RNA Template-dependent 5’ Nuclease Activity of *Thermus aquaticus* and *Thermus thermophilus* DNA Polymerases*

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DNA replication and repair require a specific mechanism to join the 3’- and 5’-ends of two strands to maintain DNA continuity. In order to understand the details of this process, we studied the activity of the 5’ nucleases with substrates containing an RNA template strand. By comparing the eubacterial and archaeal 5’ nucleases, we show that the polymerase domain of the eubacterial enzymes is critical for the activity of the 5’ nuclease domain on RNA containing substrates. Analysis of the activity of chimeric enzymes between the DNA polymerases from *Thermus aquaticus* (TaqPol) and *Thermus thermophilus* (TthPol) reveals two regions, in the “thumb” and in the “palm” subdomains, critical for RNA-dependent 5’ nuclease activity. There are two critical amino acids in those regions that are responsible for the high activity of TthPol on RNA containing substrates. Mutating glycine 418 and glutamic acid 507 of TaqPol to lysine and glutamine, respectively, increases its RNA-dependent 5’ nuclease activity 4-10-fold. Furthermore, the RNA-dependent DNA polymerase activity is controlled by a completely different region of TpPol and TthPol, and mutations in this region do not affect the 5’ nuclease activity. The results presented here suggest a novel substrate binding mode of the eubacterial DNA polymerase enzymes, called a 5’ nuclease mode, that is distinct from the polymerizing and editing modes described previously. The application of the enzymes with improved RNA-dependent 5’ nuclease activity for RNA detection using the invasive signal amplification assay is discussed.

The structure-specific 5’ nucleases are involved in DNA replication and nucleotide excision repair, where the primary function of these enzymes is to remove the RNA primers of Okazaki fragments or damaged DNA fragments, respectively (1–6). The activity of the 5’ nucleases is controlled by DNA polymerases that create for them an optimal substrate by displacing the 5’-end of the downstream strand during DNA synthesis (7–9). The precise removal of the displaced arm by the 5’ nuclease creates a nicked structure repaired by DNA ligase (9). Previously, we studied the substrate specificity of seven eubacterial and archaeal structure-specific 5’ exonucleases with DNA substrates (9). The enzymes showed very similar specificities, despite their limited level of sequence similarity and different structural organization; eubacterial 5’ nucleases are discrete domains of the DNA polymerases, whereas in archaea the 5’ nucleases are separate polypeptides. Comparison of the 5’ nuclease activities of Taq DNA polymerase (TaqPol) and its isolated nuclease domain (TaqExo) showed that the 5’ nuclease domain can function independently, although the polymerase domain influences the 5’ nuclease activity by imposing additional stringency on substrate recognition and by increasing substrate bind (9, 10).

A specific substrate for the 5’ nucleases that resembles DNA undergoing displacement synthesis can be created with synthetic oligonucleotides. This substrate, termed the “overlapping substrate,” is formed by annealing adjacent upstream and downstream oligonucleotides on a template strand, also called the target strand. The duplexes formed by the two oligonucleotides must overlap by at least one base pair for efficient cleavage by the 5’ nucleases (9–11). The upstream oligonucleotide in this substrate is completely annealed to the target except for the 3’ terminal nucleotide that may interact with the 5’ nuclease. The downstream oligonucleotide consists of the target specific region and the 5’ arm region that is not annealed to the target and cleaved by the 5’ nuclease to create the nicked substrate (9, 11). The ability of the 5’ nucleases to specifically recognize and cleave the overlapping substrate has been utilized in assays for quantitative DNA detection and single nucleotide polymorphism analysis (11–14). In this method, called the invasive signal amplification reaction, cleavage of the downstream oligonucleotide is dependent upon the presence of a particular target sequence in a sample. Each target molecule can generate more than 10^3 reporter molecules by rapid turnover of the downstream oligonucleotide at elevated temperatures (11, 12). By adding a second invasive reaction, total signal amplification can reach a factor of 10^7 (12). The invasive signal amplification reaction can also be used for quantitative RNA detection, although this would require an investigation of the ability of the structure-specific 5’ nucleases to cleave the overlapping substrate with an RNA target.

