**In vitro** transcription of the double-stranded RNA genome of maize rough dwarf virus (Reoviridae)

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An RNA-dependent RNA polymerase associated with particles of maize rough dwarf virus, a Fijivirus, was characterized using two *in vitro* assays differing in their energy regeneration systems. Optimum reaction rates occurred at pH 8.0 to 8.5 at 20 °C. The presence of virus and Mn$^{2+}$ or Mg$^{2+}$ was essential for enzyme activity; Mn$^{2+}$ stimulated more incorporation events than Mg$^{2+}$, at optimum concentrations of 2 to 4 mM and 4 mM, respectively. Incorporation was not affected by α-amanitin, actinomycin D or rifampicin. The products synthesized *in vitro* were single-stranded RNAs which hybridized specifically with the double-stranded genomic RNAs of the template virus, but not with genomic RNAs of five other reoviruses. The *in vitro* transcripts were also used to detect maize rough dwarf virus RNA in plants and in vector insects.

**Introduction**

Plant reoviruses have been placed in three different genera or subgroups on the basis of morphological differences, number of dsRNA segments encapsidated, dsRNA (Black & Knight, 1970; Kodama & Suzuki, 1982) and the species of insect vector (Francki & Boccardo, 1981; Yokoyama & Peterson, 1981; Ikegami & Francki, 1976; Reddy et al., 1987; Uyeda et al., 1987). Particles of several plant reoviruses are known to contain an RNA-dependent RNA polymerase which transcribes ssRNA from viral RNA (Black & Knight, 1970; Kodama & Suzuki, 1973; Ikegami & Francki, 1976; Reddy et al., 1977; Nuss & Peterson, 1981; Yokoyama et al., 1984; Lee et al., 1987; Uyeda et al., 1987).

In this paper we describe the properties of a transcriptase associated with purified particles of maize rough dwarf virus (MRDV; Milne & Lovisolo, 1977) which belongs to subgroup II (Fijivirus) of the plant reoviruses.

**Methods**

*Virus, plants and insects.* MRDV was purified from naturally infected maize plants harvested in the fields essentially as described by Boccardo & Milne (1984); as a modification the purified particles were subjected to a third sucrose density gradient centrifugation in a Beckman SW41 rotor at 36000 r.p.m. for 60 min in a 10 to 50% (w/v) gradient. The particles were freed from sucrose by ultracentrifugation and the final product was suspended in sterile distilled water at a concentration of 3-5 μg/ml [estimated spectrophotometrically using $E_{	ext{260}}^{	ext{1%}}$ 48 (Caciagli et al., 1985), and stored at −20 °C.

An isolate of MRDV obtained originally from naturally infected plants (Caciagli et al., 1985) was maintained in mixed cultures of barley and maize seedlings by successive transfers using the planthopper vector *Laodelphax striatellus*. Growing conditions of the seedlings and of viruliferous and healthy hoppers were as detailed by Caciagli et al. (1985).

**Assay for RNA polymerase activity.** All assays were done in triplicate. Standard reaction mixtures contained 3-5 μg of virus, 20 units of RNAsin (Promega Biotec), 100 μM-Tris–HCl pH 8.0, and 1.5 μCi of [3HTP (Amersham; 41 Ci/mmol) in 25 μl. Two methods were used for the assay. Method A: the reaction mixtures were prepared according to Yokoyama et al. (1984) and contained 5 mM-MgCl₂, 2.8 mM-phosphoenolpyruvate, 40 μg/ml of pyruvate kinase, 2 mM-ATP, 0.2 mM-CTP and -GTP, and 0.3 mM-S-adenosyl-L-methionine (SAM). Method B: reaction mixtures (Sriskantha et al., 1986) contained 5 mM-magnesium acetate, 10 mM-creatine phosphate, 20 units of creatine phosphokinase and ATP, CTP and GTP each at 1 mM. TCA-precipitable radioactivity was determined as described by Boccardo & Accotto (1988).

