KB CELL RNA POLYMERASES: OCCURRENCE OF NUCLEOPLASMIC ENZYME III

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1. Introduction

It has been established that eukaryotic DNA-dependent RNA polymerases exist in multiple forms [1, 2], since the initial studies with isolated nuclei have shown that at low ionic strength (in the presence of Mg^{2+}) RNA synthesis occurs preferentially in the nucleolus producing a ribosomal-like RNA while at higher ionic strength (in the presence of Mn^{2+}) RNA synthesis is stimulated, producing DNA-like RNA [3, 4]. According to the nomenclature proposed by Kedinger et al. [5] five different forms have been reported: AI, AII, AIII, BI and BII. These were originally designated by Roeder and Rutter as I, II and III [1] corresponding to forms AI/AII, BI/BII and AIII, respectively. RNA polymerase I is restricted to the nucleolus whereas the enzymes II and III are located in the nucleoplasm. The form II is sensitive to α-amanitin and preferentially stimulated by Mn^{2+} at high ionic strength. In contrast, forms I and III are not sensitive to α-amanitin; the enzyme I has optimum activity in low-salt conditions with Mg^{2+} ions [1, 6].

The properties of the mammalian RNA polymerase III have not been reported. During our studies on KB cells RNA polymerases we have observed that enzyme III has a different behaviour whether DEAE-Sephadex or DEAE-cellulose column chromatography is used for isolation; the former technique enabled us to detect a minor peak of RNA polymerase III activity, whereas this enzyme is not detected during DEAE-cellulose chromatography. Compared to RNA polymerases I and II, enzyme III is more labile to thermal shock, exhibits an optimal activity in a wider range of ionic strength and has a higher sedimentation rate on glycerol density gradient centrifugation.

2. Materials and methods

RNA polymerases were extracted from KB cell nuclei [7] or rat liver essentially by the method of Roeder and Rutter [8] modified by Chesterton et al. [9]. The enzymic extract was applied to a DEAE-Sephadex A-25 or DEAE-cellulose DE-52 column and eluted with a linear gradient of 0.04 M–0.5 M $(NH_4)_2SO_4$ in TGMED buffer (0.05 M Tris–HCl pH 8,25% v/v glycerol, 5 m $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol). Unless specified, enzyme activity was assayed, at optimal salt concentration, as described in fig. 1. α-Amanitin was used at 0.8 μg/ml. After incubation for 1 hr at 37°C, the reactions were stopped by adding 2 ml of ice-cold 10% trichloroacetic acid containing 0.02 M sodium pyrophosphate. The precipitate was collected on a glass fiber filter (Whatman GF/C), washed with 12 × 5 ml 2% TCA, rinsed with 2 ml of absolute ethanol, dried and counted in a toluene based scintillator. Pre-heating of RNA polymerases was performed as indicated in fig. 3 and table 1 according to Shields and Tata [10]. DNA was solubilised from KB cells by Sarkosyl–Pronase treatment, isolated by CsCl equilibrium gradient centrifugation and stored at 4°C in 0.1 × SSC (0.015 M

