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Contribution of XPF Functional Domains to the 5′ and 3′ Incisions Produced at the Site of a Psoralen Interstrand Cross-Link†

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ABSTRACT: XPF forms a heterodimeric complex with ERCC1 and is required for the repair of DNA interstrand cross-links. In association with ERCC1, it is involved in production of the 5′ incision at the site of a psoralen interstrand cross-link as well as the 3′ incision. The present study was carried out to determine the functional domains of XPF that are important in the production of the 5′ and 3′ incisions that occur at a site of a psoralen interstrand cross-link. Monoclonal antibodies (mAbs) were utilized that had been generated against polypeptide fragments of XPF and affinity-mapped to specific regions of XPF. These mAbs were examined for their ability to differentially inhibit production of dual incisions in DNA by normal human chromatin-associated protein extracts that contain XPF and ERCC1. These studies show that two regions of XPF, one N-terminal region from amino acids 12−166 and one C-terminal region from amino acids 702−854, are the most important in the production of the 5′ incision. The same N-terminal region and the C-terminal region from amino acids 702−916 are also involved in the 3′ incision, though to a much lesser extent. Since this C-terminal region corresponds to the proposed site of interaction of ERCC1 with XPF, these results suggest that binding of ERCC1 to XPF is critical for its ability to produce the 5′ and 3′ incisions at the site of an interstrand cross-link, possibly through activation or regulation of the endonucleolytic activity of the N-terminal domain of XPF.

XPF forms a heterodimeric complex with ERCC1, and this complex functions as a structure-specific endonuclease which participates in both nucleotide excision repair (NER)[1] and recombinational processes (*1*−*6*). This complex can cleave one strand of duplex DNA on the 5′ side of stem−loop, bubble, flap, and Y structures (*2*−*4*, *7*, *8*). During NER, it catalyzes the incision on the 5′ side of a lesion (*1*−*4*). Recently, XPF was found to be involved in the repair of DNA interstrand cross-links (*9*, *10*). Specifically, it functions in the production of the incisions made on the 5′ side and 3′ side of a DNA interstrand cross-link (*9*, *10*). ERCC1 is important in the production of the incisions produced by XPF (*10*). XPF can also function by itself as an endonuclease to incise DNA (*11*); however, during the repair process, ERCC1 may be needed to modulate the specificity required for production of incisions at sites of a lesion (*10*, *11*).

Unlike repair of other lesions, repair of DNA interstrand cross-links is a more complex process since incisions need to be created on both DNA strands at the site of the cross-link. In *E. coli*, this repair involves both NER and homologous recombination (*12*−*15*). The precise steps and proteins involved in interstrand cross-link repair in mammalian cells are unclear. We have previously shown that chromatin-associated protein extracts from normal human cells produce incisions on either side of a psoralen interstrand cross-link, and on both strands of DNA (*9*, *16*). That these incisions have physiological relevance is demonstrated by our finding that in Fanconi anemia complementation group A (FA-A), B (FA-B), C (FA-C), D1 (FA-D1), and G (FA-G) cells, which are deficient in ability to repair DNA interstrand cross-links, there are markedly reduced levels of these incisions (*9*, *17*, Lambert et al., in preparation). These incisions are restored to normal levels in FA-A, FA-C, and FA-G cells expressing the *FANCA*, *FANCC*, and *FANCG* cDNAs, respectively (*9*, Lambert et al., in preparation). Present in these protein extracts is a protein complex that we have shown is involved in repair of DNA interstrand cross-links (*9*, *16*−*21*). This complex contains a number of the NER proteins including XPF and ERCC1 as well as the structural protein nonerythroid α spectrin (αSpIIΣ*) (*9*, *22*, *23*). Whether all of these proteins are involved in repair of DNA interstrand cross-links is unclear; however, we have shown that XPF participates in the 5′ incision and to a certain extent the 3′ incision produced at the site of a psoralen interstrand cross-link (*9*). Studies of Kuraoka et al. have also recently shown that XPF, in association with ERCC1, is involved in the 5′ incision at the site of a psoralen interstrand cross-link as well as the 3′ incision (*10*).

In the present study, we investigated the functional domains of XPF that are important in the production of the

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[1] Abbreviations: NER, nucleotide excision repair; FA, Fanconi anemia; αSpIIΣ*, nonerythroid α spectrin; mAbs, monoclonal antibodies; TMP, 4,5′,8-trimethylpsoralen; ELISA, enzyme-linked immunosorbent asay; PBS, phosphate-buffered saline.

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5′ and 3′ incisions we have observed at a site of a psoralen interstrand cross-link. For these studies, monoclonal antibodies (mAbs) were utilized that had been generated against polypeptide fragments of XPF and affinity-mapped to specific regions of XPF. These mAbs were used to examine the importance of these regions of XPF for production of the dual incisions observed in DNA at the site of 4,5′,8-trimethylpsoralen (TMP) interstrand cross-link. Analysis of the ability of these mAbs to differentially inhibit production of the 5′ and 3′ incisions observed at the site of an interstrand cross-link has helped elucidate the domains of XPF that are critical for these incisions.

