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# Defective Interfering Particle Generated by Internal Deletion of the Vesicular Stomatitis Virus Genome

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The genome structure of the long, truncated defective interfering particle derived from the heat-resistant strain of vesicular stomatitis virus has been examined. Stocks of this defective interfering particle are shown to contain several different species having information primarily from the 3′ half of the vesicular stomatitis virus genome; the proportions of these components vary depending on the passage history of the stock. The two most abundant types have been identified and characterized. One has complementary 5′ and 3′ termini and consequently appears as a circular molecule when examined by electron microscopy. The other cannot circularize and remains linear. The circular forms are consistently 8 to 10% longer than the linear molecules. Rapid sequencing analyses reveal that both forms retain the 5′ parental viral terminal sequence, but only the linear form retains the parental 3′-terminal sequence. The circularizable RNA molecule has a nonparental 3′-terminal sequence which is the complement of the 5′ end. Hybridization experiments and electron microscopic analyses indicate that the linear form has retained 320 to 350 nucleotides of the 5′ parental sequence and was probably generated by an internal deletion of the vesicular stomatitis virus genome.

Defective interfering (DI) particles are small viral particles that accumulate in culture fluids during high-multiplicity passage of the parental virus (42). Characteristically, DI particles contain less genetic information than the parental virus, and because of this deficit they replicate only if a helper virus supplies the missing function(s) (13, 15). The genomes of a number of vesicular stomatitis virus (VSV) DI particles have been probed by hybridization to viral mRNA and found to fall into two groups: those containing information from the 5′ portion and those containing information from the 3′ portion of the parental genome (27, 38, 41). The members of the former group appear to be more numerous and to be characterized by genomes with several unusual properties. All have 5′ and 3′ termini which are complementary for 46 to 49 nucleotides, a property not shared by the parental viral genome (34, 35, 39a), and each of these DI particle genomes retains the parental 5′-terminal sequence but possesses a nonparental 3′ terminus (22-24, 35, 39a, 40).

Kolakofsky and colleagues (25, 28) and Huang (14) recently proposed models to explain the generation of DI particles having the characteristics outlined above. These authors suggested that DI particles containing information from the 5′ portion of the genome arise as the result of an aberration of replication during the synthesis of (−)-sense RNA. The origin of the minority class of DI particles—those whose genomes contain information from the 3′ portion of the parental genome—is obscure. It is possible that these DI particle genomes originate by an aberration of replication like that proposed for the majority class but occurring during the synthesis of the (+)-strand RNA. This model predicts that the 3′ terminus of the RNA of these DI particles is identical to the 3′ end of the parental genome. It also predicts that the 5′ end of the DI genome differs from the parental and is the complement of the 3′-terminal sequence. Alternatively, this type of DI particle may arise as the result of an internal deletion of the parental genome. These two models predict slightly different structures for the DI particle RNA: complementary termini versus termini that are identical to those found in the parental genome.

We have therefore analyzed the genome of the long T particle (DI-LT) derived from the heat-resistant strain of VSV (VSV-HR) (36) to determine whether its structure is, in fact, accurately predicted by one of these models. Our results indicate that many, and perhaps all, of the stocks of DI-LT currently in use contain a number of different DI particles derived from the 3′ half of the VSV genome; the proportions of these species vary depending on the conditions of growth. Nevertheless, the results clearly show that the predominant DI particle present in early-passage DI-LT stocks has retained both the paren-

tal 5'- and 3'-terminal sequences. The genome of this DI particle retains approximately 5,600 nucleotides contiguous with the 3' half of the parental genome, plus 320 to 350 nucleotides of the 5'-terminal parental sequence. We conclude from these data that this DI-LT particle was probably generated by an internal deletion of the VSV genome within the region coding for the L mRNA.

## MATERIALS AND METHODS

**Viruses and culture conditions.** Two DI-LT seed stocks were employed in this study. The first was obtained from L. Prevec in 1972 and was twice serially subcultured in this laboratory using VSV-HR as the helper. The second stock was obtained from Y. Kang and was part of the original LT stock, which had been stored frozen for 9 years. This seed stock was subcultured in BHK-21 cells that had been pretreated with actinomycin D (1 μg/ml) for 15 to 18 h, using helper virus that had been plaque purified by Y. Kang in actinomycin D-pretreated cells. Actinomycin D pretreatment has been reported to suppress the generation of new DI particles while allowing the replication of DI particles already present (20). The derivations of DI-011 and DI-T have been previously reported (26, 41).

**Preparation of RNA.** RNA labeled with [$^3$H]uridine was prepared from virus and DI particles cultured in BHK-21 cells in the presence of actinomycin D (0.6 μg/ml). The cells were not pretreated with actinomycin. The purification of VSV and its DI particles, as well as the preparation of RNA from them, has been described previously (23, 26) and is only summarized here. VSV and DI particles were precipitated from clarified culture fluids by using polyethylene glycol-6000. The resuspended particles were isopycnically banded in a glycerol-tartrate gradient and then in a linear sucrose gradient. RNA was extracted from the particles using phenol-chloroform-sodium dodecyl sulfate (SDS) and precipitated with ethanol. The RNA was fractionated by centrifugation in a linear 10 to 30% sucrose gradient in 0.1 M NaCl, 0.01 M Tris (pH 7.5), 0.001 M EDTA, and 0.5% SDS. Fractions containing the relevant RNA were precipitated with ethanol, washed with 80% ethanol, and then dried in vacuo. The RNA was finally dissolved in 0.001 M Tris (pH 7.5).

