Inverted Complementary Terminal Sequences in Single-Stranded RNAs and Snap-Back RNAs from Vesicular Stomatitis Defective Interfering Particles

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SUMMARY

Complementary single-stranded RNAs from three independent VSV defective interfering particle (DI) sources examined can anneal and give rise to monomeric and multimeric circular and linear double-stranded structures observable by electron microscopy under aqueous conditions. When the RNA from the shortest of these DI is spread from 80% formamide solutions, as many as 32% of the molecules are circular, suggesting that the single-stranded RNAs contain inverted complementary terminal sequences. This is strongly supported by the isolation of the putative terminal sequences which rapidly become RNase resistant base-paired structures after melting and quick-cooling the RNA. RNase digestion yields a major and a minor component, 60 to 70 and 135 to 170 nucleotides long respectively. Snap-back DI RNAs also contain inverted complementary sequences at both ends of the plus and minus strands of the duplexes since nicking these at the ends gives rise to double-stranded molecules which can form monomeric and multimeric circular and linear molecules. Thus, snap-back molecules most likely contain a covalent linkage between or near complementary terminal sequences on the two complementary strands as schematically shown in Fig. 5 D.

INTRODUCTION

Vesicular stomatitis virus (VSV) contains a linear, single-stranded, ‘negative’ RNA genome of mol. wt. about 3·1 to 4·0 × 10⁶ (Huang & Wagner, 1966; Mudd & Summers, 1970; Bishop & Roy, 1971; Repik & Bishop, 1973; Weber et al. 1974). VSV defective interfering particles (DI) or T particles contain various sizes of subgenomic RNA segments ranging from ~ 10 to 50% of the genome (Huang & Wagner, 1966; Petric & Prevec, 1970; Reichmann, Pringle & Follett, 1971; Roy & Bishop, 1972; Lennmann & Reichmann, 1974; Stamminger & Lazzarini, 1974; Schnitzlein & Reichmann, 1976). Depending on the particular T particle, these RNAs can be minus strands only (Roy & Bishop, 1972; Stamminger & Lazzarini, 1974), unlinked plus and minus strands (Roy et al. 1973; Lennmann & Reichmann, 1974; Reichmann et al. 1974; Schnitzlein & Reichmann, 1976; Perrault & Leavitt, 1977), or covalently linked plus and minus strands which are also called snap-back (Lazzarini et al. 1975; Perrault, 1976; Perrault & Leavitt, 1977).

Recently, the presence of complementary terminal sequences has been reported for the

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alphavirus genome and DI RNAs (Hsu, Kung & Davidson, 1973; Kennedy, 1976) and for the paramyxovirus Sendai DI RNAs (Kolakofsky, 1976). Similar sequences have been suggested for the RNA from Uukuniemi virus (Pettersson & Hewlett, 1976), a Bunyavirus, and from RNA tumour viruses (Haseltine & Baltimore, 1976). In addition, similar sequences could explain the presence of circular ribonucleoproteins in cells infected by the measles paramyxovirus (Thorne & Dermott, 1976), and in both La Crosse (Obijeski et al. 1976) and Lumbo (Samso, Bouloy & Hannoun, 1975) viruses, also members of the Bunyaviridae. Thus, as suggested earlier by Kolakofsky (1976), this structural feature which is also observed in some linear DNA animal virus genomes (Garon, Berry & Rose, 1972; Wolfson & Dressler, 1972; Koczot et al. 1973; Berns & Kelly, 1974) may be general for animal virus RNAs. The inverted complementary terminal sequences appear to be ~150 base pairs long for Semliki Forest virus RNAs (Kennedy, 1976) and ~150 to 200 base pairs long for Sendai DI RNAs (Kolakofsky, 1976), representing 1 to 2% of the total RNA.

In an earlier publication (Perrault, 1976) and in the previous accompanying paper (Perrault & Leavitt, 1977) we reported the presence of circular molecules in VSV T particle RNA preparations. We show here that three sources of VSV T particles RNAs examined can form circular and multimeric structures, probably as a result of the presence of inverted complementary terminal sequences.

