Translesion Replication by DNA Polymerase β Is Modulated by Sequence Context and Stimulated by Fork-like Flap Structures in DNA†

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ABSTRACT: Mutations in the human genome are clustered in hot-spot regions, suggesting that some sequences are more prone to accumulate mutations than others. These regions are therefore more likely to lead to the development of cancer. Several pathways leading to the creation of mutations may be influenced by the DNA sequence, including sensitivity to DNA damaging agents, and repair mechanisms. We have analyzed sequence context effects on translesion replication, the error-prone repair of single-stranded DNA regions carrying lesions. By using synthetic oligonucleotides containing systematic variations of sequences flanking a synthetic abasic site, we show that translesion replication by the repair polymerase DNA polymerase β is stimulated to a moderate extent by low stacking levels of the template nucleotides downstream of the lesion, combined with homopolymeric runs flanking the lesion both upstream and downstream. A strong stimulation of translesion replication by DNA polymerase β was seen when fork-like flap structures were introduced into the DNA substrate downstream of the lesion. Unlike for gapped substrates, this stimulation was independent of the presence of a phosphate group at the 5′ terminus of the flap. These results suggest that DNA polymerase β may participate in cellular DNA transactions involving higher order structures. The significance of these results for in vivo translesion replication is discussed.

DNA polymerase β (pol β),[1] the smallest mammalian DNA polymerase, is responsible for DNA synthesis during base excision repair (BER) (*1*), and may be involved also in recombination (*2*). The enzyme is composed of two subdomains: the 31 kDa polymerization domain and a small 8 kDa domain harboring the dRPase/AP lyase activity (*3*, *4*). The latter activity is involved in the removal of lesions during BER which creates a 1 nt gap, while the former fills this gap. Pol β replicates DNA either in a distributive fashion during replication of large gaps (>6 nt) or processively during replication of 1−6 nt long gaps (*5*). The processive mode of replication is most likely of in vivo relevance during BER, which involves filling-in replication of small gaps.

The polymerization error frequency of pol β has been measured both on plasmids and on oligonucleotides substrates and was shown to be very high compared to other polymerases (*6*−*8*). This manifestation of low fidelity by pol β has been attributed to the lack of a 3′→5′ exonuclease activity in pol β (*9*). The spectrum of mutations is dominated by −1 frameshifts, although some insertions, base substitutions, and large deletions and rearrangements (unique to pol β) were observed (*10*). The mutation spectrum of pol β as seen in large gaps (∼400 nt) is composed of hot-spot regions (*10*), suggesting that the sequence context of the template DNA has a dramatic effect on the tendency of pol β to form a mutation. In particular, −1 frameshifts and larger deletions were found mainly in homopolymeric runs, thus invoking a slippage/misalignment mechanism (*11, 12*).

Sequence context effects have also been observed during translesion replication by pol β (*13*−*15*). That is, when pol β replicates DNA containing a lesion in the template strand, the sequence in the vicinity of the lesion was found to influence the efficiency of bypass. A correlation was found between the nucleotide inserted opposite the lesion and the template base immediately downstream of the lesion, such that the efficiency of bypass was greater when the two were complementary. In addition, it was shown that bypass of DNA lesions by pol β forms predominantly −1 deletions at the site of the lesion (*13*−*15*). This led to the suggestion that bypass replication by pol β is performed via a misalignment mechanism, similar to the formation of −1 frameshifts during replication of homopolymeric runs. The ability of pol β to bypass lesions may be related to its low fidelity. That is, incorporation of a dNTP opposite a lesion and extension from that incorrect base pair may be similar to the creation of a mutation during synthesis of undamaged templates.

To dissect the structural aspects within the DNA template strand that dictate the efficiency of lesion bypass, we have employed a systematic approach to study sequence context effects. Here we present our initial screen for sequences promoting high bypass levels utilizing oligonucleotide

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[1] Abbreviations: BER, base excision repair; BSA, bovine serum albumin; DTT, dithiothreitol; nt, nucleotides; P/T, primer/template; PAGE, polyacrylamide gel electrophoresis; pol β, DNA polymerase β; pol I, DNA polymerase I.

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substrates containing variable sequences in the vicinity of a lesion. The variable sequences were chosen to represent extreme situations of stacking interactions, thermal stability, and iterated sequences.

We show that pol β had a sequence preference in lesion bypass which is distinct for this enzyme, since a different sequence preference was obtained with *E. coli* DNA polymerase I (pol I). In addition, both polymerases seemed to be affected by the stacking level of repetitive sequences. Interestingly, our approach has also revealed a surprising phenomenon by demonstrating a dramatic effect of higher-order structures in the DNA, rather than the mere linear sequence, on bypass replication by pol β. That is, we show that fork-like (flap) structures caused a substantial stimulation of bypass by pol β. This effect on pol β may be related to its unique structural mode of interaction with the DNA primer/template, and may suggest its involvement in certain DNA transactions that contain fork-like structural intermediates.

MATERIALS AND METHODS

Proteins. Recombinant rat pol β, expressed in *E. coli* and purified as described (*16*), was a generous gift of S. Wilson (NIEHS, Research Triangle Park, NC). *E. coli* pol I was from Boehringer Mannheim.

DNA Substrates. The DNA substrates were prepared as previously described (*17*, *18*), with several modifications. The DNA oligonucleotides were synthesized by the Synthesis Unit of the Biological Services Department in our institute. dSpacer CE phosphoramidite, a synthetic abasic site building block, used in the synthesis of the template DNA oligonucleotides, was from Glen Research (Sterlin, VA). Primer/template (P/T) substrates were assembled by hybridizing the 5′-^{32}P end-labeled primer oligonucleotide (using T4 polynucleotide kinase, New England Biolabs) to template oligonucleotides in a 1:1.1 ratio, respectively. The mixture containing 20 mM Tris, pH 7.5, and 150 mM NaCl was heated to 70 °C for 10 min, and allowed to cool to room temperature over a period of 3−4 h. The mixture was then purified by a BioSpin 6 gel filtration column (BioRad). Gel analysis revealed that >95% of the primer was annealed to a template oligonucleotide. Primer/template substrates containing either a gap or flap oligonucleotides were hybridized in the same fashion, except that the downstream oligonucleotide (either dephosphorylated or 5′-phosphorylated) was added to the mixture prior to annealing in a 1.1:1 excess over the template strand.

