Analyzing the Handoff of DNA from UvrA to UvrB Utilizing DNA-Protein Photoaffinity Labeling* ♦

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To better define the molecular architecture of nucleotide excision repair intermediates it is necessary to identify the specific domains of UvrA, UvrB, and UvrC that are in close proximity to DNA damage during the repair process. One key step of nucleotide excision repair that is poorly understood is the transfer of damaged DNA from UvrA to UvrB, prior to incision by UvrC. To study this transfer, we have utilized two types of arylazido-modified photoaffinity reagents that probe residues in the Uvr proteins that are closest to either the damaged or non-damaged strands. The damaged strand probes consisted of dNTP analogs linked to a terminal arylazido moiety. These analogs were incorporated into double-stranded DNA using DNA polymerase β and functioned as both the damage site and the cross-linking reagent. The non-damaged strand probe contained an arylazido moiety coupled to a phosphorothioate-modified backbone of an oligonucleotide opposite the damaged strand, which contained an internal fluorescein adduct. Six site-directed mutants of Bacillus caldotenax UvrB located in different domains within the protein (Y96A, E99A, R123A, R183E, F249A, and D510A), and two domain deletions (Δ2 and Δβ-hairpin), were assayed. Data gleaned from these mutants suggest that the handoff of damaged DNA from UvrA to UvrB proceeds in a three-step process: 1) UvrA and UvrB bind to the damaged site, with UvrA in direct contact; 2) a transfer reaction with UvrB contacting mostly the non-damaged DNA strand; 3) lesion engagement by the damage recognition pocket of UvrB with concomitant release of UvrA.

Nucleotide excision repair (NER) is the major DNA repair pathway responsible for removal of structurally diverse DNA lesions (1–5). The reaction proceeds in the following order: 1) damage recognition, 2) damage verification, 3) incision 3’ to the lesion, 4) incision 5’ to the lesion, 5) DNA re-synthesis, and 6) DNA ligation (6).

The current model for UvrABC protein function during the initial steps of bacterial NER is as follows. In solution, UvrA dimerizes to become UvrA2, and ATP binding by the UvrA monomers promotes dimer formation (5, 7). UvrA2 interacts with UvrB to form the UvrA2B complex. Although the UvrA2 dimer possesses the ability to recognize DNA lesions by itself (6, 8), it is believed that the UvrA2B complex is the DNA damage recognition complex of the NER machinery (9). We have proposed a padlock model for UvrB detection of DNA damage in which the β-hairpin of UvrB is key to the proper recognition and processing of the lesion (9–11). In this model, the UvrA2B complex binds to DNA and searches locally for DNA lesions fueled by ATP-dependent motions between domains 1α/b and the β-hairpin and domain 3 of UvrB. If no lesion is encountered, the UvrA2B complex dissociates from the DNA. If the proteins encounter a lesion, conformational changes occur such that the DNA containing the lesion, which is believed to be intimately in contact with UvrA2, is passed via an unknown mechanism to UvrB (12, 13). Thus, major conformational changes occur within the proteins as well as to the DNA (14). During this transition stage of damage verification, UvrA is believed to hydrolyze ATP and dissociate from the UvrA2B-DNA complex, or the process of UvrB engaging the lesion may in and of itself trigger the release of UvrA (15).

The padlock model predicts that UvrB binds ATP and harnesses the energy of this molecule to impose an unfavorable conformation on the DNA; the β-hairpin moiety of UvrB separates the DNA strands to facilitate incision (9). DNA in complex with UvrB has also been visualized as being bent and wrapped around the protein at this stage of the reaction (5, 16). In addition, atomic force microscopy has revealed that UvrB might function as a dimer (17). Once UvrA departs from the UvrB-DNA complex, UvrC, which has two separate catalytic sites (18, 19), can bind and execute the dual incision events. The incision mediated by the N-terminal nuclease domain of UvrC occurs 4–5 nucleotides 3’ to the lesion and precedes the incision 8 nucleotides 5’ to the site of the lesion, mediated by bovine serum albumin; XL1, 5-[(N-(4-azidotetrafluorobenzylidene-aminoxy)methylcarbamoyl)-trans-3-aminopropenyl]-1-2’-deoxyuridine-5’-triphosphate; XL2, exo-N-[(4-azido-2,5-difluoro-3-chloropyrindine-6-yl)-3-aminopropionyl]aminoethyl]-2’-deoxyycytidine-5’-triphosphate; AZ, p-azidophenacyl; FldT, single internal fluorescein; MOPS, 4-morpholinoethanesulfonic acid.
the C-terminal UvrC nuclease domain. This dual action creates a 12-nucleotide fragment containing the lesion (18–21). Classically, protein-DNA interactions have been studied via the electrophoretic mobility shift assay (EMSA). Several laboratories including our own have utilized this technique to determine whether the UvrA and UvrB proteins interact in a productive manner (22–24). This technique can readily distinguish between UvrA-DNA and UvrB-DNA complexes. However, heterotrimeric complexes such as UvrA3-DNA are poorly resolved, with the exception of complexes involving the Δβ-hairpin UvrB mutant, which lacks residues 97–112 (11, 15). In this case, a stable UvrAB-DNA complex is created, which migrates slower than the UvrA3-DNA complex. However, it is not known which protein is in direct contact with the damaged DNA in this UvrAB-DNA complex. In general with current methodologies it has been a challenge to determine where in the reaction pathway the DNA containing the lesion is transferred to UvrB.

