Analysis of the RNA Species Isolated from Defective Particles of Vesicular Stomatitis Virus

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(Accepted 20 May 1976)

SUMMARY
Serial high multiplicity passage of a cloned stock of vesicular stomatitis virus was found to generate defective interfering particles containing three size classes of RNA, with sedimentation coefficients of 31S, 23S and 19S. The 31S and 23S RNA species were found to be complementary to both the 12 to 18S and 31S size classes of VSV mRNAs. The 19S class of RNA was found to be partially base-paired. All three RNA species were found to contain ppAp at their 5' termini.

INTRODUCTION
Wild-type vesicular stomatitis virus (VSV) is a bullet shaped particle which contains a single-stranded RNA genome of mol. wt. approx. $4 \times 10^6 \text{ (}42\text{S; Wagner, 1975).}$ The virion RNA, designated as negative strand (Baltimore, 1971), is complementary to the virus mRNAs isolated from VSV infected cells. Serial undiluted passage of VSV has been shown to generate defective interfering (DI) particles which contain the same proteins and RNA of the same polarity (Schaffer, Hackett & Soergel, 1968) as the wild-type virus, but are physically smaller (Huang, 1973) and are incapable of self-replication (Crick, Cartwright & Brown, 1969). Since these particles possess only a portion of the virus genome, in most instances they are simply deletion mutants of the wild-type (Huang & Baltimore, 1970). While only one particular size class of DI particles arises when co-infected with a particular wild-type strain of VSV, a variety of DI particles from different wild-type stocks of VSV have been described in the literature (Huang, Greenwalt & Wagner, 1966; Huang & Wagner, 1966a; Petric & Prevec, 1970; Reichmann, Pringle & Follet, 1971). Leamnson & Reichmann (1974) described a particle corresponding to approx. 1/12 the size of the VSV genome RNA which self-hybridizes extensively. Similarly, Roy, Hefti & Bishop (1973) isolated RNA species from defective virions arising from a heat resistant (HR) strain of VSV, and found complementary plus and minus strand RNA to be packaged separately in the defective virions. Recently, Lazzarini et al. (1976) have isolated a unique small DI particle which contains covalently linked message and anti-message RNA.

The variety of DI particles produced suggests that the purity of the parental strain of virus or host cell used for this production may to some extent control the type of defectives generated. Exactly why a particular size of DI particle may arise during serial undiluted passages of infectious virus, is unclear. However, once the DI particles are formed, their RNAs appear to be replicated by competition with virus RNA for the replicative machineries (Stampfer, Baltimore & Huang, 1969; Wild, 1972). Experiments described in this paper

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were carried out to investigate the variety of sizes of DI particles a particular strain of plaque purified virus would generate and their relationship to the wild-type particle, and to analyse their 5' termini to begin to resolve the problem of their origin.

METHODS

Virus and cells. The original wild-type VSV strain (Indiana serotype) was obtained from Dr D. F. Summers (Albert Einstein College of Medicine, N.Y.; now at the University of Utah, Salt Lake City, Utah), and was subsequently plaque purified and grown in baby hamster kidney cells (BHK 21, clone 13) in suspension and purified as previously described (Banerjee, Moyer & Rhodes, 1974). For production of defective interfering particles, the virus was passed undiluted three times in monolayer cultures of BHK 21/13 cells maintained in Grand Island Biological Company (GIBCO) F-11 medium supplemented with 10% foetal calf serum as described below. Monolayers of BHK cells (8 × 10^6 cells) were infected at an input multiplicity of 10 p.f.u./cell, and the virus was harvested at 16 h p.i. The pools were freed from cell debris by centrifuging at 800 g for 10 min, and half the volume of the supernatant fluid (1.5 ml) was used to infect a fresh monolayer of 8 × 10^6 BHK cells. Successive undiluted passages were carried out in this fashion.

Preparation and purification of 32P-labelled DI particles. Second or third high multiplicity passage of virus was used as seed stock. No qualitative difference was observed in the types of DI particles produced with either stock, but since the yield of DI particles was higher when using second passage virus, this passage was routinely used.

