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Two separate domains within vesicular stomatitis virus phosphoprotein support transcription when added in trans

(cloning in pGEM vector/transcription–translation/trans complementation)

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ABSTRACT **The structural phosphoprotein NS of vesicular stomatitis virus, in association with the virion-associated RNA polymerase L protein, transcribes the genome ribonucleoprotein template *in vitro*. It contains an acidic N-terminal domain and two distinct domains at the C-terminal end that are involved in binding to the polymerase protein and the template RNA enwrapped with the nucleocapsid protein. In the present study, the portions of the *NS* gene that encode the N- and C-terminal domains of the protein were cloned in pGEM vectors and expressed by *in vitro* transcription and translation. It was shown that two polypeptides obtained by translation of the encoded mRNAs support RNA synthesis *in vitro* in a reconstitution reaction when they are added together in trans. Moreover, the N-terminal domain can be functionally substituted by structurally similar polypeptides.**

Transcription of the vesicular stomatitis virus (VSV) genome RNA *in vitro* and *in vivo* requires the interaction of the phosphoprotein NS with the virion-associated RNA polymerase L protein and the nucleocapsid N protein enwrapping the viral genome (referred to as the N–RNA template in the text) (1). The NS protein appears to facilitate entry and movement of the L protein on the N–RNA template and allow transcription *in vitro* (2, 3). For optimal transcription of the genome RNA, requirements of the L and the NS proteins appear to be catalytic and stoichiometric, respectively (3). To date, no enzymatic activity has been assigned to the NS protein, although it is an excellent substrate for phosphorylation by the L protein-associated protein kinase and cellular kinases (4, 5). Different phosphorylated states of the NS protein *in vivo* appear to regulate transcription (5).

Recently, using biologically active NS protein synthesized by transcription and translation of a cloned gene and subsequent deletion mapping, we have identified three unique domains within the NS polypeptide (6) that appear to play important roles in the transcription process. Domain I (Fig. 1*A*) is predominantly negatively charged and spans approximately the N-terminal half of the polypeptide. This domain also contains constitutive phosphorylation sites (7–9) and is highly nonhomologous to the corresponding domain in different VSV serotypes (10–12). The function of this domain in the transcription process is unknown. Domain II appears to contain the site involved in binding to the L protein that promotes tight association of the latter with the N–RNA template. Specific phosphorylation of serine-236 and serine-242 within the domain by the L protein-associated protein kinase regulates the binding of the NS protein with the L protein and the N–RNA template, leading to RNA synthesis *in vitro* (13). Domain III, a highly homologous basic domain within the different VSV serotypes, is not required for transcription but is involved in tight association with the N–RNA template. To gain further insight into the functions of the various domains, we separated domain I from domains II and III by molecular cloning of the specific gene segments, and demonstrated that the polypeptides encoded by the gene segments restore RNA synthetic activity when added together in trans in a transcription-reconstitution reaction. Moreover, domain I can be functionally replaced by similarly charged polypeptides—e.g., tubulin.

MATERIALS AND METHODS

Construction of Plasmid pGEM Containing Domains II and III. The pGEM-NS plasmid DNA was digested with *Eco*RI, extracted with phenol/chloroform, precipitated with ethanol, and subsequently digested with *Bsp*MII. The large fragment without domain I of NS was isolated. Twenty picomoles of the oligonucleotides 5′ AATTCCCATCATGGT 3′ and 5′ GGTAGTACCAGGC 3′ were treated with [γ-^{32}P]ATP and polynucleotide kinase. The labeled phosphorylated oligomers were then annealed, and the double-stranded DNA with *Eco*RI sites at one end and *Bsp*MII sites at the other end was isolated from 20% nondenaturing gel and purified by using a SEPPAK column. The large fragment (*Eco*RI–*Bsp*MII fragment of pGEM-NS) was phosphatase-treated and then ligated with the double-stranded DNA synthesized from the oligomers in a ratio of 1:3, respectively. The ligation mixture was used to transform competent RR1 cells and was plated on LB agar medium containing ampicillin (100 μl/ml). The positive transformants were confirmed by restriction enzyme digestion and by dideoxy sequencing of the entire region according to the Promega Biotec (Madison, WI) protocol.

