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Site-Specific Phosphorylation Regulates the Transcriptive Activity of Vesicular Stomatitis Virus NS Protein

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In vitro transcription by vesicular stomatitis virus nucleocapsids is inhibited by enzymatic dephosphorylation of the NS protein. We provide evidence that specific, partial dephosphorylation of NS molecules is the only detectable change in nucleocapsids treated with bacterial alkaline phosphatase under conditions that prevent the action of adventitious protease. Dephosphorylation appeared to affect only the rate of transcription; there were no changes in sedimentation rates of transcripts. To identify the sites of phosphorylation required for NS activity in transcription, we examined phosphopeptides produced by chymotrypsin digestion of the two electrophoretic classes of NS molecules found in virions and infected cells. The electrophoretically slower class, NS1, abundant in the intracellular soluble pool, has a lower activity in transcription; it contained six chymotryptic phosphopeptides. Five of these peptides contained both phosphoserine and phosphothreonine, indicating that this peptide cluster represents at least 11 separate sites of phosphorylation. In the electrophoretically faster nucleocapsid-associated NS2 class of molecules, which support a higher rate of transcription, another group of eight phosphopeptides was superimposed on this pattern. Two of these peptides contained both phosphoserine and phosphothreonine, so this cluster of peptides represents at least 10 additional phosphorylation sites. These sites were especially sensitive to dephosphorylation by bacterial alkaline phosphatase. One or more of them appears to be responsible for the higher transcription rates mediated by NS2 molecules.

The NS phosphoprotein of vesicular stomatitis virus (VSV) participates in viral RNA synthesis (3). A requirement for high levels of covalently bound phosphate for full activity of NS in transcription was indicated by the in vitro reconstitution experiments of Kingsford and Emerson (15). After removing NS molecules from viral nucleocapsids, these authors replaced them with several different classes of NS molecules that differed in extent of phosphorylation; only the most highly phosphorylated NS molecules restored full transcriptive activity.

These findings were confirmed by Kingsbury et al. (14), who observed inhibition of in vitro transcription by VSV nucleocapsids after digestion with bacterial alkaline phosphatase (BAP). In this paper, we document that the enzymatic dephosphorylation of NS is specific under appropriate conditions, and we identify two subsets of phosphorylated sites on the NS molecule that have different effects on its transcriptive function.

MATERIALS AND METHODS

Virus. Monolayer cultures of BHK cells were infected at an input multiplicity of 0.01 PFU per cell. For radiophosphate labeling, the cells were washed with phosphate-free Eagle minimal essential medium 4 h after infection and then incubated for 16 h in phosphate-free minimal essential medium containing 100 μCi of carrier-free $^{32}P_i$ per ml and 3% dialyzed fetal calf serum. Under these conditions, most of the cells became infected secondarily, providing time for equilibration of the intracellular ATP pool with radiophosphate before significant viral protein synthesis began. About 70% of the radioactivity added was found in the medium at the end of the incubation, indicating that an adequate supply of extracellular radiophosphate had been maintained.

[^{35}S]methionine labeling was done in the same way, except the concentration of radioactivity was 30 μCi/ml and the serum added to the medium was not dialyzed.

Enzymatic dephosphorylation. Virions were disrupted with 1% Triton X-100 in 0.15 M NaCl–0.01 M Tris-hydrochloride (pH 8.0) at room temperature. BAP from *Escherichia coli* (Worthington Diagnostics, product code BAPF; specific activity, 30 to 40 U/mg of protein) was added at the concentrations given in the figure legends, and incubation was performed for 1 h at 30°C. Aprotinin (Sigma Chemical Co.) was added to all BAP preparations in the proportion of 1 part to 10 parts of BAP by weight.

When transcriptase activity was to be measured, BAP was removed by centrifuging the nucleocapsids at 45,000 rpm (SW55 rotor) for 90 min at 8°C through a layer of 30% glycerol onto a 1.33-g/cm^3 D_2O-sucrose

cushion. Transcriptase activity was measured as described by Banerjee and Rhodes (1).

Peptide mapping. ^{32}P-labeled NS protein was isolated by polyacrylamide slab gel electrophoresis (4). NS was electrophoretically eluted from gel segments in buffer containing 300 μg of sodium dodecyl sulfate-denatured ovalbumin per ml, and both proteins were precipitated with 10 volumes of ethanol. The proteins were collected by centrifugation, denatured with guanidine-hydrochloride (9), alkylated with iodoacetamide (13), dialyzed against 0.025 M NH_4HCO_3 (pH 8.0), and digested at 37°C with *Nα-p*-tosyl-L-lysine chloromethyl ketone-treated chymotrypsin (Worthington). The enzyme was added in two portions of 30 μg/ml each, separated by an interval of 3 h, with incubation extended for 16 h after the addition of the second portion. The digest was lyophilized three times from water to remove salt, spotted on a 0.1-mm cellulose F glass thin-layer chromatography plate (E. Merck AG, Darmstadt), and electrophoresed in glacial acetic acid-pyridine-water (1:3:96, pH 5.5) at 1,000 V for 1 h on a cooling plate at 13°C. The plate was dried overnight and chromatographed in *N*-butanol–glacial acetic acid–pyridine–water (17:12:35:35, pH 4.0). Phosphopeptides were visualized by autoradiography.

