Stimulation of DNA Polymerase α Activity by Microtubule-Associated Proteins†

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ABSTRACT: Microtubule-associated protein 2 (MAP2) isolated from porcine brains stimulated the activity of DNA polymerase α immunopurified from calf thymus or human lymphoma cells, in a dose-dependent manner. This stimulation was pronounced when activated DNA or poly(dA)·$(dT)_{10}$ was used as the template-primer. DNA polymerase α bound to a MAP2-immobilized column, whereas preincubation of the enzyme with unbound MAP2 prevented binding to the column. These events suggested that a physical binding occurred between the polymerase and MAP2. Kinetic analyses revealed that MAP2 decreased the K_m value of the polymerase for deoxyribonucleotides, irrespective of the species of template-primer. A concomitant increase in V_{max} was observed; however, the extent of the increase depended on the species of template-primer. MAP2 also decreased the K_m value of the polymerase for template-primers when activated DNA of poly(dA)·$(dT)_{10}$ was used as the template-primer. Product analyses showed that MAP2 did not significantly alter the processivity of the polymerase and the increment of V_{max} is considered to be due to an increase in the frequency of initiation of DNA synthesis. The stimulation by MAP2 occurred specifically in the activity of DNA polymerase α, but not DNA polymerases β, γ, and I from *Escherichia coli*. Other MAPs, tau and 190-kDa MAP, could substitute for MAP2. Thus, the specific stimulation of DNA polymerase α by MAPs supports the notion of a possible involvement of MAPs or MAP-like proteins in DNA replication, in vivo.

Five types (α, β, γ, δ, and ε) of DNA polymerases have been identified in eukaryotes (Burgers et al., 1990). Several lines of evidence show that DNA polymerases α and δ are essential for chromosomal DNA replication whereas DNA polymerase β may be involved in DNA repair and DNA polymerase γ replicates mitochondrial DNA (Lehman & Kaguni, 1989). DNA polymerase ε is most likely involved in DNA repair in HeLa cells (Syvaoja & Zinn, 1989). Recent investigations suggested that DNA polymerases α and δ participate in the synthesis of lagging and leading strands, respectively, in the process of eukaryotic DNA replication (So & Downey, 1988; Blow, 1989). Purified DNA polymerase α is composed of several subunit polypeptides, including catalytic polypeptides of DNA polymerase α and DNA primase (Lehman & Kaguni, 1989). Since purified DNA polymerase α cannot completely mimic in vivo DNA synthesis in vitro, it is assumed that the enzyme acts in conjunction with other protein factors, forming a putative DNA replication machinery (Reddy & Pardee, 1980; Jazwinski & Edelman, 1982; Ottiger & Hübscher, 1984; Shioda, 1986; Tubo & Berezney, 1987b).

Microtubule-associated protein (MAP)[1] is the general term for accessory proteins which associate with microtubules, one of the major cytoskeletal components, and stimulate the assembly of microtubules (Dustin, 1984). MAP1, MAP2, and tau are major MAPs localized mainly in neural cells (Dustin, 1984; Vallee et al., 1984; Wiche, 1989), while 190-kDa MAP is widely distributed to nonneural cells (Kotani et al., 1988). It was noted that MAP2, tau, and 190-kDa MAP share common sequences in binding sites to microtubules (Lee et al., 1988; Lewis et al., 1988; Aizawa et al., 1989). Since microtubules are present almost exclusively in the cytoplasm, it has been considered that MAPs function in the cytoplasm as part of the microtubular structure (Dustin, 1984; Vallee et al., 1984). However, parts of MAP1 and MAP2, or proteins immunologically related to them, were found to be present in phosphorylated forms in the nuclei of actively proliferating mammalian cells in culture (Sato et al., 1985a,b, 1986, 1988). Furthermore, an association of MAP2-like proteins with the nuclear matrix was noted in rat liver cells (Nakayasu et al., 1988). These findings suggest the possibility that MAPs or MAP-like proteins may participate in DNA replication in the nucleus. Most recently, we reported that MAP2 stimulated in vitro DNA synthesis catalyzed by the isolated nuclear matrix (Shioda et al., 1989). This finding provides support for the above-mentioned possibility, since the putative DNA replication machinery containing DNA polymerase α is considered to function on the nuclear matrix (Smith & Berezney, 1982; Tubo & Berezney, 1987a,b). We postulated that MAP2 stimulated DNA synthesis by interacting with DNA polymerase α present in the nuclear matrix (Shioda et al., 1989). In the present study, we examined the direct effects of MAP2 on purified DNA polymerase α and obtained evidence that MAP2 bound to DNA polymerase α and stimulated the enzyme activity. We tentatively propose that MAPs or MAP-like proteins play a role in DNA replication.

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[1] Abbreviation: MAP, microtubule-associated protein.

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Experimental Procedures

Materials. Unlabeled deoxyribonucleoside 5′-triphosphates (dATP, dCTP, dGTP, and dTTP) were obtained from Boehringer Mannheim (Mannheim, FRG). [^{3}H]dATP (23 Ci/mmol), [^{3}H]dCTP (25 Ci/mmol), [^{3}H]dGTP (20 Ci/mmol), [^{3}H]dTTP (70 Ci/mmol), [α-^{32}P]dATP (3000 Ci/mmol), and [α-^{32}P]dTTP (3000 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, CA). Poly(dA) (average length 260 base pairs), poly(dT) (average length 770 base pairs), $(rA)_{10}$, $(dA)_{12-18}$, and $(dT)_{10}$ were from PL—Pharmacia (Uppsala, Sweden). Calf thymus DNA was obtained from Sigma Chemical Co. (St. Louis, MO) and was activated as described (Fanslar & Loeb, 1974). Affi-Gel 10 was provided from Bio-Rad (Richmond, CA).

DNA Polymerases. DNA polymerase α containing DNA primase was purified from calf thymus and Raji cells (human Burkitt's lymphoma cell line) by means of immunoaffinity column chromatography using monoclonal antibody (MT-17) directed against calf thymus DNA polymerase α, as described (Tamai et al., 1988). The purified calf thymus and Raji cell polymerase preparations had a specific activity of 1250 and 1000 units/mg (1 unit corresponds to 1 nmol of dNMPs incorporated in 1 h with activated DNA as the template-primer under the present standard assay conditions in the presence of 60 μM each of dATP, dCTP, dGTP, and dTTP), respectively, and the former was composed of 140-, 50-, and 47-kDa polypeptides and the latter of 180-, 70-, 60-, and 50-kDa polypeptides with minor bands of 165- and 140-kDa polypeptides. DNA polymerases β and γ were purified from calf thymus and human acute myelogeneous leukemia cells, respectively, as described (Yoshida et al., 1979; Tanaka et al., 1987). *Xenopus* ovarian DNA polymerase α which corresponds to DNA polymerase α–primase complex designated α_1 (Shioda et al., 1982) was a generous gift from Dr. R. M. Benbow (Department of Zoology, Iowa State University). *Physarum* DNA polymerase α was partially purified by the method of Shioda et al. (1989). *Escherichia coli* DNA polymerase I (Klenow fragment) was from PL—Pharmacia.

Preparation of MAPs. MAP2 and tau were purified from porcine brains according to the method of Kotani et al. (1985); 190-kDa MAP was purified from bovine adrenal cortex, as described (Murofushi et al., 1986).

Assay for DNA Polymerase Activity. DNA polymerase activity was determined by the incorporation of radioactive dNMPs into the acid-insoluble materials with calf thymus activated DNA or synthetic polynucleotide–oligonucleotide complex as a template-primer. The standard reaction mixture contained 25 mM Tris-HCl (pH 7.5), 20 μM each of dATP, dCTP, and dGTP, 0.5 μM TTP containing 0.5 μCi of [^{3}H]-dTTP (10 000 cpm = 1.0 pmol of dTTP), 7 mM $MgCl_2$, 40 mM NaCl, 2 mM 2-mercaptoethanol, 2 μg of calf thymus activated DNA, 10 μg of bovine serum albumin, and 10% glycerol in a final volume of 25 μL. Incubation was carried out at 37 °C for 60 min, and acid-insoluble radioactivity was determined after the addition to 25 μL of a solution containing 1.0% sodium dodecyl sulfate, 100 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 50 mM EDTA, and 2 mM ATP, as described (Shioda et al., 1980).

To determine the stimulatory activity of MAPs on DNA polymerases, the assay was carried out in the absence and presence of MAPs. The enzyme reaction was started by the addition of DNA polymerase α, and the activity of MAPs required to stimulate the enzyme activity was expressed as the ratio dNMP incorporated in the presence of MAPs to that in the absence of MAPs.

