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The limited strand-separating activity of the UvrAB protein complex and its role in the recognition of DNA damage

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The recognition by *Escherichia coli* Uvr nucleotide excision repair proteins of a variety of lesions with diverse chemical structures and the presence of helicase activity in the UvrAB complex which can displace short oligonucleotides annealed to single-stranded DNA led to a model in which this activity moves UvrAB along undamaged DNA to damaged sites where the lesion blocks further translocation and the protein–DNA pre-incision complex is formed. To evaluate this mechanism for damage recognition, we constructed substrates with oligonucleotides of different lengths annealed to single-stranded DNA circles and placed a single 2-(acetylamino)fluorene (AAF) lesion either on the oligonucleotide or on the circle. For the substrates with no lesion, the UvrAB complex effectively displaced a 22-mer but not a 27-mer or longer fragments. The presence of AAF on the oligonucleotide significantly increased the release of the 27-mer but oligomers of 30 or longer were not separated. Placing the lesion on the circular strand did not block the release of the fragments. Instead, the releasing activity of UvrAB was stimulated and also depended on the length of the annealed oligonucleotide. These observations do not agree with the predictions of a damage recognition mechanism that depends on helicase-driven translocation. Most likely, the strand-separating activity of UvrAB is a consequence of local changes occurring during the formation of a DNA–protein pre-incision complex at the damaged site and is not due to translocation of the protein along undamaged DNA to locate a lesion.

Keywords: damage recognition/DNA repair/helicase/nucleotide excision repair/UvrAB complex

Introduction

The DNA nucleotide excision repair system is present in all organisms examined to date, and has been particularly well studied in *Escherichia coli*. It has several major steps: damage recognition, dual incision, repair synthesis and ligation. The UvrA, UvrB and UvrC proteins of *E.coli* form multiple complexes which perform different activities during the course of damage recognition and incision. In contrast to repair enzymes which act on uniquely damaged nucleotides, the UvrABC nuclease repairs a wide variety of DNA lesions produced by different agents. Consequently, the recognition of many different lesions may require a mechanism where something more general than a specific chemical or structural alteration is recognized (Boyce and Howard-Flanders, 1964; Hanawalt and Haynes, 1965; Friedberg *et al.*, 1995). The observation of helicase activity of the UvrAB complex provided a basis for recognizing diverse types of DNA damage. It was shown that UvrA and UvrB, acting together in a UvrAB complex, can unidirectionally displace annealed oligonucleotides of 50 or fewer bases in length from single-stranded (ss) DNA in the presence of ATP, and that this displacement was inhibited by UV irradiation of the substrate (Oh and Grossman, 1987, 1989). This feature of UvrAB stimulated several hypotheses and models for damage recognition requiring helicase-driven translocation (Seeley and Grossman, 1990; Koo *et al.*, 1991; Thiagalingam and Grossman, 1993) and was incorporated into a prevailing model presented in a recently published book (Friedberg *et al.*, 1995). In this model, the UvrAB complex uses its helicase activity to track along DNA until the site of damage is encountered and the stable protein–DNA complex is formed. Although this might seem to be a very appealing model, some apparently relevant features of Uvr proteins are not included. For example, according to the described mechanism, the helicase activity, which requires both UvrA and UvrB, is necessary to find damage, but it is known that UvrA by itself has a significant damage-recognizing ability. UvrA preferentially binds to damaged DNA and, in fact, has been shown to give a well defined DNase I footprint at sites of damage in substrates containing a single specifically placed lesion (Seeberg and Steinum, 1982; Yeung *et al.*, 1983; Van Houten *et al.*, 1987; Bertrand-Burggraf *et al.*, 1991; Mazur and Grossman, 1991; Munn and Rupp, 1991; Visse *et al.*, 1992).

To evaluate further if the UvrAB helicase activity plays a central role in damage recognition in *E.coli*, the present study examines the protein–DNA interaction on substrates of various lengths with a single lesion at a known location. Our experiments utilize the ability of *N*-acetoxy-2-(acetylamino)fluorene (AAAF) to react specifically with guanine residues forming a bulky chemical adduct, dG-AAF [*N*-(deoxyguanosin)-2-(acetylamino)fluorene], that is an excellent substrate for incision by UvrABC (Fuchs and Seeberg, 1984; Sancar *et al.*, 1985; Seeberg and Fuchs, 1990). Substrates to measure strand displacement consisted of an oligonucleotide annealed to a ssDNA circle and were constructed to contain a single 2-(acetylamino)fluorene (AAF) lesion on either the oligomer or on the circle.

