Fanconi anemia, complementation group A, cells are defective in ability to produce incisions at sites of psoralen interstrand cross-links

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The hypersensitivity of Fanconi anemia, complementation group A, (FA-A) cells to agents which produce DNA interstrand cross-links correlates with a defect in their ability to repair this type of damage. In order to more clearly elucidate this repair defect, chromatin-associated protein extracts from FA-A cells were examined for ability to endonucleolytically produce incisions in DNA at sites of interstrand cross-links. A defined 140 bp DNA substrate was constructed with a single site-specific monoadduct or interstrand cross-link produced by 4,5,8-trimethylpsoralen (TMP) plus long wavelength (UVA) light. Our results show that FA-A cells are defective in ability to produce dual incisions in DNA at sites of interstrand cross-links. Specifically, there is defective incision on the 3’- and 5’-sides of both the furan and pyrone sides of the cross-link. This defect is correct in FA-A cells transduced with a retroviral vector expressing FANCA cDNA. At the site of a TMP monoadduct, FA-A cells can introduce incisions on both the 3’- and 5’-sides of the furan side monoadduct, but are defective in ability to produce these incisions on the pyrone side monoadduct. These studies also indicate that XPF is involved in production of the 5’ incision by the normal extracts on these substrates. These results correlate with our previous work, which showed that FA-A cells are mainly defective in ability to repair psoralen interstrand cross-links with a lesser defect in ability to repair psoralen monoadducts. This defect in endonucleolytic incision at sites of TMP interstrand cross-links could be related to reduced levels of non-erythroid α spectrin (αSpIΣ*) in the extracts from FA-A cells. αSpIΣ* could act as a scaffold to align proteins involved in cross-link repair and enhance their interactions; a deficiency in αSpIΣ* could thus lead to reduced efficiency of repair and the decreased levels of incisions we observe at sites of interstrand cross-links in FA-A cells.

Introduction

Fanconi anemia (FA) is a recessively transmitted genetic disorder characterized by bone marrow failure, a predisposition to leukemia and diverse congenital abnormalities (1–4). FA cells are particularly hypersensitive to the clastogenic and cytotoxic effects of DNA interstrand crossing agents and these agents can potentiate the spontaneous chromosomal aberrations observed in FA cells (5–8). Eight genetic complementation groups of FA (A–H) have been described (9–11) and the genes for complementation groups A, C and G have been cloned, however, the function of the proteins encoded by these genes is unknown (12–15). In cells derived from patients with FA, complementation group A (FA-A), this hypersensitivity to DNA interstrand cross-linking agents correlates with reduced ability to repair DNA damage produced by such agents (16–22). However, the precise biochemical defect in the repair of DNA interstrand cross-links in FA-A has not been delineated, although the defect has been shown to occur in the initial steps of repair of these lesions (16,17,20,23,24).

Although repair of DNA interstrand cross-links has been extensively studied in prokaryotes and the molecular basis for this process elucidated (25–28), the precise nature of these repair events in mammalian cells, specifically whether one or more than one pathway may be involved, is unclear (29–31). We have shown that chromatin-associated protein extracts from normal human cells produce dual endonucleolytic incisions at sites of DNA interstrand cross-links (32). These incisions bracket the lesion at the sites of both a 4,5,8-trimethylpsoralen (TMP) interstrand cross-link and a monoadduct and the sites of 3’ and 5’ incision depend upon the type of adduct formed (32). In contrast to these studies, Bessho et al. have found that dual incisions produced by CHO extracts or a reconstituted nucleotide excision repair (NER) system at sites of a 4’-hydroxymethyl-4,5,8-trimethylpsoralen (HMT) interstrand cross-link occur on the 5’-side of the lesion, whereas these incisions bracket the lesion at the site of a HMT monoadduct (29). It is possible that more than one pathway is involved in the repair of DNA interstrand cross-links.

The present study was undertaken in order to determine whether the inability of FA-A cells to repair psoralen interstrand cross-links is related to a defect in ability to make dual incisions on DNA at sites of these lesions. For these studies, a 140 bp substrate was constructed which contained either a single centrally located monoadduct or interstrand cross-link produced by TMP plus UVA light (32). These substrates were constructed so that incision events on either the furan side or pyrone side of both types of adduct could be examined (32). Studies utilizing extracts from xeroderma pigmentosum complementation group F (XPF) cells and a polyclonal antibody against the XPF protein indicate that, in normal cells, the XPF protein is involved in production of the 5’ incision produced at sites of both the interstrand cross-link and the monoadduct. Our results show that FA-A cells are defective in ability to make dual incisions at sites of a DNA interstrand cross-link and that this defect is corrected in FA-A cells expressing the FANCA cDNA.

