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Internal genome deletions in two distinct classes of defective interfering particles of vesicular stomatitis virus

(RNA end-labeling/hybridization/endogenous polymerase/conserved genome sequences/replication competition)

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ABSTRACT **We have characterized the genome sequences represented in two defective interfering particles derived from the heat-resistant strain of vesicular stomatitis virus by means of end-labeling and hybridization techniques. Both defective particle RNAs, which differ slightly in size, contain 5′-end sequences identical to each other and to that of the standard infectious virus genome, for at least the first ≈55 bases. In contrast, the 3′-end sequences of these two RNAs are different. The 3′-end sequence of the smaller RNA is identical to that of the standard genome for at least the first 48 bases. The 3′-end sequence of the larger RNA is an inverted complement of its 5′ end for ≈65 bases. The bulk of the sequences in both RNAs is derived from the 3′ half of the standard genome. We also show that the two defective particles differ in their *in vitro* transcription and *in vivo* replication properties. These results provide direct evidence for the presence of internal genome deletions in defective interfering particles of negative-stranded RNA animal viruses and demonstrate the existence of at least two distinct classes of these particles.**

Defective interfering virus particles (DIs) are found ubiquitously in animal virus (1). They have recently become a major focus of interest because of their powerful suppressive effects on viral infections *in vivo* and *in vitro* and because they appear to mediate long-term virus persistence in tissue culture and possibly in human and animal disease (1, 2). These naturally occurring virus particles are antigenically indistinguishable from their parent helper virus. Their important properties result from the deletions in their genomes. A structural analysis of these nucleic acids should elucidate their function as well as provide insights into the mechanisms of genome rearrangements.

The best characterized of these DI systems is that of vesicular stomatitis virus (VSV), a single-stranded, enveloped RNA virus with a genome of $\approx 3.6 \times 10^6$ daltons. The virion RNA is of negative polarity and serves as a template for the virion-bound transcriptase which generates, both *in vitro* and *in vivo*, five 5′ capped, methylated, and polyadenylylated RNA species. These in turn code for the five proteins found both in infected cells and in virions. Transcription begins at the 3′-OH terminus of the virion RNA *in vitro* and gives rise to a 48-nucleotide-long leader RNA followed sequentially by mRNA species in the order found on the genome—i.e., N, NS, M, G, and L (3–5).

Like other animal RNA and DNA viruses, VSV generates DIs at high frequency (for review, see ref. 6). Different VSV DI RNAs range from 10 to 50% of the standard genome size and generally contain sequences homologous to the large L cistron comprising the 5′ half of the standard genome (7–9). Recent studies from this laboratory (10, 11) have shown that at least the first ≈55-base sequence from the 5′ end of the several VSV DI RNAs is identical to that of the standard genome. This short segment is also present as an inverted complementary copy at the 3′ end of these DI RNAs such that hybridizing of these complementary end sequences gives rise to a short double-stranded RNA segment or stem at the base of a single-stranded loop. In contrast, the standard genome lacks this extensive terminal complementarity (11, 12). A similar situation has been described for the paramyxovirus Sendai and its DI (13, 14).

In contrast to the *in vitro* mRNA synthesis displayed by VSV standard infectious virions, several VSV DIs have been shown to synthesize only a 46-nucleotide-long product *in vitro* (15, 16). The sequence of this product has been determined (17, 18) and the product is transcribed beginning exactly at the 3′-end of the DI RNA, which is not found in the standard genome RNA (11). There is one exceptional VSV DI, denoted HR-LT, which is unusual because it contains sequences homologous to the 3′-end half of the standard genome (8, 9). This HR-LT DI was recently shown to transcribe the four mRNAs proximal to the 3′ end of the standard genome, both *in vitro* (19) and *in vivo* (20, 21). Organization of the viral RNA sequences in this unusual DI is the subject of this report.

