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Aspartic Acid Residues at Positions 190 and 192 of Rat DNA Polymerase β Are Involved in Primer Binding†

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ABSTRACT: The sequence Gly-Asp-Met-Asp, spanning positions 189–192 of rat DNA polymerase β, is similar to the sequence motif Gly-Asp-Thr-Asp that is highly conserved in a number of replicative DNA polymerases from eukaryotic cells, viruses, and phages. The role of this sequence in the catalytic function of rat DNA polymerase β was investigated by individually changing each amino acid in this region by site-directed mutagenesis. The mutant enzymes DE190 and DE192, in which aspartic acid residues at positions 190 and 192, respectively, were replaced by glutamic acid, showed about 0.1% activity of the wild-type enzyme. On the other hand, the replacement of Gly-189 by alanine or Met-191 by isoleucine or threonine only slightly affected the enzyme activity. A gel mobility shift assay showed that DNA complexes with enzyme DE190 and especially with DE192 were less stable than the corresponding complex with the wild-type enzyme. Kinetic analysis with these mutant enzymes indicate that their K_m's for primer DNA were about 10-fold higher than that of the wild type, while K_m's for deoxyribonucleoside triphosphate were not changed. Since neither DE190 nor DE192 had any significant alteration in secondary structure, our results suggest that both Asp-190 and Asp-192 are located in the active site and are involved in the interaction of DNA polymerase β with primer.

DNA polymerase β is found in a wide range of animals, from sponges to humans but not in plants or bacteria (Chang, 1976), and is believed to function in DNA repair (Friedberg, 1985) and in DNA recombination (Hirose et al., 1989). The enzyme consists of a single polypeptide of approximately 38 kDa[1] and is the smallest among known prokaryotic and eukaryotic DNA polymerases. The amino acid sequence of DNA polymerase β in various vertebrates is highly conserved (Fry & Loeb, 1986); the enzymes from rat and human are composed of 335 amino acids (including a methionine at the N terminus) deduced from cDNA and genomic nucleotide sequences (Zmudzka et al., 1986; Matsukage et al., 1987; SenGupta et al., 1986). An active recombinant rat DNA polymerase β has been overproduced in *Escherichia coli* and purified to homogeneity (Date et al., 1988).

The localization of different functional regions in rat DNA polymerase β has been suggested. The template DNA-binding acivity has been proposed to reside in the N-terminal 20% of this enzyme (Kumar et al., 1990a,b). A lysine residue at position 71 is proposed to be located in or around the nucleotide-binding pocket, since pyridoxal 5′-phosphate covalently bound to this residue inhibited the deoxyribonucleoside triphosphate binding (Basu et al., 1989). Additionally, site-directed mutagenesis indicated that Arg-183 is involved in

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[1] Abbreviations: bp, base pair(s); kDa, kilodalton; IPTG, isopropyl β-D-thiogalactopyranoside; CD, circular dichroism.

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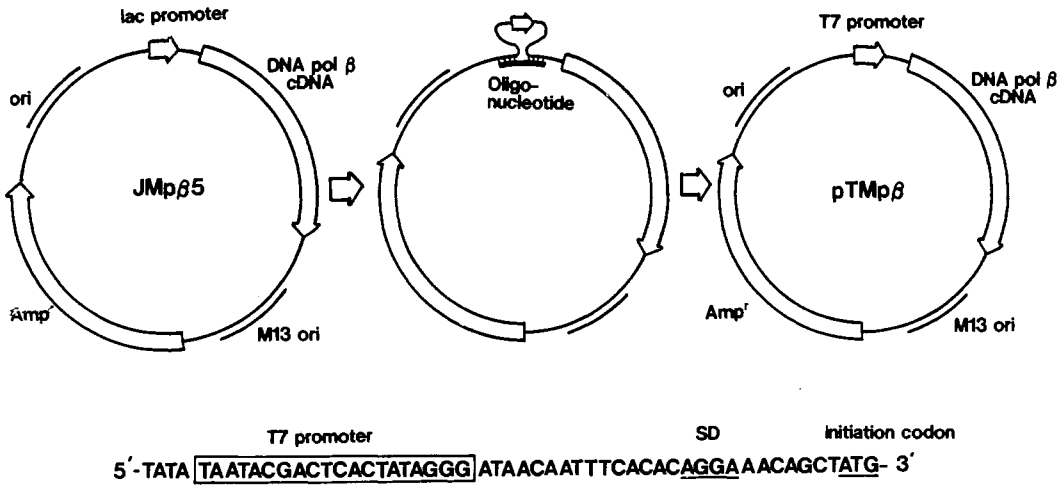

FIGURE 1: Construction of the pTMpβ plasmid for rat DNA polymerase β production. Circular single-stranded DNA was prepared from JMpβ5 plasmid and annealed with oligonucleotide 5'TGAAATTGTTATCCCTATAGTGAGTCGTATTATTGGGCGCTC3' followed by polymerization with Klenow fragment. The portions that hybridize with single-stranded DNA are underlined. The product was introduced into *E. coli* JM109 and selected by colony hybridization with use of the mutagenic primer as a probe. As a result of this promoter replacement, the vector portion is 161 bp shorter than JMpβ5. The sequence between promoter and initiation codon for rat DNA polymerase β of pTMpβ is indicated at the bottom.

