Site-Directed Mutagenesis of Recombinant Rat DNA Polymerase β: Involvement of Arginine-183 in Primer Recognition†

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ABSTRACT: By site-directed mutagenesis using synthetic oligonucleotides, amino acid residues ^{181}Phe-Arg-Arg^{183} of recombinant rat DNA polymerase β were replaced by other amino acids to clarify the roles of these residues in the DNA synthesizing reaction. Replacement of Phe-181 by alanine reduced the enzyme activity only 30%. Replacement of Arg-182 by alanine and glutamine resulted in reduction of the activity by about 67% and 95%, respectively. The Arg-182 → Gln replacement increased the binding strength to single-stranded DNA but did not significantly change the K_m's for the primer and dTTP, suggesting that Arg-182 is involved in modulation of binding to the template rather than to the primer or deoxyribonucleoside triphosphate. Replacement of Arg-183 by Gln resulted in reduction of the activity by about 95%, and this change, although causing little change in binding strength to single-stranded DNA, resulted in a 3–4-fold increase in the K_m's for the primer and deoxyribonucleoside triphosphate. A more dramatic change was observed when Arg-183 was replaced by Ala, which resulted in a 99.98% reduction of enzyme activity. Although the K_m for deoxyribonucleoside triphosphate of this mutant enzyme was hardly changed, that for the primer increased 159-fold. Therefore, it is concluded that Arg-183 occupies an important part of the primer recognition site of DNA polymerase β.

Animal cell DNA polymerase β has been thought to be involved in DNA repair (Fry & Loeb, 1986) and possibly in recombination (Hirose et al., 1989). The active form of the rat enzyme consists of a single polypeptide of 335 amino acid residues (Matsukage et al., 1987) and is smaller than any other known prokaryotic or eukaryotic DNA polymerase. Enzymologically active recombinant rat and human DNA polymerase β's have been produced in *Escherichia coli* and purified to homogeneity in large quantities (Date et al., 1988; Abotts et al., 1988). Furthermore, the structure, enzymological properties, and antigenicity of the recombinant rat DNA polymerase β are indistinguishable from those of that purified from rat cells (Date et al., 1988). The simple structure and easy purification of the recombinant enzymes have made it favorable for study of the active site of DNA polymerase.

A computer search revealed that there were extensive similarities between rat DNA polymerase β and human terminal deoxynucleotidyl transferase (terminal transferase) in their amino acid sequences as well as in the locations of putative secondary and tertiary structures (Matsukage et al., 1987). A region between amino acid positions 179 and 184 of DNA polymerase β, which contains two arginine residues, is the most conserved between these enzymes, suggesting that this region is important for DNA polymerizing activities. In this paper, we report that replacement of the arginine residue at position 182 or 183 of rat recombinant DNA polymerase β by alanine or glutamine decreased dramatically the enzyme activity. Enzymological analysis of purified mutant DNA polymerase β indicates that Arg-183 is involved in recognition of the primer.

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Materials and Methods

Site-Directed Mutagenesis. Expression plasmid JMpβ5, which contained the whole coding sequence for rat DNA polymerase β in M13 phage–plasmid chimeric vector pUC118 (Date et al., 1988), was used for this work. Isolation of single-stranded circular DNA from *E. coli* transformed by the plasmid and subsequent oligonucleotide-directed mutagenesis were performed as described previously (Date et al., 1988). The oligonucleotides used for the mutagenesis were chemically synthesized (Figure 1). By use of these oligonucleotides, phenylalanine at 181, arginine at 182, arginine at 183, or both of these two arginines were converted into alanine or glutamine(s). With the same method, the mutant enzymes were reverted into the wild-type one to exclude the possibility that sites other than the desired mutation sites were accidentally changed and that these changes might result in the reduction of the activities of mutant enzymes.

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis of Crude Extracts of Recombinant E. coli's. The recombinant plasmids were introduced into *E. coli* JM109, and transformants were grown in 20 mL of 2× YT medium containing 50 μg/mL ampicilin. When the absorbance at 600 nm of the culture reached 0.2, isopropyl β-thiogalactoside (IPTG) was added to a final concentration of 1 mM, and cells were grown for an additional 4 h. Cells were collected by centrifugation at 4000*g* for 3 min, and the precipitate was resuspended in 0.5 mL of lysing solution containing 10 mM Tris-HCl, pH 7.8, 10% sucrose, and 0.1 mg of egg white lysozyme. After incubation for 30 min at 0 °C, crude extracts were obtained by sonication for 5 min at 0 °C in a Branson Sonifire. Aliquots were taken, diluted with sample buffer for SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and then subjected to electrophoresis. In order to determine the relative amounts of 40-kDa DNA polymerase β polypeptide, the gel was stained with Coomassie Brilliant Blue R-250 and scanned by a Shimadzu gel scanner.

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Detection of DNA Polymerase β Activity in the Crude Extract by the Activity Gel Method. DNA polymerase activities of crude extracts were detected by an activity gel method described by Spanos et al. (1981) with minor modifications (Yamaguchi et al., 1983). After electrophoresis in a gel containing activated calf thymus DNA, the reaction was carried out for 30 min in Tris-HCl, pH 7.8, 6 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 μM each of dATP, dGTP, and dTTP, and 10 μCi of [α-^{32}P]dCTP (400 Ci/mmol). DNA polymerase β activity can be detected in the region of about 40 kDa in autoradiograms. The enzyme activity was quantified by counting the 40-kDa regions with an AMBIS β-scanner.

Purification of Recombinant Rat DNA Polymerase β. The recombinants were grown in 2× YT medium, and when the absorbance at 600 nm of the cultures reached 0.5, IPTG was added to a final concentration of 0.5 mM to induce synthesis of DNA polymerase β. Bacterial cells were harvested at 6 h after IPTG induction and stored at −80 °C until used.

The wild and mutant types of DNA polymerase β were purified from recombinant *E. coli* as described previously (Date et al., 1988). In brief, frozen recombinant *E. coli* (about 2 g) was suspended in 6 mL of 50 mM Tris-HCl, pH 7.8, containing 25 % (w/v) sucrose and incubated with 2.5 mg/mL egg white lysozyme for 1 h at 0 °C. EDTA was added to a final concentration of 50 mM, and the mixture was incubated for 5 min. Cells were lysed by mixing the cell suspension with 6 mL of lysing buffer containing 0.5% Nonidet P-40, 0.8 M KCl, 2 mM phenylmethanesulfonyl fluoride (PMSF) and 50 mM Tris-HCl, pH 7.8. The lysate was sonicated to shear the DNA and then clarified by centrifugation at 12000*g* for 30 min to obtain the crude extract. The crude extract was diluted by the addition of three volumes of PC buffer (50 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol), and the KCl concentration was adjusted to 0.4 M. The bulk of nucleic acid was removed from the crude extract by passing it through a DEAE-cellulose column that had been equilibrated in PC buffer containing 0.4 M KCl.

