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Regulation of MVM NS1 by Protein Kinase C: Impact of Mutagenesis at Consensus Phosphorylation Sites on Replicative Functions and Cytopathic Effects

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Minute virus of mice NS1, an 83-kDa mainly nuclear phosphoprotein, is the only viral nonstructural protein required in all cell types and it is involved in multiple processes necessary for virus propagation. The diversity of functions assigned to NS1, together with the variation of its complex phosphorylation pattern during infection, suggested that the various activities of NS1 could be regulated by distinct phosphorylation events. So far, it has been demonstrated that NS1 replicative functions, in particular, DNA-unwinding activities, are regulated by protein kinase C (PKC), as exemplified by the modulation of NS1 helicase activity by PKCλ phosphorylation. In order to determine further impact of phosphorylation on NS1 functions, including the induction of cytopathic effects, a mutational approach was pursued in order to produce NS1 variants harboring amino acid substitutions at candidate PKC target residues. Besides the determination of two additional *in vivo* phosphorylation sites in NS1, this mutagenesis allowed the segregation of distinct NS1 functions from one another, generating NS1 variants with a distinct activity profile. Thus, we obtained NS1 mutants that were fully proficient for *trans* activation of the viral P38 promoter, while being impaired in their replicative functions. Moreover, the alterations of specific PKC phosphorylation sites gave rise to NS1 polypeptides that exerted reduced cytotoxicity, leading to sustained gene expression, while keeping functions necessary for progeny virus production, i.e., viral DNA replication and activation of the capsid gene promoter. These data suggested that in the course of a viral infection, NS1 may undergo a shift from productive to cytotoxic functions as a result of a phosphorylation-dependent regulation.

Key Words: parvovirus MVM; nonstructural protein NS1; PKC phosphorylation sites; site-directed mutagenesis; replicative functions; cytopathic effects.

INTRODUCTION

Parvoviruses are small nonenveloped icosahedral particles with a single-strand linear DNA as a genome. Propagation of these viruses is strongly dependent on the proliferation and differentiation states of their host cells, in which a productive infection usually leads to cytolysis. These requirements can be fulfilled upon neoplastic transformation as apparent from the preferential multiplication of parvoviruses in some tumor cells (oncotropism) and their consequent oncolytic effect (for a review see Rommelaere and Cornelis, 1991). The 5.1-kb genome of the type species minute virus of mice (MVM) codes for two structural (VP) and at least four nonstructural (NS) proteins (for review see Cotmore and Tattersall, 1987). From the nonstructural proteins, only the 83-kDa NS1 is required for progeny virus production in all cell types (Naeger *et al.,* 1990). This multifunctional, mainly nuclear phosphoprotein is involved in many steps of the virus life cycle. In addition to having activities directly related to the production of viral components, e.g., initiation of viral DNA replication (Tullis *et al.,* 1988; Cotmore *et al.,* 1992, 1993) and induction of the capsid gene promoter (Rhode and Richard, 1987), NS1 exerts cytotoxic effects on the host cell (Caillet-Fauquet *et al.,* 1990). Furthermore, NS1 is associated with cellular ultrastructural changes, as exemplified by the accumulation of the viral product in recently described nuclear bodies (designated PAR) that are specifically induced by parvovirus infection and are sites of viral DNA replication (Cziepluch *et al.,* 2000).

The diversity of NS1 functions necessary for virus propagation suggested that this viral polypeptide might be regulated by posttranslational modifications, such as phosphorylation. Indeed NS1 was found to become phosphorylated on serine and threonine residues during an MVM infection, undergoing changes in its phosphorylation pattern concomitantly with the progress of a synchronized infection (Corbau *et al.,* 1999). The altered biochemical profile of native $NS1^{P}$ expressed from recombinant vaccinia viruses in HeLa cells, compared to its dephosphorylated ($NS1^{O}$) counterpart (Nüesch *et al.,* 1998a), further argued for the fact that NS1 may be primed for distinct functions by differential phosphoryla-

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tion altering its enzymatic activities. In particular, using a kinase-free *in vitro* replication system, NS1-dependent initiation of rolling-circle replication was shown to be dependent upon protein kinase C (PKC) phosphorylation (Nüesch *et al.,* 1998b). This defect of dephosphorylated NS1 for DNA replication could be assigned, at least in part, to regulation of NS1 DNA-unwinding activities by atypical PKCλ (Dettwiler *et al.,* 1999).

These findings prompted us to determine the impact of phosphorylation on additional NS1 functions necessary for virus propagation, besides DNA replication, and to evaluate more particularly whether cellular alterations caused by NS1 are the result of a phosphorylation-regulated process. To this end, we performed site-directed mutagenesis at candidate phosphorylation sites in NS1, changing target serine or threonine residues to alanine residues. Mutant NS1 polypeptides were then characterized in comparison with the wild-type protein for their nuclear translocation, oligomerization, and functioning in viral DNA replication, *trans* activation of the capsid gene promoter (P38), and induction of cytopathic effects. In addition, we tested whether the putative phosphorylation sites under investigation indeed served as targets for cellular kinases *in vivo* upon NS1 expression from recombinant vaccinia viruses, taking advantage of the fact that the phosphorylation pattern of NS1 produced under these conditions is very similar to that observed during the replicative phase of a genuine MVM infection (Corbau *et al.,* 1999). The present investigation allowed us to identify two *in vivo* phosphorylation sites, namely, T403 and T435, that are likely to consist of NS1 regulatory elements during the replicative phase of a virus infection, besides the previously described PKCλ site S473 (Dettwiler *et al.,* 1999). Furthermore, the mutagenesis at conserved PKC phosphorylation sites allowed the segregation of distinct NS1 activities from one another. Thus, we generated mutant forms of NS1, which are deficient for replication, but still able to *trans* activate the P38 promoter. Furthermore, some NS1 mutants mimicking partially dephosphorylated polypeptides were found to be endowed with a reduced cytotoxicity, resulting in their sustained expression over an extended period of time. This argues for the occurrence of additional regulatory elements that are activated late in infection, at times when alterations within the phosphopeptide pattern become apparent (Corbau *et al.,* 1999).

RESULTS

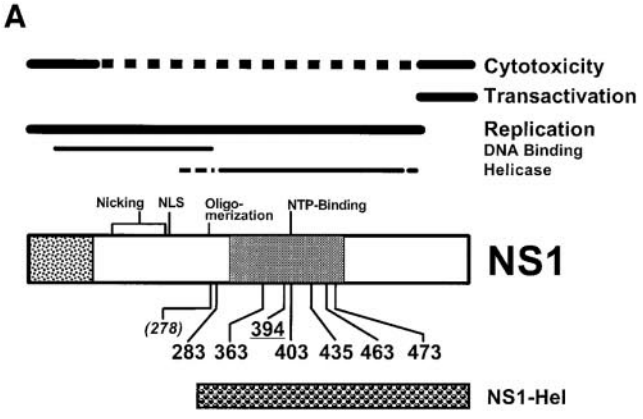
Generation of NS1 mutants harboring amino acid substitutions for putative phosphorylation sites

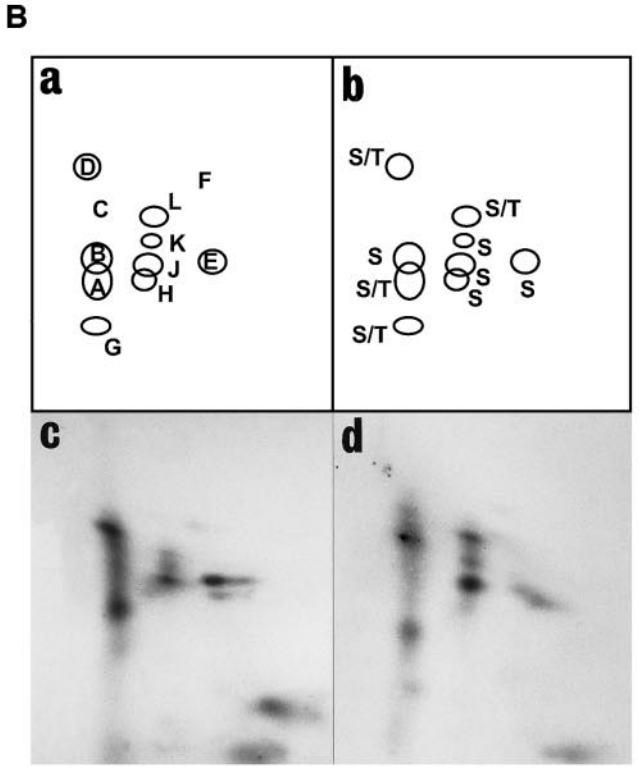
Previous investigations have shown that NS1 replicative functions are regulated by members of the PKC family (Nüesch *et al.,* 1998b; Dettwiler *et al.,* 1999). In order to determine whether an additional NS1 function(s) necessary for virus propagation might be regulated by phosphorylation, we carried out mutational analyses of NS1, targeting a number of candidate phosphorylation sites. Serine/threonine residues were changed to alanine, and the resulting NS1 variants were tested for their *in vivo* phosphorylation as well as functional profile. Previous *in vivo* analyses have shown that the majority of phosphorylation events occurring during the productive phase of an MVM infection are confined to an 18-kDa fragment spanning NS1 residues M314 to M483 (Corbau *et al.,* 1999; see Fig. 1A). This was confirmed by tryptic phosphopeptide analyses of metabolically ^{32}P-labeled NS1 deletion mutants expressed by recombinant vaccinia viruses in HeLa cells. This is illustrated in Fig. 1B, showing that NS1-Hel, a truncated NS1 polypeptide extending from M256 to the very C-terminus (Fig. 1A), contained the majority of NS1 tryptic phosphopeptides (Fig. 1B, panels a, c, and d). It should be stated that phospho-amino acid analyses of individual NS1 peptides have indicated that both serine and (to a lesser extent) threonine residues became phosphorylated during an MVM infection (Corbau *et al.,* 1999; Fig. 1B, panel b).

