DIFFERENTIAL THERMAL SENSITIVITIES OF EUKARYOTIC DNA-DEPENDENT RNA POLYMERASES

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1. Introduction

Since the original suggestion [1] of the multiplicity of nuclear DNA-dependent RNA polymerases, it has been established that eukaryotic RNA polymerases exist in at least two different forms: A and B [2] which consist of dissimilar polypeptides [3]. RNA polymerase A is preferentially stimulated by Mg^{2+} and is insensitive to inhibition by the fungal toxin α-amanitin. In contrast, form B which is preferentially stimulated by Mn^{2+} at high ionic strength, is inhibited by α-amanitin [4, 5]. In addition to response to α-amanitin, the two forms of RNA polymerase can be distinguished by resolution on ion-exchange chromatography columns [6]. Both these procedures involve drastically raising the ionic strength to supraphysiological levels in order to distinguish between RNA polymerases A and B. We felt that such variation of the ionic environment would be unsuitable for analysing the transcription of endogenous chromatin template [7] during development, since high ionic strength is known to markedly alter both chromosomal structure [8] and RNA polymerase activity [1, 2].

During our studies on animal RNA polymerases we have observed that the enzymes exhibit differential thermal sensitivities. This property offers a procedure to compare the activities of RNA polymerases A and B under identical and nearly physiological ionic conditions. It will be shown that RNA polymerase A from rat-liver and yeast nuclei is more labile to thermal shock than is RNA polymerase B. Raising the temperature from 37° to 45° causes up to 90% inhibition of polymerase A, whereas enzyme B loses only about 30% of its activity at 37°. Thus, pre-heating either whole nuclei or isolated enzymes, for short periods of time at 45–48°, prior to enzyme assay at a lower temperature, makes it possible to examine form B (the α-amanitin sensitive polymerase) in the virtual absence of the activity due to RNA polymerase A.

2. Materials and methods

Preparation of rat-liver nuclei and assay of RNA polymerase activities were as previously described [9]. RNA polymerases were extracted from rat-liver nuclei essentially by the method of Roeder and Rutter [6, 10], except that desalting of the saturated $(NH_4)_2SO_4$ fraction was achieved by passage through a Sephadex G-25 column, rather than dialysis. The protein eluate from this column was centrifuged for 30 min at 105,000 *g*, the supernatant applied to a DEAE Sephadex A-25 column (1.5 cm × 13 cm) and eluted with a linear gradient of 0.05 M to 0.5 M $(NH_4)_2SO_4$. Assay of column fractions was exactly as previously described [9].

Experiments on the thermal stability of isolated enzymes were performed by heating RNA polymerases A and B for 10 min at 45°. For extraction of RNA polymerase from pre-heated nuclei, the nuclear suspension was cooled to 0° immediately after heating and the enzymes isolated by the same procedure as above.

DNA was determined by the method of Burton [11] and RNA by the method of Fleck and Munro [12].