The eluent that contained the bulk of the DNA polymerase activity was diluted with an equal volume of PC buffer to reduce the KCl concentration and applied to a phosphocellulose column equilibrated with 0.2 M KCl containing PC buffer. Chromatography was developed with a linear KCl concentration gradient from 0.2 to 0.8 M in PC buffer. DNA polymerase β was monitored by its enzyme activity and/or SDS–PAGE. DNA polymerase β activity and/or its 40-kDa polypeptide from wild-type and mutant recombinants were eluted at a KCl concentration of between 0.45 and 0.55 M.

Pooled fractions containing the enzyme protein were diluted 3-fold with PC buffer and then applied to a denatured calf thymus DNA–cellulose column equilibrated with 0.15 M KCl–PC buffer. Chromatography was developed with a linear gradient of KCl concentration from 0.15 to 1.0 M in PC buffer. The activity and polypeptide of DNA polymerase β were monitored as described for the phosphocellulose chromatography. The purity of the preparations was usually 80% or higher with respect to the 40-kDa polypeptide, which was reactive to the anti-DNA polymerase β antibodies. Enzymological characterizations were performed with the enzyme preparations at this step.

Assay for DNA Polymerase β Activity. DNA polymerase β activity was determined with poly(rA)-oligo(dT) as a template-primer under reaction conditions that had been used for chick DNA polymerase β (Yamaguchi et al., 1980). One unit of DNA polymerase was defined as the amount catalyzing the incorporation of 1 nmol of dTMP into poly(dT) in 60 min. For the determinations of enzymological parameters, the concentration of the substrate, dTTP, or the primer, oligo-$(dT)_{12-18}$, was varied. In the assay of the enzyme with low activity, the specific activity of [^{3}H]dTTP was increased 10 times or higher.

Determination of Protein Concentration. Protein concentration was determined by a Bio-Rad protein assay kit and with bovine serum albumin as a standard according to the supplier's procedure.

Neutralization and Immunobinding of DNA Polymerase β by Anti-Rat DNA Polymerase β. Serum was obtained from a rabbit that had been immunized with the purified rat DNA polymerase β produced in recombinant JMpβ5 (Date et al., 1988). Antibodies were purified on a protein A–Sepharose column as described previously (Ei et al., 1978). Appropriate amounts of enzyme preparations that showed almost equal activities were mixed in a final volume of 10 μL with serially diluted antibody in 0.2 M KCl–PC buffer containing 1 mg/mL bovine serum albumin, and then the mixtures were incubated for 3 h at 4 °C. After incubation, 15 μL of the reaction mixture for DNA polymerase activity was added to each reaction in neutralization experiments. In the immunobinding assay, the reaction containing enzyme and antibody after the first incubation was mixed with 10 μL of 10% (w/v) formalin-fixed *Staphylococcus aureus* (*S. aureus*) Cowan I suspension. The mixture was incubated for 1 h at 4 °C, and the immune complex was precipitated by centrifugation for 10 min at 8000*g*. Ten microliters of the supernatant was mixed with 15 μL of reaction mixture for the DNA polymerase activity assay.

Immunoblotting Analysis. Anti-chick DNA polymerase β rabbit antibody, which was prepared against a highly purified preparation of the enzyme, was described previously (Yamaguchi et al., 1982). SDS–PAGE and immunoblotting analysis were carried out as described earlier (Date et al., 1988) except that antibodies against both chick and rat DNA polymerase β's were used instead of that against chick DNA polymerase β only.

Results

Site-Directed Mutagenesis of Rat DNA Polymerase β. By a site-directed mutagenesis method using oligonucleotides (Date et al., 1988), each amino acid residue in the sequence ^{181}Phe-Arg-Arg^{183} of the recombinant rat DNA polymerase β was replaced by alanine, and these mutants were designated as FA181 (Phe^{181} → Ala), RA182 (Arg^{182} → Ala), and RA183 (Arg^{183} → Ala), respectively (Figure 1). The arginine residue at position 182, that at position 183, or those at both positions were also replaced by glutamine, and these mutants were designated as RQ182 (Arg^{182} → Gln), RQ183 (Arg^{183} → Gln), and RQ182/183 (Arg^{182}-Arg^{183} → Gln-Gln), respectively.

Replacement with an alanine residue may affect the reaction properties of the enzyme dramatically, especially Arg-182 and Arg-183, if these arginine residues have important roles in the catalytic reaction. Furthermore, replacement of Arg by Gln seems to be one of the best ways to examine the effect of the positive charge of Arg on enzyme activity, because Gln has a neutral and still hydrophilic group on the terminus of the alkyl residue instead of the basic group in Arg.

E. coli JM109 cells harboring each recombinant plasmid were grown, and the synthesis of the enzyme was induced by 1.0 mM IPTG. In all recombinants, 40-kDa polypeptides were present in large quantities, and production was obviously dependent on the addition of IPTG (Figure 2). Thus, the

		181 182 183
		N-Cys Gly Ser Phe Arg Arg Gly Ala Glu-C
		5'TGC GGC AGT TTC CGA AGA GGC GCA GAG T 3'
Mutants	Replacements	Oligomers for mutagenesis
FA181	Phe →Ala	3'ACG CCG TCA CGG GCT TCT CCG 5'
RA182	Arg→Ala	3'CCG TCA AAG CGA TCT CCG CGT CT 5'
RQ182	Arg→Gln	3'G TCA AAG GTC TCT CCG CG 5'
RA183	Arg→Ala	3'TCA AAG GCT CGT CCG CGT CT 5'
RQ183	Arg→Gln	3'CA AAG GCT GTT CCG CGT 5'
RQ182/183	Arg-Arg→Gln-Gln	3'CG TCA AAG GTC GTT CCG CGT CTC A 5'
AR183	Ala→Arg	3'TCA AAG GCT TCT CCG CGT CT 5'

FIGURE 1: Oligodeoxynucleotides used for site-directed mutagenesis of rat DNA polymerase β. (Top) Partial amino acid sequence of DNA polymerase β in which the amino acid replacements were introduced and its coding nucleotide sequence. Oligonucleotides were chemically synthesized so as to contain complementary sequences to the coding sequence except for the nucleotide substitutions as indicated by underlines. By use of these oligonucleotides as primers on M13-derived single-stranded DNA containing the whole coding frame for the wild-type rat DNA polymerase β, the amino acid replacements shown in this figure were achieved. Furthermore, with the oligonucleotide at the bottom the mutant enzymes, such as RA183, can be reverted into the wild-type enzyme.

substitution of amino acids in these positions did not affect the synthesis and stability of the DNA polymerase β polypeptide in *E. coli*.

