Interaction of L and NS Proteins of Vesicular Stomatitis Virus with its Template Ribonucleoprotein during RNA Synthesis in vitro

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SUMMARY

Soluble transcriptase containing the L and the NS proteins was isolated from purified vesicular stomatitis virus and its binding with the template ribonucleoprotein containing the N protein–RNA complex was studied with respect to its ability to initiate and synthesize RNA in vitro. By using u.v.-irradiated template reconstituted with soluble transcriptase, it was shown that the synthesis of leader RNA and other small initiated mRNA sequences continued while full-length mRNA synthesis decreased by 90%. In the presence of ATP and CTP, the reconstituted complex synthesized polyphosphorylated oligonucleotides which include AC, AAC and AACA which represent 5'-terminal sequences transcribed from the leader template and genes coding for mRNAs. In the presence of arabinosyl ATP, an inhibitor of RNA synthesis in vitro, the synthesis of leader RNA was found to be inhibited considerably more than other small initiated mRNA sequences. Reconstitution of RNA synthesis with soluble transcriptase and template in the presence of viral matrix (M) protein at low ionic condition resulted in virtual cessation of leader RNA synthesis, although the synthesis of small initiated N mRNA, 11 to 14 bases, continued. These results suggest that transcriptase can bind at multiple sites on the genome template and initiate RNA chains.

Detailed studies on the mechanism of transcription in vitro by the transcribing ribonucleoprotein (RNP) of vesicular stomatitis virus (VSV) have demonstrated that the RNA synthesis is sequential, with the gene order 5' leader RNA–N–NS–M–G–L 3' (Abraham & Banerjee, 1976a, b; Ball & White, 1976). Several models were proposed for this sequential mode of VSV mRNA transcription in vitro (Banerjee et al., 1977). Some recent observations have suggested a model (Testa et al., 1980) in which the transcriptase is located at putative multiple promoter sites on the template RNP and initiates RNA chains. RNA chain elongation, however, occurs only after its 3'-proximal gene is transcribed. This constraint on the transcriptase molecules appeared to be imparted by the secondary structure of the transcribing RNP (Talib & Banerjee, 1982). Recently, Emerson (1982) using soluble transcriptase and the template N RNA complex has shown that, unlike purified virus, only pppAC was synthesized in vitro in the presence of ATP and CTP. Only after RNA synthesis had taken place in the presence of all four ribonucleoside triphosphates was the oligonucleotide AACA (representing the mRNA initiation) detected. These results suggested that the soluble transcriptase, during reconstitution, entered only at the leader template and the RNA synthesis occurred sequentially by multiple initiations (stop–start mechanism). To study this phenomenon in more detail, we initiated a series of experiments using a purified mixture of the transcriptase and studied its interactions with the RNP in vitro. The results presented in this communication suggest that the soluble transcriptase, in addition to the leader template, can also enter at internal sites and initiate RNA chains.

The template (N RNA complex) and the soluble transcriptase (L and NS) were prepared and purified as described previously (De et al., 1982). Efficient RNA synthesis occurred when template (3-4 μg) and the transcriptase (1-8 μg) were mixed in a transcription cocktail. This ratio
of enzyme to template is approximately 2.5-fold higher than that present in purified virions. The extent of RNA synthesis was approximately 50% of the yield obtained with purified undissociated RNP consisting of similar amounts of viral protein components.

To investigate whether the soluble transcriptase could bind internally on the template, experiments were performed by first irradiating the template with u.v. light for 2 and 3 min (Abraham & Banerjee, 1976a) and subsequently reconstituted with active soluble transcriptase. The RNA products were then analysed by polyacrylamide gel electrophoresis. The gels were overexposed to detect all small initiated mRNA species synthesized under these conditions. The extent of RNA synthesis (radioactivity remaining at the origin or directly acid-precipitable) was decreased significantly after 2 min and 3 min of u.v. irradiation of the template (78% and 88% respectively) (Fig. 1). In contrast, synthesis of the leader RNA and several other low mol. wt. RNA species designated RNA I to IV decreased only slightly (20% and 30% at 2 min and 3 min respectively). Each RNA species was eluted from the gel, quantified, and identified by fingerprint analyses. The bands designated leader RNA, RNA I and RNA III were confirmed to be leader RNA, small initiated N mRNA and NS mRNA, respectively, as described previously (Testa et al., 1980). RNA II was confirmed to be the G-start RNA species of 28 bases, synthesized within the N gene as observed by Schubert et al. (1982) and RNA IV, which is a mixture of three distinct bands, was confirmed to be the initiated N mRNA sequence of chain lengths 11 to 14 as previously observed by Pinney & Emerson (1982a) (data not shown). The important finding is that the relative synthesis of all small initiated RNA species during u.v. inactivation was not affected significantly when compared to the drastic inhibition of mRNA synthesis. These results suggest that each of these RNA species was initiated through independent binding of transcriptase at its putative promoter sites.

