Characterization of Snap-back RNAs in Vesicular Stomatitis Defective Interfering Virus Particles

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SUMMARY

VSV defective interfering particles of various sizes and from several independent sources frequently contain plus and minus strand RNA. In many cases some of the complementary strands are covalently linked as snap-back molecules. Infectious particles on the other hand package little or no plus strands. Snap-back molecules from the three different sources examined so far vary in size but appear to conform to the same overall linear duplex structure with cross-links at the ends only. They each contain a base sequence which is a subset of the next larger one and appear to correspond to unique sequences in the L cistron of the genome. Possible origins for these snap-back molecules are discussed.

INTRODUCTION

Vesicular stomatitis virus (VSV), a member of the 'negative-strand' Rhabdovirus group, can give rise to different size defective interfering particles (DI) within a few high multiplicity of infection (m.o.i.) passages following cloning (Reichmann, Pringle & Follett, 1971; Stampfer, Baltimore & Huang, 1971; Perrault & Holland, 1972a; Holland, Villarreal & Breindl, 1976a). These shorter truncated or T particles contain deleted forms of the genome or B RNA (Huang & Wagner, 1966; Brown et al. 1967; Schaffer, Hackett & Soergel, 1968; Roy & Bishop, 1972) which specifically inhibit the replication of the latter (Huang & Manders, 1972; Perrault & Holland, 1972b).

The properties of DI which are produced in many, if not all, virus systems (Huang & Baltimore, 1970; Huang, 1973) are becoming increasingly important since evidence now shows that they mediate virus persistence in vitro (Lehmann-Grube, 1971; Welsh & Pfau, 1972; Welsh, O'Connell & Pfau, 1972; Holland & Villarreal, 1974; Kawai, Matsumoto & Tanabe, 1975; Holland et al. 1976b; Spandidos & Graham, 1976) and may be involved in modifying the course of virus disease in vivo (Huang & Baltimore, 1970; Doyle & Holland, 1973; Spandidos & Graham, 1976). Furthermore, DI provide an important tool in the study of virus genome replication.

Some VSV T particles contain a mixture of plus and minus strand RNA whereas others appear to contain minus strand RNA only, as in the case of the infectious particle (Schaffer et al. 1968; Roy & Bishop, 1972; Roy et al. 1973; Leaman & Reichmann, 1974; Staminger & Lazzarini, 1974; Schnitzlein & Reichmann, 1976). Recently, we and others have shown that a particular clone of VSV gave rise to a T particle (referred to as DIa5 in our
previous study and as C5 ST in this paper) which contains covalently linked plus and minus strands (Lazzarini et al. 1975; Perrault, 1976). The proposed structure of these molecules was that of a hairpin (Lazzarini et al. 1975; Perrault, 1976) or a closed circle base-paired along most of its length (Perrault, 1976). The origin of these molecules is unclear since little is known regarding the generation of VSV DI RNAs in general. However, two main hypotheses have been suggested for the novel presence of a covalent linkage between the complementary strands: (1) a rare illegitimate event involving recombination or transcription across a replicative fork; and (2) a deleted form of a normal replicative intermediate structure (Lazzarini et al. 1975; Perrault, 1976). We present here evidence that snap-back RNAs are commonly found in VSV T particles and appear to conform to the same general structure.

Part of this work was presented at the Third International Colloquium on Rhabdoviruses, held in Tübingen, Germany, in July 1976.

METHODS

Cells and viruses. Baby hamster kidney cells (BHK 21) originally obtained from Flow Laboratories were employed for all virus assays and virus production. Cells and virus were grown in the presence of Eagle's minimal essential medium (MEM) containing 7% heat-inactivated calf serum. All VSV B stocks were obtained directly from Dr John Holland. WT36 B refers to the uncloned wild-type Mudd–Summers Indiana VSV originally obtained from Dr John Mudd. C5 B and C7 B are stocks derived from the fifth and seventh consecutive plaque cloning of WT36 B virus respectively. The Glasgow temperature sensitive (ts) B stocks were originally obtained from Dr M. E. Reichmann from the University of Illinois, Urbana, Ill. These were passed three or four times at low m.o.i. (<1) in our BHK 21 cells at 31 °C to obtain high titre stocks (>10^8 p.f.u./ml). The Ogden, New Jersey VSV stock was originally obtained from Dr Fred Schaffer, Naval Biological Laboratories, School of Public Health, University of California, Berkeley. After passage at low m.o.i. (<1) in BHK 21 cells, a clone was picked to produce the high titre stock employed in this study for the generation of T particles. The T particles derived from all of the above B stocks were grown by co-infesting monolayers of BHK 21 cells in 32 oz bottles with high titre B stocks (20 to 200 p.f.u./cell) and low titre T stocks obtained from the latter after two or three consecutive undiluted passages, and incubating at 37 °C for 16 to 24 h, or, in the case of ts mutants, at 31 °C for approx. 48 h. T particles from persistently infected BHK 21 cells were obtained as follows. CAR (E) B and T particles were grown from the ‘early’ CAR4 carrier cells (~6 months in culture) by co-cultivation with normal BHK 21 cells as described by Holland & Villarreal (1974). Partially purified T particles from this source (mostly carrier long T–LT) were picked from a sucrose gradient (see purification procedure below) and employed directly for co-infection of cells with a T particle-free B inoculum to grow the particles analysed in this study. The CAR (WT) T particles were obtained directly from infections carried out with co-cultivation lysates of a carrier culture established with wild-type virus and the CAR4 LT particle as described (Holland & Villarreal, 1974). The CAR (S) T particles were obtained from the stabilized two-year-old carrier culture described by Holland et al. (1976b). These were obtained after co-infection of cells with T particle-free B virus and a lysate from a second-cycle of co-cultivation.