Translesion Replication Reactions. In a typical translesion replication reaction (10 μL), 30 nM assembled primer/templates was preincubated at 37 °C for 3−5 min in EDBG buffer (20 mM Tris, pH 7.5, 0.1 mM EDTA, 8 μg/mL BSA, 4% glycerol, 5 mM DTT) containing 500 μM each of dATP, dCTP, dGTP, and dTTP, and 10 mM Mg^{2+}. Reactions were initiated by the addition of either rat pol β (150 or 380 nM) or *E. coli* pol I (30 nM). Replication was stopped by the addition of 4 volumes of formamide loading buffer (95% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyan). Samples were heated at 90 °C for 10 min prior to being loaded on 15% acrylamide (1:19 bis:acrylamide ratio) containing 8 M urea in TBE buffer (89 mM Tris−borate, pH 8.3, and 2.5 mM EDTA). Gels were run at 1800 V for 2−3 h, after which they were dried, visualized, and quantified using a Fuji BAS 1000 phosphorimager. The extent of total bypass efficiency was calculated by dividing the amount of bypass products (bands longer than 24 nt) by the amount of all extended primers. The extent of full-length bypass efficiency was calculated by dividing the amount of only products >40 nt long by the amount of all extended primers reaching the lesion (position 24).

Resolution by Native Gels. Labeled P/T (0.2 pmol) were combined with loading buffer (TBE, 5% glycerol, 0.025% bromophenol blue, 0.025% xylene cyanol) and loaded on a 12% acrylamide gel (1:30 bis:acrylamide ratio) in TBE running buffer. Gels, 10 cm long, were run for 1 h at 100 V, after which they were dried and visualized using a Fuji BAS 1000 phosphorimager. When required, $MgCl_2$ was added at a final concentration of 10 mM to the samples, to the gel matrix, and to the running buffer. Competition experiments using unlabeled P/T were performed by incubating 30 nM labeled P/T with increasing amounts of unlabeled P/T in the presence of 10 mM $MgCl_2$ for 1 h, at 37 °C. Mixtures were fractionated on 12% acrylamide gels, as above.

RESULTS

The composition and arrangement of bases comprising a particular DNA sequence dictate its detailed spatial structure. In an attempt to decipher the rules by which a certain DNA sequence, and therefore structure, affects the ability of DNA polymerases to create mutations and bypass lesions, we have employed a systematic screen of variable sequences in the vicinity of a lesion within a template DNA oligonucleotide. The tested region included five nucleotides upstream to the lesion and five nucleotides downstream to the lesion. The ability of rat pol β and *E. coli* pol I to bypass a synthetic abasic site in the environment of the variable sequences was tested. The DNA substrates used were comprised of synthetic DNA oligonucleotides assembled in a primer/template (P/T) configuration (Figure 1). The template strand contained the chemically stable abasic site analogue (tetrahydrofuran) which has been shown to have similar properties to abasic sites in blocking replication by DNA polymerases (*17*−*20*).

In choosing the variable sequences for our screen, we have focused on three categories: (1) the base stacking level of a particular sequence, with the decreasing order of stacking generally being purine-purine > purine-pyrimidine > pyrimidine-pyrimidine (*21*); (2) the thermal stability of a sequence, controlled by its G:C content; (3) reiterated sequences that increase the tendency of DNA polymerases to form deletions and insertions, most likely due to slippage (*11*).

Table 1 lists the 11 sequences that were analyzed: The first 6 sequences share low thermal stability but different levels of stacking interactions and reiterations. They are also divided into two groups: sequences 1−3 in which the variable region flanks the lesion both upstream and downstream, and sequences 4−6 in which the variable region is localized only downstream of the lesion. Sequences 7−9 share high thermal stability compared to sequences 4−6. Sequences 10 and 11 were chosen randomly as having no special features. They vary only by the two nucleotides immediately downstream of the lesion.

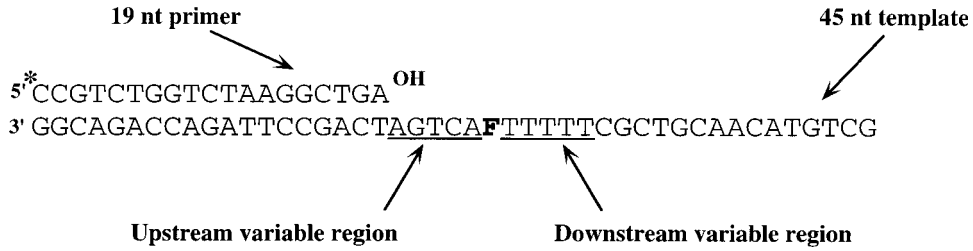

FIGURE 1: Primer/template DNA substrates used in the sequence context analysis. The 19 nucleotide long primer and 45 nucleotide long template DNA oligonucleotides used as substrates for translesion replication are shown. The position of the abasic site analogue (tetrahydrofuran) is marked (F). The sequence upstream of the lesion, which was varied in only three of the substrates, is underlined. The sequence shown is present in templates 4−11 (Table 1). The variable region (5 nucleotides long) downstream of the lesion, having a different sequence in each substrate, is also underlined. The sequence shown is that of F-T (Table 1). The position of the 5′ radiolabel is marked by an asterisk.

Table 1: Variable Sequences of 11 Primer/Templates and Their Relative Stacking, Slippage, and Thermal Stability Levels

						bypass (%)[c]	
	symbol	sequence of variable regions[a]	stacking[b]	slippage[b]	thermal stability[b]	pol β	pol I
1	T-F-T	TTTTT-F-TTTTT	+	+++	+	46	5
2	AT-F-AT	ATATA-F-ATATA	++	+	+	13	32
3	A-F-A	AAAAA-F-AAAAA	+++	+++	+	11	5
4	F-T	agtca-F-TTTTT	+	++	+	10	59
5	F-AT	agtca-F-ATATA	++	+	+	7	44
6	F-A	agtca-F-AAAAA	+++	++	+	7	8
7	F-C	agtca-F-CCCCC	+	++	+++	5	13
8	F-GC	agtca-F-GCGCG	++		+++	40	10
9	F-C	agtca-F-GGGGG	+++	++	+++	9	23
10	F-CTRL1	agtca-F-GTCAG	++	−	++	48	17
11	F-CTRL2	agtca-F-TGCAG	++	−	++	65	35

[a] Lower case letters represent the default sequence of the upstream variable region (depicted in Figure 1). [b] For each category, three relative levels [+ (low), ++ (medium), and +++ (high)] were assigned according to basic rules (see text). [c] Bypass values, expressed in %, were calculated as described under Materials and Methods, based on data taken from Figure 2A, for the 10 min reaction.

