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Analysis of the Recombination Event Generating a Vesicular Stomatitis Virus Deletion Defective Interfering Particle

FUNMEI YANG† AND ROBERT A. LAZZARINI*

Laboratory of Molecular Genetics, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205

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cDNA clones of different portions of the L cistron and 5'-terminal region of the vesicular stomatitis virus genome have been prepared and used to identify the exact site of the deletion in the defective interfering particle, DI-LT. The deletion extends from nucleotide 251 from the beginning of the L gene to a position 342 nucleotides from the end of the genome. The nucleotide sequences flanking the deletion site, as well as those at the ends of the deleted segment, did not contain any obvious vesicular stomatitis virus initiation or termination signals as had been found near the recombination sites in other defective interfering particle RNAs. The results best fit a model for the origin of this type of defective interfering particle in which the polymerase interrupts its synthesis and moves with its nascent daughter strand to a new position on the template and resumes synthesis there, further extending the nascent strand. Neither the interruption nor the resumption of synthesis appears to be in response to the template nucleotide sequence. The sequences of two partial L cistron clones also reveal open reading frames that code for amino acid sequences likely to be the amino and carboxy termini of the L protein.

The vesicular stomatitis virus (VSV) defective interfering (DI) particles can be grouped into four types on the basis of their chromosomal structure (11). Although the generation of the different types of DI particles is believed to occur by closely related copy choice mechanisms, the different genomic structures represented in the four classes indicate that special circumstances may favor the generation of one or another type of DI particle. The presence of a sequence at the 5' end of the viral chromosome that is related to the highly conserved transcription and replication initiation site is likely to play an important role in the generation of the "panhandle" type of DI particles (18). Similarly, Keene et al. (9) suggest that a sequence related to the VSV termination sequence may be important in the origin of the "compound" type of DI particle.

Analysis of the sequences flanking the site of the deletion in "simple deletion" DI particles (for example DI-LT) has not been made as yet because the relevant regions of the DI particle or parental virus chromosome are sufficiently far from the termini that the rapid RNA sequencing methods cannot be applied. Earlier studies (7, 15) established that the genome of a major DI-LT was a simple deletion which retained both parental termini. On the basis of the results of heteroduplex mapping we estimated that the site of the deletion on the most abundant DI RNA in the preparation was about 320 to 350 nucleotides from the 5' terminus and that the deletion was completely contained within the L cistron (7). In the present communication, we describe a group of cDNA clones that contain these important regions of the DI particle and parental viral genomes. By sequencing these clones, we have established that the 5' termini of the DI-LT and the parental genomes are the same for 341 nucleotides from the 5' termini. The sequence of the DI genome starting at nucleotide 342 corresponds to that found approximately 5 kilobases further down the parental genome, at a point 250 nucleotides from the beginning of the L cistron. These results, together with those reported earlier, indicate that the genomes of DI-LT and the parental virus are identical from this deletion point to their 3' termini. The sequences of the regions flanking the deletion site do not resemble the VSV initiation or termination sites, and no discernible stable secondary structure could be drawn for the sequences. Consequently, the formation of the deletion is not likely to be

† Present address: Department of Anatomy, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78284.

constrained by the primary sequence of the chromosome as suggested for the generation of the panhandle RNA structures. Other factors besides primary structures may influence DI particle generation. In particular, our analysis does not address the question of whether intra- or intermolecular alignments of the VSV chromosome in the cytoplasm may subtly influence the generation of deletion DI particles (14).

MATERIALS AND METHODS

Viruses and DI particles. The heat-resistant strain of VSV, HR, and the deletion DI particle derived from it, DI-LT, were obtained from Ludvik Prevec and Y. Kang, respectively. The DI-LT was cultured under conditions of minimal autointerference in monolayer cultures of BHK 21 cells. (Earlier analysis [7, 15] revealed that stocks derived from the DI-LT described by Petric and Prevec contained trace amounts of several other DI particles which, upon further passage, could be amplified to represent a sizeable portion of the progeny. Under these conditions, the DI-LT is the most abundant DI particle among the progeny and the DI-LT2, although present, accounts for only 10 to 15% of the total DI particles (7). The VSV DI 011, which contains covalently linked complementary RNA strands, is also of the Indiana serotype, but is derived from the Mudd-Summers strain (12). Viruses and DI particles were prepared and purified as previously described (10, 12). RNA was prepared by phenol-chloroform-sodium dodecyl sulfate extraction of purified virus particles and subsequently fractionated on sodium dodecyl sulfate-sucrose gradients. The RNA was recovered from the gradients by ethanol precipitation.