Materials and methods

Preparation of chromatin-associated protein extracts

Two FA-A (HSC 72 and HSC 99) and two normal human (GM 1989 and GM 3299) lymphoblastoid cell lines (transformed with Epstein–Barr virus)
were obtained from the Coriell Institute for Medical Research (Camden, NJ). FA-A lymphoblastoid cells (HSC 72) stably transduced with the pG1 retroviral vector expressing the FA-CA cDNA (HSC72-17) (33,34) were a gift from Dr Christopher Walsh (University of North Carolina). Cells were grown in suspension culture in RPMI 1640 medium, supplemented with 12.5% fetal calf serum (Grand Island Biological Co.) and harvested under conditions of maximal proliferation (35). All cell lines were checked for mycoplasma using a Mycoplasma PCR Test Kit (American Type Culture Collection). XFP fibroblasts transformed with SV40 (GM 08437A) were obtained from the Coriell Institute and grown by Celllex Biosciences Inc. (Minneapolis, MN). Cell nuclei were isolated and the chromatin-associated proteins were separated which may be present (39,40). The top strand was internally labeled with 32P and containing a single TMP furan side monoadduct, was reacted with 10 µg of the chromatin-associated protein extract (lane 1), 20 µg of the FA-A (HSC 72) extract (lane 2) or 10 µg of the FA-A (HSC 72) extract (lane 3). Substrates without an adduct were also incubated with 10 µg of normal (lane 4) or FA-A (lane 5) extract. Aliquots of 2000 c.p.m. of each sample were loaded per gel lane. 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’ incision are indicated by arrows. (B) A segment of the 140 bp substrate showing sites of endonucleolytic incision by FA-A extracts on the 3’ and 5’ sides of the furan side monoadduct (indicated by arrows). The adducted T is circled. The angled line extending from the T indicates linkage with the furan (F) ring of TMP. The asterisk indicates the 32P-labeled strand. Each of these experiments was repeated between four and nine times using several enzyme extracts from each of the two normal and two FA-A cell lines. For both the normal and FA-A cells results were similar between cell lines, with the differences between cell lines being <5%.

**Fig. 1.** Sites of incision by FA-A extracts on a DNA substrate containing a TMP furan side monoadduct. (A) A 140 bp DNA substrate (~100 fmol), the top strand internally labeled at the 5’-end with 32P and containing a single TMP furan side monoadduct, was reacted with 10 µg of the normal (GM 1989) chromatin-associated protein extract (lane 1), 20 µg of the FA-A (HSC 72) extract (lane 2) or 10 µg of the FA-A (HSC 72) extract (lane 3). Substrates without an adduct were also incubated with 10 µg of normal (lane 4) or FA-A (lane 5) extract. Aliquots of 2000 c.p.m. of each sample were loaded per gel lane. 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’ incision are indicated by arrows. (B) A segment of the 140 bp substrate showing sites of endonucleolytic incision by FA-A extracts on the 3’ and 5’ sides of the furan side monoadduct (indicated by arrows). The adducted T is circled. The angled line extending from the T indicates linkage with the furan (F) ring of TMP. The asterisk indicates the 32P-labeled strand. Each of these experiments was repeated between four and nine times using several enzyme extracts from each of the two normal and two FA-A cell lines. For both the normal and FA-A cells results were similar between cell lines, with the differences between cell lines being <5%.

Construction of DNA substrates containing a site-specific TMP monoadduct or interstrand cross-link

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**Construction of DNA substrates containing a site-specific TMP monoadduct or interstrand cross-link**

For examination of sites of endonucleolytic incision, a DNA substrate was used corresponding to the region from 61 to 200 of the nucleotide sequence of the 5S rRNA gene from *Lytechinus variegatus* (32). This substrate was synthesized as seven separate oligonucleotides (HHMI Biopolymer Facility and W.M.Keck Foundation Biotechnology Resource Laboratory at Yale University) and consisted of a duplex region of 132 bp with a 4 bp overhang on the 5’-end of both the top and bottom strands (32). The substrate was engineered to have a single site-specific TMP monoadduct with its furan side attached to a thymine in a centrally positioned 5’-TpA-3’ dinucleotide sequence in the top strand of the substrate (Figure 1B), since this sequence has been shown to be a hot-spot for formation of TMP adducts (37,38). Protein concentration was determined using Bradford reagent (Bio-Rad).

For construction of these substrates, an 18mer oligonucleotide containing a TMP furan side monoadduct was prepared as previously described (32). This monoadducted oligonucleotide was mixed with the three other oligonucleotides comprising the top strand and the three oligonucleotides comprising the bottom strand. These oligonucleotides were hybridized and then ligated together. This resulted in the formation of a 132 bp substrate with a 4 bp overhang on the 5’-end of both the top and bottom strands (32). The 3’-ends of the top and bottom strands were filled with dNTP (dTTP) (deoxyribonucleoside 5’-[β-thio]triphosphate) (Dupont) using the Klenow fragment of *Escherichia coli* DNA polymerase I (39,40). This resulted in the formation of a 140 bp substrate and the full-length duplex substrate was isolated as previously described (32). The presence of an α-phosphorothioate nucleotide(s) at the 3’-end of the DNA substrate confers resistance to 3’→5’ exonuclease activities which may be present (39,40). The top strand was internally labeled with γ-32P at the 5’-end (32). Irradiation of this DNA with UVA light (20 mW/cm² for 25 min) resulted in the production of a cross-link in which the furan side was adducted to the top strand of the duplex (Figure 3C) (32). For construction of pyrene side adducts, the bottom strand was internally labeled with γ-32P and the cross-link was formed as described above (32). In these substrates, the 32P label was thus on the strand to which the pyrene ring of TMP was adducted. The pyrene side monoadducted substrate was prepared by reversing the psoralen cross-link with hot alkali treatment (32). Undamaged substrates were similarly prepared. The above substrates were gel purified and cleaned up with Nensorb 20 nucleic acid purification cartridges according to the manufacturer’s protocol (NEN Research Products, Dupont) and stored in aliquots at −20°C in 10 mM Tris–HCl, pH 7.6, and 0.4 mM EDTA.

