The Role of ATP Binding and Hydrolysis by UvrB during Nucleotide Excision Repair

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We have isolated UvrB-DNA complexes by capture of biotinylated damaged DNA substrates on streptavidin-coated magnetic beads. With this method the UvrB-DNA preincision complex remains stable even in the absence of ATP. For the binding of UvrC to the UvrB-DNA complex no cofactor is needed. The subsequent induction of 3’ incision does require ATP binding by UvrB but not hydrolysis. This ATP binding induces a conformational change in the DNA, resulting in the appearance of the DNase I-hypersensitive site at the 5’ side of the damage. In contrast, the 5’ incision is not dependent on ATP binding because it occurs with the same efficiency with ADP. We show with competition experiments that both incision reactions are induced by the binding of the same UvrC molecule. A DNA substrate containing damage close to the 5’ end of the damaged strand is specifically bound by UvrB in the absence of UvrA and ATP (Moolenaar, G. F., Monaco, V., van der Marel, G. A., van Boom, J. H., Visse, R., and Goosen, N. (2000) J. Biol. Chem. 275, 8038–8043). To initiate the formation of an active UvrBC-DNA incision complex, however, UvrB first needs to hydrolyze ATP, and subsequently a new ATP molecule must be bound. Implications of these findings for the mechanism of the UvrA-mediated formation of the UvrB-DNA preincision complex will be discussed.

Nucleotide excision repair in Escherichia coli is initiated by the binding of the UvrA2B complex to DNA containing damage. Following this, UvrB is loaded onto the site of the damage, and the UvrA protein is released. The resulting UvrB-DNA preincision complex is bound by the UvrC protein, leading to incision of the DNA at the 4th or 5th phosphodiester bond on the 3’ side of the damage. This 3’ incision is immediately followed by hydrolysis of the 5th phosphodiester bond at the 5’ side of the damage. The repair reaction is completed by the action of the UvrD, polymerase I, and ligase proteins, which replace the damaged oligo with a newly synthesized strand (for reviews see Refs. 1 and 2).

ATP binding and hydrolysis play important roles throughout the repair reaction. The function of this cofactor is quite complex, which is illustrated by the presence of five ATP-binding sites in a single UvrA2B complex, two in each UvrA subunit and one in UvrB. The dimerization of UvrA is stimulated by ATP binding but not hydrolysis (3). The formation of an (active) UvrA2B complex in solution requires the hydrolysis of ATP by UvrA (4). The ATP hydrolysis by UvrA is also an important factor in discriminating between damaged and nondamaged DNA (5). In solution the UvrB protein on its own does not hydrolyze ATP, but as part of the UvrA2B complex it displays a DNA damage-dependent ATPase activity (6). This ATPase activity is associated with a limited DNA unwinding activity (7), which was shown to be important for loading the UvrB protein onto the site of the damage (8). Finally it has been shown that binding of ATP to the UvrBC-DNA complex is important for incision (9). In the latter experiments incision was monitored by the conversion of UV-irradiated supercoiled DNA substrate to the relaxed form, and therefore it could not be determined whether ATP binding is needed for 3’ incision alone or for both incision reactions.

In this paper we take a closer look at the function of ATP binding and hydrolysis in formation of the UvrB-DNA preincision complex and formation of the UvrB-DNA incision complex and in the two incision reactions. For this purpose we have constructed biotinylated damaged DNA substrates, which are used to capture repair intermediates on streptavidin-coated magnetic beads.

EXPERIMENTAL PROCEDURES

Protein Purifications—The UvrA, UvrB, and UvrC proteins were purified as described (10). Mutant proteins UvrC (D466A) (11), UvrC(L221P+F223L) (12), UvrB(G508S), and UvrB(R544H) (8) have been described and were purified according to the same procedure as the wild type proteins.

