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MutS and UvrD Proteins Stimulate Exonuclease Action: Insights into Exonuclease-Mediated Strand Repair[†]

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ABSTRACT: MutS and UvrD proteins individually stimulate *Escherichia coli* exonuclease VII activity on blunt-ended short duplex DNA substrates. Stimulation by both proteins is ATP-dependent but not mismatch-specific and is not accompanied by apparent strand separation. Under similar conditions, MutS and UvrD proteins in fact confer resistance to exonuclease VII action on ssDNA targets, thereby implying that a novel state of a double-stranded DNA intermediate, which we term a "destabilized duplex", is involved in exonuclease-mediated strand degradation. We find that DNA strands in such a destabilized duplex can be displaced by the challenge of a molar excess of homologous single- and double-stranded DNA targets, in trans. Such an action of the UvrD protein is ATP-dependent. We discuss these results in relation to the (i) directional excision repair of *E. coli* MMR, (ii) downregulation of repeat deletions by exonucleases during replication slippage, and (iii) the fork reversal function of UvrD at stalled replication forks.

Postreplicational mismatch repair (MMR)[1] is well understood in *Escherichia coli* (*1*−*3*). However, the mechanistic details of the directional excision step in *E. coli* MMR and aspects such as the strand selection mechanisms and directionality of MMR in higher eukaryotes are far from clear, although recent studies point toward some interesting possibilities largely based on the asymmetry of protein loading at the 5′ side versus the 3′ side of the nick (*4*−*7*). The role of exonuclease action in MMR is crucial (*8*−*10*). Four distinct exonucleases (RecJ, Exo VII, Exo I, and Exo X) are implicated from biochemical studies, though genetic studies of strains deficient in all four exonuclease activities show only a modest increase in mutation rate. Despite its requirement in MMR, even the quadruple mutant lacking all four nucleases confers only a 6-fold increase in mutation rate in MMR in vitro, whereas mutH, mutL, mutS, or uvrD mutations confer an at least 50-fold increase in mutation rate (*11*).

MMR is also implicated in deletions of tandem repeats in *E. coli* that ensue at rates much higher than the rate of spontaneous mutations (*12*, *13*). In addition to the length, small sequence differences between repeats reduce deletion efficiency by several orders of magnitude (*14*). Such a reduction in rate is controlled by two mechanisms: (i) destruction of the heteroduplex slippage intermediate by MMR and (ii) exonuclease digestion of a relatively unstable slippage intermediate formed during repeat expansions (*15*). In many organisms, the MMR system functions to stabilize repeats when the repeat unit is short or when sequence imperfections are present between the repeats. Exonuclease degradation of the short duplex in the slipped DNA intermediate may abort slipped misalignment, thereby preventing deletion (see Figure 2 of ref *15*). Exonuclease action is therefore expected to be a significant step in the genetic stabilization of several forms of repetitive DNA.

In light of this background, here we have investigated the role of exonuclease degradation of short blunt-ended duplexes. Short duplexes are intrinsically less stable: the duplex ends exhibit a higher propensity to fray open and therefore may structurally model instability associated with slipped duplex intermediates in deletion and/or expansion of the short repeats. We uncover interesting effects where exonuclease VII degrades the strand even in the duplex configuration under the individual influence of MutS and UvrD proteins. Interestingly, in this setting, no complete strand separation was detectable. However, we speculate that the action of UvrD results in sufficient destabilization of duplex ends that it becomes prone to branch migration by incoming competing homologous single- or double-stranded DNA added in trans. Such a result helps us rationalize certain replication fork reversal functions ascribed to the UvrD protein (Discussion). Taken together, all these results point to a novel stimulatory role of MutS and UvrD proteins in exonuclease action that may be relevant for regulating MMR as well as other repeat sequence-induced instabilities.

MATERIALS AND METHODS

Materials. Optikinase was from GE Healthcare. Exonuclease VII, single-stranded DNA binding protein, and RecA were from USB Corp. (Cleveland, OH). Nuclease free BSA and ATP were from Sigma-Aldrich. Ni-NTA resin was from Qiagen. Oligonucleotides were synthesized at DNA technology.

DNA Substrates. (1) GT-mismatched heteroduplex (121 bp) (mismatched bases are in bold) (homoduplex counterpart will have a GC base pair in the same location): top strand, 5′ TCA CCA TAG GCA TCA AGG AAT CGC GAA TCC GCC TCG TTC CGG CTA AGT AAC ATG GAG CAG **G**TC GCG GAT TTC GAC ACA ATT TAT CAG GCG AGC ACC AGA TTC AGC AAT TAA GCT CTA AGC C 3′; bottom strand, 5′ GGC

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[1]Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RFR, replication fork reversal; MMR, mismatch repair.

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TTA GAG CTT AAT TGC TGA ATC TGG TGC TCG CCT GAT AAA TTG TGT CGA AAT CCG CGA **T**CT GCT CCA TGT TAC TTAGCC GGA ACG AGG CGG ATT CGC GAT TCC TTG ATG CCT ATG GTG A 3′.