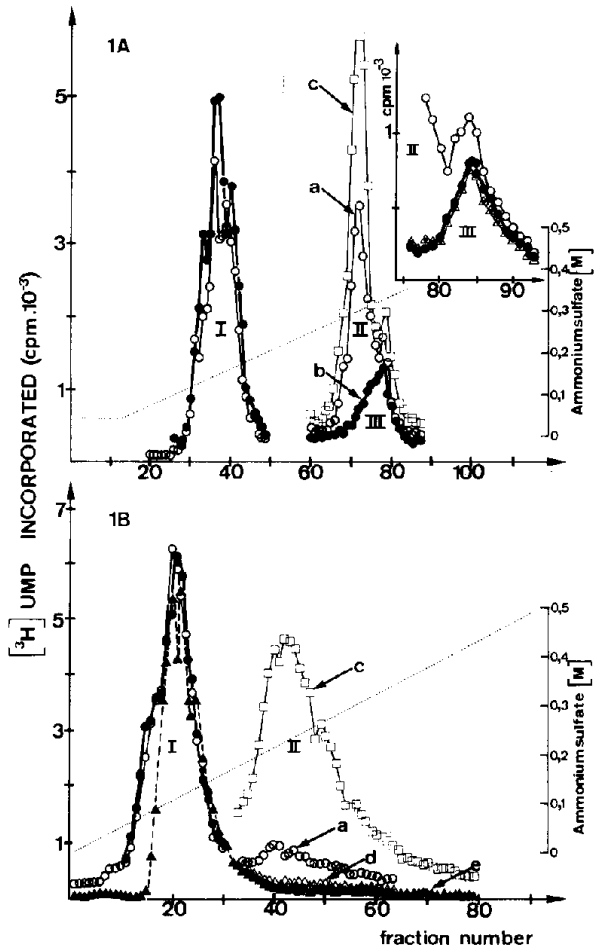

Fig. 1. Ion exchange column chromatography of KB cell RNA polymerases. A sample containing 30 mg of enzymic extract [9] was applied to a 1 × 15 cm DEAE-Sephadex A-25 (1A) or a 1.4 × 7 cm DEAE-cellulose DE-52 (1B) column. The enzymes were eluted with a 160 ml linear gradient of 0.04 M–0.5 M $(NH_4)_2SO_4$ in TGMED buffer [8] and collected in serum albumin (1 mg/ml final concn.). Column fractions (1.2–1.5 ml) were assayed (1 hr at 37°C) in a final volume of 150 μl containing 50 μl of each fraction; 0.4 mM ATP, GTP, CTP; 0.015 mM [^{3}H]UTP (1 Ci/mM); 10 μg/ml (1A) or 40 μg/ml (1B) of DNA; 6 mM $MgCl_2$; 3 mM $MnCl_2$; 50 mM Tris–HCl pH 8.0; 4 mM dithiothreitol; $(NH_4)_2SO_4$ concentration was exactly one-third of eluting concentrations, allowing optimal activity of each of the three enzymes. The activity is expressed in cpm (counts per min) of [^{3}H]UMP incorporated by aliquots (1 pmole of UMP corresponds to approx. × 10^3 cpm). RNA polymerase activity was assayed in the above conditions with: (○—○—○), native KB DNA; (●—●—●), native KB DNA and 0.8 μg/ml α-amanitin; (□—□—□), denatured calf-thymus DNA; (△—△—△), denatured calf thymus DNA and 0.8 μg/ml α-amanitin; (▲—▲—▲), native calf thymus DNA (separate DEAE-cellulose column chromatography collected without serum albumin). Inset (fig. 1A): shown for comparison of enzyme III activity with native and denatured KB DNA in the presence of 0.8 μg/ml of α-amanitin (a separate DEAE-Sephadex column chromatography. (▲—▲—▲), Denatured DB DNA.

Table 1
Thermal effect on KB and rat liver RNA polymerases isolated by DEAE-Sephadex column chromatography.

	Residual RNA polymerase activity in %		
	Enzyme I	Enzyme II	Enzyme III
KB cell enzymes	65	35 (33[a])	9
Rat liver enzymes	39	96	

[a] Enzyme isolated by DEAE-cellulose column chromatography.

KB and rat liver RNA polymerases were prepared as described in the text and fig. 1. Pre-heating of both KB and rat liver RNA polymerases [10] were for 10 min at 46°C, then enzymes were assayed at 37°C for 1 hr as described in figs. 1 and 3. Residual activities are expressed as percent of the control values at 37°C. Control values for 100 μl of unheated KB cell RNA polymerases I, II and III at 37°C were 10 500 cpm/60 min, 6 300 cpm/60 min and 3 500 cpm/60 min, respectively. Corresponding control values for the rat liver enzymes were 840 cpm/60 min for RNA polymerase I and 14 500 cpm/60 min for RNA polymerase II.

NaCl, 0.0015 M sodium citrate), by methods similar to those previously described [11]. Isolated enzymes stored at −70°C, in eluting TGMED buffer containing 1 mg/ml serum albumin, retain their initial activity for several months.

3. Results and discussion

RNA polymerases obtained from KB cell nuclei, as shown in fig. 1A can be separated into components I, II and III according to their elution order from DEAE-Sephadex [1] and corresponding to AI/AII, BI/BII and AIII, respectively [5]. Addition of α-amanitin to the assays of the column effluent results in a specific inhibition of RNA polymerase II, as shown previously by Kedinger et al. [2] and Lindell et al. [12], allowing localization of RNA polymerase III (compare curves a and b). Substitution of denatured DNA for native DNA results in stimulation of RNA polymerase II, whereas the RNA polymerase III (in the presence of α-amanitin) results in an activity of the same order as that with native DNA (see fig. 1A and inset). To assess the relative loss of enzymes II or III during the extraction procedure of nuclei, we compared yields (after DEAE-Sephadex chromatography)

