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Expression of Human DNA Polymerase β in *Escherichia coli* and Characterization of the Recombinant Enzyme

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ABSTRACT: The coding region of a human β-polymerase cDNA, predicting a 335 amino acid protein, was subcloned in the *Escherichia coli* expression plasmid pRC23. After induction of transformed cells, the crude soluble extract was found to contain a new protein immunoreactive with β-polymerase antibody and corresponding in size to the protein deduced from the cDNA. This protein was purified in a yield of 1–2 mg/50 g of cells. The recombinant protein had about the same DNA polymerase specific activity as β-polymerase purified from mammalian tissues, and template-primer specificity and immunological properties of the recombinant polymerase were similar to those of natural β-polymerases. The purified enzyme was free of nuclease activity. We studied detailed catalytic properties of the recombinant β-polymerase using defined template-primer systems. The results indicate that this β-polymerase is essentially identical with natural β-polymerases. The recombinant enzyme is distributive in mode of synthesis and is capable of detecting changes in the integrity of the single-stranded template, such as methylated bases and a double-stranded region. The enzyme recognizes a template region four to seven bases downstream of the primer 3′ end and utilizes alternative primers if this downstream template region is double stranded. The enzyme is unable to synthesize past methylated bases N^3-methyl-dT or O^6-methyl-dG.

DNA polymerase β, a DNA repair polymerase of eukaryotic cells [for a review, see Fry and Loeb (1986)], is seen as a model enzyme for structure–function analysis of the nucleotidyltransferase reaction by DNA polymerases (Tanabe et al., 1979). This enzyme is the simplest DNA polymerase known in both size and catalytic repertoire. The human and rat enzymes are polypeptides of 335 amino acids, and secondary structure predictions suggest ordinary globular structure with high α-helix content (Zmudzka et al., 1986; SenGupta et al., 1986). The purified enzyme lacks exonuclease activities and detectable reverse reactions (Tanabe et al., 1979; Fry, 1983), and the polymerase activity is fully distributive under most reaction conditions (Detera et al., 1981). Thus, the β-polymerase mechanism is a two-substrate–two-product reaction and follows ordered Bi-Bi kinetics (Tanabe et al., 1979).

To examine physical biochemical properties and structure–function relationships of mammalian β-polymerase, we overexpressed the coding region of a human β-polymerase cDNA (SenGupta et al., 1986) in the λP_L promoter-based bacterial expression system pRC23 (Crowl et al., 1985). Here, we report the purification of the recombinant enzyme in milligram quantities. Enzymatic studies revealed that the enzyme is a characteristic β-polymerase and is appropriate for structure–function studies of this enzyme.

Materials and Methods

Materials. [γ-^{32}P]ATP was obtained from New England Nuclear or ICN Radiochemicals. Homogeneous Klenow fragment from *Escherichia coli* DNA polymerase I was a generous gift from W. E. Brown. DNA polymerase β from chick embryo was purified as described (Yamaguchi et al., 1980) and was a generous gift from A. Matsukage. β-Polymerases from HeLa cells (Mosbaugh & Linn, 1983) and rat Novikoff hepatoma (Stalker et al., 1976) were purified as previously described and were generous gifts from D. W. Mosbaugh. Preparation of mouse and calf thymus DNA polymerases β and Western blotting materials were as described earlier (Swack et al., 1985; Tanabe et al., 1979; Karawya et al., 1984). T4 polynucleotide kinase, T4 DNA ligase,

and deoxynucleoside triphosphates (dNTPs)[1] were obtained from Pharmacia. ϕX174 plus strand DNA, formamide, and urea were obtained from Bethesda Research Laboratories. Sixteen-residue synthetic deoxyoligonucleotide primers complementary to positions 668–653 and 633–618 of ϕX174 plus strand (designated primer 1 and primer 2, respectively), and the synthetic templates described in Figure 7, were prepared by a solid-phase triester method and purified by anion-exchange and reverse-phase high-pressure liquid chromatography; these oligomers were generously provided by Gerald Zon. Electrophoresis-grade acrylamide and bis(acrylamide) were obtained from Bio-Rad Laboratories.

Buffers and Solutions. Buffer A was 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF, 10 mM $Na_2S_2O_5$, and 1 μg/mL pepstatin A. DEAE-cellulose (DE52) and phosphocellulose were from Whatman. Single-stranded DNA–cellulose was prepared with calf thymus DNA (Worthington) as described by Planck and Wilson (1980). *E. coli* strain JM83 was the host for initial plasmid construction, and *E. coli* strain JM101 was the host for M13 systems. *E. coli* strain RRI (Crowl et al., 1985) bearing the low copy number plasmid pRK248 cIts, encoding a ts λ repressor, was the host for the β-polymerase protein expression plasmid and was a gift from R. Crowl (Hoffmann-La Roche Inc., Roche Research Center, Nutley, NJ).

Construction of Plasmids. The construction of the expression plasmid is illustrated in Figure 2. The restriction enzyme *Kpn*I was used to cut λpolβh2 DNA (SenGupta et al., 1986), and a fragment 2.2 kb long containing 1.0 kb from λ DNA and 1.2 kb from the cDNA was subcloned in pUC19 at the *Kpn*I site. Then using *Hae*III endonuclease, we cut out a fragment of the cDNA 1.1 kb long; *Hae*III cuts at one site five nucleotides before the ATG start codon and a second site two nucleotides after the TAA stop codon. This 1.1 kb long cDNA fragment then was ligated in pRC23 DNA that had been cut with *Eco*RI, phosphatase treated, and filled-in with Klenow fragment. The resulting recombinants then were used to transform *E. coli* RRI bearing the low copy number plasmid. Plasmid DNA from the transformants was screened for the presence of the cDNA insert in the correct orientation. The resultant plasmids used here, pEX16 and pEX17, had the desired cDNA insert in the incorrect and correct orientation, respectively.

Cell Growth and Induction for DNA Polymerase β Expression. Cells transformed with various plasmids were screened by using small-scale cultures. Ten milliliters of LB broth ([ampicillin] = 50 μg/mL) was inoculated with 100 μL of an overnight culture of *E. coli* RRI containing plasmid and incubated in a 30 °C shaking water bath. When OD_{560nm} = 0.2 was reached, the cultures were transferred to a 42 °C shaking water bath and allowed to grow for 1, 2, or 3 h. Cells then were chilled on ice for 15 min, centrifuged, and washed at 4 °C with 1 mL of phosphate-buffered saline. Cells were suspended in 100 μL of buffer A containing 0.5 M NaCl and sonicated with a Braun Sonic 1510 sonifier, 3 × 10 s. An aliquot of the suspension was mixed with SDS sample loading buffer, and 10% SDS–polyacrylamide gel electrophoresis was conducted.