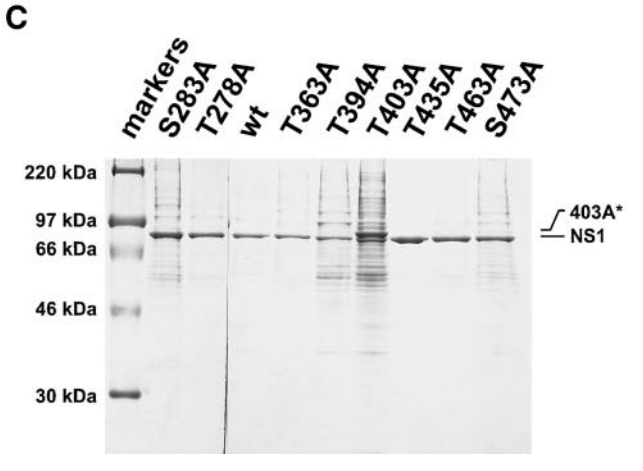
Altogether, these data led us to focus our mutagenesis on NS1 residues S283, T363, T403, T435, and T463, which are all included in a consensus PKC phosphorylation site and were changed individually to an inert alanine in order to mimic partially dephosphorylated NS1. In addition to these candidates, two NS1 mutants were included in the present work: T394A, harboring an amino acid substitution at a highly conserved PKA consensus site, and S473A, previously shown to be a PKCλ phosphorylation site mutant (Dettwiler *et al.,* 1999). For functional analyses, all mutants were expressed from various constructs, including pTHisNS1$_x$, which allows NS1 to be produced by means of recombinant vaccinia viruses and to be further purified by Ni-affinity chromatography due to an N-terminal $(His)_6$ tag (Nüesch *et al.,* 1992, 1995). As shown in Fig. 1C, all the above-mentioned NS1 derivatives could be expressed and purified in extensive amounts, arguing for the stability of the mutant polypeptides. Interestingly, NS1:T403A (carrying an amino acid substitution within the nucleotide-binding domain) consistently resolved into two distinct species after SDS–PAGE. Both species were produced from individual vaccinia virus isolates and became phosphorylated *in vivo* (data not shown), suggesting that the polypeptide chain may adopt alternative conformations during translation.

NS1 functions involved in virus propagation

Nuclear translocation. It was previously shown that wild-type NS1 is found mainly in the nuclear compartment of the cell, with a minor, yet significant, proportion (30%) of the protein being present in the cytoplasm of virus-infected host cells (Cotmore and Tattersall, 1986). This distribution was found to be altered for some NS1







variants, the naturally occurring 65-kDa NS1* and the C-terminal truncation mutant NS1*d*/C67. Thus, both polypeptides were found to be almost entirely (>95%) nuclear (Cotmore and Tattersall, 1986; Nüesch and Tattersall, 1993), while the deletion mutant NS1*d*/158, lacking a nuclear localization signal (NLS), and NS1*sb*DYKD, containing point mutations in the triple-lysine motif of the NLS, remained cytoplasmic (Nüesch and Tattersall, 1993). In order to investigate whether the cellular distribution of NS1 may be regulated by phosphorylation, as shown for SV40 large T antigen (LT) with which NS1 shares a number of similarities (Astell *et al.,* 1987), we analyzed the NS1 phosphorylation site mutants for their ability to translocate to the nuclear compartment of host cells. As previously described (Nüesch and Tattersall, 1993), A9 cells were coinfected with recombinant vaccinia viruses vTF7-3 (providing the bacteriophage T7 polymerase) and vHisNS1$_x$ (carrying the NS1 gene under control of the T7 promoter) to allow expression of NS1 proteins, fixed 5 h postinfection, and processed for the localization of NS1 by indirect immunofluorescence using αNS1$_c$ antiserum (Cotmore and Tattersall, 1988). As shown in Fig. 2, all the phosphorylation site mutants tested gave rise to intense nuclear staining, indicating that they were not impaired in nuclear translocation. In contrast, the NLS$^-$ mutant NS1*d*/158, used as a control, was found in the cytoplasm. Thus, none of the phosphorylation sites under investigation appeared to be involved in the regulation of NS1 nuclear migration.

Oligomerization. For several of its functions, NS1 is thought to act as a multimer rather than a monomeric polypeptide, as exemplified for virus propagation and intrinsic helicase function (Pujol *et al.,* 1997). Such NS1:NS1 interactions, as revealed *in vivo* through the ability of wild-type NS1 to cotransport mutant derivatives lack-

FIG. 1. Site-directed mutagenesis of MVM NS1. (A) Schematic representation of the domain structure of NS1. The NS1 residues that constitute putative targets for PKA (underlined) or PKC (other) and were chosen for mutagenesis are indicated with their amino acid numbers. Residue 278 (in parentheses) was changed in an oligomerization mutant used as a control. The 18-kDa CNBr cleavage fragment (aa 314–483), harboring major phosphorylation sites after MVM infection (Corbau *et al.,* 1999), is depicted as a shaded box; the common N-terminus of NS1 and NS2 is depicted as a dotted box. The truncated NS1-Hel polypeptide (aa 256–672) is aligned at the bottom of the scheme. (B) Analysis of the phosphorylation pattern of NS1. (a, b) Schematic representations of a typical tryptic phosphopeptide map of MVM NS1 (Corbau *et al.,* 1999) during the replicative phase of infection (a) and determination of the corresponding phospho-amino acids (b). (c, d) Tryptic phosphopeptide patterns of metabolically ^{32}P-labeled wild-type NS1 (c) and NS1-Hel (d) expressed from recombinant vaccinia virus. (C) Analysis of wild-type and mutant His-tagged NS1 proteins expressed from vaccinia viruses after one-step purification of Ni^{2+}-NTA agarose, using 10% SDS–PAGE and Coomassie blue staining. The sizes of molecular weight markers are given on the left. The position of His-tagged (wild-type) NS1 protein and the aberrant migration of the major NS1:T403A species are indicated on the right.

