Translesion Replication by DNA Polymerase δ Depends on Processivity Accessory Proteins and Differs in Specificity from DNA Polymerase β†

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ABSTRACT: Mutations caused by DNA damage lead to the development of cancer. The critical step in the formation of these mutations is the replication of unrepaired lesions in DNA by DNA polymerases, a process termed translesion replication. Using a newly developed method for preparation of gapped plasmids, containing a site-specific synthetic abasic site, we analyzed translesion replication with purified mammalian DNA polymerases δ and β. DNA polymerase δ was found to be unable to replicate through the abasic site. Addition of the sliding DNA clamp PCNA, the clamp loader RFC, and ATP caused a drastic 30-fold increase in translesion replication. Thus, similar to *Escherichia coli* DNA polymerase III, the processivity accessory proteins enable DNA polymerase δ to bypass blocking lesions. Under comparable conditions, DNA polymerase β was unable to bypass the abasic site, unless its concentration was greatly increased. Analysis of translesion replication products revealed a marked difference in the specificity of bypass: whereas 90% of bypass events by DNA polymerase δ holoenzyme involved insertion of a dAMP residue opposite the abasic site, DNA polymerase β tended to skip over the abasic site, producing mainly minus frameshifts (73%). The significance of these results for in vivo translesion replication is discussed.

A mammalian cell sustains an enormous amount of damage to its DNA every day. Several repair pathways exist that efficiently remove most of the damaged bases (*1*). Damage that has escaped the repair machinery may give rise to mutations, leading to cancer. The formation of a mutation at a lesion occurs during replication of the damaged site, a process termed translesion or bypass replication (*1*−*3*). Because most DNA lesions are miscoding, this process is inherently mutagenic. The remaining lesion in the template strand can be repaired in subsequent cycles of repair.

Three classes of DNA polymerases may be involved in mammalian translesion replication: (1) replicative DNA polymerases, during the encounter of the replication forks with an unrepaired lesion; (2) repair DNA polymerases, such as DNA polymerase β; (3) the newly emerging class of specialized DNA polymerases, whose function is to perform translesion replication. Two DNA polymerases, belonging to this family, were recently identified in mammalian cells, DNA polymerases ζ and η. In *Saccharomyes cerevisiae*, DNA polymerase ζ is responsible for error-prone translesion replication (*4*), and DNA polymerase η bypasses lesions very effectively in an error-free manner, at least for some lesions (*5*). A homologue of DNA polymerase ζ was found in human cells (*6*−*8*), and human DNA polymerase η was recently found to be encoded by the *XP*−*V* gene, which is mutated in the disease Xeroderma Pigmentosum Variant (*9*, *10*). More than one DNA polymerase type may participate in translesion replication, possibly depending on the cell cycle: replicative polymerases may do it in the S phase, whereas specialized DNA polymerases may do it at specific check points. Therefore, it is important to understand the bypass properties of the different DNA polymerases.

The current data is not sufficient to draw precise rules regarding the mechanism of mammalian translesion replication. However, it is clear from studies with purified DNA polymerases that the extent and specificity of bypass are greatly affected by the type of DNA damage and by the type of DNA polymerase (e.g., (*11*−*19*). Several studies have analyzed translesion replication during replication in crude extracts, clearly demonstrating that DNA lesions, even of the bulky type, are bypassed during SV40-dependent replication (*20*−*23*). Here, we concentrated on translesion replication by purified DNA polymerase δ, the major replicative mammalian DNA polymerase. In particular, we examined the role of processivity accessory proteins in translesion replication by human DNA polymerase δ and established the specificity of bypass by human pol δ[1] holoenzyme and rat pol β.

MATERIALS AND METHODS

Materials. Deoxynucleotides, DTT, BSA, and proteinase K were from Boehringer Mannheim; [γ-^{32}P] ATP from NEN; NA45 DEAE membranes, Schleicher & Schull; dSpacer CE phosphoramidate, a synthetic abasic site building block, was from Glen Research, Sterlin, VA. The Synthesis Unit of the Biological Services Department in our Institute synthesized all oligonucleotides.

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[1] Abbreviations: nt, nucleotide; PCNA, proliferating cell nuclear antigen; Pol β, DNA polymerase β; Pol δ, DNA polymerase δ; Pol I, DNA polymerase I; RFC, replication factor C; RPA, replication protein A.

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Proteins. Human pol δ purified from HeLa cells, recombinant human PCNA, purified from *Escherichia coli* and human RFC were a generous gift of M. O'Donnell (The Rockefeller University, New York). Recombinant rat pol β, expressed in *E. coli*, was a generous gift of S. Wilson (NIEHS, Research Triangle Park). *E. coli* pol I was from Boehringer Mannheim. Restriction nucleases, T4 DNA ligase, and T4 polynucleotide kinase were from New England Biolabs. S1 nuclease was from Promega, and T7 gp6 exonuclease was from Amersham.

