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Dynamic Phosphorylation of *Autographa californica* Nuclear Polyhedrosis Virus pp31

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***Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) pp31 is a nuclear phosphoprotein that accumulates in the virogenic stroma, which is the viral replication center in the infected-cell nucleus, binds to DNA, and serves as a late expression factor. Considering that reversible phosphorylation could influence its functional properties, we examined phosphorylation and dephosphorylation of pp31 in detail. Our results showed that pp31 is posttranslationally phosphorylated by both cellular and virus-encoded or -induced kinases. Threonine phosphorylation of pp31 by the virus-specific kinase activity was sensitive to aphidicolin, indicating that it requires late viral gene expression. We also found that pp31 is dephosphorylated by a virus-encoded or -induced phosphatase(s), indicating that phosphorylation of pp31 is a dynamic process. Analysis of pp31 fusion proteins showed that pp31 contains at least three phosphorylation sites. The amino-terminal 100 amino acids of pp31 include at least one serine residue that is phosphorylated by a cellular kinase(s). The C-terminal 67 amino acids of pp31 include at least one threonine residue that is phosphorylated by the virus-specific kinase(s). Finally, this C-terminal domain of pp31 includes at least one serine that is phosphorylated by either a host or viral kinase(s). Interestingly, site-directed mutagenesis of the consensus threonine phosphorylation sites in the C-terminal domain of pp31 failed to prevent threonine phosphorylation, suggesting that the virus-specific kinase is unique and has an undetermined recognition site.**

Reversible phosphorylation is an important posttranslational modification that can modulate the functions of many cellular enzymes and transcription factors, control protein synthesis and macromolecular assembly, and regulate cell cycle progression and signal transduction pathways (reviewed in references 4, 8, 13, and 14). The activities of many viral proteins also can be regulated by phosphorylation, and this can be an important factor in viral replication (reviewed in references 21 and 31).

The baculoviruses are rod-shaped, double-stranded DNA viruses that infect arthropods (reviewed in reference 1). One of the best-characterized baculoviruses, *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV), encodes several different phosphoproteins (7, 11, 25, 29, 41, 42), as well as its own kinase (32) and phosphatase (19, 35), which suggests that reversible phosphorylation is an important control mechanism in Ac*M*NPV replication. This idea is supported by the results of studies on the protamine-like Ac*M*NPV protein p6.9 (45) and its homolog in *Plodia interpunctella* granulosis virus, VP12 (9). Both p6.9 and VP12 are dephosphorylated prior to condensation and packaging of viral DNA, and the dephosphorylated forms of these proteins have a greater affinity for DNA and are the only forms found in mature budded virions. Consequently, it is believed that dephosphorylation of p6.9 and VP12 is necessary for viral DNA condensation and packaging and that rephosphorylation by a capsid-associated kinase decondenses and releases the viral DNA from nucleocapsids for a new round of replication (43, 44). The finding that dephosphorylated p6.9 is degraded in cells treated with cytochalasin D, which inhibits nucleocapsid morphogenesis, is consistent with the proposed role of p6.9 in viral DNA packaging and nucleocapsid assembly (29). Another Ac*M*NPV protein that appears to be regulated by phosphorylation is the transcription factor, IE1 (12). The phosphorylation pattern of IE1 is modified when it is bound to enhancer DNA, suggesting that DNA binding alters the structure of IE1 and the accessibility of one or more of its phosphorylation sites (7).

The product of the Ac*M*NPV *39K* gene, pp31, is a phosphoprotein that accumulates in the virogenic stroma and binds to DNA in a non-sequence-specific fashion (11). pp31 has three basic regions (BRs) that function interdependently as nuclear and virogenic stroma localization signals and one BR that is essential for DNA binding (5). The accumulation of pp31 in the virogenic stroma, which is the viral replication center within infected-cell nuclei, and its DNA-binding activity suggest that this phosphoprotein plays an important role in viral replication. pp31 also has been identified as a late-gene expression factor that might function in stabilization of late transcripts or as part of the transcription complex (23, 38). Since phosphorylation can regulate many of the properties attributed to pp31, including nuclear localization, DNA binding, and transcription factor activity, we have examined phosphorylation and dephosphorylation of pp31 in more detail.

MATERIALS AND METHODS

Cells and viruses. The methods and conditions used for cell culture have been described previously (30, 37). Sf9 cells are a subclone of IPLB-Sf21-AE (40) and were maintained in TNM-FH medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, Kans.), 1.25 μg of amphotericin B (Sigma Chemical Co., St. Louis, Mo.) per ml, 25 μg of gentamicin (Sigma) per ml, and 0.1% (wt/vol) pluronic F68 (BASF Corp., Parsippany, N.J.) (28). Radiolabeling medium was methionine- or phosphate-free Grace's medium supplemented with 0.5% (vol/vol) heat-inactivated fetal bovine serum. The E2 strain of wild-type Ac*M*NPV and the recombinant baculoviruses used in this study have been described previously (5, 24, 36), and the paper by Broussard et al. (5) includes a detailed description of the methods used to construct the recombinant viruses encoding pp31–β-glucuronidase (pp31-GUS) fusion proteins. Briefly, these recombinants encode all or part of the wild-type pp31 protein fused to the *Escherichia coli* GUS protein, pp31-GUS fusion proteins with mutations in the pp31 BRs, or nonfused GUS, and expression of each of these

