Formation of DNA Repair Intermediates and Incision by the ATP-dependent UvrB-UvrC Endonuclease*

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The Escherichia coli UvrABC endonuclease mediates the repair of a broad spectrum of DNA adducts during the process of nucleotide excision repair. This process can be viewed as a series of integrated steps. The UvrA protein (103.8 kDa) exists in equilibrium between a monomer and dimer; the latter interacts in solution with UvrB (76.1 kDa) to form a heterotrimer, UvrA2B (5, 6). The binding of UvrA2B to the site of damage results in a conformational change in the protein-DNA complex, the dissociation of UvrA2, and the formation of a stable preincision UvrB-DNA complex, which acts as a scaffold for the binding of UvrC, leading to subsequent incision. To understand both the contribution to incision efficiency and the nucleic acid mechanisms of the UvrB and UvrC proteins, it would be beneficial to study the activity of the UvrB and UvrC proteins in the absence of UvrA.

The UvrABC nuclease is a structure-specific, ATP-dependent endonuclease. Our study shows that UvrB is involved in damage recognition and the nuclease mechanisms of the UvrB and UvrC proteins, it would be beneficial to study the activity of the UvrB and UvrC proteins in the absence of UvrA. We have defined a series of structure-specific DNA substrates and studied the protein-DNA intermediates formed between these substrates and the UvrB and/or UvrC proteins in the presence or absence of UvrA. Results presented here demonstrate that the UvrB and UvrC proteins promote the assembly of a complex that incises DNA 5' to the adduct in an ATP-dependent manner and thus acts as a bonafide nuclease. Our study also shows that UvrB is involved in damage recognition, as part of a multilevel discrimination system, and that the 3' incision is a rate-limiting step in the overall mechanism of the UvrABC nuclease system.

MATERIALS AND METHODS

Chemicals

Tris base, boric acid, EDTA, and MgCl₂ were purchased from Sigma. Acrylamide, ammonium persulfate, N,N'-methylenebisacrylamide, and urea were obtained from Life Technologies, Inc. [γ-32P]ATP was purchased from DuPont NEN. Racemic BPDE was purchased from the National Cancer Institute Chemical Carcinogen Reference Standard Repository.

1 The abbreviations used are: BPDE, benzo[a]pyrene diol epoxide (7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene); MOPS, 4-morpholinopropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; bp, base pair; AMP-PNP, adenosine 5'-[β,γ-iminotriphosphate]; AMP-PCP, adenosine 5'-[β,γ-methylene]triphosphate.
Purification of Proteins

Purification of UvrA—UvrA was purified from E. coli strain MH1 UvrA containing the overproducing plasmid pSST10 (graciously supplied by L. Grossman, Johns Hopkins University), which is under the control of the heat-inducible PL promoter. UvrB and UvrC were overproduced from E. coli strain CH296 containing plasmids pUNC211 and pDR3274, respectively (graciously supplied by A. Sancar, University of North Carolina). The UvrA was purified to homogeneity as described previously (10). The UvrB and UvrC proteins were purified using modified procedures as described below.

