Optimal conditions for cell-free synthesis of citrus exocortis viroid and the question of specificity of RNA polymerase activity

[J. S. SEMANCIK AND K. L. HARPER]

Department of Plant Pathology and Cell Interaction Research Group, University of California, Riverside, CA 92521

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ABSTRACT

Cell-free synthesis of citrus exocortis viroid (CEV) in nuclei-rich preparations from infected Gynura aurantiaca was optimum at 18–24°C. Incubation of reaction mixtures at higher temperatures (30–36°C) resulted in an increase of CEV linear molecules and the recovery of incomplete or nicked newly synthesized RNA species. Although the Mg2+ optimum (2.5–5 mM) for CEV synthesis was lower than that for total [32P]CMP incorporation (10 mM), the response to Mn2+ ion was distinctly different. Whereas maximum total activity was observed in 1 mM Mn2+ with a pronounced reduction (80%) in 5 mM Mn2+, CEV synthesis was maintained in 1–15 mM Mn2+. Inhibition of a-amanitin-sensitive CEV synthesis in 200 mM (NH4)2SO4 resembles the reaction of RNA polymerase II on a free nucleic acid template. However, detection of trace levels of a-amanitin-resistant CEV synthesis activity inhibited by low (NH4)2SO4 concentrations (25 mM) suggests the possible involvement of RNA polymerase I and/or III-like activity.

Synthesis of the citrus exocortis viroid (CEV) in nuclei-rich cell-free preparations of Gynura aurantiaca (1) has previously been shown to be sensitive to concentrations of a-amanitin inhibitory to DNA-directed RNA polymerase II. Recently, however, the localization of viroid RNA with nucleoli-rich preparations (2) and the discovery of sequences in viroids homologous to major portions of a promoter sequence for a mouse rRNA gene (3) have suggested the possible involvement of a DNA-directed RNA polymerase I in viroid replication.

Resolution of the details involved in viroid replication is well served by the nuclei-rich cell-free system. That purified RNA polymerase II is capable of synthesizing viroid-complementary molecules when supplied with a viroid RNA template has been verified in vitro (4). However, the lack of specificity of in vitro replication systems (5) has also been evidenced by the competence of both plant RNA-directed RNA polymerase (6) and bacteriophage Qβ replicase (7) in transcription of viroid RNA sequences. Although the nuclei-rich cell-free system, similar to most nuclei or chromatin systems, probably only completes RNA molecules initiated prior to extraction, these systems offer the advantage of permeability to exogenously added cofactors and inhibitors. In addition, a more native form of replicating complex than available in purified viroid–enzyme systems is probably retained.

In this report, we describe conditions of the cell-free nuclei-rich system optimal for CEV synthesis. We also present data on effects of ionic strength and metal ion concentrations as well as on a-amanitin sensitivity to better define the nature of the RNA polymerase activity functioning in viroid replication.

MATERIALS AND METHODS

Preparation of nuclei-rich fractions essentially followed the procedure of Flores and Semancik (1) with Polytron-homogenized G. aurantiaca tissue being filtered through 40-μm nylon cloth and the 1000 × g for 10 min pellets treated with Triton X-100. Final nuclei-rich preparations, which contained intact nuclei as well as chromatin mats as observed by acetoarmine staining and light microscopy, were assayed for 2 hr at 30°C under the following standard conditions unless otherwise indicated [10 mM Tricine/NaOH, pH 8/0.3 M sucrose/5 mM MgCl2/1.6 mM CaCl2/2.5 mM MnCl2/50 mM (NH4)2SO4/20 mM mercaptoethanol/0.5 mM each unlabeled NTPs excluding the NTP added as label and 50 μCi (1 Ci = 37 GBq) of [α-32P]CTP (Amersham; specific activity, 2000–3000 Ci/mmol) or [α-32P]GTP (Amersham; specific activity, 2000–3000 Ci/mmol)].