**Determination of sites of endonucleolytic incision**

Approximately 100 fmol of the labeled duplex DNA substrate, either TMP modified or unmodified, was reacted with 5–20 µg of the chromatin-associated protein extract in a volume of 40 µl containing 50 mM Tris–HCl, pH 7.6, 10 mM MgCl₂ and 100 ng salmon sperm DNA (Strategene) for 3 h at 37°C (32). The reactions were terminated by addition of 15 mM EDTA and samples deproteinized using Nensorb 20 nucleic acid purification cartridges (32). The ATP requirement of the incision reaction was ascertained by inhibition of the dual incisions on the damaged substrates by incorporation of ATPγS (Sigma) in the incision reactions (41,42).
Just before electrophoresis, all samples (i.e. both TMP monoadducted and cross-linked substrates) were photoreversed by irradiation with 254 nm UV light (1500 µW/cm² for 5 min) at room temperature using a germicidal UV lamp (G15T8; American Ultraviolet Co.) (32). This was done in order to break the interstrand cross-link, in those substrates in which it was present, so as to allow detection of incisions on both sides of the lesion in a single assay and to remove the psoralen moiety so there would be no delay in migration of the DNA fragments upon gel electrophoresis (43–45). We have shown that this irradiation with UV light did not produce a shift in migration pattern of the DNA bands (32). Samples were prepared for sequence analysis as previously described (32) and incision sites were identified by running the normal endonuclease reactions along with the sequencing ladders of undamaged substrates on a denaturing 6% polyacrylamide gel using the method of Maxam and Gilbert (46). Sequencing gels were exposed to Dupont Reflection autoradiography film (NEF-496) with a reflection intensifying screen overnight at ~80°C. The percentage of substrate converted to incision products was quantitated from the X-ray films from each experiment using a Leica Quantimet 500 Image Analysis System with a Sony video camera. 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.

The ability of an affinity-purified polyclonal antibody against XPF protein (a gift of Dr Michael Thelen, Lawrence Livermore National Laboratory) to inhibit these incisions was examined. Normal human chromatin-associated protein extract (5–10 µg) was mixed with a 1:10 or 1:100 dilution of anti-XPF antibody or anti-rabbit IgG (Sigma Chemical Co.) as a control antibody (1:10 dilution), 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.

Results

Sites of incision on TMP furan side monoadducted DNA

FA-A chromatin-associated protein extracts were examined for sites of endonucleolytic incision on the TMP furan side monoadducted substrate. Internal labeling of the substrate with 32P at the 5′-end of the top strand allowed visualization of either the 3′ or 5′ incision events. After incubation of the substrate with the normal and FA-A extracts, the DNA was irradiated with UVC light so as to remove the TMP moiety before sequence analysis. Specific incisions were produced by FA-A extracts on both the 3′- and 5′-sides of the TMP monoadduct (Figure 1A, lanes 2 and 3). Two bands were produced which resulted from an incision made at the fifth phosphodiester bond 3′ to the added thymine and at the fifth phosphodiester bond 5′ to this same modified base (Figure 1B). No incisions were produced on undamaged DNA, demonstrating their specificity for the damaged substrate (Figure 1A, lanes 4 and 5). The distance between incisions was 9 nt. These were the same sites of endonucleolytic cleavage produced by the normal protein extracts (Figure 1A, lane 1). Minor bands that were observed on TMP added DNA following treatment with the extracts were also present on the undamaged DNA and represent incisions by non-specific nucleases.

Quantitation of the incision products showed that levels of incisions produced on TMP monoadducted DNA by extracts from two different FA-A cell lines were similar to those produced by extracts from two different normal cell lines (Table I). No significant differences were found between the normal and FA-A extracts (P > 0.1). Each of these experiments was repeated four to eight times using two to three enzyme extractions from each cell line. For both the normal and FA-A extracts, results were similar between cell lines with the differences between cell lines being <5%.

Production of the 3′ and 5′ incisions by the normal and FA-A extracts on TMP monoadducted DNA was dependent upon the concentration of the extracts used and increased as the protein concentration increased (data not shown). The rate of production of 3′ and 5′ incisions on TMP monoadducted DNA was similar between the normal and FA-A extracts (data not shown).