Purification of UvrB—E. coli cells (DH5α) containing the expression plasmid pUNC211 were grown to 1.0 OD in super broth and induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 3 h in a 5-liter fermentor, collected by centrifugation, and resuspended in 100 mM KCI buffer (50 mM MOPS, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol) at 5 ml/g of packed cells. A mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin) was added, and the cell slurry (~75 g) was brought to 20% (w/v) (NH₄)₂SO₄ saturation by the addition of solid (NH₄)₂SO₄. The E. coli cells were then disrupted by sonication at 4°C and centrifuged at 15 K for 60 min to pellet the cell debris. The supernatant was brought to 60% (NH₄)₂SO₄ saturation by the addition of solid (NH₄)₂SO₄ over 30 min. The precipitated protein was pelleted by centrifugation (12 K, 20 min), resuspended in a minimal volume of 20% saturated (NH₄)₂SO₄ in buffer A, and loaded onto a phenyl-Sepharose column. The column was developed with a 300-ml linear gradient of KCl (0.05–1 M) in buffer A; UvrB eluted off the column at 300 mM KCl. The fractions containing UvrB, as determined by SDS-PAGE, were pooled and dialyzed against 50 mM KCl in buffer A. The dialysate was loaded onto a blue Sepharose column and developed with a linear gradient of KCl (0.05–1 M) in buffer A; UvrB eluted at ~250 mM KCl. The fractions containing UvrB, as determined by SDS-PAGE, were pooled and dialyzed against 50 mM KCl in buffer A. The dialysate was loaded onto a fast protein liquid chromatography Mono Q (10/10) column that was eluted with a shallow linear gradient of KCl (50–500 mM) in buffer A; UvrB eluted off the column at 300 mM KCl. The fractions containing UvrB were pooled and brought to 20% (NH₄)₂SO₄ saturation by the addition of solid (NH₄)₂SO₄. The protein pool was loaded onto a fast protein liquid chromatography phenyl-Sepharose column (5/5) and eluted with a gradient from 20% saturated (NH₄)₂SO₄ in buffer A to 50 mM KCl in buffer A. Purity was determined by SDS-PAGE analysis of 10 μg of UvrB and Coomassie Blue staining. The fractions containing pure UvrB were pooled, dialyzed into storage buffer (50 mM Tris, pH 7.5, 100 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 50% glycerol) and stored at ~80°C.

Purification of UvrC—E. coli cells (CH296) containing the expression plasmid pDR3274 were grown to 1.0 OD, induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 3 h in a 5-liter fermentor, collected by centrifugation, and resuspended in 5 ml/g of cells 300 mM KCl buffer C (100 mM KPO₄, pH 7.5, 1 mM EDTA, 20% glycerol). A mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin) was added to the cell slurry. The E. coli cells were then disrupted by sonication at 4°C and centrifuged at 15 K for 60 min to pellet the cell debris. The supernatant was loaded onto a Q-Sepharose Fast Flow column equilibrated in 300 mM KCl buffer C. The flow-through fractions were collected and applied to a newly prepared single-stranded DNA-cellulose column (60 ml) equilibrated in 300 mM KCl buffer C. The column was developed with a 300-ml linear gradient of KCl (0.3–1.5 M) in buffer C. UvrC eluted off the column at ~600 mM KCl in buffer C. The fractions containing UvrC (as determined by SDS-PAGE) were pooled and diluted to 150 mM KCl by the addition of buffer C. The protein pool (~290 ml) was then applied to a fast protein liquid chromatography Mono S (5/5) column and eluted with a 50-ml linear gradient of KCl (0.15–1.5 M) in buffer C; UvrC eluted off the column at 200 mM KCl. Purity was determined by SDS-PAGE analysis of 10 μg of UvrC and Coomassie Blue staining. The fractions containing pure UvrC were pooled, dialyzed into storage buffer, as defined earlier, and stored at ~80°C.

Other Enzymes

All restriction and modifying enzymes were obtained from Promega or New England Biolabs unless otherwise indicated.

Construction of DNA Substrates

Oligodeoxynucleotides were synthesized on an Applied BioSystem 394 DNA/RNA synthesizer. After synthesis, all oligomers were purified by PAGE under denaturing conditions. The oligodeoxynucleotide 11-mer (Fig. 1) containing a single (+)-cis-anti-benzo[a]pyrene adduct was synthesized, purified, and characterized as described previously (15). The BPDE-adducted 11-mer was then used to construct all single adduct-containing 50-bp substrates used in this study (Fig. 1). The bold G in the sequence and the filled square in the diagram represent the base at which a single BPDE adducted in S2, S3, S5, and S6. The asterisk indicates 5′-terminal labeling with 32P. The noncomplementary 11-base sequence (TTCTTTTTTCCT) shown below the duplex used in substrates S4, S5, S6, S7, and S8. The arrow indicates a single cleaved phosphodiester bond in the DNA backbone of S6, S7, and S8. The underlined sequence represents an 11-base oligonucleotide that was ligated together with 20- or 19-base oligonucleotides to form the top strand.

