Cloning and Expression of a Viral Phosphoprotein: Structure Suggests Vesicular Stomatitis Virus NS May Function by Mimicking an RNA Template

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SUMMARY

The phosphoprotein (NS) gene from the Indiana serotype of vesicular stomatitis virus (VSV; Mudd-Summers strain) was cloned and sequenced. The NS gene encodes a protein of 265 amino acids which was expressed from a simian virus 40 vector in COS cells. The post-translational modification characteristic of viral NS, the extensive phosphorylation of a cluster of serine and threonine residues, was also evident in recombinant NS protein. The NS gene displays a property common to the phosphoprotein genes of negative-strand RNA viruses: the phosphoprotein mRNA has a second open reading frame (ORF) which could encode a small (7500 mol. wt.) protein. Both measles virus and Sendai virus employ the second ORF of their phosphoprotein gene, and the resultant proteins have an amino acid composition similar to that predicted for the VSV ORF. Comparison of phosphoproteins from different VSV strains revealed two conserved domains that we propose are critical for the function of NS in transcription and replication.

INTRODUCTION

Negative-strand RNA viruses encapsidate their genome in ribonucleoprotein particles (RNP) (Baltimore, 1971; Wagner, 1975) which can support viral transcription and replication. In vesicular stomatitis virus (VSV), these particles contain three of the five VSV proteins: the polymerase (L), the nucleocapsid protein (N) and the phosphoprotein (NS). All three proteins are absolutely required for viral transcription and replication. The majority of the protein in RNP is N, which tightly complexes with VSV RNA to form ribonuclease-resistant structures (Arnheiter et al., 1985; Blumberg et al., 1981, 1983). The remaining two proteins, L and NS, constitute the polymerase complex. The large size (241 000 mol wt.) and low abundance (approx. 50 molecules/RNP) (Harmon et al., 1985; Thomas et al., 1985) of L has recommended this protein for the catalytic functions of polymerization, capping, methylation and polyadenylation (Schubert et al., 1984; for review, see Rose & Schubert, 1987). The precise function of NS is unknown, although a structural role is suggested by its abundance in VSV-infected cells. The phosphoprotein specifically binds near the termini of VSV RNA, which implies a role in the initiation of transcription and replication (Isaac & Keene, 1982; Keene et al., 1981).

To define the role of NS in the polymerase complex, we have cloned and sequenced the phosphoprotein gene and examined its expression in eukaryotic cells. Analyses of the protein structure disclose several features strongly conserved among the four VSV phosphoprotein genes (this study; Gallione et al., 1981; Gill & Banerjee, 1985; Rae & Elliott, 1986) for which sequence data are available. We propose that the structure of two conserved domains reflects their functional interactions between the three other components of the VSV transcriptional/replica-

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tional apparatus: the RNA template, N and L. Of particular interest is a heavily phosphorylated, extremely acidic domain near the amino terminus of NS. We speculate that this domain presents a novel structure that imitates the phosphate backbone of RNA and is, therefore, capable of binding the nucleocapsid protein. Interactions between N and the phosphorylated NS domain could affect two processes. First, during elongation, NS could displace tightly bound N from the template in a localized fashion, thereby allowing the polymerase to gain access to the RNA. Second, NS can also complex with N in the cytoplasm of virus-infected cells (Bell et al., 1984; Peluso & Moyer, 1984) possibly to prevent unassembled N from self-aggregating, which occurs extensively in the absence of viral proteins (Blumberg et al., 1983; Sprague et al., 1983). Since the availability of cytoplasmic, unassembled N regulates the switch from transcription to replication (Arnheiter et al., 1985; Blumberg et al., 1981), a mechanism for maintaining N in a soluble form is required.

**METHODS**

Construction of NS expression vectors. Genomic 42S RNA isolated from the Indiana serotype of VSV (Mudd-Sommers strain) was fractionated on 10 to 30% (w/w) sucrose density gradients containing 0.5% SDS. First strand synthesis was carried out as previously described (Sprague et al., 1983) using a heat-denatured primer isolated from the 3' end of the N gene (the 70 bp *Stul*-Sa*au*3AI fragment derived from pNF4, a gift of J. Rose). Reverse transcriptase (Life Sciences) was used for both first and second strand synthesis. The resultant double-stranded cDNA was briefly treated with S1 nuclease (Boehringer Mannheim), tailed by terminal transferase (P-L Biochemicals) with [3H]dCTP (New England Nuclear) and annealed to *Pst*I cut, dG-tailed pBR322 (New England Nuclear). Competent HB101 *Escherichia coli* cells were transformed and screened on tetracycline-containing plates with nick-translated primer. Over 10% of the clones hybridized to the primer; one such positive clone (pLH1) with a 1500 bp *Pst*I insert contained the 3' end of the N gene, the entire NS gene and half of the M gene. The NS gene and its intergenic regions were sequenced by the chemical method of Maxam & Gilbert (1980) by the strategy indicated in Fig. 1.