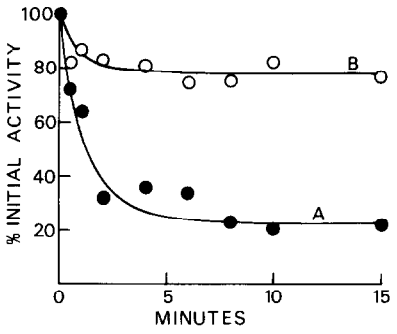

Fig. 1. Time course of thermal inactivation of isolated rat liver nuclear RNA polymerases A and B. RNA polymerases A and B, extracted from rat-liver nuclei, were isolated by a modification of the method of Roeder and Rutter [6, 10]. Nuclei were prepared from 45–50 g rat-liver by the method of Blobel and Potter [13]. The nuclei were suspended in buffer containing 1 M sucrose, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 50 mM Tris-HCl pH 7.9, at a concentration of 1 ml buffer/g equivalent of liver and sonicated for a total of 60 sec in 0.3 M $(NH_4)_2SO_4$. The suspension was centrifuged for 1 hr at 105,000 *g*; 0.42 g/ml $(NH_4)_2SO_4$ added to the supernatant and the solution stirred for 30 min. The suspension was centrifuged at 105,000 *g* for 45 min and the pellet, having been suspended in 3 ml of TGMED buffer containing 0.05 M $(NH_4)_2SO_4$, was applied to a Sephadex G-25 column. The protein eluate was centrifuged at 105,000 *g* for 30 min, the pellet discarded, and the supernatant applied to a DEAE-Sephadex A-25 column (1.5 × 13 cm). The enzymes were eluted with a linear gradient of 0.05 to 0.5 M $(NH_4)_2SO_4$ in TGMED buffer. Column fractions were assayed in a final volume of 75 μl containing 50 μl of each fraction; 0.4 mM ATP, CTP, GTP; 8 μM UTP containing 0.5 μCi [^{3}H]UTP; 100 μg/ml denatured calf thymus DNA; 2 mM $MnCl_2$; 62.5 mM Tris-HCl pH 8.0; 7% (v/v) glycerol; 50 mM KCl; 25 mM β-mercaptoethanol. A typical column elution profile is shown in fig. 3a. Fractions 50–54 inclusive were combined to give RNA polymerase A and fractions 75–78 inclusive constituted RNA polymerase B. The A_{260}/A_{280} ratios for the enzyme preparations A and B were 0.72 and 0.78, respectively. Isolated enzymes A and B were pre-heated at 45° for the times indicated in the figure, and then immediately cooled on ice. The enzymes were then incubated at 37° for 30 min with the above components to determine residual RNA polymerase activity: (•—•—•), RNA polymerase A; (○—○—○), RNA polymerase B. Incorporation of [^{3}H]UTP into RNA was determined for all experiments by a modification of the filter paper disc method of Bollum [14]. Dried discs were treated with 0.1 ml water and 0.5 ml NCS solubilizer (Amersham/Searle) and incubated at 50° for 1 hr. 10 ml of scintillant consisting of 4 g PPO per ℓ of toluene was added to each vial. This procedure achieved counting efficiencies of between 45% and 50% ^{3}H in a Packard 2425 liquid scintillation counter.

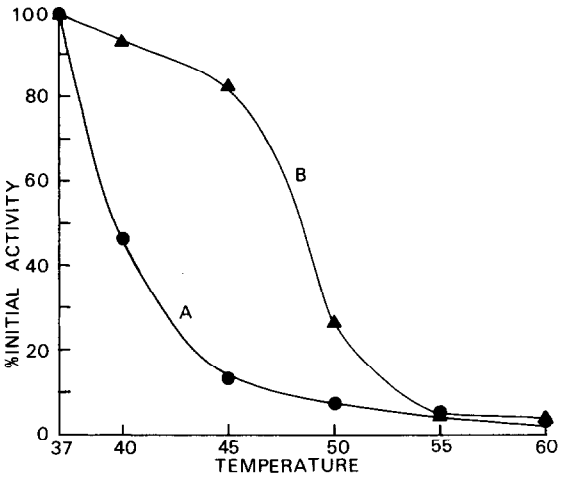

Fig. 2. Effect of pre-heating isolated rat liver RNA polymerase activities A and B at different temperatures. Enzymes A and B were isolated by DEAE-Sephadex chromatography as described in the text and fig. 1. 50 μl of enzyme preparation were incubated at the above temperatures for 10 min and then cooled on ice. The remaining enzyme activity was then assayed exactly as described in fig. 1. (•—•—•), RNA polymerase A; (▲—▲—▲), RNA polymerase B.

