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Replication of cucumber mosaic virus satellite RNA in vitro by an RNA-dependent RNA polymerase from virus-infected tobacco

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An RNA-dependent RNA polymerase purified from tobacco infected with cucumber mosaic virus catalyzes the synthesis of (−) and (+) strands of the viral satellite RNA, CARNA 5, but fails to replicate the satellite RNA of peanut stunt virus (PSV). The enzyme replicates the genomic RNAs of the three principal cucumoviruses CMV, PSV and tomato aspermy virus (TAV) with varying efficiencies. The specificity with which CMV RdRp replicates different sequence-unrelated RNA templates suggests that the site of their recognition requires secondary or higher level structural organization.

RNA-dependent RNA polymerase; Cucumovirus; Satellite RNA; Replication

1. INTRODUCTION

Cucumber mosaic virus (CMV), like other tricornaviruses [1], possesses a tripartite RNA genome and a fourth subgenomic RNA functioning as coat protein messenger RNA. In addition CMV encapsidates a satellite RNA with the designation CARNA 5 (CMV-associated RNA 5), which comprises a group of sequence-related variants 334–386 nucleotides (nt) long [2,3]. Although CARNA 5 is essentially sequence-unrelated to the CMV genome, it depends on the latter's presence for replication [4,5]. Because CARNA 5 can modify viral disease symptoms in widely varying ways, from inducing plant death at one extreme to complete amelioration of symptoms at the other (reviewed in [6,7]), and due to its usefulness in practical agriculture [8,9], this satellite has become by far the one most intensively studied among several now characterized [10].

Experimental protoplast infections have shown that CARNA 5 replication is supported by the CMV genome components 1 and 2, encoding the viral RNA-dependent RNA polymerase (RdRp) or replicase [11], which suggests that this particular helper virus lends support to satellite RNA by providing the replicase which it also uses for its own genomic RNA replication. More recently two reports described the first isolation of RdRp preparations, from CMV-infected tobacco plants, that were sufficiently pure so as to be completely dependent on an exogenous viral RNA template [12,13].

Here we provide first-time evidence that purified CMV RdRp synthesizes both (−) and (+) strands of its satellite CARNA 5, added as exogenous template, thus completing a full replication cycle. This occurs with high specificity since the enzyme fails to replicate PARNA 5, the satellite RNA of peanut stunt virus (PSV), a related member of the cucumovirus group.

Abbreviations: CMV, cucumber mosaic virus; PSV, peanut stunt virus; TAV, tomato aspermy virus; TMV, tobacco mosaic virus; CARNA 5, CMV-associated RNA 5; PARNA 5, PSV-associated RNA 5; RdRp, RNA-dependent RNA polymerase; ds, double-stranded; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis.

Mention of a commercial company or specific equipment does not constitute its endorsement by the US Department of Agriculture over similar equipment or companies not named.

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2. MATERIALS AND METHODS

2.1. *Purification of RdRp from infected tobacco*

CMV RdRp was purified from tobacco leaf tissues infected with a satellite-free preparation of CMV strain S using the procedure described previously [14], but where the DEAE-Biogel A column was substituted by a Q-Sepharose Fast Flow column (Pharmacia LKB Biotechnology Inc).

2.2. *CMV RdRp assay with different cucumoviral genomic and satellite RNAs*

CMV RdRp activity was assayed using the viral RNAs of CMV, PSV, tomato aspermy virus (TAV) (all tricornaviruses and members of the cucumovirus group [10]) and tobacco virus (TMV), as well as different CARNA 5 sequence variants and the satellite PARNA 5 (PSV-associated RNA 5) as templates. Virus was extracted for total RNA from which satellite RNAs were separated by polyacrylamide gel electrophoresis (PAGE) and gel elution [15,16]. An RdRp reaction mixture comprising a total volume of 50 μl contained 50 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM each of

GTP, CTP and ATP, 10 μM UTP, 10 units RNasin (Promega), 10 μCi [α-^{32}P]UTP (800 Ci/mmol), 10 μl of the purified CMV RdRp preparation, and 100 μg/ml viral RNAs or 20 μg/ml satellite RNA. Reaction mixtures were incubated at 28°C for 1 h. After incubation, reaction products were phenol-extracted and precipitated as described previously [13].

