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REQUIREMENTS AND FUNCTIONS OF VESICULAR STOMATITIS VIRUS L AND NS PROTEINS IN THE TRANSCRIPTION PROCESS IN VITRO

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The L and NS proteins of vesicular stomatitis virus were purified from transcribing ribonucleoprotein complex and were used to study their requirements and functions during reconstitution of RNA synthesis in vitro. The requirements for L and NS proteins for optimal RNA synthesis were found to be catalytic and stoichiometric, respectively. Addition of increasing amounts of NS protein to N-RNA template and saturating L protein, the ratio of N-mRNA to leader RNA synthesis increased linearly. In contrast, when the concentration of L protein was increased the corresponding ratio remained constant. These results, coupled with the observation that the L protein is involved in the initiation of RNA synthesis, suggest that the NS protein is involved in the RNA chain elongation step. The NS protein possibly interacts with both the L protein and the template N-RNA and unwinds the latter to facilitate the movement of L protein on the template RNA.

The transcribing ribonucleoprotein (RNP) particle of vesicular stomatitis virus is a complex that contains a linear single-stranded genome RNA (molecular weight, 4×10^6) of negative polarity tightly associated with approximately 2,500 molecules of nucleocapsid protein N (molecular weight 47,000) (1). In addition, two other dissociable minor proteins are also part of the RNP complex: there are approximately 40 molecules of a large protein L and 400 molecules of phosphoprotein NS (2) in the complex, amounts calculated from the molecular weights of 240,000 for L protein (3) and 25,000 for NS protein (4). The transcribing RNP, in the presence of the four ribonucleoside triphosphates, synthesizes sequentially five mRNA species in vitro in the order of N-NS-M-G-L (5). In addition, there is synthesis of a small leader RNA (47 bases), which precedes synthesis of N mRNA and is polyphosphorylated at its 5'-end and lacks poly(A) at its 3'-end (6).

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The RNP is rendered transcriptionally inactive by total removal of the L and NS proteins by treatment of the complex with high ionic strength conditions; efficient reconstitution of mRNA and leader RNA synthesis is achieved only when both L and NS proteins are added to the N-RNA complex (7,8). Although this phenomenon has been known for some time, the precise roles of L and NS proteins in the transcription process in vitro are still poorly understood. The extreme lability of the L protein (8) has made it difficult to purify the protein in sufficient quantity to study in detail its role in the transcription process.

We have recently shown (9) that purified L protein is involved in the initiation step of the RNA transcription process in vitro, whereas NS protein was involved in some post-initiation step. In the present studies we have studied the requirements of L and NS proteins in the transcription process in vitro and have shown that the NS protein is involved in the RNA chain elongation step.

MATERIALS AND METHODS

Purification of virus. VSV (Indiana serotype) was grown in roller bottles containing monolayers of baby hamster kidney cells (BHK-21) and purified as described previously (10).

Isolation and purification of L, NS, and N-RNA complex. The L and NS proteins were isolated from purified transcribing ribonucleoprotein (RNP) particles as described in detail recently (9). The purification of N-RNA complex from RNP has also been described (9). The protein concentrations of L, NS, and N-RNP complex were 75 µg/ml, 120 µg/ml, and 750 µg/ml, respectively.

Reconstitution of RNA synthesis in vitro. The reaction mixture (0.2 ml) contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 M NaCl, 4 mM dithiothreitol, 0.05 mM CTP, 1 mM ATP, GTP, and UTP, 20 µCi of [α-^{32}P]CTP (specific activity 410 Ci/mmole) (Amersham, Arlington Heights, IL), 2.2 µg of N-mRNA template and various concentrations of L and NS proteins as described in individual experiments in each figure. Unless otherwise stated, the reactions were carried out at 30°C for 2 hr.