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METHODS

Cells and virus. The origin and growth of cells and virus as well as T particle RNA labeling, extraction, and gel analysis were described in detail in the previous paper (Perrault & Leavitt, 1977). TsG31 ST1 and tsG31 ST2 refer to short T particles (~170S and 250S respectively) derived from the Glasgow tsG31 group III mutant. TsG11 MT and tsG31 ST refer to medium (~370S) and short (~270S) T particles from Glasgow group I and IV ts mutants respectively. C5 ST (~290S) is the previously described snap-back-containing T particle (originally referred to as DI9) derived from a fifth consecutive clone of the wild-type Mudd-Summers Indiana VSV.

Electron microscopy. Unless otherwise stated samples contained 0.5 to 1.0 µg/ml of RNA in TE buffer (10 mM-tris-Cl, 1 mM-EDTA, pH 7.6). Melting and quick-cooling was carried out in small volumes of TE buffer by incubating at 100°C for 3 min in a boiling water bath followed by immediate cooling in ice water. Aqueous spreading of RNA was exactly as described in the previous paper (Perrault & Leavitt, 1977). For formamide spreading, the samples were melted and quick-cooled in 80% formamide-containing TE buffer instead of TE buffer alone. Cytochrome c (2.5 µl; 1 mg/ml in 0.1 M-tris-Cl, 0.01 M-EDTA, pH 8.5) was then added to 45 µl of the sample and spread on to a hypophase of 50% formamide in H2O. Grids were picked and prepared for examination as for aqueous spreading. Calibration and length measurements were carried out as described in the previous paper (Perrault & Leavitt, 1977).

RESULTS

Visualization of circular and multimeric structures in VSV T particle RNAs spread under aqueous conditions

As described in the previous paper (Perrault & Leavitt, 1977) snap-back RNAs from VSV T particles can be visualized by melting and quick-cooling the RNA followed by immediate
spreading under aqueous conditions. When this procedure was carried out with a preparation of tsG31 ST2 RNA [containing \( \sim 27\% \) snap-back molecules and \( 73\% \) single-stranded (ss) RNA derived from the shorter tsG31 ST1 particle] \( \sim 4\% \) of the molecules observed were circular with a mean ± standard deviation contour length of \( 0.23 ± 0.01 \) \( \mu \)m, similar to snap-back RNA length (see previous paper). Since snap-back RNA molecules remain linear duplexes even when spread from urea-formamide solutions (Lazzarini et al. 1975; R. W. Leavitt, unpublished observations) the circular molecules observed were most probably derived from rapid intermolecular annealing of a small fraction of the complementary ssRNA in the preparation giving rise to double-stranded molecules with complementary ends (Fig. 5). It should be remembered here that approx. \( 69\% \) of the total RNA in tsG31 ST2 (including the \( 27\% \) snap-back) is double-stranded after exhaustive self-annealing (see previous paper). This interpretation was further substantiated by the experiments presented here. RNA from tsG31 ST2 particles was melted and quick-cooled as before but it was then incubated at \( 0 \) °C for 2 to 3 h in TE buffer before spreading. The rationale for this experiment was to allow the molecules to renature only partially under conditions favouring perfect duplex formation, i.e. low ionic strength, rather than network formation (Studier, 1969). Under these conditions, \( 14\% \) of the molecules (141 out of 1000 molecules scored) were circles ranging in size from \( 0.2 \) to \( 1.0 \) \( \mu \)m demonstrating that the process of circularization was time dependent as expected for a process requiring annealing of complementary ssRNAs. The length distributions of linear molecules as well as 13 circular molecules in this preparation are shown in Fig. 1 (a). Clearly, this distribution is more heterogeneous than that observed previously for snap-back molecules in tsG31 ST2 (see Fig. 1 (a), previous paper). The majority of linear molecules with a mean ± standard deviation equal to \( 0.25 ± 0.02 \) \( \mu \)m (molecules ranging from \( 0.16 \) to \( 0.36 \) \( \mu \)m) most likely represent the same snap-back molecules. Approx. \( 20\% \) of the linear molecules (\( > 0.36 \) \( \mu \)m) were clearly larger than expected for linear monomers and were arbitrarily designated putative multimers. Representative circular and linear multimer molecules are shown in Fig. 2.

