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# Action of pancreatic DNase: requirements for activation of DNA as a template-primer for DNA polymerase ∝

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## ABSTRACT

Pancreatic DNase requires both $Ca^{2+}$ and $Mg^{2+}$ for its activity as measured by formation of an activated DNA template for *in vitro* DNA polymerase α assay and by the hyperchromic shift. $Mn^{2+}$ can partially satisfy the $Mg^{2+}$ requirement of the DNase for activation of DNA but the resulting template is only 50% as active in the DNA polymerase assay. When precautions are taken to avoid divalent ion contamination, pancreatic DNase is not active in the presence of $Ca^{2+}$ or $Mg^{2+}$ alone. Analysis of the DNA by sucrose gradient centrifugation shows that only in the presence of $Ca^{2+}$ plus $Mg^{2+}$ or $Mn^{2+}$ does pancreatic DNase produce extensive strand breaks in the DNA. The activated DNA template that yields maximal DNA polymerase activity is low molecular weight material of 30,000 to 50,000 daltons.

## INTRODUCTION

The mechanism of action of pancreatic DNase on DNA has not yet been clearly defined. There has been recent evidence that indicates that the enzyme probably recognizes sequences in the DNA of at least three nucleotides in length (1). The sequence recognition is reported to be dependent on the nature of the divalent ions present. Pancreatic DNase requires divalent ions for activity and its maximal activity is reported to occur in the presence of $Mg^{2+}$ plus $Ca^{2+}$ (2-4) or with $Mn^{2+}$ (5-6). It has also been reported that pancreatic DNase produces single strand breaks in DNA in the presence of $Mg^{2+}$ and that double strand scissions occur in the presence of $Mg^{2+}$ plus $Ca^{2+}$ (7). A possible synergistic effect of $Mg^{2+}$ and $Ca^{2+}$ in pancreatic DNase activity, however, has not been generally recognized.

The *in vitro* assay of purified DNA polymerases, in general, requires a DNA template-primer that is activated by means of limited digestion by pancreatic DNase. Aposhian and Kornberg (8) introduced the use of activated DNA for *in vitro* assay of DNA

 

polymerase activity. A number of variations of this procedure have since been introduced (9-10). The net result of these procedures is believed to be the generation of an increased number of 3'-hydroxyl termini from which the DNA polymerase begins DNA synthesis in vitro. The conditions and requirements for activation of DNA as a template-primer by pancreatic DNase have not been definitely established. This has led to considerable variability in the results of preparation of activated DNA.

We have attempted to establish the requirements for preparation by pancreatic DNase of DNA as a template-primer for DNA polymerase α. As an outcome of this study we observed that the activation of DNA by pancreatic DNase can be utilized as a sensitive assay for the requirements of this DNase during the initial stage of its action on DNA.

In this report we demonstrate a requirement of $Ca^{2+}$ in addition to $Mg^{2+}$ for the preparation of activated DNA template-primer for DNA polymerase α by pancreatic DNase. In addition we show a requirement for both $Ca^{2+}$ and $Mg^{2+}$ for the production of a hyperchromic shift by the DNase and that under these conditions the enzyme produces double strand scissions in the DNA.

## MATERIALS AND METHODS

Materials. Nonradioactive deoxyribonucleoside triphosphates, crystalline bovine serum albumin, calf thymus DNA, EDTA and phenylmethylsulfonyl fluoride were purchased from Sigma. [$^3$H]-dTTP (specific activity 20 Ci/mmole) was purchased from New England Nuclear. Pancreatic DNase was purchased from Worthington (DPFF grade), Sigma (DN-CL) or P-L Biochemicals. Ethylene glycol bis (β-aminoethyl ether) N,N'-tetraacetic acid (EGTA) was from Eastman Organics, Inc. Chelex and Hepes were products of Calbiochem. Chelex was prepared and used according to the manufacturer's recommended procedures. Analytical reagent grade chloride salts of divalent ions and glycerol were from Mallinkrodt Chemical Co. DNA polymerase α from HeLa cells was purified by a modification of our published procedure (11). This includes, beyond the phosphocellulose stage, successive steps of chromatography on native and denatured DNA cellulose, at which point the DNA polymerase is free from exo- and endo-deoxyribonuclease activities.