B and T particle purification and labelling. All lysates were cleared of cell debris by low speed centrifugation at approx. 2000 g, for 15 to 20 min at 4 °C. The virus was then pelleted from these supernatants by centrifugation at 32000 g for 4 h at 4 °C in a Beckman Type
Snap-back RNAs in VSV DI

19 rotor, or for 90 min at approx. 85,000 g in a Beckman SW27 rotor. The virus pellets were resuspended in a small volume of TE buffer (10 mM-tris-Cl, 1 mM-EDTA, pH 7.6) with brief sonication (at a setting of 2, Branson Sonifier, Model LS-75 with a thin probe), layered on top of 12 ml, 5 to 20% (w/v) sucrose gradients, and centrifuged for 25 min at 30,000 rev/min and 4 °C in the SW41 Beckman rotor. The B and T particle bands were picked individually with a Pasteur pipette, layered on top of 12 ml, 10 to 40% (w/v) Na-K-tartrate gradients, and centrifuged for 2 h at 35,000 rev/min and 4 °C in the SW41 Beckman rotor. The bands were picked again and dialysed against a large volume of TE buffer overnight at 4 °C. In some cases, as indicated in the text, a second velocity centrifugation in sucrose gradients was carried out to further purify a given size T particle. The position of the particle bands relative to the B virions was marked on the tube and employed to calculate a rough sedimentation value, assuming B virus sediments at ~ 610S (Reichmann et al. 1971). T particles with S values ranging from 450 to 610, 300 to 450, and < 300 were arbitrarily designated long T (LT), medium T (MT), and short T (ST). Labelling of the various particle RNAs with 3H-uridine was carried out as described previously (Perrault, 1976) except that the concentration of 3H-uridine was raised to 200 μCi/ml in some experiments.

RNA purification, annealing, and gel analysis. RNA was extracted from the various particles by the Sarkosyl-proteinase K method described previously (Perrault, 1976). Samples were stored either at 0 °C in TE or LP buffer (1 mM-sodium phosphate, 0.1 mM-EDTA, pH 7.8) or at −20 °C after addition of ethanol in the last RNA purification step. Specific activities of the RNA were estimated roughly by measuring particle protein concentration by the Lowry method (Lowry et al. 1951) or by their extinctions (Perrault & Kingsbury, 1974) and assuming a 1% RNA content for all particles. Concentrations of unlabelled RNAs were measured by extinction.

Two different methods of melting and quick-cooling were employed to estimate 'snap-back' levels in RNA samples. In the first method, small volumes of the sample in TE or LP buffer (< 0.4 ml) were heated to 100 °C in a boiling water bath for 3 min followed by immediate cooling in ice water. In the second method, similar small volumes of sample in 6 M-urea-formamide (urea dissolved to 6 M in 99% de-ionized formamide [Malinckrodt] containing 0.02 M-sodium barbital and buffered to pH 9.0 with HCl) were heated to 70 °C for 3 min followed by immediate cooling in ice water. RNase-resistance in quick-cooled samples (20 μg/ml RNase A and 10 units/ml T1 RNase, 30 min at 37 °C) was determined by first adjusting the salt concentration to 0.3 M-NaCl or by diluting the urea-formamide samples 20-fold in TE plus 0.3 M-NaCl before addition of enzyme.