If the appearance of circular and multimeric structures in tsG31 ST2 RNA is dependent on annealing of complementary ssRNAs in the population, as suggested above, then this process should also be concentration dependent. To test this hypothesis the same RNA preparation was spread exactly as above after 2 to 3 h of incubation at \( 0 \) °C but at a sixfold lower concentration (\( \sim 0.1 \) \( \mu \)g/ml). The percentage of circles dropped to \( \sim 4\% \) (20 out of 500 molecules scored). We thus conclude that double-stranded circle formation is a concentration and time-dependent process involving annealing of complementary ssRNAs in the preparation. Although the length distribution of linear molecules was not examined in this experiment it is likely that the appearance of molecules longer than unit size is also dependent on this same process since they were only observed if the RNA was allowed to partially renature before spreading.

In order to test whether the process of circularization and multimer formation was unique to tsG31 ST RNAs we also examined tsG11 MT RNA for such a property. A preparation similar to that shown in Fig. 1 (d) of the previous paper (Perrault & Leavitt, 1977) was melted and allowed to cool freely to room temperature in TE buffer for about 2 h before spreading. As might be expected for a process involving end-to-end interactions, this larger RNA (about twice the size of ssRNA from tsG31 ST2 or ST1 particles) did not form circles as readily. Approx. \( 4\% \) of the molecules were circular and some of these are shown in Fig. 2. The length distribution of linear molecules in this slow-cooled preparation is shown in Fig. 1 (b). The results clearly indicate the appearance of \( \sim 10\% \) putative dimers and possibly a trimer molecule (compare with Fig. 1 d, previous paper). We thus conclude that a
Fig. 1. Histograms of the length distributions of (a) tsG31 ST₂ RNA, and (b) tsG11 MT RNA spread under aqueous conditions. The samples were prepared and examined as described in Methods and in the text. Circular molecules in tsG31 ST₂ RNA are represented by the black areas.
Complementary termini in VSV DI RNAs

Fig. 2. Representative circular and multimer double-stranded molecules in the tsG31 ST₄ and tsG11 MT RNAs analysed in Fig. 1. (a) to (f), tsG31 ST₄ RNA; (g) to (i), tsG11 MT RNA. (a) Two monomer circles and a linear monomer; (b) trimer circle; (c) trimer linear; (d) trimer linear; (e) hexamer circle; (f) linear dimer and linear hexamer; (g) monomer circle; (h) dimer circle; (i) trimer linear. All electron micrographs are at the same magnification.

The process of circular duplex and probable linear multimer formation can also take place with some of the complementary ssRNAs in tsG11 MT particles. Evidence presented below demonstrates that ssRNA derived from snap-back C5 ST RNA can also show this phenomenon under proper conditions. Since, in the above experiments, the fraction of the complementary ssRNA molecules which reannealed to form duplex structures could not be accurately measured, the possibility remained that end-to-end annealing could take place with only a small minority of the molecules. The following experiments support the notion that a major fraction of the ssRNA in tsG31 ST particles can participate in this kind of interaction.
Fig. 3. Histograms of the length distributions of tsG31 ST2 RNA spread in formamide. The sample was prepared and examined as described in Methods and in the text. (a) Linear molecules only; (b) circular molecules only.
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Fig. 4. Representative circular and multimer molecules in tsG31 ST$_2$ RNA spread in formamide as in Fig. 3. (a) Monomer circle; (b) linear dimer and tetramer circle; (c) linear trimer and tetramer circle; (d) monomer circle and trimer circle; (e) pentamer circle; (f) tetramer circle; (g) pentamer circle; (h) linear tetramer; (i) linear trimer.

Circle and multimer formation in the presence of formamide

In order to define more precisely the nature of the end-to-end interactions leading to circle and multimer formation described above, we examined tsG31 ST$_2$ RNA melted and quick-cooled in 80% formamide-TE buffer, and spread immediately on to a hypophase of 50% formamide in H$_2$O. Under these conditions we expect considerable, if not, complete, unfolding of non-complementary regions of ssRNA molecules (Dube et al. 1976; Petterson & Hewlett, 1976).

The majority of the observed molecules could be scored unambiguously into three classes: linear monomers, 58%; circles, 32%; and putative linear multimers, 10%. Some representative molecules are shown in Fig. 4. The length distributions of linear and circular
molecules are shown in Fig. 3 (a) and (b). The mean ± standard deviation of all linears ranging from 0.16 µm to 0.36 µm was equal to 0.26 ± 0.01 µm. All molecules of lengths above this range were again arbitrarily designated linear multimers. The contour lengths of the circular molecules clearly indicate a monomer and a dimer class as well as higher multimers (Fig. 4). The monomer circle class, however, appears to be composed of two populations with peak lengths of ~ 0.19 µm and ~ 0.23 µm.

