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Accumulation of Terminally Deleted RNAs May Play a Role in Seoul Virus Persistence

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Two independent, long-term infections were analyzed to determine whether changes in viral replication could contribute to the establishment and/or maintenance of persistent Seoul virus infections. Infected cell cultures initially contained high levels of infectious virus and intracellular viral RNA that peaked between approximately 7 to 16 days postinfection and then gradually declined until day 26. After day 26, the viral titers and the levels of the small (S), medium (M), and large (L) viral RNAs varied cyclically until the end of the studies. The changes in the concentrations of the RNAs and titer were similar in pattern and appeared to result from changes in the regulation of replication. Neither internal deletions nor an accumulation of nucleotide changes were found in the RNAs. However, fine mapping and sequence analysis revealed short deletions in some of the RNAs in the conserved complementary terminal sequences believed to contain the signals for initiation of replication and transcription. Deletions at the 3′ termini of S, M, and L virus-sense RNAs (vRNAs) accumulated during the acute phase of infection just before the time that the viral titer and the concentration of vRNAs and virus complementary-sense RNAs (cRNAs) began to decline. The absence of deletions at the 5′ termini of the S, M, and L cRNAs suggests that the 3′-deleted vRNAs may not be replication competent. Thus, as the percentage of 3′-deleted vRNAs increase in the population, they could potentially compete with standard virus and downregulate viral replication. Deletions at the 3′ L cRNA and 5′ L vRNA termini were also observed, and the proportion of these deleted RNAs varied cyclically during the infections. We propose a model in which terminal nucleotide deletions arise by nuclease activity of the viral polymerase. In addition, we speculate that cleaved terminal fragments might be used as primers during replication, resulting in the repair of some of the deleted RNAs.

The *Hantavirus* genus, in the family *Bunyaviridae*, is composed of a large group of enveloped negative-strand RNA viruses with a tripartite segmented genome (32). The three segments, small (S), medium (M), and large (L), encode the viral nucleocapsid (N), envelope glycoproteins (G1 and G2), and polymerase (L), respectively (33, 35, 36). Hantaviruses replicate in the cell cytoplasm, and replication is likely to proceed by mechanisms similar to those used by other negative-strand RNA viruses. That is, synthesis of mRNA and antigenome or virus complementary-sense RNA (cRNA) is initiated from the 3′ terminus of viral RNA templates, and the synthesis of virus-sense RNA (vRNA) is initiated from the 3′ terminus of cRNA templates. The signals for initiation of replication and transcription have not yet been defined but are believed to reside in the imperfect inverted repeat present at the termini of each genomic segment. This sequence is approximately 20 nucleotides long and is conserved among all members of the genus. Because bunyavirus ribonucleocapsids are circular (24, 26) and the terminal nucleotides of the vRNAs and cRNAs appear to be base paired, the RNAs are thought to exist as panhandle structures in cells (Fig. 1) (28). Thus, both the terminal nucleotide sequence and the structure of the panhandle may be important in proper initiation at the 3′ termini of the vRNAs and cRNAs.

Hantaviruses cause persistent infections in rodents in nature, and each strain is normally associated with a specific host species. While much is known about the geographic distribution and antigenic and genetic differences among the hantaviruses, information about persistence is limited because thus far only a few studies have described long-term infections. This is partially due to the difficulty in detecting virus, vRNA, and viral protein in infected animals (39; R. Yanagihara et al., Letter, Lancet **i:**1013, 1984). Despite these limitations, a few basic features of hantavirus persistence have emerged from experimental animal infections. As for other persistent viruses, hantavirus infections appear to consist of a short acute phase followed by a prolonged persistent phase. Acute infection seems to last about 3 to 4 weeks, and during this time animals are transiently viremic. Viremia peaks around 7 to 14 days postinfection (p.i.) (4, 12, 16, 17, 19, 40). During persistence, the levels of infectious virus or antigen-positive cells are generally lower, and in some tissues or secretions may vary or disappear and reappear several times during the course of the study in a wave-like pattern (4, 12, 16, 17, 18, 38, 40). Thus, while virus and/or antigen can be detected in many tissues and bodily fluids during the acute stage, they are often only detected sporadically from the same sites during persistence. Taken together, the reduction in virus and antigen after the acute phase of infection and the cyclic variations in infectious virus and antigen during persistence suggest that some stage of the virus life cycle is downregulated when persistence is established and at cyclic intervals during the time that persistence is maintained.

To understand the mechanism by which hantaviruses persist in cells, we investigated whether changes occur in Seoul virus (SEOV) replication during a long-term infection and, if so, how those changes could account for the apparent downregulation in viral gene expression that is associated with the establishment and maintenance of persistence in animals.

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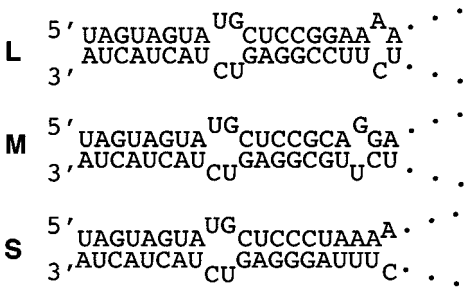

FIG. 1. The 5′- and 3′-terminal 20 nucleotides of SR-11 virus L, M, and S vRNAs shown base paired in one version of a proposed panhandle structure.

MATERIALS AND METHODS

Viral strain, cell culture, and virus assay. The SR-11 strain of SEOV was used to infect Vero-E6 cells (Vero C1008; ATCC CRL 1586) at a multiplicity of infection (MOI) of ≤0.1. The same stock of SEOV was used for both infections. Cells were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum, and the medium was changed every 3 to 4 days. Infected cells were kept in continuous culture during both infections except for the cells harvested at 46 days postinfection (p.i.) during infection 1. Those cells were split once 1:4 at 16 days p.i. Culture supernatant was harvested for virus assay, and infected cells were harvested for RNA isolation at 6, 7, 9, 12, 16, 26, and 46 days p.i. for infection 1 and at 2, 4, 6, 7, 9, 12, 16, 26, 36, 46, 56, 68, 88, 109, and 139 days p.i. for infection 2. Culture medium was changed 24 h before the medium and cells were harvested at each of the time points, so that the titer values reported reflect the number of infectious virions released during those 24 h. Viral titers were determined by plaque assay on fresh Vero-E6 cells, as previously described (14). All cells used for infections and plaque assays were from the 24th or 25th passage after establishment.

RNA isolation and quantitation. Total cellular RNA from infected cells was isolated with TRIzol reagent according to the manufacturer's instructions (Life Technologies, Grand Island, N.Y.), except that the RNA samples were extracted one to three times with phenol-chloroform and precipitated with ethanol before being dried and resuspended in RNase-free H_2O. The samples were quantitated by using absorption spectroscopy, and the A_{260}/A_{280} ratios of each were between 2.0 and 2.1, indicating highly purified preparations of RNA. The concentration of DNA in the samples was determined by using a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech, San Francisco, Calif.) and found to be <0.02% of each sample. Each sample was stored in a single tube which was repeatedly thawed and refrozen as needed for experiments. Virion RNA was isolated from virus present in the cell culture medium after 7 days of infection. Cell culture supernatant was harvested and clarified, and the polyethylene glycol-precipitated virus was pelleted as described elsewhere (34), before the virion RNA was isolated with TRIzol.

