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Vesicular Stomatitis Virus Mutants Resistant to Defective-Interfering Particles Accumulate Stable 5′-Terminal and Fewer 3′-Terminal Mutations in a Stepwise Manner

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We have studied the evolution of sequences which include the RNA polymerase binding sites at the 5′ and 3′termini of vesicular stomatitis virus mutants (Sdi$^-$) resistant to defective-interfering particles. We observed a striking stepwise accumulation of stable base substitutions within the area of replication initiation at the 5′-terminal 54 nucleotides of Sdi$^-$ mutants isolated at intervals from persistent infections and undiluted lytic passage series. Fewer mutations accumulated in the region of transcription initiation at the 3′ end and in those portions of the N and L protein coding cistrons examined. The termini changes are not strictly required to obtain the Sdi$^-$ phenotype. However, it is possible that they represent stepwise compensatory changes to accommodate Sdi$^-$ mutations affecting viral replication or encapsidation gene products or both. These results have important implications for RNA virus genome evolution.

During prolonged, persistent infections mediated by defective-interfering (DI) particles, mutants of infectious virus (Sdi$^-$ mutants) appear which are resistant to interfering effects of DI particles. This was first observed with rabies virus by Kawai and Matsumoto (14) and then with vesicular stomatitis virus (VSV) (7, 8), lymphocytic choriomeningitis virus (13), and Sindbis virus (35). Even bacteriophage F1 generates Sdi$^-$ types of mutants (4). In a recent study (9), we quantitatively characterized the biological and genetic characteristics of a variety of VSV mutants isolated during persistent infections and during serial undiluted passages. The relative DI resistance of these mutants was determined, and numerous Sdi$^-$ mutants which were no longer sensitive to interference by the DI particle present at the beginning of these infections were found to appear and disappear as virus and changing DI particle populations coevolved.

VSV is a negative-strand RNA virus with genomic RNA of approximately 11 kilobases in length (10, 25). Over half of the VSV genome has been sequenced, including complete sequences of the terminal regions (3, 6, 15, 21, 26–28, 30, 32, 33, 36). The structures of the termini of VSV and its DI particles have been reviewed recently (18, 23). These termini are important as replicase initiation sites, transcriptase initiation sites, and sites for small "leader" RNA synthesis (11, 15, 18, 23, 34). These termini are also important as nucleation sites for encapsidation of viral RNA (1), and there may be obligatory coupling between replication and encapsidation of nascent RNA strands (19). Sequence studies of the termini of one VSV mutant recovered after 5 years of persistent infection have previously shown extensive accumulation of mutations at the 5′ end (31) and fewer at the 3′ end (29). In the present report, we present 5′- and 3′-terminal sequences of a collection of Sdi$^-$ mutants of VSV isolated at intervals from persistent infections and from serial undiluted passages and show that they accumulate stable 5′ and (fewer) 3′ end-base substitutions in a stepwise manner. We discuss the possible relationship of these termini alterations to the biological interference phenotype of these Sdi$^-$ mutants and to the coevolution of virus and DI particles during serial lytic passage and during prolonged persistent infections.

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MATERIALS AND METHODS

Virus and DI particles. The isolation and quantitative properties of a collection of Sdi$^-$ mutants and their DI particles were presented earlier (9).

Purification of viral RNA. RNA was extracted from purified VSV virions by proteinase K digestion and phenol-chloroform extraction, followed by an ether extraction and repeated alcohol precipitation.

Chemical synthesis of DNA primers. Complementary DNA primers employed for sequencing the 3′- and 5′-terminal regions of viral RNA were ACAAACAGAAGCA and AGGTAAGTTAGTAAGG, respectively, These were synthesized on solid support, using the phosphotriester approach essentially as described by Miyoshi et al. (22) and Ito et al. (12).

