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Characterization of DNA Helicase II from a *uvrD252* Mutant of *Escherichia coli*

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The loss of DNA helicase II (UvrD) in *Escherichia coli* results in sensitivity to UV light and increased levels of spontaneous mutagenesis. While the effects of various *uvrD* alleles have been analyzed in vivo, the proteins produced by these alleles have not been examined in any detail. We have cloned one of these alleles, *uvrD252*, and determined the site of the mutation conferring the phenotype. In addition, the protein it encodes has been purified to homogeneity and characterized in vitro. The mutation responsible for the phenotype was identified as a glycine-to-aspartic-acid change in the putative ATP-binding domain. In comparison to wild-type DNA helicase II, the UvrD252 enzyme exhibited reduced levels of ATPase activity and a large increase in the K_m for ATP. The ability of UvrD252 to unwind DNA containing single-stranded regions, as well as DNA containing only nicks, was reduced in comparison to that of the wild-type enzyme. Possible interpretations of these results in relation to the phenotypes of the *uvrD252* mutant are discussed. This represents the first detailed analysis of the biochemical properties of a mutant DNA helicase II protein.

The *uvrD* gene of *Escherichia coli* encodes an 82-kDa protein with both ATPase and helicase activities (26, 30). This enzyme, DNA helicase II, unwinds DNA duplexes with 3′ to 5′ polarity with respect to the bound strand (13, 29) and initiates unwinding most effectively when a single-stranded region is present (1, 29). At higher enzyme concentrations, it can also initiate unwinding from a nick or blunt end (13, 42). The involvement of DNA helicase II in the postincision events of nucleotide excision repair and methyl-directed mismatch repair has been demonstrated through studies of *uvrD* mutants (23, 35, 36, 40, 47, 48, 54) and in vitro repair assays (6, 18, 32). Furthermore, expression of the *uvrD* gene is induced approximately three- to sixfold in response to DNA damage (45). Genetic and biochemical studies also suggest that DNA helicase II may play important roles in recombination (2, 11, 17, 37) and replication (22, 24, 50) as well, although the precise role of DNA helicase II in these processes has not been defined.

Strains containing deletions in the *uvrD* gene exhibit UV sensitivity and increased rates of spontaneous mutagenesis (56), reflecting deficiencies in excision repair and mismatch repair, respectively. However, the phenotypes conferred by individual alleles vary (46). One of these, *uvrD252* (formerly *recL152*) (41), was originally isolated on the basis of its effect on recombination (17). It confers the UV sensitivity associated with *uvrD* deletions but retains near-normal levels of spontaneous mutagenesis (11, 46, 56). To determine the nature of the *uvrD252* mutation and the biochemical properties of the UvrD252 enzyme, we have cloned the *uvrD252* allele and purified the protein. We have found that the mutation conferring the *uvrD252* phenotype lies in the ATP-binding domain of DNA helicase II and results in a general decrease in ATPase and helicase activities.

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MATERIALS AND METHODS

Cloning the *uvrD252* allele. *uvrD252* was cloned by using the gene replacement technique described by Hamilton et al. (15). pBWK20 (56), which carries the kanamycin-resistant (Km^r) Δ*uvrD288* deletion-insertion, chloramphenicol resistance, and a temperature-sensitive origin of replication, was introduced into SK710 (*uvrD252*) (56) and inserted into the chromosome to form a cointegrate by selecting for chloramphenicol resistance at 44°C. Five of the resulting colonies were pooled and grown for approximately 30 generations at 30°C for resolution of the cointegrates. Plasmid DNA was then extracted from this culture (19) and introduced back into SK710. Chloramphenicol-resistant transformants were screened for UV sensitivity and kanamycin resistance by replica plating (7). One UV^s Km^s isolate was chosen for further study. pBWK51, the plasmid isolated from this strain, complemented the mutator phenotype of SK7772 (Δ*uvrD291*) (56) but did not confer UV resistance (data not shown), indicating that the lesion conferring the *uvrD252* phenotype had been cloned onto the plasmid. All subsequent constructions involving this plasmid were done in either SK710 (*uvrD252*) or DH5α (*recA1*) (Bethesda Research Laboratories) in order to avoid recombination between the plasmid and chromosomal copies of *uvrD*. Wild-type *uvrD* was maintained in SK707 ($uvrD^+$) (56).

Construction of *uvrD* expression vectors. The 2.9-kb *SalI* fragments containing either the wild-type or the *uvrD252* allele were subcloned into the *SalI* site of pWSK29 (55) to produce pBWK58 and pBWK59, respectively. pWSK29 is a low-copy expression vector which allows cloned inserts to be transcribed under the control of a T7 RNA polymerase promoter. pBWK58 and pBWK59 were introduced into isogenic strains (SK707 [$uvrD^+$] and SK710 [*uvrD252*], respectively) containing pGP1-2 (49), which carries the gene for T7 RNA polymerase under the control of a λ*c*I857 repressor. This system allows *uvrD* to be maintained at a low copy number and then be overexpressed by a temperature shift as described by Tabor and Richardson (49).

Immunological methods. For the production of antibodies directed against UvrD, a New Zealand White rabbit was injected intradermally with 80 μg of wild-type UvrD protein

and twice more at 2-week intervals with 40 μg of UvrD. Serum was collected after 5 weeks and purified on a DEAE Affi-Gel Blue column (Bio-Rad). Western blots (immunoblots) (21) of a UvrD-overproducing strain revealed a single strongly reacting band, which migrated with a molecular weight identical to that of UvrD. The protein produced from the *uvrD252* clone reacted equally well with the antibodies and exhibited an electrophoretic mobility similar to that of wild-type UvrD protein.

For immunological detection of UvrD in column fractions, 200 to 400 μl of each fraction was vacuum blotted onto nitrocellulose (BA-5; Schleicher & Schuell) by using a Schleicher & Schuell dot blotter. The filter was probed with anti-UvrD antibodies and developed with alkaline phosphatase by using the materials and protocol supplied with the Promega Protoblot kit, except that BLOTTO (21) was used as the blocking agent. The developed blots were photographed, and the intensity of the antibody reaction was quantitated by scanning the negative with a Molecular Dynamics M300 scanning densitometer.

