DNA Polymerase I-Mediated Translesion Synthesis in RecA-Independent DNA Interstrand Cross-Link Repair in *E. coli*[†]

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ABSTRACT: DNA interstrand cross-links (ICLs) are mainly repaired by the combined action of nucleotide excision repair and homologous recombination in *E. coli*. Genetic data also suggest the existence of a nucleotide excision repair-dependent, homologous recombination-independent ICL repair pathway. The involvement of translesion synthesis in this pathway has been postulated; however, the molecular mechanism of this pathway is not understood. To examine the role of translesion synthesis in ICL repair, we generated a defined substrate with a single psoralen ICL that mimics a postincision structure generated by nucleotide excision repair. We demonstrated that the Klenow fragment (DNA polymerase I) performs translesion synthesis on this model substrate. This *in vitro* translesion synthesis assay will help in understanding the basic mechanism of a postincision translesion synthesis process in ICL repair.

DNA interstrand cross-links (ICLs[1]) hinder the separation of the duplex strands, thus inhibiting the critical process of DNA replication and transcription (*1, 2*). Genetic and biochemical studies of *E. coli* identified nucleotide excision repair (NER) proteins, UvrABC, a recombinase RecA, a helicase UvrD, and a repair DNA polymerase, DNA polymerase I (Pol I) as essential factors for the removal of ICLs (*3–7*). In the major ICL repair pathway in *E. coli*, UvrABC makes two incisions in one of the cross-linked strands, one on each side of the ICL. The result of the action is a short oligonucleotide (10−11 nt in length), which is still covalently attached to the complementary strand by the ICL (unhooked ICL). The 5′−3′ exonuclease activity of Pol I generates a gap from the 3′ incision site. This provides a single strand gap for the RecA-mediated strand transfer. RecA transfers a homologous strand from a sister DNA molecule to the gap. This strand transfer reaction creates a three-stranded structure with the excised and displaced ICL-containing oligonucleotide. UvrABC makes a second round of dual incisions on the three-stranded structure, releasing a short duplexed fragment with the ICL. The resulting gap is filled in by Pol I using the newly acquired strand by the strand transfer as a template. Then DNA ligase I seals the nick to complete the repair process.

In early genetic studies, it was estimated that one ICL per genome is lethal in the *uvrA*$^-$ *recA*$^-$ double mutant cells (*8*). Curiously, 2% survival was observed in *uvrA*$^+$*recA*$^-$ *E. coli*, while only 0.1% survival was detected in *uvrA*$^-$ *recA*$^-$ double mutant cells with transformation experiments using a defined psoralen ICL plasmid (*3*). The results imply that in the *recA*$^-$ cells, ICLs are repaired by a UvrABC-dependent, but homologous recombination-independent pathway. A plausible mechanism will be a coupling of NER activity with translesion synthesis (TLS) (*1, 2*). A gap generated after the first round of dual incisions in one of the two cross-linked strands by UvrABC will be filled in by a translesion DNA polymerase using the other damaged strand containing an unhooked ICL. The existence of such a repair pathway has been reported in eukaryotes. Yeast cells arrested in the G1 phase, where no sister chromatid or homologous chromosome are available for the homologous recombination repair pathway, require NER factors and a translesion DNA polymerase ζ for the cellular resistance to ICLs (*9*). It has also been demonstrated using a host-cell reactivation assay in mammalian cells that a single mitomycin C-induced ICL on a plasmid DNA, which has no apparent homology to cellular DNA, is repaired by the combined action of NER and a translesion DNA polymerase η in an error-prone manner (*10*).

As an initial attempt to understand the molecular mechanism of the UvrABC-dependent, RecA-independent ICL repair pathway in *E. coli*, we employed an *in vitro* TLS assay with a model template DNA. We chose a psoralen ICL for this study because of its chemical stability and well-established photochemistry (*11*). Psoralen is a planar, tricyclic semiaromatic compound. Psoralen preferentially induces covalent adducts with thymine, a furan-side or a pyrone-side monoadduct, at 5′-TA-3′ by UVA irradiation. The pyrone-ring of the furan-side monoadduct can form an additional covalent adduct with the thymine on the opposite DNA strand by further UVA irradiation. This photoreaction results in the formation of an ICL. The pyrone-side monoadduct is inert to form an ICL. A psoralen ICL can be quantitatively converted to a furan-side monoadduct by UVC

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[1] Abbreviations: ICL, DNA interstrand cross-link; TLS, translesion synthesis; NER, nucleotide excision repair; Pol I, *E. coli* DNA polymerase I; Kf, Klenow fragment of DNA polymerase I; Kf(exo-), 3′−5′ exonuclease-deficient Klenow fragment of DNA polymerase I; T4, T4 DNA polymerase; dNMP, deoxyribonucleoside 5′-monophosphate; dNTP, deoxyribonucleoside 5′-triphosphate.

