Strand opening by the UvrA₂B complex allows dynamic recognition of DNA damage

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Repair proteins alter the local DNA structure during nucleotide excision repair (NER). However, the precise role of DNA melting remains unknown. A series of DNA substrates containing a unique site-specific BPDE-guanine adduct in a region of non-complementary bases were examined for incision by the *Escherichia coli* UvrBC endonuclease in the presence or absence of UvrA. UvrBC formed a pre-incision intermediate with a DNA substrate containing a 6-base bubble structure with 2 unpaired bases 5' and 3 unpaired bases 3' to the adduct. Formation of this bubble served as a dynamic recognition step in damage processing. UvrB or UvrBC may form one of three stable repair intermediates with DNA substrates, depending upon the state of the DNA surrounding the modified base. The dual incisions were strongly determined by the distance between the adduct and the double-stranded–single-stranded DNA junction of the bubble, and required homologous double-stranded DNA at both incision sites. Remarkably, in the absence of UvrA, UvrBC nuclease can make both 3' and 5' incisions on substrates with bubbles of 3–6 nucleotides, and an uncoupled 5' incision on bubbles of ≥10 nucleotides. These data support the hypothesis that the *E.coli* and human NER systems recognize and process DNA damage in a highly conserved manner.

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Introduction

A major problem in nucleotide excision repair (NER) is how a broad spectrum of bulky DNA lesions, which are chemically and structurally distinct, is recognized and processed by DNA repair proteins. It has been suggested that the relative ease with which the repair proteins can process the damage to produce a common intermediate is related to the incision efficiency (Grossman *et al.*, 1988). Characterization of these protein–DNA damage-processing intermediates will yield structural clues to damage recognition. The *Escherichia coli* UvrABC nuclease serves as an excellent system for addressing these questions due to its relative simplicity and the general similarity between the *E.coli* and eukaryotic NERs.

The *E.coli* UvrABC nuclease initiates NER through a series of integrated steps which culminate in scission of the damage-containing strand at two discrete sites (Van

Houten and McCullough, 1994; Lloyd and Van Houten, 1995). The UvrA protein forms a dimer that interacts with UvrB to form a heterotrimeric protein complex, UvrA₂B (Orren and Sancar, 1989, 1990). Upon binding of UvrA₂B to the site of damage, conformational changes occur in the protein–DNA complex, leading to the dissociation of UvrA₂ and formation of a stable pre-incision UvrB–DNA complex (Oh and Grossman, 1986; Shi *et al.*, 1992). After the release of UvrA, the UvrC protein interacts with the C-terminus of UvrB in the UvrB–DNA intermediate, and is believed to trigger an endonuclease activity in UvrB which cleaves a phosphodiester bond four to seven phosphates 3' to the damage (Lin *et al.*, 1992; Moolenaar *et al.*, 1995; Zou *et al.*, 1995). This event activates a second nuclease, presumably in UvrC, which incises the eighth phosphate backbone 5' to the damaged residue (Lin and Sancar, 1992; Zou *et al.*, 1995). The oligonucleotide containing the lesion is then removed from the DNA, the gap is filled by the dual action of DNA helicase II (UvrD) and DNA polymerase I, and finally DNA ligase joins the two ends of the nick.

Although significant progress has been made in understanding the overall mechanism of *E.coli* NER, the structural aspects of damage processing remain unknown. For example, little is known about the conformation of the DNA formed in the pre-incision intermediates which is required for successful dual incision by the UvrBC nuclease. It has been suggested that formation of the pre-incision complex places all DNA lesions in a common DNA structure that facilitates the incisions (Grossman *et al.*, 1988; Van Houten, 1990). Previously, we have shown that a structure-specific Y-shaped substrate could be recognized and incised 5' to the lesion by the UvrBC nuclease, even in the absence of UvrA (Zou *et al.*, 1997). This result suggests that a specific unpaired DNA structure forms with the UvrBC proteins at the damage site. Determining the precise DNA structure in the pre-incision intermediates may also help to define the UvrBC nuclease activity at both 3'- and 5'-incision sites, and the role of UvrA in the formation of the pre-incision intermediates.

The goal of this study was to define the region of DNA which needs to be melted in order to optimize damage processing. We have constructed unique flapped and bubble substrates containing regions of heterologous DNA sequences. We have determined the characteristics of local DNA structures formed in the UvrBC–DNA pre-incision intermediates, and the structural determinants for efficient incision. We have demonstrated that the UvrBC nuclease may have two types of binding modes to the substrates, corresponding to incisions on each side of the lesion. We have also shown that the UvrBC nuclease activity requires a double-stranded DNA and, like XPF-ERCC1 in human NER, is able to perform a 5' incision on a DNA lesion in a bubble structure, independently of 3' incision. In

addition, our results indicate that a major function of UvrA in the mechanism of *E.coli* NER is to interact with UvrB to unwind the DNA at a lesion. The recruitment of UvrC to this complex then leads to the formation of a pre-incision UvrBC–DNA complex containing a 6 unpaired base structure at the lesion.

Results

Construction of the BPDE-DNA substrates

All DNA substrates used in this study are shown in Table I. These 50 bp oligodeoxynucleotide substrates have a defined sequence containing a single (+)-*cis-anti*-BPDE-N²-dG adduct in the middle of the top strand. They were made by ligating a single BPDE-modified 11mer with flanking 20mer and 19mer (or none) oligonucleotides on the 5' and 3' sides, respectively, in the presence of a 55mer complementary strand (bottom strand). After purification, the ligated strand was reannealed with the appropriate bottom strand to form the substrates with specific structures. The substrates in Table I are grouped into two sets. In the first one, flap substrates F0–F12 have a nick at a site 5 bases 3' to the adduct, and serves like a product of 3' incision, with a free 3' end. The number of mismatched or unpaired bases from the nick to the 5' end of the modified strand varies from 0 to 12. The construction of these substrates was confirmed by the different migration of the substrates on an 8% native polyacrylamide gel (Figure 1A), and their digestion with restriction enzyme *RsaI* (data not shown) (Zou *et al.*, 1995).

