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Initiation of DNA Interstrand Cross-Link Repair in Humans: the Nucleotide Excision Repair System Makes Dual Incisions 5′ to the Cross-Linked Base and Removes a 22- to 28-Nucleotide-Long Damage-Free Strand

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Most DNA repair mechanisms rely on the redundant information inherent to the duplex to remove damaged nucleotides and replace them with normal ones, using the complementary strand as a template. Interstrand cross-links pose a unique challenge to the DNA repair machinery because both strands are damaged. To study the repair of interstrand cross-links by mammalian cells, we tested the activities of cell extracts of wild-type or excision repair-defective rodent cell lines and of purified human excision nuclease on a duplex with a site-specific cross-link. We found that in contrast to monoadducts, which are removed by dual incisions bracketing the lesion, the cross-link causes dual incisions, both 5′ to the cross-link in one of the two strands. The net result is the generation of a 22- to 28-nucleotide-long gap immediately 5′ to the cross-link. This gap may act as a recombinogenic signal to initiate cross-link removal.

Many anticancer drugs, including cisplatin, mitomycin, nitrogen mustard, and psoralen, induce interstrand cross-links in DNA (31, 70). In vivo data indicate that the cross-links are eliminated from cellular DNA in both procaryotes and eucaryotes (17). However, the detailed enzymatic mechanisms of their repair are not well understood.

In *Escherichia coli*, genetic analysis indicates that both nucleotide excision repair and recombination are needed to eliminate cross-links (9). In vitro experiments with purified procaryotic enzyme systems have provided a mechanistic basis for these requirements (6, 7, 55, 62). Dual incisions by excision nuclease (excinuclease) in one strand on both sides of the cross-linked base are followed by a RecA-mediated strand transfer and ligation to generate a triple-stranded structure. This intermediate is then subjected to a second round of dual incisions by excinuclease to eliminate the cross-link and generate a gapped duplex which can be filled by using the other strand as a template.

The molecular mechanism of cross-link repair in humans is not known. However, there are several mammalian cell mutants with extreme sensitivity to cross-linking agents. Fanconi's anemia (FA) cells are hypersensitive to agents that cause interstrand cross-links; however, there appears to be a consensus in the field that FA cells are not defective in repairing cross-links (11). In Chinese hamster ovary (CHO) cells, mutations in excision repair genes *ERCC1* and *ERCC4* (*XPF*) render cells exquisitely sensitive to cross-linking agents (20). Although other excision repair mutants are also sensitive to cross-linking agents, the extreme sensitivity of ERCC1 and ERCC4 mutants suggests that these genes play a unique role in cross-link repair in addition to their role in excision repair (60). Indeed, the ERCC1 and ERCC4 (XPF) proteins form a heterodimer with structure-specific endonuclease activity (2, 35). Even though mutations in the corresponding yeast genes *RAD10* (*ERCC1*) and *RAD1* (*XPF*) do not confer extreme sensitivity to cross-linking agents, they totally eliminate cross-link repair and lead to defective mitotic recombination (17, 44), consistent with the notion that a recombination step is involved in repair of cross-links in eucaryotes as well.

In humans, nucleotide excision repair is initiated by excinuclease. This is the enzymatic activity which results from coordinated actions of six repair factors, XPA, RPA, TFIIH, XPC, XPG, and XPF · ERCC1, and which removes the damaged nucleotide by dual incisions, one (catalyzed by XPF · ERCC1) 20 ± 5 nucleotides (nt) 5′ and the other (catalyzed by XPG) 6 ± 3 nt 3′ to the damaged base (49, 66). Previous attempts to study processing of cross-links by nucleotide excision repair in vitro using plasmid DNAs containing psoralen or cisplatin cross-links have yielded results consistent with cross-link repair initiated by excinuclease (4, 13, 45). However, the data from those studies were inconclusive, in part because randomly damaged DNA was used as a substrate and because the repair synthesis assay, which is uninformative on the individual steps of the repair reaction, was used to measure repair. An attempt to detect interstrand cross-link repair with human cell extracts and a duplex with a single cisplatin cross-link failed to reveal any cross-link-specific incision of the DNA (67). In the present study, we used DNA substrates containing a site-specifically placed psoralen interstrand cross-link in in vitro systems to investigate the processing of interstrand cross-links in mammalian cells. We have obtained very surprising results which nevertheless may provide important clues to the mechanism of cross-link repair in humans.

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MATERIALS AND METHODS

Cell lines and cell extracts. The CHO cell lines used in this study were obtained from ATCC Repository (Rockville, Md.): CRL 1859 (AA8; parental cell line), CRL 1862 (UV20, ERCC1), CRL 1865 (UV5, ERCC2), CRL 1860

