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Conserved Motifs II to VI of DNA Helicase II from *Escherichia coli* Are All Required for Biological Activity

GANG ZHANG,† ENXIN DENG, LARRY R. BAUGH, CAROL M. HAMILTON,‡ VALERIE F. MAPLES, AND SIDNEY R. KUSHNER*

Department of Genetics, University of Georgia, Athens, Georgia 30602

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There are seven conserved motifs (IA, IB, and II to VI) in DNA helicase II of *Escherichia coli* that have high homology among a large family of proteins involved in DNA metabolism. To address the functional importance of motifs II to VI, we employed site-directed mutagenesis to replace the charged amino acid residues in each motif with alanines. Cells carrying these mutant alleles exhibited higher UV and methyl methanesulfonate sensitivity, increased rates of spontaneous mutagenesis, and elevated levels of homologous recombination, indicating defects in both the excision repair and mismatch repair pathways. In addition, we also changed the highly conserved tyrosine(600) in motif VI to phenylalanine (*uvrD309*, Y600F). This mutant displayed a moderate increase in UV sensitivity but a decrease in spontaneous mutation rate, suggesting that DNA helicase II may have different functions in the two DNA repair pathways. Furthermore, a mutation in domain IV (*uvrD307*, R284A) significantly reduced the viability of some *E. coli* K-12 strains at 30°C but not at 37°C. The implications of these observations are discussed.

The DNA helicase II protein of *Escherichia coli*, encoded by the *uvrD* gene, is both a DNA-dependent ATPase and an ATP-dependent DNA helicase (23, 30, 41). This enzyme unwinds DNA duplexes with a 3′-to-5′ polarity and initiates unwinding most effectively when a single-stranded region is present (29, 42). At high protein concentrations, it can also initiate unwinding on a nicked or blunt-ended DNA substrate (42, 43). Both genetic and biochemical studies have demonstrated a role for DNA helicase II in a number of DNA repair processes, including methyl-directed mismatch repair (14, 24, 34) and UvrABC-mediated excision repair (6, 20, 45). DNA helicase II may also be involved in homologous recombination (2, 27, 32, 35) and DNA replication (12, 21, 40, 52), although the precise roles of the protein in these two processes have not yet been defined.

Amino acid sequence comparisons have revealed six highly conserved motifs among 21 proteins, including UvrD, which are all involved in nucleic acid replication and/or recombination (18). The sources of these proteins range from bacteria and yeasts to plant and animal viruses. Motifs I and II, previously identified as the Walker A and B motifs (50), respectively, are conserved segments found in many nucleoside triphosphate-binding proteins and helicases. Mutations in motif I (12, 52) or motif II (3) of the UvrD protein result in significant reduction of ATPase activity and various levels of reduced helicase activities. These results suggest that these two conserved regions may function as ATP-binding and hydrolysis domains in the DNA helicase II protein.

To further understand the cellular function of DNA helicase II, as well as to better appreciate the importance of the six motifs conserved among all helicases, we introduced mutations into motifs II, III, IV, V, and VI by employing site-directed mutagenesis. Using the alanine-scanning approach described by Cunningham and Wells (9), we replaced the charged amino acid residues in each domain with alanines. In addition, we also replaced the highly conserved tyrosine residue (Y600) in motif VI (18) with a phenylalanine. By characterizing the biological phenotypes conferred by these mutations, we show here that each of these conserved motifs is essential for the function of DNA helicase II. In addition, one mutation in motif IV resulted in reduced viability at 30°C in some *E. coli* K-12 strains. The implications of these results are discussed.

* Corresponding author.

† Present address: Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

‡ Present address: Plant Science Center, Center for Advanced Technology/Biotechnology, Cornell University, Ithaca, NY 14583.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains used in this work are listed in Table 1. All strains were constructed in this laboratory via bacteriophage P1-mediated transduction (56) or plasmid transformation, unless otherwise described. The plasmids used are described in Table 2.

Strain SK4090 (*uvrDΔ294*::*kan*) was constructed as follows. Sequences approximately 200 bp immediately upstream or downstream of the *uvrD* coding region were amplified by PCR. During the PCR process, an *Eco*RI site was introduced into the 3′ end of the upstream fragment and a *Bam*HI site was introduced into the 5′ end of the downstream fragment. These two fragments were digested with *Eco*RI and *Bam*HI, respectively, and ligated with a fragment excised from pUC4K (New England Biolabs) that contained the kanamycin resistance cassette by using *Eco*RI and *Bam*HI. The ligated fragment (*kan* gene flanked with sequences homologous to the *uvrD* upstream and downstream sequences) was again PCR amplified, purified, and inserted into the *Sal*I site of pMAK705 (16). This new plasmid (pCHK103) was transformed into SK707 (Table 1), and the wild-type *uvrD* gene on the chromosome was displaced with the Δ*uvrD*::*kan* construction from the plasmid as previously described (16). The deletion on the chromosome (*uvrDΔ294*::*kan*) was verified by Southern blotting analysis (data not shown).

Media and growth conditions. Cells were grown in Luria broth (22) unless otherwise described. Ampicillin (100 μg/ml), kanamycin (20 μg/ml), or chloramphenicol (20 μg/ml) was added when necessary. Agar was added to 2% for solid medium. The minimal medium used for P1 transduction was M56/2 (56) containing 2% agar, glucose, and appropriate amino acids and vitamins. Glucose was replaced with lactose (glucose free; Sigma) for lactose minimal medium.

For assays of temperature-sensitive phenotypes, we used 30°C and 44°C as the incubation temperatures. Otherwise, the cells were grown at 37°C.

DNA techniques. Plasmid DNA was isolated according to the alkaline lysis method (44) for mini-scale preparations. The Qiagen Midikit was used for larger-scale plasmid preparations. Competent cells were transformed by electroporation (33) with a Gene-Pulser, manufactured by Bio-Rad.