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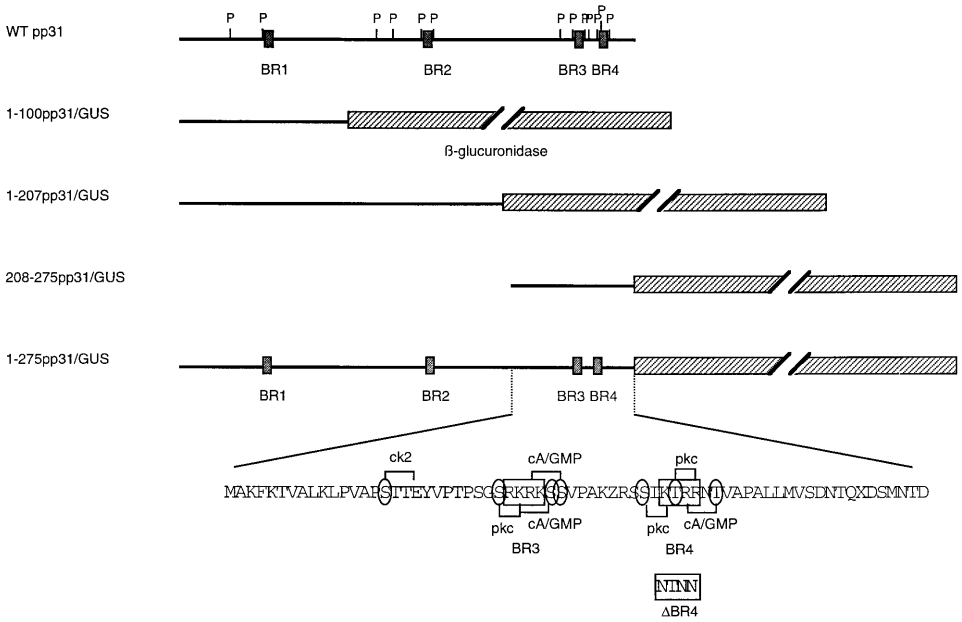

FIG. 1. Structures of pp31 and pp31-GUS fusion proteins. The thin line represents pp31, the hatched box represents GUS, and the shaded boxes represent BR 1 through BR4. In the drawing at the top of the figure, the consensus kinase recognition sites in pp31 are identified by the letter P. In the drawing at the bottom of the figure, the kinase recognition sites within the C-terminal region of pp31 are bracketed, the predicted serine and threonine phosphorylation sites are circled, BR3 and BR4 are boxed, and the mutation (*dl*BR4) used to delete the consensus threonine phosphorylation sites in the C-terminal region of pp31 is shown underneath. WT, wild type.

proteins is controlled by the polyhedrin promoter. The pp31-GUS fusion proteins, the consensus phosphorylation sites and BRs in pp31, and the C-terminal region of pp31 are shown diagramatically in Fig. 1. All baculoviruses used in this study were propagated and their titers were determined by plaque assay in Sf9 cells, as described previously (30, 37).

Metabolic labeling and biochemical fractionation. Sf9 cells were infected at a multiplicity of infection of 5 PFU per cell and labeled at various times postinfection with 100 μCi of [^{35}S]Translabel (about 80% [^{35}S]methionine and 20% [^{35}S]cysteine; ICN Radiochemicals, Irvine, Calif.) per ml or 200 μCi of $^{32}P_i$ (ICN) per ml. For some experiments, Sf9 cells were infected, incubated, and radiolabeled (18 to 24 h postinfection) in the presence of 5 μg of aphidicolin per ml. Cytosolic and nuclear fractions were prepared by the detergent-free fractionation method of Jarvis et al. (15), except that NaOH was omitted from the sample extraction buffer and equivalent samples of cytosolic and nuclear fractions were analyzed without acetone precipitation of the cytosolic fraction. Unless otherwise indicated, phosphatase inhibitors (0.2 mM sodium orthovanadate, 30 mM sodium PP_i, 50 mM NaF, and 1 mM EDTA) were included in all buffers used during subcellular fractionations, extractions, and immunoprecipitations. Samples of each fraction were immunoprecipitated with rabbit polyclonal anti-pp31 (11, 16), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20), and subjected to phosphoamino acid compositional analysis as described below.

pp31 was transiently expressed in Sf9 cells by calcium phosphate-mediated cotransfection with pBD11 (5; renamed p39K in this paper) and pAcIE1 (12) as described previously (30, 37). The transfected cells were labeled from 24 to 28 h posttransfection, separated into nuclear and cytosolic fractions, immunoprecipitated with anti-pp31, and separated by SDS-PAGE, as described above.

Phosphoamino acid compositional analysis. Phosphoamino acid analysis was performed by a modification of the procedure described by Kamps (17). At 24 h postinfection, $^{32}P_i$-labeled cells were washed from tissue culture dishes, pelleted, and divided into nuclear and cytosolic fractions, as described above, or resuspended directly in immunoprecipitation wash buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 500 kallikrein-inactivating units of aprotinin per ml). Extracts were triturated through a 27-gauge needle to shear genomic DNA, immunoprecipitated with anti-pp31, and separated by SDS-PAGE. The precipitated proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, Mass.) (39), and the membranes were rinsed three times with water, dried, and exposed to film. The radiolabeled proteins of interest were excised from the membrane, and the membrane slices were rewetted with methanol. The membrane pieces were then submerged in 6 N HCl, flushed with N_2, and hydrolyzed at 110°C for 2 h. The membrane slices were subsequently removed, and the hydrolysates were dried in a Speed Vac model A160 concentrator (Savant Instruments, Inc., Farmingdale, N.Y.) and resuspended in pH 1.9 buffer (glacial acetic acid, formic acid, water [4:1:45, vol/vol/vol], 0.3 mM EDTA). The hydrolysates were mixed with phosphoamino acid standards, spotted onto cellulose thin-layer chromatography plates, and separated by electrophoresis in the first dimension in pH 1.9 buffer and in the second dimension in pH 3.5 buffer (glacial acetic acid, pyridine, water [10:1:89, vol/vol/vol], 0.3 mM EDTA). Phosphoamino acid standards were visualized by spraying the plates with a 0.25% (wt/vol) solution of ninhydrin in acetone, incubating them at 42°C for 15 min; they were then dried and exposed to film.