Purification of DNA Polymerase β from E. coli Crude Extract. Twenty to forty grams of *E. coli* cells, induced for 2.5 h at 42 °C, was used for each purification batch. Cells were resuspended in 160 mL of buffer A containing 0.5 M NaCl (buffer D), and the suspension was sonicated for 5 × 30 s with the vessel immersed in ice water. The suspension was centrifuged at 20000*g* for 20 min at 4 °C. The supernatant fraction was diluted with buffer A to bring the NaCl concentration to 0.2 M (fraction I). Fraction I was loaded on a 200-mL DEAE-cellulose column (2.5 × 50 cm) connected in tandem with a 100-mL phosphocellulose column (2.5 × 20 cm); both columns were equilibrated and run with buffer A containing 0.2 M NaCl (buffer C). Under these conditions, DNA polymerase β passed through the DEAE-cellulose column and bound to the phosphocellulose column. The columns were washed with 600 mL of buffer C, and then the DEAE-cellulose column was disconnected and discarded. The phosphocellulose column was washed with 400 mL of buffer C, and then β-polymerase was eluted from the column with 300 mL of buffer A containing 1 M NaCl (buffer E). The columns were run at 60 mL/h at 4 °C. Fractions were assayed for the activity of DNA polymerase β, and the fractions with the peak of activity were pooled and dialyzed against buffer A containing 0.1 M NaCl (buffer B). The dialyzed fraction then was loaded on a 20-mL ssDNA–cellulose column (1 × 30 cm) equilibrated with buffer B. The column was washed with 200 mL of buffer B, and β-polymerase was eluted with 100 mL of buffer E. Fractions with the peak of activity were pooled and loaded in 5-mL portions onto a Sephacryl-S200 column (Tanabe et al., 1979). The β-polymerase enzymatic activity was recovered in a single sharp peak centered at M_r 39 000.

Preparation of Labeled DNA Primers. Synthetic DNA primers were 5′ end labeled according to the procedure described by Maxam and Gilbert (1980). DNA was extracted with phenol; residual phenol was removed with ether, and the DNA was precipitated with ethanol.

In Vitro DNA Synthesis. Primers were hybridized to ϕX DNA at a 5:1 molar ratio by heating to 100 °C and slowly cooling to room temperature. DNA synthesis reactions (20 μL) contained 50 mM Tris·HCl, pH 7.5, 100 mM NaCl, divalent metal cation as indicated, 1 mM dithiothreitol, 400 μg/mL bovine serum albumin, 20% glycerol, 30–300 μM each of dATP, dCTP, dGTP, and dTTP, and 200 fmol of hybridized primer-template. Reaction mixtures were incubated at 35 °C, and reactions were stopped by adding EDTA to a final concentration of 18 mM. A dye mixture in deionized formamide was then added to a total volume of 45 μL; 5–15-μL portions were loaded for gel electrophoresis.

Analysis of Products of DNA Synthesis. Products were analyzed by gel electrophoresis as previously described (Detera & Wilson, 1982). Electrophoresis was conducted on a 12% polyacrylamide–7 M urea gel (40 × 35 cm × 0.8 mm). The gel was prerun for 1 h at 40–45 W without cooling, and electrophoresis was performed at 40–45 W. After electrophoresis, the gel was fixed in acetic acid/methanol, transferred to 3MM paper, covered with Saran wrap, and dried under vacuum in a Bio-Rad gel dryer. Products were visualized by autoradiography with Kodak XAR-5 film at room temperature. Some exposed films were evaluated for band intensity with a Zeineh soft laser scanning desitometer (Biomed Instruments).

RESULTS

Subcloning and Expression of Recombinant β-Polymerase. We reexamined the sequence of the 5′ end of the human β-polymerase cDNA and found that the start of the coding region is 51 nucleotides 5′ of the start codon previously assigned

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; dNTP, deoxynucleoside triphosphate; Pol I, *Escherichia coli* DNA polymerase I; PMSF, phenylmethanesulfonyl fluoride; kb, kilobase(s); SDS, sodium dodecyl sulfate; ss, single stranded; kDa, kilodalton(s).

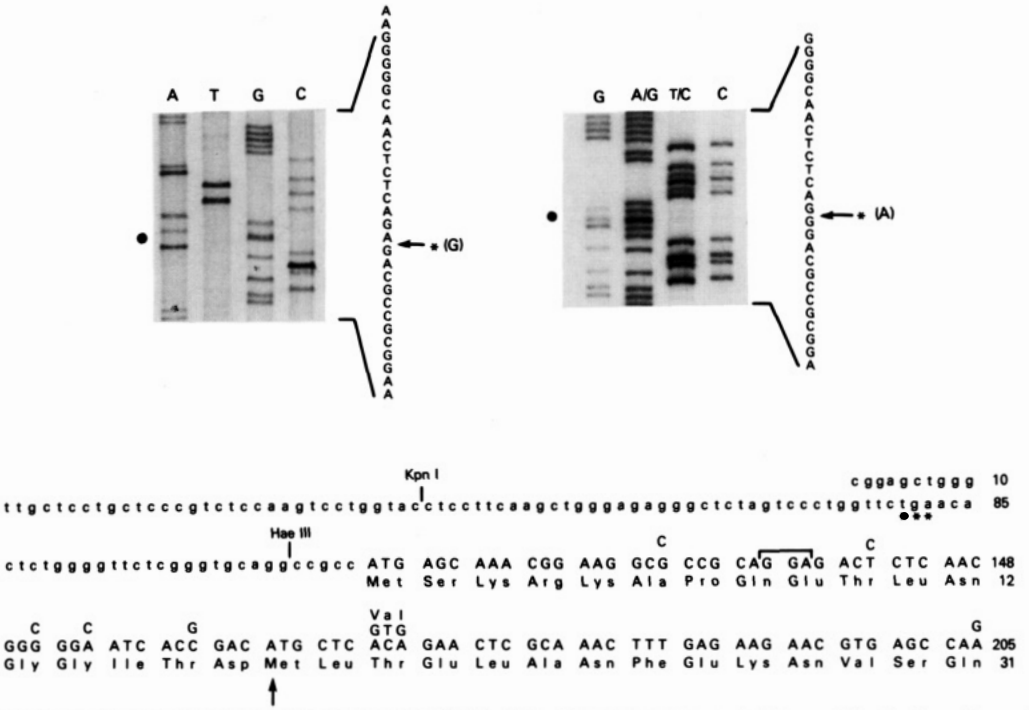

FIGURE 1: Sequence of the 5′ end of the human β-polymerase cDNA. The corrected sequence is shown at the bottom along with the deduced amino acid sequence of the first 31 residues of the 335 amino acid residue open reading frame. The bracket indicates the three-nucleotide region where the sequence reported by SenGupta et al. (1986) was corrected. Two important restriction sites mentioned below are shown. A termination codon 5′ of the Met-1 codon is indicated (asterisk), as is the start codon assigned previously (↑). Nucleotide differences with the rat β-polymerase cDNA are designated with the rat residue where different on the upper line; the single amino acid difference is indicated. The two photographs show sequencing analysis by the Sanger (1980) (left) and Maxam–Gilbert (1980) (right) methods. Band compressions (●) and the omitted residue (←*) are indicated. The corrected sequence was obtained by Maxam–Gilbert analysis of the complementary strand.

