Early RNA Synthesis in Bunyamwera Virus-infected Cells

By GORDON ABRAHAM* AND ASIT K. PATTNNAIK
School of Science, Griffith University, Nathan, Queensland 4111, Australia

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SUMMARY

RNA synthesis in Bunyamwera virus-infected cells was analysed either by sedimentation analysis in SDS-containing sucrose gradients or by hybridization procedures involving annealing with viral genome RNA (vRNA) followed by electrophoretic analysis. Using either procedure, none of the virus-specific RNAs from infected cells was found to be polyadenylated when analysed by oligo(dT)-cellulose chromatography. In addition, viral messenger RNA activity was found to be associated only with non-polyadenylated RNA species when assayed in an in vitro translation system. The infected cell RNAs could be partially resolved by sucrose gradient centrifugation, and virus-specific RNAs of each polarity were present in these preparations which indicated that the characteristic amplification of secondary transcription was occurring. In the presence of cycloheximide or puromycin, no detectable primary RNA transcription occurred. The same inhibitors, when used later in the infection cycle, caused a dramatic and almost complete inhibition of secondary RNA transcription. The inhibition of RNA synthesis caused by these drugs appeared to be fully reversible. Thus, these inhibitors of protein synthesis affect both primary and secondary RNA transcription by Bunyamwera virus indicating that this virus employs transcription mechanisms different from those known for other families of negative-stranded viruses. Hybridization of 32P-labelled vRNA from Bunyamwera virus with RNA extracted from virus-infected cells produced four duplex RNA molecules that were resolved by gel electrophoresis. Analysis by hybridization and oligonucleotide mapping showed that the two larger duplexes contained complementary (c)RNAs that were transcribed from the L and M segments of viral RNA while the cRNAs contained in the two smaller duplexes were both transcribed from the S RNA segment. Based on a comparison of their oligonucleotide fingerprints, the two latter cRNAs showed a considerable sequence overlap.

INTRODUCTION

Bunyamwera virus is the type virus of the genus Bunyavirus within the family Bunyaviridae (Bishop, 1979). The RNA genome of Bunyamwera virus is of negative polarity and segmented with three RNA species of mol. wt. 2.9 × 10^6, 1.8 × 10^6 and 0.3 × 10^6 (McPhee & Westaway, 1981; Gentsch et al., 1977). This genetic information ultimately codes for four structural proteins, a large protein (L), two glycosylated envelope proteins (G1 and G2) and a nucleocapsid protein (N). Recent reports suggest that at least one non-structural protein is synthesized in cells infected with other members of the Bunyaviridae (McPhee & Della-Porta, 1980; Ulmanen et al., 1981; Fuller & Bishop, 1982). The sum of the molecular weights of all the structural (and non-structural) proteins is significantly less than the approximately 500000 dalton coding capacity of the genome RNA of members of the Bunyaviridae. Genetic evidence shows that the smallest viral RNA segment (S) of bunyaviruses provides the coding for the nucleocapsid protein (Gentsch & Bishop, 1978) and a non-structural protein (Fuller & Bishop, 1982) while the intermediate-sized segment provides the coding for both of the structural glycoproteins (Gentsch & Bishop, 1979). Exactly how many mRNA species are involved in the genetic
expression of bunyaviruses is uncertain. Cash et al. (1979) demonstrated the presence of three duplex RNA structures derived from complementary RNAs synthesized in snowshoe hare virus-infected cells but only the RNA in the smallest could be translated in vitro to show its messenger identity. Using a different approach, Ulmanen et al. (1981) were able to show messenger activity in vitro of two and possibly three RNA species isolated from Uukuniemi virus-infected cells.

This communication approaches the problem of how many species of complementary RNA are synthesized in Bunyamwera virus-infected cells, and establishes that two species of cRNA are coded by the smallest vRNA segment.

**METHODS**

**Growth of virus.** Bunyamwera virus, supplied by Dr P. J. Wright, was originally from the American Type Culture Collection. After cloning twice by plaque isolation, virus stocks were prepared by infecting roller bottle monolayer cultures of Vero cells at a multiplicity of infection (m.o.i.) of 0.01, harvesting the supernatant growth medium after 36 h and clarifying by low-speed centrifugation. More concentrated virus stocks were prepared by direct pelleting of the virus by centrifugation for 1 h at 25000 rev/min in a Beckman SW27 rotor and resuspending in phosphate-buffered saline (PBS). Virus preparations for the extraction of RNA were obtained by polyethylene glycol precipitation from clarified growth medium (Obijeski et al., 1974), dilution in 0.01 M-Tris-HCl pH 7.5 containing 0.001 M-EDTA, and repelleting the virus by centrifugation for 1 h at 25000 rev/min in a Beckman SW27 rotor. Virus-containing pellets were resuspended and RNA recovered following phenol extraction as described previously (Abraham, 1979). [3H]Uridine-labelled virus was prepared similarly except that 25 μCi [3H]uridine/ml was included in the virus growth medium.

The infectivity of virus preparations was determined by plaque assay in Vero cells according to the procedures of Gaush & Smith (1968) except that the overlay medium contained HEPES-buffered Medium 199 (M.A. Bioproducts, Md., U.S.A.) supplemented with 5% foetal calf serum and 400 μg/ml DEAE-dextran.

