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A Point Mutation in *Escherichia coli* DNA Helicase II Renders the Enzyme Nonfunctional in Two DNA Repair Pathways

EVIDENCE FOR INITIATION OF UNWINDING FROM A NICK *IN VIVO**

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Biosynthetic errors and DNA damage introduce mismatches and lesions in DNA that can lead to mutations. These abnormalities are susceptible to correction by a number of DNA repair mechanisms, each of which requires a distinct set of proteins. *Escherichia coli* DNA helicase II has been demonstrated to function in two DNA repair pathways, methyl-directed mismatch repair and UvrABC-mediated nucleotide excision repair. To define further the role of UvrD in DNA repair a site-specific mutant was characterized. The mutation, *uvrDQ251E*, resides within helicase motif III, a conserved segment of amino acid homology found in a superfamily of prokaryotic and eukaryotic DNA helicases. The UvrD-Q251E protein failed to complement the mutator and ultraviolet light-sensitive phenotypes of a *uvrD* deletion strain indicating that the mutant protein is inactive in both mismatch repair and excision repair. Biochemical characterization revealed a significant defect in the ability of the mutant enzyme to initiate unwinding at a nick. The elongation phase of the unwinding reaction was nearly normal. Together, the biochemical and genetic data provide evidence that UvrD-Q251E is dysfunctional because the mutant protein fails to initiate unwinding at the nick(s) used to initiate excision and subsequent repair synthesis. These results provide direct evidence to support the notion that helicase II initiates unwinding from a nick *in vivo* in mismatch repair and excision repair.

The processes of replication, recombination, and oligonucleotide excision repair require that duplex DNA be transiently unwound to yield single-stranded DNA (ssDNA)[1] templates and reaction intermediates. DNA helicases are responsible for catalyzing the unwinding of double-stranded DNA (dsDNA) by disrupting the hydrogen bonds between complementary base pairs in a nucleoside 5′-triphosphate hydrolysis-dependent reaction (1–4). In *Escherichia coli*, there are at least 11 different DNA helicases, each involved in a specific reaction pathway or subset of reaction pathways (4, 5). Thus, there is a division of labor among these enzymes, and each helicase presumably makes specific protein-DNA and protein-protein contacts.

Genetic and biochemical data have demonstrated that the *uvrD* gene product, DNA helicase II, is required in both methyl-directed mismatch repair (6–10) and UvrABC-mediated nucleotide excision repair (11–14). Methyl-directed mismatch repair is utilized for repair of DNA tracts containing a mismatched base pair, a bulge or a loop (10). The UvrABC-mediated nucleotide excision repair pathway is utilized to repair various ultraviolet (UV) light-induced photoproducts and damaged DNA bases (14). Since helicase II is required in both repair pathways, helicase II null mutants are both UV-sensitive and have a mutator phenotype (15, 16). Although evidence supports a requirement for helicase II in the excision step in both reaction pathways, the details of the mechanism of excision of the tract containing the mismatched or damaged DNA base are ill-defined.

In contrast, the catalytic properties of helicase II (UvrD) have been well described. Helicase II is a ssDNA-stimulated ATPase (17) that catalyzes the unwinding of duplex DNA (18, 19) with a 3′ to 5′ polarity (20). The unwinding reaction is protein concentration-dependent (*i.e.* the amount of duplex DNA unwound is directly proportional to protein) (18, 21, 22), and helicase II coats the unwound DNA (23, 24). At high concentrations the enzyme can initiate unwinding of duplex DNA at blunt ends or nicks *in vitro* (23, 25, 26). This property may be relevant to the enzyme's roles in the cell. For example, in methyl-directed mismatch repair, helicase II has been proposed to initiate unwinding at the mismatch-provoked incision (nick) of the unmethylated strand at a hemi-methylated GATC site (9, 10). In nucleotide excision repair, helicase II releases the damaged oligomer and UvrC protein, again presumably by unwinding the nicked DNA intermediate (13, 14). However, there is no direct evidence that helicase II initiates unwinding of the duplex DNA intermediates in mismatch repair or excision repair at the site of the nick *in vivo*.

To further our understanding of the molecular genetic roles of helicase II in DNA repair, we have examined the functional significance of conserved regions of the protein (27, 28). Site-specific mutants in motifs I or II (Walker ATPase A and B sites) (29) encode UvrD proteins dramatically compromised in ATPase and helicase activities (30, 31). Expression of these *uvrD* alleles fails to complement the mutator and UV light-sensitive phenotypes of a *uvrD* deletion mutant indicating that both methyl-directed mismatch repair and nucleotide excision repair require the ATPase and/or helicase activities of helicase II.

In this study we have constructed a specific mutation in helicase motif III, a conserved segment adjacent to the Walker A and B sites. The mutant protein fails to function in either methyl-directed mismatch repair or UV excision repair as evidenced by genetic complementation studies. Biochemical characterization of the UvrD-Q251E mutant protein has revealed a

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine-5′-*0*-(3-thiotriphosphate); bp, base pair; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

significant defect in the ability of the enzyme to initiate unwinding at a nick. The elongation phase of the unwinding reaction, as measured using partial duplex DNA substrates, is nearly normal. Taken together, the data suggest that helicase II must initiate unwinding from a nick *in vivo* in both methyl-directed mismatch repair and excision repair.

EXPERIMENTAL PROCEDURES

Bacterial Strains—E. coli strain BL21(DE3) (λDE3 *omp*T r_B^- m_B^-) was obtained from Novagen, Inc. JH137 (K91 *lacZ dinD1*::MudI (Ap^r*lac*)) was obtained from P. Model (Rockefeller University). BL21(DE3)Δ*uvrD* and JH137Δ*uvrD* were previously constructed in this laboratory (30).

DNA and Nucleotides—pBluescript (Stratagene) (pBS) was grown in *E. coli* HB101 (F^- Δ(*gpt-proA*)62 *leuB6 supE44 ara-14 galK2 lacY1* D(*mrcC-mrr*) *rpsL20*(Str^r) *xyl-5 mtl-1 recA13*) and purified by alkaline-sodium dodecyl sulfate (SDS) lysis followed by banding twice on $CsCl_2$-ethidium bromide gradients. Nicked pBS was prepared by digesting supercoiled DNA with *Hinc*II restriction endonuclease in the presence of ethidium bromide (32). Reaction mixtures (20 μl) contained 1.5 μg of DNA, the appropriate reaction buffer, and ethidium bromide (0.5 μg/ml). Incubation was at room temperature for 4 h. The nicked species was resolved from the supercoiled and linear DNA molecules by electrophoresis on a 0.9% agarose gel. The band containing nicked DNA was excised, electroeluted at 60 V for 4 h, extracted with phenol-chloroform, ethanol-precipitated, and resuspended in water. To determine the concentration of nicked pBS, a portion of the resuspended DNA was linearized with *Afl*III, and the band intensity of the ethidium bromide-stained DNA was compared with a standard curve of linear pBS.

