Registry No. Ca, 7440-70-2; Mn, 7439-96-5; Sr, 7440-24-6; Ba, 7440-39-3; calpain, 78990-62-2.

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Processivity of the DNA Polymerase α–Primase Complex from Calf Thymus

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ABSTRACT: The processivity of the DNA polymerase α–primase complex from calf thymus was analyzed under various conditions. When multi-RNA-primed M13 DNA was used as the substrate, the DNA polymerase α–primase complex was found to incorporate 19 ± 3 nucleotides per primer binding event. This result was confirmed by product analysis on sequencing gels following DNA synthesis on poly(dT)·$(rA)_{10}$. The processivity depends strongly on the assay conditions but does not correlate with enzymic activity. Lowering the concentration of Mg^{2+} ions to less than 2 mM increases the processivity to 60. Replacing Mg^{2+} by 0.2 mM Mn^{2+} results in 90 nucleotides being incorporated per primer binding event. Neither the presence of ATP nor the addition of noncognate deoxynucleotide triphosphates affects the processivity of the DNA polymerase α–primase complex. Lower processivity was induced by lowering the reaction temperature, by adding spermine, spermidine, or putrescine, in the presence of the antibiotics novobiocin and ciprofloxacin, by adding *Escherichia coli* single-stranded DNA binding protein, or by adding calf thymus topoisomerase II and RNase H. Three single-stranded DNA binding proteins from calf thymus, including unwinding protein 1, do not affect processivity to any significant extent. Freshly prepared DNA polymerase α–primase complex exhibits in addition to its processivity of 20 further discrete processivities of about 55, 90, and 105. This result suggests that further subunits of the polymerase α–primase complex are necessary to reconstitute the holoenzyme form of the eukaryotic replicase.

Catalysis by polymerizing enzymes can be described in terms of two limiting cases. First, after the incorporation of one monomeric building block, the polymerase might dissociate from the substrate. This kind of synthesis has been designated as distributive. Second, after initiation, the polymerase might continue until it has reached the end of the substrate. This

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kind of polymer synthesis is fully processive. However, many polymerases display kinetic behavior that is intermediate of these two extremes. For these enzymes, the mean number of elongation steps that are performed after binding to the DNA template-primer substrate is called the "processivity".

Higher eukaryotic organisms contain at least three different DNA polymerases, namely, α, β, and γ. Of particular interest is the DNA polymerase α, because there is evidence that it is involved in cellular DNA replication (Kornberg, 1980, 1982; Fry & Loeb, 1986). Several forms of the α-type enzyme have been isolated that differ in subunit composition and template activity. The most complex forms are those which consist of at least three to four subunits (Banks et al., 1979; Grosse & Krauss, 1981, 1985; Masaki et al., 1982; Kaguni et al., 1983; Wang et al., 1984; Wahl et al., 1984; Chang et al., 1984). These multisubunit forms also have an inherent RNA primase activity and are able to form primers of 8–15 nucleotides in length on single-stranded DNA. Therefore, they should be designated as DNA polymerase α–primase complex. Although there is general agreement that the DNA polymerase α–primase complex is part of the replicative apparatus of higher eukaryotes, there is an ongoing debate as to whether the polymerase–primase by itself represents the eukaryotic holoenzyme or if there exist further undetected protein factors that are necessary for reconstitution of the eukaryotic replicase to the holoenzyme form.

The replicative enzyme from *Escherichia coli* also consists of several subunits. It has been demonstrated that upon reconstitution of the subunits to the holoenzyme form the processivity of the enzyme increases in discrete steps (Fay et al., 1981, 1982). In analogy, by monitoring the processivity of the eukaryotic counterpart, it should be possible to, first, define the current state of reconstitution of the polymerase α under investigation and, second, to facilitate the search for further still missing subunits. Independent of this approach, the determination of processivity is an important parameter of enzymic action and should be determined prior to any extended analysis on the kinetics of DNA polymerase α.

In this report, we investigate the processivity of the DNA polymerase α–primase complex under various conditions. We show that the processivity is 19 ± 3 under conditions that are optimal for enzymatic activity. This is comparable to polymerase III′ from *E. coli*. From the existence of still higher processivities within one DNA polymerase α–primase preparation, we conclude that further subunits of the eukaryotic replicase should exist.

Materials and Methods

Enzymes. The DNA polymerase α–primase complex was purified from calf thymus as outlined earlier (Grosse & Krauss, 1981). A method for the purification using affinity chromatography on monoclonal antibody columns will be described elsewhere (H.-P. Nasheuer and F. Grosse, unpublished experiments). The DNA polymerase α–primase complex from *Drosophila melanogaster* was a generous gift from S. Cotterill and I. R. Lehman, Stanford, CA. The single-stranded DNA binding protein (SSB protein)[1] from *E. coli* was a generous gift from J. Greipel, Hannover. SSB protein from calf thymus, unwinding protein 1, purified by the method of Herrick and Alberts (1976), was a kind gift from S. Spieker, Hannover. SSB-37 and SSB-35 (identical with subfractions of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase, respectively) were purified from calf thymus according to Grosse et al. (1986). Topoisomerase II from calf thymus was purified as described in our published procedure (Schomburg & Grosse, 1986). The purification procedure for calf thymus RNase H will be given elsewhere (A. Hagemeier and F. Grosse, unpublished experiments).

