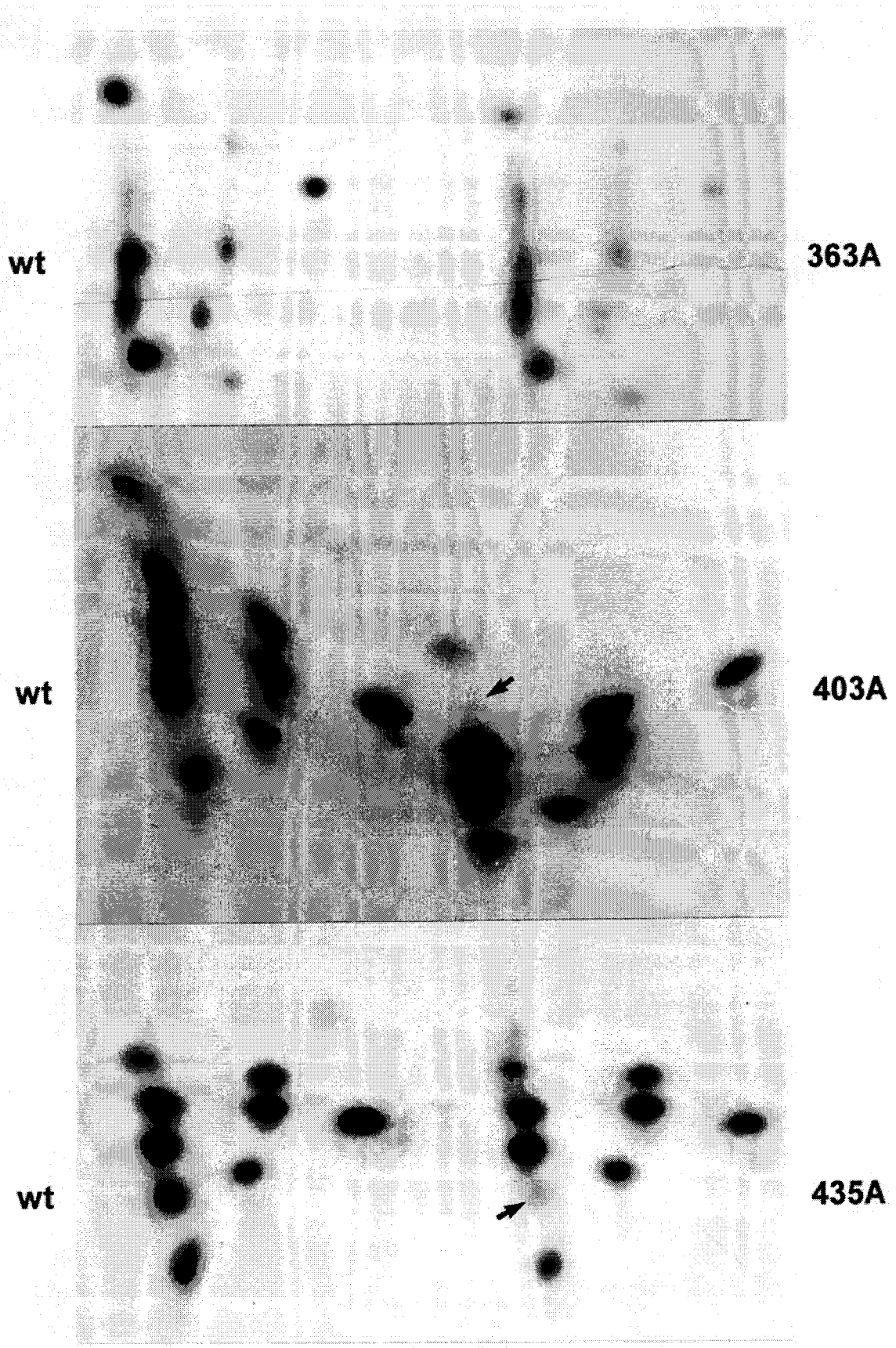

Figure 3: *In vivo* determination of NS1 phosphorylation sites. Since the NS1 phosphorylation pattern derived from vaccinia virus infection is very similar to the pattern of MVM NS1 it was suitable to analyze the presence of distinct phosphorylation sites using the vaccinia virus expression system. Wild type and mutant NS1 derivatives, which were mutated for candidate PKC phosphorylation sites (T363A, T403, and T435A, respectively) were metabolically 32P-labeled during vaccinia virus expression, isolated by immunoprecipitation, purified on SDS-PAGE and subjected to trypsin treatments. The tryptic phosphopeptide patterns of wild type and mutant NS1 were compared by two-dimensional electrophoresis/chromatography. Phosphopeptides characteristically absent due to mutagenesis as compared to the wild type NS1 analyzed in parallel, are indicated with an arrow (NS1 T403A andd T435A, respectively).

family were able to reconstitute replication activity of dephosphorylated NS1 makes T363, T403, and T435 as likely targets for regulation of viral DNA replication. This central viral function of NS1 was investigated in vitro, analyzing leftend and right-end bridge resolution (10, 13) in the presence of wild type and mutant NS1 proteins. Wild type and mutant NS1 T363A, T403A, and T435A, respectively, were expressed by recombinant vaccinia viruses in HeLa-S3 cells and purified from nuclear extracts by means of a [His]6-TAG on Ni2+-NTA agarose columns. Equal amounts of NS1 were subjected to the in vitro replication reactions using HeLa cell extracts and either pLEB711 or pREB1412 as substrates in the presence of [32P]dATP and the reaction products were immunoprecipitated with aNSN antiserum. The ScaI cleaved reaction products were then divided in NS1-attached and NS1-free DNA products by centrifugation and analyzed individually by 0.8% agarose gel electrophoresis. As presented in Fig. 4A, none of the mutant NS1 proteins were able to resolve and replicate the plasmids containing left-end dimer bridge or right-end tetramer bridge, respectively, indicating that the potential PKC phosphorylation sites T363, T403, and T435A, or crucial for the replication activity of NS1. Moreover, when plasmid substrates, containing left- or right-end origins of replication were used as substrates to detect NS1-activity to support rolling circle replication, which is a much more sensitive assay than bridge-resolution (9), the same negative results were obtained with the mutant NS1 proteins (cf. Fig. 4B).

Phosphorylation of NS1 was suggested to serve as mode for regulation of the different functions of the polypeptide during virus propagation. Besides its key-role in viral DNA replication, NS1 has been found to be a strong trans-activator of the viral P38 promoter which drives the capsid genes (40). Therefore, it was of interest to investigate whether the mutagenesis mimicking the absence of phosphorylation at the PKC-sites under investigation had an impact on this alternative NS1 function as well. Thus, we constructed the mutants T363A, T403A, and T435A, respectively, into the eukaryotic expression vector pRSV-NS1 and transfected them together with the reporter plasmid pP38-Luc into A9 cells. 48 h post transfection cells were harvested and measured for their luciferase activity and the expression of the respective NS1 polypeptide was monitored by Western blot in parallel. Fig. 5 illustrates a summary of three separate transfection experiments using various concentration of NS1-expression vector. Co-transfections with pRSV-NS1wt represents 100% activity, the reporter plasmid alone (basal activity) 0% transactivation activity. In contrast to the results observed for replication activity, the PKC-phosphorylation site mutants presented distinct phenotypes. Whereas T363A and T403A were unable to trans-activate the P38-promoter, T435A stimulated this viral promoter significantly above background, reaching almost 50% of wild type activity. This results demonstrates that at least mutant T435A is selectively impaired for replication activity of NS1 while other function(s) such as P38 trans-activation remain intact. Moreover, it also indicates that there is a possibility to selectively activate the viral multifunctional protein for discrete steps during virus propagation by phosphorylation.

