DNA misalignment occurs in homopolymer tracts during replication and can lead to frameshift mutations. Polymerase (pol) recognition of primer-templates containing bulge structures and the transmission of a bulge through a polymerase binding site or replication complex are important components of frameshift mutagenesis. In this report, we describe the interaction of the catalytic core of pol η with primer-templates containing bulge structures by single round primer extension. We found that pol η could stabilize a frayed primer terminus, which enhances its ability to extend primer-templates containing bulges. Based on methylphosphonate-DNA mapping, pol η interacts with the single strand template but not appreciably with the template strand of the DNA stem greater than two nucleotides from the primer terminus. These latter characteristics, combined with the ability to stabilize a frayed primer terminus, may explain why primer-templates containing template bulges are extended so efficiently by pol η. Although pol η could accommodate large bulges and continue synthesis without obstruction, bulge structures in the template, but not in the primer, caused termination of the T7 DNA replication complex. Terminations occurred when the template bulge neared the helix-loop-helix domain of the polymerase thumb. Terminations were not observed, however, when bulge structures approached the site of interaction of the DNA with the extended thumb and thioredoxin. At low temperature, however, terminations did occur at this site.

DNA misalignment can occur during replication and lead to frameshift mutations. Misalignment can occur within homopolymer tracts (1) or follow incorrect insertion to produce a matched primer-template. This second mechanism could account for larger deletions as well as single nucleotide frameshift deletions in mixed DNA sequences (2–4). Polymerase recognition of primer templates containing bulge structures and the transmission of a bulge through a polymerase binding site or replication complex are two important components of frameshift mutagenesis. To better understand the structure-activity relationships in the frameshift mechanism, we investigated both a DNA damage bypass polymerase and a replicative polymerase. For a DNA damage bypass polymerase, we chose the catalytic core of polymerase (pol) η from Saccharomyces cerevisiae, an enzyme that can synthesize past cis,syn-thymine dimers. For a replicative polymerase, we chose the T7 DNA polymerase-thioredoxin replication complex, which is part of the T7 bacteriophage replisome (5) and is blocked by cis/syn-thymine dimers (6). These enzymes not only differ in their catalytic properties but also in their overall design for primer-template stem recognition.

T7 DNA polymerase is an “A” family polymerase, which includes Escherichia coli DNA pol I, Bacillus DNA pol I, and the Tag pol of Thermus aquaticus (7, 8). The crystal structure of the T7 DNA polymerase complexed to processivity factor thioredoxin, a 22-mer primer/26-mer template and a ddNTP, has been solved (9). The polymerase contains an N-terminal 3’-exonuclease domain and C-terminal polymerase domain. The polymerase domain resembles a right-hand structure with palm, fingers, and thumb sub-domains. The palm contains active site residues, whereas the fingers enclose the dNTP and the templating nucleotide to provide a tight fit characteristic of high fidelity polymerase activities (9–12). This “closed” conformation is induced by dNTP binding and excludes the penultimate nucleotide and the rest of the single strand template from the active site. It is the constrained nature of this closed conformation that effectively excludes a thymine dimer from the active site (13, 14) and results in polymerase arrest and frameshift mutations in T-tracts (15).

The duplex DNA, which is distant from the active site of T7 DNA polymerase, interacts with the polymerase thumb sub-domain, which is composed of helices H and I connected by a peptide strand of 119 residues. A helix-loop-helix motif (HLH, amino acids 339–372) located within this peptide traps the primer-template stem to promote limited processive synthesis and may be drawn closer to the DNA by the interactions of conserved amino acids Asn-335 and Ser-338 (16, 17). E. coli thioredoxin also binds to this same peptide strand containing the HLH and transforms T7 DNA polymerase into a highly processive enzyme (18, 19). A recent crystal structure reveals that thioredoxin interaction helps orient positively charged amino acid residues within that peptide strand toward both the primer and template strands (20), which may be critical for function. This was verified experimentally by DNA methylphosphonate modifications designed to neutralize polymerase activity relationships in the frameshift mechanism, we investigated.

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1. The abbreviations used are: pol, polymerase; HLH, helix-loop-helix; dithiothreitol, dithiothreitol; mm, mismatch; mp, methylphosphonate; P/T, primer/template.
ase-DNA charge interactions (17). Thioredoxin interaction makes T7 DNA polymerase synthesis more prone to frameshift deletions and base substitutions, presumably the result of improved processivity (21).