The data obtained show that the strand-separating activity of UvrAB may be inappropriate to support translocation of the UvrAB complex along DNA in order to locate damage, because it is limited and is stimulated by the presence of a lesion rather than being inhibited. Our interpretation of these data is that release of annealed

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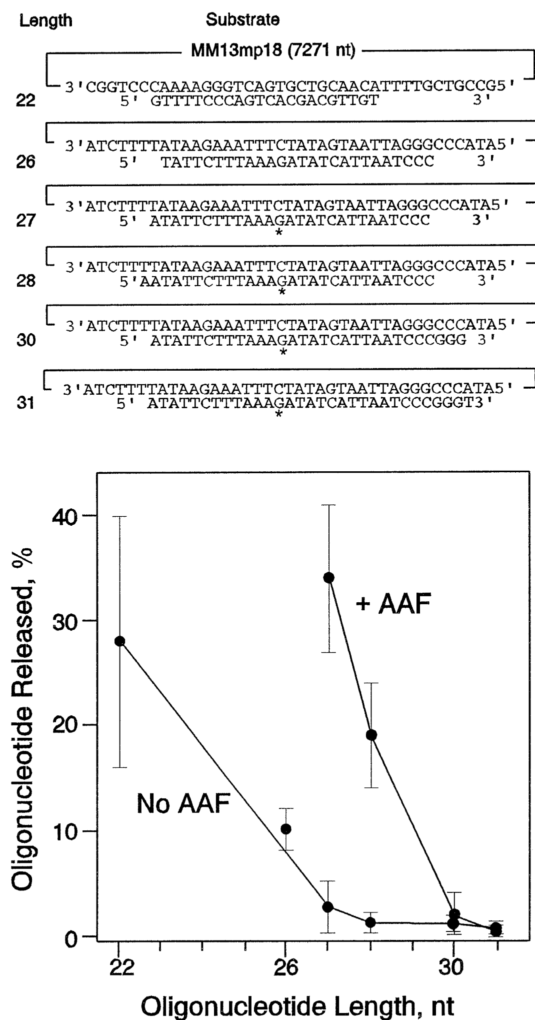

Fig. 1. Release of oligonucleotides by the UvrAB complex as a function of the length of the annealed fragment. The substrates for this experiment are shown in the top of the figure. Results of at least three separate experiments were averaged to give the data points and the standard deviations. For the curve labeled +AAF, substrates with a single AAF lesion on the G (designated with the *) in the oligonucleotides were prepared as described in Materials and methods.

oligonucleotides most likely occurs as a result of local changes caused by interactions of UvrAB with a damaged DNA site during pre-incision complex formation and is not due to helicase-driven translocation of the UvrAB complex to locate a lesion.

Results

As previously reported (Oh and Grossman, 1987, 1989), the UvrAB complex releases DNA fragments that are stably hybridized to ssDNA. To evaluate if this UvrAB activity can move the complex along DNA through distances suitable for locating damaged sites, we constructed substrates where oligonucleotides of length 22–31 bases were annealed to circular ssMM13mp18 DNA. The addition of purified UvrA and UvrB caused significant separation of a 22-mer from the circle, much less separation for the 26- and 27-mers and almost none for the 30- and 31-mer oligonucleotides (Figure 1, curve labeled 'no AAF'). This result demonstrates a sharp decrease in the ability of UvrAB to release an annealed fragment whose length is increased by only five nucleotides.

In a hypothesis for DNA damage recognition by UvrAB, the helicase-dependent translocation of the complex is used to find a damaged site while moving in a 5′ to 3′ direction along the strand to which the complex is bound (Seeley and Grossman, 1990; Thiagalingam and Grossman, 1993). A model for this hypothesis was implicit in the studies from the Grossman laboratory and was formulated more explicitly by Friedberg (Friedberg, 1994; Friedberg *et al.*, 1995). In this model, the translocating damage recognition enzyme is blocked or bound when it reaches a lesion. Accordingly, on our substrates, the UvrAB complex would presumably move along the ssDNA circle and would be expected to remove an annealed oligonucleotide without regard to the presence of a lesion on it. Conversely, the presence of the lesion on the ssDNA circle should block the progression of the complex and cause inhibition of its strand-separating activity. This model was supported by the observation that UV irradiation of substrates with short DNA sequences hybridized to ssDNA circles caused ~3-fold inhibition of the UvrAB complex strand-separating activity (Oh and Grossman, 1989). Because in those experiments the lesions were introduced randomly into the entire substrate and their exact locations were unknown, we studied substrates with a single lesion at a known location.

