UvrAB activity at a damaged DNA site: is unpaired DNA present?

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To study the activity of the *Escherichia coli* UvrA and UvrB nucleotide excision repair proteins during the formation of the pre-incision complex at a damaged DNA site, we used substrates with modifications around a single 2-(acetylaminofluorene (AAF) lesion. Based on the release of AAF-containing oligonucleotides from a single-stranded DNA circle, we conclude that during interaction with our substrates UvrAB introduces changes in DNA which are localized at the lesion and are limited to 1–3 bp. Since these changes might include a denaturation of DNA at the lesion site and, consequently, a bubble structure might be present in a pre-incision complex, we studied incision activity of UvrABC excinuclease on substrates with 1–4 unpaired bases next to an AAF adduct. Opening more than one base on either or both sides of the lesion caused a significant decrease in the incision activity of UvrABC, but did not change the position of the incision sites. We conclude that the UvrAB action leading to a pre-incision complex does not include the formation of a bubble intermediate generated by extensive denaturation of base pairs.

Keywords: DNA repair/nucleotide excision repair/pre-incision complex formation/UvrAB complex

Introduction

Many details concerning the role of Uvr proteins in nucleotide excision repair in *Escherichia coli* have been reported, and different protein–protein and protein–DNA intermediates have been seen in various stages of the UvrABC reaction. However, the actual mechanism by which the UvrABC system recognizes lesions and forms specific complexes that precisely incise only the damaged strand remains ambiguous, although it is generally agreed that UvrA and UvrB proteins interact with each other and with damaged DNA prior to the actual incision step which requires UvrC. Formation of the functional pre-incision complex as a result of this protein–DNA interaction is thought to be accompanied by conformational changes in the damaged DNA structure. Several experimental approaches have provided relevant data. First, after addition of UvrB, the size of the UvrA footprint on damaged DNA is changed and a hypersensitive DNase I site in the protein–DNA complex appears (Van Houten *et al.*, 1987; Bertrand-Burggraf *et al.*, 1991; Munn and Rupp, 1991; Snowden and Van Houten, 1991; Visse

et al., 1992). Second, electron micrographs reveal that the UvrA₂B₁–DNA complexes can be distinguished from the UvrA₂–DNA complexes, and that both significantly differ from the UvrB–DNA complexes in which the DNA is sharply bent (Shi *et al.*, 1992). The electron microscopy data were discussed mainly with regard to damage recognition, but the possibility was kept open that the bending is a result of a pre-incision complex formation. Third, because the UvrAB complex possesses a limited helicase activity that unidirectionally displaces short oligonucleotides from single-stranded (ss) DNA using the energy of ATP hydrolysis, it was proposed that this activity unwinds DNA around the lesion in the protein–DNA complex such that the DNA is conformationally primed for incision and the pre-incision complex is formed (Oh and Grossman, 1987; Orren and Sancar, 1990). All these data imply that the joint interaction of UvrA and UvrB with damaged DNA during the formation of the pre-incision complex extends over several turns of the DNA helix and might cause changes in the DNA structure such as local denaturation, unwinding, bending or kinking. However, the details of this interaction and the type of specific changes produced in damaged DNA by the UvrAB complex need further clarification.

To study the step in the UvrABC reaction during which specific ATP-dependent interaction of UvrAB with DNA generates conformational changes at a damaged site which are necessary for the formation of a pre-incision complex (we call this step High Resolution Recognition), we constructed DNA substrates with modifications around a single specifically positioned 2-(acetylaminofluorene (AAF) lesion. We used the differences in the release of the modified AAF-containing oligonucleotides annealed to circular ssM13 DNA to study the interaction of UvrAB with a damaged site during formation of a pre-incision complex. To examine whether the action of UvrAB upon the lesion results in the denaturation of base pairs, we simulated structures that might be generated by localized helicase activity of the UvrAB complex by constructing bubble substrates where DNA was artificially denatured at the AAF lesion.

We conclude that in order to position UvrB in close contact with DNA to form a functional pre-incision complex, UvrAB uses its ATPase activity during a High Resolution Recognition step to change DNA very locally in the immediate vicinity of the lesion. These changes are limited to 1–3 bp and do not include an extensive unpairing of DNA bases.