Bacteriophage M13mp18 and M13mp7 ssDNAs and their derivatives were prepared as described (33). All unlabeled nucleotides were from U. S. Biochemicals Corp. except ATPγS which was from Boehringer Mannheim. Plasmid pET81F1+ was kindly provided by Dr. P. J. Laipis (University of Florida). Plasmid pET9d was purchased from Novagen, Inc. Concentrations of DNA and nucleotides were determined by UV spectrophotometry using published extinction coefficients and are expressed as nucleotide equivalents.

Enzymes—MutL protein was kindly provided by Dr. P. Modrich (Duke University). Restriction endonucleases, DNA polymerase I (large fragment), phage T7 DNA polymerase, and phage T4 polynucleotide kinase were purchased from New England BioLabs or U. S. Biochemical Corp. The reaction conditions used were those suggested by the supplier.

E. coli DNA helicase II was purified from BL21(DE3)/pLysS cells containing the pET9d-H2wt expression plasmid (30). The UvrD-Q251E protein was purified from BL21(DE3)Δ*uvrD*/pLysS cells containing the pET81-H2Q251E expression plasmid. Ten liters of cells grown in LB media plus ampicillin (200 μg/ml) and chloramphenicol (30 μg/ml) were induced for protein expression during log phase with isopropyl-β-D-thiogalactopyranoside (0.5 mM) and harvested 4 h after induction. The procedure of Runyon *et al.* (34) was used to purify both the wild-type and mutant proteins. Final protein concentration was determined using the helicase II extinction coefficient (34).

Site-directed Mutagenesis and DNA Constructions—For mutagenesis and expression, the *uvrD* gene was cloned into pET81F1+ (35), a T7 expression vector with a bacteriophage f1 origin of replication as described previously (36). pET81-H2wt (pET81 containing the wild-type *uvrD* gene) was the target for site-directed mutagenesis using published procedures (37, 38). Oligonucleotide 5′-GTAGATTGACTCGTCGTCATC-3′ was used to alter codon 251 of *uvrD* from CAG (Gln) to GAG (Glu). The entire *uvrD* gene in pET81-H2Q251E and pET81-H2wt was sequenced on a model 373A DNA Sequencer (Applied Biosystems) using the *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems) to confirm the mutation at position 251. A 2.1-kilobase *Nde*I fragment, containing a portion of the *uvrD* gene containing the *UvrD-Q251E* mutation plus some 3′-flanking sequence, was moved from pET81-H2Q251E into pET9d-H2wt (30) to yield pET9d-H2Q251E.

Genetic Assays—The viability of bacterial strains exposed to UV light was measured as described previously (31). The spontaneous mutation frequency for each cell strain was determined as described (30).

DNA Binding Assays—A nitrocellulose filter binding assay was used to measure binding of UvrD protein to DNA (31). Binding reaction mixtures (20 μl) contained 25 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 20 mM NaCl, 5 mM 2-mercaptoethanol, the 92-bp partial duplex helicase substrate (approximately 2 μM nucleotide phosphate) (3.26×10^8 cpm μmol^{-1}), and the indicated amount of helicase II. To determine the effect of nucleotide on DNA binding, reaction mixtures were altered to contain either 3 mM rATPγS or no nucleotide as indicated in appropriate figure legends. The reaction mixture was incubated at 37 °C for 10 min, applied to a nitrocellulose filter (0.45 μM; Whatman), and processed as described previously (31).

Proteolytic Digestions of Helicase II—UvrD and UvrD-Q251E proteins were lightly digested with α-chymotrypsin (Sigma) as described previously (39). Reaction mixtures (15 μl) contained either 1.8 μM UvrD (monomer) or 1.6 μM UvrD-Q251E (monomer) and 5 ng of freshly prepared chymotrypsin. Incubation was for 2 min at 37 °C. To determine the effect of ATP, Mg^{2+}, or ssDNA on the proteolysis of helicase II, reaction mixtures contained 2 mM ATPγS, 3 mM $MgCl_2$, and/or 214 mM M13mp7 ssDNA (nucleotide phosphate). Samples were resolved on 12% polyacrylamide gels containing SDS and stained with Coomassie Blue (40).

Glutaraldehyde Cross-linking—Protein cross-linking reactions were performed at room temperature at a final concentration of UvrD or UvrD-Q251E of 2 μM (monomer). Cross-linking reaction mixtures (20 μl) contained 20 mM Tricine (pH 8.3), 50 mM NaCl, 20% glycerol, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and were processed as described previously (36). Samples were resolved on 9% polyacrylamide gels containing SDS and stained with Coomassie Blue.

DNA-dependent ATPase Assays—The hydrolysis of ATP to ADP was measured as described previously (41). ATPase reaction mixtures were identical with helicase reaction mixtures except they contained 9 μM M13mp7 ssDNA (nucleotide phosphate) or 6 μM pBS dsDNA (nucleotide phosphate) and [^{3}H]ATP (22 cpm/pmol). For k_{cat} determinations, the [^{3}H]ATP concentration was 540 μM. For K_m determinations, the [^{3}H]ATP concentration was varied between 25 and 500 μM.

Helicase Assays—The partial duplex helicase substrates were constructed as described previously (31). The 346-bp blunt-ended helicase substrate was constructed as described (30). Helicase assay reaction mixtures (20 μl) contained 25 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 20 mM NaCl, 5 mM 2-mercaptoethanol, 3 mM rATP, the indicated DNA substrate, and the indicated amount of helicase II. The concentration of the partial duplex helicase substrate in the reaction was approximately 2 μM (nucleotide phosphate). The concentration of the 346-bp blunt end duplex substrate in the assay was approximately 0.5 μM (nucleotide phosphate). Reactions were initiated by the addition of ATP and incubated at 37 °C for 10 min with the exception of the 851-bp partial duplex DNA substrate which was incubated for 20 min. Reactions were terminated by the addition of 10 μl of 50 mM EDTA, 40% glycerol, 0.5% SDS, 0.1% bromphenol blue, 0.1% xylene cyanol. The products of helicase reactions were resolved on 6 or 8% nondenaturing polyacrylamide gels as described (22). The products of the helicase reaction using the 346-bp fully duplex substrate were resolved on a 6% nondenaturing polyacrylamide gel as described (30). Polyacrylamide gels were imaged using phosphor storage technology and quantified using ImageQuant software (Molecular Dynamics).

