Deficiency in Incisions Produced by XPF at the Site of a DNA Interstrand Cross-Link in Fanconi Anemia Cells†

Kandallu R. Kumaresan,‡ Deepa M. Sridharan,‡,§ Laura W. McMahon,‡ and Muriel W. Lambert*,‡,§

Department of Pathology and Laboratory Medicine, UMDNJ—New Jersey Medical School, and the Graduate School of Biomedical Sciences, Newark, New Jersey 07103

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ABSTRACT: Repair of DNA interstrand cross-links is a multistep process, critical to which is production of incisions at the site of the lesion resulting in the unhooking of the cross-link from DNA. We have previously shown that XPF is involved in production of incisions at the site of a psoralen interstrand cross-link and that in Fanconi anemia, complementation group A (FA-A) cells, there is a deficiency in these incisions. We now demonstrate that in FA complementation group B, C, D2, F, and G cells there is also a deficiency in production of these incisions. Involvement of FA proteins in this process is demonstrated by the ability of FA cells, corrected with the appropriate *FANC* cDNAs, to produce these incisions and by inhibition of these incisions by antibodies against these proteins. This incision deficiency correlates with reduced levels of DNA repair synthesis in these cells and is not due to reduced levels of XPF. FA proteins could be influencing this incision process by interacting either with proteins involved in the unhooking step or with damaged DNA, acting as a damage sensor. The results also demonstrate that FA cells are undergoing apoptosis by 12 h after interstrand cross-link damage. It is thus proposed that the single-strand breaks known to be created in DNA during apoptosis could mask the deficiency in ability of FA cells to incise cross-linked DNA and could account for the reported discrepancy as to whether FA cells are deficient in the incision step of the repair process.

Repair of DNA interstrand cross-links (ICL[1]) is a complex process and one that may involve the coordinated effort of more than one pathway (*1*, *2*). In *Escherichia coli* and *Saccharomyces cerevisiae* there is evidence that elements of both the nucleotide excision repair (NER) and homologous recombination (HR) pathways are involved (*1*−*5*). The details of ICL repair in mammalian cells are still unclear; however, evidence points to an involvement of NER, HR, and translesion synthesis (TLS) in this repair process (*1*, *2*, *6*−*9*). An excellent model for studying repair of ICLs is the genetic disorder, Fanconi anemia (FA), which is characterized by congenital abnormalities, progressive bone marrow failure, increased cancer susceptibility, and a hypersensitivity to DNA interstrand cross-linking agents (*10*−*13*). This hypersensitivity correlates with a defect in ability to repair cross-links produced by these agents (*13*−*18*). The FA proteins are thought to play an important role in the cellular response to DNA interstrand cross-linking agents; however, the precise nature of their involvement in the repair process is unclear. Studies in our laboratory indicate that at least one of the FA proteins, FANCA, is involved in the initial damage recognition and incision steps of the repair process (*19*, *20*). Recent studies also suggest that FANCD2, FANCJ, and FANCN may, along with FANCD1 (BRCA2), play a role in HR, and FANCM has been implicated in damage sensing (*11*−*13*, *21*−*26*). These studies thus suggest that FA proteins may play a role in several steps in ICL repair.

The focus of the present paper is on the involvement of FA proteins in the initial damage recognition/incision steps in the ICL repair process. We have previously shown, using a substrate containing a site-directed psoralen interstrand cross-link, that XPF, present in chromatin-associated proteins from normal human cells, produces incisions on the 3′- and 5′-sides of a psoralen plus UVA light-induced ICL (*20*, *27*). However, in FA complementation group A (FA-A) cells, there is a deficiency in ability of XPF to produce these incisions, even though the levels of XPF are normal in these cells (*20*, *28*). This incision deficiency in FA-A cells correlates with reduced levels of unscheduled DNA synthesis (UDS) that occur in response to cross-link damage (*16*, *17*, *28*). Both the incision deficiency and the reduced levels of UDS are corrected in FA-A cells expressing the *FANCA* cDNA indicating that FANCA is needed in some manner in the production of these incisions and in the repair of DNA interstrand cross-links (*20*, *28*). There has, however, been some controversy in the literature regarding whether there is an incision defect in FA cells. There are reports that little or no defect has been observed in production of incisions in DNA in FA cells after they are damaged with a cross-linking agent (*29*−*32*). These studies have utilized either alkaline

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* Corresponding author. Phone: (973) 972-4405. Fax: (973) 972-7293. E-mail: mlambert@umdnj.edu.

‡ UMDNJ—New Jersey Medical School.

§ Graduate School of Biomedical Sciences.

[1] Abbreviations: ICL, interstrand cross-link; NER, nucleotide excision repair; HR, homologous recombination; TLS, translesion synthesis; FA, Fanconi anemia; TMP, 4,5′,8-trimethylpsoralen; 8-MOP, 8-methoxypsoralen; αSpII, nonerythroid α spectrin; UDS, unscheduled DNA synthesis.

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elution, alkaline centrifugation, or alkaline comet assays in their protocols. An important point that needs to be considered, however, when carrying out these types of assays is that FA cells undergo apoptosis after exposure to a DNA damaging agent (*33*−*35*). Apoptosis, in turn, leads to production of single- and double-strand breaks in DNA (*36*−*40*). Thus in these studies, if the methods used to damage FA cells produced apoptosis, then under the alkaline conditions employed in the assay procedures, strand breaks created by apoptotic events would be detected and would mask any defect in ability of FA cells to produce incisions during the repair process. This in turn could lead to the incorrect interpretation that FA cells can create incisions in DNA at sites of damage.

In the present study, a defined in vitro system has been utilized to examine the ability of chromatin-associated proteins from cells from an additional number of FA complementation groups (FA-B, FA-C, FA-D2, FA-F, FA-G) to incise DNA containing a site-specific DNA interstrand cross-link. The results show that cells from all of these FA complementation groups are deficient in ability to produce incisions in DNA at the site of a cross-link and that this incision deficiency is corrected when cells express the appropriate *FANC* cDNA. Antibodies against a number of the FA proteins inhibited production of these incisions. This incision deficiency correlated with reduced DNA repair in these cells. In addition, analysis of apoptosis in FA cells exposed to dosages of an interstrand cross-linking agent similar to some of those reported in the literature indicate that FA cells undergo apoptosis. This could account for reports of a lack of deficiency in production of incisions in FA cells after damage. The present results thus demonstrate that there is a defect in the unhooking step in the repair of cross-links in not only FA-A cells but also in FA-B, FA-C, FA-D2, FA-F, and FA-G cells and provide further evidence that there are deficiencies in several different and important steps in the cross-link repair process in FA.