3. Results and discussion

Pre-heating the isolated RNA polymerase A and B at 45°, for the times indicated in fig. 1, showed a major difference between the two enzyme activities, when assayed at 37°. Fig. 1. shows that RNA polymerase A is inactivated by about 80% after 5 min of heating. In other experiments, RNA polymerase A lost 80% of its activity within 3 min of heating and was 90% inactivated by 5 min pre-heating. Polymerase B, although losing some activity at 45° after 2 min pre-heating, was inactivated by about only 20% after 5 min. The reason for residual activity observed at the end of 15 min of heating is not known and may possibly be due to the presence of another RNA polymerase that is heat-resistant. It is however not due to the presence of DNA which may protect the enzyme as, a) there was less than 1% of nucleic acid present in the two enzyme preparations and b) heating the isolated enzymes in the presence of large amounts of native or denatured DNA did not alter their response to thermal shock. To ensure almost complete inactivation of RNA polymerase A whilst still being able to detect

Table 1
Effect of pre-heating isolated rat-liver nuclei on residual RNA polymerase activity.

Pre-heating temperature (°C)	RNA polymerase activity (pmoles UMP incorporated/mg DNA/10 min)		
	α-Amanitin-insensitive, assayed with		α-Amanitin-sensitive, assayed with
	Mg^{2+}	$Mn^{2+}/(NH_4)_2SO_4$	$Mn^{2+}/(NH_4)_2SO_4$
37	211	302	313
40	139	236	272
45	105	128	318
50	50	39	238
55	34	32	202

Values are from three separate experiments. Nuclei suspended in 0.25 M sucrose, 1 mM $MgCl_2$, at a concentration of approx. 2 mg DNA/ml were pre-heated for 10 min at the temperatures indicated. Assay of RNA polymerase activity was at 37° for 10 min in a final volume of 80 μl containing: 62.5 mM Tris-HCl pH 8.0; 25 mM KCl; 25 mM β-mercaptoethanol; 5% glycerol; 0.4 mM GTP, CTP, ATP; 8 μM UTP containing 0.5 μCi [^{3}H]UTP. For the Mg^{2+} activated enzyme, $MgCl_2$ was present at a final concentration of 6.25 mM. The concentration of $MnCl_2$ and $(NH_4)_2SO_4$ were 2.5 mM and 0.2 M, respectively, for the Mn^{2+} stimulated polymerase assayed at high ionic strength. When present, α-amanitin was used at a final concentration of 2 μg/ml. Results are expressed as α-amanitin sensitive ($Mn^{2+}/(NH_4)_2SO_4$ stimulated enzyme) or insensitive activity (Mg^{2+} activated enzyme). Values for α-amanitin sensitive activity were obtained by subtracting the α-amanitin resistant activity from the total activity, as described by Novello and Stirpe [15].

polymerase B activity, 10 min was chosen a convenient time for pre-heating.

The effect of pre-heating the isolated enzymes for 10 min at different temperatures can be seen in fig. 2. RNA polymerase A retains only about 10% of its initial activity if assayed after pre-heating for 10 min at 45°. RNA polymerase B, however, still retains 70–80% of its initial activity after heating at 45°, and it is only at temperatures above 50° that it is rapidly inactivated.

Having established that the isolated enzymes can be distinguished by differential thermal sensitivities, we also investigated the effect of pre-heating whole nuclei. As can be seen from table 1, the effect of pre-heating intact rat-liver nuclei was to inhibit drastically the RNA polymerase activity which is preferentially activated by Mg^{2+} at low ionic strength and would therefore correspond to RNA polymerase A. However, the activity of polymerase A, assayed in whole nuclei, required a slightly higher temperature for its inactivation than for the isolated enzymes (compare fig. 2 and table 1). The activity of RNA polymerase B assayed in whole nuclei, was only 20–25% inactivated by pre-heating at 50°. This residual heat resistant activity was identified as RNA polymerase B by its inhibition by α-amanitin. As can be seen from table 1, approx. 12%–15% of nuclear RNA polymerase is resistant to both high temperatures and to α-amanitin treatment. This residual activity implies the existence of an additional form of RNA polymerase in eukaryotic nuclei, a subject of some controversy [2, 10, 16].

