DISTINCT CYTOPLASMIC AND NUCLEAR DNA POLYMERASES FROM RAT LIVER

M.E. HAINES, A.M. HOLMES and I.R. JOHNSTON
Department of Biochemistry, University College London Gower Street, London WC1E 6BT, England

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1. Introduction

Interest in enzymes responsible for the synthesis of DNA has accelerated recently following the separation of DNA polymerases I and II in bacteria (for example [1]. Using aqueous isolation procedures the bulk of the DNA polymerase activity in mammalian systems is found in the cytoplasm [2]. It has also been found that residual activity, not easily removed by washing at low ionic strength, is present in the nucleus [3]. Our interest in pursuing a study of the nuclear and soluble (cytoplasmic) enzymes stemmed from previous work on the zonal fractionation of rat liver nuclei in which we found that nuclear DNA polymerase activity occurred predominantly in nuclei not involved in *in vivo* DNA synthesis [4]. In this paper we present evidence to show that the major soluble and the nuclear DNA polymerases of rat liver are, in fact, two distinct entities. The minor soluble polymerase appears similar in properties to the nuclear enzyme.

2. Materials and methods

DNAse I (electrophoretically purified) and calf thymus DNA (Type 1) were obtained from Sigma, Sepharose 6B from Pharmacia and phosphocellulose from Whatman. Rat liver DNA was prepared as before [4]. ^{3}H-TTP (13 Ci/mM) was from the Radiochemical Centre, Amersham, and deoxynucleoside triphosphates from Boehringer. Calf thymus DNA was activated 6-fold using DNAse I [5]. All buffers contained 20% w/v glycerol and except where stated, 1 mM 2-mercaptoethanol. Buffer A is 0.05 M Tris-HCl–1 mM EDTA, pH 7.5 (22°).

2.1. *DNA cellulose*

This was prepared as described by Litman [6] using UV light of 254 nm to bind calf thymus or rat liver DNA to Whatman CF2 cellulose powder. Activation was achieved by suspending 5 g of powder (4 mg DNA/g dry cellulose) in 25 ml 0.05 M Tris-HCl, pH 7.5 containing 1 mM $MgCl_2$ and 1 mg/ml bovine serum albumin. After adding 0.25 μg DNAse I and stirring 15 min at 22°, 100 μmoles EDTA were added, the product filtered, washed with buffer A and finally with buffer A containing 0.6 M KCl. The DNAse treatment removed about 3 mg DNA from the cellulose.

2.2 *Enzymes*

Liver nuclei (specific activity, 0.5 units/mg protein) were prepared using a 2.4 M sucrose step from 200 g albino rats [4] and non-histone protein was extracted [7]. The extract was made 2 M in NaCl, concentrated using an Amicon DIAFLO system and chromatographed on Sepharose 6B in buffer A containing 2 M NaCl, to give the nuclear enzyme free of nucleic acid (A 280/260, 1.6; 10–15-fold purified). Cytoplasmic enzyme activity from reganerating liver (29 hr after partial hepatectomy) was obtained from the 105,000 $g \times 1$ hr supernatant of a 1 in 4 homogenate in 0.25 M sucrose – 6 mM KCl. The supernatant (0.04 units/mg protein), made 0.05 M in potassium phosphate, pH 7.0, was adjusted to pH 5.0 with *N*-acetic acid [8], the resulting pre-

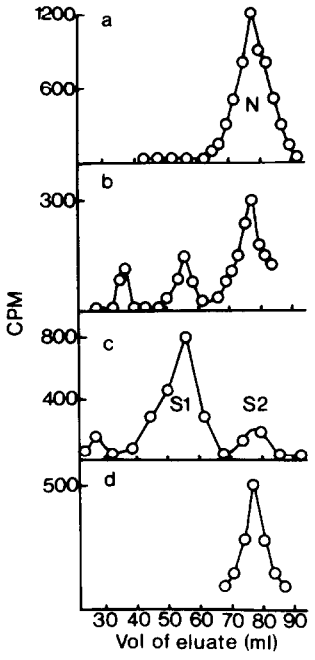

Fig. 1. Sepharose 6B chromatography of nuclear and cytoplasmic preparations. A single column (31 × 2 cm) was used in all experiments. Buffer A was used throughout. Experiments were as follows; a, nuclear enzyme, 100 mg (70 units) non-histone protein run in 2 M NaCl; b, cytoplasmic enzyme, 14 mg (initially 40 units) phosphocellulose fraction run in 2 M NaCl; c, cytoplasmic enzyme, 80 mg (25 units) pH 5.0 fraction run in 0.1 M NaCl (a similar profile was obtained using 46 mg, 157 units, phosphocellulose fraction); d, nuclear enzyme, 6 mg (20 units), sample from a, re-run in 0.1 M NaCl. For a and d, 10 μl, and for b and c 25 μl samples were used for assay. Recoveries were 80–100% of that loaded.