**Preparation of labelled virus-specific RNA.** Vero cell monolayers (10⁶ cells) were infected with Bunyamwera virus usually at an m.o.i. of 5. After adsorption for 1 h at room temperature, inocula were removed and the cells washed with PBS. Incubation at 37 °C was continued in the presence of Hanks' balanced salt solution supplemented with 15 mM-HEPES adjusted to pH 7.4, and 1 μg/ml actinomycin D. [5-3H]Uridine was added according to the protocol for each experiment, usually at a radioactivity concentration of 25 μCi/ml.

Infected cell monolayers were washed with PBS and the RNA extracted with phenol. Where appropriate, polyadenylated RNA species were selected by specific adsorption to and elution from oligo(dT)-cellulose (Abraham, 1979).

**Analysis of RNA.** Labelled RNA samples for density gradient analysis were recovered by ethanol precipitation, dissolved in water, heated to 100 °C for 1 min and rapidly chilled prior to adjusting to 0.01 M-Tris-HCl pH 7.5, 0-1 M-NaCl, 0.001 M-EDTA and 0-1% SDS. Sedimentation analysis of RNA was by centrifugation through 15 to 30% sucrose gradients (prepared in the same buffer) for 17 h at 25000 rev/min using a Beckman SW41 rotor. Gradients were fractionated from the bottom and the trichloroacetic acid-precipitable radioactivity in portions of each fraction determined.

The sensitivity of RNA samples to digestion with pancreatic ribonuclease was determined by incubation for 1 h at 37 °C in 0.3 M-NaCl, 0.01 M-Tris-HCl pH 7.8, and 40 μg/ml enzyme. The proportion of samples remaining insoluble in trichloroacetic acid was determined.

**Analysis of labelled RNA.** Labelled RNA samples from infected cells were pelleted and resuspended in unlabelled virus preparations followed the procedure described by Hay et al. (1977a). Infected cell RNA was routinely prepared in cultures of 1 × 10⁶ cells. When hybridized with viral RNA (vRNA) extracted from the standard virus yield from a culture of 15 × 10⁶ cells, saturation hybridization levels in terms of detectable double-stranded molecules were achieved. Alternatively, [3H]Uridine-labelled RNA extracted from virus preparations was hybridized similarly with unlabelled RNA extracted from virus-infected cells. In each case, the hybrid molecules so formed were treated with the single-strand-specific nuclease S₁ (1000 units/ml) and analysed by electrophoresis in a 4% polyacrylamide gel (Hay et al., 1977a). Radioactive bands were detected with pre-exposed film following the fluorographic procedures of Bonner & Laskey (1974).

**In vitro translation and electrophoresis.** Total, poly(A)⁺ and poly(A)⁻ RNA from infected or mock-infected cells were prepared as described above and used to prime a message-dependent cell-free translation system derived from a rabbit reticulocyte lysate (Amersham International). A standard translation assay (25 μl) contained reticulocyte lysate (20 μl), 0-1 to 0-4 mg/ml RNA and 1 μCi/ml [15S]methionine. Reactions were incubated at 30 °C for 1 h, terminated by the addition of an equal volume of double-strength sample preparation buffer containing SDS and subsequently analysed by electrophoresis in a 10% polyacrylamide gel (Laemmli, 1970). Radioactive bands were detected by fluorography (Bonner & Laskey, 1974).
**Preparation of samples for oligonucleotide mapping.** $^{32}$P-labelled vRNA was prepared by infecting cells at an m.o.i. of 0.1, and incubation in medium containing 1 μg/ml actinomycin D but lacking the usual phosphates. [$^{32}$P]Orthophosphate was added after 3 h to a radioactivity concentration of 2 mCi/ml and incubation continued for 24 h. The supernatant culture fluid was clarified and the virus pelleted directly (as described above). $^{32}$P-labelled vRNA was extracted with phenol and analysed by electrophoresis in a 3% polyacrylamide gel containing 7 M-urea, 90 mM-Tris–borate pH 8.3, 25 mM-EDTA and 0.5% SDS for 16 h at 8 V/cm. The three radioactive bands were located by autoradiography, cut from the gel and the RNA eluted at 45 °C for 24 h in a buffer containing 0.5 M-NaCl, 0.1% SDS and 2 mM-EDTA.

The individually labelled RNA segments were recovered by ethanol precipitation and digested with ribonuclease T1 as described by Hay et al. (1977a). The resulting oligonucleotides were resolved by two-dimensional polyacrylamide gel electrophoresis as described by De Wachter & Fiers (1972) except that the bisacrylamide concentration for the second dimension gel was reduced to 0.13% to allow drying before autoradiography.

**Materials.** Cycloheximide, puromycin and S1 nuclease were supplied by Sigma, oligo(dT)-cellulose by Collaborative Research Inc., and [$^{5}$-3H]uridine, [$^{35}$S]methionine (1290 Ci/mmol) and [$^{32}$P]orthophosphate by Amersham International. Actinomycin D was a gift from Merck, Sharp & Dohme.

