CO-FRACTIONATION OF "NICKASE" WITH RAT LIVER DNA POLYMERASE*

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Summary. DNA polymerase partially purified from rat liver nuclei or ribosomes contains alkaline endonuclease that produces single strand breaks (nicks) in rat liver nuclear DNA. The nuclease activity is specific for DNA and is active with both native and denatured DNA as substrates. The endonuclease has maximal activity in the presence of 15 mM Mg^{2+} at pH 9.0, but is inactive in the presence of Ca^{2+} at concentrations that also inactivate the DNA polymerase. The 3'-OH primer ends produced by the endonuclease may account, in part, for the native DNA primer preference of the polymerase observed in vitro.

Partially purified DNA polymerase from rat liver nuclei preferentially uses native, rather than denatured, DNA as primer (1-3). We have recently isolated a DNA polymerase from rat liver ribosome fractions and have found its physical and enzymatic properties to be identical to those of the extractable nuclear DNA polymerase (3,4). As the DNA polymerase from either source is further purified it uses native DNA primer less efficiently. The purified enzyme exhibits maximal activity only with activated DNA primer or with native DNA primer if a small amount of pancreatic DNase is present during incubation.

The partially purified DNA polymerase preparations from nuclei and ribosomes contain an alkaline endonuclease activity that produces single strand breaks in DNA (nickase). The production of 3'-OH groups in native DNA by the endonuclease may explain the ability of the partially purified DNA polymerase to use native DNA as a primer. This report describes some of the properties of the endonuclease from rat liver ribosomes.

Partial Purification of Enzymes. The procedures for the purification of rat

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liver nuclear and ribosome-associated DNA polymerase are described in detail elsewhere (3). Briefly, the procedure involves: extraction from the purified sub-cellular fractions by 1.0 M (nuclei) or 0.25 M (ribosomes) KCl-buffer A (50 mM Tris-HCl, 25 mM KCl, 5 mM Mg acetate and 1 mM dithiothreitol, pH 7.5); removal of non-extractable material by centrifugation and dialysis of the extract against buffer A, 20% ethylene glycol (EG); salting-out at 40-65% saturation of $(NH_4)_2SO_4$; chromatography on DEAE-cellulose equilibrated with buffer A, 20% EG; chromatography on phosphocellulose equilibrated with 50 mM KH_2PO_4 buffer, pH 6.8, 1 mM dithiothreitol and 20% EG. Fractions were eluted from phosphocellulose by stepwise increases in KH_2PO_4 concentration between 0.1 and 0.5 M. The endonuclease and DNA polymerase activities were both eluted with 0.5 M KH_2PO_4. The active fractions were dialyzed against buffer A, 20% EG and stored at -20°.

Results and Discussion. In the presence of Mg^{2+} partially purified DNA polymerase from ribosomes has higher activity with native than denatured DNA primer, but the activity is increased 10-fold with activated DNA as primer (Table 1). The higher activity with activated DNA may reflect a requirement of the purified polymerase for an oligonucleotide primer that is hydrogen bonded to the template strand (3).

The polymerase preparation contains some DNase activity that uses denatured DNA as substrate when assayed in the presence of Mg^{2+} by the procedure of Geiduschek and Daniels (5). However, little or no activity of the DNase could be detected with native DNA using this assay procedure. DNase activity, as well as DNA polymerase activity is optimal at pH 9.0 in the presence of 15 mM Mg^{2+} (3). Burgoyne et al (7) have reported a Ca^{2+}-stimulated endonuclease activity in rat liver nuclei and suggested that it may play a role in DNA synthesis. In contrast, the DNase we are studying is Mg^{2+}-dependent. The replacement of Mg^{2+} by Ca^{2+} at concentrations of 0.1-3 mM does not increase the DNase activity with native DNA as substrate. The nuclease activity with denatured DNA as substrate and DNA polymerase activity with either native or

Table 1. Effect of Substrate, Primer and Divalent Ions on DNase and DNA Polymerase Activities from Rat Liver Ribosomes

Primer or Substrate	Divalent Ions Present	DNase Activity (% loss/hr)	DNA Polymerase Activity (units/mg/hr)
Native DNA	Mg^{2+}	<2	3.54
Denatured DNA	Mg^{2+}	21	1.50
Activated DNA	Mg^{2+}	-	36.00
RNA	Mg^{2+}	0	0.00
Native DNA	Ca^{2+}	0	0.21
Denatured DNA	Ca^{2+}	<1	0.05
Denatured DNA	Ca^{2+} + Mg^{2+}	9	1.01

DNA polymerase activity was assayed as previously described (4). Incubation was at 37° for 60 minutes. The incubation mixture (0.5 ml) contained: 20 μmole of glycine-NaOH buffer (pH 9.0), 0.05 μmole each of dATP, dGTP, dCTP and ^{3}H-TTP (25 μC/μmole), 0.2 μmole dithiothreitol, 8 μmole $MgCl_2$ and/or 1 μmole $CaCl_2$, 100 μg of primer (calf thymus native, heat-denatured or activated DNA or rat liver ribosomal RNA), 50 μg bovine serum albumin (crystallized, Sigma) and 5 μg of protein from the phosphocellulose fraction containing DNase and DNA polymerase activities (3). One unit of activity equals 1 mμmole of ^{3}H-TMP incorporated per hour.

