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Phosphorylation of the Duck Hepatitis B Virus Capsid Protein Associated with Conformational Changes in the C Terminus

MINSHU YU AND JESSE SUMMERS*

Department of Cell Biology, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131

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The capsid protein of duck hepatitis B virus (DHBV) is phosphorylated at multiple sites during viral infection. A cluster of sites is located near the C terminus of the 262-amino-acid protein. We have used site-directed mutagenesis to show that three serines and one threonine serve as phosphate acceptor amino acids in the C terminus. An additional six potential phosphate acceptor sites in this region were apparently not utilized. Each serine or threonine that served as a phosphate acceptor was adjacent to a downstream proline, while all six serines that were not acceptors for phosphate residues lacked adjacent downstream prolines. Mutation of the downstream proline to glycine at each site had the same effect as mutating the serine itself, suggesting an SP or TP motif as an essential feature for capsid protein phosphorylation. Phosphorylation at these four sites resulted in complex shifts in electrophoretic mobility in sodium dodecyl sulfate gels of the capsid protein or of a C-terminal peptide containing the phosphorylated sites, suggesting that specific conformations of the C terminus are associated with different combinations of phosphorylated serines. We speculate that distinct functions of the C terminus may be associated with different phosphorylated domains on the intact capsid.

Hepadnaviruses make up a small group of DNA-containing viruses that replicate their DNA genomes in hepatocytes through the transcription of genomic RNAs called pregenomes (6, 10). Pregenomes are transported to the cytoplasm and together with a DNA polymerase are packaged into nucleocapsids and reverse transcribed to produce a single-stranded cDNA (minus strand). Minus-strand DNA is then copied by the endogenous DNA polymerase to produce eventually a double-stranded relaxed circular DNA. Nucleocapsids containing double-stranded viral DNA are assembled into viral envelopes and exported from the cell. The assembly of nucleocapsids into enveloped virus particles appears to depend on completion of the relaxed circular form of viral DNA, since (i) only those nucleocapsids containing mature DNA are found as extracellular particles (11, 12, 14) and (ii) mutations in the capsid protein that specifically prevent DNA maturation also prevent enveloped virus assembly (14).

The nucleocapsid of duck hepatitis B virus (DHBV) is assembled from a polypeptide species of 262 amino acids. Previous studies have revealed that this capsid protein can exist in a phosphorylated form, with phosphoserines or phosphothreonines (9) exposed on the capsid surface (7). Immature, i.e., intracellular, nucleocapsids seem to differ from mature nucleocapsids isolated from extracellular enveloped virus in the level of phosphorylation of these serines and threonines (7). In particular, it was observed that capsid protein from intracellular nucleocapsids displays electrophoretic heterogeneity in sodium dodecyl sulfate (SDS)-polyacrylamide gels as a result of differing states of phosphorylation (7, 9), while that from mature capsids isolated from extracellular virus particles is electrophoretically homogeneous (7). The heterogeneity of capsid protein from intracellular capsids can be eliminated by treatment of the intact capsids with alkaline phosphatase (7). These results led us to conclude that the capsid protein in intact virus particles is not phosphorylated or is underphosphorylated.

Schlicht et al. showed that phosphorylation sites of the capsid protein are located at the C terminus of the polypeptide (9). We have tested the ability of each of nine serines and one threonine in the C terminus of the DHBV capsid protein to be phosphorylated, as assayed by electrophoretic mobility changes, and have investigated the effects of preventing phosphorylation at each site by mutation of the potential acceptor from serine or threonine to alanine. The results indicate that phosphorylations occur at three serines and one threonine in the C terminus. All phosphorylation sites that we identified consisted of a serine or threonine followed by an adjacent proline, and phosphorylation at these sites was sensitive to mutation of the adjacent proline to glycine.

A hierarchy of effects of phosphorylation at different sites was observed, such that mutation of a single serine codon was sufficient to prevent the appearance of all electrophoretic variants. This effect, however, was due not to inhibition of phosphorylation at the other three sites but to differences in the contribution of each phosphoserine to the magnitude of phosphorylation-induced electrophoretic shifts.

