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Sites of Copy Choice Replication Involved in Generation of Vesicular Stomatitis Virus Defective-Interfering Particle RNAs

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The copy choice model for the generation of defective interfering (DI) particles of vesicular stomatitis virus suggests that during replication the polymerase prematurely terminates, moves with the nascent daughter strand to another site on the same or a different template molecule, and resumes elongation of the nascent chain. We have analyzed the sites where premature termination or resumption of replication has occurred during the generation of the deletion DI particle LT, the snapback DI particle 011, and the panhandle DI particles T, T(L), and 611. The recombination sites were identified by comparing the nucleotide sequences of the relevant regions of these DI particle RNAs to those of the vesicular stomatitis virus *L* gene (Schubert et al., J. Virol. 51:505–514, 1984). Sequence homology was not detected between these sites, which rules out the existence of a general terminator or promoter sequence involved in copy choice replication. In several cases, however, premature termination or resumption of RNA replication may be favored by specific signal sequences. The sequences immediately before the start and at the end of the deletion in DI LT contain two hexanucleotides, ATCTGA and GATTGG, in a similar spacing. In the case of DI T and 611, but not of DI T(L), the end of the 5′-terminal region bears the hexanucleotide CCUCUU. This sequence is also repeated in the stem region in all three DI particle genomes. In addition, we present data that the added 3′-terminal regions of the panhandle DI particle RNAs may differ by only one base and are 46 [DI T(L) and 611] or 45 (DI T) bases long. We suggest that each site of the vesicular stomatitis virus genome has the potential to give rise to DI particle RNAs. Specific sequences, however, may modulate this process in a quantitative way, and they favor the generation of certain types of DI particle genomes like those of the panhandle type.

Defective interfering (DI) particles of vesicular stomatitis virus (VSV) are deletion mutants which contain only a portion of the parental genome and interfere specifically with the replication of standard virus (12). With regard to the amount of (−) sense genomic RNA retained and (+) sense sequences added to these DI RNAs, four classes of DI particles have been identified: deletion, snapback, panhandle, and compound DI particles (20, 25). Despite their structural diversity, two common characteristics of all DI particle genomes analyzed to date are that part of the polymerase gene (*L*) is deleted and that their replication is dependent on the polymerase provided by the parental virus. The structure and origin of DI particles are of interest not only because these particles may play a role in the establishment and maintenance of persistent infections (8, 9) but also because they arise by aberrant replicative events and therefore reveal details of the mechanism of replication. According to the copy choice model of DI particle generation, the polymerase detaches from the template without releasing the nascent daughter chain and resumes synthesis at another position on the same or a different template, thereby extending the nascent chain (20, 25). In this study, we address the question whether or not the dissociation or reattachment of the VSV polymerase during copy choice synthesis may be promoted by sequence signals on the template. A detailed comparison of genomic recombination sites has not yet been undertaken because the locations of most of these regions are far inside the *L* gene and are therefore not easily accessible to direct RNA sequencing techniques. Nonetheless, earlier studies by Schubert et al. (31) suggested that a specific internal RNA polymerase recognition site may play a role in the generation of the most common panhandle DI particle RNAs, and Keene et al. (15) concluded that the premature termination of RNA synthesis generating the compound DI particle is specified by a sequence signal. In the case of the deletion DI particle LT, the sites of rearrangement involved in its origin did not reveal any relation to known initiation or termination sites of VSV (37).

Our complete sequence analysis of the *L* gene (30a) enabled us to study these internal regions at which termination or resumption of RNA synthesis have occurred. By comparing the sites involved in the generation of the deletion DI particle LT, the snapback DI particle 011, and the panhandle DI particles T, T(L), and 611, we found that in some, but not all, cases, the dissociation or reattachment event may be specified by the primary structure of the VSV genome. In addition, if specific sequences are involved, they share no homology and exert their effect through different mechanisms. Based on these data, we discuss the importance of sequence information for the generation of DI particle genomes in VSV.

MATERIALS AND METHODS

DI particles. DI T and T(L) derived from the San Juan strain and DI 611 derived from the Mudd Summers strain of VSV Indiana serotype were propagated and purified as described earlier (18, 21). DI T(L) was a gift from Suzanne U. Emerson, University of Virginia.