Gapped Plasmids. Preparation of gapped plasmids carrying a site-specific abasic analogue was essentially as described (*24*, *25*). Briefly, a template oligonucleotide, containing the abasic analogue, was annealed to an upstream ^{32}P-5′-labeled primer and a downstream primer, forming a gapped insert. This construct was ligated to plasmid pSKSL, a derivative of pBluescript II SK(+) (Stratagene), cleaved with restriction enzymes *Bst*XI and *Bsa*I. The synthetic insert had complementary termini to those of the cleaved plasmid. The desired gapped plasmid containing a 22 nt gap were gel purified (*25*). Enlargement of the gap was achieved by incubating the gapped plasmid with T7 gp6 exonuclease, followed by heat inactivation, phenol/chloroform extraction, and ethanol precipitation (*24*).

Translesion Replication Assay. A typical translesion replication reaction (10 μL) contained 100 ng (2 nM) gapped plasmid; 20 mM Tris·HCl, pH 7.5; 5 mM DTT; 0.1 mM EDTA; 8 μg/mL BSA; 4% glycerol, 10 mM $MgCl_2$, and 500 μM each of dATP, dGTP, dCTP, and dTTP. ATP, when present, was at a final concentration of 1 mM. The reaction mixtures were preincubated at 37 °C for 3−5 min. Reactions were initiated by the addition of either pol δ (30 nM), pol β (as stated in the text), or pol I (30 nM). Addition of the processivity subunits was accomplished by adding PCNA (600 nM) to the preincubated gapped plasmid. After a 2 min incubation, RFC (200 nM) was added, the reaction mixture was further incubated for 2 min and the DNA polymerase was than added. Reactions were terminated after 10 min by the addition of an equal volume of a solution of 0.4% SDS, 20 mM EDTA, and heat inactivated for 10 min at 80 °C. The DNA was purified by proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation, after which it was digested with restriction nucleases *Xmn*I and *Bst*XI, and fractionated by urea-PAGE as previously described (*26*). Visualization, and quantification were achieved using a Fuji BAS 1000 phosphorimager. The extent of bypass efficiency was calculated by dividing the amount of bypass products (longer than 28 nt) by the amount of all extended primers.

DNA Sequence Analysis of Translesion Replication Products. The sequence analysis was performed essentially as described (*26*) (see Results for outline).

RESULTS

The Experimental System. DNA polymerase δ is the major replicative polymerase in mammals (*27*−*29*), and as such, it is expected to encounter unrepaired lesions during the progression of the replication fork and synthesis of the leading and lagging DNA strands. The high processivity of DNA polymerase δ is acquired via binding to PCNA, an accessory protein that serves as a sliding DNA clamp that tethers the polymerase to the DNA (*30*−*32*). PCNA is a homotrimer, which forms a ring structure that encircles dsDNA (*33*, *34*). It is loaded on the DNA by the 5-subunit RFC clamp loader, after which it can freely slide on DNA (*35*−*37*). We sought to analyze the ability of pol δ to replicate through DNA lesions, and in particular, to establish the effect of its accessory proteins on bypass.

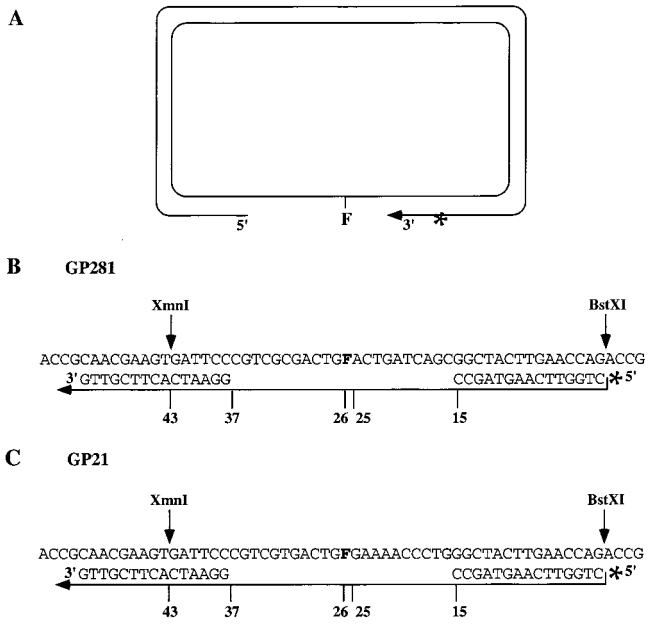

FIGURE 1: The gapped plasmid substrates. (A.) Depicted is the double-stranded plasmid, containing a single-stranded gap. The position of the abasic site analogue, tetrahydrofuran, within the gap is marked (F). The asterisk marks the internal radiolabel. The initial gap (22 nt) can be enlarged in the 5′→3′ direction (see Materials and Methods). (B.) Sequence of the gap region of gapped plasmid GP281. The location of digestion by the restriction enzymes *Bst*XI and *Xmn*I (which release the radiolabeled replication products) is marked. "F" represents the position of the lesion. Numbers below the sequence represent the length of the primer (15 nts), and common reaction products (25, 26, 37, 38, and 43 nts). (C.) Sequence of the gap region of gapped plasmid GP21. The direction of replication is marked by arrows.