To determine how the presence of a lesion affects the UvrAB-dependent release of a fragment, we first constructed substrates with a single AAF lesion in an oligonucleotide annealed to a ssDNA circle. The presence of the AAF significantly increased the release of a 27-mer by UvrAB (Figure 2). The release of this oligonucleotide after 40 min of incubation was ~12 times greater than the release of the same oligonucleotide without a lesion. Because of the effect of the AAF adduct on this release, it is relevant to consider whether the duplex region in these substrates might be too small to support damage-dependent binding of UvrAB and incision by UvrABC, since it is known that UvrABC incision is very inefficient (<1%) on 25 and 32 bp duplex substrates with a single psoralen monoadduct (Van Houten *et al.*, 1987). In our experiments, we routinely observe an efficient incision of AAF (25–50%) on substrates where the duplex region is 27–28 bp (data not shown) and conclude that our short duplexes with flanking ssDNA are much better substrates for UvrABC excinuclease than those previously studied by Van Houten *et al.* (1987).

Because the length of the annealed fragment had a profound effect on the strand-separating activity of UvrAB (Figure 1), we then determined how a lesion affected this dependence by using substrates with an AAF adduct on oligonucleotides of 27, 28, 30 and 31 bases. The presence of the lesion increased the release of the 27- and 28-mer oligonucleotides by UvrAB (Figure 1, +AAF curve). However, the presence of AAF had no appreciable effect on the release of the 30- and 31-mers: neither could be separated effectively from the ssDNA circle (Figure 1, +AAF curve). This result shows that the UvrAB-mediated release of oligonucleotides, either with or without DNA damage, is markedly length dependent, with a sharp decline in the displacement occurring over an increased length of only a few nucleotides.

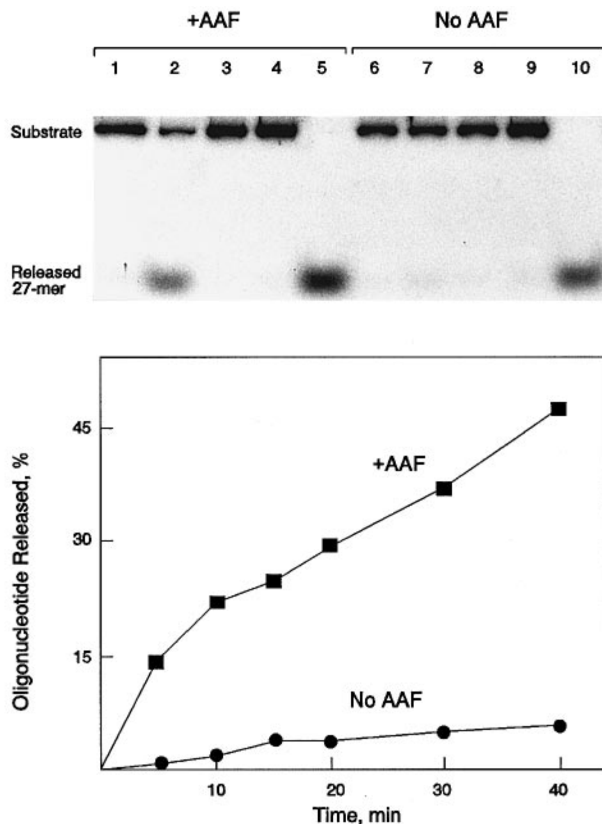

Fig. 2. Effect of an AAF lesion in a 27-mer oligonucleotide on its release by the UvrAB complex. The structure of the 27-mer substrates is shown in Figure 1. Top, autoradiogram of a non-denaturing gel showing release of annealed oligonucleotides. The standard reactions are shown in lanes 2 and 7 (30 min incubation with UvrAB). Controls are shown in lanes 1 and 6 (+UvrAB, no incubation), lanes 3 and 8 (no UvrAB, no incubation) and lanes 4 and 9 (no UvrAB, 30 min incubation). Lanes 5 and 10 show substrates denatured for 5 min at 85°C. Bottom, time course for the release of oligonucleotide from the substrate.

To study the effect of having the lesion on the circle rather than on the oligonucleotide, we constructed substrates where the single AAF lesion was on a specific residue of the ssDNA. Oligonucleotides complementary to the sequence with the AAF were annealed to the ssDNA circle (Figure 3). The substrates were prepared with the consideration in mind that the oligonucleotide must be short enough to be released by UvrAB in the absence of a lesion. As shown in Figure 1, the 26-mer is released from the substrate with an efficiency of ~10%. Consequently, we chose a 26-mer (Figure 3, substrate 1) because we could then measure inhibition of helicase activity if it occurs. Contrary to the expectation from the damage recognition model mentioned above, we did not observe inhibition of UvrAB activity on substrate 1. Instead, the release of annealed oligonucleotide was ~12 times higher compared with the same 26-mer annealed to a circle without a lesion (3 ± 1% release; three experiments). The UvrAB activity was also enhanced on the 27-mer (Figure 3, substrate 2), being ~8 times higher compared with the release of the same 27-mer from a substrate without a lesion (3 ± 2% release; three experiments). UvrAB released only 12% of the 30-mer oligonucleotide (Figure 3, substrate 3), showing a length dependence for the substrates in Figure 3 similar to that for the substrates in Figure 1. In Figure 3, the structures of the substrates are shown schematically based on the assumption from the translocation model that UvrAB moves in a 5′ to 3′ direction along the single-stranded circle until it reaches the lesion and stops there. As can be seen (Figure 3), all three substrates have the same number of base pairs on the 3′ side of the lesion. A reasonable expectation is that the activity of UvrAB would be the same on these substrates if the moving complex is blocked at the lesion. The data obtained clearly show that this is not the case, with UvrAB activity being different for all three substrates.

