Detection and Characterization of a Novel Factor That Stimulates DNA Polymerase α[†]

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ABSTRACT: A novel factor that stimulates DNA polymerase α activity on poly(dA)·oligo(dT) has been identified and partially purified from mouse FM3A cells. The assay system for the factor contained poly(ethylene glycol) 6000. The activities of DNA polymerase α on poly(dA)·oligo(dT) in the presence and absence of the stimulating factor were increased greatly by the addition of poly(ethylene glycol). Stimulation by the factor was observed at all the primer to template ratios tested from 0.01 to 0.3. The highest activity was observed at the ratio of 0.05, corresponding to about 3.3 primers on one template in the presence of the factor. The concentration of DNA polymerase α used in the assay affected the stimulation by the factor, and the stimulation became more prominent at concentrations of the enzyme lower than 0.04 unit per assay. The stimulating factor lowered the K_m value of DNA polymerase α for the template-primer, though they had no effect on the K_m value for dTTP substrate. The results of product analysis suggested that the stimulation by the factor is mainly due to the increase in the initiation frequency of DNA synthesis from the primers. The stimulating factor specifically stimulated DNA polymerase α but not DNA polymerases β and γ. Furthermore, the factor formed a complex with DNA polymerase α under a certain condition.

It is now apparent from studies with prokaryotic systems that DNA replication requires the concerted action of enzymes and nonenzymic factors in addition to DNA polymerase (Kornberg, 1980, 1982). In eukaryotic cells, DNA polymerase α is the first component of replication apparatus to be isolated and characterized in detail. However, little is known about other enzymes and factors that participate in DNA replication of eukaryotic cells. Several studies on DNA polymerase α have indicated the existence of multiple forms of the enzyme that differ in their chromatographic behavior and their molecular weights, suggesting physical association of accessory proteins with DNA polymerase α catalytic subunits (Holmes et al., 1974; Yoshida et al., 1974; Hachmann & Lezius, 1975; Matsukage et al., 1976; Pedrali-Noy & Weissbach, 1977; Brakel & Blumental, 1978; Lamothe et al., 1981; Enomoto et al., 1983).

Recently, Pritchard et al. isolated cofactors for DNA polymerase α, C_1C_2, which stimulate DNA polymerase α activity on substrates with high ratios of single-stranded DNA template to primer. By detail analysis of the mechanism of the stimulation, it was suggested that C_1C_2 complex functions as a primer recognition protein and is a candidate for one of the components of replication apparatus carrying out DNA synthesis on the extensive single-stranded replicating intermediates (Pritchard & DePamphilis, 1983; Pritchard et al., 1983).

In order to clarify the molecular mechanism of DNA replication of eukaryotic cells, we have purified several enzymes and factors that may participate in DNA replication (Kohwi-Shigematsu et al., 1978; Watanabe et al., 1982; Kawasaki, et al., 1982, 1984; Enomoto et al., 1983; Tawaragi et al., 1984). Recently, we have purified two forms of DNA polymerase α from mouse FM3A cells, one of which is associated with primase activity (Enomoto et al., 1985). More recently, we have succeeded to dissociate and reconstitute the DNA polymerase α–primase complex (Suzuki et al., 1985). The purified two forms showed very low activity on poly(dA)·$(dT)_{12-18}$ as compared to the crude enzymes. It is possible that this low efficiency reflects the lack of additional polymerase-accessory factors in the preparations of purified DNA polymerase α. Therefore, we have developed an assay system for factors that stimulate DNA polymerase α activity on poly(dA)·$(dT)_{12-18}$.

This paper describes the detection of such a novel stimulating factor and suggests that this factor is different from C_1C_2 and other factors reported previously with mammalian cells.

Materials and Methods

Materials. Poly(ethylene glycol) 6000 was purchased from Koch-Light. Poly(dA) and $(dT)_{12-18}$ were obtained from P-L Biochemicals. Activated DNA was prepared as described previously (Aposhian & Kornberg, 1962).

Cells. FM3A cells were grown in the abdominal cavity of ddY mouse and harvested as described previously (Hanaoka et al., 1981).

Buffers. Buffer 1 contained 20 mM potassium phosphate buffer, pH 7.5, 0.1 mM ethylenediaminetetraacetic acid trisodium salt (Na_3EDTA),[1] 1 mM 2-mercaptoethanol, 0.25 mM PMSF, 1% ethanol, and 2 μg/mL antipain. Buffer 2 contained all the components of buffer 1, 20% ethylene glycol, and 0.01% Triton X-100. Buffer 3 contained all the components of buffer 1 and 50% glycerol.