Two NS constructs were inserted into the pJC119 expression vector, a pML2 derivative which employs the simian virus 40 (SV40) late promoter to initiate transcription of inserted sequences and also supplies SV40 splicing and polyadenylation signals (Sprague et al., 1983). The entire NS coding region (an 800 bp *EcoRV*-AciI fragment, positions 22 to 822 in Fig. 1) was filled in and ligated to *Xho*I linkers, then ligated to *Xho*I-digested, phosphatase-treated pJC119. Both orientations of the insert were recovered in plasmids from transformed HB101 cells (pLH7, correct orientation). A fragment of NS (from positions 1 to 707 of Fig. 1) which was deleted for the last 112 bases of coding sequence was similarly cloned into the *Xho*I site of pJC119 (pLH3, correct orientation).

Transfection of COS cells. Supercoiled plasmids (10 μg/ml) were introduced into COS cells, a simian line that constitutively expresses T antigen, by the method of Parker & Stark (1979). One to 3 days following transfection, cells were prepared for immunofluorescence or immunoprecipitation. For labelling experiments, either [14S]methionine [New England Nuclear; 20 μCi/ml in low methionine (15 μg/ml) medium with 5% foetal calf serum] or [32P] (New England Nuclear; 25 μCi/ml in phosphate-free medium with 5% foetal calf serum) was added. The NS complexes were collected and washed by centrifugation, solubilized in SDS with 2-mercaptoethanol and electrophoresed on highly cross-linked polyacrylamide gels (9% acrylamide, 0.45% bisacrylamide). For complementation assays, transfected cells were superinfected with the temperature-sensitive NS (NS8) mutants G22 or 052 (Deutsch & Brun, 1978; Lafay & Benejean, 1981; Metzel & Reichmann, 1981) at a m.o.i. of approximately 1, 1 to 2 days following transfection. Virus incubation was carried out at 40°C for 4 to 5 h before the cells were washed with phosphate-buffered saline and fixed with methanol and acetone. For dual labelling, slides were successively stained with the following antibodies: (i) rabbit polyclonal anti-N antibody, (ii) anti-rabbit IgG, rhodamine conjugate (Cappel Laboratories), (iii) mouse monoclonal anti-NS antibody (either 2F2, 5D9 or 6D8, gift of M. Williams and S. Emerson) and (iv) anti-mouse IgG, fluorescein conjugate (Cappel Laboratories). Control stainings were performed on wild-type VSV-infected COS cells, NS mutants infected at 30°C and 40°C, and COS cells transfected with plasmids containing NS inserted in the backwards orientation. Fluorescence was examined with a Zeiss fluorescence microscope.

**RESULTS**

Sequence of the cloned NS and intergenic regions

NS clones were isolated from a VSV library constructed by priming genomic RNA (approx. 11 kb) with a restriction fragment from the gene directly upstream of NS (as detailed in
VSV NS protein structure and function

Fig. 1. Sequence of the Indiana (Mudd-Summers strain) NS. The amino acid sequence is shown directly below the nucleotide sequence, with the differences between the Mudd-Summers and San Juan (Gallione et al., 1981) strains noted under the appropriate amino acid. The second open reading frame present in NS mRNA is underlined. The intergenic dinucleotides are dotted. The sequencing strategy outlines the locations of the restriction sites used for end labelling (■) and the direction and extent of sequence generated from that site (arrows).

Methods. The sequence of the coding and flanking regions of one such clone (pLH1) is presented in Fig. 1. It should be noted that the protein has a coding capacity of 265 amino acids, not the 222 amino acids originally reported for the Indiana serotype (Gallione et al., 1981; J. Rose, personal communication). The Mudd-Summers strain of the Indiana serotype of VSV (Fig. 1) differed at 23 of the 795 bases in the coding region from the revised San Juan strain sequence of Rose and co-workers. Ten of these base changes resulted in an amino acid change, as shown in Fig. 1. Three of the amino acid differences altered the charge of the protein (His → Tyr at amino acid 151; Lys → Glu at amino acid 170; Asn → Asp at amino acid 237).