Purification of DNA helicase II. To overproduce DNA helicase II, 6 liters of SK8118 (SK707 [*uvrD*$^+$] containing pBWK58 [*uvrD*$^+$] and pGP1-2) or SK8119 (SK710 [*uvrD252*] containing pBWK59 [*uvrD252*] and pGP1-2) was grown at 30°C in Luria broth (25) containing 100 μg of ampicillin per ml to an optical density at 595 nm of 1.5 and induced by a 30-min temperature shift to 42°C. After 2 additional h at 37°C in the presence of 100 μg of rifampin per ml, cells were collected by centrifugation and frozen at −20°C overnight. The methods used for cell lysis and purification of DNA helicase II were a simplification of a previously described procedure (26). In brief, cells were disrupted with lysozyme and the lysate was precipitated with ammonium sulfate at 50% saturation. The resuspended ammonium sulfate pellet was sequentially eluted from a phosphocellulose column (pH 7.2) (5 by 10 cm) and a single-stranded DNA agarose column (1 by 8 cm) with NaCl gradients as previously described (27). DNA-dependent ATPase activity and immunoblots were used to detect the helicase II from wild-type cells. The UvrD252 protein, which did not exhibit detectable ATPase activity in crude extracts, was identified on the basis of immunoblots alone. The peak fractions from the final column were pooled, dialyzed against buffer A [50% glycerol, 20 mM Tris (pH 7.6), 0.1 mM EDTA, 0.5 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid, 50 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonate], and stored at −20°C. Protein concentrations were determined by the method of Bradford (4) by using the Bio-Rad protein assay reagent. The enzymatic activities of the wild-type and UvrD252 preparations were both stable for at least 1 year and were reproducible between preparations.

Helicase substrate preparation. DNA helicase substrates consisted of single-stranded M13mp18 to which ^{32}P-end-labeled fragments of various sizes were annealed (Fig. 1). Partial duplexes (12, 24, and 96 bp) were prepared as described by Matson and George (30). The 12-nucleotide fragment was a synthetic oligonucleotide (TCGAGCCATGGG) that corresponded to the sequence of the DNA lying between the *Sma*I and *Sac*I sites in the polylinker region of M13mp18. The 24-nucleotide oligonucleotide was the M13/pUC forward primer supplied by Promega. These oligonucleotides were end labeled with polynucleotide kinase and [γ-^{32}P]ATP and annealed to single-stranded M13mp18. The 96-bp fragment was isolated from a *Pvu*II digest of M13mp18, annealed to M13, and labeled with Klenow polymerase and [α-^{32}P]dCTP. Each substrate was purified over a 1.5-ml Sepharose CL-6B column (Sigma) to separate it from unincorporated nucleotides and unannealed DNA fragments (30). For quantitation, the substrates were electrophoresed on a 0.8% agarose minigel along with single-stranded M13 DNA in a dilution series ranging from 50 to 5 ng. The gel was stained with ethidium bromide and photographed with Kodak Type 55 film. The negative was then scanned with a Molecular Dynamics scanning densitometer, and the substrate concentration was determined relative to a standard curve generated from the M13 dilution series.

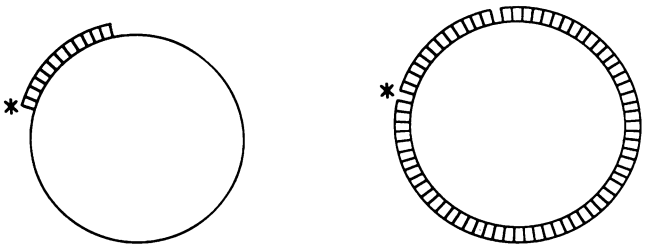

FIG. 1. General structure of substrates used in the helicase assays. DNA partial duplexes of 12, 24, and 96 bp (left, above) and a full-duplex nicked substrate (right, above) were constructed as described in Materials and Methods. The location of the 5′ ^{32}P label is denoted by an asterisk.

A fully double-stranded circular molecule containing two nicks on one strand, separated by 12 nucleotides, was constructed in two steps (Fig. 1). First, the 7,238-nucleotide linear product of an *Sac*I-*Sma*I M13mp18 digest (2.5 μg) was annealed to single-stranded 7,250-nucleotide M13mp18 (4 μg) by boiling for 3 min in 100 μl of annealing buffer (30) and cooling to room temperature over the course of 2 h. The single-stranded and double-stranded M13 were both prepared from the same culture (31), started from a single plaque, in order to minimize the possibility of noncomplementary DNA arising from small deletions during M13 growth. After the first annealing was complete, 0.06 μg of the ^{32}P-end-labeled 12-nucleotide oligonucleotide described above was added. The annealing mix was heated to 55°C, cooled slowly to 4°C over the course of 3 h, and electrophoresed on a 0.8% agarose gel (SeaKem Genetic Technology Grade) at 4°C. Nicked, supercoiled, linear, and single-stranded M13 forms were run alongside the preparative lane. The band corresponding to the nicked form was excised from the gel, electroeluted into dialysis tubing, phenol-chloroform extracted twice, and ethanol precipitated. The nicked substrate was quantitated as described for the partial duplex substrates, except that linear M13 was used to generate the standard curve.

ATPase and helicase assays. ATPase assays were performed as previously described (26). The standard ATPase reaction mixture (75 μl) contained 50 mM Tris (pH 7.5)–20 mM 2-mercaptoethanol–3 mM $MgCl_2$–15 μg of bovine serum albumin–2.5 μg of heat-denatured calf thymus DNA–500 μM [γ-^{32}P]ATP. Reactions were carried out for 10 min at 37°C and stopped by the addition of 0.5 ml of 20% Norit A in a solution containing 0.25 M NaH_2PO_4–0.25 M $Na_4P_2O_7$–0.25 M HCl. After centrifugation, 0.2 ml of the supernatant was counted in 4 ml of scintillation fluid to determine the amount of inorganic phosphate present. One unit of ATPase activity is defined as the amount of DNA helicase II needed for the hydrolysis of 1 nmol of ATP in 10 min.

