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Phosphorylation Sites on Phosphoprotein NS of Vesicular Stomatitis Virus

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The phosphoprotein NS of vesicular stomatitis virus which accumulates within the infected cell cytoplasm is phosphorylated at multiple serine and threonine residues (G. M. Clinton and A. S. Huang, Virology 108:510–514, 1981; Hsu et al., J. Virol. 43:104–112, 1982). Using incomplete chemical cleavage at tryptophan residues, we mapped the major phosphorylation sites to the amino-terminal half of the protein. Analysis of phosphate-labeled tryptic peptides suggests that essentially all of the label is within the large trypsin-resistant fragment predicted from the sequence of Gallione et al. (J. Virol. 39:52–529, 1981). A similar result has been obtained for NS protein isolated from the virus particle by C.-H. Hsu and D. W. Kingsbury (J. Biol. Chem., in press). Analysis of phosphodipeptides utilizing the procedures of C. E. Jones and M. O. J. Olson (Int. J. Pept. Protein Res. 16:135–142, 1980) enabled us to detect as many as six distinct phosphate-containing dipeptides. From these studies, together with the known sequence data, we conclude that the major phosphate residues on cytoplasmic NS protein are located in the amino third of the NS molecule and most probably between residues 35 and 106, inclusive. The studies also provide formal chemical proof that NS protein has a structure consistent with a monomer of the sequence of Gallione et al. as modified by J. K. Rose (personal communication). The low electrophoretic mobility of this protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis is not therefore due to dimerization.

The extent of phosphorylation of the protein NS of vesicular stomatitis virus (VSV) has been shown to regulate both its ability to bind to viral ribonucleoprotein and its ability to stimulate subsequent in vitro transcription (5, 12). The number and functional location of phosphorylation sites on this protein are still unknown, although estimates suggest as many as 10 or more phosphate residues per molecule (6). Hsu et al. (10; C.-H. Hsu and D. W. Kingsbury, J. Biol. Chem., in press) have observed multiple phosphorylation sites in cytoplasmic NS protein in both phosphoserine and phosphothreonine residues.

From the nucleotide sequence data of Gallione et al. (8) as modified by J. K. Rose (personal communication), there are 27 serine residues and 14 threonine residues along the length of the NS molecule. Of these, seven serines and four threonines are between the amino terminus and the first tryptophan residue at position 108. With this distribution of potential phosphorylation sites, it was feasible to utilize incomplete chemical cleavage at tryptophan residues to map the relative distribution of phosphoamino acid residues in the molecule by observing the association of ^{32}P label with the fragments generated by this cleavage. We have previously used a similar procedure to map the tryptophan positions of VSV proteins N and M (4).

The fragments produced by incomplete chemical cleavage also allow us to discriminate between monomer and dimer models of NS structure. The sequence data of Gallione et al. (8) as modified by Rose (personal communication) predict a molecular weight of 29.9 kilodaltons for the unphosphorylated NS protein. Phosphorylation of all available serine and threonine residues would increase this expected value by a maximum of three kilodaltons. In fact, depending on the concentration of bisacrylamide cross-linker (1), NS protein migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular weight of between 40 and 70 kilodaltons. It has been suggested that this may be due either to the dimerization of NS molecules or to an aberrant electrophoretic mobility due to some peculiarity of the monomer (8).

Another procedure which promises to be of some utility in the study of phosphoproteins is the demonstration by Jones and Olson (11) that limited acid hydrolysis of phosphoproteins will generate, among other products, dipeptides of the form *X*-phosphoamino acid, where *X* is any amino acid residue. An analysis of the resultant dipeptides might be expected to yield information on the number of phosphorylation sites and ultimately their location in the molecule. This procedure was applied to cytoplasmic NS protein of VSV labeled with ^{32}P.