The second set of substrates (B0–B12) has no nick at the 3' side, but forms a series of different sized bubbles starting from the second nucleotide 5' to the adduct through the ninth nucleotide 3' to the adduct (Table I). The DNase I footprinting clearly showed bubble formation as a region refractory to digestion for these substrates (Figure 1B). This approach is based on the differential nuclease activity of DNase I on single- versus double-stranded DNA. The latter is hydrolyzed 10⁴ times more rapidly than the former (Laskowski, 1971; Suck and Oefner, 1986). It should be noted that, consistent with the NMR structure (Cosman *et al.*, 1993), the (+)-*cis* BPDE-DNA adduct itself produces a limited local DNA denaturation at the adduct which inhibits the enzyme activity to generate a footprint (Zou *et al.*, 1995), as shown in lane 2 of Figure 1B. The slower migration of the 5'-labeled DNA in lanes 2–7 as compared with lane 1 is due to the presence of BPDE adduct in the DNA fragments (Figure 1B).

Incision of flap substrates by UvrABC and UvrBC nuclease

Our previous report indicated that a flap-type DNA substrate containing a site-specific adduct could serve as an analog of the DNA intermediate formed in the pre-incision protein–DNA complex (Zou *et al.*, 1997). This structure-specific DNA can be recognized and incised by the UvrBC nuclease in the absence of UvrA (Zou *et al.*, 1997). To determine the pre-incision intermediate structures at higher resolution, and to study the specific role of UvrA in facilitating the formation of these structures, we have investigated the interaction of UvrA, UvrB and UvrC with a series of structure-specific DNA substrates with different

size of flap structures (Table I). The flap structures with varying numbers of mismatched bases were designed to mimic the DNA structures induced by the action of UvrA₂B protein complex. These substrates were labeled at the 5' end of the top strand, and incised with either the complete UvrABC system or the UvrBC nuclease (Figure 2). For the complete UvrABC system, the incision efficiency increased slightly as the double-/single-stranded DNA (ds–ssDNA) junction moved away from the 3' nick. The incision efficiency reached a maximum for substrate F6, having 6 unpaired bases (Figure 2). Further strand opening of these substrates with 7 or 8 unpaired bases (F7 and F8) did not substantially increase the nuclease activity. The efficiency of incision started to decrease dramatically as the ds–ssDNA junction was nine or more nucleotides away from the 3' nick or the flap size increased to 9–12 bases (F9–F12), and the incision was accompanied by a 1–2 nucleotide shift of the 5' incision site further away from the adduct (Figure 2). As a result, little incision was observed for the flap substrate with 12 unpaired bases (F12). It should be noted that the presence of the DNA adduct is required for the efficient incision of the flap substrates (Zou *et al.*, 1997).

In comparison with the UvrABC system, the UvrBC nuclease, in the absence of UvrA, showed a completely different pattern of incisions on the substrates with the ds–ssDNA junction 0–7 nucleotides away from the 3' nick (F0–F7), but the same general pattern on those of F8–F12 (Figure 2). It can be seen that there is little or no 5' incision by UvrBC nuclease for the substrates with <7 unpaired bases. These experiments allowed us to separate the functions of UvrA and UvrBC, so that we could identify their specific roles and the intermediates formed in the NER mechanism. The same experiments with UvrABC and UvrBC have also been conducted at 24°C (data not shown). No difference in the results was observed, except that the lower temperature reactions required a longer time to produce the same amount of incision products. A gel mobility shift assay was also conducted to examine the formation of the UvrA₂B–DNA and UvrB–DNA complexes with these flap substrates in the presence of ATP as described previously (Zou *et al.*, 1997). Interestingly, the results showed that the UvrB–DNA complex formed with the substrates of F0–F6, but no such formation was observed with the substrates having a flap size >6, although F7 and F8 were cleaved most efficiently (data not shown). This suggests that the large size of flap DNA structures (with a 3' nick) destabilized the formation of the UvrB–DNA complex, and the formation did not correlate directly with the efficiency of 5' incision of these flap substrates since in this specific case, no UvrB–DNA formation was required for the 5' incision any more and the formation of the UvrBC–DNA complex become crucial. This is consistent with the fact that in the normal incision process with a normal substrate, the immediate preincision complex is UvrBC–DNA rather than UvrB–DNA. In fact, the lack of formation of the UvrB–DNA complex with the large flap substrates and no correlation between the formation and the incision confirmed the validity of using these substrates in this study.

Analysis of the data presented above strongly suggested that there was an optimized flap structure for 5' incision.

Table I. BPDE-DNA substrates constructed for the present study

Flap Substrates:	5'-incision	3'-incision	Substrates	# mismatched bases
GACTACGTACTGTTACGGCTCCATC G CTACCGCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGTAGCGATGGCGTTAGTCCGGTCTAGACG	↓	↓	F0	0
GACTACGTACTGTTACGGCTCCATC G ^{TACC} GCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGTAGCGTCTTCGTTAGTCCGGTCTAGACG	↓	↓	F4	4
GACTACGTACTGTTACGGCTCCATC G ^{CTACC} GCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGTAGCTTCTTCGTTAGTCCGGTCTAGACG	↓	↓	F5	5
GACTACGTACTGTTACGGCTCCATC G ^{CTACC} GCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGTAGTTTCTTCGTTAGTCCGGTCTAGACG	↓	↓	F6	6
GACTACGTACTGTTACGGCTCCAT C ^{GCTACC} GCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGTATTTCTTCGTTAGTCCGGTCTAGACG	↓	↓	F7	7
GACTACGTACTGTTACGGCTCCA T ^{GCTACC} GCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGTTTTTCTTCGTTAGTCCGGTCTAGACG	↓	↓	F8	8
GACTACGTACTGTTACGGCTCC A ^{TCGCTACC} GCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGCTTTTCTTCGTTAGTCCGGTCTAGACG	↓	↓	F9	9
GACTACGTACTGTTACGGCTC C ^{ATCGCTACC} GCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGTCTTTTCTTCGTTAGTCCGGTCTAGACG	↓	↓	F10	10
GACTACGTACTGTTACGGCT C ^{CATCGCTACC} GCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGATTCTTTTCTTCGTTAGTCCGGTCTAGACG	↓	↓	F11	11
GACTACGTACTGTTACGGC T ^{CCATCGCTACC} GCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGCTTCTTTTCTTCGTTAGTCCGGTCTAGACG	↓	↓	F12	12
Bubble Substrates:	5'-incision	3'-incision		
GACTACGTACTGTTACGGCTCCATC G CTACCGCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGTAGCGATGGCGTTAGTCCGGTCTAGACG	↓	↓	B0	0
GACTACGTACTGTTACGGCTCCA T ^C CTACCGCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGTCTTGATGGCGTTAGTCCGGTCTAGACG	↓	↓	B3	3
GACTACGTACTGTTACGGCTCCA T ^{CGCT} ACCGCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGTCTTTTCGGCGTTAGTCCGGTCTAGACG	↓	↓	B6	6
GACTACGTACTGTTACGGCTCCA T ^{CGCTAC} GCAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGTCTTTTCCCGTTAGTCCGGTCTAGACG	↓	↓	B8	8
GACTACGTACTGTTACGGCTCCA T ^{CGCTACCG} CAATCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGTCTTTTCCCTCTTAGTCCGGTCTAGACG	↓	↓	B10	10
GACTACGTACTGTTACGGCTCCA T ^{CGCTACCGCA} TCAGGCCAGATCTGC CTGATGCATGACAATGCCGAGGTCTTTTCCCTCACAGTCCGGTCTAGACG	↓	↓	B12	12

