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Characterization of the *Escherichia coli* Damage-independent UvrBC Endonuclease Activity*

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Geri F. Moolenaar, Merlijn Bazuine, Ingeborg C. van Knippenberg, Rob Visse, and Nora Goosen‡

From the Laboratory of Molecular Genetics, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, 2300 RA Leiden, The Netherlands

Incision of damaged DNA templates by UvrBC in *Escherichia coli* depends on UvrA, which loads UvrB on the site of the damage. A 50-base pair 3′ prenicked DNA substrate containing a cholesterol lesion is incised by UvrABC at two positions 5′ to the lesion, the first incision at the eighth and the second at the 15th phosphodiester bond. Analysis of a 5′ prenicked cholesterol substrate revealed that the second 5′ incision is efficiently produced by UvrBC independent of UvrA. This UvrBC incision was also found on the same substrate without a lesion and, with an even higher efficiency, on a DNA substrate containing a 5′ single strand overhang. Incision occurred in the presence of ATP or ADP but not in the absence of cofactor. We could show an interaction between UvrB and UvrC in solution and subsequent binding of this complex to the substrate with a 5′ single strand overhang. Analysis of mutant UvrB and UvrC proteins revealed that the damage-independent nuclease activity requires the protein-protein interaction domains, which are exclusively needed for the 3′ incision on damaged substrates. However, the UvrBC incision uses the catalytic site in UvrC which makes the 5′ incision on damaged DNA substrates.

In *Escherichia coli* nucleotide excision repair is initiated by the UvrA, UvrB, and UvrC proteins. Sequential action of these three proteins leads to two incisions in the damage-containing DNA strand, one on either side of the lesion (1–3). In solution a $UvrA_2B$ complex is formed, in which UvrA is the damage-recognizing subunit (4). After initial binding of the trimeric protein complex to the site of a DNA lesion, UvrB is loaded onto the DNA, forming a stable UvrB·DNA preincision complex (5). The actual incisions take place in a complex consisting of DNA, UvrB, and UvrC. The first incision is made at the third, fourth, or fifth phosphodiester bond 3′ of the lesion, followed by an incision at the eighth phosphodiester bond 5′ of the lesion (6). Several observations indicate that the conformation of the UvrBC·DNA complex differs for the 3′ and 5′ incision reactions. First of all, the 5′ incision can only take place when a nick is present at the 3′ incision position, introduced either enzymatically or artificially (7, 8). This strongly suggests that the DNA adopts a different conformation in the two complexes. A second observation is that the 3′ incision requires the interaction between the C-terminal domain of UvrB and a homologous internal domain of UvrC, whereas this interaction is dispensable for the 5′ incision (8, 9). Conversely, the 5′ incision requires the presence of a DNA binding domain located in the C-terminal part of UvrC, whereas this domain can be omitted for the 3′ incision (10). In the initial UvrBC·DNA complex the catalytic site for 3′ incision is most likely positioned at the scissile phosphodiester bond, incision of which triggers a transition in the complex positioning the catalytic site for the 5′ incision. The catalytic site residues responsible for 3′ incision have not yet been identified. They might be present in UvrB, in UvrC, or in both subunits. The catalytic site for 5′ incision seems to be entirely located in UvrC, because several acidic amino acids in this protein were identified to be essential (11).

It has been reported for a number of different lesions that after 5′ incision, additional cutting takes place 7 nucleotides from the normal 5′ incision site (12–14). Additional cutting was recently shown to be related to a damage-independent incision activity of the UvrABC proteins on a substrate containing a single strand-double strand junction (15). Another damage-independent incision activity of the Uvr proteins was reported by Zou *et al.* (16). This incision was observed on a specific DNA Y substrate (comparable with substrate S1 in Fig. 1*A*). In the absence of UvrA this specific DNA structure was incised by the UvrBC proteins using either ATP or ADP. The incision position was mapped three or four nucleotides 5′ to the single strand-double strand junction, and it was postulated that the UvrBC·DNA complex formed on the Y substrate is structure-specific and mimics the post-3′ incision complex on a damaged substrate (16).

We have studied Uvr(A)BC incision in damaged and non-damaged DNA using a set of defined DNA substrates of 50 base pairs. The same UvrBC nuclease activity that incises Y substrates is responsible for very efficient incision in undamaged double-stranded DNA molecules containing a nick or a 5′ single strand overhang and for the additional 5′ incision in damaged DNA. This damage-independent nuclease activity of UvrBC uses the catalytic site in UvrC that is normally responsible for the 5′ incision on damaged substrates, but in addition protein domains of UvrB and UvrC are required, which on damaged substrates are involved in 3′ incision exclusively.

EXPERIMENTAL PROCEDURES

Proteins and Antibodies—The purification of the wild-type UvrA, UvrB, and UvrC proteins (17), the UvrB* mutant (8), the UvrB mutants G509S and R544H (18), the UvrC mutant (L221P,F223L) (9), and UvrC554 (10) have been described. The clone encoding the active site mutant UvrC (D466A) was kindly provided by A. Sancar, and the protein was purified according to the procedure described for the wild-type UvrC. The polyclonal antibodies against UvrB and UvrC were raised in rabbits as described (17).

Construction of DNA Substrates—All oligonucleotides were provided by Eurogentec (Seraing, Belgium). The cholesterol "lesion" (also described in Ref. 19) is attached to a propanediol backbone instead of a nucleoside (Fig. 1*B*) and was introduced into the desired sequence by

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‡ To whom correspondence should be addressed: Laboratory of Molecular Genetics, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, P. O. Box 9502, 2300 RA Leiden, The Netherlands. Tel.: 31-71-527-4773; Fax: 31-71-527-4537; E-mail: N.Goosen@chem.Leidenuniv.nl.