MutL-stimulated helicase reactions contained 40 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM ATP, and 50 μg/ml bovine serum albumin. Both MutL and UvrD proteins were diluted into a buffer containing 20 mM KP_i (pH 7.4), 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, and 100 μg/ml bovine serum albumin.

RESULTS

To acquire a better understanding of the molecular genetic functions and biochemical properties of DNA helicase II, a specific mutation was introduced into a conserved region of the *uvrD* gene. The highly conserved glutamine in motif III was replaced with a negatively charged glutamic acid (Fig. 1*A*). The mutant protein, UvrD-Q251E, was expressed and purified from a soluble cell extract. The procedure resulted in purification of the mutant protein to apparent homogeneity (Fig. 1*B*). Limited proteolysis of UvrD-Q251E and wild-type helicase II, using either chymotrypsin (see Fig. 3*B*) or trypsin (data not shown), resulted in identical cleavage patterns for both mutant and wild-type proteins suggesting that the mutation has not drastically altered the conformation of the protein.

Genetic Characterization of the uvrDQ251E Allele—The protein product of the *uvrDQ251E* allele was genetically characterized by assessing its ability to complement the loss of the wild-type protein in two DNA repair pathways. Previous studies have shown that DNA helicase II is required in both methyl-directed mismatch repair (7, 9) and UvrABC-mediated nucleo-

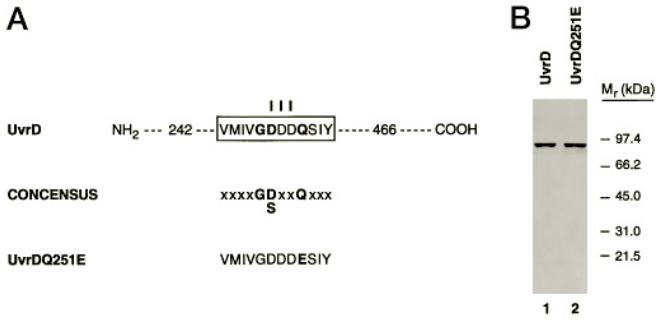

FIG. 1. *A,* the Q251E amino acid substitution in motif III of UvrD. Motif III, one of seven previously described helicase motifs, is shown for UvrD. The consensus sequence derived from an alignment of proteins belonging to superfamily I (Gorbalenya *et al.* (27); Hodgman (28)) is shown below the UvrD sequence. Shown *below* the consensus is the motif III sequence of UvrD-Q251E in which the highly conserved glutamine is replaced with a glutamic acid. *B,* Coomassie Blue-stained 9.6% SDS-polyacrylamide gel of purified UvrD proteins. Wild-type and mutant polypeptides were purified as described under "Experimental Procedures." *Lanes 1* and *2* represent 1.33 and 1.47 μg of UvrD and UvrD-Q251E proteins respectively. The marker proteins were as follows: rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa), and soybean trypsin inhibitor (21.5 kDa).

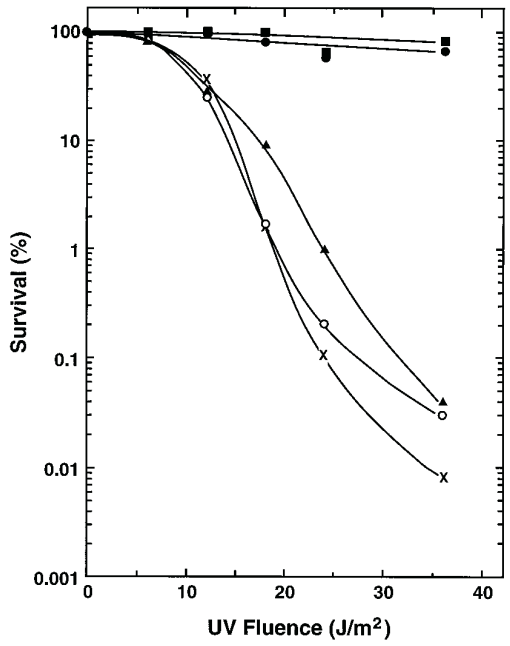

FIG. 2. **Ultraviolet light sensitivity of JH137 and JH137 derivatives.** Cell strains JH137 (●), JH137Δ*uvrD* (○), JH137Δ*uvrD*/pET9d (×), JH137Δ*uvrD*/pET9d-H2wt (■), or JH137Δ*uvrD*/pET9d-H2Q251E (▲) were irradiated using UV light as described under "Experimental Procedures." Data (percent survival) are expressed as the number of UV-irradiated cells forming colonies as a fraction of the colonies formed by unirradiated cells and represent the average of three independent experiments.

tide excision repair of pyrimidine dimers (11–13). Cells with a complete deletion of the *uvrD* gene are UV-sensitive and exhibit a mutator phenotype (15, 16). To determine the ability of the UvrD-Q251E mutant protein to function in these repair pathways, plasmids containing *uvrD* (pET9d-H2wt) or *uvrDQ251E* (pET9d-H2Q251E) were transformed into *E. coli* JH137 or JH137Δ*uvrD*. We have previously shown, using quantitative Western blots, that helicase II expression from the pET9d-H2wt plasmid (in the absence of induction) is just slightly less than that produced from the chromosome of JH137 (30). Thus the effects reported below are not due to gene dosage.

UV Sensitivity—To assess the ability of UvrD-Q251E to function in UvrABC-mediated nucleotide excision repair, we determined the relative UV sensitivity of strains expressing the mutant or wild-type proteins (Fig. 2). The *uvrDQ251E* allele failed to complement the UV-sensitive phenotype of the *uvrD* deletion strain JH137Δ*uvrD*. The UV-sensitive phenotype of JH137Δ*uvrD* was complemented by the presence of the wild-type *uvrD* allele, supplied on the same expression plasmid.