Isolation of RNA and nucleotide sequence analysis. The RNAs of DI T, T(L), and 611 were extracted as described

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(21) and labeled at the 3′ end with [^{32}P]cytidine-3′,5′-bisphosphate and T4 RNA ligase (3, 18, 31). The terminally labeled RNAs were sequenced by the chemical procedure of Peattie (24).

Computer analysis of sequences. The comparisons of the sequences of the relevant regions of the DI particle genomes with the *L* gene sequence (30a) and the search for regions with dyad symmetry were done with an IBM 370 computer, using the programs of Queen and Korn (28).

RESULTS

Mapping of the recombination sites. The structural organizations of the RNA genomes of the deletion DI particle LT, the snapback DI particle 011, and the panhandle DI particles T, T(L), and 611 have been well characterized by studies applying hybridization, partial sequencing, electron microscopic, and oligonucleotide fingerprint mapping techniques. We have recently completed the sequence analysis of the polymerase gene (*L*) of VSV (30a). Because all of the sites involved in the generation of these DI particle RNAs are located within this gene, our sequence analyses of DI T, T(L), and 611 together with those previously reported for DI LT (37) and DI 011 (33) allowed us to map precisely the positions where the homology between the parental and DI particle genome ends, i.e. the sites of recombination.

Earlier studies demonstrated that DI LT RNA consists of the entire 3′ half of the VSV genome, including the *N*, *NS*, *M*, and *G* genes, which are linked to a small portion from the 5′ terminus of the genome (4, 26). The location of the internal deletion, which is completely contained within the *L* gene (1, 4), was identified by preparing and sequencing cDNA clones of the relevant regions of the DI and the parental genome (37). Comparison of these sequences with those of the *L* gene reveals that the deleted region spans from nucleotides 341 to 6190 of the genome, involving 5,848 nucleotides (Fig. 1). The cDNA clone used for the sequence analysis of DI LT was obtained by reverse copying of a mixture of at least two DI particle RNAs of similar size: DI LT1 and DI LT2 (4, 15, 26). We cannot assign these DI particle sequences based on the data presented here, but comparison of the sequences presented by Herman (7a) with those published by Yang and Lazzarini (37) suggests that the latter belong to DI LT2.

Based on hybridization and sequence analyses, we have demonstrated earlier that the RNA of the snapback DI particle 011 consists of a region which is homologous to the genomic 5′ end and which is covalently linked by a single phosphate residue to its exact complement, so that every base has the potential of entering a duplex (21, 31, 33). Comparing our published sequence of about 70 bases before and behind the linkage or turnaround point of the DI particle RNA (33) with the *L* gene sequence (30a), we found that the last base of the (−) sense strand of DI 011 corresponds to nucleotide 1167 of the VSV genome (Fig. 1).

The genomes of the panhandle DI particles T, T(L), and 611 and of many others retain different size portions from the precise 5′ end of the VSV genome and terminate at the 3′ end with a short sequence, which is complementary to the 5′ end (16, 18, 27, 29, 30, 32, 36). This sequence—the panhandle region (Fig. 1, a′)—is 45 nucleotides long in the case of DI LT or 48 nucleotides in the case of DI T(L) and DI 611 (31). To identify the precise point where the homology between the 5′ portions of the DI particle RNAs and the standard genome ends, we labeled the 3′ ends of these DI particle RNAs (3, 18, 31) and determined their sequences for about 40 to 60 nucleotides beyond the panhandle region using the chemical methods described by Peattie (24) (data not shown). Homology to the *L* gene was found to extend to positions 2163 with DI T, 3641 with DI T(L), and 4417 with DI 611 RNA (Fig. 1). It is important to note that in the cases of DI T(L) and 611, the last two bases which are homologous to the *L* gene are identical to the first two bases of the panhandle region, whereas with DI T, the last base which is homologous to the *L* gene is clearly not part of the panhandle region. The implications of this result will be discussed later.

