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Characterization of La Crosse Virus Small-Genome Transcripts

JEAN L. PATTERSON AND DANIEL KOLAKOFSKY*

Department of Microbiology, University of Geneva Medical School, 1205 Geneva, Switzerland

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With restriction fragments from DNA clones of the La Crosse virus S genome segment, the 3′ end of the S mRNA was located by S1 nuclease mapping near a polyuridine tract, approximately 100 nucleotides from the end of the S genome. Genome replication in La Crosse virus-infected cells was abolished by the drug cycloheximide, similar to other negative-strand RNA viruses. However, the synthesis of S mRNA could not be detected in cells pretreated with cycloheximide, suggesting that ongoing protein synthesis is required for La Crosse virus genome transcription and replication. Primer extension experiments in the presence of chain-terminating nucleoside triphosphates demonstrated that the 5′ end of the La Cross virus S mRNA begins 10 to 14 nucleotides before the 3′ end of the S genome segment, suggesting that the La Crosse virus S mRNA is initiated on a host primer. A hypothesis consistent with these unexpected findings is presented.

The genome of La Crosse virus (LAC), a member of the California encephalitis serogroup of the insect-transmitted Bunyaviridae (5), consists of three segments of single-stranded RNA of negative polarity (−), each contained within a separate nucleocapsid labeled small (S), medium (M), and large (L) (26). Genetic and molecular studies have led to the following gene assignments: the S segment codes for the N protein, the M segment codes for the two surface glycoproteins, and the L segment, by elimination, codes for the L protein, which is located internally and thought to be part of the viral polymerase (15, 16). In addition to the structrual proteins, bunyavirus-infected cells also contain at least two nonstructural (NS) proteins, NS_s and NS_m, coded for by the S and M genome segments, respectively (14). The complete nucleotide sequences of the S genome of LAC and snowshoe hare virus have recently been determined (2, 6, 9, 28). In both viruses, the S segment has been shown to contain two overlapping open reading frames. The first open reading frame starts with the AUG at position 82 from the 3′ end of the (−) genome (LAC) or position 81 (snowshoe hare virus) and remains open for 235 amino acids, the expected size of the N protein. The second open reading frame starts with two AUGs in tandem at position 101 (LAC) or 99 (snowshoe hare virus) and remains open for 92 amino acids, an estimated size of the smaller NS_s. The bunyavirus S genome segment therefore appears to contain functional overlapping genes.

Little is known about the mechanism of bunyavirus transcription, for two reasons. First, although all bunyaviruses are thought to contain a virion polymerase that presumably starts the infection by primary transcription, this activity has been demonstrated in vitro only with Uukeniemi virus (33) and Lumbo virus (7), and even in these cases the polymerase activity has been too weak to be of much practical value. Thus, only intracellular viral transcripts are available for study. These intracellular mRNAs, however, do not appear to be polyadenylated as they cannot be bound to oligodeoxythymidylic acid-cellulose. It is thus difficult to prepare bunyavirus mRNAs free of genome and antigenome segments from which they differ only slightly in length. For these reasons even the chemical nature of the 5′ and 3′ ends of the bunyavirus mRNA remains unknown.

Recently, the use of synthetic oligonucleotide primers and end-labeled DNA and genome RNA probes has revealed that transcription at the beginning of the 3′ end of the LAC S (−) genome segment is more complicated than expected (29). Both primer extension and S1 nuclease mapping studies have detected the 5′ end of the major message-sized transcript near the precise 3′ end of the (−) genome template and at least seven other minor transcripts, also putative mRNAs, that start internally within a 75-nucleotide-long stretch near this end. Further, with 3′ end-labeled S genome RNA as a probe, three leader RNAs complementary to the exact 3′ end and extending for 75, 95, and 115 nucleotides were also demonstrated. The presence of these leader RNAs suggested that the putative mRNAs that start internally might be the result of reinitiation of the viral polymerase after termination of leader RNA synthesis.

This plethora of S genome transcripts and the presence of overlapping genes in this genome segment have made it difficult to formulate a coherent picture of the mechanisms by which this genetic information is expressed. We have therefore continued our studies by further defining the ends of the S genome transcripts and the mechanism of their synthesis. In this paper we report the mapping of the 3′ end of the S genome mRNA, the finding that both genome replication and transcription are dependent on ongoing protein synthesis, and the finding that the 5′ end of the major mRNA is probably derived from a host cell primer.