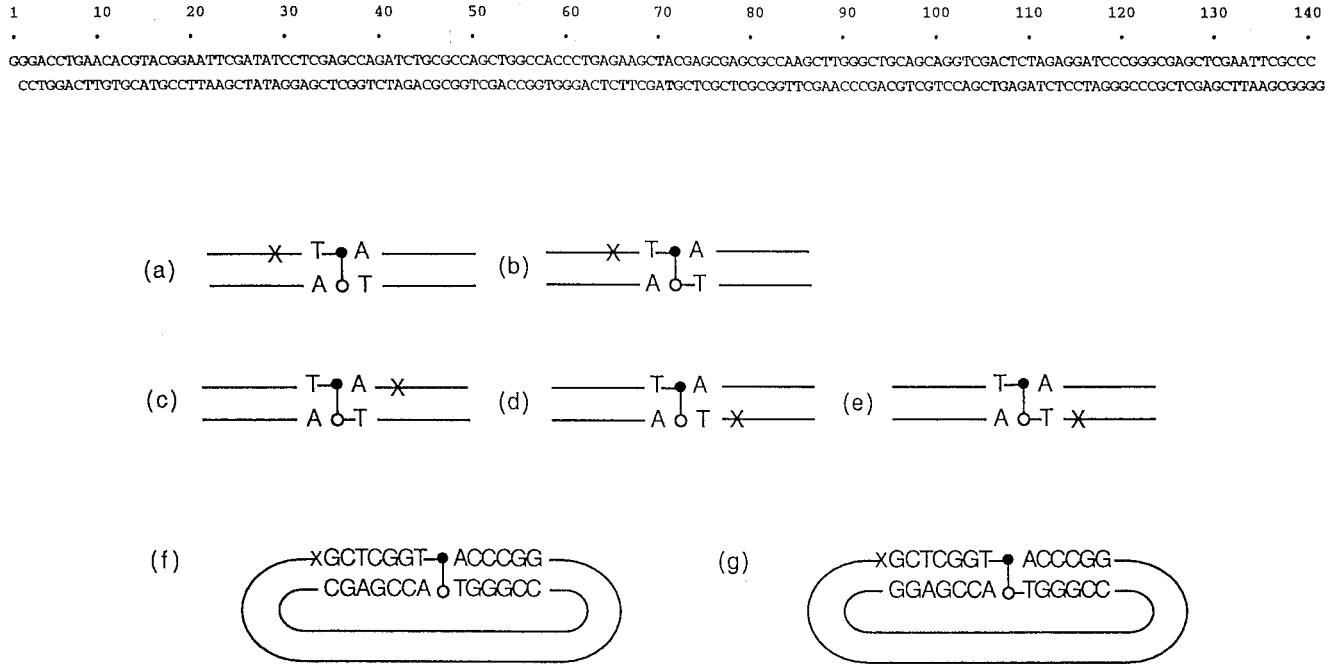

FIG. 1. Substrates used in this study. A 140-bp duplex with a psoralen in the center or plasmid DNA with a psoralen at a unique site was used. The sequence of the 140-bp duplex, with the psoralen-adducted Ts indicated (boldface), is shown at the top. The duplex contained either a furan side (closed circle)-adducted psoralen monoadduct or a cross-link in which the top strand was adducted to the furan side and the bottom strand was adducted to the pyrone side (open circle) of the psoralen molecule. The radiolabeling positions (X) used to determine the incision sites relative to the psoralen monoadduct and interstrand cross-link are shown. (a) Furan side monoadduct with radiolabel at the sixth phosphodiester bond 5′ to the adduct. (b) Interstrand cross-link with radiolabel at the sixth phosphodiester bond 5′ to the furan side-adducted T. (c) Interstrand cross-link with the radiolabel at the seventh phosphodiester bond 3′ to the furan side-adducted T. (d) Furan side monoadduct with the radiolabel at the 10th phosphodiester bond from the cross-linkable T in the complementary strand. (e) Interstrand cross-link with the radiolabel at the 10th phosphodiester bond 5′ to the pyrone side cross-linked T. (f) Circular substrate with psoralen furan side monoadduct and radiolabel at the seventh phosphodiester bond 5′ to the adduct. (g) Circular substrate with interstrand cross-link and radiolabel at the seventh phosphodiester bond 5′ to the furan side-adducted T.

(UV41, ERCC4), and CRL 1867 (UV135, ERCC5). Cell extracts were prepared as described previously (46).

Preparation of substrates. Double-stranded DNA (140-mer) was prepared by the ligation of a 12-mer oligonucleotide containing a furan side psoralen monoadduct at a specific position (a kind gift from John Hearst, University of California, Berkeley) and five partially overlapping oligonucleotides as described previously (22, 34, 54). The radiolabel was introduced into different positions in substrate DNA by subjecting one of six oligonucleotides to kinase treatment with [γ-^{32}P]ATP (see Fig. 1a to e). Cross-linked substrate was prepared by irradiating the 140-mer double-stranded substrate containing furan side psoralen monoadduct with 366-nm UV light (2.5 mW/cm^2) for 10 min at 4°C. After irradiation, cross-linked DNA was purified with a 6% denaturing polyacrylamide gel. The purity of the cross-link substrate was tested by two different gel systems (see Fig. 2). To photoreverse the cross-link, the substrate was irradiated with 254-nm light (2 mW/cm^2) for 10 min at 4°C. More than 95% of the cross-link was converted to monoadduct under these conditions (see Fig. 2A; compare lanes 3 and 4).

The plasmid substrates (see Fig. 1f and g) were prepared as described previously (58) except that pIBI25 single-stranded DNA was used as the template for second-strand synthesis. Following ligation, half of the product was converted to cross-link by 366-nm irradiation, as in the case of the linear substrates. The monoadducted and cross-linked plasmids were separated from gapped and nicked products by CsCl-ethidium bromide gradient centrifugation as described elsewhere (58). The negatively supercoiled DNA was prepared as described previously (41).

Excision assay. For repair assay with cell extracts, 80 μg of cell extract (40 μg of each mutant cell extract for cross-complementation) and 10 fmol of substrate DNA were mixed in 25 μl of reaction buffer (20 mM HEPES-KOH [pH 7.9], 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.08 mM dithiothreitol, 2 mM ATP, and 100 μM deoxynucleoside triphosphates) and incubated at 30°C for 1 h. Following organic extraction, the products were analyzed on an 8% sequencing gel.

Nucleotide sequence of the excised fragment. The 22-mer oligonucleotide excised from cross-linked substrate was isolated from the gel by electroelution, and then its nucleotide sequence was determined by the method of Maxam and Gilbert. The image of the sequence ladder was obtained by use of the Storm 860 system with ImageQuaNT Software (Molecular Dynamics, Sunnyvale, Calif.).

Excision with a reconstituted system. The excision reaction using the reconstituted excision repair nuclease was conducted as described previously (37). A typical reaction mixture contained all six factors for the excinuclease (XPA [15 ng], TFIIH [2 ng], XPC · HHR23B [7 ng], XPG [6 ng], XPF · ERCC1 [10 ng], and RPA [200 ng]) and 1 nM DNA in reaction buffer. After incubation for 1 h at 30°C, the products were analyzed on an 8% sequencing gel.

