In Vitro Synthesis of RNA That Contains Polyadenylate by Virion-Associated RNA Polymerase of Vesicular Stomatitis Virus

(poly(U) filters/oligo(dT)-cellulose/RNA gel electrophoresis/transcriptase/viral replication)

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Communicated by B. L. Horecker, August 9, 1973

ABSTRACT The RNA synthesized in vitro by the virion-associated RNA-instructed RNA polymerase of purified vesicular stomatitis virus contains polyadenylate sequences. These have been demonstrated by their partial resistance to pancreatic and T1 ribonucleases and their capacity to bind to poly(U) filters and oligo(dT)-cellulose. The polyadenylate sequences range in apparent size from 50 to 200 bases, similar to the size of the poly(A) in mRNA from vesicular stomatitis-virus-infected cells. Possible mechanisms of polyadenylation of the in vitro RNA product are discussed.

Vesicular stomatitis virus (VSV) is a bullet-shaped, membrane-maturing virus belonging to the rhabdovirus group (1). The genome of VSV consists of a single-stranded RNA with a molecular weight of approximately $4 \times 10^6$ (2, 3). Upon treatment of purified virions with nonionic detergents, an RNA polymerase is activated that, in the presence of four ribonucleoside triphosphates, synthesizes RNA species complementary to the genome RNA (4). These RNAs (referred to as product RNA) are smaller than the VSV genome RNA and include several RNA species, ranging in molecular weights from 2 to $10 \times 10^6$, that are partially resolved by electrophoresis in polyacrylamide gels (5). However, the product RNA contains sequences that are representative of the entire genome (6). The RNA isolated from polysomes of infected cells also corresponds in size to the RNA synthesized in vitro (7-11), except that an additional 28S virus-specific single-stranded RNA is present (8, 11). The RNA species made in vitro and in vivo hybridize to the genome RNA, indicating that they arise by a transcription process and presumably function as mRNA. It has been shown that mRNA isolated from VSV-infected cells contains polyadenylate sequences ranging in size from 70 to 250 bases (12). We demonstrate here that the RNA product synthesized in vitro by the virion-associated polymerase also contains polyadenylate sequences of similar length.

METHODS

Purification of VSV and [H]Adenosine-Labeled VSV. Baby hamster kidney (BHK 21, clone 13) cells adapted to suspension cultures were kindly provided by Dr. D. T. Dubin, Rutgers University. Cells were grown in Eagle’s minimum medium, supplemented with 5% fetal-calf serum, glutamine (4 mM), penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml), and twice the normal amounts of nonessential amino acids and vitamins. Cells were concentrated to a density of 10$^6$ cells per ml in the same medium and infected with VSV (Indiana serotype; kindly provided by Dr. D. Summers, Albert Ein-
taining RNA were according to the method of Sheldon et al. (16). Oligo(dT)-cellulose (17), was a kind gift from Dr. S. S. Kerwar, Roche Institute of Molecular Biology. Oligo(dT)-cellulose was packed in a column (0.5 × 2 cm) and equilibrated with high-salt buffer [0.5 M KCl, 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA]. Radioactive RNA samples in high-salt buffer were loaded onto the column and eluted with the same buffer. Ten 1-ml fractions were collected. The buffer was changed to low-salt buffer (as above, without KCl) and another ten 1-ml fractions were collected. The radioactive content of all fractions (referred to as acid-precipitable radioactivity in the text) was determined by adding 5 ml of ice-cold 5% trichloroacetic acid, filtering through Millipore filters, type HA, and counting in a toluene-based scintillation fluid in the Beckman LS-250 scintillation counter.

**RESULTS**

**Proteins in Purified VSV.** To determine the purity of the virus preparation, we subjected VSV polypeptides to electrophoresis in a 10% SDS–polyacrylamide gel. Five distinct polypeptide bands with molecular weights of 190,000, 69,000, 50,000, 45,500, and 29,000, were observed. Other workers have observed and named these bands (18). There was no significant amount of material in other bands, suggesting little, if any, contamination by cellular proteins.