For use as probes, polymerase products were synthesized overnight in conditions optimal for either method with 20 μCi of [32P]UTP (Amersham; 3000 Ci/mmol). Polymerase reactions were terminated and *in vitro* products were extracted as described by Boccardo & Accotto (1988). Comparable amounts of labelled RNAs were then incubated for 30 min at 37 °C in SSC (either 0.1 × or 2 ×) (1 × SSC is 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0), with or without pancreatic RNAs A type I (Sigma), or in 0.1 M-Tris–HCl pH 7.1, containing 10 mM-MgCl₂ with DNase type I (Sigma).

**Hybridization.** Healthy and infected barley and maize leaves, and healthy and viruliferous planthoppers were processed for dot blot experiments as described by Palukaitis et al. (1985). Portions (20 μl) of the extracts were applied to nitrocellulose membranes (BA85; Schleicher & Schüll) using a filtration apparatus (Minifold I; Schleicher & Schüll) and denatured in situ by a 45 min exposure to 50 mM-NaOH; they were then immobilized by baking for 2 h in vacuo at 80 °C. Prehybridization and hybridization conditions were as...
described by Maule et al. (1983), and post-hybridization washings were as described by Palukaitis et al. (1983). Membranes were probed with 1.5 × 10^6 to 3 × 10^6 c.p.m. of 32P-labelled in vitro polymerase products.

Phenol-extracted products synthesized overnight were denatured and electrophoresed as described by McMaster & Carmichael (1977) in 1% (w/v) agarose slabs. Genomic dsRNAs from rice gall dwarf virus (RGDV), rice dwarf virus (RDV) (both of subgroup I), MRDV and Fiji disease virus (FDV) (both of subgroup II), and rice ragged stunt virus (RRSV) (subgroup III), all from the collection of this Institute (Boccardo et al., 1985), were denatured and electrophoresed by the same method. RNAs were then transferred overnight to nitrocellulose membranes, and treated as above for dot blots. Dried filters were exposed at −80 °C.

**Results**

**Association of enzymic activity with MRDV**

After three successive sucrose density gradient centrifugations, MRDV particles were capable of incorporating radioactivity into TCA-precipitable products. [3H]UMP incorporation was negligible in the absence of virus, and was proportional to the amount of virus present in the reaction mixtures (Fig. 1). This shows that the enzyme responsible for UMP incorporation cosediments with MRDV particles in sucrose density gradients and is possibly associated with them. However, with both methods, at the highest concentrations tested, a decrease in incorporation was observed (Fig. 1), indicating that the virus preparations used may have contained some inhibitors or nucleases.

Maximum incorporation was obtained with both methods tested at 20 °C, with the enzyme losing 50% of its activity at temperatures of 30 °C or higher but retaining, with both methods tested, 80% of the activity at 10 °C (minimum temperature tested). Optimum pH was at 8-0 with Method A, whereas with Method B only 50% of maximum incorporation was obtained at this pH, the optimum being pH 8.5. Possibly this difference reflects the different pH requirements of the two energy regeneration systems used.

UMP incorporation was not affected significantly by transcriptional inhibitors such as α-amanitin, actinomycin D or rifampicin (Table 1), which indicates that the polymerase was not dependent on a DNA template. These results suggest that the enzyme is an RNA-dependent RNA polymerase.

MRDV polymerase activity was sharply dependent, with both methods tested, on the presence of divalent cations; the optimum Mg2+ concentration was 4 mM. When Mn2+ (supplied as MnCl2) replaced Mg2+, higher incorporation was obtained (Table 1); the optimum concentrations were 4 mM with Method A and 2 mM with Method B. All other divalent cations tested (supplied as CaCl2, CoCl2, CuSO4, FeSO4, and ZnSO4, at concentrations of 1, 2, 4, 10 and 20 mM) were not able to substitute for Mn2+ or Mg2+; the maximum UMP incorporation with these cations was about 10% or less than that obtained with Mn2+.

RNA polymerase activities associated with human reovirus (Levin et al., 1970) and FDV (Ikegami & Francki, 1976) have been shown to be stimulated by the addition of monovalent cations to the reaction mixtures. Monovalent cations (all supplied as chlorides at 5, 10, 20, 30, 60 and 120 mM) had no significant effect on the in vitro reaction, with the exception of an increase of approximately 30% in UMP incorporation caused by NH4+ (Table 1), the optimum concentration being 30 mM.