Northern hybridization. Samples of RNA (10 μg per lane) from infection 1 were denatured by treatment with glyoxal (21), and samples from infection 2 were denatured by treatment with formaldehyde (20) before separation on 1.75% agarose gels. After vacuum transfer in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (20) onto Duralon-UV membranes (Stratagene, La Jolla, Calif.) and cross-linking to membranes by UV exposure, the membranes were hybridized to probes according to the manufacturer's recommendations. Uniformly labeled ^{32}P probes were either single-stranded DNA (infection 1) or RNA (infection 2) and were made by asymmetric PCR or by in vitro transcription, respectively. The probes represented the region between positions 40 and 1697 of the SEOV SR-11 S segment sequence, the region between positions 157 and 3526 of the SEOV SR-11 M segment sequence (2), and a 4.4-kb region of the SR-11 L segment sequence that corresponded to positions 801 to 5202 of the SEOV 80-39 L segment sequence (1). Two identical Northern blots were made with samples from each infection; one blot was hybridized to cRNA-mRNA-specific probes, and the other was hybridized to vRNA-specific probes. Membranes with infection 1 samples were hybridized separately to S, M, or L probes after stripping of the membranes between hybridizations, whereas membranes with infection 2 samples were hybridized once to a mixture of S, M, and L probes. Human β-actin cDNA (Clontech Laboratories, Inc., Palo Alto, Calif.) was used to make the actin probe by random primer incorporation of [^{32}P]dCTP (20). Virus-specific RNA and β-actin mRNA were detected by autoradiography of membranes with Biomax MR film (Kodak). Bands in autoradiographs of Northern blots and primer extension experiments were quantitated by densitometry by using a ChemiImager 4400, version 5.04 (Alpha Innotech Corp., San Leandro, Calif.). The counts per minute (cpm) in the bands representing S, M, and L RNAs were calculated according to a standard curve consisting of samples of known counts per minute. Bands from primer extension and the Northern blot of infection 2 samples were quantitated from the same autoradiograph; bands from the Northern blot of infection 1 samples were quantitated from separate autoradiographs. Very faint bands visible to the eye were found to be below the level of detection by this method and are graphed as zero in the figures.

Reverse transcriptase-PCR (RT-PCR) restriction endonuclease mapping. One microgram of total RNA isolated 2 or 139 days p.i. was reverse transcribed by using random primers and Superscript II with the First Strand Synthesis Kit (Life Technologies). Two percent of each cDNA sample was amplified by PCR with *Pfu* polymerase (Stratagene) and pairs of S, M, or L sequence-specific primers. The forward and reverse primer pairs corresponded to the SEOV SR-11 cRNA sequence for S and M primers (2) or the cRNA sequence of Seoul 80-39 for L primers (1). S segment primers were from positions 47 to 60 and 731 to 754, 510 to 533 and 1516 to 1537, and 1261 to 1281 and 1676 to 1699. M segment primers were from positions 59 to 80 and 906 to 935, 703 to 725 and 1722 to 1745, 1511 to 1539 and 2504 to 2525, 2177 to 2206 and 3276 to 3297, and 2934 to 2956 and 3577 to 3600. L segment primers were from positions 58 to 80 and 1047 to 1070, 763 to 785 and 2531 to 2553, 2226 to 2250 and 3860 to 3884, 3569 to 3590 and 5490 to 5511, and 5179 to 5202 and 6479 to 6503. Amplification conditions were as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 1 min, with a final incubation at 72°C for 7 min. The PCR products were digested with restriction endonucleases and separated on 2% Metaphor agarose gels in TBE buffer (FMC BioProducts, Rockland, Maine).

Primer extension. RNA (1 to 7 μg) and a ^{32}P-5′-end-labeled S, M, or L sequence-specific primer were denatured in water at 70°C for 10 min and then transferred immediately to 50°C to anneal for 3 to 5 h in 0.4 M NaCl and 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4). After annealing, 2.5 U of Superscript II was added, and the reaction was incubated for 1 h at 50°C in 50 mM Tris-HCl (pH 8.3), 8.3 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 0.5 mM concentrations of deoxynucleoside triphosphates (pH 8.0). The reaction mixtures were extracted with phenol-chloroform and precipitated with 10 to 20 μg of glycogen in ethanol before resuspension in formamide and electrophoresis on 6% denaturing polyacrylamide gels. Detection was done by autoradiography with Biomax MR film (Kodak). The primers used to map the 5′ end of S cRNA and vRNA were between positions 107 and 128 and positions 1522 and 1543, respectively, of the SR-11 S cRNA sequence (2). The primers used to map the 5′ end of M cRNA and vRNA were between positions 202 and 222 and positions 3451 and 3473, respectively, of the SR-11 M cRNA sequence (2). The primers used to map the 5′ end of L cRNA and vRNA were between positions 171 and 195 and positions 6379 and 6399, respectively, of the SR-11 L cRNA sequence.

Cloning and sequencing RNA termini. RNA (0.5 to 1 μg) was decapped with tobacco acid pyrophosphatase (Epicentre Technologies, Madison, Wis.) and circularized with RNA ligase as described previously (21, 23). RNA that was phosphorylated before ligation was treated with polynucleotide kinase according to the manufacturer's directions (Roche Molecular Biochemicals, Indianapolis, Ind.) and not decapped. After circularization, the mixture was separated into six separate aliquots for cDNA synthesis. Strand-specific cDNA was made of the ligated termini with cRNA or vRNA S, M, or L sequence-specific primers and Superscript II with the First Strand Synthesis Kit. The primers corresponded to the S, M, or L SEOV SR-11 cRNA sequence (2). The vRNA and cRNA primers, respectively, were from positions 170 to 202 and 1260 to 1281 (for S), 202 to 223 and 3396 to 3418 (for M), and 314 to 337 and 6172 to 6196 (for L). Two percent of each cDNA sample was amplified by PCR with *Pfu* polymerase (Stratagene) and the conditions described for RT-PCR mapping above. PCR products were cloned into pGEM-3Z (Promega, Madison, Wis.) and sequenced by using an ABI PRISM dRhodamine Cycle Sequencing Kit and ABI 377 DNA Sequencer. The changes in the percentage of clones with deleted termini at each time were analyzed for statistical significance by a pairwise analysis with a 95% confidence interval (SAS System Package Software, version 6.12; SAS Institute, Inc., Cary, N.C.).

RESULTS

Establishment of persistent infections and collection of samples. Hantaviruses establish persistent infections in rodents and cultured cells, but the factors contributing to the establishment and/or maintenance of persistent infections are unknown. To assess whether changes in replication could have a role in hantavirus persistence, two independent infections of Vero-E6 cells were established by using the SR-11 strain of SEOV at an MOI of ≤0.1 PFU/ml. Samples of cell culture medium or total RNA from infected cells were harvested 6, 7, 9, 12, 16, 26, and 46 days p.i. during infection 1 and at 2, 4, 6, 7, 9, 12, 16, 26, 36, 46, 56, 68, 88, 109, and 139 days p.i. during infection 2. Persistently infected cells were maintained in continuous culture throughout both studies except for the sample taken at 46 days p.i. during infection 1 (see Materials and Methods). To increase experiment-to-experiment uniformity and to control for the introduction of RNases into samples

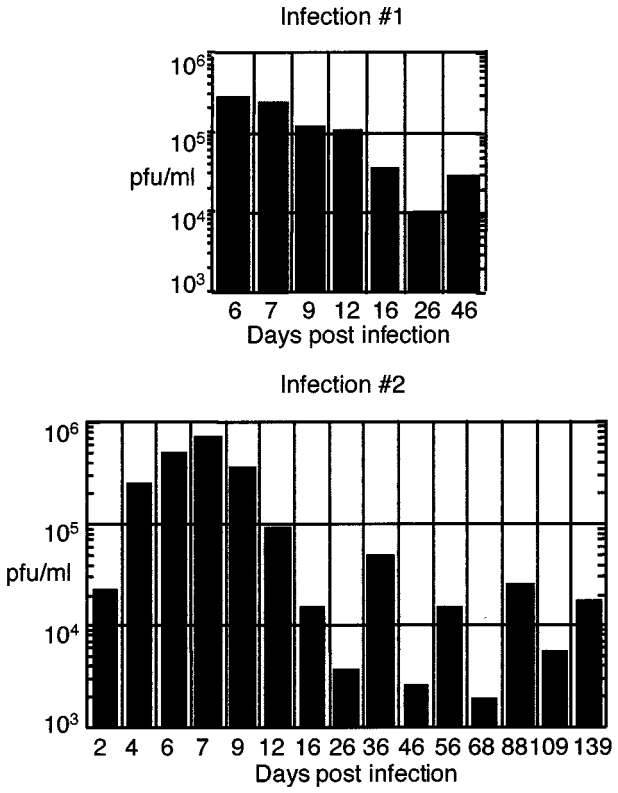

FIG. 2. Infectious titer determination. Virus was assayed by plaque assay, and results were plotted as PFU per milliliter versus days p.i. as indicated for infection 1 (top) and infection 2 (bottom). The titer represents the quantity of infectious virus found in the culture medium in the 24 h before the cells were collected for RNA isolation.

after isolation, the entire RNA sample from each time point was stored in one tube and thawed and refrozen as needed for each of the experiments. Additionally, selected samples were monitored for signs of degradation by Northern blot and primer extension analyses at the beginning and end of the studies. No evidence of RNase contamination or sample degradation was observed at any time (data not shown).

