Mutations in the Helix-Turn-Helix Motif of the *Escherichia coli* UvrA Protein Eliminate Its Specificity for UV-damaged DNA*

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The *Escherichia coli* UvrA protein possesses a stretch of amino acids, 494 to 513, that matches the consensus sequence of the helix-turn-helix motif of many sequence-specific DNA binding proteins. It also has two zinc finger motif regions and two ATP binding sites. To study the potential roles of both helix-turn-helix and zinc finger motifs in the functioning of UvrA protein, random mutations were created in these motif regions by degenerate oligonucleotide-directed mutagenesis. Using this method, 12 single substitution mutants (eight in the helix-turn-helix motif region, one in the N-terminal zinc finger region, and three in the C-terminal zinc finger region) were isolated that failed to confer UV resistance in the *E. coli* strain deleted of the uvrA gene. One “hyper” UV-resistant mutant, G275A, was identified that conferred significantly higher UV resistance than the wild-type in the MH1 strain. To further investigate the mechanism of failure of these mutant UvrA proteins to support nucleotide excision repair, two mutant UvrA proteins, G502D and V508D, were selected for purification and characterization, since they carry mutations at the positions offered as the putative constellation for the helix-turn-helix motif. The binding affinity of these two mutants for nonirradiated plasmid DNA was unaffected by the mutations. Both mutant proteins exhibited substantial ATPase activity, and together with the UvrB protein, they were capable of generating positively supercoiled plasmid DNA from the relaxed form in the presence of ATP and bacterial topoisomerase I. However, both mutant proteins failed to respond to UV damage in the filter binding assay and were incapable of forming 2 × SSC-resistant nucleoprotein complexes with UvrB protein on UV-irradiated plasmid DNA. Taking these properties together, it appears that the mutations in the helix-turn-helix motif region impaired the UvrA protein’s ability to recognize UV damage, while its other activities were largely unaffected. Interestingly, ERCC-3, a human DNA repair protein, also has a similar helix-turn-helix motif. Given the highly conserved nature of repair proteins in general, this observation raises the possibility that both procaryotes and eucaryotes might use similar mechanisms to recognize damaged sites in their genomes.

A broad spectrum of DNA damage is repaired by the nucleotide excision repair pathway in *Escherichia coli* (Van Houten, 1990). This pathway consists of five generic steps: damage recognition, incision, excision, repair DNA synthesis, and ligation to restore the structural and biological integrity to the damaged DNA. The reactions leading to incision are carried out by UvrABC endonuclease (Sancar and Rupp, 1983; Yeung et al., 1983). Regardless of the nature of the lesion, the incision almost always occurs at the 8th phosphodiester bond 5’ and at the 4th or 5th phosphodiester bond 3’ from the damaged site. Gruskin and Lloyd (1986) established a novel in vivo system for the functional analysis of DNA repair in *E. coli*. They found that UvrABC-initiated excision repair occurred by a limited processive DNA scanning mechanism, that is, a majority of the pyrimidine dimer sites on a given plasmid molecule were repaired prior to the dissociation of the UvrABC complex. They also showed that the incision reaction represented the rate-limiting step in the overall process of excision repair pathway in vivo.

One of the unique properties of UvrABC endonuclease is its ability to recognize a broad spectrum of chemically unrelated DNA-damaged sites as its substrates for incision. So far, the search for the structural determinants conferred or induced by DNA damage that are recognized by UvrABC endonuclease remains inconclusive. Van Houten (1990) suggested that DNA damage can lead to common changes in the dynamic structure of DNA by stabilizing specific conformational alterations at the site of modification, and these alterations are potential substrates for the UvrABC endonuclease. The UvrA protein is suggested to be the damage recognition subunit of the UvrABC endonuclease, since it is the only subunit of the three that can bind to double-stranded DNA by itself, and its affinity for DNA is increased when the DNA is damaged (Seeberg and Steinum, 1982; Mazur and Grossman, 1991). However, little is known about the structural properties of the UvrA protein, which enables it to recognize DNA damage. The direct approach is to solve the structure of UvrA protein by x-ray crystallography. A complementary alternative is to introduce mutations into the *uvrA* gene and then correlate the structure of the protein with its functional state(s).

Based on its homology with established consensus sequences (Fig. 1), the UvrA protein was suggested to contain two ATP binding motifs (Husain et al., 1986), two “zinc finger” motifs (Doolittle et al., 1986), and a helix-turn-helix motif (Claassen and Grossman, 1991). Supporting the existence of zinc finger motif, the purified UvrA protein was shown to contain two tightly bound zinc atoms per molecule, and each was coordinated to 4 cysteine residues (Navaratnam et al., 1989). Moreover, Claassen et al. (1991) demonstrated that zinc was absolutely required during the UvrA refolding for recovery of its activity, and therefore was a necessary com-

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Helix-Turn-Helix Motif in DNA Damage Recognition

**E. coli**

\[ \text{R I R L A S Q I G A G L V G V M Y V L D} \]

549-513

**ERCC-3**

\[ \text{S L Y A A V S V G L Q T S D I T E Y L R} \]

122-141

**Lambda Rep**

\[ \text{Q E S V A D K M G M G Q S G V G A L F N} \]

