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Properties of Two Forms of DNA Polymerase δ from Calf Thymus†

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ABSTRACT: Purified calf thymus DNA polymerases δ I and II each have an associated 3′ to 5′ exonuclease but otherwise resemble DNA polymerase α in size, biochemical kinetic parameters, and the presence of DNA primase [Crute, J. J., Wahl, A. F., & Bambara, R. A. (1986) *Biochemistry 25*, 26–36]. Here we demonstrate a functional association of polymerase and exonuclease with each δ form. Furthermore, we show that the exonuclease can be dissociated from DNA polymerase δ I but does not appear to be removable from DNA polymerase δ II. Polymerases δ I, δ II, and α are equally sensitive to the inhibitor aphidicolin, suggesting a similarity in active site structure. In comparison with DNA polymerase α and δ II, DNA polymerase δ I has intermediate sensitivity to 2-(*p*-*n*-butylanilino)-2′-deoxyadenosine 5′-triphosphate (BuAdATP) or N^2-(*p*-*n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate (BuPdGTP). The activity of the DNA primase of the δ II enzyme is insensitive to BuAdATP whereas 1.0 μM of this inhibitor will decrease the activity of the DNA primase of the α and δ I enzymes approximately 50%. Two monoclonal antibodies that potently inhibit DNA polymerase α are only slightly inhibitory to DNA polymerase δ I and are ineffective at inhibiting DNA polymerase δ II. DNA polymerase δ II had been previously found to be nearly inactive on nuclease-treated calf thymus DNA, relative to its activity on homopolymeric DNA. We find that addition of purified calf histone proteins or spermidine can greatly enhance synthesis by this enzyme on activated calf DNA.

We have recently purified two forms of DNA polymerase δ (I and II) from calf thymus using ion-exchange and substrate affinity chromatography (Crute et al., 1986a). They are polymerases that contain a 3′ to 5′ exonuclease activity and differ from each other by chromatographic behavior and template preference. They have been designated δ-class polymerases on the basis of their similarities of the δ polymerase described earlier (Byrnes et al., 1976; Lee et al., 1984; Byrnes, 1984). Examination of the δ polymerases has revealed that they share many properties with calf DNA polymerase α, which we have purified using an immunoaffinity method (Wahl et al., 1984). The three enzymes, DNA polymerases α, δ I, and δ II, have similar molecular weight and axial ratio, have an associated DNA primase activity, display similar extents of processive synthesis, and are stimulated by ATP apparently through the same mechanism (Wahl et al., 1984; Crute et al., 1986a). Crute et al. (1986b), however, demonstrated that these enzymes differ in their photosensitization to the drug hematoporphyrin derivative. Also, the compounds BuPdGTP[1] and BuAdATP have been used to differentially inhibit DNA polymerase α and δ forms (Lee et al., 1985; Crute et al., 1986a).

The increase in DNA polymerase α activity in replicating tissue and its sensitivity to the DNA replication inhibitor aphidicolin argue for its major role in DNA replication (Pedrali-Noy & Spadari, 1979). Sensitivity of DNA polymerase δ to aphidicolin (Crute et al., 1986a; Lee et al., 1984; Byrnes, 1984) is comparable to that of DNA polymerase α. However, the relative activity of DNA polymerase δ in proliferating vs. nonproliferating cells has not yet been determined. Moreover, the 3′ to 5′ exonuclease activity could be necessary for the maintenance of high-fidelity DNA replication. Therefore, DNA polymerase δ could also have a central role in DNA replication.

In general, prokaryotic DNA polymerases have an associated 3′ to 5′ exonuclease activity, which functions to remove misincorporated nucleotides during DNA replication. This

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; BuPdGTP, N^2-(*p*-*n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate; BuAdATP, 2-(*p*-*n*-butylanilino)-2′-deoxyadenosine 5′-triphosphate; DEAE, diethylaminoethyl; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IgG_1, immunoglobulin G_1; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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activity has not been found in purified DNA polymerase α from higher eukaryotes (Sedwick et al., 1975; Wang et al., 1984; Loeb & Kunkel, 1982). The error rate of DNA synthesis by the purified DNA polymerase α is approximately 1 per 30 000 nucleotides incorporated (Kunkel & Loeb, 1981). This error rate is considerably higher than the expected value for DNA replication in vivo. Although various mechanistic schemes have been proposed to account for the high fidelity of mammalian DNA replication (Bollum, 1975; Fisher & Korn, 1981; Abbotts & Loeb, 1985), little evidence has yet been developed to support them.

Our current goal has been to determine the relationship between DNA polymerases α, δ I, and δ II. Although these three DNA polymerases are similar in molecular weight, it is not possible to verify whether they share particular subunits, since they have not been purified to complete homogeneity (Wahl et al., 1984; Crute et al., 1986a). Instead, we have examined functional properties of these polymerase forms through the use of inhibitors and monoclonal antibodies. Results of these studies suggest a relationship among these polymerases.