MATERIALS AND METHODS

Cells and virus infection. BHK cells were infected and harvested (27) as described by Lindsey-Regnary (H. Lindsey-Regnary, Ph.D. thesis, Emory University, Atlanta, Ga., 1983). Actinomycin D was added at 1 μg/ml 1 h after infection. Cycloheximide was added at 50 μg/ml where indicated, and [^{3}H]uridine was added at 100 μCi per plate at various times as described in the figure legends.

Preparation of nucleocapsids and CsCl pellet RNA. Cytoplasmic extracts of uninfected and LAC-infected BHK-21 cells were prepared and fractionated into CsCl pellet and banded material as previously described (23). The CsCl pellet RNA was adjusted to a concentration of 200 optical density units per ml.

S1 analysis of LAC S clone DNA and LAC CsCl pellet mRNA. The 520-base-pair (bp) *Ava*II restriction fragment corresponding to nucleotides 600 through 982 of the LAC S genome fused to a polycytidylic acid tract and to nucleotides 3616 through 3505 of pBR322 (see Fig. 2) was isolated from

* Corresponding author.

10 μg of LAC S plasmid 4C-26 (9). This fragment, labeled with [α-^{32}P]dCTP, and the Klenow fragment of DNA polymerase I were either left double stranded or strand separated (25) as described in the figure legends. In preliminary experiments (data not shown), it was determined that the slower of the separated strands was the (−) strand since it annealed to LAC mRNA, whereas the faster band did not. The labeled (−) strand or the double-stranded fragments were eluted from the preparative gel, recovered by ethanol precipitation, and mixed with various concentrations of uninfected or LAC-infected CsCl pellet RNA (as described in the figure legends) and ethanol precipitated. For the single-stranded probe, the recovered nucleic acids were raised to a volume of 20 μl of 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–2 mM EDTA–0.4 M NaCl and hybridized at 55°C overnight as described below. The double-stranded fragments were raised to a volume of 20 μl of 40 mM PIPES (pH 6.4)–1 mM EDTA–0.4 M NaCl–80% formamide and hybridized at 50°C. After hybridization, 0.2 ml of ice-cold S1 nuclease buffer (0.28 M NaCl, 0.05 M sodium acetate [pH 4.6], 4.5 mM ZnCl, 2 mM EDTA, and 10 μg of tRNA per ml) containing 500 U of S1 nuclease per ml was added (4), and the reactions were incubated for 45 min at 37°C. The reactions were then phenol extracted, and the remaining nucleic acids were recovered by ethanol precipitation and electrophoresed on a 7 M urea–8% polyacrylamide gel (37).

Primer extension. A dodecanucleotide that corresponded to nucleotides 136 to 147 of the (−) genome was extended with reverse transcriptase in the presence of dideoxynucleotide triphosphates as previously reported (29), except that electrophoresis was carried out for a longer time on an 8% polyacrylamide gel.

RESULTS

Location of the 3′ end of the LAC S mRNA. Annealing studies with radiolabeled virion (−) genome RNA and total intracellular viral RNA have demonstrated both full-length and almost full-length transcripts of the S genome (10, Lindsey-Regnary, Ph.D. thesis). The full-length transcript was found entirely in nucleocapsids that band in CsCl density gradients, whereas the almost full-length transcript was found in the pellet of the gradient (Lindsey-Regnary, Ph.D. thesis). T1 oligonucleotide mapping studies showed that the smaller-than-full-length transcript did not protect the 5′ end of the (−) genome from nuclease digestion, suggesting that mRNA synthesis terminated before the end of the S genome template (12). As mentioned above, the LAC S genome segment has been cloned and sequenced, and the 5′ ends of numerous (+) transcripts have been mapped near the 3′ end of the (−) genome template (29). We therefore next mapped the 3′ end of the S mRNAs more precisely with S1 nuclease. The LAC S genome segment is 982 nucleotides long. To map the 3′ end of the S mRNA, 3′ end-labeled DNA restriction fragments that began at positions 600 and 736 in the viral DNA sequences and extended past the end of the S genome into the pBR322 vector sequences were used to examine the mRNA (CsCl pellet) from both uninfected and virus infected cells (Fig. 1). The results of these experiments using both double- and single-stranded DNA starting at position 600 as probes show that a single 286-bp band was obtained only when infected cell CsCl pellet RNA was used (Fig. 2, lanes 4, 5, and 9). The probe starting at position 736 confirmed this result, placing the end of the S mRNA 150 nucleotides away from the start of this probe (data not shown). These results therefore place the 3′ end of the S mRNA at approximately position 886 on the S genome segment.