MATERIALS AND METHODS

Plasmids. DHBV capsid proteins were expressed from plasmid pCMV/core/1S, which identical to one that we previously described (14) except that stop codons introduced into the envelope gene at by T→A mutations at positions 1327, 1346, and 1349 (numbered according to Mandart et al. [5]) prevented the production of functional large and small envelope proteins. The phenotype of this envelope mutation has been described previously (11, 12).

Site-directed mutagenesis. Single nucleotide substitutions at specified sites in the DHBV capsid gene were constructed by oligonucleotide-directed mutagenesis (13) by the method of Kunkel et al. (3) exactly as described previously (12). Synthetic

* Corresponding author. Mailing address: Department of Cell Biology, University of New Mexico, School of Medicine, 900 Camino de Salud N.E., Albuquerque, NM 87131. Phone: (505) 277-7979.

oligonucleotides 20 to 25 nucleotides in length were designed to cause mutations of either serine/threonine to alanine or proline to glycine. Mutant clones were identified by sequencing using Sequenase 2.0 as instructed by the supplier (United States Biochemical Corp.). Fully sequenced, mutated restriction fragments were subcloned into an otherwise wild-type expression vector in order to exclude second-site mutations.

Transfection. Capsid proteins were characterized after transient expression following DNA transfection into a chicken hepatoma cell line, LMH (2). The calcium phosphate method that we used for transfection has been previously described (12). Incubation of the calcium phosphate precipitate with the cells was for 16 to 20 h at 37°C, at which time the cells were washed with normal growth medium (F10–Dulbecco modified Eagle medium with 10% fetal bovine serum) and refed. Incubation times described below begin with the time at which DNA was added to the cells.

Extraction and analysis of capsid proteins. Transfected LMH cells were lysed 4 days following transfection by the addition of 0.5 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Nonidet P-40). The nuclei were removed by microcentrifuge centrifugation, and 5 μl of supernatant was heated at 80°C for 3 to 5 min in Laemmli loading buffer. Samples were analyzed by electrophoresis through 10% polyacrylamide gels as specified by Laemmli (4), and proteins were electrotransferred to nitrocellulose membranes (Hybond C; Amersham, Inc.). Capsid protein was detected by radioimmunostaining using a polyclonal rabbit antiserum against the capsid protein and ^{125}I-protein A (New England Nuclear) as previously described (12).

Metabolic labeling of phosphorylated capsid proteins with $^{32}P_i$. Sixty-millimeter-diameter dishes of LMH cells were transfected with capsid expression vector DNA and after 24 h were washed once with labeling medium (phosphate-free minimal essential Eagle's medium containing 10% dialyzed fetal bovine serum). Cells were then incubated in 4 ml of labeling medium containing $^{32}P_i$ (25 μCi/ml; specific activity, 9,000 Ci/mmol) at 37°C. After 24 h, the cells were washed free of labeled medium and stored at −70°C before analysis.

Immunoprecipitation. Capsids were immunoprecipitated from extracts of transfected cells by using a rabbit polyclonal antiserum against the capsid protein and protein A-Sepharose CL-4B (Pharmacia). Lysates (1 ml) were prepared as described above and adjusted to 0.1% SDS. Five microliters of antiserum was adsorbed overnight at 4°C to 25 μl (packed volume) of protein A-Sepharose, preswelled in lysis buffer, and washed three times with lysis buffer containing 0.1% SDS. The beads were blocked first by the addition of nontransfected cell lysate and overnight incubation at 4°C. The blocked beads were again washed three times and incubated with the labeled transfected cell lysate as described above. Nonadsorbed proteins were removed by further washing with SDS-containing lysis buffer. Proteins were extracted from the beads by the addition of 50 μl of Laemmli loading buffer and heating at 80°C for 3 to 5 min.