```
                                    190                 195
                  N-Glu Ser Ser Gly Asp Met Asp Val Leu Leu Thr-C
                  5'GAG TCC AGT GGA GAT ATG GAC GTT CTG CTG ACC 3'
For Mutageneses
GA181              3'TC AGG TCA CGT CTA TAC CTG CA 5'
DE190                   3'G TCA CCT CTC TAC CTG CAA GA 5'
DS190                  3'GG TCA CCT AGG TAC CTG CAA GA 5'
MI191                   3'G TCA CCT CTA TAG CTG CAA GAC GA 5'
MT191                   3'T TCA CCT CTA TGA CTG CAA GAC G 5'
DE192                        3'CT CTA TAC CTT CAA GAC GAC T 5'
DS192                     3'CA CCT CTA TAC AGG CAA GAC GAC T 5'

For Back Mutations
ED190                   3'G TCA CCT CTA TAC CTG CAA GA 5'
ED192                        3'CT CTA TAC CTG CAA GAC GAC T 5'
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FIGURE 2: Oligonucleotides used for site-directed mutagenesis of rat DNA polymerase β. Amino acid sequence from position 186 to 196 is shown at the top. Oligonucleotides are complementary to the cDNA sequence except nucleotide substitution positions, which are underlined. The oligonucleotides at the bottom were used for back mutation of DE190 and DE192 plasmids to wild type.

primer recognition (Date et al., 1990).

A computer-aided homology search indicated that rat DNA polymerase β contains long regions with a nucleotide sequence that is similar to that of human terminal transferase (Matsukage et al., 1987; Anderson et al., 1987). The primary sequence of DNA polymerase β, however, shared almost no similarity with sequences of replicative DNA polymerases except for one small region, Gly-Asp-Met-Asp (GDMD) at positions 189–192 of rat DNA polymerase β. This sequence is similar to four amino acids in the Tyr-Gly-Asp-Thr-Asp-Ser (YGDTDS) motif found in the highly conserved domain I of a number of replicative DNA polymerases (Wang et al., 1989). The sequence motif YGDTDS has been proposed to form the three-dimensional structure for binding to deoxyribonucleoside triphosphate (Gibbs et al., 1985; Bernad et al., 1987; Larder et al., 1987). However, no direct biochemical data supporting this speculation have been obtained.

In the present work, the role of the GDMD motif in rat DNA polymerase β was examined by changing each amino acid in this motif by site-directed mutagenesis and then characterizing the resultant mutant enzymes. The aspartic acid residues were found to be important for interaction with primer.

Materials and Methods

Strains and Plasmid. *E. coli* strain JM109 (Vieira & Messing, 1987) was used as a host for the construction of mutant plasmids. *E. coli* strain BL21 containing a single copy of the gene for T7 RNA polymerase in the chromosome under the *lac*UV5 promoter is a gift from Dr. Studier (Studier & Moffatt, 1986) and was used as a host for the expression of rat DNA polymerase β.

The plasmid pTMpβ, which overproduces rat DNA polymerase β, was constructed from the plasmid JMpβ5 (Date et al., 1988). Both the *lac* promoter and the residual portion of *lac*Z gene in JMpβ5 were replaced by the bacteriophage T7 promoter consisting of a 20-bp conserved sequence (McAllister & Morris, 1981; see Figure 1). Since the plasmid pTMpβ also contains both pUC and M13 replication origins and the entire coding sequence for rat DNA polymerase β, it is suitable for mutagenesis and subsequent expression experiments. The plasmid pTMpβ is more stable and produces more DNA polymerase β than did the original plasmid JMpβ5.

