An RNA-dependent RNA Polymerase Associated with the Filamentous Nucleoproteins of Rice Stripe Virus

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SUMMARY

Filamentous particles of rice stripe virus (RSV) were found to be associated with an RNA-dependent RNA polymerase activity. The enzyme catalysed the synthesis in vitro of single- and double-stranded RNAs corresponding in size with RNA found in RSV particles, as well as two other dsRNAs with mol. wt. of 1.0 × 10^6 and 0.56 × 10^6. The RSV polymerase activity required Mg^{2+}, Mn^{2+} or Fe^{2+} but not Na^{+}, K^{+} or NH_4^{+} which were inhibitory at concentrations of more than 50 mM. The optimum pH of the polymerase was around 8.0 at the optimum temperature, 40 °C. Detergent treatment of RSV particles did not enhance the polymerase activity. Purified RSV particles contained a minor polypeptide (mol. wt. 230000) as well as coat protein; the former may be the RSV polymerase. The taxonomy of RSV is discussed in relation to enveloped animal viruses with negative-stranded RNA, which also contain an RNA-dependent RNA polymerase in virus particles.

INTRODUCTION

Rice stripe virus (RSV) is a planthopper-borne plant virus that causes serious damage to Japonica-type rice varieties. RSV particles are 8 nm-wide filaments which frequently fold to form pleomorphic or branched configurations and contain ssRNA (Toriyama, 1982a, b, 1983). Other planthopper-borne viruses, such as maize stripe virus, rice grassy stunt virus and rice hoja-blanca virus have similar particles (nucleocapsids) and biological properties (Gingery et al., 1981; Morales & Niessen, 1983; Toriyama, 1985; Hibino et al., 1985). When examined, purified particles of these viruses have been found to contain four or more segments of ssRNA (Toriyama, 1982a, b, 1985; Falk & Tsai, 1984) and dsRNA was found in maize stripe virus particles (Falk & Tsai, 1984).

When sedimented, filamentous particles of RSV form three components, which contain RNA species with mol. wt. of 0.9 × 10^6 and 1.0 × 10^6 (M component), 1.4 × 10^6 (B component) and 1.9 × 10^6 (nB component). The largest RNA might be necessary for the infectivity of RSV (Toriyama, 1982a, b).

When added to either wheat germ extract or rabbit reticulocyte lysate, RNA of RSV stimulates incorporation of [3H]leucine six- to tenfold (Toriyama, 1985). However, little of the polypeptide synthesized had the size of coat protein (unpublished data); this prompted a search for an RNA polymerase in RSV particles.

METHODS

Purification of virus. RSV was purified by a modification of the method previously described (Toriyama, 1982a). The homogenate of infected rice tissues was mixed with 10% (v/v) of Diflron S3 (1,1,2-trifluoro-1,2,2-trichloroethane; Daikin Co., Tokyo, Japan) and stirred for 5 min. After low-speed centrifugation, the extract was centrifuged at 123000 g for 1.5 h on a cushion of 20% sucrose in 0.01 M-phosphate buffer pH 7.5. The pellets were dissolved in 0.01 M-phosphate buffer pH 7.5 and the suspension was made to 30% saturation with ammonium sulphate. Impurities were removed by low-speed centrifugation and, after dilution about threefold with 0.01 M-
phosphate buffer pH 7.5, the solution was made 8% in polyethylene glycol 6000 (PEG) and stirred for 1 h in an ice-bath. The precipitate was collected by centrifugation at 30000 g for 30 min and dissolved in 0.01 M-phosphate buffer pH 7.5. Further impurities were removed by precipitation from 35% saturated ammonium sulphate and virus particles were recovered by PEG precipitation and dissolved in 0.01 M-phosphate buffer pH 7.5. After low-speed centrifugation, the virus suspension was centrifuged through a linear density gradient of 10 to 40% sucrose at 68900 g for 4 h. The M, B and nB components separated in the gradients and were recovered by centrifugation and dissolved in 0.01 M-Tris-HCl pH 7.5. Virus suspensions were mixed with glycerol to 30% (v/v) and kept at −20 °C. Such RSV preparations lost no significant polymerase activity for up to 4 months.

