A Novel Type of Defective Viral Genome Suggests a Unique Strategy To Establish and Maintain Persistent Lymphocytic Choriomeningitis Virus Infections

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Defective interfering RNAs have long been thought to be a causal factor of persistent RNA virus infections. Here we describe a novel type of defective genome of lymphocytic choriomeningitis virus and the unique mechanism by which these RNAs appear to contribute to the establishment and maintenance of persistent infection. The defective genomes have short deletions in the untranslated regions at their termini and additional nontemplated terminal nucleotides. This and previous work from our laboratory suggested that the RNAs were competent for replication but not for transcription. From experiments using a technique to unambiguously determine the sequences of individual RNA termini, it appears that some truncated RNAs can be repaired. The data suggest that the loss or gain of nucleotides from the RNA termini during the course of infection is the mechanism for establishing and maintaining persistence.

Viral infection in animals or cells in culture normally is characterized by the production of defective interfering (DI) particles that contain viral genomes lacking one or more functions necessary for independent replication (2, 22, 24, 29, 40, 45). DI RNAs have an important role in down regulating viral protein expression and establishing persistent infections, probably by competing with the standard genome for replication for virus-encoded proteins. Common types of DI RNAs of RNA viruses contain sequence rearrangements or large deletions in protein-coding regions, along with at least one complete terminal sequence. This results in RNA templates that support replication but do not support transcription of mRNAs that can direct the synthesis of functional protein. Because complete terminal sequences are a minimum requirement for replication of DI RNAs for some viruses, and because these sequences are often highly conserved among any one virus group, it has been assumed that alterations in terminal sequences of an otherwise-intact genome would be detrimental. However, recent results of reverse genetic studies contradict this idea (10, 14, 19, 25-27, 38, 47, 56). Deletions and nucleotide substitutions in, or nucleotide additions to, conserved terminal sequences of otherwise-intact viral genomes do not always eliminate replication and transcription but, instead, can affect these processes in subtle ways. In addition, missing terminal nucleotides can be replaced in a nontemplated manner and nonviral terminal extensions can be copied and maintained during replication. These observations indicate that during a natural infection, the presence of viral genomes with small changes or deletions in terminal sequences has the potential to modify viral gene expression in ways that could alter the course of infection. The generation and accumulation of RNAs that are replication but not transcription competent could disrupt the virus life cycle, as is typically seen when DI RNAs are present in infected cells. Although viral genomes with terminal sequence deletions have been isolated from natural infections (11, 17, 18, 44, 55), the data in this report combined with our earlier work on infections with lymphocytic choriomeningitis virus (LCMV) are the first to suggest that viral RNAs with terminal deletions have a role in the outcome of infection. Our accumulated data suggest that truncated RNAs are a new type of DI genome that contributes to the establishment of LCMV persistence and that addition of nontemplated nucleotides to deleted RNA termini may repair the RNAs and help to maintain the persistent infection. The mechanism that produces these terminal sequence changes in the LCMV genome is unknown. However, some viral polymerases synthesize RNAs with terminal deletions and add nucleotides to RNA termini in a nontemplated fashion (3, 9, 32, 35, 37, 50). Interestingly, these activities are common in numerous other polymerases (both RNA and DNA) throughout the animal and plant kingdoms, such as the RNA-directed RNA polymerase from tomatoes, polymerase α from chicken embryos, rat polymerase β, DNA polymerase I from Saccharomyces cerevisiae, DNA polymerase I and RNA polymerase from E. coli, and DNA polymerase from Thermus aquaticus (8, 9, 23, 28, 48), begging the question of whether the generation of heterogeneous termini may have a more widespread role in modulating gene expression.

MATERIALS AND METHODS

Virus strain, cell culture, and animal infections. LCMV (Armstrong strain CA-1371) was plaque purified three times and used to infect BHK-21 cells at a multiplicity of infection of ≤1, as previously described (33). BALB/c mice (Charles River Laboratories, Inc.), VAF/Plus, were bred in an isolation room, and newborn mice were infected within 24 h of birth by intracerebral injection of 100 PFU of virus.