Site-Directed Mutagenesis. Oligonucleotides were synthesized by a Nippon Zeon polynucleotide synthesizer. Sequences of the oligonucleotides used for mutagenesis are shown in Figure 2. Single-stranded plasmid pTMpβ DNA was prepared from virion-like particles by phenol–chloroform extraction (Vieira & Messing, 1987; Date et al., 1988). Changes in the nucleotide sequence were made be use of oligonucleotides for primed synthesis on single-stranded circular pTMpβ DNA as described previously (Date et al., 1990). Positive clones

were isolated by colony hybridization with use of the mutagenic primers as a probe. In each case, the mutant plasmid was sequenced by the dideoxy method (Sanger et al., 1977) to confirm the presence of the mutation.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Determination of DNA Polymerase Activity by Activity Gels. Cells harboring wild-type or mutant pTMpβ plasmids were cultured in 20 mL of 2× YT medium (1.6% polypeptone, 1.0% yeast extract, and 0.5% NaCl, pH 7.2) containing 50 μg/mL ampicillin. When the absorbance of the culture at 600 nm approached 0.2, isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) was added to a final concentration of 1 mM and the cells were grown for an additional 4 h. Cells were harvested and disrupted by use of a sonicator as described previously (Date et al., 1990). Crude lysates were then subjected to SDS–polyacrylamide gel electrophoresis. In order to determine the relative amounts of 38-kDa DNA polymerase β polypeptide, the gel was stained with Coomassie Brilliant Blue and scanned by a Shimazu gel scanner at 540 nm.

DNA polymerase activity in crude extracts was detected by the activity gel method as described by Spanos et al. (1981) with minor modifications (Yamaguchi et al., 1982). DNA polymerase β was detected at the gel region corresponding to 38 kDa, while endogenous *E. coli* DNA polymerase I was detected at 100 kDa.

Circular Dichroism. Analyses were made with use of a Jasco J-20 recording spectropolarimeter equipped with a CD attachment and a path length of 2 mm at 20 °C. Measurements were performed at protein concentrations from 50 to 200 μg/mL. The protein concentrations were determined by the method of Lowry et al. (1951) with use of egg white lysozyme as a standard.

Purification and Assay of Recombinant DNA Polymerase β Enzymes. Mutant enzymes were purified to near homogeneity as described for the wild-type enzyme (Date et al., 1988, 1990). DNA polymerase β activity was determined with use of poly(rA)-oligo(dT) as a template–primer with reaction conditions that were used for the chick enzyme (Yamaguchi et al., 1980). For the determination of enzymological parameters, the concentration of dTTP or the primer oligo-$(dT)_{12-18}$ was varied.

Gel Retardation Assay for DNA Binding. After oligo$(dA)_{24}$ (30 ng) was end-labeled with [γ-^{32}P]ATP by use of T4 polynucleotide kinase (Takara Biochemicals), 2 μg each of unlabeled oligo$(dA)_{24}$ and oligo$(dT)_{24}$ was added in 25 μL of buffer containing 10 mM Tris-HCl, pH 7.8, and 50 mM NaCl for 3 min. The mixture was rapidly chilled to 0 °C, and an aliquot of the resultant oligo$(dA{\cdot}dT)_{24}$ (0.2 μg) was used in the gel retardation assay. The oligo$(dA{\cdot}dT)_{24}$ was incubated with 0.2 μg of the purified enzyme in 10 μL of binding buffer containing 10 mM Tris-HCl, 25 mM KCl, 0.25 mM DTT, 25% glycerol, and 0.1 mM EDTA at 37 °C for 5 min. Then, the mixture was electrophoresed through a 5% polyacrylamide gel (5 V/cm) at 4 °C for the indicated time with use of a Tris–acetate–EDTA buffer system (Fried & Crothers, 1981). The gels were prerun for 10 min in the same electric field before use. After being dried, the gels were exposed to Konica X-ray film.

Results

Site-Directed Mutagenesis. Rat DNA polymerase β contains the GDMD sequence at positions 189–192. We used site-directed mutagenesis to change Gly-189 into alanine, Asp-190 into serine or glutamic acid, Met-191 into isoleucine or threonine, or Asp-192 into serine or glutamic acid. These mutant enzymes are designated as GA189, DS190, DE190, MI191, MT191, DS192, and DE192, respectively.