The bold G with the asterisk in the sequence represents the base at which a single BPDE was adducted, the arrows indicate the major incision sites, and the bar inserted between the bases (CG) in the top strand of substrate F0 indicates a pre-nicked site.

The presence of UvrA in the reaction facilitated the formation of the optimal structure in the pre-5' incision protein-DNA complex. As shown in Figure 2, the pre-existence of unpaired bases in substrates F0–F8 increased the efficiency of the 5' incision of the UvrABC system only slightly. This slight increase is consistent with the idea that the UvrA₂B complex contains a helicase activity and opens up the DNA helix (Lin *et al.*, 1992; Moolenaar

et al., 1994) to a structure similar to one of the defined structures in our substrates. The lack of incision for the same substrates by UvrBC nuclease, in the absence of UvrA, confirms the idea that UvrA plays a critical role in activating this helicase activity. Since UvrA is believed to participate in helicase activity only in the presence of UvrB, the unpaired structures in substrates F0–F8 should only have a slight effect on the incision in the presence

Fig. 1. Formation of specific structures of BPDE-DNA substrates (Table I) used in this study. (A) Gel mobility shift of the flap substrates F0–F12. The substrates migrate differently on an 8% non-denaturing polyacrylamide gel due to the different number of mismatched bases. (B) DNase I footprint of the bubble formation in the substrates B0–B12 on a 12% polyacrylamide sequencing gel (8 M urea) under denaturing conditions. The BPDE-G represents the base (guanine) adducted by a single BPDE.

of UvrA (Figure 2, lanes 1–10), but a dramatic effect in the absence of UvrA (Figure 2, lanes 11–20). This is fully consistent with the results presented in Figure 2. The decrease in incision efficiency for F8–F12 substrates, and the shift of the incision sites further away from the lesion for both UvrABC and UvrBC nuclease, indicate that: (i) the 5' nuclease requires fully double-stranded DNA; and (ii) the incision efficiency of the nuclease depends on the relative distance between the ds–ssDNA junction and the adduct. The site of the incision was directed by the position of adduct and also partially by the junction–adduct distance. As the result of these experiments, it can be concluded that the interaction of UvrA₂B complex with a BPDE-DNA adduct introduces a local DNA structural alteration which includes 2 unpaired bases 5' to the adduct.

Incision of bubble substrates by UvrABC nuclease

The experiments with the flap DNA substrates provide important information about the local DNA structures at the 5' side of adduct formed in the pre-incision complex. To gain a full picture of the DNA structure in the complex, and to understand the nature of the 3' incision produced by the UvrBC nuclease, we have investigated the effect of DNA structural changes on the 3' incision of either UvrABC system or UvrBC nuclease by constructing a set of DNA bubble substrates, B0–B12 (Table I). Using the information gained from our previous experiments, we have engineered different size bubbles which contain the optimal two unpaired nucleotides 5' to the modified guanine and 1–9 unpaired bases 3' to the adduct.

The primary 3' incision site of a normal substrate (B0) (Zou *et al.*, 1995) is five phosphates 3' to the adduct (as indicated by the 3' side arrow in Table I). Similarly to the results from the study with the flap substrates, the 5'-terminally labeled substrates where the bubble size varied from zero to six were incised efficiently by the UvrABC system (Figure 3A). As shown in the figure, a second 5' incision, especially on substrates B3 and B6, was observed. Moolenaar *et al.* (1998) and Gordienko and Rupp (1998) have recently reported a new damage-independent nuclease activity of UvrABC or UvrBC. Our investigation indicated that this second 5' incision, primarily on the bubble substrates used in the present study, was due to the activity of the damage-independent

Fig. 2. Effect of the size of base mismatch on the incision of the BPDE-DNA flap substrates by UvrABC or UvrBC proteins. The 5'-terminally labeled flap substrates were incubated with UvrBC (UvrB 250 nM, and UvrC 50 nM) and DnaK (1.4 μ M) in the presence (lanes 1–10) and absence (lanes 11–20) of UvrA (10 nM), respectively, in the UvrABC buffer at 37°C for 30 min. The incision products were then analyzed on a 10% polyacrylamide sequencing gel. The 31mer represents the intact DNA substrates, and the 17mer and 18mer represent the incised DNA fragments.

Fig. 3. Effect of the size of base mismatch on the incision of the BPDE-DNA bubble substrates by UvrABC. The 5'- (A) and 3'- (B) terminally labeled substrates with different sizes of bubble structure were incubated with UvrABC (UvrA 10 nM, UvrB 250 nM, and UvrC 50 nM) in the UvrABC buffer at 37°C for 15 min. The 50mer in both panels represents the intact substrates, the 18mer (A) and 32mer (B) represent the 5' incision products, and the 20mer (B) is the 3' incision product. The incision products were identified on a 10 or 12% polyacrylamide sequencing gel.

nuclease, which was fully coupled to the first or damage-specific 5' incision and probably depends on the dissociation of the UvrBC from the DNA after the first incision (data not shown). This indicated that the second incision (damage-independent) occurs only after the first incision (damage-dependent). Therefore, the products of both the 5' incisions should be combined to calculate the efficiency of the damage-dependent 5' incision since the occurrence of the second incision makes the first incision become invisible in the gel.