**Transcription of VSV RNA In Vitro by the Virion-Associated Transcriptase.** Purified VSV was incubated at 30° in an incubation mixture containing four ribonucleoside triphosphates, including [α-32P]UTP as the labeled precursor (see legend of Fig. 1). Incorporation of radioactivity into RNA was linear for at least 2 hr, resulting in a 3- to 4-fold net RNA synthesis. After incubation for 2 hr, the reaction mixture was extracted with phenol–SDS, and the product RNA was separated from the radioactive precursor by Sephadex G-100 column chromatography. Labeled product RNA was denatured with (CH3)2SO, mixed with [3H]A-labeled VSV genome RNA, and layered onto a 5-30% glycerol gradient. As shown in Fig. 1, [3H] labeled VSV RNA sedimented in the position expected of 42 S; the in vitro [3H]-labeled RNA sedimented more slowly. The results show that the virus contains only 42S RNA and that the in vitro products are smaller than the genome RNA. In order to separate further the product RNA species, [3H] AMP-labeled product RNA synthesized with [3H]ATP as the labeled substrate was centrifuged in a gradient for a longer time (Fig. 2). The in vitro RNA products sedimented in a broad peak ranging predominantly from 10 to 19 S. However, 5-10% of the total acid-precipitable radioactivity applied to the gradient sedimented more rapidly than 19 S (Fig. 2).

**Polyadenylate Sequences in the RNA Product Synthesized In Vitro.** The first indication that product RNA synthesized by purified VSV contains poly(A) sequences came from the observation that [3H]AMP- and [3P]UMP-labeled product RNAs were differentially sensitive to pancreatic RNase digestion. Product RNAs were synthesized in vitro by purified VSV in separate reaction mixtures containing [3H]ATP

![Fig. 1. Glycerol gradient sedimentation of VSV genome RNA and product RNA. RNA was synthesized in vitro in an incubation mixture (0.2 ml) which contained 0.1 M NaCl, 0.05 M Tris-HCl (pH 8.0), 5 mM MgCl2, 4 mM dithiothreitol, 1 mM ATP, GTP, and CTP, 0.2 mM UTP and [α-32P]UTP (40 Ci/mmol, final specific activity = 230 cpm/pmol), 0.05% Triton N101, and 85 μg of purified VSV. Incubation was carried out at 30° (20) for 2 hr. The reaction was terminated by addition of SDS (0.5%) and the labeled RNA was extracted with phenol. The RNA was precipitated from the aqueous phase with ethanol at −20°. Unreacted [α-32P]UTP was removed by Sephadex G-100 chromatography and the RNA present in the void volume was precipitated with ethanol. The purified [3H]-labeled product RNA was denatured with (CH3)2SO (19), and precipitated with ethanol. A portion of the labeled product RNA was mixed with [3H]A-labeled VSV genome RNA and layered onto a 5-30% glycerol gradient containing 20 mM Tris-HCl (pH 8.0), 0.1 M NaCl, and 5 mM EDTA, and centrifuged at 35,000 rpm at 4° in an SW41 Spinco rotor for 5 hr. The fractions were collected from a hole pierced at the bottom of the tube and acid-precipitable radioactivity in each fraction was determined (19). In this and the following figures, the quantities indicated on the ordinate labels are the product of the experimental values and the given scale factor.**