Monolayer BHK 21/C13 cells were maintained at 37 °C in phosphate-free Eagle’s minimal essential medium buffered with 14 mM-HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid), pH 7.2, for 2 h prior to infection. The infecting virus was allowed to adsorb for 1 h in serum-free medium. Excess virus was then removed and the cells were placed in phosphate-free medium supplemented with 2% (v/v) foetal calf serum. Two hours p.i., 32PO4 (New England Nuclear) was added to 200 μCi/ml, and the cells were maintained 18 h at 37 °C. The progeny virus was treated with 25μg/ml pancreatic RNase for 30 min at 37 °C, and purified as described previously (Banerjee et al. 1974). The infectious progeny virus (B particles) was clearly separated from the DI particles in equilibrium density gradients (Huang & Wagner, 1966 b). The DI particles were further purified by re-banding in potassium tartrate equilibrium gradients.

Isolation and purification of VSV mRNA. Suspension HeLa cells were infected with VSV at a multiplicity of infection of 25. The procedures for isolation and purification are identical to those previously described for infected BHK 21 cells (Moyer et al. 1975).

Preparation of DI Particle RNA. Purified virus was suspended in SDS buffer (0.1 M-NaCl, 0.01 tris-HCl, pH 7.2, 0.01 M-EDTA, 0.5% SDS) to disrupt the virus, and the DI RNA isolated by centrifuging in 15 to 30% (w/v) sucrose gradients in SDS buffer in a Spinco SW41 rotor at 20500 rev/min for 17 h. Tritium labelled BHK 21 ribosomal RNA was present as marker RNA. The fractions designated in Fig. 3a were precipitated with 2 vol. of ethanol at -20 °C. The precipitates were extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol and re-purified by velocity sedimentation.

Analysis of the 5' termini of the DI RNAs. 32P-labelled DI RNAs were hydrolysed in 0.3 M-KOH for 18 h at 37 °C and the 5' termini were characterized by DEAE-cellulose chromatography as described previously (Moyer et al. 1975). The radioactivity eluting between charges -4 and -5 was dialysed against distilled water at 4 °C, lyophilized, and further analysed by enzymatic digestion. Digestion with P1 nuclease (200 μg/ml; Yamasa

Defective interfering particles of VSV

Fig. 1. Assay of infectious particles during successive passage of VSV. Virus was passed undiluted in monolayer cultures of BHK 21/13 cells as described in Methods. For each successive passage, samples of the supernatant fluid were assayed for infectivity (Krontiris, Soeiro & Fields, 1973).

Continuous passage of undiluted virus resulted in a reduced yield of wild-type infectious particles (Fig. 1). The infectivity of the virus was reduced by four log units after the sixth passage, presumably due to interference by the DI particles generated during the passages. In order to study the fate of the infecting virus and the DI particles produced, the following experiments were performed.

Cells were infected with wild-type virus, the progeny of which was then continuously passed through several replication cycles in fresh cells. At each passage, one half of the infected cultures were incubated in the presence of ³H-uridine in order to determine the sizes of the DI particle RNAs, and the relative amount of each RNA species generated. The
progeny virus from the unlabelled cells was used for the next passage. Following the first high multiplicity infection, only a single species of genome RNA sedimenting at 42S (presumably the wild-type genome RNA) was detected in purified virus (Fig. 2a). Upon second and third passages, however, three additional species of RNA were detected in the purified progeny virus (Fig. 2b, c). Concomitant with the appearance of these small RNA species was a corresponding loss of 42S RNA, indicating that the loss of infectivity seen in Fig. 1 was in part due to the loss of wild-type particles. The purified progeny particles from passage 2 were tested for interfering ability. Co-infection with fresh, wild-type virus resulted in a similar inhibition in the formation of infectious progeny and 42S intracellular RNA (data not shown, but similar to Fig. 1 and 2).

The three species of RNA present in the DI preparations (Fig. 2b, c) have sedimentation values of approx. 31S, 23S and 19S, as determined by comparison with ribosomal RNA markers. No qualitative difference in the sizes of the RNA was found upon repeated passages, or upon repeated experiments of undiluted passage with fresh wild-type virus, although the relative proportion of each species did tend to vary from one preparation to another. Attempts to isolate species of DI particles containing a single size class of RNA were unsuccessful.