Results

In our previous experiments in which the activity of the UvrAB complex was detected as a release of oligonucleotide from a circular ssDNA, we interpreted the results as

Fig. 1. Release of 27mer and 31mer oligonucleotides by the UvrAB complex. Top: scheme of substrates. The * shows the site of the AAF lesion. Bottom: autoradiogram of a non-denaturing gel showing release of annealed oligonucleotides. The standard reaction (30 min incubation with UvrAB) is in lane 2. Controls are in lane 1 (+UvrAB, no incubation), lane 3 (no UvrAB, no incubation) and lane 4 (no UvrAB, 30 min incubation). Lane 5 shows substrates denatured for 5 min at 85°C. (The background of free oligonucleotide in control lanes 1, 3 and 4 is higher for the 31mer because unannealed oligonucleotide is not separated from the substrate as effectively as the 27mer during the gel filtration step.)

due to specific interaction of the protein complex with damaged DNA causing destabilization of a substrate followed by the release of the annealed oligonucleotide (Gordienko and Rupp, 1997). We used the same assay in our current experiments to study the activity of the UvrAB protein complex at the site of a single AAF lesion. To check the sensitivity of our method, we constructed two substrates in which oligonucleotides of 27 or 31 nucleotides long, each with a single AAF lesion, were annealed to ssMM13mp18 DNA (Figure 1). The substrates were mixed together and the release of the annealed oligonucleotides by UvrAB was measured. As shown in Figure 1, the release of the 27mer is 35% while the release of the 31mer is so low that it is at the threshold of detection. A similar release by UvrAB was observed when the substrates were incubated separately (data not shown). Since the only difference between the substrates was the length of the DNA duplex region on the 3' side of the lesion, our results show that increasing the duplex region by only 4 bp significantly decreased the strand-separating activity of UvrAB.

To study further the effect of the size of the DNA duplex region on the activity of UvrAB at a damaged site, we constructed a set of substrates in which the length of the annealed oligonucleotide with an AAF adduct in the middle was kept at 28 nucleotides while the length of the

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Fig. 2. Effect of the length of the duplex region on oligonucleotide-releasing activity of the UvrAB complex. In substrate 1, the 28mer, 5'-AATATTCTTTAAAGATATCATTAAATCCC-3', was annealed to ssMM13mp18 DNA. Substrates 2-8 are related 28mers with one or two mismatched Cs incorporated at the indicated positions during oligonucleotide synthesis. Each substrate contains a single G-AAF lesion at the position marked by the *. Substrate 9 is a 30mer with two additional As at the 5' end and with two mismatched Cs on each side of the G-AAF. The number of contiguous base pairs is shown below each duplex region. The numbers in parentheses are the number of experiments done for each substrate.

duplex region on either side of the lesion was varied by introducing mismatches next to the adduct. As shown in Figure 2, reducing the duplex region on both sides of the lesion by 1 bp led to an increase in the oligonucleotide-releasing activity of the UvrAB complex (Figure 2, compare substrates 2, 3 and 4 with substrate 1). The substrates 2, 3 and 4 have the same duplex regions (12 bp on 5', 13 bp on 3') in equivalent positions with only the location of the AAF differing among them. Interaction of UvrAB with each of the three substrates causes similar release of the oligonucleotide (Figure 2). These data extend the results shown in Figure 1, indicating that in our experiments the length of the duplex region in DNA is more important than the exact position of the lesion in determining a substrate's stability during the interaction with the protein complex.

In the next subset of substrates (also with 28mer oligonucleotides), the position of the lesion was fixed but the length of the duplex region was reduced by 1 or 2 bp on the 3' side (Figure 2, substrates 5 and 6) or the 5' side (Figure 2, substrates 7 and 8) of the lesion. The release of oligonucleotides by the UvrAB complex was similar for substrates 5, 6 and 7 and did not differ much from substrates 2, 3 and 4 (Figure 2). However, decreasing the duplex region on the 5' side to 11 bp caused a substantial increase in the UvrAB-releasing activity (Figure 2, compare substrate 8 with substrates 1-7). Evidently, during the protein-DNA interaction, 11 paired nucleotides, even on one side of the lesion, is not enough to maintain the same level of substrate stability as it has with 12-13 bp. If this assumption is correct, then increasing the duplex

region on the 5' side of the lesion to 12–13 bp, as we have done with substrates 2–7 (even with two disrupted base pairs next to the lesion as in substrate 8), should return the UvrAB-mediated release of the oligonucleotide to the numbers observed for substrates 2–7. As shown in Figure 2, the UvrAB activity on substrate 9 (a 30mer) is 38%, which is close to the value obtained on substrate 6 which also has a 13 bp duplex region on the 5' side of the lesion and a 12 bp duplex region on the 3' side.

In substrates 1–8 in Figure 2, the annealed oligonucleotides were of the same length and the duplex region was varied by introducing mismatches around the AAF adduct. To evaluate the possible effect of mismatches on the results, in another group of substrates we varied the length of the flanking duplexes by adding or removing base pairs directly (Figure 3). The release of oligonucleotides by the UvrAB complex for these substrates was as sensitive to the length of the duplex region as for the substrates shown in Figure 2 and followed a similar pattern (Figure 3).