MATERIALS AND METHODS

Preparation of metabolically labeled NS protein. A total of 1.5×10^8 L cells were infected with VSV (Indiana serotype except where noted) at a multiplicity of 30 PFU per cell, and after an absorption period of 0.5 h the cells were incubated at 37°C for 3.5 h in minimal essential medium (GIBCO Laboratories) supplemented with 5% newborn calf serum. At this time the cells were collected by centrifugation and resuspended in either minimal essential medium containing 1/40th the normal amino acid levels (for subsequent [^{35}S]methionine labeling) or in phosphate-free minimal essential medium (for subsequent ^{32}P labeling). After 0.5 h [^{35}S]methionine (10 μCi/ml) or ^{32}P (200 μCi/ml) were added, and the cultures were incubated 1 to 1.5 h before harvesting. After pelleting, cells were dissolved in a small volume of buffer containing 5% SDS, 5% β-mercaptoethanol, 10% glycerol, and 8 M urea with 0.02% bromophenol blue, and the sample, after the

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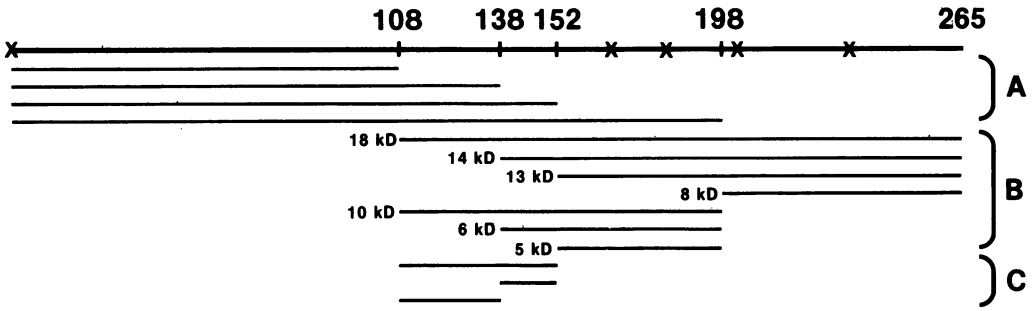

FIG. 1. Predicted fragments from incomplete chemical cleavage of NS protein at tryptophan residues. The sequence data of Gallione et al. (8) as modified by Rose (personal communication) have been used to construct this model. The sequence modification involves the deletion of one of the T residues at position 575 or 576, resulting in a substantially longer open reading frame than before. The locations of tryptophan (|) and methionine (X) residues along the NS protein are indicated in the top band. NS protein labeled only in the amino-terminal methionine generates the labeled fragments of group A. NS labeled in all methionine residues produces the labeled fragments of groups A and B. Group C fragments contain no methionine and are undetected in both of the above situations. kD, Kilodaltons.

DNA was sheared by sonication, was heated at 100°C for 1 min and layered on preparative SDS-PAGE (10% acrylamide and 0.13% *N,N*-methylene bisacrylamide). After electrophoresis for an appropriate time the gel was dried without fixation, and the labeled NS protein band was located by autoradiography and excised from the gel. The NS protein was passively eluted into Tris buffer (pH 8.9) containing 0.01% SDS.

Preparation of end-labeled NS protein. NS protein labeled at the amino terminus with [*N-formyl*-^{35}S]methionine was prepared in vitro as previously described (4). Briefly, infected L cell extracts prepared as described by Toneguzzo and Ghosh (15) were incubated with predetermined amounts of commercial reticulocyte lysates and standard protein synthesis cocktail but containing [*N-formyl*-^{35}S]methionyl tRNA and an excess of unlabeled methionine. Incubation was at 32°C for the appropriate length of time, and the resultant protein was purified on preparative SDS-PAGE as described above.

Nonlimit cleavage with NCS. NS proteins eluted from SDS-PAGE were concentrated by lyophilization and redissolved in acetic acid and *N*-chlorosuccinimide (NCS) as previously described (3). After 2 h at room temperature, carrier bovine serum albumin was added, and the products were precipitated with cold acetone. The precipitate was dissolved in sample buffer and analyzed on SDS-PAGE (15% acrylamide and 0.26% *N,N*-methylene bisacrylamide) as previously described.