The significance of the doublet length distribution for the monomer circle class is not entirely clear. It is possible that these are two classes of circles, one single-stranded and one double-stranded respectively. The smooth even thickness of some of these circles as compared to others with a more 'kinky' appearance is consistent with this possibility (Fig. 4). It should be noted again that the snap-back molecules do not appear as circular structures even when spread from urea-formamide solutions and thus fall under the linear monomer class under these conditions.

Panhandle-type structures characteristic of single-stranded RNA circles from other viruses (Hsu et al. 1973; Kolakofsky, 1976; Petterson & Hewlett, 1976) were not consistently observed and could not serve to differentiate reliably between single-stranded and double-stranded circles. Although it is likely that some of the circles observed were in fact single-stranded we cannot rule out the possibility that all circles were double-stranded since annealing of plus and minus strands could have taken place during the spreading procedure. Nevertheless, it is clear that a major fraction of the ssRNA originally present in the tsG3 ST2 RNA can give rise to circles, whether single or double-stranded, and therefore contains complementary terminal sequences. This observation also rules out the remote possibility that the circular structures observed might be due to contaminating DNA since essentially all of uniformly labelled 32P-tsG3 ST2 RNA can be degraded by RNase (data not shown).

The mean length distribution of the linear monomer molecules, 0.26 ± 0.01 µm (Fig. 3 a) is somewhat higher than that measured for snap-back molecules under aqueous conditions, 0.23 ± 0.01 µm. Although these length determinations may not be strictly comparable between formamide and aqueous spreadings it should be pointed out that the complementary ssRNA molecules present in tsG3 ST2 or ST1 preparations are ~ 15% larger than those derived from snap-back molecules after nicking with RNase (Fig. 2, previous paper).

Circle and multimer formation by RNase treated snap-back molecules

Since the ssRNA molecules present in the two T particle sources examined above were capable of end-to-end annealing interactions we also wished to examine the snap-back RNA molecules for such properties. If these hairpin duplexes also contain similar ends then two hypothetical structures as depicted in Fig. 5D and E can be considered. We are assuming here that a snap-back molecule corresponds to a continuous polynucleotide chain with a very small single-stranded region at one end of the duplex as previously proposed (Keene et al. 1977, and previous paper). The complementary termini in Fig. 5 are assumed to be inverted in the single-stranded molecules giving rise to inverted terminal repeat sequences. This assumption, which is supported by experiments presented below, is not essential for the arguments presented here which depend only on the presence of complementary termini whatever their nature. In Fig. 5E the sequences corresponding to the complementary terminal sequences in single-stranded RNAs are present only at the open end of the duplex. In Fig. 5D the complementary sequences are present at both ends of the duplex. This latter model implies that a linkage occurs between or near these complementary sequences.
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Fig. 5. Diagrammatic representation of structures proposed for the various DI RNA molecules observed. The letters abc and c'b'a' correspond to the proposed inverted complementary sequences at the ends of the RNA. (A) and (B), ssRNA of plus and minus strand polarity, respectively, in either linear or circular form; (C), linear and circular dsRNA resulting from intermolecular annealing of single-stranded plus and minus strands; (D), snap-back RNA structure with complementary terminal sequences at both ends of the duplex which can generate circular dsRNA molecules after nicking with RNase; (E), hypothetical snap-back RNA structure with complementary sequences only at the open end of the duplex which cannot generate circles after nicking with RNase. As shown below, snap-back molecules appear to correspond to structure (D).
As shown previously, snap-back RNAs can be nicked specifically at or near the cross-linking region of the molecule, leading to full size single strands after denaturation (Perrault 1976, and previous paper). If the RNase treatment does not remove the postulated complementary ends on the molecules then the alternatives suggested in Fig. 5 can be tested directly. If structure (E) is correct, only linear dimer structures can be formed after melting and renaturation. Circular structures are not possible. If structure (D) is correct, circular structures are indeed possible as well as linear structures longer than dimer size. Note that if the postulated complementary ends were present on either the plus strand or the minus
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Fig. 7. Representative circular and multimer molecules of nicked snap-back RNAs generated after melting and slow-cooling as described in Fig. 6. (a) to (e), Nicked slow-cooled C5 ST snap-back RNA. (f) to (l), Nicked, slow-cooled tsG31 ST2 snap-back RNA. (a) Linear monomer; (b) linear molecules somewhat longer than monomer probably derived from fragments; (c) trimer circle; (d) linear monomer and trimer circle; (e) linear trimer; (f) monomer circle; (g) trimer circle; (h) linear dimer; (i) hexamer circle.

