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The Effect of the DNA Flanking the Lesion on Formation of the UvrB-DNA Preincision Complex

MECHANISM FOR THE UvrA-MEDIATED LOADING OF UvrB ONTO A DNA DAMAGED SITE*

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The UvrB-DNA preincision complex plays a key role in nucleotide excision repair in *Escherichia coli*. To study the formation of this complex, derivatives of a DNA substrate containing a cholesterol adduct were constructed. Introduction of a single strand nick into either the top or the bottom strand at the 3′ side of the adduct stabilized the UvrB-DNA complex, most likely by the release of local stress in the DNA. Removal of both DNA strands up to the 3′ incision site still allowed formation of the preincision complex. Similar modifications at the 5′ side of the damage, however, gave different results. The introduction of a single strand nick at the 5′ incision site completely abolished the UvrA-mediated formation of the UvrB-DNA complex. Deletion of both DNA strands up to the 5′ incision site also prevented the UvrA-mediated loading of UvrB onto the damaged site, but UvrB by itself could bind very efficiently. This demonstrates that the UvrB protein is capable of recognizing damage without the matchmaker function of the UvrA protein. Our results also indicate that the UvrA-mediated loading of the UvrB protein is an asymmetric process, which starts at the 5′ side of the damage.

Nucleotide excision repair in *Escherichia coli* is a multi-step process that leads to the removal of a 12–13-mer oligonucleotide containing the damaged site, followed by filling in of the resulting gap by DNA polymerase I and ligation of the nick by DNA ligase (1, 2). The key intermediate of the repair process is the UvrB-DNA preincision complex, in which UvrB is tightly bound to the DNA at the site of the lesion. The UvrC protein interacts with this preincision complex, resulting in cleavage of the 4^{th} or 5^{th} phosphodiester bond 3′ to the damage. This 3′ incision is immediately followed by cleavage of the 8^{th} phosphodiester bond on the 5′ side of the damage.

The UvrB protein on its own has no significant affinity for damaged or undamaged double-stranded DNA, but very low affinity binding to single-stranded DNA carrying a lesion has been shown (3). Loading of UvrB onto damaged double-stranded DNA requires the action of the UvrA protein. A dimer of UvrA associates with UvrB in solution, and the trimeric $UvrA_2B$ complex initially binds to a damaged site in the DNA. Next, conformational changes take place in the $UvrA_2B$-DNA complex resulting in stable binding of UvrB to the DNA and the release of UvrA (4). This UvrA-directed binding of UvrB to the DNA has been shown to require ATP hydrolysis by the UvrB protein (5) and the $UvrA_2B$-associated DNA helicase activity (6, 7), suggesting that this loading of UvrB involves denaturation of the DNA near the site of the lesion.

In addition to the damage-specific DNA binding of UvrB via the action of UvrA, a complex of UvrB and UvrC (in the absence of UvrA) has been shown to specifically bind to nondamaged DNA with a double strand-single strand junction (8). These substrates are subsequently incised by the UvrBC complex at 7 nucleotides from the 3′ end of the junction. It has been proposed that the UvrBC incision of the nondamaged substrate mimics the 5′ incision event on damaged DNA (8, 9).

In this paper we investigate the role of the DNA flanking the incision positions for formation of the UvrB-DNA preincision complex and the subsequent incisions. We show that a double-stranded substrate in which DNA at the 3′ side of the damage has been removed up to the 3′ incision site can still form a stable preincision complex via the UvrA-mediated loading of the UvrB protein. Deletion of DNA on the 5′ side up to the 5′ incision position abolished the UvrA-mediated formation of the UvrB-DNA complex. In the absence of UvrA, however, the UvrB protein by itself could efficiently bind to this 5′-truncated substrate. Implications of these findings for the process of UvrA-mediated preincision complex formation will be discussed.

EXPERIMENTAL PROCEDURES

Protein Purifications—The UvrA, UvrB, and UvrC proteins were purified as described (10). For the protein-DNA binding studies an active site mutant of UvrC (D399A) was used (11). A plasmid expressing this mutant was kindly provided by Dr. A. Sancar (University of North Carolina), and the mutant protein was purified according to the same procedure as wild type UvrC. The helicase mutants UvrB(G509S) and UvrB(R544H) have been described (6). The polyclonal antibodies against UvrB and UvrC were raised in rabbits as described (10).

Construction of DNA Substrates—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. All synthesized oligonucleotides were gel purified. For 5′ labeling (indicated with an *asterisk* in Fig. 1*B*) 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 $MgCl_2$, 5 mM dithiothreitol, and 3 pmol of [γ-^{32}P]ATP (7000 Ci/mmol, 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

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substrates were constructed by hybridizing 4 pmol 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.

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 $MgCl_2$, 100 mM KCl, 0.1 μg/μl bovine serum albumin, and 1 mM ATP) as described (12). After 60 min the reaction was stopped by adding 2 μl of 2 μg/ml glycogen followed by ethanol precipitation. The incision products were visualized on a 15% acrylamide gel containing 7 M urea as described (12).

