Rabies Virus-induced RNA Synthesis in BHK21 Cells

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SUMMARY

Rabies virus polysomes contained two sizes of messenger RNAs, one of which had a sedimentation value of 30S and another which sedimented at 12 to 16S. RNA extracted from infected cultures contained virion-size RNA, 42S, as well as 30S and 12 to 16S RNA species. Hybridization studies indicated that the 30S and 12 to 16S RNAs had nucleotide sequences which were complementary to virion RNA. RNA isolated from virus polysomes contained adenylate-rich sequences which were heterogeneous in size and were determined to be about 100 to 250 nucleotides in length on the basis of their migration rates in polyacrylamide gels. Acid-urea agarose gel electrophoresis established that the 30S RNA material was composed of a single RNA species (mol. wt. > 1.65 x 10^6), whereas the 12 to 16S material could be resolved into at least four distinct species whose mol. wt. ranged from 0.28 to 0.87 x 10^6. When labelled rabies-infected cell RNAs, which were purified by oligo(dT)-cellulose chromatography, were annealed to excess unlabelled virus RNA, digested with ribonuclease T2 and the RNA duplex molecules analysed by polyacrylamide gel electrophoresis, five duplexes could be separated. The mol. wt. of these duplexes were estimated to be 3.2, 1.4, 0.96, 0.55 and 0.39 x 10^6, when compared to the known mol. wt. of vesicular stomatitis virus (VSV) RNA duplexes. This study suggests that the replicative processes of rabies virus are very similar to VSV and that rabies virus proteins are probably translated from smaller than virion-size RNAs.

INTRODUCTION

Several recent studies have provided evidence that rabies is a negative-strand Rhabdovirus, like vesicular stomatitis virus (VSV), since the virion RNA was non-infectious and purified virions contained an in vitro RNA polymerase activity (Sokol et al. 1969; Bishop & Smith, 1977; Kawai, 1977). The general scheme of rabies RNA transcription is believed to be similar to that of VSV and to proceed via the formation of several smaller than genome-size messenger RNAs (Huang et al. 1970; Mudd & Summers, 1970; Schincariol & Howatson, 1970). One report showed that rabies RNAs synthesized during infection sedimented in three major size classes of 39S, 25 to 35S, and 8 to 25S and represented about 9%, 15% and 64%, respectively, of the total radioactivity (Ermine & Flamand, 1977). This study also indicated that the 25 to 35S and 8 to 25S RNA species could hybridize to virion RNA. Villereal & Holland (1976) used denaturing gel electrophoresis and found at least seven species of rabies virus-induced RNA during acute infection in BHK21 cells. The most abundant species had an apparent mol. wt. of 0.62 x 10^6, the largest was virion-size and the smallest species detected was about 0.31 x 10^6. They also examined the RNA species in BHK21 cultures persistently infected with rabies virus for 1.5 years and found five RNA
species which ranged in mol. wt. from $1.65 \times 10^6$ to $0.45 \times 10^6$. No virion RNA was detected in the persistently infected cultures.

We describe in this study our analyses of the rabies RNA species associated with virus-specific polysomes. In addition, the RNAs induced during infection were directly analysed by sucrose-gradient centrifugation and further resolved by acid-urea agarose gel electrophoresis. By using the complementarity of mRNA to its virion RNA, we have been able to form duplex RNA molecules to separate and identify unambiguously rabies messenger RNAs.

**Methods**

**Materials.** Purified reagents for agarose and polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, Calif. All radioisotopes used in this study were supplied by New England Nuclear Corp., Boston, Mass. Absolute grade urea was from Research Plus, Inc., Denville, N.J. Worthington Biochemicals, Freehold, N.J. supplied ribonuclease (RNase) A; ribonuclease T1 and T2 was from Calbiochem-Behring Corp. La Jolla, Calif. Oligo(dT)-cellulose (type T-3) was purchased from Collaborative Research, Inc., Waltham, Mass.

**Cells, viruses, radioactive labelling and virus purification.** Baby hamster kidney (BHK21) cells were maintained as monolayer cultures and used throughout this study. Vesicular stomatitis virus (VSV) and the challenge virus stock (CVS) fixed strain of rabies virus were grown, cloned and stored as described by Coslett et al. 1980. Virus labelling conditions and purification procedures have been described (Coslett et al. 1980).

**Ribonuclease digestion and polyacrylamide gel electrophoresis of adenosine-labelled RNAs.** To isolate adenylylate-rich RNA sequences, $^3$H-adenosine-labelled RNA was suspended in 0.01 M-tris (pH 7.5), 0.3 M-NaCl, 0.001 M-EDTA and digested with ribonuclease T1 (40 units/ml) and ribonuclease A (10 µg/ml) for 45 min at 37 °C. Polyadenylate [poly(A)] fragments were purified by oligo(dT)-cellulose chromatography, concentrated with ethanol and 50 µg carrier RNA and suspended in 0.01 M-sodium phosphate (pH 7.0), 0.002 M-EDTA, 0.5% SDS and 8 M-urea. Electrophoresis was for 20 h at 3 mA/gel on 0.6 x 13 cm long 10% polyacrylamide gels (acrylamide:bis-acrylamide, 20:1) which contained 0.1 M-sodium phosphate (pH 7.0) 0.002 M-EDTA, 0.5% SDS and 8 M-urea. The electrode buffer was 0.1 M-sodium phosphate (pH 7.0) and 0.5% SDS. After electrophoresis the gels were frozen, sliced into 1 mm sections and placed into scintillation vials; the radioactivity was eluted overnight at 50 °C in 7 ml of a toluene-based scintillant, which contained 4% (v/v) TS-1 solubilizer (Research Products International, Elk Grove, Ill.).