Helicase assays were performed as previously described (30), except as noted. The standard helicase reaction mixture

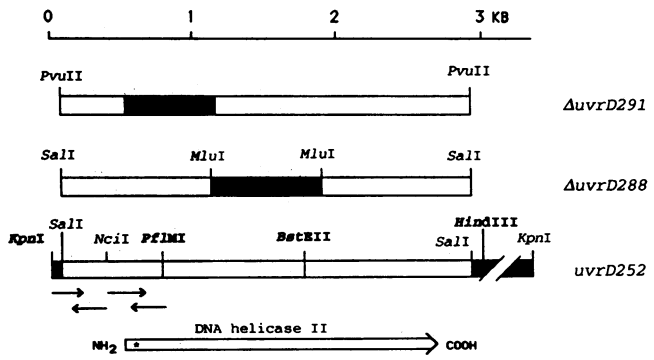

FIG. 2. Cloning and mapping the *uvrD252* mutation. The locations and extent of the deletions in the Δ*uvrD291* and Δ*uvrD288* constructs used for cloning and characterization of *uvrD252* are indicated by shaded regions. Below them is shown a restriction map of the *uvrD252* fragment (open box) and vector sequences (filled boxes) used in fragment exchanges with the wild type. The *Sal*I sites flanking the Δ*uvrD288* fragment were produced from *Pvu*II sites as previously described. *Kpn*I-*Pfl*MI, *Pfl*MI-*Bst*EII, and *Bst*EII-*Hin*dIII fragments were individually exchanged between pBWK58 (wild type) and pBWK59 (*uvrD252*). The *Kpn*I-*Nci*I and *Nci*I-*Pfl*MI fragments were made blunt with the Klenow fragment of DNA polymerase I and were subcloned into M13mp18. These clones were sequenced by the dideoxy method, with the protocol and reagents from the United States Biochemical Sequenase 2.0 kit. The direction and extent of sequencing are denoted by small arrows. The large open arrow shows the coding region for DNA helicase II. The site of the mutation is denoted by an asterisk.

(20 μl) contained 40 mM Tris (pH 7.5)–1 mM dithiothreitol–50 μg of bovine serum albumin per ml, plus $MgCl_2$ and ATP at the concentrations noted in the appropriate figure legends. Each helicase reaction mixture also contained 5 μl of buffer A (see above). To quantitate unwinding levels, helicase assay gels (10% acrylamide) were exposed to X-ray films which were then scanned on a Molecular Dynamics scanning densitometer. Percent unwinding was calculated by using the formula $[(U - C)/(B - C)] \times 100$, where U is the intensity (scan units) of the band produced by the unwinding reaction, C is the amount (scan units) of unwound DNA in the absence of enzyme, and B is the amount (scan units) of unwound DNA produced by boiling the DNA for 5 min before loading the gel.

RESULTS

Cloning the *uvrD252* allele and identification of the mutation site. The *uvrD252* allele was cloned as described in Materials and Methods. When the resolution products from the *uvrD252*-Δ*uvrD288* cointegrates (see Materials and Methods) were assayed for UV sensitivity in SK710, 75% of the plasmid transformants were Km^r UV^s, 15% were Km^s UV^r, and 10% were Km^s UV^s. The recovery of Km^s UV^r resolution products indicated that the *uvrD252* allele was probably located outside the region covered by the Δ*uvrD288* deletion-insertion (Fig. 2). Restriction fragment exchanges between the *uvrD*$^+$ and *uvrD252* plasmids indicated that the lesion responsible for UV sensitivity was contained on a 720-bp *Kpn*I-*Pfl*MI fragment (Fig. 2). This fragment complemented the mutator phenotype, but not the UV sensitivity, of a Δ*uvrD291* strain (Fig. 2). The *uvrD252* *Kpn*I-*Pfl*MI fragment was sequenced as shown in Fig. 2 and found to contain a G-to-A mutation at position 165 from the start of transcription of *uvrD*$^+$ (57), resulting in a glycine-to-aspartic-acid change at amino acid residue 30. The presence of the mutation on the chromosome of SK710 (*uvrD252*) was verified by direct sequencing of chromosomal DNA amplified by polymerase chain reaction. A DNA fragment corresponding to approximately −350 to +246 of the *uvrD252* gene was amplified, and the region spanning the site of the mutation was sequenced by the method of Seetharam and Dicker (44).

Protein	Residues	Sequence
UvrD252	27-40	VL**A**DAG**S**G**K**TRVLV
UvrD	27-40	VL**A**GAG**S**G**K**TRVLV
Rep	20-33	VL**A**GAG**S**G**K**TRVIT
RecB	21-34	IE**A**SAG**T**G**K**TFTIA
RecD	165-178	IS**G**GPG**T**G**K**TTVA

FIG. 3. Putative ATP-binding domains of wild-type UvrD, UvrD252, and related helicases. These data are summarized from Hodgman (16). The amino acid position altered in the *uvrD252* mutation is underlined. The most conserved residues in the nucleoside triphosphate binding motif (14) are shown in boldface type.

Amino acid 30 lies within the region identified as the ATP-binding domain of UvrD (12). The sequences of the ATP-binding domains of UvrD and several related ATPases (16) are shown in Fig. 3. It has also been noted (57) that the region between amino acid residues 27 and 40 exhibits some similarity to the helix-turn-helix DNA-binding motif (A/G) NNNGNNNNN(I/V) (5). However, this sequence is not conserved in related helicases (Fig. 3), and predictions of secondary structure by the GCG PEPTIDESTRUCTURE program indicated that the region did not appear to have a propensity to form the requisite alpha-helical structure (9, 20). Furthermore, comparison of the amino acid sequence of this region to a master set of helix-turn-helix proteins (5) yielded a score of 0.88, indicating that the region is not very likely to have a helix-turn-helix structure (5).

Purification and general characteristics of UvrD252. DNA helicase II was purified to homogeneity from strains overproducing the product of either the wild-type or the *uvrD252* allele. The wild-type enzyme was detected on the basis of ATPase activity and immunoreactivity, whereas UvrD252 was detected by immunoreactivity alone. The wild-type and UvrD252 enzymes exhibited roughly similar chromatographic properties throughout the purification procedure. The results of the purification are shown in Fig. 4. Both enzymes appeared to be more than 98% pure.

