Calf Thymus DNA Polymerases α and δ Are Capable of Highly Processive DNA Synthesis†

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ABSTRACT: We have demonstrated that calf thymus DNA polymerases α and δ are capable of highly processive DNA synthesis. Processivity values between 300 and 2000 nucleotides were observed when poly(dA)·oligo(dT) or singly primed single-stranded circular bacteriophage M13 DNA at pH 6.0 and 1 mM magnesium chloride was used. These conditions do not correlate with conditions, pH 7.0 and 5 mM magnesium chloride, that support the maximum synthetic rate. Lowering the pH and magnesium concentration lowers the K_m value of the reaction with respect to primer terminus concentration. Furthermore, under these same conditions, both polymerases become insensitive to dissociation from the template as a result of encountering the 5′ ends of primers. Overall, these results suggest that the affinity of the polymerases for the primer termini is higher throughout the polymerization reaction if pH and magnesium concentrations are lowered from those favoring maximum synthetic rate. Experiments with short primer templates, however, indicate that this higher affinity does not cause the DNA polymerase to remain stably bound after synthesizing up to the end of the template.

DNA synthesis occurs by a multistep mechanism involving the association of the DNA polymerase with a DNA primer-template, the addition of mononucleotides during primer elongation, and the eventual dissociation of the enzyme and DNA. Following each association, the average number of mononucleotides added prior to dissociation is the processivity. Original measurements of the processivity of DNA polymerase α, the primary enzyme thought to be involved in eukaryotic chromosomal replication (Kornberg, 1980, 1982; Fry & Loeb, 1986), produced values between 5 and 20 [Das & Fujimura, 1979; Fisher et al., 1979; Detera et al., 1981; Hockensmith & Bambara, 1981; reviewed in Fry and Loeb (1986)]. The removal of a subunit from calf DNA polymerase α was shown to decrease processivity (Hockensmith & Bambara, 1981). *Escherichia coli* single-stranded DNA binding protein was found to increase the processivity of *Drosophila melanogaster* DNA polymerase α holoenzyme from 14 to 285 nucleotides, while not affecting processivity of the catalytic subunit (Villani et al., 1981). ATP (Wierowski et al., 1983) and spermine (Mikhailov & Androsova, 1984) have also been found to increase the processivity of DNA polymerase α. Most recently, it has been shown that variations from the most commonly used assay conditions can have major affects on this parameter. Changes in pH (Tan et al., 1987) and magnesium concentration (Hohn & Grosse, 1987) produced processivity values in the hundreds for calf DNA polymerase α.

DNA polymerase δ has been shown to share some structural and functional features with DNA polymerase α (Lee et al., 1981, 1984; Byrnes, 1984; Crute et al., 1986; Wahl et al., 1986; Lee & Toomey, 1987). Calf thymus DNA polymerase δ, reported by Lee et al. (1984), has been purified to near-homogeneity as a single subunit of molecular weight 173 000. We later reported two forms of DNA polymerase δ from calf, δI and δII, that have molecular weights of 240 000 and 290 000, respectively (Crute et al., 1986). All have 3′ to 5′ exonuclease activity, but the exonuclease of DNA polymerase δI is readily removable (Wahl et al., 1986). This enzyme appears to be a mixture of forms that have not been further characterized. DNA polymerase δII and DNA polymerase δ reported by Lee et al. (1984) have similar sensitivities to inhibitors that can distinguish them from DNA polymerase α (Wahl et al., 1986; Byrnes, 1985; Lee et al., 1985). They differ in that proliferating cell nuclear antigen (or cyclin) (Bravo et al., 1987), a protein factor required for efficient Simian virus 40 replication in vitro (Prelich et al., 1987a), was found to increase the processivity of calf DNA polymerase δ from about 20 to more than 200 at pH 6.5 and 2 mM $MgCl_2$ (Prelich et al., 1987b), whereas DNA polymerase δII is unaffected by this protein (unpublished observation). Furthermore, DNA polymerase δII has an associated DNA primase (Crute et al., 1986) whereas DNA polymerase δ does not (Downey et al., 1988).

In the present report, we have further investigated the influence of assay conditions on the processivity of calf thymus DNA polymerases α and δII (henceforth called DNA polymerase δ). Appropriate conditions produce processivity values much higher than those previously measured. The significance of this finding with respect to DNA replication in vivo is discussed.

Materials and Methods

Poly(dA)$_{4000-5000}$ and poly(dA)$_{180-200}$ were purchased from Midland Certified Reagent Co. (Midland, TX). Poly(dA)$_{40-60}$ was from Pharmacia (Piscataway, NJ). Oligo(dT)$_{16}$ and the DNA pentadecamer 5′-TCCCAGTCACGACGT-3′ were

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synthesized by using an Applied Biosystems Model 380A DNA synthesizer. A one-kilobase DNA ladder was from Bethesda Research Laboratories Life Technologies, Inc. (Gaithersburg, MD). *Hae*III-digested ϕX174 RF DNA and T4 polynucleotide kinase were purchased from New England Biolabs, Inc. (Beverly, MA). M13mp9 single-stranded circular DNA was prepared according to Hayes and LeClerc (1983); 3000 Ci/mmol [γ-^{32}P]ATP and 800 Ci/mmol [α-^{32}P]dTTP were purchased from Amersham (Arlington Heights, IL). DE81 filter disks were purchased from Schleicher & Schuell (Keene, NH). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Proliferating cell nuclear antigen was kindly provided by Dr. Antero G. So (University of Miami).