We utilized a newly developed assay system for translesion replication, which is based on a gapped plasmid carrying a site-specific lesion in the single-stranded region (*24*, *25*). This long and circular DNA substrate is suitable for assaying the effects of PCNA and RFC on translesion replication, since the loading of PCNA on this circular DNA depends on RFC and requires ATP hydrolysis, much like is believed to occur under in vivo conditions. The lesion used is a synthetic abasic site (tetrahydrofuran; (*16*)), representing one of the most common lesions in DNA. Abasic sites are highly mutagenic and impose severe blocks to DNA polymerases (*15*−*17*, *19*, *38*−*43)*. The gap/lesion plasmid is shown schematically in Figure 1A. The 3′-OH primer terminus is located 11 nucleotides upstream to the abasic site and constructs with either a small gap (22 nucleotides) or a large gap (350 nucleotides) were used. In addition, an internal radiolabeled phosphate is located 15 nucleotides upstream to the 3′-OH terminus (Figure 1). Two gap/lesion plasmids were used, which differed only in the DNA sequence in the vicinity of the lesion (Figure 1B, C). Addition of a purified DNA polymerase to the gapped plasmid under replication conditions led to the extension of the 3′-OH primer terminus up to the lesion. Bypass through the lesion was monitored by

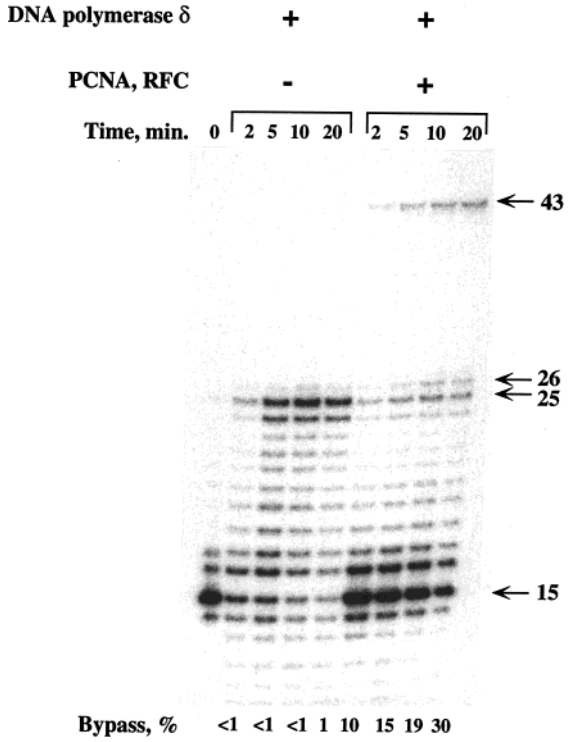

FIGURE 2: Translesion replication by DNA polymerase δ depends on PCNA, RFC, and ATP. Substrate GP281 with a large gap (350 nt) was used for replication by pol δ (30 nM) in the absence and presence of PCNA (600 nM), RFC (200 nM), and ATP (1 mM). Replication products were restricted and resolved by urea-PAGE. The length of the unextended replication primer (15 nt), and of a replication product that has passed the lesion and reached the *Xmn*I site (43 nt) is marked on the right side of the gel. The positions of replication products that had reached one nt preceding the lesion (25 nt) and across from the lesion (26 nt) are also marked. Bypass efficiencies (calculated as described in Materials and Methods) are at the bottom of each lane.

