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Characterization of cucumber mosaic virus RNA-dependent RNA polymerase

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Cucumber mosaic virus (CMV) RNA-dependent RNA polymerase (RdRp) was purified form CMV-infected tobacco. The purified enzyme is completely dependent on exogenous template. The enzyme utilizes a variety of viral RNAs and CMV satellite RNA as template for minus-strand synthesis. Cellular RNAs are not used as templates. Ribosomal RNA inhibits the viral RNA synthesis by the CMV RdRp.

Cucumber mosaic virus; RNA polymerase; Satellite RNA; Cellular RNA

1. INTRODUCTION

Cucumber mosaic virus (CMV), the type member of the cucumoviruses, is a positive-strand RNA virus with a tripartite genome. The genome organization of CMV has been elucidated by sequence analysis of the genomic RNAs [1–3]. The cucumoviruses have been grouped together with the bromoviruses (type member brome mosaic virus, BMV) and the ilarviruses (type member alfalfa mosaic virus, AlMV) as the tricornaviruses [4].

RNA 1+2 replicate in tobacco protoplasts in the absence of RNA 3 [5]. This implies the involvement in replication of the nonstructural proteins P1 and P2 encoded by RNA 1 and RNA 2, respectively. However, the molecular basis of CMV RNA synthesis is poorly understood. The development of a system capable of CMV RNA synthesis in vitro should provide more insight in this process. A prerequisite for such a system is the isolation of the key enzyme involved in viral RNA synthesis, i.e. RNA-dependent RNA polymerase (RdRp). The isolated CMV RdRp should be completely dependent on added RNA templates in order to be able to study template recognition and minus-strand RNA synthesis in vitro.

The purification of template-dependent CMV RdRp has proven to be troublesome because of the association of the enzyme with membranes and endogenous templates [6]. Moreover, the presence of a host enzyme which is greatly induced by CMV infection masks the activity of the viral RdRp [6]. Thorough analysis of the synthesized products is therefore necessary to discriminate between host and viral RdRp.

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In this paper we describe some of the characteristics of a purified, template-dependent CMV RdRp isolated from CMV-infected tobacco.

2. MATERIALS AND METHODS

2.1. *Preparation of RdRp from CMV-infected tobacco*

Tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were grown from seed at 20–22°C under fluorescent light for 16 h per day. The upper three leaves of 38-day old plants were inoculated with a 0.1 mg/ml suspension of purified CMV (S-strain). Two days after inoculation the inoculated leaves of plants were harvested for RdRp isolation. CMV RdRp was purified from 25 g of CMV-infected tobacco by Nonidet P-40/KCl solubilization and subsequent purification by glycerol gradient centrifugation and DEAE ion-exchange chromatography as described recently for BMV RdRp [7].

2.2. *RNA-dependent RNA polymerase assays*

The standard reaction mixture contained 50 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, CTP, GTP, 10 μM UTP, 2.5 μCi [α-^{32}P]UTP (400 Ci/mmol), 200 μg/ml RNA in a total volume of 25 μl. Reaction mixtures were incubated at 28°C for 60 min. After incubation, 20 μl of the reaction mixtures were spotted on phosphocellulose paper and processed as described [8]. Alternatively, reaction products were phenol-extracted, treated with nuclease S1, and analyzed by gel electrophoresis as described previously [9].

3. RESULTS AND DISCUSSION

CMV RdRp can be purified by employing essentially the same method as for purification of BMV RdRp [7]. An 8914-fold increase in specific activity was obtained by solubilization of CMV RdRP followed by subsequent purification of the solubilized enzyme (Table II). The purified enzyme is completely dependent on exogenous template. No products are observed when no RNA is added to the reaction mixture (Fig. 1, lane 5). In terms of template-dependence the CMV RdRp does not differ from the RdRps from AlMV [9] and BMV [7].