Sequence analysis of the recombination sites. Figure 2 compares the primary structures of the recombination sites within the *L* gene involved in the origins of DI LT, 011, T, T(L), and 611 RNAs. Boxed sequences represent the regions that are conserved within the DI particle RNAs (compare with Fig. 1). As can be seen, there is no sequence homology between the recombination sites. Since we cannot distinguish whether these recombinational events occurred on a template of (−) or (+) polarity, we have considered both possibilities. These data, therefore, rule out the existence of a general conserved sequence signal which might specify the termination or resumption of RNA synthesis. Further exami-

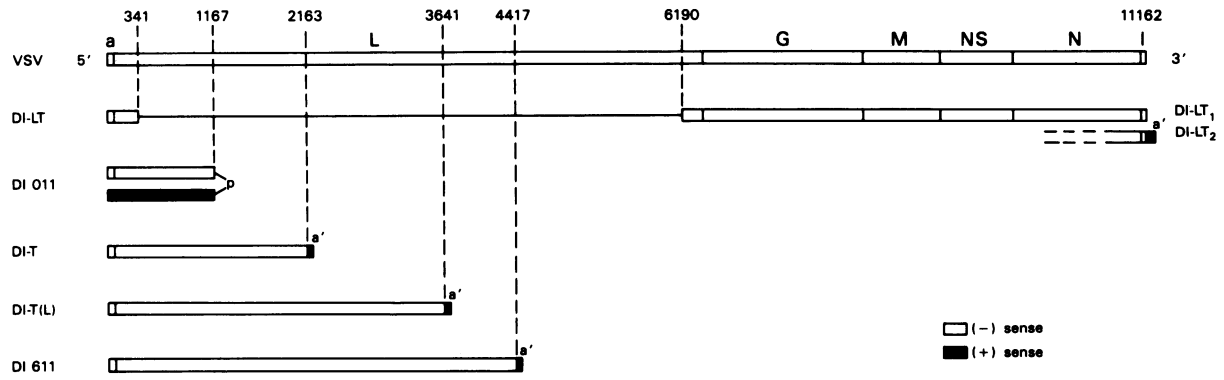

FIG. 1. Maps of the genomes of standard VSV, deletion DI particle LT, snapback DI particle 011, and panhandle DI particles T, T(L), and 611. The numbers correspond to the positions where the sequences of the DI particle genomes and the standard VSV RNA diverge. White bars represent sequences homologous to the (−) sense sequence of VSV, and black bars correspond to sequences complementary to the standard genome. Panhandle regions are marked a′.

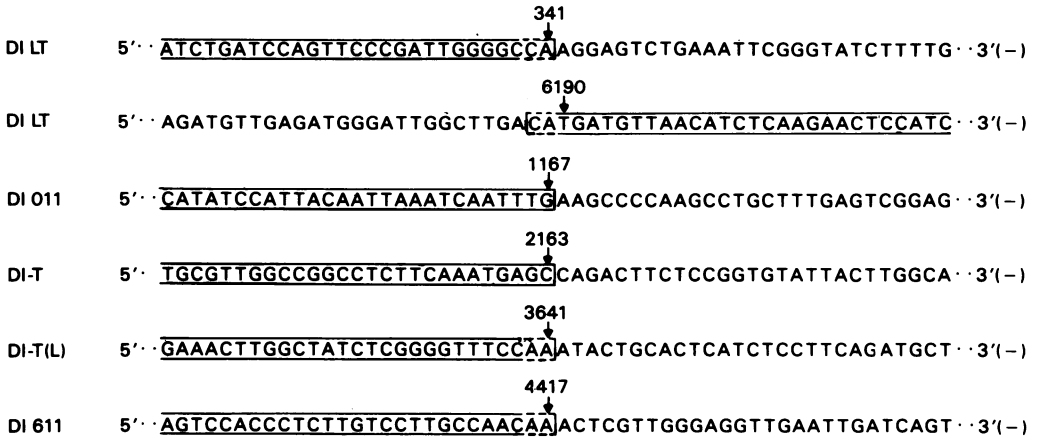

FIG. 2. Sequences of the sites of copy choice replication involved in the origin of DI LT, 011, T, T(L), and 611. All sequences are given in the (−) sense; arrows indicate the positions within the VSV *L* gene. The conserved regions in the DI particle genomes are marked by boxes.

nation of the recombination sites, however, revealed some interesting sequence features which are described below.

As schematically illustrated in the top part of Fig. 3, there exists remarkable sequence homology among those regions in the VSV genome where termination and resumption of RNA synthesis leading to the deletion DI LT have occurred. Both sequences immediately before the start of the deletion and at the end of the deletion contain a grouping of two hexanucleotides, ATCTGA and GATTGG, which do not resemble any other known sequence signals of VSV. The number of nucleotides between these two groups is in the same range (10 and 13 bases, respectively), and in addition, the distances between the sequence GATTGG and the base flanking the deletion at each site are only a little different (5 and 7 bases, respectively). This arrangement suggests a model for the origin of DI LT which may involve either weak base pairing of the nascent daughter strand and the template RNA close to the site where synthesis resumes (Fig. 3B) or a preferential association (or pausing) of the polymerase with these sequences.