The amount of RSV was determined from the absorbance assuming 1 mg/ml RSV solution has an \( A_{260} \) of 4.4 (Toriyama, 1983).

**Assay of polymerase activity.** The complete reaction mixture in a total volume of 50 μl contained 50 mM-Tris-HCl pH 8.0, 8 mM-MgCl₂, 10 mM-2-mercaptoethanol, 1 mM each of ATP, CTP, GTP and 0.1 mM-UTP, 1 μCi [³H]UTP (Amersham) and the virus preparation (10 to 20 μg). After incubation for 1 h at 40 °C, the reaction was terminated by chilling in an ice-bath and addition of 2 ml 10% TCA containing 1 mM-sodium pyrophosphate and 50 μg bovine serum albumin. After at least 30 min at 0 °C, the mixture was filtered through Whatman GF/C filters which were washed successively with 10% TCA, 5% TCA and 95% ethanol and assayed for radioactivity by scintillation counting.

The pH of the reaction mixture was adjusted at the incubation temperatures.

**Analysis of the product.**

**Preparation of product RNAs.** After incubation for 1 h at 40 °C, the reaction mixture was mixed with SDS to 0.5% and extracted with an equal volume of 90% phenol containing 10 mM-ammonium acetate, 25 μg/ml wheat germ tRNA as carrier and 4 vol. cold ethanol. RNA was re-precipitated twice from 4 M-ammonium acetate and then dissolved in 10 mM-Tris-HCl pH 7.5.

**Conditions for RNase digestion of the product RNAs.** For the detection of RNase-resistant materials in the product, it was digested with 1 μg/ml RNase A (Sigma) in 1 mg/ml wheat germ tRNA and 0.3 M-NaCl for 20 min at 30 °C, conditions which digested ssRNA, but not dsRNA, or a similar mixture containing only 0.01 M-NaCl, in which dsRNA was also digested (Zelcer et al., 1981). After the RNase treatment, the mixture was extracted with phenol and mixed with 2 vol. ethanol.

**Electrophoresis.** Polyacrylamide gels (2.5%) containing 8 M-urea (8 M-urea gel) were prepared as described (Loening, 1967; Reijnders et al., 1974) and loaded with RNA giving 10000 to 20000 c.p.m./lane. The dsRNA of rice dwarf virus was used as molecular weight markers (Reddy et al., 1974). After electrophoresis for 4-5 h at 120 V, gels were stained with 0.2% toluidine blue, destained, processed for fluorography according to Laskey & Mills (1975) and exposed to Kodak XAR-5 film at −70 °C for 2 days or more.

In some experiments, the product RNAs were glyoxalated and electrophoresed on 2.5% polyacrylamide gel for 4 h at 100 V (Carmichael & McMaster, 1980). The electrophoresis running buffer was replaced each hour. After electrophoresis, the gel was processed like the 8 M-urea gel. For molecular weight determinations, ribosomal RNA of *Escherichia coli* (mol. wt. 1.03 × 10⁶ and 0.55 × 10⁶) was used.

**Analysis of the protein.** The protein content of purified RSV components was analysed by electrophoresis in 7.5% polyacrylamide gels. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R and destained (Laemmli, 1970). The molecular weight marker protein standard kit was purchased from Bio-Rad.