ATPase activity. Although no ATPase activity was detected in crude extracts of cells overexpressing UvrD252, assays using the pure UvrD252 protein showed that the enzyme did possess ATPase activity, albeit significantly reduced in comparison to that of the wild type (Fig. 5). A total of 176 ng of UvrD252 was required for the hydrolysis of 5 nmol of ATP, in comparison to 6.9 ng for the wild-type protein. This indicated a specific activity of 28 U/μg for UvrD252 and 720 U/μg for the wild type. The latter value was in good agreement with previously determined values for DNA helicase II (26, 30). A Lineweaver-Burk plot (28) of ATPase activity as a function of ATP concentration is shown in Fig. 6. K_ms were derived from these data by the linear regression method of Cornish-Bowden and Eisenthal (8) by using the hypercard Enzyme Kinetics program (Don Gilbert, BioComputing Office, Biology Department, Indiana University). The apparent K_ms resulting from these calculations were 1.2 mM for UvrD252 and 0.05 mM for wild-type UvrD.

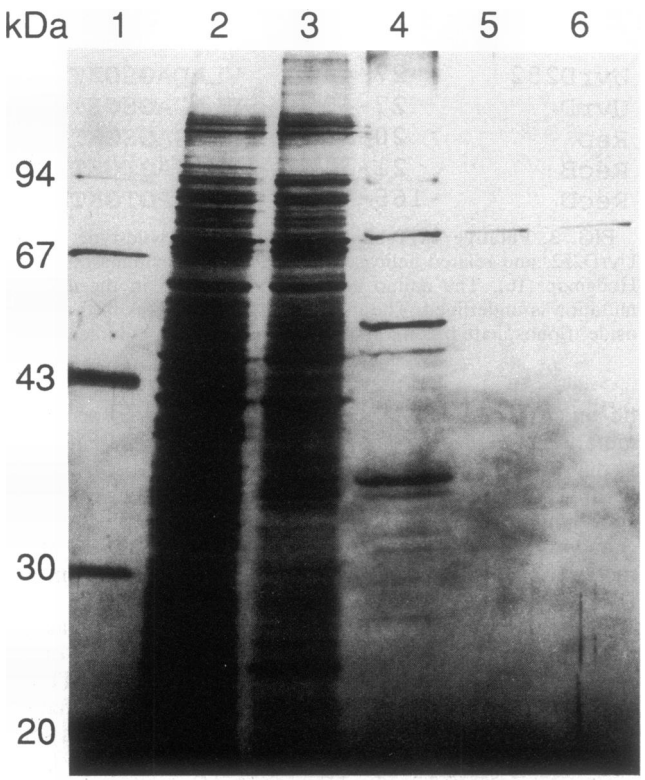

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of UvrD purification steps. Samples were electrophoresed on a 10% gel and stained with silver (34). Lane 1, molecular mass markers; lane 2, 25 μg of crude lysate (wild type); lane 3, 25 μg of ammonium sulfate fraction (wild type); lane 4, 3 μg of phosphocellulose pool (wild type); lane 5, 1 μg of DNA agarose pool (wild type); lane 6, 1 μg of DNA agarose pool (UvrD252).

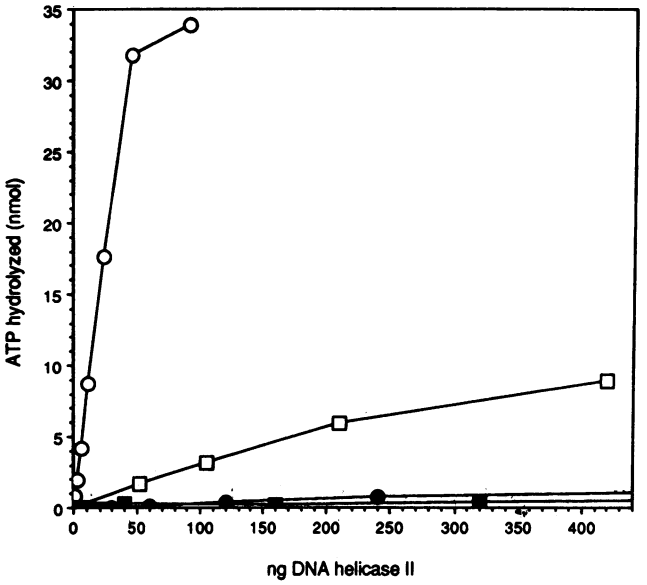

FIG. 5. ATPase activity of UvrD and UvrD252 enzymes. DNA-dependent ATPase activity of UvrD (○) and UvrD252 (□) and DNA-independent ATPase activity of UvrD (●) and UvrD252 (■) were determined as described in Materials and Methods. Reactions were performed for 10 min at 37°C.

The apparent K_m obtained for the wild-type enzyme is slightly lower than the previously reported value of 0.11 mM (30).

ATP and divalent cation requirements for helicase activity. Under previously published conditions for unwinding by helicase II (1.8 mM ATP, 4 mM $MgCl_2$) (30), the UvrD252 protein produced little unwinding of a 24-bp partial duplex even at enzyme levels as high as 130 ng (data not shown). However, alteration of the reaction conditions to include higher concentrations of ATP (Fig. 7A) and $MgCl_2$ (Fig. 7B) resulted in an increase in helicase activity. Optimal unwinding activity for UvrD252 was obtained at approximately 6 mM $MgCl_2$ and between 7 and 8 mM ATP. Therefore, subsequent helicase assays were performed under conditions of 7.5 mM ATP and 6 mM $MgCl_2$ for UvrD252 and 3 mM ATP and 1 mM $MgCl_2$ for the wild-type protein.

Unwinding of partial duplexes under optimal conditions. Under the unwinding conditions established above, the ability of UvrD and UvrD252 to unwind duplexes of various lengths was examined (Fig. 8). Approximately 0.36 ng of UvrD was required to unwind 50% of a 24-bp duplex, in contrast to 60 ng for UvrD252. For both wild-type UvrD and UvrD252, the levels of enzyme required for unwinding of a 12-bp duplex were equal to or slightly less than the amounts required to unwind a 24-bp duplex (Fig. 8). The amounts of wild-type helicase II required to unwind a 96-bp duplex were considerably greater than that required for unwinding of the 24-bp duplex. The UvrD252 enzyme, which already required large amounts of enzyme to accomplish unwinding of a 24-bp duplex, was able to produce only a small level of unwinding when confronted with a 96-bp duplex. The requirement for increased helicase II levels to unwind longer duplexes has been previously noted and examined in some detail (30).

