Participation of the Fingers Subdomain of *Escherichia coli* DNA Polymerase I in the Strand Displacement Synthesis of DNA*§

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The replication of the genome requires the removal of RNA primers from the Okazaki fragments and their replacement by DNA. In prokaryotes, this process is completed by DNA polymerase I by means of strand displacement DNA synthesis and 5'-nuclease activity. Here, we demonstrate that the strand displacement DNA synthesis is facilitated by the collective participation of Ser769, Phe771, and Arg841 present in the fingers subdomain of DNA polymerase I. The steady and presteady state kinetic analysis of the properties of appropriate mutant enzymes suggest that: (a) Ser769 and Phe771 together are involved in the strand separation via the formation of a flap structure, and (b) Arg841 interacts with the template strand to achieve the optimal strand separation and DNA synthesis. The amino acid residues Ser769 and Phe771 are constituents of the O1-helix, which together with O and O2 helices form a 3-helix bundle structure. We note that this 3-helix bundle motif also exists in prokaryotic RNA polymerase. Thus in both DNA and RNA polymerases, this motif may have been adopted to achieve the strand separation function.

Strand displacement synthesis is an essential process in the removal and replacement of RNA primer moieties of Okazaki fragments. In prokaryotes, DNA polymerase I (pol I) carries out this function by its 5'-nuclease and 5'-3' polymerase activities. Whereas early studies indicate a coordination of 5'-nuclease with the polymerase activity (1), the precise mechanism underlying this process is not clear. It appears that the participation of 5'-nuclease activity is not necessary for the strand displacement synthesis because the Klenow fragment of *Escherichia coli* DNA polymerase I has been known to catalyze strand displacement DNA synthesis (2). Thus, the strand separation activity resides in the polymerase domain of pol I.

Despite the availability of numerous DNA-bound structures of polymerases (3–8), no significant information pertaining to strand displacement could be discerned because none of these crystal structures contained sufficiently long single-stranded template overhang or a downstream double-stranded DNA. One exception to this is the DNA-bound crystal structure of mammalian DNA polymerase β, where the enzyme-DNA complex contains gapped DNA (9). However, this enzyme has no strand displacement activity. In the DNA-bound crystal structures of pol I family DNA polymerases, the immediate unpaired template nucleotide assumes a flipped conformation (6) such that it cannot pair with the incoming dNTP substrate. The base moiety of the template (n + 1) nucleotide is positioned out of the DNA helical axis by more than 90° (4, 10, 11). In the enzyme-DNA-dNTP bound ternary complex, this nucleotide rearranges its conformation and pairs with the incoming dNTP substrate (6). In the crystal structures of polymerases, because of the short length of the single-stranded template overhang, only few interactions of downstream DNA with the enzyme protein could be discerned (3, 4, 6–8, 11–13). The ternary complex crystal structure of KlenTaq shows that the 5'-phosphate group of the template nucleotide, which pairs with the incoming dNTP (referred to as n + 1 hereafter), interacts with Ser674 (equivalent to Ser769 of *E. coli* pol I) (6). In T7 DNA polymerase, the position equivalent to Ser769 is Gly527.

The phosphate backbone of the n + 1 template nucleotide in KlenTaq interacts with Arg746, which is topologically equivalent to Arg841 of KF. Arg746 has an additional interaction with next template nucleotide (n + 2) in the single stranded region. Similar interactions are also noted in T7 DNA polymerase ternary complex, where His607 occupies the position of Arg746 of KlenTaq (4, 8). In the catalytically active DNA-bound binary complex of *Bacillus stearothermophilus* (Bst) (5), Tyr19 (equivalent to Phe771 of KF) packs against the n + 2 nucleotide of the template. It has also been shown experimentally that Phe771 of KF binds the 2nd nucleotide, con-

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The abbreviations used are: pol I, E. coli DNA polymerase I; KF, Klenow fragment of *E. coli* DNA polymerase I; TP, template primer; KlenTaq, Klenow fragment equivalent of *T. aquaticus* DNA polymerase I; Bst, Klenow fragment equivalent of *B. stearothermophilus* DNA polymerase I; WT, wild-type; nt, nucleotide.

§ The numbering scheme for the template nucleotide is indicated in Scheme 1.
Strand Displacement Activity of Polymerase I Klenow Fragment

confirming the structural observation (14, 15). Therefore, it can be inferred that Ser769, Phe771, and Arg841 interact with the single-stranded template overhang. Biochemical results have confirmed some of these structural observations. Photoaffinity labeling has demonstrated that the single-stranded template overhang makes a close contact with Phe771 of E. coli pol I (14, 15). However, the mutant F771A polymerase did not exhibit significant alteration in its polymerase activity (14), suggesting a role for Phe771 in a process other than the template strand stabilization required for the nucleotidyl transfer reaction.