Phosphoamino acid identification. Phosphopeptides identified by autoradiography were scraped from thin-layer chromatography plates and hydrolyzed in 6 N HCl at 110°C for 1.5 h. After lyophilization twice from water, samples were electrophoresed on CEL 400-10 cellulose glass thin-layer chromatography plates (Macherey, Nagel and Co.) at 2,000 V for 1 h in glacial acetic acid-formic acid-water (7.8:2.5:90, pH 1.9). Autoradiography was then performed, and the positions of phosphoamino acids were compared with ninhydrin-stained marker phosphoamino acids.

RESULTS

Specific removal of phosphate by BAP. We have presented evidence that BAP can be used safely to dephosphorylate NS if a protease that contaminates BAP preparations is inhibited by the peptide Aprotinin (14). This is documented further in Fig. 1, where autoradiography reveals no breakdown of any species of virion protein after incubation with the maximum concentration of BAP used in our experiments. Also shown in Fig. 1 is the stepwise disappearance of NS2, the more highly phosphorylated electrophoretic form of NS (4, 11), at lower BAP concentrations and the partial resistance of the less phosphorylated NS1 species to even the highest BAP concentration. Under these conditions, NS2 is completely converted to NS1. This cannot be seen in the [^{35}S]methionine-labeled examples shown in Fig. 1, but it has been documented previously (11, 14).

Although the covalent integrity of the nucleocapsid proteins L, N, and NS appeared to be maintained after enzymatic dephosphorylation, it was possible that removal of phosphate from NS might secondarily alter its own affinity or the affinity of the L protein for the nucleocapsid. However, most or all of NS and L remained

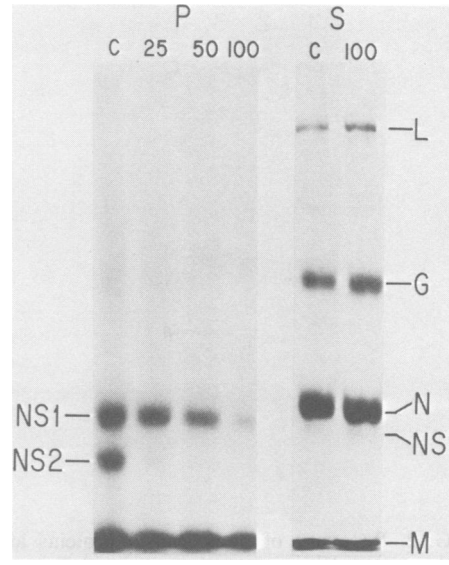

FIG. 1. Dephosphorylation of VSV NS protein. Virions labeled with $^{32}P_i$ or [^{35}S]methionine were disrupted with Triton X-100 and incubated with BAP in the presence of Aprotinin as described in the text. After incubation at 30°C for 1 h, samples were precipitated with 5% trichloroacetic acid and subjected to polyacrylamide gel electrophoresis (4). Abbreviations: P, ^{32}P-labeled virions; S, ^{35}S-labeled virions; C, control, no BAP; numerals designate micrograms of BAP per 0.25 ml.

associated with BAP-treated nucleocapsids after centrifugation through 30% glycerol (Fig. 2). We have shown elsewhere that the viral RNA within nucleocapsids had an unaltered sedimentation rate after BAP treatment (14).

Residual BAP in the transcriptase assay. Removal of BAP from treated nucleocapsids is a prerequisite for accurate estimation of remaining transcriptase activity, since BAP dephosphorylates the nucleoside triphosphate substrates of the transcriptase (18). Indeed, we found that transcription was inhibited about 30% by the addition of 10 μg of BAP per ml to the transcriptase reaction (0.3 to 0.4 U/ml), and 100 μg of BAP per ml resulted in complete inhibition (data not shown). However, BAP concentrations below 1 μg/ml were not inhibitory, and direct spectrophotometric assay (8) revealed that BAP contamination was at least 10-fold less than this level in nucleocapsids centrifuged through 30% glycerol (see above).

Products of the inhibited transcriptase. We reported that 70 to 80% of the in vitro transcriptase activity of VSV nucleocapsids is inhibited by exhaustive BAP treatment (14). The product analyses of Fig. 3 show that there is no difference in the sedimentation properties of the RNA species made after BAP dephosphorylation. On

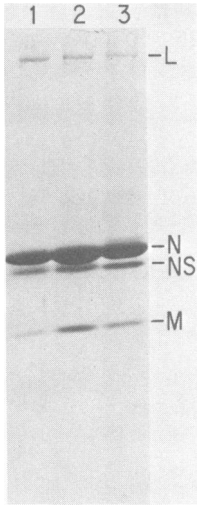

FIG. 2. Recovery of nucleocapsid proteins after BAP treatment. Virions were disrupted with Triton X-100 and treated with BAP, and nucleocapsids were isolated by centrifugation through 30% glycerol (see text). After electrophoresis (4), the gel was stained with Coomassie blue. Lanes: 1, no BAP, held at 0°C; 2, no BAP, incubated at 30°C for 1 h; 3, 400 μg of BAP per ml, incubated at 30°C for 1 h. A small amount of M protein remained attached to nucleocapsids isolated in this manner.

the one hand, the paucity of genome-size RNA molecules indicates that transcription, not replication, comprises the residual activity of dephosphorylated nucleocapsids. On the other hand, the absence of smaller RNA molecules indicates that inhibition of RNA synthesis is not due to a premature termination of nascent RNA chains and suggests that the role of phosphate in NS protein function is to enhance the rate of transcript initiation or elongation or both. More discriminating analyses are needed to decide whether transcription of individual genes or post-transcriptional RNA processing is altered by BAP.