The amount of self-annealing RNA in a given sample was determined as follows. After melting and quick-cooling, 0.2 ml samples were adjusted to a salt concentration of 0.48 M-sodium phosphate, pH 6.8, overlaid with 0.1 ml mineral oil, and incubated in small, tightly capped plastic tubes at 70 °C. Samples of 20 to 100 μl were removed at the indicated times and either assayed for total cpm/min or digested as above with RNase after a twofold dilution in H2O. All cpm/min were determined by spotting on DEAE-81 filters as described previously (Perrault & Kingsbury, 1974). Annealing of unlabelled T particle RNAs to labelled B RNA or WT30 ST RNA was carried out as above in 0.48 M-P043. Analysis of RNA samples on denaturing 6 M-urea-formamide gels was described previously (Perrault, 1976; Villarreal, Breindl & Holland, 1976). Densitometer tracings of X-ray film fluorograph strips were carried out with a Gilford spectrophotometer with a linear transport attachment and measured at 540 nm as described by Villarreal & Holland (1976). RNase treatment of samples to be analysed on gels was carried out under mild conditions with 1 μg/ml and 0.5 units/ml of
RNases A and T₁, respectively, in TE buffer containing 0·3 M-NaCl for 1 to 2 min at 37 °C. The digested RNA was re-purified by the Sarkosyl-proteinase K digestion and phenol-chloroform extraction procedure. All samples, whether RNase-treated or not, were first denatured by heating to 70 °C in 30 μl urea-formamide before applying to the gel. X-ray film was exposed to the processed gel for 2 days.

**Electron microscopy.** The procedures for aqueous spreading of RNA samples followed the techniques of Davis, Simon & Davidson (1970). Samples (0·5 to 1·0 μg/ml) were first melted and quick-cooled in TE buffer, as described above, before addition of 5 μl cytochrome c (1 mg/ml in 5 M-ammonium acetate) to 45 μl volumes followed by spreading on to a hypophase of 0·25 M-ammonium acetate. Grids were picked, stained in uranyl acetate (5 × 10⁻⁵ M), rinsed with pentane and then rotary-shadowed with platinum:palladium (80:20) at an angle of ~ 8° before examination in a Philips EM 200 electron microscope. Magnifications were determined relative to reference grids with 54 864 lines per inch. Molecules were measured and plotted with a Hewlett-Packard Model 9864A Digitizer, Model 9821A Calculator, and Model 9862A Plotter, courtesy of Dr Ronald R. Helinski of this department.

**RESULTS**

**Snap-back and self-annealing RNA in different VSV DI isolates**

Snap-back RNA molecules which remain RNase-resistant immediately following denaturation procedures have been reported previously in a unique VSV T particle isolate originating from this laboratory (Lazzarini *et al.* 1975; Perrault, 1976). The unexpected presence of these novel virus DNA molecules prompted us to examine other sources of VSV RNA for similar structures. Table I summarizes the annealing properties of purified RNA samples extracted from several sources of standard VSV B virions and T particles derived from them. The various samples are denoted ST, MT, or LT (as stated in Methods) and the percentage of snap-back RNA in a given sample was assayed by two different procedures as outlined in Methods. Prior nicking of the various snap-back-containing samples with RNase, as described previously for the C₅ ST RNA (Perrault, 1976) abolished this snap-back property as expected (data not shown). Several independent preparations of tsG₃1 ST and C₅ ST particles, labelled with ³H-uridine or ³²P, yielded similar levels of snap-back and self-annealing RNA, indicating that these properties are not a function of the label used nor are they unstable.

It should be noted that in many cases the various T particle samples do not represent homogeneous single component populations. This is particularly so in isolates producing more than one T particle where the faster sedimenting sucrose gradient bands are invariably heavily contaminated with aggregates of the slower one. In some cases, for example tsG₁₁ MT particles, the band was broader than expected for a single homogeneous component and contained at least two particles too close to each other to purify separately. The two components in tsG₃₁ were isolated from a second cycle of velocity sedimentation in sucrose and inspection of the snap-back level in these demonstrates that tsG₃₁ ST₁ was at least 90% free of the larger particle tsG₃₁ ST₂ (Table I). The tsG₃₁ ST₂ preparation, however, consisted mostly of aggregated tsG₃₁ ST₁ and of about 25% faster migrating particles (see below).

The self-annealing values reported in Table I represent the saturating levels of RNase resistance (including snap-back) obtained after long-term incubation under optimal annealing conditions.