(2) Homoduplex (61 bp): top strand, 5′ GCC TCG TTC CGG CTA AGT AAC ATG GAG CAG GTC GCG GAT TTC GAC ACA ATT TAT CAG GCG A 3′; bottom strand, 5′ TCG CCT GAT AAA TTG TGT CGA AAT CCG CGA CCT GCT CCA TGT TAC TTA GCC GGA ACG AGG C 3′.

(3) GT-heteroduplex (31 bp) (mismatched bases are in bold) (homoduplex counterpart will have a GC base pair in the same location) (31 bp strands mentioned below were used in the strand displacement assay and have the same sequence as the middle part of the 61 bp strands): top strand, 5′ AGT AAC ATG GAG CAG **G**TC GCG GAT TTC GAC A 3′; bottom strand, 5′ TGT CGA AAT CCG CGA **T**CT GCT CCA TGT TAC T 3′.

(4) Hairpin duplex DNA: 5′ GCG CAT ACT CGC TCA TAC TGC GAG TAT GCG C 3′.

All strands were purified by electrophoresis on 10% denaturing polyacrylamide gels. The full-length oligonucleotide was excised from the gel and eluted into 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA by diffusion, followed by desalting through a Seppak C18 cartridge as described by Sambrook et al. (Molecular Cloning: A Laboratory Manual. Vol. 3, 1989). The final purity was determined by 5′ end labeling using [γ-^{32}P]ATP and analysis on a 10% denaturing polyacrylamide gel. DNA concentrations expressed refer to nucleotide concentrations unless otherwise mentioned.

DNA Labeling and Annealing. The standard protocol was used to label 5′ ends of plus-strand oligonucleotide (100 μM as nucleotides) DNA using [γ-^{32}P]ATP (10 μCi) in 5 μL reaction mixtures with 5 units of Optikinase. Samples were heated at 90 °C for 5 min to inactivate the kinase. Unincorporated nucleotides were removed by using illusta ProbeQuant G-50 Micro Columns (GE Healthcare) followed by complementary strand annealing for duplexing. Annealing was accomplished in buffer [20 mM Tris (pH 7.6) and 5 mM $MgCl_2$] in a total volume of 10 μL. The sample was heated to 90 °C for 2−3 min followed by slow cooling to room temperature. Completion of annealing was assessed by analysis on a native polyacrylamide gel, which showed that all labeled strands were converted to duplexes and that no residual unannealed labeled single-stranded DNA was left. The unlabeled competitor ssDNA (in strand displacement assays) had the same sequence as the labeled strand. Concentrations are as specified in the respective figure legends.

Protein Purification. The MutS clone was obtained from L. Worth (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The mutS gene is in His tag expression vector pQE30. The protocol followed to purify MutS was described previously (*16*). The His tag was not cleaved from the protein, as it does not seem to alter the biochemical properties of MutS (*16*). The MutH and MutL clones were obtained from M. Winkler (University of Texas, Houston, TX), and His-tagged MutL and His-tagged MutH were purified as described previously (*17*). The His-tagged UvrD clone was obtained from Xi XG (CNRS) and purified as described in ref *18*. HMGB1 was purified as described in ref *19*. All proteins were checked for nuclease activity prior to use. No exo- or endonuclease activity was demonstrable in any protein preparations under the conditions tested.

Assay Conditions. All reactions were conducted in reaction buffer [20 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, and 50 μg/mL BSA] and 1.2 mM ATP when MutS was used (MutS at high ATP concentrations tends to aggregate) and 2.5 mM ATP under all other conditions, for 20 min at 37 °C. Reactions for 10% denaturing gels were stopped by using 2 × stop buffer containing 20 mM EDTA in 90% formamide. Helicase and strand displacement assays were stopped by proteinase K digestion as described by Sambrook et al. (Molecular Cloning: A Laboratory Manual. Vol. 3, 1989) and run on an 8% native PAGE gel.

RESULTS

We studied two blunt-ended oligonucleotide duplexes (31 and 61 bp) containing a single centrally placed GT mismatch (heteroduplex) and compared them with their homoduplex (no mismatch) counterparts. Most results described below pertain to homoduplexes, and where relevant, a side-by-side comparison is made with heteroduplex DNA.

ssDNA-Specific Exonuclease Action on ssDNA in the Presence of MMR Proteins. The exonuclease reaction conditions chosen were specific for ssDNA digestion: selectively labeled (5′ P^{32}) ssDNA reached limit digestion by the action of exonuclease, whereas the same strand when duplexed fully resisted the digestion (compare lane 2 with lane 12 in Figure 1). ssDNA binder proteins of MMR, namely, UvrD, MutS, MutL, and SSB, exerted contrasting effects on ssDNA-specific degradation by exonuclease: UvrD protein exerted marginal protection against exonuclease action at high concentrations, and the protection was more pronounced with a longer ssDNA (61 bases) than a shorter one (31 bases). In contrast, SSB protein caused a high degree of protection even at low protein concentrations on either strand (Figure 1). MutS and MutL proteins hardly exhibited any protection. On the other hand, the same labeled strands that were protected against exonuclease action when duplexed revealed interesting effects in the presence of MMR proteins, as described in Figure 2.

