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Specificity of the Catalytic Interaction of Human DNA Polymerase β with Nucleic Acid Substrates†

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ABSTRACT: We have employed steady-state kinetics methodology together with novel assays of the polymerization products synthesized on DNA primer-templates of known sequence to obtain new insights into the mechanism of catalysis of human DNA polymerase β. A major objective of these studies has been to define structural elements of DNA substrates that are required for catalytically productive polymerase binding. The results demonstrate very substantial differences between KB cell polymerases α and β with respect to the molecular signals that govern their specific catalytic interactions with nucleic acids. We show that substrate addition to polymerase β obeys a rigidly ordered sequential mechanism, with DNA first followed by dNTP. Under reaction conditions optimized for gapped (activated) DNA primer-template, with Mg^{2+} or Mn^{2+} as the divalent cation, polymerase β exhibits no detectable kinetic affinity for intact duplex DNA molecules, whether covalently closed circles or blunt-ended linear fragments, and both natural and synthetic, linear and circular single-stranded polydeoxynucleotides produce patterns of inhibition that are, under all conditions tested, fully and linearly noncompetitive with DNA (and dNTP) substrates. Polymerase β has a very high affinity for duplex DNA molecules that contain nicks bearing either 3′-OH or 3′-PO_4 termini, and 3′-PO_4-terminated nicked DNA is a potent inhibitor of the polymerase β reaction that is linearly competitive with DNA substrate. From a detailed examination of the interaction of polymerase β with staggered-end duplex DNA molecules of known sequence, we present strong evidence in support of the conclusion that a primary determinant of the productive binding of the polymerase to DNA is a base-paired primer moiety that must be adjacent to a very short length of (potentially) single-stranded template. Our inability kinetically to resolve separate partial reactions of primer binding and template binding suggests that in contrast to KB cell polymerase α, primer-template binding of polymerase β may occur by a concerted mechanism. The choice of divalent cation has a dramatic effect on the minimum length of template required for binding. Thus, in the presence of Mg^{2+}, the minimum template length is greater than or equal to five nucleotides, while in the presence of Mn^{2+} only a single template nucleotide is sufficient. Finally, we demonstrate that the choice of divalent cation also affects the processivity of deoxynucleotide incorporation by human polymerase β with DNA. With Mg^{2+} as cation, the polymerization mechanism is essentially distributive, with about one nucleotide inserted per binding cycle. In contrast, with Mn^{2+} as cation, the reaction mechanism is modestly processive, with insertion of four to six nucleotides in each polymerization cycle. Both the values of processivity and their response to the divalent cation are identical when measured on nicked and gapped DNA substrates.

The availability of essentially homogeneous preparations of human DNA polymerases α and β (Fisher & Korn, 1977; Wang et al., 1974, 1977), that are completely free of associated or contaminating deoxyribonuclease activities (Fisher et al., 1979; Wang & Korn, 1980), has permitted a detailed investigation of some of their enzymological properties with a variety of defined, natural and synthetic DNA primer-templates. These studies have demonstrated a number of striking differences between the two enzymes with respect to their ability to catalyze deoxynucleotide incorporation on these several DNA substrates (Eichler et al., 1977; Korn et al., 1978; Fisher et al., 1979; Wang & Korn, 1980) and have suggested that there might be equally profound differences, possibly of physiological significance, in the nature of the specific molecular signals that regulate the catalytic interactions of the two polymerases with nucleic acids.

In recent reports (Fisher & Korn, 1979a,b, 1981a,b; Fisher et al., 1981), we have employed steady-state kinetics methodology together with direct sedimentation binding assays to document several of the key features of the catalytic mechanism of KB cell DNA polymerase α. (1) The interaction of polymerase α with its substrates obeys a rigidly ordered sequential terreactant mechanism, with template (single-stranded polydeoxynucleotide) as the first substrate followed by primer as the second substrate and dNTP as the third. (2) Although

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kinetically significant dNTP binding is absolutely dependent on antecedent primer binding, specification of which dNTP can add to the enzyme is strictly determined by the template sequence. (3) Catalytically significant primer binding requires a minimum length of the primer stem of eight nucleotides, of which it appears that at least the terminal three to five nucleotides must be template complementary. (4) A single mispaired terminal primer nucleotide is sufficient to prevent correct primer binding *and also* the subsequent step of complementary dNTP addition to the polymerase. (5) The enzyme can properly bind a primer stem bearing a 3′-terminal OH or H residue, but the presence of a 3′-terminal PO_4 substituent blocks primer binding. (6) Each catalytically active polymerase molecule appears to possess at least two strongly interactive (positively cooperative) single-stranded polydeoxynucleotide binding sites [and possibly two complete active centers (Fisher & Korn, 1981a)]. On the basis of these data, we have suggested (Fisher et al., 1981; Fisher & Korn, 1981a; Korn et al., 1981) that the properties of KB cell polymerase α are compatible with those of a conformationally active protein which is capable of catalytically significant responses to signals that are generated by template sequence and transduced via template binding site(s) interactions.

A number of workers have demonstrated that DNA polymerases of the α class incorporate deoxynucleotides under a variety of reaction conditions in a moderately processive manner (Fisher et al., 1979; Das & Fujimura, 1979; Hockensmith & Bambara, 1981; Matsukage et al., 1980; Detera et al., 1981). It should be noted that for DNA polymerases that function processively, the detailed features of the product release steps of the reaction mechanism remain largely unknown (McClure & Chow, 1980).

With respect to DNA polymerase β, our understanding of at least some aspects of the catalytic mechanism, and particularly of the molecular elements that may modulate the interaction of the enzyme with nucleic acids, is much less complete. Therefore, to gain additional insights into these problems, we have carried out the experiments described in this paper. The results indicate that the order of substrate addition to human DNA polymerase β obeys a rigidly ordered sequential mechanism (DNA followed by dNTP) that we must tentatively characterize as "ordered bireactant" since we have been unable kinetically to discriminate separate template-binding and primer-binding components of the enzyme–nucleic acid interaction. In further contrast to our findings with polymerase α, the primary determinant of catalytically productive DNA recognition by polymerase β appears to be a base-paired primer moiety that must be adjacent to a minimum length of template (single-stranded polydeoxynucleotide). Both the length of adjacent template required and the processivity of deoxynucleotide incorporation are affected by the choice of divalent metal cation. Thus, in the presence of Mg^{2+}, the minimum effective template length is greater than or equal to five nucleotides, and deoxynucleotide insertion is nonprocessive, while in the presence of Mn^{2+} (Wang et al., 1977) the minimum effective template length is a single nucleotide, and the polymerization reaction can be shown to be modestly processive, with an average incorporation of four to six nucleotides per binding cycle.

Materials and Methods

Unlabeled dNTPs were from Boehringer, dideoxyTTP (ddTTP) was from P-L Biochemicals, [^{3}H]dTTP was from New England Nuclear, and [^{3}H]dATP, [^{3}H]dGTP, [^{3}H]-dCTP, [γ-^{32}P]ATP, [α-^{32}P]dATP, [α-^{32}P]dTTP, [α-^{32}P]dCTP, [α-^{32}P]dGTP, and cordycepin 5′-[α-^{32}P]triphosphate were from Amersham/Searle. All triphosphates were used without further purification. Pancreatic DNase I, micrococcal nuclease, spleen phosphodiesterase, and bacterial alkaline phosphatase (BAP) were from Worthington. Restriction endonucleases *Hae*III, *Eco*RI, and *Hin*dIII and T4 polynucleotide kinase were from BRL. *Escherichia coli* exonuclease III (exo III) was a generous gift from Dr. L. A. Loeb (University of Washington), T4 DNA polymerase was from Dr. P. Modrich (Duke University), and RNase A was from Dr. K. Cook (Stanford). The RNase A (1 mg/mL) was heated for 10 min at 100 °C before use to inactivate any contaminating DNase activity. KB cell nicking–closing activity was purified according to Vosberg & Vinograd (1976) and was free of detectable nuclease contamination. Terminal deoxynucleotidyl transferase (TdT), purified from calf thymus, was a gift from Dr. R. L. Ratliff (Los Alamos Scientific Laboratory). Human DNA polymerase β from KB cells or liver, isoelectric-focused fraction or DNA–cellulose fraction, was prepared as described (Wang et al., 1977); the two fractions were found to be enzymologically indistinguishable and were used interchangeably in these experiments. Calf thymus DNA was from Calbiochem. Homopolymers $(dA)_{\overline{100}}$, $(dT)_{\overline{100}}$, $(dC)_{\overline{100}}$, and $(dT)_{\overline{12}}$ and synthetic mixed heteropolymers were prepared and characterized as described (Fisher et al., 1981). Phage ϕX174 single-stranded circular DNA was prepared according to Franke & Ray (1970) and was found by alkaline agarose gel electrophoresis (McDonnell et al., 1977) to be at least 90% intact circles. Phage PM2 DNA (circular duplex), either ^{3}H labeled or unlabeled, and KB [^{3}H]DNA were prepared as before (Wang & Korn, 1980). Plasmid pACYC 184 was propagated in *E. coli* C600 in the presence of 25 μg/mL spectinomycin and [^{3}H]dThd; the labeled plasmid DNA [3.9 kilobase pairs (kbp); 2.6×10^6 daltons; 1862 cpm/μg] was prepared as described by Chang & Cohen (1978). Plasmid pBR 322 was propagated in *E. coli* C600 in the presence of 50 μg/mL ampicillin, as described (Bolivar et al., 1977). The sources of other reagents were as previously noted (Wang & Korn, 1980).

Preparation of Nicked Calf Thymus DNA. Native calf thymus DNA (0.5 mM nucleotide) was treated with 15 ng/mL pancreatic DNase I as described (Wang & Korn, 1980). The average number of dNMPs that could be incoporated by polymerase β at each 3′-OH primer terminus (i.e., per nick) was determined by the "ratio of extents" method of Bambara et al. (1978), as modified by Fisher & Korn (1979c). By this procedure, the DNA sample was found to contain an average of 1 nick per 1700 nucleotides.