Materials and Methods

Materials were obtained as previously described (Crute et al., 1986a) unless otherwise indicated. DNA polymerase δ I and δ II were isolated according to the method of Crute et al. (1986a) with the exception that the tailed template chromatography step was omitted. Immunoaffinity-purified DNA polymerase α was prepared as described by Wahl et al. (1984) with the following exceptions. Bacitracin was omitted as a protease inhibitor in the homogenization buffer, as some lots of bacitracin were found to inhibit the activity of DNA polymerase α on activated calf thymus DNA templates. Monoclonal anti-human KB cell DNA polymerase α–IgG_1 SJK287-38 and –IgG_1 SJK132-20 (Tanaka et al., 1982) were purified by the method of Mishell and Shiigi (1980). Western analyses were performed as described by Towbin et al. (1979) utilizing SJK132-20 IgG, biotinylated goat anti-mouse IgG, and a colorimetric peroxidase assay (Vector Laboratories). One hundred nanograms of each polymerase was used for Western analysis. Nuclease-activated calf thymus DNA was prepared by the method of Spanos et al. (1981). Histones from calf thymus (type II-A) and spermidine used to stimulate DNA polymerase δ II on nuclease-activated calf DNA were purchased from Sigma Chemical Co. (St. Louis, MO).

Enzymatic Assays. The following assays were done according to Crute et al. (1986a) with the exception that ACS (Amersham Corp.) was used as the scintillation counting solution. DNA polymerase assays utilized poly$(dA)_{4000}$·oligo$(dT)_{16}$. One unit of DNA polymerase incorporates into DNA 1 nmol of nucleotide h^{-1} at 37 °C.

To measure the effect of exonuclease inhibition by 5′-AMP on the DNA synthetic activities of DNA polymerases δ I and II, the polymerase assay was modified in the following ways. The concentration of [^{3}H]dTTP substrate was decreased to 0.5 μM, the specific activity of the [^{3}H]dTTP was increased from 4.0 to 10 Ci/mmol, and the concentration of poly$(dA)_{4000}$·oligo$(dT)_{16}$ template was decreased from 40 to 5.0 μM.

Polymerase activity on nuclease-activated calf thymus DNA was measured in a modified assay containing 20 mM Tris-HCl, pH 7.5, 5.0% glycerol, 10 mM $MgCl_2$, 5.0 mM β-mercaptoethanol, dCTP, dGTP, and dATP at 40 μM each, 2.0 mM ATP, 250 μg/mL BSA, and 25 μM 4.0 Ci/mmol [^{3}H]dTTP. When histones were added to the assay, a constant ratio of DNA/histones (60:1 w/w) was added. Also, when spermidine was added to assays, a constant ratio of DNA/spermidine (2:1 w/w) was used. Nuclease-activated DNA was treated with heparinase under the following conditions. Calcium acetate, pH 7.0, was added to 200 μL of nuclease-activated DNA to a final concentration of 100 mM. Heparinase (0.5 unit, Seikagaku Kogyo Co., Ltd., Japan) was added, followed by incubation overnight at 37 °C.

DNA primase assays utilized *Escherichia coli* DNA polymerase I Klenow fragment as a signal amplifier for the detection of DNA-dependent RNA primer synthesis. Exonuclease assays utilized poly$(dT)_{400}$·([^{3}H]dT) as a substrate.

Template-dependent generation of nucleoside monophosphates by DNA polymerases δ I and II was done essentially as described by Byrnes et al. (1976), with the reaction buffer for the synthesis reaction, 64 μM poly(dA)·oligo$(dT)_{16}$, 6 μM [^{32}P]dTTP at 63 Ci/mmol, and 0.5 unit of either DNA polymerase δ I or II. Some reactions also contained 500 μM AMP.

Separation of Exonuclease from DNA Polymerase δ I. Preparations of DNA δ I (15 000 units/1.5 mL of fraction III) and δ II (12 000 units/0.2 mL) were subjected to chromatography on DEAE-Trisacryl (0.4 × 3.5 cm, LKB Instruments, Inc.) at 4 °C preequilibrated with 30 mM Tris-HCl, pH 7.5, 20% glycerol, 20 mM NaCl, 5.0 mM dithiothreitol, 1.0 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 μg/mL pepstatin, and 0.5 μg/mL leupeptin. Subsequently, the column was eluted with an 8-mL linear gradient in which the NaCl concentration in the same buffer was increased from 20 to 200 mM. DEAE-Trisacryl chromatography was also performed in 50 mM Tris-HCl, pH 7.5, and 40 mM NaCl containing 20% glycerol, 5.0 mM dithiothreitol, 1.0 mM EDTA, 1.0 mM EGTA, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 μg/mL pepstatin, and 0.5 μg/mL leupeptin, followed by elution with an 8.0-mL linear gradient in which the NaCl concentration in the same buffer was increased from 40 to 200 mM. Two hundred microliter fractions were collected at 2.0 mL/h. DNA polymerase assays utilizing poly$(dA)_{4000}$·oligo$(dT)_{16}$ and exonuclease assays were then performed.

Phosphocellulose chromatography was used in attempts to resolve the exonuclease from DNA polymerase δ II. Preparations of DNA polymerase δ II (30 000 units/0.5 mL) were applied to a column of phosphocellulose P11 (0.4 × 6 cm, Whatman) at 4 °C, preequilibrated with 50 mM Tris-HCl, pH 7.5, and 20 mM NaCl containing 20% glycerol, 5.0 mM dithiothreitol, 1.0 mM EDTA, 1.0 mM EGTA, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 μg/mL pepstatin, and 0.5 μg/mL leupeptin. After application of DNA polymerase δ II, the column was washed with 2 column volumes of equilibration buffer containing 2.8 M urea. Polymerase was then eluted with a 20-mL linear gradient with the 2.8 M urea buffer in which the NaCl concentration was increased from 20 to 500 mM. Five hundred microliter fractions were collected at 4.0 mL/h and assayed for DNA polymerase and exonuclease activity as above.

