Purification and Characterization of DNA-dependent RNA Polymerases from Cauliflower Nuclei

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ABSTRACT

DNA-dependent RNA polymerases were solubilized from nuclei of cauliflower inflorescences and purified by agarose A-1.5m, DEAE-cellulose, DEAE-Sephadex, and phosphocellulose chromatography and sucrose density gradient centrifugation. RNA polymerases I + III were separated from II by DEAE-cellulose chromatography. Subsequent chromatography on DEAE-Sephadex resolved RNA polymerase I from III. RNA polymerases I and II were further purified to high specific activity by phosphocellulose chromatography and sucrose density gradient centrifugation. RNA polymerase I was refractory to α-amanitin at 2 mg/ml. RNA polymerase II was 50% inhibited at 0.05 μg/ml, and RNA polymerase III was 50% inhibited at 1 to 2 mg/ml of α-amanitin. The enzymes were characterized with respect to divalent cation optima, ionic strength optima, and abilities to transcribe cauliflower, synthetic, and cauliflower mosaic virus DNA templates.

Eukaryotes possess multiple forms of DNA-dependent RNA polymerase which transcribe specific DNA cistrons (3, 4, 10). The major forms of eukaryotic nuclear RNA polymerases can be grouped into three species which are referred to as either I or A, II or B, and III or C (3, 4, 10). RNA polymerase I is localized within the nucleolus (11, 13, 18), transcribes ribosomal DNA cistrons (9, 17), and is refractory to the fungal toxin, α-amanitin (11, 14). RNA polymerase II is localized within the nucleoplasm (11, 18), synthesizes heterogeneous and presumably messenger RNA (28), and is inhibited by 10⁻⁸ to 10⁻⁹ M α-amanitin (11, 14). RNA polymerase III is localized in the nucleoplasm (18, 20) and possibly the cytoplasm (2, 20, 21, 24, 26), synthesizes ribosomal SS and transfers RNAs (25), and is inhibited by 10⁻³ to 10⁻⁴ M α-amanitin in higher animals (2, 20, 21, 24, 26), but is refractory to the fungal toxin in lower eukaryotes (1, 23, 27).

Most of the studies on eukaryotic RNA polymerases have been conducted with either animal or lower eukaryotes such as yeast, while studies on higher plants have been limited (3). Plants are known to possess two major forms of RNA polymerase, I and II, and a third form has been described in rye embryos (6). Here, we describe the separation and purification of multiple forms of RNA polymerases from cauliflower. Cauliflower inflorescences offer a rich source of RNA polymerases (7, 19). The fractionation of three species of RNA polymerase from cauliflower which have properties similar to RNA polymerases I, II, and III described in animals has been achieved in this work. Although the characteristics of the plant enzymes are in general similar to the animal RNA polymerases, a 10- to 100-fold greater concentration of α-amanitin is required to inhibit cauliflower RNA polymerase III than is required to inhibit the animal form III enzyme (20, 23). The cauliflower enzymes are characterized in relation to their elution patterns from cation and anion exchange resins, α-amanitin sensitivities, Mg²⁺ and Mn²⁺ optima, ionic strength optima, and abilities to transcribe cauliflower, synthetic, and plant viral DNA templates.

MATERIALS AND METHODS

DEAE-cellulose (DE-32) and phosphocellulose (P-11) were purchased from Whatman; DEAE-Sephadex A-25 from Pharmacia; agarose A-1.5m and all components used in acrylamide gel electrophoresis from Bio-Rad; [3H]-GTP and [3H]-UTP from Schwarz-Mann; unlabeled nucleoside triphosphates, dithiothreitol, Triton X-100, and phenylmethylsulfonyl fluoride from Sigma; α-amanitin from Henley and Co., N. Y.; poly(dC) and poly(dAT) from Miles; CsCl from EM Laboratories Inc., Elmsford, N. Y.; cauliflower mosaic virus (California Salinas strain) was obtained from the American Type Culture Collection, Rockville, Md., and was used to infect Tendergreen mustard (Brassica perviridis, Bailey); cauliflower inflorescences were purchased from local food market.