Enzymes. Immunopurified DNA polymerase α was prepared according to Wahl et al. (1984). DNA polymerase δ was purified according to Kunkel et al. (1987), a modification of Crute et al. (1986).

The DNA polymerase δII purified for the present study is equivalent, by previously used criteria, to DNA polymerase δII purified by our original scheme. Both polymerase and exonuclease activities copurify through DEAE-Sephadex-A25 and phosphocellulose chromatography. Phosphocellulose chromatography was utilized in order to remove contaminating endonuclease (Kunkel et al., 1987) and to increase the specific activity 10-fold with a higher yield when compared to sucrose gradients used in the original scheme. Aphidicolin and N^2-(*p-n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate (BuPdGTP)[1] drug sensitivities are the same as reported earlier. Also, the molecular weight is similar and the specific activity is comparable to that used in our initial studies.

Processivity of DNA Polymerases α and δ. The rate of DNA synthesis and processivity of DNA polymerases α and δ were measured under conditions, where the primer-template was in excess, such that an average of less than one dTMP was incorporated per 3′ terminus. Therefore, most primers sustained no synthesis at all, and others sustained nucleotide incorporation that was the result of only one stretch of processive DNA synthesis. The length of extension of reacted primer termini then represents the length of processive DNA synthesis.

Assays (25 μL) contained 20 mM Mops at the indicated pH and $MgCl_2$ concentration as described for each experiment (see figure legends), 5% glycerol, 5.0 mM β-mercaptoethanol, 250 μg/mL BSA, 25 μM 13–19 Ci/mmol [α-^{32}P]dTTP, and 100 μM poly(dA)$_{4000-5000}$·oligo(dT)$_{16}$ (1:22). The template to primer ratios are stated as the ratio of 3′-hydroxyl termini. Reactions were initiated with 0.004 unit of DNA polymerase α, or 0.014 unit of DNA polymerase δ, and were incubated at 37 °C for 30 min followed by the addition of EDTA to a final concentration of 20 mM. Assays were also performed containing 0.36 μg of primed M13 single-stranded circular DNA. M13mp9 single-stranded circular DNA (144 μg/mL) and a 4-fold molar excess of M13mp9 universal sequencing primer (the DNA pentadecamer having the sequence 5′-TCCCAGTCACGACGT-3′) were annealed in 100 mM NaCl by heating to 55 °C for 10 min and allowing the solution to cool for 2 h. Reactions were initiated with 0.07 unit of DNA polymerase α, or 0.058 unit of DNA polymerase δ, and were incubated as above. One unit of polymerase activity is defined as the incorporation of 1 nmol of nucleotide into DNA per hour at 37 °C under conditions described previously (Kunkel et al., 1987). The radiolabeled DNA was then collected by sedimentation through centrifuge columns of Sephadex G-50 (Penefsky, 1977), and total incorporation was measured by counting of Cerenkov radiation. The product size was then determined by electrophoretic separation of aliquots containing approximately equal amounts of radioactivity on 8% denaturing polyacrylamide gels containing 100 mM Tris–borate, pH 8.3, 7 M urea, and 2 mM EDTA (LaDuca et al., 1986). Similar separations were performed on 2% alkaline agarose gels containing 35 mM NaOH and 2 mM EDTA (Villani et al., 1981). *Hae*III-digested ϕX174 RF DNA, a one-kilobase DNA ladder, and oligo(dT)$_{16}$ were used as size markers, after 5′ labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase according to Maniatis et al. (1982). After electrophoresis, gels were covered with Saran wrap and exposed at −80 °C using Kodak XAR film and a Dupont Cronex intensifying screen.

Effects of Primer Concentration on the Processivity of DNA Polymerases α and δ. The effect of primer concentration on the rate of DNA synthesis and the processivity of DNA polymerases α and δ was determined under the conditions described above. One millimolar $MgCl_2$ was used, and the Mops buffer was either pH 6.5 or pH 7.5. Each reaction contained 100 μM poly(dA)$_{4000-5000}$ template, while the oligo(dT)$_{16}$ concentration was changed to vary the nucleotide distance between primers. The average interprimer distances of 10, 29, 73, 159, 319, and 558 nucleotides (or 154, 88, 44, 22, 11, and 6 primers per DNA, respectively) were used to determine the effect of reaction parameters on processivity for DNA polymerases α and δ on the poly(dA) template during synthesis. After DNA polymerases α (0.004 unit) and δ (0.014 unit) were added to reactions, the total amount of nucleotide incorporated and product size were determined as described above.