Mutation Frequency—The UvrD-Q251E mutant protein was also examined for its ability to complement the loss of helicase II in methyl-directed mismatch repair. For this analysis the spontaneous mutation frequency at the *rpoB* locus was measured and compared with relevant strains as shown in Table I. The relative mutability values of JH137Δ*uvrD*, JH137Δ*uvrD*/pET9d-H2Q251E, and JH137Δ*uvrD*/pET9d-H2wt were found to be 185, 203, and 0.94, respectively. Thus, *uvrDQ251E* failed to complement JH137Δ*uvrD* in methyl-directed mismatch repair. The wild-type allele, expressed from the same plasmid, exhibited full complementation of the mutator phenotype. The mutation frequency of JH137 (with a wild-type copy of *uvrD* on the chromosome) transformed with pET9d-H2Q251E was also measured and found to be equivalent to that of JH137. Thus *uvrDQ251E* is recessive to the wild-type allele in methyl-directed mismatch repair. We conclude that the *uvrDQ251E* allele encodes a protein that is inactive in both repair pathways.

Biochemical Characterization of UvrD-Q251E—The inability of UvrD-Q251E to substitute for wild-type helicase II in two repair pathways suggested that the mutant protein had a biochemical defect. To determine the nature of that defect, and to gain additional insight into the role of helicase motif III, the biochemical and physical properties of UvrD-Q251E were measured.

TABLE I
Mutation frequencies of JH137 and JH137 derivatives

Strain	Relevant genotype	Mutation frequency ($\times 10^{-8}$)[a]	Relative mutability
JH137	*uvrD*$^+$	2.6 ± 1.2[b]	1
JH137Δ*uvrD*	Δ*uvrD*	488 ± 63.4	185
JH137Δ*uvrD*/pET9d-H2wt	*uvrD*$^+$	2.5 ± 1.4	0.94
JH137Δ*uvrD*/pET9d-H2Q251E	*uvrD-Q251E*	537 ± 236	203
JH137/pET9d-H2Q251E	*uvrD*$^+$/*uvrD-Q251E*	1.9 ± 0.7	0.70

[a] Mutation frequency was determined by dividing the number of resistant colonies formed on selective agar by the total number of cells plated. Relative mutability values were obtained by dividing the mutation frequency of the cell strain in question by the frequency of the wild-type strain.

[b] Value previously determined in this lab (George *et al.* (30)).

Physical Studies—To probe the conformation of UvrD-Q251E the pattern of proteolytic cleavage by chymotrypsin was examined. In the absence of ssDNA and ATPγS, both mutant and wild-type proteins were cleaved by chymotrypsin into two fragments with approximate molecular mass of 53 and 29 kDa (Fig. 3, *A* and *B*, *lane 2*). Chao and Lohman (39) have demonstrated that wild-type helicase II is protected from cleavage by chymotrypsin when ssDNA and/or ATPγS are present and presumably bound by the protein. In the presence of M13 ssDNA and/or ATPγS both wild-type helicase II and UvrD-Q251E were protected from cleavage (Fig. 3, *A* and *B*, *lanes 3, 4,* and *5*). Similar results were obtained using trypsin as the source of protease although the cleavage pattern was different (data not shown). These results indicate that the mutant protein undergoes the same or similar conformational changes as wild-type protein when bound to ssDNA and/or ATP.

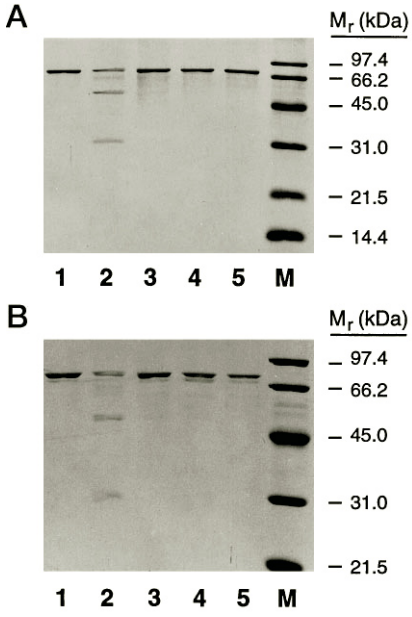

FIG. 3. **Chymotrypsin digestion of UvrD and UvrD-Q251E proteins in the presence of $MgCl_2$, ATPγS, and/or M13mp7 ssDNA.** Reaction mixtures (15 μl) contained 1.8 μM UvrD or 1.6 μM UvrD-Q251E and 5 ng of chymotrypsin. The concentrations of $MgCl_2$, ATPγS, and M13mp7 ssDNA were 3 mM, 2 mM, and 214 μM (nucleotide phosphate), respectively. Reactions were incubated at 37 °C for 2 min as described under "Experimental Procedures." Digestion products were resolved on a 12% SDS-polyacrylamide gel and stained with Coomassie Blue. Samples in *A* contained UvrD, and samples in *B* contained UvrD-Q251E. Samples in *lanes 2–5* were treated with chymotrypsin. *Lane 1*, helicase II standard; *lane 2*, helicase II; *lane 3*, helicase II + $MgCl_2$ + ATPγS; *lane 4*, helicase II + M13mp7 ssDNA; *lane 5*, helicase II + $MgCl_2$ + ATPγS + M13mp7 ssDNA. The marker proteins were as follows: rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa).

TABLE II
The k_{cat} and K_m for DNA-stimulated ATP hydrolysis catalyzed by UvrD and UvrD-Q251E

Enzyme	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	μM	s^{-1} / μM^{-1}
UvrD	123 ± 7.6	92.7 ± 35	1.3
UvrD-Q251E	4.3 ± 1.1	32.5 ± 15	0.13

Reaction conditions for DNA-dependent ATP hydrolysis assays were as described under "Experimental Procedures." For k_{cat} determinations protein concentrations were 27.2 and 200 nM for UvrD and UvrD-Q251E, respectively. The production of [^{3}H]ADP from [^{3}H]ATP was measured in a 30-μl reaction over a 5-min time course at 37 °C. Aliquots (5 μl) were taken at 1-min intervals, and the rate of ATP hydrolysis was determined from linear plots of ADP production *versus* time. In a typical reaction less than 20% of the ATP was hydrolyzed to minimize end product inhibition and substrate depletion effects. For K_m determinations protein concentrations were 2.71 and 35.9 nM for UvrD and UvrD-Q251E, respectively. Reactions mixtures (20 μl) for K_m determinations were incubated for 10 min at 37 °C.