Construction of DNA Substrates—The DNA substrates used in this study are schematically shown in Fig. 1. The cholesterol lesion was synthesized as a phosphoramidite-protected nucleoside building block as described.1 Using automated oligonucleotide synthesis this building block was directly introduced into DNA. The biotinylated oligos were purchased from Eurogentec. The bottom strands used for construction of substrates G1-bio and G2-bio contain a biotin attached to the 3’ end of the oligo. The bottom strand used for construction of G10-bio has the biotin attached to the 5’ end. For 5’ labeling 4 pmol of the appropriate oligo was incubated with 10 units of T4 polynucleotide kinase in 70 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 5 mM dithiothreitol, and 3 pmol of [γ-32P]ATP (7000 C/mmole, ICN). After incubation at 37 °C for 45 min, the reaction was terminated by incubation at 80 °C for 10 min in the presence of 20 mM EDTA. The different substrates were constructed by hybridizing 4 pmol each of the appropriate oligos in the presence of 50 mM NaCl and 1 mM EDTA. The substrates were purified from the nonincorporated nucleotides by G50 gel filtration in 50 mM Tris-HCl, pH 8.0, 50 mM NaCl.

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**Direct Incision Assay**—The DNA substrates (40 fmol) were incubated with 100 nM UvrB, 50 nM UvrC, and 2.5 nM UvrA where indicated in 20 µl of Uvr-endo buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 mM KCl, 0.1 µg/µl bovine serum albumin, and 1 mM ATP) as described (13). After the indicated times the reaction was terminated by adding 2 µM of 2 µg/µl glycogen followed by ethanol precipitation. The incubation products were analyzed on a 15% acrylamide gel containing 7 M urea.

**Isolation of UvrB-DNA Complexes with Streptavidin-coated Magnetic Beads**—The 5'-labeled biotinylated substrates G1-bio, G2-bio, or G10-bio were incubated with streptavidin-coated Dynabeads (Dynal) for 15 min at room temperature, using 10 µg of beads/40 fmol of DNA substrate in 20 µl Tris-HCl, pH 7.5, 0.5 mM EDTA, 1.0 mM NaCl. The beads were washed, first with two times with 10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA and 2 mM NaCl and next twice with 10 mM Tris-HCl, pH 7.9, 10 mM MgCl₂ and 50 mM NaCl using a magnet stand (Dynal MPC). Substrates G1-bio and G2-bio attached to the beads were incubated with 2.5 nM UvrA and 100 nM UvrB for 15 min at 37°C in Uvr-endo buffer in the presence of 1 mM ATP. The protein-DNA complexes were washed at room temperature or at 0°C as indicated, three times with 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.1 mM KCl, 0.1 µg/µl bovine serum albumin and two times with 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.1 mM KCl, 0.1 µg/µl bovine serum albumin to remove UvrA and ATP. The beads were divided into aliquots containing 40 fmol of DNA each. These aliquots were incubated with or without 1 mM nucleotide cofactor (ATP, ADP, or ATP[S]) (Roche Molecular Biochemicals) and with or without 50 nM UvrC. The G1-bio substrate was incubated at 37°C for 30 min, and the G2-bio substrate was incubated for 6 min to allow incision. Next the samples were washed with stop mix (10 mM Tris-HCl, pH 7.5, 20 mM EDTA and 2 mM NaCl) to remove the proteins. Finally 3 µl of formamide was added to the beads, and after 5 min at 90°C the mixture was loaded on a 20% acrylamide gel containing 7 M urea.

Substrate G10-bio attached to the beads was incubated for 3 min at 37°C in Uvr-endo buffer in the presence of 100 nM UvrB and 50 nM UvrC, with or without ATP as indicated. The protein-DNA complexes were washed once at room temperature with 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.1 mM KCl, 0.1 µg/µl bovine serum albumin either in the presence or absence of 50 nM UvrC. Where indicated 50 nM UvrC and 1 mM nucleotide cofactor were added, and incision was allowed for 60 min at 37°C. The reaction was terminated by glyoxal/ethanol precipitation at 37°C. The incision products were visualized on a 15% acrylamide gel containing 7 M urea.