MAP2 Affinity Column Chromatography. MAP2 (0.4 mg) dissolved in 2 mL of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (pH 6.8), 1 mM EGTA, and 0.5 mM $MgCl_2$ was mixed with 0.3 mL of Affi-Gel 10 and the preparation incubated overnight at 4 °C with gentle swirling. After the coupling reaction, the gel was blocked with 0.1 M Tris-HCl (pH 7.0). Most of the applied MAP2 was immobilized in the gel. An aliquot of the gel was packed to a column (column size 30 μL), and the column was equilibrated with 25 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 1 mM EDTA, 20% glycerol, and 1 mg/mL bovine serum albumin. For the preparation of control columns, coupling was performed in the absence of protein or in the presence of 1.3 mg/mL bovine serum albumin. To the column was applied 1.25 μg of DNA polymerase α, and the column was incubated for 15 min at room temperature. After washing out the unbound enzyme with the buffer solution used for equilibration, the adsorbed enzyme was eluted with the buffer solution containing 0.2 M NaCl.

Product Analysis by Gel Electrophoresis. In order to measure the processivity of DNA polymerase α, DNA synthesis was carried out as described above, except that template-primers and radioactive precursors were replaced as indicated in each figure legend, based on the method of Detera et al. (1981) with some modifications. ^{32}P-Labeled DNA products were isolated with phenol extraction and ethanol precipitation, according to the method of Maniatis et al. (1982), and subjected to gel electrophoresis on 25% polyacrylamide–7 M urea, as described (Maxam & Gilbert, 1977). When $(rA)_{10}$ was used as the primer, the isolated ^{32}P-labeled products was treated with 0.3 N NaOH for 17 h at 37 °C before gel electrophoresis. Gel electrophoresis was performed at 500 V/14 cm for 3–4 h. The position of newly synthesized DNA in the gel was determined by autoradiography (Maniatis et al., 1982) or by counting the radioactivity of the gel slices (3 mm each) in a liquid scintillator (Aquasol, New England Nuclear, Boston, MA).

Results

Dose-Dependent Stimulation of DNA Polymerase α Activity by MAP2. When activated DNA was used as the template-primer, the DNA polymerase α reaction was linear for at least 60 min. With the addition of MAP2, marked stimulation of the enzyme activity was observed at almost a constant rate of activation for at least 60 min; 7–10-fold activation of DNA polymerase α activity was observed by adding an excess amount of MAP2 to varying amounts of the polymerase, up to at least 200 ng. Preincubation of MAP2 with the polymerase led to no further stimulation of the activity. When the MAP2 preparation was subjected to gel filtration, the activity which stimulated DNA polymerase α was coeluted with MAP2 molecules (figure not shown). This result was identical to that for stimulation by gel-filtered MAP2 of DNA synthesis catalyzed by the nuclear matrix (Shioda et al., 1989). These results indicate that the MAP2 molecule itself, but not other minor components contaminating the MAP2 preparation, had the potential to stimulate DNA polymerase α activity. Essentially the same results as above were obtained, when Raji cell DNA polymerase α was used instead of calf thymus DNA polymerase α (figure not shown).

In the presence of a constant amount of DNA polymerase α, the enzyme activity increase linearly with increasing concentrations of MAP2 and reached a plateau level of stimulation (about 10-fold) (Figure 1A). When we increased the amount of polymerase, the minimum concentration of MAP2 required to attain the plateau level of stimulation was increased with the amount of the polymerase (Figure 1A). The plateau level

Table I: Template Specificity of the Stimulation of DNA Polymerase α by MAP2[a]

template-primer	substrate	DNA polymerase activity[b] 0.5 μM dXTP[c] −MAP2 (pmol)	+MAP2 (pmol)	stimulation (x-fold)	40 μM dXTP[d] −MAP2 (pmol)	+MAP2 (pmol)	stimulation (x-fold)
calf thymus DNA							
native	[^{3}H]dTTP + 3NTPs	0.19	0.36	1.9			
denatured	[^{3}H]dTTP + 3NTPs	0.16	0.48	3.0			
activated	[^{3}H]dTTP + 3NTPs	1.00	11.80	11.8	9.1	30.1	3.3
poly(dT)·poly(dA)	[^{3}H]dATP + 3NTPs	0.25	0.35	1.4			
poly(dT)	[^{3}H]dATP + 3NTPs	0.15	0.35	2.3			
poly(dT)·$(rA)_{10}$[e]	[^{3}H]dATP + 3NTPs	1.3	4.50	3.5	17.5	18.0	1.0
poly(dT)·$(rA)_{10}$[f]	[^{3}H]dATP + 3NTPs	1.2	2.40	2.0	15.0	17.9	1.2
poly(dT)·$(dA)_{12-18}$[e]	[^{3}H]dATP + 3NTPs	0.1	0.44	4.4			
poly(dA)·$(dT)_{10}$[e]	[^{3}H]dTTP + 3NTPs	0.23	2.37	10.3	6.4	56.8	8.9
poly(dA)·$(dT)_{10}$[f]	[^{3}H]dTTP + 3NTPs	0.21	2.00	9.5	6.3	66.2	10.5

[a] DNA synthesis was carried out using 25 ng of calf thymus DNA polymerase α in the absence (−MAP) or presence (+MAP) of MAP2 (0.1 mg/mL), as described in the text, except that the template-primer and substrates were replaced as indicated in the table; 2.0 μg of calf thymus DNA or 1.0 μg of artificial DNA per assay (25 μL) was used as the template-primer. [b] Labeled substrate incorporated. [c] In the presence of 0.5 μM labeled and 20 μM each of unlabeled substrates. [d] In the presence of 40 μM labeled and 100 μM each of unlabeled substrates. [e] Primer (micrograms)/template (micrograms) = 0.5. [f] Primer (micrograms)/template (micrograms) = 0.1.

of the stimulation rate was fairly constant, regardless of the amount of the polymerase; 625 ng of MAP2 was required to fully stimulate 25 ng of DNA polymerase α under the standard conditions, the molar ratio of MAP2 to the polymerase being 21, where the molecular masses of MAP2 and of the polymerase were assumed to be 270 and 240 kDa, respectively. The concentration of NaCl in the assay mixture affected the stimulation of the polymerase activity by MAP2 (Figure 1B). The rate of stimulation decreased by increasing the concentration of NaCl; the highest stimulation (20-fold) was noted in the absence of NaCl, and little stimulation occurred in the presence of 100 mM NaCl. With the decreased in NaCl concentration, the minimum amount of MAP2 required to attain the plateau level of stimulation was decreased (Figure 1B). The molar ratio of MAP2 to the polymerase required to attain full stimulation was 8 in the absence of NaCl in 25 ng of DNA polymerase α.

Physical Binding between DNA Polymerase α and MAP2. To search for possible physical binding between DNA polymerase α and MAP2, we made use of affinity chromatography using an MAP2-immobilized column. About 50% each of the applied polymerase was recovered in the unadsorbed and adsorbed fractions, respectively, where the recovery of the polymerase activity was almost 100% (Figure 2A). On the other hand, almost all the polymerase activity was recovered in the unadsorbed fraction with little activity in the 0.2 M NaCl eluate, when control columns with no immobilized proteins (not shown) or with bovine serum albumin (Figure 2B) were used. When the enzyme was preincubated with an excess amount of MAP2 being applied to the MAP2-immobilized column, little binding of the polymerase was observed (Figure 2C). These results show that the specific association of DNA polymerase α and MAP2 occurs under the experimental conditions we used.

