Human α Spectrin II and the FANCA, FANCC, and FANCG Proteins Bind to DNA Containing Psoralen Interstrand Cross-Links†

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ABSTRACT: Repair of DNA interstrand cross-links is a complex process critical to which is the identification of sites of damage by specific proteins. We have recently identified the structural protein nonerythroid α spectrin (αSpIIΣ*) as a component of a nuclear protein complex in normal human cells which is involved in the repair of DNA interstrand cross-links and have shown that it forms a complex with the Fanconi anemia proteins FANCA, FANCC, and FANCG. Using DNA affinity chromatography, we now show that αSpIIΣ*, present in HeLa cell nuclei, specifically binds to DNA containing psoralen interstrand cross-links and that the FANCA, FANCC, and FANCG proteins are bound to this damaged DNA as well. That spectrin binds directly to the cross-linked DNA has been shown using purified bovine brain spectrin $(\alpha SpII\Sigma1/\beta SpII\Sigma1)_2$. Binding of the Fanconi anemia (FA) proteins to the damaged DNA may be either direct or indirect via their association with αSpIIΣ*. These results demonstrate a role for α spectrin in the nucleus as well as a new function for this protein in the cell, an involvement in DNA repair. αSpIIΣ* may bind to cross-linked DNA and act as a scaffold to help in the recruitment of repair proteins to the site of damage and aid in their alignment and interaction with each other, thus enhancing the efficiency of the repair process.

Repair of DNA interstrand cross-links is a multistep process. Though the molecular basis of this process has been elucidated in prokaryotes (reviewed in *1*), the precise nature of these repair events in mammalian cells is unclear. Critical to this repair pathway is the ability of repair proteins to recognize the lesion and interact with the DNA at the site of damage. Our laboratory has shown that there is a protein in normal human cells which recognizes and binds to DNA containing interstrand cross-links produced by psoralen plus UVA[1] light (*2*). We have also demonstrated that there is a deficiency in this protein in cells from patients with Fanconi anemia (FA)[1] (*2*), a genetic disorder characterized by bone marrow failure, diverse congenital abnormalities, an increased incidence of cancer, and a marked cellular hypersensitivity to and defect in ability to repair damage produced by DNA interstrand cross-linking agents (*3*−*12*). We have recently identified the structural protein nonerythroid α spectrin (αSpIIΣ*) as a component of a chromatin-associated protein complex in the nuclei of normal human cells that is involved in repair of DNA interstrand cross-links (*13, 14*). We have also shown that αSpIIΣ* forms a complex in the nucleus with the FANCA, FANCC (*13, 14*), and FANCG proteins (Lambert et al., in preparation). Our studies have demonstrated that there is a deficiency in levels of αSpIIΣ* in FA complementation group A, B, C, D, and G cells and that this deficiency is corrected in FA-A, FA-C, and FA-G cells expressing the *FANCA*, *FANCC*, and *FANCG* genes, respectively (*13, 14*; Lambert et al., in preparation). The precise role of αSpIIΣ* in the repair of DNA interstrand cross-links, however, is unclear.

Four of the genes from the seven FA complementation groups have been identified: *FANCA*, *FANCC*, *FANCF*, and *FANCG* (*15*−*19*). However, the function of the proteins encoded by these genes is unknown. The clinical symptoms and cellular features of FA patients from the various complementation groups are similar, suggesting that the FA proteins may function in an integrated pathway (*20*). Several of these proteins have been shown to form a complex in the nucleus, which again indicates a role in a common pathway (*20*−*23*). Our studies show that there is a correlation between the ability of cells to repair DNA interstrand cross-links, the expression of the *FANCA*, *FANCC*, and *FANCG* genes, and the levels of αSpIIΣ* in these cells (*9, 12*−*14*). The exact nature of this relationship is unclear, but this correlation indicates an involvement of αSpIIΣ* and these FA gene products in the repair process.

Though nonerythroid spectrin is present in the cytoplasm, where it is involved in a number of functions, no definitive role for it in the cell nucleus is known. The present study

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[1] Abbreviations: αSpIIΣ*, nonerythroid α spectrin; FA, Fanconi anemia; TMP, 4,5′,8-trimethylpsoralen; UVA, long-wavelength ultraviolet light; UDS, unscheduled DNA synthesis.

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investigated the ability of αSpIIΣ* to bind to DNA containing psoralen interstrand cross-links and whether the FANCA, FANCC, and FANCG proteins also associate with the cross-linked DNA. Analysis of these DNA binding activities has demonstrated a role for α spectrin in the nucleus and a new role for it in the cell, an involvement in DNA repair.

EXPERIMENTAL PROCEDURES

Chromatin-Associated Protein Extracts. HeLa S3 cells were obtained from The National Cell Culture Center, Minneapolis, MN. FA-A (HSC 72) and FA-C (HSC 536) lymphoblastoid cell lines were a gift from Dr. Manuel Buchwald (Hospital for Sick Children, Toronto, Canada). FA-G (EUFA143) cell lines were a gift from Dr. Hans Joenje (Free University, Amsterdam, The Netherlands). Cell nuclei were isolated, and the chromatin-associated proteins were extracted in a series of steps as previously described (*2*, *8*, *14*). Protein concentrations were determined using the Bradford reagent (Bio-Rad).

