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*

(Received for publication, October 12, 1999, and in revised form, December 15, 1999)

Geri F. Moolenaar‡, Vania Monaco§, Gijs A. van der Marel§, Jaques H. van Boom§, Rob Visse‡, and Nora Goosen‡

From the ‡Laboratory of Molecular Genetics and the §Laboratory of Bio-organic synthesis, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, Einsteinweg 55, P.O. Box 9502, 2300 RA Leiden, The Netherlands

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' 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 4th or 5th phosphodiester bond 3' to the damage. This 3' incision is immediately followed by cleavage of the 6th 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,B complex initially binds to a damaged site in the DNA. Next, conformational changes take place in the UvrA,B-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,B-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. 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. 1B) 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 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

---

* This work was supported by the J. A. Cohen Institute for Radiopathology and Radiation Protection (IRS) and a European Community Structural Biology Framework IV Program grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† 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.: 33-715274773; Fax: 33-715274537; E-mail: N.Goosen@chem.leidenuniv.nl.

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.

**In-gel Assays**—The DNA substrates (40 fmol) were incubated with 100 nM UvrB, 50 nM UvrC, and 2.5 nM UvrA where indicated in 20 

The DNA substrate was incubated with 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 in 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).

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 5 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
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,B 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 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-
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’ end 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’ end 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 complexes formed with ATP (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
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-B complex, the UvrA-B-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-B 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-B 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, 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 non-damaged 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 UvrA2B 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.

REFERENCES
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
Geri F. Moolenaar, Vania Monaco, Gijs A. van der Marel, Jaques H. van Boom, Rob Visse and Nora Goosen

doi: 10.1074/jbc.275.11.8038

Access the most updated version of this article at http://www.jbc.org/content/275/11/8038

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 19 references, 14 of which can be accessed free at http://www.jbc.org/content/275/11/8038.full.html#ref-list-1