JOURNAL OF VIROLOGY, Jan. 1987, p. 96–103
0022-538X/87/010096-08$02.00/0

Vol. 61, No. 1

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

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Received 16 May 1986/Accepted 24 September 1986

By using methods to isolate cytoplasmic RNAs which limit degradation, the effect of drugs which inhibit protein synthesis on the accumulation of La Crosse virus plus-strand S RNAs in vivo has been studied. Cycloheximide and puromycin treatment of infected cultures caused an abortive transcript of ca. 205 nucleotides (nt) to accumulate, whereas pactamycin led to the appearance of an RNA which was slightly shorter (ca. 200 nt). Both the 205- and 200-nt RNAs contained the same range of host primers at their 5′ end, but their 3′ ends mapped at ca. positions 175 and 165, respectively. Examination of the sequence in this region and at the mature mRNA termination site (position 886) suggests that the sequence YAAAAAT(A)GCAG is involved in transcription termination.

The synthesis of bunyavirus mRNAs in vivo has long been known to be independent of cellular RNA synthesis, since drugs such as actinomycin D have little effect on virus replication (for a review, see reference 10). Recently, however, Abraham and Pattnaik (1, 14) demonstrated that when drugs such as cycloheximide or puromycin which inhibit protein synthesis are added to bunyavirus-infected cells not only genome replication but mRNA synthesis as well could no longer be detected. Although there are conflicting interpretations as to whether the small amounts of S-mRNA found late in vivo when cells are infected in the presence of puromycin means that 1° transcription does not require translation (6, 18), the effect of drugs such as cycloheximide, anisomycin, pactamycin, trichodermin, emetine, and puromycin on 2° transcription is not in dispute. More recently,we have extended the original findings of Abraham and Pattnaik by showing that cycloheximide and puromycin inhibit 2° S-mRNA accumulation in vivo in a dose-dependent manner proportional to their effect on cellular protein synthesis. In addition, since this inhibition cannot simply be explained by increased turnover of the mRNA in the presence of the drug, cycloheximide must be inhibiting S-mRNA synthesis (18).

The suggestion that ongoing protein synthesis is required for mRNA synthesis has no precedent among minus-strand RNA viruses. This suggestion also appears to be in conflict with the previous demonstration that purified virions contain a polymerase which initiates mRNA synthesis on capped primers derived from exogenous mRNAs in a cap-snatching mechanism similar to that of influenza virus (9, 12). However, further experiments on the La Crosse virus (LAC) polymerase demonstrated that although the polymerase in vitro initiates S-mRNA synthesis normally on a capped primer, this transcription terminates prematurely, at defined sites, unless it is coupled to translation by carrying out the reaction in the presence of a rabbit reticulocyte lysate in which complete and functional S-mRNA is now produced. This complete S-mRNA synthesis is also sensitive to the effects of drugs such as cycloheximide in vitro, whereas synthesis of the incomplete transcripts is not inhibited. The requirement of LAC S-mRNA synthesis for ongoing protein synthesis in vitro is thus not at the level of chain initiation but for readthrough of the polymerase at premature termination sites (3). The study reported here was undertaken to determine whether the incomplete S genome transcripts found in vitro were also present in LAC-infected cells and whether they could be shown to result from viral transcription in the absence of intracellular protein synthesis.

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MATERIALS AND METHODS

Isolation of cytoplasmic RNAs. Confluent cultures of BHK-21 cells were infected with 20 PFU of LAC per cell. At the times indicated, the cells were harvested by scraping in phosphate-buffered saline and recovered by centrifugation. Cytoplasmic extracts were prepared by vortexing the cells in 0.5% Nonidet P-40–0.15 M NaCl–10 mM Tris (pH 7.4)–1 mM EDTA, followed by centrifugation for 4 min at 4,000 × *g*. When denaturing agents were used, the cytoplasmic supernatant was immediately mixed with an equal volume of 6 M guanidine-thiocyanate–1% sarcosyl–0.2 M β-mercaptoethanol. Alternatively, 1 μg of heparin per ml was added. The RNAs were isolated from the extract either by centrifugation on a 20 to 40% CsCl density gradient or by phenol extraction.

Northern blot analysis. Total cytoplasmic RNA (20 μg) was heated for 2 min in 80% formamide plus 0.1% xylene cyanol FF and electrophoresed on a 1.5-mm-thick 4% polyacrylamide gel containing 8 M urea (19). The gel was then soaked for 10 min in TAE (10 mM Tris, 5 mM sodium acetate, 0.5 mM EDTA [pH 7.8]) and electroblotted onto zeta-probe paper in the same buffer. The paper was dried, prehybridized, and hybridized with riboprobes as described in our accompanying paper (3).

RNase mapping. Riboprobes labeled with UTP were prepared via SP64 plasmids and purified by polyacrylamide gel electrophoresis (PAGE). The radiolabeled riboprobe (ca. 10^6 cpm per sample) was coethanol precipitated with the test RNA, suspended in 15 μl of ET (1 mM EDTA, 10 mM Tris [pH 7.4]), heated for 2 min at 90°C, quick cooled, and adjusted to 0.3 M NaCl. The samples were annealed for 30 min at 70°C, raised to 50 μl with 0.4 M NaCl containing 80 μg of RNase A per ml and 0.3 U of RNase T1 per ml, and digested at 30°C for 30 min. Proteinase K (600 μg/ml) and 0.2% sodium dodecyl sulfate was then added, and the samples were incubated for 15 min at 37°C. The sodium dodecyl sulfate was then raised to 0.5%, and the RNAs were

isolated by phenol extraction and ethanol precipitation. The protected RNAs were then suspended either in ET and electrophoresed on a nonurea polyacrylamide gel or in 80% formamide and electrophoresed on a sequencing gel.