Isolation and Purification of Spectrin. Nonerythroid spectrin $(\alpha SpII\Sigma1/\beta SpII\Sigma1)_2$ was isolated from bovine brain and purified as described by Sikorski et al. (*24*). The spectrin (184 μg/mL) was in 10 mM $NaPO_4$, 15 mM sodium pyrophosphate, 1 M NaBr, 1 mM NaN_3, 0.4 mM DTT, 1 mM EGTA, pH 8.2. Purity of the spectrin was tested by SDS−PAGE on a 5% polyacrylamide gel (*25*) followed by silver staining, performed according to the manufacturer's protocol (Bio-Rad). Purified RBC spectrin was used as a marker.

DNA Substrate. A 105 bp DNA substrate was synthesized that was a modification of the region from positions 75 to 179 of the nucleotide sequence of the 5S rRNA gene from *Lytechinus variegatus*. This sequence was modified to contain a centrally located hotspot for formation of psoralen interstrand cross-links (TATAT). In addition, the sequence was modified, by changing T→ G or C and A → G or C, so that there were no AT nucleotides together, except for the hotspot, where cross-links could potentially form. The top strand, which was biotinylated on the 5′ end, and the bottom strand were synthesized by the HHMI Biopolymer Facility/W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. For construction of the duplex substrate, equimolar amounts of the top and bottom strands were mixed in TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA). Hybridization of the strands was carried out by incubating the mixture at 90 °C for 2 min and then slowly cooling it down to room temperature (25 °C). The sample was dried under vacuum (Speed Vac Concentrator, Savant) and then resuspended in sterile distilled water. For formation of psoralen interstrand cross-links, the DNA was damaged by adding 4,5′,8-trimethylpsoralen (TMP) to give a final concentration of 10 μg/mL TMP. TMP was incubated with the DNA for 10 min in the dark and then irradiated with long-wavelength ultraviolet (UVA) light from a super-high-intensity black light lamp (Spectroline model SB-100, Spectronics Corp.) for 20 min at 20 mW/cm^2 (at 4 °C). Unbound TMP was extracted with chloroform, and the DNA was precipitated with ethanol. The pellet containing the cross-linked DNA was dried under vacuum and resuspended in TE buffer. Undamaged DNA substrate was prepared in the same way except without the TMP treatment. Formation of DNA interstrand cross-links in TMP- plus UVA-treated DNA was determined as previously described by denaturing 6% polyacrylamide gel electrophoresis (*2*). Preparation of DNA containing a psoralen monoadduct was carried out as above except that the DNA was reacted with 25 μg/mL angelicin-2-oxo-[2*H*]-furo[2,3-*h*]-1-benzopyran (angelicin) (Sigma Chemical Co.) for 20 min in the dark and then exposed to UVA light (13 mW/cm^2) for 5 min as previously described (*26*).

Protein−DNA Binding Reactions. HeLa, FA-A, FA-C, or FA-G cell chromatin-associated protein extracts (25−35 μg) and a nonspecific competitor, salmon testes DNA (Sigma), were mixed in a 1:5 w/w ratio in DNA binding buffer [20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 120 mM NaCl, 0.3% Tween-20, 50 μg/mL YVAD (Ac-Tyr-Val-Ala-Asp-chloromethyl ketone) (Bachem Biochemicals), and protease inhibitor mixture (Roche Molecular Biochemicals)] and incubated on ice for 4 min. In separate reactions, purified bovine brain spectrin (7.5 μg) was mixed with the DNA binding buffer as above. For both sets of reactions, biotinylated undamaged or biotinylated TMP cross-linked DNA (7.1 μg) was then added, and the mixture was shaken slowly overnight at 4 °C. Streptavidin-coated acrylamide beads (Pierce) were blocked by shaking overnight in casein−Tris-buffered saline (Pierce), washed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10 mM $MgCl_2$, and resuspended in DNA binding buffer as a 1:1 slurry. Then 40 μL of this slurry was added per DNA binding reaction, and slow shaking was continued overnight at 4 °C. The beads/bound DNA and proteins were pelleted by centrifugation (2500*g* at 4 °C) and washed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 50 μg/mL YVAD, and protease inhibitor mixture. The pellet was resuspended in this buffer, and the bound proteins were examined by immunoblot analysis.

Competition Assays. Competition assays were carried out by adding 0, 7.1, or 21.3 μg of nonbiotinylated DNA, which was either undamaged or TMP cross-linked, to the binding reactions along with the biotinylated cross-linked DNA substrate. Incubation reactions, addition of streptavidin-coated beads, and washing were carried out as described above.

Development of Antibodies. Affinity-purified rabbit polyclonal antiserum was generated from synthetic peptides corresponding to the C-terminal regions of the FANCA, FANCC, and FANCG proteins by Bethyl Laboratories. For this, synthetic peptides corresponding to the C-terminal region of the FANCA (CRQQAAPDADLSQEPHLF) (*13*), FANCC (CEKLARELLKELRTQV), and FANCG (FLEEFRTSLPKSCDL) proteins were conjugated to KLH carrier and separately injected into rabbits. The antiserum was affinity purified using the peptides linked to agarose (Bethyl Laboratories). Verification of antibody binding to the respective FA proteins was made by Western blot analysis using FANCA polyclonal antiserum previously prepared (*13*), FANCC polyclonal antiserum (a generous gift of Dr. Christopher Walsh, University of North Carolina), and FANCG polyclonal antiserum (a generous gift of Dr. Alan D'Andrea, Harvard Medical School). A monoclonal antibody against αSpIIΣ* from normal human lymphoblastoid cells was developed as previously described (*13*).