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**Table 1. Characteristics of the RNA-dependent RNA polymerase associated with MRDV particles**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>UMP incorporation (% of standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>100</td>
</tr>
<tr>
<td>− Virus</td>
<td>6-5</td>
</tr>
<tr>
<td>− NTPs</td>
<td>16-5</td>
</tr>
<tr>
<td>− Mg2+</td>
<td>2</td>
</tr>
<tr>
<td>− Mg2+, + Mn2+ (4 mM)</td>
<td>180-8</td>
</tr>
<tr>
<td>+ NH4+ (30 mM)†</td>
<td>126-5</td>
</tr>
<tr>
<td>+ K+ (30 mM)</td>
<td>111</td>
</tr>
<tr>
<td>+ Na+ (30 mM)</td>
<td>95-5</td>
</tr>
<tr>
<td>+ Li+ (30 mM)</td>
<td>92-5</td>
</tr>
<tr>
<td>+ SAM (0 µM/500 µM)</td>
<td>93/7/108-5</td>
</tr>
<tr>
<td>+ α-Amanitin (50 µg/ml)</td>
<td>103-8</td>
</tr>
<tr>
<td>+ Actinomycin D (50 µg/ml)</td>
<td>106-3</td>
</tr>
<tr>
<td>+ Rifampicin (50 µg/ml)</td>
<td>95</td>
</tr>
<tr>
<td>+ RNase (100 µg/ml) in 2× SSC</td>
<td>12</td>
</tr>
<tr>
<td>+ RNase (100 µg/ml) in 0.01× SSC</td>
<td>1-5</td>
</tr>
<tr>
<td>+ DNase (100 µg/ml)</td>
<td>93</td>
</tr>
</tbody>
</table>

* Values given are those obtained with Method A; Method B gave slightly lower values but essentially similar relative values.
† Optimal NH4+ concentration.
The rate of RNA synthesis catalysed by *Bombyx mori* cytoplasmic polyhedrosis virus- and RRSV-associated polymerases is considerably increased in the presence of SAM or some of its analogues (Furuichi, 1974; Mertens & Payne, 1978, 1983; Lee *et al*., 1987), and a similar but smaller effect has been observed with the reovirus-associated enzyme (Faust & Millward, 1974). However, no apparent effect has been observed on the polymerases associated with other plant reoviruses (Rhodes *et al*., 1977; Uyeda *et al*., 1987), some mycoviruses (Buck, 1986) or cryptic viruses (Boccardo & Accotto, 1988; Marzachi *et al*., 1988). To examine the effect of SAM on MRDV polymerase, standard reaction mixtures containing 0, 1, 2, 3, 5, 10, 20, 100 and 500 μM-SAM were incubated overnight. With both methods tested, SAM had no significant effect on the amount of 3H-labelled products made (Table 1).

Characterization of the in vitro products

Time-course experiments revealed that UMP incorporation increased with time up to the longest time tested (64 h), without the need for additional nucleoside triphosphates or energy regeneration systems. With both methods tested about 80% of total incorporation was obtained after 32 h (data not shown).

The reaction products were resistant to DNase digestion, but were hydrolysed almost completely by RNase in buffers of both low and high ionic strength (Table 1), suggesting that [3H]UMP was incorporated mostly into ssRNA molecules. When analysed in denaturing 1% (w/v) agarose gels, the products separated into seven discrete bands, with mobilities corresponding to those of denatured MRDV dsRNAs electrophoresed under the same conditions (Fig. 2). However, the bands were less intense than expected for the amount of radioactively labelled products loaded, which suggests that a large proportion of [3H]UMP was incorporated into molecules of very small size. This may indicate incomplete transcription, or that contaminant nucleases cosediment with the virus particles in sucrose gradients. With the highest amounts of virus tested UMP incorporation was less than with lower amounts of virus (Fig. 1); therefore the latter hypothesis seems more likely. When the RNase-resistant portion of the in vitro products (Table 1) was analysed in agarose gels, nothing was detected (data not shown), a result which suggests that there may be small regions of the in vitro transcripts with some secondary structure.

