Reactivity of KB Cell Deoxyribonucleic Acid Polymerases α and β with Nicked and Gapped Deoxyribonucleic Acid†

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ABSTRACT: The experiments described in this paper address the question of the ability of human DNA polymerases α and β to use duplex DNA primer-templates that contain limited numbers of nicks or gap populations of defined average size. Although polymerase α is unreactive with nicked DNA substrates, polymerase β performs a limited synthetic reaction on such molecules to an extent of incorporation of about 15 nucleotides at each nick. We have documented the complete absence of contaminating DNase (or ATPase) activities from our polymerase β fractions at levels of 10^{-4} to 10^{-6} of the polymerizing activity. We have proved that the polymerization reaction takes place at nicks by demonstrating the abolition of primer-template capacity by prior substrate ligation and by using nondenaturing and denaturing gel electrophoresis to localize the reaction products exclusively on the appropriate form II circular and full-length linear substrate DNA species. Kinetic analysis of the nicked substrate reaction by polymerase β in Mn^{2+} yields apparent K_m values for primer termini of 4 to 5 nM and indicates that the enzyme has no measurable affinity for (intact) duplex DNA. The apparent activation energy (E_a) of the synthetic reaction by polymerase β on nicked and gapped DNA substrates in Mn^{2+} is identical and is about 35% lower than that determined for either polymerase α or β on gapped DNA in Mg^{2+}. The most reasonable interpretation of these observations is that DNA polymerase β is intrinsically capable of performing a limited strand displacement reaction to the extent of removing one or two turns from the primary DNA helix. Our examination of the utilization of specifically gapped DNA molecules as primer-templates by the two enzymes reveals that polymerase β is most reactive on short gaps about 10 nucleotides long and appears capable of filling gaps completely, while polymerase α prefers gaps of 30 to 60 nucleotides in length and is unable to fill them completely.

In recent reports we have presented the results of our initial series of examinations of the intrinsic catalytic properties of near-homogeneous preparations (Wang et al., 1974, 1977; Fisher & Korn, 1977) of human DNA polymerases α and β (Eichler et al., 1977; Fisher et al., 1979; Korn et al., 1978b; Fisher & Korn, 1979a,b). It is a major goal of these studies to obtain a detailed understanding of the reactivity of these enzymes with selected DNA substrates of defined structure and thereby develop mechanistically interpretable model assay systems with which to purify and characterize putative mammalian replication factors. The substantial purity of our polymerase fractions has permitted us to undertake these investigations without concern for the potentially highly misleading consequences of even trace levels of intrinsic or contaminating endodeoxyribonuclease or exodeoxyribonuclease activities (Fisher et al., 1979; this paper) and provides confidence that the results obtained are valid reflections of the inherent catalytic properties of the polymerase molecules themselves.

In this paper we examine the capacity of polymerases α and β to utilize duplex DNA primer-templates containing either limited numbers of nicks[1] or gaps[1] of defined mean length. We shall demonstrate that, although DNA polymerase α is totally inert on nicked DNA molecules, polymerase β does perform a readily detectable synthetic reaction on these substrates. The results are most consistent with the interpretation that this small basic protein (Wang et al., 1974) is intrinsically capable of effecting a limited degree of strand displacement to the extent of 10 to 20 nucleotides at each nick. Kinetic analysis of the polymerization reaction with gapped duplex DNA molecules indicates that polymerase α is most reactive with gaps averaging 30 to 60 nucleotides in length, while polymerase β prefers small gaps approximately 10 nucleotides long. Our data further demonstrate that polymerase β is comparable to the phage T4 polymerase in its gap-filling capacity, but, in confirmation of our earlier interpretation (Fisher et al., 1979; Korn et al., 1978a,b), polymerase α appears to be unable to fill gaps to completion.

Materials and Methods

Unlabeled deoxyribonucleotides were from Boehringer, [^{3}H]dTTP from New England Nuclear, [α-^{32}P]dTTP from Amersham/Searle, and [γ-^{32}P]ATP from ICN. Ethidium bromide was obtained from Calbiochem, CsCl from Kawecki Berylco Industries, Inc., and agarose from MC/B. Salmon sperm DNA was Calbiochem A grade and was "activated" as described (Fisher et al., 1979). PM2 form I [^{3}H]DNA (3500 or 6700 cpm/μg) was prepared by the method of Espejo et al. (1969) and M13 [^{3}H]DNA (18 cpm/pmol), according to Marco et al. (1974). [5′-^{32}P]d(pT)$_1$-d(pT)$_{\langle 200 \rangle}$ (27 600 cpm/pmol), KB [^{3}H]DNA (60 000 cpm/μg), and (dT)$_{\langle 200 \rangle}$-[^{3}H](dT)$_{\langle 4 \rangle}$ (16 800 cpm/pmol of terminal dTMP residue) were prepared as previously described (Wang et al., 1974, 1977; Fisher et al., 1979). Poly(dA) was purchased from Collaborative Research. KB and human liver DNA polymerase β (isoelectric focused fractions) were prepared as before (Wang et al., 1977; Korn et al., 1978a,b). KB DNA polymerase α (fraction VIII) was purified by P. A. Fisher (Fisher & Korn, 1977) and *Mycoplasma orale* DNA polymerase (fraction V), by L. M. Boxer (Boxer & Korn, 1979). Phage T4 DNA ligase and nuclease S1 were from BRL, pancreatic DNase was from Worthington, and polynucleotide kinase was

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[1] We define a nick as a single phosphodiester bond scission in one of the two strands of a duplex DNA molecule. The average number of nicks per DNA molecule is expressed as $\langle \bar{n} \rangle$. Since gaps are prepared by limited resection with *E. coli* exonuclease III, a distributive nuclease (Thomas & Olivera, 1978), the actual distributions of gap lengths in the DNA substrate populations are expected to be Poisson. "Mean gap lengths" are determined as described under Materials and Methods.

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from Boehringer. *E. coli* DNA ligase was a gift from Dr. I. R. Lehman (Stanford), T4 DNA polymerase from Dr. P. Modrich (Duke), and *E. coli* exonuclease III from Dr. L. Loeb (University of Washington). Bacterial alkaline phosphatase was from Worthington and was further purified according to Weiss et al. (1968) before use.

Preparation of Singly Nicked PM2 DNA. Form I PM2 DNA was treated with pancreatic DNase I in the presence of ethidium bromide, as described by Greenfield et al. (1975). The 10-mL incubation contained 62 μg/mL form I PM2 [^{3}H]DNA, 2 mM Tris-HCl, pH 8.0, 62.4 mM NaCl, 0.03% bovine serum albumin, 10 mM $MgCl_2$, 330 μg/mL ethidium bromide, and 0.1 μg/mL pancreatic DNase I. After 90 min at 30 °C, the reaction was stopped by adding EDTA to a final concentration of 24 mM. The mixture was extracted twice with equal volumes of redistilled phenol and twice with ether, and the DNA was then precipitated with ethanol and dialyzed extensively against KTE buffer (10 mM KCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0).

