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Action mechanism of ABC excision nuclease on a DNA substrate containing a psoralen crosslink at a defined position

(uniform substrate/DNA bending/damage recognition/repair model/*Escherichia coli*)

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ABSTRACT **Many carcinogenic as well as chemotherapeutic agents cause covalent linkages between complementary strands of DNA. If unrepaired, DNA crosslinks are blocks to DNA replication and transcription and therefore represent potentially lethal lesions to the cell. Genetic studies of *Escherichia coli* have demonstrated that the repair enzyme ABC excision nuclease, coded for by the three unlinked genes, *uvrA*, *uvrB*, and *uvrC*, plays a crucial role in DNA crosslink repair. To study the molecular events of ABC excision nuclease-mediated crosslink repair, we have engineered a DNA fragment with a psoralen–DNA interstrand crosslink at a defined position, digested this substrate with pure enzyme, and analyzed the reaction products on DNA sequencing gels. We find that the excision nuclease (*i*) cuts only one of the two strands involved in the crosslink, (*ii*) incises the crosslink by hydrolyzing the ninth phosphodiester bond 5′ and the third phosphodiester bond 3′ to the furan-side thymine of the crosslink, and (*iii*) does not produce double-strand breaks at any significant level. Based on these data, we present a model by which ABC excision nuclease initiates crosslink repair *in vivo*.**

Several agents have been shown to produce DNA interstrand crosslinks (1) including mitomycin C (2), nitrous acid (3), nitrogen and sulfur mustards (4), formaldehyde (5), cisplatin (6), and psoralen plus light (7, 8). Because of their predictable and highly characterized reactivity with DNA (9, 10), psoralens have been used most extensively to study the repair of DNA crosslinks in several organisms (11–17). These three-ring heterocyclic aromatic compounds (furocoumarins) contain two reactive double bonds that, upon absorption of near UV light (320–360 nm), photoreact with the 5,6 double bond in pyrimidines to form both monoadducts and interstrand crosslinked diadducts, primarily at 5′ TpA 3′ and to a lesser extent at 5′ ApT 3′ sequences (18).

Genetic and biochemical studies of *Escherichia coli* have identified several proteins necessary for the removal of psoralen crosslinks, including products of the *recA*, *uvrA*, *uvrB*, *uvrC*, *uvrD*, and *polA* genes (12, 19–26). Furthermore, it has been estimated that in the *uvrA recA* double mutant, one DNA crosslink per genome is lethal (24, 25). Based on these data, several models of psoralen crosslink repair have been proposed that incorporate components of both the RecA-dependent recombination and the nucleotide excision repair pathways (12, 23).

In *E. coli*, the incision and excision steps of nucleotide excision repair are mediated by a single enzyme, the ABC excision nuclease (see ref. 27). This enzyme is composed of three proteins UvrA (M_r, 103,874), UvrB (M_r, 76,118), and UvrC (M_r, 66,038), which act in concert to cleave both the eighth phosphodiester bond 5′ and the fourth or fifth phosphodiester bond 3′ to UV-induced cyclobutane pyrimidine dimers and 6–4 pyrimidine–pyrimidone intrastrand diadducts (28). The goal of this study was to characterize the molecular events of ABC excision nuclease-mediated DNA crosslink repair.

To this end, we have designed and constructed a DNA substrate composed of six self-complementary oligomers, which when ligated together form a 40-base-pair (bp) fragment with a 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT) furan-side (4′,5′) monoadduct at a central TpA sequence. Subsequent exposure to 360-nm light produced a crosslinked DNA substrate, which was purified on a denaturing polyacrylamide gel. We have found that ABC excision nuclease introduces dual incisions on only the furan side-adducted strand of the HMT crosslink. Furthermore, the incision pattern for the furan-side of HMT crosslink was different from those observed previously for either monoadducts or diadducts. These results are discussed with respect to the proposed structure of DNA adducts, particularly psoralen crosslinks, and a molecular model for crosslink repair in *E. coli* is presented.

MATERIALS AND METHODS

Proteins. The UvrA, UvrB, and UvrC subunits of ABC excision nuclease were purified to >95% homogeneity by the method of Thomas *et al.* (29) and were added to the reaction mixture sequentially to constitute the ABC excision nuclease.

