# Properties of Tyrosine 766 → Serine Mutant of *Escherichia coli* DNA Polymerase I: Template-Specific Effects†

Shyamal D. Desai, Virendra N. Pandey, and Mukund J. Modak*

*Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry—New Jersey Medical School, 185 South Orange Avenue, Newark, New Jersey 07103-2714*

*Received April 1, 1994; Revised Manuscript Received July 20, 1994®*

ABSTRACT: In order to determine the role of Tyr 766 of *Escherichia coli* DNA polymerase I in the catalysis of DNA synthesis, we investigated the properties of a Tyr 766 → Ser (Y766S) mutant of the Klenow fragment of *E. coli* DNA polymerase I. We found that the rates of incorporation of only dTTP but not the other dNTP substrates were affected in the reactions catalyzed by the mutant enzyme, when homopolymeric template–primers were used. The mutant enzyme exhibited a reduced rate of synthesis only with poly(rA)- or poly(dA)-directed reactions. Examination of the ability of the mutant and the wild-type enzymes to bind to dGTP and dTTP, as judged by UV-mediated cross-linking, indicated nearly identical binding efficiencies of both nucleotides. However, the ability of the mutant enzyme to bind to poly(rA)·$(dT)_{15}$ and poly(dA)·$(dT)_{15}$ was found to be significantly reduced as compared to the binding to heteropolymeric DNA. In order to further define the nature of template-mediated restriction on the catalytic activity of the mutant enzyme, its ability to copy DNA templates containing a stretch of AAAAA and ACACA sequences was compared. The results show that DNA synthesis catalyzed by the mutant enzyme is significantly retarded when it encounters the AAAAA region of the template but not the ACACA region. Product analysis of the reaction directed by the two template–primers showed that the mutant enzyme stalls/terminates synthesis upon encountering an AAAAA sequence in the template. The results suggest that (a) Tyr 766 in pol I is required for efficient binding and translocation across the template strand consisting of oligomeric A segments and (b) it is not required in the substrate dNTP binding function.

Enzymatic synthesis of DNA is a complex process comprising multiple steps. A prototype enzyme, *Escherichia coli* DNA polymerase I (pol I),[1] has served as an excellent model system to clarify the intricacies of the polymerization process. A large body of information exists on its catalytic and physical properties, including the crystal structure of the large fragment (Klenow fragment) derived from this enzyme (Ollis *et al.*, 1985; Kornberg & Baker, 1992; Joyce *et al.*, 1992; Polesky *et al.*, 1990). In our continuing efforts to understand the details of the structure–function relationship in this enzyme, we sought to clarify the role of Tyr 766, particularly in the process of substrate binding. Earlier, it was demonstrated that 8-azido-dATP cross-linked to Tyr 766 in this enzyme (Rush & Konigsberg, 1990), while results from our laboratory, using dTTP as a photoaffinity labeling reagent, showed that His 881 was the site of dTTP cross-linking (Pandey *et al.*, 1987). The availability of the crystal structure of the Klenow fragment and the 3D structural model based on the crystal structure coordinates (Ollis *et al.*, 1985; Yadav *et al.*, 1992) suggested that both Tyr 766 and His 881 lie in the vicinity of the substrate binding cleft of this enzyme. It seemed plausible that the orientation of a ring moiety of a given nucleotide (i.e., purine or pyrimidine) toward Tyr 766 or His 881 may determine the site of adduct formation upon exposure of the enzyme–substrate complex to UV irradiation. In addition, Tyr 766 is also reported to be in the vicinity of the primer terminus, since it was found to be the site for cross-linking of the primer terminus (Catalano *et al.*, 1990). In spite of these observations, a direct role for Tyr 766 in the binding of either substrate dNTP or the primer terminus seemed equivocal in view of the results of site-directed mutagenesis studies of this residue (Polesky *et al.*, 1990). The Y766S mutant was found to be catalytically active with some effect on its $k_{cat}$ and $K_m$ for dNTP (Polesky *et al.*, 1990). Interestingly, the mutant enzyme was found to be significantly more error-prone than the wild type (Carroll *et al.*, 1991). We therefore examined in detail some of the properties of this enzyme (both $exo^+$ and $exo^-$) and found a rather unique defect of the Y766S mutant to utilize certain templates, thus suggesting an additional role for Tyr 766 in the catalysis of polymerization by pol I.

## MATERIALS AND METHODS

*Materials. E. coli* DNA polymerase I (Klenow fragment) was purified from an overproducing exonuclease-deficient strain (Derbyshire et al., 1991) generously provided by Catherine Joyce of Yale University. Wild-type Klenow fragment was expressed and purified from an overproducer clone (pET-3a-K) in *E. coli* BL-21 (DE-3) as described before (Kaushik *et al.*, 1993; Pandey *et al.*, 1993). Restriction endonucleases and DNA-modifying enzymes were from Promega or Boehringer Mannheim. Homopolymeric template–primers and HPLC-purified dNTPs were obtained from Pharmacia, while tritiated and $^{32}P$-labeled dNTPs and ATP were the products of Dupont–New England Nuclear. Sequenase and other sequencing reagents were from U.S. Biochemical Corp. Synthetic self-annealing template primers (see Chart 1) were obtained from Midland Certified Reagent Co. *In vitro* mutagen kit for mutagenesis was obtained from Bio-Rad. All other reagents were of the highest available

† Supported in part by a grant from NIH (NIGMS 36307).

* Address correspondence to this author.

® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: Pol I, *Escherichia coli* DNA polymerase I; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Y766S, mutation from tyrosine to serine at position 766 of pol I.

0006-2960/94/0433-11868$04.50/0 

Chart 1

**37 mer self annealing template-primer**

```
CACGCAGTCTTCTCG -3'
TCACGTCAGAAGAGCATGCGTC -5'
```

**27 / 10 mer template-primer (AAAAA-DNA)**

```
GAGTTACAGT -3'
CTCAATGTCACAAAAACAAGGTCAGAC -5'
```

**27/10 mer template-primer (ACACA-DNA)**

```
GAGTTACAGT -3'
CTCAATGTCACACACACCAGGTCAGAC -5'
```

**21 mer (Y766S) mutagenesis primer**

```
5'-CTG ATT TCT GGC ATG AGT GCT -3'
```

purity grade and were purchased from Fisher, Millipore Corp., Boehringer Mannheim, and Bio-Rad.