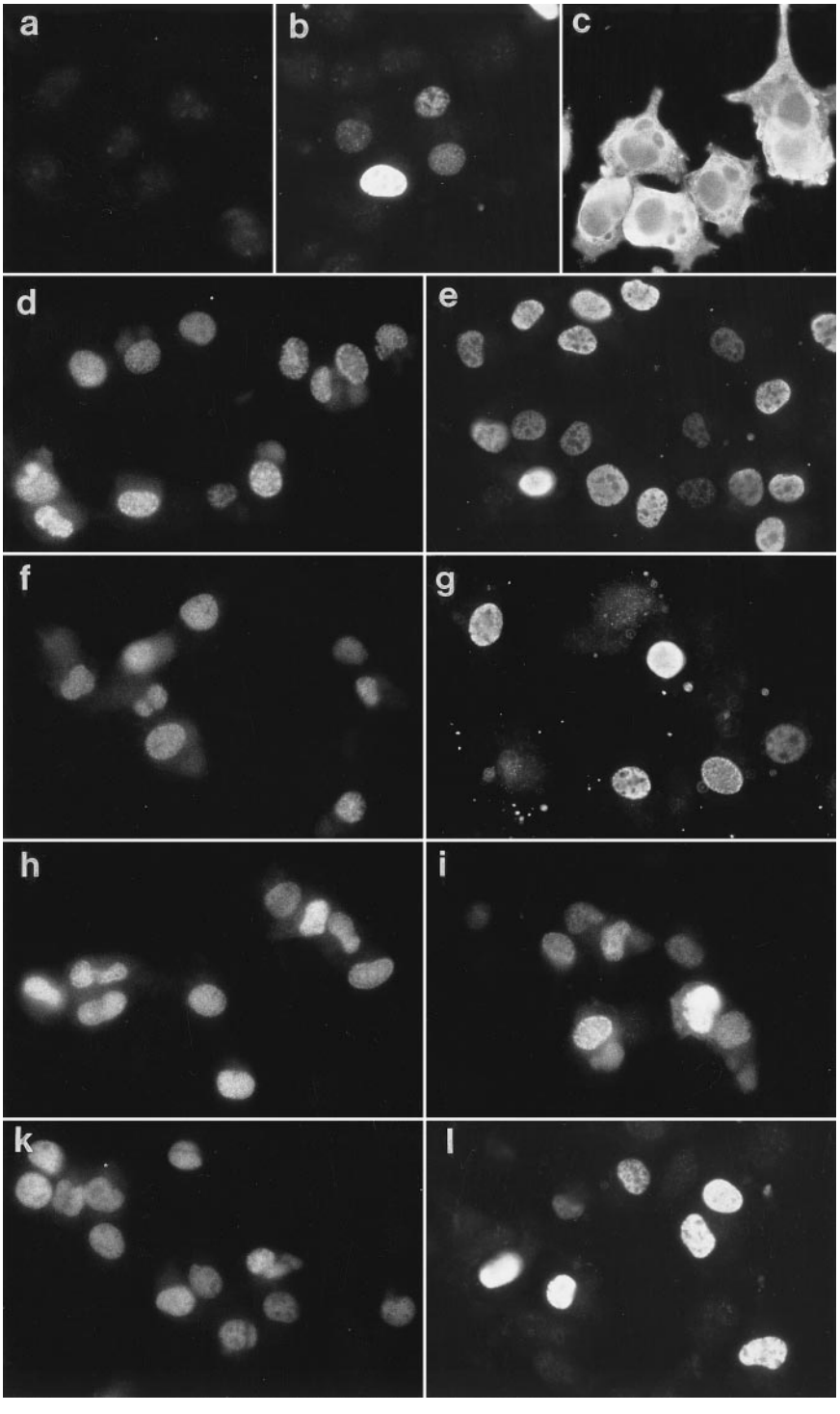


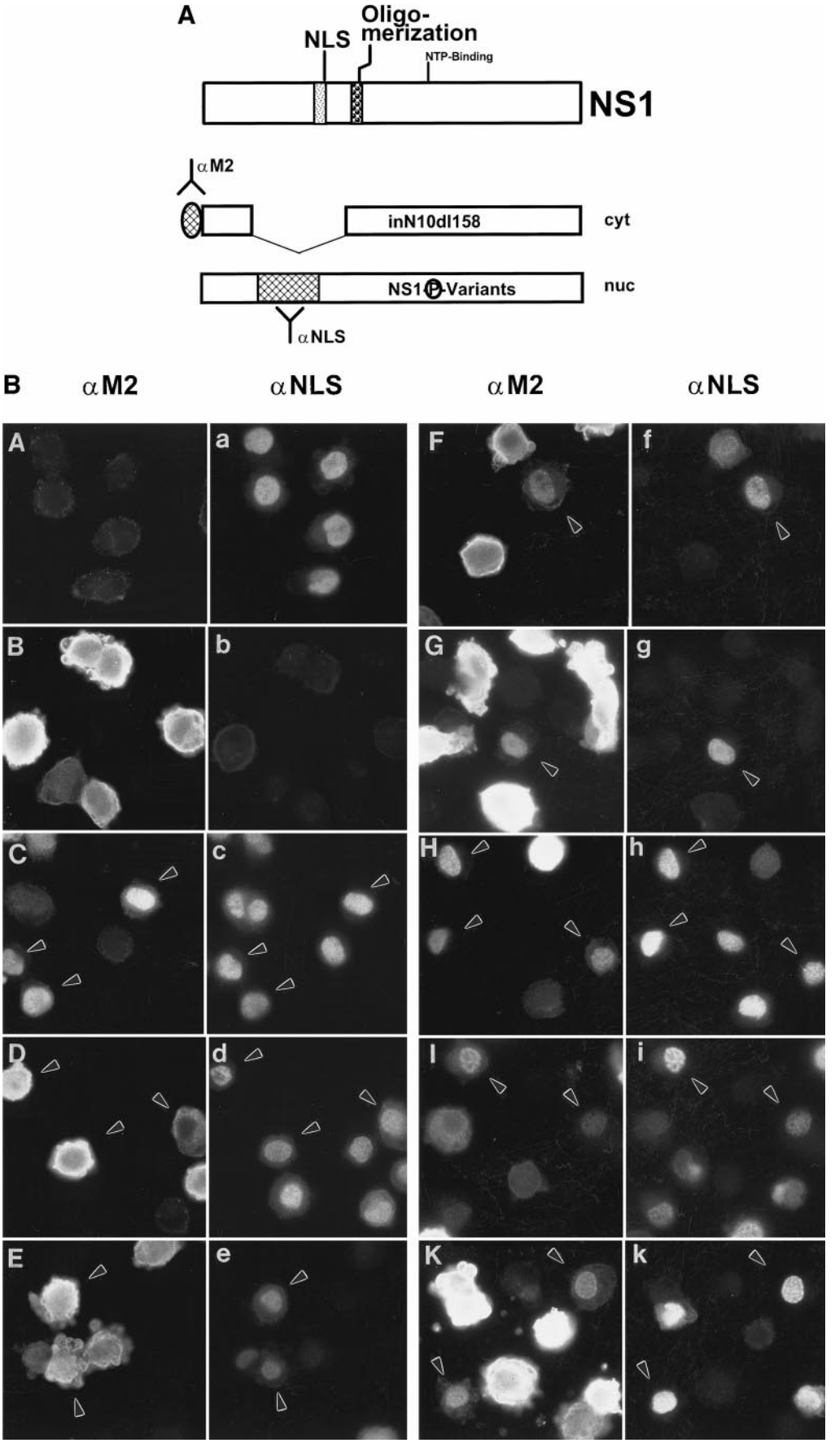
FIG. 2. Intracellular distribution of wild-type and mutant NS1 expressed in A9 cells. Immunofluorescence analyses of NS1 expressed from recombinant vaccinia viruses 5 h postinfection using $\alpha NS1_c$ antiserum for detection. (a) vTF7-3 alone (negative control); (b) vTF7-3 + vHisNS1$_{wt}$; (c) vTF7-3 + vNS1*in*N10*d*l58 (cytoplasmic control); (d) vTF7-3 + vHisNS1:**T278A;** (e) vTF7-3 + vHisNS1:**S283A;** (f) vTF7-3 + vHisNS1:**T363A;** (g) vTF7-3 + vHisNS1:**T394A;** (h) vTF7-3 + vHisNS1:**T403A;** (i) vTF7-3 + vHisNS1:**T435A;** (k) vTF7-3 + vHisNS1:**T463A;** (l) vTF7-3 + vHisNS1:**S473A**.

ing an NLS into the nuclear compartment, proved to depend on an intact ATP-binding site (Nüesch and Tattersall, 1993). This dependency on the interaction with a cofactor indicated that NS1 oligomerization consists of a regulated rather than a constitutive NS1 property. Thus, it was of interest to analyze the NS1 phosphorylation mutants under investigation for their ability to self-associate. Interactions between NS1 molecules were detected by

means of the above-mentioned cotransport assay using the cytoplasmic NS1:*in*N10*d*/158, a FLAG epitope-carrying deletion mutant as a prey. A9 cells were coinfected with the recombinant vaccinia viruses vTF7-3, vNS1:*in*N10*d*/158, and vHisNS1$_x$. As depicted in Fig. 3A, the intracellular distribution of the individual NS1 proteins was determined at 11 h postinfection by double-immunofluorescence using the mouse monoclonal antibody αM2 (recognizing specifically the FLAG epitope fused with the N-terminus of the cytoplasmic variant *in*N10*d*/158), together with the rabbit αNLS antiserum (raised against amino acids 101 to 223 of NS1, a region that is deleted in *d*/158). The reliability of the assay was tested using wild-type NS1 and mutant K405M as positive and negative controls for carrier proteins, respectively (Nüesch and Tattersall, 1993). Coexpressed wild-type NS1 and *in*N10*d*/158 were both found in the nuclear compartment of infected cells, whereas NS1:K405M (by itself mainly nuclear) was unable to direct the cytoplasmic deletion mutant into the nucleus (Fig. 3B, panels A/a to D/d). As an additional negative control, the HisNS1:T278A mutant was constructed to produce an NS1 protein that harbors an amino acid substitution within the NS1:NS1 interaction motif 261-VETTVT(X_9)IQT-278, preventing this motif from associating with NS1 in a peptide ELISA (Pujol *et al.,* 1997). Like K405M, T278A failed to alter the intracellular distribution of *in*N10*d*/158, confirming the role of NS1:NS1 interaction in the nuclear translocation of the latter mutant in the presence of wild-type NS1 (Fig. 3B, panels E/e). As exemplified for the replication-deficient NS1 variants (Fig. 3B, panels F/f to K/k) all phosphorylation mutants proved able not only to migrate to the nucleus on their own (Fig. 2), but also to induce nuclear translocation of the coexpressed *in*N10*d*/158, demonstrating that they retained the capacity for NS1:NS1 interaction. Thus none of the putative regulatory elements under investigation appeared to be involved in the modulation of NS1 self-association.

DNA replication. Using an *in vitro* replication system devoid of endogenous kinases, it has been shown that NS1 replicative functions are dependent on phosphorylation by members of the PKC family (Nüesch *et al.,* 1998b). Therefore, it was of interest to determine the impact of mutagenesis at putative target PKC phosphorylation sites on the capacity of NS1 for driving viral DNA replication. To investigate this NS1 function, we performed *in vitro* assays, analyzing NS1-dependent replication and resolution reactions of MVM origins from concatameric viral DNA. Plasmid clones of head-to-head dimer-bridge (pLEB711) and tail-to-tail tetramer-bridge (pREB1412) fragments were used as substrates in standard HeLa replication extracts, supplemented with purified wild-type or mutant NS1 proteins and [α-^{32}P]dATP as described (Cotmore *et al.,* 1992, 1993). The ^{32}P-labeled, NS1-attached replication products were immunoprecipitated using αNS$_N$ antiserum and digested with the restriction endonuclease *Sca*I. The *Sca*I cleaved reaction products were then fractionated by centrifugation into NS1-attached and NS1-free DNA products, which were then analyzed individually by agarose gel electrophoresis. As illustrated in Fig. 4, mutants T363A, T394A, T403A, T435A, and S473A were unable to resolve and replicate plasmids containing either the left-end or the right-end bridge, indicating that these residues are essential for the replication activity of NS1. Thus, these putative PKC phosphorylation sites could indeed consist of regulatory elements. In contrast, S283A and T463A retained all the NS1 activities that are necessary to carry out replication and resolution reactions initiated at both left- and right-end origins of the viral genome, albeit to a significantly reduced level compared to wild-type NS1, suggesting that phosphorylation of these residues is not absolutely required for the replicative functions of the polypeptide. Thus, phosphorylation of S283 and T463 most likely does not regulate initiation of viral DNA replication.

P38 trans activation. Phosphorylation of NS1 was shown to serve as a means to regulate different biochemical activities of the polypeptide, such as its capacity for site-specific DNA binding (Nüesch *et al.,* 1998a). Besides its key role in viral DNA replication, NS1 was found to be a strong *trans* activator of the viral P38 promoter driving the capsid genes (Rhode and Richard, 1987). Transcriptional and replicative functions have distinct requirements for the biochemical properties of the effector protein, including DNA binding. This prompted us to investigate which NS1 variant might be primed for either transcriptional or replicative functions depending on its state of phosphorylation. To this end, we analyzed the NS1 phosphorylation mutants for their ability to activate the capsid gene promoter in transient transfection assays. All mutants were expressed from pRSV-NS constructs (Spegelaere *et al.,* 1994) in A9 cells cotransfected with the reporter plasmid pP38-Luc (Vanacker *et al.,* 1996). Wild-type NS1 and mutant K405M, which has been reported to be deficient in P38 *trans* activation (Nüesch *et al.,* 1992), were used as positive and negative controls, respectively. Cells were harvested 48 h posttransfection and extracts were measured for luciferase activity. Data from multiple transfection experiments using various concentrations of NS1 expression vector are summarized in Fig. 5 and are expressed as a percentage of the value measured with NS1$_{wt}$, after subtraction of the basal activity of the reporter plasmid alone. While substitutions for S473 (as previously reported by Dettwiler *et al.,* 1999) and T463 had little influence on NS1-induced P38 *trans* activation, T363A and T403A were almost entirely negative for these functions. Intermediate phenotypes were observed with the other mutants that stimulated P38 up to 45% (T435A) or 20% (S283A, T394A) of wild-type activity. The *trans* activation capacity of T435A and S473A contrasts with their incompetence for replication, indicating that both functions of NS1 can be segregated from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each other by distinct mutations that mimic a partially dephosphorylated polypeptide. It must be mentioned, however, that some of the reported effects could result from structural/conformational abnormalities caused by amino acid substitution, rather than from the lack of phosphorylation of the target site.