RESULTS

Substrates. Cross-linked substrates were prepared with the synthetic psoralen 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (54). Psoralen is a three-ringed asymmetric aromatic compound which intercalates into DNA at alternating pyrimidine-purine sites, in particular at TA sites (8). Upon excitation with 300- to 400-nm-wavelength radiation, the furan side or the pyrone side of the molecule forms a cyclobutane adduct with the C-5 and C-6 of an adjacent thymine. Absorption of a second near-UV photon by the furan side-monoadducted psoralen can lead to covalent bond formation between the pyrone ring and the T in the complementary strand, thus forming an interstrand cross-link. Although the psoralen adducted to DNA through both the furan and the pyrone rings no longer absorbs near-UV light, it does absorb light at 254 nm, and by irradiation at this wavelength the covalent bonds between thymine and psoralen are broken, leading to reversal of the cross-link.

Figure 1 schematically shows the substrates used in this study. One class of substrates were 140-bp duplexes that were obtained by ligating a thymine-psoralen furan side monoadduct-containing oligomer with five other oligomers with partially overlapping sequences (54). To generate the cross-linked substrate, the duplex with monoadduct was irradiated with 366-nm light (5). The cross-linked DNA was purified from the residual monoadduct by denaturing polyacrylamide gel elec-

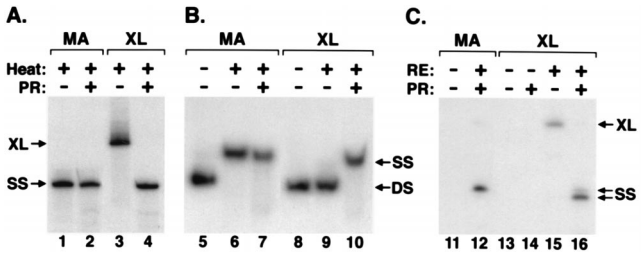

FIG. 2. Characterization of substrates containing psoralen furan side monoadduct (MA) or interstrand cross-link (XL). (A and B) The 140-bp duplexes containing monoadduct or cross-link were heat denatured or exposed to 254-nm light, which causes photoreversal (PR) of cross-links as indicated (+) and separated on 6% polyacrylamide denaturing (lanes 1 to 4) or nondenaturing (lanes 5 to 10) gels. Note that in the denaturing gel, the slowly migrating species is the cross-linked DNA (lane 3), whereas in the native gel, the slowly migrating species is single-stranded DNA (SS) and the fast-migrating species is the 140-mer duplex (DS). (C) Analysis of circular substrate. The monoadduct and cross-link substrates were digested with *Bam*HI plus *Eco*RI restriction endonucleases (RE) in lanes 12, 15, and 16, which generate a 21-bp duplex, and the products were separated on a 10% denaturing gel without (−) and with (+) photoreversal reactions as indicated.

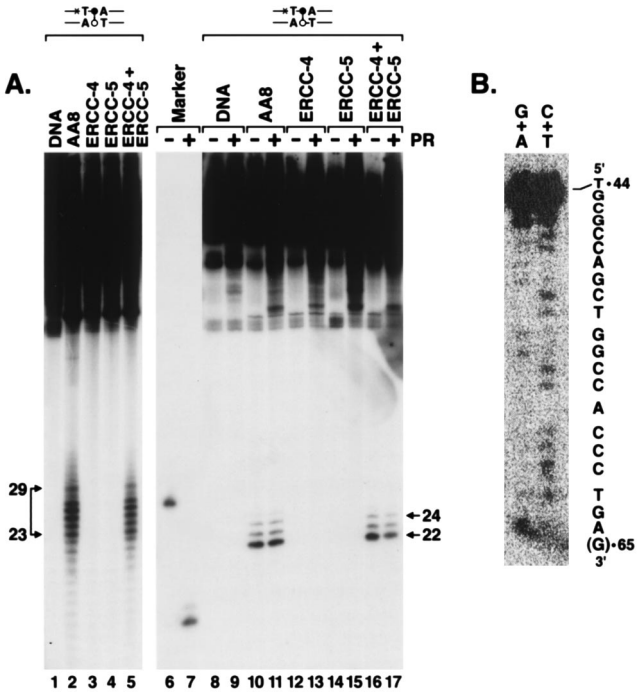

FIG. 3. (A) Excision reactions with rodent cell extracts and DNA containing psoralen monoadduct or interstrand cross-link. DNA with internal label and furan side monoadduct (lanes 1 to 5) or interstrand cross-link (lanes 8 to 17) was incubated with wild-type (AA8) or mutant cell extracts and analyzed on 8% denaturing polyacrylamide sequencing gels. The radiolabel was at the sixth phosphodiester bond 5′ to the furan side-adducted thymine in both cases. With the cross-linked DNA, the cross-link was photoreversed (PR) by 254-nm light as indicated (+) prior to being loaded onto the gel. The mutant cell lines were ERCC4 (XPF) and ERCC5 (XPG). In lanes 5, 16, and 17, complementation with the mutant extracts was performed. Lane 6 contained a 12-mer–20-mer cross-link with radiolabel at the 5′ terminus of the furan side-adducted 12-mer. Lane 7 shows the release of the 12-mer from the cross-link by photoreversal. The sizes and positions of the excised fragments are indicated (arrows). (B) Sequence of the 22-nt-long oligomer excised from the cross-linked DNA. The 22-mer generated by the AA8 cell extract was subjected to G+A and C+T chemical sequencing reactions and analyzed on a 10% polyacrylamide sequencing gel. The sequence corresponds to nt 44 through 65 of the top strand of the duplex shown in Fig. 1. Although the 22-mer arose from an internally labeled duplex, as evident from the sequencing reactions, it yields a distinct sequencing pattern, indicating that it arose from cleavage at the fifth phosphodiester 5′ to psoralen, immediately 3′ to the labeled G residue, resulting in a 22-mer with 5′ ^{32}P label at the phosphodiester between the first and second nucleotides of the oligomer (Fig. 1). Based on this, it is concluded that the 23- and 24-mers result from cleavage at the fourth and third phosphodiester bonds 5′ to the cross-linked T. Note that because of the presence of the label between the first and second 3′-terminal nucleotides, the chemical cleavage of the first nucleotide from the 3′ end does not yield a radioactive band corresponding to the 3′-terminal nucleotide (G). About 1.0% of the radiolabel was in the excision products in lane 2 and 0.5% was in lanes 10, 11, 16, and 17.

