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Structure and origin of a novel class of defective interfering particle of vesicular stomatitis virus

Stuart T.Nichol, Patrick J.O'Hara, John J.Holland and Jacques Perrault*

Department of Biology, University of California, San Diego, La Jolla, CA 92093, and *Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, MO 63110, USA

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ABSTRACT

The genome structure and terminal sequences of a 'copyback' defective interfering (DI) particle ST1, and a novel complexly rearranged 'snapback' DI particle ST2 of vesicular stomatitis virus have been determined. The ST1 DI genome RNA possesses 54 base long inverted complementary termini, the 5' end of which is homologous to the standard virus genome 5' end. Following this region of inverted complementarity the DI RNA 5' end continues to be homologous to standard virus RNA 5' sequences, whereas the 3' end diverges into sequences within the virus L gene internal sequences. ST2 DI genome RNA does not contain colinear covalently linked plus and minus sense RNA copies of the standard infectious virus RNA 5' terminus as predicted from the prototype snapback DI structure, but instead appears to be a hairpin copy of the ST1 DI RNA genome. This is the first evidence suggesting that DI particles may be generated from RNA templates other than the standard virus RNA. Generation models and the implications of these findings for RNA virus evolution are discussed.

INTRODUCTION

Nearly all RNA viruses generate DI particles, which are packaged in normal virion proteins but possess greatly deleted RNA genomes (Reviewed 1,2,3, and 4). They are important as they have been shown to be involved in the establishment and maintenance of persistent infections in vitro (5) and are thought to play a role in modulating virus infections and recovery from infections in vivo (6 and above reviews). The genome structure of these DI particles are of particular interest as although they retain only a small portion of the virus genome, they can interfere with standard virus replication and effectively out compete the standard virus replicating RNA templates. Thus, they may hold the key to which features of RNA templates are important for their efficient replication and/or encapsidation. Also, the array of DI RNA structures found gives information regarding the extent to which virus RNA polymerases are capable of synthesising 'mistake' or recombinant molecules which may play an important role in RNA virus evolution (7).

The most extensively characterized DI particles are those of vesicular

stomatitis virus (VSV), a negative strand RNA virus (with a genome ~11 kb long) of the Rhabdovirus group. Several VSV DI particles have been examined and have been grouped into a number of classes (see reviews 3,4). The vast majority of DI particles studied are derived from the 5' end of the standard virus genome and contain only portions of the L gene (polymerase gene, ~6 kb long), thus they do not give rise to translatable mRNAs and are completely dependent on standard helper virus for the proteins necessary for their replication and encapsidation. Two classes of these DI particles have been clearly defined. The first class, termed 'panhandle' or 'copyback' DI particles possess portions of the L gene 5' end linked to the 3' inverted complement of the 5' terminus to give small terminal panhandle or stem structures. It has been suggested (8,9) that such DI genomes are formed by premature termination of replication of the standard virus plus strand template RNA followed by 'copyback' near the terminus of the nascent strand. The extent of terminal complementarity of copyback DI genomes has been shown to vary from approximately 45 to 200 nucleotides (10-13).

The other well defined class of 5' derived DI particles are the 'snapback' or hairpin DI. Of these DI, only one (DI-011) has been extensively characterized and shown to possess a genome consisting of 5' terminal L gene sequences covalently linked to a plus sense copy of these sequences. This type of DI is thought to be generated by the polymerase switching templates across a replication fork without releasing the nascent strand (14). Common to all models for DI particle generation is premature termination of replication followed by the polymerase resuming synthesis elsewhere on the same RNA strand or on another RNA strand with the nascent RNA still attached (3,4).

We report here the study of the genome structure of two DI particles ST1 and ST2. Our previous studies on the genome RNA of ST1 DI particles have shown that these molecules possess inverted complementary termini of 50-60 base pairs in length suggesting they may have a 'copyback' type structure (3,10,15,16). Early electron microscope observations of ST2 DI RNA demonstrated they were 'snapback' molecules of plus and minus sense RNA strands covalently linked by a small single stranded turnaround region (16,17). When the complementary strands of ST2 genomes were separated by first nuclease nicking the turnaround link region followed by denaturation, the separated strands could form circular molecules. Based on this, it was suggested that each strand contained inverted complementary terminal sequences. It was thought that this may be a common feature of snapback DI RNA genomes, however later detailed studies (14) clearly showed that the VSV snapback DI 011, which

has become the prototype of the snapback DI class, did not possess sequences at the turnaround region which were complementary to the termini of the molecules.