<table>
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<tr>
<th>Table I. Quantitation of incisions produced by the FA-A and normal endonucleases at sites of TMP furan side adducts</th>
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<tr>
<td>Monoadduct incisions (%) of substrate</td>
</tr>
<tr>
<td>3′</td>
</tr>
<tr>
<td>Normal GM 3299</td>
</tr>
<tr>
<td>Normal GM 1989</td>
</tr>
<tr>
<td>FA-A HSC 72</td>
</tr>
<tr>
<td>FA-A HSC 99</td>
</tr>
<tr>
<td>HSC 72 (% of normal)</td>
</tr>
<tr>
<td>HSC 99 (% of normal)</td>
</tr>
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</table>

-aDNA chromatin-associated protein extract (10 µg) was incubated with 100 fmol of DNA substrate for 3 h as described in Materials and methods. Incisions are expressed as the percentage of substrate converted to incision products and were quantitated from the X-ray films from each experiment using a Leica Quantimet 500 image Analysis System. Means (± SEM) were obtained for each set of experiments.

-bEight experiments were carried out using monoadducted DNA and seven using cross-linked DNA.

-cFour experiments were carried out using monoadducted DNA and five using cross-linked DNA.

-dEight experiments were carried out using monoadducted DNA and 10 using cross-linked DNA.

-eFour experiments were carried out using monoadducted DNA and four using cross-linked DNA.

-fThe percentage is based on a comparison with the average of the number of incisions produced by both of the normal cell lines.

Defective FA-A incision at interstrand cross-links

Before TMP cross-linked DNA, which had been incubated with FA-A and normal chromatin-associated protein extracts, was examined for sites of incision by sequence analysis, the DNA was photoreacted so as to break the interstrand cross-link and release TMP from the DNA. Samples were electrophoresed on a 6% denaturing polyacrylamide gel (32). Figure 2 shows that at the UV dose used all cross-links were broken (lane 3). All substrate preparations gave the same electrophoretic pattern. Any differences observed in levels of incision between FA-A and normal extracts were, therefore, not due to incomplete or variable cross-link reversal.

Examination of the ability of the FA-A extracts to incise DNA containing a furan side interstrand cross-link showed that these extracts from both FA-A cell lines were defective in their ability to create an endonucleolytic incision on the 3′- and 5′-side of the cross-link (Figure 3A and B). Incisions produced on the 3′-side of this lesion by HSC 72 (Figure 3A, lane 2) and HSC 99 (Figure 3B, lane 2) extracts were only 36 and 31%, respectively, of those produced by extracts from the two normal cell lines (Figure 3A and B, lane 1, and Table I).

t-tests were performed and the number of incisions produced by extracts from both FA-A cell lines was significantly different from those produced by the normal extracts (P < 0.01 for both cell lines). Like the normal extracts, these incisions were at the fourth phosphodiester bond from the added thymine (Figure 3C). There was also a reduction in ability of FA-A extracts to endonucleolytically produce an incision on the 5′-side of the cross-link (Figure 3A and B, lane 2). Levels of...
incision by HSC 72 and HSC 99 extracts were 18 and 14%, respectively, of those produced by the normal extracts (Table I). These differences were significant ($P < 0.01$). The site of incision, like that produced by the normal extracts, was at the sixth phosphodiester bond from the modified thymine (Figure 3C). The distance between sites of cleavage was 9 nt. These incisions were not seen on undamaged DNA, which again indicates their specificity for the damaged substrate (Figure 3A and B, lanes 3 and 4). Other minor bands observed on cross-linked DNA following treatment with the extracts were also seen on undamaged DNA and again represent incisions by non-specific nucleases. These experiments were repeated between four and ten times for each cell line (a total of 14 experiments were carried out using FA-A extracts and 12 experiments using normal extracts). For each cell line examined, two to four enzyme extractions were utilized. Results were similar between each of the respective cell lines, with differences between FA-A and normal cell lines being <10%.

**Correction of the defect in ability to incise cross-linked DNA in FA-A cells expressing FANCA cDNA**

Studies were carried out to determine whether there was any relationship between the FANCA gene and the deficiency in the ability of the FA-A extracts to incise cross-linked DNA. Chromatin-associated protein extracts from FA-A cells transduced with a retroviral vector expressing the FANCA cDNA were examined for ability to incise the TMP cross-linked substrate. As shown in Figure 4, the deficiency in ability of the FA-A extracts to incise cross-linked DNA has been corrected in the transduced cells (lane 2) and is dependent upon the protein concentration of the extract (lanes 2 and 3). The levels of incisions are similar to those produced by the normal extracts (~100% of normal) (Figure 4, lane 1).

