

Replicative Functions of Minute Virus of Mice NS1 Protein Are Regulated In Vitro by Phosphorylation through Protein Kinase C

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NS1, the major nonstructural protein of the parvovirus minute virus of mice, is a multifunctional phosphoprotein which is involved in cytotoxicity, transcriptional regulation, and initiation of viral DNA replication. For coordination of these various functions during virus propagation, NS1 has been proposed to be regulated by posttranslational modifications, in particular phosphorylation. Recent in vitro studies (J. P. F. Nüesch, R. Corbau, P. Tattersall, and J. Rommelaere, *J. Virol.* 72:8002–8012, 1998) provided evidence that distinct NS1 activities, notably the intrinsic helicase function, are modulated by the phosphorylation state of the protein. 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 NS1 (NS1^P) supported RCR under these conditions, dephosphorylated NS1 (NS1^O) was impaired. Using fractionated HeLa cell extracts, we identified two essential protein components which are able to phosphorylate NS1^O, are enriched in protein kinase C (PKC), and, when present together, reactivate NS1^O for replication. One of these components, containing atypical PKC, was sufficient to restore NS1^O helicase activity. The requirement of NS1^O reactivation for characteristic PKC cofactors such as Ca²⁺/phosphatidylserine or phorbol esters strongly suggests the involvement of this protein kinase family in regulation of NS1 replicative functions in vitro.

Minute virus of mice (MVM) is the prototype of the genus *Parvovirus*. Members of this genus consist of nonenveloped spherical particles of about 20 to 24 nm in diameter, comprising a linear single-strand DNA genome of approximately 5.1 kb. Parvovirus DNA encodes the two structural (VP) and at least four nonstructural (NS) polypeptides, of which the 83-kDa nuclear phosphoprotein NS1 is the only viral product necessary for viral DNA replication in all cell types (22, 54). Replication of the parvovirus genome involves the formation of monomeric and concatemeric duplex DNA intermediates. These replicative forms are produced by an unidirectional, single-strand copy mechanism (for a review, see reference 16) which resembles the rolling-circle replication (RCR) mechanism described for single-stranded DNA plasmids, bacteriophages, and geminiviruses (for a review, see reference 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 group which serves as a primer for DNA synthesis (8, 13, 19, 59).

The minimal origin sequence at the left-end telomere has been mapped and consists of approximately 50 bp located

within the stem of the Y-shaped terminal structure (13). This sequence comprises binding sites for the cellular component PIF (parvovirus initiation factor) (9) and for NS1 (21) and an NS1 nick site (13). The NS1 binding and nick sites are separated by an AT-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 a functionally important mismatched "bubble," with a 5'-GAA-3' triplet on one strand opposite a 5'-GA-3' doublet on the other strand. 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 RCR, while the trinucleotide-containing counterpart remains silent (13).

Besides its key role 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 parvovirus P38 promoter that controls capsid gene expression (64). Furthermore, NS1 *trans* regulates nonparvovirus 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 posttranslational modifications such as phosphorylation. NS1 was indeed found to be phosphorylated in infected cells (2, 11, 20, 51). Moreover, recent in vitro studies have shown that HeLa cell-derived native NS1 differs from its

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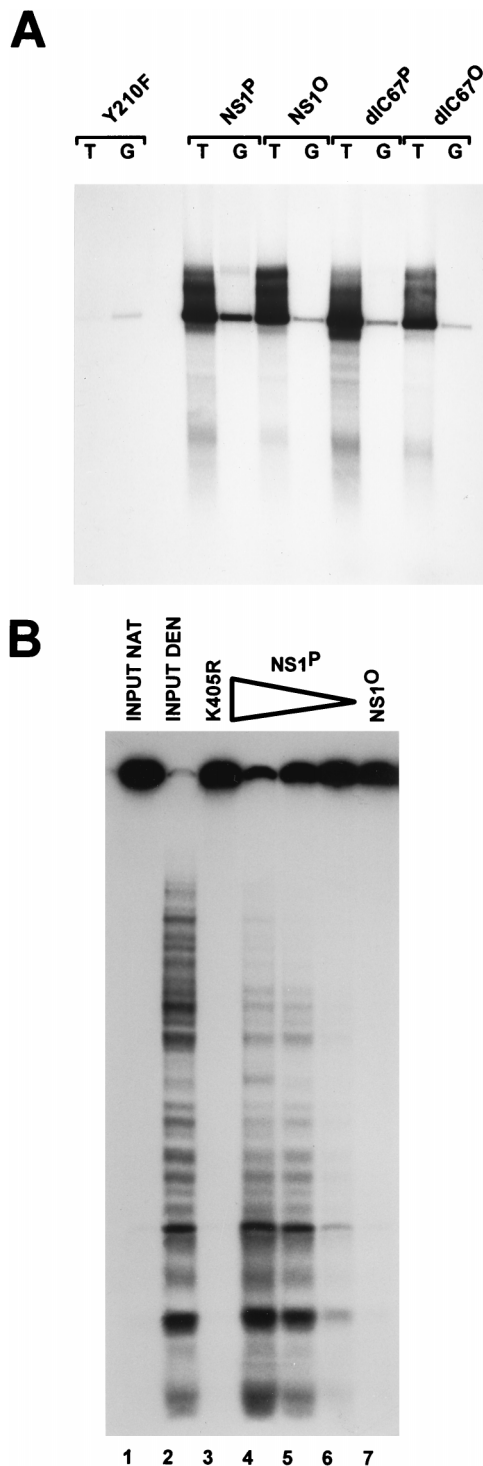


FIG. 1. Comparison of replicative functions of native NS1^P and dephosphorylated NS1^O. NS1 was expressed from recombinant vaccinia viruses in HeLa-S3 cells and harvested at 18 h postinfection by preparing nuclear extracts. Dephosphorylated NS1^O was obtained by treatment with calf intestine alkaline phosphatase. His-tagged native NS1^P and phosphatase-treated NS1^O were purified from nuclear extracts by Ni²⁺-NTA affinity chromatography. (A) NS1^P and NS1^O were compared for their capacities to support RCR in standard HeLa cell replication extracts, using plasmids containing the left-end active (T) or inactive (G) origin as substrates, in the presence of [³²P]dATP. The reaction products were linearized with *Hind*III and analyzed by 0.8% agarose gel electrophoresis. The linkage tyrosine mutant Y210F (59) served as a negative control. The *dlC67* mutant is replication competent (60) and was also analyzed in its native (P) and dephosphorylated (O) forms. (B) NS1^P (100, 30, and 10 ng [lanes 4 to 6, respectively])

antisera, and in vitro-labeled NS1 was detected by 8% SDS-PAGE and autoradiography.