In order to gain insight into the mechanism of substrate recognition by the structure-specific 5’ nucleases and to develop an enzyme for RNA detection using the invasive signal amplification assay, we compared the activity of eubacterial and archaeal 5’ nucleases using an overlapping substrate containing an RNA target. We find that only the eubacterial DNA

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1 The abbreviations used are: TaqPol, *T. aquaticus* DNA polymerase; TaqExo, 5’ nuclease domain of TaqPol; TthPol, *T. thermophilus* DNA polymerase; HPLC, high pressure liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; IL, interleukin.

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polymerases TaqPol and TthPol possess RNA template-dependent 5' nuclease activity, although it is significantly reduced compared with the 5' nuclease activity with a DNA target. Of the two enzymes, TaqPol has a higher RNA-dependent 5' nuclease activity than TaqPol. We have used this observation to investigate the activity of chimeric enzymes constructed from TthPol and TaqPol, and we have identified two regions in the polymerase domain involved in substrate recognition of 5' nucleic acid substrates. Site-directed mutagenesis studies reveal that lysine 420 and glutamine 509 of TthPol are the amino acids in these regions that are critical for the 5' nuclease activity on RNA-containing substrates. Mutating the analogous amino acids of TaqPol (G418K and E507Q) to match TthPol at those positions increases the RNA-dependent 5' nuclease activity of TaqPol 4-10-fold but has no effect on its DNA- or RNA-dependent polymerase activities. Based on these results, we propose a novel 5' nucleasename-specific mode of substrate binding by eubacterial DNA polymerases.

EXPERIMENTAL PROCEDURES

Materials—Polymerase chain reaction amplification was done with the Advantage cDNA polymerase chain reaction kit (CLONTECH). Restriction enzymes were purchased from New England Biolabs. Chemicals and buffers were from Fisher unless otherwise noted.

Cloning, Expression, and Purification of Enzymes—TThPol, TaqPol, TaqExo, and archaeal FEN1 enzymes from Archaeoglobus fulgidus, Methanococcus jannaschii, Methanobacterium thermoautotrophicum, and Pyrococcus furiosus were cloned, expressed, purified, and quantitated as described (9–11). All enzymes were dialyzed and stored in 50% glycerol, 20 mM Tris·HCl, pH 8, 50 mM KCl, 0.5% Tween 20, 0.5% Nonidet P-40, 100 μg/ml bovine serum albumin.

Site-directed Mutagenesis—Site-directed mutagenesis of the TaqPol and TthPol genes was performed with the Transformer Site-Directed Mutagenesis kit (CLONTECH) according to the manufacturer's protocol. Using site-directed mutagenesis, three new unique restriction sites, NdeI, BstBI, and NdeII, were created in the TaqPol gene at positions approximately corresponding to amino acids 328, 382, and 443, respectively. The same restriction sites were also introduced at the homologous positions 330, 384, and 443 of the TthPol gene. The NdeI sites were created by using mutagenic primers 5'–GGGCGAGGCCTGGCCGCGCCA–3' and 5'–CCCTGCACCGCCGCACCGGCCCGTGACCTGCCG–3', which correspond to the sense strands of the TaqPol and TthPol genes, respectively. The BstI and NdeII sites were introduced into both genes using sense strand mutagenic primers 5'–CTCTCTGAGCCGTTCGACACCC–3' and 5'–GTCCTGAGCCGATATGGAGGCCAG–3'. The mutated nucleotides are shown in boldface, and the corresponding restriction sites are underlined.

Construction and Purification of Chimeric Enzymes—Chimeric constructs were made by substituting homologous fragments between the cloned TaqPol and TthPol genes using the common unique restriction sites EcoRI, NdeI, BstBI, NdeII, BamHI, and SaeII and standard cloning techniques. Expression and purification of the chimeric enzymes was done as described (9–11) except that the His Bind resin chromatography purification step was replaced by affinity chromatography using an Econo-Pac heparin cartridge (Bio-Rad) and a Dionex DX 500 HPLC instrument. Briefly, the cartridge was equilibrated with 50 mM Tris·HCl, pH 8, 1 mM EDTA, and enzyme extract dialyzed against the same buffer was loaded on the column and then eluted with a linear gradient of NaCl (0–2 M) in the same buffer. The HPLC-purified protein was dialyzed and stored as described above. Expression of the TthPol–TaqPol chimeric genes yielded proteins of the expected size. The enzymes were purified to homogeneity according to SDS-polyacrylamide gel electrophoresis (9) and were shown to cleave the invasive substrates with both DNA and RNA targets.