strand only but not on both, they would be removed by RNase. The results shown in Fig. 6 indicate that structure (D) is the more likely to be correct. Two independent RNase-treated preparations of C5 ST RNA were examined microscopically under aqueous conditions. If the preparation was melted and quick-cooled, very few molecules were observed as expected since most of the RNA remains single-stranded under these conditions. Analyses of RNase-treated 3H-uridine labelled C5 ST RNA under identical conditions confirmed that ≲ 2% of the material remained double-stranded. Furthermore the length distribution of these molecules (Fig. 6a) with a mean ± standard deviation of 0.29 ± 0.02 μm is near that
of untreated C5 ST snap-back RNA (Lazzarini et al. 1975; Perrault, 1976). No circles were observed out of approx. 600 molecules scored. If, however, the RNA was allowed to renature partially by slow cooling after melting (incubation at room temperature for ~ 2 h) the distributions shown in Fig. 6(b) and (c) were obtained. Approx. 4 to 5% of the molecules observed in the first preparation were circles, including monomers, dimers and trimers, and about 10% were linear multimers. The second preparation spread at a higher RNA concentration also gave rise to about 1% circles (not shown) as well as ~ 1% linear trimers in addition to a large class of linear dimers. Representative molecules from these preparations are shown in Fig. 7. The presence of circles and linear trimers is consistent only with the model depicted in Fig. 5D. Linear dimers which were also formed in abundance in the experiment of Fig. 6(c) cannot serve to differentiate between the two models since they are expected to be produced in either case.

In addition we have examined tsG31 ST1 snap-back RNA for similar structures. Fig. 6(d) shows the results of a similar experiment. Again, it is clear that higher multimers and circles can be formed by RNase-treated snap-back molecules (Fig. 7). Furthermore the experiments confirm that these snap-back RNAs contain unique length contiguous base-paired segments since the majority of the renatured duplexes are about the same length as the original snap-back duplex or a multiple thereof. We thus conclude from the above experiments that the two snap-back RNAs examined probably contain complementary termini at both ends of the duplex as shown in Fig. 5D.

Isolation of presumptive complementary terminal sequences from single-stranded T particle RNAs

Since evidence obtained with other virus RNAs indicated the presence of inverted complementary terminal sequences (Hsu et al. 1973; Kennedy, 1976; Kolakofsky, 1976) we explored this possibility with VSV T particle RNAs by attempting to isolate the presumptive base-paired terminal structure from single-stranded circles.

For this purpose, we employed RNA from tsG31 ST1 particles which had been extensively purified away from the larger snap-back-containing particle present in tsG31 ST2. As shown previously (Table 1, previous paper) the level of RNase resistance of this preparation following melting and quick-cooling was ~ 2%. Reasoning that this resistant material might correspond to the putative terminal base-paired regions of ssRNA and not to contaminating levels of the previously characterized snap-back RNA, we examined its size on denaturing urea-formamide gels. As shown in Fig. 8, about 80% of this material migrates as a single homogeneous low mol. wt. component. The size of this RNA corresponds to 60 to 70 nucleotides relative to 4S and 5S markers. The rest of the RNase-resistant material migrates as a relatively homogeneous component, ~ 135 to 170 nucleotides long.