Analysis of RNA products by polyacrylamide gel electrophoresis. For analyses of the mRNA species, the reaction products were extracted with equal volumes of phenol and the RNA in the aqueous phase was precipitated with alcohol and the pellet suspended in water. Half of the RNA was used in a reaction mixture (40 µl) containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM dithiothreitol, 1 µg oligo$(dT)_{12-18}$ (Sigma Chemicals, St. Louis, MO), 1 unit/µl RNasin (Promega Biotec, Madison, WI), 0.5 unit RNase H (Bethesda Research Laboratories, Bethesda, MD). The reaction was incubated at 37° for 20 min, terminated by addition of sodium dodecyl sulfate (0.5%), the RNA products extracted with phenol, and the RNA removed from the aqueous layer by precipitation with ethanol. The mRNAs with poly(A) tails thus removed were

analyzed by electrophoresis in 5% polyacrylamide slab gels (32 cm x 17 cm x 0.2 cm) containing 0.16% methylene-bis-acrylamide and 7 M urea. The gels were run at 600 V for 24 hr and RNA bands were located by autoradiography on Kodak XAR film. The RNA in the other half of each sample was directly electrophoresed in 20% polyacrylamide gels containing 7 M urea (12) to determine the extent of synthesis of the leader RNA.

RESULTS

Optimum requirements of L and NS proteins for RNA synthesis in vitro. The L and NS proteins were purified by high salt treatment of RNP which initially had been purified by disruption of purified virions with Triton X-100 in the presence of 0.4 M NaCl followed by centrifugation. The L protein, which was bound to phosphocellulose and eluted at 1 M NaCl, generally retained a trace amount of the NS protein. The NS protein, on the other hand, contained a small amount (approximately 5 to 10%) of N protein. When an excess of N-RNA template (20 µg) was analyzed by polyacrylamide gel electrophoresis, a trace quantity of NS protein was discernible only after silver staining but no L protein was detectable. Addition of L protein or NS protein alone to the template produced virtually no complete mRNA species. However, addition of optimum amounts of L and NS proteins to the template resulted in stimulation of RNA synthesis by approximately 10- to 30-fold depending on the purity of the L protein preparations.

At a fixed concentration of template and a saturating concentration of NS protein, RNA synthesis was significantly stimulated by the addition of increasing concentrations of L protein (Fig. 1) . However, optimal RNA synthesis occurred at a concentration of only 0.2 µg/200 µl reaction. In contrast, the requirement of NS protein for optimal RNA synthesis was significantly higher than that for the L protein. As shown in Fig. 2, at a saturating concentration of L and the same concentration of the template as used in Fig. 1, RNA synthesis was optimal at an NS concentration of 1.4 µg/200 µl reaction. Thus, at least a seven-fold higher amount (on a weight basis) or seventy-fold higher (on a molar basis) of NS protein than the L protein was required for optimal RNA synthesis. These results indicated

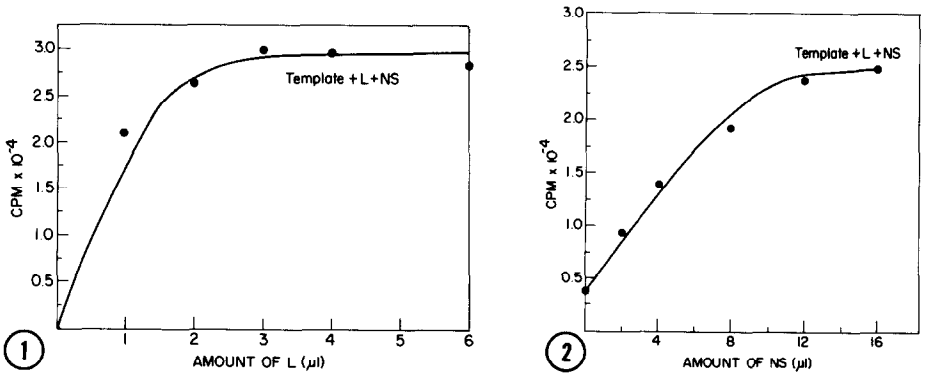

Fig. 1. Rate of RNA synthesis with saturating NS protein and increasing concentrations of L protein. RNA synthesis was carried out using template (2.2 µg), saturating concentration of NS protein (1.4 µg), and increasing amounts of L protein. The RNA synthesized at each point was determined by measuring cold trichloroacetic acid insoluble radioactivity retained on nitrocellulose filters.