*In vitro Mutagenesis and Construction of Expression Plasmids.* Initial studies were carried out with Y766S exo$^-$ mutant enzyme, kindly provided to us by Dr. Catherine Joyce of Yale University. Subsequently, mutant overproducer clones with both exo$^-$ and exo$^+$ were constructed in this laboratory as described by Kunkel *et al.* (1985). Mutagenesis at codon 766 was made on a uracil-containing M13mp19 template carrying a 1.1-kilobase *Sac*I and *Hin*dIII fragment of pET-3a-K (Pandey *et al.*, 1993) encoding the polymerase domain of pol I. Phosphorylation of the 21-mer mutagenic primer (see Chart 1), annealing, extension, and ligation were carried out in accordance with the manufacturer's protocol supplied with Mutagen M13 in vitro mutagenesis kit (Bio-Rad). After the mutation in M13 was ascertained, the 1.1-kilobase mutated fragment was subcloned from M13mp19 into a pET-3a-K cassette and introduced into *E. coli* BL-21 (DE3) for expression. The wild-type and mutant enzymes were induced by isopropyl 1-thio-$\beta$-D-galactopyranoside and purified by ammonium sulfate fractionation and Bio-Rex-70 column chromatography as described before (Pandey *et al.*, 1994a). The protein concentrations were determined by Bradford colorimetric assay (Bradford, 1976) as well as by spectrophotometric measurement using $E_{278} = 6.32 \times 10^4$ M$^{-1}$ cm$^{-1}$ (Setlow *et al.*, 1972).

An exonuclease-deficient mutation was constructed by replacing Asp424 with Ala424 (D424A) by a two-step polymerase chain reaction (PCR) using the megaprimer technique (Pandey *et al.*, 1993). The 0.7-kilobase *Nde*I and *Sac*I digest of the second step PCR product carrying the D424A mutation was cloned in the wild-type pET-3a-K cassette. After the mutation was ascertained by double-stranded DNA sequencing (Sanger *et al.*, 1977), the *Nde*I–*Sac*I fragment from exo$^-$ mutant was cloned in the pET-3a-K cassette carrying the Y766S mutation.

*Enzyme Assay.* Measurement of polymerase activity was carried out essentially as described before (Pandey *et al.*, 1990); 3′ → 5′ exonuclease activity was measured either on double-stranded DNA (Pandey & Modak, 1988) or on single-stranded DNA substrate (Derbyshire *et al.*, 1991).

*Cross-Linking of dNTP to Enzyme.* In order to examine the ability of mutant enzyme to form a binary complex with dNTP, the UV-mediated cross-linking of the enzyme with dNTP substrate was carried out. For cross-linking experiments, 1 $\mu$M mutant enzyme was incubated on ice for 10 min with 5 $\mu$M [$^{32}$P]dTTP (40 $\mu$Ci/nmol) in a buffer containing 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8, and 2% glycerol. The reaction mixture was then exposed to UV in the Spectrolinker (Spectronic Corp.) at a dose of 300 mJ/cm$^2$. Measurement of covalent attachment of [$^{32}$P]dTTP to the enzyme protein was performed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), followed by autoradiography. Quantitation of radioactivity contained in the individual band was carried out either by densitometric scanning of the autoradiogram or by excising the radioactive band from the gel and counting by Cerenkov counts per minute.

*Cross-Linking of Enzyme to Template–Primer.* We have used three heteromeric template–primers (27/10 AAAAA, 27/10 ACACA, and a self-annealing 37-mer; see Chart 1) and two homopolymeric template-primers, poly (dA)·$(dT)_{15}$ and poly (rA)·$d(T)_{15}$, for binding studies. The heteromeric template–primers were 5′-$^{32}$P-labeled either in the primer or in the template strand using [$\gamma$-$^{32}$P]ATP and $T_4$ polynucleotide kinase according to the standard protocol (Ausubel *et al.*, 1987). The homopolymeric template–primers and 5′-$^{32}$P label in the primer stand. The labeled DNA was purified on a NAP-10 column (Pharmacia) and adjusted to the required specific activity with unlabeled template–primer. The concentration of template–primers used in the cross-linking reaction was in the range of their $K_d$ values observed for the wild-type enzyme and varied from 200 nM for homopolymeric template–primer to 100 nM for heteropolymeric template–primer. For cross-linking, 1.2 $\mu$M enzyme and the desired concentration of labeled template–primer (1000 cpm/pmol) were incubated on ice for 10 min in a reaction mixture containing 50 mM Tris-HCl, pH 7.8, 1 mM DTT, 2 mM EDTA, and 5% glycerol. The template–primer cross-linked enzyme species were subjected to SDS–polyacrylamide gel electrophoresis, and the extent of cross-linking was quantitated by excising the radioactive bands and measuring the Cerenkov counts associated with them or by densitometric scanning of the autoradiogram. The cross-linked E–dNTP and E–template–primer complexes show the expected differences in gel mobility.

*Steady-State Kinetics of Polymerization.* The kinetic studies were carried out at 25 °C as described by Bryant *et al.* (1983) using homopolymeric or defined heteromeric DNA as the template–primer. Template–primers were prepared by mixing equimolar amounts of template and primer at 20 $\mu$M concentration. The reaction mixture contained 50 mM Tris HCl, pH 7.5, 2 mM $MgCl_2$, and variable concentrations of both template–primer and dNTP substrate. The concentration of enzyme from 5 nM with the wild-type enzyme to 50 nM with Y766S mutant. The reaction was initiated by the addition of $MgCl_2$ and terminated by the addition of ice-cold 5% trichloroacetic acid at the desired time point. The trichloroacetic acid-precipitable material was collected on a Whatman GF/B filter and counted for radioactivity in liquid scintillation counter as described before (Pandey *et al.*, 1987).

