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Translational Requirement of La Crosse Virus S-mRNA Synthesis: In Vitro Studies

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The exceptional requirement of La Crosse virus mRNA synthesis for ongoing protein synthesis in vivo was examined in vitro by using purified virions and a reticulocyte lysate. Transcription from the S genome produced two incomplete transcripts (110 and 205 nucleotides [nt]) in the absence of the lysate, whereas S-mRNA (900 nt) was predominantly made when the lysate was present. The addition of drugs which inhibit protein synthesis also inhibited the synthesis of S-mRNA, and in some cases led to the reappearance of the 205-nt RNA. Reconstruction experiments demonstrated that the incomplete transcripts were not the result of rapid and selective degradation of S-mRNA but were due to premature termination of the polymerase at defined sites. The requirement for ongoing protein synthesis for productive transcription in vitro is not at the level of chain initiation but for elongation of the nascent RNA beyond these sites.

La Crosse virus (LAC) is a member of the California encephalitis serogroup of the insect-transmitted bunyavirus family (22). The genome of these viruses consists of three segments of single-stranded RNA of negative polarity, each contained within a separate nucleocapsid, which are labeled small (S), medium (M), and large (L). The viral nucleocapsids have helical symmetry and often appear as circular and supercoiled structures when viewed in the electron microscope (13).

The mechanism by which bunyaviruses initiate transcription of their mRNAs is remarkably similar to that of influenza virus (12). Bunyavirus S-mRNAs in vivo contain 5′ extensions of ca. 15 nucleotides (nt) in length which are heterogeneous in sequence, presumably the result of a host cell primer used to initiate transcription (4, 16). In vitro, purified LAC virions were found to contain a transcriptase which is stimulated by natural mRNAs, such as alfalfa mosaic virus (AlMV) RNA 4 which was shown to be acting as a primer. LAC virions also contain a methylated cap-dependent endonuclease which cleaves AlMV RNA 4 at the positions expected from the length of the 5′ extensions found on the transcripts made in vitro (15). Bunyaviruses, like influenza viruses, thus apparently "snatch" capped oligonucleotides from host cell mRNAs both to prime their mRNA synthesis and to provide it with a cap group required for efficient translation (12). However, unlike influenza virus, LAC transcription takes place in the host cell cytoplasm rather than in the nucleus (25), where it uses a stable pool of mRNAs as substrates for primers, consistent with the known immunity of bunyavirus replication to drugs which disrupt host cell mRNA synthesis (14).

Abraham and Pattnaik (1, 17) have recently reported the curious finding that not only bunyavirus genome replication (as is the case for all minus-strand RNA viruses examined to date), but mRNA synthesis as well is apparently dependent on ongoing protein synthesis in vivo. Although there is no precedent for such a requirement among minus-strand RNA viruses and this result appears to conflict with the demonstration of polymerase activity in purified LAC virions, we have confirmed the results of Abraham and Pattnaik (24). To resolve this apparent conflict, we further examined the nature of LAC transcription in vitro by characterizing the products made in the absence and presence of ongoing protein synthesis.

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MATERIALS AND METHODS

In vitro polymerase reactions. The reaction conditions optimized for the influenza virion polymerase are essentially those of Plotch and Krug (21) and have been described previously (15). The reaction conditions optimized for in vitro protein synthesis containing rabbit reticulocyte lysate are essentially those of Pelham and Jackson (18) and contained 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM spermidine, 10 mM creatine phosphate, 2 mM dithiothreitol, 50 μg of rabbit liver tRNA per ml, 1 mM ATP, 0.2 mM GTP, 0.1 mM CTP, and 0.05 mM UTP. When RNA synthesis or protein synthesis was monitored directly, 25 μCi of [^{32}P]UTP or 12.5 μCi of [^{35}S]methionine per 25 μl was also added, respectively. When the lysate was included, it was present at 11 μl per 25-μl reaction; when absent, it was replaced with 11 μl of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 8.1. When AlMV RNA 4 was added to stimulate the reaction in the absence of the lysate, it was present at 2.5 μg/25 μl. When BHK cell cytoplasmic extracts (23) were present, 8 μl was added per 25-μl reaction. Purified LAC virions (15) were present in all reactions at 5 μg of protein per 25 μl, which also contained 0.05% Nonidet P-40. The lysate was prepared according to the method of Pelham and Jackson (18) and contained 2 mM dithiothreitol, 40 μg of creatine kinase per ml, and 20 mM hemin. Treatment with micrococcal nuclease, when performed, was for 5 min at 20°C with 10 μg of the enzyme per ml in the presence of 1 mM $CaCl_2$, followed by the addition of 6 mM EGTA [ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid].

After incubation for 60 min at 30°C, the reaction volume was raised to 0.5 ml containing 0.5 M NaCl, 1% Nonidet P-40, 1 mM EDTA, and 100 μl of Nonidet P-40 cytoplasmic extract of uninfected BHK cells which contained approximately 50 μg of total RNA. The diluted reaction was then