Here we describe the successful utilization of UV-activated photoreactive DNA probes containing azido groups that allow us to capture the UvrA and UvrB proteins during the NER process while they are in close proximity to these groups in dsDNA. We have taken a two-pronged approach in investigating the molecular handoff of damaged DNA from UvrA to UvrB. First, two dNTP analogs linked to terminal arylazido groups were incorporated into dsDNA using DNA polymerase. First, two dNTP analogs linked to terminal arylazido groups were incorporated into dsDNA using DNA polymerase. In this manner, these reagents served as both the site of UvrB point mutations (Fig. 1) from *B. caldotenax* and purified using the T7 IMPACT® system (New England Biolabs). UvrC was purified in a similar manner from *Thermotoga maritima* in C41(DE3) cells.

**DNA Substrates**—All DNA substrates were synthesized by Sigma-Genoys (Woodlands, TX), except for the S-1 substrate, which was synthesized by Midland Certified Reagent Co. (Midland, TX). Upon receipt, oligonucleotides were resuspended in 1 m Tris-HCl (pH 7.8) and 0.1 mM EDTA and stored at −20°C. dNTP Substrates and Cross-linking Reagents—dNTP and dCTP were obtained from Roche Applied Science. 5-(N-(4-azidotetrafluoro-3-chloropyridine-6-yl)-3-aminopropionyl)aminoethyl-2-deoxyctydine-5′-triphasophate (XLI, Fig. 2A) was synthesized and characterized as described previously (25). Synthesis of exo-N-[2-(4-azidopropylaminoethyl-2-deoxyctydine-5′-triphasophate (XLZ, Fig. 2B) will be described elsewhere. p-Azidophenacyl bromide (AZ, Fig. 2C) was purchased from Sigma.

**Radiolabeled DNA Duplex Notation**—Throughout this paper we discuss a variety of duplex DNA substrates. For clarification purposes, we wish to point out in advance that the strand listed first in the naming of a duplex oligonucleotide is 5′-3′ labeled (e.g. for substrate F26/N1, the F26 strand is 5′-labeled versus S-1AZ/F26 where the S-1AZ strand is 5′-labeled).

**Fluorescein Substrate, F26/N**—The DNA sequence of the 50-mer double-stranded substrate containing a single internal fluorescein (FidT; Fig. 3A, position in sequence indicated by red bars) adduct was: F26, 5′-GACTACGATCTGTTAGCCGCACGCACTTGCTAGCCATCGCCAGCATCTGC-3′, while the complementary non-damaged strand (ND) was 5′-GGACGTTCGAGCCTGTTACGGGCTAGATGCTGAGGAGGCGCTACATGATCGCGGCTGCCTGACGATCGGAG-3′. The F26 strand was 5′-end-labeled using OptiKinase (United States Biochemical Corp.) and γ-[32P]ATP (3000 Ci/mmol, Amersham Biosciences) according to the manufacturer’s instructions. The reaction was terminated by the addition of 20 mM EDTA, and the enzyme was heat denatured by incubation for 10 min at 65°C. After labeling, the free nucleotides were removed by gel filtration chromatography (Micro BioSpin-6, Bio-Rad). The labeled oligonucleotide (sequence identical to ND) was synthesized to contain a 45246

**Probing the DNA Exchange from UvrA to UvrB**

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of UvrABC Proteins**—The UvrA and UvrB proteins, as well as UvrB point mutants Y96A, E99A, R123A, R183E, F249A, D510A, and domain mutants Δ2 (lacks amino acids Val158-Phe244), and Δβ-hairpin (lacks amino acids Gln97 to Asp112) (15) (Fig. 1) from *B. caldotenax* were expressed in BL21(DE3) cells by standard procedures similar to those previously described by our laboratory (11) and purified using the T7 IMPACT® system (New England Biolabs). UvrC was purified in a similar manner from *Thermotoga maritima* in C41(DE3) cells.

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phosphorothioate modification at the phosphodiester linkage between the 25th and 26th nucleotide from the 5'-end (indicated by blue S-1, Fig. 3A). The 32P-labeled substrate was then modified to contain the azido-phenacyl cross-linking moiety as described by Yang and Nash (256 µl of butanol. The aqueous layer was then mixed with glycerol (1 µl, 2 mg/ml), 3 µl sodium acetate (pH 5.5) (10% of total reaction volume), and 3 additional reaction volumes of ethanol and allowed to precipitate overnight at 20 °C. The samples were then centrifuged for 60 min at 4 °C. The pellets were washed with ice-cold 70% ethanol (1 ml) and then dried under vacuum. The azidophenacyl-modified oligonucleotide (5'-32P-labeled 24-mer (5'-5'-pCTACCGCAATCAGGCCAGAT-phosphorylated 24-mer) was resuspended in TE-100 (10 mM Tris (pH 7.5), 1 mM EDTA, 100 µM KCl) and annealed to the appropriate complementary oligonucleotide as described above. The ND oligonucleotide was subjected to the same procedures described above for S-1 to gauge nonspecific incorporation of the AZ modification.