A noteworthy feature of the sequence at position 886 is the polyuridine tract 3′ (−) GUUUUU (nucleotides 886 to 892), which is similar to other (−) RNA virus termination-polyadenylation signals that also contain a U run 5 to 7 nucleotides long (18, 34, 35). It is therefore curious that LAC S mRNA is apparently not polyadenylated. It should be noted, however, that the absence of a polyadenylic acid tail on the S mRNA has not as yet been examined directly; therefore, the inability of bunyavirus mRNA to bind to oligodeoxythymidylic acid columns may be due to the shortness of the polyadenylic acid tail rather than its complete absence.

Figure 2 also demonstrates the efficacy of the CsCl density gradient technique in preparing mRNA free of genome RNA. The arrow on the right-hand side of Fig. 2 marks the position where the full-length or antigenome transcripts would be expected. Note that such transcripts cannot be detected by the exposure shown here with either double- or single-stranded DNA as the probe. In other experiments, the full-length transcript band can be detected (see Fig. 4), but only upon overexposure of the mRNA band. Figure 4 also shows what appears to be a backup termination signal, since the just detectable band at approximately position 925 (lower arrow) marks a GUUUUU sequence on the (−) genome.

Attempt to map primary S genome transcripts. The mRNAs used in the above experiments were isolated from cells 12 h postinfection (p.i.), when considerable genome amplification had taken place, and are therefore mostly secondary transcripts. We were also interested in mapping both ends of the primary transcripts, since temporal synthesis of the viral N and G proteins has been reported (Lindsey-Regnary, Ph.D. thesis), and the S genome itself codes for overlapping genes. Primary transcripts of other (−) RNA viruses are usually isolated from virion polymerase reactions in vitro. However, as mentioned above, this polymerase

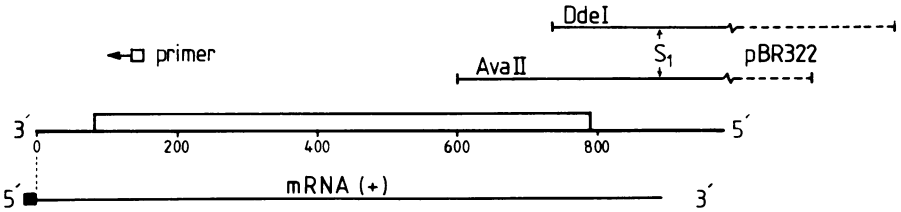

FIG. 1. Organization of the major LAC S genome transcript. The left side of the top line shows the position of the synthetic oligonucleotide used for primer extension, and the right side shows the position of the restriction fragments used to map the 3′ end of the S mRNA with S1. The middle line shows the S (−) genome with its 3′ end at position 1; the bar above the line denotes the N protein-coding sequence. The bottom line shows the structure of the major LAC S transcript deduced from these studies. The box at the left denotes the presumed host primer at the 5′ end of the mRNA.