From the data in Figures 2 and 3, we can formulate some correlations between the structure of DNA in the modified substrates and the UvrAB activity on these substrates. First, with a 12–13 bp duplex region on either side of the lesion, the strand-separating activity of the UvrAB complex is approximately the same (37–39%) regardless of the number of open bases around the lesion and the position of the lesion itself inside this opening (Figure 2, substrates 2–6 and 9). Second, when the number of base pairs on either side was decreased to 11 nucleotides (Figure 2, substrate 8; Figure 3, substrates 3 and 4), the amount of released oligonucleotide increased. Third, if the duplex region on either side of the lesion was long enough, for example 18 bp (Figure 3, substrate 6) or 25 bp (Figure 3, substrate 7), the releasing activity of UvrAB decreased substantially even though the other side was short (Figure 3, substrate 7). These results are in accordance with the interpretation that UvrAB–DNA interaction causes destabilization of the modified substrates that is reflected in systematic differences in the release of the various annealed oligonucleotides in our experiments. Therefore, we interpret our data as showing that UvrAB interacts with DNA directly at the site of damage and makes conformational changes in the DNA during the formation of a pre-incision complex. Because varying the length of the duplex region by 1–3 bp affected the UvrAB-dependent release of oligonucleotides from our substrates, we conclude that the number of base pairs that UvrAB alters near the lesion to form a pre-incision complex is not more than 1–3.

Since it has been shown that the UvrAB protein complex has a helicase activity, one possibility is that the action of UvrAB at a damaged site can result in local unwinding and/or denaturation of DNA around the lesion in preparation for incision (Oh and Grossman, 1987). To simulate potential intermediates of the UvrABC reaction with denatured DNA regions, we constructed bubble substrates which contained 1–4 mismatches around the AAF adduct on 28 or 30mer oligonucleotides annealed to a ssDNA circle. The incision activity of UvrABC on these substrates was measured. As can be seen in Figure 4, incision is observed readily and produces a 10mer in each case, which corresponds to incision between the 4th and 5th nucleotide on the 3' side of the lesion. Disruption of 1 bp

Fig. 3. Effect of the number of base pairs on each side of the lesion on the oligonucleotide-releasing activity of the UvrAB complex. Top: structure of substrates. The * shows the site of the AAF lesion. The lengths of the duplex regions on both sides of the lesion are shown below each substrate. Bottom: release of oligonucleotides by UvrAB.

on either side of the lesion (Figure 4, substrates 2 and 3), or on both sides (Figure 4, substrate 1), does not reduce incision and is consistent with the possibility that this may be a normal intermediate for the UvrABC excinuclease and that opening of 1 bp might happen routinely during the reaction. In contrast, opening 2 bp on either side of AAF (Figure 4, substrates 5 and 6) causes considerable inhibition of UvrABC incision activity while the simultaneous opening of 2 bp on both sides of the lesion (Figure 4, substrate 7) almost eliminates the incision. Since the protein–DNA interaction in these substrates seems typical in that the position of the incision site did not change, we conclude from the pattern of reduced

Fig. 4. Incision of substrates with bubble structures by the UvrABC protein complex. The center diagram shows the relevant structures of the seven substrates used for the incision experiments presented in the left panel. Substrates 1–6 have 28mer annealed oligonucleotides derived from the 28mer shown at the top. Substrate 7 has a 30mer oligonucleotide with the same 3' end but with two additional As on the 5' end. The * marks the site of the AAF lesion. In the left panel, 30 and 28 show the position of intact oligonucleotides on the denaturing gel and 10 shows the position of the marker oligonucleotide, 5'-CATTAAATCCC-3', the product of incision expected from these 3' end-labeled substrates. (In matched control substrates with no lesion, no 10mer products were present.) The numbers in parentheses are the numbers of experiments done for each substrate. In the right panel, incision by UvrABC is shown for double-stranded substrates with or without a single AAF lesion. The substrates used are the double-stranded covalent circular derivatives of structures 1, 4 and 7 in the center diagram. N shows the position of the nicked incision product and C shows the position of the original closed circle on the agarose gel.

incision seen in Figure 4 that not more than 1 bp on either or both sides of the lesion is likely to be unpaired in a pre-incision complex.

