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Vesicular Stomatitis Virus RNA Polymerase Can Read Through the Boundary Between the Leader and *N* Genes In Vitro

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Triphosphorylated in vitro transcripts of vesicular stomatitis virus were selected by mercury-Sepharose chromatography using adenosine-5′-*O*-(3-thiotriphosphate) as an affinity probe. Numerous RNAs ranging from less than 47 up to several hundred nucleotides in length were detected. Some of these contain the leader RNA covalently linked to transcripts of the *N* gene. A comparison of the published genomic sequences at the estimated termination sites of several of these RNAs reveals some homology with the sequence present at both the end of the leader and polymerase genes.

The vesicular stomatitis virus virion contains a viral specific RNA-dependent RNA polymerase which can transcribe the viral genome in vitro (2). The products of this reaction include the five capped and polyadenylated messages plus the 47-nucleotide leader, which is complementary to the precise 3′ end of the genome (4, 5, 10). UV inactivation kinetics suggest that there is only a single binding or initiation site for transcription, and the polyphosphorylated leader is thought to be the initiated RNA (1, 4). Nevertheless, additional triphosphorylated species have also been detected in vitro (7, 19). Some of these contain 5′-pppAACAG..3′, which is characteristic of the (uncapped) sequence present at the 5′ end of each message, although others of unknown origin are also found. In an effort to characterize the triphosphorylated species further, we have used the ATP analog adenosine-5′-*O*-(3-thiotriphosphate) (γ-S-ATP), containing a thio group on the γ phosphate, as an affinity probe. Those transcripts initiated with γ-S-ATP can be selectively bound to mercury-Sepharose (16), because they alone contain a sulfur atom at the 5′ end of the chain.

γ-S-ATP has little or no effect on either the rate of synthesis or the types of products synthesized by VSV in vitro (3; Herman and Lazzarini, unpublished data). In a typical experiment, 5 to 6% of the uniformly labeled RNA synthesized by VSV in the presence of γ-S-ATP bound to mercury-Sepharose, whereas only about 0.1 to 0.2% of the RNA synthesized in the presence of ATP bound to the affinity column (Fig. 1). Numerous bands were detected when the selected RNA, uniformly labeled with [α-

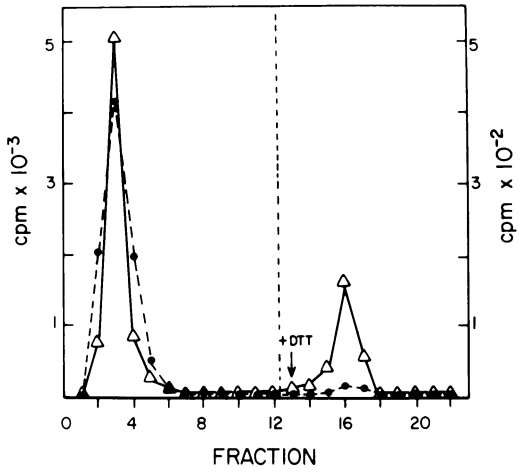

FIG. 1. *Mercury-Sepharose affinity chromatography. RNA transcripts were synthesized in vitro for 3 h at 30°C in a 0.1-ml reaction mixture containing 0.05 M Tris-hydrochloride (pH 8.0), 0.05% Triton N101, 0.1 M NaCl, 4 mM dithiothreitol (DTT), 1 mM CTP and UTP, 0.1 mM GTP, 1 mM ATP or γ-S-ATP, 5 mM $MgCl_2$, 5 μg of the heat-resistant strain of VSV (12), and 50 μCi of [α-^{32}P]GTP. The product RNA was extracted twice with phenol-chloroform and precipitated with ethanol. Unincorporated triphosphates were removed by chromatography on Sephadex G-50. The purified RNA was resuspended in TNES (0.01 M Tris-hydrochloride [pH 7.9], 0.1 M NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate) (16) and chromatographed through a mercury-Sepharose column (1 by 5 cm). Bound RNA was eluted by washing the column with 10 mM dithiothreitol in TNES. Symbols: ●, ATP; △, γ-S-ATP. The dashed line indicates change of scale.*