trophoresis. The second group of substrates consisted of covalently closed plasmid DNAs with a single furan side psoralen monoadduct or an interstrand cross-link at a defined position. The plasmid with monoadduct was prepared by primer extension as described previously (58). Part of the monoadducted plasmid DNA was converted to cross-link by irradiation with 366-nm light, as in the case of the linear substrate. The monoadducted and cross-linked substrates were purified as described previously (58). By treating the circular substrates with calf thymus topoisomerase I in the presence of 2 μg of ethidium bromide per ml, we prepared substrates with an average superhelical density of −0.05 (41).

As shown in Fig. 2, after the purification step neither the linear nor the circular substrates contained much detectable (<5%) monoadduct. These DNA preparations and other preparations of comparable purity were used in our experiments. By phosphorylating the appropriate oligomer with [γ-^{32}P]ATP, we prepared substrates that contained the radiolabel (i) at the 5′-terminal phosphate of the furan side-adducted strand, (ii) at the 6th phosphodiester bond 5′ or (iii) the 7th phosphodiester bond 3′ to the lesion of the same strand, or (iv) at the 10th phosphodiester bond 5′ to the pyrone side-adducted thymine, in order to analyze the processing of the respective strands both 5′ and 3′ to the site of the cross-link. In the case of circular DNA, the label was at the seventh phosphodiester bond 5′ to the furan side-adducted thymine.

Excision of an undamaged oligonucleotide from cross-linked DNA by cell extracts. Figure 3A shows the reaction products of monoadducted or furan-side cross-linked substrate upon incubation with cell extract from CHO cell line AA8. In agreement with previous work (58), the monoadduct is removed mainly in the form of 23- to 29-nt-long oligomers. Surprisingly, radiolabeled fragments 22 to 26 nt in length are excised from the cross-linked DNA with about the same efficiency as the oligomers from the monoadducted DNA. Because of the interstrand linkage, these fragments could not be generated by dual incisions in one strand on both sides of the lesion by the standard reaction carried out with human excinuclease. Such an incision would generate a population of 23- to 29-mers cross-linked to the other strand; only after photoreversal of the cross-link are excised oligomers of appropriate sizes expected to be released (41, 62). Hence, we considered the possibility that the fragments were excised from the cross-linked DNA not by the usual incision mode but by a double-strand break resulting from incisions on both sides of the cross-link in both strands within 6 to 7 nt of the cross-link. Such an incision pattern would release a radiolabeled oligomer with a cross-link. However, the excised oligomer did not contain a cross-link, as revealed by the fact that photoreversal by short-wavelength UV light, which converts interstrand cross-link to monoadduct quantitatively (Fig. 2), did not change the mobility of the excised fragment (Fig. 3A, compare lanes 10 and 11).

The lack of damage in the excised fragment raised the possibility that this unexpected excision mode may have been caused by nucleases not related to nucleotide excision repair. Indeed, with wild-type cell extracts or partially purified fractions, incisions at the seventh phosphodiester bonds 3′ and 5′

to a psoralen furan side monoadduct were detected by Nichols (42), and incisions at the fifth phosphodiester bond both 3′ and 5′ to a psoralen furan side monoadduct and the furan side-adducted T of interstrand cross-link were reported by Kumaresan et al. (32). However, these incisions are not related to nucleotide excision repair, because they do not require ATP. Furthermore, the relevance of these incisions to repair of monoadducts or cross-links is doubtful because there are no known mutants defective in these incisions. Hence, to prove that the excision of an undamaged oligomer from cross-linked DNA that we observe in Fig. 3 is a repair-related reaction, we tested cell extracts from mutant cell lines defective in excision repair. *ERCC4* mutant cells which are defective in the XPF · ERCC1 nuclease, which is responsible for the 5′ incision, or *ERCC5* mutant cells defective in the XPG nuclease, which makes the 3′ incision of the excinuclease, fail to excise 22- to 26-mers (Fig. 3A, lanes 12 to 15). Mixture of the two mutant cell extracts restores the excision activity (Fig. 3A, lanes 16 and 17). Thus, clearly damage-free fragments have been removed by the DNA repair excinuclease.

To identify the sites of the incisions which gave rise to these fragments, the most abundant species, the 22-nt-long fragment, was purified and sequenced. The results in Fig. 3B show that this species was generated by cleavage of the 6th and 27th phosphodiester bonds 5′ to the T residue in one of the cross-linked strands. This incision scheme predicts generation of a 43-nt-long fragment resulting from the incision at the 27th phosphodiester bond 5′ to the cross-link. Indeed, with a 5′-terminally labeled DNA, a 43-mer was observed in reactions with cell extract from AA8 (wild type) but not with cell extracts from *ERCC1*, *ERCC2*, *ERCC4*, or *ERCC5* mutants; furthermore, the defect in these mutants could be complemented by addition of the corresponding proteins (data not shown).