Plasmids. The plasmid p011-2, containing the genetic information from the 5′ end of the VSV, was prepared from DI 011 RNA by a strategy described below but detailed here. Purified double-stranded DI 011 RNA was polyadenylated with *Escherichia coli* polyadenylic acid [poly(A)] polymerase (Bethesda Research Laboratories). The reactions were carried out in 50-μl solutions which contained 50 mM Tris-hydrochloride (pH 7.9), 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.25 M NaCl, 500 μg of bovine serum albumin per ml, 5 μg of rifampin per ml, 0.25 mM [α-^{32}P]ATP (50 μCi/nmol), 150 μg of DI 011 RNA per ml, and 75 U of poly(A) polymerase per ml. Reactions were terminated after 1 h at 37°C by phenol extraction and ethanol precipitation. The tailed DI 011 RNA was nicked at the turnaround point with RNase T_1 (0.5 U/100 μg of RNA) and repurified by proteinase K digestion, phenol extraction, and ethanol precipitation.

Poly(A)-tailed, nicked DI 011 RNA was dissolved in water to a final concentration of 50 μg/ml, heated to 100°C for 45 s, quick cooled, adjusted to 0.4 M NaCl, 0.5% sodium dodecyl sulfate, 1 mM EDTA, and 1 mM Tris (pH 7.5), and passed over an oligodeoxythymidylic acid-cellulose column equilibrated with the same buffer. After extensive washing the column was eluted with 0.5% sodium dodecyl sulfate in 1 mM Tris-hydrochloride–1 mM EDTA (pH 7.5). The eluted poly(A)-tailed, positive-strand RNA was precipitated with ethanol.

DNA complementary to the 5′ terminus of VSV was synthesized by using the poly(A)-tailed DI 011 RNA as a template and a primer, T_7rAdC, which is the complement of the first two nucleotides of the positive strand of DI 011 and the first 7 As of the poly(A) tail. Reaction conditions were a modification of those previously reported (1, 20): 50 μg of RNA per ml, 3.7 μM primer, 50 mM Tris (pH 8.4), 10 mM $MgCl_2$, 50 mM KCl, 1 mM β-mercaptoethanol, 0.5 mM each deoxynucleotide triphosphate, 10 μg of actinomycin D per ml, and 500 U of avian myeloblastosis virus reverse transcriptase per ml. After 90 min of incubation at 42°C, the reaction solution was adjusted to 0.3 M NaOH and heated to 50°C for 30 min to hydrolyze the RNA. The solution was neutralized with acetic acid, passed over a Sephadex G50 column, and precipitated with ethanol. The cDNA was dried in vacuo and dissolved in water. Double-stranded DNA was synthesized under the same general reaction condition, but without primer or RNA. Double-stranded cDNA was trimmed with S1 nuclease and tailed with oligodeoxycytidylic acid as previously described (5, 25). The tailed cDNA were hybridized to oligodeoxyguanidylic acid-tailed pBR322 and introduced to *E. coli* HB101 as previously described (23). Colonies were screened by hybridization with ^{32}P-labeled DI particle RNA (6).