**Sites of incision on TMP pyrone side adducted DNA**

As we have previously reported (32), incision signals on DNA containing pyrone side monoadducts and interstrand cross-links were not strong. Levels of incisions produced by the FA-A endonucleases (Figure 5A, lane 2) on DNA containing a pyrone side monoadduct were decreased compared with incisions made by the normal endonucleases (Figure 5A, lane 1). Incisions produced on the 3'-side by extracts from two different FA-A cell lines (HSC 72 and HSC 99) were only 33 and 29%, respectively, of those produced by extracts from normal cells (Table II). The levels of incisions on the 5'-side were only 18 and 14%, respectively, of those produced by normal extracts (Table II). The differences between levels of incisions produced by the FA-A extracts compared with the normal extracts was significant ($P < 0.01$). Like the incisions produced by the normal endonucleases (Figure 5A, lane 1), these incisions were at the fifth phosphodiester bond on the 3'-side and the thirteenth phosphodiester bond on the 5'-side of the modified base (Figure 5B). The distance between sites of incision was 17 nt. These incisions were not seen on undamaged DNA (Figure 5A, lanes 3 and 4). These experiments were repeated between three and five times for each cell line. The deficiency in ability of the FA-A extracts to incise the pyrone side monoadducted DNA was corrected in FA-A cells expressing the FANCA cDNA (data not shown).

On DNA containing a pyrone side interstrand cross-link, normal endonucleolytic incisions were produced much less efficiently than on the other TMP adducted substrates. As we have previously shown, incisions were made at the fourth phosphodiester bond on the 3'-side and the fourteenth phosphodiester bond on the 5'-side of the added thymine with a distance of 17 nt between incisions (Figure 6A, lanes 1 and 2) (32). FA-A cell extracts were defective in ability to produce incisions on the 3'-' and 5'-'-sides of the pyrone side cross-link, however, levels of incision were too low to quantitate (Figure 6A, lanes 3 and 4, and Figure 6B).

**Role of ATP in these incisions**

Previous studies have shown that addition of ATP to the reactions was not needed for the incisions produced by the normal extracts (32). Studies were therefore carried out to determine if ATP could be present in the extracts themselves. The results showed that addition of the non-hydrolyzable ATP analog ATPγS to the incision reactions resulted in loss of the incisions produced by the normal extracts on both the TMP furan side monoadducted (Figure 8A) and interstrand cross-linked substrates (Figure 8B). This indicates that ATP is present in our chromatin-associated protein extracts and is needed in the incision reactions we observe.

**Examination of XPF cell extracts for production of incisions on damaged DNA**

In order to ascertain whether the XPF protein was involved in the production of the 5' incision we observed at sites of TMP interstrand cross-links and monoadducts, chromatin-associated protein extracts from XPF cells were examined for ability to incise DNA at sites of these adducts. The XPF cell extracts were defective in ability to produce incisions on the 5'- and to a certain extent on the 3'-sides of the cross-link (Figure 8, lanes 2 and 3). Levels of incisions were 16 and 0%, respectively,
Defective FA-A incision at interstrand cross-links

Fig. 3. Defect in FA-A in production of endonucleolytic incisions on DNA containing a TMP furan side interstrand cross-link. (A) A 140 bp DNA substrate (~100 fmol), in which the furan side of a TMP interstrand cross-link was adducted to the 32P-5'-end-labeled top strand, was reacted with 10 µg of normal (GM 3299) extract (lane 1) or 10 µg of FA-A (HSC 72) extract (lane 2). Undamaged substrate was incubated with 10 µg of normal extract (lane 3) or 10 µg of FA-A extract (lane 4). Aliquots of 2000 c.p.m. of each sample were loaded per gel lane. Maxam–Gilbert reaction products are shown. Notations are as in Figure 1. (B) The 140 bp substrate (100 fmol) was reacted with 10 µg of normal (GM 3299) extract (lane 1) or 10 µg of FA-A (HSC 99) extract (lane 2). Undamaged substrate was incubated with 10 µg of normal (lane 3) or 10 µg of FA-A (lane 4) extract. Aliquots of 2000 c.p.m. of each sample were loaded per gel lane. Maxam–Gilbert reaction products are shown. (C) A segment of the substrate showing sites of defective 3’/H11032 incision and 5’/H11032 incision (dotted arrows) by the FA-A extracts. Adducted T residues are circled. The angled lines extending from the T residues indicate linkage with the furan (F) or pyrone (P) ring of TMP. The asterisk indicates the 32P-labeled strand. Each of these experiments was repeated between five and ten times for each cell line using two to four enzyme extracts from each cell line with similar results.

of those produced by the normal extract (Figure 8, lane 1). They were also defective in ability to produce these incisions at sites of TMP monoadducts (data not shown). Other studies have similarly shown that XPF cell extracts are defective in ability to produce incisions on the 3’- as well as on the 5’-side of lesions (47,48).

To confirm the involvement of the XPF protein in the production of the 5’ incision we have observed, studies were carried out to determine whether an affinity-purified polyclonal antibody against XPF protein could inhibit the 5’ incision produced by the normal extracts. The results show that anti-XPF antibody inhibited the ability of the normal extracts to produce the 5’ incision and to a lesser degree the 3’ incision at the site of the TMP cross-link (Figure 9, lanes 4 and 5). Levels of incisions were 25 and 2%, respectively, of those produced when anti-XPF was not present (Figure 9, lanes 1 and 2) or in the presence of a control antibody, anti-rabbit IgG (Figure 9, lane 3). Anti-XPF similarly inhibited production of the incisions produced by the normal extracts on TMP monoadducted DNA (data not shown). Anti-ERCC1 antibodies have likewise been shown to inhibit both the 3’ and 5’ incisions at a damage site (47).