EXPERIMENTAL PROCEDURES

Extraction of Chromatin-Associated Proteins. Two normal human lymphoblastoid cell lines (GM 1989 and GM 3299) and an FA-F lymphoblastoid cell line (GM 16757) were obtained from the Coriell Institute for Medical Research (Camden, NJ). FA-B (HSC 230) and FA-C (HSC 536) lymphoblastoid cell lines were a gift from Dr. Manuel Buchwald (Hospital for Sick Children, Toronto, Canada). An FA-G lymphoblastoid cell line (EUFA 143-L) was a gift from Dr. Hans Joenje (Free University, Amsterdam, The Netherlands). An FA-D2 (PD 20) lymphoblastoid cell line was a gift from Dr. Alan D'Andrea (Harvard Medical School, Boston, Massachusetts). FA-C lymphoblastoid cells stably expressing the *FANCC* cDNA were a gift from Dr. Christopher Walsh. FA-G lymphoblastoid cells (EUFA 143-L) stably expressing the FANCG cDNA were obtained by transfecting these cells with plasmid pFAG9, which was inserted with a cDNA encoding functional FANCG (a gift from Dr. Hans Joenje), using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's directions and selection with gentomycin. Cells were grown in culture as previously described (*16*, *41*). Cell nuclei were isolated, and the chromatin-associated proteins were extracted in a series of steps as previously described (*16*, *19*, *41*).

Construction of a DNA Substrate Containing a Site-Specific TMP Interstrand Cross-Link. A 140 bp DNA substrate was constructed, as previously described, in which the top strand was internally labeled with γ-^{32}P at the 5′-end (*20*, *27*, *42*). This substrate corresponded to the region from 61 to 200 of the nucleotide sequence of the 5 S rRNA gene from *Lytechinus variegates* (*20*, *27*, *42*). The substrate was engineered to have a single site-specific 4,5′,8-trimethylpsoralen (TMP) interstrand cross-link, in which the furan ring of TMP was adducted to a centrally positioned 5′-TpA-3′ dinucleotide sequence in the top strand and the pyrone ring of TMP was adducted to a thymine on the bottom strand (*20*, *27*, *42*). 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 (*20*, *27*, *42*).

Determination of Sites of Endonucleolytic Incision. DNA substrate (100 fmol), either TMP cross-linked or unmodified, was reacted with the chromatin-associated proteins (10−20 μg) from normal or FA cells as previously described (*20*, *27*, *42*). The reactions were terminated by addition of 15 mM EDTA, and the samples were deproteinized using Nensorb 20 nucleic acid purification cartridges (NEN Research Products, Dupont) (*20*, *27*, *42*). 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 (*20*, *27*, *42*). Samples were prepared for sequence analysis as previously described (*20*, *27*, *42*), and sites of incision 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 (*20*, *27*, *42*). 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 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 since we have previously shown that incisions on the strand containing the pyrone ring of TMP are very weak (*42*).

The ability of antibodies against several of the FA proteins to inhibit the incisions produced by the normal proteins was examined. Polyclonal rabbit antibodies against FANCA, FANCC, and FANCG were prepared by Bethyl Laboratories as previously described (*43*). Normal human chromatin-associated proteins (10 μg) were mixed with 1−600 ng of anti-FANCA, anti-FANCC, or anti-FANCG antibody or antirabbit IgG (10−600 ng) (Sigma-Aldrich 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 three to four times.

Immunoblotting. Chromatin-associated proteins from the nuclei of normal and FA-A cells were separated by SDS−

PAGE on a 5−15% gradient gel as previously described (*28*, *41*). After electrophoresis, these proteins were electroporetically transferred onto nitrocellulose membranes (BioRad), which were probed with anti-XPF (an affinity-purified polyclonal antibody against XPA protein, a generous gift from Michael Thelen, Lawrence Livermore National Laboratory). Immunoblots were developed using Pierce Ultra chemiluminescent substrate (Pierce Biotechnology Inc.) and then exposed to X-ray film as previously published (*41*, *43*). Images were scanned using a Hewlett-Packard ScanJet 4c/T scanner.

Measurement of Unscheduled DNA Synthesis. Normal, FA-A, FA-C, FA-D2, FA-F, and FA-G cells in culture were treated with 8-methoxypsoralen (8-MOP) (250 ng/mL) for 10 min in the dark and then exposed to UVA light (1 mW/cm^2) for 10 min. The cells were washed with fresh media to remove the excess 8-MOP and then irradiated with a second dose of UVA light (1 mW/cm^2) for 10 min. Cells (1×10^6) were processed for UDS 15 h after damage, as previously described (*16*, *17*, *24*). Briefly, cells were resuspended in media containing [^{3}H]methylthymidine (10 μCi/mL) (ICN Biologicals), incubated for 2 h at 37 °C, washed and plated onto poly-L-lysine-coated glass chamber slides (Nalge Nunc International). The slides were fixed in Carnoy's fixative, air-dried, and dipped in Kodak NTB3 nuclear emulsion and exposed for 7 days at 4 °C. The slides were then developed with D19 developer (Kodak) and Rapid Fixer (Kodak) and stained with Fuelgen stain (Sigma-Aldrich Co.). Cells with 40−150 grains per nucleus were classified as undergoing UDS. For each experiment, 200−400 cells were counted.

Analysis of Apoptosis in Normal and FA Cells After DNA Damage. Normal human (GM3299) and an FA-A (HSC-72) lymphoblastoid cell line were treated with two different dosages of 8-MOP plus UVA light. In method 1, the cells were treated with 8-MOP (250 ng/mL) for 10 min in the dark and then exposed to UVA light (1 mW/cm^2) for 10 min. The cells were washed with PBS to remove the excess 8-MOP and then irradiated with a second 10 min dose of UVA light (1 mW/cm^2). In method 2, the cells were treated with 8-MOP (1 ng/mL) for 10 min in the dark followed by exposure to UVA (10 mW/cm^2) for 20 min. The cells are then washed with PBS to remove the excess 8-MOP and irradiated with another 30 min dose of UVA light (10 mW/cm^2). After both of these methods of treatment, the cells were incubated in a CO_2 incubator at 37 °C for 12 h and then analyzed for apoptosis using the Poly-Caspases FLICA apoptosis detection kit (Immunocytochemistry Technologies) according to the manufacturer's directions. Cells were subjected to FACS analysis using an FACSCalibur (BD Biosciences) with CellQuest Pro 5.2 software at the UMDNJ—New Jersey Medical School Core Flow Cytometry Laboratory and the caspase-positive cells determined.