Construction and Labeling of Uniquely Modified Psoralen Substrates. A full description of the synthesis and photochemical characterization of the HMT-modified oligomers is published elsewhere (30). The psoralen-modified DNA fragment, shown in Fig. 1 was made by ligating six synthetic oligomers (marked by arrows) overnight at 4°C. The central top strand oligomer 5′ TCGT(HMT)AGCT 3′ has been previously modified on the 3′ side of the internal thymine by the furan-side monoadduct of HMT. After ligation the DNA was extracted twice with phenol and then ether, ethanol-precipitated, and resuspended in 10 mM Tris·HCl (pH 7.5) buffer and irradiated with 3000 $J \cdot m^{-2}$ (at 15 $J \cdot m^{-2} \cdot sec^{-1}$), using a black light with a 320-nm cut-off filter. The crosslinked DNA and the monoadducted DNA (which had not been irradiated) were purified on a 12% polyacrylamide/7 M urea DNA sequencing gel. This gel purification step was found necessary to obtain fully ligated 40-bp DNA fragments. The crosslinked 40-bp fragments were constructed with the furan end of HMT linked to the top strand and the pyrone end linked to the bottom strand. To obtain labeled DNA fragments, the 5′ top strand oligomer was terminally 5′-labeled with [γ-^{32}P]ATP (ICN) and T4 polynucleotide kinase

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Abbreviations: bp, base pair(s); HMT, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen; pol I, DNA polymerase I.

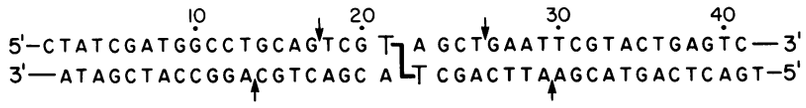

FIG. 1. Sequence of the psoralen-crosslinked DNA fragment. The 40-bp fragment, with 5′ overhanging ends, was constructed from six oligomers. The sites of ligation are indicated by arrows. The central top strand octamer was modified at the internal thymine (5′ TpA 3′) with the furan-side monoadduct of HMT. Irradiation of the modified fragment with near-UV light resulted in the formation of a HMT–DNA crosslink where the furan (4′,5′) and pyrone (3,4) sides of all the diadducts are attached to the top and bottom strands of the DNA fragment, respectively.

(Bethesda Research Laboratories) by standard methods prior to ligation. The 3′-labeled top or bottom strand was obtained by terminally labeling the 40-bp fragment with either [α-^{32}P]-dATP or [α-^{32}P]dGTP (Amersham), respectively, and the Klenow fragment of DNA polymerase I (pol I) under standard conditions. The nucleotide sequences of the 40-bp fragments were verified by the chemical method of Maxam and Gilbert.

ABC Excision Nuclease Digestion. The ABC excision nuclease digestion conditions are given in detail elsewhere (30).

RESULTS

ABC Excision Nuclease Incision Pattern for Crosslinked and Monoadducted Substrates. The crosslinked DNA was terminally labeled at the 5′, 3′, or both termini and digested with ABC excision nuclease, and the reaction products were analyzed on a DNA sequencing gel. The results are shown in Fig. 2*a*. The cutting pattern observed with the crosslink is slightly different from the patterns seen with either nucleotide mono- or di- (intrastrand) adducts. Therefore, for comparison we have included in this figure the reaction products resulting from digestion of the furan-side monoadduct containing DNA. Several conclusions regarding the action mechanism of ABC excision nuclease can be drawn from this figure.

First, the enzyme cleaves the top strand of the crosslinked fragment at the ninth phosphodiester bond 5′ and third phosphodiester bond 3′, to the furan-side-adducted thymine. The 5′ incision by ABC excision nuclease results in a band that migrates between thymine-13 and guanine-14 (Fig. 2*a*, lane 7). The 3′ incision can be observed as a band that migrates slightly slower than guanine-23. The sample in lane 8 is the digestion product of the crosslinked 40-bp substrate labeled on both the 5′ and 3′ (top strand) termini. Two bands can be observed that correspond to dual DNA incisions, 5′ and 3′ to the furan-adducted thymine of the crosslink.

Second, the incision sites with regard to furan-side monoadducted thymine are as reported (31) for DNA randomly modified with psoralen plus light (i.e., the eighth phosphodiester bond 5′ and the fifth phosphodiester bond 3′ to the adduct) and are different from those seen with the crosslink. The cutting patterns deduced from Fig. 2*a* for the furan-side monoadducted and the crosslinked 40-bp fragment are compared schematically in Fig. 3.