Preparation of Multiply Nicked PM2 DNA. Form I PM2 [^{3}H]DNA, 65 μg/mL, was treated with 10 ng/mL pancreatic DNase I as described above, but in the absence of ethidium bromide. Incubation was at 20 °C for 10 min and 15 min. The DNA product was isolated and purified as above.

Estimation of the Number of Nicks per Molecule of PM2 DNA. The average number of nicks per molecule of PM2 [^{3}H]DNA was estimated by alkaline agarose gel electrophoresis (McDonnell et al., 1977) or by alkaline sucrose gradient sedimentation (Masamune et al., 1971; Studier, 1965). For "singly nicked" DNA molecules, the average number of nicks per molecule was calculated from the ratio of radioactivity in full-length linear single-strand molecules vs. that in single-stranded circular DNA. The "multiply nicked" DNA samples were analyzed in 5 to 20% alkaline sucrose gradients that contained 0.1 N NaOH, 0.9 M NaCl, and 10 mM EDTA and overlay a 0.2-mL cushion of 80% sucrose. Centrifugation was performed in the SW 50.1 rotor at 40 000 rpm for 265 min at 20 °C. Under these conditions, the full-length linear molecules derived from singly nicked PM2 DNA had a sedimentation value of 21 S; after 10 and 15 min of nuclease treatment, the populations of linear DNA chains had mean sedimentation values of 10.9 S and 8.0 S, respectively. From these mean S values, it is possible to compute the mass-average molecular weights of the DNA chains and thus determine the average number of nicks per DNA molecule that was initially present in the nuclease-treated population (Studier, 1965). By this procedure it can be estimated that the 10-min sample contained an average of 10 nicks per PM2 DNA molecule, while the 15-min sample had an average of 21.4 nicks per molecule.

Preparation of Salmon Sperm [5′-^{32}P]DNA. Pancreatic DNase I treated salmon sperm DNA, 429 μM (nucleotide), was dephosphorylated with 2.4 units/mL *E. coli* alkaline phosphatase at 65 °C, then purified, and rephosphorylated with 20 units/mL of polynucleotide kinase and 40 μM [γ-^{32}P]ATP (3800 cpm/pmol), as described by Weiss et al. (1968). The final DNA product was purified by Sephadex G-50 gel filtration in KTE buffer, concentrated by ethanol precipitation, and resolubilized in KTE buffer. The [5′-^{32}P]DNA contained an average of one 5′-^{32}P residue per 10^3 nucleotides.

Assays of DNA Polymerases. The standard assays for DNA polymerase α with Mg^{2+} as divalent cation (Fisher & Korn, 1977) and for DNA polymerase β with either Mg^{2+} or Mn^{2+} as divalent cation (Wang et al., 1977) were performed as described. One unit of DNA polymerase activity is defined as the amount catalyzing the incorporation of 1 nmol of labeled dTMP into acid-insoluble product in 1 h under standard assay conditions with activated DNA and Mg^{2+}.

Assays of Nuclease Activities. 3′→5′-Exonuclease assays were performed as previously described (Wang et al., 1974, 1977). 5′→3′-Exonuclease assays were performed either with $(dA)_n$·[5′-^{32}P]d$(pT)_1$-d$(pT)_{(200)}$ (Wang et al., 1977) or with salmon sperm [5′-^{32}P]DNA under optimum polymerase β assay conditions with Mn^{2+} as divalent cation for 60 min at 35 °C. The lower limit of detection of exonucleolytic activities was approximately 40 fmol/h. Assays for double-stranded DNA endonuclease activity were performed with form I PM2 [^{3}H]DNA (6700 cpm/μg) under standard polymerization conditions (Mg^{2+} or Mn^{2+}) with and without dNTPs. Reactions were stopped by addition of EDTA to 10 mM, and products were analyzed either by isopycnic centrifugation in CsCl–ethidium bromide gradients (Wang et al., 1977) or by electrophoresis on nondenaturing 0.8% agarose gels (Keller, 1975) to monitor the conversion of form I to form II species. The lower limit of detection was 0.7 fmol of phosphodiester bonds incised per h.

Assays for single-stranded DNA endonuclease activity were performed with M13 [^{3}H]DNA under optimal polymerization conditions, with either Mg^{2+} or Mn^{2+} as divalent cation. Nuclease activity was monitored by electrophoresis on alkaline 2% agarose gels (McDonnell et al., 1977) to score the conversion of closed circular single-stranded DNA to linear species. The lower limit of detection was 1 fmol of phosphodiester bonds cleaved per h.

Preparation of PM2 [^{3}H]DNA Containing Gaps of Defined Mean Length. Nicked PM2 [^{3}H]DNA (65 μM, containing an average of 10 nicks/molecule) was incubated (4 mL) with 70 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 300 μg/mL gelatin, and 0.6 unit/mL exonuclease III for 10 min at 35 °C. By this procedure, the product DNA molecules could be shown (see below) to contain gaps averaging 30 to 40 nucleotides in length. For preparation of DNA molecules with longer gaps, 65 μM PM2 [^{3}H]DNA containing an average of 1.3 nicks/molecule was incubated with exonuclease III for 20 min under the conditions specified above. The gapped DNA molecules were purified by phenol and ether extraction, concentrated by ethanol precipitation, and dissolved in KTE buffer.

Estimation of Mean Gap Lengths in PM2 [^{3}H]DNA. The mean lengths of the gap populations in the exonuclease III treated PM2 DNA molecules were estimated by determining their "average template lengths" (Bambara et al., 1978) with phage T4 DNA polymerase. The data were treated by the "random nuclease equation" as described by Fisher & Korn (1979c).

Preparation of KB [^{3}H]DNA Containing Gaps of Defined Mean Length. KB [^{3}H]DNA (0.5 mM nucleotide; 21 000 cpm/nmol) was treated with 15 ng/mL pancreatic DNase I in 40 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 200 μg/mL gelatin for 8 min at 23 °C. The nicked DNA was extracted twice each with phenol and ether, concentrated by ethanol precipitation, and dialyzed extensively against KTE buffer. The nicked DNA, 0.3 mM, was then digested with 6 units/mL exonuclease III in 70 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$, and 300 μg/mL gelatin at 35 °C for 0, 10, 30, 45, 60, and 90 min. The resected DNA samples were removed and purified as above. The average fragment size of the [^{3}H]DNA samples, before and after exonuclease III digestion, was estimated by velocity sedimentation in alkaline 5 to 20% sucrose gradients that contained

0.5 M NaCl, 10 mM EDTA, and 0.2 N NaOH and overlay a 0.1-mL cushion of 60% sucrose/0.33 N NaOH. Sedimentation conditions and computation of average fragment lengths from mass-average molecular weights were according to Reynolds (1978). The average length of the fragments obtained from the original nicked DNA sample was ~826 nucleotides; the average length of the fragments obtained from all of the exonuclease III resected samples was ~800 nucleotides, providing important reassurance of the absence of significant endonuclease contamination in the exonuclease reactions. The average gap sizes in the several DNA samples were calculated from the measured acid-soluble radioactivity generated by the exonuclease treatment (exonuclease III is nonprocessive (Thomas & Olivera, 1978)) and the average fragment size in the original DNA population. By this method, we determined that the DNA samples that had been exposed to exonuclease III for 10, 30, 45, 60, and 90 min contained gap populations averaging 10, 34, 53, 80, and 130 nucleotides, respectively. Moreover, from the average fragment size of ~800 nucleotides and the known nucleotide concentration, it was also possible to estimate the number of 3′-hydroxyl termini in each of the gapped DNA samples.