RESULTS

Examination of intracellular CsCl pellet RNA by Northern blotting. Since the LAC S-mRNA (900 nucleotides [nt]) is almost the same size as the S antigenome RNA (982 nt) and cannot be isolated by oligo(dT) cellulose chromatography, we routinely used CsCl density gradient centrifugation to separate the mRNAs which pellet from the nucleocapsid RNAs (genomes and antigenomes) which band at 1.31 gm/ml. Transcripts from the 3′ end of the minus-strand S genome have previously been examined in CsCl pellet RNA isolated from cytoplasmic extracts of infected BHK cells (11). By using 3′ end-labeled S genome RNA as a probe, short plus-strand transcripts of approximately 75 to 115 nt were detected. These short transcripts were called "leader" RNAs since they represented the 5′ proximal sequences of the plus-strand S RNA. When the cytoplasmic CsCl pellet RNA was examined for plus-strand sequences downstream of this region, either by S1 mapping or by primer extension experiments, minor plus-strand transcripts whose 5′ ends started internally, at position 74 to 160, could also be detected. At the time, these leader RNAs and the downstream transcripts did not appear to be the result of random degradation since their 5′ ends were extremely well defined and the downstream transcripts all started on an A residue. Furthermore, S1 mapping experiments with a restriction fragment from the middle of the S genome segment as a control failed to give any evidence of either 5′ or 3′ ends in the S-mRNA population within this region, even on prolonged exposure of the gels (11).

To further examine the variety and origin of the S-RNAs in cytoplasmic CsCl pellet RNA, this RNA was analyzed by Northern blotting by using radiolabeled riboprobes. These riboprobes were obtained by subcloning the S genome into SP64 by using the two internal *Pst*I sites so that riboprobes representing nt 1 to 195 (A probe), nt 196 to 760 (B probe), and nt 761 to 982 (C probe) could be transcribed in either orientation. The results of such a Northern blot analysis with the minus-strand A riboprobe are shown in lane 2 of Fig. 1A and lane 4 of Fig. 1B. Besides complete S-mRNA (900 nt), several shorter bands could routinely be found. Most prominent among these shorter bands was a doublet estimated at ca. 520 nt in length, a band of ca. 310 nt which is generally the strongest of these bands, and a band of approximately 150 nt. The ratio of these shorter bands to the complete S-mRNA, however, was found to vary between different preparations. In many cases, the shorter bands were found to be as strong as or stronger than the complete S-mRNA (also see Fig. 3B, lane P), suggesting that they might indeed be due to degradation, albeit of a rather specific sort. This specificity is also indicated when identical samples were examined with the minus-strand B riboprobe. As shown in lane 4 of Fig. 1C, a different but still highly specific pattern of shorter bands was detected, two bands of ca. 560 and 725 nt. Examination of the same sample of CsCl pellet RNA with the minus-strand C riboprobe gave an identical pattern to that of the minus-strand B riboprobe (data not shown). Also shown in lanes 2 of panels B and C are the results of a virus polymerase reaction carried out in the absence of a protein synthesis system in which the products were also directly radiolabeled in vitro. Note that none of the RNAs found in the CsCl pellet fraction of infected cells comigrated with the 110- and 205-nt S-genome transcripts made in vitro. Lanes 3 of panels B and C show that complete S-mRNA is the only product seen in virion reactions carried out in the presence of a translationally active reticulocyte lysate.

These highly specific but different patterns of bands when CsCl pellet RNA is examined by Northern analysis with riboprobes from different regions of the S genome segment suggest a specific pattern of single-strand breaks. We therefore tried other methods of isolating the cytoplasmic RNAs since the shorter bands might be due to degradation which occurred during isolation. Lane 1 of Fig. 1A shows the results of immediately raising the cytoplasmic extract to 3 M guanidinium-thiocyanate–1% sarcosyl–0.1 M β-mercaptoethanol before CsCl density gradient centrifugation. These conditions have previously been described to limit degradation during isolation of total cellular RNAs (5), and they also clearly change the pattern of bands recognized by the minus-strand A riboprobe. None of the shorter bands can be detected at this exposure, and the yield of the complete S-mRNA is now considerably increased. The band above the complete S-mRNA represents the S antigenome (982 nt) which now pellets through the gradient due to the effect of the denaturing reagents (see below).