V8 protease digestion. Immunoprecipitated proteins were purified by conventional SDS-polyacrylamide gel electrophoresis (PAGE). The capsid protein bands were visualized by staining with Coomassie blue and excised from the gel. Protein was eluted by crushing the gel slice and heating it at 96°C with two successive 1.0-ml portions of elution buffer (50 mM ammonium bicarbonate, 5% 2-mercaptoethanol, 0.1% SDS) followed by overnight incubation at 37°C. Proteins were precipitated in 10% trichloroacetic acid at 4°C for 1 h and collected by microcentrifuge centrifugation. The pellets were washed with acetone, dried, and then dissolved in V8 protease digestion buffer (50 mM sodium phosphate buffer [pH 7.8], 0.5% Nonidet P-40, 0.5% Sarkosyl). V8 protease (Sigma) was added at a concentration of 50 to 100 μg/ml, and the mixture was incubated at 37°C for 8 to 12 h. We observed a strong dependence of the digestion pattern on the detergent used in the digestion buffer. Sarkosyl alone generally resulted in a C-terminal digestion product of about 6 kDa, and digestions containing SDS were inconsistent.

Two-dimensional gel electrophoresis of phosphopeptides. Digested samples were adjusted to 4 M urea and 0.1 M HCl and layered on a 10% polyacrylamide gel containing 0.9 M acetic acid (pH 3.0) and 2 M urea, preelectrophoresed to remove ammonium persulfate. Electrode buffer contained 0.9 M acetic acid. Electrophoresis of the samples toward the cathode was carried out for 4 h at 180 V. Individual lanes were excised and subjected to electrophoresis in a second dimension through a 12% polyacrylamide gel containing SDS, using the Tricine discontinuous buffer system of Schagger and von Jagow (8). Electrophoresis was carried out at 27 mA for 16 h, until the ion front reached the bottom of the gel. Two-dimensional gels were fixed in 50% methanol–10% acetic acid and dried for autoradiography. Samples analyzed in one dimension in either acid-urea- or SDS gels were adjusted with the appropriate loading buffer and loaded on the gel.

RESULTS

The C terminus of the DHBV capsid protein contains nine serines and one threonine as candidate phosphorylation sites. To assess the degree to which phosphorylation at each site contributed to the complexity of the electrophoretic pattern, we mutated each corresponding codon in the capsid gene independently to encode alanine, thus preventing phosphorylation at that site. A summary of the mutants constructed is shown in Fig. 1A. Mutated regions of the capsid gene were sequenced and cloned into a capsid protein expression vector in which transcription is driven by the immediate-early cytomegalovirus promoter as previously described (14). The expression vector was either transfected into LMH cells either alone or cotransfected with a plasmid which expressed a DHBV pregenome defective in production of the capsid protein and dependent on the capsid protein supplied in *trans* for viral DNA synthesis (1).

Phosphorylation at four SP/TP motifs. Mutant capsid proteins analyzed by SDS-PAGE and Western immunoblotting displayed various degrees of electrophoretic heterogeneity relative to the wild-type protein, depending on the site of mutation (Fig. 1B and C). Mutations at sites 230, 232, 250, 251, 252, and 253 produced no detectable change in the pattern of capsid proteins, while mutations at threonine 239, serine 245, serine 257, and serine 259 resulted in reductions in the degree of complexity of the pattern, suggesting that these amino acids serve as phosphate acceptors. The most profound effect was seen with the mutant S259A protein, in which a single electrophoretic species was observed comigrating with the most rapidly migrating species present in the wild-type pattern. Individual upstream mutations of serine 257, serine 245, and threonine 239 resulted in progressively more complex patterns with electrophoretic mobility shifts of greater magnitude.