**RESULTS**

**Properties of RSV polymerase activity**

Incubation of RSV particles in the complete reaction mixture led to the incorporation of [³H]UMP into material insoluble in TCA. In a typical experiment the amount of radioactivity incorporated was about 30000 to 40000 c.p.m. M, B and nB components were all active. Omission of the nucleoside triphosphates or the inclusion of RNase A at 50 μg/ml completely abolished activity but neither actinomycin D nor rifampicin, each at 100 μg/ml, inhibited incorporation. S-Adenosyl methionine at 2 mM did not affect incorporation but DNase, thought to be free of RNase, at 50 μg (125 U)/ml inhibited incorporation 27% and a less pure grade of DNase inhibited incorporation 90%. Both results may have been caused by RNase contamination of DNase. NP40 at 0.3% had no effect on the activity, whereas although sodium deoxycholate had no effect at 0.03% it abolished activity at 0.1% or higher concentrations.
Fig. 1 shows that incorporation was proportional to the amount of RSV in the reaction mixture between 2.5 and 37.5 μg. However, the incorporation in a reaction containing 50 μg RSV was 465 pmol which suggests that the enzyme was saturated. The polymerase activity per unit weight RSV protein in different preparations of RSV differed and seemed to depend on the condition of the plant material from which the virus had been purified.

Requirements for divalent and monovalent cations

The incorporation of [3H]UMP was dependent on Mg²⁺, Mn²⁺ or Fe²⁺ being in the reaction mixture. The optimum concentrations were 8 to 10 mM-Mg²⁺, 1 mM-Mn²⁺ and 10 to 12 mM-Fe²⁺ (Fig. 2a, b, c). The activity at the optimum concentration of Mn²⁺ was higher than that attainable in Mg²⁺ and the addition of 0.1 to 1.0 mM-Mn²⁺ to the complete reaction mixture containing 8 mM-Mg²⁺ enhanced the polymerase activity to the level found with 1 mM-Mn²⁺ alone. Neither Ca²⁺ nor Cu²⁺ could activate the [3H]UMP incorporation at concentrations of 1, 3 or 10 mM.
Fig. 3. Kinetics of $[^{3}H]$UMP incorporation at different temperatures: 26 °C (○), 30 °C (●), 34 °C (□), 38 °C (■), 42 °C (△) and 46 °C (▲).

**Table 1. Effects of monovalent cations on the incorporation of $[^{3}H]$UMP by the RSV polymerase**

<table>
<thead>
<tr>
<th>Salt</th>
<th>0 mM</th>
<th>50 mM</th>
<th>100 mM</th>
<th>150 mM</th>
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<td>32.7</td>
<td>27.3</td>
<td>12.5</td>
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<tr>
<td></td>
<td>42.8</td>
<td>42.2</td>
<td>31.0</td>
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<td>ND</td>
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<tr>
<td>NH$_4$Cl</td>
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<td>39.0</td>
<td>35.0</td>
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<td>ND</td>
</tr>
<tr>
<td>KCl</td>
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<td>32.6</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>37.4</td>
<td>31.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CH$_3$COOK</td>
<td>40.9</td>
<td>39.7</td>
<td>32.6</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, Not determined.

Monovalent cations Na$^+$, K$^+$ and NH$_4^+$ did not enhance the polymerase activity and were inhibitory at concentrations of more than 50 mM (Table 1).

Analysis by gel electrophoresis of the products synthesized *in vitro* showed that those made in 8 mM-Mg$^{2+}$, 1 mM-Mn$^{2+}$ or 8 mM-Mg$^{2+}$ with 50 mM-Na$^+$ added, differed little but that products made in 8 mM-Fe$^{2+}$ contained relatively little RNA corresponding to RNA 2 and RNA 3 of RSV.

**pH optimum and temperature optimum**

At 35 °C incubation, the incorporation began to be activated at around pH 7.3 and the optimum pH was 7.6. However, the optimum shifted to around pH 8.0 at 40 °C. Kinetic analysis of RSV polymerase activity was performed for 30 min at each of six different temperatures between 26 and 46 °C. The optimum temperature was about 40 °C; activity was suppressed at over 40 °C. The highest incorporation of $[^{3}H]$UMP was obtained at 38 °C after incubation for 1.5 h (Fig. 3).