To minimize the problem of RNA degradation during isolation, all subsequent experiments not requiring separation of nucleocapsid and nonnucleocapsid RNA were carried out by adding these reagents to the cytoplasmic extract as soon as possible. These additions were found to limit degra-

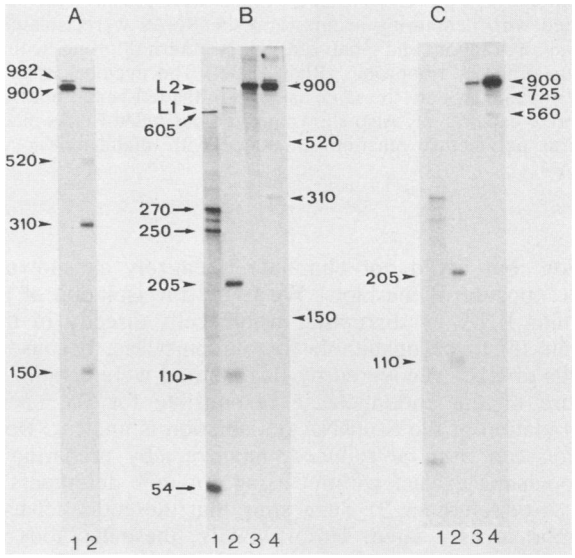

FIG. 1. Northern blot analysis of cytoplasmic LAC plus-strand S RNAs isolated with and without denaturing agents. (A) Duplicate samples of cytoplasmic extract which contained either no additions (lane 2) or 3 M guanidinium-SCN–0.5% sarcosyl–0.1 M β-mercaptoethanol (lane 1) were centrifuged on CsCl density gradients. The pelleted RNAs were analyzed by Northern blotting by using the minus-strand A riboprobe (nt 1 to 195). (B and C) Lanes: 1, SP6-transcribed RNAs as length markers; 2 and 3, reaction products of a virus polymerase reaction stimulated with alfalfa mosaic virus RNA 4 or a rabbit reticulocyte lysate, respectively, which are also directly radiolabeled in vitro; 4, cytoplasmic RNAs isolated without denaturing agents. Panels B and C were probed with the minus-strand A and B (nt 196 to 767) riboprobes, respectively. The lengths of the cytoplasmic RNAs (arrowheads) were estimated by using the riboprobe markers (arrows) as well as S-mRNA (900 nt) and antigenome RNA (983 nt).

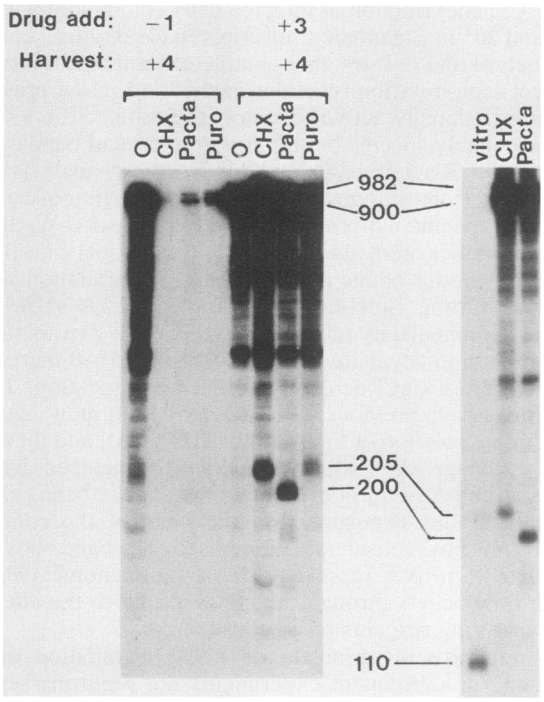

FIG. 2. Effect of inhibitors of protein synthesis on accumulation of LAC plus-strand S RNAs in vivo. (Left panel) Cycloheximide (CHX) (50 μg/ml), pactamycin (Pacta) (50 μg/ml), puromycin (Puro) (100 μg/ml), or no (0) drugs were added to cultures either 1 h before infection or 3 h p.i. Cytoplasmic extracts were prepared at 4 h p.i., treated with denaturing agents, and the RNAs were isolated by phenol extraction and analyzed by Northern blotting with the minus-strand A riboprobe. (Right panel) The cycloheximide and pactamycin lanes are the same as those in the left panel, but from a separate experiment. Also shown are the unlabeled RNAs made in a virus polymerase reaction stimulated with alfalfa mosaic virus RNA 4.

dation, but could not eliminate it entirely as shown by overexposure of the blots. We found the isolation of total cellular RNA by disrupting whole cells directly in these agents (5) to be unsuitable for our purposes, because the yields of RNA recovered by this method were erratic. The nature of the endonuclease responsible for the specific degradation of the S-mRNA on isolation is unclear. Degradation can best be reduced, however, by preparing the cytoplasmic extract without using nonionic detergents (cf. Fig. 6 of reference 3), suggesting that the endonuclease is membrane associated. Unfortunately, these methods also gave nonuniform recovery of S-mRNA.

Effect of cycloheximide, puromycin, and pactamycin on the accumulation of S-genome incomplete transcripts in vivo. We next examined whether the addition of drugs which inhibit protein synthesis affected the pattern of plus-strand S RNAs found in LAC-infected cells. In these experiments, parallel cultures of LAC-infected BHK cells were either not treated or treated with inhibitors of protein synthesis and the cytoplasmic RNAs were isolated and analyzed by Northern blotting. The drugs pactamycin, cycloheximide, and puromycin were chosen for their known effects of inhibiting chain initiation (20), slowing translocation (7, 8), and promoting premature chain termination (16), respectively. We first examined the effect of adding these drugs to the cultures at 1 h before infection, so that genome replication would be restricted and only the transcripts from the infecting genomes should be seen. As shown in Fig. 2, all three drugs dramatically decreased the amount of both S-mRNA and antigenomes found in these cells at 4 h postinfection (p.i.) relative to the untreated control, but only in the case of cycloheximide did this effect appear absolute. Some S-mRNA and antigenomes are made in the presence of pactamycin and especially puromycin, presumably due to drug escape (18). When the lower region of the Northern blot is examined, only in the case of pactamycin can an S genome incomplete transcript be detected, but just barely. This RNA however migrates slightly faster than the 205-nt RNA noted previously.