RESULTS

Time course of pp31 synthesis and phosphorylation. A previous study had shown that pp31 is phosphorylated, accumulates in the virogenic stroma, and binds to double-stranded DNA (11). However, no further information was available on the timing of pp31 phosphorylation during infection or on the nature of the kinases that modify pp31. To begin to address these questions, we examined the time course of pp31 synthesis and phosphorylation during Ac*M*NPV infection. Sf9 cells were infected with wild-type Ac*M*NPV, pulse-labeled with either [^{35}S]Translabel or $^{32}P_i$ at different times postinfection, separated into nuclear and cytosolic fractions, and immunoprecipitated with rabbit polyclonal anti-pp31. SDS-PAGE analysis of the immunoprecipitates showed that pp31 was synthesized and phosphorylated throughout infection (Fig. 2A). However, phosphorylation of pp31 appeared to lag slightly behind synthesis, because labeling with ^{35}S was first detected at 0 to 4 h postinfection whereas labeling with $^{32}P_i$ was first detected at 4 to 8 h postinfection. Previously, it had been reported that $^{32}P_i$-labeled pp31 was detected only in the nuclear fraction (11, 18), but we clearly detected phosphorylated pp31 in both the cytosolic and nuclear fractions in this study (Fig. 2B). This apparent discrepancy can be explained by the fact that phosphatase inhibitors were included in the extraction buffers in this but not in the previous studies. Several proteins coprecipitated with nuclear pp31 at later times of infection, and at least one of these was heavily phosphorylated. None of these pro-

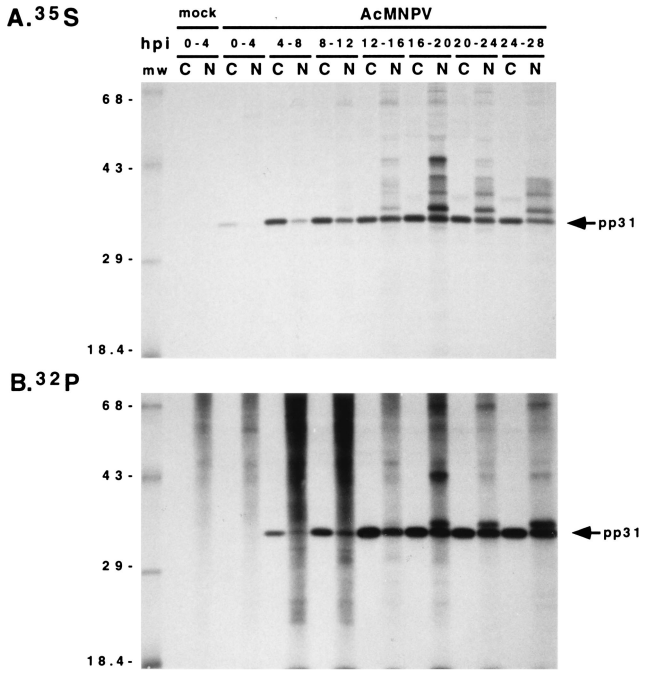

FIG. 2. Time course of pp31 biosynthesis and phosphorylation. Ac*M*NPV- or mock-infected Sf9 cells were metabolically labeled at the indicated times postinfection and separated into nuclear (N) and cytosolic (C) fractions. The fractions were immunoprecipitated with anti-pp31, the immunoprecipitates were separated by SDS-PAGE (12% acrylamide), and the gels were dried and exposed to film. In both panels, the numbers on the left indicate the molecular sizes (in kilodaltons) of protein standards and the arrow on the right marks the position of pp31. (A) Cells labeled with [^{35}S]Translabel. (B) Cells labeled with $^{32}P_i$.

teins was recognized by anti-pp31 in immunoblots (data not shown), and their identity is unknown.

Phosphoamino acid analysis of the pp31 immunoprecipitated from Ac*M*NPV infected-cell lysates showed that it contained both phosphoserine and phosphothreonine but no detectable phosphotyrosine (Fig. 3).

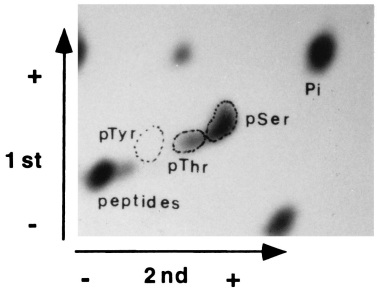

FIG. 3. Phosphoamino acid composition of pp31. Ac*M*NPV-infected Sf9 cells were labeled with 200 μCi of $^{32}P_i$ per ml from 18 to 24 h postinfection, and pp31 was immunoprecipitated from total-cell lysates, resolved by SDS-PAGE (12% acrylamide), transferred to polyvinylidene difluoride membranes, and hydrolyzed as described in Materials and Methods. The hydrolysate was mixed with phosphoamino acid standards, spotted onto a cellulose thin-layer chromatography plate, and separated by high-voltage electrophoresis in two dimensions, as indicated by the arrows. The phosphoamino acid standards were visualized by staining with ninhydrin, and the plate was dried and exposed to film. The positions of the phosphotyrosine (pTyr), phosphoserine (pSer), and phosphothreonine (pThr) standards are circled and labeled. The positions of free phosphate and partial-hydrolysis products are indicated by P_i and peptides, respectively.

