Interstrand DNA cross-link damage is a severe challenge to genomic integrity. Nucleotide excision repair plays some role in the repair of DNA cross-links caused by psoralens and other agents. However, in mammalian cells there is evidence that the ERCC1-XPF nuclease has a specialized additional function during interstrand DNA cross-link repair, beyond its role in nucleotide excision repair. We placed a psoralen monoadduct or interstrand cross-link in a duplex, 4–6 bases from a junction with unpaired DNA. ERCC1-XPF endonucleolytically cleaved within the duplex on either side of the adduct, on the strand having an unpaired 3′ tail. Cross-links that were cleaved only on the 5′ side were purified and reincubated with ERCC1-XPF. A second cleavage was then observed on the 3′ side. Relevant partially unwound structures near a cross-link may be expected to arise frequently, for example at stalled DNA replication forks. The results show that the single enzyme ERCC1-XPF can release one arm of a cross-link and suggest a novel mechanism for interstrand cross-link repair.

An important structure-specific DNA nuclease family in eukaryotes is represented by the ERCC1-XPF complex in mammalian cells and by the Rad1-Rad10 complex in Saccharomyces cerevisiae. These enzymes specifically cleave DNA near junctions between single-stranded and duplex DNA in cases where the single strand has a 5′→3′ polarity as it moves away from the junction (1–3). The ability of these enzymes to cut such bubble, flap, and Y structures is necessary for many DNA transactions. In mammalian cells, the heterodimeric ERCC1-XPF nuclease is formed by tight association of ERCC1 and XPF subunits (2, 4). Nucleotide excision repair (NER) uses nucleotide excision repair (NER)1 uses this nuclease to cleave a damaged DNA strand on the 5′ side of an opened “bubble” intermediate formed around a lesion. Some strategies for repair of DNA double-stranded breaks in S. cerevisiae involve the use of Rad1-Rad10, sometimes in a complex with Msh2 and Msh3 (5). In these cases the nuclease aids in resolution of recombination intermediates by clipping off non-homologous 3′ single-stranded tails. In Drosophila, the XPF homolog mei9 is implicated in meiotic recombination (6). In the fission yeast Schizosaccharomyces pombe, the XPF homolog rad16 (swi9) and ERCC1 homolog swi10 are involved in the recombination events that lead to mating-type switching (7).

NER-defective cells are sensitive to agents such as psoralen, which causes interstrand DNA cross-links (8). Beyond its participation in NER, however, ERCC1-XPF appears to have an additional role in the repair of interstrand DNA cross-links. It is known, for example, that ERCC1-defective and many XP group F cells are much more sensitive to DNA cross-linking agents than are other NER mutants (9, 10). Very little is known about the mechanism of repair of interstrand DNA cross-links. A number of pathways for cross-link repair can be envisaged. One possibility would mirror the model of Cole (11) for cross-link repair in Escherichia coli, which involves sequential NER and homologous recombination steps. A comparable model in mammalian cells would not account for the additional role of ERCC1-XPF in cross-link repair beyond any function in NER. An alternative possibility is that ERCC1-XPF participates in a pathway in which it clips off strands with 3′ tails, arising for example from recombination intermediates following introduction of double-strand breaks on either side of a cross-link. However, the action of ERCC1-XPF on DNA structures containing a cross-link is an unexplored area. Here we studied the action of ERCC1-XPF on oligonucleotides containing a specifically located interstrand psoralen-DNA cross-link and found that the enzyme has an unexpected ability to cleave DNA on both sides of a psoralen cross-link, suggesting a novel mechanism for initiation of cross-link repair.