Nucleic Acids and Nucleotides. Poly(dT), $(rA)_{10}$, $(dA)_{10}$, $(dA)_{20}$, and poly(rA) were purchased from Pharmacia. The chain lengths of poly(dT), as determined by gel electrophoresis, spanned a range between 750 and 850 nucleotides. Poly(rA) was treated with 0.1 M NaOH for 10 min at 56 °C in order to produce a ladder of oligonucleotides. *Hae*III-digested pBR322 DNA, which was purchased from Boehringer Mannheim, served as an additional molecular weight standard. Dephosphorylation and 5′-labeling were performed as described by Maniatis et al. (1982). Single-stranded M13 DNA was purified according to the procedure of Yamamoto et al. (1970). Multi-RNA-primed M13 DNA was produced exactly as described by Villani et al. (1981). Nucleoside triphosphates were from Boehringer Mannheim, ultrapure deoxynucleoside triphosphates were from PL-Pharmacia, and the ATP analogue adenosine 5′-(α,β-methylenetriphosphate) (AMP-CPP) was from Miles. All radiochemicals were from Amersham.

Measurement of Processivities. Processivities on single-stranded M13 DNA were measured by using a modification of the method of Bambara et al. (1978), under conditions that were optimized for the calf thymus replicase. The assay mixture (50 μL) contained 20 mM Tris–acetate, pH 7.3, 75 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 50 μM (nucleotide) multi-RNA-primed M13mp8 DNA, and 0.1 mM sample of each dNTP (ultrapure). For the determination of the cycling times, 75 μM (nucleotides) M13mp8 single-stranded DNA served as the inhibitor. The limited reaction was performed with the omission of dCTP. The radioactively labeled nucleotide was [α-^{32}P]dATP (10 and 0.1 μCi/mL for the limited and the unlimited reaction, respectively). After the four assay mixtures were prewarmed to 37 °C, the reactions were started through the addition of 1–2 ng of DNA polymerase α–primase complex. Aliquots were withdrawn from the linear part of the incorporation kinetics, typically after 5 and 10 min. Acid-precipitable radioactivity was determined as outlined earlier (Grosse & Krauss, 1980).

The primer extension assay was performed following the method of Detera et al. (1981), but again under conditions that were optimized for the DNA polymerase α–primase complex. The reaction mixture contained 20 mM Tris–acetate, pH 7.3, 75 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 20 μM dATP, 1–3 μCi of [α-^{32}P]dATP, 1.6 μg of $(dT)_{800}$, 20 ng of $(rA)_{10}$, and 1–2 ng of homogeneous DNA polymerase α–primase complex in a total volume of 20 μL. Following incubation at 37 °C, aliquots were withdrawn, and acid-precipitable radioactivity was determined. For each point, a second aliquot was removed and the DNA precipitated through the addition of 2.5 volumes of ice-cold ethanol containing 250 mM ammonium acetate. After centrifugation, the pellets were dried and then redissolved in formamide containing 0.5% xylene cyanol FF and 0.5% bromophenol blue. After denaturing at 96 °C for 3 min, the samples were electrophoresed on a 20% polyacrylamide gel in 8 M urea (Aristar, BDH), 0.1

[1] Abbreviations: AMP-CPP, adenosine 5′-(α,β-methylenetriphosphate); Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; Pol-α, DNA polymerase α; SDS, sodium dodecyl sulfate; SSB protein, single-stranded DNA binding protein; Tris, tris(hydroxymethyl)aminomethane; UP1, unwinding protein 1; kDa, kilodalton(s).

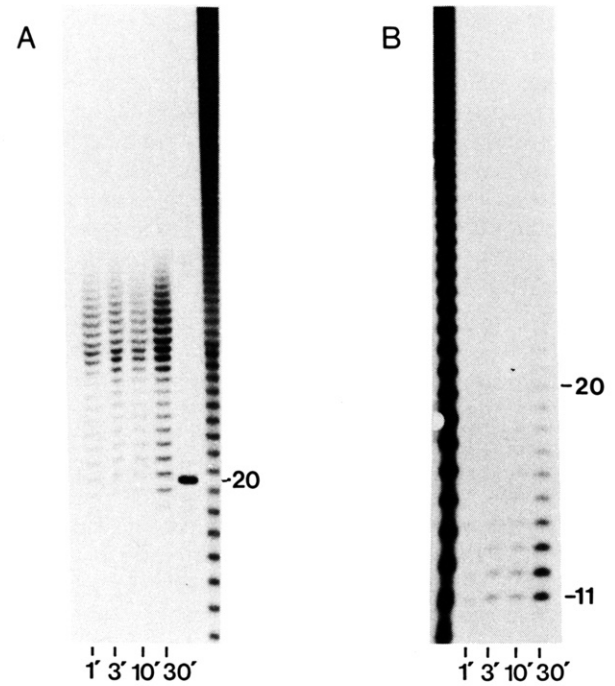

FIGURE 1: Product analysis following DNA synthesis on poly(dT). (A) Priming with $(rA)_{10}$; (B) priming with $(dA)_{10}$. Reaction conditions are given under Materials and Methods. Approximately equal amounts of radioactivity (1 μCi) were loaded onto each slot.

M Tris–borate, pH 8.3, and 1 mM EDTA. Gels were 16 cm wide, 40 cm long, and 0.5 mm thick. Electrophoresis was carried out, at 1500 V with thermostating at 50 °C, until the bromophenol blue tracking dye had migrated to about two-thirds of the gel's length. After removal of the glass plates, the gels were wrapped with saran, overlaid on X-ray film (Cronex 4, Du Pont), and exposed overnight at −80 °C. Densitometry scans were performed with a Vitatron densitometer.

Results

In this work, we have analyzed processivities by two independent methods. First, we have determined the mean processivities on natural DNA by adapting the method of Bambara et al. (1978) to multi-RNA-primed single-stranded M13 DNA. Second, we have performed a primer extension analysis on poly(dT)·$(rA)_{10}$ as already described by Detera et al. (1981). Those authors demonstrated that DNA synthesis on poly(dT)·$(rA)_{10}$ allows only one round of DNA synthesis after binding of DNA polymerase α to the RNA primer.

