Expression of Active Rat DNA Polymerase β in *Escherichia coli*†

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ABSTRACT: A recombinant plasmid for expression of rat DNA polymerase β was constructed in a plasmid/phage chimeric vector, pUC118, by an oligonucleotide-directed mutagenesis technique. The insert contained a 1005 bp coding sequence for the whole rat DNA polymerase β. The recombinant plasmid was designed to use the regulatory sequence of *Escherichia coli lac* operon and the initiation ATG codon for β-galactosidase as those for DNA polymerase β. The recombinant clone, JMpβ5, obtained by transfection of *E. coli* JM109 with the plasmid, produced high levels of DNA polymerase activity and a 40-kDa polypeptide that were not detected in JM109 cell extract. Inducing this recombinant *E. coli* with isopropyl β-thiogalactopyranoside (IPTG) yielded amounts of 40-kDa polypeptide as high as 19.3% of total protein. Another recombinant clone, JMpβ2-1, which was constructed by an oligonucleotide-directed mutagenesis to use the second ATG codon for the initiation codon, thus deleting the first 17 amino acid residues from the amino terminus, produced neither high DNA polymerase activity nor the 40-kDa polypeptide. The evidence suggests that this amino-terminal structure is important for stability of this enzyme in *E. coli*. The DNA polymerase was purified to homogeneity from the IPTG-induced JMpβ5 cells by fewer steps than the procedure for purification of DNA polymerase β from animal cells. The properties of this enzyme in activity, chromatographic behavior, size, antigenicity, and also lack of associated nuclease activity were indistinguishable from those of DNA polymerase β purified from rat cells, indicating the identity of the overproduced DNA polymerase in the JMpβ5 and the rat DNA polymerase β.

Eukaryotic cells as well as prokaryotes contain multiple species of DNA polymerases (Fry & Loeb, 1986). Those of animal cells, designated DNA polymerase α, β, and γ, are involved in different DNA synthesizing reactions (Fry & Loeb, 1986). These DNA polymerases have common reaction properties: recognition of base on template nucleic acid, recognition of the 3′-OH of primer, binding of dNTP with complementary base to template, and formation of a phosphodiester bond between the 3′-OH of primer and the 5′-α-phosphate of dNTP. Except for *Escherichia coli* DNA polymerase I, little has been clarified in the way of how these functions are related to structures of DNA polymerase molecules. The lack of knowledge about the structures of DNA polymerase has been caused mainly by difficulty in the large-scale preparation of pure enzymes and in the method for modification of the enzyme structures.

Of three eukaryotic cellular polymerases, DNA polymerase β, which has been suggested to be involved in DNA repair (Fry & Loeb, 1986), appears to be the most useful in elucidating the structure–function relationships. The important reasons are that DNA polymerase β is the smallest in size among all known DNA polymerases, and the active enzyme consists of a single polypeptide of about 40 kDa (Yamaguchi et al., 1980b; Tanabe et al., 1979; Stalker et al., 1976; Chang, 1973; Wang et al., 1974). Furthermore, this enzyme is different from prokaryotic DNA polymerase I and III in that it does not contain intrinsic exonuclease (Wang et al., 1974; Kunkel et al., 1978; Chang & Bollum, 1973). In addition, the reaction mechanism of DNA polymerase β is highly distributive (Chang, 1975; Bambara, 1978; Matsukage et al., 1981), in contrast to DNA polymerase α (Willani et al., 1981; Das & Fujimura, 1979) and γ (Matsukage et al., 1981; Yamaguchi et al., 1980a). These simplicities in the structure and reaction properties of DNA polymerase β have encouraged us to study this enzyme.

Previously, we had obtained the cDNA clone for the rat DNA polymerase β and suggested the presence of a 954-nucleotide coding region for this enzyme (Zmudzka et al., 1986). More recently, we have analyzed the structural organization of the 5′-terminal region of the rat DNA polymerase β gene and transcription initiation sites (Yamaguchi et al., 1987). By combining this result with that obtained with the cDNA, we suggested a 1005 bp complete coding sequence for a polypeptide with 335 amino acid residues (Matsukage et al., 1987) instead of the 318 amino acid residues that were previously suggested by the cDNA sequence (Zmudzka et al., 1986). In the present work, we constructed a recombinant plasmid that carried the complete coding region and used it to transform *E. coli*. The DNA polymerase produced in the recombinant was indistinguishable from the rat DNA polymerase β. Furthermore, we employed an oligonucleotide-directed mutagenesis technique to delete 17 amino acid residues from the amino terminus of the enzyme and found that this region was important for stability of the enzyme molecule in *E. coli*.

Materials and Methods

Construction of Expression Plasmids and Recombinant E. coli. Recombinant plasmids pUC9-10F and pUC9-10S were prepared previously and contain the 437-bp 5′-terminal fragment and the 764-bp 3′-terminal fragment, respectively, of the 1197 bp cDNA for rat DNA polymerase β (Zmudzka et al., 1986). Plasmid pUC118, phage M13KO7, *E. coli* strains MV1304 [Δ(lac-proAB), thi, rpsL (strep^r), erdA, sbcB15,

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hspR4, (srl-recA) 306::Tn10 (tet^r) F′: traD36, proAB, lacI^{q}Z M15)] and JM109 [(recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-pro AB9) F′: traD36, proAB, lacI^{q}Z M15)] were purchased from Takara Biotec Co. Synthetic oligodeoxynucleotides were made by the phosphoamidine method using a nucleic acid synthesizer (Nippon Zenon).

The procedure for the construction of expression plasmids is illustrated in Figure 1. The 437-bp cDNA fragment of pUC9-10F was purified by *Eco*RI digestion, agarose gel electrophoresis, and phenol extraction. This fragment was then ligated at the *Eco*RI site of pUC118 and was used to transfect *E. coli* MV1304. Transformants were selected from a plate containing 50 μg/mL ampicillin by colony hybridization using the cDNA probe. The orientation of cDNA inserts was determined by restriction mapping; clones containing cDNA in correct orientation were isolated. The recombinant *E. coli* was grown and infected with helper phage MV13K07. Progeny phages containing the recombinant plasmid DNA in a single-stranded circular form were recovered, and DNA was purified by phenol extraction.