**RESULTS**

**Sedimentation analysis of RNAs from infected cells**

Preliminary experiments indicated that at an m.o.i. of 5 the time of maximal RNA synthesis in infected cells (in the presence of actinomycin D) was between 6 h and 8 h after infection. In order to radiolabel virus-specific RNA species synthesized at earlier times, cultures of infected Vero cells were incubated in the presence of actinomycin D and [$^{3}$H]uridine from 2 h to 8 h after infection. Whole cell RNA extracts were analysed by sedimentation in SDS-containing sucrose density gradients as shown in Fig. 1. In these conditions host cell mRNA synthesis is significantly reduced (Fig. 1 a) as shown by the relatively low level of [$^{3}$H]uridine incorporation. In contrast, virus-specific RNAs could be resolved into four size classes (Fig. 1 b), three of which co-sedimented with RNA extracted from purified Bunyamwera virus particles, the sedimentation positions of which are shown in Fig. 1 (a). The RNAs in these four size classes were in a single-stranded form prior to centrifugation, as shown by the ribonuclease sensitivity profile (Fig.1 b).

Affinity chromatography on oligo(dT)-cellulose was used to determine whether the virus-specific RNA species were polyadenylated like most cellular mRNAs. Fig. 1 (c) shows that RNAs synthesized in the infected cells are not polyadenylated; only 1.6% of the $^{3}$H-labelled RNA specifically bound to and was eluted from the oligo(dT)-cellulose. This result is consistent with the observations of Ulmanen et al. (1981) who were unable to show polyadenylation of Uukuniemi virus-induced RNAs.

In order to try to restrict RNA synthesis to only the primary type of transcription one infected culture was labelled in the presence of actinomycin D and [$^{3}$H]uridine incorporation. In contrast, virus-specific RNAs could be resolved into four size classes (Fig. 1 b), three of which co-sedimented with RNA extracted from purified Bunyamwera virus particles, the sedimentation positions of which are shown in Fig. 1 (a). The RNAs in these four size classes were in a single-stranded form prior to centrifugation, as shown by the ribonuclease sensitivity profile (Fig.1 b).

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In order to try to restrict RNA synthesis to only the primary type of transcription one infected culture was labelled in the presence of the protein synthesis inhibitor, cycloheximide, before the onset of amplified transcription. No RNA products corresponding in size distribution to those observed in untreated infected cells were detected (Fig. 1 d). The low amount of acid-precipitable radioactivity present in the gradient could not be shown to hybridize specifically with RNA extracted from Bunyamwera virus (data not shown). Similar gradient profiles to that shown in Fig. 1 (d) were obtained if another protein synthesis inhibitor, puromycin, was used or if cycloheximide was added after the onset of amplified transcription (unpublished results).

**The polarity of RNAs synthesized in infected cells**

The results in Fig. 1 (b) show that virtually all labelled RNA extracted from infected cells was sensitive to digestion with pancreatic ribonuclease prior to sedimentation analysis. To establish the nature of the RNAs that sedimented somewhat broadly in Fig. 1 (b), four pools were collected corresponding to the size classes of the sedimenting RNA (fraction numbers 7 to 23). These pools were designated 1, 2, 3 and 4 as shown in Fig. 1 (b). Portions of the material in each pool were denatured and self-annealed before analysis for pancreatic ribonuclease resistance of the RNA (Table 1). The larger-sized RNA was up to 86% ribonuclease-resistant but the RNA...
Fig. 1. Sucrose density gradient analysis of intracellular RNAs. Parallel cultures of Vero cells were either uninfected (a) or infected with Bunyamwera virus at an m.o.i. of 5. After 1-75 h incubation (in the presence of 1 µg/ml actinomycin D), cycloheximide was added to one culture to 100 µg/ml (d). [3H]Uridine (30 µCi/ml) was added to all cultures 2 h after infection for a further 6 h. Labelled RNA samples were extracted and analysed on SDS-containing sucrose gradients (see Methods). (a) Radioactivity profile of RNA from uninfected cells; (b) the corresponding profiles of RNA from infected cells either before (○) or after (●) ribonuclease digestion; (c) radioactivity profiles of RNA that was either polyadenylated (●) or non-polyadenylated (○); (d) RNA labelled in the presence of cycloheximide. Arrows in (a) indicate the corresponding positions after sedimentation of the three segments of Bunyamwera virus RNA. Sedimentation is from right to left.
Table 1. Ribonuclease resistance of RNA samples isolated from sucrose gradient fractions

<table>
<thead>
<tr>
<th>Gradient pool number</th>
<th>Initial acid-precipitable radioactivity (ct/min)</th>
<th>Treatment of RNA</th>
<th>Ribonuclease-resistant radioactivity (ct/min) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6404</td>
<td>Undenatured</td>
<td>1747 (27%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured</td>
<td>74 (1%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured and self-annealed</td>
<td>5542 (86%)</td>
</tr>
<tr>
<td>2</td>
<td>7928</td>
<td>Undenatured</td>
<td>2219 (28%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured</td>
<td>141 (2%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured and self-annealed</td>
<td>4234 (53%)</td>
</tr>
<tr>
<td>3</td>
<td>10184</td>
<td>Undenatured</td>
<td>3450 (34%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured</td>
<td>217 (2%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured and self-annealed</td>
<td>3869 (38%)</td>
</tr>
<tr>
<td>4</td>
<td>8417</td>
<td>Undenatured</td>
<td>2779 (33%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured</td>
<td>238 (3%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured and self-annealed</td>
<td>3629 (43%)</td>
</tr>
</tbody>
</table>

* 3H-labelled RNA from the four sucrose gradient pools indicated in Fig. 1(b) was recovered and portions digested with pancreatic ribonuclease (see Methods) either directly, after denaturation by boiling for 1 min (and rapid chilling), or after denaturation and self-annealing. The proportion of radioactivity (as a percentage of the undigested initial sample) which remained acid-precipitable in each case was determined.

from pool 4 was only 43% resistant. All samples that had been denatured and digested with ribonuclease without annealing were less than 3% resistant to digestion. These results show that RNA species of both polarities are synthesized in significant amounts during the labelling period of this experiment, and are consistent with the results of Bouloy & Hannoun (1976) who showed that ribonucleoprotein particles isolated from Lumbo virus-infected cells contained RNA of both polarities.