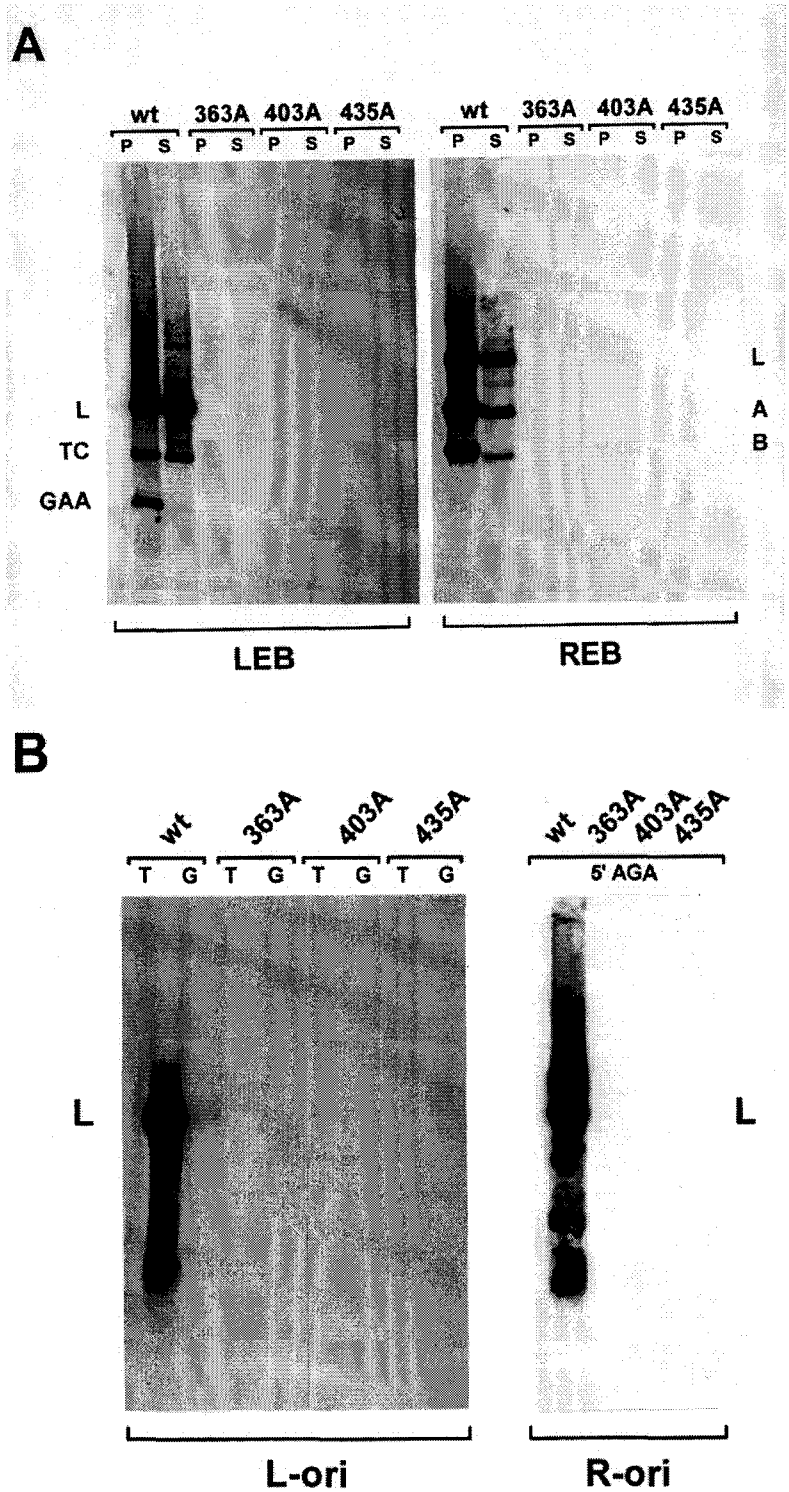

Figure 4: Analyses of wild type and mutant NS1 replicative functions at the left- and right-end origin of replication. (A) Wild type and mutant NS1 proteins were subjected to replication assays in standard HeLa extracts using pLEB711 (LEB) , pREB1412 (REB) as substrates for resolution and replication reactions of concatemeric form viral DNA. The reactions were performed as described previously (10, 13) in the presence of α[32P]dATP and [His]6-TAG purified NS1. The reaction products were immunoprecipitated with αNSN, cleaved with ScaI and the NS1 attached part of the plasmid (P) further separated from the NS1-free part (S) by centrifugation. The deproteinized cleavage products were then analyzed by 0.8% agarose electrophoresis and autoradiography. L: linearized input plasmid; TC: Plasmid-half containing the TC-arm of the dimer-bridge after resolution by NS1 and cleavage with ScaI; GAA: GAA-containing half of the plasmid. A and B: Large and small half of pREB1412 after resolution and cleavage with ScaI. (B) Rolling circle replication of plasmids containing left- (L-ori) and right-end (R-ori) origins. Replication reactions were performed in crude HeLa replication extracts as described in the presence of α[32P]dATP and His-TAG purified NS1 using the circular plasmids pL1-2TC (T), pL1-2GAA (G), and p5'AGA, respectively as substrates. The NS1-attached replication products were immunoprecipitated and cleaved with HindIII. The deproteinized products were analyzed by 0.8% agarose gel electrophoresis. L, migration of the linearized plasmid.

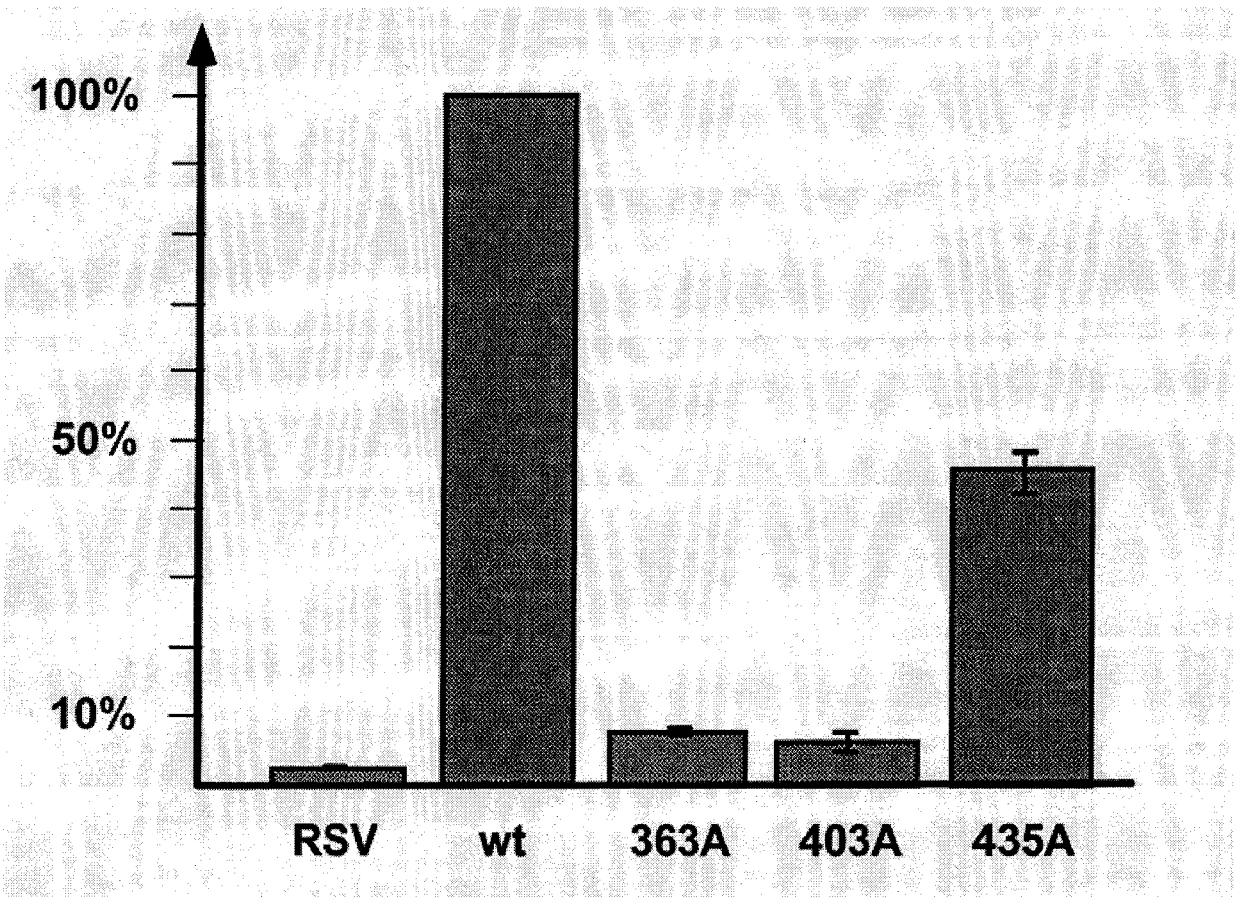

Figure 5: P38 trans-activation of wild type and mutant NS1 polypeptides. To measure the potential of wild type and mutant NS1 polypeptides to trans-activate the promoter driving the capsid genes, pRSV-NSx expression plasmids producing wild type or mutant NS1 derivatives were co-transfected with the reporter plasmid pP38-Luc into A9 cells. 48 h post transfection cells were harvested and analyzed for their luciferase activity. The presented data summarize three independent experiments performed in triplicate with various concentration of the expression plasmids. The reporter plasmid alone represents 0%, pRSV-NSwt was set to 100% activity. The empty expression vector as well as the constructs expressing mutant NS1 were presented as percentage of wild type trans-activation.

Effects of mutagenesis in PKC-phosphorylation sites of NS1 on its biochemical functions.

