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Structure and Origin of a Snapback Defective Interfering Particle RNA of Vesicular Stomatitis Virus

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The nucleotide sequence of the region which covalently links the complementary strands of the "snapback" RNA of vesicular stomatitis virus, DI 011, is

Both strands of the defective interfering (DI) particle RNA were complementary for their full length and were covalently linked by a single phosphate group. Because the strands were exactly the same length and complementary, template strand and daughter strand nucleocapsids generated during replication of DI 011 were indistinguishable on the basis of sequence, a property not shared by other types of DI particle RNAs. Treatment of the RNA with RNase T1 in high-ionic-strength solutions cleaved the RNA only between positions 1 and 1'. These results and the availability of the guanosine residue in position 1' to kethoxal, a reagent that specifically derivatizes guanosines of single-stranded RNA, suggest that steric constraints keep a small portion of the "turnaround" region in an open configuration. The sequence of the turnaround region was not related in any obvious way to the sequences at the 3' and 5' termini and limited the number of possible models for the origin of this type of DI particle RNA. Two models for the genesis of DI 011 RNA are discussed. We favor one in which the progenitor DI 011 RNA was generated by replication across a nascent replication fork.

Comparative studies of primary structures of the genomes of defective interfering (DI) particles are one approach to understand the role of specific regions of the viral genome in transcription, replication, encapsidation, and maturation of the virus. A large number of DI particles of vesicular stomatitis virus (VSV) have been described which contain RNAs of different length and of different regions of the parental VSV genome (2, 3, 8, 9, 12, 15, 16, 21, 24, 26, 27, 34). All of the DI particle RNAs characterized so far share the following structural features: (i) there are deletions in the polymerase cistron; (ii) 5' termini are of minus sense and are identical to that found on the parental genome (13, 25, 29, 32); and (iii) 3' termini of DI particle RNAs are also highly conserved and are identical to the 3' terminals of either the VSV minus- or plus-sense genomic RNA (4, 5, 8, 13, 14, 30, 31). A corollary of these properties is that DI particle RNAs that have plus-sense 3'-terminal sequences have complementary 3'- and 5'-terminal sequences. These structures have been identified by hybridization and by direct sequencing (23, 25, 29). Models for the genesis of this latter and most abundant type of DI particle have been proposed which involve a copy back of the 5'-terminal sequences (9, 17) starting at a specific internal RNA polymerase recognition site approximately 48 nucleotides away from the 5' end (29).

Little is known about the generation of DI particle RNAs which contain extensive self-complementary regions (15, 21, 22). The two complementary strands of these RNAs are covalently linked and therefore rapidly self anneal (snap back). We have studied the structure of a small (approximately 860 base pairs long) snapback RNA isolated from DI 011 and have determined the nucleotide sequence of the region which covalently links the two strands. Surprisingly, both strands are completely complementary for their full length and are linked by a single phosphate group. Because the strands are exactly the same length and complementary, template strand and daughter strand nucleocapsids generated during replication of this DI particle are indistinguishable on the basis of sequence, a property not shared by other types of DI particle RNAs.

The sequence of the turnaround point is

unique and unrelated to those of the 3′ and 5′ termini (28, 29). Consequently, the suggestion by others (23) that this type of DI particle RNA contains inverted complementary terminal sequences at the union of the plus and minus strands is not confirmed. Models for the genesis of this RNA are discussed which involve template switching by the viral polymerase during replication. We suggest that DI 011 RNA was generated by reading across a nascent replication fork.

MATERIALS AND METHODS

Virus growth and RNA purification. The DI particles DI 011 and DI-T were derived from the Mudd-Summers and the San Juan isolates, respectively, of VSV (Indiana). Propagation of the particles as well as the extraction of the RNAs have been described earlier (14, 15, 20). Reovirus RNA was a generous gift from J. D. Keene, Duke University, Durham, N.C.

^{32}P labeling of the 5′ termini. The 5′ terminus of DI 011 RNA was labeled with [γ-^{32}P]ATP and polynucleotide kinase after removal of the terminal triphosphate (11) with calf intestinal phosphatase as described earlier (29). In other experiments, DI 011 RNA was first treated with nucleases and the newly generated 5′ terminus of the plus strand was labeled. For these experiments, 1 μg of DI 011 RNA was incubated for 30 min at 37°C in 20 μl of 10 mM Na_2HPO_4-NaH_2PO_4 (pH 6.8)–0.4 M NaCl in the presence of either RNase T1 (5 U/ml) or RNase A (1 μg/ml). The RNases were removed by adding 2 μg of proteinase K and incubating for 15 min at 37°C. The volume of the reaction mixture was increased to 400 μl, using 10 mM Tris-hydrochloride (pH 7.5), 0.4 M NaCl, and 0.2% sodium dodecyl sulfate. The mixture was extracted with phenol, and the RNA was precipitated with ethanol, washed with 80% ethanol, and dried in vacuo. The newly generated 5′-hydroxy termini were labeled with [γ-^{32}P]ATP and polynucleotide kinase as described earlier (29). The residual ATP was removed by gel filtration over Sephadex G-150, and the labeled RNAs were precipitated in the presence of 50 μg of carrier tRNA.

Nuclease treatment of DI 011 RNA. For nuclease treatment, 5′ terminally labeled DI 011 RNA together with 25 μg of carrier tRNA was incubated in 50 μl of 10 mM Na_2HPO_4-NaH_2PO_4 (pH 6.8)–0.4 M NaCl for 10 min at 37°C in the presence of RNase T1 (2 U/ml) and RNase A (10 μg/ml). The RNases were inactivated by adding proteinase K as described above.

Partial digestion conditions. Terminally labeled RNAs were heated for 1 min at 100°C and quickly cooled in 15 μl of 20 mM sodium citrate (pH 5)–1 mM EDTA. RNase U2, T1, or Phy1 was added, and the digestions were carried out for 15 min at 50°C as described previously (7, 29, 30). Random partial hydrolysates were obtained either at 90°C, pH 9 (7), or at 100°C in formamide (33).