In order to construct a recombinant plasmid containing the complete amino-terminal structure of DNA polymerase β molecule, we employed an oligonucleotide-directed mutagenesis originally reported by Gillam et al. (1980) with modifications (Figure 1C). We prepared a synthetic 30-mer in which the 5′-terminal 13 nucleotides were complementary to the region extending from the ribosome-binding site (Shine–Dalgarno sequence) to the first ATG of the *E. coli* β-galactosidase gene; the 3′-terminal 11 nucleotides were complementary to the 5′-terminal sequence of the DNA polymerase β cDNA. Furthermore, between the 13-mer and the 11-mer, a 6-nucleotide segment that was complementary to the 5′-terminal coding sequence lacking in cDNA was added to obtain the complete coding sequence for DNA polymerase β. By this procedure, the initiation codon for lac Z gene was converted into that for rat DNA polymerase β, keeping exactly the same distance between the Shine–Dalgarno sequence and the initiation ATG codon in the lac gene.

Single-stranded circular DNA (0.9 μg) and phosphorylated 30-mer oligonucleotide (7 ng) in 10 μL of buffer containing 120 mM NaCl, 30 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.9, 6 mM $MgCl_2$ and 10 mM β-mercaptoethanol were heated in a sealed capillary tube for 1 min at 100 °C. Incubation was then continued for 3 h as the temperature was gradually lowered from 60 to 28 °C. The solution was transferred to a plastic tube and was mixed with 1.6 μL each of 2 μM dATP, dGTP, dCTP, dTTP, and rATP, followed by 1 μL of DNA polymerase I Klenow large fragment (80 units) and 1 μL of T4 DNA ligase (5000 units). This mixture was incubated at 28 °C for 2 h; the reaction was then terminated by adding 3 μL of phenol. After extraction with ether 3 times, an aliquot (2 μL) containing heteroduplex DNA was used for transformation of *E. coli* HB101 by a calcium chloride procedure (Maniatis et al., 1982). The transformant-containing mutagenized sequence was selected by colony hybridization using a ^{32}P-labeled 30-mer as a probe. Colony hybridization was performed by the method of Grunstein and Hogness (1975) as modified by Hollenberg et al. (1984) for synthetic oligonucleotide probes. Since positive clones might contain both the original and the mutant-type plasmids, plasmids were extracted to transform JM109, and positive clones were again isolated. The obtained clones now exclusively contained the mutant-type plasmid, which was confirmed from evidence that the mutant-type plasmid had one *Eco*RI site instead of the two sites in the original type.

By use of the same method and an oligodeoxyribonucleotide 26-mer, the recombinant plasmid in which the second ATG of DNA polymerase β gene [the first ATG present in the cloned rat cDNA fragment (Zmudzka et al., 1986)] was used for the initial codon. Therefore, in the polypeptide coded by the insert in this recombinant, 17 amino acids were deleted from the amino terminus of the intact enzyme molecule (Figure 1C). In this recombinant, 42 5′-terminal nucleotides of the cDNA as well as the β-galactosidase coding sequence and *Eco*RI linker were deleted.

Two types of plasmid DNAs, thus prepared, were cut by *Eco*RI at the 3′-terminal end of 10F cDNA inserts. The 10F cDNA fragment obtained from pUC19-10F by *Eco*RI digestion and gel electrophoresis was ligated to complete the whole DNA polymerase β coding sequence, and then the obtained plasmids were transfected into JM109. Clones containing plasmids with correct orientation were selected. Clones with sequences coding 335 and 318 amino acid residues were designated JMpβ5 and JMpβ2-1, respectively.

Purification of DNA Polymerase from Recombinant E. coli JMpβ5. JMpβ5 cells were cultivated at 37 °C in 1 L of 2 × YT medium supplemented with 50 μg/mL ampicillin. When the absorbance at 600 nm of the culture reached 0.5, isopropyl β-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and culturing continued for an additional 7 h before cell harvest. The wet weight of obtained *E. coli* was about 3 g.

All procedures for enzyme purification were carried out at 0–5 °C unless otherwise indicated. Two cell extraction methods were investigated.

Method 1. E. coli (1.6 g) was suspended in 8 mL of the extraction buffer [50 mM Tris-HCl at pH 7.6, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 10% glycerol, 0.5 M KCl, and 1 mM phenylmethanesulfonyl fluoride (PMSF)], and cells were disrupted by sonication for 1 min. The extract was clarified by centrifugation for 20 min at 12000*g*.

Method 2. About 1.6 g of *E. coli* was suspended in a 5-mL solution containing 50 mM Tris-HCl, pH 7.8, and 25% sucrose, and then 12.5 mg of egg white lysozyme was added. The cell wall was digested by incubating for 1 h at 4 °C. Then EDTA was added to a final concentration of 50 mM, and the mixture was incubated for an additional 5 min. Cells were lysed by mixing the cell suspension with 5 mL of lysing solution containing 0.5% Nonidet P-40, 0.8 M KCl, 2 mM PMSF, and 50 mM Tris-HCl, pH 7.8. The lysate was clarified by centrifugation at 12000*g* for 30 min. DNA in the supernatant was sheared by using a syringe with a 21-gage needle. The yield of DNA polymerase activity and 40-kDa polypeptide in method 2 was 5-fold or more greater than the yield in method 1.