The plasmids pLT-86, pLT-54, and pHR-9 carry VSV inserts that begin at the end of the G cistron and extend into the L cistron in the case of pHR-9 or the remnant of the L cistron in the case of pLT-86 and pLT-54. All three plasmid inserts extend beyond the deletion point. The inserts were prepared by using either DI-LT RNA or VSV HR RNA as a template and extending a 49-nucleotide-long primer cut from pG65 (16). Double-stranded cDNA was prepared with these primers and templates under the conditions described above, except that the concentration of the primer was 1.2 μg/ml. Ampicillin-sensitive clones were screened by colony hybridization with ^{32}P-labeled DI 611 (24) and ^{32}P-labeled primer. The three plasmids, pHR-9, pLT-86, and pLT-54, containing inserts larger than 400 base pairs were selected for further study. The primer used in the synthesis of the cDNAs extends from the *Hin*fI site at position 1,584 to the *Hae*III site at position 1,632 of the G cistron. Consequently, the clones obtained with this primer contain the primer followed by a 41-base-pair sequence that corresponds to the end of the G, the polyadenylation site, and the intercistronic dinucleotide preceding the L cistron.

Restriction endonuclease mapping and sequencing. Restriction endonucleases were obtained from either Bethesda Research Laboratories, New England Biolabs, or PL Biochemicals and used under conditions suggested by the vendor or Davis et al. (3). Restriction endonuclease fragments were labeled at their 3′ termini with cordycepin [^{32}P]triphosphate by using terminal deoxynucleotide transferase (5). The fragments were cleaved with a second endonuclease, and the uniquely labeled fragments were separated by polyacrylamide gel electrophoresis. Labeled DNAs were recovered from the gel by extraction of the crushed gel slices (13). Uniquely labeled DNA fragments were sequenced by the chemical sequencing method of Maxam and Gilbert (13).

RESULTS

L-cistron clones. To analyze the sequences that may have been important in determining the

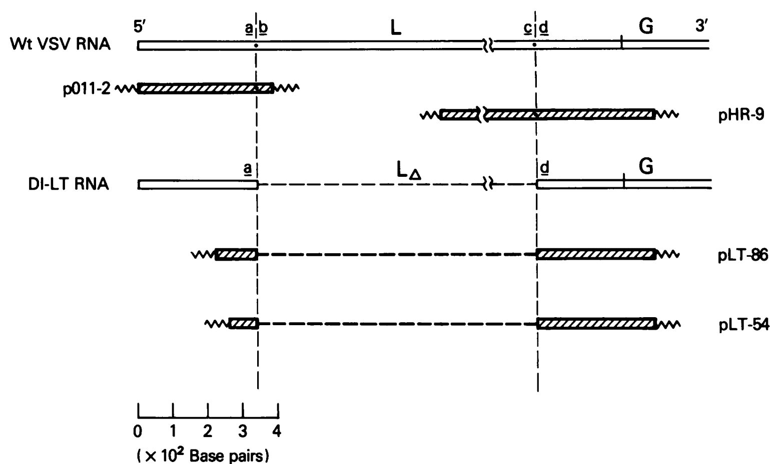

FIG. 1. Comparison of the physical maps of VSV DI-LT and the cloned segments of VSV. Open bars depict viral RNA. Hatched bars represent the VSV inserts in pHR-9, pLT86, and pLT54. Jagged lines represent the oligodeoxycytidylic acid-oligodeoxyguanidylic acid tails introduced during cloning.

site and size of the deletion in DI-LT, we obtained cDNA clones that represented the relevant regions of the VSV and the DI-LT genomes. In Fig. 1 we schematically compare RNA genomes of DI-LT and VSV to the cloned cDNA's that were analyzed. For simplicity, we will refer to the relevant regions as a, b, c, and d. The a region lies between the 5' terminus of the DI particle or parental viral chromosome and the deletion site at position 341 from the 5' terminus (see below). The b region abuts the a region in the parental genome, but is deleted in DI-LT. Similarly, the d region starts at the deletion site and extends upstream toward the beginning of the L cistron. It, too, is present in both the DI-LT and the parental viral genomes. The c region is deleted in the DI particle and abuts the d region in the parental viral genome. These sequences define the environment of the polymerase in any hypothetical copy choice recombination event whether it occurred while replicating the positive or the negative strand of the genome.