**Preparation and analysis of rabies intracellular RNAs.** Monolayer cultures of BHK21 cells (∼1 x 10⁶ cells) were inoculated with rabies virus at an input multiplicity of about 20 to 40 p.f.u./cell. After 45 min adsorption at 36 °C, the inoculum was removed and MEM containing 2% dialysed foetal calf serum was added. The cultures were incubated at 36 °C and at various intervals were pulsed with 100 µCi/ml $^3$H-uridine or $^3$H-adenosine in the presence of actinomycin D (2 µg/ml) after a 30 min pre-treatment with the drug. Cells were harvested at the end of the pulse period by scraping, collected by centrifugation, and washed twice with cold TSE buffer (0.02 M-tris, pH 7.6, 0.15 M-NaCl, 0.002 M-EDTA). The cells were resuspended in 2.0 ml cold TSE buffer and a cytoplasmic extract was prepared by adding Nonidet P40 (NP40) and sodium deoxycholate (DOC) to 1% and incubating for 20 min on ice. Nuclei and particulate debris were removed by centrifugation (5000 rev/min, 5 min, 4 °C) and the supernatant was treated with 750 µg/ml autodigested pronase for 30 min at 37 °C after being adjusted to 0.1 M-tris, pH 9.0, 0.01 M-EDTA, 1% (w/v) SDS and 0.5% (v/v) 2-mercaptoethanol. The preparation was extracted three times with an equal vol. of phenol:chloroform:isoamyl alcohol (50:49:1) and the RNA was precipitated from the aqueous phase with 2 vol. ethanol and 0.2 M-LiCl. The RNA was recovered by centrifuga-
Rabies virus mRNAs

Purified RNA was stored under ethanol at −70 °C until needed.

RNAs were analysed by centrifugation at 23000 rev/min (SW41 rotor) for 16 h at 20 °C in 15 to 30 % (w/w) linear gradients of sucrose containing 0.1 M-NaCl, 0.002 M-EDTA and 0.1 % SDS in 0.02 M-tris, pH 7.6. Fractions (0.4 ml) were collected from the bottom of the gradient, and portions were used to determine trichloroacetic acid-insoluble radioactivity before or after RNase treatment (RNase A, 20 µg/ml, incubated for 20 min at 37 °C).

In some experiments it was necessary to purify RNAs which contained poly(A) sequences. For this, the RNAs were suspended in binding buffer [0.01 M-tris, pH 7.5, 0.5 M-LiCl, 0.5 % lithium dodecyl sulphate (LiDS), 0.002 M-EDTA; Hay et al. 1977] and applied to 1.0 g oligo(dT)-cellulose equilibrated in binding buffer in a 0.5 × 10 cm glass column. The column was washed with 10 ml binding buffer and the bound RNA was eluted with 2 ml of 0.01 M-tris, pH 7.5, 0.05 % LiDS, 0.002 M-EDTA. The eluted RNA was precipitated either with 0.2 M-LiCl and ethanol or by the CTAB procedure.

Electrophoresis of RNA in acid-urea agarose gels. RNA samples dried under vacuum were suspended in 0.025 M-sodium citrate buffer (pH 3.3), 7 M-urea, 98 % formamide and 0.006 % bromophenol blue, and were heated at 75 °C for 2 min before loading (Freeman et al. 1979). Samples were analysed by electrophoresis for 20 h at 4 V/cm at 4 °C on 1.5 % agarose submersed horizontal slab gels containing 6 M-urea and 0.025 M-sodium citrate buffer, pH 3.3 (Lehrach et al. 1977; Wertz & Davis, 1979). After electrophoresis, the RNA was fixed and the urea removed by five washes in 10 % (v/v) acetic acid. The slab was dehydrated and impregnated with 2,5-diphenyloxazole (PPO) in a single step by treating the slab for 2 h at 20 °C with 3 vol. of 66 % (v/v) glacial acetic acid containing 30 % (v/v) dioxane, 4 % (v/v) hexadecane, 6 % (w/v) naphthalene and 16 % (w/v) PPO. The PPO was precipitated in the gel by several washes in cold water. The gel was dried onto chromatography paper and was used to expose duPont Cronex-4 medical X-ray film at −70 °C with the aid of a duPont Cronex Hi-Plus intensifying screen.