MutS and UvrD Stimulate ssDNA-Specific Exonuclease Action on dsDNA Substrates. Foremost, addition of all MMR proteins in the absence of any exogenous nuclease showed no digestion (lane 6, Figure 2), indicating that MMR proteins were relatively free of contaminating exonuclease activity. Exo-

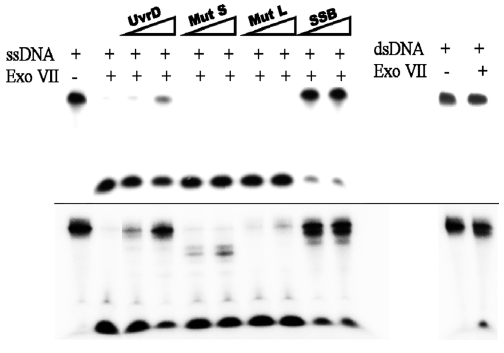

FIGURE 1: ssDNA-specific action of Exo VII. Denaturing PAGE (10%) analyses of Exo VII digestion products of ssDNA in the presence of MMR proteins (0.25 and 1.0 μM each in a 10 μL reaction volume) (5′-labeled 31mer oligo in the top panel and 5′-labeled 61mer oligo in the bottom panel). Lanes 1 and 2 are positive controls showing digestion of ssDNA (0.5 μM nucleotides) by Exo VII. Lanes 11 and 12 are negative controls showing dsDNA (0.5 μM nucleotides) treated with Exo VII. Lanes containing ssDNA treated with Exo VII in the presence of MMR proteins: UvrD (lanes 3 and 4), Mut S (lanes 5 and 6), Mut L (lanes 7 and 8), and SSB (lanes 9 and 10).

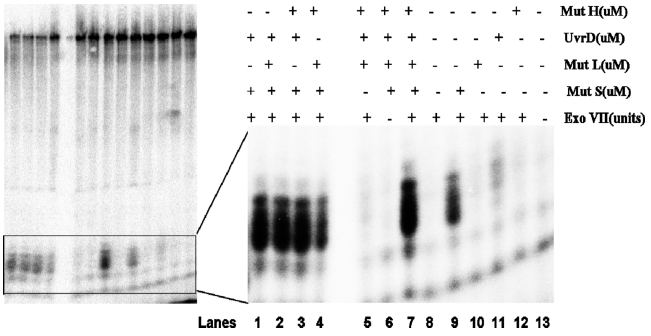

FIGURE 2: MutS protein specifically stimulates Exo VII action on blunt-ended duplex DNA. Denaturing PAGE (10%) analyses of Exo VII digestion products of 5′-labeled (top strand) blunt-ended heteroduplex DNA (121mer) (1 μM nucleotide) in the presence of MMR proteins (0.5 μM each in a 10 μL reaction volume). The left panel is the full gel autoradiogram, while the right panel shows the zoomed up version of the boxed region containing only the limited digestion products (+ and − indicate the presence and absence of the specific protein, respectively).

nuclease control without any MMR proteins also showed no digestion (lane 8), testifying that the nuclease action under study is entirely ssDNA-specific and does not act on dsDNA. MMR proteins, in the presence of exogenous exonuclease (exonuclease VII), gave rise to stimulation of strand digestion (lane 7, ~40%). When MMR proteins were tested individually, only MutS (lane 9, ~15%) and UvrD (lane 11, ~5%) showed measurable stimulation of strand degradation. MutH and MutL proteins showed no stimulation of exonuclease action (lanes 12 and 10 with 2 and 1%, respectively). Expectedly, omission of only MutS from the MMR set led to almost complete elimination of exonuclease stimulation (lane 5, 5%). Similarly, omission of UvrD led to a marginal decrease in exonuclease stimulation (lane 4, 15%), whereas the absence of MutH or MutL gave rise to no decrease in the level of strand degradation (lanes 2 and 3 with 38 and 42%, respectively). As expected, the combined presence of MutS and UvrD without MutH/L (lane 1) stimulated digestion (44%) that was equivalent to those without MutH (lane 2), without MutL (lane 3), or with all four proteins present together (lane 7). All these controls, put together, suggest that the protein components that are largely responsible for exonuclease stimulation are MutS and UvrD.

UvrD-Mediated Stimulation of Exonuclease Action on dsDNA Is Not Accompanied by Strand Separation. We studied UvrD-stimulated nuclease action further: its action was critically protein concentration-dependent. At sufficiently low protein concentrations, UvrD stimulated nuclease sensitivity in dsDNA and was also dependent on DNA length. At a high UvrD level, the nuclease action was partially protected. The nuclease protection was more pronounced in a longer dsDNA (61 bp) than in a shorter one (31 bp) (Figure 3A,B). Nuclease sensitive behavior of both duplexes (31 and 61 bp) in the presence of UvrD protein was not accompanied by any detectable strand separation. The same samples in native PAGE analyses revealed no traces of separated strands (Figure 3C,D). Under the same conditions, where the blunt-ended duplex showed no strand separation by UvrD, the double-tailed duplex, a classical substrate used often, revealed strand separation products reflective of bona fide helicase activity of UvrD protein (Figure 3E). At the lowest UvrD concentration, ~60% of the starting duplex underwent strand separation that reached ~75% at the highest protein concentration.

