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NS Phosphoprotein of Vesicular Stomatitis Virus: Subspecies Separated by Electrophoresis and Isoelectric Focusing

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The NS protein of vesicular stomatitis virus is the only phosphorylated nucleocapsid protein. The amount of NS phosphorylation appears to regulate the activity of the protein in the transcription of the virus genome. Several methods have been used to separate NS subspecies containing different amounts of phosphate, but the relationships among the subspecies separated by different workers have been unclear. We report that the isoelectric points of NS molecules were abnormally acidic in some commercial ampholytes, but favorable ampholytes resolved multiple phosphorylated NS subspecies with isoelectric points ranging from pH 6.8 to 7.2. The most highly phosphorylated NS molecules had more acidic isoelectric points, and they exhibited greater electrophoretic mobilities in two previously employed electrophoretic systems.

NS is the major phosphorylated protein species in vesicular stomatitis virus (VSV) virions and infected cells. The protein functions in virus-specific RNA synthesis, and a regulatory role of phosphate in the function of the protein has long been suspected (11). Recently, direct evidence for such a role was obtained by Kingsford and Emerson (7), who separated several forms of NS containing different amounts of phosphate and found that the most highly substituted species are significantly more active in a reconstituted cell-free viral transcription system. Independently, Kingsbury et al. (6) showed that enzymatic dephosphorylation of nucleocapsid-associated NS molecules markedly reduced the rate of in vitro transcription.

The exact number of phosphorylated NS subspecies is not known, but there is potential for a great variety: the deduced amino acid sequence indicates that the commonly phosphorylated residues, serine and threonine, constitute 33 of the 222 residues in the molecule (4). In attempting to sort out NS subspecies, different workers have used different separation methods, and the relationships among the fractions obtained have been unclear (1-3, 5, 7, 8). In addition, one procedure, isoelectric focusing, gave anomalous results: NS molecules containing the most phosphate had the least acidic isoelectric points (5). Therefore, we systematically compared several of these methods. We have learned that the isoelectric focusing behavior of the NS becomes rational in appropriate ampholytes and that the separation obtained by isoelectric focusing can be related logically to the NS subspecies separated by two methods of polyacrylamide gel electrophoresis (3, 7).

Suspecting that the abnormal isoelectric focusing of NS molecules reflects ampholyte-protein interactions, we examined the behavior of NS molecules in ampholyte preparations from several manufacturers. In all of the ampholyte mixtures tested, with the exception of Pharmalytes, purchased from Pharmacia Fine Chemicals, Inc., after 1979, NS molecules reached equilibrium between pH 5.0 and 4.0, as previously reported (5). In contrast, Pharmalyte samples purchased in 1980 (Table 1) resolved NS in two-dimensional O'Farrell (9, 10) gels (Fig. 1A) as a heterogeneous band, from pH 7.2 to 6.8, just beneath the three major subspecies of nucleocapsid structure unit N. According to Pharmacia, there have been no changes in the manufacturing process, but lots of Pharmalyte made since 1979 are more highly purified (J. Ritchie, personal communication). It is noteworthy that NS is the only VSV protein that has exhibited markedly different behavior in different ampholyte systems. The isoelectric focusing patterns of proteins G and N, which are both heterogeneous, are noticeably sharper in recent lots of Pharmalyte, but these proteins occupy the same pH ranges in all ampholytes that we have tested (5, 10).

Evidence for the accuracy of the isoelectric pattern of NS molecules shown in Fig. 1A is presented in Fig. 1B and C. Progressive dephosphorylation with increasing amounts of bacterial alkaline phosphatase (BAP) produced a cathodic shift of the cohort of NS molecules relative to