MATERIALS AND METHODS

Cells, Virus, and DI. The BHK_{21} cell line used in these studies as well as methods of infection, labeling of standard VSV and its DI with [^{3}H]uridine, purification of particles, and RNA extraction have been described (22). The DI 0.22 OR (Orsay) and DI 0.10 GL (Glascow) used here correspond to the WT_{MS} ST and tsG31 ST_1 used before (17). The HR-LT DI were kind gifts of R. A. Lazzarini (National Institutes of Health) and C. Y. Kang (University of Texas, Southwestern Medical School). Experiments involving size (Fig. 1) or structural analyses (Figs. 2 and 3) of DI RNAs were carried out with DIs separated from standard virus by velocity sedimentation in sucrose gradients (22). The purification of total released particles for the competition experiment described in Fig. 4 was carried out by a modification of previous procedures. Resuspended crude virus pellets were centrifuged through 2 ml of 20% (wt/vol) sucrose solution, buffered as described (22) and layered on top of 2 ml of 60% (wt/vol) sucrose cushion, for 1.5 hr at 40,000 rpm in the SW 50.1 Beckman rotor. The virus band on top of the bottom sucrose layer was then centrifuged again through 1 ml of 10% (wt/vol) potassium tartrate solution, layered on top of 1 ml of 45% (wt/vol) solution of the same, for 2 hr at 45,000 rpm in the same rotor. The purified virus was then dialyzed as described (22).

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Abbreviations: DI, defective interfering virus particle; VSV, vesicular stomatitis virus; bp, base pair.

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End-Labeling and Hybridization of RNA. DI RNA 5′-end labeling (polynucleotide kinase and [γ-^{32}P]ATP) and purification were carried out as described (17, 23). Efficiency of labeling ranged from ≈5 to 50% in different experiments. Standard and DI RNA 3′-end labeling with RNA ligase (P-L Biochemicals) and cytidine 3′,5′-bis[^{32}P]phosphate (New England Nuclear) was carried out essentially as described by England and Uhlenbeck (24) except for increasing the ATP concentration to 20 μM. Labeling efficiency, ≈20–50%, was similar for all preparations. Hybridization conditions and RNase (A and T1) digestion were as described (10) except for substitution of 10mM Tris·HCl/1 mM EDTA, pH 7.8/0.6 M NaCl for the hybridization buffer. Unlabeled DI 0.10 stems were obtained as before (10, 11); [^{3}H]uridine-labeled transcriptase (RNA nucleotidyltransferase, EC 2.7.7.6) products of standard virus and DI 0.46 were purified free of template by pelleting in CsCl (25) after 3 hr of reaction under standard conditions (17).

Gel Analyses. Glyoxal-denatured RNAs (30 min, 50°C) were electrophoresed in 1.1% agarose (SeaKem, Rockland, ME) horizontal slab gels for 3–5 hr at ≈3 V/cm in the phosphate buffer system as described by McMaster and Carmichael (26) and then processed for fluorography. Small duplex RNAs were analyzed in 20% acrylamide gels as described (17). Gels were exposed to Kodak X-Omat R film at 4°C after being wrapped in SaranWrap or at −70°C after drying.

RESULTS

Heterogeneity of DI RNAs in HR-LT DI Isolates. To examine the representation of genome sequences in the unique HR-LT DI isolate by hybridization techniques, it was first necessary to assess the homogeneity of our starting material. Unexpectedly, at least three RNA species, differing slightly in size, were observed on electrophoresis of glyoxal-denatured RNA samples from purified HR-LT DI particles, grown from two different inoculum sources. When either one of these inoculae was passaged more than once in our BHK_{21} cells before the DI particles were purified for analysis, the RNA profile appeared similar, with a major species of $\approx 1.80 \times 10^6$ daltons and a minor component of $\approx 1.70 \times 10^6$ daltons (Fig. 1, lanes b and c). The third and smallest RNA species, $\approx 1.65 \times 10^6$ daltons, was seen only in DI obtained from the first undiluted passage of the original HR-LT DI obtained from C. Y. Kang (Fig. 1, lane d) and in DI appearing after growth of a single plaque isolate from this same original HR-LT inoculum (Fig. 1, lane e). An explanation for the selective loss of the smallest

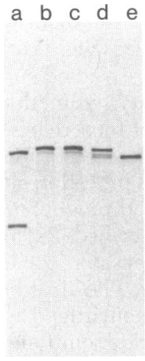

FIG. 1. Agarose gel analysis of [^{3}H]uridine-labeled and glyoxal-denatured HR-LT DI RNAs. Lanes: a, HeLa cell ribosomal RNA molecular weight markers; b, RNA from purified DI obtained after a few passages of an HR-LT inoculum obtained from R. A. Lazzarini; c, original HR-LT DI (from C. Y. Kang) after two undiluted passages in our cells; d, same original HR-LT (from C. Y. Kang) DI inoculum, first undiluted passage; e, DI obtained after growth of a single plaque isolated from the original HR-LT DI inoculum.