**The binding of intracellular RNA species to oligo(dT)-cellulose**

The results of the density gradient analysis in Fig. 1(c) suggested that the intracellular RNA species being synthesized were not polyadenylated. To try to confirm this result by another type of analysis, intracellular RNAs from Bunyamwera virus-infected cells were again labelled with [3H]uridine from 4 h to 8 h after infection, and the extracted RNA was chromatographed through oligo(dT)-cellulose using conditions that favoured the binding of polyadenylated mRNA molecules. In these conditions, mRNAs synthesized by influenza virus in cycloheximide-treated cells specifically bound to and could be eluted from the column. The RNAs that specifically bound (and were eluted), and the unbound samples were recovered and hybridized with an excess of RNA extracted from the corresponding virus (vRNA). After digestion of any unhybridized or single-stranded species with the single-strand-specific S1 nuclease, the residual double-stranded molecules were resolved by polyacrylamide gel electrophoresis and detected by fluorography (see Methods). The results in Fig. 2 show that four double-stranded hybrid species could be detected in the fraction that failed to bind to oligo(dT)-cellulose, while none could be detected in the small amount of RNA that bound to oligo(dT)-cellulose. Preliminary experiments to quantify the sensitivity of the fluorography procedure used to detect hybrid RNA species indicated that a sample containing 3% of the radioactivity present in Fig. 2, lane 1 would have been detected at this exposure level. The absence of detectable radioactivity in polyadenylated RNAs in this analysis confirms the previous observation (Fig. 1c) that no polyadenylated RNAs can be detected in cells infected with Bunyamwera virus.

Included in Fig. 2 is a similar analysis (lane 4) of a sample prepared in the reverse way, by hybridization of [3H]uridine-labelled vRNA with unlabelled RNA extracted from infected cells. This analysis shows that four hybrid species representing Bunyamwera virus complementary RNA show the same electrophoretic mobilities as those derived by the alternative method using [3H]uridine-labelled RNA extracted from infected cells (lane 1).

The four species of hybrid RNA molecules detected are significantly different in size. The electrophoretic mobilities in the same conditions of both the largest (2.79 x 10⁶ daltons) and the
Fig. 2. Polyacrylamide gel electrophoresis of [3H]uridine-labelled hybrid RNA species derived from Bunyamwera virus-infected cells. Two parallel cell cultures were infected with Bunyamwera virus at an m.o.i. of 5, and incubated in the presence of 1 µg/ml actinomycin D. [3H]Uridine was added to the cultures at 4 h and incubation continued for a further 4 h, prior to the extraction of total cellular RNA. The RNA from one culture was fractionated into portions that either bound specifically, or failed to bind to oligo(dT)-cellulose. Both fractionated samples, together with the unfractionated RNA from the parallel culture, were hybridized with vRNA (Methods). After digestion with S1 nuclease, each sample was analysed by electrophoresis and the radioactive bands detected by fluorography. Lane 1, hybrid RNA molecules derived from whole infected cells. Lanes 2 and 3, hybrid molecules from RNA which either failed to bind, or bound to oligo(dT)-cellulose. Lane 4, hybrid molecules produced in the reverse experiment in which [3H]uridine-labelled vRNA was hybridized with unlabelled RNA from cells infected as described above and incubated for 6 h. Arrows indicate the positions after electrophoresis of the largest and the smallest RNA segments of reovirus type III.

Fig. 3. Polyacrylamide gel electrophoretic analysis of in vitro translated and in vivo labelled Bunyamwera virus-specific proteins. [35S]Methionine-labelled translation products, without exogenous RNA (lane 1), with poly(A)+ and poly(A)−-containing RNA from mock-infected cells (lanes 2 and 3 respectively) or with total, poly(A)+ and poly(A)− RNA from infected cells (lanes 4, 5 and 6 respectively) were prepared as described in Methods and analysed by electrophoresis in a 10% polyacrylamide gel. Lanes 7 and 8 contain [3H]leucine-labelled proteins synthesized in vivo in either Bunyamwera virus-infected cells between 7 and 23 h after infection, or in mock-infected cells. After electrophoresis, the radioactive bands were detected by fluorography. The arrow indicates the position of the most prominent virus-specific polypeptide.

Protein-coding capacities of polyadenylated and non-polyadenylated viral RNAs

Confirmation that non-polyadenylated RNAs act as mRNAs in Bunyamwera virus-infected cells was attempted by translation of these RNAs in a cell-free protein synthesizing system. RNA was extracted from either mock-infected or virus-infected cells and fractionated by oligo(dT)-cellulose chromatography into polyadenylated and non-polyadenylated species (see
Bunyamwera virus RNA synthesis

Radioactively labelled proteins translated in vitro were resolved by polyacrylamide gel analysis (Fig. 3). Polyadenylated mRNAs from uninfected host cells were successfully translated to produce a wide range of polypeptides as expected (Fig. 3, lane 2). In contrast, non-polyadenylated RNAs from host cells were not translated efficiently. When RNA from virus-infected cells was used, no products corresponding to virus-specific polypeptides were translated from non-polyadenylated RNA species (Fig. 3, lane 5). However, one (possibly two) polypeptide band(s) tentatively identified as being virus-specific on the basis of its electrophoretic mobility was translated from non-polyadenylated RNAs present in virus-infected cells (Fig. 3, lane 6).