Transcription, Translation of mRNAs, and Reconstitution of *VSV RNA* Synthesis. *In vitro* transcription reactions were performed with the Riboprobe system as described (6). *In vitro* translation of mRNAs was carried out by using a rabbit reticulocyte lysate, and the protein was purified from ribosomes as detailed (6). Transcription-reconstitution reactions using *in vitro* synthesized proteins, purified L protein, and N–RNA template were carried out as detailed (6).

Binding and Phosphorylation of NS Proteins and Its Derivatives with L Protein and the N–RNA Template. Binding of *in vitro* synthesized NS protein or its derivatives with the N–RNA template in the presence and absence of the L protein was performed as detailed (6). Phosphorylation of these proteins was carried out as described (13).

RESULTS

Expression of Recombinant Plasmids Containing *NS* Gene. The recombinant phage SP6 transcription plasmid pGEM-NS, containing the full-length gene encoding the NS protein

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Abbreviation: VSV, vesicular stomatitis virus.

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(New Jersey serotype) (6), was used to construct two recombinant plasmids containing specific segments of the *NS* gene (Fig. 1*A*). The plasmid pRA-NS (codes for a 27-kDa polypeptide referred to as RA NS protein) contained the entire *NS* gene from which the sequence encoding domain II polypeptide segment was specifically deleted (13). The plasmid pEB-NS (codes for a 10-kDa polypeptide, referred to as EB NS protein) contained only the sequence encoding domains II and III and the 5'-flanking 100 nucleotides (encoding 33 amino acids) of domain II. Each of the above plasmids was linearized by digestion with *Bam*HI restriction enzyme, and run-off transcription products were synthesized and subsequently translated in rabbit reticulocyte lysate. The control plasmid (pGEM-NS) containing the full-length *NS* gene (6) was digested separately with *Bam*HI or *Bst*NI, yielding DNA fragments coding for the full-length NS protein (31-kDa polypeptide, referred to as *Bam*HI NS protein) and a 22-kDa polypeptide (referred to as *Bst*NI NS protein). The migration of the run-off transcripts and their corresponding translation products in PAGE are shown in Fig. 1 *B* and *C*. It is quite apparent from the migration rates of the polypeptides (Fig. 1*C*) that the full-length NS protein (*Bam*HI protein), RA NS protein, and *Bst*NI NS protein with predicted molecular weights of 31, 27, and 22 kDa, respectively, migrated anomalously during electrophoresis (14). In contrast, the EB NS protein (10 kDa) migrated correctly according to its predicted molecular weight. These data support the notion that the acidic amino acids within domain I probably interact nonuniformly with negatively charged $NaDodSO_4$, resulting in the anomalous migration of the polypeptides in polyacrylamide gel electrophoresis (8, 9). The EB NS protein, on the other hand, being highly basic, interacts with $NaDodSO_4$ and migrates appropriately.

Functional Analyses of Truncated NS Proteins. The translated NS polypeptides, obtained as described above, were assayed for their (*i*) capacity to bind to the N–RNA template in the presence and absence of the L protein, (*ii*) degree of phosphorylation with the L protein and the N–RNA template, and (*iii*) RNA synthetic ability when reconstituted with purified L protein and the N–RNA template (6). The control *Bam*HI NS protein and the EB NS protein bound to the N–RNA template efficiently in the presence or absence of the L protein, whereas RA NS and *Bst*NI NS proteins failed to bind to the template under similar conditions (Table 1). In addition, like *Bam*HI NS protein, the EB NS protein was effectively phosphorylated by the L protein–(N–RNA) tem-