The substrates used for the incision experiments consisted of ssDNA circles with an annealed short oligonucleotide and differed from a normal physiological substrate of UvrABC which is double-stranded (ds) DNA. Taking this into consideration, we constructed three substrates in which the structures of substrates 1, 4 and 7 from Figure 4 were embedded in covalent circular dsMM13mp18 DNA. The efficiency of incision on these synthetic double-stranded substrates did reproduce the relative order of UvrABC incision on the original substrates with the corresponding oligonucleotides annealed to the ssDNA circles (Figure 4). This result indicates that the data obtained with the model substrates do reflect events occurring in normal dsDNA.

Discussion

The UvrA and UvrB proteins of *E. coli* are both required during the early steps of nucleotide excision repair to form a DNA-protein complex at the damaged site that allows incision (with UvrC) to occur. In this study, we

were interested in the specific step where conformational changes in damaged DNA are taking place while the UvrA and UvrB proteins work together in an ATP-dependent reaction to bring UvrB into close contact with DNA to form a functional protein-DNA pre-incision complex. The existence of such a step can be inferred from various published data. For example, after the addition of UvrB, the UvrA-UV-damaged DNA complex is converted to a much more stable complex, containing UvrB (Yeung *et al.*, 1986a). During the interaction of UvrAB with a DNA-psoralen monoadduct, DNA-DNA cross-linking with the complementary strand is prevented, while the monoadduct can react directly with UvrB to generate a protein-DNA cross-link (Orren *et al.*, 1992). Van Houten *et al.* (1987) concluded that, in the presence of UvrB, the binding affinity of UvrA for the substrate with a psoralen monoadduct increases and observed that a DNase I-hypersensitive site appears on the 11th nucleotide 5' to the lesion. This hypersensitive site was attributed to bending of DNA at that position (Lin *et al.*, 1992). [It was shown by electron microscopy that DNA is bent or kinked by 130° after formation of the UvrAB ternary complex (Shi *et al.*, 1992).] In the DNase I footprint, UvrA alone protects 33–37 nucleotides on psoralen- or

cisplatin-modified DNA, while UvrA and UvrB together protect 19–20 nucleotides (Van Houten *et al.*, 1987; Munn and Rupp, 1991; Visse *et al.*, 1992) although a value of 45 nucleotides for UvrAB protection has also been reported by Bertrand-Burggraf *et al.* (1991). The possibility that a significant single-stranded region might be present in DNA is suggested from topological data which were interpreted as demonstrating that Uvr protein–DNA complex formation results in DNA helix unwinding estimated to be about one helical turn (Oh and Grossman, 1986; Seeley and Grossman, 1990). Experiments with diethylpyrocarbonate (DEPC), which reacts with unpaired adenines, supported the idea that UvrAB could cause unpairing since DEPC-sensitive sites were observed in the immediate vicinity of the lesion after interaction with UvrAB (Lin *et al.*, 1992; Visse *et al.*, 1994).

A common feature of the available data is that they describe the final structures resulting from the action of UvrA and UvrB. Our approach differs from that of others in that the method used is responsive to the stability of the substrate during the time when the interaction of UvrAB with damaged DNA is taking place. In our experiments on the substrates with an oligonucleotide of the appropriate length, the protein–DNA interaction causes destabilization of the structure leading to the release of the annealed oligonucleotide (Gordienko and Rupp, 1997). Assuming that UvrAB-mediated changes occur at the lesion site, we modified DNA around the adduct to simulate destabilization that might be produced by protein activity. If the destabilization by the enzyme is similar or less than that introduced by the synthetic construction, further destabilization after addition of UvrAB is not expected and oligonucleotide release should not be increased. However, if synthetic modification involves more base pairs than those that are destabilized during the normal UvrAB reaction, the release of the oligonucleotide after interaction with UvrAB will be increased. The data presented in Figure 2 demonstrate that in the substrates with 28mers, a duplex region shorter than a total of 25 bp (with 12–13 bp being present on each side of the lesion) causes additional destabilization of the substrate during the protein–DNA interaction. Since 25 of the 28 nucleotides need to be paired (with 12–13 bp required on each side of the lesion), this means that alteration of Watson–Crick pairing by UvrAB activity is limited to three nucleotide pairs (including the nucleotide with an adduct). Thus, although the interactions of UvrA and UvrB with DNA extend over several turns of the helix (footprinting experiments, for example), the significant protein-induced destabilizing changes resulting in oligonucleotide release are much more limited.