Detection of Activities of Mutated DNA Polymerase β. The crude extracts of *E. coli* with wild-type and mutant enzymes were examined in an activity gel to detect DNA polymerase activity. As shown in Figure 3A, enzyme activities at the 40-kDa region were different among recombinants, while the activities of the larger size, which indicates *E. coli* DNA polymerase I, were almost identical in all extracts. Although the extracts of FA181 and RA182 had activities similar to that of the wild one, replacement of Arg-183 by alanine (RA183) extensively reduced the enzyme activity. Mutant enzymes of RQ182 and RQ183 also had much lower activities than the wild-type one. The RQ182/183 enzyme, in which two arginine residues were replaced by glutamines, did not have any detectable enzyme activity with this method (data not shown). Since the enzyme activities of some mutant enzymes (RQ182, RQ183, RQ183, and RQ182/183) were much lower than that of the wild-type one in chromatographies as shown later (see Figures 4 and 5), the low activities of these mutant enzymes in the activity gel were not caused from the low efficiencies in renaturation in the gel but caused from the amino acid substitutions.

In order to rule out the possibility that the reduction of enzyme activities by mutation was caused from accidental change(s) in site(s) other than the directed sites, we have tried to revert mutant enzymes into the wild one using an oligonucleotide with the wild-type sequence. An example is shown in Figure 3C. Reversion of Ala-183 in RA183 into the original Arg (AR183) resulted in a high enzyme activity similar to that of the wild one.

Since the incorporation of [α-^{32}P]dCMP at the 40-kDa region in the activity gel was proportional to the amount of crude extract of wild-type recombinant (Figure 3B), the relative specific activity of each mutant enzyme could be roughly calculated by measuring the radioactivity and the amount of 40-kDa polypeptide in each preparation in the dye-stained gel (Figure 2). The obtained specific activities relative to that of the wild-type enzyme are as follows: FA181, 70%; RA182, 33%; RQ182, 13%; RA183, 0.1%; RQ183, 5%; RQ182/183, <0.1%. In the activity gel method, the concentration of ^{32}P-labeled dCTP in the reaction mixture was extremely low. However, since the K_m values of these mutant enzymes for deoxyribonucleoside triphosphate and primer are

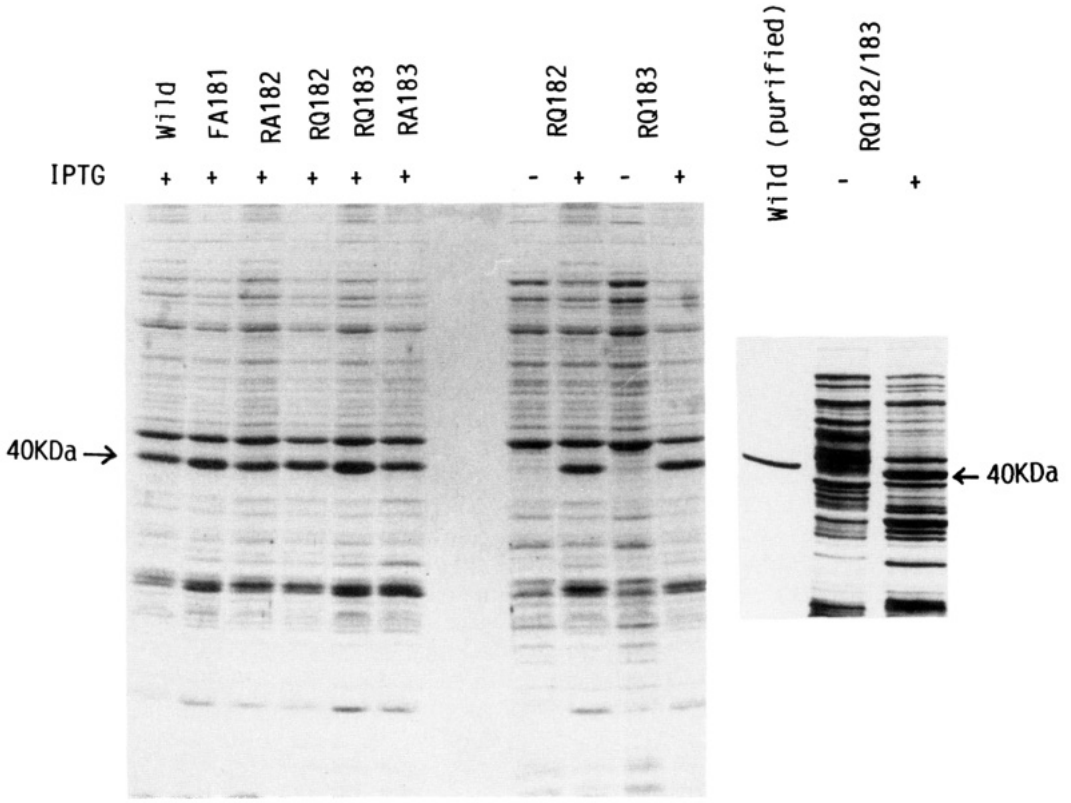

FIGURE 2: SDS–polyacrylamide gel electrophoretic analysis of extracts of *E. coli* JM109 which were transformed by plasmids containing cDNA for wild and mutant types of DNA polymerase β. Cells were cultured, and production of enzyme was induced by IPTG. Extracts were made as described under Materials and Methods. After electrophoresis, the gels were stained by Coomassie Brilliant Blue. With some mutants (RQ182, RQ183, and RQ182/183), samples with and without IPTG induction were compared, indicating that synthesis of the 40-kDa polypeptides for DNA polymerase β was dependent on the induction.