33-52

**Lambda Cro**

\[ \text{Q T K T A K D L G V Y Q S A I N K A I H} \]

16-35

**CAP Protein**

\[ \text{R Q E I G Q I V G C S R E T V R I L K} \]

169-188

**FIG. 1.** Comparison of the segments of **E. coli** UvrA and ERCC-3 (Weeda et al., 1990) with homologous helix-turn-helix motifs of the λ repressor (Pabo and Lewis, 1982), λ Cro protein (Anderson et al., 1981), and **E. coli** CAP protein (McKay and Steitz, 1981). The amino acid residues in the **boldface** type represent the positions offered as the diagnostic constellation of helix-turn-helix motif.

| 1 | TTC GCC TGC CCA ATT TGC GGC TAC AGT ATG CGT GAA CGG CAG CTG TTT |
|---|---|
| 2 | CGG CGA CTG TTT TCG TTT AAC AAC CGC GGC GGC GGC TGC CCG ACC TGC GAG |
| 3 | GAA GCA CAG GGT ATT CGT CCG AGC CAG ATT GTT GGC GCC |
| 4 | AGT CGG CCG GGC TGT GTT ATG TAC CGT GCG GAC CGG |
| 5 | GTA CGC TGC GAG GGC TGT CAG GCG GAT GCG GTG ATC AAA GTG GAG ATG CAC |
| 6 | GTG GAG ATG CAC TTT CGG GAT TAC TGC CGG TAC GAC CAG GAG AAG GGT |

in the C-terminal zinc finger region, were identified that failed to confer UV resistance in the **E. coli** strain MH1-ΔA in which the entire wrrA gene was deleted. To further investigate the mechanism that the mutations in the helix-turn-helix motif region caused the loss of excision repair activity, two mutant UvrA proteins, G502D and V508D, which carry mutations at these diagnostic positions of helix-turn-helix motif were used to create random mutations in these three motif regions, and 12 single substitution mutants, one in the N-terminal zinc finger region, eight in the helix-turn-helix motif region, and three transformed cells from a minimal-media plate were grown at 30 °C in 2 × YT media supplemented with 0.25 mg/ml uridine until A₅₉₅ was about 0.8, and then infected with M13K07 helper phage at a multiplicity of about 10. The culture was grown at 30 °C for 1 h after standing at room temperature for 15 min. Kanamycin was added to the culture at a final concentration of 75 mg/ml to kill the uninfected cells. The culture was incubated at 30 °C overnight with vigorous shaking, and single-stranded DNA template was prepared from the supernatant.

**Localised Random Mutagenesis Using Degenerate Oligonucleotides**—The following six degenerate oligonucleotides were synthesized on an Applied Biosystems 380A automated synthesizer by Scott Morrow (The Johns Hopkins University). Oligonucleotides 1 and 2 were designed to introduce mutations into the N-terminal zinc finger region, 3 and 4 into the helix-turn-helix motif region, and 5 and 6 into the C-terminal zinc finger region as follows.

**EXPERIMENTAL PROCEDURES**

**E. coli Strains**—**E. coli** strain C236 (dut ung thi relA/ pCJ105 F', Cmu') was used to generate uracil-containing single-stranded DNA for mutagenesis. The **uvrA** deletion strain MH1-ΔA (araD139 Δara, leu) 7697, lacX74, galU1, galK, StrA, ΔuvrA) was obtained from J. A. Brandsma (Leiden University, The Netherlands).

**JM109** (recA1, endA1, gyrA96, thi1, hsdR17, supE44, relA1, λ, Δlac-proB), F' (traD36, proA1, lacP2ZM(15)) was obtained from P. Gearhart (The Johns Hopkins University).

**Molecular Cloning**—pSST1 (for single-stranded DNA preparation) and pSST70 (for **UvrA** protein expression) plasmids were originally constructed by Dr. Sambasivamoorthy Thiagalingam (Thiagalingam and Grossman, 1991). Single-stranded DNA Preparation from pSST7 Plasmid—**E. coli** strain C236 (dut ung) was used to generate uracil-incorporated single-stranded DNA template for mutagenesis. Briefly, pSST7-

To produce a maximal number of molecules with a single substitution mutation, the percentage of wild-type base and an equimolar mixture of the remaining three bases used in the synthesis of underlined positions for each oligonucleotide was calculated as 96/4/0/0 for 1: 36/5/4/5 for 2: 36/5/5/5 for 3: 96/4/5/5 for 4: 36/5/4/8 for 5: and 36/6/4.5/5 for 6 (McNeil and Smith, 1985). The degenerate oligonucleotides were annealed to the single-stranded DNA template and then converted into the double-stranded form following the procedure described by Kunkel et al. (1987).

**Subcloning of Mutations from pSST71 to pSST70 Plasmid—**StuI/ StyI DNA fragments from the mutant pSST7 plasmids were purified using low-melting agarose and were used to replace the same fragments from pSST70 plasmid for the subcloning of mutations created in the N-terminal zinc finger region. Similarly, Styl/AosI and Stul/Sall fragments were used for the subcloning of the mutations in the helix-turn-helix motif region and C-terminal zinc finger region, respectively.