The extract obtained in either method was diluted by PC buffer (50 mM Tris-HCl at pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) to adjust KCl concentration to 0.4 M and applied to a (diethylamino)ethylcellulose (DEAE-cellulose) column (1 × 7 cm) that had been equilibrated with 0.4 M KCl–PC buffer. This process was to remove nucleic acids. The eluent that contained DNA polymerase activity was diluted by an equal volume of PC buffer to reduce KCl concentration and applied to a phosphocellulose column (1 × 10 cm) equilibrated by 0.2 M KCl–PC buffer. After extensive washing, protein was eluted with a 150-mL linear gradient of KCl from 0.2 to 0.7 M in PC buffer. Fractions of 4 mL were collected, and DNA polymerase activity in a 1-μL aliquot of each fraction was determined. Although a

minor portion of DNA polymerase activity was present in fractions passed through the column, the majority of the enzyme activity was retained with the column and was recovered as a single peak at about the 0.4 M KCl region. Most of the DNA polymerase in the unbound fraction, although not confirmed, might have been *E. coli* DNA polymerase I. The KCl concentration required for the elution of the major activity was almost identical with that for elution of chick DNA polymerase β from a phosphocellulose column (Yamaguchi et al., 1980b). Pooled active fractions were diluted 3-fold with PC buffer and then applied to a denatured calf thymus DNA–cellulose column (0.6 × 5 cm) equilibrated with 0.15 M KCl–PC buffer (20% glycerol). After extensive washing, DNA polymerase activity was eluted with 0.6 M KCl–PC buffer (20% glycerol). A single peak of DNA polymerase activity was recovered. Purified enzyme was stored at −20 °C after glycerol was added to a final concentration of 50%.

The content of the 40-kDa polypeptide at this purification stage was about 95%, as judged by a $NaDodSO_4$–polyacrylamide gel electrophoresis. Further purification was carried out by using gel filtration and the second DNA–cellulose column chromatography with elution using a linear gradient system. The enzyme preparation at the first DNA–cellulose step was applied to a 1.2 × 50 cm column made of Sephadex G-150, and chromatography was developed with PC buffer containing 0.15 M KCl. Fractions containing high DNA polymerase activity were pooled and applied to a DNA–cellulose column (0.6 × 5 cm) that had been equilibrated with 0.15 M KCl in PC buffer. Protein was eluted by a 30-mL linear KCl gradient (0.15–0.7 M in PC buffer). Fractions with high DNA polymerase activity were pooled and supplemented with glycerol to a final concentration of 50%. The final preparation was stored at −20 °C.

Assay for DNA Polymerase and Nucleases. DNA polymerase activity was determined in the reaction conditions that had been used for chick DNA polymerase β (Yamaguchi et al., 1980b). Briefly, the reaction (25-μL final volume) contained 50 mM Tri-HCl (pH 8.8 at 37 °C), 1 mM dithiothreitol, 0.5 mM $MnCl_2$, 40 μg/mL $(rA)_n$, 40 μg/mL $(dT)_{12-18}$, 0.1 mM [^{3}H]dTTP (15 or 60 cpm/pmol), 15% (v/v) glycerol, 400 g/mL bovine serum albumin, 120 mM KCl, and 1–2 μL of enzyme solution. After incubation at 37 °C for 10 or 30 min, radioactive DNA product was collected on a DEAE-cellulose paper (DE 81) disc as described by Lindell et al. (1970). Radioactivity was measured with a scintillation counter. One unit of DNA polymerase was defined as the amount catalyzing the incorporation of 1 nmol of deoxyribonucleotides in polymer DNA for 60 min. The units of Klenow's large fragment of DNA polymerase I were also calculated by this definition in this study, although the reaction condition for this enzyme was different from that for DNA polymerase β.

For 3′–5′ exonuclease assay, 3′-terminal ^{32}P-labeled DNA was made as follows. A 2347 bp DNA fragment was obtained from plasmid pML-1 by cleaving with restriction endonuclease *Hin*dIII and *Sal*I and by agarose gel electrophoresis fractionation. The 3′-region of the DNA was ^{32}P-labeled at the cleavage sites by using [α-^{32}P]dCTP and Klenow's large fragment. The reaction (50-μL final volume) contained 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 20 mM NaCl, 7 mM β-mercaptoethanol, 100 μg/mL gelatin, 10^5 cpm of ^{32}P-labeled DNA, and 60 units of DNA polymerase purified from JMpβ5 or 72 units of Klenow's large fragment. Incubation was carried out at 37 °C. At indicated times, 10-μL aliquots were taken, and the reaction was terminated by mixing with 3 μL of 0.24 M EDTA/0.1% $NaDodSO_4$. An aliquot of each sample was analyzed by 0.5% agarose gel electrophoresis and autoradiography.

Endonuclease activity was measured by detecting the conversion of a closed circular form of pUC19 DNA into nicked circular form. Reaction mixtures (25-μL final volume) were identical with that for exonuclease assay, except that 2 μg of pUC19 DNA (content of closed circular form was 80–90%) was added instead of [^{32}P]DNA. In this experiment, 120 units of JMpβ5 DNA polymerase or 80 units of Klenow's large fragment were used. Incubation was at 37 °C, and 5-μL aliquots were taken at indicated times. The reaction was terminated by mixing with 2 μL of dye mixture for agarose gel electrophoresis. DNA was analyzed by electrophoresis in an 0.6% agarose gel, followed by ethidium bromide staining.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis and Immunowestern Blot Analysis. Protein was analyzed by electrophoresis in a 10% polyacrylamide gel containing $NaDodSO_4$. Protein in gel was stained with Coomassie brilliant blue or subjected to an immunowestern blot analysis. In the latter experiment, protein was transferred electrophoretically from gel to a nitrocellulose membrane by using a blotting apparatus for 15 h at 2000 V at 4 °C in a buffer containing 25 mM Tris, 192 mM glycine (pH 8.3), and 20% methanol. The membrane was blocked with TBS solution (50 mM Tris-HCl at pH 8.3 and 150 mM NaCl) containing 20% fetal calf serum for 30 min at room temperature and then incubated with 500-fold-diluted anti-chick DNA polymerase β rabbit antiserum (Yamaguchi et al., 1982) for 2 h at room temperature. After washing with TBS, the membrane was incubated with 500-fold-diluted anti-rabbit immunoglobulin G goat antibody conjugated with biotin, followed by avidin conjugated with horseradish peroxidase for 2 h at room temperature. Color was developed in the TBS solution containing 0.05% 4-chloronaphthol, 0.015% H_2O_2, and 16.5% methanol.