DI RNA species on further passage will become apparent from the results presented below. In accordance with a forthcoming codification of nomenclature for VSV DI we will refer to the above DI RNAs in order of decreasing molecular weight as DI 0.50 OR (HR), DI 0.47 OR (HR), and DI 0.46 OR (HR). For the experiments described in this paper DI RNA preparations identical to those in lanes b (≈95% DI 0.50) and e (≈99% DI 0.46) of Fig. 1 were used.

Analysis of 5′-End-Labeled DI RNAs. The approach used for comparing sequences at the 5′ ends of standard genome and DI RNA molecules was similar to that used previously (11). Briefly, probe molecules were labeled with [^{32}P]phosphate at their 5′ ends by using [γ-^{32}P]ATP and polynucleotide kinase, after phosphatase digestion to remove unlabeled 5′-terminal phosphate groups. These probes were then self-hybridized to allow intramolecular reassociation or hybridized to an excess of some other test RNA molecules. The label was then assayed for protection against an RNase specific for single-stranded RNA, and the size of the RNase-resistant duplex molecules was determined by electrophoresis in polyacrylamide gels. Because RNase cleaves any unmatched or mismatched region in a double-stranded molecule, the detection of such a labeled duplex indicates the presence of sequences complementary to the labeled 5′-terminal sequence of the probe. By determining the size of this labeled duplex, we also obtained a measure of the distance between the labeled 5′ terminus and the first site at which complementarity ceased. The reliability of this method for detecting even minor mismatches in a duplex RNA molecule is documented below.

In the experiments described here, we also used DI stem sequences, isolated from the small VSV DI GL (ts31) RNA, as a probe for the 5′-end genome sequence. As shown previously, the inverted complementary terminal sequences of this DI RNA, which readily hybridize to form stems, can be isolated in pure form by RNase digestion (10). By using the method described above, this small homogeneous duplex RNA species, ≈55 base pairs (bp) long, was previously shown to contain the sequence complementary to the first ≈55 bases at the 5′ end of the genome (11).

Self-hybridizing of 5′-end-labeled DI 0.50 RNA yielded a labeled duplex RNA ≈65 bp long relative to the marker ≈55-bp stem RNA (Fig. 2*A*, lane a). Hybridization in the presence of an excess of these marker stem sequences (0.2 μg of 55-base complexity is equivalent to 20 μg of ≈5000-base complexity) (Fig. 2*A*, lane b) yielded an additional duplex that comigrated with marker stems. We conclude from this experiment that DI 0.50 RNA contains within itself sequences complementary to ≈65 bases at its 5′ end. Furthermore, the first 55 bases or so at the 5′ end of this DI RNA are identical, within the resolution of this analysis, to those found at the 5′ end of the genome. It should be denoted here that DI 0.50 RNA, in common with several other VSV DI RNA preparations previously analyzed (10), contains a mixture of plus and minus strands (15–20% plus strands in this case). This does not interfere with the analysis described here because the conditions used (Fig. 2*A*, lane a) allow only for the detection of rapid and concentration-independent intramolecular rehybridization.

In contrast to DI 0.50 RNA, no significant self-hybridization of 5′-end-labeled DI 0.46 RNA was observed (Fig. 2*B*, lane c). Addition of DI stems to the mixture (Fig. 2*B*, lane b) yielded a labeled duplex that comigrated with marker stems. We conclude from these results that DI 0.46 RNA lacks 5′-terminal inverted complementary sequences but that, in common with DI 0.50 RNA and other VSV DI RNAs (11), this 5′-end sequence is identical to the genome 5′-end for at least ≈55 bases.