**UV Survival**—The **UV**-streak test was carried out following the procedures described by Thiagalingam and Grossman (1991). Briefly, 2 × YT media containing 50 µg/ml carbenicillin was inoculated with the overnight culture, which was then grown at 30 °C until A₅₉₅ was about 0.8. The culture was streaked across a 2 × YT/carbenicillin plate. The calculated exposure to 254-nm UV light was achieved by moving an opaque shield perpendicular to the bacterial streaks from right to left. The UV dose was determined with a digital UV meter (Hoefer). The plate was incubated overnight at 30 °C in the dark.

**Gel Electrophoresis and Western Blotting**—The gel systems used were the denaturing gels of Laemmli (1970), with a stacking gel at a concentration of 5% acrylamide, and a separating gel at 7.5%.
same gel systems were used to prepare the protein products for Western blotting. Following electrophoresis, proteins were transferred onto nitrocellulose, probed with a rabbit α-UvrA polyclonal antibody, and developed by alkaline phosphatase-linked goat α-rabbit IgG according to the method of Dickstein et al. (1988).

**Protein Purification**—The mutant G502D, V508D, and wild-type UvrA protein purified from Escherichia coli were used for Western blots or mutant pSST10 plasmids for UvrA protein expression. Individual colonies of appropriately transformed MH1-ΔA cells were used to inoculate 8 liters of 2 X YT media containing 50 μg/ml carbenicillin. The cultures were grown to saturation at 30°C for about 18 h; this was followed by inoculation of the above K5 strain, and 2 X YT media containing 50 μg/ml carbenicillin, 10 mM MgCl2, 1 mM DTT, 100 μg/ml BSA, 5% (v/v) glycerol, and 0.3 M KCl, after the UvrA preparations were concentrated by a microconcentrator (Centricron 30, Amicon). The concentrations of the purified proteins were determined by the Bradford (1976) method, using BSA as the concentration standard.

**DNA Binding Assay**—The formation of UvrA nucleoprotein complexes was determined by nitrocellulose filter binding (Clausen and Grossman, 1991). The 100-μl reaction mixtures contained 60 fmol of 32P-labeled plasmid DNA, 5 μg/ml cellular DNA, 5 μM MgCl2, 1 mM DTT, 100 μg/ml BSA, 5% (v/v) glycerol, 2 mM ATP, and an ATP-regenerating system (4 mM phosphoenolpyruvate and 500 units/ml pyruvate kinase from Boehringer Mannheim). After equilibration at 30°C for 10 min, the UvrA proteins were added to initiate the reaction. After 30 min of incubation, the DNA-protein complexes were trapped by filtration over nitrocellulose filters (HAWP025, Millipore) that had been soaked in filter buffer (50 mM MOPS, pH 7.6, 100 mM NaCl, and 10 mM MgCl2). The amount of DNA bound to filters via nucleoprotein complexes was determined.

**ATPase Assays**—The hydrolysis of ATP by UvrA proteins (wild-type or mutants) was assayed by the thin-layer chromatography method. The reaction mixture contained 50 mM K+ MOPS, pH 7.6, 100 mM NaCl, 15 mM MgCl2, 50 μg/ml bovine serum albumin, 2 mM ATP, and an ATP-regenerating system (4 mM phosphoenolpyruvate and 500 units/ml pyruvate kinase). The reaction was initiated by adding the plasmid DNA and continued at 37°C for 30 min. The reaction mixtures were then challenged with 5 μl of ice-cold 2 X SSC solution, and filtered after 5 min on ice over nitrocellulose filters that had been soaked in 2 X SSC solution. The amount of DNA bound to the filters via nucleoprotein complex formation was determined, which was then converted to units of fentomoles of nucleoprotein complexes formed according to the Poisson distribution calculation in the same manner.

**RESULTS**

**Mutation Screening**—The mutant selection procedure consisted of four steps:

(i) UV-sensitivity screening: flat-ended toothpicks were used to pick up individual colonies and make replicates on two 2 X YT/carbenicillin plates, one as master plate and the other for UV irradiation at 15 J/m2. The UV-sensitive colonies that were killed by irradiation were inoculated from the master plates and grown up. The cultures were tested by UV-streak test to confirm the phenotype (Fig. 2).

(ii) Western blotting was used to screen for those UvrA sensitive clones which expressed soluble UvrA proteins to levels comparable to that of wild type.

(iii) After the plasmids were prepared, the mutations were identified in the corresponding regions by sequencing. (iv) Subcloning of mutations from pSST1 to pSST10 plasmids to further amplify the mutant UvrA protein fragments containing the identified mutations was done from pSST1 plasmid and used to replace the same fragment from pSST10 plasmid. After the phenotypes (UV sensitivity and UvrA expression) were confirmed following transformation of the competent MH-1-ΔA cells, the entire region corresponding to the fragment being subcloned was sequenced to exclude the existence of secondary mutations for several mutant plasmids (G502D, V508D, H754Y, and C763F). Abundant UvrA protein (Brinkmann) that had been prespotted with 1 μl of 100 mM ATP/ADP markers. The thin-layer chromatography plates were developed in a solution of 1 M formic acid and 0.5 M LiCl. The dried plates were visualized with shortwave UV light, and the spots corresponding to ATP and ADP were excised to determine the percentage of ATP hydrolysis in scintillation fluid (Bio-Safe NA, Research Products International). The initial rates were derived from linear regression analyses of time points taken before 10% of the substrate had been consumed. The kinetic parameters were calculated from Lineweaver-Burke plots.