Both viral functions examined above, P38 trans-activation and even more pronounced DNA replication require several distinct biochemical activities of the NS1 protein. In this respect, site-specific interaction with a cognate DNA-motif [ACCA]2-3 and the formation of higher order oligomers are required for both, whereas NS1 site-specific endonuclease activity and intrinsic helicase function are specifically necessary during reproduction of the viral DNA. Since NS1 functions are easily abolished by mutagenesis irrespective whether a distinct property of the protein is targeted, we first examined common biochemical functions of the polypeptide such as site-specific DNA binding activity. Site-specific interaction with the target DNA-motif [ACCA]2 contained in the left-end origin of replication was investigated using purified wild type and mutant NS1 proteins which were subjected to 3'end-labeled Sau3AI/NarI cleaved pL1-2TC in the presence of g-S-ATP (17). NS1/DNA complexes were then immunoprecipitated with aNSN and analyzed after proteinase K digestion by PAGE in the presence of 0.1% SDS. The linkage tyrosine mutant Y210F served as a negative control. As shown in Fig. 6, all of the PKC-site mutants were able to interact specifically with the DNA-fragment containing the left-end origin of replication. This result clearly demonstrated that the proteins did not become grossly altered due to the amino acid substitution, since they were able to exert a characteristic function described for NS1. Interestingly, mutant T363A presented a significantly higher affinity to the [ACCA]-element than wild type NS1. However, since this PKC-site has not been identified in vivo as a major target phosphorylation site it is questionable whether it reflects regulation by phosphorylation or whether it exerts the "dephosphorylated" phenotype (36) by coincidence.

Replicative functions of dephosphorylated NS1O could be restored in the presence of activated members of the PKC family in vitro. Therefore, it is likely that target protein kinase C phosphorylation sites in NS1 are used to regulate biochemical activities of the polypeptide which are selectively required for viral DNA replication. A crucial step during viral DNA amplification consists in the production a primer to initiate DNA synthesis. This step is achieved by NS1 through its endonuclease activity, introducing site- and strand-specific nicks into the origins in order to produce the necessary free 3'OH. We first investigated the involvement of selective PKC-sites in regulation of initiation by subjecting wild type and mutant NS1 proteins to 3'end-labeled origin-containing DNA fragments in the presence of ATP under low salt concentration (35). Since NS1 becomes covalently attached to the 5'end of the nicked DNA, we isolated the nicked DNA products by immunoprecipitation with aNSN and analyzed the recovered DNA after proteinase K digest by PAGE in the presence of 0.1% SDS. The specificity of the reaction was ascertained by the use of the nicking impaired NS1 mutant Y210F and by substitution of ATP with the unhydrolysable analogue g-S-ATP. As presented in Fig. 7A, mutants 363A and T435A

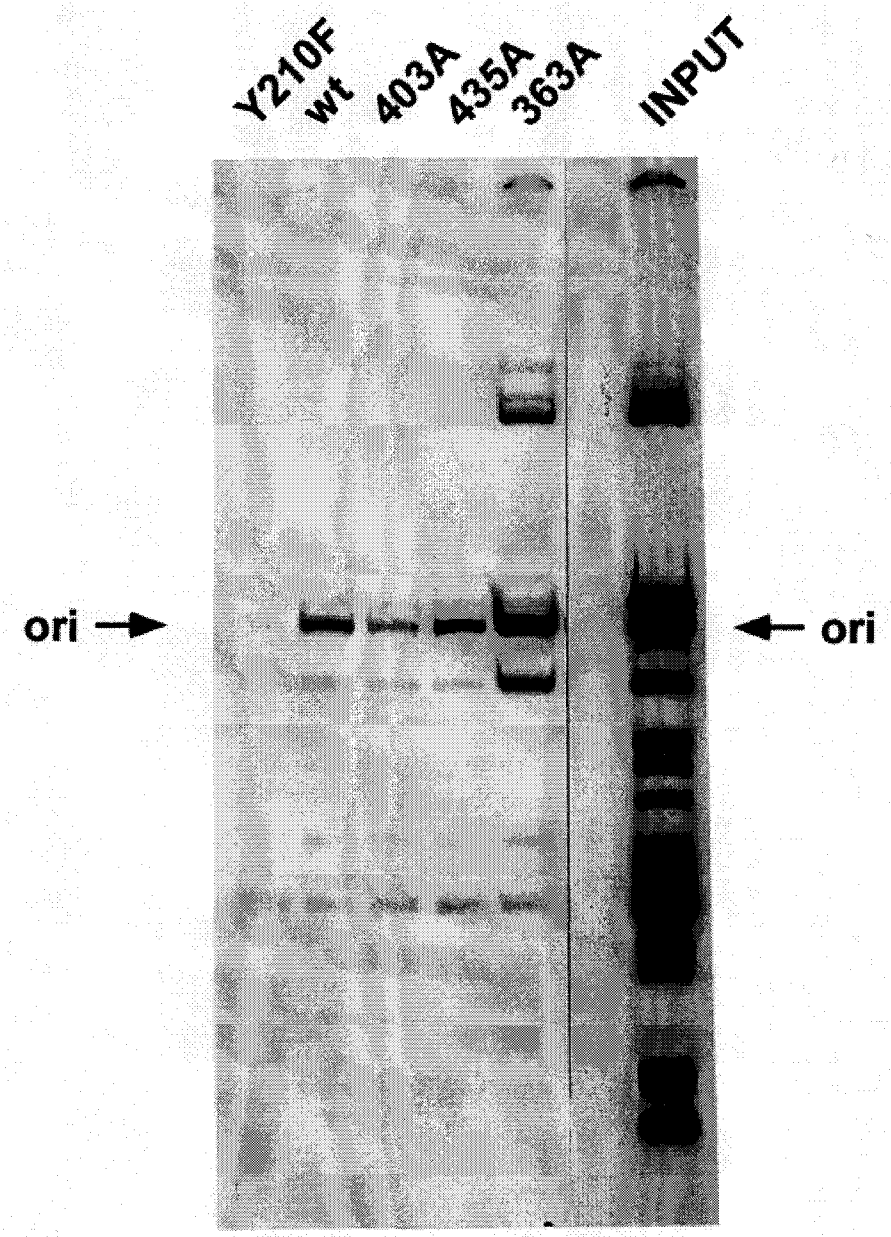

Figure 6: Site-specific interaction of wild type and mutant NS1 to the left-end origin. The ability of wild type and mutant NS1 protein to interact site-specifically with the left-end origin was analyzed as described (17) using 3'end-labeled, Sau3AI/NarI digested pL1-2TC as substrate (INPUT). The labeled substrate was subjected to interaction with NS1 in the presence of non-hydrolysable γ-S-ATP and competitor oligo[dI,dC]. Fragments which specifically bind to NS1 were immunoprecipitated with αNSN the complexes deproteinized, analyzed by 7% PAGE in the presence of 0.1% SDS and revealed by autoradiography. The migration of the fragment containing the left-end origin is indicated. Y210F served as a negative control.