EXPERIMENTAL PROCEDURES

ERCC1-XPF Protein—The XPF and ERCC1 genes were expressed as a dicistronic construct from pET30b. XPF had an N-terminal His tag and was expressed before ERCC1 with a C-terminal His tag. E. coli BL21 (DE3) recA host cells were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 20 °C for 22 h. Cells were lysed by sonication in buffer A (20 mM Hepes-KOH (pH 8.0), 2 mM MgCl₂, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 0.02% NaN₃). Clarified supernatant was loaded on a column of nickel-agarose in buffer A containing 0.5 M KCl and 5 mM imidazole, and ERCC1-XPF eluted in a gradient from 25–400 mM imidazole. CHAPS was added to 0.02%, and pooled fractions were concentrated on a Centriprep 50 and loaded onto an S-200 column in buffer B (20 mM Hepes-KOH (pH 7.4), 0.5 mM EDTA, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM diithiothreitol, 0.5 mM KCl, 2 mM MgCl₂, 0.01% CHAPS, and 0.02% NaN₃). Pooled fractions were dialyzed against buffer A containing 0.5 M KCl and 5 mM 2-mercaptoethanol and loaded on a Talon column. The column was eluted with a 10–500 mM imidazole gradient in the same buffer. Pooled fractions were concentrated, dialyzed into buffer A plus 50 mM KCl and 5 mM 2-mercaptoethanol, and loaded on an fast protein liquid chroma-
Incision on Both Sides of a Cross-link by ERCC1-XPF

RESULTS AND DISCUSSION

ERCC1-XPF Cleaves Structures with a Psoralen Cross-link

Located at the Junction between Duplex and Single-stranded DNA—ERCC1-XPF enzyme was purified in recombinant form as a complex of its two subunits (Fig. 1A). The complex produced in E. coli is functional and can correct the nucleotide excision repair defect of XPF-defective human cell extracts (data not shown). Native ERCC1-XPF enzyme from HeLa cells was found to cleave a stem-loop structure near the 5′ junction between loop and stem (2), and this same pattern of cleavage was observed for recombinant enzyme (Fig. 1B). It is noteworthy that although ERCC1-XPF can cleave Y structures (3) it does not require a free 3′ single-stranded end for cleavage.

To study the ability of ERCC1-XPF to cleave near a psoralen adduct, a 26-mer oligonucleotide was synthesized containing a furan-side psoralen monoadduct on thymine residue 11 (Fig. 2A). This strand was 5′-labeled with [32P]ATP and annealed to a complementary oligonucleotide to give the structure DS-MA (Fig. 2A) or to a partially complementary oligonucleotide, forming the structure Y1-MA with a 12-base pair (bp) duplex region and a 3′ single-stranded tail (Fig. 2C). ERCC1-XPF could cleave the Y structure containing a monoadduct, cutting within the duplex near the junction. The main cleavage site was 3 phosphodiester bonds away from the junction, releasing a labeled 9-mer (Fig. 2D, left). This same major cleavage site was observed for a Y substrate without a psoralen adduct (not shown). Cleavage was dependent on the Y structure, as a fully duplex molecule containing a monoadduct was not cleaved (Fig. 2B, left).

The psoralen monoadduct was located in a 5′-TA-3′ sequence (Fig. 2A) and was convertible to a cross-link by irradiation with near-UV light (12), which converted about 90% of the duplexes to a cross-linked form. The purified cross-linked DNA migrated more slowly on a denaturing polyacrylamide gel (Fig. 2B, right). Several bands are often observed with cross-links, probably reflecting different possible conformations of denatured arms. DNA Y1-CL containing the single cross-link was also cleaved by ERCC1-XPF at the same 5′ position as the monoadduct, with some minor cleavage products slightly further from the junction (Fig. 2D, right). DNA containing a cross-link in the absence of a junction was not cleaved by the enzyme (Fig. 2B, right).