Site-directed mutagenesis was carried out with the U.S.E. mutagenesis kit from Pharmacia Biotech. A 2.9-kb *Sal*I DNA fragment containing the *uvrD* gene cloned in pWSK29 (51) was the target for site-directed mutagenesis (pGZK20). The oligonucleotide primers used in this work, with the altered codons underlined, were as follows: oligonucleotide 5′-GGTATCCTGGAATGCGGCCACC AGGATATTGG-3′ was used to alter codons 220 and 221 (D220A E221A) to

TABLE 1. Strains used in this work

Strain	Relevant genotype	Reference or source
AB3505	F$^-$ *uvrD*$^+$ *rep*$^+$ *argH1 hisG4 ilvD188 metE46 lacY1* (or *lacZ4*)	54
C600	F$^-$ *uvrD*$^+$ *rep*$^+$ *thr-1 leuB6 thi-1 lacY1*	1
MC1061	F$^-$ *uvrD*$^+$ *rep*$^+$ Δ(*araA-leu*)*7697* Δ(*codB-lac*) *rpsL150*	7
MG1693	F$^-$ *uvrD*$^+$ *rep*$^+$ λ$^-$ *thyA715 rph-1*	15
W3110	F$^-$ *uvrD*$^+$ *rep*$^+$ λ$^-$ IN(*rrnD-rrnE*) *rph-1*	17
KC11	*uvrD*$^+$ *rep*Δ*11*::*kan*	8
SK707	F$^-$ *uvrD*$^+$ *rep*$^+$ *argH1 hisG4 ilvD188 metE46 lacMS286* φ80dII*lacBK1*	52
SK4090	F$^-$ *uvrD*Δ*294*::*kan rep*$^+$ *argH1 hisG4 ilvD188 metE46 lacMS286* φ80dII*lacBK1*	This work
SK7712	Hfr *uvrD*$^+$ *rep*$^+$ *hisG1 thi*	52
SK7757	F$^-$ *uvrD*$^+$ *rep*Δ*11*::*kan argH1 hisG4 ilv*$^+$ *metE46 lacMS286* φ80dII*lacBK1*	P1 (KC11 × SK707) *rep*Δ*11*::*kan* transductant
SK9040	SK4090 (*uvrD*Δ*294*::*kan*) transformed with pWSK29 (vector)	This work
SK9041	SK4090 (*uvrD*Δ*294*::*kan*) transformed with pGZK20 (*uvrD*$^+$)	This work
SK9042	SK4090 (*uvrD*Δ*294*::*kan*) transformed with pGZK22 (*uvrD305*)	This work
SK9043	SK4090 (*uvrD*Δ*294*::*kan*) transformed with pGZK23 (*uvrD306*)	This work
SK9044	SK4090 (*uvrD*Δ*294*::*kan*) transformed with pGZK24 (*uvrD307*)	This work
SK9045	SK4090 (*uvrD*Δ*294*::*kan*) transformed with pGZK25 (*uvrD308*)	This work
SK9046	SK4090 (*uvrD*Δ*294*::*kan*) transformed with pGZK26 (*uvrD309*)	This work
SK9047	SK4090 (*uvrD*Δ*294*::*kan*) transformed with pGZK27 (*uvrD310*)	This work

generate *uvrD305*; oligonucleotide 5′-GTAGATTGACTGGGCGGCAGCACC GACGATCATCAC-3′ was used to alter codons 248, 249, and 250 (D248A D249A D250A) to generate *uvrD306*; oligonucleotide 5′-GCTGGTAGAGGCG TAGTTTTGCTCC-3′ was used to alter codon 284 (R284A) to generate *uvrD307*; oligonucleotide 5′-CTGCGGGAACGCCAGGCCTGCCGCCGAGT GTAGC-3′ was used to alter codons 563 (K563A) and 566 (E566A) to generate *uvrD308*; oligonucleotide 5′-GGTTACGCCAACGAAGGCCAGACGGCG-3′ was used to alter codon 600 (Y600F) to generate *uvrD309*; and oligonucleotide 5′-CTGCATCGCGGCGGTTACGCCAAC-3′ was used to alter codon 605 (R605A) to generate *uvrD310.*

All mutations were confirmed by DNA sequence analysis. Sequencing was performed with the fmol DNA cycle sequencing system from Promega. Since the entire 2,900-bp *uvrD* gene was not sequenced for each mutant, in order to rule out possible second-site alterations, each mutagenesis procedure was performed at least twice, independently. In each experiment the mutant phenotype was 100% linked to the mutation introduced. Any colony exhibiting a wild-type phenotype did not contain a nucleotide alteration in the motif being examined.

Genetic assays. The UV survival of bacterial strains was measured as follows. Two hundred to 400 μl of an overnight standing culture of each strain was inoculated into 10 ml of fresh Luria-Bertani (LB) medium containing the appropriate antibiotic. Cells were grown at the designated temperatures to a concentration of 10^8 cells/ml before they were harvested by centrifugation and resuspended in the same volume of M56/2 buffer. The cell suspensions were incubated at the designated temperature for 60 min before dilution with prewarmed M56/2 buffer. Appropriate dilutions were plated on prewarmed LB agar plates. These plates were subsequently subjected to UV irradiation at varying doses (calibrated by a UVX digital radiometer manufactured by UVP Inc., San Gabriel, Calif.) and incubated overnight in the dark at the appropriate temperature. Surviving colonies were typically counted after 20 h of incubation. Colony numbers on duplicate plates without UV irradiation were used to calculate the survival rate.

Methyl methanesulfonate (MMS) sensitivity assays were performed in liquid medium by the method of Siegel (46).