The results presented here clearly show that the ST2 DI particles represent a novel class of snapback DI in that they have a very different structure to that of the prototype snapback DI 011 (14). Furthermore, the striking structural similarity of 3' proximal sequences of ST1 and ST2 DI RNAs strongly suggests that ST2 DI is a hairpin form of the ST1 genome RNA from which it most likely originated. These structural similarities also probably account for the very similar biological properties of these two DI particles.

MATERIALS AND METHODS

Virus DI particle RNAs

The origins of the clonal pool of the temperature sensitive mutant tsG31 of the Glasgow strain of VSV Indiana which was used to generate the two DI particles ST1 and ST2 and used to initiate undilute virus passages and (in conjunction with DI) long term persistent infection has been described elsewhere (5,16,18). The method by which DI particles are grown, purified and RNA extracted has been described previously (16).

RNA labelling, electrophoresis and sequencing

DI RNAs were ^{32}P labelled internally or terminally at the 5' or 3' end using standard procedures (19,20,21). Labelled molecules were analyzed on glyoxal denaturation agarose gels essentially as described (22) or on nondenaturing 5% polyacrylamide tris-borate EDTA (TBE) buffered gels (23). Preparative gel electrophoresis was carried out using either non-denaturing gels as above or TBE buffered 4% polyacrylamide gels containing 8 M urea (23). On the latter gels snapback molecules have a characteristic mobility running much faster than expected based on their molecular weight due to migration as double strand structures, for example intact ST2 RNAs migrate approximately 2.5 times faster than T1 nuclease unlinked separated strands or than ST1 DI RNA (14, 24, and Nichol, unpublished results). Where 5' and 3' end direct RNA sequences are presented for a molecule these were derived from bands which comigrated exactly. Labelled RNA bands were excised and electroeluted essentially as described elsewhere (25). Direct RNA sequencing was carried out by standard enzymatic (for 5' ends) or chemical (for 3' ends) methods (20,26-29). ST1 5' end RNA sequences were obtained by chemical DNA sequencing (23) of a reverse transcript synthesized off ST1 RNA template using a 5' labelled synthetic oligonucleotide AGGTAAGTTAGTAAGG complementary to sequence between

positions 183 and 198 from the 5' end. Primer was synthesized and extended as described earlier (30). Sequences were determined on 20%, 8% and 6% polyacrylamide-8M urea gels (23). Sequences were compared against known VSV sequences with the aid of a computer program developed earlier (31).

RNA annealing and ribonuclease digestion conditions

Digestion conditions for nicking the turnaround region of ST2 DI RNA were as follows unless otherwise stated: 125 µg/ml RNA (including both DI RNA and carrier yeast total RNA), 250 u/ml T1 RNAase (Sankyo), 0.6 M NaCl, 10mM Tris-Cl, pH 7.7, 1 mM EDTA, 30 min, 0°C. Samples were first denatured at 100°C for 2 min, in the same buffer minus NaCl and enzyme. By monitoring the extent of 5' end-labelling following nicking with decreasing amounts of T1, we observed $\leq$ 50% nicking at 5 u/ml of enzyme. The size of the labelled duplex remained the same at all concentrations of enzyme employed including conditions nicking only ~5% of the molecules (data not shown).

The denaturation, annealing, and nuclease digestion protocols employed for detecting intrastrand annealing in ST2 DI RNA (Figure 4) were similar to the above except for keeping the viral RNA concentration below 25 µg/ml and digesting with 100 u/ml T1 RNAase for 30 min at 37°C.

RESULTS

Structural analysis of ST1 and ST2 DI RNAs

The genomic RNA of ST1 DI particles has previously been shown to be an apparent 'copyback' type molecule possessing inverted complementary terminal stems of 50-60 base pairs in length (see Introduction). Glyoxal denatured 3' end-labelled ST1 and ST2 DI RNAs were analyzed on agarose gels. Figure 1 shows glyoxal denatured ST1 DI RNA to be a single species approximately 900 nucleotides in length relative to marker ribosomal RNAs. ST2 DI RNA was shown earlier to be a snapback molecule of plus and minus sense RNA strands covalently linked by a small turnaround single strand region (see Introduction). Figure 1 shows glyoxal denatured ST2 DI RNA before and after T1 ribonuclease nicking of the single strand turnaround region linking the plus and minus sense RNA strands. End-labelling of nicked molecules was carried out following phosphatase treatment to remove the 3' end phosphate groups. Both plus and minus strands should therefore be labelled and end-base analysis yielded roughly equal amounts of labelled G and U residues as expected (data not shown). The unlinked RNA strands appear to comigrate with an apparent size of approximately 900 nucleotides (lane a). Intact snapback molecules migrated with an apparent size of approximately 1650 nucleotides. Identical results were obtained whether RNAs were 5', 3' or internally labelled. The