Primer extension using reverse transcriptase. Gel-purified DNA primer (1 to 10 ng) end labeled with ^{32}P in the 5′ position was annealed to 2 μg of viral RNA and then extended by using 3 U of avian myeloblastosis virus reverse transcriptase (obtained from Life Sciences, Inc.) in 20-μl reactions containing 50 mM Tris-hydrochloride (pH 8.3), 70 mM KCl, 8 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.5 mM each of the four deoxynucleoside triphosphates at 37°C for 1 h. Product DNA was separated from excess unextended primer on Sephadex G-100 or G-75 and sequenced by the method of Maxam and Gilbert (20).

Virion transcriptase assay. Virion in vitro transcriptase activities of a number of Sdi$^-$ mutant viruses were compared with that of the standard *tsG*31 virus, using purified virion cores prepared and assayed essentially as described earlier (2). Virus pools were all grown, purified, and assayed in parallel.

RESULTS

Sequencing strategy. The method employed to obtain comparative sequences of a large variety of VSV mutants

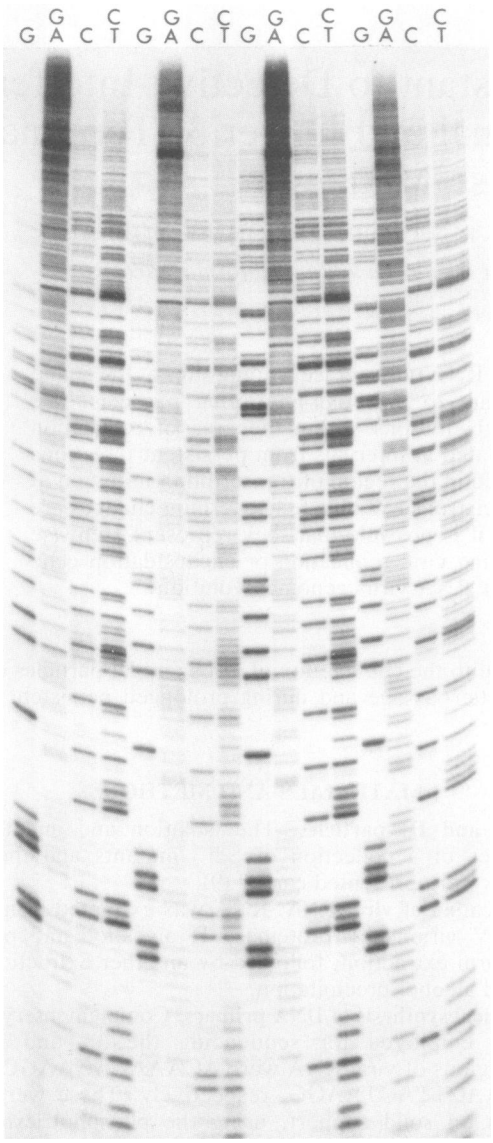

FIG. 1. DNA sequencing gel of DNA molecules extended from synthetic oligonucleotide primer exactly complementary to the first 13 bases at the 3′ end of VSV isolates. The DNA sequencing method of Maxam and Gilbert (20) was employed on single-stranded DNA after primer extension by reverse transcriptase of ^{32}P-labeled dACAAACAGAAGCA. The sequencing patterns for four mutants are shown. For each mutant, the lanes from left to right display G, G+A, C, C+T.

was to synthesize a specific deoxynucleotide primer complementary to known sequences 3′ proximal to one region of interest, extend the primer with reverse transcriptase, and sequence the reverse transcript. The dideoxy chain termination technique is usually the method of choice because of its relative ease compared with the method of Maxam and Gilbert (20). However, in our reverse transcriptase reaction with VSV RNA templates, we observed strong stops which led to ambiguities in sequence determination by the dideoxy method. Most of these ambiguities can be eliminated by utilizing chemical cleavage sequencing which yields 3′ phosphate-containing fragments that can be electrophoretically resolved from the 3′ hydroxyl-containing premature termination products. Therefore, we employed a sequencing approach involving reverse transcriptase extension of a 5′-labeled DNA primer followed by chemical sequencing by the technique of Maxam and Gilbert (20). The infrequent ambiguities in the sequences presented below are mostly due to strong stops which could not be resolved from the chemical cleavage product. Figure 1 shows an autoradiograph of a sequencing gel employing Maxam-Gilbert cleavages of an extended 3′ end primer.