EXPERIMENTAL PROCEDURES

Chromatin-Associated Protein Extracts. Two normal human (GM 1989 and GM 3299) lymphoblastoid cells lines, obtained from the Coriell Institute for Medical Research (Camden, NJ), were grown in culture as previously described (*9*, *18*). Cell nuclei were isolated, and the chromatin-associated proteins were extracted in a series of steps as previously described (*9*, *19*, *24*). Protein concentrations were determined using the Bradford reagent (Bio-Rad).

DNA Substrate Containing a Site-Specific TMP Interstrand Cross-Link. A 140 bp DNA substrate was constructed that corresponded to the region from 61 to 200 of the nucleotide sequence of the 5S rRNA gene from *Lytechinus variegatus* (*9*, *16*). The top strand was internally labeled with γ-^{32}P at the 5′ end at position 81 (*9*, *16*). The substrate was engineered to have a single site-specific TMP interstrand cross-link, in which the furan ring of TMP was adducted to a thymine at position 132 on the top strand and the pyrone ring of TMP was adducted to a thymine at position 133 on the bottom strand (*9*, *16*). An α-phosphorothioate nucleotide was present at the 3′ ends of the DNA substrate that conferred resistance to 3′ → 5′ exonuclease activities that may be present (*9*, *25*, *26*).

Assay for Sites of Endonucleolytic Incision. DNA substrate (100 fmol), either TMP cross-linked or unmodified, was reacted with the chromatin-associated protein extract (10 μg) from normal human cells as previously described (*9*, *16*). The reactions were terminated by addition of 15 mM EDTA and the samples deproteinized using Nensorb 20 nucleic acid purification cartridges (*9*, *16*). Just before electrophoresis, TMP cross-linked substrates were photoreversed by irradiation with 254 nM UV light (1500 μW/cm^2 for 5 min) so as to break the interstrand cross-link and release TMP from the DNA (*9*, *16*). Samples were prepared for sequence analysis as previously described (*9*, *16*), and incision sites were identified by running the normal endonuclease reactions along with the sequencing ladders of undamaged substrates on a denaturing 5% polyacrylamide gel using the method of Maxam and Gilbert (*9*, *16*, *27*). Sequencing gels were exposed to Dupont Reflection autoradiography film (NEF-496) with a reflection intensifying screen overnight at −80 °C. Images were scanned using a Hewlett-Packard Scan Jet 4cT scanner, and for each experiment, the percentage of substrate that was added to each lane and converted to incision products was analyzed using ImageQuant (Molecular Dynamics). Background was subtracted from each lane using a lane containing substrate and no protein. Calibration curves were carried out including determination of a linear relationship between amount of radioactivity per lane and density of the 3′ and 5′ bands. Incisions were only examined on the DNA strand to which the furan ring of TMP was adducted. This was because we have previously shown that incisions on the strand containing the pyrone ring of TMP are very weak (*16*).

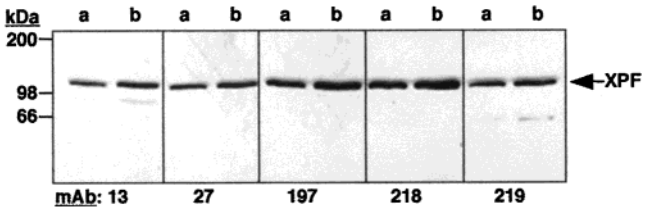

FIGURE 1: Detection of XPF in HeLa cell extracts by purified mAbs. HeLa S3 cell extracts, containing 50 μg (lanes a) or 100 μg (lanes b) of total protein, were separated on a 10% polyacrylamide−SDS gel and transferred to nitrocellulose. The nitrocellulose strips shown were incubated with the purified mAbs (each at 0.25 μg/mL) and further incubated with a secondary anti-mouse IgG (Fab-specific) peroxidase conjugate. The immunoblot was processed for ECL detection, and a scan of a developed film is presented. Protein molecular mass standards are shown on the left; an arrow indicates the position of the XPF protein; mAbs are identified on the bottom. A similar immunoblot using mAb 6D12 has been previously reported (*6*).

For antibody inhibition of incision activity, normal human chromatin-associated protein extract (10 μg) was mixed with 3, 5, or 10 μg of anti-XPF antibody or anti-rabbit IgG (10 μg) (Sigma Chemical Co.) as a control antibody, incubated on ice for 5 min, and mixed with the substrate. The samples were then incubated at 37 °C for 3 h and incisions examined as described above. Each of these experiments was carried out 4 times. For each of the values obtained, the standard error of the mean (SEM) was determined.

