

Identification of Four Complementary RNA Species in Akabane Virus-Infected Cells

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The analysis of RNA extracted from purified Akabane virus demonstrated the presence of three size classes of single-stranded RNAs with sedimentation coefficients of 31S (large, L), 26S (medium, M), and 13S (small, S). Molecular weights of these RNA species were estimated to be 2.15×10^6 , 1.5×10^6 , and 0.48×10^6 for the L, M, and S RNAs, respectively. Hybridization analysis involving viral genomic RNA and RNA from virus-infected cells resulted in the identification of four virus-specific cRNA species in infected cells. These cRNAs were found to be nonpolyadenylated by their inability to bind to oligodeoxythymidylate-cellulose. Kinetic analysis of cRNA synthesis in infected cells at various times postinfection suggested that cRNA synthesis could be detected as early as 2 h postinfection and that maximal synthesis occurred at 4 to 6 h postinfection. The RNAs synthesized in infected cells could be partially resolved by sucrose density gradient centrifugation. The RNA fraction that cosedimented with the S segment of viral genomic RNA yielded two duplex RNA species when hybridized with viral genomic RNA, suggesting the presence of two small cRNA species. Specific hybridization with individual viral genomic RNAs confirmed that two species of cRNA are coded by the S RNA segment. Analysis of cRNA synthesis in the presence of the protein synthesis inhibitors cycloheximide and puromycin indicated that cycloheximide completely inhibited virus-specific RNA synthesis early and late in infection, whereas a very low level of synthesis occurred in the presence of puromycin. The inhibitory effects of these drugs were found to be reversible when the drugs were washed from the cells. It is concluded that continued protein synthesis is required for cRNA synthesis to proceed in Akabane virus-infected cells.

Akabane virus is a member of the genus *Bunyavirus* and family *Bunyaviridae* (7). All bunyaviruses so far characterized contain three unique segments of single-stranded RNA (large [L], medium [M], and small [S]) of negative polarity with a total molecular weight of approximately 4×10^6 to 6×10^6 (3). These RNAs reside within the virion as three helical and circular nucleocapsids, being complexed with multiple copies of a nucleocapsid protein (N) and a few copies of a large protein (L), believed to be a transcriptase component (5). Two external glycoproteins (G1 and G2) form the spikes on the viral envelope; G1 protein contains a site for binding to cellular receptors and also functions as the viral hemagglutinin, whereas the function of G2 still remains to be demonstrated (15). A recent report (25) also suggests the presence of a fifth virion-associated polypeptide of molecular weight 16,000 (16K). In addition to these structural proteins, the viral genome also codes for several nonstructural proteins (12). Recombination studies (4, 13, 14) show that the small viral

RNA contains coding information for the N protein, whereas the M RNA segment contains the information for both G1 and G2. It is therefore inferred that the L protein is coded by the L RNA segment.

Although more than five virus-specific proteins have been identified, the mechanism of bunyavirus gene expression in infected cells remains to be explained. It is not known how many mRNAs mediate the expression of three genome segments into more than five virus-specific polypeptides. Using duplex RNA analyses, Cash et al. (9) identified the presence of three cRNA species in snowshoe hare virus-infected cells but could only detect messenger activity in the smallest of the species. By fractionating RNA from Uukuniemi virus (a member of the family *Bunyaviridae*)-infected cells in sucrose gradients, Ulmanen et al. (26) detected four virus-specific RNAs, two of which directed the synthesis of virus-specific polypeptides in vitro. The mRNA species that cosedimented with the M RNA segment directed the synthesis

of a 110K-molecular-weight protein (found to be the precursor to G1 and G2). In addition, a 12S RNA species directed the synthesis of the N protein and a 30K nonstructural protein.

This communication reports the identification of four nonpolyadenylated cRNA species synthesized in Akabane virus-infected cells and the sensitivity of their synthesis to the protein synthesis inhibitors cycloheximide and puromycin.

MATERIALS AND METHODS

Cells and virus stock. Akabane virus (strain R7947) was obtained from the Queensland Institute of Medical Research, Brisbane, Australia, and was cloned by plaque isolation twice before use. Vero cells were grown in minimal essential medium containing 5% fetal calf serum. Virus stocks were usually prepared by infecting confluent monolayers of Vero cells in roller bottles at a multiplicity of infection (MOI) of 0.01 PFU per cell. High-titer stocks were prepared by directly pelleting virus from the clarified culture fluids and resuspending it in phosphate-buffered saline containing 0.2% bovine serum albumin and 0.001% DEAE-dextran.

Growth of virus and preparation of vRNA. Subconfluent monolayer cultures of Vero cells in roller bottles were infected with Akabane virus at an MOI of 0.001 PFU per cell. After 1 h of virus adsorption at room temperature, inocula were removed and replaced with virus growth medium and incubated at 37°C for about 35 h. Virus was concentrated from clarified supernatant by polyethylene glycol precipitation (22) or by direct pelleting and purification by centrifugation in potassium tartrate-glycerol gradients (22). After repelleting the virus, viral genomic RNA (vRNA) was extracted with sodium dodecyl sulfate (SDS) and phenol (1).

³²P-labeled vRNA was prepared similarly from virus grown in phosphate-free medium containing 1 mCi of ³²P_i per ml. The vRNA segments were resolved by electrophoresis in a 3% polyacrylamide gel (120 V for 16 h) containing 6 M urea and 90 mM Tris-EDTA-borate buffer, pH 8.3. The individual RNA segments were detected by autoradiography, excised, eluted from the gel (in 10 mM Tris-hydrochloride, pH 7.5, 1 mM EDTA, 0.5% SDS, 0.5 M NaCl), and precipitated with ethanol.