Immunoblotting. The DNA binding reactions were separated on SDS−PAGE gels (7.5 × 8.5 cm) following the method of Laemmli (*25*) and proteins analyzed by immu-

noblotting as previously described (*12*−*14*). Immunoblots were developed using Pierce Ultra chemiluminescent substrate and then exposed to X-ray film (Pierce). The primary antibodies used were anti-αSpIIΣ*, anti-FANCA, anti-FANCC, anti-FANCG, and anti-XPF, an affinity-purified polyclonal antibody against the XPF protein that was a generous gift of Dr. Michael Thelen, Lawrence Livermore National Laboratory. When blots were reprobed, they were stripped using the Re-Blot Western Recycling Kit (Chemicon) and reblocked as above before reprobing with the secondary antibody. Images were scanned using a Hewlett-Packard ScanJet 4c/T scanner and analyzed with ImageQuant (Molecular Dynamics).

Incision Reactions. A 140 bp DNA substrate was constructed, which corresponded to the region from 61 to 200 of the nucleotide sequence of the 5S rRNA gene from *Lytechinus variegatus* (*27*). The top strand was internally labeled with γ-^{32}P at the 5′-end (*27*). The presence of an α-phosphorothioate nucleotide at the 3′-end of the DNA substrate conferred resistance to 3′ → 5′ exonuclease activities which may be present (*28, 29*). A single site-specific TMP interstrand cross-link was produced, as previously described, in which its furan side was attached to a thymine in a centrally positioned 5′-TpA-3′ dinucleotide sequence in the top strand of the substrate (*27*). Incision reactions were carried out by incubating the DNA substrate (100 fmol), either TMP cross-linked or unmodified, with chromatin-associated protein extract (10 μg) from normal human lymphoblastoid cells (GM 3299) as previously described (*12, 27*). Incision sites were identified using the sequencing method of Maxam and Gilbert (*12, 27*). For examination of the effects of anti-αSpIIΣ* on endonucleolytic incisions produced at sites of TMP interstrand cross-links, the protein extract was preincubated with either IgM (300 ng) or varying concentrations of anti-αSpIIΣ* (100, 200, 300 ng) before carrying out the incision reactions. For examination of the influence of purified bovine spectrin on the incisions at sites of DNA interstrand cross-links, varying concentrations of spectrin (36 or 53 ng) were added.

Immunoprecipitation. For immunoprecipitation (IP), chromatin-associated protein extracts from HeLa cells were utilized. For anti-α spectrin IPs, anti-α spectrin (specific for mammalian nonerythroid α spectrin and chicken α spectrin) (Chemicon, mAb 1622) or anti-mouse IgG_1 (Sigma) was bound to protein G-coated agarose beads (Sigma), and the binding reactions and immunoprecipitations were carried out as previously described (*14*). For immunoprecipitation, anti-α spectrin was used because our anti-αSpIIΣ* is of the IgM class and cannot be used effectively in immunoprecipitations (*14*). For anti-XPF immunoprecipitation, anti-XPF was bound to protein A-coated agarose beads (Sigma), and immunoprecipitations were carried out as described (*14*). The IPs were subjected to SDS−PAGE, transferred to nitrocellulose, and immunoblotted as previously described (*14*).

RESULTS

A 105 bp DNA substrate containing a hotspot for formation of DNA interstrand cross-links induced by psoralen plus UVA light was synthesized and utilized as a probe for determining whether αSpIIΣ* and the FANCA, FANCC, and FANCG proteins bind to DNA containing interstrand cross-links. The extent of interstrand cross-linking in the treated substrate was determined, as previously described (*2*), by denaturing gel electrophoresis. The fraction of DNA containing interstrand cross-links was 98%.

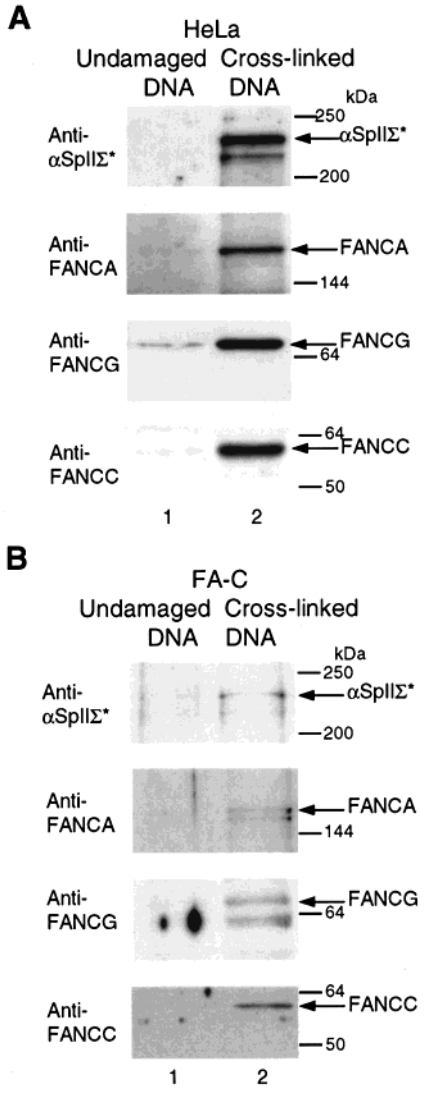

FIGURE 1: Binding of αSpIIΣ* and the FANCA, FANCC, and FANCG proteins to DNA containing a TMP interstrand cross-link. (A) Chromatin-associated proteins (30 μg) from HeLa cells were reacted with undamaged DNA (7.1 μg) (lane 1) or TMP cross-linked DNA (7.1 μg) (lane 2), and protein binding to DNA was visualized by immunoblot analysis using either anti-αSpIIΣ* (top panel), anti-FANCA (second panel), anti-FANCG (third panel), or anti-FANCC (bottom panel). All of the Western blots shown are from the same DNA binding experiment and the same Western blot, which was stripped and reprobed. These experiments were repeated 25 times, using 4 different HeLa cell extractions and 3 different DNA substrate preparations, with similar results. (B) Chromatin-associated proteins (30 μg) from FA-C cells were reacted with undamaged (lane 1) or TMP cross-linked (lane 2) DNA (7.1 μg). Immunoblot analysis of protein binding to DNA was carried out using either anti-αSpIIΣ* (top panel), anti-FANCA (second panel), anti-FANCG (third panel), or anti-FANCC (bottom panel). All of the Western blots shown are from the same DNA binding experiment and the same Western blot, which was stripped and reprobed. These experiments were repeated 3 times, with similar results.