Phosphorylation of candidate NS1 residues. In order to substantiate the relevance of phosphorylation for the regulation of NS1 functions involved in virus amplification, we determined whether the NS1 residues under investigation were indeed targets for phosphorylation *in vivo.* The wild-type and mutant forms of NS1 proteins produced by vaccinia virus infection were used to perform comparative tryptic phosphopeptide analyses, taking advantage of the fact that the phosphorylation pattern of NS1 expressed by recombinant vaccinia viruses closely mimics the pattern observed during the productive phase of an MVM infection (Corbau *et al.,* 1999). HeLa cells were coinfected with vTF7-3 and vHisNS1$_x$, and metabolically ^{32}P-labeled NS1 was analyzed as previously described (Corbau *et al.,* 1999). Three of the functionally impaired NS1 mutants described above, T403A (Fig. 6, middle), T435A (Fig. 6, bottom), and S473A (Dettwiler *et al.,* 1999), could indeed be distinguished from the wild-type protein by the absence of one major phosphopeptide each, arguing for the role of phosphorylation at the respective positions in the regulation of the viral product. In contrast, all phosphopeptides previously determined for wild-type NS1 were also detected when analyzing the other mutants, as exemplified for T363A (Fig. 6, top), suggesting that their functional alterations resulted from the amino acid substitution per se rather than their incomplete phosphorylation. It should be stated, however, that mutants S283A, T363A, T394A, and/or T463A might be deficient in phosphorylation and consequent regulation during the early or late phases of infection, which have been shown to differ regarding the NS1 phosphorylation pattern from the replicative phase as well as the NS1 expressed from vaccinia viruses (Corbau *et al.,* 1999).

NS1-induced cell alterations

In the course of an MVM infection, the NS1 phosphorylation pattern has been shown to change significantly toward the end of the virus cycle when cytopathic effects become apparent (Corbau *et al.,* 1999). This raises the possibility that phosphorylation may also regulate the capacity of NS1 to induce cellular alterations. To investigate such a possibility, we compared wild-type NS1 with the phosphorylation mutants for their respective cytopathic effects. This was determined by means of transient transfection experiments using an effector/reporter construct designed to keep the genuine viral environment as close as possible. As depicted in Fig. 7A, NS1 was expressed from its genuine parvoviral cassette directed by the early promoter P4. To identify transiently transfected cells, the EGFP (enhanced green fluorescent protein)-encoding gene was coexpressed from the same construct under the control of the early promoter of either cytomegalovirus (in constructs producing NS1$_{wt}$, G404V, and T403A) or MVM P4- (in constructs producing no NS1 or NS1$_{wt}$, T278A, S283A, T363A, T394A, T435A, T463A, and S473A). This type of effector/reporter construct allows NS1-expressing cells to be identified after transient transfection and their analysis for cytopathic effects in the absence of drugs (used to isolate stable transfectants or to induce conditional promoters) and without the need for cell fixation (thereby producing artifacts due to cell dehydration). This assay does not rely on the amplification of the construct or the presence of NS2 and is therefore suitable for comparing NS1 mutants for their intrinsic toxicity irrespective of their competence to produce progeny virus particles. In fact, all constructs under investigation were unable to replicate in A9 cells, most likely due to the absence of full-length NS2 (data not shown). Thus, A9 cells grown on spot slides were transfected with the various NS1/GFP constructs using lipofectamine and examined by fluorescence microscopy in daily intervals up to day 7 posttransfection. These time-course experiments are illustrated in Fig. 7B, showing representative cell populations. In the absence of NS1, no significant morphological changes could be detected in A9 cells expressing EGFP over a 7-day period (Fig. 7B, row 1). In contrast, in the presence of wild-type NS1 (rows 2 and 5), cells started to round up from day 2 posttransfection onward, with the numbers of affected

FIG. 3. Oligomerization of NS1 proteins. The ability of wild-type and mutant NS1 polypeptides to self-associate was measured through their ability to transport the cytoplasmic variant NS1:*in*N10*d*/158 into the nucleus. (A) Schematic representation of the constructs used in the cotransport assay. Full-length NS1 is shown on the top, with the location of the nuclear localization signal (NLS), a peptide involved in NS1:NS1 interaction (Pujol *et al.,* 1997), and the NTP-binding site. The NS1:*in*N10*d*/158 mutant (cyt) is impaired for nuclear translocation by itself unless cotransported through its association with a translocation-competent NS1 polypeptide (nuc) serving as a carrier. The artificial FLAG epitope recognized by αM2 and the NS1 epitope used to produce αNS1$_{NLS}$ are cross-hatched in the respective drawings and were used for the separate detection of the cyt mutant and its potential nuc carrier. (B) Double-immunofluorescence analysis of A9 cells coinfected with vTF7-3, vNS1:*in*N10*d*/158, and vHisNS1$_x$ at 11 h postinfection using αM2 and αNS1$_{NLS}$. Mouse monoclonal M2 antibodies were detected with FITC-conjugated goat anti-mouse IgGs (panels marked with uppercase letters); rabbit NS1$_{NLS}$ polyclonal antibodies were revealed by means of Texas red-conjugated goat anti-rabbit IgGs (panels marked with lowercase letters). Cells coexpressing both NS1:*in*N10*d*/158 and NS1$_x$ are indicated with arrowheads. (A/a) vTF7-3 + vHisNS1$_{wt}$; (B/b) vTF7-3 + NS1:*in*N10*d*/158; (C/c) vTF7-3 + NS1:*in*N10*d*/158 + vHisNS1$_{wt}$ (positive control); (D/d) vTF7-3 + NS1:*in*N10*d*/158 + vHisNS1:**K405M** (negative control); (E/e) vTF7-3 + NS1:*in*N10*d*/158 + vHisNS1:**T278A;** (F/f) vTF7-3 + NS1:*in*N10*d*/158 + vHisNS1:**T363A;** (G/g) vTF7-3 + NS1:*in*N10*d*/158 + vHisNS1:**T394A;** (H/h) vTF7-3 + NS1:*in*N10*d*/158 + vHisNS1:**T403A;** (I/i) vTF7-3 + NS1:*in*N10*d*/158 + vHisNS1:**T435A;** (L/l) vTF7-3 + NS1:*in*N10*d*/158 + vHisNS1:**S473A**.

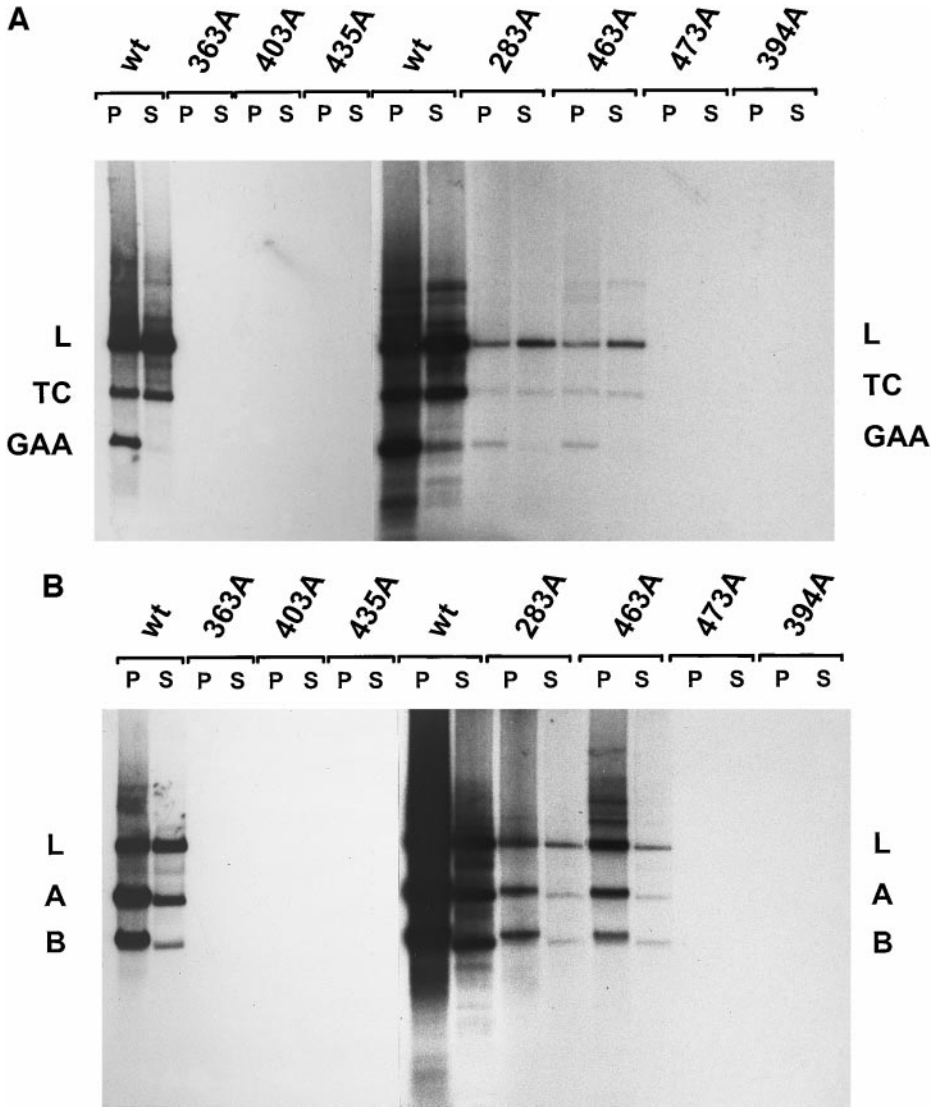

FIG. 4. Initiation of replication and resolution of MVM origins by wild-type and mutant NS1. Purified wild-type and mutant NS1 proteins were tested for their activity in replication and resolution assays, using standard HeLa extracts supplemented with cloned left-end (pLEB711) or right-end (pREB1412) origins in the presence of ^{32}P-labeled nucleotides. Reaction products were immunoprecipitated with αNS_N, cleaved with *Sca*I, and subdivided into NS1-attached (pellet, P) and NS1-free (supernatant, S) fractions by centrifugation. The deproteinized cleavage products were then analyzed by 0.8% agarose gel electrophoresis and autoradiography. (A) Replication reactions using pLEB711 as a substrate. L, linearized input plasmid; TC and GAA, plasmid parts containing the TC and GAA arms of the dimer bridge, respectively, after resolution by NS1 and cleavage with *Sca*I. (B) Replication and resolution reactions using pREB1412 as a substrate. L., linearized input plasmid; A and B, large and small parts of pREB1412 after resolution and cleavage with *Sca*I.