Phosphorylated sites in NS molecules. NS molecules with higher levels of phosphorylation are more abundant in virions than in infected cells (10), and they are more active in transcription in vitro (15). To obtain more information about the structural basis of NS phosphorylation and its relationship to transcriptase activity, we digested denatured NS molecules with proteolytic enzymes and separated the resulting phosphopeptides by two-dimensional electrophoresis and chromatography. Preliminary trials with trypsin digestion yielded smeared and poorly resolved spots (data not shown). We were more successful with chymotrypsin, which gave more discrete products (Fig. 4). As many as 10 phosphopeptide spots were seen in a digest of the more highly phosphorylated subspecies, NS2 (Fig. 4D). However, two of these spots, labeled 1 and 2, were disproportionately large, indicating that they represented groups of phosphopeptides with similar mobilities. This suspicion was confirmed when three subspecies were resolved in each of these spots on a different cellulose matrix (Fig. 5). The peptides in groups 1 and 2 were present in every sample of NS that we examined, including NS1 from the soluble pool of infected cells (Fig. 4A), NS1 from intracellular nucleocapsids (Fig. 4B), and both electrophoretic forms of NS from virions (Fig. 4C and D). The ubiquity and relative resistance to dephosphorylation of these phosphopeptides (see below) led to the designation, primary cluster. Migration toward the anode and a fairly low mobility in the ascending chromatographic dimension indicate that this cluster is relatively acidic in net charge and hydrophilic.

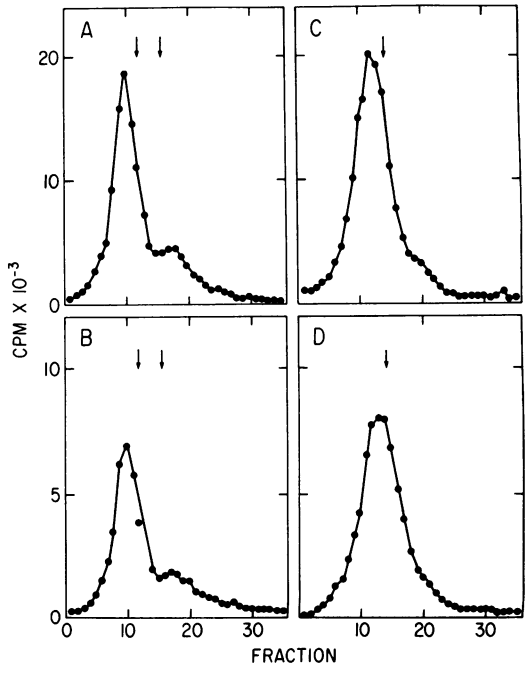

FIG. 3. Sedimentation analysis of RNA transcripts made after BAP treatment. VSV nucleocapsids were incubated at 30°C for 2 h in a transcriptase reaction mix containing [^{3}H]UTP (1). Product RNA was isolated by phenol-sodium dodecyl sulfate extraction and centrifuged in 15 to 30% sucrose gradients. Before centrifugation half of each sample was denatured with glyoxal (16). Panels: (A) RNA made by nucleocapsids not treated with BAP; (B) RNA made by BAP-treated nucleocapsids; (C) as (A), but glyoxylated; (D) as (B), but glyoxylated. Vertical arrows represent rRNA markers; these did not separate in glyoxylated samples. Sedimentation is shown from left to right.