Two models have been discussed to explain the exact complementarity of the 5′ and 3′ halves of the genome of the snapback DI particle 011 (33). The examination of the genomic recombination site (Fig. 4) allows us to distinguish between these two possibilities. The prerequisite of one of these models is the presence of a small potential hairpin in the ribonucleoprotein template of the VSV genome. An unencapsidated transcript of this region could potentially form a duplex, resulting in the detachment of the polymerase from the template followed by copy back synthesis of the nascent chain. Our sequencing data of the *L* gene clearly rule out this model since the sequences in the template before

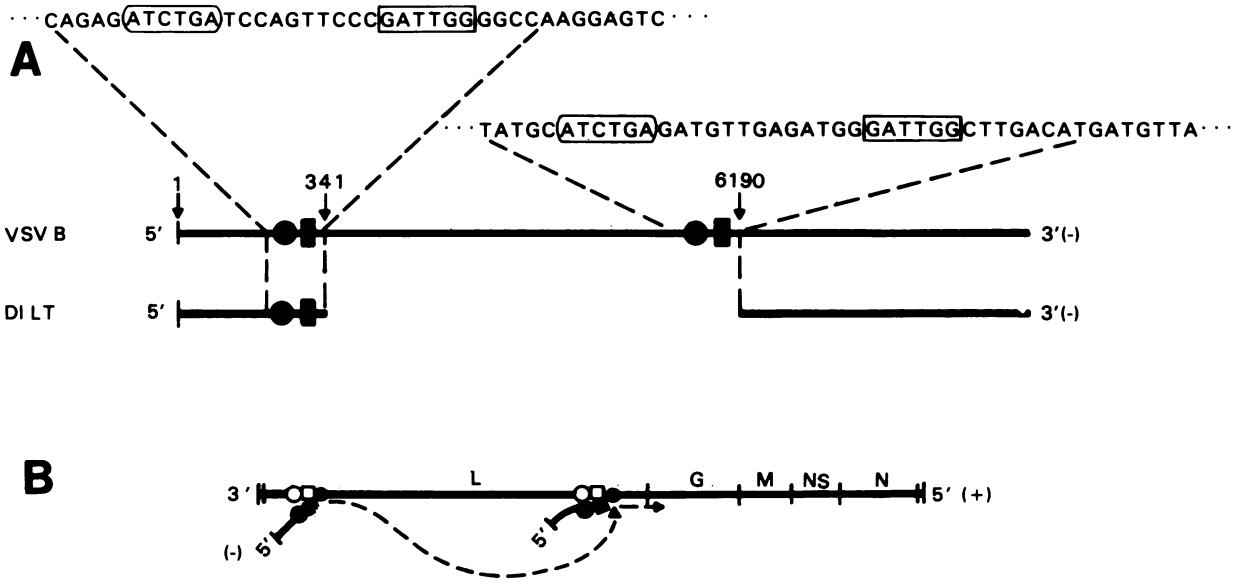

FIG. 3. Sequence homologies between the genomic recombination sites involved in the generation of the deletion DI particle LT. The sequences immediately before the start of the deletion and at the end of the deletion contain two hexanucleotides ATCTGA and GATTGG in a similar spacing (A). This arrangement may be involved in the origin of DI LT (B).