Several important conclusions can be derived from these results: (1) B virion RNA from
Table 1. Snap-back and self-annealing RNA in VSV B and T particles

<table>
<thead>
<tr>
<th>Source of particle†</th>
<th>Approximate</th>
<th>% Snap-back</th>
<th>% self-annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>size</td>
<td>Aqueous</td>
<td>Urea-formamide</td>
</tr>
<tr>
<td>C5 B</td>
<td>610</td>
<td>1.2</td>
<td>—</td>
</tr>
<tr>
<td>C5 LT</td>
<td>540</td>
<td>7.9</td>
<td>3.9</td>
</tr>
<tr>
<td>C5 ST</td>
<td>290</td>
<td>7.9</td>
<td>81</td>
</tr>
<tr>
<td>C7 B</td>
<td>610</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>WTms ST</td>
<td>290</td>
<td>&lt;1.0</td>
<td>—</td>
</tr>
<tr>
<td>tsG11 LT</td>
<td>460</td>
<td>3.0</td>
<td>25</td>
</tr>
<tr>
<td>tsG11 MT</td>
<td>370</td>
<td>4.5</td>
<td>40</td>
</tr>
<tr>
<td>tsG22 MT</td>
<td>340</td>
<td>5.6</td>
<td>62</td>
</tr>
<tr>
<td>tsG31 B</td>
<td>610</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>tsG31 ST2</td>
<td>250</td>
<td>27</td>
<td>—</td>
</tr>
<tr>
<td>tsG31 ST1</td>
<td>170</td>
<td>2.0</td>
<td>—</td>
</tr>
<tr>
<td>tsG41 MT</td>
<td>350</td>
<td>44</td>
<td>40</td>
</tr>
<tr>
<td>tsG41 ST</td>
<td>270</td>
<td>37</td>
<td>32</td>
</tr>
<tr>
<td>CAR(E) B</td>
<td>610</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>CAR(E) LT</td>
<td>520</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>CAR(E) MT2</td>
<td>420</td>
<td>2.3</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>CAR(E) MT1</td>
<td>360</td>
<td>5.5</td>
<td>2.5</td>
</tr>
<tr>
<td>CAR(S) MT</td>
<td>—</td>
<td>7.9</td>
<td>2.9</td>
</tr>
<tr>
<td>CAR(S) ST</td>
<td>—</td>
<td>42</td>
<td>23</td>
</tr>
<tr>
<td>CAR(WT) MT</td>
<td>370</td>
<td>55</td>
<td>—</td>
</tr>
<tr>
<td>WTns ST</td>
<td>290</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Snap-back and self-annealing levels were determined as described in Methods. Samples were labelled with 3H-uridine with sp. act. of 200 to 400 K cp/min/μg RNA for C7B, WTms ST1, tsG31 ST1, and tsG31 ST2, and 20 to 40 K cp/min/μg for all the rest. Except for the former, RNA concentrations of 1 to 2 μg/ml and time of annealing, ~72 h, were identical for all samples. The values reported for the C7B and WTms ST samples, both at 0.1 to 0.2 μg RNA/ml are for 48 h of annealing and are identical to 24 h values (not shown) indicating that saturation had been reached. The tsG31 ST1 and tsG31 ST2 samples were tested over a 50-fold range of concentration, ~0.02 μg/ml to ~1 μg/ml, and annealed for 48 h with no change in the values obtained.

† See Methods.
‡ These are only rough approximations of sedimentation value estimated as described in Methods.

any source shows little if any snap-back or self-annealing; (2) several T RNAs show significant levels of snap-back; (3) an even greater number of T RNAs contain unlinked plus and minus strands which can self-anneal; (4) snap-back and/or self-annealing RNA can be found in T particles derived from wild-types, temperature-sensitive mutants, persistently infected cells, and both Indiana and New Jersey serotypes.

Characterization of tsG31 and tsG11 T snap-back RNAs by electron microscopy

Electron microscopy of C5 ST RNA, melted, quick-cooled and spread under aqueous conditions, was shown previously to yield simple linear molecules about 0.33 μm in length (Lazzarini et al. 1975; Perrault, 1976). We chose to examine the structural features of two additional sources of snap-back RNA. Accordingly, tsG31 ST2 RNA (particles purified twice in sucrose velocity gradients) and tsG11 MT RNA (two particles similar in size and purified together on one gradient only), containing approx. 27% and 40% snap-back respectively, were spread exactly as described previously (Perrault, 1976). Both of these RNA preparations, Fig. 1(a) and (b), contained simple linear duplex molecules with the
Fig. 1. Electron micrographs and length distributions of snap-back RNA molecules under aqueous spreading conditions from (a) and (c), tsG31 ST2 particles; (b) and (d), tsG11 MT particles.

Length distributions shown in Fig. 1 (c) and (d). The tsG31 ST2 snap-back RNA duplexes are clearly a very homogeneously sized population with a mean length ± standard deviation equal to 0.23 ± 0.01 μm. The tsG11 MT RNA appears to contain two relatively homogeneously sized components differing in size by about 0.04 μm only. Taken together these
two components (molecules ranging from 0.36 to 0.66 μm) show a mean ± standard deviation equal to 0.51 ± 0.03 μm.