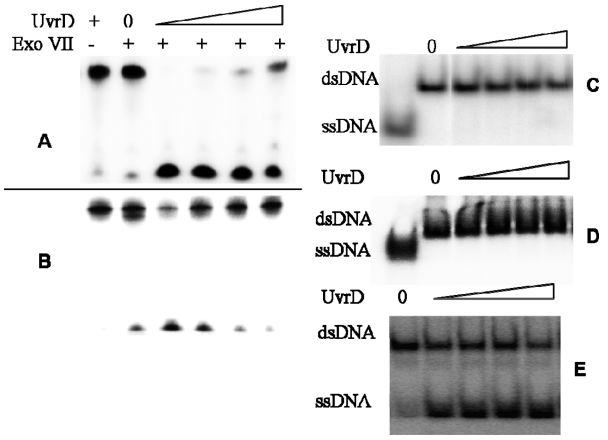

FIGURE 3: UvrD protein renders blunt-ended duplex DNA sensitive to Exo VII action even in the absence of apparent strand separation. (A) Denaturing PAGE (10%) analyses of Exo VII digestion products of 5′-labeled (top strand) blunt-ended 31mer homoduplex (1 μM nucleotide). (B) Denaturing PAGE (10%) analyses of Exo VII digestion products of blunt-ended 61mer homoduplex (1 μM nucleotide). (C and D) Helicase assays on a native 8% PAGE gel of the samples in panels A and B, respectively, without Exo VII. (E) Helicase assay as in panel C but performed on a double-tailed duplex [61mer oligo annealed with a labeled 31mer oligo (1 μM nucleotide)].

UvrD-Mediated Stimulation of Exonuclease Action on dsDNA Is Accentuated by MutS and SSB Proteins but Not by MutL. To test whether MutS-based stimulation of nuclease sensitivity is evident even in the presence of UvrD action, we conducted the following analyses. The UvrD level chosen under these conditions was just sufficient to elicit a marginal nuclease action over which the action of MutS was tested. We analyzed nuclease sensitivity as a function of MutS and MutL proteins, separately. While the presence of MutL showed an only feeble effect on UvrD-induced nuclease sensitivity, the same with MutS was clearly discernible (Figure 4A,B) (~72 and ~76% digestion at the highest MutS concentration in panels A and B, respectively). The combined presence of MutS and MutL proteins did not show any further augmentation over that of MutS (data not shown). Stimulation of nuclease sensitivity by UvrD and MutS proteins was again not accompanied by any strand separation as tested by native gel analyses (data not shown). Interestingly, in the same reaction system, the SSB protein showed a marginal but reproducible level of stimulation of UvrD-induced nuclease sensitivity on dsDNA [compare lane 4 (~42% digestion) with lane 8 (~59% digestion)] (Figure 4C), which contrasted with SSB-mediated protection of nuclease action on a ssDNA substrate (Figure 1). However, at low concentrations of UvrD, the stimulatory role of SSB was lost and the latter even appeared to inhibit the former, reflecting the fact that these two proteins perhaps compete with each other for binding to DNA [compare lane 3 (~50% digestion) with lane 7 (~30% digestion)] (Figure 4C). The presence of MutL had no discernible effect on SSB action in UvrD-mediated nuclease stimulation (lanes 9 and 10 are highly comparable to lanes 7 and 8, respectively). Native PAGE analyses of all these reactions revealed no strand separations (Figure 4D).

UvrD- and MutS-Mediated Stimulation of Exonuclease Action on dsDNA Does Not Depend on the Presence of a Mismatch. We compared UvrD- and MutS-stimulated nuclease sensitivity between homo- and heteroduplex DNA substrates. Both substrates differed only in the presence of a centrally placed GC versus GT base pair in an otherwise identical sequence