Preparation and analysis of tryptic and acid hydrolysis peptides. For analysis of tryptic peptides, NS proteins, labeled with either ^{35}S or ^{32}P as required, were eluted from SDS-PAGE and subjected to complete tryptic digestion after oxidation, all as described previously (2). One-dimensional electrophoretic analysis was carried out on cellulose CEL 300 thin layers (Brinkman, Inc.) in pyridine-acetate (pH 3.5) at 400 V for 1.5 to 2 h.

For dipeptide isolation by acid hydrolysis the procedures of Jones and Olson (11) were used. Specifically, labeled, purified NS proteins were transferred in pyridine to glass ampules and after lyophilization resuspended in 2 N HCl under a reduced nitrogen atmosphere. After hydrolysis for 4 h at 110°C, the samples were filtered through glass wool and concentrated by lyophilization. The resultant products were separated and analyzed by thin-layer electrophoresis and chromatography. First-dimension electrophoresis was at pH 1.9 in formic acid-acetic acid, whereas the second-dimension electrophoresis was at pH 3.5 as described above. Chromatography was in *n*-butanol–pyridine–acetic acid at right angles to the second electrophoretic dimension.

Subtractive Edman degradation. The putative phosphodipeptide fraction which remained at the origin after electrophoresis at pH 1.9 was eluted from the thin layer with water and reduced to dryness by lyophilization. The peptides were subjected to one cycle of Edman degradation (7). Briefly, phenylisothiocyanate was coupled to the peptides in pyridine at 37°C for 30 min. Volatile reagents were removed under vacuum, and cleavage was effected by incubation in anhydrous trifluoroacetic acid under nitrogen for 30 min at 37°C. The acid was removed under vacuum, and the sample was dissolved in water and extracted with three cycles of ethyl acetate. The organic phases were dried and assayed for radioactivity, and the material in the aqueous phase was concentrated by lyophilization and analyzed by electrophoresis and chromatography as described above.

RESULTS

Linear mapping of phosphorylated residues in NS protein. The sequence data of Gallione et al. (8) as modified by Rose (personal communication) predicts that partial cleavage at tryptophan residues of monomeric NS protein should generate 14 fragments in addition to the uncleaved NS protein (Fig. 1). Only four of these (group A) will be radioactively labeled if derived from NS protein labeled only at the amino terminus. NS protein labeled at all five methionine positions could generate seven additional labeled fragments (group B). The smallest three fragments (group C) are unlabeled in both cases. NS protein metabolically labeled with [^{35}S]methionine or ^{32}P was purified from infected cytoplasmic extracts, whereas NS protein labeled with [^{35}S]methionine only at the amino terminus was synthesized in vitro as described above. The three independently labeled NS species were reacted with NCS under conditions which yield nonlimit cleavage at tryptophan residues, and the products were analyzed on SDS-PAGE.

NS protein labeled at the amino terminus yielded no more than four labeled cleavage fragments in addition to the uncleaved protein (Fig. 2, lane C). The 43,000-molecular-weight (43K), 34K, 31K, and 25K fragments are thought to result from partial cleavage of tryptophan at residues 198, 152, 138, and 108, respectively (Fig. 1, group A). The molecular weights of the four fragments as estimated from protein standards are all larger than the corresponding molecular weights predicted from the sequence data. This

observation, which is consistent with that seen in the intact NS molecule, would suggest that the region responsible for the anomolous electrophoretic migration of this protein resides in the amino-terminal half of the protein. The efficiency of cleavage by NCS at any particular tryptophan residue is known to be a function of the local amino acid environment. On the assumption that cleavage at tryptophan residues 138 and 152 is very inefficient, the observed result is not inconsistent with that expected for a monomeric NS protein.

Cleavage of NS protein labeled at all methionine positions yielded labeled fragments of smaller size in addition to the four amino terminus-containing fragments (Fig. 2, lane A). All of the observed fragments (18K, 14K, 11K, and 8K) are compatible in size with predicted fragments; however, a number of other fragments predicted from Fig. 1 were not observed. A number of possibilities to explain these discrepancies are considered below.