Discussion

This study shows that at sites of TMP interstrand cross-links, chromatin-associated protein extracts from FA-A cells are defective in ability to make endonucleolytic incisions in DNA that bracket the lesion on both the furan side and pyrone side of the cross-link. This deficiency correlates with the reduced levels of unscheduled DNA synthesis (UDS) that occur in FA-A cells in response to 8-methoxypsoralen plus UVA light (17,18). Both the incision defect and the decreased UDS (34) are corrected in FA-A cells expressing FANCA cDNA. These studies emphasize the importance of the incisions we observe in the repair of DNA interstrand cross-links and indicate that
the FANCA gene plays a role in repair of this lesion. The ability of FA-A extracts to incise DNA containing TMP monoadducts depended upon the type of monoadduct. Normal levels of incisions were observed on either side of the furan side monoadduct, however, there was a defect in production of these incisions at sites of the pyrone side monoadduct. The pattern of incisions was similar on both monoadducted and cross-linked DNA with the distance between sites of incision.

Table II. Quantitation of incisions produced by the FA-A and normal endonucleases at sites of TMP pyrone side monoadducts

<table>
<thead>
<tr>
<th>Incisions (% of substrate)</th>
<th>3'</th>
<th>5'</th>
</tr>
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<tbody>
<tr>
<td>Normal GM 3299</td>
<td>3.1 ± 0.4</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Normal GM 1989</td>
<td>3.6 ± 0.1</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>FA-A HSC 72</td>
<td>1.2 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>FA-A HSC 99</td>
<td>1.0 ± 0.1</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>HSC 72 (% of normal)</td>
<td>33</td>
<td>18</td>
</tr>
<tr>
<td>HSC 99 (% of normal)</td>
<td>29</td>
<td>14</td>
</tr>
</tbody>
</table>

aDNA chromatin-associated protein extract (10 µg) was incubated with 100 fmol of DNA substrate for 3 h as described in Materials and methods. Incisions are expressed as the percentage of substrate converted to incision products and were quantitated as in Table I. Means (± SEM) were obtained for each set of experiments.
bFour experiments were carried out.
cThree experiments were carried out.
dThe percentage is based on a comparison with the average of the number of incisions produced by the normal cell line GM 3299 since experiments with these normal and FA-A cell lines were carried out at the same time.
eThe percentage is based on a comparison with the average of the number of incisions produced by the normal cell line GM 1989 since experiments with these normal and FA-A cell lines were carried out at the same time.

Fig. 4. Incision of DNA containing a TMP furan side interstrand cross-link by extracts from a FA-A cell line transduced with a cDNA expressing the FANCA cDNA. DNA substrate, as in Figure 3, was reacted with 10 µg of normal (GM 1989) extract (lane 1) or 10 (lane 2) or 5 µg (lane 3) of transduced FA-A extract. Undamaged substrate was incubated with 10 µg of normal (lane 4) or transduced FA-A (lane 5) extract. Aliquots of 2000 c.p.m. of each sample were loaded per gel lane. Maxam–Gilbert reaction products are shown. Notations are as in Figure 1.

Fig. 5. Defect in FA-A in production of endonucleolytic incisions on DNA containing a TMP pyrone side monoadduct. (A) A 140 bp DNA substrate (~100 fmol), in which the pyrone ring of TMP was adducted to the 32P-5'-end-labeled bottom strand, was reacted with 20 µg of normal (GM 3299) extract (lane 1) or 20 µg of FA-A (HSC 72) extract (lane 2). Undamaged substrate was incubated with 20 µg of normal extract (lane 3) or 20 µg of FA-A extract (lane 4). Aliquots of 2000 c.p.m. of each sample were loaded per gel lane. Maxam–Gilbert reaction products are shown. Notations are as in Figure 1. (B) A segment of the substrate showing sites of defective 3' incision and 5' incision (dotted arrows) by the FA-A extracts. The adducted T is circled. The angled line extending from the T indicates linkage with the pyrone (P) ring of TMP. Each of these experiments was repeated three or four times using two enzyme extracts from each of the two normal and two FA-A cell lines. For both the normal and FA-A cells, results were similar between cell lines.
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**Fig. 6.** Defect in FA-A in production of endonucleolytic incisions on DNA containing a TMP pyrone side cross-link. (A) A 140 bp DNA substrate (~100 fmol), in which the pyrone ring of TMP interstrand cross-link was adducted to the $^{32}$P-5'-end-labeled bottom strand, was reacted with 20 µg of normal (GM 3299) extract (lane 1), 20 µg of normal (GM 1989) extract (lane 2), 20 µg of FA-A (HSC 72) extract (lane 3) or 20 µg of FA-A (HSC 99) extract (lane 4). Aliquots of 2000 c.p.m. of each sample were loaded per gel lane. Maxam–Gilbert reaction products are shown. Notations are as in Figure 1. (B) A segment of the substrate showing sites of defective 3' incision and 5' incision (dotted arrows) by the FA-A extracts. The adducted T residues are circled. The angled lines extending from the T residues indicate linkage with the furan (F) ring or pyrone (P) ring of TMP. The asterisk indicates the $^{32}$P-labeled strand. Each of these experiments was repeated three or four times using two enzyme extracts from each of the two normal and two FA-A cell lines. For both the normal and FA-A cells results were similar between cell lines.