cells increasing with time until most of the cultures showed this phenotype (usually after day 4). It has previously been reported that the nucleotide-binding site mutant NS1-G404V (pULB3241) is nontoxic in a colony formation assay measuring the ability of NS1 to inhibit stable transformation of human cells by cotransfection with a neomycin expression cassette (Legendre and Rommelaere, 1992). In order to determine whether our data correlated with this toxicity assay, the same mutant was included in our analyses and indeed proved incompetent for the induction of cell rounding-up (row 3). An additional negative control was provided by the oligomerization mutant T278A, which was found to be unable to cause morphological alterations of the host cell, arguing for the dependence of these disturbances on NS1 oligomerization. We then analyzed the impact of alanine substitutions for various (putative) phosphorylation sites of NS1 on the occurrence of cytopathic effects, in a first attempt to correlate these changes with a possible phosphorylation-dependent regulation and distinct functions of NS1. As shown in Fig. 7B (rows 6, 8, and 9), some NS1 mutants (S283A, T394A, and T403A) were able to induce A9 cells to round up and, like the wild-type protein, started to detach as early as 2 days posttrans-

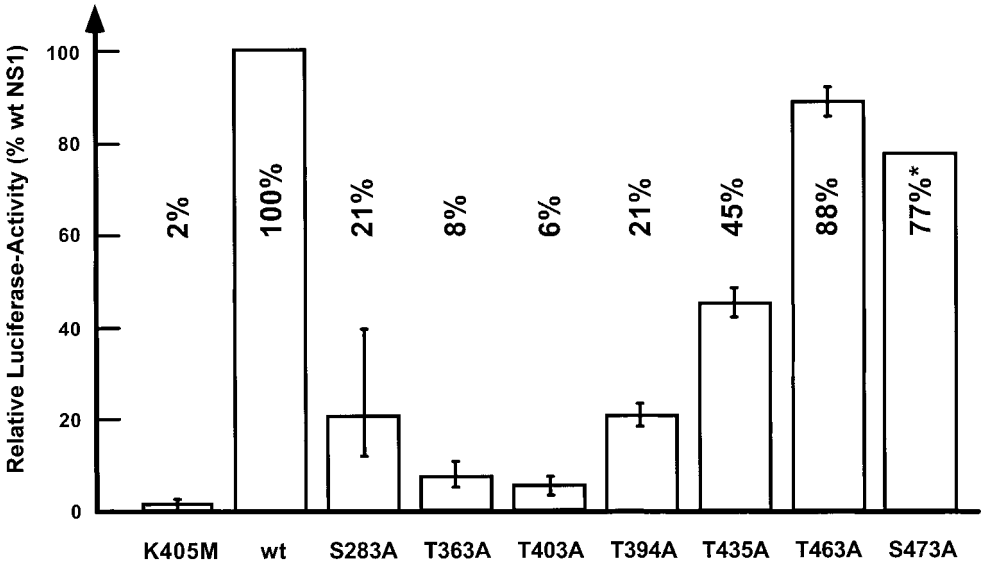

FIG. 5. *Trans* activation of the capsid gene promoter by wild-type and mutant NS1 polypeptides. The ability of wild-type and mutant NS1 polypeptides to *trans* activate the P38 promoter was measured after cotransfection of A9 cells with pP38-Luc and various amounts of pRSV-NS_x as reporter and effector plasmids, respectively. At 48 h posttransfection, cells were harvested and analyzed for luciferase activity. The data presented are average values with standard deviation bars from multiple independent experiments and are expressed as percentages of the activity induced by pRSV-NS_{wt}, after subtraction of the background value measured in the absence of effector plasmid. * The value obtained for S473A was previously determined by Dettwiler *et al.* (1999).

fection. This process was even accelerated in S283A-expressing cells, indicating that NS1 is endowed with a greater cytotoxic potential than that exerted by the wild-type protein in normal circumstances, possibly due to its down-modulation by distinct phosphorylation events. It is also worth noting that the capacity of NS1 for the altering of the cell morphology could be segregated from its replication and *trans* activation functions, as exemplified by T403A, suggesting that the latter activities are not required for at least some facets of NS1 cytotoxicity. In contrast, T435A, T463A, and S473 were unable to induce cell rounding-up efficiently (Fig. 7B, rows 10–12) and did not appear to prevent transfected cells from growing, as suggested by the increase in the fraction of fluorescent cells with time (data not shown). Double-fluorescence staining of transfected cells was performed to test whether expression of the "nontoxic" NS1 phosphorylation variants (T435A, T463A, and S473A, respectively) indeed persisted over extended periods of time. Seven days posttransfection, cultures were fixed, and GFP-positive cells were tested for the expression of NS1 by indirect immunofluorescence using $\alpha NS1_c$, an antiserum raised against the 16 C-terminal amino acids of NS1 (Cotmore and Tattersall, 1988). As illustrated in Fig. 8, the majority of GFP-positive cells transfected with P4-NS1: (T435A, T463A, or S473A)-P4-GFP stained positive for NS1 as well, demonstrating that the maintenance of these cells in culture resulted from the lack of toxicity, rather than the lack of expression or rapid degradation of the mutant forms of NS1. Thus, several NS1 phosphorylation site mutants, one being active for both replicative and transcriptional functions (T463A) together with two mutants competent for *trans* activation (T435A and S473A) that proved to be underphosphorylated *in vivo,* failed to show any cytopathic activity within the time period tested. Besides confirming that NS1 toxicity is not only a by-product of the replicative and *trans* activating properties of the protein, these data confirm the possibility that distinct phosphorylation events prime NS1 to perform functions involved in virus multiplication and cell alterations, respectively. Further investigations, however, will be necessary to determine whether one or several of these residues are indeed phosphorylated *in vivo* late in infection. It is also worth noting that the T363A mutant exhibited a phenotype intermediate between that of wild-type NS1 and that of the nontoxic mutants. Indeed T363A-expressing cells did not round-up but underwent unique morphological changes consisting of their spreading out and forming "axon-like" structures, some of which exceeded twice the length of a normal cell. Such changes are also observed upon MVM infection of A9 cells (data not shown) and may therefore represent a physiological outcome of the viral life cycle.

DISCUSSION

The changes within the phosphorylation pattern of MVM NS1 in the course of a viral infection (Corbau *et al.,* 1999) argue for the regulation of this multifunctional polypeptide by phosphorylation. This possibility is supported by the fact that NS1 needs to be phosphorylated by PKC in order to exert its replicative functions *in vitro* (Nüesch *et al.,* 1998b; Dettwiler *et al.,* 1999). It is still unknown, however, whether other NS1 functions, be-

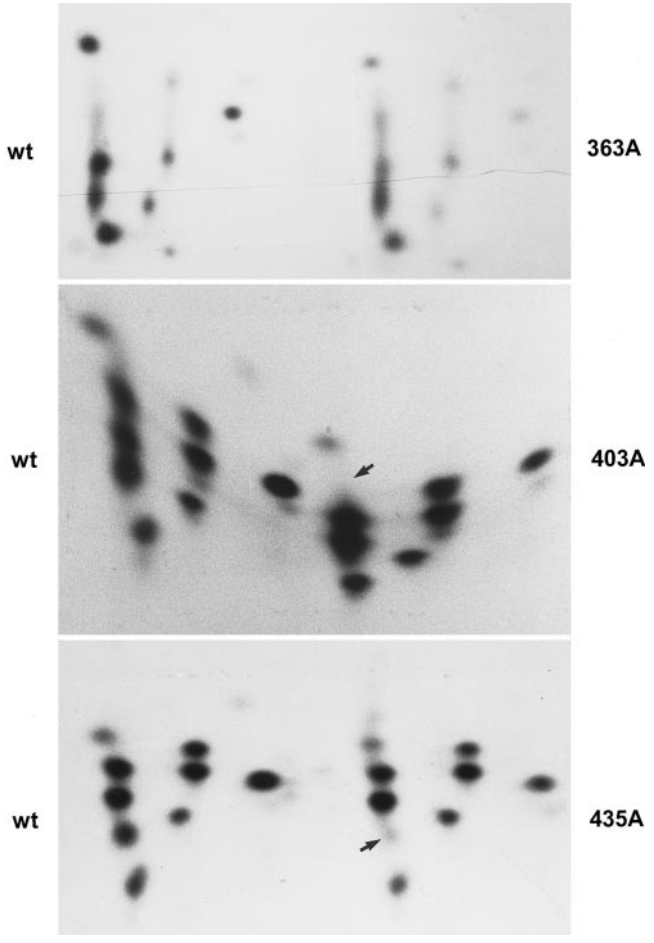

FIG. 6. *In vivo* determination of NS1 phosphorylation sites. NS1 phosphorylation sites were identified by comparing the phosphorylation patterns of wild-type and mutant NS1 proteins expressed from recombinant vaccinia viruses in HeLa cells. NS1 polypeptides were metabolically ^{32}P-labeled, purified by immunoprecipitation with αSP8 followed by SDS–PAGE, and digested with trypsin. The tryptic phosphopeptides were then analyzed by two-dimensional thin-layer electrophoresis/chromatography and revealed by autoradiography. Phosphopeptides that are missing in the mutant (compared to the wild type) NS1 pattern are indicated with an arrow.

sides replication, are regulated in a positive or negative way through posttranslational modifications. If this is indeed the case, these modifications may prime the multifunctional NS1 protein for distinct tasks in the course of a viral infection, in keeping with the altered biochemical profile of dephosphorylated versus native NS1 (Nüesch *et al.,* 1998a). While still lacking direct experimental evidence, this possibility is strongly supported by the present analyses of NS1 phosphorylation site mutants for their functional profile as summarized in Table 1 and further substantiated by analyses of these mutants at the biochemical level (Nüesch *et al.,* manuscript in preparation). Indeed, some of the residues targeted for mutagenesis were found to be phosphorylation sites *in vivo.* These mutants, which mimic partially dephosphorylated NS1, proved to be impaired in their respective replicative functions, while two of these were still competent for *trans* activation. Nevertheless, some limitations in the use of site-directed mutants for mimicking partially (de)phosphorylated NS1 must be recognized, since structural modifications due to the amino acid substitutions may modify the function of the protein irrespective of its phosphorylation. Further investigations, using dominant-negative protein kinase mutants *in vivo,* will be necessary to prove the involvement of these phosphorylation sites in the regulation of NS1 activities.