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[1] Abbreviations: BSA, bovine serum albumin; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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Assay of DNA Polymerase α Stimulating Factor. The reaction mixture (30 μL) contained 20 mM Tris-HCl, pH 8.0, 3 mM dithiothreitol, 8% poly(ethylene glycol) 6000, 100 μM [^{3}H]dTTP (0.5 Ci/mmol), 7 mM $MgCl_2$, 40 μg/mL BSA, 6.7 μg/mL poly(dA)·$(dT)_{12-18}$ (20:1), and 0.02–0.04 unit of purified FM3A DNA polymerase α_2 (3110 units/mg of protein). Incubations were carried out for 30 min at 37 °C. The reaction was terminated by chilling the reaction mixture to 0 °C and by adding 0.2 mL of 0.15 M sodium pyrophosphate, 0.3 mL of 1 mg/mL carrier DNA, and 2 mL of 5% TCA containing 1% sodium pyrophosphate. TCA-insoluble materials were collected by filtration on a Whatman GF/C glass filter. The filter was washed 5 times with 3 mL of cold 5% TCA containing 1% pyrophosphate and 2 times with ethanol and dried. One unit of the stimulating activity is defined as a 100% stimulation of DNA polymerase α activity in this assay.

Assay of DNA Polymerase α. The standard reaction mixture (30 μL) contained 20 mM Tris-HCl, pH 8.0, 3.3 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 0.2 mg/mL BSA, 100 μM each of dATP, dCTP, and dGTP, 50 μM [^{3}H]dTTP (0.1 Ci/mmol), and 500 μg/mL activated calf thymus DNA. Incubations were carried out for 30 min at 37 °C in a plastic immunological microtitration tray. Each reaction mixture was spotted on a 1-in. square of DEAE paper (Whatman DE 81), and the paper was washed with 5% Na_2HPO_4 3 times and then with water twice and with ethanol once. One unit of the enzyme activity is defined as the amount catalyzing the incorporation of 1 nmol of dNMP into acid-insoluble materials in 1 h at 37 °C.

Assay of Other Enzymes. Assay of DNA-dependent ATPase was carried out as described previously (Tawaragi et al., 1984). DNA polymerase β and γ activities were assayed as described previously (Enomoto et al., 1983). Protein E was assayed as described by Kawasaki et al. (1984). Primase activity was assayed as described previously (Enomoto et al., 1985).

Purification of DNA Polymerases α_1 and α_2. DNA polymerases α_1 and α_2 were purified from mouse FM3A cells essentially according to the methods as described previously (Enomoto et al., 1985) by sequential column chromatography on DEAE-cellulose (Brown), DE-52 (Whatman), phosphocellulose, hydroxylapatite, and single-stranded DNA–cellulose.

Purification of DNA Polymerase β. DNA polymerase β was purified from FM3A cells by sequential column chromatography on DEAE-cellulose, DEAE-cellulose, phosphocellulose, hydroxylapatite, and native DNA–cellulose to a specific activity of 7.2×10^4 units/mg of protein. One unit of the enzyme activity is defined as the amount catalyzing the incorporation of 1 nmol of dNMP into acid-insoluble materials in 1 h at 37 °C.

Purification of DNA Polymerase γ. DNA polymerase γ was purified from FM3A cells by DEAE-cellulose column chromatography, ammonium sulfate precipitation, and a second DEAE-cellulose, phosphocellulose, and hydroxylapatite column chromatography, successively to a specific activity of 9.8×10^4 units/mg of protein. One unit of the enzyme activity is defined as the amount catalyzing the incorporation of 1 nmol dTMP into acid-insoluble materials with poly(rA)·$(dT)_{12-18}$ (5:1) as template-primer in 1 h at 37 °C.

Purification of the Factor. All operations were carried out at 0–4 °C. A frozen stock of 5×10^{10} FM3A cells was thawed, suspended in 400 mL of buffer 1, and sonicated 3 times for 10 s each with 20-s intervals by a Branson sonifier Model 185 (40 W). The sonicate was made 0.3 M in KCl by the addition of one-tenth volume of buffer 1 containing 3.3 M KCl. Extraction was performed for 30 min with stirring. The extract was centrifuged for 30 min at 15000*g*, and the supernatant was recentrifuged for 1 h at 125000*g*. The supernatant was loaded onto a DEAE-cellulose column (500 mL, 4.5 × 29 cm) equilibrated with 0.3 M KCl in buffer 1. The flow-through fractions were combined and dialyzed against 50 mM KCl in buffer 2 after the addition of Triton X-100 at a final concentration of 0.01%. The dialysate was centrifuged for 30 min at 15000*g*. The supernatant was loaded onto a DE-52–cellulose column (250 mL, 4.3 × 18.5 cm) equilibrated with 50 mM KCl in buffer 2. The column was washed with 2.5 column volumes of the above buffer, and the proteins adsorbed to the column were eluted with 10 column volumes of a linear gradient of KCl from 50 to 400 mM in buffer 2. An activity that stimulated DNA polymerase α activity on poly(dA)·$(dT)_{12-18}$ was eluted from the column at about 150 mM KCl, and the peak of the activity coincided with the peak of DNA polymerase α activity. The peak fractions of the stimulating activity were combined and dialyzed against 50 mM KCl in buffer 2. The dialysate was loaded onto a phosphocellulose column (75 mL, 2.4 × 16.6 cm). The column was washed with 50 mM KCl in buffer 2 and eluted with 10 column volumes of a linear gradient of KCl from 50 to 800 mM in buffer 2. The stimulating activity was eluted from the column at 600 mM KCl, being separated from DNA polymerase α activity. The peak fractions were pooled and loaded onto hydroxylapatite column (10 mL, 1.8 × 4 cm) equilibrated with 400 mM KCl in buffer 2. The proteins bound to the column were eluted with 8 column volumes of a linear gradient of potassium phosphate buffer, pH 7.5, from 20 to 200 mM in buffer 2 containing 400 mM KCl. The activity was eluted from the column at 100 mM potassium phosphate. The active fractions were pooled and dialyzed against 50 mM KCl in buffer 2. The dialysate was applied onto native DNA–cellulose column (4 mL, 1.2 × 4 cm). The column was washed with 3 column volumes of 50 mM KCl in buffer 2 and eluted with 10 column volumes of a linear gradient of KCl from 50 to 600 mM in buffer 2. The stimulating factor was eluted from the column at 200 mM KCl, being separated from the residual contaminating activity of protein E, which was eluted from the column at 120 mM KCl (Kawasaki et al., 1984). The active fractions were pooled and dialyzed against 50 mM KCl in buffer 3. The dialysate was stored at −20 or −80 °C until use. The specific activity of the purified factor was 2.7×10^4 units/mg of protein.