We have previously shown (Chanda & Banerjee, 1981) that purified virions are capable of synthesizing pppAC, pppAAC and pppAACA in the presence of ATP and [α-32P]CTP as the only source of RNA precursors. To study whether similar results are obtained when soluble transcriptase was added to the template, the oligonucleotides synthesized in the presence of ATP and [α-32P]CTP were analysed (Chanda & Banerjee, 1981) by electrophoresis in a 20% polyacrylamide gel. As shown in Fig. 2(a), no oligonucleotide was detected when purified template was incubated with ATP and CTP. When reconstitution was carried out at two salt concentrations (0.02 M- and 0.1 M-NaCl), two distinct oligonucleotides (oligo I and II) were seen (Fig. 2b, c). The oligonucleotides from each experiment were excised from the gel, eluted, and counted for radioactivity. It was found that ionic conditions had a profound effect on the synthesis of oligo I and II (an increase of fivefold and tenfold, respectively, at the lower ionic strength). However, total RNA synthesis by the reconstituted RNP under these two salt concentrations was identical (De et al., 1982). The oligo I (Fig. 2) was confirmed to be ApC by the methods used by Chanda & Banerjee (1981). The oligo II (Fig. 3b, d) was found to be a mixture of oligonucleotides which included AAC (30%) and AACA (10%). In addition, two other oligonucleotides were identified: ACC (50%) and ACAA (10%) (data not shown). These results indicate that both RNP (lane d) and reconstituted RNP (lane b) synthesized AC (leader initiation), AAC and AACA (mRNA initiations), suggesting that the transcriptase could bind at multiple sites on the template. However, the origin of additional oligonucleotides, such as ACC, and ACAA remains unclear.

We next studied the effect of arabinosyl ATP (ara-ATP) on the synthesis of RNA and the small initiated mRNA sequences during reconstitution (Chanda & Banerjee, 1980). The RNA synthesized at two different concentrations of ara-ATP (0.25 and 0.5 mM) was analysed by polyacrylamide gel electrophoresis. Total RNA synthesis and the leader RNA synthesis were decreased by more than 80% at a concentration of 0.5 mM-ara-ATP (Fig. 3c). In contrast, there was a differential effect of ara-ATP on the synthesis of other small mRNA sequences. RNAs I and IV were less affected by ara-ATP (30% and 20% decrease, respectively). Specifically, the synthesis of RNA IV (initiated N mRNA) remained virtually constant at 0.25 mM concentration of the inhibitor. These results indicate that the transcriptase complexes bound to leader template and at internal sites react differently to ara-ATP. This differential effect of the inhibitor on the synthesis of leader RNA and initiated N mRNA sequences suggests that
Fig. 1. RNA synthesis after reconstitution of u.v.-inactivated template and purified transcriptases. Template containing N protein–RNA complex and the transcriptase containing the L and NS proteins were isolated from purified virus (VSV, Indiana serotype) as detailed elsewhere (De et al., 1982). The template and the enzymes were at 680 µg/ml and 360 µg/ml, respectively. Equal aliquots of purified template (3-6 µg) in 10 mM-Tris-HCl pH 8.0 in 50 µl were irradiated with u.v. light for 0 min (a), 2 min (b) or 3 min (c) as described by Abraham & Banerjee (1976a). Reconstitution was carried out by incubation with 1.8 µg of soluble transcriptase at 30 °C for 2 h in the presence of standard transcription cocktail containing [α-32P]CTP (400 Ci/mol) as the labelled precursor (De et al., 1982). The RNA was extracted with phenol and directly analysed in a 20% polyacrylamide gel containing 8 M-urea as previously described (Chanda & Banerjee, 1981). Migration positions of leader RNA, RNA species I to IV, xylene cyanol (XC) and bromophenol blue (BPB) dyes are shown.