The T7 DNA polymerase 3′-exonuclease activity is required for high fidelity DNA replication. Polymerase and exonuclease sites are separated by 35 Å (9), similar to the Klenow fragment (22). The exonuclease activity of T7 polymerase is fast (kexoc > 700 s⁻¹) and about equal to the transfer rate back to the polymerase site to ensure that only a few nucleotides are released per encounter (12, 23). We used an exonuclease-deficient T7 DNA polymerase in this study. T7 DNA polymerase lacking exonuclease activity is more prone to frameshift mutations (15, 24).

Y family polymerases (formerly, the UmuC/DinB/Rev1/Rad30 superfamily) are specific for translesion replication. These polymerase activities include prokaryotic pol IV and V, eukaryotic pol η, ι, and κ, and Rev 1 (25, 26). S. cerevisiae pol η catalytic core (residues 1–501) from the Rad30 family (27), DinB family human polymerase κ (28), polymerase Dhb from Sulfolobus solfataricus P1 (29, 30), and Dpo4 from S. solfataricus P2 (31) all have been crystallized.

Pol η non-mutagenically synthesizes past cis/syn-thymidine dimers (32), and mutations in the human variant result in a condition known as xeroderma pigmentosum (33). Human pol η is also known to generate frameshift mutations in vitro (34–36). Pol η lacks an exonuclease domain, but the overall structure resembles a right hand similar to T7 DNA polymerase with palm, fingers, and thumb but with an additional structure termed the “polymerase-associated domain,” which is a four-β-sheet-structure with two α-helices (27), analogous to structures referred to as the “little finger” (31) or “wrist” (29, 30). A peptide strand connects the thumb to the polymerase-associated domain. Pol η has not been crystallized with a primer template and ddNTP, but there is a ternary complex available for Dpo4 (31). The thumb and little finger sandwich the DNA and span 8 bp of primer-template. The thumb binds to the minor groove and contacts both primer and template while the little finger fits into the major groove on the opposite side of the DNA. The tether linking the domains wraps around the DNA and may allow the little finger to occupy variable positions relative to the palm. The yeast pol η catalytic core, which we use in this study, is missing 121 C-terminal residues that extend from the polymerase-associated domain (27). In vivo, the full-length protein interacts with the host replication complex, which targets pol η to the replication fork through an interaction between proliferating cell nuclear antigen (37) and residues 621–628 near the 632 C terminus of pol η (38).

Here, we describe the interaction of the catalytic core of yeast pol η and T7 DNA polymerase with DNA bulge structures by single round primer extension. Because bulge migration can occur in homopolymer tracts, we have limited this study to intra- or extra-helical bulge structures locked in position by sequence context that have been structurally characterized by NMR analysis (39–41). We also utilized methylphosphonate-modified DNA to neutralize polymerase-DNA charge interactions in the template stem and single-strand overhang. The T7 DNA polymerase-thioredoxin complex was used as a model system to test primer and template bulge transmission through a replication complex.

**EXPERIMENTAL PROCEDURES**

**Proteins and Enzymes**—Thioredoxin crystals (U.S. Biochemical Corp.) were dissolved in 50% glycerin with 20 mM Tris-HCl (pH 7.5) at 5 mg/ml and stored at −20 °C. T4 polynucleotide kinase was from New England Biolabs. Calf intestine phosphodiesterase I crystals (Sigma) were dissolved in 50% glycerin and 10 mM Tris-HCl (pH 7.5) at 4 mg/ml and stored at −20 °C. T7 phage gene 5 (18) exo− T7 DNA polymerase was a double mutant (D5A,E7A) (10) fused at the N terminus to the His₆ tag containing peptide MGSSHHHHHSSGLPRGS. The gene 5 DNA was cloned into a pET-28a vector (Novagen) through the NdeI and Xhol restriction sites (17), and the plasmid was overproduced in cell line BL2(DE)pLysS (Stratagene).

The yeast pol η catalytic core containing residues 1–513 of the native protein was used in this study (27). The polymerase was fused at the N-terminal end to the same His₆ tag as described for the T7 DNA polymerase. The plasmid was overproduced in cell line BL21-CodonPlus (DE3)-RIL (Stratagene). Cell growth, lysis, polymerase purification by nickel-nitriolotriacetic acid-agarose column (Qiagen), and polymerase storage and stability have been described previously (17).

**Oligodeoxynucleotides**—Unmodified oligodeoxynucleotides were purchased from IDT (Coralville, IA). An oligodeoxynucleotide template containing a cis/syn-thymidine dimer was synthesized as described previously (42), as well as were oligodeoxynucleotides containing methylphosphonates (17).