![Diagram of DNA substrates](image-url)
UvrA, UvrB, and UvrC, or with UvrB and UvrC in the UvrABC buffer accompanied the oxidation of NADH. From the linear change in absorbance at 340 nm over 30 min which by the same specific activity of the 32P-labeled substrates as quantified by liquid scintillation counting (Beckman LS6000SC) and the DNA concentration.

The substrates were sequenced by standard Maxam-Gilbert procedures (16) with a DuPont NEN sequencing kit.

DNA Binding Assay

Binding of the 50-bp DNA substrates by UvrA, UvrB, and UvrC proteins was assessed qualitatively by gel mobility shift. Typically, the substrate (2 nm) was incubated with the indicated concentrations of UvrA, UvrB, UvrC, or some combination at 37 °C for 15 min in 20 nL of UvrABC buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl$_2$, 5 mM dithiothreitol) in the presence or absence of 1 mM ATP. After incubation, 2 nL of 80% (v/v) glycerol was added, and the mixture was loaded immediately onto a 3 or 3.5% native polyacrylamide gel in TBE buffer (10 mM Tris borate, pH 8.1, 1 mM EDTA) and electrophoresed at 80 volts at room temperature. The gel was dried and exposed for an appropriate time to an x-ray film with intensifying screen or a PhosphorImaging screen (Molecular Dynamics).

DNA Incision Assay

The 5′-terminally labeled DNA substrate (2 nm) was digested with UvrA, UvrB, and UvrC, or with UvrB and UvrC in the UvrABC buffer († 1 mM ATP) at 37 °C for 15 min. Uvr subunits were diluted and premixed into storage buffer before mixing with DNA. Reactions were terminated by adding EDTA (20 mM) or heating to 90 °C for 3 min. The samples were denatured with formamide (50% v/v) and heated to 90 °C and then quick chilled on ice. The digested products were analyzed by electrophoresis on 12% polyacrylamide sequencing gel under denaturing conditions as described before. The substrates (2 nM) were incubated with the indicated concentrations of the UvrA2B-DNA complex with incision of DNA damage by UvrABC nuclease. The reaction mixture (0.5 mL) was allowed equilibrate to 33 °C, and the assay was initiated by the addition of ATP (0.5 mM). The rate of ATP hydrolysis was calculated from the linear change in absorbance at 340 nm over 30 min which accompanied the oxidation of NADH.

Western Blotting Analysis

After the gel mobility shift assay described above, the complex formed by Uvr proteins interacting with the DNA substrates was identified by Western blotting analysis. Briefly, the gel was blotted to a nitrocellulose immobilization membrane (Schleicher & Schuell) using a Hoefer electrotransfer unit and the manufacturer's instructions. The membrane was then treated with UvrB antisera (graciously supplied by L. Grossman, John Hopkins University) or UvrC (graciously supplied by E. Tang, University of Texas M.D. Anderson Cancer Center) antisera for ECL Western blotting (Amersham).

Quantification of Incision and DNA Binding Products

Incision products were quantified using a PhosphorImager 425 (Molecular Dynamics) as described previously (10).

RESULTS

Incision of Defined Substrates by UvrABC Nuclease—To probe the structure of protein-DNA intermediates that form during the assembly of the UvrABC nuclease, we have synthesized a series of eight structure-specific DNA substrates; substrates 2, 3, 5, and 6 contain a single BPDE adduct at position 26 in the 50-bp sequence (Fig. 1). The double-stranded character of all substrates was confirmed by a restriction assay. All substrates were 5′-terminally labeled on the top strand except for S8, which was 5′-terminally labeled on the bottom strand. Incision of each of the 5′ end-labeled BPDE-DNA substrates (S1–S6) by the UvrA, UvrB, and UvrC proteins is shown in Fig. 2. Incision was not observed for the two nondamaged substrates S1 and S4. Although S4 contains a region of 11 mismatched bases, it is not a substrate for the UvrABC nuclease excision repair system. However, the addition of a BPDE adduct in the middle of the unpaired region (S5) results in normal incision at the eighth phosphodiester bond (P8) 5′ to the adduct as reported previously (10). The mismatched region did not affect the incision efficiency, with S2 and S5 being incised at 1.3 and 1.1 fmol/min, respectively. Compared with the normal substrate (S2), the substrate containing a preexisting 3′ nick (S3), which is one nucleotide further away 3′ from the adduct than the major 3′ incision (P5; 10), shows a increase in its 5′ incision efficiency, 2.3 fmol/min (Fig. 2). The same observation has been obtained by Moolenaar et al. (11) for a cisplatin-GG adduct. In spite of the shift of the 3′ incision site 1 base in the 3′ direction, the 5′ incision of the substrate remains at the eighth phosphodiester, as in the normal substrate S2 (Fig. 2), which is consistent with the results reported previously (10, 11). The fact that a 3′ nick facilitates the 5′ endonuclease activity suggests that there is a kinetically slow step through which the UvrABC nuclease system must proceed to make the 3′ incision.

