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Structure and Function of the UvrB Protein*

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UvrB plays a central role in (A)BC excinuclease. To identify the regions of UvrB which are involved in interacting with UvrA, UvrC, and DNA, deletion mutants, point mutants, and various fusion forms of UvrB were constructed and characterized. We found that the region encompassing amino acid residues 115–250 of UvrB binds to UvrA, while the region encompassing amino acid residues 547–673 binds to both UvrA and UvrC. In addition, the region between these two domains, which contains the helicase motifs II-VI, was found to be involved in binding to DNA. Within this DNA-binding region, two point mutants, E265A and E338A, were found to be unable to bind DNA while two residues, Phe-365 and Phe-496, were identified to interact with DNA. Furthermore, fluorescence quenching studies with mutants F365W and F496W and repair of thymine cyclobutane dimers by photoinduced electron transfer by these mutants suggest that residues Phe-365 and Phe-496 interact with DNA most likely through stacking interactions.

In *Escherichia coli,* nucleotide excision repair is initiated by the (A)BC excinuclease which excises a wide variety of DNA damages in a dodecanucleotide (Sancar and Tang, 1993; Grossman and Thiagalingam, 1993). This activity results from the coordinated actions of the UvrA, UvrB, and UvrC proteins. Specifically, UvrA is a damage recognition protein as well as a molecular matchmaker (Sancar and Hearst, 1993); it forms an A_2B_1 complex with UvrB and guides UvrB to a lesion in the DNA. Upon binding to the lesion, the DNA is bent by approximately 130° (Shi *et al.*, 1992) and the area around the lesion is unwound by about 5 bp[1] (Lin *et al.*, 1992; Visse *et al.*, 1994b). UvrA must then dissociate from the lesion before UvrC can bind to the UvrB-DNA complex to induce the dual incisions (Orren and Sancar, 1989, 1990; Visse *et al.*, 1992). Upon binding of UvrC to the UvrB-DNA complex, UvrB makes the 3′ incision after which the 5′ incision is made by UvrC (Lin *et al.*, 1992; Lin and Sancar, 1992). As evident from the ongoing presentation, UvrB is the central component of the entire excision repair process; it must interact with UvrA, bind specifically to damaged DNA, interact with UvrC, make the 3′ incision, and following the dual incisions, it must then interact with both helicase II and polymerase I to complete the repair process (Orren *et al.*, 1992). Despite its central role in excision repair, there is only a limited information on the structure-function relationship of UvrB.

In the present study, we have investigated the structural basis for the multiple interactions of UvrB during nucleotide excision repair. By assaying deletion mutants, point mutants, and various fusion forms of this protein for protein-protein interaction, DNA binding, and excision activities, the UvrA, UvrC, and DNA-binding domains of UvrB have been identified.

EXPERIMENTAL PROCEDURES

Materials—UvrA, UvrB, and UvrC proteins were purified as described by Thomas *et al.* (1985). *E. coli* strains DR153 (*recA1* Δ*uvrB*)/F′lacI^q and DR1984 (*recA1* UvrC34)/F′lacI^q were used as hosts for plasmids overproducing the mutant or wild-type proteins. T4 polynucleotide kinase, T4 DNA ligase, and restriction enzymes were purchased from Life Technologies, Inc. [γ-^{32}P]ATP (7000 Ci/mmol) was obtained from ICN Biomedicals, Inc.

UvrB Mutants and Fusion Protein—Site-specific and deletion mutants of the *uvrB* gene were made by the method of Kunkel *et al.* (1987) using the Muta-gene M13 *in vitro* mutagenesis (Bio-Rad) Kit. The *Eco*RI-*Pst*I fragment of pUNC211, which contained all but the first 21 bp of UvrB (Thomas *et al.*, 1985), was cloned into M13 mp18 (New England Biolabs). Mutations were then generated by oligonucleotide-directed mutagenesis and identified by single strand sequencing using the Sequenase DNA sequencing kit (U. S. Biochemicals) and/or restriction enzyme digestions. After the mutated gene was cloned back into the expression plasmid pUNC211, mutant constructs were reconfirmed by double-strand DNA sequencing.

Fusions of UvrB with the maltose-binding protein (MBP) were constructed by ligating different fragments of the *uvrB* gene into the pMAL-c2 expression vector (New England Biolabs). Briefly, a derivative of pUNC211, called pUNC211a, was constructed which introduced the restriction site for *Hin*cII, GTTAAC, at position 747 and a restriction site for *Hin*dIII, AAGCTT, at position 2072 of the *uvrB* gene (Arikan *et al.*, 1986). Afterwards, three different fusion proteins of UvrB to MBP were constructed: MBP/UvrB(115–250), corresponding to amino acid residues 115–250 of UvrB, was made by cloning the *Hin*cII fragment of pUNC211a from positions 342 to 750 into the *Xmn*I site of pMal-c2. MBP/UvrB(251–547), corresponding to residues 251–546 of UvrB, was made by cloning the *Hin*cII-*Eco*RV fragment of pUNC211a from positions 751 to 1783 into the *Xmn*I site of pMal-c2. MBP/UvrB(547–673), corresponding to residues 547–673 of UvrB, was made by cloning the *Eco*RV-*Hin*dIII fragment of pUNC211a from positions 1784 to 2075 into the *Xmn*I/*Hin*dIII site of pMal-c2. Fusion constructs were identified by restriction enzyme digestions.

The fusion proteins were purified as follows. Single colonies of DR153 harboring the appropriate plasmid were inoculated in 500 ml of LB with the required antibiotics and grown at 30 °C until an O.D. of 0.6 was reached. The culture was then induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside and grown for an additional 4 h. Afterwards cells were harvested and resuspended in 10 ml of lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 10% sucrose) per liter of culture. Cells were lysed by sonicating 10 × for 10 s each with a Branson Sonicator. The lysate was spun for 1 h at 40,000 × *g* at 4 °C and the supernatant was loaded onto an amylose column equilibrated with Buffer B (100 mM Tris, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 20% glycerol) + 0.1 M KCl. The column was then washed with 5 column volume of Buffer B + 0.1 M KCl. Bound proteins were then eluted with 1 column volume of Buffer B + 0.1 M KCl + 10 mM maltose. Purified proteins were dialyzed and stored in storage buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 50% glycerol).

Since fusion proteins were used for most of the studies to identify

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[1] The abbreviations used are: bp, base pair(s); MBP, maltose-binding protein; TRCF, transcription-repair coupling factor; T<>T, thymine cyclobutane dimer; T<>HMT, thymine-furanside monoadduct of 4′-hydroxymethyl-4,5′,8-trimethylpsoralen; ABPD, 2-aminobutyl-1,3-propanediol; ssDNA and dsDNA, single- and double-stranded DNA, respectively; PAGE, polyacrylamide gel electrophoresis.

structural domains in UvrB it was essential to establish that these proteins were "well behaved" with regard to their solubility and quaternary structures. All of the constructs used were as soluble as the wild-type protein and those that were poorly soluble (*e.g.* MBP/UvrB(Δ114–120) and MBP/UvrB(Δ207–225) were only characterized genetically. In addition, the constructs used for biochemical studies behaved as monomers as determined by gel permeation chromatography under conditions employed to determine the oligomerization status of the wild-type protein. Thus, we conclude that the interactions we detect for the fusion constructs are intrinsic to the UvrB part of the fusions and are not compounded by competing interactions that arose from newly created protein interfaces.

Protein Affinity Chromatography—Protein-protein interactions between wild-type and mutant UvrBs and UvrA, and UvrC were detected by affinity chromatography. UvrA, UvrB, or UvrC proteins were covalently cross-linked to the Affi-Gel-10 resin as recommended by the manufacturer (Bio-Rad). In general, a typical column contained 3–5 mg of protein per 500 μl of resin. The following procedure was then used to detect protein-protein interaction with purified proteins. 1.0 μg of the test protein (or cell-free extract from 1–32 ml of an appropriate culture) was loaded onto an affinity column that had been equilibrated with Binding Buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, and 20% glycerol) + 0.1 M KCl and washed with 10 column volumes of Binding Buffer + 0.1 M KCl at a flow rate of 2 ml/h. Bound fractions were then eluted with 2 column volumes of Binding Buffer + 0.5 M KCl. Fractions of 250 μl were collected and analyzed by SDS-PAGE and silver staining.