Fig. 2. Rate of RNA synthesis with saturating L protein and increasing concentrations of NS protein. RNA synthesis was carried out using template (2.2 µg), saturating concentration of L protein (0.2 µg), and increasing amounts of NS protein as indicated. The RNA synthesized at each point was determined by measuring cold trichloroacetic acid insoluble radioactivity retained on nitrocellulose filters.

that the requirement for NS protein for RNA synthesis was stoichiometric whereas L protein requirement was catalytic.

Role of NS protein in RNA chain elongation. We have previously demonstrated that the L protein alone was capable of synthesizing initiated oligonucleotides representing the 5' termini of the leader RNA and mRNA sequences (9), whereas NS protein lacked such properties. In order to better understand the role of NS protein in the transcription process, we performed a series of reconstitution experiments using limiting to saturating amounts of NS or L proteins and analyzed the RNA products synthesized under each condition. Each reaction product was analyzed for synthesis of individual mRNA species and leader RNA by electrophoresis through 5% and 20% polyacrylamide gels, respectively. Figures 3 and 4, and Table 1 show the results of such experiments using various concentrations of NS protein and L protein. When L protein was kept at a constant saturating concentration and the NS protein concentration was increased from limiting (0.24 µg/200 µl) to saturating (1.4 µg/200 µl) (Fig. 3), the ratio of N mRNA to leader RNA synthesis increased linearly with the increase of NS protein concentration

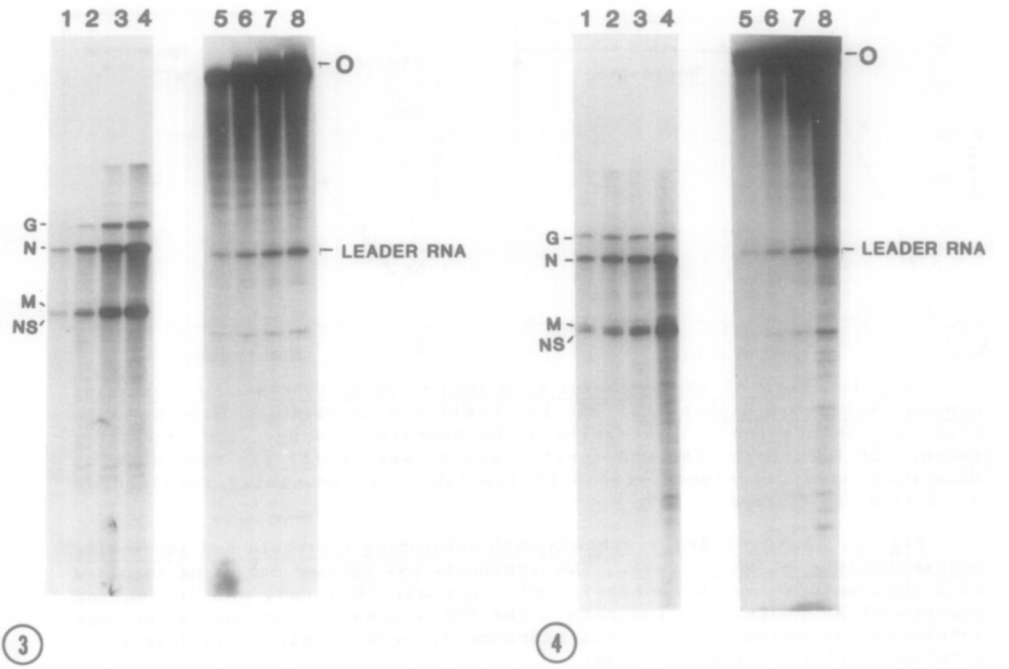

Fig. 3. Analysis of the RNA products synthesized at saturating L protein concentration and increasing amounts of NS protein. The mRNAs (lanes 1 to 4) and leader RNA (lanes 5 to 8) synthesized in vitro in reconstituted reactions containing template (2.2 µg), L protein (0.2 µg) and various amounts of NS protein were analyzed by electrophoresis, as described in Materials and Methods. Amounts of NS protein in lanes 1 and 5 were 0.24 µg; lanes 2 and 6 were 0.48 µg; lanes 3 and 7 were 0.96 µg; and lanes 4 and 8 were 1.4 µg. The migration positions of the mRNAs for G, N, M, NS, and leader RNA are shown. O represents the origin of the gel.