Isolation of 5′ ^{32}P-labeled T1 oligonucleotides. One microgram of DI 011 or DI-T RNA was heated for 1 min at 100°C in 20 μl of 7 M urea–4 mM EDTA (pH 5). The RNAs were digested by adding RNase T1 (200 U/ml) and incubating for 30 min at 50°C, 15 min at 60°C, and 3 min at 90°C. After adjusting to 0.4 M NaCl, the resulting oligonucleotides were precipitated with ethanol, washed with 80% ethanol, and specifically labeled at their 5′ termini with [γ-^{32}P]ATP and polynucleotide kinase as previously described (29). Then 15 μl of 8 M urea, bromophenol blue, and xylene cyanol dye markers were added, and the sample was applied directly onto a 12% polyacrylamide gel.

Specific derivatization of non-base-paired guanosine (G) residues using kethoxal. For isolation of the region that links the two strands of DI 011 RNA, 1 μg of the RNA was incubated for 60 min at 37°C in 20 μl of 100 mM sodium cacodylate (pH 7), 10 mM $MgCl_2$, and 15 mM kethoxal (3-ethoxy-2-oxobutyraldehyde; Accurate Chemical and Scientific Corp., Hicksville, N.Y.) (10, 18). Kethoxal was removed by ethanol precipitation of the RNA after adjusting to 0.4 M NaCl. The RNA was subjected to RNase T1 digestion as outlined above.

Separation of the RNAs. The RNAs or RNA fragments were separated in one-dimensional 2.5, 12, or 20% polyacrylamide gels buffered with Tris-borate, pH 8.3, and containing 7 M urea, as previously described (7). The ratio of acrylamide to bisacrylamide was 20:1. For two-dimensional separations, a modification (19) of the gel system first described by De Wachter and Fiers (6) was used. The Tris-borate concentration in the second-dimensional gel was 160 mM rather than 90 mM (J. S. Robertson, personal communication).

RESULTS

We have previously shown that the 5′ and 3′ termini of DI 011 RNA are complementary and that the strand which carries the 5′-terminal triphosphate is of minus sense and identical to the 5′ terminus of VSV RNA for its full length, approximately 1 kilobase (29). We suspected that only a minute single-stranded region joined the two strands in the intact RNA since no difference in migration of the RNA on agarose-polyacrylamide composite gels was detected after treatment of the RNA with single-strand-specific RNases.

Size analysis of nicked and 5′-labeled DI 011 RNA. To determine the size of the RNase-susceptible region, we compared the electrophoretic mobilities of DI 011 before and after nuclease treatments on a 2.5% polyacrylamide gel. Double-stranded reovirus RNA segments of defined length (1) were used to estimate the size of DI 011 RNA. Under the conditions used, RNAs differing by approximately 10 to 20 nucleotides in size could be distinguished (Fig. 1a).

DI 011 RNA was terminally labeled with [γ-^{32}P]ATP and polynucleotide kinase after removal of the original 5′-terminal triphosphate (11) with calf intestinal phosphatase. The labeled RNA was either left intact (Fig. 1, lanes 2