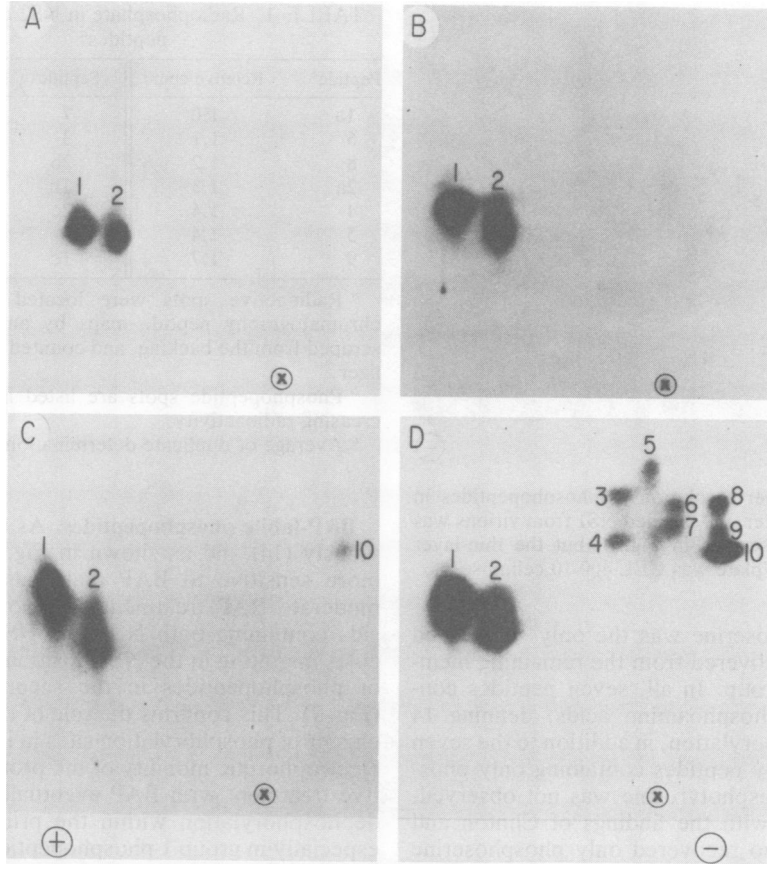

FIG. 4. Phosphopeptides in NS molecules. $^{32}P_i$-labeled NS molecules from infected cells and virions were isolated by gel electrophoresis and digested with chymotrypsin. The digests were separated by two-dimensional electrophoresis and chromatography in cellulose F thin layers (see text), and autoradiograms were prepared. Panels: (A) NS1 from the soluble intracellular pool; (B) NS1 from free intracellular nucleocapsids; (C) NS1 from virions; (D) NS2 from virions. Symbols: ⊗, site of sample application; ⊕, anode; ⊖, cathode. Electrophoresis was in the horizontal dimension.

NS1 is the only form of NS that we observed in the soluble pool; Kingsford and Emerson (15) have shown that NS molecules from the soluble pool do not restore full transcriptive activity when added to nucleocapsid templates. This indicates that phosphorylation of primary cluster sites does not fully activate NS for its role in transcription.

The phosphopeptides in spots 3 to 10 were characteristic of NS2 molecules (Fig. 4D). This group of phosphopeptides is relatively basic and hydrophobic. The fact that it was superimposed upon the primary cluster earned the designation, secondary cluster. Since molecules with the electrophoretic mobility of NS2 activate transcription fully (14, 15), the sites responsible for this activity evidently reside in the secondary cluster.

There were marked differences in the intensities of the radioactive spots in Fig. 4 and 5. Quantitatively, 60% of the total radiophosphate in NS2 molecules was in the primary cluster, and the relative incorporation into individual peptides within this cluster spanned a 13-fold range (peptides 1a and 1c in Table 1). The range of radioactivity in the secondary cluster was about half as great. Since steady-state labeling conditions were employed (see above), we conclude that the efficiency of phosphorylation of different phosphorylation sites or their stability varies considerably within both clusters and that the majority of molecules, even in the more highly phosphorylated NS2 class, are incompletely phosphorylated.

Phosphoamino acid analyses (Fig. 6) showed that both phosphothreonine and phosphoserine were present in most members of the primary cluster (spot 1a appeared to lack phosphothreonine). In the secondary cluster, phosphothreonine and phosphoserine were seen in spots 3 and

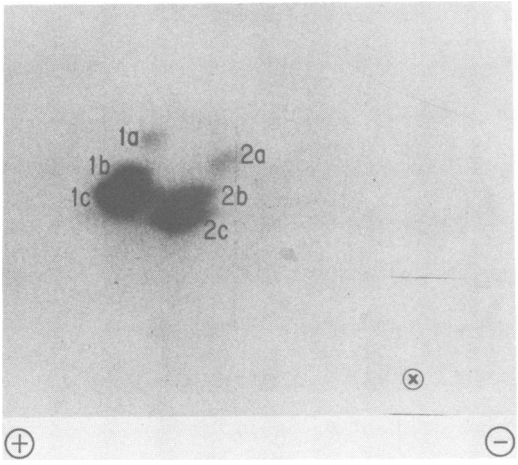

FIG. 5. Further resolution of phosphopeptides in the primary cluster. $^{32}P_i$-labeled NS1 from virions was examined as described in Fig. 4, but the thin-layer chromatography plate was CEL 400-10 cellulose.

4, but phosphoserine was the only substituted amino acid recovered from the remaining members of this group. In all, seven peptides contained both phosphoamino acids, defining 14 sites of phosphorylation, in addition to the seven sites defined by peptides containing only phosphoserine. Phosphotyrosine was not observed, in agreement with the findings of Clinton and Huang (5), who recovered only phosphoserine and phosphothreonine from NS molecules phosphorylated in vivo.

TABLE 1. Radiophosphate in NS2 chymotryptic peptides[a]

Peptide[b]	Relative cpm[c]	Peptide[b]	Relative cpm[c]
1a	1.0	7	2.0
6	1.1	3	2.8
8	1.2	2b	3.9
2a	1.3	1b	4.3
4	1.4	2c	5.7
5	1.4	10	7.0
9	1.7	1c	13.0

[a] Radioactive spots were located on thin-layer chromatography peptide maps by autoradiography, scraped from the backing, and counted in PCS solubilizer.