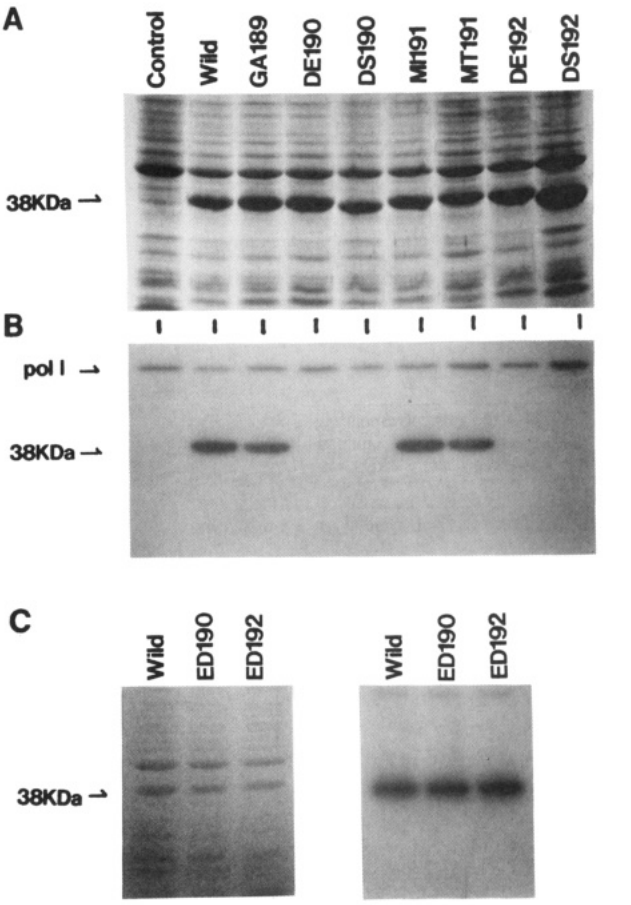

FIGURE 3: SDS–polyacrylamide gel electrophoresis analysis of extracts from IPTG-induced *E. coli* BL21 harboring expression plasmids for rat DNA polymerase β and the detection of DNA polymerase activity by an activity gel method. Cells were cultured and synthesis of enzyme was induced by IPTG. Proteins were stained by Coomassie Brilliant Blue (A). Equal volumes of cell lysate were also electrophoresed under the same conditions except with use of a gel containing active calf thymus DNA. After renaturation of the protein by removal of SDS from the gel, DNA synthesis reactions in situ were carried out with [^{32}P]dCTP (B). Incorporation of [^{32}P]dCTP by *E. coli* DNA polymerase I is detected at about the 100-kDa position. The dye-stained gel and the autoradiogram of the activity gel of revertants from DE190 and DE192 are shown in (C).

Site-directed mutagenesis was carried out by use of synthetic oligonucleotides (Figure 2) to prime synthesis on a single-stranded pTMbβ template, which contains the full-length coding sequence for rat DNA polymerase β under control of the T7 promoter. After identification of colonies harboring the desired mutated plasmids by colony hybridization, the plasmid DNAs were extracted and introduced into *E. coli* BL21 strain, a host strain used for the overproduction of rat DNA polymerase β. The synthesis of mutant DNA polymerase β was induced by IPTG, and crude cell lysates were analyzed by SDS–polyacrylamide gel electrophoresis. As shown in Figure 3A, mutant enzymes represented about 20% of total *E. coli* protein.

Activities of Mutant Enzymes. To detect the DNA polymerase activities of mutant enzymes in crude extracts, *E. coli* lysates were examined by an activity gel method. High-level incorporation of dCTP was observed with the extracts of recombinants producing the wild-type and the GA189, MI191, and MT191 mutant enzymes at the appropriate region of the gel, while very low-level incorporation was observed with those for DS190, DE190, DS192, and DE192 (Figure 3B).

Table I: Activities of Wild-Type and Mutant DNA Polymerase β[a]

DNA polymerases	incorporation of [^{32}P]dCTP (a) (cpm)	staining intensity (b)	a/b	relative activity (%)
wild	4364	24250	0.178	(100)
GA181	2481	42773	0.058	32
DE190	9	45882	0.00020	0.1
DS190	5	27714	0.00018	0.1
MI191	6436	31101	0.206	116
MT191	2448	26419	0.0927	52
DE192	8	33233	0.00024	0.1
DS192	9	50097	0.00018	0.1
		revertants		
wild	2660	15448	0.172	(100)
ED190	2500	15130	0.165	96
ED192	2594	15236	0.170	99

[a] Regions of the gels corresponding to 38-kDa fragments were excised and counted. After staining by Coomassie Brilliant Blue, the gel was scanned at 540 nm and the peak at 38 kDa was quantified.