Effect of Premature Termination on the Rate of DNA Synthesis. The rate of DNA synthesis was measured under the described conditions at pH 6.5 or 7.5, in reactions containing 2 or 10 mM $MgCl_2$. DNA templates (100 μM) poly(dA)$_{4000-5000}$·oligo(dT)$_{16}$ (1:80), poly(dA)$_{180-200}$·oligo(dT)$_{16}$ (1:4), and poly(dA)$_{40-60}$·oligo(dT)$_{16}$ (1:1) were utilized to measure the effect of premature chain termination on the rate of DNA synthesis by polymerases α and δ.

Reactions were initiated by the addition of 0.009 unit of DNA polymerase α or 0.015 unit of DNA polymerase δ. After incubation, the amount of nucleotide incorporated was determined by collecting the radiolabeled DNA on DE81 filters according to LaDuca et al. (1986).

Results

Rate of DNA Synthesis and Processivity of DNA Polymerases α and δ on Poly(dA)·Oligo(dT). The rate of DNA synthesis and processivity were measured on poly(dA)·oligo(dT) for DNA polymerases α and δ under conditions of varying pH and $MgCl_2$ concentration. As can be seen in Figure 1A,B, DNA polymerases α and δ have a maximum synthetic rate in reactions at pH 7.0 and 5 mM $MgCl_2$. High concentrations of $MgCl_2$ (e.g., 10 mM) were found to inhibit DNA polymerase α and to a lesser amount DNA polymerase δ.

The length of processive synthesis was determined by analyzing the products of DNA polymerases α and δ on 8% denaturing polyacrylamide gels. As can be seen in Figure 2, the length of the products synthesized by DNA polymerase α is highly sensitive to pH and $MgCl_2$ concentration. Decreasing the $MgCl_2$ concentration to 1 mM and the pH to 6.0 resulted in the synthesis of very long DNA products. The average length increases from less than 25 nucleotides long at pH 8.0 and 10 mM $MgCl_2$ (lane w) to between 300 and 2000 nu-

[1] Abbreviations: Mops, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BuPdGTP, N^2-(*p-n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate.

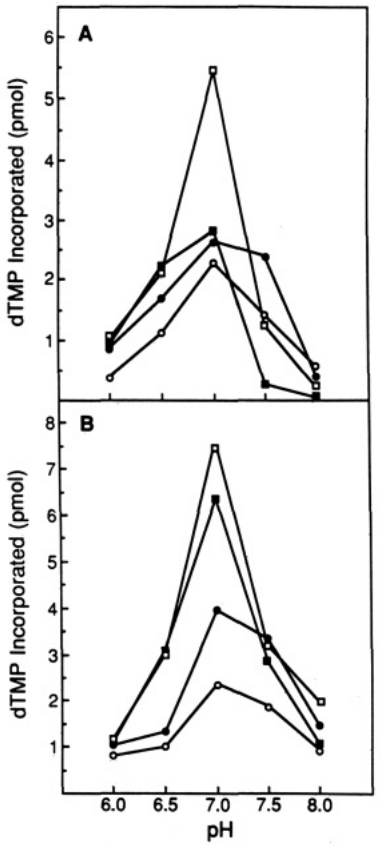

FIGURE 1: Influence of assay conditions on the rate of DNA synthesis of DNA polymerases α and δ. Rate of DNA synthesis was determined on poly(dA)$_{4000-5000}$·oligo(dT)$_{16}$ (1:22) as described under Materials and Methods for DNA polymerases α and δ, panels A and B, respectively. The Mops buffer was varied from pH 6.0 to 8.0 as indicated in the figure. The $MgCl_2$ concentration was 1 mM (open circles), 2 mM (closed circles), 5 mM (open squares), and 10 mM (closed squares).

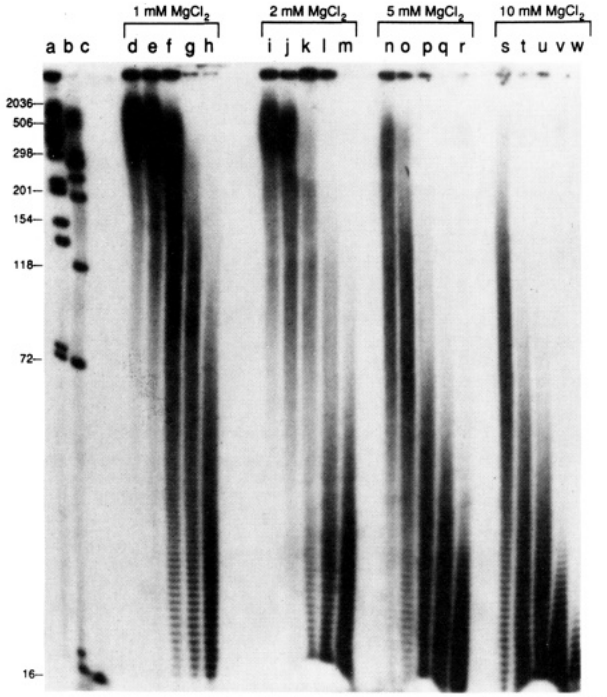

FIGURE 2: Analysis of the DNA products synthesized by DNA polymerase α. DNA products synthesized by DNA polymerase α, on poly(dA)$_{4000-5000}$·oligo(dT)$_{16}$ (1:22) (see Materials and Methods), were analyzed on denaturing, 8% polyacrylamide gels. The $MgCl_2$ concentration was varied from 1 to 10 mM as indicated in the figure. Lanes d–h, i–m, n–r, and s–w indicate a series of increasing pH values of the Mops buffer in half-unit increments from pH 6.0 to 8.0. (Lane a) A one-kilobase DNA ladder, (lane b) *Hae*III-digested ϕX174 RF DNA, and (lane c) oligo(dT)$_{16}$ were used as size markers.

cleotides long at pH 6.0 and 1–2 mM $MgCl_2$ (lanes d and i).