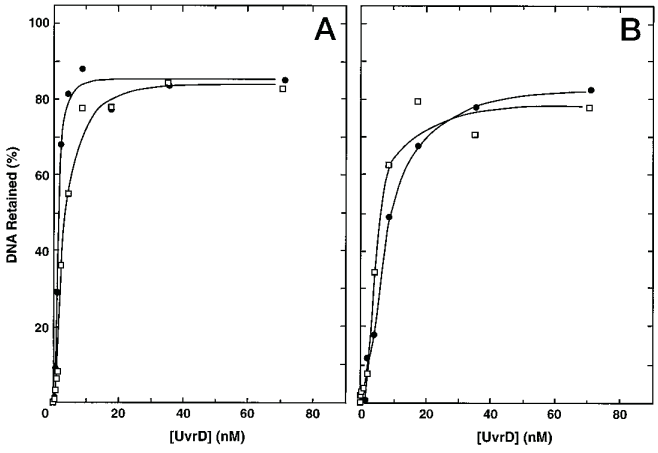

FIG. 4. **DNA binding by UvrD-Q251E and UvrD.** Binding assays were performed as described under "Experimental Procedures" using the indicated amounts of UvrD (□) or UvrD-Q251E (●). DNA binding incubations were performed in the presence of 3 mM ATPγS (*A*) or no nucleotide (*B*). Background values were typically less than 2% and have been subtracted from the reported data. These data represent the average of at least three independent determinations.

Helicase II has been shown to self-assemble to form a dimer or higher order oligomer in solution (34). Upon binding DNA the dimeric form of helicase II is stabilized. To test the ability of UvrD-Q251E to oligomerize, protein dimer formation was examined by treating the UvrD-Q251E mutant protein with glutaraldehyde and detecting cross-linked dimers on denaturing SDS-polyacrylamide gels. At a concentration of 2 μM (monomer) both UvrD-Q251E and UvrD formed dimers and higher order oligomers in solution (data not shown). In the presence of 2 mM ATPγS and 4.2 μM $(dT)_{10}$, the extent of dimer formation was enhanced for both proteins.

ATPase Activity—The ssDNA-dependent ATPase activity of UvrD-Q251E was determined and compared with the activity of the wild-type protein. The ATP hydrolysis kinetic constants k_{cat} and K_m are shown in Table II. UvrD-Q251E exhibited a k_{cat} value of 4.3 s^{-1}. This is approximately 3.5% that of the wild-type protein. No significant change in the apparent K_m for ATP was detected for UvrD-Q251E compared with the wild-type protein. The specificity constant (k_{cat}/K_m) was reduced 10-fold for the mutant protein. Thus, the replacement of Gln-251 with a glutamic acid negatively impacts the ATP hydrolysis reaction catalyzed by helicase II but apparently does not significantly alter the interaction of ATP with the enzyme.

DNA Binding—The reduction in the DNA-stimulated ATP hydrolysis reaction catalyzed by UvrD-Q251E led us to examine the DNA binding properties of the mutant protein. Nitrocellulose filter binding assays, using the 92-bp partial duplex DNA as a ligand, were performed with both the UvrD-Q251E and the wild-type proteins (Fig. 4). In the presence of the poorly hydrolyzed ATP analog, ATPγS, or in the absence of nucleotide, UvrD-Q251E demonstrated a binding isotherm similar to that measured for the wild-type enzyme. Similar binding of the mutant and wild-type proteins was also observed in the presence of ATP and ADP (data not shown). Thus, the reduction in DNA-stimulated ATP hydrolysis is not due to an inability to interact with DNA.

Helicase Activity—To determine the impact of the Q251E mutation on helicase activity the unwinding reaction catalyzed by the mutant protein was examined using both partial duplex and blunt duplex DNA substrates. Titrations of the UvrD and UvrD-Q251E proteins with partial duplex substrates are shown in Fig. 5. Wild-type helicase II, at a concentration of 0.34 nM (monomer), unwound 39% of the 20-bp partial duplex DNA substrate in a 10-min reaction (Fig. 5*A*). The UvrD-Q251E mutant protein achieved a comparable level of unwinding (36%) at a 5.5-fold higher protein concentration (1.87 nM monomer). Wild-type helicase II unwound 39% of the 92-bp partial duplex substrate at a concentration of 17 nM (monomer) (Fig. 5*B*). The Q251E-catalyzed reaction required a 5.3-fold higher concentration of enzyme (90 nM monomer) to unwind approximately 57% of the 92-bp partial duplex. Similarly, unwinding of the 343- and the 851-bp partial duplex DNA substrates required approximately a 4-fold higher concentration of the mutant enzyme to achieve a level of unwinding comparable with

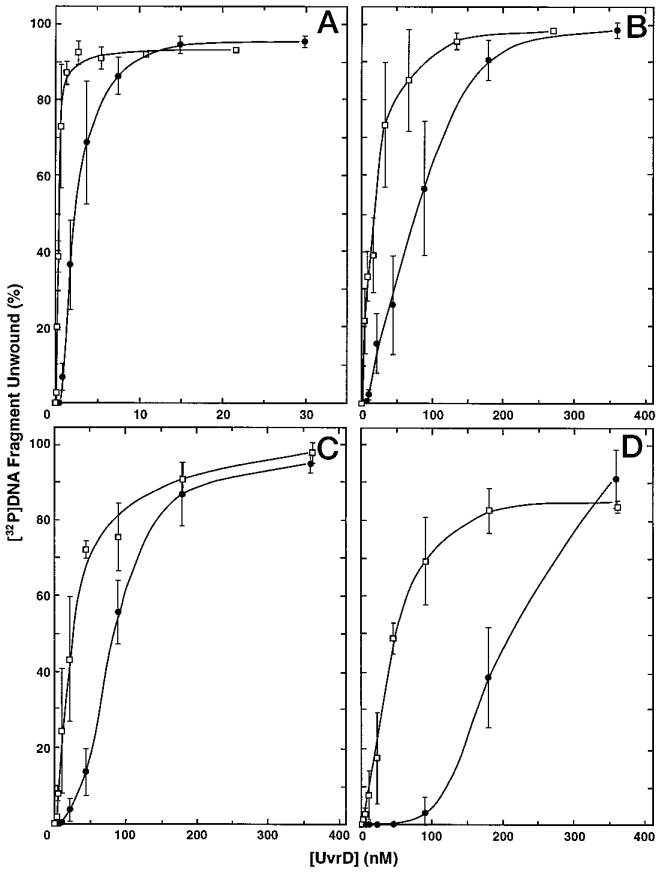

FIG. 5. **The unwinding reaction catalyzed by the UvrD and UvrD-Q251E proteins on partial duplex DNA substrates of various lengths.** Helicase reactions using partial duplex DNA substrates containing 20 (*A*), (*B*), 343 (*C*), or 851 bp (*D*) of duplex DNA were as described under "Experimental Procedures." Reactions were initiated by the addition of the indicated amounts of UvrD-Q251E (●) or the wild-type enzyme (□) and incubated at 37 °C for 10 min (*A–C*) or 20 min (*D*). The data presented represent the average of three or more experiments. *Error bars* represent the standard deviation about the mean.