Helicase assays. Helicase assays were performed as described previously (59, 60) with M13-VAR as a template. Reaction mixtures with 10 to 100 ng of purified NS1 were incubated for 40 min at 37°C. For reactivation experiments, titrated amounts of protein extracts were added to the reaction mixtures together with one or more of the following PKC cofactors: 2 mM Ca²⁺, 1 μg of L-α-phosphatidyl-L-serine (PS) per μl, or 5 nM TPA. None of these PKC cofactors alone had any influence on the helicase function of native NS1^P, dephosphorylated NS1^O, or mutant NS1 proteins used as negative controls.

Western blot analyses. Protein extracts were fractionated by discontinuous 10% SDS-PAGE, blotted on nitrocellulose membranes, and revealed with rabbit antibodies directed against the most conserved domain of PKC (αPKCγ and αPKCζ), or with mouse antibodies specific for the atypical PKCε (Transduction Laboratories). The αPKCγ and αPKCζ polyclonal antibodies were affinity purified on peptide columns, used at 0.6 mg of IgG per ml, and revealed with [¹²⁵I]-labeled protein A (ICN; 0.2 μCi/ml). Mouse αPKCε antibodies were used at a 1:2,500 dilution, and bound antibodies were revealed with a 1:5,000 dilution of horseradish peroxidase-conjugated anti-mouse IgGs by using the ECL system (Amersham).

RESULTS

NS1 phosphorylation is required for RCR. Previous investigations comparing biochemical activities of dephosphorylated NS1 (NS1^O) and native NS1 (NS1^P), both derived from HeLa cells, revealed modulation of site-specific binding to the left-end origin, site-specific nicking, and helicase and ATPase activities. In contrast, no significant difference between NS1^O and NS1^P could be observed in their respective capacities for dimer-bridge resolution in an in vitro replication assay with HeLa cell extracts (60). It was suggested that NS1^O became phosphorylated by kinases present in cell extracts, leading us to develop 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 only a short stretch of labeled DNA. This prompted us to use a more simple assay, which consists of the NS1-dependent initiation of RCR of nonpalindromic 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 a plasmid containing an active origin of replication, this assay leads to the synthesis of several kilobases of labeled DNA, which makes it far more sensitive than dimer-bridge resolution. In addition, in the 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, NS1^P was compared with NS1^O in regard to the ability to support RCR from a parvovirus origin in standard HeLa cell extracts. Purified NS1^P and NS1^O 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 which is proficient in helicase activity (59), served as a negative control for NS1 replicative function. As shown in Fig. 1A, NS1^O was able to support RCR in crude HeLa cell extracts almost as efficiently as NS1^P, confirming previously reported findings with cloned

and NS1^O (100 ng [lane 7]) were compared for their intrinsic helicase activities, using M13-VAR template, for 40 min at 37°C in the presence of 2 mM ATP. The ATP-binding site mutant K405R (57) served as a negative control. Reaction products were analyzed by native 7% PAGE in the presence of 0.1% SDS. Lanes 1 and 2, native (NAT) and denatured (DEN) input DNA, respectively.

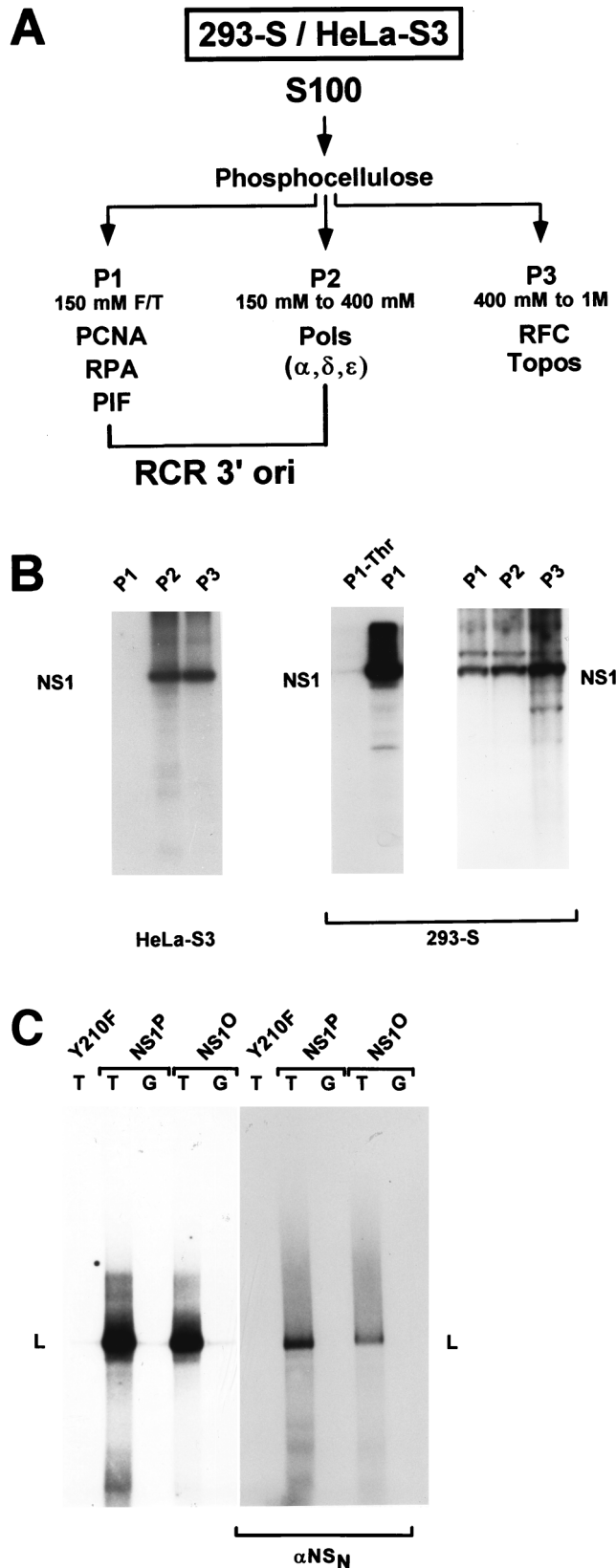


FIG. 2. Fractionation of cell extracts by phosphocellulose chromatography. S100 replication extracts were prepared from 293-S or HeLa-S3 cells and fractionated on phosphocellulose columns as described by Tsurimoto and Stillman (68). (A) Scheme of replication extract fractionation into P1, P2, and P3, with the expected distribution of known replication factors (8, 55, 68). The NaCl

dimer-bridge as a substrate (60). Indeed, the replication activities of both wild-type NS1 and the replication-competent NS1 mutant *dIC67* were reduced to only a small extent by dephosphorylation; i.e., NS1^O sustained approximately 60% of the level of [³²P]dATP incorporation into newly synthesized DNA as did NS1^P (data not shown). To verify dephosphorylation, we also tested NS1^O 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, NS1^O helicase activity was indeed reduced more than 10-fold compared to that of the native polypeptide, in contrast to the significant activity of NS1^O in replication assays. The helicase-deficient NS1 mutant K405R served as a negative control in these experiments.