Ligation of Nicked DNA. Nicked PM2 [^{3}H]DNA (82 μM nucleotide; $\overline{1.3}$ or $\overline{1.5}$ nicks/molecule) was incubated with 200 units/mL *E. coli* DNA ligase for 30 min at 30 °C under the conditions described by Modrich & Lehman (1973). Alternatively, DNA samples were incubated with 20 units/mL phage T4 DNA ligase in 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.5 mM ATP, and 200 μg/mL gelatin for 120 min at 26 °C. Reactions were quenched by addition of EDTA to 50 mM and NaOH to 0.3 N. DNA products were analyzed by centrifugation in 5 to 20% sucrose gradients in 0.3 N NaOH, 0.7 M NaCl, and 10 mM EDTA over a cushion of 1.7888 g/cm^3 CsCl in 0.3 N NaOH and 10 mM EDTA. Sedimentation was in the SW 50.1 rotor for 150 min at 35000 rpm. Recovery of [^{3}H]DNA from the gradients was 80–90%. When the ligatability of the polymerase β product on nicked substrate DNA was tested, 41 μM PM2 [^{3}H]DNA containing $\overline{1.5}$ nicks/molecule was incubated with 1.4 units/mL KB DNA polymerase β under standard polymerization conditions with Mn^{2+} as divalent cation. After 60 min of incubation, when an average of 8.5 pmol of dNMPs had been incorporated at each 3′-hydroxyl (nick) terminus, the reaction products were precipitated with ethanol, dissolved in KTE buffer, then incubated either with *E. coli* or T4 DNA ligase, and analyzed as above. In a parallel control reaction, nicked PM2 DNA was incubated under identical polymerization conditions, but without polymerase β, and similarly exposed to ligase. For examination of the effect of preligation on the primer-template capacity of nicked DNA, 29 μM PM2 [^{3}H]DNA containing $\overline{1.5}$ nicks/molecule was first treated with 200 units/mL *E. coli* DNA ligase, as above. The DNA product was recovered by ethanol precipitation and resolubilized in KTE buffer, and a portion (20%) was analyzed by alkaline sucrose-gradient sedimentation to determine the percentage of closed circular duplex DNA. The remainder of the treated DNA was then tested for primer-template capacity with 2.5 units/mL polymerase β under standard (Mn^{2+}) incubation conditions. In a parallel control reaction, a sample of nicked PM2 DNA was carried through the identical procedure, but without exposure to the ligase.

Assay of DNA-Dependent ATPase Activity. The reaction was carried out under optimal DNA polymerase β conditions in the presence of Mn^{2+} and 100 μM [γ-^{32}P]ATP (1384 cpm/pmol). The release of $^{32}P_i$ was measured by chromatography on poly(ethylenimine)-impregnated cellulose thin-layer plates (polygram Cel 300 PEI, Brinkmann Instruments) as described by Kornberg et al. (1978).

Digestion of DNA Products with S1 Nuclease. Nicked PM2 [^{3}H]DNA (75.4 μM, $\overline{1.3}$ nicks/molecule) was incubated with 0.38 unit of polymerase β under standard (Mn^{2+}) conditions at 35 °C. Additional polymerase β (0.08 unit) was added every 60 min until, after 240 min of incubation, a stable extent of incorporation (average of 21 dNMPs/DNA molecule) had been reached. The reaction was adjusted to 100 mM sodium acetate, pH 5.0, 180 mM NaCl, 3 mM $ZnSO_4$, 5% glycerol, 300 μg/mL gelatin, and 250 units/mL of S1 nuclease, according to Lowell et al. (1978), and incubation was continued for 30 min at 20 °C. Incubation samples were then assayed for acid-precipitable radioactivity. In a parallel control, the polymerization product was first heat denatured and then treated with S1 as above.

Other Methods. T4 DNA polymerase was assayed as described by Nossal (1974) and *Mycoplasma orale* DNA polymerase, as described by Boxer & Korn (1979). Urea–polyacrylamide gel electrophoresis was performed according to Maniatis et al. (1975).

Results

Reactivity of DNA Polymerases α and β with Nicked PM2 DNA. Circular duplex PM2 DNA molecules containing $\overline{1.3}$, $\overline{10}$, or $\overline{21}$ nicks/molecule, respectively, were examined for their capacity to serve as substrates with human polymerases α and β. DNA polymerase α (Figure 1A) is essentially unreactive with these nicked primer-templates; thus, with PM2 DNA containing $\overline{1.3}$ nicks/molecule, dNMP incorporation by polymerase α is $<10^{-4}$ to 10^{-5} of that detected with activated DNA at comparable nucleotide concentrations. This lack of reactivity cannot be attributed to the low concentration of 3′-hydroxyl termini in the nicked DNA substrate. We have previously (Fisher et al., 1979) noted that the apparent K_m of DNA polymerase α for primer termini in activated DNA is ~75 nM. In a separate experiment (not shown) we could demonstrate that an amount of polymerase α that could incorporate 300 to 400 pmol of dNMP on 26 μM (nucleotide) activated salmon sperm DNA (50 nM 3′-hydroxyl termini) could incorporate <0.7 fmol of dNMP on 680 μM (nucleotide) PM2 DNA containing $\overline{1.3}$ nicks/molecule. In the latter reaction, the concentration of 3′-primer termini was 50 nM.

In sharp contrast to the above results, we observed that DNA polymerase β, prepared from either KB cells or human liver, could perform a readily detectable incorporation reaction with nicked PM2 DNA in the presence of Mn^{2+} as divalent cation (Figure 1B). Thus, under conditions in which polymerase β incorporated 6.2 pmol of dTMP in 15 min with activated DNA, the enzyme incorporated 0.86, 3.6, and 4.3 pmol of dTMP with PM2 DNA containing $\overline{1.3}$, $\overline{10}$, and $\overline{21}$ nicks/molecule, respectively. The reactions were linear with time for the first 30 min, and the rate of incorporation appeared to be proportional to the concentration of primer-termini (nicks) in the incubation. (As an additional control, we tested the ability of homogeneous *M. orale* DNA polymerase (Boxer & Korn, 1979) to utilize these three nicked PM2 DNA substrates and found that enzyme to be unreactive with all of them ($<10^{-4}$ of the incorporation detected in parallel incubations with activated DNA).)