Gel Retardation Assay—The DNA substrates (40 fmol) were incubated with 100 nM UvrB, with or without 2.5 nM UvrA and or 50 nM UvrC in Uvr-endo buffer. The mixture was incubated at 37 °C as described (10), and subsequently 1 μl of antiserum was added where indicated. Analysis of the protein-DNA complexes in the absence of ATP was done by loading the samples on a 3.5% native polyacrylamide gel in 0.5× Tris borate/EDTA. For analysis of the complexes in the presence of ATP, 1 mM ATP, and 10 mM $MgCl_2$ were included in the gel and in the running buffer as described (10). The gels were run at room temperature at 9 mA for gels without ATP and 15 mA for gels with ATP, and the protein-DNA complexes were visualized using autoradiography.

DNase I Footprinting—Substrate G6 was incubated with or without 2.5 nM UvrA and 100 nM UvrB in UV endo buffer for 15 min. Next to each incubation mixture 1 μl of 50 mM $CaCl_2$, and 1 μl of DNase I was added (a 240× dilution of 10 units/μl in 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 10% glycerol). After incubation for 5 min at 20 °C, the reaction was terminated by addition of 2 μl of 0.5 M EDTA. Next the DNA was precipitated with glycogen/ethanol and loaded on a 20% acrylamide gel containing 7 M urea.

RESULTS

Analysis of UvrB-DNA and UvrBC-DNA Complexes on a 50-mer Substrate Containing a Cholesterol Lesion—A DNA lesion consisting of a cholesterol attached to the ribose of a nucleoside (Fig. 1*A*) is a good substrate for UvrABC. The incisions on such a substrate take place at the 5th phosphodiester bond 3′ to the lesion and at the 8th phosphodiester bond 5′ to the lesion. Consequently, incubation of a 5′-labeled 50-mer substrate containing a cholesterol lesion at position 27 (G1) with UvrABC results in a 19-nucleotide 5′ incision product (Fig. 2, *G1*). This 5′ incision product subsequently is substrate for the damage-independent nuclease activity of UvrBC (8), resulting in an additional incision product of 12 nucleotides (Fig. 2, *G1*).

Using the classic Uvr gel retardation assay, *i.e.* with ATP included in the gel and in the running buffer (10), a stable UvrB-DNA complex with substrate G1 can be visualized (Fig. 3, *G1*). This complex reacts specifically with anti-UvrB antibodies, retaining the complex in the slot of the gel. The addition of UvrC to the incubation mixture results in a UvrBC-DNA complex that specifically reacts with UvrC antibodies. Analysis of the same incubation mixtures on a gel without ATP reveals only a small amount of UvrB-DNA complexes (Fig. 4, *G1*). Apparently during electrophoresis the ATP is lost from UvrB, and as a consequence the protein dissociates from the DNA. In the accompanying paper (13) we show that immobilizing UvrB-DNA complexes on magnetic beads followed by removal of the ATP by extensive washing does not result in dissociation of the UvrB-DNA complex. This means that UvrB can remain attached to damaged DNA without ATP but seemingly in a different conformation, resulting in dissociation during electrophoresis. The UvrBC-DNA complex, in contrast, is very stable in a gel without ATP (Fig. 4, *G1*). This indicates that either the binding of UvrC prevents the dissociation of ATP or that the protein-DNA complex is stabilized by additional contacts of UvrC with UvrB and the DNA.

The Role of 3′-Flanking Sequences on the Formation of UvrB-DNA and UvrBC-DNA Complexes—As shown before (14) a DNA substrate with a nick at the 3′ incision position is very efficiently incised at the 5′ position by UvrABC (Fig. 2, *G2*).