Results

Construction of Expression Recombinants. The construction of expression recombinant, clone JMpβ5, was carried out as shown in Figure 1. In this recombinant, the insert DNA capable of encoding whole rat DNA polymerase β was arranged so as to use the regulatory region for *E. coli lac* operon; the expression can be induced by adding IPTG to the culture medium. The initial codon for β-galactosidase was converted into that for DNA polymerase β by a synthetic oligonucleotide-directed mutagenesis. Therefore, messenger RNA of DNA polymerase β might be as effectively translated as that of β-galactosidase. In addition, we constructed the recombinant, JMpβ2-1, in which 51 nucleotides at the 5′-terminal of the above insert were deleted by a oligonucleotide-directed mutagenesis, because the second ATG codon was previously thought to be the initial codon for this enzyme (Zmudzka et al., 1986).

DNA Polymerase Activity in Extracts of the Recombinant E. coli. Recombinants JMpβ5 and JMpβ1-2 and the host *E. coli* JM109 were cultivated in 6 mL of 2 × YT medium containing 50 μg/mL ampicillin. When absorbance at 600 nm reached 0.5, each culture was divided into two equal portions: one portion was supplemented with IPTG to a final concentration of 1 mM. After shaking for an additional 6 h at 37 °C, cell extracts were made in a procedure similar to extraction method 1 and used for determination of DNA polymerase activities (Table I). IPTG-induced JMpβ5 extract yielded DNA polymerase activity of about 20-fold that of JM109 extract. Although JMpβ5 cells produced high DNA polymerase activity in the absence of IPTG, its amount was

