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Concurrent Sequence Analysis of 5′ and 3′ RNA Termini by Intramolecular Circularization Reveals 5′ Nontemplated Bases and 3′ Terminal Heterogeneity for Lymphocytic Choriomeningitis Virus mRNAs

BARBARA J. MEYER* AND PETER J. SOUTHERN

Department of Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

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We have used a technique of RNA circularization coupled with polymerase chain reaction amplification for simultaneous analysis of the 5′ and 3′ termini of subgenomic mRNAs derived from the S RNA of lymphocytic choriomeningitis virus during an acute infection of BHK cells. These mRNAs possess 1 to 7 nontemplated nucleotides of apparently random sequence at their 5′ ends. The predominant mRNA species have 4 or 5 nontemplated nucleotides. The 5′ termini of the mRNAs also have properties consistent with the presence of a 5′ cap structure. The 3′ termini of the mRNAs lack poly(A) tails, and we have shown that transcription termination occurs at heterogeneous positions within the intergenic region of the S RNA. The identification of several distinct termini in the vicinity of a putative stem-loop structure in the RNA templates suggests that transcription termination may be mediated by a structural signal rather than a precise sequence signal.

The genome of lymphocytic choriomeningitis virus (LCMV) consists of two single-stranded RNA segments, designated L and S, that are both organized in an ambisense coding arrangement (29, 30, 34) (Fig. 1). Two primary translation products are derived from each genomic RNA segment via the synthesis of subgenomic mRNAs. The coding regions are separated by short intergenic regions that have the potential to form significant stem-loop structures (1, 30). On the basis of length estimates and hybridization studies (30, 31), the mRNAs are thought to terminate within the intergenic regions. This organizational scheme suggests a simple model for temporal regulation of viral gene expression whereby, in the newly infected cell, transcription of the incoming genomic L and S RNAs by the LCMV RNA-dependent RNA polymerase would produce mRNAs for the L protein (part or all of the viral polymerase) and the nucleocapsid protein (NP), respectively (12, 13). Once these proteins begin to accumulate, then replication would proceed via the synthesis of full-length genomic complementary RNAs. These full-length RNAs serve both as replication intermediates in the formation of newly synthesized genomic RNAs and as templates for subgenomic mRNA synthesis.

At the present time, there is only limited information available concerning the regulatory processes that discriminate between the initiation of replication and initiation of transcription and the mechanism of transcription termination that generates subgenomic mRNAs. From the positions of translation initiation and termination codons, transcription initiation could occur (i) internally on the RNA template, (ii) precisely at the terminus of the RNA template, or (iii) beyond the terminus of the RNA template by using nontemplated bases as a transcription primer. Likewise, for transcription termination, three theoretical locations in which termination could occur exist: (i) at the proximal side of the intergenic hairpin, just beyond the translation termination codons; (ii) within the hairpin; and (iii) on the distal side of the hairpin. Because the LCMV mRNAs are not polyadenylated (33), the presence of a hairpin structure at the 3′ ends of the mRNAs might provide an alternative mechanism of protection from RNase digestion in the cell. We therefore set out to identify the precise 5′ and 3′ termini of the LCMV mRNAs as a first step towards understanding transcriptional control for LCMV. By using a technique that allows simultaneous identification of the sequences at both the 5′ and 3′ termini, we were able to determine the terminal sequences of both the NP and glycoprotein (GP) mRNAs. Additionally, we present evidence which suggests that these mRNAs have 5′ cap structures. Our findings for the mRNAs derived from the S RNA segment are consistent with independent studies that were performed with different RNA mapping procedures for another arenavirus, Tacaribe virus (15–17, 26), and are highly suggestive of a generalized scheme for arenavirus transcriptional control.

MATERIALS AND METHODS

Virus strain and cell cultures. BHK-21 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. At 50 to 75% confluency, the monolayers were infected with LCMV (Armstrong strain CA-1371) (7) at a multiplicity of infection of 1. Total cellular RNA was extracted 24 h after infection.

Probes. Purified DNA consisting of NP or GP coding sequences (34) or a 450-bp DNA fragment of the rabbit globin gene was used in making probes by the random primer method using digoxigenin-labeled dUTP (Genius System; BMB).

mRNA isolation. RNA was harvested from infected cultures by gently lysing cells in detergent and STE (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, containing 0.75% each of TX-100, Na deoxycholate, and Nonidet P-40), followed by centrifugation through a 20 to 40% CsCl density gradient to separate the S and L genomic and antigenomic RNAs (in nucleocapsids) from the viral mRNAs (26). The pelleted material contained the rRNAs plus the viral and

* Corresponding author.