2.3. *Analysis of CMV RdRp reaction products*

RdRp reaction products from the use of viral RNA templates were analyzed by 3% PAGE. Reaction products from the use of CARNA 5 and PARNA 5 templates were analyzed using a PAGE procedure which efficiently separates the previously denatured complementary strands of the double-stranded (ds) forms of CARNA 5 or PARNA 5 [17] with slight modifications. The RdRp reaction products were resuspended in 10 μl of 30% DMSO, 1 mM EDTA, 0.1% each of Bromophenol blue and xylene cyanol, and heated for 3 min at 90°C followed by quick cooling. The denatured samples were subjected to 7% PAGE (acrylamide:bisacrylamide, 50:1 w/w) in 0.05 M Tris-borate, pH 8.3, 2 mM EDTA. Electrophoresis was at 4 V/cm for 16 h or until the xylene cyanol had reached the bottom of the gel. The gels were dried under pressure and exposed to X-ray film with intensifying screens.

3. RESULTS

3.1. *Synthesis of (−) and (+) strands of CARNA 5 by CMV RdRp*

The purified CMV RdRp replicated two CARNA 5 sequence variants with high specificity. This is shown in Fig. 1 where 1-CARNA 5 (334 nt [18]), Y-CARNA 5 (369 nt [19]), and PARNA 5 (393 nt [20]) were offered as templates, and the reaction products analyzed by PAGE. While the phenol-extracted reaction mixture showed ^{32}P incorporation into a ds product prior to denaturation (data not shown), after denaturation and PAGE ^{32}P label was found in 2 bands with mobilities corresponding to that of the (−) and (+) strands of the respective CARNA 5 variants (Fig. 1, lanes B2 and 4). This indicates that the CMV RdRp catalyzed the complete replication of CARNA 5, which could not be demonstrated unequivocally in a previous report [13]. No ^{32}P incorporation whatsoever was found with the PARNA 5 template (Fig. 1, lane B6), even after increasing its concentration to 100 μg/ml (data not shown). When, in a separate test, CARNA 5 and PARNA 5 were offered as a mixture of templates, only CARNA 5 (−) and (+) strands were synthesized (Fig. 2). This result also eliminates the possibility that impurities in the PARNA 5 preparation inhibited its ability to serve as a template for the RdRp. When no unlabelled nucleotide triphosphates were included in the reaction using CARNA 5 there was no ^{32}P incorporation, indicating that the radioactive labeling in the reaction products could not have resulted from end-labeling by any contaminating terminal transferase (data not shown).

From the intensity of the two bands in Fig. 1 (lanes B2 and 4) or Fig. 2 (lanes 1 and 3), it can be concluded that generally more (−) than (+) strands of CARNA 5 were synthesized over the length of the experiment,