Models for nucleotide excision repair are often drawn to show substantial unpairing of nucleotides in the pre-incision complex (Van Houten, 1990; Friedberg *et al.*, 1995). These models presumably originate from the estimate based on topological studies (Oh and Grossman, 1986) and also from the observation that the UvrAB protein complex has a helicase activity which dissociates short duplexes of DNA (Oh and Grossman, 1987). Because genetic experiments in which mutations were introduced into the ‘ATPase’ and ‘helicase’ motifs of UvrB have shown a strong correlation between the capacity to displace an oligonucleotide in the helicase assay and the ability to

form a pre-incision complex (Seeley and Grossman, 1989, 1990; Moolenaar *et al.*, 1994), it was reasonable to incorporate helicase activity into repair models. A possibility, suggested by Friedberg *et al.* (1995), is that when a site of damage is encountered, UvrAB unwinds DNA using its DNA helicase activity to form a protein–DNA pre-incision complex in which the DNA is conformationally primed for incision. If we assume that the essential activity of UvrAB is opening of DNA near the lesion to allow formation of the pre-incision complex, then unpaired DNA might persist until UvrABC incision occurs. To check the hypothesis that unpaired nucleotides are part of the normal pathway of nucleotide excision repair, we performed UvrABC incision experiments on bubble substrates with unpaired nucleotides around the lesion to simulate disruption of base pairs that might be introduced by UvrAB helicase activity. The rationale is that if UvrAB normally introduces an open region into DNA, the efficiency of incision by UvrABC on a substrate with a synthetic bubble of the same size should be comparable with that on the normal substrate with no bubble. If the size of the synthetic bubble is larger than the one introduced by UvrAB or if disruption of base pairs is not the change which the protein normally makes in the DNA around the lesion site, we expect incision to be less efficient. As we show in Figure 4, disrupting more than 1 bp on either side of the lesion decreases AAF incision by UvrABC.

Because of the idea that a bubble structure can be an intermediate on the pathway of nucleotide excision repair, other attempts have also been made to simulate relevant reactions. For example, experiments with Rad1 and Rad10 proteins of *Saccharomyces cerevisiae* and human XPG protein were designed to study if the junction of the duplex and the bubble is a recognition element for incision (O'Donovan *et al.*, 1994; Davies *et al.*, 1995). On a substrate with no lesion, it was shown that these proteins interact with a synthetic bubble structure 30 nucleotides long and incise the DNA at the ends of the bubble. In contrast to those results with eukaryotic repair proteins, our data suggest a much smaller upper limit for the size of a bubble that might be formed by the combined action of UvrA and UvrB. It is unlikely that a bubble of more than two nucleotides is produced during the interaction of UvrAB with an AAF lesion: in Figure 2, the introduction of any bubble (even one base in addition to the lesion) causes a destabilization of the substrate during the UvrAB reaction. (We would not anticipate such destabilization if the normal UvrABC incision pathway includes the generation of a bubble structure.) The extent of destabilization (as measured by UvrAB release of oligonucleotides in Figure 2) does not correlate with the bubble size, but does correlate with the length of the duplex region in the substrates. Since incision with UvrABC occurs at the ‘correct’ site on both the 3′ side (Figure 4) and the 5′ side (data not shown) of the AAF lesion in these substrates, we take this as evidence that the interaction of the Uvr proteins with modified substrates is not anomalous. The situation with the five nucleotide bubble (the AAF lesion plus two mismatched bases on each side) is particularly noteworthy. When a substrate containing this bubble is constructed with a 28mer oligonucleotide, the duplex structure is too unstable to permit meaningful experiments as 60% of the substrate dissociates under the normal

reaction conditions in the absence of UvrAB (and >90% in the presence of UvrAB) (data not shown). When this five nucleotide bubble is included in a 30mer, the increased stability of the substrate allows experiments to be done with the result that the release of oligonucleotide carried out by UvrAB is comparable with the other substrates containing duplex regions of similar lengths but with smaller bubbles (Figure 2, substrate 9). Although this substrate is incised poorly by UvrABC, the position of the cleavage site did not change, indicating that the enzyme does carry out a successful reaction on this substrate, although with considerably more difficulty than with a normal substrate containing no bubble (Figure 4, substrate 7), and is able to incise at the same distance from the AAF even though the lesion is present in the middle of a bubble region.

In the experiments with UvrABC, we observe up to 50% incision on the substrates where the oligonucleotides are 28mers (Figure 4). This argues strongly against a model in which an extensive bubble is created and incision occurs at a duplex–bubble junction. Applying such a model to our case, a bubble of ~12 nucleotides long would have to be generated to accommodate UvrABC incision at the correct locations, and we already have pointed out the extreme instability generated by a much smaller bubble of five nucleotides in the middle of a 28mer. Taken together, we interpret our data as meaning that a bubble is not an obligatory intermediate for incision and that, if there is a partially denatured or open DNA structure around the lesion in the pre-incision complex, it does not exceed one or two nucleotides.