From these observations we conclude that the snap-back RNA molecules in tsG31 MT and tsG31 ST2 appear as linear duplex molecules with lengths corresponding roughly in size to the particles from which they were extracted (Table 1). Furthermore, large amounts of single-stranded RNA (ssRNA) which remain collapsed under these spreading conditions do not appear to be visibly associated with the duplex structures. The limits of detection of such collapsed ssRNA regions, however, remain uncertain, particularly if they are small (less than a few hundred base pairs). Nevertheless it is clear that all three VSV snap-back RNAs examined so far appear to have the same general duplex structure, differing only in length.

Out of 134 tsG31 ST2 RNA molecules measured in Fig. 1(c), seven circular molecules were observed. The contour length of these circles ranged from 0.22 to 0.25 μm with a mean ± standard deviation equal to 0.23 ± 0.01 μm. The nature and origin of these structures will be discussed in the following paper (Perrault & Leavitt, 1977).

Denaturing gel analysis of tsG31 and tsG11 T particle RNAs. The results above suggested to us that snap-back RNA from various sources might contain a unique duplex region resistant to RNase as shown previously for C5 ST RNA (Lazzarini et al. 1975; Perrault, 1976). We tested this hypothesis directly by analysing the nicked and non-nicked snap-back RNAs on denaturing urea-formamide gels as described previously (Perrault, 1976). Fig. 2
shows that both tsG31 ST1 and tsG31 ST2 particle preparations (same samples as in Table 1) apparently contain the same major denatured RNA component with a mol. wt. estimate of 0.44 ± 0.04 × 10^6 relative to markers. In addition, tsG31 ST2 also contains a slower migrating band representing ~ 25% of the total RNA. Clearly, the major component reflects the migration of most or all of the ssRNA characteristic of tsG31 ST1 particles. As mentioned previously the tsG31 ST2 preparation contains mostly aggregated tsG31 ST1 particles. Therefore, the minor RNA component probably represents snap-back molecules which originate from the faster sedimenting particles. However, this remains tentative since we have previously shown that C5 ST snap-back molecules migrate anomalously in this gel system (Perrault, 1976). This putative snap-back component in tsG31 ST2 also appears to migrate more heterogeneously in some cases (Fig. 2 c).

Fig. 2 (c) and (d) shows the results obtained with a preparation of tsG31 ST2 RNA (particles purified only once in sucrose gradients and containing ~ 22% snap-back) before and after RNase digestion as described in Methods. The rationale for this experiment was to examine the nature of the snap-back duplex RNA after digesting away all the ssRNA present in the mixture, either in association with snap-back containing molecules or separate from these. RNase was added immediately following melting and quick-cooling in order to avoid duplex formation by unlinked complementary strands in the sample. The RNase treatment employed was very mild in order to avoid overdigestion and possible internal nicking of duplex RNA. This treatment, however, was sufficient to remove all the ssRNA since the amount of resistant RNA recovered (~ 21%) was essentially the same as that obtained under more severe digestion conditions (Table 1). Since the duplex length of tsG31 ST2 snap-back RNA is about 70% that of C5 ST (0.23 μm against 0.33 μm, see Fig. 1 c and Perrault, 1976) RNase digestion of these molecules should yield denatured ssRNAs with mol. wt. in the same ratio if the duplexes are uninterrupted by ssRNA regions. This prediction is confirmed within experimental error since RNase-treated tsG31 ST2 RNA yields a homogeneous band of mol. wt. 0.38 ± 0.04 × 10^6 which is about 80% that of RNase-treated C5 ST snap-back RNA (Perrault, 1976). In addition to this large RNA, a much smaller RNA peak representing about 18% of the total RNase-resistant material is obtained after digestion of tsG31 ST2 RNA (Fig. 2 d). Experiments outlined in the following paper (Perrault & Leavitt, 1977) show that this minor component does not originate from the snap-back molecules but most likely represents complementary terminal inverted sequences derived from the ssRNA component of tsG31 ST1.

A similar gel analyses of tsG11 MT RNA shows that all of the material obtained after RNase treatment of a melted and quick-cooled sample migrates as a single homogeneous denatured component of mol. wt. 0.80 ± 0.08 × 10^6 which falls within the expected mol. wt. range assuming nicking does not occur within the duplex region (data not shown).