Preparation of RNA from infected cells. Confluent monolayers of Vero cells were infected with Akabane virus at a usual MOI of approximately 7. After 1 h of virus adsorption at room temperature, inocula were removed and the monolayer was washed with warm phosphate-buffered saline and incubated in the presence of Hanks balanced salt solution supplemented with 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) adjusted to pH 7.4. Actinomycin D (2 µg/ml), [³H]uridine (25 to 50 µCi/ml), cycloheximide (100 µg/ml), and puromycin (100 µg/ml) were added at appropriate times postinfection according to the protocol of each experiment. After completion of the labeling period, cell monolayers were washed with cold phosphate-buffered saline and dissolved in buffer containing 0.5% SDS, and the RNA was extracted with phenol and precipitated with etha-

nol. Polyadenylated and nonpolyadenylated RNAs were separated by oligodeoxythymidylate [oligo(dT)]-cellulose chromatography (1).

RNA-RNA hybridization and electrophoresis. Labeled cRNAs from infected cells were hybridized with an excess of unlabeled vRNA (17). The hybrid molecules thus formed were digested with single strand-specific nuclease S1 (1,000 U/ml) at 37°C for 2 h and the residual double-stranded RNAs (dsRNAs) recovered by ethanol precipitation were analyzed by electrophoresis on a 4% polyacrylamide gel at 40V for 15 to 20 h (17). The gel was processed for fluorography (8), and the radioactive bands were detected by exposing the dried gel to X-ray film at -70°C (19).

To determine the excess of unlabeled vRNA required by hybridization, fixed amounts of [³H]cRNA from the virus-infected cells were hybridized with increasing amounts of unlabeled vRNA. The trichloroacetic acid-precipitable radioactivity in portions of the hybridized RNAs before and after S1 nuclease digestion was determined (Table 1). This analysis indicated that saturation levels of hybridization were achieved by using at least 6 µg of partially purified vRNA.

Sucrose density gradient analysis. Labeled RNAs for sucrose density gradient analysis were dissolved in water, denatured by heating at 100°C for 1 min, immediately chilled, and adjusted to 10 mM Tris-hydrochloride (pH 7.5)-100 mM NaCl, 1 mM EDTA-0.1% SDS. RNA samples were then analyzed by sedimentation through 15 to 30% (wt/vol) sucrose density gradients (prepared in the same buffer) in a Beckman SW41 rotor (25,000 rpm, 16 h, 20°C). Fractions (0.4 ml) were collected from the bottom of the gradient, and trichloroacetic acid-precipitable radioactivity in portions of each fraction was determined. Where appropriate, RNAs were recovered from the gradient by ethanol precipitation of the pooled peak fractions.

Materials. Cycloheximide, puromycin, and nuclease S1 were obtained from Sigma Chemical Co., oligo(dT)-cellulose from Collaborative Research Inc., and [³H]-uridine from the Radiochemical Centre, Amersham, England. Actinomycin D was a gift from Merck Sharp & Dohme, Australia. [³H]uridine-labeled dsRNA from Wallal virus was a gift from P. J. Walker, Queensland Institute of Medical Research, Brisbane, Australia.

TABLE 1. Determination of the saturating level of unlabeled vRNA required for hybridization with [³H]cRNA

Amt (µg) of unlabeled vRNA hybridized with [³ H]cRNA from 2 × 10 ⁶ cells	% Radioactivity resistant to S1 nuclease digestion ^a
0	32
1	41
2	50
4	67
6	82
8	83
10	82

^a Determined by precipitating a portion of the hybridized RNAs before and after S1 nuclease digestion.

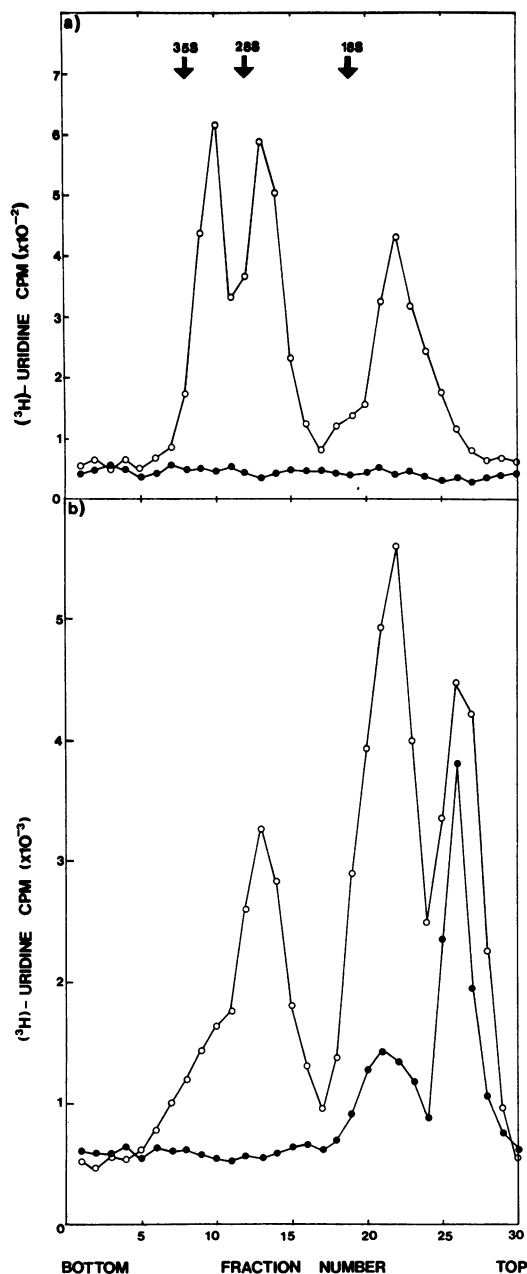


FIG. 1. (a) Sucrose density gradient centrifugation of RNA from purified Akabane virus. ^3H -labeled RNA from purified virus was denatured and sedimented through a 15 to 30% SDS-sucrose density gradient as described in the text. Fractions (0.4 ml) were collected, and trichloroacetic acid-precipitable radioactivity in each fraction was determined. Symbols: \circ , RNA from purified virus; \bullet , RNA from purified virus digested with pancreatic RNase (20 $\mu\text{g}/\text{ml}$ in 10 mM Tris-hydrochloride [pH 7.5], 0.3 M NaCl at 37°C for 30 min). Arrows indicate the sedimentation position of ^3H -labeled 35S poliovirus RNA and 28S and 18S