In comparison with the non-bubble normal substrate B0, the opening of the DNA strands up to 6 bases (B6) around the BPDE-modified site moderately increased the incision efficiency with the increase more for B0–B3 than for B3–B6. These results strongly suggest that a region of at least 6 bases may be melted during the interaction with the UvrABC system. However, further opening of the substrate to form an 8-base bubble structure led to a dramatic reduction of the incision as the opposite effect (Figure 3A, lane 8). It seems that UvrABC did not recognize a substrate with a bubble size >6 or 7 bases, probably because the size of the artificial bubble is larger than that of the bubble normally made by UvrABC. While we expected to see even worse incision on substrates with the bubble size >8 bases, the 5' incision was completely restored when the bubble was enlarged to 10–12 bases (B10 and B12). One possible explanation for these results is that these large bubble structures may allow the UvrBC to perform a 5' incision in the absence of 3' incision.

To confirm this idea, the same substrates B0–B12 were 3'-terminally labeled and incised with the UvrABC system (Figure 3B). Similarly, a poor 3' incision on the 8-base bubble substrate was observed, compared with that on the substrates with bubble size up to 6 bases (Figure 3B). Exactly as expected, moving the region of unpaired bases beyond the normal 3' incision site (4 bases 3' to the adduct) led to a dramatic inhibition of 3' incision, but an efficient 5' incision. It has been generally accepted that the UvrABC nuclease system makes dual incisions, first at the 3' site and then at the 5' site. In other words, the 5' incision is always coupled with the 3' incision. Since the substrates used in this experiment were labeled at the 3' end, we would expect to see only the 3' incision products in Figure 3B. The surprising appearance of the 5' incision products indicated that the 5' incision occurred without a 3' incision. This strongly suggested that the larger sized bubble structures at DNA damage triggered an independent or uncoupled 5' incision. Figure 3A and B showed the same pattern of incisions for both the 5'- and 3'-labeled substrates, and the results in these two cases are fully complementary to each other.

As shown in Figure 3B, the 3' incision occurred at multiple sites, including P5, P6 and P7 which are five, six and seven phosphates 3' to the BPDE-modified guanine, respectively. This is consistent with our previous observation (Zou *et al.*, 1995). Since substrate B8 has a bubble size ending at six phosphates 3' to the adduct (Table I), the dramatically reduced incision efficiency at P5 and P6 but not P7 of substrate B8 (compared with substrate B6) indicates that the 3'-nuclease activity of UvrBC is dependent on double-stranded DNA. This incision activity occurred at least 1 base 3' to the ds-ssDNA junction. These results, together with the fact that substrate B6 is

incised efficiently by the 3'-nuclease, suggested that the bubble size introduced by the action of UvrA₂B on a normal substrate (B0) in the pre-incision protein-DNA intermediate should be 6 bp. Furthermore, the lack of uncoupled 5' incisions for B0–B6 (Figure 3B) suggested that the UvrBC nuclease carried out 5' incisions of substrates B0–B6 only coupled to the 3' incision. The observation of the simultaneous inhibition of both 5' and 3' incisions of substrate B8 strongly supported the suggestion (Figure 3A and B). In summary, these results indicate that the 5' incision is triggered by strand opening 3' to the adduct. This strand opening can occur by 3' incision or, in this present study, by an extended unpaired region.

Incision of bubble substrates by UvrBC nuclease

As described above, the interaction of UvrABC with the BPDE-modified DNA led to formation of a 6-base bubble DNA intermediate. Data presented above indicated that the UvrABC was able to make an uncoupled 5' incision which was dependent upon a large DNA bubble structure. To learn more about the uncoupled 5'-nuclease activity, and the DNA structures formed with UvrBC in the pre-incision complex, we examined the interaction of UvrBC nuclease with the bubble substrates B0–B12, in the absence of UvrA. As shown in Figure 4A and B using substrates labeled at the 5' and 3' ends, respectively, UvrBC, unlike the UvrABC system, made little or no incision at either the 5' or the 3' side for substrate B0, and a small amount for B3 even though B3 contains a 3-base bubble at the adduct. This result is consistent with the role of UvrA in the development of the structure-specific DNA intermediates leading to the incisions. However, further opening of the DNA helix to 6 bases (substrate B6) resulted in efficient 5' and 3' incisions by UvrBC nuclease. A 5' and 3' incision pattern for the UvrBC nuclease similar to that for UvrABC was observed for the other substrates, B8–B12. Specifically, the construction of an 8-base bubble in the DNA resulted in a dramatic decrease in incision efficiency, and enlarging the bubble to 10 or 12 bases resulted in efficient uncoupled 5' incision, without any 3' incision. These results strongly support the idea that a 6-base bubble is required for an efficient 3' incision coupled to 5' incision by UvrBC nuclease. The lack of incision of non-bubbled or inefficient incision of smaller-bubbled substrates by UvrBC nuclease, compared with their incision by UvrABC, indicated that neither UvrB nor UvrC alone nor their combination had helicase activity, but the presence of UvrA provided the necessary strand-opening activity to build a 6-base bubble structure of DNA for incision. It was also evident that the nuclease for the uncoupled 5' incision was totally independent of UvrA, and depended only on the formation of an appropriate size of DNA bubble structure. Furthermore, this incision was fully dependent on a modified base, since neither UvrABC nor UvrBC was able to make incision on a bubble substrate without damage (Zou *et al.*, 1997; data not shown).

Chemical footprinting of the Uvr protein-DNA complexes

Formation of the bubble intermediates was also probed by the permanganate footprinting assay (Borowiec *et al.*, 1988; Visse *et al.*, 1994; Evans *et al.*, 1997), although

Fig. 4. Effect of the size of base mismatch on the incision of the BPDE-DNA bubble substrates by UvrBC in the absence of UvrA. The 5'- (A) and 3'- (B) terminally labeled substrates with different sizes of bubble structure were incised by UvrBC (UvrB 250 nM, and UvrC 50 nM) in the UvrABC buffer at 37°C for 15 min. The 18mer (A) and 32mer (B) represent the 5' incision products and the 19mer and 20mer (B) are the major 3' incision products. The incision products were identified on a 10 or 12% polyacrylamide sequencing gel.