Analysis of 3′-End-Labeled DI RNAs. A similar analysis of

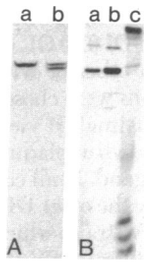

FIG. 2. Polyacrylamide gel electrophoresis of small duplex RNAs recovered from 5′-end-labeled DI RNAs self-hybridized or hybridized with DI stems followed by RNase A and T1 digestion. (*A*) 5′-End-labeled DI 0.50 RNA; ≈1 μg (28,000 cpm, 7% RNase resistance before hybridization) in 0.5 ml of hybridization buffer, 70°C, 2.5 hr (near-saturation hybridization by 15 min). Lanes a, self-hybridization (61% resistant); b, hybridization with ≈0.2 μg of DI 0.10 stems (80% resistant). Labeled DI 0.10 stems comigrated with the lower band (not shown). (*B*) 5′-End-labeled DI 0.46 RNA; ≈0.6 μg (300,000 cpm, 4.6% resistant before annealing) in 50 μl of hybridization buffer, 70°C, 20 min. Lanes: a, marker DI 0.10 stems; b, hybridization with ≈0.1 μg of unlabeled DI 0.10 stems (43% resistant) but only 1/13th of the total sample loaded on the gel; c, self-hybridized with an additional 5 μg of unlabeled DI 0.46 RNA (6.2% resistant). The size of these duplexes was estimated after glyoxal denaturation and electrophoretic migration relative to similarly treated HeLa cell 4S and 5S RNAs (not shown).

3′-end-labeled DI 0.50 RNA (Fig. 3*A*, lane a) shows that the RNA self-hybridizes at the 3′ end and yields a duplex ≈65 bp long relative to marker stems. We thus conclude that DI 0.50 RNA contains inverted terminal complementary sequences or stems ≈65 bp long. No other band was observed when the hybridization was carried out with added standard genome RNA (Fig. 3*A*, lane b), indicating that most likely all of this longer stem structure represents a contiguous sequence from the genome 5′ end and that the DI RNA 3′ end does not represent a longer contiguous complement of the genome 5′ end. As expected, addition of the ≈55-bp-long DI stems to this annealing mixture gave rise to some labeled duplex which comigrated with the shorter marker stems and to a major band ≈21 bp long (Fig. 3*A*, lane c). This latter band most likely results from RNase cleavage at a single A·C bp mismatch. We have previously shown, from base sequence data, the presence of a single base difference at position 21 from the 5′ end of the standard genome sequence between Indiana Orsay and Glascow strains, which correspond to the sources of DI 0.50 and DI 0.10, respectively (17). The corresponding single bp mismatch in Fig. 2*A* is the relatively stable G·U wobble bp, which most likely partially resists nuclease attack. These data provide strong evidence for the reliability of this RNase method for detecting minor mismatches in a duplex RNA molecule.

In contrast to DI 0.50 RNA, no significant self-hybridization of 3′-end-labeled DI 0.46 RNA was observed, whereas addition of DI stems to the mixture did not protect the label from RNase digestion, as expected (data not shown). These observations, as well as those in Fig. 2*B*, suggest strongly that DI 0.46 RNA does not contain self-complementary sequences which include either its 5′- or 3′-end terminal residues.

To determine whether DI 0.46 RNA might contain the standard genome 3′-end sequence at its 3′ end, we used *in vitro* synthesized transcription products from standard virus and DI 0.46 particles. As shown below, DI 0.46 particles synthesize the four VSV mRNAs proximal to the 3′ end of the genome *in vitro*. These products should therefore contain the 48-nucleotide-long leader RNAs which are complementary to the 3′ terminus of the standard genome RNA (3, 19). 3′-End-labeled DI 0.46 RNA yielded the expected 48-nucleotide-long duplex when hybridized to these products (Fig. 3*B*, lanes b and c). 3′-End-labeled standard virus RNA behaved identically to DI 0.46 RNA (Fig. 3*B*, lanes d and e), as expected. We therefore conclude that DI 0.46 RNA has conserved at least the first 48 bases from the 3′ end of the genome at its own 3′ end.