Excision of undamaged DNA from covalently closed circles. The linear substrate may be considered a cross-linked DNA with a double-strand break (ends of the fragment) within 70 bp of the cross-link. Hence, it has certain limitations as a model for cross-links within human genomic DNA. To better approximate repair of the interstrand cross-links in vivo, we used plasmid DNAs containing a monoadduct or a cross-link as substrates. The results of excision experiments with these substrates are shown in Fig. 4. As in the case of linear DNA, covalently closed circular DNA yielded similar types of products from monoadduct- and cross-link-containing plasmids. As expected, the fragments excised from monoadducted DNA contained the psoralen-thymine adduct which blocks the 3′-to-5′ exonuclease activity of T4 DNA polymerase, whereas those excised from the cross-linked substrate (23 to 28 nt long) were free of damage and hence digested to completion by the 3′-to-5′ exonuclease (Fig. 4, lanes 13 to 16). Thus, clearly, the human excinuclease is capable of removing an oligomer from the 5′ side of the cross-link without a need for a free end. Furthermore, the same excision pattern, with about the same efficiency, was observed when the DNA was negatively supercoiled (Fig. 4, lanes 11 and 12). Thus, the removal of undamaged DNA from the 5′ side of the cross-link is an intrinsic property of human excinuclease and is not affected by the topological status of the substrate.

Excision of undamaged DNA occurs from both strands. Although the TA sequence at which the cross-link is formed is symmetrical, the psoralen molecule itself is asymmetric, and as a consequence the thymines are attached to the furan or pyrone side of the three-ring molecule (8). Hence, it was conceivable that the furan side- and pyrone side-adducted strands would be processed differently by the human excinuclease, as has been found for the *E. coli* excinuclease, which greatly

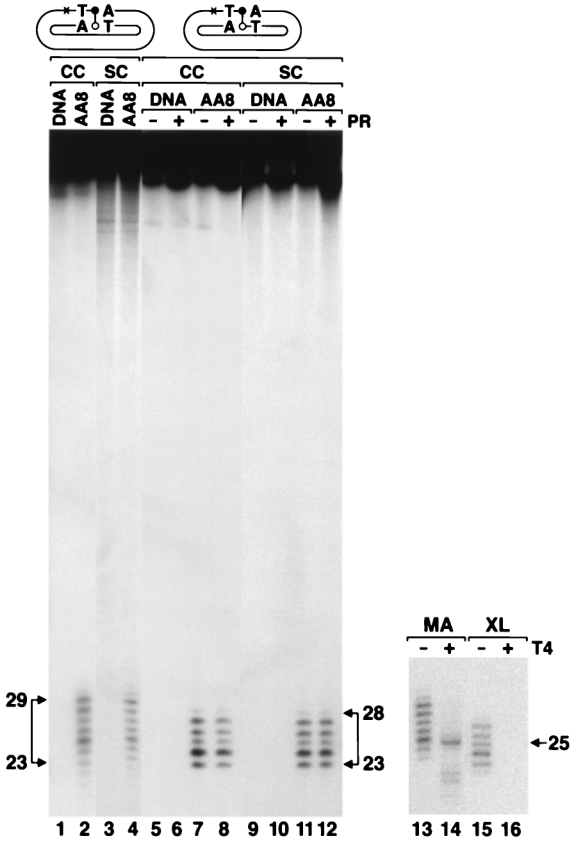

FIG. 4. Excision of undamaged DNA from the 5′ side of the cross-link in a circular substrate. Covalently closed relaxed (CC) or supercoiled (SC) plasmid DNAs containing psoralen monoadduct (lanes 1 to 4) or interstrand cross-link (lanes 5 to 12) were treated with cell extract from the AA8 (wild-type) cell line, and the products were analyzed on an 8% denaturing polyacrylamide sequence gel. The fractions of radiolabel in the excised fragments were as follows: lane 2, 0.7%; lane 4, 0.6%; lane 7, 0.9%; lane 8, 0.8%; lane 11, 0.6% and lane 12, 0.6%. The presence or absence of psoralen adduct in the excision products was verified by treatment with the 3′-to-5′ exonuclease of T4 DNA polymerase. The excision products from lane 2 (monoadduct [MA]) and lanes 7 and 8 (cross-link [XL]) were eluted from the gel, treated with T4 DNA polymerase (3′-to-5′ exonuclease) as indicated above the lanes, and reanalyzed on a 10% polyacrylamide sequencing gel (lanes 13 to 16). The major stop site of the exonuclease with the oligomers from the monoadduct is indicated (arrow). The major 5′ incision site with the psoralen monoadduct in this sequence context is at the 25th phosphodiester bond; hence, 3′-to-5′ exonuclease degradation generates a 24-nt fragment terminating at the psoralen monoadduct. This fragment comigrates with a 25-nt-long marker.

favors the furan side-adducted over the pyrone side-adducted strand (28, 62). By placing the radiolabel at the appropriate sites in either strand 3′ or 5′ to the cross-link, we wished to address the following specific questions: (i) whether the human excinuclease removes a 22- to 26-mer from the 3′ side of the cross-link of the furan side-adducted strand and (ii) whether the enzyme removes undamaged DNA from the 5′ side of the pyrone side-adducted thymine. The results of experiments which address these issues are shown in Fig. 5 and 6. No specific incision on the 3′ side of the cross-link can be detected (Fig. 5). In contrast, fragments of 22 to 26 nt are excised from the 5′ side of the pyrone side-adducted strand (Fig. 6). Thus, the excinuclease cuts out a single-stranded DNA fragment from both strands only on the 5′ side of the cross-link, regardless of the orientation of the cross-link.

