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# A specific 3′ exonuclease activity of UvrABC

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**Specific cutting of undamaged DNA by UvrABC nuclease is observed. It occurs seven nucleotides (nt) from the 3′ terminus of oligonucleotides annealed to single-stranded M13 DNA circles. Although the location of the UvrABC cut on undamaged DNA is similar to that of the cut on the 5′ side of a damaged DNA site during the dual incision reaction, the cut of undamaged DNA is not an intermediate in the dual incision step. On DNA duplexes with a single AAF adduct, the anticipated cut at the eighth phosphodiester bond 5′ of the lesion is present, but extra cuts at 7-nt increments are observed at the 15th and 22nd phosphodiester bonds. We suggest that these additional cuts are made by the UvrABC activity observed on undamaged DNA; such activity is referred to as ABC 3′ exonuclease and may play a significant role by providing a suitable gap for RecA-mediated recombinational exchanges during repair of interstrand crosslinks and closely opposed lesions. This ABC 3′ exonuclease activity depends on higher concentrations of Uvr proteins as compared with dual incision and may be relevant to reactions that occur when UvrA and UvrB are increased during SOS induction.**

*Keywords*: DNA repair/RecA-mediated exchanges/ UvrABC 3′ exonuclease/UvrABC 5′ side cut/SOS response

## Introduction

The UvrABC nuclease is the central enzyme of *Escherichia coli* involved in nucleotide excision DNA repair which occurs in all living organisms. It initiates the multistep repair of a variety of DNA lesions with different structures and origins (Van Houten, 1990; Van Houten and Snowden, 1993; Friedberg *et al.*, 1995; Sancar, 1996). Extensive studies of UvrA, UvrB and UvrC and their complexes have revealed many details of the activities of these proteins during damage recognition and incision when UvrABC makes two coupled cuts in a DNA strand on each side of the lesion (Sancar and Rupp, 1983; Yeung *et al.*, 1983; Lin and Sancar, 1992; Lin *et al.*, 1992; Moolenaar *et al.*, 1995). Then, UvrD, Pol I and DNA ligase carry out repair synthesis and ligation and the repair of damaged DNA is completed (Caron *et al.*, 1985; Husain *et al.*, 1985; Kumura *et al.*, 1985; Yeung *et al.*, 1986a; Van Houten *et al.*, 1988; Orren *et al.*, 1992). However, when both DNA strands are damaged by two closely opposed lesions or with an interstrand crosslink, additional steps are required for repair, starting with a UvrABC dual incision on one DNA strand, followed by RecA-mediated strand exchange, and then by a second UvrABC dual incision on the second DNA strand. Repair synthesis and ligation complete the process (Sancar and Sancar, 1988; Friedberg *et al.*, 1995). In order for the recombinational strand exchange to take place, a suitable gap must be present in the DNA. The role for providing such a gap has been attributed to the 5′ → 3′ exonuclease activity of Pol I (Sladek *et al.*, 1989). In this study we describe a new activity of the UvrABC protein complex which results in additional cutting of DNA after dual incision. In a reaction which resembles the 5′ side incision step of normal dual incision, this 3′ exonuclease activity of UvrABC provides a gap which can be used by RecA for recombination without requiring Pol I which acts during repair synthesis. Dependence of this newly recognized activity on higher concentrations of the Uvr proteins suggests that it is relevant to reactions in SOS-induced cells when the levels of UvrA and UvrB are increased.

## Results and discussion

The UvrABC nuclease of *E.coli* initiates the repair of a variety of DNA lesions by making two coupled cuts in a damaged DNA strand on each side of the lesion. This reaction includes damage recognition, preincision complex formation and actual incision steps. During studies in which we examined the nuclease activity of UvrABC at damaged sites on model substrates with a short oligonucleotide annealed to MM13 single-stranded circles, we were surprised to observe specific cutting of the undamaged oligonucleotides used as controls. The experiments presented in Figure 1 show the activity of UvrABC on undamaged DNA substrates. As seen in Figure 1A, only one major new product band is observed in each experiment. For the substrate shown in lane 1 in which the 27-mer is labeled at the 3′ terminus, the product is a 7-mer. The UvrABC treatment of other 3′-labeled DNA substrates with oligonucleotides of different lengths always generates a 7-mer cleavage product (data not shown). When substrates with 5′ end-labeled oligonucleotides are examined (Figure 1A), the cleavage product of the substrate with a 30-mer is a 23-mer (lane 2) while that generated from a 26-mer is a 19-mer (lane 3). Taken together, data in Figure 1A demonstrate that the action of UvrABC on the substrates with no damage is to make a single cut 7 nucleotides (nt) from the 3′ end of the annealed oligomer. This cut is a function of all three UvrABC proteins.