**5'-Terminal labeling of RNA.** RNA was labeled at the 5' terminus with [$\alpha$-$^{32}$P]GTP using the guanylyl transferase from vaccinia virus (32). One picomole of [$^3$H]RNA was incubated at 37°C for 15 min in 100 μl containing 50 mM Tris-hydrochloride (pH 7.6), 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM ATP, 20 μM *S*-adenosylhomocysteine, 10 μM [$\alpha$-$^{32}$P]GTP (932 Ci/mmol), and 5 μl of vaccinia guanylyl transferase (generously provided by B. Moss). The reaction was terminated by the addition of SDS to a final concentration of 0.1%, and the capped RNA was separated from the unincorporated GTP by chromatography over Sephadex G-150. The RNA was precipitated with ethanol, washed with 80% ethanol, dried, and dissolved in distilled water. The efficiency of capping of the RNA was approximately 17%.

**3'-Terminal labeling of RNA.** RNA was labeled at the 3' terminus with [5'-$^{32}$P]cytidine-3',5'-bisphosphate using RNA ligase as described previously (11, 23). The ligation reaction was carried out either overnight at 4°C or for 30 min at 37°C. The reaction was terminated and the RNA was isolated as described above for 5'-terminal labeling.

**Rapid RNA sequencing.** 5'- or 3'-terminally labeled RNA was partially hydrolyzed with alkali or RNase $T_1$ or $U_2$ and then analyzed by polyacrylamide gel electrophoresis as described by Donis-Keller et al. (10). Autoradiography was performed using Kodak XR-2 X-ray film and Lanex or Cronex Lightning Plus intensifying screens.

**Formation and sizing of heteroduplexes between DI-011 and DI-LT RNA.** 3'-Labeled DI-011 RNA was nicked with RNase A (0.5 μg/ml) in 0.4 M NaCl–0.01 M Tris-hydrochloride (pH 7.5) for 30 min at 37°C. The nuclease was inactivated by digestion with proteinase K (100 μg/ml) for 30 min at 37°C, followed by phenol extraction. The RNA was ethanol precipitated, washed with 80% ethanol, dried, and dissolved in 0.01 M Tris (pH 7.5). Duplexes between the labeled (+)-sense portion of DI-011 RNA and DI-LT RNA were formed by mixing the RNAs, heating at 100°C for 30 s, and annealing for 2 h at 60°C in 0.4 M NaCl–0.01 M Tris (pH 7.5). Single-stranded RNA was digested with RNases A and $T_1$ at either 0.5 μg + 0.1 U (respectively) per ml, 2.5 μg + 0.5 U per ml, or 12.5 μg + 2.5 U per ml, for 30 min at 37°C. The nuclease was inactivated by digestion with proteinase K (100 μg/ml) at 37°C for 30 min, followed by incubation with 0.2% (final concentration) diethyl pyrocarbonate for 30 min at 37°C. Carrier tRNA was added, and the duplexes were precipitated with ethanol. The precipitate was washed with 80% ethanol, dried, and dissolved in distilled water. Portions were mixed with an equal volume of electrophoresis buffer containing bromophenol blue, xylene cyanol FF, and 40% sucrose. Duplexes were analyzed by electrophoresis on 2% polyacrylamide, 0.5% agarose composite gels (9). Uniformly labeled *Hin*dII + III restriction fragments of simian virus 40 DNA (kindly provided by G. Khoury and R. Dhar) were used as size markers.

**Isolation of nucleocapsid RNA.** BHK-21 monolayers were infected with VSV at a multiplicity of 10. [$^3$H]uridine (5 μCi/ml) and actinomycin D (0.6 μg/ml) were added 30 min postinfection. Cells were cultured at 37°C for 4 h and harvested, and cell-free extracts were prepared as described earlier (41). Nucleocapsids were purified using a cesium chloride gradient (29), and RNA was prepared from them by SDS-phenol-chloroform extraction. By the amount of self-annealing, it was estimated that 25% of the RNA in this preparation was (+)-sense 42S RNA.

**In vitro mRNA synthesis.** Viral mRNA was synthesized in vitro by VSV-HR as previously described (4) using [$^3$H]UTP as the labeled precursor in a 2-ml reaction mixture. The RNA was extracted twice with phenol-chloroform-SDS and purified by chromatography on Sephadex G-50 and oligodeoxythymidylic acid-cellulose. The RNA that bound to the column was denatured with dimethyl formamide and dimethyl sulfoxide and sedimented in a 15 to 30% sucrose gradient in 0.01 M Tris(pH 7.4), 0.001 M EDTA, and 0.5%

SDS (8). The 12-17S RNA was pooled and precipitated with ethanol.

**Hybridization of DI particle RNA to mRNA.** DI particle RNA (10,000 cpm), uniformly labeled with [$^{32}$P]orthophosphate, was annealed for 3 h at 60°C in 10 μl of 0.4 M NaCl–0.01 M Tris (pH 7.5)–0.2% SDS with increasing amounts of 12-17S polyadenylated mRNA synthesized in vitro by VSV-HR. The samples were diluted into 1.1 ml of 0.3 M NaCl–0.01 M Tris (pH 7.5), and two 0.5-ml portions were taken. RNase A (20 μg/ml final concentration) and RNase $T_1$ (28 U/ml) were added to one portion of each sample, and both portions were incubated at 37°C for 30 min. RNase-resistant material was precipitated with trichloroacetic acid and collected on Millipore membrane filters.

**Electron microscopy and heteroduplex mapping.** DI-LT RNA at a concentration of 5 to 20 μg/ml was self-annealed or annealed to a mixture of (+) and (−) 42S nucleocapsid RNA for 20 min at 37°C in 44% formamide, 2 M urea, 0.2 M tricine-NaOH (pH 8.3), and 0.036 M EDTA in glass capillaries. The RNA was diluted into 12 volumes of 83% formamide, 3.8 M urea, 0.1 M tricine-NaOH (pH 8.3), and 0.01 M EDTA and spread onto a hypophase of water. Grids were prepared and shadowed as previously described (31). Samples were examined in a Philips EM201 electron microscope at 40 kV. Tracing of the molecules and methods of data processing have been described (43). Magnifications were determined relative to a grating replica with a spacing of 416.7 μm.