These data, showing that no mRNA activity could be demonstrated using polyadenylated RNA species from Bunyamwera virus-infected cells, is consistent with the previous analyses that the viral mRNAs are not polyadenylated.

Four hybrid RNA species can also be isolated by sucrose gradient sedimentation

Another portion of the sample used for the analysis in Fig. 1(b) was sedimented in a preparative sucrose gradient, similar to those used in Fig. 1. Fractions corresponding to those shown in Fig. 1(b) were pooled and recovered by ethanol precipitation. The RNA in each pool was then hybridized with vRNA (as described previously) and the hybrid species so formed analysed by electrophoresis (Fig. 4). The RNA from the pool with the highest sedimentation coefficient gave rise to the hybrid molecule with the slowest electrophoretic mobility (lane 1), together with a small amount of the hybrid with the next slowest electrophoretic mobility. The RNA from pool 2 in Fig. 1(b) gave rise to only one hybrid RNA species by gel electrophoresis (lane 2). The RNA from pool 3 in the sucrose gradient did not produce any detectable hybrid molecules in this analysis. However, in a subsequent similar analysis, labelled RNA with the same sedimentation properties produced hybrid RNAs corresponding to both of the faster migrating species, yielding a profile similar to that shown in lane 4 (which was derived from the RNA in pool 4). The sample in lane 5 shows the RNA hybrids derived from total infected cell RNA. In addition, in Fig. 4, lane 5, a band resembling a very large RNA hybrid can be seen which is believed to be an artefact because it was seen only in this experiment. Thus, by using fractionated portions of the RNA of infected cells, four hybrid RNAs showing the same electrophoretic mobilities as those found in whole cell extracts can be identified.

Attempted differentiation between primary and secondary transcription

Results above have shown that RNAs of both polarities are being synthesized at relatively early times after infection. In order to try to restrict RNA transcription to the primary type only, methods that are standard for this purpose in the study of other negative-stranded viruses were adopted. It is well known that inhibitors of protein synthesis such as cycloheximide and puromycin prevent translation of primary mRNA transcripts in families such as the Orthomyxoviridae (Bean & Simpson, 1973), Rhabdoviridae (Marcus et al., 1971) and Paramyxoviridae (Robinson, 1971) and, in so doing, prevent the normal viral replication process from advancing beyond the primary type of transcription to secondary (amplified) transcription.

Cells were infected with Bunyamwera virus at a higher than usual m.o.i. (50) in order to make the detection of primary transcription more likely. Cultures were labelled with [3 H]uridine for either 3 h or 6 h after infection in either the presence or absence of cycloheximide. In cultures labelled in the presence of cycloheximide, no radioactive RNA that hybridized to viral RNA was detected even after 6 h of labelling (Fig. 5a, c). In both of the control cultures, the same four virus-specific RNA hybrid species found previously were seen (Fig. 5b, d). The fluorography procedure used in this experiment (Fig. 5) was sensitive enough to detect radioactive bands in samples (a) and (c) containing 3% of the radioactivity present in sample (b) (data not shown).

Cycloheximide could possibly have had an unknown toxic effect on the virus-associated RNA transcriptase. In order to determine firstly, if this effect was specific for cycloheximide, and secondly whether it had any similar effects on the enzyme(s) responsible for secondary transcription in Bunyamwera virus-infected cells, the following experiment was done. Cells were infected and the normal infection processes were allowed to proceed for 4-5 h. Preliminary
Fig. 4. Polyacrylamide gel electrophoresis of hybrid RNA molecules derived from samples after density gradient separation. A parallel sucrose gradient analysis of Bunyamwera virus-specific RNAs similar to that in Fig. 1(b) was used to prepare four pools of RNA corresponding to the four regions indicated in Fig. 1(b). Each pool was hybridized with an excess of vRNA and the products treated and analysed by electrophoresis as described for those in Fig. 2. Lanes 1 to 4 correspond to the gradient pools 1 to 4. Lane 5 shows hybrid RNAs derived from the original labelled RNA preparation used for the sucrose gradient after it had been hybridized with vRNA and treated similarly.

Fig. 5. Polyacrylamide gel electrophoresis of Bunyamwera virus-specific hybrid RNA molecules labelled in the presence or absence of protein synthesis inhibitors. Four parallel cultures (a to d) were infected at an m.o.i. of 50. Two cultures (a and c) were treated from 0 h post-infection with cycloheximide (100 μg/ml) and all four cultures were immediately labelled with [3H]uridine. Cultures (a) and (b) were harvested after 3 h of labelling while (c) and (d) were harvested after 6 h. Another five cultures (e to i) were infected at the usual m.o.i. of 5 and the infection allowed to proceed for 4 to 5 h. Cycloheximide (100 μg/ml) was added to (e) and (f) while puromycin (100 μg/ml) was added to (g) and (h). Culture (i) served as an untreated control. After 15 min, the drugs were removed from cultures (f) and (h) and the cell monolayers washed twice with warmed Hanks' solution. At 5 h post-infection, [3H]uridine was added to these five cultures for a further 2 h. The RNA from all nine cultures was hybridized with vRNA and analysed as described for the samples in Fig. 2. Radioactive bands were detected by fluorography.