RESULTS

As we have previously shown (*20*, *42*), chromatin-associated proteins from normal human cells produced incisions on the 3′- and 5′-side of a TMP interstrand cross-link (Figure 1A, lane 1). These incisions, which we have shown are produced by XPF (*20*, *27*), occurred at the fourth phosphodiester bond 3′ to the adducted thymine and at the sixth phosphodiester bond 5′ to this modified base, with a distance of 9 nucleotides between sites of incision (Figure 1C). Bands representing the 3′- and 5′-incisions comprised 4.0% and 2.9% of the substrate, respectively (Table 1). These incisions were not seen on undamaged DNA (Figure 1A, lane 4). Examination of the ability of chromatin-associated proteins from FA-B cells to incise the cross-linked DNA showed that they were defective in ability to create an endonucleolytic incision on both the 3′- and 5′-side of this lesion (Figure 1A, lane 2). Incisions produced on the 3′- and 5′-sides of the cross-link were only 33% and 10% of those produced by the normal proteins (Table 1). When the amount of protein from FA-B cells incubated with the substrate was doubled to twice that of the normal protein, the incisions increased but were still less than those produced by the normal proteins; the 3′- and 5′-incisions were 60% and 21%, respectively, of normal (Figure 1A, lane 3) (Table 1). The sites of incision produced by the FA-B proteins were the same as those produced by the normal proteins. Other minor bands observed on the cross-linked DNA following treatment with the normal and FA-B proteins were also seen on undamaged DNA and represent incisions by nonspecific nucleases.

Chromatin-associated proteins from FA-C cells were similarly defective in production of dual incisions by XPF at the site of a TMP interstrand cross-link (Figure 1B, lane 2). Incisions produced on the 3′- and 5′-side of this lesion were only 26% and 10%, respectively, of those produced by the proteins from the normal cells (Figure 1B, lane 1) (Table 1). Doubling the amount of FA-C cell protein added to the reaction increased the levels of incisions only slightly so that the 3′- and 5′-incisions were 34% and 14%, respectively, of normal levels (Figure 1B, lane 3) (Table 1). In order to determine whether a functional *FANCC* gene is needed for production of these incisions, studies were carried out using FA-C cells expressing the *FANCC* cDNA. Chromatin-associated proteins from the corrected FA-C cells were able to incise the cross-linked DNA. When the amount of protein used was equal to that of the normal proteins, incisions on both the 3′- and 5′-side of the cross-link were 58% and 31%, respectively, of those of normal (Figure 1B, lane 4) (Table 1). When the amount of protein was doubled, these incisions were 100% of those of normal (Figure 1B, lane 5) (Table 1). Proteins from corrected FA-C cells did not produce incisions on undamaged DNA (Figure 1B, lane 6). These results indicate that FANCC is needed for the production of these incisions.

FA-F and FA-G cells also had a deficiency in ability to produce dual incisions at the site of a TMP cross-link. Incisions produced by the proteins from FA-F cells on the 3′- and 5′-side of the cross-link were 28% and 33%, respectively, and for the FA-G proteins they were 22% and 8%, respectively, of normal levels (Figure 2A, lane 3 and Figure 2B, lane 3) (Table 1). Increasing the amount of FA-F and FA-G cell proteins used in the reactions to twice that of normal raised the levels of incisions but did not increase them to normal levels. The levels of the 3′- and 5′-incisions were 38% and 44%, respectively, for FA-F and 30% and 12%, respectively, for FA-G (Table 1). However, when FA-G cells were transduced with a cDNA expressing the *FANCG* cDNA, chromatin-associated proteins from these cells were able to incise the cross-linked DNA indicating that the *FANCG* gene is needed for production of these incisions.