Initiation of helicase action at a nick. In both excision repair and methyl-directed mismatch repair, it has been proposed that UvrD may initiate at a nick to unwind DNA prior to repair synthesis (29, 32). It has previously been demonstrated in vitro that in addition to unwinding partial duplexes, helicase II can also unwind nicked circular or blunt-ended DNA (43). In comparison to unwinding reactions using partial duplexes, efficient unwinding of nicked or blunt-ended DNA required either higher levels of enzyme or a reduction of the $MgCl_2$ concentration from 2.5 to 0.5 mM.

To determine the ability of UvrD252 to initiate unwinding at a nick, a double-stranded DNA substrate containing two nicks separated by 12 nucleotides (Fig. 1) was utilized. This substrate is similar to the product expected from the incision step catalyzed by the UvrABC complex (52). Under the reaction conditions that were optimal for unwinding of partial duplexes by UvrD and UvrD252, both enzymes were able to unwind the nicked substrate (Fig. 9). Surprisingly, the levels of helicase II necessary to unwind the nicked duplex were considerably lower than that required for unwinding of a 12- or 24-bp partial duplex (cf. Fig. 8). Fifty percent unwinding of the 12-bp partial duplex required either 0.36 ng of UvrD or 94 ng of UvrD252, whereas 50% unwinding of the nicked substrate required either 0.043 ng of UvrD or 3.45 ng of UvrD252. Thus, the ability of UvrD252 to unwind a 12-bp partial duplex is reduced by 260-fold in comparison to the wild type, whereas its ability to unwind at a nick is reduced 80-fold in comparison to the wild type. The lower levels of enzyme required for unwinding of the nicked duplex may result from the fact that the vast majority of the DNA in the 12-bp partial duplex reaction is single stranded and may be coated with DNA helicase II, reducing the local

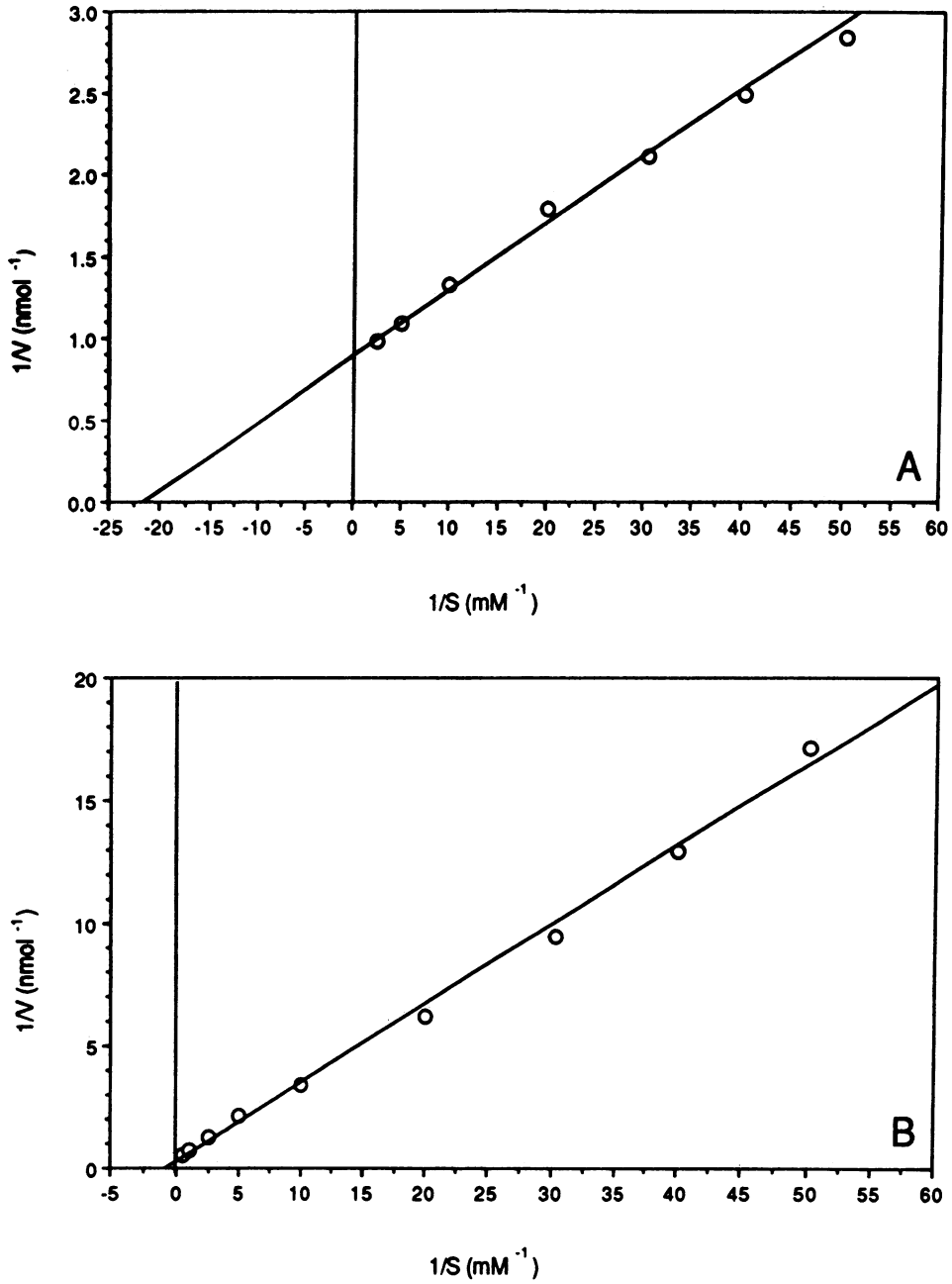

FIG. 6. Double-reciprocal plot of ATPase activity as a function of ATP concentration. A total of 1 ng of wild-type UvrD (A) or 30 ng of UvrD252 (B) was used in each reaction. Reactions were performed for 10 min at 37°C as described in Materials and Methods.

concentration of enzyme available for actual unwinding. Unlike the reaction conditions described by Runyon and Lohman (43), the reaction conditions that we used to assay unwinding of nicked DNA did not include single-stranded DNA.