**DNase I Footprinting**—G1-bio and G2-bio attached to the beads were incubated with UvrA and UvrB and were washed five times as described above. Aliquots of the resulting beads-bound UvrB-DNA complexes (20 µl, containing 40 fmol DNA) were incubated for 3 min at room temperature with or without 1 mM nucleotide cofactor. To each sample 1 µl of 50 mM CaCl₂, 1 µl of 60 µg/ml supercoiled pUC18 plasmid DNA, and 1 µl of DNase I was added (a 147-fold dilution of 10 units/µl in 10 mM Tris-HCl, pH 7.5, 10 mM CaCl₂, 10 mM MgCl₂, and 10% glycerol). After incubation for 10 min at 20°C the reaction was terminated by addition of an equal volume of 20 mM Tris-HCl, pH 7.5, 100 mM EDTA, 500 mM NaCl. The beads were first washed in 10 mM Tris-HCl, pH 7.5, 20 mM EDTA, and 2 mM NaCl to remove the proteins, followed by a wash with 10 mM Tris-HCl, pH 7.5, 250 mM NaCl, 20 mM EDTA to reduce the salt concentration. Finally 3 µl of formamide was added to the beads, and after 5 min at 90°C the mixture was loaded on a 20% acrylamide gel containing 7 M urea.

**Gel Retardation Assay**—The DNA substrates (40 fmol) were incubated with 100 nM UvrB, with or without 50 nM UvrC in Uvr-endo buffer containing 1 mM of the indicated cofactor. The mixture was incubated at 37°C, and the protein-DNA complexes were analyzed by loading the samples on a 3.5% native polyacrylamide gel in 0.5× Tris borate/EDTA.

**RESULTS**

**ATP Binding but Not Hydrolysis Is Required for the 5’ Incision**—To study the role of ATP in the incision reaction it is important to isolate repair reaction intermediates and to separate them from free ATP, which initially needs to be present for the activity of UvrA and UvrB. In the past this was done by purifying UvrB-DNA complexes via gel exclusion chromatography (9). The disadvantages of this method are that it is time consuming and that protein-DNA complexes may dissociate during the procedure. We have chosen a different approach by immobilizing repair intermediates on streptavidin-coated magnetic beads via coupling of biotin to a 50-mer damaged DNA substrate.

Substrate G1-bio (Fig. 1) was bound to the beads and incubated with UvrAB in UV-endo buffer with ATP, allowing formation of the UvrB-DNA complex. The free proteins and ATP were removed by washing the beads with the appropriate buffer, and the retained complex was incubated with UvrC, in the presence or absence of different cofactors (Fig. 2A). Without cofactor no incision takes place (lane 3), indicating that all the cofactor has been removed from the preincision complex during the washing procedure. Adding UvrC together with ATP resulted in incision, which is as efficient as direct incision of substrate G1-bio with UvrABC and ATP (compare lanes 1 and 4). This shows that during the washing procedure, no UvrB-DNA complex was lost, indicating that the complex is very stable even in the absence of cofactor. Addition of UvrC in the presence of the nonhydrolyzable analog ATPγS resulted in proficient incision as well (lane 6), whereas ADP only promoted residual incision (lane 5). Clearly ATP binding, but not hydrolysis is required for the 5’ incision reaction. The residual level of incision found with ADP is probably due to ATP contamination of commercially available ADP preparations.

**Both ATP and ADP Stimulate the 5’ Incision Reaction**—To study the cofactor requirements for the 5’ incision independent from the 3’ incision, we constructed substrate G2-bio containing a single strand nick at the 3’ incision position (Fig. 1). The substrate was bound to the beads and the UvrB-DNA complex was isolated as described above. Subsequent incubation with UvrC in the presence of ATP, ATPγS, or ADP promoted 5’ incision (Fig. 2B, lanes 4–6), as efficient as direct incubation of the substrate with UvrABC and ATP (lane 1). In the absence of cofactor a very low amount of incision was observed (lane 3). This could mean that, in contrast to substrate G1-bio, a residual amount of cofactor remains bound after washing. More likely, incision of the prenicked substrate can take place, albeit inefficiently, in the absence of cofactor. In any case, the results with G2-bio indicate that (i) the UvrB-DNA complex on the

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2 The abbreviation used is: ATPγS, adenosine 5’-O-(thiotriphosphate).
3'-nicked substrate is also stable in the absence of ATP and (ii) the 5' incision is greatly stimulated by the binding of a cofactor, which can be either ADP or ATP.