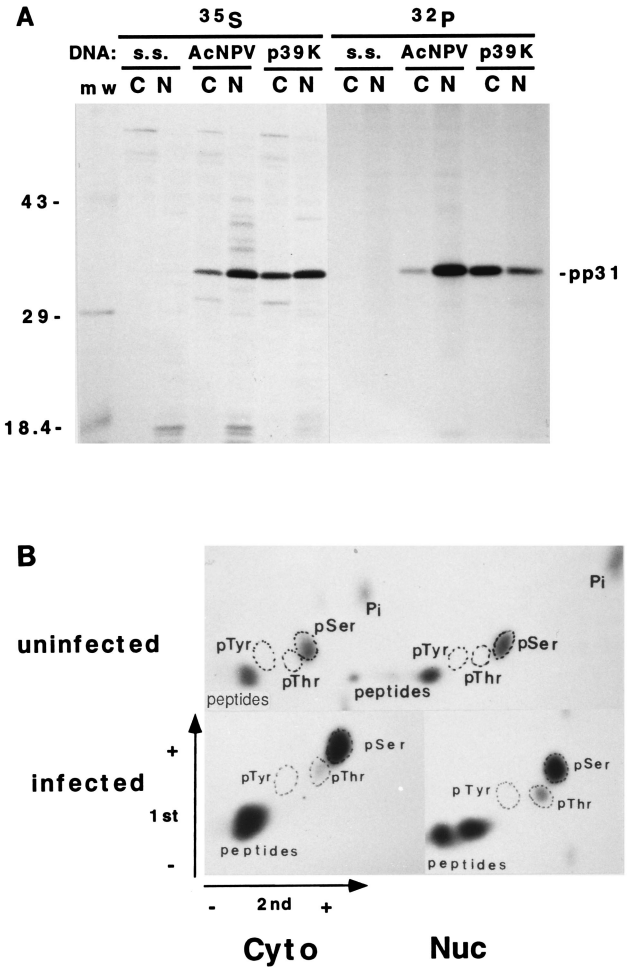

FIG. 4. Differential phosphorylation of pp31 in infected and uninfected cells. Sf9 cells were transfected with Ac*M*NPV DNA (AcNPV), a plasmid encoding pp31 (p39K), or salmon sperm DNA (s.s.) and labeled with [^{35}S]Translabel or $^{32}P_i$ from 20 to 24 h posttransfection. The cells were fractionated, and pp31 was immunoprecipitated from the nuclear (N, Nuc) and cytosolic (C, Cyto) fractions and used for SDS-PAGE and phosphoamino acid compositional analysis as described in the legend to Fig. 3. (A) SDS-PAGE analysis (12% acrylamide). The numbers on the left indicate the molecular sizes (in kilodaltons) of protein standards. The location of the pp31 bands is indicated on the right. (B) Phosphoamino acid analysis of the pp31 bands from panel A.

Differential phosphorylation of pp31 in infected and uninfected cells. Transient-expression assays were used to compare pp31 phosphorylation in uninfected and infected Sf9 cells. Pulse-labeling, radioimmunoprecipitation, and SDS-PAGE analysis showed that pp31 could be labeled with $^{32}P_i$ in uninfected Sf9 cells (Fig. 4A). The proportion of both newly synthesized and phosphorylated pp31 in the cytosolic fraction appeared to be slightly higher in uninfected than in infected cells, but these results were not quantitated. Phosphoamino acid analysis showed that pp31 from uninfected cells contained only phosphoserine (Fig. 4B) whereas pp31 from infected cells contained both phosphoserine and phosphothreonine (Fig. 4B), as before (Fig. 3). It is difficult to see the phosphothreonine spot in the cytosolic pp31 sample from infected cells in Fig. 4B, but we know that this subpopulation contains phosphothreonine, because it was seen very clearly in other experiments (Fig. 5 and data not shown). The radiolabeling results shown in Fig. 2B and 4A clearly indicated that pp31 is phos-

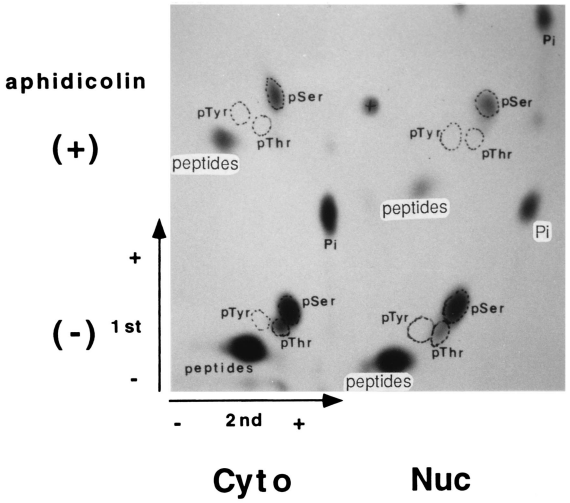

FIG. 5. Influence of late viral gene expression on phosphorylation of pp31. Sf9 cells were infected with Ac*M*NPV and radiolabeled with 200 μCi of $^{32}P_i$ per ml from 18 to 24 h postinfection in the presence (+) or absence (−) of aphidicolin. pp31 was isolated from the cytosolic (Cyto) and nuclear (Nuc) fractions and used for phosphoamino acid analyses as described in the legend to Fig. 3.

phorylated in both infected and uninfected Sf9 cells, suggesting that it must be phosphorylated by a cellular kinase(s). Furthermore, the difference in the phosphoamino acid compositions of pp31 from uninfected and infected cells suggests that pp31 is additionally phosphorylated by a virus-encoded or -induced kinase.