Glycerol Gradient Sedimentation. Two hundred microliters of the native DNA–cellulose column fraction concentrated with a small phosphocellulose column was layered onto a 15–35% glycerol gradient in a solution containing 50 mM potassium phosphate, pH 7.5, 100 mM KCl, 2 mM 2-mercaptoethanol, 0.5% ampholine, pH 6–8, 10 mM $NaHSO_3$, 2 μg/mL antipain, and 0.25 mM PMSF. The gradient was centrifuged for 22 h at 216000*g* at 4 °C.

Formation of the DNA Polymerase α and Stimulating Factor Complex. Purified DNA polymerase α_2 was mixed with the purified factor. The mixture, the purified DNA polymerase α, and the DNA polymerase α of the DE-52 column fraction were added with BSA at a final concentration of 0.2 mg/mL and dialyzed against 20 mM Tris-HCl, pH 7.8 50 mM KCl, 7 mM $MgCl_2$, 2 μg/mL antipain, 0.25 mM PMSF 1 mM 2-mercaptoethanol, and 20% ethylene glycol for 9 h at 4 °C. Two hundred microliters each of the dialysates was layered onto 15–35% glycerol gradients in a solution containing 20 mM Tris-HCl, pH 7.8, 50 mM KCl, 7 mM $MgCl_2$, 0.2 mg/mL BSA, 2 μg/mL antipain, 0.25 mM PMSF,

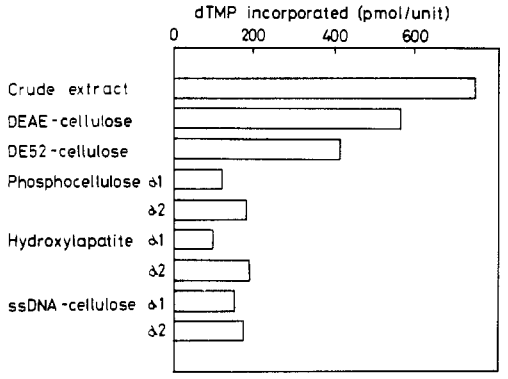

FIGURE 1: Decrease of the activity on poly(dA)·$(dT)_{12-18}$ during purification of DNA polymerase α. The DNA polymerase α fractions at various stages of purification were prepared as described under Materials and Methods. The unit of DNA polymerase α was determined by α assay using activated calf thymus DNA. DNA polymerase activity on poly(dA)·$(dT)_{12-18}$ was assayed under conditions for stimulating factor assay without purified DNA polymerase α.

and 1 mM 2-mercaptoethanol. Centrifugation was performed for 18 h at 216000g at 4 °C.

Analysis of Chain Length of Products by Polyacrylamide Gel Electrophoresis. DNA was synthesized in 90 μL of the standard reaction mixture for the stimulating factor assay except that [α-^{32}P]dTTP (1 Ci/mmol) and $(dT)_{10}$ were used instead of [^{3}H]dTTP and $(dT)_{12-18}$, respectively. After incubation at 37 °C for 10 min, the reaction was stopped by the addition of 18 μL of 2.4% SDS and 40 mM Na_3EDTA at 0 °C, and ethanol precipitation was carried out twice in the presence of 10 mM $MgCl_2$ and 2 M ammonium acetate. The precipitates were dissolved in 20 μL of a solution containing 90% formamide, 89 mM Tris–borate, pH 8.0, 1 mM Na_3EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol. The solution was heated at 90 °C for 10 min and quickly chilled in ethanol–ice bath. The samples were loaded on a 23% polyacrylamide (20:1) slab gel (40 × 18 × 0.1 cm) containing 7 M urea, 89 mM Tris–borate, pH 8.0, and 1 mM Na_3EDTA. Electrophoresis was performed at 600 V until two dyes had separated from each other by between 8 and 12 cm. The electrophoresis running buffer was 89 mM Tris–borate, pH 8.0, containing 1 mM Na_3EDTA. Autoradiography was performed at −80 °C using Kodak X-Omat AR films.

