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Adenylate-Rich Sequences in Vesicular Stomatitis Virus Messenger Ribonucleic Acid

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Vesicular stomatitis virus (VSV)-specific messenger ribonucleic acid (mRNA) species contain sequences of adenylate-rich RNA which are more heterogeneous in their migration through sodium dodecyl sulfate-polyacrylamide gels than the corresponding fractions from HeLa cell mRNA. VSV virion RNA contains no adenylate-rich sequences. The possible role of such sequences in the mRNA species of a cytoplasmically replicating virus is discussed.

Vesicular stomatitis virus (VSV) is a member of the rhabdovirus group (12) that contains a single-stranded ribonucleic acid (RNA) genome with a molecular weight of about 3.6×10^6 (14, 20) and replicates entirely in the cytoplasm of susceptible cells. During its replicative cycle, virus-specific messenger RNA (mRNA) is synthesized in the cytoplasm; most of this mRNA has an approximate sedimentation coefficient of 13*S* and is complementary to the RNA contained in the virion (13, 19, 24, 29). At least part of this 13*S* mRNA is likely synthesized by a virus-specific transcriptase contained within the infectious virus particle which has been shown to carry out in vitro synthesis of complementary VSV RNA with sedimentation properties similar to VSV mRNA in vivo (2, 4, 5).

Recently, several laboratories have reported that animal cell mRNA and heterogeneous nuclear RNA contain sequences of adenylate-rich RNA of about 150 to 200 nucleotides in length (8, 9, 18, 26). One study has provided some evidence that this poly-A sequence is added after transcription of the RNA (7). At least two deoxyribonucleic acid-containing viruses, vaccinia and adenovirus, specify the synthesis of RNA containing similar segments of adenylate-rich RNA (16, 23); two RNA-containing viruses (1); and RNA tumor viruses contain this sequence in their genomes (10, 11, 17).

The function of the terminal adenylate-rich sequence on cellular RNA molecules is unknown; it has been suggested that these sequences may play some role in the processing and transport of mRNA from the nucleus to the cytoplasm or in translation of the mRNA on polysomes (1, 7), or both. Preliminary evidence from this laboratory suggested that VSV-specific RNA isolated from the polysomes of infected cells and which hybridized with virion RNA contained a base composition which was higher in adenylic acid than was complementary to the uridylic acid content of virion RNA. This mRNA fraction hybridized to polyribouridylic acid such that 5 to 7% of ^{3}H-adenosine-labeled RNA was resistant to ribonuclease digestion (19). Since VSV mRNA apparently requires neither processing nor nuclear transport prior to its translation, and since infection by VSV effectively inhibits cellular protein synthesis (20), we have compared the 13*S* VSV mRNA with HeLa cell mRNA with respect to ribonuclease-resistant adenylate-rich sequence.

MATERIALS AND METHODS

Cells and virus. Conditions used for growth of HeLa S3 cells in suspension cultures, and for the growth, purification, and assay of VSV (Indiana serotype) have been described (6, 20). Conditions for infection of HeLa cells were as in Cohen et al. (6).

Radioactive labeling of HeLa cell and VSV RNA. Cells (10^8; 4×10^6 cells/ml) were infected with VSV; actinomycin D (5 μg/ml) was added to the culture at 1 hr postinfection; fetal calf serum (5%) was added at 1.5 hr postinfection; and 5 mCi of ^{3}H-adenosine or 5 mCi of ^{3}H-uridine (New England Nuclear; specific activity, 5 Ci/mmole and >20 Ci/mmole, respectively) was added at 3 hr postinfection. ^{32}P labeling was carried out similarly by the addition of 10 mCi of carrier free H_3-$^{32}PO_4$ (New England Nuclear) to the infected culture suspended in minimal essential medium missing phosphate. At 5 hr postinfection, the infected cells were placed at 4 C and washed once with cold Earle's solution, and cytoplasmic extracts were prepared and sedimented through sucrose gradients (19). When uninfected cells were used, 0.06 μg of actinomycin D per ml was added to the cell culture 30 min prior to addition of radiolabeled nucleosides to stop synthesis of cell

ribosomal RNA species but not mRNA or transfer RNA (tRNA) (22).

Extraction and purification of 13S VSV mRNA. The polysome region of sucrose gradients was pooled and diluted three- to fivefold with RSB (0.01 M NaCl, 1.5 mM Mg^{2+} and 0.01 M tris[hydroxymethyl]-aminomethane [Tris], *p*H 7.4). The material in this pooled fraction was then pelleted by centrifugation in a Spinco no. 30 rotor at 100,000 × *g*, 5 C, for 3 hr. The pellet was suspended in 1 ml of NETS buffer (0.1 M NaCl, 0.01 M ethylenediaminetetraacetate [EDTA], 0.01 M Tris [*p*H 7.4], and 0.2% sodium dodecyl sulfate [SDS]), and layered over a 36-ml, 15 to 30% linear sucrose gradient in NETS. Conditions of centrifugation are described in figure legends.