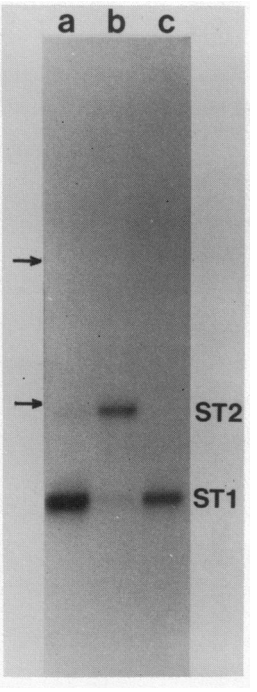

Fig. 1. Size determination of glyoxal denatured ST1 and ST2 DI RNAs. Unnicked samples were directly 3' end-labelled with ^{32}pCp and RNA ligase following extraction from purified DI particles. A portion of the ST2 RNA sample was first nicked at the turnaround region and phosphatase treated before labelling. All preparatons were gel purified on non-denaturing polyacrylamide gels before glyoxal denaturation and analysis on agarose gels (see Materials and Methods). Arrows indicate positions of Hela 28S and 18S ribosomal RNAs run on a parallel lane. Lane a, nicked ST2 RNA; lane b, ST2 RNA, lane c, ST1 RNA.

slightly lower than expected estimate of intact ST2 molecular size presumably reflects the inability of the glyoxal denaturation conditions to hold the snapback structure open in a completely denatured form. Previous results have indicated that the intact ST2 RNA molecules are indeed twice the size of T1 nuclease cut, unlinked strands when analyzed under methyl mercury complete denaturation conditions (16). Clearly there is a striking similarity in size between the unlinked RNA strands of ST2 genomes and ST1 RNA genomes.

Sequence analysis of ST1 and ST2 DI RNA termini

The terminal sequences of ST1 and ST2 DI genomic RNAs were determined by a number of procedures. A representative gel of each of the sequencing methods employed is shown in Figure 2. The genomic RNA of ST1 DI is predominantly of the negative sense (16). The 5' sequence was obtained by Maxam-Gilbert DNA sequencing of a reverse transcript of ST1 RNA synthesized using a synthetic primer (see Materials and Methods). Sequence analysis showed the 5' end of ST1 RNA to be homologous to the 5' end of standard virus RNA for the 177 bases examined (Figure 3).

Direct chemical RNA sequence analysis of 3' end-labelled ST1 RNA showed the terminal 54 bases to be the inverted complement of the 5' terminus, con-