## RESULTS

**Circular and linear forms of DI-LT RNA.** One of the hallmarks of most of the VSV DI particle RNAs examined to date is the complementarity of the 5′ and 3′ termini. If DI-LT were formed by a mechanism similar to that described by Leppert et al. (28) and Huang (14), it would be expected to retain the original parental 3′-terminal sequence and to have a nonparental 5′-terminal sequence that is complementary to its 3′ end. DI particle RNAs with termini that are complementary for as little as 46 to 49 nucleotides can circularize and, under the appropriate conditions, be visualized as circular RNA molecules in the electron microscope (34). On the other hand, RNA molecules that possess the parental 5′ and 3′ termini do not circularize under the same conditions, since the complementarity between these sequences is not sufficient to hold the RNA in a closed form (6, 22, 23, 39, 39a, 40; B. L. Semler, J. Perrault, and J. J. Holland, Nucleic Acids Res., in press).

Table 1 shows the results of electron microscopic analyses of three preparations of DI-LT RNA, which were derived from two different seed stocks of virus. After self-annealing, all three preparations appear to be mixtures of linear and circular RNA molecules. RNA preparations 1 and 2 were derived from a seed stock that was obtained from Y. Kang. This stock was a portion of one of the earliest preparations of DI-LT and had been stored frozen for 9 years. When cells were infected with this stock at low multiplicity, the progeny were approximately equal numbers of infectious virus and DI particles, and the 28S DI particle RNA (preparation 1) was almost devoid of circles. However, when the same stock was used at high multiplicity, the progeny were almost exclusively DI particles, and the 28S DI particle RNA (preparation 2) contained significant numbers of circular forms. Preparation 3 was derived from a seed stock that was originally obtained from L. Prevec in 1972 and was serially passaged twice during the intervening years. The RNA prepared from this stock contains approximately equal numbers of linear and circularizable molecules (Table 1). Nevertheless, although the proportions of linear and circularizable forms can vary greatly, their lengths remain essentially unchanged (Table 1).

TABLE 1. *Length distribution of DI-LT RNA*

| Prepn | Circular | | Linear | |
|---|---|---|---|---|
| | Size (μm)[a] | Percent[b] | Size (μm)[a] | Percent[b] |
| 1 | 2.23 ± 0.01 (8) | 5 | 1.99 ± 0.07 (42) | 95 |
| 2 | 2.17 ± 0.07 (22) | 24 | 2.06 ± 0.10 (69) | 76 |
| 3 | 2.17 ± 0.01 (23) | 58 | 1.93 ± 0.02 (25) | 42 |

[a] One micrometer corresponds to approximately 3,000 nucleotides. Data are mean ± one standard deviation. Parentheses indicate number of molecules measured.

[b] At least 100 molecules were counted.

Since the proportions of linear and circularizable RNA molecules can vary depending upon both the origin of the seed stock and the conditions of growth, it is unlikely that the linear molecules observed in the electron microscope are simply open forms of a molecule with complementary ends. (Control experiments with DI-611 RNA, which is of similar size and known to contain complementary 5′ and 3′ termini [39a] showed that, under the conditions used here, at least 75% of the molecules appear as circles in the electron microscope.) The histograms of the length measurements for RNA preparations 1 and 3 (Fig. 1 and 2, respectively) indicate that it is also unlikely that the linear RNAs are merely broken molecules. Panel (A) of each figure shows the length distribution of the closed forms, and (B) shows the length distribution of the linear forms in these two preparations. All RNA molecules from 1.2 to 3.0 μm in length were included in these histograms to avoid subjective skewing of the data. The measurements, which are summarized in Table 1, indicate that the circles are consistently 8 to 10% longer than the linear forms. The length distributions of the linear molecules in preparations 1 and 3 are also not

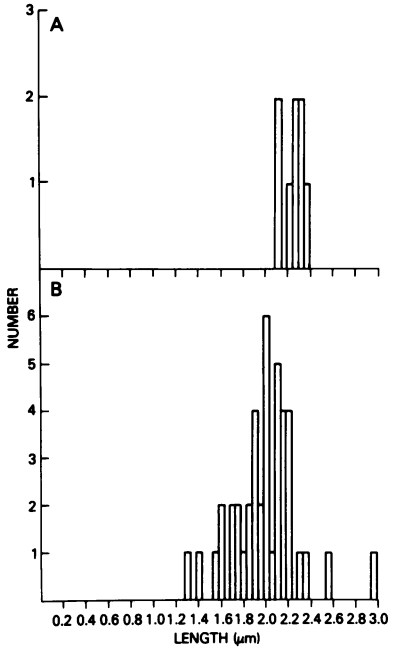


FIG. 1. *Length distribution of DI-LT RNA. DI-LT RNA (preparation 1) was self-annealed for 20 min at 37°C as described in the text. RNA was spread onto a hypophase of distilled water. (A) Circular, (B) linear forms. Molecules measuring between 1.2 and 3.0 μm were included in the histogram.*

those that would be predicted for randomly fragmented RNA molecules. These results indicate that the preparations of DI-LT contain at least two species of DI particles, only one of which can circularize. The relative abundance of these RNAs in different preparations suggests that the species containing the RNA that can circularize enjoys a selective advantage when the stock is passaged at high multiplicity.