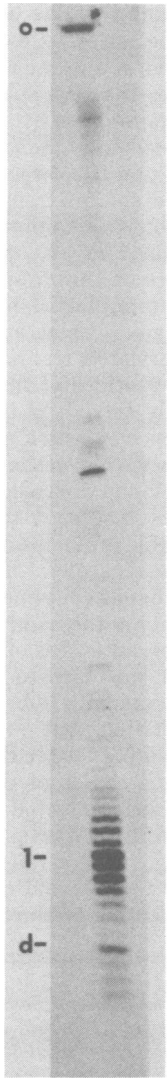

FIG. 2. *Polyacrylamide gel electrophoresis of RNA bound to mercury-Sepharose. Product RNA, uniformly labeled with [α-^{32}P]GTP, was prepared and purified as outlined in the legend to Fig. 1. The RNA was resuspended in 5 μl of 0.01 M Tris-hydrochloride (pH 7.4), 1 mM EDTA, and 0.5% sodium dodecyl sulfate; 20 μl of dimethyl sulfoxide was added, and the sample was warmed at 37°C. TNES (1 ml) was added and affinity chromatography was carried out as described in the legend to Fig. 1. The thiotriphosphorylated RNA was precipitated with ethanol and resuspended and denatured with dimethyl sulfoxide before rebinding to mercury-Sepharose. The eluted RNA was precipitated with ethanol, resuspended in sample buffer, and heated for 1.5 min in a boiling-water bath before electrophoresis in a 40-cm 12% polyacrylamide gel containing 8 M urea (11). Electrophoresis was for 18 h at 650 V. Autoradiography of the wet gel was against Kodak X-Omat R film with an intensifying screen. o, Origin of the gel; d, location of the xylene cyanol tracking dye; and l, position of the 47-nucleotide leader RNA.*

^{32}P]GTP, was resolved in a 40-cm, 12% polyacrylamide gel containing 8 M urea (11) (Fig. 2). The most prominent band migrated slightly slower than the xylene cyanol dye and contained the 47-nucleotide leader RNA (3). Nevertheless, other RNAs, both larger and smaller than the leader, were also visible. In addition, some radioactivity remained at the origin of the gel. Denaturation of the RNA in 80% dimethyl sulfoxide before chromatography on mercury-Sepharose neither eliminated the material which failed to enter the gel nor altered the pattern of bands resolved in it; at least 80 to 90% of the denatured RNA which bound to the column could rebind after a second cycle of dimethyl sulfoxide treatment and affinity chromatography (data not shown). This implies that VSV synthesizes numerous triphosphorylated species in vitro and that some of these transcripts are quite large. (Dimethyl sulfoxide denaturation and multiple cycles of affinity chromatography were routinely used during the purification of all the thiotriphosphorylated RNA for these experiments.)

To determine which of these species arose by transcription of the 3′ end of the genome, we annealed [5-^{3}H]UTP-labeled, mercury-Sepharose-selected RNA to the 42S viral genome which had been labeled at its 3′ end with [5′-^{32}P]cytidine-bis-diphosphate (6, 8). RNase T_2 nearest-neighbor analyses demonstrated that the vast majority of the radioactivity was released from the ligated genome as 5′-Up-3′ (data not shown). This is exactly the result expected for the specific labeling of the 3′ end of the 42S VSV RNA which terminates with a uridine residue (8). Therefore only those species which are complementary to the precise 3′ end of the genome should protect the radioactive phosphate. After hybridization, the resultant partial duplexes were trimmed with RNases A and T_1 and then heat denatured before electrophoresis in a 12% polyacrylamide gel (Fig. 3). The leader RNA and several other species among the 5-h in vitro transcription products were detected by this specific probe (Fig. 3, lane b). Some of these are less than 47 nucleotides long and may arise by a premature termination of transcription. The others are larger than the leader, and some radioactivity even remained at the origin of the gel. Trimming of the partial duplexes with RNase T_2 together with RNases A and T_1 had little or no effect on this gel profile (data not shown). In contrast, when the region of the terminally labeled genome protected by the leader was eluted from a gel and applied to a second gel, only one doublet band was detected

