In vitro Transcription by RNA Polymerase Associated with Rice Gall Dwarf Virus

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(Accepted 7 November 1983)

SUMMARY
An RNA-dependent polymerase associated with rice gall dwarf virus (RGDV), a new Phytoreovirus, was characterized. Optimum polymerase activity was found between pH 8.0 and 8.5 at 25 °C. Mg2+ was essential for the activity, the optimum concentration being 2 mM. Mn2+ could replace Mg2+. The RNA products synthesized in vitro hybridized with RGDV RNA, but not with RNA of rice dwarf virus which is in the same subgroup of plant reoviruses. Electrophoretic analysis of the products showed at least nine bands, the sizes of which are discussed.

INTRODUCTION
Rice gall dwarf virus (RGDV), recently found in Thailand, is a member of the plant reovirus group (Phytoreovirus) (Omura et al., 1982). The virus particles contain 12 double-stranded RNA (dsRNA) segments with molecular weights ranging from 0·3 × 10^6 to 3·1 × 10^6 (470 bp to 4800 bp) (Hibi et al., 1981).

One of the characteristic properties of members of the reovirus group is to have virion-associated RNA polymerase activity which transcribes single-stranded RNA from viral dsRNA. Rice dwarf virus (RDV) and wound tumour virus (WTV) have been reported to have the virion-associated enzyme (Kodama & Suzuki, 1973; Black & Knight, 1970).

In this paper, we describe the properties of an RNA polymerase activity associated with RGDV particles and of its reaction products.

METHODS
Virus, insects and plants. The source of RGDV, rice plants (cultivar Taichung Native I), the propagation of the virus in the plants using viruliferous Nephotettix nigropictus and the growing conditions of the infected plants were as described by Omura et al. (1982).

Virus purification. RGDV and RDV were purified by the method of Omura et al. (1982), except that virus particles were precipitated with 6% (w/v) polyethylene glycol and 0·3 M-NaCl containing 1% Triton X-100. The final pellets were resuspended in 0·1 M-glycine, 0·01 M-MgCl2, adjusted to pH 7·0 with 0·1 M-NaOH. Virus concentration was estimated spectrophotometrically using A260nm = 60 for 1 mg/ml of virus (the value for RDV) (Kodama & Suzuki, 1973).

Assay for RNA polymerase activity. The suspension of virus, stored at −80 °C, was thawed at 37 °C, and the particles were pelleted by centrifugation at 25,000 × g for 15 min using a Beckman Airfuge. They were resuspended to make the appropriate concentration just prior to the assay of enzyme activity. The standard mixture, 50 μl, contained 100 mM-Tris–HCl (pH 8·5), 2 mM-MgCl2, 2 mM-ATP, 0·2 mM-GTP, 0·2 mM-CTP, 0·045 mM-[5-3H]UTP (sp. act. 0·22 Ci/mmol), 2·8 mM-phosphoenol pyruvate, 40 μg/ml pyruvate kinase, 240 μg/ml bentonite and 0·5 mg/ml purified RGDV. The reaction mixtures were incubated at 25 °C for 30 min. The reaction was stopped by adding 1 ml of 5% trichloroacetic acid (TCA) followed by standing on ice for 30 min. The precipitate was collected by filtration on glass fibre filters (Whatman GF/C), washed with cold TCA, dried and counted in a liquid scintillation spectrometer.

Extraction of dsRNA and radioactive RNA products. dsRNA was extracted from purified RGDV and RDV particles using phenol–SDS. In order to obtain radioactive RNA products, the reaction mixture for analysing the enzyme activity containing [5-3H]UTP or [α-32P]UTP was incubated at 25 °C for 2 h, and SDS was added to a concentration of 0·5% after removal of bentonite by centrifugation at 10,000 rev/min for 2 min. RNA products

Key words: RGDV/RNA polymerase/dsRNA
were extracted once with water-saturated phenol and three times with ether, and then precipitated with 2 vol. ethanol at −20 °C. The precipitate was dissolved in water.

Hybridization technique. dsRNA from RGDV or RDV was denatured by heating at 100 °C for 5 min in 50% formamide. The denatured RNA and about 4000 cts/min of 3H-labelled RNA product were incubated in 4 × SSC buffer (1 × SSC: 0.15 M-NaCl and 0.015 M-sodium citrate) containing 50% formamide at 42 °C for 2 h. The extent of hybridization is expressed as the percentage of 3H-labelled RNA that remained TCA-precipitable after a 60 min incubation with 25 μg/ml of RNase A in 2 × SSC at 37 °C.