The interactions between pol I and the downstream double-stranded DNA have not been characterized. T7 RNA polymerase is homologous to the pol I family of DNA polymerases, and its structure with a double-stranded downstream DNA has been solved (16). The superposition of the 3-helix bundle from pol I and T7 RNA polymerase shows that Phe771 of pol I is structurally homologous to Phe644 of T7 RNA polymerase. Phe644 of T7 RNA polymerase is located at the DNA fork, implying its role in DNA strand separation although there are no reported studies of Phe644 mutation.

We propose tentative interactions of Ser769, Phe771, and Arg841 of pol I with a nicked DNA generated after the gap-filling reaction, prior to strand displacement (Fig. 1). This proposal suggests that some rearrangements in the interactions of Ser769, Phe771, and Arg841 would be required as the enzyme encounters the nicked DNA situation. Based on this proposal, we carried out the biochemical characterization of the alanine mutant enzymes of these residues with respect to their strand displacement activity. The mutant enzymes were also examined for their ability to synthesize DNA on gapped DNA substrates. Our observations clearly show that Ser769, Phe771, and Arg841 of pol I are required for strand displacement synthesis. Arg841 is required for stabilizing the template strand, while Ser769 and Phe771 play a role in initializing the process of strand separation.

EXPERIMENTAL PROCEDURES

Materials—The PCR grade dNTPs were from Roche Applied Science. Radiolabeled nucleotides were obtained from PerkinElmer Life Sciences. The DNA extraction kit was from Qiagen, whereas DNA oligodeoxynucleotides were either from MWG-Biotechnologies or from the Molecular Resources Facility at the New Jersey Medical School, Newark, NJ. All 32P 5′-end-labeled oligomers were purified by denaturing polyacrylamide-urea gel electrophoresis.

In Vitro Site-directed Mutagenesis and Purification of WT and Mutant Enzymes—The PCR-based protocol using PFU-Turbo polymerase, as described in Stratagene’s QuikChange site-directed mutagenesis kit was used to generate the desired mutations of KF. The plasmid pCJ141 (17, 18) (provided by Dr. Catherine Joyce of Yale University) was used for the generation of KF protein and to construct the desired mutant derivatives. This plasmid contains the E. coli KF gene carrying a mutation, D424A. This mutation abolishes 3′–5′ exonuclease activity of pol I. The wild-type KF used for this study and its Ser769, Phe771, and Arg841 mutants are therefore exonuclease-deficient. Plasmid DNA from mutant clones, after confirming desired mutation by DNA sequencing, was used to transfected an expression strain, E. coli CJ376 (19). Isolation and purification of S769A, F771A, R841A, and S769A/F771A (double mutant) enzymes were carried out as described before (20). The WT pol I and the double mutant S769A/F771A (double mutant) enzymes were carried out as described before (20). The WT pol I and the double mutant S769A/F771A (double mutant) enzymes were carried out as described before (20).
reaction, the rates of single nucleotide incorporation were determined under single turnover conditions by rapid mixing of enzyme\textsuperscript{32}P-radiolabeled template primer blocker (32/14/14-mer; see Scheme 1) complex with Mg\textsubscript{2+}/H\textsubscript{18528}dNTP, followed by quenching of the reaction by EDTA. All assays were carried out in 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl\textsubscript{2}, 1 mM dithiothreitol, 0.01% bovine serum albumin, and 10% glycerol at 25 °C. Preincubated KF (wild-type or mutant enzymes) (60 nM) and template primer blocker (20 nM) were loaded into one sample loop, and dATP (50 nM) and MgCl\textsubscript{2} (5 mM) were loaded into the other sample loop. Reactions were quenched at desired times (5 ms to 5 s) with 0.3 M EDTA, and the products were electrophoresed on 16% polyacrylamide gel containing 8M urea. The intensities of the product and substrate bands here and in all other assays were quantitated with a PhosphorImager and ImageQuant version 5.2 (Molecular Dynamics). To ensure reproducibility, the assays were repeated at least two times. The amplitudes and rate constants for the duplicate experiments were in very close agreement.

Data Analysis—The amount of product formed (P) was graphed as a function of time (t), and the data were fit by nonlinear regression to the burst Equation 1.

