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CHARACTERIZATION OF AN α-AMANITIN INSENSITIVE RNA POLYMERASE FROM CAULIFLOWER

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Abstract—α-Amanitin insensitive RNA polymerase (polymerase I) isolated from apical parts of the cauliflower inflorescence was highly stable for several months at −18°. The DEAE-cellulose fraction was more effective in utilizing denatured DNA than native DNA as a template. Optimum pH for RNA synthesis was *ca* 7 in the reaction mixture with Tris–HCl or with Tris–maleate buffer. From the properties examined, it seems that DNA-dependent RNA polymerase I of cauliflower differs from other eucaryotic RNA polymerases.

INTRODUCTION

Multiple forms of DNA-dependent RNA polymerase have been detected in a number of eucaryotic organisms [1–4]. At least one enzyme (I or A) is derived from the nucleolus [5], and is insensitive to α-amanitin [6–8]. Generally, the nucleolar enzyme is unstable and comprises a small proportion of the total nuclear enzyme [9,10]. In a previous study on DNA-dependent RNA polymerase from cauliflower inflorescence, we have demonstrated that the polymerase I fraction possesses a high synthesizing activity of polyguanylic acid and polyadenylic acid [11]. The cauliflower inflorescence exhibits a peculiar hypertrophic differentiation with continuous nuclear and cell divisions until floral primordia have developed [12]. In order to characterise the cauliflower RNA polymerase, we have studied the *in vitro* RNA synthetic properties of the polymerase I fraction.

RESULTS

Separation and stability of RNA polymerase I

As described in previous papers [11,13], DNA-dependent RNA polymerase is easily solubilized from the apical parts of cauliflower inflorescence. DEAE-cellulose column chromatography resolved the enzyme activity into two main peaks called in order of elution polymerase I and II. A greater amount of polymerase I was always obtained. In our preliminary report [13], three peaks of enzyme activity were recognized. The peaks 1 and 2 were close together and frequently fused when eluted on a linear gradient of KCl on a DEAE-cellulose column. Both peaks being insensitive to α-amanitin are treated as a single enzyme, polymerase I in the present report. Polymerase II was sensitive to α-amanitin.

DEAE-polymerase I eluted in TGMED-buffer (50 mM Tris–HCl pH 8, 5 mM $MgCl_2$, 0·1 mM EDTA, 1 mM dithiothreitol, 25% glycerol) was highly stable compared to polymerase II (Table 1), with only a slight loss of activity observed after several months at −18°.

Experimental conditions affecting RNA polymerase activity

The reactions in the standard assay system were closely dependent on the enzyme concentration. The experiment with the DEAE-gradient-enzyme gave a linear activity curve up to 70 μg of protein examined. The DEAE-stepwise-enzyme also showed activities proportional to the enzyme concentration up to 340 μg of protein in the assay system. Further assays were conducted in a range where activity depended linearly on the protein concentration. A broad optimum was found around pH 7 in Tris–maleate buffer [14]

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Table 1. Stability of cauliflower RNA polymerase stored in TGMED-buffer

Expt.	Assayed	Polymerase I		Polymerase II	
		Activity (pmol)	%	Activity (pmol)	%
1	Freshly prepared	790	100	36·2	100
	After 4 months	676	85·8	7·1	19·6
2	Freshly prepared	305	100	56·5	100
	After 6 months	146	47·8	9·1	16·1

DEAE-enzyme fractions in TGMED-buffer were stored at −18°, and the enzyme activity was assayed in the standard assay system (0·125 ml) with 75 μl of the respective enzyme solution. The activity was expressed as pmol of ^{14}C-labeled uridine-monophosphate incorporated for 10 min. Experiment 1; DEAE-stepwise-enzyme containing 4·3 mg of protein/ml in polymerase I and 0·96 mg/ml in polymerase II. Experiment 2; DEAE-gradient-enzyme containing 0·94 mg of protein/ml in polymerase I and 1·3 mg/ml in polymerase II.

and in Tris–HCl buffer pH 7 was also optimal. In a parallel experiment, the optimum pH of *E. coli* RNA polymerase in Tris–HCl buffer was 8·0–8·5 as shown previously by Kitano [15].

The rate of RNA synthesis was investigated at various concentrations of uridine-5′-triphosphate-[4-^{14}C] from 0·025 to 0·4 mM. A substrate concentration of 0·2 mM was sufficient for the reaction. The K_m value for the substrate was 0·08 mM in the assay system.

The time course of radioactivity incorporation was surveyed at various temperatures; 30, 35, 40, 44 and 50°. The incorporation was almost linear during the first 10 min, then increased gradually except that incubation at 50° gave a decreased incorporation after 10 min.