BUFFERS

Buffer A contained 25 mM 2-(N-morpholino)ethane sulfonic acid, (pH 6), 20 mM MgCl₂, 20 mM KCl, 0.25 M sucrose, 10 mM 2-mercaptoethanol, and 40% glycerol. Buffer B contained 0.05 M tris-HCl (pH 8), 0.1 mM EDTA, 10 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% dimethylsulfoxide, 0.1% Triton X-100, and 30% glycerol. Dithiothreitol and phenylmethylsulfonyl fluoride in 100% dimethylsulfoxide were added immediately before use of buffer B.

PREPARATION OF ION EXCHANGE RESINS

DEAE-cellulose was prepared for chromatography as described by Whatman and was equilibrated with buffer B containing 0.05 M ammonium sulfate. Phosphocellulose and DEAE-Sephadex were prepared for chromatography as described previously (8) and equilibrated with buffer B containing 0.05 M ammonium sulfate.

REFRACTIVE INDEX AND PROTEIN DETERMINATIONS

Ammonium sulfate concentrations were measured with a Bausch and Lomb refractometer. Protein concentrations were determined by the method of Lowry et al. (15) after the samples were precipitated with trichloroacetic acid.

DNA ISOLATION FROM CAULIFLOWER INFLORESCENCES

DNA was extracted from cauliflower inflorescences by homogenization in a detergent buffer containing 6% (w/v) p-
aminoacetic acid and 1% (w/v) triisopropanolamine-sulfonic acid, extraction with chloroform and phenol, and digestion with ribonuclease and pronase as described (12). Final purification was achieved by banding the DNA on 25 ml CsCl (initial density 1.70 g/cm³) equilibrium density gradients. The gradients were formed by centrifugation at 35,000 rpm at 20°C for 60 hr in a Spincţm type 60 rotor. The CsCl purified DNA was dialyzed against 1 × SSC containing 0.1 mM EDTA, precipitated with 2 volumes of ethanol, and dissolved (1 mg/ml) in 0.05 M NaCl, 0.05 M tris (pH 8), and 0.1 mM EDTA (buffer C).

**ISOLATION OF CAULIFLOWER MOSAIC VIRUS DNA**

Cauliflower mosaic virus was isolated from infected mustard leaves by the method described by Shepard et al. (22). Cauliflower mosaic virus DNA was isolated from the purified virus as described (22) except that ribonuclease digestion and phenol extraction were omitted to prevent any possible nicking of the viral DNA. Following pronase digestion, the DNA was banded twice on 25 ml CsCl (initial density 1.70 g/cm³) equilibrium density gradients as described above for cauliflower inflorescence DNA. The CsCl-purified DNA was dialyzed against 1 × SSC containing 0.1 mM EDTA, precipitated with 2 volumes of ethanol, and dissolved (1 mg/ml) in 1 × SSC containing 0.1 mM EDTA. The DNA solution was layered onto a 5 ml, 5 to 20% convex exponential sucrose gradient (16) and centrifuged in a Spinco SW 50.1 rotor for 3 hr at 48,000 rpm at 2°C to remove any small degraded DNA from high mol wt viral DNA. The high mol wt DNA was collected, dialyzed against 1 × SSC containing 0.1 mM EDTA, precipitated with 2 volumes of ethanol, and dissolved (200 μg/ml) in buffer C.

**RNA POLYMERASE PURIFICATION METHODS**

A nuclear fraction from cauliflower inflorescences (5 kg tissue) was prepared with buffer A as described by Chen et al. (5). All purification procedures were conducted at 0 to 4°C. RNA polymerase was solubilized from the nuclei by suspending the purified nuclear fraction in buffer B containing 0.5 M ammonium sulfate, shearing the chromatin for 5 min at high speed with a Polytron (Brinkmann Instruments), and sonicating the chromatin for 3 min (1-min intervals) at maximum power with a Bronson microtip sonicator. The solubilized RNA polymerase was recovered in the supernatant after centrifugation for 60 min at 50,000 rpm with a Spincţm type 60 rotor. The supernatant was then diluted to 0.1 M ammonium sulfate with buffer B and recentrifuged for 60 min at 50,000 rpm. The RNA polymerase was precipitated from the supernatant by the gradual addition of solid ammonium sulfate (0.35 g/ml) and stirring for 60 min. The protein precipitate was recovered by centrifugation for 60 min at 50,000 rpm and was suspended in 40 ml of buffer B containing 0.5 M ammonium sulfate.

