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The Fidelity of DNA Polymerase β during Distributive and Processive DNA Synthesis*

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During base excision repair, DNA polymerase β fills 1–6-nucleotide gaps processively, reflecting a contribution of both its 8- and 31-kDa domains to DNA binding. Here we report the fidelity of pol β during synthesis to fill gaps of 1, 5, 6, or >300 nucleotides. Error rates during distributive synthesis by recombinant rat and human polymerase (pol) β with a 390-base gap are similar to each other and to previous values with pol β purified from tissues. The base substitution fidelity of human pol β when processively filling a 5-nucleotide gap is similar to that with a 361-nucleotide gap, but "closely-spaced" substitutions are produced at a rate at least 60-fold higher than for distributive synthesis. Base substitution fidelity when filling a 1-nucleotide gap is higher than when filling a 5-nucleotide gap, suggesting a contribution of the 8-kDa domain to the dNTP binding pocket and/or a difference in base stacking or DNA structure imposed by pol β. Nonetheless, 1-nucleotide gap filling is inaccurate, even generating complex substitution-addition errors. Finally, the single-base deletion error rate during processive synthesis to fill a 6-nucleotide gap is indistinguishable from that of distributive synthesis to fill a 390-nucleotide gap. Thus the mechanism of processivity by pol β does not allow the enzyme to suppress template misalignments.

DNA polymerase β (pol β)[1] is the smallest of the mammalian cellular DNA polymerases. Evidence suggests that it can participate in several DNA transactions *in vivo*, including DNA replication (1, 2), recombination (3, 4), and base excision DNA repair (reviewed in Ref. 5). Base excision repair is needed to replace damaged nucleotides that are depurinated, deaminated, oxidized, or methylated as a result of normal cellular metabolism or environmental insult (reviewed in Ref. 6). There are at least two forms of base excision repair (BER) in mammalian cells (5). The predominant form is "simple" BER (7), involving excision of a single damaged nucleotide and its replacement catalyzed primarily by pol β (8, 9). Alternatively, BER may occur by excision of 2 to 6 nucleotides involving proliferating cell nuclear antigen and flap endonuclease 1, and DNA synthesis may be catalyzed by pol β (10, 11) or by pol δ or pol ε (12, 13). This pathway is referred to as "alternate" BER.

Early studies of the fidelity of DNA synthesis by pol β employed a long single-stranded template within a 390-nucleotide gap (14). The average pol β error rate for the 12 possible base substitution errors was 7×10^{-4} (15), while the average error rate for single-base deletion errors was $3–9 \times 10^{-4}$ (16). These rates are substantially higher than those measured for the other cellular DNA polymerases that perform the bulk of genomic DNA replication (17). These higher error rates seem consistent with the fact that pol β lacks an intrinsic 3′ → 5′ proofreading exonuclease activity. The observations that pol β has low frameshift fidelity and synthesizes DNA in a distributive manner during primer extension to copy long single-stranded templates suggested that deletion errors within mononucleotide template runs may result from strand slippage initiated during the dissociation-reassociation phase of a polymerization reaction (18). This hypothesis has been supported by numerous subsequent observations (*e.g.* Ref. 19 and references therein; reviewed in Ref. 20) and is central to the present study (see below).

Given that perhaps as many as one million damaged nucleotides per cell per day may be repaired by BER (21), any low fidelity DNA synthesis during BER could potentially introduce a number of mismatches into the eukaryotic genome. Thus, it is important to understand the fidelity of the pol β synthetic reaction. However, recent studies suggest that pol β synthesis to fill a short gap may differ significantly from that during a distributive primer extension reaction. Pol β contains a polymerase domain that binds to duplex template-primer DNA and a unique 8-kDa processivity domain (22) that contains the active site for AP lyase activity (23–26). This 8-kDa domain binds to the 5′-phosphate in short gap substrates (27) and to single-stranded DNA (28), such that DNA synthesis by pol β to fill gaps of 2–6 nucleotides is processive rather than distributive (29). The crystal structure of a pol β·DNA·ddNTP complex revealed that the 5′-terminal phosphate in a single nucleotide gap is bound near the AP lyase active site (25, 26, 30). A 90° kink in the DNA template is also observed, precisely at the 5′-phosphodiester linkage of the template base in the polymerase active site. This sharp bend exposes the base pair on the downstream side of the active site, allowing that template-strand base to stack with a histidine residue in the 8-kDa domain rather than with its neighboring base in the active site. This alteration in base stacking interactions has been suggested to be relevant to the fidelity of a single-nucleotide gap-filling reaction (31). Moreover, cross-linking studies suggested that the 8-kDa domain also contributes to dNTP binding in the polymerase active site (32, 33). Thus, unlike distributive synthesis on a long single-stranded template, pol β fills gaps of up to 6 nucleotides in a processive manner, and the 8-kDa domain participates in this reaction in multiple ways.

Since the processive gap-filling reaction may be more rele-

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[1] The abbreviations used are: pol β, DNA polymerase β; BER, base excision repair; nt, nucleotide.

vant to the fidelity of BER than the earlier studies of distributive primer extension, we have developed in the present study three short gap substrates with which to measure fidelity. One substrate is a model for the predominant role of pol β in simple BER. This substrate contains a single template adenine in a single-nucleotide gap, similar to the gapped substrates used in recent kinetic studies of misinsertion by rat pol β (34, 35). The other two gapped DNA substrates are models for synthesis in the alternate BER pathway; one contains a 5-base gap and a target for scoring eight different base substitution errors, including the same three misincorporations opposite template adenine as in the 1-base gap (this substrate was used earlier to identify pol β mutants with strongly reduced base substitution fidelity (36)); the other substrate contains a 6-nucleotide target to monitor the single-base deletions within a TTTT run during processive synthesis.

The results reveal that pol β has low fidelity when filling short gaps, and generates errors not previously observed during distributive synthesis, including clustered multiple substitutions and complex additions. Base substitution error rates are similar during filling of a >300-nucleotide gap and a 5-nucleotide gap, but are lower for three mispairs during synthesis to fill a single-nucleotide gap. Single-base deletion error rates in a homopolymeric run are similar during distributive and processive synthesis. These data are discussed in light of a model wherein the 8-kDa domain of pol β influences fidelity during the filling of short gaps.