Using DNA affinity chromatography, it was found that αSpIIΣ* from chromatin-associated protein extracts from HeLa cell nuclei bound to the TMP cross-linked substrate. Western blot analysis of the bound proteins showed that

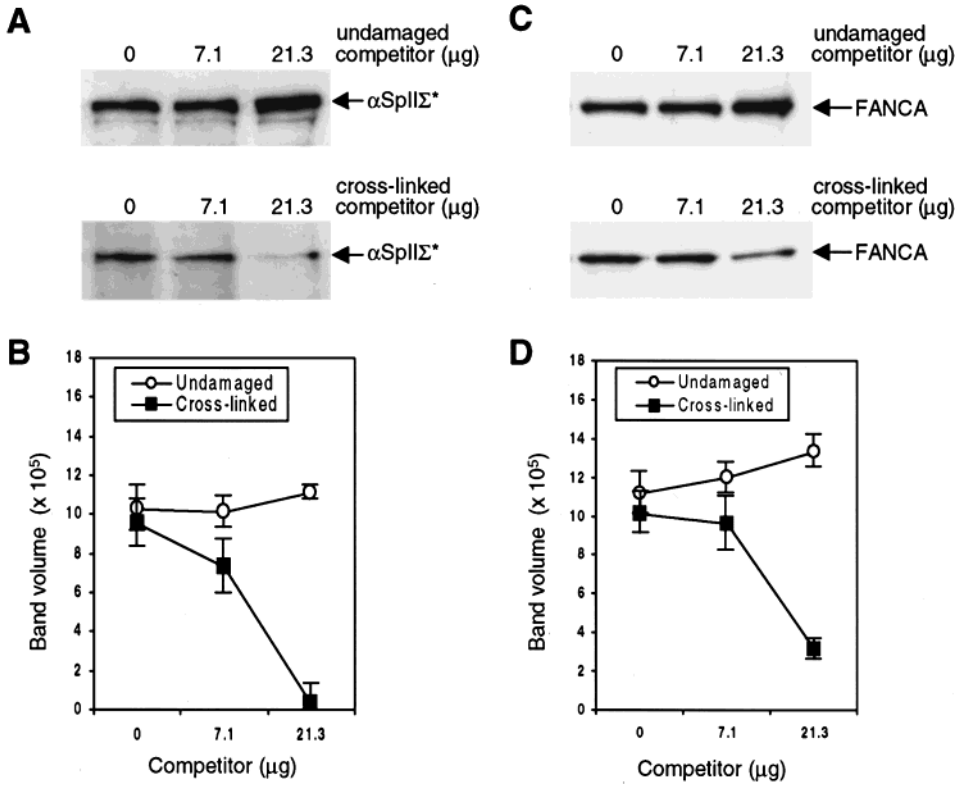

FIGURE 2: Competition experiments showing the preferential binding of αSpIIΣ* and FANCA to TMP cross-linked DNA. (A) Increasing amounts (0, 7.1, and 21.3 μg) of undamaged (top panel) or TMP cross-linked (bottom panel) nonbiotinylated DNA were added as a competitor to binding reactions containing HeLa cell chromatin-associated proteins (30 μg) and TMP cross-linked biotinylated DNA (7.1 μg). Binding of αSpIIΣ* to the damaged DNA during these competition reactions was examined by immunoblot analysis using anti-αSpIIΣ*. (B) Quantitation of the results shown in (A). Vertical lines represent ±SEM. (C) The specificity of binding of FANCA to TMP cross-linked DNA was examined. Undamaged (top panel) or TMP cross-linked (bottom panel) nonbiotinylated DNA was added to the binding reactions. Immunoblot analysis of these competition reactions was carried out using anti-FANCA. This immunoblot is a reprobe of the same blot shown in (A). (D) Quantitation of the results shown in (C). Vertical lines represent ±SEM. These experiments were carried out 3 times with similar results.

αSpIIΣ* had a much higher affinity for the cross-linked substrate (Figure 1A, top panel, lane 2) than for undamaged DNA (Figure 1A, top panel, lane 1). Western blot analysis of the bound proteins with anti-FANCA, anti-FANCC, and anti-FANCG indicated that the FANCA (Figure 1A, second panel), FANCC (Figure 1A, fourth panel), and FANCG (Figure 1A, third panel) proteins also bound to the cross-linked substrate with a higher affinity (Figure 1A, lane 2) than for the undamaged substrate (Figure 1A, lane 1). All of the Western blots shown in Figure 1A are from the same DNA binding experiment and the same Western blot, which was stripped and reprobed. These binding experiments were repeated at least 25 times, using four different HeLa cell extractions and three different DNA substrate preparations, with similar results. For the results shown in Figure 1A, the buffer used to wash the beads/bound DNA/protein mixture contained 150 mM NaCl. If the concentration of the NaCl in the wash buffer was raised to >150 mM, protein binding to the damaged DNA was no longer observed (data not shown).