Results

Detection of a Stimulating Factor for DNA Polymerase α Activity on Poly(dA)·$(dT)_{12-18}$. In our previous study, we have purified two forms of DNA polymerase α from mouse FM3A cells (Enomoto et al., 1985). One form designated as α_1 was tightly associated with primase activity, and the other form, α_2, contained no primase activity. The purified two forms showed very similar properties in their sensitivity to several inhibitors and their preference for template-primers. They utilized poly(dA)·$(dT)_{12-18}$ less effectively than the crude enzyme fractions did, suggesting that factors which stimulate DNA polymerase α activity on poly(dA)·$(dT)_{12-18}$, were lost during purification. As shown in Figure 1, the activities of α_1 and α_2 on poly(dA)·$(dT)_{12-18}$ as a template-primer were reduced to very low levels after the step of phosphocellulose column chromatography. In order to detect stimulating factors that facilitate the DNA synthesis by purified DNA polymerase α on poly(dA)·$(dT)_{12-18}$, an assay system has been developed. The assay system contains 8% poly(ethylene glycol) 6000. The addition of poly(ethylene glycol) increased the activity on poly(dA)·$(dT)_{12-18}$ by more than 100-fold (Table I). Polyvinyl alcohol showed a similar effect. With this assay system, an

Table I: Specificity of the Stimulating Factor for DNA Polymerase α[a]

DNA polymerase	template: activated DNA (pmol)	template: poly(dA)·$(dT)_{12-18}$ −factor (pmol)	template: poly(dA)·$(dT)_{12-18}$ +factor (pmol)	x-fold
FM3A DNA pol α	8.9	1.3 (0.01)[b]	14.3 (0.06)[b]	11
FM3A DNA pol β	10.5	69.2	72.1	1.04
FM3A DNA pol γ	6.9	5.2	6.5	1.3
E. coli DNA pol I	8.4	22.4	13.1	0.58

[a] Reactions were carried out in the standard DNA polymerase α assay mixture and in the standard stimulating factor assay mixture. DNA polymerases were prepared as described under Materials and Methods. [b] The number in the parentheses indicates the incorporation of dTMP in the absence of 8% poly(ethylene glycol).

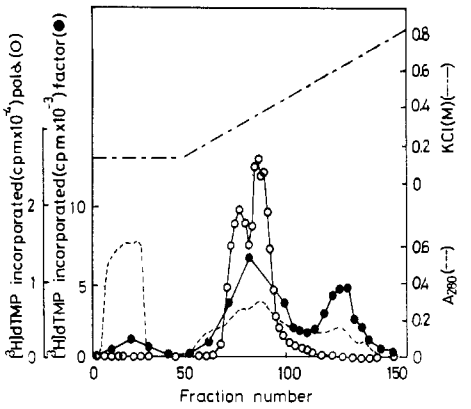

FIGURE 2: Detection of a stimulating factor on a phosphocellulose column. The second DEAE-cellulose column fraction was chromatographed on a phosphocellulose column. Five and one microliters of each fraction were assayed for DNA polymerase α (○) and the stimulating factor activity (●), respectively. Absorbance at 280 nm (---); concentration of KCl (– – –).

activity that stimulates purified DNA polymerase α_1 and α_2 activities on poly(dA)·$(dT)_{12-18}$ was detected in the phosphocellulose column fraction (Figure 2). The stimulating activity was eluted from the column at about 0.6 M KCl, and DNA polymerase activities of α_1 and α_2 were eluted at 0.38 and 0.31 M KCl, respectively. The stimulating factor was further purified by successive column chromatography on hydroxylapatite and native DNA–cellulose to a specific activity of 27 000 units/mg of protein as described under Materials and Methods.

Properties of the Stimulating Factor. The partially purified factor had no detectable activities of DNA polymerase α, β, and γ, primase, and DNA-dependent ATPase. It sedimented at 1.8 S in a glycerol gradient containing 0.1 M KCl and 0.05 M potassium phosphate buffer, pH 7.5. The stimulating activity was completely inactivated by the treatment of heat at 100 °C for 10 min or 500 μg/mL proteinase K at 37 °C for 30 min, suggesting that the stimulating activity is protein nature.

In order to know whether this factor has protein E activity previously reported by us (Kawasaki et al., 1982, 1984), which stimulates DNA polymerase α activity on heat-denatured DNA, the factor was assayed under conditions for E activity in addition to the factor assay. The results showed that the factor had no protein E activity.

Characterization of and Optima for Stimulation Assay. The factor stimulated of both DNA polymerases α_1 and α_2. Therefore, DNA polymerase α_2 was used for the following experiments. The activities of the DNA polymerase α on poly(dA)-$(dT)_{12-18}$ in the presence and absence of the factor were increased by more than 100-fold by the addition of

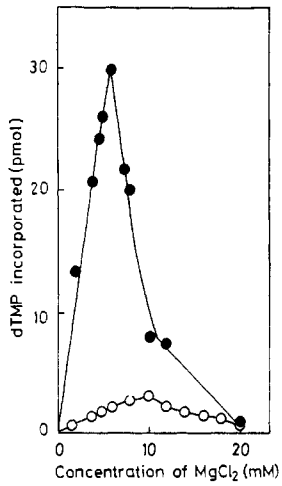

FIGURE 3: Effect of $MgCl_2$ concentration on the stimulation. Assays were performed under conditions for the stimulating factor assay in the presence of the indicated concentrations of $MgCl_2$. DNA polymerase α (0.04 unit) alone (○); DNA polymerase α (0.04 unit) and purified stimulating factor (13 units) (●).