The effect of a specifically placed AAF lesion on the UvrAB release of a 27-mer annealed to a ssDNA circle is summarized in Figure 4. We observe that the presence of an AAF lesion on the oligonucleotide or the presence of the same lesion on the ssDNA circle stimulates the strand-separating activity of the UvrAB complex. These observations seem to contradict the predictions of a model for damage recognition in which an ATP-dependent helicase drives the UvrAB complex along undamaged DNA until it reaches a lesion where translocation is inhibited.

Discussion

The recognition of damage in DNA and the dual incision at those sites by UvrA, UvrB and UvrC proteins is accomplished by a complex series of reactions. An ATP-dependent helicase activity that releases oligonucleotides annealed to long ssDNA molecules, one of the reactions observed for UvrA and UvrB proteins (Oh and Grossman, 1987, 1989), is associated with an important step during damage recognition prior to incision because the site-specific conversion of Lys45 in the ATPase motif of UvrB protein inactivated helicase activity and prevented the dual incisions and the interaction of the mutant protein with damaged sites, but did not interfere with the interaction of UvrB with UvrA and of the UvrAB complex with undamaged DNA (Seeley and Grossman, 1990). It was concluded that this was a 'key step in nucleotide excision repair involving the UvrB ATPase-dependent translocation of nucleoprotein complexes from undamaged to damaged DNA sites' (Seeley and Grossman, 1990). Because of the putative key role of helicase activity in damage recognition, we examined the ability of the UvrAB complex to remove annealed oligonucleotides of different lengths and determined the effect of specifically placed lesions on this removal.

The UvrAB protein complex shares some similarities with known helicases: it separates DNA strands unidirectionally from 5′ to 3′ in a reaction requiring ATP hydrolysis, and it needs at least 10 nucleotides of ssDNA to initiate its separation activity (Oh and Grossman, 1987, 1989). However, in marked contrast to other helicases, the UvrAB activity has a steep dependence on the length of the annealed fragment and does not release oligonucleotides longer than 30 bases (Figure 1). Typically, helicases separate stretches of duplex DNA ranging from 50 to 25 000 nucleotides long and use the energy of NTP hydrolysis to move unidirectionally along DNA, disrupting the hydrogen bonds linking the two strands (Matson and Kaiser-Rogers, 1990; Matson, 1991). The helicase activity