DNA Binding—The binding of UvrB to DNA was quantified by gel retardation assays. Substrates tested were terminally labeled 12- or 13-nucleotide long oligomers containing either an unmodified base or a modified mono- or dinucleotide in the center. The following modified bases were tested: (i) thymine-monoadduct of 4′-hydroxymethyl-4,5′-8-trimethylpsoralen (T<>HMT): 5′-GCTCGG(T<>HMT)ACCCGG-3′; (ii) 1,2-d(GpG) cisplatin [cis-platinum(II)diammine dichloride] diadduct: 5′-TCTA(G G)CCTTCT-3′; (iii) synthetic "AP" site (2-aminobutyl-1,3-propanediol, ABPD): 5′-GAAGC(ABPD)ACGAGC-3′.

The psoralen and cisplatin containing oligomers were kindly provided by Drs. John E. Hearst (University of California, Berkeley) and Stephen J. Lippard (Massachusetts Institute of Technology). Oligomers without a modified base or with a synthetic AP site were obtained from Operon Biotechnologies. Binding assays of UvrB to these oligonucleotides were done as follows. 1 nM ^{32}P-labeled oligomers were incubated with increasing concentrations of UvrB at 22 °C for 20 min in 50 μl of ABC buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.1 M KCl, 1 mM dithiothreitol, and 10% glycerol). The reaction mixtures were then loaded onto a 10% polyacrylamide gel and electrophoresed at 80 V for 5 h at 4 °C. Binding of UvrB to DNA was then quantified by AMBIS scanning. Since the binding of UvrB to DNA is of low affinity, the bound fraction does not always yield a sharp band; in fact, quite often it appears as a smear above the free (unbound) DNA. Thus, the fraction of DNA not in the band corresponding to free DNA was considered bound in our analysis.

Fluorescence Quenching—UvrB mutants containing a tryptophan residue at position 88, 107, 187, 216, 365, 496, or 527 were used to investigate DNA binding by fluorescence quenching. In these mutants, phenylalanine residues were individually replaced by tryptophans at the indicated positions (Phillips, 1994). Since wild-type UvrB does not contain any tryptophan, any fluorescence detected in these mutants is a result of the single amino acid replacement. Fluorescence quenching studies were done as follows: 3 μM mutant UvrB protein was incubated with 130 μM (in nucleotide) calf thymus DNA in 500 μl of ABC buffer for 5 min at 4 °C. All spectras were taken with a 1-cm path length quartz cuvette at 15 °C and measured with an excitation wavelength of 295 nm with a Shimadzu model RF5000U spectroflurometer.

Repair of Thymine Dimer by UvrB—The proximity of the tryptophan residues, which were introduced into UvrB, to DNA was determined by the splitting of T<>T by the bound protein. A single strand 49-mer containing a centrally located T<>T (Svoboda *et al.*, 1993) was terminally labeled with [γ-^{32}P]ATP. 5.0 μM mutant proteins were incubated with 1.0 nM substrate in 250 μl of ABC buffer for 20 min at 22 °C. The samples were then irradiated with 293 nm light in a Quantacount monocoromator at a fluence rate of 500 microwatts/cm^2 for 30 min. The DNA was extracted with phenol/cholorform, ethanol precipitated, annealed with a 5-fold molar excess of the complementary strand, treated with *Mse*I endonuclease, and then electrophoresed on a 12% denaturing gel. The photodimer is located within a *Mse*I recognition sequence, T<>TAA; thus photoreversal of the dimer restores the susceptibility of this sequence to the restriction endonuclease (Li *et al.*, 1993) and repair is measured by the extent of *Mse*I digestion.

DNA-Protein Cross-linking—Cross-linking of the UvrB protein to a psoralen-adducted oligomer was conducted as described previously (Orren *et al.*, 1992) to investigate the binding of UvrB to DNA. Briefly, 1 nM ^{32}P-labeled 13-mer oligonucleotide with a psoralen adduct was incubated with 10 μM wild-type or mutant UvrB in 30 μl of ABC buffer for 20 min at 22 °C. Then, the reaction mixture was irradiated for 30 min with 366 nm light from a Sylvania black lamp at a fluence rate of 3 milliwatts/cm^2. The samples were electrophoresed on SDS-PAGE and the level of cross-linking was estimated by autoradiography.

Incision Assay—A 137-mer duplex with a centrally located furanside thymine-psoralen monoadduct (T<>HMT) (Van Houten *et al.*, 1987) was labeled with ^{32}P at both termini on the damaged strand and used in an incision assay as described previously (Lin *et al.*, 1992). Briefly, 1 nm of substrate, 5 nM UvrA, 80 nM UvrB, 40 nM UvrC, and 0.1 μg of unmodified pBR322 DNA were incubated in 25 μl of ABC buffer containing 2 mM ATP for 20 min at 37 °C; the reaction was stopped by adding 2 μl of a 1:1 mixture of 0.25 M EDTA and 5 mg/ml oyster glycogen, precipitated with 60 μl of ice-cold ethanol and analyzed on 8% denaturing gels. The incision products observed were a 66-mer representing the 5′ incision, a 60-mer representing the 3′ incision, and a 78-mer representing the 3′ uncoupled incision.

RESULTS

Protein-Protein Interactions—Previous studies failed to detect any UvrB-UvrC interaction off DNA; therefore, it was assumed that UvrB, upon binding to damaged DNA, undergoes a conformational change which enables UvrC to bind to the UvrB-damaged DNA complex (Orren and Sancar, 1989). However, these studies employed hydrodynamic methods which are not practical for detecting weak protein-protein interactions between a large number of protein pairs. To study a possible, direct interaction between UvrB and UvrC, protein affinity columns of UvrB and UvrC were employed. When *E. coli* cell-free extract from a strain overproducing the UvrB protein was passed through the UvrC affinity column, the UvrB protein was retained on the column (Fig. 1*A*). However, in addition, RNA polymerase was also retained on the column with relatively high affinity raising some doubt about the specifity of binding. A control column without any cross-linked protein was used to further clarify the specificity of these bindings. UvrB did not bind to the control column, but some RNA Pol did (data not shown), suggesting that the binding of UvrB to the UvrC column is specific, while the binding of the subunits of RNA Pol to the UvrC column is primarily nonspecific. To further ascertain that the UvrB-UvrC interaction revealed by this assay was specific, *E. coli* cell-free extract from a strain overproducing the UvrC protein was passed through an UvrB affinity column. Fig. 1*B* shows that in this case, only the UvrC protein is retained on the column. Thus, we conclude that UvrB and UvrC do interact off DNA specifically, but with lower affinity than the UvrA-UvrB interaction, which can be easily detected by hydrodynamic methods. More importantly, since these columns were able to detect weak but specific protein-protein interactions, they were used subsequently to identify the UvrA and UvrC binding domains of UvrB.