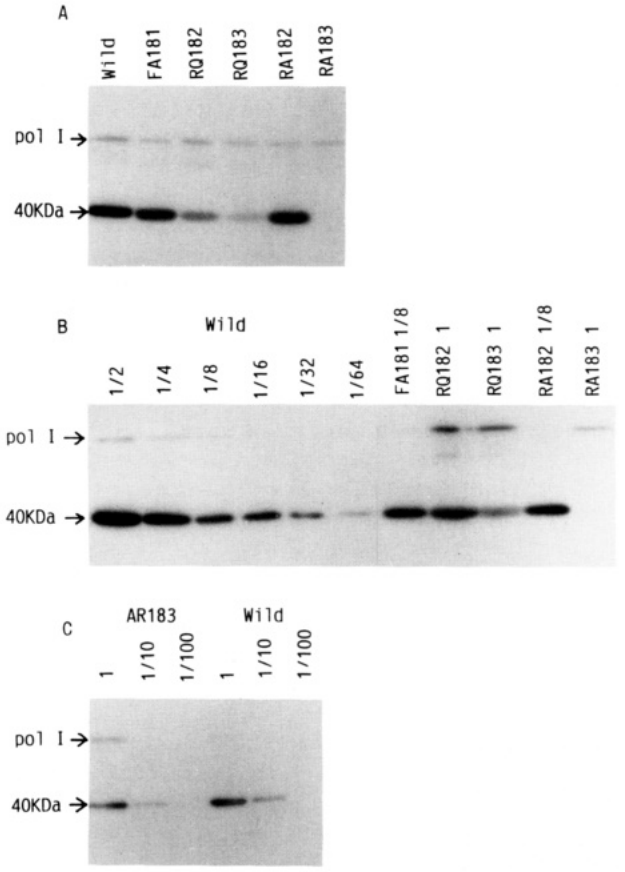

FIGURE 3: Detection of DNA polymerase activities in the cell extracts of wild, mutant, and revertant recombinants by an activity gel method. The same extracts used in Figure 2 were used. (A) Equal volumes (5 μL) of extracts were electrophoretically separated in activated DNA-containing gels, and after renaturation of enzymes by removal of SDS from gels, DNA synthesis reaction in situ was carried out in the reaction mixture containing [^{32}P]dCTP. (B) Serially diluted wild-type extract and appropriately diluted or nondiluted mutant extracts were analyzed to compare their relative activities. By normalizing the obtained values with the amounts of 40-kDa polypeptide shown in Figure 2, apparent specific activities of mutant enzymes relative to that of the wild-type enzyme could be estimated. (C) 5-, 0.5-, and 0.05-μL extracts of the revertant from RA183 (AR183) and original wild-type recombinant *E. coli*'s were applied to an activity gel.

higher than those in this reaction as mentioned below, these specific activities are indefinite. Absolute values of specific activities at their V_{max} will be described later.

Enzymological Characterizations of Mutant DNA Polymerase β's with Reduced Activities. Mutant DNA polymerase β's with significantly reduced activities (RQ-182, RA-183, RQ-183, and RQ-182/183) were purified, and their enzymological properties were compared with those of the wild-type enzyme.

The results of phosphocellulose column chromatographies are shown in Figure 4. The mutant DNA polymerases are all eluted by 0.45–0.55 M KCl similarly to the wild-type one. Despite the use of similar amounts of recombinat cells as starting materials, the amounts of mutant enzyme activities were much less than that with the wild-type enzyme. Although the enzyme activity of mutant RQ182/183 was not detected, the 40-kDa polypeptide, which was detected by SDS–polyacrylamide gel electrophoresis, was eluted at the position corresponding to the DNA polymerase β activities of the other samples (Figure 4G).

The fractions containing DNA polymerase β activity and/or 40-kDa polypeptide were chromatographed in a denatured DNA–cellulose column (Figure 5). After this step, purified

Table I: Properties of Wild and Mutant DNA Polymerase β's

DNA polymerase	KCl concn (M) for elution from P-cellulose	KCl concn (M) for elution from DNA–cellulose	K_m's for dTTP (μM)	K_m's for oligo(dT) (μg/mL)	sp act. at V_{max} (units/mg of protein)
wild	0.45	0.35	39 (45)[a]	2.1	2.3×10^6
RQ182	0.45	0.50	43	2.5	8.9×10^4
RA183	0.45	0.30	270 (48)[a]	333	3.7×10^2
RQ183	0.45	0.40	130	10.3	1.4×10^5
RQ182/183	0.45	0.35	ND[b]	ND[b]	<10

[a] These values were obtained in the reactions with high primer concentrations (120 μg/mL) instead of 20 μg/mL as in other experiments. [b] ND: not determined.

enzyme preparations were obtained [Figure 5F,G and also Date et al. (1988)]. All the mutant DNA polymerase β's as well as the wild one retained DNA binding activity. Although the DNA polymerase activity in the wild-type sample was eluted at 0.35 M KCl, some of the mutant DNA polymerase β's were eluted from columns with higher KCl concentrations: RQ182, 0.50 M; RQ183, 0.40 M. However, the RA183 and RQ182/183 enzymes or polypeptides were eluted from this column with 0.3 and 0.35 M KCl, respectively, almost the same concentration as for elution of the wild-type enzyme. These results indicate that the arginine residues at positions 182 and 183 are not essential for DNA binding of DNA polymerase β, but this region, especially Arg-182, is possibly important for modulation of the binding strength to DNA.

DNA polymerase activities of both RQ182 and RQ183 recombinants as well as the wild-type one were neutralized and immunologically precipitated by a rabbit antibody prepared against wild-type rat DNA polymerase β produced in *E. coli* (Figure 6). Since the amounts of mutant enzyme protein used in this experiment were more than 10 times that of the wild one to obtain similar amounts of activities as for wild-type enzyme, the antibody concentrations required for neutralization and precipitation of the mutant enzymes were higher than those required for the wild-type one. Considering this difference, these mutant enzymes might have antigenicities similar to that of the wild one. Because the activity was too low, we did not carry out neutralization or immunoprecipitation experiments using the RQ182/183 and RQ183 enzymes. However, by an immunoblotting analysis, they were demonstrated to have antigenicity to the anti-rat and anti-chicken DNA polymerase β antibodies similar to that of other enzymes (Figure 7).