Intrachromosomal recombination was analyzed by measuring the number of Lac$^+$ revertant colonies resulting from genetic recombination between two nonoverlapping partially deleted lactose operons (*lacMS286*φ80dII*lacBK1*) in the bacterial chromosome (57). Cells were grown to 10^8 cells per ml in L broth and resuspended in M56/2 buffer after two washes with the same buffer. Various dilutions were plated on lactose minimal agar plates. Appropriate dilutions were also plated on Luria agar plates to determine the number of viable cells. After 48 h of incubation at 37°C, the numbers of colonies on both sets of plates were counted.

Determination of conjugational recombination frequencies was performed as described by Willetts et al. (56). SK7712 (52), the Hfr donor, transferred *hisG1* early, while the recipient strains carried a *hisG4* allele. Following a 50-min mating period, cells were vigorously vortexed, washed, and resuspended in M56/2 buffer and plated onto minimal medium containing necessary supplements but no histidine. The resulting colonies were counted after 48 h of incubation at 37°C.

Mutation frequencies were determined by the method of Luria and Delbruck (26). A 10-ml culture was started from a single colony and grown to a density of 10^8 cells per ml. Subsequently, 100 μl of a 10^{-5} dilution were inoculated into each of 10 separate culture tubes containing 1 ml of Luria broth each. After 40 h of incubation without shaking at 37°C, 100 μl of each 1-ml culture was plated onto Luria agar plates containing 50 μg of spectinomycin/ml. In addition, the contents of five randomly picked tubes were pooled and plated at appropriate dilutions on Luria agar plates for determination of viable counts. The resulting colonies were counted after 48 h of incubation at 37°C. The results presented are averages of three separate experiments. For nonmutator strains that produced few or no colonies by the method described above, the procedure was modified by inoculating 10-ml cultures instead of 1-ml cultures and by plating the entire content of each tube (10 ml concentrated to 0.2 ml by centrifugation).

Protein analysis. One milliliter of fresh overnight culture of each strain was centrifuged in 1.5-ml Eppendorf tubes for 2 min at 3,000 rpm in a MSE Micro Centaur. After removing the supernatant, cell pellets were resuspended in 200 μl of cracking buffer (48) and heated in boiling water for 10 min. After centrifuging at 10,000 rpm for 2 min, aliquots from each supernatant, containing equal amounts of total protein (10 mg), were applied to a sodium dodecyl sulfate–10% polyacrylamide gel. After electrophoresis, the proteins in the gel were transferred to a polyvinylidene difluoride transfer membrane as described previously (38). The membranes were probed with anti-UvrD antibodies (53) and developed with the ECL Western blotting kit from Amersham Life Science. Signals

TABLE 2. Plasmids used in this work

Plasmid	Description[a]	Reference or source
pWSK29	Ap^r low-copy-number vector	51
pGZK20	Ap^r low-copy-number *uvrD*$^+$ plasmid	*uvrD*$^+$ cloned into the *Sal*I site of pWSK29
pGZK22	Ap^r *uvrD305* (D220A E221A) substitution in pGZK20	Site-directed mutagenesis
pGZK23	Ap^r *uvrD306* (D248A D249A D250A) substitution in pGZK20	Site-directed mutagenesis
pGZK24	Ap^r *uvrD307* (R284A) substitution in pGZK20	Site-directed mutagenesis
pGZK25	Ap^r *uvrD308* (K563A E566A) substitution in pGZK20	Site-directed mutagenesis
pGZK26	Ap^r *uvrD309* (Y600F) substitution in pGZK20	Site-directed mutagenesis
pGZK27	Ap^r *uvrD310* (R605A) substitution in pGZK20	Site-directed mutagenesis

[a] Mutationally altered *uvrD* genes were isolated as described in Materials and Methods. Items in parentheses indicate the positions of amino acid substitutions.

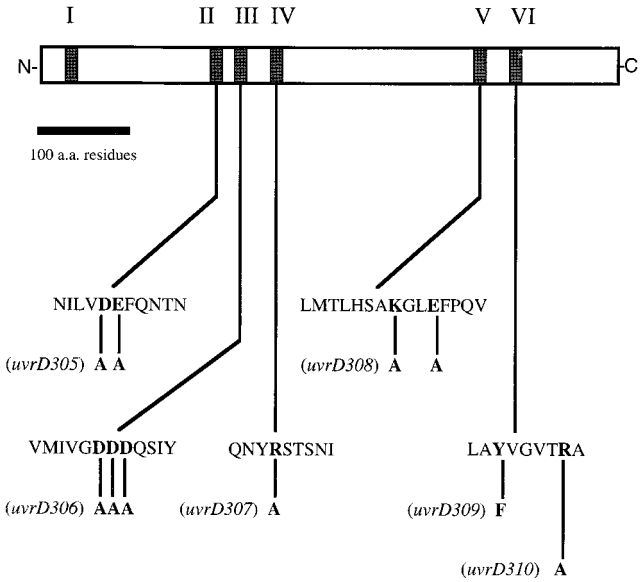

FIG. 1. Schematic representation of mutations in motifs II to VI of DNA helicase II. Mutations in these regions were introduced via site-directed mutagenesis as described in Materials and Methods. Motifs I to VI are designated distinct regions of homology characterized by conserved amino acids (18). The amino acid substitutions that were introduced into the five conserved motifs are presented below the diagram. The changed amino acid residues are indicated in boldface type.

produced by the UvrD protein were quantified with a scanning densitometer (Molecular Dynamics).