One possible explanation for the replication competence of NS1^O, despite the lack of helicase function, might be the presence of protein kinases within the cell extracts used in the former but not in the latter assay, which would lead to NS1^O rephosphorylation and consequent reactivation. To test this possibility, and eventually to establish RCR in absence of endogenous protein kinases, we first determined the presence of NS1^O-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 RCR 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 be confined to fractions 2 and 3, with no detectable activity in the P1 flowthrough (Fig. 2B). Yet, HeLa cell P1 failed to support significant replication in the presence of P2 and native NS1^P (data not shown) and hence could not be used in subsequent experiments. Therefore, we prepared P1 from 293 cells as previously described (8), in order to obtain sufficient replication factors therein to drive RCR. In contrast to HeLa cell P1, 293 cell P1 significantly phosphorylated NS1^O, 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 cell P1, this fraction was purified over an L-threonine affinity column, resulting in P1-Thr, which was essentially free of NS1^O-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 com-

concentrations used for elution are indicated. F/T, flowthrough; PCNA, proliferating cell nuclear antigen; RPA, replicator protein A (also designated RF-A or human single-stranded-DNA-binding protein); PIF, parvovirus initiation factor; Pols, eukaryotic DNA polymerases; RFC, replication factor C; Topos, eukaryotic topoisomerases. As indicated, P1 plus P2 have been shown to contain all cellular components necessary to support NS1-dependent RCR from the active left-end (3') origin (ori) (8). (B) Detection of NS1^O-phosphorylating protein kinases in the distinct phosphocellulose fractions. NS1^O was incubated with the indicated fractions in the presence of [³²P]ATP for 30 min at 37°C and analyzed by SDS-PAGE after immunoprecipitation with αNS_N (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 NS1^P and NS1^O in RCR assays with 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 NS1^O 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 cofactors Ca²⁺ and PS. The replication-deficient NS1 mutant Y210F was used as a negative control. Linearized, labeled reaction products (L) were analyzed by 0.8% agarose gel electrophoresis, either directly after proteinase K treatment (left panel) or after immunoprecipitation with αNS_N (right panel).

ponents allowing NS1-mediated RCR. As seen in Fig. 2C, NS1^P 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 a small extent when the inactive (GAA) origin was used as a substrate. Furthermore, as seen previously with standard HeLa extracts, NS1^O was also able to support RCR under these conditions, i.e., in the presence of the sole protein kinases present in HeLa cell P2. Under these conditions, NS1^O achieved close to 50% of the RCR activity of NS1^P (Fig. 2C). The inactivity of the replication-deficient NS1 mutant Y210F, used as a negative control, and the immunoprecipitation of labeled DNA products with NS1 antiserum confirmed the specificity of the RCR reaction (Fig. 2C). Together, these results indicate that the phosphocellulose P3 fraction, as well as the protein kinases therein, are dispensable for RCR initiated by NS1 at the left-end origin.

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 decided to replace the whole fraction P2 by purified bacterial or bacteriophage polymerases. With native NS1^P, no replication products were obtained when either P1 or P2 was omitted from the reaction (Fig. 3, lanes 1 and 2). On the other hand, DNA synthesis took place when the P1-Thr fraction was supplemented with *Escherichia coli* DNA polymerase I or the Klenow fragment thereof (data not shown) or with DNA polymerase 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 a unidirectional single-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 α NS antiserum. When *E. coli* Klenow fragment, DNA polymerase I (data not shown), or bacteriophage T7 polymerase (Fig. 3, lanes 8 to 12) was 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 α NS1 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 simian virus 40 (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 NS1^P (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 α NS1-immunoprecipitable replication products in the presence of wild-type NS1^P but not Y210F (Fig. 3, lanes 3 and 4).

Since we were able to obtain a specific RCR reaction by using a kinase-free P1-Thr and T4 DNA polymerase in the presence of native NS1^P, we further investigated the require-

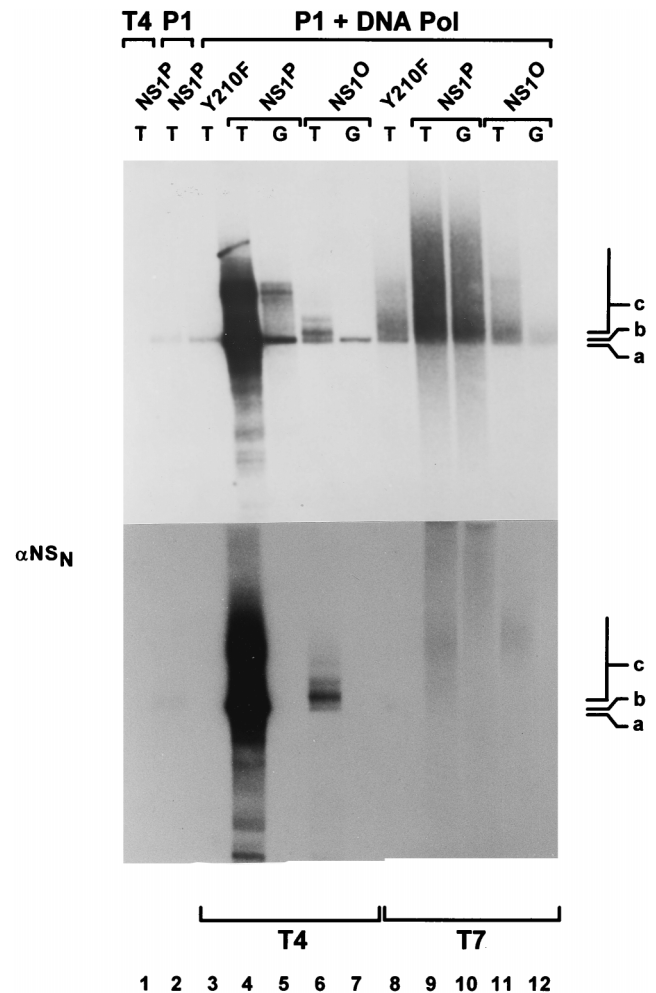


FIG. 3. NS1-dependent RCR in a kinase-free in vitro system. Plasmids containing the active (T) or inactive (G) left-end origin were used as substrates to measure the capacity of NS1^P and NS1^O to support RCR in absence of endogenous protein kinases, in a system based on P1-Thr and bacteriophage DNA polymerases. T4 and T7, 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 with α NS_N (bottom panel). The positions of the linearized plasmid (a) and slower-migrating products (b and c) are indicated.