**Positive Supercrossing Assay**—Relaxed pT718R plasmid DNA was prepared by treating the negatively supercoiled form with E. coli topoisomerase I (Koo et al., 1991). Then, 40 ng of relaxed plasmid DNA, either nonirradiated or irradiated with 254-nm UV light for 600 J/m2, was incubated in a 20-μl reaction mixture containing 5 μM UvrA, 5 μM UvrB, 80-ng E. coli topoisomerase I, 20 mM Heps, pH 7.5, 50 mM KCl, 5 mM MgCl2, 4 mM ATP, 1 mM DTT, and 50 μg/ml BSA. The reaction was allowed to proceed at 37°C for 1 h and was stopped by adding 25 mM EDTA and 0.5% SDS. Protease K (300 μg/ml) was then added and the digestion continued at 37°C for 2 h. The reaction products were subjected to analysis in a 1% agarose gel in 0.5 X TBE buffer. The gels were then dried and hybridized in situ using pT718R plasmid DNA that had been nick-translated with [α-32P]dCTP as probe.

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1 The abbreviations used are: DTT, dithiothreitol; ERCC-3, excision repair cross-complementing-3; MOPS, 4-morpholino propanesulfonic acid; BSA, bovine serum albumin.
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UV Dose (J/m²)

| UV Dose (J/m²) | 1 | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 |
|----------------|---|---|----|----|----|----|----|----|----|----|
| S269P          | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| R494P          | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| A494E          | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| G502D          | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| L505R          | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| V506D          | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| V508D          | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| Y510F          | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| D513H          | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| C740F          | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| C742Y/K750I    | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| H754Y          | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| C763F          | 2 | 1 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |

*Fig. 2. UV survival curves of E. coli MH1-ΔA strain transformed by wild-type or mutant pSSST10 plasmids or by pTZ18R vector. The UV dose is in J/m².*

**TABLE I**

Screening summary

| Oligonucleotide screened | UV-sensitive colonies | UvrA expression mutants | Single mutation |
|--------------------------|-----------------------|-------------------------|-----------------|
| 1 | 5242 | 86 | 1 | 0 |
| 2 | 4994 | 44 | 6 | 3 |
| 3 | 5444 | 51 | 6 | 5 |
| 4 | 4944 | 90 | 12 | 6 |
| 5 | 4266 | 128 | 7 | 2 |
| 6 | 4464 | 106 | 4 | 3 |
| Total | 19464 | 488 | 36 | 19 |

**TABLE II**

Characterization of the UvrA mutants

| Mutant | Residue change | Codon change | UV sensitivity* | Independent isolate, n |
|--------|----------------|--------------|-----------------|------------------------|
| 2-13  | Ser<sup>269</sup> to Pro | TCG to CCG | S | 3 |
| 3-15  | Arg<sup>294</sup> to Pro | CGT to CCT | S | 1 |
| 3-12  | Ala<sup>299</sup> to Glu | GCC to CAG | S | 2 |
| 5-5   | Gly<sup>390</sup> to Asp | GGT to GAT | S | 1 |
| 4-27  | Leu<sup>400</sup> to Arg | CTG to CCG | S | 1 |
| 4-13  | Val<sup>406</sup> to Asp | GGT to GAT | S | 2 |
| 4-42  | Val<sup>406</sup> to Asp | GGT to GAT | S | 1 |
| 4-7   | Tyr<sup>412</sup> to Phe | TAC to TTC | S | 1 |
| 4-4   | Asp<sup>413</sup> to His | GAC to CAC | S | 1 |
| 5-12  | Cys<sup>419</sup> to Phe | TGC to TTC | S | 1 |
| 5-7   | Cys<sup>424</sup> to Tyr | AAA to ATA | S | 1 |
| 5-7   | Lys<sup>425</sup> to Ile | TGT to TAT | S | 1 |
| 6-14  | His<sup>434</sup> to Tyr | CAC to TAC | S | 1 |
| 6-12  | Cys<sup>439</sup> to Phe | TGC to TTC | S | 2 |
| 6-61  | Cys<sup>439</sup> to Ser | TGC to TCC | R | 1 |

* S, sensitive; R, resistant (see Fig. 2 for detail).
* Mutants are named using two numbers. The first number represents the oligonucleotide used to create the mutation; the second number represents the number used in screening.

was expressed from pSSST10 plasmid for purification.