**Preparation of purified species of DI RNA and analysis of their 5' termini**

DI particles labelled with $^{32}$PO$_4$ were purified from various passages and finally layered on to 15 to 30 % SDS-sucrose gradients for separation of the individual species of RNA. The three species of RNA were collected as indicated in Fig. 3(a) and further purified by velocity sedimentation (Fig. 3b to d). A portion of each purified RNA species was used for the analysis of their 5' termini and the remainder used for annealing studies.

In order to isolate the 5' termini, the three species of RNA were digested with KOH (Fig. 3b to d) and the digestion products analysed by chromatography on DEAE-cellulose (Fig. 4). The elution profile for all three products was similar. Most of the $^{32}$P eluted as mononucleotides with a net negative charge of approx. $-2$. A portion of each (0·1 % for the 31S, 0·2 to 0·25 % for both 23S and 19S) eluted with a net negative charge of approx. $-4·5$, presumably containing the 5' terminal nucleotide. The materials eluting at $-4·5$
Defective interfering particles of VSV

Fig. 3. Isolation of individual size classes of RNA following undiluted passage of VSV. (a) Size classes of RNA produced following third undiluted passage. The indicated fractions were collected, and the RNA precipitated in ethanol. (b) Sedimentation profile of the RNA collected from fractions 9 to 12 in (a). (c) Sedimentation profile of the RNA collected from fractions 16 to 20 in (a). (d) Sedimentation profile of the RNA collected from fractions 22 to 25 in (a). Sedimentation was in 15 to 30% (w/v) sucrose-SDS at 20,500 rev/min for 17 h, except for (d) at 33,000 rev/min. Sedimentation is from right to left.

Fig. 4. DEAE-cellulose chromatography of the products of alkaline digestion of the RNA isolated from DI particles. Purified DI RNA species were digested with KOH, and analysed by DEAE-cellulose chromatography as described in Methods. (a) Analysis of 31 S RNA (Fig. 3 b). (b) Analysis of 23 S RNA (Fig. 3 c). (c) Analysis of 19 S RNA (Fig. 3 d).

charge were pooled, desalted by dialysis and analysed by enzymatic digestions followed by high voltage paper electrophoresis. When analysed by paper electrophoresis, the 5' terminal material of the 31 S DI RNA migrated ahead of the pppA marker (Fig. 5 a). For further characterization, portions of the material were digested with either P1 nuclease, an enzyme which degrades RNA to 5' mononucleotides and also contains a 3'-phosphatase activity (Abraham, Rhodes & Banerjee, 1975), or bacterial alkaline phosphatase. As shown in
Fig. 5. Paper electrophoretic analysis of enzymatic digests of the 5'-terminal fragments of DI particle RNA. The radioactive material eluting between -4 and -5 (Fig. 4) was isolated and analysed by high voltage electrophoresis. (a) Undigested KOH product; (b) digestion with nuclease P₁; (c) digestion with alkaline phosphatase.

Fig. 5(b), 67% of the labelled products resulting from P₁ nuclease treatment migrated with the ppA marker, and 33% as Pi. Digestion of the original material with bacterial alkaline phosphatase released all the converted radioactivity as Pi (Fig. 5c). These results show that the 5' termini of the 31S DI RNA is probably ppAp..., since digestion with P₁ nuclease released one-third of the radioactivity as Pi, and two-thirds as ppA (Fig. 5b). Identical results were obtained when 23S and 19S DI RNA were analysed as above (data not shown).