**The putative polypeptide of RSV polymerase**

Purified preparations of RSV contain, in addition to coat protein (32K), small amounts of a polypeptide of mol. wt. 230K (Fig. 4). This polypeptide was detected in particles of M, B and nB components. RSV preparations, purified by a method used previously (Toriyama, 1982a), also
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Fig. 4. Polyacrylamide gel electrophoresis of protein from particles purified using chloroform (Toriyama, 1982a) (lanes 1 to 3) or purified as described in this paper (lanes 4 to 6). Lanes 1 and 4, M components; lanes 2 and 5, B components; lanes 3 and 6, nB components. The arrow indicates the minor peptide of mol. wt. 230K; CP, coat protein of RSV (32K).

Fig. 5. Product RNAs synthesized in vitro by the RSV polymerase, analysed in 2.5% polyacrylamide gel containing 8 M-urea. (a) Products synthesized in vitro using the B component of RSV which contained RNAs 2, 3 and 4. The positions of these RNAs, shown by toluidine blue staining, are indicated as 2, 3 and 4 on the left side. (b) After the in vitro synthesis, the products were digested with RNase A in 0.3 M-NaCl (lanes 1 to 4) or 0.01 M-NaCl (lanes 5 to 8). The concentration of RNase A in the digestion mixture was 0 (lanes 1 and 5), 0.1 (lanes 2 and 6), 0.3 (lanes 3 and 7) or 1.0 μg/ml (lanes 4 and 8). The mol. wt. of bands a to c are given in the text. Lane 9, dsRNAs of rice dwarf virus, stained with toluidine blue. The mol. wt. (×10^6) from the top are 3.1, 2.5, 1.9, 1.8, 1.15 (1.13), 0.86, 0.78, 0.75 and 0.52 (0.52) (Reddy et al., 1974).

had polymerase activity, but it was three- to fourfold lower than in the preparations described in this paper. The 230K polypeptide was also found in both types of preparation, but was more prominent in the more active preparations purified by the present method (Fig. 4).

**ss- and dsRNAs synthesized in vitro**

Fig. 5(a) shows the products of a polymerase reaction analysed in a gel containing 8 M-urea. The major bands corresponded in position to each species of RSV RNA and much labelled material remained at the top of the gel. The major bands disappeared completely following RNase treatment, suggesting that they are ssRNA (Fig. 5b). In contrast, minor bands (a, b and c) in the untreated products (Fig. 5a) were not digested by RNase in 0.3 M-NaCl but were digested in 0.01 M-NaCl. The mol. wt. of these presumed dsRNAs were 2.7 × 10^6, 2 × 10^6 and 1.6 × 10^6 respectively, which are twice the sizes of ssRNAs 2, 3 and 4 of RSV. Also, two other RNase-resistant discrete bands (d and e), mol. wt. 1.0 × 10^6 and 0.56 × 10^6, appeared when the products were treated with RNase A. These two dsRNAs seemed to arise from the digestion of the major bands in Fig. 5(a).

**Analysis of product RNAs denatured by glyoxalation**

To determine whether the ssRNAs synthesized in vitro, observed in the 8 M-urea gel, were genome-length RNAs or replicative intermediates containing short newly synthesized RNA molecules, the product RNAs were analysed after denaturation with glyoxal. That glyoxalated dsRNAs of rice dwarf virus migrated as ssRNAs was determined by comparisons with the migration of RSV RNAs 2, 3 and 4 and the ribosomal RNAs of *E. coli* (Fig. 6a, lane 1). Glyoxal-denatured product RNAs migrated in bands which corresponded in position with RSV RNAs 2, 3 and 4 (Fig. 6a, b, lanes 2, 4 and 5). In addition, a dense band corresponding to a mol. wt. of about 0.5 × 10^6 (Fig. 6b, lanes 2, 3 and 5) and often, but not always, a less distinct band corresponding to a mol. wt. of 0.28 × 10^6 were seen. It is possible that these RNAs originated
Fig. 6. Product analysis, after denaturation with glyoxal, by electrophoresis in 2.5% polyacrylamide gel in 0.01 M-sodium phosphate buffer. (a) Stained with toluidine blue, (b) 3H-labelled newly synthesized products. Lanes 1, RNAs of rice dwarf virus (see Fig. 5); lanes 2, 3 and 5, products synthesized in vitro; lanes 3, RNase-digested in 0.3 M-NaCl; lanes 4, RSV RNA. Positions of RNAs 2, 3 and 4 are shown on the right side; d' and e', mol. wt. about $0.5 \times 10^6$ and $0.28 \times 10^6$, respectively.