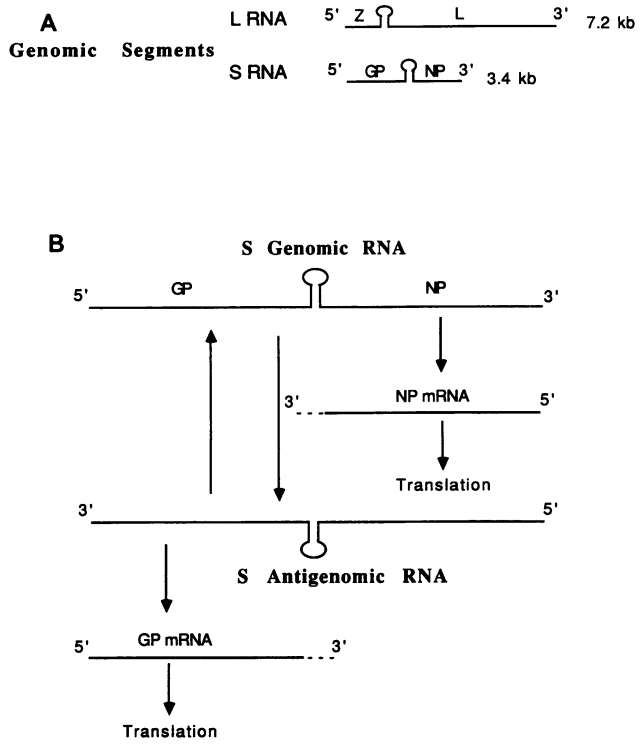

FIG. 1. Replication and transcription scheme for arenaviruses. (A) Diagram of the L (7.2-kb) and S (3.4-kb) genomic segments; coding regions for the Z, L, GP, and NP proteins are shown flanking the proposed intergenic hairpin loop structures. (B) Proposed strategy for LCMV replication, transcription, and translation is shown for the S segment. NP mRNA is transcribed from the S genomic sense viral RNA and translated into the viral nucleoprotein. The full-length complementary S antigenomic sense RNA is synthesized from the S genomic template, which is then transcribed into GP mRNA and translated. The 64 nucleotides between the NP and GP coding regions have the potential to form a hairpin structure with a perfectly matched 21-bp-long stem (see Fig. 6).

cellular mRNAs and was recovered separately from the nucleocapsids that formed a band in the gradient. S and L RNAs were purified from the nucleocapsid band fraction by digestion with proteinase K and phenol-chloroform extraction. The RNAs were resuspended in H_2O and quantitated by spectrophotometry.

Northern (RNA) blot hybridization. RNAs were electrophoresed on 1.5% glyoxal gels (21) and transferred by vacuum onto magnagraph membranes (MSI). The RNAs were detected by hybridization to specific probes and chemiluminescence (Genius System).

Boronate affinity chromatography. RNA from the CsCl gradient pellet fraction (20 μg) plus 15 ng of purified rabbit globin mRNA (Novagen) was either left untreated or decapped with tobacco acid pyrophosphatase (TAP) (18) before application to a 2- to 3-ml column of boronate-derivatized polyacrylamide gel (Affi-Gel 601; Bio-Rad) (35). The column fractions were analyzed by Northern blot hybridization.

Hybrid selection. Either NP or GP DNA derived from the NP or GP coding region (34) was denatured, bound to nitrocellulose filters, and used to hybrid select NP- or GP-specific mRNA (20).

Primer extension. Approximately 50 ng of hybrid-selected NP or GP mRNA was annealed to a 30-fold molar excess of ^{32}P-end-labeled oligonucleotide primers and extended with avian myeloblastosis virus reverse transcriptase (BMB) (22). The primers corresponded to S RNA nucleotide positions 3272 to 3292, 5′-GCTTGCGTCCATTGGAAGCTC (N6), and the complement of positions 89 to 111, 5′-GCAGAG CCTCAAACATTGTCAC (G4).