5′ End sequences. Since the sequence of the terminal 200 nucleotides was previously determined for the Mudd-Summers strain of VSV Indiana by Keene et al. (15), we confirmed the identity of this sequence for the Glasgow strain, from which most of these mutants were derived. For this purpose, we gel purified an RNase T_1 oligonucleotide product of virion RNA homologous to the sequence between 19 and 40 nucleotides from the 5′ end. We labeled the oligonucleotide at the 5′ end with ^{32}P and annealed the end-labeled oligonucleotide to the products of a randomly primed reverse transcriptase reaction of *tsG*31 genomic RNA. We determined the sequence of the resulting cDNA by the dideoxy chain termination method as utilized by Zimmern and Kaesberg (37). The sequence obtained by this method was identical to the previously published sequence of the Mudd-Summers strain (15), with the exception of a G→A substitution at position 124 which is unique to the Glasgow strain (data not shown).

We therefore synthesized a 16-base deoxyoligonucleotide primer complementary to the sequence between positions 183 and 198 from the 5′ end by the chemical synthesis procedure outlined above. We labeled the primer with ^{32}P by using polynucleotide kinase and confirmed the sequence of the 5′ end-labeled primer by the method of Maxam and Gilbert (20).

For all subsequent 5′ end sequencing by primer extension, we annealed the end-labeled primer to genomic RNAs of a large number of virus variants whose biological properties were presented previously (9). The primer annealed specifically to the desired site on the genomes of all viruses examined, since a homogeneous primer extension sequence exactly 198 bases in length was seen for all of the isolates. An extremely minor additional priming site was seen in some reactions since a gross overexposure of autoradiographs revealed a small amount of cDNA larger than 198 nucleotides, probably due to very low-level priming at a second site on the genome which contained homology with some mismatches. In any case, the minor product was rare, so it never interfered with sequence determinations.

Comparative sequences from position 2 to position 170 are shown in Fig. 2 together with the relative biological activity of the wild-type (*tsG*31) DI particles in eliciting interference with each mutant (9). The 5′-terminal nucleotide could not be identified since it could not be resolved from the intense band of undegraded full-size (198 nucleotides long) cDNA. Only two nucleotide substitutions were observed in the terminal sequences of the L mRNA among all the mutants sequenced. The G→A at position 115 was observed in the 75-day CAR4 isolate and in all subsequent isolates from CAR4, and the T→C changes at position 146 do not appear until 5 years of persistence. In the open reading frame (from base 100 inward) defined by Yang and Lazzarini (36), one of these substitutions is a neutral third-base change.

The most striking feature of the 5′ end sequences in Fig. 2 is the clustering of mutations in the genome terminal 54 nucleotides. Among all of the Sdi$^-$ mutants sequenced to date, there are 11 different positions within the first 54 which