A common feature of each putative phosphorylation site was the presence of a proline immediately downstream of the acceptor amino acid. Sites that were not apparently phosphorylated, as measured by the gel shift assay, are lacking prolines in this relative position. To determine whether these prolines were required elements in recognition of the active phosphorylation sites by the responsible protein kinase(s), we mutated each of the four relevant prolines to glycine (Fig. 2A). The

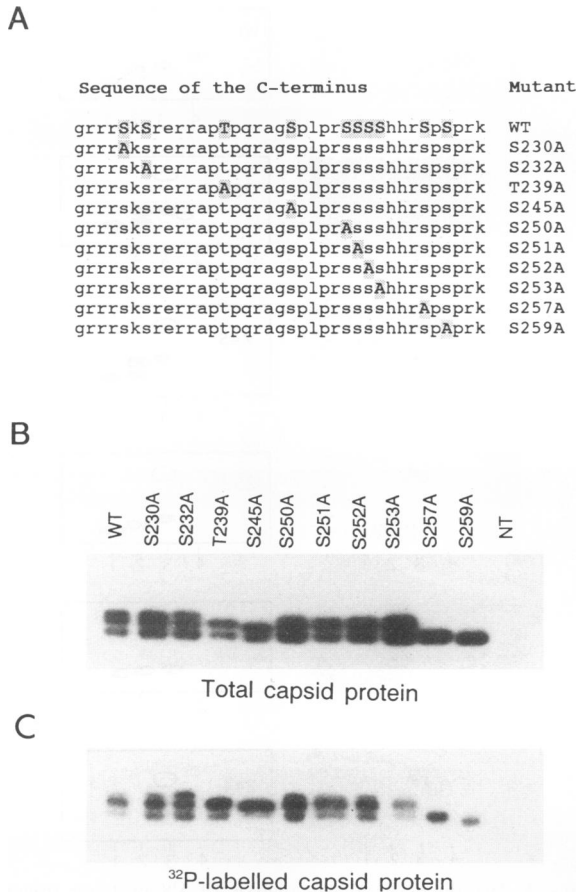

FIG. 1. Effects of serine- or threonine-to-alanine substitutions in the C terminus of the DHBV capsid protein. (A) Sequences of the C-terminal 37 amino acids of the wild-type (WT) capsid protein and various mutant proteins. Mutants are named according to the amino acid change resulting from the mutation. (B and C) Analyses of capsid proteins from the various mutants in panel A by SDS-PAGE. (B) Each lane corresponds to 1/50 of the capsid protein produced by transfection of a 60-mm-diameter tissue culture dish of LMH cells and detected by Western blotting. The faster-moving protein band is due to cross-reactivity of the antiserum with a cellular protein. (C) Capsid proteins labeled in vivo with $^{32}P_i$ and isolated by immunoprecipitation. Each lane contains one-third of the labeled protein isolated from a 60-mm-diameter tissue culture dish of LMH cells. NT, not transfected.

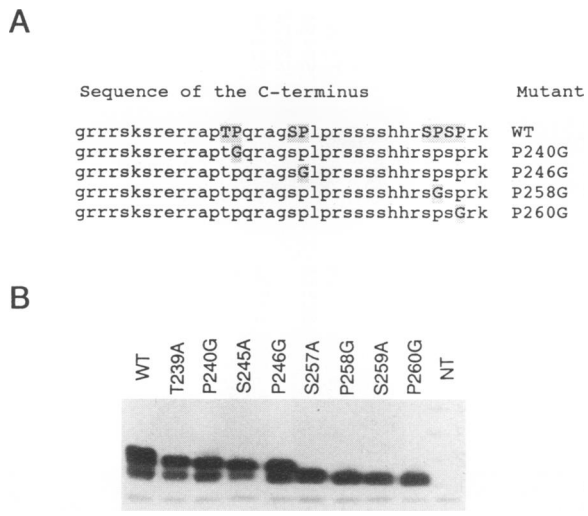

FIG. 2. Effect of proline-to-glycine substitutions in the C terminus of the DHBV capsid protein. (A) Sequences of the C-terminal 37 amino acids of the wild-type (WT) and mutant capsid proteins, with mutants designated as in Fig. 1A. (B) Capsid proteins from mutants substituted at either the serine or proline residue of the four SP motifs, analyzed by SDS-PAGE and Western blotting. NT, not transfected.

resulting effect on phosphorylation appeared to be identical to that observed when the acceptor amino acid itself was mutated (Fig. 2B). Thus, the SP or TP motif appeared to be an essential part of the mechanism by which the four amino acid residues were selected for phosphorylation.