When partial cleavage products of NS protein labeled with ^{32}P were examined (Fig. 2, lane B), a pattern identical to that of end-labeled protein was observed. The fact that ^{32}P label was present only in those fragments containing the amino terminus shows that the major phosphorylation sites of the NS protein are wholly located on the smallest fragment also containing the amino terminus. The major phosphorylation sites are therefore within the 108 amino acids between the amino terminus and the first tryptophan residue.

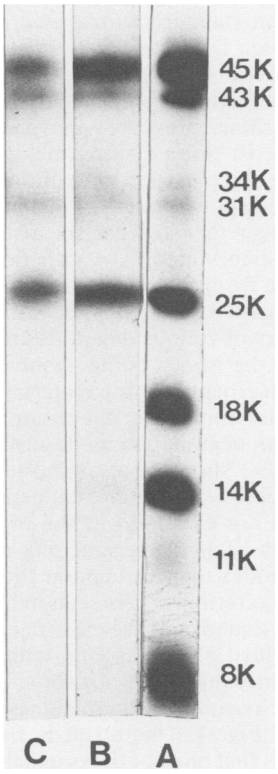

FIG. 2. Fragments produced by incomplete chemical cleavage of NS at tryptophan residues. NS protein from the cytoplasmic fraction of infected cells after labeling with [^{35}S]methionine (A) or $^{32}P_i$ (B) or from in vitro synthesis with [*N-formyl*-^{35}S]methionine (C) was purified on SDS-PAGE and subjected to partial chemical cleavage with NCS as described in the text. The resultant fragments were analyzed on 10% SDS-PAGE, and labeled fragments were visualized by autoradiography.

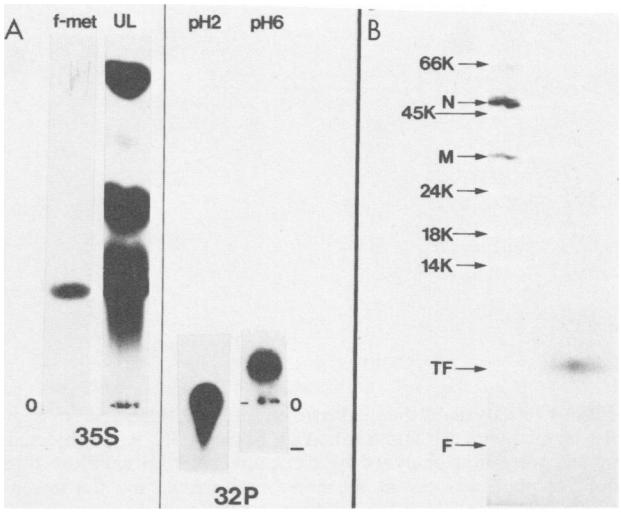

FIG. 3. (A) Electrophoresis of tryptic peptides from NS protein. NS proteins labeled with [^{35}S]methionine or $^{32}P_i$ were produced as described in the legend to Fig. 2 and after oxidation were digested to completion with trypsin. The resultant peptides were analyzed by electrophoresis on cellulose thin-layer plates. Peptides from proteins labeled solely at the amino terminus (f-met) or labeled at all methionine positions (UL) were analyzed at pH 3.5. Peptides from ^{32}P-labeled preparations were analyzed at pH 2 and 6 as noted. (B) SDS-PAGE analysis of the ^{32}P-labeled tryptic fragment of NS protein. The ^{32}P-labeled tryptic fragment remaining at the origin of cellulose thin layer was analyzed on 15% SDS-PAGE. The dye front is indicated (F), and the size of the tryptic fragment (TF) was estimated at 5.5 kilodaltons by extrapolation from the labeled and unlabeled marker proteins indicated.

The same essential features of this pattern were observed when the NS protein was purified from infected human KB cells or when the VSV serotypes of New Jersey or Piry were used as a source of NS protein (data not shown).