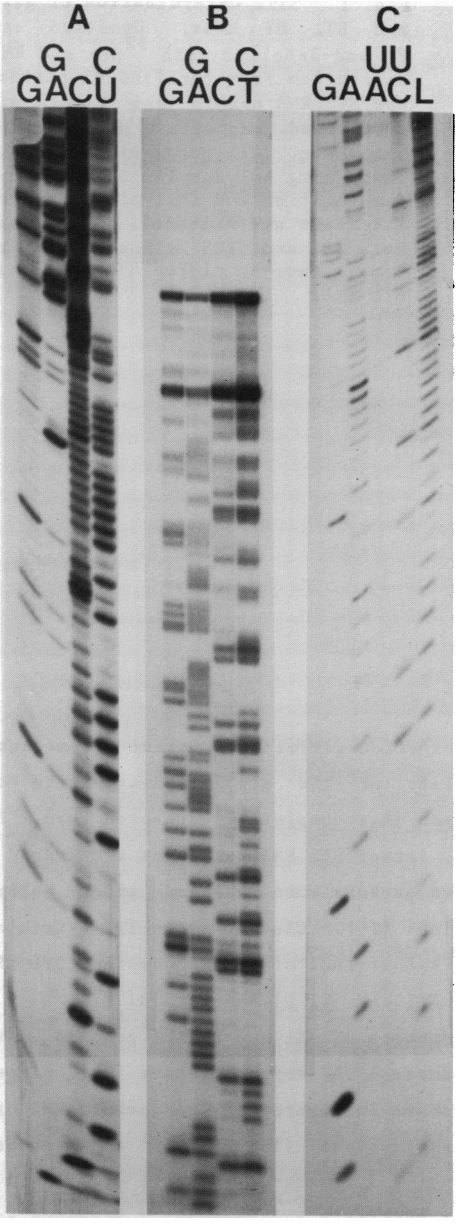

Fig. 2 Gel analysis of RNA samples sequenced by various procedures. Panel A shows 3' end labelled ST2 RNA sequenced by direct chemical methods, panel B shows ST1 RNA analyzed by chemical sequencing of a primed cDNA transcript, and panel C shows 5' labelled ST2 RNA sequenced by direct enzymatic methods (see Materials and Methods).

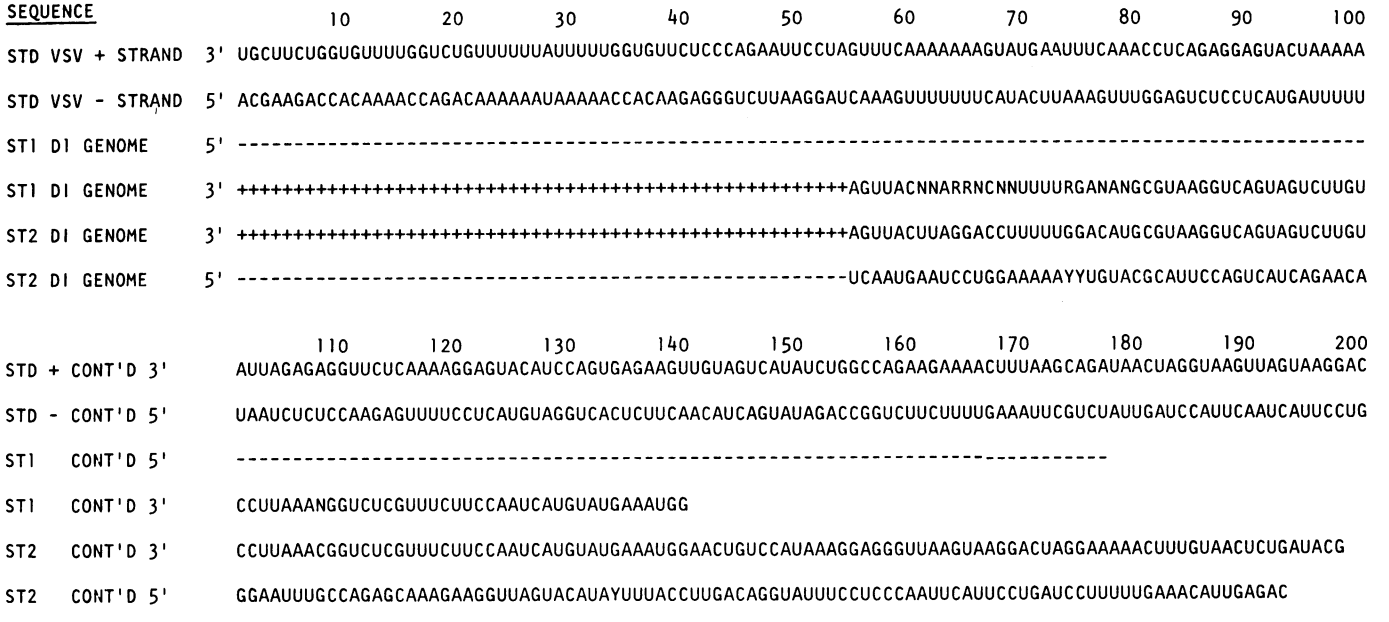

Fig. 3. The 5′ and 3′ terminal nucleotide sequences of ST1 and ST2 DI RNAs are compared with the previously published sequence of the standard virus minus strand RNA 5′ end (41) and its complement, the plus strand 3′ end. - denotes homology with the minus sense 5′ end, and + denotes homology with its complement, the plus sense 3′ end. N denotes not determined, Y pyrimidines and R purines.

firming that ST1 RNA is a copyback type DI possessing terminal panhandle or stem structures. A partial sequence was obtained for the next 86 bases adjacent to the stem structure (Figure 3). Computer analysis of the complete sequence of the VSV standard virus genome showed this sequence to be homologous to that 866 nucleotides from the 5' end of the standard virus genome (32-41, and Manfred Schubert, personal communication). As ST1 DI RNA is approximately 900 nucleotides in length (Figure 1), the location of the sequence adjacent to the 54 nucleotide stem is consistent with this region being the point where premature termination of replication occurred and ST1 having a simple copyback structure (see Figure 6).