Ligation, amplification, and cloning of the 5′-3′ junction sequence. NP or GP hybrid-selected mRNA (50 ng) was decapped with 10 U of TAP (Epicentre Technologies) and subsequently circularized by ligation (19) for 1 h at 37°C. After deproteinization and concentration, the RNA was heated in a volume of 13 μl for 2 min at 65°C with 1 μg of random primers (Promega), chilled on ice, and used for cDNA synthesis by incubating in a total volume of 20 μl (50 mM Tris-HCl [pH 8.3] at 42°C, 140 mM KCl, 10 mM $MgCl_2$, 4 mM dithiothreitol, 1.25 mM each deoxynucleoside triphosphate) with 25 to 40 U of RNasin (Promega) and 25 U of reverse transcriptase (BMB) at 42°C (32). After 1 h of incubation, the reaction mixture was heated at 95°C for 3 min and chilled on ice, and an additional 12 U of reverse transcriptase was added for a second 1-h incubation. One twenty-fifth of the cDNA product was amplified in a polymerase chain reaction (PCR) using 0.4 μM each primer in 50 mM KCl–10 mM Tris-HCl (pH 8.3)–1 mM $MgCl_2$–250 μM deoxynucleoside triphosphates. The primers (see Fig. 5) corresponded to nucleotide positions in the genomic RNAs, for S (N or G primers) or L (Z or L primers), as follows: N1, complement of 3272 to 3292, 5′-GAGCTTCCAATGGACGC AAGC; N2, 2845 to 2865, 5′-TACCACACCACTTGCACC CTG; N6, see "Primer extension" above; N7, complement of 1693 to 1714, 5′-CCCAGATCTGAAAACTGTTCAC; G1, 38 to 59, 5′-CTCTAGATCAACTGGGTGTCAG; G3, complement of 678 to 700, 5′-CAGCCACTCCTCATGTATT TCC; G4, see "Primer extension" above); G5, 1522 to 1543, 5′-GTTGTGGTGCATTTAAGGTGCC. The cycle conditions were as follows: 95°C, 7 min; 85°C until 2 U of AmpliTaq DNA polymerase (Perkin Elmer) was added to each reaction mixture; 54°C, 3 min; 40 cycles at 94°C for 1 min, 54°C for 1.5 min, and 72°C for 1.5 min; and 72°C for 10 min. The PCR products were separated by electrophoresis, and DNA bands were recovered by using Gelase (Epicentre Technologies).

Restriction fragment length mapping. The NP and GP PCR junction fragments were cut asymmetrically with *Fsp*I and *Xba*I, respectively; end labeled by a replacement or fill-in reaction with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP (32); and electrophoresed on a 6% denaturing polyacrylamide gel, adjacent to a dideoxy sequencing ladder of a cloned NP PCR junction fragment.

Sequencing. Plasmid preparations of individual PCR junction fragment clones were sequenced by the Sequenase Version 2.0 DNA Sequencing System (USB). To reduce artifact bands from the sequencing reactions, the products were extended with terminal deoxynucleotidyl transferase (BMB) (9) before electrophoresis.

RESULTS

Preparation of NP and GP mRNAs. Analysis of the 5′ termini of LCMV mRNAs is dependent on effective separation between mRNAs and genomic and antigenomic RNAs of the same polarity (Fig. 1). Since LCMV mRNAs lack 3′ poly(A) tails (33), the mRNAs were separated away from genomic-sized RNAs in nucleocapsid structures by CsCl density gradient centrifugation. Nucleocapsids formed a

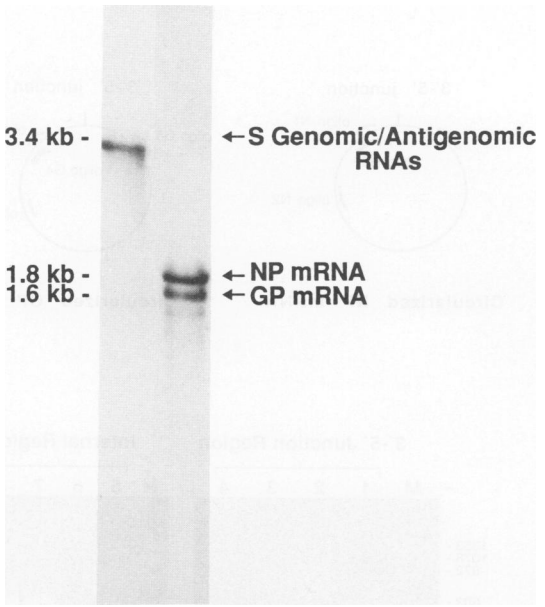

FIG. 2. Separation of LCMV mRNAs from genomic and antigenomic RNAs. Samples of the CsCl gradient-separated fractions of viral nucleocapsid S RNAs (band) and NP and GP mRNAs (pellet) were examined by Northern blot hybridization by using mixed NP and GP probes. The 3.4-kb nucleocapsid S RNAs (left lane) contain both genomic and antigenomic S RNA (0.2 μg loaded). Right lane, 18 μg of pellet fraction RNA containing the NP (1.8-kb) and GP (1.6-kb) mRNAs.

band in the gradient, while all other intracellular RNAs were pelleted (26). There was no detectable cross-contamination between the viral S genomic-length and NP and GP mRNAs from the band and the pellet fractions, respectively, when samples of each fraction were analyzed by Northern blot hybridization (Fig. 2). The NP and GP mRNAs were subsequently separated from each other and the remaining RNAs in the pellet fraction by hybrid selection procedures (20) (data not shown).