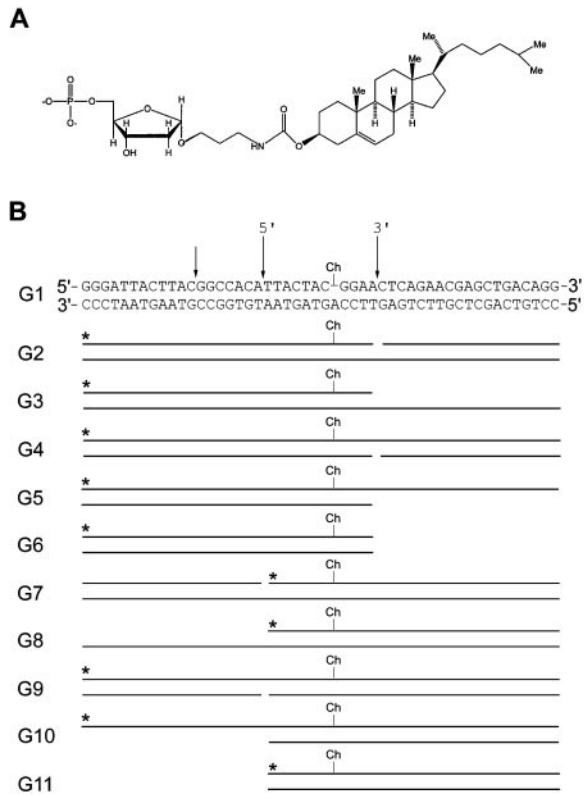

FIG. 1. **DNA substrates used in this study.** *A*, structure of the cholesterol lesion attached to the ribose of a nucleoside. *B*, DNA sequence of the 50-mer double-stranded DNA fragment with the cholesterol lesion at position 27 (*G1*) and schematic representation of the derivatives of this substrate (*G2–G11*). The position of the cholesterol is indicated with *Ch*. The *asterisk* indicate 5′ end labeling with ^{32}P. The *long arrows* indicate the positions of the 3′- and 5′ incision sites. The *short arrow* indicates the cleavage site of the damage-independent UvrBC activity.

The substrate gives rise to a clear UvrB-DNA complex, even in a gel without ATP, indicating that under these conditions the complex is stabilized by the presence of the nick (Figs. 3 and 4, *G2*). A clear UvrBC-DNA complex is also observed in both types of retardation gels. Substrate G3, which lacks the top strand beyond the 3′ incision position, behaves exactly the same as the 3′ prenicked substrate (G2). The efficiencies of 5′ incision in G2 and G3 are the same (Fig. 2, *G2* and *G3*), and also the UvrB-DNA and UvrBC-DNA complexes are formed with comparable efficiencies and stabilities (Figs. 3 and 4, *G2* and *G3*). Obviously the DNA sequences in the damaged strand flanking the 3′ incision are not involved in essential protein contacts with UvrA, UvrB, or UvrC.

Next we analyzed substrates with a nick (G4) or a gap (G5) at similar positions in the bottom strand. Both substrates again give rise to stable UvrB-DNA and UvrBC-DNA complexes (Figs. 3 and 4, *G4* and *G5*). Surprisingly, only an extremely low level of incision is obtained after incubation of the two substrates with UvrABC (Fig. 2, *G4* and *G5*). Extension of the bottom strand of substrate G5 with 3 nucleotides, giving a total of 7 base pairs to the right of the lesion, did not improve the incision efficiency (results not shown), indicating that it is not the single-strandedness of the incision site that hampers the incision reaction. Because substrate G4 has the same potential for making DNA contacts as substrates G1 and G2, the inability to incise has to be explained by the DNA conformation in the UvrBC-DNA complex that is adapted as a result of the nick in the bottom strand. This DNA conformation is apparently different from the one that results from a nick in the top strand, because the latter not only allows 5′ incision but is in fact a

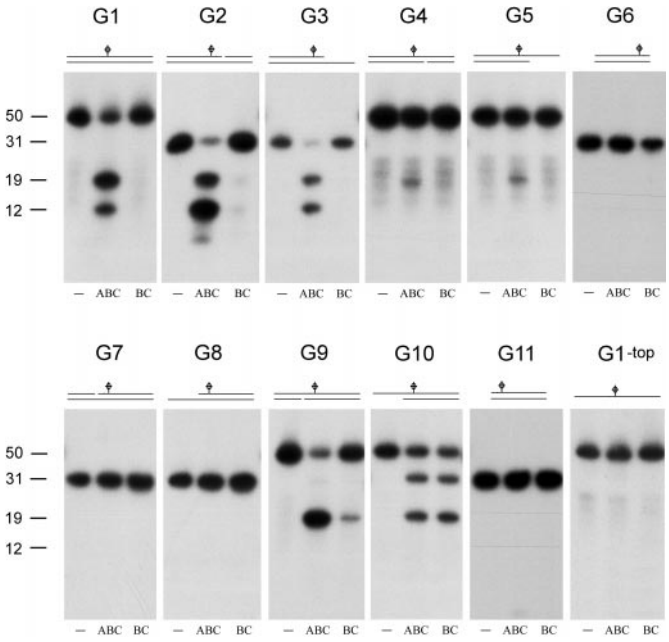

FIG. 2. **Incision of substrates G1 to G11 with Uvr(A)BC.** The substrates used are indicated above each panel. The 5′ end-labeled DNA substrates were incubated with UvrABC or UvrBC as indicated. DNA fragments of 31 nucleotides correspond to incision at the 3′ site. Fragments of 19 nucleotides correspond to incision at the 5′ site. Fragments of 12 nucleotides are the result of the additional 5′ incision by UvrBC.