The extent of deoxynucleotide incorporation by polymerase β on PM2 DNA with $\overline{1.5}$ nicks/molecule is illustrated in Figure 1C, where it is demonstrated that a stable extent is

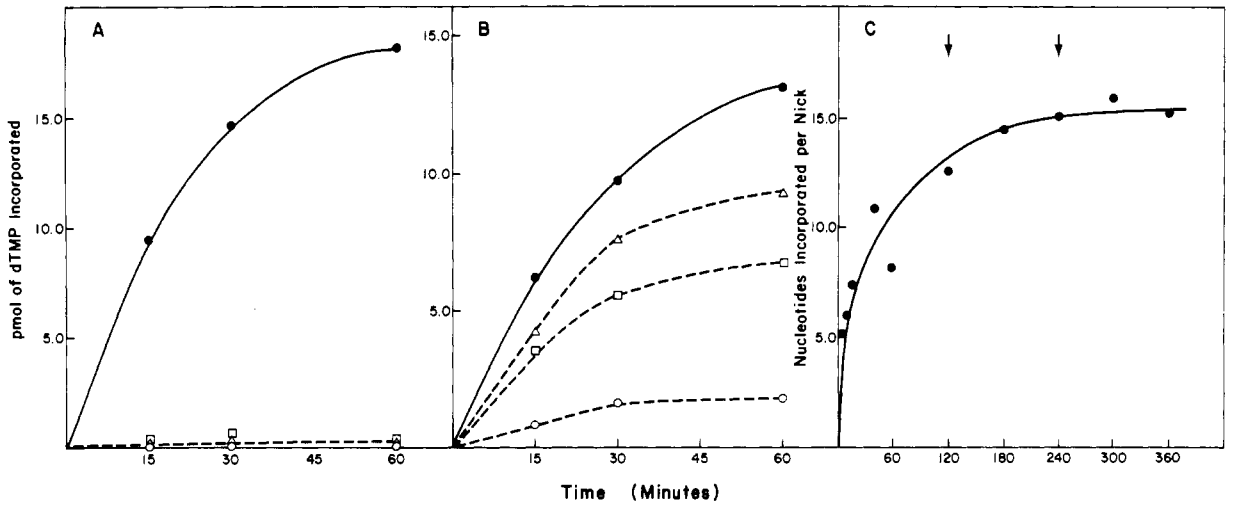

FIGURE 1: Utilization of nicked PM2 DNA by human DNA polymerases α (A) and β (B and C). (A) Polymerase α reactions contained 40 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol, 200 μg/mL gelatin, 4 mM $MgCl_2$, 25 μM each dNTP, [α-^{32}P]dTTP at 3000 cpm/pmol, 39 μM (nucleotide) DNA, and 3 units/mL KB DNA polymerase α. Incubations were at 37 °C. Identical results were obtained if Mg^{2+} was replaced by Mn^{2+} over a concentration range of 25 μM to 8 mM. (B) Polymerase β reactions contained 50 mM Tris-HCl, pH 8.9, 100 mM KCl, 300 μg/mL gelatin, 25 μM each dNTP, [α-^{32}P]dTTP at 3250 cpm/pmol, 1 mM $MnCl_2$, 75 μM (nucleotide) DNA, and 1.9 units/mL human liver DNA polymerase β. Incubations were at 35 °C. Incorporation is expressed as pmol of dTMP per 0.1 mL. (●) Activated DNA; (○) PM2 DNA with $\overline{1.3}$ nicks/molecule; (□) PM2 DNA with $\overline{10}$ nicks/molecule; (△) PM2 DNA with $\overline{21}$ nicks/molecule. (C) Reaction conditions were as in (B) and contained 41 μM (nucleotide) PM2 DNA with $\overline{1.5}$ nicks/molecule and 3 units/mL KB DNA polymerase β. At 120 (↓) and 240 min (↓) fresh polymerase β was added to the incubation to a final concentration of 3 units/mL.

reached after 2–3 h at an average incorporation of ~15 nucleotides/nick. Additional experiments were performed with PM2 DNA substrates containing $\overline{10}$ nicks/molecule, and whether computed conventionally as in Figure 1C, or by the modified (Fisher & Korn, 1979c) "ratio of extents" method of Bambara et al. (1978), stable extents of incorporation averaging 15 to 16 nucleotides/nick were reproducibly observed. The excellent agreement between the estimates of extent derived from the two different methods of analysis provides both powerful confirmation of the absence of even trace levels of endonuclease contamination in our polymerase β fractions and a strong indication that the polymerase is recognizing and using every 3′-hydroxyl site in the nicked DNA population.[2]

Exclusion of Contaminating Enzymatic Activities from Polymerase β Preparations. The data presented above suggested that human DNA polymerase β possesses the capacity to perform a limited synthetic reaction on nicked DNA, presumably (and tentatively) by a strand-displacement mechanism. Critical analysis of these findings demanded, first, rigorous exclusion of contaminating enzymatic activities, particularly 5′→3′-exonuclease, from our polymerase fractions, and, second, proof that the observed reaction was in fact taking place at nicks. The KB and human hepatic polymerase β preparations were tested for contaminating nuclease activities by the highly sensitive assays described under Materials and Methods. The results are summarized in Table I and document the absence of endodeoxyribonuclease and exodeoxyribonuclease activities from our fractions at levels of 10^{-4} to 10^{-6} of the polymerase activity. (Additional evidence of the absence of various nuclease contaminants is implicit in the results in Figures 1C and 2A and Table II.) The polymerase β fractions were also tested for intrinsic or contaminating DNA-dependent ATPase activities, also with negative results. Thus, in an incubation with 41 μM PM2 DNA containing $\overline{1.5}$ nicks/molecule, 0.05 unit of polymerase β, and 100 μM [γ-^{32}P]ATP, under conditions in which 1 pmol of dNMP could be incorporated, <7 fmol of $^{32}P_i$ was released after 60 min at 35 °C. Furthermore, addition of ATP has no detectable effect on the reaction of polymerase β with nicked DNA.

Table I: Exclusion of Nuclease Activities from Human DNA Polymerase β[c]

substrate	specificity of assay	exclusion limit[a] (nuclease/polymerase)
PM2 [^{3}H]DNA	DS[b] endonuclease	1×10^{-6}
M13 [^{3}H]DNA	SS endonuclease	3×10^{-5}
$(dT)_{<200>}$-[^{3}H]$(dT)_{<4>}$	SS 3′-exonuclease	4×10^{-4}
$(dA)_n \cdot (dT)_{<200>}$-[^{3}H]$(dT)_{<4>}$	DS 3′-exonuclease	4×10^{-4} 4×10^{-4}
[5′-^{32}P]$d(pT)_1$-$(dT)_{<200>}$	SS 5′-exonuclease	6×10^{-6}
$(dA)_n \cdot$[5′-^{32}P]$d(pT)_1$-$(dT)_{<200>}$	DS 5′-exonuclease	6×10^{-6}
[5′-^{32}P]DNA	DS 5′-exonuclease	1×10^{-5}

[a] Polymerase activity was defined under strand assay conditions with Mg^{2+} as cation. [b] Abbreviations: DS, double strand specific; SS, single strand specific. [c] Nuclease assays were performed as described under Materials and Methods. In all cases, reactions were for 60 min at 35 °C in the presence and absence of dNTPs and under optimal polymerization conditions for activated DNA or homopolymers with either Mg^{2+} or Mn^{2+} as divalent cation.