**Gapped Heteroduplex Substrates, Gap_25 and Gap_26**—To generate gapped heteroduplex substrates, both a 25-mer oligonucleotide (5'-GACTACGTACTGTTACGGCTCCATC-3') and a 24-mer oligonucleotide (5'-GACTACGTACTGTTACGGCTCCATC-3') were 5'-end-labeled with OptiKinase (United States Biochemical Corp.) and [γ-32P]ATP (3000 Ci/mm, Amersham Biosciences) as described above. The reaction was terminated by the addition of 20 mM EDTA, and the enzyme was heat-denatured by incubation for 10 min at 65 °C. The entire reaction volume was then passed through a Micro Bio-Spin 6 column (prewashed four times with 10 mM NH4OAc). The column eluent was evaporated to dryness. The 5'-labeled 25-mer was resuspended in 1 mM Tris-HCl (pH 7.8) and 0.1 mM EDTA and mixed at an equimolar ratio with a 5'-phosphorylated 24-mer (5'-pCTACCGCAATCAGGCCAGAT-CATG-3', the second half of the "top" strand) and a 50-mer (5'-CGTC- TAGAGCCGACTGCCTCCTCTCCTGCTTGGTGCTGATCAGCA-G-3') by heating at 90 °C for 5 min in the presence of 100 mM KCl. The 5'-labeled 24-mer was resuspended in 1 mM Tris-HCl (pH 7.8) and 0.1 mM EDTA and mixed at an equimolar ratio with a 5'-phosphorylated 25-mer (5'-pCTACCGCAATCAGGCCAGATCGT-3', the second half of the "bottom" strand) and a 50mer (5'-CGTCGATCCGG- AAACCCGCAATCCCTCTCCTGCTTGGTGCTGATCAGCA-G-3').

**Nicked Heteroduplex Substrates, dTnp_50, dCnp_30, XL1_20, and XL2_20**—Nicked heteroduplex substrates were prepared by incubating the gapped heteroduplex substrates Gap_25 and Gap_26 (22 pmol) with 100 pmol of dTTP and XL1, or 400 pmol dCTP and XL2, respectively, in the UvrABC incision buffer (50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl2, 1 mM ATP) for 20 min at 55 °C. The products were resolved on a 10% denaturing polyacrylamide gel, and the respective oligonucleotide mixtures were annealed by slow cooling to room temperature.

**RESULTS**

**Experimental Strategy**—Oligonucleotides for this study (see Fig. 3A for general sequence) were designed to incorporate lesions such as fluorescein (F26) and/or photoaffinity reagents (XL1, XL2, AZ; Fig. 2) in a centrally located position (i.e. on or near base 25 or 26) of a 50-base pair fragment of dsDNA. Previous studies have shown that a centrally located lesion such as fluorescein (F26/NDB) is well recognized by the UvrABC NER system (11, 15). By placing cross-linking reagents on either the damaged or non-damaged strands of the DNA, this study aims to capture the regions of the proteins that are in closest proximity to the lesion during protein assembly for NER and assess the handoff of damaged DNA from UvrA to WT UvrB and UvrB mutants from the perspective of both the damaged site and the non-damaged strand.

Photoaffinity labeling has been used to study protein-DNA interactions within a variety of biochemical systems (27–29). Orren and Sancar (30) showed, using a psoralen monoadduct, that UvrB forms intimate contacts with the damage site and that UvrD (helicase II) is necessary to dissociate the excised oligonucleotide from the post-incision protein-DNA complex. It was suggested that within this complex, UvrB remains bound to the non-damaged strand and was released by the action of DNA polymerase I. In a similar manner, Reardon and Sancar (31) probed the molecular anatomy of the preincision complexes of human nucleotide excision repair proteins.

Base-substituted analogs of dNTP carrying photoreactive arylazo groups (32) have been utilized previously to design photoreactive DNAs to study the molecular interactions of select base excision repair proteins (33, 34) and to study other proteins such as human replication protein A (35, 36). Similarly, a variety of reactive dNTPs with photoreactive azide functional groups have been surveyed for their ability to cross-link DNA to yeast RNA polymerase in their transcription complexes (37). Phosphorothioester backbone modifications have been utilized to successfully couple an azidophenacyl moiety to oligonucleotides and cross-link proteins such as integration host factor and transcription factor IIIB to DNA (26, 38). By incorporating both photoreactive azide-containing dNTPs and the azidophenacyl cross-linking moieties into oligonucleotides in this study, we sought to gain new insight into the interaction of UvrA and UvrB with damaged DNA. We have included a graphic representation of our modified DNA substrates superimposed on the crystal structure of B. caldotenax UvrB Y96A, Fig. 3E, (10, 15). As stated above, we have strategically designed our substrates to contain a cross-linking probe on either
the damaged or non-damaged strand (XL126 and XL225 or S-1AZ/F26) of the DNA.

**Incorporation and Characterization of Cross-linking Reagents**—Both DNA base and backbone modifications were applied in this study. Base modification required the synthesis of base-substituted dNTPs with an arylazide functional group. A, FABC-dUTP (XL1, \(5'-(N-(4-azido-tetrafluorobenzylideneaminooxy)methylcarbamoyl)-trans-3-aminopropenyl-1'-2'-deoxyuridine-5'-triphosphate). B, Photo-reactive dNTP analogs: FAB-dCTP (XL2, exo-\(N\)-(4-azido-2,5-difluoro-3-chloro-pyridine-6-yl)-3-aminopropionyl]-aminomethyl]-2'-deoxyctydine-5'-triphosphate). C, a photo reactive AZ functional group was coupled to the backbone of a phosphorothioate-containing (at the phosphodiester linkage, S-1) oligonucleotide. The terminal azido group (\(N_3\)) in each reagent is activated by exposure to UV light (365 nm) allowing DNA-protein cross-linking to occur via a highly reactive nitrene intermediate.

**FIG. 2. Cross-linking reagents.** The structures of the photo reactive cross-linking reagents used in this work are shown. A, FABC-dUTP (XL1, \(5'-(N-(4-azido-tetrafluorobenzylideneaminooxy)methylcarbamoyl)-trans-3-aminopropenyl-1'-2'-deoxyuridine-5'-triphosphate). B, Photo-reactive dNTP analogs: FAB-dCTP (XL2, exo-\(N\)-(4-azido-2,5-difluoro-3-chloro-pyridine-6-yl)-3-aminopropionyl]-aminomethyl]-2'-deoxyctydine-5'-triphosphate). C, a photo reactive AZ functional group was coupled to the backbone of a phosphorothioate-containing (at the phosphodiester linkage, S-1) oligonucleotide. The terminal azido group (\(N_3\)) in each reagent is activated by exposure to UV light (365 nm) allowing DNA-protein cross-linking to occur via a highly reactive nitrene intermediate.