Polymerase Activities in DI 0.50 and DI 0.46. Because HR-LT DI had been reported previously to synthesize the four VSV mRNAs proximal to the 3′ end of the genome (19) we tested which, if any, of our HR-LT DI preparations displayed this activity. Under standard polymerase assay conditions (17), only DI 0.46 incorporated labeled precursor at a rate similar to that with standard virus (18.2 and 17.2 nmol of UTP per mg

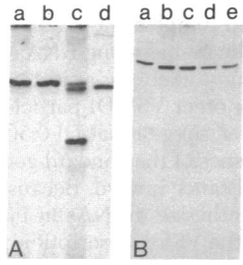

FIG. 3. Polyacrylamide gel electrophoresis of small duplex RNAs recovered from 3′-end-labeled DI and standard virus RNAs under various conditions of hybridization. (*A*) 3′-End-labeled DI 0.50 RNA; ≈0.4 μg (≈48,000 cpm) in 50 μl of hybridization buffer, 70°C, 20 min. Lanes: a, self-hybridized (70% RNase-resistant); b, hybridized with 5 μg of DI 0.50 RNA and 10 μg of standard virus genome RNA (resistance not measured); c, hybridized with ≈0.1 μg of DI 0.10 stems (56% resistant); d, marker DI 0.10 stems. (*B*) 3′-End-labeled DI 0.46; ≈0.5 μg (≈15,000 cpm; 22% resistant before hybridization but no specific duplexes were observed on gels not shown here) or 3′-end-labeled standard genome RNA, ≈1 μg (≈15,000 cpm, 8.3% resistant; no specific duplex) in 50 μl of hybridization buffer, 70°C, 1.5 hr. Lanes: a, marker DI 0.10 stems; b, end-labeled DI 0.46 RNA plus ≈10 μg of standard virus transcriptase products; c, end-labeled DI 0.46 RNA plus 1 μg of DI 0.46 transcriptase products; d, end-labeled standard genome RNA plus ≈10 μg of standard virus transcriptase products; e, end-labeled standard genome RNA plus ≈1 μg of DI 0.46 transcriptase products.

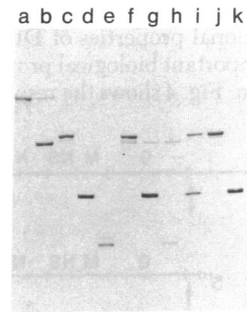

FIG. 4. Replication competition between DI 0.46 and other DIs in coinfected cells as measured by agarose gel electrophoresis of [^{3}H]uridine-labeled RNAs from released virus particles. All infections were carried out in parallel with a standard VSV (heat-resistant strain) at ≈15 plaque-forming units per cell and with various purified DI inocula adjusted to give approximately maximal yield of each DI singly. The interference with plaque-forming units yield is indicated in parentheses after each DI listed. Lanes: a, standard only (yield, ≈10,000 plaque-forming units per cell); b, DI 0.46 (≈80%); c, DI 0.50 (≈92%); d, DI 0.22 (≈93%); e, DI 0.10 (≈87%); f, DI 0.46 plus DI 0.50 (≈92%); g, DI 0.46 plus DI 0.22 (≈90%); h, DI 0.46 plus DI 0.10 (≈94%); i, DI 0.50 plus DI 0.22 (≈96%); j, DI 0.50 plus DI 0.10 (≈93%); k, DI 0.22 plus DI 0.10 (≈93%).

of protein per hr, respectively), whereas DI 0.50 showed a low activity comparable to that of DI 0.10 which maps within the 5′ half of the genome (1.7 and 3.4 nmol of GTP per mg of protein per hr, respectively).

As expected from the previously reported characterization of HR-LT DI polymerase products (19), both standard and DI 0.46 products were identical when analyzed electrophoretically (as in Fig. 1), except for a minor, high molecular weight band (presumably corresponding to the *L* gene transcript) present only in the standard virus products (data not shown). The DI 0.50 and DI 0.10 products (analyzed in 20% acrylamide gels as in Fig. 2) also were similar to each other and corresponded to the 46-nucleotide-long product RNA (data not shown) described earlier in this laboratory for DI 0.10 (17). We conclude that only DI 0.46 RNA, which has conserved the 3′ end of the standard genome, can serve as a template for mRNA transcription.