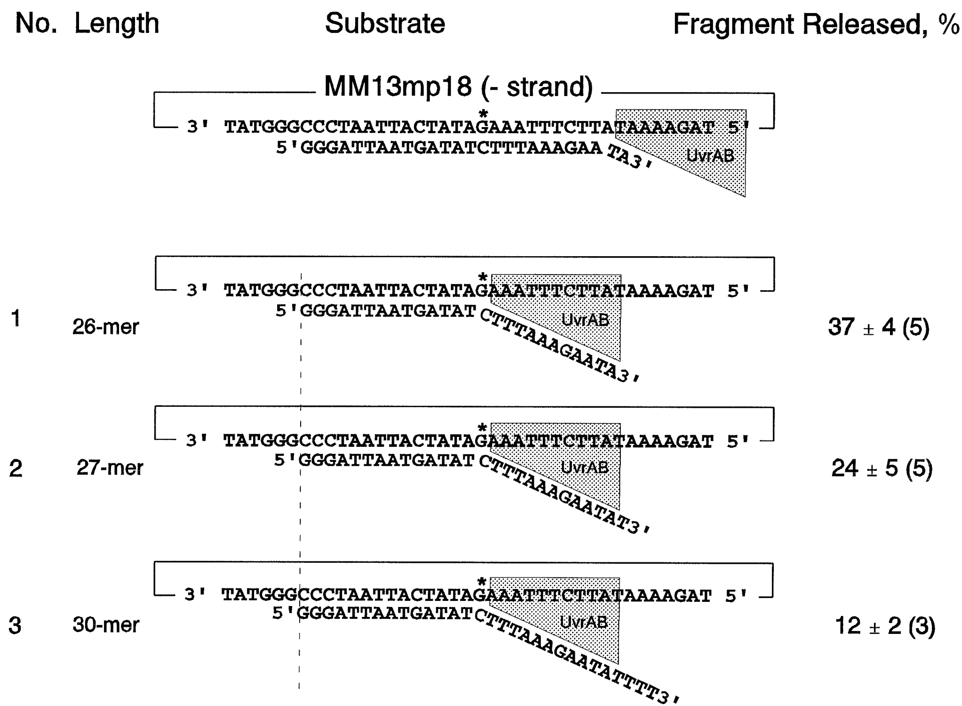

Fig. 3. Release of annealed oligonucleotides by the UvrAB complex from substrates with an AAF lesion placed on the ssDNA circle. A single AAF lesion (designated with the *) was placed on the minus strand of MM13mp18 as described in Materials and methods. The numbers in parentheses are the number of experiments done for each substrate. The substrates are presented here in the form of a model in which the UvrAB complex binds to the ssDNA circle and moves along that strand in a 5′ to 3′ direction displacing the annealed strand until becoming blocked at the site of damage in the circle. For these substrates, the identical length of duplex extends beyond the lesion on its 3′ side (with regard to the circular strand). Instead of inhibiting or preventing release of the annealed fragments, the AAF lesion in the circular strand actually increases the release of the fragments, in contrast to the predictions from this model.

of the UvrAB complex was classified as being distributive (Matson, 1991). A length dependence for fragment separation has been seen in distributive helicases, but it is not as marked as we have observed with UvrAB, where an increase in length of only 5–8 nucleotides can prevent release of an oligonucleotide from the duplex almost completely. For example, Rep protein and helicase IV unwind 119-mers from the duplex regions less effectively than 71-mers (Yancey-Wrona *et al.*, 1992). However, the decrease in activity observed for these helicases is much less dramatic than the decrease in the activity of UvrAB from 28% for a 22-mer to 2.5% for a 27-mer (see Figure 1). It is noteworthy that not all proteins that can release an annealed oligonucleotide are automatically called helicases. For example, a strand-separating activity, also requiring ATP hydrolysis and an initial ssDNA region, was described for the RecA protein (Bianchi *et al.*, 1985). However, because it did not unwind duplexes longer than 30 nucleotides, RecA was not classified as a helicase and its unwinding activity was attributed to cooperative binding to ssDNA. Likewise, the inability of UvrAB to unwind duplexes longer than 30 nucleotides distinguishes its activity from that of typical helicases.

We have shown that the presence of a lesion on the annealed 27-mer oligonucleotide sharply increased the strand-separating activity of UvrAB from 2.5 to 34%. This increase was also length dependent: the release of a 30-mer was ~2% and was only marginally affected by the lesion. When the lesion was placed on the single-stranded circle, the release of the 27-mer was stimulated to 24%, but the release of the 30-mer was much less. This

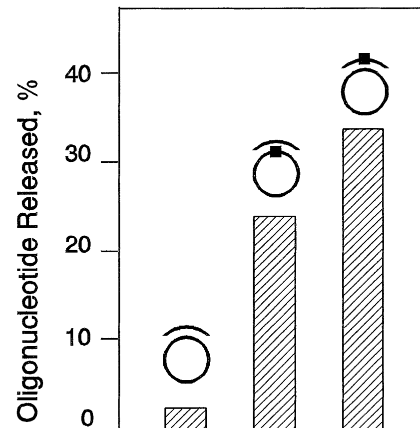

Fig. 4. Summary of the effect of a single AAF lesion present on either the oligonucleotide or the single-stranded circle on the release of an annealed fragment by the UvrAB complex. Data are taken from Figures 1 and 3 for substrates with a 27-mer oligonucleotide annealed to the ssDNA circle. In either configuration, a lesion significantly increases the release of the oligonucleotide. Key: left, no lesion; center, single lesion present on the circle; and right, single lesion present on the oligonucleotide.

stimulation of release of the annealed fragment due to the presence of a lesion on either the oligonucleotide or on the single-stranded circle seems inconsistent with a damage recognition mechanism where the protein translocates along a DNA strand and locates a lesion by being stopped at that site. In comparison, Rad3 protein of *Saccharomyces cerevisiae* has helicase activity and is proposed to be a damage recognition protein in nucleotide excision repair,