All buffers and solutions used in this study were prepared in glass distilled water that had been passed over a Chelex column to remove divalent ions. Dialysis tubing was boiled in 0.1M $NaHCO_3$ and 1mM EDTA-$Na_2$ and was stored in 1mM EDTA plus trace amounts of $CHCl_3$ at 4°. Bovine serum albumin, 1 mg/ml in Chelex treated distilled water, was heated at 55-60° for 3 hours before use. DNA solutions were dialyzed versus 10mM Tris, pH 7.5 at 25°, 1mM EGTA followed by dialysis against 10mM Tris, pH 7.5. Pancreatic DNase was prepared at a concentration of 1 mg/ml in 10mM Hepes buffer, pH 7.5 containing 1 mg/ml of heated bovine serum albumin and dialyzed versus 10mM Hepes, pH 7.5, 1mM EGTA followed by dialysis against 10mM Hepes buffer pH 7.5 containing 1 mg/ml phenylmethylsulfonyl fluoride, and 50% glycerol. Under these conditions the DNase solution could be stored at -20° for at least 2-3 months without significant loss in activity.

Activation of DNA template. Activation of DNA by DNase was by a modification of the procedure of Aposhian and Kornberg (8). The complete digestion mixture contained in a volume of 1 ml, except as indicated with individual experiments; 10mM Hepes, pH 7.5, 2.5mM $MgCl_2$, 0.05mM $CaCl_2$, 50 µg of heated bovine serum albumin, 10 µg of phenylmethylsulfonyl fluoride, 1 mg of calf thymus DNA and 1 µg of DNase. Incubation was at 25° or 37° for the times indicated with the individual experiments. The digestion was terminated by addition of EGTA to a concentration of 0.1mM followed by heating at 60° for 5 minutes.

Hyperchromicity Assay. DNase activity was also measured by the hyperchromicity assay of Price (4). A Gilford recording spectrophotometer equipped with a water jacketed cuvette holder was used for the assay.

DNA Polymerase Assay. HeLa DNA polymerase α activity was assayed with the activated DNA template by our published procedure (11). One unit of activity is equal to 1 nmole of total deoxynucleoside triphosphate incorporated into acid-insoluble material in 1 hour at 35°.

Sucrose Gradient Analysis of DNA. DNA after digestion by pancreatic DNase was analyzed by centrifugation in neutral or alkaline 10-20% sucrose gradients. Neutral sucrose gradients were prepared in 0.05M Tris-HCl, pH 7.5, 0.8M KCl and 0.01M EDTA-

$Na_2$. Alkaline sucrose solutions, pH 13, contained in addition 0.3N KOH. 10-20% sucrose gradients were lowered over 0.5 ml cushions of 60% sucrose in the appropriate buffers in polyallomer tubes. Centrifugation was at 40,000 RPM in an SW 50.1 rotor at $4^{\circ}$ for 12 or 16 hours. Fractions of 0.2 ml were collected by puncturing the bottoms of the tubes and the absorbance at 260 nm was recorded in microcuvettes of 0.2 ml capacity.

## RESULTS

$Ca^{2+}$ and $Mg^{2+}$ requirements for Template Activation. Activation of DNA by pancreatic DNase to yield a template-primer for DNA polymerase activity *in vitro* requires the presence of both $Ca^{2+}$ and $Mg^{2+}$ ions during the incubation (Fig. 1). Maximal activation under these conditions occurs between 10 and 13 minutes of incubation at $25^{\circ}$ or 2 and 6 minutes at $37^{\circ}$ (Fig. 1, inset).