RESULTS

Introduction of mutations by site-directed mutagenesis. Sequence comparison (18) among 21 functionally related proteins (including UvrD) has revealed seven conserved motifs which may contribute significantly to the biological function of these proteins. We employed the alanine-scanning mutagenesis method, first used by Cunningham and Wells (9), to introduce mutations into five of these six conserved regions. We specifically targeted the charged amino acids in motifs II, III, IV, V, and VI and replaced them with alanines (motif I was not altered because it is the well-characterized ATPase site [12, 53]). In addition, we also changed a noncharged amino acid, Tyr, to Phe in motif VI, because this Tyr residue is highly conserved in this gene family (18). These mutations are diagrammed in Fig. 1. All were verified by direct sequence analysis, and each allele produced a stable full-length UvrD protein as judged by Western blotting analysis (Fig. 2). Plasmids carrying the mutant alleles were kept in a strain carrying a complete chromosomal *uvrD* deletion (SK4090 [*uvrDΔ294*::*kan]; see Materials and Methods) to prevent gene conversion.*

Effects of mutations in motifs II to VI on excision repair. DNA helicase II is known to be involved in the UvrABC-mediated excision repair pathway, which is responsible for efficient removal of UV-induced photoproducts. It has been suggested that DNA helicase II is required for the turnover of UvrC after incision on both sides of the damaged DNA (37). *uvrD* deletion mutants, as well as the majority of other *uvrD* alleles, cause increased sensitivity to UV (3, 5, 46, 52, 53). To assess the abilities of our mutants to function in this pathway, we measured their UV sensitivities in a *uvrDΔ294*::*kan* genetic background. Mutations in motif II (*uvrD305*), motif III (*uvrD306*), motif IV (*uvrD307*), and motif V (*uvrD308*) all

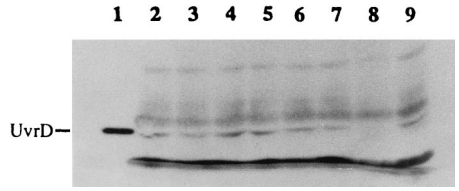

FIG. 2. Western blotting analysis of the expression of various *uvrD* alleles. Experiments were carried out as described in Materials and Methods. Lane 1 contains 75 ng of purified UvrD$^+$ protein. Lanes 2 through 9 contain 10 mg of total cell protein from strains carrying various *uvrD* alleles, as follows: lane 2, SK9042 (*uvrDΔ294 uvrD305*); lane 3, SK9043 (*uvrDΔ294 uvrD306*); lane 4, SK9044 (*uvrDΔ294 uvrD307*); lane 5, SK9045 (*uvrDΔ294 uvrD308*); lane 6, SK9046 (*uvrDΔ294 uvrD309*); lane 7, SK9047 (*uvrDΔ294 uvrD310*); lane 8, SK9040 (*uvrDΔ294*); lane 9, SK9041 (*uvrDΔ294 uvrD*$^+$).

led to levels of UV sensitivity comparable to those observed in the strain with the complete structural gene deletion (*uvrDΔ294*::*kan*^{r}) (Fig. 3). By contrast, one mutant allele in motif VI (*uvrD310*) was slightly less sensitive to UV light than the deletion allele (*uvrDΔ294*::*kan*^{r}), and the other allele in motif VI (*uvrD309*) displayed only a moderate increase in UV sensitivity (Fig. 3).

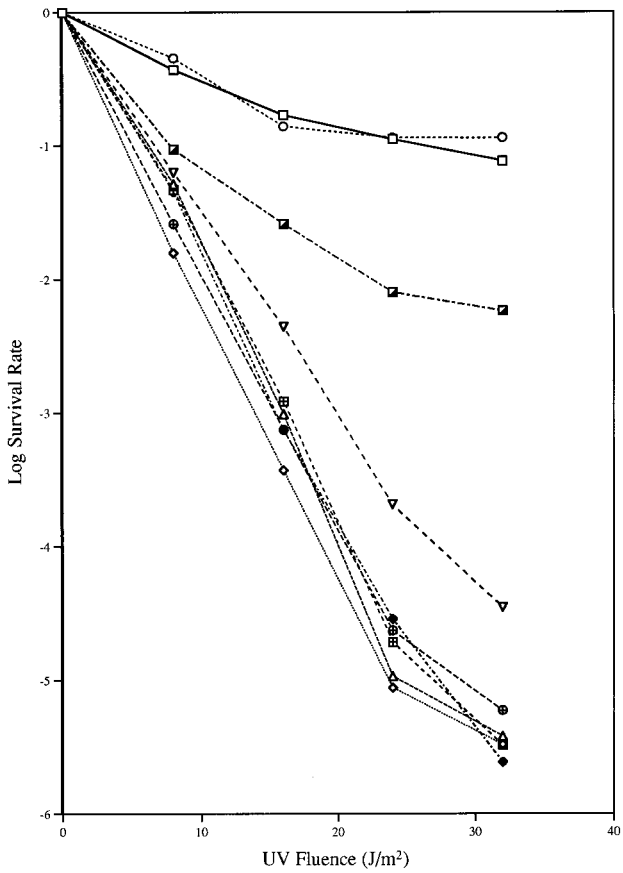

FIG. 3. UV sensitivity of various *uvrD* strains. UV survival experiments were carried out at 37°C as described in Materials and Methods. Data (log survival) are expressed as logarithmic functions of the results of viable UV-irradiated cells forming colonies versus cells forming colonies on unirradiated plates. Symbols: □, SK707 (*uvrD*$^+$); ◇, SK9040 (*uvrDΔ294* vector); ○, SK9041 (*uvrDΔ294 uvrD*$^+$); △, SK9042 (*uvrDΔ294 uvrD305*); ⊞, SK9043 (*uvrDΔ294 uvrD306*); ◆, SK9044 (*uvrDΔ294 uvrD307*); ⊕, SK9045 (*uvrDΔ294 uvrD308*); ◪, SK9046 (*uvrDΔ294 uvrD309*); ▽, SK9047 (*uvrDΔ294 uvrD310*).