Figure 3 indicates that DNA polymerase δ also has the capability of synthesizing products between 300 and 2000 nucleotides long (lanes a–k and p). The processivity of DNA polymerase δ, however, is less affected by pH compared to that of DNA polymerase α. The observed decreases in product length begin to compare to DNA polymerase α as the pH is decreased from 6.0 to 8.0 at 10 mM $MgCl_2$ (lanes p–t).

Similar measurements were performed using a bacteriophage M13mp9 template primed in a single location. With both DNA polymerases α and δ, an equivalent increase in the length of processive DNA synthesis was observed, as found with the homopolymeric DNA (data not shown).

The length of the products synthesized by DNA polymerases α and δ cannot be completely resolved on 8% polyacrylamide gels. We, therefore, analyzed DNA products on 2% alkaline agarose gels to determine the maximum processive length of DNA synthesized. Figure 4 shows that DNA polymerases α and δ are capable of synthesizing products between 300 and 2000 nucleotides with an average of about 700 nucleotides in length (e.g., lanes b and c for DNA polymerase α and lanes h and k for DNA polymerase δ).

These results demonstrate that conditions for maximum DNA synthesis do not correlate with conditions for highly processive synthesis. The optimum synthetic conditions observed are pH 7.0 and 5 mM $MgCl_2$ for both enzymes, whereas optimum processivity is observed at pH 6.0 and 1 mM $MgCl_2$.

Effects of High Primer Concentrations on Dissociation of the DNA Polymerases from the Primer-Template. Results

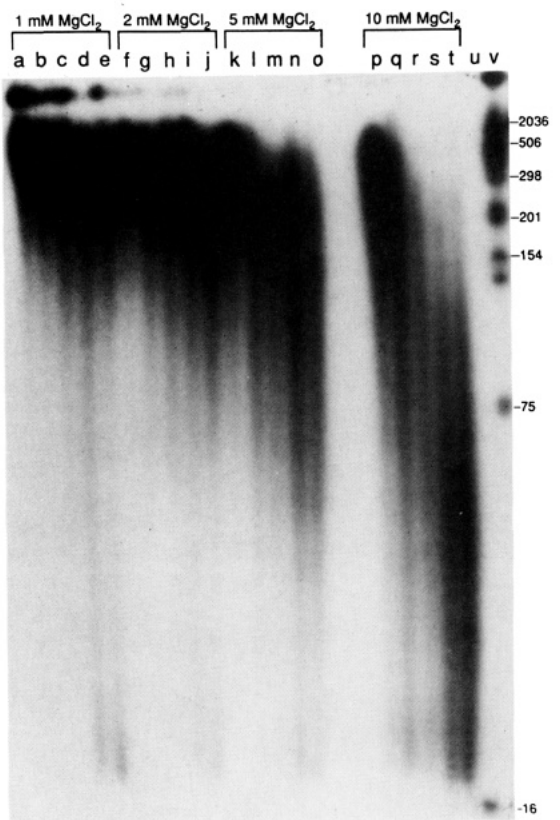

FIGURE 3: Analysis of the DNA products synthesized by DNA polymerase δ. Reaction conditions and analysis are described in Figure 2 except that DNA polymerase δ was used. Lanes a–e, f–j, k–o, and p–t each indicate a series of increasing pH values of the Mops buffer in half-unit increments from pH 6.0 to 8.0. (Lane u) Oligo(dT)$_{16}$ and (lane v) a one-kilobase ladder were used as size markers.