DNA polymerase α activity *in vitro* is not increased when the native DNA template has been incubated with DNase in the presence of $Ca^{2+}$ alone or with $Mg^{2+}$ plus the $Ca^{2+}$-chelating agent EGTA. Activation of native DNA template by DNase was not observed at various concentrations of $Mg^{2+}$ from 0.1 to 10mM in the presence of 0.1mM EGTA. In agreement with others (8-10), we have observed activation of a DNA template for DNA polymerase by incubation with pancreatic DNase, from several commercial sources, in the presence of $Mg^{2+}$ alone. However, in all cases this activation of the template was abolished by either pre-dialysis of the DNase in the presence of EGTA or by inclusion of 0.1mM EGTA during the incubation.

$Mn^{2+}$ can partially replace $Mg^{2+}$ in the presence of $Ca^{2+}$. The optimal $Mn^{2+}$ concentration is five-fold lower (.05mM) than that of $Mg^{2+}$ (2.5mM). However the activated DNA template is much less effective in the DNA polymerase α assay (Fig. 1). Unlike the case of $Mg^{2+}$ only, a slight, but significant, activation of DNA occurs in the presence of $Mn^{2+}$ plus EGTA.

The inclusion of 0.1 mM EGTA during the incubation of native DNA with DNase in the presence of $Ca^{2+}$ plus $Mg^{2+}$ completely inhibits the activation of the template for DNA polymerase α (data not shown). The addition of 0.1mM EGTA completely inhibits the reaction in less than 30 seconds. As shown in Table I, EGTA has

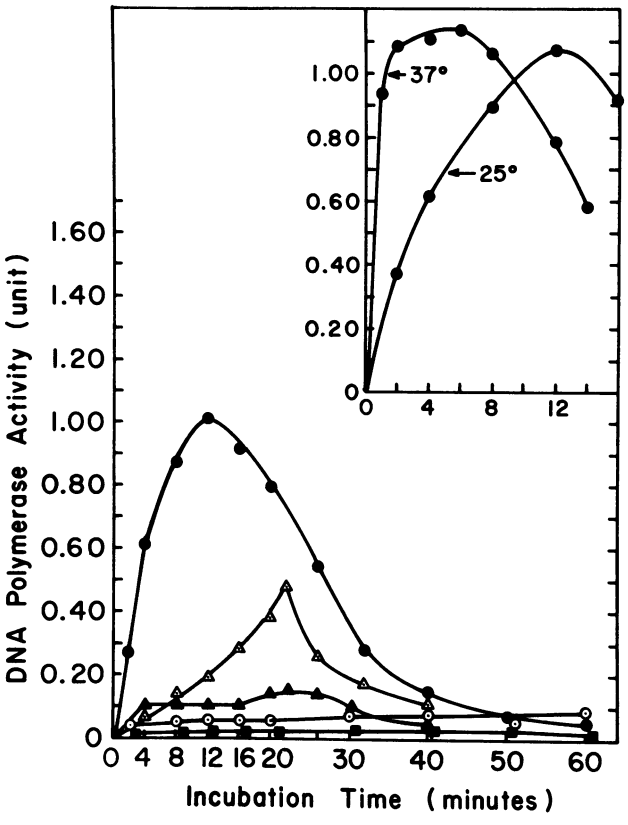


Fig. 1. Effects of Divalent Ions and Temperature on the Activation of DNA by DNase. Calf thymus DNA was incubated with pancreatic DNase at 25° in the presence of the designated divalent ions for activation of DNA as described in Methods. The activated DNA was used as a template-primer in the DNA polymerase α assay according to our published procedure (11). ●——● 2.5mM $Mg^{2+}$ plus 0.05mM $Ca^{2+}$, o——o 2.5mM $Mg^{2+}$ plus 0.1mM EGTA, ■——■ 0.05mM $Ca^{2+}$, ▲——▲ 0.05mM $Mn^{2+}$ plus 0.1mM EGTA and Δ——Δ 0.05mM $Mn^{2+}$ plus 0.05mM $Ca^{2+}$. Inset. Calf thymus DNA was incubated with DNase in the presence of 2.5mM $Mg^{2+}$ plus 0.05mM $Ca^{2+}$ at 37° ●——● or 25° ●——● for activation of DNA as described in Methods.