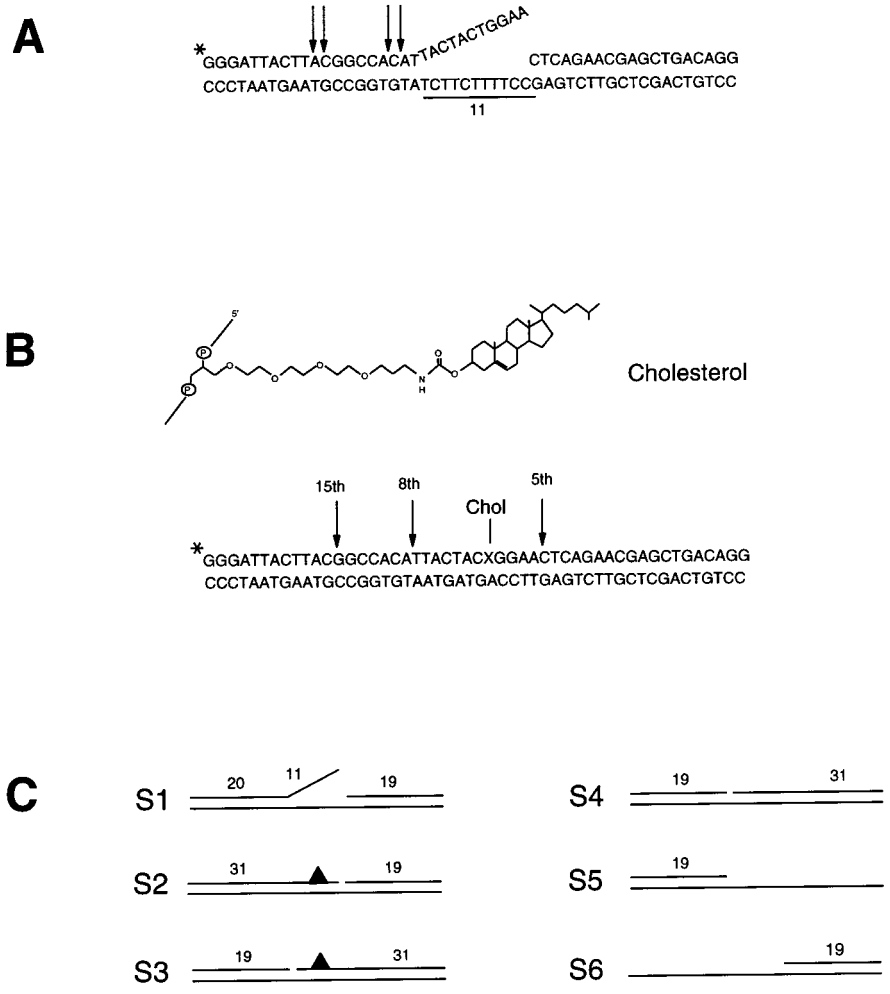

FIG. 1. **The 50-base pair DNA substrates used in this study.** *A*, DNA sequence of substrate S1. The *asterisk* indicates the 5′-terminal ^{32}P label. The *arrows* indicate the incision positions. The *underlined* 11 nucleotides of the *bottom strand* are noncomplementary with the *top strand*. *B*, DNA sequence of substrates S2–S6. The structure of the cholesterol lesion is shown. The cholesterol (*Chol*) modification is introduced at position 27 (*X*) in the *top strand*. The *arrows* indicate the incision positions. The *asterisk* indicates the 5′-terminal ^{32}P label. *C*, schematic representation of DNA substrates S1–S6. The *numbers* indicate the lengths of the different *top strand* oligonucleotides. The cholesterol lesion in *S2* and *S3* is represented by a *filled triangle*.

standard phosphoramidite chemistry at position 27 of the top strand. The presence of the cholesterol compound was verified using mass spectrometry (Eurogentec) and denaturing polyacrylamide gel electrophoresis. All "damaged" oligonucleotides contained 100% cholesterol. The purity of the nondamaged oligonucleotides was checked using denaturing gel electrophoresis. The nucleotide sequence of the Y substrate is shown in Fig. 1*A*. All other DNA substrates are of the sequences shown in Fig. 1*B*. Construction of the substrates was carried out by the following procedure. The 5′ top strand oligo (4 pmol) was terminally labeled using T4 polynucleotide kinase (10 units) in 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 5 pmol of [γ-^{32}P]ATP (7000 Ci/mmol, ICN). After incubation at 37 °C for 45 min, the reaction was terminated at 90 °C for 10 min. The labeled top strand was hybridized to the bottom strand (4 pmol), and additional top strand oligonucleotides (4 pmol each) when indicated in the presence of 50 mM NaCl. The substrate was purified by G-50 gel filtration from the nonincorporated nucleotides in 50 mM Tris-HCl (pH 8.0) and 50 mM NaCl.

Incision Assay—The DNA substrates (40 fmol) were incubated with 2.5 nM UvrA, 100 nM UvrB, and 50 nM UvrC in 20 μl of Uvr-endo buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 0.1 μg BSA/μl) containing 1 mM ATP and 85 mM KCl. The UvrBC endonuclease activity was tested using 100 nM (mutant) UvrB and 50 nM (mutant) UvrC in 20 μl of Uvr-endo buffer containing 1 mM ADP or 1 mM ATP and 85 mM KCl. After 60 min at 37 °C the incision reaction was terminated by glycogen-ethanol precipitation. The precipitated DNA was collected by centrifugation and resuspended in 10 μl of H_2O. The volumes of the samples were reduced to 2 μl using a Speedvac concentrator (Savant) and 2 μl of formamide/dyes was added. The samples were run on a 15% acrylamide gel containing 6 M urea. Incision reactions according to Gordienko and Rupp (15) were performed in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM NaCl, 2 mM ATP, and 5 mM dithiothreitol using 100 nM UvrA, 100 nM UvrB, and 100 nM UvrC. Incisions were quantified using a Betascope 603 blot analyzer (Betagen Corp., Waltham, MA). The incision frequencies are the averages of at least three independent experiments.

Bandshift Assay—The UvrBC protein-DNA complexes were formed in Uvr-endo buffer containing 95 mM KCl without cofactor or when indicated in the presence of either 1 mM ADP or 1 mM ATP in a total volume of 10 μl. After 5 min at 37 °C 1 μl of serum was added when indicated, or the samples were directly loaded on a 3.5% native polyacrylamide gel in 0.5× Tris-borate EDTA. The gel was run at room temperature at 8 mA, and the protein-DNA complexes were visualized using autoradiography.

Visualization of UvrBC Complex on Native Gel—2 μM (mutant) UvrB and 2 μM UvrC were incubated in Uvr-endo buffer containing 100 mM KCl, and after 6 min at 37 °C the incubation was loaded on a 3.5% native polyacrylamide gel (without nucleotide cofactor). After the run the gel was bound to a Whatman No. 3MM filter and stained with Coomassie Brilliant Blue R-250. The proteins were visualized after destaining, and the filter was dried.

RESULTS

The Damage-independent UvrBC Endonuclease Activity Is Responsible for the Extra 5′ Incision on Damaged DNA Substrates—Incubation of a 50-base pair double-stranded DNA substrate containing a cholesterol adduct attached to the phosphodiester backbone at position 27 in the top strand (Fig. 1*B*) with UvrABC results in incision at the fifth phosphodiester bond 3′ of the lesion and at the eighth phosphodiester bond 5′ of the lesion (results not shown). On the same cholesterol substrate containing an artificial nick at the 3′ incision position (Fig. 1*C*, substrate *S2*), the 5′ incision (producing a 19-nucleotide DNA fragment) is also efficiently induced (Fig. 2*A*, *lane 2*), confirming previous data that the 5′ incision is independent from the 3′ incision (7, 8). In addition, a 12-nucleotide incision product is observed, corresponding to an extra 5′ incision at 7 nucleotides from the first 5′ incision position. Such an addi-

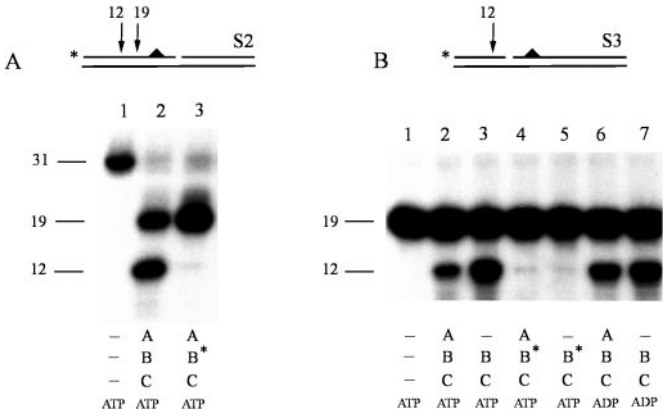

FIG. 2. **Incision of substrates S2 and S3 by Uvr(A)BC.** The 5′ end-labeled DNA substrates were incubated with the indicated proteins at concentrations of 2.5 nM (UvrA), 100 nM (UvrB or UvrB*), and 50 nM (UvrC) for 60 min at 37 °C in Uvr-endo buffer with 1 mM ADP or 1 mM ATP. The incision products were analyzed on a 15% denaturing acrylamide gel. The incision products of 19 and 12 nucleotides are indicated. *A*, incision of substrate S2. *B*, incision of substrate S3.