Several lines of evidence indicate that this material corresponds to inverted complementary terminal sequences. First, the 60 to 70 nucleotide long RNA has been examined on high percentage polyacrylamide gels and > 90% of it is homogeneous in size within a resolution of 2 or 3 nucleotides (data not shown). It is thus unlikely to be derived from a non-specific breakdown product of the larger snap-back molecules. Furthermore, the latter do not fragment significantly, let alone to a number of identical size pieces, even after extensive RNase digestion (Fig. 6d). As shown in the previous paper when a mixture of tsG31 ST1 ssRNA and tsG31 ST2 snap-back RNA are melted, quick-cooled, digested with RNase, and the resistant material examined on denaturing gels, both the presumptive inverted complementary terminal sequences derived from the ssRNA and the larger ssRNA derived from snap-back are recovered (see Fig. 2d, previous paper). Secondly, the recovery
of presumptive terminal sequences from tsG31 ST1 ssRNA varies in different experiments from ~ 2% to ~ 8% and does not correlate with the presence of the larger snap-back molecules (data not shown). If all the tsG31 ST1 ssRNA molecules contain these terminal sequences and the uridine content of the latter is assumed to be similar to the whole molecule, then the yield can be calculated to vary between ~ 20% and ~ 80% of the theoretical value (the size of tsG31 ST1 RNA was estimated to be ~ 0.44 x 10^6 daltons in the previous manuscript). Lastly, we have obtained preliminary evidence that the 60 to 70 nucleotide material isolated as above contains a phosphorylated 5′ terminus and we are currently determining the base sequence of the whole fragment (B. Semler, J. Perrault, J. Abelson, R. Leavitt & J. Holland, unpublished data).

The results outlined above strongly suggest that these isolated low mol. wt. components correspond to the putative complementary terminal sequences. It is also likely that other T particle RNAs contain similar sequences since very small amounts of low mol. wt. component(s) in the 4S marker region of the gel were observed in other similarly RNase-treated RNAs from tsG11 MT, C5 ST, and tsG41 ST particles (data not shown). The lower yields of the putative terminal sequences from these latter RNAs are not surprising in view of the lower probability of interaction between the ends of larger molecules following melting, quick-cooling, and immediate RNase digestion.

Fig. 8. Densitometer tracing of urea-formamide slab gel electropherogram of RNase-treated tsG31 ST1 RNA. 100 K ct/min of 3H-uridine labelled RNA was melted in urea-formamide solution at 70 °C for 2 min as described in the previous paper (Perrault & Leavitt, 1977), diluted 20-fold in ice-cold 0.3 M-NaCl-TE buffer containing 5 μg/ml RNase A and 2.5 units/ml RNase T1 and then incubated at 37 °C for 5 min. The RNase-resistant material (~3000 ct/min) was recovered by the Sarkosyl-protease K digestion and phenol-chloroform extraction procedure and analysed on a slab gel as described previously (Perrault & Leavitt, 1977). The positions of HeLa cell ribosomal, 5S and 4S markers run in a parallel gel slot are indicated by the arrows. The film was exposed to the gel for 7 days.
DISCUSSION

It is clear from the results presented in this paper that the three DI RNA sources examined can form circular and multimeric structures. These were readily observed in the electron microscope as double-stranded molecules originating from annealing of complementary ssRNAs present in these DI. The nature of the complementary ends in these double-stranded molecules probably involves inverted terminal repeat sequences. This interpretation is strongly supported by the isolation of the putative inverted complementary terminal sequences from single-stranded DI RNAs after RNase digestion under conditions allowing only intramolecular annealing. Although the data do not prove whether both plus and minus single-stranded DI RNAs contain these sequences this seems very likely since snap-back DI RNAs do have them on both strands. Likewise, it is not yet clear whether they are present in all DI RNA molecules or in B virion RNA although this is also probable in view of the results obtained by others which show that both DI and infectious particle RNAs of Semliki Forest virus contain such terminal sequences (Kennedy, 1976).

The main component of the putative complementary ends in tsG31 ST1 ssRNA was estimated to be 60 to 70 nucleotides by analysis on denaturing gels. It is not yet clear what the 135 to 170 nucleotides long minor component represents. Similar heterogeneity in the RNase-resistant material obtained from Sendai virus DI ssRNAs which corresponds to complementary terminal sequences has also been observed (D. Kolakofsky, personal communication). It has not yet been determined whether the complementary ends on other VSV DI RNAs are identical in size and/or sequences but preliminary results indicate that small RNAs in the same size range can be isolated from at least three of these after RNase treatment of melted and quick-cooled preparations (unpublished observations). The small size of this RNA is consistent with our inability to visualize clear-cut panhandle or rabbit-ear structures in circular molecules. It is interesting to note that the recently described leader sequence obtained from endogenous virion transcriptase reactions is also in this same size range (Colomno & Banerjee, 1976). Since the leader sequence is presumably initiated at the 3' end of the template it may be identical or closely related in base sequence to one of the strands from the terminal sequences isolated in this study. Furthermore, the close similarity in size suggests that the postulated cleavage site on the in vitro synthesized plus strand precursor(s) may occur at or near the junction between the complementary terminal sequences and the start of the N protein mRNA.