Third, ABC excision nuclease performs its dual incisions on the furan-side monoadducted or crosslinked DNA in a

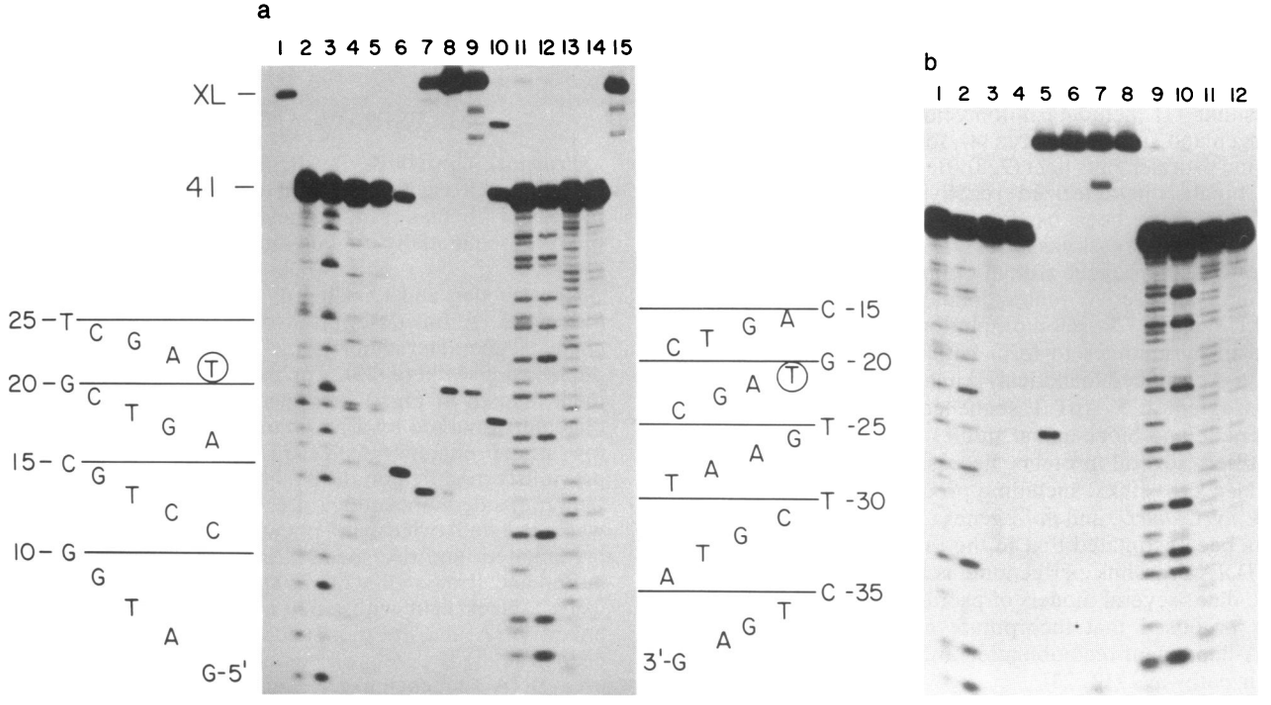

FIG. 2. ABC excision nuclease digestion pattern of the psoralen-modified 40-bp DNA fragment. HMT-modified DNA was digested with the excision nuclease and the reaction products, following deproteinization, were analyzed on a sequencing gel. (*a*) In all cases, the label is on the top strand. Lane 1, 5′-labeled crosslinked (XL) DNA; lanes 2–5, A+G, G, C+T, and C Maxam–Gilbert sequencing reactions of the 5′-labeled top strand DNA. The DNA samples in lanes 6–10 were digested with ABC excision nuclease. Lane 6, 5′-labeled furan-side monoadducted DNA; lane 7, 5′-labeled crosslinked DNA; lane 8, 5′- and 3′-labeled crosslinked DNA; lane 9, 3′-labeled crosslinked DNA; lane 10, 3′-labeled furan-side monoadducted DNA; lanes 11–14, A+G, G, C+T, and C Maxam–Gilbert sequencing reactions of the 3′-labeled top strand; lane 15, 3′-labeled crosslinked DNA. (*b*) Lanes 1–4 and 9–12 show the Maxam–Gilbert sequencing reactions on the 3′-labeled top and bottom strands, respectively. Lanes 5 and 6 contain 3′ top strand-labeled crosslinked DNA plus or minus ABC excision nuclease digestion, respectively. Lanes 7 and 8 are 3′ bottom strand-labeled crosslinked DNA plus or minus ABC excision nuclease digestion, respectively.