Fig. 2

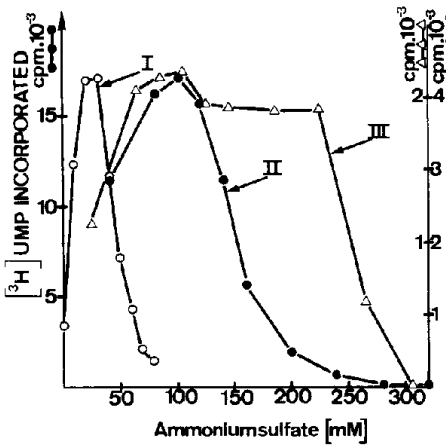

Fig. 3

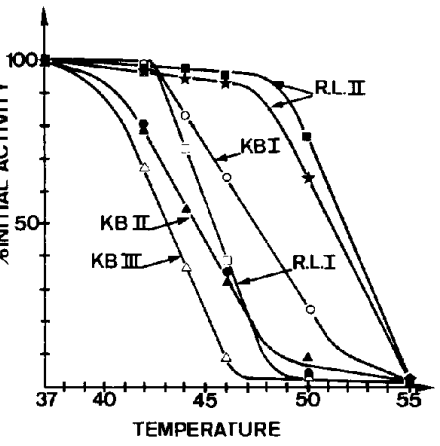

Fig. 2. Effect of ammonium sulfate on KB RNA polymerases I, II and III. DEAE-Sephadex enzyme fractions (containing 1 mg/ml albumin) were dialyzed overnight against TGMED without $(NH_4)_2SO_4$. Enzyme activity was assayed with native KB cell DNA (50 μg/ml) as described in fig. 1. Enzyme III was assayed in the presence of 0.8 μg/ml α-amanitin. (○—○—○), RNA polymerase I; (●—●—●), RNA polymerase II; (△—△—△), RNA polymerase III.

Fig. 3. Thermal sensitivities of KB and rat liver RNA polymerases. Enzymes were isolated by ion exchange column chromatography as described in Materials and methods and fig. 1. 100 μl Of enzyme fraction, containing 1 mg/ml serum albumin, were incubated at indicated temperatures for 10 min, cooled on ice [10], then remaining enzyme activity was assayed as indicated in fig. 1 and expressed as percent of the control values at 37°C. DEAE-Sephadex enzymes: KB RNA polymerases I (○—○—○), II (●—●—●), III (△—△—△) assayed with 20 μg/ml native KB DNA, and rat liver RNA polymerases I (□—□—□), II (■—■—■) assayed with 50 μg/ml denatured calf thymus DNA. Rat liver RNA polymerase II (★—★—★) was also assayed at higher salt concentration than that required for optimal activity, as described by Shields and Tata [10]. DEAE-cellulose enzyme II from KB cells was assayed with 20 μg/ml of denatured KB DNA (▲—▲—▲).

of these enzymes with those obtained from intact KB cells, as well as from a mixture of intact KB cells and isolated nuclei. No significant differences were found in the activity of these enzymes. These results also show that the origin of enzyme III is rather nucleus than cytoplasm, as already pointed out by Roeder and Rutter [1]. The same enzymic extract as that used in fig. 1A applied on a DEAE-cellulose column does not show any appreciable activity peak of enzyme III in the presence of native KB DNA. Enzyme II also shows a very low activity (see fig. 1B, curve a). With denatured DNA substituted for native KB DNA, a prominent peak of enzyme II is observed, although addition of α-amanitin does not reveal any detectable amount of RNA polymerase III (fig. 1B, curve c and d). Fractions from a separate DEAE-cellulose column chromatography, not collected in the presence of serum albumin, do not show any detectable polymerase II activity with native DNA (see fig. 1B, curve e).

The behaviour of RNA polymerase III on these two ions exchangers shows that this enzyme cannot be detected by DEAE-cellulose column chromatography. The reasons for the inability of enzyme II obtained on DEAE-cellulose to transcribe efficiently native DNA are unknown. Additional proteins involved in transcription by RNA polymerase II might be lost during DEAE-cellulose column chromatography.

Changes in ionic strength markedly influenced the RNA polymerase III activity as observed with other polymerases; form III exhibits maximum activity in a wide range between 60 and 225 mM ammonium sulfate while form I and II exhibit optimal activity at 20–40 and 80–120 mM salt, respectively (see fig. 2). The effect of various salt concentrations on the activity of the KB cell enzymes is similar to that for the three sea urchin [1] and yeast [13] enzymes, as well as for rat-liver [1] and calf thymus [14] RNA polymerases I and II. It should be pointed out that ammonium sulfate concentrations required for optimal activity of KB enzymes I and II in the presence of intact Adenovirus 2 DNA are lower compared to those required in the presence of native KB DNA (unpublished results).