Primer extension to map 5′ termini. Samples of NP and GP mRNA, obtained by hybrid selection, were annealed with labeled, sequence-specific oligonucleotide primers and incubated with reverse transcriptase to generate primer extension products (22). By comparison with sequence ladders generated from NP or GP DNA (see below), the primer extension products were 1 to 7 nucleotides longer than the genomic and antigenomic templates (Fig. 3). The predominant species, for both NP and GP mRNAs, were 4 or 5 nucleotides longer than their respective templates.

Detection of 5′ caps. It has been assumed, but not previously demonstrated, that the LCMV mRNAs possess 5′ caps, and we set out to investigate this issue with two independent experimental approaches. Capped RNAs are known to interact with boronate groups by virtue of a positive charge adjacent to two *cis* diols which are present in the cap structure (35). Under conditions of high salt, binding is specific for mature caps which contain a positively charged m^7G residue (35). We therefore established conditions for boronate affinity chromatography using a boronate derivatized polyacrylamide gel (Affi-Gel 601; Bio-Rad) and RNAs from the pellet fractions of CsCl gradients. As a control to monitor the specificity of binding to the column matrix, purified capped globin mRNA (8, 18) was mixed with

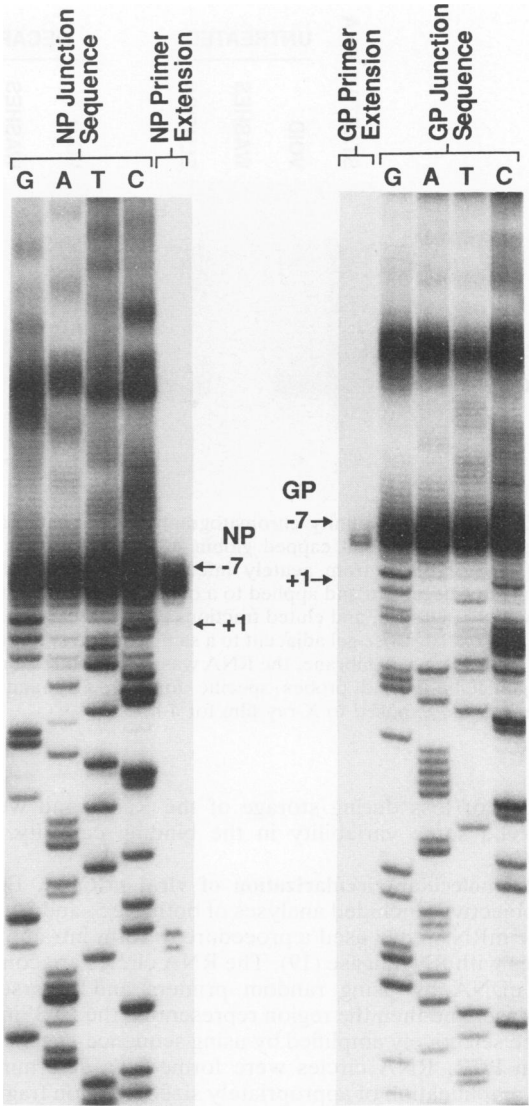

FIG. 3. Mapping the 5′ termini of the NP and GP mRNAs by primer extension. Hybrid-selected NP and GP mRNAs were annealed to ^{32}P-end-labeled primers N6 and G4, respectively (see Fig. 5), and analyzed by primer extension. Lengths of the products were determined by comparing them with adjacent sequencing ladders of either NP or GP PCR 5′-3′ junction fragments with N6 or G4 primers, respectively (see Fig. 5), and read from the 3′ (bottom of gel) to the 5′ (top of gel) terminus for both NP and GP. The sequence is distinct up to the +1 at the arrows, which corresponds to the 5′ terminal nucleotide of the S genomic or antigenomic templates.

the viral RNA samples before loading onto the columns. Part of the viral RNA/globin mRNA mixture was also treated with TAP to remove cap structures (8, 18) prior to the chromatography. The results show that approximately 50% of the untreated starting RNAs were specifically retained by the boronate affinity gel and that predigestion with TAP effectively eliminated all binding to the column (Fig. 4). The finding that NP and GP mRNAs partitioned with the capped globin mRNA in these experiments is consistent with the interpretation that the viral mRNAs possess 5′ caps. The failure to obtain quantitative binding for either the viral or globin mRNAs remains unexplained, but there may be cap