Only the strand having the 3′-unpaired tail was cleaved, as shown by labeling the opposite strand with [32P]ATP at the 5′ terminus (Fig. 2E). ERCC1-XPF cleavage of the cross-link (Fig. 2F, lanes 1–3) produced a slow migrating labeled product (Fig. 2F, bracketed) but no labeled short products. An identical set of reaction products was irradiated with UVC (peak 254 nm) light after ERCC1-XPF cleavage, a procedure that efficiently reverses the psoralen cross-link back to monoadduct form (12). The labeled slower migrating products disappeared (Fig. 2F, lanes 4–6), confirming that they arose from cross-linking of the intact labeled strand with a cleaved unlabeled strand. Y1 DNA containing a cross-link was actually cleaved more efficiently than the DNA with a monoadduct, suggesting that the stabilization of the duplex part of the structure by the cross-link favors recognition or cleavage. These results show that, surprisingly, a cross-link between the cutting site and the junction does not prevent ERCC1-XPF cleavage at the correct position.

Y Structures Can Be Cleaved on Either Side of Psoralen Adducts More Distant from the Junction—We next asked whether cleavage could occur on the 3′ side of a psoralen adduct. Structure Y3 was formed by annealing the 5′-labeled strand with the psoralen monoadduct to a partial complement, giving a 17-bp duplex with 6 bp between the adduct and the junction (Fig. 3A). Two main cleavage sites were observed when this structure contained a monoadduct (Fig. 3B, lanes 1–6). One was 3 phosphodiester bonds 5′ to the adduct, at the same sequence seen with structure Y1, releasing a labeled 9-mer. The second site was located 3 phosphodiester bonds on the 3′ side of the adduct (4 phosphodiester bonds from the junction), releasing a labeled 13-mer. This shows that ERCC1-XPF is capable of cutting on either side of a monoadduct in Y structures.

With a psoralen cross-link, two types of ERCC1-XPF cleav-
same position observed with the Y3-MA monoadduct substrate. The second type were slow migrating species (Fig. 3B, lanes 7–12, bracket), hypothesized to be products of cleavage on the 3′ side of the cross-link. To verify this notion, an identical set of reaction products was irradiated with UVC (peak 254 nm) light after ERCC1-XPF cleavage to reverse the psoralen cross-link back to monoadduct form. This reversal liberated a 13-nt product, demonstrating cleavage on the 3′ side of the cross-link, closer to the junction (Fig. 3B, lanes 13–18).

To confirm the identity of these cleavage sites, the strand with the 3′-single-stranded tail was labeled with 32P at the 3′ terminus (Fig. 3D). ERCC1-XPF cleavage of this cross-linked Y3-CL substrate gave a 13-nt product from cleavage at the expected position on the 3′ side of the cross-link and a slower-migrating product, presumably arising from cleavage on the 5′ side of the cross-link (Fig. 3E, left). Reversal of the cross-link by UVC irradiation converted the slower migrating product to a 17-mer, the expected size for cleavage on the 5′ side of the cross-link (Fig. 3E, right). We note that with these Y structures, ERCC1-XPF cleavage is endonucleolytic, with little or no exonuclease activity.

Some cleavage on the 3′ side of monoaducts or cross-links was also observed with a structure designated Y2, formed by annealing the same labeled oligonucleotide with a complement to give a 14-bp duplex where the adduct was only 4 bp from the junction. The extent of 3′ cleavage was less than with structure Y3 (data not shown).

It was significant that with higher ERCC1-XPF enzyme concentration there was a decrease in yield of labeled 3′ cleavage product and a continuing increase in labeled 5′ cleavage product arising from cross-linked Y3 molecules (Fig. 3C). This suggested that some molecules were being cleaved twice, on both the 3′ and 5′ sides of the cross-link. This possibility was tested in the next set of experiments.

Sequential Cutting by ERCC1-XPF on the 5′ and 3′ Sides Releases One Arm of a Psoralen Cross-link—The cross-linked products bracketed in Fig. 3B were excised from a polyacrylamide gel and reincubated with ERCC1-XPF. Significantly, this second treatment with ERCC1-XPF generated 9-mers (Fig. 3F, lane 2). This result demonstrates that ERCC1-XPF can cleave the same Y structure molecule on both the 3′ and 5′ sides of an interstrand cross-link. Without enzyme, two other bands appeared after purification (Fig. 3F, lane 1). One was a small amount of 13-mer, resulting from a limited amount of spontaneous cross-link reversal during isolation of the intermediates. The second was a 26-mer, probably arising as a minor contaminant during the purification.