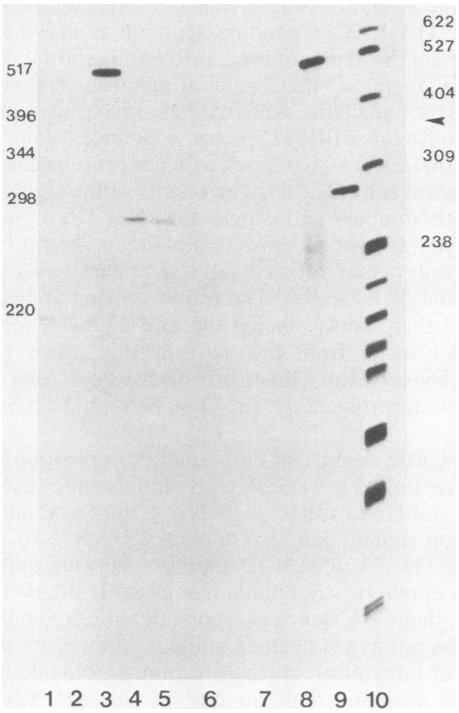

FIG. 2. S1 nuclease mapping of the 3′ end of LAC S mRNA. The 520-bp *Ava*II restriction fragment (nucleotide 600 of LAC S genome to nucleotide 3505 of pBR322, see Fig. 1) was annealed to 2 or 1 optical density units of LAC-infected CsCl pellet RNA (lanes 4 and 5, respectively) and 2 optical density units of uninfected CsCl pellet RNA (lane 2) overnight in 80% formamide at 50°C and digested with S1 nuclease as described in the text. Lane 3 shows the undigested restriction fragment. The same *Ava*II restriction fragment was also strand separated, and the (−) strand was annealed to 2 optical density units of LAC-infected CsCl pellet RNA (lane 9) or 2 optical density units of uninfected CsCl pellet (lane 7) at 55°C overnight without formamide (see the text). Lane 8 shows the undigested single-stranded DNA fragment. Lane 1 shows an *Hin*fI digestion of pBR322, and lanes 6 and 10 are *Msp*I digestions of pBR322. The numbers at the side denote the lengths of the restriction fragment markers. The arrow on the right refers to the region where a full-length transcript would be expected.

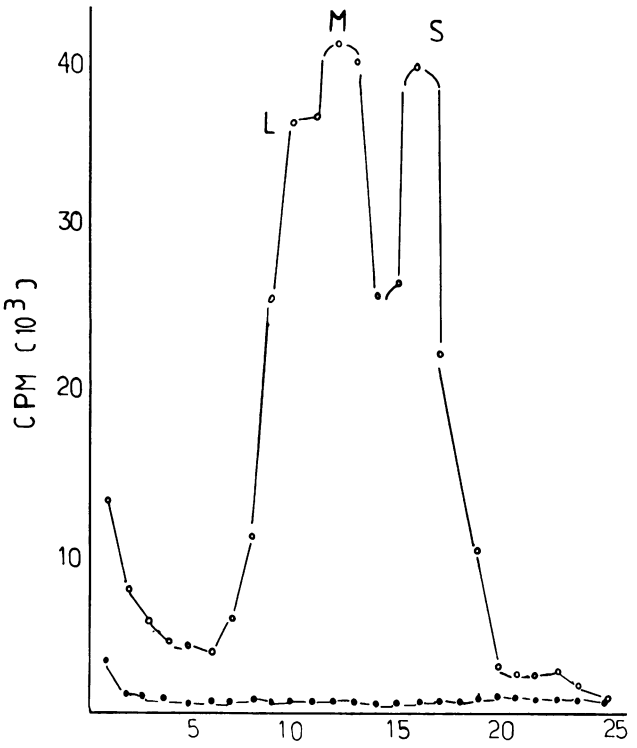

FIG. 3. Effect of cycloheximide on the synthesis of nucleocapsid RNA in BHK cells infected with LAC. Nucleocapsids were isolated from four plates of LAC-infected BHK cells that were either untreated or cycloheximide treated as described in the text. The nucleocapsid bands were diluted with 3 ml of TNE and pelleted by centrifugation for 1 h at 59,000 rpm in the SW 60 rotor. The nucleocapsid pellets were dissolved in 0.2 ml of TNE containing 0.1% sodium dodecyl sulfate, phenol extracted, and sedimented directly on 5 to 23% sucrose gradients containing 100 mM LiCl, 20 mM Tris-hydrochloride (pH 7.4), 1 mM EDTA, and 0.1% sodium dodecyl sulfate for 90 min at 12°C and 59,000 rpm in an SW 60 rotor. The gradients were fractionated by puncturing the bottom of the tube and then counted for radioactivity. Symbols: (○) untreated LAC-infected BHK cells labeled with [^{3}H]uridine 5 to 7 h p.i.; (●) LAC-infected BHK cells treated with cycloheximide at 50 μg/ml 4 h p.i. and labeled identically.

activity has not as yet been demonstrated with purified LAC virions. Primary transcripts of other (−) RNA viruses can also be detected intracellularly by treating cells before infection with inhibitors of protein synthesis (3, 13, 25, 36), which prevents genome replication, but does not affect transcription. Under these conditions, only transcripts from the infecting genomes are made.