(SenGupta et al., 1986). Sequence analysis of this region using the Sanger method had been complicated by a reproducible band compression, such that adjacent G residues were scored as a single residue. Aberrant gel migration for this region also was observed with the Maxam/Gilbert method. Our corrected sequence of the first 205 nucleotide residues of the human cDNA is shown in Figure 1, along with a comparison with the sequence of the rat β-polymerase cDNA (Zmudzka et al., 1986). The 5′ end of the rat cDNA corresponds to the C residue in the Arg-4 codon of the human cDNA. From this C residue to the ATG codon for Met-18, the start codon assigned previously, each nucleotide difference between human and rat is silent, consistent with the idea that coding properties of this sequence were conserved through protein function. The five nucleotides upstream of the codon for Met-1 have the canonical sequence CCGCC for translation initiation sites (Kozak, 1984). An in-frame termination codon, TGA, occurs 30 nucleotides upstream from the Met-1 codon. The presence of this termination codon was confirmed in experiments to be described below.

The cDNA contains only two *Hae*III sites, one five nucleotides 5′ of the start codon (Figure 1) and the other two nucleotides 3′ of the termination codon. Hence, the coding region was excised from a plasmid subclone using *Hae*III, and the resulting fragment was ligated into pRC23 (Figure 2). The construction of the final plasmid, pEX17, was confirmed by sequencing ~50 residues of the 5′ and 3′ ends of the cDNA.

E. coli transformed with pEX17 and induced did not contain an abundant new dye-stained peptide by SDS–polyacrylamide gel electrophoresis of a crude soluble extract. However, when Western blots of such gels were evaluated with a β-polymerase antiserum, an epitope peptide was detected at molecular weight ~39 000 (Figure 3). This peptide was not observed in the absence of heat induction, when the cDNA was in reverse orientation in pRC23, or with pRC23 alone. Further immunoblotting experiments revealed that approximately half of the epitope peptide in the cells was recovered in the soluble extract (not shown). A minor recombinant polypeptide with a reactive epitope is also seen at molecular weight ~27 000 (Figure 3).

Demonstration of Upstream Stop Codon. The pRC23 expression system was used to evaluate the presence of a termination codon upstream of the Met-1 codon (Figure 1). Confirmation of this in-frame termination codon would strongly suggest there is no further coding region upstream of Met-1. A *Kpn*I fragment containing the coding region and sequence 5′ of the Met-1 codon (Figure 1) was excised and subcloned into the variable reading frame derivative of pRC23 (Crowl et al., 1985). This construction would lead to termination of translation at the upstream termination codon (Figure 1), corresponding to residues 80–82 of the cDNA. As a control in the experiment, the *Hae*III fragment of the cDNA described above also was cloned into the variable reading frame vector. Transformation of *E. coli* with these plasmids resulted in production of an epitope peptide with the *Hae*III fragment, as expected, but not with the longer fragment. This suggests that the termination codon 30 nucleotides 5′ of the Met-1 (Figure 1) is indeed correct. Finally, we found that the amount of epitope peptide in the induced cells was the same with our standard construct in pRC23, pEX17, and with the variable reading frame construct.

Purification and Characteristics of Recombinant β-Polymerase. Recombinant β-polymerase was purified (Table I) from the soluble extract by using step elutions from phosphocellulose and ssDNA–cellulose columns and then Sephacryl-S200 gel filtration (Figure 4a). The purification was monitored by using DNA polymerase assays for β-polymerase activity, including poly(dA)·oligo(dT) as template-primer, Mn^{2+}, and pH 8.8. The enzymatic activity formed a sharp symmetrical peak

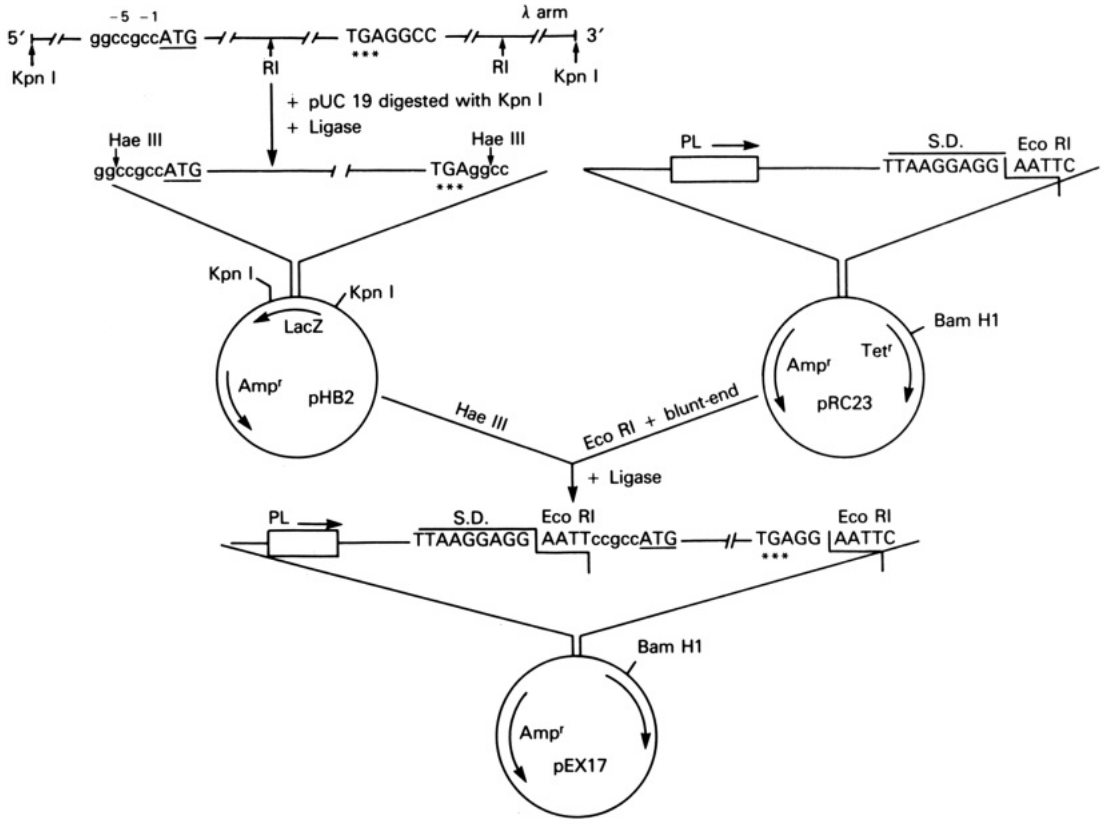

FIGURE 2: Diagram showing the construction of expression plasmid for human β-polymerase. The cDNA coding region contained an internal *Eco*RI site; therefore, the coding region was removed from the λ recombinant clone (SenGupta et al., 1986) with *Kpn*I. The coding region was excised from the *Kpn*I fragment with *Hae*III and subcloned in pUC19 to yield the intermediate plasmid pHB2. The insert junctions (about 50 nucleotides) in pHB2 were sequenced to confirm the construct, and the insert then was subcloned in pRC23 as illustrated. Another plasmid, pEX16, with the coding region in reverse orientation also was constructed.