UvrA-binding Domains of UvrB—Protein sequence of the Mfd protein (transcription-repair coupling factor, TRCF) revealed a stretch of 137 amino acids which displayed a high degree of sequence homology to amino acid residues 115–250 of the UvrB protein (Selby and Sancar, 1993). Since both TRCF and UvrB bind to the UvrA protein, it was hypothesized that this region of homology may be involved in binding to UvrA. In addition, a screen for dominant negative *uvrB*$^-$ mutants generated by random mutagenesis yielded a mutant expressing only the amino-terminal half of UvrB (Moolenaar *et al.*, 1994), further supporting the idea that residues 115–250 of UvrB binds to UvrA. To test these predictions and narrow down the binding site, a fusion of the maltose-binding protein with amino acid residues 115–250 of UvrB ((MBP/UvrB(115–250)) was con-

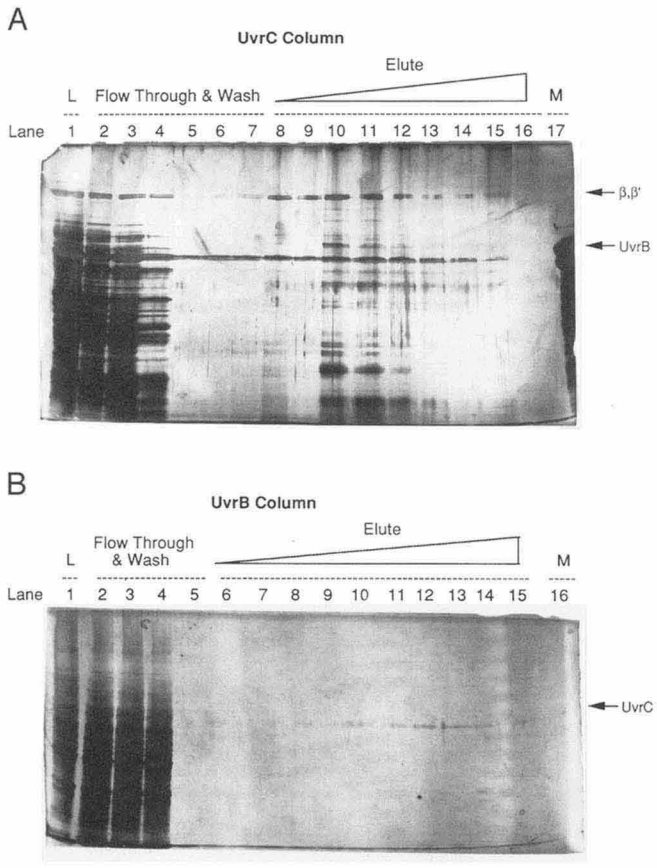

FIG. 1. *A*, binding of UvrB protein to UvrC affinity column. Cell-free extract from 30 ml of DR153/pUNC211 culture overexpressing the UvrB protein was loaded onto an UvrC affinity column (0.5 ml) containing 3 mg of UvrC protein. The column was washed with 10 column volumes of binding buffer + 0.1 M KCl and bound fractions were eluted with a continuous gradient of binding buffer + 0.1 M KCl to binding buffer + 1.0 M KCl. Fractions of 0.25 ml were collected and analyzed by SDS-PAGE gels and silver staining. *L*, load; *M*, purified UvrB as a marker; *β*, *β'*, large subunits of RNA polymerase. *B*, binding of UvrC protein to UvrB affinity column. Cell-free extract from 1.2 ml of DR1984/pUNC3274 culture overexpressing the UvrC protein was loaded onto an UvrB affinity column (0.4 ml) containing 5 mg of UvrB protein. The column was washed and bound proteins were eluted and analyzed as in *panel A*.

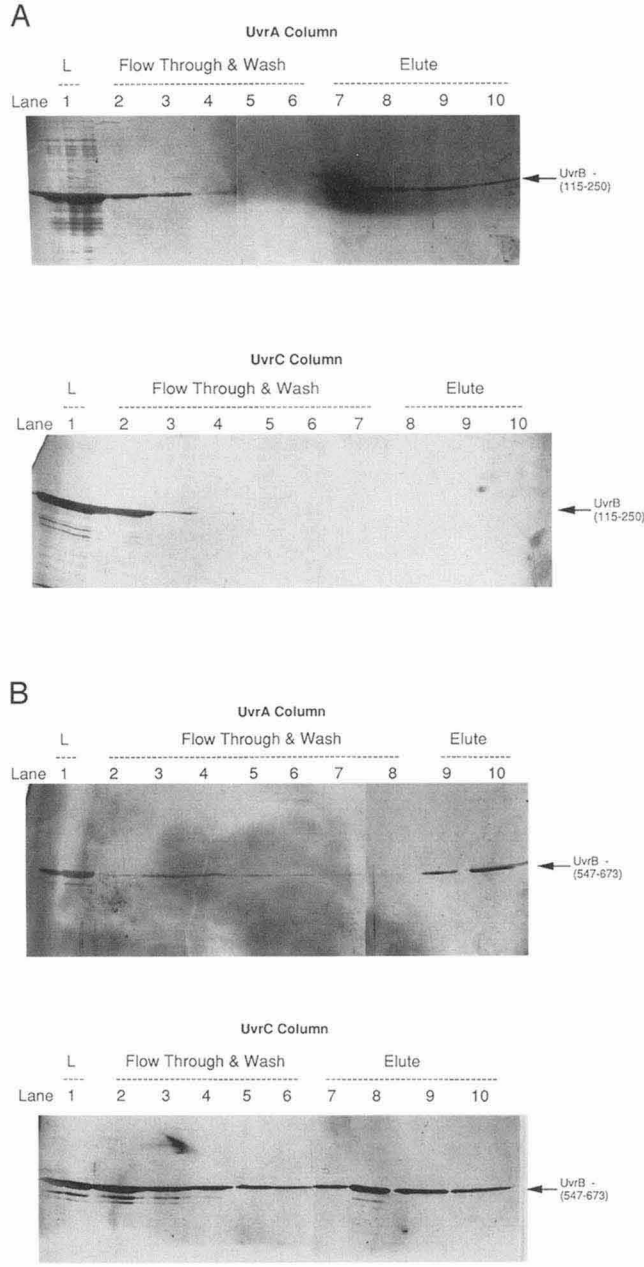

FIG. 2. *A*, binding of MBP/UvrB(115–250) to UvrA and UvrC affinity columns. 1.0 μg of MBP/UvrB(115–250) protein was loaded onto either an UvrA (*top panel*) or an UvrC (*bottom panel*) affinity column. The column was washed with 10 column volumes of binding buffer + 0.1 M KCl and bound fractions were eluted with binding buffer + 0.5 M KCl. Fractions of 0.25 ml were collected and analyzed by SDS-PAGE gels and silver staining. *L*, load; *M*, purified UvrC as a marker. *B*, binding of MBP/UvrB(547–673) to UvrA and UvrC affinity columns. 1.0 μg of MBP/UvrB(547–673) was loaded onto either an UvrA (*top*) or an UvrC (*bottom*) affinity column. The column was washed and bound proteins were eluted and analyzed as in *panel A*.

structed and applied to the UvrA and UvrC affinity columns. Fig. 2*A* shows that this fusion protein binds to the UvrA affinity column but not to the UvrC column, suggesting that this region of UvrB specifically interacts with UvrA. In addition, 4 deletion mutants of UvrB within this region were constructed (MBP/UvrB(Δ114–120), MBP/UvrB(Δ136–145), MBP/UvrB-(Δ166–173), and MBP/UvrB(Δ207–225)) and tested for complementation *in vivo*. All these deletion mutants failed to complement a *uvrB*$^-$ mutation, presumably because of failure to bind to UvrA. However, these mutant proteins had low solubility and were not amenable to *in vitro* analysis.

UvrC-binding Domains of UvrB—To identify the region of UvrB which binds to UvrC, two other MBP fusion proteins of UvrB were made. One, MBP/UvrB(251–546), was comprised of the so-called helicase motifs II-VI of UvrB, while the other, MBP/UvrB(547–673), consisted of the carboxyl-terminal 126 amino acids of UvrB. Analysis of these fusions using the UvrA and UvrC affinity columns revealed that MBP/UvrB(547–673) bound to the UvrC column (Fig. 2*B*) and interestingly enough, also bound to the UvrA column with an affinity comparable to that for the MBP/UvrB(115–250) fusion (Fig. 2, *A versus B*). Thus, it appears that while UvrC binds only to the carboxyl-terminal domain of UvrB, UvrA binds to both the amino-terminal and carboxyl-terminal domains of UvrB, suggesting that the binding domains of UvrA and UvrC in the carboxyl-terminal half of UvrB may overlap. Furthermore, the UvrA- and UvrC- binding regions on UvrB appear to be restricted to the areas defined by these fusion proteins as MBP/UvrB(251–546) failed to bind to either the UvrA or UvrC affinity column (data not shown).