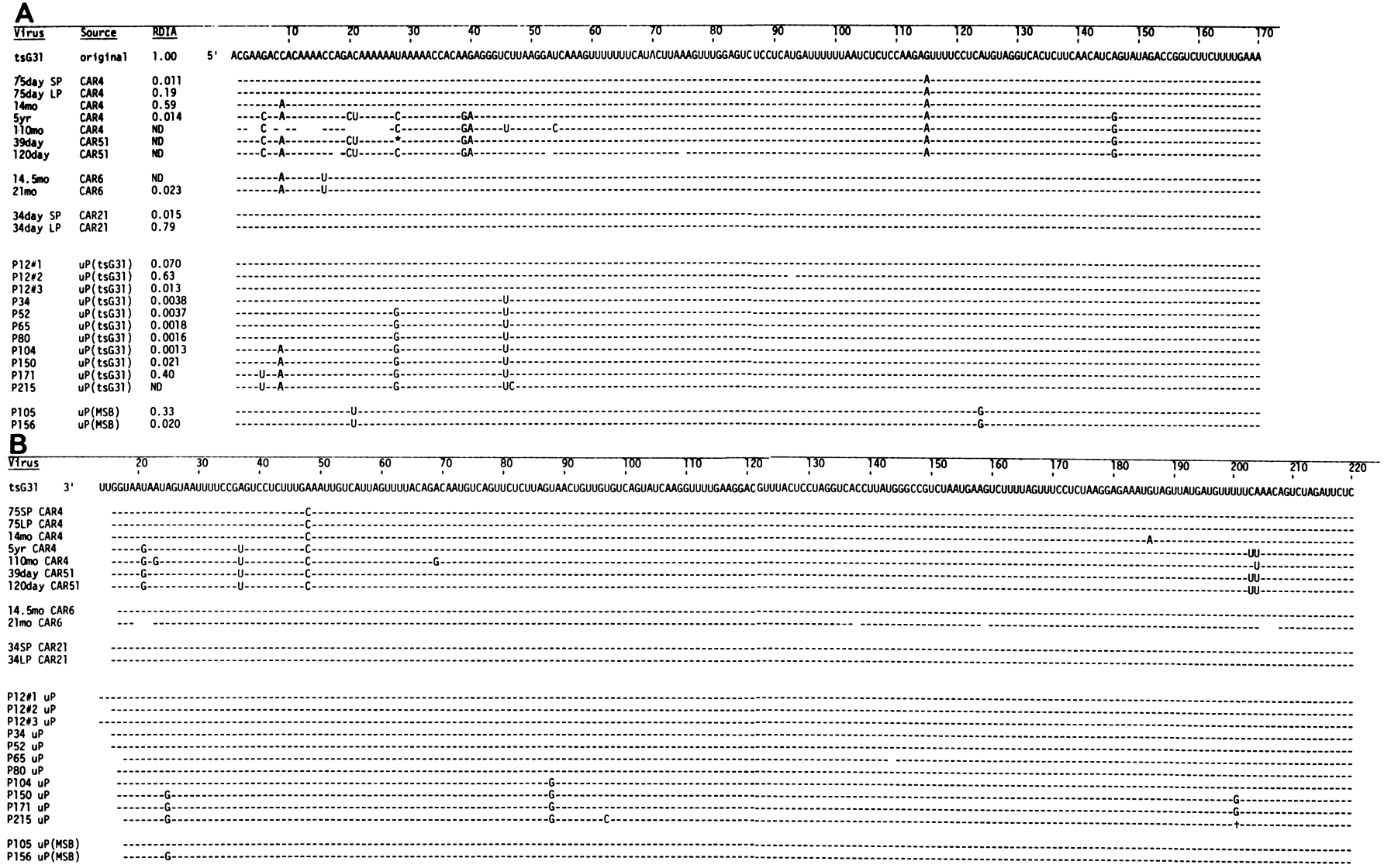

FIG. 2. (A) Comparative sequences of the 5′ terminus of genomic RNA of VSV mutants. The relative DI activity (RDIA) of the *tsG*31 DI particle against these viruses isolated from persistent infections and undiluted lytic passage series was determined and presented previously (9). Abbreviations: uP, undiluted passages; SP, small plaque (mutant) phenotype; LP, large plaque (original) phenotype. Confirmed sequence identities are designated by dashes. Ambiguities due to strong stops are noted by spaces. Position from the 5′ end of VSV RNA is indicated by numbers above the sequence. CAR4, CAR6, and CAR21 designate independently initiated VSV persistent infections of BHK-21 carrier cells (7), whereas CAR51 designates BHK-21 carrier cells which were originally persistently infected (day 1) by a cloned VSV mutant isolated from CAR4 carrier cells after 5 years (plus its homologous DI particles). In the isolates obtained from MSB (P105 and P156), the U at position 21 and the G at position 124 are the same as wild-type MSB. The asterisk indicates that either a G or C (or both) is present at position 28 of 39-day CAR51 genomes (B) Comparative sequences of the 3′ termini of genomic RNA of VSV mutants. Position from the 3′ end of VSV RNA is indicated. Additional sequence information (out to as far as 330 bases) was obtained for many of these mutants, but no changes from the sequence reported by Gallione et al. (6) were observed. The dagger indicates that either a G or U (or both due to microheterogeneity) is present at position 101 in passage 215 genomes. ND, Not determined.