Table I

Activity of CMV RdRp using various RNA templates

Template RNA	[^{32}P]UMP incorporation[a] (cpm)
AIMV	41 388
BMV	44 717
CMV	42 104
TMV	12 834
CMV satellite RNA	19 910
none	1 743
tRNA	1 491
cellular RNAs	1 698

[a]Incorporation of [^{32}P]UMP in double-stranded products corrected for background

In contrast, the CMV RdRp does not exhibit the strict template specifity that is observed with AIMV RdRp [9] and BMV RdRp [7]. CMV RdRp recognizes and utilizes a variety of viral RNAs as template for minus-strand synthesis leading to the formation of double-stranded RNAs corresponding to viral RNAs 1, 2 and 3 and subgenomic RNAs (Fig. 1, lanes 1–4). Virion RNAs from BMV and CMV act as equally efficient templates (Fig. 1, lanes 2 and 3; Table I). This is not surprising in view of the fact that in addition to a very similar genome organization both BMV and CMV possess a tRNA-like structure at the 3′ termini of the genomic and subgenomic RNAs [10]. It has been shown that this 3′ tRNA-like structure is essential for the initiation of BMV RNA synthesis (for reviews see [11,12]). AIMV RNAs are also utilized as templates by the CMV RdRp with an efficiency very similar to CMV and BMV RNAs (Fig. 1, lane 1; Table I). This is somewhat surprising because AIMV RNAs do not carry a 3′ tRNA-like structure. Double-stranded products of intermediate sizes are synthesized when AIMV RNAs are used as templates as can be concluded from the presence of multiple bands in lane 1 of Fig. 1. This suggests that CMV RdRp also initiates RNA synthesis on AIMV RNAs internally. Virion RNA of the non-tricornavirus tobacco mosaic virus (TMV) carries a 3′ tRNA-like structure and is also used as a template, albeit with a lower efficiency than with tricornaviral RNAs (Fig. 1, lane 4; Table I). This indicates that the presence of a 3′ tRNA-like structure is not sufficient for efficient RNA

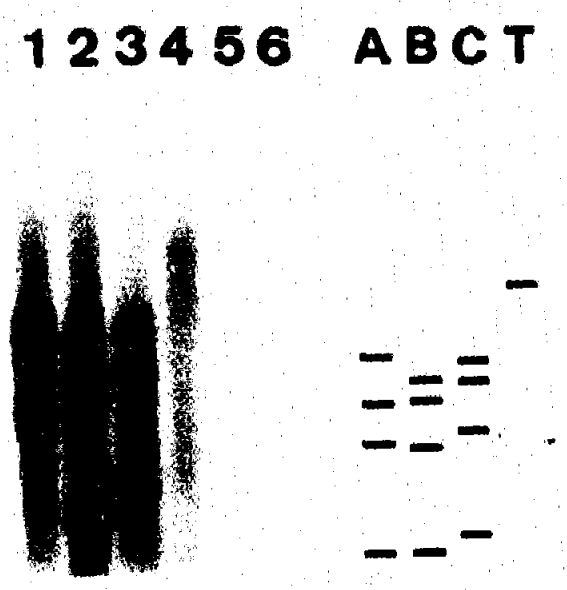

Fig. 1. Double-stranded RNA products synthesized by CMV RdRp in standard reaction mixtures containing AIMV RNAs (lane 1); BMV RNAs (lane 2); CMV RNAs (lane 3); TMV RNA (lane 4); no RNA (lane 5); cellular RNAs from tobacco (lane 6). In lanes A, B, C, T the positions of AIMV, BMV, CMV and TMV virion RNAs are indicated.

synthesis by the CMV RdRp. Sequences and/or structural features specific for tricornaviral RNAs seem to be required as well. The observation that TMV RNA can be used as a template by the CMV RdRp is consistent with the observation that TMV can replicate in rice protoplasts, which normally do not support TMV replication, if coinoculated with CMV [13].