These same binding experiments were repeated using chromatin-associated protein extracts from FA-A, FA-C, and FA-G cells. Western blot analysis of bound proteins from FA-C extracts showed that αSpIIΣ* bound to the cross-linked DNA (Figure 1B, top panel, lane 2) with a greater affinity than for undamaged DNA (Figure 1B, top panel, lane 1). However, levels of binding were much lower than for αSpIIΣ* present in the normal extracts (Figure 1A, top panel). The FANCA (Figure 1B, second panel, lane 2), FANCC (Figure 1B, fourth panel, lane 2), and FANCG (Figure 1B, third panel, lane 2) proteins also showed greater binding to the cross-linked DNA than to undamaged DNA (Figure 1B, lane 1), though again these levels were lower than those from normal cells. All of the Western blots shown in Figure 1B are from the same DNA binding experiment and the same blot which was striped and reprobed. Western blot analysis of bound proteins from FA-A cells indicated that αSpIIΣ* had some increased binding for damaged compared to undamaged DNA; no binding to cross-linked DNA was observed for FANCA or FANCG, and only a slight amount of binding of FANCC was seen (data not shown). These levels of binding to damaged DNA were much lower than those from HeLa extracts. Western blot analysis of proteins from FA-G cells that bound to the cross-liked DNA showed that there was little to no binding of αSpIIΣ* FANCA, FANCC, or FANCG to either the damaged or the undamaged substrate (data not shown). For FA-A, FA-C, and FA-G extracts, lower levels of binding of αSpIIΣ* and the FA proteins to cross-linked DNA correlate with lower levels of these proteins in these cells (see Discussion).

Preferential binding of αSpIIΣ* to the cross-linked DNA was determined by carrying out competitive binding experiments. As shown in Figure 2, protein binding to the damaged DNA was competed out by addition of nonbiotinylated TMP cross-linked substrate (7.1 and 21.3 μg) (Figure 2A, bottom panel). By contrast, undamaged nonbiotinylated DNA did

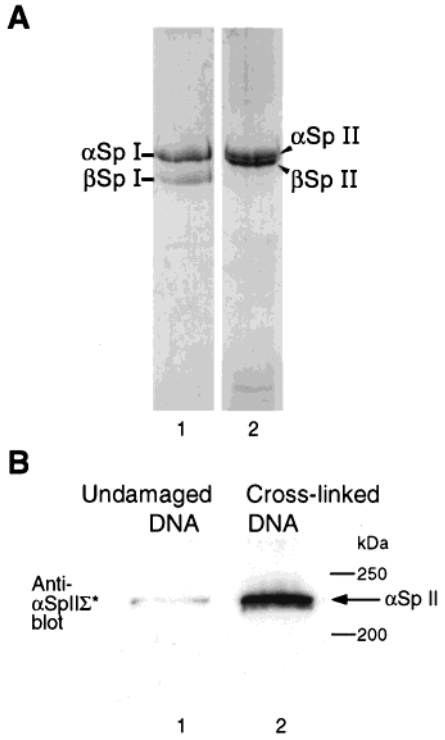

FIGURE 3: Binding of bovine brain spectrin to cross-linked DNA. (A) Purity of bovine brain spectrin (αSpIIΣ1/βSpIIΣ1)$_2$ used in the DNA binding reactions was analyzed by SDS−PAGE on a 5% polyacrylamide gel followed by sliver staining (lane 2). Purified RBC spectrin separated in the same manner and silver stained is shown to compare migration and purity (lane 1). 2 μg of both spectrins was electrophoresed. Spectrin subunits are labeled using the nomenclature of Winkelman and Forget (*31*) without specifying splice form. (B) Purified bovine brain spectrin (7.5 μg) was reacted with undamaged DNA (7.1 μg) (lane 1) or TMP cross-linked DNA (7.1 μg) (lane 2) and protein binding to DNA visualized by immunoblot analysis using anti-αSpIIΣ*.

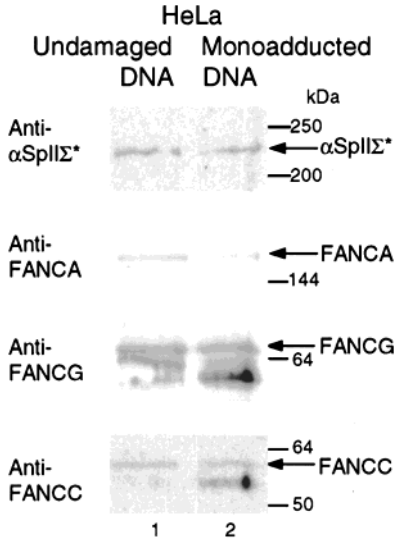

FIGURE 4: Binding of αSpIIΣ* and the FANCA, FANCC, and FANCG proteins to DNA containing a psoralen monoadduct. Chromatin-associated proteins (30 μg) from HeLa cells were reacted with undamaged DNA (7.1 μg) (lane 1) or DNA containing an angelicin monoadduct (7.1 μg) (lane 2), and protein binding to DNA was visualized by immunoblot analysis using either anti-αSpIIΣ* (top panel), anti-FANCA (second panel), anti-FANCG (third panel), or anti-FANCC (bottom panel). All of the Western blots shown are from the same DNA binding experiment and the same Western blot, which was stripped and reprobed. These experiments were repeated 3 times with similar results.