**Aagarose A-1.5 M Chromatography.** The soluble RNA polymerase was loaded onto a column (90 × 2.5 cm) of agarose A-1.5M equilibrated with buffer B containing 0.5 M ammonium sulfate. The enzyme was eluted with buffer B containing 0.5 M ammonium sulfate and 5-M fractions were collected at a flow rate of 0.25 ml/min. Fractions were assayed for RNA polymerase activity and the absorbance at 280 nm was determined. The peak fractions were combined and precipitated by the gradual addition of 0.35 g/ml solid ammonium sulfate and stirring for 60 min. The protein pellet was collected by centrifugation at 50,000 rpm for 60 min.

**DEAE-cellulose Chromatography.** The ammonium sulfate precipitate from the agarose fractions was suspended in buffer B and adjusted to 0.05 M ammonium sulfate. The solution was loaded onto a column (40 × 1.5 cm) of DEAE-cellulose equilibrated with buffer B containing 0.05 M ammonium sulfate. The column was washed with 1 column volume of the same buffer, and the enzymes were eluted with a 0.05 to 0.3 M linear gradient of ammonium sulfate in buffer B. The gradient was 3 column volumes, and 2-ml fractions were collected. Fractions from under each peak of RNA polymerase were combined, diluted with 1.5 volumes of buffer B, and rechromatographed separately on DEAE-Sephadex.

**DEAE-Sephadex Chromatography.** Conditions of DEAE-Sephadex chromatography were those described for DEAE-cellulose except that the gradient was 0.05 to 0.5 M ammonium sulfate. Fractions under each peak of RNA polymerase were collected and adjusted to 0.05 M ammonium sulfate with buffer B.

**Phosphocellulose Chromatography.** RNA polymerase peak fractions from DEAE-Sephadex were individually chromatographed on columns (10 × 0.9 cm) of phosphocellulose equilibrated with buffer B containing 0.05 M ammonium sulfate. The columns were washed with 2 column volumes of the same buffer and the enzymes eluted with 0.05 to 0.5 M ammonium sulfate gradients in buffer B. The gradients were 2.5 column volumes and 1-ml fractions were collected.

**Sucrose Density Gradient Centrifugation.** RNA polymerase peaks from phosphocellulose were diluted with 1.5 volumes of buffer B, loaded on phosphocellulose columns (5 × 0.9 cm) as described above. The enzymes were eluted with 0.3 M ammonium sulfate in buffer B and 0.5-m1 fractions were collected. Approximately 80% of the RNA polymerase activity from each column eluted in a single fraction, and this fraction was layered directly onto a 5 to 20% convex exponential sucrose gradient in buffer B containing 0.25 M ammonium sulfate and 1 mM MgCl₂. The gradients were centrifuged for 36 hr in an SW 50.1 rotor at 50,000 rpm.

**POLYACRYLAMIDE GEL ELECTROPHORESIS**

Electrophoresis of the purified RNA polymerases was performed according to the method described previously (8) except that for native gels the electrophoresis buffer contained 30% glycerol and 10 mM dithiothreitol.

**RNA POLYMERASE ASSAY**

Unless indicated otherwise, RNA polymerase was assayed in a reaction volume of 500 μl containing 5 to 20 μl of enzyme and 0.05 M tris-HCl (pH 8), 25% glycerol, 1 mM MnCl₂, 25 mM (NH₄)₂SO₄, 50 μg heat-denatured cauliflower DNA, 0.4 mM each of the three unlabeled nucleotides and 0.04 mM ³H-UTP or ³H-GTP (1 μCi) at 28°C for 30 min. Reactions were terminated, collected on Whatman GF/A filters, and radioactivity determined as described (8). A unit of activity is 1 nmol ³H-U-MP or ³H-GMP incorporated into RNA/mg protein·30 min at 28°C.