In order to obtain exact specific enzyme activities, the exact protein concentrations and DNA polymerase activities were determined in the experiments as shown in Figure 8. Furthermore, the obtained values of specific enzyme activities were calibrated by the ratio of the maximal velocities to the velocities at the substrate concentration (50 μM) used in this experiment (see Figure 9). This is important for precise comparison, because the K_m values of mutant enzymes are different. The results are summarized in Table I. The specific activity of the wild-type enzyme was as high as 2.3×10^6 units/mg of protein, while those of RQ182, RQ183, and RA183 are 8.9×10^4, 1.4×10^5, and 3.7×10^2 units/mg of protein, respectively. Even if we employed a large quantity of the RQ182/183 enzyme preparation and [^{3}H]dTTP with high specific radioactivity, we obtained little activity. Thus, its specific activity was estimated as less than 10 units/mg of protein. This evidence indicates that at least one of the arginine residues at positions 182 and 183 is essential for DNA polymerase β activity. It should be pointed out that the absence of significant DNA polymerase activity in the RQ182/183 preparation ruled out the contamination of de-

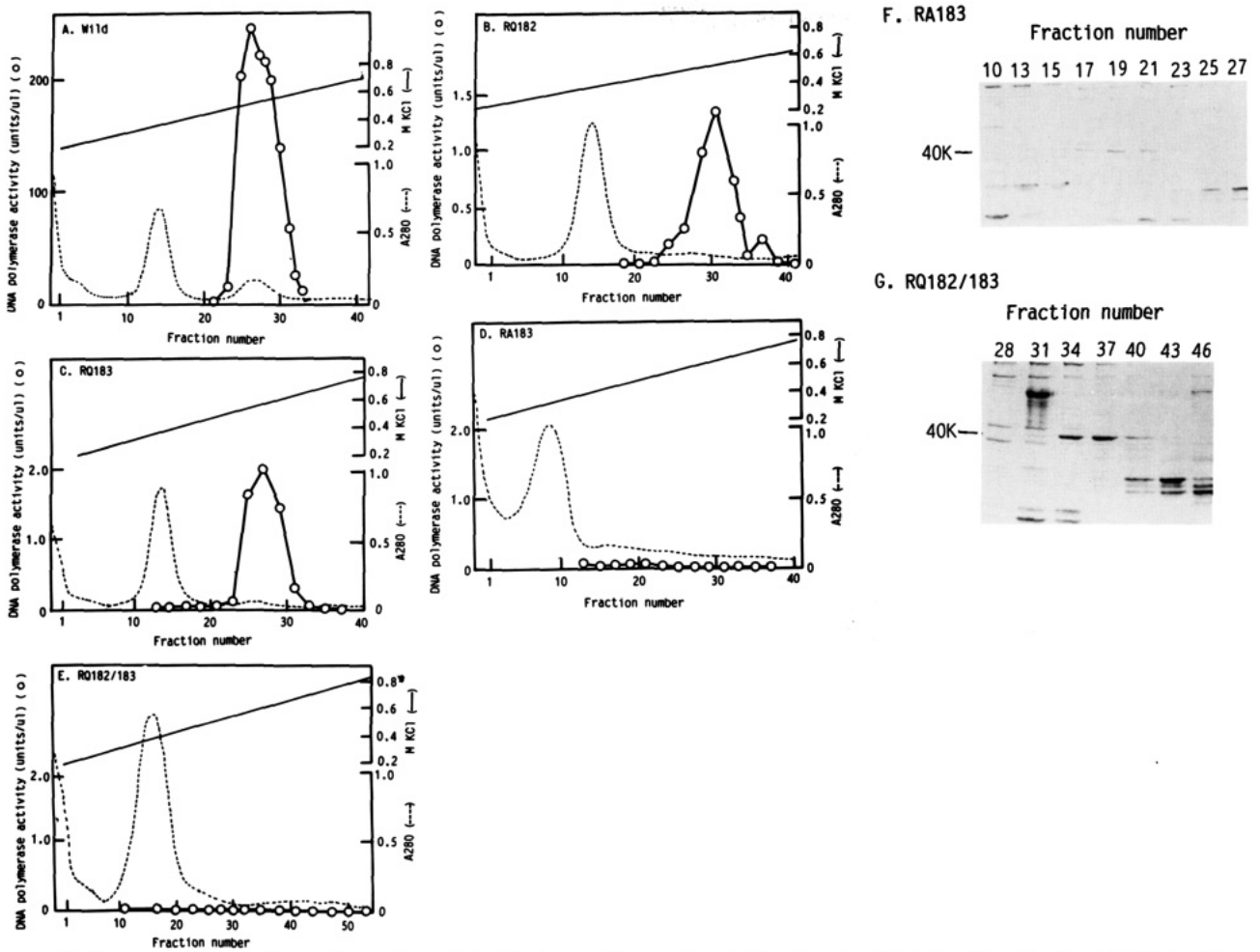

FIGURE 4: Phosphocellulose column chromatographies of extracts of recombinant *E. coli*'s with plasmids with sequences encoding wild- and mutant-type DNA polymerase β's. Cells were grown and IPTG induced. The crude extract was passed through a DEAE-cellulose column to remove nucleic acids and then applied to a phosphocellulose column. Chromatography was developed with a linear gradient of KCl concentration. DNA polymerase activity was determined in diluted (wild type) or nondiluted (others) eluents and expressed in unit number per microliter of eluents. Although little activity was detected in the RA183 (D) and RQ182/183 (E) extracts, polypeptides were analyzed by SDS–polyacrylamide gel electrophoresis (F and G). KCl concentration was determined by measuring conductivity.

tectable amounts of *E. coli* DNA polymerases in the enzyme preparations at the final step.

For further insight into the effects of the mutations, enzymological parameters of the RQ182, RQ183, and RA183 enzymes were compared with those of the wild-type enzyme (Figures 9 and 10). Although the K_m values for dTTP and the primer of the RQ182 enzyme are almost identical with those of the wild-type enzyme, the values of RQ183 are about 3–4-fold higher than those of the wild type. More dramatic increases in K_m values were observed when Arg-183 was replaced by alanine (RA183); K_m's for dTTP and primer [oligo(dT)] are 270 μM and 333 μg/mL, respectively, which are 6.9- and 159-fold, respectively, of those of the wild-type enzyme. We were afraid of the possibility that the requirement of a high primer concentration by RA183 might depend on a terminal transferase like activity generated by mutagenesis. It seemed possible because DNA polymerase β has extensive homology with terminal transferase (Matsukage et al., 1987). However, this was not the case, because the activity of the RA183 enzyme, like the wild-type one, was completely dependent on both primer and template (data not shown).