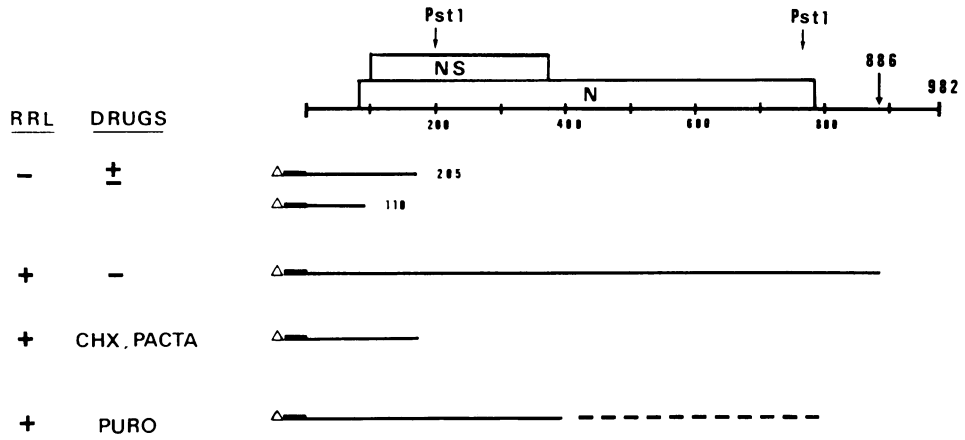

FIG. 1. Schematic diagram of the LAC S genome segment and the in vitro polymerase products. Above is shown the LAC S genome segment, including the N and the NS overlapping reading frames, the *PstI* sites used in the subcloning into SP64, and the 3′ end of the in vivo mRNA at position 886. Below are shown the transcripts made in vitro under various conditions. The open triangles and thick bars at the left of the transcripts indicate the capped primer derived from other mRNAs which are ca. 15 nt in length. RRL, Presence or absence of the rabbit reticulocyte lysate.

placed onto 3.5 ml of a 20 to 40% CsCl density gradient and centrifuged for 16 h in an SW60 rotor. The gradient supernatant, including the virion nucleocapsids which band in the middle, was carefully removed, and the pelleted RNAs were suspended in 50 μl of ET (1 mM EDTA, 10 mM Tris, pH 7.4). For electrophoretic analysis of the reaction products, the recovered RNAs were made 80% in formamide containing 0.1% xylene cynanol FF, were treated for 2 min at 70°C, and were analyzed on either a 6% sequencing gel (27) or a 1.75% agarose gel containing 6 M urea, and also in both gels in TBE (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3).

Hybrid selection of in vitro reaction products. Cytoplasmic extracts from 12 plates of LAC-infected BHK-21 cells were prepared and fractionated on a CsCl density gradient as previously described (16). The nucleocapsid band was isolated and then fractionated into L, M, and S RNAs by sucrose gradient centrifugation (13). Then, 5% of the L, M, and S RNAs was denatured with 50% formamide and TAE (10 mM Tris, 5 mM sodium acetate, 0.5 mM EDTA) for 10 min at 60°C and dotted onto 0.5-cm squares of zeta-probe membranes. Prehybridization and hybridization was done according to the instructions of the manufacturer.

The entire procedure was carried out in Eppendorf tubes which each contained four filters containing either L, M, and S genome and antigenome RNAs or 10 μg of *Escherichia coli* tRNA (to monitor hybridization specificity) and one-fourth of the [^{32}P]UTP polymerase reaction products. After hybridization, the filters were washed according to the method of Curran et al. (8), and the hybrid-selected RNAs were eluted by heating twice at 98°C in ET containing 0.1% sodium dodecyl sulfate and recovered by ethanol precipitation.

Northern analysis of the in vitro reaction products. Reaction products were recovered by centrifugation through CsCl density gradients (see above). One-third of each reaction was treated for 2 min at 70°C in 50% formamide and electrophoresed on a 1.5-mm-thick 4% polyacrylamide gel (27). The gel was then soaked in TAE for 10 min and electroblotted onto zeta-probe paper in the same buffer. Prehybridization, hybridization, and washing were carried out as previously described (6), except that the final stringent wash was at 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) at 70°C for 30 min.

RESULTS

Effect of ongoing protein synthesis on LAC in vitro polymerase products. In vivo, the LAC genome segments are transcribed unequally, and the S-mRNA is by far the most abundant species. The LAC S-mRNA begins on a nontemplated primer of ca. 15 nt, and its 3′ end, which is apparently not poly(A)$^+$, has been mapped at position 886 (16; Fig. 1). The LAC S-mRNA is thus ca. 900 nt in length and codes for both the nucleocapsid protein N and the small nonstructural (NS) protein(s) in overlapping reading frames (2, 7).

To examine the possible effect of ongoing protein synthesis on LAC transcription in vitro, virion polymerase reactions were first carried out under conditions optimized for in vitro protein synthesis by using a rabbit reticulocyte lysate (but without the lysate), and the reaction products were examined by gel electrophoresis. In the absence of added mRNA which can serve as a primer, no RNA products could be detected (Fig. 2A and B, lanes 4). When AlMV RNA 4 was added to the reaction (Fig. 2A and B, lanes 5), two bands of approximately 110 and 205 nt (see Fig. 2C) and a much larger doublet marked L1 and L2 were made. For comparison, lanes 6 (Fig. 2A and B) show the products of a reaction carried out under conditions we had previously found optimal (15), but in which [^{32}P]UTP incorporation was actually 5- to 20-fold lower (Table 1). When rabbit reticulocyte lysate was added to the reaction, a remarkable change in the pattern of products was found (Fig. 2A and B, lanes 3). The 110- and 205-nt transcripts, as well as L1 and L2, were no longer detectable. The major reaction product was now a transcript estimated to be 900 nt in length which comigrated with S-mRNA isolated from infected cells (data not shown). A second band which migrated above the 1,800-nt DNA marker and which might represent the M genome mRNA was also seen on the sequencing gel (panel A), but this band was not reproducible. A control reaction (Fig. 2A and B, lanes 2) shows that the lysate by itself incorporates no detectable [^{32}P]UTP.