DNA Synthesis on Poly(dT). To further analyze the observations of Detera et al. (1981), we have compared the kinetics of DNA synthesis on poly(dT) primed with $(dA)_{10}$ or with $(rA)_{10}$. With $(dA)_{10}$ as the primer, dAMP incorporation increases linearly with time. With $(rA)_{10}$ priming, the initial rate is 200-fold higher than the rate observed when priming with $(dA)_{10}$. With the ribonucleotide primer, dAMP incorporation is linear for the first 3 min and then levels off after about 10 min, leading to the incorporation kinetics of oligo(dA)-primed DNA synthesis (data not shown). The reason why the polymerase α–primase complex synthesizes faster with the poly(dT)·oligo(rA) combination is not understood [cf. Wilson et al. (1977) for a more extended discussion of this phenomenon]. Analysis of the products of both reactions on denaturing polyacrylamide gels revealed that the $(rA)_{10}$ primers were elongated in one step with a maximum of mean product lengths at 29 nucleotides (Figure 1A). This corresponds to a processivity of 19 (the primer length of 10 must be subtracted from the mean product lengths). The elongation products are already visible after 1 min of incubation. Prolonged times yield only an increase in intensity and no change in the total length of the products. In the case of $(dA)_{10}$ priming, elongation occurs in a step-by-step fashion (Figure 1B), indicating a completely distributive mode of action on this template-primer. From these observations, and from the fact that we can resolve processivities as low as 1 nucleotide incorporation (corresponding to a product length of 11) on poly(dT)·$(rA)_{10}$, it appears that for the DNA polymerase oligo(rA)-primed poly(dT) is an appropriate substrate. However, a riboprimer that has been elongated by any number of dAMPs will be a poor substrate for initiation and further elongation. Therefore, it is possible to determine the processivity simply by monitoring the saturation level of dAMP incorporation per molecule of $(rA)_{10}$ primer.

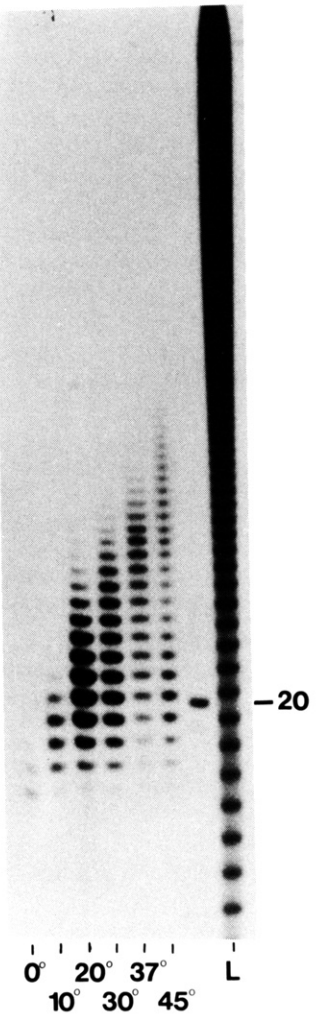

FIGURE 2: Influence of the reaction temperature on the processivity of the DNA polymerase α–primase complex.

Measurement of the Processivity under Various Conditions. As shown in Figure 1A and Table I, the DNA polymerase α–primase complex exhibits under conditions of optimal DNA synthesis a mean processivity of 19 ± 3, both on natural single-stranded DNA and also on poly(dT)·$(rA)_{10}$. To further clarify the enzymic mechanism of the replicase, we systematically varied different parameters affecting the DNA synthesis and analyzed the resulting processivities.

Temperature. The influence of the incubation temperature is given in Figure 2. At a temperature of 0 °C, the processivity is 6 ± 1. The processivity increases with increasing reaction temperatures, showing a distribution around 18–19