RNA polymerase activity associated with nuclei-rich preparations was determined by spotting aliquots of the reaction mixture onto Whatman GF/C discs (1). The nucleic acid species contained in the remainder of the assay mixture were extracted and analyzed as previously noted (1) with the modification that the viroid band was removed from native gels (8), placed onto a second gel containing 8 M urea, and electrophoresed under fully denaturing conditions (9). With this procedure a more definitive separation of viroid-related molecular forms is achieved, resulting in cleaner autoradiographs.

RESULTS

Temperature Optimum and the Kinetic Relationship Between CEV Circular and Linear Forms. The nuclei-rich preparation capable of producing labeled CEV (1) must be considered as a heterogeneous fraction composed of not only RNA-synthesizing but also RNA-degrading activities. Therefore, the temperature optimum for polymerase activity may not necessarily be consistent with the temperature of maximum recovery of the specific viroid RNA product. When reaction mixtures were held for 2 hr at various temperatures ranging from 12°C to 42°C, a pronounced biphasic response curve for incorporation of [32P]CMP resulted (Fig. 1). Samples held at 12–24°C reached levels of isotope incorporations of about twice the value of those held at 30–36°C with the maximum trichloroacetic acid precipitable activity recovered in the range of 1.7–6.8 × 10^6 cpm. Incorporation into CEV RNA at the various temperatures reflected the pattern for total RNA synthesis.

To determine the distribution of isotope incorporation into the circular and linear forms of CEV (CEVC and CEVL, respectively) over extended intervals at low and high temperatures, reaction mixtures were incubated at either 18°C or 36°C and sampled over a 22-hr period. Fig. 2 presents the

Abbreviations: CEV, citrus exocortis viroid; CEVC, circular CEV; CEVL, linear CEV.
and at 1-4 preparations; and 7), length CEVL autoradiographed by polyacrylamide bromide staining, removed, electrophoresis (B).

The CEVL, of the treatment temperature was allowed to migrate under denaturing conditions (Fig. 2A and B). Total RNA patterns from samples held at 18°C for 22 hr (Fig. 2B, lane 4) displayed less heterogeneity than RNA extracted from samples held at 36°C for only 6 hr (Fig. 2B, lane 7), as evidenced by the polydisperse quality of the low molecular weight region of the latter RNA pattern.

When the CEV bands were excised from the native gel and allowed to migrate under fully denaturing conditions, the relative concentration of CEVc and CEVL, as identified by coelectrophoresis with purified viroid, was constant with the obvious exception of the CEV population recovered from the sample held at 36°C for 22 hr. This prolonged high-temperature treatment visually appeared to result in an increase of CEVL, perhaps at the expense of CEVc, molecules.

A more striking differential recovery in the recently synthesized CEV-related molecules was observed in the autoradiograph (Fig. 2D) of the denaturing gel (Fig. 2C). Even though the total viroid population was relatively resistant to degradation during incubation, synthesis of both CEVc and CEVL was clearly superior at 18°C (Fig. 2D, lanes 1–4) than at 36°C (Fig. 2D, lanes 5–8). Short incubations (0.5 hr), either

**Fig. 1.** Incorporation of $[^{32}P]_{\text{CMP}}$ into an acid-insoluble fraction after incubation of a nuclei-rich preparation from CEV-infected *G. aurantiaca* for 2 hr at various temperatures. Aliquots from reaction mixtures were applied to glass-fiber discs (Whatman GF/C) and washed with cold trichloroacetic acid/1% sodium pyrophosphate (three times), ethanol, and ethyl ether. After drying the discs, radioactivity was determined in a Beckman LS-3133 liquid scintillation counter. The maximum count level in three independent experiments (○, ●, □) reported was 1.7–6.8 × 10⁴ cpm.