The lysate used in the above experiment had been briefly treated with micrococcal nuclease to eliminate endogenous translation. This treatment had little effect on the ability of the lysate to support LAC transcription, presumably because the fragmented mRNAs could still serve as substrates

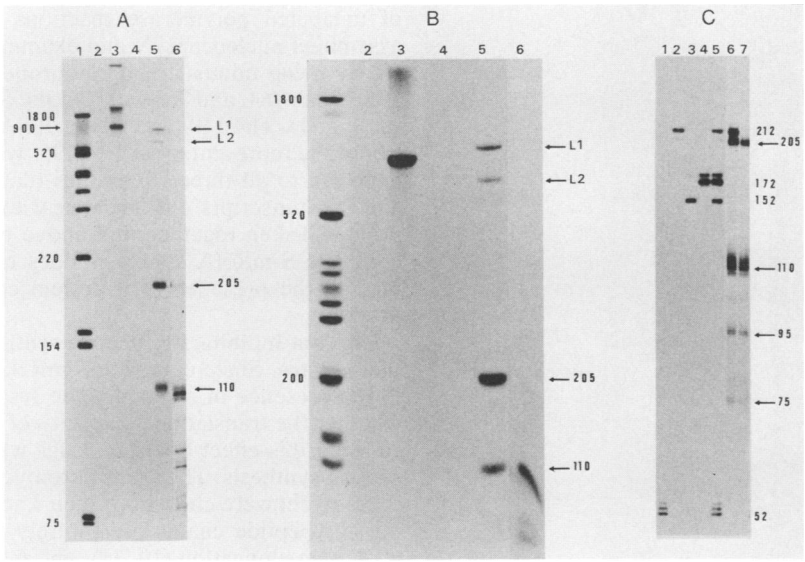

FIG. 2. Analysis of the LAC polymerase products by gel electrophoresis. (A and B) LAC polymerase reactions were carried out under either the conditions optimized for the influenza virion polymerase or for in vitro protein synthesis, by using rabbit reticulocyte lysate. The products were recovered and analyzed on either a 6% polyacrylamide sequencing gel (A) or a 1.75% agarose–6 M urea gel (B). Lanes: 1, DNA restriction fragments as length markers; 2 and 3, reticulocyte lysate conditions including the lysate, without and with LAC virions, respectively; 4 and 5, reticulocyte lysate conditions without added lysate and without and with the addition of AlMV RNA 4, respectively; 6, influenza virion polymerase conditions with the addition of AlMV RNA 4; 6, exposed three times as long as the other lanes. Numbers without arrows refer to length markers, those with arrows refer to LAC polymerase products. (C) The reaction products shown in lanes 5 of panels A and B were also electrophoresed on a 6% sequencing gel along with RNAs made in vitro from SP64 plasmids as markers. Lanes: 1 to 4, 52-, 212-, 152-, and 172-nt marker RNAs, respectively; 5, a mixture of all four markers; 6 and 7, in vitro LAC polymerase products with and without the 212-nt RNA marker, respectively. The numbers following arrows on the right refer to the estimated lengths of the LAC polymerase products. None of the RNA markers contained G-C tails.

for the virion endonuclease. When the coupled reaction was carried out with [^{35}S]methionine to see whether the in vitro transcripts could promote the synthesis of viral proteins, both the N protein (25 kilodaltons) and a group of polypeptides whose size is consistent with the NS proteins could also be detected (Fig. 3). These translation products would appear to be due to the in vitro-made S-mRNA, since Northern analysis of the purified virions could not detect any preexisting S-mRNA (data not shown). It is not clear whether any of the expected translation products of the M and L genome mRNAs (i.e., the glycoprotein precursor, ca. 110 kilodaltons, and the L protein, ca. 200 kilodaltons) have

TABLE 1. Effect of puromycin on LAC S-mRNA synthesis in vitro

	Puromycin (μg/ml)	UTP incorporated (cpm)[a]	Protected by nt 1 to 195[b]	
			%	cpm
LAC + RRL		90,759	14.2	12,887
LAC + RRL	50	77,534	ND[c]	ND
LAC + RRL	100	70,201	16.5	11,583
LAC + AlMV 4[d]		41,214	70.4	29,014
LAC + AlMV 4[d]	100	29,531	80.2	23,638
LAC + ApG[d]		2,630	ND	ND

[a] Determined after the reaction products were pelleted through a CsCl density gradient.

[b] The percentage of the reaction products protected against ribonuclease digestion was determined as previously described (25), by using an excess of unlabeled RNA representing nt 1 to 195 as minus-strand RNA which was made in vitro via SP6.

[c] ND, Not done.

[d] The AlMV RNA 4 and ApG were used at 0.37 and 600 μM, respectively.

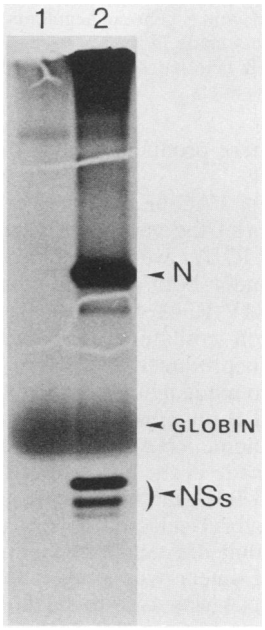

FIG. 3. LAC proteins made in the coupled system in vitro. The reactions in lanes 1 and 2 are identical to those in lanes 2 and 3 of Fig. 2A, respectively, except that the [^{32}P]UTP was replaced with [^{35}S]methionine. After incubation, the reactions were electrophoresed on a 15% sodium dodecyl sulfate-urea-polyacrylamide gel (29). The band marked N was found to comigrate with N protein in LAC-infected BHK cells (not shown).