RESULTS

Sucrose density gradient analysis of vRNA. ^3H uridine-labeled RNA was extracted from purified virus, and the denatured products were analyzed by sedimentation in an SDS-containing sucrose density gradient. A profile of the acid-precipitable radioactivity in the gradient fractions revealed three discrete peaks (Fig. 1a) representing RNA species with sedimentation coefficients of 31, 26, and 13 for the L, M, and S RNAs, respectively. Molecular weights of 2.15×10^6 , 1.5×10^6 and 0.48×10^6 for these three RNA species were calculated. The single-stranded nature of the RNA was shown by its sensitivity to prior digestion with pancreatic RNase (Fig. 1a). These data confirmed that Akabane virus RNA shows the characteristic properties of RNA from a member of the *Bunyaviridae*.

Identification of cRNA species in infected cells. RNA species synthesized in mock-infected or Akabane virus-infected cells were labeled with ^3H uridine from 2 to 8 h postinfection (p.i.) in the presence of actinomycin D. Total RNA was extracted and analyzed by density centrifugation in SDS-containing sucrose density gradients (Fig. 1b). Incorporation of radioactivity into mock-infected cell RNA was considerably less than into infected cell RNA and was concentrated at the top of the gradient. Two of the major peaks of radioactivity in the infected cell RNA sample corresponded with the sedimentation positions of the M and S vRNA segments (Fig. 1a), whereas a shoulder of radioactive material (fractions 6 to 10) corresponded to the sedimentation position of the L vRNA segment.

To demonstrate the virus-specific and complementary nature of the RNA synthesis, we hybridized ^3H uridine-labeled RNAs from either mock- or virus-infected cells with unlabeled vRNA. Preliminary experiments had shown that saturation levels of hybridization were achieved in standardized experiments using approximately 7 μg of crude vRNA extracted from partially purified virus. After removal of unhybridized

rRNAs. (b) Analysis of RNA from Akabane virus-infected or mock-infected cells. The virus-infected or mock-infected cells were labeled with ^3H uridine from 2 to 8 h p.i. in the presence of 2 μg of actinomycin D per ml (added at 0 h p.i.). Total labeled RNA was extracted from each culture and analyzed by centrifugation on 15 to 30% SDS-sucrose density gradients. Fractions (0.4 ml) were collected and the trichloroacetic acid-precipitable radioactivity in portions of each fraction was determined. Symbols: \circ , RNA from the virus-infected cells; \bullet , RNA from mock-infected cells.