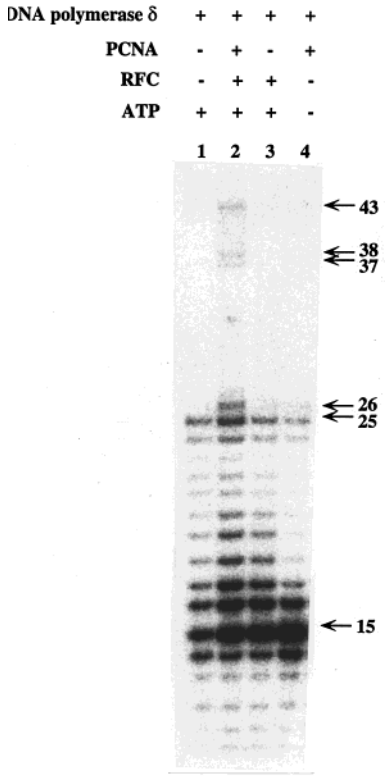

FIGURE 3: Replication reactions by pol δ and different combinations of the replication accessory machinery. Reaction were performed for 10 min, as described in the legend to Figure 2, except that the concentration of dNTPs was 100 μM, and GP281 with a short gap (22 nt) was used as substrate. The lower dNTPs concentration, which has no effect on the pattern of translesion replication, was used here to diminish the hydrolysis of dNTPs by RFC as an energy source instead of ATP. The numbers on the right side of the gel mark the position of the 15 nts long primer, the 25 and 26 nts long pausing products at the lesion, the 37 nts long pausing products at the end of the gap (see Figure 1), and long products beyond the gap cleaved by *Xmn*I (43 nts long).

following the extension of the nascent DNA strand beyond the abasic site. The replication products were cut with restriction nucleases *Bst*XI and *Xmn*I (Figure 1), the fragments were separated by urea-PAGE and visualized and quantified by phosphorimaging.

Bypass by pol δ Depends on PCNA, RFC, and ATP. Addition of human pol δ to a gap/lesion plasmid (GP281, Figure 1B) with a large ssDNA gap in the presence of Mg^{2+} and dNTPs promoted replication of the single-stranded region, as indicated by the extension of the radiolabeled 15-mer oligonucleotide, representing the unextended primer. As can be seen in Figure 2, replication was strongly inhibited at the site of the lesion, with the major termination site being at the nucleotide preceding the lesion (25-mer). Thus, under our reaction conditions, pol δ was unable to bypass the abasic site. Upon addition of PCNA, RFC, and ATP, a dramatic stimulation of up to 30-fold of bypass by pol δ was observed. This is manifested by the accumulation of the 43 nt long replication product (Figure 2). The 43 nts long product is the result of cleavage of much longer replication products by the restriction enzyme *Xmn*I (Materials and Methods, and Figure 1B). The efficiency of bypass by pol δ in the presence of PCNA and RFC reached 30% after 20 min (Figure 2). Notice that at shorter times (2 and 5 min), bypass was observed, while most of the primer was yet unextended. This suggests that lesion bypass by pol δ−PCNA was processive, i.e., occurred without dissociation at the lesion, since dissociation would have favored binding to and extension of the unextended primers, which are present in excess over the primers located at the lesion. A similar bypass reaction was observed when a substrate with a small gap of 22 nucleotides was used (Figures 3 and 4). The only difference between replication on the small (22 nt) and the enlarged (~350 nt) gap was the appearance of extra product bands in the former (Figure 3, lane 2, & Figure 4, lane 6). The length of these products (37 and 38 nts; Figure 3, lane 2) suggest that they are the result of replication pause sites caused by the end of the gap. That is, in the absence of a DNA helicase, the pol δ−PCNA complex failed to displace the nontemplate strand efficiently, and only a small fraction of replication complexes was able to extend the primer beyond the gap (as indicated by the presence of 43 nts long products (Figure 3; see also Figure 4, lane 6).

Omission of PCNA (Figure 3, lane 3) abolished any bypass replication, suggesting that PCNA is crucial for this reaction and consistent with the suggested processive mechanism of bypass. Omission of RFC and ATP (Figure 3, lane 4) or PCNA (Figure 3, lane 1), resulted in no detectable bypass. Taken together, these results demonstrate the total dependence of pol δ on PCNA, RFC, and ATP in order to perform translesion replication. Therefore, a properly assembled replication complex had most likely been formed on the

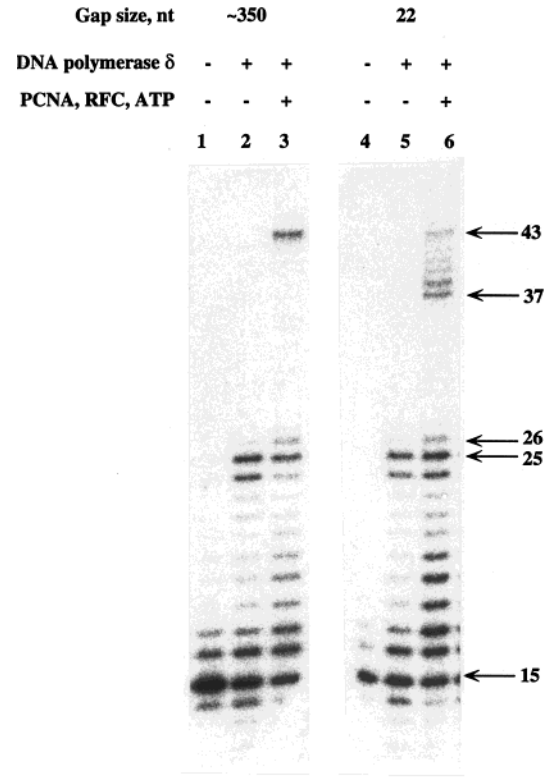

FIGURE 4: The gap size does not affect translesion replication by DNA polymerase δ holoenzyme. Reactions were performed and analyzed as described in the legend to Figure 2, with gapped plasmid GP281 preparations, which had a gap of either 22 nts, or approximately 350 nts. The numbers on the right side of the gel mark the position of the replication products, as detailed in Figure 3.