To examine LAC primary transcripts by this latter approach, we first examined whether LAC genome replication was indeed inhibited by the drug cycloheximide. One half of a duplicate culture of LAC-infected BHK cells was treated with cycloheximide at 4 h p.i., and both cultures were labeled with [^{3}H]uridine in the presence of actinomycin D for 5 to 7 h p.i. Genome and antigenome nucleocapsids were then isolated by banding in CsCl density gradients, and their RNAs were examined by sedimentation in velocity sucrose gradients. Cycloheximide treatment completely abolished the synthesis of genome and antigenome nucleocapsids (Fig. 3), whereas the control culture showed the expected trisegmented pattern. This experiment, however, did not exclude the possibility that genome-length RNAs were synthesized in the presence of the drug, but were simply not encapsidated because of the lack of available nucleocapsid protein. Under these conditions, the genome-length RNA would be expected to pellet through the CsCl gradient. To control for this possibility, replicate cultures of LAC-infected cells were treated with cycloheximide at 2, 4, and 6 h p.i., and the 3′ ends of the LAC transcripts in the CsCl pellet RNA from these cultures (harvested at 8 h p.i.) were examined by S1 mapping as above. The results of this experiment (Fig. 4) demonstrated that full-length transcripts or antigenomes, which are barely detectable in the untreated control culture (Fig. 4, upper arrow in lane 3), did not increase upon addition of cycloheximide from 2 to 6 h p.i. (Fig. 4, lanes 4 to 6). Thus, genome-length transcripts, i.e., genome replication, in LAC-infected cells is dependent on ongoing protein synthesis similar to that in other (−) RNA viruses.

Figure 4 also demonstrates the effect of cycloheximide at various times after infection on the steady-state concentration of mRNA at harvest time. Note that the addition of the drug at 2 h p.i. led to a dramatic decrease in the amount of intracellular mRNA at 8 h p.i., but that the addition of the drug at 4 or 6 h p.i. had little, if any, noticeable effect. If one

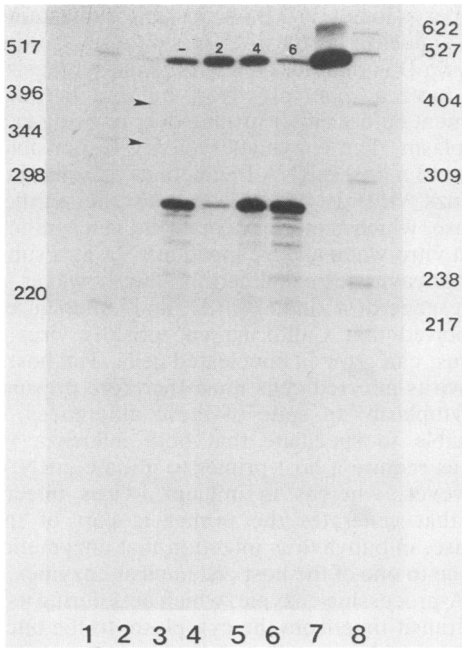

FIG. 4. Effect of cycloheximide on the accumulation of LAC S genome transcripts in the CsCl pellet. The 520-bp *Ava*II restriction fragment was annealed to 20 optical density units of uninfected CsCl pellet RNA (lane 2), untreated LAC-infected CsCl pellet RNA (lane 3), and LAC-infected CsCl pellet RNA from cells treated with cycloheximide at 2 h p.i. (lane 4), 4 h p.i. (lane 5), and 6 h p.i. (lane 6). All cultures were harvested 8 h p.i. Lane 7 shows the undigested restriction fragment. Lane 8 shows an *Msp*I digestion of pBR322, and lane 1 shows an *Hin*fI digestion of pBR322. The numbers alongside refer to the lengths of the restriction fragment. The upper arrow shows the expected position of the full-length transcripts; the lower arrow marks approximately position 925 on the (−) genome. The figures above lanes 3 to 6 denote the time after infection when cycloheximide was added.

assumes that the absence of ongoing protein synthesis does not affect mRNA synthesis, as is the case for other (−) RNA viruses (31, 39), then the results in Fig. 4 would suggest that all S genome amplification has already taken place by 4 h p.i. However, previous experiments have demonstrated that S genome replication under these conditions continues at maximal rates well past 4 h p.i. (Lindsey-Regnary, Ph.D. thesis). In addition, all attempts to detect primary transcripts in LAC-infected cells pretreated with cycloheximide by the above technique with both single- and double-stranded DNA as probes have yielded only negative results. These negative results suggest that either the intracellular primary transcripts are below the level of detection with end-labeled probes or that ongoing protein synthesis is also required for transcription. While this work was in progress, Abraham and Pattnaik (1, 30) reported that they also failed to detect primary transcripts in both Bunyamwera and Akabane virus (two other members of the bunyavirus family)-infected cells that had been pretreated with cycloheximide. More importantly, they demonstrated that ongoing protein synthesis is clearly required for secondary transcription. The above results with LAC-infected cells are consistent with these findings.