In addition to mutants affecting the production of viral components, replication and/or *trans* activation-competent mutants were generated, which were deficient in the induction of cytopathic effects, providing a first indication that at least some facets of NS1 toxicity may also be subject to regulation through phosphorylation. Together with our previous observations showing that NS1 undergoes striking changes in its phosphorylation pattern during the successive early, replicative, and late phases of infection (Corbau *et al.,* 1999), the present observations

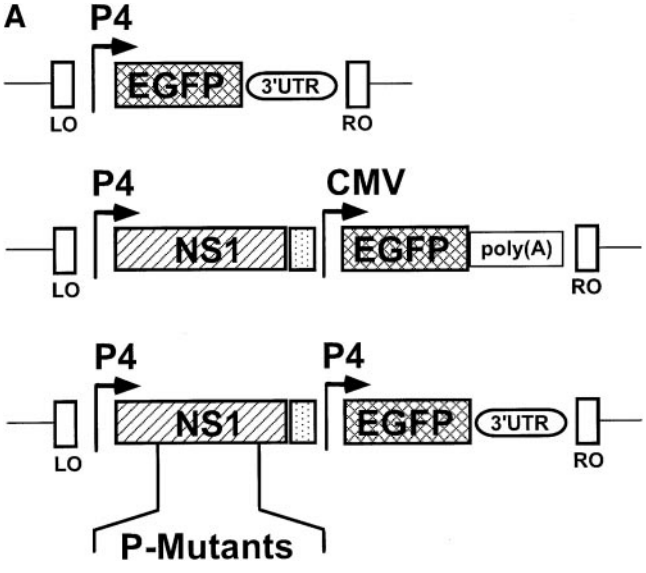

FIG. 7. NS1-induced changes in host cell morphology. NS1 was tested for its cytopathic effect after transfection of A9 cells with pP4-NS1$_x$-CMV-GFP or pP4-NS1$_x$-P4-GFP, in comparison with pP4-GFP used as a negative control. Successfully transfected cells were identified through their positive GFP fluorescence and monitored at daily intervals. (A) Schematic representation of the individual constructs, showing the indicator (EGFP) and effector (NS1) genes with their respective promoters and 3′ untranslated regions. P4, genuine MVM NS-gene promoter; CMV, cytomegalovirus early promoter; 3′UTR, genuine MVM polyadenylation signal; poly(A), SV40 polyadenylation signal. LO and RO, left- and right-end origins of MVM DNA replication, respectively. The NS1 gene, the truncated NS2 coding body, and the GFP gene are represented as hatched, dotted, and cross-hatched boxes, respectively (B). Analysis of representative populations of GFP-expressing cells at indicated times posttransfection. (1) pP4-GFP (negative control); (2) pP4-NS1$_{wt}$-CMV-GFP; (3) pP4-NS1:**G404V**-CMV-GFP; (4) pP4-NS1$_{wt}$-P4-GFP; (5) pP4-NS1:**T278A**-P4-GFP; (6) pP4-NS1:**S283A**-P4-GFP; (7) pP4-NS1:**T363A**-P4-GFP; (8) pP4-NS1:**T394A**-P4-GFP; (9) pP4-NS1:**T403A**-CMV-GFP; (10) pP4-NS1:**T435A**-P4-GFP; (11) pP4-NS1:**T463A**-P4-GFP; (12) pP4-NS1:**S473A**-P4-GFP.