where it is supposed to scan the DNA strand to which it is bound until it is blocked by a lesion (Harosh *et al.*, 1989; Naegeli *et al.*, 1992, 1993; Friedberg, 1994; Friedberg *et al.*, 1995). This mechanism resembles the one proposed for UvrAB (Friedberg, 1994; Friedberg *et al.*, 1995). The helicase activity of Rad3, however, differs from the strand-separating activity of the UvrAB complex in several important aspects. First, Rad3 displaces much longer duplexes (>800 nucleotides) than UvrAB and does not seem to have a defined upper limit to the length of duplex which it can unwind (Sung *et al.*, 1987). Second, the inhibition of Rad3 helicase activity by DNA lesions is strand specific and corresponds to the strand along which the protein presumably translocates. The extent of release by Rad3 was the same for both an unirradiated and a UV-irradiated 206-mer annealed to a ssDNA circle. UV irradiation of the ssDNA circle markedly inhibited the helicase activity of Rad3, and the same inhibition was observed when the entire partial duplex substrate was irradiated (Naegeli *et al.*, 1992). The activity of the *E.coli* UvrAB complex was inhibited by UV irradiation of the helicase substrate with an 18-mer annealed to ssDNA (Oh and Grossman, 1989). However, irradiation of only the ssDNA prior to annealing with the same 18-mer did not cause inhibition of UvrAB-mediated release of the fragment (I.Gordienko and W.D.Rupp, unpublished observation), although the inhibition is expected by extrapolation from the data on a similar substrate with the Rad3 protein of *S.cerevisiae*. These results show that helicase activity of Rad3 is uniquely sensitive to damage in the DNA strand on which it is presumed to be bound during translocation, thus providing a mechanism to explain how it could be a damage recognition helicase. Since in our experiments the presence of a lesion on either strand stimulated UvrAB strand-separating activity (see Figure 4), this indicates that the role for UvrAB is probably different from that of a damage recognition helicase that tracks along one DNA strand until it is blocked by a lesion.

The observation by Koo *et al.* (1991) that positive and negative supercoiled domains were introduced into double-stranded (ds) DNA in the presence of UvrAB was interpreted as evidence for the translocation of the protein complex along DNA. However, the supercoiling in their experiments was stimulated by UV irradiation, which, according to the damage recognition model, should stop the movement of the translocating complex and decrease the supercoiling activity. An alternative explanation could be that local specific changes during the assembly of a UvrAB-mediated complex at the damaged site generate the reported changes in supercoiling and that these interactions result in the release of annealed oligonucleotides measured in the helicase assay.

Additional support for the idea that the UvrAB complex acts locally at a damaged site comes from DNA footprinting experiments in which UvrA alone has been shown to leave a footprint of ~33–37 nucleotides on psoralen- or cisplatin-modified DNA. (This shows that UvrA without UvrB is able to locate DNA damage and suggests that the activity that requires both UvrA and UvrB acting together is something other than the initial recognition of a damaged site.) Meanwhile, UvrA and UvrB together protect only 19–20 nucleotides, which, in fact, may be due to UvrB alone (Van Houten *et al.*, 1987; Bertrand-Burggraf *et al.*, 1991; Munn and Rupp, 1991; Visse *et al.*, 1992, 1994). Other information shows that in the UvrAB–DNA complex, UvrB is in close contact with the damaged site (Orren *et al.*, 1992), and that the DNA in the UvrB–DNA complex is sharply bent (Shi *et al.*, 1992). Since UvrB itself has little or no affinity for dsDNA (Kacinski and Rupp, 1981; Yeung *et al.*, 1986a; Caron and Grossman, 1988; Hsu *et al.*, 1995), it is likely that the formation of a stable UvrB–DNA complex requires significant local conformational changes at the UvrAB-damaged DNA binding site. During this process, UvrAB presumably uses ATP hydrolysis as an energy source in preparing a damaged site for successful incision (Moolenaar *et al.*, 1994). Accordingly, the defect in the UvrB protein caused by mutation at Lys45 in the ATPase motif can be interpreted as interfering with the UvrAB-mediated pre-incision complex formation rather than as a defect in helicase-driven translocation along undamaged DNA to a damaged site as previously suggested (Seeley and Grossman, 1990). Our data are consistent with the concept that local activity of the protein complex at the lesion causes the release of annealed oligonucleotides by UvrAB. First, this release is about the same with AAF being placed either on the oligonucleotide or on the ssDNA. Second, the limitations on the length of the oligonucleotide that can be released are similar regardless of the strand on which the lesion is located. Thus, we conclude that it is unlikely that damage recognition is accomplished by a mechanism involving helicase-linked translocation of UvrAB along undamaged DNA. UvrAB strand-separating activity (originally termed 'helicase' activity) is probably a manifestation of an intermediate step in DNA repair that occurs after the initial recognition of the damaged site but before incision. We call this step 'high resolution recognition' and consider it to be a part of a multistep damage recognition process (Gordienko and Rupp, 1997, accompanying paper). During this step, the UvrAB complex, while hydrolyzing ATP, acts locally at the damaged site to load and precisely position UvrB so that incision with UvrC can then occur. This 'high resolution recognition' of damage may require opening of the hydrophobic interior of the DNA molecule to allow appropriate positioning of UvrB. The opening may be associated with localized conformational changes limited to a few nucleotides. The activity of UvrAB may also include bending of DNA and local unwinding of dsDNA. Any of these could lead to destabilization of the substrates used in our experiments, with the resulting release of the annealed oligonucleotide. Thus, we suggest that what was termed 'helicase activity' is, in fact, a release that occurs as a result of specific recognition and positioning of Uvr proteins at a damaged site and that this activity is not evidence for a mechanism of damage recognition that requires helicase-mediated translocation of UvrAB along undamaged DNA.