Among the substrates incised by the UvrABC system, S6 displayed 5′ incision at a site one nucleotide further away 5′ (P9) from the adduct and a much weaker incision site at P10 two nucleotides further away, versus the other substrates, which were incised at the P8 position 5′ from the adduct (Fig. 2); S6 was cleaved with the same incision efficiency as the normal substrate (S2). Given that S6 contains a nicked unpaired 11-base region around the adduct and that UvrB unwinds and bends the DNA helix at the adduct when forming the preincision complex UvrB-DNA, the shift of the 5′ incision site may imply that the position and size of the unpaired region play a role in determining the position of the 5′ incision.

Binding of Defined Substrates by UvrA and UvrB Proteins—To examine any correlation between the formation of stable protein-BPDE-DNA complexes and incision, binding of UvrA and/or UvrB to the defined substrates (Fig. 1) was examined by gel mobility shift assays. Fig. 3 shows the interactions of the UvrA and UvrB proteins with the S2, S4, and S5 substrates. Comparison with the incision results in Fig. 2 suggested that the lack of incision of S4 by UvrABC is due to the lack of formation of the UvrB-DNA complex. The data in Fig. 3 suggest that the S2 and S5 substrates are incised equivalently (Fig. 2), probably because they support the production of approximately equal amounts of UvrB-DNA complex.

Comparison of the protein complexes formed on substrates S2 and S3 indicates that the 3′-nicked substrate, S3, supported the formation of more UvrB-DNA complex than S2, whereas in contrast S2 generated much more UvrA$_{2B}$-DNA complex (Fig. 4A). This dramatic increase in the formation of the UvrB-DNA complex for S3 is most likely due to the 3′ nick causing a destabilization of UvrA$_{2B}$ from the protein-DNA complex, so that UvrA$_{2B}$-S3 is less stable than UvrA$_{2B}$-S2. Incision of the same substrates by UvrABC nuclease (under these experimental conditions) (Figs. 4B) showed that S3 supports a much higher incision efficiency than for S2 (5-fold), which formed much more UvrA$_{2B}$-DNA complex than S3 (5-fold, Fig. 4A). These results suggest a direct correlation of the formation of UvrB-DNA complex with incision of DNA damage by UvrABC nuclease.
Incision of Defined Substrates by UvrB and UvrC Proteins—The major role of UvrA in nucleotide excision repair is to provide about 3 orders of magnitude in damage discrimination and to load UvrB to the damage site. Previous studies of interaction of UvrB and UvrC with damaged DNA have only been possible in the presence of UvrA. To probe the interaction of the UvrB and UvrC proteins with damaged DNA, we wanted to find substrates that do not require UvrA involvement. If the UvrA2B interaction leads to a locally unwound and melted structure, we reasoned that the need for UvrA in the incision process might be obviated by placing the damaged site in a locally unpaired region (S5, S6). Thus it may be possible to demonstrate a direct interaction of UvrB and/or UvrC with certain DNA structures resembling preincision intermediates. To test this hypothesis, UvrB and UvrC were allowed to react with the 5'-terminally labeled substrates S2–S6 and S8 (Fig. 1) in the absence of UvrA (Fig. 5). S6, the Y substrate containing a nicked unpaired region surrounding the BPDE-DNA adduct, was the only substrate that supported incision by the UvrBC nuclease (lane 5). Although S3 is a 3'-nicked substrate, it contains a fully base paired region surrounding the damage (in contrast to S6), and no incision was observed (lane 2). These results also show that the Y substrate-specific incision was strand-specific because no incision was observed on the other strand (S8, lane 7). Furthermore, the lack of incision in the absence of ATP (lane 6) indicated that ATP hydrolysis or binding of hydrolyzed products to the proteins was necessary for UvrBC incision. In control reactions, (Fig. 6), no 5’ incision was produced by the individual Uvr proteins. DNA sequence analysis indicated that the UvrBC nuclease incised the Y substrate at the 9th and 10th phosphodiester bonds 5’ (Fig. 5, lane 5, and Fig. 6, lane 7), which is identical to the incision sites by UvrABC (Fig. 6, lane 6). These sites have been found to be located in the double-stranded region three and four nucleotides 5’ to the junction of the duplex and the unpaired region.