Production and Isolation of Monoclonal Antibodies. Six week old female Swiss Webster mice were immunized by subcutaneous injection of 50 μg of purified XPF protein (*11*) in Ribi adjuvant (Ribi ImmunoChem Research, Inc.). The mice received one priming dose and two booster doses at 7 and 21 days and the serum tested for XPF-specific antibodies by enzyme-linked immunosorbent assay (ELISA) as described below. To establish hybridomas, an immunized mouse was injected intravenously with 25 μg of XPF protein in phosphate-buffered saline (PBS). Three days afterward, the mouse was sacrificed, the spleen was removed, and splenocytes were collected and fused with SP2/0 myeloma cells using standard methods (*28*). For hybridoma selection, fused cells were cultured in ISDM containing 20% FCS and 1% HAT supplement (*28*) and were kept in humidified air with 5% CO_2 at 37 °C for 2 weeks. Screening for XPF-specific antibodies surviving hybridoma colonies was performed by ELISA. XPF was precoated onto Nunc-Immuno PolySorp 96 well plates by incubating 16 h at 4 °C with 0.5 μg of XPF per well in 100 μL of 50 mM sodium carbonate buffer, pH 9.6, containing 0.1% NaN_3. Wells were rinsed with PBS containing 0.05% Tween 20 blocked with 100 μL of PBS containing 0.5% BSA for 1 h. The wells were then incubated with 50 μL of hybridoma supernatant for 1 h at 37 °C. Wells were washed 3 times with PBS−Tween and incubated with 50 μL of alkaline phosphatase-conjugated goat anti-mouse IgG (BioRad) diluted 1:3000 in PBS containing 0.5% BSA and 0.05% Tween 20. After washing 3 times as before, wells were incubated with 100 μL of

A.

mAb	XPF polypeptides N166	N260	N378	N702	584C	667-854	region (aa)
27	+	+	+	+	-	-	12-166
6D12	-	+	+	+	-	-	166-260
197	-	-	-	+	-	-	378-584
219	-	-	-	+	+	-	584-667
13	-	-	-	-	+	+	702-854
218	-	-	-	-	+	-	854-916

B

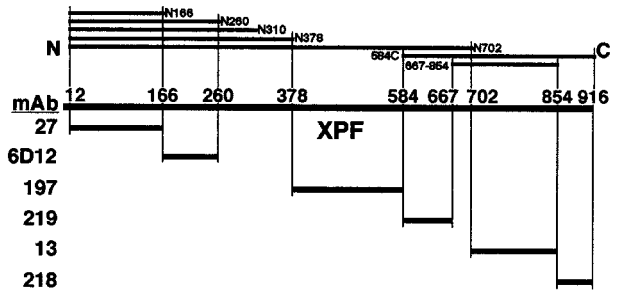

FIGURE 2: Determination of the mAb binding region within XPF. (A) Each mAb was affinity-mapped by Western blot analysis of recombinant XPF polypeptides (*11*). Binding regions were deduced by the least common overlapping fragment detected: +, detected; − not detected. (B) The recombinant XPF polypeptides used are shown schematically (top), and the regions of XPF recognized by mAb are shown as lines (bottom). Numbers along the central bar indicate XPF amino acid position.

phosphatase substrate prepared according to the manufacturer (Sigma). Absorbance was measured at 405 mm in a microplate spectrophotometer. Cells derived from positive wells were cloned by limiting dilution. Several clones producing XPF-specific mAbs were confirmed by Western blotting, and these positive clones were gradually expanded over several days into hybridoma medium. To purify individual mAbs, cells were removed by centrifugation, and the supernatant was collected. IgG was precipitated with 50% ammonium sulfate and then purified on Protein-A Sepharose (Pharmacia) and examined for purity by SDS−PAGE. Each purified mAb was tested at equal concentration against a collection of recombinant XPF polypeptides (*11*) by Western blot analysis, and specific antibody binding regions within XPF were deduced by the shortest common overlapping polypeptide fragment detected.

RESULTS

To probe for functional sites in the XPF protein, a collection of mAbs was developed and mapped to regions within the XPF protein. Six mAbs were purified and characterized, and each was initially tested for its sensitivity in detecting XPF in an extract from HeLa cells. As shown in Figure 1, each of the mAbs, 27, 197, 219, 13, and 218, bound to XPF in these extracts. Binding of mAb 6D12 to XPF in HeLa cell extracts has been previously described (*6*). These mAbs were then examined for specificity in binding to a series of seven recombinant XPF polypeptides (*11*) using Western blot analysis. Each mAb was affinity-mapped to a distinct, nonoverlapping region of XPF (Figure 2). A summary of these qualitative results given in Figure 2A indicates that mAb 27 recognizes the region comprising residues 12−166, mAb 6D12 recognizes region 166−260, mAb 197 recognizes region 378−584, mAb 219 recognizes regions 584−667, mAb 13 recognizes region 702−854, and mAb 218 recognizes region 854−916.