Preparation of Gapped Calf Thymus DNA. The nicked calf thymus DNA (0.6 mM nucleotide) was digested with 1.2 units/mL exo III in 70 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$, and 300 μg/mL bovine serum albumin at 35 °C for intervals ranging from 0 to 180 min. The resected DNA samples were purified and tested for their relative primer-template capacities for polymerase β (Wang & Korn, 1980). Maximum reactivity was observed with the 60-min sample, and this condition was used to prepare an optimally gapped DNA substrate. The mean gap length of this substrate was estimated by determining the "average template length" (Bambara et al., 1978) with polymerase β; the data were treated by the "no nuclease" equation of Fisher & Korn (1979c). The mean gap length was thus found to be $\sim$14 nucleotides.

Micrococcal Nuclease Treatment of Calf Thymus DNA. Native calf thymus DNA (1250 μg/mL) was incubated for

60 min at 22 °C with 35 ng/mL micrococcal nuclease in 10 mM Tris-HCl, pH 8.9, 10 mM $CaCl_2$, and 200 μg/mL bovine serum albumin. The sample was extracted twice with equal volumes of redistilled phenol and twice with ether, precipitated with ethanol, and extensively dialyzed againt KTE buffer (10 mM KCl, 20 mM Tris-HCl, pH 8.0, and 1 mM EDTA). The average number of nucleotides per 3′-phosphate (nick) site was estimated by 5′-^{32}P labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP (13 000 cpm/pmol) (Weiss et al., 1968). By this method, the DNA sample was found to contain approximately one 3′-PO_4 terminus per 2870 nucleotides.

Preparations of Form IV (Covalently Closed, Relaxed) Plasmid pACYC 184 DNA. Form I plasmid [^{3}H]DNA (377 μM nucleotide) and KB nicking–closing activity (6380 units/mL) were incubated for 60 min at 35 °C in 15 mM Tris-HCl, pH 8.0, 200 mM KCl, 0.2 mM EDTA, 1 mM spermidine, and 150 μg/mL bovine serum albumin. The reaction was stopped by addition of sodium dodecyl sulfate to a final concentration of 0.5%, brought to a final density of 1.460 g/cm^3 with CsCl and 100 μg/mL ethidium bromide, and the sample was centrifuged to equilibrium at 38 000 rpm for 48 h in a Beckman type 65 fixed-angle rotor. The form IV plasmid DNA band was collected, passed over a 1-mL column of Dowex 50Wx8 to remove the dye, and then dialyzed extensively against KTE buffer to remove the CsCl. The recovered DNA was completely relaxed (form IV) as determined by nondenaturing agarose gel electrophoresis (Keller, 1975).

Restriction of PM2 DNA. PM2 [^{3}H]DNA (92 μg) was restricted with *Hae*III endonuclease, and the products were analyzed as described (Fisher & Korn, 1979a). PM2 DNA (670 μg) was restricted with 50 units of *Hin*dIII endonuclease for 100 min at 36 °C in a 10-mL reaction that contained 20 mM Tris-HCl, pH 7.5, 7.7 mM $MgCl_2$, 30 mM NaCl, and 100 μg/mL gelatin. The reaction was terminated by adding EDTA to 50 mM, and the DNA products were collected by ethanol precipitation. Under these reaction conditions, the DNA is completely restricted into seven fragments that range from ~100 to >6000 base pairs in length (Brack et al., 1976), as resolved on nondenaturing agarose gel electrophoresis.

*Preparation of Staggered-End Duplex of PM2 DNA Fragments by Exonuclease III Resection. Hin*dIII-restricted PM2 DNA (487 μg) was incubated with 1.5 units/mL exo III at 36 °C in a 2-mL reaction containing 70 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$, and 60 μg/mL gelatin. At 10, 20, and 30 min, aliquots (660 μL) were removed, the reaction was stopped by adding EDTA to 50 mM, and the sample was heated for 10 min at 65 °C to inactivate the exonuclease. After the sample was slowly cooled, the DNA products were recovered by ethanol precipitation. The mean lengths of the staggered ends in these resected DNA samples were estimated by measuring the extent of dNMP incorporation with T4 DNA polymerase. Under reaction conditions in which the T4 enzyme incorporated four nucleotides at extent on the original *Hin*dIII-restricted DNA fragments, the exo III treated samples were found to contain staggered ends averaging 5.5, 6.5, and 7.4 nucleotides in length, respectively, after 10, 20, and 30 min of exo III digestion. [These length values are interpreted as mean values of a Poisson distribution, since exo III is a nonprocessive exonuclease (Thomas & Olivera, 1978)].

Preparation of 5′-^{32}P-Labeled EcoRI- and *HindIII-Restricted pBR 322 DNA Marker Fragment.* pBR 322 DNA (26 μg) was restricted with *Eco*RI, dephosphorylated with BAP, and decontaminated of RNA by preparing a reaction (100 μL) that contained 100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 50 mM NaCl, 2.5 μg of RNase A, 0.06 unit of BAP, and 50 units of *Eco*RI, and incubating for 60 min at 37 °C. The DNA sample was extracted twice each with phenol and ether, precipitated with ethanol, and then 5′-phosphorylated in a reaction (25 μL) that contained 50 mM sodium–glycine, pH 9.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 5% glycerol, 1.2 units of T4 polynucleotide kinase, and 200 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). The incubation was for 60 min at 37 °C. The reaction was stopped by adding an equal volume of 2 M NH_4OAc and 20 mM EDTA. Unincorporated [γ-^{32}P]ATP was removed by Sephadex G25 gel filtration, and the labeled DNA sample was concentrated by ethanol precipitation. The 5′-^{32}P-labeled, *Eco*RI-restricted DNA was then further restricted with 45 units of *Hin*dIII in a reaction (50 μL) that contained 20 mM Tris-HCl, pH 8.0, 7 mM $MgCl_2$, and 60 mM NaCl at 37 °C for 60 min. After the digestion was terminated by addition of an equal volume of 2 M NH_4OAc, 20 mM EDTA, and 30 μg of carrier sRNA, the DNA sample was recovered by ethanol precipitation and heat denatured at 100 °C for 1 min in 80% (v/v) formamide, 50 mM Tris–borate, pH 8.3, 1 mM EDTA, 0.1% (w/v) xylene cyanol (XC), and 0.1% (w/v) bromophenol blue (BPB). The denatured DNA fragments were electrophoresed in a 20% polyacrylamide gel slab (16.5 cm × 26.5 cm × 0.2 cm) containing 7 M urea (Maxam & Gilbert, 1977) at 1000 V until the two dyes had separated by 8 cm. The wet gel was then transferred to Kodak XAR-5 film for autoradiography. The labeled DNA band that corresponded to the single *Eco*RI–*Hin*dIII restriction fragment of 29 nucleotides in length was recovered from the gel by electroelution and was used as a marker fragment in subsequent gel analyses.

*Preparation of Cordycepin 3′-[^{32}P]Phosphate Labeled HindIII- and EcoRI-Restricted pBR 322 DNA Marker Fragments. Hin*dIII restricted pBR 322 DNA (7 μg), prepared as described above, was 3′-end labeled with 50 μCi of cordycepin 5′-[α-^{32}P]triphosphate (3000 Ci/mmol), using 420 units of TdT, 37 °C, for 60 min under described conditions (Tu & Cohen, 1980). The labeled DNA was recovered by ethanol precipitation and was further restricted with 25 units of *Eco*RI, also as described above. The final labeled fragment was analyzed by electrophoresis in a 20% polyacrylamide gel containing 7 M urea and demonstrated four bands of 30, 29, 28, and 27 nucleotides in length. [The appearance of labeled bands shorter than the single expected 30-nucleotide fragment (a single residue of cordycepin is added to the 29-nucleotide-long starting fragment) is due to a trace amount of 3′-exonuclease contamination in the TdT.]

*Preparation of Exonuclease III Resected, HindIII-Restricted pBR 322 DNA Substrate. Hin*dIII-restricted pBR 322 DNA (22 μg) was incubated with 0.03 unit/mL exo III for 10 min at 37 °C as described above. Resection was terminated by the addition of 0.1 volume of 2 M NH_4OAc and 20 mM EDTA, and the sample was heated at 65 °C for 10 min and slowly cooled. The DNA was recovered by ethanol precipitation, dialyzed against KTE buffer, and used as a primer-template for polymerase β.

Cordycepin 3′-[^{32}P]Phosphate Labeling of Exonuclease III Resected, HindIII- and EcoRI-Restricted pBR 322 DNA Marker Fragments. The exo III digested, *Hin*dIII-restricted pBR 322 DNA (1.8 μg) was labeled with cordycepin 5′-[α-^{32}P]triphosphate as above. The labeled DNA was recovered by ethanol precipitation and further restricted with *Eco*RI, also as described earlier. The final sample was precipitated

with ethanol in the presence of 5 μg of carrier sRNA and analyzed on a 7 M urea–20% polyacrylamide gel.

Determination of Extent of dNMP Incorporation of DNA Polymerase β on Exonuclease III Resected, HindIII-Restricted pBR 322 DNA. Exo III resected, *Hin*dIII-restricted pBR 322 DNA (54 μM nucleotide) was reacted with 12 units/mL human DNA polymerase β (isoelectric-focused fraction) under the conditions described in Figure 1A, with Mg^{2+} as the divalent cation and all four α-^{32}P-labeled dNTPs at a total specific activity of 18 400 cpm/pmol. Every 30 min, an aliquot of the incubation containing 0.27 nmol (nucleotide) of DNA was removed for assay of dNMP incorporation, and a fresh aliquot of reaction mixture that contained polymerase β at 12 units/mL (but no primer-template) was added to the incubation until dNMP incorporation had reached a stable extent. After 330 min, a total of 2.82 units of enzyme had been added, and an average of 8.3 dNMP residues had been incorporated on each available 3′-OH terminus. The reaction products were recovered by adding an equal volume of 2 M NH_4OAc, 20 mM EDTA, and 20 μg of carrier sRNA, followed by ethanol precipitation. The DNA products were then further restricted with 50 units of *Eco*RI and analyzed by gel electrophoresis.