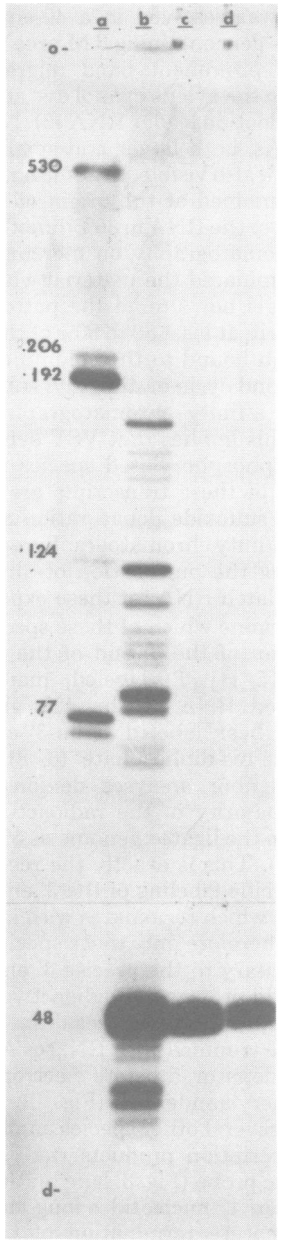

FIG. 3. *Size distribution of triphosphorylated RNA transcribed from the 3′ end of the genome. RNA was synthesized in vitro in a 0.4-ml reaction mixture containing 0.1 mM UTP, 1 mM each CTP, GTP, and γ-S-ATP, 20 μg of the heat-resistant strain of VSV, 50 μCi of [5-^{3}H]UTP, and the salts indicated in the legend to Fig. 1 for 5 h at 30°C. The product RNA was purified as described in the legend to Fig. 1 and then twice denatured with dimethyl sulfoxide and bound to mercury-Sepharose. The 42S virion RNA was labeled uniquely at the 3′ end with [5′-^{32}P]cytidine-bis-diphosphate using bacteriophage T4 RNA ligase (6, 8). Hybridizations between an excess of thiotriphosphorylated RNA and the terminally labeled genome were performed at 60°C for 1 h in 0.4 M NaCl–0.01 M Tris-hydrochloride (pH 7.5). The partial duplexes were trimmed with RNase A (2.5 μg/ml) and RNase T_1 (0.5 U/ml) in the same buffer at 37°C for 30 min. Digestion was terminated by adding diethylpyrocarbonate. The RNase-resistant duplexes were precipitated with ethanol in the presence of 10 μg of tRNA. The precipitated RNA was resuspended in sample buffer and heated in a boiling water bath for 1.5 min before electrophoresis in a 12% polyacrylamide gel (lane b). Uniformly labeled in vitro transcripts of bacteriophage lambda were used as size markers (lane a). Their lengths (in nucleotides) are shown at the left of the figure. As a marker for 48 nucleotides, the region of the terminally labeled genome protected by the leader was eluted from a 12% polyacrylamide gel after electrophoresis as either a single-stranded (lane c) or double-stranded RNA (lane d) and then heat denatured before being applied to this gel. Electrophoresis was for 18 h at 650 V. Symbols are explained in the legend to Fig. 2.*

(Fig. 3, lanes c and d). (The band is a doublet most probably because 5′-Cp-3′ had been removed from some of the molecules by RNase A.) This indicates that the species larger than 48 nucleotides did not arise by artifactual aggregation of the protected sequence.