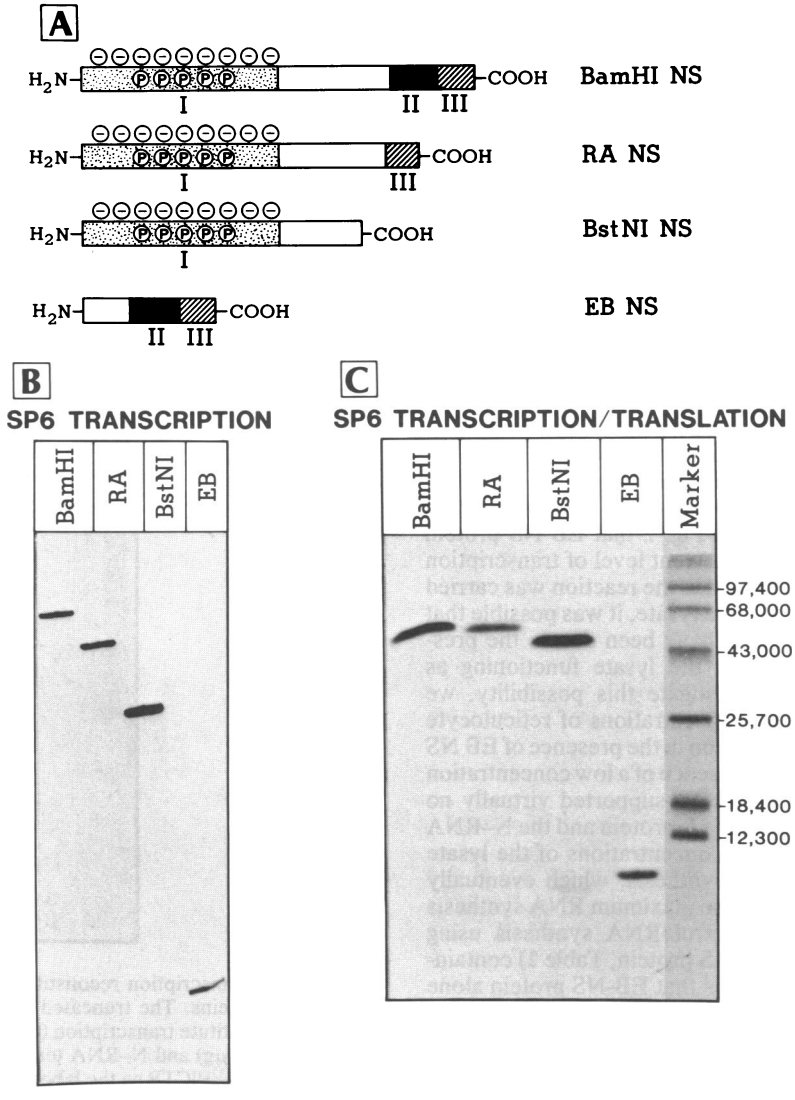

FIG. 1. (*A*) Diagramatic representation of the truncated NS proteins. The various truncated *NS* genes in pGEM-NS vector were transcribed *in vitro* in the presence of [^{3}H]UTP and were subsequently translated in the presence of [^{35}S]methionine as detailed (5). The RNA (*B*) and the protein products (*C*) were analyzed by 7 M urea/5% polyacrylamide gel electrophoresis and $NaDodSO_4$/10% polyacrylamide gel electrophoresis, respectively, and were fluorographed as described (6).

Table 1. Binding, transcription reconstitution, and phosphorylation of various NS constructs

NS construction(s)	NS functional domain(s)	Binding with N–RNA, %		Transcription	Phosphorylation by L/N–RNA
		− L	+ L		
Control NS (*Bam*HI)	I, II, III	70	100	100	+
RA NS	I, III	4	4	0	−
EB NS	II, III	53	90	9	+
*Bst*NI NS	I	5	8	4	−
RA NS + EB NS				66	
*Bst*NI NS + EB NS				30	

plate, whereas the RA NS and *Bst*NI NS proteins were not. These results show that domains II and III, when removed from the NS protein, are still active and bind efficiently to the L protein and the N–RNA template (6). As expected, the RA NS and *Bst*NI NS proteins failed to reconstitute RNA synthesis; however, EB NS protein by itself produced a basal level of RNA synthesis (9%) under similar conditions (Table 1).