The three drugs were also added to cultures at 3 h p.i. when s-mRNA synthesis was well established, and the cultures were harvested 1 h later and analyzed. As shown in Fig. 2, cycloheximide inhibition of protein synthesis led to the accumulation of an incomplete transcript which comigrates with the 205-nt RNA made in vitro in the absence of a reticulocyte lysate. The 110-nt RNA which is also made in vitro under these conditions would not be expected to appear in vivo, since addition of cytoplasmic extracts to the virion reaction, whether translationally active or not, effectively suppressed synthesis of the 110-nt RNA and led to a concomitant increase of the 205-nt RNA (3). In control cultures which were not drug treated, the 205-nt RNA could not be detected, even on the prolonged exposure shown for this experiment. When pactamycin was used to inhibit protein synthesis, an incomplete S genome transcript also accumulated, which again migrated slightly ahead of the 205-nt RNA, at ca. 200 nt. When puromycin was used, some 205-nt RNA also accumulated due to the effect of this drug, but its amount was considerably less than that which occurred on cycloheximide inhibition.

In the above experiment, the cytoplasmic RNAs were isolated by phenol extraction of extracts which had been immediately raised to 3 M guanidine-SCN to limit degradation, but some degradation still occurred. However, it is unlikely that the 205- and 200-nt RNAs found as a consequence of cycloheximide and pactamycin treatment were a result of this degradation, since these two bands represented the only clear differences that could be seen relative to the control culture. Nevertheless, to further control for this possibility, cytoplasmic RNAs were also isolated after guanidine-SCN treatment of the cytoplasmic extract followed by CsCl density gradient centrifugation, as described in Fig. 1. When cycloheximide was added at 4 h p.i. and the RNAs were analyzed 1 h later, the 205-nt RNA again accumulated in vivo relative to the untreated control (Fig. 3A, B, and D). In addition, the amount of 205-nt RNA found was proportional to the amount of cycloheximide used (panel C). Under these conditions of RNA isolation, slightly less RNA degradation appeared to take place and the 205-nt RNA was the strongest band seen on these exposures except for the S-mRNA and antigenome RNA. We also examined the effect of increasing the incubation time after drug addition on the amount of the 205-nt RNA which accumulated intracellularly. As shown in Fig. 3B, less 205-nt RNA was actually found 2 or 3 h after drug addition than 1 h after drug addition. In this experiment, two smaller transcripts which migrated on either side of the 110-nt RNA made in vitro also accumulated due to cycloheximide treatment, but the appearance of these two bands was not as reproducible as was that of the 205-nt RNA. The finding that the amount of the 205-nt RNA found did not increase upon prolonged incubation of the culture after cycloheximide addition suggests that

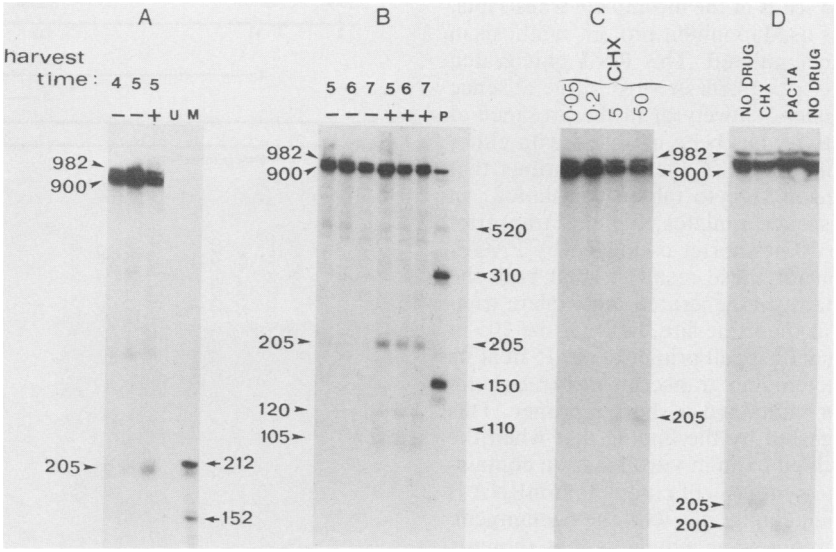

FIG. 3. Effect of cycloheximide on accumulation of LAC plus-strand S RNA in vivo. Cytoplasmic extracts were prepared from control or cycloheximide-treated cultures, denaturing agents were immediately added, and the RNAs were recovered through a CsCl density gradient and analyzed by Northern blotting with the minus-strand A riboprobe. (A and B) + and −, Presence or absence of 50 μg of cycloheximide per ml added at 4 h p.i.; U, uninfected cell RNA; M, RNA length markers made via SP6; P, CsCl pellet RNA from virus-infected cells isolated without addition of denaturing agents. (C) Infected cultures at 4 h p.i. were treated with increasing concentrations (μg/ml) of cycloheximide (CHX) as indicated, and the RNAs were isolated and analyzed as above at 5 h p.i. (D) Infected cultures were either not treated or treated with 50 μg of either cycloheximide or pactamycin per ml at 4 h p.i. and harvested 1 h later. The RNAs were isolated and analyzed as above.

this RNA may be turning over in vivo under these conditions.