DNase activity was assayed according to Geiduschek and Daniels (5). The incubation mixture (0.5 ml) was the same as for the polymerase assay except that the deoxynucleoside triphosphates were eliminated and native or heat-denatured ^{3}H-labeled rat liver nuclear DNA (30 μg, 5,000 CPM) or ^{3}H-labeled rat liver RNA (20 μg, 10,000 CPM) was used. Incubation was for 2 hours at 37°.

denatured DNA primers are markedly lowered when Ca^{2+} replaces Mg^{2+} at a concentration of 1 mM. The Mg^{2+}-dependency of both the DNA polymerase and DNase activities is further shown by the fact that both activities are partially restored when Mg^{2+} is added in the presence of Ca^{2+}.

The DNase must act as an endonuclease, as shown by gel filtration on Sephadex G-100 of the product resulting from incubation of DNA with the enzyme (Fig. 1A,B). No detectable deoxyribonucleotides were released during a 6 hour incubation of native or denatured DNA with the enzyme. In addition, gel filtration on agarose (Biogel A-5) showed that either native or denatured DNA

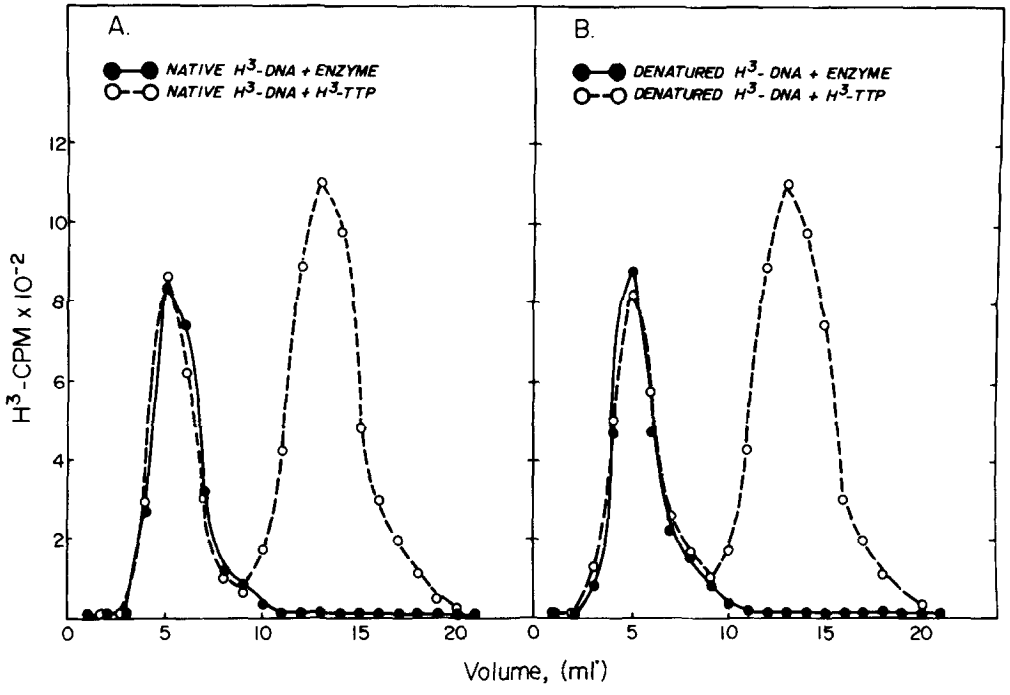

Figure 1. Gel filtration on Sephadex G-100 of A. native and B. denatured ^{3}H-labeled rat liver nuclear DNA after incubation for 6 hours with ribosome-associated DNase. The incubation mixture for DNase activity is described in Table 1. After incubation at 37°, the samples were heated for 5 minutes at 100°, cooled and loaded onto a 0.9 x 10 cm column of Sephadex G-100 equilibrated with 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0. One ml fractions were collected and the OD_{260} mμ monitored. CCl_3COOH insoluble radioactivity was determined as described previously (4) and aliquots of CCl_3COOH soluble fractions were counted in 10 ml of Aquasol (New England Nuclear). The elution volumes for DNA and deoxyribonucleotides were determined by filtration of a 0.5 ml mixture of ^{3}H-labeled DNA (30 μg, 5,000 CPM) and ^{3}H-TTP (10,000 CPM).

remained of relatively high molecular weight after incubation with the DNase.[1] This suggests that the number of breaks introduced into the DNA must be severely limited.