![Fig. 2. Glycerol gradient centrifugation of [3H]AMP-labeled product RNA. RNA was synthesized in vitro as described in Fig. 1 except that the concentrations of ATP, GTP, and CTP were each 0.5 mM, [α-32P]UTP was replaced by [3H]ATP (1.2 Ci/mmol, final specific activity = 35 cpm/pmol), and 13 μg of purified VSV was used. The [3H]-labeled RNA product was purified as described in Fig. 1. A portion of the RNA was layered onto a glycerol gradient as in Fig. 1 and centrifuged at 26,000 rpm for 18 hr at 5°. Arrows indicate the positions of the three size classes of [3H]A-labeled reovirus mRNAs, 25 S, 19 S, and 12 S, synthesized in vitro by the virion-associated transcriptase (19) and centrifuged under the same conditions. Acid-precipitable radioactivity in each fraction was determined.]
To ascertain if the poly(A) sequences are covalently linked to VSV-specific product RNA and if all of the RNA species contain these sequences, we performed the following experiment. Product RNA labeled with \([^3H]AMP\) was denatured and layered onto a 5-30% glycerol gradient and centrifuged. Acid-precipitable radioactivity was determined in each of the collected fractions. In a similar gradient individual fractions were collected and digested with pancreatic RNase before measuring the acid-precipitable radioactivity. In additional samples, the labeled product RNA was treated with RNase before gradient centrifugation. Fig. 3 shows the results of this experiment. When individual gradient fractions of the \([^3H]AMP\)-labeled product RNA were treated with RNase, the profile of radioactivity resistant to RNase and precipitable by acid coincided with that of the undigested RNA (Fig. 3B). This suggests that if there are free molecules of poly(A) present, they are of the same size as the in vitro VSV-specific product RNA. However, this was found not to be the case, since when \([^3H]AMP\)-labeled product RNA was treated with pancreatic RNase (or both pancreatic and T1 RNases) before centrifugation, the RNase-resistant radioactivity sedimented in the 4S region of the gradient (Fig. 3B), showing that these sequences arise from the RNA species sedimenting from 10 to 19 S. In a similar experiment, the radioactivity in \([^3P]UMP\)-labeled product RNA was completely sensitive to RNase digestion when treated before or after centrifugation (Fig. 3A). The results suggest that most, if not all, RNA species synthesized in vitro by the VSV-associated RNA polymerase contain covalently linked poly(A) sequences.

**Table 1. Hybridization of \([^3H]AMP\)- and \([^3P]UMP\)-labeled product RNAs with VSV genome RNA**

<table>
<thead>
<tr>
<th>Virion RNA (μg/ml)</th>
<th>Treatment</th>
<th>Acid-insoluble (cpm)</th>
<th>RNase resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Hydridized</td>
<td>[^3H]RNA 4306</td>
<td>4353</td>
</tr>
<tr>
<td>None</td>
<td>Hydridized</td>
<td>[^3P]RNA 1500</td>
<td>35</td>
</tr>
<tr>
<td>None</td>
<td>Hydriized</td>
<td>[^3H]RNA 1920</td>
<td>357</td>
</tr>
<tr>
<td>0.1</td>
<td>Hydridized</td>
<td>[^3H]RNA 1982</td>
<td>450</td>
</tr>
<tr>
<td>0.2</td>
<td>Hydridized</td>
<td>[^3H]RNA 2224</td>
<td>655</td>
</tr>
<tr>
<td>0.5</td>
<td>Hydridized</td>
<td>[^3H]RNA 2329</td>
<td>1447</td>
</tr>
<tr>
<td>1.0</td>
<td>Hydridized</td>
<td>[^3H]RNA 2766</td>
<td>1898</td>
</tr>
<tr>
<td>5.0</td>
<td>Hydridized</td>
<td>[^3H]RNA 3959</td>
<td>3506</td>
</tr>
<tr>
<td>10.0</td>
<td>Hydridized</td>
<td>[^3H]RNA 4244</td>
<td>3762</td>
</tr>
</tbody>
</table>

\[^3H]AMP\)- and \([^3P]UMP\)-labeled RNAs were synthesized in *vitro* and purified as described in the legends of Figs. 1 and 2. Both products were denatured with (CH3)2SO and precipitated with ethanol. Aliquots of each product were hybridized for 2 hr at 60° with unlabelled VSV genome RNA in 0.01 M Tris-HCl (pH 7.4) containing 0.4 M NaCl in 0.2 ml (6). RNA samples were digested with 1 μg of pancreatic RNase (Worthington Biochemical Co.) for 30 min at 37° in 0.4 M NaCl buffer as indicated. Acid-precipitable radioactivity in each reaction mixture was determined.