DNA Binding by UvrB—It was previously believed that UvrB could not bind DNA by itself, but that upon loading of UvrB by UvrA onto DNA, a stable UvrB-DNA complex is formed (Orren and Sancar, 1989). However, when a 13-nucle-

otide long oligonucleotide with a psoralen adduct was mixed with the UvrA, UvrB, and UvrC proteins, it was discovered that upon irradiation with 366 nm light, only UvrB became cross-linked to the psoralen adduct (Orren *et al.*, 1992). This suggested that UvrB could perhaps interact with damaged DNA in the absence of UvrA. Thus, we decided to investigate the direct binding of UvrB to DNA in more detail.

Binding to Damaged Oligomers—The cross-linking of UvrB to a 13-nucleotide long oligomer containing a psoralen furanside monoadduct raised the possibility that UvrB may bind specifically to ssDNA containing a lesion. Using the standard gel retardation assay, we tested the binding of UvrB to single-strand oligomers with three lesions known to be repaired by the (A)BC excinuclease: thymine-psoralen monoadduct, 1–2-d(GpG) cisplatin diadduct, and a synthetic AP site. The results are shown in Fig. 3. UvrB is indeed a DNA-binding protein, albeit with lower affinity than most specific DNA-binding proteins. It binds with higher affinity to psoralen or cisplatin-modified DNA ($K_d \sim 5 \times 10^{-6}$ M) than unmodified DNA or DNA containing an AP site ($K_d > 10^{-5}$ M). Furthermore, it appears that UvrB binds with higher affinity to lesions that are efficiently removed by (A)BC excinuclease (psoralen and cisplatin) than lesions that are poorly removed by this enzyme (AP site).

To determine if UvrB also bound specifically to double-strand DNA with a lesion, gel retardation assays with single- and double-stranded DNAs containing a thymine-psoralen monoadduct were conducted. Fig. 4 shows that UvrB binds to ssDNA with T<>HMT but has no affinity for dsDNA containing the same adduct. Furthermore, it also appears that UvrB cannot discriminate between ssDNA and dsDNA when the DNA has no lesion (Fig. 5), which further supports the notion that UvrB binds specifically only to ssDNA with a lesion.

Fluorescence Quenching—Quenching of tryptophan fluorescence by DNA is a commonly used and sensitive method for studying DNA-protein interactions (see Ferrari *et al.* (1994)). Wild-type UvrB does not contain any tryptophan, but does contain 23 phenylalanine residues scattered throughout the protein (Arikan *et al.*, 1986; Backendorf *et al.*, 1986). By replacing phenylalanines with tryptophans through site-directed mutagenesis, we generated fluorescent UvrB derivatives in order to investigate UvrB-DNA interaction by fluorescence quenching.

Seven phenylalanine residues that were distributed throughout the primary structure of UvrB were replaced individually with tryptophans to obtain UvrB mutants with a Trp residue at positions 88, 107, 187, 216, 365, 496, or 527. Since the Phe to Trp substitution is a conservative change, the mutant proteins were fully functional and behaved identically to wild-type UvrB protein in every aspect including *in vivo* complementation. The mutant proteins were purified and the quenching of Trp fluorescence of these mutants by ssDNA was investigated.

The results of these studies are shown in Fig. 6. Several features of this data should be noted. First, the Trp emission maxima of all the mutants are between 330 and 340 nm, which is lower than the emission maximum of 355 nm for free tryptophan. The blue shift of the Trp emission typically occurs when the tryptophan is in a hydrophobic environment. Thus, it appears that all of the Trp residues and by extension, all of the Phe residues which were replaced by Trp residues, are located in a hydrophobic environment within UvrB. Second, according to fluorescence intensity, these mutants fall into three classes; F107W with the lowest intensity; F88W and F527W with intermediate intensity; and F187W, F216W, F365W, and F496W with the highest level of intensity. The environments of the various Trp residues influence the deactivation of the excited tryptophans by non-emissive pathways to different degrees and

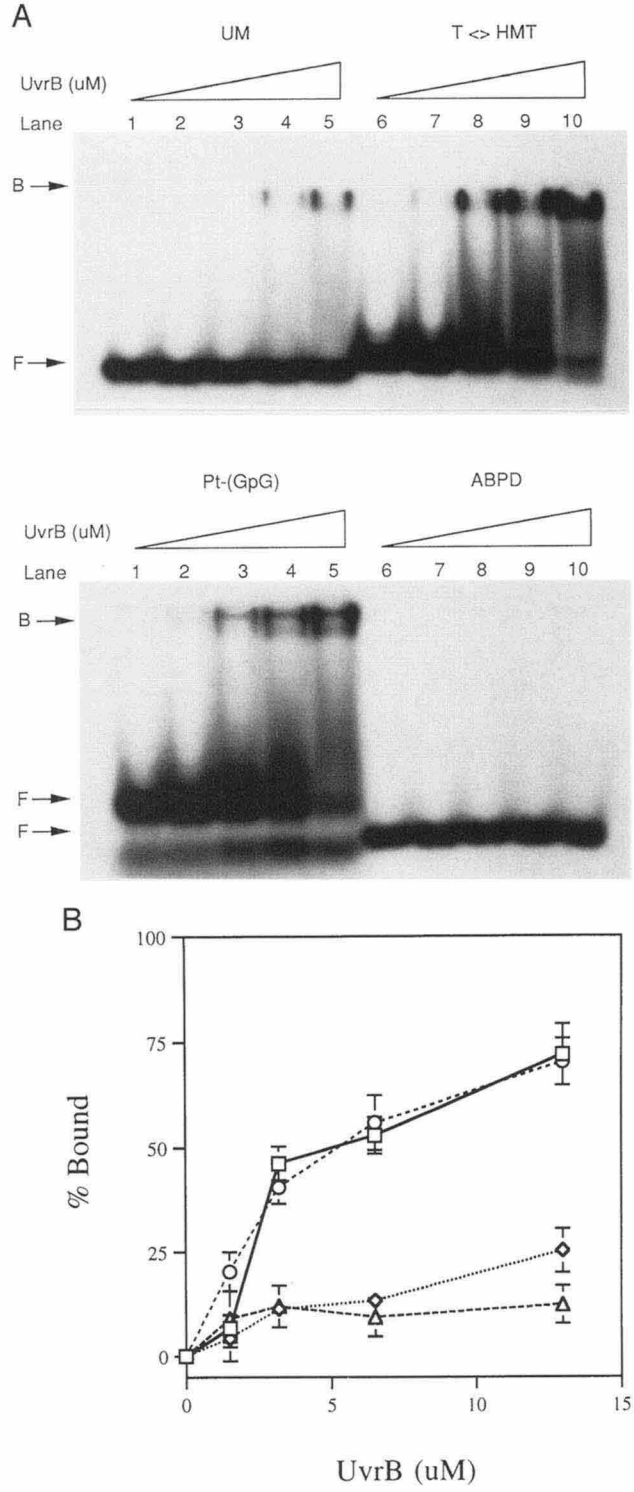

FIG. 3. *A*, binding of UvrB protein to ssDNA containing a lesion. 5′-labeled substrates (1 nM) were incubated in a 50-μl reaction mixture containing increasing concentrations of UvrB in ABC buffer for 20 min at 22 °C. Samples were then loaded onto a 10% ploy acrylamide gel and electrophoresed at 80 V for 5 h at 4 °C. The specific activities of the substrates were as follows: T<>HMT, 14.8 Ci/nmol; Pt-(GpG), 18.6 Ci/nmol; ABPD, 7.4 Ci/nmol; and unmodified (*UM*), 7.6 Ci/nmol. *F*, free DNA; *B*, bound DNA band. The UvrB concentrations in the reaction mixtures were: *Lanes 1* and *6*, 0 μM; *lanes 2* and *7*, 1.5 μM; *lanes 3* and *8*, 3.2 μM; *lanes 4* and *9*, 6.5 μM; and *lanes 5* and *10*, 13 μM. *B*, quantitative analysis of the binding data. The percent binding was determined by quantification of the radioactivity in the unbound fractions. Data from *panel A* and two other experiments conducted under identical conditions were averaged. *Error bars* show standard deviation. *Square*, T<>HMT; *circle*, Pt-(GpG); *triangle*, APBD; *diamond*, unmodified.