**FIG. 3. Photoaffinity substrates for probing the DNA handoff from UvrA to UvrB during nucleotide excision repair.** All DNA substrates used in this study were double-stranded and 50 base pairs in length. The red asterisk indicates the position of the 5'-\(^{32}\)P label in each substrate, and the general sequence for all substrates is shown in A. Positions 25 and 26 of the labeled strand are highlighted in blue and red, respectively, to denote the location in the oligonucleotide sequence where modifications were incorporated. S-1 (in blue) indicates the position of the phosphorothioate modification in the backbone of the complementary strand. Three different types of substrates were prepared to incorporate and utilize the cross-linking reagents, XL1 and XL2. B, Gap25/26 indicates a single base pair gap at position 25 or 26 in the labeled strand of the heteroduplex oligonucleotide. A lowercase “p” indicates the position of a 5'-phosphate. Single nicked substrates without cross-linking functionality (dC25 or dT26 + Nick, not ligated, small gray box) and with cross-linking functionality (XL126 and XL225 + Nick, not ligated) were prepared via incorporation of dCTP or XL2 at position 25 and dTTP or XL1 at position 26 into the appropriate Gap25/26 heteroduplex substrates using DNA pol β. C, the F26/ND substrate, our standard UvrABC NER damaged oligonucleotide, contains a centrally located fluorescein adducted thymine (FldT, yellow oval). D, the S-1AZ/F26 substrate also contains the FldT lesion as well as the AZ (blue box) moiety in the non-damaged strand at the S-1 position, which is 5' to the FldT lesion on the opposite strand. ND,AZ/F26 was treated with AZ in the same manner as the S-1,AZ/F26 substrate to assess nonspecific incorporation of the AZ group in the absence of a specific phosphorothioate modification. E, putative DNA binding model based on the crystal structure of *B. caldovenax* UvrB Y96A (9, 10, 15) with a graphic representation of the potential points of contact for our cross-linking reagents. The fragment of dsDNA (red) is shown separated by the β-hairpin region of UvrB (blue Ca trace) with the non-damaged strand of DNA clasped between the β-hairpin and domain 1b. Undisturbed base pairs are represented with yellow and green spokes within the DNA. The points of 3'- and 5'-incision are indicated with black arrows.
(25) followed by incorporation of a photoreactive dNMP moiety into a gapped DNA substrate using DNA pol/β in reaction buffer for 1 h at 37 °C. Parallel reactions without pol β served as negative controls. The reaction mixtures were analyzed on a 10% PAGE-urea gel. Incorporation of dNMPs (dC, dT, and XL1 or XL2) went essentially to completion as indicated by the slower migrating species observed in lanes 2, 3, 5, and 6. A DNA size marker is shown to the right of the gel. Cross-linking conditions for the XL modified substrates were optimized for the following conditions: UvrA concentration (n = 3, mean ± S.D.) with UV exposure held constant for 10 min (B), length of UV (365 nm) exposure (n = 2, mean) with UvrA concentration held constant at 200 nM for XL126 (black boxes) and XL225 (gray diamonds) (C), and UvrB concentration in the presence of 200 nM UvrA (n = 3, mean ± S.D.) (D). A molecular mass protein marker is shown to the left of the full gel in D. The concentration of DNA was 2 nM, and the distance from the UV source to the samples was 5 cm. Optimal cross-linking conditions were 200 nM UvrA, 1000 nM UvrB, and 5-min UV exposure.

**Design and Optimization of Damage-dependent Cross-linking Reagents (XL1 and XL2)—**The photoreactive dTTP or dCTP analogs (XL1 or XL2) contain an arylazido group projecting into the major groove from either the C5 or N4 positions, respectively. The 5'-32P-labeled, heteroduplexed, 50-base pair dsDNA substrates with a gap at position 25 or 26 (Fig. 3B, Gap25) were incubated with dCTP or XL2 and dTTP or XL1, respectively, and human pol β in reaction buffer for 1 h at 37 °C. A DNA size marker is shown to the right of the gel. Cross-linking conditions for the XL modified substrates were optimized for the following conditions: UvrA concentration (n = 3, mean ± S.D.) with UV exposure held constant for 10 min (B), length of UV (365 nm) exposure (n = 2, mean) with UvrA concentration held constant at 200 nM for XL126 (black boxes) and XL225 (gray diamonds) (C), and UvrB concentration in the presence of 200 nM UvrA (n = 3, mean ± S.D.) (D). A molecular mass protein marker is shown to the left of the full gel in D. The concentration of DNA was 2 nM, and the distance from the UV source to the samples was 5 cm. Optimal cross-linking conditions were 200 nM UvrA, 1000 nM UvrB, and 5-min UV exposure.