highly coupled process because bands corresponding to a lone 3′ incision on the 5′-labeled fragment or a lone 5′ incision on 3′-labeled fragments were not seen at any significant levels. Overexposure of the gel did reveal this type of anomalous incision pattern for the furan-side monoadducted DNA, albeit at <1% of the total incisions (data not shown).

Fourth, under our assay conditions, in which the enzyme was in excess, the DNA was not digested to completion. We believe this may be due to the formation of stable "nonproductive" AB or ABC complexes on the DNA that bind tightly to the substrate but fail to incise it. Filter binding assays as well as preliminary DNase "footprinting" studies suggest that the ternary A·B·DNA complex is quite stable. The dissociation of these nonproductive complexes may require additional protein factors, such as helicase II and DNA pol I; these have been found to be necessary for the dissociation of the three ABC excision nuclease subunits following cleavage (32). It is also conceivable that the DNA analog used in this study is of insufficient length and thus makes nonproductive complexes with the enzyme readily.

Finally, the data in Fig. 2*b* show that ABC excision nuclease acts only on the DNA strand that is attached to the furan-side of a HMT crosslink at a 5′ TpA 3′ site. If ABC excision nuclease incised either the furan- or the pyrone-adducted strands of the crosslink, then two types of digestion products would have been observed: incision of the labeled strand would yield a faster migrating band, while incision of the nonlabeled strand would result in a band representing the excised 11- to 12-nucleotide oligomer crosslinked to the full-length labeled 41-mer. ABC excision nuclease digestion of the crosslinked DNA, terminally 3′-labeled in the top strand (furan-adducted strand), produced a single faster migrating band (Fig. 2*b*, lane 5), while digestion of crosslinked DNA, terminally 3′-labeled in the bottom strand (pyrone-adducted strand), produced only one band, which migrated between the crosslinked DNA and the full-length 41-mer (Fig. 2*b*, lane 7). Note the complete absence of a faster migrating band in this lane, evidence that the nuclease does not incise the pyrone-adducted strand (lane 7). These two digestion patterns are only consistent with ABC excision nuclease cleavage occurring exclusively in the furan-adducted strand of the crosslink.

To further confirm that ABC excision nuclease does not make incisions on both the pyrone and furan sides of the crosslink, which would result in double-strand breaks, we analyzed the digested DNA on a nondenaturing polyacrylamide gel (data not shown). No change in mobility was observed for the crosslinked fragment when it had been digested with ABC excision nuclease, thus indicating that the enzyme did not introduce closely spaced nicks on opposite strands. These data as well as the data presented in Fig. 2 lead us to conclude that ABC excision nuclease in the absence of other factors acts only on the furan side of a HMT crosslink.

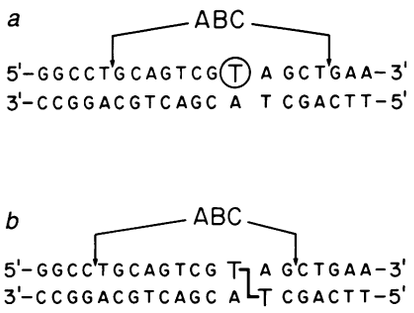

FIG. 3. Incision sites of ABC excision nuclease on psoralen-monoadducted or crosslinked DNA. The positions of the ABC excision nuclease incision sites were obtained from Fig. 2, for the furan-side monoadducted (*a*) and the crosslinked (*b*) 40-bp fragment (only the relevant nucleotides of the 40-bp fragment are shown).

DISCUSSION

The results presented in this paper reveal certain features regarding the action mechanism of ABC excision nuclease on psoralen-adducted DNA and form a basis for a molecular description of crosslink repair in *E. coli*.