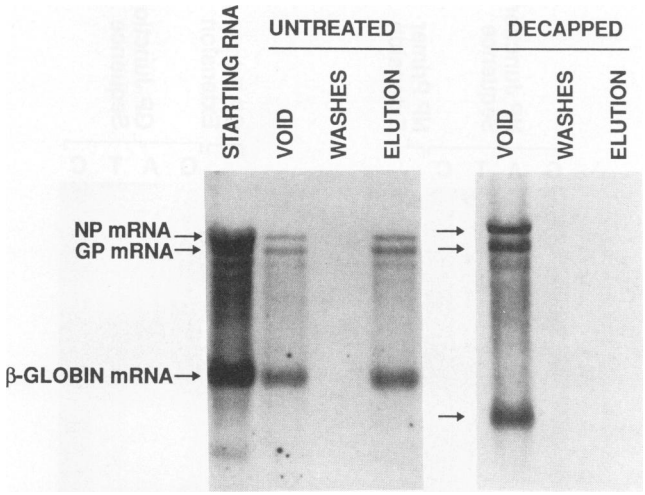

FIG. 4. Boronate affinity chromatography of untreated and decapped mRNA. Purified capped globin mRNA (15 ng) and CsCl pellet RNA (20 μg) from acutely infected cells were either left untreated or decapped and applied to a column of Affi-Gel 601. The void volume, washes, and eluted fractions were electrophoresed on a 1.5% glyoxal agarose gel adjacent to a sample of the starting RNA. After transfer to a membrane, the RNA was hybridized to a mixture of digoxigenin-labeled probes specific for NP, GP, and globin sequences and exposed to X-ray film for 4 h.

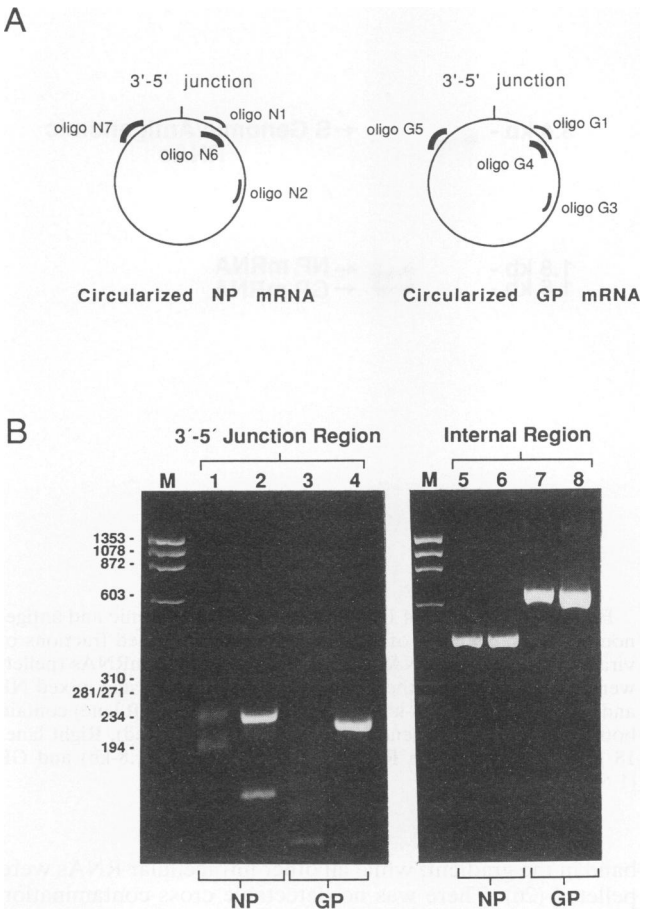

FIG. 5. PCR amplification of the untreated or decapped NP and GP mRNAs after circularization. (A) Diagram of the experimental strategy and the PCR primers used. Primer sets N6 and 7 and G4 and 5 amplify the sequence spanning the 3′–5′ junction region, and the N1 and 2 and G1 and 3 primers amplify internal regions. (B) Ethidium bromide-stained agarose gel showing the PCR products derived from mRNA that had been left untreated (lanes 1, 3, 5, and 7) or decapped (lanes 2, 4, 6, and 8) for NP and GP mRNAs, as indicated. The panel on the left shows the products resulting from PCR synthesis across the 3′–5′ junction with primers N6 and N7 (NP reaction) or G4 and G5 (GP reaction). The panel on the right shows the products from control reactions with primers that span internal regions from oligonucleotide N1 to N2 and G1 to G3 for NP and GP, respectively.

damage or loss during storage of the RNA, and we have observed some variability in the binding capacity of the Affi-Gel.

Intramolecular circularization of viral mRNAs. Because our objectives included analyses of both the 5′ and 3′ termini of the mRNAs, we used a procedure to form intramolecular circles with RNA ligase (19). The RNA circles are converted into cDNA by using random primers and reverse transcriptase, and then the region representing the 5′–3′ junction can be selectively amplified by using sequence-specific primers in PCR. RNA circles were formed (as determined by PCR amplification of appropriately sized junction fragments, approximately 215 bp for NP and 200 bp for GP) with the hybrid-selected NP and GP mRNA preparations only when the starting RNAs had been predigested with TAP (Fig. 5). A second pair of primers was used to amplify an internal region of 448 bp (NP) or 663 bp (GP) of each of the respective cDNA preparations, demonstrating that all of the samples contained cDNA and that successful PCR amplification of the 5′–3′ junction correlated with TAP digestion (Fig. 5). Because TAP is known to eliminate 5′ cap structures by cleavage of the pyrophosphate linkage and leave a 5′ phosphate group (necessary for ligation of RNA termini), these results are also consistent with the presence of caps at the 5′ termini of the NP and GP mRNAs.