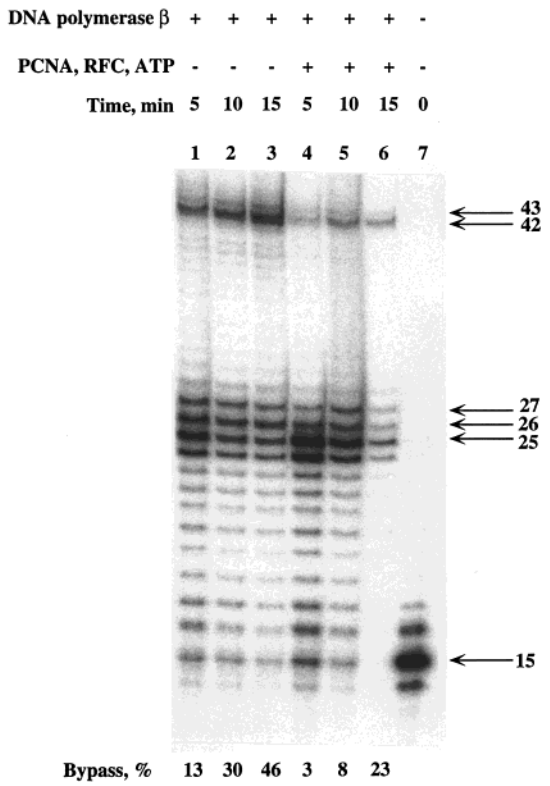

FIGURE 5: Translesion replication by DNA polymerase β is independent of PCNA and RFC. A. Replication with 485 nM pol β using GP281 with an enlarged gap, was performed in the absence (lanes 1−3) or presence (lanes 4−6) of PCNA (600 nM), RFC (200 nM), and ATP (1 mM). Reactions were stopped after 10 min and the products were resolved by urea-PAGE. Bypass efficiencies (calculated as in Materials and Methods) are at the bottom of each lane, while the length of replication products (in nt) is marked on the right side of the gel.

gapped plasmid substrate and was responsible for the ability of the polymerase to bypass the lesion.

It was previously shown that bypass of an abasic site by *E. coli* DNA polymerase III holoenzyme was more efficient in a short 22 nt gap, as compared to a long 350 nt gap (*24*). A similar examination of bypass by pol δ−PCNA showed that the size of the gap had no effect on bypass by pol δ in the presence or absence of PCNA and RFC (Figure 4). The addition of human RPA, the mammalian single-stranded binding protein, had no effect on either replication or lesion bypass in our model system (data not shown).

Pol β is Less Proficient in Bypass Than pol δ, and Its Bypass is Independent of PCNA, RFC, and ATP. To confirm that PCNA and RFC were stimulating bypass by pol δ through specific interactions with this polymerase, we have tested the effect of these processivity factors on translesion replication by rat pol β. Using the same gapped substrate plasmid, pol β was able to bypass the abasic analogue unassisted (Figure 5, lanes 1−3). Addition of PCNA, RFC, and ATP to a pol β replication reaction showed no stimulation (Figure 5, lanes 4−6). In addition, some inhibition of these proteins on pol β's bypass was detected (Figure 5, lanes 4−6). As can be seen in Figure 6, high percents of bypass were obtained with pol β only when it was present at high concentrations (>190 nM, Figure 6, lane 5). Even at pol β concentrations three times higher than that used with pol δ (30 nM), in the presence of PCNA and RFC, very low bypass with pol β was observed (Figure 6, lane 4).

Pol δ and pol β Each have a Distinct Bypass Specificity. A striking difference in translesion replication products between pol δ and pol β can be seen in Figure 7A. Both polymerases synthesized the same length of products from undamaged templates (Figure 7A, lanes 2 and 5). However, while pol δ−PCNA produced the same lengths of products independent of the presence of a damage in the template strand (Figure 7A, compare lanes 1 and 2), pol β synthesized a product shorter by one nucleotide than the full length, when replicating through the synthetic abasic site (Figure 7A, compare lanes 3 and 4 to lane 5). These results suggested that pol δ−PCNA incorporated a nucleotide opposite the synthetic abasic site producing a base substitution mutation, while pol β skipped over the abasic analogue, producing a −1 deletion.