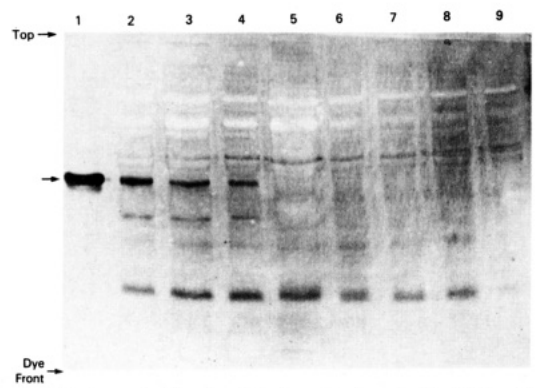

FIGURE 3: Photograph showing Western blot analysis of *E. coli* crude soluble extracts and partially purified recombinant human β-polymerase. Ten-milliliter cultures of cells were centrifuged, and cell pellets were extracted with 100 μL of extraction buffer; 35 μL of the soluble extract was electrophoresed. A blot of a 10% SDS–polyacrylamide gel was probed with anti-β-polymerase IgG; the bound IgG was detected with a horseradish peroxidase based dye system. Lane 1, 5 μg of fraction IV recombinant β-polymerase; lanes 2, 3, 4, and 5, pEX17 induced at 42 °C for 3, 2, 1, or 0 h, respectively; lanes 6 and 7, pEX16 induced at 42 °C for 3 or 0 h, respectively; lanes 8 and 9, pRC23 induced at 42 °C for 3 or 0 h, respectively. Marker proteins and respective kilodalton values were myosin (200), β-galactosidase (116), phosphorylase *b* (92.5), serum albumin (66), ovalbumin (45), carbonic anhydrase (31), and soybean trypsin inhibitor (21.5). The arrow on the left designates the position of M_r 39 000 in the gel. An induced peptide of M_r ~27 000 with a reactive epitope is also seen as a minor band in lanes 2–4.

in each column profile. In the Sephacryl-S200 column fractions, the only DNA polymerase activity present formed a symmetrical peak corresponding to a globular protein of M_r 39 000 (Figure 4a). Immunoblotting of the enzyme after the ssDNA–cellulose column is shown in Figure 3, lane 1. The

Table I: Purification of β-Polymerase from *E. coli* Carrying pEX17 and Grown for 2.5 h at 42 °C

description	fraction	volume (mL)	total protein (mg)	β-Pol (units)
crude lysate[a]	I	100	650	
low-speed supernatant fraction	II	90	400	
phosphocellulose eluate	III	10	25	100
ssDNA–cellulose eluate	IV	10	2	100
Sephacryl S-200 peak (pool)	V	16	0.5	50

[a] The experiment was conducted with 10 g of frozen cell paste. One unit is 1 μmol of dNMP incorporated per hour.

epitope peptide gave a strong signal corresponding in size to the epitope peptide in the crude extract. SDS–polyacrylamide gel analyses of several fractions from the gel filtration column are shown in Figure 4b. The main protein in these fractions was an M_r ~39 000 polypeptide; this polypeptide in the final fraction reacted with the β-polymerase antibody in immunoblotting (Figure 4c). In the final fraction, the recovery of the M_r 39 000 protein from the soluble extract routinely was ~2 mg/40 g of cells.

The DNA polymerase specific activity of the final fraction (Table II) was similar to that of the enzyme purified from mouse myeloma (Tanabe et al., 1979). The recombinant enzyme exhibited template-primer specificity characteristics of a β-polymerase, but unlike *E. coli* Pol I (Table II). This was not surprising as the recombinant enzyme had been separated cleanly from the abundant Pol I activity in the extract during the phosphocellulose column step and no higher molecular weight polymerase activity was present in the final gel

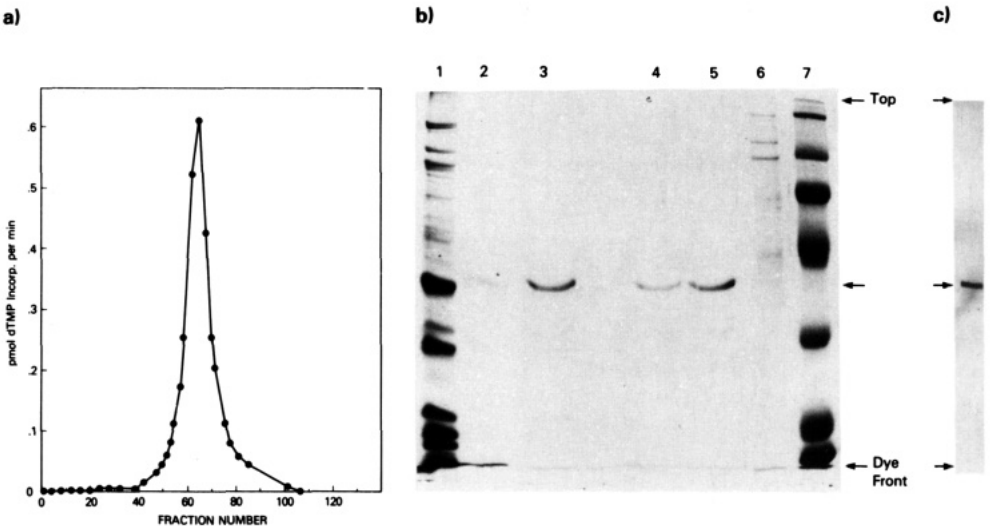

FIGURE 4: Sephacryl S-200 gel filtration and SDS–polyacrylamide gel analysis of purified recombinant human β-polymerase. (Panel b) Photograph of a 10% gel after staining with Coomassie Blue. Lane 1, 30 μg of protein fraction IV (after ssDNA–cellulose chromatography); lanes 2–5, fractions from the Sephacryl S-200 column chromatography shown in panel a; lane 2, 1 μg of protein from a fraction at the leading edge (higher molecular weight) of the enzyme activity peak; lane 3, 3 μg of protein from a fraction at the trailing edge (lower molecular weight) of the activity peak; lanes 4 and 5, 1 and 3 μg of protein, respectively, from the activity peak (i.e., fraction V); lanes 6 and 7, protein markers as described in Figure 3. The arrow on the right notes the position of M_r 39 000 in the gel. (Panel c) Photograph of Western blot analysis of 1 μg of protein of fraction V. Blots were probed with anti-β-polymerase IgG or with preimmune rabbit IgG, which did not show a signal (not shown).