cipitate (pH 5.0 fraction; 15 fold purified) dissolved in 0.05 M potassium phosphate, pH 7.0, clarified by centrifugation (12,000 *g* × 10 min) and adsorbed onto phosphocellulose in the same buffer. After washing the column with 0.1 M potassium phosphate, pH 7.0, all of the polymerase activity (50-fold purified) was eluted with 0.5 M potassium phosphate, pH 7.0 and dialysed and concentrated immediately against buffer A containing 0.1 M NaCl containing 30% w/w polyethylene glycol. Either the pH 5.0 or phosphocellulose fractions were then run on Sepharose 6B in buffer A containing 0.1 M NaCl. The A 280/260 was 1.5–1.6 for peak tubes of activity. When phosphocellulose fraction was run on Sepharose 6B, the main (S1) peak was 300-fold purified over the supernatant. Enzyme from normal liver supernatant was purified in exactly the same way.

A sample of cytoplasmic enzyme, omitting the use of pH 5.0 and phosphocellulose steps, was prepared by loading 5 ml of supernatant containing 1 mM EDTA directly on to a DNA cellulose column (8 × 1.3 cm) in buffer A. After washing with more buffer A to remove most of the protein, the activity was recovered in good yield on eluting with 0.6 M NaCl in buffer A. This enzyme (30-fold purified) was used in the experiments of fig. 2.

2.3. *DNA polymerase assay*

Activity was assayed in 0.125 or 0.25 ml incubation mixtures. The following were contained in 0.25 ml; Tris-HCl, pH 7.5, 15 μmole; $MgCl_2$, 2.5 μmole; dATP, dGTP, dCTP and ^{3}H-TTP (40 μCi/μmole), 25 nmole each; 2-mercaptoethanol, 0.25 μmole; activated calf thymus DNA, 50 μg; bovine serum albumin, 25 μg; and enzyme protein. After incubating 1 hr at 37°, the reaction was stopped and radioactivity processed and counted for 10 min as before [4], except that precipitates were collected by filtration on Whatman GF/C circles. Both peaks N and S1 (fig. 1) were totally dependent on DNA and Mg^{2+}, and required all four triphosphates for maximal activity. Using only ^{3}H-TTP in the assay, peaks N and S1 (the latter 300-fold purified) showed 50% and 30% respectively, of the activity with four triphosphates. The products of both enzymes were DNAse sensitive.

1 unit of activity is 1 nmole of ^{3}H-TMP incorporated per hr. Incorporation was linear with time and protein for both enzymes.

2.4. *Protein estimation*

This was by the Lowry procedure or by ultraviolet absorption [9].

3. Results

3.1. *Agarose gel chromatography*

The first indication of a difference between the enzymes from cytoplasm and nuclei came from experiments using Sepharose 6B. The results are shown

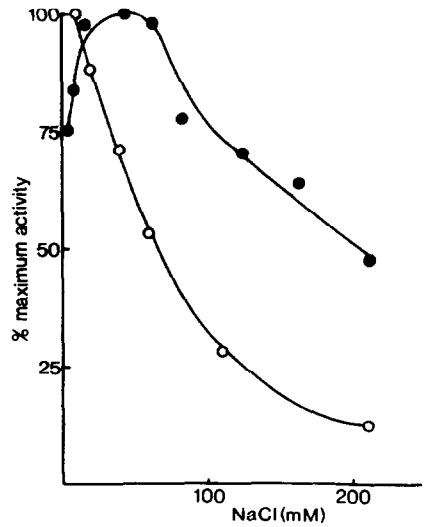

Fig. 2. Effect of salt on enzyme activity. NaCl was added to standard assay mixtures. o——o, phosphocellulose fraction (0.08 units per assay), was used in the experiment shown. pH 5.0 fraction, peak S1 or DNA cellulose enzyme (ex-cytoplasm, see 2.2) gave almost identical results. •——•, peak N (0.09 units per assay) was used. Peak S2 gave a similar result.