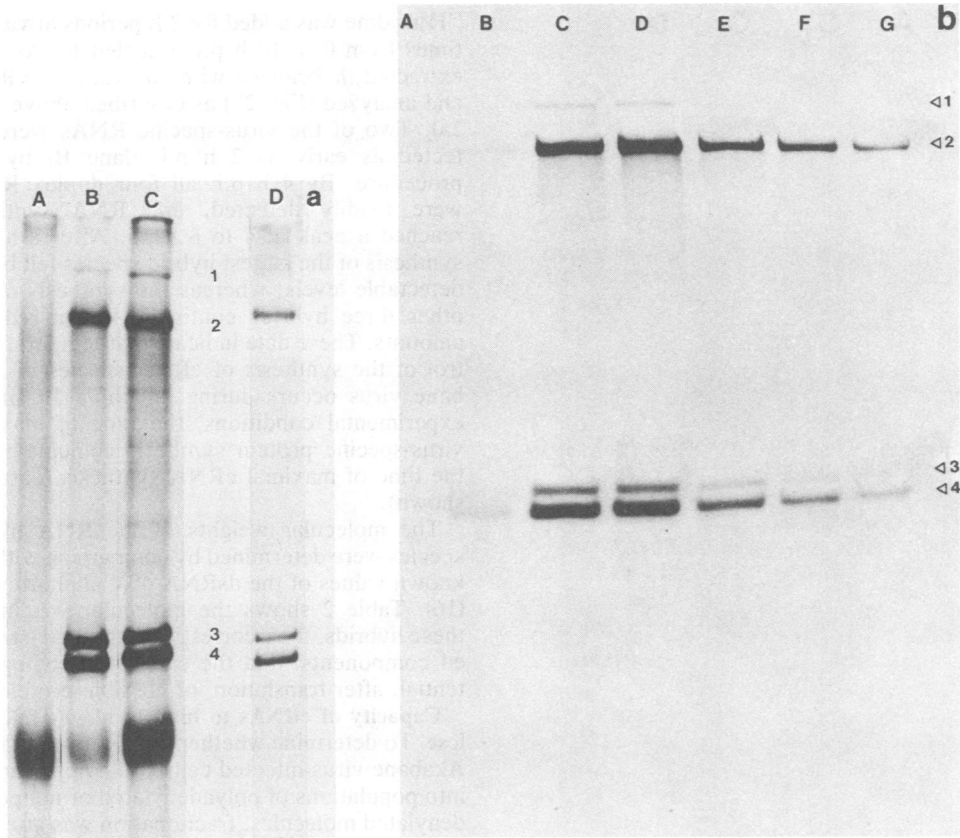


FIG. 2. Polyacrylamide gel electrophoresis of virus-specific dsRNA hybrids. (a) Total RNA from mock-infected (lane A) or virus-infected (lanes B and C) cells labeled with [3 H]uridine at 2 to 8 h p.i. were either hybridized with unlabeled vRNAs (lanes A and B) or self-annealed (lane C), digested with nuclease S1, and analyzed by electrophoresis on a 4% polyacrylamide gel as described in the text. Lane D represents the dsRNAs produced by hybridization of [3 H]uridine-labeled vRNAs with unlabeled RNAs from virus-infected cells at 8 h p.i. After electrophoresis, the radioactive bands were detected by fluorography. Arrowheads with arbitrary numbers represent the four virus-specific dsRNA hybrids. (b) Infected or mock-infected cultures were incubated in presence of actinomycin D at 0 h p.i. and labeled with [3 H]uridine for 2-h periods at various times starting at 0 h p.i. The RNAs were then hybridized with unlabeled vRNAs, and the duplexes formed were analyzed as above. Duplexes in lanes B to G were derived from infected cells labeled at 0, 2, 4, 6, 8, and 10 h p.i., respectively. Lane A corresponds to a mock-infected culture labeled similarly.

RNAs by digestion with S1 nuclease, the double-stranded hybrid molecules were resolved by polyacrylamide gel electrophoresis (Fig. 2a). Four radioactive bands representing four dsRNA hybrids (arbitrarily numbered 1 to 4) were detected in extracts from virus-infected cells (lane B) but not from mock-infected cells (lane A). When a portion of 3 H-labeled RNA from infected cells was self-annealed, digested with nuclease S1, and analyzed similarly, the same four hybrid RNAs were detected (Fig. 2a, lane C). This result indicated that RNA of both polarities was synthesized in infected cells during the period 2 to 8 h p.i. To confirm that hybridization to vRNA was occurring in these

experiments, the reverse procedure of hybridizing [3 H]uridine-labeled vRNA to unlabeled RNA from infected cells was done (Fig. 2a, lane D). Four hybrid RNAs with the same electrophoretic mobilities as seen in lane B were detected. All four cRNA species in infected cells were shown to be cytoplasmic rather than nuclear in their location (data not shown).

Coding origin of cRNA species. The results in Fig. 2 demonstrated the presence of four cRNA species in the virus-infected cells. To identify the coding origins of these cRNAs, vRNA uniformly labeled with 32 P was prepared, and the three single-stranded RNA segments were resolved by polyacrylamide gel electrophoresis (as

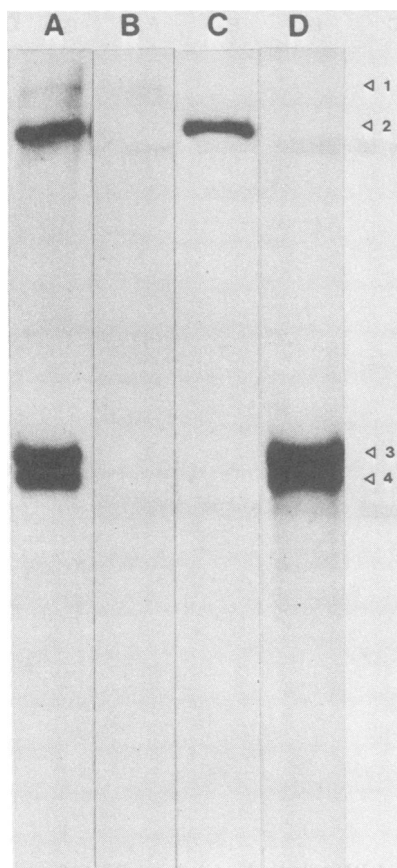


FIG. 3. Coding origin of the induced cRNA species. Uniformly labeled [^{32}P]vRNA (total or individual segments) was hybridized with an excess of unlabeled cRNAs from virus-infected cells and analyzed by electrophoresis as in Fig. 2 and detected by autoradiography. Hybrids were produced with total [^{32}P]vRNA (lane A), L RNA segment (lane B), M RNA segment (lane C), and S RNA segment (lane D).

described above). The recovered individual RNA segments were each hybridized with an excess of unlabeled cRNA from infected cells, digested with S1 nuclease, and analyzed by polyacrylamide gel electrophoresis. The unfractionated [^{32}P]vRNA upon hybridization produced the four typical dsRNA hybrids (Fig. 3, lane A). The L RNA upon hybridization produced hybrid 1, whereas the M RNA gave rise to hybrid 2 (lanes B and C, respectively). In contrast, the S RNA yielded both hybrid 3 and hybrid 4 (lane D) upon hybridization.

Kinetics of cRNA synthesis. To follow the kinetics of synthesis of cRNA species after infection, we infected parallel cultures at an MOI of 7 PFU per cell and incubated in the presence of 2 μg of actinomycin D per ml.

[^3H]uridine was added for 2-h periods at various times from 0 to 10 h p.i. Labeled RNAs were extracted, hybridized with an excess of vRNA, and analyzed (Fig. 2b) as described above (Fig. 2a). Two of the virus-specific RNAs were detected as early as 2 h p.i. (lane B) by this procedure. By 4 h p.i. all four duplex RNAs were readily detected, and RNA synthesis reached a peak at 4 to 6 h p.i. After 8 h, the synthesis of the largest hybrid species fell below detectable levels, whereas the synthesis of the other three hybrids continued but in reduced amounts. These data indicate that temporal control of the synthesis of cRNA species of Akabane virus occurs during infection. In similar experimental conditions, the time of maximal virus-specific protein synthesis coincided with the time of maximal cRNA synthesis (data not shown).

The molecular weights of the RNA hybrid species were determined by comparison with the known values of the dsRNA of Wallal orbivirus (16). Table 2 shows the molecular weights of these hybrids, their corresponding single-stranded components, and the calculated coding potential, after translation, of possible proteins.