To verify that the nicked substrate did not contain gaps on either side of the 12-nucleotide oligonucleotide, the DNA was reacted with T4 DNA ligase and ATP and, after heat inactivation of the ligase, reacted with helicase II. As shown in Fig. 10, the ligated substrate was not unwound at enzyme levels which allowed 100% unwinding of the nicked substrate by either UvrD or UvrD252. To prove that the substrate was a circular DNA molecule, it was digested with the restriction enzyme *Alw*NI at room temperature and electrophoresed on a 0.8% agarose gel along with undigested substrate and M13mp18 in its linear, single-stranded, nicked, and supercoiled forms. The gel was then stained with ethidium bromide and dried onto Whatman paper, and the positions of the unlabeled control DNAs were marked under UV light. Exposure of the gel to film (data not shown) indicated that the radiolabeled substrate migrated with the same mobility as the nicked double-stranded M13 and that the *Alw*NI-digested radiolabeled substrate comigrated with the linear M13.

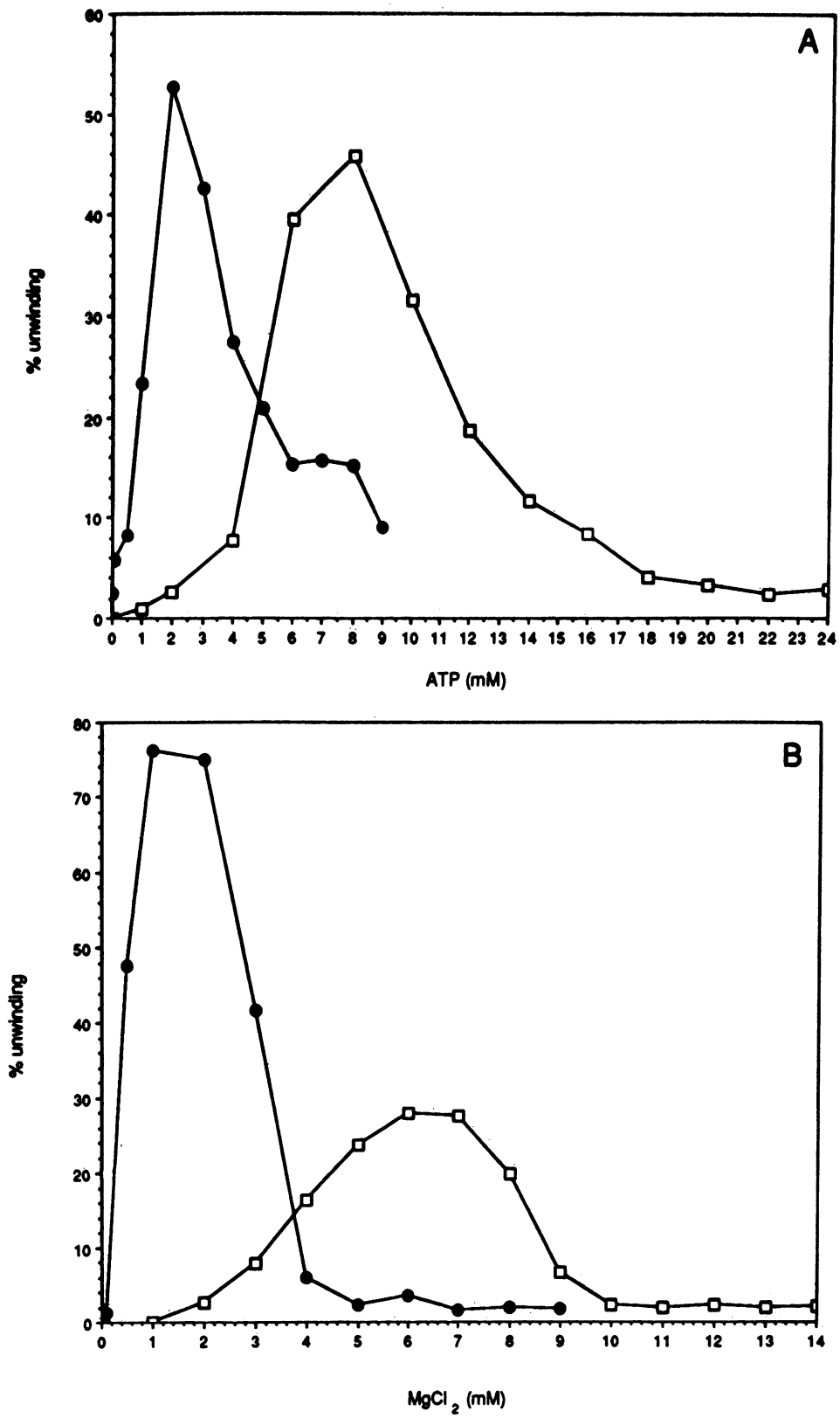

FIG. 7. Unwinding of a 24-bp partial duplex as a function of ATP and $MgCl_2$ concentrations. Reactions were performed for 10 min at 37°C as described in Materials and Methods, with either 0.3 ng of wild-type DNA helicase II (●) or 130 ng of UvrD252 (□). (A) ATP concentration was varied in the presence of 1 mM $MgCl_2$ (wild type) or 6 mM $MgCl_2$ (UvrD252). (B) $MgCl_2$ concentration was varied in the presence of 3 mM ATP (wild type) or 7.5 mM ATP (UvrD252).