To directly test the mutational spectrum produced by pol δ−PCNA and pol β at the site of the lesion, we utilized a methodology previously used in this laboratory for the analysis of mutational spectra generated by DNA polymerase III holoenzyme and SOS proteins (*24*, *26*). Briefly, the products of the translesion replication reactions were amplified by PCR, cloned into a vector, and the vector was propagated in *E. coli* cells. Individual clones were picked and subjected to DNA sequence analysis. In addition to applying this analysis to reactions using GP281 (Figure 1B), we have also analyzed the mutation spectrum produced using a different template, GP21 (Figure 1C). As can be seen in Figure 7B, pol δ−PCNA and pol β produced on substrate GP21 dramatically different bands from each other, similar to the patterns obtained with gapped plasmid GP281 used

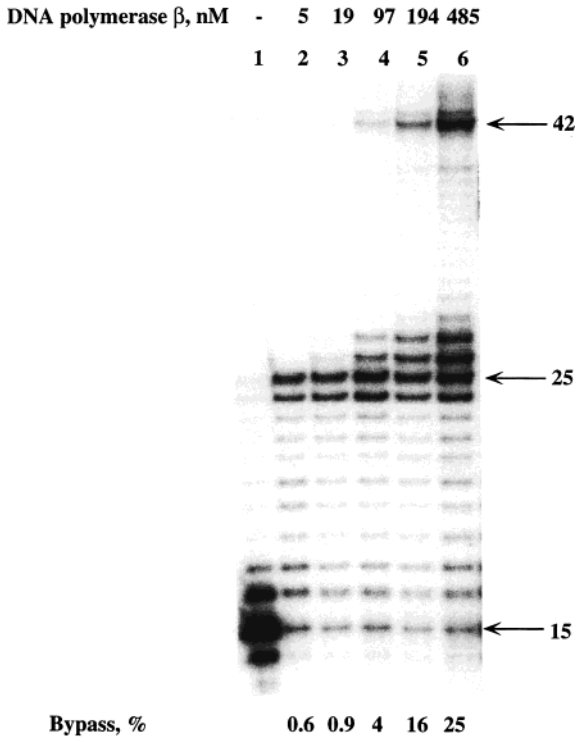

FIGURE 6: Effect of increasing amounts of DNA polymerase β on translesion replication. Reactions were performed and analyzed as described in the legend to Figure 5, but in the absence of PCNA and RFC, and with the indicated concentrations of pol β.

in Figures 2−7A. Therefore, the difference between the polymerases is not specific to a particular DNA sequence context. *E. coli* DNA polymerase I was used here as a reference polymerase. Clearly, *E. coli* pol I produced bypass products with base substitution mutations. In addition, pol I created what seemed to be −2 deletions (Figure 7B).

DNA sequence analysis of translesion replication products from both GP21 and GP281 is shown in Table 1. All the mutations detected were targeted to the site of the lesion. DNA polymerase I showed a strong preference for inserting a dAMP residue opposite the abasic site (20/21; 95% for GP281, and 17/23; 74% for GP21). This fits nicely with the known bypass specificity of pol I (*15*, *38*, *43*), and with the "A rule" (*44*). In addition, 5 out of 23 mutations (22%) obtained for the GP21 template (Table 1), contained a −2 deletion opposite the abasic site, in accordance with the gel analysis. For pol β, 15 out of 23 (65%) and 18 out of 25 (72%) of the GP281 and GP21 sequences, respectively, were −1 deletions opposite the abasic site, while the rest were A and G base substitutions. In striking contrast to pol β, pol δ−PCNA produced mostly base substitutions, comprised entirely of A insertions opposite the synthetic abasic site: 31/32 (97%) with GP21, and 15/19 (79%) with GP281 (Table 1).