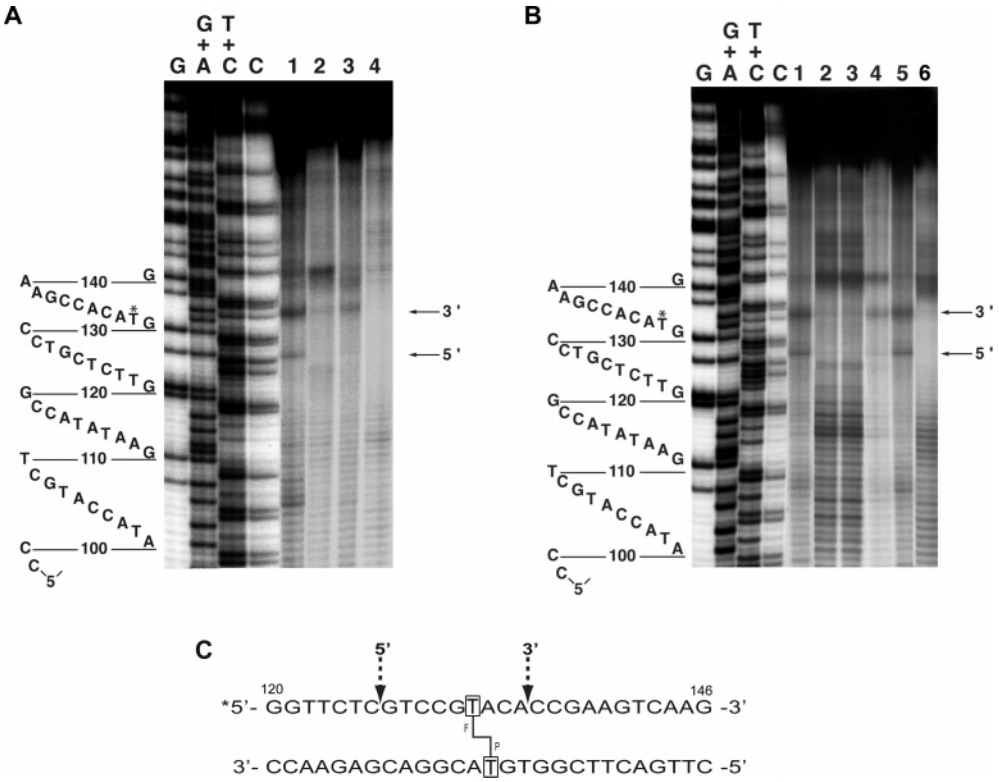

FIGURE 1: Defect in FA-B and FA-C cells in production of dual endonucleolytic incisions in DNA at the site of a TMP interstrand cross-link. (A) A 140 bp DNA substrate (~100 fmol), which corresponds to the region from 61 to 200 of the nucleotide sequence of the 5 S rRNA gene from *L. variegates*, was constructed and used. The furan side of a TMP interstrand cross-link was adducted to the ^{32}P-5′-end-labeled top strand, which was reacted with 10 μg of normal chromatin-associated proteins (lane 1), 10 μg of FA-B (lane 2), or 20 μg of FA-B (lane 3) chromatin-associated proteins. Undamaged substrate was incubated with 10 μg of normal proteins (lane 4). (B) The 140 bp DNA substrate (~100 fmol) was reacted with 10 μg of normal chromatin-associated proteins (lane 1), 10 μg (lane 2) or 20 μg (lane 3) of FA-C chromatin-associated proteins, and 10 μg (lane 4) or 20 μg (lane 5) of chromatin-associated proteins from corrected FA-C cells. Undamaged substrate was incubated with 10 μg of corrected FA-C cell proteins (lane 6). Aliquots of 2000 cpm 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′-incisions are indicated by arrows. Each of these experiments was repeated three to four times for each cell line. (C) A segment of the substrate showing sites of defective 3′- and 5′-incision (dotted arrows) by the FA chromatin-associated proteins. Adducted T residues are boxed in. The angled line extending from the T residues indicates linkage with the furan (F) or pyrone (P) ring of TMP. Numbers on the segment refer to the corresponding numbers in the nucleotide sequence from *L. variegates*. The asterisk indicates the ^{32}P-5′-labeled strand.

Incisions on the 3′- and 5′-side of the cross-link were 84% and 104% of normal levels (Figure 2B, lane 4) (Table 1), and when the amount of protein used was twice the normal amount, these incisions increased to 97% and 154% of normal levels (Figure 2B, lane 5) (Table 1). These results indicate that correcting the deficiency in levels of FANCG in FA-G cells restores the ability of XPF to incise the cross-linked DNA substrate.

FA-D2 cells had a deficiency in the production of the 3′- and 5′-incisions at the site of a TMP cross-link; however, the defect was not as great as it was for cells from the other FA complementation groups, particularly as regarded the 3′-incision. There was a greater defect in the production of the 5′-incision than the 3′-incision, with levels of incisions 24% and 70%, respectively, of normal (Figure 3, lanes 1 and 3) (Table 1). The level of the 3′-incision was approximately 2−2.5 greater than the 3′-incision produced by FA-B, FA-C, FA-F, or FA-G cells, which again indicates that in FA-D2 cells there is less of an incision deficiency. Chromatin-associated proteins from FA-D2 cells did not produce any incisions on undamaged DNA (Figure 3, lane 4).

To confirm the involvement of FA proteins in the production of the dual incisions at the site of a cross-link, studies were carried out to determine whether antibodies against these proteins could inhibit these incisions. The results show that, in a concentration-dependent manner, anti-FANCA (Figure 4A, lanes 3 and 4), anti-FANCC (Figure 4B, lanes 3 and 4), and anti-FANCG (Figure 4C, lanes 3−5) inhibited the 3′- and 5′-incisions at the site of a TMP cross-link. At the highest concentration of antibody used, the levels of the 3′- and 5′-incisions were decreased to 16% and 13%, respectively, for anti-FANCA, 27% and 10%, respectively, for anti-FANCC, and 10% and 0%, respectively, for anti-FANCG compared to those produced by the normal proteins in the absence of these antibodies (Figure 4A, lane 1, Figure 4B, lane 1, Figure 4C, lane 1). The control antibody, antirabbit IgG, had no effect on these incisions (Figure 4A, lane 2, Figure 4B, lane 2, Figure 4C, lane 2).

A study was also carried out to determine whether the decreased levels of incisions produced by XPF present in the chromatin-associated proteins from FA cells were due to decreased levels of XPF in these cells. Western blot analysis of chromatin-associated proteins from normal, FA-C, and FA-G cells showed that the level of XPF in these FA cells lines (Figure 5, lanes 2 and 3) was similar to that present in normal cells (Figure 5, lane 1). We have previously shown that the levels of XPF in FA-A cells are also similar to normal (*28*).

Table 1. Quantitation of Endonucleolytic Incisions Produced by the FA and Normal Cells at Sites of a TMP Interstrand Cross-Link

		incisions (% of substrate)[a]		incisions (% of normal)[b]	
		3′	5′	3′	5′
normal	10 μg	4.0 ± 0.3	2.9 ± 0.2		
FA-B	10 μg	1.3 ± 0.1	0.3 ± 0.1	33	10
	20 μg	2.4 ± 0.2	0.6 ± 0.1	60	21
normal	10 μg	3.8 ± 0.2	2.9 ± 0.1		
FA-C	10 μg	1.0 ± 0.1	0.3 ± 0.1	26	10
	20 μg	1.3 ± 0.1	0.4 ± 0.1	34	14
corrected	10 μg	2.2 ± 0.2	0.9 ± 0.1	58	31
FA-C	20 μg	3.8 ± 0.3	2.9 ± 0.2	100	100
normal	10 μg	4.1 ± 0.3	3.3 ± 0.2		
FA-D2	1 μg	0.8 ± 0.2	0.2 ± 0.1	20	6
	10 μg	2.9 ± 0.3	0.8 ± 0.2	71	24
normal	10 μg	4.0 ± 0.2	2.7 ± 0.2		
FA-F	10 μg	1.1 ± 0.1	0.9 ± 0.1	28	33
	20 μg	1.5 ± 0.2	1.2 ± 0.2	38	44
normal	10 μg	3.7 ± 0.3	2.4 ± 0.2		
FA-G	10 μg	0.8 ± 0.2	0.2 ± 0.1	22	8
	20 μg	1.1 ± 0.2	0.3 ± 0.1	30	12
corrected	10 μg	3.1 ± 0.2	2.5 ± 0.2	84	104
FA-G	20 μg	3.6 ± 0.3	3.7 ± 0.4	97	154

[a] Chromatin-associated proteins from normal and FA cells were incubated with DNA containing a TMP interstrand cross-link. Incisions are expressed as the percentage of substrate converted to incision products. Means (±) SEM were obtained for three to four experiments. [b] This percentage is based on a comparison with the number of incisions produced by the normal chromatin-associated proteins.