**5'-Terminal sequence of DI-LT RNA.** The genomes of VSV and of all of the DI particles that have been examined bear an adenosine di- or triphosphate at the 5' terminus (2, 16). RNA terminating in a polyphosphate can be specifically labeled at the 5' end with [$\alpha$-$^{32}$P]GTP using the guanylyl transferase enzyme from vaccinia virus to form a cap structure (32). We have labeled the 5' termini of DI-LT (preparation 3) and VSV-HR RNA in this fashion and then determined the positions of the adenosine and guanosine residues in the terminal region of each RNA by rapid sequencing techniques (10). Figure 3 shows an autoradiogram of a 20% polyacrylamide gel in which partial hydrolysates of the 5'-end-labeled DI-LT and VSV-HR RNA were resolved. Comparison of the sizes of the fragments generated by RNase $T_1$ or $U_2$ demonstrates that the positions of the purines are identical for at least 60 nucleotides from the 5' end. Even though the DI-LT RNA used in this experiment (preparation 3) contained almost equal numbers of linear and circularizable RNAs, no differences in the locations of the purines were observed between the DI-LT and the VSV-HR RNAs. In addition, the ladder generated by limited alkaline cleavage of the DI-LT RNA showed no signs of sequence heterogeneity (39a). These results strongly indicate that the 5'-terminal sequences of both species of DI-LT RNA are identical to that of VSV-HR RNA.

Based on the models that have been proposed for the generation of DI particles by VSV (14, 25, 28), a DI particle containing the 3' half of the genome might be expected to have the complement of the parental 3'-terminal sequence at the 5' end of its genome. Such a sequence would be identical to that of the leader RNA, which has been shown to be the complement of the precise 3' end of the VSV genome (4–6). The leader sequence has guanosine residues in positions 3, 6, 33, and 34 but does not have a G in position 19. The analysis of the 5' terminal sequence of

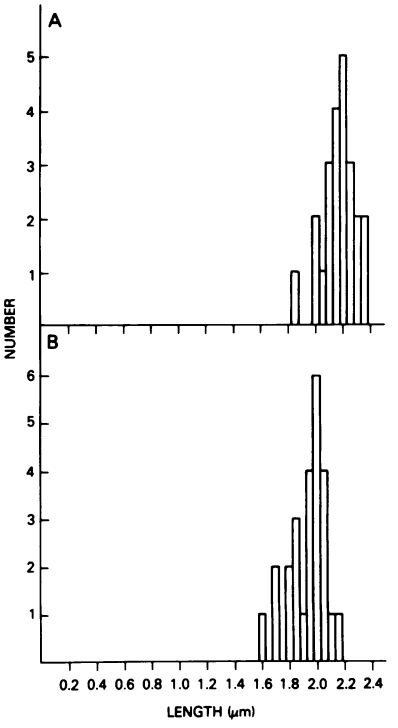


FIG. 2. *Length distribution of DI-LT RNA. DI-LT RNA (preparation 3) was self-annealed for 20 min at 37°C and spread as described in the text. (A) Circular, (B) linear forms. Molecules measuring between 1.2 and 3.0 μm were included.*

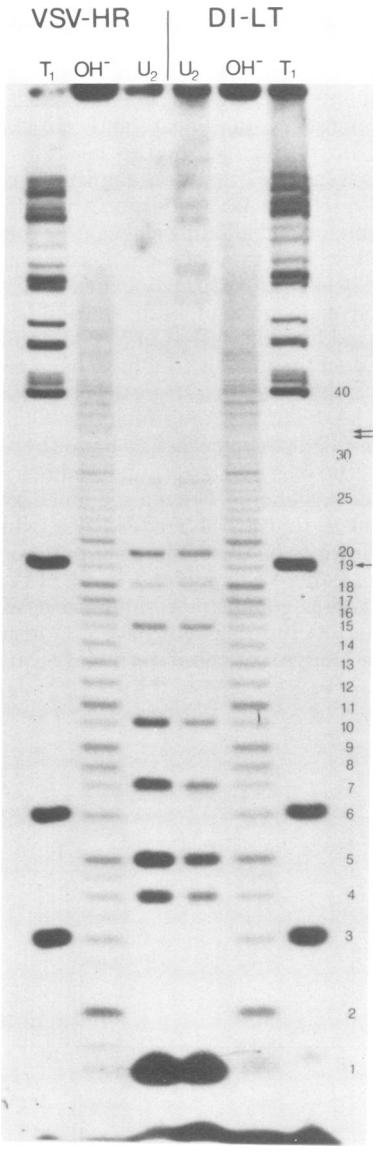


FIG. 3. *5′-Terminal sequence of DI-LT and VSV-HR RNA. DI-LT RNA preparation 3 and VSV-HR RNA were specifically labeled at the 5′ end with [α-$^{32}$P]GTP using the vaccinia virus guanylyl transferase (32). Each RNA was partially hydrolyzed with alkali, RNase $T_1$, or RNase $U_2$. Fragments were resolved by electrophoresis in a 20% polyacrylamide gel (10). The numbers indicate the length in nucleotides of the fragments. The arrows are explained in the text.*

DI-LT RNA (preparation 3) shows no evidence of a guanosine in either position 33 or 34 but does show the presence of a G in position 19. (The relevant areas of the sequencing gel in Fig. 3 are illuminated with arrows.) We conclude from these results that the majority of linear and circularizable DI-LT RNAs have 5′-terminal sequences similar to that of the VSV-HR. Furthermore, the positions of the adenosine and guanosine residues found here in both VSV-HR and DI-LT RNA correspond exactly to those found by Schubert et al. (39a) and Semler et al. (in press) for the locations of the purines at the 5′ end of the Mudd-Summers strain of VSV.