\textbf{Observed Catalytic Rates (k\textsubscript{obs}) of Single Nucleotide Incorporation of WT and Mutant Enzymes with Nicked DNA—}

The assays to determine the catalytic rates of single nucleotide incorporation were performed under the identical conditions mentioned above except for the DNA substrate, which was a nicked DNA consisting of a 28-mer template annealed with two 14-mer oligonucleotide (Scheme 1). To determine the DNA binding affinity of the wild-type and mutant enzymes for nicked DNA, the enzymes (60 nM) were preincubated with nicked DNA (final concentration of 10–160 nM), were loaded into one sample loop, and dATP (50 nM) and MgCl\textsubscript{2} (5 mM) were loaded into the other sample loop. After rapid mixing, the reactions were quenched with 0.3 M EDTA at various reaction time intervals. The products were resolved by urea-polyacrylamide gel electrophoresis. The quantitation of products was carried out as above.

\textbf{Data Analysis—}

The amount of product formed (P) was graphed as a function of time (t), and the data were fit by nonlinear regression to the burst Equation 1.
TABLE 1

Polymerase activity parameters of WT and mutant Klenow fragment enzymes

| Enzyme       | DNA polymerase activitya | K\textsubscript{cat} \textsuperscript{b} | K\textsubscript{D,DNA} \textsuperscript{b} |
|--------------|--------------------------|------------------|------------------|
|              | Gapped DNA | Nicked DNA | Nicked DNA |
|               | % | s\textsuperscript{-1} | s\textsuperscript{-1} | M | M |
| WT           | 100 | 150 | 0.19 | 0.29 | 0.55 | 7.1 |
| S769A        | 81  | 128 | 0.11 | 0.27 | 0.88 | 10.5 |
| F771A        | 70  | 149 | 0.07 | 0.30 | 0.96 | 9.8  |
| S769A/F771A  | 65  | 143 | 0.03 | 0.30 | ND\textsuperscript{c} | 11.9 |
| R841A        | 65  | 90  | 0.04 | 0.40 | 2.10 | 13.0 |

a The polymerase activity was determined by standard trichloroacetic acid precipitation assay. The 100% activity of KF equals 1.21 \times 10\textsuperscript{4} units/mg of protein. One unit is defined as the activity necessary to incorporate 1 nmol of dNMP into acid-insoluble material in 30 min at 37 °C.

b The K\textsubscript{D} value given in this table is determined from the single nucleotide incorporation assay on the gapped DNA substrate by dividing the constant k\textsubscript{ss} (steady state rate constant) by the amplitude (A) in Equation 1.

c The DNA binding affinity (K\textsubscript{D,DNA}) for the Template Primer (TP) was determined by gel retardation assay as described by Singh and Modak (20). The K\textsubscript{D,DNA} for the nicked DNA substrate was determined by a single nucleotide incorporation assay as described under “Experimental Procedures.”

d Not determined.

where A is the amplitude of the burst phase, k\textsubscript{1} is the rate constant of the burst phase, and k\textsubscript{ss} provides the rate of the linear (steady-state) phase. The amplitudes of the bursts (A) were graphed as a function of total DNA concentration ([DNA]), and the data were fit to the quadratic Equation 2,

\[ A = 0.5(K_D + [\text{pol}] + [\text{DNA}]) \]

\[ - \sqrt{0.25(K_D + [\text{pol}] + [\text{DNA}])^2 - ([\text{pol}] [\text{DNA}] / K_D)} \]

where K\textsubscript{D} is the dissociation constant for the pol-DNA complex, and [pol] is the concentration of active polymerase molecules.

Strand Displacement DNA Synthesis by WT and Mutant Enzymes—The strand displacement DNA synthesis by the WT and mutant enzymes was assayed by incubating 100 nm gapped and/or flap structured DNA with 50 nm of enzyme in a buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 0.01% bovine serum albumin, and 10% glycerol, at 25 °C. The DNA synthesis was initiated by the presence of all dNTPs and Mg\textsuperscript{2+} as the metal cofactor. None of the mutants showed any significant decrease in DNA synthesis activity (Table 1), suggesting that the individual residues Ser769, Phe\textsuperscript{771}, and Arg\textsuperscript{841} do not play a critical role in the polymerase function.

Results

Mutant proteins, F771A, S769A, R841A, and S769A/F771A were prepared to investigate the role of each amino acid in DNA synthesis and strand displacement DNA synthesis. All the enzymes used here including the WT and the mutants were exonuclease-deficient enzymes, because of the D424A mutation, which was introduced to eliminate the 3′→5′ proofreading exonuclease activity and to accurately measure DNA synthesis activity.