no effect on the activity of HeLa DNA polymerase α even at concentrations of 1 to 5mM. Thus, EGTA apparently inhibits activation of the DNA template by $Ca^{2+}$ chelation but does not secondarily inhibit the DNA polymerase itself.

Hyperchromicity Assay of DNase Activity. In agreement with Price (4) we have also observed a requirement of $Ca^{2+}$ plus $Mg^{2+}$ ions for DNase activity as measured by the hyperchromicity assay.

Table I. Effect of EGTA on DNA Polymerase α Activity

| Addition | DNA polymerase α activity (Units) |
|---|---|
| None | 1.02 |
| 0.1 mM EGTA | 1.08 |
| 1.0 mM EGTA | 1.01 |
| 5.0 mM EGTA | 1.07 |

Native DNA was activated in the presence of 2.5 mM $MgCl_2$ plus 0.05 mM $CaCl_2$ as described in Methods. The activated DNA was used in the DNA polymerase α assay (11) in the presence of the designated amounts of EGTA.

No hyperchromicity is observed when native DNA is incubated with DNase in the presence of $Ca^{2+}$ alone or with $Mg^{2+}$ plus EGTA. The inclusion of 0.1mM EGTA in the assay containing 0.05mM $Ca^{2+}$ plus 2.5mM $Mg^{2+}$ completely inhibits DNase activity (Fig. 2a). The addition of 0.1mM EGTA to the reaction inactivates the DNase in less than 30 seconds (Fig. 2b). DNase activity can be restored by addition of 1mM $Ca^{2+}$ to the reaction (data not shown).

Optimal Conditions for Template Activation. The optimal $Mg^{2+}$ concentration for activation of the DNA template (1 mg/ml) for DNA polymerase α by DNase is 1 to 4mM in the presence of $Ca^{2+}$. The optimal $Ca^{2+}$ concentration in the presence of $Mg^{2+}$ is approximately 0.5mM. This is approximately one-half the optimal $Ca^{2+}$ concentration in the presence of $Mg^{2+}$ for DNase activity as measured by the hyperchromicity assay. There is a decrease in the required $Ca^{2+}$ concentration with increasing concentrations of DNA in the assay over the range of 0.01 mg/ml to 1 mg/ml.

The maximal activation of the DNA template by DNase at the optimal $Mg^{2+}$ and $Ca^{2+}$ concentrations occurs at pH 7.5.

Under optimal conditions maximum activation of the DNA template occurs within 4 to 6 minutes at 37° (Fig. 3). The hyperchromic shift under these conditions occurs more slowly and reaches its maximum after maximal activation of the DNA template has resulted.

Heating Required for Maximal Activation. Heating the DNA to at least 60° following its partial hydrolysis by DNase is essential for its maximal activation as a template-primer for DNA