EXPERIMENTAL PROCEDURES

Materials—Bacterial strains, M13mp2 bacteriophage derivatives, and the preparation of the gapped substrate for the forward mutation assay have been described (37). DNA polymerase β from rat Novikoff hepatoma, homogenous fraction VI (38), was generously provided by Dale W. Mosbaugh, Oregon State University, Corvallis, OR. Recombinant wild-type rat and human pols β were expressed in *Escherichia coli* and purified as described (39). ATP and dNTPs were from Amersham-Pharmacia Biotech, Inc. T4 DNA ligase, *Sph*I, and *Eco*RI were from New England Biolabs. Sequenase version 2.0 and sequencing reagents were from U. S. Biochemical Corp. (Cleveland, OH). Synthetic oligonucleotides were from Genosys Biotechnologies, Inc. (The Woodlands, TX).

Forward Mutation Assay—The forward assay scores errors in the *lacZα* gene in M13mp2 (37). Correct polymerization to fill a 390-nucleotide gap produces DNA that yields blue plaques upon introduction into an *E. coli* α-complementation strain and plating on indicator plates. Errors are scored as light blue or colorless plaques. Sequence analysis of DNA extracted from mutants defines the *lacZ* mutations.

Construction of Short Gapped Substrates for Reversion Assays—Short gapped DNA substrates were constructed in which the gap contains a portion of the *lacZα*-complementation sequence of M13mp2 which has been modified by the introduction of an in-frame *opal* codon (Fig. 2, *1ntGap* and *5ntGap*) or a template TTTT sequence (Fig. 2, *6ntGap*). Construction of the 5ntGap and 6ntGap reversion substrates began with addition, by oligonucleotide-directed mutagenesis (40), of a single T at position 59 (where position 1 is the first transcribed base of the gene). This disrupts the reading frame to yield a colorless plaque phenotype. Following alkaline lysis and Qiagen (Chatsworth, CA) purification, the RF I DNAs were purified by CsCl equilibrium density gradient centrifugation to ensure that they were free of single-strand DNA that could compete with the exogenously added single-strand DNA template for hybridization to the primer during formation of gapped substrates. The replicative form DNA was then linearized with *Eco*RI to generate the primer strand DNA for construction of the 5ntGap and 6ntGap substrates. Single-stranded DNA with the T nucleotide inserted at position 59 was used as the template for additional oligonucleotide-directed mutagenesis to generate the template strand DNAs for the 5- and 6-nucleotide gapped substrates. For the 5ntGap substrate, 5 nucleotides were inserted at position 58 of the *lacZα* coding region, restoring the reading frame and introducing an in-frame *opal* codon (5′-GTTGA-3′). Template DNA for the 6ntGap substrate was constructed by the addition of 6 nucleotides at the same position, maintaining the +1 reading frame and introducing a short homopolymeric run (5′-GTTTTA-3′). Gapped substrates were formed from the linear primer and the single-stranded circular plus-strand DNAs as described (37). Substrates were separated from excess primer and single-strand DNA by preparative agarose gel electrophoresis.

TABLE I
Frequency of various classes of observed mutations for DNA polymerases β while conducting distributive DNA synthesis

Mutational event	Rat DNA polymerase β		Human DNA polymerase β	
	Number	MF[a]	Number	MF[a]
		$\times 10^{-4}$		$\times 10^{-4}$
Base substitutions, total	35	83	25	58
Base substitutions minus T → G @ 103	25	59	18	42
−1 Base frameshifts	64	150	68	160
+1 Base frameshifts	2	4.7	1	2.3
−2 Base frameshifts	3	7.1	1	2.3
317-bp deletion	1	2.6	3	6.9
Other mutations[b]	12	28	13	31
Total	106	250	104	240
T → G @ 103	10	24	7	16
−T @ 70	19	45	25	58

[a] MF, mutation frequency.
[b] For the rat enzyme this includes 4 deletions ranging from 5 to 38 base pairs as well as a large deletion with one unknown endpoint and 7 DNA molecules with two widely separated single-base point mutations. For the human enzyme this number includes 6 deletions ranging from 5 to 378 base pairs and a 43-bp insertion resulting from loop back synthesis and 6 DNA molecules with two or more widely spaced single-base point mutations.

For the 1ntGap substrate, oligonucleotide-directed mutagenesis was employed to construct an *Sph*I site at position 58 of the *lacZα* complementation gene. This disrupts the gene with an overall +2 nucleotide insertion. Replicative form DNA was purified as described above and linearized with *Sph*I to generate primer DNA for the 1ntGap reversion substrate. Further mutagenesis was carried out on single-strand DNA containing the *Sph*I site to insert an A nucleotide at position 2 of the *Sph*I site. This generates a template in the normal reading frame but containing an *opal* codon. Gapped substrate was formed and purified as above. These substrate DNAs each have a colorless plaque phenotype. In the 5ntGap and 1ntGap assays, base substitution errors that restore α-complementation result in a blue plaque phenotype. With the 6ntGap substrate, deletions (−1, −4) or insertions (+2) that restore the reading frame result in blue plaques.

Short Gap Fidelity Assays—Fidelity was determined in reaction mixtures (20 μl) containing 20 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 25 mM NaCl, 10 mM $MgCl_2$, 5% glycerol, 1 mM ATP, 150 ng (32 fmol) of gapped DNA, 500 μM each dATP, dCTP, dGTP, and dTTP, 400 units of T4 DNA ligase and pol β. Human DNA polymerase β was used at a 25:1 molar ratio of enzyme to DNA. Following incubation at 37 °C for 60 min, reactions were stopped by adding EDTA to 15 mM and the products were separated on agarose gel. Gel slices containing covalently closed circular DNA products were electroeluted from gel slices and concentrated. DNA samples were introduced into *E. coli* MC1061 by electroporation and plated as described (37). After scoring revertant and total plaques, revertants were replated to confirm the phenotype and then reversion frequencies were calculated. Sequence analysis of revertants was performed to define the sequence responsible for the blue plaque phenotype.