Samples of each gradient fraction were analyzed for radioactivity, and the 13*S* peak of VSV-specific RNA was pooled (*see* Fig. 1B), 100 μg of purified HeLa cell ribosomal RNA was added as carrier, the solution was made 0.2 M in NaCl and 65% ethanol, and the RNA was allowed to precipitate at −85 C for 16 hr.

The RNA was collected by centrifugation in a Sorvall SS-34 rotor (4 C, 45 min, at 27,000 × *g*), and the pellet was dissolved in an appropriate buffer for further analysis.

Agarose gel filtration of radiolabeled RNA. A column (1.5 by 85 cm) containing sepharose 2B (2% agarose beads, Pharmacia Fine Chemicals) was prepared and equilibrated at room temperature with a solution containing 0.01 M NaCl, 0.001 M EDTA, 0.01 M Tris (*p*H 7.4), and 0.5% SDS. Radiolabeled RNA (dissolved in 1 ml of the eluting buffer) was applied to the column; elution was carried out with a pressure of 15 cm of water, yielding a flow rate of about 3.5 ml/hr. Void volume for the column was about 30 ml. Fractions from the column were pooled and made 0.2 M in NaCl; carrier HeLa cell RNA was added, and the RNA was precipitated with cold ethanol as above.

Ribonuclease digestion. Precipitated RNA was dissolved in 1 ml of buffer containing 0.01 M Tris (*p*H 7.4), 0.2 M NaCl, 0.01 M EDTA; 50 units of T_1 ribonuclease (Calbiochem) and 10 μg of pancreatic ribonuclease (Calbiochem or Worthington) was added, and the solution was incubated at 37 C for 60 min. The reaction was stopped by the addition of 0.5% SDS, and ribonuclease-resistant oligonucleotides were precipitated with ethanol.

Polyacrylamide gel electrophoresis. Precipitated RNA was dissolved in 0.15 ml of a solution containing 0.01 M $NaPO_4$ (*p*H 6.8), 0.01 M NaCl, 0.02 M EDTA, 0.1% SDS, 10% glycerol, and bromophenol blue dye as marker, and was layered over an 18-cm column of 10% polyacrylamide gel (acrylamide-bisacrylamide; 20:1). Electrophoresis buffer was the same as above except the NaCl was 0.1 M. Electrophoresis was carried out for 18 hr, 10 mA/gel at room temperature. Gels were fractionated and assayed for radioactivity as previously described (27).

Nucleotide analyses. Ribonuclease-resistant ^{32}P RNA was extracted from fractionated acrylamide gels, and the base composition was determined by the method of Sebring and Salzman (25).

RESULTS

The sedimentation pattern of polyribosomes from cytoplasmic extracts of VSV-infected HeLa cells were indistinguishable from those of uninfected cells. After 2 hr of incubation of infected cells with radioactive adenosine or uridine in the presence of actinomycin D, radioactivity was found in the polysome region of the sucrose gradient (Fig. 1A). The labeled RNA extracted from the pooled polysome fractions contained a heterogeneous population of molecules that sedimented primarily at approximately 13*S* (Fig. 1B). An RNA peak sedimenting at approximately 26 to 30*S* was also observed. This fraction has been shown to contain ribonuclease-resistant material (19), and its relationship to mRNA is not clear. The highly labeled peak at fraction 12 (Fig. 1A) is comprised primarily of viral cores and contains predominantly 42*S* RNA. These fractions were excluded for the further preparation of viral mRNA.

Approximately 5 to 10% of the 13*S* viral RNA obtained from the polysome region of sucrose gradients showed properties of double-stranded RNA. It was soluble in 2 M LiCl, highly resistant to ribonuclease digestion in 0.2 M NaCl, and excluded from 2% agarose beads. This contaminating double-stranded RNA was removed from all preparations by gel filtration on sepharose 2B (Fig. 2). Single-stranded RNA eluted from the agarose column retained its sedimentation coefficient of 13*S* when resedimented through sucrose density gradients.

Radioactivity in single-stranded RNA that had been labeled with uridine was completely digested by pancreatic and T_1 ribonuclease. However, adenosine-labeled mRNA always yielded a ribonuclease-resistant fraction of 13 to 20% (Fig. 3). The double-stranded RNA recovered from the void volume of the sepharose column was always greater then 50% resistant to these conditions of nuclease digestion, regardless of whether the radioactivity was present as purine or pyrimidine nucleoside.