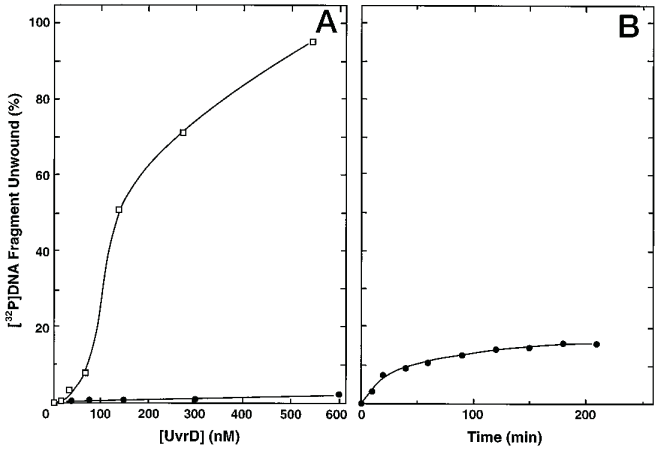

FIG. 6. **UvrD-Q251E catalyzes a slow unwinding reaction on a blunt duplex DNA substrate at high enzyme concentration.** Helicase reactions using a 346-bp blunt duplex DNA substrate were as described under "Experimental Procedures." *A*, reactions (20 μl) were initiated by the addition of the indicated amounts of UvrD-Q251E (●) or the wild-type enzyme (□) and incubated for 10 min at 37 °C. *B*, the UvrD-Q251E reaction mixture was increased to 240 μl, and a 20-μl aliquot was removed at time = 0. The reaction was initiated by the addition of UvrD-Q251E at a final concentration of 599 nM (monomer), and incubation was at 37 °C. Aliquots were removed at the indicated times and analyzed by polyacrylamide gel electrophoresis as described under "Experimental Procedures." The data represent the average of at least three independent experiments.

the wild-type protein (Fig. 5, *C* and *D*). These data demonstrate that the Q251E mutant protein is only modestly compromised as a DNA helicase on partial duplex DNA substrates.

Recently MutL protein has been shown to stimulate the unwinding reaction catalyzed by helicase II.[2] We examined the effect of MutL protein on the helicase reactions catalyzed by UvrD-Q251E and wild-type UvrD using a 92-bp partial duplex DNA substrate. The unwinding reactions catalyzed by UvrD and UvrD-Q251E were both stimulated by MutL protein (data not shown). Thus, the Q251E mutation has not altered interactions helicase II may have with the mismatch repair protein MutL.

Helicase II has also been shown to unwind blunt-ended duplex DNA substrates (23, 25, 26), and we tested the ability of the UvrD-Q251E mutant protein to unwind a 346-bp blunt duplex DNA substrate (Fig. 6). Wild-type helicase II, at a concentration of 136 nM (monomer), unwound approximately 50% of the 346-bp duplex in a 10-min reaction (Fig. 6*A*). Less than 1% of the 346-bp blunt duplex was unwound by the Q251E mutant at a comparable concentration of protein (150 nM monomer). An increase in wild-type helicase II concentration to 543 nM (monomer) resulted in the unwinding of 95% of the 346-bp duplex. At a comparable mutant enzyme concentration of 599 nM (monomer), less than 2% of the 346-bp blunt duplex was unwound.

The kinetics of the blunt duplex unwinding reaction catalyzed by the UvrD-Q251E mutant protein were also examined (Fig. 6*B*). At a concentration of 599 nM (monomer) UvrD-Q251E achieved a plateau with approximately 14% of the 346-bp blunt duplex unwound in 210 min. Both the rate and extent of unwinding of the 346-bp blunt duplex DNA substrate were dramatically reduced for the UvrD-Q251E mutant protein compared with wild-type helicase II. This is in contrast to the slightly compromised unwinding of the partial duplex DNA substrates.

Helicase II has also been shown to unwind nicked duplex DNA molecules (23, 25, 26). In this case the unwinding reaction is initiated at the nick (23), and the rate-limiting step in the reaction is the initiation event (26). Since the UvrD-Q251E mutant protein exhibits nearly wild-type unwinding of a partial duplex DNA substrate, and a significantly compromised unwinding of blunt duplex DNA, we were interested in the ability of the protein to unwind a nicked DNA substrate. This has been suggested to be the substrate unwound by helicase II *in vivo* in DNA repair (9, 10, 13, 14), and a significant defect in unwinding nicked DNA could explain the inability of UvrD-Q251E to function in excision repair and methyl-directed mismatch repair.

The kinetics of an unwinding reaction initiated on a nicked DNA substrate were examined by measuring ssDNA-dependent ATP hydrolysis. The reaction catalyzed by wild-type helicase II exhibited a lag phase during the 1st min of the reaction followed by a linear steady-state rate from 2 to 5 min (Fig. 7*A*). These data are completely consistent with previous results indicating that ATP hydrolysis catalyzed by helicase II is the result of unwinding the nicked substrate to yield ssDNA products (26). At an 8-fold higher protein concentration the UvrD-Q251E mutant failed to catalyze a detectable ATP hydrolysis reaction using a nicked DNA effector. This is in direct contrast to the results observed using a ssDNA effector in an ATP hydrolysis assay (Fig. 7*B*). In this case the UvrD-Q251E mutant catalyzes an easily detectable ATPase reaction. We con-

[2] P. Modrich, personal communication.