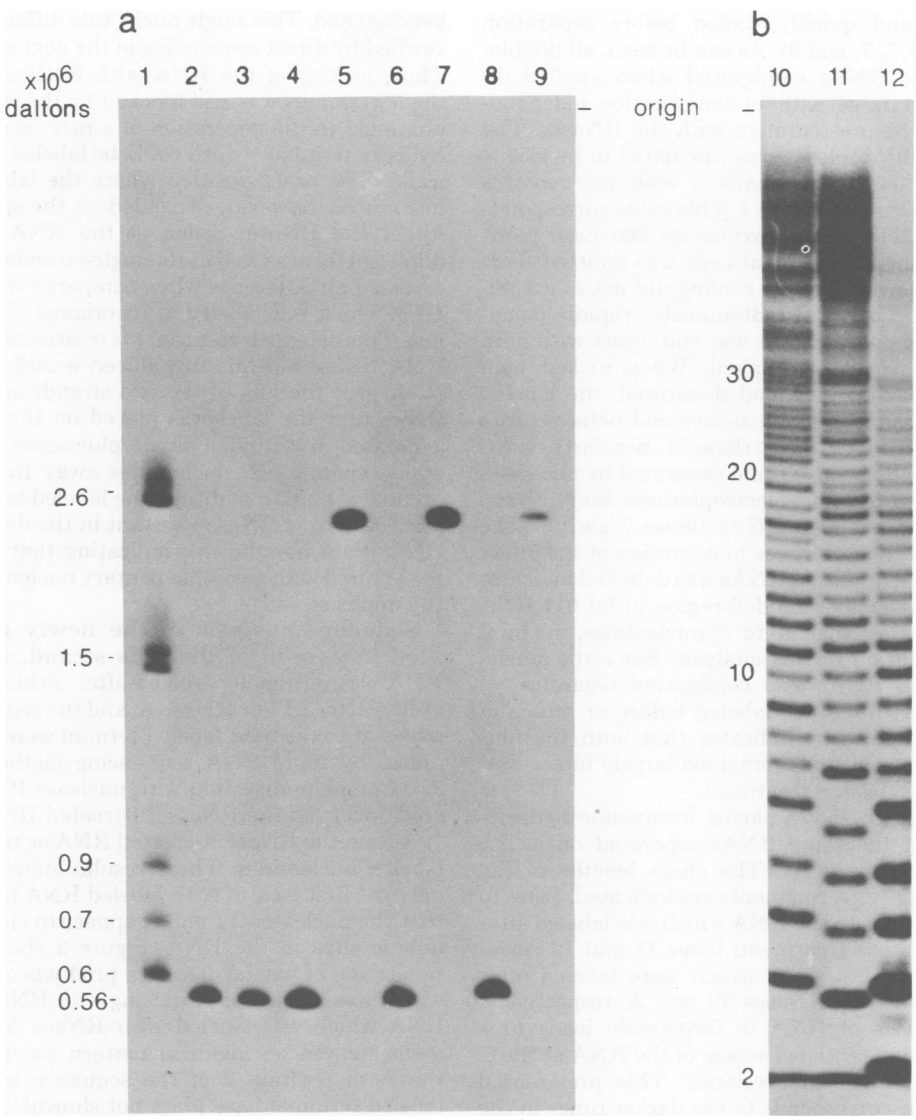


FIG. 1. *Size and nucleotide sequence analyses of 5' terminally labeled DI 011 RNA. (a) DI 011 was labeled with [γ-^{32}P]ATP and polynucleotide kinase after removal of the terminal triphosphate. Samples of the labeled RNA were either untreated (lanes 2 and 3) or nicked with RNases T1 and A (lanes 4 and 5). Other samples of DI 011 RNA were labeled at the 5' termini generated by either RNase T1 (lanes 6 and 7) or RNase A (lanes 8 and 9). The terminally labeled RNAs either were applied directly on a 2.5% polyacrylamide gel (lanes 2, 4, 6, and 8) or were denatured for 1 min at 100°C (lanes 3, 5, 7, and 9) before electrophoresis. Three-prime terminally labeled double-stranded reovirus RNA segments served as molecular weight standards (lane 1). The molecular weights of the segments are indicated. (b) Portions of the 5' terminally labeled RNAs used in lanes 2, 6, and 8 were partially hydrolyzed at 100°C with formamide and were separated on a 20% polyacrylamide gel in the same order (lanes 10, 11, and 12). The chain lengths of the RNA fragments are indicated.*

and 3) or treated with RNases T1 and A (lanes 4 and 5). Other samples of DI 011 RNA which still bore the original triphosphate termini were labeled after treatment with RNase T1 (lane 6 and 7) or RNase A (lane 8 and 9). Under these conditions, the original 5' terminus could not be labeled and the new 5' termini generated by the nuclease cleavage were specifically labeled. The RNAs were then either directly applied on the gel (lanes 2, 4, 6, and 8) or heated for 1 min at

100°C and quickly cooled before separation (lanes 3, 5, 7, and 9). As can be seen, all double-stranded RNAs comigrated when applied directly to the gel without denaturation, independent of the pretreatment with the RNases. The size of the duplexes was calculated to be 0.56×10^6 daltons by comparison with the reovirus RNA segments in lane 1. This value corresponds to an RNA of approximately 860 base pairs. When the RNase treatment was omitted (lane 3), heating and quick cooling did not affect migration. This RNA self-annealed rapidly (snapback), showing that it was still intact with both strands covalently linked. When nicked with RNases T1 and A and denatured, the labeled RNA migrated much slower and behaved as a single-stranded RNA (lane 5). Similarly, when RNAs labeled at termini generated by nucleases were heated before electrophoresis, they also ran as single-stranded RNAs (lanes 7 and 9). The fact that no difference in migration of the intact or nicked duplexed RNAs was detected indicates that the single-stranded region in DI 011 RNA must be less than 10 to 20 nucleotides, the limit of resolution of this analysis. Since the single-stranded RNAs also comigrated regardless of whether they were labeled before or after nuclease treatment indicates that both the plus and minus strands remained largely intact during the nuclease treatment.

Figure 1b shows partial formamide hydrolysates of the same RNAs separated on a 20% polyacrylamide gel. The chain lengths of the resulting RNA fragments are indicated. Lane 10 corresponds to the RNA which was labeled after phosphatase treatment; lanes 11 and 12 correspond to the RNAs which were labeled after digestion with RNases T1 and A, respectively. Hydrolysis of RNA in formamide leads to a slight preferential cleavage of the RNA at the 5′ side of adenosine residues. This preferential cleavage corresponds to the darker rungs in the formamide ladder in Fig. 1. In addition, the spacing between successive rungs of a ladder is a characteristic of the nucleotide difference between the two RNA fragments. Thus, addition of a guanosine (G) to a fragment will retard its electrophoretic mobility much more than the addition of a cytidine (C) to the same fragment. Consequently, the patterns of darker rungs and the spacing between rungs are characteristics of the RNA sequence. The patterns in lanes 10, 11, and 12 are clearly different from one another However, the patterns of lanes 11 and 12 would be identical to one another if the pattern of lane 11 were shifted down by one nucleotide. This relationship suggests that the RNA of lane 11 has one additional nucleotide at its 5′ (label-bearing) end. This single nucleotide difference is verified by direct sequencing in the next section. Thus, nicking of the RNA with RNases while the 5′ terminus was still blocked by the triphosphate led to the generation of a new unique 5′-hydroxy terminus which could be labeled specifically. The exact position where the label was introduced, however, depended on the specificity of the RNase. Sizing of the RNA in its duplexed form as well as its single-stranded form revealed no difference when compared with the RNA which was labeled at its original 5′ terminus. This demonstrates that pretreatment of the RNA before labeling introduced a nick in the RNA near the link of the two strands and also shows that the label was placed on the newly generated 5′ terminus of the plus-sense strand approximately 860 nucleotides away from the original 3′ end. In addition, the labeled terminal nucleotide was RNase resistant in the duplexed RNA (data not shown), indicating that it was base paired with a complementary nucleotide on the minus strand.

Sequence analysis of the newly generated 5′ termini of the plus strand. DI 011 RNA was terminally labeled after nicking with either RNase T1 or RNase A, and the sequences adjacent to the new, labeled termini were determined by rapid RNA sequencing methods (7, 33). Complete digestion with nuclease P1 liberated *pC from the RNase T1-treated RNA and *pA from the RNase A-treated RNA as the only labeled nucleotides. These results indicate not only the first base of each labeled RNA but also that the nucleases T1 and A appear to cleave at unique sites in the RNA. Figure 2 shows the separation of partial digestion products of RNA which was labeled after nicking with RNase T1. RNA which was labeled after RNase A treatment showed an identical pattern except that the A in position 2 of the sequence was the labeled terminal base (data not shown). Pyrimidine assignments were made or confirmed by two-dimensional polyacrylamide gel analyses. Partial hydrolysates of the RNA were separated according to base composition in a 10% gel at pH 3.5 followed by a separation by size in a 20% gel at pH 8.3 (Fig. 3) (6, 19). The sequence of the first 68 nucleotides from the 5′ terminus generated by RNase T1 was determined and is as summarized between position 1 and 68 of Fig. 7.