Assay of infectious virus. The infectious virus released during the 24 h before cells were harvested for RNA isolation was quantitated by plaque assay of cell supernatants on fresh Vero-E6 cells (14). The concentration of infectious virus was highest at the end of the first week of infection and then gradually declined until day 26 in both infections 1 and 2 (Fig. 2). This period probably represents the approximate length of the acute phase of infection and the time when persistence was established. After day 26, the amount of infectious virus varied in a cyclic pattern but did not return to the higher levels observed during the acute phase.

Analysis of the S, M, and L genomic segments. Equal amounts of total RNA isolated from infected cells at 6, 7, 9, 12, 16, 26, and 46 days p.i. during infection 1 and at 2, 7, 12, 26, 56, 68, and 139 days p.i. during infection 2 were examined by Northern hybridization by using probes to detect either cRNA and mRNA (top panels) or vRNA (middle panels) (Fig. 3). The S, M, and L RNAs in infection 1 blots tended to trail off rather than run as tight bands, and L vRNA appeared to consist of more than one band. To address whether subgenomic L RNAs were present in these samples, RNA from day 9 p.i. was mapped by RT-PCR by using five pairs of overlapping L-specific primers that encompassed the region between positions 58 and 6503. No evidence of internal deletions was seen (data not shown). We subsequently discovered that the glyoxal did not completely denature the vRNAs under the conditions that were used and likely accounts for their appearance in these blots (data not shown). Samples from infection 2 were therefore denatured with formaldehyde and the S, M, and L RNAs migrated as discrete bands.

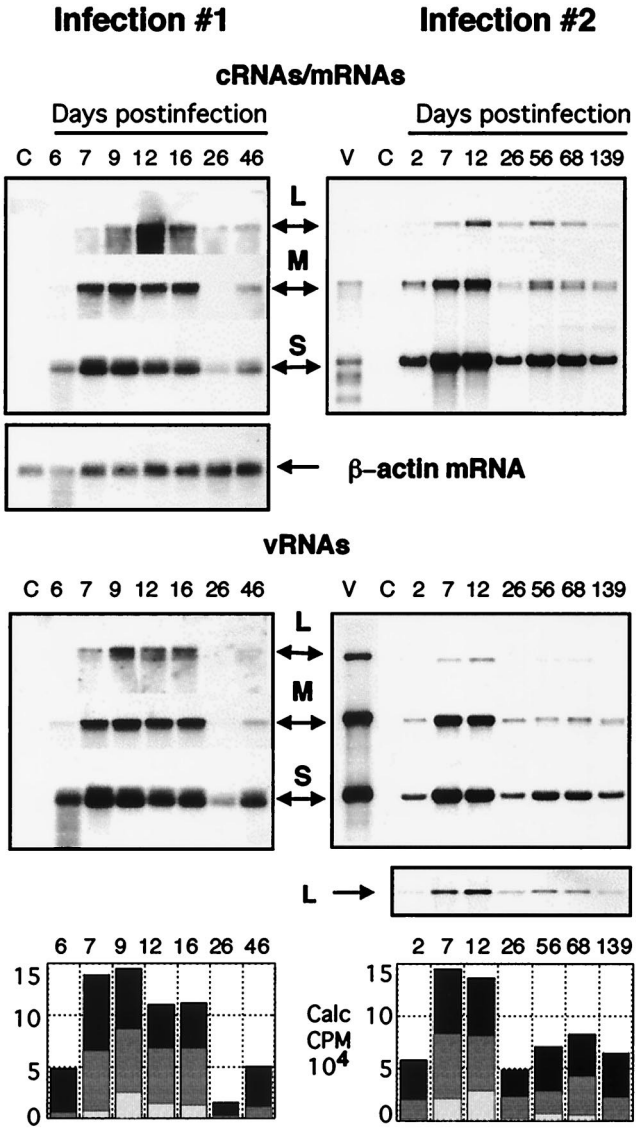

FIG. 3. Northern hybridization analysis of SR-11 vRNA during acute and persistent infections for infection 1 (left panels) and infection 2 (right panels). Total RNA (10 μg/lane) from infected Vero-E6 cells isolated at the times designated above the lanes was separated on agarose gels and, after transfer to solid support, hybridized to ^{32}P-labeled single-stranded probes specific for S, M, or L cRNA and mRNA (top panels) or vRNA (middle panels). The cRNA blot from infection 1 was hybridized to a β-actin probe after the virus-specific probes were removed. A longer exposure of L vRNA from infection 2 is shown below the right vRNA panel. Lanes: C, 10 μg of total RNA from uninfected cells; V, 3 μg of RNA isolated from virions in cell culture supernatant. S, M, and L RNAs were present at all time points, although they were not visible in all lanes of the autoradiographic exposures shown. Bottom panels show the calculated cpm from densitometry readings of the S (black bars), M (dark gray bars), and L (light gray bars) bands detected in the vRNA blots.

The results from both infections showed that the concentra-

tions of the S, M, and L RNAs peaked between 7 to 16 days p.i. and declined by day 26. Hybridization of an infection 1 blot to cellular β-actin mRNA indicated that the decrease in viral RNA at day 26 was virus specific. After day 26, the concentrations of the S, M, and L RNAs varied but were consistently lower than their peak concentrations during the acute stage of infection. While not visible in the exposures shown in Fig. 3, S-, M-, and L-specific RNA was present at all of the time points examined. The concentrations of the vRNAs also appeared to vary in concert with their respective cRNAs and mRNAs. Densitometry was used to quantitate the S, M, and L RNAs in the vRNA blots of both infections (bottom panels) and showed a cyclic rise and fall in the concentrations of the vRNAs after day 26 in infection 2. The changes in vRNA concentrations at each time point seemed to lag slightly behind but otherwise paralleled the changes observed in the viral titer, except at day 68 and 139 p.i. in infection 2. This lag could have been due to the fact that the titer reflected the amount of infectious virus produced slightly before the cells were harvested for RNA isolation and could have contributed to the data from days 68 and 139 appearing out of phase. However, there is no reason that all of the vRNAs and cRNAs-mRNAs must change in the same way and at the same time as the titer, since only one small change in any of the RNAs, such as L, could initiate a cascade of events resulting in a change in replication and a drop or rise in the amount of infectious virus released into the medium. Subgenomic RNAs missing 150 or more nucleotides, the estimated limit of detection in these experiments, were not detected at any time points, indicating that defective interfering (DI) RNAs with large deletions were not associated with the persistence of SEOV.

RNAs with small deletions might also act as DI RNAs. To determine whether small deletions accumulated in the vRNAs during persistence, RNA isolated 2 and 139 days p.i. during infection 2 was assessed by RT-PCR and restriction endonuclease digestion (Fig. 4). cDNA was synthesized with random primers so that both vRNAs and cRNAs of S, M, and L could be mapped simultaneously. S cDNA was amplified by PCR with three pairs of primers (corresponding to lanes A to C, top panel in Fig. 4). M and L cDNA were each amplified with five pairs of primers (corresponding to lanes A-E, middle and bottom panels, respectively, Fig. 4). We examined all but the terminal 40 to 60 nucleotides of each RNA by this method. The PCR products overlapped by at least 240 bp; thus, if part of the population of RNAs contained a deletion at the position of one primer so that only the RNA without a deletion was amplified, then the deleted RNA would be detected by amplification with adjacent primer pairs. The purified PCR products were subsequently digested with restriction endonucleases to reduce their length to less than 600 bp for size analysis in the agarose gels. The limit of resolution for the smallest fragments in these gels was approximately 2 bp and for the largest fragments was approximately 20 bp. As shown in Fig. 4, the products derived from RNA isolated at 139 days p.i. were the same size as those derived from RNAs isolated at 2 days p.i., indicating that internal deletions larger than 2 to 20 nucleotides did not accumulate by the end of the study in the S, M, or L RNAs.