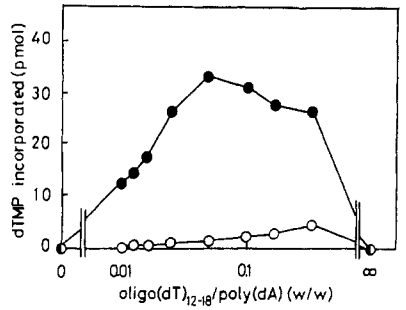

FIGURE 4: Stimulating activity as a function of the ratio of oligo(dT) to poly(dA). Reactions were performed under standard conditions for 30 min in the presence of poly(dA)·$(dT)_{12-18}$ with various ratios of oligo(dT) to poly(dA) as indicated. The total amount of poly(dA) and oligo(dT) was fixed at 6.7 μg/mL. DNA polymerase α (0.03 unit) alone (○); DNA polymerase α (0.03 unit) and purified stimulating factor (10 units) (●).

poly(ethylene glycol) 6000. In the presence of the stimulating factor, a maximum activity was obtained with 8% poly(ethylene glycol).

The reaction required a divalent cation. A sharp peak of activity was obtained with 8 mM $MgCl_2$ in the presence of the factor, and a broader peak of activity between 4 and 14 mM was observed in the absence of the factor (Figure 3).

Figure 4 shows the effect of primer to template ratio on the activities of DNA polymerase α in the presence and absence of the stimulating factor. The activity in the absence of the factor increased gradually in proportion to the ratio up to 0.33. On the other hand, the activity in the presence of the factor reached a maximum at the ratio of 0.05 and slightly decreased at higher primer concentrations. Stimulation by the factor was observed in all the ratios tested, and large stimulatory effect was observed between 0.016 and 0.05 corresponding to about 1 and 3.3 primers on one template, respectively.

The concentration of DNA polymerase α used in the assay affected the stimulation by the factor. As shown in Figure 5, stimulation became more prominent at the concentration of DNA polymerase α lower than 0.04 unit per assay.

Under optimal conditions for the stimulation, DNA synthesis in the presence of the factor continued linearly for about 60 min and at a diminishing rate during further incubation. The initial rate and the extent of DNA synthesis in the presence of the factor were higher than those in the absence of the factor.

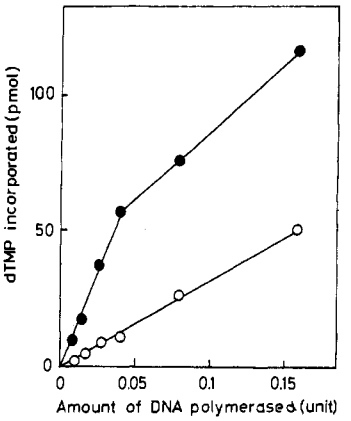

FIGURE 5: Effect of the amount of DNA polymerase α on the stimulation. DNA polymerase activity on poly(dA)·$(dT)_{12-18}$ was measured in the presence (●) or absence (○) of purified stimulating factor (13 units) with various amount of DNA polymerase α as indicated in the figure.

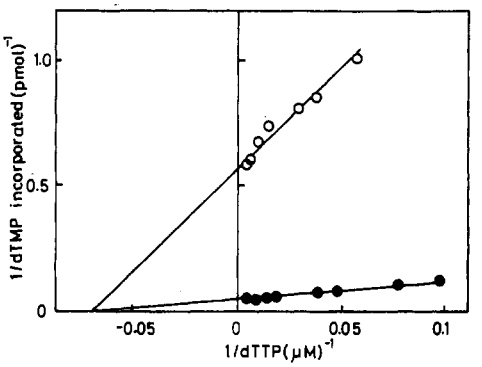

FIGURE 6: Determination of the K_m for the deoxyribonucleotide substrate. The K_m values for dTTP were determined by varying the concentration of dTTP up to 200 μM. Double-reciprocal plots obtained in the absence (○) and in the presence of the stimulating factor (13 units) (●) are indicated.