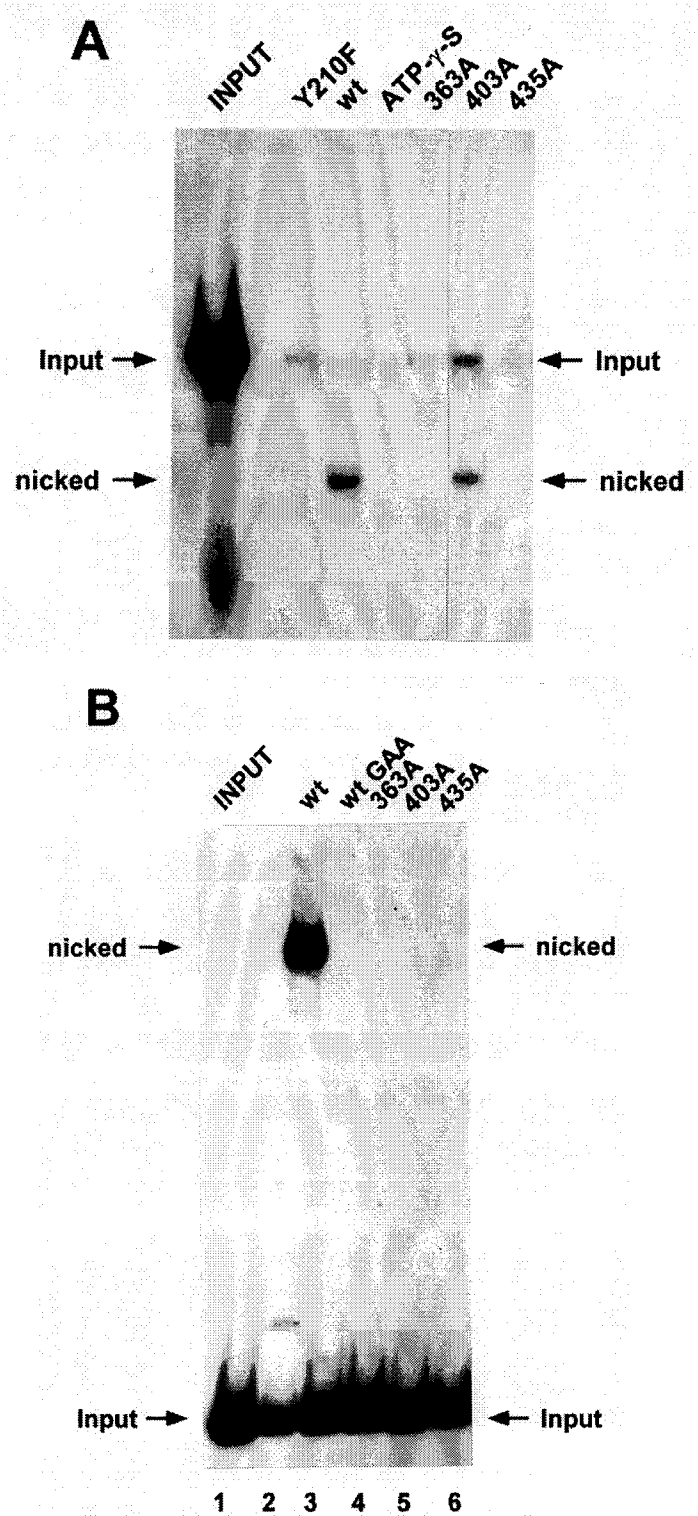

Figure 7: NS1 site- and strand-specific endonuclease activity using the left-end origin as a substrate. The ability of wild type and mutant NS1 to initiate replication at the left-end origin by site- and strand-specific nicking was investigated in absence of any additional protein(35) or in the presence of the parvovirus initiation factor PIF (5). A 3'end-labeled, 95 bp EcoRI fragment derived from pL1-2TC, containing the left-end origin served as a substrate. (A) Wild type and mutant NS1 was subjected to the nicking reaction in the presence of 2 mM ATP (35). The products covalently attached to NS1 were isolated by immunoprecipitations with αNSN, deproteinized, analyzed by 7% PAGE in the presence of 0.1% SDS and revealed by autoradiography. The linkage tyrosine mutant Y210F and a reaction performed in the presence of the non-hydrolysable γ-S-ATP and wild type NS1 served as negative controls. The migration of the input fragment and 53 nt nicked DNA are indicated. (B) Similar nicking reactions were performed in the presence of the parvovirus initiation factor PIF in the presence of 100 mM NaCl (5). The reaction products were heat-denatured, analyzed by 7% PAGE in the presence of 0.1% SDS and revealed by autoradiography. The nicking inactive GAA-origin served as a negative control. Covalently attached NS1-DNA complex migrate as a discrete species in the top of the gel as indicated. The migration of the input DNA is indicated as well.

were unable to nick the origin, whereas T403A was almost as efficient as the wild type protein for this reaction. Since this reaction is very inefficient, and due to low stringency salt concentration occurs independently of site-specific binding to the [ACCA]-sequence (35), we also performed the nicking assay in the presence of the parvovirus initiation factor PIF under physiological conditions (5). The same substrate was subjected to purified NS1 in the presence of ATP, 100 mM NaCl, non-specific carrier DNA, and recombinant purified PIF. The reaction products were then boiled and analyzed by PAGE in the presence of 0.1% SDS, presenting the NS1-attached DNA-fragment as a distinct species with lower mobility than the input fragment. Again, performed now under more genuine conditions, the mutants T363A and T435A were still unable to nick the left-end origin. Surprisingly, in contrast to the assay under low salt stringency, where there is no specificity for the "TC"-origin, T403A was unable to nick under these more physiological conditions in the presence of an accessory protein (Fig. 7B). Since T403A was able to bind site-specifically to the origin (cf. Fig. 5), it is tempting to postulate a novel, as yet undefined NS1-activity involved during site-specific nicking of the origin. Moreover, the fact that T403 has been shown to be phosphorylated in vivo (cf. Fig. 2), it could serve as a regulatory site to control initiation of DNA replication from the left-end origin.

NS1 unwinding function is an additional important feature of the polypeptide to support viral DNA replication. Besides unwinding the origin of replication to allow nicking to occur it has been proposed that NS1 serves as a helicase unwinding the double-strand template in order to allow the replication fork to proceed. This feature can be measured easily with a general helicase assay (35, 43). Using this assay it has been shown that NS1 needs to be phosphorylated for DNA unwinding (36), and moreover, dephosphorylated NS1O was reactivated in the presence of atypical PKC (20, 37). Thus, it was of interest whether this replicative function of NS1 is regulated through one of the target PKC phosphorylation sites under investigation. First, we examined mutants T403A and T435A, respectively, for their ability to unwind various length fragments from a circular M13-template and compared their activity in titration experiments with native wild type NS1 (35). The nucleotide binding-site mutant K405R served as a negative control. As shown in Fig. 8, mutant T403A was significantly active for this NS1 function, exerting only a three fold reduction compared to the wild type polypeptide. In contrast, T435A was unable to unwind even the smallest fragments from the template, demonstrating the requirement of this amino acid residue for this specific NS1 function. Moreover, the inability to unwind double-strand DNA could also explain the impairment of this polypeptide in nicking assays, since it is suggested that nicking and covalent attachment of replicator protein occurs on single-strand level of nicking consensus sequence (23, 42).

The coincidence of a PKC-site, which is a target for phosphorylation in vivo, and the finding that mutagenesis of this particular amino acid leads to a selective loss of DNA unwinding function,