Capacity of cRNAs to bind to oligo(dT)-cellulose. To determine whether the cRNA species in Akabane virus-infected cells could be separated into populations of polyadenylated or nonpolyadenylated molecules, fractionation was attempted by oligo(dT)-cellulose chromatography. [^3H]uridine-labeled RNA was extracted from infected cells at either early or late times after infection, and the polyadenylated RNA species were separated from the nonpolyadenylated RNA. When actinomycin D (2 $\mu\text{g}/\text{ml}$) was included during the labeling period (4 to 8 h p.i.), less than 4% of the acid-precipitable radioactivity was found to bind specifically to and elute from oligo(dT)-cellulose. In contrast, a similar

TABLE 2. Molecular weights of Akabane virus-specific cRNAs and their coding potentials

Hybrid RNA species	Mol wt ($\times 10^6$)		Approximate coding potential ($\times 10^3$) ^b
	dsRNA ^a	Corresponding single-stranded RNA	
1	3.8	1.9	210
2	2.76	1.38	153
3	0.25	0.125	14
4	0.21	0.105	12

^a Molecular weights of the dsRNAs were calculated by using the double-stranded RNAs of Wallal orbivirus (16) as molecular weight standards.

^b The coding potential of each of the cRNA species was calculated as one-ninth of their respective molecular weights.

fractionation of host cell RNA labeled in the absence of actinomycin showed that 35% of the incorporated radioactivity was in a polyadenylated form.

Evidence that virus specific cRNAs were present in the fraction that failed to bind to oligo(dT)-cellulose was provided by hybridization experiments. Polyadenylated and nonpolyadenylated RNAs from infected cells labeled either early (0 to 4 h p.i.) or late (4 to 8 h p.i.) were isolated and hybridized with an excess of vRNA and analyzed as described above. The virus-specific dsRNA hybrids could only be seen in the lanes containing products derived from nonpolyadenylated RNAs (Fig. 4, lanes A and C). Hybrid RNA 1 species could be detected early after infection by longer exposure of the gel to X-ray film (lane A), but was clearly present when labeling was done at the time of maximal RNA synthesis (lane C). The broad band of radioactivity seen in the lower part of the gels is a residual host component as actinomycin D was not included in this experiment. Preliminary experiments to test the sensitivity of this fluorography method showed that if virus-specific radioactivity in the polyadenylated samples had exceeded 2% of that in the nonpolyadenylated samples then discrete bands would have been visible in lanes B and D (Fig. 4). Additional experiments were done to try to detect virus-specific polyadenylated RNA species in Akabane virus-infected cells by analyses in sucrose density gradients. When the small amount of polyadenylated RNA that could be isolated was analyzed as described in Fig. 1b, no radioactive peaks corresponding to viral RNAs could be detected (data not shown). Thus, by either method of detection, no virus-specific polyadenylated RNA species could be found in Akabane virus-infected cells.

Virus-specific RNA synthesis in the presence of protein synthesis inhibitors. Results of self-annealing of RNAs from infected cells (Fig. 2a) and the partial resistance of such intracellular RNAs to digestion with pancreatic RNase (data not shown) indicated that RNAs of both polarities were being synthesized even at early times after infection. It has been well established (2, 20, 23) with other negative-strand viruses that inhibitors of protein synthesis prevent the translation of mRNA synthesized early in infection and so restrict viral RNA synthesis to that of only the primary type. In such conditions, only cRNAs that correspond to viral mRNAs are produced. The classical protein synthesis inhibitors cycloheximide and puromycin were used to treat Akabane virus-infected cells from the time of infection. [³H]uridine was added at 2 h p.i. for a further 4 h. After extraction and denaturation, the labeled RNAs were analyzed by sucrose

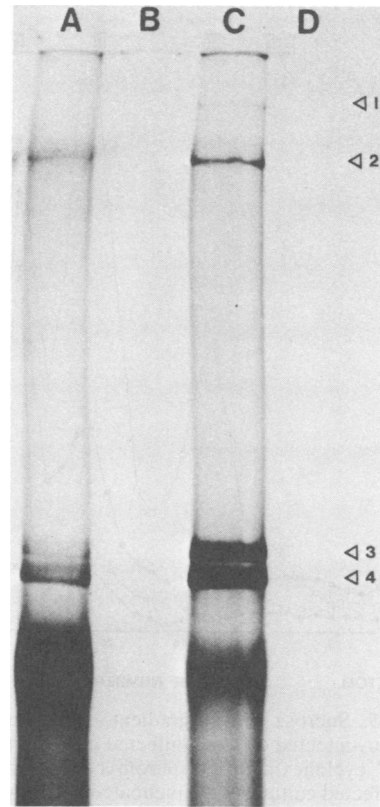


FIG. 4. Electrophoretic analysis of dsRNAs formed by hybridization of polyadenylated or nonpolyadenylated RNAs from the virus-infected cells. [³H]uridine-labeled RNA from the virus-infected cells at 0 to 4 h (lanes A and B) or 4 to 8 h (lanes C and D) were fractionated into polyadenylated and nonpolyadenylated RNAs by oligo(dT)-cellulose chromatography and hybridized to vRNA and analyzed as described in the legend to Fig. 2. Lanes A and C contain duplexes derived from nonpolyadenylated RNAs, whereas lanes B and D contain duplexes from polyadenylated fractions.

density gradient centrifugation (Fig. 5). The gradients containing mock-infected cell RNAs or infected cell RNAs (in the absence of cycloheximide or puromycin) produced profiles similar to that seen in Fig. 1b. However, the RNA profiles from cultures treated with either of the drugs failed to reveal any radioactive peaks corresponding to the sedimentation positions of the L and M vRNA segments. Minor peaks of radioactivity were seen in these gradients, corresponding to the sedimentation position of the S vRNA segment, which was also the region to which some labeled host RNAs sedimented (see the mock-infected profile, Fig. 5). These results indicated that RNA synthesis in the presence of