An analysis of levels of repair of DNA interstrand cross-links in normal and FA cells, in vivo, was carried out by measuring UDS in these cells after exposure to 8-MOP plus UVA light. UDS was measured at a time point 15 hours after damage since we have previously shown that colocalization of αIISp, FANCA, and XPF into damage-induced nuclear foci peaks during this period of time (*44*). At this time point, FA-A, FA-C, FA-D2, FA-F, and FA-G cells showed reduced levels of UDS compared to normal cells. Levels were between 34% and 43% of normal depending upon the complementation group with slightly lower levels of UDS observed in FA-A and FA-G cells compared to the other complementation groups (Figure 6).

Studies were carried out to determine whether, under two different sets of conditions for DNA damage that have been used in repair assays described in the literature, early apoptotic events could be detected in FA cells. The assay used to examine apoptosis measures active caspases in a cell, which are detected using a fluorescent-labeled caspase inhibitor. This inhibitor binds to the active caspase heterodimer, and binding can be measured using flow cytometry. The data obtained from this assay was expressed as the percentage of cells that were caspase positive. FA-A cells were utilized for these studies, and the results were compared with those obtained from normal cells. At 12 h after damage with 8-MOP plus UVA light, using method 1, the percentage of caspase-positive normal cells was 22% and the percentage of caspase-positive FA-A cells was 51% (Figure 7A). At this same time point, using method 2, the percentage of positive normal cells was 37% and the percentage of positive FA-A cells was 71% (Figure 7A). Thus, the higher dosage of UVA light that was used in method 2 led to a higher level of apoptosis compared to method 1, where the UVA dosage was lower. With both methods, levels of apoptosis in FA-A cells were approximately double those in normal cells. At either concentration used, 8-MOP by itself produced little apoptosis in normal or FA-A cells (Figure 7A). Either dose of UVA light by itself produced only a slight increase in apoptosis in normal cells, but in FA-A cells it led to a slightly greater increase in apoptosis, particularly at the higher UVA dosage (Figure 7A). A histogram of the results of a representative set of experiments is shown in Figure 7B. Caspase-negative and -positive cells are seen as two distinct peaks with the left peak showing caspase-negative cells and the right peak showing the caspase-positive cells. As seen in Figure 7B, parts a and b, respectively, undamaged normal and FA-A cells show only one peak of caspase-negative cells. With the use of method 1, 12 h after damage with 8-MOP plus UVA light a small proportion of the normal cells show a small second peak of caspase-positive cells (Figure 7B, part c) and this proportion increases in FA-A cells (Figure 7B, part d). With the use of method 2, a larger proportion of the normal cells are caspase-positive than when method 1 was used (Figure 7B, part e) and the majority of the FA-A cells are caspase positive (Figure 7B, part f). These results thus demonstrate that 12 h after damage with 8-MOP plus UVA light, and particularly at higher dosages of UVA light, FA-A cells have undergone apoptosis.

DISCUSSION

The present results, combined with our previous studies (*20*), demonstrate that cells from a number of FA complementation groups (FA-A, FA-B, FA-C, FA-D2, FA-F, and FA-G) are deficient in ability to produce dual incisions at the site of a DNA interstrand cross-link. Recognition of the sites of damage and production of incisions that result in unhooking of the cross-link are one of the initial events in the repair process (*1*, *2*). We have previously shown that in normal human cells XPF is involved in the generation of these incisions: antibodies specific for XPF inhibit incisions on the 3′- and 5′-side of a psoralen cross-link, and in XPF cells, which are deficient in XPF, there is also a deficiency in ability to produce these incisions (*20*, *27*). The decreased ability of FA cells to incise DNA at the sites of an interstrand cross-link is not due, however, to reduced levels of XPF since we have shown both previously (*28*) as well as in the present study that XPF levels are normal in these cells. This would indicate, therefore, that FA core complex proteins (FANCA, FANCB, FANCC, FANCF, and FANCG) as well as FANCD2 are, in some manner, involved in this initial damage recognition/incision process. In support of this are the present findings which show that in corrected FA cells, expressing the appropriate *FANC* cDNA, levels of incisions are similar to normal and that antibodies against these individual FA proteins can inhibit production of these incisions.

Repair of DNA interstrand cross-links in mammalian cells is a complex, multistep process. In the initial events, there is evidence that the endonucleolytic activity of XPF is modulated by ERCC1, which forms a heterodimeric complex with XPF (*6*, *45*−*47*). We have shown that the ability of XPF to incise DNA at the site of a DNA interstrand cross-link is inhibited by an mAb that specifically recognizes and binds to the C-terminal domain of XPF, which is the domain that interacts with ERCC1 (*45*−*47*). Thus, ERCC1 may play an important role, along with XPF, in the incision process.