A summary of the UvrA mutant screening is listed in Tables I and II. Of the 36 UV-sensitive clones that overexpressed UvrA proteins, 19 of them carried single substitution mutation, and the other 17 clones carried either double or triple mutations. This procedure was successfully used to select the desired mutants, since we were able to devise a simple UV sensitivity test that enabled us to screen a large number of colonies. This procedure could also be used to introduce random mutations into other regions of uvrA genes, or into selected regions of uvrB and uvrC genes with minimal modification to probe their structure-function relationship. The mutations this procedure created appeared to be random. The majority of the substitutions were transversions, and this phenomenon might be the result of the selection of the UV-sensitive phenotype. Of the 12 mutants, eight appeared to confer no UV resistance in the MH1-ΔA strain, while the other four conferred a little over the background level of UV resistance, judged by UV-streak test (Fig. 2). Since the folding mutants were likely to be degraded quickly in vivo, Western blotting was used to screen out this type of mutants. All but one of these 12 mutants were expressed to the level comparable to that of wild-type, judged by Western blotting and Coomassie-stained SDS-polyacrylamide gel electrophoresis (data not shown). For mutant Y510F, the expression level was greatly reduced, barely detectable by Western blotting. The most likely cause was the instability of this mutant UvrA polypeptide in vivo, but other explanations could not be excluded.

In Vivo Properties of the Mutant UvrA Proteins—E. coli strain JM109 (recA<sup>-</sup> uvr<sup>C</sup>) was used to investigate the in vivo properties of these identified mutant UvrA polypeptides. JM109 strain carries the wild-type uvrA, uvrB, and uvrC genes in its chromosome, and is resistant to low doses of UV irradiation. All but one (Y510F) of these mutants produced negative complementing proteins that reduced the activity of chromosomally specified wild-type proteins (Fig. 3). Hence, the mutant uvrA genes must specify polypeptides that were able to interact with and inactivated the wild-type UvrA protein produced from the chromosomal uvrA<sup>+</sup> allele, presumably by forming heterodimers. Since the mutant uvrA genes were present on a high copy number plasmid with a strong promoter, there was a large excess of mutant UvrA protein compared to the wild-type encoded by the chromosomal allele (data not shown). Thus, if the mutant UvrA protein could associate with wild-type UvrA randomly, there should be relatively few homodimers of the wild-type UvrA. Hence, the negative complementation conferred by these mutant UvrA proteins suggested that the heterodimers could form in vivo, because of the wild-type conformation at the subunit contact

![Fig. 3. Negative complementation of the repair capacity of E. coli JM 109 strain by the mutant UvrA proteins](image-url)

The UV exposure and streaks of JM 109 cells carrying the different plasmids are indicated.
sites, but they could not lead to incision because of the mutations. Y510F mutant did not show negative complementation, and this was consistent with the observation that the expression level of this mutant UvrA protein was minimal.

"Hyper" UV-resistant Mutant Screening—The mutant UvrA library generated above was used to screen for a UvrA mutant that could confer greater UV resistance than the wild type in the MH1-ΔΔ strain. Using the procedure described above, one mutant, G275A, was found to fit into this category. This "hyper" UV-resistant phenotype was confirmed using the UV-streak test (see Fig. 4). SDS-polyacrylamide gel electrophoresis showed that the G275A UvrA protein was expressed to a level similar to that of wild type in the MH1-ΔΔ strain. Using the procedure described above, one mutant, G275A, was found to fit into this category. This "hyper" UV-resistant phenotype was confirmed using the UV-streak test (see Fig. 4). SDS-polyacrylamide gel electrophoresis showed that the G275A UvrA protein was expressed to a level similar to that of wild type in the MH1-ΔΔ strain (data not shown). A similar approach to isolate this kind of mutation in the helix-turn-helix motif and C-terminal zinc finger regions was unsuccessful.

Purification of Wild-type and Mutant UvrA Proteins—Wild-type and mutant UvrA proteins expressed from pSST10 construct in MH1-ΔΔ strain were mostly in soluble form. Eight-liter cultures of MH1-ΔΔ/pSST10(wt), MH1-ΔΔ/pSST10(G502D), and MH1-ΔΔ/pSST16(V508D) were grown for the preparation of cell extracts. Using the procedure described above, wild-type and two mutant UvrA proteins were all purified to greater than 90% homogeneity (Fig. 5).

The chromatographic behavior of these two mutant UvrA proteins was similar to that of the wild-type in all four columns of the purification procedure. The original UvrA purification procedure of Thiagalingam and Grossman (1991) was modified for the purification of another mutant UvrA protein, C763F, which failed to bind to single-stranded DNA column. To be consistent, both G502D and V508D mutant UvrA proteins were purified using this modified procedure, although they bind to single-stranded DNA column as strongly as the wild-type UvrA protein.