The results with RQ183 and RA183 indicate that Arg-183 is involved in the recognition of the primer and/or dTTP. For DNA polymerization activity by DNA polymerase, the formation of a ternary complex with these substrates at the active site(s) of the enzyme is necessary. The reduction of the affinity to the primer may affect the requirement for a high deoxynucleoside triphosphate concentration and vice versa. In order to clarify whether the primer or deoxynucleoside triphosphate binding activity is the primary cause of high K_m values in these two substrates, the dependence of dTTP concentration was determined in the reactions with a high primer concentration (120 μg/mL) instead of the usual concentration (20 μg/mL). As shown in Figure 11, the K_m of RA183 for dTTP was identical with that of wild-type enzyme under this condition. Therefore, the primary function affected by substitution of Arg-183 with Ala is primer binding rather than dTTP binding.

Discussion

DNA polymerase is thought to have multiple subfunctions to perform the DNA polymerizing reaction: (1) binding to template DNA and recognition of the base on the template, (2) recognition of the 3′-OH of the primer, (3) recognition and binding of deoxynucleoside triphosphate with the base complementary to the base of the template, and (4) phosphodiester bond formation between the primer 3′-OH and 5′-α-phosphate of deoxyribonucleoside triphosphate. The present study was undertaken to clarify the structures of DNA polymerase β responsible for these subfunctions. The cDNA for rat DNA polymerase β was cloned, and the active enzyme was successfully expressed in *E. coli* (Date et al., 1988). Furthermore, the vector used for the overexpression is pUC118,

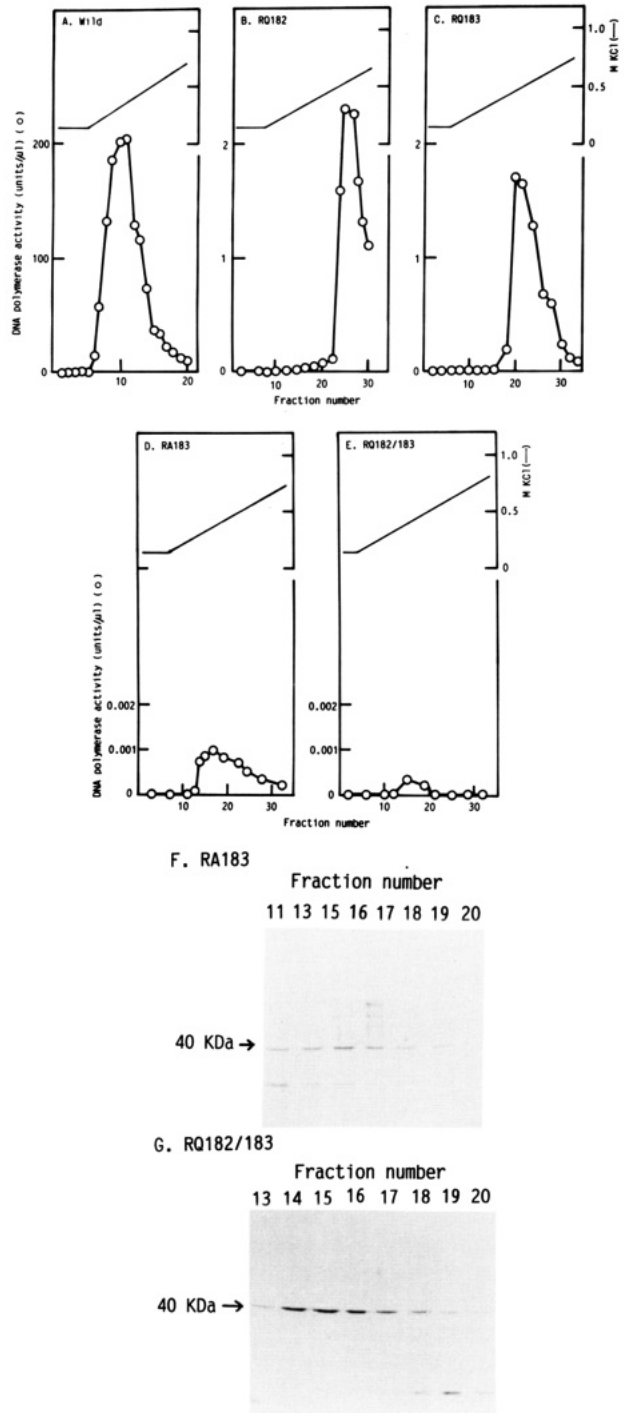

FIGURE 5: DNA–cellulose column chromatographies of wild and mutant types of rat DNA polymerase β. Pooled fractions containing DNA polymerase β activities and/or its 40-kDa polypeptide after phosphocellulose column chromatography were applied to denatured calf thymus DNA–cellulose columns, and chromatography was developed by a linear KCl gradient. DNA polymerase activity and polypeptide were analyzed as described in Figure 4.

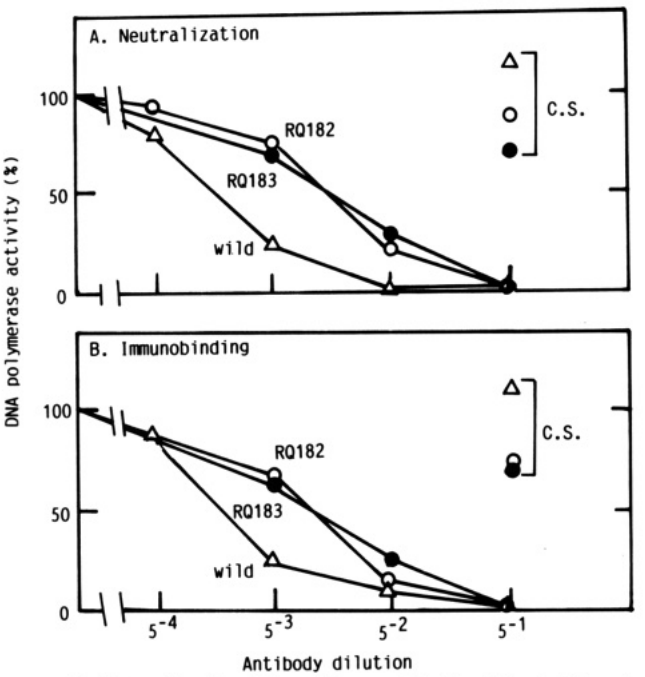

FIGURE 6: Neutralization (A) and immunobinding (B) of wild and mutant types (RQ182 and RQ183) of DNA polymerase β with anti-rat DNA polymerase β antibody. Almost equal activities (about 4 units) of DNA polymerase β's were mixed with the serially diluted antibody or immunoglobulin from preimmune serum (C.S.). After incubation, half of each mixture was mixed with the reaction mixture for DNA polymerase activity (A), and the remaining portion was mixed with a suspension of formalin-fixed *S. aureus* Cowan-1 to remove the immune complex. An aliquot of the supernatant was then mixed with the reaction mixture for DNA polymerase activity (B). In (A), 100% values for wild, RQ182, and RQ183 were 1.26, 1.53, and 1.94 units, respectively, while those in (B) were 0.26, 0.59, and 0.19 unit, respectively, because of general inactivation of the enzyme activity during incubations and treatments.