To exclude the possibility that the observed stability of the UvrB-DNA complexes is a consequence of trace amounts of UvrA that might remain after washing and that could reload UvrB after dissociation from the DNA, we did the following experiment. Unlabeled G2-bio substrate was bound to the beads and incubated with UvrAB, and the sample was subsequently washed to remove free protein in an identical way as in the procedure described above. Next the remaining protein-DNA complex was incubated with labeled G2 substrate together with UvrB, UvrC, and ATP with (Fig. 2C, lane 1) or without (lane 2) UvrA. In the absence of UvrA no incision of the labeled G2 substrate can be detected after this procedure, confirming that indeed the washing procedure removes all UvrA protein.

**Binding of ATP to the UvrB-DNA Complex Induces a Conformational Change in the DNA**—A common feature of UvrB-DNA preincision complexes formed on a variety of lesions is the appearance of one or more DNase I-hypersensitive sites at the 5' side of the damage (13–16). These hypersensitive sites are indicative for a local widening of the minor groove. We studied the effect of the cofactor on the appearance of this enhanced cleavage site in the UvrB-DNA complex. Substrates G1-bio and G2-bio were incubated with UvrAB and ATP, after which the free protein and the cofactor were removed by washing. The resulting UvrB-DNA complex was incubated with DNase I with or without cofactor (Fig. 3). The addition of ATP or ATPγS to the UvrB-DNA complexes clearly induce an enhanced cleavage site both in the double-stranded and in the 3'-nicked substrate (lanes 3, 4, 9, and 10). This enhanced site is at position 16, which is 11 phosphodiester bonds 5' to the damage. In the absence of cofactor or in the presence of ADP the DNase I sensitivity of this position is much less (lanes 1, 2, 6, and 7), whereas the protection of the DNA from DNase I cleavage by UvrB is the same as in the complex with ATP. Clearly there is a correlation between the appearance of the enhanced cleavage site and the 3' incision, because both need binding of ATP but not hydrolysis. Therefore, it is very likely that the specific conformation of the DNA induced by the ATP-UvrB complex is a prerequisite for the 3' incision. The prenicked substrate binding of ATP induces a similar conformation in the DNA, but because 5' incision can take place with ADP, this specific conformation apparently has no effect on the incision.