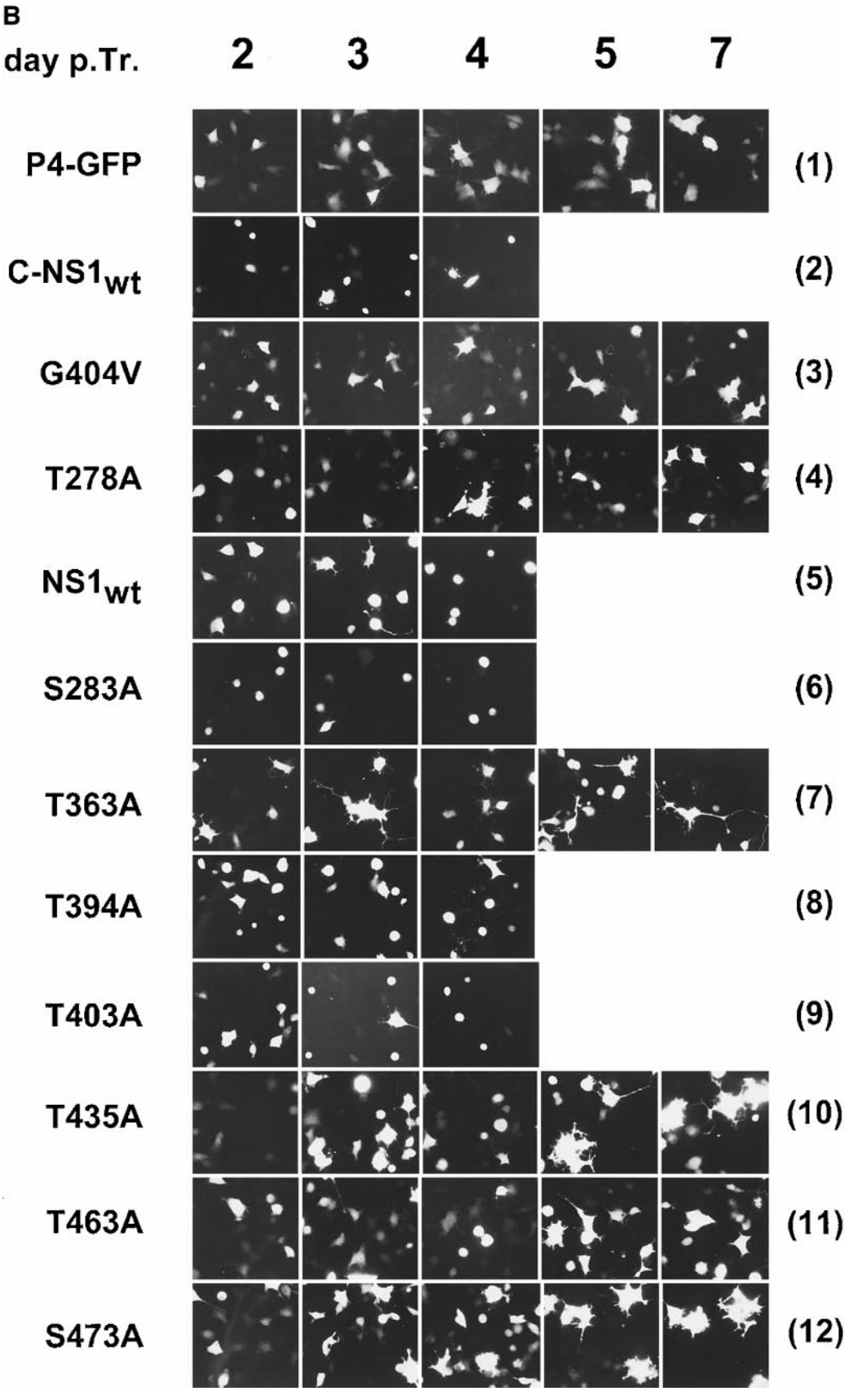

FIG. 7—*Continued*

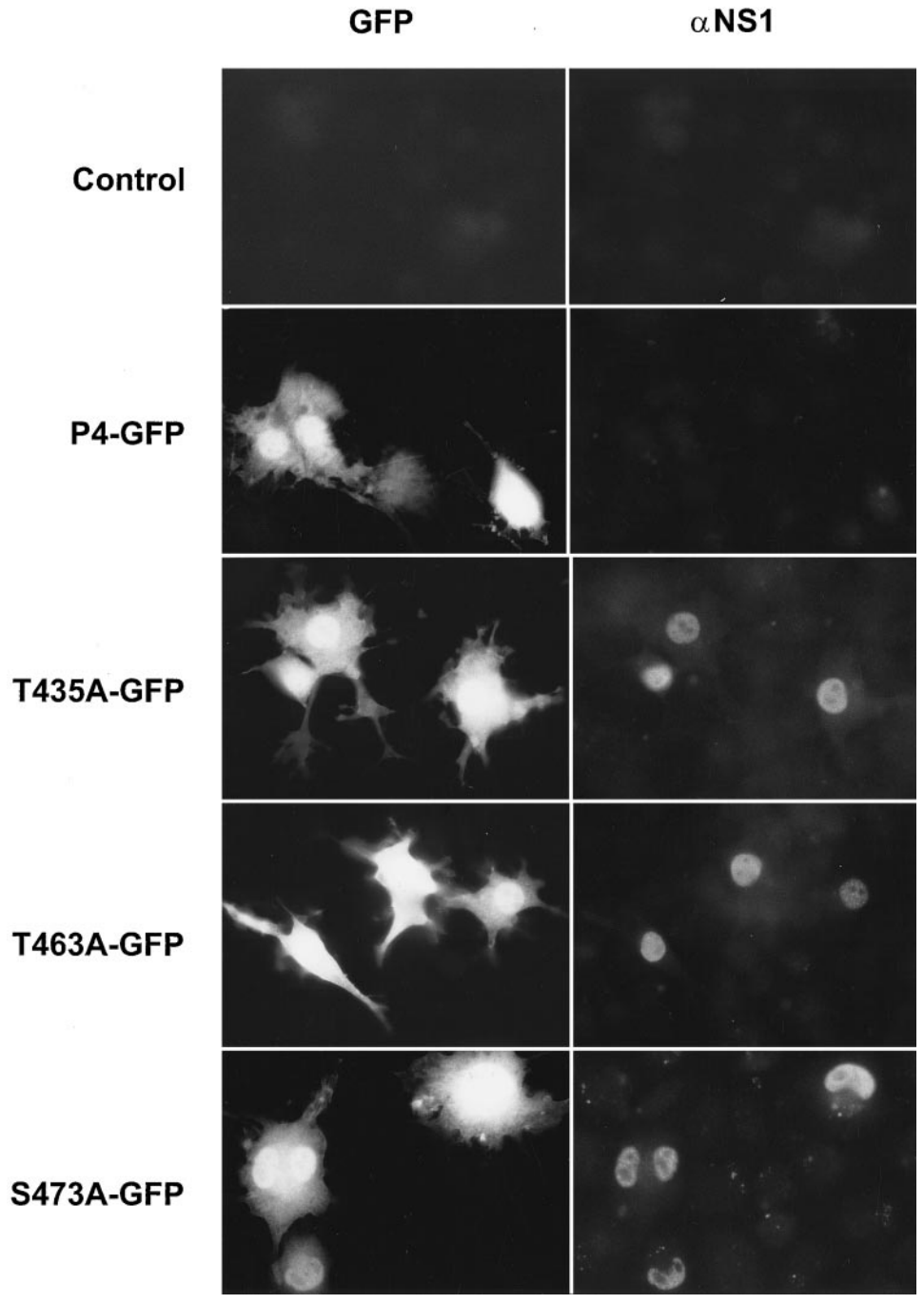

FIG. 8. Sustained expression of less cytopathic mutant forms of NS1. A9 cells were mock-treated and transfected with pP4-GFP or pP4-NS1$_x$-P4-GFP. At 7 days posttransfection cultures were fixed, and GFP-fluorescent cells (identified in the FITC channel) were analyzed for the expression of NS1 by immunofluorescence using αNS1$_c$ antiserum and Texas red-conjugated goat anti-rabbit IgGs.

suggest a role of selective phosphorylation events in the timely coordinated regulation of NS1 activities.

Although in certain cell lines parvovirus H-1 infection was shown to induce apoptosis (Rayet *et al.,* 1998; Oshima *et al.,* 1998; Ran *et al.,* 1999), the general mechanisms of parvovirus cytotoxicity, eventually leading to the lysis of productively infected cells as seen for the prototype strain MVMp in A9 cells, is still elusive. The NS1 protein proved to be a major effector of parvovirus toxicity (Caillet-Fauquet *et al.,* 1990; Li and Rhode, 1990; Mousset *et al.,* 1994), and several NS1 activities are candidates for the induction of cell disturbances (Legendre and Rommelaere, 1992; Op De Beeck *et al.,* 1995; Op De Beeck and Caillet-Fauquet, 1997; Anouja *et al.,* 1997). Some of these activities might be necessary to establish a productive infection and could occur already early in infection, with a possible delayed effect on host cell survival. Thus, NS1 was shown to *trans* regulate not

TABLE 1

Effects of Mutagenesis at Conserved Phosphorylation Sites

	NS1 Property[a]					
Mutation	Phos[b]	Nuc[c]	Oligo[d]	Rep[e]	P38-TA[f]	Tox[g]
Wild type		+	+	+	+	+
S283A	No	+	+	+	±	++
T363A	No	+	+	−	−	(+[h])
T394A	No	+	+	−	±	+
T403A	Yes	+	+	−	−	+
T435A	Yes	+	+	−	+	−
T463A	No	+	+	+	+	−
S473A	Yes	+	+	−	+	−

[a] NS1 functions as determined by various assays are graded "−" for no detectable activity, "+" for activity measured, "++" exceeding wild-type activity.

[b] Original residue identified as an *in vivo* phosphorylation after vaccinia virus expression (mimicking the replicative phase of MVM infection).

[c] Nuclear translocation.

[d] Oligomerization.

[e] *In vitro* replication and resolution.

[f] Transactivation of the capsid gene promoter.

[g] Induction of cell rounding-up and detachment.

[h] Particular cellular alterations observed.

only viral but also cellular promoters (Rhode and Richard, 1987), which may contribute to the dysregulation of cellular gene expression, as exemplified by the NS1-mediated activation of specific genes (Vanacker *et al.,* 1996) and alteration of cellular kinase activity (Anouja *et al.,* 1997). Another by-product of NS1 replicative functions may consist of the induction of nicks into chromosomal DNA (Op De Beeck and Caillet-Fauquet, 1997) and cell cycle arrest in late S-phase (Op De Beeck *et al.,* 1995; Op De Beeck and Caillet-Fauquet, 1997; Corbau *et al.,* 1999). Yet the present data show that some mutant forms of NS1 failed to induce morphological alterations of the host cell, while being fully competent for replicative and *trans* activating functions, indicating that at least some properties of NS1 leading to cell lysis after infection may be uncoupled from the replicative phase of the virus life cycle and represent intrinsically toxic processes regulated by phosphorylation. The latter processes may be induced late in infection in order to promote efficient release of progeny virus particles.

Cytotoxicity of NS1 was first demonstrated through the capacity of the viral product for inhibiting cell colony formation (Caillet-Fauquet *et al.,* 1990; Li and Rhode, 1990), which could be assigned to cytocidal and/or cytostatic effects. The initial occurrence of the latter effect is supported by the observation of cell cycle arrest upon MVM infection (Op De Beeck *et al.,* 1995; Corbau *et al.,* 1999). In addition, by analysis of cell lines stably transfected with a conditional NS expression cassette, cells were found to accumulate in late S-phase upon induction (Op De Beeck and Caillet-Fauquet, 1997). Moreover, the induction of NS1 in such cell lines led to morphological alterations, such as cytoplasmic extensions and rounding-up (Caillet-Fauquet *et al.,* 1990; Mousset *et al.,* 1994), similar to those described in the present work. Interestingly, the thereby reported impairment or incompetence of some NS1 phosphorylation mutants for the induction of these alterations argues for the regulation of the responsible NS1 function(s) through phosphorylation. It is worth noting in this respect that the cytotoxicity of NS1 in the above-mentioned cell lines proved to depend on the physiological state of the target cells, being exacerbated upon cell transformation with various oncogenes (Mousset *et al.,* 1994). The demonstrated involvement of protein kinases in the signaling pathways associated with neoplastic transformation (Mishak *et al.,* 1993) and the hypophosphorylation of several nontoxic NS1 mutants under *in vivo* conditions strongly suggest that NS1 cytotoxicity is, at least in part, a regulated phenomenon responding to the host physiology through NS1 post-translational modifications.

The mechanism by which NS1 induces changes in the cell morphology is currently a matter of speculation. The analyses of nontoxic NS1 mutants for their known biochemical activities have not yet allowed the correlation of these cell disturbances with distinct NS1 activities besides oligomerization (see Table 1). The NS1:T463A mutant proved to be competent for all NS1 activities under investigation (nuclear translocation, oligomerization, viral DNA replication, P38 *trans* activation), yet its failure to induce the characteristic cell rounding-up and detachment indicates the requirement for an additional NS1 function(s). Conversely, the replicative and *trans* activating functions of NS1 do not appear to be essential to the morphological alterations of target cells, which can indeed be achieved by mutants (T394A, T403A) deficient in both functions. Altogether, these data argue for a component of NS1 cytotoxicity being executed by the viral product independent of its other functions. It should be stated that the three nontoxic NS1 mutants identified in the present work all map within a 40-amino-acid region, raising the possibility that the cell disturbances observed may involve the interaction of a distinct NS1 domain with a cellular target protein(s).

Prior attempts to modify NS1 by site-directed mutagenesis led to the generation of polypeptides that were severely impaired at the functional level (Li and Rhode, 1990; Skiadopoulos and Faust, 1993; Nüesch *et al.,* 1995). This is most likely due to the choice of target NS1 motifs that are required for multiple functions, such as the NTP-binding site (Li and Rhode, 1990; Nüesch *et al.,* 1992), or are shared by overlapping domains, such as the active-site tyrosine, NLS, and site-specific DNA-binding region (Nüesch and Tattersall, 1993; Nüesch *et al.,* 1995; Mouw and Pintel, 1998). A more suitable modulation of NS1 could be achieved using the present approach, which was focused on

putative regulatory elements suspected of contributing to the timely coordination of the various NS1 functions. This study led to the identification of mutants exhibiting an interesting segregation of distinct properties, e.g., retaining the competence for replication, while being less cytotoxic. Some of these mutants can be expected to be useful for therapeutic applications of parvoviruses or vector derivatives, for which it is desirable to tip the balance between NS1 replication and cytotoxic activities in favor of one or the other function.

MATERIALS AND METHODS

Viruses and cells

Recombinant vaccinia viruses were constructed using pTM-1 (Moss *et al.,* 1990) derived constructs as described and the recombinant viruses were propagated in monolayer cultures of BSC-40 or HeLa cells and purified over a sucrose cushion, except for the release of the virus from cells by three cycles of freezing and thawing instead of sonification (Nüesch *et al.,* 1992). MVMp was propagated in A9 cells. HeLa, BSC-40, and A9 cells were grown as monolayers in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. HeLa-S3 cells were grown in suspension, using spinner bottles and Joklik's medium supplemented with 5% fetal calf serum.