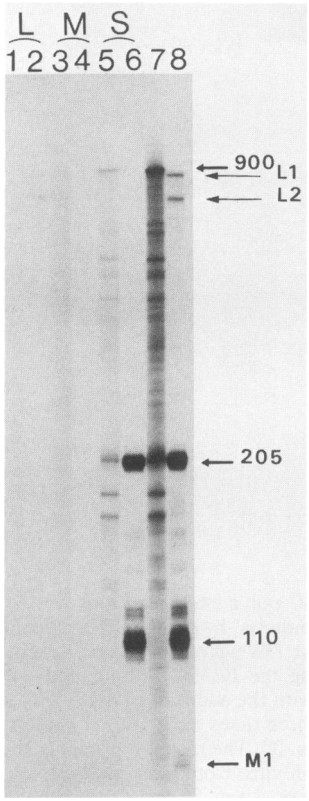

FIG. 4. Segmental origin of LAC polymerase products. LAC polymerase reactions were carried out either in the presence of the lysate (lane 7) or without the lysate but with AlMV RNA 4 (lane 8). The recovered reaction products were then hybrid selected with disks containing either the L, M, or S genome and antigenome RNAs and electrophoresed on a 6% sequencing gel. Lanes: 1, 3, and 5, reaction products made in the presence of the lysate hybrid selected with the L, M, and S genome segments, respectively; 2, 4, and 6, reaction products made in the absence of the lysate selected with the L, M, and S genome segments, respectively; 7 and 8, unselected reaction products.

been made, since these products would be lost in the smear at the top of the gel.

Characterization of LAC in vitro transcripts. To examine the segmental origin of the various transcripts seen, the L, M, and S genome RNAs were used to hybrid select the transcripts made under both conditions, namely reactions stimulated with AlMV RNA 4 in the absence of the lysate and reactions which contained reticulocyte lysate. This latter reaction again produced the 900-nt transcript as the major band, but also made a number of smaller bands which may be the result of contaminating nuclease (Fig. 4, lane 7). As shown, the S genome RNA hybrid selected both the 110- and 205-nt RNAs made in the absence of the lysate (lane 6) as well as the 900-nt RNA made in its presence (lane 5). The M genome RNA hybrid selected a very minor band (M1) from the reaction that did not contain the lysate (lane 4). Only a background smear was visible on the gel (lane 3) when M genome RNA was used to select the products of a reaction containing the lysate. Interestingly, bands L1 and L2, products made only in the absence of the lysate, were hybrid selected by the L genome RNA (lane 2).

The 110- and 205-nt S genome transcripts and L1 and L2 would appear to represent the promoter proximal sequences since their synthesis is dependent on added mRNA. To further characterize the S genome transcripts, the products of unlabeled polymerase reactions were isolated free of assembled nucleocapsids and examined by Northern analysis by using minus-strand riboprobes representing nt 1 to 195, 196 to 764, and 765 to 982 of the S genome segment (also see Fig. 1). The 110- and 205-nt RNAs annealed only to the riboprobe representing nt 1 to 195, whereas the 900-nt RNA annealed to all three riboprobes (data not shown). The L1 and L2 transcripts did not anneal to any of the S genome probes. Taken together, the above evidence indicates that complete S-mRNA synthesis does take place in vitro, but only in the presence of a system capable of synthesizing protein.

Effect of inhibitors of protein synthesis on LAC transcription in vitro. Since complete S-mRNA is made in vitro only in the presence of a reticulocyte lysate, we next examined whether the translational capacity of the lysate was responsible for this effect by using drugs which specifically inhibit protein synthesis. The drugs puromycin, cycloheximide, and pactamycin were chosen for their known effects of terminating polypeptide chains prematurely (19), slowing polypeptide chain elongation (10, 11), and inhibiting chain initiation (28), respectively. The effect of these drugs in diminishing [^{35}S]methionine incorporation in the same lysate which was not treated with micrococcal nuclease was also determined in parallel. In reactions which contain the lysate, all three drugs diminished the synthesis of complete S-mRNA as a function of drug concentration roughly proportional to their effect on protein synthesis (Fig. 5). To examine the effect of these drugs on the virion polymerase itself, the drugs were also added to reactions stimulated with AlMV RNA 4 in the absence of the lysate. As shown in the right hand lanes of each panel, in the same experiment the three drugs had little or no effect on either the level or nature of the transcripts made in the absence of the lysate. Two conclusions can be drawn from these experiments. (i) These drugs do not appear to exert their effect on the virion polymerase itself. (ii) The finding that three drugs which have different mechanisms of action all have similar effects in diminishing the synthesis of complete S-mRNA suggests that it is the translational capacity of the lysate rather than a specific factor that is required.

Fig. 5 also shows that the three drugs nevertheless have different effects on the pattern of transcripts which result from inactivation of the lysate. In the case of pactamycin for example, the loss of the 900-nt transcript is paralleled by the appearance of the 205-nt transcript, but in vastly greater than equivalent amounts. In an accompanying paper (26), we mapped the 3′ end of the 205-nt RNA to ca. position 175. This transcript would then contain only $\frac{175}{886}$ or 20% of the radioactivity that the complete S-mRNA does. In the presence of 5 μg of pactamycin per ml, we calculate that approximately 15 times more moles of the 205-nt transcript accumulate than do moles of S-mRNA whose synthesis was suppressed. Pactamycin apparently stimulates initiation of the viral polymerase in the presence of the lysate, but these transcripts all terminate prematurely at position 175. When cycloheximide was added to the coupled reaction, the 205-nt transcript again appears, but now in equimolar amounts. Cycloheximide therefore does not appear to alter the rate of polymerase initiation either in the presence or absence of the lysate.