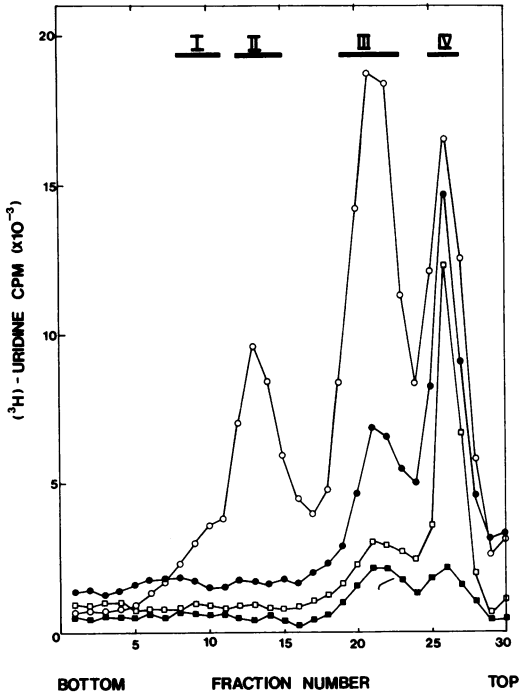


FIG. 5. Sucrose density gradient analysis of RNA from virus-infected or mock-infected cells in the presence of cycloheximide or puromycin. Infected or mock-infected cultures were incubated in the presence of actinomycin D and either cycloheximide (100 $\mu\text{g}/\text{ml}$) or puromycin (100 $\mu\text{g}/\text{ml}$) from the time of infection. RNAs labeled with [^3H]uridine from 2 to 6 h p.i. were extracted and analyzed by centrifugation on 15 to 30% SDS-sucrose density gradients as described in the legend to Fig. 1. RNA from mock-infected cells (\bullet) and RNA from Akabane virus-infected cells either directly (\circ) or in the presence of cycloheximide (\square) or puromycin (\blacksquare).

either of these inhibitors was below detectable levels in this analysis. Even if the MOI in such experiments was increased to 50 PFU per cell, no RNA synthesis was detectable in cells in which protein synthesis had been stopped by these inhibitors (see below).

To determine whether the radiolabeled RNA species in Fig. 5 were virus specific, the RNAs in four regions of the gradients (numbered I, II, III, and IV) were recovered by ethanol precipitation and hybridized with an excess of unlabeled vRNA. When the resulting dsRNAs after S1 nuclease digestion were analyzed by polyacrylamide gel electrophoresis, virus-specific RNAs could be demonstrated only in fractions I to III of the gradient containing RNA from the control infected cells (data not shown). No virus-specific products were detected in gradients containing RNA from either cycloheximide- or

puromycin-treated cells (data not shown), which confirmed that primary RNA transcription in such treated cells was below detectable levels.

Since the inhibitory effects of puromycin and cycloheximide on protein synthesis are fully reversible, it was of interest to determine whether RNA synthesis in treated infected cells would recommence if the drugs were removed. To make the detection of any primary transcription in such cells more likely, an MOI of 50 PFU per cell was used. Cycloheximide and puromycin were added to four cultures at the time of infection. At 1.75 h p.i., the drugs were thoroughly washed from two of the cultures over a period of 10 min. All four cultures (together with an untreated control culture) were labeled with [^3H]uridine from 2 to 6 h p.i. The labeled RNA from each culture was hybridized with vRNA and analyzed as described above (Fig. 6a). No dsRNAs were seen when cycloheximide was retained in the culture (lane B), but a faint band corresponding to the fourth viral hybrid RNA could be seen in the corresponding puromycin-treated sample (lane D). When the inhibitors were washed from the cultures, cRNA synthesis was restored as shown by the presence of the typical pattern of four dsRNAs in lanes C and E. However, the inhibitory effect of cycloheximide could not be completely reversed within the labeling period used as judged by a comparison of the intensities of the radioactive bands (lanes A and C).

Since in the presence of cycloheximide and puromycin primary transcription was below detectable levels, an experiment was done to show their effects on secondary (amplified) RNA transcription when synthetic rates are much higher. Five cell cultures were infected (at the usual MOI of 7 PFU per cell) and incubated for 4 h to allow RNA synthesis to reach its maximal rate. Cycloheximide and puromycin were added to two cultures each and then washed from two cultures at 5.75 h p.i. [^3H]uridine was added for a further 4 h before preparing and analyzing samples as described for Fig. 6a.

Results observed (Fig. 6b) indicated that both cycloheximide and puromycin had a dramatic inhibitory effect on secondary RNA transcription also (lanes B and D) and that this inhibition could be readily reversed by washing the drugs from the cell cultures (lanes C and E). However, the effect of puromycin on secondary transcription was not complete, as a faint band of radioactivity corresponding to hybrid RNA 4 can be seen in lane D, Fig. 6b. The sensitivity of the fluorography procedure used could just detect bands containing 2% of the radioactivity of control bands (lane A), and so an estimate of the effect of puromycin on RNA synthesis in this experiment is that it is at least 90% complete.