Fig. 5. Opening of the BPDE-damaged DNA interacted with the Uvr proteins probed by the permanganate footprinting. The DNA substrates (4 nM) 5'-terminally labeled on the damaged strand (A) or the non-damaged complementary strand (B) were incubated with UvrA (10 nM) and UvrB (2.5 μ M) with or without UvrC (500 nM) in the presence of 1 mM ATP at 37°C for 15 min. After the incubation, the samples were probed with 60 mM KMnO₄ and analyzed on a 12% denaturing polyacrylamide gel. Incisions occurred with UvrABC as indicated. The T₂₄ and T₂₈ in (A) stand for the thymine residues at positions 24 and 28 counted from the 5'-terminally labeled end on the damaged strand. The T₂₂ in (B) stands for the thymine residue 22 counted from the 5'-terminally labeled end on the non-damaged strand. The band T₂₂* migrating more slowly than T₂₂ in (B) was due to the incomplete deletion of the permanganate-modified T₂₂ by piperidine (data not shown).

the selective attack of only single-stranded thymine by permanganate is constrained by sequence context and thus limits the resolution of defining the bubble size. As shown in Figure 5A, where the substrate 5'-terminally labeled on the damage strand was treated with the permanganate after incubation with UvrAB or UvrABC, the two most intense bands (T₂₄ and T₂₈) were observed at the thymine residues, implying that these thymines were probably unpaired in the protein-DNA complex. The relatively lower intensity of these two bands in the case of UvrABC as compared with that of UvrAB was due to the 5' incision which made the permanganate cleavage invisible in the gel. The mild KMnO₄ sensitivity observed here was expected because it has been reported previously that interaction of UvrABC with the cisplatin-adducted DNA substrates produced no specific KMnO₄ sensitivity on the damaged strand (Visse *et al.*, 1994). To obtain more information on the intermediate structures, the same

Fig. 6. Binding of UvrA and UvrB proteins to the BPDE-DNA bubble substrates. The substrates (4 nM) with different sizes of bubble structure were incubated with UvrA (10 nM) and UvrB (100 nM) at 37°C for 15 min in the UvrABC buffer with 1 mM ATP. After the incubation, the samples were loaded immediately onto a 4% native polyacrylamide gel for electrophoresis. ND, non-damaged DNA without bubble; UvrAB- and UvrB-DNA, the protein-DNA complexes formed with UvrAB and UvrB, respectively.

experiments were performed with the BPDE-DNA substrate 5'-terminally labeled on the non-damaged strand as shown in Figure 5B. Unlike the case with the labeling on the damage strand, there was chemical sensitivity only for the UvrABC-DNA interaction but not for the UvrAB-DNA interaction. The KMnO₄ footprint for the UvrABC-DNA interaction indicated that the single-stranded thymine (T₂₂) was involved in formation of a bubble. The lack of the footprint for the UvrAB-DNA interaction, which may include the formation of the UvrAB-DNA and UvrB-DNA complexes, was probably because the thymine was involved in the protein-DNA interactions and could be shielded from attacking by the permanganate. This is consistent with the idea that UvrA makes direct contact with the non-damaged strand (Van Houten *et al.*, 1986). The good KMnO₄ sensitivity on the non-damaged strand is consistent with that observed previously (Visse *et al.*, 1994). Sequence identification indicated that these KMnO₄-sensitive thymines (T₂₄ and T₂₈ on damaged strand and T₂₂ on non-damaged strand) were located exactly in the bubble region of 6 unpaired bases in the B6 substrate. These results strongly support the determination of the DNA structures formed in repair intermediate as described above.

Binding of Uvr proteins to the DNA bubble substrates

It has been shown that several different protein-DNA complexes formed during DNA damage recognition (Orren and Sancar 1989, 1990; Van Houten and Snowden, 1993; Zou *et al.*, 1995). Of these intermediates, the UvrA-dependent formation of the UvrB-DNA complex is believed to play an important role in DNA damage recognition, since the specific binding of UvrA to the damage has not been correlated to incision efficiency (Zou *et al.*, 1998a). Figure 6 shows a gel mobility shift assay for the binding of UvrA and UvrB to these bubble substrates. It can be seen that all substrates except the non-damaged DNA facilitated the formation of the UvrB-DNA complex. The size change of the DNA bubble at the 3' side of the adduct did not affect the formation of UvrB-DNA complex catalyzed by UvrA, a result suggesting that the existence of double-stranded DNA 3' to the adduct did not contribute significantly to the complex formation. No complexes were observed with any bubble substrate with just UvrB (data not shown). The stabilization

of the UvrB–DNA complex with the bubbles >6 bases, and the dramatic reduction of 3' incision, support the idea that the 3' incision activity of UvrBC nuclease is dependent upon double-stranded DNA, although double-stranded DNA is not required at the 3' incision site for stable UvrB–DNA binding. These results indicated that it was the UvrBC–DNA complex that ultimately determined the incisions, and implied that a conformational change occurs after UvrC binds to the UvrA-loaded UvrB–DNA complex in order to initiate the 3' incision which requires a double-stranded DNA region.

In addition, analysis of these results and those from the study with the flap substrates suggests that the UvrBC nuclease may have two binding modes, one leading to the 3' incision and another to the 5' incision. In the binding mode for 3' incision, the UvrBC nuclease driven by UvrA may form a stable complex with the substrate containing a bubble structure with 6 unpaired bases and a double-stranded region at the 3' incision site. However, the formation may be destabilized when the 3' ds–ssDNA junction of the bubble moved further away from the adduct. On the other hand, the opening of the bubble with at least >5 bases at the 3' side such as that in the B10 and B12 substrates seems to allow UvrBC to form a second binding mode for the 5' incision.

Discussion

During the process of the NER mediated by the *E.coli* UvrA, UvrB and UvrC proteins, UvrB forms a stable protein–DNA complex with the help of UvrA (Orren and Sancar, 1989; reviewed in Van Houten, 1990) and plays an important role in DNA damage recognition. The formation of the UvrB–DNA complex leads to the recruitment of UvrC to the damage site to form the UvrBC–DNA pre-incision complex which catalyzes the dual incisions. Characterization of these pre-incision complexes is not only critical in determining the mechanism of UvrABC nuclease, but also helpful in understanding the common structure of the protein–DNA complexes formed during NER. UvrB alone shows no helicase activity, and is able to bind to single-stranded DNA damage only at high concentrations (Hsu, *et al.*, 1995). The fact that UvrA₂B complex contains a limited helicase activity which loads UvrB to the damaged site to form a stable UvrB–DNA intermediate (Oh and Grossman, 1987, 1989; Orren and Sancar, 1989; Van Houten, 1990) suggests that: (i) this process alters the conformation or structure of the DNA and (ii) it is actually part of the damage recognition process. It has been shown that formation of the UvrB–DNA complex results in unwinding and bending of the DNA helix at the adduct (Lin *et al.*, 1992; Shi *et al.*, 1992; Moolenaar *et al.*, 1994). The possible existence of a single-stranded region in the UvrA₂B–DNA complex has also been suggested (Oh and Grossman, 1986; Seeley and Grossman, 1990). However, the details of the DNA structures within these pre-incision complexes and the mode of action of the UvrBC nuclease have remained unclear. In this study, by designing structure-specific DNA substrates and using the power of the UvrA-independent UvrBC nuclease, we have been able to determine some important structural and functional aspects of the DNA repair intermediates.