We also examined whether the 205-nt transcript which accumulated on cycloheximide treatment was assembled into nucleocapsids intracellularly. In this experiment, 1 μg of heparin per ml was added to the infected cells before disruption to limit degradation during isolation, but denaturing agents were not added to the cytoplasmic extract. The vast majority of the genome and antigenome nucleocapsids thus continued to band at 1.31 g/ml and were not included in the pellet RNA. Equal amounts of RNA (in cytoplasm equivalents) from both the pellet fraction (P) and the banded nucleocapsid fraction (C) were then examined by Northern analysis by using all three riboprobes as both plus and minus strands, along with total cytoplasmic RNA isolated in the presence of denaturing agents (Fig. 4, lane T) as a control. The 205-nt RNA was found only in the pellet fraction and not in the nucleocapsid RNAs and hybridized only to the minus-strand A probe (Fig. 4).

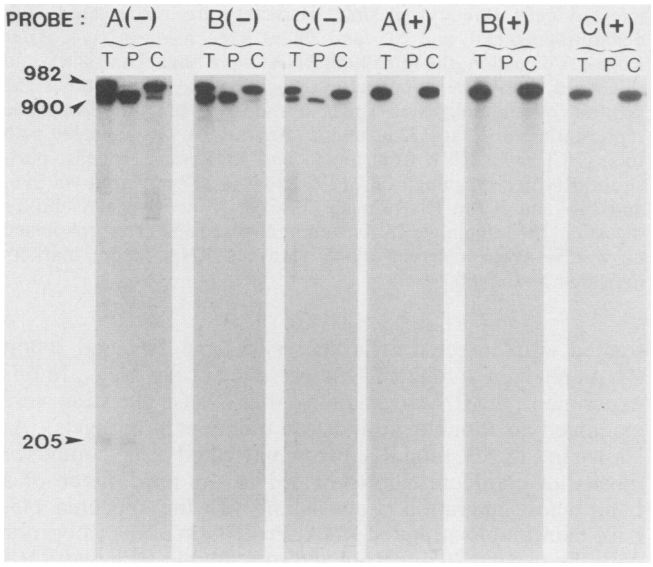

FIG. 4. Separation of cytoplasmic LAC S RNAs by CsCl density gradient centrifugation. LAC-infected cultures were treated with 50 μg of cycloheximide per ml at 4 h p.i., and cytoplasmic extracts were prepared 1 h later. One-third of the extract was treated with denaturing agents, and the total RNAs (T) were recovered as CsCl pellets. The remaining two-thirds of the extract was treated with 1 μg of heparin per ml, and the RNAs were separated into pelleted RNA (P) or banded nucleocapsid RNA (C) on CsCl density gradients. The banded RNAs were then recovered by pelleting followed by phenol extraction. The RNAs were analyzed by Northern blotting by using riboprobes from different regions of the S genome as both plus and minus strands as indicated.

The results in Fig. 4 also show the efficacy of the CsCl density gradients as a method of separating S-mRNAs from S antigenome and genome RNAs. The CsCl pellet fraction was found to contain the vast majority of the 900-nt mRNA, but was often contaminated with small but noticeable amounts of the 982-nt antigenome RNA (also see Fig. 1B and C, lanes 4). Curiously, we could not detect any minus-strand genome RNA in these pellet preparations even on prolonged exposure of the gels. The material which was found as a hypersharp band at 1.31 g/ml contained the vast majority of the antigenome RNA and all of the minus-strand genome RNA. These results indicate that all of the minus-strand genome RNA intracellularly is assembled into nucleocapsids. The small amount of plus-strand antigenome RNA which does not band but pellets through the CsCl density gradient may represent unassembled antigenomes intracellularly, or antigenome nucleocapsids may be more sensitive to the relatively high salt concentrations of the gradient and a small portion may have disassembled during isolation.

Mapping of the 5′ and 3′ ends of the incomplete transcripts. When cycloheximide was used to inhibit protein synthesis in vivo, the 205-nt RNA accumulated. This RNA comigrated with the incomplete transcript made in vitro in the absence of a protein synthesis system entirely or in the presence of such a system whose activity has been inhibited with either cycloheximide or pactamycin. It is therefore curious that when pactamycin is used in vivo to inhibit translation, an incomplete transcript also accumulates, but this transcript would appear to be 5 to 10 nt shorter as judged by PAGE. One possible explanation for these results is that both the cycloheximide- and pactamycin-generated incomplete transcripts were terminated at the same site, but that the 205-nt RNA contained the normal host cell primer of ca. 15 nt at its 5′ end, whereas the pactamycin transcript either did not contain a host primer or contained a shorter primer. This possibility was also suggested by the finding that when the cap analog mGpppG is added to an in vitro reaction containing the reticulocyte lysate synthesis of complete S-mRNA is inhibited and a band which comigrates with the pactamycin-generated transcript in vivo now accumulates (not shown).