Samples of the PCR-amplified 5′–3′ junction were taken directly from the PCR mixtures and sequenced to assess the nucleotide heterogeneity of the whole population of the PCR products. The sequence of the 5′ ends of the NP and GP mRNAs derived from the population of PCR products precisely matched the sequences predicted from S RNAs up to the +1 residue (Fig. 3), the reported terminus of the genomic and antigenomic S RNA templates (31). The unreadable sequence beyond the +1 residue presumably reflected the mixed base composition from the 1 to 7 additional nucleotides at the 5′ ends of the mRNAs. The sequence representing the 3′ ends of the NP and GP mRNAs was distinct until the start of the hairpin region (not shown), reflecting the start of heterogeneity in this region.

To obtain more precise terminal sequence information, the amplified junction fragments were cloned into the pCR1000 plasmid vector (Invitrogen) and sequences across the 5′–3′ junction were determined for a number of independent clones. To assign nucleotides to either mRNA terminus, the junction sequences representing the 3′ mRNA termini were aligned with the genomic S RNA sequence, and at the first position of divergence from the genomic sequence, all subsequent nucleotides were inferred to belong to the 5′ mRNA extensions. However, the formal possibility remains that a nucleotide placed by this method to the 3′ end could actually be derived from the 5′ end. By this approach, all of the

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A 5'                          Hairpin loop                    3'
  CGCACAGUGG.....GCCUCCCUGACUCUCCACCUCUUUCGAGGUGGAGAGUCAGGGAGGC...
   U.........................UGACUCUC
  GU.........................ACUCUCCA
  UC.....................................UCUUUCGA
 ACU.....................................UUCGAGGU
AAAU.................GGGCCUCC
AUCG.....................UCCCUGAC
UGCG.....................................................UCAGGGAG
CACG.....................................................AGGGAGGC