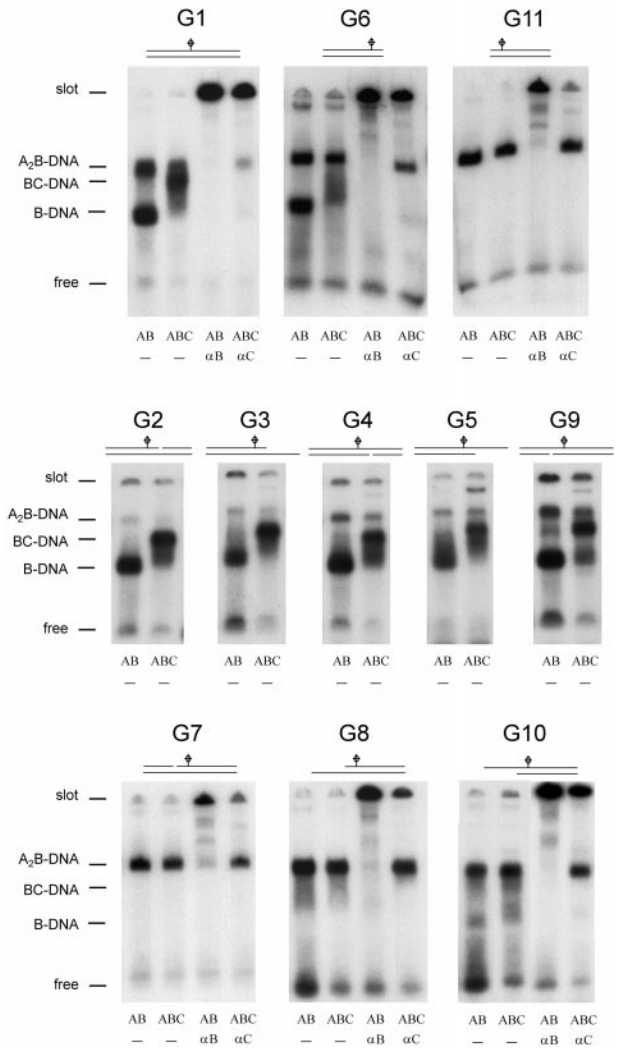

FIG. 3. **Complex formation of the different DNA substrates with the Uvr proteins visualized in a gel containing ATP.** The substrates used are indicated above each panel. The 5′ end-labeled DNA substrates were incubated with UvrAB or UvrABC as indicated. Addition of antibodies against UvrB or UvrC is indicated with αB and αC, respectively. The position of the different Uvr-DNA complexes is shown. Binding of an antibody to a specific complex retains this complex in the slot. The αC antibody has previously been shown to give a small amount of cross-reactivity with the UvrB protein (14), which for substrates G11, G7, and G8 (*lanes 4*) results in a small amount of supershift, even though no UvrC binding occurs.

prerequisite for this event (15). Finally we tested substrate G6, in which both the top and bottom strands flanking the 3′ incision site have been deleted. Also this truncated substrate gives rise to very stable UvrB-DNA and UvrBC-DNA complexes (Figs. 3 and 4, *G6*). DNase I footprinting shows (Fig. 5) that the UvrB-DNA complex on G6 has the characteristic DNase I hypersensitive site, which is indicative for the formation of an active preincision complex (12, 17–19). Still no 5′ incision can be detected after incubation of G6 with UvrABC (Fig. 2, *G6*). Substrate G3, which has the same top strand as G6, is incised very efficiently, which shows that the presence of the bottom strand "to the right" of the 3′ incision position is essential for triggering the 5′ incision event.

In summary the results with substrates G2 to G6 show that for the UvrA-mediated loading of UvrB and the subsequent stable binding of UvrB to the site of the damage, no contacts with DNA beyond the 4th base pair 3′ of the damage are required. For induction of the 3′ incision, however, the bottom strand has to extend beyond at least the 7th base pair 3′ of the damage, and this DNA should not contain a nick. For induction of the 5′ incision the top strand flanking the 3′ incision position can be omitted, but at least part of the corresponding bottom strand is required.

The Role of 5′-Flanking Sequences on the Formation of UvrB-DNA and UvrBC-DNA Complexes—In a similar way the effect of DNA flanking the 5′ incision site on protein-complex formation and DNA incision was analyzed. Substrate G7, carrying a nick at the 5′ incision position, and G8, in which the top strand flanking this position has been deleted, are efficiently recognized by the $UvrA_2B$ complex (Figs. 3 and 4, *G7* and *G8*). However, no UvrB-DNA complexes can be detected in a retardation assay, neither in the presence (Fig. 3) nor in the absence of ATP (Fig. 4). Apparently the UvrA protein is no longer capable of loading UvrB onto the site of the damage. Because all potential UvrB contact points are present in G7, this means that somehow the nick interferes with the loading process. As a result of the inability to form a UvrB-DNA complex, also no UvrBC-complexes (Figs. 3 and 4) and no 3′ incisions are observed with substrates G7 and G8 (Fig. 2).