To examine the length of the host primer at the 5′ end of the cycloheximide and pactamycin incomplete transcripts generated in vivo, unlabeled 205- and 200-nt RNAs were isolated from a polyacrylamide gel containing in vivo RNA from cycloheximide- and pactamycin-treated cultures, respectively, by using radiolabeled 205-nt RNA made in vitro as a guide. This unlabeled RNA was then used as a template to extend a radiolabeled primer representing nt 38 to 50 as minus-strand DNA with reverse transcriptase. The length of the extended primer can then be used to determine the position of the 5′ ends of the 205- and 200-nt RNAs relative to the 3′ end of the minus-strand S genome RNA. The position of the 3′ end of the minus-strand genome was determined in this experiment by primer extension on antigenome RNA (12). As shown in Fig. 5A, on both the 205- and 200-nt RNAs made in vivo due to cycloheximide and pactamycin treatment, the primer is extended to a ladder of bands at ca. position −15. For comparison, the primer also extended on unlabeled 205-nt RNA made in vitro in the presence of a reticulocyte lysate inhibited with cycloheximide. A similar result is obtained with the in vitro-made 205-nt RNA, except that the ladder is more restricted. As a control, the primer was also extended on RNA isolated from the 200- and 205-nt regions of the gel of the cycloheximide- and pactamycin-treated cultures, respectively, but no primer extension could be detected (data not shown). The difference in mobility on PAGE of the incomplete transcripts which accumulated in vivo due to cycloheximide and pactamycin treatment therefore could not be accounted for by differences in length of the host primer used to initiate these transcripts.

The 3′ ends of these in vivo transcripts were then examined by RNase mapping experiments in which the same unlabeled RNA was used to protect highly radiolabeled riboprobes from RNase digestion. Since the 205-nt RNA (estimated by PAGE) contains ca. 15 nontemplated nt at its 5′ end, we would expect that this RNA would be terminated at ca. position 190. To precisely determine the 3′ ends of these transcripts, two experiments were done. In the first experiment, an SP6 plasmid containing nt 1 to 195 in the negative orientation was linearized with *Hinc*II and transcribed in vitro so that a riboprobe containing 20 nt of vector sequence followed by nt 195 to 43 as minus-strand RNA was made (see Fig. 5). This riboprobe was then annealed against total cytoplasmic RNA from uninfected and non-drug-treated virus-infected cells, as well as the 205- and 200-nt RNAs made in vivo due to the presence of the drugs. In this experiment, the RNAs remaining after RNase digestion were examined as double strands on nondenaturing gels. As shown in Fig. 5B, total RNA from untreated cells, composed mostly of complete S-mRNA, led to the appearance of a band whose migration is consistent with the expected 145-base-pair double-stranded RNA. Protection of the riboprobe with the in vivo 205-nt RNA led to a double-stranded RNA which migrated slightly faster, and the 200-nt RNA led to a band which migrated again slightly faster than the band caused by the 205-nt RNA. This experiment indicates that the 3′ end of the 200-nt RNA is before that of the 205-nt RNA and that both are before position 195.

To more exactly map these 3′ ends, the same SP6 plasmid was linearized with *Sau*3A so that only nt 195 to 136 of the

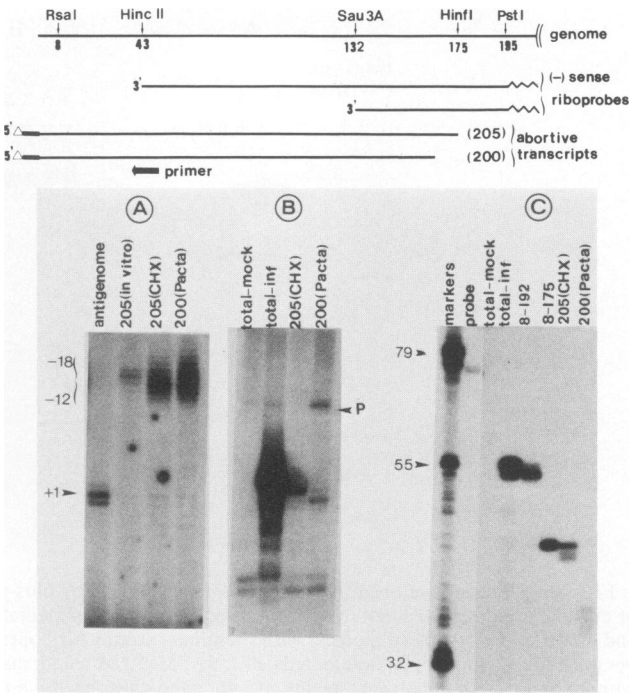

FIG. 5. Mapping of the 5′ and 3′ ends of the 205- and 200-nt in vivo RNAs. (A) 5′ End-labeled oligodeoxynucleotide primer representing nt 50 to 38 as minus-strand DNA was extended with reverse transcriptase (12) on intracellular nucleocapsid RNA which was heat denatured (antigenome), 205-nt RNA made in vitro in the presence of a reticulocyte lysate inhibited with cycloheximide (CHX), and 205- and 200-nt RNA made in vivo in the presence of cycloheximide and pactamycin, respectively. The extended primers were phenol extracted and separated on a 12% sequencing gel. The positions −12 to −18 were determined by counting the ladder background relative to position 1 (determined with antigenome RNA). (B) Riboprobe representing nt 195 to 43 as minus-strand RNA (see drawing at top) was annealed with total CsCl pellet RNA from mock- and LAC-infected cells (the cytoplasmic extracts were not treated with denaturing agents), and 205- and 200-nt RNA made in vivo. After RNase A digestion, the remaining RNAs were phenol extracted and separated on a 10% nondenaturing acrylamide gel. P shows the position of an undigested single-stranded probe. (C) Riboprobe representing nt 195 to 132 as minus-strand RNA was annealed with total CsCl pellet RNA from mock- and virus-infected cells, plus-strand RNAs containing nt 8 to 192 and 8 to 175 prepared via SP6, and 205- and 200-nt RNAs made in vivo. After RNase A and T1 digestion, the remaining RNAs were recovered and electrophoresed on a 12% sequencing gel along with the RNA length markers indicated on the left.