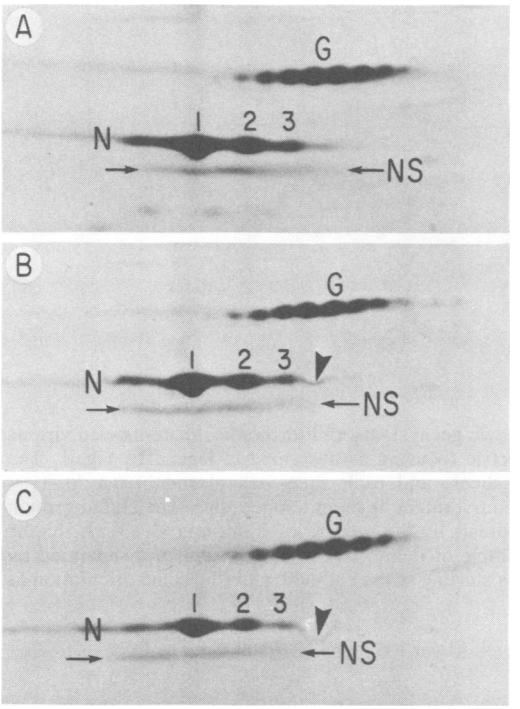

FIG. 1. Isoelectric focusing of VSV proteins. [^{35}S]methionine-labeled virions were disrupted with 1% Triton X-100 in 0.01 M Tris-hydrochloride–0.15 M NaCl (pH 8.0) and incubated with BAP (Worthington Diagnostics) at 30°C for 1 h in the presence of the protease inhibitor Aprotinin (6). Virus proteins were precipitated by 9 volumes of ethanol, collected by centrifugation, and dissolved in 0.1 M Tris-hydrochloride (pH 6.8) containing 5% SDS. Samples were incubated at 60°C for 20 min, cooled to room temperature, and saturated with urea crystals to displace the SDS. We added 2 volumes of isoelectric focusing sample buffer (10) and layered 20-μl portions onto polyacrylamide gels containing a mixture of ampholytes (Table 1). Other conditions and the procedure for second-dimension electrophoresis are given elsewhere (5, 10). Although the pH range of the isoelectric focusing was from 8.0 to 4.0, only the relevant portion of each autoradiogram is shown, from pH 7.6 at the left to pH 6.1 at the right. G, N, and NS designate VSV polypeptide species. The horizontal arrows indicate the limits of the band of NS components. Arrowheads indicate the position of BAP. Numerals 1, 2, and 3 refer to charge variants of the N protein (5). (A) Control, not treated with BAP; (B) 200 μg of BAP per ml; (C) 800 μg of BAP per ml.

the other proteins. BAP itself, although not labeled, made its presence known by displacing a group of radioactive N molecules that focused at a slightly more acidic pH than that at which component 3 of the N protein focused (arrowheads, Fig. 1B and C).

A more dramatic example of the effect of

TABLE 1. Ampholytes giving accurate isoelectric focusing of VSV proteins

pH range	Relative vol[a]	Lot no.[b]
3.5–10	1	EF 11760
4.0–6.4	2	EF 12920
6.0–8.0	2	EF 12688

[a] Proportion in polyacrylamide isoelectric focusing gels (Fig. 1 through 3).

[b] Lot numbers of Pharmalyte (Pharmacia Fine Chemicals).

enzymatic dephosphorylation is shown in Fig. 2, which shows a gel in which the second dimension was modified to separate NS molecules into two electrophoretic classes (7). The class that migrated more rapidly in electrophoresis, which we designated NS2 (see below), was converted to the slower class, NS1, by BAP treatment, in agreement with previous findings (6); the converted molecules participated in the shift of the NS1 band to the cathode. The disappearance of NS2 was due only to changes in its electrophoretic and isoelectric focusing behaviors: total NS protein, relative to N or G protein, did not change after BAP treatment (6; unpublished data).

To improve resolution in the second dimension and to make a direct comparison between

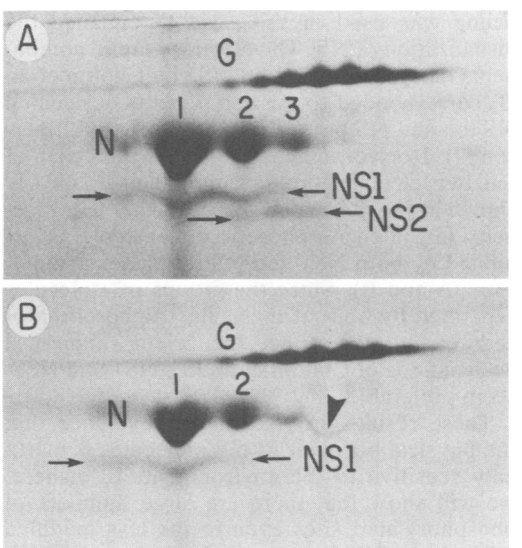

FIG. 2. Separation of NS molecules in two dimensions. [^{35}S]methionine-labeled virions were treated with BAP, and isoelectric focusing was performed as described in the legend to Fig. 1. However, in this case, electrophoresis in the second dimension was performed as described by Kingsford and Emerson (7). (A) Control; (B) 800 μg of BAP per ml. The designations are explained in the legend to Fig. 1.