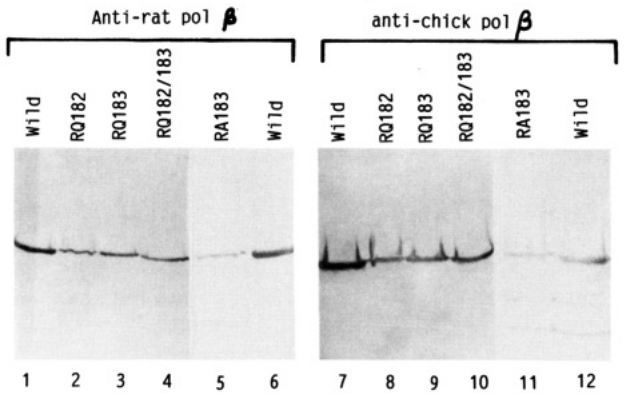

FIGURE 7: Immunoblotting analysis of wild and mutant types of DNA polymerase β. Purified DNA polymerase β samples containing 0.2–0.5 μg of protein were electrophoretically separated and blotted onto nitrocellulose membranes. Antigenic polypeptides were detected with anti-rat recombinant DNA polymerase β (A) or anti-chick DNA polymerase β (B) antibody as a primary antibody.

which can be easily converted into a single-stranded form because it has the replication origin of M13 phage DNA (Date et al., 1988). This makes it simple to mutagenize the enzyme by use of oligonucleotides.

We attempted substitutions by glutamine or alanine of the phenylalanine residue at position 181 and of the arginine residues at positions 182 and 183 of rat DNA polymerase β, which are highly conserved between this enzyme and human terminal transferase (Matsukage et al., 1987). Although the substitution of Arg-182 by Ala reduced the enzyme activity to only a limited extent, that by Gln reduced the activity more extensively. Since the enzyme molecule with substitution of the Arg-182 by Gln has a much higher affinity to single-stranded DNA than the wild-type enzyme but almost identical K_m values for primer and dTTP with those of the wild type, Arg-182 might be involved in the region modulating the template binding activity. The reaction mechanism of DNA polymerase β is highly distributive (Wang & Korn, 1980, 1982; Mosbough & Linn, 1983; Matsukage et al., 1983). Therefore, after finishing polymerization of one nucleotide, this enzyme is released from the site of DNA elongation and rebinds to the next primer site in a random manner. Too strong of

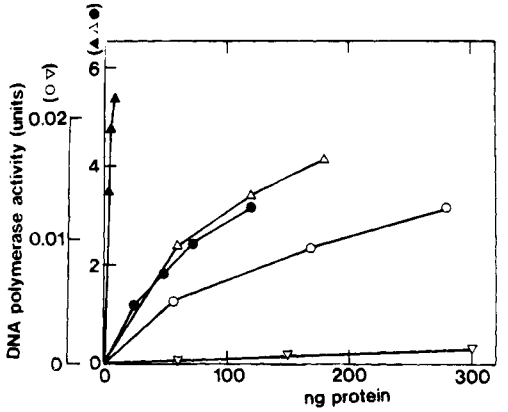

FIGURE 8: Relationships between protein amounts and DNA polymerase activities in wild and mutant enzyme preparations. Protein concentrations in the enzyme preparations after the denatured DNA–cellulose chromatography were determined, and the appropriate volumes of these enzymes were used for determination of the activity. In the reactions of RA183 and RQ182/183, radioactive substrate with a high specific activity was added to obtain high sensitivity. The enzyme activities were plotted against protein amounts. (▲) Wild; (●) RQ182; (△) RQ183; (○) RA183; (▽) RQ182/183.

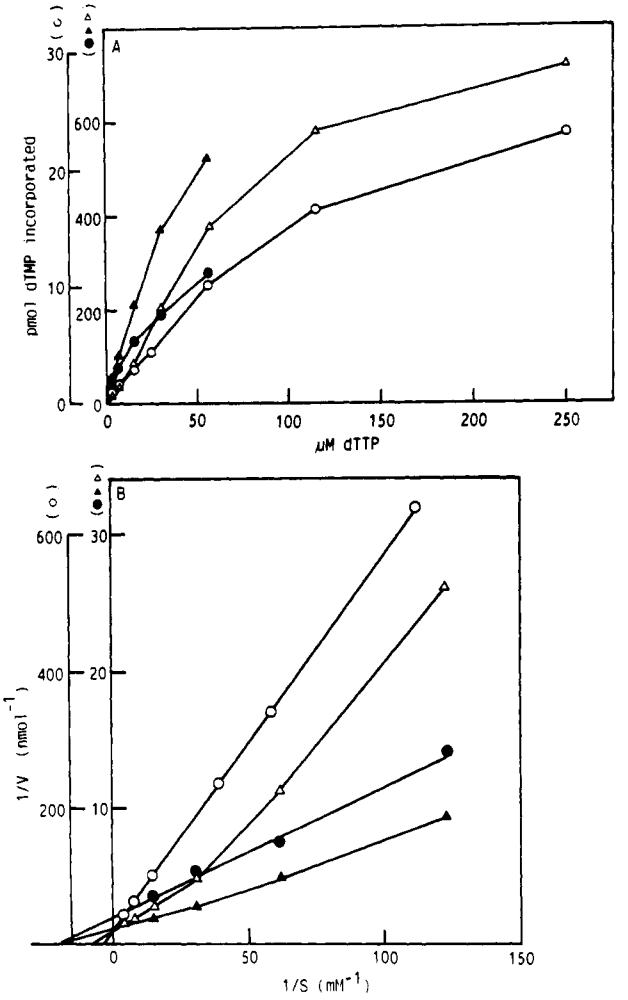

FIGURE 9: Dependence on dTTP concentration of wild and mutant types of DNA polymerase β. In the presence of 40 μg/mL poly(rA) and 20 μg/mL oligo(dT), the dTTP concentration was varied as indicated (A). Lineweaver–Burk plots are shown in (B). (▲) Wild; (●) RQ182; (△) RQ183; (○) RA183.

binding to the template might result in a low reaction turnover.