However, the important finding was that when RA NS or *Bst*NI NS protein was added in saturating amounts to the reaction mixture containing the EB NS protein, RNA synthesis was stimulated significantly (Table 1 and Fig. 2). RA NS protein was more effective than the *Bst*NI NS protein. These results clearly indicate that two domains within the NS protein have separate functions and act independently with regard to their interactions with the L protein and the N–RNA template. The EB NS protein (10 kDa) is a necessary domain that interacts with the L protein and allows the complex to bind with the N–RNA template. However, this complex was not able to carry out transcription efficiently. On the other hand, RA NS protein (lacking domain II) or *Bst*NI NS protein (lacking domains II and III) failed to bind functionally with the L protein and N–RNA template but supported transcription only when the EB NS protein was present in the reaction mixture. These results indicate that domain I may have a direct role in facilitating L polymerase, which is bound to N–RNA template via domains II and III, to continue transcription on the template. The above results further show that domain I can be separated from domains II and III and that both polypeptides are active for RNA synthesis when added together in trans.

Role of Negatively Charged Polypeptides in Transcription Reconstitution. It can be seen in Fig. 2 that EB NS protein alone can support a low but consistent level of transcription in the reconstitution reaction. Since the reaction was carried out in the presence of reticulocyte lysate, it was possible that the observed transcription may have been due to the presence of some component(s) in the lysate functioning as domain I polypeptide. To investigate this possibility, we studied the effect of various concentrations of reticulocyte lysates on the transcription reaction in the presence of EB NS protein. EB NS protein in the presence of a low concentration (3 μl) of the lysate (Fig. 3, lane D) supported virtually no transcription when mixed with the L protein and the N–RNA template. However, increasing concentrations of the lysate (up to 20 μl) stimulated RNA synthesis, which eventually plateaued (Fig. 3, lanes E–H). The maximum RNA synthesis was 22% compared to the control RNA synthesis using full-length NS protein (*Bam*HI NS protein, Table 1) containing 20 μl of lysate. Thus, it seems that EB-NS protein alone can bind efficiently to the L protein and the template but is incapable of supporting RNA synthesis. The observed basal level of transcription (Fig. 2) was primarily due to the addition of excess reticulocyte lysate, which stimulated RNA synthesis. Stimulation of transcription of VSV by cell extracts has been reported previously (15–18).

To investigate whether the function of domain I can be replaced by a similarly charged polypeptide, we chose tubulin and the corresponding domain of NS protein of VSV Indiana serotype which is highly nonhomologous but structurally similar (10, 11). Tubulin, a constituent polypeptide of cellular microtubules, is highly negatively charged with a cluster of glutamic acid aspartic acid residues at the C-terminal portion of the polypeptide (19, 20). In addition, β-tubulin has been shown to be a positive transcription factor of VSV RNA synthesis *in vitro* (21). Both β-tubulin and NS(VSV Indiana serotype) protein supported RNA synthesis to the extent of 20% and 30%, respectively, only when EB NS protein was present in the reaction mixture. These results show that