Table II: Template-Primer Specificity of Recombinant Human β-Polymerase

template-primer system	rel amount of dNMP incorpd[b] for DNA polymerase: recombinant β-polymerase[a]	calf β-polymerase	Pol I large fragment
activated DNA; Mg^{2+}	100	100	100
poly(dA)·oligo(dT); Mn^{2+}	240	280	10
poly(rA)·oligo(dT); Mn^{2+}	5	10	80
poly(dT)·oligo(rA); Mg^{2+}	15	10	20

[a] Enzyme specific activity in the poly(dA)·oligo(dT) system was 90 μmol of dNMP incorporated h^{-1} (mg of protein)$^{-1}$. [b] 100 represented, from left to right above, 1, 1, and 4 pmol of dNMP incorporated per 10 min.

filtration step in the purification (Figure 4a).

Enzymatic activity by the 39-kDa polypeptide itself was further confirmed by SDS–polyacrylamide gel electrophoresis followed by precise slicing, elution, and renaturation of the 39-kDa polypeptide. We found that ~40% of the DNA polymerase activity of the starting sample was recovered in the renatured 39-kDa polypeptide. This recovery was similar to that obtained with calf thymus β-polymerase used as a reference and was 3–4-fold higher than observed with Pol I. These results indicate that the 39-kDa polypeptide, and not some minor contaminant, was responsible for the DNA polymerase activity in the preparation. Hence, on the basis of its size, the presence of β-polymerase antibody epitope, and its template-primer specificity and specific activity, the recombinant enzyme was identified as β-polymerase.

In experiments not shown, we found that the purified recombinant enzyme did not contain nicking activity against supercoiled plasmid DNA and did not degrade double-stranded DNA. The preparation also was completely free of 5′- or 3′-exonuclease activity, as measured with a 16-residue single-stranded deoxyoligonucleotide substrate or with this oligonucleotide substrate annealed to complementary DNA.

Processivity, Pause Sites, and Gap Filling by Recombinant Human β-Polymerase. We evaluated the following three questions about the DNA polymerase activity of recombinant human β-polymerase: Is the enzyme capable of processive synthesis? Does the enzyme have "pause sites" when replicating a long single-stranded template? Can the enzyme fill a short single-stranded gap in double-stranded DNA? Processivity was evaluated by using a template-primer system composed of φX174 DNA (Figure 5). A 16-residue oligonucleotide primer labeled with ^{32}P in the 5′ end was annealed

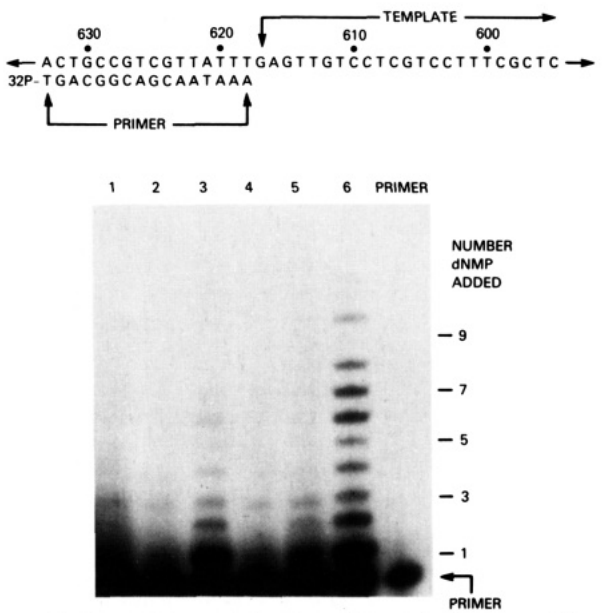

FIGURE 5: Processivity analysis of recombinant β-polymerase. Shown at the top is the synthetic primer labeled with ^{32}P on the 5′ end and hybridized to φX174 (+) strand DNA. The numbers correspond to the φX174 map. DNA synthesis reactions were carried out as described under Materials and Methods with a 1:40 molar ratio of polymerase β to template. Products were displayed by electrophoresis and visualized by autoradiography, and a photograph is shown. Reaction conditions for each lane were as follows: lane 1, 30 μM dNTPs, 5 mM $MgCl_2$, 200-s incubation; lane 2, 280 μM dNTPs, 6 mM $MgCl_2$, 200-s incubation; lane 3, 280 μM dNTPs, 6 mM $MgCl_2$, 20-min incubation; lane 4, 30 μM dNTPs, 0.5 mM $MnCl_2$, 200-s incubation; lane 5, 280 μM dNTPs, 2 mM $MnCl_2$, 200-s incubation; lane 6, 280 μM dNTPs, 2 mM $MnCl_2$, 20-min incubation.

to single-stranded ϕX174 DNA; the single-stranded template region 95 nucleotides downstream of the primer is considered to be secondary structure free on the basis of computer-derived predictions and previous enzymatic studies (Weaver & DePamphilis, 1982). The DNA polymerase incubations were conducted for 200 s at a 40 to 1 molar ratio of template-primer to enzyme. Products then were separated by gel electrophoresis, visualized by autoradiographs, and quantified. Under these incubation conditions, most of the product molecules represented one cycle of enzyme binding, synthesis, and termination. This system allows one to distinguish increased processivity from increased polymerase activity under different conditions, since the size distribution of products is distinct for the two modes of synthesis (Detera & Wilson, 1982).

In the presence of magnesium and 30 μM dNTP, the enzyme was distributive, adding one nucleotide residue to a primer and then dissociating (Figure 5, lane 1). In the presence of magnesium and a high level of dNTP (280 μM), a pattern of product molecules indicative of distributive synthesis again was observed (Figure 5, lane 2). Even after a 20-min incubation allowing for many cycles of enzyme binding, synthesis, and termination, the large majority of products still represent just one nucleotide addition (Figure 5, lane 3). In the presence of manganese and 30 μM dNTP, synthesis again was distributive (Figure 5, lane 4). However, with manganese and 280 μM dNTP, the enzyme now exhibited a very low level of processive synthesis (Figure 5, lane 5). In the reaction mixtures incubated for 20 min, approximately 5% of the extended primer molecules represented processive addition of from 6 to 10 residues. These results indicate the recombinant enzyme, like the natural enzymes, is generally distributive in its mode of synthesis, and the results are partially consistent with a report by Wang and Korn (1982), who found by kinetic methods that β-polymerase from human KB cells was processive for incorporation of approximately five residues in reactions containing Mn^{2+}. The low level of processive synthesis found here could not have been detected by kinetic methods.