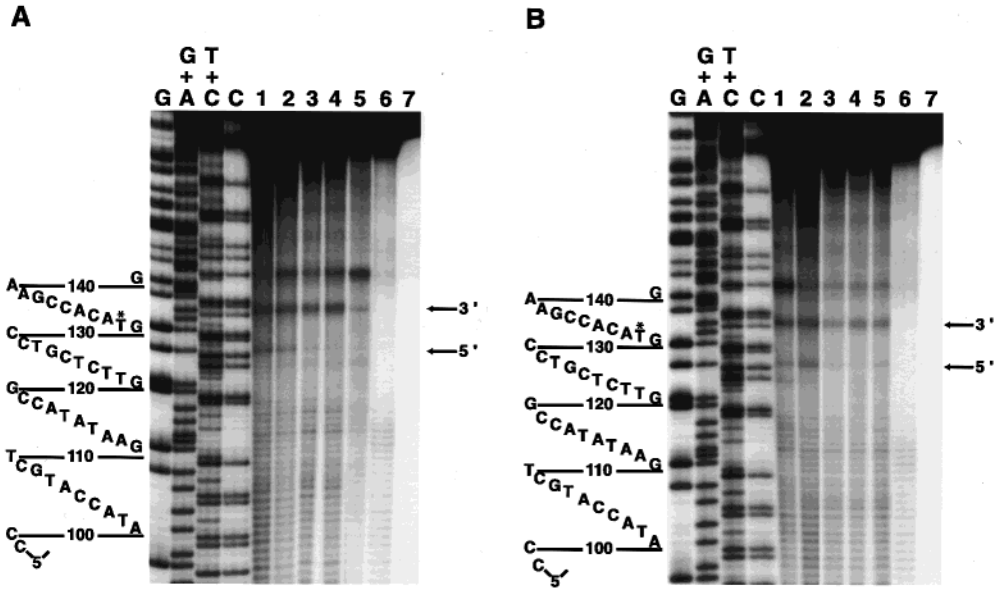

FIGURE 3: Inhibition by mAbs 27 and 6D12 of production of incisions produced at sites of DNA interstrand cross-links. (A) A 140 bp DNA substrate (100 fmol), in which the furan side of a TMP interstrand cross-link was adducted to the ^{32}P-5′-end-labeled top strand, was reacted with a chromatin-associated protein extract (10 μg) from normal human lymphoblastoid cells (lane 1) or a chromatin-associated protein extract (10 μg) that had been preincubated with 10 μg of anti-rabbit IgG (lane 2), 3 μg of mAb 27 (lane 3), 5 μg of mAb 27 (lane 4), or 10 μg of mAb 27 (lane 5). Undamaged substrate was incubated with 10 μg of normal extract (lane 6) or 10 μg of mAb 27 (lane 7). Endonucleolytic incisions produced at the sites of the interstrand cross-link were examined using the sequencing method of Maxam−Gilbert. The position of the TMP-adducted thymine is indicated by an asterisk. Products of Maxam−Gilbert sequence reactions are shown. Sites of 3′ and 5′ incisions are indicated by arrows. (B) The 140 bp DNA substrate was reacted with chromatin-associated protein extract (10 μg) from normal cells (lane 1), or the protein extract (10 μg) that had been preincubated with 10 μg of anti-rabbit IgG (lane 2), 10 μg of mAb 6D12 (lane 3), 5 μg of mAb 6D12 (lane 4), or 3 μg of mAb 6D12 (lane 5). Undamaged substrate was incubated with 10 μg of normal extract (lane 6) or 10 μg of mAb 6D12 (lane 7). Endonucleolytic incisions produced at the sites of the interstrand cross-link were examined using the sequencing method of Maxam−Gilbert. Notations are the same as in (A).