The presence of a nick in the bottom strand opposite the 5′ incision site (G9) does not interfere with the loading of UvrB onto the DNA, because the retardation gel in the presence of ATP reveals a clear UvrB-DNA complex (Fig. 3, *G9*). This UvrB-DNA complex resembles the complex formed on G1, in that it is much less stable in a retardation gel without ATP (Fig. 4, *G9*). Apparently a nick opposite the 5′ incision site does not have a stabilizing effect on the UvrB-DNA complex. This is in contrast to the effect of a nick at the 3′ side of the lesion (substrates G2 and G4). Subsequent binding of UvrC to the UvrB-DNA complex on G9 results in a clear UvrBC-DNA complex (Figs. 3 and 4, *G9*) and a very efficient incision like that found for G1 (Fig. 2, *G1* and *G9*). The only difference between substrates G1 and G9 is the absence of the additional 12-nucleotide incision product with the latter substrate. This 12-nucleotide product is the result of the damage-independent incision activity of UvrBC on substrates with a single strand-

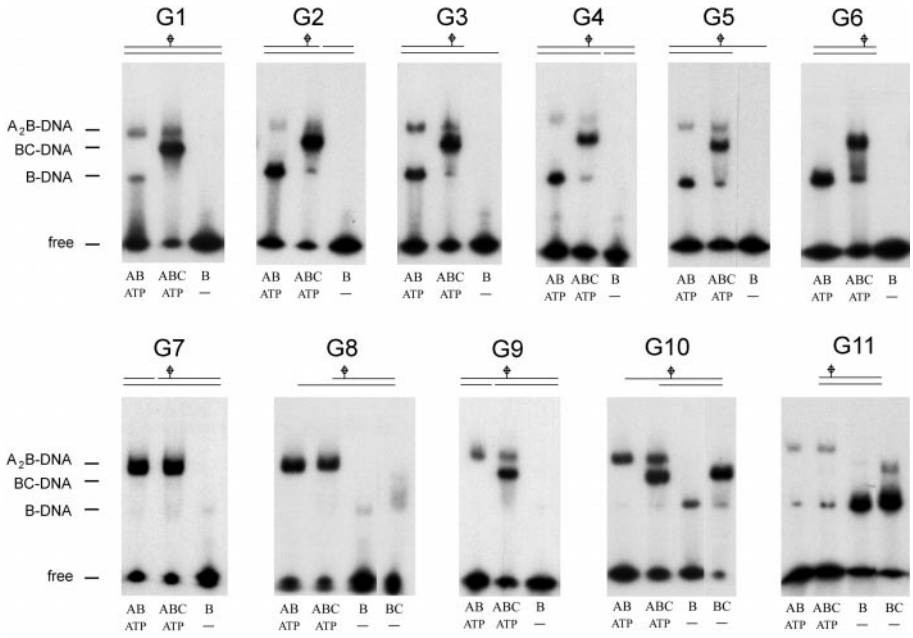

FIG. 4. **Complex formation of the different DNA substrates with the Uvr proteins visualized in a gel without ATP.** The substrates used are indicated above each panel. The 5′ end-labeled DNA substrates were incubated with UvrAB, UvrABC, UvrB, or UvrBC with or without ATP as indicated. The position of the different Uvr-DNA complexes is shown.

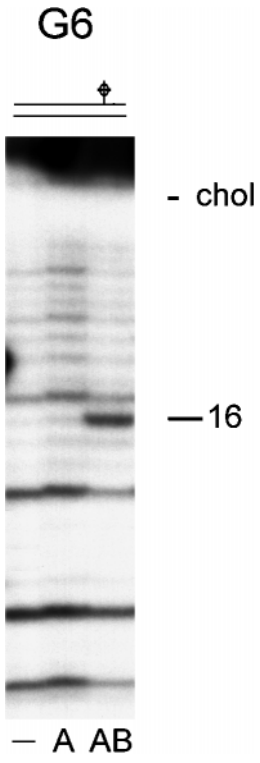

FIG. 5. **DNase I footprint of substrate G6.** Substrate G6 was incubated without protein (*lane 1*), with UvrA (*lane 2*), or UvrAB (*lane 3*) after which the complexes were treated with DNase I. The *arrow* indicates the DNase I hypersensitive site.

double strand junction (8), and, as a consequence of the nick in G9, the 5′ incision no longer produces such a structure.