   5'                          Hairpin loop                    3'
B CGCACCGGGG.....GCCUCCCUGACUCUCCACCUCGAAAGAGGUGGAGAGUCAGGGAGGC...
 AUA.....................................................CAGGGAGG
ACUG.....................................................GAGUCAGG
AGCG.....................................................UCAGGGAG
UAUA.....................................................UCAGGGAG
AUUG.....................................................CAGGGAGG
AGGCG....................................................UCAGGGAG
```

FIG. 6. Sequences of individual clones of the NP and GP PCR junction fragments. Above the sequences of each group of clones (A, NP; B, GP) is the reported sequence (boldface type) of the 5′ and hairpin regions of the genomic and antigenomic S RNA templates, respectively. Below each S sequence and to the left of the dotted lines are the nucleotides which represent the nontemplated bases found at the 5′ termini of the individual clones derived from the NP or GP mRNAs. The 3′ mRNA termini determined from these clones are indicated to the right of the dotted lines, and all end within the sequence of the S intergenic hairpin shown directly above. The 4-nucleotide loop of the 21-base paired hairpin stem is underlined.

clones were found to have one or more nucleotides representing 5′ nontemplated mRNA extensions, and these sequences showed considerable variability among the clones (Fig. 3 and 6). The lengths of the 5′ extensions, up to 5 nucleotides beyond the end of the template, were entirely consistent with the primer extension results (Fig. 3). The 3′ termini corresponded to several positions within the hairpin region; the six GP mRNA termini were very tightly clustered, whereas the eight NP mRNA termini were scattered throughout the hairpin (Fig. 6).

Population analysis of 3′ mRNA termini. To assess the representative nature of the 3′ termini in the clones, we devised a strategy to visualize the extent of the mRNA 3′ terminal heterogeneity represented in the population of PCR-amplified junction molecules. The PCR products were digested with a restriction enzyme that cleaved each fragment once asymmetrically (*Fsp*I for NP and *Xba*I for GP) such that all of the size variability derived from the 5′-3′ junctions was included in the larger restriction fragments (Fig. 7A). The restriction fragments were then end labeled with [^{32}P]dCTP and Klenow DNA polymerase, and the reaction products were separated on a standard 6% sequencing gel (Fig. 7B). Several distinct size classes of the longer restriction fragments, containing the 5′-3′ junctions, were identified. Since the 5′ termini had relatively few additional nucleotides, most of the variability in the lengths of these restriction fragments was due to the 3′ terminal heterogeneity. By alignment with an S cDNA sequencing ladder, the clusters of termini were shown to correspond to sites within the hairpin region (Fig. 8). In general, the distal or 3′ side of the hairpin appeared to be the preferred site for transcription termination for both NP and GP mRNAs, as indicated by the upper, most intensely labeled bands in the figure for each experiment, but it was clear from this analysis that other

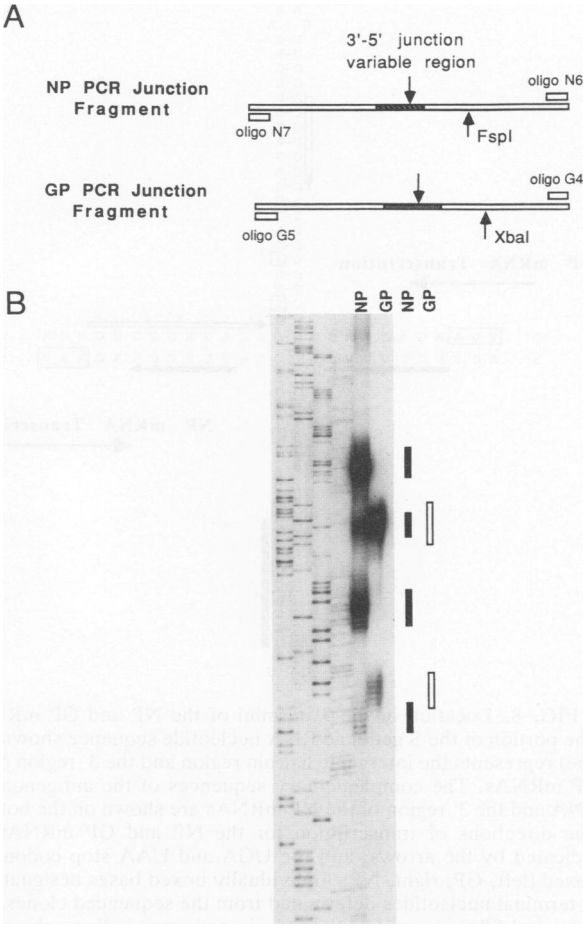

FIG. 7. Restriction fragment length mapping of the NP and GP mRNA 3′ termini represented in the PCR junction fragments. (A) Diagram of the PCR junction fragments and the strategy used in mapping. (B) Electrophoresis of labeled products adjacent to a sequencing ladder of NP DNA. Only the larger labeled restriction fragments containing the variable region are shown; the smaller labeled restriction fragments (*Fsp*I to N6 and *Xba*I to G4) are not in this portion of the gel. Filled rectangles indicate the groups of bands resulting from NP junction region variability, and the open rectangles indicate the groups of bands from GP variability. These bands correlate to mRNA termination sites in the regions indicated by the filled and open rectangles shown on the sequence in Fig. 8.

termination sites were also represented in the population of molecules.

DISCUSSION

We are interested in defining the regulatory mechanisms that influence and discriminate between viral transcription and replication in LCMV-infected cells. In an acute infection of cultured BHK cells, transcription and replication proceed efficiently and progeny virions are released after a lag time of about 16 h. However, late in the acute, noncytolytic infection (72 to 96 h postinfection), there is a marked reduction in transcription, replication, and release of infectious virions, and the cells progress towards a persistent state of infection (34). In a previous study, we observed an accumulation of the viral L protein late in the acute infection (14). The L protein is thought to be part or all of the virus-encoded

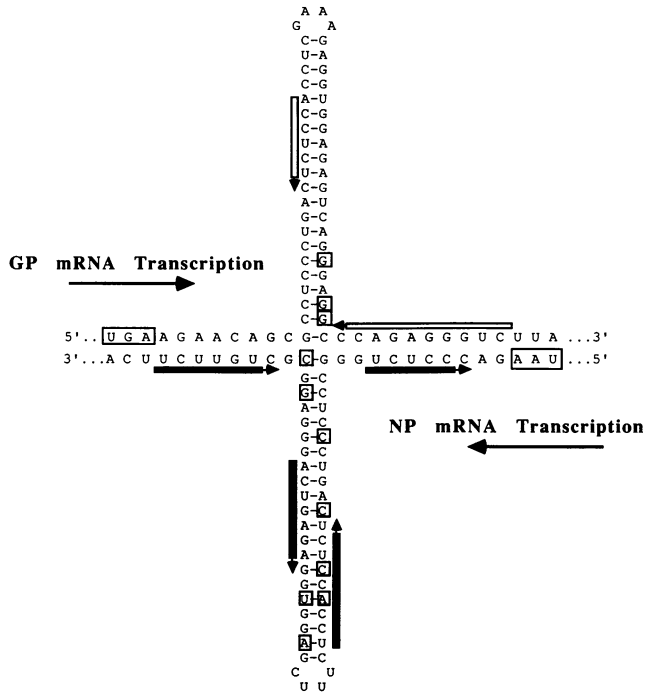