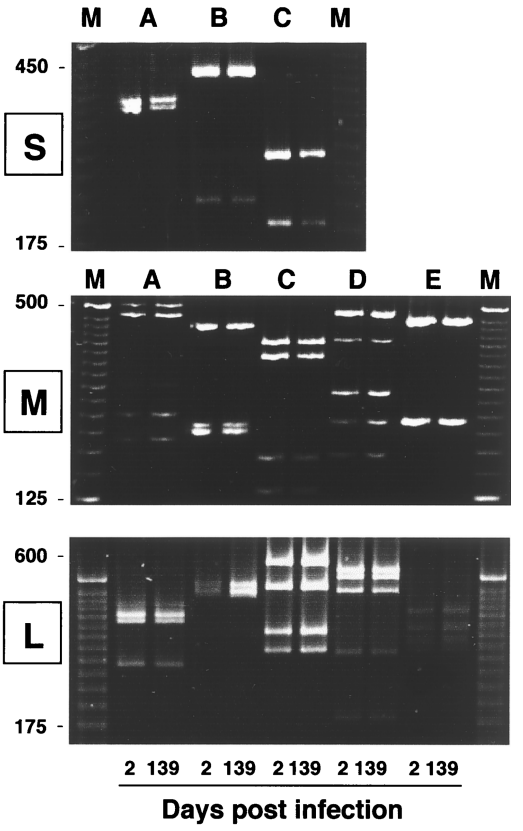

FIG. 4. RT-PCR restriction endonuclease mapping of S, M, and L RNA. Restriction endonuclease digestion products from RT-PCR-amplified RNA were separated on 2% Metaphor agarose gels adjacent to 25-bp DNA markers. RNA was isolated at 2 or 139 days p.i. as indicated below the gels and amplified by three pairs (lanes A to C) of S-specific (top panel) or five pairs (lanes A to E) of M (middle panel)- or L (bottom panel)-specific primers as described in Materials and Methods.

Primer extension analysis of the 5′ termini of S, M, and L vRNAs. The 5′ termini of the S, M, and L RNAs were mapped by primer extension by using samples from the same time points examined by Northern hybridization (6, 7, 9, 12, 16, 26, and 46 days p.i. from infection 1 and 2, 7, 12, 26, 56, 68, and 139 days p.i. from infection 2) (Fig. 5). Primer extension of S vRNA resulted in two products (Fig. 5B, top panels) showing that two populations of S vRNAs were present during infection. One population had full-length 5′ termini and is represented by the lower band in both groups of samples. The other population, represented by the upper band, is eight nucleotides longer. Sequencing studies identified the longer RNAs as S vRNAs with an identical eight-base insertion (5′-AGAAGGT T-3′) in the untranslated region 70 nucleotides from the 5′ end of the vRNA. This sequence was unique and not present elsewhere in the S, M, or L genomic segments. Because this insertion was also present in RNA that was recovered from cell supernatants presumably containing packaged virions (lane V), it may have been simply carried in the RNA population. Whether it has another role in the viral life cycle is unknown.

Unlike the 5′ termini of S vRNAs, primer extension experiments revealed that both the intracellular M vRNAs and those recovered from virions were full length, as shown by the single band next to the arrows in Fig. 5B (middle panels). Since the concentration of M vRNA from day 26 onward was very low (see Fig. 3), only faint bands the size of full-length 5′ termini were detected in the amount of total RNA mapped in these experiments. In contrast, the L vRNA population was composed of two groups (Fig. 5B, lower panels). One portion of the population had full-length 5′ termini (top arrow), while the other portion had heterogeneous 5′ termini that were missing from approximately 9 to 15 nucleotides (between bottom two arrows) in both infections 1 and 2. Deletions of approximately 10 bases were most common. L vRNAs packaged into virions

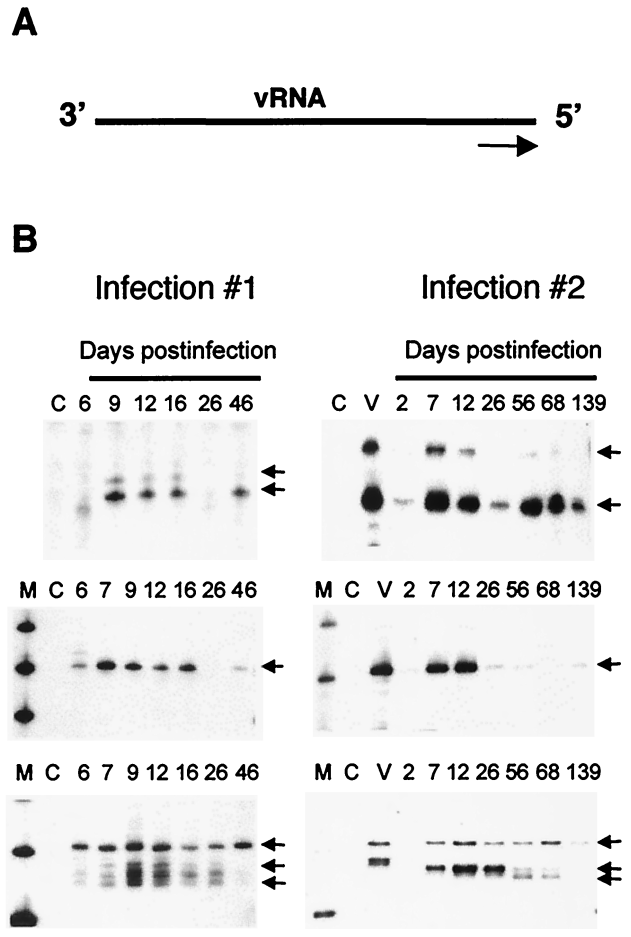

FIG. 5. Primer extension mapping of the 5′ termini of S, M, and L vRNAs. (A) Diagram of the region mapped. The arrow indicates the labeled primer extension products. (B) Total RNA isolated from infected cells at the times indicated above the lanes was mapped by using ^{32}P-end-labeled S (top panels)-, M (middle panels)-, or L (bottom panels)-specific primers. Samples from infection 1 (left panels) and from infection 2 (right panels) are shown. Lane C, RNA isolated from uninfected cells; lane V, RNA isolated from virions; lane M, 25-bp DNA markers. The arrows indicate products that are the size of full-length S vRNA termini (240 nucleotides) or S RNAs that have gained 8 bases (top panels), full-length M vRNA termini (201 nucleotides) (middle panels), or full-length L vRNA termini (152 nucleotides) (bottom panels), as well as the group of L vRNAs below that are missing approximately 9 to 15 terminal bases.

also had 5′ deletions, but the deletions were shorter (seven to nine bases) than those detected at the termini of the intracellular RNAs. Both the proportion of intracellular deleted RNAs to full-length RNAs and the sizes of the deletions appeared to change over the course of the infection. To ascertain whether these changes occurred in a specific pattern, samples of RNA isolated at all of the time points during infection 2 were examined by primer extension, and the bands were quantitated by densitometry (Fig. 6). The results of this experiment showed that both the proportion of the deleted L vRNAs in the total L vRNA population as well as the sizes of the deletions changed over the course of infection in a cyclic manner. The deleted and full-length populations at day 2 p.i. and the deleted population at day 139 p.i. were barely detectable in longer exposures of the autoradiographs shown in Fig. 6 and so were present only at very low levels. From days 2 to 12 the deleted population steadily increased, then declined until days 26 and 36, and then increased once more, followed by a slow decrease at the remaining time points. The large increase in the terminally deleted L RNA population from days 7 to 12 coincided with the peak and subsequent decline in the viral titer. The proportion of RNAs with larger deletions also rose steadily as the acute stage progressed, decreased by day 26 p.i., and increased again at day 46. The full-length RNAs also varied in a cyclic manner during the infection, and we suspect that if samples had been taken more frequently, the deleted population would continue to vary cyclically as well.

Primer extension analysis of the 5′ termini of S, M, and L cRNAs and mRNAs. Mapping the cRNAs and mRNAs by primer extension showed that the S, M, and L cRNAs had full-length 5′ termini (Fig. 7B, lower band next to arrow in each panel). The upper arrow to the right of each panel points to a short ladder of products that was 15 to 19, 10 to 13, or 11 to 15 nucleotides longer for the S, M, and L RNAs, respectively. Sequence analysis of the cloned RNAs showed that these bands represented mRNAs with 5′-nontemplated leader sequences that were heterogeneous in both sequence and length (data not shown) and believed to be acquired by hantaviruses by a "cap-snatching" mechanism (8, 11).