exhibit base substitutions, and base substitutions accumulated in a stepwise manner in the CAR4 carrier cells (and in the CAR51 carrier cells derived from 5-year CAR4 virus). Once a base substitution occurred in these Sdi$^-$ mutants, the changed base remained stable for years thereafter as further substitutions accumulated in these evolving genomes. This suggests that selective pressures maintain these changed bases. This stepwise accumulation of base substitutions is seen even more strikingly in the Sdi$^-$ mutants obtained from the undiluted passage series with *tsG*31 virus. Five base changes occurred, one at a time, with each of the earlier changes again remaining stable as subsequent changes appeared (Fig. 2). Remarkably, four of these changes occurred in the same position as did mutations in the later CAR4 virus isolates, which were derived from long persistence rather than lytic passages. The C→A change at position 9 and a C→U change at position 46 were identical changes. Two nucleotide substitutions were observed in virus isolated from CAR6, including the identical C→A change at position 9 and an additional change at position 16. No mutations were observed in virus from CAR21 after 34 days, nor from the virus isolated after 156 undiluted passages initiated with the Mudd-Summers strain of VSV.

Isaac and Keene (11) presented evidence suggesting that the NS protein component of VSV RNA polymerase contacts a site 17 to 37 bases from the 3′ end of some DI RNAs. This sequence is present in the plus strand of the standard genome and is templated by the sequence 17 to 37 bases from the 5′ end of the minus strand (see Fig. 2). Within this sequence, we observed changes at bases 20, 21, and 28 in the later isolates from persistent infection and undiluted passage series.

Note in Fig. 2 that the Sdi$^-$ phenotype does not strictly require the concurrent presence of 5′-terminal mutations, since early-appearing Sdi$^-$ mutants, such as the 75-day small plaque virus from CAR4 carrier cells and the 34-day small plaque virus from CAR21 carrier cells, show no terminal base substitutions. This is true also for passage 12 plaque isolates numbers 1 and 3 from the undiluted passage series of *tsG*31 virus.

3′ End sequences. The 3′-terminal sequence of the Glasgow strain of VSV Indiana was previously determined to be identical to the Mudd-Summers strain (21, 28). A 13-nucleotide-long primer, complementary to the exact 3′ terminus of the viral genomic RNA, was employed to sequence the 3′ termini. This primer provided a single homogeneous sequence in extended primers with all Sdi$^-$ mutants tested. This indicates that mismatches are not present at the extreme 3′ end in any of these mutant viral RNAs, in agreement with previous studies which have shown that the 5-year CAR4 virion RNA is identical in sequence to the original *tsG*31 virus for 20 nucleotides at the 3′ end (29). The sequence information obtained by using this primer begins at position 14 to 18 from the 3′ end and is shown from position 1 to position 220. Our wild-type (*tsG*31) 3′ sequence is identical to previously published sequences of VSV Indiana (6, 21, 28).

Fewer changes than those observed at the 5′ end occurred at the 3′ ends of Sdi$^-$ mutants (Fig. 2). The 3′ terminus is involved with both replication and transcription and may be more strongly conserved than the 5′ end. Only one change (A→C at position 48) occurred within the 3′-terminal 50 (leader RNA coding) bases during the first 14 months in the CAR4 carrier cells, and this was seen in the 75-day CAR4 carrier cell isolates as well. By 5 years, there were two additional 3′ end-base substitutions; a fourth mutation appeared by 110 months, and the other three remained. In contrast, no 3′ end changes occurred in the CAR6 and CAR21 persistent infections and a single different change (A→G at position 25) occurred in late undiluted passages of both *tsG*31 virus and Mudd-Summers strain virus. However, of the changes that did occur, many accumulated in a stable manner, indicating a selective pressure to maintain these mutations. As is the case for the 5′ end mutations, the 3′ end mutations are not required for the Sdi$^-$ phenotype.