DNA-binding Properties of the Mutant Proteins—UvrA protein is the only subunit of UvrABC endonuclease that can bind to double-stranded DNA by itself. It has a preference for damaged over undamaged DNA, although the specificity is only 10^3 to 10^4 (Mazur and Grossman, 1991), which is much lower than the specificity of 10^7 for some sequence-specific DNA binding proteins, such as the lac repressor. In the filter binding assay, both G502D and V508D mutant UvrA proteins bind to UV-irradiated and nonirradiated plasmid DNA with similar affinities (Fig. 6). To further investigate this loss of specificity of the mutant UvrA proteins for damaged DNA, the percentage of plasmid DNA retained on the filter via nucleoprotein complex formation was plotted as a function of UV dosage used to irradiate the plasmid DNA. Wild-type UvrA protein bound to damaged DNA with an increased affinity that increased with UV fluency. Both mutant UvrA proteins bound to undamaged DNA as strongly as the wild type, but their affinity for the plasmid DNA damaged with increasing doses of UV irradiation remained at the level as for the nonirradiated plasmid DNA, which suggested that the mutant UvrA proteins could no longer recognize the UV adducts on the plasmid DNA (Fig. 7). 2×SSC-resistant Nucleoprotein Complex Formation—To define the reaction intermediates leading to incision, 2×SSC was used to distinguish nucleoprotein complexes of UvrA and UvrB proteins selectively associated at the damaged sites of the DNA substrate. UvrA-DNA complexes are extremely sensitive to 2×SSC challenge (t<sub>0.5</sub> = 5 s), so are the complexes of UvrA and UvrB formed on undamaged plasmid DNA. The nucleoprotein complexes of UvrA and UvrB formed on damaged DNA substrate are uniquely resistant to such a 2×SSC challenge, with a resident half-life of approximately 55 min (Yeung et al., 1986). The mechanism of this discrimination remains unclear, but that this discrimination can be mimicked by diluting complexes into an excess of 50 mM EDTA indicates that citrate, a weak metal chelator in 2×SSC, may be responsible for this effect, potentially by disrupting the tertiary or quaternary structure of UvrA protein anchored by the two zinc atoms. In this assay, wild-type UvrA protein was capable of forming 2×SSC-resistant complexes with UvrB on UV-damaged pH26 plasmid DNA substrate, and this reaction was dependent on UV irradiation of the substrate. Some 2×SSC-resistant complexes did form on the nonirradiated DNA substrate, possibly due to the endogenous DNA damage introduced during the plasmid preparation. Under similar conditions, neither G502D nor V508D mutant UvrA proteins exhibited any detectable activity to form a damage-dependent, 2×SSC-resistant complex with UvrB protein (Fig. 8). This result further supported the finding that these two mutant UvrA proteins lost their capability to recognize UV damage. Since these two mutants retained their native DNA binding ability and were capable of forming UvrA-B complexes that could translocate along the DNA helix (see the supercoiling assay below), the failure to detect 2×SSC-resistant complexes had to be attributed to the loss of their damage recognition capabilities.
Helix-Turn-Helix Motif in DNA Damage Recognition

FIG. 6. DNA binding of wild-type or mutant UvrA proteins. The pHE6 plasmid DNA was either non-irradiated or irradiated with 254 nm UV light for 600 J/m². A, binding of G502D UvrA protein to UV-irradiated versus nonirradiated pHE6 plasmid DNA as compared to wild-type UvrA protein. B, binding of V508D UvrA protein to UV-irradiated versus nonirradiated pHE6 plasmid DNA as compared to wild-type UvrA protein.

FIG. 7. Damage recognition by wild-type or mutant UvrA proteins. Wild-type or mutant UvrA proteins (all at 5 nM) was equilibrated with either nonirradiated pHE6 plasmid DNA or the same DNA that had been damaged with 254-nm UV light at 120 J/m²/min for the filter binding assay (see “Experimental Procedures”).

ATP Hydrolysis by the Mutant UvrA Proteins—UvrA protein is a DNA-independent ATPase, although DNA does modulate its kinetic properties. Site-directed mutagenesis studies of the Walker consensus sequences suggested that both of the UvrA's ATPase sites were functionally required in nucleotide excision repair (Thiagalingam and Grossman, 1991; Myles et al., 1991). The apparent $K_m$ for the wild-type UvrA ATPase was reported to be 150–200 μM. But in this study, its apparent $K_m$ was measured at 103 μM. The $K_m$ values for G502D and V508D mutant UvrA proteins were
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FIG. 8. Damage dependence of 2 × SSC-resistant complex formation. The pHE6 plasmid DNA was either non-irradiated or irradiated with 254-nm UV light at 120 J/m²/min.

**TABLE III**

ATPase properties of the wild-type and mutant UvrA proteins

The initial rates of ATPase activity were measured as described under "Experimental Procedures." For wild-type UvrA, the reaction mixture contained 50 nM of purified protein, and 25–300 μM ATP was used as substrate. For mutants, the reaction mixtures contained 61.5 μM of V508D UvrA or 100 nM of G502D UvrA, and 150–700 or 100–600 μM ATP was used as substrate, respectively.

| Kinetic parameters | UvrA protein | G502D | V508D |
|--------------------|--------------|-------|-------|
| \( K_m (\mu M) \)  | 103          | 220   | 232   |
| \( K_m (\text{min}^{-1}) \)  | 73.7         | 22.0  | 23.4  |
| \( K_m/K_a (\mu M^{-1} \text{min}^{-1}) \) | 7.16 \times 10^{-1} | 1.00 \times 10^{-1} | 1.01 \times 10^{-1} |

determined to be 220 and 232 μM, respectively. The turnover number for ATP hydrolysis by wild-type UvrA protein under the conditions used in this study was about 73.7 per min, consistent with 16–125 per min of previous reports (Van Houten, 1990; Thiagalingam and Grossman, 1991). Under the same conditions, both G502D and V508D mutants exhibited substantial ATPase activities (Table III).