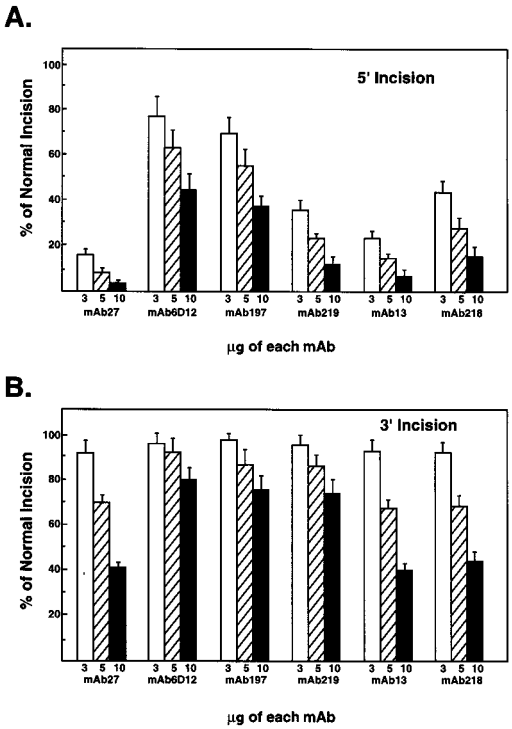

FIGURE 4: Quantitation of the effect of the six mAbs against specific regions of XPF on the production of endonucleolytic incisions by chromatin-associated protein extracts from normal human cells on DNA containing a site-directed TMP interstrand cross-link. The results shown in Figures 3 and 4−6 have been quantitated and incisions expressed as percent of incisions produced when no mAb was present. (A) Quantitation of the 5′ incisions produced. (B) Quantitation of the 3′ incisions produced. Vertical lines represent ±SEM. These experiments were carried out 4 times.

These mAbs were then used to define which domains in XPF are involved in the production of the 5′ and 3′ incisions that are observed at sites of DNA interstrand cross-links. Using a 140 bp DNA fragment containing a single site-directed TMP interstrand cross-link, each mAb was tested for ability to inhibit the incisions produced on both the 3′ and 5′ sides of the cross-link by chromatin-associated protein extracts from normal human cells. Examination of the influence of these mAbs on the 5′ incision showed that each of the mAbs differentially affected levels of this incision. Incubation of the normal extracts with 3, 5, and 10 μg of mAb 27 resulted in significantly reduced levels of the 5′ incision (Figure 3A, lanes 3−5) compared to these levels when mAb 27 was not present (Figure 3A, lane 1). When 3 μg of mAb 27 was used, the level of incision was 16% of normal (Figure 4A). As the concentration of mAb increased to 5 and 10 μg, the level of incision decreased to 8% and 3% of normal (Figure 4A). The control antibody, anti-rabbit IgG, had no effect on these incisions (Figure 3A, lane 2). As we have previously shown (*9*, *16*), the 3′ and 5′ incisions were at the fourth and sixth phosphodiester bonds, respectively, from the adducted thymine. Minor bands that were observed on TMP cross-linked DNA following treatment with the extracts were also present on the undamaged DNA (Figure 3A, lane 6) and represent incisions by nonspecific nucleases. mAb 27 by itself produced no incisions on undamaged DNA (Figure 3A, lane 7).

Significantly less of a reduction in the level of the 5′ incision was observed when mAb 6D12 (Figure 3B, lanes 3−5) or mAb 197 (Figure 5A, lanes 3−5) was used. Examination of the results of preincubation of 5 or 10 μg of mAb 6D12 with the normal extracts (Figure 3B, lanes 4 and 3) showed that levels of incisions were 65% and 46%, respectively, of normal (Figure 4A). Preincubation with 5 or 10 μg of mAb 197 with the normal extracts (Figure 5A, lane 3) showed that levels of incisions were 57% and 38%, respectively, of normal (Figure 6A). The level of inhibition of the incisions depended on the concentration of the mAb and decreased as the concentration of each of these mAbs in the reaction decreased (Figure 4A). Comparison of the results obtained when 10 μg of mAbs 6D12 and 197 was used versus 10 μg of mAb 27 shows that mAb 27 had over a 10-fold greater inhibitory effect on the production of the 5′ incision than did than mAbs 6D12 or 197.

Similarly, mAbs 219, 13, and 218 had a greater inhibitory effect on the production of the 5′ incision than did mAbs 6D12 and 197. Incubation of the protein extracts with 10 μg of mAbs 219 (Figure 5B, lane 5), 13 (Figure 6A, lane 5), and 218 (Figure 6B, lane 3) resulted in levels of incisions that were 12%, 7%, and 16% (Figure 4A), respectively, of normal (lane 1 in Figures 5B, 6A, and 6B). As with the other mAbs, the level of inhibition was dependent on the concentration of the mAb in the reaction, and as the concentration of each of the mAbs decreased, so did the level of inhibition of the 5′ incision. Of these three mAbs, the greatest inhibition occurred with mAb 13. The level of inhibition produced by mAb 13 was significantly less than that produced by mAb 219 ($p < 0.02$) or mAb 218 ($p < 0.01$). Of all six mAbs, mAb 27 had the greatest inhibitory effect on the 5′ incision at the site of the cross-link, followed by mAb 13.