**RESULTS**

Solubilization of Cauliflower RNA Polymerase. Cauliflower inflorescences offer a very rich source of nuclei containing active RNA polymerases. The nuclei are readily obtained by gentle homogenization in a buffer similar to that described by Chen et al. (5) for the isolation of soybean nuclei. The nuclei possess approximately equal amounts of α-amanitin-sensitive and α-amanitin-insensitive RNA polymerase activities when assayed in vitro under conditions previously described (5). The nuclear RNA polymerase is solubilized by shearing the chromatin in buffer B containing 0.5 M ammonium sulfate. After the viscosity of the chromatin suspension is reduced by sonication, the fraction of chromatin which is not solubilized under these conditions is pelleted and discarded. The RNA polymerase activity remains in the supernatant. Total activity recovered in the supernatant is about 2-fold greater than the total activity observed with nuclei; this result may be due to removal of inhibitors or ribonuclease or may reflect repression of the enzymes on the chromatin tem...
plate. Dilution of the supernatant from 0.5 to 0.1 M ammonium sulfate and subsequent centrifugation results in an increase in specific activity with no detectible loss in total activity of soluble RNA polymerase. At this stage of purification little activity is observed in the absence of an added DNA template.

**Chromatography and Sucrose Gradient Purification.** A greater than 2-fold purification of the crude soluble RNA polymerase from cauliflower is achieved by gel filtration on agarose A-1.5m under high salt conditions (0.5 M ammonium sulfate). RNA polymerase activity elutes as a single peak several fractions after the void volume (Fig. 1a). Nearly 50% of the protein loaded onto the column elutes in several turbid fractions with the void volume in front of the RNA polymerase peak. The RNA polymerase activity recovered from the agarose column is about 50% inhibited at 1 μg/ml of α-amanitin.

Following agarose gel filtration, the RNA polymerase is fractionated on a DEAE-cellulose column into two discrete peaks of RNA polymerase activity (Fig. 1b). The first peak elutes at 0.13 M ammonium sulfate and is refractory to 0.1 μg/ml of α-amanitin. The second peak which elutes at 0.21 M ammonium sulfate is inhibited by 0.1 μg/ml of α-amanitin. Thus the first peak has the chromatographic and α-amanitin insensitivity properties of RNA polymerase I described in other systems while the second peak has the properties of RNA polymerase II.

When the DEAE-cellulose RNA polymerase peaks are subsequently fractionated on DEAE-Sephadex columns, peak I from DEAE-cellulose resolves into two peaks eluting at 0.14 and 0.30 M ammonium sulfate (Fig. 1c) while peak II from DEAE-cellulose chromatographs as a single peak eluting at 0.25 M ammonium sulfate (Fig. 1d). In animal systems, the RNA polymerase I peak from DEAE-cellulose has been reported to resolve into RNA polymerases I and III on DEAE-Sephadex (2, 21, 23, 24). These two enzymes can be distinguished by the inhibition of RNA polymerase III at 10⁻² to 10⁻⁴ M α-amanitin while RNA polymerase I is refractory to the toxin (2, 21, 23). Figure 2 shows the α-amanitin sensitivities of the three different

![Fig. 1. Chromatography of cauliflower RNA polymerases.](image-url)

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cauliflower RNA polymerase fractions obtained by DEAE-Sephadex chromatography. The cauliflower enzymes like the animal enzymes are identified as RNA polymerases I, II, and III which refers to their sequence of elution from DEAE-Sephadex. RNA polymerase I is refractory to α-amanitin even at toxin concentrations of 2 mg/ml; RNA polymerase II is 50% inhibited by α-amanitin at 0.05 μg/ml; RNA polymerase III is 50% inhibited at 1 to 2 mg/ml. Thus RNA polymerases I and II have sensitivities to α-amanitin similar to the animal enzymes; RNA polymerase III from cauliflower requires a 10- to 100-fold greater concentration of the toxin than is required for a similar inhibition in animals.

RNA polymerases I and II from DEAE-Sephadex were further purified by chromatography on phosphocellulose. Peak I from DEAE-Sephadex is resolved into two peaks on phosphocellulose which elute, respectively, at 0.18 and 0.23 M ammonium sulfate (Fig. 1e). The first peak is referred to as RNA polymerase Ia and the second peak as RNA polymerase Ib. When assayed in the presence of denatured cauliflower DNA or a completely denatured template such as poly(dC), both peaks are observed on phosphocellulose; however, if the column fractions are assayed in the presence of native cauliflower DNA or a native template such as poly(dAT) only peak Ib is observed. Thus native templates are transcribed very poorly if at all by RNA polymerase Ia while RNA polymerase Ib transcribes both native and denatured templates. RNA polymerase II chromatographs as a single peak on phosphocellulose, eluting at 0.12 M ammonium sulfate (Fig. 1f).