*Determination of Rate Constants for the Incorporation of First and Second Nucleotide by the Wild-Type and Mutant Enzymes.* Two different 27/10-mer template–primers containing a stretch of AAAAA or ACACA in the template region were used in this study (see Chart 1). The first and second nucleotides are dC and dA in both the template–primers. Rates of incorporation of the first nucleotide were measured by using individual 27/10 template–primers in a standard reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 $\mu$M [$\alpha$-$^{32}$P]-dGTP (2 $\mu$Ci/nmol), and 5–50 nM enzyme. The reaction was initiated at 25 °C by addition of $MgCl_2$, aliquots were withdrawn at desired time intervals, and the incorporation of radiolabeled substrate was determined by acid precipitation. The rates of second nucleotide addition on the above template–primers were determined in a similar manner except that the reaction mixture contained 5 $\mu$M $^{32}$P-labeled dTTP in addition to 10 $\mu$M unlabeled first nucleotide (dGTP). The steady-

state rate constant ($k_{cat}$) for polymerization of dGMP and dTMP for both the mutant and the WT enzyme was determined from the ratio of slope *versus enzyme* concentration as described by Bryant *et al.* (1983).

*Processivity Measurement.* Two different template-primers (27/10, see Chart 1) containing a stretch of either AAAAA or ACACA in the template region were used for a qualitative measurement of processivity of DNA synthesis by the mutant and the wild-type enzymes. The 10-mer primer labeled with $^{32}P$ at the 5′ terminus was annealed with 27-mer template at an equimolar ratio. The appropriate enzyme was first incubated with the labeled template–primer in an incubation mixture containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2.5 nM primer termini, and 100 nM enzyme in a total volume of 3 μL. After 60 s of incubation at 25 °C, the polymerase reaction was initiated by the addition of a 3-μL solution containing 200 μM dNTP (50 μM each), calf thymus DNA trap (Joyce, 1989) equivalent to 50 μM primer termini, 50 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$. The reaction was allowed to continue at 25 °C, and 2.5-μL samples were removed after 1- and 10-min incubations and mixed with 2 μL of stop solution containing 0.5% SDS and 100 mM EDTA (Polesky *et al.*, 1990). Samples were kept frozen in dry ice until ready for gel analysis. The products were analyzed on a denaturing 20% polyacrylamide gel containing 7 M urea followed by autoradiography.

## RESULTS

*Polymerase Activity of Klenow Fragment and Its Mutant Derivative Y766S.* In the initial characterization of the catalytic abilities of control and mutant enzyme, we used two template–primers, poly(rA)·$(dT)_{15}$ and poly(dC)·$(dG)_{15}$. An interesting observation was the difference in enzyme activities with the two template–primers; i.e., the mutant enzyme showed only about 20% of the control activity with poly(rA)·$(dT)_{15}$ as a template–primer, while exhibiting nearly identical activity with poly(dC)·$(dG)_{15}$. We therefore compared the polymerase activity of the control and mutant Klenow fragment with a variety of synthetic template–primers, which were as follows: poly(dA)·$(dT)_{15}$, poly(rA)·$(dT)_{15}$, poly(dC)·$(dG)_{15}$, poly(dC)·poly(dG), poly(dT)·$(dA)_{15}$, poly(dAT), and activated calf thymus DNA. The activity of the Y766S mutant was significantly reduced when poly(rA), poly(dA), and activated DNA were used as the the templates. However, the activities of the mutant and control enzymes with the other template–primers were nearly identical (see Figure 1). The activity of the Y766S mutant with exo$^+$ was also significantly reduced when poly(rA) and poly(dA) were used as the templates (results not shown). These results suggested that the catalytic deficiency of the mutant enzyme may be restricted to the polymerization of substrate dTTP. However, the observation that dTTP incorporation with poly(dAT) as a template is unaffected implied that the defect in the mutant enzyme may not be related to dTTP recognition per se.

In order to further investigate if the defect in the mutant enzyme is in the recognition of TTP substrate, incorporation of TTP using a 37-mer self-annealing heteropolymeric DNA template–primer was examined. In this template primer, addition of only a single dTMP is possible provided only dTTP is present. Results presented in Figure 1 show that dTTP incorporation by the mutant and the Klenow fragment with this DNA is nearly identical.

*Steady-State Kinetic Parameters for Y766S (exo$^-$ and exo$^+$) and the Wild-Type Klenow Fragment.* Since the mutant Y766S (both exo$^-$ and exo$^+$) showed significantly reduced activity with poly(rA)·$(dT)_{15}$ and poly(dA)·$(dT)_{15}$ but not

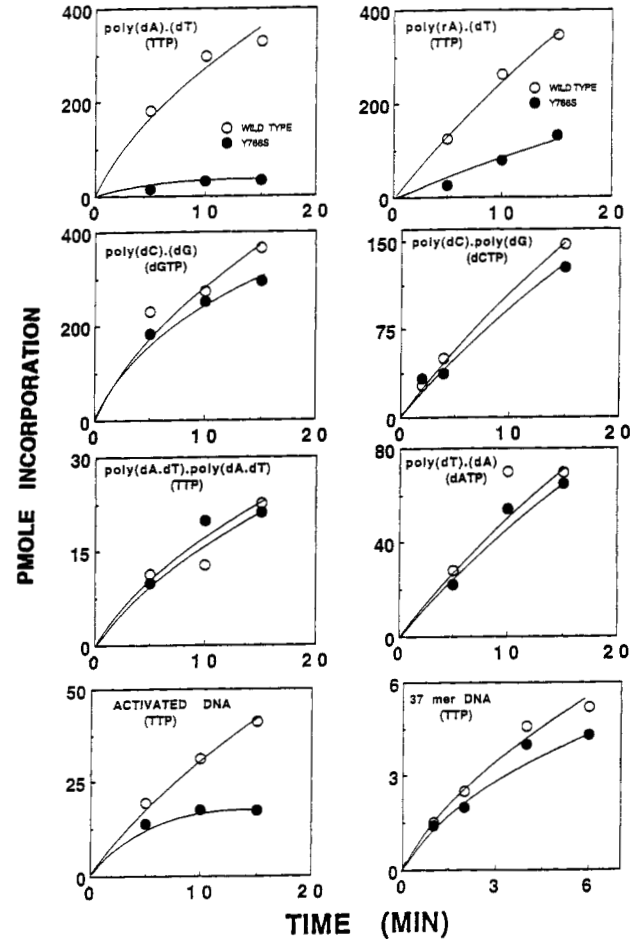


FIGURE 1: DNA polymerase activity of the Klenow fragment and its mutant derivative Y766S on different template–primers. Ten nanograms of Klenow fragment or mutant Y766S was incubated at 37 °C in a reaction mixture in a final volume of 100 μL containing 50 mM Hepes, pH 7.8, 10 μg of bovine serum albumin, 50 mM NaCl, 20 μM appropriate [$^3$H]dNTP (1 μCi), and 0.5 μg of desired template–primers. Activated DNA containing reaction mixture contained all four dNTPs at 20 μM concentrations. Reactions were initiated by the addition of $MgCl_2$ to a final concentration of 10 mM. An aliquot of the reaction mixture was removed at desired time intervals and placed in tubes containing ice-cold 5% trichloroacetic acid. Incorporation of radioactive precursors was then determined as described in Materials and Methods.