Synthesis was evaluated further by using the same template-primer system as in Figure 5, except that in contrast to the processivity assay, the enzyme to template-primer ratio and time of incubation were adjusted to obtain many cycles of enzyme binding, synthesis, and termination. Nevertheless, the polymerase to template-primer ratio was 1 to 4 rather than a stoichiometric range of 1 to 1 or higher. This precaution was used to reduce secondary effects of the enzyme on the template–primer complex, such as nucleic acid binding protein effects or protein–protein interaction. The results are shown in Figure 6. With the long single-stranded template, β-polymerase added as many as about 50 nucleotides to the primer during a 20-min incubation and then extended these molecules into much longer products after a 60-min incubation. Nine prominent bands of product molecules corresponding to so-called "pause sites" were noted after 20-min incubation. Yet, these and the other shorter products were almost completely absent after 60-min incubation. Most of the pause sites corresponded to an incoming G residue. Of the first seven cases where G was the incoming nucleotide, five were pause sites. This was a distinctly different pattern than that seen here with *E. coli* Pol I (not shown) or reported for α-polymerase (Weaver & DePamphilis, 1982).

Gap filling synthesis was examined by annealing an unlabeled oligonucleotide of 16 bases to the template beginning 20 bases in front of the labeled primer. In the presence of this downstream oligonucleotide, the products of DNA synthesis

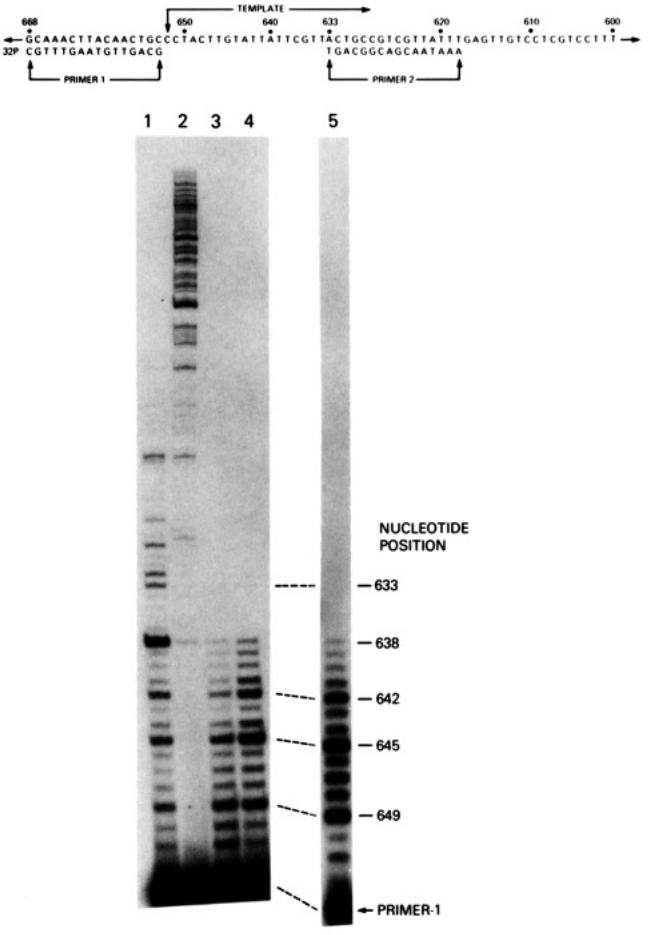

FIGURE 6: Pause site and gap filling analysis of recombinant β-polymerase with ϕX174 DNA as template-primer. Photographs of autoradiograms are shown. At the top is shown synthetic primer 1 5′ end labeled and hybridized to ϕX174 DNA. DNA synthesis reactions were carried out as described in Figure 5, except that in lanes 3–5, both primer 1 and unlabeled primer 2 were hybridized to ϕX174 DNA. DNA synthesis reactions contained 20 mM Tris·HCl, 2 mM $MnCl_2$, 280 μM dNTPs, a 1:4 ratio of polymerase to template, and 10 mM NaCl for lanes 1–4. Additional conditions were as follows: lane 1, only primer 1 hybridized, 20-min incubation; lane 2, conditions identical with lane 1, 60-min incubation; lane 3, both primers hybridized, 20-min incubation; lane 4, both primers hybridized, 60-min incubation; lane 5, both primers hybridized, reaction contained 100 mM NaCl, 60-min incubation. Position indicated on the right refers to template nucleotide opposite the last nucleotide of the product.

from the labeled primer were not elongated beyond position 638, leaving a gap of four to seven nucleotides before the 5′ terminus of the downstream oligomer. After the incubation had been continued for a total of 60 min, the pattern of labeled product molecules was unchanged, and only a very small amount of limited strand displacement of the downstream oligomer was observed (Figure 6, lanes 4 and 5). Raising the sodium chloride concentration of the reaction mixture to 100 mM failed to alter the pattern of product molecules. When the experiment in Figure 6, lanes 1 and 2, was repeated, but with primer 2 labeled instead of primer 1, similar elongation to that with labeled primer 1 was observed, as expected. We found that annealing unlabeled primer 1 to the template strand has no effect on elongation of primer 2. This confirms the presence of active enzyme in the reaction mixtures shown in Figure 6, lanes 3–5, even though the four to seven nucleotide gap is not filled. In experiments not shown, Pol I large fragment, even at 10-fold lower enzyme concentration than β-polymerase, was able to fill the single-stranded gap and displace the downstream oligomer.