Incision Mechanism of ABC Excision Nuclease on Monoadducted DNA. Several studies have demonstrated that in *E. coli* both the incision and excision steps of nucleotide excision repair are mediated by a multisubunit enzyme, ABC excision nuclease (27). This enzyme was shown to have an unusual nuclease activity, producing dual DNA incisions seven nucleotides 5′ and four or five nucleotides 3′ to UV-induced DNA damage (28). Furthermore, this incision motif has been shown to be consistent with potential monoadduct-forming sites in DNA randomly damaged with *N*-acetoxyaminofluorene and psoralen plus UV (31). While these studies have provided important information regarding the enzyme's mode of action, the results were indirect because the DNA substrates were not uniformly modified at specific sites, and there was no independent measure of the level of modification at the nucleotide level. The HMT furan-side monoadducted and crosslinked 40-bp DNA fragments represent suitable substrates to confirm the action mechanism of ABC excision nuclease on chemically modified DNA. In Fig. 2, we have verified the proposed incision pattern for monoadducted DNA—namely, the enzyme mediates the cleavage of the eighth phosphodiester bond 5′ and the fifth phosphodiester bond 3′ to the HMT furan-side monoadduct. Unlike incisions due to UV-induced DNA damage, 3′ incision of the fourth phosphodiester bond was not detected for the HMT-monoadducted substrate.

Possible Recognition Signals for ABC Excision Nuclease. It was necessary to map the location of the ABC excision nuclease-mediated incisions before a detailed model of crosslink repair could be proposed. The experiments presented in Fig. 2 yielded two unexpected results. First, ABC excision nuclease produced dual DNA incisions in only the furan-adducted strand of the crosslink, even though the enzyme recognizes both the furan- and pyrone-side monoadducts with equal affinity (30). Second, the incision pattern for the crosslink differs from those observed for either diadducts or monoadducts, in that ABC excision nuclease cleaves the ninth phosphodiester bond 5′ and the third phosphodiester bond 3′ to the furan-adducted thymine of the crosslink. What is the significance of the different mode of action of ABC excision nuclease on these two substrates?

It has been proposed that ABC excision nuclease recognizes the helical distortion created by a DNA adduct rather than the adduct itself (28, 31). It is conceivable that the extent of DNA perturbation could affect the position of the incisions with respect to the damaged nucleotide, as well as the distance between the two incisions. Using x-ray diffraction data from psoralen-adducted thymidine monoadducts and isolated pyrimidine dimers together with energy minimization calculations, Pearlman *et al.* (33) have proposed molecular models of DNA containing either a pyrimidine dimer or a psoralen crosslink. These models show several common features that may be recognized by repair enzymes including a local unwinding of the helix, displacement of the helix axis, and a bending of the DNA helix into the major groove. We have extended the helices of these models to examine the positions of the phosphodiester bonds that are hydrolyzed by ABC excision nuclease. These model structures are shown in Fig. 4.

Studies of pyrimidine dimer incision by ABC excision nuclease in the presence of the *E. coli* photoreactivating