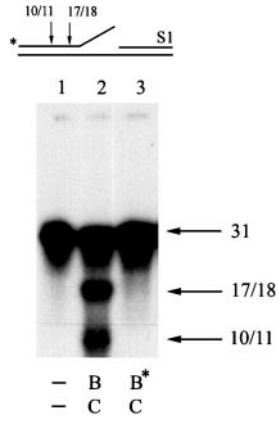

FIG. 3. **Incision of substrate S1 by UvrBC.** The 5′ end-labeled S1 substrate was incubated with 100 nM UvrB (*lane 2*) or UvrB* (*lane 3*) and 50 nM UvrC for 30 min at 37 °C in Uvr-endo buffer and 1 mM ATP. The incision products were analyzed on a 15% denaturing acrylamide gel. The uncut oligonucleotide (*31*) and the products from the first incision (*17/18*) and the second incision (*10/11*) are indicated.

tional 5′ incision has been reported for a variety of DNA lesions (12–14). UvrB*, a truncated form of UvrB lacking the C-terminal domain, was shown to be defective in 3′ incision on a damaged DNA substrate but fully proficient in 5′ incision (8). Incubation of substrate S2 with UvrA, UvrB*, and UvrC again results in an efficient first 5′ incision but the second 5′ incision is extremely low (Fig. 2*A*, *lane 3*). With respect to the UvrB protein, apparently the requirements for the first and second 5′ incision events are different. To study the additional 5′ incision reaction independently from the first 5′ incision, substrate S3 was constructed having an artificial nick at the position of the first 5′ incision site (Fig. 1*C*). When substrate S3 is incubated with UvrABC, the 12-nucleotide incision product is observed, indicating that the first 5′ incision event itself is not needed for induction of the second 5′ incision (Fig. 2*B*, *lane 2*). Again only a trace amount of incision (<1%) is observed when wild-type UvrB is replaced by UvrB* (Fig. 2*B*, *lane 4*). No incision is detected with UvrC or UvrB* alone (results not shown). When substrate S3 is incubated with only UvrB and UvrC, the 12-nucleotide incision product is also formed and with an even higher efficiency (30%) than in the presence of UvrA (13%; Fig. 2*B*, *lanes 2* and *3*). This means that the second 5′ incision is induced by a UvrA-independent UvrBC-associated nuclease. Also the UvrB*C complex induces residual amount of incision in the absence of UvrA (Fig. 2*B*, *lane 5*).

A UvrA-independent UvrBC endonuclease activity has also been demonstrated on a specific Y DNA substrate (16), in this paper referred to as substrate S1 (Fig. 1, *A* and *C*). We constructed a similar Y substrate, and incubation of this substrate with UvrBC results in two incision products (Fig. 3, *lane 2*). Subsequent analysis on a high resolution gel (results not shown) reveals that the upper band consists of two fragments of 17 and 18 nucleotides corresponding to incisions events at the third and second phosphodiester bonds 5′ to the single strand-double strand junction, respectively, which is comparable with the results already described (16), although the incision frequency in our assay seems much higher. The lower band also consists of two fragments of 10 and 11 nucleotides representing a second 5′ incision event 7 nucleotides 5′ to the first incision sites. The positions of these second incision events are similar to the position of the additional incision in substrates S2 and S3. The additional 5′ incisions on the Y substrate were not reported by Zou *et al.* (16), but because in their assay the frequency of the first incision was much lower, the second incision was probably below the level of detection. When the S1 substrate is incubated with UvrB* and UvrC, no incision products are observed (Fig. 3, *lane 3*), indicating that the UvrC binding domain of UvrB is essential for the structure-specific incision by UvrBC. Likewise a UvrC mutant carrying two substitutions (L221P,F223L), which were shown to abolish the interaction of UvrC with the C-terminal part of UvrB (9), is equally disturbed in incision of the S1 substrate (results not shown). This demonstrates that the structure-specific incision of the Y substrate requires the same protein-protein interaction between UvrB and UvrC as was found for the extra 5′ incision event on damaged DNA.

To further investigate the similarities of UvrBC-induced incision on the Y substrate (S1) and the UvrBC-induced extra 5′ incision on damaged substrates, we assayed substrate S3 in the presence of ATP or ADP (Fig. 2*B*). It has been shown before (16) that both ATP and ADP can facilitate the UvrBC incision of the Y substrate, albeit ADP with lower efficiency (77% relative to ATP). Incision of substrate S2 is UvrA- and ATP-dependent (results not shown), which is expected because the first 5′ incision requires the UvrA- and ATP-dependent formation of the preincision complex. Comparable with the Y substrate, incision of substrate S3 is UvrA-independent and also occurs in the presence of ADP (Fig. 2*B*, compare *lanes 2* and *3* with *6* and *7*). With S3 also the efficiency in the presence of ADP is somewhat lower (24%) compared with that with ATP (30%). Taken together these results strongly indicate that the UvrBC incision reaction on a Y substrate without damage and the extra 5′ incision reaction on a damaged substrate are identical.

Substrate Requirements for UvrBC Incision—To test whether the presence of a DNA lesion in S3 is essential for the extra 5′ incision, a similar undamaged substrate was constructed (Fig. 1*C*, substrate *S4*). This substrate is incised by the UvrBC endonuclease in the presence of ATP (43%) and ADP (40%) (Fig. 4*A*, *lanes 5* and *6*), which is even more efficient than with the damaged DNA substrate S3 (30 and 24%, respectively). Apparently the DNA lesion is inhibiting rather than stimulating the incision activity of the UvrBC nuclease.

We also constructed substrate S5, which lacks the 3′ top strand 31 mer (Fig. 1*C*). As shown in Fig. 4*A* (*lanes 2* and *3*), incision of this substrate is very efficient (65% in the presence of ATP and 60% in the presence of ADP) and significantly higher than either of the nicked substrates. This shows that DNA containing a single strand-double strand junction is better recognized by the UvrBC endonuclease than nicked DNA; therefore, substrate S5 was used in all further experiments.

