Sequence Heterogeneity in the Termini of Lymphocytic Choriomeningitis Virus Genomic and Antigenomic RNAs

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Sequence analysis of lymphocytic choriomeningitis virus L and S RNAs has revealed evidence of heterogeneity within the termini of the genomic and antigenomic RNAs. The RNAs are missing from 0 to 38 bases, show characteristic patterns of deleted nucleotides at both 5' and 3' termini, and often have a nontemplated base at the terminus. The same deletions, at either the 5' or the 3' terminus of the genomic L and S RNAs, are frequently found in the complementary strand of antigenomic RNA, suggesting that RNAs with deleted termini may be recognized as functional templates for replication. RNAs extracted from virions, or viral nucleocapsids isolated from acutely infected cells, are similar in the nature and extent of terminal heterogeneity that have been observed. This finding brings into question the function of the conserved sequences located at the termini of arenavirus genomic RNAs. Our data suggest that, while replication and packaging of the genomic and antigenomic RNA molecules can occur with terminally deleted molecules, mature transcripts may be derived only from full-length templates containing the conserved terminal sequence.

The 3'-terminal sequences of the genomic single-stranded L and S RNAs of lymphocytic choriomeningitis virus (LCMV) are identical for 17 of the first 19 nucleotides (2). This sequence is conserved at the 3' terminus of genomic RNAs for other members of the family *Arenaviridae* (1–4, 8, 11, 15, 16, 20, 27, 29, 30, 34). Furthermore, the sequences at the 3' and 5' termini of arenavirus genomic RNAs are complementary (3, 4, 7, 11, 15, 19, 27, 28, 30, 34), and it has been suggested that the termini may be involved in RNA replication (7, 28), perhaps by representing part or all of a polymerase-binding site. The simplest replication scheme for the ambisense arenavirus RNAs requires synthesis of a full-length complementary (antigenomic) RNA intermediate from a genomic RNA template (4, 11, 12). Sequence complementarity between the 3' and 5' termini of the genomic RNAs would provide a mechanism to preserve the putative binding site at the 3' end of antigenomic templates for initiation of de novo genomic RNA synthesis. Transcription of viral subgenomic mRNAs also initiates at the 3' termini of the genomic and antigenomic RNAs. For the S-derived transcripts, the mRNAs are transcribed with a complete 5'-terminal sequence plus one or more additional nontemplated nucleotides at the 5' end, with respect to the 3' termini of the genomic and antigenomic templates (24). Thus, the conserved sequences at the 3' and 5' termini of the genomic L and S RNAs have a role in both the initiation of replication and transcription, although details regarding these events remain to be resolved.

In order to determine whether the terminal sequences are absolutely required and conserved during LCMV replication, we sequenced the 5' and 3' termini of several individual genomic and antigenomic RNA molecules and examined the termini of RNA populations by RNase protection analysis. The antigenomic RNAs were of particular interest, given the virtual absence of existing terminal-sequence information for these RNAs. The 5' antigenomic terminal sequence has been reported for Tacaribe virus S RNA (13), but 3' antigenomic

sequences have only been inferred from complementarity to genomic RNAs.

Antigenomic and genomic RNAs were obtained by isolating intracellular viral nucleocapsids 24 h after BHK cells were infected, with a virus stock that had been triple plaque purified to ensure homogeneity of the infecting virus (26). To compare the RNA termini from intracellular RNAs with those packaged in virions, we also examined genomic and antigenomic RNAs isolated from purified LCM virions harvested 48 h postinfection (7). The overall experimental approach was based on modifications to our recently published procedure for cloning and sequencing 5'-3' RNA termini contained in ligated, circularized RNA molecules (24). RNA samples were treated with tobacco acid pyrophosphatase (TAP) to remove extra 5' phosphates or 5' caps (23) and leave a 5' monophosphate terminus for ligation. The RNA was circularized by incubation with RNA ligase, and the first strand of cDNA was synthesized across the ligated 5'-3' junction. The remaining RNA in the reaction mixtures was subsequently removed with RNase A to eliminate the possibility that the input RNA could be amplified by *Taq* polymerase in later steps (31).