When puromycin was used to inhibit the translational capacity of the lysate, no other RNA band, and in particular the 205-nt RNA, could be seen. This effect could be due to the inability of the polymerase to initiate in the lysate, or alternatively, to the polymerase initiating normally but terminating too heterogenously so that discrete bands are not

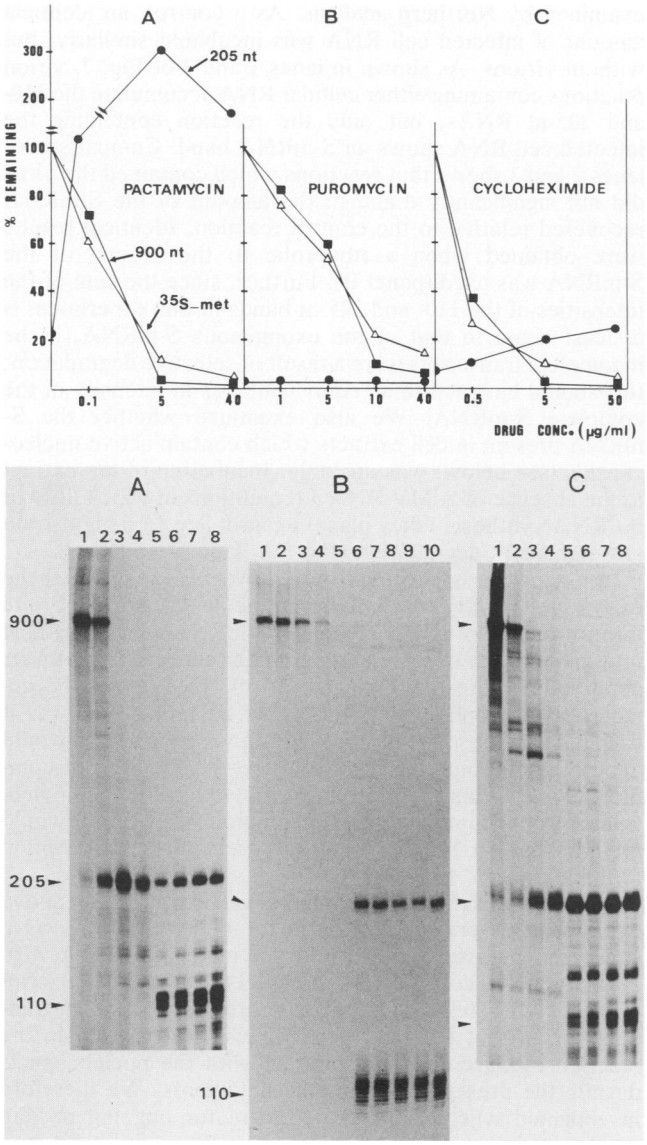

FIG. 5. Effect of drugs which inhibit protein synthesis on products of the LAC polymerase reactions. (Lower panels) Pactamycin, puromycin, and cycloheximide were added at increasing concentrations to LAC polymerase reactions carried out in the presence of the lysate (lanes 1 to 4 of panels A and C, lanes 1 to 5 of panel B) or in the absence of the lysate but with AlMV RNA 4 (lanes 5 to 8 of panels A and C, lanes 6 to 10 of panel B). The reaction products were then recovered through CsCl density gradients, and equal fractions of each reaction were analyzed on a 6% polyacrylamide sequencing gel. The drug concentrations used are indicated on the upper panels. The lysate used in these experiments was not treated with micrococcal nuclease. (Upper panels) Effect of the drugs on incorporation of [^{35}S]methionine into RNAse A-resistant, TCA-precipitable material was determined in parallel reactions as shown in panels A, B, and C, in which drug concentrations are plotted on an arbitrary scale. The autoradiograms shown in the lower panels were scanned by densitometry, and the areas under the 900- and 205-nt bands are indicated, with the zero drug concentration of the 900-nt band as 100% for each experiment.

seen. This latter possibility is suggested by the numerous incomplete transcripts above ca. 400 nt in length when low concentrations of cycloheximide are used (Fig. 5, lanes 2 and 3). We therefore repeated the puromycin experiment and in addition to polyacrylamide gel electrophoretic analysis of the products, we also determined both total RNA synthesis and the fraction of this RNA which represents nt 1 to 195 by hybridization as a measure of the level of initiation. As shown in Table 1, the addition of puromycin to the lysate inhibits RNA synthesis only slightly and the total amount of RNA representing nt 1 to 195 has not changed. Puromycin therefore does not alter the rate of polymerase initiation in the lysate. Polyacrylamide gel electrophoretic analysis of the reaction products (Fig. 6) shows that puromycin has again almost completely inhibited complete S-mRNA synthesis, but also led to the appearance of multiple bands above ca. 400 nt in length. A small amount of 205-nt RNA is also visible in this experiment, but its amount increases only slightly due to presence of the drug. Inhibition of the translational capacity of the lysate with high concentrations of puromycin does not prevent the polymerase from reading through position 175, but still causes the polymerase to terminate prematurely (see below), at multiple sites beyond ca. position 400.

The requirement for ongoing protein synthesis for bunyavirus mRNA synthesis found in vivo can thus also be reproduced in vitro. In addition, these studies demonstrate that protein synthesis is not required for the initiation of mRNA synthesis, which takes place normally on a capped primer either in the absence of the lysate or in the presence of a lysate inactivated by cycloheximide, puromycin, or

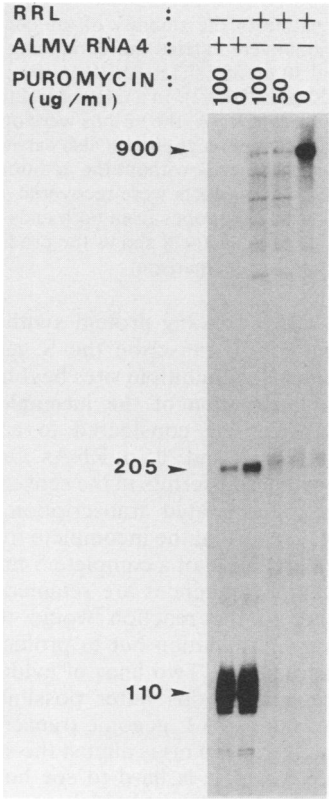

FIG. 6. Effect of puromycin on LAC polymerase reactions. Polymerase reactions were carried out either in the presence of a reticulocyte lysate (RLL) or without a lysate but with the addition of AlMV RNA 4, as indicated. Puromycin at 50 or 100 μg/ml was added to some of the reactions. The reaction products were recovered through a CsCl density gradient, and an equal amount (10,000 cpm) was analyzed on a 6% sequencing gel. Total UTP incorporation and the percent RNA representing nt 1 to 195 are reported in Table 1.