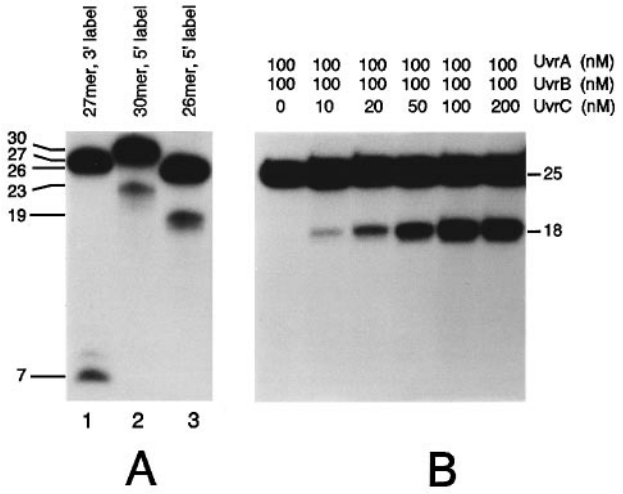



**Fig. 1.** Specific cutting of undamaged oligonucleotides by UvrABC excinuclease. Each substrate consists of synthetic oligonucleotide annealed to MM13 single-stranded circle. (**A**) UvrABC cutting of undamaged oligonucleotides of different lengths. The 3′-labeled 27-mer in lane 1 is 5′-ATATTCTTTAAAGATATCATTAATCCC-3′. The 5′-labeled 30-mer in lane 2 is 5′-AAAATATTCTTTAAAGAT-ATCATTAATCCC-3′. The 5′-labeled 26-mer in lane 3 is 5′-ATATT-CTTTAAAGATATCATTAATCC-3′. The protein concentrations in the reaction mixtures are: UvrA, 100 nM; UvrB, 100 nM; UvrC, 100 nM. (**B**) UvrABC cutting of undamaged oligonucleotide at different UvrC concentrations. The annealed 5′-labeled 25-mer is 5′-ATATTCTTTAAAGATATCATTAATC-3′.

Addition to the substrates of UvrA alone, UvrB alone, UvrC alone, or combinations of UvrA and UvrB or UvrA and UvrC, does not cause cutting of DNA although in some experiments we have observed a very low level of activity with a mixture of UvrB and UvrC (data not shown). When the UvrA and UvrB concentrations are kept constant and UvrC is varied, this cut is observed at all UvrC concentrations tested from 10 nM up to 200 nM (Figure 1B). The extent of cutting of undamaged DNA at different UvrC concentrations follows the same general pattern as normal dual incision by UvrABC on similar substrates containing a single AAF lesion with the reaction conditions and requirements for the cutting of undamaged DNA being similar to those for UvrABC dual incision at a lesion (data not shown). The similarity of the conditions for UvrABC activity on undamaged and damaged DNA raises the question of the relation of these events.

During dual incision at a wide variety of unifilar DNA lesions, the cut on the 5′ side of the lesion occurs on the eighth phosphodiester bond 7 nt from the lesion, the same distance as observed for the length of the fragment generated in our experiments on undamaged substrates. Since the reaction we study in some ways mimics the reaction that occurs on the 5′ side of a lesion, one possibility is that our substrates containing a 3′ undamaged terminus are recognized and cut by UvrABC because they resemble an intermediate that occurs during normal dual incision at a lesion (Rupp and Gordienko, 1994). To investigate this possibility further, we constructed several substrates, with or without the AAF lesion, in which the 3′ termini are incorporated into structures possibly present during the UvrABC dual incision at a damaged site (Figure 2A). The scheme at the top of Figure 2A summarizes the incision sites that occur during normal dual incision when the 3′ side cut is made first on the fifth phosphodiester bond 4 nt from the lesion by UvrBC (Lin *et al.*, 1992; Moolenaar *et al.*, 1995), while the 5′ side cut follows on the eighth phosphodiester bond 7 nt from the lesion and is made by UvrBC using a catalytic site in UvrC (Lin and Sancar, 1992). In Figure 2A, substrate A has an annealed oligonucleotide with a single AAF specifically placed on the fifth nucleotide from the 3′ end to simulate the product formed after the first UvrABC cut on the 3′ side of the lesion. When substrate A was treated with UvrABC, two significant observations were made: first, incision of this substrate was efficient even though the lesion was near the end of the oligonucleotide; and second, the position of the incision was 7 nt from the AAF, at exactly the position expected for the 5′ side cut during a normal dual incision reaction. In the same figure, substrate B is identical to substrate A except that it does not have an AAF adduct. In this case, the efficiency of cutting with UvrABC is lower, and this cutting occurs 7 nt from the 3′ end of the annealed oligonucleotide, at a position different from that observed for substrate A, but at the same position observed for the substrates in Figure 1.