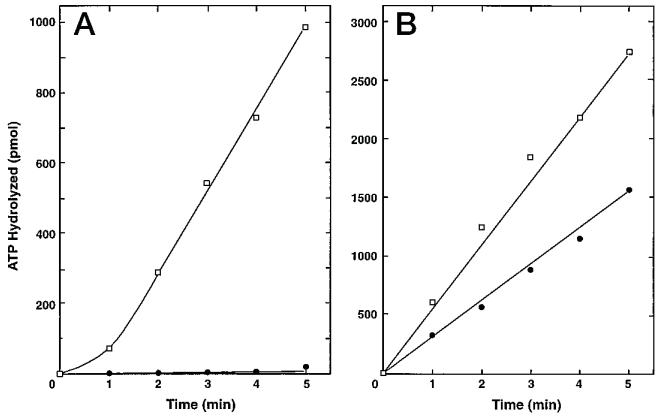

FIG. 7. **Kinetics of ATP hydrolysis in the presence of nicked duplex or ssDNA as the DNA effector.** DNA-dependent ATPase assays were conducted using nicked DNA (6 μM nucleotide phosphate) (*A*) or M13mp7 ssDNA (9 μM nucleotide phosphate) (*B*) as described under "Experimental Procedures." Reactions (30 μl) contained 0.54 mM [^{3}H]ATP. *A*, reactions contained 72 nM UvrD (monomer) (□) or 599 nM UvrD-Q251E (monomer) (●). *B*, reactions contained 27 nM UvrD (monomer) (□) or 200 nM UvrD-Q251E (monomer) (●). Reactions were initiated by the addition of the DNA effector, and aliquots (5 μl) were removed at 1-min intervals and processed as described previously (41).

clude that the UvrD-Q251E mutant fails to initiate an unwinding reaction on a nicked DNA substrate.

DISCUSSION

To define further the functional role of helicase II in nucleotide excision repair and methyl-directed mismatch repair, we have characterized a site-specific *uvrD* mutant that fails to function in these pathways *in vivo* (see Fig. 2 and Table I). The Q251E mutation in helicase II replaces an invariant glutamine located in motif III of a superfamily of DNA helicases and putative helicases (SF1) (27, 28) with a glutamic acid. Our objectives were to determine why UvrD-Q251E is defective in DNA repair and extend our understanding of the mechanism of tract excision by helicase II in the cell. In addition, these studies have provided insight into the functional significance of motif III in DNA helicases.

To determine the defect responsible for the failure of UvrD-Q251E to function in DNA repair, the physical and biochemical properties of the mutant enzyme were examined. The UvrD-Q251E and wild-type proteins exhibit identical cleavage patterns upon limited treatment with chymotrypsin or trypsin suggesting that the two proteins are similarly folded in solution. In the presence of ATP and/or ssDNA UvrD-Q251E was protected from cleavage by protease indicating that the mutant protein undergoes conformational changes induced by ligand binding as previously reported for wild-type helicase II (39). Cross-linking experiments demonstrated that UvrD-Q251E has the ability to form dimers and higher order oligomers in solution. Moreover, the dimeric species is favored in the presence of DNA and ATP indicating that UvrD-Q251E was capable of making the necessary protein-protein contacts between monomers to dimerize. The nucleotide and DNA-induced conformational changes of UvrD-Q251E, and the ability of UvrD-Q251E to dimerize, suggested that the mutant enzyme might retain catalytic activity. However, the failure of UvrD-Q251E to function in DNA repair *in vivo* suggested that the biochemical properties of the enzyme were somehow compromised.

A kinetic analysis of ssDNA-stimulated ATP hydrolysis demonstrated a 29-fold reduction in k_{cat} for UvrD-Q251E compared with the wild-type protein. The K_m for ATP was approximately the same for both proteins suggesting that the affinity of the mutant enzyme for ATP has not been altered. That the Q251E mutant protein retained its ability to bind ATP was further supported by (i) the observation that the mutant enzyme bound [^{3}H]ATP as assayed by gel exclusion chromatography,[3] and (ii) UvrD-Q251E undergoes the appropriate ATP-induced conformational change as shown by the partial proteolysis studies.

To address the possibility that the reduction in ssDNA-dependent ATPase activity was due to a defect in DNA binding, the DNA binding activity of both mutant and wild-type proteins was examined. Results from nitrocellulose filter binding assays demonstrate that the UvrD-Q251E mutant retained DNA binding activity similar to that of wild-type UvrD. These results are in agreement with the partial proteolysis studies which indicate that the UvrD-Q251E protein undergoes conformational changes upon binding DNA. Therefore, the reduced ATPase activity of UvrD-Q251E is not due to a grossly impaired interaction with DNA or ATP.

A similar reduction in k_{cat} for ATP hydrolysis has been observed for another motif III helicase II mutant, UvrD-D248N (36). Replacement of the highly conserved aspartic acid, three amino acids N-terminal to the invariant glutamine (see Fig. 1*A*), with an asparagine markedly reduced the k_{cat} of UvrD-D248N to approximately 4% that of the wild-type enzyme. This mutation also impaired the protein's ability to form stable binary complexes with ATP or ssDNA. The fact that the Q251E mutation has not perturbed the enzyme's ability to bind ATP or DNA suggests that the nature of this mutation is distinct from the D248N mutation. However, the nearly equivalent k_{cat} values for the two mutant proteins, and the close proximity of the mutations within a conserved motif, suggest that the two mutations may compromise ATPase activity by a related mechanism. We have suggested that Asp-248 may have an indirect role in the DNA-dependent hydrolysis of ATP, perhaps as a determinant of the relative orientation of the ssDNA binding domain and the ATP binding domain (36). It is possible that Gln-251 plays a similar role. The specificity constant (k_{cat}/K_m) is reduced 10-fold for the Q251E mutant as compared with reductions of >250-fold for mutations in motifs I and II which directly impact the hydrolysis of ATP (30, 31). This is consistent with the notion that the mutation impacts ATP hydrolysis by an indirect mechanism. Structural data will be useful in determining more precisely the functional significance of these two residues in motif III.

A decrease in helicase activity might be expected due to the decrease in ATP hydrolysis since ATP hydrolysis is coupled to the unwinding reaction. However, it is not clear how tightly these two reactions are coupled. Thus it is possible that a decreased k_{cat} for ATPase activity would result in a relatively small change in helicase activity. Indeed, this seems to be the case. The specific ATPase activity of the mutant protein was reduced 29-fold with only a 4–5-fold reduction in the unwinding of partial duplex substrates. This suggests that a portion of the ATP hydrolysis catalyzed by the wild-type enzyme is not efficiently coupled to strand separation during the elongation phase of an unwinding reaction.