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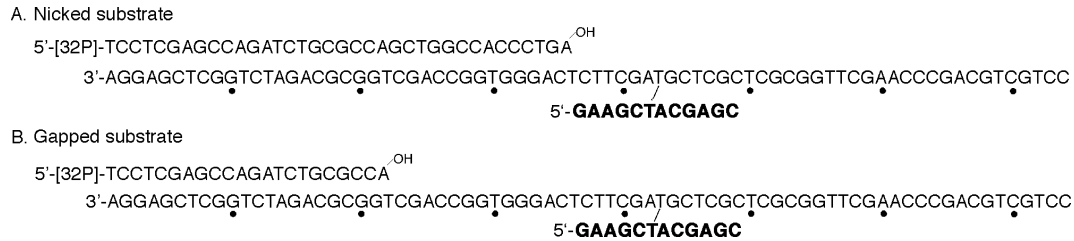

FIGURE 1: Substrates used in this study. A 12-mer with a single psoralen furan-side monoadduct (shown in bold) was annealed to a 74-mer. The partially duplexed DNA was exposed to UVA light to generate an ICL. The 5′-^{32}P-labeled primers were annealed to the cross-linked template: (A) a 36-mer for a nicked substrate and (B) a 22-mer for a gapped substrate. Dots indicate every tenth nucleotide.

irradiation (photoreversal reaction). Psoralen has been used to generate defined ICL substrates to study prokaryotic and eukaryotic ICL repair (*2, 12*).

A 12-mer oligonucleotide was attached to a 74-mer template by a psoralen ICL (Figure 1). A primer was annealed to the template with the ICL to mimic a DNA structure after the first round of NER action. We investigated whether Pol I displays TLS activity on this template. The gap filling reaction by Pol I occurs concomitantly with the dual incisions by UvrABC in NER. Therefore, Pol I could be the first DNA polymerase to encounter the ICL attached to a template strand. We discovered that Pol I displaces the incised oligonucleotide attached to the template by the ICL efficiently and is capable of bypassing the damage, albeit with a low efficiency. Remarkably, a limited sequencing analysis of the TLS products showed that the bypass was error-free. Therefore, we provide biochemical evidence of the direct involvement of Pol I in the UvrA-dependent, RecA-independent ICL repair pathway.

EXPERIMENTAL PROCEDURES

Enzymes. E.coli Pol I, Klenow fragment (Kf), Klenow fragment (exo-), and T4 DNA polymerase (T4) were purchased from New England BioLabs.

Substrate Preparation. Primer oligonucleotides (100 pmol) were 5′-labeled using T4 polynucleotide kinase and γ-^{32}P ATP (1 mCi) and purified from a 10% sequencing gel. One hundred pmol of a 12-mer oligonucleotide (5′-GAAGC-TACGAGC-3′) with a psoralen furan-side monoadduct at the 5′-TA-3′ in the middle (*13*) was phosphorylated with cold ATP by T4 polynucleotide kinase, and annealed to 100 pmol of a 74-mer oligonucleotide (5′-CCTGCTGCAGCCCAAGCT-TGGCGCTCGCTCGTAGCTTCTCAGGGTG-GCCAGCTGGCGCAGATCTGGCTCGAGGA-3′). The partially duplexed DNA was exposed to UVA light (366 nm) for 10 min to convert a monoadduct to an ICL (*11, 14*). The cross-linked substrate was purified from a 10% sequencing gel and annealed to a 5′-^{32}P-labeled 22-mer primer (5′-TCCTCGAGCCAGATCTGCGCCA-3′) to generate a gapped substrate or a 36-mer primer (5′-TCCTCGAGCCAGATCT-GCGCCAGCTGGCCACCCTGA-3′) to generate a nicked substrate. The substrate was then purified from a 6% native gel (Supporting Information, Figure 1). Typically, the ICL substrate contains less than 0.5% noncross-linked DNA (Supporting Information, Figure 1). For the nondamaged substrate, the ^{32}P- labeled 22-mer primer was annealed to the 74-mer oligonucleotide (Figure 1).