Table II: Properties of Wild-Type and Mutant DNA Polymerase β

DNA polymerase	sp act. at V_{max} (units/mg of protein)	relative activities (%)	K_m dTTP (μM)	K_m oligo(dT) (μg/mL)
wild	470000	100	39	2.1
DE190	88	0.019	40	18.2
DE192	63	0.013	67	25.0

The activity of mutants at Asp-190 or Asp-192 was detectable only after prolonged exposure (data not shown). Back-mutation of both DE190 and DE192 into the wild-type sequence using oligonucleotides yielded wild-type DNA polymerase activity, indicating that the low activity of both DE190 and DE192 was solely due to the mutations at the desired position. Table I shows the relative activity of mutant enzymes calculated from the radioactivity in the activity gel and the dye-staining densities of the 38-kDa polypeptides in the gel shown in Figure 3. Under the conditions used, the incorporation of [^{32}P]dCTP was proportional to the amount of protein used (data not shown). The relative activities of DE190, DS190, DE192, and DS192 were about 0.1% of the wild-type enzyme. It should be especially pointed out that DE190 and DE192 in which either of these aspartic acids was converted into glutamic acid, the amino acid with the most similar property to aspartic acid among the other 19 amino acids, resulted in extensive inactivation. Thus, it is strongly suggested that aspartic acid residues at these sites are stringently required for DNA polymerase β activity. It was also noticed that the MT191 mutant enzyme having the GDTD motif found in replicative DNA polymerases maintained an activity similar to the wild-type enzyme with the GDMD sequence.

Purification of Mutant Enzymes. For further enzymatical characterization of DE190 and DE192, the overproduced proteins were purified to apparent homogeneity. These mutant enzymes were in the soluble fraction of crude lysates and eluted from phosphocellulose columns at the same ionic strength (about 0.5 M KCl) as the wild-type enzyme. The specific activity of these enzymes measured under standard assay conditions indicated that each activity was reduced by more than 3 orders of magnitude in comparison with the wild-type enzyme (Table II). The relative activity of purified DE190 and DE192 is similar to that obtained by the activity gel method (Table I). Since *E. coli* DNA polymerase I was removed during purification (Date et al., 1990), these results indicate that low residual activity in DE190 and DE192 preparations is not due to low-level contamination of other DNA polymerases but due to the intrinsic activity of the mutant enzymes.

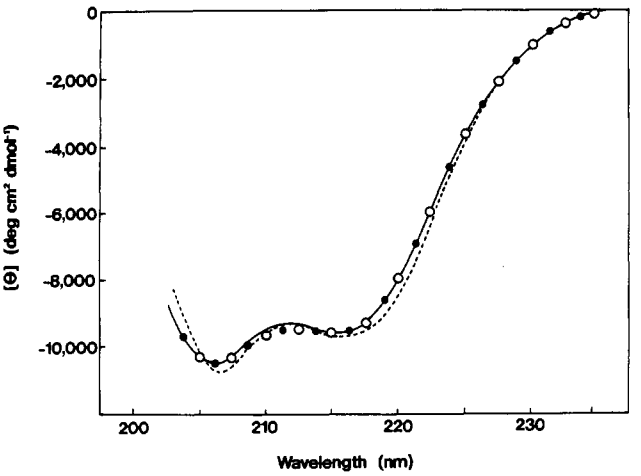

FIGURE 4: Circular dichroism spectra of recombinant rat DNA polymerase β. The solid line, open circles, and closed circles show the spectra of wild-type, DE190, and DE192, respectively. The dashed line shows the computed spectrum of protein containing 24% α helix and 10% β structure.

Secondary Structure of DNA Polymerase β. The CD spectra of wild-type and two mutant enzymes were measured in physiological aqueous solution. Double minima at 207 and 220 nm indicate that the enzyme contains a helical structure (Figure 4). On the basis of the difference curve (Chen et al., 1972), the secondary structure of the wild-type enzyme was computed as 24% helical structure and 10% β structure. No significant difference in CD spectra between wild-type enzyme and the mutant enzymes was observed, indicating that both DE190 and DE192 mutations produced no significant conformational alterations in the structure of the polypeptide backbone as detected by this method.

DNA-Binding Reaction. To clarify whether the decreased polymerase activity in these two mutant enzymes is due directly to the alteration of DNA-binding activity, a gel retardation assay was carried out. After each enzyme was incubated with oligo(dA·dT) in the presence of 1 mM EDTA, the mixture was electrophoresed through a gel for 0.5, 1.5, and 2.5 h. As shown in Figure 5A, the amount of DE192–DNA complex decreased markedly with increasing time of electrophoresis while that of the DE190–DNA complex was slightly reduced when compared with the wild-type enzyme. The estimated half-lives of the DE190 and DE192 complexes are about 4.0 and 1.5 h, respectively (Figure 5C). On the other hand, when single-stranded oligo(dA) was used, no detectable difference was observed among wild-type, DE190, and DE192 proteins. These complexes of single-stranded DNA and DNA polymerase β were more unstable than the corresponding complex with double-stranded DNA, since no complex was visible after 1.5 h of electrophoresis (Figure 5B).