Single-stranded complementary copies of the genomic and antigenomic RNAs were synthesized by using primers that annealed specifically to one or the other of the RNAs in separate reaction mixtures (Fig. 1A), and the polarity of these cDNAs was assessed by asymmetric (one-sided) PCR amplification (Fig. 1B). In these reactions, a primer either complementary to the anticipated cDNA product or of the same polarity as the cDNA product was used to amplify a portion of each cDNA sample in the presence of ^{35}S-dATP. Primers complementary to the genomic or antigenomic cDNA template (G1 and N1 for S cDNA and Z2 and L2 for L cDNA) would be expected to amplify the region between the primer and the 5' end of the complementary cDNA. When primers of the same polarity as the cDNA are used (G4 and N6 for S genomic and antigenomic cDNAs, respectively, and Z1 and L1 for genomic and antigenomic L cDNAs, respectively), no significant amplification product should be produced. The results of this experiment showed that a PCR product of the expected size was produced with the first, complementary primer (Fig. 1B) whereas no major PCR product was synthe-

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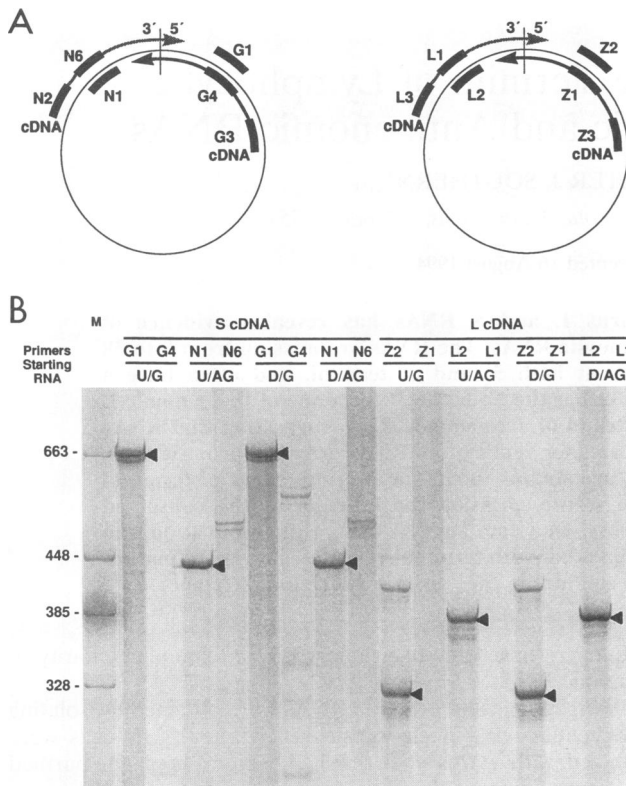
A

B

FIG. 1. Asymmetric PCR analysis to assess cDNA polarity. (A) Location of the oligonucleotide primers used for cDNA synthesis, asymmetric PCR amplification, and standard PCRs (Fig. 2) with respect to the original RNA templates. Positions of the cDNA primers, in a 5'-to-3' orientation, with respect to the sequence of the S or L genomic template were as follows: G3, 700 to 679 (S RNA); N2, 3013 to 3032 (S RNA); Z3, 362 to 342 (L RNA); L3, 6753 to 6774 (L RNA). Primers used for PCR amplification were as follows: G1, 38 to 59 (S RNA); G4, 111 to 90 (S RNA); N1, 3292 to 3272 (S RNA); N6, 3272 to 3292 (S RNA); Z1, 115 to 95 (L RNA); Z2, 38 to 59 (L RNA); L1, 7117 to 7137 (L RNA); and L2, 7137 to 7117 (L RNA). (B) Products from the asymmetric PCRs. Single-stranded cDNAs synthesized from genomic (G) or antigenomic (AG) RNA templates were amplified by using the indicated primers. Following amplification, the products were electrophoresed on a 6% denaturing polyacrylamide gel. The starting RNAs for these reactions had been either treated with TAP (D) or left untreated (U) prior to ligation. Primers G1, N1, Z2, and L2 are complementary to the anticipated cDNA in the reaction mixtures and should generate specific-length products; primers G4, N6, Z1, and L1 are of the same polarity as that of the expected cDNA. Amplification products resulting from the use of primers complementary to the cDNAs present in the reaction mixtures, which amplified the region between the 3' terminus and the 5' terminus of the cDNA template, are indicated (arrowheads). Lane M, markers.