Earlier in the Discussion, we briefly mentioned data that have been interpreted as supporting the unwinding and/or unpairing of DNA as an important part of the nucleotide excision repair mechanism. The topological changes caused by interaction of UvrA and UvrB with a lesion result in a different linking number (after treatment with a topoisomerase) that is approximately equivalent to unwinding one helical turn of DNA. It is these data of Oh and Grossman (1986) that primarily are cited as the evidence for significant bubble formation at a lesion by UvrAB. As pointed out by Cozzarelli *et al.* (1990), such topological changes can be due to wrapping DNA around protein or other conformational changes in DNA structure that do not require unpairing of bases or local denaturation.

Chemical probing experiments (Lin *et al.*, 1992; Visse *et al.*, 1994) with DEPC have not revealed an extensive denatured region, but have shown that some nucleotides near a lesion became sensitive to DEPC after interaction with UvrAB. However, DEPC data have to be interpreted with some reservations. The reaction is limited by sequence restraints because of its specificity for As. In addition, it is clear that a number of different changes in DNA structure can result in sensitivity to DEPC, so this sensitivity is not conclusive evidence that unpairing has occurred (Johnston and Rich, 1985; Toth, 1991). Failure to react with DEPC is also not necessarily evidence that the target As are in a duplex, since protein binding can prevent access of the chemical to normally sensitive sites (Hagler and Shuman, 1992). The interpretation of experiments on DNA–protein complexes is complicated further because proteins are typically more DEPC-reactive than DNA by several orders of magnitude (Saluz and Jost, 1993).

Fig. 5. Model for the activity of the UvrAB protein complex at sites of DNA damage. Proteins (UvrA₂ or A₂B) interact non-specifically with DNA (I) forming a weakly bound complex. This complex might partially dissociate without complete separation and then reassociate with DNA at a nearby site (limited diffusion, sliding) or it might separate from the DNA completely before attaching to a new site (dissociation–reassociation). More specific complex formation happens during the step which we designate Low Resolution Recognition (II). It occurs at a site where the helix is broken, more flexible or 'weak'. At these sites, the slight bending, initially present as a result of protein binding, may occur more easily, resulting in the protein–DNA complex being more stable at such a location. (If UvrB is not already in the protein complex, it can enter at this point.) The next step, which we designate as High Resolution Recognition (III), occurs when the UvrAB complex, formed during the Low Resolution Recognition step, uses ATP hydrolysis to bend DNA. There are at least two possible consequences of this step. F results in successful formation of a protein–DNA pre-incision complex (IV). At some sites, bending will result in the formation of a stable complex with UvrB in the bent DNA. The interaction of UvrC with this complex results in incision. R shows unsuccessful formation of a protein–DNA pre-incision complex. In some cases, UvrB will not be positioned properly in the DNA. This leads to dissociation of UvrAB from this particular site either by direct dissociation or by diffusion along DNA to another position. The balance between successful and unsuccessful pre-incision complex formation determines whether a particular site or lesion is a good substrate for incision. The circle represents a site or lesion that is incised efficiently, while the triangle represents a site or lesion at which the initial protein–DNA complex dissociates before incision occurs. Coupling several different selective steps together in a cascade greatly increases the specificity of the overall reaction so that the selectivity attained can be much greater than the discrimination that occurs between a 'specific' and a 'non-specific' site at any single step.

However, conformational changes other than unwinding and/or local denaturation can be generated in DNA by UvrAB activity. As shown by electron microscopy, DNA is bent or kinked sharply after interaction with UvrA and UvrB proteins (Shi *et al.*, 1992). In further studies, electron microscopy and flow linear dichroism were employed to probe the DNA conformation in protein–DNA complexes formed with mutant UvrB(D478A) protein and showed that DNA in these complexes is not bent or kinked (Hsu *et al.*, 1994). The properties of this mutant protein are consistent with its having a defect at the step in which UvrAB conformationally changes DNA during the formation of the pre-incision complex: it associates with UvrA normally and results in a normal sized footprint at a damaged site but does not produce the DNase I-hyper-

sensitive site and is defective in the UvrABC incision reaction (Lin *et al.*, 1992). Although bending was suggested to occur at the hypersensitive site 11 nucleotides from the lesion, the release of an annealed oligonucleotide in our assays is probably a consequence of the UvrB loading process and DNA bending at the lesion site, and is a manifestation of the specific step carried out at the lesion by the UvrAB protein complex in the presence of ATP, a step which we call High Resolution Recognition. Bending of DNA is presumably the reaction that is associated with destabilization of the DNA helix and might be accompanied by local denaturation of DNA bases. In this case, denaturation will depend largely on lesion structure and/or on distortion in DNA caused by a lesion. Data by Visse *et al.* (1994), which showed that the DNA structure in the pre-incision complexes formed on two different cisplatin lesions was sensitive to DEPC in one case but not in the other, are consistent with this interpretation.