Kinetics of Mutant Enzymes. The above results strongly suggest that Asp-190 and Asp-192 are involved in primer or template DNA binding reactions. In order to distinguish these possibilities, the K_m for primer was determined. Figure 6 shows the dependency on the primer concentration for mutant and wild-type enzymes, indicating that the K_m's of DE190 and DE192 enzymes for the primer were 18.2 μg/mL and 25 μg/mL, respectively, which were almost 10-fold higher than that of the wild-type enzyme (2.1 μg/mL). These results are consistent with those of the gel retardation assay.

On the other hand, the K_m's of DE190 and DE192 for deoxyribonucleoside triphosphate were 40 μM and 67 μM, respectively, which are in the same range as that of the wild-type enzyme (39 μM) (Figure 7). These results strongly

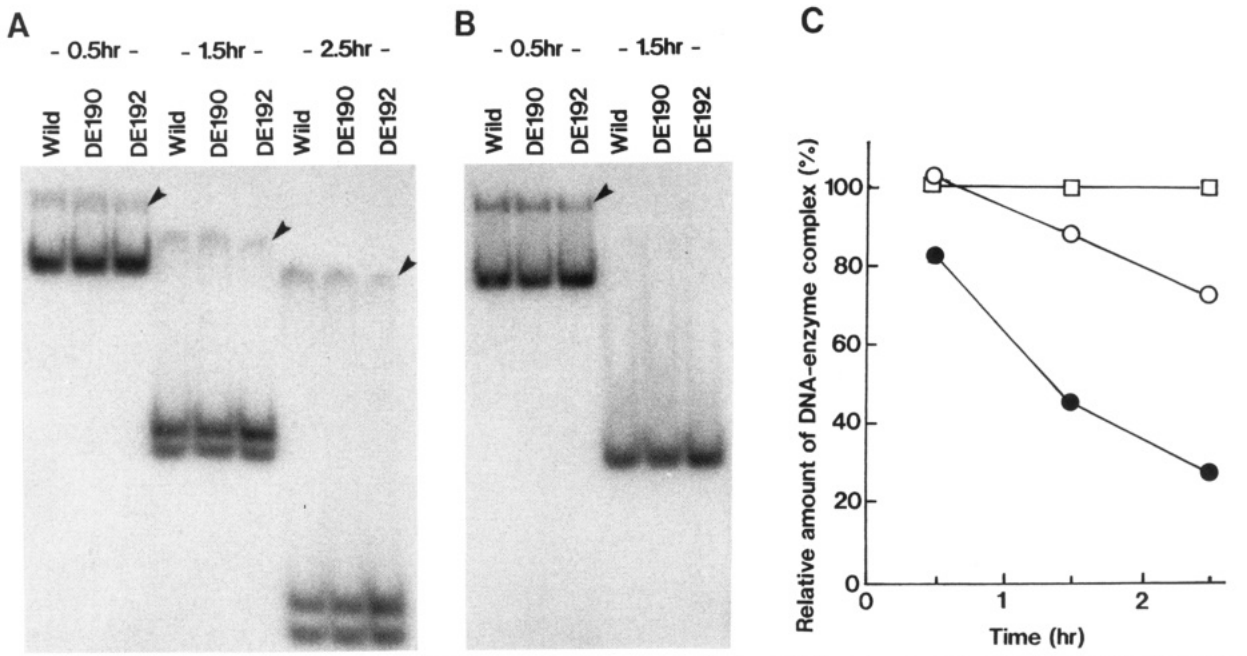

FIGURE 5: Gel retardation assay of the complex with DNA and wild-type or mutant rat DNA polymerase β. After incubation of the enzyme with ^{32}P-labeled oligo(dA·dT) (A) or oligo(dA) (B), samples were loaded on a gel and electrophoresed for the indicated time under the conditions described in Materials and Methods. Arrows indicate DNA–enzyme complexes. The front and the following unretarded oligonucleotides in (A) are single-stranded and double-stranded oligonucleotides, respectively. Regions of the gels corresponding to the retarded complex were excised and counted. Percentages of relative binding compared with corresponding wild-type binding are shown for oligo(dA·dT) (C): (□) wild-type; (○) DE190; (●) DE192.

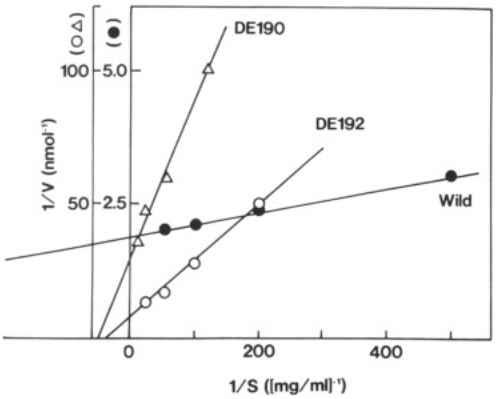

FIGURE 6: Dependence of oligo(dT) concentration on wild-type and mutant DNA polymerase β enzymatic activity. The concentration of oligo(dT) was varied from 2 to 40 μg/mL in the presence of a constant concentration of poly(rA) (40 μg/mL). Lineweaver–Burk plots of wild-type enzyme, DE190, and DE192 are shown. The value of 25 on the transversal axis is the point where the ratio of primer to template is 1.