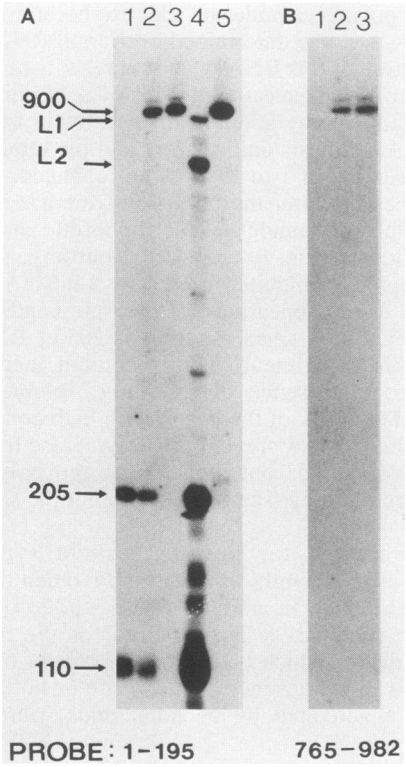

FIG. 7. Examination of the stability of the complete S-mRNA. Polymerase reactions were carried out containing purified virions, AlMV RNA 4, and 30 μg of CsCl pellet RNA from uninfected (lane 1) or infected BHK cells (lane 2). In a control reaction containing 30 μg of infected CsCl pellet RNA, the virions were omitted (lane 3). A nonradioactive polymerase reaction was also carried out containing extracts of infected BHK cells without the addition of AlMV RNA 4 (lane 5). The reaction products were recovered and examined by Northern analysis with riboprobes from both ends of the S genome segment (panels A and B). Lane 4 shows the products of a directly radiolabeled reaction for comparison.

pactamycin. In vitro, ongoing protein synthesis would appear to be required to transcribe the S genome segment beyond nt 175 and also at multiple sites beyond position 400.

Mechanism of generation of the incomplete transcripts. Two mechanisms can be considered to account for the accumulation of the 110- and 205-nt RNAs. One possibility is that they are abortive transcripts in the sense that they result from prematurely terminated transcription. An alternate possibility, however, is that the incomplete transcripts result from the rapid degradation of a complete S-mRNA such that only the 5′ proximal sequences are retained. The effect of adding the lysate to the reaction would then not be to prevent premature termination but to protect the complete mRNA from degradation. Two lines of evidence, however, appear to argue against this latter possibility. (i) In the absence of the lysate, two L genome transcripts are found, the larger of which (ca. 850 nt) is almost the same size as the complete S-mRNA. (ii) It is hard to see how pactamycin, cycloheximide, and puromycin, which act on protein synthesis by such different mechanisms, all cause the lysate to lose its ability to stabilize the mRNA.

Nevertheless, to examine this possibility directly, we investigated the stability of S-mRNA under our reaction conditions. Cytoplasmic RNA from either uninfected or LAC-infected BHK cells was added to nonradioactive virion polymerase reactions (minus the lysate), and the RNAs were examined by Northern analysis. As a control, an identical amount of infected cell RNA was incubated similarly, but without virions. As shown in lanes 1 and 2 of Fig. 7, virion reactions containing either cellular RNA accumulate the 110- and 205-nt RNAs, but only the reaction containing the infected cell RNA shows an S-mRNA band. Comparison of lanes 2 and 3 shows that reactions which contained the virus did not significantly diminish the amount of the S-mRNA recovered relative to the control reaction. Identical results were obtained when a riboprobe to the 3′ end of the S-mRNA was used (panel B). Further, since the sum of the intensities of the 110- and 205-nt bands in this experiment is at least equal to that of the exongenous S-mRNA, if the incomplete transcripts were a result of selective degradation, this should have led to an equivalent loss in intensity of the complete S-mRNA. We also examined whether the S-mRNA present in cell extracts which contain active nucleocapsids (see below) was unstable. Incubation of the extract in the absence of AlMV RNA 4 (conditions in which little or no RNA synthesis takes place) again failed to demonstrate any significant degradation (Fig. 7A, lane 5).

In a separate experiment (not shown), we examined the early kinetics of RNA synthesis in the absence of the lysate in an attempt to detect complete S-mRNA as an unstable intermediate. By using reaction times as short as 1 min under conditions where the 110- and 205-nt RNAs could be detected, no complete S-mRNA could be found, nor was it found at any subsequent time. The above experiments would appear to rule out rapid and selective degradation of complete S-mRNA as the mechanism by which the incomplete transcripts accumulate. The incomplete transcripts would then appear to be the result of premature transcription termination.

In vitro transcription with infected cell extracts. The above experiments were carried out with purified virions as a source of the viral genome and polymerase. Such in vitro systems can be considered as a model for primary transcription in vivo. They differ however from intracellular transcription in that the virion envelope is still present in the reaction mixture and may interact with the nucleocapsids despite the presence of nonionic detergents. We therefore investigated whether the requirement for ongoing protein synthesis for complete S-mRNA synthesis in vitro was peculiar to the use of complete virions or could also be demonstrated with infected cell extracts.