Results had indicated that at this time, the rate of RNA synthesis was approaching its maximum and undoubtedly involved secondary (amplified) transcription. Either cycloheximide or the alternative protein synthesis inhibitor, puromycin, were added to parallel cultures. After 10 min, the inhibitors were removed from two cultures prior to the addition of [3H]uridine. The resulting virus-specific RNAs labelled were selected by hybridization and analysed in the usual way. Again, the presence of cycloheximide in an infected culture (Fig. 5e) caused the level of amplified RNA synthesis to fall below detectable levels. Thus, secondary RNA transcription (as
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Fig. 6. Polyacrylamide gel electrophoresis of single-stranded (a) or double-stranded (b) RNAs derived from Bunyamwera virus. Uniformly $^{32}$P-labelled RNA extracted from Bunyamwera virus was analysed by electrophoresis in denaturing conditions in a 3% polyacrylamide gel (a). The three radioactive bands located by autoradiography were cut out and the eluted RNA segments separately hybridized with the total RNA extracted from $5 \times 10^6$ Vero cells 6 h after infection with Bunyamwera virus at an m.o.i. of 5. After hybridization, the preparations were digested with S$_{1}$ nuclease, analysed by electrophoresis in a 4% polyacrylamide gel, and the bands of radioactivity detected by autoradiography. Lane T shows duplex species detected following hybridization of total $^{32}$P-labelled vRNA with complementary RNAs, and identified arbitrarily as hybrids 1 to 4. Lanes L, M and S show hybrid products derived by hybridization of the individual genome segments with complementary RNAs.

well) appeared to be inhibited by this drug. Puromycin had a similar effect on RNA synthesis, although the inhibition did not appear to be as complete as with cycloheximide (Fig. 5g), as some radioactivity is seen corresponding to the smallest hybrid.

The effects that both of these drugs have on protein synthesis are fully reversible once the drug is removed. It was of interest, therefore, to see whether RNA synthesis could resume after having been stopped by the action of these drugs. The results in Fig. 5(f, h) show that virus-specific RNA synthesis returned to approximate pre-inhibition values once the two drugs were removed from the cultures.

The coding origins of the four cRNA species

Uniformly $^{32}$P-labelled RNA was extracted from Bunyamwera virus (vRNA) and resolved into its three characteristic components by electrophoresis (Fig. 6a). Individual L, M and S RNAs were eluted from the gel, separately hybridized with the total RNA extracted from Bunyamwera virus-infected cells and the resultant duplex molecules analysed by electrophoresis
Fig. 7. Oligonucleotide fingerprint analyses of Bunyamwera viral RNA. Uniformly $^{32}$P-labelled RNA corresponding to the L, M and S RNA segments of Bunyamwera virus were eluted from a preparative polyacrylamide gel similar to that shown in Fig. 6(b), recovered by ethanol precipitation and digested with ribonuclease T$_1$. The resulting oligonucleotides were resolved by two-dimensional polyacrylamide gel electrophoresis as described (Methods). The fingerprint obtained for the S RNA segment is shown on the left-hand side. Double-stranded hybrid molecules were prepared and resolved as described for Fig. 6, lane T. Individual radioactive bands were eluted from the gel and analysed similarly except that the duplex molecules were denatured by boiling for 1 min prior to ribonuclease T$_1$ digestion. Four fingerprint analyses corresponding to the hybrid species numbered 1 to 4 in Fig. 6(b) were prepared and those for hybrids 3 and 4 are shown in the right-hand panels. Crosses indicate the positions of the marker dyes xylene cyanol and bromophenol blue after electrophoresis. Arrows in fingerprints on the right-hand side indicate the corresponding positions of oligonucleotides present in the single-stranded RNA segment but absent from the duplex analyses.
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(Fig. 6b). Lane T shows the four species of double-stranded RNA (designated 1 to 4) obtained by hybridization of total $^{32}$P-labelled vRNA with RNA from infected cells. Duplex RNA produced by hybridization with the L RNA segment (lane L) had an electrophoretic mobility corresponding principally to the largest of the four hybrid species detected in the total viral RNA analysis. Similarly, duplex RNA derived from RNA segment M corresponded to hybrid 2. However, RNA segment S upon hybridization gave rise to two hybrid species corresponding in electrophoretic mobilities to hybrids 3 and 4 (lane S).

Confirmation of the coding contained in each of the three vRNA segments was obtained by comparative oligonucleotide mapping. Individual $^{32}$P-labelled L, M and S RNAs were digested with ribonuclease T1 and the resulting oligonucleotides resolved by two-dimensional gel electrophoresis. Each RNA segment showed a characteristic fingerprint, but only that for RNA segment S is illustrated (Fig. 7).

Preparations of individual $^{32}$P-labelled RNA hybrids 1 to 4 were obtained by elution from an electrophoretic separation similar to that shown in Fig. 6(b), lane T. Following heat denaturation, oligonucleotide maps for each hybrid species were prepared (Fig. 7). The unique oligonucleotides in the fingerprint prepared from hybrid 1 corresponded to those found in the L segment fingerprint, confirming the hybridization data that the cRNA found in hybrid 1 is transcribed from the L RNA segment (data not shown). Similarly, all of the unique oligonucleotides found in the fingerprint for hybrid 2 were represented in the fingerprint for RNA segment M (data not shown). The results for the analysis of hybrids 1 and 2 confirm the observations of Cash et al. (1979) that segments L and M of bunyaviruses each give rise to a single complementary RNA species.