**Binding of ATP to the UvrB-DNA Complex Is Not Needed for UvrC Binding**—A possible role for the ATP-induced conformational change in the DNA might be that it is needed for stable binding of UvrC to the preincision complex. To test this possibility we analyzed UvrC binding in the presence and absence of cofactor. UvrB-DNA complexes on substrates G1-bio and G2-bio were isolated using magnetic beads as described above. Next UvrC was added to these complexes in the presence or absence of ATP and the mixture was kept on ice for 5 min, allowing UvrC to bind. Free UvrC protein was removed by a second wash, and finally incision was monitored by incubating at 37 °C with or without ATP and/or UvrC (Fig. 4). *Lanes* 1 and 3 confirm that both the first and the second wash remove the ATP from the UvrB-DNA complex. Comparable with the results of Fig. 2, no incision for G1-bio (Fig. 4A, *lanes* 1 and 3) or very little incision for G2-bio (Fig. 4B, *lanes* 1 and 3) is observed when no ATP is added to the final incubation. On the double-stranded DNA substrate UvrC binds to the UvrB-DNA complex in the absence of ATP, because addition of ATP after washing of the UvrBC-DNA complex results in incision (Fig. 4A, *lane* 5). The incision is lower than the incision after direct incubation of the UvrB-DNA complex with UvrC and ATP (lane 2). This means that some of the UvrC dissociates from the UvrB-DNA complex during the washing procedure. The UvrB complex that remains after the wash does not contain ATP, as can be seen from the lack of incision in *lane* 4, which demonstrates that the ATP-induced conformation of the UvrB-DNA complex is not essential for UvrC binding. Whether UvrC binding in the presence of ATP would be more stable could not be tested, since the subsequent washing step removes the ATP from the complex (lane 6). On the other hand this experiment shows that UvrC does not stabilize ATP binding in the UvrBC-DNA complex. With the 3'-nicked substrate UvrC binding occurred in the absence of cofactor as well (Fig. 4B, *lane* 5). This binding seems to be more stable than with the double-stranded substrate, because incision after preincubation with UvrC and...
subsequent washing was as efficient as incision after direct incubation with UvrC and ATP (Fig. 4B, lanes 2 and 5). The very low incision in lane 4 confirms the results described above that the 5’ incision is greatly stimulated by binding of the cofactor. Strikingly the 5’ incision of the 3’-nicked substrate with UvrC and ATP was readily induced at 0 °C (Fig. 4B, lane 7). The double-stranded substrate does not show any incision at 0 °C (Fig. 4A, lane 7), even though UvrC can bind at this temperature (Fig. 4A, lane 5). This probably means that association of UvrC with the preincision complex directly positions the active site for 5’ incision at the scissile phosphodiester bond, whereas for 3’ incision correct positioning of the active site requires the input of thermal energy.

**ATP Hydrolysis by UvrB Must Precede ATP Binding for 3’ Incision**—In the accompanying paper (17) we have shown that a substrate in which the bottom strand “to the left” of the 5’ incision site is missing (Fig. 1, G10) is incised by UvrBC in the absence of UvrA. Gel retardation experiments showed that UvrB on its own binds specifically to the damage of G10 with- out the need for a cofactor. This allows us to directly examine the cofactor requirement for the UvrBC incision of this substrate. In the absence of cofactor, G10 is not incised by UvrBC (Fig. 5A, lane 5). In the presence of ATP, incision occurs (lanes 2 and 6), yielding two incision products. The 31-mer is the result of an uncoupled 3’ incision, and the 19-mer stems from the 5’ incision. The uncoupled 3’ incision product most likely is the consequence of the dissociation of the protein-DNA complex before the 5’ incision could take place. We have shown that ATPase/helicase-deficient mutants of UvrB can also form a stable complex on substrate G10 (17). The same mutants that have been shown to bind ATP (19) do not display any incision in the presence of UvrC and ATP (Fig. 5A, lanes 3 and 4), indicating that the UvrBC-mediated incision of G10 requires ATP hydrolysis. This is confirmed by the observation that incubation of G10 with wtUvrBC in the presence of ADP or ATPγS does not result in any incision either (lanes 7 and 8).

To test at what stage ATP hydrolysis is important for the incision of G10, we isolated protein-DNA complexes on this substrate using the magnetic beads. For this purpose G10-bio was constructed, with a biotin attached to the 5’ end of the bottom strand (Fig. 1). First we attempted to separate UvrB-DNA complexes from the free UvrB protein with the streptavidin-coated magnetic beads in a similar way as described for G1-bio and G2-bio. The UvrB-DNA complexes of G10, however, appeared to be too unstable, either with or without cofactor, because no incision is observed after subsequent incubation with UvrC and ATP (Fig. 5B, lanes 6 and 5–8). Preincubation of G10 with UvrC and ATP followed by incubation with buffer containing UvrC protein (see “Experimental Procedures”). After the wash different cofactors were added, and the incision was monitored on a gel (Fig. 5B). Preincubation of G10 with UvrBC in the presence of ATP for 3 min does not result in detectable incision (lane 7). After washing of the complex and subsequent incubation for 60 min in the presence of ATP incision is observed (lane 5). As observed before, in addition to the 5’ incision product (31-mer) also the uncoupled 3’ incision product (31-mer) is visible. The total incision is much less than after direct incubation of G10 with UvrBC and ATP (Fig. 5A), indicating that a large part of the UvrBC-DNA complex dissociates during the washing procedure. A similar preincubation with UvrBC and ATP followed by incubation without ATP after the wash does not result in incision (lane 4), confirming that the ATP is removed from the UvrBC-DNA complex during the washing procedure. When the nonhydrolyzable ATPγS is added after the preincubation with UvrBC and ATP, a similar amount of incision is observed as with ATP (compare lanes 5 and 6). Like with the double-stranded substrate G1, incision of G10 apparently requires ATP binding and not hydrolysis. The incision in the presence of ATPγS, however, does not occur when the preincubation with UvrBC is carried out in the presence of ATPγS (lane 8). These results demonstrate that incision of G10...
by UvrBC first requires ATP hydrolysis followed by the binding of a new ATP molecule, which does not have to be hydrolyzed.