Results

Effect of Exonuclease Inhibition on DNA Synthetic Activity. Purified DNA polymerases δ I and δ II from calf thymus have three associated activities. These are DNA polymerase activity, DNA-dependent RNA polymerase (primase) activity, and 3′ to 5′ exonuclease activity (Crute et al., 1986a). Selective inhibition of the exonuclease of DNA polymerase δ I and δ II is useful for the independent study of polymerase function and can further clarify the interdependence of these associated activities. The 3′ to 5′ exonuclease

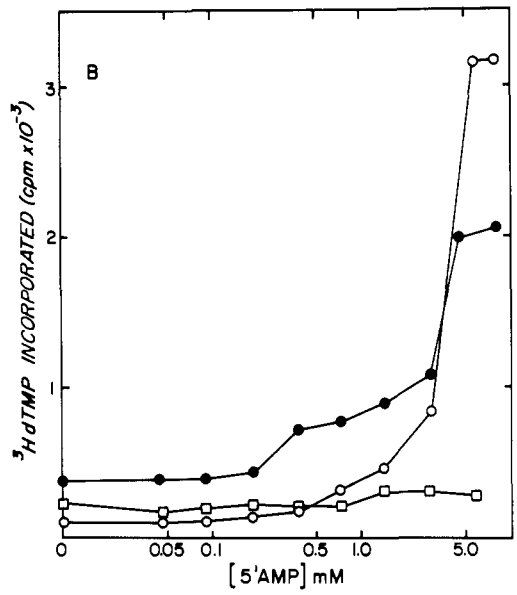

FIGURE 1: Effect of 5′-AMP on DNA polymerase activity. Polymerase activity of DNA polymerase δ I (●), δ II (○), and α (□) was determined by a modified polymerase assay as described under Materials and Methods. Poly(dA)$_{4000}$·oligo(dT)$_{16}$ was decreased to 5.0 μM, [^{3}H]dTTP decreased to 0.5 μM, and its specific activity increased to 10 Ci/mmol. 5′-AMP was added to reactions, as indicated, prior to the addition of enzyme (0.02 unit).

associated with *E. coli* DNA polymerase I, and DNA polymerase δ purified from rabbit erythroid hyperplastic bone marrow, can be inhibited 50% by 200 μM 5′-AMP (Byrnes et al., 1977). Five millimolar 5′-AMP was also shown to inhibit the 3′ to 5′ exonuclease associated with DNA polymerases δ I and δ II purified from calf thymus (Crute et al., 1986a). A dose response of the exonuclease activities associated with DNA polymerases δ I and δ II to increasing concentrations of 5′-AMP shows a 50% inhibitin of either exonuclease by the addition of 500 μM 5′-AMP and 90% inhibition by 3 mM 5′-AMP.

We have previously demonstrated that the exonuclease associated with DNA polymerases δ I and II degrades single-stranded DNA to nucleoside monophosphates in a 3′ to 5′ direction (Crute et al., 1986a). Consistent with the findings of Byrnes and colleagues (Byrnes et al., 1976; Byrnes, 1984), either DNA polymerase δ I or II will catalyze the conversion of dTTP to dTMP in the presence of primed template DNA. However, as expected, addition of 500 μM inhibits dTMP generation (data not shown).

To measure the effect of exonuclease inhibition on the DNA synthetic activity of DNA polymerase δ I and δ II, the deoxynucleoside triphosphate level in the polymerase assay was decreased below the K_m for DNA synthesis (approximately 5 μM for DNA polymerases δ I and δ II). Under these conditions, there is a competition between incorporation of deoxynucleoside monophosphates into nascent DNA by the polymerase and their removal by the associated exonuclease. Inhibition of the exonuclease by 5′-AMP would then increase the rate of nucleotide incorporation into DNA. Figure 1 shows the effect of increasing concentrations of 5′-AMP on nucleotide incorporation by DNA polymerases α, δ I, and δ II at sub-K_m levels of deoxynucleoside triphosphates. Since there is no exonuclease associated with DNA polymerase α, its synthetic activity is not affected by 5′-AMP. However, at concentrations above 500 μM 5′-AMP, the nucleotide incorporation by DNA polymerases δ I and δ II increases several fold. Similar results were obtained for DNA polymerase δ purified from rabbit erythroid hyperplastic bone marrow (Byrnes et al., 1977). At 5′-AMP concentrations above 6.0 mM, this apparent stimulatory effect is reversed as the nucleoside monophosphate begins to inhibit the function of the polymerase.