Northern hybridization. RNA was electrophoresed on 1.5% glyoxal gels, transferred by vacuum onto MagnaGraph membranes (Micron Separations, Inc.), and detected by hybridization to 32P-labeled randomly primed probes representing the entire S segment and the majority of the L segment sequence (33).

RNA isolation. RNA was isolated from brains aseptically removed from infected mice at 3, 5, 7, 9, 12, 14, 19, 21, and 28 days and 2 years postinfection (p.i.). The tissue was homogenized in guanidine thiocyanate, and the RNA was recovered as the pellet fraction after centrifugation through a 5.7 M cesium chloride cushion (7). Infected BHK-21 cells were harvested and gently lysed in detergent, and viral RNA was isolated from nucleocapsids (containing only genomic and antigenomic RNAs) purified on a 20 to 40% CsCl gradient, as previously described (33).
Cloning of terminal sequences. Nucleocapsid RNA (0.5 μg) was decapped with tobacco acid phosphatase (Epiconcept Technologies) as previously described (33, 34). The 5’ RNA termini were ligated with RNA ligase (New England Biolabs) to a synthetic 43-mer RNA oligonucleotide (4), the sequence from position 58 to 78, and the products were separated on denaturing polyacrylamide gels (34). Probes were synthesized with either T7 or SP6 polymerase from plasmids containing cloned terminal sequences of the S and L RNAs. The S probes used to map the 5’ terminus of antigenomic RNA and the 3’ terminus of genomic RNA were synthesized from a clone containing a 5’ antigenomic RNA terminus shown in Fig. 4B, with the extra nucleotides 5’AAG 3’ at the ligated junction. The L probes were synthesized from a clone isolated previously (34). The probes consisted of nucleotides complementary to each terminus (5’ S genome, 365 bases; 5’ S antigenome, 306 bases; 5’ S antigenome, 306 bases; 3’ L genome, 104 bases; 5’ L antigenome, 104 bases; and 3’ L antigenome, 117 bases) flanked by 10 to 48 nucleotides that were not complementary to LCMV RNA. The sizes of the products were calculated according to the distance that RNA markers of known size migrated in the gel. The sizes of bands in control experiments (reactions with the probe alone, reactions with the probe after RNase treatment, and mock reactions where RNA synthesized in vitro was substituted for RNA isolated from infected cells) were within 1 nucleotide of actual size, indicating that the number of missing and additional nucleotides designated in Fig. 3 are correct to within 1 nucleotide. The control experiments were cloned into the multilonging site of pGEM-3Z and sequenced as previously described (33, 34).

RESULTS

LCMV infection of newborn BALB/c mice progresses from an initial acute phase of active virus replication and virion formation to a life-long persistent infection that is associated with sustained but lower levels of virion formation (12, 30, 39, 51). The primary molecular characteristics of persistent infections are the accumulation and maintenance of high levels of intracellular LCMV RNAs and an uncoupling of the synthesis of the major structural proteins, nucleoprotein (NP) and glycoprotein (GP) (36, 52). NP is uniformly distributed throughout the cytoplasm of persistently infected cells, whereas surface expression of GP is significantly down regulated relative to that in acutely infected cells. DI particles have been implicated in LCMV persistence (42, 53, 54), but no detailed molecular explanations are presently available to account for the derivation and functioning of putative LCMV DI RNAs. We have therefore begun to characterize intracellular LCMV RNAs that appear during the transition from acute to persistent infection in order to determine whether altered intracellular viral RNA templates (Fig. 1) might account for the attenuation of viral gene expression that occurs during persistent LCMV infections.

Newborn BALB/c mice were injected intracerebrally with 100 PFU of LCMV (Armstrong strain), and total cellular RNA was isolated from brains of infected mice at 3, 5, 7, 9, 12, 14, 15, 21, and 28 days and 2 years p.i. (Fig. 2) of RNAs isolated from mice showed that the S genomic segment increased in concentration during persistence, indicating that replication continued during the time that viral GP expression and the release of infectious virions are normally reduced (36, 52). The synthesis of NP and GP mRNAs transcribed from S also increased in concentration, although to a lesser extent than S RNA. L RNA was much less abundant than S RNA and was not detectable in this experiment at the early time points. The S and L RNAs, as well as the mRNAs, appeared to be full length throughout the infection. Additional exposures of the blots showed that the heterogeneously sized RNAs detected at late time points were not uniquely associated with persistence. Discretely sized subgenomic RNAs, typical of DI RNAs, were not detected (16), indicating that if substantial numbers of RNAs with deletions were present and contributed to down regulation of viral protein expression, the deletions would be relatively small.