In Vitro DNA Polymerase Assay. All of the reactions (10 μL) were conducted at 37 °C in the following buffer: 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5 nM substrate and all four dNTPs at 100 μM. For T4 polymerase, BSA was also added at 50 μg/mL. Typically, 0.15 nM of Kf, 0.15 nM of Kf (exo-), or 0.017 nM of T4 DNA polymerase was used. The amount of each DNA polymerase was determined to give similar primer-extension efficiency on a nondamaged template (Figure 2A). After the incubation for the indicated time, the reactions were terminated by phenol/chloroform extraction. The reaction products were isolated by ethanol precipitation and analyzed on an 8% sequencing gel. The dried gel was exposed to a PhosphorImage screen, an image was obtained by scanning the screen with the Typhoon 9410, and the products were quantitated by ImageQuant. Bypass activity is defined as the amount of the 74 nt full-length product. Strand displacement activity is defined as the total amount of the products with the sizes of 37 - 42 nt. The 42 nt fragment is the product of the inhibition of polymerase activity at one nucleotide before the ICL. Error bars represent the standard deviation of three independent experiments.

Determination of dNMP Incorporated Across the ICL. Kf (180 nM) was incubated with 10 nM of the nicked substrate at 37 °C for 30 min. The majority of the chain elongation reaction was terminated at one nucleotide before the ICL (Figure 4B, lane 1). The reaction products were isolated and 75 nM of Kf (exo-) was incubated with 1/7 of these terminated products for 10 min at 37 °C in the presence of 100 μM of dATP, TTP, dGTP, or dCTP. The reaction products were analyzed by an 8% sequencing gel.

Sequencing Analysis. A polymerase assay was performed with Kf or Kf (exo-) using 10 nM of the nicked ICL substrate. The fully extended 74 nt fragment was isolated from an 8% sequencing gel. The product was amplified with 10 cycles by ExTaq (TaKaRa) with the following primers: 22-mer (5′-TCCTCGAGCCAGATCTGCGCCA-3′) and 22-mer reverse (5′-CCTGCTGCAGCCCAAGCTTGGC-3′). The unused primers and dNTPs were removed by a MicroSpin G-50 column (GE Healthcare). The cleaned products were cloned into the pCR4-TOPO vector (Invitrogen) and transformed into TOP10 competent cells. Thirteen clones from the Kf reaction and ten clones from the Kf (exo-) reaction were identified that had the insert and were sequenced. As a control experiment for PCR-mediated error, the 74-mer oligonucleotide was amplified by PCR with the same primers for 20 cycles. Eight clones were sequenced and all gave the correct sequence.

RESULTS AND DISCUSSION

We generated a defined template that mimics a DNA structure after the first round of dual incisions by UvrABC to study a NER-dependent, TLS-mediated ICL repair. This