RNA annealing. RNA-RNA hybridization and RNA duplex analysis were based on the method developed by Ito & Joklik (1972) as modified by Freeman et al. (1977) and Rhodes et al. (1977). Unlabelled virus RNA (10 to 15 µg) was mixed with labelled RNA, which was purified by oligo(dT)-cellulose chromatography (1 to 2 µg), in 0.03 ml of 0.001 M-EDTA, pH 7.6. The RNAs were denatured by adding 0.270 ml of sterile deionized dimethyl sulphoxide (DMSO) and heating at 45 °C for 30 min in 1.5 ml polypropylene tubes. The solution was adjusted to 60 % DMSO, 0.02 M-tris, pH 7.6, 0.03 M-NaCl, 0.003 M-EDTA and incubated at 37 °C for 14 to 18 h. The RNAs were precipitated by adding 0.05 ml of 3 M-NaCH₃COO/0.1 M-Mg(C₂H₅O₃)₂ and 1.0 ml of absolute ethanol, then mixing and chilling in a dry ice–ethanol bath for 5 min. The RNAs were recovered by centrifugation in a Beckman microfuge at 10000 rev/min for 5 min. The RNA was resuspended in 0.3 ml of 0.3 M-NaCH₃COO (pH 4.7) and re-precipitated with 1 ml ethanol. After collecting the RNA by centrifugation it was washed twice with ethanol; the supernatant was removed and residual ethanol eliminated under vacuum.

Annealed RNA was dissolved in 0.04 ml sterile H₂O. After adding 0.05 ml of 0.1 M-NaCH₃COO (pH 4.5), 0.02 ml of 1 M-NaCl and 1 unit of RNase T₂, the mixture was incubated at 37 °C for 30 min. Digestion was stopped with 0.01 ml of 10 % (w/v) SDS and the RNA was prepared for electrophoresis by adding 0.002 ml 20 × TPE (0.6 M-tris-base, 0.6 M-NaH₂PO₄, 0.02 M-EDTA), 0.025 ml glycerol and 0.002 ml 1 % bromophenol blue. RNA duplexes were separated on 5 % cylindrical (0.6 × 10 cm) polyacrylamide gels in 1 × TPE buffer by electrophoresis for 20 h at 3 mA/gel. After electrophoresis the gels were frozen, sliced into 1 mm fractions and processed for liquid scintillation spectrometry.

Preparation of polysomes. Rabies-infected monolayer cultures of BHK21 cells (3 to 5 ×
Fig. 1. Velocity sedimentation analysis of polysomes from rabies infected BHK21 cells. Monolayer cultures were infected with rabies virus at an input multiplicity of 20 to 40 p.f.u./cell and labelled with $^3$H-uridine from 8 to 12 h p.i. in the presence of actinomycin D (2 μg/ml). Cytoplasmic extracts were prepared as described in the Methods section; 40 ml were loaded on 15 to 45% (w/w) linear sucrose gradients which were centrifuged for 3.5 h at 27000 rev/min in a Beckman SW27 rotor. After centrifugation each fraction was monitored for absorbance at 260 nm (solid line) and for TCA-insoluble radioactivity before (○—○) or after (▲—▲) treatment with RNase A (20 μg/ml, 20 min, 37°C).

Fig. 2. Gradient analysis of RNA from rabies polysomes. Fraction A, B, and C from the gradient in Fig. 1 were separately pooled, diluted with TKM-S buffer and centrifuged in a Beckman SW27 rotor (3.5 h, 27000 rev/min, 4°C). The pellets were resuspended in 0.5 ml of TSE buffer, treated with SDS (1%, w/v) and 2-mercaptoethanol (1%, v/v) and layered over 15 to 30% (w/w) linear sucrose gradients in 0.02 M-tris (pH 7.6), 0.002 M-EDTA, 0.1 M-NaCl and 0.2% SDS. After centrifugation in a Beckman SW41 rotor for 16 h at 23000 rev/min and 20°C, samples (0.4 ml) were collected and the TCA-insoluble radioactivity in each was determined. The arrows mark the positions of rabies virion RNA, and BHK21 28S and 18S ribosomal RNA centrifuged in a separate gradient.

$10^7$ cells were scraped into TKM-P buffer (0.02 M-tris, pH 7.6, 0.15 M-KCl, 2 mM-MgCl$_2$), centrifuged for 3 min at 1500 rev/min, washed twice with buffer and resuspended in 4 ml of cold TKM-P buffer. After 2 min, 0.4 ml of a 1:1 mixture of 10% (v/v) NP40 and 10% (w/v) DOC was added and the cells were incubated for 15 min on ice with occasional shaking. The lysates were clarified as above and the supernatant centrifuged at 10000 rev/min for 10 min. The supernatant was removed, layered on to a 32 ml 15 to 45% (w/w) linear sucrose gradient in TKM-S buffer (0.02 M-tris, pH 7.6, 0.05 M-KCl, 2 mM-MgCl$_2$) and centrifuged for 3.5 h at 27000 rev/min at 4°C in a Beckman SW27 rotor. After centrifugation the gradients were fractionated through a recording spectrophotometer into 0.9 ml aliquots and saved for further analyses. In some experiments, infected cells were washed in TKM-S
buffer, incubated in the same buffer for 30 min at 4 °C and a cytoplasmic extract prepared by homogenization in a stainless steel Dounce homogenizer. After clarification the extract was centrifuged as above to display the polysome profile.