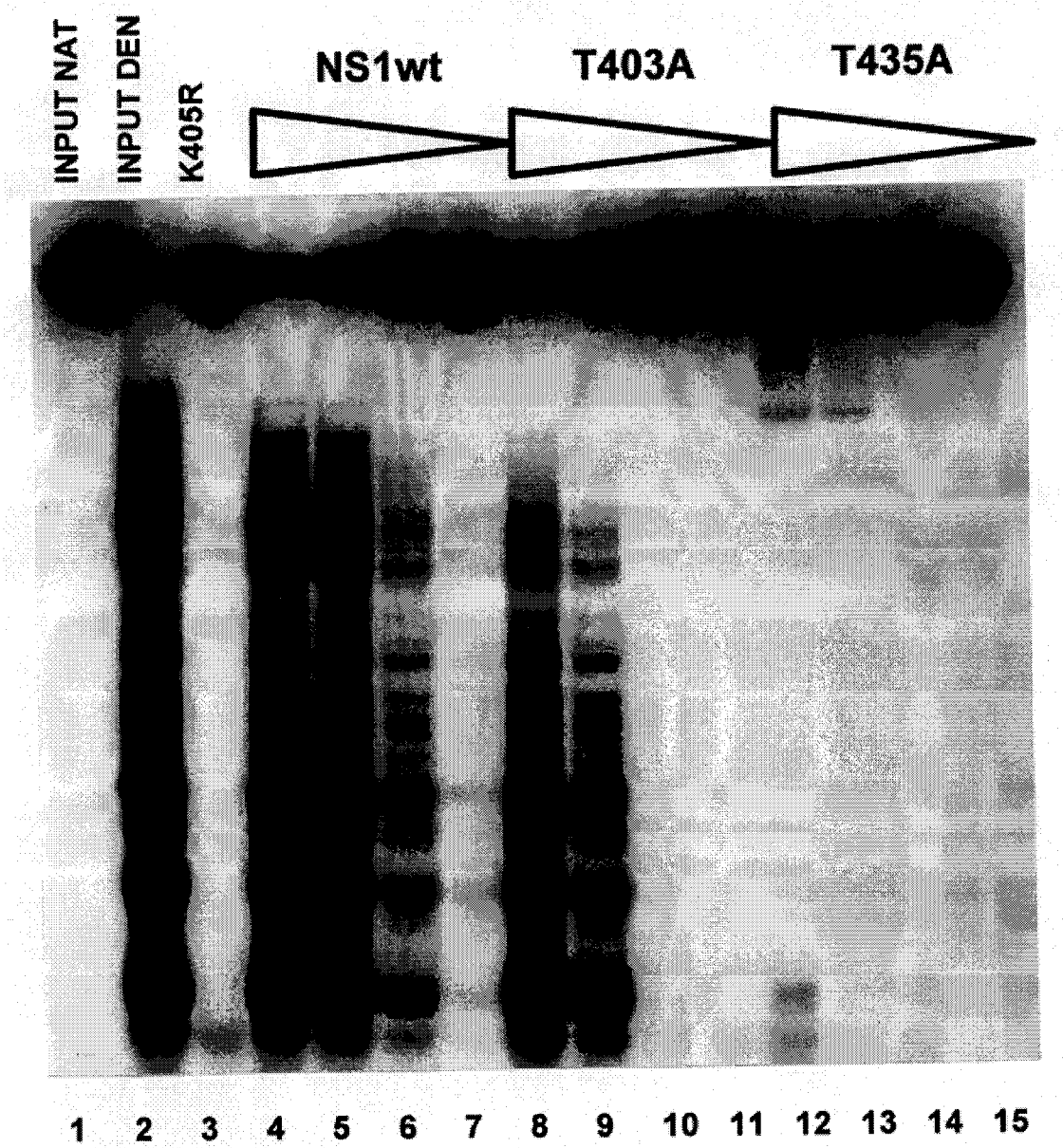

Figure 8: Helicase activity of wild type and mutant NS1. Unwinding activity of serial dilutions of wild type and mutant NS1 proteins were investigated in standard helicase assays using M13-VAR as a substrate in the presence of 2 mM ATP. The reaction products were analyzed by 7% PAGE in the presence of 0.1% SDS. Lanes 1 and 2, native and heat-denatured input substrate; lane 3, NS1:K405R serving as a negative control; lanes 4-7, 100 ng, 30 ng, 10 ng, and 3 ng of wild type NS1; lanes 8-11, serial dilution of T403A; lanes 12-15, serial dilution of T435A.

makes T435 a likely residue for regulation of NS1 replicative functions. Previously, we have shown that NS1 helicase function is regulated in vitro by atypical PKC either present in semi-purified fractions derived from HeLa cells (37), or supplied as a recombinant PKCl (20). In order to examine, whether reactivation in vitro by PKCl and the phosphorylation invivo on residue T435 are complementary, we performed alignments of phosphorylation pattern obtained in vitro and the phosphorylation pattern of native NS1. 32P-labeled NS1 either from metabolic labeling during vaccinia virus expression, or by in vitro kinase assays using activated HA-1 as a protein kinase. Proteins were then purified, subjected to trypsin digestion, and analyzed by two-dimensional electrophoresis and chromatography. As shown in Fig. 9A; the phosphorylation sites targeted in vitro by atypical PKC overlapped exactly with the phosphopeptide determined to contain phosphorylated T435. Moreover, we further tested whether T435 serves as target phosphorylation site in vitro for atypical PKC, by comparison of the phosphorylation patterns obtained with wild type and mutant NS1 proteins. Purified dephosphorylated wild type NS1, as well as the mutants T363A and T435A, were 32P-labeled in vitro by activated HA-1, purified on SDS-PAGE and subjected to chymotrypsin digestion. The resulting phosphopeptide patterns were then compared by two-dimensional electrophoresis and chromatography. As shown in Fig. 9B, in vitro phosphorylation of NS1 by atypical PKC lead to three major phosphopeptides which were targeted in wild type NS1 and T363A. In contrast, one distinct phosphopeptide was missing when T435A was analyzed, demonstrating that this PKC-site serves as a target phosphorylation site of atypical PCK. These data together with the reported findings about reactivation of NS1O helicase activity by atypical PKC implicate regulation of NS1 unwinding function through phosphorylation of T435.

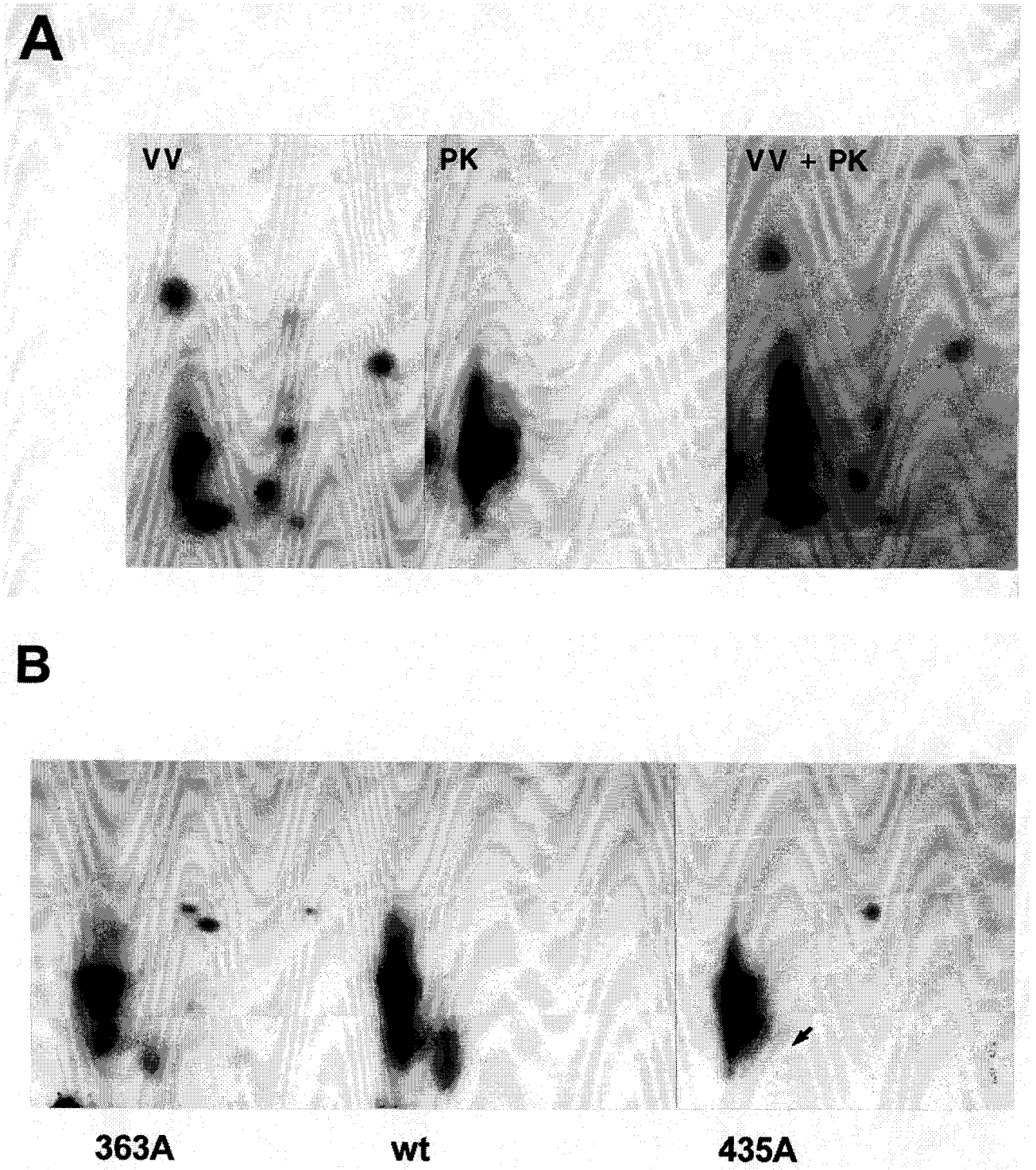

Figure 9: Characterization of atypical PKC phosphorylation sites in NS1. (A) Alignment of 32P-labeled NS1 derived from metabolic labeling during vaccinia virus expression in HeLa cells (VV) with *in vitro* phosphorylated NS1O by atypical PKC present in HA-1 (PK), a protein kinase fraction which is able to reactivate NS1O helicase activity (Nuesch et al., 1998b). Tryptic phosphopeptides of 32P-labeled VV-NS1 and PK-NS1 were analyzed in parallel or in an "overlap"-experiment (VV+PK) to confirm the comigration of the aligned phosphopeptides by two-dimensional electrophoresis/chromatography. (B) Comparative phosphopeptide analysis of *in vitro* 32P-labeled wild type and mutant NS1 protein. Wild type and mutant NS1O were subjected to phosphorylation in the presence of γ[32P]ATP and activated HA-1, purified by SDS-PAGE and subjected to chymotrypsin digestion. The resulting phosphopeptides were analyzed by two-dimensional electrophoresis/chromatography. The phosphopeptide characteristically missing due to the site-directed mutagenesis of T435 is indicated with an arrow.

Discussion

NS1 has been shown to be activated for its replicative functions in vitro by phosphorylation through members of the protein kinase C family (20, 36, 37). However, no evidence about the regulation of NS1 (positive or negative) for other, non-replicative functions has been obtained so far, and it remained unclear whether the multifunctional polypeptide is selectively regulated for different tasks during the course of a viral infection as postulated. A first indication about such possible modulation was obtained by the differential phosphorylation pattern of NS1 during the course of a natural MVM infection (7). In addition, since, the majority of phosphorylated serine and threonine residues have been localized on a 18 kDa CNBr-fragment (amino acids 315-482), covering a main part of the putative NS1 helicase domain (7) it is intriguing to postulate a selective activation for NS1 replicative functions by phosphorylation at target PKC sites within this region. The analyses performed during this study strongly support such a possibility.