**Fig. 4.** Incorporation and characterization of damage-dependent cross-linking reagents (XL1 and XL2). A. The 5'-32P-labeled, heteroduplexed, 50-mer dsDNA substrates with a gap at positions 25 and 26 were incubated with dCTP or XL2 and dTTP or XL1, respectively, and human pol β in reaction buffer for 1 h at 37 °C. Parallel reactions without pol β served as negative controls. The reaction mixtures were analyzed on a 10% PAGE-urea gel. Incorporation of dNMPs (dC, dT, and XL1 or XL2) went essentially to completion as indicated by the slower migrating species observed in lanes 2, 3, 5, and 6. A DNA size marker is shown to the right of the gel. Cross-linking conditions for the XL modified substrates were optimized for the following conditions: UvrA concentration (n = 3, mean ± S.D.) with UV exposure held constant for 10 min (B), length of UV (365 nm) exposure (n = 2, mean) with UvrA concentration held constant at 200 nM for XL126 (black boxes) and XL225 (gray diamonds) (C), and UvrB concentration in the presence of 200 nM UvrA (n = 3, mean ± S.D.) (D). A molecular mass protein marker is shown to the left of the full gel in D. The concentration of DNA was 2 nM, and the distance from the UV source to the samples was 5 cm. Optimal cross-linking conditions were 200 nM UvrA, 1000 nM UvrB, and 5-min UV exposure.
creased, the cross-linking to UvrA is decreased consistent with the handoff of damaged DNA from UvrA to UvrB. It is important to note that no DNA-protein cross-linking occurs with either XL reagent in the absence of UV exposure (Fig. 4B, lane 2, and Fig. 4C, lane 1) or in the absence of either XL reagent (Fig. 4B, lane I, and 3D, lane 2), and no DNA cross-linking to UvrB occurs in the absence of UvrA (Fig. 4D, lane 5). The full gel shown in Fig. 4D, representative of all cross-linking experiments described in this paper, clearly shows cross-linking bands specific to UvrA and UvrB and that as cross-linking bands appear, the amount of free DNA decreases accordingly. Bovine serum albumin (BSA) was used as a control to check for nonspecific cross-linking to proteins with no known DNA damage recognition functions.

The cross-linking bands that are shown and quantitated in all figures of this study are attributed to DNA substrate cross-linked to *B. caldotenax* UvrA (~105 kDa) or *B. caldotenax* UvrB (~76 kDa) protein monomers. While it is conceivable that the slower migrating band observed in the full-length gels shown in Figs. 4D and 5, A and B, could correlate with UvrA still bound to the intein-chitin binding domain tag (total MW ~161 kDa), this is unlikely based on the purity of UvrA used in these studies (Fig. 7A). Since the exact nature of the faint, slower migrating cross-linking band is currently unknown, the counts associated with this band are not included in the analysis.

**Design and Optimization of Non-damaged Strand Cross-linking Probe (ND*AZ/F26* Versus S-1*AZ/F26*)**—Cross-linking conditions for the AZ-treated DNA substrates were also optimized prior to assaying the UvrB mutants for their ability to interact with UvrA and DNA. The concentration of UvrA (Fig. 5A) and length of UV (365 nm) exposure (Fig. 5B) were varied.
while cross-linking to azidophenacyl-treated substrates S-1AZ/F26 and NDAZ/F26. The concentration of DNA in each reaction was 2 nM for all experiments. Again, optimal cross-linking conditions were determined to be 200 nM UvrA and 5-min UV exposure (365 nm) at 5 cm below the UV source. Both S-1AZ/F26 and NDAZ/F26 substrates were treated identically with the AZ reagent but only the S-1AZ/F26 substrate contains a specific phosphorothioate modification. Surprisingly, the NDAZ/F26 substrate exhibited cross-linking activity indicating that the azidophenacyl bromide reacts with DNA, regardless of specific phosphorothioate modifications, at some background level. This background reactivity of azidophenacyl bromide has not apparently been reported previously. We speculate that the nonspecific modification observed for the NDAZ/F26 substrate may be due to mild nucleophilic reactivity of purines with azidophenacyl bromide. Therefore, for each cross-linking reaction involving AZ, we probably overestimate the contribution of cross-linking due to AZ incorporation at nonspecific (i.e. non-phosphorothioate) sites within the DNA substrate. The percentage of nonspecific cross-linking observed to each protein was subtracted as “background” for each reaction utilizing the AZ-treated substrates.

Recognition of Substrates via UvrABC Incision Assay—It was necessary to ensure that the substrates prepared for this study, despite their initial ability to cross-link to the UvrA and UvrB proteins, were well recognized and incised as a DNA adduct by the UvrABC NER machinery. Therefore, each substrate was subjected to our standard UvrABC incision assay (11). Each 5'-32P-end-labeled substrate (2 nM) was incubated with 20 nM B. caldotenax UvrA, 100 nM B. caldotenax WT UvrB, and 50 nM T. maritima UvrC for 30 min at 55 °C in reaction buffer. The reactions were terminated with 20 mM EDTA, and the incision products were analyzed on a 10% denaturing polyacrylamide gel. DNA size markers are indicated to the left of the gels. C, graphic comparison of the incision activity of the UvrABC system on each substrate: F26/ND (n = 5, mean ± S.D.); substrates Gap25 through XL25 (n = 3, mean ± S.D.); F26/S-1, F26/S-1AZ, and S-1AZ/F26 (n = 2, mean ± S.D.). Refer to the legend to Fig. 3 for complete description of each substrate.

FIG. 6. Recognition of substrates by the UvrABC NER system. A and B, each 5'-32P-end-labeled (*) substrate (2 nM) was incubated with 20 nM B. caldotenax UvrA, 100 nM B. caldotenax WT UvrB, and 50 nM T. maritima UvrC for 30 min at 55 °C in reaction buffer. The reactions were terminated with 20 mM EDTA, and the incision products were analyzed on a 10% denaturing polyacrylamide gel. DNA size markers are indicated to the left of the gels. C, graphic comparison of the incision activity of the UvrABC system on each substrate: F26/ND (n = 5, mean ± S.D.); substrates Gap25 through XL25 (n = 3, mean ± S.D.); F26/S-1, F26/S-1AZ, and S-1AZ/F26 (n = 2, mean ± S.D.). Refer to the legend to Fig. 3 for complete description of each substrate.
reduced overall incision efficiency relative to F26/ND (55% versus 70%, respectively; Fig. 6B, lanes 2 and 4 versus Fig. 6A, lane 2). Thus, at least 80% of the UvrABC system complexes assembled on the F26 strand of the DNA substrate.