Recently a damage-independent incision on DNA substrates

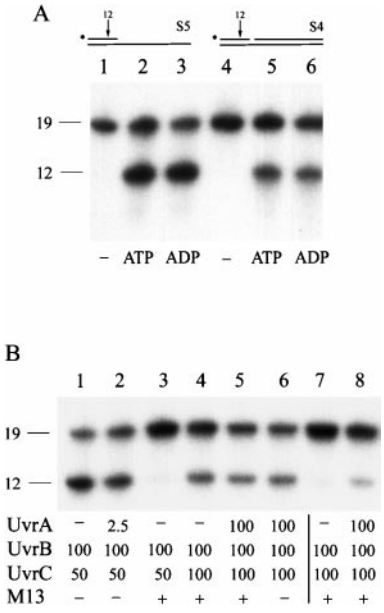

FIG. 4. **Incision of substrates S4 and S5 by Uvr(A)BC.** The substrates were 5′ end-labeled in the *top strand*. *A*, substrates S4 (*lanes 4–6*) and S5 (*lanes 1–3*) were incubated with 100 nM UvrB and 50 nM UvrC for 60 min at 37 °C in Uvr-endo buffer with 1 mM ATP or ADP as indicated. *B*, substrate S5 was incubated with the indicated amounts (nM) of UvrA, UvrB, and UvrC for 60 min at 37 °C in Uvr-endo buffer with 85 mM KCl and 1 mM ATP (*lanes 1–6*) or in Uvr-endo buffer with 150 mM NaCl and 2 mM ATP (*lanes 7* and *8*) in the presence or absence of 8 fmol of single-strand M13 DNA.

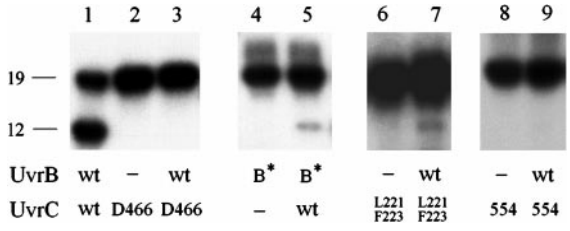

FIG. 5. **Incision of substrate S5 with (mutant) UvrBC proteins.** The 5′ end-labeled S5 substrate was incubated with 100 nM UvrB (*lanes 1, 3, 7,* and *9*) or UvrB* (*lanes 4* and *5*) and 50 nM UvrC (*lanes 1* and *5*) or UvrC (D466A) (*lanes 2* and *3*) or UvrC (L221P,F223L) (*lanes 6* and *7*) or UvrC554 (*lanes 8* and *9*) for 60 min at 37 °C in Uvr-endo buffer with 1 mM ATP. *wt*, wild type.

containing a single-stranded-double-stranded junction also has been reported (15). The substrates used in these experiments were short oligonucleotides (25 or 26 nucleotides long) annealed to single-stranded M13 DNA circles. Comparable with our damage-independent UvrBC incision activity, the incision in these substrates took place at 7 nucleotides from the 3′ terminus of the oligonucleotide. In contrast to our results, however, the incision was dependent on UvrA and occurred with a much lower efficiency (1–5%). The assay reported by Gordienko and Rupp (15) differs from ours in the presence of a relatively large amount of single-stranded DNA (M13) and different protein and salt concentrations. Addition of a comparable amount of M13 single-stranded DNA (8 fmol) to our incubation mixture indeed results in a severe drop in the UvrBC nuclease activity (Fig. 4*B*, compare *lanes 1* and *3*). This inhibitory effect of single-stranded DNA seems to be caused by the sequestering of UvrC protein from the incision reaction, because the use of a higher concentration of UvrC partly restores the UvrBC nuclease activity (Fig. 4*B*, *lane 4*). With our incubation conditions, however, no stimulatory effect of UvrA, either in the presence (Fig. 4*B*, *lanes 4* and *5*) or absence (Fig. 4*B*, *lanes 1, 2*, and *6*) of M13 single-stranded DNA, can be detected. The presence of UvrA rather seems to inhibit incision (Fig. 4*B*, compare *lanes 1* and *5*). Using the incubation conditions described by Gordienko and Rupp (15) (*i.e.* 150 mM NaCl instead of 85 mM KCl), only a very low incision is obtained (Fig. 4*B*, *lane 7*), and indeed with this high salt concentration the incision becomes UvrA-dependent (Fig. 4*B*, *lane 8*). Because both UvrC and UvrA are single-stranded DNA-binding proteins, it is possible that with the conditions used, UvrA prevents the sequestering effect of the single-stranded DNA by competing with UvrC for its binding. In any case, our experiments clearly show that UvrA is not directly involved in the damage-independent incision reaction.

Protein Domains Needed for UvrBC Incision—The extra 5′ incision on the 5′-nicked cholesterol-containing substrate S3 is dependent on the presence of the UvrC binding domain in the C-terminal part of UvrB (Fig. 2*B*, *lanes 4* and *5*). Likewise the UvrBC incision on substrate S5 is disturbed when UvrB* lacking this domain is used (Fig. 5, *lane 5*). With a mutant of UvrC carrying two substitutions in the corresponding UvrB binding domain (L221P,F223L), a severely reduced damage-independent UvrBC incision is also obtained (Fig. 5, *lane 7*). Apparently, the interaction between the homologous domains of UvrB and UvrC, which is normally needed exclusively for the 3′ incision in damaged DNA substrates, is also involved in the damage-independent UvrBC nuclease activity. To determine whether UvrBC uses the catalytic site for 3′ or 5′ incision for the damage-independent incision, we also tested the activity of UvrC (D466A). UvrC (D466A) has a substitution in the catalytic site for the 5′ incision reaction, because incubation of damaged DNA substrates with UvrABC (D466A) results in a normal 3′ incision but no detectable 5′ incision (11). Incubation of substrate S5 with UvrBC (D466A) also does not show any incision activity (Fig. 5, *lane 3*). As a control the same UvrC mutant was assayed on the double-stranded cholesterol-containing fragment in the presence of UvrA and UvrB, and indeed only the 3′ incision product was produced (results not shown). This means that the damage-independent UvrBC incision activity uses the catalytic site of UvrC for 5′ incision, but in addition it requires an interaction between UvrB and UvrC, which normally has no function in 5′ incision.

The C-terminal domain of UvrC has also been found to be specifically involved in 5′ incision on damaged substrates. This domain, which is homologous to the C-terminal domain of the human ERCC1 protein, contains a helix-hairpin-helix DNA binding motif. A truncated UvrC protein lacking this C-terminal domain is disturbed in DNA binding and 5′ incision. It has been postulated that this motif is needed to position the catalytic site for the 5′ incision reaction (10). As shown in Fig. 5, *lane 9*, UvrC554 is also not able to incise substrate S5, suggesting that the function of the C-terminal domain in the damage-independent incision is the same as for the 5′ incision.