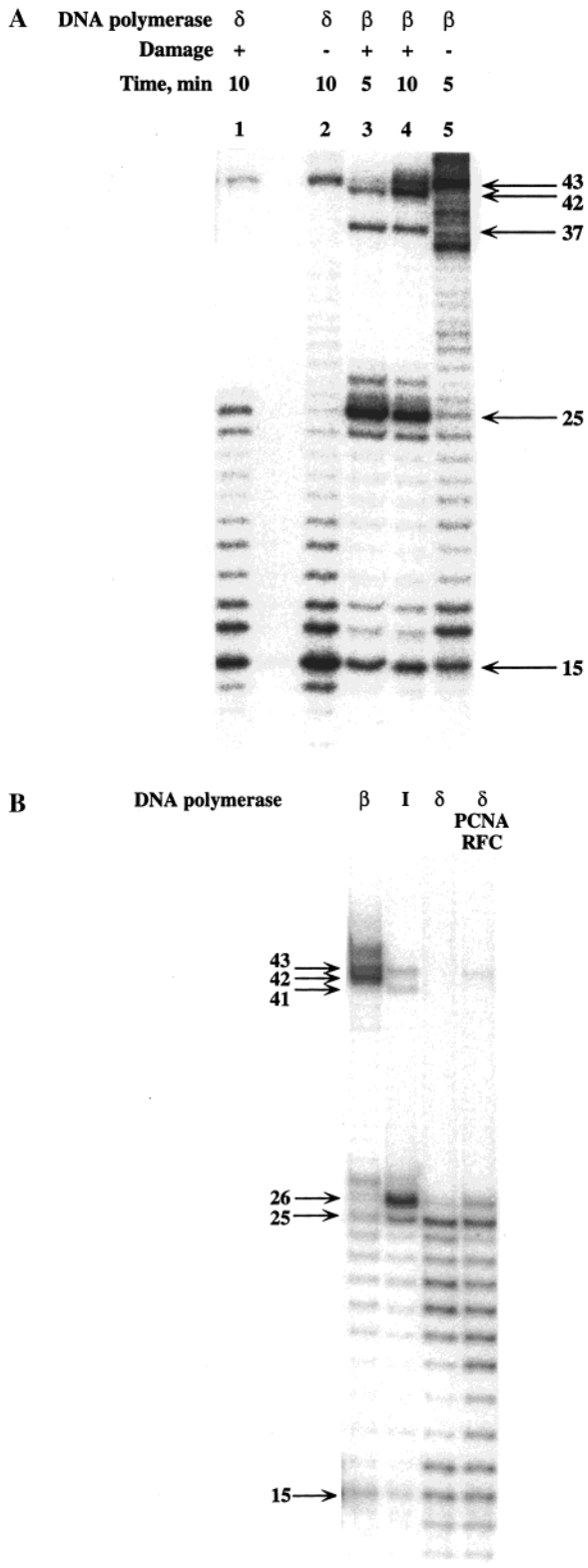

FIGURE 7: DNA polymerase β and δ exhibit different bypass specificities. A. Comparison of replication products by pol δ and pol β using GP281 with or without the lesion. Restricted reaction products were resolved by urea-PAGE. Reaction times are indicated on top of the gel. B. Translesion replication reactions by either pol β (485 nM), pol δ (30 nM), or *E. coli* pol I (30 nM) were carried out for 10 min and were used for sequence analysis (Materials and Methods and Table 1), were resolved by urea-PAGE. The gapped plasmid used was GP21. Size of products (in nt) is on the side of the gels.

DISCUSSION

The recent development of a system that produces large amounts of gapped plasmid containing a uniquely positioned lesion within a gap whose size can be varied (*24*, *25*), provided us with the opportunity to assay the properties of translesion replication by the mammalian replicative pol δ holoenzyme. Our results show that translesion replication by pol δ depended on the presence of PCNA, RFC, and ATP.

Table 1: Specificity of Mutations Generated During in Vitro Translesion Replication

	DNA polymerase		
mutation type	Pol β	Pol I	Pol δ, PCNA & RFC
substrate GP281			
−1	**15 (65%)**		3 (16%)
−2	1 (4%)	1 (5%)	
A	6 (26%)	**20 (95%)**	**15 (79%)**
other	1 (4%)		1 (5%)
substrate GP21			
−1	**18 (72%)**		1 (3%)
−2		5 (22%)	
A	4 (16%)	**17 (74%)**	**31 (97%)**
G	3 (12%)	1 (4%)	
total			
−1	**33/48 (69%)**		4/51 (8%)
−2	1/48 (2%)	6/44 (14%)	
A	10/48 (21%)	**37/44 (84%)**	**46/51 (90%)**
G	3/48 (6%)	1/44 (2%)	
other	1/48 (2%)		1/51 (2%)

This suggests that the proper assembly on DNA of the processive form of pol δ, complexed with PCNA is crucial for translesion replication. The results presented are in partial agreement with a previous analysis with oligonucleotide substrates, showing that PCNA alone stimulated bypass by pol δ through an abasic site (*45*) and a thymine photodimer (*46*). The lack of requirement for RFC and ATP in both previous studies may stem from the use of short oligos, where PCNA can slide on by diffusion (*47*). Previous results from our laboratory showed that bypass of a synthetic abasic site by the *E. coli* DNA polymerase III depended on its β subunit processivity sliding clamp (*24*). PCNA and the β subunit are structural and functional homologues, acting to endow high processivity on the replicative DNA polymerases (*48*). Thus, there seems to be a conservation between the human and the *E. coli* replicative DNA polymerases in the requirement for the processivity subunit in translesion replication.