**Fig. 2.** Incorporation of $[^{32}P]_{\text{CMP}}$ into an acid-insoluble fraction (A) and CEV (D) after incubation of a nuclei-rich preparation from CEV-infected *G. aurantiaca* at 18°C or 36°C for various intervals. Total $[^{32}P]_{\text{CMP}}$ incorporation was determined as in Fig. 1. The remainder of the reaction mixtures was extracted with phenol/2 M LiCl. Nucleic acids were treated with deoxyribonuclease I (ca. 140 μg/ml) for 1 hr at 30°C and fractionated by 5% polyacrylamide gel electrophoresis (B). The CEV RNA band was detected by ethidium bromide staining, removed, and directly electrophoresed in a second 5% polyacrylamide gel under fully denaturing conditions (C) and autoradiographed by exposure of Kodak XR-5 film for assay of CEV synthesis (D). Lanes: 5, purified CEV-containing tRNA standard preparations; 1–4 and 5–8, extracts of mixtures incubated at 18°C and at 36°C, respectively for 0.5 hr (1 and 5), 1.5 hr (2 and 6), 6 hr (3 and 7), and 22 hr (4 and 8). Arrowheads indicate shorter than unit-length CEVL forms.
at 18°C or 36°C, clearly produced the cleanest population of newly synthesized viroid products. Long incubations (6–22 hr), especially at the high temperature (36°C), were characterized by the recovery of three or four RNA species that were shorter than unit-length CEV₁₃ forms (Fig. 2D, lanes 6–8). These small RNAs appear similar to the short viroid transcription products detected when viroid RNA was provided as a template to purified DNA-dependent RNA polymerase II (4). Since the denaturing gel pattern (Fig. 2C and D) was produced by molecules migrating in the viroid region of 5% native gels known to contain no RNA species in healthy extracts, it must be assumed that the small RNA species detected here must have resulted from structures migrating under nondenaturing conditions as full-length viroid RNA. These molecules probably contain specific hidden breaks in the polynucleotide chain. Furthermore, since the nonradioactive patterns (Fig. 2C) of CEVc and CEV₁₃ forms do not appear dramatically altered, these presumed breaks probably did not result from random nuclease action on the total viroid RNA population but, instead, from either sensitivity of the partially completed viroids to nuclease nicking or inability of the polymerase to complete the CEV RNA. Although the relative concentration of these short RNA species varied with incubation temperatures (Fig. 2D, lanes 4 and 6–8), the similar sizes suggest common semistable viroid-related products.

The cell-free CEV-synthesizing system might be characterized as a progression of stages with increasing incubation time. Good viroid synthesis fidelity seems to occur during about the first 3 hr of incubation, and this is followed by evidence of increasing product heterogeneity (3–6 hr) and finally by a decaying system (6–22 hr) in which RNase activity and/or polymerase incompetence prevails. The incubation temperature constitutes a significant factor in the timing of this progression.

In spite of these limitations it has been possible to investigate relationships that can exist between CEVc and CEV₁₃ forms. Quantitation of the incorporation rate of [³²P]CMP into CEVc and CEV₁₃ made by direct analysis of excised bands from gels is displayed in Fig. 3. During the initial interval (0–3 hr) of incubation at 30°C, synthesis of CEVc prevailed. With extended intervals, levels of [³²P]CMP detected in the region of CEV₁₃ became predominant, suggesting that at least some portion of the linear viroid population results from specific degradation of circular forms. This is not, however, to suggest that all linear molecules present during the early stages of cell-free synthesis result from degradation; preliminary experiments indicate infectivity of CEV₁₃, as well as coordinated synthesis of CEVc and CEV₁₃ (unpublished).

Differential Mn²⁺-Stimulated CEV Synthesis. Divalent metal cations are required for activity of all known plant RNA polymerases, with optimum concentrations of Mn²⁺ and Mg²⁺ usually 1–2 and 5–10 mM, respectively (10). The optimum Mn²⁺/Mg²⁺ activity ratio has also been used to discriminate among the properties of purified nuclear RNA polymerases (11). Total [³²P]CMP incorporation was optimal for the nuclear-rich RNA-synthesizing system at 1 mM Mn²⁺ and 10 mM Mg²⁺ (Fig. 4A). The Mn²⁺/Mg²⁺ activity ratio at the optimal concentration of each ion was about 1.5, suggesting the principal action of RNA polymerase I- and/or III-like activity.