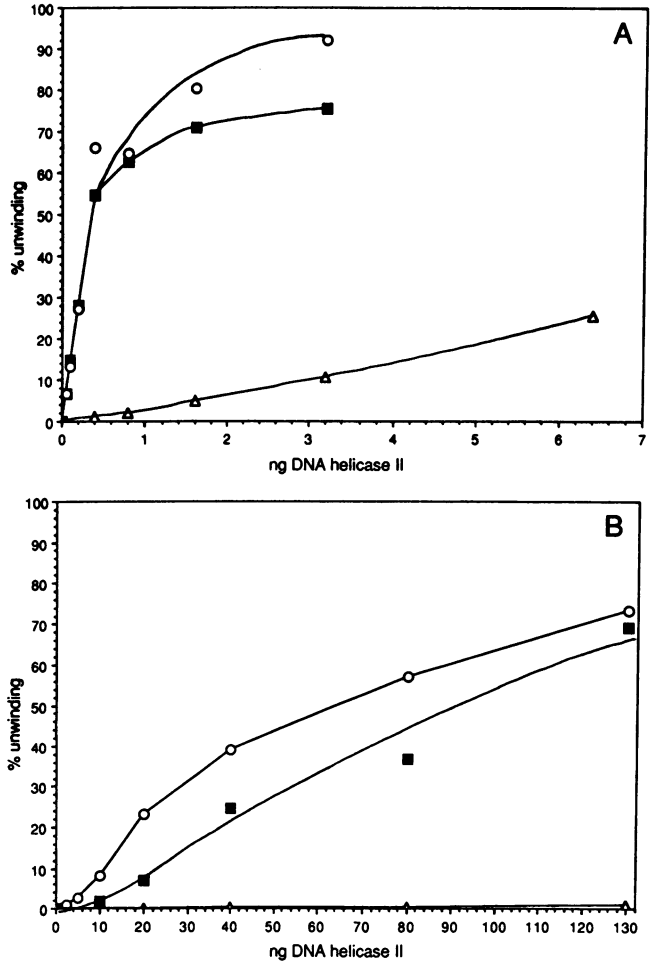

FIG. 8. Unwinding of partial duplexes. UvrD (A) and UvrD252 (B) were reacted with duplexes of 12 (■), 24 (○), and 96 (△) bp for 10 min at 30°C as described in Materials and Methods. Wild-type reaction mixtures contained 3 mM ATP–1 mM $MgCl_2$. UvrD252 reactions contained 7.5 mM ATP–6 mM $MgCl_2$.

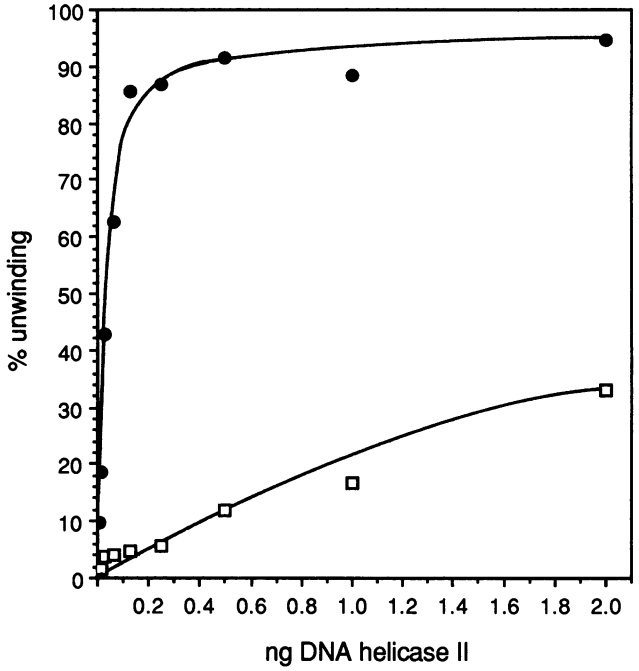

FIG. 9. Unwinding at a nick by UvrD and UvrD252. Reactions were performed for 10 min at 30°C as described in Materials and Methods. Wild-type reaction mixtures contained 3 mM ATP–1 mM $MgCl_2$. UvrD252 reaction mixtures contained 7.5 mM ATP–6 mM $MgCl_2$. The reaction mixtures containing UvrD and UvrD252 were loaded on separate gels but were exposed to the same piece of film. Percent unwinding was determined as described in Materials and Methods. A total of 4 ng of wild-type enzyme produced 100% unwinding, whereas the UvrD252 protein produced a maximum level of unwinding (85%) with approximately 30 ng. ●, UvrD; □, UvrD252.

DISCUSSION

DNA helicases are required for various aspects of DNA metabolism, including DNA repair, replication, and recombination. Genetic and biochemical studies have implicated DNA helicase II in all of these processes (17, 23, 35, 36, 40, 41, 46–48, 54, 56). Although the effects of *uvrD* mutations on the cell have been examined in detail, the only *uvrD* mutant proteins whose biochemical activities have been extensively characterized are ones that produce no discernible phenotype in vivo (38, 39). Of the well-characterized alleles which do confer phenotypes, one which might be expected to retain some biological activity is *uvrD252*, since a *uvrD252* strain does not share the mismatch repair proficiency associated with a *uvrD* deletion (56).

To ascertain which, if any, enzymatic activities are associated with DNA helicase II produced by various *uvrD* alleles, several laboratories have measured the UvrD-associated ATPase activities in crude extracts prepared from *uvrD* mutants. However, the results of these studies have been difficult to interpret. When fractionated on phosphocellulose, extracts from a *uvrD252* strain, as well as *uvrD210*, *uvrD156*, and *uvrD502* strains, did not produce a detectable peak of ATPase activity corresponding to DNA helicase II (39, 51). However, fractionations of *uvrD252*, *uvrD100*, and *uvrD3* extracts on DNA agarose all produced peaks of DNA-dependent ATPase activity that were equal to or greater than that of wild-type extracts (27).

Because the assays of crude extracts gave such variable results, we chose to investigate the biochemical properties of UvrD252 enzyme which was purified to homogeneity on the basis of immunoreactivity. In agreement with the results of Richet et al. (39), we could not detect the UvrD252 protein in crude extracts on the basis of ATPase assays. Assays using purified UvrD252 confirmed that the ATPase activity of UvrD252 is severely reduced in comparison to that of the wild type. The identification of the *uvrD252* mutation in the ATP-binding domain of the protein suggests that the decrease in ATPase activity results from an inability to bind and/or hydrolyze ATP. The extremely high K_m for ATP is consistent with the conclusion that the UvrD252 protein has a reduced ability to bind ATP. Although the altered glycine residue is not absolutely conserved in related ATPases (Fig. 3), it apparently has a major impact on the ability of DNA helicase II to bind ATP.