sized in the presence of the second primer, indicating that each cDNA was of the expected polarity and single stranded. These cDNAs were therefore appropriate to determine the terminal sequences of the genomic and antigenomic viral RNAs for both the S and the L segments.

Single-stranded cDNAs (Fig. 1) were amplified in standard PCRs using sequence-specific pairs of forward and reverse primers which spanned the ligated 5'-3' RNA junctions. The amplification products were separated by agarose gel electrophoresis and visualized by staining the gel (Fig. 2). For the S genomic and antigenomic cDNAs, the PCR products migrated

as broad bands in agarose gels and were produced only when the starting RNA had been treated with TAP before ligation. This requirement for TAP treatment indicates that the S genomic and antigenomic RNAs from both the nucleocapsid and the virion must not terminate with 5' monophosphate nucleotides. These termini most likely have either additional phosphates or a cap structure on the 5' nucleotide. We have previously shown that LCMV mRNAs also required TAP treatment for ligation and that the viral mRNAs appeared to terminate with a 5' cap (24).

Amplification of the L cDNAs, unlike that of the S cDNAs, yielded two discrete PCR products (Fig. 2). One PCR product was the size expected for a full-length or nearly full-length ligated template and was generated only when the RNA had first been treated with TAP. A substantial amount of a second, smaller PCR product was also produced, but this product appeared irrespective of TAP treatment. These two products reflected the presence of two distinct 5'-terminal nucleotides on the L RNAs, with the larger one having additional phosphates or a 5' cap structure and the smaller one having a 5' monophosphate terminus. On the basis of size estimates (170 bp) and restriction enzyme mapping studies (data not shown), the smaller L PCR product appears to represent a subset of truncated L RNAs with 10- and 38-base deletions (−10/−38) at their termini, which were detected after the PCR product was cloned and sequenced (see below and Fig. 3).

The S PCR products and the larger of the two L PCR products from the genomic and antigenomic nucleocapsid and virion cDNAs (Fig. 2) were isolated by preparative gel electrophoresis and cloned into pCRII (Invitrogen), and several independent clones were sequenced (Fig. 3). The resulting sequences of the cloned S and L RNAs were similar whether the RNA was derived from nucleocapsids or virions. The sequences revealed that the S and L RNAs were missing from 0 to 38 nucleotides at their termini and that the terminal deletions reflected in genomic clones were frequently the complement of the deletions in the antigenomic clones. The different lengths of the RNA terminal sequences are comparable to the sizes of the original PCR products. In addition to terminal deletions, nontemplated nucleotides were also present at the 5'-3' junctions of the ligated RNAs. RNA degradation does not appear to account for the terminal deletions, since degradation would be unlikely to generate precise complementary deletions on the genomic and antigenomic strands, especially when the deletions are flanked by nontemplated terminal nucleotides. Interestingly, nucleotide changes were also detected within the conserved terminal sequences (Fig. 3) but not in the regions adjacent to the termini (data not shown).

The sequences from the L clones showed that L RNA appears to have two major types of terminal deletions, with one type consisting of short, various-size deletions similar to the S clones and another type that had exactly 10 and 38 bases missing from the 5' and 3' termini, respectively (Fig. 3). These clones also had nontemplated nucleotides at the ligated 5'-3' RNA junctions. The size of the clones with −10/−38 deletions is consistent with the size of the smaller L PCR product in the gel in Fig. 2. Although only the large L PCR product was isolated and cloned, there was apparently some cross-contamination with the smaller PCR product.