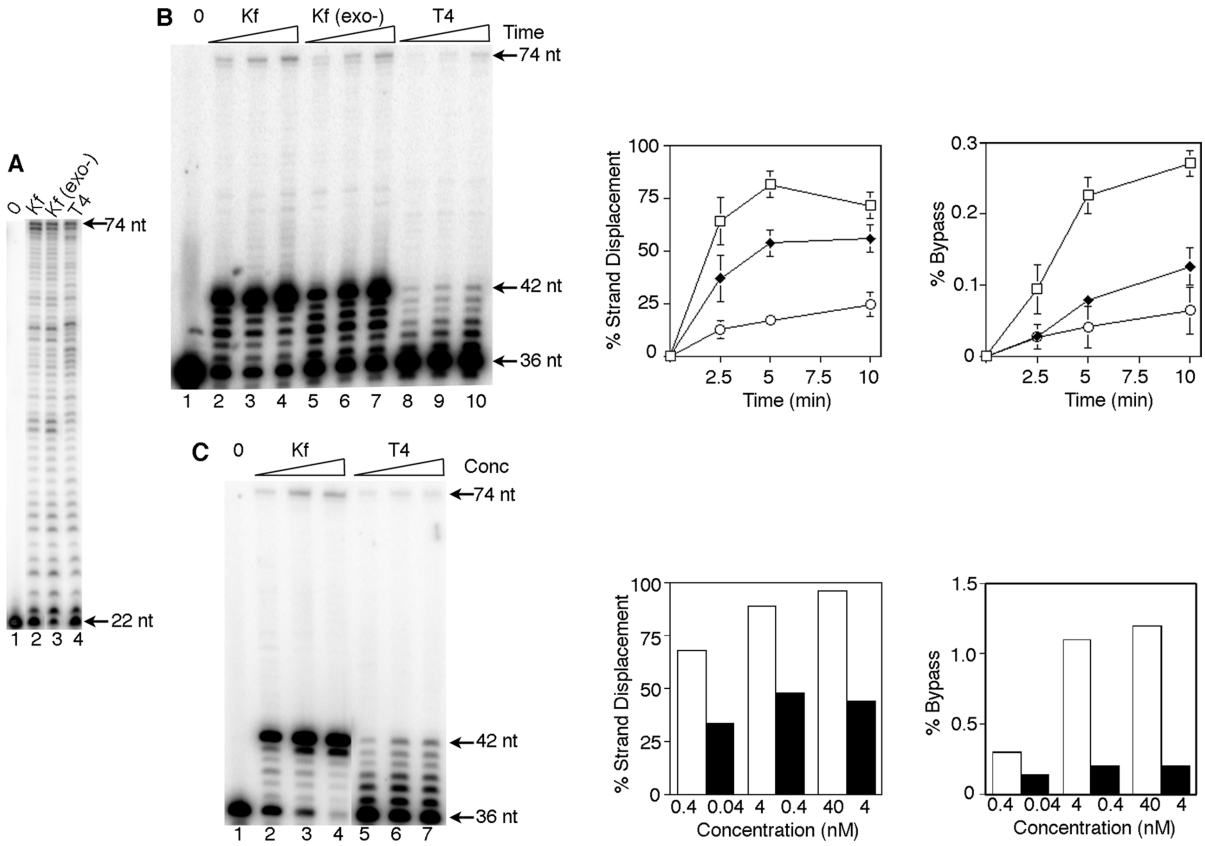

FIGURE 2: TLS of a psoralen ICL attached to a template *in vitro*. (A) *In vitro* DNA polymerase activity on a nondamaged template. DNA polymerase was incubated with 0.5 nM of nondamaged substrate (5′-^{32}P-labeled 22-mer annealed to the 74-mer) for 10 min at 37 °C. Lane 1, no polymerase; lane 2, Kf (0.15 nM); lane 3, Kf (exo-) (0.15 nM); and lane 4, T4 (0.017 nM). Sixty-five percent, 83%, and 70% of the primer was extended in lane 2, 3, and 4, respectively. (B) *In vitro* TLS activity. DNA polymerase was incubated with 0.5 nM of the nicked ICL substrate for 2.5, 5, or 10 min at 37 °C. The amount of polymerase that gave a similar primer-extension activity on a nondamaged template was used. Lane 1, no polymerase; lanes 2−4, Kf (0.15 nM); lanes 5−7, Kf (exo-) (0.15 nM); and lanes 8−10, T4 (0.017 nM). The percentage of the TLS and the strand displacement products were determined and depicted in the graphs next to the gel. Kf (□) showed strong strand displacement activity, while T4 (○) had a much weaker activity as indicated by the extension from the 36-mer primer. Most primer extension by the polymerase was terminated at one nucleotide before the cross-linked site (42 nt). Kf read through the unhooked ICL and gave the 74 nt full-length product with low efficiency. T4 showed little TLS activity. Kf (exo-) (◆) showed lower TLS activity as well as strand displacement activity than Kf. Error bars represent the standard deviation of three independent experiments. (C) T4 DNA polymerase lacks TLS activity. Increasing amounts of Kf (0.4, 4, 40 nM) and T4 (0.04, 0.4, 4 nM) were incubated with 0.5 nM of the nicked ICL substrate for 10 min at 37 °C. Lane 1, no polymerase; lanes 2−4, Kf; lanes 5−7, T4. The percentage of the TLS and the strand displacement products were determined and depicted in the graphs next to the gel. White bars, Kf; black bars, T4. Very few TLS products were detected in the reaction with T4 with the highest amount (lane 7).