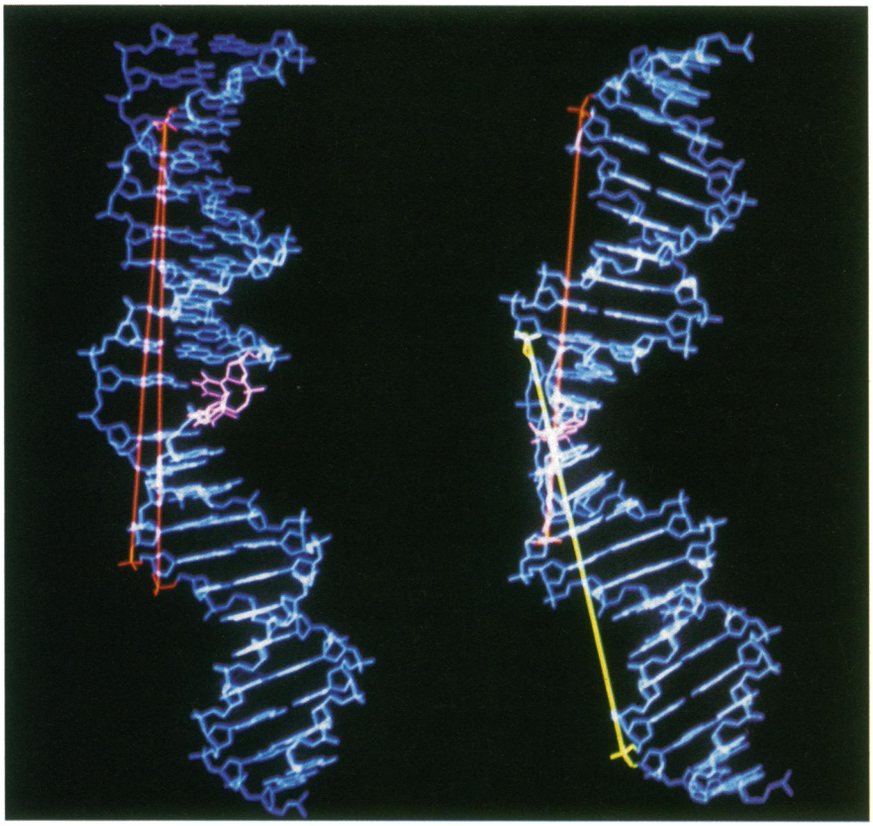

FIG. 4. Incision sites of ABC excision nuclease on DNA containing pyrimidine dimer or psoralen crosslink. The energy-minimized structures for double-stranded DNA containing a thymine cyclobutane dimer (*Left*) or a psoralen crosslink (*Right*) from Pearlman *et al.* (33) were extended to accommodate the positions of the phosphodiester bonds hydrolyzed by ABC excision nuclease in these substrates. (These DNA sequences differ from the sequence given in Fig. 1, although in each case the crosslink is contained within a 5′ TpA 3′ site.) The structures have been oriented to display maximum bending of the DNA. The dimer produces a helical kink of 27° and a total unwinding of 19.7°, while the psoralen crosslink unwinds the helix by 87.7° and produces a 46.5° helical kink. The color code for the vector drawing is as follows: DNA, blue; phosphates cleaved by the excision nuclease (along the furan-side strand of psoralen-crosslinked DNA and along the strand containing the thymine dimer) for the UV damaged DNA, red; phosphates along the pyrone-side strand of the psoralen crosslinked DNA, but not incised, yellow-green; psoralen crosslink and thymine dimer, magenta. The linear distances between the dual cleavage sites are 45.1 Å, and 47.8 Å for the two incision motifs (the eighth phosphate 5′ and the fourth or fifth phosphate 3′) for the pyrimidine dimer, and 45.0 Å for the furan-side strand of the crosslink. (The distance for the pyrone-side strand that is not cleaved would be 44.3 Å, assuming a similar incision motif.)

enzyme (photolyase), have revealed certain features of nuclease binding (34). The fact that photolyase, which is known to bind to both the cyclobutane ring and to two phosphates surrounding the dimer (35), does not interfere with (but actually stimulates) ABC excision nuclease incision (34), supports the idea that photolyase and the excision nuclease bind to opposite faces of the DNA. This observation combined with preliminary methylation protection experiments (unpublished results), when interpreted in terms of the proposed structure of the dimer-containing DNA, suggests a model for ABC excision nuclease action: the enzyme binds to the convex side of the DNA (opposite the photolyase contact sites), recognizing and contacting the major groove both 5′ and 3′ to the dimer-induced bend, and makes the dual incisions at appropriately spaced phosphates on this side of the DNA (see Fig. 4). Assuming that this enzyme utilizes a similar mode of binding to DNA containing other types of damage as to DNA containing a pyrimidine dimer, then one would predict that ABC excision nuclease would also recognize the major grooves immediately 5′ and 3′ to the psoralen-induced bend, again making incisions at the phosphodiester sites on the convex face of the DNA that are topologically equivalent to those cleaved in DNA containing a pyrimidine dimer. This model explains the preference for incision on the DNA strand linked to the furan of psoralen, since the observed phosphodiester incision sites are spatially in a similar location to those of DNA containing a pyrimidine dimer, while the equivalent sites on the opposite strand are not in the proper arrangement. The calculated distance between incision sites for the psoralen crosslinked DNA (45.0 Å) is close to that calculated for the dimer-containing DNA model (45.1 Å, 47.8 Å), even though the incision pattern is different in terms of nucleotides away from the damage site. This may be due to the greater bending and unwinding postulated for the psoralen DNA when compared to DNA containing a pyrimidine dimer.