with poly(dC)·$(dG)_{15}$, steady-state kinetic parameters were determined for the mutant enzyme using poly(dA)·$(dT)_{15}$ and poly(dC)·$(dG)_{15}$ as template–primers. As the enzyme utilizes two substrates, i.e., dNTP and template–primer, pseudo-first-order conditions were established by maintaining the concentration of one substrate at saturating level, while the concentration of the second substrate was varied. The initial velocity of the reaction was determined by measuring the rate of incorporation of $^3$H-labeled dNTP substrate into the homopolymeric template–primer as a function of both Mg-dNTP and template–primer concentration. The $K_m$ values for dNTP and template–primer substrate and the $V_{max}$ for the polymerase reaction catalyzed by the wild type and its mutant derivative were determined by Eadie–Hofstee plots constructured from the initial velocity data. The results summarized in Table 1 show that replacement of tyrosine with serine (Y766S) causes a 3–4-fold reduction in the $K_m$ value for poly(dA)·$(dT)_{15}$ without any significant change in the apparent affinity ($K_m$) for dNTP substrate, while the efficiency of polymerization [$k_{cat}/K_m$(dNTP)] was decreased by 20-fold. Most interestingly, with poly(dC)·$(dG)_{15}$, both the wild-type and mutant enzymes exhibited identical kinetic parameters and efficiency of polymerization. These results indicate that

Table 1: Kinetic Parameters of WT and Y766S Mutant Derivative with Homopolymeric Template–Primer and a Single dNTP Substrate[a]

| template–primer | dNTP substrate | enzyme | $K_m$ (DNA) (nM) | $K_m$ (dNTP) ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ (dNTP) ($M^{-1}\ s^{-1}$) |
|---|---|---|---|---|---|---|
| poly(dA)·$(dT)_{15}$ | dTTP | WT | 4.5 | 2.2 | 3.90 | $1.8 \times 10^6$ |
| | | Y766S | 15.0 | 3.0 | 0.26 | $0.086 \times 10^6$ |
| poly(dC)·$(dG)_{15}$ | dGTP | WT | 3.6 | 1.2 | 2.0 | $1.7 \times 10^6$ |
| | | Y766S | 5.2 | 1.2 | 2.0 | $1.7 \times 10^6$ |

[a] Kinetic constants were calculated from the initial velocity data where poly(dA)·$(dT)_{15}$ and poly(dC)·$(dG)_{15}$ were used as the template–primers and the respective dNTP (dTTP or dGTP) was used as the substrate.

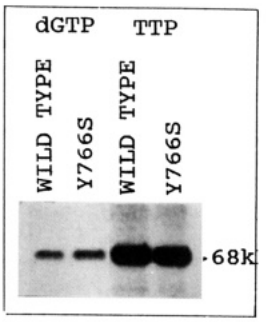


FIGURE 2: Cross-linking of [$\alpha$-$^{32}$P]dGTP and [$\alpha$-$^{32}$P]dTTP to the Klenow fragment and Y766S mutant enzyme. A standard irradiation mixture in a final volume of 50 $\mu$L contained 5 $\mu$Ci of [$\alpha$-$^{32}$P]dTTP or [$\alpha$-$^{32}$P]dGTP (final concentration 1.6 $\mu$M, with specific activity 57.9 Ci/mmol) and 3.5 $\mu$g of Klenow fragment or Y766S. The mixture was subjected to UV irradiation as described in Materials and Methods. The extent of cross-linking of dNTP to enzyme was determined by SDS–polyacrylamide gel electrophoresis followed by autoradiography of the gel.

the Y766S mutant specifically lacked the ability to read dA template, while its efficiency to copy dC template remained unaffected.

*Formation of E–dNTP and E–(Template–Primer) Binary Complexes.* The drastic reduction in the efficiency of the polymerase reaction catalyzed by the mutant enzyme with poly(dA)·$(dT)_{15}$ suggested that the mutant enzyme may be specifically defective in binding to the dTTP substrate. However, the $K_m$ value of dTTP substrate with the Y766S mutant was similar to that of the wild-type enzyme, indicating that the affinity for dNTP substrate was not altered. In order to further confirm that the defect in mutant enzyme is indeed not related to dTTP recognition, the abilities of wild-type and Y766S mutant enzymes to form a binary complex with two different dNTP substrates were compared by measuring the extent of UV-mediated cross-linking of the two enzymes to dGTP and dTTP. The concentration of each dNTP substrate in the cross-linking reaction mixture was kept at subsaturating levels and within close range of its $K_m$ value. The extent of binary complex (E–dNTP) formation was found to be nearly identical with both the mutant and wild-type enzymes (Figure 2). These results correlate well with the kinetic constants, suggesting that mutation Y766S does not affect the binding of either dGTP or dTTP substrate and that the low efficiency of polymerization of dTTP by the mutant enzyme is not due to the defective binding of this nucleotide.