Isolation of a 33-nucleotide-long T1 oligonucleotide representing the 3′ end of the minus strand. The data shown in Fig. 1 strongly indicate that the nuclease-generated 5′ termini are at or near the middle of the intact DI 011 sequence. However, it is conceivable that, because of complex secondary or tertiary struc-

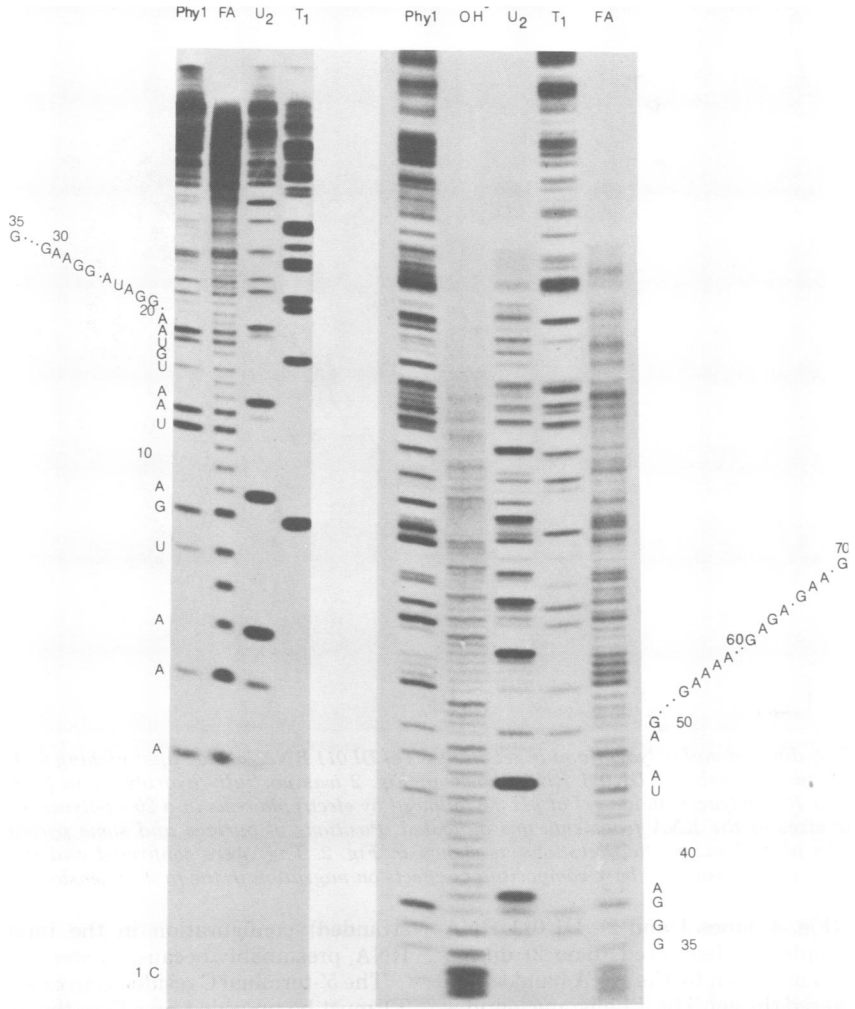


FIG. 2. *Partial RNase digestions of DI 011 RNA labeled after nicking with RNase T1 DI 011 RNA was nicked with RNase T1 and labeled at its new 5′ terminus with [γ-^{32}P]ATP and polynucleotide kinase. The RNA was heat denatured, and samples were partially digested with RNases Phy1, U2, and T1. The RNA was randomly cleaved by hydrolysis either in formamide (FA) or at pH 9 (OH⁻) as described earlier (7, 33). The RNA fragments were separated on a 20% (left panel) or on a 12% (right panel) polyacrylamide gel.*

tures, the sites exposed to the nucleases were actually part of the plus- or minus-sense strand and slightly removed from the true transition point. To establish that the sequence adjacent to the nuclease-generated 5′ termini was part of the plus sense, we identified the complement of this sequence in the 5′ quarter of the VSV genome, thus establishing that the complement was of the minus sense. From the sequence determined in the previous section (see Fig. 7), we deduced that if the complement were present in VSV genomic RNA, part of it would yield a 33-base-long T1 oligonucleotide. This 33′mer should also be present in the RNA of a small DI particle (DI-T) that contains the genetic information from the 5′ quarter of the VSV genome (27, 29, 34). VSV RNA contains four large oligonucleotides (2, 3, 9, 12) which can be separated from the bulk of T1 oligonucleotides by means of a single one-dimensional polyacrylamide gel electrophoresis. As shown earlier, not all of these large oligonucleotides are present in DI particle RNAs since DI particle RNAs are subsets of the parental genome (2, 3, 9, 12). DI-T and DI 011 RNA were digested with RNase T1, and the resulting T1 oligonucleotides were labeled at their 5′ termini with [γ-^{32}P]ATP and polynucleotide kinase and run directly on a 12% polyacryl-