RESULTS

Rabies virus specific polysomes

When cytoplasmic extracts of rabies virus-infected cells were analysed by velocity sedimentation in sucrose gradients, two minor peaks (Fig. 1, fractions A and B) and one major peak (C) of radioactivity were found. The major peak C sedimented at about 120S and was about 80 to 90 % ribonuclease-resistant. By electron microscopy it was identical in appearance to the nucleocapsid isolated from purified virus (not shown). In some experiments cytoplasmic extracts were prepared by Dounce homogenization instead of detergent treatment. The sedimentation profiles of these extracts were the same as shown here (Fig. 1); however, the major peak of radioactivity sedimenting at 120S was decreased by 15 %. Three regions from the rabies virus polysome gradient (Fig. 1) fractions A, B and C were separately pooled, pelleted and extracted with EDTA and SDS, and the RNAs analysed by SDS-sucrose gradient centrifugation. These analyses (Fig. 2) indicated that the larger polysomes in fraction A contained mostly RNA sedimenting at about 12 to 16S and some RNA sedimenting at 30S (Fig. 2a). Fraction B contained about equal amounts of 30S and 12 to 16S RNA, whereas the major radioactive peak (fraction C) contained a small amount of 12 to 16S but a large amount of 42S material. These experiments suggested that the majority of rabies virus-specific mRNAs were associated with polysome fractions A and B (Fig. 1) and that fraction C contained primarily virion-size 42S RNA which was probably not associated with ribosomes.

RNA extracted from rabies-infected cells

Direct analysis of the RNAs synthesized during rabies virus infection was done by pulse labelling cultures for 3 h intervals throughout the growth cycle and resolving the RNA species by SDS-sucrose gradient centrifugation. At the earliest pulse period examined, 6 to 9 h (Fig. 3b) essentially all of the label sedimented in the 12 to 16S region of the gradient. At later times during infection 42S and 30S RNA species were also found (Fig. 3c, d, e). The 12 to 16S RNA material was present in all labelling periods tested. Essentially all of the 3H radioactivity was tendered acid-soluble after ribonuclease treatment.

To determine if the RNA species synthesized during rabies infection contained nucleotide sequences complementary to virion RNA (i.e. were mRNA), three regions (fractions, I, II, III) from a gradient similar to that in Fig. 3(e) were separately pooled and annealed to excess 42S unlabelled rabies virion RNA (Table 1). Self-annealing of each fraction ranged from 23 % to 42 %. After addition of saturating amounts of cold virus RNA the RNase resistance of fraction II and III gradient material increased to about 80 %. RNase resistance of self-annealed fraction I material was 41.8 %, which increased by approx. 10 % after hybridization to virion RNA. It appeared that fraction I contains both virion (minus strand) and complementary (plus) RNA species. Perhaps full-length, virion-size complementary RNA strands were responsible for the rather high self-annealing of fraction I material. Full-size 42S RNA complementary to virion RNA has been observed during VSV virus replication (Morrison et al. 1975). Alternatively, fraction I material could be contaminated with the smaller complementary RNA species (i.e. 30S and 12 to 16S). This is unlikely, however, since fraction I material analysed by acid-urea agarose gel electrophoresis, was relatively homogeneous (see Fig. 5).
Fig. 3. Rate zonal centrifugation of RNA from rabies virus-infected cells. BHK21 cultures (30 mm Petri dishes) were infected with rabies virus (20 to 40 p.f.u./cell) and labelled with $^3$H-uridine from (b) 6 to 9 h, (c) 9 to 12 h, (d) 12 to 15 h and (e) 15 to 18 h p.i. in the presence of actinomycin D (2 μg/ml). Cytoplasmic extracts were prepared with NP40 and DOC and treated with SDS (1%, w/v) and 2-mercaptoethanol (1%, v/v) and the RNA was separated by centrifugation on 15 to 30% (w/w) linear sucrose gradients as described in Fig. 2. The distribution of TCA-insoluble radioactivity in each fraction was determined directly (○---○) or after incubation with 20 μg RNase A per 0.1 ml sample (▲—▲). The RNAs extracted from non-infected BHK21 cells labelled for 3 h without actinomycin D treatment are shown in (a). The positions of 28S and 18S BHK21 cell ribosomal RNAs are noted.
Rabies virus mRNAs

Table I. Annealing of RNAs from rabies-infected cells with unlabelled rabies virus RNA

<table>
<thead>
<tr>
<th>No added rabies virus RNA</th>
<th>With rabies virus RNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Labelled RNA</strong></td>
<td><strong>Ct/min</strong></td>
</tr>
<tr>
<td>From SDS-sucrose gradient</td>
<td></td>
</tr>
<tr>
<td>Region I†</td>
<td>3268</td>
</tr>
<tr>
<td>Region II†</td>
<td>2279</td>
</tr>
<tr>
<td>Region III†</td>
<td>4126</td>
</tr>
</tbody>
</table>

Bound to oligo(dT)-cellulose column‡

- Total cytoplasmic RNA from rabies virus-infected cells, pulsed from 9 to 12 h p.i. with 3H-uridine was applied to an oligo(dT)-cellulose column. The bound RNA was eluted, ethanol precipitated and a portion was resuspended in 2 × SSC containing 0.1 % SDS and mixed with 9 μg of virus RNA (final vol. 0.4 ml). The mixture was boiled, annealed and treated with RNase, as described below.

* Unlabelled rabies virus RNA was extracted from purified virus with SDS and 2-mercaptoethanol, and the RNA was purified by SDS-sucrose gradient centrifugation. Virion RNA was used after self-annealing for 150 min at 68 °C. Before annealing, rabies virus RNA was somewhat resistant to RNase treatment (about 8 to 13 %). After self-annealing, it ranged between 15 % and 19 %. Others have reported similar findings (Bishop & Smith, 1977; Saghi & Flamand, 1979).