**ATP Hydrolysis by UvrB Can Take Place in the Absence of UvrA and UvrC, and It Alters the Conformation of the UvrB-DNA Complex**—The experiments described above clearly illustrate that for formation of an active UvrBC-DNA incision complex on substrate G10, first hydrolysis of ATP by UvrB is needed, followed by the exchange of ADP for ATP. For the UvrABC-mediated incision of the “normal” substrate G1, a similar order of events is likely to occur, with the exception that for the induction of the ATPase of UvrB on this substrate the UvrA protein is required.

The ATP hydrolysis of UvrB bound to substrate G10 can be monitored by gel retardation. UvrB binds to G10 in the absence of cofactor (Fig. 6A, lane 2). Addition of ADP or ATPγS does not alter the stability of the complex, resulting in retarded bands of similar intensity (lanes 4 and 5). Upon addition of ATP, however, the UvrB-DNA complex can no longer be detected in the retardation gel (lane 3). This indicates that ATP hydrolysis alters the protein-DNA interactions in such a way that the complex dissociates during electrophoresis. This experiment not only demonstrates a hydrolysis-induced change in the UvrB-DNA complex, it also shows that the UvrB protein can hydrolyze ATP in the absence of any other Uvr protein. As expected the UvrB-DNA complex of the ATPase/helicase deficient mutants is not altered in the presence of ATP, and as a result the complex remains stable in the retardation assay (Fig. 6B).

When UvrC is added to the UvrB-DNA complex formed on substrate G10, a UvrBC-DNA complex is detected in the gel retardation assay, irrespective of the presence of a hydrolyzable or nonhydrolyzable cofactor (Fig. 6C). Again this shows that UvrC binding to the UvrB-DNA complex is dependent neither on binding nor on hydrolysis of ATP. The amount of UvrBC-DNA complex in the gel (Fig. 6C) is higher than that of UvrB-DNA (Fig. 6A). This indicates a stabilizing effect of UvrC on the complex, even when ATP is hydrolyzed.