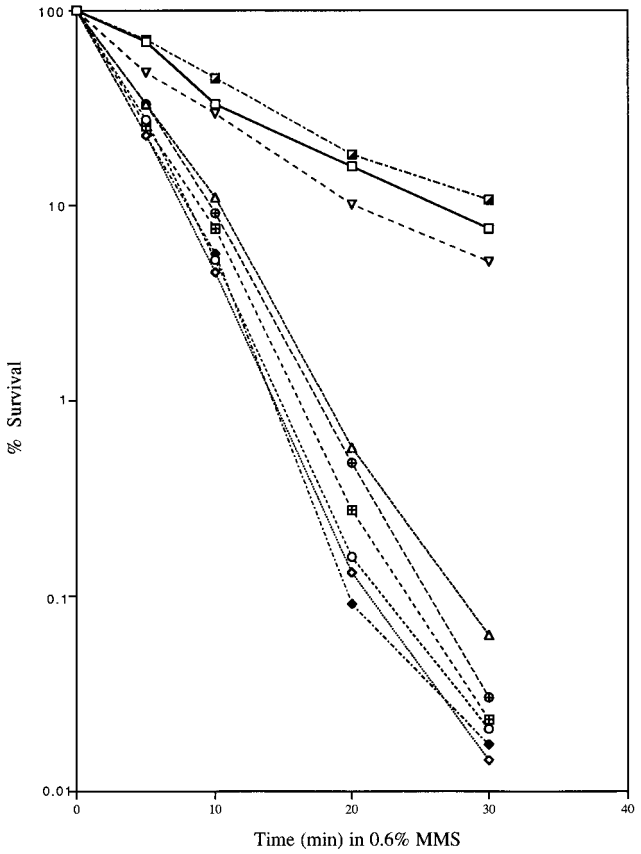

FIG. 4. MMS sensitivity of various *uvrD* mutants. The MMS sensitivity assay was performed at 37°C as described in Materials and Methods. Data (percent survival) are expressed as percentages of MMS-exposed cells forming colonies versus unexposed cells forming colonies. Symbols: □, SK707 (*uvrD*$^+$); ◇, SK9040 (*uvrDΔ294* plus vector); ◪, SK9041 (*uvrDΔ294 uvrD*$^+$); ○, SK9042 (*uvrDΔ294 uvrD305*); △, SK9043 (*uvrDΔ294 uvrD306*); ⊞, SK9044 (*uvrDΔ294 uvrD307*); ◆, SK9045 (*uvrDΔ294 uvrD308*); ▽, SK9046 (*uvrDΔ294 uvrD309*); ⊕, SK9047 (*uvrDΔ294 uvrD310*).

Effects of mutations in motifs II to VI on MMS sensitivity. Although the specific mechanism is still unclear, DNA helicase II has also been shown to be involved in the repair of DNA lesions caused by alkylating agents such as MMS (47, 52). As shown previously for *uvrD* partial-deletion strains (52), the complete loss of the *uvrD* gene led to a significant increase in MMS sensitivity. Mutations in motifs II (*uvrD305*), III (*uvrD306*), IV (*uvrD307*), and V (*uvrD308*) exhibited MMS sensitivities comparable to *uvrDΔ294*::*kan* (Fig. 4). Of particular interest was the differential MMS sensitivity displayed by the motif VI mutations. The arginine-to-alanine substitution (*uvrD310*) led to a significant increase in MMS sensitivity, while the change of tyrosine to phenylalanine (*uvrD309*) resembled the wild type (Fig. 4).

Effects of mutations in motifs II to VI on mismatch repair. Many years ago, the *uvrD156* allele (formerly *mutU4*) was isolated solely on the basis of its mutator phenotype (46). This and most other *uvrD* alleles increase the rate of spontaneous mutagenesis by as much as 200-fold (47). It appears that the failure to correctly repair mismatched bases is the primary mechanism by which *uvrD* mutations increase spontaneous mutation rates (28, 36). DNA helicase II has hence been assigned a role in methyl-directed mismatch repair (34). However, not all *uvrD* alleles act as mutators. The *uvrD3* and *uvrD252* strains are reported to have near-normal levels of spontaneous mutagenesis (10, 28, 47, 53), which suggests that the function of UvrD in excision repair can be separated from its function in mismatch repair.

TABLE 3. Mutation frequencies in various *uvrD* mutants

Strain	Relevant genotype	Mutation frequency (10^{-10})[a]	Relative mutability[b]
SK707	*uvrD*$^+$	1.9 ± 1.1	1.0
SK9040	*uvrDΔ294*/pWSK29	490 ± 230	258
SK9041	*uvrDΔ294*/pGZK20 (*uvrD*$^+$)	1.3 ± 0.6	0.7
SK9042	*uvrDΔ294*/pGZK22 (*uvrD305*)	400 ± 25	210
SK9043	*uvrDΔ294*/pGZK23 (*uvrD306*)	450 ± 130	237
SK9044	*uvrDΔ294*/pGZK24 (*uvrD307*)	360 ± 70	189
SK9045	*uvrDΔ294*/pGZK25 (*uvrD308*)	160 ± 80	84
SK9046	*uvrDΔ294*/pGZK26 (*uvrD309*)	0.8 ± 0.4	0.4
SK9047	*uvrDΔ294*/pGZK27 (*uvrD310*)	530 ± 160	279

[a] Mutation frequencies for spectinomycin resistance were calculated by dividing the number of colonies growing on each spectinomycin plate by the number of total cells originally applied to the plate. The data presented are averages of three independent experiments ± standard deviations. All experiments were performed at 37°C as described in Materials and Methods.

[b] Relative mutability was calculated by dividing the mutation frequency of each strain by that of SK707 (*uvrD*$^+$).