Functional Association of Nuclease and Polymerase Activity. As a control, a modification of the experiment described above was used to demonstrate that the exonuclease activity in preparations of DNA polymerase δ is functionally associated with the polymerase and does not represent a contaminating exonuclease. This approach was based on the assumption that an increase in DNA concentration would distribute a separate exonuclease primarily to 3′ termini other than those occupied by the polymerase. An associated exonuclease, however, would always accompany the polymerase irrespective of the DNA concentration. DNA polymerase assays, containing stimulating levels of 5′-AMP, were performed over a range of substrate DNA concentrations. If the polymerase and exonuclease were not associated, DNA polymerization would be affected by the exonuclease to a lesser extent as the DNA concentration was increased. Therefore, the *x*-fold stimulation of polymerase activity by 5′-AMP would be decreased at the highest DNA concentration. At 5, 10, and 15 μM poly(dA)$_{4000}$·oligo(dT)$_{16}$ substrate, the *x*-fold stimulation for DNA polymerase δ I in the presence of 2.0 mM 5′-AMP was 2.4, 2.5, and 2.8, respectively.[2] Identical experiments done with DNA polymerase δ II resulted in stimulations of 5.9-, 4.8-, and 6.3-fold. Clearly, the *x*-fold stimulation was unaffected by DNA concentration for either enzyme. This indicates that the exonuclease and polymerase activities of both δ forms are physically associated during catalysis.

Separation of Exonuclease from DNA Polymerase δ I. During the initial purification of DNA polymerases δ I and δ II, the low charge density resin DEAE-Sephadex A-25 was utilized. This resin did not disrupt the polymerase/exonuclease complex (Crute et al., 1986a). Chromatography of DNA polymerase δ I on a higher charge density anion exchanger has revealed that exonuclease activity can be partially resolved from polymerase activity. When DNA polymerase δ I was applied to a column of DEAE-Trisacryl at a low salt concentration (30 mM Tris-HCl, pH 7.5, 20 mM NaCl), both polymerase and exonuclease activities coeluted from the column at a position corresponding to 100 mM added NaCl (data not shown). However, when the equilibration salt concentration was raised to 50 mM Tris-HCl, pH 7.5, and 40 mM NaCl, most of the exonuclease did not bind to the resin whereas most of the polymerase was retained and subsequently eluted at a position corresponding to 70 mM added NaCl (Figure 2). We observed that the elutions of polymerase and exonuclease that are retained do not coincide. This may mean that the exonuclease-deficient polymerase elutes at a position slightly different from that of the polymerase/exonuclease complex. We also observed that under the same conditions DNA polymerase δ II exonuclease is not dissociated (data not shown).

DNA polymerase δ II differs from DNA polymerase δ I in that exonuclease is tightly associated with the polymerase. Chromatography on phosphocellulose in the presence of 2.8 M urea does not resolve exonuclease from the polymerase activity. Both activities coelute by a linear salt gradient at 400 mM NaCl (data not shown).

Effect of ddTTP on Synthetic Activities of DNA Polymerases δ I, δ II, and α. The nucleotide analogue 2′,3′-di-

[2] These experiments are performed near the K_m for DNA. In the presence or absence of 5′-AMP, the rate of DNA synthesis rises by 40% and 30% for DNA polymerases δ I and II, respectively, when the DNA concentration is raised from 5 to 15 μM.

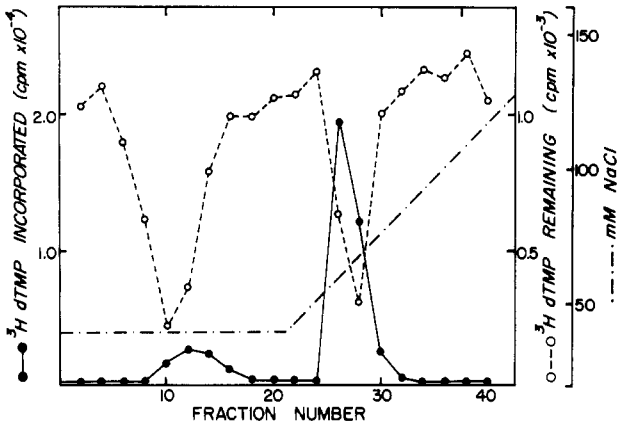

FIGURE 2: DEAE-Trisacryl chromatography of DNA polymerase δ I showing separation of exonuclease activity from polymerase activity. After DNA polymerase δ I application (15 000 units), the column was washed with equilibration buffer followed by a linear gradient from 40 to 200 mM NaCl. Fractions were collected and assayed for DNA polymerase with poly(dA)$_{4000}$·oligo(dT)$_{16}$ as template (●) and exonuclease (○) as described under Materials and Methods.

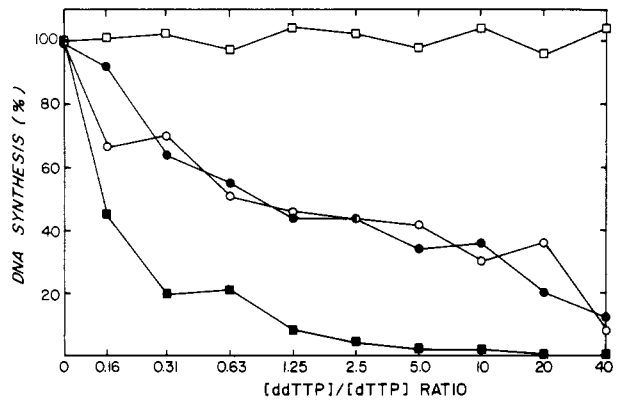

FIGURE 3: Effect of ddTTP on polymerization activity. The enzymes shown are DNA polymerases α (□), δ I (●), δ II (○), and β (■). Assay conditions were as described under Materials and Methods with poly(dA)$_{4000}$·oligo(dT)$_{16}$ as template except that ddTTP was added to reaction mixtures prior to the addition of enzyme (0.02 unit).