Mutant UvrA Proteins in Positive Supercoiling Reaction—

UvrA protein, together with UvrB protein, is capable of partitioning the DNA template into both positively and negatively supercoiled domains in an ATP-dependent manner. The presence of bacterial DNA topoisomerase I (ω protein) in the reaction, which removes negative supercoils specifically, results in an accumulation of positive supercoils in the DNA template (Koo et al., 1991). This discovery is consistent with the fact that the UvrA-B complex is a DNA helicase that can displace a short (20- to 50-mer) oligonucleotide in a 5' to 3' direction (Oh and Grossman, 1987). Both the supercoiling and helicase activities of the UvrA-B complex require ATP hydrolysis. While UV damage inhibits the helicase activity of the UvrA-B complex, it stimulates the supercoiling activity significantly, presumably by providing an "anchoring" site for one-half of the duplex and thereby enhancing the supercoiling process physically (Koo et al., 1991). It is possible that these two assays may monitor different aspects of the same reaction, which most likely involves the UvrA-B complex translocation along the DNA helix. Using this supercoiling assay, both G502D and V508D mutant UvrA proteins were found to be active in generating the positive supercoils from the relaxed form in the presence of ATP and bacterial DNA topoisomerase I (Fig. 9). This result suggested that both mutants were capable of associating with the UvrB protein and the mutant UvrA-B complexes could hydrolyze ATP effectively as a "power stroke" to translocate along the DNA helix. The positively supercoiled products were not quantitated, since this assay was only used to demonstrate that the mutant UvrA proteins were active in participating in the supercoiling reaction.

**DISCUSSION**

To study the structure-function relationship of UvrA protein, Claassen and Grossman (1991) constructed a serial of C-terminal deletion mutants. Results from the purified deletion mutants suggested that the DNA binding domain of UvrA protein lies within the first 900 of its 940 amino acid residues. Two damage recognition domains were detected. The first domain, which coincides with the DNA binding region, is required to detect the damage. The second domain, located on or near the C-terminal 40 amino acids, stabilizes the protein-DNA complex when damage is encountered. As a continuation of the deletion study, we created a pool of random mutations in the helix-turn-helix and zinc finger motif regions and developed a novel procedure to select the mutants that were no longer capable of supporting the nucleotide excision repair. There have been many reports dealing with site-directed mutagenesis of zinc finger proteins, in
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which the histidine or cysteine residues were replaced with either serine, alanine, or glycine. In most cases, these changes inactivated the mutant proteins (Severne et al., 1988; Redemann et al., 1988). Three single mutants (C740F, H754Y, and C763F) and one double mutant (C742Y/K750I) in the C-terminal zinc finger motif were found to be excision repair defective. It is expected that C740F, C742Y/K750I, and C763F mutant UvrA proteins lost their activity, because 1 of the 4 zinc-bonding cysteine residues was replaced by a bulky amino acid residue. The other two mutations occurred at the positions of lysine 750 and histidine 754, the only 2 basic amino acid residues on the finger part of the C-terminal zinc finger motif region. C763F mutant UvrA protein failed to bind to the single-stranded DNA column during the purification process, and preliminary results from the purified mutant protein suggested that this mutation caused a loss of the DNA-binding activity. The only mutant that we were able to isolate in the N-terminal zinc finger region involved the replacement of serine 269 by proline, and this S269P UvrA mutant was isolated independently three times. The pyrrolidine ring of proline constrains its $\phi$ dihedral angle to values near $-60^\circ$, so the effect of this mutation was most likely caused, not by the loss of wild-type side chain, but by insertion of a destabilizing proline residue where significantly different backbone torsional angles were required. Extensive efforts to isolate other mutants in this motif region was not successful. Cysteine 277 was converted to either alanine or phenylalanine using site-directed mutagenesis, and its effect on the mutant protein was found to be minimal, since both C277A and C277F mutant UvrA proteins conferred UV resistance similar to that of the wild-type in the MH1-DA strain (data not shown). Perhaps the N-terminal zinc finger motif region might be involved in maintaining the tertiary conformation of the UvrA protein, instead of being involved in one of its specific activities.

The concept of "helix-turn-helix motif" was proposed when the three-dimensional structures of $\lambda$ Cro protein, $\lambda$ repressor, and E. coli CAP protein were solved by x-ray crystallography in the early 1980s (Pabo and Sauer, 1984). All three proteins are sequence-specific DNA binding proteins and bind to their cognate sequences as dimers. They share a common structure composed of two $\alpha$-helices separated by a relatively sharp $\beta$ turn, and this structure is termed as the helix-turn-helix motif. Six positions offer a diagnostic constellation for the helix-turn-helix motif (Johnson and McKnight, 1989). The 4th residue of helix 2 almost always contains a hydrophobic side chain, and the 5th residue is either glycine or alanine. The $\beta$ turn begins most frequently with glycine, and is invariably followed by a hydrophobic amino acid residue. The 4th and 7th residues of helix 3 are almost always occupied by hydrophobic amino acid residues. Using this consensus sequence, the homeobox-containing proteins were noted to possess a helix-turn-helix motif, although the homologies were relatively weak (Porter and Smith, 1986; Laughon and Scott, 1984). This observation was later confirmed to be correct after the three-dimensional structures of the yeast MATa2 protein and Drosophila engrailed protein were solved by x-ray crystallography (Wolberger et al., 1991; Kissinger et al., 1990).