Formation of the UvrBC Protein-DNA Complex—It has been shown that incubation of a Y substrate with UvrB and UvrC results in the formation of a distinct protein-DNA complex in a bandshift gel (16). In these studies the presence of UvrB in the complex was confirmed, but the presence of the UvrC protein could not be determined. We assayed UvrBC·DNA complex formation on substrate S5 and used UvrB and UvrC antibodies to identify the proteins in the complex. In Fig. 6 a protein-DNA complex is shown, which is formed in the presence of both UvrB and UvrC (*lane 4*) but not with either protein alone (*lanes 2* and *3*). Although there is some reaction of the DNA with the preserum (*lane 5*), the protein-DNA complex is clearly specifically retarded in the gel when either antibodies against UvrB (*lane 6*) or antibodies against UvrC (*lane 7*) are added, showing that indeed both proteins are present in the complex. The UvrB*

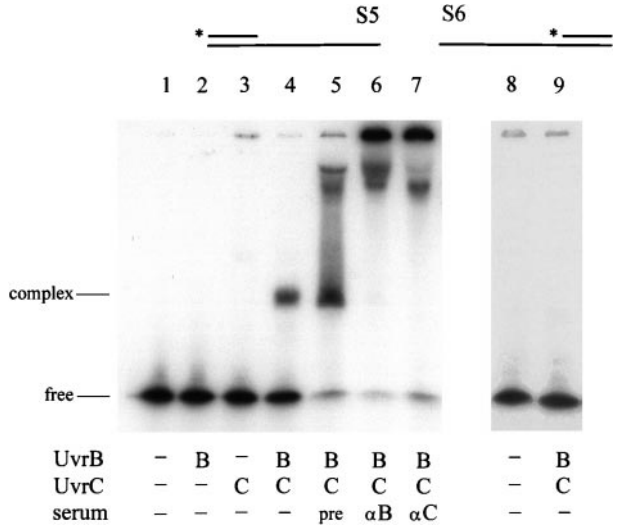

FIG. 6. **Complex formation of UvrBC with substrates S5 and S6.** Substrate S5 (*lanes 1–7*) and substrate S6 (*lanes 8* and *9*) were incubated with or without 100 nM UvrB and 50 nM UvrC as indicated for 5 min at 37 °C in Uvr-endo buffer without nucleotide cofactor. Next, 1 μl of preserum (*lane 5*), anti-UvrB serum (*lane 6*), anti-UvrC serum (*lane 7*) was added, and the mixture was loaded on a 3.5% polyacrylamide native gel.

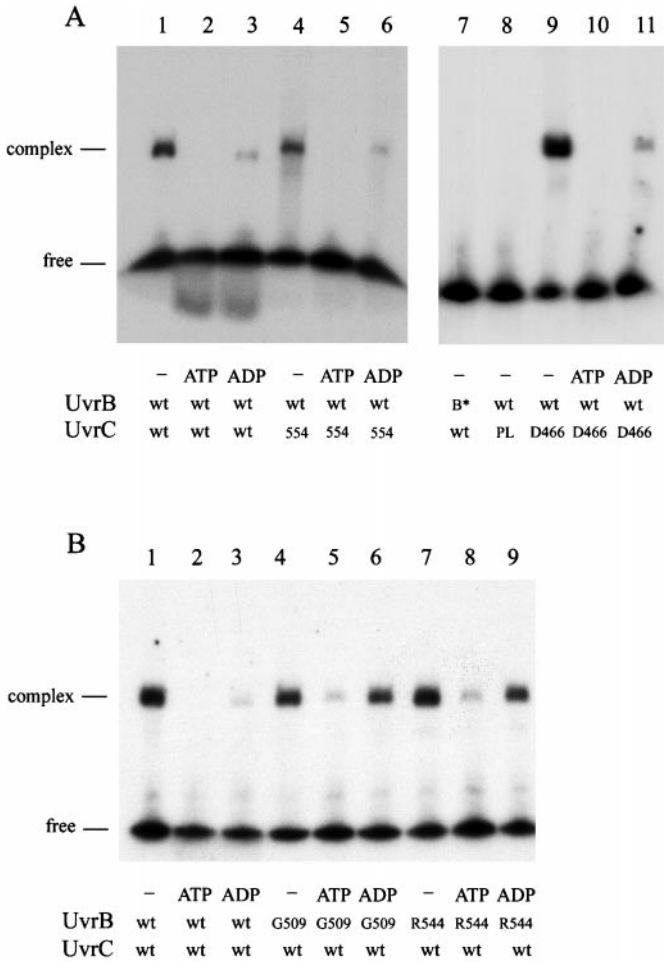

FIG. 7. **Complex formation of (mutant) UvrBC with substrate S5 in the presence or absence of nucleotide cofactor.** *A*, substrate S5 was incubated with 100 nM UvrB (*lanes 1–6,* and *8–11*) or UvrB* (*lane 7*) and 50 nM UvrC (*lanes 1–3* and *7*), UvrC554 (*lanes 4–6*), UvrC (L221P,F223L) (*lane 8*), or UvrC (D466A) (*lanes 9–11*), and 1 mM ADP or ATP as indicated. *B*, substrate S5 was incubated with 100 nM UvrB (*lanes 1–3*), UvrB (G509S) (*lanes 4–6*), or UvrB (R544H) (*lanes 7–9*) and 50 nM UvrC (*lanes 1–9*) with 1 mM ADP or ATP as indicated. The complexes were analyzed on a 3.5% polyacrylamide native gel. *wt*, wild type.

protein and the UvrC (L221P,F223L) mutant do not give rise to the UvrBC·DNA complex under the same bandshift conditions (Fig. 7*A*, *lanes 7* and *8*), showing the importance of these UvrB-UvrC protein interaction domains. In contrast, the active site mutant of UvrC (D466A) does still form the complex (Fig. 7*A*, *lane 9*), indicating that its defect in UvrBC nuclease activity is indeed attributable to the inability to catalyze the incision reaction itself and not to a defect in substrate binding. UvrC554 is also able to form the protein-DNA complex (Fig. 7*A*, *lane 4*), but the "smearing" of the complex band indicates the UvrBC554 binding is somewhat less stable then in the wild-type complex. This means that if the helix-hairpin-helix motif of UvrC binds DNA in the complex, this interaction only marginally contributes to the stability of the complex, but it seems absolutely essential for the incision event.

The specificity of DNA binding was tested further using substrate S6 (Fig. 1*C*). This DNA substrate contains the same lengths of single- and double-stranded DNA as substrate S5, but the single strand overhang is on the 3′ side. No UvrBC-induced incision can be found using this substrate, neither in the presence nor in the absence of ADP or ATP (results not shown), and in accordance, on the bandshift gel no specific protein-DNA complex is detectable (Fig. 6, *lane 9*). Apparently UvrBC is not merely binding to the single- or double-stranded part of the DNA substrate, but it seems capable of specifically recognizing the 3′ end of the single strand-double strand junction.

Interaction between UvrB and UvrC in the Absence of DNA—From the bandshift assay described above it is clear that in the UvrBC·DNA complex the UvrB and UvrC proteins make contact via their homologous domains. To find out whether this protein-protein interaction is induced by the DNA or whether it also occurs in solution, a "bandshift" experiment was done without addition of the DNA substrate in the reaction mixtures and after electrophoresis the protein was stained with Coomassie Brilliant Blue.