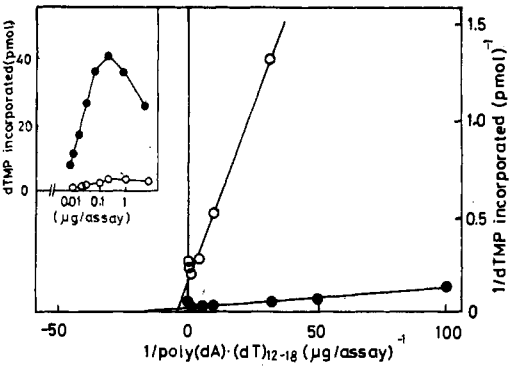

FIGURE 7: Determination of the K_m for poly(dA)·$(dT)_{12-18}$ (20:1). DNA polymerase α (0.03 unit) was incubated in the presence of the stimulating factor (13 units) (●) and in the absence of the factor (○) with various concentrations of poly(dA)·$(dT)_{12-18}$ up to 10 μg per assay under the standard conditions for factor assay. Double-reciprocal plots of the results are shown. (Inset) Initial velocity vs. poly(dA)·$(dT)_{12-18}$ concentration for DNA polymerase α alone (○) and DNA polymerase α with the factor (●).

Analysis of the Mechanism of Stimulation. In order to investigate the mechanism of the stimulation, key parameters of the DNA synthesis in the presence and absence of the stimulating factor were measured under the standard stimulating assay condition. As shown in Figure 6, the stimulating factor did not change the K_m value of DNA polymerase α for dTTP (13 μM). The K_m value for poly(dA)·$(dT)_{12-18}$ (20:1) in the absence of the stimulating factor was reduced by about 4-fold (11 to 3 μg/mL) by the addition of the factor (Figure

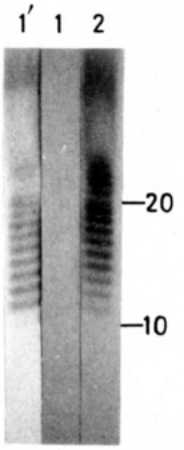

FIGURE 8: Sizes of the products in the presence and absence of the stimulating factor. Reactions were carried out for 10 min at 37 °C in the standard reaction mixture (90 μL) with 100 μM [α-^{32}P]dTTP (1 Ci/mmol), 6.7 μg/mL poly(dA)·$(dT)_{10}$ (20:1), and 0.09 unit of DNA polymerase α either in the presence of purified stimulating factor (39 units) or in the absence of the factor. Synthesized products were analyzed by electrophoresis in a 23% polyacrylamide slab gel containing 7 M urea as described under Materials and Methods. Positions of 10 and 20 nucleotides in length are indicated. Lane 1, −factor, 3-day exposure; lane 2, +factor, 3-day exposure; lane 1′, −factor, 8-day exposure.

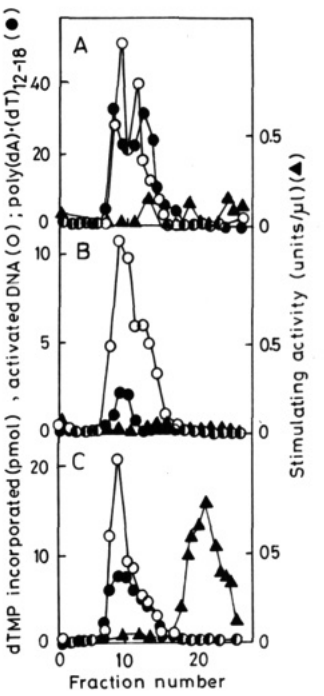

FIGURE 9: Formation of the complex between DNA polymerase α and the stimulating factor. The purified DNA polymerase α was mixed with the purified factor. The mixture, the purified DNA polymerase α alone, and DNA polymerase α of the second DEAE-cellulose column fraction were dialyzed against the buffer containing 50 mM KCl and 7 mM $MgCl_2$ and centrifuged as described under Materials and Methods. Seven-microliter aliquots of each fraction were assayed for DNA polymerase activity with activated DNA (O) and poly(dA)·$(dT)_{12-18}$ (●). One-microliter aliquot of each fraction was assayed with poly(dA)·$(dT)_{12-18}$ for stimulating factor activity (▲). (A) Second DEAE-cellulose column fraction of DNA polymerase α; (B) purified DNA polymerase α; (C) purified DNA polymerase α with stimulating factor.

7). Similar results were obtained with poly(dA)·$(dT)_{12-18}$ (100:1) (data not shown).

We next analyzed the size of the products. The products were fractionated by polyacrylamide gel electrophoresis under conditions that resolve single nucleotide difference in length. As shown in Figure 8, very faint bands were detected with the products synthesized in the absence of the factor after exposure for 3 days (lane 1), while distinct bands were observed with the products synthesized in the presence of the factor (lane 2). A long exposure of lane 1 revealed several distinct bands (lane 1′). It is obvious from the results that the sizes of the products in the presence of the stimulating factor are not so much different from those in the absence of the factor.

Specificity of the Stimulation for DNA Polymerase α. The stimulating factor was tested for its ability to stimulate the activity of various DNA polymerases on poly(dA)·$(dT)_{12-18}$. DNA polymerases α, β, and γ and *Escherichia coli* DNA polymerase I were assayed under the stimulating factor assay condition with or without the stimulating factor. The factor stimulated DNA polymerase α but did not stimulate DNA polymerases β and γ and *E. coli* DNA polymerase I (Table I).