RESULTS

Fidelity of Recombinant Pol β during Distributive DNA Synthesis—Previous studies had established the error rate and specificity during synthesis to fill a 390-base gap by pol β from rat hepatoma and chick embryo (14–16). We began this study by examining the fidelity of recombinant rat and human polymerases β during distributive synthesis to fill this long gap where the polymerases exhibited *lacZ* total mutation frequencies of 250×10^{-4} and 240×10^{-4}, respectively (Table I). Error specificity was defined by sequence analysis of greater than 100 mutants. The error spectra for the two enzymes are summarized in Fig. 1 and Table I. As was found for the native rat and chick enzymes (14), about two-thirds of the errors by the recombinant enzymes are −1 base frameshifts (Table I), with half occurring at a previously identified pol β hot spot, the template TTTT residues at positions +70 through +73 (Fig. 1). As be-

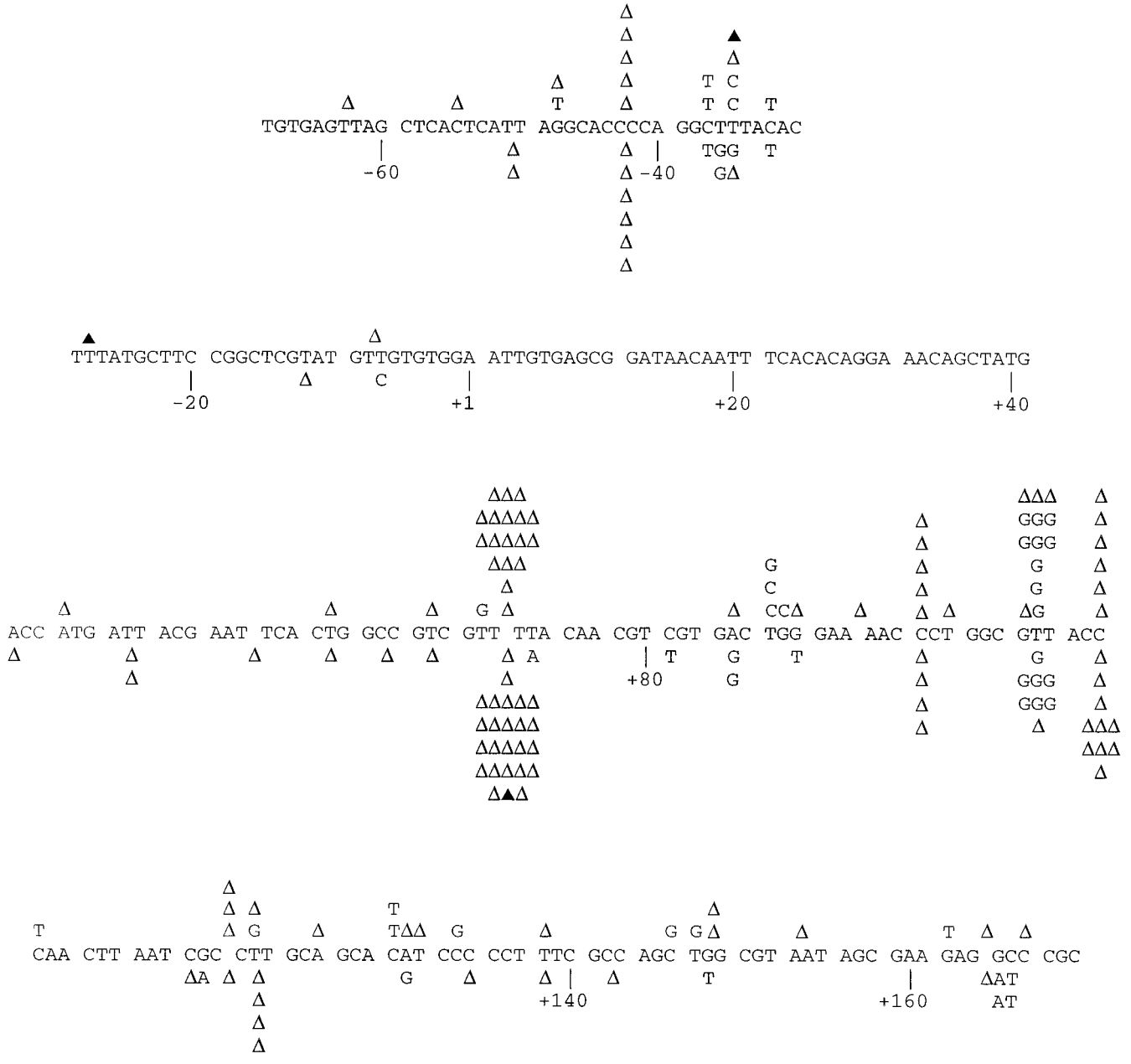

FIG. 1. **Spectrum of single mutations produced by pol β during long gap-filling synthesis.** Four lines of primary wild-type DNA sequence are shown. The *upper two lines* of primary DNA sequence (of the viral (+)-template strand) are the regulatory regions for the *lacZα* gene carried in M13mp2. Position +1 is the first transcribed base. The *lower two lines* are the first 129 bases (43 codons) of the *lacZα* gene. This figure presents mutations produced by recombinant *rat* pol β shown *above* each line of wild-type sequence and mutations generated by recombinant *human* pol β shown *below* each line. The *letters* used for base substitutions indicate the new base found in the viral template strand DNA sequence, and is indicated directly *above* or *below* the wild-type base. For frameshift events, the loss of a base is indicated by an *open triangle* (Δ) directly *above* or *below* the base lost, while the addition of a base is indicated by a *filled triangle* (▲). When a frameshift occurs in a run of 2 or more of the same base, it is not possible to assign the event to an individual base. Therefore, the *symbol* is *centered above* or *below* the run. For both the rat and human enzymes, there were mutants with widely separated sequence changes in the same molecule. Such linked mutations are not shown in this figure.

fore, −1 base frameshifts are generated much more often than are plus 1-base frameshifts. Also as before, a T → G hot spot is observed at position +103. Evidence suggests that this error results from a dislocation mechanism (41, 42). This single T → G transversion accounts for nearly 30% of all the base substitutions in the spectra, even though 241 different substitution errors can be scored in the 250-nucleotide target sequence (37). Both *lacZ* mutant collections contained deletions of 317 base pairs (Table I). These occur between a 5-base direct repeat sequence and are characteristic of the two previous pol β error spectra (14). The mutation frequencies for a variety of different errors are shown in Table I. Overall, the results reveal that the rat and human polymerases β have error rates similar to each other and to earlier determinations with the native rat and chick polymerases β. This lends confidence that the recombinant polymerases are acceptable models for the enzymes isolated from eukaryotic tissues.