deoxythymidine 5′-triphosphate (ddTTP) has been useful in DNA sequence analysis because it can be incorporated into nascent DNA by *E. coli* DNA polymerase I and can cause termination of synthesis since it lacks a 3′-OH. Waqar et al. (1978) reported that although mammalian DNA polymerases β and γ are inhibited at 1:1 ratios of ddTTP:dTTP, the activity of DNA polymerase α remains unaffected at up to a 50-fold excess of ddTTP. They also found that replicative DNA synthesis was not affected at these concentrations of ddTTP. They concluded that DNA polymerase α is the only polymerase required for cellular DNA replication. The data in Figure 3 show the sensitivities of various DNA polymerases purified from calf thymus tissue to increasing ratios of ddTTP to dTTP, during DNA synthesis. Calf thymus DNA polymerase β synthetic activity was inhibited 50% at a ddTTP:dTTP ratio of 0.15:1. It retained only 5% of its original activity at a ratio of 2.5:1. In sharp contrast, DNA polymerase α retains nearly 100% of its activity at ddTTP:dTTP ratios as high as 40:1, reflecting a high selectivity of the enzyme for the natural substrate over the analogue. The sensitivities of DNA polymerase δ I and δ II to ddTTP are intermediate between those of DNA polymerase β and DNA polymerase α. DNA polymerase δ I and δ II are inhibited 50% by a ddTTP:dTTP ratio of 0.75:1. However, both enzymes retain >30% of their DNA synthetic activities at ddTTP:dTTP ratios of 10:1. The sensitivities of both of these enzymes are much less than that

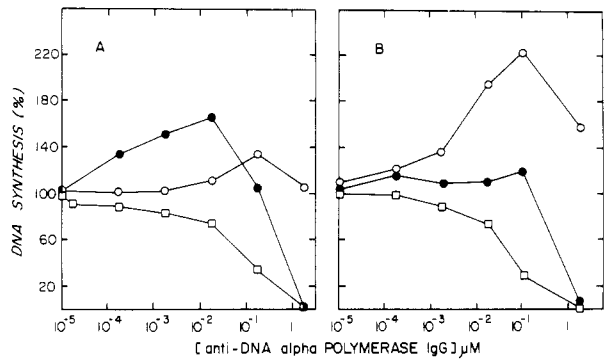

FIGURE 4: Effect of anti-α polymerase IgG titration on DNA polymerase activity. DNA polymerases α (□), δ I (●), and δ II (○) were assayed for DNA synthetic activity in the presence of serial dilutions of the anti-α polymerase antibody SJK132-20 IgG (panel A) or SJK287-38 IgG (panel B). The standard polymerase assay using poly(dA)$_{4000}$·oligo(dT)$_{16}$ as template and 0.1 unit of each enzyme was used with the addition of antibody dilutions prior to enzyme addition.

observed with DNA polymerase β and reported sensitivities of DNA polymerase γ (Waqar et al., 1978) but are still far greater than that of DNA polymerase α.

Antibody Sensitivity of DNA Polymerases δ I, δ II, and α. The anti-α polymerase antibodies purified from hybridoma cell lines SJK287-38 and SJK132-20 (Tanaka et al., 1982) are effective inhibitors of calf DNA polymerase α and have been used successfully for immunoaffinity purification of that enzyme (Wahl et al., 1984). To examine the relative antibody sensitivities of DNA polymerases δ I, δ II, and α, the DNA synthetic activity of each enzyme was assayed in the presence of increasing concentrations of antibody. The results using either SJK132-20 IgG or SJK287-38 IgG are shown in panels A and B of Figure 4, respectively. DNA polymerase α is inhibited 50% by 70 nM of either antibody and shows a typical dose response to further dilution of these antibodies. Ten times this concentration, or approximately 0.75 μM, of either antibody is required to inhibit DNA polymerase δ I. DNA polymerase δ II is not inhibited by either antibody at the highest (micromolar) concentrations. It is interesting to note that, in addition to the reduced sensitivity to antibody by either DNA polymerase δ, antibody at some concentrations is stimulatory to their DNA synthetic activity. This effect occurs with other unrelated proteins such as serum albumin. Therefore, the stimulation noted here is thought to result from increased stabilization of the polymerase by additional protein and evidently is not the result of specific antibody–antigen interaction. Consistent with these results, we have demonstrated, using Western analysis, that SJK132-20 IgG binds to the 180-kDa polypeptide of DNA polymerase α. However, this antibody does not bind sufficiently to any of the polypeptides associated with either DNA polymerase δ I or δ II for detection by Western analysis.