That the effect of pre-heating is due to intrinsic differential thermal sensitivities of the RNA polymerases was further demonstrated by experiments in which RNA polymerases were solubilized from pre-heated rat liver nuclei and then resolved by ion-exchange column chromatography. It can be seen from the elution profiles of RNA polymerases extracted from nuclei pre-heated to 48° (fig. 3) that the peak of activity of enzyme A was absent but that enzyme B is still present, although at reduced activity. The elution profile of the unheated control nuclear extract was very similar to that previously described from our laboratory [9, 17] as well as by others [2, 6]. The small peak of enzyme activity which is eluted as a shoulder immediately before the peak of enzyme A, and which we have previously found to be α-amanitin resistant [9], is also heat sensitive, since it was not recovered from pre-heated nuclei. The enzyme activity from pre-heated nuclei which elutes before the salt gradient is applied to the DEAE-Sephadex column i.e. in tubes 15–20 (in fig. 3B), may have arisen, however, as a result of heating.

Preferential heat inactivation of polymerase A does not seem to be a phenomenon confined exclusively to rat liver. We have examined the effects of heating on isolated yeast RNA polymerases prepared by a modification of the method of Ponta et al. [17] and found similar results to those for rat liver polymerases described above. These findings, summarized in table 2, show that the selective thermal sensitivity of form A polymerase is more marked for yeast RNA polymerase than for the mammalian enzyme, the yeast polymerase B being completely stable even after 10 min

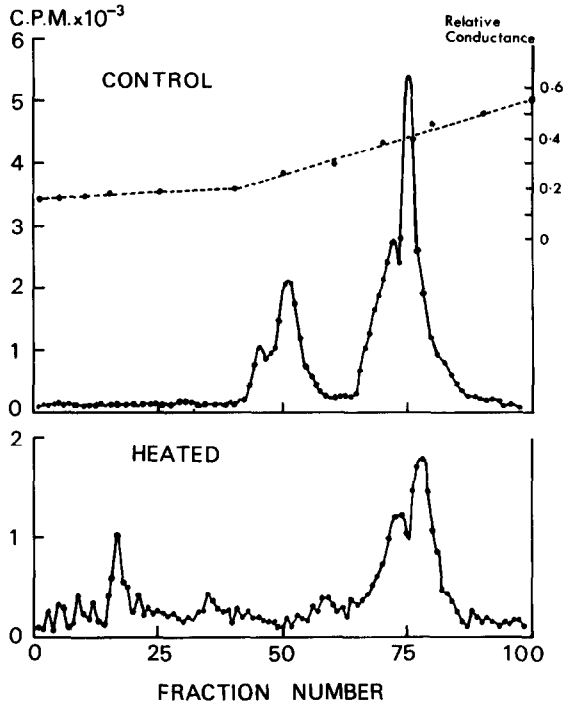

Fig. 3. Extraction of RNA polymerase A and B from heated and unheated rat liver nuclei. Nuclei prepared from 45 g of rat liver [13] were suspended in 1.0 M sucrose containing 5 mM β-mercaptoethanol, 5 mM $MgCl_2$, 50 mM Tris-HCl pH 7.9, at a concentration equivalent to 1 ml/g equivalent of liver, and heated at 48° for 10 min. After heating, the nuclear suspension was cooled on ice prior to sonication in 0.3 M $(NH_4)_2SO_4$. From this stage onwards control (unheated) and heated enzyme extractions and assay were performed in a manner identical to that described in fig. 1 and in the text. A) Elution profile of RNA polymerases from control nuclei. B) Elution profile of enzymes extracted from nuclei that had been pre-heated at 48° for 10 min.

heating at 50°. If such differential thermal sensitivity is applicable to all eukaryotes, then the observation [19] that heating ascites tumour cells at 44.5° for 30 min caused a preferential cessation of the synthesis of nucleolar 45 S RNA may be explained by the selective loss of RNA polymerase A activity at 45°.

In this context, it is important to note that enzymes A and B are predominantly segregated into nucleolar and extranucleolar compartments [2] so that in the intact cell the two enzymes are associated with different templates, factors, etc.