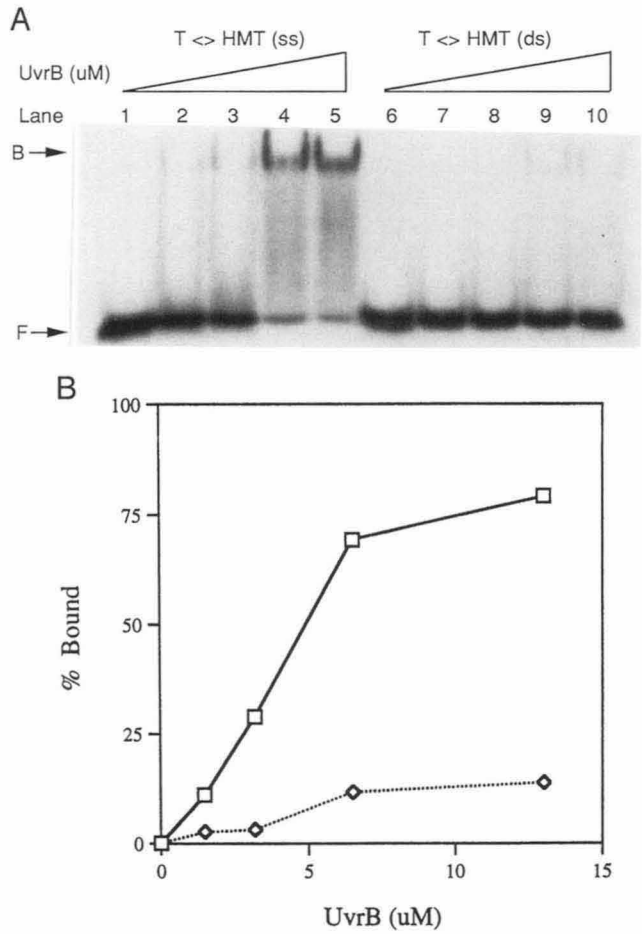

FIG. 4. *A*, binding of UvrB protein to ssDNA and dsDNA containing a thymine-psoralen monoadduct. 5′ labeled single- and double-strand DNA containing a thymine-psoralen monoadduct were incubated in a 50-μl reaction mixture containing increasing concentrations of UvrB in ABC buffer for 20 min at 22 °C. Samples were then loaded onto a 10% polyacryamide gel and electrophoresed at 80 V for 5 h at 4 °C. *F*, free DNA; *B*, bound DNA band. The UvrB concentrations in the reaction mixtures were: *Lanes 1* and *6*, 0 μM; *lanes 2* and *7*, 1.5 μM; *lanes 3* and *8*, 3.2 μM; *lanes 4* and *9*, 6.5 μM; and *lanes 5* and *10*, 13 μM. *B*, quantiative analysis of data in *panel A*. The percentage of binding was determined by quantification of the radioactivity in the unbound fractions from *panel A*. *Square*, ssDNA-T<>HMT; *diamond*, dsDNA-T<>HMT.

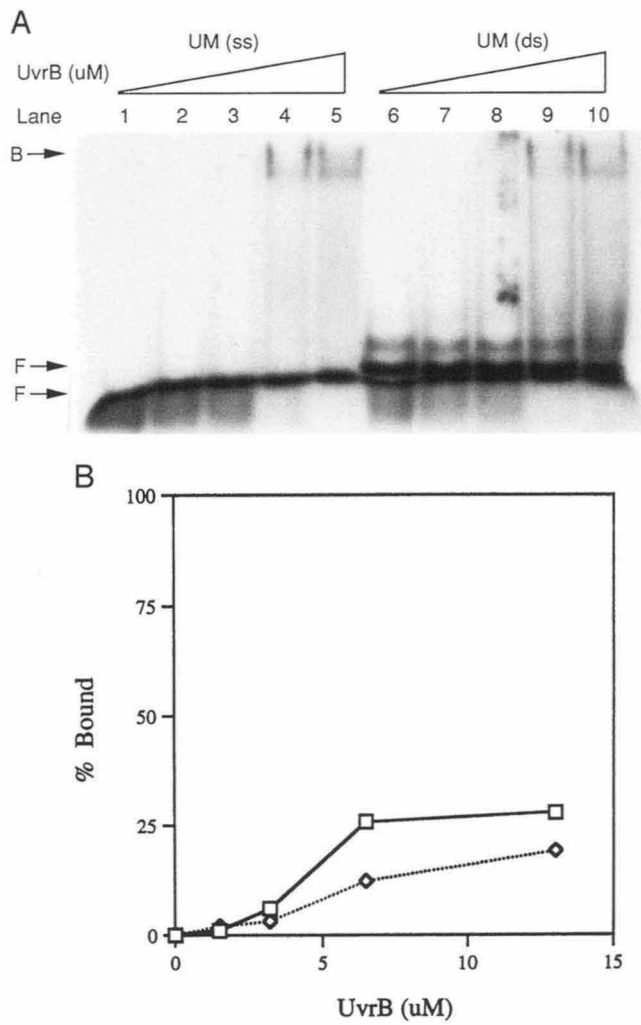

FIG. 5. *A*, binding of UvrB protein to single-strand and double-strand oligonucleotide. 5′ labeled single- and double-strand DNA were incubated in a 50-μl reaction mixture containing increasing concentrations of UvrB in ABC buffer for 20 min at 22 °C. Samples were then loaded onto a 10% polyacrylamide gel and electrophoresed at 80 V for 5 h at 4 °C. *F*, free DNA; *B*, bound DNA band. The UvrB concentrations in the reaction mixtures were: *Lanes 1* and *6*, 0 μM; *lanes 2* and *7*, 1.5 μM; *lanes 3* and *8*, 3.2 μM; *lanes 4* and *9*, 6.5 μM; and *lanes 5* and *10*, 13 μM. Note that the dsDNA contains some ssDNA contaminant, and a slower migrating minor species of unknown origin. *B*, quantitative analysis of data in *panel A*. The percentage of binding was determined by quantification of the radioactivity in the unbound fractions from *panel A*. *Square*, ssDNA; *diamond*, dsDNA.

as a result, different fluorescence intensities are observed. To a first approximation, tryptophans more accessible to solvent or polar amino acids are more likely to decay by non-emissive pathway and hence, have lower quantum yield of fluorescence. Table I shows the quantum yields of Trp fluorescence for the various UvrB mutants. Finally, ssDNA specifically quenches the fluorescence of three mutants, F187W, F365W, and F496W. Quenching can be caused by either direct contact of the DNA with these tryptophans or as a result of a conformational change that may have occurred upon DNA binding which affects the solvent accessibility to these residues. In order to differentiate between these two possibilities, we conducted photoinduced dimer splitting by wild-type and mutant proteins.

Pyrimidine Dimer Splitting by UvrB—In model studies on photosensitized splitting of pyrimidine dimers, it has been found that excitation of free indoles in solution (Helene and Charlier, 1977), tethered to the dimer (Kim *et al.*, 1990), or as Trp residues in single-strand DNA-binding proteins (Helene and Charlier, 1977), all lead to the cleavage of the cyclobutane ring by photoinduced electron transfer (see Kim *et al.*, 1992). In addition, for the reaction to occur from a DNA-bound protein with reasonable efficiency, the tryptophan must be in contact with the DNA (see Kim *et al.*, 1992). Therefore, to determine if the fluorescence quenching of F187W, F365W, and F496W resulted from contact of the Trp residue with DNA or from a conformational change upon binding, the abilities of these mutants to split dimers were investigated.

Wild-type and mutant UvrB proteins were mixed with a 49-nucleotide long single-strand DNA with a centrally located T<>T within the *Mse*I recognition site T<>TAA (Svoboda *et al.*, 1993) and irradiated with 293 nm light. Fig. 7 shows that none of the mutants which did not display any fluorescence quenching could photoreverse the dimer. Of the three mutants which displayed fluorescence quenching, F365W and F496W were capable of repairing the dimer, while F187W was not. We conclude that the quenching of fluorescence observed with F365W and F496W is the result of direct contact of the tryptophans in these mutants with the DNA and hence, these residues must be located within the DNA-binding domain of UvrB.