Analysis of RNA termini. To determine whether small changes in the RNA templates were present, which could account for continued replication and a decrease in detectable viral protein, we examined the terminal regions of S and L RNAs. Sequence changes in the terminal highly conserved 19-nucleotide sequence, proposed to contain the sites for initiation of replication and/or transcription in arenaviruses, would likely have a significant effect on viral replication and protein expression (Fig. 1) (1, 46, 51). If such RNAs accumulated, they also would have the potential to alter the course of infection.

Using RNase protection assays, the terminal 104 to 365 nucleotides of each 5’ and 3’ genomic and antigenomic S and L RNA were examined, in samples isolated 3, 7, and 28 days and 2 years p.i. (Fig. 3). At 3 and 7 days p.i. the sizes of the protected S RNAs (Fig. 3) indicated that the RNA population was composed of a mixture of RNAs with full-length termini, RNAs approximately 4 nucleotides longer at their terminus than unit length, and RNAs missing only a few terminal nucleotides. The sequences of the probes protecting the 3’ termini of the S genomic RNAs and the complementary 5’ termini of the S antigenomic RNAs indicated that the 4 additional nucleotides at these termini must be 3’ UUUCC 5’ and 5’ AAAG 3’, respectively (see Materials and Methods). The sequences of these extensions are also complementary to each other. Although the probes complementary to the 5’ termini of genomic and antigenomic RNAs are also complementary to the 5’ termini of the viral mRNAs, mRNAs are a relatively small proportion of the population (Fig. 2). Because mRNAs also have been shown to have very heterogeneous 5’ extensions (34), the specific 4-nucleotide extension detected is most likely one which is attached to genomic and antigenomic RNAs. The intensity of the gel bands corresponding to these products indicated that a sizable portion of these S RNA termini had additional nucleotides of this sequence. The 5’ end of S genomic RNA and the complementary 3’ end of S antigenomic RNA were most often missing the first 3 terminal nucleotides. These results coincided with earlier work in our laboratory, where we found that the virus stock as well as a small portion of the S and L RNA population isolated during the first 24 h of an acute infection of BHK-21 cells were missing a few terminal nucleotides or had additional nontemplated nucleotides at their termini (34).

The termini of the L RNAs at 3 and 7 days p.i. (Fig. 3) were more varied in length than those of the S RNAs. The population of L RNAs was composed primarily of molecules missing up to 14 nucleotides, consistent with the types of deletions previously found during acute infection of BHK-21 cells (34). A small proportion of 5’ genomic RNA and the complementary 3’ terminus of the antigenomic RNA strand had 4-nucleotide extensions complementary to nucleotides in the multicloning region of the vector (Fig. 3), which were 3’ U GGA 5’ and 5’ ACCU 3’, respectively. These 4 nucleotides are also complementary in sequence to each other. In general, most of
the L RNA termini were missing part or all of the highly conserved 19-nucleotide terminal sequence.

At 28 days and 2 years p.i., the proportion of full-length or longer S RNA termini decreased and the proportion of deleted termini increased from those detected at 3 and 7 days p.i. Only 30 to 40% of the viral RNA population at 28 days and 2 years p.i. (with the exception of the 3' terminus of the S antigenomic RNA) had retained full-length or longer termini (Fig. 3). The remaining S RNAs in the population (60 to 70%) were missing up to 25 nucleotides. L RNA showed a similar pattern at most termini, where RNAs with terminal deletions of up to 40 or more nucleotides accumulated. However, the 5' end of L antigenomic RNA appeared to gain 1 to 6 terminal nucleotides at late time points, and these RNAs also accumulated during persistence.

Many of the same-size deletions on S and L RNAs were present at 28 days p.i. and 2 years later. Mapping the termini by primer extension showed that the RNAs were truncated rather than having sequence mismatches with respect to the probes used in RNase protection experiments (data not shown).