Extension of the 5′-radiolabeled primer and bypass of the abasic site analogue by pol β were analyzed by urea−PAGE, and typical results of replication from 7 (of the 11) P/T are shown in Figure 2A. Clearly, the amount of replication products that had passed the lesion varied among the sequences. This effect can be seen quantitatively for all 11 P/Ts in Figure 2B and Table 1.

Another striking difference between the 11 P/Ts is the pattern of products that have passed the lesion: for some sequences, pol β formed mainly long products, that had reached the end of the template (e.g., F-T, F-A, F-C, and F-G in Figure 2A). For others (F-CTRL1, F-CTRL2, and F-GC), short products (in the range of 25−29 nt long products) which seem to be strong pause sites of replication past the lesion appeared in addition to the long products. A quantitative analysis both of the total amount of bypass (short plus long products, Table 1) and of only the short products (data not shown) suggested that the sequence elements that had stimulated long product formation were also responsible for the creation of pauses.

To examine whether this effect was specific to pol β, we examined bypass by *E. coli* pol I using the same set of DNA substrates. Indeed, the observed sequence preference by pol β seemed to be specific for this polymerase, since pol I produced a different sequence preference when analyzed in a similar fashion: The highest bypass was observed with sequences F-T, F-AT, F-CTRL2, and AT-F-AT (Figure 2B and Table 1). Most notably, three of these sequences, which were bypassed very effectively by pol I (F-T, F-AT, and AT-F-AT), showed low bypass with pol β (Figure 2B and Table 1). In addition, pol I did not seem to pause past the lesion at any of the P/T substrates (data not shown). Thus, the two enzymes seem to respond to different structural elements in the DNA.

As can be seen in Figure 3A, among the sequences with overall high thermal stability (sequences 7−9, Table 1), both polymerases showed no trend which can be explained by the simplified rules formulated in Table 1. For pol β, P/T F-GC showed the highest bypass efficiency, while F-C and F-G demonstrated similar values. Pol I exhibited bypass efficiencies in the order F-G > F-C > F-GC. This order does not fit with the stacking tendency (Figure 3A), nor does it correlate with the level of sequence repetition which is similar in all three P/Ts.

Among the sequences with low thermal stability (sequences 1−6, Table 1), some correlation between bypass efficiencies and the stacking level appeared: As can be seen in Figure 3B, pol I showed the tendency F-T > F-AT > F-A, which is in reverse correlation to the stacking level. However, this correlation was not reproduced using substrates 1−3 (Figure 3C), whose order of efficiencies there was AT-F-AT > A-F-A > T-F-T. Whether a repetitive sequence is flanking the lesion or just positioned downstream to it seems therefore to have a different effect on the ability of pol I to bypass the lesion (see Discussion). For pol β, five of the six sequences with low thermal stability (sequences 2−6) exhibited overall low levels of bypass efficiencies (ranging from 7 to 13%; Figure 3 B,C and Table 1). One sequence, however (sequence 1, T-F-T), did show high bypass levels (46%, Table 1 and Figure 3C). According to