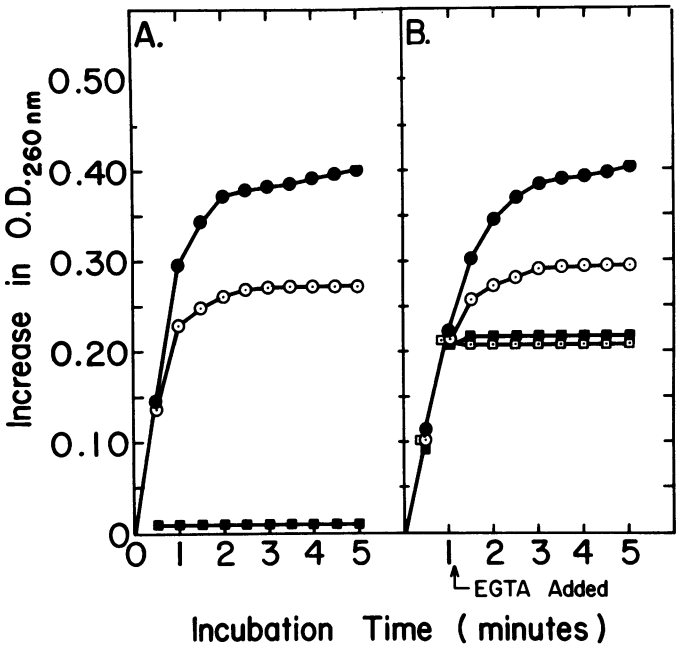


Fig. 2. EGTA Inhibition of Hyperchromic Effect by DNase. A. Calf thymus DNA (40 µg/ml) was incubated in the presence of 2.5mM $Mg^{2+}$ plus 0.05mM $Ca^{2+}$ and the designated amounts of EGTA at 25° by the procedure described for the hyperchromicity assay of DNase in Methods. ●—● none, o—o 0.1mM EGTA and ■—■ 1mM EGTA. B. Calf thymus DNA (40 µg/ml) was incubated at 25° with pancreatic DNase by the procedure of the hyperchromicity assay of DNase as described in Methods. After one minute of incubation the designated amounts of EGTA were added and incubation at 25° was continued. ●—● none, o—o 0.1mM EGTA, ■—■ 1mM EGTA and □—□ 10mM EGTA.

polymerase α. As shown in Table II, omission of the heating step markedly reduces the effectiveness of the DNA as a template-primer for DNA polymerase.

Sucrose Gradient Analysis of DNA Product. The activated DNA template for the assay of DNA polymerase α was analyzed by alkaline and neutral sucrose gradient centrifugation. The analysis showed that under conditions for maximal activation of the template in the presence of $Ca^{2+}$ plus $Mg^{2+}$, the DNase produced extensive breaks in both strands of the DNA (Fig. 4). Gel filtration analysis of the activated DNA gave an estimated molecular weight range of 30,000 to 150,000 daltons (data not shown). However, maximal DNA polymerase α activity is obtained with low

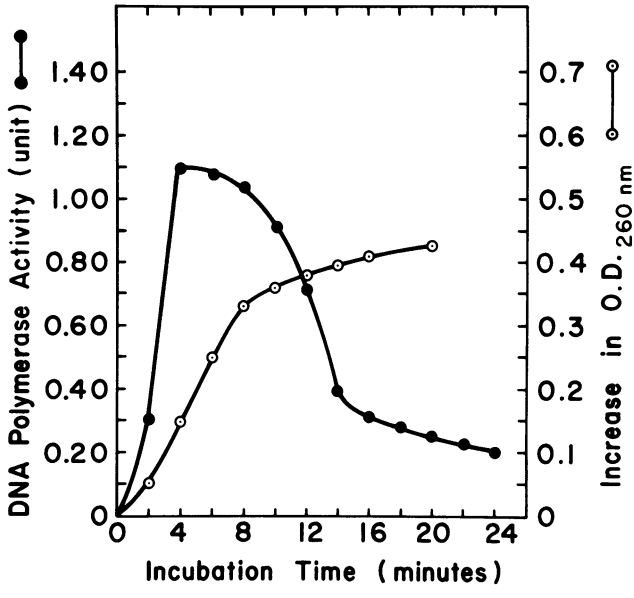


Fig. 3. Time Course of DNA Activation and Hyperchromicity by DNase. DNA (1 mg/ml) was incubated with pancreatic DNase at 37° in the presence of 2.5mM $Mg^{2+}$ plus 0.05mM $Ca^{2+}$ by the procedures for activation and hyperchromicity assays as described in Methods. ●——● units of HeLa DNA polymerase α activity with activated DNA template-primer. o——o increase in $O.D._{260}$ nM.