The experiments performed in our present work suggest that driven by UvrA, the interaction of UvrB and then UvrBC with BPDE-damaged DNA results in formation of a 6-base bubble structure with 2 bases 5' and 3 bases 3' to the adduct. Why is the formation of the bubble required in the pathway? It has been widely accepted that formation of a stable pre-incision UvrB–DNA complex is a necessary intermediate leading to incision. UvrB by itself, however, does not recognize or bind to a double-stranded damaged DNA. It has a weak but higher binding affinity (of micromolar order) for single-stranded DNA containing an adduct than for non-damaged DNA (Hsu *et al.*, 1995). This affinity, however, cannot account for the formation of the highly stable UvrB–DNA intermediate, implying that other factors are also involved. Our incision data presented here show that formation of the 6-base bubble leads to the highly efficient coupled incisions of the BPDE adduct by the UvrBC nuclease. A general principle of chemical reactions is that the more stable the intermediate, the faster the reaction will proceed, suggesting that the specific bubble formation stabilizes the UvrB–DNA or UvrBC–DNA intermediate. These experiments also help to clarify the role of UvrA in damage recognition. Although UvrA has been widely believed to act as a 'molecular matchmaker' (Orren *et al.*, 1992; Sancar and Hearst, 1993; Zou *et al.*, 1997) and participate with UvrB as a helicase, no direct evidence has been presented to show its strand-opening activity involved in formation of the intermediates. Our data strongly suggest that UvrA₂B with its strand-opening activity, driven by ATP hydrolysis, denatures the DNA around the adduct allowing direct access of UvrB to the adduct. This process results in a dynamic recognition of DNA damage by the UvrABC system.

It should be noted that the findings reported in this study are not consistent with the results from a previous study by Gordienko and Rupp (1997), who concluded that during the interaction with the DNA substrates containing a single 2-(acetyl-amino)fluorene (AAF) lesion, UvrAB introduced DNA changes localized within only 1–3 bp. This discrepancy could be due to the different types of DNA damaged substrates, sequence context and different conditions that were used in their experiments. Since the UvrA₂B complex can fully displace short oligonucleotides (Oh and Grossman, 1987; Gordienko and Rupp, 1997), the oligonucleotides (28–30mer) with a region of mismatched bases of 0–4 containing an adduct (AAF) annealed to a circular DNA used in their study might be too short to form pre-incision complexes which are sufficiently stable to support incisions. Although the substrates with an adduct in a covalently closed circular DNA with an unpaired region up to 5 bases were also constructed, the experiments were performed using a relatively high background nicking assay. On the other hand, it is also possible that the optimal unwinding for a AAF adduct is up to 3 bp, whereas for a BPDE adduct, the melting structure of 6 bp is the most stable pre-incision conformer.

Interestingly, formation of a similar open DNA complex was observed in human NER, although the bubble structure was determined at lower nucleotide resolution. The size of the unwound region was reported by Evans *et al.* (1997) to be ~25 nucleotides, but by Mu *et al.* (1997) to be ~20 nucleotides, both much larger than that we determined for

Fig. 7. Schematic representation of the pre-incision complex formed between UvrBC and damaged DNA. With the early participation of UvrA, the damaged DNA is unwound around the adduct, and an open DNA structure forms with UvrBC proteins, which is characterized by 2 unpaired bases 5' and 3 unpaired bases 3' to the adduct. The 3' and 5' double-stranded nuclease activities of UvrBC incise the damage 1 base 3' and 6 bases 5' to the open structure, respectively. In addition, we propose a direct interaction between the UvrBC and the adducted base. The yellow, violet, green and blue colored sticks in the DNA strands represent the bases cytosine, thymine, adenine and guanine, respectively. The red stick represents the adducted guanine.

the UvrABC system in this study. The dramatic difference in the bubble size between the *E.coli* and human NER systems is consistent with their difference in the size of the damaged DNA fragment excised, which is ~12–14 nucleotides in *E.coli* and 27–29 nucleotides in human. This may also partially explain why two helicases XPB and XPD (Wood, 1997) must participate in human NER to produce a larger bubble structure. Because of the large size of the unwound region in human NER, the dual incisions at the 3' and 5' sites, unlike in *E.coli*, are carried out by two physically separate nuclease activities, XPG and XPF-ERCC1, respectively. The multiprotein human transcription factor IIH (TFIIH), therefore, is required in the pre-incision complex probably as a factor to maintain the large size of the bubble structure after the strand opening.

Our results combined with those from previous studies (reviewed in Van Houten, 1990; Sancar, 1996; Wood, 1997) demonstrate the important mechanistic similarities between *E.coli* and human NER systems. In general, in both systems, there is the formation of bubble intermediates, the involvement of structure-specific nucleases, dual incisions in which the 3' incision occurs prior to the 5' incision, and the occurrence of uncoupled 5' incision in special situations. More specifically, like the UvrA dimer, the XPC-hHR23B complex recognizes DNA damage and initiates the global genome NER process (Van Houten, 1990; Sugawara *et al.*, 1998). Also like the UvrA dimer, the XPC-hHR23B complex acts like a molecular matchmaker to help the assembly of the excision nucleases

but is absent in the pre-dual incision complex (Orren *et al.*, 1992; Sancar and Hearst, 1993; Wakasugi and Sancar, 1998; Zou *et al.*, 1998b). In addition, the UvrBC in *E.coli* and the XPG and XPF-ERCC1 in humans contain structurally specific nucleases for 3' and 5' incisions which can be observed separately with structure-specific substrates even in the absence of other repair factors.