[b] Phosphopeptide spots are listed in order of increasing radioactivity.

[c] Average of duplicate determinations.

BAP-labile phosphopeptides. As reported previously (14) and as shown in Fig. 1, NS2 was more sensitive to BAP than was NS1. After moderate BAP treatment of virion nucleocapsids containing both NS1 and NS2, NS molecules migrating in the NS1 position were devoid of phosphopeptides in the secondary cluster (Fig. 7). This confirms the role of the secondary cluster of phosphorylation sites in increasing the electrophoretic mobility of the protein. Exhaustive treatment with BAP eventually resulted in dephosphorylation within the primary cluster, especially in group 1 phosphopeptides (Fig. 7B). More work will be needed to determine the relative importance of BAP-sensitive and BAP-

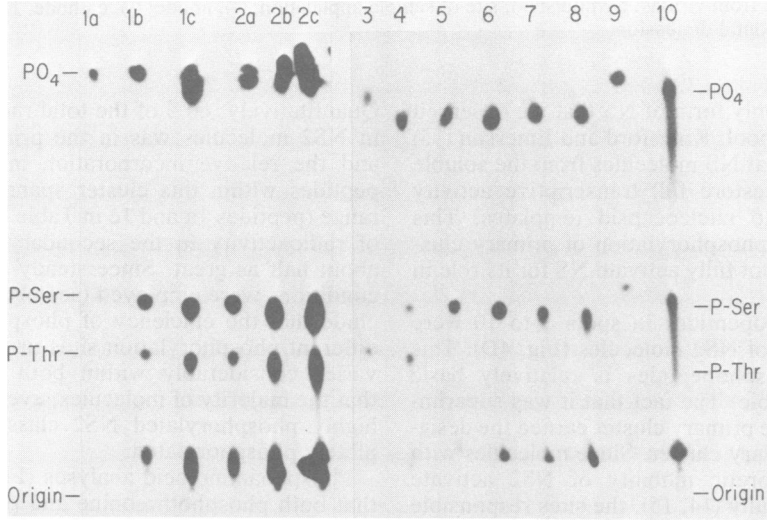

FIG. 6. Phosphoamino acids in NS chymotryptic peptides. Thin-layer electrophoresis was performed after acid hydrolysis (see text) of ^{32}P-labeled peptides isolated from two-dimensional maps. The number at the head of each lane designates peptide spots identified in Fig. 4. Abbreviations: PO_4, P_i; P-Ser, phosphoserine; P-Thr, phosphothreonine. Incomplete hydrolysis left some slowly migrating radioactivity near the origin.

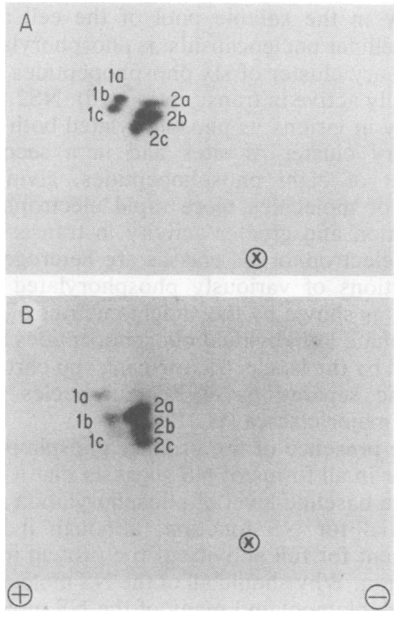

FIG. 7. Phosphopeptides remaining after BAP treatment. Nucleocapsids from 100 μg of virions were treated with BAP for 1 h at 30°C in a volume of 0.5 ml as described in the text. After treatment, only NS1 molecules were seen on polyacrylamide gel electrophoresis. Panels: (A) 100 μg of BAP; (B) 400 μg of BAP.

resistant sites within the primary cluster for transcriptive activity, but it is clear that a baseline level of activity representing about 20% of maximum (14) remains when dephosphorylation is as severe as shown in Fig. 7B.

Superphosphorylation and rephosphorylation. VS virions contain a protein kinase that is capable of superphosphorylating NS as well as virion envelope protein M (12, 17). This kinase appeared to have a marked preference for sites in the primary cluster, judging by the electrophoretic and chromatographic behavior of the phosphopeptides resulting from in vitro phosphorylation (Fig. 8). In addition, the small amount of radiophosphate appearing in the relatively basic and hydrophobic peptides corresponded poorly to the pattern of the secondary cluster seen in molecules phosphorylated in vivo (Fig. 4). All of these results indicate that the virion kinase has protein substrate specificities different from the intracellular kinases that phosphorylate NS molecules.