Template-Primer Specificity of the Stimulation of DNA Polymerase α by MAP2. Template-primer specificity of the stimulation of DNA polymerase α activity by MAP2 is summarized in Table I. When calf thymus DNA was used, the stimulation rate was low (1.9-fold) with native DNA, moderate (3.0-fold) with denatured DNA, and high (11.8-fold) with activated DNA. A similar tendency was observed with the artificial template-primers, in the following order: double-strand DNA such as poly(dT)·poly(dA), single-stranded DNA such as poly(dT), and single-stranded DNA with oligonucleotide primer such as poly(dT)·$(rA)_{10}$, poly(dT)·$(dA)_{12-18}$, and poly(dA)·$(dT)_{10}$. In the absence of MAP2, DNA polymerase activity was higher with poly(dT)·$(rA)_{10}$ than with poly(dA)·$(dT)_{10}$; however, activation of the enzyme by the addition of MAP2 was greater with poly(dA)·$(dT)_{10}$ than with poly(dT)·$(rA)_{10}$. The rate of stimulation by MAP2 with the former was comparable to the case of calf thymus activated

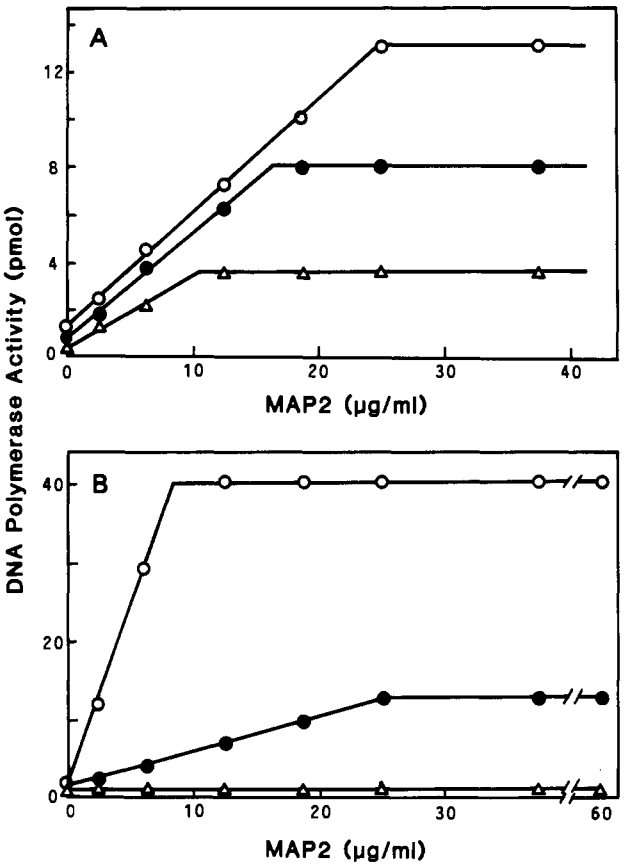

FIGURE 1: Dose-dependent stimulation of DNA polymerase α activity by MAP2. (A) Relationship between stimulation and the concentration of MAP2 with various amounts of DNA polymerase α. DNA synthesis was carried out in the presence of the indicated concentration of MAP2, using 25 (○), 12 (●), and 6 ng (△) of calf thymus DNA polymerase α, as described in the text. (B) Effect of NaCl on the stimulation. DNA synthesis was carried out in the presence of the indicated concentration of MAP2 using 25 ng of calf thymus DNA polymerase α, as described in the test, except that the NaCl concentration in the reaction mixture was changed to 0 (○), 50 (●), or 100 mM (△).

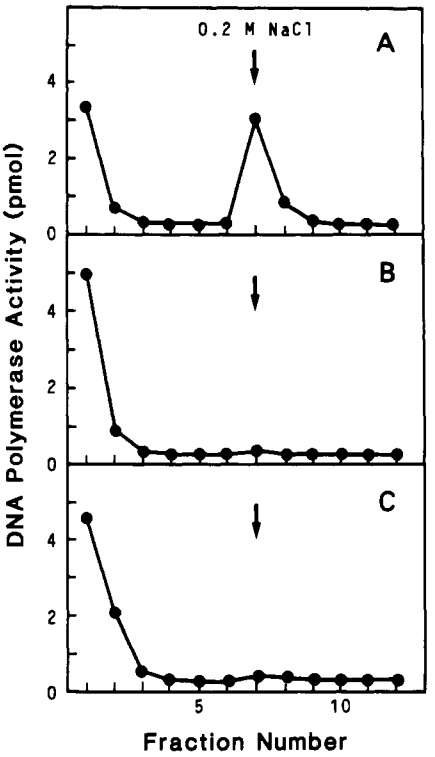

FIGURE 2: Binding of DNA polymerase α to an MAP2-immobilized affinity column. An MAP2-immobilized (A and C) or a bovine serum albumin immobilized (B) column (volume, 30 μL) was prepared and equilibrated as described in the text. Calf thymus DNA polymerase α (1.25 μg) was applied to each column. In the case of (C), the polymerase was preincubated with 13 μg of MAP2 (final concentration, 0.5 mg/mL) for 15 min at room temperature in 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM 2-mercaptoethanol, and 20% glycerol and applied to the MAP2-immobilized column. After incubation of the column for 15 min at room temperature, unadsorbed enzyme was recovered by washing the column with 180 μL of 25 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mg/mL bovine serum albumin, and 20% glycerol, followed by elution of the bound enzyme with 180 μL of the above solution containing 0.2 M NaCl. Three drops each of the fractions were collected (30 μL). Samples (2.5 μL) of the fractions were mixed with 2.5 μL of MAP2 (1 mg/mL) and assayed for DNA polymerase activity, as described in the text. Arrows show the fraction from which elution was carried out with the buffer solution containing 0.2 M NaCl.

DNA. Almost the same stimulation rates were observed regardless of the length of the single-stranded region in poly-(dA)·$(dT)_{10}$ or poly(dT)·$(rA)_{10}$, determined by the ratio of the amounts of the template to the primer. These results are interpreted to mean that stimulation occurred depending on the species of primers rather than those of templates and the length of the single-stranded region of the templates.

In the standard assay conditions, a low concentration (0.5 μM) of dNTP was used to enhance the stimulatory effect of MAP2; however, DNA polymerase α was not fully active in these conditions. Thus, the stimulation was also examined in the presence of a higher (40 μM) concentration of dNTPs, under which the polymerase was fully active (Table I). Under this condition, a similar tendency of template preference was observed, but the degree of stimulation was only one-third of that in the presence of 0.5 μM dNTPs, when activated DNA and poly(dT)·$(rA)_{10}$ were used. However, in the case of poly(dA)·$(dT)_{10}$, a similar degree of stimulation was noted, regardless of the concentration of dTTP.

Mode of Stimulation. (a) Effect of MAP2 on the K_m of DNA Polymerase α for dNTPs. Figure 3 shows the relationship between the concentration of dNTPs and the stimulation of DNA polymerase α activity by MAP2 with activated

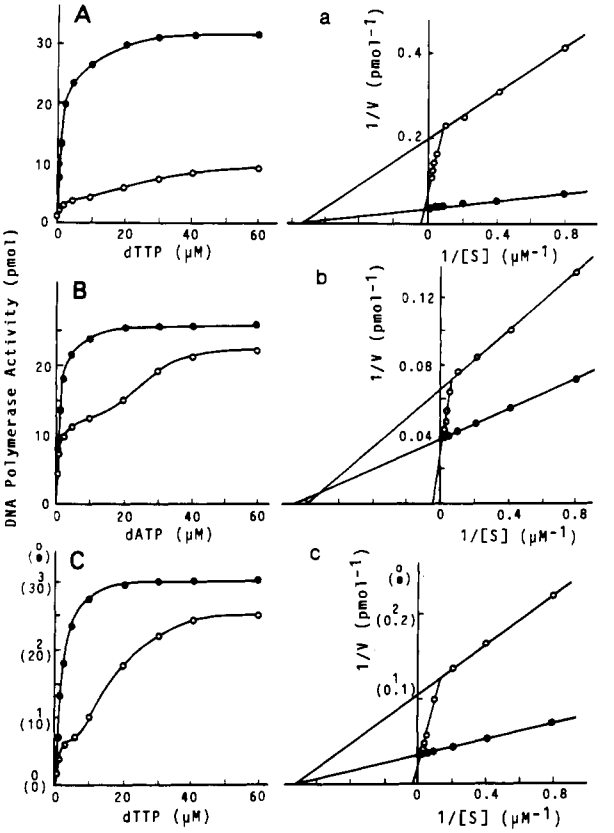

FIGURE 3: Effect of MAP2 on the relationship between the concentration of dNTPs and DNA polymerase α activity. Calf thymus DNA polymerase α (125 ng) was assayed with the indicated concentrations of dNTPs in the absence (○) and presence (●) of MAP2 (0.1 mg/mL) as described in the text. Activated DNA (A), poly-(dT)·$(rA)_{10}$ (B), or poly(dA)·$(dT)_{10}$ (C) was used as the template-primer. In all cases, the reaction mixture contained the indicated species and concentrations of a dNTP containing radioactivity (1 pmol = 1000 cpm) and a 100 μM sample of each of the other three dNTPs. Panels a, b, and c correspond to the double-reciprocal plots of panels A, B, and C, respectively. K_m values (and corresponding V_{max} values) for dTTP, dATP, and dTTP in panels A, B, and C, respectively, determined by the plots were as follows: (A) 1.3 μM (5.0 pmol/h) and 25.0 μM (12.5 pmol/h) in the absence of MAP2; 1.25 μM (29.5 pmol/h) in the presence of MAP2; (B) 1.25 μM (15.0 pmol/h) and 20.0 μM (28.0 pmol/h) in the absence of MAP2; 1.14 μM (27.1 pmol/h) in the presence of MAP2; (C) 1.3 μM (0.95 pmol/h) and 25.0 μM (3.85 pmol/h) in the absence of MAP2; 1.4 μM (29.4 pmol/h) in the presence of MAP2.