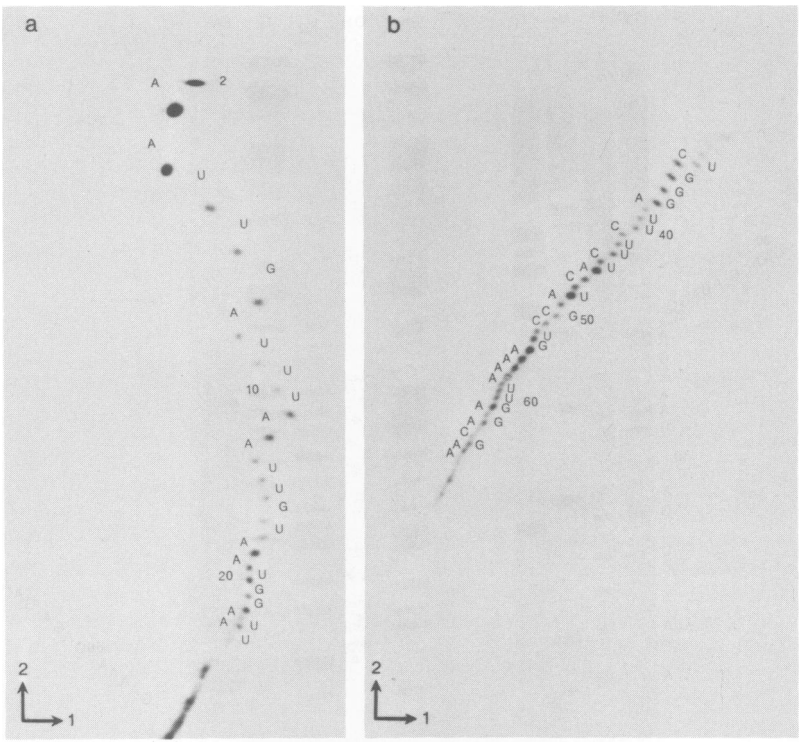


FIG. 3. *Two-dimensional polyacrylamide gel analyses of DI 011 RNA labeled after nicking with RNase T1. Five-prime terminally labeled DI 011 RNA shown in Fig. 2 was partially hydrolyzed in formamide and separated in a 10% polyacrylamide gel at pH 3.5 followed by electrophoresis in a 20% polyacrylamide gel at pH 8.3. The sizes of the RNA fragments are indicated. Positions of purines and some pyrimidines were determined by partial enzymatic digestions as shown in Fig. 2. They were confirmed and the remaining pyrimidines were determined by base compositional effects on migration in the first dimension.*

amide gel (Fig. 4, lanes 1 and 2). DI 011 RNA was not completely denatured (lane 2) during this digestion as shown by the RNA band which had just entered the gel. The T1 oligonucleotides marked a and b were excised and eluted, and their sequences were determined (data not shown). Band a in lane 1 (DI-T) and lane 2 (DI 011) had identical sequences. They corresponded exactly to the deduced 33'mer, which was complementary to the first 33 nucleotides from the nuclease-generated 5' end of DI 011 RNA. Since the 33'mer was found in DI-T RNA, it must be of minus sense. Band b RNA, which was 40 nucleotides long, was only detected in DI 011 RNA. Its sequence was also unique, and it presumably originated from the plus strand of DI 011 RNA.

Isolation of the region that links the two strands. The results described in the second section indicate that RNase A cleaves the dinucleotide ApC occupying positions 1 and 2 in the intact DI 011 but not in RNA previously nicked with RNase T1. These results show that this region of the molecule is held in an open (single-stranded) configuration in the intact DI 011 RNA, presumably because of steric constraints.

The 5'-terminal C residue generated by RNase T1 must be preceded by a G in the intact molecule. We postulated that this G residue might be identical to the 3'-terminal G residue in the 33'mer described above (Fig. 7, position 1'). If this were so, this G residue should not be base paired in the intact RNA because of steric constraints there and should be uniquely accessible to kethoxal, a reagent that specifically modifies G residues in single-stranded RNA (10, 18). If our speculations are correct, then kethoxal-treated DI 011 RNA should yield a new specific 40-nucleotide-long T1 oligonucleotide that contains the sequence of the 33'mer extended at its 3' side by seven nucleotides (see Fig. 7) and contain the modified G. The seven nucleotides of this oligonucleotide should represent the first seven nucleotides from the 5' end of the plus strand, starting with its terminal C residue and extending to the G residue in position 7.

DI 011 RNA was incubated in the presence of kethoxal as described in Materials and Methods.

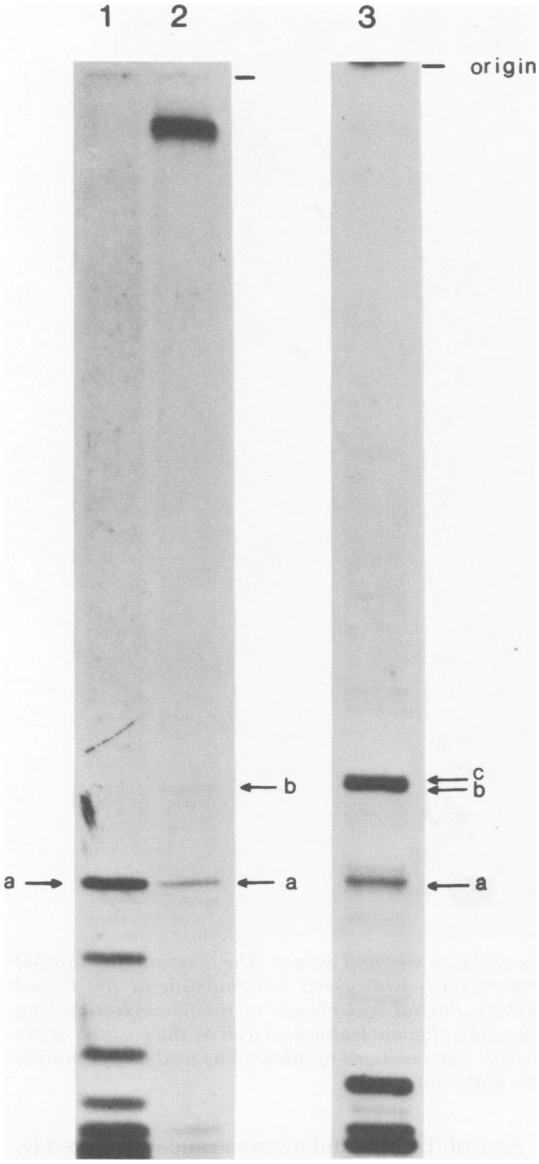


FIG. 4. *Isolation of unique RNase T1-resistant oligonucleotides from DI 011 RNAs. DI-T and DI 011 RNAs were digested with RNase T1. The resulting oligonucleotides were labeled at their 5′ termini with [γ-^{32}P]ATP and polynucleotide kinase and directly applied onto a 12% polyacrylamide gel (lanes 1 and 2). Lane 3 shows the oligonucleotides which were derived from a complete RNase T1 digest of kethoxal-treated DI 011 RNA. The origins of the oligonucleotides marked a, b, and c are discussed in the text.*