Reexamination of the precise 5′ end of the major S mRNA. The curious and unexpected finding that ongoing protein synthesis in bunyavirus-infected cells is apparently required for transcription as well as genome replication led us to reexamine the 5′ ends of the LAC S mRNA more precisely. If ongoing protein synthesis in LAC-infected cells is, indeed, required for primary transcription (see below), then this requirement must be for a host function, as no viral mRNA is present in LAC-infected cells before primary transcription. The only other (−) RNA virus that is known to require a host function for mRNA synthesis is influenza virus. In this case, mRNA synthesis is initiated on a primer derived from a host cell mRNA (19, 32).

As mentioned above, we have previously examined the 5′ ends of the S genome mRNAs by both S1 mapping and primer extension. Both techniques detected the major transcript at or near the precise 3′ end of the S genome template. The primer extension experiment was also carried out in the presence of dideoxynucleotide triphosphates so that the nucleotide sequence could not be read directly from the gel. This experiment (29) precisely mapped the 5′ ends of the minor transcripts at positions 74 to 123 with reference to the cloned S genome, but the sequence within the first 30 to 40 nucleotides of the major transcript was too weak to read. Furthermore, relative to restriction fragment markers, the major transcript appeared to be slightly longer (156 to 160 bp) than predicted from the S genome clone (147 bp).

We have therefore repeated this experiment with the same 5′ end-labeled primer (positions 147 to 136), except that more radioactivity was used and the sequence gel was electrophoresed longer to better display this region (Fig. 5). The dots beside the sequence ladders in this figure denote the predicted sequence at the 5′ end of the major S transcript as determined from the cloned DNA. Note that the dideoxynucleotide triphosphate sequence obtained from the CCCC at positions 61 to 64 to the C at position 2 is exactly that expected, except for ambiguities at positions 4, 22, and 33, which may be due to the limited extension of this primer on uninfected cell RNA (Fig. 5, right-hand T lane). However, note that the extended primer does not stop at position 1 [the precise 3′ end of the (−) genome template], but continues beyond the end of the (−) template and stops heterogeneously at positions −10 to −14 (just above the 154-bp *Hin*fI marker). The sequence between positions +1 and −10 is uninterpretable; the A and G lanes contain a band at every position, whereas the C and T lanes are too weak to read. Such a result is consistent with a heterogeneous sequence at positions +1 to −10. These results suggest that the first 10 to 14 nucleotides at the 5′ end of this mRNA cannot be coded for by the virus genome and that they are heterogeneous both in sequence and length, similar to the 5′ ends of influenza virus mRNAs (11, 22, 32). It therefore appears that the major LAC S mRNA initiates on a 10- to 14-nucleotide-long primer, presumably of host origin.

DISCUSSION

The work reported in this paper presents two unexpected findings. Ongoing protein synthesis in LAC-infected cells is required for mRNA synthesis and for genome replication, as recently shown for two other bunyaviruses, Bunyamwera and Akabane viruses (1, 30). Further, the 5′ end of the major S genome mRNA extends approximately 10 to 14 nucleotides beyond the exact 3′ end of the (−) template, presumably on a host primer.

The finding of Abraham and Pattnaik that secondary transcription is inhibited by cycloheximide is clearly the strongest of the evidence cited in this regard, since the amount of mRNA measured here is large relative to primary transcription and well within the limits of the techniques used. The evidence presented by both ourselves and Abra-