When denatured genomic dsRNAs from various reoviruses were probed with 32P-labelled reaction products, only the template viral RNAs were recognized (Fig. 3). This shows that the polymerase products were transcribed from dsRNAs encapsidated within MRDV particles, and that little if any nucleotide sequence homology exists between MRDV and the other reoviruses tested.

*In vitro* transcripts were assayed for their ability to detect, by dot blot experiments, MRDV infection in
barley and maize seedlings and in planthopper vectors. It was found that the transcripts could be used for diagnostic purposes (Fig. 4), with a sensitivity 10 to 20-fold higher than that attained by ELISA (Caciagli et al., 1985) on the same samples before RNA extraction.

Discussion

Plant reoviruses, similar to their animal counterparts (for a review, see Joklik, 1983), have been shown to possess a virus-associated RNA-dependent RNA polymerase, which transcribes ssRNA from the template dsRNA and thus acts as a transcriptase. We have shown that MRDV, a member of the plant reovirus group (Reoviridae) also has such an enzyme.

Proteolytic processing of the reovirus outer shell by chymotrypsin is required for transcriptase activity (Borsa et al., 1973), but such treatment had little or no effect on the polymerases of plant reoviruses, with the exception of that of RRSV (Lee et al., 1987). The MRDV-associated enzyme was active without the need for any further pretreatment (data not shown). However, the process of freezing and thawing causes degradation of MRDV particles to subviral particles (Milne et al., 1973). In most cases, electron microscopic observation of the preparation used for in vitro polymerase reactions of plant reoviruses belonging to subgroups I and II has revealed that the particles were more or less damaged (Kodama & Suzuki, 1973; Ikegami & Francki, 1976; Reddy et al., 1977; Yokoyama et al., 1984; Uyeda et al., 1987). Hence it would appear that for such viruses there is no need for pretreatment in order to elicit enzymic activity because the labile outer shell will already have been completely or partially removed.

Optimum pH and divalent cation requirements were found to be in the range of those reported for other viruses of the group, whereas the optimum temperature for the MRDV transcriptase appears to be lower than those of the other enzymes of the same viruses (Black & Knight, 1970; Kodama & Suzuki, 1973; Ikegami & Francki, 1976; Reddy et al., 1977; Uyeda & Shikata, 1984; Yokoyama et al., 1984; Lee et al., 1987; Uyeda et al., 1987). Although Mn\(^{2+}\) can substitute partially for Mg\(^{2+}\) for some reovirus-associated polymerases (Reddy et al., 1977; Lee et al., 1987), there is no case where a plant reovirus transcriptase has been shown to give higher incorporation with Mn\(^{2+}\) than with Mg\(^{2+}\), as was the case in our experiments.

With the exception of FDV (Ikegami & Francki, 1976), little attention has been paid to the effect of monovalent cations on the transcriptases of other plant reoviruses. MRDV-associated transcriptase activity was stimulated by NH\(_4\)^+, although less dramatically than that of FDV (Ikegami & Francki, 1976). Also, the optimum concentration of NH\(_4\)^+ for the MRDV enzyme was about one-sixth of the optimum for the FDV transcriptase.

Stimulation of polymerase activity by the presence of SAM in the reaction mixture has been found only for the RRSV (subgroup III) enzyme among the plant reoviruses (Lee et al., 1987). In this respect the MRDV polymerase appears to be similar to those of other viruses belonging to subgroups I and II (Kodama & Suzuki, 1973; Ikegami & Francki, 1976; Reddy et al., 1977; Yokoyama et al., 1984; Uyeda et al., 1987).

Comparison of glyoxal-denatured MRDV polymerase products with similarly processed viral dsRNAs showed a correspondence in their sizes (Fig. 2) and Northern blots confirmed the specificity of the products (Fig. 3). The possibility of using in vitro transcripts as diagnostic probes should be investigated further. Our limited experiments suggest that such probes could be more sensitive than serological tests, as would be expected. Some samples from hoppers exposed to the virus, but which did not transmit it and which proved negative in ELISA tests, did react with MRDV transcripts (data not shown). Clearly more work is needed to evaluate the significance of these findings.

References


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