To determine whether the deletions in the clones were representative of RNAs existing in the total population, we performed RNase protection analysis on the virion RNA (Fig. 4) (5). Uniformly labeled RNA probes, complementary to the 5' or 3' termini of genomic or antigenomic RNAs of either the S or the L segments, were annealed separately to portions of
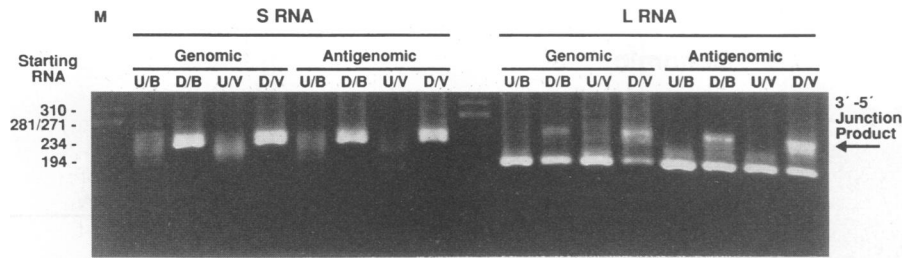
FIG. 2. PCR amplification across the ligated 5'-3' RNA termini. The strand-specific cDNAs assayed by asymmetric PCR (Fig. 1) were amplified with primers that spanned the ligated 5'-3' terminal junctions by using primer pairs G4-N6 for S cDNAs and Z1-L1 for L cDNAs. The starting RNAs were extracted either from nucleocapsids (B) or from virions (V) and were either left untreated (U) or treated with TAP (D) prior to ligation and cDNA synthesis. M, markers.

the same virion RNA stock used for cloning. Since areas of nucleotide mismatches between the probe and protected RNA are also subject to RNase digestion, any nucleotide errors in the viral RNA will result in this analysis slightly underrepresenting the longer RNAs in the population. Results of these experiments (Fig. 4; Table 1) indicated that the RNA termini present in the viral population were deleted to a similar extent as the individually cloned and sequenced nucleocapsid and virion RNAs shown in Fig. 3. The 5' ends of S genomic RNAs and 3' ends of S antigenomic RNAs were primarily full length, while the opposite ends of the RNAs were more variable, as in the sequenced clones. The sizes of the L RNA termini also were similar to the clone data and revealed that a substantial proportion of the RNA population had the same $-10/-38$ deletions as the clones. When the sizes of the protected RNAs were longer than full length, the RNAs must have had extra terminal nucleotides that were complementary to the probe sequence used in those experiments.

Terminal deletions for LCMV have not been reported previously. The presence of deleted molecules in the RNA population may have been overlooked since the original stud-ies identified the sequence of the majority of the RNA population or longest sequence (2, 27–30). For example, when conventional reverse transcription and cDNA cloning experiments with viral RNA templates are used, the observation of 5' sequence heterogeneity in independent clones is more readily explained by incomplete reverse transcription rather than template heterogeneity. Sequence heterogeneity is more easily detected, however, when terminal sequences are examined specifically, as for the viral RNAs in leishmania (33), for hepatitis C virus (17) and in this study.

Our data show that the population of RNA molecules in virions and individually cloned RNAs had deletions in the conserved 19-nucleotide terminal sequence similar to the deletions in the RNAs isolated from nucleocapsids during active viral replication and that these RNAs are also packaged, apparently without selection for full-length molecules. The presence of the same deletions in samples prepared at different times, irrespective of the RNA source, suggests that the deletions were generated by some specific replicative mechanism. The fact that the deletions in both the genomic and the antigenomic RNAs are frequently complementary strongly