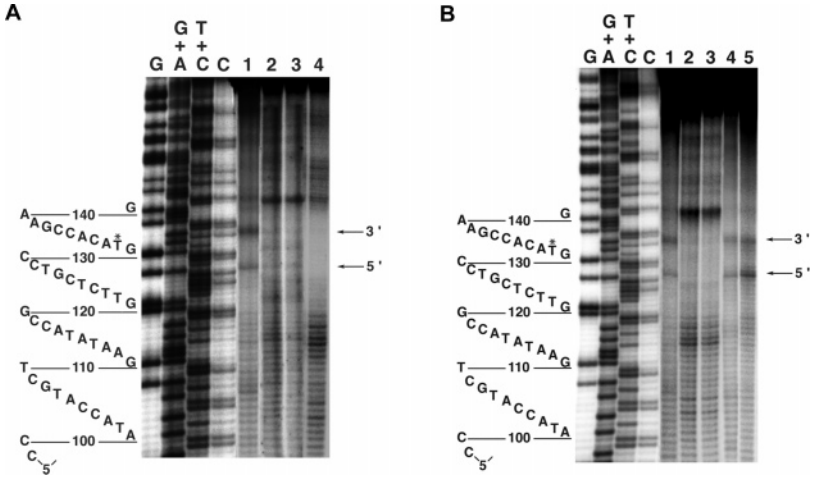

FIGURE 2: Defect in FA-F and FA-G cells in production of dual endonucleolytic incisions in DNA at the site of a TMP interstrand cross-link. (A) A 140 bp DNA substrate containing a TMP interstrand cross-link, as in Figure 1, was reacted with 10 μg of normal chromatin-associated proteins (lane 1), 20 μg of FA-F (lane 2), or 10 μg of FA-F (lane 3) chromatin-associated proteins. Undamaged substrate was incubated with 10 μg of FA-F chromatin-associated proteins (lane 4). (B) The 140 bp DNA substrate was reacted with 10 μg of normal chromatin-associated proteins (lane 1), 20 μg of FA-G (lane 2), or 10 μg of FA-G (lane 3) chromatin-associated proteins, and 10 μg (lane 4) or 20 μg (lane 5) of chromatin-associated proteins from corrected FA-G cells. Aliquots of 2000 cpm of each sample were loaded per gel lane. Notations are as in Figure 1. Each of these experiments was repeated three to four times for each cell line.

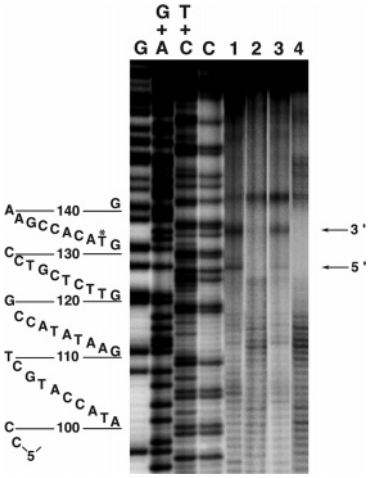

FIGURE 3: Defect in FA-D2 cells in production of dual endonucleolytic incisions in DNA at the site of a TMP interstrand cross-link. A 140 bp DNA substrate containing a TMP interstrand cross-link, as in Figure 1, was reacted with 10 μg of normal chromatin-associated proteins (lane 1), 1 μg of FA-D2 (lane 2), or 10 μg of FA-D2 (lane 3) chromatin-associated proteins. Undamaged substrate was incubated with 10 μg of FA-D2 chromatin-associated proteins (lane 4). Aliquots of 2000 cpm of each sample were loaded per gel lane. Notations are as in Figure 1. Each of these experiments was repeated three to four times for each cell line.

Repair of DNA interstrand cross-links has been shown to occur in the S phase of the cell cycle (*1*, *2*, *32*, *48*). It is also possible that in mammalian cells, just as in yeast, repair of cross-links occurs in the G1 phase of the cell cycle as well (*49*). Repair of cross-links is proposed to involve an NER step followed by HR and/or TLS past the incised intermediate (*1*, *2*, *6*−*9*, *48*, *49*). In FA cells, there is evidence that the FA pathway is activated in response to DNA replication fork arrest in S phase at the site of an interstrand cross-link (*8*, *10*−*13*). A number of models have been proposed as to how repair of cross-links proceeds at these stalled replication forks (*8*, *11*, *12*, *45*, *50*−*52*). In the majority of these models, unhooking of the cross-link on one strand of the DNA is one of the initial steps and is needed in order for repair to proceed. XPF is one of the proteins proposed to be involved in this process. This is followed by a series of steps involving HR and/or TLS, and a number of proteins including the FA proteins, FANCD2, FANCD1, FANCJ, and FANCN have been proposed to be involved (*11*, *12*, *48*, *50*−*52*). A second set of incisions, possibly involving XPF, then removes the unhooked ICL (*11*, *12*, *48*, *50*−*52*). The present results indicate that in FA cells there is a deficiency in unhooking of the ICL by XPF, and they suggest that in some manner FA core complex proteins as well as FANCD2 are involved in this process. This incision deficiency is shown, in the present study, to correlate with reduced levels of DNA repair in vivo in FA-A, FA-C, FA-D2, FA-F, and FA-G cells after cross-link damage.

We have previously demonstrated that the structural protein nonerythroid α spectrin (αIISp) is involved in the repair of DNA interstrand cross-links and plays an important role in the initial damage recognition/incision step of the repair process (*28*, *41*, *43*, *44*, *53*). Purified αIISp binds to DNA containing a psoralen interstrand cross-link (*43*), purified αIISp enhances the incisions produced by XPF at sites of a TMP interstrand cross-link (*43*), antibodies against αIISp inhibit the production of dual incisions by XPF at sites of a cross-link (*43*), αIISp coimmunoprecipitates with XPF (*43*, *53*), and after normal cells are damaged with 8-MOP plus UVA light, αIISp colocalizes with XPF and FANCA in damage-induced nuclear foci (*44*). We have proposed a model in which αIISp acts as a scaffold to aid in the recruitment of repair proteins to the site of a DNA interstrand cross-link, thus enhancing the efficiency of the repair process (*43*, *44*, *53*). In cells from a number of FA complementation groups, there are reduced levels of αIISp (*41*), which in turn correlate with the reduced levels of UDS observed in these cells after cross-link damage. The levels of αIISp mRNA in these FA cells, however, are similar to those in normal cells (*54*). We have, therefore, proposed that the reduced levels of αIISp in FA cells are due to reduced stability of this protein, rather than to reduced expression, and that this in turn is due to deficiencies in one or more of the FA proteins that are important in its stability (*54*). The FA proteins could