Little effect of the nuclease on native DNA could be detected by the assay procedure of Geiduschek and Daniels (5). This method depends on retention of long chains of denatured DNA on nitrocellulose filters and a limited number of breaks in the DNA would not be detected. However, even a limited number of breaks should be detectable by alkaline sucrose gradient centrifu-

[1] Unpublished observations.

gation. Native, ^{3}H-labeled, rat liver DNA was incubated with the enzyme and analyzed by neutral and alkaline sucrose gradient centrifugation. As shown in Figure 2, there was no significant difference in the sedimentation rate of the DNA in a neutral sucrose gradient following a 4 hour incubation in the presence or absence of the enzyme. However, DNA incubated with the enzyme

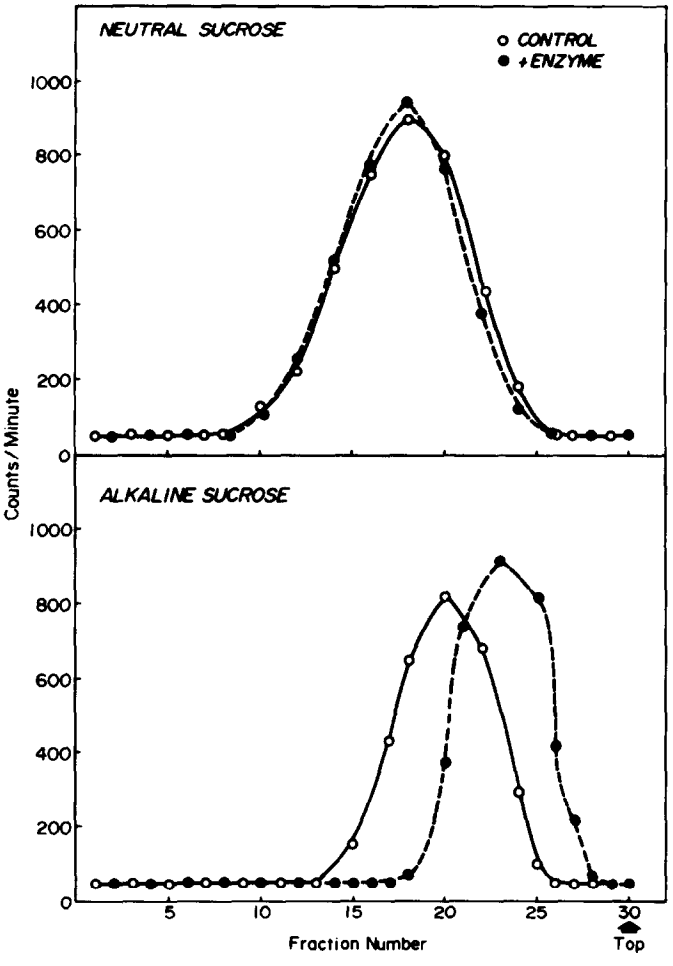

Figure 2. Neutral and alkaline sucrose gradient centrifugation of native, ^{3}H-labeled, rat liver nuclear DNA after incubation for 4 hours in the presence (closed circles) or absence (open circles) of the phosphocellulose fraction containing ribosome-associated DNase and DNA polymerase. The incubation mixture was the same as that described for DNase activity in Table 1. After incubation NaCl and EDTA were added to final concentrations of 0.9 and 0.001 M respectively. Alkaline and neutral sucrose gradient centrifugation was performed according to Studier (6). Centrifugation was for 16 hours at 4° and 25,000 RPM (SW 25.1). One ml aliquots were collected from the bottom of the tube, precipitated with 10 % CCl_3COOH and the acid insoluble radioactivity counted as previously described (4).

sedimented more slowly than the control DNA in an alkaline sucrose gradient. These data indicate that the endonuclease produces single strand breaks (nicks) in native DNA that were not detected by less sensitive endonuclease assay procedures.

Co-fractionation of DNA polymerase activity that prefers native DNA as primer and an endonuclease that nicks native DNA suggests, but does not prove, a role of the endonuclease in the primer preference of the polymerase. The similar responses of the DNA polymerase and endonuclease activities to pH, Mg^{2+} and Ca^{2+} concentrations provide some support for this contention.

The requirement of activated DNA primer for maximal activity of the partially purified polymerase, however, suggests that additional factors, possibly exonucleases, must also be involved and are removed during purification of the enzyme. We are investigating the nature of the factor(s).

Burgoyne et al (7), from studies with isolated, intact, rat liver nuclei, have suggested that a Ca^{2+}-dependent endonuclease may be involved in initiation of DNA synthesis. Their assays were performed in the presence of excess Mg^{2+}. Any relationship of the partially purified, Mg^{2+}-dependent endonuclease (nickase) reported here to the endonuclease activity observed by Burgoyne et al (7) must still be demonstrated.

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