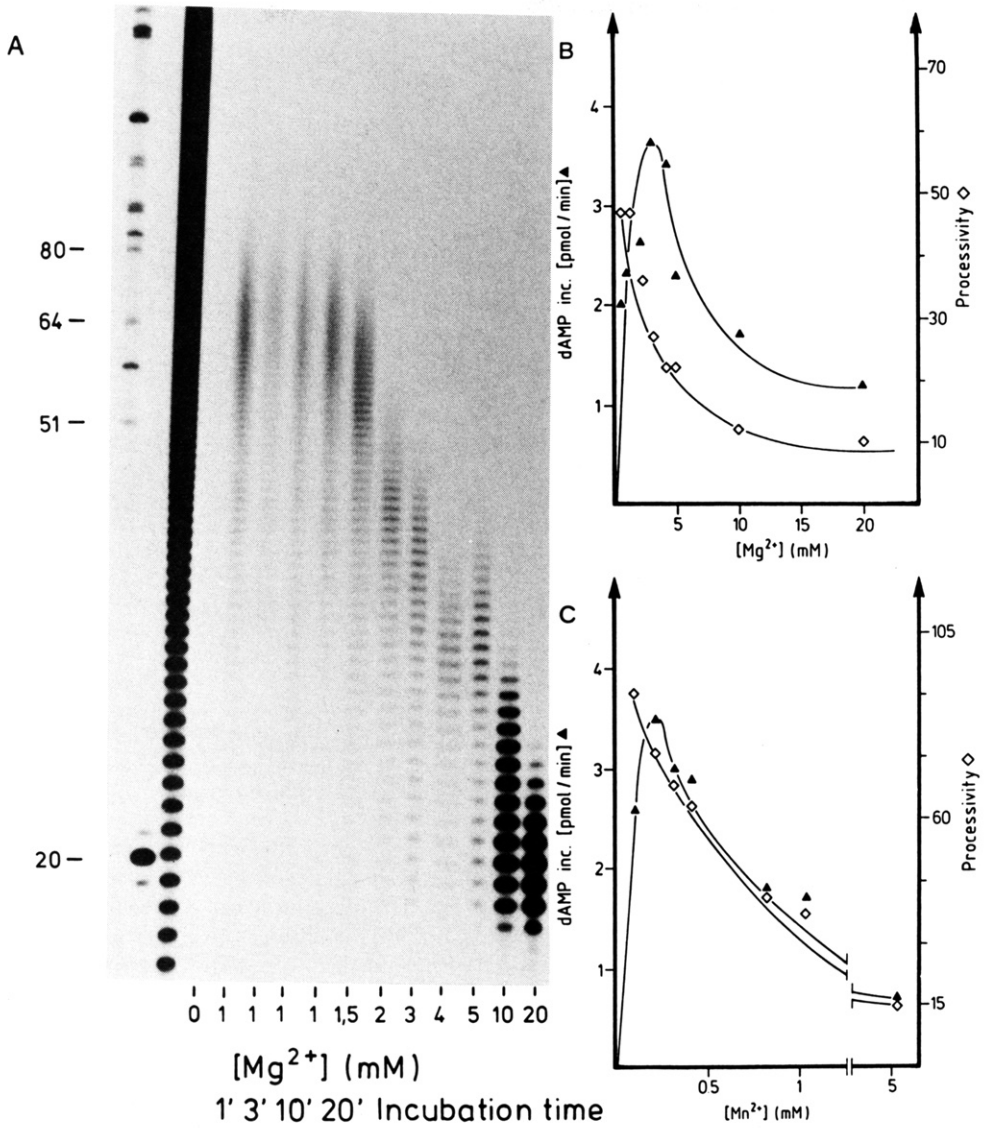

FIGURE 3: Influence of the divalent cation concentration on the processivity of the DNA polymerase α–primase complex. (A) Analysis of the product lengths at different concentrations of Mg^{2+}. If not stated otherwise, the incubation time was 3 min. (B) Comparison of enzymic activity and processivity at various concentrations of Mg^{2+}. (C) Comparison of enzymic activity and processivity at various concentrations of Mn^{2+}.

at 37 °C and a bimodal distribution with maxima centered around 10 and 21 at 45 °C. A similar effect was observed with natural DNA (Table I).

Salts. Potassium acetate had only a marginal effect on the processivity of the eukaryotic replicase. Increasing the concentration from 0 to 50 mM caused an increase in processivity from 13 to 18. However, at higher concentrations, to 200 mM, the processivity remained constant. In contrast, increasing concentrations of KCl decreased the processivity, from 18 at 50 mM KCl to 9 at 200 mM (gels not shown).

More dramatic effects were observed when the concentration of the essential divalent cation was varied. The processivity is markedly increased when the concentration of the divalent cation is lowered. As shown in Figure 3A, magnesium concentrations less than 2 mM cause the processivity to increase to about 60 incorporated nucleotides per binding event. Within the concentration range of 0.2–1 mM, the effect was even more pronounced when Mg^{2+} was replaced by Mn^{2+}. At a manganese concentration of 0.2 mM, which is optimal for the activity of DNA polymerase α on poly(dT)·$(rA)_{10}$ (Figure 3C), the observed processivity was centered around 90. Since the product bands are visible after incubation times of 1 min and do not change their lengths after prolonged times of incubation (lanes 4–7 in Figure 3A), this dramatic increase in processivity is certainly not due to the binding of enzyme to an elongated primer. A similar picture emerges from DNA synthesis on multi-RNA-primed single-stranded M13 DNA in the presence of low concentrations of Mg^{2+} and Mn^{2+} (Table I). It seems worthwhile to point out that an increase in processivity must not necessarily be accompanied by an increase in polymerase activity (Figure 3B,C).

Polyamines. The biogenic amines spermine, spermidine, and putrescine might be involved in the process of cellular DNA replication (Tabor & Tabor, 1984). For this reason, we included these amines in our study on the processivity of the eukaryotic DNA polymerase α–primase complex. As can be seen from Figure 4, spermine decreases the processivity in a concentration-dependent manner from 18 down to 4 ± 2 at a concentration of 10 mM. A very similar behavior was observed for spermidine and putrescine (Table I).

Deoxynucleoside Triphosphates. We determined the influence of noncognate nucleotides on the processivity of the DNA polymerase α–primase complex in order to mimic "pool bias" conditions. For DNA synthesis on poly(dT)·$(rA)_{10}$, the addition of dGTP, dTTP, and dCTP up to concentrations of 3 mM (vs. 20 μM dATP) did not markedly influence the

Table I: Processivity of DNA Polymerase α–Primase Complex under Various Conditions[a]