Effects of single serine mutations on ^{32}P labeling of the capsid protein. Since the hierarchy of effects caused by mutations of the phosphorylation sites might be explained by a cascade in which phosphorylation of each serine would have been dependent on prior phosphorylation of the adjacent C-terminal serine, we tested the effects of serine mutations on the total ^{32}P incorporation in the capsid protein. Wild-type and mutant proteins were metabolically labeled with [^{32}P]phosphate, and the capsid proteins were recovered from the cell lysates by immunoprecipitation. The results of gel electrophoresis and autoradiography showed patterns similar or identical to those detected by immunostaining (Fig. 1C). Moreover, the absence of heterogeneity in the mutant capsid protein bands did not apparently correspond to a reduction in phosphate labeling. This result suggested that phosphorylations that do not result in mobility changes could occur in the mutant and perhaps in the wild-type capsid protein.

Phosphopeptide analysis of the wild-type and mutant capsid proteins. Digestion of the protein with V8 protease, which cuts specifically to the carboxyl side of glutamyl and aspartyl residues, should result in the release of a 3.8-kDa peptide from the C terminus of the protein. This peptide would contain all of the residues that had been identified as phospho acceptors, and the levels of phosphorylation of this peptide could be directly determined by relative mobilities in acid-urea-gel electrophoresis. We determined conditions for complete release of the predicted C-terminal peptide by V8 protease by digesting a purified recombinant wild-type capsid protein and a protein in which the C-terminal 18 amino acids were deleted (244Δ) and analyzing the V8 protease digestion products on SDS-polyacrylamide gels, with Tricine as the trailing ion in the cathode buffer. We found that while the C-terminal fragment could be quantitatively released by digestion under some conditions, other potential sites for digestion were not cleaved under any conditions tested (data not shown). The C-terminal fragment migrated relative to markers at the expected position, and a set of phosphate-labeled peptides showing the same relative electrophoretic complexities as the various wild-type and mutant capsid proteins was found in this region when the radiolabeled protein was digested and subjected to SDS-PAGE (Fig. 3a). This set of bands was missing in the C-terminal deletion mutant and could therefore be assigned to the wild-type C-terminal peptide. The pattern of phosphorylated species of the C-terminal peptide and the effects of individual serine substitutions seemed identical to those of the intact protein. This result indicated that the components of the protein that resulted in phosphorylation-induced mobility shifts were present in the C-terminal 28 amino acids. In addition, a phosphate-labeled peptide migrating at the position of approximately 6 kDa was present and labeled to approximately the same extent in all samples tested. The presence of

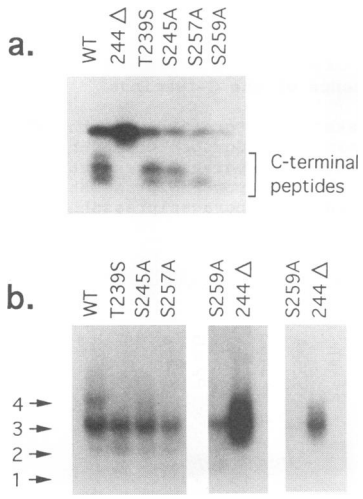

FIG. 3. Effects of alanine substitutions on the mobility of phosphorylated V8 protease-generated fragments of the capsid protein. (a) V8 protease digestion was carried out on ^{32}P-labeled, gel-purified capsid proteins, and the fragments generated were separated by electrophoresis through a 12% polyacrylamide gel in the presence of SDS with Tricine instead of glycine in the cathode buffer chamber (8). The gel was dried and exposed for autoradiography. (b) V8 protease digestion products prepared as described above were separated on a 12% polyacrylamide gel containing 2 M urea and 0.9 M acetic acid (pH 3.0). The mutant 244Δ expressed a capsid protein in which the C-terminal 18 amino acids were deleted, and this protein was used to identify the C-terminal 28-amino-acid fragments derived from the full-length proteins. The gel was dried and exposed for autoradiography. Positions of the C-terminal fragments with phosphorylations at one, two, three, and four of the four sites identified, deduced from the two-dimensional analysis shown in Fig. 4, are indicated on the left. The right-hand panel is a short exposure of the middle panel. WT, wild type.