Table II. Effect of Heating on Activation of DNA Template

| Condition | DNA polymerase α activity (Units) |
|---|---|
| Control (Native DNA) | 0.03 |
| Activated without heating step | 0.48 |
| Activated with heating: | |
| 45° for 10 min. | 0.52 |
| 50° for 10 min. | 0.78 |
| 60° for 10 min. | 1.16 |
| 70° for 10 min. | 1.19 |

Native DNA was incubated with pancreatic DNase in the presence of 2.5 mM $MgCl_2$ plus 0.05 mM $CaCl_2$ under the conditions for activating DNA described in Methods. 10 µg of the respective templates were used in the DNA polymerase α assay (11).

molecular weight DNA of about 30,000 to 50,000 daltons.

In the presence of $Mg^{2+}$ plus EGTA, or of $Ca^{2+}$ alone, activation of the DNA template did not occur and the DNase produced only limited if any strand breaks in the DNA (Fig. 4). Neutral and alkaline sucrose gradient analysis indicates that the DNase also produced very limited strand breaks in the presence of $Mn^{2+}$ plus EGTA. However, extensive breaks in both strands were pro-

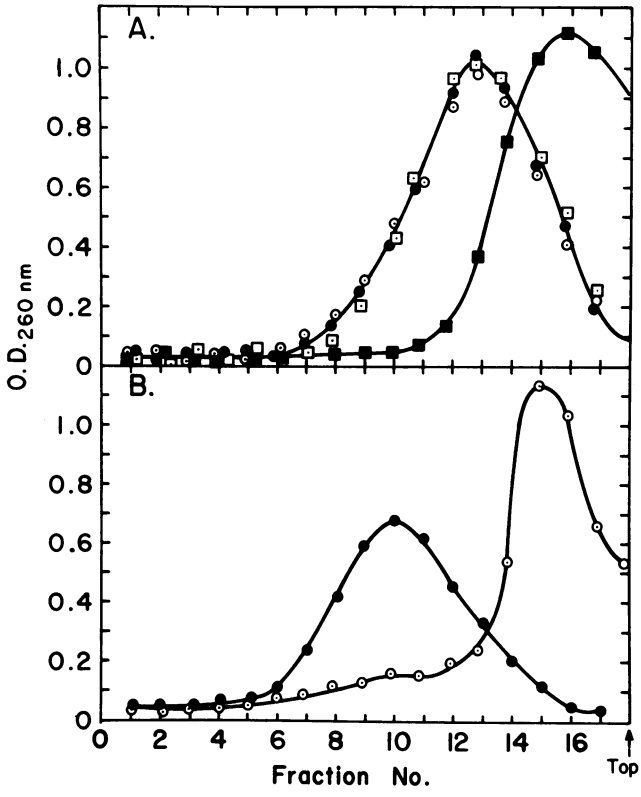


Fig. 4. Analysis of Product from Activation of DNA with $Mg^{2+}$.
A. 10 to 20% alkaline sucrose gradient analysis of DNA digestion by DNase in the presence of the designated divalent cations by the procedure for activation of DNA at 25°. Centrifugation was at 40,000 RPM in the SW 50.1 rotor for 12 hours at 4°. ●—● control (denatured DNA) and DNA activated in the presence of: o—o 2.5mM $MgCl_2$ plus 0.1mM EGTA, □—□ 0.05mM $CaCl_2$ and ■—■ 2.5mM $MgCl_2$ plus 0.05mM $CaCl_2$.
B. 10 to 20% neutral sucrose gradient analysis of the activated DNA product. Centrifugation was at 40,000 RPM in the SW 50.1 rotor for 12 hours at 4°. ●—● control (native DNA) and o—o DNA activated in the presence of 2.5mM $MgCl_2$ and 0.05mM $CaCl_2$.