Protein-induced DNA bending is a feature of many DNA binding proteins. It is likely that bending acts by facilitating protein-protein or protein-DNA contacts in complexes (Kahn and Crothers, 1992). Another possible role for bending to increase specificity has been discussed recently. For example, studies with Cro protein have shown that DNA in the protein-DNA complex at both specific and non-specific binding sites is bent (Erie *et al.*, 1994). Since bending requires significant energy input, it has been proposed that bending of DNA at non-specific sites increases the energy difference available for discrimination between specific and non-specific sites, thus resulting in better selectivity for the specific site (Erie *et al.*, 1994). Specificity also would be enhanced if a protein were designed to recognize a stable local structure such as a DNA bend or kink, or if the protein bends the DNA as it binds, or if the specific sites were more flexible, i.e. more easily bent, than non-specific sites (Pabo and Sauer, 1992). Any of these advantages of bending can be used by UvrABC excinuclease as a tool to increase the specificity for finding and repairing a damaged site. In conclusion, we propose two stages in the interaction of UvrA and UvrB with damaged DNA which might be linked together in a well synchronized process to accomplish damage recognition.

As a first stage, UvrA or UvrAB protein binds DNA forming a DNA-protein complex. This binding might result in some slight bending of the DNA backbone. If this is the case, then a preferred location for the DNA-protein complex will be a position where there is either a pre-existing 'weak spot' in the DNA helix or a latent one that is only revealed by binding of the protein. These sites of preferential positioning of the UvrA (or UvrAB) protein will include many of the lesions that are known to be substrates for UvrABC, but can also include other structures, such as mismatches (Gordienko and Rupp, manuscript in preparation) that may be poor or marginal substrates for the enzyme. This first step of preferential association at a 'weak spot' is the one that we call the Low Resolution step of damage recognition.

A second stage is that in which the specific activity of UvrAB is required to position the UvrB protein in close contact with DNA so that incision (with UvrC) can actually occur. During this step, energy-dependent DNA

bending takes place which might be accompanied by very localized and limited unpairing along with exposure of the hydrophobic DNA interior that finally results in the proper installation of UvrB in a sharply bent functional pre-incision complex. During this step, only a subset of those sites forming an association with the Uvr enzyme during the initial Low Resolution Recognition step will be processed further into functional pre-incision complexes. This is the step that we call High Resolution Recognition and which we studied here. We incorporate these stages into a model (Figure 5) in which local denaturation of DNA at the damaged site is not an obligatory intermediate of nucleotide excision repair.

Materials and methods

Enzymes

UvrA, UvrB and UvrC 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 and Klenow fragment of DNA polymerase I 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 single-stranded DNA. *N*-acetoxy-2-(acetylamino)fluorene (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 specifically AAF-modified oligonucleotides were gel purified as described previously (Hansson *et al.*, 1989), annealed to ssMM13mp18 and used as a substrate or as a primer for further extension.

Construction of substrates with a single AAF lesion in the dsDNA molecule

The 25 nucleotide long DNA oligomer, 5'-ATATCTTTAAAGATAT-CATTAATC-3', was modified with AAAF to have a single adduct and annealed with ssMM13mp18 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). EDTA was added to 20 mM after the reaction was completed. The synthesized covalent circular dsDNA molecules, containing the AAF adduct, were separated by electrophoresis in 0.8% low melting agarose (FMS) with 1 μ g/ml of ethidium bromide and purified by butanol and phenol extractions. After ethanol precipitation, the DNA was treated with β -agarase I and ethanol precipitated again.

Preparation of DNA substrates with an oligonucleotide annealed to a ssDNA circle

We used established procedures (Oh and Grossman, 1987) with some modifications. Synthetic AAAF-modified oligonucleotide (0.8 pmol) was mixed with 0.8 pmol of ssMM13mp18 in a sequencing buffer (40 mM Tris-HCl, pH 7.5; 10 mM MgCl₂; 50 mM NaCl) in a 10 μ l reaction. The mixture was incubated 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 μ l reaction in sequencing buffer and 5 mM dithiothreitol (DTT), 50 μ Ci [α -³²P]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 μ l with TE buffer and phenol extracted. Unincorporated label and unannealed oligonucleotides were removed by passing the mixture through two G50 Sephadex columns (Boehringer Mannheim).