conditions	processivity	cycling time	n
Pol-α (calf thymus)			
1 mM $MgCl_2$	54.3 ± 13.1	0.731 ± 0.117	4
5 mM $MgCl_2$[b]	19.7 ± 3.1	1.035 ± 0.269	4
temp, 0 °C[c]	7.5 ± 0.7	1.355 ± 0.362	3
polyamines			
spermine[d]			
10 mM	4 ± 2	1.221 ± 0.142	4
spermidine			
1 mM	9		
5 mM	7		
10 mM	6		
putrescine			
1 mM	16		
5 mM	8		
10 mM	7		
ATP			
1 mM	18.4	1.132	1
3 mM	20		
5 mM	19		
AMP-CPP, 3 mM[e]	14.3 ± 3.4	1.147 ± 0.378	3
antibiotics			
novobiocin			
0.4 mg/mL	13		
1.0 mg/mL	8		
ofloxacin			
up to 1 mg/mL	19		
ciprofloxacin			
0.2 mg/mL	12		
0.8 mg/mL	8		
aphidicolin, 0.05 mg/mL	19		
dimethyl sulfate (10% v/v)	35		
proteins			
DNA topoisomerase II			
2 ng	18		
5 ng	8/18[f]		
10 ng	8		
10 ng	10.5 ± 3.5	1.136 ± 0.313	2
RNase H			
1 ng	17		
4 ng	13		
8 ng	11		
E. coli SSB protein[g]			
33% covering	19		
50% covering	12 ± 2.9	0.915 ± 0.283	4
100% covering	1 ± 1.5	0.874 ± 0.325	4
calf thymus SSB proteins			
SSB-35 (=LDH) (0.04 mg/mL)	16		
SSB-37 (=GAPDH) (0.01–0.08 mg/mL)	19		
SSB-24 (UP1) (0.02–10 μg/mL)	19		
Pol-α (*D. melanogaster*)			
1 mM $MgCl_2$	31.3 ± 8.7	1.028 ± 0.268	3
5 mM $MgCl_2$	20.3 ± 6.1	0.847 ± 0.185	3
E. coli SSB protein			
50% covering	153 ± 36.1	1.101 ± 0.13	3

[a] Processivities were measured by using the modification of the Bambara et al. (1978) method given under Materials and Methods. Where cycling times (T_3/T_4) are reported, the mean standard deviations are indicated when more than one determination was made. The number of determinations is listed under n. In cases where no cycling times are reported, processivities were determined solely by direct product analysis following DNA synthesis on $(rA)_{10}$-primed poly(dT). [b] Further data displayed in Figure 3. [c] Further data displayed in Figure 2. [d] Further data displayed in Figure 4. [e] Further data displayed in Figure 5. [f] Bimodal. [g] 100% covering corresponds to 1 μM tetrameric SSB protein per 50 μM (nucleotides) M13 DNA.

processivity, as long as care was taken to increase the concentration of Mg^{2+} in a mole per mole stoichiometry to the nucleoside triphosphate. This was done in order to take into account the complexing behavior of the dNTPs for divalent

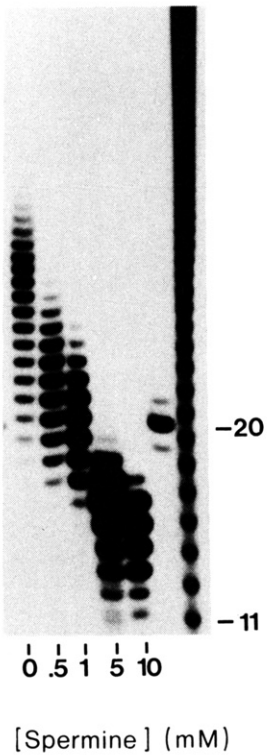

FIGURE 4: Influence of spermine on the processivity of the DNA polymerase α–primase complex.

cations. Aphidicolin, a competitive inhibitor of dCTP in DNA synthesis catalyzed by DNA polymerase α (Oguro et al., 1979), did not influence processivity either (Table I).

ATP. The influence of varying concentrations of ATP on the processivity of the DNA polymerase α–primase complex was studied on multi-RNA-primed and unprimed M13 DNA. Processivities on unprimed single-stranded DNAs can be measured because ATP by itself is sufficient for allowing an effective priming (Grosse & Krauss, 1984, 1985). In both cases, varying concentrations of ATP did not show any significant effect, as long as care was taken to compensate for the chelating effect of the triphosphate on divalent cations (Table I). The situation was quite similar with poly(dT)·$(rA)_{10}$ (gels not shown). The inhibition of the primase function with high concentrations of the nonhydrolyzable analogue AMP-CPP causes lower processivities (Figure 5).

Antibiotics. Several antibiotics of the class that inhibit prokaryotic gyrase have been shown to affect components of the eukaryotic replicational machinery (Nakayama & Sugino, 1980; Rusquet et al., 1984). As an extension of an earlier study on the interaction of novobiocin and 4-quinolones (Hussy et al., 1986), we investigated the effect of some of these drugs on the processivity of the DNA polymerase α–primase complex. Novobiocin, at concentrations where it becomes inhibitory to the eukaryotic replicase (0.4 mg/mL), decreases the processivity from 20 to 13, and at 1 mg/mL to 8 nucleotides incorporated per primer binding event. Ofloxacin does not show any effect on the processivity of the DNA polymerase α–primase complex in the same concentration range. Ciprofloxacin diminishes the processivity from 20 to 12 at a concentration of 0.2 mg/mL and from 20 to 8 at 0.8 mg/mL (Table I). Since the solvent for the antibiotics, dimethyl sulfoxide, has a stimulatory effect on the processivity (Table I), we corrected the given values for this influence.

Proteins. We have measured the influence of several proteins thought to be involved in DNA replication on the processivity of the DNA polymerase α–primase complex. The

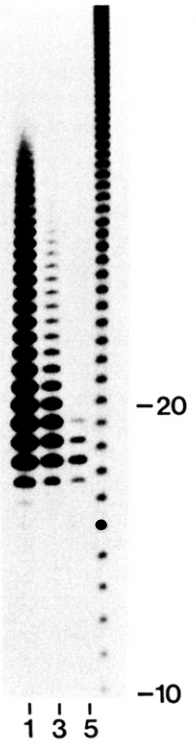

FIGURE 5: Influence of AMP-CPP on the processivity of the DNA polymerase α–primase complex.

addition of SSB protein from *E. coli* to both template-primer systems clearly inhibits the processivity of DNA polymerase α–primase complex from calf thymus in a concentration-dependent manner. In contrast, the processivity of DNA polymerase α–primase from *Drosophila melanogaster* (Kaguni et al., 1983) is stimulated by the heterologous SSB protein (Table I). Recently, we have purified two different single-stranded DNA binding proteins (SSB-37 and SSB-35) from calf thymus which affect the homologous DNA polymerase α–primase complex in both an inhibitory and a stimulatory mode. They were subsequently identified as subfractions of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase (Grosse et al., 1986). Neither of these "SSB proteins" influenced the processivity of the eukaryotic replicase to a significant extent (Table I). Also, a third tentatively assigned SSB protein, unwinding protein 1, did not affect the processivity of the DNA polymerase α–primase complex either. This confirms recent findings that showed that the processivity of the A_2 subassembly of DNA polymerase α was not affected by unwinding protein 1 (Lawton et al., 1984).