Cytoplasmic extracts (S-10) of infected and uninfected BHK cells were prepared by hypotonic swelling and Dounce homogenization in the absence of detergents. Although such extracts of other cells prepared similarly are competent for translation (23), we found that our BHK extracts were extremely inefficient in protein synthesis and did not synthesize detectable amounts of RNA. Nevertheless, these infected BHK cell extracts did contain active nucleocapsids. When uninfected BHK cell extracts were added to a reticulocyte lysate, no de novo RNA synthesis could be detected (Fig. 8, lane 2). Infected BHK extracts, although weaker than purified virions, clearly led to the synthesis of the 900-nt transcript and a small amount of the 205-nt transcript simultaneously (lanes 3 and 6). When cycloheximide was added to these reactions (lanes 4 and 5), the synthesis of the 900-nt transcript was again abolished, but the 205-nt transcript continued to be made. The 110-nt transcript was not detected with infected cell extracts. These experiments demonstrate that the requirement of complete S-mRNA synthesis for ongoing protein synthesis in vitro is not peculiar to the use of purified virions.

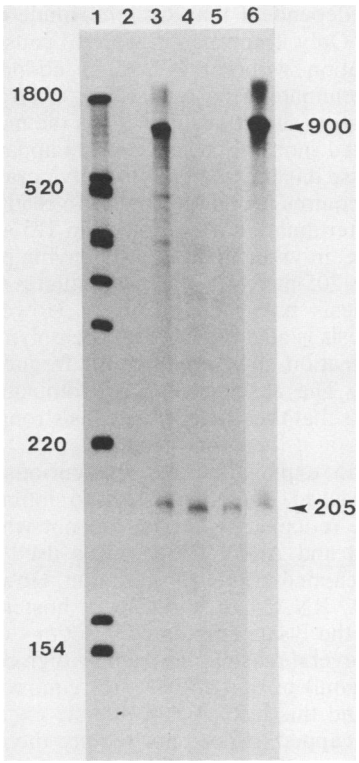

FIG. 8. Analysis of polymerase reactions by using LAC-infected cell extracts. Polymerase reactions were carried out in the presence of rabbit reticulocyte lysate containing either mock-infected BHK cell extracts (lane 2) or LAC-infected BHK cells extracts (lanes 3 and 6) or with LAC-infected cell extracts in the presence of 5 or 50 μg of cycloheximide per ml (lanes 4 and 5, respectively). Lane 1, *Hin*fI digest of pSEN-8 as markers.

Effect of uninfected cell extracts on the LAC polymerase reaction. When purified virions are stimulated with AlMV RNA 4, the ratios of the 110- and 205-nt RNA were found to vary considerably (cf. Fig. 2, 4–7, and 9). Further, when the virion reaction is carried out in the presence of a reticulocyte lysate inhibited with drugs or when AlMV RNA 4 is used to stimulate an infected BHK cell extract rather than virions (not shown), only the 205-nt transcript can be detected. It would appear that termination of the polymerase at the earlier of these two termination regions is variable in the absence of cytoplasmic extracts and that the presence of these extracts effectively suppresses termination at the earlier site.

To directly examine this point, we carried out AlMV RNA 4-stimulated virion polymerase reactions in the absence and presence of an uninfected BHK cell extract. The presence of the cytoplasmic extract almost completely suppresses synthesis of the 110-nt RNA, with a concommitant increase in the intensity of the 205-nt RNA, and also two bands whose migration is consistent with that of L1 and L2 (Fig. 9). However, since the migration of L1 and complete S-mRNA are rather close to each other, unlabeled reaction products were also examined by Northern analysis by using a riboprobe representing nt 1 to 195. A similar shift of the 110- to the 205-nt RNA due to the presence of the extract was noted, but no material which migrated more slowly than the 205-nt RNA, including complete S-mRNA, could be detected. These experiments indicate that the presence of cytoplasmic extracts effectively suppresses termination of the polymerase before position 175, but cannot relieve the premature polymerase termination at this site. The effect of cytoplasmic extracts on the virion polymerase reaction does not seem to be a nonspecific effect of protein concentration, since bovine serum albumin at 1 mg/ml has no effect (data not shown).

DISCUSSION

When ongoing protein synthesis is interrupted in LAC-infected cells by the addition of drugs such as pactamycin, cycloheximide, or puromycin, mRNA synthesis as well as genome replication is inhibited. The requirement for ongoing protein synthesis for genome replication, which has been found for all minus-strand RNA viruses studied to date, is thought to reflect the coupling of genome/antigenome synthesis and nucleocapsid assembly, since genomes and antigenomes probably exist in vivo only as assembled nucleocapsids. This requirement is thought to be for the continued synthesis of a functional protein product. In the case of vesicular stomatitis virus genome replication, for example, this product has been demonstrated to be at least in part unassembled nucleocapsid protein (3, 5). In the case of LAC S-mRNA synthesis, however, the existence of such a protein seems problematical. The hypothetical protein cannot be virus coded since minus-strand RNA viruses must first transcribe their genomes to initiate the infectious cycle,