Materials and methods

Enzymes

UvrA and UvrB were purified by published procedures (Sancar and Rupp, 1983; Yeung *et al.*, 1986b). T4 polynucleotide kinase and β-agarase I were purchased from New England Biolabs. T4 DNA polymerase was the generous gift of W.Konigsberg, Yale University

School of Medicine. The T4 accessory proteins, the 44/62 complex and the 45 protein, were purified in our laboratory by M.Munn using published procedures (Morris *et al.*, 1979; Nossal, 1979; Rush *et al.*, 1989). T4 DNA ligase, Klenow fragment of DNA polymerase I and uracil-DNA glycosylase were purchased from Boehringer Mannheim.

Construction of DNA for substrates with a single AAF lesion on the oligonucleotide

A derivative of M13, designated MM13mp18, was constructed in our laboratory by M.Munn in collaboration with E.Ackerman and T.Jenkins at NIH. The polylinker region of M13mp18 was modified to contain a single AAF target site and additional restriction sites. This DNA was used to transform *E.coli* TG1 cells for the preparation of both replicative form and ssDNA. AAAF reacts specifically with guanine residues in DNA, predominantly forming a covalent bond between the 2-amino group of AAAF and C-8 of guanine (Kriek *et al.*, 1967). The synthetic DNA oligomer, complementary to the target region of the ssMM13mp18, was reacted with AAAF to form a dG-C8-AAF adduct at the single guanine residue. The AAAF-modified oligonucleotides were gel purified as described previously (Hansson *et al.*, 1989). The specifically modified oligonucleotides or the unmodified oligonucleotides were annealed to ssMM13mp18 and used as a substrate or as a primer for further extension.

Construction of DNA for substrates with a single AAF lesion on the single-stranded circle

To place an AAF lesion at a specific position on ssDNA, we started with MM13mp18 phage grown in Luria broth with uridine [host strain CJ236 (*dut⁻ung⁻*F′)] and obtained ssMM13mp18 DNA with uracil replacing the thymine according to standard procedures (Cormack, 1994). The 25 nucleotide long DNA oligomer, 5′-ATATTCTTTAAAGATAT-CATTAATC-3′, was modified with AAAF to have an adduct and annealed to uracil-containing MM13mp18 ssDNA at 37°C for 30 min. These primed circles were converted to covalently closed duplex circles using the T4 DNA polymerase and its accessory proteins plus T4 DNA ligase (Kodadek and Gamper, 1988). The proteins were removed by phenol extraction and DNA was ethanol precipitated. After dissolving in H_2O, DNA was passed through a G50 Sephadex column (Boehringer Mannheim), equilibrated with glycosylase incubation buffer [60 mM Tris–HCl, pH 8.0; 1 mM EDTA; 1 mM dithiothreitol (DTT); 0.1 mg/ml bovine serum albumin (BSA)] and digested with uracil-DNA glycosylase in order to remove the uracil (U)-containing strand. The synthesized minus strand, containing the AAF adduct, was separated by gel electrophoresis in 0.8% low melting agarose with 1 µg/ml of ethidium bromide and purified by phenol extraction. After ethanol precipitation, the DNA was treated with uracil-DNA glycosylase again to remove traces of U-containing DNA and closed circles were separated from open circles by electrophoresis on a 1.8% low melting agarose gel. The final product, ssDNA circles with the single AAF adduct at a defined position, was purified from the agarose gel by digestion with β-agarase I and ethanol precipitated.

Preparation of helicase DNA substrates with no lesions or with one lesion on the oligonucleotide

For preparation of helicase substrates, we used established procedures (Oh and Grossman, 1987, 1989) with some modifications. Synthetic oligonucleotide (0.8 pmol) was mixed with 0.8 pmol of MM13mp18 in a sequencing buffer (40 mM Tris–HCl, pH 7.5; 10 mM $MgCl_2$; 50 mM NaCl) in a 10 µl reaction. The mixture was hybridized for 30 min at 37°C. The annealed substrates were labeled and extended with 5 U of Klenow fragment of DNA polymerase I in a 20 µl reaction in sequencing buffer and 5 mM DTT, 50 µCi [α-^{32}P]dNTP (3000 Ci/mmol, Amersham), together with 1 mM dNTP, if necessary. After incubation for 15 min at room temperature, the reaction was quenched with 50 mM EDTA, brought up to 50 µl with TE buffer and phenol extracted. Unincorporated label and unannealed oligonucleotides were removed by passing the mixture through two G50 Sephadex columns.