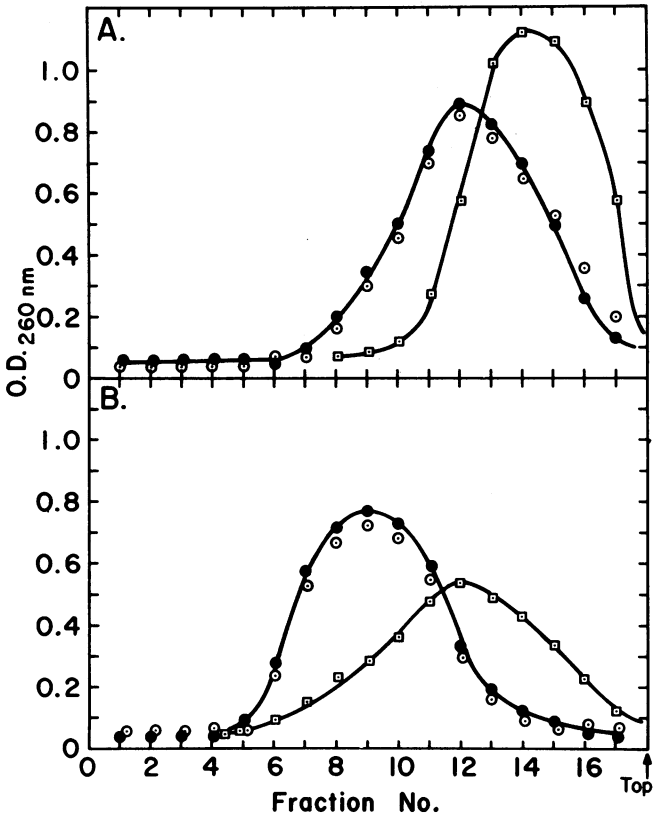


Fig. 5. Analysis of Product From Activation of DNA with $Mn^{2+}$.
A. 10 to 20% alkaline sucrose gradient analysis of DNA digestion by DNase in the presence of the designated divalent cations by the procedure for activation of DNA at 25°. Centrifugation conditions were as described in Fig. 5a. ●——● control (denatured DNA) and DNA activated in the presence of o——o 0.05mM $MnCl_2$ plus 0.1mM EGTA and □——□ 0.05mM $MnCl_2$ plus 0.05mM $CaCl_2$.
B. 10% to 20% neutral sucrose gradient analysis of the activated DNA product. Centrifugation conditions were as described in Fig. 4b. ●——● control (native DNA) and DNA activated in the presence of: o——o 0.05mM $MnCl_2$ plus 0.1mM EGTA and □——□ 0.05mM $MnCl_2$ plus 0.05mM $CaCl_2$.

duced under conditions for maximal activation of the template in the presence of $Mn^{2+}$ plus $Ca^{2+}$ (Fig. 5).

DNA Activated by Different Procedures. A comparison of DNA polymerase α activity with equal amounts of DNA activated as a template-primer by different procedures using pancreatic DNase is shown in Table III. Activated DNA prepared in the presence of

Table III. Comparison of DNA Activated by Different Procedures

| Template | DNA Polymerase α activity (Unit) |
|---|---|
| Native DNA | 0.02 |
| Denatured DNA | 0.05 |
| DNA activated with $Mg^{2+}$ plus $Ca^{2+}$ | 1.26 |
| DNA activated by procedure of Aposhian and Kornberg (8) | 0.86 |
| DNA activated by procedure of Oleson and Koerner (9) | 0.55 |