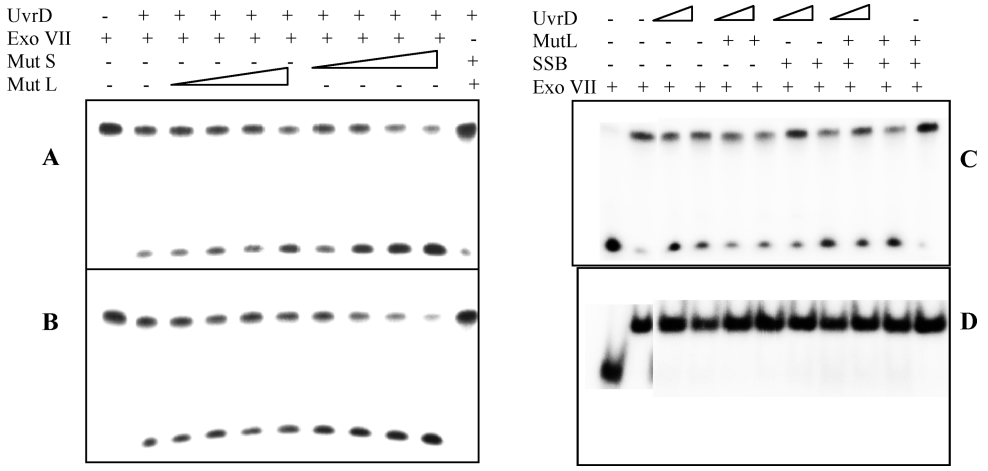

FIGURE 4: UvrD mediated Exo VII sensitization of blunt-ended duplex DNA is augmented by MutS and SSB proteins. Denaturing PAGE (10%) analyses of Exo VII digestion products of 5′-labeled (top strand) blunt-ended 31mer duplex DNA (1 μM nucleotide) at varying MutS or MutL protein concentrations (0.25, 0.5, 0.75, and 1.0 μM each with a suboptimal UvrD concentration of 0.1 μM): (A) homoduplex and (B) heteroduplex. (C) Denaturing PAGE (10%) analyses of Exo VII digestion products of blunt-ended 61mer duplex DNA (1 μM nucleotide) at varying UvrD protein concentrations (0.1 and 0.25 μM) in the presence or absence of SSB (1.0 μM) or MutL (1.0 μM). The first lane contained a ssDNA control (61mer top strand) sample. All other lanes contained duplex samples. (D) Same samples as in set C analyzed via 8% native PAGE but without Exo VII treatment. The first lane contained a ssDNA control (61mer top strand) sample. All other lanes contained duplex samples.

background (see Materials and Methods). A side-by-side comparison revealed that MutS-stimulated exonuclease sensitivity was very similar in homo- and heteroduplex DNA configurations (panel A vs panel B of Figure 4) (~72 and ~76% digestion at the highest MutS concentration in panels A and B, respectively). The MutS concentration range tested in this experiment (0.25, 0.5, 0.75, and 1.0 μM) was sufficiently broad that it covered a range of exonuclease stimulation. Below 0.25 μM, there was hardly any effect of MutS, and above 1.0 μM, the effect had reached its maximum (data not shown). At no concentration of MutS was any enhanced stimulation observed in heteroduplex over that of homoduplex DNA. This experiment suggested that MutS loading on either short duplexes was similar, rendering both equally sensitive to nuclease action (see Discussion).

In summary, it appears that UvrD loading of blunt-ended duplexes renders them nuclease sensitive even without any detectable strand separation. This reaction was augmented further by the presence of MutS or SSB proteins, but not by MutL protein. These results, taken together, suggest that the UvrD protein acts on duplex DNA such that the distorted duplex (see Discussion) was rendered nuclease sensitive. To study this aspect further, we conducted the following analyses.

Directionality of Exonuclease Stimulation. The short duplexes used in this study do not enable us to address whether exonuclease VII stimulation observed here was due to either its 5′, its 3′, or both actions of the enzyme. The high processivity of digestion in either direction could lead to the limit digestion observed, and no intermediate products would be discernible. In fact, when we tested a short hairpin duplex that has only a single 5′ and 3′ end, even with a highly processive 3′ exonuclease (*E. coli* exonuclease I), we observed the same limit digestion products. Hairpin duplex DNA that was 5′ ^{32}P labeled exhibited limit digestion products by the action of exonuclease I only when MutS was present (data not shown). The stimulation by MutS was dependent on protein concentration. A similar stimulation was observed with UvrD protein also (data not shown). In none of the lanes where exonuclease I digestion was observed were the partial products of 3′ to 5′ action evident. Studies involving long duplex substrates have already demonstrated that even though exonuclease VII is intrinsically active in either direction (5′ or 3′), its action on duplexes in MMR is much more pronounced in 5′ repair than in 3′ repair (Figure 4 of ref *20*). In fact, the highly pronounced 5′ action of exonuclease VII was so overwhelming that in their earlier in vitro study involving purified components, the repair catalyzed by this nuclease was restricted only to 5′ ends (*21*).

The UvrD Protein Destabilizes dsDNA. The results described above suggest that UvrD action on blunt-ended duplexes renders the substrate nuclease prone without separating the strands in the substrate duplex. Might this involve transient opening and reannealing of strands dynamically such that no strand separation is observed at a steady-state level? Recent single-molecule analyses of the mode of action of UvrD captured on λ DNA show that the enzyme catalyzes steps of strand separation as well as reannealing dynamically which are subject to complex events of duplex destabilization forces (*22*, *23*). In the following assays, we tested duplex destabilization by the UvrD protein using a novel strand competition assay.