DNA (Figure 3A), poly(dT)·$(rA)_{10}$ (Figure 3B), or poly-(dA)·$(dT)_{10}$ (Figure 3C) used as the template-primer. When activated DNA was used in the absence of MAP2, the polymerase activity was enhanced with increasing concentrations of dTTP, in a biphasic manner (Figure 3A). Double-reciprocal plots of these results clearly show the biphasic relationship between the enzyme activity and the concentration of dTTP (Figure 3a). Thus, the polymerase had two apparent K_m values for dTTP: the lower K_m value of 1.3 μM (V_{max} = 5.0 pmol/h) and the higher K_m value of 25.0 μM (V_{max} = 2.5 pmol/h). On the other hand, in the presence of MAP2, DNA polymerase α activity increased markedly in a monophasic manner, and a plateau level was reached with about 20 μM (Figure 3A). Double-reciprocal plots gave a single linear line (Figure 3a). K_m and V_{max} for dTTP were calculated to be 1.25 μM and 29.5 pmol/h, respectively. Thus, it is apparent that the addition of MAP2 alters the biphasic feature of the polymerase for dTTP to a monophasic one, with a concomitant disappearance of the higher K_m value. Accompanying the alteration of K_m for dTTP, MAP2 increased the V_{max} severalfold. Consequently, the pronounced stimulation of the polymerase activity

Table II: Changes in K_m Values of DNA Polymerase α for dNTPs by MAP2[a]

substrate	K_m value for dNTPs	
	−MAP2 (μM)	+MAP2 (μM)
dATP	1.1 ± 0.2 (3)	1.7 ± 0.7 (3)
	23.3 ± 8.5 (3)	
dCTP	0.9 ± 0.2 (3)	1.5 ± 0.9 (3)
	24.0 ± 6.1 (3)	
dGTP	1.3 ± 0.3 (4)	2.2 ± 1.3 (3)
	22.8 ± 5.3 (4)	
dTTP	1.1 ± 0.1 (4)	1.6 ± 0.7 (9)
	24.0 ± 2.4 (4)	

[a] DNA synthesis was carried out using 125 ng of calf thymus DNA polymerase α, as described in the legend of Figure 3A, except that concentrations of the indicated dNTPs were changed in the presence of 100 μM each of the other three dNTPs and the apparent K_m value was estimated. Data are the mean ± SE. Numbers in parentheses represent the number of experiments.

Table III: Changes in K_m Values of DNA Polymerase α for Template-Primer by MAP2[a]

template-primer	−MAP2		+MAP2	
	K_m	V_{max} (pmol/h)	K_m	V_{max} (pmol/h)
activated DNA	10.3 μg/mL	190	2.5 μg/mL	600
poly(dT)·(rA)$_{10}$[b]	0.97 μM[c]	220	0.95 μM[c]	250
poly(dA)·(dT)$_{10}$[b]	20.0 μM[c]	125	2.1 μM[c]	1600

[a] DNA synthesis was carried out using 125 ng of calf thymus DNA polymerase α in the absence (−MAP2) or presence (+MAP2) of MAP2 (0.1 mg/mL), as desribed in the legend of Figure 3, except for varying the concentrations of the template-primers indicated in the table and fixing the concentration of each of four dNTPs to 40 μM. When activated DNA, poly(dT)·(rA)$_{10}$, and poly(dA)·(dT)$_{10}$ were used as the template-primer, incorporation of [^{3}H]dTMP, [^{3}H]dAMP, and [^{3}H]dTMP (1.0 pmol = 1000 cpm in each case) was measured, respectively, and the kinetic parameters were estimated by double-reciprocal plot analysis. [b] Primer (micrograms)/template (micrograms) = 0.5. [c] Values represent the concentration of primer.

by MAP2 with a low concentration of dTTP can be ascribed to the decrease in the K_m value for dTTP and to an increase in V_{max}, while moderate stimulation with a high concentration of dTTP stemmed mainly from the increase in V_{max}. The same alteration by the addition of MAP2 occurred with respect to other deoxyribonucleotides, dATP, dCTP, and dGTP (Table II).

When poly(dT)·(rA)$_{10}$ (Figure 3B) or poly(dA)·(dT)$_{10}$ (Figure 3C) was used in the absence of MAP2, the polymerase activity changed biphasically with the concentration of dATP or dTTP. The K_m values for dATP (1.25 and 20 μM) or dTTP (1.3 and 25.0 μM) were practically equal to those obtained in the case of activated DNA (Figure 3A). In the presence of MAP2, a monophasic profile was obtained, and the K_m values for dATP with poly(dT)·(rA)$_{10}$ as the template-primer and for dTTP with poly(dA)·(dT)$_{10}$ were calculated to be 1.1 and 1.4 μM, respectively. These values were close to those obtained in the case of activated DNA in the presence of MAP2. Thus, the K_m values for dNTPs were practically equal, and the mode of alteration by the addition of MAP2 was the same regardless of the species of template-primer.

On the other hand, in the absence of MAP2, V_{max} values depended on the species of the template-primer. An increase in the V_{max} occurred by adding MAP2; however, the degree of the increase depended on the species of template-primer: 7.8-fold 2.4-fold, and 1.0-fold with poly(dA)·(dT)$_{10}$, activated DNA, and poly(dT)·(rA)$_{10}$, respectively, in the presence of 40 μM dNTPs. Therefore, it can be concluded that stimulation of DNA polymerase α activity by MAP2 is due to the decrease in the K_m for dNTPs, irrespective of the template-primer, and the increase in V_{max}, depending on the species of template-primer.

Essentially the same results as above were obtained when Raji cell DNA polymerase α was used instead of calf thymus DNA polymerase α: K_m values (corresponding V_{max}) for dTTP, dATP, and dTTP, when activated DNA, poly(dT)·(rA)$_{10}$, and poly(dA)·(dT)$_{10}$ were used as the template-primers, respectively, were 1.1 μM (8.7 pmol/h) and 23 μM (16 pmol/h) in the absence of MAP2 and 0.8 μM (30 pmol/h) in the presence of MAP2, 1.7 μM (16 pmol/h) and 20 μM (34 pmol/h) in the absence of MAP2 and 2.0 μM (36 pmol/h) in the presence of MAP2, and 1.1 μM (1.5 pmol/h) and 27 μM (4.2 pmol/h) in the absence of MAP2 and 1.0 μM (32 pmol/h) in the presence of MAP2, respectively.

(b) Effect of MAP2 on the K_m's of DNA Polymerase α for Template-Primers. The relationship between DNA polymerase α activity and the concentration of the template-primers was examined in the presence of a high concentration (40 μM each) of dNTPs, using activated DNA, poly(dT)·(rA)$_{10}$, or poly(dA)·(dT)$_{10}$ as the template-primer. In the absence of MAP2, the polymerase activity was enhanced with increasing concentrations of each of the template-primers. Double-reciprocal plot analyses showed a monophasic relationship between the enzyme activity and the concentration of the template-primer (data not shown). In the presence of MAP2, a similar monophasic relationship with stimulation of the enzyme activity was obtained (data not shown). Changes in kinetic parameters caused by the addition of MAP2 are summarized in Table III. The K_m value for the template-primer tended to decrease by adding MAP2; however, the extent of the decrease depended on the template-primer: 4-fold, 1-fold, and 10-fold with activated DNA, poly(dT)·(rA)$_{10}$, and poly(dA)·(dT)$_{10}$, respectively. In the presence of MAP2, V_{max} increased 3.2-fold, 1.1-fold, and 12.8-fold with activated DNA, poly(dT)·(rA)$_{10}$, and poly(dA)·(dT)$_{10}$, respectively, roughly in parallel with the degree of the decrease of K_m.

When Raji cell DNA polymerase α was used instead of calf thymus DNA polymerase α, K_m values of the enzyme for activated DNA, poly(dT)·(rA)$_{10}$, and poly(dA)·(dT)$_{10}$ were estimated to be as follows: 11.1 μg/mL in the absence of MAP2 and 3.1 μg/mL in the presence of MAP2, 0.75 μM in the absence of MAP2 and 0.80 μM in the presence of MAP2, and 25.0 μM in the absence of MAP2 and 1.8 μM in the presence of MAP2, respectively, where the values were represented as the amount of primer in the case of poly(dT)·(rA)$_{10}$ and poly(dA)·(dT)$_{10}$.