Reactions performed with Mn^{2+} as the divalent cation contained 21.5 μM (nucleotide) substrate DNA and were carried out under the conditions described in Figure 6B; the incubations contained 12 units/mL polymerase β (isoelectric-focused fraction) and all four α-^{32}P-labeled dNTPs at a total specific activity of 114 000 cpm/pmol. The experimental protocol was otherwise as described above. After 330 min, a total of 3 units of polymerase had been added, and an average of 12 dNMPs had been incorporated on each available 3′-primer terminus. The reaction products were resolved by gel analysis as described above.

Analysis of Polymerase β Products by Polyacrylamide Gel Electrophoresis. The products of the polymerase β reaction on staggered-end DNA substrates, together with DNA marker fragments, were suspended in a final volume of 5 μL of 80% formamide, 50 mM Tris–borate, pH 8.3, 1 mM EDTA, 0.1% XC, and 0.1% BPB, boiled for 1 min, and quick-chilled in ice. The samples were applied directly to a 20% polyacrylamide gel slab that contained 7 M urea, 50 mM Tris–borate, pH 8.3, and 1 mM EDTA. The gel was prerun for at least 2 h at 1000 V before sample loading. Electrophoresis was carried out at room temperature at 1000 V until the two dyes had separated by 8 cm; the wet gel was then autoradiographed on Kodak XAR-5 film at −70 °C.

Other Methods. Standard assays for DNA polymerase β with either Mg^{2+} or MN^{2+} as divalent cation, and the definition of the unit of polymerase activity, were as previously described (Wang et al., 1977). The processivity of dNMP incorporation by polymerase β was determined by the procedure of Das & Fujimura (1979); as those authors have reported, the micrococcal nuclease and spleen phosphodiesterase preparations (both from Worthington) are completely free of contaminating phosphatase or nucleotidase activities, as assayed with [^{3}H]dTTP by thin-layer chromatography. We have reported (Fisher et al., 1981) that with KB cell DNA polymerase α and activated DNA, measurements of processivity by this method give results essentially identical with those determined (Fisher et al., 1979) by the methodology of Bambara et al. (1978). All steady-state kinetics experiments were performed under initial velocity conditions; straight line analyses of the kinetics data were generated by the method of least squares. [The experimental rationale and methodology employed in our steady-state kinetics studies of the KB cell DNA polymerases are presented in detail in Fisher et al. (1981).]

Results

Human DNA Polymerase β Exhibits a Rigidly Ordered Sequential Mechanism of Substrate Addition. From their steady-state kinetics studies of a near-homogeneous preparation of murine DNA polymerase β, Tanabe et al. (1979) concluded that the enzyme demonstrated an ordered bi-bi mechanism of polymerization. That conclusion was derived from substrate kinetics patterns and product (PP_i) inhibition experiments and was, at least in part, based on the assumption that the incorporation of dNMPs by the polymerase was nonprocessive. Although the interpretation of an ordered sequential mechanism of substrate addition appears to be strongly supported by the data of Tanabe et al., the interpretation of the product release steps of the reaction is less secure, since the question of the processivity vs. the nonprocessivity of DNA polymerases β of diverse origins is somewhat controversial (Chang, 1975; Bambara et al., 1978; Matsukage et al., 1979; Das & Fujimura, 1979; also see later section of these Results), and observations may vary with different incubation conditions.

To confirm and extend these observations, we have examined the mechanism of substrate addition to KB cell polymerase β by using a dead-end competitive substrate inhibitor, ddTTP. This approach offers several theoretical advantages over product inhibition studies (particularly when product release steps are uncertain) both for discriminating random from ordered sequential mechanisms and for indicating whether an apparently ordered sequence is "rigidly ordered" (i.e., quantitatively the only available reaction pathway) or simply the consequence of a random mechanism with a relatively preferred binding order (Cleland, 1970; Fromm, 1979). The results of these experiments are displayed in Figure 1. As we (Fisher et al., 1979) and others (Edenberg et al., 1978; Waqar et al., 1978) have reported, ddTTP is a potent inhibitor of the polymerase β reaction that is linearly competitive with dTTP (Figure 1C); in contrast, the analogue is purely uncompetitive with respect to DNA primer-template, as analyzed by Lineweaver–Burk (Figure 1A) or Hanes (Figure 1B) plots. These patterns are most consistent with an ordered sequential mechanism of substrate addition (DNA followed by dNTP), as postulated for the murine enzyme, and they further suggest that this ordered sequence is quantitatively the most significant (or only) pathway of substrate addition to polymerase β.

Interaction of DNA Polymerase β with Intact Duplex DNA. We have recently documented the capacity of human DNA polymerase β to carry out a limited strand displacement reaction on nicked duplex DNA substrates, to the extent of incorporating an average of 15 nucleotides at each nick (Wang & Korn, 1980). Preliminary kinetic analysis had indicated that the apparent activation energy (E_a) of the polymerization reaction on nicked and gapped DNA was identical and that the enzyme appeared to demonstrate a measurable affinity only for 3′-primer termini (or primer moieties) but none for the duplex DNA backbone. To explore the implications of these initial observations in more detail, and to attempt kinetically to resolve the separate components of primer binding and template binding that might be involved in the polymerase–nucleic acid interaction (Fisher & Korn, 1981a,b), we examined the interaction of polymerase β with several classes of DNA molecules of defined structure.

The effect of intact duplex DNA molecules on the polymerase β reaction was tested with form IV (duplex circular relaxed) plasmid pACYC 184 DNA, which contains no ter-

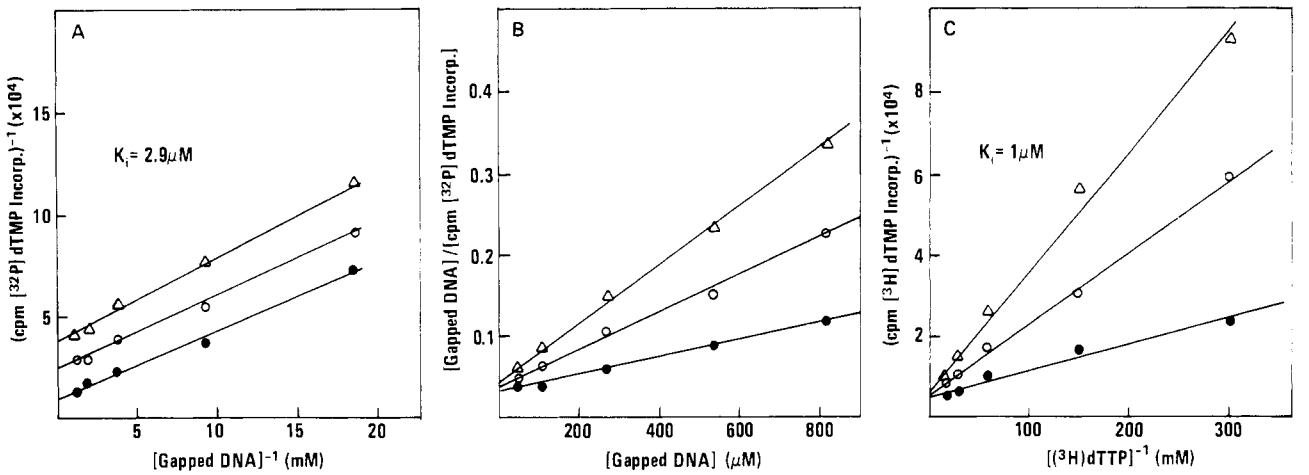

FIGURE 1: Steady-state kinetics analysis of the mechanism of substrate addition to DNA polymerase β. The panels show the patterns of inhibition produced by the competitive dead-end substrate inhibitor, ddTTP. Reactions (40 μL) contained 50 mM Tris-HCl, pH 8.9, 100 mM KCl, 300 μg/mL bovine serum albumin, 50 μM dNTP, 20 mM $MgCl_2$, and 0.5 unit/mL polymerase β and were incubated for 10 min at 35 °C. (A) Lineweaver–Burk plot, v^{-1} vs. $[DNA]^{-1}$. Concentrations of gapped DNA were as shown in reciprocal form on the abscissa; [α-^{32}P]dTTP was at 2020 cpm/pmol; ddTTP concentrations were (●) 0, (○) 4, and (△) 8 μM. (B) Hanes plot, S/v vs. S, of the data shown in panel A. (C) Lineweaver–Burk plot, v^{-1} vs. $[dTTP]^{-1}$. Reactions were formulated as in panel A, with gapped DNA at 540 μM (nucleotide). The concentrations of [^{3}H]dTTP (8730 cpm/pmol) were as indicated in reciprocal form on the abscissa; ddTTP concentrations were (●) 0, (○) 1, and (△) 2 μM.