Rephosphorylation of BAP-treated NS molecules was minimal and restricted to two phosphopeptides of a hydrophobic and basic character (Fig. 8C). Extensive dephosphorylation may change the conformation of NS molecules markedly, reducing the availability of sites to the virion kinase. Alternatively, the residual protein

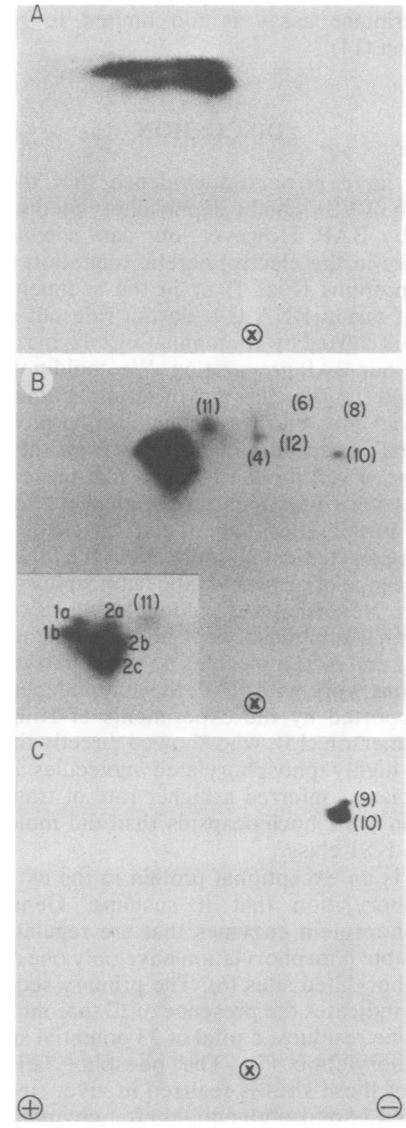

FIG. 8. Superphosphorylation and rephosphorylation of NS protein. Numbers in parentheses represent tentative peptide assignments (Fig. 4). Virions were disrupted with Triton X-100 and were phosphorylated in vitro by endogenous protein kinase (14). Panels: (A) NS1; (B) NS2 (the inset is a shorter exposure of the primary cluster of phosphopeptides); (C) after extensive dephosphorylation with BAP (Fig. 7B), nucleocapsids were isolated by centrifugation through 30% glycerol and rephosphorylated in vitro by the protein kinase that accompanied them (14). The radioactivity migrated on electrophoresis as NS1.

kinase that remained attached to isolated nucleocapsids may have been insufficient to rephosphorylate effectively. In either case, the limited amount of phosphorylation obtained is insufficient to restore the electrophoretic migration of these molecules to the NS2 position, confirming that rephosphorylation during the transcriptase assay is too limited to restore function (14).

DISCUSSION

We have presented evidence that the NS protein of VSV can be dephosphorylated specifically by BAP. However, our data showing no changes in the electrophoretic migration of the viral proteins (Fig. 1) or in the sedimentation rate of virion RNA (14) do not rule out subtle changes caused by contaminating enzyme activities in our BAP preparation. We could not have detected limited cleavage of terminal amino acids from any of the nucleocapsid proteins or removal of terminal nucleotides from the virus genome. Even limited changes like these might have severe functional consequences, so until these possibilities have been checked, there must be an element of doubt about the functional significance of our enzymatic dephosphorylation results. Nevertheless, our conclusions about the functional relationships of phosphorylated sites in NS1 and NS2 molecules do not depend entirely on our work with BAP; they are independently supported by the experiments of Kingsford and Emerson (15), who showed directly that the more highly phosphorylated molecules of the NS2 class conferred a higher rate of transcription on VSV nucleocapsids than did molecules of the NS1 class.

NS is an exceptional protein in the extent of phosphorylation that it sustains. Generally, phosphoprotein enzymes that are regulated by reversible phosphorylation have only one or two phosphorylated sites (6). The primary sequence of NS indicates the presence of 12 threonine and 21 serine residues, a total of 33 potential sites of phosphorylation (7). The phosphorylation of most of these sites is realized in vivo, since we resolved 14 phosphopeptides after chymotrypsin digestion and 7 of these peptides contained both phosphoserine and phosphothreonine, identifying at least 21 phosphorylated sites. Further proteolytic digestion of NS may well uncover additional sites within the chymotryptic peptides that we resolved.

Our chymotryptic peptide maps indicate that there are two classes of phosphorylated sites in NS that define the two major electrophoretic and functional classes of NS molecules previously described (4, 11, 15). NS1, which is found mainly in the soluble pool of the cell and in intracellular nucleocapsids, is phosphorylated in a primary cluster of six phosphopeptides and is not fully active in transcription (15). NS2, found mainly in virions, is phosphorylated both in the primary cluster of sites and in a secondary cluster of eight phosphopeptides, giving this class of molecules more rapid electrophoretic migration and greater activity in transcription. Both electrophoretic classes are heterogeneous collections of variously phosphorylated molecules, as shown by the stoichiometries of radiophosphate in individual phosphopeptides (Table 1) and by the isoelectric focusing and chromatographic separations of NS subspecies within these major classes (11, 15).