These six mAbs had much less of an effect on the production of the 3′ incision. Again, levels of inhibition depended on the mAb and occurred in a concentration-dependent manner. mAbs 27, 13, and 218 had the greatest inhibitory effect on the 3′ incision (Figure 4B). When 10 μg of mAb 27 (Figure 3A, lane 5), mAb 13 (Figure 6A, lane 5), and mAb 218 (Figure 6B, lane 3) was incubated with the chromatin-associated protein extracts, the levels of incision produced were 41%, 40%, and 45% (Figure 4B), respectively, of normal (lane 1 in Figures 3A, 6A, and 6B). These three mAbs had approximately twice the inhibitory effect of 10 μg of mAbs 6D12, 197, and 219 on levels of the 3′ incision (Figure 4B). These levels of inhibition were significantly different ($p < 0.01$) than those produced by mAbs 6D12, 197, and 219. When 10 μg of mAbs 6D12 (Figure 3B, lane 3), 197 (Figure 5A, lane 3), and 219 (Figure 5B, lane 3) was included in the incision reactions, levels of incisions were 82%, 77%, and 75% (Figure 6B), respectively, of normal (lane 1 in Figures 3B, 4A, and 4B).

In comparing the effect of these six mAbs on production of the 5′ and 3′ incisions produced at a site of a TMP interstrand cross-link, it can therefore be seen that the greatest inhibitory effect is on the 5′ incision, rather than on the 3′ incision. Comparing, for example, the two mAbs that have the greatest inhibitory effect on the 5′ incision, mAb 27 and 13, levels of inhibition are approximately 14- and 6-fold greater, respectively, on the 5′ side than on the 3′ side.

DISCUSSION

XPF has been shown to be involved in the 5′ incision in NER (*1*−*4*) and in the 5′ and also the 3′ incisions at the site

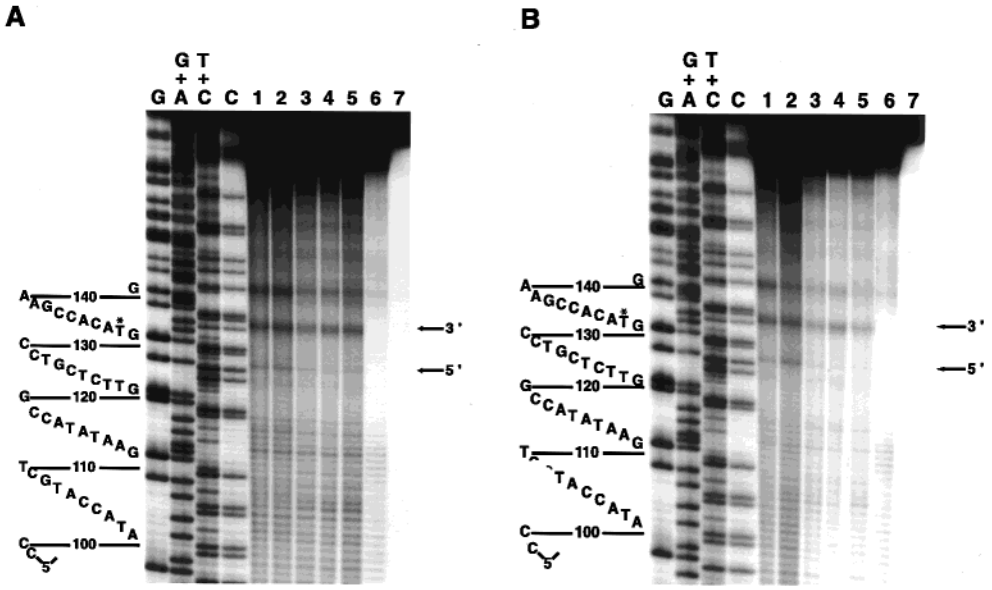

FIGURE 5: Inhibition by mAbs 197 and 219 of production of incisions produced at sites of DNA interstrand cross-links. (A) The 140 bp DNA substrate (100 fmol), containing a TMP interstrand cross-link, was reacted with a chromatin-associated protein extract (10 μg) from normal human lymphoblastoid cells (lane 1) or a chromatin-associated protein extract (10 μg) that had been preincubated with 10 μg of anti-rabbit IgG (lane 2), 10 μg of mAb 197 (lane 3), 5 μg of mAb 197 (lane 4), or 3 μg of mAb 197 (lane 5). Undamaged substrate was incubated with 10 μg of normal extract (lane 6) or 10 μg of mAb 197 (lane 7). Endonucleolytic incisions produced at the sites of the interstrand cross-link were examined using the sequencing method of Maxam−Gilbert. Products of Maxam−Gilbert sequence reactions are shown. Notations are as in Figure 3. (B) The 140 bp DNA substrate was reacted with chromatin-associated protein extract (10 μg) from normal cells (lane 1), or the protein extract (10 μg) that had been preincubated with 10 μg of anti-rabbit IgG (lane 2), 3 μg of mAb 219 (lane 3), 5 μg of mAb 219 (lane 4), or 10 μg of mAb 219 (lane 5). Undamaged substrate was incubated with 10 μg of normal extract (lane 6) or 10 μg of mAb 219 (lane 7). Endonucleolytic incisions produced at the sites of the interstrand cross-link were examined using the sequencing method of Maxam−Gilbert. Notations are the same as in (A).