Sequence analysis of S, M, and L vRNA and cRNA termini. To determine whether the 3′ termini of the S, M, and L RNAs were full length or deleted and to identify the nature of the two ends of the same RNA molecule, the 5′ and 3′ termini were cloned and their nucleotide sequences were determined. Samples of S, M, and L RNAs from days 2 and 7 (acute phase) and day 139 (last point of the study) of infection 2 were examined, as well as one additional sample of L RNA from the midpoint of the infection (day 68).

Analysis of the clones revealed that a substantial proportion of the population of S, M, and L vRNAs had 3′-terminal deletions of one or more nucleotides (Table 1). Those RNAs with 5′-nontemplated leader sequences, and thus mRNAs, were not included in these data. The percentage of vRNAs with 3′ deletions increased after day 2 and constituted a large portion of the population by day 7. The increase in the population of 3′-deleted RNAs by day 7 coincided with the time when viral titers peaked and subsequently began to decline, which was quickly followed by a decline in the total concentration of vRNAs (see Fig. 3). By day 7, 29% of the cloned S RNAs, 45% of the cloned M RNAs, and 63% of the cloned L RNAs had 3′ deletions. Approximately 80% of the deletions were of ≤20 nucleotides and fell within the terminal panhandle region (Table 2). A pairwise analysis of the data from all possible combinations of time points showed that the increase in 3′ vRNA deletions between days 2 and 7 for S, M, and L vRNAs was statistically significant with a 95% confidence interval. Likewise, the decrease in 3′ deletions seen for M vRNAs between days 7 and 139 was statistically significant.

Unlike the deletions at the 3′ termini of the vRNAs, deletions of the 3′ termini of cRNAs were primarily found on L cRNAs (Table 1). Of the L cRNAs, 18 to 38% were missing 3′-terminal nucleotides at 2, 7, 68, and 139 days p.i., but the changes in the proportion of the deleted population over time did not represent a statistically significant accumulation as they did for the S, M, and L vRNAs. All but one of the 3′ L cRNA deletions were more than 12 nucleotides long (23 of 24 clones), and the deletions were similar in size to those found at the 5′ terminus of L vRNA by primer extension. Few S cRNA (7 of 65) and M cRNA (2 of 48) clones had 3′ deletions, and all were ≤8 nucleotides long and within the panhandle and triplet repeat portion of the terminal sequence.

Consistent with the primer extension studies, sequence analysis revealed that the 5′ termini of S, M, and L cRNAs and S and M vRNAs were also primarily intact at all time points

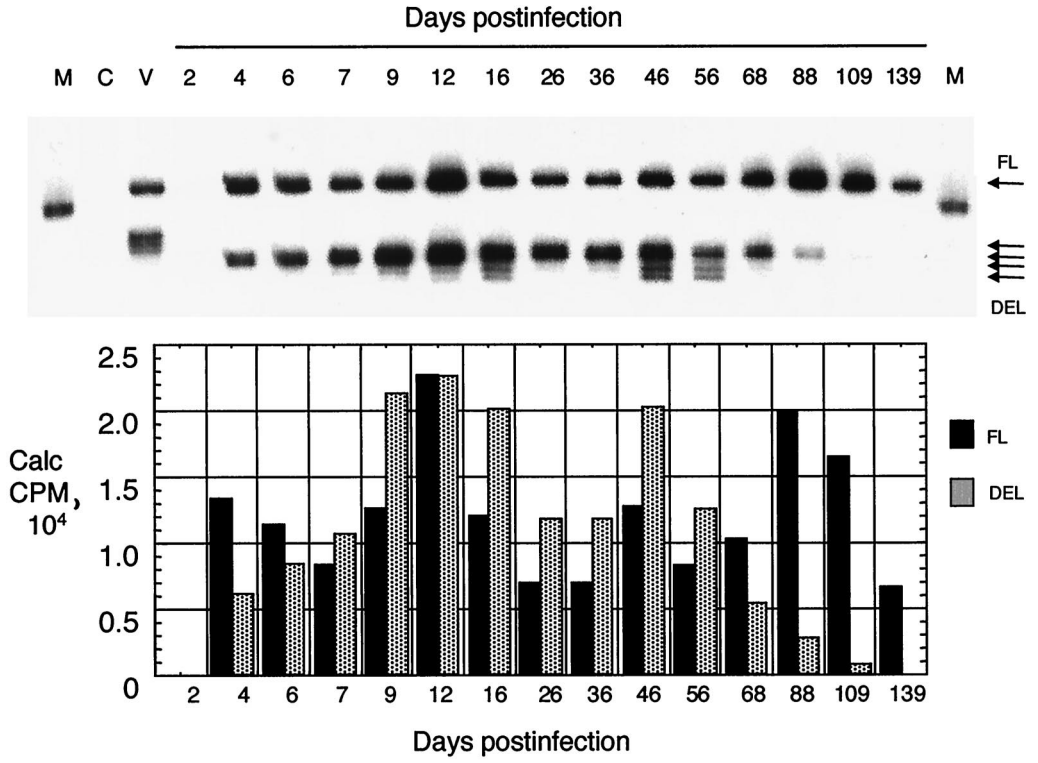

FIG. 6. Primer extension mapping and quantitation of the 5′ termini of L vRNAs. RNA from all time points collected during infection 2 was mapped with the L-specific primer as in Fig. 5B. The top arrow indicates products from full-length (FL) vRNA, and the bottom group of arrows indicates products from terminally deleted (DEL) vRNAs. Lane C, RNA isolated from uninfected cells; lane V, RNA isolated from virions; lane M, 25-bp DNA markers. The calculated counts per minute values of the bands representing full-length (solid bars) and deleted (stippled bars) RNAs from densitometry readings of the autoradiograph are presented.

examined (Table 2). Unexpectedly, however, the majority of L vRNAs had full-length 5′ termini and, of those with deletions, all but one were missing more than eight nucleotides. This finding is in contrast to the results from primer extension experiments which indicated that a sizable portion of the population had 5′ deletions at days 7 and 68, that the 5′ deletions were approximately 9 to 15 nucleotides long, and that deletions of 10 bases were most abundant. While the small number of clones may account for this apparent difference, an alternate possibility could be that the deleted RNAs had hydroxyl groups rather than monophosphates at their 5′ termini and thus were unavailable for ligation. To address this possibility, L vRNA was recloned with day 7 RNA that was phosphorylated before ligation. Of 17 clones recovered from this experiment, 6 were found to have 5′ deletions of 1, 3 (two clones), 7, 8, and 14 nucleotides. However, the increase in 5′ L vRNA deletions in phosphorylated RNA over that recovered from RNA that was not phosphorylated was not statistically significant.

Another possible reason for the difference between the proportion of 5′ L vRNA deletions observed by sequencing and that observed by primer extension may be due to a nucleotide preference of the RNA ligase. Several studies have shown that the efficiency of the joining reaction is dependent on the particular donor (5′) and acceptor (3′) terminal nucleotides and the secondary structure of the termini (6, 7, 13, 25, 30). While purine acceptors and pyrimidine donors are generally believed to be the optimum substrates and could account for the large percentage of full-length L vRNAs ligated, not all types and combinations of oligoribonucleotides have been examined. In our study, a total of 23 S, M, and L clones had 5′ deletions (with or without 3′ deletions). The majority had an A or a C residue acceptor (21 of 23) and a U or an A residue donor (22 of 23). No C residues and only one G residue were found in a donor position. In contrast, primer extension data indicated that C and G residues should predominate in the donor positions of L vRNAs having deletions of 9 to 15 nucleotides at their 5′ termini, resulting in a ratio of 3 C to 3 G to 1 U donor nucleotides in the cloned RNAs (Fig. 1). Thus, it appears that a bias in ligation of the 5′-deleted L vRNAs was present and that sequence analysis underestimated the number of 5′-deleted termini. Despite this potential bias, our sequence results suggested that L vRNAs with deletions at only their 5′ termini were rarer than L vRNAs with deletions at only their 3′ termini or at both termini.