† RNA from regions I, II or III from a sucrose gradient similar to that in Fig. 3(e) was pooled separately, ethanol precipitated, resuspended in 2 × SSC (0.3 M-NaCl-0.03 M-Na citrate) containing 0.1 % SDS and mixed with 9 μg of virus RNA (final vol. 0.4 ml). The mixture was boiled, annealed and treated with RNase, as described below.

‡ Total cytoplasmic RNA from rabies virus-infected cells, pulsed from 9 to 12 h p.i. with 3H-uridine was applied to an oligo(dT)-cellulose column. The bound RNA was eluted, ethanol precipitated and a portion was resuspended in 2 × SSC containing 0.1 % SDS. Virion RNA (9 μg) was added and the mixture (0.4 ml) was boiled for 6 min and then annealed for 150 min at 68 °C. The sample was divided into two equal portions; one was treated with 40 μg/ml RNase A and 20 units/ml RNase T1 for 30 min at 30 °C. Both samples were precipitated with TCA and analysed for radioactivity.

In another experiment, total unfractionated RNA synthesized during a 3 h pulse 9 to 12 h after rabies virus infection was purified by oligo(dT)-cellulose chromatography. Self-annealing of this material was low (13.7 %), and RNase-resistance increased to almost 90 % after hybridization with virus RNA.

Presence and size of polyadenylate [poly(A)] sequences in rabies intracellular RNAs

Poly(A) tracts have been identified at the 3'-ends of almost all mRNAs of eukaryotic origin (Darnell, 1976) and virus mRNAs (Bachrach, 1978), including VSV (Ehrenfeld & Summers, 1972). Since the mode of replication of rabies virus appeared to be similar to that of VSV, it was necessary to determine whether rabies mRNA, like VSV, contained poly(A) tracts and to determine its size. An earlier report by Erminie & Atanasiu (1978) provided evidence that rabies virus-infected cell RNAs contained poly(A) tracts. RNA species from rabies virus polysomes were extracted and applied to oligo(dT)-cellulose columns. About 86 % of the total radioactivity bound to the affinity support. By comparison, about 5 % of the virion RNA from purified rabies virus or VSV was bound. Almost 92 % of 32P-labelled 35S poliovirus RNA bound to the column (Table 2).

To estimate the size of poly(A) sequences in rabies virus mRNA species, cells were labelled with 3H-adenosine at 8 to 12 h p.i. and the virus polysomes were separated by velocity sedimentation in sucrose gradients (see Fig. 1). Fractions A and B from the polysome gradient were pooled, concentrated by centrifugation and the RNAs were extracted with SDS and phenol. The RNAs were digested with RNase A and T1, and the adenosine-labelled RNAs were analysed by polyacrylamide gel electrophoresis. They were heterogeneous in size and were about 100 to 250 nucleotides long when compared to BHK 4S RNA (Fig. 4).
Table 2. Binding of RNAs to oligo(dT)-cellulose columns

<table>
<thead>
<tr>
<th>RNA sample from:</th>
<th>TCA radioactivity, ct/min</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Rabies polysomes†</td>
<td>256157</td>
</tr>
<tr>
<td>Poliovirus‡</td>
<td>765146</td>
</tr>
<tr>
<td>Rabies virus§</td>
<td>237500</td>
</tr>
<tr>
<td>Vesicular stomatitis virus§</td>
<td>161752</td>
</tr>
</tbody>
</table>

* Mean of three determinations.
† Rabies-infected BHK-cultures were labelled with *H-uridine from 9 to 12 h after infection in the presence of actinomycin D (2 µg/ml). Cytoplasmic extracts were prepared and the virus polysome fractions A and B (see Fig. 1), isolated by velocity centrifugation, were pooled and the RNA extracted. The RNAs were suspended in binding buffer and chromatographed through oligo(T)-cellulose columns as described in Methods.
‡ 3P-labelled 3S poliovirus (Mahoney) RNA was provided by B. Nottay.
§ *H-uridine-labelled VSV and rabies virus RNA were purified by SDS-sucrose gradient centrifugation.

Fig. 4. Polyacrylamide gel analysis of ribonuclease-resistant RNA fragments isolated from rabies virus polysomes. Rabies virus-infected cultures were labelled with *H-adenosine 100 µCi/ml from 8 to 12 h p.i. in the presence of actinomycin D (2 µg/ml). Cytoplasmic extracts were prepared and virus polysomes isolated by sucrose-gradient centrifugation as described in Fig. 1. Polysome fractions A and B (see Fig. 1) were collected, extracted with phenol and the RNAs were digested with RNase A and T1, as described in the Methods section. RNA fragments, which contained polyadenylate tracts, were purified by oligo(dT)-cellulose chromatography, concentrated with ethanol, and mixed with 14C-uridine-labelled 4S RNA from BHK21 cells and the RNAs were applied to 13 cm columns of 10% polyacrylamide containing 8 M-urea. After electrophoresis for 20 h at 3 mA/gel, the gels were frozen, sliced into 1 mm fractions and the radioactivity in each was determined. ○—○, 3H-adenosine-labelled RNA; ——, 14C-uridine-labelled 4S RNA.
Rabies virus mRNAs