For purposes of direct comparison to results obtained during short gap-filling synthesis (below), we also determined the base substitution fidelity of recombinant human pol β for distributive synthesis opposite template TGA *opal* codons within a 361-nucleotide gap. When a TGA codon at template nucleotides 87–89 was used (43), the average error rate was 5.5×10^{-4}. When a 361-nucleotide gap substrate was used that contained

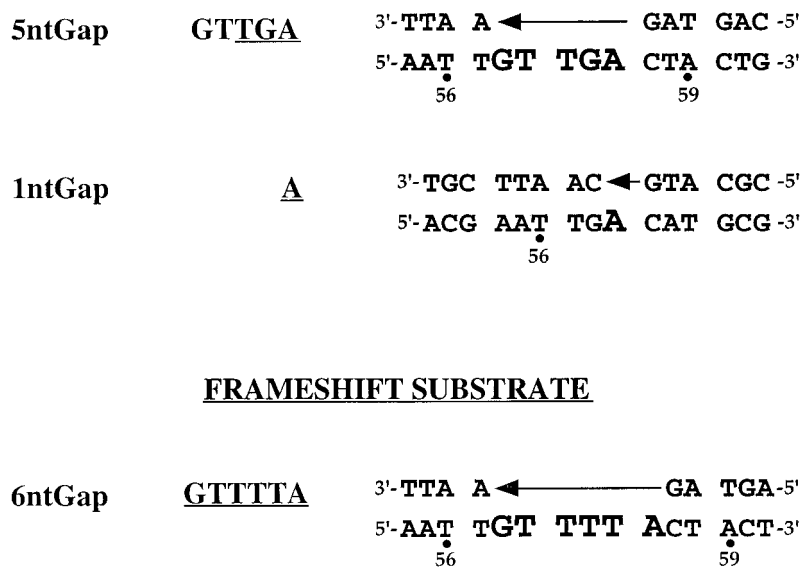

FIG. 2. **Substrates for short gap-filling reversion assays.** The reversion targets and surrounding sequences are shown for the 5ntGap and 1ntGap base substitution assays and the 6ntGap frameshift assay. The inserts are located near the amino terminus of the wild-type M13mp2 *lacZα* complementation gene; *numbers below* the template sequence (*lower strand*) indicate nucleotide position in the wild-type M13mp2 sequence (where +1 is the transcription start). The direction of DNA synthesis is indicated by the *arrows*. The template nucleotides to be copied by pol β are larger. The position of the *A* nucleotide in the 5- and 1-nt targets is the same; it is the first nucleotide synthesized.

the same TGA codon used to construct the 5-nucleotide gap (Fig. 2), an error rate of 5.5×10^{-4} was again observed.

Base Substitution Fidelity during Short Gap-filling Synthesis—The base substitution error rate of recombinant human pol β was determined during synthesis to fill the 5ntGap substrate (Fig. 2). The reversion frequency for the DNA products of this synthesis[2] was 2600×10^{-6} (Table II), at least 460-fold higher than for the uncopied template DNA. Thus, human pol β generates synthesis errors at easily detectable rates during gap-filling DNA synthesis. Sequence analyses of 49 *lacZ* mutants recovered from filling the 5-base gap indicated that pol β produced substitution errors at each of the three phenotypically detectable target nucleotides (Table III), involving seven of the eight detectable mispairs. The overall average error rate for all substitutions at the TGA target was 13×10^{-4}. However, substitutions did not occur randomly; no revertants consistent with a T·dTMP mispair were observed while 29 of 49 revertants were consistent with a T·dGMP mispair. Thus, rates for specific errors ranged from $\leq 0.9 \times 10^{-4}$ for two transversion mispairs (T·dTMP and A·dAMP) to 29×10^{-4} for the T·dGMP mispair (Table III). An unexpected result was the recovery of *lacZ* mutants containing two closely-spaced misincorporations (Fig. 3). These represented 13% (6/49) of the collection. In contrast, such substitutions have not been observed during synthesis by pol β (or any other polymerase) using the long-gap forward mutation assay and undamaged DNA (14, 41, 42, the present study). The error rate for these closely-spaced double misincorporations during synthesis to fill a 390-base *versus* a 5-base gap differs by at least 60-fold (Table IV, compare 180×10^{-6} to $\leq 3.1 \times 10^{-6}$).

Base Substitution Fidelity during Simple BER Synthesis—The base substitution error rate of recombinant human pol β was next examined during synthesis to fill the gap containing a single template adenine (Fig. 2). As above, the reversion frequency for the DNA products of this synthesis was well above the background value for the uncopied template DNA

TABLE II
Mutation frequencies for human pol β while conducting short gap-filling DNA synthesis

DNA sequence in gap[a]	Background MF[b]	Pol β MF	Number of determinations	Number of mutants sequenced
		$\times 10^{-6}$		
GTTGA[c]	5.6	2600 ± 970	9	49
A	5.1	320 ± 92	4	47
GTTTTA (−1, −4, +2, +5)	3.4	3700 ± 1000	5	31

[a] Target nucleotides in the base substitution gaps are underlined and the substrates are illustrated in Fig. 2.
[b] Background mutation frequency (MF) is from transfection of the single-stranded template DNA used to construct each of the short gapped DNA substrates.
[c] Data from Beard *et al.* (36).

(Table II). However, the reversion frequency was 8-fold lower than for synthesis with the 5-base gap (Table II). Sequence analysis revealed that 19 of 47 revertants contained substitutions resulting from misinsertions opposite the template adenine, corresponding to an average base substitution error rate of 2.2×10^{-4}. Again, the rates for the three mispairs that could be scored differed (Table III), being highest for the A·dCMP transition mispair. A comparison of error rates for these three mispairs during synthesis to fill the 1-base and 5-base gap reveals consistently higher apparent fidelity for 1-base gap-filling; the differences are 2.2-fold for the A·dCMP mispair, 9.1-fold for the A·dGMP mispair, and 3.8-fold for the A·dAMP mispair (Table III).

Surprisingly, 19 of 47 revertants generated by human pol β during synthesis to fill the single-base gap contained 4 nucleotides in place of the template adenine (Fig. 3). Such errors have not been observed during synthesis by pol β (or any other polymerase) using the long-gap forward mutation assay (14, 41, 42, the present study) or during synthesis to fill the 5-base gap. Thus, the origin of these errors is unique to filling a single-base gap.[3]

[2] These data are taken from Beard *et al.* (36). They are shown here for direct comparison to the results with the other assays, and to emphasize the recovery of the "closely-spaced" double mutations, which were only briefly mentioned in the previous article that focused on pol β mutator polymerases.

[3] Eight of the nine remaining revertants had a base substitution mutation at one of the other two positions of the TGA codon. The origin of these revertants is uncertain, since these nucleotides were not included as single-stranded template bases for DNA synthesis.