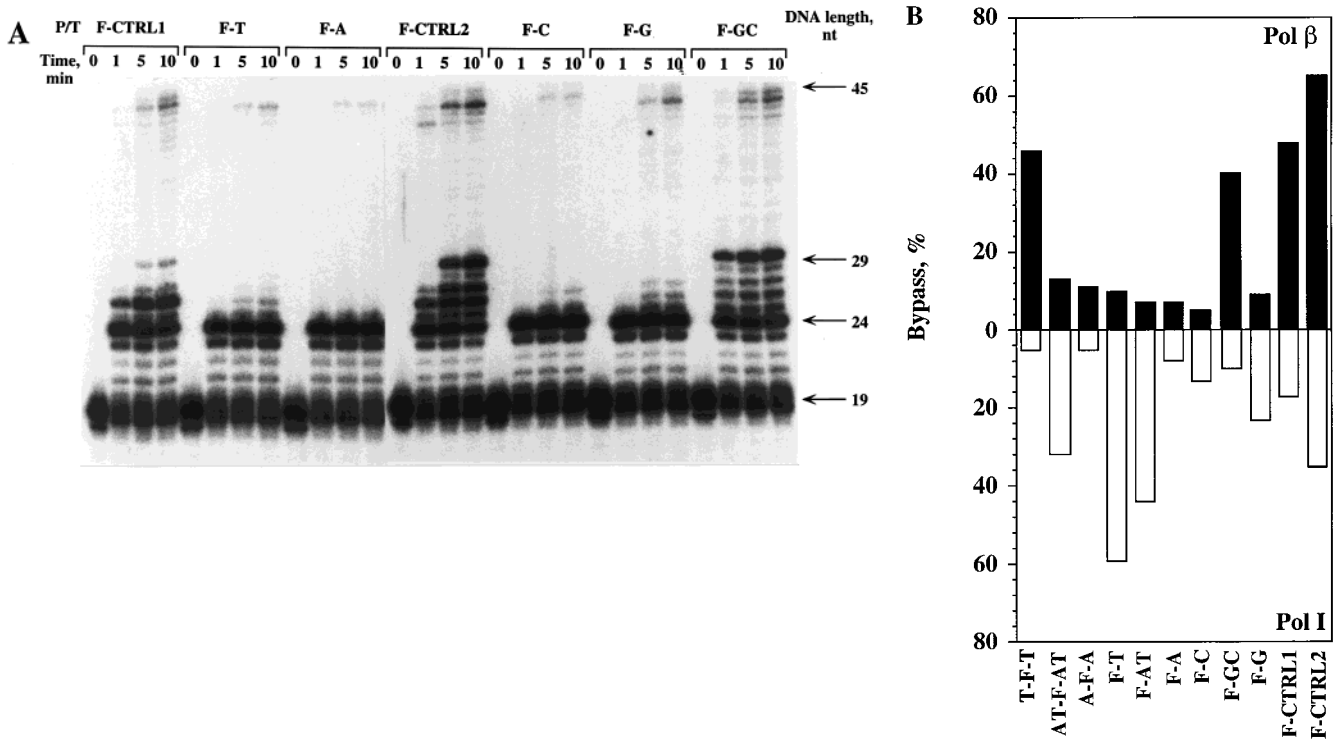

FIGURE 2: DNA polymerase β and DNA polymerase I produce distinct patterns of translesion replication products on the various primer templates. (A) Primer extension reactions initiated by 380 nM pol β were carried out for 1, 5, and 10 min, and were resolved by urea−PAGE. Shown are products of 7 of the 11 primer/templates (listed in Table 1) that were used as DNA substrates. Marked on the right side of the gel are the positions of the 19 nucleotide long primer, a 24 nucleotide long replication product (due to pausing at the nucleotide preceding the lesion), a 29 nucleotide long paused translesion product, and the full-length (45 nucleotides) translesion product. (B) The efficiencies of bypass by pol β were determined for the total translesion products produced after 10 min as shown in (A) (all products that are >24 nucleotides long divided by the total amount of primer extended, expressed in percent) and plotted for each of the 11 primer/templates used (marked at the bottom of each bar). A similar analysis was conducted for pol I, and the results are plotted below the pol β bars to demonstrate the different patterns.

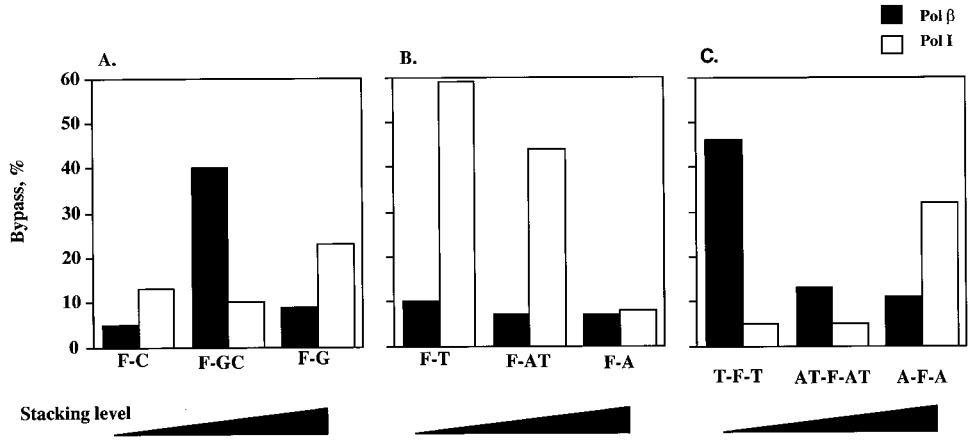

FIGURE 3: Correlating translesion replication by DNA polymerase β and DNA polymerase I to base stacking, thermal stability, and homopolymeric runs. The data are taken from Table 1 and Figure 2. (A) P/T with high thermal stability and increasing base stacking downstream of the lesion; (B) P/T with low thermal stability and increasing base stacking downstream of the lesion; (C) same as in (B), but extending both upstream and downstream of the lesion.

Table 1, this sequence exhibits the lowest stacking levels and the highest slippage tendency among our sequence collection.

The above analysis suggested that no simple rules can be formulated using our initial screen for sequence context effects on lesion bypass. However, this initial set had pointed out four sequences that demonstrated intriguing results: F-CTRL1, F-CTRL2, F-GC, and T-F-T all show high levels of total bypass (>40%). Pol β also produced replication pause sites after lesion bypass, when replicating on three of these four sequences (F-CTRL1, F-CTRL2, and F-GC; see Figure 2A), but not using T-F-T (not shown). Based merely on the rules that we have formulated (Table 1), it was difficult to find common features among these four sequences, which may explain the high bypass levels exhibited in those, and not other, sequences.

A clue to the factor(s) that may be common to the sequences for which pol β demonstrated high levels of bypass was revealed when the DNA substrates were analyzed by native PAGE. In the absence of Mg^{2+} in the gel, P/T

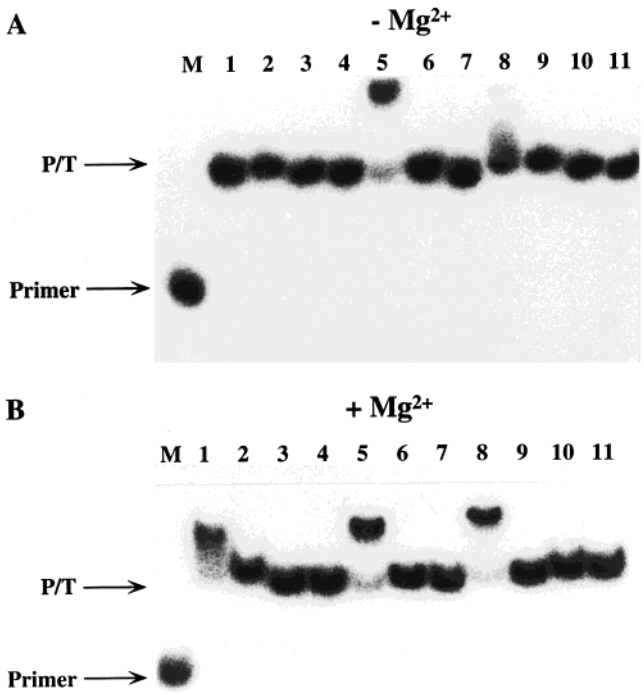

FIGURE 4: Abnormal migration of some of the primer/template substrates in native gels. 5′ end-labeled primer was annealed (Materials and Methods) to each of the 11 templates (Table 1) and resolved by PAGE, either in the absence (panel A) or in the presence (panel B) of 10 mM Mg^{2+} in the gel and running buffer (Materials and Methods). Lane M, free primer. Lanes 1−11, primer annealed to template F-CTRL1, F-T, F-A, F-AT, F-CTRL2, F-C, F-G, F-GC, T-F-T, A-F-A, and AT-F-AT, respectively. The position of migration of free primer and annealed primer/template is marked on the left side of the gel.