CEV RNA synthesis activity displayed a dramatically different divalent metal cation profile. In the absence of all other divalent cations, the optimum Mg²⁺ concentration was 1–10 mM (Fig. 4B). More surprising was the marked lack of sensitivity to inhibition by high Mn²⁺ concentrations. CEV synthesis was detected over a broad concentration range (0.5–15 mM), with CEVc molecules maintaining high levels even when total [³²P]CMP incorporation had been reduced by 90% (Fig. 4C). These properties are atypical of all well-characterized nuclear RNA polymerases as well as of plant RNA-directed RNA polymerase activity (10, 11).

Ionic Strength and the Response to α-Amanitin Inhibition. Viroid synthesis in both protoplasts (12) and cell-free extracts (1) has been shown to be sensitive to inhibition by α-amamin. A similar inhibition of in vitro transcription of viroid RNA by purified RNA polymerase II has also been reported (4). Since cells may not be freely permeable to α-amamin, making intracellular concentrations difficult to determine, and our previous report (1) presented evidence for a trace level of CEV synthesis even at high α-amamin concentrations (10–1000 nM), these data may conceal a low-level RNA polymerase I- and/or III-like activity. Therefore, total [³²P]CMP incorporation and CEV synthesis was monitored in the presence and absence of α-amamin and as affected by ionic strength.

The broad optimum of (NH₄)₂SO₄ concentration, ranging from 0 to 100 mM, for total [³²P]CMP incorporation is shown in Fig. 5A. The biphasic nature of the response over these concentrations might be expected for combined RNA polymerase I- and II-like activities (11). Preincubation of the reaction mixtures with α-amamin at 4 μg/ml did not completely reduce the total incorporation (Fig. 5B).

In the absence of α-amamin, maximum CEV synthesis was observed in an (NH₄)₂SO₄ range of about 25–75 mM (Fig. 5C), characteristic of purified RNA polymerase II (10). Additional support for the action in viroid synthesis of polymerase II-like activity is provided by the marked inhibition of CEV synthesis by α-amamin (4 μg/ml) (Fig. 5D). This inhibition, however, is not complete since a trace level of CEV synthesis can be observed in the presence of α-amamin.

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**FIG. 3.** Incorporation of [³²P]CMP into CEVc and CEV₁₃ molecules as well as the relationship of CEVc/CEV₁₃ (A) after incubation of a nuclei-rich preparation from CEV-infected G. aurantiaca at 30°C for various times. CEVc and CEV₁₃ molecules were extracted and separated and radioactivity determined by direct counting of radioactivity in the bands.
**FIG. 4.** Incorporation of \(^{32}\)P]CMP into an acid-insoluble fraction (A) and CEV (B and C) after incubation of a nuclei-rich preparation from CEV-infected *G. aurantiaca* for 2 hr in the presence of various concentrations of Mg\(^{2+}\) [A (●) and B] or Mn\(^{2+}\) [A (○) and C]. Total incorporation and incorporation into CEV were determined as shown in Fig. 1 and Fig. 2.

**DISCUSSION**

RNA polymerase activity and the identification of the action of specific nuclear polymerases can be affected by the distinctly different properties in intact nuclei or chromatin systems as opposed to the behavior of purified polymerase transcribing free nucleic acid templates (10). The host-dependent

**FIG. 5.** Incorporation of \(^{32}\)P]CMP into an acid-insoluble fraction (A and B) and CEV (C and D) after incubation of a nuclei-rich preparation from CEV-infected *G. aurantiaca* for 2 hr in the presence of various concentrations of (NH\(_4\))\(_2\)SO\(_4\) without [A, B (●), and C] and with [B (○) and D] α-amanitin at 4 µg/ml. This activity appears to be salt sensitive, with an (NH\(_4\))\(_2\)SO\(_4\) optimum of 0–25 mM. Although incomplete penetration or interaction of α-amanitin with RNA polymerase II must be considered, the distinct (NH\(_4\))\(_2\)SO\(_4\) concentration maxima for the α-amanitin-sensitive and -resistant CEV-synthesizing activities suggest a combined effect of two independent systems. Therefore, consideration of the possible involvement of both RNA polymerase I- and/or III-like and II-like activities in viroid replication appears warranted.
nuclear synthesis of the nontranslated viroid RNA, which is characterized by a unique structure and conformation, might also influence the definition of polymerase activity.