**The Same UvrC Molecule Is Involved in 3' and 5' Incision**—The requirements for the 3' and 5' incision of damaged DNA are very different, not only with respect to the cofactor as we show in this paper but also with respect to protein domains that are involved. The binding of UvrC to the C-terminal domain of UvrB via a coiled-coil interaction (18) is essential for 3' incision but not for 5' incision (12, 20). On the other hand active site mutants of UvrC that no longer induce 5' incision do allow normal 3' incision (11). These observations clearly indicate that the UvrBC-DNA complexes leading to each of the two incisions must be structurally and functionally different. An explanation for the formation of two such different complexes could be that the UvrB-DNA complex is bound by two UvrC molecules, one for each incision event. To test this hypothesis we performed a competition experiment using two different UvrC mutants. UvrC(D466A) is an active site mutant that no longer promotes 5' incision but allows normal 3' incision (11). UvrC(L221P + F223L) contains two substitutions in the UvrB binding domain, and as result the 3' incision is disturbed but the 5' incision is unaffected (12). Incision of substrate G1 with UvrC(D466A) results in an uncoupled 3' incision product (Fig. 7A, lane 3), whereas with UvrC(L221P + F223L) no incision is shown at all (lane 4). Incision of the preincubated substrate G2 with UvrC(D466A) hardly gives incision (Fig. 7B, lane 3), whereas mutant UvrC(L221P + F223L) results in incision comparable with UvrC(wt) (lanes 1 and 2). The only difference is that with UvrC(L221P + F223L) the damage-independent additional incision, which was shown to require the coiled-coil interaction between UvrB and UvrC (19), is absent. When substrate G1 was preincubated with UvrC(D466A) after which UvrC(L221P + F223L) was added, still uncoupled 3' incision is observed (Fig. 7A, lane 5). Apparently the binding of UvrC(D466A) to the UvrB-DNA complex prevents the induction of 5' incision by the other mutant. Also for substrate G2, which is very efficiently incised by UvrC(L221P + F223L), a preincubation with UvrC(D466A) severely inhibits this incision (Fig. 7B, lane 4). This means that the UvrBC-DNA complex does not allow the binding of a second UvrC molecule. When substrate G1 is first incubated with UvrC(D466A) and subsequently with UvrC(wt), some 5' incision does occur (Fig. 7A, lane 6). This can be explained by the fact that UvrC(wt) in contrast to UvrC(L221P + F223L) has an intact UvrB binding domain, and therefore the wild type protein can compete with UvrC(D466A), partially displacing it from the complex. Preincubation with UvrC(L221P + F223L) does not affect incision of G1 by UvrC(wt) (Fig. 7C), which shows that the coiled-coil interaction between UvrB and UvrC indeed is the most important determinant for UvrBC-DNA complex formation. In conclusion the competition experiments show that the coiled-coil interaction between UvrC and UvrB remains after the 3' incision and that the same UvrC molecule also induces 5' incision, although it has been shown that for this 5' incision the coiled-coil interaction is not essential (12, 20).

**DISCUSSION**

On a double-stranded damaged DNA substrate the loading of UvrB onto the site of the damage requires the action of UvrA
and ATP. Several observations have suggested that the resulting UvrB-DNA preincision complex is stable in the presence of ATP only: (i) Isolation of UvrB-DNA complexes by column chromatography at room temperature was only possible if the chromatography buffers contained ATP (9). (ii) Separation of preincision complexes by gel retardation results in a much higher yield when ATP is included in the gel and the electrophoresis buffer (10, 17). In this paper we show that a stable UvrB-DNA complex can be isolated in the absence of ATP by capture of a biotinylated damaged DNA substrate on streptavidin-coated magnetic beads. The complex survived multiple washes in buffer without ATP even at room temperature. Apparently the stability of the UvrB-DNA complex is highly dependent on the method used to separate it from the other components of the reaction mixture.