This finding raised the following question: do the two excisions 5′ to the cross-link occur in the same DNA molecule? If

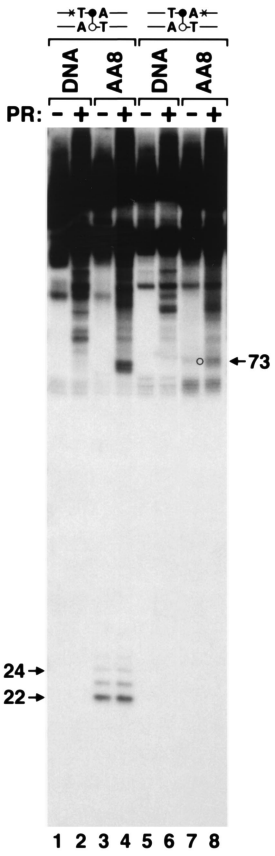

FIG. 5. Excinuclease does not make dual incisions 3′ to the cross-link. Substrates with the radiolabel 5′ (lanes 1 to 4) or 3′ (lanes 5 to 8) to the furan side-adducted thymine were incubated with AA8 (wild-type) cell extract, and the DNA was analyzed with (+) or without (−) photoreversal (PR) as indicated. The enzyme system excises 22- to 24-mers from the 5′ side of the cross-link (lanes 3 and 4) but not from the 3′ side (lanes 7 and 8). Note that with the substrate with label on the 3′ side of the cross-link (substrate c), photoreversal gives rise to a 73-mer (open circle) which corresponds to incision 3 nt 5′ to the cross-link, in agreement with the data obtained with the 5′ labeling scheme (substrate b) in lanes 3 and 4.

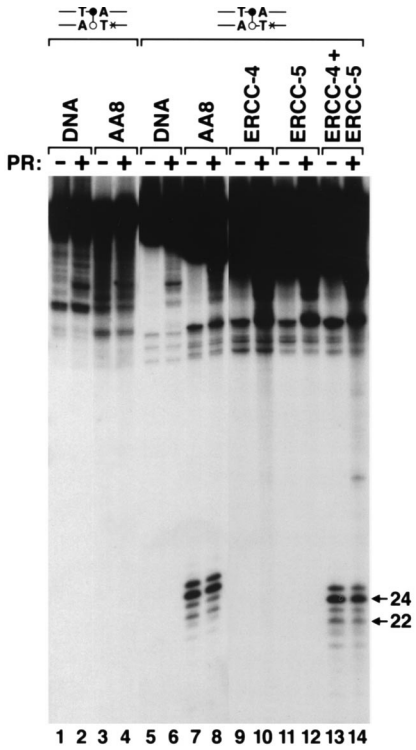

FIG. 6. Dual incisions 5′ to the pyrone side-adducted thymine of psoralen cross-link. Monoadducted or cross-linked DNA with radiolabel in the bottom-strand 10th phosphodiester bond 5′ to the cross-linkable (substrate d, lanes 1 to 4) or cross-linked (substrate e, lanes 5 to 14) thymine was incubated with cell extracts from the indicated rodent cell lines, the DNA was extracted and was (+) or was not (−) subjected to photoreversal (PR), and the products were analyzed on 8% denaturing polyacrylamide sequencing gels. No excision from the undamaged strand occurs (lanes 3 and 4). Upon cross-linking, 20- to 25-mers are excised from the pyrone side-adducted strand. These oligomers are free of psoralen because photoreversal does not change the fragment size.

this were to occur in our experiments, photoreversal of the cross-link following the reaction would generate a double-strand break. Hence, after treatment of the cross-linked DNA with cell extracts, the cross-link was photoreversed and the products were analyzed on a nondenaturing gel. No double-strand breaks were seen (data not shown); therefore, the two sets of dual incision events must occur independently of each other. In fact, we think it is very unlikely that 5′ gaps can be produced in both strands in the same molecule. Productive assembly of human excinuclease requires about 40 bp of duplex DNA 3′ to the damage (23). When a gap is made 5′ to the cross-link in one strand, it is not expected that the excinuclease system can assemble and make the dual incisions 5′ to the cross-link in the complementary strand.

Excision in reconstituted excinuclease system. The data presented so far convincingly show that dual incisions 5′ to the cross-link occur in cell extracts in a manner characteristic of the dual incisions of excinuclease which remove intrastrand mono- or diadducts. However, considering the unorthodox nature of the incisions that occur with cross-linked substrate, we reasoned that in addition to the six general nucleotide excision repair factors, XPA, RPA, TFIIH, XPC, XPG, and XPF · ERCC1, additional factors which are present in the cell extracts might be necessary for this unusual mode of dual incisions. Hence, we conducted the excision reaction with cross-linked DNA using the human excinuclease system reconstituted with the six repair factors purified to homogeneity (38). The results are shown in Fig. 7. The six factors are necessary and sufficient for making the dual incisions 5′ to the cross-link which lead to the removal of 22- to 24-nt-long fragments that do not contain any damage. Omission of ATP (Fig. 7, lane 5) or of the individual components of the excinuclease (lanes 7 to 11) abolishes the excision reaction. Thus, we conclude that human excinuclease is capable of generating a single-stranded gap of 22 to 28 nt on the 5′ side of the cross-linked base in either strand. This gap may initiate a recombination reaction which eventually eliminates the cross-link.

DISCUSSION

In this study, we have determined the mode of incision of psoralen cross-links by the mammalian nucleotide excision repair system (Fig. 8). We believe that this pattern of incision is applicable to cross-links made by other agents as well and that dual incisions 5′ to the cross-link are the first step in a cross-link repair pathway in humans.

Cross-link repair in model systems. In *E. coli* and in *Saccharomyces cerevisiae*, the repair of cross-links appears to depend on both nucleotide excision repair and homologous recombination (9, 26, 33). In vitro experiments have provided a

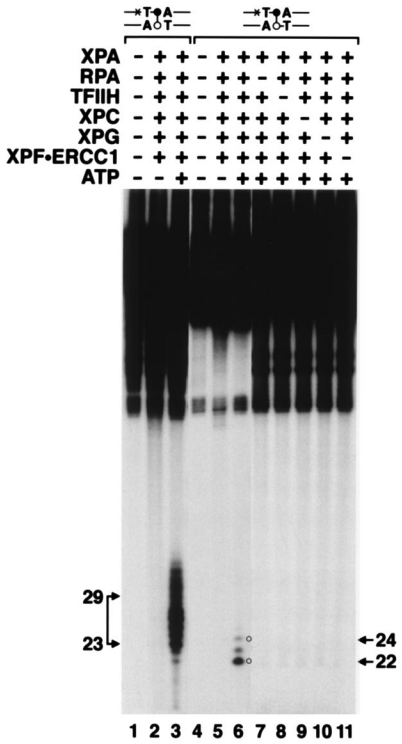

FIG. 7. Effect of reconstituted human excinuclease on DNA with psoralen monoadduct and cross-link. Substrates a and b were incubated with the entire set of the repair factors with (+) or without (−) ATP as shown, and the requirement for individual repair factors was tested for the cross-link substrate. The reconstituted system excises the psoralen monoadduct mainly in the form of 23- to 29-mers and removes psoralen-free 22- to 24-mers (circles) from the cross-link substrate. Excision of undamaged DNA from the cross-link substrate requires ATP and all of the excision repair factors (lanes 7 to 11; data for XPA not shown). The percentages of label excised were 1.2 and 0.4% in lanes 3 and 6, respectively.