not effectively compete with the damaged biotinylated DNA substrate for binding of αSpIIΣ* (Figure 2A, top panel). Quantitation of these results is shown in Figure 2B. This indicates that αSpIIΣ* preferentially binds to the cross-linked DNA. Preferential binding of the FANCA protein to the cross-linked DNA was determined by similar competitive binding experiments. FANCA binding to the damaged DNA was competed out by addition of nonbiotinylated cross-linked DNA (7.1 and 21.3 μg) (Figure 2C, bottom panel). Undamaged nonbiotinylated DNA did not compete with the damaged DNA for binding of FANCA (Figure 2C, top panel). The immunoblot shown in Figure 2C is a reprobe of the same blot shown in Figure 2A. Quantitation of these results is shown in Figure 2D. Similar results were seen with the FANCC and FANCG proteins (data not shown). These results show that the FANCA, FANCC, and FANCG proteins bind preferentially to the damaged DNA.

To determine whether the binding of αSpIIΣ* to the cross-linked DNA was direct or not, purified bovine brain spectrin (αSpIIΣ1/βSpIIΣ1)$_2$ was used in the binding reactions. Analysis of the purity of the spectrin by SDS−PAGE showed that only two bands were present which represent αSpIIΣ1 and βSpIIΣ1 (Figure 3A, lane 2). Purified RBC spectrin was used as a marker where αSpI migrates with a molecular mass of 240 kDa (*30, 31*) and βSpI migrates with a molecular mass of 220 kDa (*30, 31*) (Figure 3A, lane 1). By densitometry, the purity of the brain spectrin was >95%. Western blot analysis of the binding of purified brain spectrin to the cross-linked DNA, using anti-αSpIIΣ*, showed that spectrin bound in greater amount to the damaged DNA (Figure 3B, lane 2) than to the undamaged DNA (Figure 3B, lane 1). Titration of the amount of spectrin used in the binding reactions showed that maximum binding to damaged DNA occurred at a protein/DNA ratio of 7.5 μg/7.1 μg (Figure 3B, lane 2).

To ascertain the specificity of binding of αSpIIΣ* and the FA proteins for cross-linked DNA, binding of these proteins to DNA containing a psoralen monoadduct was examined under the same conditions used for cross-linked DNA, except that the substrate contained an angelicin monoadduct rather than a TMP interstrand cross-link. Western blot analysis of bound proteins from HeLa chromatin-associated protein extracts showed that αSpIIΣ* had no greater affinity for the angelicin monoadducted substrate (Figure 4, top panel, lane 2) than for the undamaged DNA (Figure 4, top panel, lane 1). Similarly, Western blot analysis indicated that FANCA (Figure 4, second panel), FANCC (Figure 4, fourth panel), and FANCG (Figure 4, third panel) proteins also had no greater binding affinity for the monoadducted DNA (Figure 4, lane 2) than for undamaged DNA (Figure 4, lane 1). Increasing NaCl concentrations to >150 mM NaCl in the wash buffer reduced the overall protein binding to DNA. When much less stringent conditions were used in the binding step (i.e., when the NaCl concentration was reduced to <50 mM), some increased binding of αSpIIΣ* to the monoadducted DNA compared to undamaged DNA was observed (data not shown).

Studies were carried out to further examine the involvement of αSpIIΣ* in the endonucleolytic incisions we observe at sites of TMP interstrand cross-links in normal cells. Examination of the influence of anti-αSpIIΣ* on the ability