The identification of T403 and T435 as target phosphorylation sites in vivo and the functional analysis of the mutant polypeptides in comparison with wild type NS1 suggest that indeed NS1 might be activated for replication by phosphorylation through at T435, while in absence of this phosphorylation event transcriptional functions might be achieved. This is certainly substantiated by the findings that T435A is active for P38 trans-activation, but severely impaired for replication activities. Moreover, the properties of NS1:T435 additionally support such a regulation. Both NS1 functions, replication as well as P38 trans-activation, require the ability of the polypeptide to interact site-specifically with its cognate DNA recognition element [ACCA]2-3 (4, 17, 26), a property of NS1 that is achieved by the mutant polypeptide as efficiently as the wild type protein. In contrast, all functions of NS1, which require DNA unwinding activity of the polypeptide, a property of NS1 that does not support its role as a transcription factor, were strongly impaired by mutagenesis of this PKC phosphorylation site. However, DNA unwinding functions seem to consist of a major activity of NS1 during viral DNA replication. At first DNA unwinding is required for local unwinding of the origin to allow site- and strand-specific nicking to occur on single-strand level, a mechanism extensively studied for bacteriophage "Rep"-proteins (23), which contain similar sequence motifs within the active site (35). Further support is provided by the finding that the helicase negative mutants T363A and T435A are also impaired for site-specific nicking. In addition, after establishing a replication fork, NS1-helicase activity is also essential to unwind the double-strand template to allow the replication machinery to proceed during viral DNA amplification (22, 33, 35, 37). This NS1 function, which could not be substituted for by endogenous cellular helicases (37) reflects an NS1 activity that is activated by phosphorylation in vitro (20, 36, 37). Further evidence for a positive regulation of NS1 replicative functions derived from alignments of NS1-phosphopeptides from in vivo phosphorylated NS1 and in vitro 32P-

labeled NS1 using atypical PKC which in turn are able to activate the dephosphorylated polypeptide for helicase activity. Indeed, the target in vivo phosphorylation site T435 consists also of a target phosphorylation site of atypical PKCs in vitro. Hence, it is likely that NS1 becomes regulated for replicative and transcriptional functions by selective phosphorylation at T435 through its DNA unwinding activity. It has to be mentioned that mutational analyses of S473, an alternative PKCl phosphorylation site in vitro (20) has presented similar regulation in respect to DNA unwinding function of NS1. Whether phosphorylation at both sites is required to activate NS1 for replication or whether a selective regulation occurs between the to target phosphorylation sites remains to be shown.

Besides T435 which seems to be a regulatory element for NS1 unwinding activity, a second PKC phosphorylation site, T403 which is located within the P-loop of the NTP-binding site in NS1, consists of an additional, alternative candidate to coordinate NS1-functions. Interestingly, mutagenesis of this in vivo phosphorylation site did not impair intrinsic biochemical activities of NS1, such as site-specific DNA binding, endonuclease, and helicase activity, but severely abolished more complex reactions requiring the involvement of cellular components, such as the site-specific nicking of the left-end origin under high stringency in the presence of the cellular accessory protein PIF. An intriguing explanation for this failure of T403A to nick under physiological conditions would be a possible, but so far not proven interaction with its accessory factor. However, this explanation could not explain the impairment of this mutant to support replication at the right-end origin which requires members of the high mobility group (HMG) proteins for efficient initiation of replication instead of PIF (8). An indication for the impact of this particular mutagenesis might consist in the conformation of the polypeptide. Post-translational modifications such serine/threonine phosphorylation are thought to induce conformational changes within a polypeptide rather introducing a negative charge like tyrosine phosphorylations. Such conformational alteration can be detected in SDS-PAGE as multiple species, a phenomenon that has been described for MVM NS1 as well (7). Analyzing purified T403A on SDS-PAGE it was consistently observed that this particular mutant migrates as a distinct double-band, detected by Coomassie-blue staining and western blot analyses (32). Therefore it is possible that the mutation in T403 could affect the conformational flexibility of the polypeptide revealed in the two species with different biochemical properties. Since all investigations of NS1 activities have been performed with this "mixture" of two species it is possible that either "isoform" was able to achieve distinct activities, while more complex functions, like replication or trans-activation activities were impaired by the lack of conformational flexibility within the polypeptide in order to allow the same NS1 to perform a sequel of biochemical reactions.

Despite the lack of evidence for in vivo phosphorylation at T363, the mutant at this particular PKC phosphorylation site presented an interesting feature. Previous investigations have shown that dephosphorylated NS1O has a significantly higher specific affinity to the cognate DNA-recognition element [ACCA]2-3. This property has been attributed to a lower ATP turnover rate, which has been reported for the dephosphorylated NS1 as well (36). Mutagenesis of T363 to an alanine exerted the same phenotype as the overall dephosphorylated polypeptide, despite the fact that there is no direct connection to residues that could interact with the trinucleotide. An alternative explanation could consist in the interplay between different domains of NS1 which have to function in concert to achieve the various functions. This was shown by site-directed mutagenesis demonstrating that an intact NTP-binding site is essential for oligomerization (34), site-specific binding to the origin (17), P38-transactivation (25, 33), and cytotoxicity (24), as well as a variety of described functions requiring energy in form of ATP-hydrolysis (22, 25, 33, 35), while the active sites are located within different parts of the protein (cf. Fig. 1). It is certainly possible that T363 affects the ATP-turnover rate of NS1, however, due to the location of the amino acid residue it is more likely that the mutagenesis between the helicase/ATPase domain and the site determined to serve for NS1:NS1 interactions (39), could cause a (stable) conformational shift within the polypeptide-chain that exposes either the oligomerization sequence and/or the domain which interacts site specifically with the [ACCA]-repeats independently of ATP. Such a constitutive conformational change within the protein would certainly negatively affect other (more complex) functions, what is demonstrated analyzing T363A for replication and P38 trans-activation.

In vitro analyses of NS1 regulation for replication by phosphorylation have shown the involvement of members of the protein kinase C family (20, 37). This in vitro regulation becomes more substantial towards the in vivo situation by the finding that two PKC consensus sequences are target phosphorylation sites in vivo. Since PKC are present throughout the whole infectious cycle (7) it is certainly possible that regulation of NS1 functions as reported from in vitro modulations (20, 37) and the mutational analysis of these target phosphorylation sites, could occur by members of this protein kinase family. This is of particular interest, since these kinases are known to be stimulated in transformed cells (3, 29)and are induced by substances, which cause neoplastic transformation (28, 38) a feature shared with parvoviruses in tissue culture. However, despite the coincidence of the in vivo features with our findings obtained in vitro it remains to be shown that indeed members of the PKC family are regulating NS1 replicative functions during viral infection. In addition, so far we have obtained data from only two phosphorylation sites which are targeted in vivo. This reflects only a little part of the whole complex phosphorylation pattern of NS1. As indicated by the comparison of the phophorylation patterns between NS1 obtained after infection in A9 cells and the vaccinia expressed polypeptide,

which presented a high similarity of phosphorylation, it is certainly possible to use this expression system to identify additional target phosphorylation sites. Moreover, it could also help to examine non-permissive viral/cell system in this respect and eventually to use such system in complementation assay to study the regulation of NS1 functions. Last but not least, it has to be mentioned that not just a small part of possible post-translational regulatory elements in NS1 have been identified so far, but their is also a lack of information of NS1 functions to achieve progeny virus production. It would certainly be beneficial for the study of NS1 regulation by post-translational modifications, if other than replicative functions could be dissected on molecular levels in order to get insight about the differential regulation of NS1 functions during the virus cycle.

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Discussion

NS1, the major non-structural protein of the parvovirus MVM, is a 83 kDa polypeptide that plays key roles in multiple processes during the course of a viral infection, ranging viral DNA amplification, promoter regulation, to cell killing. This great variety of activities exerted by a single polypeptide chain, can be assumed to be regulated so that the different tasks are co-ordinated temporally. Changes in the properties of a polypeptide, can occur through multiple mechanisms, such as interaction with heterologous partner proteins, self-assembly to higher order oligomers, association with co-factors, or most intriguingly, post-translational modifications. All of these mechanisms have been found to apply to MVM NS1 and are implicated in the control of at least some of the multiple tasks of this protein. For a decade it is been known that NS1 becomes phosphorylated in MVM infected A9 cells. However, the multiplicity of the functional domain of NS1(some of which have to work in concert (Fig.7)), together with high number (~ 12) of sites on NS1 serving as targets for phosphorylation *vivo* made it very difficult to assess the functional relevance of this post-translational modification. Recent developments, allowing the expression and purification of recombinant NS1 and the concomitant establishment of *in vitro* assays for the biochemical activities of this polypeptides, open new ways to investigate the role of NS1 phosphorylation. In the framework of my Ph D thesis, I combined *in vivo* (natural MVMp infection of A9 mouse cells) and *in vitro* (biochemical assays with partially purified native and dephosphorylated NS1) approaches that lead me to conclude that the multifunctional viral protein NS1 is indeed regulated by phosphorylation, in particular with respect to its replication function.