The 3' and 5' termini of intact ST2 genome RNAs were sequenced by direct chemical and enzymatic RNA sequencing respectively (see Materials and Methods). Surprisingly, ST2 snapback molecules were found to possess the same 54 base inverted complement of the 5' terminus of standard virus RNA and the identical adjacent sequence as ST1 RNA 3' termini (Figures 3 and 6). Similar to ST1 DI RNA, the sequence beginning at nucleotide 55 from the 3' end of ST2 RNA is also homologous to that 866 nucleotides from the 5' end of the standard genome except for 3 single base mismatches (Manfred Schubert, personal communication). As expected the 5' terminal sequence of ST2 RNA was found to be completely complementary to that of the 3' terminus for at least 193 bases (Figures 3 and 6). Thus, the 3' terminus of the copyback ST1 DI was identical to that of the snapback ST2 DI sharing very specific features of the exact same length complementary terminal stem and identical recombination point (see Figure 6). Clearly the RNA structure of the ST2 DI genome is very different to that of the prototype snapback DI particle DI-011 (4,14).

Structural analysis of T1 nuclease nicked ST2 DI RNA

Previous electron microscope studies suggested that plus and minus strands of ST2 snapback DI RNA unlinked by nuclease digestion of the single strand turnaround region were capable of forming circular structures indicating that they each possessed complementary terminal panhandles or stems (16,17). A more detailed investigation of this property is shown in Figure 4. Snapback ST2 RNA molecules were specifically ^{32}P labelled at either the original 5' end or the 5' end generated by T1 nuclease nicking the single strand turnaround region. The specific labelling of the original 5' end requires first the removal of the terminal phosphate group whereas the T1 cut site can be specifically 5' labelled without prior phosphatase treatment as ribonuclease T1 generates a 5' hydroxyl group upon cutting. Results indicate that the 5' terminus generated by T1 nuclease nicking the single strand turnaround