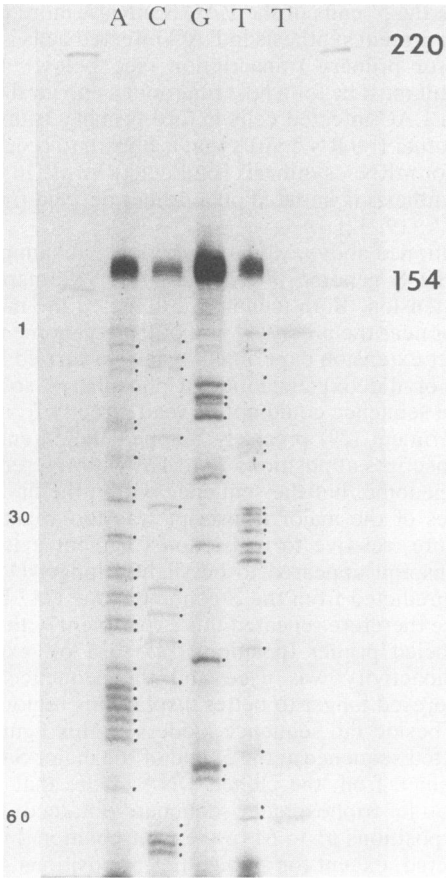

FIG. 5. Dideoxy sequence of the 5′ end of the major S mRNA. The 5′ end-labeled duodecamer (see text) was primer extended on uninfected (right hand T lane) or LAC-infected CsCl pellet RNA (four left hand lanes) as described in the text in the presence of individual dideoxynucleotide triphosphates. Lanes surrounding the sequence are *Hin*fI digestions of pBR322. The numbers on the right-hand margin refer to the lengths of the restriction fragment markers. The sequence lanes are marked with their appropriate dideoxynucleotide added, and the dots to the right of the ladder bands show the sequence expected at the 5′ end of the major S mRNA. The numbers on the left-hand margin refer to the nucleotide position from the 3′ end of the S (−) genome.

ham and Pattnaik (1, 30) concerning the requirement for ongoing protein synthesis for primary transcription must be considered more tentative, since the relative amount of primary transcripts expected is unknown; for example, if primary transcription were limited to 1 mRNA per cell, this probably would not have been detected. Nevertheless, should the requirement for ongoing protein synthesis for primary transcription be confirmed with even more sensitive probes, this requirement must therefore be for a host function, presumably a host protein, as no viral mRNA is present in the infected cell before primary transcription.

This host function must be unstable, as its synthesis is continually required, because it is either constantly turning over in or being exported from the cytoplasm. Thus, the second unexpected finding, that the major LAC S mRNA appears to start on a host primer, possibly like influenza mRNA, may offer a clue to the mechanism of bunyavirus transcription. In the case of influenza virus infections, mRNA synthesis takes place in the nucleus (20, 21) and uses as a primer the 5′ capped end of a recently made host mRNA 10 to 15 bases long (19, 32). A possible explanation for the fact that influenza virus mRNA synthesis is sensitive to actinomycin D is that host mRNAs, once synthesized in the nucleus, have a relatively short half-life in this cellular compartment before either turning over or being exported to the cytoplasm. The enzymatic activity responsible for the generation of a host mRNA fragment as a primer in the case of influenza virus is clearly viral, as the purified virion polymerase, which is primer dependent, can synthesize viral mRNA in vitro when given capped mRNA as a substrate (8, 38). All bunyaviruses examined to date, however, replicate in the presence of actinomycin D, and Goldman et al. (17) have reported that California encephalitis virus, another bunyavirus, can grow in enucleated cells. The host function in bunyavirus-infected cells must therefore presumably act in the cytoplasm. In spite of these differences, it is not unreasonable to speculate that both influenza virus and bunyavirus require a host primer to initiate mRNA synthesis. However, whereas in influenza virus infections the enzyme that generates the primer is part of the virion polymerase, in bunyavirus infection that enzymatic activity may be due to one of the host cell nuclear enzymes, possibly an mRNA-processing enzyme, which acts during its relatively short transit time from the cytoplasm to the nucleus.

Although highly speculative, this hypothesis is testable. For example, as a (−) RNA virus, LAC should contain a virion polymerase since the host cells presumably do not contain enzymes capable of transcribing nucleocapsids. The fact that this activity has not as yet been demonstrated in vitro may, like the influenza virion polymerase, simply reflect its primer dependence for initiation. However, the LAC virion polymerase may in addition require uninfected cell extracts to provide the putative host functions.

LAC-infected cells have also been shown to contain three S genome leader RNAs (29). We have previously suggested that these leader RNAs may be involved in allowing the viral polymerase to reinitiate transcripts internal to the 3′ end of the (−) genome template, creating mRNAs whose 5′ proximal AUG would start the NS_s open reading frame. In the light of the above discussion, the LAC leader RNAs may also represent abortive replication events in the absence of sufficient N protein to allow genome and antigenome synthesis, analogous to vesicular stomatitis virus defective interfering particle polymerase activity in vitro (23). In this case, these leader RNAs, unlike the mRNA, should be initiated independently at the precise 3′ end of the genome. Experiments to test this hypothesis are now in progress.

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