**Fig. 7.** Inhibition of endonucleolytic incisions on the furan side monoadducted or cross-linked substrate by ATPγS. (A) DNA containing a TMP furan side monoadduct, as in Figure 1, was reacted with 10 µg of normal (lane 1) extract, 10 µg of normal extract plus 1 mM ATPγS (lanes 2 and 4) or 10 µg of normal extract plus 2 mM ATPγS (lane 3). Undamaged substrate was incubated with 10 µg of normal (lane 5) extract. (B) DNA substrate containing a TMP furan side interstrand cross-link, as in Figure 3, was reacted with 10 µg of normal (lane 1) extract, 10 µg of normal extract plus 2 mM ATPγS (lane 2) or 10 µg of normal extract plus 3 mM ATPγS (lane 3).
dependent upon whether the furan ring or pyrone ring of TMP was adducted to DNA. The greater distance that was observed between incisions on the strand containing the pyrone side adduct could be due to the fact that, since TMP is an asymmetric molecule, more distortion may occur in DNA at the site where the pyrone side of TMP is adducted to DNA compared with the furan side (40,49–51).

The present results corroborate our previous in vitro studies which showed that FA-A cells are mainly defective in ability to incise DNA containing psoralen interstrand cross-links, with a much smaller defect in ability to incise DNA containing psoralen monoadducts (17,18). These findings are in agreement with those of Averbeck et al. (16), Papadopoulo et al. (20) and Rousset et al. (21), who showed that in FA-A cells incision of DNA containing psoralen interstrand cross-links or removal of psoralen cross-links from DNA was significantly less than that observed in normal cells. As we (17,34) as well as others (52,53) have previously reviewed, there is variability in the reported degree of the DNA repair defect in FA cells. Some of this variability could be due to the fact that a number of these investigations were carried out before classification of cell lines by complementation analysis was available and that a number of different types of assay systems have been utilized. In addition, in studies in which repair of psoralen adducts in DNA is examined, the ratio of psoralen monoadducts to cross-links and of the furan side to the pyrone side monoadduct need to be taken into account since, as is shown in the present study, FA-A cells have different abilities to repair these adducts. It has also been shown that ~25% of patients with FA have evidence of spontaneously occurring mosaicism in which some circulating cells have reverted to the wild-type and have normal mitomycin C sensitivity (54,55). It is possible that this type of reversion of the FA phenotype could account for some of the variability reported regarding the DNA repair defect in FA cells.

Repair of DNA interstrand cross-links is more complex than for other types of DNA damage since both strands of DNA need to be repaired at a single location. In Escherichia coli (25,27,28,56) and yeast (57–60) both NER and homologous recombination have been shown to be needed in this repair process. Whether a combination of these processes or different mechanisms are involved in repair of interstrand cross-links in mammalian cells is unclear. There is evidence that NER plays a role (61). However, there are differences in the reported patterns of incisions observed at sites of DNA interstrand cross-links (29,32). It is possible that there is more than one pathway for repair of these cross-links in mammalian cells and that recombination is involved (31,62,63). There may also be overlap in some of the proteins that take part in both processes (29,31,64). A study by Bessho et al., in which excision of DNA was examined, indicated that the pattern of incisions created by CHO cell extracts and by a reconstituted NER system on HMT cross-linked DNA was different than that produced on monoadducted DNA (29). Sites of incision on the cross-linked DNA were at the sixth and twenty-seventh

Fig. 8. Defect in production of endonucleolytic incisions by chromatin-associated protein extracts from XPF cells on DNA containing a TMP furan side interstrand cross-link. DNA substrate, as in Figure 3, was reacted with 10 µg of normal (GM 1989) extract (lane 1) or 5 (lane 2) or 10 µg (lane 3) of XPF cell extract. Undamaged substrate was incubated with 10 µg of normal extract (lane 4). Aliquots of 2000 c.p.m. of each sample were loaded per gel lane. Maxam–Gilbert reaction products are shown. Notations are as in Figure 1.