Polymerase β Products Covalently Attached to the PM2 DNA Substrate. The products of the reaction of polymerase β with PM2 DNA containing $\overline{1.3}$ nicks/molecule were analyzed directly by agarose gel electrophoresis as shown in Figure 2. On the native gel (Figure 2A), only the expected form II DNA molecules were revealed by ethidium bromide stain, and all of the incorporated radioactivity migrated with these nicked molecules. On the denaturing gel (Figure 2B), once again only the expected closed-circular and full-length linear single-

[2] It is important to note that the results of the "ratio of extents" experiments on the nicked DNA substrates also serve to rule out absolutely any possibility that the observed incorporation might be due to some sort of non-template-directed end-addition reaction. For such a reaction, the ratio of incorporation measured with 4 and <4 dNTPs would be equal to 1.0. We have addressed this hypothetical issue in an earlier report (Wang et al., 1977) and noted that we have never observed such end-addition reactions (as had been encountered by Chang (1973) with a calf thymus polymerase β preparation) with our human KB or hepatic enzyme fractions.

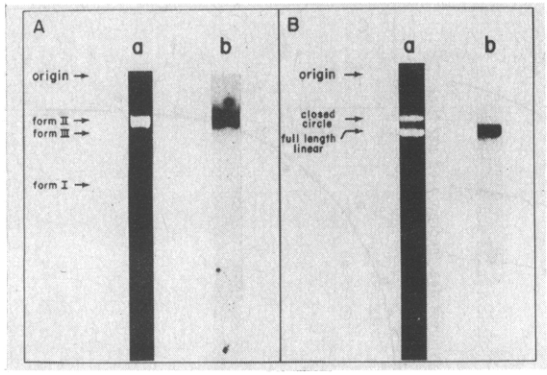

FIGURE 2: Agarose gel electrophoretic analysis of the reaction product of DNA polymerase β with nicked PM2 DNA. Reaction conditions were as described in Figure 1B and contained 125 μM (nucleotide) PM2 DNA with $\overline{1.3}$ nicks/molecule and 3.8 units/mL hepatic polymerase β. After incubation for 2 h at 35 °C, an average of 12 dNMPs had been incorporated per DNA molecule. Aliquots (25 μL) containing 660 cpm of incorporated [^{32}P]dTMP were loaded directly onto a nondenaturing 0.8% agarose gel (A) or an alkaline 2% agarose gel (B) and electrophoresed for 24 h at 20 °C. The gels were stained with 50 μg/mL ethidium bromide, photographed (lanes a), dried, and autoradiographed on Kodak X-omat R film for 48 h (lanes b). The positions of the marker species in both panels were obtained from ethidium bromide staining of unreacted PM2 DNA samples run in parallel lanes.

Table II: Reaction of DNA Polymerase β Takes Place at Nicks[d]

ligation of DNA substrate[a]	closed circular duplex DNA[b] (%)	dNMP incorporation[c] (%)
none	2.0	100
prior to polymerase β incubation	90.0	3.8
after reaction with polymerase β	2.0	(100)

[a] Ligation was carried out as indicated under Materials and Methods. [b] Percentage of closed circular duplex DNA was determined by alkaline sucrose-gradient sedimentation as described under Materials and Methods. [c] Polymerase assays were performed as described under Materials and Methods. 100% represents 10.5 pmol of dNMP incorporated per pmol of DNA (molecules) in 24 min. [d] The reaction of DNA polymerase β was performed as described in Figure 1B with PM2 DNA that initially contained $\overline{1.5}$ nicks/molecule.

stranded DNA molecules were seen by stain, and all of the radioactive product was present in the linear species. The results of the gel analyses prove that the polymerization reaction is taking place on form II PM2 DNA molecules and not on contaminating DNA fragments that might have been copurified inadvertently with the substrate.

Polymerization Reaction Occurs at Nicks. Proof that the polymerase β reaction was occurring at nicks was obtained from a series of experiments that exploited the stringent substrate requirements of DNA ligase (Lehman, 1974) and are presented in Table II. Prior to reaction with the polymerase, about 90% of the nicked (initially containing $\overline{1.5}$ nicks/molecule) PM2 DNA molecules could be ligated by either the *E. coli* or the phage T4 DNA ligase, and such ligated molecules could no longer serve as polymerase substrates. Conversely, after reaction with polymerase β, the form II DNA product molecules (Figure 2) were no longer ligatable.

Kinetic Analysis of the Polymerase β Reaction with Nicked DNA. Kinetic parameters were obtained from Lineweaver–Burk analysis of the reaction of polymerase β with PM2 DNA molecules containing $\overline{1.3}$, $\overline{10}$, and $\overline{21.4}$ nicks/molecule, respectively. The results are presented in Table III. As the mean number of nicks per DNA substrate molecule increased, the apparent K_m of the polymerase for DNA (nucleotide) progressively decreased, but the values of apparent K_m for 3′-hydroxyl termini remained constant at 4 to 5 nM. Similarly, values of V_{max} computed for the three nicked substrates also remained relatively constant at 220 to 260 fmol of dTMP incorporated per min. The kinetic results further indicated that under these experimental conditions, polymerase β demonstrated a measurable affinity only for primer termini but not for duplex DNA. This interpretation was confirmed in separate experiments (data not shown) in which we observed that intact linear (phage T7) and circular (PM2; supercoiled and relaxed) duplex DNA molecules did not inhibit the polymerization reaction with nicked substrate.

Table III: Kinetic Parameters of Reaction of DNA Polymerase β with Nicked DNA[a]

mean number of nicks per DNA molecule	apparent K_m for DNA: nucleotide (μM)	apparent K_m for DNA: 3′-OH termini (nM)	V_{max} (fmol of dTMP per min)
1.3	58	4.3	256
10.0	9	5.1	266
21.4	3.7	4.5	220

[a] Reactions were carried out as described in Figure 1B, with 1.9 units/mL hepatic polymerase β; incubations were for 10 min at 35 °C. Kinetic parameters were calculated from Lineweaver–Burk plots by least-squares analysis.

Effect of Temperature on the Rate of Synthesis by DNA Polymerases α and β. The limited extent of the synthetic reaction performed by polymerase β on nicked DNA and the absence of detectable affinity of the polymerase for duplex polydeoxynucleotides suggested that the capacity of the enzyme to utilize the nicked substrate might be particularly sensitive to localized melting of the substrate at the nicked sites and thus that the reaction might have an unusual (and informative) response to temperature variation. Accordingly, we measured initial velocities of incorporation by polymerases α and β on activated and nicked DNA with Mg^{2+} or Mn^{2+} as divalent cation at 10, 20, 30, 35, 40, and 45 °C. The results of these experiments are presented as Arrhenius plots in Figure 3. In the presence of Mg^{2+} and activated DNA (Figure 3A), the velocity of the polymerase α reaction increases smoothly between 10 and 40 °C; the break in the curve between 40 and 45 °C is most likely due to the onset of thermal inactivation of the enzyme. From the slope of the Arrhenius plot, one can calculate an apparent activation energy (E_a) of 15.9 kcal/mol and a Q_{10} of 2.33. Under comparable incubation conditions, the Arrhenius plot of the polymerase β reaction (Figure 3A) shows a sharp transition at about 35 °C, indicating that the thermolability of the purified polymerase is even greater than had previously been inferred (Dube et al., 1977) from preincubation experiments at higher temperatures with polymerase β preparations from several mammalian sources. The values of the thermal constants computed for the polymerase β reaction in Mg^{2+} are quite comparable to those for polymerase α: apparent E_a = 17.5 kcal/mol; Q_{10} = 2.7. It may be noted that these values of apparent E_a for the polymerization reaction on activated DNA in the presence of Mg^{2+} are similar to those previously recorded for *E. coli* DNA polymerase I (McClure & Jovin, 1975), 18 kcal/mol, and phage T5 polymerase (Das & Fujimura, 1977), 23 kcal/mol, respectively.