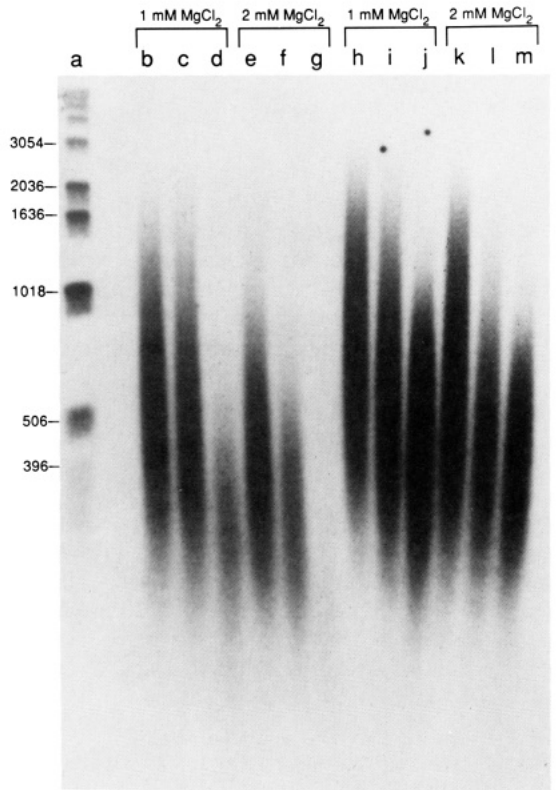

FIGURE 4: Analysis of the DNA products synthesized by DNA polymerases α and δ by alkaline agarose gel electrophoresis. Products of DNA polymerase α (lanes b–g) and DNA polymerase δ (lanes h–m) on poly(dA)$_{4000-5000}$·oligo(dT)$_{16}$ (1:22) were analyzed by 2% alkaline agarose gel electrophoresis. The $MgCl_2$ concentration was either 1 or 2 mM as indicated in the Figure. Lanes b–d, e–g, h–j, and k–m each indicate a series of increasing pH values of the Mops buffer in half-unit increments from pH 6.0 to 7.0. (Lane a) A one-kilobase DNA ladder was used as a size marker.

shown in Figures 2–4 demonstrate that DNA polymerases α and δ have the capacity to synthesize products much longer than the interprimer distance of 159 nucleotides. This suggests that primers have little effect on the elongation reaction. In an attempt to magnify the effects of encountering primers, synthetic rate and processivity were measured at high primer concentrations. As shown in Figure 5, the rate of synthesis by both polymerases at pH 6.5 is unchanged as primer concentration is increased. At pH 7.5, there is a considerable increase in rate with primer concentration. This indicates that lowering the pH lowers the K_m with respect to primer termini such that reactions at pH 6.5 are near maximum velocity at all primer terminus concentrations used. This result is consistent with the interpretation that there is a higher affinity of the polymerase for 3′ termini caused by the lower pH.

Analysis of DNA product lengths at various primer concentrations (Figure 6) demonstrates that primers have some capacity to interfere with chain elongation. As can be seen for DNA polymerase α at pH 6.5 and 7.5 (lanes a–f and g–l, respectively), products become shorter as the primer concentration is increased. However, at pH 6.5, where DNA polymerase α is highly processive, products greater than 500 nucleotides in length are made even at primer concentrations having an average interprimer distance of 10 (lane f). This suggests that during highly processive synthesis DNA polymerase α encounters many primers before dissociation. At pH 7.5, the polymerase, being intrinsically less processive, is evidently much more sensitive to interference by primers. In this case, only a few primers are encountered before dissociation occurs.

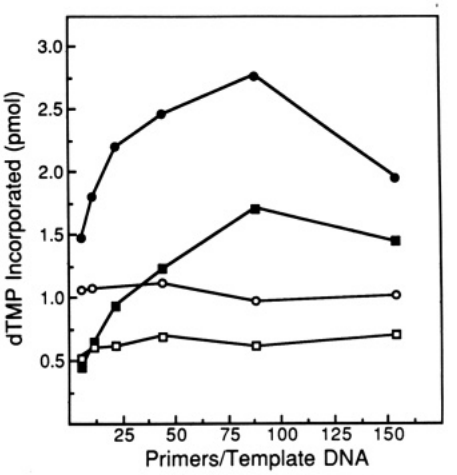

FIGURE 5: Effect of increasing primer concentration on the rate of DNA synthesis of DNA polymerases α and δ. Rates were determined on poly(dA)$_{4000-5000}$ in a series of reactions having increasing concentration of oligo(dT)$_{16}$ as indicated in the figure for DNA polymerase α at pH 6.5 (open circles) and pH 7.5 (closed circles), and for DNA polymerase δ at pH 6.5 (open squares) and pH 7.5 (closed squares). The number of primers per DNA molecule in the reaction series was 6, 11, 22, 44, 88, and 154, which is equivalent to an interprimer distance of 558, 319, 159, 73, 29, and 10 nucleotides, respectively.