Since the junction of the duplex unpaired region appears to be a structural characteristic recognized by UvrB-UvrC proteins, it was reasonable to ask whether the nondamaged Y structure such as S7 (Fig. 1) is a target of UvrB-UvrC nuclease. As shown in Fig. 6, S7 was incised by UvrB-UvrC proteins at the same positions as was S6 substrate but with lower efficiency.

Binding of UvrB and UvrC to Nicked Unpaired Substrate—UvrBC nuclease incision of the Y structures (substrates S6 and S7) strongly suggests that the UvrB-UvrC heteromeric complex is able to recognize and bind to the DNA substrates under appropriate conditions. As shown in Fig. 7A, the interaction of UvrA, UvrB, and/or UvrC proteins with the Y structure, substrate S7, occurred in the absence of ATP. The nonadducted Y structure was recognized by UvrA, as shown in lanes 2 and 3. However, neither UvrB nor UvrC bound alone to the substrate (lanes 4 and 5). Similar to the normal damaged substrate S2, UvrA and UvrB together bound to the S7 substrate to form UvrA2B-DNA complex (lane 6). Most importantly, a complex
formed when both the UvrB and UvrC proteins were incubated with the S7 substrate (lanes 7–10). Since UvrC was found to cause DNA aggregation, a 5:1 ratio of UvrB to UvrC was used for the assay. Western blotting with UvrB antiserum (Fig. 7B) revealed that UvrB was a participant in the complex with DNA. Unfortunately the antibodies to UvrC also cross-reacted with UvrB, so we could not rule out the presence of UvrC in this complex. The observed formation of UvrB/UvrC-DNA complex agrees with the results of the 5' incision made by UvrB and UvrC proteins on the S6 and S7 substrates.

ATP Hydrolysis in the UvrBC Incision—The results described above (Fig. 5) indicated that incision of the nicked unpaired Y substrate by the UvrB and UvrC proteins is an ATP-dependent process. Therefore, incision experiments were performed in the absence and presence of ATP and its analogs including ADP, AMP-PNP, and AMP-PCP, to determine whether ATP binding or hydrolysis was necessary for the incision process. We found that both ATP and ADP can facilitate incision, 100 and 77%, respectively, whereas no incision was observed in the presence of nonhydrolyzable AMP-PNP or AMP-PCP (data not shown). The near full activity of incision with ADP implies that binding of ADP may be involved in the incision mechanism.

An ATP hydrolysis assay was also employed to monitor the ATPase activity of the UvrB protein during the incision process. Analysis of the sequence of UvrB displays one Walker-type ATPase motif (GKT) near the NH₂ terminus of the protein. It has been reported that native UvrB shows no ATPase activity unless it is cleaved at residues 607–610 near the COOH terminus, yielding a 69-kDa protein, UvrB* (11, 17). The truncated UvrB protein is still able to form normal preincision complexes with UvrA on DNA (5, 11, 18) but loses its nuclease activity. The data shown in Table I indicate that the ATPase activity of the native UvrB is activated when the protein inter-
acts with UvrC or the Y structure alone and is stimulated greatly by the addition of both UvrC and the Y structure, which is consistent with the incision results shown above. These data, combined with the fact that UvrB and UvrC interact to form a protein-DNA complex on the S6, S7, and S8 in the absence of ATP and that ATP hydrolysis is required for incision, suggest that ATP hydrolysis or binding of ADP to UvrB is required for 5′ incision produced by the UvrABC system.