In conclusion, this report, which presents evidence of differential thermal sensitivities of RNA polymerases A and B, constitutes further evidence that the

Table 2
Differential thermal inactivation of isolated RNA polymerases A and B extracted from rat liver nuclei and yeast nuclei.

Pre-heating temperature (°C)	Rat liver RNA polymerase		Yeast RNA polymerase	
	A	B	A	B
37	–	–	–	–
40	59	25	18	–
45	85	30	53	–
50	93	87	73	5

Rat liver RNA polymerases were prepared as described in the text and fig. 1. RNA polymerases from yeast nuclei were prepared essentially by the method of Ponta et al. [17]. Preheating of both rat liver and yeast RNA polymerases was for 10 min at the temperatures mentioned above, the enzymes were subsequently incubated at 37° for 30 min as described in fig. 1. RNA polymerases from both species were assayed in exactly the same way, (see figs. 1 and 2) using denatured calf thymus DNA (100 μg/ml) as template. Results are expressed as percentage inhibition of the control values at 37°. Control values for 50 μl of unheated rat liver RNA polymerases A and B at 37° were 8900 cpm/30 min and 11,700 cpm/30 min, respectively. Corresponding control values for the enzymes extracted from yeast nuclei were 3000 cpm/30 min for RNA polymerase A and 4000 cpm/30 min for polymerase B.

two major eukaryotic nuclear RNA polymerases are distinct species of the enzyme. The higher sensitivity of RNA polymerase A to heating at 45° makes it possible to distinguish between the two (perhaps three) forms of RNA polymerases, either as they exist in intact nuclei or after their extraction. This forms the basis of a simple and effective diagnostic tool for assessment of the multiple RNA polymerases without raising the ionic strength and in the absence of α-amanitin.

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References

[1] C.C. Widnell and J.R. Tata, Biochim. Biophys. Acta 87 (1964) 513;
C.C. Widnell and J.R. Tata, Biochim. Biophys. Acta 123 (1966) 478.

[2] See numerous articles in: Cold Spring Harbor Symp. Quant. Biol. 35 (1970).

[3] F. Gissinger and P. Chambon, European J. Biochem. 28 (1972) 277;
C. Kedinger and P. Chambon, European J. Biochem. 28 (1972) 283.

[4] F. Stirpe and L. Fiume, Biochem. J. 105 (1967) 779.

[5] T.J. Lindell, F. Weinberg, P.W. Morris, R.G. Roeder and W.J. Rutter, Science 170 (1970) 447.

[6] R.G. Roeder and W.J. Rutter, Nature 224 (1969) 234.

[7] P.H.W. Butterworth, R.F. Cox and C.J. Chesterton, European J. Biochem. 23 (1971) 229.

[8] H. Harris, Nucleus and Cytoplasm (Oxford Univ. Press, 1968) p. 81.

[9] J.R. Tata, M.J. Hamilton and D. Shields, Nature New Biology 238 (1972) 161.

[10] R.G. Roeder and W.J. Rutter, Proc. Natl. Acad. Sci. U.S. 65 (1970) 675.

[11] K. Burton, Biochem. J. 62 (1956) 315.

[12] A. Fleck and H.N. Munro, Biochim. Biophys. Acta 55 (1962) 571.

[13] G. Blobel and V. Potter, Science 154 (1966) 1662.

[14] F.J. Bollum, Methods in Enzymology 12 Part B (1968) 169.

[15] F. Novello and F. Stirpe, FEBS Letters 8 (1970) 57.

[16] R. Price and S. Penman, J. Mol. Biol. 70 (1972) 435.

[17] E.A. Smuckler and J.R. Tata, Biophys. Biochem. Res. Commun. 49.

[18] H. Ponta, V. Ponta and E. Wintersberger. European J. Biochem. 29 (1972) 16.

[19] R. Simard, F. Amalric and J.P. Zalta, Exp. Cell Res. 55 (1969) 359;
F. Amalric, R. Simard and J.P. Zalta, Exp. Cell Res. 55 (1969) 370.