Substrate Preparation and Purification—The downstream and upstream oligonucleotides and the IL-6 DNA target were synthesized on a PerSeptive Biosystems instrument using standard phosphoramidite chemistry (Glen Research). The synthetic RNA–DNA chimeric 171 nt target was synthesized using 2-AC RNA chemistry (Dharmacon Research). The 2′-protecting groups were removed by acid-catalyzed hydrolysis according to the manufacturer's procedure. The downstream probes labeled with 5'-fluorescein or 5'-tetrachlorofluorescein at their 5' ends were purified by reverse phase HPLC using a Resource Q column (Amersharm Pharmacia Biotech). A fragment of human IL-6 cDNA (nucleotides 64–691 of the sequence published in Ref. 15) was cloned using a TOPO-TA Cloning Kit (Invitrogen), and the 640-nucleotide IL-6 RNA target was synthesized by T7 RNA polymerase run-off transcription of the cloned fragment using a Megascript Kit (Ambion). All oligonucleotides were finally purified by separation on a 20% denaturing polyacrylamide gel followed by excision and elution of the major bands. The concentration of the major bands was determined by measuring absorption at 260 nm. The biotin-labeled IrT target was incubated with a 5-fold excess of streptavidin (Promega) in a buffer containing 10 mM MOPS, pH 7.5, 0.05% Tween 20, 0.05% Nonidet P-40, and 10 μg/ml RNA at room temperature for 10 min.

RESULTS

DNA and RNA Template-dependent 5' Nuclease Activities of Structure-specific 5' Nucleases—The 5' nuclease activity assay was designed similarly to the invasive signal amplification reaction described previously (11). The IL-6 substrate was used in these experiments to confirm the presence of the downstream and upstream oligonucleotides annealed with either RNA or DNA target (the terms "target" and "template" will be used interchangeably) as shown in Fig. 1A. The fluorescencelabeled downstream oligonucleotide, also called the probe, is specifically cleaved by the 5' nucleic acid at the site where the upstream oligonucleotide overlaps or "invases" the probe (9, 10). The invasive signal amplification reaction is carried out under conditions of limiting target and excess probe. At the optimal reaction temperature, which occurs at the melting temperature of the probe, a single
target molecule gives rise to multiple cleaved probes due to a rapid probe exchange. The cleavage rate of this reaction is determined as the number of the probe molecules cleaved per target molecule per minute.

In agreement with the previous results, all enzymes showed high cleavage activity with the IL-6 DNA target (Fig. 1B). When the DNA target was replaced with the IL-6 RNA target, only TthPol and TaqPol were able to cleave the probe, although at a reduced rate compared with the DNA target. The cleavage site of the probe is indicated by an arrow. Sequence of the IL-6 DNA target is shown below. B, cleavage of IL-6 substrate containing 0.05 mM IL-6 DNA target and a 0.5 μM concentration of each downstream and upstream oligonucleotide with 0.28 μM TaqExo (lane 1), 28 μM TthPol (lane 2), 28 μM TmPol (lane 3), 0.28 μM archaeal FEN1 enzyme from P. furiosus (Pfu) (lane 4), 0.28 μM archaeal FEN1 enzyme from A. fulgidus (Afu) (lane 5), 0.28 μM archaeal FEN1 enzyme from M. jannaschii (Mja) (lane 6), and 0.28 μM FEN1 enzyme from M. thermoautotrophicum (Mth) (lane 7). Lane 8, no enzyme (NE) control. Reactions were carried out at 60 °C for 30 min as described under “Experimental Procedures” except that the reaction buffer (10 mM MOPS, pH 7.5, 0.05% Tween 20, 100 mM KCl) contained no MgSO₄. C, cleavage of the IL-6 substrate with the RNA target was done as described in B, except that the IL-6 RNA target concentration was 1 nM and the reactions were performed at 57 °C for 60 min.