ments of this reaction for NS1 phosphorylation. As illustrated in Fig. 3, the patterns of labeled DNA products generated in the presence of NS1^O and NS1^P could be distinguished in three respects. (i) 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. (ii) Major products of the RCR reaction taking place in the presence of NS1^P 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 lengths (13). These intermediates, marked c in Fig. 3, were not formed efficiently in the presence of NS1^O, 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 DNA replication). This conclusion is in agreement with the previously reported main deficiency of NS1^O in the helicase activity, which is thought to fa-

ilitate unwinding of the double-stranded template to allow the replication fork to proceed (Fig. 1B) (60). (iii) The predominantly labeled DNA product obtained with NS1^O (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 the absence of extensive strand displacement synthesis. This species b was not detected when replication-competent NS1^P was used, suggesting that the lack of NS1 phosphorylation is associated with an impairment of growing-strand elongation in already initiated DNA molecules. It should also be stated that besides this modulation of the elongation step, the initiation of parvovirus DNA replication also appears to be stimulated by NS1 phosphorylation. Indeed, recently reported *in vitro* assays have shown that although it is proficient in site-specific nicking, NS1^O is less efficient than NS1^P for this function (60).

Reactivation of NS1^O for RCR by members of the PKC family. Since NS1^O was able to support RCR in the presence of cell extracts containing endogenous protein kinases but distinguished itself from NS1^P in its low capacity to achieve this reaction in the kinase-free *in vitro* replication system, we further attempted to reactivate NS1^O for RCR by providing exogenous cellular components. Previous investigations using commercially available PKC preparations indicated the involvement of this protein kinase family in regulation of NS1 helicase activity (60). Therefore, we performed the following reactivation experiments in the presence of Ca²⁺ and PS, which are known cofactors for PKC (44, 61). Indeed, under these conditions, NS1^O replication activity could be stimulated to a significant extent by supplying the reaction mixture with limited amounts of whole HeLa cell replication extracts (data not shown). This result encouraged us to fractionate HeLa cell extracts in order to characterize the NS1^O-activating components, and in particular to determine whether the rescue of NS1^O replication activity cosegregated with NS1 phosphorylation by specific protein kinases as postulated.

Figure 4 gives the purification scheme for HeLa cell extracts used for these reactivation experiments. Individual fractions were tested for their ability to phosphorylate NS1^O. In parallel, various protein concentrations were used to determine the capacity of individual or combined HeLa cell fractions for reactivating the RCR function of the underphosphorylated polypeptide. Native wild-type NS1^P and the replication-deficient mutant Y210F served as positive and negative controls in these assays, respectively. The specificity of the reactions was also ascertained by using active (pL1-2TC) versus inactive (pL1-2GAA) origin-containing substrates and by immunoprecipitating NS1-bound replication products with α NS_N antiserum. Chromatography steps 1 and 2 were designed to achieve a first bulk segregation of the multiple protein kinases which are able to phosphorylate NS1^O. As illustrated in Fig. 2B and C, more than 50% of the NS1-targeted kinase activity segregated in phosphocellulose fraction P3 (step 1) and proved to be non-essential, since NS1^O was almost as efficient as NS1^P 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 NS1^O in the kinase-free replication system (data not shown). This result indicated that specific rather than random phosphorylation of NS1 was necessary for the replicative functions of the viral product. After further purification of P2 on anion-exchange columns (step 2), NS1^O-stimulating activity was recovered in the low-affinity DE-1 fraction, eluting between 50 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,

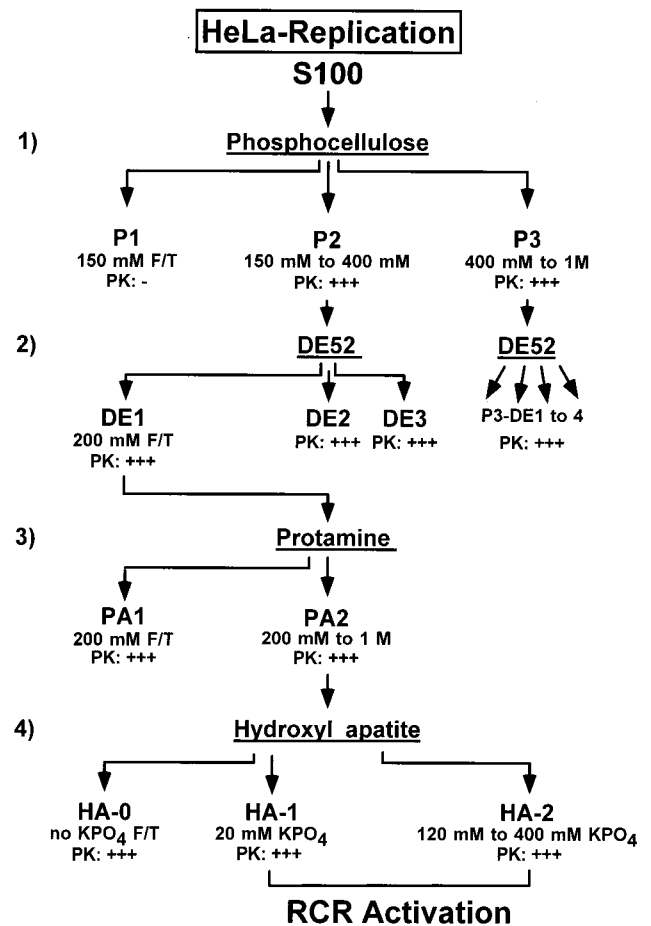


FIG. 4. Purification scheme used to identify NS1^O-activating protein kinases. HeLa cell replication extracts were analyzed for protein kinases which are able to phosphorylate and activate NS1^O for RCR in a kinase-free system. Chromatography steps 1 (phosphocellulose) and 2 (anion exchange) allow the bulk separation of protein kinases. Steps 3 (protamine) and 4 (hydroxylapatite) are performed to further purify members of the PKC family. Selective elution of the fractions under investigation from rows 1, 2, and 3 was achieved with the indicated NaCl concentrations, while the hydroxylapatite-bound components (row 4) were eluted in phosphate buffer. PK: - and PK: +++, undetectable and strong protein kinase activities, respectively, assayed *in vitro* with NS1^O as a substrate. F/T, flowthrough.

despite their ability to phosphorylate NS1^O to a significant extent *in vitro* (data not shown).