Our study clearly demonstrates that the UvrBC complex contains a nuclease which can make 5' incisions in the absence of 3' incisions. Regarding the uncoupled 5' incision, the UvrBC complex appears to be a UvrA-independent nuclease, since the addition of UvrA to the reaction had no effect on incision efficiency. Therefore, the UvrBC complex may either have two different types of nuclease activities for coupled and uncoupled 5' incisions, respectively, depending on the size of the bubble, or only one type of nuclease, which can be triggered by two different mechanisms. Reconciliation of these two possibilities ultimately will require structural and functional analysis of UvrB and UvrC by X-ray diffraction studies combined with site-specific mutagenesis. We hypothesize, however, that the UvrBC complex has the same nuclease for both coupled and uncoupled 5' incisions, and the 3' incision is required for the coupled 5' incision only because it can produce a DNA structure functionally equivalent to a bubble with at least 10 bases. This helps to explain why 3' incisions always precede the 5' incision during normal UvrABC NER.

Analysis of our experimental data indicates that three

stable intermediates may form between UvrB/UvrC and damaged DNA during the process of UvrABC incision. These are the UvrB–DNA complex, the UvrBC–DNA complex for 3′ incision and the UvrBC–DNA for 5′ incision. The formation of the UvrB–DNA complex requires UvrA, a damaged base and a melted region of DNA, particularly on the 3′ side of the adduct. This picture is based on the fact that UvrB bound specifically to the damaged single-stranded DNA, but with very low affinity (Hsu *et al.*, 1995), and on our gel mobility shift assay, in which UvrB, in the presence of UvrA, bound specifically and tightly to the bubble substrates with no effects from the DNA denaturing 3′ to the adduct (Figure 6). Our experiments suggested that UvrBC may have two DNA damage-binding modes, one for 3′ and another for 5′ incisions. In the mode leading to 3′ incision, the binding of UvrBC to the substrate seems to include a double-stranded DNA region 3 bases 3′ to the adduct, because further opening of the 3′ DNA region resulted in little or no incision. However, binding of UvrBC, which leads to the 5′ incision, may require a larger number of unpaired bases 3′ to the adduct. It is possible that this opening provides access for the domains of the UvrBC proteins into the bubble to interact with the single-stranded DNA.

Combining the results from our present and previous studies, we therefore propose a dynamic recognition model for the mechanism of UvrABC system, with new insights on intermediate structures and the roles of proteins involved. In this model, UvrA loads UvrB onto the damaged DNA site. The interaction of UvrA₂B with the damage causes unwinding, denaturing and opening of the local DNA duplex at the adduct. It is the bubble formation that allows UvrB direct access to the adducted base, resulting in the formation of a stable UvrB–DNA open complex. Due to the hydrophobic property of a modified base, a hydrophobic pocket or interaction surface on UvrB may also dock to the adduct molecule. The affinity of the binding is sequence and lesion dependent and is an important determinant for the stability of the UvrB–DNA intermediate. Interaction of the N-terminal half of UvrC with the C-terminus of UvrB in the complex (Moolenaar *et al.*, 1995, 1997) results in a conformational transition of the proteins, leading to the formation of a stable UvrBC–DNA pre-incision intermediate with 2 unpaired bases 5′ and 3 unpaired bases, 3′ to the adduct (Figure 7). The binding of UvrBC to a double-stranded region 3′ to the adduct appears to be essential for the stability of the intermediate. The 3′ incision releases the DNA stress and provides a further opening for the two strands originally in the bubble. It is possible that this DNA structural change allows UvrBC to be able to interact with the substrate in a different mode, resulting in the 5′ incision. This interaction would include a double-stranded DNA binding by UvrBC 5′ to the adduct.

Materials and methods

Protein purification

UvrA was purified from *E. coli* strain MH1 ΔUvrA containing the overproducing plasmid, pSST10 (graciously supplied by L. Grossman, Johns Hopkins University), in which the *uvrA* gene is under the control of the heat-inducible PL promoter. UvrC was overproduced from *E. coli*

strain CH296 containing plasmid pDR3274 (graciously supplied by A. Sancar, University of North Carolina). The two proteins were purified to homogeneity as described previously (Zou *et al.*, 1995).

UvrB was purified in one step through a 20 ml chitin column from *E. coli* strain XL-1 Blue transformed with the overexpressing plasmid pUTG97 containing the *uvrB* gene under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-induced P_{tac} promoter. The pUTG97 was constructed by subcloning the *uvrB* gene from plasmid pUNC211 (graciously supplied by A. Sancar, University of North Carolina) into an IMPACT system (New England BioLabs). Briefly, the *uvrB* gene in pUNC211 was amplified by PCR with *Pfu* DNA polymerase (Stratagene) and two primers containing an *Nde*I and a *Sma*I site, respectively, which flank the gene. The PCR products were purified with a PCR purification kit (Qiagen) by following the procedures suggested by the manufacturer. Both the PCR-purified product and the plasmid vector pCYB2 from the IMPACT system (New England BioLabs) were subjected to restriction with *Nde*I and *Sma*I. After purification with the same PCR purification kit, the restricted vector pCYB2 was dephosphorylated by alkaline phosphatase to increase the subsequent ligation efficiency. The products were purified again with the same DNA purification kit. For the ligation, the dephosphorylated vector (3.5 ng) and the purified PCR-generated *uvrB* gene fragment (0.4 ng) were incubated with 10 U of T4 DNA ligase (Gibco-BRL) in 60 μl of ligation buffer at 16°C for 12 h, then at 37°C for 1 h. One-twentieth of the ligation products were then transfected into competent *E. coli* DH5α cells (Gibco-BRL) in 2× YT medium by electroporation. To reduce the background from self-ligation of the vector further, before transfection the ligated samples were digested with the restriction enzyme *Xba*I, whose site is deleted from the polylinker flanked by *Nde*I and *Sma*I in the original pCYB2 vector and is absent in the *uvrB* gene. The transfected cells were plated on 2× YT-agar with 100 μg/ml ampicillin and incubated at 37°C overnight. Colonies were screened for those containing the plasmid encoding the *uvrB* gene by both restriction and PCR assays after growing to appropriate amount in 3 ml of 2× YT culture, and purified with Qiagen Plasmid Mini Kit. The construct from the cloning finally was identified by full-length DNA sequencing of the *uvrB* gene. It should be noted that the cloning into the *Sma*I site left an additional glycine residue attached to the C-terminus of the UvrB protein.