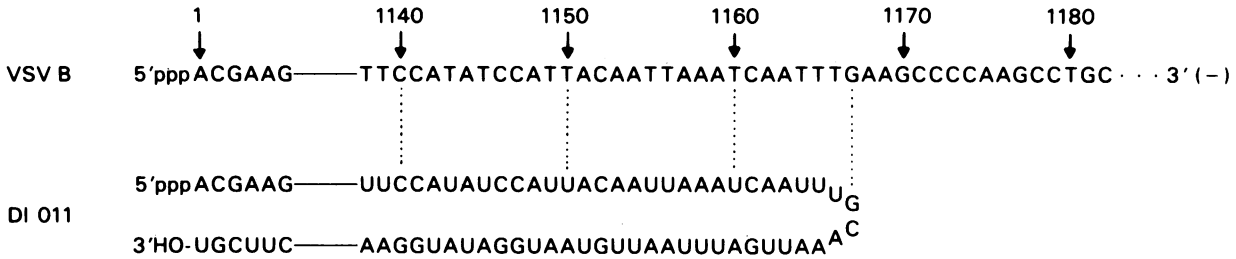

FIG. 4. Nucleotide sequence around the turnaround point of the snapback DI particle 011 (33) and primary structure of the corresponding genomic recombination site. The absence of dyad symmetry in this region of the VSV genome is consistent with a model in which this DI particle is generated by replication across a replication fork (33).

and behind the position corresponding to the turnaround point of the DI RNA are not self-complementary (Fig. 4). The second model which suggests that the two complementary strands originated by symmetrical replication across a replication fork (33) is, however, consistent with our data.

Figure 5 represents the structures of the panhandle DI particles T, T(L), and 611 together with the sequences of the genomic recombination sites involved in their generation. Analysis of these sequences reveals that the two A residues in positions 47 and 48 from the 3′ terminus of the DI particle genomes either could have originated during the synthesis of the panhandle region or may correspond to positions 3640 and 3641 or positions 4416 and 4417 of the VSV genome in the cases of DI T(L) or DI 611, respectively. In the case of DI T, the boundary of the panhandle and the *L* gene region can be precisely defined since the sequences at the recombination sites are not homologous. The added 3′ terminal (+) sense regions of these panhandle DI particle genomes could, therefore, be 46 [DI T(L) and 611] or 45 (DI T) nucleotides long. This result raises the question whether the (−) strand leader RNA, which predominantly consists of 46 nucleotides (32, 34), may be involved in the generation of these panhandle-type DI particle genomes. At the end of the 5′ region, we detected a hexanucleotide CCUCUU in the case of DI T and 611 but not in that of DI T(L). Interestingly, this sequence is also repeated at the beginning of the stem region in all three