DNA Synthesis on a Template Primer Substrate—The DNA synthesis activity of the mutants was determined initially on a heteropolymeric (47/17-mer) template primer substrate in the presence of all dNTPs and Mg\textsuperscript{2+} as the metal cofactor. None of the mutants showed any significant decrease in DNA synthesis activity (Table 1), suggesting that the individual residues Ser\textsuperscript{769}, Phe\textsuperscript{771}, and Arg\textsuperscript{841} do not play a critical role in the polymerase function.

Rate Constant of Single Nucleotide Addition—Further characterization of DNA synthesis was carried out by measuring the rate constant of single nucleotide addition on a defined DNA substrate. WT and mutant polymerases were preincubated with a radiolabeled gapped DNA substrate (Fig. 2) under conditions of excess enzyme over DNA. Reaction, which was introduced to eliminate the 3′→5′ proofreading exonuclease activity and to accurately measure DNA synthesis activity.

FIGURE 2. Single nucleotide incorporation kinetics of the WT and mutant KF enzymes. Presteady-state kinetics of dNTP incorporation with gapped DNA was monitored by preincubating WT or mutant KF (60 nm) and \textsuperscript{32}P-end-labeled gapped DNA (20 nm) (see Scheme 1) followed by mixing with dATP (50 μM) and Mg\textsuperscript{2+} (5 mM) using a rapid chemical quench flow instrument for various reaction times. The data for WT and mutant enzymes (○) WT, (●) S769A, (▼) F771A, (▼) S769A/F771A, and (■) R841A were fit to the burst equation as described under “Experimental Procedures.”
**Strand Displacement Activity of Polymerase I Klenow Fragment**

A

DNA substrate with 5-nt gap: 3' 61-mer self annealing template

Time (Min): 1 3 5 1 3 5

1-mer 3-mer 19-mer (Primer)

WT S769A F771A S769A/F771A R841A WT S769A/F771A

Klenow Fragment DNA pol I

B

DNA substrate with RNA blocker: 3' 51-mer (Template) 5' 14-mer 32-mer (RNA Blocker)

Time (Min): 1 3 5 1 3 5

1-mer 3-mer 19-mer (Primer) 14-mer (RNA Blocker) 32-mer (RNA Blocker)

WT S769A F771A S769A/F771A R841A WT S769A/F771A

Klenow Fragment DNA pol I

FIGURE 3. The pattern of strand displacement DNA synthesis by WT Klenow fragment, WT E. coli DNA pol I, and the indicated mutant enzymes. This figure shows the strand displacement DNA synthesis by WT (KF and pol I) with a gapped DNA substrate containing DNA as blocker shown on top of the figure (A) and RNA as a blocker also shown on the top of the figure (B). The strand displacement DNA synthesis shown in this and in subsequent assays was carried out in a buffer containing 50 mM Tris-HCl, pH 7.8 and 0.1% bovine serum albumin. The enzymes (50 nM) were incubated with DNA substrate (100 nM), and the reactions were initiated by the addition of MgCl₂ (5 mM) and dNTPs (50 μM) together. The reactions were quenched at 1, 3, and 5 min by addition of Sanger’s gel loading buffer, containing 98% deionized formamide, 0.05% xylene cyanol, and 20 mM EDTA. Products were analyzed by gel electrophoresis on 16% polyacrylamide gel containing 8 M urea, and visualized using a phosphorimager. Gapped DNA substrate consisted of a partially self-annealing template of 61 nucleotides, which was further annealed with 14-mer primer to generate a gapped duplex DNA. However, the gapped DNA substrate shown above contained a 51-mer DNA template annealed to the 14-mer DNA primer and 32-mer RNA blocker. The primer extension reaction requires the displacement of the self-annealing 5'-terminal sequence or RNA strand. The product of the 5'-nuclease gap-filling reaction was 19-mer in either case, whereas that of strand displacement was 61-mer for substrate used in A and 51-mer for the substrate used in B. Note that both the WT and mutant enzymes completed the gap-filling synthesis within 1 min. However, only the WT KF, pol I, and S769A mutant enzymes were able to complete the strand displacement DNA synthesis. The mutant enzymes F771A, R841A, and the double mutants (S769A/F771A) of either KF or pol I failed to do so. The identical profile of strand displacement reactions were initiated by rapidly mixing the polymerase-DNA complex with the next correct nucleotide dATP at 50 μM concentration with Mg²⁺ (5 mM) in a rapid quenched-flow instrument. The reactions were quenched after 5 ms to 1 s, and the quenched reactions were run on a sequencing gel to separate the radiolabeled 15-mer product from the starting 14-mer primer. The amount of 15-mer was quantified and graphed as a function of time, and the resulting kinetic profiles (Fig. 2) were fit to obtain the rate constant of dAMP addition by each polymerase. The observed single nucleotide addition rate constants determined from these data for the WT, S769A, F771A, S769A/F771A, and R841A mutant enzymes were 150, 130, 150, 140, and 90 s⁻¹, respectively. Except for the R841A mutant, the rate constants of single nucleotide addition by all the polymerases are very similar, indicating that these amino acid residues do not contribute in any major way to the nucleotide transfer catalytic apparatus of the polymerase.