F-CTRL2 had a retarded migration in the gel (Figure 4A, lane 5). A small smeared retardation could also be observed for P/T F-GC (Figure 4A, lane 8). The addition of Mg^{2+} to the gel, to a similar concentration as found in our replication reactions, increased this effect such that three P/Ts now stood out in their retarded migration: F-CTRL1, F-CTRL2, and F-GC (Figure 4B, lanes 1, 5, and 8, respectively). These are three of the four sequences which showed the highest bypass levels by pol β, and for which replication pause sites were formed (Figure 2).

A close examination of the sequences downstream to the lesion within the P/Ts F-CTRL1, F-CTRL2, and F-GC revealed inadvertent inverted repeats (Figure 5). The retardation observed in the native gel could therefore be due to an inter- or an intramolecular interaction, as depicted graphically in Figure 5. The putative intramolecular structure seemed unlikely due to the lack of sequences that can form a complete hairpin and could therefore fail to form stable structures (Figure 5). To examine these possibilities experimentally, increasing amounts of unlabeled DNA substrates were added to the radiolabeled P/Ts, followed by resolution of the mixture by native PAGE (Figure 6A). In this approach, inter- and intramolecular interactions should give rise to different patterns: the addition of unlabeled DNA P/Ts is expected to change the migration of the labeled P/Ts only if an intermolecular (bimolecular) interaction is responsible for the retardation, but should have no effect if the interaction is intramolecular (unimolecular). We chose three sequences (F-T, F-CTRL1, and F-CTRL2) to test this hypothesis, representing P/Ts with no direct repeats, a 4 nt direct repeat, and a 6 nt direct repeat, respectively (Figure 5). Addition of unlabeled P/Ts to their respective labeled P/Ts, followed by a 1 h incubation at 37 °C, had no effect on the migration of F-T, but retarded the migration of F-CTRL1 and F-CTRL2 (Figure 6A). These results suggested that F-CTRL1, F-CTRL2, and F-GC P/Ts are found in solution as dimers, with a putative structure as depicted in Figure 5.

It seemed therefore likely that upon binding to these three P/Ts, a DNA polymerase encounters a fork-like (flap) structure (Figure 5). We reasoned that the stimulation of bypass by pol β observed for these three P/Ts was due to the effect that a flap structure, rather than their actual sequence, might have on pol β's ability to bypass the lesion. This model was tested by designing flap structures composed of three oligonucleotides, depicted schematically in Figure 6B. This oligonucleotide construct would create a fork-like structure for P/Ts such as F-T, for which no inadvertent inverted repeats exist. Our prediction was that such a flap structure would stimulate bypass at the F-T sequence, but should not affect bypass efficiencies at, e.g., P/T F-CTRL2, the latter existing in a flap structure even when P/Ts were used. As a control, we assayed also a gapped substrate, that was previously shown to have a stimulatory effect on pol β's synthesis (*5*) and bypass replication (*22*). A urea−PAGE analysis of translesion replication reactions using F-T P/Ts in which a flap was either present or absent showed that bypass by pol β on the F-T template was greatly stimulated by the flap structure (Figure 7A,B; compare P/T and $Flap^P$). In contrast, the control F-CTRL2 template was hardly affected by the predesigned flap (data not shown), as expected. This result is consistent with the formation of a flap structure by intermolecular annealing in the F-CTRL1, F-CTRL2, and F-GC P/Ts, without needing a third flap oligonucleotide. The gap structure had a comparable stimulatory effect on pol β as the flap in total bypass efficiency (Figure 7). However, displacement synthesis required for pol β to reach the end of the template was favored on the flap structure (Figure 7).

Gaps have been shown to stimulate synthesis by pol β primarily by the 5′-phosphate (*5*). Therefore, we tested the possibility that the stimulation by the flap was due merely to the phosphate at its 5′-end. This was done by using similar constructs as in Figure 7, except that the downstream oligonucleotides forming the gap and flap structures were not phosphorylated. As can be seen in Figure 8, bypass on the gapped substrate in the absence of the 5′-phosphate in the gap was 4−5-fold slower than in its presence. For example, bypass after 2 min in the presence of a 5′-phosphate was 64% (Figure 7), whereas in the absence of a 5′-phosphate was only 13% (Figure 8). In contrast, lesion bypass on the flap-containing substrate was hardly affected by the lack of a 5′-phosphate on the flap. For example, after 2 min of reaction, bypass reached 32% with the phosphorylated flap (Figure 7), and 34% with the nonphosphorylated flap (Figure 8). Thus, unlike gaps, fork-like flap structures stimulate translesion replication by pol β through the synthetic abasic site, regardless of the presence of a 5′-phosphate group on the flap.