FIG. 1. Diagram of the LCMV genome and proposed replication strategy for arenaviruses. (A) The two ambisense single-stranded genomic segment RNAs, L (7.2 kb) and S (3.4 kb), encode two proteins each (hatched bars), L (viral polymerase), Z (viral protein of unknown function), NP, and GP. Each segment is flanked by a 19-nucleotide complementary inverted repeat (black boxes), which is conserved among all arenaviruses and believed to contain the sites for initiation of replication and transcription (1, 46, 51). (B) After entry into the cell, genomic S RNA (shown) and L RNA (not shown) are used as templates for the synthesis of an antigenomic, complementary RNA copy. Synthesis of additional genomic RNA and the GP mRNA initiates at the 3' terminus of antigenomic RNA, while NP mRNA is transcribed from the 3' terminus of the genomic strand. Transcription for both NP and GP mRNA terminates at random points between the coding regions, in an area predicted to form a highly stable hairpin loop structure (33).
The deleted termini were probably not generated from cleavage by cellular or contaminating sequence-specific RNases, because little homology is present in the sequence flanking the deletions of the differently sized truncated RNAs. Random exoribonuclease digestion also would not generate terminally truncated RNAs with specific-size deletions. No evidence of other sequence mutations in the S and L RNAs were detected during the 2-year period. The lack of mutations may have been due to a lack of replication; however, the increase in RNA concentration from 3 days to 2 years p.i. indicates that replication continued throughout the course of infection. Because genomes of other viruses have a relatively short half-life when replication is blocked (6, 13), LCMV RNAs having the same-size terminal deletions (or additions) would not be expected to persist from 28 days to 2 years p.i. Thus, the accumulation of molecules with identically sized deletions in RNA isolated from individually infected mice over the 2-year period indicates that either these RNAs were replication competent or RNAs with these specific deletions were continuously synthesized during persistence and suggests that the presence of terminally truncated RNAs is an important component of persistence.

Location of terminal nontemplated nucleotides. We previously found that nontemplated nucleotides were joined to LCMV RNA termini, but we were not able to identify which termini due to the cloning method used (34). To understand

![FIG. 2. Northern blot analysis of LCMV RNA during acute and persistent infection of BALB/c mice. Total RNA from the brains of infected mice was isolated from 3 days (d) to 2 years (y) p.i., as indicated above each lane, separated on glyoxal-agarose gels (20 μg per lane), and hybridized to 32P-labeled randomly primed probes representing the entire S segment sequence and the majority of the L segment sequence. L, L RNA; S, S RNA; NP and GP, NP and GP mRNAs, respectively.](http://jvi.asm.org/)

![FIG. 3. Ribonuclease protection analysis of the termini of the S and L RNAs isolated at 3, 7, and 28 days (d) and 2 years (y) p.i. Uniformly labeled 32P-RNA probes complementary to either the 5′ or 3′ termini of the S and L genomic and antigenomic RNAs were annealed separately to portions of the RNA samples shown in Fig. 1, and after RNase digestion, protected products were detected on denaturing polyacrylamide gels. Numbers at the right of the bands in each panel indicate the number of nucleotides missing from (−) or added to (+) the terminus.](http://jvi.asm.org/)
what function the addition of nontemplated nucleotides might have in replication, we devised a different cloning method to determine whether extra nucleotides were present at all or at only specific termini. If present at all termini, then their addition could serve the purpose of repairing truncated RNAs to fully functional genomes. Because it was not possible to cleanly separate nucleocapsid RNA (containing genomic and antigenic RNAs) from mRNA in infected tissues, as it is from cultured cells, sequences of individual termini were obtained by directly ligating RNA recovered from nucleocapsids isolated from BHK-21 cells 24 h p.i. to either RNA or DNA (Fig. 4A and B). Intermolecular ligation before cDNA synthesis eliminated the possibility of terminal sequence artifacts caused by reverse transcriptases, where nontemplated nucleotides are added to the 3′ ends of cDNAs after the 5′ termini of RNAs have been copied. The sequences of termini cloned by this method showed that 1 to 3 nontemplated nucleotides were present at all 5′ and 3′ termini of the S genomic and antigenic RNAs (Fig. 4C). The nontemplated nucleotides present at the 5′ termini of the antigenic RNAs, 5′ AAG 3′, confirms the results from ribonuclease protection experiments where the longer sequence 5′ AAAG 3′ (plus or minus 1 5′ nucleotide according to size estimates from gels) was predicted. This cloning method revealed somewhat different terminal sequences than when intramolecular ligation was used (34), but the results are consistent with studies which have shown that efficient ligation of donor and acceptor RNAs is influenced by the terminal nucleotides available for ligation (references 15, 20, 21, 31, and 49 and unpublished data).