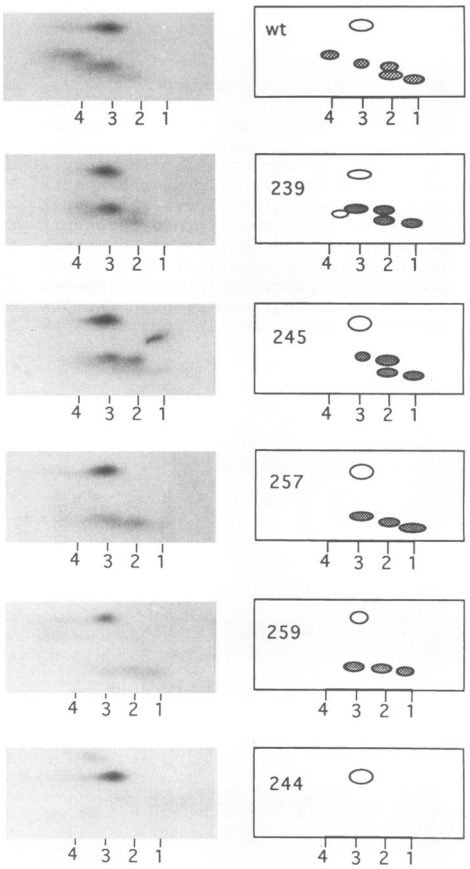

FIG. 4. Two-dimensional gel analysis of V8 protease fragments labeled with [^{32}P]phosphate. Each digested capsid protein was first separated by acid-urea-gel electrophoresis as in Fig. 3b (left to right), and the lane was then excised and subjected to electrophoresis in a second dimension through an SDS-containing gel as in Fig. 3a (top to bottom). Gels were dried and exposed for autoradiography (right-hand panels). The mutant number is indicated in each panel, and tracings of the patterns are shown on the left. The C-terminal fragments are indicated by shading, and the positions of the peptides containing one, two, three, and four phosphates, as identified in Fig. 3, are shown in the bottom scale.

this phosphorylated peptide indicated that one or more sites of phosphorylation were present at an internal position in the capsid protein. This internal phosphorylation site was not present in the adjacent upstream V8 protease fragment (consisting of 21 amino acids), since partial digestion products seen in some digestions (not shown) could be separated from the labeled internal fragment. In addition, the absence of a labeled 21-amino-acid fragment in any of the samples confirmed that serines 230 and 232 were not utilized as phosphorylation sites in the capsid protein.

Analysis of the same digests by electrophoresis through acetic acid-urea-containing polyacrylamide gels revealed the presence of multiple phosphorylated species, migrating at positions determined by their respective charge-to-mass ratios (Fig. 3b). To identify which phosphorylated peptides observed in the acid-urea gels were derived from the C-terminal V8 protease fragment, we subjected the peptides to electrophoresis in a second dimension of SDS-PAGE, using Tricine in the cathode buffer. In this analysis, it was apparent that the C-terminus-derived peptides, identified in the SDS-Tricine dimension, resolved into a set of at least four species based on their total level of phosphorylation (Fig. 4). The four species were judged to contain one, two, three, and four phosphates, respectively, from comparison of their mobilities relative to an unphosphorylated fragment released by V8 protease digestion of capsid protein produced in bacteria (not shown). Although single serine mutations had very different effects on the complexities of the patterns in the SDS dimension, the only effect noted in the acid-urea dimension was the elimination of the most highly phosphorylated species. The result indicated that prevention of phosphorylation of each single residue at position 239, 245, 257, or 259 by alanine substitution did not eliminate phosphorylation at other sites in the C terminus. This result ruled out a phosphorylation cascade as the explanation for the effects of alanine substitutions in the C terminus on mobility shifts in SDS gels.