The subsequent purification steps (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 structurally related PKC isoforms can be separated on hydroxylapatite columns, based on their various requirements for Ca²⁺ ions and affinities to phosphate groups (32, 62). As expected, NS1^O-activating components were retained on the protamine column and recovered in fraction PA-2 after high-salt elution (data not shown). A further concentration of these components was achieved by hydroxylapatite chromatography, yielding an inactive flowthrough and two bound fractions of low (HA-1) and high (HA-2) affinity (eluting at 20 and 120 to 400 mM KPO₄, respectively). Though inactive on their own, the HA-1 and HA-2 fractions were able to reactivate NS1^O when supplied in combination in RCR assays (Fig. 5, lanes 9 to 14; Table 1). The

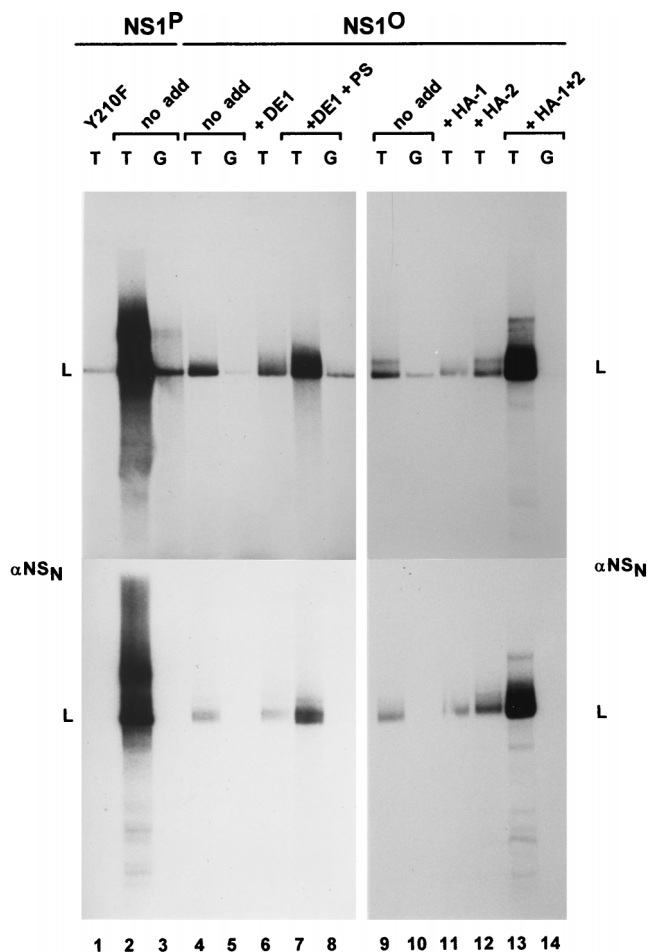


FIG. 5. Activation of NS1^O for RCR by fractionated HeLa cell extracts. NS1-dependent RCR of plasmids containing the left-end active (T) or inactive (G) origin was determined in a kinase-free *in vitro* system based on P1-Thr and T4 DNA polymerase. Activation of NS1^O (lanes 4 to 14) was achieved by using the indicated protein components (see Fig. 4) in the presence or absence of protein kinase C cofactors. Only some of the protein-cofactor combinations that have been tested are illustrated, as most representative. Lanes 4 to 8 show the dependency of NS1^O activation on PKC cofactors. PS, Ca²⁺ plus PS. Lanes 9 to 14 show the segregation of protein components allowing the reactivation of dephosphorylated NS1. The illustrated reactions were carried out in presence of the PKC activators Ca²⁺ and PS (HA-1) or TPA (HA-2 and HA-1 plus HA-2). It should be mentioned that TPA-stimulated HA-1 or Ca²⁺+PS-stimulated HA-2 also failed to activate NS1^O (data not shown), while the combined protein components (HA-1 plus HA-2) were able to rescue NS1^O in the presence of Ca²⁺+PS instead of TPA. The NS1 mutant Y210F served as a negative control (lane 1). Native NS1^P was used as a phosphorylated, replication-competent standard (lanes 2 and 3). L, migration of the linearized plasmid. The lower panel presents reaction products after immunoprecipitation with αNS_N.

reaction stimulated by HA-1 and -2 was a specific NS1-dependent RCR process. Indeed, no significant DNA synthesis was observed when the inactive GAA origin was used 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 panel, lane 13). To further evaluate the active components within fractions HA-1 and HA-2, we performed the reactivation experiments in the presence or absence of characteristic cofactors for PKCs (44, 61). As summarized in Table 1 and illustrated in Fig. 5 and 6, reactivation of NS1^O in replication assays was strictly dependent upon addition of PS, Ca²⁺+PS, TPA, or PS + TPA. This strongly argues for the involvement of members of the PKC family in modulation of NS1 replicative functions *in vitro*.

TABLE 1. Dependence of the reactivation of NS1^O replicative function on PKC cofactors

Column fraction	Reactivation of ^a :							
	NS1 ^O RCR activity				NS1 ^O helicase activity			
	No co-factors	PS	TPA	Ca ²⁺ -PS	PS-TPA	No co-factors	PS	TPA
HA-1	-	-	++	-	+	-/+	+++	-/+
HA-2	-	-	-	-	-	-	-	-
HA-1 plus HA-2	-	+	++	++	+++	ND ^b	ND	ND

^a The protein fraction-induced reactivation of NS1^O functions in the presence of cofactors (PS, TPA, and Ca²⁺) is graded from - (no effect) to +++ (maximal stimulation). The following PKC isoforms are known to be typically activated by the cofactors: PS, atypical isoforms; TPA, classical and novel isoforms; no cofactors, proteolytically derived catalytic domain; Ca²⁺-PS, classical and atypical isoforms; PS-TPA, classical, atypical, and novel isoforms.

^b ND, not determined.

The reactivation experiments described 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 of NS1^O activation for PKC cofactors. Unlike standard HeLa cell 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 can serve as PKC activators (61). Assuming that the results obtained with the kinase-free reconstituted system can be extrapolated to mammalian DNA polymerases, these fractions should be supplemented with PKC activators in order to render NS1^O competent for RCR. This prediction was tested by comparing NS1^P and NS1^O for RCR with P1-Thr plus P2-pol in the presence or

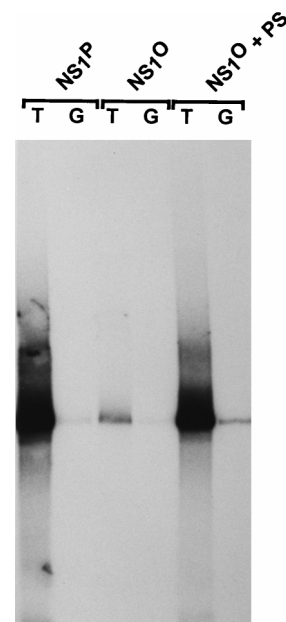


FIG. 6. Effect of PKC cofactors on activation of the NS1^O replicative functions in an entirely eukaryotic replication system. NS1^P and NS1^O were compared for RCR of plasmids containing the left-end active (T) or inactive (G) origin of replication by using P1-Thr plus P2-pol (containing endogenous DNA polymerases). NS1^O activity was tested in either the absence or presence (+ PS) of 1.5 μg of PS. All reactions were performed in the presence of 2 mM CaCl₂.