During the UvrABC dual incision, the 3′ side cutting presumably occurs first (Lin *et al.*, 1992) and is, by inference, the rate-limiting step (Rupp and Gordienko, 1994); however, it is unclear what factors determine the location of the second cut on the 5′ side of the lesion. It is, in fact, logically plausible that the structure or features determining the 5′ side cut may be something other than the lesion itself. Perhaps a cut only 4 nt from the AAF causes sufficient fraying of the DNA on the 3′ side of the lesion and generates a transient structure that constitutes the substrate for the 5′ side cut. With this possibility in mind, substrate C with four mismatched nucleotides on the 3′ side of the AAF adduct was constructed (Figure 2A). The results of UvrABC incision with this substrate were similar to those with substrate A in Figure 2A: cutting was efficient and at the position expected for cutting during normally coupled dual incision. In the case of substrate D (Figure 2A), which is identical to substrate C but without a lesion, no detectable cutting with UvrABC occurred, thus arguing against the possibility mentioned above that the second incision might be triggered by a structure that is independent of whether the lesion is there. (This comparison of the two substrates is not completely satisfactory because it is clearly not possible to duplicate the exact changes in a DNA structure caused by the presence of the lesion. However, even with this caveat in mind, it is obvious that the four Cs at the 3′ terminus have little or no effect on the UvrABC incision of the substrate with AAF and that the presence of just the unpaired nucleotides does not provide a satisfactory signal for incision when there is no lesion on the molecule.) These particular results with substrates C and D agree with our previous observation that unpairing of nucleotides at the lesion site is not necessary for UvrABC incision and that the presence of a ‘bubble’ structure is an inhibitory element for normal incision (Gordienko and Rupp, 1997a). These results contrast with data obtained with the eukaryotic repair proteins, yeast Rad1-Rad10 and human XPG, where the junction between duplex double-stranded DNA and the two single-stranded arms is sufficient to trigger cleavage near the junction (Bardwell *et al.*, 1994;