Aphidicolin Sensitivity of Purified DNA Polymerases δ I, δ II, and α. Aphidicolin is a known inhibitor of chromosomal replication and cell division (Ohashi et al., 1978; Ikegami et al., 1978). It is well documented that DNA polymerase α is sensitive to this drug (Holmes, 1981). This sensitivity has been used to implicate DNA polymerase α as the major DNA replication enzyme in higher eukaryotes (Pedrali-Noy & Spadari, 1979). It has subsequently been reported that DNA polymerase δ is also inhibited by aphidicolin (Lee et al., 1984; Crute et al., 1986a). To determine the relative sensitivities of the purified enzymes to this drug, polymerase activities of immunoaffinity-purified DNA polymerase α and DNA polymerases δ I and δ II were determined on the substrate

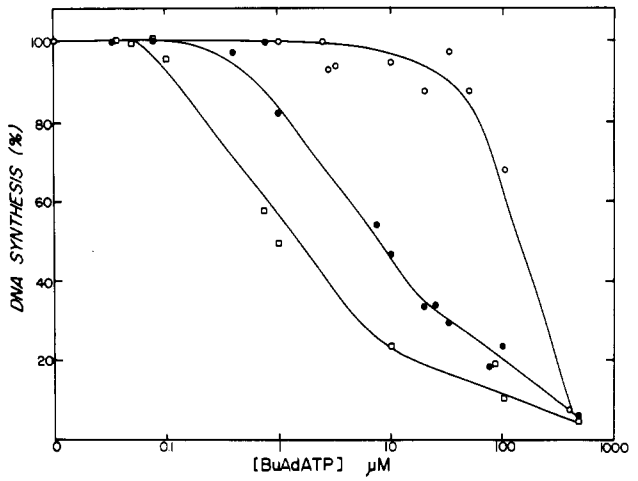

FIGURE 5: Effect of BuAdATP on polymerase activity. Polymerase activities of DNA polymerases δ I (●), δ II (○), and α (□) were assayed as described under Materials and Methods with poly-$(dA)_{4000}$·oligo$(dT)_{16}$ as template except for the addition of BuAdATP, as indicated, prior to enzyme addition (0.02 unit).

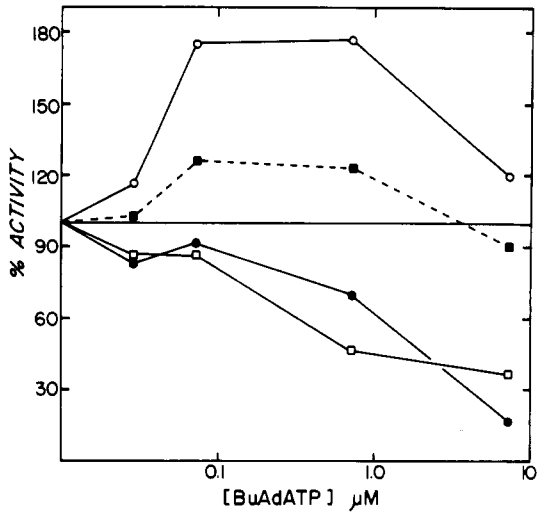

FIGURE 6: Effect of BuAdATP on DNA primase activity. Primase activities were assayed for 0.1 unit of DNA polymerases δ I (●), 0.01 unit of δ II (○), and 0.022 unit of α (□) as described under Materials and Methods except for the addition of BuAdATP as indicated prior to enzyme additions. The effect of BuAdATP on the synthetic activity of *E. coli* DNA polymerase I is shown as a control for the efficiency of the coupled DNA primase activity assay.

poly$(dA)_{4000}$·oligo$(dT)_{16}$ in the presence of increasing concentrations of aphidicolin. The activities of DNA polymerase α, δ I, and δ II were inhibited 50% in the presence of 2.0, 2.5, and 4.0 μM aphidicolin, respectively, demonstrating essentially identical sensitivities.

Decreasing the concentration of dTTP substrate for this reaction 2-fold (from 25 to 12.5 μM) increased the sensitivity of DNA polymerases α, δ I, and δ II by approximately 2-fold. This increase in the percent inhibition indicates that aphidicolin is a competitive inhibitor of DNA polymerases α and δ with respect to dTTP on poly$(dA)_{4000}$·oligo$(dT)_{16}$ as a template. Under the same assay conditions, DNA polymerase β from calf thymus tissue is relatively insensitive to aphidicolin, retaining greater than 90% of its activity in the presence of 200 μM aphidicolin.

BuAdATP Sensitivity of Purified DNA Polymerases δ I, δ II, and α. BuPdGTP (Kahn et al., 1984) and BuAdATP (Kahn et al., 1985) have been shown to be potent inhibitors of mammalian DNA polymerase α. To further assess the relationship among DNA polymerases δ I, δ II, and α, the relative sensitivities of these purified DNA polymerases were determined on poly$(dA)_{4000}$·oligo$(dT)_{16}$.

The polymerase activities associated with each enzyme are differentially affected by BuAdATP (Figure 5). DNA polymerase α is at least 100 times more sensitive than DNA polymerase δ II (50% inhibition at 1.0 and 100 μM, respectively). DNA polymerase δ I is intermediate in its sensitivity to BuAdATP (50% inhibition at 10 μM). In this respect, calf DNA polymerase δ I resembles the DNA polymerase δ isolated from rabbit bone marrow (Byrnes, 1985; Khan et al., 1985), which has a 10-fold greater sensitivity to this drug than does the rabbit DNA polymerase α. DNA polymerase δ II, on the other hand, is similar to DNA polymerase δ from human placenta (Lee et al., 1985).

When the associated DNA primase activity of DNA polymerase δ I, δ II, and α was determined in the presence of BuAdATP, a similar trend in sensitivities was observed (Figure 6). Surprisingly, the primase activity associated with DNA polymerase δ II is insensitive to BuAdATP over the concentration range tested. In fact, there appears to be a 1.5-fold stimulation of primase activity over the value in the absence of BuAdATP. Taken together, the results shown in Figures 5 and 6 suggest that, with regard to sensitivity to BuAdATP, DNA polymerase δ I and α are similar while DNA polymerase δ II shows very distinctive attributes.