The amino acid sequences of TaqPol and TthPol share about 87% identity and greater than 92% similarity. We took advantage of this high degree of sequence similarity between the enzymes to construct a series of chimeric enzymes between TthPol and TaqPol. The chimeric constructs shown in Fig. 2 were created by swapping DNA fragments defined by the restriction endonuclease sites, EcoRI and BamHI, common for both genes, the SalI site in the cloning vector, and the new sites, NdeI, BstBI, and NdeI, created at the homologous positions of both genes by site-directed mutagenesis (see “Experimental Procedures” and Fig. 2). Since TthPol has a 4-fold higher cleavage rate with the IL-6 RNA template than TaqPol (Fig. 1C), the activities of the chimeric enzymes were rated relative to TthPol and used as a parameter to identify the region(s) affecting RNA template-dependent 5’ nuclease activity.

**RNA-dependent 5’ Nuclease Activity of the Chimeric Enzymes**—The activity of each chimeric enzyme was evaluated using the invasive signal amplification assay with the IL-6 RNA target (Fig. 1A); the cleavage rates shown in Fig. 3 were determined as described under “Experimental Procedures.” Comparison of the cleavage rates of the first two chimeras, TaqTth(N) and TthTaq(N), created by swapping the polymerase and 5’ nuclease domains at the NdeI site (Fig. 2), shows that TaqTth(N) has the same activity as TthPol, whereas its counterpart TthTaq(N) retains the activity of TaqPol (Fig. 3). This result indicates that the higher cleavage rate of TthPol is associated with its polymerase domain, which is consistent
with an important role for the polymerase domain in the 5′ nuclease activity (9, 10).

The next step was to identify the minimal region of TthPol polymerase domain that would give rise to the TthPol-like 5′ nuclease activity when substituted for the corresponding region of the TaqPol sequence. To this end, we selected the TaqThr(N) chimera to generate a series of new constructs by replacing its TthPol sequence with homologous regions of TaqPol. First, we substituted the N-terminal and C-terminal segments of the TaqPol polymerase domain for the corresponding regions of TaqThr(N) using the common BamHI site as a breaking point to create TaqThr(N-B) and TaqThr(B-N) chimeras, respectively (Fig. 2). TaqThr(N-B), which has the TthPol sequence between amino acids 328 and 593, is approximately 3 times more active than the TaqThr(B-N) and 40% more active than TthPol (Fig. 3). This result demonstrates that the NotI–BamHI region of the TthPol polymerase domain is responsible for the high 5′ nuclease activity of TthPol with RNA targets.

The NotI–BamHI region of TthPol was further subdivided into two approximately equal parts using NdeI (Fig. 2), and the effect of the substitution of each of these sequences on the cleavage rate of TaqPol was investigated by measuring the activities of the TaqThr(N-Nd) and TaqThr(Nd-B) chimeras. Each of these chimeric enzymes showed TthPol-like activity (Fig. 3), suggesting that both NotI–NdeI and NdeI–BamHI regions of TthPol contain amino acids indispensable for the high cleavage rate of TthPol. The substitution of the BstBI–BamHI region of TthPol for the homologous sequence of TaqPol produced the TaqThr(bs-B) chimera, which exhibited the same activity as TthPol (Fig. 3). Thus, this study of the chimeric enzymes limited the portion of the TthPol sequence determining its high RNA template-dependent 5′ nuclease activity to the BstBI–BamHI region located approximately between amino acids 382 and 593 (Fig. 2).

Site-directed Mutagenesis of the Chimeric Enzymes—Comparison of the TthPol and TaqPol amino acid sequences between the BstBI and BamHI sites reveals only 25 differences (Fig. 4A). Among these, there are 12 conservative substitutions and 13 substitutions resulting in an alteration of charge. Since the analysis of the chimeric enzymes suggested that the critical mutations are located in both BstBI–NdeI and NdeI–BamHI regions of TthPol, we used chimeric enzymes that have TthPol sequence in one of these regions and introduced TthPol-specific amino acids by site-directed mutagenesis in the other region that has TaqPol sequence. For example, six TthPol-specific substitutions changing amino acid charge were created in the BstBI–NdeI region of the TaqThr(Nd-B) chimera by single or double amino acid mutagenesis (Fig. 4A). Only one of these substitutions, double mutation W417L/G418K, was able to restore the TthPol activity with the IL-6 RNA target, whereas the other four mutations were neutral (Fig. 4B and data not shown). Similarly, all 12 TthPol amino acid substitutions were introduced at the homologous positions of the NdeI–BamHI region of the TaqThr(Nd) chimera, and only one, E507Q, increased the cleavage rate to the TthPol level, whereas the other 10 mutations were neutral, and one, G499R, showed a smaller increase (Fig. 4B and data not shown).