Since the Y766S mutant exhibited low efficiency of polymerization only with polymeric rA or dA template, it seemed plausible that the enzyme may be selectively defective in its ability to bind to these template–primers. In order to assess this premise, the ability of the mutant enzyme to form an E–(template–primer) complex was examined by photochemical cross-linking of the complex. For this purpose, a 5′-$^{32}$P end-labeled 37-mer self-annealing template–primer as

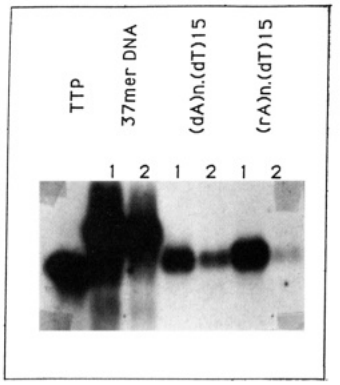


FIGURE 3: Cross-linking of poly[(dA)·($^{32}$P-$dT_{15}$)] and 5′-$^{32}$P-37-mer template–primers to the Klenow fragment and Y766S mutant. The cross-linking of template–primer to enzyme was carried out as described in the legend to Figure 2 except that no divalent cations were present in the irradiation mixture. Lanes 1 and 2 represent wild-type and Y766S enzyme.

well as poly(rA). $^{32}$P$(dT)_{15}$ and poly(dA)·$^{32}$P$(dT)_{15}$ were used as ligands. Since $K_d$ values of homopolymeric template–primers for wild-type enzyme are known ($2 \times 10^{-7}$ M; Bryant et al., 1983), we determined the $K_d$ value for 37-mer self-annealing template–primer (60 nM; Pandey *et al.*, 1994b) in order to evaluate the cross-linking efficiencies at the subsaturating concentration of each template–primer. As judged by the extent of cross-linking, and also by densitometric scanning of the autoradiograms, the mutant enzyme showed some reduction (~25%) in binding affinity for the 37-mer heteromeric self-annealing template–primer, while its binding affinity to both poly(dA)·$(dT)_{15}$ and poly(rA)·$(dT)_{15}$ was significantly reduced (~75–95%) as compared with the WT enzyme (Figure 3).

As Tyr 766 of the Klenow fragment has been shown to be the site for primer cross-linking (Catalano et al., 1990), it is likely that the reduction in cross-linking observed with poly(dA)·$^{32}$P$(dT)_{15}$ could be simply due to mutation of the primer cross-linking residue, i.e., Tyr at the 766 position. In contrast, relatively less effect on the cross-linking of self-annealing 37-mer template–primer by the mutant enzyme may be due to its cross-linking at sites other than Tyr 766 (Pandey *et al.*, 1994b). Therefore, the differential cross-linking results obtained with homopolymeric poly(dA)·$(dT)_{15}$ and self-annealing heteropolymeric DNA may not represent a template-specific binding defect but may be indicative of reduced interaction with the primer binding region of the enzyme. This possibility was ruled out by the following experiments, where the cross-linking efficiency of the mutant enzyme was compared on two template–primers containing either a stretch of A residues or the same stretch containing dA residues alternated with dC (ACACAC) in the template region.

In one set of experiments, each of the template–primers was labeled with $^{32}$P at the 5′ end of either the template strand or the primer strand and the other set was prelabeled at the 3′ terminus by converting 27/10 to 27/11 using [$\alpha$-$^{32}$P]dGTP and the WT Klenow fragment. The results shown in Figure 4 indicate that WT enzyme had equal affinity for both template–primers (Figure 4, lanes 1–4). In contrast, a drastic reduction in the cross-linking of mutant enzyme was observed selectively with -AAAAA-containing template–primer (Figure 4, lanes 5–8). A more pronounced effect on the binding affinity was observed with 27/11 template–primers with dA as the first template base. The binding affinity of mutant enzyme for 27/11-AAAAA template–primer was reduced almost to background level, while WT enzyme exhibited an identical affinity for both template–primers (results not shown). Thus the cross-linking results with AAAAA and ACACA templates

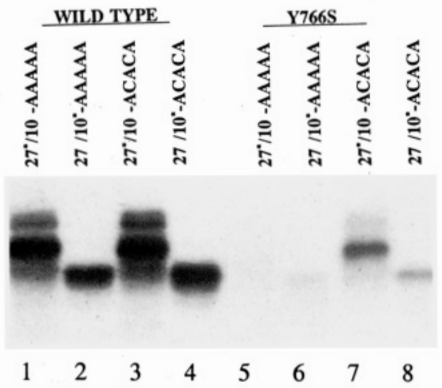


FIGURE 4: Cross-linking of 27/10 template–primers containing AAAAA or ACACA region on the template strand. The cross-linking reaction mixture in a final volume of 50 $\mu$L contained 50 $\mu$L Tris-HCl, pH 7.8, 30 nM template–primer with 5′-$^{32}$P label on either template or primer strand, 3 $\mu$M WT or mutant enzyme, and 2 mM $MgCl_2$. Following UV irradiation, the extent of cross-linking of individual template–primer to enzyme was determined by SDS–PAGE and autoradiography as described in the Materials and Methods. The asterisk indicates the $^{32}$P labeling of the template or primer strand of 27/10 template–primers.

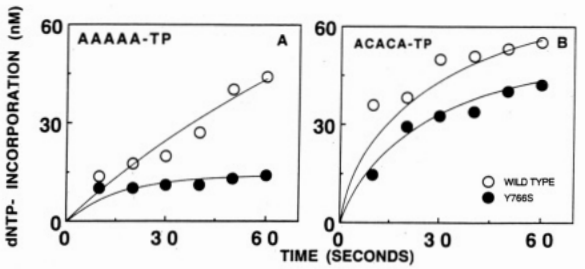


FIGURE 5: Time course of polymerase activity of mutant and WT enzymes on 27/10 template–primers. The two template–primers containing AAAAA (panel A) or ACACA (panel B) sequences in the template strand (see Chart 1) were used to measure the rate of incorporation of dNTP catalyzed by WT and Y766S mutant enzymes. The reaction mixture contained 200 nM template–primer, 5 nM mutant or WT enzyme, 6 $\mu$M each dATP, dGTP, dCTP, and [$\alpha$-$^{32}$P]-dTTP (1 $\mu$Ci/nmol), and 2 mM $MgCl_2$. The reactions were initiated at 25 °C by the addition of divalent metal ion and were terminated at desired time intervals by placing the aliquots into tubes containing ice-cold 5% TCA. The acid-insoluble radioactivity was determined by scintillation spectroscopy.

have clearly shown a template-specific defect in the mutant enzyme, which is reflected in the loss of its ability to form an E–(template–primer) complex.