Fig. 9. Inhibition by anti-XPF antibody of production of endonucleolytic incisions by chromatin-associated protein extracts from normal cells on DNA containing a TMP furan side interstrand cross-link. DNA substrate, as in Figure 3, was reacted with 5 (lane 1) or 10 µg (lane 2) of normal (GM 1989) extract, normal extract (10 µg) preincubated with rabbit anti-IgG (1:10 dilution) (lane 3) or a 1:10 (lane 4) or 1:100 dilution (lane 5) of anti-XPF antibody. Aliquots of 2000 c.p.m. of each sample were loaded per gel lane. Maxam–Gilbert reaction products are shown. Notations are as in Figure 1.
phosphodiester bonds, both 5’ to the adducted thymine residue, and this pattern of incisions was observed with equal efficiency on both the furan side and pyrone side adducted strand (29). On monoadducted DNA, in contrast, these incisions bracketed the adduct (29,65). These observations are in contrast to the present and our previous studies (32), which show that the pattern of incisions on TMP monoadducted and cross-linked substrates is similar and that on both substrates incisions bracket the lesion. Sites of incision similar to the ones we observed on monoadducted DNA were obtained by Nichols (66) using HeLa cells. In this study a nuclease activity was shown to incise DNA at the seventh phosphodiester bonds 3’ and 5’ to a furan side HMT monoadduct and cyclobutane thymine dimer (66).

We have shown that a number of the proteins involved in NER are present in the endonuclease complex that has specificity for DNA interstrand cross-links, indicating an involvement of NER in the repair of this lesion (34). These include XPA, RPA, TFIIH (XPB, XPD, p62 and p44), XPG, XPF, ERCC1 and PCNA (34). Whether they all play a role in the pattern of incisions we have observed is not clear at present. The present study shows that the XPF protein is involved in production of the 5’ incision we observe at sites of TMP interstrand cross-links and monoadducts. XPF–ERCC1 has been shown to be responsible for production of the 5’ incision in NER (67,68) and has been implicated in repair of DNA interstrand cross-links and recombination (31,62). Our studies show that the levels of the XPF protein in FA-A cells are similar to those in normal cells (34), which indicates that the repair defect in FA-A is not due to a deficiency in the XPF protein. The present investigation also indicates that ATP is needed for the incisions we observe at sites of interstrand cross-links. Since addition of ATPγS to the reactions inhibited the production of these incisions, this indicates that ATP is present in our extracts and is needed for these incisions. This method has been used similarly in other studies to show that ATP is present in cell-free extracts (41). The pattern of incisions observed in the present study shares some similarities with those produced by the UvrABC nuclease. Dual incisions created by the UvrABC nuclease bracket the psoralen monoadduct and cross-link with a distance of 12 nt between incisions on both the furan and pyrone side adducted substrates (25,27,28,69,70). Also, in both our system and the UvrABC system incision signals on psoralen cross-linked substrates are stronger and are produced more efficiently on the furan side compared with the pyrone side adducted strand (69,70).

In addition to the NER proteins, we have recently shown that another protein present in the normal endonuclease complex, with specificity for DNA interstrand cross-links, is non-erythroid α spectrin (αSpIΠ*) (71). Several lines of evidence, from both our previous studies (34,71) and from the present investigation, indicate that αSpIΠ* is involved in repair of DNA interstrand cross-links: (i) a monoclonal antibody against αSpIΠ* inhibits production of the endonucleolytic incisions we observe at sites of DNA interstrand cross-links; (ii) there are decreased levels of αSpIΠ* in FA-A as well as in FA-B, FA-C and FA-D cells, which correlate with reduced levels of UDS and reduced levels of endonucleolytic incision in these cells in response to DNA interstrand cross-linking agents; (iii) the reduced levels of αSpIΠ* in FA-A cells are restored to normal when these cells are transduced with a retroviral vector expressing the FANCA cDNA, indicating a role for the FANCA gene in the stability or expression of αSpIΠ*. This correlates in turn with restoration of normal levels of UDS and endonucleolytic incision in the transduced cells in response to cross-linking agents. αSpIΠ* has been shown to be associated with the nuclear matrix (72); a number of studies suggest that the nuclear matrix is important in DNA repair (73–75). It is possible that αSpIΠ* acts as a scaffold or meshwork to align the proteins involved in repair of DNA interstrand cross-links so as to enhance their interactions and the efficiency of repair. Such an alignment could be of particular importance if a recombination step is involved. Reduced levels of αSpIΠ* in the nucleus would thus be expected to reduce the efficiency of the repair process, rather than inhibit it altogether (71), consistent with our findings of reduced levels of UDS in FA-A cells (17,18,34) and reduced levels of incisions produced at sites of cross-links by FA-A extracts.

The present study clearly demonstrates that in FA-A cells there is a defect in the incision step of the pathway we have described for processing of TMP interstrand cross-links. Specifically, there is a deficiency in the production of dual incisions at the site of this lesion. The importance of the FANCA gene in this repair process is demonstrated by our finding that expression of the FANCA cDNA in FA-A cells corrects this deficiency. The role of the FANCA gene in repair of DNA interstrand cross-links may be via its effect on the level of αSpIΠ* in the nucleus, which is reduced in FA-A cells, where there is a defect in the FANCA gene (71). The precise relationship between the decreased incisions we observe at sites of DNA interstrand cross-links in FA-A cells, the decreased levels of αSpIΠ* in these cells and the FANCA gene is currently under investigation.

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