Three of the clones (pHR-9, pLT-86, and pLT-54) used in this study were obtained by extending a 49-nucleotide-long primer cut from pG-65, a clone described by Rose and Gallione (16). The primer spans positions 1,584 to 1,632 of the G cistron and lies 41 nucleotides from the beginning of the L cistron. pHR-9 is a clone obtained with RNA from the parental virus, VSV HR. The clone extends from the primer across the d and c region to a position about 1,300 nucleotides into the L cistron. pLT-86 and pLT-54 are cDNA clones of DI-LT RNA and extend from the primer across the deletion site to positions 238 and 265 from the 5' end of the DI-LT genome. Therefore, they contain regions a and d.

To obtain a clone of the VSV 5' terminus we employed the RNA of the "snap-back" DI particle, DI 011. This RNA is perfectly self-complementary (21) and folds into an approximately 860-base-pair hairpin duplex. The 5' arm of this duplex is homologous to the 5' portion of the wild-type VSV genomic RNA for its full length (20), and consequently the 3' arm is the complement of the 5' portion of the VSV chromosome for its full length. DI 011 RNA is unique in that it forms a duplex with only a single 3' terminus, and polyadenylation of DI 011 RNA with *E. coli* poly(A) polymerase introduces a poly(A) chain only on the positive sense arm. After mild nuclease digestion to nick the hairpin RNA at the turnaround point, the polyadenylated (positive-sense) strand was collected by adsorption to and elution from oligodeoxythymidylic acid-cellulose. The recovered polyadenylated RNA served as a template for avian myeloblastosis virus reverse transcriptase by using T_7rAdC to specifically prime synthesis adjacent to the original 3' terminus of the DI RNA. After second-strand DNA synthesis, the DNA was inserted into the *Pst*I site of pBR322 by oligodeoxyguanidylic acid-oligodeoxycytidylic acid tailing. Transformation of *E. coli* HB101 with the DNA mixture yielded colonies containing the VSV 5' terminal sequence. These were identified by colony hybridization with DI particle product RNA—a 46-nucleotide-long transcript whose sequence is identical to that of the 5' terminus of VSV (11, 19, 21, 22). From among the positive colonies, p011-2 was selected for further study.

Sequence divergence between clones p011-2,

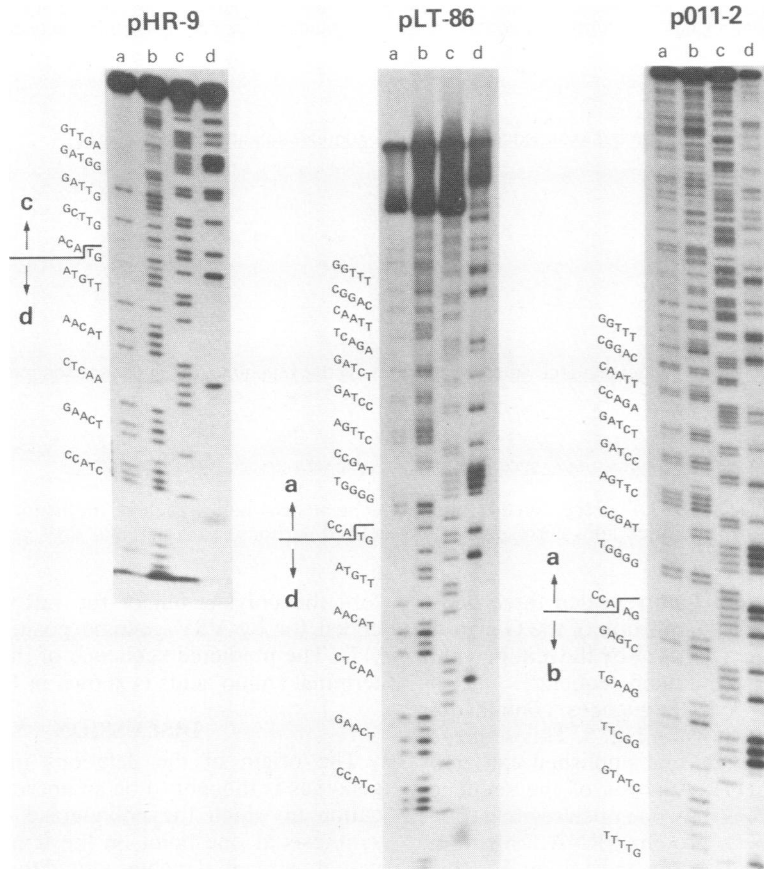

FIG. 2. Autoradiograms of sequencing gels that illustrate the sequence divergence between the clones. a, b, c, and d refer to the regions of the VSV chromosome defined in Fig. 1.