**Primer-Templates**—Primers were 5′-end-labeled with [γ-³²P]ATP and T4 polynucleotide kinase. Primer-templates were made by adding 3-fold excess template to the labeled primer before heating and slow cooling. Numbering of nucleotides and phosphates within the primer-template stem begins with the 3′-end of the primer or the complementary position in the template as shown in Scheme 1.

**Single-Round Synthesis**—Reactions were carried out at 23 °C unless stated otherwise in 10 μl of 10 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol with either 5 mM 5′-³²P-labeled primer-template for the pol η reactions or 0.5 mM for the T7 DNA polymerase reactions. Reactions with the T7 DNA polymerase included 0.5 μM thioredoxin. After 8 min preincubation, 30 μl containing 300 μg of sonicated single-strand calf DNA was added as a trap with 10 mM Tris-HCl (pH 7.5), 10 mM MgCl, and 500 μM dNTPs. All four dNTPs were used in extension reactions opposite mixed sequence DNA, but TTP alone was used opposite homopolymer da-tracts. The reactions were allowed to proceed for 10 s before quenching with 80 μl of formamide containing xylene-cyanol dye, 25 mM EDTA, 0.1% SDS, and 0.5 μg of cold primer. The trap efficiency was tested with each primer-template by adding the DNA trap to the reaction mix prior to the addition of polymerase. After primer extension, the samples were heated to 100 °C for 8 min before loading 5 μl onto a

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**Scheme 1**

| Primer | 5'-³²PpN | pN | pN | pN | pN | pN-3' |
|-------|---------|----|----|----|----|------|
| Template | 3'-X | pX | pX | pX | pX | pX | pT | T-T-5' |
|        | 6     | 5  | 4  | 3  | 2  | 1    | +1 | +2    |

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**Bulge Structure Bypass**
**RESULTS**

**Effect of Single Nucleotide Bulge Position on Primer Extension**—The goal of this study was to investigate the interaction of pol η and T7 DNA polymerase with primer templates containing bulge structures. Single-round primer extension was used as an assay, because it measures binding of a catalytically competent polymerase with a primer-template. In this assay, the DNA polymerase was pre-equilibrated with the primer-template, after which, dNTPs were added together with excess sonicated single-strand calf thymus DNA as a trap for free polymerase. The primer-templates used are shown in Fig. 1. The primer-template P7/T7, without a thymine dimer photoproduct, was used in preliminary experiments for later comparison with the same primer-template sequence containing a thymine dimer. Single nucleotide additions in the P7 primer were made to create single nucleotide bulges in the template, whereas, single nucleotide additions were made to make bulges in the primer when annealed with template T7. PY/TY and PX/TX were used to test the effect of a single nucleotide bulge near the primer-template terminus but located at different positions.

For pol η, the amount of primer extension was low with a single nucleotide bulge near the primer-template terminus but much higher with a single nucleotide bulge located beyond the third base pair (Table 1). With a bulge located between the third and fourth positions there was 58% primer extension with the bulge in the template and 53% with the bulge in the primer, which were both close to the 64% value observed with the P7/T7 control without any bulge. In contrast, T7 DNA polymerase primer extensions were significantly lower with bulge structures located at these same positions. Primer extensions were also much lower with the primer bulge structures except when located between the fourth and fifth positions.

**Design of the Primer-Templates**—Single-round synthesis with pol η was high enough with the bulge located between the third and fourth primer-template positions to obtain reproducible apparent $K_d$ values (Table 1). Based on the crystal structure of the related Dpo4 complex with DNA (31), such a bulge structure would be located within the pol η recognition site for the template-primer stem. Because bulge migration can occur in homopolymer tracts, we used primer-templates with bulge structures known to be either extra- or intrahelical that were locked in position by sequence context. These two types of bulge structures have different structural characteristics, which could have different affects on flanking hybrid stability and polymerase recognition. To test the effect of an extra-helical T bulge structure, we used the primer-template sequence d(GCGAA*AAG)-d(CTTTTCGC) with a T inserted at the position designated by the asterisk in the da-tract. A single nucleotide T (in boldface) inserted in that sequence context,
d(GCGAATAAGCG)-d(CGCTTTTCGC), has been shown to be extra-helical in solution by NMR analysis (39). We eliminated the underlined nucleotides so that the primer-template bulge would close with only a 3-bp stem at the primer terminus. Switching the sequence of the primer and template strands puts the bulge in the template as in d(CGCTTTTC)-d(GAATAAGCG). In addition to a single T, we tested two and three T nucleotide bulges, although there is no structural information available on these structures.