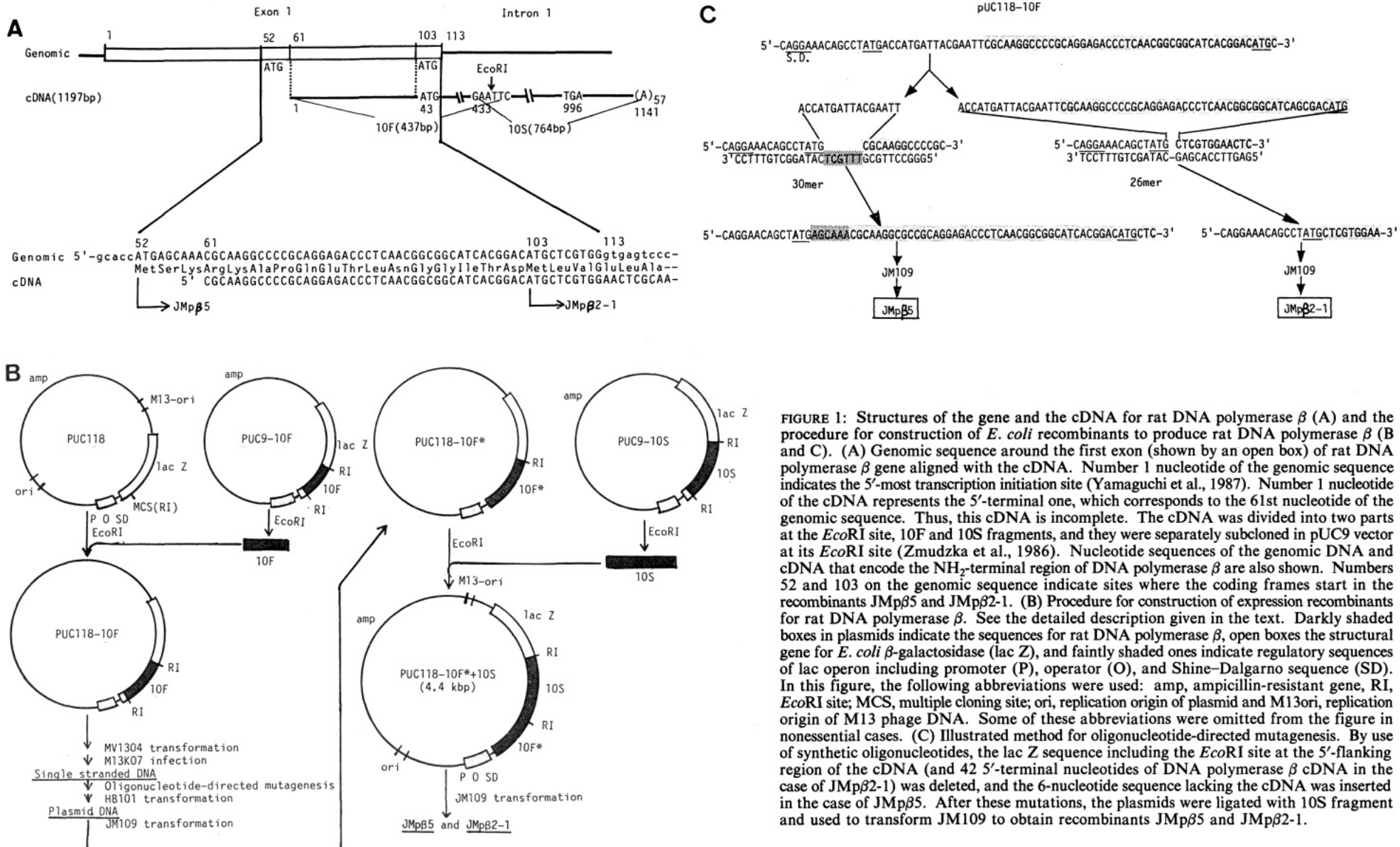

FIGURE 1: Structures of the gene and the cDNA for rat DNA polymerase β (A) and the procedure for construction of *E. coli* recombinants to produce rat DNA polymerase β (B and C). (A) Genomic sequence around the first exon (shown by an open box) of rat DNA polymerase β gene aligned with the cDNA. Number 1 nucleotide of the genomic sequence indicates the 5′-most transcription initiation site (Yamaguchi et al., 1987). Number 1 nucleotide of the cDNA represents the 5′-terminal one, which corresponds to the 61st nucleotide of the genomic sequence. Thus, this cDNA is incomplete. The cDNA was divided into two parts at the *Eco*RI site, 10F and 10S fragments, and they were separately subcloned in pUC9 vector at its *Eco*RI site (Zmudzka et al., 1986). Nucleotide sequences of the genomic DNA and cDNA that encode the NH_2-terminal region of DNA polymerase β are also shown. Numbers 52 and 103 on the genomic sequence indicate sites where the coding frames start in the recombinants JMpβ5 and JMpβ2-1. (B) Procedure for construction of expression recombinants for rat DNA polymerase β. See the detailed description given in the text. Darkly shaded boxes in plasmids indicate the sequences for rat DNA polymerase β, open boxes the structural gene for *E. coli* β-galactosidase (lac Z), and faintly shaded ones indicate regulatory sequences of lac operon including promoter (P), operator (O), and Shine–Dalgarno sequence (SD). In this figure, the following abbreviations were used: amp, ampicillin-resistant gene, RI, *Eco*RI site; MCS, multiple cloning site; ori, replication origin of plasmid and M13ori, replication origin of M13 phage DNA. Some of these abbreviations were omitted from the figure in nonessential cases. (C) Illustrated method for oligonucleotide-directed mutagenesis. By use of synthetic oligonucleotides, the lac Z sequence including the *Eco*RI site at the 5′-flanking region of the cDNA (and 42 5′-terminal nucleotides of DNA polymerase β cDNA in the case of JMpβ2-1) was deleted, and the 6-nucleotide sequence lacking the cDNA was inserted in the case of JMpβ5. After these mutations, the plasmids were ligated with 10S fragment and used to transform JM109 to obtain recombinants JMpβ5 and JMpβ2-1.

Table I: DNA Polymerase Activity in Crude Extracts of Recombinant and Host *E. coli* Cells[a]

strain	1 mM IPTG in culture	DNA polymerase activity (units/μL of extract)
JM109	−	6.2
	+	8.8
JMpβ2-1	−	12.3
	+	15.2
JMpβ5	−	34.9
	+	152.2

[a] Each *E. coli* strain was cultivated and IPTG induced as described in the text. Cells in 6 mL of culture were collected by centrifugation and suspended in 200 μL of extraction buffer (50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.5 M KCl), and cells were disrupted by sonication. The extract was obtained by centrifugation at 1000*g* for 15 min at 2 °C, and 5 μL of the 50-fold-diluted extract was mixed with the reaction mixture to determine the DNA polymerase activity. DNA polymerase activities were expressed as unit numbers per microliter of extract.

increased by adding IPTG. JMpβ2-1 cell extracts seemed to contain slightly higher DNA polymerase activity than those of JM109; however, we have not determined whether this increased amount of activity was due to overproduced recombinant DNA polymerase or the difference between the amounts of *E. coli* DNA polymerase in JMpβ2-1 and JM109. An approximate estimation of specific DNA polymerase activity with respect to the 40-kDa polypeptide in the JMpβ5 extract (estimated from the value in Table I) and the amount of the 40-kDa polypeptide in the crude extract (lane 1 of Figure 4A) was about 6×10^5 units/mg of protein. This value was similar to that of the homogeneous preparation of chick embryo DNA polymerase β (11×10^5 units/mg) (Yamaguchi et al., 1980b). The evidence suggests that most of the enzyme extracted from recombinant JMpβ5 cells has enzyme activity similar to that of animal cells.

Analysis of Protein from the Recombinant E. coli. Recombinant *E. coli* cells were cultivated as above and collected by centrifugation. Cells were resuspended in $^1/_{15}$ the original volume of sample loading solution for $NaDodSO_4$–polyacrylamide gel electrophoresis. After cells were lysed by heating for 2 min at 100 °C, protein was analyzed by electrophoresis. As shown in Figure 2, the JMpβ5 cell contained a predominant polypeptide band of about 40 kDa: its amount accounted for 19.3% of the total extracted protein after IPTG induction. The 40-kDa polypeptide was not detectable in either JMpβ2-1 or the host strain JM109 cells even after cultivation in the presence of IPTG.

Purification of DNA Polymerase Produced in Recombinant JMpβ5 Cells. DNA polymerase was purified from the extract of IPTG-induced JMpβ5 cells by ion-exchange column chromatographies, gel filtration, and DNA–cellulose column chromatography as described under Materials and Methods. The behavior of DNA polymerase activity along with the 40-kDa polypeptide in this procedure was almost identical with that of chick DNA polymerase β (Yamaguchi et al., 1980b). Gel filtration (Figure 3A) demonstrated that DNA polymerase activity is associated with a protein of about 40 kDa, obviously different from *E. coli* DNA polymerase I or III, but identical with animal cell DNA polymerase β (Yamaguchi et al., 1980b; Tanabe et al., 1979; Stalker et al., 1976; Chang, 1973; Wang et al., 1974). After only three steps, a considerably pure DNA polymerase preparation was obtained; that is, the purity of 40-kDa polypeptide was about 95% (Figure 4B). Further purification using gel filtration (Figure 3A) and a second DNA–cellulose column chromatography (Figure 3B) yielded a preparation almost homogeneous with respect to the 40-kDa polypeptide (Figure 4B). Specific DNA polymerase activity

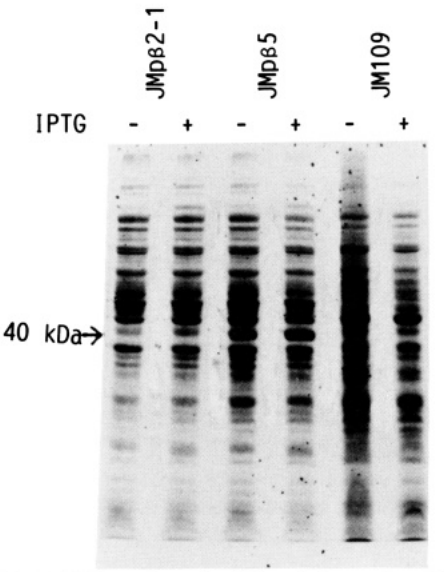

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of proteins in extracts of the expression recombinants JMpβ5 and JMpβ2-1 and the host *E. coli* JM109. These recombinant *E. coli* strains were cultivated in the presence or absence of IPTG. Cells were lysed in the presence of $NaDodSO_4$ and β-mercaptoethanol, and protein was analyzed. Extracts made of 0.25-mL original cultures that contained approximately 20 μg of protein were loaded to the gel. Proteins were stained with Coomassie brilliant blue.

was estimated as about 7.5×10^5 units/mg of protein on the basis of enzyme activity and protein content.