in fig. 1. Nuclear enzyme in 2 M NaCl, in which it was completely stable, eluted at 77 ml (peak N, fig. 1a). However, dialysis of the cytoplasmic enzyme against 2 M NaCl leads to large (75%) losses of activity, not recoverable on removal of salt, even before chromatography; separation of the resulting dialysed preparation (fig. 1b) revealed that the largest peak of activity eluted in the same position as the nuclear peak N. The exact position of this peak was re-checked using ^{3}H-TTP in the assay at four times the specific activity (160 μCi/μmole) normally used. Chromatography of cytoplasmic preparations in 0.1 M NaCl (fig. 1c) gave a different profile, with the appearance of a major enzyme peak (S1) at 56 ml, together with a peak (S2) eluting at 77 ml (cf. fig. 1b). A trace of activity eluting near the breakthrough volume (29 ml) could be aggregated, or membrane-bound [10], material and has not been further studied. Although the experiments in fig. 1b and c were carried out on enzyme from regenerating liver, a distribution of enzyme activity, identical (except for the incorporation of less ^{3}H-TMP) to that in fig. 1c was observed for the

Table 1
The binding of nuclear and soluble DNA polymerases to DNA cellulose.

Experiment no.	1	2	3	4	5	6
Enzyme	S1	S1	S1	N	N	S2
pH	7.5	7.5	6.5	7.5	9.0	7.5
Ionic strength	Recovery of enzyme (%)					
0.048	–	26*	8*	–	–	–
0.20	73*	30	21	0*	0*	0*
0.65	12+	14+	43+	25	90	20
2.05	0	0	0	65	12	80
Total recovery of enzyme (%)	85+	70+	72+	90	102	100

S1 = soluble, N = nuclear enzyme.
Values are percentages of loaded material. The asterisk indicates the ionic strength at which a particular enzyme was loaded on to the same column (1.3 × 8 cm) of rat liver DNA cellulose prepared as described under Materials and methods. After loading and standing 15', the column was washed with 20–25 ml of the same buffer followed by similar volumes of buffers of increasing ionic strength as shown. For expts. 1, 2, 3 and 6, buffer A (*I*, 0.048) was used; for expt. 4, 0.0286 M sodium phosphate – 1 mM EDTA, pH 6.5 (*I*, 0.048); for expt. 5, 0.05 M 2-amino-2-methylpropan-1,3-diol (Ammediol) HCl – 1 mM EDTA, pH 9.0, (*I*, 0.032) was used. In each case ionic strengths were adjusted to the stated values using NaCl. All buffers and enzymes loaded, contained 1 mM dithiothreitol and 0.5 mg/ml bovine serum albumin. The latter did not influence binding of polymerase. Although assays were carried out in about 20 mM NaCl, in expts. 1, 2 and 3, recoveries are low due to instability of enzyme at higher salt concentrations. Amounts of enzyme loaded in expts. 1 to 6 were 11.0, 11.0, 10.0, 15.3, 13.5 and 12.0 units respectively.

phosphocellulose fraction obtained from a normal rat liver supernatant remaining after the usual nuclear isolation procedure. Further, a phosphocellulose fraction prepared directly from a regenerating liver, 105,000 *g* supernatant (omitting pH 5.0 step) also gave rise to the profile seen in fig. 1c.

Finally, it was shown that the eluting position of peak N was the same (77 ml) in both low and high salt conditions (cf. fig. 1a and d).

3.2. *Salt effects*

Nuclear activity is activated by low salt concentrations and is relatively resistant to inhibition by

higher concentrations (fig. 2). In the case of cytoplasmic activity, pH 5.0 fraction, phosphocellulose fraction and enzyme isolated directly on DNA-cellulose (see 2.2) all showed no activation and marked inhibition by salt. These inhibitory effects were produced equally by NaCl, KCl and potassium phosphate over the same ionic strength range. Further, when peaks S1 and S2 (fig. 1c) were assayed in the presence of increasing levels of NaCl, S1 was strongly inhibited whereas S2 responded in a similar way to peak N, suggesting that N and S2 may be the same enzyme. Finally it should be mentioned that the reason for the low level of S1 activity in fig. 1b cannot be due solely to high salt inhibition (NaCl in assay, 100 mM) but predominantly to the irreversible loss of activity which occurs at higher ionic strengths (e.g. standing in, or dialysis against, 1–2 M NaCl in buffer A, 0.5 M potassium phosphate, pH 7.0).