DISCUSSION
Experiments performed in this study illustrate several important points regarding incision of the defined substrates by the UvrABC nuclease system (Fig. 2). 1) Neither of the undamaged S1 and S4 substrates is incised by the UvrABC nuclease, even though the latter contains an unpaired region of 11 bases. 2) The presence of an adduct in the middle of the unpaired region (S5) supports incision equivalent to that of the normal adducted substrate S2. 3) S3, which contains a nick 3′ to the adduct, was incised about 3–5 times as efficiently as the normal substrate S2. 4) S6, a nicked unpaired Y substrate, is incised as well as S2, the normal substrate. 5) Incision of the Y substrate occurs in the absence of a DNA adduct although with lower efficiency than either S2 or S6.

It has been shown that the local conformation of the DNA adduct is an important determinant for UvrABC nuclease recognition and incision (10, 19). Formation of the preincision complex of UvrB-DNA results in unwinding and bending of the DNA helix at the adduct (9, 20). It seems that DNA conformational changes induced by the modified base and by protein-DNA complex formation play an important role in the process of damage recognition and nucleotide excision repair. In this

## Table I

| Substrate | UvrB | UvrC |
|-----------|------|------|
| S8        | 0.66 | 0.16 |

* Rate was calculated from the linear decrease in absorbance at 340 nm and equals mol of ATP hydrolyzed/mol of UvrB/min.

* The concentration of UvrB was 200 nM for all experiments.
* Below detection.
* The concentration of S8 was 2 nM for all experiments.
* The concentration of UvrC was 20 nM for all experiments.
report, we studied the interaction of UvrABC with DNA substrates containing partially single-stranded structure (S4 and S5). Although the 11-base unpaired substrate, S4, is recognized by UvrA nearly as well as the BPDE and nicked substrates (Fig. 3), no incision of this substrate was observed (Fig. 2A). This result indicates that UvrA recognizes configurational alterations in the DNA duplex and confirms earlier suggestions that there is no direct correlation between specific binding of UvrA to DNA and UvrABC incision (13). However, positioning a BPDE adduct in the middle of the unpaired region (S5, Fig. 1) results in the same incision efficiency as for S2 (Fig. 2). These results strongly suggest that in addition to the adduct-induced DNA conformation change, the presence of a bulky foreign (non-base) molecule in the DNA sequence is essential for recognition by the UvrABC nuclease. This indispensable requirement is probably indicative of a direct interaction of Uvr proteins with the modified base. Results of these experiments also indicate that large departures from normal B form DNA in the vicinity of the adduct (11-base unpaired region) do not affect recognition and incision by the UvrABC nuclease.

The binding of UvrB to damaged DNA has been suggested as being involved in the recognition (10, 13, 21). In this study, we demonstrated the direct correlation between the formation of the UvrB-DNA complex and the incision (Figs. 2–4). We propose that there are at least two levels of discrimination. 1) The specific binding of UvrA detects a general DNA distortion, and this interaction helps bring UvrB to the site of the damaged base, since UvrB itself normally has no ability to recognize DNA damage in double-stranded DNA; 2) The formation of the UvrB-DNA complex through the UvrA2B-DNA intermediate provides the second level of recognition which monitors modified bases, especially intercalating adducts. The binding of UvrB to the damaged DNA may be mediated through hydrophobic stacking interactions between the aromatic amino acid side chains and the bases (10, 12, 21). These two levels, plus a third level of discrimination, as discussed below, determine the specificity of the 3′ incision near the adduct.