When UvrC alone is loaded on the gel, the protein becomes visible as a Coomassie Brilliant Blue-stained band in the slot, indicating that UvrC is not able to migrate into the gel (Fig. 8, *lane 2*). UvrC has a calculated pI of 9.88; therefore, in this gel system (pH 8.5) it is not expected to be negatively charged. UvrB (pI 4.99) does migrate into the gel (Fig. 8, *lane 1*). When both UvrB and UvrC are present, the UvrB no longer enters the gel, indicating that it is retained in the slot by UvrC in a UvrB·UvrC complex (Fig. 8, *lane 3*). That this retention is indeed indicative for specific complex formation could be shown by repeating the experiment with UvrB*. The UvrB* protein migrates into the gel both in the absence (Fig. 8, *lane 4*) and in the presence of UvrC (Fig. 8, *lane 5*). Apparently, also in the absence of DNA a UvrBC complex is formed, via interaction of the C-terminal domain of UvrB and the homologous domain of UvrC.

Role of the Nucleotide Cofactor in UvrBC Incision—The damage-independent UvrBC incision can take place only in the presence of ATP or ADP but not without a cofactor (Fig. 4*A*). Likewise it has been shown that incision of a Y substrate can occur in the presence of ATP or ADP but not with a nonhydrolysable form of ATP (16). Apparently the ADP-bound form of the UvrBC complex is the active one for incision.

Kinetic analysis of the incision on substrate S5 shows that incision is very fast: in the presence of ATP 40% incision is reached within 2 min at 37 °C (Fig. 9*A*, *lane 2*). Incision also takes place at room temperature, which is as efficient as obtained at 37 °C (Fig. 9*A*, compare *lanes 10* and *5*). During the

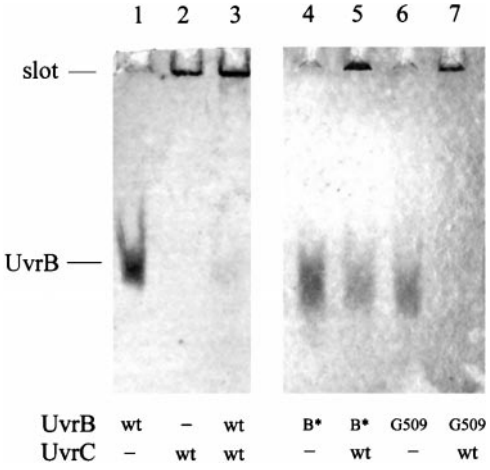

FIG. 8. **Analysis of UvrBC complex formation.** 2 μM UvrB (*lanes 1* and *3*), UvrB* (*lanes 4* and *5*), or UvrB (G509S) (*lanes 6* and *7*) was incubated with (*lanes 2, 3, 5,* and *7*) or without (*lanes 1, 4,* and *6*) 2 μM UvrC for 6 min at 37 °C in Uvr-endo buffer with 100 mM KCl. The incubation mixtures were loaded on a 3.5% polyacrylamide native gel. After the run the gel was bound to a Whatman No. 3MM filter, and the proteins were stained with Coomassie Brilliant Blue R-250. *wt*, wild type.

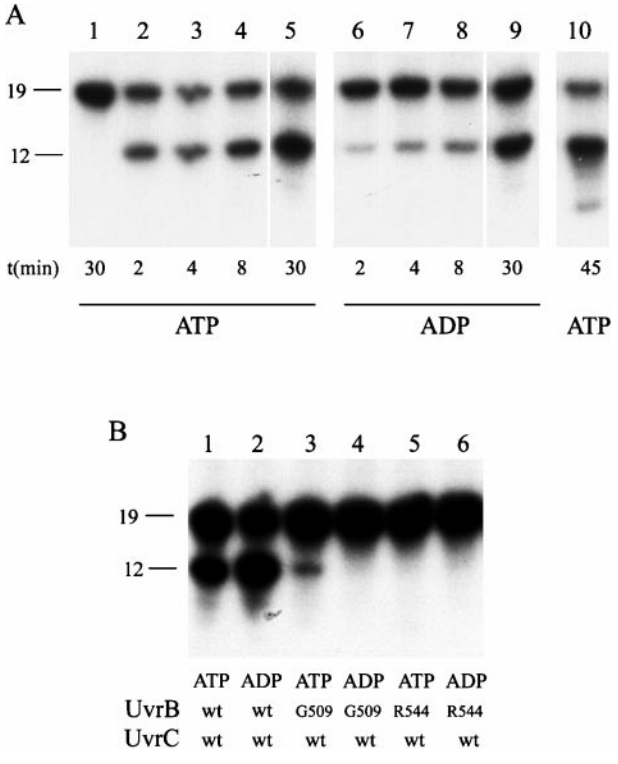

FIG. 9. **Incision of substrate S5 by (mutant) UvrBC nuclease.** *A*, substrate S5 was incubated with 100 nM UvrB and 50 nM UvrC in Uvr-endo buffer with 1 mM ATP (*lanes 1–5,* and *10*) or 1 mM ADP (*lanes 6–9*) at 37 °C (*lanes 1–9*) or at room temperature (*lane 10*). After incubation during the indicated times the incision products were analyzed on a 15% denaturing polyacrylamide gel. *B*, substrate S5 was incubated with 100 nM UvrB (*lanes 1* and *2*), UvrB (G509S) (*lanes 3* and *4*), or UvrB (R544H) (*lanes 5* and *6*) and 50 nM UvrC for 60 min at 37 °C in Uvr-endo buffer with 1 mM ATP (*lanes 1, 3,* and *5*) or 1 mM ADP (*lanes 2, 4,* and *6*). *wt*, wild type.

normal nucleotide excision repair reaction ATP hydrolysis of UvrB is induced by UvrA and damaged DNA, but apparently the hydrolysis can also be very efficiently induced by UvrC and nondamaged DNA, even at room temperature. In the presence of ADP incision is much slower, 10% after 2 min, and after 30 min the incision reaches a level that is slightly reduced (45%; Fig. 9*A*, *lane 9*) compared with that found with ATP (67%; Fig. 9*A*, *lane 5*). After 60 min of incubation no difference in incision is observed anymore (Fig. 4*A*, *lanes 2* and *3*). This difference in kinetics probably reflects a lower binding affinity of UvrB for ADP compared with ATP.

In the bandshift assay as described in Fig. 6, neither ADP nor ATP was present in the incubation mixture or in the gel onto which the complexes were loaded. Because for incision the presence of a cofactor is essential, we also tested the UvrBC·DNA complex formation using ATP or ADP in the incubation mixture (but not in the gel or gel buffer). Substrate S5 was incubated with UvrB, UvrC, and cofactor for 5 min at 37 °C before loading on the bandshift gel. Fig. 7*A* shows that in the presence of ATP (*lane 2*) and also in the presence of ADP, albeit to a lesser extent (*lane 3*), the UvrBC·DNA complex dissociates. At the same time a band below the unbound DNA becomes apparent, indicating that incision has taken place. However, the UvrBC·DNA complexes of the UvrC active site mutant D466A (Fig. 7*A, lanes 10* and *11*) and of mutant UvrC554 (Fig. 7*A, lanes 5* and *6*) also dissociate in the presence of ATP or ADP, showing that it is not the incision itself that destabilizes the complex but the binding of the cofactor. The interaction between UvrB and UvrC in solution occurs both in the absence of nucleotide cofactor and in the presence of ADP or ATP (Fig. 6 and results not shown), indicating that cofactor binding does not influence the UvrB-UvrC interaction but, rather, the interaction of the UvrBC complex with the DNA.