Calf thymus DNA (1 mg/ml) in 10mM Tris-HCl pH 7.5 was denatured by heating at 100° for 10 minutes followed by rapid cooling at 40° in an ice-water bath. DNA was activated as a template-primer for DNA polymerase α by pancreatic DNase in the presence of 2.5mM $MgCl_2$ plus 0.05mM $CaCl_2$ as described in Methods or by the procedures of Aposhian and Kornberg (8) or Oleson and Koerner (9). In the latter two procedures the DNase was not pre-dialyzed versus EGTA and activation of DNA was performed in the presence of 5mM $MgCl_2$ but without addition of $CaCl_2$. In each case the DNA was activated to yield maximal DNA polymerase activity in the assay. The DNA activated by the procedure of Aposhian and Kornberg (8) was heated at 77° for 5 minutes and cooled in an ice-water bath. DNA activated according to the procedure of Oleson and Koerner (9) was precipitated with ice-cold 95% ethanol and dried at room temperature. 10 µg of the respective templates were used in the DNA polymerase assays (11).

$Mg^{2+}$ plus $Ca^{2+}$ by the procedure described here is most effective as a template for DNA polymerase α activity. This procedure also produces a more effective template-primer for the assay of DNA polymerase β and E. coli DNA polymerase I activities (data not shown). Also, in our experience the degree of activation of the DNA template is much more reproducible between preparations by this method than it is by the other procedures that we have used.

## DISCUSSION

In this report we show that $Ca^{2+}$ in addition to $Mg^{2+}$ is essential for pancreatic DNase activity in the preparation of activated DNA as template-primer for DNA polymerase α. $Mn^{2+}$ can partially replace $Mg^{2+}$ in the reaction but the resulting DNA template-primer is only 50% as active in the DNA polymerase assay. Activated DNA that yields maximal DNA polymerase activity is low molecular weight of about 30,000 to 50,000 daltons as estimated by gel filtration.

The role of divalent ions in the activity of pancreatic DNase is not readily discerned. Although it is known that divalent ions combine with DNA there is also considerable evidence

that divalent ions affect the active site of pancreatic DNase either directly or indirectly. For example, it has recently been shown that pancreatic DNase recognizes sequences of at least 3 nucleotides in length in DNA (1). The sequence recognition by DNase was shown to differ in the presence of $Mg^{2+}$ or $Mn^{2+}$ ions.

A synergistic effect of $Ca^{2+}$ and $Mg^{2+}$ or $Mn^{2+}$ in pancreatic DNase activity has been reported by Price (4). Our results, based on three independent methods of analysis, are in agreement with this observation. When precautions are taken to control the divalent ion concentration it becomes apparent that pancreatic DNase requires $Ca^{2+}$ in addition to $Mg^{2+}$ or $Mn^{2+}$ for its maximal activity. That pancreatic DNase activity has generally been observed in the presence of $Mg^{2+}$ alone is probably due to low levels of $Ca^{2+}$ contamination in the assays. We, as well as Price (4), find that this activity is abolished when precautions are taken to reduce the level of possible $Ca^{2+}$ contamination.

Maximal activation of DNA as a template requires a brief heating period at $60^{o}$ to $70^{o}$ following its partial hydrolysis by pancreatic DNase in the presence of $Mg^{2+}$ and $Ca^{2+}$. The heating requirement is not related to inactivation of the DNase since it is necessary even in the presence of concentrations of EGTA that completely inactivate the DNase. Soave et al. (12) have shown that during the initial phase of spleen DNase action the melting temperature of the DNA decreases before any hyperchromic shift is observed. We have also observed a $15^{o}$ to $20^{o}$ displacement of the $T_m$ for the DNA activated as a template by pancreatic DNase (data not shown). Thus, it is probable that the heating step in the activation of DNA as a template increases single strand regions at which synthesis by DNA polymerase can occur.

Activated DNA is extensively used as a template-primer for the in vitro assay of DNA polymerase activity. In this study we have defined the requirements for activation of DNA as a template by pancreatic DNase. This has resulted in a simple but highly-reproducible procedure for activating DNA as a template by pancreatic DNase. Activation of DNA as a template for DNA polymerase is also a sensitive assay of the requirements for pancreatic DNase activity. It may have particular utility in studying the mechanism of action of this enzyme during the initial phase of its hydrolysis of DNA.

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