S Genomic Sequences

```
        +1                                    3376       Terminal Nucleotides
    5' CGCACCGGGG.......................CCACUGUGCG  3'    Deletions  Additions
                                                          5'    3'
    n  CGCAC..........................CCACUGUG             -2    +G
    n  CGCAC..........................CCACUGUGCG
    n  CGCAC..........................CCACUGUG             -2    +G
    n  CGCAC..........................CCACUGUGCG
    n* CaCAC..........................CCACUGUGCG
    n  CGCAC..........................CCACUGUGCG
    n  CGCAC..........................CCACUGUGCG

    v  CGCAC..........................CCACUG         -4
    v  CGCAC..........................CCACUG         -4
    v  CGCAC..........................CCACUGUGCG
    v  CGCAC..........................CCACUGUGCG
    v     CGGGG...............AAAUGCCU          -5   -15
    v  CGCAC..........................CCACUG         -4    +G
    v* CGCAC..........................CCACUGaGCG     -4
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S Antigenomic Sequences

```
        3376                                      +1
    3' GCGUGGCCCC.......................GGUGACACGC  5'
    n  GCGUG.........................GGUGACAC        -2    +C
    n  GCGUG.........................GGUGACACGC
    n  GCGUG.........................GGUGACACGC
    n  GCGUG.........................GGUGAC          -4
    n  GCGUG...................CCUAGG                -8    +C
    n  GCGUG.........................GGUGACAC        -2    +C

    v  GCGUG.................GAGUUCU                -36    +C
    v  GCGUG.........................GGUGACAC        -2    +C
    v  GCGUG.........................GGUGACACGC            +C
    v* GCGUG................GUUAGU                  -22    +C
    v  GCGUG.........................GGUGACACGC
    v  GCGUG.........................GGUGACACGC            +C
    v  GCGUG.........................GGUGACAC        -2    +C
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L Genomic Sequences

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        +1                                    7220       Terminal Nucleotides
    5' CGCACCGGGGAUCC.......................CCUCGGUGCG  3' Deletions  Additions
                                                          5'    3'
    n  CGCAC.............................CUCGGUG          -2    +G
    n     AUCCUA.........GAUUUC                    -10   -38
    n     AUCCUA.........GAUUUC                    -10   -38
    n  CGCAC.............................UCCUCGG         -4    +G
    n  CGCAC.............................CUCGGUGCG             +ACTG

    v  CGCAC.............................CUCGGUG          -2
    v     AUCCUA.........GAUUUC                    -10   -38
    v     AUCCUA.........GAUUUC                    -10   -38
    v  CGCAC.............................CUCGGUG     -2    +G
    v     UAGGCG.........UCAUCC                     -14   -34
    v  CGCAC.............................CUCGGUG     -2    +G
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L Antigenomic Sequences

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        7220                                        +1
    3' GCGUGGCCCCUAGG.......................GGAGCCACGC  5'
    n     UAGG.............CUAAAG                 -38   -10
    n     UAGG.............CUAAAG                 -38   -10
    n     UAGG.............CUAAAG                 -38   -10
    n     UAGG.............CUAAAG                 -38   -10
    n  GCGUGG......................GGAGCC          -4    +CG
    n     AAUCAA..........AGUUUUU                 -19   -21
    n  GUGG.........................GGAGCCACGC     -2    +UGCGCG