Eight single substitution mutants were identified in the helix-turn-helix motif region of the UvrA protein, with four at the diagnostic positions. Since five of these eight mutations involved replacement of neutral amino acids by charged ones, it raised the possibility that the effect of these mutations was caused by the disruption of the overall structure of UvrA protein. But all the evidence that has been gathered so far argued against this possibility. First, misfolding appears to be rare. Most mutant proteins that have been studied thus far have conformations that are similar to wild type, with structural differences occurring only at or near the site of mutation. On the other hand, whether a single substitution mutation can cause nonlocal conformational changes may have little to do with how conservative the substitution is. For example, a Glu$^{65}$ to Asp substitution in staphylococcal nuclease causes detectable changes at residues as far as 30 Å away (Wilde et al., 1988), while in other cases, mutants with charged amino acids replacing the neutral ones retain wild-type-like activity (Reidhaar-Olson and Sauer, 1988). Second, random mutagenesis in the helix-turn-helix motif regions of $\lambda$ Cro protein and E. coli trp repressor also produced a large number of neutral to charged amino acid substitutions (Kelley and Yanovsky, 1985; Pakula et al., 1986). Third, most importantly, the in vivo complementation experiment suggested that these mutant UvrA proteins were capable of forming complexes with the wild-type UvrA protein. Furthermore, in vitro experiments using the purified G502D and V508D mutant UvrA proteins suggested that their activities other than damage recognition capability was largely unaffected.

The mutations in the helix-turn-helix motif region had little measurable effect on the binding affinity of mutant UvrA proteins for undamaged plasmid DNA, suggesting that the structure of UvrA protein for nonspecific DNA binding was not affected by these mutations. But unlike the wild-type, the mutant UvrA proteins carrying mutations in the helix-turn-helix motif region lost their capability to respond to UV damage in both the filter binding assay and the 2 x SSC-resistant complex formation assay. The purified G502D and V508D UvrA proteins, together with UvrB protein, were still capable of using the energy of ATP hydrolysis as a "power stroke" (Jencks, 1982) to track along the DNA helix, presumably searching for the damage sites for repair. This reaction

| DNA          | GS02D | V508D | Wild-Type |
|--------------|-------|-------|-----------|
| UV-DNA       | -     | +     | -         |
| UvrA         | +     | +     | +         |
| UvrB         | -     | +     | +         |
depends on ATP hydrolysis. It also depends on the complex formation of UvrA and UvrB proteins, since neither protein can catalyze this reaction by itself. The stoichiometry of UvrA-UvrB complex is determined to be 2 molecules of UvrA and 1 molecule of UvrB (Orren and Sancar, 1989), presumably because the UvrA protein needs to be in the form of a dimer before it can associate with UvrB protein. Both mutant UvrA proteins were active in supporting the supercoiling reaction. This result suggested that the mutant UvrA, UvrB complex could still form and track along the DNA helix. This conclusion was also supported by the finding that both mutant UvrA proteins negatively complemented the wild-type when transformed into *E. coli* JM109 strain, which suggested that the mutant UvrA proteins were capable of dimerizing with and titrating the wild-type protein into inactive form.

If the helix-turn-helix motif region is the damage recognition unit of UvrA protein, one might be able to isolate a mutant UvrA protein carrying a mutation in this motif region, which could recognize a DNA adduct that wild-type UvrA protein does not recognize. Such as, N-methyl-N'-nitro-N-nitrosoguanidine-induced DNA adducts are very poor substrates for UvrABC endonuclease in *vivo* (Van Houten and Sancar, 1987). It would be interesting to see if an assay can be developed to screen for a UvrA mutant that can lead to efficient repair of N-methyl-N'-nitro-N-nitrosoguanidine-induced DNA adducts in *vivo*.

Gruskin and Lloyd (1988) showed that UvrABC-initiated excision repair occurred by a limited processive mechanism, that is, the enzyme actively "scanned" DNA for dimer sites. This is consistent with the finding that UvrA bound to a DNA helix and it tracks along the DNA helix searching for damage (Oh and Grossman, 1987; Koo et al., 1991). More importantly, they showed that approximately all but one of the dimer sites on a given plasmid molecule were repaired prior to the dissociation of the UvrABC complex. The dimer left unrepaird might be the anchor for UvrABC endonuclease to scan the DNA helix (Koo et al., 1991). From partial in *vitro* reactions, it was suggested that UvrA acted catalytically to deliver UvrB to a damaged site and then dissociates from the complex (Orren and Sancar, 1988, 1989; Visse et al., 1992). It is feasible that supercoiling-translocation may precede the "loading" onto a damaged site. The *in vitro* interaction of proteins with nontarget DNA is electrostatic in nature and sensitive to the salt concentration of the reaction. Such as, endonuclease V binds to unirradiated DNA in 10 mM NaCl but not in 100 mM NaCl. As a result, it incises pyrimidine dimers by a processive mechanism at low salt concentration and switches to a distributive mechanism at high salt concentration (Lloyd et al., 1980; Ganesan et al., 1986), although this enzyme acts by a processive mechanism in *vivo*. It would be interesting to look at the effect of salt concentration on the mechanism by which UvrABC endonuclease acts.

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