Nonterminal Sequences in DI 0.46 and DI 0.50. As pointed out (19), the template RNA of the transcriptionally active HR-LT DI (i.e., DI 0.46) must comprise the entire 3′ end of the 42S genome RNA through the 17S mRNA region (G protein cistron) and must terminate shortly thereafter in the L protein cistron. We therefore estimate that the extent of genome 5′ end sequence representation in this DI RNA is very small (≤200 bases including the ≈55 bases measured).

The template activity and gross stem structure of DI 0.50 RNA are shared by most VSV DIs so far examined (11, 17) but, in contrast, most of its sequences originate from the 3′ half of the genome. To demonstrate this, we took advantage of the fact that uniformly labeled DI 0.50 RNA self-hybridizes 30–40% (15–20% plus strands), whereas DI 0.46 RNA self-hybridizes 1.5% or less. Hybridization of ≈0.1 μg of [^{3}H]uridine-labeled DI 0.46 RNA in the presence of ≈2 μg of the double-stranded fraction from DI 0.50 RNA resulted in ≈97% protection of the label from RNase digestion. This indicates that a minimum of 85% of DI 0.50 sequences are derived from the 3′ half of the standard genome. Furthermore, similar hybridization experiments under conditions minimizing RNA fragmentation (100 μg of RNA per ml, 70°C, 15 min) yielded RNA duplex molecules which, when denatured with glyoxal and analyzed on agarose gels as in Fig. 1, gave rise to some RNAs as large as 97% of the size of DI 0.46 RNA (data not shown).

***In Vivo* Interference with DI 0.46 Replication by Other VSV DIs.** Finally, we examined the possibility that the unique structural and functional properties of DI 0.46 might be reflected in its most important biological properties—i.e., interference or replication. Fig. 4 shows the results of one of several similar experiments in which three other DI particles—DI 0.50 OR (HR), DI 0.22 OR, and DI 0.10 GL (ts 31)—were competing for replication with each other or with DI 0.46 in the same BHK_{21} host cell. Conditions were chosen for each DI infection to give approximately maximal DI yields and similar levels of interference with standard virus plaque-forming unit yields as indicated in the figure legend. In all cases, DI 0.46 replication was strongly suppressed by the other DIs, even when competing with DI 0.10 (Glascow VSV strain) which does not compete well with DI 0.50 or DI 0.22 (Orsay VSV strain). We have also observed repeatedly that purified DI 0.46 inocula, in contrast to all other VSV DIs we have so far examined, are difficult to maintain as relatively pure stocks free of other DI even when passaged one additional time (see Fig. 4, lane b). These results indicate that the *in vivo* replication of DI 0.46 is exceptional because it is sensitive to interference by other VSV DIs.

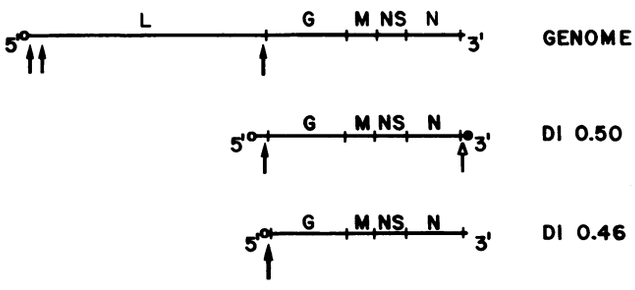

FIG. 5. Proposed structures of VSV DI 0.46 and DI 0.50 RNAs. Open circles stand for the first ≈65 bases at the 5′ end of all the molecules; the solid circle in DI 0.50 RNA represents an inverted complementary copy of this short end sequence. The solid arrows mark the sites of recombination generating the internal deletions. The open arrow in DI 0.50 indicates that a small 3′-end genome segment (≈150 bases) could conceivably be deleted at the site linking the large 3′-end genome segment to the stem sequences (see text).