After removal of the kethoxal and denaturation, the RNA was completely digested with RNase T1. The resulting oligonucleotides were labeled at their 5′ ends with [γ-^{32}P]ATP and polynucleotide kinase and subjected to electrophoresis on a 12% polyacrylamide gel (Fig. 4, lane 3). In addition to bands a and b, a new oligonucleotide marked c was generated migrating slightly above the 40′mer (Fig. 4b). This RNA fragment as well as bands a and b were recovered from the gel. The RNAs in bands a and b were identical to those in lanes 1 and 2 and corresponded to the 33′mer and the 40′mer present in the untreated RNA (data not shown).

The T1 oligonucleotide in bands a and c were partially hydrolyzed in formamide, and the resulting fragments were separated on a two-dimensional gel system (Fig. 5). Pyrimidine assignments in the sequence were made from the two-dimensional gel electrophoresis as before. The A residues were determined by partial digestion with RNase U2 (data not shown). As can be seen, the 33′mer (Fig. 5a) and the newly generated RNase T1-resistant 40′mer (Fig. 5b) had identical sequences for the first 33 nucleotides as indicated by the shifts of the fragments as well as by the position of some prominent spots which result from the preferred cleavage at XpA bonds by formamide. The 40′mer, however, had seven additional nucleotides following the G residue in position 33 which were identical to the first seven nucleotides at the 5′ end of the plus strand (see Fig. 7, positions 1 through 7). Therefore, both strands of DI 011 RNA were complementary, including the last nucleotides, and must be linked by a single phosphate group between the G residue in position 1′ and the C in position 1. To confirm that the G residue was actually RNase T1 resistant due to modification with kethoxal, we subjected the 40′mer to two different amounts of RNase T1, one which we normally use for partial degradation (Fig. 6, lane 2) and one which we use for complete digestion (lane 3), and separated the products next to partial hydrolysates of the 40′mer (lane 1) as well as of the 33′mer (lane 4) on a 12% polyacrylamide gel. As can be seen, the G residue in position 33 of the 40′mer was only cleaved in the presence of an excess of RNase T1 and then only to a small extent. This indicates that modification of this G residue with kethoxal led to a highly RNase T1-resistant bond which was the prerequisite for the isolation procedure of the 40′mer. Limited degradation using high amounts of RNase might reflect either a very low sensitivity to RNase T1 or partial dissociation of the kethoxal from the G residue during the isolation of the oligonucleotide.

DISCUSSION

In this communication we report the primary structure of the region joining the complemen-

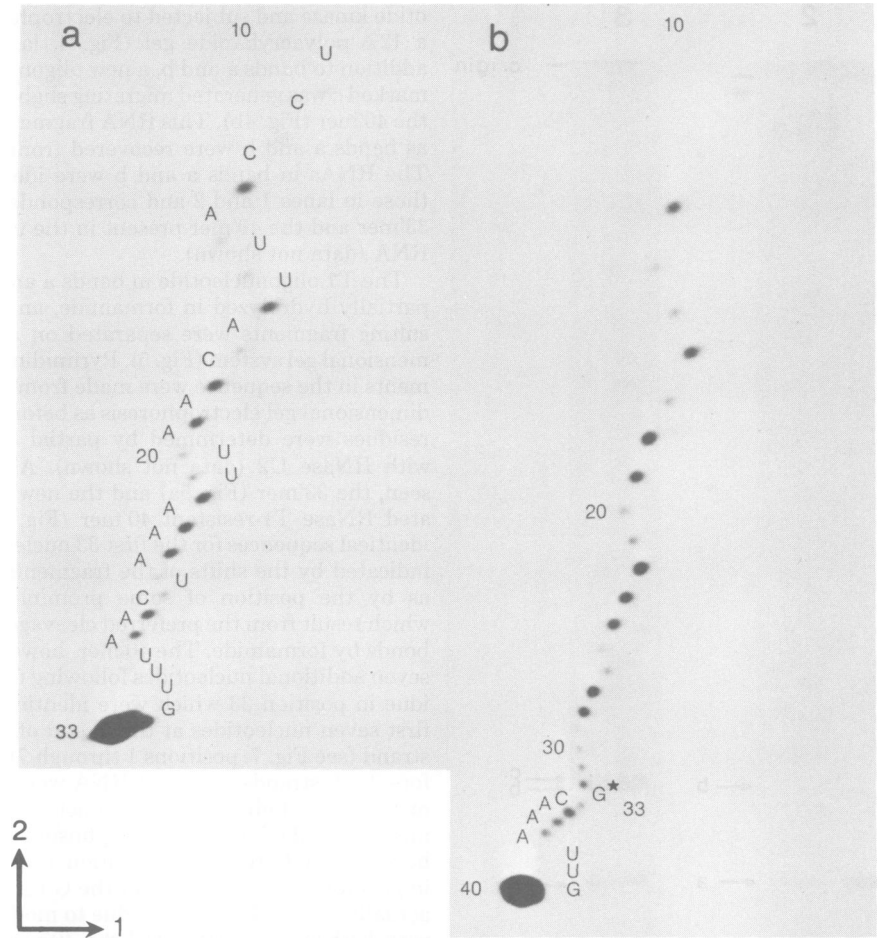


FIG. 5. *Two-dimensional polyacrylamide gel analyses of the 33'mer and 40'mer. The 5' terminally labeled oligonucleotides marked a and c in Fig. 4 (lane 3) were partially hydrolyzed in formamide at 100°C and separated in a two-dimensional polyacrylamide gel system. Partial hydrolysate of the 33-nucleotide-long RNA fragment a (a) and fragment c (b) in Fig. 4. The size of the oligonucleotides as well as the position of the modified G residue (position 33) in the 40'mer are indicated. The positions of adenine as well as pyrimidine residues were determined by partial enzymatic digestions (data not shown).*

tary strands of the hairpin-type DI 011 RNA as summarized in Fig. 7.