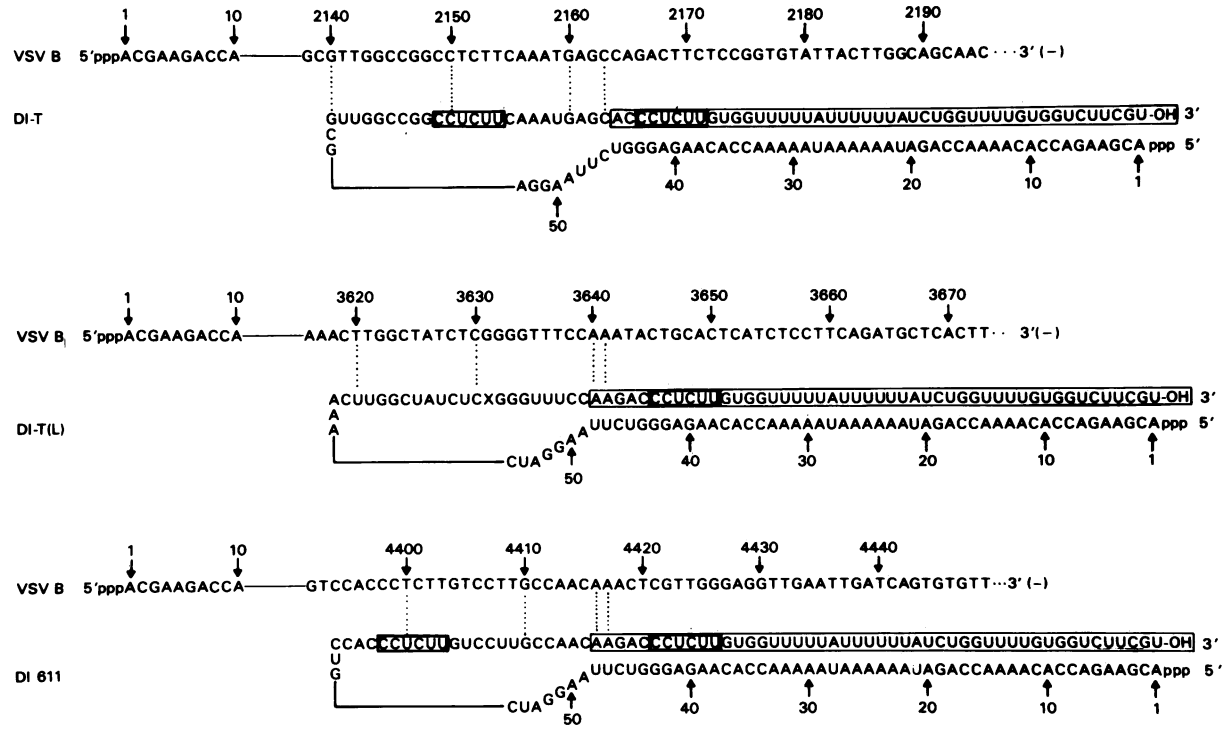

FIG. 5. 5′- and 3′-terminal sequences of panhandle DI particles T, T(L), and 611 and the primary structure of the corresponding genomic recombination sites. The 3′-terminal sequences are complementary to the 5′ terminus for 45 (DI T) or 48 [DI T(L) and 611] bases. Because of the presence of two adenosine residues at the termination or resumption sites within the *L* gene (positions 3640, 3641 and 4416, 4417), the added 3′-terminal regions of DI T(L) and 611 may only be 46 bases long instead of 48 bases. The same region of DI T consists of 45 nucleotides. At the end of the 5′ region, we found the hexanucleotide CCUCUU in the case of DI T and 611, but not of DI T(L). This sequence is also repeated at the beginning of the stem region in all three DI particle RNAs.

DI particle RNAs. Its significance for DI particle generation, however, is unclear since it is not present in the corresponding region of DI T(L).

DISCUSSION

The precise mapping of the positions where the sequences of the parental and the DI particle genomes diverge extends earlier studies about the structural organization of the RNAs of the deletion DI particle LT, the snapback DI particle 011, and the panhandle DI particles T, T(L), and 611. In fact, in the case of DI 011, we indirectly determined the first complete sequence of a DI particle RNA of VSV. This conclusion is based on hybridization studies, which established that the 5′ half of the DI 011 RNA is homologous to the 5′ terminus of the standard genome for its entire length (33). The locations of the recombination sites within the *L* gene of the RNAs of DI T, T(L), and 611 are consistent with the overall size of the DI particle genomes based on hybridization data and oligonucleotide fingerprint mapping (2, 7, 29, 35, 36). In addition, the 5′ terminal regions of the genomes of these DI particles are identical to the 5′ terminus of VSV RNA for at least 1,167 bases (31). Therefore, we assume that no additional severe rearrangements have occurred in the RNAs of these DI particles. The complete sequences of the genomes of DI T, T(L), and 611 can therefore be deduced from the primary structure of the *L* gene (30a). Table 1 summarizes the sizes of the different DI particle genomes, which range from 5,314 bases (47.6% of the total genome) for DI LT to 2,208 bases (19.8% of the total genome) for DI T.

The comparative analysis of the primary structure of the recombination sites within the *L* gene (Fig. 2) excludes the existence of a general signal sequence which alone or together with other factors may be responsible for the generation of DI LT, 011, T, T(L), and 611. In addition, no potential stable secondary structures can be drawn of these regions, which rules out the involvement of secondary structures in the generation of these DI RNAs, including the snapback DI 011 RNA. Interestingly, analyses of subgenomic RNAs of influenza virus led to the same conclusion (5, 13). Our results, however, do not imply that the construction of DI particle RNAs of VSV occurs in all cases independently of the primary structure of the genome, as will be outlined below.

At the sites of the aberrant replication event leading to the deletion DI LT, we found the hexanucleotides ATCTGA and GATTGG (Fig. 3). Moreover, the spacings of these sequences in both regions are very similar. An arrangement consisting of these two hexanucleotides should be found about once in 1.6×10^7 bases, assuming random distribution. The repeat of this highly rare sequence feature at the recombination sites does not seem to be a random occurrence but may indicate its involvement in the generation of DI LT. During its generation, replication of the (−) strand RNA was interrupted, and the nascent 341-base RNA chain was elongated at the same or another template of (+) polarity starting closely downstream from a site which is homologous to the site where premature termination occurred (Fig. 3). Unique for this scheme is the fact that a sequence at the end of the nascent RNA strand may have determined the site of resumption for the polymerase. Although we cannot distinguish whether the 341-base RNA was synthesized first, as exemplified here, or last, in both cases the two hexanucleotides may have been involved by promoting either the resumption or the termination of replication.