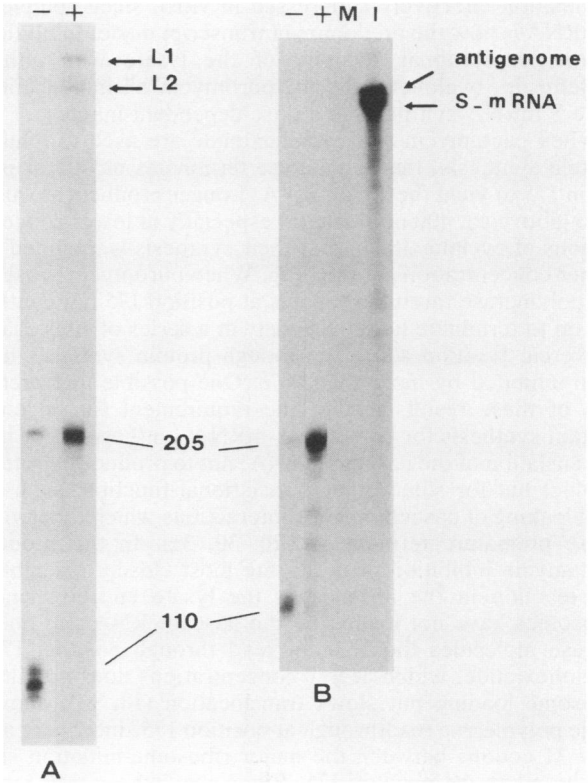

FIG. 9. Effect of cytoplasmic extracts on virion polymerase reactions. Virion polymerase reactions stimulated with AlMV RNA 4 were carried out in the presence or absence of uninfected BHK cytoplasmic extracts as indicated. (A) Directly radiolabeled reactions analyzed on a 6% sequencing gel. (B) Nonradioactive reactions separated on a 4% acrylamide gel and analyzed by Northern blotting with a riboprobe representing nt 1 to 195 at minus-strand RNA. Also shown are the results from 20 μg of CsCl pellet RNA from mock-infected (M) and virus-infected (I) BHK cells.

and it is unlikely that it is an unstable host protein since complete S-mRNA is made in the presence of a reticulocyte lysate whose endogenous protein synthesis has been effectively abolished by micrococcal nuclease. The requirement for ongoing protein synthesis here would then seem to be not for a newly made protein but presumably for some other function associated with active protein synthesis.

The experiments reported here were undertaken to investigate the apparent conflict between the requirement of LAC S-mRNA synthesis for ongoing protein synthesis in vivo and the finding that purified virions contain an active polymerase. This conflict appears to be resolved by the finding that in the absence of ongoing protein synthesis in vitro, the polymerase initiates S-mRNA synthesis normally but terminates prematurely before position 886.

Interestingly, the positions on the S genome template where the polymerase terminates prematurely in the absence of ongoing protein synthesis are nonrandom. In the absence of the reticulocyte lysate entirely, the polymerase terminates predominantly at two sites to produce the 110- and 205-nt RNAs. The 3′ end of the 205-nt RNA is well defined and has been mapped to within 2 or 3 nt at position 175 (26). Addition of cytoplasmic extracts which have poor or undetectable translational activity suppresses the termination which yields the 110-nt RNA and increases the amount of 205-nt RNA found, but cannot suppress termination at position 175. Only when transcription is carried out in the presence of a translationally active rabbit reticulocyte lysate is premature termination effectively suppressed in vitro, since complete S-mRNA is now the predominant transcript made. Inhibition of the translational capacity of the lysate with either pactamycin, cycloheximide, or puromycin all inhibit complete S-mRNA synthesis in a dose-dependent manner.

When pactamycin and cycloheximide are used to inhibit protein synthesis, the polymerase terminates mostly at position 175 to yield the 205-nt RNA. Longer products are also seen (above ca. 400 nt in length) especially at lower concentrations of cycloheximide, but their synthesis is inhibited at higher concentrations of the drug. When puromycin is used, the polymerase rarely terminates at position 175 but continues on to terminate heterogenously at a series of sites again above ca. position 400, even though protein synthesis has been inhibited by more than 90%. One possible interpretation of these results is that the requirement for ongoing protein synthesis for complete S-mRNA synthesis is for the cotranslation of the nascent mRNA, not to produce a protein product but for some other translational function, such as the blocking of nascent mRNA interactions which otherwise cause premature termination (20, 30, 31). In this model, pactamycin inhibition of the lysate most closely resembles the reaction in the absence of the lysate entirely, since ribosomes have not loaded on the nascent RNA and polymerase molecules then cannot read through position 175. Cycloheximide, which at low concentrations does not alter ribosome loading but slows translocation (10, 11), allows some polymerase readthrough at position 175 since there are only 31 codons between the major ribosome-initiation site (position 82) and position 175. Ribosomes whose translocation has been only partially slowed might still be able to disrupt the RNA interactions which cause the polymerase to terminate at position 175. Higher concentrations of cycloheximide, which more strongly slows ribosome translocation (and possibly also inhibits initiation) would then be more effective in preventing the ribosomes from disrupting nascent mRNA interactions. Puromycin, which is the translational equivalent of ddNTP in a Sanger sequencing reaction, is even more dependent than cycloheximide on target size for its action. Only if puromycin were to cause polypeptide chain termination within the first 31 codons would the polymerase terminate at position 175.

The termination site at position 175 is the most prominant site we detected, not only because of its apparent strength, but also because it is the first that the polymerase encounters for which it requires protein synthesis to read through. The nature of the termination site at position 175 is unclear, but it also operates in vivo. In an accompanying paper (26), we show that the 205-nt RNA also accumulates in vivo when protein synthesis has been inhibited. However, ongoing protein synthesis is also required for the polymerase to read through termination sites which occur frequently after ca. positions 400. The absence of a predominant site in this region suggests that these sites are not as strong as the site at position 175.

We have no explanation for the curious finding that pactamycin caused the polymerase to initiate more frequently in the reticulocyte lysate, but not when the lysate was ommitted and AlMV RNA 4 was used to provide a source of the capped primer for initiation. However, the use of pure AlMV RNA 4 to stimulate a host mRNA is not equivalent to the lysate-stimulated reactions or the in vivo situation for several reasons. Extremely high concentrations (100 to 200 μg/ml) of this mRNA are required for effective stimulation, and this mRNA is a notable exception in that although it is capped, it does not require the cap group for efficient translation (9). The mRNAs in the lysate and in vivo which are used as a source of the primer to initiate mRNA synthesis may also already be associated with proteins involved in ribosome initiation. It would be of interest to examine the requirements of the LAC endonuclease on mRNAs other than AlMV RNA 4 and to determine the effect ribosome-initiation factors, especially cap-binding factors, have on this reaction.

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