The results obtained with snap-back DI RNAs reveal an important structural feature of these molecules. Complementary ends appear to be present on both ends of the duplex after RNase treatment (as in Fig. 5 D). This strongly suggests that at least one linkage occurs between or near these complementary sequences on the plus and minus strands. It cannot be determined from these experiments whether both sets of complementary sequences are the same but they must be related at least to the extent of forming stable base-paired regions under the conditions employed. This structural feature of snap-back molecules is consistent with a mechanism of generation which introduces a linkage during the initiation of replication of one or both complementary strands (see previous paper).

The presence of inverted complementary terminal sequences on VSV RNAs bears some important implications regarding the replication of these RNAs and the generation of DI. DI RNAs are clearly not generated by simple end deletions as suggested from earlier studies (Schnitzlein & Reichmann, 1976). More likely this process involves some yet undefined recombinational mechanism which preserves both ends of the genome RNA as suggested for poliovirus and Semliki Forest virus DI RNA generation (Villa-Komaroff et al. 1975;
Kennedy, 1976). The cleavage enzyme(s) postulated for the processing of VSV mRNA precursors (Colonno & Bannerjee, 1976) and putative related base sequences recognized by this enzyme could conceivably play a role in VSV DI generation. Intriguingly, the endogenous polymerase activity of several VSV T particles synthesizes similar small RNA products very near in size to the leader sequence and the complementary termini (Reichmann et al. 1974; B. Semler & J. Holland, personal communication). It is thus plausible that many if not all T particle RNAs contain a recombinational site at or near the junction between the genome 3' end complementary terminal sequence and the start of the N gene which leads to abortive transcription.

The presence of identical sequences at the 3' ends of both plus and minus strands does suggest that initiation of transcription and replication involve, at least in part, the same recognition sites. Likewise, the 5' ends of both strands might serve in part as recognition sites for initial binding of nucleocapsid proteins. Regulation of transcriptive and replicative functions would thus depend on additional factors as suggested from other studies (Perlman & Huang, 1974). Furthermore, the mechanism of interference by VSV DI which has been postulated to occur at the level of B RNA replication (Huang & Manders, 1972; Perrault & Holland, 1972) could possibly depend on some difference in base sequence and/or secondary structure between inverted complementary terminal sequences on DI RNA versus B genome RNA. Further work is in progress to characterize these terminal sequences.

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Note added in proof. We have recently established that only the 5' end, and not the 3' end, terminal sequence of 60 to 70 nucleotides of VSV DI RNAs is found in the full size genome RNA (J. Perrault, B. Semler, R. Leavitt & J. Holland, unpublished data). Leppert et al. (Cell, in the press) have reached similar conclusions for Sendai virus and its DI.

REFERENCES
KENNEDY, S. I. T. (1976). Sequence relationships between the genome and the intracellular RNA species of
in plus or minus strands of adenovirus-associated virus DNA. Proceedings of the National Academy of
PERLM~I, S. M. & ICANG, A. S. (1974). Virus-specific RNA specified by the group I and group IV temperature-
and specific RNA. Virology 40, 615–630.
PETTERSON, R. F. & HEWLETT, M. J. (1976). The structure of the RNA of Uukuniemi virus, a proposed Bunya-
ROY, P. & BISHOP, D. H. L. (1972). The genome homology of vesicular stomatitis virus and defective T particles,
and evidence for the sequential transcription of the virion ribonucleic acid. Journal of Virology 9, 946–955.
virus particle RNAs in relation to homotypic and heterotypic interference. Journal of Molecular Biology
101, 307–325.
Journal of Molecular Biology 24, 199–209.
RNA in a eukaryotic cell-free system. Proceedings of the National Academy of Sciences of the United
States of America 72, 4157–4161.
the National Academy of Sciences of the United States of America 69, 3054–3057.

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