The nearly wild-type unwinding reaction observed using partial duplex substrates suggests that UvrD-Q251E is dysfunctional in DNA repair for a reason other than poor unwinding activity. Additional support for this notion derives from the fact that the D248N motif III mutant is more defective as a helicase than UvrD-Q251E yet complements in both repair pathways (36). An alternative possibility is that the Q251E mutant protein is defective in key protein-protein interactions that occur during DNA repair. MutL protein stimulates the unwinding reaction catalyzed by helicase II.[2] The unwinding reaction cat-

[3] R. M. Brosh, Jr. and S. W. Matson, unpublished results.

alyzed by UvrD-Q251E was also stimulated by MutL protein suggesting that the mutant protein is capable of engaging in important protein-protein interactions. Moreover, it seems unlikely that a single amino acid change would abolish key protein-protein interactions in two separate repair pathways each utilizing a different spectrum of proteins.

In contrast to what was observed using partial duplex DNA substrates, the unwinding reaction catalyzed by UvrD-Q251E was drastically reduced on a blunt duplex DNA substrate. This is likely due to a defect in the initiation phase of the helicase reaction. Runyon and Lohman (26) have provided evidence to suggest the initiation of unwinding on blunt duplex DNA is the rate-limiting step. Apparently the Q251E mutant protein is able to efficiently initiate a helicase reaction on a DNA substrate with a ssDNA flanking region but performs poorly in initiation of unwinding at a blunt end.

The poor unwinding of blunt duplex DNA by UvrD-Q251E suggested that the mutant protein might be defective in initiating unwinding from a nick. A kinetic analysis of ssDNA-dependent ATP hydrolysis using a nicked circular duplex as the DNA effector provided direct evidence that the Q251E mutant is defective in the initiation of unwinding at a nick. In this assay ATP hydrolysis reflects unwinding of the duplex since ATP hydrolysis is ssDNA-dependent. Unlike the strand displacement helicase assay, which measures complete unwinding of a DNA duplex, the ATP hydrolysis assay has the ability to measure partial unwinding. The failure to detect ssDNA-dependent ATP hydrolysis at relatively high UvrD-Q251E concentrations in the presence of nicked DNA indicates that the enzyme failed to generate ssDNA, *i.e.* catalyze unwinding from the nick to any significant extent.

Taken together the biochemical and genetic data suggest that the UvrD-Q251E mutant protein fails to function in either methyl-directed mismatch repair or nucleotide excision repair due to its inability to initiate unwinding at a nick. *In vivo* complementation assays have indicated that motif I and II *uvrD* mutants, which encode catalytically inactive proteins, fail to function in nucleotide excision repair or methyl-directed mismatch repair. Thus the ATPase and/or helicase reaction catalyzed by UvrD is required for excision of the oligonucleotide containing the damaged or mismatched base (30, 31). The observation that the *uvrDQ251E* allele fails to complement in DNA repair might be explained by a loss of helicase activity since the unwinding reaction is presumed to be required for the excision step in both DNA repair pathways. However, the unwinding data obtained using partial duplex DNA substrates (see Fig. 5) indicates that the helicase activity of UvrD-Q251E mutant is only modestly reduced on duplexes ranging from 20 to 851 bp. The fact that the UvrD-D248N mutant protein catalyzes less unwinding of partial duplex DNA substrates than UvrD-Q251E, yet retains its ability to complement the mutator and UV light-sensitive phenotypes of a *uvrD* deletion mutant (36), further supports the notion that UvrD-Q251E fails to complement due to a defect other than compromised helicase activity. The failure to initiate unwinding at a nick, on the other hand, provides a ready explanation for the inability of UvrD-Q251E to function in DNA repair. The fact that helicase II can initiate unwinding of duplex DNA at a nick at high protein concentrations *in vitro* has suggested that the enzyme may function in this manner *in vivo*. It is important to note that helicase II is an abundant protein in the cell, present at approximately 300 to 800 copies per cell (30). Moreover, expression of the *uvrD* gene is induced by the SOS response, and helicase II protein levels increase 3–6-fold upon induction (42–45). These observations suggest that relatively high concentrations of helicase II exist in the cell to initiate unwinding of duplex DNA at nicks. The nick provides the access point for helicase II to initiate tract excision. The biochemical and genetic studies presented here provide evidence consistent with the idea that DNA helicase II loads at a nick *in vivo* and that initiation of unwinding at a nick is a critical event in both DNA repair pathways. We point out, however, that helicase II is likely to require other proteins in the cell to facilitate loading on the nicked DNA intermediate that arises during methyl-directed mismatch repair or excision repair. Moreover, the mechanism of helicase II action in the cell and the nature of its interaction with other proteins are not well understood. These may also contribute to the repair defect observed for the UvrD-Q251E mutant protein.

The fact that the glutamine in motif III is conserved for all helicases of superfamily I should be noted (27, 28). If the glutamine residue (and perhaps motif III) has a critical role in the initiation of unwinding at a blunt end or a nick, it is reasonable that the conserved residue(s) may serve a similar function in other DNA helicases. A number of DNA helicases from *E. coli* belonging to SF1 potentially initiate unwinding of dsDNA at a nick or blunt end *in vivo*. Rep protein catalyzes processive unwinding of ϕX174 DNA that has been previously nicked with phage-encoded CisA protein (46). Helicase I, which catalyzes site- and strand-specific nicking at *oriT* (47), has a role in the transfer of F plasmid from donor to recipient during bacterial conjugation (48). Presumably the unwinding activity of helicase I is required to drive transfer of the ssDNA from donor to recipient initiating from the nick (5). Lastly, RecBCD enzyme, known to have roles in DNA repair (49, 50) and recombination (51), catalyzes a processive unwinding reaction from a blunt duplex DNA end (52–54).

A number of proteins from a related superfamily (SF2) may also have roles in unwinding nicked DNA intermediates. ERCC6 (55) and RAD16 (56) are involved in eukaryotic nucleotide excision repair. RAD54 (57) has been shown to be involved in recombinational repair. These DNA repair proteins and other proteins involved in diverse cellular processes such as transcriptional regulation (MOT1, SNF2, and BRM) and maintenance of chromosome stability during mitosis (LDS) all have a conserved glutamine in the motif III sequence that aligns with the invariant glutamine located in motif III of SF1 proteins (55, 58). All of these proteins, like UvrD, may have a role in unwinding DNA from a nick or blunt end *in vivo*. The absolute requirement for helicase II to initiate unwinding at a nick in methyl-directed mismatch repair and nucleotide excision repair suggests that this step of the unwinding reaction is critical to helicase II function in both of these repair pathways.

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