A novel type of RNase H from calf thymus, which seems to bind stoichiometrically to the DNA polymerase α–primase complex (A. Hagemeier and F. Grosse, unpublished experiments), decreases the processivity from 18 to 8 (Table I). Also, we found a significant decrease in processivity from 18 to 8 when an approximately stoichiometric amount of the homologous DNA topoisomerase II was added (Table I).

DNA Polymerase α–Primase Complex with Higher Processivities. In order to search for protein factors that increase the processivity of the DNA polymerase α–primase complex, we determined the processivities at each step of purification. This approach was affected by the presence of nucleases and other not yet identified components. However, DNA polymerase α–primase complex that was immediately assayed for processivity after the final purification step was performed

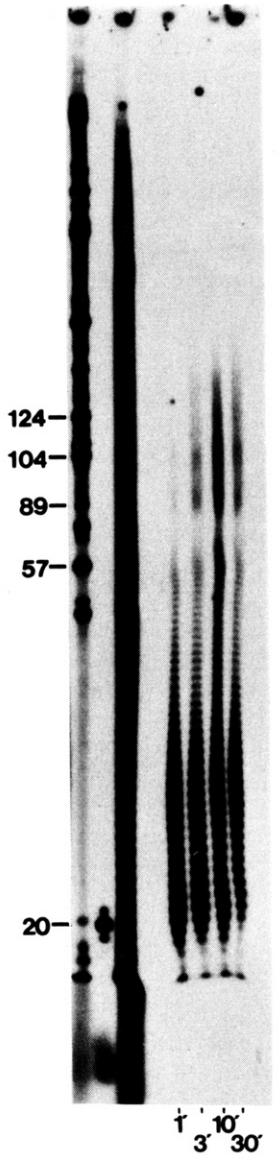

FIGURE 6: Freshly purified DNA polymerase α–primase complex exhibits further discrete processivities upon DNA synthesis on poly(dT)·$(rA)_{10}$. Reaction conditions were identical with those shown in Figure 1A. The only exception was that DNA polymerase α–primase complex was assayed immediately after ultracentrifugal separation [step VII enzyme according to Grosse and Krauss (1981)].

(preparative ultracentrifugation) (Grosse & Krauss, 1981) showed reproducibly higher processivities with discrete bands at 55, 90, and 105 in addition to the always observed distribution at around 20 (Figure 6). To further substantiate this effect, we measured the processivity of DNA polymerase α–primase complex purified to apparent homogeneity on a monoclonal antibody column within 12 h of isolation. Again, this preparation showed the additional processivities at 55, 90, and 105 that were observed after step VII of the conventional purification method. With both preparations, the higher processivities (but not enzymatic activities) seem to be extremely labile and sensitive to freezing. After the purified enzyme has been frozen to −20 °C, in the presence or absence of glycerol or ethylene glycol, the higher processivities were no longer observed.

Discussion

Several experimental designs have been employed to measure the processivity of DNA polymerases. These include template challenge experiments (McClure & Jovin, 1975;

Chang, 1975; McKune & Holmes, 1978), comparison of reaction rates of limited and unlimited reactions in the presence and absence of an inhibitor DNA (Bambara et al., 1978), and direct methods that visualize product lengths after one initiation event (Das & Fujimura, 1979; Detera et al., 1981; McClure & Chow, 1982). In this work, we have employed two independent methods. In order to determine the processivity on natural single-stranded M13 DNA, we have used the approach of Bambara et al. (1978) and adapted it to allow multi-RNA-primed templates. This technique is very convenient for the determination of processivity on natural single-stranded DNA. However, it requires a careful adjustment of the reaction conditions. We found large apparent variations in processivity depending on the preparation of inhibitor DNA. This problem was solved by Hockensmith and Bambara (1981) by the utilization of activated poly(dC)·(dG) as inhibitor DNA or, as in our case, by using unprimed single-stranded M13 DNA as an inhibitor. In order to achieve a higher resolution, we have measured DNA synthesis on poly(dT)·$(rA)_{10}$ and analyzed the product lengths after one processive cycle on DNA sequencing gels. This method not only yields mean processivities but also allows an analysis of the product length distribution. Moreover, it resolves possible different processivities that are shown by one enzyme population. Poly(dT)·$(rA)_{10}$ was chosen as a template-primer, because this system allows only one round of DNA synthesis after binding of DNA polymerase to the RNA primer. This property renders the system extremely useful for the accurate measurement of processivity. It was possible to directly determine the processivity simply by monitoring the saturation level of dAMP incorporation on poly(dT)·$(rA)_{10}$. However, different reaction conditions might influence the assay in a way that might make a terminal dAMP more acceptable to the DNA polymerase α–primase complex. Therefore, we always determined the processivities by direct visualization of the elongation products from the linear part of the incorporation kinetics. However, during the course of this study, no discrepancies were observed between incorporation measurements on poly(dT)·$(rA)_{10}$, direct visualization of product bands on DNA sequencing gels, and indirect determination of processivities on multi-RNA-primed single-stranded M13 DNA. Thus, for the first rapid screening of enzyme processivity and to test the influence of various factors, incorporation measurements on poly(dT)·$(rA)_{10}$ should provide a very effective and simple analytical tool.