Presumably as a result of its decreased ability to utilize ATP, the UvrD252 protein exhibits reduced ability to unwind partial duplexes in comparison to the wild-type enzyme. An increase in ATP concentration to 7.5 mM alleviates this deficiency to some extent. The additional requirement for increased magnesium in these reactions may

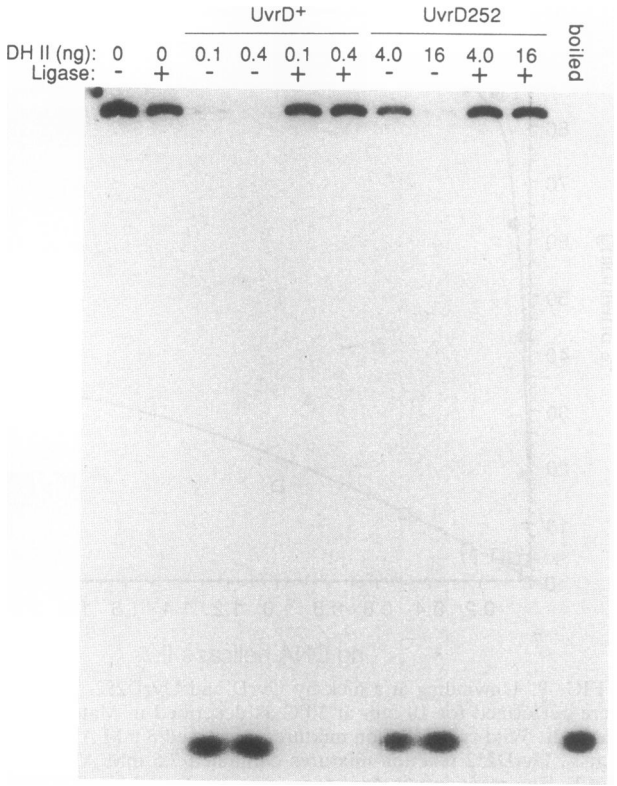

FIG. 10. Autoradiogram of the unwinding reaction produced by helicase II (DH II [DNA helicase II]) on a nicked substrate following ligation. Nicked substrate (4 ng) was incubated in 10 μl of ligation buffer (50 mM Tris [pH 7.0], 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP) containing either 0.5 μl (0.5 U) of T4 DNA ligase or 0.5 μl of distilled water. After 4 h at 22°C, samples were diluted to 35 μl in distilled water, and the sample containing ligase was heated for 15 min at 75°C and cooled to room temperature. Five microliters of this DNA was used in each helicase reaction. To obtain 100% unwinding, sample was boiled for 5 min prior to electrophoresis.

reflect the higher levels required for the formation of MgATP complexes in the presence of high levels of ATP (33).

UvrD252 also showed a reduced ability to initiate at a nick to unwind a 12-nucleotide oligonucleotide, although the proportional difference between the ability of wild-type and that of mutant proteins to unwind nicked DNA was somewhat less than that for a partial-duplex substrate. The ability of DNA helicase II to initiate unwinding at a nick may be particularly relevant to its proposed roles in DNA repair. In the repair of DNA by the nucleotide excision repair pathway, DNA helicase II and DNA polymerase I are required for release of the 12- to 13-bp oligonucleotide produced on either side of the adduct by the UvrABC incision reaction and for release of the components of the incision complex from the DNA (6, 18). This could be accomplished by initiation of unwinding at a nick produced by the incision step, releasing both the DNA fragment and the bound proteins (29). In another model, DNA polymerase I is responsible for displacing the oligonucleotide and the role of UvrD is to release UvrB and/or UvrC from the displaced oligonucleotide (53).

In mismatch repair, the actual substrate for UvrD initiation is also unclear. Possibilities include initiation at the nick produced at the GATC site by MutH (32) or loading onto the DNA at the site of the mismatch, perhaps in an area of localized unwinding produced by MutS (37). In each case, DNA helicase II presumably unwinds the intervening DNA. Since the UvrD252 enzyme initiates at a nick more efficiently than on a partial duplex, it seems unlikely that a specific defect in the ability of UvrD252 to unwind starting at a nick is sufficient to explain the differential ability of UvrD252 to participate efficiently in mismatch repair but not excision repair.

There has been speculation that UvrD might interact with DNA polymerase I during the resynthesis step of excision repair (40), or with components of the UvrABC complex before (29) or after (52) displacement of the oligonucleotide from the DNA. In view of the nature of the *uvrD252* mutation and its effect on unwinding, it seems unlikely that the inability of UvrD252 to participate in excision repair is a result of a deficiency in protein-protein interactions.

One way to explain the phenotype of the *uvrD252* strain on the basis of the observed properties of the enzyme is to suppose that the decrease in helicase activity has caused it to lose the ability to remove proteins that are bound to DNA. It has previously been demonstrated that the ability to displace a protein that is bound to DNA is not a universal property of helicases. For example, the bacteriophage T4 Dda protein can displace a bound RNA polymerase to facilitate DNA replication, whereas the T4 gene 41 helicase is unable to accomplish this (3). In the case of UvrD, an inability to displace proteins would prevent the recycling of the UvrABC complex and would be expected to lead to UV sensitivity.

Since the UvrD252 enzyme apparently participates in mismatch repair in vivo, we must assume that the UvrD252 enzyme unwinds DNA more efficiently in vivo than it does by itself in vitro. It seems likely that the presence of auxiliary factors which are not present in vitro would allow higher levels of unwinding in vivo. Possible candidates for additional proteins could include single-stranded DNA binding protein or Rep helicase stimulatory factor, both of which have been shown to stimulate unwinding by DNA helicase II in vitro (30, 58). Other factors may also contribute to increased unwinding efficiency. For example, Rep helicase unwinds processively in the presence of φX174 CisA protein and DNA polymerase III but unwinds in a rather limited fashion in their absence (10, 58, 59). UvrD252 may unwind more efficiently when its helicase activity is coupled with the DNA resynthesis activity of DNA polymerase III, or with the exonuclease activities shown to be involved in mismatch repair (32). Alternatively, DNA helicase II simply may have a different function in mismatch repair that is retained in the *uvrD252* mutant.

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