Comparable analyses of the polymerase β reaction with activated and nicked DNA in the presence of Mn^{2+} (Figure

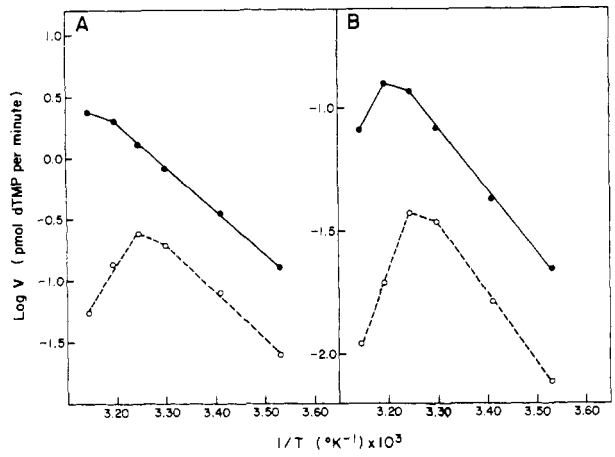

FIGURE 3: Effect of temperature on polymerization rate of DNA polymerases α and β. The data are displayed in Arrhenius plots. (A) Mg^{2+} as divalent cation. Polymerase α reactions (●) were as described in Figure 1A and contained 860 μM (nucleotide) activated DNA and 6 units/mL polymerase α. Incubations were for 5 min. Polymerase β reactions (○) contained 50 mM Tris-HCl, pH 8.9, 100 mM KCl, 10 mM $MgCl_2$, 300 μg/mL gelatin, 100 μM each dNTP with [α-^{32}P]dTTP at 3800 cpm/pmol, 860 μM (nucleotide) activated DNA, and 1.2 units/mL hepatic polymerase β. Incubations were for 10 min. (B) Mn^{2+} as divalent cation. Polymerase β reactions were as described in Figure 1B and contained 52 μM (nucleotide) DNA and 1.2 units/mL hepatic polymerase β. Incubations were for 10 min. (●) Activated DNA; (○) PM2 DNA with 1.3 nicks/molecule.

Table IV: Analysis of Polymerase β Product on Nicked DNA by S_1 Nuclease Digestion[a]

	[^{3}H]DNA		[^{32}P]dNMP	
treatment	nmol of nucleotide	% resistant	pmol	% resistant
control	1.2	(100)	1.7	(100)
S_1 nuclease	1.2	98	1.1	62
denature, S_1	0.3	25	0.35	20

[a] Incubations were carried out with polymerase β and nicked PM2 [^{3}H]DNA as described in Figure 1C. The reaction products were then subjected to digestion with S1 nuclease as indicated under Materials and Methods.

3B) revealed, first, that the value of apparent E_a for the reaction with activated DNA in Mn^{2+} was strikingly lower than that determined with the same substrate in Mg^{2+} and, second, that computed values of apparent E_a (11.5, 11.4 kcal/mol) and Q_{10} (1.93, 1.90) were identical with the two different primer-template species. These results indicated that the frequency of productive interactions of polymerase β with 3′-hydroxyl termini at nicks and gaps (in the presence of Mn^{2+}) is indistinguishable and suggested that localized destabilization of the 5′-terminated DNA strand at the nick site does not contribute significantly to the rate-determining step(s) of the polymerization reaction.

Characterization of the Polymerase β Product on Nicked DNA with S1 Nuclease. DNA polymerase β was allowed to react to extent with PM2 [^{3}H]DNA containing 1.3 nicks/molecule, and the reaction products were tested for susceptibility to digestion by the single-strand-specific S1 nuclease under the stringent conditions described under Materials and Methods. The results are shown in Table IV. If the sample was first heat denatured, about 75% of the primer-template molecules (^{3}H) and 80% of the polymerization products (^{32}P) could be degraded by the nuclease to acid-soluble fragments. In the absence of thermal denaturation, essentially all of the primer-template label and about half of the product label were

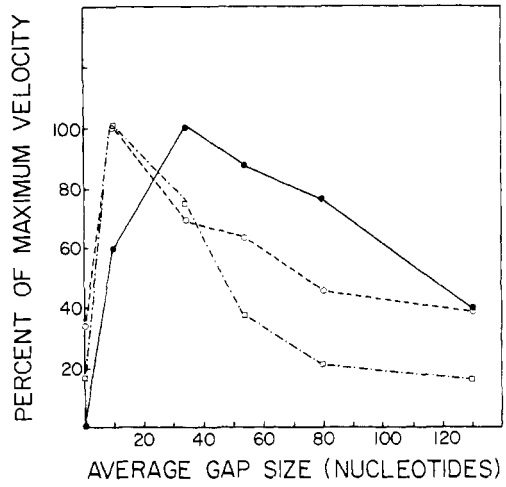

FIGURE 4: Relationship of maximal velocity of polymerization to mean gap length in substrate DNA. DNA polymerase α (●): polymerase α, 0.11 unit/mL, was reacted for 5 min at 37 °C under the conditions described in Figure 1A. DNA samples of known mean gap lengths (see Materials and Methods) were used at 17 to 560 μM (nucleotide). All data were treated by Lineweaver–Burk plots and analyzed by the method of least squares; 100% of V_{max} equals 470 fmol of dTMP incorporated per min. DNA polymerase β with Mn^{2+} as divalent cation (○): hepatic polymerase β, 0.6 unit/mL, was incubated for 5 min at 35 °C under conditions described in Figure 1B. DNA concentrations ranged from 3 to 60 μM (nucleotide); 100% of V_{max} equals 78 fmol of dTMP incorporated per min. DNA polymerase β with Mg^{2+} as divalent cation (□): polymerase β, 1.3 units/mL, was incubated for 5 min at 35 °C under conditions described in Figure 3A. DNA concentrations varied from 20 to 500 μM (nucleotide); 100% of V_{max} equals 830 fmol of dTMP incorporated per min. Note that polymerase β is capable of utilizing nicked duplex DNA in the presence of Mg^{2+}.

resistant to nuclease digestion. A sample of the reaction product that had been treated with S1 nuclease without prior heating was further examined by gel electrophoresis. On native agarose gels, most of the S1 nuclease-resistant product label was present in form II DNA molecules; a small amount could be detected in form III species [under these S1 digestion conditions (Lowell et al., 1978) there is only limited endonucleolytic cleavage of nicked duplex DNA molecules]. On urea–15% polyacrylamide gels, all of the S1-resistant product label was in species that were too large (i.e., >100 nucleotides) to enter the gel. Although the small extent of the polymerization reaction precludes definitive product analysis, these results indicate that, on average, about one-half of the product synthesized at extent by polymerase β on form II DNA molecules is covalently attached to the primer-template in a relatively stable duplex configuration. We believe these findings are most consistent with the limited-strand displacement mechanism that is discussed below.