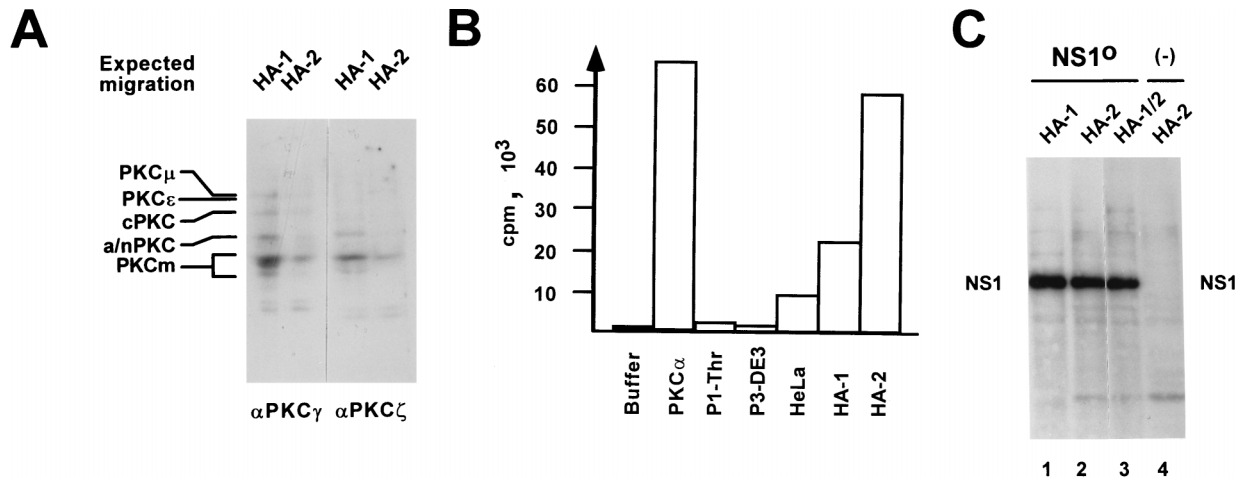


FIG. 7. Detection of PKC within fractions HA-1 and HA-2 from HeLa cell extracts. (A) Western blot analysis of HA-1 or HA-2 after 10% SDS-PAGE, using peptide affinity-purified polyclonal antisera that were raised against the most-conserved regions of classical PKCs (PKC γ , amino acids 416 to 569; PKC ζ , amino acids 310 to 444) and revealed the various PKC isoforms by cross-reaction. The expected migration positions of known PKC isoforms are given on the left: cPKC, classical PKC α , - β , and - γ (approximately 80 kDa); a/nPKC, atypical PKC δ/λ and - ζ (70 to 72 kDa) and novel PKC δ , - η , and - θ (72 to 78 kDa). PKC ϵ (90 kDa) and PKC μ (115 kDa), higher-molecular-mass novel PKCs; PKCm (approximately 45 kDa), PKC catalytic domains produced by proteolytic cleavage. (B) PKC activity assays (Amersham) of fractions used for reactivation of NS1^O in replication reactions. Buffer, negative control in the absence of added protein components; PKC α , 10 ng of His tag-purified recombinant PKC α produced by recombinant vaccinia virus expression in HeLa cells; P1-Thr, fraction containing no NS1^O-targeted protein kinase activity (see Fig. 2B); P3-DE3, fraction which proved able to phosphorylate but failed to reactivate NS1^O (see Fig. 4); HeLa, standard HeLa cell replication extract; HA-1 and HA-2, NS1^O-activating fractions in RCR assays (see Fig. 5). The values are expressed as transferred ³²P-labeled substrate per 10 ng (recombinant PKC α) or 10 μ g (HeLa cell fractions) of total effector proteins. (C) In vitro phosphorylation of 1 μ g of NS1^O with protein fractions HA-1 and HA-2, used individually or in combination in the presence of PKC cofactors. The reactions were performed in the presence of [γ -³²P]ATP for 30 min at 37°C, and radiolabeled NS1 was revealed by autoradiography after SDS-PAGE. In lane 4, the NS1^O substrate was omitted. The migration of NS1 is indicated.

absence of PKC cofactors. As shown in Fig. 6, the ability of NS1^O (but not NS1^P) to support RCR of pL1-2TC templates under these conditions was dependent upon addition of the PKC cofactors Ca²⁺ and PS, despite the fact that P2-pol contains, besides DNA polymerases, multiple protein kinases which do not require additional cofactors for activation. This experiment clearly demonstrates that dephosphorylated NS1^O is not irreversibly inactivated with regard to its replication function, and it extends the above-mentioned results to suggest that phosphorylation by distinct protein kinases is required for NS1 activity in a purely eukaryotic DNA replication system.

The purification profile and the cofactor requirements of the NS1^O-reactivating components in replication assays strongly suggested PKC to be an essential kinase(s) for NS1 activation in vitro. In order to ascertain the presence of active PKC within the reactivating HeLa cell fractions, we produced polyclonal antibodies against the most-conserved regions of PKC, which are between amino acids 416 and 569 in PKC γ and between amino acids 310 and 444 in PKC ζ , and performed Western blotting with these immune affinity-purified antisera. As illustrated in Fig. 7A, multiple proteins with the sizes of known PKC isoforms were indeed immunodetected within fractions HA-1 and HA-2, in addition to a major product of lower molecular weight corresponding to PKCm, the proteolytically cleaved catalytic domain of PKC. Moreover, as seen in Fig. 7B, both fractions HA-1 and HA-2 were enriched in PKC activity compared with crude HeLa cell extract or the negative controls P1-Thr (which does not contain significant protein kinase activity), and P3-DE3 (for which NS1^O constitutes a target for in vitro phosphorylation but not replication reactivation [data not shown]), as determined with a commercially available PKC detection system (Amersham). Furthermore, by performing in vitro kinase assays in the presence of [γ -³²P]ATP under conditions used in replication assays, NS1^O was found to serve as a substrate for semipurified protein kinases contained in HA-1 and/or HA-2 (Fig. 7C). Surprisingly, overall NS1 phosphory-

lation was only slightly stimulated upon addition of PKC cofactors (data not shown). This may be because either additional kinases were still present within the HA-1 and -2 fractions or the cofactors target PKC on a specific phosphorylation site(s) within the NS1 polypeptide. Together, these results clearly demonstrated that cellular factors able to reactivate the replicative functions of NS1^O copurified with highly active PKCs contained within both fractions HA-1 and HA-2. Furthermore, the requirement of HA-1 and -2-induced RCR reactivation for specific PKC cofactors substantiated the role of one or more PKC isoforms from HA-1 and/or HA-2 in the upmodulation of NS1 replicative functions.