Since progression from the early to the late phase of Ac*M* NPV infection can be blocked by inhibiting DNA synthesis, we examined the phosphoamino acid compositions of pp31 expressed in the presence or absence of aphidicolin. The results showed that pp31 produced in aphidicolin-treated cells lacked phosphothreonine, which indicates that this modification of pp31 requires late viral gene expression (Fig. 5). It is not clear from these data, however, whether the late viral gene product(s) influences threonine phosphorylation of pp31 directly or indirectly.

Dephosphorylation of pp31 in infected and uninfected cells. Ac*M*NPV encodes a protein tyrosine/serine-threonine phosphatase (19, 35), and it was of interest to determine whether pp31 is a target for this phosphatase activity. Ac*M*NPV-infected Sf9 cells were labeled with $^{32}P_i$, divided into cytosolic and nuclear fractions, and immunoprecipitated in the presence or absence of phosphatase inhibitors. In the absence of phosphatase inhibitors, phosphorylated pp31 was detected only in the nuclear fraction (Fig. 6A), as reported previously (11, 18). By contrast, $^{32}P_i$-labeled pp31 was seen in both fractions when the cells were fractionated and immunoprecipitated in the presence of phosphatase inhibitors (Fig. 6A). Phosphoamino acid analyses of pp31 showed that pp31 isolated in the absence of phosphatase inhibitors contained only phosphoserine whereas pp31 prepared in the presence of inhibitors contained both phosphoserine and phosphothreonine (Fig. 6B). These results indicated that phosphorylated amino acids in cytosolic pp31 and phosphorylated threonines in nuclear pp31 are sensitive to dephosphorylation by a phosphatase activity in Ac*M* NPV-infected cells.

Analysis of $^{32}P_i$-labeled pp31 expressed in uninfected cells showed that phosphorylated pp31 could be detected in both the cytosolic and nuclear fractions, irrespective of the addition of phosphatase inhibitors (Fig. 6C). pp31 isolated from uninfected cells always contained phosphoserine but never contained phosphothreonine or phosphotyrosine (Fig. 4B and data not shown). These data suggest that the phosphothreonine(s) on pp31 is reversibly phosphorylated by virus-encoded or -induced kinase and phosphatase activity(ies).

Phosphorylated domains in pp31. pp31 was analyzed with the Motifs subroutine of the University of Wisconsin Genetics Computer Group package (10). Thirteen consensus kinase recognition sites were identified, most of which were clustered around BR1 to BR4 in pp31 (Fig. 1). To begin to map the phosphorylation sites in pp31, we examined the phosphoamino acid compositions of several pp31-GUS fusion proteins (5). Sf9 cells were infected with recombinant baculoviruses encoding fusion proteins composed of the amino-terminal 100 or 207 amino acids of pp31 fused to GUS, the C-terminal 67 amino acids (208 to 275) of pp31 fused to GUS, or the full-length pp31 protein fused to GUS (shown diagramatically in Fig. 1). A recombinant baculovirus that expresses nonfused GUS was used as a negative control.

When expressed in infected cells, the four fusion proteins were labeled with $^{32}P_i$ but nonfused GUS was not (Fig. 7A), indicating that each fusion protein contained phosphorylation sites derived from pp31. Phosphoamino acid analysis showed that the full-length pp31-GUS fusion, like wild-type pp31, contained both phosphoserine and phosphothreonine (Fig. 7F). The fusion protein containing only the C-terminal 67 amino acids of pp31 also contained both phosphoserine and phosphothreonine (Fig. 7E). However, the fusion proteins containing the first 100 or 207 amino acids of pp31 contained only phosphoserine (Fig. 7C and D). These results suggest that the infected cell-specific threonine phosphorylation site in pp31 is located within its C-terminal 67 amino acids. These results also suggest that the N-terminal 100 amino acids and C-terminal 67 amino acids of pp31 each contain at least one serine phosphorylation site.

The computer analysis of potential kinase recognition sites within pp31 had identified four putative threonine-containing phosphorylation sites at Thr-120, Thr-150, Thr-252, and Thr-256 (Fig. 1). From this analysis, we predicted that Thr-252 and/or Thr-256 might be the residue(s) phosphorylated by the virus-encoded or -induced kinase during Ac*M*NPV infection. We tested this hypothesis by analyzing the phosphoamino acid compositions of various pp31-GUS fusion proteins containing mutations in BR4. BR4 overlaps the consensus kinase recognition sites for Thr-252 and Thr-256, and the mutations in BR4 eliminate both of these sites. Interestingly, 208–275pp31/GUSΔBR4 still contained both phosphoserine and phosphothreonine (Fig. 8G). This result suggests that the virus-encoded or -induced kinase which phosphorylates the threonine(s) within the C-terminal 67 amino acids of pp31 is a novel kinase with a unique recognition site.

Analysis of additional pp31-GUS fusion proteins with BR mutations that altered consensus serine phosphorylation sites showed that 1–100pp31/GUSΔBR1, 1–207pp31/GUSΔBR1, 1–207pp31/GUSΔBR2, and 1–207pp31/GUSΔBR1/2 all contained phosphoserine (Fig. 8A to E). Thus, these mutations were unable to conclusively reveal whether any of these sites are used for phosphorylation of pp31.