TABLE III
Base substitution specificity for human pol β while conducting short gap-filling DNA synthesis

Template nucleotide	Mismatch (template · dNTP)	Revertant nucleotide	GTTGA[a] (5ntGap) Number of mutants	GTTGA[a] (5ntGap) Error rate	A (1ntGap) Number of mutants	A (1ntGap) Error rate
				$\times 10^{-4}$ [b]		$\times 10^{-4}$ [b]
T	T·dGTP	C	32	29		
	T·dCTP	G	2	1.8		
	T·dTTP	A	0	≤0.9		
G	G·dATP	T	2	1.8		
	G·dGTP	C	2	1.8		
A	A·dCTP	G	3	2.7	11	1.2 (2.9)[c]
	A·dGTP	C	7	6.2	6	0.68 (0.24)[c]
	A·dATP	T	1	0.88	2	0.23 (0.24)[c]

[a] From Beard *et al.* (36).
[b] Error rates, defined as errors per detectable nucleotide incorporated, were calculated by dividing the total number of revertants obtained for each substitution error by the total number of mutants sequenced, and then multiplying by the total mutant frequency. This value was then divided by 0.6, the probability of expressing an error in *E. coli* (37).
[c] From Ahn *et al.* (35).

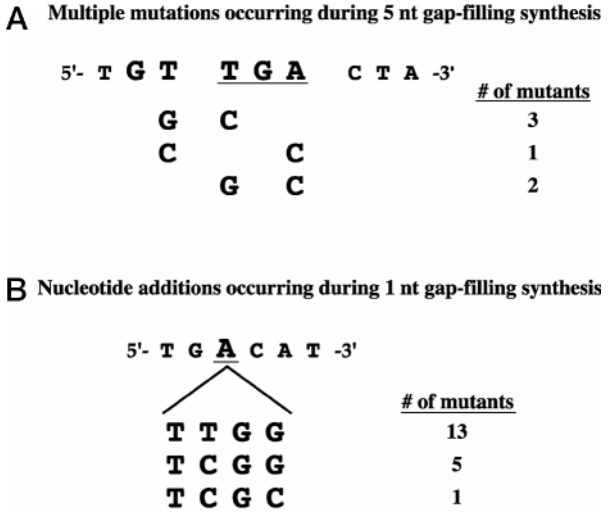

FIG. 3. **Multiple mutations and nucleotide additions occurring during shorter gap-filling DNA synthesis.** *A,* the template (large type) and surrounding sequence in the 5-nt gap-filling substrate is shown on the *upper line*; the reversion target, *TGA*, is indicated. The *letters* on the *lines below* indicate new bases found in the template strand DNA sequence of the revertants. In 6 of 49 revertants, there were multiple DNA synthesis errors made by pol β while copying the 5-nt target. *B,* the template/target nucleotide, *A*, and surrounding sequence in the 1-nt gap-filling substrate is shown on the *upper line*. On the *lines below* is shown the template strand DNA sequence for 19 of the 47 mutants analyzed. The sequence of these revertants indicates that while copying the 1-nt template, pol β frequently makes complex base substitution/addition errors. The single *A* nucleotide in the template has been replaced by four nucleotides.

Frameshift Fidelity during Short Gap-filling Synthesis—The single-base deletion error rate of recombinant human pol β was examined during synthesis to fill the 6-base gap (Fig. 2). The 5′ -GTTTTA-3′ template sequence corresponds to that of the hot spot for −1 base frameshift errors by pol β in the forward mutation assay (Fig. 1). Processive DNA synthesis by pol β to fill this 6ntGap results in a reversion frequency of 37×10^{-4}, more than 1000-fold higher than that of the control uncopied template (Table II). Sequence analysis of 31 revertants revealed that all had lost one T in the TTTT run. This yields a error rate of one deletion for every 670 template T residues copied (Table IV). This result is similar to the deletion error rate when the TTTT run at nucleotides 70–73 in the *lacZ* coding sequence is copied by rat or human pol β during distributive synthesis to fill the 390-base gap in the forward assay (Table I, Table IV).

DISCUSSION

DNA polymerase β plays a key role in mammalian base excision repair. Pol β is a small nuclear DNA polymerase with a host of biochemical properties that make this enzyme well suited for its role in base excision repair. Pol β carries both gap-tailoring (23) and gap-filling enzymatic activities (29, 44), and it physically interacts with at least 3 other base excision repair proteins: AP endonuclease (45), DNA ligase I (46, 47), and XRCC1 (48, 49). Structure-function analysis revealed that the enzyme has a processivity domain which functions only on short-gapped substrates (27). In recent years, additional roles of pol β have been suggested, most involving synthesis to fill relatively short-gapped intermediates in recombination (3, 4) and replication (1, 2). A noteworthy feature of pol β is that it does not have an intrinsic proofreading exonuclease activity (50).

In mammalian cells, several base excision repair pathways have been identified, involving filling of a single nucleotide gap and a gap of 2 to 6 nucleotides. Evidence indicates that pol β performs gap-filling in both of these BER pathways (8, 10, 11), and that synthesis to fill gaps of 2 to 6 nucleotides can be processive (29). To better understand the fidelity of these base excision repair processes, as well as fidelity during primer extension to copy long single-stranded templates that may be relevant to pol β's participation in some types of replication (1) and recombination events (3, 4), we describe here the base substitution and single-base deletion fidelity of recombinant pol β using substrates containing gaps of varying sizes. Data obtained with the 390-base gapped substrate (Table I, Fig. 1) demonstrate that error rates and error specificities during distributive synthesis by the recombinant rat and human enzymes are similar to earlier studies of native pol β purified from several eukaryotic sources (14–16, 18). The error specificities of all the pols β tested to date include the same hot spots for single-base deletions and for dislocation base substitution errors. These data indicate that the recombinant polymerases β are valid models for understanding principles that govern the fidelity of the native polymerases. The error rates of the recombinant rat and human enzymes are also similar to each other (Table I). Although there are 14 amino acid differences between the rat and human polymerases β, all are located on the surface of the enzymes in the crystal structures (31), and the data in Table I and Fig. 1 indicate that the differences do not influence fidelity when these enzymes synthesize DNA in the absence of other BER proteins.