On the basis of ionic strength optimum and response to α-amanitin, a compelling argument can be made for CEV synthesis mediated principally by RNA polymerase II and also, to a lesser extent, possibly by RNA polymerase I and/or III. Furthermore, the inhibition of α-amanitin-sensitive CEV synthesis by 200 mM (NH₄)₂SO₄ resembles the action of RNA polymerase II on a free nucleic acid template (13). This might be taken as evidence that the viroid and/or the viroid complement does persist as a free RNA in vitro.

In sensitivity of CEV synthesis to high concentrations of Mn²⁺ is unlike all known nuclear polymerases but is suggestive of the reduced template specificity of Qβ replicase in the presence of Mn²⁺ (14). Therefore, one might be inclined to think in more exclusive terms of RNA polymerase I- and/or III-like or RNA polymerase II-like activities when considering viroid replication, the action of a minor yet distinct RNA-synthesizing system must still be entertained. Reports of atypical polymerase activities such as α-amanitin-sensitive polymerase I activity (15), electrophoretic variants of polymerase II, and rRNA-encoding DNA transcription by polymerase II (16) may further complicate this definition.

Recent reports of the association of viroids with nucleolar pellets (2) as well as homology between viroid RNA and the promoter sequence of a mouse ribosomal RNA gene (3) suggest the involvement of RNA polymerase I in viroid biosynthesis. Although data presented here support this proposition in principle, the overwhelming bulk of viroid-synthesizing activity is α-amanitin sensitive and therefore RNA polymerase II-like. Subnuclear localization of viroid RNA can be instructive in defining possible replicative processes and biological activities. However, fundamental questions concerning the leakage problems of isolated nuclei and non-specific association of viroid RNA with host structures, particularly membranes (17), must be addressed in these studies.

In support for the involvement of RNA polymerase I, Palukaitis and Zaitlin (3) have pointed out the absence in potato spindle tuber viroid of the consensus sequence (“TATA” or “CCAAAT”) associated with transcription by RNA polymerase II. Even though the RNA nature of the viroid molecule would obviate the existence of such specific sequences, the experimental evidence for involvement of DNA-directed RNA polymerase II as defined by α-amanitin sensitivity remains firm. The ability of polymerase II to accept either a DNA or an RNA template may require some modification in the consensus sequences such as substitution of UMP for the TMP components or perhaps rendering of the terminal TMP in the “CCAA-T” sequence as nonessential in viroid RNA transcription. It is interesting to note that the core tetranucleotide, CCAA, is contained in the central conserved region of all viroid RNAs, including the unusual avocado sunblotch viroid (18), which shares only a hexanucleotide continuous sequence with all other viroids for which sequences have been reported.

Alternatively, the properties we have described here may simply reflect a process involving a total lack of polymerase specificity, with all nuclear polymerases competent in transcribing a truly unusual RNA structure. Since RNA polymerase II exists in the highest concentration in vivo and is potentially most available in the nucleoplasm, it may emerge as the polymerase of choice for CEV synthesis. That is not to say that, provided an increased availability of polymerase I or sufficient concentrations of polymerase III, these enzymes might not also function in viroid replication. The existence of common subunits in all three forms of nuclear RNA polymerases (19) may also suggest a core activity capable of recognizing specific regions of the viroid RNA. Testing the efficacy of purified polymerase I, II, and III with a viroid template in vitro might contribute to the resolution of this question.