The substrate with a 22-mer was made by annealing a 17-mer ‘universal primer’ (USB), labeling it with dGTP and extending with dTTP. The substrate with the 27-mer was made by annealing a 25-mer and labeling and extending it with dCTP. For the substrate with the 28-mer, a 26 nucleotide long oligomer was annealed to a ssDNA circle and labeled and extended with dCTP. For the substrate with the 30-mer oligonucleotide, a 25-mer was annealed to the ssDNA, labeled with dCTP and extended with dGTP. The substrate with the 31-mer was made by annealing of a 25-mer to ssDNA circle, labeling it with dCTP and extending it with dGTP and dTTP.

The substrate with the 26-mer was labeled at the 5′ end of the fragment. We labeled 0.8 pmol of oligonucleotide with 20 µCi of [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and T4 polynucleotide kinase in a 10 µl reaction. Then NaCl to 50 mM and 0.8 pmol of MM13mp18 ssDNA were added. After the mixture was hybridized at 37°C for 30 min, we added 50 mM EDTA, brought the volume of reaction up to 50 µl with TE and passed the mixture through a G50 Sephadex column to remove any unincorporated label. After phenol extraction, the mixture was passed through a G50 Sephadex column again. Approximately 8 fmol of the substrate were used for one helicase reaction.

Preparation of DNA substrates with a lesion on the ssDNA circle

Eight pmol of oligonucleotide were labeled at the 5′ end with 100 µCi of [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and T4 polynucleotide kinase in a 10 µl reaction. After phenol extraction, unincorporated isotope was removed with a P6 polyacrylamide gel column (Bio-Rad). Then 0.8 pmol of oligonucleotide was annealed to 0.16 pmol of AAAF-modified ssMM13mp18 for 30 min at 37°C. The substrate was passed through two G50 Sephadex columns and ~1.6 fmol of this substrate were used for one helicase reaction. We brought up the amount of substrate to the usual ~8 fmol by adding an equivalent amount of ssDNA in the form of oligonucleotide (59-mer) to each reaction.

Helicase assay

The reaction mixture contained ~8 fmol (in ssDNA circles) of DNA substrate in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT and 2 mM ATP. The reaction (20 µl volume) was initiated by addition of UvrA and UvrB to a final concentration of 100 nM each and incubated at 37°C for 30 min. The reaction was quenched with 5 µl of stop solution [50% (v/v) glycerol, 1% SDS, 100 mM EDTA and 0.25% bromphenol blue]. The entire sample was then loaded onto a 12% non-denaturing polyacrylamide gel equilibrated with TBE running buffer. Electrophoresis was carried out at 120–150 V for 1–2 h. The gels were covered with plastic wrap and autoradiographed. Radioactivity was quantified by cutting out bands and counting them using Cerenkov radiation.

Every reaction mixture in the experiment had two controls: (i) a complete reaction stopped with no incubation; and (ii) a reaction without UvrA and UvrB incubated for 30 min at 37°C. The higher number obtained for oligonucleotide release in these controls was subtracted from the number obtained for reaction with UvrA and UvrB after 30 min of incubation. The percent of released fragment was calculated as: $[\text{product}_{\text{reaction}} - \text{product}_{\text{control}}] \times 100\% / [\text{product}_{\text{denatured for 5 min at 85°C}}]$.

Melting temperature of DNA substrates

Melting temperature (T_m) was estimated by incubating the helicase reaction mixtures containing DNA substrates without proteins for 5 min at different temperatures and quantifying the results as described for the helicase reaction. A plot of percent of fragment released as a function of temperature was used to estimate the temperature at which 50% of the substrate was dissociated (T_m). The T_m determined by this procedure was estimated to have an error of about ±1°C. The T_m of substrates without a lesion was 59–60°C for the 22-mer, 54°C for the 26-mer, 56–57°C for the 27- and 28-mers and 62°C for the 30–31mers. The T_m of substrates with a lesion on the oligonucleotide was 49–50°C for the 27- and 28-mers, 57°C for the 30-mer and 57–58°C for the 31-mer.

In experiments with short annealed oligonucleotides, we are aware that differences in the T_m of substrates might influence the results. Analyzing the data (Figure 1), we do not find a correlation between the UvrAB-mediated oligonucleotide release and T_m of the substrates. For example, the T_m of the 26-mer is lower than the T_m of the 22-mer, but the 26-mer is released by UvrAB less efficiently. The 27- and 28-mers with AAF have approximately the same T_m, but UvrAB activity on these oligonucleotides differs almost 2-fold. Release of the 27-mer with a lesion does not differ much from the 22-mer, but their T_ms differ by ~10°C. The T_m of the 22-mer is higher than that of the 27- and 28-mers without AAF, but UvrAB-mediated release of the 22-mer is almost 10 times higher than that of the 27- and 28-mers.

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