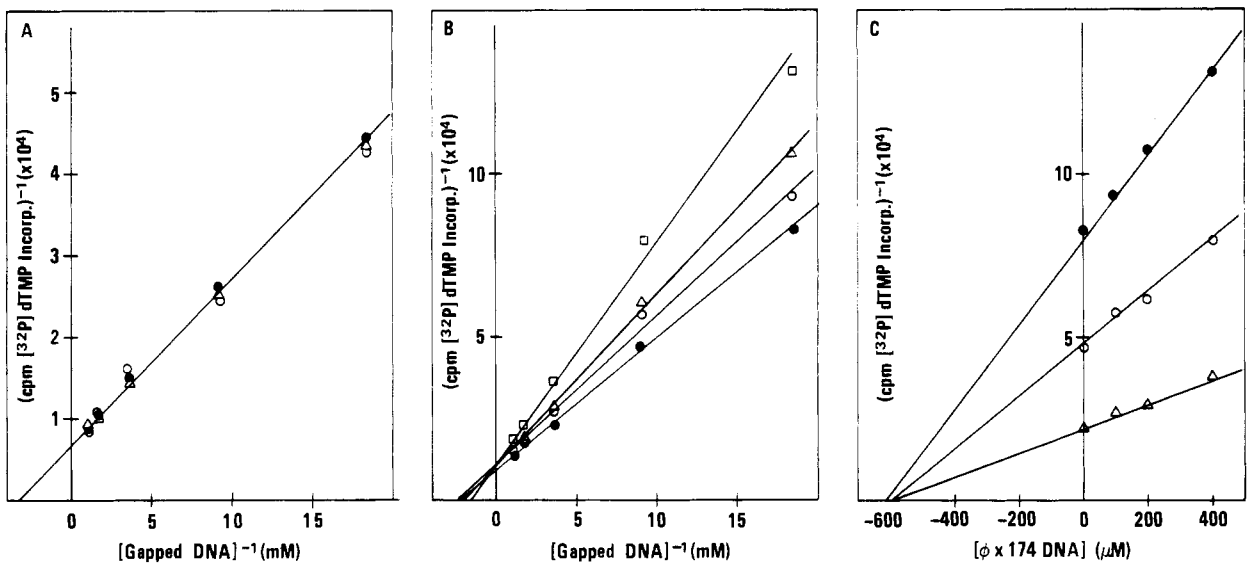

FIGURE 2: Kinetic analysis of the effect of intact duplex DNA (A) and of circular single-stranded φX174 DNA (B and C) on the utilization of DNA substrate by DNA polymerase β. (A) Lineweaver–Burk plot. The effect of *Hae*III-restricted (blunt-ended, duplex) PM2 DNA fragments. Reactions were formulated as in Figure 1A and contained [α-^{32}P]dTTP at 2030 cpm/pmol, gapped DNA (substrate) concentrations as indicated on the abscissa, and PM2 DNA at (●) 0, (○) 318, and (△) 636 μM (nucleotide). (B) Lineweaver–Burk plot. Reactions were formulated as in Figure 1A, with optimally gapped DNA substrate, [α-^{32}P]dTTP at 2420 cpm/pmol, and inhibitor φX174 DNA at (●) 0, (○) 100, (△) 200, and (□) 400 μM (nucleotide). Slope and intercept replots of the data in panel B yielded, respectively, values of the apparent K_{is} of 600 μM and of the apparent K_{ii} of 1600 μM. (C) Dixon plot. Reactions were formulated as in panel B, with concentrations of φX174 DNA as indicated on the abscissa, and with gapped substrate DNA at (●) 54, (○) 180, and (△) 270 μM (nucleotide).

mini (data not shown), and with a *Hae*III restriction digest of PM2 DNA (Figure 2A), which is comprised of a mixture of 15 blunt-ended 3′-OH- and 5′-PO_4-terminated linear duplex fragments that range from ≤100 to approximately 1900 base pairs in length (Fisher & Korn, 1979a). Both populations of duplex DNA molecules were without detectable effect on the polymerization reaction with gapped DNA substrate, whether tested in the presence of Mg^{2+} or Mn^{2+} (not shown) as divalent cation. These observations confirm the interpretation of our earlier study of the polymerization reaction on nicked DNA, and they further demonstrate the ability of polymerase β to distinguish between internal (nick) and flush-ended 3′-OH termini, both of which may be assumed, on a simple first approximation, to present comparable base-paired configurations. This discriminatory capacity contrasts with the behavior of *E. coli* DNA polymerase I, which has been shown (Erglund et al., 1968) to bind readily to the ends of duplex DNA molecules.

Interaction of DNA Polymerase β with Single-Stranded DNA. The strong (and cooperative) interaction of KB cell DNA polymerase α with single-stranded natural and synthetic DNA molecules provided an important clue to the resolution of the ordered sequential mechanism of substrate addition to that enzyme (Fisher & Korn, 1979a, 1981a). Accordingly, we examined the effects of a variety of single-stranded polydeoxynucleotides [circular φX174 DNA and linear denatured fragments of KB DNA, $(dA)_{\overline{100}}$, $(dT)_{\overline{100}}$, $(dC)_{\overline{100}}$, and $(dA, dG, dC)_{\overline{51}}$ as well as its dideoxyterminated homologue] on the reactivity of polymerase β with optimally gapped DNA sub-

strate. The results in Figure 2B,C demonstrate, in sharp contrast to the findings with polymerase α (Fisher & Korn, 1979a), that single-stranded, circular ϕX174 DNA produces only a very slight inhibition of the polymerase β reaction, and the patterns obtained by Lineweaver–Burk (Figure 2B) and Dixon (Figure 2C) plots indicate that the mechanism of inhibition is fully and linearly noncompetitive with DNA substrate. The values of the apparent K_{is} and K_{ii}, derived from linear slope and intercept replots of the data in Figure 2B, suggest that under these reaction conditions single-stranded circular DNA has a very low apparent affinity for the enzyme and that the affinity for free polymerase (K_{is} = 600 μM) is about 3-fold greater than that for the polymerase·DNA substrate complex (K_{ii} = 1600 μM). Very similar results were obtained with linear denatured KB DNA fragments averaging 1700 nucleotides in length; thus, again in contrast to the results with polymerase α (Fisher & Korn, 1979a,b), the presence of potentially base-pairable (by fold back) 3′-OH termini appeared not to enhance the affinity of polymerase β for single-stranded DNA.

When the inhibitory effects of ϕX174 DNA were examined as a function of dNTP concentration, both at subsaturating and saturating concentrations of substrate DNA (the first substrate in the ordered bi mechanism), the patterns obtained from Lineweaver–Burk plots were again consistent with a fully linear noncompetitive mechanism of inhibition with respect to dNTPs. The intersection of the lines on the abscissa indicated that under these conditions $K_{is} \approx K_{ii}$ and suggested that the kinetically estimated affinity of single-stranded DNA for E·DNA (substrate) and E·DNA·dNTP complexes was very similar.

The effects of a variety of reasonably well-defined (Fisher et al., 1981) linear synthetic polydeoxynucleotides on the polymerase β reaction were closely analogous to those observed with natural, single-stranded heteropolymeric DNA molecules. Thus, for example, $(dA)_{\overline{100}}$ produced only very slight inhibition under these reaction conditions. The pattern obtained from Lineweaver–Burk analysis indicated that the mechanism of inhibition was fully and linearly noncompetitive with the DNA substrate, and the calculated values of the apparent K_{is} (2600 μM) and the apparent K_{ii} (3620 μM) again suggested a slightly greater kinetic affinity of the single-stranded inhibitor for free enzyme (E) than for the E·DNA (substrate) complex. Entirely similar results were obtained with the inhibitor molecules $(dT)_{\overline{100}}$ and $(dC)_{\overline{100}}$, as well as with (dA, dG, dC)$_{\overline{51}}$ and its dideoxyterminated homologue.

We (Wang et al., 1975, 1977) and others [for example, Change (1974)] have previously described the brisk reactivity of DNA polymerase β on a variety of synthetic homopolymer primer-templates, but under optimum incubation conditions that differ from those required with activated DNA. For example, with KB cell polymerase β, maximum activity with synthetic $(dA)_m \cdot (dT)_n$ substrates is obtained at pH 7.5 with 5 mM Mg^{2+}; the conditions used in the present experiments were optimized for the DNA substrate and employed pH 8.9 and 20 mM Mg^{2+} [all other reaction components are identical (Wang et al., 1977)]. Thus, in spite of the almost identical behavior of the several natural and synthetic single-stranded polydeoxynucleotide inhibitors, it was important to document the capacity of polymerase β to replicate synthetic primer-templates under incubation conditions identical with those used in the inhibition studies. With the primer-template $(dA)_{\overline{100}} \cdot (dT)_{\overline{12}}$, the polymerization reaction demonstrated simple saturation kinetics and yielded an apparent K_m value of 910 μM (expressed in terms of template nucleotide concentration). Although the apparent affinity of the polymerase for this primer-template was greatly reduced under these nonoptimal conditions [at pH 7.5, 5 mM Mg^{2+}, the apparent K_m of polymerase β for poly(dA)·$(dT)_{\overline{16}}$ is about 7 μM (nucleotide) (Wang et al., 1977)], it was nonetheless significantly higher than that for the $(dA)_{\overline{100}}$ inhibitor, and the polymerization reaction on the synthetic substrate was readily demonstrable.

Finally, when Mn^{2+} (1.6 mM) was substituted for Mg^{2+} (20 mM) under otherwise identical conditions, the patterns of inhibition produced by ϕX174 DNA, $(dT)_{\overline{100}}$, and $(dA)_{\overline{100}}$, were qualitatively unchanged; i.e., they continued to be fully and linearly noncompetitive with respect to the DNA substrate. However, in the presence of Mn^{2+}, the apparent affinity of the polymerase for these inhibitor molecules was substantially increased; thus, K_m (substrate DNA) = 2.7 μM; ϕX174 DNA, K_{is} = 8.5 μM, K_{ii} = 36 μM; $(dA)_{\overline{100}}$, K_{is} = 380 μM, K_{ii} = 1280 μM. [These changes in the kinetic affinity for single-stranded polydeoxynucleotides that are noncompetitive either with DNA or with dNTP substrates suggest that the dramatic enhancement of the apparent affinity of polymerase β for primer-templates in the presence of Mn^{2+} (Wang et al., 1977) may be due to relatively nonspecific changes in the polymerase (and/or nucleic acid) rather than to specific alterations in the active center of the enzyme.] We conclude that although the relative affinity of polymerase β for different natural and synthetic single-stranded polydeoxynucleotide inhibitors may vary significantly with incubation conditions, the pattern of inhibition (linear noncompetitive) that is produced by these molecules is internally consistent and remarkably invariant.