Substrate G10, in which the bottom strand flanking the nick in G9 is missing, does not give rise to stable UvrB-DNA complexes in a retardation gel with ATP (Fig. 3, *G10*). The amount of UvrBC-DNA complexes on G9 and G10 as detected in a gel without ATP, however, are comparable (Fig. 4, *G9* and *G10*). This indicates that it is not the formation of the UvrB-DNA complex *per se* that is impaired with G10 but that that this complex is less stable. Incubation of G10 with UvrABC leads to incision, but the total incision is lower compared with that of substrate G9 (Fig. 2, *G9* and *G10*), suggesting that also the UvrBC-DNA complexes formed with substrate G10 are less stable. This is reflected in the retardation assay with an ATP-containing gel (Fig. 3). Incubation with UvrABC does not result in a clear UvrBC-DNA complex, but a broad smearing band is observed instead (Fig. 3, *G10*, *second lane*). In the presence of UvrC antibodies, which stabilize the complex, a significant supershift is observed (*lane 4*), confirming that indeed UvrBC-DNA complexes are present. About half of the incised molecules of G10 appear to be cut at the 3′ site only resulting in an uncoupled incision product of 31 nucleotides (Fig. 2, *G10*). Labeling of the damaged strand of substrate G10 at the 3′ end confirmed that all molecules cut at the 5′ site were also cut at the 3′ site (not shown). The uncoupled incision observed indicates that following the 3′ nick half of the protein-DNA complexes has fallen apart before 5′ incision could take place. As a result of the missing part of the bottom strand, UvrB-DNA and/or UvrC-DNA contacts important for the stability of the complex must be lacking. Finally, substrate G11, in which both top and bottom strands have been truncated, does not give any UvrB-DNA complexes in a retardation gel with ATP (Fig. 3, *G11*). The residual complex formation observed in a gel without ATP appears to be the result of an UvrA-independent reaction (see below) indicating that like for substrates G7 and G8 the UvrA-mediated loading of UvrB is severely impaired. As a result, substrate G11 is not incised by UvrABC (Fig. 2, *G11*). Summarizing the results of substrates G7 to G12, it can be concluded that for the formation and the stability of UvrB-DNA complexes the DNA "to the left" of the 5′ incision position is much more important than DNA "to the right" of the 3′ incision position.

Damage-specific UvrB Binding in the Absence of UvrA—The truncation of both top and bottom strand in substrate G11 clearly impairs the UvrA-mediated loading of UvrB (Fig. 3). Strikingly, incubation of substrate G11 with UvrB alone results in a high level of UvrB-DNA complex formation (Fig. 4). This complex is the result of damage-specific binding of UvrB, because a similar substrate without the cholesterol damage does not give any retardation with UvrB (not shown). Substrate G10, in which only the bottom strand has been truncated also specifically binds UvrB alone, albeit to a lesser extent (Fig. 4). With G8, in which part of the top strand has been deleted, a very faint band at the position of the UvrB-DNA complex is

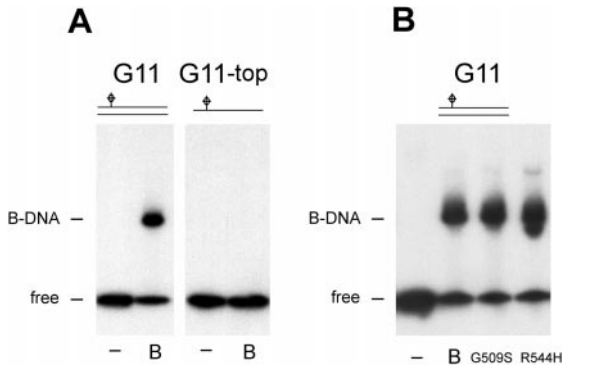

FIG. 6. **Complex formation of (mutant) UvrB with substrates G11 and the top strand of G11.** *A*, binding of UvrB to the double-stranded DNA and the single-stranded top strand of G11. *B*, binding of wtUvrB and the helicase mutants G509S and R544H to substrate G11. Note that the preparation of R544H protein contains the proteolytic product UvrB*, which results in a second retarded band that migrates faster as result of the reduced molecular weight.

observed (Fig. 4). Neither the fully double-stranded 50-mer (G1) nor any of the derivatives with truncations at the right-hand side of the lesion (G2 to G6) bind UvrB in the absence of UvrA (Fig. 4). This shows that deletion of the DNA specifically at the left-hand side of the cholesterol adduct circumvents the need for UvrA to put UvrB onto the site of the damage. Specific binding of UvrB alone to single-stranded DNA containing damage has previously been shown (3). This, however, was a very low affinity binding as it could only be observed at UvrB concentrations above 5 μM. With G11 the observed UvrB-DNA complex formation occurs with the same UvrB concentration normally used for the UvrA-mediated loading of UvrB (100 nM). When the damage-containing top strand of G11 was incubated with 100 nM UvrB, we could not detect any UvrB binding (Fig. 6*A*). This means that the binding of UvrB to substrate G11 is much more specific than the binding to the damage-containing single strand.

Addition of UvrC to the UvrB-DNA complex of G10 results in a clear UvrBC-DNA complex (Fig. 4). The addition of UvrC also leads to 3′ and 5′ incisions, which are identical to the incisions of the same substrate with UvrABC (Fig. 2). Not only are the incision efficiencies equal, but also uncoupling of the 3′ incision is observed. This shows that the UvrBC-DNA complexes on G10 formed in the absence and presence of UvrA must be structurally and functionally identical. This, together with the observation that no stable UvrB-DNA complex is formed in the presence of UvrA, strongly suggests that the protein-DNA complexes obtained after incubation of G10 with UvrAB(C) are in fact the result of the UvrA-independent UvrB(C) reaction.