Oligonucleotide maps prepared from hybrid RNA species 3 and 4 were compared with that obtained for RNA segment S (Fig. 7). All oligonucleotides except one in the fingerprint prepared from hybrid 3 were found also in the fingerprint of the S RNA segment, indicating that almost total protection for the S segment was provided by hybridization with this particular species of cRNA molecule. A similar fingerprint was prepared from RNA hybrid 4 except that at least five different oligonucleotides were absent when compared with the fingerprint of the S RNA segment. This result shows that a shorter complementary RNA species is present in RNA hybrid 4 which protects the RNA segment S from nuclease digestion to a lesser degree. However, the majority of the unique oligonucleotides were common to both hybrid RNA species 3 and 4, as well as to the parental molecules, segment S.

**DISCUSSION**

Two aspects of Bunyamwera virus RNA synthesis described in this report differentiate it from that seen with other better-studied families of negative-stranded viruses. Firstly, its mRNAs do not appear to be polyadenylated and secondly, both primary and secondary transcription are very sensitive to the action of inhibitors of protein synthesis. Hybridization of complementary RNAs isolated from infected cells with an excess of vRNA revealed four electrophoretically distinct species of double-stranded RNA after removal of single-stranded or unhybridized regions enzymically. The use of the alternative enzymes, ribonuclease T1 or T2, did not alter the profile of hybrid molecules detected (data not shown). It has not been shown that these hybrid RNAs represent four separate mRNA species, but the results are, nevertheless, consistent with this possibility. It is not known how many mRNAs are involved in the translation of at least five proteins in bunyavirus systems. This is because the mRNAs do not appear to be polyadenylated (Ulmanen et al., 1981; data above), are synthesized in relatively low amounts, and have not all been translated satisfactorily in *in vitro* protein-synthesizing systems.

It was surprising to find four double-stranded hybrid RNAs in Bunyamwera virus-infected cells since Cash et al. (1979) using similar procedures detected only three cRNA species by hybridization of snowshoe hare bunyavirus RNAs. The hybridization data in this report and the oligonucleotide mapping data (not shown) confirm the observations of Cash et al. (1979) that both the L and M RNA segments of bunyaviruses each give rise to one species of cRNA. However, both hybridization data and oligonucleotide mapping indicate that two cRNA species are transcribed from the S RNA segment in Bunyamwera virus-infected cells.
Inspection of the fingerprint for hybrid 3 (Fig. 7) shows that all but one of the unique oligonucleotides in the S RNA have been protected by hybridization, indicating that the cRNA species involved in this hybrid is almost a full-length copy of the genome segment. The fingerprint from hybrid 4 is somewhat less complex indicating the involvement of a smaller cRNA species. However, both fingerprints for hybrids 3 and 4 have a significant number of large oligonucleotides in common with each other and also with the genome segment S, indicating that considerable overlap occurs in the coding of these cRNAs. This information is consistent with the reports by Clerx-van Haaster et al. (1982) and Bishop et al. (1982) that the S segments of bunyaviruses have sequences that allow for the potential expression of two gene products by using overlapping reading frames. In addition, it appears likely that both the N and NS proteins of snowshoe hare virus are unrelated, yet both are coded within the S RNA genome (Fuller & Bishop, 1982). If the cRNAs represented in hybrid RNAs 3 and 4 in this report are mRNAs, then they would have overlapping sequences which could allow the translation of two separate proteins by using different reading frames.

It is known for several bunyaviruses that the N protein is coded by the S segment of viral RNA (Gentsch & Bishop, 1978; Cash et al., 1979) and recent evidence suggests that the coding information for a small NS protein of snowshoe hare virus comes also from the S RNA segment (Fuller & Bishop, 1982; Bishop et al., 1982). In addition, Ulmanen et al. (1981) have successfully translated in vitro two Uukuniemi virus-specific proteins (N and NS) using low molecular weight RNAs from infected cells. However, attempts in this laboratory to translate the two cRNAs derived from the S RNA of Bunyamwera virus in vitro into other than the N protein have been unsuccessful.

An alternative explanation for the two transcripts from S segment RNA is that the larger represents a template used in S segment replication while the smaller is a functional mRNA. Such a situation exists, for example, in influenza virus replication (Hay et al., 1977b). However, it is difficult to establish this point in the Bunyamwera virus system as mRNAs cannot be selected on the basis of polyadenylation, nor can RNA transcription be satisfactorily restricted to that of the primary type only by using inhibitors of protein synthesis.

The sizes of the four Bunyamwera virus hybrids described were estimated by comparison with both the largest and the smallest RNA segments of reovirus (Fig. 2). The two larger Bunyamwera hybrid species migrated slower than the largest reovirus segment, suggesting a size greater than $2.79 \times 10^6$ daltons, while the two smaller hybrids migrated significantly faster than the smallest reovirus segment, suggesting a mol. wt. less than $0.61 \times 10^6$ (Shatkin et al., 1977). It follows that if either of the two smaller Bunyamwera virus hybrid species is derived from a single viral messenger RNA, then its maximum protein coding potential would be significantly less than that coded by the smallest reovirus segment, approximately 33000 daltons. Since both of the G_1 and G_2 proteins of the Bunyaviridae are coded by the one viral RNA segment (Gentsch & Bishop, 1979) then it appears that the coding potential contained in either of the two smaller Bunyamwera virus hybrids is insufficient to code for the G_1 and G_2 proteins. One of the hybrid species probably contains the coding for the small protein N, while the other could conceivably code for a small non-structural protein as has been reported for other bunyaviruses (McPhee & Della-Porta, 1980; Ulmanen et al., 1981; Fuller & Bishop, 1982).