Template Specificity of DNA Polymerases δ I and II. We reported earlier that DNA polymerase δ I could utilize both nuclease-activated calf DNA and poly$(dA)_{4000}$·oligo$(dT)_{16}$ for DNA synthesis with similar efficiency (Crute et al., 1986a). DNA polymerase δ II, which preferentially utilized homopolymeric DNA, was found to have little activity on activated calf DNA. Others (Byrnes, 1984; Lee et al., 1984) have also found that DNA polymerase δ has little activity on activated calf DNA. We now find that DNA polymerase δ II activity on nuclease-activated calf DNA is highly dependent upon appropriate nuclease activation, the DNA concentration, and the presence of purified calf histones (or spermidine) in the DNA polymerase assay. DNA polymerase activity of DNA polymerase δ II reaches an optimum at 500 μM activated calf thymus DNA and decreases above this DNA concentration. However, in the presence of a constant ratio of purified calf histones to the DNA substrate, the reaction rate constantly increases with DNA concentration. A Lineweaver–Burk double-reciprocal plot of the reaction rate in the absence of histones suggests substrate inhibition by the DNA, whereas the inhibition disappears in the presence of histones. Similar results are obtained if spermidine is added to DNA polymerase assays.

Discussion

Our recent development of methods to purify calf thymus DNA polymerases δ I, δ II (Crute et al., 1986a), and α (Wahl et al., 1984) have allowed us to examine physical and functional properties of all three enzymes. Their similarity in size, shape, biochemical kinetics, processivity, capacity for stimulation by ATP, and associated DNA primase activity (Crute et al., 1986a) led us to suspect that these enzymes are structurally related. We have now examined more properties of this enzymes that lend further support to this supposition and additionally demonstrate the ways that these enzymes differ.

At sub-K_m levels of dTTP substrate, inhibition of the DNA polymerase δ I and II exonucleases by 5′-AMP increases the net nucleotide incorporation into DNA. Increasing the concentration of DNA, the substrate for both exonuclease and polymerase, did not change the x-fold stimulation of polymerase activity in the presence of 5′-AMP. This indicates a

physical association of each form of DNA polymerase δ with its 3′ to 5′ exonuclease, as explained earlier. Addition of 5′-AMP to reactions consistently stimulates DNA polymerase δ II to a greater extent than it stimulates DNA polymerase δ I. This suggests a fundamental difference in the properties of the exonuclease and polymerase in each enzyme, but could also imply some inactivation or loss of the exonuclease associated with DNA polymerase δ I during purification. In fact, under appropriate conditions (Figure 2) the exonuclease of DNA polymerase δ I can be partially resolved from the polymerase, suggesting that these two activities are loosely associated. On the other hand, the exonuclease of DNA polymerase δ II appears to be tightly associated even under high-salt and denaturing conditions. By this criterion DNA polymerase δ II resembles the DNA polymerase δ from calf reported by Lee et al. (1984) and from rabbit bone marrow (Goscin & Byrnes, 1982) in which the polymerase and exonuclease appear to reside on the same polypeptide. Our studies using inhibitors suggest both similarities and differences among DNA polymerases δ I, δ II, and α. The enzymes are differentially inhibited by hematoporphyrin derivative and light, with DNA polymerase δ II most sensitive, while DNA polymerase δ I and α are less sensitive (Crute et al., 1986b). DNA polymerase δ I and δ II show intermediate sensitivity to dideoxythymidine triphosphate compared to DNA polymerase α (insensitive) and DNA polymerase β (sensitive). Both DNA polymerase δ forms have little sensitivity to monoclonal SJK287-38 or SJK132-20 IgG, originally raised against human DNA polymerase α. DNA polymerase δ I exhibits some antibody sensitivity but only at levels of IgG 10-fold higher than those required to produce similar inhibition of DNA polymerase α. DNA polymerase δ II is resistant to all tested levels of antibody. Since both of these antibodies were produced against a polymerase devoid of exonuclease, this finding is consistent with the possibility that the presence of an associated exonuclease acts to sterically block antibody–polymerase interaction with similar epitopes on each enzyme.

Measurement of BuAdATP inhibition indicates that the polymerase and associated primase activities of DNA polymerases δ I and α have a similar sensitivity while the activities of DNA polymerase δ II are insensitive. In fact, the primase activity of DNA polymerase δ II is stimulated by this drug.

Recently, we reported (Crute et al., 1986a) that DNA polymerase δ II was nearly inactive on nuclease-activated calf thymus DNA. We have now demonstrated significant activity of this DNA polymerase on activated calf DNA but find it is highly dependent on the DNA concentration, the appropriate nuclease activation, and the presence of purified calf histones or spermidine in the polymerase assays.

It should be noted that the template activity of activated calf thymus DNA for DNA polymerases α or δ can be increased by pretreatment of DNA with heparinase as indicated under Materials and Methods. The amount of stimulation is variable, depending on the source and lot of commercial calf thymus DNA (data not shown). DNA polymerases α and δ have been shown to bind heparin agarose (Lee & Toomey, 1985). Sulfated polysaccharides, sensitive to degradation by heparinase and capable of binding to and inhibiting the polymerase, may, therefore, be present in some DNA preparations.