RNA polymerases Ib and II were purified further by sucrose density gradient centrifugation in the presence of high salt (0.25 M ammonium sulfate). A summary of the purification of the cauliflower enzymes is presented in Table I. The specific activities of the sucrose-purified cauliflower enzymes are comparable to those described for the highly purified eukaryotic RNA polymerases (3, 4, 27).

The putative polypeptide subunit composition of purified RNA polymerases Ib and II on acrylamide gel electrophoresis under denaturing conditions are shown in Figure 3. The protein bands observed on the denatured gel of RNA polymerase Ib have the following mol wt: a: 185,000 (0.4); b: 168,000 (0.6); c: 135,000 (1.0); d: 70,000 (0.5); e: 56,000 (0.5); f: 45,000 (1.0); and g: 28,000 (1.9). The molar ratios are given in parentheses. The mol wt of RNA polymerase II putative subunits are: a: 170,000 (0.9); b: 140,000 (1.0); c: 42,000 (0.9); and d: 27,000 (1.7). The above gel patterns are not meant to represent absolute nor complete subunit structures of the cauliflower RNA polymerases. Polypeptides of mol wt below 25,000 daltons are not resolved on these gels. The gel patterns do represent, however, putative RNA polymerase subunits to which the case of RNA polymerase Ib, several of the subunits are similar in mol wt to soybean RNA polymerase I (8) and animal RNA polymerase I enzymes (3, 4). The electrophoretic gel pattern of RNA polymerase Ib under denaturing conditions indicates that RNA polymerase Ib may be contaminated by RNA polymerase Ia and may represent two forms of the enzyme which differ only in the mol wt of their largest subunit. RNA polymerase I subunits a and b added together are at a molar ratio equal to subunit c. In addition, on gels electrophoresed under non-denaturing conditions the purified RNA polymerase Ib migrates as two very closely running bands. In the case of RNA polymerase II, the putative subunit pattern is strikingly similar to the nuclear form of the enzyme purified from soybean and differs from the cytoplasmic form of the enzyme purified from soybean only in respect to the largest subunit (Guilfoyle and Key, manuscript in preparation). Several smaller putative subunits of both soybean and cauliflower RNA polymerase II are resolved on 8.75 to 15% linear gradient gels, and these have mol wt of 20,000, 16,000 and 14,000 daltons. On native gels, cauliflower RNA polymerase II migrates as a single band following the sucrose step of purification. The high specific activities of RNA polymerases Ib and II (Table I) provide additional evidence along with the native and denatured gel patterns that the enzymes are at a high state of purity.

Cauliflower RNA polymerases Ib, II, and III have been characterized with respect to divalent cation optima, ionic strength optima, and ability to transcribe cauliflower, synthetic, and cauliflower mosaic virus DNA templates. All three enzymes have similar Mg2+ and Mn2+ optima which are 2 to 10 mM and 0.5—1 mM, respectively (Fig. 4a to c). RNA polymerase I utilizes Mg2+ and Mn2+ equally well; RNA polymerase II shows a preference for Mg2+; and RNA polymerase III shows a slight preference for Mn2+.

Table I. Summary of Purification of Cauliflower RNA Polymerases

<table>
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<th>Fraction</th>
<th>RNA Polymerase</th>
<th>Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
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<tr>
<td>I + III</td>
<td>113</td>
<td>80</td>
<td>0.71</td>
<td></td>
<td></td>
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<tr>
<td>II</td>
<td>68</td>
<td></td>
<td>0.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I + III</td>
<td>182</td>
<td>2.40</td>
<td>100</td>
<td></td>
<td></td>
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<tr>
<td>Crude soluble</td>
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<td></td>
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<tr>
<td>Agarose A-1.5m</td>
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<td></td>
<td>4.10</td>
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<tr>
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<td>40</td>
<td>205</td>
<td>22</td>
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</tr>
</tbody>
</table>
Mn\textsuperscript{2+}. Little difference is observed in the effect of salt concentration on the RNA polymerase activities (Fig. 5, a to c). RNA polymerase III is least effected by changes in salt concentration while RNA polymerase I is most sensitive, especially to high salt inhibition. Greater differences in salt optima of the three RNA polymerases were observed if denatured DNA was replaced with native DNA (data not shown).