DISCUSSION

The known properties of the Ac*M*NPV pp31 protein, including its localization in the nucleus and virogenic stroma, its non-sequence-specific DNA-binding activity, and its capability to stimulate the expression of late genes, suggest that it plays an important role in Ac*M*NPV replication (5, 11, 38). Further-

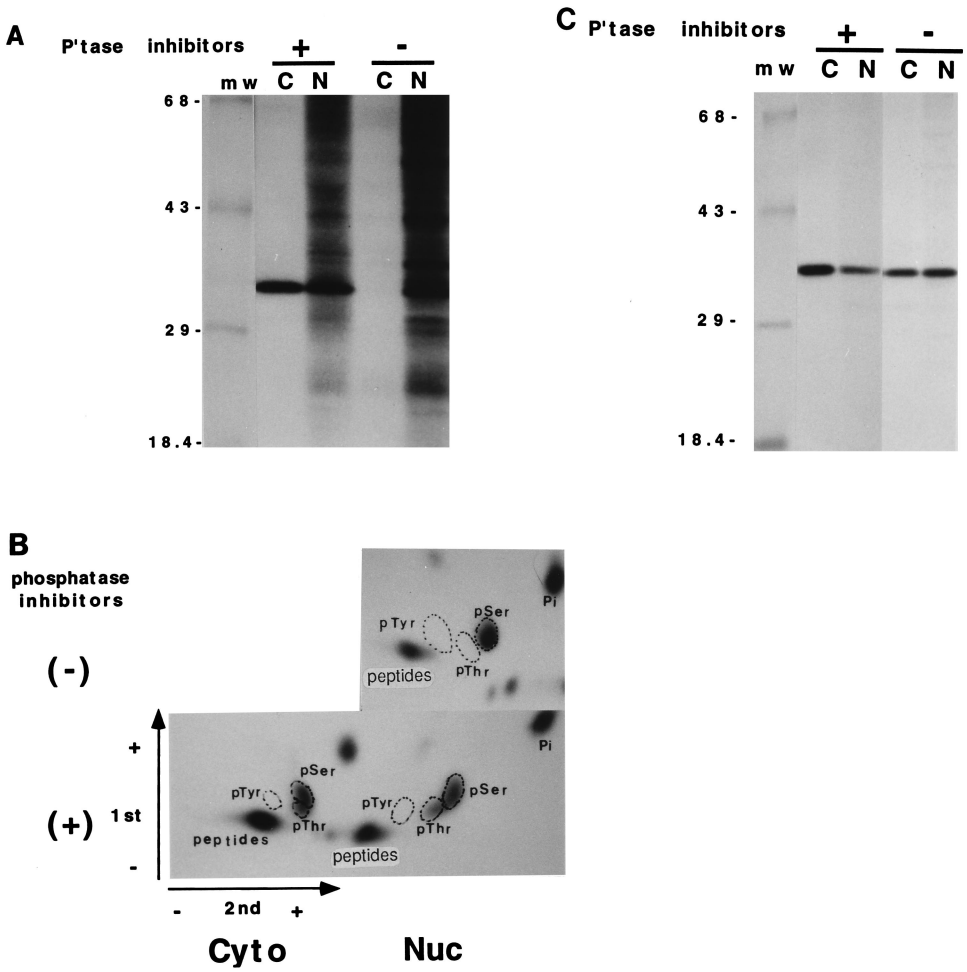

FIG. 6. Dephosphorylation of pp31 in infected and uninfected cells. Sf9 cells were infected with AcMNPV or transfected with a plasmid encoding pp31 and radiolabeled with $^{32}P_i$. The labeled cells were then separated into nuclear (N, Nuc) and cytosolic (C, Cyto) fractions, and the fractions were immunoprecipitated with anti-pp31 in the presence (+) or absence (−) of phosphatase (P'tase) inhibitors. pp31 was isolated from each fraction and used for phosphoamino acid analyses, as described in the legend to Fig. 3. (A) SDS-PAGE analysis of pp31 from infected cells. (B) Phosphoamino acid analysis of pp31 bands from panel A. (C) SDS-PAGE analysis of pp31 isolated from transfected cells.

more, pp31 is a phosphoprotein, and since phosphorylation can influence any or all of these properties, it is reasonable to expect that the function of pp31 might be regulated by reversible phosphorylation. To begin to address this possibility, we have examined phosphorylation and dephosphorylation of pp31 in detail.

A time course analysis showed that pp31 is phosphorylated throughout AcMNPV infection, but pp31 synthesis was detectable before phosphorylation, which is consistent with the expectation that phosphorylation is a posttranslational event. The detection of pp31 phosphorylation at early times (4 to 8 h) after infection indicated that it must be modified by a cellular and/or early viral kinase(s). A role for a host cell kinase(s) in the phosphorylation of pp31 was supported by the demonstration that pp31 can be phosphorylated in uninfected Sf9 cells. Also, there was a striking difference in the phosphoamino acid compositions of pp31 produced in uninfected and infected cells. While pp31 from uninfected cells contained only phosphoserine, pp31 from infected cells contained both phosphoserine and phosphothreonine. Thus, pp31 must be additionally modified by a virus-encoded or -induced kinase during AcMNPV infection. No phosphotyrosine was detected in pp31 expressed under any conditions, which is consistent with the results of previous studies showing that other baculovirus proteins contained only phosphoserine and phosphothreonine, not phosphotyrosine (18, 25, 26, 29).