To confirm that the W417L, G418K, and E507Q substitutions are sufficient to increase the TaqPol activity to the TthPol level, TaqPol variants carrying these mutants were created, and their cleavage rates with the IL-6 RNA target were compared with that of TthPol. Fig. 4C shows that the TaqPol W417L/G418K/E507Q and TaqPol G418K/E507Q mutants have a 1.4 times higher activity than TthPol and more than a 4-fold higher activity than TaqPol, whereas the TaqPol W417L/E507Q mutant has the same activity as TthPol. Thus, these results provide strong evidence that the RNA-dependent 5′ nuclease activity of TaqPol with the RNA IL-6 target can be significantly increased by just two critical amino acid substitutions, G418K and E507Q.

Characterization of the TaqPol G418K/E507Q Mutant—Next, we compared the TaqPol G418K/E507Q, TaqPol, and TthPol enzymes in the RNA template-dependent 5′ nuclease assay while varying temperature and the concentrations of salt and divalent ions. The upstream DNA oligonucleotide and the RNA target of the substrate used in this study were linked into a single molecule, called the IrT target, as shown in Fig. 5A, and the labeled probe was present in large excess. The 5′-end of the IrT target was blocked with a biotin-streptavidin complex to prevent nonspecific degradation by the enzyme during the reaction (18, 19). The cleavage rates for TaqPol G418K/E507Q, TaqPol, and TthPol are plotted versus temperature in Fig. 5B. The activity difference between TthPol and TaqPol with the IrT target is even greater than that found with the IL-6 RNA target. The G418K/E507Q mutations increase the activity of TaqPol more than 10-fold and by 25% as compared with TthPol. All three enzymes show a typical temperature profile of the invasive signal amplification reaction and have the same optimal temperature. We found no significant effect of the G418K/E507Q mutations on DNA template-dependent 5′ nuclease activity of TaqPol with an all-DNA target analogous to IrT under the same conditions (data not shown).

The effects of KCl and MgSO4 concentrations on the 5′ nuclease activity of TaqPol G418K/E507Q, TaqPol, and TthPol with the IrT target are shown in Fig. 5, C and D. The activities of all enzymes have similar salt dependences with an optimal KCl concentration of 100 mM for TaqPol G418K/E507Q and TthPol and 50 mM for TaqPol. The optimal MgSO4 concentration for all enzymes is approximately 8 mM. The analysis of the data presented in Fig. 5 suggests that the properties of TaqPol G418K/E507Q are much closer to those of TthPol than TaqPol, confirming the key role of the G418K/E507Q mutations in the recognition of the substrate with an RNA target.

To understand the mechanism of the reduction of the 5′ nuclease activity in the presence of an RNA versus a DNA target, we determined the Michaelis constant, Km, and the maximal catalytic rate, kcat, of TaqPol, TthPol, and TaqPol G418K/E507Q using an excess of the IrT target and the probe and a limiting enzyme concentration as described under “Experimental Procedures.” It was found that all three enzymes have similar Km values in the range of 200–300 nM and kcat...
values of approximately 4 min$^{-1}$ for TaqPol and TthPol and of 9 min$^{-1}$ for TaqPol G418K/E507Q (data not shown).

**Polymerase Activities of TthPol, TaqPol, and TaqTth Chimeric Enzymes**—To determine whether the RNA template-dependent 5' nuclease activity of the *Thermus* DNA polymerase enzymes is related to their RNA-dependent polymerase activity, we have reversed the D785N and D787N mutations used to create the polymerase-deficient versions of TaqPol and TthPol by site-directed mutagenesis. The polymerase activities were then evaluated by extension of a dT$_{25}$–35-oligonucleotide primer with fluorescein-labeled dUTP in the presence of either poly(dA) or poly(A) template as described under “Experimental Procedures.” As shown in Fig. 6, the DNA-dependent polymerase activities are very similar for all constructs used in this experiment, whereas the RNA-dependent polymerase activities of TthPol, TaqTth(N-B), and TaqTth(Nd-S), and TthPol. The cleavage rates were measured for the IL-6 substrate (Fig. 1A) containing 1 nM IL-6 RNA target and 0.5 μM downstream and upstream oligonucleotides as described under “Experimental Procedures.” Reactions were carried out with a 28 nM concentration of each enzyme at 57 °C for 60 min. C, the G418K/E507Q mutations increase the cleavage rate of TaqPol to the level of TthPol. The cleavage rates with the IL-6 RNA target were measured as described for B.