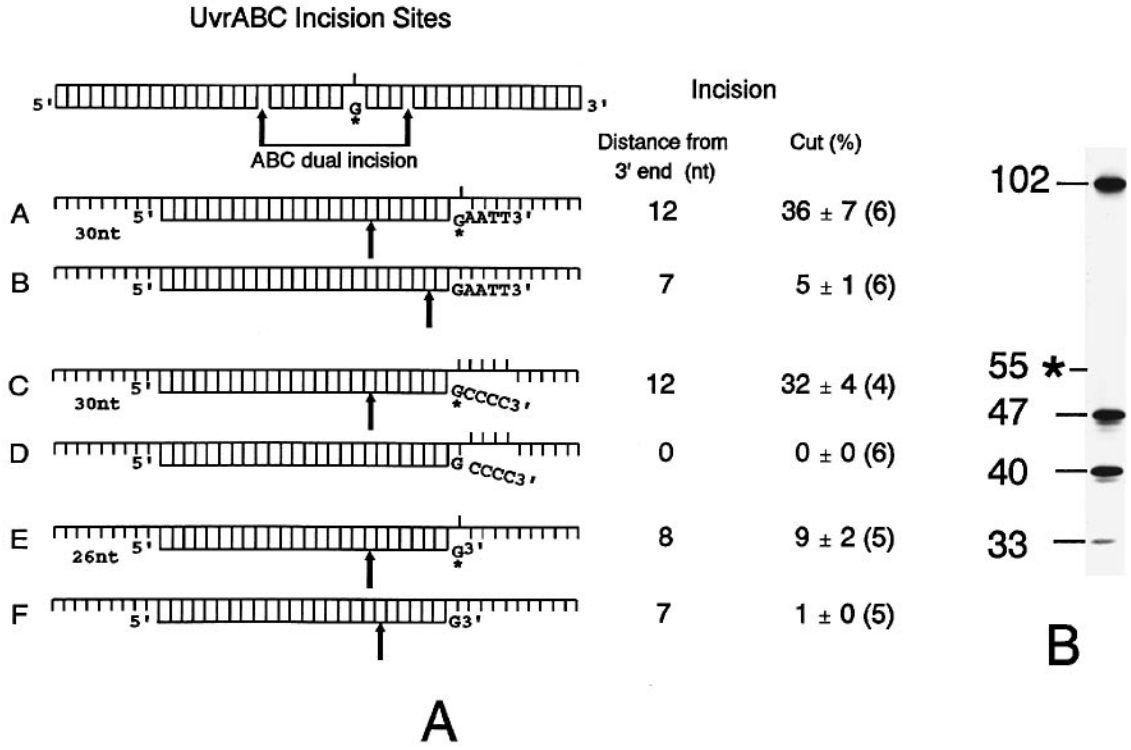



**Fig. 2.** Substrate specificity for UvrABC cutting of undamaged and damaged DNA. (**A**) UvrABC incision reaction on substrates related to potential intermediates of dual incision. The scheme of UvrABC dual incision at the AAF lesion is shown on the top. Substrates consist of the indicated oligonucleotides annealed to MM13 single-stranded circles. The sequence of the first 26 nt in the oligonucleotides in substrates A, B, C and D and of the entire 26-mer in substrates E and F is 5′-TTAATTACATTTAACAATTTCATTTG-3′. All substrates are labeled at the 5′ end of the annealed oligonucleotide. Substrates A, C and E contain a single G-AAF lesion at the position marked by the *. Arrows show the position of UvrABC cutting on each substrate. The numbers in parentheses are the number of experiments done for each substrate. (**B**) UvrABC incision reaction on 102-mer DNA duplex. The substrate is a 5′-labeled oligonucleotide with an AAF lesion at position 55 annealed to an unlabeled 102-mer complementary strand. The position of the AAF lesion is marked with a *. 102 shows the position of the uncut labeled strand in the denaturing gel. 47 marks the position of the 47-mer resulting from the 5′ side cut of normal dual incision. 40 and 33 show the positions of the first and second extra cuts made by UvrABC after dual incision. Considering that the substrate for the additional cuts is not available until the previous cut occurs, the frequency of the extra cuts is very high. Adding UvrD into a reaction mixture did not increase the efficiency of extra cuts (data not shown).

O'Donovan *et al.*, 1994; Davies *et al.*, 1995; Evans *et al.*, 1997). In a recent report, a very low level of cutting at undamaged 'Y' structures has been observed with UvrBC, but it is not yet clear whether or not this cutting is related to the 5′ side cut made by UvrABC during dual incision at a lesion (Zou *et al.*, 1997).

Next, we examined the effect of having an AAF at the 3′ terminus of the oligonucleotide (Figure 2A, substrate E) and observed that significant cutting by UvrABC occurred 8 nt from the 3′ terminus at the normal position for dual incision (i.e. 7 nt on the 5′ side of the AAF adduct). The corresponding substrate without a lesion (substrate F, Figure 2A) was cleaved 7 nt from the 3′ terminus, at the position observed for cutting of the undamaged DNA in Figure 1.

Taken together, the data in Figure 2A give us significant insight into what features UvrABC recognizes in making cuts during the incision reaction. Clearly, the recognition and incision of AAF lesions does not require that they be present in a lengthy duplex region of DNA, as accurate recognition and incision is achieved even when the lesion is present at or near the 3′ terminus of an oligonucleotide that is annealed to a longer complementary single-stranded DNA. AAF lesions, even when present on the terminal nucleotide, take precedence over undamaged ends, and the position of the lesion itself determines the site for the 5′ side incision. On the substrates with no AAF, UvrABC-mediated cutting occurs 7 nt from the 3′ end of the annealed oligonucleotide. The elimination of this reaction by the terminal four mismatched Cs indicates that the preferred structure in the strand being cut at the 3′ end is the one that is fully base-paired (Figure 2A and data not shown).

All substrates in Figure 2A were labeled at the 5′ end and did not allow us to see whether any breaks adjacent to the lesion occurred during normal incision with UvrABC. Next, we constructed a substrate identical to substrate A in Figure 2A except with the label at the 3′ terminal nucleotide of the 30-mer. When this substrate was treated with UvrABC, the 12-mer product resulting from a normal 5′ side incision 7 nt from the AAF lesion was observed, but there was no trace of either a 4-mer or 5-mer that would have been formed by cutting immediately adjacent to AAF on either side (data not shown). These results show no indication for the presence of intermediate structures formed by hypothetical additional cuts inside the normal product of UvrABC dual incision. Thus, taken together, these data and data in Figure 2A suggest that even though the UvrABC-mediated cuts of undamaged DNA are made at precise positions 7 nt from 3′ termini and the resulting incised fragment has the same number of nucleotides as between the lesion and the 5′ incision

site during dual incision, there is no intermediate present during normal dual incision at the lesion which could be attributed to the activity we observe on undamaged DNA.