3.3. *Inhibition experiments*

Aliquots of nuclear enzyme, peak N (further purified for this experiment on phosphocellulose, to about 80-fold) and S1, after dialysis against 0.05 M Tris-HCl, pH 7.5 containing 0.1 M NaCl to remove thiols, were incubated with *p*-chloromercuribenzoate (10^{-4} M final concentration). At intervals up to 20 min portions were removed into 0.01 M 2-mercaptoethanol and incubated in the standard assay. After 20 min, with 10^{-4} M PCMB, S1 was 85% and N, 30%, inhibited. Prior addition of DNA to S1 offered no protection against *p*-chloromercuribenzoate but for N, when previously bound to DNA, inhibition was reduced to about half.

3.4. *DNA-binding studies*

The nuclear (peak N) and soluble (S1) enzymes also differ in their ability to bind to DNA. Table 1 shows that the soluble enzyme, when applied to DNA-cellulose, has a tendency either to fail to bind, or to elute substantially with ionic strengths of 0.2 (expts. 1, 2 and 3). In contrast, the nuclear enzyme binds completely when loaded at an ionic strength of 0.2, and is only eluted by much higher ionic strengths (expts. 4 and 5). Although a certain degree of electrostatic interaction must inevitably occur between a nucleic acid polymerase and its template, this alone is insufficient to account for binding of the nuclear enzyme as is shown by a comparison of expts. 1 and 5 where both enzymes have a net negative charge (judged by their ability to bind to QAE Sephadex). These experiments reveal that the bulk (73%) of the soluble enzyme fails to bind to the column at *I* 0.2 whereas the nuclear enzyme binds completely. Further, in expts. 3 and 4, when both enzymes have a net positive charge (ability to bind to phosphocellulose), binding is tighter in both cases as judged by salt levels required for elution. However, under conditions of temperature (4°), actual pH (8.0), ionic strength (0.65) and eluting ion (Na^+) identical to those of expt. 4, nuclear enzyme is eluted completely from a phosphocellulose column, whereas *I* 0.65 removes only 25% from DNA-cellulose (which has 1% of the exchange capacity of the phosphocellulose used).

Results similar to those in table 1 have also been obtained using calf thymus DNA cellulose. Prior incubation of cytoplasmic enzyme with RNAse or low levels of DNAse, or addition of Mg^{2+}, did not increase the affinity of the enzyme for DNA. It is also unlikely that nucleic acid in the preparation is interfering with binding since the activity in the 105,000 *g* supernatant (which is at pH 6.8 and *I* approx. 0.05) will bind almost completely to DNA cellulose and then elute in a similar pattern to that in expt. 3.

Since it is possible that the characteristics of binding to DNA seen in table 1 are a function of the UV treatment the DNA receives in preparing DNA cellulose, binding experiments were repeated using activated calf thymus and rat liver DNAs, on a column (25 × 1 cm) of Sepharose 6B (buffer A + 0.1 M NaCl). The results, for both N and S1, confirmed the pattern of binding already observed in table 1.

4. Discussion

From the results presented above, N and S1 appear to be distinct enzymatic entities. In the purification procedures used so far they are obtained in 60–80% yield (for S1 this is based on pH 5 fraction) and appear to account for a substantial part of the DNA polymerase activity. Although there appears to be some nuclear enzyme in the cytoplasm, we cannot detect S1 in nuclear preparations probably because

the method of extraction, using high ionic strength, inactivates it. For the moment we are unable to elaborate on the different DNA binding abilities of S1 and N. However, it is of interest to record that in further experiments using molecular weight markers (β-galactosidase (*E. coli*), catalase, lactate dehydrogenase and bovine serum albumin) on Sepharose 6B, peak S1 has a molecular weight of 400,000 ± 10% and peak N of 65,000 ± 10%.

We are pleased to note that recent work on rat liver supports our own observations [10]. Observations on HeLa cells [11] also indicate the existence of distinct nuclear and cytoplasmic DNA polymerases.

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