**Fig. 3.** Glycerol gradient analysis of the nuclease-resistant fragments in product RNAs. \([^3H]AMP\)- and \([^3P]UMP\)-labeled product RNAs were separately synthesized in *vitro* and denatured as detailed in Figs. 1 and 2. Portions of the \([^3P]UMP\)-labeled product (A) and \([^3H]AMP\)-labeled product (B) were directly layered onto 5-30% glycerol gradients and centrifuged under conditions described in Fig. 2. Acid-precipitable radioactivity in each fraction was assayed (○). A second aliquot of \([^3P]\)- or \([^3H]\)-labeled RNA product was also centrifuged in a separate gradient. The salt concentration of each of the collected fractions was adjusted to 0.3 M NaCl, and pancreatic RNase (5 μg/ml) was added. The fractions were incubated for 30 min at 37° and acid-precipitable radioactivity was determined (△). Other aliquots of \([^3P]\)- or \([^3H]\)-labeled product RNAs were incubated at 37° for 30 min with pancreatic RNase (5 μg/ml) in buffer containing 0.01 M Tris-HCl (pH 7) and 0.4 M NaCl, and the digest was layered onto the gradient and centrifuged. Acid-precipitable radioactivity in each fraction was determined (○). In each case the samples were adjusted to 0.2% SDS before layering onto gradients.

or \([α-[^3P]]UTP\) as the labeled precursor. After the reaction, the two mixtures were extracted separately with phenol-SDS and the product RNAs were isolated by Sephadex G-100 column chromatography. \([^3H]\)- and \([^3P]UMP\)-labeled product RNAs were then denatured with (CH3)2SO to eliminate secondary structure before digestion with RNase. As shown in Table 1, line 2, the pancreatic RNase resistances of \([^3H]AMP\)- and \([^3P]UMP\)-labeled products were markedly different. Only 3% of the \([^3P]UMP\)-labeled product RNA was RNase-resistant, whereas 35% of the \([^3H]AMP\)-labeled product RNA was resistant. The same results were obtained when the labeled product RNAs were treated with both pancreatic and T1 RNases. Both of the labeled products hybridized to the VSV genome RNA to 90-100% at a genome RNA concentration of 5 μg/ml (Table 1, line 8). The results indicate that the product RNAs are copied from the genome RNA and the RNase-resistant radioactivity in the \([^3H]AMP\)-labeled product RNA is apparently not due to the presence of double-stranded regions, but instead to the presence of either free poly(A) or poly(A) sequences in the product RNA.
Poly(U) Filter and Oligo(dT)-Cellulose Binding of Product RNA. Poly(U) immobilized on glass filters and oligo(dT) covalently attached to cellulose have been used to detect poly(A) sequences in RNA molecules (16, 17). Under appropriate salt conditions, both poly(U) and oligo(dT) form duplexes with poly(A). Product RNA was tested for the presence of covalently linked poly(A) by both of these binding tests. Both [3H]AMP and [32P]UMP-labeled product RNAs, which were differentially RNase-resistant (35 and 3%, respectively), were similarly and efficiently bound to oligo(dT)-cellulose and poly(U) filters (Table 2). In control experiments, VSV mRNA isolated from infected cells, which was previously shown to contain poly(A) sequences (12), was bound to poly(U) filters and oligo(dT)-cellulose to a similar extent (60%). [3H]Adenosine-labeled VSV genome RNA, which was 3% RNase-resistant, bound only 0.4% to poly(U) filters and 6% to oligo(dT)-cellulose. The results indicate that the product RNA contains poly(A) sequences, and confirm that the genome RNA (12) contains insufficient adenine-rich sequences to allow binding to poly(U) filters or to oligo(dT)-cellulose. It can be seen from Table 2 that about 30% of the [3H]AMP-labeled product RNA and 36% of the [32P]UMP-labeled product RNA do not bind to oligo(dT)-cellulose. This could be due to the absence of poly(A) sequences from some RNA species or to partial degradation of some RNA by nucleases during the transcription reaction.