To test intra-helical dA bulges, we used the primer-template sequence d(CATCG*GCT)-d(AGCCGATG) with dAs inserted between the two dGs in the sequence as indicated by the asterisk. The dA (in boldface) in the duplex, d(CATCGAGCTAC)-d(GTAACGGCGATG), as well as 2 and 3dA bulge structures inserted in the same sequence context have been previously determined to be intra-helical (41). The underlined sequence was also eliminated to yield a primer-template bulge that would close with a 3-bp stem at the primer terminus. The single-strand portion of the template was a homopolymer dA tract, which cannot base pair with the primer termini, except for d(CATCG*GCT), which terminates with a single 3A.

We found that both pol η and T7 DNA polymerase could bind to the blunt ends of the DNA primer-templates and interfered with extension of the desired bulge-containing primer end when the primer-templates were too short. Primer-templates containing a 20-bp stem were long enough not to cause interference for pol η but not for the T7 DNA polymerase-thioredoxin complex, which recognizes ~20 bp. To circumvent this problem for T7 polymerase, we used when possible a template that formed a stem-loop at one end (6-bp stem with a 6-base loop) designed to block polymerase binding to that end.

We also found that excess 6-bp template-stem also serves as a primer-template for pol η, but that this did not interfere with Kd estimations. The Kd for pol η is ~100 nM, and the primer-template concentration used was 5 nM. Primer templates were made by annealing a 3-fold excess template, which would leave an additional 10 nM template that was unlabeled. The total primer template of both labeled primer template and that contributed by the free unlabeled template was 15 nM, well below the Kd for pol η.

**Fraying of the Primer-Template Terminus**—Based on DNA mfold calculations (43), the primer-templates with the 3N bulge structures should be significantly frayed at 23 °C. This was investigated experimentally with phosphodiesterase I, a single-strand-specific 3′-exonuclease. A “frayed” primer-template termi

**FIGURE 2. Assaying the extent of fraying of primer termini.** The 3′-exonuclease activity of phosphodiesterase I was used to assay fraying of the 3′-primer termini of P1/T1, P1/T1+3T, P1, and P1/T1 mm (mm = mismatch). Partial sequences are shown. The lane designated “no” is without enzyme. Lanes 1–5 have 20, 2, 0.2, 0.02, and 0.002 μg/ml enzyme.

Bulge-induced Termination of Synthesis by T7 Polymerase—*In vivo*, transmission of a bulge structure through the replication complex would occur after pol η primer extension. To study the propagation of defined bulge structures through a replication complex, we used the exonuclease-deficient T7 DNA polymerase together with its processivity factor E. coli thioredoxin. Previously, we reported that a primer-template with a 3T bulge caused T7 DNA polymerase termination. The bulge was located between the third and fourth positions in template strand. Termination occurred after the synthesis of two nucleotides, presumably when the bulge neared the HLH structure in the polymerase thumb domain (17). This analysis was extended to include site-specific 1, 2, or 3dN extra- or intra-helical bulge structures located in either the template or primer strands of P1/T1, P2/T2, P3/T3, and P4/T4.

Some terminations occurred with the single nucleotide bulges, but two- and three-nucleotide template bulges terminated synthesis sharply after addition of only two nucleotides. No termination was detected with these same bulges in the primer strand, but the amount of primer extension was much lower. The sites of termination were the same with T or dA bulge structures. Primer extensions with the dA bulge structures are shown in Fig. 3 (A and B). Termination also occurred at sites in the DNA where the bulge would be expected to interact with the thioredoxin-thumb complex of the polymerase (20). This was tested with P5/T5 and P6/T6 with or without a 3T bulge located between the ninth and tenth base pair positions in either the template or primer at 4 °C. With P5/T5+3T, containing a template bulge, there was very slight termination after the synthesis of 10 nucleotides, which would position the bulge at template position 20 (Fig. 3C). With P6+3T/T6, con...
Bulge Structure Bypass

A. P3 - TAGC GCA
   T3 - ATGC*GCTA
   no 1 2 3 4 5 6 7 8
   P3 + 1A
   T3 + 1A
   P3 + 2A
   T3 + 2A
   P3 + 3A
   T3 + 3A

B. P4 - ATGC*GCT
   T4 - TAGC CGAA
   no 1 2 3 4 5 6 7 8
   P4 + 1A
   T4 + 1A
   P4 + 2A
   T4 + 2A
   P4 + 3A
   T4 + 3A