Because in the UvrBC·DNA complex ATP is hydrolyzed to ADP, we can not yet conclude whether it is only the ADP-bound UvrB that causes destabilization of the UvrBC·DNA complex or also the ATP-bound form. Two different UvrB ATPase mutants were analyzed for protein complex formation and UvrBC-induced incision. Mutant G509S, with a substitution in helicase motif V of UvrB, has been shown to induce ATP hydrolysis at a reduced level (28% of wild type), and mutant R544H, with a substitution in helicase motif VI, has been shown to be completely defective in ATP hydrolysis (18). As a consequence, neither of these UvrB mutants is capable of forming a preincision complex on damage-containing double-stranded DNA fragments or of inducing incision on these same substrates. Both in the absence and in the presence of nucleotide cofactor the two UvrB mutants can bind to UvrC in solution (Fig. 8 and results not shown). In the absence of cofactor both UvrB mutants also form a UvrBC·DNA complex with substrate S5 (Fig. 7*B*, *lanes 4* and *7*). In the presence of ATP the complexes of both ATPase mutants dissociate (Fig. 7*B*, *lanes 5* and *8*). Because mutant R544H is fully deficient in ATPase activity, this means that also the ATP-bound form of UvrB destabilizes the UvrBC·DNA interaction. In the presence of ADP the UvrBC·DNA complexes of the ATPase mutants are stable (Fig. 7*B*, *lanes 6* and *9*), suggesting that the mutant UvrB proteins bind ADP very poorly or not at all. In accordance with this the two UvrB mutants do not show any UvrBC incision of substrate S5 in the presence of ADP (Fig. 9*B*, *lanes 4* and *6*), whereas mutant G509S, which still has residual ATPase activity, does partly incise S5 in the presence of ATP (Fig. 9*B*, *lane 3*). The inability of mutant R544H to hydrolyze ATP and to catalyze incision (Fig. 9B, *lane 5*) confirms the observation by Zou *et al*. (16) that incision can only take place when ADP and not ATP is bound to UvrB.

In summary, our results indicate that in the absence of nucleotide cofactor the UvrBC complex, which is already formed in solution, can form a stable complex with the S5 substrate. Whether the protein-DNA interactions in this complex are mediated via UvrB, UvrC, or both is still not clear. The binding of ATP (or ADP) to UvrB induces a conformational

change in the protein complex in such a way that the interaction with the DNA is less stable. In solution the complex is expected to still be present, because in the presence of cofactor incision takes place. After entry of the complex in the bandshift gel, however, dissociation occurs probably as a consequence of the propensity of the UvrBC-protein complex not to migrate into the gel. For incision to occur ATP needs to be hydrolyzed, implicating another conformational change, which probably positions the active site of UvrC at the incision site.

DISCUSSION

In this paper we have shown that in the absence of both DNA damage and UvrA, UvrBC efficiently incises a double-stranded DNA molecule containing a 5′ single strand overhang (S5) and, with a somewhat lower efficiency, a double-stranded DNA molecule containing a nick in one strand (S4). This damage-independent incision appears to be responsible for the additional 5′ cutting that takes place after dual incision of damaged DNA. The same UvrBC nuclease activity was also shown to be responsible for the structure-specific incision of a Y structure (Ref. 16 and this paper).

In the absence of cofactor UvrBC forms a stable complex with substrates S5 and and S4 (this paper and results not shown). Substrate S6, which differs from S5 in the polarity of the double strand-single strand junction is not bound by UvrBC at all, suggesting that the presence of a 3′ end at a double strand-single strand junction, even if this junction is merely the result of a nick, is important for substrate recognition. The Y substrate, which also forms a stable complex with UvrBC (Ref. 16 and results not shown), however, does not contain such a 3′ end. There are no obvious other common denominators in the three substrates that can explain the binding specificity of the UvrBC complex. Possibly in S4 and S5 the 3′ end of the junction forms the initial recognition site, and next, as a consequence of the UvrBC binding, the DNA might adopt a structure similar to that of the Y substrate. The Y substrate, already having this structure, would then "fit" right in the UvrBC complex and might therefore not need the initial recognition site. Although the requirements for UvrBC incision of substrates S4 and S5 very much resemble those for the incision of the Y substrate, the position of the incision site is different. With substrates S4 and S5 incision takes place at 7 nucleotides from the 3′ terminus of the strand, whereas with the Y substrate the incision positions are located 13 and 14 nucleotides from the 3′ end. This implies that the incision position is not dictated by the terminus of the strand to be incised but more by the structure of the DNA in the complex. In the Y substrate incision is in the double-stranded portion of the molecule at the second and third phosphodiester bonds 5′ to the double strand-single strand junction. If indeed binding of UvrBC to S4 and S5 results in partial unwinding of the two DNA strands, this would create a double strand-single strand junction that subsequently can be incised at a similar position as in the Y substrate.

The UvrBC damage-independent incision uses the same catalytic site that on damaged DNA induces the 5′ incision. In addition it requires the presence of the C-terminal DNA binding domain of UvrC, which has also been shown to be specifically essential for the 5′ incision event on damaged DNA (10). These common features argue that there might be structural similarities between the UvrBC·DNA complex, which incises nondamaged DNA, and the UvrBC·damaged DNA complex, in which the 5′ incision takes place. Incision of the undamaged DNA requires that UvrB in the complex is associated with ADP. For incision of damaged supercoiled plasmid DNA, it has been shown that addition of UvrC to purified UvrB·DNA preincision complexes results in incision of the DNA only when ATP or ATPγS is present in the incubation mixture but not with ADP (21). This would suggest that incision of damaged DNA requires the ATP-bound form of UvrB. In these studies, however, incision was monitored by the conversion of supercoiled to relaxed DNA. Because the induction of the 3′ incision alone would also generate relaxed DNA, it can therefore only be concluded that the 3′ incision event requires ATP. It is very well possible that after the 3′ incision this ATP is hydrolyzed and that subsequently the 5′ incision is induced by the ADP-bound form of UvrB, as for the UvrBC cutting of undamaged DNA.

An important difference between the incision of undamaged DNA and the 5′ incision is the role of the C-terminal domain of UvrB. The presence of this domain is essential for the damage-independent reaction but not for the damage-dependent incision event. We favor the hypothesis that the UvrC binding domain is not required for the incision of the undamaged DNA itself but for the loading of UvrB, in complex with UvrC, onto the DNA. In damaged DNA this loading is achieved by UvrA, for which the UvrC binding domain can be omitted. For the loading of UvrBC onto undamaged DNA, it seems that UvrB and UvrC first form a complex in solution for which the interaction between the C-terminal domain of UvrB and its homologous domain in UvrC is required.