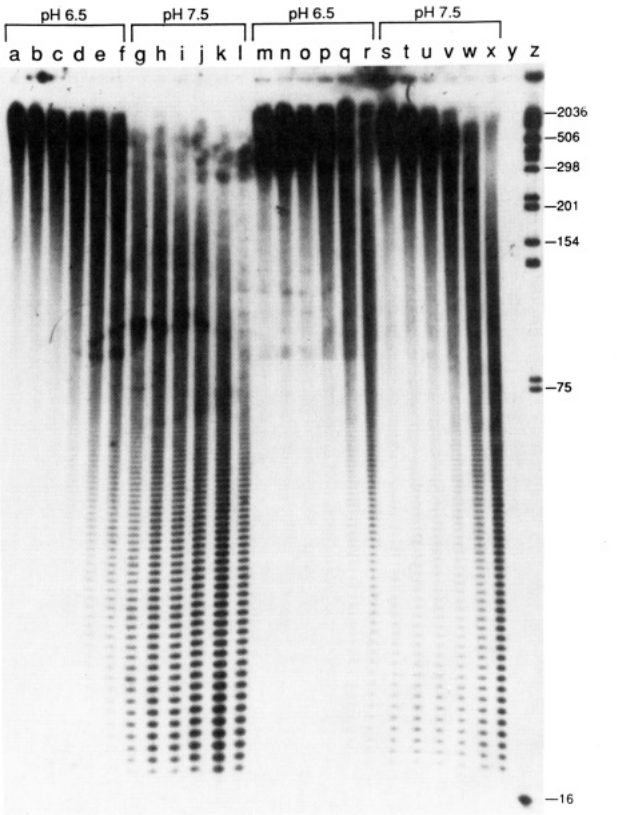

FIGURE 6: Effect of primer concentration on the processivity of DNA polymerases α and δ. DNA products synthesized by DNA polymerase α (lanes a–l) and DNA polymerase δ (lanes m–x), in the presence of increasing primer concentration, were analyzed by 8% denaturing polyacrylamide gel electrophoresis. Assays were performed as described under Materials and Methods at pH 6.5 or 7.5 as indicated in the figure. Lanes a–f, g–l, m–r, and s–x indicate a series of reactions having primer concentrations at 6, 11, 22, 44, 88, and 154 primers per DNA molecule, which are equivalent to interprimer distances of 558, 319, 159, 73, 29, and 10 nucleotides, respectively. (Lane y) Oligo(dT)$_{16}$ and (lane z) a one-kilobase DNA ladder were used as size markers.

A similar result was observed when the product sizes of DNA polymerase δ were analyzed with increasing primer concentration. At pH 6.5 and 7.5 (Figure 6, lanes m–r and

Table I: Premature Termination of Processive DNA Synthesis Utilizing DNA of Varying Lengths[a]

	DNA polymerase α		DNA polymerase δ			
	2 mM $MgCl_2$		2 mM $MgCl_2$		10 mM $MgCl_2$	
pH	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5
$poly(dA)_{40-60}$	0.6[b]	1.1	0.2	0.2	0.4	0.2
$poly(dA)_{180-200}$	1.6	3.4	0.9	2.1	2.8	2.8
$poly(dA)_{4000-5000}$	1.9	3.4	1.5	4.0	3.4	3.2

[a] DNA synthesis was measured on 100 μM $poly(dA)_{40-60}$·$oligo(dT)_{16}$, $poly(dA)_{180-200}$·$oligo(dT)_{16}$, and $poly(dA)_{4000-5000}$·$oligo(dT)_{16}$ at ratios of 1:1, 1:4, and 1:80, respectively. [b] The values are reported as picomoles of nucleotides incorporated by DNA polymerase α (0.009 unit) and DNA polymerase δ (0.015 unit) in 30 min at 37 °C. Each number is the average of duplicate determinations.

s–x, respectively), the DNA products become shorter as the primer concentration increases. DNA polymerase δ appears to be only slightly more sensitive to primers at pH 7.5 versus pH 6.5 since only a slight increase in smaller products was observed.

Effects of Premature Termination on the Rate of DNA Synthesis. Results described above suggest that DNA polymerases α and δ are more tightly bound to the DNA template at pH 6.5 than at pH 7.5. Under conditions favoring highly processive synthesis, if the reaction were prematurely terminated (prior to the time that normal-length products are synthesized) the polymerase might be expected to remain bound for a longer period of time before dissociation. Dissociation would then be the rate-limiting step and would effectively decrease the rate of synthesis.

An experiment was designed, using poly(dA) templates with an average length of 50, 190, and 4500 nucleotides, to examine the effects of premature termination. Synthesis was performed at a high primer-template concentration (100 μM) such that DNA synthesis is occurring near maximum velocity. Under these conditions, the elongation and dissociation rates should have the maximum effect on the overall reaction rate. On the shorter templates, the highly processive polymerase was expected to synthesize to the 5′ end of the template strand and remain bound to the 3′-OH of the newly synthesized strand, whereas the nonprocessive polymerase (being more loosely bound) was expected to dissociate and quickly reassociate with another primer terminus. If this were occurring, a comparison of reaction rates on long versus short templates should reveal a relatively slower rate on the short template at pH 6.5 but little difference at pH 7.5. Table I shows the results comparing the synthetic rate of DNA polymerases α and δ on $poly(dA)_{50}$, $poly(dA)_{190}$, and $poly(dA)_{4500}$. The percent change of rate when synthesizing on $poly(dA)_{4500}$ versus $poly(dA)_{190}$ or $poly(dA)_{50}$ is similar for either DNA polymerase α or DNA polymerase δ at both pH 6.5 and pH 7.5 at the indicated $MgCl_2$ concentrations. Therefore, the premature termination of DNA polymerase α or δ did not decrease the rate as expected. Evidently, when the polymerases reach the end of the template, factors which maintained the unique high affinity seen at pH 6.5 during primer elongation are no longer operative.