Size of the Poly(A) Sequences. To determine the approximate length of the poly(A) sequences in the product RNA, in vitro [3H]AMP-labeled product RNA and in vivo [3H]-adenosine-labeled mRNA were digested with pancreatic and T1 ribonucleases. The resistant fragments were subjected to electrophoresis in 20% polyacrylamide gels containing 8 M urea. Radioactive poly(A) fragments derived from both in vitro and in vivo RNAs showed a heterogenous distribution (Fig. 4). From the positions of RNA markers it was estimated that the poly(A) from RNase-treated in vitro RNAs is approximately 50-200 bases (12). In view of the similarity of the mobilities of the RNase-resistant fragments, we conclude that the poly(A) in RNA products synthesized in vitro is also 50-200 bases long.

Transcriptase assay conditions were the same as described in Fig. 2, except that [3H]ATP (40 Ci/mmol) was used as the labeled substrate and the concentration of unlabeled ATP was changed and UTP, GTP, and CTP were omitted as indicated in the table. 32 μg of purified VSV was used in each reaction.

**Table 2. VSV mRNA binding to poly(U) filters and oligo(dT)-cellulose**

<table>
<thead>
<tr>
<th>Sample</th>
<th>RNase resistance (%)</th>
<th>Poly(U) filter</th>
<th>Oligo(dT)-cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro RNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]AMP-labeled product</td>
<td>35</td>
<td>68</td>
<td>70</td>
</tr>
<tr>
<td>[32P]UMP-labeled product</td>
<td>3</td>
<td>52</td>
<td>64</td>
</tr>
<tr>
<td>In vivo RNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]A-labeled mRNA</td>
<td>44</td>
<td>63</td>
<td>66</td>
</tr>
<tr>
<td>[3H]A-labeled virion RNA</td>
<td>3</td>
<td>0.4</td>
<td>6</td>
</tr>
<tr>
<td>[3H]poly(A)</td>
<td>99</td>
<td>79</td>
<td>100</td>
</tr>
</tbody>
</table>

[3H]AMP- and [32P]UMP-labeled product RNAs were synthesized in vitro and purified as described in the legends of Figs. 1 and 2. Aliquots of each RNA product were denatured with (CH3)2SO and tested for RNase resistance. Poly(U) filter binding was carried out as described (15), while oligo(dT)-cellulose binding was as described in Methods. [3H]Poly(A) (11.6 Ci/mol; S0.15 = 8.8) was from Miles Laboratories, and [3H]A-labeled VSV mRNA was isolated from infected cells as described in Methods.

**Fig. 4. Polyacrylamide-gel analysis of polyadenylate sequences in RNA product synthesized in vitro and in infected cells.** [3H]AMP-labeled product RNA was synthesized in vitro and purified as described in Fig 2. A portion of the labeled RNA was incubated with pancreatic RNase (10 μg) and T1 RNase (1 μg) in 0.15 M Tris-HCl (pH 7.4) containing 0.005 M EDTA and yeast tRNA (100 μg). The incubation was carried out at 37° for 60 min. The RNase-resistant fragments were precipitated with two volumes of ethanol at −20° for 18 hr (15). The precipitate was collected and electrophoresed in a 20% polyacrylamide gel containing 8 M urea (32) for 16 hr at 130 V at room temperature. [3H]Adenosine-labeled VSV mRNA isolated from infected cells was similarly processed and electrophoresed was carried out in a separate gel. The gels were fractionated in a Gilson automatic gel crasher. Each gel fraction (1 mm) was dissolved in H2O and counted in Aquasol (New England Nuclear Corp.) Migrations of labeled tRNA from Bombyx mori (kindly provided by Dr. L. P. Gage, Roche Institute of Molecular Biology) and bromphenol blue dye marker are shown by the arrows. In vitro product RNA (O); in vivo mRNA (●).