pLT-86, and pHR-9. p011-2, pLT-86, and pLT-54 were sequenced in their entirety by the chemical sequencing methods of Maxam and Gilbert (13). Clone pHR-9 was partially characterized, and only the portions relevant to this study were sequenced. As expected only a portion of the sequence of pLT-86 was homologous to the sequence of clone p011-2. The autoradiogram shown in Fig. 2 illustrates sequence divergence between the two clones. On the right side are the results obtained with a *Hpa*II to *Pst*I fragment of p011-2 labeled at the terminal *Pst*I site. The results presented in the center of the figure were those obtained with *Taq*I to *Pst*I fragment of pLT-86 labeled at the *Taq*I site. The autoradiogram on the far left shows the sequence of a fragment of pHR-9 labeled at the *Taq*I site that corresponds to the one used in the pLT-86 sequencing. In all three cases, the sequences are read from the virion sense strand with the G cistron-proximal positions at the bottom of the figure. The points of sequence divergence and the regions of the genome represented (a, b, c, and d) are indicated in the figure. The point of sequence divergence between p011-2 and pLT-86 clones corresponds to position 342 from the 5′ end of the viral or DI particle chromosome. The sequence of clone pLT-86 and pHR-9 diverge at a position 251 nucleotides from the beginning of the L cistron. The relevant portions of the VSV sequence are shown in Fig. 3. For ease of analysis, the sequences corresponding to the antigenomes are also presented.

Nucleotide sequences of the beginning and end of the L cistron and the deduced amino acid sequences of the NH_2 and COOH termini of the L protein. The sequence found for pHR-9 (Fig. 4) agrees with that of pLT-86 up to the site of the deletion. The sequence exhibits only one open reading frame. The amino acid sequence deduced for the beginning of the L protein is presented below the nucleotide sequence in Fig. 4.

Comparison of the nucleotide sequence obtained for VSV HR with those published for the end of the G cistron and the beginning of the L

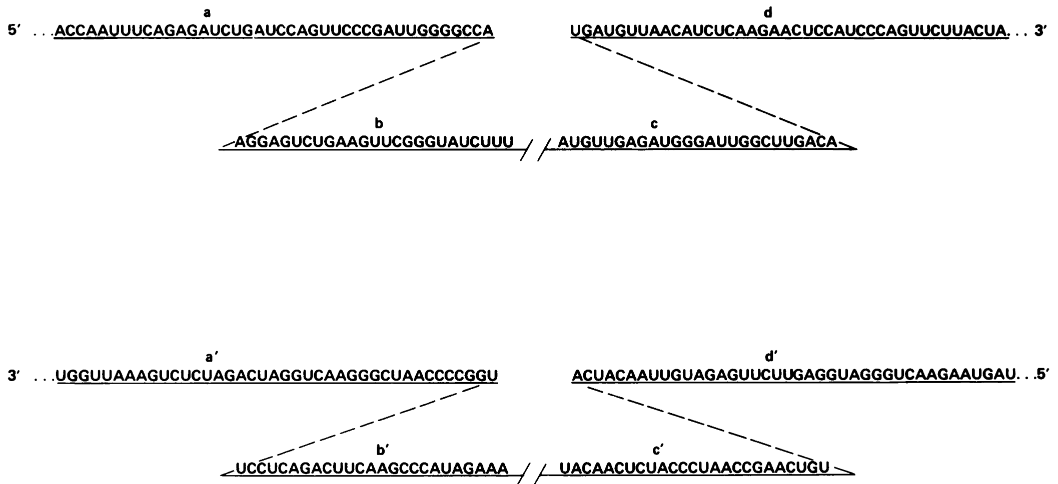

FIG. 3. Sequence of DI-LT at the deletion point. The sequences below each of the main sequences and connected to them with dashed lines correspond to the deleted portions of the parental VSV genome.

cistron of VSV San Juan revealed three differences in the noncoding portion of the G cistron. These are indicated in Fig. 4 by the letters within parentheses above the main sequence.