Our data (Figures 1 and 2A, and data not shown) show that UvrABC cutting of undamaged DNA occurs on those DNA structures which have 3′ termini with the 3′ end nucleotides paired. In the UvrABC reaction such a structure is available after dual incision. Thus, there is a possibility that cutting of undamaged DNA in the UvrABC reaction occurs only when the primary dual incision at a lesion creates the substrate for additional cuts. With the duplex substrate 102 base pairs long with a single AAF at position 55 on the 5′-labeled strand (Figure 2B), in addition to the normal product of UvrABC incision, a band of approximately the same intensity is seen 7 nt from the normal 5′ side incision site and a weaker band is seen 14 nt from the normal 5′ side incision site (i.e. at the 15th and 22nd phosphodiester bonds from the lesion). We interpret the additional cutting that occurs at intervals of 7 nt to be the result of the UvrABC exonuclease activity on undamaged DNA that we have described in Figures 1 and 2A. These cuts are due to additional events that occur sequentially when a 3′ terminus is made available by UvrABC dual incision at a lesion.

There have been previous observations of extra bands in gels of UvrABC reaction products that are not due to typical dual incision. In some cases the unusual bands were ignored (Van Houten *et al.*, 1987) or described as of unknown origin (Lin *et al.*, 1992); in other cases they have been described as being due to high UvrA or UvrC concentrations (Visse *et al.*, 1991, 1992; Lin *et al.*, 1992), UvrABC activity at guanines inadvertently damaged during oligonucleotide synthesis by the phosphotriester method (Bertrand-Burggraf *et al.*, 1991), anomalous endonucleolytic cutting of undamaged DNA (Caron and Grossman, 1988), or secondary cleavage at pyrimidine–cytosine photoproducts (Kumura *et al.*, 1985). We now suggest that the 3′ exonuclease activity of UvrABC on undamaged DNA can produce the unexpected products. We call this activity a 3′ exonuclease according to the nomenclature of Frenkel and Richardson (1971), since the UvrABC cutting activity described here occurs near the 3′ end of a substrate. [It might also be appropriate to consider the 5′ incision of the normal dual incision pair to be an exonuclease activity, as it is thought to require the previous occurrence of the 3′ incision (Lin *et al.*, 1992).]

Extra bands which can be interpreted as corresponding to UvrABC 3′ exonuclease activity were seen on substrates damaged with cis-platinum (Visse *et al.*, 1991, 1992, 1994a,b; Moolenaar *et al.*, 1994), psoralen monoadduct (Van Houten *et al.*, 1987; Bertrand-Burggraf *et al.*, 1991; Lin *et al.*, 1992), AAF (Figure 2B) and UV photoproducts (Kumura *et al.*, 1985). Since the described activity is observed on a number of different lesions and the frequency of these extra cuts is quite high (especially when it is taken into account that the substrate is not present until generated by the 5′ side incision) (Figure 2B), an intriguing possibility is that the observed reaction, producing additional cuts in association with dual incision at lesions, is a natural function of UvrABC excinuclease. While it is unclear how these additional cuts would be needed for the repair of damage limited to one DNA strand, such cuts can play an important role in the efficient repair of closely opposed lesions and of DNA interstrand crosslinks. Little is known about the mechanism of repair of closely opposed lesions except that when UvrABC acts on duplex substrates with closely opposed thymine dimers, double-strand breaks are not produced and UvrABC incision occurs on only one strand (Svoboda *et al.*, 1993). The repair of interstrand crosslinks has been studied more extensively. In addition to UvrABC it requires RecA and is postulated to occur in a sequential incision–recombination–incision reaction that starts with the UvrABC dual incision of one strand, followed by RecA-mediated exchange, and then finishes with a second dual incision by UvrABC to remove the residual half crosslink from the other original strand (Cole, 1973; Cole *et al.*, 1976; Van Houten *et al.*, 1986a,b; Cheng *et al.*, 1988). In experiments with DNA synthetic substrates simulating the structure after the first UvrABC dual incision on the furan strand of a psoralen crosslink, it was demonstrated that the RecA-mediated exchanges did occur only when a gap was generated in the substrate by the 5′→3′ nuclease of Pol I (Sladek *et al.*, 1989). In the model summarized in Figure 3, we suggest that the function of the additional cuts produced by the UvrABC 3′ exonuclease activity is to generate a gap that provides the site required for the initiation of recombinational exchanges. In studying repair synthesis in a substrate crosslinked with psoralen, Cheng *et al.* (1991) observed that repair synthesis extended beyond the expected UvrABC 5′ side incision site. While they attributed this to exonuclease activity of Pol I, it could have been caused instead by the UvrABC activity described in this paper.