C. P5 - N3TT TTCCG TGGCC
   T5 - N3AA*AAAGGCA CGA
   no 1 2 3 4 5 6 7 8
   P5 + 3A
   T6 + 3A
   P6 + 3A
   T6 + 3A

D. P6 - N3AA*AAAGGCA CGA
   T6 - TTCCG TGGCC
   no 1 2 3 4 5 6 7 8
   P6 + 3A
   T6 + 3A
   P6 + 3A
   T6 + 3A

FIGURE 3. Mapping sites of bulge-induced termination of T7 DNA polymerase-thioredoxin synthesis. All the reactions are single-round synthesis with the T7 DNA polymerase-thioredoxin complex. A, primer extensions with P3/T3, P3/T3 + 1A, P3/T3 + 2A, or P3/T3 + 3A. A partial sequence of P3/T3 is shown in the figure. The asterisk designates the template bulge position for one, two, or three dAs. B, primer extensions with P4/T4 (partial sequence shown), P4 + 1A/T4, P4 + 2A/T4, or P4 + 3A/T4. Lanes designated "no" are without enzyme. Lanes 1–8 are with 0.36, 0.49, 0.73, 0.98, 2, 4, 7.5, and 15 nm T7 DNA polymerase-thioredoxin, respectively. C, primer extensions at 4 °C with 15 nm enzyme and P5/T5 (partial sequence shown) or P5/T5 + 3T. D, primer extensions at 4 °C with 15 nm enzyme and P6/T6 (partial sequence shown) or P6 + 3T/T6. The lane designated "no" is without enzyme and is the P6 + 3T primer.

taining a primer bulge, termination occurred after the synthesis of 6 nucleotides, which would put the bulge at primer position 16 in the stem (Fig. 3D). Terminations did not occur at 23 °C with these primer-templates nor with the same primer-templates containing a mixed single-strand template at 4 °C. No terminations were observed with pol η with any of the primer-templates containing 1–3dN bulge structures at any temperature (data not shown).

Affinity of pol η for Primer and Template Strand Bulges—Apparent $K_d$ values for the perfect hybrid (P1/T1) and the primer template containing a 3T template bulge (P1/T1 + 3T) were close in value (95 ± 11 and 107 ± 11 nm, respectively). The maximum primer extension for P1/T1 + 3T was 82% relative to P1/T1. (From this point on in this report, the percent primer extension refers to the maximum primer extension value relative to the control without the bulge.) The apparent $K_d$ values for the same bulge in the primer (P2 + 3T/T2) were 50% higher (143 ± 15 nm), and primer extension was significantly lower at 33% (Table 2). Data for the fully complementary primer-templates P2/T2 as well as P3/T3, P4/T4, P3a/T3a, and P4a/T4a, used as controls in subsequent experiments, are not shown, but all behaved similarly to P1/T1 (Table 2 and Fig. 4A).

It was possible that the lower amount of primer extension for P2 + 3T/T2 might be due to the formation of an alternate hybrid structure. The 3Ts in the bulge, in theory, might base pair with any of the primer-templates nor with the same primer-templates containing a 3T template bulge (P1/T1). The apparent extension refers to the maximum primer extension value relative to the control without the bulge. The percent primer extension was 28%, close to the 33% observed with P2 + 3T/T2 (data not shown).

### TABLE 2
Relative ability of pol η to extend primer-templates containing a three-nucleotide bulge located between the third and fourth positions of the primer or the template

| P/Ts     | Description | Apparent $K_d$ | Relative extension |
|----------|-------------|----------------|--------------------|
| P1/T1    | No bulge    | 95 ± 11        | 100                |
| P1/T1 + 3T | 3 T in P   | 107 ± 11       | 82                 |
| P2 + 3T/T2 | 3 T in T   | 143 ± 15       | 33                 |
| P3/T3 + 3A | 3 A in T  | 95 ± 16        | 77                 |
| P4 + 3A/T4 | 3 A in P  | 257 ± 67       | 44                 |
| P3a/T3a + 3A | 3 A in T | 122 ± 20       | 45                 |
| P4a + 3A/T4a | 3 A in P | 291 ± 109      | 18                 |
| P1/T*     | No bulge, single base overhang | 105 ± 13 | 86 |
| P1/T+ + 3T | 3 T in T, single base overhang | 137 ± 26 | 53 |