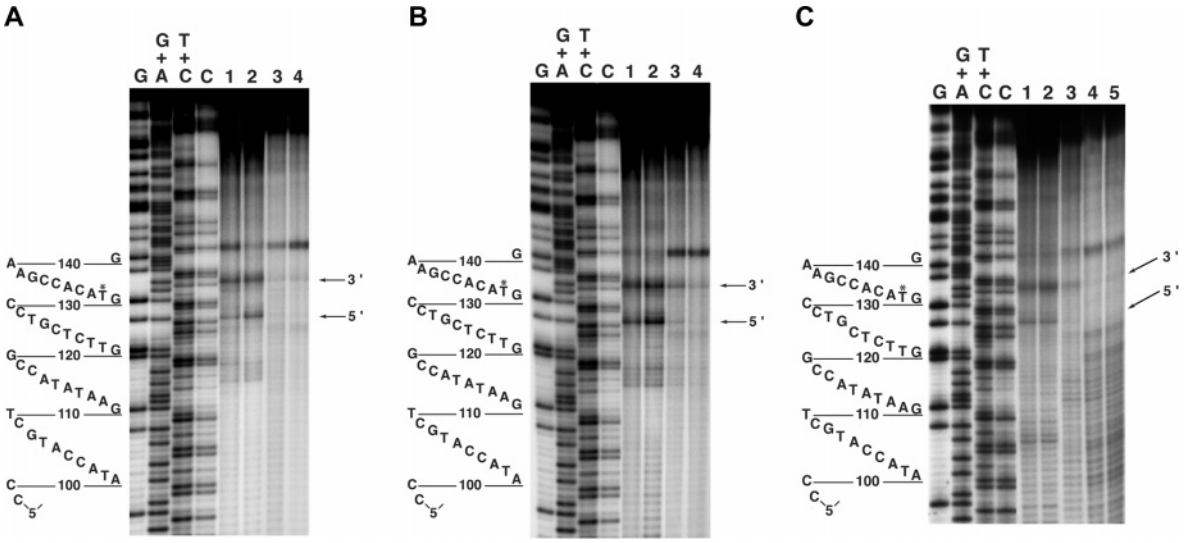

FIGURE 4: Inhibition of incisions in DNA at the site of a TMP interstrand cross-link by antibodies against FANCA, FANCC, and FANCG. (A) DNA containing a TMP interstrand cross-link, as in Figure 1, was reacted with 10 μg of normal chromatin-associated proteins (lane 1), and in addition 600 ng of IgG (lane 2), or 600 ng of anti-FANCA (lane 3), or 60 ng of anti-FANCA (lane 4). (B) DNA containing a TMP interstrand cross-link was reacted with 10 μg of normal chromatin-associated proteins (lane 1), and in addition 600 ng of IgG (lane 2), or 60 ng of anti-FANCC (lane 3), or 600 ng of anti-FANCC (lane 4). (C) DNA containing a TMP interstrand cross-link was reacted with 10 μg of normal chromatin-associated proteins (lane 1), and in addition 10 ng of IgG (lane 2), or 1 ng of anti-FANCG (lane 3), or 6 ng of anti-FANCG (lane 4), or 10 ng of anti-FANCG (lane 5). Notations are as in Figure 1. Each of these experiments was repeated three times for each antibody.

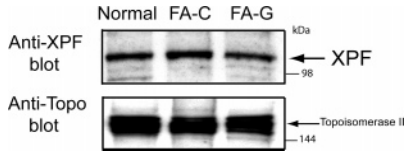

FIGURE 5: Western blot analysis of levels of XPF in FA-C and FA-G cells. Chromatin-associated proteins from FA-C and FA-G cells were separated by SDS−PAGE and analyzed by western blot analysis. The blot was probed with an antibody against XPF and reprobed with an mAb against topoisomerase II, which was used as a loading control.

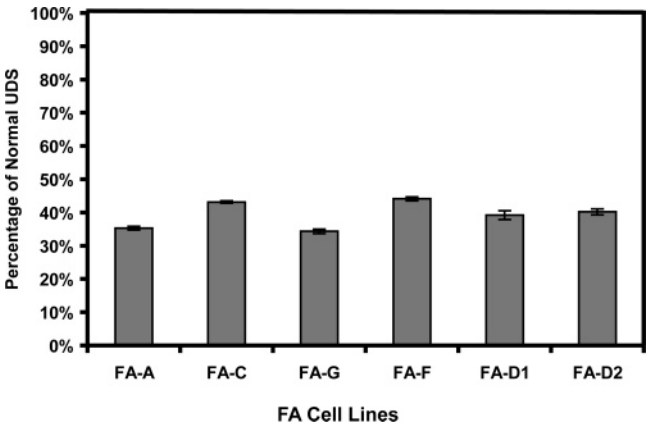

FIGURE 6: UDS in FA cells treated with 8-MOP plus UVA light. FA-A, FA-C, FA-G, FA-F, FA-D1, and FA-D2 were treated with 8-MOP (250 ng/mL) and two doses of UVA irradiation (1 mW/cm^2) for 10 min each. UDS was examined in these cells 15 h after damage. Results are expressed as percentage of normal UDS. Vertical lines represent ±SEM for three to four separate experiments.

thus be indirectly involved in the production of incisions at the site of a cross-link via their effect on the stabilization of αIISp, which is needed in the incision process, or via interaction with other proteins involved in the damage recognition/incision step. These could include XPF, ERCC1, or possibly the endonuclease Mus81-Eme1, which has been proposed to have a role in processing stalled DNA replication forks at the site of a cross-link (*9*, *55*). It is also possible that one or more of these FA proteins may interact directly with the damaged DNA itself, acting as a damage sensor or in influencing the incision process.

There has been disagreement as to whether FA cells are deficient in the initial incision step of the ICL repair process. A number of studies, including ours, have shown that there is a deficiency in ability of FA cells to produce incisions at sites of ICLs (*14*−*20*, *56*−*58*). Other studies using an in vitro cross-link repair synthesis assay have shown that extracts from FA cells display an intermediate to wild-type level of activity on a cross-linked substrate depending on the complementation group (*59*, *60*). There are also studies which indicate that there is no incision deficiency in FA cells (*29*−*32*). These latter studies have utilized methods that involve either alkaline elution, alkaline sucrose gradients, or alkaline comet assays. Alkaline conditions have been used in these assays so that the DNA strands can be separated and single-strand breaks in DNA can be detected. These single-strand breaks are attributed to incisions occurring in DNA during the repair process. With the use of these methodologies, the levels of DNA single- and double-strand breaks occurring in FA cells after cross-link damage have been shown to be similar to normal or slightly higher than normal (*29*−*32*). This has led to the conclusion that FA cells are not deficient in ability to incise cross-linked DNA (*29*−*32*). However, there is an important consideration that needs to be taken into account when carrying out these types of assays using FA cells. FA cells show increased apoptosis after DNA damage (*33*−*35*), and it is well documented that apoptosis can lead to the production of single-strand breaks in DNA, particularly in the early stages of apoptosis (*36*−*40*). Thus, in those studies in which alkaline conditions have been used in the measurement of single- and double-strand