*Polymerase Activity of Y766S and Control Enzyme with Two Template–Primers.* The defect in the binding of AAAAA templates seen above may well be the rate-limiting step in the formation of a productive E–(template–primer)–dNTP ternary complex when the mutant enzyme encounters the dA region in the template strand. To test this possibility, we measured the time course of the polymerase activity of the mutant and WT enzyme on AAAAA and ACACA templates. Results shown in Figure 5A clearly indicate that, with template–primer containing a stretch of AAAAA sequences, the incorporation of dNTP by mutant enzyme levels off within the first minute, in contrast to the progessive incorporation with time by WT enzyme. This unusual pattern could be due to either formation of nonproductive binary and ternary complexes or slow translocation of the mutant enzyme along the dA template. The rate of dNTP incorporation by both control and mutant enzymes with ACACA template remained nearly identical (Figure 5B). The rate constants for steady-state incorporation estimated from these data indicated a 5-fold decrease in $k_{cat}$ for the mutant enzyme (Y766S, 0.033 $s^{-1}$; WT, 0.16 $s^{-1}$) with AAAAA template, whereas with ACACA

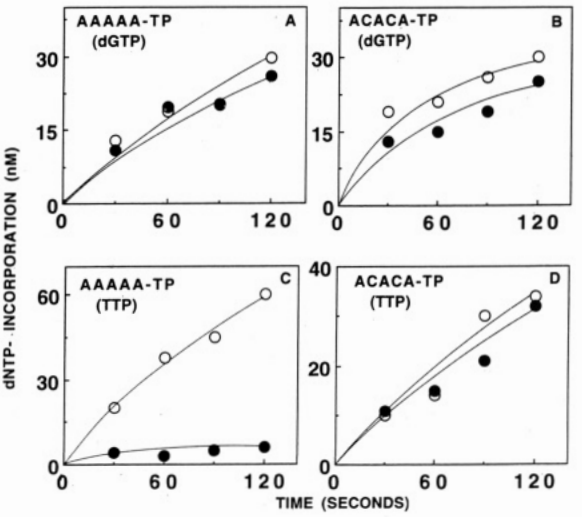


FIGURE 6: Determination of rates of incorporation of first and second nucleotide in two template–primers by mutant and wild-type enzymes. The rates of incorporation of the first (dGTP, panels A and B) and second nucleotide (dTTP, panels C and D) on 27/10 AAAAA and ACACA template–primers were determined as described in Materials and Methods. Because of very poor polymerase activity of Y766S with AAAAA-containing template–primer, the concentration of enzyme in the reaction was increased 10-fold to obtain statistically significant incorporation data for determining the rate constants. Aliquots of the reaction mixture were withdrawn at 5-s intervals. The incorporation data obtained at each time point with the mutant enzyme were normalized to the concentration of WT enzyme (5 nM). The number of time points in the figure was also reduced so that they could be accommodated in the frame. The curve shown in panel C represents the value after normalization.

template the $k_{cat}$ value for Y766S mutant was close to that for the WT enzyme (Y766S, 0.20 $s^{-1}$; WT, 0.22 $s^{-1}$).

*Rate Constants for Addition of First and Second Nucleotides by the Mutant and Wild-Type Enzymes.* The low efficiency of the polymerase reaction catalyzed by the Y766S mutant with a template–primer containing a stretch of dA nucleotides in the template region seemed to be unusual and could be due to formation of an unstable E–(template–primer) or E–(template–primer)–dTTP complex at the dA template region. This prompted us to examine the rate of dT addition on two AAAAA and ACACA template–primers preceded by dC as the first template base (see Chart 1). The rates of incorporation of dGMP as the first nucleotide and of subsequent dTMP nucleotides by the mutant and the wild-type enzymes were compared. The results shown in Figure 6B,D indicate that with the ACACA template (27/10-ACACA) the rates of incorporation of the first nucleotide (dGTP) and subsequent dTTP nucleotides were identical with both the mutant and the wild-type enzyme, whereas with the template containing an oligo(dA) region (27/10-AAAAA) a large difference in the rate of incorporation was observed only with the mutant enzyme (Figure 6A,C). From the data shown in Figure 6, the steady-state rate constants ($k_{cat}$) for polymerization of dGMP and dTMP for both the mutant and WT enzymes were determined from ratio of slope *versus enzyme* concentration as described by Bryant et al. (1983). As shown in Table 2, the ratio of efficiency of incorporation (WT/Y766S) of dGMP varied within a close range from 1.3 to 1.7 with both the template–primers, whereas for dTMP incorporation the ratio of efficiency varied from 1.8 with the ACACA template–primer to 11.4 with the AAAAA template–primer. Thus an 11-fold decrease in the rate of dTMP incorporation observed only with the template containing a stretch of dA sequences suggests that the mutant enzyme is unable to go through the oligomeric dA region either due to reduced affinity or nonproductive binding to such a region or

Table 2: Steady-State Rate Constant for Polymerization of dGTP and dTTP Nucleotides on Defined Template–Primers[a]

| template–primer | dNTP | WT | | Y766S | | ratio of efficiency (WT/Y766S) |
|---|---|---|---|---|---|---|
| | | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}$ $s^{-1}$) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}$ $s^{-1}$) | |
| 27/10-<br>-AGT<br>-TCACAAAAAC- | dGTP<br>dTTP | 0.221<br>0.357 | $0.18 \times 10^6$<br>$0.16 \times 10^6$ | 0.171<br>0.042[b] | $0.14 \times 10^6$<br>$0.014 \times 10^6$ | 1.3<br>11.4 |
| 27/10-II<br>-AGT<br>-TCACACACAC- | dGTP<br>dTTP | 0.207<br>0.242 | $0.17 \times 10^6$<br>$0.11 \times 10^6$ | 0.128<br>0.178 | $0.1 \times 10^6$<br>$0.06 \times 10^6$ | 1.7<br>1.8 |

[a] The $k_{cat}$ values for the first and second nucleotide incorporation on both the 27/10 template–primers were calculated from the slope of the incorporation curve (slope/[enzyme]) shown in Figure 6 (Bryant *et al.*, 1993). $K_m$ values for dGTP and dTTP were taken from Table 1. [b] Because of the rather unusual time course pattern of the mutant enzyme with 27/10-AAAAA template-primer (see Figure 6C), the $k_{cat}$ value determined from the initial time point may represent a value higher than the actual value.

due to rapid dissociation following an encounter with the oligomeric dA region on the template strand.