**DISCUSSION**

Whereas all structure-specific 5' nucleases tested in this work efficiently cleave the overlapping substrate with a DNA target, only TthPol and TaqPol enzymes retain partial activity with the substrate in which the DNA target is replaced with an RNA target (Fig. 1). These were the only enzymes that had both 5' nuclease and DNA polymerase domains, thereby implying an important role for the polymerase domain in substrate recognition. This conclusion agrees with our previous finding that the polymerase domain of TaqPol affects the activity and specificity of its 5' nuclease domain and that both TthPol and TaqPol bind the overlapping DNA substrate more strongly than the archaeal 5' nucleases and the isolated 5' nuclease domain of TaqPol (9, 10). Using the chimeric constructs between TthPol and TaqPol and site-directed mutagenesis, we identified two groups of amino acids that are important for the RNA template-dependent 5' nuclease activity and that are possibly involved in recognition of the target strand of the overlapping substrate. In the TthPol sequence, the first group includes leucine 419 and lysine 420, and the second one cont-
with DNA. Deletion of a 24-amino acid portion of the tip of the Klenow fragment of DNA polymerase I (21) and TaqPol (22) was previously suggested by the binary complex structures of the thumb and the minor groove of the DNA primer-template and template strands, respectively. The interaction between 3.8 Å and 18 Å from the backbone phosphates of the primer located at the tip of the thumb subdomain at a nearest distance of 25 Å from the template DNA (20) are shown in Fig. 7. The E507Q mutation is domain of TaqPol (Klentaq1), dideoxynucleotide, and a primer-template DNA duplexes (20, 21, 22). The primer-template regions of the polymerase domain are indicated. The *asterisks* denote TaqPol and TthPol sequences, respectively. The 5' nucleic acid and primer-template DNA duplexes (20, 21, 22). The DNA- and RNA-dependent DNA polymerase activities were determined as a percentage of incorporated dUTP labeled with fluorescein under conditions described under “Experimental Procedures.”

The positions of the W417L, G418K, and E507Q mutations in TaqPol (W417L, G418K, and E507Q) mutations present in the palm region of TaqPol (Fig. 7) are located approximately 25 Å from the nearest phosphates of the primer-template duplex according to the binary/ternary complex structures of TaqPol with DNA bound in polymerizing mode (20, 22). The same distance was observed between the analogous W513 and P514 amino acids of Klenow fragment and the template strand of DNA bound in the editing mode (21). Thus, no interactions between amino acids 417–418 of TaqPol and the overlapping substrate can be suggested from the available binary complex studies.

To explain the data described here, we propose that the amino acids at positions 417 and 418 in the palm region of TaqPol interact with the upstream duplex of the overlapping substrate only when the enzyme functions as a 5' nuclelease but no interaction of the substrate with these amino acids occurs when TaqPol switches into polymerizing mode. This hypothesis suggests a novel mode of substrate binding by DNA polymerases, which we call the 5' nuclease mode. Several lines of evidence support this hypothesis. The chimeric enzyme study clearly separates regions of the polymerase domain involved in the 5' nuclease and polymerase activities. Accordingly, the W417L and G418K mutations, together with the E507Q mutation, affect the 5' nuclease activity of TaqPol on substrates having an RNA target strand (Fig. 4C) but have no effect on either RNA- or DNA-dependent DNA polymerase activities (Fig. 6). Conversely, mutations in the active site of TaqPol, such as R573A, R587A, E615A, R746A, N750A, and D785N, which correspond to substitutions in Klenow fragment that affect both polymerase activity and substrate binding in the 5' nuclease mode (24–26), have little or no effect on the 5' nuclease activity (data not shown). Superposition of the polymerase domains of TaqPol (22), Escherichia coli DNA polymerase I (21), and Bacillus stearothermophilus DNA polymerase I (27) using DALI (28, 29) and Insight II (Molecular Simulation Inc.) programs shows that a portion of the palm subdomain of TaqPol between amino acids 402 and 451, including Trp417 and Gly418, is highly conserved between the three polymerases, although there is no structural similarity between the rest of the palm subdomains. This observation sug-
suggests an important role for this region in eubacterial DNA polymerases.