Stimulation of NS1^O helicase activity by members of the PKC 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 of 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, NS1^O was found to be heavily impaired for this biochemical activity in standard helicase assays (Fig. 1B) (60). In order to investigate whether the intrinsic helicase function of NS1 might be regulated by the same components as those identified with the in vitro RCR system, the HA- fractions were also tested for their ability to rescue NS1^O in helicase assays. As illustrated in Fig. 8A, one of these fractions, HA-1, was able to stimulate the helicase function of NS1^O to a significant extent, whereas HA-2 or the flowthrough (HA-0) had no detectable effect. None of the hydroxylapatite fractions exerted helicase activity by itself, even when tested in a 100-fold excess over the amount used to stimulate the NS1^O helicase function (Fig. 8A, lanes 4 to 6), indicating that this stimulation resulted from the activation of NS1^O rather than 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 in vitro. PKCs have been subdivided into three sub-

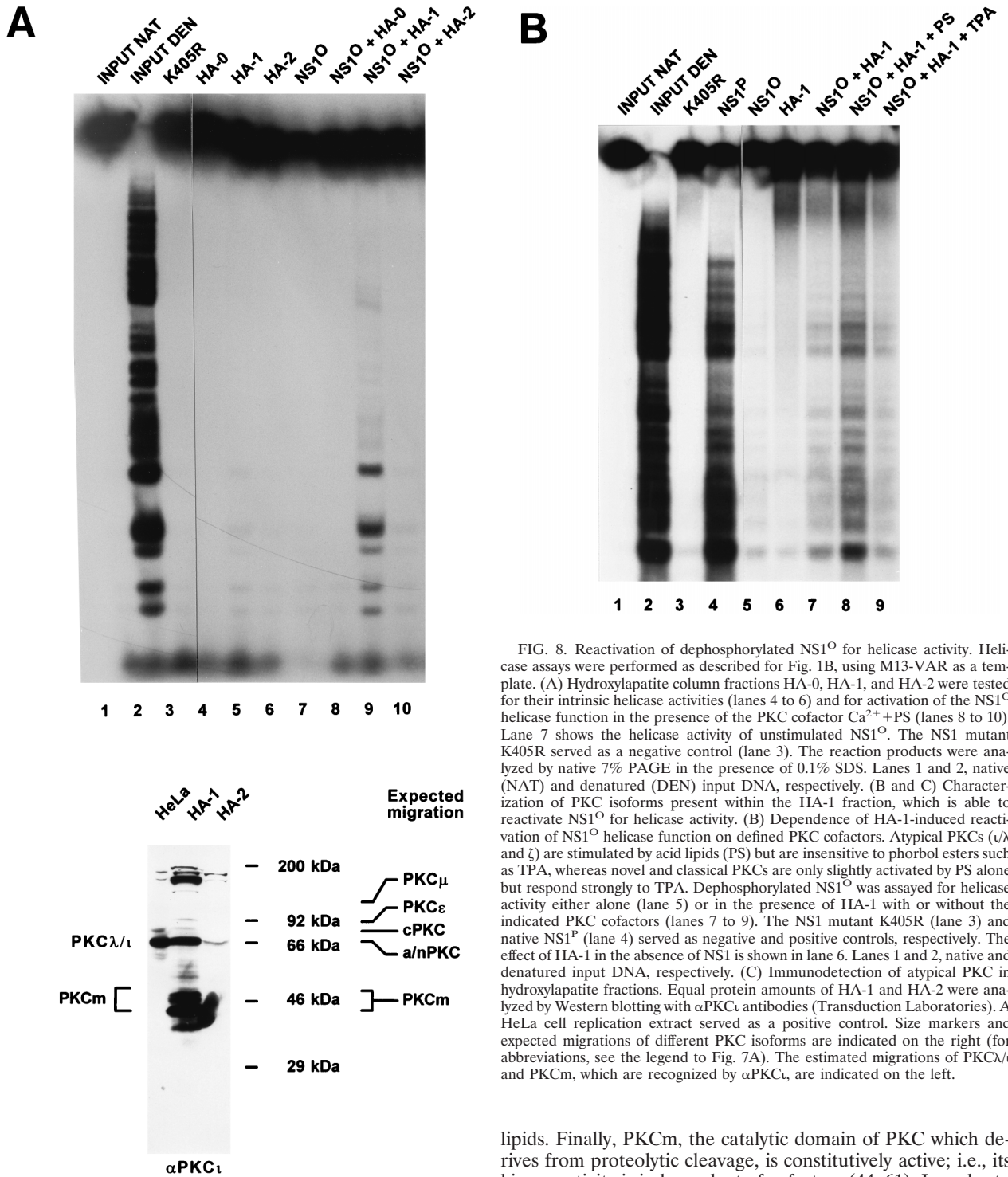


FIG. 8. Reactivation of dephosphorylated NS1^O for helicase activity. Helicase assays were performed as described for Fig. 1B, using M13-VAR as a template. (A) Hydroxylapatite column fractions HA-0, HA-1, and HA-2 were tested for their intrinsic helicase activities (lanes 4 to 6) and for activation of the NS1^O helicase function in the presence of the PKC cofactor Ca²⁺+PS (lanes 8 to 10). Lane 7 shows the helicase activity of unstimulated NS1^O. The reaction products were analyzed by native 7% PAGE in the presence of 0.1% SDS. Lanes 1 and 2, native (NAT) and denatured (DEN) input DNA, respectively. (B and C) Characterization of PKC isoforms present within the HA-1 fraction, which is able to reactivate NS1^O for helicase activity. (B) Dependence of HA-1-induced reactivation of NS1^O helicase function on defined PKC cofactors. Atypical PKCs (ι/λ and ζ) are stimulated by acid lipids (PS) but are insensitive to phorbol esters such as TPA, whereas novel and classical PKCs are only slightly activated by PS alone but respond strongly to TPA. Dephosphorylated NS1^O was assayed for helicase activity either alone (lane 5) or in the presence of HA-1 with or without the indicated PKC cofactors (lanes 7 to 9). The NS1 mutant K405R (lane 3) and native NS1^P (lane 4) served as negative and positive controls, respectively. The effect of HA-1 in the absence of NS1 is shown in lane 6. Lanes 1 and 2, native and denatured input DNA, respectively. (C) Immunodetection of atypical PKC in hydroxylapatite fractions. Equal protein amounts of HA-1 and HA-2 were analyzed by Western blotting with α PKC ι antibodies (Transduction Laboratories). A HeLa cell replication extract served as a positive control. Size markers and expected migrations of different PKC isoforms are indicated on the right (for abbreviations, see the legend to Fig. 7A). The estimated migrations of PKC λ/ι and PKCm, which are recognized by α PKC ι , are indicated on the left.