Since the majority of the information contained in the DI-LT RNA comes from the 3′ half of the parental genome (27, 41), the 5′-terminal sequence must diverge from that of the parent virus at some point. We have made use of DI-011 RNA to locate this point of divergence. The DI-011 genome contains about 1,000 bases that are identical (or very nearly identical) in sequence to the 5′ terminus of the VSV genome, but these nucleotides are also covalently linked to the complementary (+)-sense sequence (26, 33). By specifically labeling the 3′ end of the DI-011 RNA, nicking the region linking the (+) and (−) sequences with nuclease, and dissociating the strands, we have made a (+)-sense hybridization probe that is specific for the 5′ kilobase of VSV RNA. This probe was annealed to DI-LT RNA preparation 1 (95% linear molecules), and the partial duplexes were trimmed of single-stranded RNA with RNases A and $T_1$. The length of the resultant duplex is determined by the position at which the two sequences diverge, provided that it occurs within the first kilobase from the 5′ terminus. If the DI-LT RNA diverges after that point, a limit size duplex of approximately 1,000 base pairs will be obtained. The results of this experiment are shown in Fig. 4. Two sizes of duplex were obtained after annealing the 3′-terminally labeled DI-011 RNA with DI-LT RNA. The more abundant duplex comigrates with a simian virus 40 DNA restriction fragment that is 350 base pairs long (12); the less abundant duplex comigrates with the self-annealed DI-011 RNA, and its size may be limited by the length of the (+)-sense portion of the DI-011 genome. The larger duplex is detected by an increase above the control in the amount of material that comigrates with self-annealed DI-011 RNA (Fig. 4). The amount of each duplex recovered is unchanged even when the RNase concentration is varied over a 25-fold range. This suggests that these are perfect or near-perfect duplexes and that neither is the result of incomplete digestion. In parallel control experiments (not shown), 3′-labeled DI-011 RNA was annealed to RNA from VSV-HR, the virus from which DI-LT was derived. Only the expected limit size duplex was obtained, and this demonstrates that the formation of the shorter duplex with DI-LT RNA is not a result of strain differences between the parental viruses. Because two duplexes of different size are generated by the

annealing between DI-011 and DI-LT RNA, we again conclude that there is more than one type of particle within the DI-LT stock. One type contains approximately 350 nucleotides of the 5′-terminal sequence of the parental viral genome, whereas another contains at least one kilobase of this sequence. (It should be noted that these size estimates are only approximate since they are based on a comparison of the migration of double-stranded RNA with double-stranded DNA.)

**3′-Terminal sequence of DI-LT RNA.** The presence of an RNA in the DI-LT preparations that is capable of circularizing suggests that some of the molecules must have complementary ends. Since only the parental 5′-terminal sequence was detected (Fig. 3), we examined the 3′ termini to determine whether the complement of this sequence was present. Work by Colonno et al. (7) has shown that the VSV leader RNA could be annealed to the DI-LT RNA. This suggested that at least some of the molecules possessed the parental 3′-terminal sequence. However, those experiments did not exclude the possibility that other 3′ termini were also present in the same population. The sequence of the parental 3′ terminus and that of the complement of the 5′ terminus have been directly and indirectly determined (6, 23, 39, 40). We therefore compared the 3′-terminal sequences of the genomes of DI-LT, VSV-HR, and DI-T. (DI-T is a particle derived from the 5′ portion of the genome whose 3′ terminus had previously been shown to be the complement of the parental 5′ sequence [39a]. For this comparison we terminally labeled the RNAs at the 3′ end with [5′-$^{32}$P]cytidine-3′,5′-bisphosphate and determined the locations of the purines by rapid sequencing techniques. Since RNases $T_1$ and $U_2$ are specific for single-stranded RNA, it is possible to examine the sequences present in single-stranded RNA separately from those sequestered in any duplexes that may also be present. The latter are detected by the rapid sequencing techniques only if the RNA is heat denatured before digestion with the nucleases. We compared the sequence of purines in DI-LT RNA (preparation 2) that is obtained before or after heat denaturation with those obtained from heat-denatured VSV-HR and DI-T RNA (Fig. 5). It can be readily seen that the locations of the purines near the 3′ end of the unheated DI-LT RNA are identical to those in VSV-HR RNA for at least 38 nucleotides. Both RNAs have guanosine residues in positions 8, 12, 16, 17, 26, 36, and 38. Both also have adenosine residues in positions 19, 20, 22, 23, 25, 28, and 29. However, when the DI-LT RNA is heat denatured prior to digestion, three additional bands appear, which are characteristic of the complement of the 5′-terminal sequence as it is found in DI-T. These correspond to guanosine residues in positions 9 and 11 and an adenosine residue in position 21. These

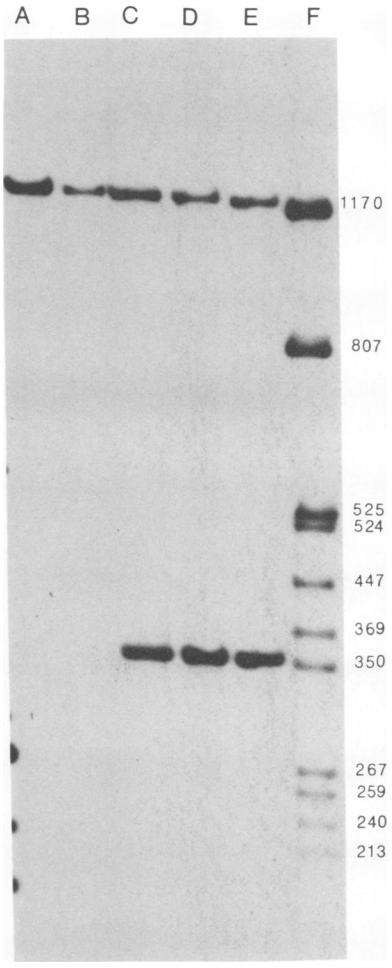


FIG. 4. *Heteroduplex formation with DI-011 RNA. Samples containing 4,000 cpm of nicked DI-011 RNA labeled at its 3′ terminus with [5′-$^{32}$P]cytidine-3′,5′-bisphosphate were mixed with 1 μg of DI-LT RNA (preparation 1) in 600 μl of 0.01 M Tris (pH 7.6), heated to 100°C for 60 s, quickly cooled, adjusted to 0.4 M NaCl, and annealed for 1 h at 60°C. The resultant duplexes were treated with increasing amounts of RNases A and $T_1$ as described in the text before electrophoresis on a 40-cm 2% polyacrylamide–0.5% agarose composite gel. (A) Control, untreated 3′-labeled DI-011 RNA; (B) DI-011 RNA nicked, denatured, and self-annealed prior to digestion with RNase A (2.5 μg/ml) + $T_1$ (0.5 U/ml); (C) heteroduplex between DI-011 and DI-LT RNA digested with RNase A (0.5 μg/ml) + $T_1$ (0.1 U/ml); (D) heteroduplexes digested with RNase A (2.5 μg/ml) + $T_1$ (0.5 U/ml); (E) heteroduplexes digested with RNase A (12.5 μg/ml) + $T_1$ (2.5 U/ml); (F) uniformly labeled simian virus 40 HindII + III restriction endonuclease fragments, whose lengths in base pairs are indicated at right (12).*