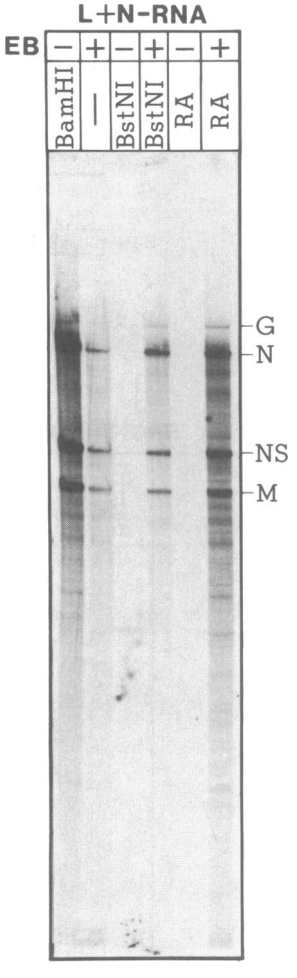

FIG. 2. Transcription reconstitution *in vitro* with various truncated NS proteins. The truncated NS proteins, as indicated, were used to reconstitute transcription (0.2 ml) in the presence of purified L protein (1.6 μg) and N–RNA template (4 μg) in a total volume of 200 μl with [α-^{32}P]CTP as the labeled precursor as detailed (6). The amount of reticulocyte lysate in each reaction mixture was kept constant at 20 μl. The RNA products synthesized in 2 hr were analyzed by 7 M urea/5% polyacrylamide gel electrophoresis for mRNA and subsequently autoradiographed. Migration positions of mRNAs coding for the G, N, NS, and M proteins are shown.

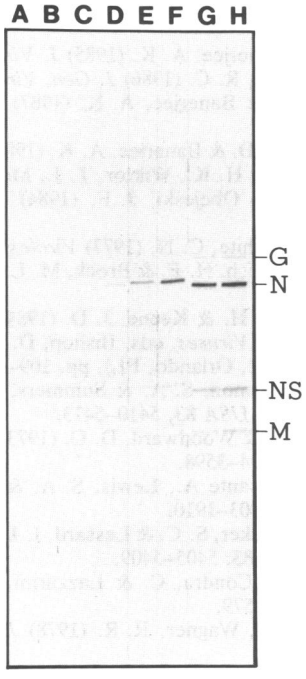

FIG. 3. Effect of ribosome-free reticulocyte lysate on transcription reconstitution with EB NS protein. Transcription reconstitution with purified L protein and N–RNA template in the absence of EB NS protein was carried out in the presence of 5 μl (lane A), 10 μl (lane B), and 20 μl (lane C) of reticulocyte lysate. EB NS protein (60 pg/3 μl of reticulocyte lysate) was added to the L protein and N–RNA template (lane D). Additional ribosome-free reticulocyte lysate was added to the transcription mixtures containing EB NS protein: 2 μl (lane E), 5 μl (lane F), 10 μl (lane G), and 20 μl (lane H) (4). The [α-^{32}P]CMP-labeled RNA synthesized in 2 hr was analyzed as described in Fig. 2.