A comparison of purified JMpβ5 DNA polymerase and rat DNA polymerase β by $NaDodSO_4$–polyacrylamide gel electrophoresis demonstrated that their sizes are indistinguishable (Figure 5A) and further suggests that the rat enzyme is also composed of 335 amino acids instead of 318. An immunowestern blotting analysis indicated that anti-chick DNA polymerase β antibody (Yamaguchi et al., 1982) reacted well with the 40-kDa polypeptide of JMpβ5 DNA polymerase as with those of rat and chick DNA polymerase β (Figure 5B).

Check of Associated Nuclease. Some DNA polymerases are associated with exonuclease activity. *E. coli* DNA polymerase I, for example, is associated with both 3′–5′ and 5′–3′ exonuclease activity (Kornberg, 1974). *E. coli* DNA polymerase III is also associated with 3′–5′ exonuclease activity (Bachmair, 1986). Animal cell DNA polymerases α and β, on the other hand, do not contain intrinsic nuclease activity (Wang et al., 1974; Kunkel et al., 1978; Chang & Bollum, 1973). Therefore, we checked nuclease activity in the purified JMpβ5 DNA polymerase to obtain further supporting evidence of the identity of this enzyme as DNA polymerase β. As shown in Figure 6A, the JMpβ5 enzyme at the purification stage after the first DNA–cellulose column chromatography did not significantly reduce the radioactivity from 3′-^{32}P-labeled DNA, whereas Klenow's large fragment associated with 3′–5′ exonuclease digested this substrate extensively. Although the Klenow enzyme preparation converted very slightly the form I plasmid DNA into form II, the JMpβ5 DNA polymerase did not (Figure 6B), indicating that the JMpβ5 DNA polymerase preparation did not contain detectable endonuclease activity.

Discussion

The properties examined thus far of DNA polymerase produced in the recombinant *E. coli* JMpβ5 were indistinguishable from those of rat DNA polymerase β. However, very minor differences between these enzymes have not yet been ruled out. One possible difference is NH_2-terminal

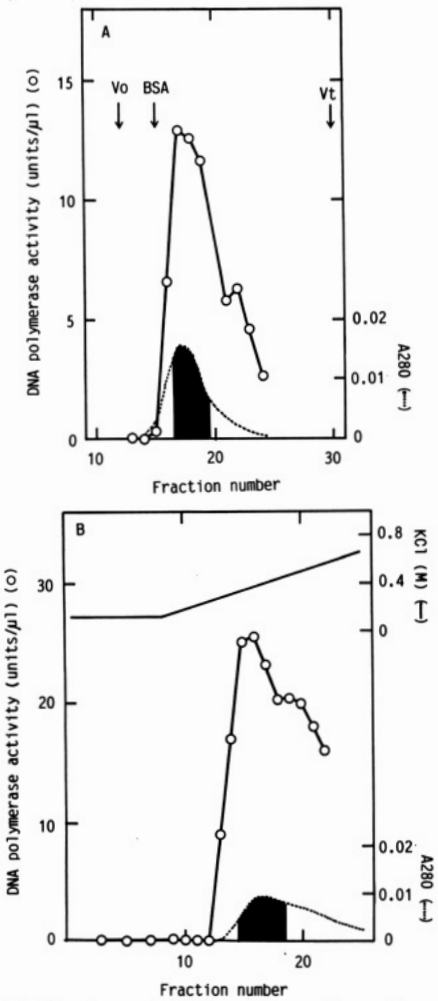

FIGURE 3: Gel filtration and DNA–cellulose column chromatography of JMpβ5 DNA polymerase. (A) DNA polymerase preparation after the first DNA–cellulose column chromatography was applied to a Sephadex G-150 column (1.2 × 50 cm), and chromatography was developed with PC buffer containing 0.15 M KCl. A 1-μL aliquot of each fraction of 4 mL was used to determine DNA polymerase activity. Shaded fraction were pooled and further purified. The chromatography was calibrated by using blue dextran, bovine serum albumin (68 000 Da), and dATP, whose elution positions are indicated by Vo, BSA, and Vt, respectively. (B) DNA polymerase after gel filtration were applied to a denatured calf thymus DNA–cellulose column (1 × 5 cm), and protein was eluted with a 30-mL linear KCl gradient from 0.15 to 0.7 M in PC buffer. Fractions of 1 mL were collected, and a 1-μL aliquot of each fraction was used to determine DNA polymerase activity. Under these conditions, the incorporation of dTMP was not linearly correlated with enzyme amounts, presumably due to shortage of substrate and/or template primer. Thus, the values over 15 units were underestimated in this figure. Eventually, the pooled fractions (indicated by shading) contained more than 50 units of DNA polymerase/μL.