mechanistic framework for the requirement of these two enzyme systems for elimination of cross-links from DNA in *E. coli*. The *E. coli* excinuclease makes dual incisions, one on each side of the cross-linked base in one strand, generating an 11-nt-long oligomer attached to the complementary strand through the cross-link (62). The resulting structure is recombinogenic and, in a RecA-catalyzed reaction, can be invaded by a homologous third strand to generate a three-strand intermediate (7, 55) in which the fragment produced by the dual incision is flipped out of the duplex. This structure is then recognized by the excision repair system, which, in a second round of dual incisions, releases the cross-linked oligomer (6, 55). At present, there is no in vitro yeast system for processing cross-links. However, genetic studies show that also in yeast the majority, if not all, of the cross-links are processed by the joint actions of nucleotide excision repair (26, 33) and homologous recombination (10, 47). This model seems insufficient to explain the behavior of the several classes of mammalian cell lines with increased sensitivity to cross-linking agents, as discussed below.

Mammalian mutants hypersensitive to cross-links. Four classes of mutations are associated with hypersensitivity of mammalian cells to cross-linking agents.

FA cell lines are the best-known example of mutant mammalian cells with hypersensitivity to cross-linking agents (18, 50). Although the extreme sensitivity of FA cells to cross-linking agents is well established, the reports by some groups that these cells are defective in cross-link repair (18, 19, 69) have not been confirmed by others (14, 16, 30). Currently, it is thought that FA is not a repair defect disease (11). Furthermore, the identical processing of the cross-link by cell extracts and the reconstituted excinuclease system in our study rules out any involvement of FA proteins in the nucleotide excision repair-mediated incision of cross-linked DNA. Hence, the possible roles of FA proteins in the cellular response to cross-links will not be discussed further.

Mutations in recombination genes, including those encoding DNA-PK catalytic and regulatory subunits (27), and in *XRCC2* and *XRCC3* genes, which encode RAD51 homologs (60), also confer sensitivity to cross-linking agents. These genes encode proteins which presumably catalyze recombination, and the sensitivity of these mutants to cross-linking agents in addition to ionizing radiation underscores the role of recombination in cross-link repair (29, 60, 68).

XP mutants constitute the third group of mammalian cells with increased sensitivity to cross-linking agents (17, 20, 60). Cell lines of all XP complementation groups tested, including XPA (30, 65) and XPD (3) mutants and UV-sensitive rodent cell lines with mutations in the *XPB*, *XPD*, and *XPG* genes, are three- to fivefold more sensitive to cross-linking agents than the wild-type cells (20). While all of the interstrand cross-linking agents produce intrastrand lesions, which are likely substrates for excinuclease and hence the increased sensitivity could be ascribed to defective intrastrand adduct repair solely, it has been reported that XP mutants either exhibit no detectable cross-link repair (30, 65) or remove cross-links at a lower rate than wild-type cells (20, 69). Thus, at least one pathway for removing cross-links in mammalian cells involves the nucleotide excision repair system.

The fourth group of mammalian genes known to control cross-link repair are the *ERCC1* and *XPF* genes, whose products form a heterodimer (43) with structure-specific endonuclease activity (2, 34). The XPF · ERCC1 heterodimer is an essential component of human excinuclease (37, 38). However,

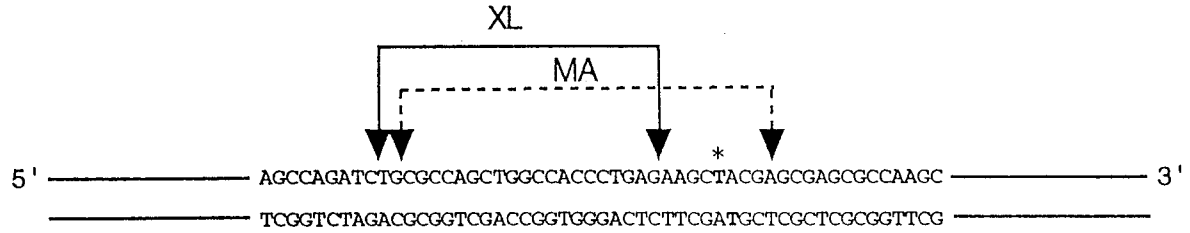

FIG. 8. Modes of action of human excinuclease on psoralen monoadduct (MA) and cross-link (XL). The enzyme makes dual incisions one on each side of the monoadducted T (asterisk) but makes both incisions on the 5′ side of the cross-link (formed between the Ts shown in boldface). The incision sites for both monoadducts and cross-links show some variability. For simplicity, only one of the major incision sites is shown for each of the dual incisions. In the cross-linked DNA, excision from either strand occurs with about equal frequency.