The complete VSV sequences contained in clone p011-2 are shown in Fig. 5. This sequence confirms and extends that published earlier by Schubert et al. (17). Analysis of the sequence reveals that there is only one open reading frame in this part of the L protein mRNA. This frame terminates at the UAA coded for by positions 102 to 100 from the 5′ end of the genome. If this translation terminator is functional, the VSV L-protein synthesis terminates 36 nucleotides before the poly(A) tail of the mRNA, which is coded for by VSV genome positions 60 to 66 (17). The predicted sequence of the 88 carboxy terminal amino acids is shown in Fig. 5.

DISCUSSION

The origin of the deletions in DI particle genomes is thought to be an aberration of replication in which the polymerase interrupts its synthesis at one point on the template, moves with its nascent daughter strand to another position of the template, and resumes synthesis, further extending the incomplete nascent chain. It seems likely that the polymerase remains

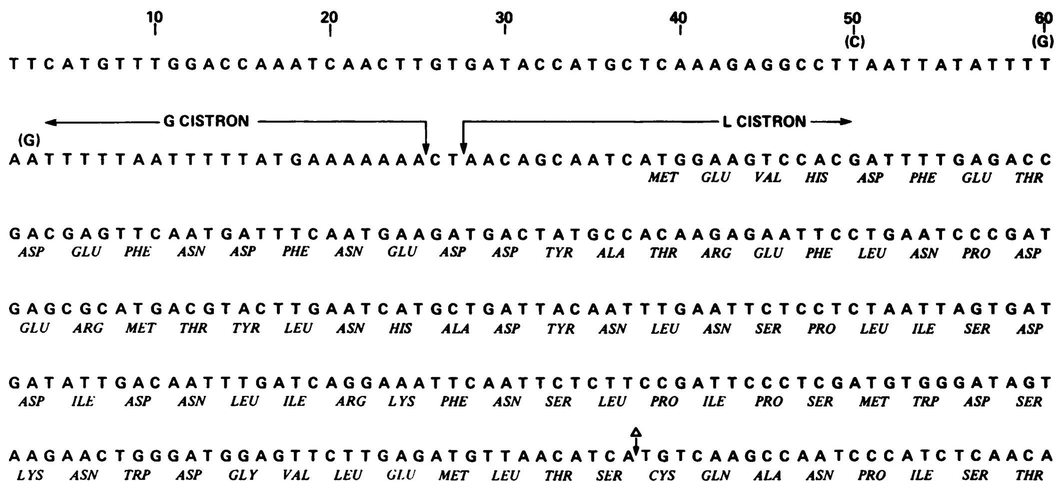

FIG. 4. Sequence of the first 360 nucleotides of the pHR-9 insert. The predicted amino acid sequence for the NH_2 terminus of the L protein is given in italics under the nucleotide sequence. The site of the deletion is indicated by △.

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                                                            337                           307
A A A G A T A C C C G A A C T T C A G A C T C C T T G G C C C C A A T C G G G A A C T G G A T C A G A T C T C T G G A A
 LYS   ASP   THR   ARG   THR   SER   ASP   SER   LEU   ALA   PRO   ILE   GLY   ASN   TRP   ILE   ARG   SER   LEU   GLU

                                                            277                           247
T T G G T C C G A A A C C A A G T T C G T C T A A A T C C A T T C A A T G A G A T C T T G T T C A A T C A G C T A T G T
 LEU   VAL   ARG   ASN   GLN   VAL   ARG   LEU   ASN   PRO   PHE   ASN   GLU   ILE   LEU   PHE   ASN   GLN   LEU   CYS