We thus investigated whether the mutations we describe here can complement mismatch repair in a strain carrying a complete chromosomal deletion of *uvrD* (SK4090 [*uvrDΔ294*::*kan*]). As shown in Table 3, the complete deletion of the *uvrD* coding sequence led to a 250-fold increase in spontaneous mutation frequency. Mutations in motifs II to V also led to significant increases in spontaneous mutation rate (189- to 237-fold [Table 3]). Motif VI alleles again showed an interesting divergence in their properties. *uvrD310* (R605A) was a strong mutator which resulted in a 279-fold increase in the spontaneous mutation rate. In contrast, *uvrD309* (Y600F) caused a small reduction in the rate of spontaneous mutation (Table 3).

Effects of mutations in motifs II to VI on homologous recombination. Previous studies have implicated *uvrD* in the RecF pathway of recombination (19). DNA helicase II may also be involved in the RecBCD homologous recombination pathway, since *uvrD* mutants generally exhibit increased recombination frequencies, with *uvrD3* as an exception (2, 10, 11, 25, 52, 58). Therefore, we examined how mutations in motifs II to VI affect two types of homologous recombination. We analyzed recombination frequencies either by measuring intrachromosomal recombination between two nonoverlapping partially deleted *lac* operons (57) or by measuring recombination following Hfr mating (56). The results obtained with mutations in motifs II to V (Tables 4 and 5) indicated an increase in homologous recombination frequencies which paralleled the increased rates of spontaneous mutation (Table 3).

Again, we saw different effects by the two motif VI mutants on genetic recombination. There was a 12.5-fold increase in conjugal recombination for the *uvrD310* allele, while the *uvrD309* allele resulted in a 5-fold reduction in the same measurement (Table 4). Intrachromosomal recombination was affected in a similar fashion (Table 5).

Certain wild-type strains carrying the *uvrD307* allele exhibit reduced viability at 30°C. Besides the well-documented functions of DNA helicase II in DNA repair and recombination, it has long been argued whether DNA helicase II also plays a role in DNA replication (12, 31, 49). Although *uvrD* is not an essential gene in *E. coli* (52), a *uvrD* mutant allele in motif I that resulted in a dominant-negative phenotype was reported (12), suggesting that DNA helicase II may participate in an essential pathway in *E. coli*, possibly DNA replication. To

TABLE 4. Conjugal recombination in various *uvrD* mutants

Strain	Relevant genotype	No. of *his*$^+$ recombinants/ml[a]	Relative recombination proficiency[b]
SK707	*uvrD*$^+$	$(5.1 \pm 0.7) \times 10^3$	1.0
SK9040	*uvrD*Δ*294*/pWSK29	$(8.5 \pm 0.7) \times 10^4$	16.9
SK9041	*uvrD*Δ*294*/pGZK20 (*uvrD*$^+$)	$(3.0 \pm 0.7) \times 10^3$	0.6
SK9042	*uvrD*Δ*294*/pGZK22 (*uvrD305*)	$(3.9 \pm 0.6) \times 10^4$	7.6
SK9043	*uvrD*Δ*294*/pGZK23 (*uvrD306*)	$(1.2 \pm 0.3) \times 10^5$	23.5
SK9044	*uvrD*Δ*294*/pGZK24 (*uvrD307*)	$(7.2 \pm 0.6) \times 10^4$	14.1
SK9045	*uvrD*Δ*294*/pGZK25 (*uvrD308*)	$(6.9 \pm 0.8) \times 10^4$	13.5
SK9046	*uvrD*Δ*294*/pGZK26 (*uvrD309*)	$(1.2 \pm 0.2) \times 10^3$	0.2
SK9047	*uvrD*Δ*294*/pGZK27 (*uvrD310*)	$(6.4 \pm 0.6) \times 10^4$	12.5

[a] Data represent averages of at least three independent experiments ± variation from the mean.
[b] Recombination assays were performed at 37°C as described in Materials and Methods. Relative recombination proficiency is the recombination frequency of each strain tested divided by that of SK707 (*uvrD*$^+$).

assess the effects of *uvrD* mutations in motifs II to VI on cell viability, plasmids carrying either wild-type or mutant *uvrD* alleles were transformed into several common *E. coli* laboratory strains of differing origins (Table 1). Cells were grown in LB medium at 37°C until the concentration reached approximately 10^8 cells/ml and were diluted in prewarmed LB medium. One hundred-microliter aliquots were plated in a pairwise manner on LB agar plates (with ampicillin, 50 μg/ml) and incubated at 37°C and 30°C. Colonies were counted after 48 h of incubation. As shown in Table 6, the presence of *uvrD307* (R284A) resulted in a 10-fold reduction of viability at 30°C in MC1061 and more than a 250-fold reduction in MG1693. In other strains it caused little difference of viability between the two temperatures. No further reduction in viability was observed at temperatures below 30°C (data not shown).

DISCUSSION

Amino acid sequence analysis has revealed that there are seven conserved domains (motifs IA, IB, and II to VI) in UvrD that have high homology among a family of proteins that are involved in DNA metabolism (18). Motifs IA and IB, formerly known as the Walker A sequence (50), are considered to be involved in nucleotide binding and/or hydrolysis. Mutations in this domain significantly reduce the catalytic activity of ATP hydrolysis (12) and the apparent K_m for ATP binding (53). It has recently been reported that mutations in motif II resulted in substantial reduction in k_{cat} for ATP hydrolysis (3), indicating that motif II may also participate in the catalytic domain of ATPase activity.