**Strand Displacement Synthesis by the WT KF, DNA pol I, and Their Mutant Derivatives**—Strand displacement DNA synthesis was measured using the gapped DNA substrate (Fig. 3A) that contained a 5-nt single-stranded region immediately following the 3'-end of the primer, followed by a downstream double-stranded DNA region. To copy the entire template strand, the polymerase needs to separate the downstream double-stranded DNA (strand displacement DNA synthesis). The 5'-end of the downstream non-template DNA strand was phosphorylated to mimic in vivo conditions. To assess the extent of strand displacement DNA synthesis by the WT and the mutant enzymes S769A, F771A, and R841A, DNA synthesis was measured in the presence of all dNTPs. The kinetics of DNA synthesis (Fig. 3A) showed that all the polymerases were able to fill the gap within 1 min of reaction time to make the marked 19-mer product. WT and S769A polymerases were able to displace the blocking strand and synthesize a full-length 61-mer DNA product after longer incubation or reaction times. In contrast, F771A, S769A/F771A, and R841A mutant polymerases were unable to copy the template DNA past the gap, even after 5 min of reaction. These results indicate that F771A, R841A, and double mutant S769A/F771A proteins have a defect in strand displacement DNA synthesis activity.

We examined if the same mutations in complete pol I would have similar properties. A double mutant (S769A/F771A) of pol I was generated and studied alongside the KF mutant enzymes. No difference was noted between WT KF and WT pol I in their ability to catalyze strand displacement DNA synthesis (Fig. 3A). Similarly, double mutation of Ser²⁷⁶ and Phe²⁷¹ in KF as well as pol I rendered the polymerase inactive in catalyzing strand displacement DNA synthesis. All the polymerases were capable of catalyzing the gap-filling reaction with comparable efficiencies.

DNA synthesis by WT KF and pol I confirms that 5'-nuclease domain does not contribute to the separation of the non-template DNA strand of downstream double-stranded DNA. The significant amount of primer left (at 14-mer position) is caused by the fact the ratio of radiolabeled primer to template in this case was 2 to 1, whereas the reverse ratio was used for the self annealing 61-mer template and 14-mer primer.
Because, the biological activity of pol I is to perform gap-filling DNA synthesis and to further displace and cleave the RNA primer that initiate lagging strand DNA synthesis, we investigated DNA synthesis on a substrate containing an RNA blocker. WT KF and WT pol I were able to complete strand displacement DNA synthesis on the substrate with the RNA blocker (Fig. 3B). The single and double mutant enzymes, however, failed to perform strand displacement DNA synthesis on the substrate with the RNA blocker, which is similar to the results observed with the DNA blocker except S769A mutant enzyme, which displayed the strand displacement activity similar to WT KF on DNA blocker. These results, indicate that substrates with DNA and RNA blockers behave in general, in a similar manner, and therefore all further studies were carried out with the DNA blocker substrate, and using KF as the model enzyme.

**Strand Displacement DNA Synthesis under Presteady State Conditions**—To measure the relative efficiencies of gap-filling and strand displacement DNA synthesis, we measured the kinetics of DNA synthesis under single turnover reaction conditions. The time course of DNA synthesis by WT and F771A (as a representative of mutant enzymes) at 50 μM dNTPs and 5 mM MgCl₂ is shown from 5 ms to 5 s (Fig. 4A). Both WT and F771A complete the gap-filling synthesis reaction to make the 19-mer product within 25 ms, but both pause upon encountering the DNA blocker. The WT polymerase makes the full-length DNA by strand displacement synthesis by 1 s, but the F771A fails to do so even after 5 s. Similar behavior was observed with the mutant polymerases, S769A, R841A, and S769A/F771A (data not shown), indicating that the mutant polymerases are defective in strand displacement DNA synthesis.

In the WT reaction, the product of the gap-filling reaction 19-mer is extended to longer DNAs by strand displacement synthesis. To extract the rate constants of formation of 19-mer and its elongation by strand displacement DNA synthesis, the amount of 19-mer was quantified at various reaction times (Fig. 4B). WT and F771A mutant polymerases show a rapid initial increase in 19-mer, but its subsequent decrease with time is more prominent in the WT reaction compared with the F771A reaction. The data were fitted to a double exponential equation (Equation 3; see “Experimental Procedures”).