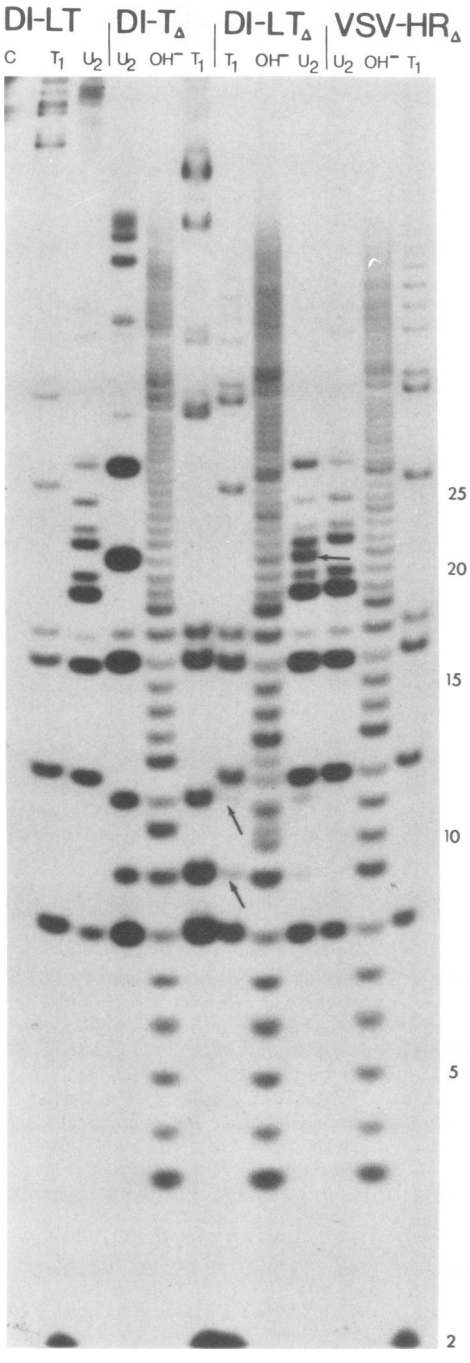


FIG. 5. *Comparison of 3′-terminal sequences. RNA from DI-LT (preparation 2), DI-T, and VSV-HR was specifically labeled at the 3′ end with [5′-$^{32}$P]cytidine-3′,5′-bisphosphate. Unheated or heated DI-LT RNA and heated DI-T and VSV-HR RNAs were partially hydrolyzed with alkali, RNase T$_1$, or RNase U$_2$. The fragments were resolved by electrophoresis in a 20% polyacrylamide gel. Lane C (control) contained undigested DI-LT RNA; △ indicates that the RNA was heat denatured before digestion. The arrows mark the locations of the bands that appear only after the DI-LT RNA is heated. The numbers indicate the length in nucleotides of each fragment. Mononucleotides have been run off the bottom of the gel.*

bands are identified by arrows in Fig. 5. The results indicate that there are two different 3′-terminal sequences present in the DI-LT RNA. One sequence is identical to that of the parental viral RNA. The other, which is revealed only if the RNA is denatured before digestion, has a purine sequence, suggesting that it is the complement of the parental 5′-terminal sequence.

We have attempted to estimate the proportions of the two types of 3′-terminal sequence in the following manner. After complete digestion of self-annealed 3′-labeled DI-LT RNA (preparation 1) with RNases A and T$_1$, the RNase-resistant duplex stems were isolated. These stems were found to contain 7.3% of the end-specific [$^{32}$P]phosphate originally present in the undigested RNA. This number is quite close to that anticipated for an RNA population in which 5% of the molecules can form circles. The size of the stem (less than 75 base pairs) and the locations of the guanosine residues within it are similar to those found for stems isolated from DI particles containing information from the 5′ portion of the genome.

**Hybridization of DI-LT RNA to mRNA.** The mixture of 3′-terminal sequences confirms that there are at least two different DI particles present in the preparation. However, it also presents the possibility that one of these DI particles may contain genetic information exclusively from the 5′ half of the parental genome. To investigate this possibility we annealed $^{32}$P-labeled DI-LT RNA, prepared identically to preparations 1 and 3, and $^{32}$P-labeled DI-T RNA to increasing amounts of purified 12-17S polyadenylated mRNA which was synthesized in vitro by VSV-HR. As shown in Fig. 6, the two preparations of DI-LT RNA annealed to an identical extent with the mRNA, even though they differed greatly in the relative proportions of linear and circularizable RNA molecules. In contrast, the DI-T RNA, which contains sequences exclusively from the 5′ end of the genome, did not anneal to this mRNA. These results clearly indicate that both species of RNA in the DI-LT preparations contain sequences from the 3′ half of the VSV genome, despite the differences in their terminal sequences. Furthermore, electron microscopic examination of hybrids formed between the mRNA and DI-LT RNA (preparation 3) revealed the presence of circular RNA mole-

cules that had annealed to all four of the 12-17S messages (data not shown).