It is estimated that the three most prominent triphosphorylated species transcribed from the 3′ end of the genome are approximately 95, 120, and 170 nucleotides long by comparison of their mobilities to those of uniformly labeled in vitro transcripts of bacteriophage lambda (Fig. 3, lane a). Since the *N* gene begins at position 51 (9), these triphosphorylated transcripts contain up to about 120 nucleotides of the *N* message sequence covalently linked to the leader RNA. (Several larger species which are not apparent in Fig. 3 were also visible on the autoradiogram.) A direct comparison of the radioactivity in these bands is possible since each thiotriphosphorylated RNA molecule protects only a single radioactive phosphate regardless of its length. The three prominent bands contained about 8 to 10% of the radioactivity in the band representing the leader RNA when an excess of the thiotriphosphorylated RNA was used to drive the terminally labeled genome into hybrids.

It should be noted that (+)-sense in vitro transcripts containing leader linked to *N* message or full-length copies of the viral genome have been previously detected using analogs of either GTP or ATP (17, 18). In those experiments, the analogs reduced the rate of transcription by up to 80 to 85%, and only a small proportion of the resulting product RNA contained

the leader linked to larger species. In contrast, the γ-S-ATP used here has very little effect on the rate of transcription and allows us to enrich for those triphosphorylated species normally synthesized by VSV in vitro.

An experiment similar to that shown in Fig. 3 was also carried out with RNA that had been selected by mercury-Sepharose chromatography after in vitro transcription for only 30 or 90 min. No obvious differences between the gel profiles were observed when these thiotriphosphorylated RNAs were annealed to the terminally labeled genome, even though the amount of RNA which bound to the column increased approximately twofold between 30 and 90 min (data not shown). This suggests that the species transcribed from the 3′ end of the genome are all synthesized rapidly and continuously during in vitro transcription.

The data presented here show that VSV synthesized numerous triphosphorylated RNAs in vitro. In contrast to previously published results (19), virtually all of the leader RNA synthesized in these experiments contained a triphosphate terminus, as judged by the ability to bind to mercury-Sepharose (data not shown). Some of the triphosphorylated RNAs contained the leader convalently linked to transcripts of the *N* gene. These may be either incomplete and unprocessed precursors to monocistronic messages or, perhaps, aborted attempts at replication of the genome in vitro. The fact that only a limited number of discrete species was detected suggests that the polymerase may encounter specific sequences in the genome or sites in the nucleocapsid which cause a premature termination of transcription. A comparison of the published genomic sequences (13, 14) at or near to positions 95, 120, and 170 from the 3′ end reveals some elements of similarity. In general, all three of these regions are uridine-rich (38 to 53%), as is the end of the leader gene (43%). Furthermore, two of the genomic sequences contain 3′..UGAAG..5′, whereas the third contains 3′..UCAAG..5′ within approximately 5 to 10 nucleotides of the estimated termination sites. We do not currently know if these sequences have some functional role in the normal regulation of transcription, but it is interesting to note that they are quite similar to the sequences found at both the end of the leader and polymerase genes (9, 15) (Table 1). However, additional data will be required to determine what function these triphosphorylated molecules have in normal transcription-replication and whether sequences such as 3′..UGAA..5′ do, in fact, play a physiological role in the regulation of VSV RNA synthesis.

TABLE 1. *Comparison of sequences at estimated termination sites*

Position[a]	Region	Sequence	Reference
End of leader gene	44–51	3′..UUUGAAAU..5′	9
95	103–110	3′..UAUCAAGG..5′	13, 14
120	112–119	3′..UUUGAAGG..5′	13
170	153–160	3′..AAUGAAGU..5′	13
End of *L* gene	65–58[b]	3′..UUUGAAAC..5′	15

[a] From the 3′ end of the genome.
[b] From the 5′ end of the genome.

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