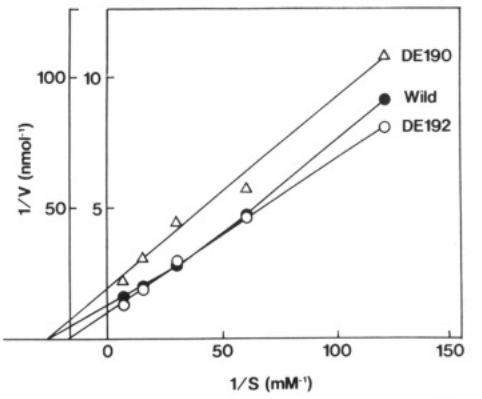

FIGURE 7: Dependence of dTTP concentration on wild-type and mutant DNA polymerase β activity. In the presence of 40 μg/mL poly(rA) and 20 μg/mL oligo(dT), the dTTP concentration was varied from 8 to 128 μM. Lineweaver–Burk plots of wild-type enzyme, DE190, and DE192 are shown.

suggest that the reduced activity of DE190 or DE192 enzyme was caused by the alteration in the primer-binding region of DNA polymerase β.

Discussion

Although the primary sequence of rat DNA polymerase β shares significant similarity with that of human terminal transferase (Matsukage et al., 1987; Anderson et al., 1987), no obvious sequence similarity with replicative DNA polymerases is evident except one small region containing GDMD sequence motif spanning from position 189 to 192. As shown in Table III, a GDTD sequence is highly conserved in both prokaryotic and eukaryotic DNA polymerases involved in DNA replication. The two aspartic acids are also conserved in terminal transferase. In order to elucidate the function of the GDMD of DNA polymerase β, each of these amino acids was substituted by an other amino acid by site-directed mutagenesis and the enzymological properties of these mutant enzymes were investigated.

As shown in Table II, the two aspartic acids in the GDMD domain play critical roles in the DNA polymerization reaction; the activities of mutant enzymes DE190 and DE192 were reduced by more than 1000-fold in comparison with the wild-type enzyme. The gel retardation assay suggested that the mutation of either aspartic acid reduced the binding affinity for the template or primer. Kinetic analysis of the residual activities of these mutant enzymes indicated that the mutations reduced the primer-binding affinity by more than 10-fold but did not alter the affinities for deoxyribonucleoside triphosphate, suggesting that the two aspartates in the conserved sequence are at the least involved in the primer DNA binding reaction. It is possible that these two aspartates also play an important role in the catalytic reaction or the other unidentified process because V_{max} was also decreased by the mutation. Recently we showed that Arg-183 is involved in

Table III: Conserved Gly-Asp-X-Asp Sequences in DNA Polymerases[a]

DNA polymerase	Position	Amino acid sequence	References
Rat and human pol β	183	R G A E S S **G D** M **D** V **L** L **T** H	(Zmudzka et al., 1986)
EBV	750	R V I Y **G D** T **D** S **L** F I E	(Gibbs et al., 1985)
Adeno 2	865	K S V Y **G D** T **D** S **L** F V T	(Gibbs et al., 1985)
HSV-1	881	R I I Y **G D** T **D** S I F V L	(Gibbs et al., 1985)
HCMV	961	R V I Y **G D** T **D** S V F V R	(Kouzarides et al., 1987)
VZV	846	K V I Y **G D** T **D** S V F I R	(Bernad et al., 1987)
Vacc.	724	R S V Y **G D** T **D** S V F **T** E	(Earl et al., 1986)
φX29	451	R I I Y **C D** T **D** S I H L T	(Larder et al., 1987)
PRDI	423	R P L Y **C D** T **D** S I I C R	(Barnad et al., 1987)
Human pol α	997	E V I Y **G D** T **D** S V M I N	(Wong et al., 1988)
Dros. pol α	1007	D V V Y **G D** T **D** S **L** M I N	(Hirose et al., unpub. data)
Yeast pol I	990	L V V Y **G D** T **D** S V M I D	(Pizzagalli et al., 1988)
T4	613	F I A A **G D** T **D** S V Y V C	(Spicer et al., 1988)
Human TdT	339	R G K K M G H **D** V **D** F **L** I **T** S	(Peterson et al., 1985)

[a] The amino acids in common with rat or human DNA polymerase β are boxed. The single amino acid code is used. The numbers indicate the position of the first residue in each sequence.