is the first biochemical study to examine DNA polymerase activity on this unique DNA repair intermediate. Because Pol I is the first DNA polymerase to encounter an unhooked ICL, we attempted to use Pol I in our TLS assay. We observed TLS activity of Pol I under some conditions; however, Pol I also degraded the labeled primer by 5′−3′ exonuclease activity with higher concentrations (Supporting Information, Figure 2). Therefore, we used Kf in our studies in place of Pol I. Kf displayed strong strand displacement activity on the substrate. Nearly 80% of the extended primer was terminated at 42 nt (Figure 2B and C), one nucleotide before the cross-linked thymine (Figure 1A). Importantly, we detected a weak but measurable amount of a 74 nt length bypass product by Kf (Figure 2B and C). T4 DNA polymerase, which lacks strand displacement activity, gave very little bypass products (Figure 2B and C). These data also demonstrated that the displaced cross-linked strand does not inhibit the chain elongation activity of Kf, but the lesion does, as is the case for a psoralen monoadduct Supporting Information, Figure 3). Thus, strand displacement activity and TLS activity are required for TLS on the template with an unhooked ICL.

We also generated a defined substrate with a 14 nt gap 5′ to the unhooked ICL to facilitate access for DNA polymerase to a primer end (Figure 1B). All three polymerases tested showed slightly better TLS activity on the gapped template than those on the nicked template (Figure 3). Interestingly, the enhanced TLS activity by Kf (exo-) and T4 DNA polymerase was associated with the elevated level of strand displacement activity. These results also emphasize the requirement of strong strand displacement activity for TLS of the unhooked ICL.

Many translesion DNA polymerases lack a 3′−5′ exonuclease activity and the contribution of the proofreading activity to the outcome in TLS is well documented (*15*). To investigate a role for the 3′−5′ exonuclease activity in TLS of the unhooked ICL, Kf (exo-) was used. Kf (exo-) was able to insert an additional dNMP across the ICL with higher concentrations (Figure 4A). To identify the dNMP inserted across the ICL, we utilized the products from the reaction

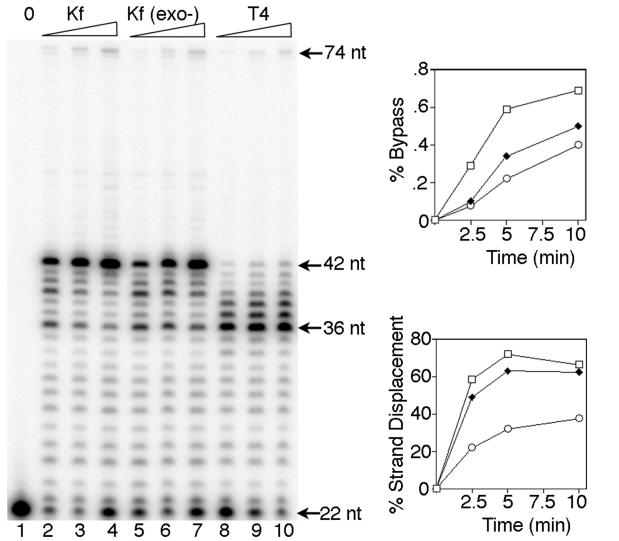

FIGURE 3: Effect of a gap 5′ to an unhooked ICL on TLS. DNA polymerase was incubated with 0.5 nM of the gapped ICL substrate for 2.5, 5, and 10 min at 37 °C. The elongation of the primer by the polymerase was inhibited at 36 nt when the polymerase encountered the 5′ end of the cross-linked 12-mer strand. The primer extension to 42 nt indicates strand displacement activity. Lane 1, no polymerase; lanes 2−4, Kf (0.15 nM); lanes 5−7, Kf (exo-) (0.15 nM); lanes 8−10, T4 (0.017 nM). The percentage of the TLS and the strand displacement products were determined and depicted in the graphs next to the gel. Kf, □; Kf (exo-), ◆; T4, ○. All three polymerases displayed a 2 to 3-fold better TLS activity on the gapped ICL substrate compared to the nicked ICL substrate.