Interaction of DNA Polymerase β with Nicked Duplex DNA. The kinetics of the interaction of DNA polymerase β with nicked duplex DNA are demonstrated in the experiments that are summarized in Figure 3. Duplex, linear calf thymus DNA molecules that contained an average of one 3′-OH-terminated nick per 1700 nucleotides (see Materials and Methods) were tested both as substrates (Wang & Korn, 1980) and as alternate product inhibitors of the polymerization reaction, in the presence of Mg^{2+} and Mn^{2+} as divalent cations. As substrates, these nicked DNA molecules exhibited simple saturation kinetics (data not shown) and yielded apparent K_m values in Mg^{2+} of 100 μM (nucleotide) and 60 nM (3′-OH termini) and in Mn^{2+} of 8 μM (nucleotide) and 4 nM (3′-OH termini). These results are entirely in accord with our previous reports (Wang et al., 1977; Wang & Korn, 1980). When the same population of nicked DNA molecules was added to polymerization reactions containing optimally gapped DNA (Figure 3A,B), they behaved as linearly competitive inhibitors with respect to primer-template, and values of the apparent K_i, as well as their response to the divalent cations Mg^{2+} and Mn^{2+}, were identical with those of the apparent K_m.

When a population of duplex calf thymus DNA molecules that contained an average of one 3′-PO_4-terminated nick per 2870 nucleotides (see Materials and Methods) was tested as dead-end inhibitors in reactions containing gapped DNA, the results obtained (Figure 3C,D) were qualitatively identical with those observed with the 3′-OH-terminated, nicked molecules. Values of the apparent K_i were again responsive to the divalent cation and measured 293 μM (nucleotide) and 100 nM (3′-PO_4 termini) in Mg^{2+} and 30 μM (nucleotide) and 10 nM (3′-PO_4 termini) in Mn^{2+}.

These results document, in sharp contrast to the observations with KB cell polymerase α (Fisher & Korn, 1979a,b), both

Table I: Comparison of Primer-Template Capacity of Specific Staggered-End DNA Molecules[a]

primer-template	mean no. of nucleotides per staggered end	app K_m (μM nucleotide)	V_{max} (cpm/ 10 min)
optimally gapped DNA		200–400	13900
nicked DNA		100	960
*Hin*dIII-restricted PM2 DNA	4		
exo III resected, *Hin*dIII-restricted PM2 DNA			
substrate A	5	930	8900
substrate B	6	830	9100
substrate C	7	730	9100

[a] Reactions were formulated as in Figure 1A, with [^{3}H]dATP, [^{3}H]dGTP, [^{3}H]dTTP, and [^{3}H]dCTP each at 50 μM and 8400 cpm/pmol. Incubations were for 10 min at 35 °C. The specific DNA substrates were prepared and characterized as described under Materials and Methods. Kinetic parameters were calculated from Lineweaver–Burk plots by the method of least squares.

the very high affinity of polymerase β for nicked duplex DNA and the ability of the enzyme to bind with roughly comparable avidity[1] to 3′-OH and 3′-PO_4 nick termini.

Interaction of DNA Polymerase β with Staggered-End DNA. The data presented thus far suggested that a base-paired primer moiety was a primary determinant of catalytically productive binding of polymerase β to DNA substrates. Although the results obtained with duplex intact, duplex blunt-ended, and single-stranded DNA molecules provided no clues that might have helped to resolve partial reaction steps of template binding and primer binding, the ability of the polymerase to discriminate internal nicks from flush-end termini argued that some additional structural elements in the nucleic acid substrate were required; i.e., that the primer moiety, although necessary, was not sufficient.

To pursue this problem, we examined the interaction of the polymerase with *Hin*dIII-restricted PM2 DNA. The restriction digest is comprised of a mixture of seven duplex fragments, ranging from ~100 to 5000 base pairs in length; each fragment contains 5′-PO_4-terminated, four-nucleotide-staggered ends. In the presence of Mg^{2+} as divalent cation, these fragments were inactive as primer-templates, and they had no detectable inhibitory effect on the polymerization reaction with gapped DNA (Figure 4A); i.e., these molecules were kinetically indistinguishable from blunt-ended duplex fragments. The *Hin*dIII-restricted PM2 DNA fragments were then subjected to very limited resection with exonuclease III to produce populations of staggered-end fragments containing 5′-terminal single-stranded template lengths that ranged from four nucleotides (original population) to an average of five, six, and seven nucleotides, respectively. When these resected molecules were tested for primer-template capacity with polymerase β, we obtained the results shown in Table I. Although the initial restriction fragments were kinetically inert, fragments that had been only minimally resected and contained staggered ends of five to seven nucleotides proved to be surprisingly good substrates that exhibited simple saturation

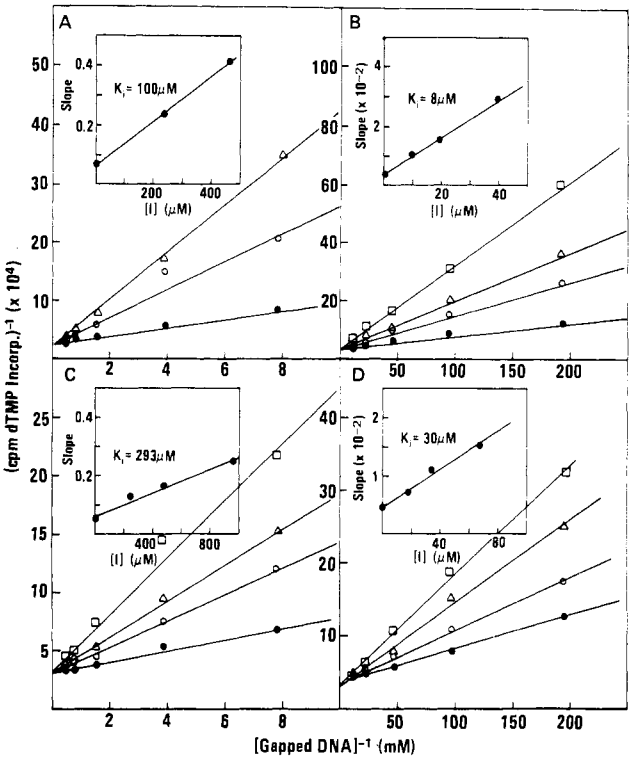

FIGURE 3: Kinetic analysis of nicked duplex DNA molecules as inhibitors of DNA polymerase β. The data are displayed in Lineweaver–Burk plots. (A) Inhibition of polymerase β by 3′-OH nicked DNA in the presence of Mg^{2+}. Reactions were formulated as in Figure 1A, with [^{3}H]dTTP at 930 cpm/pmol, gapped (substrate) DNA concentrations as indicated on the abscissa, and pancreatic DNase I nicked DNA at (●) 0, (○) 233, and (△) 466 μM (nucleotide). The data were calculated by subtracting from each set of samples the amount of dNMP incorporation that was measured in the presence of nicked DNA alone from that determined in the presence of optimally gapped DNA substrate. In reactions with Mg^{2+}, the magnitude of the correction was ~10%; with Mn^{2+}, ~30%. (B) Inhibition of polymerase β by 3′-OH nicked DNA in the presence of Mn^{2+}. Reactions were formulated as in panel A, with [α-^{32}P]dTTP at 4010 cpm/pmol and 1.6 mM $MnCl_2$ in place of $MgCl_2$; nicked DNA was present at (●) 0, (○) 10, (△) 20, and (□) 40 μM (nucleotide). (C) Inhibition of polymerase β by 3′-PO_4 nicked DNA in the presence of Mg^{2+}. Reactions were formulated as in panel A, with [^{3}H]dTTP at 1350 cpm/pmol, gapped DNA as indicated on the abscissa, and micrococcal nuclease nicked DNA at (●) 0, (○) 243, (△) 485, and (□) 920 μM (nucleotide). (D) Inhibition of polymerase β by 3′-PO_4 nicked DNA in the presence of Mn^{2+}. Reactions were formulated as in panel B, with [α-^{32}P]dTTP at 4010 cpm/pmol, gapped DNA as indicated on the abscissa, and 3′-PO_4 nicked DNA at (●) 0, (○) 17, (△) 34, and (□) 67 μM (nucleotide). In all panels, the inserts show slope replots of the primary data, from which values of the apparent K_i were computed.

[1] It is not possible to compare quantitatively the apparent affinity of polymerase β for 3′-OH vs. 3′-PO_4 nick termini from the inhibitor studies presented in Figure 3. Aside from the uncertainties inherent in the estimation of nick densities in the two populations of DNA molecules (see Materials and Methods), the values of the apparent K_i determined with these two populations are themselves not directly comparable. Thus, 3′-PO_4 nicked DNA molecules are dead-end inhibitors, and values of the apparent K_i should reflect true dissociation constants (K_D). In contrast, the 3′-OH nicked DNA molecules are alternate product inhibitors, and values of the apparent K_i must contain downstream rate constants in addition to the on-rate and off-rate constants that characterize enzyme–DNA binding. In terms of a simplified Michaelis–Menten formalism:

$$E + I(3'\text{-}PO_4) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \quad (1)$$

$$E + I(3'\text{-OH}) \underset{k_{2'}}{\overset{k_{1'}}{\rightleftharpoons}} E{\cdot}I \xrightarrow{\text{“}k_3\text{”}} E + (\text{alternate})P \quad (2)$$

(where “k_3” represents all of the several individual rate constants between E·S and E + P). In case 1 (3′-PO_4), app $K_i = k_2/k_1$. In case 2 (3′-OH), app $K_i = k_{2'} + \text{“}k_3\text{”}/k_{1'}$, and we do not know the relative magnitudes of the constants $k_{1'}$, $k_{2'}$, and “k_3” [although in the case of the polymerization reaction on nicked DNA substrates, there is reason to believe that the “k_3” terms may be small (Wang & Korn, 1980)]. In spite of this uncertainty, it is evident that polymerase β does in fact exhibit substantial affinity for 3′-PO_4-terminated nicks, with K_D values of the order of 10–100 nM, depending on the divalent cation; what is not known is the absolute binding affinity of the enzyme for 3′-OH termini.