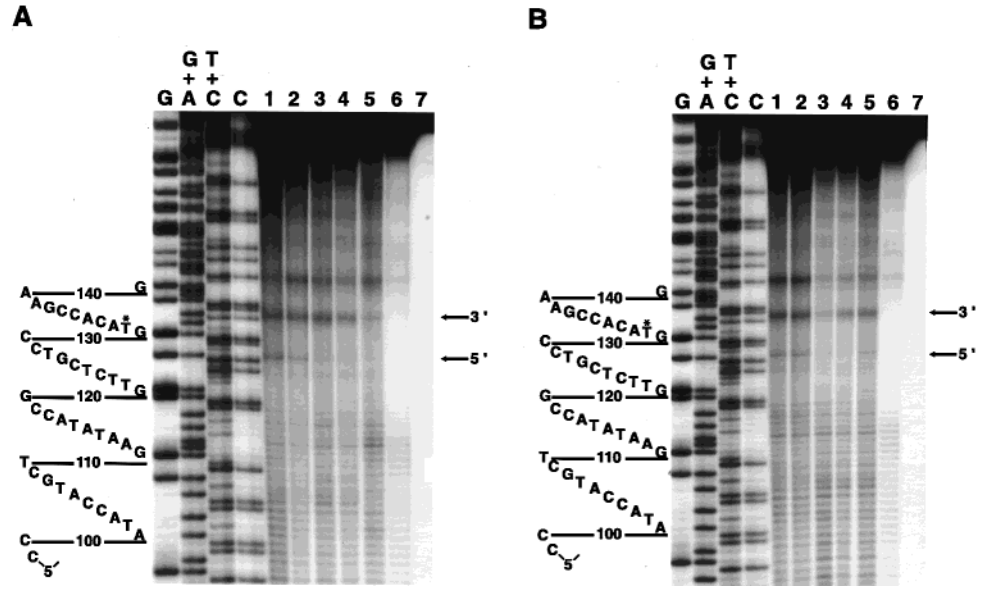

FIGURE 6: Inhibition by mAbs 13 and 218 of production of incisions produced at sites of DNA interstrand cross-links. (A) The 140 bp DNA substrate (100 fmol), containing a TMP interstrand cross-link, was reacted with a chromatin-associated protein extract (10 μg) from normal human lymphoblastoid cells (lane 1), or a chromatin-associated protein extract (10 μg) that had been preincubated with 10 μg of anti-rabbit IgG (lane 2), 3 μg of mAb 13 (lane 3), 5 μg of mAb 13 (lane 4), or 10 μg of mAb 13 (lane 5). Undamaged substrate was incubated with 10 μg of normal extract (lane 6) or 10 μg of mAb 13 (lane 7). Endonucleolytic incisions produced at the sites of the interstrand cross-link were examined using the sequencing method of Maxam−Gilbert. Products of Maxam−Gilbert sequence reactions are shown. Notations are as in Figure 3. (B) The 140 bp DNA substrate was reacted with chromatin-associated protein extract (10 μg) from normal cells (lane 1), or the protein extract (10 μg) that had been preincubated with 10 μg of anti-rabbit IgG (lane 2), 10 μg of mAb 218 (lane 3), 5 μg of mAb 218 (lane 4), or 3 μg of mAb 218 (lane 5). Undamaged substrate was incubated with 10 μg of normal extract (lane 6) or 10 μg of mAb 218 (lane 7). Endonucleolytic incisions produced at the sites of the interstrand cross-link were examined using the sequencing method of Maxam−Gilbert. Notations are the same as in (A).

of a DNA interstrand cross-link (*8, 10*). ERCC1, which forms a heterodimeric complex with XPF, is important in these reactions (*1, 2, 10*). The focus of the present study was to investigate the region(s) in XPF that is (are) important in the 5′ and 3′ incisions observed at sites of DNA interstrand cross-links. mAbs, which have been affinity-mapped to

specific regions of XPF, were utilized in an in vitro incision assay to determine their ability to inhibit the 5′ and 3′ incisions produced by chromatin-associated proteins from normal human cells on DNA containing a site-directed TMP interstrand cross-link. The results show that two regions of XPF, one N-terminal region from amino acids 12−166 and one C-terminal region from amino acids 702−854, are the most important in the production of the 5′ incision. This was demonstrated by the finding that mAbs affinity-mapped to these two regions (mAbs 27 and 13, respectively) significantly reduced the level of 5′ incision produced by the chromatin-associated protein extracts, and produced the greatest levels of inhibition of the 5′ incision on the cross-linked DNA. Regions flanking amino acids 702−854, which were amino acids 584−667 and 854−916, also had some involvement in the 5′ incision as indicated by the ability of mAbs affinity-mapped to these two regions (mAbs 219 and 218, respectively) to also partially inhibit this incision. Though the level of inhibition was less than that produced by mAb 13, these flanking sequences could still play a role in the incision reaction.