We thus conclude from this analysis that in all three snap-back RNAs examined so far the duplex regions consist of a unique length, contiguously base-paired segment. This conclusion is also strongly supported by experiments presented in the following paper (Perrault & Leavitt, 1977) which show that the denatured strands of nicked snap-back molecules can re-anneal to form duplexes of about the same length as the original snap-back molecules. Whether untreated tsG31 and/or tsG11 snap-back RNAs also contain associated ssRNA regions at one or both ends of these duplexes will be discussed below.

Mapping of snap-back RNAs by hybridization

Although previous studies have shown that C5 ST snap-back RNA contains sequences homologous to B virion RNA, the extent of genomic representation was not investigated
Table 2. Annealing of snap-back containing T particle RNAs to labelled genome and WT<sub>ms</sub> ST RNAs*

<table>
<thead>
<tr>
<th>Source of RNA</th>
<th>Concentration (~ µg/ml)</th>
<th>% annealing of labelled RNA</th>
<th>B genome</th>
<th>WT&lt;sub&gt;ms&lt;/sub&gt; ST†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snap-back RNAs‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tsG&lt;sub&gt;31&lt;/sub&gt; ST&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2.0</td>
<td>11, 11</td>
<td>42, 38</td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;5&lt;/sub&gt; ST</td>
<td>2.0</td>
<td>16, 15</td>
<td>55, 50</td>
<td></td>
</tr>
<tr>
<td>tsG&lt;sub&gt;11&lt;/sub&gt; MT</td>
<td>2.0</td>
<td>24, 24</td>
<td>71, 69</td>
<td></td>
</tr>
<tr>
<td>tsG&lt;sub&gt;31&lt;/sub&gt; ST&lt;sub&gt;3&lt;/sub&gt; + C&lt;sub&gt;5&lt;/sub&gt; ST</td>
<td>2.0</td>
<td>—</td>
<td>53, 49</td>
<td></td>
</tr>
<tr>
<td>tsG&lt;sub&gt;31&lt;/sub&gt; ST&lt;sub&gt;3&lt;/sub&gt; + tsG&lt;sub&gt;11&lt;/sub&gt; MT</td>
<td>2.0</td>
<td>—</td>
<td>74, 69</td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;5&lt;/sub&gt; ST + tsG&lt;sub&gt;11&lt;/sub&gt; MT</td>
<td>2.0</td>
<td>—</td>
<td>73, 81</td>
<td></td>
</tr>
<tr>
<td>tsG&lt;sub&gt;31&lt;/sub&gt; ST&lt;sub&gt;3&lt;/sub&gt; + C&lt;sub&gt;5&lt;/sub&gt; ST + tsG&lt;sub&gt;11&lt;/sub&gt; MT</td>
<td>3.0</td>
<td>—</td>
<td>71, 69</td>
<td></td>
</tr>
</tbody>
</table>

* Annealing and RNase assays were carried out as described in Methods. The labelled genome C<sub>7</sub> B RNA and WT<sub>ms</sub> ST RNAs, both at 0.1 to 0.2 µg/ml, correspond to the ones analysed in Table 1. Each sample containing labelled RNA only or labelled RNA plus cold RNA was melted and quick-cooled before annealing. The % annealing values listed correspond to 24 h and 48 h or 48 h only.

† The self-annealing value of this RNA alone without added cold RNA is 22% (Table 1). Therefore, 22% of the cts/min were subtracted from both control and RNase-treated samples to obtain the corrected values listed.

‡ The purified, nicked snap-back RNAs were obtained by first melting and quick-cooling the total T particle RNA followed by immediate RNase digestion in TE plus 0.3 M-NaCl with 20 µg/ml RNase A and 10 units/ml T<sub>1</sub> RNase for 20 min at 37 °C. The RNA was then recovered by Sarkosyl-proteinase K digestion and phenol-chloroform extraction as before.

directly (Lazzarini et al. 1975; Perrault, 1976). A priori snap-back RNAs could represent many copies of a short segment of the genome, either in tandem or in some other arrangement, or a unique subset of sequences of defined length. The results shown in Table 2 demonstrate that the latter is the case. Small amounts of labelled genome RNA were annealed to an excess of unlabelled, nicked snap-back RNAs and the fraction of the genome in the duplex determined. This kind of experiment examines only the duplex fraction of snap-back RNAs. The kinetics of such a reaction with C<sub>5</sub> ST snap-back RNA, as well as the kinetics of the self-annealing reaction, are shown in Fig. 3.

Clearly, the fraction of the genome represented in the various snap-back RNA duplexes is proportional to their size in comparison to that of the genome which has a mol. wt. of approx. 3·2 to 3·8 x 10<sup>6</sup> (Huang & Wagner, 1966). As shown in Table 3 this conclusion is in excellent agreement with the estimates of snap-back duplex lengths by electron microscopy and denaturing gel analysis.