It is known that both the *uvrA* and *uvrB* genes are among the genes under SOS control and are induced by DNA-damaging treatments (Kenyon and Walker, 1980, 1981; Fogliano and Schendel, 1981). However, the physiological significance of their induction is enigmatic since the direct measurement of the excision of lesions from the DNA in UV-irradiated *E.coli* did not demonstrate an increase in the rate of lesion removal over that found with the constitutive levels of UvrA and UvrB (Swenson and Setlow, 1966; Cooper, 1982). The experiments presented in Table I were done in order to learn how changes in UvrA, UvrB and UvrC concentrations affect the 3′ exonuclease activity of UvrABC described in this communication. The results show that the additional cutting that occurs 7 nt from the normal 5′ side dual incision site is, in fact, more sensitive to protein concentrations than dual incision and requires more protein. As shown in Table IA, on the 102-mer duplex DNA with a single AAF, levels of dual incision and 3′ exonuclease activity are observed with ⩾50 nM UvrA, ⩾100 nM UvrB and ⩾100 nM UvrC where different protein ratios and concentrations cause only a slight variation in the actual numbers. Reducing the concentrations of UvrA or UvrB to 30 nM and 50 nM respectively does not change the level of dual incision, but noticeably decreases the frequency of the 3′ exonuclease cuts. Further decreasing the UvrA or UvrB concentrations (to 10 nM and 20 nM respectively) decreases the efficiency of the dual incision and reduces the 3′ exonuclease activity to as low as 2–3%. In Table IB we use a complementary approach by studying the effects of different DNAs added to the reaction mixture. Using the same 102-mer duplex with a single AAF as the labeled

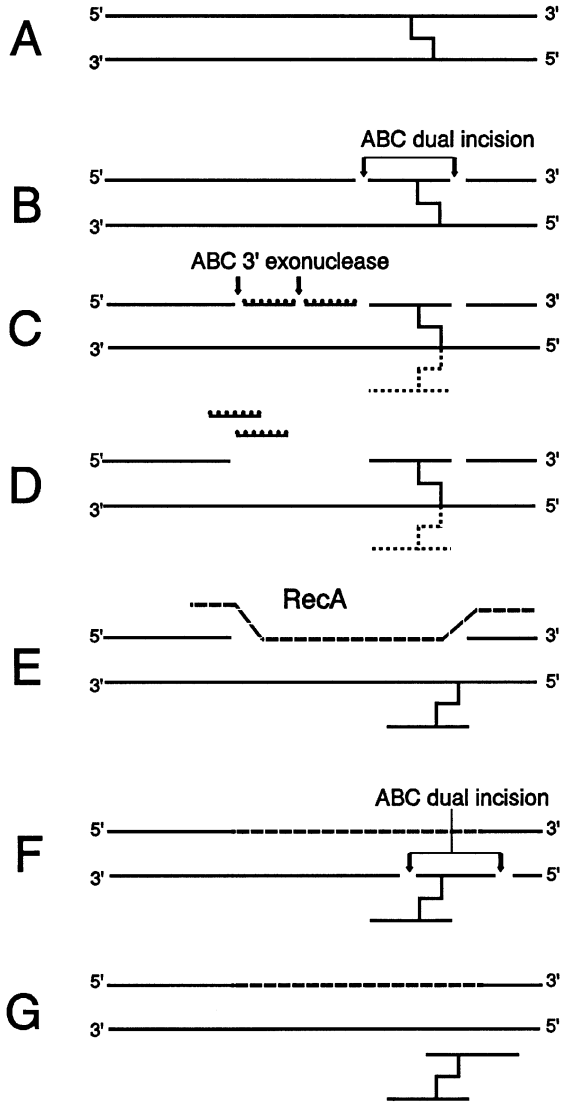



**Fig. 3.** Model of DNA interstrand crosslink repair mediated by UvrABC. (**A**) Interstrand crosslinked DNA. (**B**) On one of the damaged DNA strands UvrABC excinuclease makes a dual incision flanking the crosslink. (**C**) The UvrABC 3′ exonuclease activity makes an additional cut or cuts after dual incision producing 7-nt fragments. (The dotted structure indicates an unpaired alternate structure for the partially excised crosslink because the duplex stability of the oligonucleotide between the initial dual incision sites is not known.) (**D**) These short fragments will dissociate from the duplex and a gap at least 14 nt long is generated 5′ to the site of the crosslink. (**E**) This gap is used as a preferred site for RecA-mediated recombinational exchanges to reconstruct the incised strand. (**F**) Once a recombinational exchange is completed, the residual half crosslink is incised by UvrABC to complete the removal of the crosslinked structure. (**G**) Repair synthesis and ligation finish the repair process.