On the other hand, Arg-183, although localized very closely to Arg-182, is thought to be primarily involved in binding to the primer, because the substitution of this site by glutamine or alanine induced a dramatic increase in the K_m for the

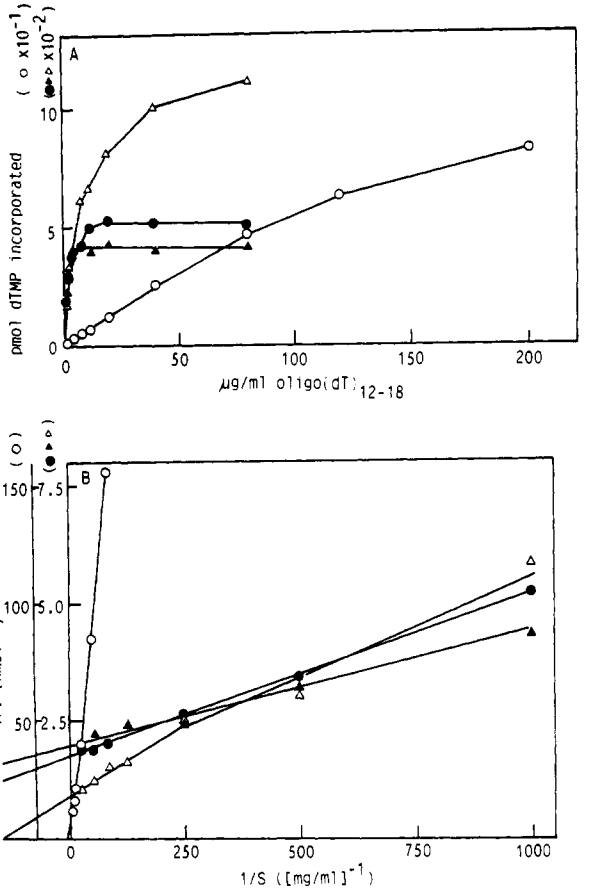

FIGURE 10: Dependence on oligo(dT) concentration of wild and mutant types of DNA polymerase β. The concentration of oligo(dT) was varied as indicated in the presence of a constant concentration of poly(rA) (40 μg/mL) (A). The Lineweaver–Burk plot is shown in (B). (▲) Wild; (●) RQ182; (△) RQ183; (○) RA183.

primer. This interpretation is conceivable given the previous finding that the reaction mechanism of DNA polymerase β is an ordered Bi-Bi mechanism in which the enzyme binds primer first and then deoxyribonucleoside triphosphate (Tanabe et al., 1979). The lowered affinity to the primer might cause a requirement for a higher concentration of deoxyribonucleoside triphosphate in order to compensate for the reaction efficiency.

The deoxyribonucleoside triphosphate binding site seems to be located in a region different from that dealt with in this work, because the K_m's for deoxyribonucleoside triphosphate of the mutant DNA polymerase β's in this work were not significantly different from that of the wild-type enzyme. Recently, two papers were published dealing with this site, although observations of them are still contraversial. By a photoaffinity labeling method Evans and Coleman (1989) determined that the region of positions 209–233 of terminal transferase β-subunit contains the site for deoxyribonucleoside triphosphate binding. This region corresponds to positions 188–217 of DNA polymerase β. Since these regions of the two enzymes have similar secondary structures as well as primary structures (Matsukage et al., 1987), that of DNA polymerase β might be responsible for the deoxyribonucleoside triphosphate binding. On the other hand, Basu et al. (1989) concluded, from modification with pyridoxal 5′-phosphate, that the Lys at position 71 of rat DNA polymerase β is important in the deoxyribonucleoside triphosphate binding pocket. In order to obtain the final organization of the active sites of DNA polymerase β, information on X-ray diffraction analysis of the crystallized enzyme is required. We are now pursuing

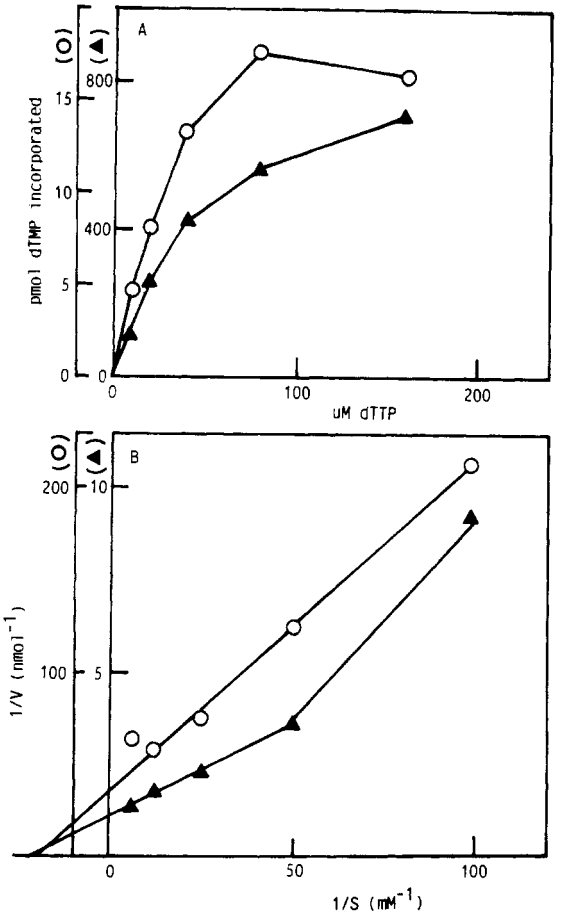

FIGURE 11: Dependence on dTTP concentration of wild and RA183 DNA polymerase β in the presence of high concentrations of the primer. The experiment was carried out as described in Figure 8 except for an oligo(dT) concentration of 120 μg/mL (A). The Lineweaver–Burk plot is shown in (B). (▲) Wild; (○) RA183.

this line of studies using a homogeneous recombinant rat polymerase β preparation.

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