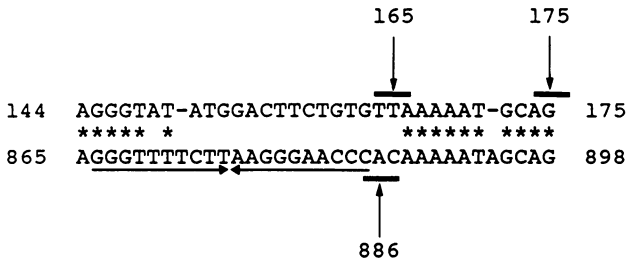

FIG. 6. Homologies between the premature and mature S-mRNA termination regions. The sequences from nt 144 to 175 and 865 to 896 of the S genome segment (2, 4) were aligned to maximize homology, as indicated by asterisks. The heavy bars above refer to the termination sites determined in this paper; the heavy bar below refers to the termination site of the mature LAC or snowshoe hare virus S-mRNA, as determined previously (6, 13). The region of dyad symmetry (886 to 885) is indicated by opposing arrows underneath the sequence.

viral sequence was transcribed as a riboprobe and the protected fragments were analyzed as single strands on a sequencing gel along with other RNAs as length markers. The nondigested riboprobe, which should be 79 nt long, migrated slightly ahead of another 79-nt RNA marker, presumably due to differences in base compositions. When this riboprobe was protected against both RNase A and T1 digestion by intracellular S-mRNA, we expected a fragment of 59 nt, and we found a band which migrated just slightly behind a 55-nt maker. When the riboprobe was protected by an unlabeled RNA representing nt 8 to 192 of the S genome as plus-strand RNA (via SP6), a 55-nt protected fragment was expected and we found a band which migrated just ahead of the 55-nt marker. Total RNA from uninfected BHK cells did not protect any of the riboprobe. When the 205-nt RNA was used to protect the riboprobe, a group of 3 bands 45 to 47 nt in length was found, estimated by counting the ladder bands in the marker lane. When the riboprobe was protected with unlabeled RNA representing nt 8 to 175 as plus-strand RNA (also made via SP6), a band of 42 nt was expected and a single band was found which comigrated with the top band of the triplet formed when the 205-nt RNA was used. The 3′ end of the 205-nt RNA can thus be mapped to position 175 to 177 by this technique. When the 200-nt RNA was used to protect the riboprobe, a triplet band was again found whose length is estimated at 35 to 37 nt relative to the markers. Since this triplet migrated 10 nt faster than that generated from the 205-nt RNA, we estimate the 3′ end of the 200-nt RNA at ca. position 165 to 167. These experiments demonstrate that the difference in mobility between the 205- and 200-nt RNAs is due to the different positions of their 3′ ends.

DISCUSSION

Synthesis of complete LAC S-mRNA in vivo requires ongoing protein synthesis (1, 14, 18). In an accompanying paper (3), we have shown that S-mRNA can be made in vitro, but only in the presence of a translationally active reticulocyte lysate. In the absence of ongoing protein synthesis, the polymerase synthesizes only incomplete transcripts. We investigated the mechanism by which drugs which inhibit translation also inhibit S-mRNA synthesis in vitro. We found that pactamycin, cycloheximide, or puromycin inhibition of the lysate did not inhibit the initiation of S-mRNA synthesis on a capped primer, but caused the polymerase to terminate prematurely. In the case of cycloheximide and pactamycin, the polymerase terminated predominantly at position 175 (the 205-nt RNA), whereas when puromycin was used, the polymerase rarely terminated at this site, but continued on to terminate heterogeneously at a number of sites after ca. position 400. The lack of efficient termination at position 175 in the case of puromycin was ascribed to the mechanism of action of this drug and to the small number of codons between the AUG at position 82 and the termination site at position 175. In the virion-reticulocyte lysate reaction, ongoing protein synthesis is thus required for the polymerase to read through a termination site at position 175 as well as multiple sites after position 400.

The work described in this paper was undertaken to investigate whether ongoing protein synthesis was also required in vivo to prevent premature polymerase termination. We found that cycloheximide inhibition of translation in vivo led to the accumulation of a 205-nt RNA which comigrated with the RNA made in vitro in the absence of translation and which also annealed only to probes representing the first 195 nt of the S genome segment. Very little, if any, 205-nt RNA could be found in cells which were not drug treated. The 205-nt RNA found in cycloheximide-treated cells was not assembled into nucleocapsids, its level was proportional to the amount of drug used, and the finding that its level did not increase with prolonged incubation after drug addition suggested that it is unstable under these conditions. When puromycin was used to inhibit translation, a much smaller amount of 205-nt RNA was found to accumlate than when cycloheximide was used. As mentioned above, puromycin was also inefficient in causing termination at position 175 in vitro. However, since puromycin inhibited complete S-mRNA synthesis in vivo, it is likely that the polymerase has terminated heterogenously after position 175 and that these transcripts are not detectable above the background.