The same interaction between the homologous domains of UvrB and UvrC has previously been shown to be important for the specific binding of UvrC to the UvrB·DNA preincision complex and the subsequent 3′ incision (8). Binding of UvrC to a UvrAB·DNA complex could not be demonstrated. It was postulated that the conformational change in UvrB that accompanies the formation of the preincision complex exposes the C-terminal domain of UvrB for UvrC to bind to (18). It is now clear, however, that UvrC can also bind to UvrB when it is not in the preincision complex. The inability of UvrC to bind to a UvrAB·DNA complex is therefore more likely the result of shielding the UvrC binding domain of UvrB by UvrA. In agreement with this, it has been found that a maltose-binding protein fusion with the last 126 amino acids of UvrB binds not only to UvrC but also to UvrA (20). If indeed the binding of UvrB to either UvrA or UvrC is mutually exclusive, this explains why in our assays UvrA inhibits the UvrBC nuclease activity.

In the presence of ADP or ATP the UvrBC·DNA complex is less stable, and as a result it can no longer be detected in a gel retardation assay. This does not necessarily mean that the complex no longer exists in solution. A Coomassie Brilliant Blue-stained gel shows that in the absence of DNA the UvrBC complex is retained in the slot, probably as a result of a lack of negative charge of the UvrC protein at the pH of the gel buffer. Therefore, only when firmly bound to the DNA can the complex profit from the negative charge of the DNA to enter the gel. When this interaction is much weaker, as a result of a conformational change induced by the nucleotide cofactor, the DNA is expected to be stripped from the complex during electrophoresis. Destabilization not only occurs in the ADP-bound complex but also in the ATP-bound form, as was shown with ATPase mutants of UvrB. Because only the ADP-bound complex seems incision-proficient, this implies that ATP hydrolysis induces yet another conformational change, which probably positions the catalytic residues at the incision site.

What, if any, might be the *in vivo* function of the observed damage-independent UvrBC incision? Because it is clear that UvrA inhibits the UvrBC incision by competing with UvrC for UvrB binding, an *in vivo* function can only be expected if in the cell there is an excess of UvrB molecules with respect to UvrA dimers. Several conflicting reports on the amount of Uvr proteins in the cell have appeared. In unirradiated cells the

amounts of molecules per cell have been reported to be 20 molecules of UvrA and 150–200 molecules of UvrB (22), 1000 molecules of UvrA and 250 molecules of UvrB (23), and 200 molecules of UvrA and 400 molecules of UvrB (24). In induced cells the determined quantities were 2200 molecules of UvrA and 1500 molecules of UvrB (23) and 1200 molecules of UvrA and 2000 molecules of UvrB (24). If the values as determined by Crowley and Hanawalt (24) are correct, it should be possible to form UvrBC complexes in the cell, both under induced and noninduced conditions.

In this paper we have shown that the damage-independent UvrBC nuclease is responsible for the induction of additional 5′ incisions on damaged substrates. It has been postulated that the function of these additional incisions is to generate a gap upstream from the DNA lesion, which can subsequently be used as entry site for RecA-mediated recombination repair (15). This could in particular be important for the repair of interstrand cross-links and of closely opposed lesions. A possible function of the UvrBC nuclease in processes other than DNA repair, however, should also be considered. Interesting in this respect is the observation already made a long time ago that the combination of a mutation in the *uvrB* gene and a mutation in the *polA* gene is lethal to the cell (25), suggesting a possible role of UvrB in DNA replication. Whether this role is associated with the UvrBC nuclease activity described in this paper awaits the analysis of the viability of a Δ*uvrC, polA* double mutant.

REFERENCES

1. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) *DNA Repair and Mutagenesis*, ASM Press, Washington, D. C.
2. Sancar, A. (1996) *Annu. Rev. Biochem.* **65,** 43–81
3. Goosen, N., Moolenaar, G. F., Visse, R., and van de Putte, P. (1998) in *Nucleic Acids and Molecular Biology: DNA Repair* (Eckstein, F., and Lilley, D. M. J., eds), pp. 103–123, Springer Verlag, Berlin
4. Yeung, A. T., Mattes, W. B., Oh, E. Y., Yoakum, G. H., and Grossman, L. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 6157–6161
5. Orren, D. K., and Sancar, A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5237–5241
6. Van Houten, B. (1990) *Microbiol. Rev.* **54,** 18–51
7. Lin, J.-J., Phillips, A. M., Hearst, J. E., and Sancar, A. (1992) *J. Biol. Chem.* **267,** 17693–17700
8. Moolenaar, G. F., Franken, K. L. M. C., Dijkstra, D. M., Thomas-Oates, J. E., Visse, R., van de Putte, P., and Goosen, N. (1995) *J. Biol. Chem.* **270,** 30508–30515
9. Moolenaar, G. F., Franken, K. L. M. C., van de Putte, P., and Goosen, N. (1997) *Mutat. Res.* **385,** 195–203
10. Moolenaar, G. F., Schoot Uiterkamp, R., Zwijnenburg, D. A., and Goosen, N. (1998) *Nucleic Acids Res.* **26,** 462–468
11. Lin, J.-J., and Sancar, A. (1992) *J. Biol. Chem.* **267,** 17688–17692
12. Visse, R., de Ruijter, M., Brouwer, J., Brandsma, J. A., and van de Putte, P. (1991) *J. Biol. Chem.* **266,** 7609–7617
13. Bertrand-Burggraf, E., Selby, C. P., Hearst, J. E., and Sancar, A. (1991) *J. Mol. Biol.* **219,** 27–36
14. Van Houten, B., Gamper, H., Sancar, A., and Hearst, J. E. (1987) *J. Biol. Chem.* **262,** 13180–13187
15. Gordienko, I., and Rupp, W. D. (1998) *EMBO J.* **17,** 626–633
16. Zou, Y., Walker, R., Bassett, H., Geacintov, N. E., and Van Houten, B. (1997) *J. Biol. Chem.* **272,** 4820–4827
17. Visse, R., de Ruijter, M., Moolenaar, G. F., and van de Putte, P. (1992) *J. Biol. Chem.* **267,** 6736–6742
18. Moolenaar, G. F., Visse, R., Ortiz-Buysse, M., Goosen, N., and van de Putte, P. (1994) *J. Mol. Biol.* **240,** 294–307
19. Matsunaga, T., Mu, D., Park, C.-H., Reardon, J. T., and Sancar, A. (1995) *J. Biol. Chem.* **270,** 20862–20869
20. Hsu, D. S., Kim, S.-T., Sun, Q., and Sancar, A. (1995) *J. Biol. Chem.* **270,** 8319–8327
21. Orren, D. K., and Sancar, A. (1990) *J. Biol. Chem.* **265,** 15796–15803
22. Sancar, A., and Sancar, G. B. (1988) *Annu. Rev. Biochem.* **57,** 29–67
23. Lin, C.-L. G., Kovalsky, O., and Grossman, L. (1997) *Nucleic Acids Res.* **25,** 3151–3158
24. Crowley, D. J., and Hanawalt, P. C. (1998) *J. Bacteriol.* **180,** 3345–3352
25. Shizuya, H., and Dykhuizen, D. (1972) *J. Bacteriol.* **112,** 676–681

Characterization of the *Escherichia coli*Damage-independent UvrBC Endonuclease Activity

Geri F. Moolenaar, Merlijn Bazuine, Ingeborg C. van Knippenberg, Rob Visse and Nora Goosen

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