Reactivity of Polymerases α and β with DNA Substrates Containing Gaps of Defined Mean Length. Our previous studies of the enzymological properties of KB cell DNA polymerase α (Fisher et al., 1979; Korn et al., 1978a,b) had suggested that this enzyme has an unusual selectivity in its capacity to utilize gapped DNA and an inability to fill gaps to completion. To develop those initial observations in more detail and to gain comparable insight into the catalytic properties of DNA polymerase β, we have examined the reactivity of both enzymes on a series of duplex DNA substrates that contained gaps of defined mean length (see Materials and Methods). The first set of experiments comprised a series of kinetic analyses from which we were able to compute values of V_{max} for each polymerase with each of six gapped KB [^{3}H]DNA populations (Figure 4). In incubations with po-

lymerase α in which DNA substrate was present in concentrations ranging from 17 to 560 μM nucleotide (20 to 680 nM 3′-hydroxyl termini), we observed that the enzyme could utilize gap populations ranging from 10 to 130 nucleotides in average length; however, the polymerase showed a pronounced preference for gaps 30 to 60 nucleotides long. With all of the DNA samples tested, apparent K_m values for primer-template were relatively similar, varying from 30 to 50 μM (nucleotide).

In parallel experiments with polymerase β (Figure 4), a strong preference was demonstrated for the smallest gap tested, about 10 nucleotides long, and the relative utilization of the different gapped substrates by this enzyme was generally similar in the presence of either Mg^{2+} or Mn^{2+} as divalent cation (although longer gaps were used somewhat better with Mn^{2+}). In incubations performed with Mn^{2+}, DNA concentrations ranged from 3 to 60 μM nucleotide (3.5 to 75 nM 3′-hydroxyl termini), and apparent K_m values for primer-template were $\sim$10 μM (nucleotide) with all substrates. In reactions with Mg^{2+}, DNA concentrations varied from 20 to 500 μM nucleotide (25 to 600 nM 3′-hydroxyl termini), and apparent K_m values for DNA decreased as average gap size increased, e.g., from 1200 μM with the 10-nucleotide gap substrate to 240 μM with the 130-nucleotide gap substrate. With all DNA samples, however, the apparent K_m of polymerase β for primer-template was 25- to 100-fold higher in Mg^{2+} than in Mn^{2+}, in agreement with our previous report (Wang et al., 1977).

The capacity of polymerases α and β to fill gaps was measured with two samples of duplex PM2 [^{3}H]DNA containing gap populations averaging about 35 and 50 nucleotides, respectively (see Materials and Methods). The experiments were performed by the appropriate modifications (Fisher & Korn, 1979c) of the "ratio of extents" method (Bambara et al., 1978) for determining "effective template length", and phage T4 DNA polymerase was used as the control (Masamune et al., 1971). The results demonstrated that polymerase β was comparable to the phage T4 enzyme in its gap-filling ability but that polymerase α showed extents of deoxynucleotide incorporation that were only 50 to 60% of those achieved by the former enzymes. That is, polymerase α appeared reproducibly to leave an unfilled stretch of 19 $\pm$ 4 nucleotides (1 SD; two experiments on each of the two gapped substrates). This observation is in excellent agreement with our earlier interpretation of the limited gap-filling capability of DNA polymerase α (Fisher et al., 1979; Korn et al., 1978a,b).

Discussion

The considerable progress that has been made in recent years in reconstituting in vitro prokaryotic DNA replication systems has been substantially abetted by detailed understanding of the intrinsic catalytic capabilities, and limitations, of purified DNA polymerases (Kornberg, 1974; Wickner, 1978). In particular, studies with nicked DNA substrates have been of major importance to the successful resolution of the bacteriophage T4 and T7 DNA replication apparatuses (Nossal, 1974; Nossal & Peterlin, 1979; Liu et al., 1978; Scherzinger et al., 1977; Kolodner & Richardson, 1978; Kolodner et al., 1978).

Only two DNA polymerases have previously been shown to be capable of utilizing nicked DNA primer-templates, *E. coli* DNA polymerase I and the phage T5 polymerase, and with both enzymes deoxynucleotide incorporation on such substrates can be substantial. With the T5 polymerase, the reaction proceeds exclusively by a strand displacement mechanism and at a rate that is curiously identical at 25 and 37 °C (Fujimura & Roop, 1976). With the *E. coli* enzyme, the polymerization reaction is complicated by the associated 5′→3′-exonuclease activity and the predominance of nick translation (Kelly et al., 1970) during the initial phase of the reaction (Masamune & Richardson, 1971); at later stages, synthesis occurs primarily by a strand displacement mechanism. The latter phase is not dependent on the former, however, since the polymerase I "large fragment", that lacks the 5′→3′-exonuclease activity (Brutlag et al., 1969; Klenow & Henningsen, 1970), is itself capable of effecting strand displacement synthesis (Setlow et al., 1972; Masamune & Richardson, 1971). With both polymerases, the products of extensive synthesis on nicked circular duplex substrates are structurally complex due to strand-switching and self-priming by the newly synthesized DNA strand, which lead to the formation of single-stranded, duplex and partially duplex tails (Masamune & Richardson, 1971; Fujimura & Roop, 1976).

In this report, we have demonstrated that although DNA polymerase α is incapable of using nicked DNA primer-templates, DNA polymerase β has the unexpected capacity to perform a limited, but entirely reproducible, synthetic reaction with such substrates. The limit of incorporation is about 15 nucleotides/nick, an extent that corresponds to the unwinding of 1 or 2 turns of the primary DNA helix. We have taken particular care to document the complete absence of contaminating DNase (or ATPase) activities from our polymerase fractions, and the data in Figures 1C and 2 and Tables I and II provide assurance that the reaction is an intrinsic property of polymerase β and that it does in fact occur at nicks.

Several features of this synthetic reaction are of interest. Kinetic analysis in the presence of Mn^{2+} indicates that the polymerase possesses measurable affinity only for the 3′-hydroxyl (nick) termini (apparent K_m = 4 to 5 nM) but none for duplex polynucleotide. The results of the thermal experiments (Figure 3) show that the apparent E_a of the polymerization reaction in Mn^{2+} is identical for nicked and gapped substrates and is substantially lower than the apparent E_a of the corresponding reaction of either polymerase α or β with gapped primer-templates in Mg^{2+}. The identity of E_a values suggests that the much lower V_{max} of the synthetic reaction with nicked vs. gapped substrates (Figure 4) may well reflect the slow rate of (unassisted) primary helix unwinding.