The presence of the primary phosphopeptide cluster in all forms of NS suggests that it represents a baseline level of phosphorylation that is essential for NS function, although it is not sufficient for full activity of the protein in transcription. Why should all of the NS molecules in the soluble pool and many of the NS molecules in nucleocapsids be limited to this primary level of phosphorylation? A possible explanation is that a protein kinase with specificity limited to the primary cluster is the only kinase that NS encounters within the soluble pool or in nucleocapsids that are far from the cellular membrane, where virion budding occurs. Secondary site phosphorylation may be mediated by a membrane- or cytoskeleton-associated protein kinase with a different specificity. Another factor that may prevent kinase action on free NS molecules is the association of the protein with nucleocapsids. Binding to nucleocapsids may alter the conformation of NS molecules, rendering previously hidden sites available for kinase modification.

Speculations about the regulatory significance of NS phosphorylation have focused on qualitative mechanisms, such as switching the synthesis of RNA from transcription to replication. For example, Clinton et al. (3) made observations on the distribution of electrophoretic species of NS in infected cells, suggesting that more highly phosphorylated NS molecules were less likely to bind to nucleocapsids and that the binding of less phosphorylated NS molecules increased the rate of RNA replication versus transcription. Independently, Testa and co-workers (20) arrived at a similar hypothesis; they suggested that dephosphorylation of a nucleocapsid-associated protein (presumably NS) favors replication, based on results of in vitro transcription experiments with an ATP analog that cannot act as a γ-phosphate donor. Our in vitro studies provide no support for these ideas, since we observed no increases in the sizes of RNA products made by partially dephosphorylated VSV nucleocapsids.

Alternatively, phosphorylation might regulate transcription exclusively, in a quantitative way. As we saw in our in vitro experiments, nucleocapsids containing dephosphorylated NS exhibited significant levels of residual transcriptive activity. Such levels might be appropriate for intracellular secondary transcription, which must be balanced against replication, but suboptimal for primary transcription, which is a prerequisite for gene expression at the beginning of infection by a negative-strand RNA virus. Increased phosphorylation of NS molecules in nucleocapsids destined for virions would ensure that infecting nucleocapsids transcribe at the highest possible rate. This hypothesis can be tested by comparing the relative transcriptive activities of infecting nucleocapsids and progeny nucleocapsids within the infected cell.

The predicted secondary structure of NS suggests that many potential phosphorylation sites are located at β-turns that also possess basic amino acid residues (Fig. 9). These are structural features that characterize externally located sites of regulatory phosphorylation in other proteins (19). Therefore, we expect to find secondary cluster sites at such β-turns when we determine the locations of the relevant phosphopeptides in the primary sequence of NS. We also plan to continue to probe the relationships of phosphorylation sites to NS protein function by examining the transcriptive activity of nucleocapsids treated with lesser amounts of BAP. This may reveal whether any of the secondary cluster of sites are dispensable for full NS activity and whether there is a hierarchy of sites within the secondary cluster that determines accessibility to dephosphorylation and levels of transcriptase activity.