The adenosine-labeled ribonuclease-resistant core from single-stranded VSV mRNA was analyzed by polyacrylamide gel electrophoresis and compared with the adenosine-rich sequence isolated from uninfected HeLa cell mRNA (Fig. 4). The HeLa cell adenylate-rich sequence migrates more slowly through 10% polyacrylamide gels than 4*S* tRNA and is more heterogeneous in mobility than ribosomal RNA or tRNA markers in similar gels, in confirmation of reports by other laboratories (8, 9; Fig. 4A). Treatment of this RNA with dimethyl sulfoxide before and during nuclease digestion did not alter the mobility of

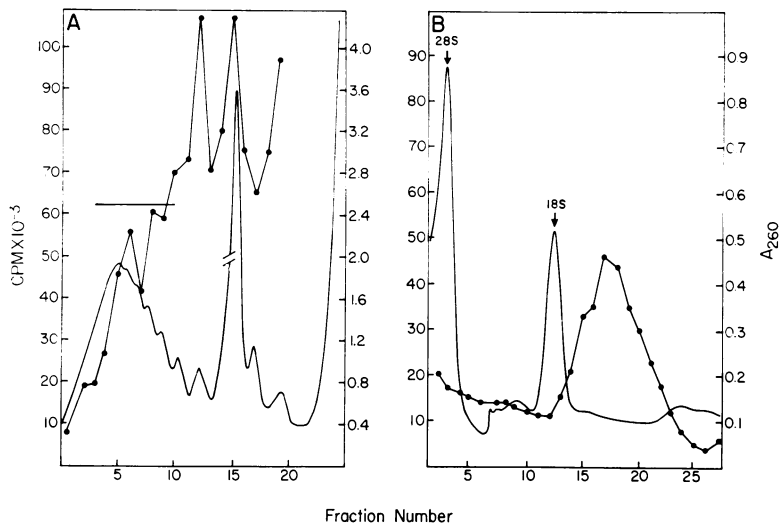

FIG. 1. *Isolation of VSV mRNA. Approximately 1 × 10⁸ HeLa cells were infected and labeled with ³H-adenosine (5 mCi) for 2 hr as described. Cells were collected by centrifugation, and cytoplasmic extracts were layered over linear 7 to 52% (w/w) 36-ml sucrose gradients in RSB, which were centrifuged in a Spinco SW27 rotor (4 C, 16 hr, 16,000 rev/min). Samples (50 μliters) of each fraction were assayed for radioactivity (A). The top of the gradient was not assayed for radioactivity since it contained large amounts of acid-soluble radioactivity. Fractions of the gradient indicated by the bar were pooled, diluted with RSB, and centrifuged in a Spinco no. 30 rotor (3 hr, 30,000 rev/min, 4 C). The pellet was suspended in 1 ml of NETS buffer, layered over a 15 to 30% linear sucrose gradient in NETS, and centrifuged at 23 C, in a Spinco SW27 rotor at 27,000 rev/min for 17 hr. Samples (50 μliters) of each fraction were assayed for radioactivity, and appropriate fractions of 13S RNA were pooled and precipitated with cold ethanol (B).*

the resulting product (*unpublished results*). The length of HeLa cell adenylate-rich sequences has been estimated at between 150 to 200 nucleotides (8, 9).

Figure 4C shows a similar electrophoresis pattern of the ribonuclease-resistant VSV mRNA fraction. The material is even more heterogeneous than the corresponding HeLa cell fraction and frequently falls into two apparent size classes, both of which migrate more slowly than 4*S* tRNA. As expected, no oligonucelotides containing uridine were detected by gel electrophoresis after ribonuclease digestion (Fig. 4B).

To determine the nucleotide composition of the nuclease-resistant sequences, infected cells were labeled with $^{32}PO_4$ under the same conditions as described for uridine or adenosine labeling of RNA. Polysome RNA was purified by sucrose gradient sedimentation and agarose gel column chromatography, and the eluted single-stranded RNA was subjected to polyacrylamide gel electrophoresis. Alternate gel fractions were analyzed for radioactivity, and all remaining fractions from the heterogeneous peak were pooled and separated from the polymerized polyacrylamide by centrifugation. The recovered oligonucleotides were hydrolyzed and analyzed for base composition, yielding 93% adenosine monophosphate (Table 1). ^{32}P-labeled double-stranded RNA recovered from the sepharose column yielded a mixture of all four labeled ribonucleotides. Base ratios were not precisely complementary since replicative intermediates are also excluded from the sepharose column.