Keene et al. (17) suggested that the sequence 16 to 30 nucleotides from the 3′ terminus may be involved in the initiation of transcription. Within this region, we observed base changes at positions 21, 23, and 25. These changes appeared late in persistent infections and undiluted passage series.

Among all of the Sdi$^-$ mutants, we observed a total of seven base substitutions in the first 170 bases encoding the N terminus of the N protein. Three of these are silent mutations, and four cause amino acid substitutions. It is not yet clear whether any of these are related to any Sdi$^-$ phenotype (the N protein is the encapsidation protein). However, it is clear that none of these are strictly required for the earliest Sdi$^-$ phenotypes to appear during either persistent infections or undiluted passages, since all changes appeared well after the Sdi$^-$ mutants appeared and there were no common mutations among different sources of the Sdi$^-$ phenotypes.

Virion transcriptase alterations. It is possible that Sdi$^-$ mutants have altered replication or encapsidation machinery or both (see below). Because virus polymerase proteins probably are involved in both replication and transcription, mutation of the replicase activity of the complex due to DI pressure may be expected to affect the transcriptase function of the polymerase complex. Alterations in transcriptase activity were found for all Sdi$^-$ mutants tested from the undiluted passage series, including ones with no terminal mutations (Fig. 3). We have not yet explored the molecular

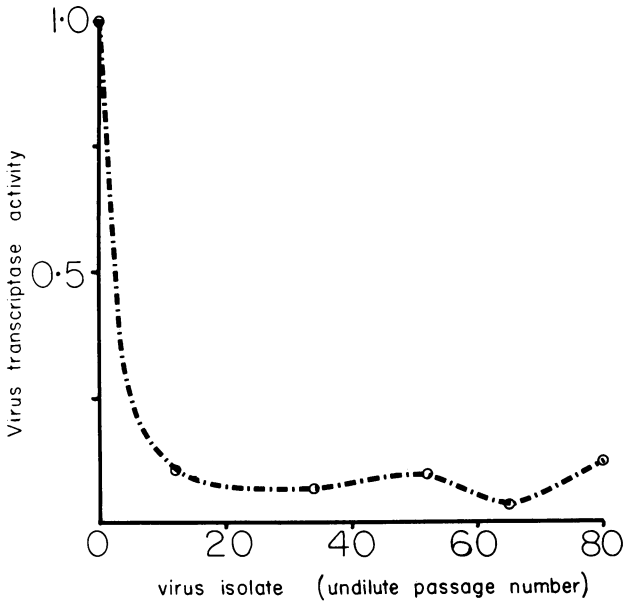

FIG. 3. Virion transcriptase activities of *tsG*31 and Sdi$^-$ mutants derived from serial undiluted passage were assayed as described in the text. Trichloroacetic acid-precipitable counts were measured after 3 h of incubation, and relative activities are normalized to the original *tsG*31 virus.

basis for these altered transcriptase levels, but similar changes in in vivo levels of transcriptase have been reported for VSV mutants from persistent infections (5).

DISCUSSION

The results presented here and in a previous paper (9) constitute a detailed study of the evolution of a virus in a predominantly intracellular environment in which the principal evolutionary pressure is the presence of DI particles. DI particles are known to specifically interfere with the replication of homologous standard virus genomes. The DI genomes are thought to disrupt the function of the standard virus replicase in such a way as to outcompete the standard virus genomic RNAs for available viral replicase complexes (18, 23). In response to this pressure, during persistent infections or undiluted passage series, mutant viruses (Sdi^-) arise which are no longer sensitive to interference by DI particles originally present in these infections (9).