DISCUSSION

We have observed that prevention of serine or threonine phosphorylation by individual substitution of 4 of the 10 potential phospho-acceptor amino acids in the C terminus of the capsid protein altered the complexity of the electrophoretic pattern of the protein in SDS-polyacrylamide gels. We interpret these effects as due to the selective elimination of phosphorylated species, suggesting that these particular amino acids were targets for phosphorylation in the wild-type protein. This interpretation was confirmed in analyses of a V8 protease-generated C-terminal peptide in acid-urea gels. Each mutation

resulted in the disappearance of the most highly phosphorylated species, with the remaining three phosphorylated species unchanged. The four sites that were sensitive to alanine substitution were each adjacent to downstream proline residues, while those sites that were insensitive to alanine substitution lacked adjacent downstream prolines. Substitution of glycine for proline at these four sites resulted in the same effects on the electrophoretic complexity in SDS gels as did replacement of the acceptor amino acid itself. This result could be explained by a requirement for SP or TP as a recognition motif for phosphorylation. Alternatively, an adjacent downstream proline might be required for phosphorylation at each particular site to cause a mobility shift.

In addition, amino acid substitution at each apparent phosphorylation site produced a more profound effect than a similar substitution at the neighboring upstream site. This effect was found to be due to differences in the effects of phosphorylation at the four different sites on the magnitude of the mobility changes induced by phosphorylation at the remaining sites. The order of strength of the magnifying effect was S259 > S257 > S245 > T239. Thus, although three different phosphorylated peptides were produced by mutant S259A, the peptides all migrated at approximately the same position in SDS gels. If our interpretation is correct, then some information about the identity of the phosphorylated forms of the wild-type capsid protein can be inferred. Because of these differing effects of phosphorylation at different sites on mobility, any multiphosphorylated species in the acid-urea dimension could potentially resolve into different electrophoretic forms in the SDS dimension if different combinations of phosphorylation sites were used. This was not generally the case except for the diphosphorylated species, which resolved into two electrophoretic forms in the SDS dimension.

The results indicated that the various phosphorylated species of capsid protein may not have been random combinations of phosphorylation at the four sites identified. We were unable, however, to assign identities to the species comprising the mono-, di-, and triphosphorylated species. The total number of radiolabeled spots generated from the C terminus of the wild-type protein was at least four and probably five. If we include a nonphosphorylated species, our data would suggest that with respect to phosphorylation, at least five separate species of capsid protein were present in preparations of intracellular viral nucleocapsids. We do not know whether single nucleocapsids contained capsid proteins with all combinations of phosphorylation or whether populations of nucleocapsids with distinct phosphorylation combinations were present in our preparations.

The mobility differences in SDS gels caused by phosphorylation could be assigned to the C terminus of the protein, since the C-terminal V8 protease peptide displayed behavior identical to that of the intact protein in SDS gels. It is not known why phosphorylation at selected sites in a polypeptide can change the electrophoretic mobility in SDS gels, while phosphorylation at other sites has no effect. Phosphorylation at specific sites might alter the quantity of SDS bound to the protein, thus reducing its charge and electrophoretic mobility. Alternatively, phosphorylation may produce a conformational change of the SDS-bound protein. Our experiments do not distinguish between these alternatives.

Functional analyses of the effects of the mutations that we have described have shown that different mutants can be assigned distinct phenotypes (15). The S245A and S259A mutations caused defects in viral DNA synthesis. The S259A mutation also inhibited virus infection of primary duck hepatocytes, and an aspartic acid substitution at serine 257, which was intended to mimic constitutive phosphorylation, resulted in a defect in utilization of the nucleocapsid for viral covalently closed circular DNA amplification. The evidence for distinct phosphorylation patterns of the C terminus in the nucleocapsid is consistent with our genetic evidence for distinct functions associated with phosphorylation-deficient mutations. That is, separate regions on the surface of the nucleocapsid may have unique functions that are associated with conformations that are determined by particular patterns of phosphorylation. The use of phosphorylation to stabilize distinct conformations on the surface of the nucleocapsid may provide a solution to the biological problem of creating multiple functional domains on an otherwise homogeneous surface of repeating units.

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