Fig. 5. Fluorogram of rabies and VSV intracellular RNA species separated in acid-urea agarose gels. Rabies infected cultures were labelled from 6 to 9 h, 9 to 12 h, and 12 to 16 h p.i., and VSV cultures were labelled 1 to 3 h and 3 to 6 h after infection with 3H-uridine; the cellular RNAs were separated into fractions I, II and III on sucrose gradients as described in Fig. 3. Each of the three fractions for each virus was separately pooled, concentrated with ethanol and the RNA species in each fraction resolved on 1.5% agarose acid-urea gels. After electrophoresis at 4 V/cm for 20 h at 4°C the gel was prepared for fluorography as described in the Methods section. The VSV mRNA bands were designated L, G, N, NS and M, to reflect their coding capacity, as recommended by Freeman et al. (1979) and Wertz & Davis (1979). The rabies RNA species resolved were consecutively numbered 1 to 5 in increasing order of their mobilities toward the anode. The insert *5 is a longer exposure of the same gel, so that band 5 could be better visualized. The mol. wt. of the VSV mRNAs: L $\approx$ 1.65 x 10^6, G = 0.70 x 10^6, N = 0.55 x 10^6, NS and M = 0.28 x 10^6, have been determined by Rose & Knipe (1975). Band R is 3H-uridine-labelled 28S and 18S BHK21 cell ribosomal RNA that was purified by SDS-sucrose gradient centrifugation.
Initial studies indicated that rabies intracellular RNA species could be separated into at least two mRNA classes (i.e. 30S and 12 to 16S; see Fig. 2, Fig. 3e and Table 1). To resolve and characterize RNA species that may be present in these two size classes, rabies virus-specific RNAs were labelled for 3 h intervals from 6 to 9 h, 9 to 12 h and 12 to 15 h p.i., and the RNA was separated into fractions I, II and III by SDS-sucrose gradient centrifugation (see Fig. 3e). Although not shown here, VSV-infected BHK21 cultures were labelled from 1 to 3 h and 3 to 5 h p.i. and the RNAs separated into fractions I, II and III as was done for rabies virus (Stampfer et al. 1969; Wild, 1971). From the various pulse intervals each of the three fractions were separately pooled and prepared for acid-urea agarose gel electrophoresis. Since the five VSV mRNAs species have been well characterized and their mol. wt. determined (Rose & Knipe, 1975; Freeman et al. 1979), they provided an excellent control for comparison with rabies cell RNAs. When a portion of fraction I material from the VSV SDS-sucrose gradients was examined, a single RNA species of virion-size was seen (Fig. 5). Fraction II material contained the mRNA species coding for the VSV L protein, whereas fraction III material could be resolved into the mRNAs coding for the G, N, NS & M proteins. Separation of the VSV mRNAs and their protein coding assignments have been established by others (Morrison et al. 1975; Rose & Knipe, 1975; Freeman et al. 1977; Wertz & Davis, 1979).

When fraction I and II from rabies SDS-sucrose gradients were analysed, a single major band was found in each fraction (Fig. 5). The RNA species from gradient fractions I and II of either rabies or VSV had the same mobilities (Fig. 6). Purified rabies and VSV virions RNA co-migrated in the acid-urea agarose gel system and migrated to the same position in the gel as the RNA species in fraction I material (Fig. 6). Fraction III RNA from rabies gradients contained two prominent RNA species, 3 and 4, and two minor species, 2 and 5 (Fig. 5). Band 5 was difficult to detect during short exposure of the X-ray film. However, the insert (*5) is from a longer exposure of the same gel, in which the presence of RNA species 5 can be readily seen.

In about one half of our experiments an additional band, which migrated between band 1 and 2, was seen when fraction II gradient material was analysed. This band was present only in fraction II material isolated from SDS-sucrose gradients and appeared to decrease at the later pulse periods (compare fraction II, 9 to 12 h and 12 to 16 h with 6 to 9 h, Fig. 5). Although actinomycin D was used during all the pulse periods, it is reasonable to suggest that a small amount of 28S ribosomal RNA was being transcribed since this additional band appeared to migrate to the same position in the gel as did purified 28S BHK21 cell ribosomal RNA (Fig. 5, lane R).

The mol. wt. of the rabies RNA species 1, 2, 3, 4 and 5 are summarized in Table 3. They were estimated by using the previously reported mol. wt. for the L, G, N, NS and M VSV mRNAs (Rose & Knipe, 1975) and 28S and 18S BHK21 ribosomal RNA (Loening, 1968). The mol. wt. ranged from $0.28 \times 10^6$ for the smallest species, 5, to $\geq 1.65 \times 10^6$ for band 1.