Depending on the pretreatment of the RNA, ^{32}P label could be placed specifically at three different positions in the RNA by using [γ-^{32}P]-ATP and polynucleotide kinase. Position a (Fig. 7) was labeled after phosphatase treatment, and positions b and c were labeled after nicking the RNA with RNases T1 and A, respectively. The labeled, duplexed RNAs all comigrated on a low-percentage polyacrylamide gel, irrespective of the nuclease treatment, indicating that the size of the single-stranded region in the intact RNAs is not larger than 10 to 20 bases, the limit of resolution of the method (Fig. 1). Similarly, the sizes of the plus and minus strands generated by nuclease treatment were shown to be the same. We have previously shown that the triphosphate-bearing 5' terminus belongs to the minus strand of the RNA which is conserved from VSV RNA for its entire length (29), and we now estimate this to be about 860 bases. This corresponds to 7 to 8% of the total VSV genome. The 5'-terminal nucleotide sequence of the plus strand started with a C residue and lacked other C residues until position 34 (Fig. 7). This suggested the presence of a large T1 oligonucleotide on the complementary strand spanning from positions 1' to 33'. This 33-nucleotide-long oligonucleotide was isolated after cleavage at po-

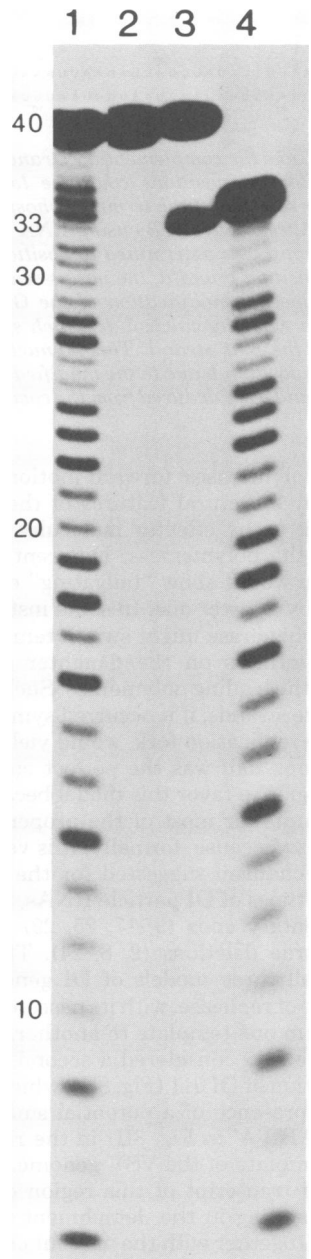


FIG. 6. *RNase T1 resistance of the modified G residue in position 33 of the 40'mer. The 5' terminally labeled 40'mer generated after kethoxal treatment of DI 011 RNA was incubated for 15 min at 50°C in the presence of either 0.5×10^{-3} U of RNase T1 per μg of RNA (lane 2, partial digestion condition) or 40×10^{-3} U of RNase T1 per μg of RNA (lane 3, complete digestion condition). The RNAs were separated on a 12% polyacrylamide gel next to partial hydrolysates of both the 40'mer (lane 1) and 33'mer (lane 4) RNAs. The sizes of the fragments are indicated.*

sitions b and d, and its nucleotide sequence was confirmed. DI-T RNA which is of minus sense and carries only short complementary terminal regions also had this 33'mer, demonstrating that the oligonucleotide was indeed of minus sense. Since pancreatic RNase was able to cleave at position c (Fig. 7) although the C residue in position 1 could base pair and was protected in the nicked RNA suggested that base pairing is not possible in the intact RNA. In addition, the C residue in position 1 could be labeled after RNase T1 treatment and so was preceded by a G residue in the intact RNA. These observations indicate that the single-stranded loop which links the two strands had to be less than four to six nucleotides long and that at least the terminal G and C residues were part of this region. We postulated that the C and G residues in positions 1 and 1' might actually be the termini that are linked by a single phosphate group. To test this possibility, we specifically modified the G residue in position 1' by using the single-strand-specific G residue-modifying compound kethoxal. After modification and complete RNase T1 digestion, we detected a new 40-base-long oligonucleotide. This 40'mer contained at its 5' side the nucleotide sequence of the 33'mer mentioned above followed by seven nucleotides identical to those from positions 1 through 7 on the plus strand. This oligonucleotide was generated by cleavage at positions d and e without the cleavage at site b, since the G in that position was modified. These data demonstrate that both strands of DI 011 RNA are complementary to the last nucleotides and are linked by a single phosphate group. For sterical reasons at least, the last two nucleotides on both strands are not base paired.