Analyses of VSV RNA extracted from purified virions demonstrated no adenylate-rich sequences contained in the viral RNA. All radioactivity from uridine-, adenosine- or ^{32}P-labeled RNA was sensitive to ribonuclease digestion, and no oligonucleotides migrating more slowly than bromophenol blue dye marker could be detected on polyacrylamide gels.

DISCUSSION

VSV-specific mRNA extracted from polysomes of infected cells labeled in the presence of actinomycin D contain sequences of oligonucleotides that are resistant to pancreatic and T_1 ribonuclease digestion at moderate salt concentrations and which are comprised of approximately 93% adenylic acid. The adenylate-rich sequences are relatively more heterogeneous than the corresponding regions which have been described for HeLa cell mRNA and hemoglobin mRNA (8, 9, 21) and

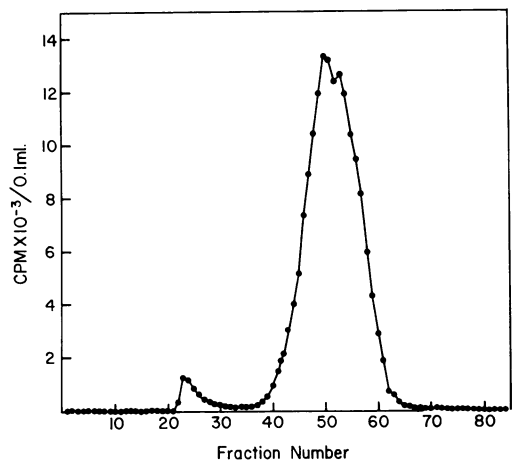

FIG. 2. *Sepharose gel filtration of 13S VSV RNA. 13S RNA precipitated from fractions of a sucrose gradient (see Fig. 1B) was dissolved in 1 ml of column buffer and was applied to a 2% sepharose column as described. Samples (100 μliters) of each fraction were assayed for radioactivity.*

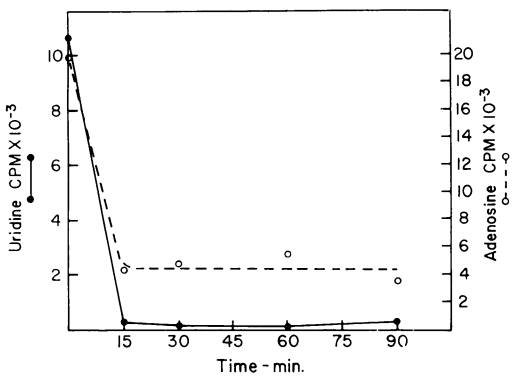

FIG. 3. *Ribonuclease digestion of single-stranded 13S VSV RNA. Single-stranded VSV 13S RNA, labeled either with ^{3}H-uridine or ^{3}H-adenosine, was precipitated with cold ethanol after elution from a sepharose column (see Fig. 2). The RNA was dissolved in 1 ml of 0.2 M NaCl buffer (see Materials and Methods) and was incubated with pancreatic and T_1 ribonucleases at 37 C. Samples (10 μliters) were taken at the indicated times and were assayed for trichloroacetic acid-insoluble radioactivity.*