    v  GCGUGG........................AGUUUUU      -19    +CU
    v  GCGUGG........................AGUUUUUC     -18
    v  GCGUGG........................GUAGUU       -22    +C
    v  GCGUGG........................GUAGUU       -22    +C
    v     UAGG.............CUAAAG                 -38   -10
    v  GCGUGG........................AGUUUU       -20    +C
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FIG. 3. Sequences of the terminal nucleotides of the S and L genomic and antigenomic RNAs from individual clones. The 5' and 3' termini of the published sequence (boldfaced) are shown above each group of clones. Sources of RNA: n, nucleocapsid; v, virion. Clones containing a nucleotide change (lowercased and boldfaced) are indicated (★). Although only the terminal several nucleotides of each clone are shown, the adjacent 40 to 50 nucleotides were also sequenced and had no nucleotide changes relative to the published LCMV sequence. The dots in the clone sequences represent the fact that the sequenced termini came from the ends of the same original RNA molecule, although the interior region of the molecule was not entirely sequenced. The numbers of missing bases at the 5' or 3' termini and the nontemplated bases found at the 5'-3' junctions are indicated on the right.
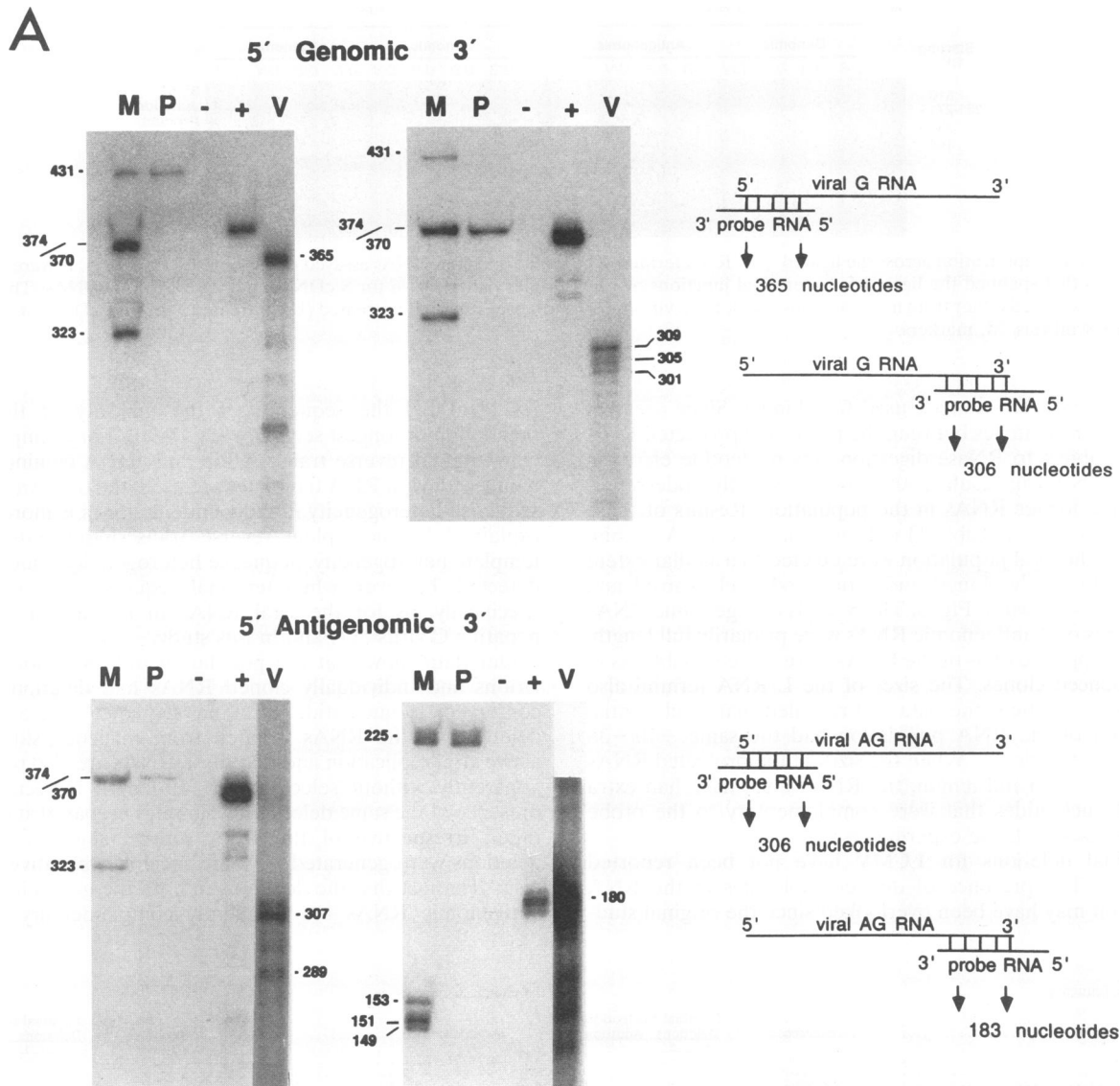
FIG. 4. RNase protection analysis of virion genomic (G) and antigenomic (AG) S and L RNA termini. RNA extracted from virions was annealed separately to RNA probes uniformly labeled with ^{32}P which were complementary to each of the four ends of the S and L RNAs, as shown in the diagrams on the right. After RNase digestion with RNase A and T_1, products were electrophoresed on 6% denaturing polyacrylamide gels, and the sizes of bands were determined on the basis of the migration of adjacent RNA markers uniformly labeled with ^{32}P. (A) S RNA probes; (B) L RNA probes. Lanes: M, RNA markers; P, probe alone (P$\star$, probes were not present on the section of the gel shown); −, probe after RNase digestion; V, viral RNA after RNase digestion. Differences between the sizes of the observed major protected bands and the predicted products are listed in Table 1.