(c) Product Analysis by Gel Electrophoresis. DNA synthesis was performed with poly(dT)·(rA)$_{10}$ or poly(dA)·(dT)$_{10}$ as the template-primer in the presence of 40 μM dNTPs, under conditions in which the molar ratio of the incorporated dNMP to the primer was less than 1/100, in order to decrease the frequency of reinitiation of DNA synthesis on the same primer to a low level. In the case of poly(dT)·(rA)$_{10}$, incorporation of dAMP increased linearly with incubation time, and the rates of incorporation in the absence and presence of MAP2 were not so different. Gel electrophoresis of the ^{32}P-labeled DNA product showed that synthesized DNA was 10–25 bases regardless of the incubation time, at least up to 60 min (Figure 4A), thereby indicating that the processivity of the polymerase was 10–25 bases with poly(dT)·(rA)$_{10}$, under the conditions used. The addition of MAP2 led to no significant change in size of the synthesized DNA (Figure 4A); thus, MAP2 apparently had no effect on the processivity.

When poly(dA)·(dT)$_{10}$ was used as the template-primer, the rate of incorporation of dTMP into DNA in the presence of

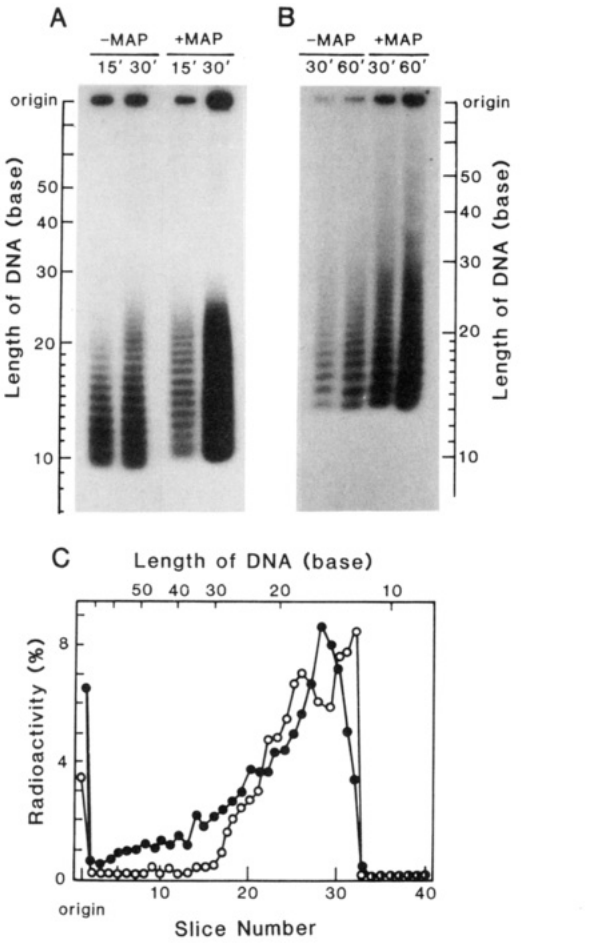

FIGURE 4: Effect of MAP2 on the processivity of DNA polymerase α. (A) Processivity with poly(dT)·$(rA)_{10}$. DNA synthesis was carried out using 20 ng of calf thymus DNA polymerase α in the absence (−MAP) or presence (+MAP) of 0.1 mg/mL MAP2, as described in the text, except that 4.0 μg of poly(dT)·$(rA)_{10}$ [the primer (micrograms) to template (micrograms) ratio was 0.1, and the amount of primer was 120 pmol] and 40 μM each of four kinds of dNTP containing 10 μCi of [α-^{32}P]dATP were used as the template-primer and substrates, respectively. After incubation for 15 and 30 min at 37 °C, ^{32}P-labeled products were isolated, as described in the text, followed by treatment with 0.3 N NaOH for 17 h at 37 °C to degrade the $(rA)_{10}$. The amount of dAMP incorporated into DNA was 0.4 (−MAP2, 15-min incubation), 0.8 (−MAP2, 30-min incubation), 0.5 (+MAP2, 15-min incubation), and 1.1 pmol (+MAP2, 30-min incubation). About one-eighth of the ^{32}P-labeled products was subjected to electrophoresis using a 25% polyacrylamide–7 M urea gel at 500 V/14 cm for 3 h, 30 min and autoradiography, as described in the text. (B) Processivity with poly(dA)·$(dT)_{10}$. DNA synthesis was carried out using 100 or 10 ng of calf thymus DNA polymerase α in the absence (−MAP) or presence (+MAP) of MAP2 (0.1 mg/mL), respectively, for 30 and 60 min, as described in the text, except that 4.0 μg of poly(dA)·$(dT)_{10}$ [the primer (micrograms) to template (micrograms) ratio was 0.1, and the amount of the primer was 120 pmol] and 40 μM each of four kinds of dNTP containing 15 μCi of [α-^{32}P]dTTP were used as the template-primer and substrates, respectively. The amounts of dTMP incorporated into DNA were 0.4 (−MAP2, 30-min incubation), 0.8 (−MAP2, 60-min incubation), 0.5 (+MAP2, 30-min incubation), and 0.9 pmol (+MAP2, 60-min incubation). About one-fourth of the ^{32}P-labeled products was analyzed by gel electrophoresis, without NaOH treatment, as described in (A). Gel electrophoresis was performed at 500 V/14 cm for 3 h, 15 min. (C) Quantitative analysis of processivity. After gel electrophoresis and autoradiography, as described in (B), the gels were cut into 3-mm slices, and the radioactivity of each slice was counted, as described in the text. (●) and (○) represent the results of 15-min incubation with and without MAP2, respectively. Total radioactivities of the former and the latter were 2368 and 9476 cpm, respectively.

Table IV: Stimulatory Effect of MAP2 on Various Kinds of DNA Polymerase[a]

DNA polymerase (source)	DNA polymerase activity −MAP2 (pmol)	+MAP2 (pmol)	stimulation (x-fold)
α (human, Raji cell)	0.24	2.20	9.2
α (calf thymus)	0.76	7.80	10.3
α (*Xenopus* ovary)	0.10	1.10	11.0
α (*Physarum polycephalum*)	0.20	2.00	10.0
β (calf thymus)	0.56	0.60	1.1
γ (human leukemia cell)	0.36	0.32	0.9
I (*E. coli*)	0.60	0.61	1.0

[a] Various kinds of DNA polymerase were prepared as described in the text. DNA synthesis was carried out using the DNA polymerase indicated in the standard reaction mixture in the absence (−MAP2) and presence (+MAP2) of MAP2 (0.5 mg/mL) as described in the text.

MAP2 was 10-fold higher than that in its absence. The size of the synthesized DNA, including a 10-base primer in the absence of MAP2, was 13–30 bases long, regardless of the incubation time up to 60 min (Figure 4B). Thus, the processivity of the polymerase is 3–20 bases. The size of the synthesized DNA in the presence of MAP2 was not significantly altered, except for the appearance of low amounts of longer DNA as smear bands, regardless of the incubation time (Figure 4B). Figure 4C shows a quantitative representation of the results of Figure 4B. In the absence of MAP2, 96% of the synthesized DNA was distributed in the region of 13–30-base DNA, while in the presence of MAP2, 86% was in the same region, with 14% distributed in the region of DNA longer than 30 bases. These results suggest that MAP2 increased the processivity to some extent, but the major part of the synthesized DNA remained unchanged in length. Thus, an increase in the incorporation of dTMP by MAP2 is considered to be mainly due to increases in the frequency of initiation of DNA synthesis.

When Raji cell DNA polymerase α was used instead of calf thymus DNA polymerase α, MAP2 did not affect the processivity of the enzyme; the processivity of the enzyme was estimated to be 10–20 and 2–35 bases with poly(dT)·$(rA)_{10}$ and poly(dA)·$(dT)_{10}$ as the template-primer, respectively, in the absence and presence of MAP2.

Stimulatory Effect of MAP2 on Various DNA Polymerases. MAP2 stimulated the activity of DNA polymerase α from a wide variety of eukaryotic cells (human lymphoma cells, calf thymus, *Xenopus* ovary, and *Physarum polycephalum*), whereas no such stimulation was detected in DNA polymerases β, γ, and I from *E. coli* (Table IV). All of the DNA polymerase α preparations represented in the table had two apparent K_m values for dTTP in the absence of MAP2, while they had only the lower K_m value for dTTP in the presence of MAP2 (data not shown), the results being similar to those in Figure 3A. These results suggest that the stimulatory effect of MAP2 is specific for DNA polymerase α, which has two apparent K_m values for dNTPs, irrespective of the cell species from which the enzymes were prepared.

Stimulatory Effect on DNA Polymerase α by Various MAPs. The stimulatory effect on DNA polymerase α activity was examined in excess amounts of various MAPs (Table V). Significant stimulation of the polymerase activity was detected in MAP2, tau, and 190-kDa MAP. Although the maximal rate of stimulation was not the same in each case, the ability to stimulate DNA polymerase α was shared by these MAPs. On the other hand, bovine serum albumin, tubulin, and actin which were used as controls did not significantly stimulate the enzyme activity.