Discussion

It has been generally accepted that DNA polymerases α and δ have low processivity values between 5 and 20 [Crute et al., 1986; Prelich et al., 1987b; reviewed in Fry and Loeb (1986)]. These findings are surprising in view of results obtained from well-studied prokaryotic DNA polymerases. *E. coli* DNA polymerase III holoenzyme (McHenry, 1985) and the polymerases from bacteriophages T4 (Mace & Alberts, 1984) and T7 (Tabor & Richardson, 1987) were all shown to synthesize DNA in vitro with processivity values in the thousands.

As discussed earlier, small molecules, proteins, and reaction conditions have been shown to influence the processivity of DNA polymerases α and δ. We performed most experiments examining the effects of reaction conditions on the processivity of these enzymes using the primer-template poly(dA)·$oligo(dT)_{16}$ (1:22). This substrate is particularly appropriate because of the following reasons. (a) It has little or no secondary structure, such that the distribution of products representing processivity is not influenced by blockages in primer elongation. (b) Comparative values have already been obtained with *E. coli* DNA polymerase III holoenzyme. (c) It is efficiently utilized by both calf DNA polymerases α and δ. Our results have shown that calf DNA polymerases α and δ are both capable of highly processive synthesis. Values between 300 and 2000, with an average of about 700, are obtained for each polymerase by decreasing the pH and magnesium concentration below the optimal values determined for maximum synthetic rate. Additional measurements using single-stranded M13mp9 DNA, primed in a single location, verify that equivalent effects of reaction conditions on processive synthesis are observed on native DNA.

The investigation of *E. coli* DNA polymerase III holoenzyme in vitro has shown that the enzyme is capable of transforming from a form displaying low processivity (5–20) to one having high processivity (more than 1000). The most efficient transformation requires the hydrolysis of ATP and the presence of a molar excess of the β subunit [reviewed in McHenry (1985)]. The conformational transition occurs without the synthesis of intermediate-length products (Crute et al., 1986). This indicates that full-length highly processive synthesis takes place before the highly processive enzyme form can shift back to the low processivity state. The mode of transition is apparently different when calf DNA polymerases α and δ are converted into states displaying high processivity. As the pH and magnesium concentration are lowered, products of intermediate length (i.e., 50–500 nucleotides) are observed. This suggests that there is a rapid equilibrium between enzyme states at conditions favoring intermediate values of processivity. Therefore, the transition back to a low processivity state is more rapid than the length of time to complete maximum potential primer elongation. Mechanistically, this situation could be attributed to the change in the enzyme from one state to the other by the titration of a pH-sensitive group on an amino acid. An alternative possibility is that the enzyme itself passes through several conformational states, each capable of more highly processive DNA synthesis, as the pH and magnesium concentration are lowered. At an intermediate pH, for example, the enzyme form capable of intermediate processivity would account for the majority of synthetic activity.

Although DNA polymerase III holoenzyme is capable of highly processive primer elongation, it is easily dissociated by encountering the 5′ ends of primers. In fact, on poly(dA)·$oligo(dT)_{10}$, product sizes rarely exceed twice the average gap size between primers (Crute et al., 1983). Processive primer elongation by DNA polymerases α and δ is less sensitive to primer density at all conditions tested. Conditions supporting highly processive synthesis favor the maximum resistance to dissociation induced by primers. These results suggest that the affinity for the primer-template is increased throughout elongation as these polymerases become more processive.

Our experiments were designed such that the increase in primer density resulted in an increase in the molar concentration of 3′ termini. For both polymerases, the reaction rate

was found to increase with primer concentration at high, but not low, pH. Evidently, when the enzymes are not very processive, the elongation and dissociation steps of the reaction are relatively rapid compared to the primer binding step. This is an expected consequence, if the affinity of the enzymes for DNA were low throughout the reaction. Under conditions favoring high processivity, the converse appears to be the case.

It is notable that at high pH, the reaction rate declines at the highest primer terminus concentration. Earlier work with DNA polymerase α suggested that once the gap size on the primer-template is decreased below 20 nucleotides, the enzyme has difficulty utilizing the primer terminus for synthesis (Korn et al., 1979; Fisher et al., 1979; Wang & Korn, 1980; Hockensmith & Bambara, 1981; Mosbaugh & Linn, 1984). This phenomenon is not observed at low pH, indicating that the enzymes can efficiently utilize primer-templates with short gaps under these conditions.

Since the elongation and dissociation steps appear to limit the rate of reaction during highly processive synthesis, it is possible that if elongation were terminated, the polymerases would remain bound for a significant amount of time before dissociating. This phenomenon was observed with DNA polymerase III holoenzyme (Kwon-Shin et al., 1987) in an experiment measuring the effect of increasing concentrations of dideoxythymidine triphosphate on the rate of synthesis. Dideoxythymidine is efficiently incorporated and terminates elongation on poly(dA)·oligo(dT). At a dideoxythymidine triphosphate concentration sufficient to limit elongation to about 10 nucleotides, the reaction rate of DNA polymerase III core (an enzyme processive for 5–10 nucleotides) was only slightly inhibited. DNA polymerase III holoenzyme, under conditions favoring high processivity, was severely inhibited. It was concluded that the highly processive enzyme form tends to remain bound for a substantial time, at the point of termination.