FIG. 8. Location of the 3′ termini of the NP and GP mRNAs. The portion of the S genomic RNA nucleotide sequence shown (top line) represents the intergenic hairpin region and the 3′ region of the GP mRNAs. The complementary sequences of the antigenomic S RNA and the 3′ region of the NP mRNAs are shown on the bottom. The directions of transcription for the NP and GP mRNAs are indicated by the arrows, and the UGA and UAA stop codons are boxed (left, GP; right, NP). Individually boxed bases designate the 3′ terminal nucleotides determined from the sequenced clones. The open and filled arrows on the hairpin sequence indicate the corresponding regions of 3′ transcription termination determined by mapping the labeled restriction fragments shown in Fig. 7. Nontemplated nucleotides at the 5′ end of individual mRNAs would shift these regions 1 to 7 bases in the direction of the arrowheads.

RNA-dependent RNA polymerase, and, at least in acutely infected BHK cells, there is an inverse correlation between the level of intracellular L protein and the viral polymerase activity estimated by the in vitro assay (14). In addition to monitoring polymerase activity, we have characterized the intracellular viral RNAs that arise under different conditions of infection (10, 11, 34). The current study was undertaken to map the 5′ and 3′ termini of subgenomic mRNAs transcribed from the genomic S RNA segment for eventual comparison to similarly sized subgenomic RNAs that are detected in persistent infections.

Our results establish that, in acutely infected BHK cells, the LCMV mRNAs for NP and GP extend beyond the 5′ ends of the genomic RNA templates and possess 5′ terminal structures that are consistent with the presence of a 5′ cap. The extensions of 1 to 7 nucleotides are heterogeneous in sequence composition, and the predominant species appear to have 4 or 5 additional nucleotides. The source of the 5′ nontemplated nucleotides and caps is currently unknown, but three distinct possibilities can be identified. The process of "cap stealing" has been described for influenza viruses (3, 5, 25) and certain bunyaviruses (2, 6, 23), but in these cases the capped 5′ nontemplated extensions are more typically 10 to 15 residues rather than the 1 to 7 detected for LCMV. Alternately, the LCMV polymerase may utilize random short cellular oligoribonucleotides to initiate transcription and may add caps in the cytoplasm by using a virus-encoded activity. Such oligoribonucleotides could be postulated to arise during the normal process of RNA turnover. Finally, the LCMV polymerase complex may be capable of synthesizing short primers in the apparent absence of a template and may then subsequently add the 5′ caps. This general description of the capped nontemplated 5′ ends of the LCMV NP and GP mRNAs agrees very closely with a similar study of Tacaribe virus mRNAs (15). A minor difference between our findings and those of Garcin and Kolakofsky is that the extensions reported for Tacaribe virus were 1 to 4 nucleotides whereas the LCMV extensions were detected in the range of 1 to 7 nucleotides. The convergence of these two independent investigations strongly suggests a general mechanism for initiation of arenavirus transcription, although many details remain to be resolved.

This study also identified the positions of NP and GP mRNA transcription termination. From the positions of the translation termination codons relative to the intergenic region, it was highly likely that the 3′ termini of the subgenomic mRNAs would be positioned in the vicinity of the hairpin structure. One published report for Tacaribe virus, using S1 nuclease mapping, concluded that transcription termination did occur in the intergenic hairpin (17), but the intergenic region for Tacaribe virus is somewhat different from LCMV in that two distinct hairpin structures are postulated. We therefore sought an alternative strategy to map the 3′ termini of the LCMV subgenomic mRNAs because we were concerned that the stable intergenic hairpin structure in LCMV RNA might generate nuclease protection artifacts. The cumulative information derived from our analysis of cloned sequences and the direct analysis of populations of molecules indicates that there is substantial terminal heterogeneity at the 3′ ends of both the NP and GP mRNAs with no apparent consensus termination sequence. This result also emerged from other RNase protection studies of Tacaribe virus transcription (16). The process of transcription termination may then be mediated through RNA secondary structure rather than specific sequence signals, as suggested previously for Tacaribe virus (17). For the majority of LCMV NP and GP mRNAs, it appears that there is a 3′ terminal overlap of approximately 20 nucleotides that arises when the mRNAs each terminate on the 3′ side of the intergenic hairpin. The mechanism that causes transcription termination versus continued RNA synthesis during genome replication is still unknown, but antitermination, perhaps via NP binding to disrupt the hairpin, has been suggested (4, 17). The process of LCMV mRNA termination described here is reminiscent of rho-independent transcription in prokaryotes, in which termination also occurs at several positions 3′ to a hairpin structure (24, 27, 28).

ACKNOWLEDGMENTS

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