substrate for incision, we add UV-irradiated or untreated double-stranded MM13 DNA. The rationale is that the presence of additional substrate for DNA repair in the form of UV-irradiated double-stranded DNA might decrease the amount of protein available for incision of a lesion on a 102-mer duplex and change the efficiency of either the dual incision or the 3′ exonuclease on the 102-mer duplex. As shown in Table IB, neither untreated double-stranded DNA nor UV-irradiated double-stranded DNA changes the efficiency of the UvrABC dual incision of the 102-mer substrate. However, the UvrABC 3′ exonuclease activity decreases 3-fold when the double-stranded DNA is UV irradiated. From the results in Table IA and B it is seen that the UvrABC 3′ exonuclease activity described here requires more UvrA and UvrB than is needed for dual incision at a lesion and, thus, might be anticipated to occur in a cell when the levels of UvrA and UvrB are induced to higher levels. It is worth noting that although the overall removal of UV-induced pyrimidine dimers does not seem to be affected by SOS induction of UvrA and UvrB, the distribution of repair patch sizes does show a difference that is under SOS regulation and depends on Rec-Lex control (Cooper and Hanawalt, 1972; Cooper, 1982). In UV-irradiated cells, ~99% of the lesions are repaired by the short patch process which is not affected by SOS-inducing treatments. The remaining 1% of the events are of the long patch type, are under Rec-Lex control, and require SOS induction (Cooper, 1982). Considering that with biological doses of radiation closely opposed photodimers can be present in DNA at a surprisingly high frequency of 1% of the total number of photodimers (Minton and Friedberg, 1974; Lam and Reynolds, 1986a,b, 1987; Livneh *et al.*, 1993), it may be relevant that the conditions of high UvrA and UvrB in our reaction system favor the additional cuts observed with UvrABC, just as SOS-inducing conditions seem to be required for the long patch mode of UvrABC-dependent 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 was purchased from New England Biolabs. T4 DNA ligase and Klenow fragment of DNA polymerase I were purchased from Boehringer-Mannheim.

### *Preparation of DNA substrates with an oligonucleotide annealed to a single-stranded DNA circle*

A derivative of M13, designated MM13mp18, was constructed in our laboratory as described by Gordienko and Rupp (1997a,b) and used for single-stranded DNA circle preparation. Modification of synthetic oligonucleotides with *N*-acetoxy-2-(acetylamino)fluorene (AAAF) is described by Gordienko and Rupp (1997a,b).

For substrates labeled at the 3′ end of the oligonucleotide, 0.8 pmol of single-stranded MM13mp18 was mixed with 0.8 pmol of synthetic oligonucleotide in a sequencing buffer (40 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl) in a 10 µl reaction. The mixture was incubated for 30 min at 37°C. The annealed substrates were labeled with 5 units of Klenow fragment of DNA polymerase I in a 20 µl reaction in sequencing buffer with 5 mM DTT and 50 µCi [$\alpha$-$^{32}$P]dNTP (3000 Ci/mmol, Amersham). After incubation for 15 min at room temperature, the reaction was stopped by adding EDTA to 50 mM, brought up to 50 µl with TE buffer and phenol-extracted. Unincorporated label and unannealed oligonucleotides were removed by passing the mixture through two G-50 Sephadex columns (Boehringer-Mannheim).

For substrates labeled at the 5′ end of the oligonucleotide, first, 0.8 pmol of oligonucleotide was labeled with 20 µCi of [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol, Amersham) and T4 polynucleotide kinase in a volume of 10 µl. NaCl (to 50 mM) and 0.8 pmol of MM13mp18 single-stranded DNA were then added. After incubation of the mixture at 37°C for 30 min, EDTA was added to 50 mM, the volume brought to 50 µl with TE, and the mixture passed through a G50 column to remove any unincorporated label. After phenol extraction the mixture was re-passed through a G50 column. Approximately 8 fmol of the appropriate substrate was used for each reaction.

### *Preparation of a 102-mer duplex DNA substrate with a single AAF lesion*

Synthetic oligonucleotides used for duplex preparation were related to each other as shown:

**Table I.** Influence of UvrA and UvrB concentrations on the dual incision activity of UvrABC and on the 3′ exonuclease activity of UvrABC

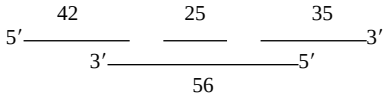
**A**

| Protein (nM) added to reaction mixture | | | UvrABC dual incision (%) | UvrABC 3′ exonuclease incision (%) |
|---|---|---|---|---|
| UvrA | UvrB | UvrC | | |
| 100 | 100 | 100 | 38 | 23 |
| 100 | 100 | 200 | 36 | 26 |
| 100 | 100 | 300 | 33 | 26 |
| 100 | 200 | 100 | 35 | 28 |
| 200 | 100 | 100 | 40 | 17 |
| 200 | 200 | 100 | 32 | 32 |
| 50 | 100 | 100 | 38 | 21 |
| 30 | 100 | 100 | 37 | 9 |
| 100 | 50 | 100 | 39 | 13 |
| 10 | 100 | 100 | 19 | 2 |
| 100 | 20 | 100 | 27 | 3 |