The dramatic increase in the 5′ incision of a BPDE-damaged substrate containing a 3′-preincised substrate (S3) suggests that a rate-limiting step in the UvrABC mechanism of nucleotide incision has been overcome (Figs. 2 and 4). This suggestion is supported strongly by the fact that the UvrB-nicked DNA complex is much more stable than the UvrA2B-DNA complex (Fig. 4A). These data suggest that the 3′ incision is a rate-limiting step and is a consequence of successful damage recognition. It has been reported that the 3′ incision is triggered by the coiled-coil domain interaction of the COOH terminus of UvrB with UvrC (11). It appears that the slow formation of the UvrBC-DNA complex may provide the third level of damage recognition.

Although the UvrABC nuclease has been studied widely as a model of nucleotide excision repair, much remains to be determined regarding the mechanism of the post-3′ incision process, especially the direct interaction of UvrB and/or UvrC with the DNA. In this study, structure-specific DNA substrates containing nicked unpaired Y structures (S6–S8) were used to investigate the mechanism of the post-3′ incision process. The data presented here demonstrate for the first time that UvrB and UvrC proteins work together to form a protein-DNA complex with the nicked unpaired Y substrates in the absence of UvrA and ATP (Fig. 7). Formation of this UvrB-containing protein-DNA intermediate seems to be specific to the Y structure, as no similar complex formation was been observed for other substrates (S1–S5) used in this study (data not shown). As a result, the formation of this Uvr-DNA complex leads to efficient 5′ incision of the adduct in the presence of ATP (Fig. 5), which occurs at the double-stranded region 3–4 bases to the duplex-single strand junction. This endonuclease activity is strand-specific because no incision has been observed on the other (bottom) strand, thus the incision is correlated to the specific feature of the duplex-single strand junction and is strand orientation-dependent. The specific recognition of the duplex-single strand feature is confirmed further by the 5′ incision of the nonadded Y substrate (substrate S7) by the UvrB and UvrC proteins (Fig. 6). Similar results have been reported for the Rad1-Rad10 protein complex in Saccharomyces cerevisiae (22, 23). It has been shown that the Rad1-Rad10 nuclease plays a role both in the cleavage of specific recombination intermediates and in the 5′ incision of DNA damage during nucleotide excision repair (22, 23). The functional similarity between UvrB-UvrC and Rad1-Rad10 nucleases in the cleavage of duplex-single strand junctions suggests a conservation in function between E. coli UvrB-UvrC and yeast Rad1-Rad10 complexes.

The requirement of ATP for the 5′ endonuclease activity is consistent with that for the 3′ incision by the Uvr proteins. The involvement of ATP hydrolysis was confirmed further using ATP analogs in the study. Although previous studies have indicated that protease-truncated UvrB* has ATPase activity, intact UvrB was believed not to have an associated ATPase in the absence of UvrA. Our results indicate that the silent ATPase activity in UvrB can be activated by UvrC and/or a Y type DNA structure in the process of producing a 5′ incision in DNA. The data also show that the hydrolysis occurs between the β and the γ phosphorus atoms. There are two possible roles for ATP hydrolysis in the final incision: 1) hydrolysis provides energy for the incision reaction, and 2) the binding of ADP to UvrB activates the nuclease.

In conclusion, we have presented the novel finding that the UvrB-DNA complex, in the absence of UvrA, can bind to and perform the 5′ incision on the Y type structure in an ATP-dependent manner. We have shown that damage discrimination and incision by the UvrABC nuclease system occur in several steps. UvrA recognizes helical distortions and helps bring UvrB to the site of conformational change. If the distorted region contains no adduct, the UvrAB complex dissociates. However, if the altered DNA contains an adducted base, there is a slow conformational change in the protein-DNA complex (19) such that the DNA is bent, becomes unpaired, and interacts strongly with UvrB. This causes UvrA to dissociate, leaving behind a stable UvrB-DNA complex. Recognition of the UvrB-DNA complex by UvrC causes an allosteric change in the protein-DNA complex to induce an incision 3′ to the adducted base. That the 5′ incision that rapidly follows dissociation of UvrA is favored by a substrate containing a nick 3′ to the adduct indicates that the rate-limiting step in the incision process is the formation of the preincision UvrB-DNA complex and/or UvrC binding. We favor the hypothesis that UvrA must dissociate before the binding of UvrC.

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