The substrate with a 27mer (Figure 1 and substrate 2 in Figure 3) was made by annealing a 25mer to a ssDNA circle and labeling and extending it with dCTP. The substrate with the 31mer (Figure 1 and substrate 6 in Figure 3) was made by annealing a 25mer, labeling it with dCTP and extending it with dGTP and dTTP. For the substrates with the 28mers in Figures 2, 3 (substrate 1) and 4, a 26mer was annealed to a ssDNA and labeled and extended with dCTP. The substrate with the 30mer in Figures 2 and 4 was made by annealing a 28mer and labeling and extending it with dCTP. Substrate 5 in Figure 3 with the 27mer was made by annealing a 26mer and labeling and extending it with dTTP.

Substrates 3, 4 and 7 in Figure 3 were labeled at the 5' end of the fragment. First, 0.8 pmol of oligonucleotide was labeled with 20 μ Ci of [γ - 32 P]ATP (3000 Ci/mmol, Amersham) and T4 polynucleotide kinase in a 10 μ l reaction. Then NaCl to 50 mM and 0.8 pmol of MM13mp18 ssDNA were added. After the mixture was incubated at 37°C for 30 min, EDTA was added to 50 mM, the volume was brought to 50 μ l with TE and the mixture was passed through a G50 column to remove any unincorporated label. After phenol extraction, the mixture was passed through a G50 column again. Approximately 8 fmol of the appropriate substrate were used for each reaction.

Oligonucleotide-releasing assay

The reaction mixture contained ~8 fmol (in ssDNA circles) of DNA substrate in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 5 mM DTT and 2 mM ATP). The reaction (20 μ l volume) was initiated by addition of UvrA and UvrB to a final concentration of 100 nM each. After incubation at 37°C for 30 min, the reaction was quenched with 5 μ l of stop solution [50% (v/v) glycerol, 1% SDS, 100 mM EDTA and 0.25% bromophenol 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 percentage of released fragment was calculated as: $[\text{product}_{\text{reaction}} - \text{product}_{\text{control}}] \times 100\% / [\text{product}_{\text{denatured for 5 min at 85°C}}]$.

Incision reaction on substrates with an AAF-containing oligonucleotide annealed to ssDNA circle

The reaction mixture contained ~8 fmol (in ssDNA circles) of DNA substrate in buffer A. The reaction (20 μ l volume) was initiated by addition of UvrA and UvrB to a final concentration of 100 nM each and incubated at 37°C for 2 min. Then UvrC was added to 100 nM and the incubation was continued for 30 min. The reaction was quenched with 5 μ l of stop solution, heated for 2 min at 85°C and the entire sample was then loaded onto a 15% denaturing polyacrylamide gel equilibrated with TBE running buffer. Electrophoresis was carried out at 800–1000 V for 3–4 h. The gels were covered with plastic wrap and autoradiographed. Radioactivity was quantified by cutting out bands and counting them using Cerenkov radiation. The extent of incision was calculated as the ratio of the reaction product to the sum of product and unreacted substrate. Every reaction mixture in the experiment had at least two controls: (i) a reaction containing only UvrA and UvrB with UvrC omitted; and (ii) a reaction containing UvrB and UvrC with UvrA omitted. The controls were incubated like the complete reaction and showed that AAF incision required all three proteins: UvrA, UvrB and UvrC.

Incision reaction on double-stranded substrates

Reaction mixtures contained 100 ng of synthetic covalent circular dsDNA substrate per reaction in buffer A. UvrA and UvrB were added to final concentrations of 100 nM each. After incubation at 37°C for 2 min, UvrC was added to 100 nM. Aliquots of 20 μ l were removed at 2, 5, 10, 20 and 30 min after UvrC addition and reactions were stopped with 5 μ l of stop solution. The zero time point was removed before addition of UvrABC and was not incubated. The entire samples were then loaded onto 0.8% agarose-TBE gels containing 1 μ g/ml of ethidium bromide. Electrophoresis was carried out at 100–120 V for 1–2 h. The gels were then washed for 30 min and photographed in UV light with Polaroid Type 55 positive/negative film.

Melting temperature (T_m) of DNA substrates

The T_m was determined as described (Gordienko and Rupp, 1997) and estimated to have an error of about $\pm 1^\circ\text{C}$. The T_m of substrates in Figure 1 was 49–50°C for the 27mer and 57–58°C for the 31mer. The T_m of substrates in Figure 2 was 49–50°C for substrate 1, 47°C for substrates 3 and 4, 47–48°C for substrates 5 and 7, 46–47°C for substrate 2, 46°C for substrates 6 and 8 and 43–44°C for substrate 9. The T_m of substrates in Figure 3 was 49–50°C for substrates 1 and 2, 56°C for substrate 7, 57–58°C for substrate 6, 46–47°C for substrate 3 and 44–45°C for substrate 4.

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