Plasmids, mutagenesis, and reporter constructs

Wild-type and mutant forms of pTHisNS1. Plasmid pTHis-NS1$_{wt}$ has been described previously (Nüesch *et al.,* 1995) and consists of a modification of pTM1-NS1 (Nüesch *et al.,* 1992) that expresses a $(His)_6$-TAG followed by an enterokinase cleavage site at the N-terminus of the MVM NS1 polypeptide. Mutant forms of NS1 were obtained by replacement of the *Eco*RI (nt 1085) to *Bst*EII (nt 1885) fragment of the NS1 gene by an equivalent PCR fragment containing an appropriate mutation. All PCR-derived products were verified for the correct coding capacity by sequencing. Site-directed substitution mutagenesis was performed by chimeric PCR as described (Nüesch and Tattersall, 1993) using the external primers 5′-CTAAGCGCGGCAGA-3′ (rightward) and 5′-GGTAGATTGGTAACCATTC-3′ (leftward) together with two internal overlapping primers harboring the appropriate mutation. The following primer pairs were used to substitute alanine (mutation in italics) for the target serine/threonine: T363 (5′-GCCTGACACAAGA*G*C*C*TGCAG-3′ and 5′-CTGCAGG*C*TCTTGTGTCAGGC-3′), T394 (5′-GACACAAGA*G*C*C*TCTGT-3′ and 5′-ACAGAGG*C*TCTTGTGTC-3′), T403A (5′-GGACCAGCCAGC*G*CAGGCAAATC-3′ and 5′-GATTTGCCTG*C*GCTGGCTGGTCC-3′), T435A (5′-TAATGACTGT*G*CCAACAAGAAC-3′ and 5′-GTTCTTGTTGG*C*ACAGTCATTA-3′), and T463 (5′-GGTCAA*G*CTATTCGCATTGAT-3′ and 5′-GCGAATAG*C*TTGACCAGAGC-3′). Alanine substitutions for T278 and S283 were achieved by single PCR using the leftward primer 5′-GGTAGATTGGTAACCATTC-3′ and either of the mutation harboring primers 5′-CGGCAGAATTCAA*G*CTAAAAAAGAAGT-3′ (T278A) or (5′-CGGCAGAATTCAAACTAAAAAAGAAGTT*G*CTATTAAAACTACACTTAAAG-3′ (S283A).

Other NS1 clones. The mutations were transferred from pTHisNS1 to other NS1 clones [pRSV-NS (Spegelaere *et al.,* 1994); pP4-NS1-P4-GFP] by exchange of the *Eco*RV (nt 385) to *Bst*EII (nt 1885) fragment of MVM DNA.

Reporter constructs. The MVM origin-containing plasmids pLEB711 and pREB1418 serving as substrates in *in vitro* replication assays have been described (Cotmore *et al.,* 1992, 1993). The pP38-Luc reporter used to measure *trans* activation of the capsid gene promoter has been described by Vanacker *et al.* (1996). vNS1*in*N10*d*l158, a recombinant vaccinia virus encoding a cytoplasmic FLAG-tagged NS1 deletion mutant used for *in vivo* oligomerization assays (Nüesch and Tattersall, 1993), and pP4-NS1-P4-GFP/pP4-NS1-CMV-GFP were used to measure the persistence and cytopathic effects of NS1 expression in transfected cells. To construct the latter clones, pP4-GFP was first produced by inserting the *Nco*I to *Xba*I fragment of pEGFP (Clontech) into *Nco*I- and *Xba*I-cleaved pdbMVP (Kestler *et al.,* 1999). The NS1-containing pP4-NS1-P4-GFP derivative was then obtained by insertion of the *Pme*I to *Xba*I fragment of pP4-GFP into *Stu*I and *Xba*I-cleaved pdbMVP. In some experiments, pP4-NS1-CMV-GFP was used instead of pP4-NS1-P4-GFP, as obtained by inserting the CMV-GFP cassette in the form of a *Bgl*II fragment into *Bgl*II-cleaved pdbMVP. It should be stated that pdbMVP was deleted for its *Stu*I fragment (nt 2375 to 2498) in order to prohibit the production of NS2.

In vivo ^{32}P-labeling and tryptic peptide and phosphoamino acid analyses

Metabolic ^{32}P-labeling of vaccinia virus-expressed NS1 was performed as described (Nüesch *et al.,* 1998a; Corbau *et al.,* 1999) by coinfecting adherent HeLa cell cultures with 15 PFU/cell of each vTF7-3 (a vaccinia virus expressing the phage T7 RNA polymerase) and the appropriate recombinant vaccinia virus containing the NS1 gene under control of the bacteriophage T7 promoter. Labeling was performed by incubation in the presence of [^{32}P]orthophosphate (ICN; 10^{-10} Ci/cell) for 4 h, starting from 5 h postinfection. Cells were harvested directly into RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% sodium-deoxycholate, 1% Triton X-100) containing protease and phosphatase inhibitors, and immunoprecipitations were carried out with the NS1-specific antiserum αSP8 (Brockhaus *et al.,* 1996). Immune complexes were further purified on SDS–PAGE and blotted on polyvinylidene fluoride membranes (Millipore), and the band corresponding to NS1 was excised. Membrane-bound NS1 was digested with 50 units of

trypsin (Promega) for 18 h at 37°C and purified ^{32}P-labeled peptides were analyzed by two-dimensional electrophoresis/chromatography on thin-layer cellulose (TLC) plates (Nüesch *et al.,* 1998a). Phospho-amino acid analyses from individual phosphopeptides were performed as described for full-length NS1 by Corbau *et al.* (1999). Phosphopeptides were scraped off TLC plates hydrolyzed in 6 M HCl at 110°C for 1 h, and the products were analyzed on TLC plates by electrophoresis in two dimensions using pH 1.9 and pH 3.5 buffer followed by autoradiography. The positions of phosphoserine, phosphothreonine, and phosphotyrosine residues were monitored by loading 1 μg each of the unlabeled purified amino acids together with the ^{32}P-labeled digests and subsequent detection by ninhydrin reaction.

Production and purification of wild-type and mutant NS1

NS1 was produced from recombinant vaccinia viruses in suspension cultures of HeLa-S3 cells (Nüesch *et al.,* 1998a) using 15 PFU/cell each of vTF7-3 (Fuerst *et al.,* 1986) together with the appropriate recombinant vaccinia viruses containing the NS1 gene under control of the bacteriophage T7 promoter (Nüesch *et al.,* 1992, 1995). Infected cultures were harvested 18 h postinfection, nuclear extracts were prepared, and His-NS1 was purified using Ni^{2+}-NTA agarose (Qiagen) columns (Nüesch *et al.,* 1995). 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 being used as controls.

In vitro resolution and replication reactions with plasmids containing MVM origins

Resolution and replication reactions were performed as previously described (Cotmore *et al.,* 1992, 1993) using pLEB711 and pREB1412 as substrates containing the right-end and left-end origins of MVM replication, respectively. HeLa replication extracts were supplied with approximately 100 ng of purified NS1 that had been expressed from HeLa cells. The reaction mixture was incubated for 2 h at 37°C in the presence of dNTPs including [α-^{32}P]dATP, ATP, and an ATP-regenerating system. The NS1-attached labeled products were recovered by immunoprecipitations with αNS_N (Cotmore and Tattersall, 1986) and digested with *Sca*I, and the resulting fragments were further separated into NS1-bound and NS1-free fractions by centrifugation. The ^{32}P-labeled DNA was then analyzed by 0.8% agarose gel electrophoresis and revealed by autoradiography.

P38 *trans* activation

To measure the ability of wild-type NS1 and mutant derivatives to *trans* activate the P38 promoter, 2×10^5 A9 cells grown in monolayer cultures were cotransfected with 50 ng of the reporter plasmid pP38-Luc and various amounts of the NS1 expression plasmid pRSV-$NS1_x$. At 48 h posttransfection, cells were harvested into lysis buffer (1.5 mM glyc-glycine, pH 7.8; 15 mM $MgSO_4$, 0.4 mM EGTA, 1 mM DTT, 1% Triton X-100, 10% glycerol) and tested for luciferase activity as described (Vanacker *et al.,* 1996). Luciferase activities achieved by NS1 mutants were expressed as percentages of the value obtained with wild-type NS1, after subtraction of the background measured in the absence of any expression vector.

Intracellular localization of NS1 by indirect immunofluorescence

For determination of the subcellular localization of NS1, A9 cells were grown on spot slides, infected with the recombinant vaccinia viruses vTF7-3 and vHis$NS1_x$ (15 PFU/cell each), fixed with paraformaldehyde 5 h postinfection, and immunostained for NS1 protein expression using $\alpha NS1_c$ antiserum and FITC-conjugated anti-rabbit IgG (Cotmore and Tattersall, 1988). For detection of NS1 in the presence of GFP, cells were fixed in paraformaldehyde (25 min), followed by neutralization in 50 mM NH_4Cl (6 min), permeabilization with 0.2% Triton X-100 (2 min), and a second fixation in paraformaldehyde (10 min) to avoid the loss of GFP. Oligomerization of a given NS1 product was determined by measuring its capacity for cotransporting the cytoplasmic NS1:*in*N10*d*/158 mutant into the nucleus (Nüesch and Tattersall, 1993). A9 cells grown on spot slides were infected with 15 PFU/cell of each of the recombinant vaccinia viruses vTF7-3, v$NS1_{Nuc}$, and vNS1*in*N10*d*/158, producing T7 RNA polymerase required for NS1 expression, wild-type or mutant nuclear NS1 to be assayed for transport, and cytoplasmic reporter NS1, respectively. Infected cells were fixed with paraformaldehyde 11 h postinfection and NS1 proteins were detected by double-immunofluorescence. The cytoplasmic deletion mutant NS1:*in*N10*d*/158 was specifically revealed with a 1:100 dilution of mouse αM2 monoclonal antibodies (Sigma), due to an N-terminal FLAG-epitope (*in*N10) and stained using FITC-conjugated anti-mouse secondary antibodies. Full-length NS1 polypeptides (wild type and phosphorylation-site mutants) were recognized specifically by means of a 1:100 dilution of $\alpha NS1_{NLS}$, a rabbit antiserum raised against a peptide that corresponds to amino acids 101 to 213 of NS1 (Cotmore and Tattersall, 1986) and is located within the region deleted from *d*/158. Bound $\alpha NS1_{NLS}$ antibodies were detected with Texas red-conjugated anti-rabbit secondary antibodies.

Determination of morphological changes induced by NS1

A9 cells grown on spot slides were transfected with 200 ng per spot of pP4-$NS1_x$-P4-GFP, pP4-GFP, or pP4-$NS1_x$-CMV-GFP, using 1 μl of lipofectamine (Gibco BRL)

in the presence of OPTI-MEM. At 5 h posttransfection, the medium was changed to DMEM containing 10% FCS, and living cells expressing GFP were monitored for morphological changes at 24-h intervals.

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