Nucleotide changes during infection. To assess whether nucleotide changes accumulated in the terminal regions of the genome during the 139-day infection, the sequences of the cloned S, M, and L cRNAs and vRNAs isolated 2 and 139 days p.i. were aligned with CLUSTAL W 1.7. We expected that changes in the noncoding region would be most important for influencing replication; therefore, all of the noncoding regions of the S, M, and L RNAs were examined except for the 13 nucleotides preceding the M segment start codon and 213 of the 437 nucleotides following the predicted S segment stop codon (Fig. 8). In addition, the first 74 or 116 nucleotides of the coding regions of S and L, respectively, were aligned. Likewise, for L, which has a very short 3′ noncoding region, the final 114 nucleotides of the coding region of the polymerase protein were aligned, and for M the final 47 nucleotides of the coding region of the G2 protein were aligned. Of the nucleotides aligned, 60% were in noncoding regions and 40% were in coding regions. The nucleotide changes identified (other than

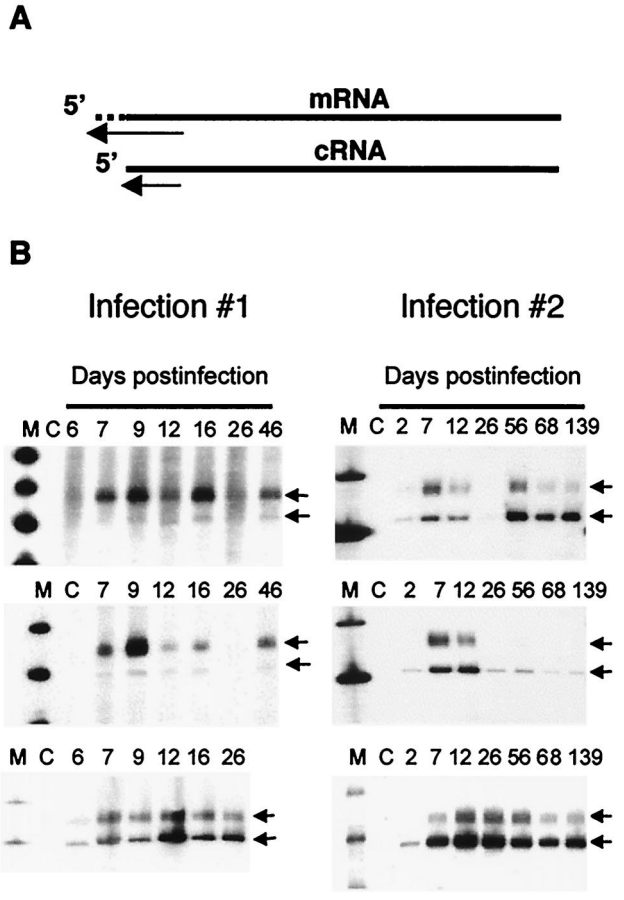

FIG. 7. Primer extension mapping of S, M, and L cRNAs and mRNAs. (A) Diagram of the region mapped. The dotted line represents the 5′ leader sequences at the terminus of mRNAs, and arrows represent the labeled primer extension products of mRNA (top) or cRNA (bottom). (B) The experiment was identical to that described in Fig. 5, except that cRNA-specific primers were used to map the S (top panels), M (middle panels), and L (bottom panels) RNAs. Lower arrows indicate the products that are the size of full-length termini that are 128 nucleotides long for S RNA, 222 nucleotides long for M RNA, and 195 nucleotides long for L RNA. The upper arrows indicate the heterogeneous group of products that map mRNAs.

the terminal deletions and the eight-base insertion in S vRNA) were in both the coding and noncoding regions and were generally equally distributed among the day 2 and 139 clones and clones derived from vRNA and cRNA (Table 3). Because many of the same nucleotide changes were present in multiple clones, some changes may simply represent a natural variation in the population. Only six clones of S and M RNAs had unique base changes, and in three of those the changes were in the conserved terminal sequence at positions 9 and 10, position 1760 (10 bases from the 3′ cRNA terminus), and position 19. The presence of nucleotide changes at positions 9 and 10 and position 1760 would allow the formation of base pairs at positions not normally base paired in the panhandle structure shown in Fig. 1, as would a change at position 20 in 6 of the 37 S clones. The number of changes found among the cloned RNAs may be artificially high, considering that the RNA had been copied into cDNA and amplified and that the accuracy rate for the software used in sequence analysis was 98.5% (5). Regardless, the data do not show that nucleotide changes accumulated during the 139-day infection in the region of the genome aligned.

TABLE 1. 3′-Terminal deletions of S, M, and L vRNAs and cRNAs as determined by sequence analysis

RNA	No. of clones/total no. (%)[a] at day p.i.:			
	2 days	7 days	68 days	139 days
vRNA				
S	1/23 (4)	6/21 (29)	–[b]	5/11 (45)
M	1/13 (8)	9/20 (45)	–	0/19 (0)
L	1/6 (16)	12/19 (63)	11/15 (73)	6/9 (67)
cRNA				
S	0/6 (0)	6/45 (13)	–	1/14 (7)
M	0/6 (0)	2/31 (6)	–	0/11 (0)
L	4/22 (18)	9/24 (38)	5/16 (31)	6/32 (19)

[a] Number and percentage of clones with terminal deletions/total clones sequenced.
[b] –, RNA not cloned.

DISCUSSION

In this study we analyzed hantavirus replication during two long-term infections to determine whether changes occurred in the viral genome during the establishment and maintenance of persistence. Earlier work showed that hantavirus infections in animals are composed of two phases, an acute phase, lasting about 3 to 4 weeks, where viremia peaks between approximately 7 to 14 days p.i., followed by a persistent phase, where the concentration of virus and/or viral antigen or RNA is lower and varies in amount in certain tissues and secretions (4, 12, 16, 17, 18, 19, 38, 40). Our cell culture infections mimicked rodent infections in that they also were characterized by an initial acute phase where the viral titer peaked at 6 to 7 days p.i., followed by a persistent phase where the viral titer varied cyclically over time. Our data suggest that for SEOV the acute phase ended and persistence was established sometime around day 26 p.i. Like the viral titer, the concentration of the S, M, and L RNAs also varied during infection. The RNAs were most abundant during the acute stage between 7 to 16 days p.i. and, after day 26, varied in concentration but at a decreased level. The concentrations of the vRNAs and cRNAs also appeared to change in concert at most of the time points. Because this study was performed in cultured cells that do not produce interferon due to a chromosomal deletion, the changes could not be due to either the effects of a host immune system or induction of interferon (15). Changes in RNA stability also are unlikely to have caused the changes in RNA concentration and titer that we observed. Changes in stability could result from a change in the RNA sequence or a change in expression of a host cell factor that destabilized the vRNAs (31). However, these changes would have to vary many times during the course of infection to result in the decreasing concentrations of the S, M, and L RNAs at those times. In addition, the changes would have to cause a simultaneous increase or decrease in RNAs that have different sequences, i.e., the cRNAs and vRNAs. Instead, the cyclic changes in the concentrations of the vRNAs and viral titer appear to be a natural feature of the persistent infection. Taken together, the data suggest that changes in the levels of RNA and titer are the result of changes in the regulation of synthesis of the vRNAs and that viral replication is downregulated during infection. This conclusion is supported by a recent study of another hantavirus, Black Creek Canal virus, in its natural host, in

TABLE 2. Number of nucleotides missing from the cloned 5′ and 3′ S, M, and L vRNA and cRNA termini[a]

RNA type and days p.i.	vRNA			cRNA		
	No. of clones sequenced	5′-Deletion size (nt)	3′-Deletion size(s) (nt)	No. of clones sequenced	5′-Deletion size (nt)	3′-Deletion size(s) (nt)
S						
2	21	0	0	6	0	0
	1	0	6			
	1	6	0			
7	15	0	0	39	0	0
	5	0	1, 7, 9, 34, 39	4	0	1
	1	8	6	1	0	6
				1	1	8
139	6	0	0	13	0	0
	2	0	2, 3	1	1	8
	2	0	11			
	1	1	13			
M						
2	12	0	0	6	0	0
	1	0	3			
7	11	0	0	29	0	0
	5	0	3, 8, 18, 22, 23	2	0	1
	2	0	7			
	1	4	8			
	1	1	3			
139	19	0	0	11	0	0
L						
2	5	0	0	18	0	0
	1	4	8	4	0	1
7	7	0	0	13	0	0
	3	0	6, 8, 29	4	0	1
	2	0	1	4	0	9, 10, 11, 12
	1	0	4	2	3	0
	3	0	3	1	1	8
	1	16	20			
	1	8	6			
	1	1	8			
68	4	0	0	10	0	0
	7	0	1, 3, 4, 10, 22, 31, 39	2	0	1
	1	8	6	2	0	11, 39
	1	6	19	1	3	0
	1	4	43	1	4	8
	1	1	39			
139	2	0	0	26	0	0
	2	0	19, 36	2	0	1
	1	8	0	2	0	3
	2	0	3	1	0	11
	1	1	8	1	4	8
	1	5	26			

[a] nt, nucleotides.

which the concentration of cRNA, as a measure of replication, also varied cyclically in certain tissues during a 150-day infection (12). A logical extension of these observations is that one or more of the factors important in establishing and maintaining persistence might also change cyclically.