Both methods employed showed that DNA polymerase α–primase complex from calf thymus exhibits a quasi-processive behavior with 19 ± 3 nucleotides incorporated per primer binding event. A similar moderate processivity was observed for the DNA polymerase α–primase complex from *Drosophila melanogaster* (Villani et al., 1981) and for the α-polymerase subspecies A_1 from calf thymus (Hockensmith & Bambara, 1981). In contrast, proteolytically degraded forms of the α-polymerase and forms that consist only of the DNA polymerizing subunit typically exhibit a processivity of 5–10 (Fisher et al., 1979; Detera et al., 1981; Villani et al., 1981).

The direct visualization of the product bands on DNA sequencing gels revealed that in addition to the mean processivity of 19 there is a more or less pronounced discrete processivity of 8. The distribution of products around both maxima was Poisson-like, as judged from a densitometrical analysis of the relative intensities of the product bands. This points to an all-or-none mechanism for chain elongation after DNA synthesis has been initiated. Furthermore, the bimodal distribution of product lengths, which is more pronounced under certain circumstances (e.g., 45 °C, higher Mg^{2+} concentrations, or DNA polymerase α–primase complex after prolonged storage), points to the presence of a further discrete enzyme species in the polymerase–primase complex. As the 5.7S form of DNA polymerase α does not contain smaller subunits (Grosse & Krauss, 1980) and its processivity is 8 (data not shown), it is conceivable that the large-core subunit of the α-polymerase has an intrinsic processivity of 8 and at least one of the other three subunits is required to enhance the processivity to 20.

Several factors were demonstrated to affect the processivity of the α-polymerase–primase complex in vitro. Increasing temperatures, increasing concentrations of potassium salts to up to 50 mM, or the addition of dimethyl sulfoxide to up to 10% increase both the activity and the processivity of the DNA polymerase α–primase complex. Biogenic amines at concentrations of more than 0.5 mM, some antibiotics, and potassium acetate as well as potassium chloride at higher concentrations of 150 mM inhibit both the activity and also the processivity. Nonetheless, a linkage between processivity and enzymatic activity obviously does not exist. This was illustrated most impressively by the influence of the essential divalent cation Mg^{2+}. At a concentration of 1 mM, the activity of DNA polymerase α is lowered by a factor of 2, but the processivity is increased from 18 to 60. Such behavior was postulated by Fisher and Korn (1981) from a thorough study on the DNA polymerase α–DNA interaction. Even higher processivities (correlating to a further decrease in enzymatic activity) were observed in the presence of 0.1 mM Mn^{2+}. It is not yet known whether these enhanced processivities at low concentrations of divalent cations reflect any physiological situation.

This study further revealed that ATP did not influence the processivity of the DNA polymerase α–primase complex to any significant extent. Actually, such an influence has been shown to occur in different subassemblies of the calf thymus enzyme (Wierowski et al., 1983) and also the α-polymerase–primase complex from *Xenopus laevis* (Riedel et al., 1984). The possibility exists that those preparations contained further subunits that might be responsible for the different observations. However, a conceivable alternative is that a lower Mg^{2+} concentration, provoked by the chelating feature of nucleoside triphosphates, might be responsible for the observed ATP effect.

The addition of noncognate deoxynucleoside triphosphates to the poly(dT) assay did not markedly influence the processivity either. Therefore, measurements on the accuracy of DNA polymerase α under pool bias conditions (Grosse et al., 1983) are reliable. Also, there was no indication of a possible effector function of one of the deoxynucleoside triphosphates on the DNA polymerase α–primase complex.

Since the processivity of the DNA polymerase α–primase complex is a very sensitive parameter of the enzymic action, it is a good probe to test the influence of proteins which have been hypothesized to play a role in cellular DNA replication. The heterologous single-stranded DNA binding protein (SSB protein) from *E. coli* has been shown to increase the processivity of the DNA polymerase α–primase complex from *Drosophila melanogaster* substantially (Villani et al., 1981). This result was completely reproducible in our hands (Table I). By contrast, the processivity of the mammalian DNA polymerase was diminished by increasing concentrations of *E. coli* SSB protein and led to a completely distributive mode at 100% covering of the DNA template. Three putative single-stranded DNA binding proteins from calf thymus, namely,

SSB-35 (lactate dehydrogenase), SSB-37 (glyceraldehyde-3-phosphate dehydrogenase), and unwinding protein 1 (a proteolytically degraded form of the ribonuclear protein A1), all of which affect the activity of the DNA polymerase α–primase complex (Grosse et al., 1986), exhibit no significant influence on the processivity of the homologous polymerase. This strongly argues against participation of any of those proteins in the process of DNA replication but underlines the analytical usefulness of processivity measurements.

Two further proteins, which are thought to be involved in the process of cellular DNA replication, affect the activity and processivity when present in stoichiometric concentrations with the α-polymerase. A novel form of calf thymus RNase H stimulates DNA synthesis by the DNA polymerase α–primase complex 3–5-fold (unpublished results) but decreases the processivity. DNA topoisomerase II both inhibits the activity and decreases the processivity. Since these effects are occurring when the proteins are in a stoichiometric relationship with the DNA polymerase α, it is tempting to speculate that topoisomerase II and RNase H might form a direct complex with the eukaryotic polymerase–primase. However, as we expect that a correct reconstitution of the replicase should lead to higher processivities, our findings with RNase H and topoisomerase II suggest that a still more complicated complex exists. We therefore conclude that further protein factors are necessary for a correct assembly of a multifunctional (and highly processive) replicase complex.