DNA primer recognition (Date et al., 1990). Therefore, together with our previous finding, it is strongly suggested that Arg-183, Asp-190, and Asp-192, in the segment from Arg-183–Asp-192, are required for interaction with the primer. Arg-183 probably binds to the backbone phosphate of the primer by electrostatic interaction, whereas the aspartic acids may interact with either an oxygen atom of the deoxyribose backbone or the 3′-hydroxyl group of deoxyribose by hydrogen bonding.

Recently, the template-binding region of rat DNA polymerase β was proposed by Kumar et al. (1990). Under the controlled conditions, the protein was cleaved at position 82 or 88 by trypsin and small and large fragments were produced. The smaller fragment consisted of the NH_2-terminal 20% of the protein and possessed a strong binding activity for single-stranded DNA, while the larger fragment did not. They also reported that positions 1–14 were not involved in the template-binding reaction. Since no single-stranded DNA binding activity was changed by mutation of Asp-190 and Asp-192, these two aspartic acid residues may not be involved in template binding. Therefore, the template-binding domain is localized to the region between positions 15 and 81, whereas the primer-binding site includes the region spanning amino acids 183–192 of rat DNA polymerase β.

Site-specific or linker-insertion mutagenesis of the YGDTDS region in human adenovirus DNA polymerase, herpes simplex virus DNA polymerase, and φX29 DNA polymerases resulted in a reduction of activity (Chen & Horwitz, 1989; Wang et al., 1989; Bernad et al., 1990). On the basis of site-specific mutagenesis results using φX29 DNA polymerase and its homology with known or putative metal-binding amino acid sequences, Bernad (1990) proposed that the YGDTDS consensus motif is part of the deoxyribonucleoside 5′-triphosphate binding site, which is important for the catalytic activities of the nucleic acid polymerases. Crystallographic analysis of the Klenow fragment of *E. coli* DNA polymerase I indicated that two carboxylate groups of Asp-355 and Glu-357, located in homologous regions, are involved in metal binding and thereby associate with deoxyribonucleotide triphosphate (Derbyshire et al., 1988). Since conformation of the YGDTDS region is predicted to be a β structure, two carboxylate groups would be oriented in the same direction. Thus, prediction of potential metal-binding function in this region is acceptable. This possibility was supported by photolabeling experiments using the β subunit of bovine terminal transferase. After the protein was photolabeled with [γ-^{32}P]-8-azido-dATP, Evans et al. (1989) determined that a 25 amino acid tryptic fragment containing corresponding GDTD motif was covalently bound to the substrate analogue (1989). However, interpretation of these results is complicated since the fragment also contains three nucleotide-binding motifs that are found in adenylate kinase but not in other DNA polymerases. In contrast to these predictions, it seems to be unlikely that both Asp-190 and Asp-192 of rat DNA polymerase β coordinate the metal ion associated with the substrates because the mutation caused no significant change in K_m for deoxyribonucleoside triphosphate. Crystallographic analysis should unequivocally resolve this conflict.

The question is raised whether the function of the GDMD domain of rat DNA polymerase is the same as the GDTD motif in replicative DNA polymerases. There are some common features in two motifs besides sequence similarity. First, both motifs are located at the C terminus of the protein. Second, both motifs are predicted to form β structure (Matsukage et al., 1987; Argos, 1988). Third, site-specific or linker-insertion mutagenesis of this region in adenovirus, herpes simplex virus, and φX29 DNA polymerases resulted in inactivation of these enzymes (Chen & Horwitz, 1989; Wang et al., 1989; Bernad et al., 1990). As proposed by Argos (1988), mutagenesis of φX29 DNA polymerase indicated that the second aspartate was critical, whereas the first aspartate was not absolutely required for catalysis (Bernad et al., 1990). Our results contrast with their data with regard to polymerization activity but agree with the primer-binding activity. Fourth,

replacement of the GDMD methionine by threonine in rat DNA polymerase β only reduces the activity about 2-fold. Furthermore, basic amino acids are found on the N-terminal side of the motif in most replicative DNA polymerases (Table III), suggesting a possibility that they have the Arg-183-like function of rat DNA polymerase β, which is involved in the primer-binding reaction. The question whether the function of the GDTD motif in replicative DNA polymerases is the same as that of the GDMD motif in DNA polymerase β will be elucidated by further biochemical and biophysical characterization of these enzymes.

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Registry No. Asp, 56-84-8; 5'-TTP, 365-08-2; $d(T)_{24}$, 83381-52-6; DNA polymerase, 9012-90-2.

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