Tryptic digestion of Indiana NS protein. When [^{35}S]methionine-labeled NS protein is digested with trypsin and analyzed on cellulose thin layers, three or four clearly defined labeled peptides are resolved (4). One-dimensional electrophoresis at pH 3.5 also resolved at least three major methionine-containing tryptic peptides (Fig. 3). Of these the most slowly migrating peptide corresponds to the tryptic peptide derived from end-labeled NS protein (Fig. 3). This proves, as anticipated from the results shown in Fig. 2, that the amino-terminal methionine of cytoplasmic NS remains associated with the mature protein. When ^{32}P-labeled NS protein was similarly digested with trypsin and analyzed by electrophoresis, the ^{32}P-labeled peptides either failed to leave the origin (pH 2) or moved slowly toward the anode (pH 6). A similar result has been reported by Hsu et al. (10) with their strain of Indiana VSV. The sequence data of Gallione et al. (8) indicate a possible trypsin-insensitive 7.5-kilodalton peptide located between residues 35 to 109. It is possible that this peptide contains the major phosphorylation sites of the VSV NS protein. Consistent with this possibility is our observation that SDS-PAGE analysis of the material which remained at the origin on electrophoresis yielded a labeled peptide which we estimate from markers to be at least 5.5 kilodaltons (Fig. 3).

Partial acid hydrolysis of NS protein. Several authors (6, 10) have identified serine and threonine residues as the sites of phosphorylation in Indiana NS protein produced in infected BHK cells. The right-hand lane of Fig. 4 demonstrates that both of these residues (PS and PT) are also

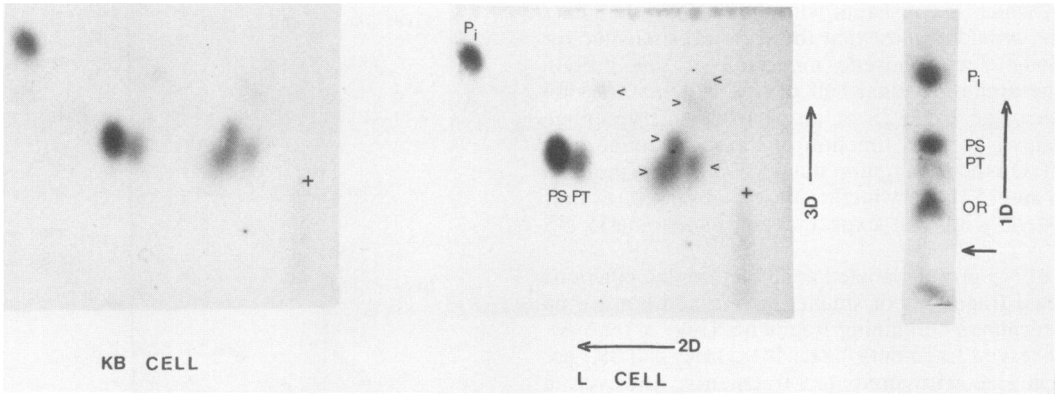

FIG. 4. Analysis of the acid hydrolysate of ^{32}P-labeled NS protein. Cytoplasmic NS protein, from either infected L cells or infected KB cells, labeled with ^{32}P and purified on SDS-PAGE, was subjected to acid hydrolysis, all as described in the text. The resultant hydrolysis products were first analyzed by electrophoresis on cellulose thin layers at pH 1.9, as shown in the rightmost panel (1D). The resultant thin-layer plate was cut at the horizontal arrow, and the lower material was discarded. The remaining material was next subjected to electrophoresis at pH 3.5 as indicated (2D) and then separated by chromatography in the perpendicular dimension (3D). Positions of phosphoserine and phosphothreonine were determined from stained authentic markers. Abbreviations: L cell, NS protein from infected L cells; KB cell, NS protein from infected human KB cells; PS, phosphoserine; PT, phosphothreonine; OR and +, point of sample application.

labeled with ^{32}P in NS protein from L cells and human KB cells. Other ^{32}P-labeled products of limited acid hydrolysis of NS protein are P_i, positively charged phosphopeptides (below the horizontal arrow in this figure), and material remaining at the origin (OR). The material at the origin corresponds to the fraction containing phosphodipeptides of the form amino acid-phosphoamino acid, as described by Jones and Olson (11).