(*i*) *Single-Strand Displacement Assay*. A blunt-ended duplex with a 5′ label on one strand was treated with UvrD protein followed by challenging the reaction mixture with a molar excess of unlabeled ssDNA (the same strand that was labeled in duplex DNA). We surmised that if the blunt-ended duplex was partially unwound or destabilized enough at the ends by UvrD protein, the unlabeled competitor strand would simply compete with the labeled homologous strand and displace the same by branch migration, which can be scored by the release of labeled strand via native PAGE. When we analyzed the products of such a competition assay, we observed the release of the labeled strand from the duplex in the presence of competitor challenge only under specific conditions: the strand release was catalyzed by UvrD specifically when ATP was present (lane 4 vs lane 5, Figure 5). In the absence of either ATP or UvrD protein, no traces of strand release were evident (lanes 2 and 5). Expectedly, in the absence of UvrD, strand release was evident only following heat denaturation and/or reannealing of duplex DNA (lane 3). This reaction was reminiscent of the classical branch migration assay and prompted us to compare the same with RecA. As

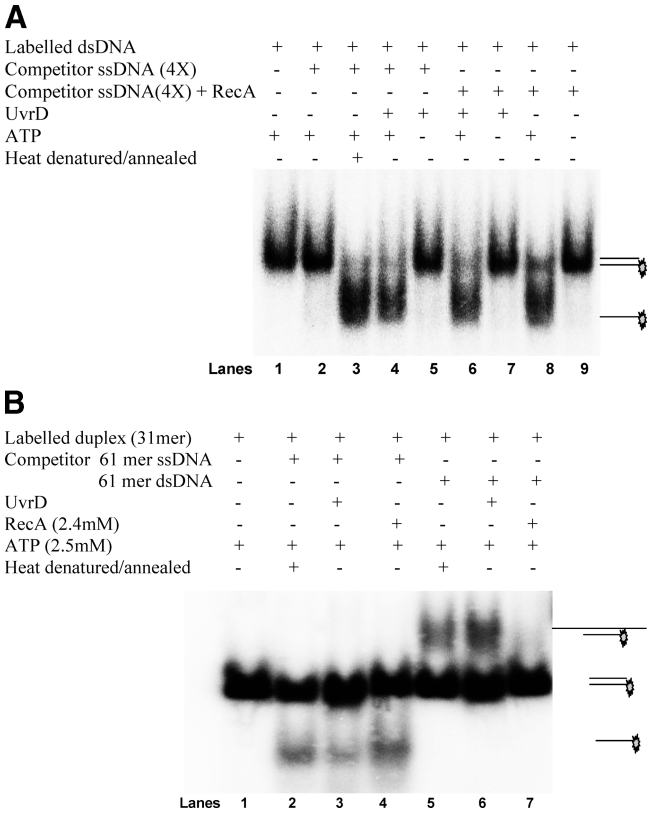

FIGURE 5: (A) Duplex DNA undergoes strand exchange by the action of UvrD protein when challenged by ssDNA (single-strand displacement assay). Labeled 31mer (top strand) duplex DNA (2 μM nucleotide) was incubated with UvrD protein (2.4 μM each) (see Materials and Methods), challenged with unlabeled 31mer top strand (4 μM nucleotide) followed by further incubation at 37 °C for 20 min. For samples in which RecA was used (lanes 6−9), ssDNA competitor was precoated with RecA (2.4 μM each) before labeled duplex DNA was added. Samples were deproteinized and analyzed via 8% native PAGE: controls without protein (lanes 1 and 2) and heat-denatured DNA control (lane 3). (B) Duplex DNA undergoes strand exchange by the action of UvrD protein when challenged by dsDNA (four-stranded reciprocal exchange assay). Labeled 31mer (top strand) duplex DNA (2 μM nucleotide) was incubated with either UvrD or RecA protein (2.4 μM each) (see Materials and Methods) and challenged with unlabeled 61mer top strand (4 μM nucleotide) or 61mer duplex DNA (8 μM nucleotide) followed by a further incubation at 37 °C for 20 min. Samples were deproteinized and analyzed via 8% native PAGE: control without protein (lane 1) and heat-denatured DNA controls (lanes 2 and 5).

expected, a similar level of strand release was evident by the action of RecA protein specifically in the presence of ATP (lane 8 vs lane 9), and no synergistic stimulation was observed by the combined presence of both UvrD and RecA protein (lanes 6 vs lane 8). There appeared to be no mutual inhibitory effect either. No strand displacement was evident even in the presence of UvrD or RecA with ATP when the competing unlabeled ssDNA was fully nonhomologous (data not shown). When the homology was restricted to either the 3′ end or the 5′ end of the competing strand, the observed level of strand displacement was not significantly reduced as compared to that of the fully homologous strand. Quantification revealed that the 3′ homology-restricted strand (5′ heterology) exhibited an efficiency that was ~80% of that of the homologous set.