Fig. 4. Analysis of RNA products synthesized at saturating NS proteins and increasing concentrations of L protein. The mRNA products (lanes 1 to 4) and leader RNA (lanes 5 to 8) synthesized at saturating NS protein (1.4 µg), and increasing L protein amounts were analyzed by electrophoresis as in Fig. 3. Amounts of L protein in lanes 1 and 5 were 0.02 µg; in lanes 2 and 6 were 0.04 µg; in lanes 3 and 7 were 0.08 µg, and lanes 4 and 8 were 0.2 µg, respectively. The migration positions of the mRNAs for G, N, M, NS, and leader RNA are shown. O represents the origin of the gel.

(Table 1). In contrast, at a constant saturating concentration of NS protein, the increase of L protein concentration from limiting (0.02 µg/200 µl) to saturating (0.2 µg/200 µl) (Fig. 4) resulted in the corresponding N mRNA to leader RNA ratio remaining virtually constant over this concentration range (Table 1). These results indicated that at saturating concentrations of L protein, all RNA chains were rapidly initiated, whereas chain elongation was rate limiting, due to limiting concentration of NS protein. On the other hand, when NS protein was in excess, the rate limiting step was the initiation

TABLE 1

Expt.	Additions N-RNA µg/200 µl reaction	L	NS	Radioactivity incorporated (cpm) Leader RNA	N-mRNA	Ratio N-mRNA / Leader RNA
A	2.2	0.2	0.24	791	652	0.8
	2.2	0.2	0.48	1111	1937	1.7
	2.2	0.2	0.96	1398	6139	4.4
B	2.2	0.02	1.4	176	642	3.6
	2.2	0.08	1.4	269	1044	3.9
	2.2	0.2	1.4	373	1253	3.4

Reconstitution reactions were carried out using indicated amounts of N-RNA complex, L, and NS proteins. The mRNAs and leader RNAs synthesized in Expt. A and B were analyzed by polyacrylamide gel electrophoresis as described in Fig. 3 and Fig. 4, respectively. The labeled bands representing the N mRNA and the leader RNA under each condition were excised from the gel, and radioactivity was quantitated by Cerenkov counting.

of RNA synthesis by L protein. Thus, all initiated RNA chains were quickly extended and completed due to the presence of excess of NS protein.

In order to further investigate the role of NS protein in the RNA chain elongation step, heat inactivation experiments were performed. Purified L or NS proteins were heated at 40°, 60°, and 80°C for 2 min and used at saturating concentrations for RNA synthesis in reconstitution experiments and the RNA products were analyzed by polyacrylamide gel electrophoresis. Heating purified L protein at various temperatures virtually abolished synthesis of both completed mRNA (Fig. 5, lanes 4, 5, and 6) and leader RNA (lanes 11, 12, and 13). In contrast, heated NS preparations were able to synthesize leader RNA as well as a series of small, preterminated RNA species migrating between the origin and the leader RNA (lanes 8, 9, and 10), but synthesis of completed mRNA decreased sharply with the increase of temperature of heating. These results were consistent with the interpretation that heat treatment of NS protein effectively eliminated its capability to elongate and complete the large mRNA chains but had less effect on its capacity to complete small RNA chains. In contrast L, being thermolabile, failed to initiate RNA chains