To resolve the material in the putative phosphodipeptide fraction, the acid hydrolysate was fractionated by three-dimensional analysis. The first dimension was electrophoresis at pH 1.9 (Fig. 4, left-hand panel). The positively charged phosphopeptides were removed by trimming off the lower edge of the cellulose sheet below the horizontal line. The remaining material was first subjected to electrophoresis at pH 3.5 and then to chromatography as described above. The positions of phosphoserine and phosphothreonine were confirmed by ninhydrin staining of unlabeled marker phosphoamino acids. As seen in the two rightmost panels of Fig. 4, the ^{32}P-labeled material from hydrolysates of NS from either L or KB cells resolved into similar patterns, strongly suggesting that the same sites are phosphorylated in both preparations. At least three distinct major spots and three minor spots are resolved in each preparation. Assuming that each of the resolved spots is due to a distinct phosphodipeptide, it would appear that at least six distinct sites on the molecule are phosphorylated. The variation in labeling intensity may result from variations in the phosphorylation pattern of individual NS molecules, or it may simply reflect differential recovery of various dipeptides by this procedure.

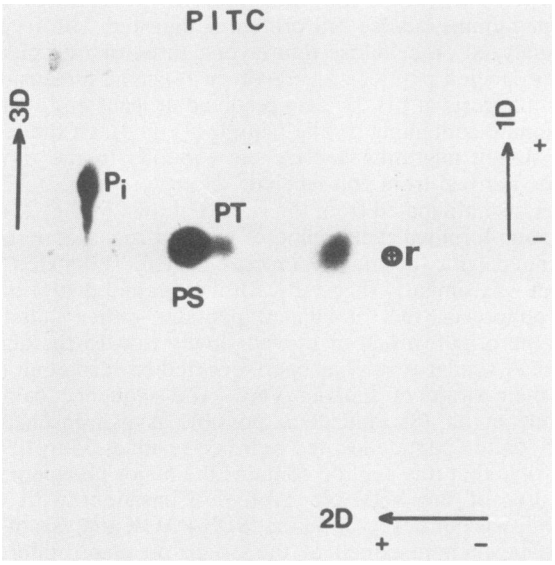

FIG. 5. Analysis of phosphodipeptides after Edman degradation. The material remaining at the origin after electrophoresis at pH 1.9 of an acid hydrolysate of NS protein (see the rightmost panel of Fig. 4) was subjected to one cycle of Edman degradation and then analyzed by the same three-dimensional analysis as described in the legend to Fig. 4. The point of sample application was the middle of the circle in the abbreviation or. All other abbreviations are defined in the legend to Fig. 4.

To verify that the spots being resolved were in fact principally phosphodipeptides, the material remaining at the origin of the first electrophoretic dimension was subjected to one cycle of Edman degradation as described above. Mamrack et al. (13) have shown that amino-terminal phosphoamino acids form derivatives after treatment with phenylisothiocyanate which are extracted in the organic phase. We did not observe any ^{32}P in the organic phase, suggesting that the putative dipeptides did not contain the phosphorylated residue at the amino terminus. As seen in Fig. 5, analysis of the material in the aqueous phase after one cycle of phenylisothiocyanate yielded phosphoserine with a small amount of phosphothreonine, together with some material that migrates to the same position as the major cluster of putative dipeptides seen in Fig. 4. This result is entirely consistent with the hypothesis that most of the original starting material was dipeptides of the form *X*-phosphoamino acid and that the Edman degradation was incomplete.