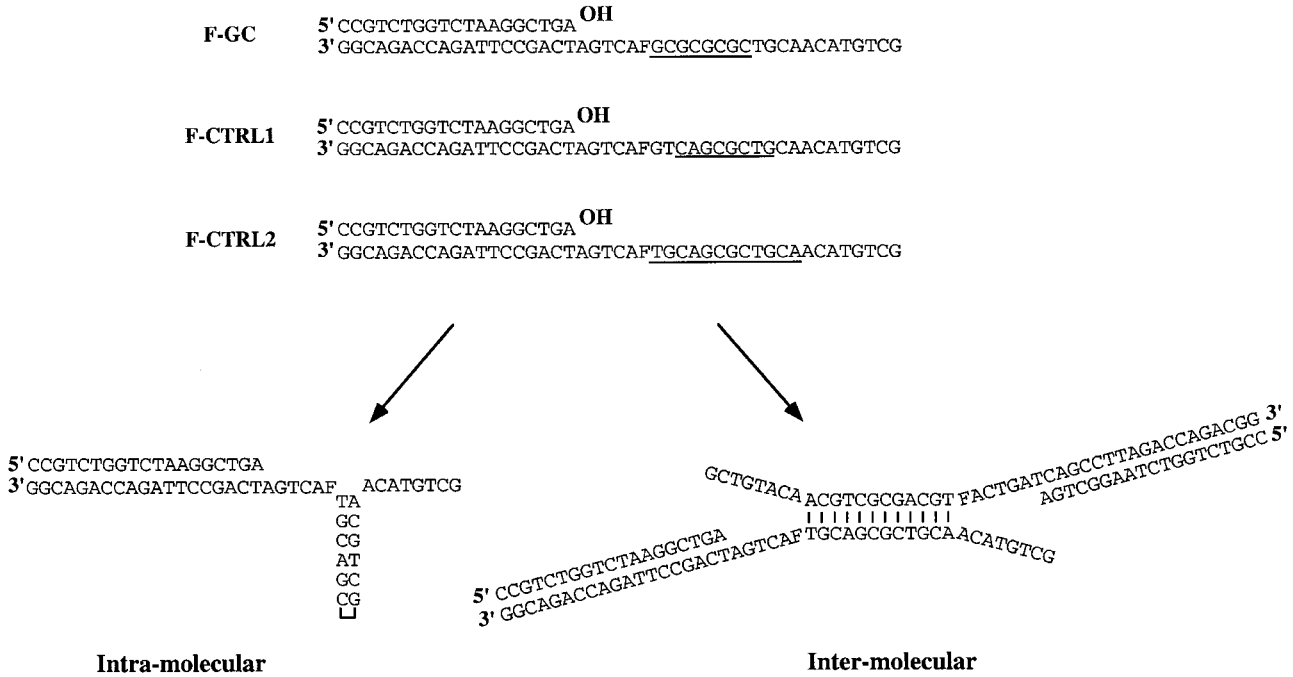

FIGURE 5: Putative secondary structures in the DNA substrates may be responsible for stimulation of translesion replication by pol β. The sequences of three P/Ts each containing an inverted repeat downstream of the lesion (underlined) are shown. F-GC, F-CTRL1, and F-CTRL2 contain 4 nucleotide, 4 nucleotide, and 6 nucleotide inverted repeats, respectively. Intramolecular interactions, forming a stem structure downstream of the lesion, are depicted for F-CTRL2. Also shown is a putative structure formed by intermolecular interactions between two F-CTRL2 molecules.

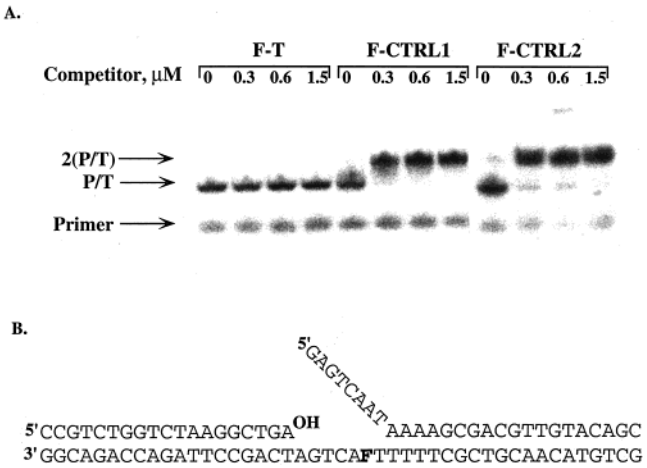

FIGURE 6: Primer/templates containing inverted repeats form intermolecular interactions. (A) Mixtures of increasing amounts of unlabeled F-T, F-CTRL1, and F-CTRL2 (competitor DNAs), added to their corresponding labeled P/T, were incubated at 37 °C (Materials and Methods), and resolved by native gels, containing Mg^{2+}. The concentration of the competitor DNA is marked at the top of each lane. The positions of free primer, P/T, and a P/T dimer are shown on the left side of the gel. (B) Detailed sequence of a DNA substrate composed of 3 oligonucleotides, designed to form a fork-like flap structure.

DISCUSSION

The existence of mutational hot-spots in the human genome suggests that some sequences are more prone to accumulate mutations than others. These sequences may be either damaged more frequently, repaired slower by error-free repair mechanisms, or processed faster by error-prone tolerance mechanisms such as translesion replication. Deciphering the underlying principles that lead to the formation of mutational hot-spots is crucial to our understanding of cancer formation and prevention. In this study we have focused on the effect that sequence variations have on translesion replication by pol β, by devising an initial screen for sequences that promote bypass. A different approach to search for sequences which promote bypass by T4 DNA polymerase was recently utilized by Hatahet et al. (*23*), who used a pool of substrates with randomized sequences in the vicinity of the lesion, combined with PCR amplification of bypass products. Further research, using these and other approaches, will help to formulate the rules governing sequence context effects of translesion replication.

Using our assay, we found two main effects of sequence variations on translesion replication: (1) subtle effects related to the stacking and slippage level of a particular sequence, for which clear and direct rules are difficult to formulate (although some commonalties already emerged from our study, see below); (2) a more apparent phenomenon, related to the tertiary structure of the DNA downstream of the lesion, that affects the ability of pol β to bypass the synthetic abasic site.

Base Stacking and Slippage Affect Bypass Replication. High levels of stacking interactions between adjacent bases in the template strand may interfere with bypass replication if the latter occurs by flip-out of the lesion out of the helical plane of the DNA. That is, a more ordered structure of the DNA immediately downstream of the lesion may inhibit such a flip-out and would therefore prevent bypass replication via a misalignment mechanism, in which the polymerase does not incorporate a nucleotide opposite the lesion, but rather opposite the nucleotide downstream of the lesion. This explanation is in accordance with the most prevalent mechanism accepted for pol β's mode of lesion bypass and formation of -1 deletions during large gap-filling (*11, 14*).

In addition, X-ray crystal structures of pol β−DNA complexes suggest that stacking interactions play an impor-