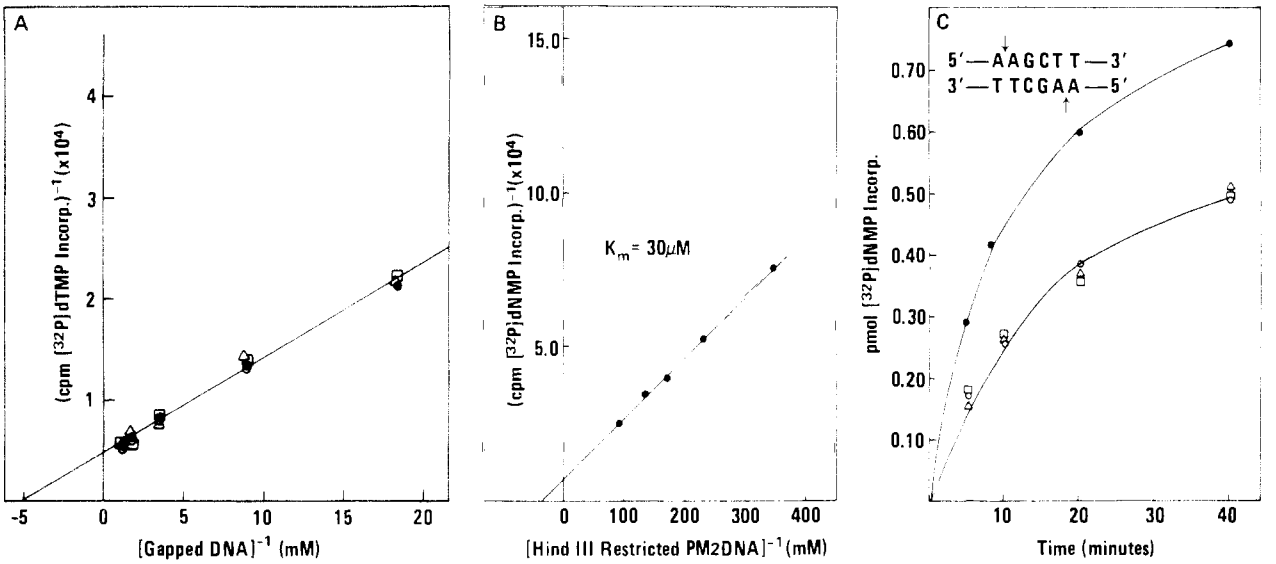

FIGURE 4: Interaction of DNA polymerase β with duplex DNA molecules that contain four-nucleotide staggered ends. (A) Effect of *Hin*dIII-restricted PM2 DNA on the polymerization reaction with optimally gapped DNA substrate in Mg^{2+}. The data are displayed in a Lineweaver–Burk plot. Reactions were formulated as in Figure 1A, with [α-^{32}P]dTTP at 4660 cpm/pmol, gapped (substrate) DNA as indicated on the abscissa, and *Hin*dIII-restricted PM2 DNA at (●) 0, (○) 217, (△) 434, and (□) 868 μM (nucleotide). (B) Utilization of *Hin*dIII-restricted PM2 DNA as primer-template by polymerase β in the presence of Mn^{2+}. The panel shows a Lineweaver–Burk plot. Reactions were formulated as in Figure 3B, with all four [α-^{32}P]dNTPs at 21 420 cpm/pmol, 1.5 units of polymerase β per mL, and *Hin*dIII-restricted DNA (substrate) as indicated on the abscissa. (C) Estimation of minimal effective template length that can be copied by polymerase β in Mn^{2+}. Four separate reactions (110 μL each) were formulated as in Figure 3B, with 1.5 units/mL polymerase β, 25 μM each of the four dNTPs, and 11 μM (nucleotide) *Hin*dIII-restricted PM2 DNA. A different labeled dNTP was added to each reaction at a final specific activity of 7200 cpm/pmol: (●) [α-^{32}P]dATP; (○) [α-^{32}P]dTTP; (□) [α-^{32}P]dGTP; (△) [α-^{32}P]dCTP. After 0, 5, 10, 20, and 40 min of incubation at 36 °C, an aliquot (20 μL) was removed from each reaction for assay of dNMP incorporation. The *Hin*dIII staggered-end sequence is depicted in the upper left corner of the panel.

kinetics. The affinity of these fragments for polymerase β was relatively poor, but V_{max} values were ~65% of that measured with optimally gapped DNA. These data suggested that in the presence of Mg^{2+}, catalytically productive binding of polymerase β to DNA might require a 3′-primer stem adjacent to a short length of single-stranded template of greater than or equal to five nucleotides.

In contrast to above, the presence of Mn^{2+} as divalent cation permitted the original *Hin*dIII-restricted PM2 DNA fragments to be utilized as effective primer-templates by polymerase β (Figure 4B). The polymerization reaction again exhibited simple saturation kinetics; the apparent K_m of 30 μM (nucleotide) or 23 nM (3′-OH termini) was from 3- to 6-fold higher than values obtained with 3′-OH nicked or gapped DNAs, but the V_{max} was in fact comparable to that measured with optimally gapped substrate. The results in Figure 4B suggested that in Mn^{2+} polymerase β might be capable of completely filling in staggered-end DNA substrates; they also indicated that the minimum template length required for catalytically productive binding of the enzyme appeared to be strongly modulated by the choice of divalent cation.

To clarify these questions, we first used the *Hin*dIII restriction fragments as primer-templates in four separate incubations, each containing a different labeled dNTP. The rationale was based on the known sequence of the four-nucleotide stagger (template), viz., T-C-G-A. The results (Figure 4C) demonstrated that all four dNMPs could be incorporated. Although the rate of incorporation of the *first* product nucleotide, dAMP, was somewhat greater than those of the other three dNMPs, the important observation was that incorporation of the *last* product nucleotide, dTMP, was readily detectable, and at a rate indistinguishable from those of the penultimate nucleotides, dGMP and dCMP. These data thus provided strong support for the conclusion that in the presence of Mn^{2+}, polymerase β was capable of accomplishing the complete repair of staggered-end DNA primer-templates.

Documentation of Minimum Effective Template Length and Extent of Incorporation of DNA Polymerase β on Staggered-End DNA Substrates. To resolve conclusively the questions of the divalent cation effect on minimum template length and the extent of deoxynucleotide incorporation on staggered-end primer-templates, we devised the experiments that are outlined in Figure 5 and presented in Figure 6. The principle of the experiments was to use as polymerase substrate a sequenced DNA molecule, pBR 322, that was known to contain single *Eco*RI and *Hin*dIII restriction sites that are separated by a distance of 29 nucleotides (Figure 6C, lanes 1 and 6) (Sutcliffe, 1978).

Primer-template molecules were prepared by first restricting pBR 322 DNA with *Hin*dIII to convert the closed-circular molecules to full-length linears that contained four-nucleotide staggered ends and then gently resecting these molecules from their 3′-OH termini with exonuclease III, thereby generating a population of staggered-end molecules, each of which contained a single *Eco*RI site 29 nucleotides upstream from the original *Hin*dIII cleavage site. From the known distributivity of exonuclease III (Thomas & Olivera, 1978), these DNA molecules were expected to represent an approximately Poisson distribution of template lengths (Figure 6C, lane 3). This DNA preparation was used as primer-template in reactions with polymerase β that were allowed to reach extent, either with Mg^{2+} (Figure 6A) or with Mn^{2+} (Figure 6B) as divalent cation. The polymerization products *at extent*, after restriction with *Eco*RI to release the product chains of interest (i.e., the single population of fragments originally bounded by the *Eco*RI and *Hin*dIII sites), were then analyzed by high-resolution polyacrylamide gel electrophoresis (Maxam & Gilbert, 1977). [Note that polymerase products synthesized from the primer terminus on the opposite DNA strand are not released by *Eco*RI. They remain covalently linked to the large pBR 322 DNA molecules and fail to enter the gel. A similar fate befalls reaction products synthesized at random nick sites that

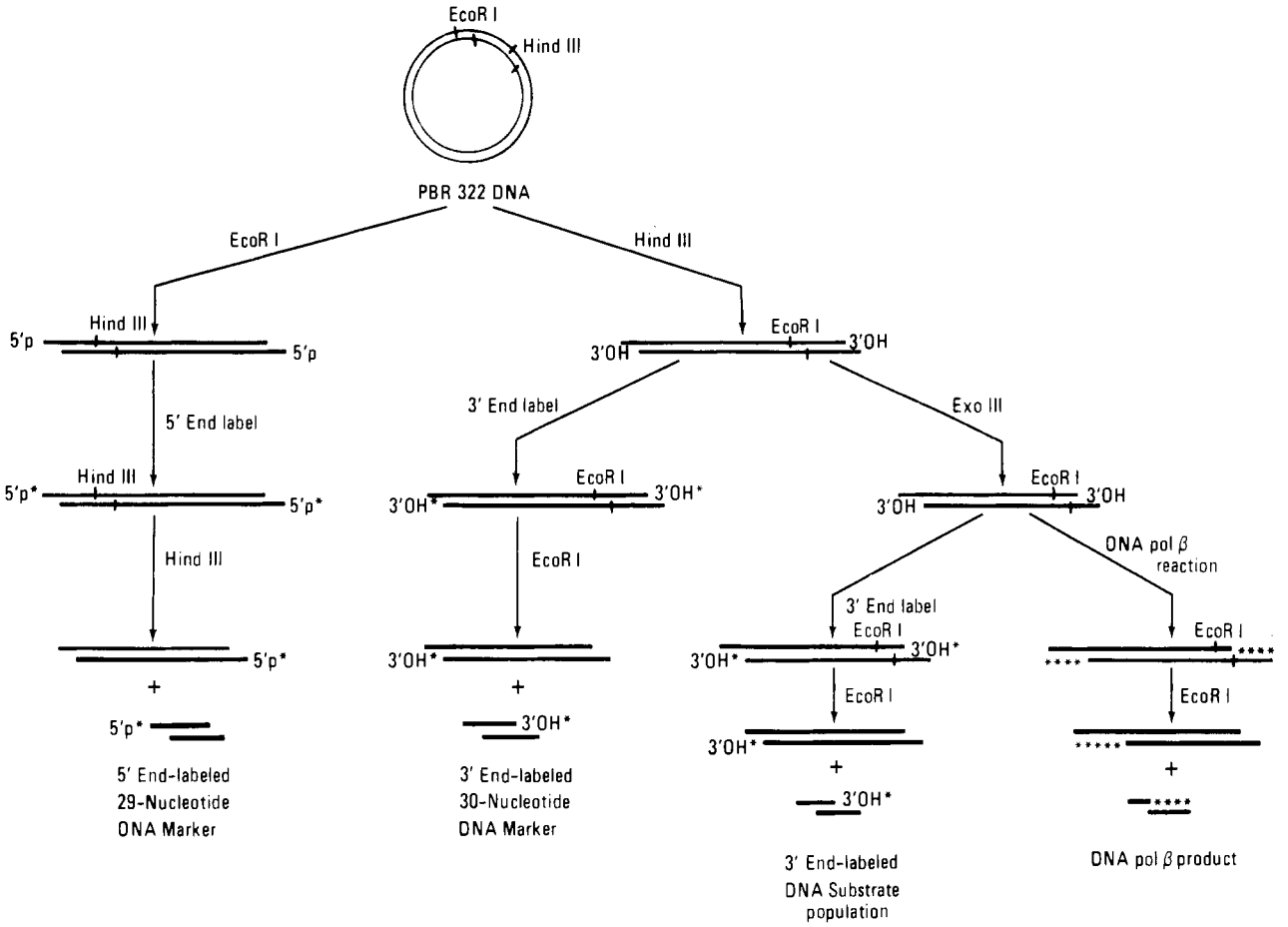

FIGURE 5: Schematic outline of experimental protocol designed to determine the minimum effective template length and the extent of incorporation by DNA polymerase β on staggered-end duplex pBR 322 DNA primer-templates.