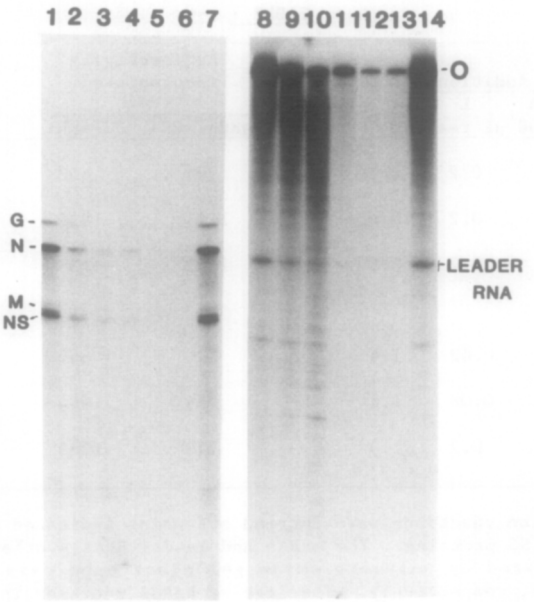

Fig. 5. Effect of temperature of the L and NS proteins on mRNA and leader RNA synthesis in in vitro reconstitution reactions. The mRNAs (lanes 1 to 7) and leader RNA (lanes 8 to 14) were synthesized using heated L (0.2 µg) or NS proteins (1.4 µg) and template (2.2 µg) and analyzed as described in Fig. 3. NS protein was heated at 40°C for 2 min (lanes 1 and 8); 60° for 2 min (lanes 2 and 9); 80° for 2 min (lanes 3 and 10). L protein was heated at 40°C for 2 min (lanes 4 and 11); 60° for 2 min (lanes 5 and 12); 80° for 2 min (lanes 6 and 13). Lanes 7 and 14 represent control reactions at 30°C for 2 hr. The migration positions of mRNAs for G, N, M, NS, and leader RNA are shown. O represents the origin of the gel.

after being heated; consequently, no RNA was synthesized even though excess NS protein was present.

DISCUSSION

In this communication, we have demonstrated that the requirement of NS protein for an optimal rate of transcription is approximately 7-fold higher on a weight basis (or approximately 70-fold higher on a molar basis) than the L protein. This conclusion is different from that reported earlier (8,11) where a 1:1 molar ratio of L and NS proteins was found to be required for optimal RNA synthesis *in vitro*. This discrepancy may be due to the extreme lability of the L protein used in the previous report (8) which may have complicated the stoichiometry studies. The difference in the degree of thermolability between the L proteins of the reported one (8) and our preparations may also be due to the different strains of VSV used in these studies. In a similar

reconstitution experiment, using L, P, and the NP-RNA complex of Newcastle disease virus (NDV), Hamaguchi et al. (14) have also observed that the requirement for P protein (the analogue of NS in NDV) was higher than that for the L protein for optimal RNA synthesis in vitro.

If the molecular weights of L, NS, and N proteins of 240,000 (3), 25,000 (4), and 47,000 (4), respectively, are used, and if it is assumed that 2,500 molecules of N protein are bound per molecule of genome RNA (2), the optimum molar ratio of RNA:L:NS determined here would be 1:40:2800. The corresponding molar ratio in the purified virion has been reported to be approximately 1:40:400 (ref. 2, corrected for the molecular weights of L and NS). It is interesting to note that, like the higher requirements for NS in vitro, the concentration of NS protein and its mRNA are also present in high molar amounts in the infected cells (15,16). Moreover, in a cell-free replication system, the molar ratio of newly synthesized NS protein associated with the nucleocapsid was found to be considerably higher (greater than 100-fold) than the L protein (17). Perhaps, the high concentration of NS protein might facilitate continued synthesis of RNA from progeny RNP in the infected cells, but during the process of virus maturation, a suboptimal level of NS protein is packaged within the virion.

We have demonstrated previously that L protein alone, when added to N-RNA template, was unable to synthesize full-length mRNAs (9). However, using incomplete reactions containing [α-^{32}P]ATP and CTP, it was shown that L protein alone can efficiently initiate RNA chains, e.g. AC and AACA, etc., whereas the NS protein, lacked the ability to initiate RNA chains. A chain elongation function for NS protein is directly demonstrated by the fact that when the concentration of NS protein was increased, in the presence of an excess of L protein (Fig. 3), the ratio of N mRNA/leader increased linearly (Table 1). In sharp contrast, the corresponding ratio remained constant when NS protein was in excess and L protein concentration was increased (Fig. 4, Table 1). Thus, it seems that L protein, when present in excess, may have initiated RNA chains but failed to complete them due to lack of NS protein

which was the rate limiting step in the reaction. Thus, a linear increase of the ratio of the mRNA synthesis with respect to leader RNA occurred with the increase of NS protein concentration. When NS protein was present in excess, all RNA chains which had been initiated by the L protein were rapidly brought to completion by the NS protein, thus maintaining a constant ratio of N-mRNA to leader RNA synthesis. The heat inactivation studies (Fig. 5) clearly showed that NS protein lost its capability to complete full-length mRNA but still retained the capacity to complete leader RNA as well as numerous aborted RNAs larger than the leader RNA, but smaller than the message species.