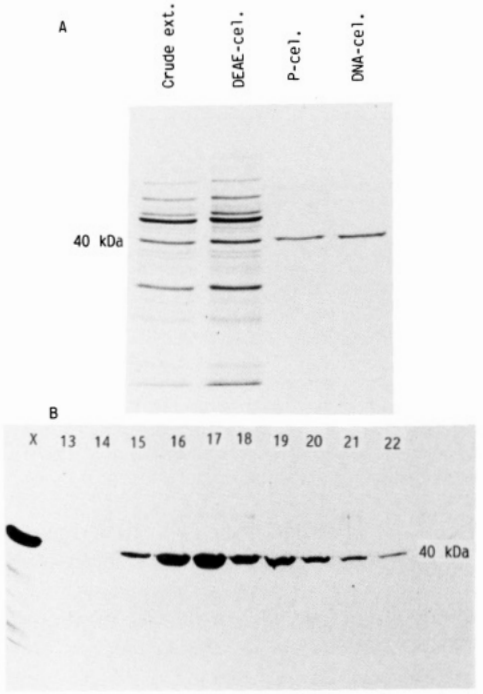

FIGURE 4: NaDodSO$_4$–polyacrylamide gel electrophoresis of proteins in JMpβ5 DNA polymerase preparations. Polypeptides in the pooled fractions at various purification steps were analyzed (A). Since the crude extract used in this experiment was made by method 1, the amount of 40-kDa polypeptide was not high in comparison with that shown in Figure 2. Extract made by method 2 contained 5 times more 40-kDa polypeptide than did extract made by method 1 (data not shown). (B) Analysis of polypeptide in 50-μL aliquots of fractions obtained in the second DNA–cellulose column chromatography. The lane numbers correspond to those fractions in Figure 3B. Lane X shows the preparation after the first DNA–cellulose step. Small amounts of polypeptides other than the 40-kDa one were detected. Most of these contaminants were removed after the second DNA–cellulose step.

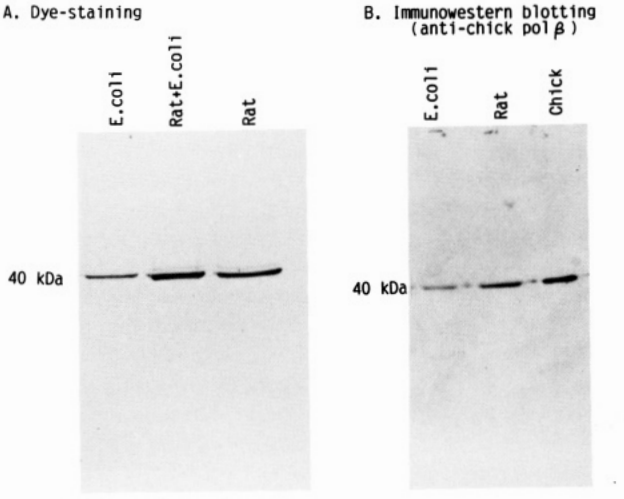

FIGURE 5: Comparison of JMpβ5 DNA polymerase with rat DNA polymerase β. (A) NaDodSO$_4$–polyacrylamide gel electrophoresis of the purified JMpβ5 DNA polymerase (lane indicated by *E. coli*), rat DNA polymerase β (rat), and the mixture of two enzymes (rat and *E. coli*) were analyzed. (B) Immunowestern blotting analysis of JMpβ5 DNA polymerase (*E. coli*), rat DNA polymerase β (rat), and chick embryo DNA polymerase β (chick) were electrophoresed and electrophoretically blotted onto a nitrocellulose membrane. Antigenic substances were then visualized by using anti-chicken DNA polymerase β rabbit serum and horseradish peroxidase conjugated second antibody. Cross-reactivity of this antibody to murine DNA polymerase β was reported previously (Tanabe et al., 1984).

structure. Rat and chick DNA polymerase β are both blocked at this site so that no amino acid was recovered by Edman's reaction (Zmudzka et al., 1986). The NH_2 terminus of the recombinant-produced enzyme, however, was not blocked (our preliminary data).

An interesting question is what structure causes the difference in the stabilities of enzymes produced by JMpβ5 and JMpβ2-1. The high degree of stability of the former seems to be supported by the additional 17 amino acid residues at the amino terminus that are absent in the product of the latter. Recently, Bachmair et al. (1986) proposed a "N-end rule" suggesting the half-lives of proteins in yeast are dependent on the species of amino acids at their amino termini: protein with Met, Ser, etc. as the terminal residue had a half-life exceeding 20 h, whereas protein with Leu, Phe, etc., had a half-life of about 3 min. They also suggested that the N-end rule might

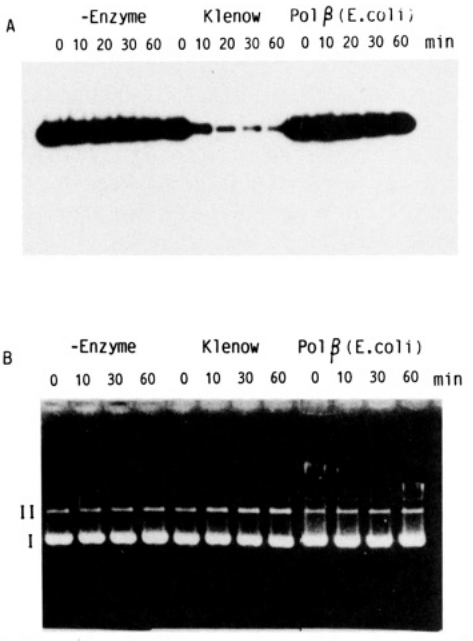

FIGURE 6: Detection of 3′–5′ exonuclease (A) and endonuclease (B) in the preparations of JMpβ5 DNA polymerase and Klenow's large fragment of DNA polymerase I. In these experiments, the recombinant enzyme preparation after the first DNA–cellulose step was used. (A) DNA polymerases were mixed with 3′-terminal labeled DNA fragment. Aliquots of the reactions were withdrawn at indicated times of incubation and analyzed by agarose gel electrophoresis and autoradiography. The amount of radioactivity in the DNA band decreased in the reaction with Klenow's large fragment, which is associated with 3′–5′ exonuclease, whereas no significant reduction of radioactivity was detected in the reaction with the recombinant enzyme. (B) Enzymes were incubated with pUC19 plasmid DNA (form I content of about 80%), and the reaction products were analyzed by agarose gel electrophoresis with ethidium bromide staining.

be applicable to prokaryotes. Our preliminary results of the amino-terminal sequencing of the JMpβ5 product indicate that the (formyl)Met might be removed from the terminus and that Ser was detected. If the (formyl)Met is also removed from the product of JMpβ2-1, the terminal amino acid is Leu. Thus, the difference in the stabilities of these two products may reflect the different amino acids at the amino termini.