**Electrophoretic Mobility of UvrAB Complexes**—Stable protein-DNA interactions can be visualized by employing the EMSA technique; however, transient or labile protein-DNA interactions are not easily resolved by this technique. Prior to EMSA, each protein utilized in this study was examined on a SDS-PAGE gel to ensure that stock solutions of all proteins were essentially pure and of the same concentration (1.25 M, Fig. 7A). WT or mutant UvrB proteins were then subjected to EMSA analysis in the presence of *B. caldotenax* UvrA (Fig. 7B).

The UvrA₂-DNA complex can be readily identified in most lanes. However, in some lanes there is a significant upward smearing pattern indicative of dissociation of labile larger complexes, probably the UvrA₂B-DNA complex; compare the compact band of the lane containing only UvrA₂ with the lanes of UvrA₂ plus WT UvrB or Y96A (Fig. 7B, lane 2 versus lanes 3 or 4). The addition of a UvrB protein results in a band that migrates slightly slower compared with the pattern of UvrA₂ alone. More readily distinguishable from the UvrA₂-DNA complex is the stable, a slower migrating UvrAB-DNA complex formed in the presence of the UvrB mutant Δβ-hairpin (Fig. 7B, lane 11).

Last, we can visualize the UvrB-DNA complex as the faster migrating species in our EMSAs. WT UvrB in the presence of UvrA and damaged DNA produces a UvrB-DNA complex (26%). The F249A mutant forms a UvrB-DNA (30.5%) complex comparable with WT UvrB. Weaker complexes (~50% or less of WT complex formation but still detectable) are observed for R123A (14.6%) and R183E (7.8%). A very weak complex is observed for E99A (1.4%). The remaining mutants examined here, Y96A, D510A, Δ2, and Δβh, are completely defective in producing a UvrB-DNA complex suggesting that these regions of UvrB are essential for the transfer of damaged DNA from UvrA to UvrB.

**Probing the Damage Recognition Domains of UvrA and UvrB**—DNA substrates XL126 and XL225 (2 nM) were incubated with UvrA (200 nM) and WT UvrB or point mutants (1000 nM) for 30 min at 55 °C. BSA (lane 1) was utilized as a control. The reaction mixtures were analyzed by 4% native PAGE (19:1) using 0.5× Tris-borate-EDTA running buffer with 1 mM ATP and 10 mM MgCl₂. A graphic representation of the percentage of DNA shifted to form a UvrA₂-DNA or UvrAB-DNA complex (white bars) or B complex (black bars), n = 3, mean ± S.D. We are investigating the nature of an additional protein-DNA complex (*) whose composition is unknown.
observed for the XL126 and XL225 photoreactive DNA substrates to UvrA and WT and mutant UvrBs. No DNA-protein cross-linking is observed to BSA (lane 1), no DNA-protein cross-linking occurs in the absence of UV irradiation (lane 2), and no DNA-protein cross-linking is observed to WT UvrB in the absence of UvrA (lane 4). The percentage of DNA cross-linked to protein is summarized in Fig. 8, C and D, for the XL126 and XL225 substrates, respectively. In general, the percent of DNA cross-linked to protein was nearly 2-fold higher for the XL225 substrate compared with the XL126 substrate (average cross-linking to UvrA was 14.8% for XL225 versus 8.3% for XL126).

The presence of WT UvrB caused a decrease in the cross-linking to UvrA of ~50% for the XL126 substrate (Fig. 8, A and C, lanes 3 and 5). The XL225 substrate cross-linked to UvrA with the same efficiency in both the presence and absence of WT UvrB (Fig. 8, B and D, lanes 3 and 5). All UvrB proteins, with the exception of the Δ2 mutant, were captured to some extent (1.3–6.7%) by the XL225 substrate (Fig. 8D). Only WT and F249A UvrB were captured with XL126 (Fig. 8C). Cross-linking to UvrA is enhanced by the presence of the Δ2 and Δ9b mutants (40% increase for XL126 and 70% increase for XL225). No cross-linking is observed to UvrB in the absence of UvrA (lane 4). Percentage of DNA cross-linked to protein is reported in C and D for the XL126 and XL225 substrates, respectively (n = 3, mean ± S.D.). Gray bars indicate percent cross-linked to UvrA; black bars indicate percent cross-linked to WT or mutant UvrB.

Probing the Non-damaged Strand with S-1AZ/F26—DNA substrates NDAZ/F26 and S-1AZ/F26 (2 nM) were incubated with UvrA (200 nM) and WT or mutant UvrB (1000 nM) for 30 min at 55 °C in the dark. Samples were exposed to 5 min of UV and processed as described under “Experimental Procedures.” A and B are representative gels that display the DNA-protein cross-linking bands observed for the XL126 and XL225 substrates, respectively. No cross-linking is observed to BSA (lane 1), no cross-linking occurs in the absence of UV irradiation (lane 2), and no cross-linking is observed to WT UvrB in the absence of UvrA (lane 4). Percentage of DNA cross-linked to protein is reported in C and D for the XL126 and XL225 substrates, respectively (n = 3, mean ± S.D.). Gray bars indicate percent cross-linked to UvrA; black bars indicate percent cross-linked to WT or mutant UvrB.