DISCUSSION

Positive-stranded RNA animal virus DI particles have been shown previously to contain internal genome deletions (27, 28). This communication describes internal genome deletions in DI of negative-stranded RNA animal viruses. Evidence for this rests on the detection of conserved standard genome 5′- and 3′-end sequences at or near the ends of each of two different VSV DI RNA molecules. Fig. 5 depicts our proposed structures for these two DI RNAs. A minimum of ≈55 contiguous bases from the 5′ end of the standard genome are clearly conserved in each of these two RNAs (Fig. 2 *A* and *B*). For DI 0.46 RNA (≈5.1 kb) this 5′-end genome segment cannot be much larger (≤200 bases) because the size of the 3′-end genome segment coding for the four mRNAs and conserved in this RNA is near the size of the whole DI molecule. In the case of the larger DI 0.50 RNA (≈5.5 kb), hybridization data show that ≈88% of its sequences correspond to a contiguous segment also present in DI 0.46 RNA. We infer that the remaining 12% (except for ≈65 bases at the 3′ end) consists of a contiguous 5′-end genome segment. This is supported by preliminary experiments that show that a ≈500-bp-long duplex is obtained when 5′-end-labeled genome RNA is hybridized to a mixture of plus and minus strands of DI 0.50 RNA (unpublished observations). The 3′-terminal sequence of DI 0.50 RNA, in contrast to DI 0.46 RNA, does not correspond to that of the standard genome but represents an inverted complement of its 5′ end, ≈65 bases long (Fig. 3 *A* and *B*). The 46-base-long RNA synthesized *in vitro* by this DI particle most likely has as its template the 3′ end as shown previously for other VSV DI particles (17). Preliminary experiments, however, suggest that DI 0.50 RNA also contains the 3′-terminal sequence of the standard genome, not at its own 3′ end but a short distance inward. Because DI 0.46 particles, but not DI 0.50, synthesize mRNAs *in vitro*, this intriguing result suggests that the VSV transcription promoter sequence remains silent if present internally in the ribonucleoprotein template.

The generation of inverted-terminal complementary sequences or stems, which are not found in the standard genome of negative-stranded RNA viruses (11, 14), is thought to occur via a "strand-switching and copy-back" mechanism during synthesis of progeny RNA molecules (6, 14). VSV DI 0.46 RNA, in contrast to DI 0.50 RNA, lacks stems (Figs. 2*B* and 3*B*). These structures are therefore not an absolute requirement for generation of a negative-stranded virus DI. It is presently unclear whether DI 0.46 represents (*i*) an intermediate structure that might acquire stem sequences upon further passage (and give rise to DI RNAs analogous in structure to the larger DI 0.50 RNA) or, (*ii*) a rare, but stable, particle that disappears upon further passage because of the independent formation of stem-containing DI.

We have pointed out previously (11) that the interference properties of VSV DI may be explained at least in part by the presence of stems on DI RNAs. These would be expected to provide a replicative advantage to DI RNAs if the initiation of genome plus strand synthesis, which involves recognition of a different 3′-end template sequence, is rate limiting. Our observation that DI 0.46 RNA replication is also sensitive to interference by DI containing RNAs with stems (Fig. 4) is consistent with this interpretation. However, this cannot be the case for the interference of standard virus RNA replication mediated by DI 0.46. Moreover, this lack of DI transcriptive ability, which could also play a role in interference with genome RNA replication by most DI, cannot account for DI 0.46 interference either. It is thus more likely that interference by DI 0.46 occurs via a different mechanism.

In conclusion, we propose that existence of at least two distinct classes of negative-stranded virus DI. DIs of class I, which includes most DIs so far examined, characteristically (*i*) contain RNAs with stem structures, (*ii*) lack transcriptive ability *in vitro* and *in vivo*, and (*iii*) interfere strongly with the replication of standard virus and class II DIs, at least in BHK_{21} cells. In contrast, DIs of class II, typified by VSV DI 0.46, (*i*) contain RNAs with ends identical to the standard genome, (*ii*) are transcriptionally active *in vitro* and *in vivo*, and (*iii*) interfere with standard virus replication but are also subject to strong interference by class I DIs in BHK_{21} cells.

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