**Hybridization of rabies intracellular RNAs with virion RNA and separation of RNA duplexes molecules**

The data presented in the previous section (Fig. 5 and 6) provided strong supportive evidence that at least five individual RNA species and virion-size RNA could be resolved from rabies virus-infected cells. Although the separations appeared adequate, we could not be completely assured that all RNA species were resolved by the acid-urea gel system. Therefore, we hybridized labelled mRNA species, isolated by oligo(dT)-cellulose chromato-
Fig. 6. Fluorogram of rabies and VSV virion and gradient-separated intracellular RNAs. Rabies and VSV virion RNAs were labelled during infection of BHK21 cells, purified by SDS-sucrose gradient centrifugation and prepared for acid-urea gel electrophoresis. Infected BHK21 cultures of rabies virus or VSV were pulsed with ³H-uridine from 12 to 16 h or 1 to 3 h p.i., respectively. The RNAs were separated into fractions I, II, or III by SDS-sucrose gradient centrifugation (see Fig. 3). Fractions I and II for each virus were separately pooled and prepared for acid-urea agarose gel electrophoresis. The virion RNAs of rabies and VSV were co-electrophoresed in lane 4. The intracellular RNAs from fractions I and II of rabies virus (lane 1) and VSV (lane 3) infected cultures were separately mixed and electrophoresed. In lane 2 a mixture of rabies virus and VSV fraction I and II material were co-electrophoresed. Electrophoresis was for 22 h at 4 V/cm in 1.1 % agarose acid-urea gels. These conditions caused the smaller RNAs to electrophorese out of the gel. The gel was prepared for fluorography and was used to expose X-ray film as described. Lane 4 became distorted during the drying of the gel which caused the image of the RNA species to appear skewed.
Table 3. Estimated molecular weights and coding capacities of rabies single- and double-stranded RNA species

<table>
<thead>
<tr>
<th>Single-stranded RNA species</th>
<th>Rabies proteins</th>
<th>Duplex RNA species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band number* × 10⁻⁴†</td>
<td>Estimated coding capacity x 10⁻⁸§</td>
<td>Species × 10⁻⁸</td>
</tr>
<tr>
<td>1</td>
<td>≥ 1.65</td>
<td>≥ 182.9</td>
</tr>
<tr>
<td>2</td>
<td>0.87</td>
<td>93.8</td>
</tr>
<tr>
<td>3</td>
<td>0.65</td>
<td>68.7</td>
</tr>
<tr>
<td>4</td>
<td>0.38</td>
<td>37.9</td>
</tr>
<tr>
<td>5</td>
<td>0.28</td>
<td>26.5</td>
</tr>
</tbody>
</table>

* Refer to numbers assigned to rabies RNA species in Fig. 5.
† Mol. wt. were determined by using the apparent mol. wt. determined for the VSV L mRNA (≥ 1.65 × 10⁶), G mRNA (0.70 × 10⁶), N mRNA (0.55 × 10⁶), NS and M mRNAs (0.28 × 10⁶) (Rose & Knipe, 1975)
‡ Determined by assuming that the average size of the polyadenylate tracts associated with rabies mRNA were 150 long (see Fig. 4) and that the average mol. wt. of a nucleotide was 321 and an amino acid, 110.
§ Mol. wt. of rabies proteins taken from Coslett et al. 1980.
¶ Determined by assuming that the average mol. wt. of a nucleotide is 321 and an amino acid, 110.
** Refer to numbers assigned to rabies duplex RNAs in Fig. 7.

Fig. 7. PAGE analysis and mol. wt. determination of rabies duplex RNAs. Rabies or VSV intracellular RNAs, differentially labelled with ³²P or ³H-uridine, respectively, were extracted from infected cultures and purified by oligo(dT)-cellulose chromatography. Portions of each labelled RNA were annealed to their corresponding unlabelled genome RNA and digested with RNase T₁; the duplex RNAs were co-electrophoresed on 5% polyacrylamide gels for 20 h at 3 mA/gel. After electrophoresis the gel was frozen, sectioned into 1 mm pieces and the radioactivity in each piece was monitored. The migration rates of the VSV duplex RNAs (1, 2, 3, 4, 5) were used to construct a semi-log plot of mol. wt. versus distance moved. Mol. wt. of the VSV duplex RNAs (1 = 3.52 × 10⁶, 2 = 1.16 × 10⁶, 3 = 0.82 × 10⁶, 4 = 0.46 × 10⁶, 5 = 0.40 × 10⁶) were taken from Freeman et al. (1977), and represent gene assignments for the L, G, N, NS and M VSV proteins. Mol. wt. for the rabies duplex RNAs (I, II, III, IV, V; X—X) were estimated from the plot of the VSV RNAs and are summarized in Table 3. ○—○, ³²P-labelled rabies duplex RNAs; ●—●, ³H-labelled VSV duplex RNAs.
Rabies virus mRNAs

graphy, to unlabelled purified virion RNA. After digestion with RNase T2 the RNA duplex molecules were separated by gel electrophoresis. Differentially labelled VSV duplexes were included as a control and could be separated into five major peaks, 1, 2, 3, 4, 5 (Fig. 7), which are the RNA duplexes for the L, G, N, NS and M proteins as suggested by Freeman et al. (1977) and by Rhodes et al. (1977). Five major peaks of radioactivity were also observed for rabies virus and were designated I, II, III, IV and V (Fig. 7). Four of these RNA duplexes (i.e., II, III, IV and V) did not co-migrate with the VSV duplexes.

The mol. wt. of the rabies duplex RNAs were estimated by using the mol. wt. of the VSV duplexes determined by Freeman et al. (1977) and by assuming that the relative mobilities of the duplex RNAs during electrophoresis were inversely proportional to the logarithm of their mol. wt. When the five RNA duplexes of VSV are plotted in this manner, four of the duplexes, 2, 3, 4 and 5, fall close to a straight line (Fig. 7a); however, the largest VSV duplex, 1, did not. This line was used to estimate the mol. wt. of the rabies duplex RNAs based on their migration rate in the gel. This information, as well as the coding capacity of the rabies duplex RNAs and the apparent mol. wt. of the rabies proteins, is summarized in Table 3.