suggests that, once made, the deleted molecules are subsequently maintained by replication. Replication is also suggested by the presence of genomic and antigenomic molecules which have complementary deletions internal to complementary nontemplated nucleotide additions at the terminus. For Tacaribe virus, the finding of a nontemplated G at the 5′ end of the genomic and antigenomic S RNAs has led to the proposal of a novel "primer-slippage" mechanism for the initiation of Tacaribe virus RNA replication (13, 14). However, for LCMV, the nontemplated bases on the genomic RNAs are usually complementary to those on the antigenomic RNAs, rather than identical. The complementarity of the extra bases for LCMV is more consistent with a model in which a

nontemplated nucleotide is added to the terminus of one strand during replication and is then copied onto the other strand. Thus, once synthesized, the complementary nontemplated terminal nucleotides and complementary terminal deletions could be maintained during subsequent rounds of LCMV replication.

Whether the terminally deleted RNAs have a function in the viral life cycle is currently unclear. Replication of viral RNAs containing small deletions or substitutions in the conserved terminal sequences has been seen in other viral systems (9, 18, 21, 22). However, for LCMV, the data suggest that the conserved terminal sequence can be dispensed with entirely, as in the case of the −10/−38 LCMV RNAs. Clearly, replication
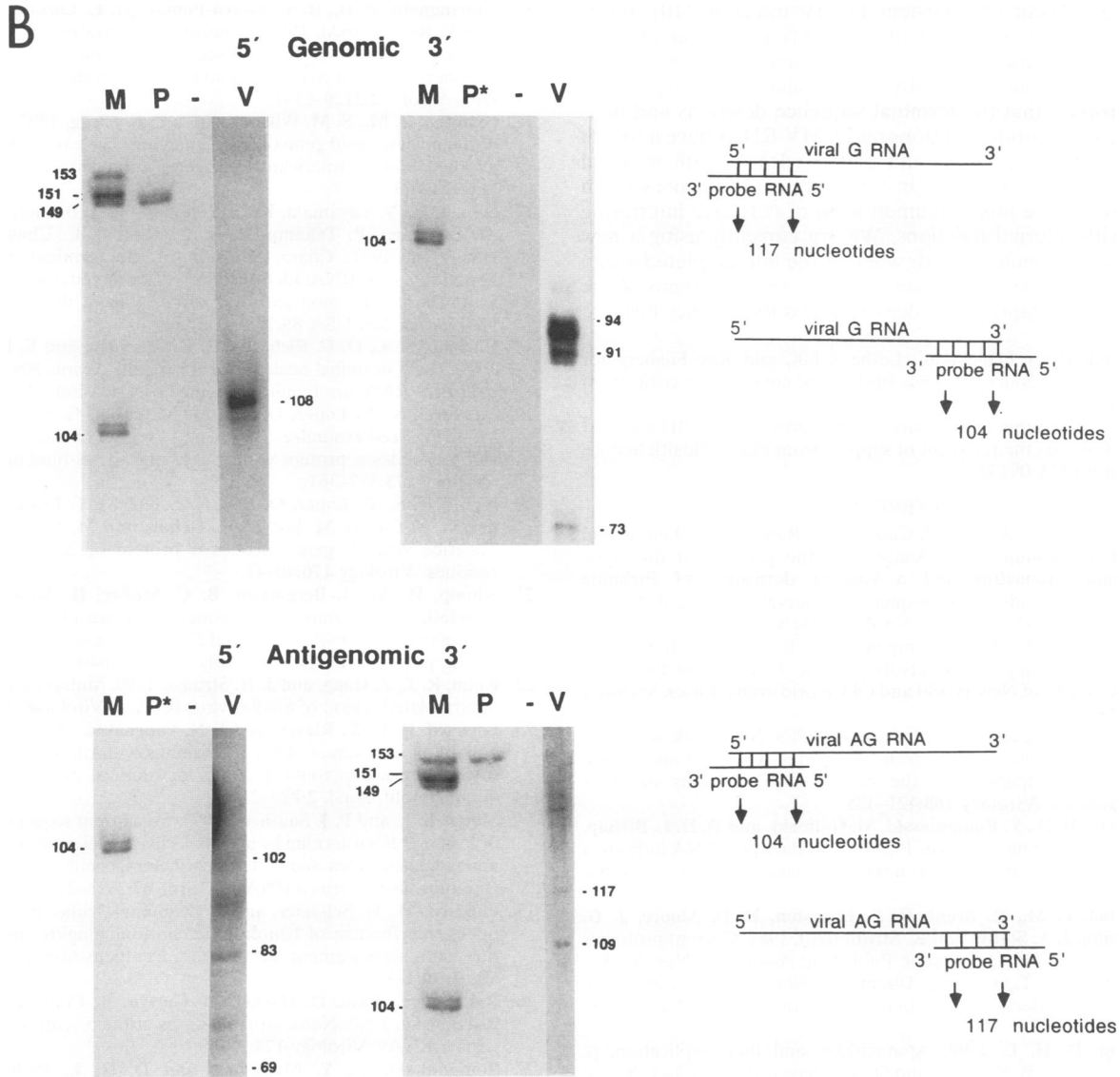
FIG. 4—Continued.