Denaturation of dsRNA by glyoxal. dsRNA of RGDV was denatured with glyoxal essentially according to the method of McMaster & Carmichael (1977). RNA was dried by a concentrator (EC-10, Tomy Seiko Co., Ltd), and dissolved in a mixture containing 21 μl of dimethyl sulphoxide, 5 μl of 40% glyoxal purified by passing through ion-exchange resin just prior to use, 1 μl of 1 M-sodium phosphate buffer (pH 7.2) and 15 μl of water. The reaction mixture was sealed in a glass tube and incubated at 50 °C for 1 h. After incubation, 10 μl of 1 M-potassium acetate solution and 120 μl of cold ethanol were added and the mixture was allowed to stand overnight at −20 °C. The precipitate was collected by centrifugation and dried.

Gel electrophoresis of RNA. Composite gels consisting of 2.0% acrylamide and 0.4% agarose in Tris-acetate buffer (pH 7.8) containing 2 mM-Na2EDTA (Loening, 1967) were used for analysis of transcribed 32P-labelled RNA products, purified dsRNA and glyoxal-treated RNAs. Electrophoresis was carried out at 10 V/cm at 4 °C. Gels for RNA not treated with glyoxal were stained with 3 μg/ml of ethidium bromide, and examined on a short-wavelength u.v. transilluminator and photographed. Gels for single-stranded RNA denatured by glyoxal were stained with silver (Bio-Rad).

Chemicals. [5-3H]UTP and [α-32P]UTP were obtained from Amersham. Nucleoside triphosphates, phosphoenol pyruvate and pyruvate kinase were purchased from Boehringer Mannheim, α-amanitin from Boehringer Ingelheim, yeast RNA and RNase A from Sigma, DNase I from P-L Biochemicals (cat. no. 0512), and mixed bed ion-exchange resin, AG 501-X8, from Bio-Rad. All other chemicals used were reagent grade, and distilled water was sterilized for all experiments. Phenol was used after distillation. Rifampicin and cucumber mosaic virus (CMV) RNA were the kind gifts of Dr F. Sakai and Dr K. Hanada, respectively.

RESULTS

Association of RNA polymerase activity with RGDV particles

On centrifugation in a 10 to 40% sucrose density gradient, RGDV particles separate into three bands. Incorporation of nucleotides (polymerase activity) was detected in the middle and lower bands (Fig. 1 a); no activity was detected in the upper band which included empty particles (data not shown). When the particles in the middle band were further subjected to quasi-equilibrium centrifugation in a 40 to 60% sucrose gradient, a single band, which corresponded to the main polymerase activity, was obtained (Fig. 1 b).

Characterization of RNA polymerase activity

The activity of the polymerase was not significantly affected by transcriptional inhibitors such as α-amanitin, actinomycin D or rifampicin (Table 1). Addition of RNase A to the reaction mixture without bentonite markedly inhibited the synthesis of the products, but DNase did not. This suggests that it is an RNA-dependent RNA polymerase.

The polymerase activity was significantly affected by the pH of 0.1 M-Tris–HCl, with 0.01 M-MgCl₂, the optimum pH being 8.0 to 8.5; the optimum temperature, tested over the range 20 to 45 °C, was 25 °C. The polymerase activity required divalent cations, the optimum concentration of Mg²⁺ being 2 mM. Mg²⁺ could be replaced by Mn²⁺, of which the optimum concentration was also 2 mM. In this experiment, we could not eliminate a combined effect with Mg²⁺, because the virus stock solution carried 0.5 mM-Mg²⁺ into the reaction mixture.

The time course of the reactions using various amounts of RGDV particles (Fig. 2) shows that the polymerase activity increased in proportion to the amount of RGDV in the reaction mixture. Fig. 2 also shows the time course of incorporation by RDV particles. Even though the optimum temperature of activity for RDV is 35 °C (Kodama & Suzuki 1973), its activity was much higher than that of RGDV at 25 °C.

Characterization of RNA products synthesized in vitro

In order to determine whether the products synthesized in vitro were transcripts from dsRNA, 3H-labelled products were hybridized to virus RNA. As shown in Fig. 3, the RNA products
Fig. 1. Cosedimentation of RGDV particle and RNA polymerase activity. (a) The crude virus preparation was layered on a 10 to 40% (w/v) linear sucrose gradient in Gly-Mg (0.1 M glycine, 0.01 M MgCl$_2$, pH 7.0) and centrifuged for 50 min at 20000 rev/min in a Hitachi RPS-27 rotor. Fractions (approx. 1 ml) were assayed, and the absorbance at 260 nm (○) and polymerase activity (●) were determined. (b) Fractions from the middle band in (a) were recovered, layered on a 40 to 60% (w/v) linear sucrose gradient in Gly-Mg and centrifuged for 15 h at 16000 rev/min in a Hitachi RPS-27 rotor. Fractions (approx. 1 ml) were assayed as above. Sedimentation was from left to right (arrows).
Fig. 2. Time courses of RNA synthesis. To the standard reaction mixtures, various amounts (in μg) of RGDV (●) or 96 μg of RDV (○) were added. The inset represents RNA syntheses over a 60 min period with RGDV added at the amounts noted.