While these bent models are helpful in visualizing the spatial arrangement of the incision sites, it should be noted that the actual conformation of the damaged DNA could be considerably different than that depicted here, and therefore these models should be viewed with caution. Furthermore, the structure of the crosslink might undergo an additional conformational change when bound by ABC excision nuclease. This model is testable by studying ABC excision nuclease-mediated incisions on psoralens with various orientations at 5′ TpA 3′ and 5′ ApT 3′ sequences. Furthermore, footprinting experiments with ABC excision nuclease bound

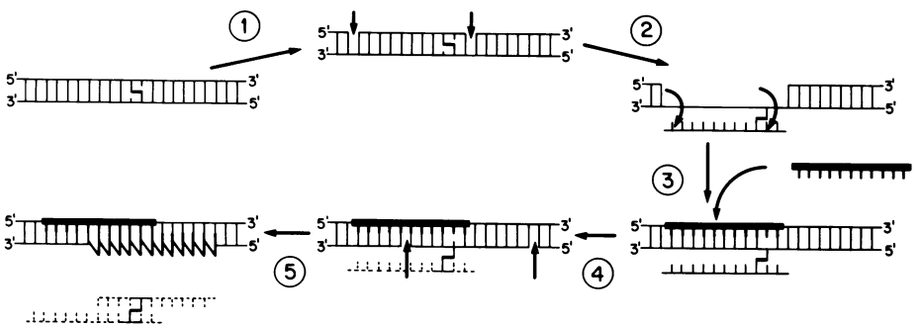

FIG. 5. Model for psoralen crosslink repair. Step 1, ABC excision-mediated dual incisions; steps 2 and 3, gap filling by a recombination event mediated by the RecA protein; step 4, second round of ABC excision nuclease incisions; step 5, the ABC nuclease and the crosslink dissociate, with concomitant gap filling by the combined action of pol I and helicase II.

to a defined substrate should help to elucidate the sites of DNA contact.

The question of whether a psoralen crosslink actually induces a bend at the site of the adduct has not been resolved. In a study of a DNA fragment randomly modified with five to seven crosslinks per molecule, polyacrylamide gel electrophoresis and differential decay of birefringence failed to detect a DNA bending like that observed for a known bent DNA fragment from trypanosome kinetoplast DNA (36). In the future, more detailed physical studies on DNA fragments, like those described here with one DNA crosslink per molecule at a predetermined site, should help elucidate the conformation of damaged DNA as well as determine the structural requirements for recognition by ABC excision nuclease.

Model for DNA Crosslink Repair. In *E. coli* the majority of the lethal effects of psoralen crosslinks are removed by a combination of both the RecA recombination and DNA excision repair pathways, although a minor repair mechanism has been proposed that is independent of the Uvr repair system (37). The incision mechanism observed with the psoralen-crosslinked substrate *in vitro* combined with previous *in vivo* work demonstrating that crosslink repair is absolutely dependent on a functional RecA protein in *E. coli* suggests a model for the repair of crosslinks in *E. coli* (Fig. 5). This model contains elements of an earlier model first proposed by Cole (12).

In step 1, ABC excision nuclease produces dual incisions in only the DNA strand, which is covalently linked to the furan ring of HMT. The resulting excised oligomer is displaced by the RecA protein (step 2), which mediates strand invasion by a sister duplex (step 3). [Alternatively, in what presumably would be a mutagenic event, the excised oligomer may be displaced by the dual action of helicase II (UvrD protein) and a polymerase that could perform translesion DNA synthesis so as to fill in the gap.] A triple-strand intermediate having two fully complementary strands attached to the excised oligomer via the psoralen crosslink is produced and, in step 4 this new structure is cut by ABC excision nuclease on both sides of the pyrone side-adducted thymine. The dangling oligomer may be trimmed by nonspecific *E. coli* nucleases. The resulting excision product, which consists of two oligomers covalently linked by psoralen, is displaced by the joint action of DNA polymerase I plus helicase II (37) and the excision gap is filled in and ligated (step 5). Presumably, crosslinks caused by other agents are repaired by a mechanism similar to that used for psoralen crosslinks. However, when the crosslinking agent creates a symmetrical structure (e.g., nitrous acid), either one of the strands may be incised in the initial step to generate the structure shown in step 1 of Fig. 5. An absolute requirement for a recombinational step in crosslink repair in both *E. coli* and mammalian cells is further supported by the fact that only cells containing multiple genomes are capable of undergoing crosslink repair (37–39), suggesting that the model proposed here for *E. coli* may be applicable to other organisms as well.

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