Fig. 2. Oligonucleotides synthesized by RNP and reconstituted complex in the presence of ATP and CTP. Partial transcription reactions were carried out in a reaction mixture containing ATP (1 mM) and [α-32P]CTP (30 µM) (Chanda & Banerjee, 1981), using reconstituted RNP at 0.02 M-NaCl (b), 0.1 M-NaCl (c) and RNP prepared from Triton-disrupted virus (Abraham & Banerjee, 1976b) (6 µg) at 0.02 M-NaCl (d) and 0.1 M-NaCl (e). The reaction products were extracted with phenol, treated with calf intestinal alkaline phosphatase and analysed by electrophoresis in a 20% polyacrylamide gel as described by Chanda & Banerjee (1981). Lane (a) corresponds to the transcription mixture with only template at 0.02 M-NaCl. Electrophoresis was carried out for 12 h at 500 V. Oligonucleotide bands were located by autoradiography on Kodak XR-5 films with intensifying screens at 4 °C.

possibly two polymerase activities, mediated by L or NS or in combination (Emerson & Yu, 1975), are involved in the synthesis of these two types of RNA species. NS protein in the virion has been shown to exist in different phosphorylated forms (Kingsford & Emerson, 1980; Hsu & Kingsbury, 1982). It is not known whether different species of NS proteins, in combination with the L protein, are involved in the synthesis of leader RNA and mRNA.

In the above studies (Fig. 1, 3), the synthesis of the leader RNA was not totally inhibited. Thus, it was not clear whether synthesis of complete leader RNA was a prerequisite for binding
Fig. 3. RNA synthesis by reconstituted RNP in the presence of ara-ATP. Reconstitution reactions were carried out as described in Fig. 1 except that ara-ATP was included in the reaction mixture: (a) no ara-ATP, (b) 0.25 mM-ara-ATP, (c) 0.5 mM-ara-ATP (Chanda & Banerjee, 1980). Similar transcription reactions were also carried out using RNP purified from Triton-disrupted virus in the absence (d), or in the presence of 0.25 mM-ara-ATP (e), or 0.5 mM-ara-ATP (f). The RNA products were extracted with phenol and analysed by electrophoresis in a 20% polyacrylamide gel containing 8 M-urea as in Fig. 1.

Fig. 4. RNA synthesis by reconstituted RNP in the presence of M protein. A soluble enzyme fraction containing L, NS, M and G was prepared as described earlier (De et al., 1982). Reconstitution with template RNP (3.6 μg) and the soluble fraction described above (1.5 μg) was carried out under standard transcription conditions at 0.02 M-NaCl (b), or 0.12 M-NaCl (c). Transcription reactions were carried out also with Triton-disrupted virus (20 μg) under standard reaction conditions at 0.02 M-NaCl (d) or 0.12 M-NaCl (e). RNA products were extracted with phenol and analysed by electrophoresis in a 20% polyacrylamide gel as in Fig. 1. Lane (a) represents the transcription reaction with template only.
mRNA chains. Initiation of the N gene, however, continues. This demonstrates again that the internal site on the template RNP is available for binding of the transcriptase.

Thus, the above findings suggest that the transcriptase molecules can interact with template N RNA complex at internal sites and synthesize RNA chains by multiple initiations. In a recent report, Emerson (1982) using similar reconstitution studies, has shown that the soluble transcriptase binds exclusively on the leader template. Only after RNA synthesis has taken place does the transcriptase move along the template and remain bound at putative internal promoter sites. Single entry by the polymerase was shown by the sole synthesis of the dinucleotide AC in the presence of ATP and [α-32P]CTP. However, we have routinely detected, in addition to AC, synthesis of AAC and AACA in a similar partial reaction (Fig. 2). The reasons for this discrepancy are presently unclear. We believe that once the transcriptase enzymes are removed from the template, re-binding of the enzymes at the original sites, in the absence of RNA synthesis, depends on various parameters such as ionic conditions (Fig. 2), concentrations of enzyme and template, and differential affinity of enzymes for different binding sites (e.g. leader or internal binding sites). On the other hand, when RNA synthesis occurs (in the presence of all four ribonucleoside triphosphates) the interaction of the transcriptase with the template is greatly facilitated with restoration of the original RNP structure. Identification of different types of oligonucleotides in a partial reaction may have been due to different enzyme binding conditions having been used in our studies and the reported one (Emerson, 1982). Thus, it seems that the results obtained from partial reactions alone may not be definitive in the understanding of the mechanism of transcription in vitro. For that reason, we have chosen conditions where binding of polymerase to the template can be studied in the presence of all four ribonucleoside triphosphates (Fig. 1, 3 and 4). The detection and synthesis of small initiated RNA transcripts, including the leader RNA, under different transcription conditions as described above, gave us an opportunity to study binding of polymerase to the template. Although the role of these initiated RNA species in mRNA synthesis remains unclear, their synthesis probably reflects interaction of transcriptase with the template. Clearly, further studies are needed along this line to shed light on the precise mechanism of the VSV transcription process in vitro.

REFERENCES


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