Protein kinase activity has been detected in occluded and budded virions of AcMNPV (26) and in nucleocapsids of *Plodia interpunctella* granulosis virus (43), and kinase activity is induced in cells infected with *Bombyx mori* nuclear polyhedrosis virus (46). A putative serine/threonine protein kinase gene, *pk1*, has been identified in AcMNPV (32) and in *Lymantria dispar* nuclear polyhedrosis virus (3). The AcMNPV *pk1* gene is classified as late or very late, and both viral *pk1* genes encode proteins with in vitro kinase activity. AcMNPV also has a truncated protein kinase gene, *pk2*, which is classified as an early gene (22). Thus, there is substantial evidence that baculoviruses encode their own protein kinases. Since threonine phosphorylation of pp31 was detected only in infected cells and was sensitive to aphidicolin, which blocks late-gene expression, it is reasonable to conclude that threonine phosphorylation of pp31 is probably carried out by AcMNPV PK1, which is a late-gene product. It also is possible that AcMNPV PK2 is involved in serine phosphorylation of pp31 early in infection,

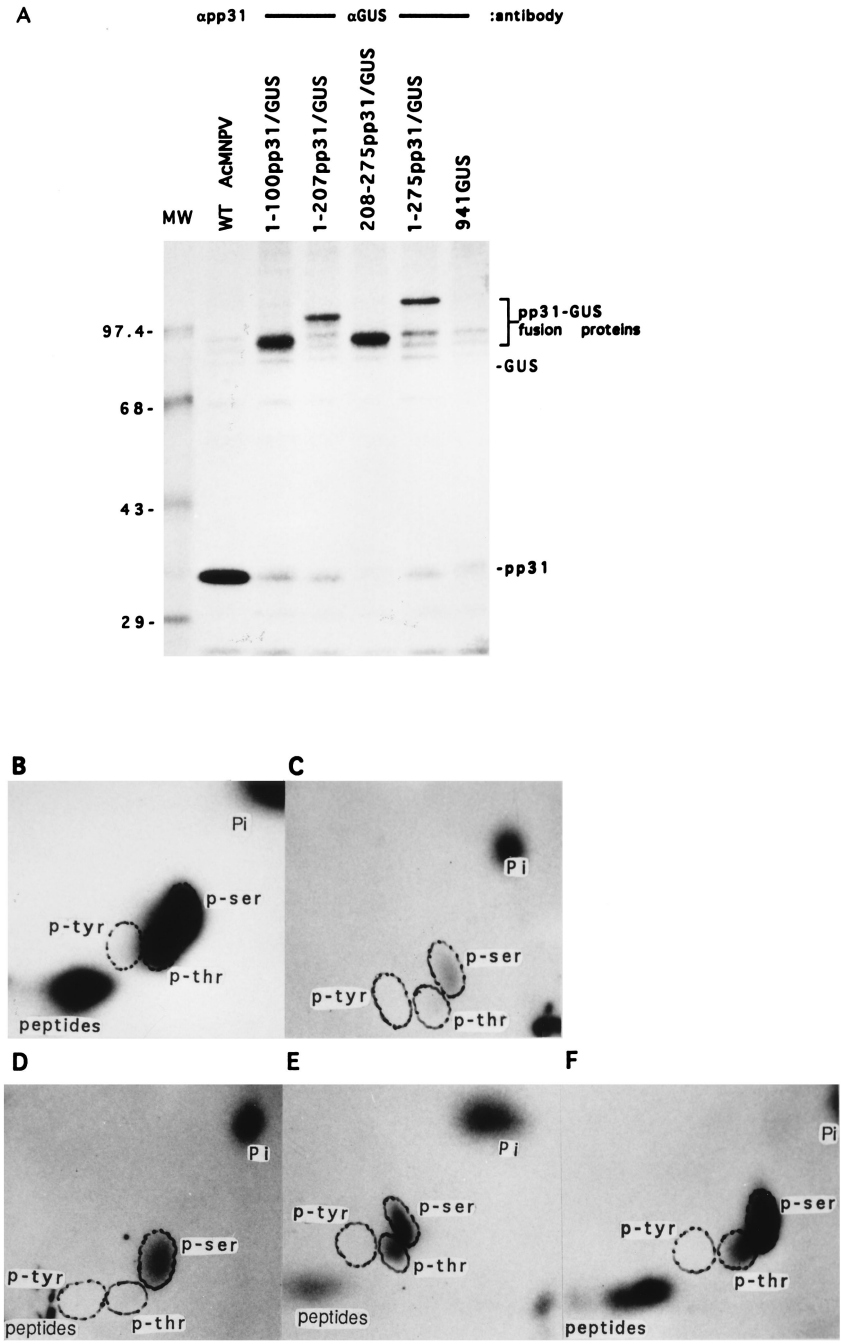

FIG. 7. Mapping phosphorylated domains in pp31. Sf9 cells were infected with wild-type (WT) AcMNPV or recombinant baculoviruses encoding part or all of the pp31 protein fused to GUS. pp31 or pp31-GUS fusion proteins were immunoprecipitated from infected-cell lysates and used for SDS-PAGE and phosphoamino acid analyses as described in the legend to Fig. 3. (A) SDS-PAGE analysis (10% acrylamide). The antisera used for immunoprecipitation and the viruses used for infection are indicated at the top, the numbers on the left indicate the molecular sizes (in kilodaltons) of protein standards, and the locations of pp31, the pp31-GUS fusion proteins, and GUS are indicated on the right. (B to F) Phosphoamino acid compositions of pp31 (B), 1–100pp31/GUS (C), 1–207pp31/GUS (D), 208–275pp31/GUS (E), and 1–275pp31/GUS (F).

but this seems less likely, because PK2 lacks several motifs found in eucaryotic kinases and probably has no kinase activity (22). Definitive tests of these preliminary conclusions will require analysis of the phosphoamino acid composition of pp31 expressed by Ac*M*NPV mutants lacking functional *pk1* and *pk2* genes.