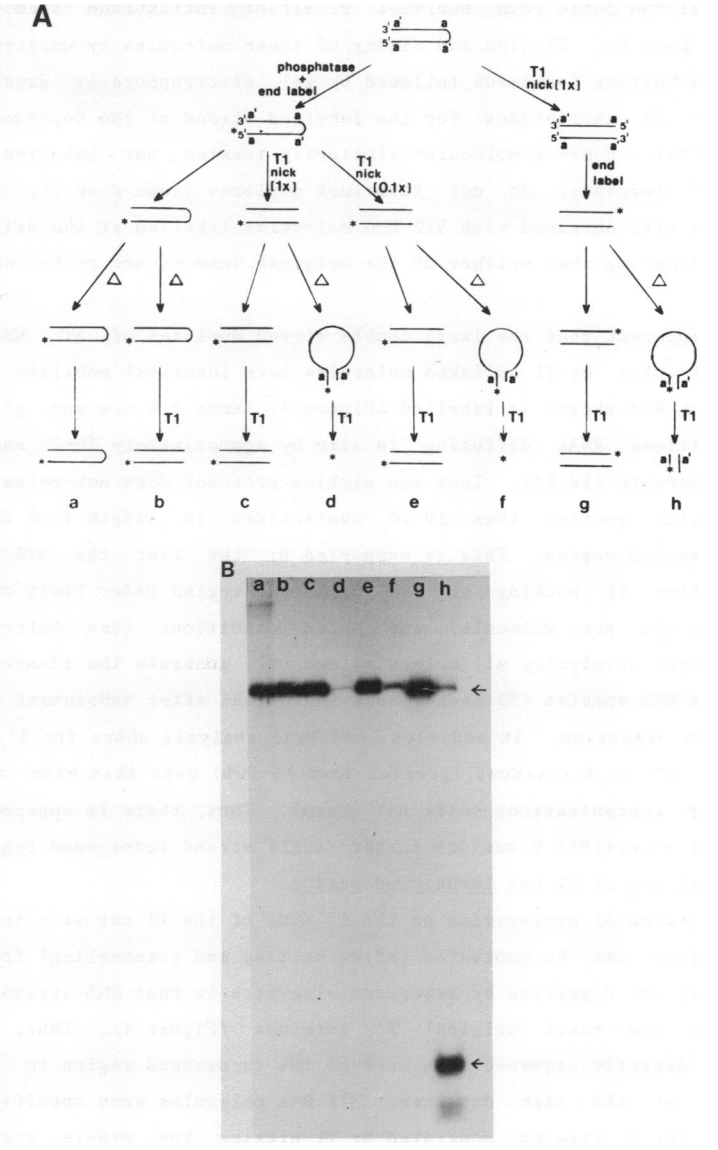

Fig. 4. Structural analysis of DI ST2 RNA by means of 5' end-labelling and RNAse T1 digestion. A) Diagrammatic representation of the protocol employed in preparing samples a through h. Standard conditions for the T1 nicking reaction (1X) as well as the denaturation step (Δ) and subsequent T1 digestion are outlined in Materials and Methods. Samples e and f were nicked with a tenfold lower concentrations of T1. The * symbol denotes the position of the ^{32}P end-label. B) The samples prepared as in A) were analyzed on a non-denaturing 5% acrylamide gel. The arrows point to the positions of the large ST2 RNA duplex and the resulting small labelled duplex stem in sample h.

region of ST2 RNA could form nuclease resistant intrastrand stem duplexes (Figure 4, Lane h). Elution and sizing of these molecules by uniform partial digestion in boiling formamide followed by gel electrophoresis gave a size estimate of 53 nucleotides for the labelled strand of the duplexes (unpublished results). However molecules similarily treated but labelled at the original 5' terminus, do not form such duplexes (lane d or f). Identical results were also obtained with ST2 RNA molecules labelled at the original 3' terminus indicating that neither of the original termini are protected in stem duplexes.

It is apparent that the large double strand duplexes of ST2 RNA intact hairpin molecules or T1 unlinked molecules have identical mobility irrespective of which RNA strand is labelled (Figure 4, lanes a,b,c,e and g). Under these conditions RNAs differing in size by approximately 10-20 nucleotides would be separable (14,24). Thus the nicking protocol does not release a T1 oligonucleotide greater than 10-20 nucleotides in length from the single strand turnaround region. This is supported by the fact the ST2 RNAs 5' labelled after T1 nicking of the turnaround region under limit conditions (less than 1 hit per molecule) and under conditions (see Materials and Methods) where virtually all molecules are cut, generate the identical major small duplex RNA species (53 nucleotides in length) after subsequent annealing and nuclease digestion. In addition, end base analysis shows the 5' label to be virtually all in A residues (greater than 80-90%) over this wide range of T1 nuclease concentrations (data not shown). Thus, there is apparently only one major T1 accessible G residue in the single strand turnaround region.

Sequence analysis of T1 cut turnaround region.

An estimated 53 nucleotides on the 5' side of the T1 cut site in the turnaround region can be protected (after melting and reannealing) from subsequent T1 nuclease digestion by sequences elsewhere in that RNA strand which do not include the exact original 3' terminus (Figure 4). Thus, it was of interest to directly sequence this area of the turnaround region to determine the origin of the stem duplexes. ST2 RNA molecules were specifically ^{32}P labelled at the 5' terminus generated by T1 nicking the single strand turnaround region (see previous section and Figure 6) then enzymatically sequenced directly or after duplex formation as in Figure 4, lane h (Figure 5). Analysis of ST2 molecules sequenced directly (Figure 5, line 3), gave the partial sequence of the first 138 bases on the 5' side of the turnaround region. Clearly direct homology with the standard virus genome 5' end can be seen over this region (Figures 5 and 6). Complementarity with the 3' terminus