The N:NS and NS:M intergenic regions were sequenced to determine whether the unusual GU dinucleotide observed at the San Juan strain NS:M junction (Gallione et al., 1981) was unique to the NS:M junction or was simply another variation of the San Juan strain. The spacer regions of VSV were well conserved and included a sequence (5'-UAUGAAAAAAA-3') present at the mRNA poly(A) junction in each mRNA, followed by the CU dinucleotide whose complement did not appear in mRNA and constituted the intergenic region (Rose, 1980). The N:NS junction featured the conserved sequence (Fig. 1), but the NS:M intergenic region of the Mudd-Summers strain of VSV was identical to that of the San Juan strain. These results document the unique NS:M intergenic sequence that may be responsible for the inefficient
Table 1. Amino acid composition of the second open reading frame encoded by NS mRNA

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<tr>
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<th>Indiana serotype</th>
<th>New Jersey serotype</th>
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<tr>
<td></td>
<td>Mudd-Summers (%)</td>
<td>San Juan* (%)</td>
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<td></td>
<td>Ogden† (%)</td>
<td>Hazlehurst‡ (%)</td>
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</tr>
<tr>
<td>Serine and threonine</td>
<td>13</td>
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* Gallione et al. (1981).
† Gill & Banerjee (1985).
‡ Rae & Elliot (1986).

termination observed at this site, as reflected in the production of transcripts containing both NS and M (Herman et al., 1980).

NS mRNA may encode a second protein

A common feature of VSV NS genes is a second open reading frame (ORF; underlined in Fig. 1 for the Indiana serotype, Mudd-Summers strain) which could encode a small (65 or 67 amino acid) basic protein. The phosphoprotein genes of two other negative-strand viruses, measles and Sendai viruses, also contain other ORFs; moreover, these internal ORFs are translated efficiently in vitro and during viral infection (Bellini et al., 1985; Giorgi et al., 1983; Curran et al., 1986). Although the precise function of this class of protein (named C protein) is unknown, the co-localization of the measles C protein with the nucleocapsid protein suggests a role in replication or transcription (Bellini et al., 1985). The amino acid composition of the second ORF encoded by NS mRNA is presented in Table 1. Despite their nucleotide sequence divergence, all four NS genes sequenced (Fig. 1; Gallione et al., 1981; Gill & Banerjee, 1985; Rae & Elliott, 1985, 1986) have the potential to encode proteins which have a remarkably similar amino acid composition. Moreover, this pattern is exhibited by the basic measles virus C protein (Bellini et al., 1985): 16% basic amino acids, 7% acidic amino acids, 44% hydrophobic amino acids, 18% serine plus threonine. As is the case for the NS protein, the protein coded by the second ORF displays a high degree of amino acid identity within a serotype (93% for Indiana strains and 88% for the New Jersey strains), while only a low degree of conservation between serotypes (27%, considering conservative amino acid replacements). The predicted proteins are too small (approx. 7500 mol. wt.) to have been detected on the standard composition gels previously used to visualize VSV proteins.
Expression of NS in COS cells

The full-length NS gene subcloned into an SV40 expression vector, pJC119, was transfected into COS cells. NS protein was detected 1 to 3 days following transfection by immunofluorescence, as shown in Fig. 2. All three NS monoclonal antibodies, which react with different epitopes of the NS molecule (M. Williams & S. Emerson, personal communication), stained transfected cells. The NS staining appeared diffuse throughout the cytoplasm, in contrast to the combination of particulate and partially diffuse NS staining in VSV-infected cells previously noted by Ohno et al. (1985). NS produced in a viral infection was present both in nucleocapsids (particulate staining) and a free cytoplasmic pool (diffuse staining), while NS synthesized in a transfection was found, as expected, only as a cytoplasmic pool. No staining was detectable in cells transfected with NS cloned in the backwards orientation in pJC119.

To determine whether the NS produced in transfected cells was phosphorylated, cells were labelled with $[^{35}S]$methionine or $^{32}$P, and the immunoprecipitated NS was resolved on polyacrylamide gels. Although only 2 to 5% of cells were transfected and these cells produced approximately fourfold less NS than VSV-infected cells, $^{32}$P-labelled NS could be detected in transfected cells (1.5% of labelled protein; Fig. 3). Methionine-labelled NS was not reproducibly detected in transfected cells, despite the presence of four methionines in the protein. Transfected cells displayed phosphorylated NS of the same mol. wt. as the major form found in VSV-infected COS cells (Fig. 3, lanes 2 and 4). An additional higher mol. wt. band was also specifically precipitated from transfected cells, and may have represented another phosphorylation state of the expressed NS. No bands were apparent in uninfected COS cells or in cells transfected with the shortened NS plasmid (pLH3). At present, we do not know whether the failure to detect the truncated NS was due to a diminished phosphorylation of the protein or a reduced intracellular concentration of protein compared to full-length NS.