We also noted from the data shown in Fig. 4A, the initial fraying of the blocker strand from a nicked DNA is a slow step. Once a few bp of the blocker DNA was unwound, the WT polymerase catalyzed strand displacement DNA synthesis very efficiently. The F771A mutant also extended one or two bp inside the double-stranded region, but DNA synthesis beyond that was blocked. These results indicate that F771A is deficient in continued unwinding of the downstream double-stranded DNA.  

**Single Nucleotide Addition Rate on Nicked Substrates**—To determine the efficiency of DNA synthesis on a nicked substrate, the single turnover kinetics of nucleotide addition was measured for the WT and each of the mutant proteins. The kinetics showed that a single nucleotide is added in the nicked substrate at a rate constant that is ~1000-fold less compared with that of the single nucleotide incorporation in the gap-filling reaction (Table 1). This suggests a significant difference in the kinetics of strand displacement DNA synthesis compared with the gap-filling synthesis. The rate constant of nucleotide incorporation by the mutant enzymes was lower compared with that of the WT enzyme. The rate difference varied between 2–7-fold depending upon the mutant polymerase used (Table 1), indicating the requirement of Ser⁷⁶⁹, Phe⁷⁷¹, and Arg⁸⁴¹ in the strand separation by pol I. This difference in the catalytic activity was not due to the defect in DNA binding of mutant polymerases. The $K_{D,\text{DNA}}$ determined by plotting the amplitude of the burst phase against DNA concentration of WT and mutant enzymes was 7.1, 10.5, 9.8, 11.9, and 13.0 nM for WT, S769A, F771A, S769A/F771A, and R841A mutant enzymes, respectively (Fig. 5, B–E). It may be pointed out here that the amplitude of the burst-phase in the R841A mutant reaction is significantly low. One possible reason may be that the amount of productive R841A-DNA complex is significantly less compared with other enzymes.
FIGURE 5. Single nucleotide incorporation by WT and mutant Klenow fragment enzymes with nicked DNA under presteady state conditions. A, single nucleotide incorporation by the wild-type and mutant enzymes (as indicated) with nicked DNA substrate, under presteady state conditions, was carried out essentially under same conditions as those used for gapped DNA (see legend for Fig. 2). B, incorporation of dNTP by WT enzyme (60 nM) with indicated concentrations of the nicked DNA substrate ranging from 5 to 100 nM for various reaction times is shown in this panel. The solid lines represent the best fits to the burst equation. C, depicts the plot of the amplitudes of the burst phases in B as a function of DNA concentration. The solid line represents the best fit to the quadratic equation with a $K_{D,\text{DNA}}$ for the WT KF-DNA complex equal to $7.1 \pm 0.05$ nM and an active site concentration equal to $56 \pm 3$ nM. D shows the results obtained with double mutant KF (S769A/F771A) (60 nM) and concentrations of the DNA substrate identical to those used for the WT KF (B). The amplitudes of the burst phases in D were plotted as a function of DNA concentration. The plot (E) represents the best fit to the quadratic equation with a $K_{D,\text{DNA}}$ for the S769A/F771A-DNA complex equal to $11.9 \pm 0.04$ nM and an active site concentration equal to $57 \pm 2$ nM. The same assays were also carried out for S769A, F771A, and R841A mutant enzymes (See supplemental materials). However, the $K_{D,\text{DNA}}$ values obtained for these enzymes are shown in Table 1.
Requirement of the 5′-Flap in the Blocker for Strand Displacement Synthesis—The pausing of DNA synthesis prior to the separation of the downstream double-stranded DNA and the slow rate constant of nucleotide addition in nicked substrates indicate that initiation of strand separation is a very slow process. To understand this process better, we made gapped DNA substrates with flaps of 0, 1, 2, and 4 nt at the 5′-end in the blocker DNA and measured DNA synthesis in the presence of all dNTPs. Interestingly, as the length of the flap increases, the pausing by the WT polymerase after 19-mer synthesis decreases (Fig. 6). WT polymerase therefore shows efficient strand displacement DNA synthesis on a substrate with a 4-nt flap in the blocker.

Using the gapped DNA substrate with or without a 4-nt flap, we measured the strand displacement DNA synthesis activity of the mutant polymerases. The presence of a flap sequence at the 5′-end of the blocker clearly enhances the strand displacement DNA synthesis activity of S769A and F771A polymerases, but not that of the double mutant (S769A/F771A) and R841A polymerases (Fig. 7).