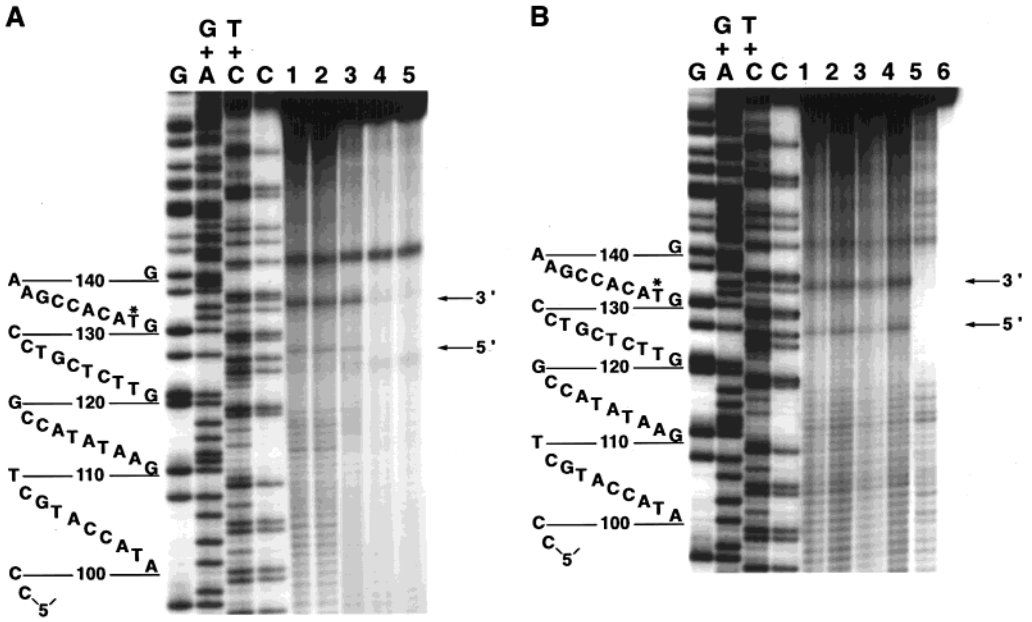

FIGURE 5: Influence of anti-αSpIIΣ* and purified bovine brain spectrin on incisions produced at sites of DNA interstrand cross-links. (A) A 140 bp DNA substrate (100 fmol), in which the furan side of a TMP interstrand cross-link was adducted to the ^{32}P-5′-end-labeled top strand, was reacted with a chromatin-associated protein extract (10 μg) from normal human lymphoblastoid cells (GM 3299) (lane 1), or a chromatin-associated protein extract (10 μg) that had been preincubated with 300 ng of IgM (lane 2), 100 ng of anti-αSpIIΣ* (lane 3), 200 ng of anti-αSpIIΣ* (lane 4), or 300 ng of anti-αSpIIΣ* (lane 5). The effect of anti-αSpIIΣ* on endonucleolytic incisions produced at sites of interstrand cross-links was 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′ incision are indicated by arrows. (B) The influence of purified bovine brain spectrin on incisions produced at sites of DNA interstrand cross-links was also examined. The TMP cross-linked substrate (100 fmol) was reacted with either 5 μg (lane 1) or 10 μg (lane 2) of normal chromatin-associated protein extract. To reactions containing 5 μg of normal extract was added either 36 ng of spectrin (lane 3) or 54 ng of spectrin (lane 4). As controls, 5 μg of normal extract was reacted with undamaged DNA (100 fmol) (lane 5), and 54 ng of spectrin was reacted with the cross-linked DNA (100 fmol) (lane 6). Notations are as in (A). Each of these experiments was repeated 2−3 times with similar results.

of the normal chromatin-associated protein extracts to produce incisions at sites of TMP furan side interstrand cross-links showed that anti-αSpIIΣ*, in a concentration-dependent manner, decreased levels of the 3′ and 5′ incisions produced (Figure 5A, lanes 3−5). Levels of incisions were decreased compared to those produced when anti-αSpIIΣ* was not present (Figure 5A, lane 1). The influence of purified bovine brain spectrin on these dual incisions was also examined. Purified brain spectrin was found to enhance the level of the 3′ and 5′ incisions on the TMP cross-linked DNA (Figure 5B, lane 4) compared to the level of these incisions produced on damaged DNA alone (Figure 5B, lane 1). As we have previously shown (*27*), the 3′ and 5′ incisions were at the fourth and sixth phosphodiester bonds, respectively, from the adducted thymine. Increasing the concentration of spectrin from 36 ng (Figure 5B, lane 3) to 54 ng (Figure 5B, lane 4) resulted in a 2-fold increase in the endonucleolytic incisions produced on the cross-linked DNA. Spectrin by itself did not produce any incisions on cross-linked DNA (Figure 5B, lane 6). Minor bands that were observed on TMP adducted DNA following treatment with the extracts were also present on the undamaged DNA (Figure 5B, lane 5) and represent incisions by nonspecific nucleases.

In addition, studies were carried out to determine whether there was any association between αSpIIΣ* and XPF, the protein involved in the production of the incisions we observe at sites of DNA interstrand cross-links (*12*). Immunoprecipitation with anti-α spectrin and immunoblotting with either anti-αSpIIΣ* (Figure 6A, upper panel, lane 1) or anti-XPF (Figure 6A, lower panel, lane 1) showed that XPF coimmunoprecipitated with αSpIIΣ*. Likewise, when immunoprecipitation was carried out using anti-XPF, immunoblotting with anti-αSpIIΣ* (Figure 6B, upper panel, lane 1) or anti-XPF (Figure 6B, lower panel, lane 1) showed that αSpIIΣ* coimmunoprecipitated with XPF.

DISCUSSION

Identification of sites of damage on DNA by specific proteins is an essential component of the repair process. In the current study, using DNA affinity chromatography, αSpIIΣ*, present in HeLa cell nuclei, has been shown to specifically bind to DNA containing TMP- plus UVA-induced interstrand cross-links with little affinity for psoralen monoadducted DNA. That this binding is due to direct binding of αSpIIΣ* to the cross-linked DNA has been demonstrated using purified bovine brain spectrin. We have previously identified, in normal human cells, a DNA binding protein with specificity for DNA containing interstrand cross-links produced by TMP plus UVA light which has an apparent isoelectric point of 5.5 (*2*). Spectrin has an isoelectric point of 5.6 (*32*). It is thus possible that these two proteins are identical. Additionally, we have shown that the previously identified binding protein is defective in FA-A cells (*2*). This correlates with a deficiency in αSpIIΣ* in FA-A cells (*13*, *14*). The FANCA, FANCC, and FANCG proteins, present in HeLa cell nuclei, were also found to bind to cross-linked DNA. However, whether this binding is direct or indirect, via the association of these FA proteins with αSpIIΣ* (*14*), is not yet clear.