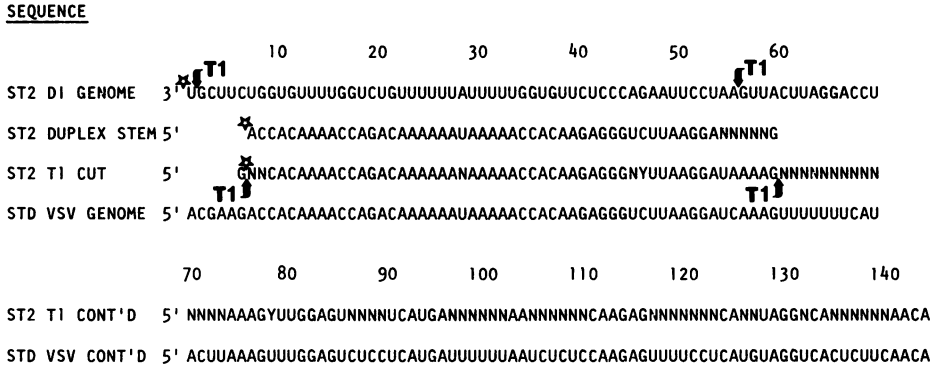

Fig. 5. The nucleotide sequence of the 5' side of the T_1 cut turnaround region of ST2 DI RNA analyzed directly (line 3) or after duplex stem formation (line 2), compared against previously published standard virus genome RNA 5' end sequences (line 4) (41). Stars denote points of labelling, arrows denote the predicted length of the T_1 nuclease resistant stem generated by intrastrand annealing of unlinked minus strand ST2 RNA and Y denotes pyrimidines. The 3' end sequence of ST2 RNA (line 1) is that of Figure 2 and is shown for comparison.

of ST2 RNA molecules (excluding the exact terminal 6 nucleotides) can be seen and annealing of the regions followed by T1 digestion would give rise to duplex stems containing a 53 base long minus strand (Figure 5, line 3) and a 54 base long plus strand (Figure 5, line 1). This is in good agreement with earlier sizing experiments, and was directly confirmed by the sequencing of

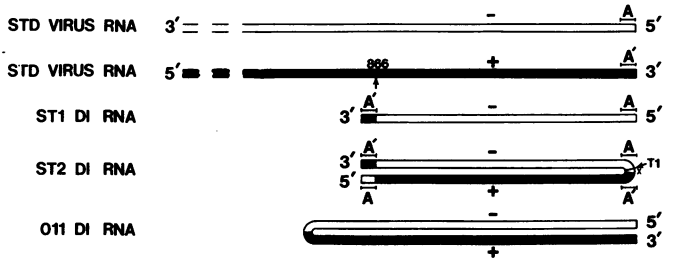

Fig. 6. A diagrammatic representation of the structure of ST1 and ST2 DI genome RNAs with reference to approximately the last 1.5 kb at the 5' end and 3' end of the standard VSV genome minus and plus strands respectively and the genome of DI-011 (14). Areas of complementarity are indicated by primed and non-primed letters, and black and white shading. X denotes a small number of nucleotides in the ST2 turnaround region, the exact sequence of which is unknown. The arrow on the standard genome plus strand indicates the site of premature termination of minus strand synthesis presumed to give rise to ST1 DI RNA. The arrow in ST2 RNA indicates the site of T1 RNAase nicking of the turnaround region.

the 5' labelled strand of isolated duplex stems (Figure 5, line 2). The RNA genome structure of ST1 and ST2 DI particles is summarized in Figure 6.

Attempts to sequence the 3' side of the T1 cut site in the turnaround region (X in Figure 6), have failed mainly due to T1 nuclease leaving a 3' phosphate group after cutting. Thus 3' labelling of ST2 RNA molecules after phosphatase treatment generates molecules labelled at both the 3' end generated by T1 cutting the turnaround region and the original 3' terminus, generating a mixed population of labelled molecules which does not permit sequence analysis. Efforts to separate the labelled strands (approximately 900 nucleotides in length) using a wide range of denaturing and non-denaturing gel eletrophoresis conditions have failed indicating that the size difference between the T1 unlinked separated strands is less than 5-10 nucleotides. Assuming only standard virus type sequences extend through the turnaround region, then only another 4 nucleotides could exist in the single strand turnaround region based on the known sequence and absence of any other major T1 accessible G in this region (see Figure 5).

Regardless of the exact identity of the few remaining bases in this region, clearly sequences are present at the turnaround region of ST2 molecules which are homologous (and complementary) to the standard virus 5' terminal region sequences and will form duplex stems in conjunction with sequences close to their termini (Figure 6). This is in marked contrast to the prototype snapback DI, DI-011 which does not possess such rearranged structural features (14).

DISCUSSION

ST1 DI RNA appears to be of the copy back class, possessing inverted complementary termini of 54 bases in length, the 5' and 3' termini of which are homologous and complementary, respectively, to the standard virus RNA 5' end (see Figure 6). Sequences adjacent to the stem at the 5' end are identical to standard virus 5' end sequences for at least another 123 bases. Sequences adjacent to the stem at the 3' end are no longer complementary to standard virus 5' end sequences but represent internal L gene sequences presumably read immediately preceding premature termination of replication during formation of the DI genome (see Introduction and Figure 6).