Recombinant human pol β generates 1 base substitution error for every 13,000 nucleotides polymerized during synthesis to fill a 390-nucleotide gap. This average rate is for the 12 possible mispairs that can be scored at 125 detectable template nucleotides in the *lacZ* target. The error rate varies markedly by mispair and position (Fig. 1). During synthesis to fill gaps of >300 nucleotides, pol β is less accurate than average at either of two *opal* codons, generating 1 base substitution error for every 1,800 nucleotides polymerized (Table IV). With all three long gaps, no downstream 5′ terminus is adjacent to the site at which errors are scored, and synthesis is distributive. The average single-base substitution error rate at the *opal* codon during synthesis to fill a 5-nucleotide gap is even higher, 1/770 (13×10^{-4}, Table IV). This value is similar to the misinsertion rates for G·dTMP and G·dAMP mispairs determined by Chagovetz *et al.* (34) using a 6-nucleotide gap. In our study with a 5-nucleotide gap with the template nucleotides for scoring

TABLE IV
Comparative error rates for DNA polymerases β—long versus short gap-filling DNA synthesis

Gap size	Target sequence	Number of errors in class / Total number of mutants sequenced	Mutant fraction	Target size	Average error rate[a]
nucleotides			$\times 10^{-4}$	*nucleotides*	
Base substitutions					
391	*lacZα* gene	25/104	240	125	0.77×10^{-4}
361	TGA_{89}[b]	174/170,000[c]	10	3	5.5×10^{-4}
413	TGA[d]	11/11,000[c]	10	3	5.5×10^{-4}
5	TGA	43/49	26	3	13×10^{-4}
1	A	19/47	3.2	1	2.2×10^{-4}
Closely-spaced base substitutions					
391	*lacZα* gene				
	Rat	0/106	250	125	$\leq 3.1 \times 10^{-6}$
	Human	0/104	240	125	$\leq 3.1 \times 10^{-6}$
5	TGA				
	Human	6/49	26	3	180×10^{-6}
Frameshifts					
391	*lacZα* gene[e]				
	Rat	19/106	250	4	1.9×10^{-3}
	Human	25/104	240	4	2.4×10^{-3}
6	GTTTTA				
	Human	31/31	37	4	1.5×10^{-3}

[a] Average error rates were calculated by dividing the total numbers of errors in a class by the total number of mutants sequenced, then multiplying by the mutant fraction, and then dividing by the target size. This value was then divided by 0.6, the probability of expressing an error in *E. coli* (37).
[b] The substrate used is described in Kunkel and Soni (41).
[c] For these two assays, the number in this column is the ratio of blue plaques to total plaques.
[d] This substrate was made with the same single-strand template and RFI DNAs as the 5ntGap. However, the linear primer was generated by restricting the replicative form DNA with PvuII resulting in a substrate with a single-stranded gap of approximately 400 nucleotides.
[e] Loss of a T residue at position 70–73 in the *lacZα* gene.

errors only 2, 3, or 4 nucleotides distant from the 5′ terminus (Fig. 1), we also observe multiple base substitution errors (Fig. 3) at a rate much higher than during distributive filling of longer gaps (Table III). Closely-spaced substitution errors have not been observed in the spectra of errors generated *in vitro* by other DNA polymerases. Key to considering their origins and the generally low fidelity of pol β are biochemical (36) and structural information suggesting that the ability of a polymerase to form hydrogen bonds or van der Waal contacts with atoms in the minor groove of the nucleic acid is important. In the structures of the Pol I family polymerases, the DNA near the polymerase active site is A-form-like with a wide, shallow minor groove, and these polymerases form hydrogen bonds with minor groove O-2 and N-3 atoms (51–53). However, in the ternary complex of pol β with a single nucleotide gap, the duplex DNA is B-form and there are fewer hydrogen bonds between the polymerase and the duplex primer stem upstream of the active site (30). If the DNA is also B-form when pol β is bound to a 5-nucleotide gap, it may be less able than other polymerases to sense correct base pairing geometry in the active site and/or at the primer terminus. This might account for the overall low base substitution fidelity of pol β and for generation of multiple mutations during 5-nucleotide gap filling. The ability of pol β to generate multiple, closely-spaced substitutions is also consistent with a model (Fig. 4*A*), wherein the 8-kDa domain binds to the downstream 5′ terminus to promote processive extension of misinserted nucleotides that cannot be proofread because pol β lacks 3′-exonuclease activity. Consistent with this idea, the kinetic constant for DNA synthesis during filling of short gaps is greater than that for product release (35).

In experiments in which UV-irradiated shuttle vectors containing a bacterial suppressor tRNA gene were employed as a mutagenic target (54–57), a significant number of multiple substitution mutations were observed. The data presented here suggest that these errors could have resulted from gap-filling synthesis *in vivo* by DNA polymerase β. More recently, we have found that the spectrum of somatic hypermutations within an immunoglobulin gene in mismatch repair-deficient *pms2(−/−)* mice is characterized by a high proportion of tandem double-base substitutions that could result from error-prone filling of short gaps by pol β (58).

During synthesis to fill a 1-nucleotide gap, human pol β makes one mistake for every 4,500 nucleotides polymerized at the *opal* codon (Table IV). This average rate is about 6-fold lower than for filling a 5-nucleotide gap (Table IV), with rates for individual misincorporations opposite template adenine being 2–9-fold lower than during 5-nucleotide gap filling (Table III). These differences could at least partly be explained by decreased T4 DNA ligase activity when mispairs are present at the nick. The relatively low base substitution fidelity of pol β and the small differences in fidelity for filling a 1-nucleotide gap *versus* a longer gap are consistent with kinetic determinations of misinsertion fidelity reported by Ahn *et al.* (35). They found (see values in parentheses in Table III) that pol β was not highly accurate for the same three mispairs (albeit in a different sequence context), and that the fidelity of pol β for single-nucleotide gap-filling varied no more than 10-fold from that observed during synthesis with a non-gapped template-primer. However, our results are in contrast to observations by Chagovetz *et al.* (34), who report much higher fidelity for filling a single-nucleotide gap. As one example, they reported that pol β misinserts TMP opposite template guanine (typically one of the most frequent errors by DNA polymerases) at a rate of only 1×10^{-5}, a rate 180 times lower than during synthesis to fill the 6-nucleotide gap (34). There are several possible explanations for the difference between this study and ours, including the fact that different mispairs were examined and these were in different neighboring sequence contexts. Nonetheless, we conclude from the present study, and that of Ahn *et al.* (35), that the average base substitution fidelity of pol β is low during single-nucleotide gap filling. The fidelity of BER *in vivo* may