*Mode of DNA Synthesis on Two Template Regions by Y766S and WT Enzymes.* In order to clarify the nature of the defect in Y766S mutant in copying the oligo(dA) region of the template strand, an experiment was carried out to analyze the mode of DNA synthesis (i.e., processive vs distributive) with AAAAA and ACACA template–primers. The 10-mer primer was labeled at the 5′ position and annealed with the 27-mer template in a molar ratio of 1:1. Wild-type Klenow and its mutant derivatives were incubated with the labeled template–primer to allow the formation of an enzyme–(template–primer) complex. The initiation of the polymerization reaction was carried out by the addition of all four dNTPs and a calf thymus DNA trap in $(50 \times 10^3)$-fold molar excess over the labeled template–primer. The higher concentration of the calf thymus DNA trap present in the reaction would prevent the enzyme from rebinding to the labeled template–primer molecule from which it has dissociated after completing one processive cycle. Results shown in Figure 7 indicate that the Y766S mutant is less processive on AAAAA-containing template (Figure 7, lanes 3 and 4) as compared to ACACA-containing template (Figure 7, lanes 9 and 10). In contrast, the WT enzyme exhibits a processive mode of synthesis with both template–primers (Figure 7, lanes 1 and 2 and lanes 7 and 8). These results support the contention that Y766S mutant enzyme can catalyze the polymerase reaction in the processive mode as efficiently as the WT enzyme, provided the template strand is devoid of oligomeric dA nucleotides. When the mutant enzyme encounters region containing a stretch of dA on the template strand, the enzyme rapidly dissociates from the template–primer and is unable to reinitiate the synthetic cycle, resulting in the appearance of a pseudodistributive mode of synthesis.

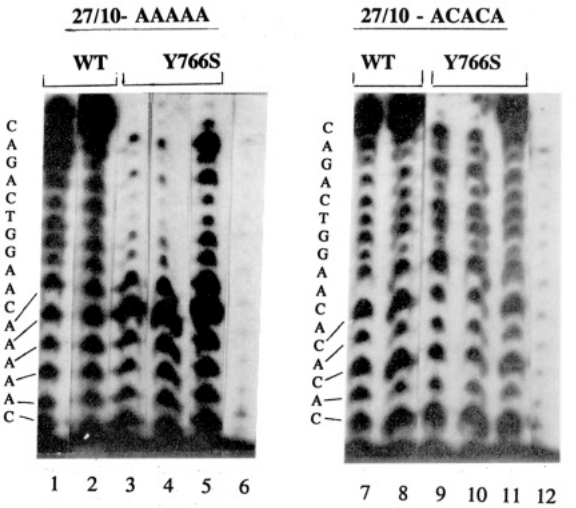


FIGURE 7: Mode of DNA synthesis by Y766S and WT enzymes on two template–primers containing AAAAA and ACACA in the template region. The 5′-$^{32}$P-labeled 10-mer primer was annealed to 27-mer template containing either AAAAA (lanes 1–6) or ACACA (lanes 7–12) at template positions 2–6 (Chart 1). Labeled DNA (2.5 nM) was mixed with 100 nM wild-type or mutant enzyme. The reaction was initiated with the addition of 50 $\mu$M of each dNTP together with a DNA trap (see Materials and Methods) and terminated by the addition of 1% SDS and 100 mM EDTA. The products were analyzed on a 20% polyacrylamide gel containing 7 M urea followed by autoradiography as described in Materials and Methods. In lanes 1–4 and 7–10, the DNA trap was added along with dNTP and the product length generated represents a single processive cycle during 1 min of incubation (lane 1, 7 with WT; lane 3, 9 with Y766S) and 10 min of incubation (lane 2, 8 with WT; lane 4, 10 with Y766S) with AAAAA and ACACA template–primers, respectively. Lanes 5 and 11 represent the DNA synthesis by Y766S mutant in the absence of a DNA trap. Lanes 6 and 12 demonstrate the effectiveness of the DNA trap by including the trap at the binding step and carrying out the reaction as above for 10 min after dNTP addition. The letters on the left side of each panel indicate the template sequence in both template–primers.

## DISCUSSION

Tyrosine 766 of *E. coli* DNA polymerase has been identified as an important residue which participates in the catalysis of DNA synthesis. It was first identified as a site of cross-linking to azido-dATP (Rush & Konisgsberg, 1990) as well as to the primer terminus (Catalano *et al.*, 1990). Site-directed mutagenesis of tyrosine 766 (to serine 766) was then shown to result in the reduction of catalytic activity (Polesky *et al.*, 1990), and the mutant Y766S was also reported to exhibit increased infidelity during the catalysis of DNA synthesis (Carroll *et al.*, 1991). The location of Tyr 766 in the active-site domain was clearly visible in the 3D model structure (Yadav *et al.*, 1992) as well as in the recently described crystal structure of the binary complex of the Klenow fragment and substrate dNTP (Beese et al., 1993). The report that Tyr 766 is the cross-linking site for dATP (Rush & Konigsberg, 1990) was of particular interest to us since we had found that histidine 881 of pol I was the site of cross-linking to dTTP (Pandey *et al.*, 1987). It thus appeared that purine and pyrimidine nucleotides may (selectively) bind to Tyr 766 and His 881, respectively. In order to assess this possibility, we examined the catalytic activity of Y766S mutant with homopolymer templates. We found that the mutant enzyme exhibited different catalytic activity with poly(dA)- and poly(dC)-templated reactions as judged by the polymerization of dTTP and dGTP, respectively (Figure 1). It was only the synthesis directed by poly(dA) as well as poly(rA) that was severely affected. In fact, the activity of Y766S with templates lacking oligo/poly(A) did not seem to differ from that of the wild-type enzyme to any significant extent. The presence of tyrosine at the 766 position is essential for reading through a stretch of A sequences in the template strand since alternating copolymer poly(dA)-(dT)·(dA)-(dT) and heteropolymeric DNA without an oligo(dA) region in the template support