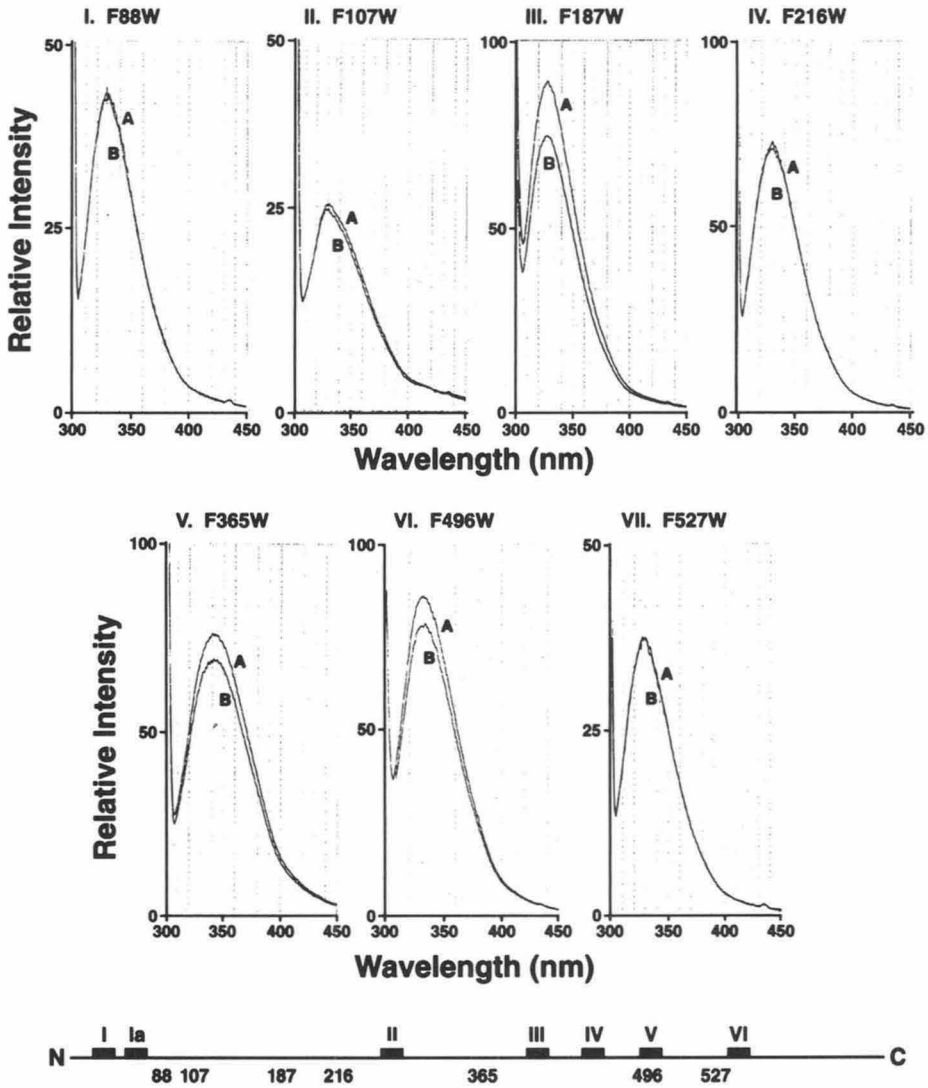

FIG. 6. **Fluorescence quenching of UvrB-Trp mutants.** 3 μM mutant UvrB protein was incubated with 130 μM (in nucleotides) calf-thymus DNA (ssDNA) in 500 μl of ABC buffer. Fluorescence emission spectra were measured with excitation at 295 nm. *A* and *B* indicates fluorescence emission before and after the addition of DNA, respectively. The *diagram* in the *bottom* represents the primary sequence of the UvrB protein, the location of the helicase domains I-VI (*boxes*), and the sites of the F → W substitutions. The slit width was adjusted for each mutant to obtain an optimal signal, hence the relative intensities in different panels are not directly comparable.

TABLE I
Fluorescence Properties of UvrB (F→W) Mutants

The reaction mixtures contained 3 μM of Trp either in UvrB or dissolved in buffer and 130 μM (in nucleotides) calf thymus DNA (ssDNA).

Mutant	λ_{max} (nm)	ϕ_F[a]	% Fluorescence quenching by DNA	% T⟨⟩T repair
F88W	328	0.01	2	0
F107W	329	0.02	4	0
F187W	328	0.10	22	0
F216W	329	0.01	2	0
F365W	342	0.06	12	35
F496W	332	0.01	10	20
F527W	329	0.02	2	0
Tryptophan	353	0.12	0	0

[a] ϕ_F is fluorescence quantum yield in ABC buffer.

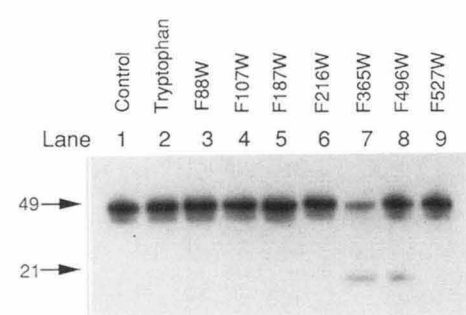

FIG. 7. **The repair of thymine dimer by mutant UvrB proteins.** A single-stranded 49-mer (1 nM) containing a centrally located T<>T was incubated with 5 μM mutant UvrB protein in 250 μl of ABC buffer and irradiated with 293 nm light at a fluence rate of 500 microwatts/cm^2 for 30 min. After irradiation, the DNA was extracted with phenol/chloroform, annealed with the complementary strand, and treated with *Mse*I restriction endonuclease, which digests only the repaired DNA, to generate the 21-mer. This is an autoradiograph of a 10% polyacrylamide gel.

In contrast, repeated attempts to repair dimers with F187W failed even though this mutant was quenched by DNA twice as efficiently as the other two mutants. It appears that upon binding to DNA, UvrB undergoes a significant conformational change such that residue 187 becomes substantially exposed to the solvent leading to drastic quenching of fluorescence of F187W.

Psoralen-mediated Cross-linking and Gel Retardation of UvrB Mutants to Oligonucleotides—Furanside thymine-psoralen monoadduct is photoreactive and thus an oligonucleotide containing this adduct can be photocross-linked with 366 nm irradiation to proteins which bind this DNA (Orren *et al.*, 1992). In order to further define the DNA-binding region of UvrB, photocross-linking experiments were done on *UvrB*$^-$ mutants which were isolated by alanine scanning mutagenesis within the putative DNA-binding domain of UvrB. In addition, two other mutants that had been previously characterized as

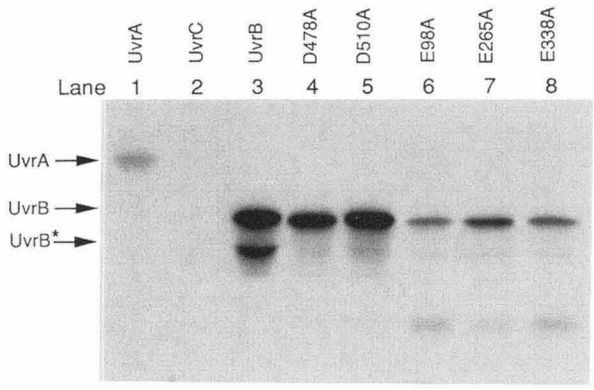

FIG. 8. **Psoralen-mediated cross-linking of mutant UvrB proteins.** 1 nM terminal labeled psoralen adducted 13-mer oligonucleotide was incubated with 10 μM UvrA, UvrC proteins, or wild-type or mutant UvrB proteins in 30 μl of ABC buffer for 20 min at 22 °C. The mixture was irradiated with 366 nm light at a fluence rate of 3 milliwatts/cm^2 for 30 min at 4 °C. The products were analyzed on 15% SDS-PAGE followed by autoradiography.

either a DNA bending mutant, D478A (Lin *et al.*, 1992; Hsu *et al.*, 1994), or binding mutant, D510A (Lin *et al.*, 1992), were tested as controls. The results (Fig. 8) revealed that three mutants, E98A, E265A, and E338A, had greatly reduced cross-linking efficiency compared to the wild-type protein. In addition, the D478A mutant cross-linked to DNA with an efficiency comparable to that of the wild-type UvrB protein, which is in agreement with the earlier conclusion that this mutant bound DNA normally but was unable to bend it. Unexpectedly, the D510A mutant which cannot be loaded onto DNA by UvrA, was also cross-linked quite efficiently. To investigate the DNA binding properties of these mutants further, we conducted gel retardation experiments with the same set of mutants.