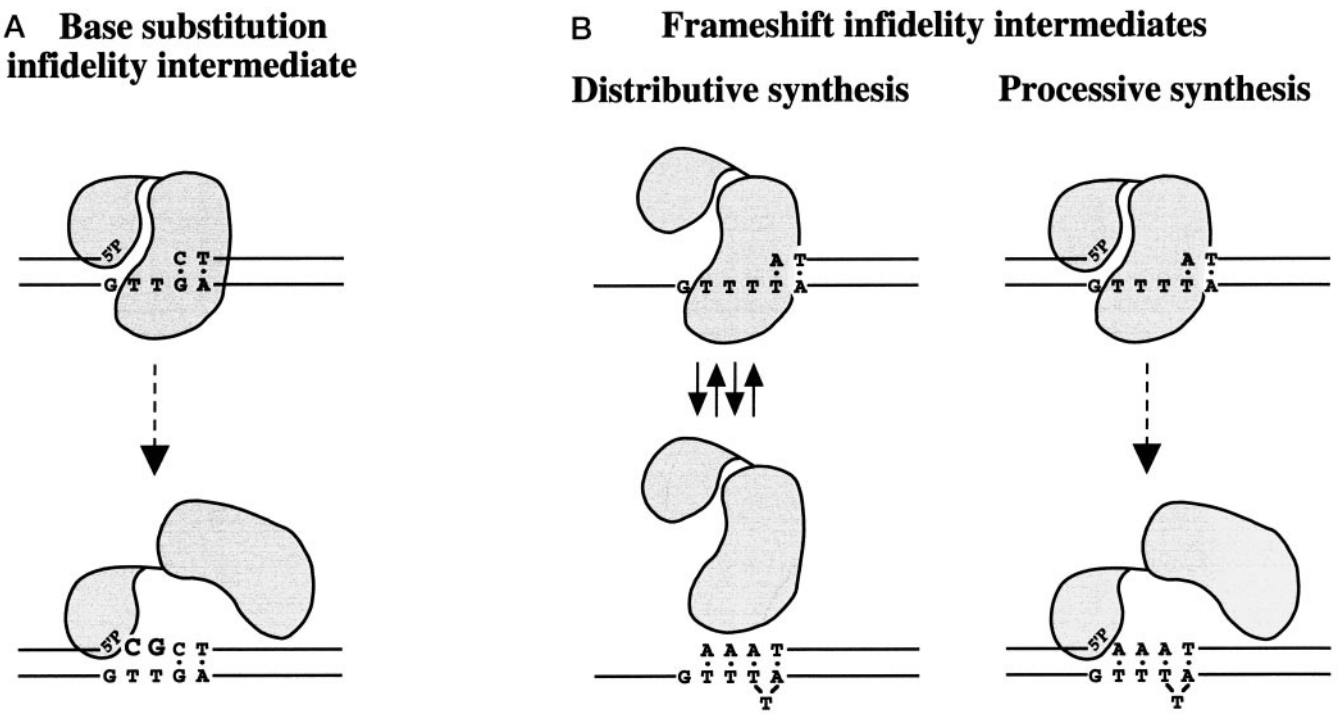

FIG. 4. **Model depicting the base substitution and frameshift infidelity intermediates of DNA polymerase β during short gap-filling synthesis.** *A,* during shorter gap-filling, the 8-kDa domain of pol β is bound to the 5′-phosphate moiety at the 3′-end of the gap. Because the polymerase domain is tethered to the substrate, synthesis by pol β proceeds in a processive manner (*dashed arrow*). Analysis of revertants from shorter gap-filling synthesis by pol β demonstrates that pol β can misincorporate a nucleotide and then continue synthesis from the mispaired template-primer terminus. Multiple misincorporations are facilitated due to processive synthesis by DNA polymerase β resulting in tandem and clustered base substitution errors. *B,* misalignment of the template and primer strands during DNA synthesis can lead to frameshift errors resulting in the addition or deletion of one or more nucleotides. During distributive synthesis, pol β binds to the DNA substrate, conducts synthesis, dissociates, and then rebinds (*solid arrows*). When the polymerase is not bound, template-primer misalignment can occur, leading to frameshift errors. In the presence of 5′-phosphorylated downstream DNA, pol β will remain tethered to the substrate and synthesis will be processive (*dashed arrow*). The data presented here indicate that the rate of frameshift errors during distributive and processive synthesis by pol β is nearly the same. This suggests that either unpaired bases can migrate through the run while the polymerase is bound to the template-primer or the polymerase domain dissociates from the DNA to allow misalignment as illustrated.

still be higher since other BER proteins could enhance pol β selectivity or inefficient ligation of mispairs could allow removal of misinsertions by a 3′-exonuclease not intrinsic to pol β.

This study and the Ahn *et al.* (35) and Chagovetz *et al.* (34) reports describe one or more examples of higher fidelity for filling a one-nucleotide gap than for synthesis with substrates having distant or no downstream 5′ termini. A possible explanation is that the 8-kDa domain, which binds to the downstream 5′ terminus, contributes to polymerase selectivity during synthesis of the final nucleotide in a gap. Biochemical evidence for this comes from cross-linking studies which suggest that the 8-kDa domain contributes to dNTP binding in the polymerase active site (32, 33). The crystal structure of a pol β·DNA·ddNTP complex (30) reveals a 90° kink in the DNA at the 5′-phosphodiester linkage of the template residue in the polymerase active site. This exposes the (n+1) base pair on the downstream side of the active site, which stacks with His-34 in the 8-kDa domain rather than with the active site template base (Fig. 5*A*). Elimination of base-base stacking could prevent nonspecific stabilization of incorrect incoming dNTPs, thus providing a selective advantage for correct incorporation. Stacking of the (n+1) base with His-34 is not observed in the structure of a rat pol β·DNA·ddNTP complex which lacks a downstream primer (59) (Fig. 5*B*). Moreover, the latter structure reveals minimal interactions of the 8-kDa domain with the polymerase active site, such that it may not contribute to dNTP binding (Fig. 5*B*).

Synthesis to fill a single-nucleotide gap also generated *lacZ* mutants containing four nucleotides in place of the template adenine (Fig. 3). The number of additional nucleotides observed in the *lacZ* revertants (3 rather than 1, 2, 4, 5, etc.) is partly defined by the requirement to maintain the correct reading frame for blue plaque phenotype. Thus, other complex errors may be generated in this reaction but remained undetected. Examination of the sequence surrounding the template adenine did not reveal an obvious sequence that could be used to template, by simple strand displacement, the three examples of this type of error that were observed (Fig. 3). Such errors have not been found in previous studies of other polymerases, during synthesis by pol β to fill long gaps (14, 41, 42), or using any other gapped substrate in this study. Moreover, no such *lacZ* mutants were generated during single-nucleotide gap-filling by exonuclease-deficient Klenow polymerase or by several mutant derivatives of this enzyme (63). Thus, to date, these complex errors are unique to single-nucleotide gap-filling by pol β. Their origin may eventually be explained by the unique features of the pol β DNA synthesis reaction, involving a second DNA binding site in the 8-kDa domain and strong reconfiguration of the single-nucleotide gapped substrate (30).