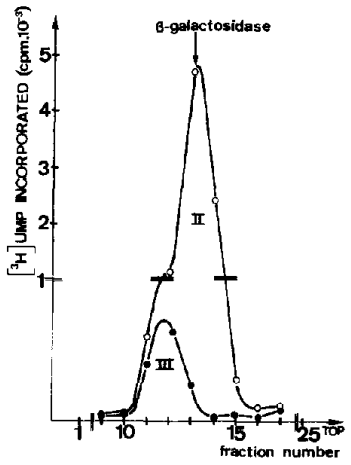

Fig. 4. Relative sedimentation rates of KB RNA polymerases II and III. A mixture of enzymes II and III, isolated by DEAE-Sephadex column chromatography, was dialysed against 50 mM Tris–HCl buffer (pH 8), 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 100 mM $(NH_4)_2SO_4$, then layered (0.5 ml) over 11 ml linear 10–40% (w/v) glycerol gradient made up in the same buffer [9, 14] containing 500 μg/ml serum albumin [17]. Centrifugation was made at 36 000 rpm in a Spinco SW 41 rotor for 15 hr at 0°C. β-Galactosidase (marker) was detected as previously described [18]. RNA polymerase activity was assayed at 120 mM $(NH_4)_2SO_4$ as indicated in fig. 1, in the presence of 50 μg/ml native KB DNA with (•—•—•) and without (○—○—○) 0.8 μg/ml α-amanitin.

Pre-heating KB RNA polymerases for 10 min at different temperatures, according to the conditions of Shields and Tata [10], showed differences in the degree of sensitivity to heat between the three enzymes. Polymerase III was more sensitive than enzymes I and II (fig. 3). As shown in table 1, enzyme III retains less than 10% of its initial activity after pre-heating at 46°C for 10 min, whereas polymerase I, the most resistant in the same conditions, still retains about 65% of its initial activity. It is noteworthy that 'DEAE-Sephadex enzyme II' in the presence of native KB DNA and 'DEAE-cellulose enzyme II' which almost exclusively transcribes denatured DNA, exhibit identical inactivation curves, suggesting that the heat-denaturation does not affect additional proteins in the DEAE-Sephadex enzyme preparation that might be responsible for double-stranded DNA transcription. Relative heat sensitivity of KB enzymes can change during storage if the low temperature (−70°C) is not maintained, althouth the highest thermal sensitivity was always attained for enzyme III. The enzymes from rat liver nuclei, isolated under similar conditions (including DEAE-Sephadex chromatography) showed heat denaturation curves quite different from those of the KB enzymes. The difference was particularly pronounced for enzyme II, (fig. 3 and table 1). In agreement with recently reported data by Shields and Tata [10] rat liver enzyme II exhibits a higher thermal stability than enzyme I. In our conditions, the enzymes showed higher overall heat stability, presumably due to the protecting effect of serum albumin added to prevent enzyme inactivation during fractionation and storage (see Materials and methods). Changes in the concentration of ammonium sulfate, ranging from 30–130 mM for enzyme I and 30–250 mM for enzyme II, had little effect on the heat stability (using treatments at 46°C for 10 min). The residual activity for rat liver enzyme I varied from 35–38%, and that for rat liver enzyme II from 92–99%. So far, enregistered heat sensitivity of RNA polymerases may not be entirely due to their intrinsic properties, but could also be accounted for by an incidental action of proteases on these enzymes.

As reported by Chambon et al. [14] and Chesterton et al. [9] RNA polymerase II sedimented slightly more rapidly than polymerase I and *E. coli* RNA polymerase (in the presence of 100 mM ammonium sulfate). After DEAE-Sephadex column chromatography, a fraction of enzyme II, containing a sufficient amount of enzyme III, was run on a glycerol gradient at 100 mM ammonium sulfate. Fig. 4 shows that enzyme III activity (measured in the presence of α-amanitin) sediments faster than enzyme II whose molecular weight is close to that of β-galactosidase, i.e. about 595 000 [15] (the value for RNA polymerase II is similar to those obtained for the BI and BII enzymes from calf thymus [16]).

The unique characteristics of RNA polymerase III (sedimentation rate, influence of salt concentration on activity, differential heat inactivation) show that this enzyme presents a distinct entity in the KB cell nucleus. The exact role that this enzyme may play is unknown. Zylber and Penman have shown a nucleoplasmic amanitin-resistant activity in HeLa cell nuclei resembling polymerase III [19]. Their data suggest that RNA polymerase III as well as enzyme II, produce nuclear heterogeneous RNA.

Acknowledgements

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