To purify UvrB, the XL-1 blue cells containing pUTG97 were grown at 30°C to an A₆₀₀ of 0.6 in 10 l of Luria–Bertani medium supplemented with ampicillin (100 μg/ml). The IPTG was then added to a final concentration of 0.7 mM for 2.5 h of induction. The cells were harvested and spun down at 5000 *g* for 10 min at 4°C. The cell pellet was resuspended and lysed by sonication in ice-cold lysis/column buffer containing 20 mM Tris–HCl pH 8.0, 500 mM NaCl, 0.1 mM EDTA and 0.1% Triton X-100. Clarified extracts were obtained after centrifugation at 12 000 *g* for 30 min. The extracts were then slowly (<0.5 ml/min) loaded onto a column packed with chitin beads which were fully pre-equilibrated with column buffer. After loading, the column was washed thoroughly with column buffer (at least 30 column volumes). The on-column cleavage of UvrB protein from the intein–chitin-binding domain fusion was induced by flushing the column quickly with three column volumes of the cleavage buffer [20 mM Tris–HCl pH 8.0, 100 mM NaCl, 0.1 mM EDTA and 30 mM dithiothreitol (DTT)]. The flow was stopped immediately and the cleavage was allowed to continue at 4°C overnight. UvrB protein was eluted and collected with the cleavage buffer without DTT, then dialyzed against storage buffer (50 mM Tris–HCl pH 7.8, 100 mM KCl, 0.1 mM EDTA, 0.1 mM DTT and 50% glycerol).

The biological activities of the UvrB protein purified from the IMPACT system were confirmed in both DNA damage-binding and incision assays (data not shown), as compared with those of UvrB purified directly from the plasmid pUNC211 as described previously (Zou *et al.*, 1995).

DNA substrate construction

The 50 bp oligonucleotides containing a single BPDE adduct, (+)-*cis-anti*-BPDE, were prepared as described previously (Zou *et al.*, 1995) with minor modification. Briefly, the phosphorylated BPDE-11mers (30 pmol, generously supplied by Dr Nick Geacintov, Department of Chemistry, NYU) were ligated with stoichiometric quantities of 20mer and phosphorylated 19mer (or none), using T4 DNA ligase in the presence of a 55mer bottom strand containing the complementary sequence of 50 bases and an additional 5-deoxythymidine at the 3′ end in a 30 μl solution containing 50 mM Tris–HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP and 50 μg/ml of bovine serum albumin (BSA). The ligation was carried out at 16°C for 12 h. After ligation, the products were purified and then reannealed with various 50mer bottom strands to

make appropriate substrates as shown in Table I. The annealed substrates were purified on a non-denaturing 8% polyacrylamide gel. The double-stranded character and homogeneity of the 50 bp substrates were examined by a restriction assay (Zou *et al.*, 1995) and analyzed on a 12% polyacrylamide sequencing gel under denaturing conditions with TBE as the running buffer (50 mM Tris-HCl, 50 mM boric acid, 1 mM EDTA, pH 8.0).

DNase I footprinting assays

The 3'-terminally-labeled DNA bubble substrates (4 nM) in 10 µl of the buffer containing 50 mM Tris-HCl pH 7.8, 50 mM KCl, 10 mM MgCl₂, 10 mM CaCl₂, and 1 mM DTT were digested with 1 ng of DNase I (Sigma) at room temperature for 1 min. The reactions were terminated by adding 1 µl of 250 mM EDTA. The samples were mixed with 10 µl of formamide DNA-denaturing buffer and heated to 90°C for 5 min, then immediately chilled on ice. The samples were then subjected to electrophoresis in 12% polyacrylamide sequencing gel (8 M urea) under denaturing conditions as above. The gel was dried and exposed to Kodak XAR5 X-ray film.

Permanganate footprinting assay

The chemical footprinting assay was conducted by following the procedures described previously (Borowiec *et al.*, 1988; Visse *et al.*, 1994; Evans *et al.*, 1997). Briefly, the (+)-*cis*-BPDE-DNA substrate (4 nM) or non-damaged DNA was incubated with UvrA (10 nM) and UvrB (2.5 µM) with or without UvrC (500 nM) at 37°C for 15 min in the UvrABC buffer in the presence of 1 mM ATP. After the incubation, the samples were added with KMnO₄ to a final concentration of 60 mM and allowed to react for 2 min at room temperature. The reactions were stopped by mixing with 1 M 2-mercaptoethanol, 20 mM EDTA and 0.3 mg/ml of tRNA. The DNA was precipitated with 65% ethanol and then incubated with 25 µl of 1 M piperidine at 90°C for 25 min. Samples finally were speed-vacuumed and analyzed on a 12% sequencing gel under denaturing conditions.

Gel mobility shift assays

Binding of the various DNA bubble substrates by UvrA and UvrB, or UvrB and UvrC proteins was determined qualitatively by gel mobility shift assays. Typically, the substrate (4 nM) was incubated with UvrA (10 nM) and UvrB (100 nM), or UvrB (100 nM) and UvrC (20 nM), at 37°C for 15 min in 20 µl of UvrABC buffer (50 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM MgCl₂, 5 mM DTT) in the presence (for UvrAB) or absence (for UvrBC) of 1 mM ATP. After incubation, 2 µl of 80% (v/v) glycerol was added and the mixture was loaded immediately onto a 4% native polyacrylamide gel in TBE running buffer and electrophoresed at room temperature. For the binding with UvrA and UvrB, ATP (1 mM) and MgCl₂ (10 mM) were added to the TBE running buffer.

Incision assays

The 5'- or 3'-terminally labeled DNA substrates (4 nM) were incised by UvrABC or UvrBC (UvrA 10 nM, UvrB 250 nM, and UvrC 50 nM) in the UvrABC buffer (1 mM ATP) at 37°C for 15–30 min. The Uvr subunits were diluted and pre-mixed into storage buffer before mixing with DNA. The reactions were terminated by adding EDTA (20 mM) or heating to 90°C for 3 min. The samples were denatured with formamide and heated to 90°C for 5 min and then quick-chilled on ice. The digested products were analyzed by electrophoresis on a 10–12% polyacrylamide sequencing gel under denaturing conditions with TBE buffer.

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