**B**

| DNA added to reaction mixture | UvrABC dual incision (%) | UvrABC 3′ exonuclease incision (%) |
|---|---|---|
| Synthetic ds MM13 (No UV) | 45 | 9 |
| Synthetic ds MM13 (+ UV) | 42 | 3 |
| MM13 RF (No UV) | 34 | 24 |
| MM13 RF (+ UV) | 46 | 8 |

(**A**) The amount of UvrA and UvrB available for incision at a single lesion on the labeled substrate is changed by adding different concentrations of Uvr proteins to the incision reaction mixtures. The reaction mixture (in buffer A) contains the same 5′ end-labeled 102-mer duplex substrate with a single AAF lesion as used in Figure 2B. Percentage of dual incision and 3′ exonuclease incision is calculated as the ratio of the 47-mer product of the reaction (dual incision), or the 40-mer product of the reaction (3′ exonuclease) to the initial substrate concentration.
(**B**) The amount of UvrA and UvrB effectively available for incision at a single lesion on the labeled substrate is changed by adding UV-damaged unlabeled double-stranded DNA (a substrate for DNA repair) to the incision reaction mixture. As the labeled substrate for incision we use the same 102-mer duplex with a single AAF as in (A). The reaction mixture contains labeled substrate and UvrA (100 nM), UvrB (100 nM) and UvrC (100 nM) in buffer A. Synthetic double-stranded MM13 is prepared as described (Gordienko and Rupp, 1997b). Approximately 100 ng of untreated or UV-irradiated (120 J/$m^2$) synthetic double-stranded and RF MM13 is added to each reaction. Percentage of dual incision and 3′ exonuclease incision is calculated as in (A). The rationale to have two types of DNA in this experiment is to show that the effect of UV-irradiated synthetically prepared DNA or UV-irradiated DNA extracted from the cell is to reduce the 3′ exonuclease 3-fold in both cases while having little effect on the dual incision.




The sequences are: 25-mer, 5′-ATATTCTTTAAAGATATCATTAATC-3′; 56-mer, 5′-CGGTACCCGGGATTAATGATATCTTTAAAGAATAT-TTTCTAGAGTCGAC-3′; 42-mer, 5′-AAAAATCGATAAGCTTGCAT-GCCTGCAGGTCGACTCTAGAAA-3′; 35-mer, 5′-CCGGGTACC-GAGCTCGAATTCAGGGCCCAGATCTG-3′. The 25-mer oligonucleotide was modified with AAAF to have a single adduct as described previously (Gordienko and Rupp, 1997a). The 35-mer oligonucleotide was phosphorylated and labeled with ATP and T4 polynucleotide kinase for 30 min at 37°C. The reaction was stopped by heating at 65°C for 10 min. Into ligation buffer (40 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, 50 μg/ml BSA) with 20 units of T4 ligase were added the 25-mer with a single AAF (phosphorylated after modification with AAAF), phosphorylated and labeled 35-mer, 42-mer and 56-mer in the ratio 1 (0.6 nmol):3:3:3 respectively. After incubation for 16 h at 16°C, the mixture was ethanol-precipitated with NaCl and the dry DNA pellet resuspended in 40 μl of TE buffer. Resuspended DNA was run on a 10% sequencing gel with an equal volume of formamide (labeled 102-mer was used as a marker). The band corresponding to 102-mer in a marker lane was cut out and soaked overnight in 5 ml of TE buffer at 37°C to extract DNA. After purification on a Sep-pak column, DNA was resuspended in 20 μl of TE buffer. The resulting 102-mer oligonucleotide with a single AAF lesion was annealed with a 2-fold excess of phosphorylated complementary 102-mer in buffer (100 μl) containing 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 100 mM NaCl. The mixture was incubated 5 min at 90°C and slowly cooled to room temperature. 102-mer duplex was separated from 102-mer oligonucleotides by hydroxyapatite chromatography (Sambrook *et al.*, 1989). Purified 102-mer duplex was resuspended in 20 μl of TE buffer and labeled with T4 polynucleotide kinase at the 5′ end of the AAF-containing strand which was not phosphorylated.

### *UvrABC incision reaction*

The reaction mixture contained ~8 fmol (in single-stranded DNA circles or in 102-mer duplexes) of DNA substrate in buffer A (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 2 mM ATP). The reaction (20 μl volume) was started by addition of UvrA and UvrB to a final concentration of 100 nM each (unless indicated differently) and incubated at 37°C for 2 min. UvrC was then added to 100 nM (unless indicated differently) and incubation continued for 30 min. The reaction was quenched with 5 μl of stop solution and heated for 2 min at 85°C, after which the entire sample was loaded on to 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 products 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 as for the complete reaction and showed that AAF incision required all three proteins, UvrA, UvrB and UvrC.

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