Interestingly, CMV satellite RNA which does not resemble tricornaviral RNAs nor possesses a 3′ tRNA-like structure is used as a template by CMV RdRp (Fig. 2, lane 1). The identity of the double-stranded products synthesized on CMV satellite RNA was established by hybridization with a CMV satellite-specific cDNA probe (data not shown). This result supports the hypothesis that satellite RNAs are multiplied by the RdRp of the helper virus [14]. Small-sized products characteristic of

Table II

Purification of CMV RdRp from CMV-infected tobacco

Enzyme fraction[b]	RdRp[a] activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Purification factor (fold)
Particular fraction[b]	247	127	1.95	1
Glycerol gradient fractions	3214	0.72	4464	1380
DEAE Biogel A fraction	3825	0.22	17382	8914

[a]One unit of RdRp activity is defined as 1 fmol of UMP incorporated in 60 min at 28°C using 200 μg/ml CMV RNAs as templates. From this value the incorporation of UMP by the enzyme in absence of added templates was subtracted. This was done in order to obtain the true amount of template-dependent CMV RdRp activity

[b]Resuspended 30 000 × *g* membrane pellet

Fig. 2. Double-stranded RNA products synthesized by CMV RdRp in standard reaction mixtures containing CMV satellite RNA (lane 1); tRNA (lane 2).

the host RdRp which is induced by viral infection [6,15] were not observed (Fig. 2, lane 2), indicating that this enzyme is separated from the viral RdRp during the purification procedure. Furthermore, in similar preparations from mock-inoculated tobacco, no RdRp activity on any template could be detected (data not shown).

Neither tRNA nor total cellular RNA from healthy tobacco is utilized as template by CMV RdRp (Fig. 1, lane 6; Fig. 2, lane 2). Moreover, when mixed with CMV RNAs cellular RNAs strongly inhibit CMV RdRp activity on viral templates (Fig. 3). Identical amounts of tRNA or BMV RNAs added to CMV RNAs did not inhibit CMV RdRp activity (data not shown), indicating that the inhibition is caused specifically by cellular RNAs. Inhibition of CMV RdRp activity was still observed after the total cellular RNA preparation was passed over a poly(U)-Sepharose column in order to remove polyadenylated mRNAs (Fig. 3), suggesting that the inhibitory effect is caused by ribosomal RNAs.

We hypothesize that the CMV RdRp complex like Qβ replicase [16] contains a ribosomal protein similar to prokaryotic ribosomal S1 protein essential for RdRp activity. This protein would bind to ribosomal RNA with high affinity and thus block the transcription of CMV RNAs. However, definite proof of this hypothesis awaits further experimentation.

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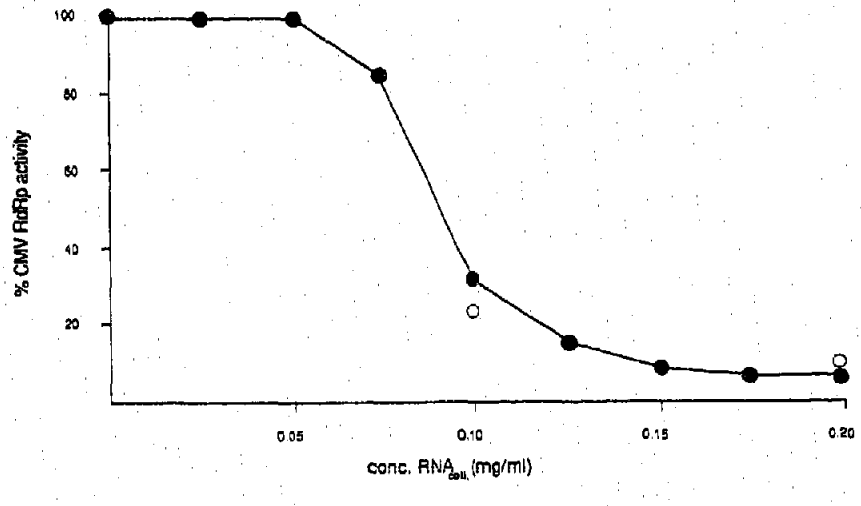

Fig. 3. Inhibition of CMV RdRp-mediated viral RNA synthesis by cellular RNAs. To reaction mixtures containing a fixed amount (200 μg/ml) of CMV RNAs (•) cellular RNA from uninfected tobacco or (○) total cellular RNA passed over a poly(U)-Sepharose column was added to concentrations indicated on the graph.

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