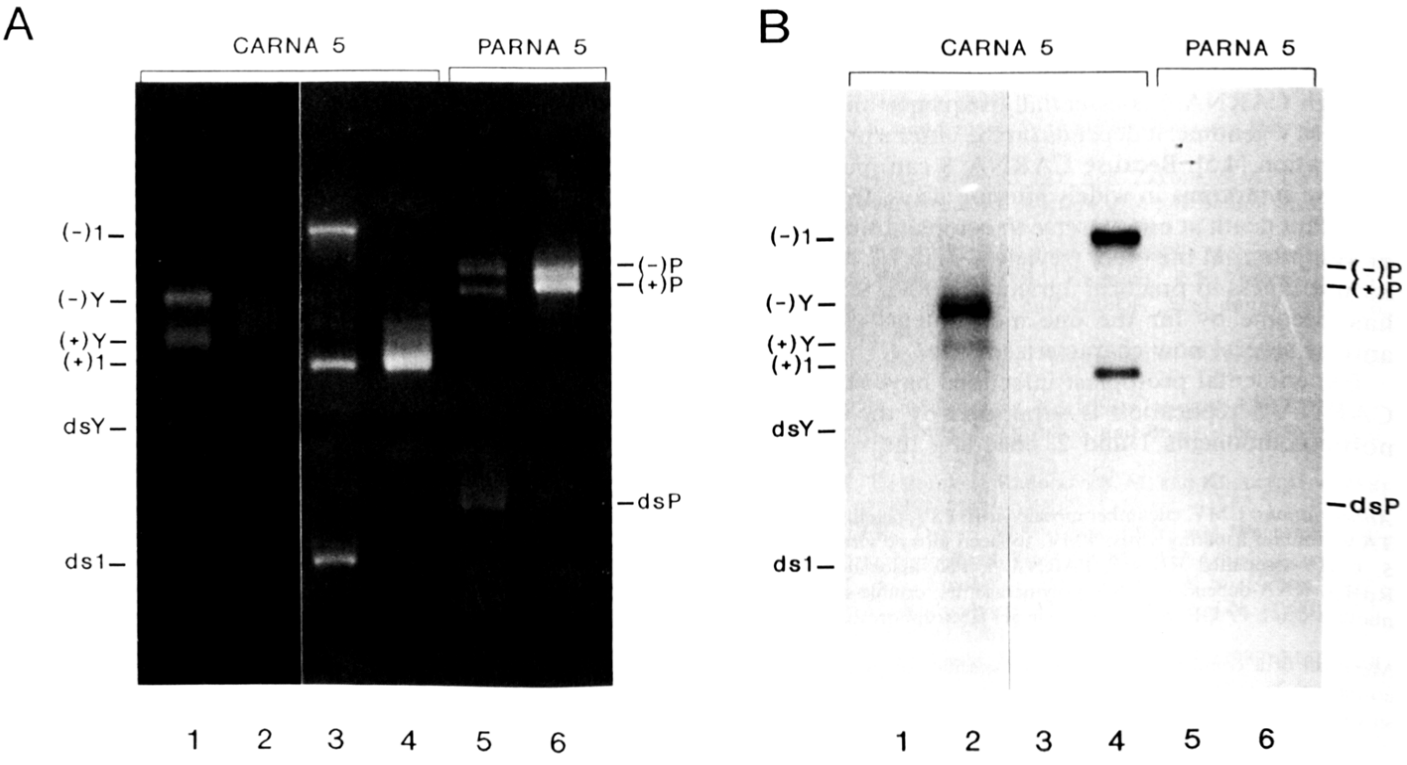

Fig. 1. 7% PAGE separating complementary RNA strands followed by (A) ethidium bromide staining and (B) autoradiography of products synthesized by CMV RdRp in reactions containing Y-CARNA 5 (lanes A2 and B2), 1-CARNA 5 (lanes A4 and B4) and PARNA 5 (lanes A6 and B6) as templates. Denatured double-stranded forms of Y-CARNA 5 (lane A1), 1-CARNA 5 (lane A3) and PARNA 5 (lane A5) were used as markers.

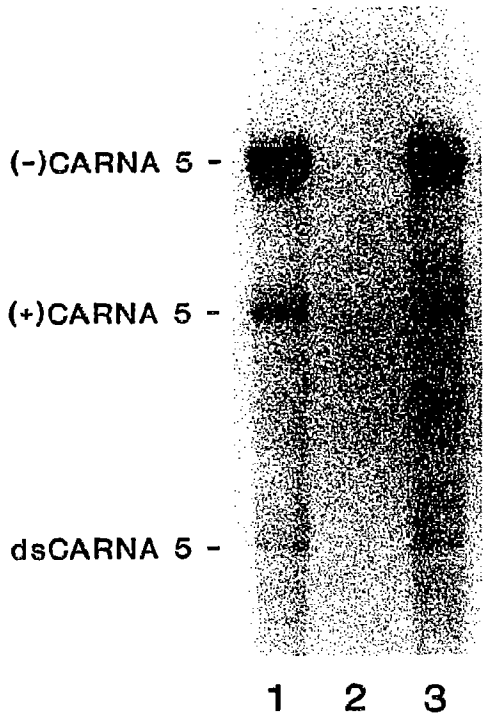

Fig. 2. 7% PAGE and autoradiography of ^{32}P-labeled products synthesized bij CMV RdRp in reactions containing 1-CARNA 5 (lane 1), PARNA 5 (lane 2) and a mixture of 1-CARNA 5 and PARNA 5 as templates.

whereas usually this proportion is reserved under in vivo conditions [21]. This discrepancy probably resulted from general imperfection of in vitro conditions, with (exogenously added) (+) strand templates greatly outnumbering newly synthesized (−) strands. The occasional absence of labeled ds CARNA 5 reaction product after denaturation and PAGE analysis (Fig. 1, lane B2 and 4), as opposed to the presence of some non-dissociated or re-annealed ds CARNA 5 in the controls (Fig. 1, lanes A1 and 3), was due to the low product concentration, as per separate denaturation test of a ds CARNA 5 dilution series (data not shown).