Stimulating Factor Physically Interacts with DNA Polymerase α. In order to investigate the possibility that the stimulating factor directly interacts with DNA polymerase α, we tried to construct the DNA polymerase α–stimulating factor complex. Purified DNA polymerase α was mixed with an excess amount of the stimulating factor and dialyzed against a medium containing 7 mM $MgCl_2$ as described under Materials and Methods. The mixture was subjected to glycerol gradient centrifugation. The purified DNA polymerase α alone and the DNA polymerase α of the second DEAE-cellulose column fraction were also centrifuged in parallel gradients. The DNA polymerase α of the second DEAE-cellulose column fraction showed high activity on poly(dA)·$(dT)_{12-18}$ (Figure 9A). In contrast, the purified DNA polymerase α alone showed very low activity on the template-primer (Figure 9B). Relatively high activity to utilize poly(dA)·$(dT)_{12-18}$ was observed with the purified DNA polymerase α which had been mixed with the factor, indicating the formation of the DNA polymerase α–stimulating factor complex (Figure 9C). Excess stimulating factor sedimented at 1.8 S as the free form.

The reaction to form the complex required the presence of $MgCl_2$ and was sensitive to salts. In the presence of 0.3 M KCl, the factor did not form the complex. Furthermore, under the above condition, the DNA polymerase α of the second DEAE-cellulose column fraction was converted into free DNA polymerase α and stimulating factor.

Existence of a Similar Factor as a Free Form. We detected a similar activity in the flow-through fractions of the second DEAE-cellulose column, which contained DNA polymerase β but not DNA polymerase α activity. At this stage of purification, it was not clear whether or not the stimulating activity was really due to stimulating factors because it is difficult to evaluate the stimulating activity with the sample containing DNA polymerase β activity. The DNA polymerase β contained in the flow-through fraction was separated from the stimulating activity by phosphocellulose column chromatography. The factor recovered from the flow-through fractions of the second DEAE-cellulose column behaved similarly as the factor described above on phosphocellulose, hydroxylapatite, and native DNA–cellulose columns and had the same sedimentation coefficient of 1.8 S. In addition to the above observations, the optima of pH, divalent cation, salt, and DNA concentration for their stimulation assay were the same between the two factors (data not shown). Furthermore, when one factor was added to the reaction mixture containing a saturating level of the other factor, no additional stimulation was caused by the addition. The factor recovered from the flow-through fraction also reduced the K_m for the template-

primer, specifically stimulated DNA polymerase α, and formed a complex with the enzyme as the factor described in the previous section.

Discussion

A novel factor that stimulates DNA polymerase α activity on poly(dA)·$(dT)_{12-18}$ has been isolated from mouse FM3A cells. The results of product analysis revealed that the stimulation by the factor was mainly due to the increase in the initiation frequency of DNA synthesis from the primers (Figure 8). The above observation can be explained by the fact that the factor directly interacts with DNA polymerase α to form a complex (Figure 9) and accentuates the affinity of the polymerase for template-primer (Figure 7).

Several factors influencing DNA polymerase α activity have been isolated from mammalian cells. One type of the factors, designated as single-stranded DNA binding protein or helix destabilizing protein, requires stoichiometric interaction with DNA for stimulation, affecting its secondary and higher order conformations (DePamphilis & Wasserman, 1980). These proteins preferentially bind to single-stranded DNA, whereas the stimulating factor reported here binds both single-stranded and double-stranded DNA (unpublished observation). The other type includes the factors that stimulate DNA polymerase α activity with a DNA template-primer which contains extended single-stranded regions, functioning as primer recognition protein. C_1C_2 (Pritchard & DePamphilis, 1983; Pritchard et al., 1983) and protein E (Kawasaki et al., 1984) belong to this type of stimulating factor. Protein E, which we have reported previously, stimulates DNA polymerase α activity on heat-denatured DNA by increasing the initiation frequency of DNA synthesis from 3′-hydroxyl termini of the DNA. However, the factor has no detectable protein E activity. In addition, the factor and protein E were separated from each other by native DNA–cellulose column chromatography.

C_1C_2 complex, which has been isolated from CV-1 and HeLa cells and characterized in detail by Pritchard et al. (1983), stimulates DNA polymerase α activity not only on heat-denatured DNA but also on any template-primer such as poly(dT)·$(dA)_{10}$ (100:1) as long as the ratio of template to primer is high. It has been suggested that C_1C_2 enhances the ability of DNA polymerase α to initiate DNA synthesis from the termini of primers. The mechanism of the stimulation by the factor reported here seems to be similar to that by C_1C_2; however, the factor differs from C_1C_2 in several respects. (1) The sedimentation coefficient of the factor is 1.8 S compared to 4.7 and 3.5 S for C_1 and C_2, respectively. (2) The stimulating factor binds to the native DNA–cellulose column, but C_1C_2 does not. (3) The factor showed no detectable C_1C_2 activity under the conditions optimized for C_1C_2 assay.

Recently, Sapp et al. (1985) isolated single-strand-specific DNA binding proteins from calf thymus, which increase the probability for an association of DNA polymerase α with 3′-OH primer ends. These proteins are distinguished from the factor reported here by the fact that the proteins almost totally ignore double-stranded DNA.