Changes in the regulation of vRNA synthesis could be due to a number of events, such as the production of DI RNAs or an accumulation of mutations in the genome. Both factors have been reported to play a major role in establishing and maintaining persistent infections (reviewed in reference 10). However, typical types of DI RNAs (RNAs with large internal deletions, RNAs with sequence rearrangements, and snapback RNAs) were not detected. Likewise, changes in the nucleotide sequence in the region surrounding the termini did not accumulate during the 139-day infection. Instead, we found that the vRNAs and cRNAs had short deletions at their termini in the region believed to contain the sequence and/or structural features necessary for initiation of replication and transcription. More than 80% of the deleted RNAs were missing ≤20 nucleotides. For S and M RNAs, deletions were common only at the 3′ termini of vRNA. In contrast, for L RNA, deletions were observed at both the 3′ and 5′ termini of vRNA and also at the 3′ terminus of cRNA. Terminally deleted RNAs were previously proposed to have a role in downregulating viral gene expression for a persistent virus in the arenavirus family, lymphocytic choriomeningitis virus (LCMV) (22, 23).

The S, M, and L vRNAs with 3′ deletions that were longer than a few nucleotides probably were not replication competent because deletions were rarely observed at the 5′ termini of the S, M, and L cRNAs. Defective RNAs that have lost the

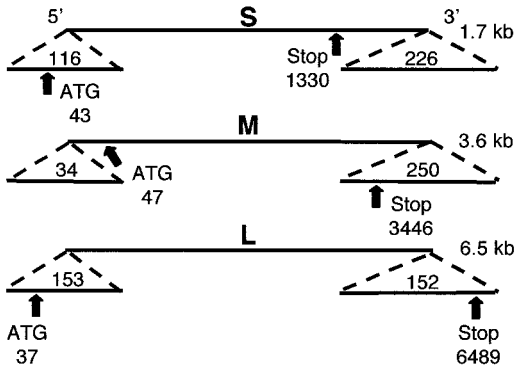

FIG. 8. Diagram of the cloned S, M, and L RNAs regions aligned. The terminal 116 and 226 bases of S RNA, the terminal 34 and 250 bases of M RNA, and the terminal 153 and 152 bases of L RNA were aligned by using CLUSTAL W 1.7. The positions of the start (ATG) and stop codons are indicated.

ability to replicate and/or transcribe could have a role in downregulating replication by accumulating in the population and thus reducing the percentage of replication-competent vRNAs. This is consistent with our sequence data showing that the accumulation of 3′-deleted S, M, and L vRNAs in the population by day 7 p.i. occurred just before the time when the viral titer and the concentration of viral RNA declined. Because a higher percentage of deletions were present at the 3′ end of L vRNA compared to M and S, this also could result in fewer L templates and lower levels of L protein compared to N, G1, and G2. Such a reduction in polymerase protein could contribute to the downregulation of replication and the maintenance of persistence. RNAs with 3′ deletions could also downregulate replication by competing with standard virus for binding N protein. Encapsidation of terminally deleted RNAs with N protein is supported by our finding that L RNAs with short terminal deletions are found in virions. Further support is provided by two earlier studies that showed that terminally truncated RNAs of other bunyaviruses could bind N protein in vitro (9, 29). Soluble N protein not bound in nucleocapsids is required for the replication of influenza virus (3, 37) and is believed to be necessary for bunyavirus replication as well. Thus, as the percentage of 3′-truncated vRNAs increased in the population and bound N, the pool of soluble N protein would become limiting and replication would be downregulated.

TABLE 3. Nucleotide and amino acid changes in clones

Clone type	No. of clones[a]	Nucleotide position[b]	Nucleotide/amino acid[c]	
			WT	Change
S[d]	6/37	20*	G/nc	A
S vRNA	1/48	1689	T/nc	C
S vRNA	1/48	1719	C/nc	A
S cRNA	1/48	1760*	C/nc	T
M vRNA	1/47	9–10*	GA/nc	TG
M vRNA	1/47	19*	G/nc	T
M vRNA	1/47	3433	G/Lys	T/Asn
L cRNA	7/59	75	G/Glu A/Glu	
L cRNA	4/67	6403	G/Val	A/Met
L[d]	44/67	6478	T/Ser	C/Pro

[a] Number of clones with change/number of clones aligned.
[b] Numbering is from the 5′ terminus of the cRNA sequence. An asterisk indicates there is a nucleotide change in the conserved terminal sequence.
[c] The nucleotide indicated corresponds to the sequence of the cRNA strand. WT, wild type; nc, noncoding region.
[d] Mixture of cRNA and vRNA clones.

Although vRNAs with large 3′ deletions would not be expected to replicate, vRNAs with very short 3′ deletions potentially could do so, according to a recently proposed prime and realign model for Hantaan virus initiation (8). In that model, RNA synthesis is initiated by alignment of GTP with the third or sixth C residue of the triplet repeat at the 3′ end of vRNA. After a few nucleotides are added, the nascent strand is proposed to slip backwards and realign with the preceding identical triplet repeat. This would result in an overhanging G residue, which is then postulated to be cleaved by the viral polymerase to yield a U monophosphate at the 5′ termini of cRNAs. The process of internal initiation and realignment predicts that if RNA templates were missing a few 3′ nucleotides replication may not be affected and that some of those 3′ deletions might not appear at the 5′ terminus of the nascent cRNA. In contrast, support for limited replication of 3′-deleted templates comes from the observation that when the 3′ terminus of the ambisense S RNA of Rift Valley fever virus is missing one nucleotide, replication is only slightly affected, and when two nucleotides are missing, replication decreases to 30% (27). In our study, we found several clones with 3′-terminal deletions that were less than six nucleotides long, which could have been used as templates in a prime and realign mechanism. We also found a few S and L cRNAs with short 5′ deletions that could have arisen by replication of 3′-deleted S and L vRNAs. However, the majority of RNAs had larger 5′ and 3′ deletions, and replication of those 3′-truncated RNAs and the occurrence of those 5′-truncated RNAs cannot be explained by this model.

The deletions at the 3′ termini of the vRNAs could arise from events such as early termination of the viral polymerase due to limiting components needed for replication when the synthesis of vRNA was increasing. This idea is supported by the observation that as the concentration of total vRNA increased between days 2 and 7 p.i. (Fig. 3), so did the proportion of 3′-deleted S, M, and L vRNAs identified by cloning (Table 1). The decrease in concentration of the vRNAs and cRNAs by 26 days p.i. and at other times during persistence could in turn be due to the eventual degradation of the 3′-deleted vRNAs that could not be replicated. Loss of 3′-truncated RNAs would relieve competition for limiting factors and/or result once again in a higher proportion of full-length RNAs in the population. This, in effect, would reverse the process of downregulation and most likely result in a burst of replication until the cycle repeated itself. If correct, this theory predicts that RNAs with 3′ deletions could downregulate RNA synthesis and play an important role in holding virus replication to a low enough level to allow persistence.