The mechanism by which PCNA stimulates bypass has yet to be deciphered. It is very likely that the increased stability of the polymerase−DNA complex due to the processivity accessory proteins leads to an increased residence time at the lesion, therefore, enabling completion of the slow kinetic steps involved in lesion bypass. This is indicated by the apparent processive nature of the bypass reaction (Figure 2A). In addition, PCNA may inhibit the 3′→5′ exonuclease (proofreading) activity of pol δ, thus reducing its fidelity, which may prevent futile idling at the site of lesion, and a more productive extension of the abnormal base pair. This hypothesis is in accordance with recent results suggesting that PCNA reduces the levels of pol δ's fidelity during normal replication (*49*).

Pol δ−PCNA showed a strong preference for insertion of a dAMP residue opposite the abasic site (90% of all mutations; 46/51). This is in a striking contrast to the specificity of bypass by the *E. coli* pol III holoenzyme, which produced primarily −1 deletions opposite the lesion (*24*, *26*). Thus, although both human and *E. coli* replicative DNA polymerases require their processivity clamps for bypass, they differ drastically in their mutagenic outcome. In fact, the tendency of pol δ−PCNA to insert a dAMP opposite the abasic site resembles that of *E. coli* pol I (Table 1). This specificity seems to be directed by a mechanism different than misalignment, since there is no T downstream to the abasic site that can serve as a template base for the incoming A (Figure 1, B and C). Rather, the specificity of bypass seems to be directed by misincorporation opposite the abasic site.

The sequence analysis of pol β's bypass products is a direct demonstration of pol β's tendency to bypass abasic lesions via a skipping (misalignment) mechanism (69% of the mutations; 34/48). Pol β was shown to form −1 deletions and even larger deletions at high frequencies during replication of undamaged templates (*50*). In addition, a mechanistic study of translesion replication by pol β using synthetic oligonucleotides showed a correlation between the extent of bypass and the sequence context in the vicinity of the lesion (*51*). That is, the most efficient bypass was achieved when the dNTP complementary to the base immediately downstream to the lesion was added to the reaction. In this study, Efrati et al. (*51*) invoked, using this kinetic analysis, a misalignment mechanism in which the incoming nucleotide stabilizes the extrahelical abasic site, thus favoring a deletion, although the direct formation of −1 deletions was not shown. Our results support the suggestion of Efrati et al (*51*) and directly prove that most of the mutations formed by pol β during bypass are indeed −1 deletions.

Interestingly, a substantial fraction of the mutations formed by pol β contained A and G insertions opposite the lesion (13/48; 27%). Since the two nearest neighbors of the lesion were purines in both DNA substrates (Figure 1), this implies that they could not have instructed the incorporation of dAMP or dGMP. This excludes misalignment as a bypass mechanism in these cases and implies that pol β has at least one additional mechanism for bypass, possibly direct misinsertion opposite the abasic site, similarly to human pol δ and *E. coli* pol I.

Our results suggest that *E. coli* pol I can also utilize a misalignment mechanism in combination with a default A insertion. Examination of the sequence flanking the lesion (Figure 1C) suggests that the −2 deletion was formed due to an initial insertion of an A opposite the lesion, which is sometimes stabilized by a misalignment event of two bases. That is, for pol I it seems that nucleotide selection and incorporation are the first step in the synthesis cycle, sometimes followed by misalignment. This conclusion can be tested by a systematic variation of sequence context elements surrounding the lesion.

The dependence of pol δ's translesion replication on PCNA renders it a likely candidate for a control point of this process. In the past few years, there have been an accumulation of proteins, shown to interact with PCNA. Some of these proteins, through interactions with PCNA, may be regulating translesion replication. That is, through interactions with PCNA, translesion replication may be tied to damage detection and cell cycle regulation, e.g., through interactions with p21 (*52*, *53*), Gadd45 (*54*), and pol ϵ (*55*, *56*), and to repair processes such as long patch base excision repair and mismatch repair (e.g., through interactions with FEN1 (*57*), and MSH2/MLH1 (*58*), respectively).

Data regarding the frequency and specificity of mutations caused by abasic sites in mammalian cells in vivo is scarce, and the few studies that have been published produced conflicting results. Some claimed that abasic sites directed mostly base substitution mutations with no apparent preference for a particular nucleotide (*59*). Others suggested that

abasic sites induced, in addition to base substitution, deletion mutations (*60*). Finally, one study found that there was a tendency for a preferential A insertion opposite abasic sites in mammalian cells, as was found for *E. coli* (*61*). Clearly, more studies need to be done, and our model gap/lesion plasmid may be a convenient tool for achieving such goals. One of the challenges is in trying to study the process of translesion replication at distinct and well-defined points within the cell cycle, since it is very likely that more than one polymerase is performing translesion replication in vivo.

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