Since the original discovery that both L and NS proteins are required for efficient transcription of the N-RNA complex in vitro (8,10,13), little progress has been made to elucidate which of the two proteins (L or NS) constitutes the RNA polymerase. The L protein, being a large polypeptide (240,000 daltons, ref. 3), seems to be the proper candidate for the RNA polymerase, although no direct evidence has been presented demonstrating that viral mRNAs are synthesized by the interaction of only the L protein and the N-RNA template. The above results support the conclusion that the L protein is probably the RNA polymerase which has the capacity to initiate small RNA chains but cannot extend them to full length mRNA sizes. The NS protein possibly interacts with the L protein as well as with the N protein and prepares the template such that the L protein may move along it. Perhaps NS protein is an RNA unwinding protein which displaces the N protein from the genome RNA for L protein to gain access to the genome. This possibly explains the high molar requirement of NS relative to L. For each L protein molecule, on the order of 70 NS molecules might be required to interact with the region of N-RNA preceding the moving site of new RNA synthesis. It should be interesting to study whether any other agent, for example, cellular or synthetic protein, may replace the NS function in vitro. Stimulation of RNA synthesis in vitro by host cell components has been reported (18). It remains to be seen whether the putative host factors facilitate NS function or can

replace it *in vitro*. Future studies along this line would certainly help to firmly establish the functions of L and NS in the transcription process.

REFERENCES

1. Wagner, R.R. (1975) in, Comprehensive Virology (H. Fraenkel-Conrat and R.R. Wagner, eds.), pp. 1-80, Plenum Press, New York.
2. Bishop, D.H.L., and Roy, P. (1972) J. Virol. 10, 234-243.
3. Schubert, M., Harmison, G.G., and Meier, E. (1984) J. Virol. 51, 505-514.
4. Gallione, C.J., Greene, J.R., Iverson, L.E., and Rose, J.K. (1981) J. Virol. 39, 529-535.
5. Banerjee, A.K., Abraham, G., and Colonno, R.J. (1977) J. Gen. Virol. 34, 108.
6. Colonno, R., and Banerjee, A.K. (1978) Nucl. Acids Res. 5, 4165-4176.
7. Abraham, G., and Banerjee, A.K. (1976) Virology 71, 230-241.
8. Emerson, S.U., and Yu, Y.H. (1975) J. Virol. 15, 1438-1356.
9. De, B.P., and Banerjee, A.K. (1984) J. Virol. 51, 628-634.
10. Banerjee, A.K., Moyer, S.A., and Rhodes, D.P. (1974) Virology 61, 527-558.
11. Naito, S., and Ishihama, A. (1976) J. Biol. Chem. 251, 4307-4314.
12. Thornton, G.B., De, B.P., and Banerjee, A.K. (1984) J. Gen. Virol. 65, 663-668.
13. Emerson, S.U., and Wagner, R.R. (1972) J. Virol. 10, 297.
14. Hamaguchi, M., Yoshida, T., Nishikawa, K., Naruse, H., and Nagai, Y. (1983) Virology 128, 105-117.
15. Hsu, C., Kingsbury, D.W., and Murti, K.G. (1979) J. Virol. 32, 304-313.
16. Villarreal, L.P., Briendl, M., and Holland, J.J. (1976) Biochemistry 15, 1663-1667.
17. Patton, J.T., Davis, N.L., and Wertz, G.W. (1983) J. Virol. 45, 155-164
18. Rose, J.K., Lodish, H.F., and Brock, M.L. (1977) J. Virol. 21, 683-693.