Characteristics

The incorporation of uridine-5′-triphosphate-[4-^{14}C] into acid-insoluble material was dependent on the presence of all four ribonucleoside triphosphates, denatured DNA and a divalent metal ion (Table 2). The effect of Mn^{2+} and Mg^{2+}, added separately, on the rate of radioactivity incorporation was investigated. Optimum concentrations of Mg^{2+} and Mn^{2+} were 2 and 1 mM, respectively. The activity ratio at the optimum ionic concentration for Mn^{2+} to Mg^{2+} was *ca* 0·9. High concentrations of divalent metal ion were inhibitory, Mn^{2+} more so than Mg^{2+}. In the presence of 2 mM Mg^{2+}, Mn^{2+} stimulated RNA synthesis at lower concentrations but 1 mM Mn^{2+} hardly increased RNA synthesis compared to that in the absence of Mn^{2+}.

The effect of ionic strength on the enzyme activity was examined with KCl or $(NH_4)_2SO_4$. The activity reached a peak in the presence of 20 mM $(NH_4)_2SO_4$ and was markedly inhibited at higher concentrations. Less inhibition, however, was found with KCl, though a broad optimum range was obtained.

The effectiveness of various templates during a 10 min incubation was surveyed (Table 2). Denatured calf-thymus DNA was most effective

Table 2. Requirements for RNA polymerase I

Assay system	Enzyme activity (%)	Assay system (mM)		Enzyme activity (%)
Complete	100	Mn^{2+}*	0	94·9
−DNA	10·1		0·25	122·8
−DNA, +native DNA	20·9		0·5	120·4
−DNA, +yeast *r*RNA	7·7		2·0	69·5
−DNA, +yeast *t*RNA	11·1	$+(NH_4)_2SO_4$	20	110·8
−ATP	28·8		100	62·3
−CTP	20·9		200	4·4
−GTP	16·0	+KCl	20	116·7
$-Mg^{2+}$, $-Mn^{2+}$	0·2		100	119·6
$-Mg^{2+}$	84·4		200	92·7

Complete reaction mixture (0·125 ml) was as decribed in Experimental.
* Respective concentrations of Mn^{2+} were used instead of 1 mM Mn^{2+} in the complete system.

Table 3. Inhibitors affecting RNA polymerase activity

Addition		Enzyme activity (%)
Complete		100
+α-amanitin	8 μg/ml	100·5
	20 μg/ml	101·2
	40 μg/ml	97·4
+Pancreatic RNase	80 μg/ml	28·1
+Rifampicin	20 μg/ml	103·4
+Cycloheximide	200 μg/ml	101·0
+Sodium phosphate	0·5 mM	99·9
	1·0 mM	95·6
+Sodium pyrophosphate	0·5 mM	94·5
	1·0 mM	64·7
+Actinomycin D	10 μg/ml	67·8

Complete reaction mixture (0·125 ml) was as described in Experimental.

but Yeast ribosomal and transfer RNAs could not replace it. The kinetics of the reaction was examined with denatured calf-thymus DNA at various concentrations from 1 to 40 μg/ml and the K_m value was 5 μg/ml in the system. The enzyme activity was proportional to the amount of denatured DNA at the lower concentration and was almost saturated at about 40 μg/ml of DNA. In contrast, native DNA was markedly less effective than denatured DNA, and no increase was observed even when a high concentration was added to the reaction mixture.

The effect of various polymerase inhibitors on the cauliflower RNA polymerase activity was measured *in vitro* (Table 3). Rifampicin, an inhibitor of bacterial DNA-dependent RNA polymerase, and cycloheximide had no inhibitory effects on the activity. Inorganic phosphate was added to the incubation medium at a final concentration of 0·5 mM and 1 mM; no inhibition occurred with orthophosphate, while considerable inhibition was observed with 1 mM pyrophosphate.

The nucleotide composition of RNA synthesized *in vitro* was estimated using the four labeled nucleoside triphosphates, comparing the cauliflower RNA polymerase with *E. coli* RNA polymerase, with denatured calf-thymus DNA as template. The incorporation of each nucleotide into RNA was measured and molar ratios were calculated. Almost the same ratios of incorporation were observed for the four nucleotides with both RNA polymerases. In cauliflower RNA polymerase these were: uridine 32·6%; adenine 25·5%; guanine 20·8%; and cytosine 21·1%. This base ratio is in accord with the base composition of calf-thymus DNA, though UTP-incorporation is a little high.

DISCUSSION

Isolation of highly stable RNA polymerase I from the apical parts of the cauliflower inflorescence has permitted the characterization of the RNA synthetic reaction. The enzyme requires the presence of four nucleoside triphosphates, a divalent metal ion, and a denatured DNA template to catalyze RNA synthesis. Orthophosphate does not inhibit incorporation of labeled substrate, but pyrophosphate does. This suggests that a significant portion of the ribonucleotide incorporation was not due to the contamination of polynucleotide phosphorylase activity in the preparation. Rifampicin had no inhibitory effect on the enzyme activity, suggesting that the activity was not due to bacterial contamination. Cycloheximide had no inhibitory effect, and this enzyme appeared to be unlike RNA polymerase I from aquatic fungi [16,17] and also that from rat liver [18].