The Sdi^- phenotype entails an acquired ability of the mutant virus replication machinery to discriminate between itself and the original DI particle in a way that the original Sdi^+ standard virus was unable to do. Previous studies with chimeric DI particles (9) showed that the inability of the original DI to interfere with Sdi^- mutant standard viruses was not due to the protein component of these DIs, indicating that the change in standard virus-DI particle discrimination observed in Sdi^- mutants is not at the level of the DI protein component. It might be expected that mutations in the standard viral replication initiation site would allow Sdi^- mutant virus replicase to distinguish between its own RNA and that of the original DI RNA. However, the emergence of the Sdi^- phenotype precedes the appearance of any terminal nucleotide changes in several Sdi^- mutants (Fig. 2). Previous work with the 5-year Sdi^- mutant showed peptide map alterations in each of the VSV proteins, including those involved with replication and encapsidation (29). It is possible that the Sdi^- replication machinery has mutated in such a way that original DI replicative structures can be distinguished from Sdi^- replicative structures in some way other than on the basis of altered replicase initiation sites. Alterations in transcription-replication coupling, altered ratios of plus and minus templates generated, or changes in ribonucleoprotein configurations are some possibilities for standard virus-DI particle discrimination. The later stepwise accumulation of stable mutations in the region of replication initiation might be compensatory changes in response to constraints imposed by interaction with mutationally altered replicase proteins.

Fewer mutations were found to accumulate at the 3′ end of Sdi^- virus RNA, in agreement with previous observations on a single 5-year virus isolate (29, 31). The limited number of VSV proteins (24) and the extent of terminal complementarity (14 of 17 nucleotides) at the ends of the viral RNA (16) suggest that the transcriptase complex and the replicase complex share some identical virus-coded proteins. The clustering of mutations at the 5′ end of the viral RNA indicates that a greater genetic variability may be possible at the replicase binding site, in contrast to tighter constraints imposed by transcriptase complex requirements at the 3′ end of the minus strand. As is the case with the 5′ end, a number of Sdi^- mutants have no 3′ terminus change, and we conclude again that 3′ end mutations are not required for the Sdi^- phenotype. However, possible changes in the replicase function of the virus polymerase complex due to DI pressure may affect the transcriptase function of the complex, leading to altered transcriptase activity and, considerably later, to compensatory changes in the transcriptase binding site. Consistent with this hypothesis, altered transcriptase activity was observed in each of the Sdi^- mutants tested (Fig. 3), and later 3′ end changes did accumulate in the region (17) of transcription initiation. In agreement with findings presented here for Sdi^- mutants from undiluted passage series, Frey and Youngner (5) have also seen the appearance of mutants with apparently altered transcriptase activity in vivo occurring during persistent VSV infections of L cells.

It is interesting that few of the 11 base substitutions that we have observed in the first 54 nucleotides from the 5′ end would greatly disrupt the multiple stem and loop self-complementary structure of the 5′ end of virion RNA proposed by Schubert et al. (30). Only three changes occur at internal positions within stems (at positions 20, 47, and 46), and the latter changes an unpaired to a paired base within a stem. It will be important to learn whether this stem-loop secondary structure is generally maintained as we select greater numbers of Sdi^- mutants. The complement of this proposed structure would be a similar structure at the replicase initiation sites of the 3′ termini of virus plus strand RNA and of plus and minus strands of most DI particle RNAs (18, 23).

It will now be important to obtain the 5′ and 3′ terminal RNA sequences of DI particles from our persistent infections and passage series. Particularly, those DI particles showing altered interactions with Sdi^- mutants (9) will provide considerable information regarding DI particle interference requirements for terminal sequence specificities in replication-encapsidation interactions. However, it will be necessary to employ a different sequencing strategy or a modification of the primer extension approach employed here for virus RNA. The presence of long complementary stems or panhandles (or complete "snapback" self-complementarity) (18, 23) strongly interferes with DNA primer extension sequencing.

Finally, Blumberg et al. (1) recently proposed that the encapsidation nucleation site at the 5′ ends of VSV RNAs is a five-times-repeated A residue at every third position from the 5′ end. Our data are consistent with this suggestion since none of the viral 5′ end (minus strand) mutations reported here alter any of these A residues. The specific priming by our 3′ end primer (which includes all of these A residues) with every one of our VSV mutants indicates that there are no changes in the 5′ end of the plus strand RNA complements of these 3′ ends either.

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