Calf DNA polymerases α and all forms of δ do not use dideoxythymidine triphosphate efficiently as a substrate (Lee et al., 1981; Wahl et al., 1986). Consequently, we designed a similar experiment in which highly processive synthesis would be terminated by using short templates. We compared the rates of reactions containing either a long template with many primers or a shorter template with less primers. In these reactions, primer and template concentrations in nucleotides were unchanged; only the template length was varied. The reactions were performed at high primer concentration to maximize the probability that dissociation would limit the reaction rate. Table I shows that the ratio of reaction rates on the long versus short templates was independent of pH and magnesium concentration. Therefore, a shift from conditions favoring low processivity did not induce the formation of a stable dissociation complex after premature termination on the short templates. This result suggests that dissociation rates at the end of templates are relatively independent of the processivity.

Finally, it is notable that conditions favoring highest processivity do not match those favoring maximum reaction rate. A possible explanation for this is that the highly processive enzyme state may be predominant in vivo but not produced by low pH and low magnesium concentrations. An alternative possibility is that association of the DNA polymerases with other DNA replication proteins produces a microenvironment similar to that induced by changes in pH and magnesium concentration. If so, high processivity and high activity may be simultaneously possible in vivo. However, it is not clear whether highly processive DNA synthesis is an important aspect of eukaryotic DNA replication in vivo. The fact that eukaryotic Okazaki fragments are rather short (135 nucleotides in length; Anderson & DePamphilis, 1979) suggests that highly processive DNA synthesis may not actually be required in vivo. Nevertheless, we have shown that these enzymes are capable of highly processive primer elongation, a fact that must be considered in proposing models of DNA replication in eukaryotes.

Registry No. Mg, 7439-95-4; poly(dA)·oligo(dT), 24939-09-1; DNA polymerase, 9012-90-2.

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Selective Strand Scission by Intercalating Drugs at DNA Bulges†

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ABSTRACT: A bulge is an extra, unpaired nucleotide on one strand of a DNA double helix. This paper describes bulge-specific strand scission by the DNA intercalating/cleaving drugs neocarzinostatin chromophore (NCS-C), bleomycin (BLM), and methidiumpropyl-EDTA (MPE). For this study we have constructed a series of 5′-^{32}P end labeled oligonucleotide duplexes that are identical except for the location of a bulge. In each successive duplex of the series, a bulge has been shifted stepwise up (from 5′ to 3′) one strand of the duplex. Similarly, in each successive duplex of the series, sites of bulge-specific scission and protection were observed to shift in a stepwise manner. The results show that throughout the series of bulged duplexes NCS-C causes specific scission at a site near a bulge, BLM causes specific scission at a site near a bulge, and MPE·Fe(II) causes specific scission centered around the bulge. In some sequences, NCS-C and BLM each cause bulge-specific scission at second sites. Further, bulged DNA shows sites of protection from NCS-C and BLM scission. The results are consistent with a model of bulged DNA with (1) a high-stability intercalation site at the bulge, (2) in some sequences, a second high-stability intercalation site adjacent to the first site, and (3) two sites of relatively unstable intercalation that flank the two stable intercalation sites. On the basis of our results, we propose a new model of the BLM/DNA complex with the site of intercalation on the 3′ side (not in the center) of the dinucleotide that determines BLM binding specificity. It appears that specific scission at DNA bulges can be employed as a general assay for intercalation and binding orientation.

DNA double helices that contain single nucleotide bulges are of interest in part because of evidence that bulges are intermediates in the process of frameshift mutagenesis (Streisinger et al., 1966). A bulge, defined as an extra, unpaired nucleotide on one strand of the double helix, is thought to be stabilized by intercalating drugs (Nelson & Tinoco, 1985). Thermodynamic stabilization of bulges may be the basis of the frameshift mutagenic properties of many intercalators (Brockman & Goben, 1965; Ames & Whitfield, 1966).

We have investigated the scission specificity near DNA bulges of a series of drugs that intercalate into and cleave DNA. The data provide information on binding in the vicinity of a bulge. The results are consistent with a new model describing intercalation stabilities at several sites in the vicinity of a bulge. From this model of bulged DNA, we have deduced an unexpected orientation of the bleomycin/DNA complex. It appears that specific scission at DNA bulges can be employed as a general assay for intercalation and binding orientation of any agent that cleaves DNA.

The following three drugs have been employed in this study: (1) Neocarzinostatin chromophore (NCS-C) is an antitumor antibiotic that damages DNA by causing O_2- and thiol-dependent single-strand breaks (Kappen & Goldberg, 1978; Burger et al., 1978). DNA strand scission, with specificity thymine > adenine ≫ cytosine > guanine (Hatayama et al., 1978; D'Andrea & Haseltine, 1978; Takeshita et al., 1981), occurs via free radical abstraction of a hydrogen from the 5′ carbon of the deoxyribose (Kappen & Goldberg, 1985). It has been shown that the substituted naphthoate moiety of NCS-C intercalates into DNA (Povirk et al., 1981), and this is expected to anchor the drug such that the active site is in

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