**Determination of homology of DI RNA species**

Samples of each size class of the DI RNA (Fig. 3b to d) were tested for ribonuclease resistance prior to annealing with virus messenger or genome RNA (Table 1). It was found that the 31S and 23S DI RNA size classes remained totally sensitive to pancreatic ribonuclease, indicating that the RNA species were single stranded. However, the 19S RNA size class did exhibit considerable resistance to ribonuclease which varied from one preparation to another, but was always a minimum of approx. 40%, indicating complementary strands were present in the 19S preparation. The 19S DI RNA was rendered completely sensitive to
Defective interfering particles of VSV

Table 1. Ribonuclease resistance of the DI RNA species

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acid-precipitable</th>
<th>% resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>31S RNA</td>
<td>4100</td>
<td></td>
</tr>
<tr>
<td>+ Pancreatic RNase (25 µg/ml)</td>
<td>28</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>+ KOH</td>
<td>24</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>23S RNA</td>
<td>3400</td>
<td></td>
</tr>
<tr>
<td>+ Pancreatic RNase (25 µg/ml)</td>
<td>42</td>
<td>1%</td>
</tr>
<tr>
<td>+ KOH</td>
<td>34</td>
<td>1%</td>
</tr>
<tr>
<td>19S RNA</td>
<td>4150</td>
<td></td>
</tr>
<tr>
<td>+ Pancreatic RNase (25 µg/ml)</td>
<td>1675</td>
<td>40%</td>
</tr>
<tr>
<td>+ KOH</td>
<td>31</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>+ Denatured in 90% DMSO and pancreatic RNase digested</td>
<td>367</td>
<td>4.5%</td>
</tr>
</tbody>
</table>

* Designated DI RNA samples were treated as described in the Table.
† Pancreatic RNase digestion was carried out as described elsewhere (Moyer & Banerjee, 1975)
‡ Alkaline hydrolysis consisted of incubation at 37 °C for 16 h in 0.3 M-KOH.
§ (CH₃)₃SO denaturation was for 30 min at 37 °C.

Table 2. Annealing of the DI RNA species with VSV mRNAs

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Acid-precipitable</th>
<th>% RNase resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>42S RNA</td>
<td>2050</td>
<td>—</td>
</tr>
<tr>
<td>+ Sat. 12 to 18S mRNA†</td>
<td>828</td>
<td>40%</td>
</tr>
<tr>
<td>+ Sat. 31S mRNA</td>
<td>812</td>
<td>40%</td>
</tr>
<tr>
<td>+ 12 to 18S; 31S mRNA</td>
<td>1582</td>
<td>78%</td>
</tr>
<tr>
<td>31S DI RNA</td>
<td>2911</td>
<td>—</td>
</tr>
<tr>
<td>+ Sat. 12 to 18S mRNA</td>
<td>1685</td>
<td>58%</td>
</tr>
<tr>
<td>+ Sat. 31S mRNA</td>
<td>1060</td>
<td>36%</td>
</tr>
<tr>
<td>23S DI RNA</td>
<td>4022</td>
<td>—</td>
</tr>
<tr>
<td>+ Sat. 12 to 18S mRNA</td>
<td>1521</td>
<td>38%</td>
</tr>
<tr>
<td>+ Sat. 31S mRNA</td>
<td>2105</td>
<td>52%</td>
</tr>
</tbody>
</table>

* Annealing reactions were carried out as described in Methods.
† Sat. = saturating quantities of mRNA.

Ribonuclease when it was denatured with DMSO prior to digestion. These results indicate that the RNA contains extensive base pairing with complementary strands. Annealing experiments were carried out to determine whether the DI RNAs were complementary to virus mRNAs, and if so, which size class(es) they represented. Purified VSV mRNAs were isolated from infected HeLa cells and the unlabelled 31S and 12 to 18S virus mRNA size classes provided 40% protection of the 42S genome RNA, and these values were additive (Table 2). These results suggested that base sequences complementary to at least 80% of the genome RNA were present in the total mixture of the mRNA species.