The time course of strand displacement DNA synthesis activity of WT and F771A mutant enzymes on the flap-structured DNA substrate was measured under presteady state conditions. The time course of strand displacement DNA synthesis between 5 ms and 5 s (Fig. 8A) shows little or no stall after gap-filling synthesis. The average rate of DNA synthesis by the WT and F771A mutant polymerase was very similar (13.20 and 12.42 s⁻¹, respectively) (Fig. 8B). The strand displacement DNA synthesis rates (k₂ in Equation 3) for the DNA substrate containing a 4-nt long flap, for WT and F771A mutant enzymes were 1.23 and 0.89 s⁻¹, respectively (Fig. 8C), providing a defect of ~1.38-fold in the strand displacement activity of F771A mutant enzyme. This difference is significantly lower compared with the one observed for the substrate containing no flap (~17-fold) (Fig. 4). In summary, these results indicate that the initiation of strand displacement synthesis is a very slow process. Both WT and the single mutants are able to catalyze efficient strand displacement synthesis when provided with a pre-made flapped substrate. The double mutant and R841A, however, cannot catalyze strand displacement synthesis on the flapped DNA substrate, which indicates that the combined action of residues Ser⁷⁶⁹ and Phe⁷⁷¹ and that of Arg⁸⁴¹ is required for DNA strand displacement.

DISCUSSION

Properties of Ser⁷⁶⁹ and Phe⁷⁷¹—As outlined under the Introduction, Ser⁷⁶⁹ of KF interacts with the phosphate moiety of the n + 1 template nucleotide (5). This nucleotide governs the selection of incoming substrate in KF. The interaction of Ser⁷⁶⁹ with the phosphate backbone is nonsequence-specific. Therefore, Ser⁷⁶⁹ can be attributed to participate in positioning the template nucleotide at the active site. The mutations S769A and F771A individually or together (double mutant) do not significantly affect the DNA synthesis activity as long as the template is single-stranded. As shown in Table 1, the mutant enzymes retained polymerase activity under steady state conditions as shown by standard trichloroacetic acid precipitation assays. Similar results were obtained in single nucleotide incorporation assays under presteady state conditions. These results indicate that the side chains of Ser⁷⁶⁹ and Phe⁷⁷¹ are
clearly indicated a role for Phe\(^{771}\) in strand displacement (or flap-structure generation). Very similar results were obtained with pol I, which indicates that the 5’-nuclease activity associated with pol I has little or no effect on strand displacement DNA synthesis. The double mutant (S769A/F771A) was defective in displacement activity compared with either of the single mutation carrying enzymes (S769A or F771A), implying the participation of both residues (Ser\(^{769}\) and Phe\(^{771}\)) in the process of strand displacement possibly through the formation of the flap-structured DNA.

The role of Phe\(^{771}\) becomes obvious when strand displacement activity of the F771A mutant enzyme is compared with the WT KF under presteady state conditions. WT KF removed the block generated after the gap-filling reaction, whereas the F771A mutant enzyme failed in this activity. In fact, the rate of block removal by F771A mutant enzyme was nearly 17-fold lower than the WT enzyme. Because no significant difference was noted in the binding affinity for the nicked DNA by the WT and mutant enzyme, the only defect that can be attributed to mutant enzyme (F771A) is its deficiency in performing the strand displacement activity.

**Properties of Arg\(^{841}\)**—As described before, this residue has two contacts with the template strand (6). Thus, the likely role of Arg\(^{841}\) is to stabilize the template strand during the nucleotide incorporation reaction as well as during the strand displacement reaction. However, the participation of Arg\(^{841}\) in the nucleotide incorporation does not appear to be essential as R841A mutant enzyme showed significant polymerase activity (Table 1). Interestingly, the strand displacement properties of R841A mutant enzyme were as severe as those of the double mutant S769A/F771A enzyme including the DNA displacement synthesis with a DNA substrate. These results suggest that Arg\(^{841}\) is an essential component of KF for optimal strand displacement DNA synthesis.

**Comparison with Other Polymerases and Structural Evolution of the 3-Helix Bundle**—A primary amino acid sequence comparison of pol I family enzymes has shown a high degree of conservation of these two residues (Ser\(^{769}\) and Phe\(^{771}\)). However, in the enzymes that are devoid of strand