Model for Initiation of Cross-link Repair by ERCC1-XPF—Two possible models for repair of an interstrand cross-link are presented in Fig. 4. Part A shows a model involving a sequence of NER events and homologous recombination. The essential features of this model were originally proposed by Cole (11) for repair of psoralen interstrand cross-links by E. coli. It is consistent with the genetic dependence of repair in that system and recombination within this pathway with purified E. coli enzymes (13–15). In mammalian cells, attempts to visualize first NER incision at an interstrand cross-link as in Fig. 4 (ii) have been unsuccessful (16), although NER incisions can occur flanking a psoralen-thymine monoaduct (17).

In part B, a model incorporating the ability of ERCC1-XPF to cleave on either side of a cross-link is shown. A single-strand duplex junction near the cross-link could be created by several mechanisms, for example at a stalled replication fork, elongating transcription complex, or by action of a DNA helicase. Digestion of the bottom strand in Fig. 4B, (i) by a 5′−3′ exonuclease would also create a single-strand/duplex junction.

Fig. 2. Cleavage near psoralen adducts located at a Y junction. A, a 26-mer duplex (DS-MA) containing a furan-side psoralen monoaduct (top) can be converted to a cross-linked form (DS-CL) by irradiation with 360 nm of light (bottom). B, polyacrylamide gel showing that incubation of DS-MA or DS-CL with ERCC1-XPF gives no significant cleavage of these duplexes. Increasing ERCC1-XPF concentrations are required for cleavage reactions in lanes 1–3 with 360 nm of light (peak 254 nm) light. C, polyacrylamide gel showing that incubation of DS-MA or DS-CL with ERCC1-XPF gives no significant cleavage of these duplexes. Increasing ERCC1-XPF concentrations are required for cleavage reactions in lanes 1–3 with 360 nm of light (peak 254 nm) light. D, polyacrylamide gel showing cleavage products after incubation with increasing concentrations of ERCC1-XPF nuclease (0, 4, 8, 12, and 24 nt in each group of six lanes). E, Y structures containing a monoaduct (Y1-MA), indicated by a star, or a cross-link (Y1-CL). The strand adducted to the furan side of the psoralen was labeled at the 5′ terminus with 32P. Arrows show main sites of cleavage. F, polyacrylamide gel showing cleavage products after incubation with increasing concentrations of ERCC1-XPF nuclease (0, 4, 8, 12, and 24 nt in each group of six lanes). E, Y structure containing a cross-link (Y1-CL). The strand adducted to the 5′ terminus with 32P. Irradiation with 254 nm UVC light leads to reversal of the cross-link as shown. F, polyacrylamide gel showing cleavage products after incubation of the cross-linked Y structure in E with ERCC1-XPF nuclease. Lanes 1 and 4, 0 nt; lanes 2 and 5, 8 nt; lanes 3 and 6, 24 nt. Lanes 1–3, cleavage of cross-linked structure. The bracket indicates the slowly migrating product in lanes 2 and 3. Lanes 4–6, labeled material after irradiation of the cleavage reactions in lanes 1–3 with UVC light.
FIG. 3. Cleavage on both sides of a psoralen cross-link by ERCC1-XPF. A, Y structures containing a monoadduct (Y3-MA) or cross-link (Y3-CL) placed more distant from the junction than in Fig. 2. The strand adducted to the furan side of the psoralen was labeled at the 5′ terminus with 32P. Arrows show main sites of cleavage deduced from the gel in B. B, lanes 1–6, products formed on both the 5′ and 3′ sides of a monoadduct by ERCC1-XPF cleavage of structure Y3-MA. Lanes 7–12, cleavage of cross-linked structure Y3-CL generates a 9-nt product of 5′ cleavage and slower migrating products indicated by the bracket. Lanes 13–18, UVC reversal of the cross-link cleavage products after incubation with ERCC1-XPF, showing that the slower migrating molecules represent molecules cleaved on the 3′ side to give a 13-nt product. In each group of six lanes, the ERCC1-XPF concentrations used were 0, 4, 8, 12, 16, and 24 nM. C, graph showing yields of 9- and 13-nt products with increasing enzyme concentration. D, Y structure containing a cross-link (Y3-CL) with the strand adducted to the furan side of the psoralen labeled at the 3′ terminus with 32P. Arrows show main sites of cleavage deduced from the gels in E. E, lanes 1–6, ERCC1-XPF cleavage of structure Y3-CL yields a 13-nt product of incision on the 3′ side of the product and a slower migrating product. Lanes 7–12, UVC reversal of the cross-link cleavage products after incubation with ERCC1-XPF, showing that the slower migrating molecules represent molecules cleaved on the 5′ side to give a 17-nt product. In each group of six lanes, the ERCC1-XPF concentrations used were 0, 4, 8, 12, 16, and 24 nM. F, the products of 3′ incision bracketed in B were purified (lane 1) and reincubated with 16 nM ERCC1-XPF enzyme (lane 2), generating a 9-nt product of 5′ incision.
ERCC1-XPF can cleave on the 3’ side (Fig. 4A, (ii)). A Y structure is created near a cross-link, for example by steps iv–vi. Steps can then take place to complete repair according to vi. After resolution of the heteroduplex, a second NER incision can take place to incise the other arm of the cross-link (iii), releasing the cross-link and allowing gap-filling by repair synthesis (v). ERCC1-XPF can cleave on the 3’ side of one arm of the cross-link (ii) and then on the 5’ side (iii). After fork collapse, recombination and NER steps can then take place to complete repair according to steps iv–vi.