What could be the reason for a 5'9'nuclease binding mode? As we discussed above, the 5' nuclease and polymerase activities should be precisely synchronized in order to create a nicked structure rather than a gap or an overhang that could result in a deletion or an insertion during Okazaki fragment processing or DNA repair. According to the previously proposed model (9), the 3' terminal nucleotide of the upstream strand in the overlapping substrate is sequestered by the 5' nuclease domain to prevent its extension, thus halting synthesis. This interaction with the 3' nucleotide activates the 5' nuclease, which then endonucleolytically removes the displaced 5' arm of the downstream strand by precise incision at the site defined by the 3' nucleotide to create the nick. This model requires a substantial rearrangement of the substrate-enzyme complex that may include a translocation of the complex to the 5' nuclease mode to separate the primer-template duplex from the polymerase active site.

The hypothesized translocation into the 5' nuclease mode could be accomplished through an interaction of the downstream duplex formed between the template and downstream strands with the crevice formed by the finger and thumb subdomains. Such an interaction could force conformational transitions in the thumb that would bring the upstream duplex into close contact with the Trp417 and Gly418 amino acids. Significant flexibility of the thumb has been previously reported that might explain such changes (17, 20–22, 30, 31). Additional conformational changes in the fingers domain that might help to open the crevice, such as the transition from the "closed" to the "open" structure described by Li et al. (20), are consistent with this model. To answer the question as to why the 5' nuclease binding mode was not observed in any of the published co-crystal structures of a DNA polymerase I, we would argue that the majority of the structures were solved for the polymerase domain only and with a primer-template substrate rather than with an overlapping substrate.

The $K_m$ values of 200–300 nM determined in this work for TaqPol, TthPol, and TaqPol G418K/E507Q for the RNA-containing substrate are much higher than the $K_m$ value of <1 nM estimated previously for TthPol with an all-DNA overlapping substrate,2 suggesting that the RNA target adversely affects substrate binding. The reduced affinity for RNA-containing substrates can be explained by the unfavorable interaction between the enzyme and the A-form duplex adopted by the substrate with an RNA target or by inhibition of binding by the ribose 2' hydroxyls of the RNA target. Among these two factors, the latter looks more attractive, since the 5' nuclease of eubacterial DNA polymerases can efficiently cleave substrates with an RNA probe (18), which would presumably have an A-form, and since the binary complex structural studies suggest that the primer-template duplex partially adopts a conformation close to A-form in its complex with DNA polymerase (20, 22, 27). The G418K/E507Q mutations increase the $k_{cat}$ of TaqPol more than 2-fold but have little effect on $K_m$. Such an effect would be expected if the mutations positioned the sub-

![Fig. 7. Location of the W417L/G418K/E507Q mutations in the crystal structure of a ternary complex of Klentaq1 with dideoxynucleotide and primer-template DNA in the polymerizing mode determined by Li et al. (20). Amino acids Trp417, Gly418, and Glu507 are displayed in a space-filling view, and the rest of the polypeptide is shown as a white ribbon. Heavy atoms of the DNA template and primer strands are shown in green. A portion of the tip of the thumb, corresponding to amino acids 494–518, and a part of the structurally conserved region of the palm subdomain (see "Discussion") corresponding to amino acids 402–435 are shown as red ribbons.](image-url)
strate in an orientation more appropriate for cleavage rather than simply increasing the binding constant.

In conclusion, we have identified specific mutations in two regions of the polymerase domain of TaqPol important for RNA template-dependent 5′ nuclease activity. We propose a novel 5′ nuclease mode of substrate binding that is distinct from the previously described editing and polymerizing modes. According to the model, the transition of the enzyme-substrate complex from the polymerizing to the 5′ nuclease mode requires the presence of the 5′ nuclease domain and a specific overlapping substrate. The transition is probably accompanied by a dislocation of the thumb subdomain that brings the substrate in direct contact with the structurally conserved portion of the palm subdomain. This work opens up the possibility of development of 5′ nucleases that can specifically cleave signal probes in the presence of an RNA target and, therefore, can be used for RNA analysis with the invasive signal amplification reaction.

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