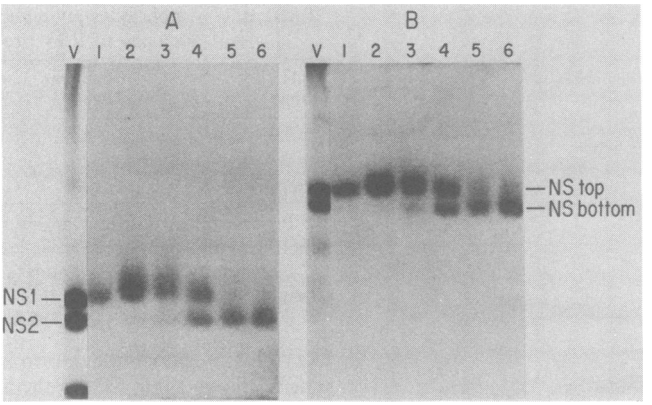

FIG. 3. Electrophoretic separation of NS subspecies in two gel systems. [^{32}P]orthophosphate-labeled virions (5) were disrupted with 5% SDS and subjected to isoelectric focusing as described in legend to Fig. 1. The cylindrical isoelectric focusing gel was cut into 5-mm slices, and each slice was placed in 0.1 M Tris-hydrochloride (pH 8.0) containing 0.1% SDS and incubated overnight at room temperature. The eluted protein was precipitated with ethanol and subjected to electrophoresis by the method of Clinton et al. (3) (A) or the method of Kingsford and Emerson (7) (B). V, Control sample of radioactive virion proteins not separated by isoelectric focusing; lanes 1 through 6, successive gel slices starting at the cathodic end (the same orientation as in Fig. 1 and 2).

two electrophoretic systems that separate NS molecules into two classes (3, 7), we sliced an isoelectric focusing gel perpendicular to the pH gradient; then we eluted the proteins and electrophoresed them (Fig. 3). Radiophosphate labeling was used in this case to enhance the visualization of NS. The electrophoretic components designated NS1 and NS2 by Clinton et al. (3) corresponded to the components designated NS top and NS bottom by Kingsford and Emerson (7). Isoelectric heterogeneity within each of the two electrophoretic components is clearly shown in Fig. 3A and B. Also, further heterogeneity in the electrophoretic dimension was exhibited by both NS1 and NS top (lanes 2 and 3, Fig. 3A and B). Since the groups of molecules separated by both of these electrophoretic procedures are the same, the briefer numerical designations of Clinton et al. (3), NS1 and NS2, seem preferable.

These results provide further evidence that the physical behavior of NS molecules is markedly sensitive to the environment. Elsewhere, we will show that there are large numbers of phosphorylated sites even in the less modified NS1 class of molecules (C.-H. Hsu and D. W. Kingsbury, submitted for publication); perhaps the high density of negative charges in NS molecules favors ionic binding to basic groups in some types of ampholyte molecules, producing the anodic shift that we and others have observed (1, 5, 8). A high density of negative charges may also affect the conformation of NS and may explain its abnormal electrophoretic migration in sodium dodecyl sulfate (SDS) gels (4).

The isoelectric focusing patterns shown here indicate that there are many NS subspecies that differ from one another in extent of phosphorylation. This conclusion is supported by previous chromatographic and electrophoretic separations (7) and by phosphopeptide analyses that have identified a large number of phosphorylated sites in the NS population (Hsu and Kingsbury, submitted for publication). Further investigation of the chemical basis of this heterogeneity and its functional consequences should be informative. However, sites that determine the electrophoretic behavior of subclasses NS1 and NS2 occur in two readily distinguished groups of peptides (Hsu and Kingsbury, submitted for publication), and the two subclasses seem to be functionally distinct (6, 7); therefore, the electrophoretic separation is not fortuitous and is likely to be useful for many purposes.

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LITERATURE CITED

1. **Bell, J. C., and L. Prevec.** 1979. Proteins of vesicular stomatitis virus. V. Identification of precursor to the phosphoprotein of Piry virus. J. Virol. **30**:56–63.
2. **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.

3. **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.
4. **Gallione, C. J., J. R. Greene, L. E. Iverson, and J. K. Rose.** 1981. Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus N and NS proteins. J. Virol. **39**:529–535.
5. **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.
6. **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 virus. Developments in cell biology, vol. 7. Elsevier/North-Holland Publishing Co., New York.
7. **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.
8. **Maack, C. A., and E. E. Penhoet.** 1980. Biochemical characterization of the tsE1 mutant of vesicular stomatitis virus (New Jersey). Alterations in the NS protein. J. Biol. Chem. **255**:9249–9254.
9. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
10. **Raghow, R., A. Portner, C.-H. Hsu, S. B. Clark, and D. W. Kingsbury.** 1978. Charge heterogeneity in polypeptides of negative strand RNA viruses. Virology **90**:214–225.
11. **Sokol, F., and H. F. Clark.** 1973. Phosphoproteins, structural components of rhabdoviruses. Virology **52**:246–263.