We believe that the results of the S1 nuclease digestion studies (Table IV) are most consistent with the limited strand displacement model of the reaction that is illustrated in Figure 5, which suggests that the extent of polymerization may be determined by branch migration reactions of the displaced template strand (Lee et al., 1970) that establish an equilibrium between structures whose limit conformations are shown as II and III. According to this scheme, with each dNMP that is incorporated the number of energetically equivalent states in which the DNA substrate molecules can exist can be considered at first approximation to be increased by one. With increasing elongation of the primer strand, therefore, the probability is progressively decreased that the single state will be present that can be utilized by the polymerase, i.e., that in which the 3′-terminus is stably base paired to the template. This model is also consistent with our demonstration that the polymerization product is no longer ligatable (Table II). Although the results of the S1 nuclease experiments and consideration of physicochemical constraints imposed by the small size of the product suggest that strand switching (structure IV) and self-priming by the newly synthesized DNA do not occur to a significant degree in this reaction, the

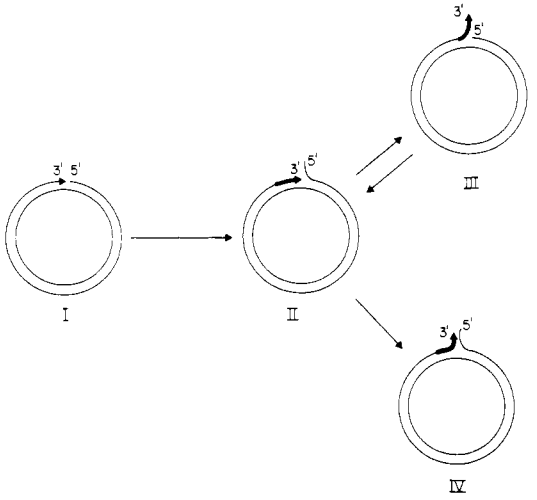

FIGURE 5: Postulated mechanism of limited reaction of DNA polymerase β with nicked DNA. The newly synthesized polymer is indicated by the heavy arrow. The model is adapted from that of Masamune & Richardson (1971).

available data certainly do not permit us to exclude them entirely. It is of interest that the limit of incorporation by polymerase β on a nick is substantially less than that observed on primed duplex circles (D-mt DNA: Eichler et al., 1977; Korn et al., 1978a,b), a formally similar primer-template, most probably because primary helix unwinding with the closed circular substrate is facilitated by the removal of negative superhelical turns (Bauer & Vinograd, 1968; Eichler et al., 1977). With the latter primer-template, possibly because of the greater extent of primer strand elongation or of topological constraints, there is also a prominent strand-switching component of the polymerase β reaction that is not observed with nicked duplex substrates.

In conclusion, we have documented in this paper several enzymological properties of DNA polymerases α and β that provide exploitable catalytic parameters for the design of reconstitution assays. Moreover, the ability of polymerase β to initiate DNA synthesis at nicks and its pronounced preference for filling small gaps are catalytic properties that are intuitively compatible with the proposed in vivo functioning of this enzyme species primarily in DNA repair (Weissbach, 1977; Waser et al., 1979; Hanawalt et al., 1979).

Acknowledgments

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Phenobarbital Induction of NADPH-Cytochrome *c* (P-450) Oxidoreductase Messenger Ribonucleic Acid†

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ABSTRACT: Total RNA isolated from polysomes tightly bound to the rat liver endoplasmic reticulum directed the synthesis of NADPH-cytochrome *c* oxidoreductase (EC 1.6.2.4) by means of a rabbit reticulocyte protein synthesizing system. The oxidoreductase was immunoprecipitated by a monospecific antibody from total translation products and shown to be identical with the enzyme synthesized in vivo in terms of molecular weight and peptide fingerprint patterns, indicating that the oxidoreductase is not synthesized in a form larger than the native enzyme. RNA isolated from free polysomes and polysomes loosely bound to the endoplasmic reticulum did not direct the synthesis of the enzyme in the cell-free system. Phenobarbital administration resulted in a threefold increase in translatable levels of oxidoreductase mRNA; this maximum level was reached 4 h after a single dose of the drug. Cordycepin decreased this response by 50% when administered 20 min before phenobarbital. After reaching its maximum level, induced oxidoreductase mRNA declined with an estimated half-life of 9 h. Phenobarbital administration also resulted in a threefold increase in the in vivo rate of incorporation of [^{3}H]leucine into oxidoreductase 3 h after the maximal increase in oxidoreductase mRNA was obtained. The rate of incorporation of isotope into total microsomal protein, however, did not increase. Cytochrome b_5, an intrinsic microsomal membrane protein not inducible under these conditions, was synthesized from free and loosely membrane-bound polysomal RNA, and translatable levels of its mRNA did not increase upon phenobarbital administration. Serum albumin, which was synthesized from tightly membrane-bound polysomal RNA, also was not increased by phenobarbital administration. These data demonstrate that two intrinsic microsomal membrane proteins, both of which are anchored to the membrane via hydrophobic segments of their polypeptide chain, are synthesized on separate populations of polysomes. Induction of enzymes by phenobarbital does not result solely from a stabilization of protein or mRNA or from an increase in the rate of translation of certain mRNAs. Accumulation of mRNA results from either an increased rate of transcription or posttranscriptional processing and nucleocytoplasmic transport of specific mRNAs.

Chronic phenobarbital administration results in an increased rate of oxidative drug metabolism which is attributed to an increase in activity of the endoplasmic reticulum mixed function oxidase system (Conney et al., 1960; Remmer & Merker, 1963; Orrenius et al., 1965). This change is correlated with a proliferation of smooth endoplasmic reticulum and an increase in the level of certain enzymes involved in drug metabolism (Ernster & Orrenius, 1965; Orrenius & Ericksson, 1966; Conney, 1967; Lu et al., 1969). The mechanism of phenobarbital induction is poorly understood, however. Maximum increase in an inducible enzyme is obtained only after daily administration of the drug and peaks between 4 and 5 days after the first inoculation (Jick & Shuster, 1966; Kuriyama et al., 1969). This is in contrast to another class of drug metabolism inducing agents, which includes the polycyclic aromatic hydrocarbons, in that a maximal increase in specific activity of inducible enzymes occurs 1 to 2 days after a single dose of the inducer with no increase in smooth endoplasmic reticulum (Conney et al., 1957; Conney, 1967; Parke, 1976). The mechanism of these inducing agents appears to be similar to that of steroid hormones, where a receptor-ligand complex influences a battery of genes by promoting transcription (Poland & Glover, 1976). No direct evidence for a phenobarbital receptor, however, has been demonstrated. Another important biochemical distinction between the two types of inducing agents is that phenobarbital preferentially induces certain enzymes in the endoplasmic reticulum but does not induce their nuclear envelope counterparts; yet, polycyclic aromatic hydrocarbons induce certain enzymes in both membrane systems (Kasper, 1971; Khandwala & Kasper, 1973; Fahl et al., 1978).

The enzyme NADPH-cytochrome *c* oxidoreductase has been extensively studied with respect to its induction by phenobarbital. Early reports demonstrated that an increase in the rate of synthesis of the enzyme occurred early after a single dose of the drug; however, the rate of synthesis of another membrane-bound protein, cytochrome b_5, did not increase (Jick & Shuster, 1966; Kuriyama et al., 1969). In

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