The results are shown in Fig. 9. Three mutants, E98A, E265A, and E338A had no detectable affinity for DNA; D510A had lower affinity, and D478A had affinity for DNA comparable to that of wild-type. The discrepancy between the earlier conclusion that D510A is a DNA binding mutant (Lin *et al.*, 1992) and the current result can be reconciled by assuming that the main defect in this mutant is in the loading step (which is what was measured previously) rather than formation of a complex with the single-stranded region of the DNA after the loading reaction. In contrast, the three mutants, E98A, E265A, and E338A, are true DNA binding mutants which are defective in maintaining a stable complex subsequent to loading. In agreement with this conclusion, it was not possible to isolate UvrB-DNA complexes of these mutants by gel exclusion chromatography (data not shown), suggesting that these mutants either have no affinity for DNA or bind DNA very transiently.

To investigate the latter point, incision assays were performed with (A)BC excinuclease reconstituted with the mutant proteins. The results are shown in Fig. 10. Unlike wild-type UvrB, only the 3′ incision was made by the (A)BC excinuclease reconstituted with the E98A, E265A, and E338A mutants. Since all available data suggest that UvrC makes the 5′ incision (Lin and Sancar, 1992) and that the 5′ incision, under certain conditions, is the rate-limiting step (Visse *et al.*, 1994a), these results suggest that a transient UvrB-DNA complex is formed with the mutant UvrBs and when UvrC interacts with this complex, UvrB is able to make the 3′ incision. However, it also appears that these complexes are so transient that UvrB dissociates from the DNA before UvrC can make the 5′ incision.

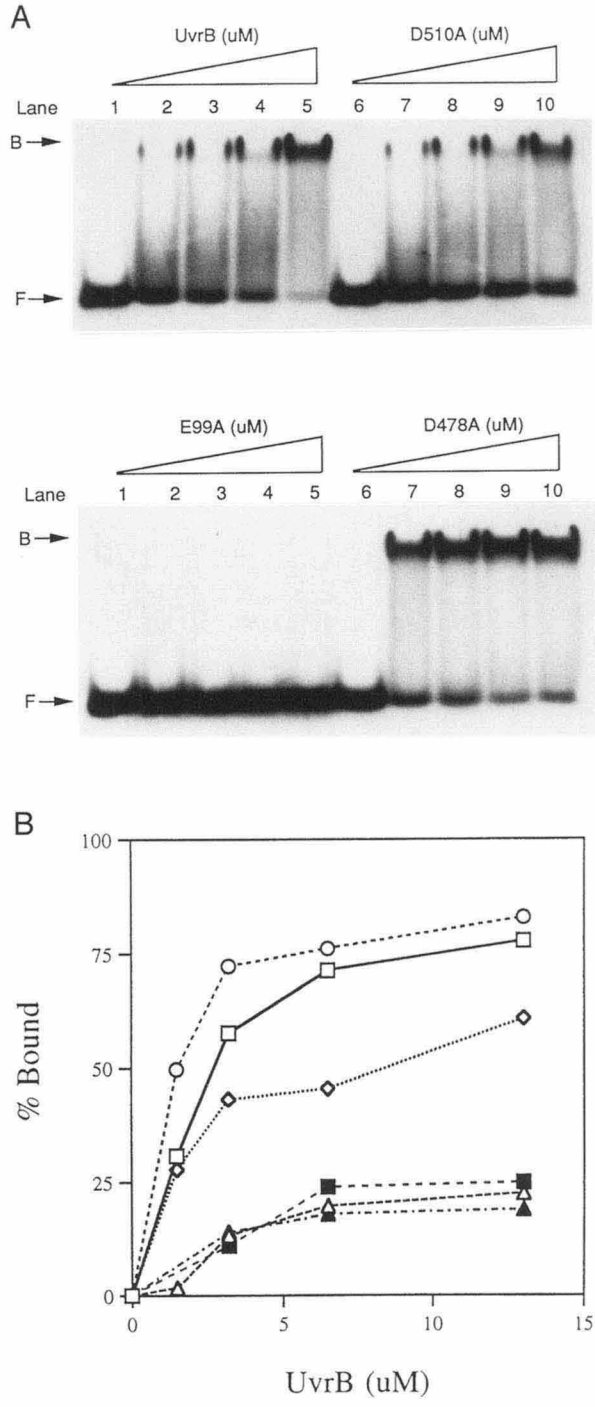

FIG. 9. **Binding of mutant UvrB protein to oligonucleotide containing a thymine-psoralen monoadduct.** 5′ labeled ssDNA (1 nM) containing a T<>HMT was incubated in 25 μl of ABC buffer containing wild-type or mutant UvrB proteins at the indicated concentrations for 20 min at 22 °C. Samples were then loaded onto a native 10% polyacrylamide gel and electrophoresed at 80 V for 5 h at 4 °C. *A*, autoradiographs of representative gels. WT or mutant UvrB concentrations were as follows: *lanes 1* and *6*, 0 μM; *lanes 2* and *7*, 1.25 μM; *lanes 3* and *8*, 2.5 μM; *lanes 4* and *9*, 6 μM; and *lanes 5* and *10*, 12.5 μM. *B*, quantitative analysis of the binding data. Data points from three experiments were averaged. The standard errors for all of the data points were less than 10% and hence error bars are not shown for clarity. *Square*, wild-type; *diamond*, D510A; *circle*, D478A; *triangle*, E98A; *closed square*, E265A; and *closed triangle*, E338A.

DISCUSSION

UvrB plays a central role in nucleotide excision repair. It interacts with both the UvrA and UvrC proteins in addition to binding, bending, and incising DNA. In this work, we have attempted to identify the regions of UvrB involved in interacting with UvrA, UvrC, and DNA. In light of our findings, the

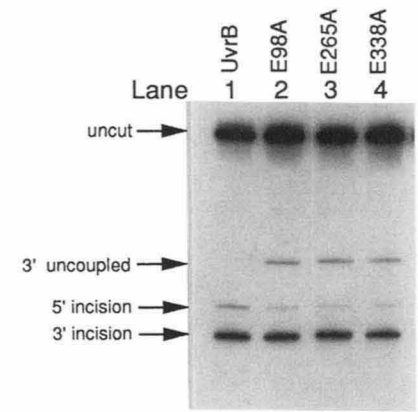

FIG. 10. **Incision of a 137-mer duplex with a centrally located furanside thymine-psoralen monoadduct by (A)BC excinuclease reconstituted with wild-type and mutant UvrB proteins.** 1 nM substrate labeled at both termini of the damaged strand was incubated in 25 μl of ABC buffer + 2 mM ATP containing 5 nM UvrA, 80 nM wild type or mutant UvrB, 40 nM UvrC, and 0.1 μg of pBR322 for 20 min at 37 °C. The reaction was stopped by adding 2 μl of a 1:1 mixture of 0.25 M EDTA, 5 mg/ml oyster glycogen, and 60 μl of EtOH. The precipitate was collected by centrifugation, resuspended in formamide/dye mixture, and analyzed on a 8% polyacrylamide DNA sequencing gel. The products generated by the dual incisions are a 66-mer indicated by *5' incision*, and a 60-mer indicated by *3' incision*, respectively. The band marked *3' uncoupled* is a 78-mer which can only be generated when the enzyme makes the incision 3' to the lesion but fails to make the incision 5' to the damage (Lin and Sancar, 1992).

following structure-function model for UvrB is proposed (Fig. 11).