It still remains unclear if several different polymerases are required for effective replication of the eukaryotic genome. Since aphidicolin has been used to implicate DNA polymerase α as the major DNA replication enzyme (Pedroli-Noy & Spadari, 1979), we examined the possibility that DNA polymerase δ I and δ II are affected by this inhibitor. Each polymerase indeed shows a similar dose response to aphidicolin in the micromolar range.

DNA polymerase δ I is reported here to have an intermediate sensitivity, between that of DNA polymerases δ II and α, to various DNA polymerase inhibitors. We further find that the exonuclease activity can be removed from DNA polymerase δ I but not from DNA polymerase δ II. DNA polymerase δ I, therefore, has properties similar to those expected of DNA polymerase α plus a loosely associated nuclease, whereas DNA polymerase δ II has properties of DNA polymerase α with a tightly associated or covalently attached nuclease.

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Registry No. DNA polymerase δ I, 9012-90-2; DNA primase, 64885-96-7; 3′→5′-exonuclease, 79393-91-2; spermidine, 124-20-9.

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DNA Binding Specificity of a Series of Cationic Metalloporphyrin Complexes†

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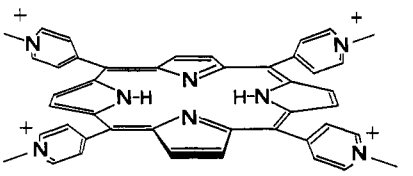
ABSTRACT: The sequence specificities of a series of cationic metalloporphyrins toward a 139 base pair restriction fragment of pBR-322 DNA have been studied by DNase I footprinting methodology. Analysis using controlled digests and quantitative autoradiography/microdensitometry revealed that the 5- and 6-coordinate complexes of *meso*-tetrakis(*N*-methyl-4-pyridiniumyl)porphine, MT4MPyP, where M is Mn, Fe, Co, and Zn, were found to bind to AT regions of DNA. Footprinting analysis involving the radiolabel on the opposing strand of restriction fragment showed site skewing in the direction of the 3′ end of the fragment, indicating that the porphyrins bind in the minor groove of DNA. The significant increase in DNase I catalyzed hydrolysis observed in various regions of the fragment appeared to be primarily due to a decrease in available substrate DNA upon porphyrin binding with possible contributions from structural changes in DNA caused by ligand binding. The complexes NiT4MPyP and CuT4MPyP were found to bind to both AT and GC regions of the fragment, producing different degrees of inhibition in the two regions. Since the outside-binding porphyrins can neither intercalate or effectively hydrogen bond to DNA, they appear to read sequence by responding to steric and/or electrostatic potential effects located in the minor groove of DNA.

The interaction of metalloporphyrins with DNA has been the subject of a number of investigations. The basis for these studies is in part related to the observation that certain porphyrins can serve as photo sensitizers for destruction of tumor cells. This effect, termed photodynamic therapy, has been useful for treatment of a number of tumors that occur in man (Kelly et al., 1975; Dougherty et al., 1979). In addition to the photodynamic properties of the porphyrins, the Fe^{3+} complex of protoporphyrin IX, hemin, is believed to play an important role in the differentation of Friend erythroleukemia cells (Lo et al., 1981). Since porphyrin-mediated DNA damage may take place during this process (Scher & Friend, 1978), efforts have been made to detect porphyrin-induced DNA strand scission in vitro. In this regard it has been shown that hemin in the presence of 2-mercaptoethanol and oxygen can efficiently degrade the closed circular plasmid pBR-322 (Aft & Mueller, 1983).

Metalloporphyrins have also been reported to possess antitumor properties (Lown et al., 1984). Covalent attachment of the DNA binding groups acridine or acodazole to hemin results in bleomycin-like porphyrin compounds, which in addition to exhibiting in vivo antitumor effects are capable of binding to and degrading PM2-DNA (Lown & Joshua, 1982). Studies of the degradation process with natural DNAs of definded sequence have shown that these compounds cleave DNA in a sequence neutral manner (Lown et al., 1986). Nuclease activity has also been observed for ferric porphyrins possessing the appended potent mutagen 2-amino-6-methyldipurido[1,2-*a*:3′2′-*d*]imidazole (Hashimota et al., 1984). However, in this case studies with a restriction fragment from pBR-322 DNA revealed that porphyrin-induced DNA cleavage is sequence specific and is similar to that of the anticancer agent bleomycin (Dabrowiak, 1983).

The most actively studied DNA binding metalloporphyrins are the metal complexes of the tetracationic water-soluble compound *meso*-tetrakis(*N*-methyl-4-pyridiniumyl)porphine, H_2T4MPyP[1] (Fiel et al., 1985; Pasternack et al., 1984; Kelly et al., 1985; Marzilli et al., 1986). Kinetic (Pasternack et



al., 1983a,b), optical (Carvlin & Fiel, 1983), and other physiochemical evidence (Dougherty et al., 1985) suggests that the nature of the coordinated metal ion controls the porphyrin-binding mechanism and its DNA base preference. At low

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[1] Abbreviations: T4MPyP, dianion of *meso*-tetrakis(*N*-methyl-4-pyridiniumyl)porphine; MPE, methidium–propyl–iron ethylenediaminetetraacetic acid; DNase, deoxyribonuclease; TPP, *meso*-tetraphenylporphine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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