As a probe for the existence of further protein factors, we tried to determine the processivity of the DNA polymerase α at various stages during the purification. However, the presence of nucleases in the earlier fractions interfered with this approach. Nevertheless, we found in freshly harvested preparations of DNA polymerase α–primase complex strong indications for the existence of these factors. Actually, the occurrence of further discrete processivities at 55, 90, and 105 strongly resembles the behavior of DNA polymerase III after reconstitution of polymerase III′ and III* subassemblies. An estimate of the relative amount of the DNA polymerase α–primase complex that displayed this higher processivity revealed that not more than 1% of our preparation contained the presumptive protein factors. This small amount is unfortunately not detectable by means of SDS–polyacrylamide gel electrophoresis. We expect that affinity chromatography on immobilized DNA polymerase α–primase should provide a powerful tool in further purifying these factors. On the other hand, it is conceivable that the higher processivities might arise from less degraded forms of the DNA polymerase α–primase complex. This seems to be unlikely for the following reasons: first, although preparations of the immunopurified enzyme from calf thymus contain in addition to the 148-kDa subunit up to 30% of an α-subunit of molecular mass 180 kDa, this preparation does not exhibit a higher processivity than freshly prepared α-polymerase that contains only the 148-kDa α-subunit. Second, DNA polymerase α–primase complex from *Drosophila melanogaster*, whose α-subunit consists of one polypeptide with a molecular mass of 182 kDa (Kaguni et al., 1983), produces a comparable product pattern with only a maximum centered around 20 (Table I).

In conclusion, this study has revealed that the DNA polymerase α–primase complex, prepared either by conventional means or by immunopurification, is most likely not a holoenzyme like the DNA polymerase III holoenzyme, although it does efficiently copy long stretches of single-stranded DNA. Therefore, we suggest that a definition of a eukaryotic holoenzyme should also take into account the processivity of the enzyme under consideration. It is conceivable to assume that the eukaryotic holoenzyme should exhibit a processivity which comprises at least the length of one eukaryotic Okazaki fragment, i.e., 100–300 nucleotides. Furthermore, a eukaryotic holoenzyme should be able to perform DNA synthesis under physiological salt conditions with near in vivo rates, as already demonstrated for the prokaryotic counterpart (Kornberg, 1982). To our knowledge, there is at present no preparation of the eukaryotic DNA polymerase α available that fulfills these more stringent requirements for defining a eukaryotic holoenzyme. To achieve this goal, processivity measurements will be a powerful tool, both for characterization of the current state of reconstitution of the eukaryotic replicase and as a probe for further factors and subunits that comprise the eukaryotic holoenzyme.

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Registry No. RNase H, 9050-76-4; DNA topoisomerase, 80449-01-0; AMP-CPP, 7292-42-4; Me_2SO, 67-68-5; Mg, 7439-95-4; Mn, 7439-96-5; spermine, 71-44-3; sperimdine, 124-20-9; putrescine, 110-60-1; novobiocin, 303-81-1; ciprofloxacin, 85721-33-1.

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Protein-Dependent Conformational Behavior of DNA in Chromatin†

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ABSTRACT: Information from circular dichroism (CD) and DNA thermal denaturation has been used in concert to study the conformational behavior of DNA in the extended 11-nm fiber of chromatin isolated from HeLa nuclei. The histone-dependent conformational states of the system were investigated by selectively removing the hydrophilic histone domains with trypsin. These were compared to acetylated chromatin from the same source. The integrated intensity of the positive CD band for DNA above 260 nm is found to increase with the content of relatively unstressed B-form DNA. This same increase is observed along the series of whole, H1-stripped, and trypsinized chromatin samples as protein is removed. Hence, the ratio of percent hyperchromicity to integrated CD band intensity of the respective melting transitions provides useful information on the conformational state of DNA in the three principal regions of the chromatin fiber: the central loop and flanking nucleosomal regions and the linker. Results from this study suggest that central loop DNA in both hyperacetylated and control chromatin relaxes as protein is removed. However, hyperacetylated chromatin shows significantly less dependence than control chromatin upon core histone hydrophilic domains in the flanking and linker regions. Thus, histone hyperacetylation evidently relaxes DNA in chromatin with no major overall conformational changes. A possible role of histone hyperacetylation may therefore be to reduce cooperativity in the unfolding transition in chromatin and thus provide for greater localized control of unfolding during transcription.

Trypsin proteolysis has been used to probe the structural function of the N-terminal and C-terminal histone protein domains in chromatin [reviewed in Bohm and Crane-Robinson (1984)]. Trypsin digests of chromatin yield a stable series of peptides at maximal proteolysis which have been identified as core histone C-terminal regions with the N-terminal regions preferentially cleaved (Crane-Robinson & Bohm, 1985). At this level of proteolysis, some C-terminal residues of histone H2A (and at times H3) are also cleaved.

Earlier trypsin digestion studies have shown that the highly conserved C-terminal core histone domains are capable of folding DNA into compact nucleosome-like particles (Whitlock & Stein, 1978). These trypsinized core particles appear to unfold from the DNA ends on either side of the 80 base pair central core loop (Lilley & Tatchell, 1977). The unfolding process seems to be reversible, and the transition occurs at about 0.1 M sodium ion concentration (Grigoryev & Krasheninnikov, 1982). Other studies with chromatin fragments have shown that the hydrophilic domains of the core histones are necessary to condense chromatin into the 30-nm solenoid (Allan et al., 1982; Marion et al., 1983). The N-terminal domains of core histones, which also contain the in vivo acetylation sites, therefore appear to play a structural role in

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