Fig. 7. (a) RNAs of M, B and nB components of RSV, separated in 2.5% polyacrylamide gel containing 8 M-urea and (b) the products synthesized in vitro by each component. After long exposure, three minor bands (a, b and c in Fig. 5) were visible.

from the dsRNAs of mol. wt. $1.0 \times 10^6$ and $0.56 \times 10^6$ which were observed in the RNase-treated products. Although samples containing almost the same amount of dsRNAs were loaded on lanes 2, 3 and 5 in Fig. 6, only weak bands were seen in the positions of RSV RNAs 2, 3 and 4 in the glyoxal-denatured product dsRNA (Fig. 6b, lane 3). It seems that the genome-size RNAs synthesized in vitro came from the ssRNAs, which were synthesized in vitro and RNase-sensitive, but not from the glyoxal-denatured dsRNAs of genome size.

These results suggest strongly that ssRNAs with the full length of genome-size RNAs were synthesized in vitro by the RSV polymerase.

The products synthesized by M, B and nB components

The components of RSV used in these experiments had not been purified by more than one cycle of sucrose density gradient centrifugation and were therefore contaminated, particularly by slower sedimenting components.

Fig. 7(a) shows that all three components contained RNAs 2, 3 and 4, but that the M component contained relatively little RNA 2 and that only the nB component contained RNA 1, as described before (Toriyama, 1982a). Fig. 7(b) shows clearly that the products synthesized in vitro
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Fig. 8. The products of synthesis in vitro, analysed in 2.5% polyacrylamide gel containing 8 M-urea. The mixture was unseparated (lanes 1 and 2) or separated by centrifugation into pellet fraction (lanes 3 and 4) and supernatant fraction (lanes 5 and 6). The samples in lanes 2, 4 and 6 were digested with RNase A in 0.3 M-NaCl.

vitro by the B component contained relatively large amounts of newly synthesized RNA 2, compared with the M component products, and that the products of the nB component contained RNA 1 synthesized in vitro. These results show that each RNA species in the M, B and nB components can act as a template for the RSV RNA polymerase.

Association of the RNAs synthesized in vitro with RSV nucleoproteins

To separate RSV nucleoproteins from the incubated reaction mixture, it was centrifuged for 1.5 h at 117,800 g. More than 90% of the incorporated radioactivity was pelleted by this procedure. Fig. 8 (lanes 3, 4) shows the labelled RNA in the pellet fraction before and after RNase treatment. No great difference was found between unfractionated product and the pellet fraction product, except that the dsRNA of mol. wt. 2.6 × 10^6 seemed to be slightly more abundant in the latter. However, no bands were detected in the supernatant fraction (Fig. 8, lanes 5, 6). These results show that most of the RNAs synthesized in vitro associated tightly with RSV nucleoproteins even after the completion of viral and complementary RNA synthesis.

DISCUSSION

The rate of incorporation of [3H]UMP per unit weight of RSV protein is close to that reported for vesicular stomatitis virus (Baltimore et al., 1970). The RSV polymerase absolutely requires Mg^{2+}, Mn^{2+} or Fe^{2+} for enzyme activation. Mn^{2+} is required for activity by the polymerases of influenza virus (Bishop et al., 1971), Pichinde virus (Carter et al., 1974), Lumbo virus (Bouloy & Hannoun, 1976) and Uukuniemi virus (Ranki & Pettersson, 1975) but inhibits the polymerases of vesicular stomatitis virus, Sendai virus (Robinson, 1971), lettuce necrotic yellows virus (Toriyama & Peters, 1980) and broccoli necrotic yellows virus (Toriyama & Peters, 1981). Mn^{2+} added to Mg^{2+} enhanced the incorporation by RSV polymerase as shown with Lumbo virus (Bouloy & Hannoun, 1976) and Mn^{2+} was dominant in the activation of RSV polymerase. As with Lumbo virus (Bouloy, 1978), high concentrations of Na^+ and other monovalent cations inhibited RSV polymerase. The optimum temperature for RSV polymerase was about 40°C and similarly high optimum temperatures are found with Lumbo virus, Uukuniemi virus and Pichinde virus (Bouloy & Hannoun, 1976; Ranki & Pettersson, 1975; Carter et al., 1974).