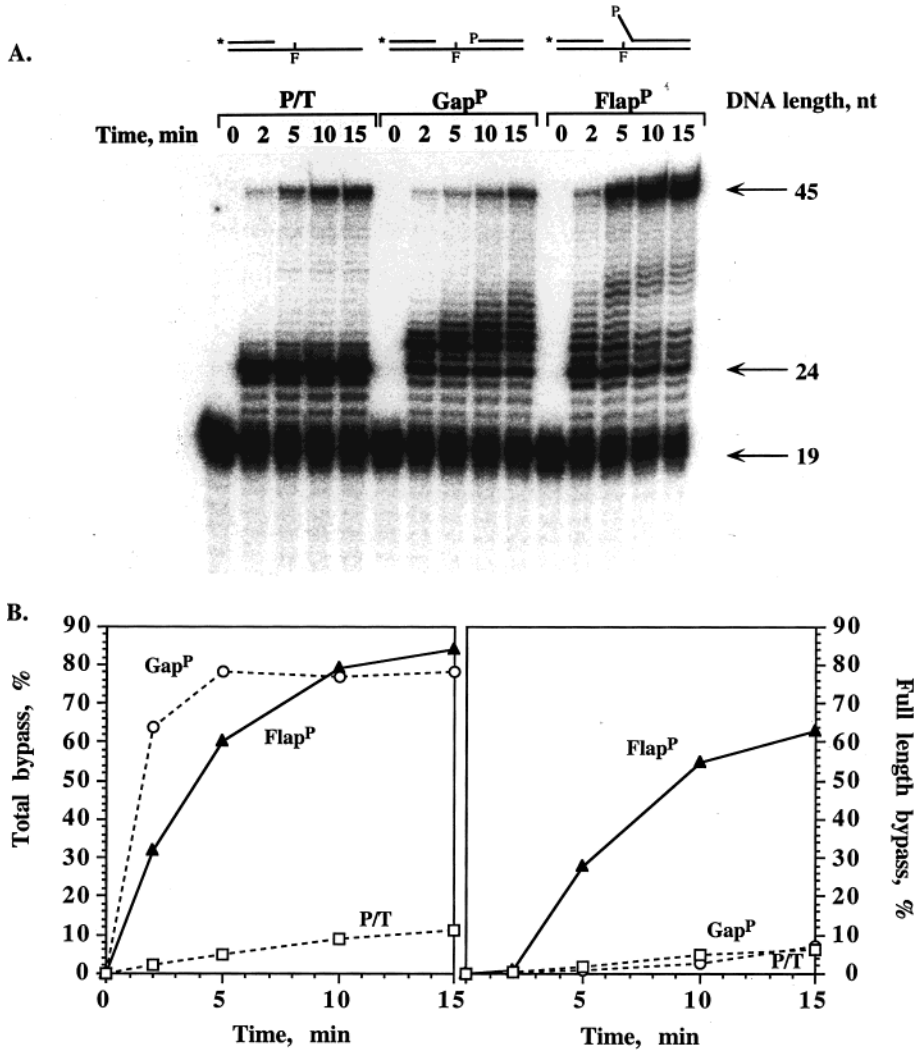

FIGURE 7: A predesigned flap structure stimulates translesion replication by pol β. (A) F-T DNA substrates were used in translesion replication reactions as either P/T, gapped, or flapped P/T. The structure of the flap substrate is as shown in Figure 6B. The gap substrate is similar to a flap except that all the noncomplementary bases in the downstream (flap) oligonucleotide were omitted. Reactions were initiated by the addition of 150 nM pol β and stopped after 2, 5, 10, and 15 min and resolved by urea−PAGE. The substrate used is marked and depicted schematically on top of the gel, P representing the 5′-phosphate of the downstream oligonucleotide and F representing the position of the tetrahydrofuran (abasic) lesion. The product lengths (in nucleotides) are marked on the right side of the gel. Gap^P and Flap^P represent gap and flap structures, respectively, with 5′-phosphorylated downstream oligonucleotide. (B) The left panel demonstrates total bypass efficiencies (total translesion products >24 nt divided by the total amount of primer extended) that were calculated for the reactions shown in (A) and plotted as a function of time. Results are shown for P/T substrates (open squares), Gap^P substrates (open circles), and Flap^P substrates (closed triangles). Full-length bypass efficiencies (right panel) were calculated by dividing the amount of products >40 nt long by the total amount of primer extended that had reached position 24. Gap^P and Flap^P represent gap and flap structures, respectively, with 5′-phosphorylated downstream oligonucleotide.

tant role in the mechanism of replication by pol β (*24*). The picture emerging is that the template base immediately 3′ to the template base which is in the active site stacks with a histidine residue in the 8 kDa domain of pol β, rather than with its neighboring base. It therefore seems that this protein−DNA stacking interaction would be favored when the template bases have low stacking energies.

Despite the observed low bypass levels common to the sequences containing homopolymeric runs (Table 1, sequences 1−9, and Figure 2), two sequences from this group did show significant bypass efficiencies by pol β: T-F-T and F-GC. We believe that the effect of the latter sequence is attributed to higher order structures, as discussed below. The T-F-T sequence seems to mimic the common T-run hotspots formed during gap-filling synthesis by pol β (*10*). That is, it seems that the dominant sequence effect for this P/T may be attributed to slippage at homopolymeric runs, at a low stacking environment. In addition, it seems that the F-T P/T, unlike T-F-T, does not provide the sufficient structural requirement to promote slippage. Slippage seems therefore to occur when the homopolymeric run flanks the lesion. The significant difference between bypass efficiencies at the T-F-T and the A-F-A sequence may be explained by the difference in stacking levels, but can also stem from different bending angles of A:T as opposed to T:A stretches. In conclusion, it seems that a low stacking level or a high slippage potential is not sufficient, when they exist separately, to produce bypass stimulation, and they are only effective when combined.

E. coli pol I exhibited a slightly more significant correlation between stacking levels and bypass efficiencies than pol β, in particular for P/Ts F-T, F-AT, and F-A. However, it is possible that the high bypass efficiencies seen for F-T, and perhaps even for F-AT, may be attributed not just to low