FIGURE 4. Apparent $K_d$ plots for pol η extension of primer-templates containing a 3dA bulge. A, apparent $K_d$ plots with P1/T1 (X), P3/T3 + 3A (squares), P3a/T3a + 3A (diamonds), P4 + 3A/T4 (circles), or P4a + 3A/T4a (+ signs). B, examples of single-round primer extension reactions at various pol η concentrations. The lane designated "no" is without enzyme, and lanes 1–12 are with 7.5, 15, 25, 35, 56, 75, 112, 150, 250, 375, 562, and 750 nm, respectively. C, partial sequences of primer-templates, P3/T3 + 3A and P3a/T3a + 3A are the same except for the underlined base pair at the second primer-template position. Similarly, P4 + 3A/T4 and P4a + 3A/T4a are the same except for the underlined base pair.
Similarly, a primer-template with a 3dA bulge structure in the template strand (P3/T3 + 3A) showed higher primer extension than the primer-template with the same bulge in the primer (P4 + 3A/T4) (76 and 44%, respectively). The apparent 

\[ K_d \] \text{ was also lower for P3/T3 + 3A (95 ± 16 nM) than for P3 + 3A/T3 (237 ± 67 nM) (Fig. 4A and Table 2). The higher apparent } K_d \text{ values for the primer-templates containing a primer bulge appear to correlate with lower percent primer extensions and might be explained by faster dissociation of the polymerase from the primer bulge substrates. However, other factors related to bulge distortions near the active site might also contribute to lower catalytic efficiency.}

The 3dA Bulge Causes More Fraying at the Primer-Template Terminus—The closing sequence for primer templates with the 3T bulge consisted of two dA:Ts and one dG:dC, whereas the closing sequence for the 3dA bulge primer templates consisted of one dA:T and two dG:dCs. The resulting difference in hybrid stabilities could have affected the maximum primer extension values. Therefore, we tested the 3dA bulge with primer templates closing with two dA:Ts and one dG:dC for comparison. Primer extensions for P3a/T3a + 3A and P4a + 3A/T4a were 45 and 18%, respectively, both much lower than the values obtained with the 3T bulge structure and suggests the 3dA bulge causes more fraying. (Fig. 4A and Table 2).

Probing Phosphate Contacts between pol \( \eta \) and the Primer-Template—Primer extensions were greater with template bulges, which could indicate less polymerase interaction with the template (Table 2). To investigate electrostatic interactions between the polymerase and the template strand near the primer-template terminus we replaced key phosphates with neutral methylphosphonates. Although methylphosphonates do not affect hybrid stability (45), they do have other effects. They narrow the minor groove of DNA (46), and neighboring phosphates tend to collapse toward the neutral charge causing a 5° bend in the duplex DNA per neutralized residue (47, 48). The neutralized charge also affects DNA flexibility (49). Therefore, modifications near the primer terminus will not only affect polymerase charge interaction with the phosphate-DNA backbone, but the distortion might also affect interactions with the bases. For this reason, we mainly tested templates with single phosphate modifications, but two templates contained multiple consecutive modifications.

The template sequence T2 was synthesized with single methylphosphonate modifications either in second, third, or fourth template positions. These are designated 2mp, 3mp, or 4mp, respectively. The primer-templates are P2/T2-2mp, P2/T2-3mp, and P2/T2-4mp. To test a methylphosphonate at position 1 and +1 in a template we used the T2-2 template with the P2 primer shortened by one or two nucleotides (P2a and P2b, respectively). The primer-templates were designated P2a/T2-2mp and P2b/T2-2mp. Part of the primer-template sequence is shown on the \( K_d \) plots (Fig. 5). For simplicity, we refer here to these primer-templates by the specific template modifications (1, 2, 3, 4, +1, or +2). Apparent \( K_d \) values for the primer-templates with modifications at template phosphates 3 and 4 (124 ± 12 and 108 ± 9 nM, respectively) were similar to the primer-template control sequence without modification (data not shown). Primer extension values were also near 100%.

However, higher apparent \( K_d \) values were observed for modifications at positions 1 and 2 (186 ± 30 and 203 ± 24 nM, respectively) as well as lower primer extensions (40%). In addition to the single phosphate template modifications, we tested a template containing multiple modifications. P11/T11 contains two consecutive methylphosphonates at template positions 3 and 4. The apparent \( K_d \) value was 109 ± 22 nM, and primer extension was close to 100%. Also, there was no difference in \( K_d \) between a normal primer-template and one containing 10 consecutive modifications at template phosphates 4–14 (P10/T10) (data not shown). These results indicate there is little pol \( \eta \) interaction beyond the second phosphate in the template stem.

pol \( \eta \) interaction with the single-strand template also affects binding. Phosphate modification at +1 showed much lower primer extension (6%) and an approximate apparent \( K_d \) value of 300 ± 99 nM. In contrast, modification at +2 had no detectable effect on \( K_d \) or primer extension but synthesis mostly terminated after one nucleotide (data not shown). The kinetic data with methylphosphonate-modified primer templates is summarized in Table 3.