Complementation of NS<sup>ts</sup> VSV mutants

To assay for function of the expressed protein, we tested whether the transfected NS could complement the defective/absent NS of NS<sup>ts</sup> VSV mutants. Cells transfected with the NS expression vector (pLH7) were superinfected with either of two NS<sup>ts</sup> VSV mutants and examined by dual label fluorescence. None of the cells which expressed NS displayed staining for other VSV proteins (N, L or G) at 1 to 2 days post-transfection. The absence of complementation could be due to incompletely phosphorylated or insufficient NS, or to a distortion in the ratio of NS to the other viral proteins. Indeed, a critical balance of viral proteins has been noted by M. Schubert and co-workers in complementation assays with recombinant L protein (Schubert et al., 1985).

DISCUSSION

Recombinant NS protein was expressed in COS cells, as was evident from immunofluorescent staining with three NS monoclonal antibodies (Fig. 2) that recognize different portions of the NS protein. Expressed NS was phosphorylated and displayed the same mobility as NS from
Fig. 3. Phosphorylation of NS expressed in transfected cells. Immunoprecipitates of \(^{32}\)P-labelled extracts from mock-transfected COS cells (lane 1), VSV-infected COS cells (lane 2), pLH3-transfected COS cells (lane 3) and pLH7-transfected COS cells (lane 4) are shown. The M protein, which is usually co-precipitated with NS from infected cells and displays a phosphorylated form, is 26000 mol. wt. NS migrates at 43000 mol. wt. on these gels.

Fig. 4. Conserved features of the NS protein. The 265 amino acid sequence of NS is shown in linear form with the phosphorylated portion of Domain I (P) and the positively charged Domain II expanded below. Asterisks mark the phosphorylated positions in the Indiana serotype of VSV (Bell & Prevec, 1985; Hsu & Kingsbury, 1985; Marnell & Summers, 1984); + marks the positively charged residues of Domain II. Identical (\(\square\)) and similar (\(\square\)) amino acids between the Indiana (Mudd-Summers and San Juan strains) and New Jersey (Ogden strain; Gill & Banerjee, 1985) serotypes are noted. The Hazlehurst strain of New Jersey differs from the Ogden sequence of Domain II at position 252, which is a histidine in the Hazlehurst subtype (Rae & Elliott, 1986).

VSV-infected cells on SDS-polyacrylamide gels (Fig. 3). Previous estimates indicated that NS migrates as multiple species in the 40000 to 50000 mol. wt. range on acrylamide gels (Clinton et al., 1978; Kingsford & Emerson, 1980). The multiple species reflect different phosphorylation states of the protein (Clinton et al., 1978; Hsu et al., 1982; Kingsford & Emerson, 1980), while the anomalous mobility of this protein (expected 30000 mol. wt.) may result from the presence of an extremely negatively charged domain (Domain I of Fig. 4) and as yet undefined structural features of the protein. The major species of NS in virus-infected cells is phosphorylated at the sites indicated in Fig. 4 (Domain I), and these appear to be constitutively phosphorylated in all
NS molecules (Bell & Prevec, 1985; Hsu & Kingsbury, 1985; Marnell & Summers, 1984). The other forms of NS have additional, more labile phosphates that are readily lost during isolation or by phosphatase treatment (Bell & Prevec, 1985; Hsu & Kingsbury, 1985; Hsu et al., 1982). Hyperphosphorylated forms of NS predominate in the virion which has an associated kinase activity (Bell et al., 1984; Witt & Summers, 1980). The recombinant NS protein was phosphorylated in the absence of any VSV proteins, probably at the constitutive sites, by a cellular protein kinase.

The NS sequence of the Indiana serotype, Mudd–Summers strain of VSV differs from the San Juan strain (Gallione et al., 1981) at 3% of the bases (3.8% of the amino acids). This amount of divergence is not unusual for different isolates of a VSV serotype (Rose & Schubert, 1987). However, Indiana serotype NS is remarkably different from New Jersey NS; the two proteins are only about 30% homologous (Fig. 1; Gallione et al., 1981; Gill & Banerjee, 1985; Rae & Elliott, 1985, 1986). The striking divergence between the two proteins highlights the few common features that may be critical for NS function. These are illustrated in Fig. 4. All NS proteins have two characteristic domains: Domain I, an extremely acidic region near the amino terminus of the protein, which is flanked by the major sites for phosphorylation of the protein, and Domain II, the basic carboxy terminus in which the amino acid sequence is well preserved between strains (as previously noted by Gill & Banerjee, 1985). Domain I is composed of a stretch of 38% negatively charged amino acids in which only the phosphorylated region displays amino acid conservation between strains (Fig. 4). Several groups have identified the phosphorylated residues in NS (as noted in Fig. 4) and shown that the New Jersey and Indiana serotypes are phosphorylated in the same region (Bell & Prevec, 1985; Hsu & Kingsbury, 1985; Marnell & Summers, 1984). Despite the lack of amino acid homology in the remainder of the NS molecule, the relative charge distribution and hydropathy are preserved between serotypes (Gill & Banerjee, 1985; Rae & Elliott, 1986).