All above observations indicate that the factor reported here is a new factor, which is different from the DNA polymerase α stimulating factors ever reported with mammalian cells. Ganz and Pearlman (1980) reported M protein which stimulates *Tetrahymena* DNA polymerase activity on poly(dA)·oligo(dT). However, it is not clear whether or not this protein corresponds to the factor reported here.

Fisher and Korn (1979a,b) have demonstrated that the binding of DNA polymerase α to DNA is absolutely dependent on the presence of single-stranded regions and that the recognition of the 3′-hydroxyl primer terminus occurs only in concert with, or subsequent to, the binding of the enzyme to single-stranded regions. Therefore, we must take into consideration "nonproductive" binding of DNA polymerase α to single-stranded regions. Then a possibility can be postulated for the stimulation by the factor that the factor eliminates nonproductive binding of the enzyme to single-stranded DNA, allowing it to slide along the template until it recognizes a primer. The above mechanism of the stimulation of DNA polymerase α has been proposed for the stimulating factors C_1C_2 and the single-strand-specific DNA binding proteins from calf thymus. However, the stimulating factor reported here is distinguished from these proteins as described.

At present, it is not clear whether the stimulating factor directly increases the affinity of DNA polymerase α for the template-primer or indirectly increases it by eliminating nonproductive binding. Whatever the precise mechanism of the stimulation by the factor is, it must be emphasized that the factor specifically interacts with DNA polymerase α to form a complex with the enzyme and stimulates the enzyme activity by increasing the initiation frequency of DNA synthesis from the 3′-hydroxyl termini of the primers. The above is suggestive that the stimulating factor is one of the components of the DNA polymerase α holoenzyme complex.

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Registry No. Mg, 7439-95-4; dTTP, 365-08-2; poly(dA)·(dT), 24939-09-1; DNA polymerase, 9012-90-2; poly(ethylene glycol), 25322-68-3.

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Differential Effects of Captan on DNA Polymerase and Ribonuclease H Activities of Avian Myeloblastosis Virus Reverse Transcriptase†

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ABSTRACT: Captan was used as an inhibitor of avian myeloblastosis virus reverse transcriptase to study the polymerase and RNase H catalytic activities. With purified enzyme, RNase H activity was 10-fold more sensitive to captan than was either the DNA-dependent or RNA-dependent DNA polymerase activity. Inhibition of the RNA-dependent polymerase activity could be prevented by dTTP. Conversely, inhibition of this polymerase activity was enhanced by template/primer. The calculated K_{dTTP} of the uninhibited reaction was 5.6 μM. Kinetic studies allow for the proposition of a model for the interaction of captan with the polymerase active center. RNase H activity showed a sigmoidal relationship between activity and substrate concentration. Nuclease activity decreased in V_{max} with no change in the Hill coefficient in the presence of captan. Addition of dithiothreitol to the incubation cocktail prevented inhibition by captan of both RNA-dependent polymerase and RNase H activities, suggesting that the (trichloromethyl)thio moiety of captan is involved in the inhibitory action. Captan inhibition suggests the presence of essential amino residues in both polymerase and RNase H active centers.

Reverse transcriptases are unique enzymes. They are capable of polymerizing DNA from an RNA template (Baltimore, 1970; Temin & Mizutani, 1970) or from a DNA template (Verma, 1977). Many also have a ribonuclease H activity that is used to processively degrade, in either a 5′ → 3′ or a 3′ → 5′ direction, the RNA strand of an RNA–DNA hybrid (Perbal, 1984; Molling et al., 1971). AMV[1] reverse transcriptase is composed of two structurally related polypeptides, an α subunit of approximately 65 kDa and a β subunit of 95 kDa assembled into an $\alpha\beta$ holoenzyme (Grandgenett et al., 1973). This dimer is thought to be generated by proteolytic cleavage of a minor, less active $\beta\beta$ precursor (Gibson & Verma, 1974). It has been reported that the polymerizing activity is associated with the β subunit whereas both polymerase and ribonuclease H activities are observed in connection with the α subunit (Grandgenett et al., 1973). Little is known concerning the relationship of the active sites to each other or to the enzyme as a whole, although both polymerase and ribonuclease H activities arise from a single gene product (Verma et al., 1974). Whether RNase H and polymerase activities reside in different areas of the enzyme or have overlapping active centers has been a subject of debate (Jacob, 1983). Another area of active research is the identification of amino acid residues that are important to catalysis.

Captan is a known inhibitor of RNA and DNA polymerases (Dillwith & Lewis, 1980; Dillwith & Lewis, 1982a; Dillwith & Lewis, 1982b). Captan has also proved to be an inhibitor of AMV reverse transcriptase. In this study, we present evidence that captan preferentially inhibited RNase H as compared to polymerase function, indicating that DNA polymerase and RNase H activities have independent active sites. In this way captan is acting unlike other reported chemical modifiers of this enzyme (Gorecki & Panet, 1978; Brewer & Wells, 1974; Srivastava et al., 1981). Inhibition data also support the hypothesis that lysine is involved both in the dTTP binding

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[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; captan, *N*-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide; AMV, avian myeloblastosis virus; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Me_2SO, dimethyl sulfoxide.

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