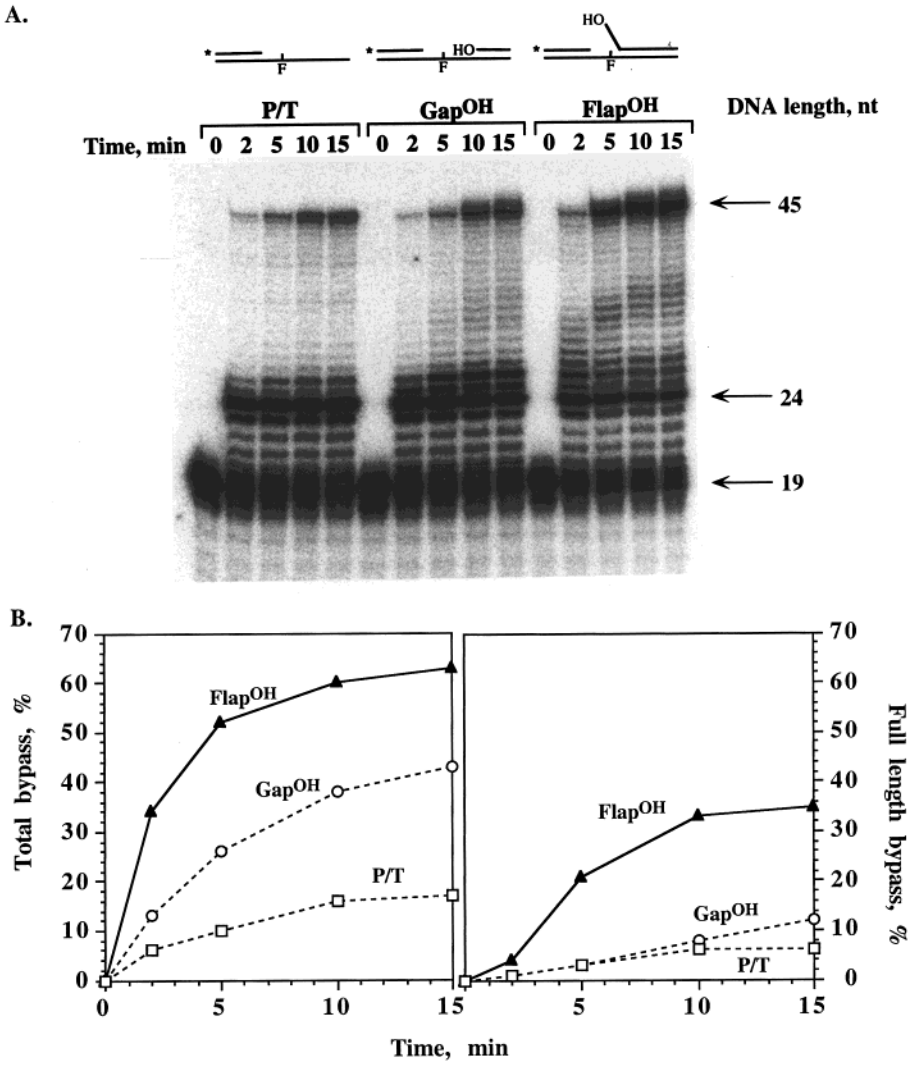

FIGURE 8: Stimulation by a flap structure is independent of the presence of a 5′-phosphate. Reactions were performed (A) and analyzed (B) as in Figure 7, except that the downstream oligonucleotide within the gap and flap substrates was not phosphorylated (Gap^{OH} and $Flap^{OH}$, respectively).

stacking, but also to the tendency of this polymerase to insert an A opposite abasic sites (*18*, *25*, *26*). That is, the template T, positioned 1 or 2 nt downstream of the lesion, may be stabilized by the incoming A nucleotide, and therefore promote bypass. P/T F-CTRL2, which also contains a template T immediately downstream of the lesion, does fit this hypothesis. The fact that the P/T T-F-T demonstrated low bypass efficiencies does not support that notion, and is therefore puzzling. However, it is possible that the inhibition of bypass seen on P/T T-F-T compared to F-T may be related to effects of DNA bending, rather than merely to the factors that we were initially considering.

Fork-like DNA Structures Stimulate Bypass Replication by Pol β. Our results suggested that a putative fork-like structure, formed in some P/Ts, stimulated bypass replication by pol β. This hypothesis was supported by using DNA substrates with a predesigned flap (Figures 7 and 8). The flap-containing substrate indeed demonstrated significantly higher bypass levels than a substrate lacking a flap but sharing the same sequence (Figures 7 and 8). The basis for the stimulatory effect of the flap on lesion bypass is not clear. It is possible that the fork-like structure confers a certain structure upon the template DNA similar to that found in the X-ray crystal structure of pol β−P/T complexes (*27*). In this detailed structure, the template strand was found to be bent at a 90° angle at the P/T junction. In addition to bypass stimulation, the flap structure seemed to create a difficulty to pol β in replicating the sequences downstream of the lesion, as seen by the appearance of pause sites (short bypass products; Figures 2 and 3). This difficulty was probably a manifestation of the inefficient displacement synthesis performed by pol β, especially in G:C-rich regions (F-CTRL1, F-CTRL2, F-GC). The amount of pausing in the predesigned flap of substrate F-T was only minor. In addition, displacement synthesis in flap structures was much better than in gap structures (Figures 7 and 8).

It was previously reported that bypass replication of cisplatin and *N*-2-acetylaminofluorene adducts, promoted by a crude cell extract, occurred on fork-like DNA templates, but not on single-stranded DNA templates (*28*). However, subsequent experiments performed by the same researchers indicated that at least part of the apparent bypass DNA products were not formed by translesion replication as originally thought (*29*). Thus, the results of the present study provide the first clear example that higher order fork-like flap structures stimulate translesion replication. In addition, our results (Figures 7 and 8) suggest that the phosphate at the 5′ flap does not contribute to bypass stimulation,

strengthening our conclusion that the structure sensed by pol β, when approaching a flap, is different than a gap.

Mammalian cells contain a multiplicity of DNA polymerases, including some which are specialized for lesion bypass, i.e., DNA polymerases ζ (*30*−*33*) and η (*34*, *35*). However, it was recently reported that expression of a mutant pol β in mice cells caused a mutator phenotype (*36*). This suggests that despite the multiplicity of DNA polymerases in mammalian cells, pol β can affect their overall mutability. Pol β might be participating in lesion bypass in vivo at regions where it is engaged in repair reactions, and therefore its local concentration is high. Specifically, repair of a cross-linked lesion such as formed by bleomycin (*37*) is expected to involve bypass replication, since filling in the gap left after excision of one side of the lesion invokes bypass of the lesion that is left on the opposing strand.

The finding that fork-like structures stimulate bypass may indicate that pol β participates in processes in which a fork-like structure serves as an intermediate. It is possible that these structures mimic intermediates formed during short and long patch BER (*38*). Indeed, it was recently reported that pol β is required not only for short patch BER but also for long patch BER (*39*, *40*). Some DNA damaging agents, such as γ radiation, produce clusters of lesions in the DNA (*41*). In such regions with a high density of lesions, it is possible to envision how a fork structure, formed during repair of one lesion, encounters a downstream lesion positioned on the opposing strand. In this context, our results suggest that pol β bypasses lesions efficiently. Further analysis of the effect of flap structure on bypass replication by pol β, as well as its effect on replication of undamaged templates, is required in order to elucidate the mechanism of bypass stimulation, and perhaps reveal new processes in which pol β participates in.

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