There are two regions of UvrB which are responsible for binding to UvrA. The first region, encompassing amino acid residues 115–250, is homologous to a region in the amino-terminal half of TRCF which also binds UvrA (Selby and Sancar, 1993). The second region, encompassing amino acid residues 547–673, is located in the carboxyl terminus of UvrB. Previous studies have shown that cleavage of the 43 carboxyl-terminal amino acids of UvrB generates UvrB* (Arikan *et al.*, 1986), which is catalytically inactive (Lin *et al.*, 1992) but binds both UvrA (Orren and Sancar, 1989) and UvrC (Lin *et al.*, 1992) with normal affinity. Therefore, the carboxyl-terminal region that is involved in binding to UvrA can be narrowed down to amino acid residues 547–630.

Recently, the 135-amino acid long region in the TRCF, which has 25% sequence identity with amino acids 115–250 of UvrB, was found to be sufficient for high affinity binding of TRCF to UvrA (Selby and Sancar, 1995). Since UvrB makes a tighter complex with UvrA compared to TRCF, it is possible that the region in the carboxyl-terminal half of UvrB, which also interacts with UvrA, contributes to the formation of a more stable UvrA-UvrB complex. Significantly, the carboxyl-terminal domain of UvrB which interacts with UvrA is also essential for formation of a UvrB-UvrC complex. This finding, therefore, explains why a ternary complex containing all three subunits cannot be isolated, and why UvrA must dissociate from the A_2B_1-DNA complex before UvrC can bind to the UvrB-DNA complex and initiate the dual incisions.

UvrB is a member of a family of proteins which require the assistance of a molecular matchmaker to bind DNA (Sancar and Hearst, 1993). In the case of UvrB, UvrA is required to load UvrB onto DNA with a lesion. It has been suggested that many of the proteins which bind to DNA by this mechanism can actually form a DNA-protein complex in the absence of the matchmaker under special conditions such as extremely high protein concentrations or the inclusion of macromolecular crowding compounds in the reaction mixture. For example, the β-clamp of DNA Pol III which is loaded onto DNA by the γ complex (Kuriyan and O'Donnell, 1993) can associate with DNA directly when a 100-fold molar excess over the DNA Pol III holoenzyme of the β subunit are used in the reaction (Crute *et al.*, 1983; Kwon-Shin *et al.*, 1987). Similarly, the gp45 protein, which is the polymerase clamp for T4 DNA polymerase (gp43) and is loaded onto DNA by the gp44/62 complex (Huang *et al.*, 1981; Nossal and Alberts, 1984), can be loaded directly onto DNA by using either a high concentration of gp45 (Reddy *et al.*, 1993) or by including polyethylene glycol in the reaction mixture (Sander *et al.*, 1994). Here we show that this property of proteins which use the aid of molecular matchmakers also applies to UvrB. Specifically, we demonstrate that at high concentrations of UvrB, the protein can bind to ssDNA with a lesion in the absence of UvrA. Furthermore, based on the limited number of lesions we tested, it appears as though the affinity of UvrB to a lesion correlates with how efficiently that lesion is excised by the (A)BC excinuclease.

Upon loading UvrB to a lesion, UvrA dissociates from the DNA, leaving a stable UvrB-DNA complex that is bent by about 130 °C and in addition, is locally denatured by about 5–6 bp around the lesion (Sancar and Tang, 1993). This suggests that UvrB is the "ultimate damage recognition subunit" of the (A)BC excinuclease which determines the efficiency of removal of a lesion from DNA. Hence, this study proposes that in contrast to UvrA (the proximal damage recognition subunit), which recognizes damage in a duplex, UvrB binds to the area of denaturation around the lesion to form a UvrB-DNA complex and the formation of this complex then determines whether or not incision occurs.

It has also been reported previously that although the (A)BC excinuclease recognizes a wide variety of damages, the level of excision is widely different for each type of damage (Huang *et al.*, 1994). This study now shows that the affinity of UvrB to a particular lesion is correlated with how efficiently that lesion is excised and thus further supports the notion that UvrB is the ultimate damage recognition subunit of the (A)BC excinuclease.

This conclusion, then, raises the question of how UvrB binds to damaged ssDNA. Several observations bear on this question. First, mutations in the charged residues of the so-called helicase motifs interfere with binding (Lin *et al.*, 1992; Moolenaar *et al.*, 1994; Seeley and Grossman, 1990) suggesting that these motifs constitute at least part of the DNA-binding domain. Second, these studies suggest that ionic interaction play an important role in the formation of a UvrB-DNA complex. However, seemingly parodoxically, the UvrB-DNA complexes are very stable in high-ionic strength buffers (Orren and Sancar, 1989, 1990) suggesting that the main interaction between UvrB and DNA is hydrophobic. Finally, in this study we have demonstrated by fluorescence quenching and photoinduced electron transfer that residues Phe-365 and Phe-496 of UvrB are presumably in direct contact with the DNA bases. All these observations combined suggest the following model for the formation of UvrB-DNA complexes: the initial contact with the DNA which is aided by UvrA, is mainly ionic in nature. Afterwards, upon ATP hydrolysis, UvrB undergoes a conformational change which expose residues Phe-365, Phe-496, and possibly other aromatic residues for direct interaction, perhaps by intercalation, with the bases.

The proposed mode of interaction may also explain the "specific" binding of UvrB to a lesion. Since the (A)BC excinuclease repairs virtually all lesions in DNA, previously we and others have argued that the subunits of the enzyme cannot be making direct contact with an essentially infinite number of chemical groups which constitute a "lesion" and hence it has been generally assumed that the enzyme recognizes the backbone dis-

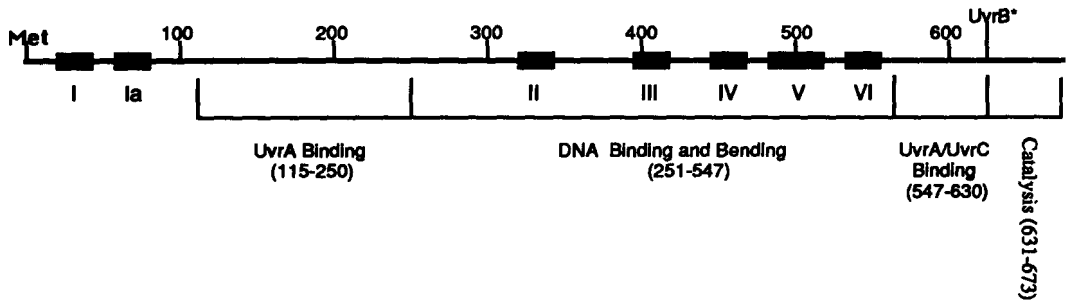

FIG. 11. **Structure-function model for UvrB.** The *black boxes numbered* with *Roman numerals* are the so-called helicase motifs. UvrA binds to two well defined domains in the amino- and carboxyl-terminal halves of the protein. The UvrA-binding region in the amino-terminal domain is homologous to the UvrA-binding region of TRCF. The UvrA-binding region in the carboxyl-terminal domain overlaps the UvrC-binding site. The helicase motifs participate in DNA binding and within this DNA-binding domain, residues Glu-265, Glu-338, and Asp-510 contact DNA possibly through salt bridges with phosphates counterions while residues Phe-365 and Phe-496 presumably intercalate into the DNA. The carboxyl-terminal 43 amino acids contain the catalytic residue Glu-639 (Lin and Sancar, 1992).

tortions of the DNA (Sancar and Tang, 1993; Grossman and Thiagalingam, 1993). In light of our finding of direct binding of UvrB to lesions, we would like to propose the following model: UvrB in fact does have a lesion binding pocket where the modified bases fit in. We propose that this is a hydrophobic pocket and because of lack of requirements for specific H-bond donors or acceptors or for formation of salt bridges of unique orientations, a vast number of chemical groups can be accommodated within this pocket. Presumably the degree of hydrophobicity, the size as well as some other, as yet to be determined, factors contribute to the relative affinities of various side groups for UvrB and hence their susceptibilities to function as substrates.

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