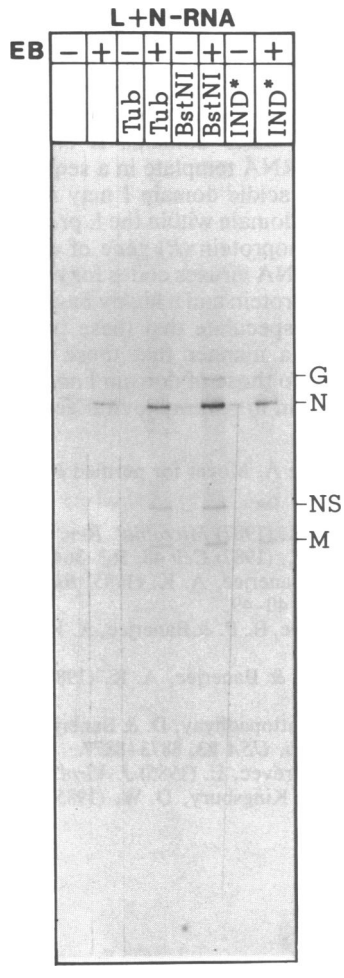

FIG. 4. Effect of negatively charged polypeptides on transcription reconstitution. Transcription reconstitution was carried out in the presence of purified L protein, N–RNA template, and 60 pg of EB NS protein in 3 μl of reticulocyte lysate. Lanes: Tub, β-tubulin (10 μg); *Bst*NI, *Bst*NI NS proteins synthesized as described in Fig. 1 (350 pg/7 μl of reticulocyte lysate); IND*, the N-terminal half of NS protein of VSV Indiana serotype similarly prepared from a truncated clone in pGEM vector (P. Paul and D.C., unpublished data) (300 pg/7 μl of reticulocyte lysate). The final amount of ribosome-free reticulocyte lysate in each reaction mixture was 10 μl.

unrelated, yet structurally similar, polypeptides can replace the function of domain I of the NS protein. However, domains II and III interact with the L protein and N–RNA template in a specific manner.

DISCUSSION

The above studies have provided a deeper insight into the structure and function of the phosphoprotein of VSV. This highly stable protein, which is a necessary component of the VSV transcription process, has evolved in such a manner that two functional domains can be unlinked, and the separated domains are active when added together in trans. More importantly, one of the domains (domain I), which is highly nonhomologous but structurally similar among the VSV serotypes, can be functionally substituted by a similarly charged protein—e.g., tubulin—and possibly by other cellular proteins with similar properties. It seems that the highly acidic domain I possibly mimicks RNA (22) and interacts with the L protein and the N–RNA template facilitating movement of the polymerase on the template. It is interesting to note that RA NS protein (containing domain III) displays the highest stimulatory activity, whereas *Bst*NI NS protein (containing 90% of the acidic amino acids but lacking domain III) or tubulin and NS(VSV Indiana serotype) protein (Fig. 4) stimulate RNA synthesis by only 20% to 30%. This indicates that domain III must have some role in maintaining the structure of domain I so that it interacts with the L protein and the template efficiently. On the other hand, the proteins that can substitute RA NS protein, such as tubulin, may interact with the L protein and the N–RNA template solely on the basis of being highly acidic and thereby transcribe inefficiently. It is possible that L protein has some basic domains within the polypeptide that interact with the acidic domains of these proteins. In fact, strong association of L protein with tubulin in VSV-infected cells has been demonstrated (21). It is interesting to note that VSV transcription (23) as well as Sendai virus transcription (24) are stimulated *in vitro* by the addition of polyanionic compounds. Moreover, we have recently observed that polyglutamic acid also can partially replace the function of domain I *in vitro* (unpublished results).

Thus, it seems that the NS protein may have two binding regions for the L protein. Domain II probably is the region that specifically links the L protein to the template to initiate transcription. Phosphorylation of serine-236 and serine-242 by the L protein within domain II may directly regulate its binding with the domain and the template (12). Domain I may bind to the L protein through interaction between the highly acidic region of the former with the basic domain(s) of the latter, thus facilitating elongation of RNA chains on the template. The latter contention is supported by the findings that negatively charged tubulin can partially replace the function of domain I *in vitro* (Fig. 4), and it has been found to be tightly associated with the L protein in the VSV-infected cells (21). Consistent with the above observations,

we have found that the requirements for EB NS protein (domains II and III) and RA NS protein (domains I and III) for optimal transcription are catalytic and stoichiometric, respectively (unpublished observations). Moreover, the net pI of RA NS and EB NS proteins are 5.3 and 10.2, respectively. Thus, the basic domains II and III may interact directly with the RNA template in a sequence-specific manner, whereas the acidic domain I may interact electrostatically with a basic domain within the L protein. Curiously, the analogous phosphoprotein (*P*) gene of other nonsegmented negative-strand RNA viruses codes for two distinct proteins; a highly acidic P protein and a highly basic C protein (25–27). It is tempting to speculate that these two genes may have evolved in such a manner that these proteins may have similar functions to those of domain I and domains II and III of VSV NS protein in paramyxoviral genome transcription.

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