Since the stem adjacent sequences correspond to position 866 from the 5' end of the standard genome and the size of ST1 RNA is ~900 nucleotides long (Figure 1), the simplest possible structure for this DI RNA is that of a con-

tiguous 5' end minus strand genome segment 866 bases long, followed by a 54 base-long plus strand stem sequence as shown in Figure 6. However, further genome sequence rearrangements in the body of ST1 RNA for which we have no direct sequence determination can not be ruled out. The ST2 DI particle is a snapback DI, but represents a novel class of DI particle as it possesses a quite different structure to the prototype DI particle of the snapback class, DI-011 (4,14). ST2 snapback RNA does not possess linked minus and plus sense RNA strands which are colinearly homologous and complementary, respectively, to standard virus genome RNA as in DI-011, but contains internal sequences which are the inverted complement of sequences near the termini of the molecule. In addition the orientation of the ST2 hairpin structure relative to the standard virus genome is the opposite of that of DI-011 (see Figure 6) suggesting a different mode of generation. It should be noted here that, in contrast to DI ST2, the larger C5ST snapback DI (~2200 bases long) which we have previously partially characterized appears to be very similar or identical to DI-011 (3).

The length of different DI RNA genomes varies widely and rarely are DI particles found possessing the same length terminal inverted complementary stems or panhandles (3,4,10,11,12,13). In addition no two DI particles previously studied have been found that share both inverted terminal panhandle length and homologous sequences adjacent to this region indicating an identical point of premature replication termination and reinitiation of RNA synthesis during generation. This would suggest that these processes are relatively independent of sequence specificity. Thus it was to our surprise that the 3' end of ST2 DI RNA was found to be identical to that of ST1 DI RNA including the very specific features of the same 54 base length inverted complementary panhandle sequence and adjacent sequences (see Figure 6). This strongly suggests that the generation of these DI particles was closely linked. A number of models for this are possible.

Since the T1 nuclease separated ST2 RNA strands are virtually identical in length to ST1 RNA molecules and the 5' side of the turnaround region of ST2 molecules contains sequences homologous with ST1 5' end sequences (Figure 6), it seems likely that ST2 DI RNA is a hairpin copy of ST1 DI RNA molecules. If copyback DI RNA molecules such as ST1 DI RNA originate from standard virus RNA templates as hypothesized (8,9) then ST2 DI RNA molecules probably originate not from standard virus RNA, but from a DI RNA template, specifically DI ST1. This would occur for example, if the virus polymerase, on nearing completion of replication on the ST1 minus sense RNA template prematurely terminates syn-

thesis. Resumption of synthesis (without release of the nascent strand) could occur either directly back on the nascent strand, or near the 3' terminus of an ST1 plus strand RNA template in close proximity. Other models are possible including both ST1 and ST2 originating from a common ancestral RNA having already undergone genome rearrangement.

This is the first time evidence has been found suggesting that DI RNA genomes may not always be constrained to being generated from a viable standard virus RNA template but can originate from an already deleted and rearranged RNA molecule. This may allow some DI particles to more easily and quickly accumulate complex novel genome rearrangements and sequence recombinations like those now observed for a number of RNA viruses (30,42-47 and this publication). Such pools of novel virus sequence rearrangements may be of use to RNA viruses continuously evolving in response to their environment (7). These findings are in good agreement with the general 'leaping or jumping replicase complex' model for DI generation which predicts a great variety of genome rearrangements including the possibility that once generated DI molecules can undergo further rearrangements (3).

No biological difference has been seen between ST1 and ST2 DI particles in that neither out competes the other in mixed infections and both are equally ineffective in interfering with the Sdi^- standard virus mutants which accumulate during persistent infection or high multiplicity passage series (48). This indicates that neither structure is favoured over the other in terms of DI particle RNA replication and interference properties. The main structural features these DI particles share in common are the identical length of panhandle or stem sequences (54 bases) and large stretch of homologous bases in the 3' proximal region, suggesting that in this case these regions may play a more important role in modulating DI particle RNA replication and/or encapsidation than other areas of the genome.

In conclusion we have shown that ST2 DI particles represent a complexly rearranged novel class of snapback DI containing a hairpin copy of the simpler ST1 DI genome from which it was most likely derived.

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