TABLE 5. Intrachromosomal recombination in various *uvrD* mutants[a]

Strain	Relevant genotype	No. of *lac*$^+$ recombinants/ plate	Relative recombination proficiency
SK707	*uvrD*$^+$	76 ± 10	1.0
SK9040	*uvrD*Δ*294*/pWSK29	502 ± 35	6.4
SK9041	*uvrD*Δ*294*/pGZK20 (*uvrD*$^+$)	96 ± 16	1.2
SK9042	*uvrD*Δ*294*/pGZK28 (*uvrD305*)	342 ± 55	4.5
SK9043	*uvrD*Δ*294*/pGZK29 (*uvrD306*)	418 ± 78	5.5
SK9044	*uvrD*Δ*294*/pGZK30 (*uvrD307*)	437 ± 44	5.8
SK9045	*uvrD*Δ*294*/pGZK31 (*uvrD308*)	286 ± 36	3.8
SK9046	*uvrD*Δ*294*/pGZK32 (*uvrD309*)	59 ± 28	0.8
SK9047	*uvrD*Δ*294*/pGZK32 (*uvrD310*)	479 ± 69	6.3

[a] Recombination tests were performed as described in Material and Methods. Equal amount of cells for each strain were plated (approximately 2×10^7 cells/plate) on lactose minimal plates. All experiments were conducted at 37°C. Data are expressed as described in Table 4, footnotes *a* and *b*.

TABLE 6. Viability ratio of strains carrying various *uvrD* alleles[a]

Strain	Plasmid	Avg no. of colonies on plates incubated at[b]:		Viability ratio (30°C/37°C)
		37°C	30°C	
AB3505	pGZK20 (*uvrD*$^+$)	757 ± 17	703 ± 14	92.8
AB3505	pGZK24 (*uvrD307*)	667 ± 16	604 ± 21	90.5
C600	pGZK20 (*uvrD*$^+$)	1,024 ± 18	953 ± 23	93.0
C600	pGZK24 (*uvrD307*)	795 ± 21	748 ± 16	94.1
SK707	pGZK20 (*uvrD*$^+$)	774 ± 18	732 ± 25	94.6
SK707	pGZK24 (*uvrD307*)	712 ± 12	676 ± 14	94.9
MC1061	pGZK20 (*uvrD*$^+$)	881 ± 11	782 ± 16	88.7
MC1061	pGZK24 (*uvrD307*)	881 ± 11	82 ± 6	9.3
MG1693	pGZK20 (*uvrD*$^+$)	920 ± 16	824 ± 11	89.6
MG1693	pGZK24 (*uvrD307*)	994 ± 22	3 ± 2	0.3
W3110	pGZK20 (*uvrD*$^+$)	584 ± 17	541 ± 12	92.6
W3110	pGZK24 (*uvrD307*)	550 ± 16	503 ± 12	91.2

[a] Cells were grown at 37°C in Luria broth (with 50 mg of ampicillin per ml if required) to approximately 10^8 cells/ml and diluted in prewarmed Luria broth. Aliquots (100 ml) of appropriate dilutions from each strain were plated in a pairwise manner on LB agar plates and incubated at 30°C and 37°C. Colonies were counted after 48 h of incubation.
[b] Values are averages ± variation from the mean.

To further understand the functional significance of the conserved motifs in *uvrD*, as well as to better appreciate the structure-function relationships among DNA helicases and their closely related homologs, we employed the genetic approach of systematically introducing mutations into motifs II through VI of DNA helicase II via site-directed mutagenesis, specifically replacing the charged amino acids with alanines (Fig. 1). The alanine substitution method has been widely used for systematic mutagenesis in many laboratories. In the majority of cases the replacement of a charged amino acid with alanine has resulted in minimal misfolding or global structural alterations (9, 55). In the case of each mutant allele described here, densitometric analysis of Western blots, such as shown in Fig. 2, showed that protein was produced in levels comparable to the wild-type control. Nevertheless, it must be noted that the solubility of the mutant proteins in vivo may have been altered, which could contribute to the phenotypes observed.

The results presented here show that all five motifs are essential for the biological functions of DNA helicase II. As shown in Fig. 3, mutations in motifs II to V all exhibited strong UV sensitivity, similar to that of a *uvrD* deletion strain (SK9040 [*uvrD*Δ*294*::*kan*]). The *uvrD310* (R605A) allele in motif VI was only slightly less sensitive than SK9040, and the other mutation in motif VI (*uvrD309* [Y600F]), which did not involve a charged amino acid replacement, also exhibited a moderate but clearly detectable increase in sensitivity to UV light. These results suggest that the mutations in each of the conserved motifs in DNA helicase II render cells defective in the excision repair pathway.

MMS, like other alkylating agents, causes base modifications, which are toxic lesions, in the DNA. DNA helicase II has previously been shown to be required for the efficient repair of DNA damage caused by MMS (46), and *uvrD* deletion strains display an increase in MMS sensitivity (52). As shown in Fig. 4, all mutations in motifs II to VI (except for *uvrD309* [Y600F]) exhibited a level of MMS sensitivity comparable to the *uvrD* deletion strain (SK9040 *uvrD*Δ*294*::*kan*), indicating that the mutant proteins were also defective in repairing alkylated bases in DNA.

DNA helicase II is also known to be involved in methyl-directed mismatch repair (35). Consequently, mutations in *uvrD* usually result in an elevated level of spontaneous mutagenesis (3, 5, 47, 52). As shown in Table 3, cells carrying the mutant alleles *uvrD305* (motif II), *uvrD306* (motif III), *uvrD307* (motif IV), and *uvrD310* (R605A, motif VI) showed the same increase in the spontaneous mutation rate as the *uvrDΔ294* deletion allele, reflecting a deficiency in the mismatch repair pathway. The *uvrD308* (motif V) allele also displayed a mutator phenotype, although to a lesser extent than did the *uvrD* deletion strain (Table 3). In contrast, the spontaneous mutation frequency for *uvrD309* (Y600F, motif VI) decreased more than 50% compared to the *uvrD*$^+$ strain (Table 3), suggesting a more efficient mismatch repair process. However, the observed higher UV sensitivity in the same strain (Fig. 3) suggested a deficiency in excision repair. Although this result seems contradictory, it is possible that different activities, or different levels of the same activity associated with DNA helicase II, may be required in these two repair processes. Consequently, a mutation in one particular domain may not cause a global effect, but rather affect only one repair pathway. Further genetic and biochemical studies are needed to test this hypothesis.