TABLE 1. Size of the DI particle RNA genomes

DI particle	No. of nucleotides	% of total genome[a]
DI LT[b]	5,314	47.6
DI 611	4,463	40.0
DI T(L)	3,687	33.0
DI 011	2,334	20.9
DI T	2,208	19.8

[a] Length of total genome, 11,162 nucleotides (30a).
[b] Numbers refer to the defective RNA of DI LT1 or DI LT2.

For the generation of the panhandle DI particles T, T(L), and 611, a copy back model was proposed (11, 19, 23) which, if correct, would involve a specific internal polymerase recognition site with the sequence GGUCUU located 43 to 48 bases from the 5′ end of the VSV genome (31). After polymerization of the large 5′ portion of the DI particle RNA, the polymerase copies back the nascent chain starting at this specific internal polymerase recognition site and forms the 3′ terminal stem region. Comparison of the recombination sites of the three panhandle DI particle RNAs, however, suggests that a different mechanism for the origin of these DI particle RNAs which does not involve copy back synthesis may be considered. We found that the two A residues at the end of the panhandle regions of DI T(L) and 611 may not have been introduced by copy back synthesis but are simply conserved from the *L* gene (Fig. 5). In this case, the added 3′-terminal regions of all three panhandle DI particle RNAs differ only by one base and are either 45 (DI T) or 46 [DI T(L) and 611] bases long. This length corresponds closely to the size of the predominantly 46-nucleotide long (−) strand leader RNA (32, 34), suggesting that the synthesis of this (−) strand leader RNA may be the first step in the construction of these three panhandle DI particle genomes. Instead of releasing the (−) leader transcript, the polymerase detaches with it from a (+) strand template and resumes synthesis at a (−) strand template, extending it by several thousand nucleotides. The observed synthesis of this (−) strand leader RNA in relatively high molar amounts in vivo (22), together with the presence of the (−) strand template, which is four times more abundant than the (+) strand template, would favor the resumption of RNA synthesis of the (−) leader RNA-polymerase complex on a (−) strand template. Interestingly, this copy choice mechanism is similar to that proposed for the generation of the compound DI LT2 (15). In both cases, a short (−) strand leader-type RNA is synthesized first, followed by the resumption of RNA synthesis either starting at the 3′-terminal promoter site, as in the case of DI-LT2, or starting at different positions internally, as in the case of DI T, T(L), and 611. The abrupt termination of RNA synthesis after 46 nucleotides presumably can be addressed to the sequence 3′ . . . GAA(A) . . . 5′, which is believed to operate together with other yet unknown factors as a stop signal in the replication process of VSV (6, 15, 17). Although the copy back model and the model described here are contrary with respect to which part of the DI particle RNA is first synthesized, the unifying feature of these two schemes is that a sequence signal is involved that specifies either the resumption or the termination of RNA synthesis.

Another sequence element possibly involved in the genesis of DI T and 611, but not DI T(L), is the hexanucleotide CCUCUU. Based on random distribution, a given hexanucleotide should be found only about three times in the VSV

genome. The occurrence of the hexamer CCUCUU at the recombination sites of two different panhandle DI particles has, therefore, a low probability. In addition, the repeat of this sequence in the stem regions of these DI particle RNAs emphasizes that this hexanucleotide may be an important signal in the genesis of DI T and 611.

In summary, the analysis of the recombination sites reveals that in some, but not all, cases, the dissociation or reattachment of the polymerase leading to DI particle genomes involves mechanisms that are dependent on the primary structure of the VSV genome. If sequences are involved, they share no homology and exert their influence through different mechanisms. We do not know whether these sequences alone are sufficient for termination or reattachment of the polymerase or whether other factors such as host proteins (10, 14) or the partial removal of N proteins may contribute in this process. We propose that each site in the genome has the potential to give rise to DI particles through a copy choice mechanism unless other unknown factors interfere. To account for the discussed sequence specificities at the recombination sites involved in the origin of DI LT, T, T(L), and 611, we propose that specific sequences may modulate the process of DI particle generation in a quantitative manner in that they favor the construction of a particular type of DI particle. The large number of panhandle DI particles with stem regions of 45 to 55 bases (27, 31), for example, may be explained by this model.

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