(*ii*) *Double-Strand Displacement Assay in the Four-Strand Reaction*. We also tested the strand displacement activity by UvrD and RecA in another competition reaction where the unlabeled challenger was duplex DNA rather than ssDNA. To differentiate the putative products from the reactant duplexes, we used a longer competitor duplex (61 bp) in molar excess. Interestingly, the reaction revealed that UvrD protein had rendered the labeled duplex competent enough to bring about a reciprocal branch migration with added unlabeled competitor duplex DNA that was even longer. Consequently, the short labeled strand in the duplex was exchanged with a long unlabeled homologous strand (from the competitor duplex), thereby generating a labeled tailed duplex (lane 6, Figure 5), the reaction that mimicks four-stranded branch migration. The position of the labeled tailed duplex product was authenticated by generating the same in a control where the duplexes were heat denatured followed by annealing (lane 5). UvrD reaction depended on ATP nucleotide cofactor. Strikingly enough, a similar reaction performed with RecA alone yielded no reciprocal products (lane 7). RecA reaction was unproductive, reflecting poor loading of protein on these substrates. Typically, RecA-mediated four-strand exchanges are observed more efficiently only when the RecA-coated DNA is a gapped circular duplex (see Discussion). However, RecA-mediated strand displacement occurred when the competitor was ssDNA as opposed to dsDNA (lane 4). Expectedly, UvrD protein-mediated strand displacement ensued whether the competitor was ssDNA (lane 3) or dsDNA (lane 6). In the absence of proteins, strand displacement was evident only following heat denaturation and/or reannealing of labeled duplex DNA (lanes 2 and 5) (Figure 5). As expected, no strand displacement was evident even in the presence of UvrD and ATP when the competing unlabeled dsDNA was fully nonhomologous (data not shown).

In summary, the results reported here demonstrate that UvrD protein loading on blunt-ended duplex substrate leads to interesting consequences: the destabilized duplex, perhaps through its ends, becomes nuclease sensitive as well as competent in exchanging strands with its homologue by branch migration. This reaction has important implications in both MMR and the replication restart function of UvrD protein, as discussed below. All these results are also discussed in the framework of exonuclease function relevant for DNA repair in MMR-dependent pathways.

DISCUSSION

Here we show that short blunt-ended duplex is digested by exonuclease VII only when either MutS or UvrD is present (Figures 2−4). The controls testify that MMR proteins used here are free of any intrinsic exonuclease contamination activity. As expected of its specificity, extrinsically added exonuclease VII was unable to digest dsDNA but acted only on ssDNA targets (Figure 1). However, upon addition of MutS or UvrD proteins, dsDNA was rendered sensitive to exonuclease VII. The sensitivity was independent of any mismatch in the DNA. MutS is a bona fide binder of not only mismatches but also dsDNA ends (*24*). Some of eukaryotic MMR proteins even have instrinsically associated nuclease activity (*25*) and physically recruit extrinsic exonuclease function (*5*, *8*−*10*). Similar collaboration between MutS and MutL proteins and UvrD and exonuclease activities is a foregone conclusion in *E. coli* based on the elegant demonstration of various mechanistic steps of MMR, in vitro (*11*, *20*, *21*, *26*, *27*). However, to date, there has been no experimental demonstration of direct physical interaction or recruitment of exonuclease VII or any other exonuclease function by either MutS, MutL, or UvrD protein at the repair site. On the other hand, there is evidence that MutL, the key protein in MMR, could directly interact with the individual subunits of clamp loader

protein in DNA polymerase III, thereby strongly coupling MMR components to DNA replication machinery (*28*). It is presumed that following UvrD-mediated strand separation from the nicked site at hemimethylated GATC, exonuclease activity degrades the strand toward a mismatch. The precise molecular basis of directionality selection of strand repair is also uncertain. Our study provides some useful insights in this direction, as described below.

In the absence of evidence that MutS/MutL or UvrD proteins physically recruit an exonuclease function, the next simple model that can rationalize repair toward and up to the site of mismatch requires that the exonuclease activity be positively regulated by MutS, MutL, and UvrD proteins. Such a modulation of exonuclease activity would then facilitate strand degradation preferentially toward mismatch rather than away from it, because MutS and UvrD binding density (from the nicked GATC site) is likely to be high toward the mismatch. Such a model provides a basis of strand degradation up to the site of mismatch along the MutS, MutL, and UvrD protein tract that extends toward mismatch. This model is consistent with our earlier evidence that MutS binding at a mismatch leads to ATP-dependent expansion of the MutS tract on the duplex (*29*) that is presumably driven by the intrinsic ability of the MutS protein to aggregate on DNA (*30*). We also propose that Exo VII action as observed in our experiments is highly consistent with its preferred nuclease for 5′ repair in MMR as suggested previously (*20*, *21*). The model we are proposing here is also consistent with the results of a recent study which showed that MutH activation (and perhaps subsequent strand repair) strongly depends on the cis action of MutS and MutL from the site of mismatch involving signal transmission along the helix contour (*27*).