The recombinant with the coding region for 335 amino acid residues, but not that for 318 residues, produced active DNA polymerase in a stable form. This evidence in combination with the identical sizes of the rat and the recombinant-produced enzyme indicates that the rat DNA polymerase β consists of 335 (or 334) amino acid residues and has a molecular weight of about 38 300. This value is larger than the value reported previously (Zmudzka et al., 1986).

Although the recombinant strain JMpβ5 produced DNA polymerase β in large quantities in the presence of IPTG, cell growth was identical (data not shown) with that of the host strain JM109, indicating that the production of active enzyme is neither deleterious nor fatal. It will be interesting to determine whether the DNA polymerase β produced in *E. coli* can replace the DNA repair function of DNA polymerase I in DNA polymerase I-negative and radiation-sensitive mutants.

We have established a simple procedure to obtain a homogeneous preparation of DNA polymerase β in a nuclease-free form from recombinant JMpβ5 cells. Since the amount of DNA polymerase β polypeptide accounted for almost 20% of total protein in IPTG-induced recombinant cells, it may be possible to obtain about 50 mg of DNA polymerase β from *E. coli* cells cultured in 1 L of medium. Availability of a large quantity of purified DNA polymerase β opens various fields of applications. For example, X-ray diffraction analysis of the crystallized enzyme in free or complex form with DNA or dNTP may resolve the difficulties concerning structural organization of active sites of this enzyme. Another interesting approach is site-directed mutagenesis, which may identify sites of the enzyme responsible for various functions, for example, binding to template, recognition of primer, binding to dNTP, signaling for the nuclear localization, etc. In this respect, the multicopy plasmid pUC118 used in this study seems to be a useful expression vector: it has a strong *lac* promoter as well as a multicloning site downstream of the initiation codon for β-galactosidase gene. This plasmid also can be converted into a single-stranded circular form that simplifies the mutagenesis by using synthetic oligonucleotide. Using this technique in this study, we designed the expression plasmid that has the complete original *lac* sequence at the 5′-untranslated region, facilitating translation of rat DNA polymerase β mRNA. Finally, overproduced DNA polymerase β may be useful in place of Klenow's large fragment of DNA polymerase I in recombinant DNA technology. The DNA polymerase activity free from exonuclease activity seems to allow the 3′-OH termini of DNA to effectively form blunt ends.

Registry No. DNA polymerase, 9012-90-2.

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Rabbit Liver Factor D, a Poly(thymidine) Template Stimulatory Protein of DNA Polymerases: Purification and Characterization†

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ABSTRACT: Factor D, a DNA binding protein that enhances the activities of diverse DNA polymerases with a common restricted set of templates, was initially characterized in mouse liver but has resisted extensive purification. In this paper, we report that a similar stimulatory activity can be obtained in highly purified form from nuclei of rabbit hepatocytes. The rabbit liver protein increases the rates at which several DNA polymerases copy sparsely primed natural DNA templates and primed synthetic poly(dT), but it has no effect on the rates of copying of activated DNA or of poly(dG), poly(dA), and poly(dC). Direct binding of the purified stimulatory protein to an oligomer that contains a $(dT)_{16}$ base stretch is visualized by retardation of the nucleoprotein complex on nondenaturing electrophoretograms. In the presence of the enhancing factor, Michaelis constants, K_m, of responsive polymerase for singly primed bacteriophage M13 DNA and for poly(dT), but not for poly(dA), are decreased. Product analysis of M13 DNA primer extension indicates that the rabbit factor augments the apparent processivity of DNA polymerase by decreasing the extent of enzyme pausing at a tract of four consecutive thymidine residues in the template. Gel filtration of the native stimulatory protein yields an apparent relative molecular size of 58 ± 2 kilodaltons. Stimulatory activity is readily inactivated by heat or by trypsin digestion, but it is resistant to micrococcal nuclease, *N*-ethylmaleimide, or calcium ions.

Studies on *Escherichia coli* indicate that DNA binding proteins are required for the replication of DNA (Kornberg, 1980). Several DNA binding proteins from animal cells have been shown to enhance the activities of DNA polymerases [reviewed by Chase and Williams (1986), Richter et al. (1986), and Fry and Loeb (1986)]. However, in contrast to such proteins in prokaryotes, the role of single-strand DNA binding proteins in the replication of eukaryotic cell DNA is debatable (Chase & Williams, 1986; Richter et al., 1986). Yet, analysis of the modes of enhancement of DNA polymerase activity by eukaryotic DNA binding proteins affords insight into the molecular details of DNA synthesis. A unique template-selective DNA polymerase stimulating protein, that has been designated factor D, was isolated recently from regenerating liver of the mouse (Fry et al., 1985). By selectively increasing the affinities of DNA polymerases from diverse sources for sparsely primed DNA and for the synthetic template poly(dT), factor D enhances the rates of their copying (Fry et al., 1987a). The murine stimulatory protein has no significant effect, however, on the efficacies of copying of activated DNA or of poly(dA), poly(dG), poly(dC), poly[d(A-T)], or poly[d(G-C)] (Fry et al., 1985, 1987a). It was demonstrated recently that factor D enhances the copying of singly primed bacteriophage M13 DNA by increasing the efficiency at which either *E. coli* pol I or bovine polymerase α traverses a cluster of at least four contiguous thymidine residues in this template (Fry et al., 1987b). Hence, by binding to DNA, factor D decreases the frequency at which polymerases pause at stretches of consecutive dT residues in synthetic or natural templates, thus increasing the apparent processivity of the enzymes (Fry et al., 1987b).

Definitive studies on factor D and on analogous activities require their purification to a very high degree. Purification of factor D has been hindered, however, by the limited amounts of extractable mouse tissue as well as by the instability of the partially purified murine protein. In this paper, we report the isolation and extensive purification of a DNA binding protein from rabbit liver that stimulates DNA polymerases in a template-specific manner analogous to that of mouse factor D.

Materials and Methods

Materials. Tritium- or α-^{32}P-labeled deoxynucleoside 5′-triphosphates ([α-^{32}P]dNTPs), and adenosine 5′-[γ-^{32}P]tri-

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