Pactamycin treatment of infected cells also caused an incomplete transcript to accumulate, but this transcript (ca. 200 nt) was unexpectedly found to be slightly shorter than the 205-nt RNA. We determined the positions of the 5′ and 3′ ends of the 205- and 200-nt RNAs so as to be able to account for this difference. We found that both RNAs contain a host primer at their 5′ ends of ca. 12 to 18 nt in length, but that the 3′ ends of the 205- and 200-nt RNAs map to ca. positions 175 and 165, respectively. For technical reasons, it is difficult to demonstrate whether the incomplete transcripts made in vivo are the result of premature termination rather than specific degradation, but specific degradation has been ruled out as the mechanism by which these transcripts accumulate in vitro. Considering that the 205-nt RNAs made in vivo and in vitro contain the same 3′ ends, it seems likely that premature termination is also responsible for the incomplete transcripts found in vivo.

The finding that cycloheximide and pactamycin both apparently cause the polymerase to terminate prematurely, but at two distinct sites which are relatively close to each other, prompted us to examine the sequence in this region, and to compare it to the termination site for complete S-mRNA at position 886 (Fig. 6). Interestingly, the sequence at positions 165 to 175 YAAAAATGCAG is also found as an almost perfect repeat at position 887 to 898 YAAAAATAGCAG (except for the underlined A) but nowhere else in the S genome segment. Just upstream of the mature mRNA termination site lies a region of almost perfect dyad symmetry GGGTTTTCTT/AAGGGAACCC (886 to 895) which can be folded into a relatively stable hairpin loop (Δ G, −8.4

kcal/mol), but the region preceding position 165 does not contain similar structural features. Regions of dyad symmetry which immediately precede termination sites are common features of procaryotic transcription, in which they are thought to function both as polymerase pause sites and as barriers to exonucleolytic attack at the 3′ end of the mRNA (17, 21, 22).

The coupling of transcription to translation is possible in eucaryotic cells only in special cases such as infection with viruses which replicate in the cytoplasm. No such limitation exists in procaryotes, however, in which this coupling has been well documented to control gene expression (17, 22). Two mechanisms which control expression by premature transcription termination in procaryotes, attenuation and polarity, effectively couple transcription to translation via ribosomal masking or exposure of signals in the nascent mRNA. In attenuation of the amino acid biosynthetic operons of bacteria, translation of a leader peptide containing cognate amino acids governs formation of a distal hairpin terminator in a mechanism that requires the progress of the polymerase to be coordinated with that of the ribosome (22). The current view of polarity is that rho-dependent termination sites exist within operons, but these sites are not susceptible to rho as long as the nascent mRNA is concurrently translated (17). The present evidence regarding the requirement for ongoing protein synthesis for complete LAC S-mRNA synthesis suggests that this phenomenon more closely resembles polarity in procaryotes. Termination sites exist within the S-genome segment which are similarly not expressed when transcription is coupled to translation. These termination sites, however, are unlikely to be factor dependent, since efficient termination occurs in vitro with purified virions.

It is tempting to speculate that the sequence YAA AAAT(A)GCAG is involved in transcription termination, but it is also clear that this sequence by itself cannot be sufficient, nor is it unique. For example, the S-mRNA of the closely related snowshoe hare virus also terminates near position 886, whose sequence in this region is identical to that of LAC (as is most of the S genome segment [2]), but the snowshoe hare virus M-mRNA terminates at a site which has no homology to the above sequence and does not contain a region of dyad symmetry just upstream (6). Further, it is hard to imagine how pactamycin and cycloheximide inhibition of translation in vivo would cause the polymerase to terminate at either end of this sequence if this sequence alone were involved. The precise nature of bunyavirus transcription termination signals thus appears elusive but similar to rho-dependent terminators (21, 22). In this system, the termination endpoints do not appear to correspond to any specific sequence requirements and are sometimes multiple, and it is also clear that the sequence contained within the termination region itself cannot alone be responsible for termination. We favor the view that for bunyavirus termination the sequence of the termination region represents only part of the signal and that other features, such as RNA interactions involving the nascent mRNA chain (17, 21, 22), must also play a role. Ongoing protein synthesis could then be responsible for interrupting these interactions which otherwise would lead to termination. Ribosomes, however, would not be able to interrupt the interactions near position 886, since translation is terminated 100 nt upstream.

One of the more puzzling aspects of this work has been the finding that pactamycin causes the polymerase to terminate at ca. position 165 in vivo, but when this drug is used to inhibit the reticulocyte lysate, the polymerase efficiently reads through this site and terminates 10 nt downstream. This latter site is also used predominantly in the absence of the lysate or when cycloheximide is used both in vivo and in vitro. The polymerase can, however, efficiently terminate at position 165 as well in vitro, for example, when mGpppG was used to inhibit the virion-reticulocyte lysate reaction (data not shown). There appear to be two regulatable or nonconstitutive termination sites at ca. positions 165 and 175 which are recognized by the virus polymerase when protein synthesis is inhibited, but which of these sites predominates presumably depends on exactly how translation has been affected. It should be noted in this context that the mature mRNA termination site at position 886 is also regulatable, in that the polymerase must read through this site during antigenome synthesis. It can only be hoped that further investigation of the other features believed to also play a role in the termination signal, such as the possible interactions of nascent mRNA, will provide a basis for the suppressibility of these termination sites.

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