the extreme sensitivity to cross-linking agents (about 60-fold more sensitive than the wild type) of rodent cells with mutation in either of these genes (20) indicates that their role in cross-link repair goes beyond their participation in nucleotide excision repair. Genetic and biochemical studies with the homologs of these genes in other organisms suggest that the XPF · ERCC1 complex plays a unique role in cross-link repair because of its involvement in certain recombination reactions. Thus, even though the yeast RAD1 (XPF) and RAD10 (ERCC1) mutants, in contrast to their mammalian counterparts, are not extraordinarily sensitive to cross-linking agents (26), they are deficient in mitotic recombination (51, 52). Similarly, the *Drosophila* XPF homolog MEI-9 is involved in meiotic recombination (53). The role of RAD1 · RAD10 in mitotic recombination in yeast has been investigated in some detail. By analysis of the recombinational products of HO endonuclease-induced double-strand breaks, it was established that the RAD1 · RAD10 complex, independently of other excision repair proteins, promotes recombination by trimming the 3′ single-stranded termini of recombining molecules (15, 25). The properties of purified RAD1 · RAD10 complex, which include single-stranded endonuclease (57, 61) and junction endonuclease (1) activities, are consistent with such a role. All these data support the notion that XPF · ERCC1 plays a role in recombination which, depending on the recombinational systems of the particular organisms, may or may not be important for cross-link repair. In mammalian cells, XPF · ERCC1 appears to play a significant role in a reaction pathway which is not initiated by the excision repair system and which eliminates a substantial fraction of cross-links. Inactivation of this complex essentially abolishes cross-link repair by eliminating both excision repair-dependent and excision repair-independent cross-link repair systems because XPF · ERCC1 is a part of both systems.

Mechanistic basis of unusual dual incisions at cross-links by human excinuclease. Human excinuclease removes damage from DNA by incising the damaged strand on both sides of the lesion (21). Our data show that when presented with a cross-link, the enzyme system makes both incisions to one side of the damaged base, excising an undamaged oligomer in a seemingly futile reaction. This finding raises two interrelated questions: why does the excinuclease fail to make dual incisions in the regular manner, and does this unorthodox incision-excision lead to eventual elimination of the cross-link from DNA?

The answer to the first question is provided by the structure of the DNA in the preincision complex. Unwinding of DNA 5′ and 3′ to the lesion is an early step in the pathway for dual incisions (12, 39, 40). If unwinding is a prerequisite for both 3′ and 5′ incisions, the 3′ incision is more likely to be inhibited by the cross-link, since a cross-link interferes with unwinding of the duplex in the immediate vicinity of the cross-link and since the 3′ incision is within 6 ± 3 nt of the lesion, whereas the 5′ incision is at 20 ± 5 nt of the damage (21, 39, 58). It is conceivable that during the assembly of the excinuclease the DNA is unwound in the 5′-to-3′ direction with respect to the excised oligomer. Such a reaction would create an excision bubble which initiates at the vicinity of the 5′ incision site and terminates near the cross-link. In such a structure, the active site of XPG is positioned immediately 5′ to the cross-link where the 3′ incision is made, followed by the 5′ incision at about the 27th phosphodiester bond 5′ to the cross-linked base by the XPF · ERCC1 nuclease. It must be noted that this incision pattern was observed in three sequence contexts (Fig. 1). Even though the sequence context affects the precise incision sites by 2 or 3 nt (Fig. 3, 5, and 7), the dual incisions are 5′ to the cross-link in all cases, and this pattern of incision is not affected by the linking number of the plasmid substrate (Fig. 4, lanes 1 to 12).

Whether formation of a gap immediately 5′ to the cross-link leads to repair cannot be ascertained from our data. It is noteworthy, however, that in vivo, even though psoralen cross-links are removed faster than monoadducts from transcribed sequences (63, 64), the rates of removal of the two lesions from nontranscribed DNA are about equal (24, 63, 64). In our in vitro system in which the substrate is not transcribed, the efficiency of monoadduct removal is about the same as that of formation of a gap 5′ to the cross-link. We take this as an indication that the 5′ gap is on the pathway to cross-link repair.

Model for excinuclease-initiated cross-link repair. How a 22- to 26-nt-long gap 5′ to the cross-link leads to the eventual elimination of the cross-link cannot be predicted from our data. Several possibilities exist. (i) The gap in one strand is filled in by repair synthesis but the resulting nick cannot be ligated because of proximity to a cross-link. The other strand of the resulting structure is then acted on by the same enzyme system to generate a duplex with nicks 5′ to the cross-link in both strands, as has been suggested by Miller et al. (36) for cross-link repair in yeast. This structure is then acted upon by homologous-recombination enzymes to eliminate the cross-link (56, 59). (ii) Only one set of dual incisions occurs in either strand, and the resulting gap initiates recombination, which would be blocked early during the strand transfer step. The blocked intermediate could then be acted on by a structure-specific endonuclease, resulting in either cleavage on the other side of the cross-link in the same strand, yielding an *E. coli*-like recombinational cross-link repair mechanism (7, 55), or cleavage of the unbroken strand, leading to a double-strand break. (iii) Dual incisions are made in only one strand, and the resulting gap provides an entry site for an exonuclease which digests the gapped strand past the cross-link, thus creating a gap containing a cross-linked dinucleotide. This structure could then be processed by homologous recombination dependent on XRCC2 and XRCC3 or could be filled in by translesion synthesis, as has been proposed previously to explain the mutation spectrum of psoralen cross-links in rodent cells (48).

Clearly, this is not an exhaustive list of the possible pathways that can be utilized to convert a duplex with a gap 5′ to a cross-link to a repaired duplex with or without a mutation at the cross-link site. Regardless of the precise mechanism for eliminating the cross-link from a nicked or gapped DNA, it is important that in contrast to the bacterial system (28, 55, 62), in mammalian cells the dual incision is made on one side of the cross-link and is not the rate-limiting step. Furthermore, in vivo data suggest that there are two pathways for cross-link repair in humans. These two pathways overlap in some of their enzymatic requirements, notably, recombination functions (XRCC2 and XRCC3) and XPF · ERCC1, but not in others, such as XPA, XPD, and XPG proteins. Hence, the cross-link repair initiated by the 5′ gap described here is only one of the two pathways for repairing cross-links in humans. Further work is required to understand the contributions of the various pathways and the roles of strand transfer and double-strand break repair proteins in these pathways.

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