Substrate G8, which only exhibited a very weak UvrB binding, does not show clear UvrBC-DNA complexes in a retardation gel (Fig. 4), and consequently no incision is observed (Fig. 2). Substrate G11, which does allow very stable UvrB binding, does not give rise to stable UvrBC-DNA complexes either (Fig. 4), and as a result also this substrate is not incised by UvrBC (Fig. 2). Apparently the top strand adjacent to the 5′ incision position is important for stable UvrC binding and for subsequent 3′ incision.

The UvrA-independent binding of UvrB to substrates G10 and G11 occurs in the absence of ATP. In the accompanying paper (13), we examine the effect of ATP and show that addition of ATP destabilizes the UvrB-binding. As a result the amount of UvrB-DNA complexes obtained with (UvrA and) UvrB in the presence of ATP (Fig. 4, *G10* and *G11*, *first lanes*) is much lower than in the absence of ATP (*third lanes*).

In the past we have shown that for the UvrA-dependent binding of UvrB to the site of the damage, the ATPase activity of the UvrB protein is essential (6). UvrB mutants with substitutions in helicase motifs V (G509S) or VI (R544H) are disturbed in their ATPase/helicase activities (6), and as a consequence they can no longer be loaded onto a damaged site by UvrA. The same mutants, however, do bind efficiently to substrates G10 (not shown) and G11 (Fig. 6*B*) in the absence of UvrA, confirming that the ATPase/helicase activity is not needed for the damage recognition by UvrB *per se*. The UvrA-independent incision of G10 by UvrBC, however, does require the hydrolysis of ATP (see Ref. 13). Apparently the damage-specific binding of UvrB as such is not sufficient for the generation of an incisable preincision complex, but ATP hydrolysis is needed to induce the proper protein and/or DNA conformations. In summary our results show that in the absence of UvrA and ATP the UvrB protein can recognize and bind a damaged site when this site is close to the 5′ end of a DNA fragment.

DISCUSSION

Binding of UvrB to a damaged site in double-stranded DNA has been generally believed to require the "molecular matchmaker" function of UvrA. Here we show for the first time that UvrB by itself is capable of recognizing and binding to the site of a lesion, if this lesion is located close to the end of a double-stranded fragment. This damage-specific binding of UvrB alone seems to have a polarity, occurring when the damage is close to the 5′ end of the damaged strand but not when it is close to the 3′ end. This indicates that for the direct binding of UvrB, the protein needs to "approach" the damage from the 5′ side. This directionality is likely to be a reflection of the mechanism that UvrA uses to load UvrB onto a damaged site. After initial recognition of damage by the UvrA protein in the $UvrA_2B$ complex, the $UvrA_2B$-associated helicase activity might transiently unwind the DNA region at the 5′ side of the damage. As a result of this, an "entry site" for UvrB is created close to the 5′ side of the damage, to which it will subsequently bind via its own damage-recognizing determinants. Several experiments described in this paper are in agreement with such a mechanism of UvrB loading: (i) On a truncated DNA fragment, which has 7 base pairs at the 5′ side of the damage, UvrA can no longer load the UvrB protein. A deletion at the other side leaving only 4 base pairs at the 3′ side of the damage, however, does not interfere with this process. Apparently for the loading process the 5′-flanking region is more important than the 3′-flanking region, which would be in agreement with an entry site function of this 5′ region. (ii) Mutants of UvrB that affect the ATPase/helicase activity of the $UvrA_2B$ complex can no longer be loaded onto a damaged site via UvrA (6). The same mutants, however, do bind to the damaged site when it is close to the end of the DNA fragment. This shows that the DNA unwinding activity *per se* is not needed for the damage recognition, but it is more likely creating a DNA conformation that allows UvrB to access the damage, *i.e.* opening up of the DNA helix in the 5′ region. When the damage is located close to the end, this active opening up is no longer required. (iii) The introduction of a single-stranded nick at the 5′ incision position completely abolishes the UvrA-mediated loading of UvrB. Gel retardation experiments showed that the nick does not inhibit the damage-specific binding of the $UvrA_2B$ complex. Because the presence of a nick does not alter the number of possible DNA contacts with the UvrA and/or UvrB proteins, its effect must be topological. The DNA conformation in the protein-DNA complex after the postulated unwinding of the 5′ region would be different when a nick is present. The 19-mer top strand might even dissociate completely from the complex. A nick in the bottom strand does not interfere with the UvrA-mediated loading of UvrB, although this nick is expected to influence the topology of the DNA after unwinding as well. This

could indicate that after the unwinding of the 5′-flanking region by $UvrA_2B$, the UvrB protein mainly interacts with the top strand of this region. The presence of a nick in this strand would then prevent the access of UvrB to the damaged site.