Table 1. Characteristics of the RNA polymerase activity associated with RGDV

<table>
<thead>
<tr>
<th>Reaction mixture*</th>
<th>Activity (%)†</th>
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<tbody>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>Complete - bentonite</td>
<td>100</td>
</tr>
<tr>
<td>+ α-amanitin, 9.2 × 10⁻³ μg/ml</td>
<td>103</td>
</tr>
<tr>
<td>+ α-amanitin, 9.7 μg/ml</td>
<td>98</td>
</tr>
<tr>
<td>+ actinomycin D, 10 μg/ml</td>
<td>97</td>
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<td>+ DNase, 10 μg/ml</td>
<td>85</td>
</tr>
<tr>
<td>+ DNase, 50 μg/ml</td>
<td>81</td>
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* Standard assay conditions were used as described in Methods.
† The value is expressed as the percentage of the activity in the complete system; in the case of nuclease treatments, reaction mixture without bentonite was used as the complete system.
Fig. 3. Hybridization of the RNA products with RGDV and RDV RNA. 3H-labelled RNA products and denatured RGDV (●) or RDV (○) RNA, as well as yeast RNA (△) as control, were annealed. The samples were then assayed for RNase resistance.

Fig. 4. Analysis of RNA products synthesized in vitro. 32P-labelled RNA products were synthesized as described in Methods. The electrophoresis was carried out in a 2-0% acrylamide-0.4% agarose composite slab gel. Lane (a) contained dsRNA of RGDV. Lane (b) contained 32P-labelled RNA product. Exposure time was 4 h (b) or 16 h (b'). Dots indicate positions of bands. The mol. wt. × 10⁻³ of CMV RNA standards (RNA 3: 850000; RNA 4: 350000; RNA 5: 120000) are shown by arrows. Lane (c) contained 32P-labelled product treated with glyoxal. Bars indicate positions of 11 bands of glyoxal-denatured RGDV RNA.

hybridized with RGDV RNA, but not with RDV RNA nor with yeast RNA. The results confirmed that the RNA products were transcribed from dsRNA encapsidated within the virus particles.

When the 32P-labelled product was analysed by gel electrophoresis, we detected at least nine bands, whose molecular weights, estimated using CMV RNAs as standards, ranged from 0-096 × 10⁶ to 1-52 × 10⁶ (Fig. 4, lane b); the estimate of 1-52 × 10⁶ involves an extrapolation from the standards. We also found two faint but distinct bands, when exposure time for autoradiography was prolonged (Fig. 4, lane b'). The sizes of these bands were apparently greater than those expected to be transcribed from the largest segment of RGDV RNA.

The sizes of the RNA products showed no correspondence with those of RGDV RNA reported by Hibi et al. (1981). As McMaster & Carmichael (1977) reported that glyoxal denaturation is a reliable method for the molecular weight estimation of single- and double-stranded nucleic acids, dsRNA obtained from virus particles and the RNA products were denatured with glyoxal and compared. As shown in Fig. 4, lane (c), at least five bands of the RNA products detected on the gel corresponded to denatured virus RNA segments.

**DISCUSSION**

Three viruses, WTV, RDV and RGDV, have so far been grouped as Phytoreoviruses and the properties of the RNA polymerase associated with two of them, WTV and RDV, have been reported (Black & Knight, 1970; Reddy et al., 1977; Kodama & Suzuki, 1973). In this paper, we demonstrate that RGDV also has an RNA polymerase associated with the virus particles, and show that the properties of the enzyme are similar to those of WTV and RDV. However, there
appear to be some differences. The optimum activity of RGDV is at 25 °C whereas that of RDV is at 35 °C (Kodama & Suzuki, 1973). RGDV also appears to have much less activity per unit weight of virus particles than does RDV. Divalent cations are indispensable for the RNA polymerase activity. The optimum concentration of Mg$^{2+}$ for RGDV (2 mM) is lower than that reported for other viruses belonging to the reovirus group (Shatkin & Sipe, 1968; Lewandowski et al., 1969; Reddy et al., 1977). We could not recover the intact virus particles by centrifugation at this low magnesium concentration (data not shown): this suggests that the divalent cation at low concentration not only stimulates the polymerase activity but also weakens the integrity of the virus particles.

Comparison of glyoxal-denatured polymerase products with similarly treated dsRNA from virions showed a correspondence in the size of at least some of the products with virion RNAs. However, among the species of non-denatured product RNA are ones with sizes greater than would be expected from transcripts from the largest virion dsRNA. The nature of these large products is, at the moment, unclear.

The hybridization of transcribed products from RGDV with RDV RNA showed that there is no sequence homology between these two RNAs, though both viruses belong to the same subgroup.

REFERENCES


(Received 2 August 1983)