A first indication of the role of NS1 phosphorylation, besides the finding that NS1 is indeed a phosphoprotein, was obtained through the biochemical comparison of native (phosphorylated) $NS1^{P}$ derived from mammalian cells with its dephosphorylated counterpart $NS1^{o}$ for various biochemical activities. While the un(der)phosphorylated $NS1^{O}$ was able to bind site-specifically to its cognate DNA-recognition motif with even higher affinity than the native polypeptide, functions requiring energy from ATP-hydrolysis, such as site-specific endonuclease and intrinsic helicase activities, were significantly reduced in $NS1^{O}$. These alteration of $NS1^{O}$ biochemical properties were reflected by the impairment of the un(der)phosphorylated polypeptide to initiate rolling circle replication (RCR) of plasmids carrying the left-end origin of replication. This impairment was overcome, at least in part, upon addition of distinct protein kinases, demonstrated that NS1 is regulation by phosphorylation *in vitro*. In parallel, I attempted to evaluate the impact of NS1

phosphorylation during the course of an MVM infection of A9 cells. As previously mentioned, the phosphorylation pattern of NS1 is rather complex *in vivo*, and so far I have not been able to identify phosphorylation sites in purified MVM NS1 by mass-spectrum analyses. This failure of a method that has been proven very successful for other proteins, might be due to additional post-translational modification in NS1, which is suggested by the heterogeneity of the polypeptide in two-dimensional analyses using isoelectric focusing and electrophoresis. Since this straight forward approach did not succeed, I took advantage of findings obtained *in vitro*, showing that members of the protein kinase C family are involved in the regulation of NS1 replicative functions. Moreover, I could also make use of a vaccinia virus expression system allowing the production of NS1 molecules whose phosphorylation pattern is almost identical to the one observed during the most active phase of a natural infection, and which proved to be competent to support replication in a kinase-free assay. With the help of these tools, I was able to assign major *in vivo* phosphorylation sites to the putative helicase domain of NS1, in keeping with the fact that the helicase function of NS1 is most affected by dephosphorylation of the polypeptide. Three candidate target amino acids, T363, T403, and T435, were mutated individually to inert alanines, and the mutants were compared with wild type NS1 regarding that phosphorylation *in vivo*. This allowed me to identify T403 and T435 as *in vivo* phosphorylated residues. The biochemical analysis of the mutant NS1 proteins confirmed the importance of these residues, presumably through their phosphorylation, for NS1 functioning. Moreover, I performed phosphopeptide alignments of *in vivo* ^{32}P-labelled NS1 with NS1 phosphorylated *in vitro* with atypical PKC and using wild type and mutant NS1. Atypical PKCs were chooses because related studies in our laboratory revealed the ability of these kinases to up-regulate the helicase functions of NS1. These analyses showed that T435 is as a phosphorylation site for this particular class of kinases. This observation together with the helicase deficient phenotype of T435A make this PKC phosphorylation site a prime candidate for the regulation of NS1 replicative functions. Since the T435 mutant was still able to trans-activate the viral P38 promoter driving the capsid genes, the regulating role of this phosphoresidue may be specific for replication. As the other *in vivo* phosphorylation site (T403) is concerned, we still lack significant information. The T403 mutant polypeptide is able to achieve all intrinsic biochemical functions that are performed in absence of accessory proteins (helicase, DNA binding and DNA nicking (under non-physiologic conditions)), but is heavily impaired in functions requiring the concerted action of cellular partner proteins (DNA nicking reaction performed under physiologic condition with PIF). So far, the capacity of this mutant for interacting with partner proteins has not been investigated. Yet, the possibility, deserves to be considered that NS1 regulation by protein-protein interaction is also affected by phosphorylation, in particular at the level of the T403 residue.

There are still a number of unsolved questions concerning the regulation of NS1 by phosphorylation. Enough is to say that the role of 10 additional phosphorylation sites is unclear to date. Nevertheless, I would like to propose a hypothetical model for the regulation of NS1 by differential phosphorylation through the regulatory sites I have identified during my thesis. Fig. 19 illustrates the infectious cycle of MVM and presents the putative "phosphorylation state" of