Alternatively, since both of the two larger Bunyamwera virus hybrids migrate slower than the largest reovirus segment (Fig. 2) one strand of either appears to have sufficient coding potential for the translation of either the L protein ($1.45 \times 10^5$ to $2 \times 10^5$ daltons) or both of the G_1 and G_2 proteins (total $1.36 \times 10^5$ to $1.5 \times 10^5$ daltons).

All mRNAs described for other families of negative-stranded viruses and most eukaryotic mRNAs are polyadenylated at the 3′-termini. However, specific data concerning this aspect of mRNAs coded by members of the Bunyaviridae are not available. Cash et al. (1979) were able to translate in vitro RNA that had bound specifically to oligo(dT)–cellulose to give only the N protein of snowshoe hare virus. However, by using three different types of analysis following oligo(dT)–cellulose chromatography, the evidence is conclusive in this report that no polyadenylated RNAs can be found in Bunyamwera virus-infected cells. In the experiment described in Fig. 1(a), most of the 3H-labelled RNA was virus-specific as actinomycin D was
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used to suppress host RNA synthesis. However, less than 2% of this labelled RNA specifically bound to and was eluted from the oligo(dT)-cellulose. This value undoubtedly represents low levels of host cell mRNA synthesis or other experimental variables associated with the chromatography. An alternative procedure involving hybridization (Fig. 2) was used to try to demonstrate polyadenylation of mRNAs but the only detectable radioactivity was found in samples derived from RNA that had failed to bind to oligo(dT)-cellulose. Similar results were found for another member of the Bunyaviridae, Akabane virus (unpublished results). The translation in vitro of RNAs isolated from Bunyamwera virus-infected cells (Fig. 3) indicated that messenger activity was associated with poly(A)− RNA rather than poly(A)+ RNA species. Thus, the failure to demonstrate polyadenylated RNA in Bunyamwera virus-infected cells is consistent with the observations of Pettersson and co-workers who have been unable to find polyadenylated mRNAs in Uukuniemi virus-infected cells (Ulmanen et al., 1981).

Inhibitors of protein synthesis are known to interrupt the replication of most negative-stranded viruses at an early stage such that the only known synthetic event that occurs is the production of mRNAs by the particle-associated enzyme (for reviews, see Wagner, 1975; Choppin & Compans, 1975). This event is known as primary transcription and protein synthesis is necessary for the progression of the infectious cycle to the stage of amplified RNA synthesis known as secondary transcription. Once this stage of the infection cycle is reached, continued RNA synthesis is again independent of protein synthesis (Scholtissek & Rott, 1970; Robinson, 1971). However, the behaviour of Bunyamwera virus in experiments of this type was unlike that found for members of the Orthomyxoviridae (Bean & Simpson, 1973), the Rhabdoviridae (Marcus et al., 1971) or the Paramyxoviridae (Robinson, 1971). Using an m.o.i. of 50 and an RNA labelling period of up to 6 h (in the presence of cycloheximide) no complementary RNA synthesis could be detected (Fig. 5). Such conditions considerably exceed those required to detect primary transcription with other negative-stranded viruses (e.g. Hay et al., 1977a; Marcus et al., 1971).

Cycloheximide had a profound effect on RNA synthesis during secondary transcription (Fig. 5). The inhibitory effect on RNA synthesis of cycloheximide at this concentration was very fast and was estimated to be more than 90% complete within 10 min of addition of the drug (unpublished results). Similar results were observed with the alternative inhibitor, puromycin, but the results were not absolute in all experiments. This could be because the level of inhibitor used (100 μg/ml) was insufficient for complete cessation of RNA synthesis. However, it does show that a more general effect is probably responsible for the inhibition of RNA synthesis in this system, rather than a drug-specific toxicity for the enzyme(s).

The most likely explanation for these results is that continued protein synthesis is necessary for RNA transcription. Since both of these drugs exhibit a parallel reversibility for the inhibition of both protein synthesis and Bunyamwera viral RNA synthesis (Fig. 5), it seems possible that the two functions are linked.

Previous reports have indicated that very low levels of primary transcription can be detected in bunyavirus-infected cells in the presence of protein synthesis inhibitors. Using snowshoe hare virus and hybridization procedures, Vezza et al. (1979) were unable to show an increase in primary transcription products from 2 to 8 h after a high-multiplicity infection in the presence of puromycin, but could show a slight increase in hybridization levels when cycloheximide was used. Kascsak & Lyons (1977) demonstrated that in Bunyamwera virus-infected cells in the presence of cycloheximide, incorporation of [3H]uridine into virus-specific RNAs was about twice the background level found for mock-infected cells. Transcription levels in both of these systems were minimal and it is possible that the procedures used in this report were not sensitive enough to detect such low levels of primary transcription. However, the dramatic and reversible effects both cycloheximide and puromycin have on secondary RNA transcription (Fig. 5) lend support to the alternate hypothesis presented that, in this system, transcription does not occur in the absence of protein synthesis.

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