The UvrB protein by itself is capable of specifically binding to the damage of substrate G10 in the absence of ATP. The subsequent stable binding of UvrC can occur in the absence of a cofactor too. However, for the incisions to take place, ATP is indispensable, indicating that for the formation of an active UvrBC-DNA incision complex an ATP-induced conformational change of UvrB is required. The role of the cofactor in formation of the incision complex is further discussed in the accompanying paper (13).

Once UvrA has loaded UvrB onto the DNA, the stability of the resulting preincision complex is influenced by the conformation of the damage-flanking regions. The gel retardation experiments in the absence of ATP indicate that the UvrB-DNA complex is stabilized by introduction of a single-stranded nick in either top or bottom strand at the 3′ side of the lesion. This indicates that in the UvrB-DNA complex without ATP the DNA is in a state of torsional tension. Nicking of the DNA will release this tension thereby stabilizing the complex. Because the presence of a nick at the 5′ side of the lesion (in the bottom strand) does not stabilize the UvrB-DNA complex, the DNA helix seems mainly distorted at the 3′ side of the damage. In a retardation gel with ATP, the UvrB-DNA complex of substrate G1 is much more stable than in a gel without ATP. This indicates that ATP alters the conformation of the UvrB-DNA complex such that the DNA tension in the 3′ region is (partially) released.

Stable binding of UvrC to the preincision complex is largely determined by protein-protein interactions between the homologous domains of UvrB and UvrC (14). Additional interaction of UvrC with the DNA contributes to the stability (16). Here we show that the DNA region at the 5′ side of the lesion is important for stable UvrC binding. Substrate G11, in which this region is missing, efficiently binds UvrB by itself, but the subsequent association with UvrC is very weak. Truncation of the other end of the DNA substrate, leaving only 4 base pairs at the 3′ side of the lesion does not destabilize UvrC binding significantly. The interaction of UvrC with the 5′-flanking region seems to be mainly with the top strand, because substrate G10, in which only the bottom strand of this region is missing forms a substantially more stable UvrBC-DNA complex than G11. An interaction with the top strand is not unexpected, because it is this strand that is incised by the UvrC protein (11).

Although nicking or truncation of DNA at the 3′ side of the lesion does not seem to have large effects on the formation of the UvrBC-DNA complex, it does influence both incision reactions. The introduction of a single-stranded nick opposite the 3′ incision site, which seemingly allows normal UvrB-DNA and UvrBC-DNA complex formation, almost completely blocks 3′ incision. Apparently the topology of the DNA is extremely important for this incision reaction. As argued above, the DNA in the UvrB-DNA complex (without ATP) seems to be distorted at the 3′ side of the lesion. Possibly this specific conformation is essential for the eventual positioning of the catalytic site for 3′ incision in the UvrBC-DNA incision complex. When a damaged DNA substrate is incised at the 3′ position, this immediately triggers induction of the 5′ incision. Relaxation of the DNA as a result of the 3′ incision, however, cannot be the only determinant for this second event, because a nick in the nondamaged strand (G4) does not induce 5′ incision. Apparently, specific protein-DNA contacts need to be established before 5′ incision can take place. The bottom strand adjacent to the 3′ incision position appears to be very important for these contacts, either with UvrB or with UvrC because G3 containing this region is incised very efficiently, whereas G6 lacking this strand is not incised at all.

Combining our results we come to the following model. After initial recognition of damage by the $UvrA_2B$ complex, the DNA at the 5′ side of the damage is unwound, creating an entry site for the UvrB protein, which subsequently binds to the site of the damage. In the resulting UvrB-DNA complex the DNA is distorted, creating stress mainly at the right-hand side of the damage. The UvrC protein binds to the UvrB-DNA complex via the coiled-coil interaction between the C-terminal domain of UvrB and the homologous domain of UvrC (14). In addition UvrC makes contacts with the DNA at the left-hand side of the damage, mainly with the damaged strand. For 3′ incision to occur, a specific DNA conformation in the region of the incision site seems essential. The subsequent incision possibly opens up the DNA in this region, thereby exposing the bottom strand at the right-hand side of the lesion. Interaction of UvrB and/or UvrC with this bottom strand triggers the 5′ incision event. In agreement with this last part of the model, it has recently been found that a damaged DNA substrate containing 2 unpaired bases 5′ and 7 or 9 unpaired bases 3′ to the lesion is efficiently incised by UvrBC at the 5′ site, without 3′ incision (20). Probably the unpairing of the DNA around the 3′ incision site sufficiently exposes the bottom strand for UvrB and/or UvrC to bind to.

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The Effect of the DNA Flanking the Lesion on Formation of the UvrB-DNA Preincision Complex: MECHANISM FOR THE UvrA-MEDIATED LOADING OF UvrB ONTO A DNA DAMAGED SITE

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