**Heteroduplexes formed between DI-LT RNA and (+) 42S VSV RNA.** The conclusions drawn from the results presented above indicate that most of the RNA in DI-LT preparation 1 has retained both the parental viral 5′ and 3′ termini and diverges from the parental 5′ sequence at a point approximately 350 nucleotides from the 5′ end. These results suggest that this DI particle was generated by an internal deletion of the VSV genome. We have pursued this possibility by annealing an excess of DI-LT RNA (preparation 1) to a mixture of (+) and (−) 42S RNA isolated from CsCl-purified intracellular nucleocapsids (29). The resultant heteroduplexes were examined in the electron microscope under conditions where single- and double-stranded RNA can be readily distinguished. Numerous partial and full-length duplex structures were visible. In some molecules, a duplex the size of the DI-LT genome was interrupted by a large looped-out single-stranded region. Three examples of such heteroduplexes are shown in Fig. 7 together with line drawings. The single-stranded looped-out regions are sequences in the (+) 42S RNA that have no complement in the DI-LT genome. Eighteen such heteroduplex molecules were measured, and their maps are shown in Fig. 8. The length of each horizontal line represents the sum of the lengths of the double-stranded region and any single-stranded tail protruding beyond the end of the duplex. (Two of the 18 molecules had single-stranded tails which presumably resulted from fragmentation.) The small vertical bars mark the positions of the single-stranded loops. It can be seen that there are several classes of heteroduplexes. The most abundant class (14 of 18 molecules) has loops that are located about 0.11 μm or approximately 320 nucleotides from the nearest end of the duplex. The remaining class(es) has loops located at a considerable distance from the end of the molecule, indicating that these RNAs have points of divergence much farther from the 5′ end. These RNAs would yield a limit size duplex of one kilobase when annealed to the (+) strand of DI-011 (Fig. 4). The average size of the looped-out single-stranded regions in all of the molecules examined is estimated to be about 6,000 nucleotides.

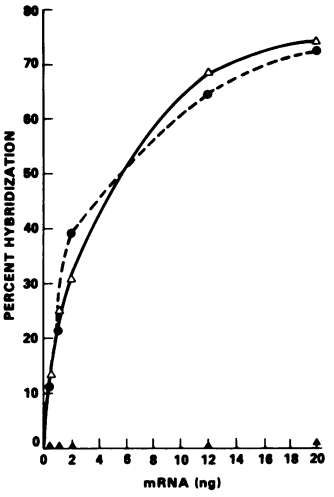


FIG. 6. *Saturation hybridization of DI-LT and DI-T RNA with mRNA. DI particle RNA uniformly labeled with [$^{32}$P]orthophosphate was annealed for 3 h at 60°C with increasing amounts of 12-17S polyadenylated RNA as described in the text.* (△) *DI-LT preparation 1;* (●) *DI-LT preparation 3;* (▲) *DI-T. RNase resistance in the absence of added mRNA (6.7% for preparation 1, 11.1% for preparation 3, and 12.2% for DI-T) was subtracted from each value. mRNA concentrations were determined based on the specific activity of the [$^{3}$H]UTP in the in vitro transcription mixture.*

## DISCUSSION

Previous analyses of DI particles showed that several major types could be identified on the basis of the structure of their genomic RNA (21, 27, 38, 41). The most abundant class contains RNA derived from the 5′ half of the parental genome and has complementary 5′ and 3′ termini. DI particles such as DI-011, having a totally self-complementary RNA which forms "hairpin" structures when deproteinated, may be a subset of this class (33). A second class of DI particles is typified by DI-LT, which contains RNA derived principally from the 3′ half of the VSV genome. The current work demonstrates that the DI-LT stocks commonly used are mixtures of particles in which two species predominate but in which minor components can also be identified. The genomes of the two major species can be differentiated in the electron microscope on the basis of their relative size and conformation. Linear forms of DI-LT have a mean length of about 1.99 μm, whereas circular forms have a mean length of 2.19 μm. These two species can also be distinguished because they have different terminal sequences. One has the parental 5′ and 3′ termini, whereas the other has the parental 5′-terminal sequence and the complement of this sequence at the 3′ end. Both forms were found to have information from the 3′ half of the VSV genome despite the differences in the structures of their RNAs.

Heteroduplex mapping of the preparation 1 RNA shows convincingly that this DI particle