might inadvertently have been introduced into the pBR 322 DNA during preparation of the primer-template (note radioactivity at the tops of lanes 4 and 5 in Figure 6C).]

The experimental results are displayed in Figure 6A,B and lanes 4 and 5 of Figure 6C. In the presence of Mg^{2+} (Figure 6A), the polymerization reaction reached extent after an average of 8.3 dNMPs had been incorporated per initial 3′-primer terminus. When these extent products were subjected to gel analysis (Figure 6C, lane 4), the only labeled chains detected were those between 26 and 30 nucleotides in length. The most abundant products were the 27-mer and the 29-mer; only minor amounts of 26-mer and 30-mer were present, and no fragments longer than 30 nucleotides were seen. In incubations performed with Mn^{2+} (Figure 6B), the polymerization reaction reached extent after an average of 12 nucleotides had been incorporated per initial primer terminus. Gel analysis of these products (Figure 6C, lane 5) revealed only two fragments, 32 and 33 nucleotides long, with the longer fragment the more abundant product.

Since the theoretical maximum length of product chain that can be synthesized in these reactions is 33 nucleotides (i.e., the distance between the 5′ boundary of the *Eco*RI cut on the *primer strand* and the 5′ boundary of the *Hind*III cut on the *template strand*), it is clear that in the presence of Mn^{2+} DNA polymerase β is capable of filling in a staggered-end primer-template completely. The results displayed in lane 5 of Figure 6C are in excellent agreement with those presented in Figure 4B,C, and they support the interpretation that with Mn^{2+} as cation the minimum template length required by polymerase β is of the order of a *single* nucleotide. In sharp contrast, with Mg^{2+} as divalent cation, polymerase β is unable to copy the terminal three or four nucleotides of a staggered-end template. Again, the results shown in lane 4 of Figure 6C are entirely consistent with the kinetic data presented in Figure 4A and Table I, and they permit the interpretation that in the presence of Mg^{2+} polymerase β requires a minimum template length of at least five nucleotides for catalytically productive binding to DNA.

Processivity of Human DNA Polymerase β. Several workers have addressed the question of the processivity of DNA polymerases β of diverse origins and have arrived at contradictory conclusions. When different reaction conditions and very different methods of analysis were used, reported values of processivity for this enzyme have varied between 1 (i.e., a nonprocessive mechanism of catalysis) (Chang, 1975; Bambara et al., 1978; Matsukage et al., 1979) and 9–10 (i.e., a moderately processive mechanism of catalysis) (Das & Fujimura, 1979). In light of this disagreement, and in order to further assess the effects of Mg^{2+} and Mn^{2+} on the catalytic properties of human polymerase β, we have examined the processivity of the KB enzyme on nicked and gapped DNA substrates in the presence of these two divalent cations. The results (Table II) demonstrate that the processivity of human polymerase β on nicked and gapped DNA substrates is very similar, if not identical. In the presence of Mg^{2+}, polymerization is essentially distributive, with an average of one or two nucleotides incorporated per binding cycle. In the presence of Mn^{2+}, however, the mechanism of polymerization becomes modestly processive, with an average incorporation of four to six nucleotides per cycle.

Discussion

The experiments described in this paper provide new insights into the mechanism of catalysis of human DNA polymerase

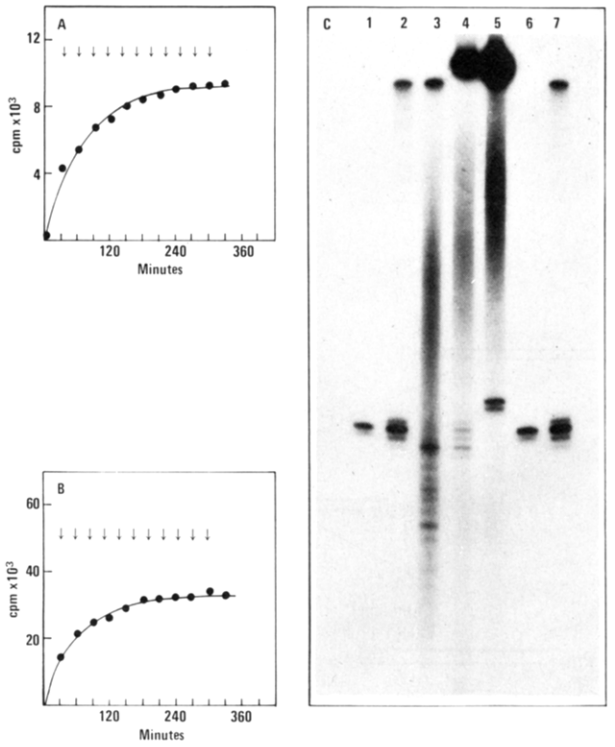

FIGURE 6: Determination of minimum effective template length and the extent of incorporation by DNA polymerase β on staggered-end duplex DNA primer-templates. (A) Extent of dNMP incorporation on *Hin*dIII-restricted, exonuclease III resected pBR 322 DNA with Mg^{2+} as divalent cation. The experimental procedure is described under Materials and Methods and is schematically outlined in Figure 5. Every 30 min (↓), fresh incubation mixture containing 12 units/mL polymerase β, but no primer-template, was added to the reaction until a stable extent of dNMP incorporation had been reached. (B) Extent of dNMP incorporation on *Hin*dIII-restricted, exonuclease III resected pBR 322 DNA with Mn^{2+} as divalent cation. The experimental procedure is described under Materials and Methods. The arrows (↓) indicate the addition of fresh enzyme to the reaction as in panel A. (C) Polyacrylamide gel autoradiographic analysis of the products synthesized by polymerase β *at extent* on the pBR 322 DNA molecules described in panels A and B. The preparation of 5′ end labeled and 3′ end labeled marker DNA fragments, and of reaction products, is described under Materials and Methods and is schematically outlined in Figure 5. (Lanes 1 and 6) 5′-^{32}P-Labeled, *Eco*RI- and *Hin*dIII-restricted marker fragments, 29 nucleotides long. (Lanes 2 and 7) Cordycepin 3′-[^{32}P]phosphate labeled, *Eco*RI- and *Hin*dIII-restricted marker fragments, 27–30 nucleotides long. (Lane 3) *Hin*dIII-restricted, exonuclease III resected pBR 322 DNA molecules were 3′ end labeled with cordycepin [^{32}P]phosphate (see Materials and Methods) and then further restricted with *Eco*RI to generate a ladder that reflects the Poisson distribution of mean fragment lengths that results from exonuclease III digestion. The *Hin*dIII-restricted, exonuclease III resected DNA molecules comprise the primer-template population that was used in the experiments in panels A and B which are analyzed in lanes 4 and 5. (Lane 4) Polymerase β products after 330 min of incubation with Mg^{2+} as cation (panel A), followed by restriction with *Eco*RI. (Lane 5) Polymerase β products after 330 min of incubation with Mn^{2+} as cation (panel B), followed by restriction with *Eco*RI. The autoradiogram was exposed for 3 days.

β and demonstrate a number of profound differences between KB cell DNA polymerases α and β with respect to the molecular signals that appear to govern their specific interactions with nucleic acid substrates. In performing these studies, we have continued to exploit the power of classical steady-state kinetics (Fisher & Korn, 1979a,b, 1981a,b; Fisher et al., 1981) to suggest features of polymerase–nucleic acid binding interactions that cannot presently be attacked by more direct physicochemical methods because of the extreme limitation of homogeneous enzyme proteins. By employing DNA molecules of known sequence and novel methods of polymerization product analysis, we have, as in the earlier studies, been able to obtain unambiguous corroboration of the principal interpretations derived from the kinetics experiments and thereby alleviate at least some of the legitimate concerns that arise from the indirect nature of kinetics studies, particularly in complex systems. From the data presented here, we can identify the following major features of the catalytically productive polymerase β–nucleic acid binding reaction.

Table II: Processivity of DNA Polymerase β[a]

primer-template	divalent cation	processivity
gapped DNA	Mg^{2+}	1.9
	Mn^{2+}	5.6
nicked DNA	Mg^{2+}	1.5
	Mn^{2+}	3.7

[a] Polymerase processivity was measured by the method of Das & Fujimura (1979). Reaction conditions were as described in Figures 1A (Mg^{2+}) and 6B (Mn^{2+}). Incubations (50 μL) contained either 2.5 mM (nucleotide) gapped DNA (~65 pmol of usable 3′-primer termini per incubation) or 2.5 mM (nucleotide) nicked DNA (~63 pmol of usable primer termini per incubation). In reactions with Mg^{2+}, each of the four [^{3}H]dNTPs was present at 50 μM and 40000 cpm/pmol; ~7 pmol of [^{3}H]dNMP was incorporated into gapped DNA products and ~2.6 pmol into nicked DNA products for analyses. In reactions with Mn^{2+}, each of the four [^{3}H]dNTPs was present at 50 μM and 31600 cpm/pmol; ~7.5 pmol of [^{3}H]dNMP was incorporated into gapped DNA products and ~3 pmol into nicked DNA products for analyses.