Consequently such opened structures at a cross-link may be expected to occur frequently, and these would not be a substrate for repair by the NER pathway, which requires duplex DNA. However, the ERCC1-XPF enzyme could cleave the junction on the 3’ side of the cross-link (Fig. 4B, (ii)). Next, as shown here, ERCC1-XPF could cleave on the 5’ side (Fig. 4B, (iii)), perhaps facilitated by further unwinding. The net effect would be to release one arm of the cross-link. This intermediate is poised for a second step of recombination with a homologous sequence, after which the other arm of the former cross-link could be removed by NER. Attractions of this model are that there is no loss of genetic information and that it directly involves the unique properties of ERCC1-XPF in repair of a cross-link. A homologous recombination step is consistent with genetic evidence for the involvement of XRC2 and XRC3 in cross-link repair in mammalian cells (18, 19), two proteins of the RAD51 homologous pairing protein family. Other models are also possible, for example a scheme involving recombination by a single strand annealing pathway. A double-strand break on either side of a cross-link could remove it. Exonuclease digestion followed by annealing between partly homologous sequences could produce 3’ tails for processing by ERCC1-XPF, extrapolating from properties of the homologous Rad1-Rad10 enzyme of S. cerevisiae. However, if two double strand breaks are required to remove cross-linked DNA, deletion of the intervening DNA would often cause loss of genetic information in mammalian cells.

The pathway in Fig. 4B is also consistent with the following observations. First, ERCC1 and some XPF cell lines are more sensitive to DNA interstrand cross-linking agents than any other NER-deficient cell lines. Second, cells from ERCC1-deficient mice show a reduced frequency of S phase-dependent illegitimate chromosome exchange (20), suggesting that ERCC1-XPF is important to maintain correctly operating replication forks in S phase. The interaction of ERCC1-XPF with the single-stranded DNA binding replication protein A may be important in this regard (21). Finally, studies of deletion formation in ERCC1 knockout cells (22) suggest that the ERCC1 gene product is normally involved in a pathway leading to nonmutagenic processing of heteroduplex intermediates in recombination.

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Repair of an Interstrand DNA Cross-link Initiated by ERCC1-XPF Repair/Recombination Nuclease
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