Table V: Stimulatory Effect of Various Kinds of MAP on DNA Polymerase α[a]

protein	DNA polymerase α activity (−) (pmol)	(+) (pmol)	stimulation (x-fold)
MAP2	1.2	13.1	10.9
tau	1.5	10.5	7.0
190-kDa MAP	1.2	11.0	9.2
tubulin	1.2	1.7	1.4
actin	1.0	1.3	0.9
bovine serum albumin	1.3	1.3	1.0

[a] DNA synthesis was carried out using 25 ng of calf thymus DNA polymerase α under the standard reaction conditions, as described in the text, in the absence (—) and presence (+) of various kinds of protein (0.1 mg/mL).

Discussion

The present study revealed that MAP2 stimulated DNA polymerase α activity (Figure 1), thereby supporting our assumption that DNA polymerase α is the target of MAP2 in the stimulation of DNA synthesis catalyzed by the nuclear matrix (Shioda et al., 1989). It is considered that the stimulation occurs through direct binding of MAP2 to the polymerase, since physical binding between them was noted when we used MAP2-immobilized affinity column chromatography (Figure 2). The minimal molar ratio of MAP2 to DNA polymerase α was 8:1, a ratio necessary to obtain full stimulation of the polymerase activity (Figure 1). It has been reported that MAP2 purified by the conventional method is heterogeneous in terms of posttranscriptional modification such as phosphorylation (Sloboda et al., 1975; Herrmann et al., 1985; Wiche, 1989). Therefore, it may be considered that some species of MAP2 are active and others are inactive or less active in the stimulation of DNA polymerase activity. The minimal molar ratio of the "active" MAP2 to DNA polymerase α required for the full stimulation of the enzyme activity is probably less than 8. In fact, our preliminary experiments of further fractionation of purified MAP2 with DEAE-cellulose column chromatography and determination of the specific activity of each fraction to stimulate DNA polymerase α activity revealed at most a 10-fold difference in the specific activity between fractions (unpublished results). We assume that the difference in the specific activity of the DEAE-cellulose fractions required to stimulate DNA polymerase α may be due to differences in modification, presumably the phosphorylation, of MAP2. It has been reported that MAP2 is phosphorylated at multiple sites by a variety of protein kinases (Yamauchi & Fujiwara, 1982; Theurkauf & Vallee, 1983; Kadowaki et al., 1985; Akiyama et al., 1986). In a preliminary experiment, we noted that phosphorylation by protein kinase C of MAP2 with low specific activity to stimulate DNA polymerase α produced a MAP2 preparation with a higher specific activity (unpublished results). Therefore, we consider that the phosphorylated form of MAP2 is essential to stimulate DNA polymerase α activity.

Kinetic analyses (Figure 3 and Table II) revealed that MAP2 changes the higher one of the two apparent K_m's of DNA polymerase α for dNTPs to the lower value, thereby stimulating polymerase activity. This alteration occurred in the preparation of DNA polymerase α which had biphasic features for dNTPs. However, all the DNA polymerase α we tested did not have the biphasic feature for dNTPs; i.e., some of the polymerases, especially old preparations, had only a low K_m value for dNTPs, and no significant stimulation of the polymerase activity by MAP2 was observed (unpublished results). At present, it is unclear whether the polymerase preparation we used consists of two forms of DNA polymerase α with different K_m values for dNTP or whether the preparation contains only one form of the polymerase with two different K_m values. Calf thymus DNA polymerase α used in the present experiments was composed of 140-, 50-, and 47-kDa polypeptides, values different from those reported by other workers (Nasheur & Grosse, 1987). Therefore, it might be that the stimulation of enzyme activity by MAP2 is restricted to the enzyme composed of those kinds of subunits. However, this is not the case, because the stimulation occurred in DNA polymerase α purified from Raji cells mainly composed of 180-, 70-, 60-, and 50-kDa polypeptides with a slight contamination of 165- and 140-kDa polypeptides, the subunit composition being comparable to those reported (Lehman & Kaguni, 1989). On the other hand, the potentiality of the calf thymus enzyme to be activated by MAP2 was lost by long storage of the polymerase with the disappearance of the biphasic feature for dNTPs, i.e., the loss of the higher K_m for dNTPs. In this case, a change in the size of the subunits did not occur (unpublished results). This would suggest that differences in the conformation or modification of DNA polymerase exist between MAP2-sensitive polymerase, which has high and low K_m's for dNTPs, and MAP2-insensitive polymerase, which has only a low K_m for dNTPs. Racine and Morris (1978) found that one of two forms of DNA polymerase α in sea urchin embryos has high (31 μM) and low (0.75 μM) K_m's, for dNTPs, and pointed out that the high K_m tended to be ignored. Hence, the presence of DNA polymerase α with high and low K_m's for dNTPs is not unusual. Further studies are required to elucidate the kinetic nature of DNA polymerase α's from various sources.

Accompanying the alternation of the K_m for dNTPs, MAP2 decreased the K_m for the template-primer with a concomitant increase in V_{max} (Table III). These changes in kinetic parameters seem to be related to an increase in the frequency of the initiation of DNA synthesis, but not the increase in processivity, since the size of the synthesized DNA was little changed despite the marked increase in the amount of dNMP incorporated into DNA (Figure 4). It was also shown that the addition of MAP2 increased the V_{max} and decreased the K_m for the template-primer; however, the degree of the changes depended on the species of template-primer used (Table III). A change in the K_m values for dNTPs was observed irrespective of the species of template-primer (Figure 3). Thus, the essential effect of MAP2 on DNA polymerase α activity is presumably the alteration of the K_m value for dNTPs.

To date, several protein factors that stimulate DNA polymerase α or DNA polymerase α–primase activity have been identified. Accessory proteins of DNA polymerase α that are concerned with primer recognition in mammalian cells have been purified and characterized (Lamothe et al., 1981; Pritchard & DePamphilis, 1983; Pritchard et al., 1983; Jindal & Vishwanatha, 1990). These primer recognition proteins are thought to stimulate homologous DNA polymerase α activity at low primer to template ratios by eliminating nonproductive binding of the enzyme to single-stranded DNA, allowing the enzyme to slide along the template until it recognizes a primer (Pritchard et al., 1983). These proteins decrease the K_m of DNA polymerase α for the primer without changing the processivity of the polymerase (Pritchard et al., 1983). These properties partially resemble those for the MAP2 described here (Table III and Figure 4). However, stimulation of DNA polymerase α by these primer recognition proteins is not observed with activated DNA that has high primer to template ratios (Pritchard et al., 1983), whereas stimulation by MAP2 takes place with activated DNA (Table I). This property

distinguishes these primer recognition proteins from MAP2. In addition, these primer recognition proteins do not affect the K_m of DNA polymerase α for deoxyribonucleotides (Pritchard et al., 1983), whereas MAP2 decreases it (Figure 3 and Table II). These data suggest that there is a considerable difference in the mode of action between these primer recognition proteins and MAP2. MAP2 also seems to differ from a factor in mouse FM3A cells described by Kawasaki et al. (1986), which partially resembles the above primer recognition proteins, since this factor does not change the K_m of DNA polymerase α for deoxyribonucleotides.

Factor D isolated from mouse (Fry et al., 1987a,b) or rabbit (Sharf et al., 1988) liver and factor C isolated from rabbit liver (Asna et al., 1989) stimulate DNA polymerase α activity by decreasing the K_m of the polymerase for the template-primer, causing an increase in the processivity of the polymerase. This mode of stimulation is distinct from that described here for MAP2 (Table III and Figure 4). Stimulation by factor D occurs with primed natural DNA and poly(dA)-primed poly(dT), but not with activated DNA (Sharf et al., 1988). Factor C stimulates DNA polymerase α with poly[d(G-C)] and poly(dG)-primed poly(dC), but not with activated DNA (Asna et al., 1989). Thus, the template-primer preference of these factors also differs from that of MAP2 (Table I). Moreover, stimulation of these factors is not specific for DNA polymerase α (Sharf et al., 1988; Asna et al., 1989), whereas MAP2 stimulated DNA polymerase α, but not DNA polymerases β, γ, and I (Table IV). These properties distinguish factors D and C from MAP2.

A recently characterized accessory protein of DNA polymerase α, designated AAF, in mouse cells (Goulian et al., 1990; Goulian & Heard, 1990), has been suggested to increase the affinity of DNA polymerase α–primase for the DNA template. AAF stimulates DNA polymerase α and primase activities with poly(dA)·$(dT)_{12-18}$ and with poly(dT) and unprimed single-stranded fd DNA, respectively, but not with activated DNA. On the other hand, MAP2 stimulated DNA polymerase α activity with poly(dA)·$(dT)_{10}$ and activated DNA (Table I), but it did not stimulate the primase activity with unprimed single-stranded fd DNA (unpublished result). AAF also increases the processivity of the polymerase and allows the polymerase to traverse double-stranded regions of the DNA template. Judging from the template preference and the effect on the processivity, AAF is apparently different from MAP2.