A constant amount of ³²P DI RNA was annealed with increasing amounts of 31S virus mRNA (40 µg/ml) or 12 to 18S virus mRNA (20 µg/ml), and the amount of ribonuclease resistant product was determined (Table 2). The results showed that approx. 60% of the 31S DI RNA species was rendered ribonuclease resistant with saturating amounts of the 12 to 18S VSV mRNAs, and 40% to the 31S mRNA species. On the other hand, the 23S DI RNA species was 40% complementary to the 12 to 18S mRNA, and approx. 60% complementary to the 31S mRNA species.
DISCUSSION

Following serial undiluted passage of a cloned stock of vesicular stomatitis virus, defective interfering particles were generated and contained three different size classes of RNA. Since DI particles of different sizes were not separated in the present studies, it cannot be concluded at this stage whether the three classes of RNA are packaged in one DI particle or exist as individual entities. However, previous studies with the uncloned wild-type VSV strain generated a relatively homogeneous size class of defective particles containing a 21 S RNA species (Mudd & Summers, 1970; Leamnson & Reichmann, 1974). In contrast, the present study indicates that upon plaque purification of this wild-type strain, completely different types of DI particles were generated which contain 31 S, 23 S and 19 S RNA species. These results demonstrate that the generation of DI particles from a particular strain depends on the genetic history of the virus, and possibly also on the host cell used for propagation. Presumably, the uncloned wild-type VSV was contaminated by DI particles containing 21 S RNA which were amplified upon successive passages to yield a homogeneous class of the DI particles. The present data indicate that upon purification, the cloned stock of virus (presumably free of contaminating 21 S DI particles) will now generate DI particles of different sizes, the nature of which will depend on random processes by which DI particles are produced. The fact that our cloned VSV stock always produces the three varieties of DI particles suggests that this virus stock is now contaminated with these DI particles. Successive plaque purification of this cloned VSV will presumably result in DI particles by different random processes from the ones we observed. Similar results have been obtained in another laboratory (Lazzarrini et al. 1976; R. A. Lazzarrini, personal communication). The 31 S and 23 S RNA of the DI species were found to be complementary to both the 31 S and 12 to 18 S VSV mRNA species (Table 2). These results again differ from the previous reports (Leamnson & Reichmann, 1974; Stamminger & Lazzarrini, 1974), where it was shown that the majority of the DI RNA species from different stocks of VSV strains hybridized exclusively with the 31 S VSV mRNA species, with the exception of the heat-resistant DI RNA which hybridizes only with 12 to 18 S VSV mRNA species. These observations suggest that DI particles are produced in a more or less random manner and depend on the virus strains or the host cells.

Examination of the smallest class of DI RNA generated (19 S) revealed this species to be partially double-stranded (Table 1). Although the exact quantity of self-complementarity tended to vary from one preparation to another, 40 to 70 % of the sequences were always resistant to RNase digestion. The resistance was not simply due to adventitious virus mRNA adhering to the DI particles and annealing to the minus strands during phenol extraction because (1) denaturation of this RNA species did not result in release of 23 or 31 S RNA. (2) Only the 19 S class of DI RNA was found to be partially double-stranded and RNase treatment of the particles prior to isolation did not alter the RNase resistance of the 19 S DI RNA species. At this time we have not determined whether or not the complementary strands are packaged individually, or within the same nucleocapsid. The biosynthesis of this type of DI particle containing + and − strands packaged in the virion, is not clear and requires further investigation.

Analysis of the 5′ termini of the three size classes of RNA reveals that the terminal nucleotide to be identical to all three cases and consist of ppAp . . . . The γ-phosphate is presumably removed by the virion-associated phosphohydrolase (Roy & Bishop, 1971). A similar 5′ terminal nucleotide is also present at the 5′ terminus of the wild-type 42 S genome (Hefti & Bishop, 1975; Moyer et al. 1975). Further sequencing from the 5′ end will determine
whether this is coincidental, or whether extensive base homologies exist between the 42S
RNA and the DI particle RNAs. The results will throw light on the possible mechanism of
the DI RNA synthesis. Furthermore, the presence of 5' terminal polyphosphates suggest
that these RNA species are replicating molecules, and not simply the result of the random
nicking of a larger precursor RNA. Since the DI particles contain the same structural
proteins as the wild-type VSV, the above results indicate that the presumptive DI particle
replicase may be the same as the wild-type one and may recognize common sequences
present in both types of RNAs. Thus, it is possible that the 3' terminal sequence of the wild-type
genome is also conserved in the DI RNAs. Studies along this line are currently under
investigation.

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(Received 10 March 1976)