                                                            217                           187
C G T A C A G T G G A T A A T C A T T T G A A A T G G T C A A A T T T G C G A A G A A A C A C A G G A A T G A T T G A A
 ARG   THR   VAL   ASP   ASN   HIS   LEU   LYS   TRP   SER   ASN   LEU   ARG   ARG   ASN   THR   GLY   MET   ILE   GLU

                                                            157                           127
T G G A T C A A T A G A C G A A T T T C A A A A G A A G A C C G G T C T A T A C T G A T G T T G A A G A G T G A C C T A
 TRP   ILE   ASN   ARG   ARG   ILE   SER   LYS   GLU   ASP   ARG   SER   ILE   LEU   MET   LEU   LYS   SER   ASP   LEU

                                                            97                             67
C A C G A G G A A A A C T C T T G G A G A G A T T A A A A A A T C A T G A G G A G A C T C C A A A C T T T A A G T A T G
 HIS   GLU   GLU   ASN   SER   TRP   ARG   ASP   END

                                                            37                              7
A A A A A A C T T T G A T C C T T A A G A C C C T C T T G T G G T T T T T A T T T T T T A T C T G G T T T T G T G G T

C T T C G T
```

FIG. 5. Sequence of VSV insert in p011-2. The sequence shown is the positive sense and is numbered from the 5′ terminus of the positive VSV RNA. The amino acid sequence predicted for the carboxy terminus of the L protein is presented in italics.

loosely associated with the template during this replication leap since the same viral chromosome appears to direct the synthesis of both halves of most deletion DI particle RNAs (2, 11). By analogy with the variations of the copy choice models proposed to explain the other types of DI particle RNAs one might expect to find specific sequences near the position where synthesis was interrupted or resumed, e.g., sequences similar to those found at termination or initiation sites. The putative, aberrant replicative event leading to the generation of a deletion might take place while copying either a positive or a negative template. Analysis of the sequences shown in Fig. 3 does not reveal any relation to the known initiation or termination sites. Furthermore, the sequences do not suggest any (potentially) strong secondary structures, but we cannot rule out interactions between these regions and others that are at a distance. However, in the absence of any evidence to the contrary, it seems likely that the generation of the simple deletions may proceed by mechanisms that are independent of the primary sequence at the site of deletion.

It is interesting to note that recent analyses of influenza virus DI particle RNAs led to the same conclusion. Nayak and associates examined four DI particle RNAs and found that the sequences that flank the deletion are different in different DIs and do not appear to be related to influenza virus termination or initiation sequences (2, 4; Nayak, personal communication). These studies have been greatly extended by Robertson and associates, who have sequenced 36 influenza DI particle RNAs. All were found to be simple deletions with no apparent sequence constraints on the formation of the deletion (Robertson, personal communication).

In a previous publication, Herman and Lazzarini (8) reported the synthesis of an unusual mRNA, G* mRNA, by a DI-LT particle. This polyadenylated RNA was shown to contain sequences from both the G and L cistrons. Both the size and the composition of the mRNA are consistent with it being a product of readthrough transcription of the G cistron and the remnant of the L cistrons with polyadenylation only at the end of the L sequences. The proposed failure of the polymerase to polyadenylate and terminate at the end of the G cistron might be attributed to alterations of the sequences that specify these processes. The sequence presented in Fig. 4 includes the polyadenylation site of the end of the G cistron in DI-LT and VSV HR. Three differences between the DI-LT sequence and the VSV San Juan were found in this region. However, the relevance of these to the G* mRNA is not clear, since both the parental VSV HR and the DI-LT sequences are the same in this region, although only the DI-LT synthesizes appreciable amounts of the G* mRNA. In making these comparisons, one must bear in mind that the DI-LT stocks are mixtures of particles and that during each amplification of the stock, the composition may change. Consequently, there is no assurance that the DI particle giving rise to G* mRNA had a genomic RNA identical

to that copied in the preparation of the clones described here. However, we have sequenced two independent clones prepared from DI-LT RNA, and both are deleted for precisely the same sequence, suggesting that the clones are copies of identical RNAs that are present in reasonable abundance.

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