synthesis comparable with the wild-type enzyme. Examination of the ability of Y766S mutant enzyme to cross-link to dTTP, dGTP, and template–primers showed that the mutant enzyme was able to form a binary complex (E–dNTP) as well as the wild-type enzyme (Figure 2). The functional significance of the binary complex between dNTP and the Klenow fragment is indicated by the following observations: the pyridoxylation of lysine 758 affects the formation of the binary as well as the ternary complex with dNTP (Basu et al., 1987). Similarly, the K758A mutant exhibits very low catalytic activity and shows significant loss in its ability to form the binary complex. Therefore, the binary complex between dTTP or dGTP and Y766S may indicate that the mutant is not defective in substrate binding activity. Binding of the 37-mer template–primer to the Y766S mutant enzyme was only slightly reduced, while the binding of poly(dA)·$(dT)_{15}$ and poly(rA)·$(dT)_{15}$ to the enzyme seemed to be severely affected (Figure 3). The steady-state kinetic parameters of WT and Y766S mutant enzymes, however, indicated that the affinity for dNTP substrate remained unchanged with both poly(dA)·$(dT)_{15}$ and poly(dC)·$(dG)_{15}$ template–primers. Nevertheless, the efficiency of polymerization by Y766S mutant was found to be severely reduced only with poly(dA)·$(dT)_{15}$, while it remained unaffected with poly(dC)·$(dG)_{15}$ template–primer. Further insight into the restriction on the catalytic activity of Y766S imposed by A templates was obtained through the use of two synthetic 27/10-mer template–primers where template nucleotides 11–16 contained the sequence CAAAAA or CACACA. Rates of incorporation of dGTP (first nucleotide) and dTTP (second nucleotide) by wild type and Y766S were compared with these two template–primers. Results clearly indicated that it is only the dTTP addition with AAAAA-containing template–primer that was severely affected in the mutant-catalyzed reaction. Thus, encounter with an oligo(A) sequence in the template seemed to be the major obstacle for the Y766S enzyme. Further support for this contention came from an experiment to determine the processivity of DNA synthesis catalyzed by the mutant and wild-type enzymes (Figure 7). It was observed that with 27/10 ACACA template–primer the mutant enzyme exhibited processivity identical to the WT enzyme, while with 27/10 AAAAA template–primer it showed a distinct pause pattern upon encountering the oligo(dA) region of the template.

Results from our laboratory with the K758A mutant, which also lies on the same O-helix where Tyr 766 is located, have indicated a similar template-dependent defect in reading dA templates (Pandey *et al.*, 1994a). These results strongly suggest the possibility that the entire O-helix surface may be involved in the recognition and/or translocation across the oligo(A) templates.

It had been reported earlier that the mutant Y766S exhibits a 2–3-fold increase in both $K_m$(dTTP) and in $k_{cat}$, suggesting the involvement of this residue in the binding of dNTP (Polesky *et al.*, 1990). Although our results appear qualitatively similar to those reported by Polesky *et al.* (1990), detailed analyses with different template–primers has shown a clear effect of the Tyr 766 mutation on template binding function, especially when a oligo(A) sequence is encountered. The observed increase in $K_m$ for dTTP is only marginal and may be due to secondary effects caused by impaired translocation following the nucleotidyl transferase reaction.

In conclusion, on the basis of the above-discussed observations, Tyr 766 of *E. coli* DNA polymerase I appears to play an important role in the binding and translocation of the enzyme, especially to a stretch of A nucleotides in the template strand. This conclusion may be generalized to imply that the ability of the polymerase class of enzymes to pass/copy certain DNA templates with structural deformities would require the presence of specific residues.

## ACKNOWLEDGMENT

We thank Dr. Catherine Joyce of Yale University for generously providing the purified mutant protein used in the initial studies. We also thank Stephan Sarafianos for many useful discussions.

## REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. S., Smith, J. A., & Struhl, K. (1987) *Current Protocols in Molecular Biology*, pp.2.4.1–2.4.2, Greene Publishing Associates & Wiley-Interscience, John Wiley & Sons, New York.

Basu, A., & Modak, M. J. (1987) *Biochemistry 26*, 1704–1709.

Beese, L. S., Friedman, J. S., & Steitz, T. A. (1993) *Biochemistry 30*, 14095–14101.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bryant, F. R., Johnson, K. A., & Benkovic, S. J. (1983) *Biochemistry 22*, 3537–3546.

Carroll, S. S., Cowart, M., & Benkovic, S. J. (1991) *Biochemistry 30*, 804–813.

Catalano, C. E., Allen, D. J., & Benkovic, S. J. (1990) *Biochemistry 29*, 3612–3621.

Derbyshire, V., Grindley, N. D. F., & Joyce, C. M. (1991) *EMBO J. 10*, 17–24.

Joyce, C. M. (1989) *J. Biol. Chem. 264*, 10858–10866.

Joyce, C. M., Sun, C. X., & Grindley, N. D. F. (1992) *J. Biol. Chem. 267*, 24485–24500.

Kaushik, N., Sanzgiri, R. P., Patil, M. S., & Pandey, V. N. (1993) *Curr. Sci. 64*, 102–107.

Kornberg, A., & Baker, T. (1992) in *DNA Replication*, 2nd ed., W. H. Freeman & Co., New York.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367–382.

Laemmli, U. K. (1970) *Nature 277*, 680–685.

Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G., & Steitz, T. A. (1985) *Nature 313*, 762–766.

Pandey, V. N., & Modak, M. J. (1988) *J. Biol. Chem. 263*, 6068–6073.

Pandey, V. N., Williams, K. R., Stone, K. L., & Modak, M. J. (1987) *Biochemistry 26*, 7744–7748.

Pandey, V. N., Kaushik, N. A., Pradhan, D. S., & Modak, M. J. (1990) *J. Biol. Chem. 265*, 3679–3684.

Pandey, V. N., Kaushik, N., Sanzgiri, R., Patil, M. S., Modak, M. J., & Barik, S. (1993) *Eur. J. Biochem. 214*, 59–65.

Pandey, V. N., Kaushik, N. K., & Modak, M. J. (1994a) *J. Biol. Chem. 269*, 13259–13265.

Pandey, V. N., Kaushik, N., & Modak, M. J. (1994b) *J. Biol. Chem.* (in press).

Polesky, A. H., Steitz, T. A., Grindley, N. D. F., & Joyce, C. M. (1990) *J. Biol. Chem. 265*, 14579–14591.

Rush, J., & Konigsberg, W. (1990) *J. Biol. Chem. 265*, 4821–4827.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A 74*, 5463–5467.

Setlow, P., Burtlag, D., & Kornberg, A. (1972) *J. Biol. Chem. 247*, 224–231.

Yadav, P. N., Yadav, J., & Modak, M. J. (1992) *Biochemistry 31*, 2879–2886.