Probing the Non-damaged Strand with S-1AZ/F26—DNA substrates NDAZ/F26 and S-1AZ/F26 (2 nM) were incubated with UvrA (200 nM) and WT or mutant UvrB (1000 nM) for 30 min at 55 °C in the dark. Samples were exposed to 5 min of UV and processed as described under “Experimental Procedures.” A and B are representative gels that display the DNA-protein cross-linking bands observed for the XL126 and XL225 substrates, respectively. The amount of non-target (NDAZ) cross-linking observed in Fig. 9A was subtracted from the specific (S-1AZ) cross-linking observed in Fig. 9B to yield the percentage of DNA cross-linked to each protein reported in Fig. 9C. As for the XL reagents, no DNA-protein cross-linking is observed to BSA. Due to the proposed orientation of the AZ reagent in these two substrates (see Fig. 3E), we believe that most (80% or greater) protein “captured” would be closest to the non-damaged strand of the DNA. Cross-linking of AZ containing substrates to UvrA in the presence and absence of WT UvrB or mutants is 1.5 to 3 times lower compared with the XL126 and XL225 substrates, respectively (average cross-linking to UvrA for S-1AZ/F26 was 5.4%). Approximately 2–4% cross-linking to UvrB mutants (except Δ2) is achieved with the S-1AZ/F26 substrate (Fig. 9C). Again, cross-linking to UvrA is enhanced in the presence of Δ2 UvrB, while no cross-linking to Δ2 is achieved. In contrast to both the XL-containing substrates, the Y96A mutant is captured (~2%) by the AZ-containing substrates. Finally, the DNA backbone modified substrate S-1AZ/F26 consistently captures (~2–4%) a majority (eight out of nine) of the UvrB proteins examined here, whereas the damaged strand cross-linking reagents, XL126 and XL225, capture two and seven out of nine UvrB proteins, respectively.

**DISCUSSION**

To probe the molecular handoff of damaged DNA from UvrA to UvrB during NER, we have developed two types of photoaffinity labeled DNA substrates that probe the regions of proteins making contact along the damaged or non-damaged...
strands. Two of these DNA-protein cross-linkers are arylazido-modified dNTPs, FABC-dUTP (XL1) and FAB-dCTP (XL2), reagents that function as both the lesion and the cross-linker within the DNA substrate (Fig. 2, A and B). The third DNA-protein cross-linking substrate consisted of a phosphorothioate (S-1) DNA backbone modified with AZ (Fig. 2C) on the strand of DNA opposite a fluorescein adduct at position 26. Site-specific mutants and domain deletion mutants of UvrB were used to systematically address how the DNA travels from UvrA to UvrB during the process of nucleotide excision repair. In the course of this study we found that the XL substrates (XL1 and XL2, Fig. 6C) were incised 82 and 79%, respectively, compared with a single nucleotide gapped DNA substrate (Gap26 and Gap25, 69 and 65% incised, respectively) and fluorescein-containing DNA substrate (F26/ND; 71% incised) indicating that these DNA-XL substrates were well recognized by the NER proteins. Surprisingly, we also discovered that substrates containing a single nick were good substrates for the thermophilic UvrABC NER machinery (Fig. 6C). Goosen and co-workers (40) have shown previously that the UvrBC nuclease efficiently incised DNA gaps and nicks in a UvrA-independent manner. In contrast, we find UvrA to be required for efficient incision of nicked or gapped substrates. Thus we believe our thermophilic UvrABC proteins are sensing a nick or a gap, which at 55 °C might be sufficiently dynamic and partially denatured, to be recognized as a DNA lesion.

B. caldotenax UvrA and B. caldotenax UvrB were efficiently cross-linked (3.7–20.9% and 1.3–6.7%, respectively) to the two types of photoaffinity probes. Experiments with UvrA and WT UvrB indicated that UvrA was cross-linked about 2-fold more efficiently than WT UvrB. With respect to the XL1 and XL2 containing substrates, results indicate that UvrA makes more intimate contacts with the major groove azido moiety and that the azido-DNA adduct is further removed from UvrB, despite its tight binding to DNA. Furthermore, cross-linking DNA to UvrB via FAB-dCTP (XL2) was two to four times more efficient than FABC-dUTP (XL1) indicating that the cross-link arm of XL1, which projects from the five position of uridine into the

**Fig. 9.** Cross-linking data for non-damaged strand cross-linker (AZ). DNA substrates ND$_{26}$/F26 and S-1$_{26}$/F26 (2 nM) were incubated with UvrA (200 nM) and WT or mutant UvrB (1000 nM) for 30 min at 55 °C in the dark. Samples were exposed to 5 min of UV and processed as described under "Experimental Procedures." A and B are representative gels that display the DNA-protein cross-linking bands observed for the ND$_{26}$/F26 and S-1$_{26}$/F26 substrates, respectively. For each protein, the amount of nonspecific (ND$_{26}$) cross-linking observed in A was subtracted from the specific (S-1$_{26}$) cross-linking observed in B to yield the percentage of DNA cross-linked to protein reported in C (n = 3 for S-1$_{26}$/F26 experiments, n = 4 for ND$_{26}$/F26 experiments; percent cross-linked is the mean ± S.D.). Gray bars indicate percent cross-linked to UvrA; black bars indicate percent cross-linked to WT or mutant UvrB.
**Probing the DNA Exchange from UvrA to UvrB**

Fig. 10. Model of DNA handoff from UvrA and UvrB showing points of arrest for various UvrB mutants. A = B. caldotenax UvrA, B = B. caldotenax UvrB, C = T. maritima UvrC. Amino acids determined to be important for a particular step are indicated over the reaction arrows. A blocked arrow indicates where a particular UvrB domain or point mutation results in a defective step in the DNA handoff pathway. Brackets indicate transient protein-DNA complexes that are observed when the DNA handoff pathway stalls in the presence of the specified UvrB mutant. The white arrow in step 4 indicates a gain of function for Phe169 when mutated to alanine. DNA* in step 4 indicates an altered conformation of DNA in which the DNA is bent and unwound and that the B-DNA* complex is capable of supporting incision (42). In step 5, DNA and DNA indicate DNA containing 3' and both 3' and 5' incisions, respectively.