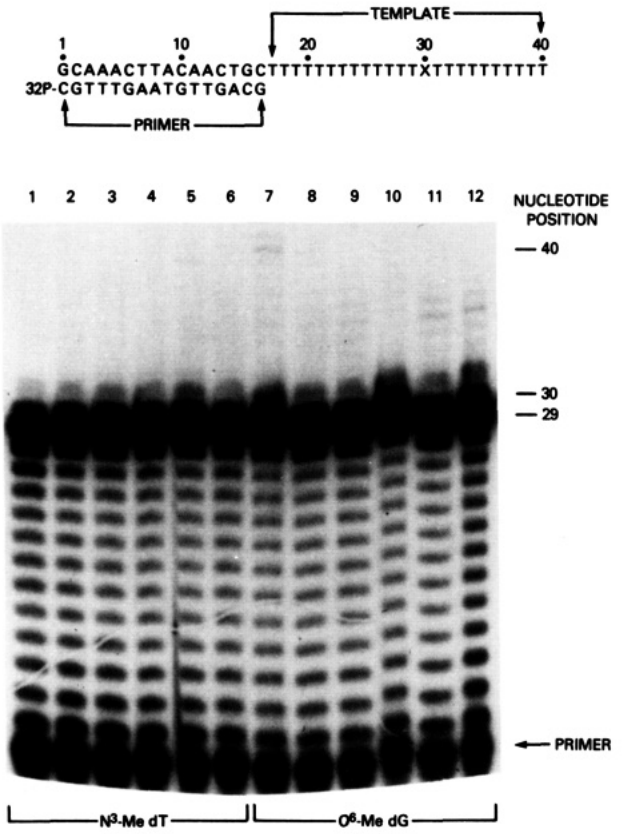

FIGURE 7: Pause site analysis of recombinant β-polymerase with a synthetic homopolymer template containing one methylated base residue. A photograph of an autoradiogram is shown. The template-primers are illustrated at the top. DNA synthesis was carried out as described under Materials and Methods. Reactions contained 400 fmol of template, hybridized at a 2.5:1 molar ratio of primer 1 to template, and 2 mM $MnCl_2$. Incubation times were 60 min. For lanes 1–6, the template contained N^3-methyl-dT at position 30; for lanes 7–12, the template contained O^6-methyl-dG at position 30. All reactions contained 300 μM dATP. Other dNTP concentrations were as follows: lanes 1 and 7, no additional dNTPs; lanes 2 and 8, 600 μM total dATP; lanes 3 and 9, 300 μM dCTP; lanes 4 and 10, 300 μM dGTP; lanes 5 and 11, 300 μM dTTP; lanes 6 and 12, 100 μM each of dCTP, dGTP, and dTTP.

In order to compare these results with a template-primer consisting of single-stranded nicks in plasmid DNA, we prepared a substrate using plasmid pML2. A single-stranded nick was introduced by *Eco*RI activity on ethidium bromide treated DNA. The recombinant β-polymerase, at a molar ratio of 1:4 polymerase to nick, is completely unable to synthesize on this DNA molecule, and after the incubation, we found that the DNA could be ligated by T4 DNA ligase. T7 DNA polymerase strongly synthesizes on this nicked substrate, and the products are not substrates for ligase (Lechner et al., 1983).

Modified Template Residues and β-Polymerase Elongation. We examined the effect on chain elongation of selected base modifications that changed hydrogen-bonding properties between the template and the incoming nucleotide. A synthetic template containing the sequence complementary to primer 1 used above plus 24 residues of single-stranded homopolymer template was used. Modified versions of the template included O^6-methyl-dG and N^3-methyl-dT. Recombinant β-polymerase was unable to synthesize past these two modified residues efficiently, even in the presence of all four dNTPs (Figure 7). Product molecules did not accumulate upstream from the modified template residue, indicating that the enzyme did not detect the presence of the template modification until it attempted to incorporate an incoming nucleotide opposite the modification. At that point, the enzyme showed difficulty incorporating. Even when the polymerase did incorporate at position 30 opposite the modified base, it was not able to further elongate at a significant frequency. Recombinant β-polymerase was able to synthesize to the end of the template with a normal T nucleotide replacing the methyl derivative at position 30 (data not shown).

Discussion

The purification of enzymatically active β-polymerase from *E. coli* transformed with an expression plasmid is unambiguous proof of the authenticity of the cDNA. This is not a trivial point since the identity of the cDNA had been based upon microscale sequencing of enzyme tryptic peptides. Immunobinding and enzymatic studies strongly suggested that recombinant β-polymerase had been obtained. However, proof that the recombinant enzyme indeed had been purified was provided by renaturation experiments after SDS–polyacrylamide gel electrophoresis of the final fraction. The 39-kDa polypeptide in this fraction was extracted from the gel and renatured to an active β-polymerase with good yield relative to the enzyme activity of the starting sample.

The size of the recombinant human enzyme appeared to be the same in the initial crude extract and the final fraction of purified enzyme, indicating there was no major truncation during purification. The crude extract did contain, however, a minor ~27-kDa recombinant polypeptide with a reactive epitope. It should be interesting to evaluate this peptide as a possible proteolysis-resistant domain of β-polymerase.

Our present analysis of catalytic properties of β-polymerase falls into two categories. (1) Is the recombinant enzyme similar to natural β-polymerases? (2) Making use of the purified recombinant enzyme, what are the synthesis properties of β-polymerase on precisely defined template-primer made of natural DNA? In the first category, we found that the specific activity of the recombinant enzyme purified from *E. coli* was about the same as that of natural enzymes purified extensively in this laboratory (Table II; Tanabe et al., 1979; Swack et al., 1985). This suggests that β-polymerase does not require a eukaryotic cell posttranslational modification for activity. Similar observations on expression of another mammalian β-polymerase in *E. coli* have been made recently by Matsukage and co-workers (personal communication). Compared with results described here, those investigators used a different strategy for subcloning a rat cDNA and a different expression system. Yet, they were able to purify a fully active recombinant β-polymerase from an extract of transformed *E. coli*.

The recombinant human enzyme purified from *E. coli*, like the natural enzymes, does not have exonuclease or endonuclease activity. The recombinant enzyme has the same general template-primer specificity as the natural enzyme and, of course, has a reactive epitope for anti-β-polymerase IgG. Thus, the recombinant and natural β-polymerases are very similar. Yet, in spite of the essential similarity of these enzymes, differences were observed in pause site analysis between the recombinant polymerase and the natural enzymes. The chick and calf enzymes showed identical patterns of pause sites on the φX174 template, and their patterns differed slightly from that of the recombinant enzyme. The early strong pause sites for these enzymes are summarized in Table III; some sites are common to all polymerases examined, but differences are also seen. The significance of these differences is unknown. The differences could possibly be due to contaminating proteins in the natural enzyme preparations; all of the natural enzymes tested, calf thymus, HeLa, chick embryo, and Novikoff hepatoma, were less pure than the recombinant enzyme. An

Table III: Pause Sites for β-Polymerase in the First 40 Nucleotides Synthesized on the φX174 Template

nucleotide position,[a] pause site for recombinant enzyme	incoming dNTP	nucleotide position, pause site for chick and calf enzymes
	T	650
649	G	649
645	A	
	T	644
642	A	
638	G	638
633	G	
632	A	
629	G	629
626	G	626
617	T	

[a] Nucleotide position refers to the template residue opposite the end of the product, as in Figure 6. DNA synthesis was carried out as described in Figure 6, and autoradiograms were scanned by densitometry. Bands were compared with their several neighboring bands, and those designated strong stops showed intensities at least twice as great.

alternative explanation could be differential posttranslational modification between the polymerase expressed in bacteria and enzymes synthesized in eukaryotic cells.