The nucleotide sequence of this region of DI 011 RNA limits the number of possible mechanisms for the generation of this hairpin-type RNA. Three different mechanisms were discussed earlier (15): the ligation of complementary RNA strands; the chance fragmentation of some natural duplex intermediate of VSV replication; and the replication across a replication fork. The fact that both strands of DI 011 RNA are complementary, including the last nucleotides, makes it highly unlikely that it arose by a random ligation reaction since the substrate RNAs for such ligation would have to be perfectly complementary RNAs of precisely the same length. There is also no evidence for covalently linked plus- and minus-sense RNA intermediates in VSV replication or transcription which might serve as a potential precursor for a hairpin-type DI particle RNA. The third possibility, that of replication across a replication

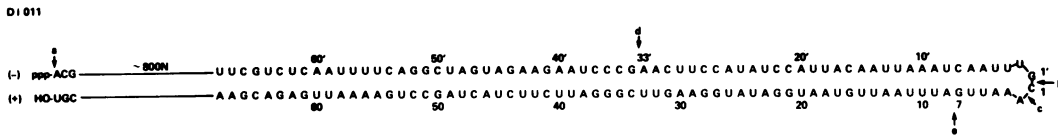


FIG. 7. *Summary of the nucleotide sequence of the region which links the complementary strands of DI 011 RNA. Depending on the pretreatment of DI 011 RNA, the following positions could be labeled with [γ-^{32}P]ATP and polynucleotide kinase: position a was labeled after removal of the terminal phosphates, and positions b and c were labeled after nicking with RNases T1 and A, respectively. By using RNA with the 5′-terminal label at position 1, the nucleotide sequence of the plus strand was determined to position 68. After complete digestion of the RNA with RNase T1 and cleavages at position b and d, the minus-sense oligonucleotide (33′mer) was isolated and its sequence was confirmed. Specific modification of the G residue in position 1′ followed by complete RNase T1 digestion gave rise to a new oligonucleotide which spanned the region from position 33′ on the minus strand up to position 7 on the plus strand. This 40-nucleotide-long fragment was generated by cleavages at positions d and e, omitting position b due to the modified RNase T1-resistant G residue at position 1′. Positions 34′ to 68′ on the minus strand were deduced from the complementary strand.*

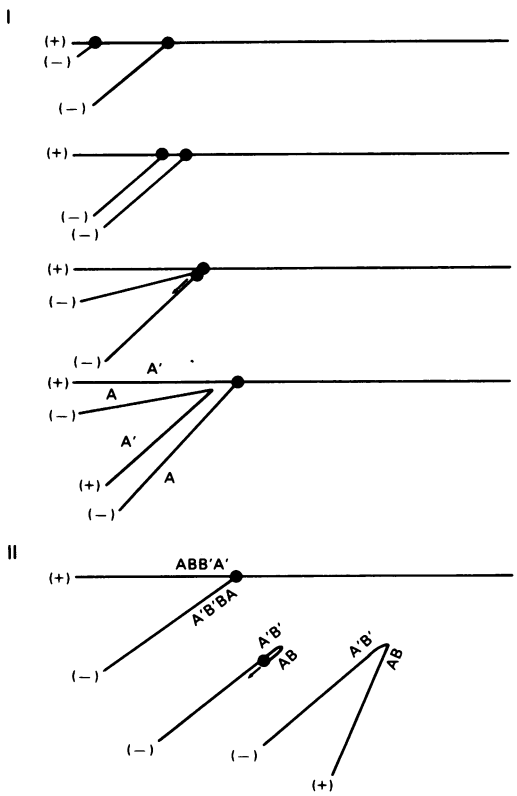


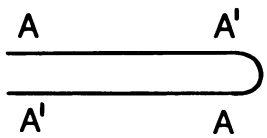
FIG. 8. *Models for the generation of DI 011 RNA. (I) Transcription across a nascent replication fork. (II) Potential hairpin in the nascent transcript leads to detachment of the nascent chain from the template and copyback replication. For details see Discussion.*

fork, easily accounts for the known properties of DI 011 RNA (Fig. 8I). This model requires that more than one replicase copy a single template at the same time and that the movement of the replicase along the template can vary. Conceivably the polymerases' forward motion might be affected by structural features of the template or product or by effector molecules acting directly on the polymerases. Different speeds in replication would allow "tailgating" of a slower replicase by a faster one. In some instances, the trailing polymerase might switch templates and resume synthesis on the daughter strand attached to the leading polymerase. Such a change in template strands, if it occurred symmetrically across the replication fork, would yield an RNA in which one half was the perfect complement of the other. We favor this model because it not only accounts for most of the properties of DI 011 but also because, formally, it is very similar to the mechanism suggested for the origins of two other types of DI particle RNAs, those with complementary ends (9, 17, 25, 29) and those that are true deletions (2, 8, 24). The central event in all three models of DI genesis is the movement of replicase, with its nascent daughter in tow, from one template to another.

We have also considered a second model for the formation of DI 011 (Fig. 8II), which depends upon the presence of a potential small hairpin (marked ABB′A′ in Fig. 8II) in the ribonucleoprotein template of the VSV genome. An unencapsidated transcript of this region could self-anneal, resulting in the detachment of the polymerase (together with the nascent chain) from the template. The template-free polymerase might then copy back on the encapsidated part of its own nascent chain, yielding a hairpin RNA. If VSV RNA contained such a small hairpin in this position, then part of the sequences of the nuclease-generated 5′ terminus of the plus strand would be minus sense (the AB portion in Fig. 8). We have tested this possibility by annealing the minus-sense 33-nucleotide-long RNA fragment mentioned above to VSV L message which carries a complement of the 33′mer.

The 33'mer annealed readily to L message for its full length without any indication of competition caused by a hairpin in the sequence (data not shown). In addition, when the 40'mer, which consists of the 33'mer linked to the first seven nucleotides of the plus strand, was annealed to L message, the resulting duplexes were either 34 or 32 nucleotides long, depending on the RNases used. However, no larger duplexes were detected (data not shown). These experiments, although not totally conclusive, strongly indicate that a potential hairpin loop is not present at this position in the VSV RNA, a feature that is a prerequisite for the second model.

Earlier studies by electron microscopy on the structure of hairpin DI particle RNAs by Perrault and Leavitt (23) suggested that nicked and denatured hairpin RNAs could enter into circular and multimer structures. The authors interpreted these results as indicating that the DI particle RNAs had the structure:



where A and A' are complementary sequences, However, the nucleotide sequences of the 3' and 5' termini as well as the turnaround portion of DI 011 have been determined by us (this communication; 28, 29), and no discernible relationship exists between the termini and the turnaround region. Consequently, DI 011 RNA does not conform to that proposed structure. The possibility remains that there are several types of snapback DI particle RNAs and that one has the sequence complementarity predicted from the electron microscope studies.

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