of these RNAs is inconsistent with a function for the conserved sequence in polymerase binding and replication. This, combined with previous data from our laboratory (24), suggests that, although the conserved terminal sequences may not be strictly required for replication, they may be absolutely required for the synthesis of mature transcripts. When S-derived

TABLE 1. Terminal deletions and additions in S and L RNAs corresponding to the major protected bands in Fig. 4

RNA	Deletion or addition[a] (nucleotides)	
	5′	3′
S genomic	0 to −5	+3 to −5
S antigenomic	+3 to +1, 0 to −17	0, −3 to −36
L genomic	−10 to −12	−10 to −13, −31
L antigenomic	0 to −2, −7 to −13, ≥−21	0, −8

[a] Indicated by − and +, respectively.

mRNAs (from the same infected BHK cells used to obtain the nucleocapsid RNAs examined in these experiments) were sequenced, all of the 5′ termini of the cloned mRNAs were intact, in addition to having one or more 5′-terminal nontemplated nucleotides. This suggests that mature transcripts may be derived only from full-length templates containing the conserved terminal sequence but that replication and packaging of the genomic and antigenomic RNA molecules can occur with terminally deleted molecules. Since the conserved terminal sequence appears to have a role in replication and transcription different from the role previously suspected, the presence of truncated RNAs during replication may have a biological function in a different aspect of the viral life cycle. For LCMV, the truncated RNAs could be acting as defective interfering RNAs by interfering with transcription (6). Interfering activity has been well documented for LCMV (25, 32), but there is as yet no convincing evidence for conventional defective interfering RNAs with large internal deletions as a

causal component of persistent LCMV infection (10). Interference might be due to lack of efficient transcription, or if the mRNAs are transcribed they may be altered in stability or defective in translation, thereby downregulating viral expression.

We propose that the terminal sequence deletions and nontemplated nucleotide additions on LCMV RNAs have a role in the life cycle of the virus and that the RNAs with terminal deletions may be defective in transcription and represent an alternative to the more common form of defective interfering RNAs with internal deletions. We are currently using a new strategy to determine directly whether the nontemplated nucleotides in the clones are located at the 5' or 3' terminus of the original RNA template in order to address these issues further.

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