In order to determine which region of the genome is represented in these snap-back RNA duplexes we carried out a similar protection experiment using labelled WT<sub>ms</sub> ST RNA which has been shown previously to map in the L cistron in common with most other VSV T particle RNAs (Leamnson & Reichmann, 1974). This map location has been independently corroborated in this laboratory since sequences complementary to this WT<sub>ms</sub> ST RNA appear late in endogenous virion transcription reactions, and the T<sub>1</sub> oligonucleotide fingerprint of the particle RNA, in common with several other T particles, differs greatly from that of HR LT RNA which maps in the N to G gene regions of the genome (D. Kohne & J. Holland, personal communication). Clearly, most of the snap-back RNA duplex sequences also map in this T particle RNA (Table 2). Furthermore, all the snap-back RNAs appear to be subsets of one another since the percentage of protection with various combinations never exceeds that of the largest component present. Assuming tsG<sub>31</sub> ST<sub>3</sub> snap-back RNA is totally included within the sequences in WT<sub>ms</sub> ST, then the mol. wt. of the
Fig. 3. Kinetics of nicked and non-nicked C5 ST RNA self-annealing and annealing to genome RNA. The conditions for annealing to genome RNA are as described in Table 2. C5 ST RNA self-annealing was monitored by adding small amounts of high specific activity (~ 200 K cpm/µg) ^3H-uridine labelled C5 ST RNA, non-nicked or RNase-treated as in Table 2, to the corresponding unlabelled, 2 µg/ml, C5 ST RNA solutions. □—□, labelled, non-nicked C5 ST RNA self-annealing; ○—○, labelled, nicked C5 ST RNA self-annealing; ■—■, non-nicked C5 ST RNA annealing to labelled B genome; •—•, nicked C5 ST RNA annealing to labelled B genome.

Table 3. Comparative size estimates of T particle snap-back molecules

<table>
<thead>
<tr>
<th>Snap-back source</th>
<th>Duplex length (µm)*</th>
<th>Single strand mol. wt. x 10^6†</th>
<th>% genome represented‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>tsG31 ST</td>
<td>0.23 ± 0.01</td>
<td>0.38 ± 0.04</td>
<td>11</td>
</tr>
<tr>
<td>C5 ST</td>
<td>0.33 ± 0.02</td>
<td>0.47 ± 0.05</td>
<td>15</td>
</tr>
<tr>
<td>tsG11 MT</td>
<td>0.51 ± 0.03</td>
<td>0.80 ± 0.08</td>
<td>24</td>
</tr>
</tbody>
</table>

* Electron microscopy under aqueous conditions, Fig. 1.
† Denaturing gel analysis of nicked, denatured snap-back molecules, Fig. 2 and text.
‡ Annealing of labelled genome RNA to nicked, denatured snap-back RNA, Table 2.

latter can be calculated to be ~ 0.95 ± 0.10 x 10^6, which is in good agreement with previous estimates (Leamnson & Reichmann, 1974). Similarly, most, if not all, of C5 ST snap-back RNA is included within the WTMS ST sequences since the protection level observed, ~ 49 to 53 %, is very near the expected 50 % value on the basis of the nicked, snap-back C5 ST ssRNA mol. wt. of 0.47 ± 0.05 x 10^6 (Perrault, 1976). Furthermore, the ratio of WTMS ST RNA protection to B RNA protection is the same for both C5 ST and tsG31 ST snap-back RNAs. Based on similar arguments, assuming a mol. wt. of 0.80 ± 0.08 x 10^6 for nicked snap-back tsG11 MT ssRNA, we conclude that 80 to 95 % of the sequences in this RNA are also found in WTMS ST RNA. A model for the origin of these various RNA sequences is presented in the Discussion.

**DISCUSSION**

Snap-back RNA molecules are found frequently in VSV DI. Seven out of ten independent sources of VSV B virions examined in this study gave rise to a number of different sizes of T particles at least one of which contained high levels of snap-back RNA. These included
Snap-back RNAs in VSV DI

Fig. 4. Suggested genome map location of snap-back RNAs. The central feature of this model is the common location of a cross-link at a site corresponding to the 5' end of the genome RNA and the 3' end of its complementary plus strand.

wild-type, temperature sensitive, Indiana and New Jersey serotypes as well as persistently infected cells. It should be noted that this latter source involved the continual selection of different DI over two years of culture (Holland et al. 1976 b) indicating that such snap-back-containing particles probably do not differ significantly from other DI in their interference properties.