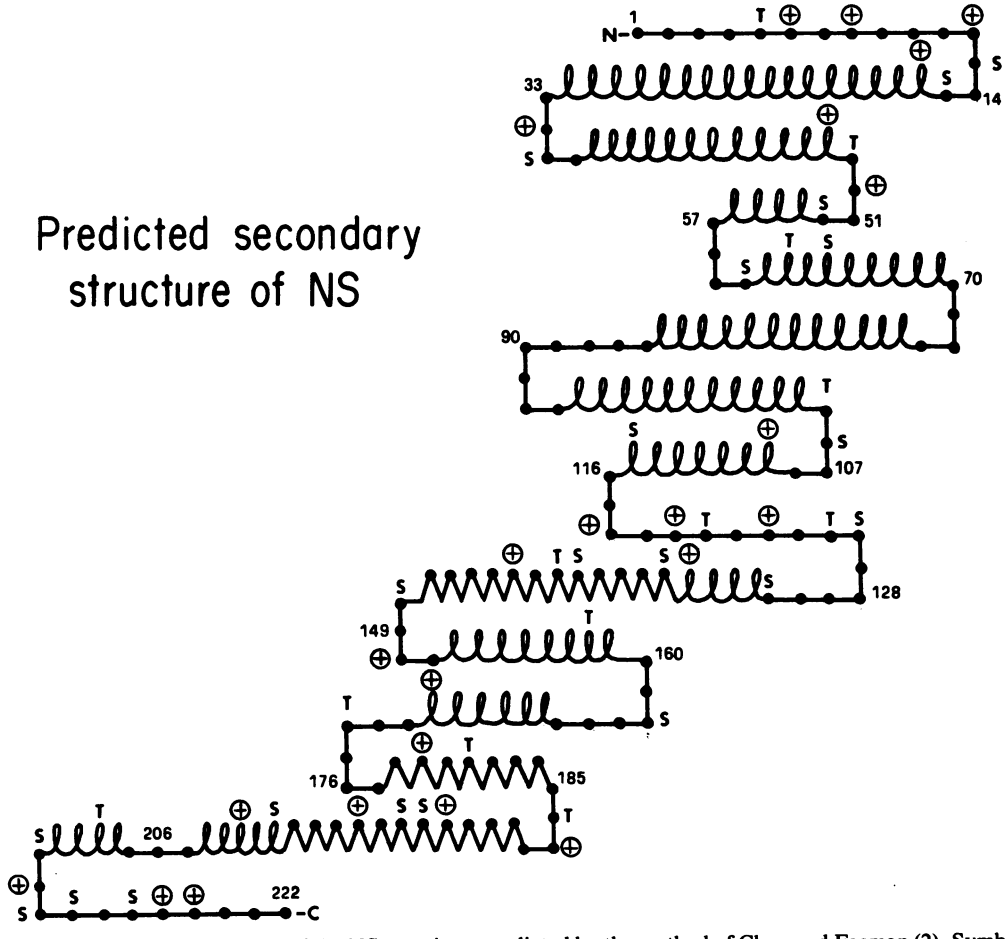

FIG. 9. Secondary structure of the NS protein as predicted by the method of Chou and Fasman (2). Symbols: ℓℓℓ, α-helix; ∿∿∿, β-strand; •—•—•, random coil; ⊕, a basic amino acid within four residues of a threonine or serine. β-Turns are indicated by chain reversals. Numerals refer to amino acid residues (7). Abbreviations: S, serine; T, threonine.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Banerjee, A. K., and D. P. Rhodes.** 1973. *In vitro* synthesis of RNA that contains polyadenylate by virion-associated RNA polymerase of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **70**:3566–3570.
2. **Chou, P. Y., and G. D. Fasman.** 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. **47**:45–148.
3. **Clinton, G. M., B. W. Burge, and A. S. Huang.** 1978. Effects of phosphorylation and pH on the association of NS protein with vesicular stomatitis virus cores. J. Virol. **27**:340–346.
4. **Clinton, G. M., B. W. Burge, and A. S. Huang.** 1979. Phosphoproteins of vesicular stomatitis virus: identity and interconversion of phosphorylated forms. Virology **99**:84–94.
5. **Clinton, G. M., and A. S. Huang.** 1981. Distribution of phosphoserine, phosphothreonine and phosphotyrosine in proteins of vesicular stomatitis virus. Virology **108**:510–514.
6. **Cohen, P.** 1980. Well established systems of enzyme regulation by reversible phosphorylation, p. 1–10. *In* P. Cohen (ed.), Recently discovered systems of enzyme regulation by reversible phosphorylation. Molecular aspects of cellular regulation, vol. 1. Elsevier/North Holland, Amsterdam.
7. **Gallione, C. J., J. R. Greene, L. E. Iverson, and J. K. Rose.** 1981. Nucleotide sequences of the mRNA's encoding the vesicular stomatitis virus N and NS proteins. J. Virol. **39**:529–535.
8. **Garen, A., and C. Levinthal.** 1960. A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characterization of alkaline phosphatase. Biochim. Biophys. Acta **38**:470–483.
9. **Gracy, R. W.** 1977. Two-dimensional thin-layer methods. Methods Enzymol. **47**:195–204.
10. **Hsu, C.-H., and D. W. Kingsbury.** 1980. Vesicular stomatitis virus morphogenesis is accompanied by covalent protein modifications, p. 613–622. *In* B. N. Fields, R. Jaenisch, and C. F. Fox (ed.), Animal virus genetics, ICN-UCLA Symposia on Molecular and Cellular Biology, vol. 18. Academic Press, Inc., New York.
11. **Hsu, C.-H., and D. W. Kingsbury.** 1982. NS phosphoprotein of vesicular stomatitis virus: subspecies separated by electrophoresis and isoelectric focusing. J. Virol. **42**:342–345.
12. **Imblum, R. L., and R. R. Wagner.** 1974. Protein kinase and phosphoproteins of vesicular stomatitis virus. J. Virol. **13**:113–124.
13. **Jacobson, M. F., J. Asso, and D. Baltimore.** 1970. Further evidence on the formation of poliovirus proteins. J. Mol. Biol. **49**:657–669.
14. **Kingsbury, D. W., C.-H. Hsu, and E. M. Morgan.** 1981. A role for NS-protein phosphorylation in vesicular stomatitis virus transcription, p. 821–827. *In* D. H. L. Bishop and R. W. Compans (ed.), The replication of negative strand viruses. Developments in cell biology, vol. 7. Elsevier/North Holland, New York.
15. **Kingsford, L., and S. U. Emerson.** 1980. Transcriptional activities of different phosphorylated species of NS protein purified from vesicular stomatitis virions and cytoplasm of infected cells. J. Virol. **33**:1097–1105.
16. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. U.S.A. **74**:4835–4838.
17. **Moyer, S. A., and D. F. Summers.** 1974. Phosphorylation of vesicular stomatitis virus in vivo and in vitro. J. Virol. **13**:455–465.
18. **Reid, T. W., and I. B. Wilson.** 1971. *E. coli* alkaline phosphatase, p. 373–415. *In* P. D. Boyer (ed.), The enzymes, vol. 4, 3rd ed. Academic Press, Inc., New York.
19. **Smith, J. A., and L. G. Pease.** 1980. Reverse turns in peptides and proteins. CRC Crit. Rev. Biochem. **8**:315–399.
20. **Testa, D., P. K. Chanda, and A. K. Banerjee.** 1980. *In vitro* synthesis of the full-length complement of the negative-strand genome RNA of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **77**:294–298.