DISCUSSION

The labeled fragments of cytoplasmic NS protein produced by chemical cleavage at tryptophan residues with NCS show that the majority of phosphate groups in this

protein are located on residues in the amino-terminal half of the molecule. The results of trypsin hydrolysis suggest that most of this phosphorylation is located on the large trypsin-resistant fragment predicted from the sequence data of Gallione et al. (8). Essentially the same result was obtained for the Piry and New Jersey serotypes as is described for NS protein from Indiana VSV in either L or KB cells. This consistency supports the notion that phosphorylation in the amino-terminal half of the molecule is structurally significant to the NS phosphoprotein. These conclusions regarding the cytoplasmic NS protein are corroborated by the studies of Hsu and Kingsbury on viral protein NS1 (in press; personal communication). Although our studies were carried out on total cytoplasmic NS protein, we observed the same major phosphorylation pattern for cytoplasmic NS proteins either in the free or RNP-bound state and on occasion observed minor phosphorylation at other sites in the molecule as well (data not shown). Our results would support the hypothesis of Hsu and Kingsbury (personal communication) that the phosphorylation sites identified in these studies correspond to core phosphorylations exhibited by all NS proteins.

The ^{32}P-labeled products of acid hydrolysis of NS protein include phosphoserine, phosphothreonine, and phosphopeptides. The phosphopeptides can be further subdivided by electrophoresis at pH 1.9 into positively charged and neutral fractions. The work of Jones and Olson (11) with other phosphoproteins showed that the neutral fraction consisted of a heterogeneous population of molecules of the form *X*-phosphoamino acid where *X* is any amino acid. The sequence data of Gallione et al. (8) shows that six of the seven serines and all four threonines in the amino-terminal half of the NS protein have a distinctive amino-terminal neighbor. Our analysis of the radiolabeled species constituting the neutral fraction from acid hydrolysates of NS protein shows that at least six distinct phosphorylated species can be resolved. Since Edman degradation of the putative phosphodipeptides yielded only a small fraction of phosphothreonine compared with phosphoserine, most of the dipeptides must contain the latter phosphoamino acid. Since the tryptic digest suggests that all of the phosphates are on the large trypsin-resistant fragment and since this fragment contains five serine residues, we would conclude that at least three and perhaps all of these residues are capable of being phosphorylated. By using synthetic marker dipeptides it should be possible to determine the nearest neighbor residue in each dipeptide. Work to this end is in progress.

Finally our observations present formal chemical proof that NS protein which migrates on SDS gels with an apparent molecular mass of 40 to 60 kilodaltons is in fact a monomer of the sequence described in Fig. 1. Incomplete NCS cleavage of NS protein produces no more than four cleavage fragments (Fig. 2). Even the simplest dimer of NS, one joined by a cross-link in the carboxy-terminal fragment, would be expected to generate eight new partial cleavage products. The number of labeled fragments observed by cleavage of NS uniformly labeled with [^{35}S]methionine is also totally inconsistent with the dimer model.

The observed molecular weights of the amino terminus-containing fragments show the same anomoly as complete NS protein. It seems, therefore, that the aberrant electrophoretic mobility is associated with a region in the amino-terminal half of the molecule. The relatively lesser amounts of the 31K and 34K fragments may be the result of reduced cleavage at tryptophan residues 136 and 152. It is known that the efficiency of NCS mediated cleavage is influenced by the local amino acid environment (14).

The fragments of 18K, 14K, and 8K resulting from cleavage of uniformly labeled NS protein correspond to three of the four predicted carboxy terminus-containing fragments (Fig. 1, upper four fragments of group B). Since the 14K fragment is generated by cleavage at tryptophan residue 152, a site we suggested was poorly cleaved in the native protein, we must postulate that this site is efficiently cleaved after prior cleavage at tryptophan residue 108. We cannot, at this time, explain our failure to observe a significant amount of the predicted 10K, 6K, and 5K labeled fragments. Although the gel presented in Fig. 2 does not include the lower range, we have never observed a labeled fragment in a position equivalent to 5K or 6K. Our observations are most compatible with a NS molecule of the type presented in Fig. 1 but lacking the methionine residues at positions 168 and 184.

Our data are taken from studies with the HR Toronto strain of the Indiana serotype, whereas the sequencing data is from the San Juan strain. Whether this is the source of discrepancies between the observed and predicted results remains to be determined. In any case our conclusions are derived from sequences in the amino terminus and are not affected by the noted discrepancies.

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