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major groove, may not be in optimal juxtaposition in the UvrB-DNA complex for efficient cross-linking.

Analysis of photoaffinity cross-linking of several UvrB point mutations and domain deletions that arrest repair at specific points has given molecular insight into the transition of a UvrAB-DNA complex to a UvrB-DNA complex (Fig. 10). In this model, UvrA and UvrB are shown to bind to the adducted DNA in a series of discrete steps. After forming a UvrA-B complex (step 1), which is dependent upon domain 2 of UvrB, the initial contact with DNA damage occurs through UvrA (step 2). Step 3 involves Uvr isomerization where the DNA is physically moved from UvrA to UvrB. Step 4 is characterized by the release of UvrA and engagement of the DNA damage by UvrB to form the UvrB-DNA propereincision complex (41) followed by the formation of the UvrB-DNA* complex, which is incision-competent (42). UvrC is then recruited to the preincision complex near the site of DNA damage to perform both 3' and 5' incisions (step 5). The positions of the major defects for each of the UvrB mutants are indicated by a blocked arrow in each step of Fig. 10 and described below.

Based on our previous work (15), domain 2 and Arg183 of UvrB are essential for forming a productive UvrA-B complex (Fig. 10, step 1). However, based on EMSA (Fig. 7B) (15) and cross-linking data (Figs. 8 and 9), it would appear that another region of UvrB can interact with UvrA to shift the equilibrium of UvrA from monomer to dimer, facilitating DNA binding. It is possible that the C-terminal coiled-coiled region of UvrB, while helping to promote interaction with UvrA, helps to stabilize UvrA dimer formation but is not sufficient for interaction with UvrA, in the absence of DNA (15). Our data also suggest that conversion of Arg183 to glutamic acid (R183E) is a "leaky" mutation in that it greatly reduces, but does not completely block, UvrA-UvrB inter-
than 5% of WT. In addition, this mutant fails to form a stable UvrB-DNA complex (Fig. 7B, lane 5). We have proposed that Glu99 is involved in a salt-bridge contact or charge repulsion at the tip of the β-hairpin. When mutated, the β-hairpin may not be in the proper orientation to facilitate the opening of DNA (9–11). It is interesting to note that E99A UvrB did not form a cross-link with XL225 but cross-linking was achieved to this mutant with XL225 and S-1A/FP26, albeit 40 and 28% less than WT UvrB, respectively. This indicates that each of the three photoaffinity substrates appear to be probing a different region of UvrB and that Glu99 is essential for strong engagement of the adduct but not as essential for the non-damaged DNA contact.

Arg123 is located in domain 1a, close to domain 2. Very poor incorporation and very weak cross-linking with XL225 and S-1A/FP26 (Fig. 8B, lane 8, and Fig. 9B, lane 6) to the R123A mutant suggest that this residue plays a key role in the handoff of damaged DNA from UvrA to UvrB. Arg123 may provide an attractive force with the negatively charged phosphate on the non-damaged strand. In this manner, the mutant is trapped between the transition from binding the non-damaged strand and forming a tight UvrB-DNA complex. This residue may also be important in communications with domain 2, which interacts with UvrA (15).

The final residue examined in this study was Phe249, which is located in the beginning of domain 1b. Mutation of Phe249 to alanine enhanced both incision and UvrB-DNA complex formation (Fig. 7B, lane 8). This mutant was also cross-linked up to 2-fold better to the photoaffinity substrates than WT UvrB. The highest amount of cross-linking to this mutant was achieved with XL225 (12.9% UvrA and 6.7% FP249 UvrB; Fig. 8B, lane 10). These results indicate that Phe249 may play a key role in DNA binding. Phe249 is located near the DNA binding pocket of UvrB. Replacement of the larger phenylalanine residue with a smaller residue results in UvrB incapable of forming a stable DNA preincision complex (Fig. 10, step 4, white arrow), which is consistent with the results achieved in this study.

CONCLUSIONS

A new assay to study the interactions of NER proteins with damaged DNA during the DNA repair process has been successfully implemented and described. DNA-protein photoaffinity cross-linking has allowed a visualization of the architecture of the DNA when in complex with UvrA and UvrB proteins such that we can now dissect the molecular handoff of DNA from UvrA to UvrB in discrete steps, the most important step being Uvr isomerization in which UvrB comes into close contact with the adduct within the UvrA-B complex, prior to UvrA dissociation. We believe that the β-hairpin deletion and Y96A mutants are arrested during this normally transient step that proceeds by movement of the non-damaged strand into the pocket between the β-hairpin and domain 1b. This is followed by engagement of the damaged strand at the base of the β-hairpin through hydrophobic residues, primarily Tyr286. The DNA probes containing photoactive groups on both the damaged and non-damaged strands and assays described here provide a novel approach to identify and dissect specific regions of the UvrA and UvrB proteins that are in closest proximity to the site of DNA damage during NER. Experiments utilizing tandem liquid chromatography-mass spectrometry are currently in progress to further characterize the specific peptide regions of the proteins that are cross-linked.

2 B. Van Houten and M. Skovragna, manuscript in preparation.