Two of the clones generated by this method contained nucleotide differences with respect to the LCMV sequence (Fig. 4). These differences were found specifically within the terminal 8 nucleotides of the genomic and antigenic RNAs and not in the adjacent 50 or more nucleotides. We have previously found small numbers of changes only in this 8-nucleotide region of the genomic and antigenic S RNAs (34) and not in the termini of the NP and GP mRNAs, which were cloned by identical methods (33). These results suggest that errors in this region are the result of a specific event during replication.

**DISCUSSION**

We have shown that replication occurs during persistent LCMV infections and that viral RNA accumulates when the production of infectious virus and viral GP is characteristi-

FIG. 4. Location of nontemplated terminal nucleotides at the termini of S RNAs. (A) Intermolecular ligation strategy for cloning 5′ terminal RNA was decapped with tobacco acid pyrophosphatase (TAP) and ligated to a 43-mer synthetic RNA oligonucleotide, and cDNA was synthesized across the ligated junction. Specific primers were used to amplify either genomic or antigenomic cDNA by PCR. (B) Intermolecular ligation strategy for cloning 3′ terminal. Untreated viral RNA was ligated to EcoRI-cut pGEM-3Z DNA, the 3′ end of the staggered EcoRI-cut plasmid was used to prime synthesis of cDNA, and specific primers were used to amplify either genomic or antigenic cDNA by PCR. (C) Sequences of the cloned S genomic RNAs (top) and antigenic RNAs (bottom). Sequences in boldface type above each group of clones are the published sequences of the terminal 10 nucleotides at each end of the S RNA strands. Sequences of clones are aligned below according to the number of nucleotides missing from each RNA terminus, except where termini were missing 16 or more nucleotides and are indicated with negative numbers. The nucleotides at the far left and right of the clone sequences and separated by a space represent the nontemplated bases found at the S termini. Clones marked by asterisks contain nucleotide errors, which are indicated by lowercase letters.

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tary genomic and antigenomic termini from acutely infected BHK-21 cells (34).

Matched deletions or additions at complementary termini suggest that the RNAs either are copied from each other during replication (end-to-end replication) or are continuously synthesized from full-length templates. The latter possibility would require the use of multiple replication initiation and termination sites in the genomic and antigenomic RNAs. Initiation sites would have to be at the same position as the termination sites on the opposite, template, strand in order for subsequent rounds of replication to generate deletions of the same size on the complementary genomic and antigenomic termini. Furthermore, during persistence, use of internal sites would have to increase over the use of terminal sites to result in a rise in the concentration of terminally truncated RNAs. Although these events could occur, the alternate possibility, that truncated RNAs may be capable of replication, has more support: terminally truncated RNAs are found only in nucleocapsids containing replicating RNAs and are not found in the nonreplicating mRNA fraction of the cell; terminally truncated RNAs are packaged into virions, and when virions containing RNAs with terminal deletions are used to infect mice or BHK-21 cells, the same-size deletions are present after infection and the deletions are maintained (33, 34); and genomes of other viruses having terminal nucleotide additions or deletions are known to be faithfully replicated (27, 47). Therefore, the most likely interpretation is that terminally truncated RNAs are copied from each other during replication and that initiation occurs at the termini of all or most of the RNAs present in the nucleocapsid: those with deleted termini, those with extended termini, and those with normal termini.

Although the conserved 19-nucleotide terminal sequence may not be necessary for initiation of replication, it does appear to be necessary for initiation of transcription because truncated termini are not present in the sequences of cloned viral NP and GP mRNAs or have not been detected when the 5' termini of mRNAs have been mapped by primer extension (17, 33, 41). Accumulation of truncated, transcriptionally incompetent genomes in cells would be likely to down regulate protein synthesis and the production of infectious progeny virions. This would result in infections with the same characteristics as those previously reported for LCMV persistence and attributed to LCMV DI activity (5, 12, 30, 36, 39, 42, 52–54).