Consistent with the affinity of pol \( \eta \) for the single-strand section of a primer template, a 6-bp stem-loop primer-template containing no single-strand template (PT6B) was 51% less effective as a steady-state inhibitor than PT6, which contained a two base single-strand template overhang (sequences described in Fig. 1). Initial rates of synthesis were compared with 1 nM pol \( \eta \), 100 nM 5',3''P-labeled PT6 as the primer-template, and with either unlabeled 200 nM PT6 or PT6B as inhibitors (data not shown). We also compared pol \( \eta \) binding with P1/T1, which contains a nine-nucleotide single strand template, to P1/T1*, that contains only a single nucleotide template overhang by the
single round synthesis assay. $K_d$ values for both primer-templates were similar, with that for P1/T1* being slightly higher (95 ± 12 and 107 ± 13 nM, respectively), whereas the maximum primer extension for P1/T1* was 86% relative to P1/T1. The lower value for primer extension could be related to a slight decrease in binding affinity, which was reproducible in four separate experiments. A much larger difference in primer extension related to single-strand template length was found for P1/T1* + 3T, which contains a 3T template bulge. The maximum primer extension, in this case, relative to P1/T1, was only 53%. This value was much lower than that of 82% for P1/T1 + 3T, which has a nine base single strand template. The apparent $K_d$ for P1/T1* + 3T was also higher than P1/T1 + 3T (137 ± 26 and 107 ± 11 nM, respectively, Table 2).

**Thymine Dimer Bypass**—We tested misalignment-mediated synthesis as a mechanism for thymine dimer bypass by pol η. We found this enzyme utilizes this mechanism to a greater extent when synthesizing past the 5′-T of the dimer than the 3′-T. The template T7 contains a thymine dimer in the sequence d(AGTAT = TATGA). P7 anneals with T7 to form a perfectly complementary duplex in which the terminal dA of the primer base pairs with the 3′-T of the dimer. P7-3 is missing dA at the third position and creates a single T bulge between the second and third template positions when annealed to T7. Similarly, the P8 primer base pairs with the 5′-T of the dimer and P8-3 is missing a T at the third primer position, which creates a dA bulge between the second and third template positions. Each primer-template contained a template bulge structure closing with two dA:T base pairs. Primer extension for P7-3/T7 relative to control P7/T7 was 9%, whereas extension was higher (21%) for P8-3/T7 relative to P8/T7 (data not shown).

**DISCUSSION**

We investigated pol η recognition of primer-templates containing bulge structures by single-round primer extension experiments. Primer extension was high with a 3T template bulge structure (Table 2), despite predictions based on Zuker mfold calculations and enzymatic analysis with phosphodiesterase I that suggest the primer-template terminus was substantially frayed (Fig. 2). Because pol η does not efficiently extend mismatched primer termini in single-round primer extension reactions, we conclude pol η must stabilize the DNA duplex stem at the primer terminus. This phenomenon may be common among polymerases lacking a proofreading mechanism. An extreme example may be pol λ, which appears to stabilize a primer-template closing with a single base pair duplex stem (50). We could not study bulge structures closer to the primer terminus than 3 bp, because single-round synthesis was low, even with single nucleotide bulge structures located between the second and third positions in the primer-template (Table 1). In these latter cases, the extent of fraying would be greater (51), and polymerase interactions would also be limited by fewer base pairs in the closing duplex.

Based on methylphosphonate modification experiments, there may be only weak pol η interactions with phosphates beyond the first few with the primer-template stem (Table 3). Modeling of a primer-template bound to the structure of the catalytic core of pol η, assuming no protein conformational change, also suggests there may be little direct polymerase contact with the distal part of the primer (Fig. 6A). Therefore, a bulge structure positioned beyond the first few base pairs from the primer terminus may not directly interfere with existing polymerase-DNA interactions. This characteristic, combined with the ability to stabilize a frayed primer terminus, could explain why the catalytic core of pol η extends primer templates with distal bulge structures so efficiently.