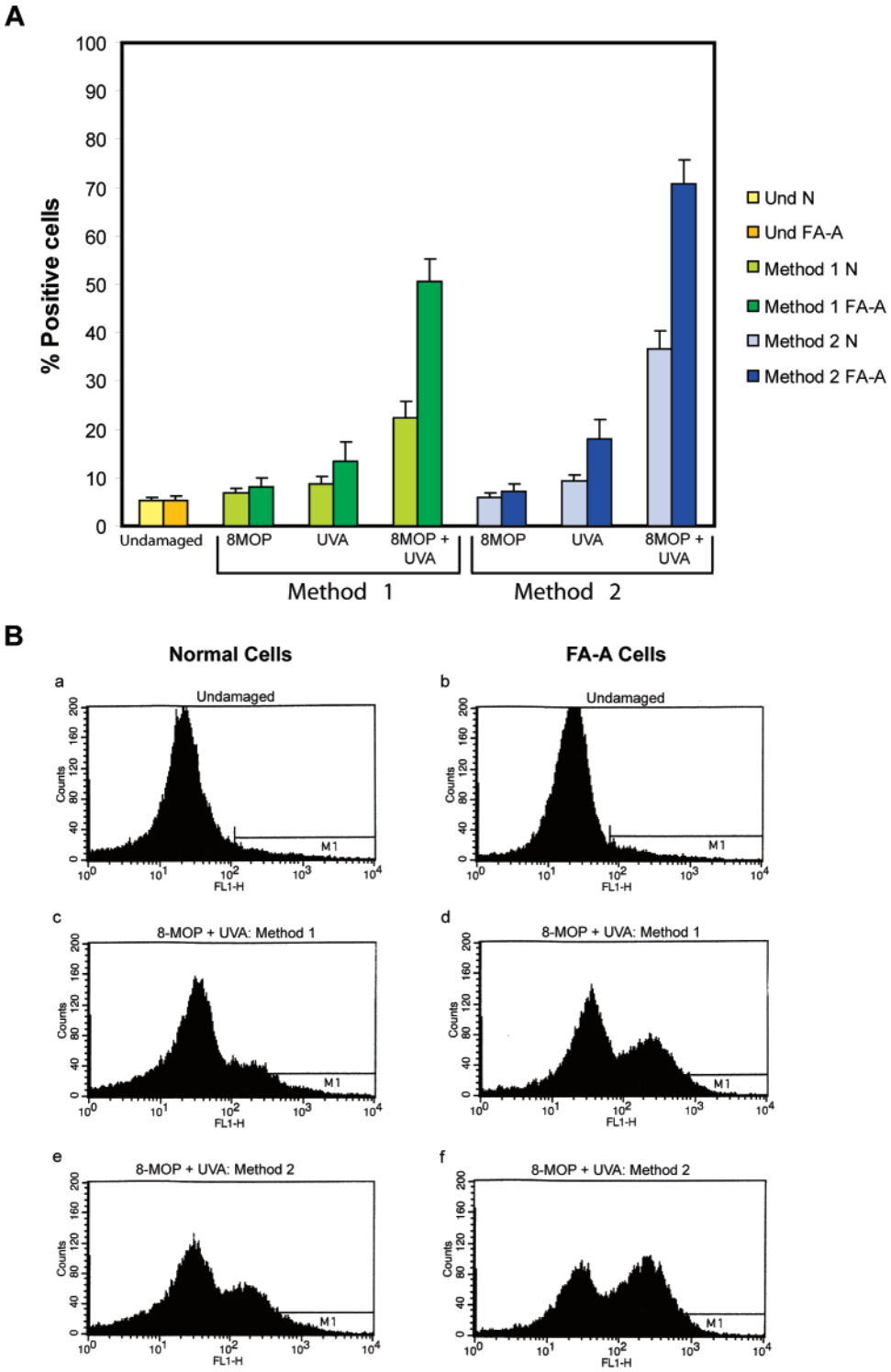

FIGURE 7: Analysis of apoptosis in normal and FA-A cells after treatment with two different dosages of 8-MOP plus UVA light. In method 1, cells were treated with 250 ng/mL 8-MOP plus two doses of UVA light (1 mW/cm^2) for 10 min each. In method 2, cells were treated with 1 ng/mL 8-MOP plus two doses of UVA light (10 mW/cm^2) for 20 min and 30 min each. (A) Apoptosis was measured using FACS analysis as percentage of caspase-positive cells 12 h after damage. Results are shown for both normal and FA-A cells. Vertical lines represent ±SEM for seven separate experiments. (B) A representative histogram of the FACS analysis of one set of experiments showing undamaged normal (a) and FA-A cells (b), normal (c) and FA-A (d) cells damaged using method 1, and normal (e) and FA-A (f) cells damaged using method 2.

breaks in DNA, a number of these breaks could be due to apoptosis occurring in the FA cells rather than to incisions produced during repair events. In the present study, assays were carried out to ascertain whether apoptosis could be detected in FA cells after cross-link damage when methods of damage were used that were similar to those described using these types of alkaline assays, in particular, during the period of time after damage that these other described repair assays have been carried out, many of which have been at 24 h after damage (*29*−*32*). The results showed that 12 h after damage with 8-MOP plus UVA light, using either of the two methods examined, there was a higher level of

apoptosis in FA-A cells compared to normal cells. The level of apoptosis in FA-A cells was approximately twice that of normal cells. In addition, the levels of UVA light used in conjunction with 8-MOP had an effect on apoptosis, with higher levels producing more apoptosis. Since single-strand breaks in DNA have been shown to occur during apoptosis (*36*−*40*), the present study suggests that a number of the strand breaks observed in FA cells, when assays employing alkaline conditions were used for analysis, could have been due to apoptosis and not to incisions occurring during the DNA repair process. Thus, the present results strengthen the viewpoint that FA cells are deficient in the ability to produce incisions at the site of a cross-link and thus in the unhooking step in the repair process. The precise role of FA core complex proteins as well as FANCD2 in these damage recognition/incision events and whether they interact with other repair proteins and/or damaged DNA is not clear. Further studies will be needed to determine this. However, these results emphasize that FA proteins play an important role in several different steps in the cross-link repair process, among which is the damage recognition/incision step.

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