Studies of McCutchen-Maloney et al. have shown that purified recombinant XPF, independent of ERCC1, has endonuclease activity on undamaged DNA and can bind to this DNA (*11*). Further studies indicated that the endonuclease activity was located within the first 378 amino acids of XPF (*11*). This suggested a catalytic domain within the N-terminus of the XPF protein (*11*). In support of this is the present finding that the N-terminal region of XPF from amino acids 12−166 appeared to have significant involvement in production of the 5′ incision at the site of a TMP interstrand cross-link. Similarly, studies using a mAb with specificity for the N-terminal 237 amino acid residues in the *S. cerevisiae* homologue of XPF, RAD1, indicated that this region is critical for the nuclease activity of RAD1 (*29*).

Examination of the C-terminal domain of XPF has shown that this is the region that interacts with ERCC1 (*11*, *30*). de Laat et al. reported that the interaction domain of XPF with ERCC1 was in the C-terminal 91 amino acids of XPF, which corresponds to amino acids 825−916 (*30*). McCutchen-Maloney et al. showed that the C-terminal 214 residues (amino acids 702−916) are capable of binding specifically to ERCC1 (*11*). They proposed that a broader C-terminal region than previously described (*30*) governs the interaction of these two proteins (*11*). In the present study, the C-terminal region of XPF that is most important in production of the 5′ incision at the site of the TMP interstrand cross-link (amino acids 702−854) contains a 29 amino acid overlap (amino acids 825−854) with the 2 reports on the segment of XPF that binds to ERCC1. This region could thus be of critical importance in the production of the 5′ incision at the site of a cross-link. It is very likely that binding of ERCC1 to XPF is critical for its ability to produce the 5′ incision. A similar type of functional interaction has been observed between RAD1 and RAD10, the *S. cerevisiae* homologue of ERCC1 (*4*), where the C-terminal domain of RAD1, which is important for its nuclease activity (*29*), binds to RAD10 (*31*, *32*).

Studies using mAbs mapped to the regions of XPF flanking amino acids 702−854, i.e., 584−667 (mAb 219) and 854−916 (mAb 218), indicate that these regions are also involved in the production of the 5′ incision. The inhibitory action of mAb 218 could be via its effect on a region of XPF that is also thought to govern the interaction between XPF and ERCC1 (*11*, *30*). The inhibitory effect of mAb 219 could be due to its binding to a region of XPF that interacts with another protein involved in the incision activity of XPF on cross-linked DNA, such as nonerythroid α spectrin (*17*, *23*). The two regions of XPF that were least affected by mAb binding to them, from amino acids 166−260 (mAb 6D12) and 378−584 (mAb 197), have been shown to bind to DNA but not to have nuclease activity (*11*). Since binding of XPF to DNA must accompany the incisions it produces at sites of cross-links, the small inhibitory effect that these mAbs have on incision activity could indicate that binding of XPF to DNA plays a role in its ability to produce the 5′ incision.

We have previously shown that XPF is involved not only in the 5′ incision at the site of a TMP interstrand cross-link but also to some extent in the 3′ incision as well (*9*). Kuraoka et al. have subsequently reported similar results using purified XPF-ERCC1 (*10*). The present results also indicate that XPF has some involvement in the incision on the 3′ side of the interstrand cross-link. At the highest concentration of mAb tested, mAbs against the N-terminal domain, amino acids 1−166 (mAb 27), and the C-terminal domain, amino acids 702−916 (mAbs 13 and 218), significantly decreased production of the 3′ incision ($p < 0.01$) compared to the other mAbs, whose inhibitions are probably nonspecific. Levels of inhibition of the 3′ incision by these mAbs were approximately 3−10-fold less than those they produced on the 5′ incision. Thus, while XPF appears to have some involvement in the 3′ incision, it is to a lesser extent than its role in the 5′ incision.

The present studies thus indicate that there are two main functional domains of XPF that are critical for production of the 5′ incision that occurs at a site of a TMP interstrand cross-link. One is in the N-terminal domain (amino acids 12−166), which has been proposed to have catalytic activity (*11*), and the other is in the C-terminal domain (amino acids 702−854), which contains the ERCC1 binding site (*11*, *30*). These results suggest that the binding of ERCC1 to XPF is critical for its ability to produce the 5′ incision at the site of an interstrand cross-link, possibly through activation or regulation of the endonucleolytic activity of the N-terminal domain of XPF. These same domains also appear to be involved, though to a limited degree, in the 3′ incision at the site of an interstrand cross-link. This indicates that, in addition to XPF, another protein(s) may be important in the production of the 3′ incision.

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