We outline two structural roles for NS in VSV transcription and replication, and propose that the two domains shown in Fig. 4 mediate these functions. NS might initiate replication or transcription by binding to a specific site on genomic or anti-genomic VSV RNA, as previously suggested by Keene and co-workers (Isaac & Keene, 1982; Keene et al. 1981). This site was identified by foot-printing as a 15 base region located 16 nucleotides from the 3' terminus of genomic and anti-genomic RNA (Isaac & Keene, 1982; Keene et al. 1981). According to our model, NS bound to the initiation site would be recognized by L, which is unable to bind template in the absence of NS (Mellon & Emerson, 1978), and polymerization could commence. The carboxy terminus of NS (Domain II in Fig. 4) is a likely site of interaction with the RNA template and the VSV polymerase because it is the only region of conserved amino acid sequence (90% homology between strains; Gill & Banerjee, 1985). Since the sequence of the VSV RNA termini containing the putative initiation site does not vary between VSV strains (Rose & Schubert, 1987), one would predict that the NS domain which binds RNA would be similarly preserved. Also, the carboxy terminus is very basic, as anticipated for a nucleic acid-binding domain. Although no common structural features have yet been identified for protein domains that bind RNA, it is worth noting that the carboxy domain contains an eight amino acid stretch (from residue 255 to 262) that is conserved between two prokaryotic anti-terminators, which also interact both with polymerase and RNA (Franklin, 1985).

The second function proposed for NS is to bind the nucleocapsid protein that enwraps the VSV template. Transcription and replication in negative-strand RNA viruses require that the polymerase functions on a template that is continually coated with nucleocapsid (N). There are approximately 1300 N molecules spread throughout an RNP (Thomas et al., 1985), which corresponds to 1 nucleocapsid protein per 8 or 9 bases of RNA. We speculate that the heavily phosphorylated NS can mimic an RNA template and temporarily displace N from the template to facilitate polymerase entry. As the NS : L complex traverses the template, the local denuding of RNA accomplished by nucleocapsid binding primarily to the phosphorylated, extremely acidic Domain I of NS would allow the polymerase to transcribe RNA. In fact, the N protein appears to bind RNA primarily via the phosphate moieties (Isaac & Keene, 1982; Keene et al. 1981); therefore, the heavily phosphorylated NS domain might be expected to attract N
similarly. Highly phosphorylated forms of NS have been isolated which, in addition to the core, constitutively phosphorylated residues shown in Fig. 4, have uncharacterized phosphorylated residues. The hyperphosphorylated forms have increased activity in transcription assays (Hsu et al., 1982; Kingsford & Emerson, 1980) possibly due to an enhanced ability to interact with nucleocapsids. There are 11 possible phosphorylation sites (serine or threonine residues) conserved between the New Jersey and Indiana serotypes in the region between Domains I and II (Fig. 4). The regular spacing of these conserved sites is of interest since phosphorylation at these positions would result in an NS molecule that would more closely mimic an RNA structure.

Complexes between N and NS have been detected in the cytoplasm of VSV-infected cells (Bell et al., 1984; Peluso & Moyer, 1984) and in vitro using a combination of monoclonal antibodies directed against N or NS (Arnheiter et al., 1985; Wertz et al., 1985). Purified N or N synthesized in the absence of any viral proteins forms large aggregates (Blumberg et al., 1981, 1983; Sprague et al., 1983), which may not be suitable for assembly into RNP. The binding of unassembled N to the phosphorylated region of NS would discourage aggregate formation, thereby ensuring a supply of N for encapsidation.

Our model suggests that NS is required for both the initiation of transcription and the subsequent elongation of transcripts. Recent electron micrographic observations of negative strand nucleocapsids support a role for the phosphoprotein in elongation (Harmon et al., 1985; Portner & Murti, 1985). The polymerase and NS proteins appeared uniformly distributed throughout the VSV RNP with twice as many NS molecules than L molecules present per RNP (Harmon et al., 1985). A possible dimeric structure for NS is of interest since the NS binding site (Keene et al., 1981) consists of two 5 base repeats separated by 4 bases and may therefore provide binding sites for two NS molecules.

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