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**FIGURE 8.** Presteady state pattern of strand displacement DNA synthesis by the WT and F771A mutant KF enzymes with a gapped DNA substrate containing the 4-nucleotide flap. This figure shows the strand displacement DNA synthesis by the WT and F771A mutant enzymes under presteady state conditions with a DNA substrate containing a 4-nucleotide flap at the 5'-end of the non-template strand (A). The reaction conditions were essentially the same as those described in the legend for Fig. 4. The reactions were quenched by the addition of 50 mM EDTA at various reaction times (5 ms to 5 s), and the products were analyzed by 16% polyacrylamide-urea gel electrophoresis. The rate of multiple nucleotide incorporation was estimated from the DNA synthesis pattern shown in A. To determine the rate of multiple nucleotide incorporation, the intensity of each band was multiplied by a number representing the events of incorporation. The sum of these values was divided by the sum of the intensities of individual bands in a given lane. The method is similar to that described by Derbyshire et al. (27) for the determination of the exonuclease rate. The data were plotted against time, and the slope of the straight line provides the average rate of nucleotide incorporation (B). In C, the amount of 18-mer product generated by the WT KF and F771A mutant enzyme (representing the site of strand separation) was plotted against time, and the data were fit to the double exponential as described in the legend to Fig. 4. The rates of disappearance of the 18-mer product by the WT and mutant enzyme were 1.23 and 0.89 s\(^{-1}\), respectively, providing a ratio of 1.38-fold in contrast to 17-fold observed in the absence of flap (see Fig. 4).

not essential for the nucleotide addition activity of KF or pol I (14, 15). The double mutant S769A/F771A showed a significant decrease (~7-fold) in single nucleotide incorporation activity on nicked DNA compared with WT polymerase. Because no significant difference was noted in the affinity for nicked DNA by WT and mutant enzyme, the only reason for the lower polymerase activity is the inability of mutant enzymes to displace the non-template strand, which is a prerequisite for the nucleotide incorporation. These observations led us to extensively characterize the mutant enzymes for their strand displacement DNA synthetic activity.

WT and S769A mutant had a similar strand displacement DNA synthesis activity on a substrate with a DNA or an RNA blocker. Both polymerases pause after filling the gap before continuing to copy the template by displacement of the blocker. On the other hand, F771A and S769A/F771A mutant polymerases nearly stop after filling the gap, which
displacement activity, the equivalent positions are occupied by non-homologous residues. For example, in T7 DNA polymerase, which is deficient in strand displacement activity (23), the positions of Ser\textsuperscript{769}, Phe\textsuperscript{771}, and Arg\textsuperscript{841} are occupied by glycine, glutamic acid, and histidine, respectively. Thus the presence of serine, phenylalanine, and arginine at defined positions in the pol I family of enzymes may be prerequisite for the expression of strand displacement synthesis. Ser\textsuperscript{769} and Phe\textsuperscript{771} are located on the O1-helix of DNA pol I. It has previously been reported that the structure formed by O, O1, and O2 helices of pol I shares structural homology with a novel DNA binding motif (24) found in transcription factor Mrf-2 and in some DNA repair proteins such as *E. coli* endonuclease III (25) and T4 RNase H (24, 26). This structural homology suggested the involvement of this motif in the recognition of double-stranded nucleic acid. However, for the DNA polymerase I family of enzymes, it was suggested that this region may be important in the binding of the ssDNA template overhang (24).

In this context, the occurrence of the 3-helical structural motif in pol I and T7 RNA polymerase suggests that this motif is clearly utilized in the binding and separation of two strands of DNA. In both enzymes, the strand with 3′–5′ polarity serves as template for the synthesis of appropriate nucleic acid. The superposition of the 3-helix bundle from pol I onto the structurally homologous region of T7 RNA polymerase is shown in Fig. 9. In addition to the excellent superposition of all 3 helices, the nucleotide substrate binding residues of T7 RNA polymerase and pol I (Arg\textsuperscript{754} and Lys\textsuperscript{758} equivalent residues) also superpose extremely well. Additionally, Phe\textsuperscript{771} of pol I appears to be structurally conserved with Phe\textsuperscript{644} of T7 RNA polymerase (16), which is located at the DNA fork in the transcription complex (Fig. 9), implying that this residue participates in separation of the two DNA strands. Collectively, these observations suggest that the 3-helix bundle is a functional unit that recognizes or induces altered DNA structure. The placement of an aromatic residue at the apex of the 3-helix bundle as seen in pol I (or T7 RNA polymerase) may facilitate the strand separation.

In summary, the biochemical experiments described above clearly demonstrate that Ser\textsuperscript{769}, Phe\textsuperscript{771}, and Arg\textsuperscript{841} are required for strand displacement synthesis. The first two amino acids provide a means to separate the double-stranded DNA structure, which is essential for the rapid strand displacement DNA synthesis. The structural analyses further indicate that Ser\textsuperscript{769} and Phe\textsuperscript{771} are the components of a novel 3-helix bundle structure that functions in strand separation reactions.

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