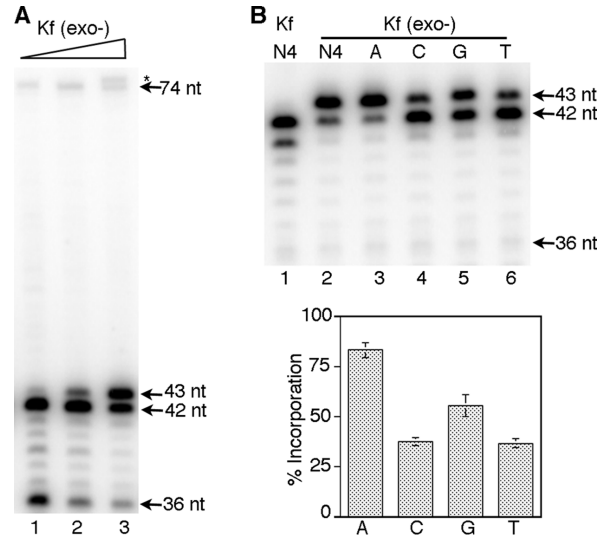

FIGURE 4: Incorporation of dAMP across the ICL. (A) Kf (exo-) inserts a dNMP across the psoralen ICL. Increasing amounts of Kf (exo-) (lane 1, 0.4 nM; lane 2, 4 nM; and lane 3, 40 nM) were incubated with 0.5 nM of the gapped ICL substrate for 10 min at 37 °C. Kf (exo-) was able to insert a nucleotide across from the ICL. Kf (exo-) added one nucleotide to the 74 nt product as marked by the asterisk. This TdT activity of Kf (exo-) is often observed with a high amount of Kf (exo-). (B) Preferential incorporation of dAMP across the ICL by Kf (exo-). A partially triple-stranded ICL substrate with a 42 nt primer was isolated from the reaction with Kf and the nicked ICL substrate (lane 1). This substrate was incubated with 75 nM of Kf (exo-) in the presence of only one dNTP at 100 μM. Lane 1, control with Kf; lane 2, Kf (exo-) with all four dNTPs at 100 μM; lane 3, with dATP; lane 4, with dCTP; lane 5, with dGTP; and lane 6, with TTP. The percentage of the extension from 42 nt to 43 nt was depicted in the graph. Error bars represent the standard deviation of three independent experiments.

with Kf and the nicked ICL substrate. The *in vitro* polymerization by Kf on the nicked ICL substrate was predominantly terminated at one nucleotide before the ICL (Figure 2B and C). Thus, these products are suitable for studying the insertion of a dNMP across the ICL. The products from the reaction with Kf were isolated and incubated with Kf (exo-) in the presence of only one dNTP. The preference of the incorporation across the ICL by Kf (exo-) is dAMP > dGMP > dCMP = TMP (Figure 4B). However, the 3′−5′ exonuclease activity had no significant influence on the efficiency of the bypass (Figure 2B and C). Kf and Kf (exo-) showed very similar bypass efficiency when the two polymerases gave a similar amount of strand-displacement products (compare lanes 2 and 7 in Figure 2B).

These results imply another important feature of the TLS of an unhooked ICL. Eukaryotic DNA polymerase ζ is capable of extending a primer end across a lesion in TLS (*15*). Kf (exo-) incorporates a dNMP across from the ICL and generates a primer end for an extender DNA polymerase; however, it cannot extend the primer end by itself. Because Kf (exo-) also cannot extend a primer end across a psoralen monoadduct (Supporting Information, Figure 3), the primer-extension reaction at the cross-linked nucleotide could be a rate-limiting step in the TLS of the psoralen ICL. In addition, a complete displacement (dislocation) of the unhooked strand might also influence the efficiency of TLS. The unhooked strand may not be fully displaced by the polymerase because the polymerase is stalled at one nucleotide before the ICL or at the ICL, and the cross-linked strand ahead of the polymerase is not yet displaced. A strand attached 3′ to an ICL might have a negative impact on a primer-extension reaction at a cross-linked nucleotide.