Several lines of evidence support the notion that the overall structure of the three snap-back RNA molecules examined so far is similar. The duplex regions of these molecules consist of simple contiguously base-paired regions of a unique size and sequence for each snap-back RNA source. This is supported by size determinations by electron microscopy and denaturing gel analyses as well as annealing data. It has been established previously that C5ST snap-back RNA molecules contain little, if any, covalently associated ssRNA apart from small RNase-sensitive regions which link the complementary strands near one or both ends (Perrault, 1976). This is also probably true for tsG31 and tsG11 snap-back RNAs for the following reasons. Firstly, electron microscopy under aqueous conditions or formamide denaturing conditions (see following paper) does not show any major fraction of ssRNA in association with the duplexes. Secondly, the duplex lengths of the three snap-back sources roughly correspond to the sizes of the particle from which they were extracted. Thirdly, tsG11 as well as tsG41 RNA, which also contains snap-back, give rise to two peaks in KI density gradients corresponding to single-stranded and double-stranded RNA as shown previously for C5 ST (Perrault, 1976; our unpublished observations). Lastly, the following paper shows that at least some, if not all, C5 ST and tsG31 snap-back duplexes contain small inverted terminal repeat sequences following digestion of all ssRNA by RNase. Since terminal inverted complementary sequences are conserved at the ends of the ssRNAs as well, it is likely that the ends of untreated snap-back RNAs also correspond at least in part to these same terminal sequences. Thus it is probable that all snap-back VSV DI RNAs correspond to the structure previously proposed, i.e. a self-complementary molecule base-paired along most of its length (Lazzarini et al. 1975; Perrault, 1976). Recent evidence indicates that several DI RNAs (including a snap-back RNA) contain the same 3' and 5' termini as the parent B virion RNA (Keene, Rosenberg & Lazzarini, 1977). Thus, VSV snap-back RNA appears to differ somewhat from the self-complementary, covalently-closed circular structures recently proposed for plant viroid RNAs (Sänger et al. 1976).

Analysis of the base sequences present in these snap-back molecules leads us to conclude that they mostly represent unique non-permuted subsets of the L cistron region of the
genome. Essentially all the sequences within the two smaller snap-back RNAs and at least 80% of the largest are present within WT<sub>st</sub> ST RNA, which presumably maps entirely within the L cistron (Leamnson & Reichmann, 1974). Since all the snap-back sequences are subsets of one another the simplest model would place these at the 5' end of the L cistron as shown in Fig. 4. A similar inference has recently been made by Schnitzlein & Reichmann (1976) for the origin of VSV DI single-stranded minus RNAs which map in the L cistron. It should be pointed out that this suggestion is based on a minimum of genomic sequence rearrangements. As mentioned above, the following paper (Perrault & Leavitt, 1977) presents evidence that both ends of the snap-back duplex RNAs as well as other single-stranded VSV DI RNAs contain small inverted complementary sequences. Thus, the extent of genomic sequence rearrangements in DI RNAs by recombination and/or other unknown mechanisms may be more extensive than indicated by this simple model.

One important feature of the model, however, suggests a possible origin for the snap-back molecules. If we make the above assumption that all snap-back molecules originate from the 5' end of the genome RNA then each of these probably contains a common cross-link corresponding to the original 5' end of the minus strand genome RNA linked to the 3' end of its plus strand complement (Fig. 4). As discussed in the following paper (Perrault & Leavitt, 1977) the cross-link in snap-back molecules probably occurs between a set of complementary sequences which most likely correspond to conserved terminal sequences in VSV DI and B RNAs. Thus, this cross-link could conceivably be a regular feature of DI and possibly B minus strand RNA replication. Such a replication scheme suggests a self-priming type of mechanism which has found strong support recently in the replication of parvovirus DNA (Tattersall, Crawford & Shatkin, 1973; Bourguignon, Tattersall & Ward, 1976; Gunther & May, 1976; Straus, Sebring & Rose, 1976; Tattersall & Ward, 1976). A self-priming mechanism has also been proposed recently for the synthesis of duplex DNA in RNA tumour virus replication (Haseltine & Baltimore, 1976). Alternatively, the origin of snap-back RNAs could result from some recombinational process involving terminal complementary sequences and related sequences (see following paper). We are currently investigating these various possibilities.

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Note added in proof. Mol. wt. estimates of untreated tsG31 and C5 DI snap-back RNAs in totally denaturing methyl mercury containing gels (carried out in collaboration with Dr Luis Villarreal, Dept. of Biochemistry, Stanford University) confirm our proposed structure for these snap-back molecules. Their size corresponds to twice that of the nicked, denatured molecules, indicating the presence of only one very small single-stranded region linking the two complementary strands.
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