3.2. *Template specificity for genomic RNAs of two other cucumoviruses*

CMV RdRp recognized and utilized both PSV-RNA (Fig. 3, lane 2) and TAV-RNA (Fig. 3, lane 3) as a template for ^{32}P incorporation into RNA products with electrophoretic mobilities of ds RNAs 1, 2 and 3 and subgenomic RNA 4. With TMV-RNA there was no incorporation of radioactivity (Fig. 3, lane 4). However, while TAV-RNA incorporated label equally strongly as the homologous CMV-RNA (Fig. 3, lane 1 and 5), PSV-RNA was apparently synthesized with lesser efficiency.

4. DISCUSSION

The data reported in this publication constitute the first unequivocal evidence that the RdRp encoded by a

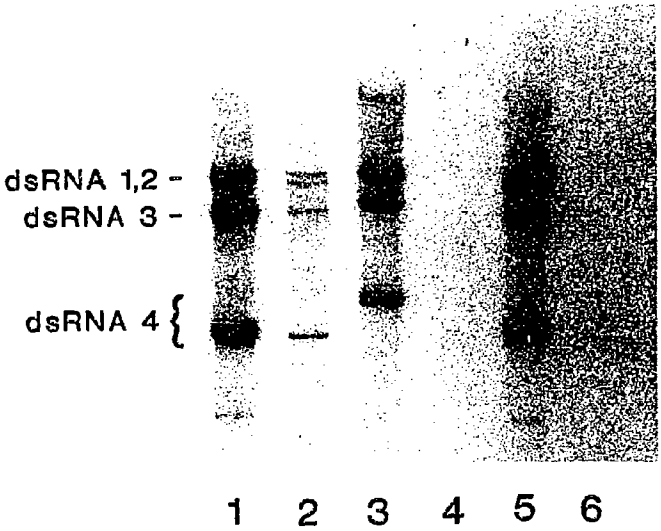

Fig. 3. 3% PAGE and autoradiography of ^{32}P-labeled products of CMV RdRp reactions containing viral RNAs of CMV-1 (lane 1), PSV (lane 2), TAV (lane 3), TMV (lane 4), CMV-S (lane 5) and no RNA (lane 6) as templates.

(+)-stranded RNA virus, which catalyzes the replication of its homologous RNA with high efficiency, can also replicate its encapsidated, sequence-unrelated satellite RNA. The high specificity of satellite template recognition by the enzyme is obvious from Fig. 1, where two CARNA 5 variants with significant differences in length and sequence are replicated with equal efficiency, but PARNA 5, the satellite RNA of PSV, is not. On the other hand, CMV RdRp could replicate the genomic RNAs of this taxonomically related cucumovirus, albeit with lower efficiency. The genomic RNA of TAV, the third member of the cucumovirus group, which does support the replication of CARNA 5 in vivo [22], was replicated with equal efficiency by the CMV RdRp as its homologous substrate CMV-RNA (Fig. 3). No sequence information is presently available for PSV-RNA, but partial sequence data for TAV-RNA suggest it has a close evolutionary relationship with CMV-RNA, as well as with the RNA of brome mosaic virus (BMV) [23]. These findings are also consistent with previous observations on the replication efficiency of this enzyme with BMV-RNA [13]. They do suggest that in order for the RdRp to catalyze the replication of specific viral and satellite RNAs certain sequence domains must be recognized that are structurally organized at a higher level than that of primary structure alone.

The experimental demonstration that the RdRp of CMV also replicates its homologous satellite has validated the basic premise of a previously proposed biochemical mechanism that rationalizes the disease-regulatory properties of CMV satellite RNAs by way of their competition with viral RNAs for the same virus-encoded replicase [24]. In addition, due to the relative simplicity of viral satellite RNAs, this report opens up

new approaches for identifying and studying structural domains in replicating RNA molecules that are recognized with subtle specificity by their replicative enzymes.

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