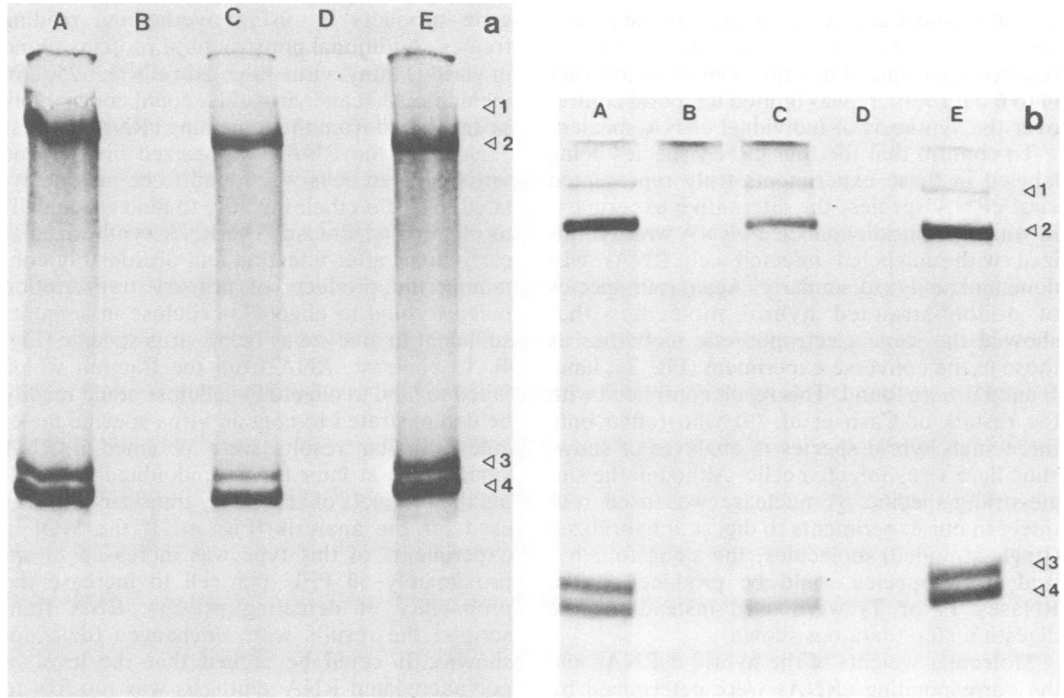


FIG. 6. Effect of cycloheximide and puromycin on RNA synthesis in virus-infected cells. (a) Cultures infected with Akabane virus at a multiplicity of infection of 50 PFU per cell were treated with either cycloheximide (100 $\mu\text{g/ml}$) or puromycin (100 $\mu\text{g/ml}$) at 0 h p.i. At 1.75 h p.i., drugs from one set of cultures were washed out and the cells were labeled with [^3H]uridine from 2 to 6 h p.i. The RNAs were extracted, hybridized with unlabeled vRNAs, and analyzed by polyacrylamide gel electrophoresis. Duplexes were derived from infected cells (lane A), in the presence of cycloheximide or puromycin (lanes B and D, respectively), or when cycloheximide and puromycin were washed out (lanes C and E, respectively). (b) Similar analysis of the duplexes when cell cultures were infected at an MOI of 7 PFU per cell were treated with cycloheximide or puromycin at 4 h p.i., and the drugs were washed out at 5.75 h p.i. RNA was labeled with [^3H]uridine from 6 to 10 h p.i. Duplexes were from infected cells (lane A) in the presence of cycloheximide or puromycin (lanes B and D, respectively) or when cycloheximide and puromycin were washed out (lanes C and E, respectively).

DISCUSSION

Analysis of the RNA contained in Akabane virus particles indicated that it was typical of that found for other bunyaviruses in that the genome RNA was single stranded and in three segments, although the measured sizes of these segments were slightly smaller than those found for other members of the family (5). However, Akabane virus was found to differ from the general properties shown by negative-strand viruses in that no polyadenylated cRNA species could be detected in infected cells, and established secondary RNA transcription was sensitive to the action of inhibitors of protein synthesis. In addition, four species of cRNA were detected in Akabane virus-infected cells, which differs from the three found previously for snowshoe hare bunyavirus (9).

The synthesis of [^3H]uridine-labeled cRNA in Akabane virus-infected cells was followed by

hybridization with unlabeled vRNA and analysis of the ^3H -labeled hybrid species so formed by gel electrophoresis. This was necessary because the usual experimental procedures used with negative-strand viruses to analyze only primary RNA transcripts resulted in undetectable levels of [^3H]uridine incorporation. Kinetic studies of cRNA synthesis at various times after infection at an MOI of 7 PFU per cell (Fig. 2b) showed that detectable synthesis was established as early as 2 h p.i. At this time, only RNA hybrids 2 and 4 were visible (Fig. 2b, lane B). Synthesis of cRNAs increased as the infection proceeded, reaching a maximal rate at 4 to 6 h p.i. At this time, all four hybrid species representing the four cRNA species were detected readily (lane D). The synthesis of the cRNA species leading to the formation of hybrid 1 was the first to decrease to below detectable levels (lane E), whereas synthesis of the cRNA species leading to the formation of hybrid 3 persisted along with

hybrids 2 and 4 until after 10 h p.i. These results show that although all four cRNA species reached their time of maximal synthesis together (4 to 6 h p.i.), there was limited temporal control over the synthesis of individual cRNA species.

To confirm that the four cRNA species being labeled in these experiments truly represented viral cRNA species, the alternative experiment in which [³H]uridine-labeled vRNA was hybridized with unlabeled infected cell RNAs was done and analyzed similarly. Again four species of double-stranded hybrid molecules that showed the same electrophoretic mobilities as those in the converse experiment (Fig. 2a, lanes B and D) were found. This result contrasted with the results of Cash et al. (9) who found only three such hybrid species in analyses of snowshoe hare virus-infected cells. Although the single-strand-specific S1 nuclease was used routinely in our experiments to digest unhybridized (single-stranded) molecules, the same four hybrid RNA species could be produced if the RNases T₁ or T₂ were used instead for the digestion step (data not shown).