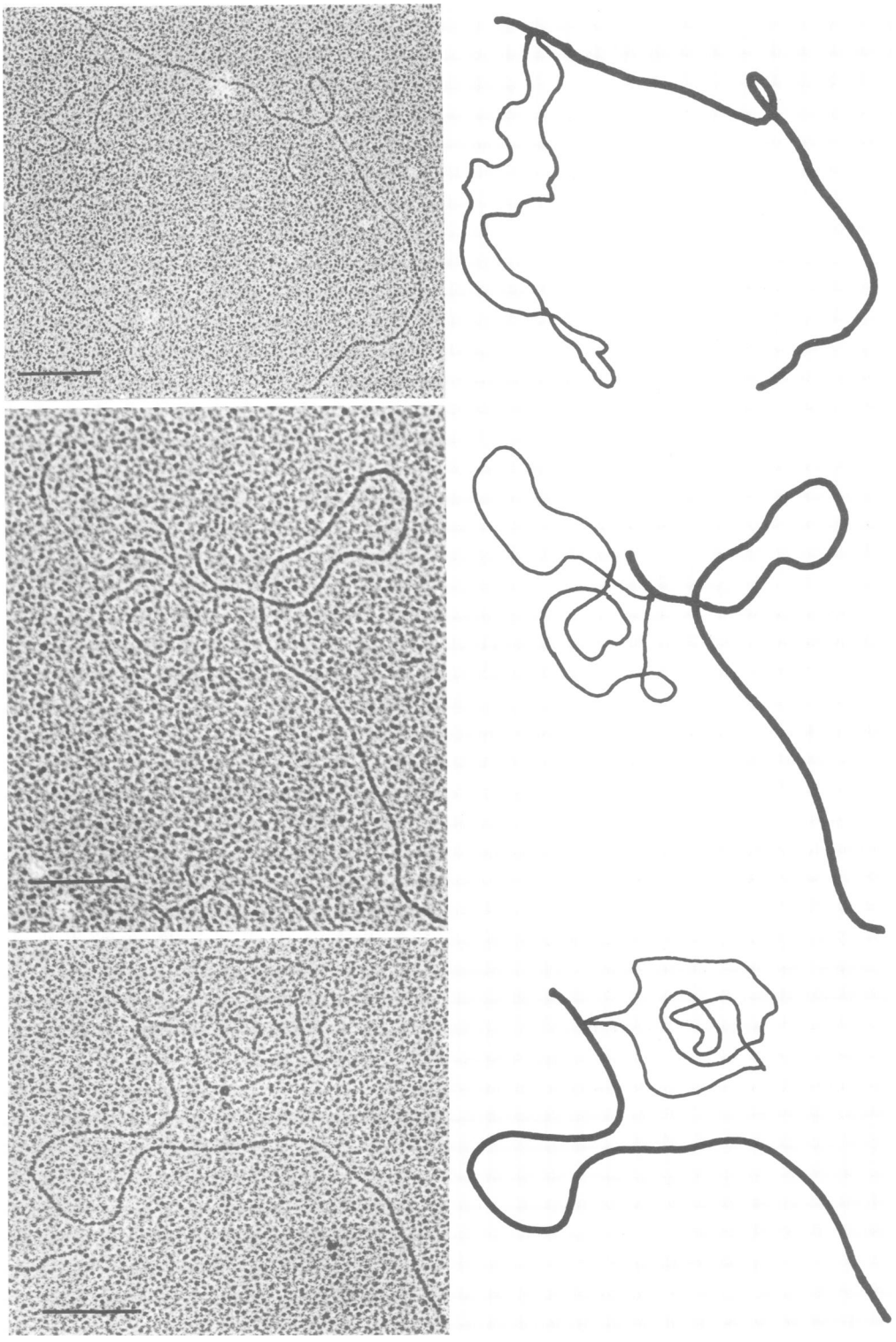

FIG. 7. *Heteroduplexes between DI-LT and (+) 42S nucleocapsid RNA. RNA isolated from CsCl-purified nucleocapsids was mixed with an excess of DI-LT RNA (preparation 1), heated at 60°C for 15 s, and then annealed for 20 min at 37°C. The RNA was spread and shadowed as described in the text. The bars represent 0.2 μm. Magnifications are relative to a grating replica with a spacing of 416.7 μm.*

has retained sequences from both ends of the parental genome but has deleted about 6,000 nucleotides from the center. It is estimated from the electron microscopic analysis that the most common deletion begins about 320 nucleotides from the 5′ end. This is in excellent agreement with the estimate of 350 nucleotides that was obtained by annealing the DI-LT RNA with the (+)-sense portion of the DI-011 genome. Recent sequencing results (Schubert et al., submitted for publication) show that the L gene ends 60 nucleotides from the 5′ terminus of the VSV genome. Thus, from the estimated size of the deletion, the nucleotides deleted from the DI-LT must all come from within the L gene. Vestiges of the 5′ and 3′ regions of this gene are therefore likely to be present in the DI-LT genome. Electron microscopic analysis demonstrated that a portion of the L message does, in fact, anneal to DI-LT RNA (R. Herman, unpublished data). This confirms the findings of Stamminger and Lazzarini (41), who detected low but significant levels of hybridization between DI-LT and the L message. Both the annealing experiment (Fig. 4) and the heteroduplex maps (Fig. 8) suggest that minor components in the DI-LT RNA preparations have deletions that begin farther than 320 to 350 nucleotides from the 5′ end. However, at present we do not know whether these particles were derived from one another or whether they were generated in separate and parallel events. The circularizable form was most probably generated by a multistep process.

The changes in the composition of the DI particle population with passage history and multiplicity of infection suggest that a DI particle whose RNA has complementary termini may have a selective advantage over those having parental termini. This may occur because DI particles with complementary termini interfere with DI particles having parental termini in the same manner as they do with the parental virus itself. However, the observation that the RNA of some New Jersey serotype DI particles has the same termini as the parental virus suggests that the competitive advantage enjoyed by these DI particles must be derived from sequences other than the terminal 20 nucleotides (22). Furthermore, DI particles may not all interfere by the same mechanism.

A number of distinctive biological properties have been attributed to the DI-LT. These particles have been shown to interfere with both Indiana and some New Jersey serotype VSV (1, 37, 38). They have been shown to transcribe in vitro (7) and in vivo (3, 17, 18), to genetically complement temperature-sensitive mutants (19), and to kill the host cell (30). The fact that

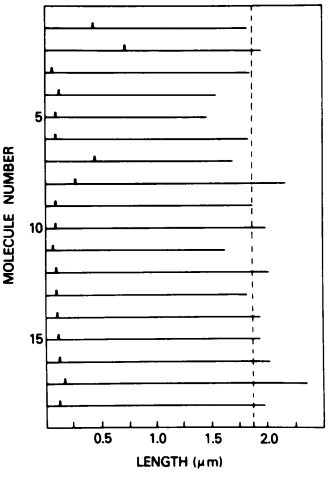


FIG. 8. *Heteroduplex maps. Eighteen heteroduplexes formed between DI-LT and (+) 42S nucleocapsid RNA (Fig. 7) were mapped as previously described (31, 43). The positions of the single-stranded looped-out regions are marked by the vertical bars. All molecules have been aligned with the loop closest to the end on the left. The dashed vertical line indicates the mean length of the duplexed region of these molecules. The average size of the single-stranded regions is estimated to be about 6,000 nucleotides. (One micrometer is equivalent to approximately 3,000 nucleotides. No corrections have been applied for any differences in spreading of single- and double-stranded RNA.)*

the preparations used in all of those studies were probably mixtures of two major and perhaps several minor components raises the question whether all of these activities can be ascribed to a single type of DI particle or whether the different types are capable of only some of these activities. A final resolution of this problem must await the cloning of the individual DI particles.

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