**Table 3. Ribonucleoside triphosphate requirements for in vitro RNA synthesis**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>ATP (mM)</th>
<th>Incorporation (nmol/2 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>– UTP</td>
<td>1.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>– CTP</td>
<td>1.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>– GTP</td>
<td>1.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>– UTP, CTP, GTP</td>
<td>1.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>– UTP, CTP, GTP</td>
<td>0.1</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Transcriptase assay conditions were the same as described in Fig. 2, except that [3H]ATP (40 Ci/mmol) was used as the labeled substrate and the concentration of unlabeled ATP was changed and UTP, GTP, and CTP were omitted as indicated in the table. 32 μg of purified VSV was used in each reaction.
carried out in the presence or absence of various ribonucleoside triphosphates with [H]ATP as the only labeled precursor. As shown in Table 3, the in vitro transcriptase reaction was dependent on the presence of four ribonucleoside triphosphates and there was virtually no polymerization of [H]AMP when ATP was the only substrate present. Thus purified VSV, under these conditions, does not synthesize free poly(A) or add AMP residues to endogenous viral RNA.

**DISCUSSION**

The virion-associated transcriptase in purified VSV synthesizes, in vitro, RNA complementary to genome RNA. When labeled with [H]AMP, the in vitro product RNA was 35% resistant to pancreatic and T1 RNases, whereas [CP]UMP-labeled product RNA was only 3% resistant. RNA products labeled in vitro with either precursor bind efficiently to poly(U) filters and oligo(dT)-cellulose, as does in vivo mRNA. On the other hand, [H]adenosine-labeled VSV genome RNA is only 3% resistant to RNase and binds poorly to poly(U) filters or oligo(dT)-cellulose. These results show that in vitro synthesized RNA species contain covalently linked poly(A) sequences and confirm that the genome RNA contains very little, if any, poly(A) (12). The size of the poly(A) sequences is similar to that of mRNA from VSV-infected cells, 50–200 bases. Purified VSV does not possess detectable poly(A)-polymerase activity and does not add AMP residues to endogenous genome RNA.

There are several possible mechanisms by which adenylylation of product RNA might occur in vitro. First, virion-associated polymerase may transcribe poly(U) sequences located in the viral genome, resulting in the synthesis of poly(A). A similar mechanism was suggested for adenylylation of vaccinia RNA synthesized in vitro by transcription of thymidine-rich regions in vaccinia DNA by the core-associated transcriptase (21). Second, AMP residues may be added sequentially to the 3' ends of RNA chains in the absence of a poly(U) template, presumably by an enzyme similar to that reported by Edmonds and Abrams (22). Such post-transcriptional modification has been suggested for adenylylation of mRNAs isolated from eukaryotic cells as well as from cells infected with DNA- or RNA-containing viruses (23–26). Third, virion-associated transcriptase may synthesize poly(A) by repeated transcription of short tracts of uridine residues in the RNA template by a mechanism similar to that reported in the E. coli DNA-dependent RNA polymerase system (27), where the enzyme synthesizes poly(A) from ATP by slipping on oligo(dT) sequences in the denatured DNA template in vitro. Similar results were also obtained with phage T3 RNA polymerase and denatured T3 DNA (28). In the VSV system, poly(A) is found covalently associated with newly synthesized RNA chains and no free poly(A) is synthesized or is added to the endogenous viral RNA in the presence of ATP alone. Recently, it has been shown that VSV genome RNA does not contain U-rich sequences (29), although uridylic acid is the predominant base in VSV genome RNA, comprising 31 mole percent (7). From these observations, it appears that adenylylation of VSV product RNA in vitro is not mediated by transcription of U-rich regions in the genome RNA template, but possibly by a slippage mechanism from short uridine tracts. It is also possible that the poly(A) sequences in product RNA may be synthesized by an enzyme similar to that reported by Edmonds and Abrams (22) with newly synthesized RNA acting as a primer for AMP incorporation. The synthesis of poly(A) could be mediated by the viral transcriptase alone or in combination with any of the other viral structural proteins.

While this work was in progress, we learned that cell-free extracts from VSV-infected L-cells also synthesize RNA containing poly(A) sequences (30). Similar observations have also been made with purified Newcastle disease virus (31).

We thank Drs. Aaron J. Shatkin and C. Martin Stoltzfus for reviewing the manuscript.