It has also been noted that most *uvrD* mutations lead to an increase in the level of homologous recombination (2, 10, 52, 58). Although the exact mechanism is not clear, it has been proposed that the mismatch repair system functions as an antirecombinase (39), causing recombination to be aborted if the invading strand contains mismatches (40). In this process, DNA helicase II is assumed to be responsible for unwinding the invading strand from the complementary recipient strand. Thus, a functional loss of DNA helicase II could result in an increase in the rate of recombination. Consistent with the model described above, all of the alleles showing a mutator phenotype (Table 3) also had higher levels of conjugal recombination (Table 4) and intrachromosomal recombination (Table 5). On the other hand, the *uvrD309* allele (Y600F, motif VI) which showed reduced spontaneous mutagenesis (Table 3) also displayed a lower level of genetic recombination (Tables 4 and 5).

In conclusion, the mutations in each of the five motifs we studied render DNA helicase II either defective or altered in both the excision repair and mismatch repair pathways. As a corollary, they also lead to increased levels of homologous recombination. The phenotype of *uvrD305* (D220A E221A) (Fig. 3, Table 3) agrees well with a different *uvrD* allele (*uvrDD220NE221Q*) isolated independently in another laboratory (3). Recently, Brosh and Matson (4, 5) have also isolated and characterized mutations in motif III. One *uvrD* allele (*uvrDQ251E*) with a single amino acid substitution in motif III (VMIVGDDD**Q**SIY [Fig. 1]) led to a level of UV sensitivity and spontaneous mutagenesis comparable to that of a *uvrD* deletion strain (5). Another allele (*uvrDD248N*) produced a partially functional UvrD protein defective in forming stable binary complexes with ATP or DNA and resulted in a moderate increase in UV sensitivity and spontaneous mutation rate (4). The mutation in motif III (*uvrD306*) that we have reported here is different because it has three adjacent substitutions (D248A D249A D250A [Fig. 1]). It resulted in UV sensitivity and spontaneous mutagenesis levels comparable to Δ*uvrD*. It is conceivable that all three aspartic acid residues are involved in the function of forming stable binary complexes with DNA and ATP, and a mutant protein with one residue alteration could remain partially functional, while removing all three negatively charged residues would completely abolish the function in this domain.

Our results with mutations in motifs IV, V, and VI provide the first evidence that these motifs also make indispensable contributions to the biological function of DNA helicase II in vivo. The replacement of charged with uncharged amino acids in each of these motifs abolished the normal function of UvrD in excision repair, mismatch repair, and homologous recombination, suggesting that many, if not all, of the motifs may function cooperatively. Moreover, the importance of these motifs is not restricted to the charged amino acids. At least in one case, the replacement of tyrosine(600) with phenylalanine in motif VI showed an increase in UV sensitivity. Interestingly, the MMS sensitivity remained close to that of the wild-type strain, while the level of spontaneous mutagenesis and homologous recombination was actually reduced. In contrast, the replacement of arginine(605) with alanine six amino acids downstream completely abolished the activity of UvrD. Further genetic and biochemical studies are necessary to discern whether these two mutations affect two different enzymatic activities or the same activity with different levels of severity.

Another very interesting observation of this work is that a mutation in motif IV (*uvrD307* [R284A]) led to cold sensitivity in two *E. coli* K-12 strains but not in several other K-12 strains (Table 6). At 37°C, *uvrD307* (R284A) had no significant impact on the growth and viability in strains MC1061 (7) and MG1693 (15). However, the viability of these strains decreased 10-fold and 250-fold, respectively, when incubated at 30°C. We also transformed pGZK24 (*uvrD307*) into several other commonly used *E. coli* K-12 strains such as AB3505 (54), C600 (1), and W3110 (17), in addition to SK707 (an AB3505 derivative [52]). In these genetic backgrounds, no reduction of viability was observed (Table 6). A parallel experiment was also conducted with pGZK20, a *uvrD*$^+$ plasmid otherwise isogenic to pGZK24, and no significant difference in cell viability between 30°C and 37°C was observed in any of the strains described above (Table 6).

Although DNA helicase II has been implicated in several important genetic pathways affecting DNA metabolism, deletion of the coding sequence in *uvrD* has no discernible effect on cell viability (52). However, a lysine-to-methionine mutation in conserved motif I (*uvrDK35M*) has been reported to be lethal, reducing the cell viability even with modest expression (12). It has since been speculated that DNA helicase II may be involved in an essential process in vivo, in addition to the well-documented excision repair and mismatch repair pathways. The conditional lethal effect observed with the *uvrD307* allele provides additional evidence for an essential role of DNA helicase II in cell viability. It also implies specific importance of motif IV among the six conserved motifs in *uvrD*. Since *uvrD307* did not cause cold sensitivity in all strains tested, an unidentified factor(s) may be required along with UvrD307 to produce the phenotype. Alternatively, suppressor mutations in certain genetic backgrounds might eliminate the lethal effect of UvrD307. Although we are unable to explain this intriguing observation at the present time, it represents a new opportunity to explore additional roles of DNA helicase II in vivo.

Finally, it is worth noting that Graves-Woodward et al. (13) have recently shown that domains IV to VI of the herpes simplex virus type 1 helicase-primase, a eukaryotic member of the helicase superfamily to which DNA helicase II belongs, are also required for biological activity.

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