The ability of RNA polymerases I, II, and III to transcribe various templates is summarized in Table II. All three enzymes prefer denatured over native cauliflower DNA. Synthetic templates are transcribed more efficiently than cauliflower DNA. RNA polymerase I shows an especially strong preference for poly(dC) similar to that of RNA polymerase I from soybean (8). RNA polymerase II is able to transcribe cauliflower mosaic virus DNA at very low DNA concentrations relative to the transcription on denatured or native cauliflower DNA at a similar concentration. It is especially interesting that RNA polymerase II from cauliflower is so efficient in the transcription of the viral DNA since this is native rather than single-stranded DNA (22); generally RNA polymerase II enzymes demonstrate a much stronger preference for single-stranded DNA (3, 4). This may suggest that the viral DNA contains strong promoter sites for cauliflower RNA polymerase II.

**DISCUSSION**

Cauliflower inflorescences offer a rich supply of enzymically active nuclei for the isolation of multiple forms of RNA polymerase. Several forms of cauliflower RNA polymerase can be solubilized and separated on cation and anion exchange resins. Two forms of RNA polymerase I were separated on phosphocellulose, and one of these forms transcribes native templates very poorly. Both type I enzymes are refractory to \(\alpha\)-amanitin even at concentrations of 2 mg/ml. RNA polymerase II chromatographs as a single peak on both cation and anion exchange resins used in this study. The form II enzyme from cauliflower is 50% inhibited at an \(\alpha\)-amanitin concentration of 0.05 \(\mu\)g/ml. Thus both RNA polymerases I and II from cauliflower have similar properties to animal RNA polymerases I and II with respect to chromatography on cation and anion exchange resins and sensitivity to \(\alpha\)-amanitin. A third form of cauliflower RNA polymerase, type III, was separated from the DEAE-cellulose RNA polymerase I peak by rechromatography on DEAE-Sephadex. This procedure of chromatography on DEAE-cellulose followed by chromatography on DEAE-Sephadex has also been used successfully in animal systems to demonstrate RNA polymerase III activity (2, 21, 23, 24). RNA polymerase III activity is typically much less than either RNA polymerase I or II activity and has thus proved difficult to detect in a single chromatographic procedure (20, 26). When DEAE-cellulose is used, RNA polymerase III usually co-elutes with RNA polymerase I whereas with DEAE-Sephadex the form III enzyme is obscured by the RNA polymerase II peak (2, 23). However, by combining these two chromatographic steps RNA polymerase II is first separated from I and III by DEAE-cellulose while rechromatography of peak I on DEAE-Sephadex completely separates RNA polymerase I from...
III. Thus in chromatographic properties RNA polymerase III from cauliflower is similar to RNA polymerase III described in several animal systems (2, 21, 23, 24); however, the enzyme from cauliflower is 10- to 100-fold less sensitive to α-amanitin than higher animal RNA polymerase III enzymes (2, 21, 23, 24). While RNA polymerase III from higher animals (i.e., vertebrates) is inhibited 50% by 20 to 200 μg/ml of α-amanitin, the enzymes from Bombyx mori (23) and lower eukaryotes (i.e., yeast) (1, 27) are refractory to the fungal toxin.

Unlike some of the animal RNA polymerase enzymes (3, 4, 10), the cauliflower RNA polymerases are not easily distinguished by either divalent cation preference or ionic strength optima. The cauliflower enzymes are somewhat similar to animal RNA polymerases in template preferences; i.e., RNA polymerase II shows a stronger preference for denatured DNA than either form I or III (3, 10) and RNA polymerase I shows a very strong preference for poly(dC) (8). However, unlike animal RNA polymerase III (20, 23), cauliflower RNA polymerase III is not easily distinguished from cauliflower form I and II by using a poly(dA:T) template.

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LITERATURE CITED