In the second category mentioned above, we note that all of the earlier work on detailed characterization of β-polymerase for processivity, gap filling, and strand displacement synthesis was conducted before product chain length analysis and defined natural template-primers were readily available. Therefore, with the availability of such approaches, along with known quantities of pure enzyme, we undertook the present work. Processivity of the recombinant β-polymerase was examined by using a φX174 single-stranded DNA template considered to be free of secondary structure. Under all conditions examined, most of the products of synthesis represented the addition of one nucleotide, indicating a distributive enzyme. In the presence of manganese and higher concentrations of dNTPs (280 μM), however, a small portion of product molecules represented moderately processive synthesis. These findings are partially consistent with an earlier report that polymerase β is moderately processive in the presence of manganese (Wang & Korn, 1982). In addition, Kunkel and Loeb (1981) found that β-polymerase conducts more in vitro replication in φX reversion assays in the presence of supersaturating levels of dNTP. Under these conditions, the mechanistic explanation for the effects of manganese compared with magnesium and of 280 μM dNTP compared with 30 μM dNTP is not clear. We note that high concentrations of dNTP are required to saturate the nucleotide binding site of *E. coli* Pol I in equilibrium dialysis experiments (Kornberg, 1969) and this same phenomenon could be true of β-polymerase. Saturation of a low-affinity binding site for dNTP could drive the enzyme toward forming a productive complex with the template-primer terminus. These ideas remain to be tested experimentally.

Products of DNA synthesis also were analyzed on a φX174 DNA template with larger, but less than stoichiometric, amounts of enzyme and longer incubation times, allowing many cycles of enzyme binding, synthesis, and termination. Under these conditions, annealing a second primer, with its 5′ end 20 nucleotides downstream of the first primer's 3′ end, dramatically blocked extension of product molecules (Figure 6). Polymerase β left a gap of four to seven nucleotides on most products but continued to synthesize from the downstream primer. We were surprised by these results, since the literature contains reports of the ability of β-polymerase to both fill single-stranded gaps and conduct synthesis at nicks (Wang & Korn, 1982; Siedlecki et al., 1981; Mosbaugh & Linn, 1983). We, therefore, tested natural β-polymerases purified from chick and calf, as well as the preparation from HeLa cells for which strand displacement synthesis had been reported (Mosbaugh & Linn, 1983). Using equal amounts (activity) of these enzymes, we observed that each showed behavior on the gapped φX174 template similar to that seen with the recombinant enzyme; annealing the second primer dramatically inhibited extension off the upstream primer. When autoradiograms were overexposed, all enzymes, including the recombinant, showed some very minor synthesis into the region of the downstream primer, but significant gap filling and displacement of this primer were not seen. Hence, it seems possible that the results observed are explained on the basis of primer preference: As the single-stranded gap size was reduced by elongation of primer 1, the second primer and its elongation products are strongly preferred for productive enzyme binding and synthesis. There was little change in the size of the single-stranded gap after 20-min incubation, as the polymerase preferred to extend the downstream primers rather than fill the DNA gap. These results may have implications for DNA repair. Auxiliary proteins may be necessary to assist β-polymerase in completely filling single-stranded gaps or may direct this polymerase to gapped regions of DNA until they are filled completely.

Experiments using synthetic templates showed that β-polymerase incorporates poorly opposite the modified bases N^3-methyl-dT and O^6-methyl-dG (Figure 7). Strong pause sites were seen immediately before these modified bases, both of which disrupt normal hydrogen binding. When the polymerase did incorporate at position 30 opposite the modified base, it was not able to extend synthesis at a significant frequency. This indicates that the enzyme could not use a "loopout" mechanism to synthesize past the modified base and that the normal base pairing at the 3′-OH end of the primer is required for activity. Larson et al. (1985) had previously shown that methylation produces pause sites for Pol I and other enzymes, but to our knowledge, this is the first demonstration that methylated bases altering hydrogen bonding cause pause sites with β-polymerase. Our results, and those of Larson et al. (1985), suggest that methyl groups must be removed from damaged DNA bases before DNA repair synthesis can occur. That polymerases stall at sites opposite a methylated base may provide the cell with a defense against misincorporation, allowing alkyl removal before the extension of synthesis.

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Comparison of the Crystal and Solution Structures of Calmodulin and Troponin C†

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ABSTRACT: X-ray solution scattering data from skeletal muscle troponin C and from calmodulin have been measured. Modeling studies based on the crystal structure coordinates for these proteins show discrepancies between the solution data and the crystal structure that indicate that if the size and shape of the globular domains are the same in solution as in the crystal, the distances between them must be smaller by several angstroms. Bringing the globular domains closer together requires structural changes in the interconnecting helix that joins them.

Calmodulin is a small (M_r 16 700), highly conserved protein that is present in all eukaryotic cells and plays a regulatory role in a diverse set of calcium-dependent cellular processes (Cheung, 1980). Troponin C (M_r 18 000) is a calcium-dependent regulator in muscle systems that shows strong sequence homology with calmodulin. These proteins can cross-react in their respectively biological systems [Means and Dedman (1980) and references cited therein]. The crystal structures of calmodulin (Babu et al., 1985) and skeletal troponin C (Herzberg & James, 1985; Sundaralingam et al., 1985a) have been solved, and they are very similar. Both structures show four Ca^{2+} binding sites each with the helix–loop–helix structural motif that has also been found in parvalbumin (Wery et al., 1985; Moews & Kretsinger, 1975) and in the vitamin D dependent calcium regulating protein from bovine intestine (Szebenyi et al., 1987).

The overall tertiary structures of troponin C and calmodulin are unusual in that they each show two globular domains (each having two Ca^{2+} binding sites) connected by a single α-helix, of about eight turns. The interdomain connecting helix is mostly exposed to solvent and forms few contacts with the rest of the molecule. Questions have been raised as to what stabilizes the interconnecting helix in these structures in the crystal forms and what rearrangements might occur in solution (Herzberg & James, 1985; Sundaralingam et al., 1985b). It has also been suggested that structural changes specifically in the interconnecting helix may be important in the calcium regulation (Babu et al., 1985; Herzberg & James, 1985; Sundaralingam et al., 1985a; Seaton et al., 1985).

Changes in the structure of the interconnecting helix can result in large effects on the overall shape of these proteins. Both troponin C and calmodulin are acidic proteins with p*I* values around 4.5, and the crystals used for the crystallographic analyses were grown at low pH (pH 5.0 and 5.5, respectively). In view of the potential flexibility in the interconnecting helix, it is important to examine the effects of a solution environment as well as changes in pH on the overall structure of these proteins. Fluorescence energy transfer measurements (Wang et al., 1986; Wang & Cheung, 1985) on skeletal troponin C suggest that it undergoes a large conformational rearrangement involving a change in the distance between the two calcium

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