Factor T purified from calf thymus (Yoshida et al., 1989), which may be an accessory protein of DNA polymerase α, has the ability to help the polymerase read through pause sites in the template. This factor is relatively heat-stable, as is MAP2. However, factor T is distinct from MAP2, since it does not alter the K_m of DNA polymerase α for either template-primers or deoxyribonucleotides.

Single-stranded DNA binding proteins isolated from calf thymus by Sapp et al. (1985) are thought to block nonproductive polymerase binding sites on single-stranded DNA sequences. These proteins stimulate DNA synthesis catalyzed by DNA polymerase α only in the presence of an excess of activated DNA as template-primers. By contrast, stimulation by MAP2 was apparent at low concentrations of activated DNA (Table III), implying that these proteins also do not correspond to MAP2.

Replication factor A (RF-A) (Fairman & Stillman, 1988), alternatively designated single-stranded DNA binding protein (Wobbe et al., 1987) or replication protein A (Wold & Kelly, 1988), and replication factor C (RF-C), which are essential components for SV40 DNA replication, independently stimulate DNA polymerase α (Tsurimoto & Stillman, 1989). RF-A, which has been suggested to function in the initiation of DNA replication and chain elongation, increases the processivity of DNA polymerase α (Tsurimoto & Stillman, 1989). RF-C, which may be an accessory protein of DNA polymerase α, affects both the processivity of DNA synthesis and the frequency of primer recognition (Tsurimoto & Stillman, 1989). These properties distinguish RF-A and RF-C from MAP2.

Among RNase H's that are thought to be involved in DNA replication (Karwan et al., 1983; DiFrancesco & Lehman, 1985; Hagemeier & Gross, 1989), those found in yeast (Karwan et al., 1983) and calf thymus (Hagemeier & Gross, 1989) have the ability to stimulate the activity of their homologous DNA polymerases. However, these proteins apparently differ from MAP2, since MAP2 has no RNase H activity (unpublished result).

As described above, MAP2 differs from all previously well-characterized protein factors that stimulate DNA polymerase α. It is worth noting that MAP2 is unique in its ability to alter the K_m of DNA polymerase α for deoxyribonucleotides. Therefore, we conclude that MAP2 is a novel type of factor with the ability to stimulate DNA polymerase α.

Although we used MAP2 as a model for the stimulation of DNA polymerase α activity, it may be unlikely that MAP2 itself participates in DNA replication, since this MAP localizes predominately in neural cells which are completely inactive in DNA replication (Dustin, 1984; Vallee et al., 1984; Wiche, 1989). However, stimulation of DNA polymerase α activity was observed not only in MAP2 but also in tau and 190-kDa MAP (Table V). The 190-kDa MAP was found to be present in a wide variety of proliferating cells (Kotani et al., 1988). Therefore, it is possible that the 190-kDa MAP or other MAPs present in the proliferating cells are involved in DNA replication, in vivo. MAP2, tau, and 190-kDa MAP have sites with a common amino acid sequence (Lee et al., 1988; Lewis et al., 1988; Aizawa et al., 1989). The finding that all these MAPs have the potential to stimulate DNA polymerase α activity suggests that the common site of these MAPs is essential for the stimulation. Immunohistochemical studies showed that parts of the MAPs are located in the nucleus of proliferating cells (Sato et al., 1985a,b, 1986, 1988). However, biochemical evidence of the existence of MAPs in the nucleus has not been reported. Whether or not MAPs participate in DNA replication in vivo remains an open question. Even if authentic MAPs are not involved in DNA replication in vivo, there may exist a protein factor in the nucleus which has the same partial amino acid sequence as those of MAPs and has the stimulatory activity for DNA polymerase α. If such is indeed the case, then the factor would be a regulator of DNA replication by altering the K_m value of DNA polymerase α for dNTPs, or would be an accessory protein of DNA polymerase α which had been removed from the polymerase in the course of the enzyme purification. We are now searching for the regulator protein of DNA polymerase α which participates in DNA replication in vivo. If it is an authentic MAP or an MAP-related protein, then elucidation of the mechanism of the stimulation of DNA polymerase α will pave the way to understanding the regulation of DNA replication in eukaryotic cells.

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REFERENCES

Aizawa, H., Kawasaki, H., Murofushi, H., Kotani, S., Suzuki, K., & Sakai, H. (1989) *J. Biol. Chem. 264*, 5885–5890.

Akiyama, T., Nishida, E., Ishida, J., Saji, N., Ogawara, H., Hoshi, M., Miyata, Y., & Sakai, H. (1986) *J. Biol. Chem. 261*, 15648–15651.

Asna, N., Weisman-Shomer, P., Waldman, E., & Fry, M. (1989) *J. Biol. Chem. 264*, 5245–5252.

Blow, J. J. (1989) *Trends Genet. 5*, 134–136.

Burgers, P. M. J., Bambara, R. A., Campbell, J. L., Chang, L. M. S., Downey, K. M., Hübscher, U., Lee, M. Y. W. T., Linn, S. M., So, A. G., & Spadari, S. (1990) *Eur. J. Biochem. 191*, 617–618.

Detera, S. D., Becerra, S. P., Swack, J. A., & Wilson, S. H. (1981) *J. Biol. Chem. 256*, 6933–6943.

DiFrancesco, R. A., & Lehman, I. R. (1985) *J. Biol. Chem. 260*, 14764–14770.

Dustin, P. (1984) *Microtubules*, 2nd ed., pp 40–46, Springer-Verlag, Berlin.

Fairman, M. P., & Stillman, P. (1988) *EMBO J. 7*, 1211–1218.

Fansler, B. S., & Loeb, L. A. (1974) *Methods Enzymol. 29*, 53–70.

Fry, M., Sharf, R., Weisman-Shomer, P., Evers, P. C., & Loeb, L. A. (1987a) *J. Biol. Chem. 262*, 8868–8874.

Fry, M., Weisman-Shomer, P., Lapidot, T., & Sharf, R. (1987b) *J. Biol. Chem. 262*, 8861–8867.

Goulian, M., & Heard, C. J. (1990) *J. Biol. Chem. 265*, 13231–13239.

Goulian, M., Heard, C. J., & Grimm, S. L. (1990) *J. Biol. Chem. 256*, 13221–13230.

Hagemeier, A., & Grosse, F. (1989) *Eur. J. Biochem. 185*, 621–628.

Herrmann, H., Dalton, J. M., & Wiche, G. (1985) *J. Biol. Chem. 260*, 5797–5803.

Jazwinski, S. M., & Edelman, G. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3428–3432.

Jindal, H. K., & Vishwanatha, J. K. (1990) *Biochemistry 29*, 4767–4773.

Kadowaki, T., Fujita-Yamaguchi, T., Nishida, E., Takaku, F., Akiyama, T., Kathuria, S., Akanuma, Y., & Kasuga, M. (1985) *J. Biol. Chem. 260*, 4016–4020.

Karwan, R., Blutsh, H., & Wintersberger, U. (1983) *Biochemistry 22*, 5500–5507.

Kawasaki, K., Enomoto, T., Suzuki, M., Seki, M., Hanaoka, F., & Yamada, M. (1986) *Biochemistry 25*, 3044–3050.

Kotani, S., Nishida, E., Kumagai, H., & Sakai, H. (1985) *J. Biol. Chem. 260*, 10779–10783.

Kotani, S., Murofushi, H., Maekawa, S., Aizawa, H., & Sakai, H. (1988) *J. Biol. Chem. 263*, 5385–5389.

Lamothe, P., Baril, B., Chi, A., Lee, L., & Baril, E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4723–4727.

Lee, G., Cowan, N., & Kirshner, M. W. (1988) *Science 239*, 285–288.

Lehman, I. R., & Kaguni, L. S. (1989) *J. Biol. Chem. 264*, 4265–4268.

Lewis, S. A., Wang, D., & Cowan, N. J. (1988) *Science 242*, 936–939.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560–564.

Murofushi, H., Kotani, S., Aizawa, H., Hisanaga, S., Hirokawa, H., & Sakai, H. (1986) *J. Cell Biol. 103*, 1911–1919.

Nakayasu, H., Ueda, K., & Berezney, R. (1988) *Cell Struct. Funct. 13*, 646.

Nasheur, H., & Grosse, F. (1987) *Biochemistry 26*, 8458–8466.