Binding of ATP to the UvrB-DNA complex induces a conformational change in the DNA as was shown by DNase I footprinting. In the presence of ATP the UvrB-DNA complex shows a DNase I-hypersensitive site at the 5’ side of the damage. This site is generally believed to be characteristic for the formation of the preincision complex. The DNase I-hypersensitive site is also apparent in the presence of ATPγS but not with ADP, indicating that it is the ATP binding and not the hydrolysis that induces the specific DNA conformation. We will refer to the UvrB-DNA complex prior to the binding of ATP as the pro-preincision complex and with bound ATP as the preincision complex. The appearance of the DNase I-hypersensitive site fully correlates with the induction of 3’ incision after addition of UvrC to the isolated complexes; efficient incision occurs in the presence of ATP and ATPγS, but not with ADP or without cofactor. The binding of UvrC to the UvrB-DNA complex does not require a cofactor, suggesting that the ATP-induced conformational change is needed for the 3’ incision itself. The ATP-induced conformational change is not required for the 5’ incision. Although on a 3’ prenicked substrate ATP and ATPγS still specifically induce the DNase I-hypersensitive site, the 5’ incision is as efficient with ADP, which does not give this conformational change. The 5’ incision can even take place in the absence of cofactor, albeit at a very low level. Strikingly, addition of UvrC to isolated UvrB-DNA complexes formed on a 3’ prenicked substrate resulted in a very efficient 5’ incision at 0 °C, even after incubation for only 3 min. Apparently the binding of UvrC to the preincised complex directly docks the 5’ incision position into the catalytic site of the protein. The 3’ incision event, in contrast, appears to be much more difficult to achieve. In the accompanying paper (17) we have shown that in a UvrB-DNA complex without ATP the DNA region of the 3’ incision is under torsional stress, resulting in the instability of this pro-preincision complex as discussed above. This deformation of the DNA is important for the eventual 3’ incision, because relaxation of the DNA by introduction of a single strand nick opposite the 3’ site completely abolishes incision (17). Subsequent binding of ATP to the pro-preincision complex not only induces the DNase I-hypersensitive site as we show here, but it also seems to release or compensate for the torsional stress in the 3’ region, because it stabilizes the complex in a retardation gel (17). These observations indicate that 3’ incision requires two consecutive conformational changes in the 3’ region of the DNA, the first one made during formation of the pro-preincision complex and the second one because of subsequent ATP binding. Moreover the 3’ incision appears also to require thermal energy, because addition of UvrC to preformed preincision complexes does not give any 3’ incision at 0 °C. Taken together, the exposure of the 3’ incision site to the catalytic residues seems to need a very specific protein-DNA conformation in which the DNA helix is likely to be considerably distorted. Recently we have shown that the UvrC protein contains two catalytic sites, one for the 3’ incision and one for the 5’ incision (21). From the results in this paper, it is clear that both incisions are made by the same UvrC molecule. The competition experiments indicate that the coiled-coil interaction between the C-terminal domain of UvrB and the homologous domain of UvrC is maintained after the 3’ incision has occurred, even though it has been shown that it is not essential for the 5’ incision (20).

Damage recognition by the UvrB protein per se does not require ATP (17), which is why UvrB can specifically bind to the site of the damage in substrate G10 in the absence of cofactor. Before incision can occur, however, the UvrB-DNA complex on this substrate first needs to hydrolyze ATP, and then a new ATP molecule must be bound. These two ATP-dependent reactions are most likely required to induce the two consecutive conformational changes associated with formation of the pro-preincision complex and the preincision complex, respectively, as discussed above. On a double-stranded DNA substrate, ATP hydrolysis by UvrB is also needed in a prior step to trigger the DNA helicase activity of the UvrA-B complex, which is required for the loading of UvrB onto the damaged site (6–8). In the accompanying paper (17) we have shown that this helicase activity presumably unwinds the DNA at the 5’ side of the damage, thereby allowing UvrB access to the damage.

Taken together we come to a model in which UvrB hydrolyzes multiple ATP molecules during the repair reaction (Fig. 8). First ATP hydrolysis by the UvrA-B complex is needed for opening up the DNA helix to bring UvrB close to the damage. Next the UvrB protein binds to this damaged site, and in the resulting UvrB-DNA complex a second round of ATP hydrolysis is triggered, thereby inducing the conformational changes that lead to formation of the relatively unstable pro-preincision complex. The experiments with substrate G10 have shown that this ATP hydrolysis can occur in the absence of UvrA. Therefore UvrA might be released from the complex during formation of the initial UvrB-DNA complex, although we cannot exclude the possibility that this dissociation occurs at a later stage. The binding of ATP to the pro-preincision complex induces formation of the preincision complex, which after binding of UvrC can be incised at the 3’ site. Finally, for the 5’ incision no further ATP binding or hydrolysis is needed. We have shown in this paper that the UvrC protein is capable of binding to all
three UvrB-DNA intermediate complexes. In the normal chain of events the UvrB-DNA complex formed after loading of UvrB, and the pro-preincision complex are expected to be very short-lived. Therefore in vivo UvrC will most probably bind when the preincision complex is formed.

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