The other deletions that were common among the viral RNAs were those at the 5′ terminus of L vRNA and at the 3′ terminus of L cRNA. The deletions at the 5′ terminus of the L vRNAs and at the 3′ terminus of L cRNAs appeared to vary in a cyclical manner during infection. Such RNAs could function similarly to the 3′-deleted vRNAs in establishing and maintaining persistence by downregulating replication. However, deletions at the 5′ termini of L vRNAs could not arise by premature termination as could deletions at 3′ termini. Because the majority of 5′ L vRNA deletions detected by primer extension experiments were 9 to 15 nucleotides long and because the prime and realign model would only account for 5′ deletions of less than six nucleotides, as discussed above, the deletions must have been created by another mechanism. One possibility is de novo initiation at any nucleotide in a 3′-deleted RNA, combined with a means to generate a 5′ monophosphate

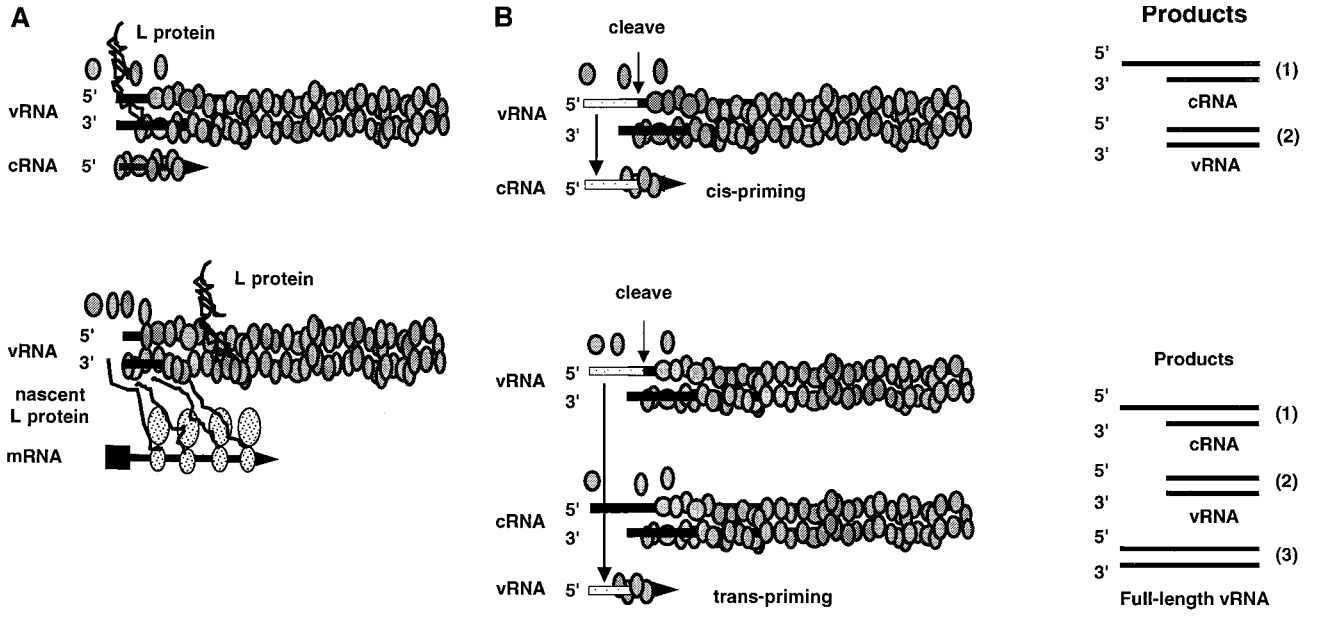

FIG. 9. Diagram of a self-priming model. (A) Replication of L cRNAs and transcription and translation of L mRNAs. As more L protein is synthesized, the increase in polymerase concentration in the microenvironment around the L nucleocapsids leads to cleavage of the L RNA termini. (B) In *cis* priming (top), a primer is cleaved from the 5′ terminus of a 3′-deleted vRNA and used as a primer to synthesize a 3′-deleted cRNA (right) from the same or another 3′-deleted vRNA template. The remaining product of this reaction is a 5′- or 3′-deleted vRNA. In *trans* priming (bottom), a primer cleaved from a 3′-deleted vRNA is used as a primer to synthesize full-length vRNA (right) from a 3′-deleted cRNA template. The other products of this reaction are a 5′- or 3′-deleted vRNA and a 3′-deleted cRNA.

terminus, such as with an RNA 5′ triphosphatase. The existence of some mechanism to replicate RNAs with these larger 3′ deletions is supported by the fact that many deletions at the 3′ terminus of L cRNA are similar in size to the deletions at the 5′ L vRNA terminus. Precedent for replication of RNAs with these larger-sized deletions is found in a natural infection with LCMV, where terminally truncated RNAs appeared to be capable of replication (22).

As an alternative way to explain how the 5′ L vRNA deletions might arise during replication by a mechanism other than prime and realign or de novo synthesis from 3′-truncated templates, we propose the following model. In our model (Fig. 9A), the viral polymerase (L protein) first functions to unwind the termini and to transiently dissociate the RNA from protein to allow base pairing of the nascent oligoribonucleotides. As L vRNA is copied to produce mRNA, ribosomes may load immediately on the nascent mRNA and begin synthesis of additional L protein. As more L protein is synthesized, its concentration would increase in the microenvironment around the L RNAs but would not do so around the S and M RNAs. Because bunyavirus L proteins have a nuclease activity that is used to acquire 5′-capped oligoribonucleotide primers from host mRNAs for transcription initiation, an increase in the concentration of L protein around replicating L RNAs could lead to the cleavage of fragments from the termini of those RNAs. The sizes of the deletions found at the 5′ L vRNA termini by primer extension (approximately 9 to 15 nucleotides) and the presence of G residues at positions 10, 15, and 16 are consistent with the features of cap-snatching by the viral polymerase. The polymerase commonly cleaves primers from host mRNAs that are 10 to 14 nucleotides long, and cleavage frequently occurs after G residues (8, 12). Both 5′ and 3′ deletions could occur via L protein nuclease activity or, as already discussed, 3′ deletions could occur by premature termination. While one might expect that the 5′ L cRNA termini would also be cleaved, the sequence of the 5′-terminal 20 nucleotides (and perhaps the resulting panhandle structure) of L vRNA differs slightly from that of L cRNA (and S and M RNA) and might in itself alter the affinity or function of the L protein interaction (see Fig. 1). Clearly, the presence of deletions at the 5′ terminus of L vRNA and the 3′ terminus of L cRNA and the absence or near absence of deletions at those termini in the S and M RNAs suggests that the mechanism of L RNA synthesis may be very different from that of S and M RNA.

By analogy to the mechanism of cap snatching, after the viral polymerase cleaves the 5′ L vRNA terminal fragments the fragments could be used as primers to initiate synthesis from either full-length or 3′-truncated RNAs in a self-priming mechanism. Although a number of variations of this model could be imagined, Fig. 9B shows the simplest version, where synthesis is initiated from 3′-truncated L vRNAs or cRNAs. The model could operate in the following way. The viral polymerase cleaves the 5′ terminus of a 3′-deleted L vRNA to provide a primer to initiate synthesis from itself by *cis* priming or from a 3′-deleted cRNA by *trans* priming. The 5′ primer could be cleaved from a 3′-deleted L vRNA or a full-length L vRNA, but the latter choice would generate vRNAs with a 5′ deletion and not a 3′ deletion, and few of those were cloned. For self-priming in *trans* the primer could be any length, and the products resulting from this reaction were abundantly represented among the RNAs cloned in this study: 5′- or 3′-truncated vRNAs, 3′-truncated cRNAs, and full-length vRNAs. Self-priming in *cis* would be more limited than *trans* priming because the primer would need to be $\leq$8 bases to maintain the sequence differences between cRNAs and vRNAs at positions 9 and 10 in their 5′ ends (see Fig. 1). The abundance of 5′ L vRNA deletions 9 to 15 bases long, and the restriction of primer size in *cis* priming ($\leq$8 bases long) suggests that *trans* priming would be favored. A self-priming mechanism would be consistent with the wave-like changes detected in the population of 3′-truncated L cRNAs and 5′-truncated L vRNAs and could provide a mechanism of fine control over L protein expression. Self-priming could also provide a mechanism to

assure the continued production of full-length L vRNAs in the cell when levels might be critically low.

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