Molecular weights of the hybrid dsRNAs and the corresponding cRNAs were determined by comparison with the genome RNAs of the described orbivirus, Wallal (16). The derived sizes of the cRNA species (Table 1) were smaller than the values measured for the vRNAs, indicating that none represented full-length transcripts of the genome RNAs. The coding potentials of the cRNAs (calculated as one-ninth of the molecular weight of the cRNA) were found to be approximately 210×10^3 , 153×10^3 , or 14×10^3 daltons respectively for the L, M, or S transcripts (Table 1). Either of the cRNAs represented in hybrid species 1 and 2 appear to have sufficient coding information for either the L protein (121×10^3 daltons) or the G₁ and G₂ proteins together (140×10^3 daltons, total). In contrast, the cRNAs present in hybrid species 3 and 4 do not appear to be large enough to code for either the L or G₁ plus G₂ proteins. Within the approximations used for calculating the coding potentials of these cRNAs, it seems possible that one of cRNA species present in either hybrid 3 or 4 codes for the N protein. This conclusion has been supported by preliminary *in vitro* translation of the RNA species in region III, Fig. 5, which has resulted in the translation of only the polypeptide corresponding to the N protein (unpublished results). Hybrid RNA species 3 and 4 are similar in size and may have overlapping sequences since hybridization shows that they are both derived from segment S of viral RNA (Fig. 3). If so, this information is consistent with the report of Bishop and co-workers (6) that the S segments of bunyaviruses have sequences that allow for the potential expression of two

gene products by using overlapping reading frames. Additional nonstructural proteins found in various bunyavirus-infected cells (12, 25, 26), including Akabane virus (21), could conceivably be translated from the remaining cRNA species.

None of the RNAs synthesized in Akabane virus-infected cells was found to be polyadenylated, based on their inability to bind specifically to oligo(dT)-cellulose. The RNA synthesized at early times after infection and presumably containing the products of primary transcription failed to bind to oligo(dT)-cellulose in amounts sufficient to analyze as being virus specific (Fig. 4). In contrast, RNA from the fraction which failed to bind to oligo(dT)-cellulose could readily be demonstrated to contain virus-specific molecules. Similar results were obtained if RNA synthesized at later times (undoubtedly including the products of secondary transcription) was used for the analysis (Fig. 4). If the MOI in experiments of this type was increased to approximately 50 PFU per cell to increase the probability of detecting primary RNA transcripts, the results were unchanged (data not shown). It could be argued that the level of polyadenylated RNA synthesis was too low to detect in these experiments. However, the data in Fig. 4 combined with measurements of the sensitivity of the fluorography detection procedure indicate that the synthesis of virus-specific nonpolyadenylated RNA was at least 30 times higher than that of polyadenylated RNA in Akabane virus-infected cells. Such a situation is unique among the negative-strand viruses in general, but is consistent with work reported previously for another bunyavirus, Uukuniemi virus (26). However, it conflicts with the results of Cash et al. (9), who were able to show that both polyadenylated and nonpolyadenylated RNA fractions from snowshoe hare virus-infected cells could be translated *in vitro* to produce the nucleocapsid protein N. Preliminary studies in our laboratory with *in vitro* translation systems suggest mRNA activity is associated only with nonpolyadenylated RNA in Akabane virus-infected cells, thus supporting our contention that in this system no polyadenylated RNAs can be detected.

It is accepted that the first new synthetic event in cells infected with a negative-strand virus is the production of mRNA by the virus-associated RNA polymerase. This primary transcription is independent of protein synthesis. However, the change to secondary (amplified) transcription is dependent on the expression of viral proteins, and this transition can be prevented by the use of inhibitors of protein synthesis (10, 11, 28). Thus, cycloheximide and puromycin were used to treat Akabane virus-infected cells from the time of infection to examine

primary RNA transcription products. When analyzed either by sedimentation in SDS-containing sucrose density gradients or by hybridization plus gel electrophoresis, no virus-specific RNA products could be detected (Fig. 5 and 6). Similar results were obtained if the inhibitors were not added until the time of maximal RNA synthesis, when secondary transcription was undoubtedly occurring. This was a surprising result as secondary transcription with other negative-strand viruses has not been reported to be dependent upon continuing protein synthesis (23, 24). Similar effects were observed if an alternate protein synthesis inhibitor, emetine, was used (data not shown). Studies on the primary transcription of bunyaviruses in the presence of cycloheximide or puromycin have been reported previously. In snowshoe hare virus-infected cells at a high MOI, low levels of RNA synthesis could be detected by hybridization in the presence of cycloheximide but not in the presence of puromycin (27). Similar results also were obtained by using mutants of this virus (27). RNA synthesis in Bunyamwera virus-infected cells in the presence of cycloheximide has been examined (18) and again [³H]uridine incorporation levels were only marginally above background levels, and the products were not shown to be complementary to vRNA. Thus, in both of these reports and the results presented in this communication, primary RNA transcription by bunyaviruses in the absence of protein synthesis has not been demonstrated conclusively.

The synthesis of protein and RNA in Akabane virus-infected cells appeared to be linked. Since the inhibitory effects of cycloheximide and puromycin on protein synthesis can be readily reversed by the washing of treated cells, it was of interest to see whether secondary RNA transcription was affected similarly. The results in Fig. 6a and b show that secondary transcription can be readily established after the removal of either inhibitor (Fig. 6a) and can recommence and approach former synthetic rates if interrupted by the action of these drugs (Fig. 6b). Although it is not known whether the effects of cycloheximide and puromycin on Akabane viral RNA synthesis are direct or indirect, it seems that the latter alternative is possible because of the correspondence between the effects on protein and RNA synthesis and the reversible nature of each (Fig. 6b). The conclusion follows that secondary RNA transcription (and perhaps primary transcription as well) in Akabane virus-infected cells is dependent on continuing protein synthesis.

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