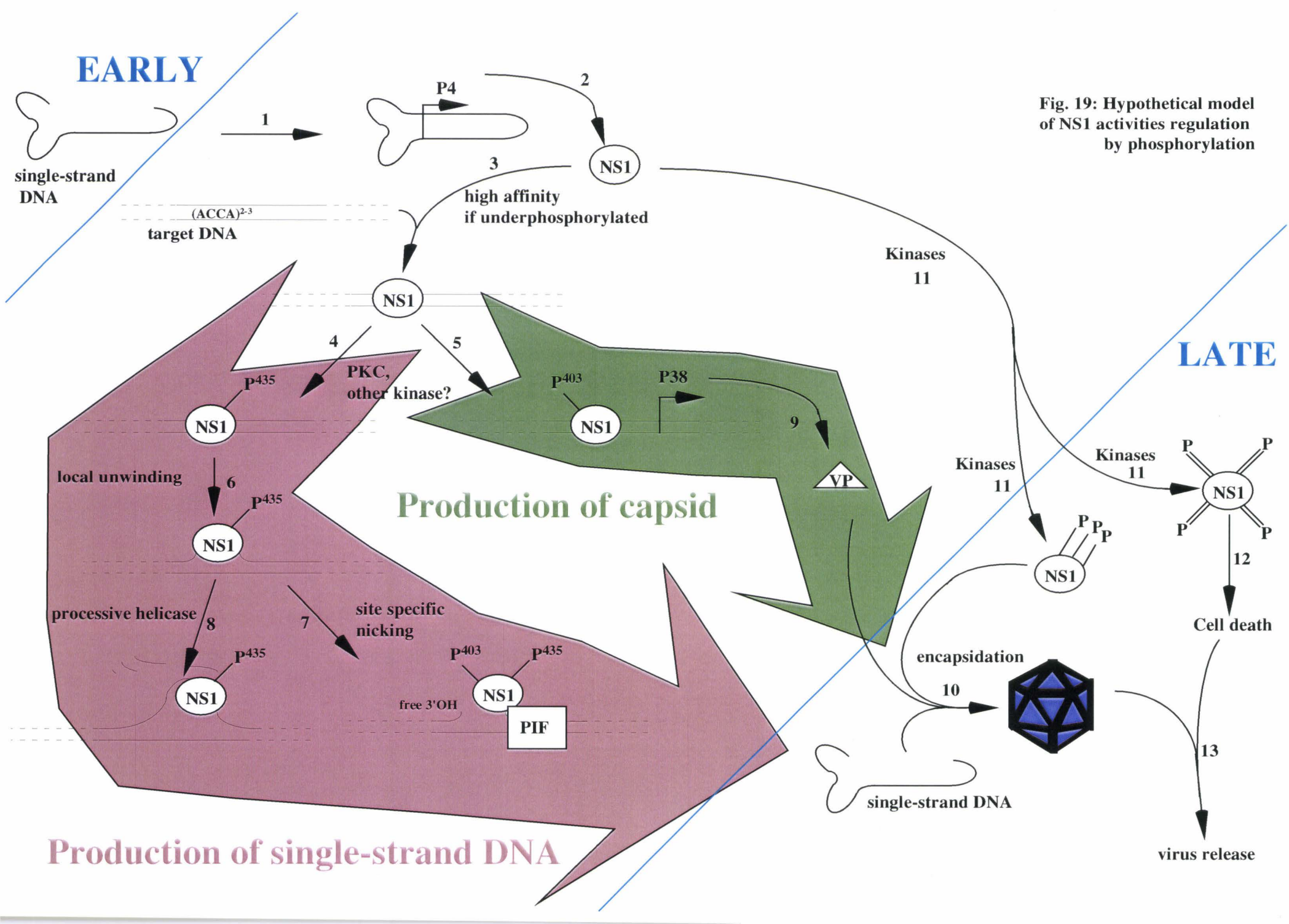

Fig. 19: Hypothetical model of NS1 activities regulation by phosphorylation

NS1 required to achieve these steps as determined by mutagenesis. After virus entry into the cell and transfer of the single-strand genome into the nucleus, the cell is latently infected until it enters S-phase. At the G1/S phase transition, the single-strand genome is converted a the first monomer duplex serving as a template for transcription (step 1), allowing the production of NS1 from P4-driven transcription (step 2). Newly synthesised NS1 is unphosphorylated and able to bind with high affinity to its cognate DNA recognition element which is present at multiple location through the genome (step 3). This high affinity interaction with the viral DNA might result in the local concentration of NS1 in putative replication centres, a feature that has been discussed from immunofluorescence and *in situ* hybridisations observations during MVM infection. In this way, the underphosphorylated DNA-bound protein is ready to be recruited for either transcription or replication, possibly as a function of its subsequent differential phosphorylation (steps 4 and 5). For both tasks, transcription as well as replication, NS1 has to be phosphorylated on T403 (in steps 9 and 7). As stated above, this phosphorylation event may allow the polypeptide to act in concert with accessory proteins (like PIF in step 7). Whether T403 phosphorylation allows NS1 to initiate the assembling of the transcription/replication machinery or whether it is required for NS1 to be included in a pre-assembled complex, is a matter of speculation. A possible scenario, would be that upon phosphorylation at T403, NS1 is targeted on the TAR element and interacts with SP1 bound to the GC-box of the P38 promoter, leading NS1 to serve as transcription factor (steps 5 and 9). Alternatively, when it is unphosphorylated at the T403 residue but phosphorylated at T435 the NS1 polypeptide may be targeted on the viral origins of replication (step 4) and unwind duplex DNA (steps 6 and 8). To allow replication to proceed, a the second phosphorylation event at T403 would then be required to initiate viral DNA replication by site- and strand-specific nicking in concert with the cellular accessory proteins PIF (Parvovirus Initiation Factor) or HMG (High Mobility Group protein), at the left-end and right-end origins respectively (step 7). For further action as a helicase in front of the replication fork, only phosphorylation at T435A appeared to be required (step 8). During the course of a viral infection, NS1 becomes increasingly phosphorylated (steps 11). These additional, so far undefined phosphorylation events might serve for the regulation of single-strand DNA production and encapsidation (step 10), as well as cell death (step 12) allowing the progeny virus particles to be released (step 13).

The up-regulation of NS1 replicative functions by protein kinase C phosphorylation has been well documented *in vitro*, indicating that at least two different components are involved in NS1 activation for rolling circle replication. Further evidence of the involvement of this protein kinase family was obtained *in vivo* by inhibition of these kinases by peptides corresponding to the pseudosubstrate region. These peptides were given to MVM-infected A9 cells and found to inhibit the phosphorylation of NS1 at position T435 (Corbau unpublished observations) arguing for the involvement of this kinase in NS1 phosphorylation under *in vivo* conditions. Phosphorylation is

likely to be one determinant of the host cell specificity of the virus. This prompted me to investigate the phosphorylation pattern of NS1 in transformed permissive cells in comparison with the non-transformed resistant parental line. Preliminary datas show difference in the NS1 phosphorylation pattern between normal and transformed cells raising the possibility that the differential phosphorylation capacity of both type of cells may contribute to their differential permissiveness for virus replication.

So far, our evidence of a role of phosphorylation in the regulation of NS1, concerns the replicative and, to a more limited extend, the transcriptional activity of the viral polypeptide. The knowledge of the toxic action of NS1 on the host cell remains very poor. Since this property of NS1 is of major interest for the development of parvovirus based vectors for gene therapy, it certainly deserves further attention, in particular with respect to the role of phosphorylation in the modulation of NS1 cytotoxicity. An additional important prerequisite for the use of parvoviruses vectors in gene therapy approaches concerns the permissiveness of the target cell. The work presented in my thesis clearly demonstrates that phosphorylation of NS1 is crucial for virus propagation, and hence it is essential to determine whether a target cell is able to modify NS1 properly if replication of the recombinant vectors is desirable. The findings that members of the protein kinase C family are involved, at least *in vitro* could explain in part the preferential oncotropism of parvoviruses, since members of this kinase family have been associated with cell growth, differentiation, and cell transformation. Indeed, PKC activity is stimulated by phorbol esters, which are known tumour promoters and it was shown that some PKC isoforms are induced upon cell transformation. Thus, the dependency of parvovirus replication on (a) selected protein kinase(s) regulated as a function of the physiological state of the target cell, may restrict the host cell range of parvovirus-derived vectors, which may be advantageous for certain application and disadvantageous for others. The work presented in this thesis uncovered an additional component of the complex control exerted by the host cell over a (parvo)viral infection, namely the post-translational modification of the viral NS1 protein, a key regulator of the virus life-cycle. It is obvious that although phosphorylation was clearly shown to affect NS1 functioning, we are only beginning to understand this important aspect of parvovirus regulation. It should be remembered that the majority of phosphorylation sites in NS1 still require to be identified, and little is known about the involvement of NS1 in the molecular mechanisms of cell death and release of progeny viruses. Whether NS1 phosphorylation is involved as well during these late stages of infection remains to be shown.

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La phosphorylation de NS1 est un événement clef durant la propagation du virus minute de la souris (MVMp).

RESUME

Le virus minute de la souris (MVM) est un petit virus possédant un génome d' ADN linéaire simple brin. Une propriété remarquable de ces virus consiste en leur réplication lytique préférentielle dans des cellules transformées. La majorité des fonctions virales de régulation sont assurées par la protéine multifonctionnelle NS1. Elle est impliquée dans la réplication de l'ADN, le contrôle transcriptionnel, ainsi que la cytotoxicité du virus. Afin de rendre compte de la coordination de ces diverses fonctions, il a été suggéré que l'activité de NS1 est modulée par des modifications post-traductionnelles. Durant ma thèse, je me suis attaché à tester cette possibilité en analysant l'influence de la phosphorylation sur diverses propriétés intrinsèques de cette protéine. Nous avons montré que NS1 est phosphorylé de manière différentielle lors de la progression de l'infection virale, et que la forme phosphorylée de NS1 est impliquée dans la réplication de l'ADN viral. Ces donnés furent confirmées par des expériences *in vitro* montrant que l'initiation de l'amplification des formes réplicatives d'ADN parvoviral dépend de la phosphorylation de NS1 par la protéine kinase C. Ainsi, les fonctions de NS1 dépendantes de l'ATP, comme la coupure ou le déroulement de l'ADN, nécessitent la phosphoryation préalable de NS1, alors que sa liaison au niveau d'une séquence spécifique d'ADN est stimulée en absence de phosphorylation. Deux sites majeurs de phosphorylation ont été identifiés et modifié par mutagenèse dirigée. L'un de ces sites (Thr 435) est la cible d'événements de phosphorylation induits par la PKC in vitro. Les deux sites de phosphorylation (Thr 403 et 435) sont essentiels pour les fonctions réplicatives de NS1, alors que seul le résidu Thr 403 semble impliqué dans la capacité de NS1 à trans-activer le promoteur P38. Ces observations étayent l'hypothèse selon laquelle la phosphorylation différentielle de NS1 confère des propriétés distinctes au polypeptide viral.

NS1 phosphorylation is a key-event during the course of Minute Virus of Mice (MVMp) propagation.

Minute virus of mice, a parvovirus, is a small virus with a single-strand linear DNA as a genome. A striking property of these viruses consists in their preference to replicate in transformed cells and thus exert an oncolytic activity. Due to the size of the parvoviruses genome (5.1 kb), the majority of viral functions besides the production of the capsid shell are concentrated in the major non-structural protein NS1, a multifunctional protein involved in DNA replication, promoter regulation and cytotoxicity. In order to coordinate all these functions, it was thought to be regulated by post-translational modifications, such as phosphorylation. During my Ph D Thesis I investigated this possibility analyzing the properties of this protein *in vivo* and *in vitro*. We have shown that NS1 is differentially phosphorylated during the course of a viral infection and that phosphorylated NS1 is involved in viral DNA replication. This is substantiated by *in vitro* findings, demonstrating that initiation of replication is regulated by phosphorylation through protein kinase C. Detailed biochemical analysis of native and dephosphorylated NS1 presented that ATPase dependent functions such as site-specific nickase and helicase activity require phosphorylation, while site-specific binding to the cognate DNA motif is enhanced in absence of phosphorylation. Using site-directed mutagenesis we identified two target phosphorylation sites *in vivo* that are prime candidates for differential regulation NS1. Both phosphorylation sites T403 and T435 are essential for replication, while mutant T435A was still able to trans-activate the late viral promoter, indicating that differentially phosphorylated NS1 is able to exert alternative functions during the course of a viral infection. Moreover, since T435 is regulating NS1 helicase activity *in vitro* through its phosphorylation by PKC, this makes this residue a prime candidate for NS1 regulation.

Mots clés: *Parvovirus, Phosphorylation, NS1, Réplication.*
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