What is the basis of MutS- and UvrD-mediated activation of exonuclease VII or even exonuclease I? It is unclear how MutS alters duplex ends to render them exonuclease sensitive. Does MutS binding to duplex ends increase the propensity of strand "breathing" at the duplex ends? We are currently investigating the same using time-resolved fluorescence anisotropy studies using 2-aminopurine as a function of its position in DNA (unpublished observations and ref *31*). This aspect of MutS binding is relevant given its high affinity for not only mismatches but also duplex ends (*24*). Perhaps this explains why MutS effects are not strictly dependent on the presence of a mismatch in short duplexes where the binding preference of the protein for heteroduplex over homoduplex is only marginally higher. Therefore, we were not surprised at the lack of difference between hetero- and homoduplexes in the level of MutS-mediated exonuclease stimulation (Figure 4). Additional interesting speculation relating to short size of duplexes studied here is in order: We believe that short blunt-ended duplexes might well model unstable ends encountered frequently during strand slippage events across short repeats during replication, even though replication slippages ensue at ssDNA−dsDNA junctions (reviewed in ref *32*). The repeat-induced deletions and expansion mutations, which occur at high frequency in *E. coli*, are negatively regulated by the MMR system (*15*). Current genetic and molecular studies reveal that such negative regulation exerted by MMR is best rationalized by exonuclease-mediated degradative loss of deletion and expansion intermediates (Figure 2 of ref *15*). We believe that our current biochemical study offers an experimental justification of such a model.

UvrD protein being a classical helicase has the ability to unwind the duplex, facilitating stable strand separation in tailed duplexes (Figure 3E) and perhaps transient separation of strands at the ends of blunt-ended duplexes. The latter leads to sufficient destabilization of duplex ends to render them sensitive to exonuclease VII action (Figure 3A−D). Most interestingly, the destabilizing effect of UvrD at duplex ends also leads to a novel effect: the duplex is rendered competent to exhibit branch migration with single- and double-stranded sequences (Figure 5A,B). We believe that the ability of UvrD protein to catalyze branch migrations of this type is relevant for its hitherto unexplained function in replication fork reversal, as explained below. We believe that the activity of UvrD described here perhaps is a simple consequence of its duplex end destabilization and reannealing function followed by spontaneous branch migration, and not to be confused with bona fide actions of a recombinase. We previously had used this assay to unravel the function of another DNA binding protein, human Translin that essentially stabilizes DNA ends from fraying open (*33*). The finding that UvrD unwinds a tailed duplex that can be observed by a gel assay (Figure 3E), but not with the blunt-ended duplex (Figure 3C,D), can be rationalized by its highly efficient unwinding in the former and inefficient unwinding in the latter duplex. Consequently, the steady-state level of strand separation (unwinding) in the blunt-ended duplex is too low to detect but discernible enough at the duplex ends that ssDNA-specific nuclease action can be elicited (Figure 3A,B), and even branch migrations can be evoked by excess competitor strands in the presence of UvrD and ATP where the latter simply anneals to the transiently separated duplex ends followed by branch migration (Figure 5). Unlike that of a classical recombinase protein, the ssDNA competitor with restricted homology at one end (3′ or 5′) was essentially as effective as the fully homologous strand, thereby suggesting that UvrD effects are a consequence of overall destabilization in duplex ends followed by reannealing of strands, driven by partial homology. In fact genetically, UvrD functions as an anti-recombinase rather than a recombinase.

The need for UvrD in Pol IIIts mutants only when RecQ, RecJ, RecFOR, and RecA are all present led Lestini and Michel (*34*) to propose that UvrD antagonizes deleterious actions of RecQ-, RecJ-, and RecFOR-dependent RecA binding to arrested forks, which prevents replication fork reversal (RFR) (Figure 1F,G of ref *33*). In vitro results that UvrD can undo RecA−ssDNA filaments and RecA-mediated recombination

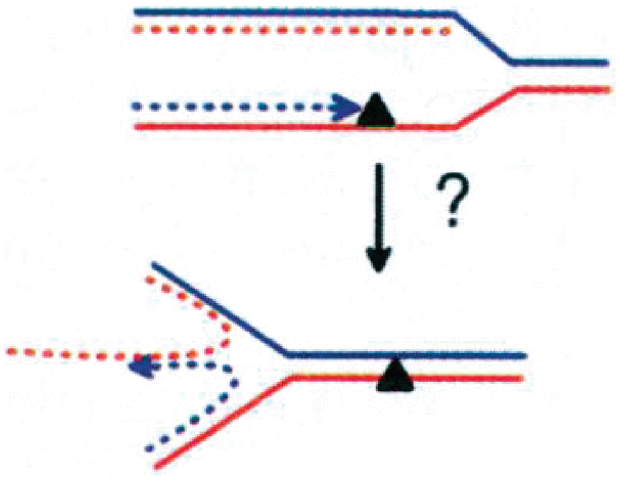

FIGURE 6: Model of replication fork reversal promoted by UvrD via its strand exchange function (adapted from ref *34*). We propose that the unknown activity in the model (?) must be UvrD.

intermediates (*35*, *36*) suggested that UvrD acts by clearing RecA from halted forks, thereby facilitating replication fork reversal (RFR) (*37*). We propose that UvrD that has cleared the fork of RecA indeed can actually facilitate reciprocal branch migrations required for active RFR (unknown entity proposed in Figure 6 by Lestini and Michel in ref *34*) (Figure 6). Branch migration by UvrD appears to be counterintuitive to its anti-recombinase functions envisaged previously by others. However, this role of UvrD is a perfect missing link proposed in earlier models for RFR involving UvrD. The protein has the uncanny ability to translocate along the duplex separating the strands and switch back to reanneal the same, a facet that is just right for facilitating RFR as proposed in the model (Figure 6).

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