It is known that nucleolar RNA polymerase is quite unstable, and is usually contained in smaller amounts in the nucleus than is nucleoplasmic RNA polymerase [6]. It is also known that growth stimulating hormones produce increased activity of the nucleolar RNA polymerase in plant [19,20] and animal tissues [21–23], and that virus infection results in the increase of nucleolar RNA polymerase activity [24]. Cacace and Nucci [25] have observed that two of three RNA polymerases in calf-liver nuclei were insensitive to α-amanitin. The cauliflower RNA polymerase I is completely insensitive to α-amanitin, and is probably a nucleolar enzyme. It is more stable than polymerase II, and the optimum pH of the enzyme is 7 in the reaction mixture. It seems that cauliflower RNA polymerase I has somewhat different properties from other eucaryotic RNA polymerases [26,27]. Sasaki *et al.* [28] also separated two RNA polymerases from the cauliflower inflorescence with DEAE-Sephadex column chromatography and obtained a slightly different elution pattern from that obtained by us with DEAE-cellulose column chromatography. The difference may be attributed to the procedures used for enzyme extraction.

Cauliflower RNA polymerase I is more effective in utilizing denatured DNA than native DNA.

This template preference is an intrinsic property of the enzyme since it cannot be attributed to saturation of the system by DNA. The marked preference for denatured DNA template in cauliflower RNA polymerase I resembles the action in maize [3], soybean [8] and yeast RNA polymerases [29].

It is interesting, in spite of the different properties of cauliflower RNA polymerase described above, that the base components of RNA synthesized *in vitro* resemble the base ratio of RNA synthesized by *E. coli* RNA polymerase, when calf-thymus DNA was used as template.

EXPERIMENTAL

Preparation of RNA polymerase I. DNA-dependent RNA polymerase I was isolated by almost the same procedures as described in a previous paper [11]. 100 g of frozen tissues were homogenized with TGMED-buffer (50 mM Tris–HCl pH 8, 5 mM $MgCl_2$, 0·1 mM EDTA, 1 mM dithiothreitol, 25% glycerol) containing 0·6 M $(NH_4)_2SO_4$ and 5 g of PVP. PVP (20 g) was mixed with 200 ml of the homogenate and centrifuged at 15000 *g* for 40 min. The supernatant was centrifuged again at 105000 *g* for 60 min. The supernatant was brought to 50% $(NH_4)_2SO_4$ saturation by addition of saturated $(NH_4)_2SO_4$ soln (prepared at 25° and adjusted to pH 8 with NH_4OH). The ppt. was dialyzed 18 hr against TGMED-buffer. The dialysate was centrifuged at 15000 *g* for 40 min and the supernatant was loaded on a DEAE-cellulose column (1·5 × 15 cm) previously equilibrated with TGMED-buffer. The enzymes were eluted, with a linear gradient concentration of KCl from 0 to 0·4 M in TGMED-buffer (DEAE-gradient-enzyme). In some cases, stepwise elution was done with 150 ml of TGMED-buffer containing 0·12 M KCl (DEAE-stepwise-enzyme), followed by a linear gradient of KCl from 0·12 to 0·4 M in TGMED-buffer. *E. coli* (A-19) RNA polymerase was purified to the extent of fraction 4 in Ref. [30].

Assay for RNA polymerase activity. Enzyme activity was assayed as described previously [12]. The standard reaction mixture (0·125 ml) contained: 40 mM Tris–HCl pH 7·9; 1 mM $MnCl_2$; 2 mM $MgCl_2$; 12 mM 2-mercaptoethanol; 0·4 mM each of adenosine-5′-triphosphate; guanosine-5′-triphosphate; cytidine-5′-triphosphate; and 0·2 mM uridine-5′-triphosphate-[4-^{14}C] (8 μCi/μmol), 40 μg/ml heat-denatured calf-thymus DNA and enzyme soln. After incubation at 40° for 10 min, 100 μl of the incubation mixture were put onto a filter paper-disc (24 mm), and the radioactivity was measured with a scintillation counter. Enzyme activity was expressed as pmol of nucleotides incorporated under the respective assay conditions. Corrections of the activity values were made for zero-time controls. Radioactive ^{14}C-labeled nucleoside triphosphates (Radiochemical Centre, Amersham), unlabeled nucleoside triphosphates, calf-thymus DNA (type I) and yeast RNA (type XI) (Sigma Chemical Co.), yeast transfer RNA and α-amantin (Boehringer Co.), pancreatic ribonuclease (3000 units/mg) (Worthington Biochemical Co.), Rifampicin (Rifampin) (Calbiochem, Inc.), actinomycin D (Schwarz–Mann.) and DEAE-cellulose DE-52 (Whatman Co.) were used.

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