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**TABLE 3**

| P/T          | Position | Apparent $K_d$ (μM) | Relative extension (%) |
|--------------|----------|---------------------|------------------------|
| P2/T2-2mp    | 2d       | 203 ± 24            | 50                     |
| P2/T2-3mp    | 3d       | 124 ± 12            | 94                     |
| P2/T2-4mp    | 4h       | 108 ± 9             | 100                    |
| P2a/T2       | 1st      | 186 ± 30            | 83                     |
| P2b/T2       | +1       | 300 ± 99            | 6                      |

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[3 V. J. Cannistraro and J.-S. Taylor, unpublished observation.]
We also found that pol η interacts with the single-strand section of the template. A modified primer-template containing a single methylphosphonate preceding the templating base was extended ~10-fold less in single-round synthesis compared with the unmodified control and indicates some polymerase interaction with that phosphate group (Table 3). Although this interaction improves binding it may also help orient the templating base or in some other way affect the catalytic mechanism. In addition, polymerase interaction with distal portions of the single-strand section of the template increased the level of primer extension with a primer-template containing a 3T template bulge (see “Methylphosphonate Modification of the Template Strand”), pol η may be more effective in stabilizing the DNA duplex stem of primer-templates containing template bulge structures because of the additional interactions with the single-strand portion of the template. Polymerase-template interactions in the stem near the primer terminus, together with polymerase interactions with the single-strand section of the template, may both contribute to the overall stability of the DNA duplex stem. This could explain the observed higher primer extension values with template bulges than with primer bulges (Table 2), but this model needs to be tested further. Polymerase-template “overhang” interactions have been suggested for the Klenow fragment based on primer-template competition experiments (52). The crystal structures of T7 DNA polymerase and Dpo4 ternary complexes with DNA and ddNTP also indicate interactions with the single-strand template. Both the T7 DNA polymerase and Dpo4 interact with the templating base and preceding phosphate in “closed” conformations. It is unknown, however, whether these or different interactions would persist in “open” complexes with DNA, as described in our single-round primer extension assay.

No terminations of synthesis were detected with pol η with any bulge structure tested at any temperature. It was suggested that the little finger of the related Dpo4 polymerase may vary in position (53) and, if also true for pol η, may also explain the ability of pol η to accommodate large bulge structures and, in part, the lack of termination of synthesis. In contrast, termination of synthesis was observed with the T7 DNA polymerase-thioredoxin complex. Previously, we reported that a 3T template bulge caused termination when the structure neared the HLH of the T7 DNA polymerase-thumb (17). We now find 2N and to a lesser extent 1N template bulges also terminate at the HLH structure (Fig. 3, A and B). Terminations also occurred when a 3T bulge neared sites of interaction for the extended thumb and E. coli thioredoxin with the DNA. These occurred when a bulge neared primer position 16 (near thioredoxin Arg-73 and polymerase Lys-290) and template position 20 (near polymerase Lys-300) (Fig. 3C, 3D, and 6B). The stops do not occur during replication of mixed DNA but are exaggerated with da-tract synthesis at 4 °C, which may slow processive synthesis. Therefore, the terminations are probably not biologically relevant but are consistent with the sites of protein-DNA contacts observed in a recent crystal structure (20) (Fig. 6B).

T7 DNA polymerase uses a proofreading mechanism to suppress frameshift mutations (24, 54), which may work more efficiently with the bulge located in the primer. Even in the absence of a functioning exonuclease activity, primer partitioning to the exonuclease active site could explain why we observe lower primer extension values with 1–3N bulges located in the primer strand (Fig. 3). We used the exonuclease-deficient mutant T7 DNA polymerase (D5A,E7A), which can no longer bind magnesium, but we suspect the exonuclease domain still retains significant affinity for DNA, similar to the Klenow fragment (55). In addition, primer release from the exonuclease site may be slower in the exonuclease-deficient T7 DNA polymerase. The rapid release of the primer strand from the exonuclease site probably depends on catalysis, which removes a 3’-nucleotide. By analogy, E. coli ribonuclease II, a 3’-exonuclease specific for RNA, dissociates from single strand DNA with a half-life measured in hours but dissociates rapidly from single-strand DNA containing short 3’-RNA tails (44, 56). In contrast, there may be less primer partitioning to the exonuclease site with the template bulge structures, but the propagation of these structures, unlike primer bulges, are blocked by the HLH structure that would cause the termination of synthesis and lead to repeat efforts at proofreading.

Although the thioredoxin-thumb of T7 DNA polymerase is not blocked by bulge structures, it appears to be a different situation for yeast polymerase δ complexed to proliferating cell nuclear antigen (37, 38). The size of the sliding clamp opening and the size or any possible alterations to the bulge structure appear to be additional variables related to bulge transmission through a processivity ring (57). On the other hand, yeast pol δ may be similar to the T7 DNA polymerase as a frameshift mutation filter, because it also appears to favor partitioning of primers with insertions to the exonuclease site (58).

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