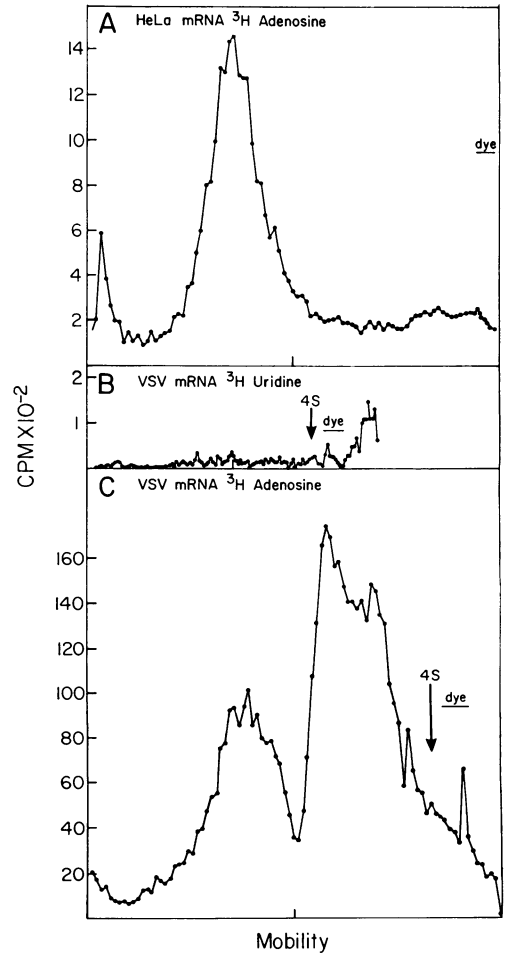

FIG. 4. *Polyacrylamide gel analysis of ribonuclease-resistant sequences in HeLa and VSV mRNA. A, ^{3}H-adenosine-labeled HeLa mRNA was prepared and digested with ribonuclease as described. The nuclease-resistant oligonucleotides were precipitated with ethanol, dissolved in 0.1 ml of gel buffer, and applied to an 18-cm column of 10% polyacrylamide gel. Electrophoresis was for 18 hr at 10 mA/gel. ^{14}C-Uridine-labeled 4S RNA from HeLa cells was subjected to electrophoresis on a parallel gel. The bromophenol dye marker was retained at the end of the gel. Migration is from left to right. ^{3}H-Uridine-labeled (B) or ^{3}H-adenosine-labeled (C) VSV 13S single-stranded RNA was prepared as described in Materials and Methods and was digested with ribonuclease, and samples were subjected to electrophoresis as in (A).*

apparently range from approximately 70 to 250 nucleotides in length, based on their rate of migration in polyacrylamide gels. However, the interpretation of the precise size of ribonuclease-resistant poly-A fragments must be made with caution, since, even under salt and concentration conditions where no loss of trichloroacetic acid-precipitable radioactivity in commercially prepared polyadenylic acid occurs, a sharp decline in viscosity is detected (15), indicating preferential endonucleolytic attack of longer poly-A molecules. A decrease in the average size of commercial poly A occurs under the conditions employed in

TABLE 1. *Nucleotide composition of ribonuclease-resistant sequences from ^{32}P-labeled VSV 13S mRNA, and of ^{32}P-labeled double-stranded polysomal VSV RNA*

Determinations	Counts/min	% Total counts/min
13S mRNA[a]		
AMP	379	92.5
UMP	6	1.5
GMP	6	1.5
CMP	19	4.5
Double-stranded RNA[b]		
AMP	705	31.3
UMP	650	28.9
GMP	355	15.8
CMP	539	24.0

[a] The ^{32}P-labeled VSV 13S mRNA fraction was obtained from polyacrylamide gel fractions as described in the text. Abbreviations: AMP, adenosine monophosphate; UMP, uridine monophosphate; GMP, guanosine monophosphate; CMP, cytosine monophosphate.

[b] Double-stranded RNA was obtained directly from the void volume of a sepharose 2B column similar to that shown in Fig. 2.

these experiments (*unpublished results*); however, the rate of attack seems to be highly dependent upon the concentration of poly A available as substrate (3). The concentration of adenylate-rich sequences from VSV mRNA in the experiments is not known.

The function of poly A sequences in VSV mRNA, as in other viral and cellular mRNA species, is not known. Although most if not all mRNA species contain these sequences, they are apparently not essential for translation to occur, since neither cellular histone mRNA (M. Adesnik, *personal communication*) nor reovirus mRNA (28) contain them. If adenylate-rich sequences function in the processing, selection, or transport of mRNA from the nucleus, it is perhaps surprising that a cytoplasmically replicating virus such as VSV would contain them, although competition for protein-synthesizing factors in the cell might require that VSV mRNA be made chemically similar to cellular mRNA, even though the sequences may be functionless except for mimicry. Poliovirus and Eastern equine encephalitis virus, both cytoplasmically replicating RNA viruses whose mRNA species are probably identical to virion RNA, contain poly A at the 3′ terminus of the virion RNA, but the sequence is only about 50 to 70 nucleotides long (1) and may be quite heterogeneous (E. Wimmer, *personal communication*).

The adenylate-rich sequence represents approximately 13 to 20% of the adenosine label in VSV mRNA. Since all detectable VSV-specific proteins are synthesized at all times during the replication cycles (20), it is likely that all species of mRNA are synthesized during the 2-hr labeling period employed. However, it is not possible to determine whether all species of VSV-mRNA contain adenylate-rich sequences or whether the various mRNA species differ in this respect.

The addition of adenylate sequences to cellular RNA appears to occur in the nucleus sequentially after transcription (7). If the synthesis of poly A on VSV mRNA also occurs after transcription, the enzymatic activity responsible must also occur in the cytoplasm of infected cells, regardless of whether it is viral or cellular in origin. Alternatively, VSV virion RNA might contain complementary regions of polyuridylic acid. These questions are currently under investigation.

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