Pritchard, C. G., & DePamphilis, M. L. (1983) *J. Biol. Chem. 258*, 9801–9809.

Pritchard, C. G., Weaver, D. T., Baril, E. F., & DePamphilis, M. L. (1983) *J. Biol. Chem. 258*, 9810–9819.

Ottiger, H.-P., & Hübscher, U. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3993–3997.

Racine, F. M., & Morris, P. W. (1978) *Nucleic Acids Res. 5*, 3959–3973.

Reddy, G. P., & Pardee, A. B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3312–3316.

Sapp, M., Konig, H., Riedel, H. D., Richter, A., & Knippers, R. (1985) *J. Biol. Chem. 260*, 1550–1556.

Sato, C., Tanabe, K., Nishizawa, K., Nakayama, T., Kobayashi, T., & Nakamura, H. (1985a) *Exp. Cell Res. 160*, 206–220.

Sato, C., Nishizawa, K., Nakayama, T., & Kobayashi, T. (1985b) *J. Cell Biol. 100*, 748–753.

Sato, C., Nishizawa, K., Nakayama, T., Hirai, R., & Nakamura, H. (1986) *Exp. Cell Res. 167*, 281–286.

Sato, C., Nishizawa, K., Nakayama, T., Ohtsuka, K., Nakamura, H., Kobayashi, T., & Inagaki, M. (1988) *Exp. Cell Res. 175*, 136–147.

Sharf, R., Weisman-Shomer, P., & Fry, M. (1988) *Biochemistry 27*, 2990–2997.

Shioda, M. (1986) *Eur. J. Biochem. 160*, 571–578.

Shioda, M., Nagano, H., & Mano, Y. (1980) *Eur. J. Biochem. 108*, 345–355.

Shioda, M., Nelson, E. M., Bayne, M. L., & Benbow, R. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7209–7213.

Shioda, M., Murofushi, H., Murakami-Murofushi, K., & Sakai, H. (1989) *Biochem. Biophys. Res. Commun. 159*, 834–841.

Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L., & Greengard, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 177–181.

Smith, H. C., & Berezney, R. (1982) *Biochemistry 21*, 5857–5865.

So, A. G., & Downey, K. M. (1988) *Biochemistry 27*, 4591–4595.

Syvaoja, J., & Linn, S. (1989) *J. Biol. Chem. 264*, 2489–2497.

Tamai, K., Kojima, K., Hanaichi, T., Masaki, S., Suzuki, M., Umekawa, H., & Yoshida, S. (1988) *Biochim. Biophys. Acta 950*, 263–273.

Tanaka, M., Kimura, K., & Yoshida, S. (1987) *Cancer Res. 47*, 5971–5974.

Theurkauf, W. E., & Vallee, R. B. (1983) *J. Biol. Chem. 258*, 7883–7886.

Tsurimoto, T., & Stillman, B. (1989) *EMBO J. 8*, 3883–3889.

Tubo, R. A., & Berezney, R. (1987a) *J. Biol. Chem. 262*, 1148–1154.

Tubo, R. A., & Berezney, R. (1987b) *J. Biol. Chem. 262*, 5857–5865.

Vallee, R. B., Bloom, G. S., & Luca, F. C. (1984) in *Molecular Biology of the Cytoskeleton* (Borisy, G. G., Cleveland, D. W., & Murphy, D. B., Eds.) pp 111–130, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Wiche, G. (1989) *Biochem. J. 259*, 1–12.

Wobbe, C. R., Weissbach, L., Borowiec, J. A., Dean, F. B., Murakami, Y., Bullock, P., & Hurwitz, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1834–1838.

Wold, M. S., & Kelly, T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2523–2527.
Yamauchi, T., & Fujiwara, H. (1982) *Biochem. Biophys. Res. Commun. 109*, 975–981.
Yoshida, S., Yamada, M., & Masaki, S. (1979) *J. Biochem. (Tokyo) 85*, 1387–1395.
Yoshida, S., Tamai, K., Umekawa, H., Suzuki, M., & Kojima, K. (1989) *J. Biochem. (Tokyo) 106*, 389–395.

Interaction of Ribosomal Protein S1 and Initiation Factor IF3 with the 3′ Major Domain and the Decoding Site of the 30S Subunit of *Escherichia coli*†

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ABSTRACT: We have studied the effect of the binding of ribosomal protein S1 and initiation factor IF3 on the accessibility of nucleotide residues 584–1506 in the small subunit of the *Escherichia coli* ribosome. Protein S1 strongly decreases RNase V1 attack at G1164, in hairpin 40 of the 3′ major domain, and weakly decreases DMS attack at C1302, in the central loop of the 3′ major domain, and at A1503, in the 3′ minor domain. It also weakly increases the DMS reactivity of A1004, in the 3′ major domain, and of A901, in the central domain. Factor IF3 strongly decreases RNase V1 attack (but not dimethyl sulfate attack) at A1408, in the decoding site, and weakly protects A1500, in the 3′ minor domain and near the colicin E3 cleavage site. Neomycin does not interfere with this effect of IF3, but IF3 interferes with the protective effect of neomycin against dimethyl sulfate attack at A1408.

Protein S1 is by far the largest of all ribosomal proteins. It appears to have only one site for RNA binding (Lipecky et al., 1977; Thomas et al., 1978; Yuan et al., 1979; Mulsch et al., 1981) and another site to interact with the 30S subunit or the all-protein phage-induced Qβ RNA replicase. The binding site of S1 on the 30S subunit is dependent on S9 (Laughrea & Moore, 1978b), more sensitive to trypsin than to RNase, insensitive to aurintricarboxylic acid (Boni et al., 1982; Odom et al., 1984), and salt-sensitive, unlike its interaction with RNA, which is very stable in 1 M salt (Carmichael, 1975; Draper & von Hippel, 1978b). Studies with fragments of S1 have shown that, by and large, residues 224–309 constitute the RNA binding domain of S1 while residues 1–193 constitute the ribosome and the Qβ replicase binding domain (Subramanian et al., 1981; Guerrier-Takada et al., 1983; Subramanian, 1985; Giorginis & Subramanian, 1980). However, residues 1–193 do not bind to 30S subunits as well as S1; they are required for its nucleic acid unwinding property, and they possess some salt-sensitive poly(U) binding activity (Thomas et al., 1979; Giorginis & Subramanian, 1980; Subramanian et al., 1981), not unlike S1's site I (Draper & Von Hippel, 1978a). Thus, S1 may possess a second RNA binding site with an association constant of less than 10^5 M^{-1}. Draper and Von Hippel (1978a,b) had earlier concluded the existence of two possibly noncontiguous RNA binding sites on S1.

In this context, it seemed justified to assess the impact of S1 on the chemical and enzymatic reactivity of a large number of nucleotide residues within the 16S RNA of the ribosome or the 30S subunit. We were stimulated by the knowledge that all other 30S ribosomal proteins (several of which not being known as RNA binders) have some impact on the reactivity of 16S RNA (Stern et al., 1988d). S1 is the only 30S protein left to be footprinted. However, our goal is different from that of Noller's laboratory: not so much to locate rRNA segments within the 30S subunit but rather to delineate the zone of influence of mammoth S1.

Though it is no longer believed that initiation factor IF3 plays a direct role in binding mRNA to the 30S subunit (Canonaco et al., 1989), IF3 has a number of attributes which justifies a simultaneous study of the interaction of IF3 and S1 with the 30S subunit. Both proteins unfold RNAs (Schleich et al., 1980; Bear et al., 1976; Thomas & Szer, 1982; Wickström et al., 1986), can be cross-linked to the 3′ end of 16S RNA without actually needing it for binding to the ribosome [see Laughrea et al. (1978a) and references cited therein], may induce small conformational changes in the 30S subunit (Michalski et al., 1978; Laughrea et al., 1978b; Beaudry et al., 1976; Pon et al., 1982; Gualerzi & Pon, 1990), and cooperate during the initiation of R17 RNA-directed coat protein synthesis (Steitz et al., 1977). IF3 seems to inspect the correctness of the interaction between the mRNA initiator codon and the anticodon stem–loop of the P-site-bound tRNA (Hartz et al., 1990; Gualerzi & Pon, 1981), and S1 differentially increases the affinity of the 30S subunit for various mRNAs (Steitz et al., 1977; Roberts & Rabinowitz, 1989; Boni et al., 1991). In contrast to S1, the binding site of IF3 on the 30S subunit is likely to be dominated by 16S RNA. It is sensitive to kethoxal, RNase, or low concentrations of aurintricarboxylic acid (Pon & Gualerzi, 1976; Gualerzi & Pon, 1973; Pon et al., 1972; Sabol et al., 1973), independent of the presence or absence of many ribosomal proteins (Pon

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