The calculated mol. wt. for the five rabies virus duplex RNAs and their estimated coding capacity were in reasonable agreement with the mol. wt. for the recognized rabies proteins, L, G, N, M1 and M2.

DISCUSSION

These results provide evidence that rabies virus genome transcription results in the synthesis of at least five mRNA species. Acid-urea agarose gel electrophoresis of rabies intracellular RNAs resolved virion-size, 42S, RNA and five smaller ssRNAs (bands 1, 2, 3, 4, 5, Fig. 5). The estimated mol. wt. of the five RNA species totalled $3.83 \times 10^6$ which would account for the complete coding capacity of a genome size of $3.8 \times 10^6$. The virion RNA of rabies virus was determined to be the same size as that of VSV (i.e. $3.8 \times 10^6$) on the basis of their co-migration in the acid-urea gel system (Fig. 6). The mol. wt. of VSV genome RNA has been calculated by oligonucleotide analysis to be $3.82 \pm 0.14 \times 10^6$ (Bishop et al. 1975). Previously, rabies virion RNA was estimated to be $4.6 \times 10^6$ on the basis of its sedimentation properties in sucrose gradients (Sokol et al. 1969).

Hybridization of rabies virus genome RNA with complementary mRNAs followed by RNase digestion resulted in five RNA duplex molecules, which could be separated by polyacrylamide gel electrophoresis (Fig. 7). The duplex RNA molecules each contain a region of genome and its complementary mRNA [without poly(A)], which presumably codes for each of the known rabies proteins. These results support our observation that rabies structural proteins synthesized in infected cells are derived by the independent translation of five monocistronic mRNA species (Coslett et al. 1980). There seems to be sufficient correspondence between the coding capacities of the RNA duplex species and the mol. wt. of the known rabies structural proteins to suggest that the five duplex RNAs contain the messengers for L, G, N, M1 and M2 (Table 3). The results of others have already convincingly established this relationship for the five VSV duplex RNAs (Freeman et al. 1977; Rhodes et al. 1977).

The RNAs from rabies virus-infected cell extracts could be separated into three size classes with sedimentation values of 42S, 30S and 12 to 16S (Fig. 3). The smallest class, 12 to 16S, was detected as early as 6 h p.i. and was present in infected cell extracts at 18 h. Virion-size 42S RNA was observed only at later times in the infectious growth cycle. Small amounts of 30S RNA species were detected by 9 h p.i. Hybridization studies indicated that the 30S and 12 to 16S RNA species contained complementary sequences to virion RNA (Table 1). In addition, these RNAs were found associated with virus polysomes and contained heterogeneous poly(A) tracts of about 100 to 250 nucleotides. Heterogeneous poly(A) tracts have been found in VSV mRNAs isolated from infected cells (Ehrenfeld & Summers, 1972;
Soria & Huang, 1973) and in mRNA transcripts synthesized in vitro (Banerjee et al. 1974). Ermine & Flamand (1977) were also able to separate three size classes of RNA in rabies virus-infected cells with sedimentation values of 39S, 25 to 35S and 8 to 25S. Only the 25 to 35S and 8 to 25S RNAs were complementary to virion RNA. These RNA species probably correspond to the 42S, 30S and 12 to 16S RNA classes described in this study.

Although we have been able to detect five ssRNAs smaller than virion size in infected cells (Fig. 5) and have separated five RNA duplex molecules (Fig. 6), we cannot dismiss the possibility that other minor mRNA species which are not resolved by these methods are transcribed from rabies genome RNA.

Intracellular protein studies and transcriptional mapping analyses have established the existence of at least five major rabies virus proteins (Madore & England, 1977; Flamand & Delagneau, 1978; Coslett et al. 1980). However, Naito & Matsumoto (1978) have detected the presence of two minor proteins in their purified virus preparations. One of these, P42, was a host protein, actin, which was incorporated into virus particles during maturation. Another, P40, which migrated slightly slower than virion protein M1 in polyacrylamide gels and represented about 2% of the total virus protein, appeared not to be of host origin. Its tryptic peptide maps were different from the virion proteins G and N. Peptide maps of rabies virion proteins M1 and M2 were not done. The P40 protein could be analogous to the minor proteins, a and b, sometimes found in VSV preparations (Bishop & Smith, 1977). A more reasonable interpretation is that P40 is the same as protein M1I, which also migrates slightly slower than virion protein M1 recently observed in some purified rabies preparations (Dietzschold et al. 1979). The tryptic peptide maps of M1I and virion M1 proteins were identical except for an additional peptide in M1I. Apparently, protein M1I contains about threefold more phosphorous than M1, which may account for its lower electrophoretic mobility.

Our results suggest that the transcription processes of rabies virus and VSV are very similar. In addition, we would anticipate that each of the major rabies proteins are encoded by the five RNA species. Preliminary in vitro cell-free translation studies indicated that the 30S RNA species from sucrose gradients programmed the synthesis of virion protein L and that the 12 to 16S RNAs directed the synthesis of virion N, M1, M2 proteins as well as unglycosylated G protein (D. Pennica & J. F. Obijeski, unpublished data).

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REFERENCES


Rabies virus mRNAs