These general features of RSV polymerase suggested that it is a virion-associated enzyme rather than a polymerase from infected tissue, such as that of cowpea mosaic, turnip yellow
mosaic and brome mosaic viruses (Dorssers et al., 1984; Mouches et al., 1984; Hardy et al., 1979).

Protein from purified RSV particles contained a 230K peptide as well as coat protein. The relative amount of the 230K polypeptide and the polymerase activity of the particles were less in particles purified using 20% chloroform (Toriyama, 1982a). The 230K polypeptide therefore seems to be a likely candidate for being the RSV polymerase. Such a polypeptide could be coded for by $1.9 \times 10^6$ RNA, which is contained in the nB component of RSV, the component considered to be essential for RSV infectivity (Toriyama, 1982a).

As shown in Fig. 7, each RSV component (M, B and nB) had polymerase activity and produced RNA corresponding in size to the RNA in the particles. This further suggests that RSV polymerase is associated closely with each nucleoprotein component of RSV.

The nucleoprotein complexes which were obtained from vesicular stomatitis virus (Szilágyi & Uryvayev, 1973), Sendai virus (Robinson, 1971), Lumbo virus (Bouloy, 1978) and Pichinde virus (Leung et al., 1979) have polymerase activity. In vesicular stomatitis virus the L and NS proteins, which constitute the polymerase, remain in the nucleoprotein complex even after the disruption of virions with Triton N-10 and CsCl. No detectable loss of the enzyme activity occurred during the disruption of virus particles (Szilágyi & Uryvayev, 1973). The filamentous particle-associated polymerase of RSV appears to be analogous to the polymerase activity in nucleocapsids of enveloped animal viruses. Of the plant rhabdoviruses, only lettuce necrotic yellows and broccoli necrotic yellows viruses are known to possess a virion-associated polymerase activity (Toriyama & Peters, 1980, 1981).

Animal viruses with virion-associated polymerase and ssRNA genomes are all enveloped and have negative-stranded RNA (Bishop, 1977). RSV has filamentous structures which resemble the structures of nucleocapsids isolated from enveloped arenavirus and bunyavirus particles (Bishop, 1977; Bishop & Shope, 1979; Rawls & Leung, 1979) and RSV RNA translates poorly in vitro. These observations seem to suggest that RSV, which has an associated RNA-dependent RNA polymerase, might be a negative-strand virus. An ambisense genome, which has been found in Pichinde virus (Auperin et al., 1984) and Punta Toro virus (Ihara et al., 1984), is also a possibility for RSV.

In maize stripe virus, which is serologically related to RSV, ss- and dsRNAs were detected in the purified nucleoproteins and it was suggested that negative- and positive-sense RNAs might be separately encapsidated (Falk & Tsai, 1984). The ss- and dsRNAs newly synthesized in vitro by RSV polymerase were associated closely with RSV nucleoproteins, which suggests another possible explanation of why ss- and dsRNAs were detected in maize stripe virus nucleoproteins.

In a preliminary study, it was found that rice grassy stunt virus also has an RNA-dependent RNA polymerase activity in the nucleocapsid-like particles (S. Toriyama, unpublished data). It is possible that RSV, rice grassy stunt virus and other similar viruses are defective forms of spherical enveloped viruses such as tomato spotted wilt virus (Je, 1982), but equally they might be related evolutionarily with the enveloped viruses possessing an RNA-dependent RNA polymerase inside their virions.

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