Ac*M*NPV also encodes a protein tyrosine/serine-threonine phosphatase (19, 35). Since the phosphothreonine in pp31 is sensitive to dephosphorylation in Ac*M*NPV-infected but not uninfected Sf9 cells, it is reasonable to conclude that the phosphothreonine in pp31 can be dephosphorylated by the virus-encoded protein tyrosine/serine-threonine phosphatase. A subpopulation of the phosphoserines in pp31 also was sensitive to dephosphorylation by a virus-encoded or -induced phos-

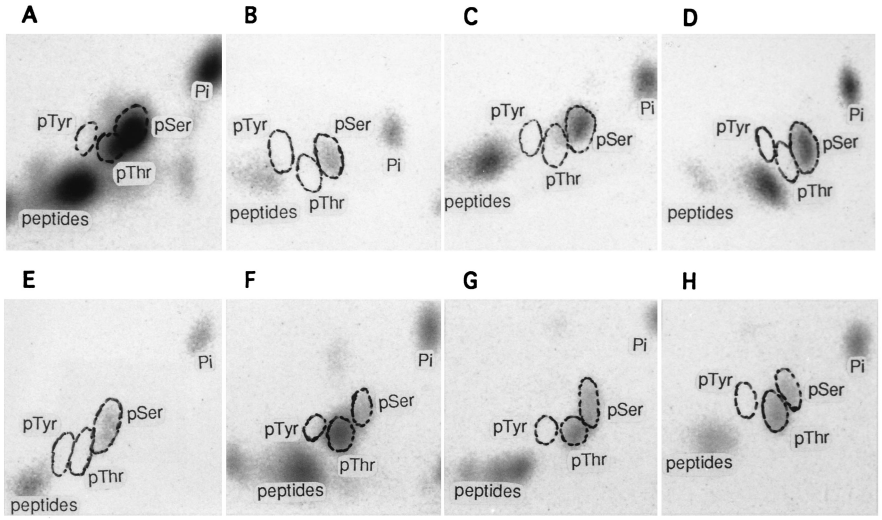

FIG. 8. Effects of BR mutations on phosphorylation of pp31. Sf9 cells were infected with wild-type AcMNPV or recombinant baculoviruses encoding pp31-GUS fusion proteins with various BR mutations. pp31 or the pp31-GUSΔBR fusion proteins were isolated from total lysates of infected cells and used for phosphoamino acid analyses as described in the legend to Fig. 3. (A) pp31; (B) 1–100pp31/GUSΔBR1; (C) 1–207pp31/GUSΔBR1; (D) 1–207pp31/GUSΔBR2; (E) 1–207pp31/GUSΔBR1/2; (F) 208–275pp31/GUSΔBR3; (G) 208–275pp31/GUSΔBR4; (H) 208–275pp31/GUSΔBR3/4.

phatase, because the cytosolic fraction of pp31 from infected cells lacked detectable phosphoserine. Overall, these results lead us to conclude that phosphorylation of pp31 is a dynamic process that is governed, at least in part, by a virus-encoded or -induced kinase(s) and phosphatase(s). However, the functional significance of these reversible phosphorylation events, if any, is unclear.

To begin to map the phosphorylation sites in pp31, we expressed various pp31-GUS fusion proteins in recombinant baculovirus-infected Sf9 cells, determined whether they could be labeled with $^{32}P_i$, and examined their phosphoamino acid compositions. Fusion proteins containing amino acids 1 to 100, 1 to 207, or 208 to 275 of pp31 could be labeled with $^{32}P_i$. The fusion proteins containing amino acids 1 to 100 or 1 to 207 of pp31 contained only phosphoserine, and the former fusion protein also could be $^{32}P_i$ labeled in uninfected Sf9 cells (data not shown). By contrast, fusion proteins containing full-length pp31 or amino acids 208 to 275 of pp31 contained both phosphoserine and phosphothreonine. Together, these results indicated that pp31 is phosphorylated on at least three sites. Amino acids 1 to 100 and 208 to 275 of pp31 each include at least one serine phosphorylation site that can be phosphorylated by a cellular kinase. Amino acids 208 to 275 also includes a threonine phosphorylation site that can be phosphorylated by a virus-encoded or -induced kinase. Interestingly, mutations within the two consensus threonine kinase recognition sites within amino acids 208 to 275 failed to prevent threonine phosphorylation. This suggests that one or more of the six additional threonines in this region of pp31 must lie within an undefined recognition site that can be phosphorylated by the virus-encoded or -induced kinase, perhaps PK1. Mutagenesis of additional threonine residues in this region of pp31 should allow us to identify the virus-specific phosphorylation site(s).

From the results of this study, we propose a working model to describe how phosphorylation might influence the activity of pp31. This model states that pp31 is initially synthesized as an unphosphorylated precursor ($pp31_0$), which is posttranslationally phosphorylated by a cellular kinase(s) that recognizes at least one serine phosphorylation site within amino acids 1 to 100 and produces a partially phosphorylated intermediate, $pp31_1$. During the late stage of AcMNPV replication, $pp31_1$ is additionally phosphorylated by PK1, which recognizes at least one threonine residue in a novel recognition site within amino acids 208 to 275 of pp31 and produces $pp31_2$. Phosphorylation of a threonine(s) and some serines in pp31 is dynamic, subject to reversal by the action of the AcMNPV protein tyrosine/serine-threonine phosphatase. At some point, cellular and/or viral kinases must phosphorylate at least one additional serine located within amino acids 208 to 275 of pp31.

Previous studies have demonstrated that reversible phosphorylation can modulate the functions of proteins with key roles in viral replication and gene expression. For example, the rate of nuclear localization (33) and the DNA-binding specificity (6, 31, 34) of simian virus 40 large T antigen are regulated by phosphorylation. Phosphorylation of the phosphoprotein (p) subunit of the vesicular stomatitis virus RNA polymerase by both cellular and viral kinases is required for transcriptional activation (2). Although this study did not reveal what influence, if any, phosphorylation and dephosphorylation might have on any of the known properties of pp31, it seems likely that these modifications will have functional significance, and future studies will be designed to test this working hypothesis.

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