Previous studies have shown that during distributive primer extension without a downstream 5′ terminus, pol β generates single-base frameshift errors at a relatively high rate (14, 16, 18, 41). These and subsequent studies of other DNA polymerases, especially HIV-1 reverse transcriptase (19, 60–62), have revealed a correlation between processivity and the rate of single-base frameshift errors in homopolymeric runs. This correlation led to the suggestion that these errors may be initiated by strand slippage that occurs during polymerase dissociation-reassociation with the template-primer. The ob-

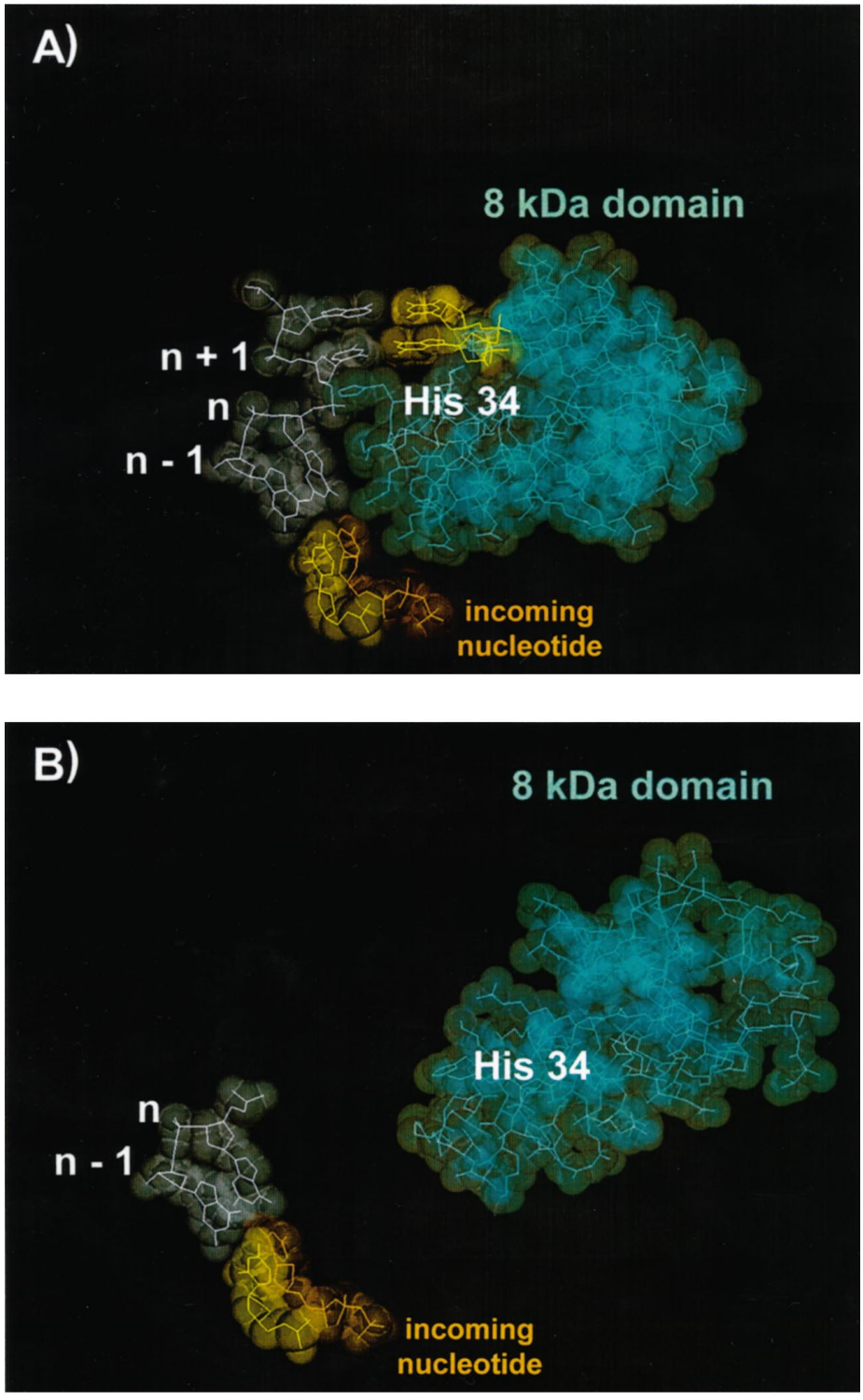

FIG. 5. **The role of the 8-kDa domain of pol β in DNA synthesis fidelity.** *A,* in the crystal structure of human pol β complexed with single nucleotide gapped DNA and an incoming ddNTP, the 8-kDa domain is bound to the 5′-terminal phosphate in the gap. A 90° kink is present in the DNA, located precisely at the 5′-phosphodiester linkage of the template residue (*n*) in the active site (30). The base pair on the downstream side of the active site is exposed, so that this template-strand base (n+1) is stacked with His-34 rather than with its neighbor in the active site (*n*). The 8-kDa domain of pol β also contributes to the dNTP binding pocket in the polymerase active site. *B,* in a rat pol β·DNA·ddNTP ternary complex with a single-stranded DNA template and no 5′-phosphorylated downstream DNA (59), the position of the 8-kDa domain is not consistent with its contribution to the dNTP binding pocket.

servation that pol β fills short gaps processively (29) offered an opportunity to examine this hypothesis. When frameshift fidelity was monitored in a 6-nucleotide gap containing a template TTTT run, pol β was highly error prone (Table II). This represents the first reported measurement of the frameshift fidelity of a short-gap filling polymerization reaction and is consistent with the possibility that the infidelity of BER may contribute to microsatellite instability. This low frameshift fidelity is also consistent with the structural data discussed above (and reviewed in Ref. 53), that reveals limited minor groove contacts of the enzyme with the duplex template-primer stem. The pol β single-base deletion error rate per detectable nucleotide polymerized to fill the 6-nucleotide gap was not different than for distributive primer extension without a 5′ terminus adjacent to the run (Table IV). One possible explanation is that pol β remains fully engaged with the template-primer during processive synthesis, and an unpaired template base can migrate through the run to form the misaligned intermediate. Alternatively, we favor the model shown in Fig. 4*B*, in which the polymerase domain dissociates from the DNA allowing misalignment, but pol β remains tethered to the DNA for processive synthesis through interactions of the 8-kDa domain with the adjacent 5′ terminus. This concept of error-prone synthesis initiated by strand slippage due to polymerase dissociation-

reassociation and promoted by virtue of a second DNA binding site conferring processivity to the reaction is supported by a recent study of the frameshift fidelity of T4 DNA polymerase with and without its processivity factor, the gp45 sliding clamp (20). In that study, the error rate for 1-nucleotide deletions in a homopolymeric run was similar for the T4 polymerase alone or with its accessory proteins. This led to the idea that the polymerase could dissociate from the template-primer to allow misalignment but remained tethered to the DNA for processive synthesis through polymerase interactions with the topologically bound sliding clamp.

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