The frequency of nontemplated nucleotide additions at both the 5' and 3' RNA termini suggests that the additions may have an important role in the viral life cycle. Because the probes used in RNase protection experiments were not designed to detect nucleotide extensions, our results may underestimate the number of RNAs in the population having terminal extensions. One possible function of an activity that adds extra nucleotides to RNA termini may be to repair the termini of truncated RNAs to their full-length, functional status. Clearly, the concentration of L antigenomic RNAs that have gained nucleotides at their 3' termini increased from 3 to 28 days and 2 years p.i. (Fig. 3). Further support comes from the fact that nucleotide differences are found only in the terminal 8 nucleotides and not elsewhere in the genome. If, during the course of replication, RNAs with terminal deletions have a one in four chance of gaining the next correct nucleotide by random addition, then the chance that termini would gain nucleotides in the correct order when deletions were larger than 10 nucleotides would be extremely low. If addition produced too many errors, the molecules would likely be nonfunctional and subject to degradation, perhaps contributing to the heterogeneously sized RNAs detected by Northern hybridization late in infection (Fig. 2). RNAs that had been correctly repaired could not be distinguished from intact, unrepaired molecules. Only RNAs that were imperfectly repaired but still replication competent would exist as evidence of repair. These RNAs probably would have few errors, and the errors would be in RNAs that previously had only short terminal deletions, exactly matching the characteristics of the molecules that we have found. Interestingly, no errors have been found in mRNA termini, so incorrectly repaired RNAs may not be transcription competent (33). Because the proportions of truncated to full-length RNAs (at most termini) are relatively equal between 28 days and 2 years p.i., the truncated RNAs may be critical in maintaining persistence by keeping viral protein expression low. Likewise, occasional repair of deleted RNA termini may shift the balance of truncated to full-length RNAs slightly and thus lead to a transient increase in protein expression and the production of infectious virus, helping to maintain the infectious state. This hypothesis is consistent with the observations that persistence is characterized by cyclical variations in interference, antigen-positive cells, and the production of infectious virus (39, 42). Repair of truncated RNAs also would explain the

Fig. 5. Molecular model proposed for the establishment and maintenance of persistence. G, genomic RNA; AG, antigenomic RNA. See Discussion for model description.
puzzling observation that LCMV DI particles do not become increased over standard virus during persistence (42).

Our data are consistent with numerous other observations of LCMV infections from other laboratories. The presence of small numbers of truncated RNAs at 3 and 7 days p.i. in mice and during the first 24 h in tissue culture infections correlates with reports of a DI activity, appearing during the first cycle of replication after infection, which is tightly associated with replication of standard virus (34, 39). The large number of intact 3′ termini on the S genomic RNA, the transcriptional initiation site for NP mRNA synthesis, and the lack of many full-length termini at the 3′ end of S antigenic RNA, the transcriptional initiation site for GP mRNA synthesis, are both consistent with the relative abundance of NP and lack of GP in persistently infected cells.

The data support the following model for the establishment and maintenance of persistent LCMV infections (Fig. 5). During replication in acutely infected cells, some viral RNAs lose and gain small numbers of nucleotides at their termini, though the presence of large numbers of full-length genomes results in high expression of viral proteins and the release of infectious virus. Terminally truncated RNAs replicate but are not used as templates for transcription. After 7 to 9 days, more terminal nucleotides are lost or nucleotide gain does not balance this loss, and a new equilibrium of terminally truncated to full-length RNAs is established, initiating persistence. The presence of high concentrations of transcriptionally incompetent RNAs interferes with the amount of viral protein expressed and the number of infectious virions produced. Addition of nontemplated nucleotides occasionally repairs some truncated RNAs to the full-length sequence, upsetting the balance of transcriptionally competent and incompetent RNAs and leading to a transient increase in the production of viral proteins. Fluctuations in the concentration of full-length and truncated RNAs, resulting from nucleotide loss and gain, provide a continuing, low-level, and sporadic source of newly functional RNAs which help to maintain the persistent state and perpetuate the long-term infection for the lifetime of the host.

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