(1) Like polymerase α, human DNA polymerase β has no kinetically detectable affinity for intact duplex DNA molecules, whether covalently closed circles or flush-ended linear fragments.

(2) Under the reaction conditions used in these studies (optimized for gapped DNA substrate with Mg^{2+} or Mn^{2+}), polymerase β exhibits only a relatively low apparent affinity for single-stranded natural or synthetic polydeoxynucleotide inhibitors. That affinity is not enhanced by the presence of potentially base-pairable 3′-OH termini (although it does vary with the choice of the divalent metal cation), and the pattern of inhibition produced by these polymers under all conditions tested is fully and linearly noncompetitive with substrate DNA (or with dNTPs). *In each of these respects*, the kinetics of the interaction between polymerase β and the single-stranded deoxypolymers are dramatically different from those exhibited by KB cell polymerase α (Fisher & Korn, 1979a,b; Fisher et al., 1981).

(3) DNA polymerase β, but not polymerase α, can perform a limited strand-displacement synthesis on nicked duplex DNA molecules (Wang & Korn, 1980). We have demonstrated in this paper the extremely high affinity of polymerase β for such molecules, the ability of 3′-PO_4-terminated nicked DNA to behave as a linearly competitive inhibitor with respect to gapped DNA substrate, and the roughly comparable affinity of the enzyme for nick sites bearing 3′-OH or 3′-PO_4 residues. This affinity is on the order of $\leq$10 nM (expressed in terms of nicks). Again in striking contrast, KB cell polymerase α has no detectable affinity for nicked duplex DNA, whether assessed kinetically or by direct sedimentation binding assays (Fisher & Korn, 1979a); moreover, the presence of a 3′-terminal PO_4 group blocks primer binding by polymerase α (Fisher & Korn, 1979a, 1981b).

In the studies summarized above, it had been anticipated that the kinetic patterns of the polymerase β–nucleic acid interactions might help to resolve separate partial reactions of primer binding and template binding and thereby permit a more complete mechanistic description of the reaction of the

polymerase with DNA. However, the sought for clues did not emerge. Although the data strongly suggested that a base-paired primer moiety was an important signal for polymerase–DNA binding, the indifference of the enzyme to blunt-ended DNA termini indicated that some nucleic acid structural elements in addition to the primer stem must be involved, yet the patterns of the polymerase–single-stranded polydeoxynucleotide interaction did not provide kinetic evidence of a discrete template-binding step.

In an effort to resolve this paradox, we performed a detailed study of the ability of the polymerase to recognize staggered-end DNA molecules, with the intention of defining a minimum template length that might be required for catalytically productive binding. The data presented in Figures 4 and 6 and Table I clearly demonstrate that in the presence of Mg^{2+} polymerase β appears to require a minimum template length of greater than or equal to five nucleotides. Thus, the enzyme cannot recognize DNA molecules with four-nucleotide staggered ends (*Hin*dIII-restriction digest), whether tested as polymerization substrates or inhibitors; however, minimal resection of these molecules with exonuclease III, to produce staggered ends averaging five to seven nucleotides in length, converts the molecules into acceptable substrates that yield V_{max} values >50% of those obtained with optimally gapped DNA. Finally, when polymerization was carried out to extent on a staggered-end DNA population containing a Poisson distribution of template lengths (L), $4 \leq L \leq 33$ nucleotides, the only product chains detected on high-resolution polyacrylamide gels were those between 26 and 30 nucleotides in length. The predominant products were the 27-mer and the 29-mer, and only trace quantities of the 26-mer and the 30-mer were observed.

When these same experiments were carried out in the presence of Mn^{2+} (Figures 4 and 6), dramatic and unanticipated differences were observed. Thus, the four-nucleotide staggered-end DNA molecules produced by *Hin*dIII restriction were now acceptable substrates for polymerase β, and by using different labeled dNTPs corresponding to the known sequence of the *Hin*dIII restriction site (template), it appeared that the polymerase was capable of repairing these staggered-end molecules completely. This interpretation was confirmed in the experiments displayed in Figure 6, in which only two product chains were detected, a predominant species 33-nucleotides long, corresponding to a full-length template copy, and a less abundant 32-mer. These results, together with the complete lack of reactivity of polymerase β with blunt-ended DNA molecules in Mn^{2+}, support the conclusion that in reactions containing Mn^{2+} the minimum template length required for catalytically productive binding of the polymerase to DNA is of the order of a single nucleotide. On the basis of these observations, it is reasonable to suggest that the high avidity of KB cell polymerase β for nicked duplex DNA molecules must likely reflect the ability of this small basic protein to accomplish limited melting of the DNA helix at nick sites, sufficient to expose the short length of single-stranded template that is required for binding. [In this regard, it is of interest to recall that the E_a of the polymerase β reaction on nicked and gapped DNA substrates is identical (Wang & Korn, 1980)].

We have also demonstrated in this report that the processivity of polymerization effected by human DNA polymerase β represents another catalytic parameter, in addition to the apparent K_m, V_{max}, E_a (Wang et al., 1977; Wang & Korn, 1980), and minimum required template length, that is significantly altered by the substitution of Mn^{2+} for Mg^{2+} as the divalent metal activator. Thus, in Mg^{2+}, the polymerization mechanism is essentially distributive, while in Mn^{2+}, from four to six nucleotides are inserted in each binding cycle. It is important to point out that this change in processivity does *not* explain the change in minimum template length required for enzyme binding that is discussed above. In support of this conclusion are the results in Figure 4, in which substitution of Mn^{2+} for Mg^{2+} converts a population of *Hin*dIII-restricted DNA molecules from completely inert status to readily utilizable substrates, and those in Figure 6, in which, given the Poisson distribution of template lengths available in the initial DNA substrate, the processivity argument would predict that the extent products in Mn^{2+} must include chains of 29–33 or 30–33 nucleotides in length, in roughly comparable amounts.

The results presented in Figure 1 indicate, in agreement with the earlier conclusion of Tanabe et al. (1979), that the addition of substrates to DNA polymerase β follows a rigidly ordered sequential pathway, with DNA first followed by dNTP. Although separate partial reactions of primer binding and template binding have not been demonstrated, the data described here do support the conclusion that a major signal for productive DNA binding by polymerase β is a base-paired primer stem adjacent to a minimum length of (potential) single-stranded template that may be as short as a single nucleotide with Mn^{2+} and must be greater than or equal to five nucleotides with Mg^{2+}. The data further suggest that with polymerase β, in contrast to KB cell polymerase α (Fisher & Korn, 1981a), the primer-binding and template-binding steps of the nucleic acid interaction may take place by a concerted mechanism. In further distinction to polymerase α, primer recognition by polymerase β is not blocked by a 3′-terminal PO_4 group. This fact, together with the recognized capacity of polymerase β to incorporate dNMPs on primers that contain one to three terminally mispaired nucleotide residues (Wang et al., 1974; Chang, 1973), suggests that the requirements for primer recognition by polymerase β may be substantially less stringent than those of polymerase α with respect at least to the 3′-terminal primer domain.

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A Thioamide Substrate of Carboxypeptidase A†

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ABSTRACT: Carbobenzoxythioglycyl-L-phenylalanine [CbzNHCH$_2$C(=S)Phe, Z-Gly^S-Phe] was synthesized as a thioamide analogue of Z-Gly-Phe, a known substrate of carboxypeptidase A (CPA). By use of a ninhydrin-based assay and Z-Gly-Gly-Phe as the substrate, Z-Gly^S-Phe was shown to be a weak, competitive inhibitor of CPA (K_i = 1.4 mM). The L isomer (but not the D) of Z-Gly^S-Phe proved to be a substrate for CPA (K_m = 1.1 mM and k_{cat} = 5.3 s^{-1} at pH 7.5), binding with comparable affinity to, but hydrolyzing at 10% the rate of, the oxo analogue Z-Gly-Phe. The CPA-catalyzed hydrolysis of Z-Gly^S-Phe was shown to involve only C–N bond cleavage, to give carbobenzoxythioglycine and phenylalanine.

Carboxypeptidase A (CPA)[1] has been the subject of intense investigation for many years, and more is known of its properties and behavior than any other zinc-containing peptidase (Lipscomb, 1980, and references cited therein; Kaiser & Kaiser, 1972; Hartsuck & Lipscomb, 1971; Pĕtra, 1970; Vallee & Riordan, 1968). Crystal structures have been determined for inhibitor complexes as well as for the free enzyme (Hartsuck & Lipscomb, 1971; Lipscomb, 1974; Rees & Lipscomb, 1980; Rees et al., 1980), and its activity with peptide and ester substrates, at various pHs and with various metals in place of zinc, has been extensively explored (Auld & Holmquist, 1974; Turk & Marshall, 1975; Makinen et al., 1979; Auld & Vallee, 1970a,b, 1971; Pĕtra, 1970; Coleman & Vallee, 1960). There remains, however, a degree of uncertainty about the actual mechanism by which a peptide

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[1] Abbreviations: CPA, carboxypeptidase A; Z-Gly, carbobenzoxyglycine; Z-Gly^S, carbobenzoxythioglycine; Z-Gly-Phe, carbobenzoxyglycyl-L-phenylalanine; Z-Gly^S-Phe, carbobenzoxythioglycylphenylalanine (unless otherwise indicated, this refers to the L isomer); Z-Gly-Gly-Phe, carbobenzoxyglycylglycyl-L-phenylalanine; Phe, phenylalanine; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; DCC, dicyclohexylcarbodiimide; IR, infrared spectrum; ^{1}H NMR, proton magnetic resonance spectrum; UV, ultraviolet spectrum; MS, mass spectrum.

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