groups according to their cofactor requirements. Classical (α , β , and γ) and novel (δ , ϵ , θ , η , and μ) PKC isoforms are stimulated through binding of phorbol esters (such as TPA) and only to a minor extent by binding of acid lipids (such as PS). Full activation of classical PKC is also achieved by Ca²⁺ in addition to PS, while novel PKCs do not contain a Ca²⁺-binding site. Members of the last group, designated atypical PKC isoforms (ι/λ and ζ), lack a calcium-binding domain and do not respond to TPA either, but they are 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 cofactors (44, 61). In order to characterize the activating kinase(s) present in fraction HA-1, we further tested NS1^O reactivation for helicase function in the presence of HA-1 together with selected PKC cofactors. As seen in Fig. 8B, the HA-1 fraction had a moderate, constitutive stimulatory effect in absence of cofactors (lane 7), which may be ascribed to PKCm and/or other cofactor-independent components. Interestingly, this activation was significantly enhanced upon supply of PS (lane 8), while TPA failed to increase the capacity of HA-1 to stimulate the helicase function of NS1^O (lane 9). As summarized in Table 1, this PS respon-

siveness and TPA insensitivity of the HA-1-induced rescue of NS1^O point to an atypical PKC(s) as the effector(s) mediating the dependence of NS1 helicase activity on phosphorylation. This conclusion was substantiated by Western blot analysis of fractions HA-1 and HA-2 with specific antibodies recognizing the atypical PKC ϵ . As shown in Fig. 8C, the atypical PKC ϵ segregated mainly to fraction HA-1 during the purification procedure, while HA-2, which was unable to activate NS1^O in helicase assays, did not contain substantial amounts of this PKC isoform. The PS responsiveness of the NS1 helicase-stimulating factor(s) and its cosegregation with PKC ϵ into HA-1 strongly suggested that atypical PKCs are involved in the upmodulation of the NS1 DNA-unwinding function.

DISCUSSION

NS1, the major nonstructural protein of 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 cofactors (61), and/or to become posttranslationally modified. The last possibility can provide an original polypeptide with functional heterogeneity through the addition of various molecule groups, catalyzed with high efficiency by enzymes present in rather small amounts, targeted on a large protein pool. All of the above-mentioned modes of regulation have been assigned to NS1, including interactions with cellular partner proteins (24, 36), oligomerization (58), and phosphorylation (20). Interestingly, NS1 oligomerization has been implicated in the control of NS1 replicative functions, in particular helicase activity (63). It is worth noting that NS1 self-assembly to produce higher-order oligomers is dependent on an intact nucleoside triphosphate-binding domain (58) and might thus be regulated by association with this cofactor. 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 turn, the NS1-associated ATP turnover was recently found to be 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 functions (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 obtained evidence that a central NS1 function necessary for progeny virus production, namely, replication initiated at the left-end origin, is upmodulated by phosphorylation of NS1 and that members of the PKC family participate in this stimulation. The phosphorylation dependence of NS1 is reminiscent of the regulation of large T antigen (LT), the initiator protein for SV40 DNA replication (71). LT, which is expressed in nondividing cells and is able to drive quiescent cells into S phase, becomes activated for rep-

lication 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), hence the restriction of parvovirus multiplication *in vivo* to proliferating tissues (43). Yet, in contrast to the case for LT, NS1 production is limited during G₀/G₁ (25, 66), and parvoviruses fail to drive quiescent cells into S phase (15). Moreover, extracts derived from cells arrested in G₀ are able to activate the replicative functions of dephosphorylated NS1 *in vitro* (56). Therefore, posttranslational modifications of NS1 do not account for the S phase dependency of parvovirus DNA replication. Another feature of parvoviruses is their striking oncotropism (65). This tropism could be mimicked in various cell culture systems, where the 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 that PKC activators, such as phorbol esters, also exert strong effects on cell proliferation, differentiation, and, most intriguingly, tumor promotion (44, 61). Moreover, it has been reported that overexpression of PKC ϵ , a novel PKC isoform which is activated by phorbol esters and has been detected in A9 cells (a natural host cell line permissive for MVMp [26]), leads to cell transformation *in vitro* (6, 49). These correlations of oncogenic transformation with changes in both 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 NS1^P was able to initiate RCR, leading to extensive strand displacement synthesis in the absence of protein kinases. In contrast, NS1^O had a restricted phenotype that was characterized by the formation of a distinct NS1-bound replication intermediate. This intermediate migrated in agarose gels as a molecule in which DNA synthesis was initiated but became arrested prior to strand displacement synthesis, suggesting 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 NS1^O 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 NS1^O could be corrected by supplementing solely the HA-1 fraction, while RCR reactivation of NS1^O required both protein fractions HA-1 and HA-2. Thus, more than one protein kinase seem to be necessary to switch NS1^O on for DNA replication. The additional replicative functions of NS1 which are regulated by phosphorylation, besides helicase activity, are as yet undefined. One candidate is the nicking reaction, which is achieved by NS1^O to a detectable, albeit reduced level compared to NS1^P, both in the RCR assay and in an *in vitro* nicking assay performed in the absence of additional cellular components (60). However, this reduction could also be a consequence of the defect of NS1^O in DNA unwinding, since the latter is thought to facilitate single-strand nicking (35, 67). Other potential phosphorylation-dependent NS1 replicative functions might be considered by analogy with SV40 LT. LT binds to the origin of replication in the absence of phosphorylation (45, 46, 50) and is able to interact with components of the basic replication machinery such as replicator protein A (48) or polymerase α

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, since it is known that LT- and NS1-driven *in vitro* replications share specific requirements for eukaryotic DNA polymerases or the related T4 DNA polymerase (69) and for template DNA unwinding by the helicase activities 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 indeed 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 cell replication extract were able to activate NS1^O for replication activity, pointing to the involvement of specific kinases and phosphorylation events in NS1 regulation. Based on the cofactor requirements for NS1^O reactivation and the purification properties of effector kinases, we were able to assign, at least in part, the capacity for NS1 phosphorylation and reactivation *in vitro* to members of the PKC family. In particular, the NS1 unwinding function might be modulated 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 to involve more than one protein kinase, as indicated by the fact that rescue of NS1^O for RCR required at least two protein components that could be separated by hydroxylapatite chromatography. When combined, the active HA- fractions were able to rescue the RCR functions of NS1^O 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 of these kinases appears to be one of the classical or novel PKC isoforms (which respond to TPA) and controls an as-yet-undefined NS1 replicative function, while the other is a TPA-insensitive atypical PKC isoform that regulates the DNA-unwinding activity of NS1. The latter PKC may be activated by the former, thereby accounting for the sole requirement of TPA as a cofactor to rescue the RCR capacity of NS1^O, 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, should contribute to understanding the posttranslational regulation of NS1 activities.

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