To examine the accuracy of TLS by Kf, we attempted to sequence the 74 nt bypass products. Due to the low amount of the 74 nt products, we took a PCR-based sequencing analysis, which was employed to examine the bypass of abasic lesions by Dpo4 DNA polymerase from *S. solfataricus* (*16, 17*) (Supporting Information, Figure 4). The labeled 74 nt fragments were isolated from an 8% sequencing gel. The purified fragments were subjected to the PCR reaction using primers matched to the sequences of the 5′- and 3′-end of the template. The PCR was limited to only 10 cycles to avoid an unnecessary side effect with PCR by ExTaq DNA polymerase. The template attached with the 12-mer would not be in the purified fragments because it migrates much slower than the nondamaged 74 nt fragment. The PCR products were cloned directly into the pCR-vector using the TA-cloning system. A limited sequencing analysis of 13 clones from the reactions with Kf and 10 clones from the reactions with Kf (exo-) revealed that there were no mutations, point or frameshift mutations, in the TLS products. The results might suggest that only the primer terminus with dAMP across the lesion can be extended by Kf or Kf (exo-) to give TLS products. We concluded that the TLS of the unhooked ICL by Kf is not significantly mutagenic with > 92% accuracy (no mutation found in 13 clones derived from the TLS products by Kf).

Our data showed that Pol I performs TLS on an unhooked ICL with a low efficiency *in vitro*. The detected TLS activity could not be the result of the polymerization products of uncross-linked DNA that exist in our ICL substrate preparation due to the following reasons: (a) less than 0.5% of the uncross-linked template exists in our ICL substrate (Supporting Infor-

mation, Figure 1) and this amount can not explain the 1% TLS activity observed with Kf (Figure 2C); (b) the incubation of the ICL substrate under the TLS conditions without DNA polymerase did not generate any non cross-linked DNA from the cross-linked substrate (i.e., the psoralen ICL is stable under the reaction conditions) (Supporting Information, Figure 1); and (c) the increasing amounts of T4 DNA polymerase in the reaction, which gave better DNA polymerase activity on nondamaged template compared to Kf, failed to improve the bypass activity (Figure 2C).

The low TLS activity of Kf implies that there are likely other factors to stimulate the TLS activity of Pol I. An attractive candidate is UvrD. UvrD is an ATP-dependent DNA helicase and an essential component of NER. It removes a damage-containing oligonucleotide generated by UvrABC, and then exposes a gap accessible for Pol I to perform repair synthesis during NER. This function of UvrD might coordinate with the strand displacement activity of Pol I and consequently stimulate the bypass activity of Pol I in RecA-independent ICL repair.

Another possibility is that multiple DNA polymerases are required for efficient TLS. The inserter−extender mechanism in TLS has been well documented in eukaryotic lesion bypass (*15*). A similar mechanism might be applied to the TLS of an unhooked ICL. There are five DNA polymerases in *E. coli*, Pol I, II, III, IV, and V. Candidates, which are involved in ICL repair other than Pol I, are Pol II, IV, and V. Host-reactivation experiments with a defined psoralen ICL plasmid showed that the induction of the SOS response in host cells improves cell-survival (2.6-fold) with increased mutagenesis (1.5-fold) (*8*). The increased mutagenesis was largely due to elevated point mutations (*8*). These results implicate the involvement of error-prone TLS in ICL repair during the SOS response. The expression of Pol II, IV, and V are induced in the SOS response and these three polymerases have TLS activity past various types of DNA damage (*18*). Furthermore, a Pol II-deficient mutant (Δ*polB*) is sensitive to mechorethamine that generates nitrogen-mustard ICLs, a Δ*polB recA*$^-$ double mutant is more sensitive than a *recA*$^-$ mutant and shows a similar sensitivity to a *uvrA*$^-$ mutant (*19*). In addition, the Δ*polB* mutant displayed a significantly reduced replication activity of plasmid with a defined nitrogen-mustard ICL (*19*). Collectively, these results indicate that Pol II plays a pivotal role in a UvrABC-dependent, RecA-independent ICL repair. Thus, Pol II, IV, and V are somehow involved in TLS during ICL repair in *E. coli*. A stalled Pol I at the cross-linked site might be the signal to recruit these polymerases to facilitate TLS. Our *in vitro* TLS assay should be a powerful tool to examine these possibilities to define the role of different polymerases in the TLS of an unhooked ICL.

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SUPPORTING INFORMATION AVAILABLE

Figures 1−4. This material is available free of charge via the Internet at http://pubs.acs.org.

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