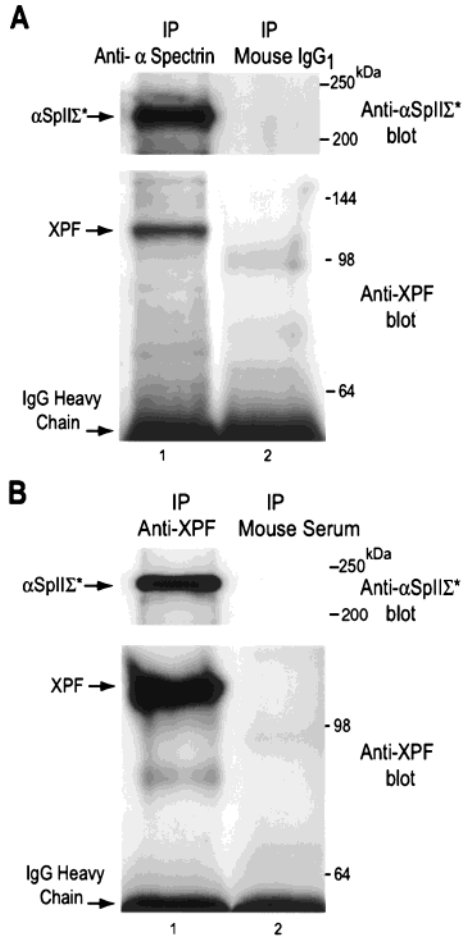

FIGURE 6: Binding of XPF to αSpIIΣ* in the nucleus. (A) Chromatin-associated proteins from HeLa cells were immunoprecipitated with anti-α spectrin (lane 1) or mouse IgG_1 (lane 2) and immunoblotted with anti-αSpIIΣ* (upper panel) or anti-XPF (lower panel). (B) Chromatin-associated proteins from HeLa cells were immunoprecipitated with anti-XPF (lane 1) or mouse serum (lane 2) and immunoblotted with anti-αSpIIΣ* (upper panel) or anti-XPF (lower panel).

When FA-A, FA-C, and FA-G chromatin-associated proteins were used in these binding studies, αSpIIΣ*, FANCA, FANCC, and FANCG were also observed to bind to cross-linked DNA; however, the levels of binding were less than those found in HeLa cells and correlated with the decreased levels of these proteins in the three FA complementation groups. Electrophoretic analysis has shown that the levels of αSpIIΣ* in FA-A, FA-C, and FA-G cells are decreased compared to those in normal cells, with the level of deficiency in FA-C cells less than in FA-A or FA-G cells (*13*, Lambert et al., in preparation). Studies indicate that there are reduced levels of FANCA, FANCC, and FANCG in FA-A, FA-C, and FA-G cells, with the relative levels of these proteins dependent on the cell line (*21*, *33*). This has suggested an interdependence of these proteins on each other (*20*, *21*). Therefore, in FA-A, FA-C, and FA-G cells, the reduced amount of binding of the FANCA, FANCC, and FANCG proteins to cross-linked DNA could be due to the reduced levels of these proteins in these FA cell lines, or, if the binding of the FA proteins is indirect, it could be due to the reduced levels of αSpIIΣ* in these cells.

Spectrin in nonerythroid cells has been shown to be involved in a number of different functions in the cytoplasm. It is found on the cytoplasmic surface of the plasma membrane and all organelle membranes (*34*). Spectrins are components of a membrane-associated organizing center where membrane and cytosolic proteins are gathered and their interactions coordinately stabilized and regulated (*35*−*38*); spectrin forms a skeleton associated with the Golgi that is involved in protein sorting and trafficking in the secretory pathway (*37*, *39*−*41*); and brain spectrin (*β*SpIIΣ1) plays a key role in synaptic transmission in neurons where it interacts with synaptic vesicles and is important in their alignment at the presynaptic plasma membrane (*24*, *30*, *42*). The function of spectrin in the nucleus, however, is unclear. α spectrin has been shown to be associated with the nuclear matrix and the nuclear envelope (*43*, *44*), and a protein cross-reacting with α and *β* spectrin antibodies has been detected in nucleoli of amphibian oocytes (*45*). We have recently identified αSpIIΣ* in the nuclei of normal human lymphoblastoid cells and HeLa cells and have shown that it is a component of a nuclear protein complex involved in repair of DNA interstrand cross-links (*13*, *14*) and that it also forms a complex with the FANCA, FANCC, and FANCG proteins (*14*, Lambert et al., in preparation). The present finding that αSpIIΣ*, as well as purified bovine brain spectrin, binds to DNA containing interstrand cross-links is the first report of interaction of α spectrin with DNA. Structurally nonerythroid α spectrin is made up of an extended array of triple α-helical repeat units (*30*, *31*). Analysis of the crystal structure of spectrin shows that the α-helices of individual repeats contain substantial numbers of polar residues on the surface of the helices (46, PDB entry 2SPC). Most of the charged residues appear to be ion-paired to residues at one helix turn apart (three to four residues), confirming that these residues are on the surface of the helices (*46*, PDB entry 2SPC). This indicates that α spectrin has the potential to bind to DNA. Since the number of positive and negative residues in α spectrin is comparable (PDB entry 2SPC), the interaction of spectrin with DNA through the DNA backbone seems unlikely. Interaction of α spectrin with DNA could occur through hydrogen bonding interaction between its protein side chains and the base atoms (N_3 of purines and O_2 of pyrimidines) in the minor groove of DNA. Enhanced binding of α spectrin to cross-linked DNA could possibly be due to the enhanced ability of this protein to interact with DNA when the minor groove of DNA opens up due to the formation of the psoralen interstrand cross-link (*47*).

The evidence presented in the present study that αSpIIΣ* binds preferentially to DNA containing interstrand cross-links further points to a role for αSpIIΣ* in DNA repair. In addition, several other findings in the present study also indicate an involvement of αSpIIΣ* in the repair of DNA interstrand cross-links: anti-αSpIIΣ* decreases the levels of the incisions we observe at sites of cross-links, purified bovine spectrin enhances the levels of these incisions, and αSpIIΣ* coimmunoprecipitates with XPF, a protein shown to be involved in repair of DNA interstrand cross-links, in particular production of the incisions observed at these sites of damage (*12*, *48*). Different isoforms of α spectrin have been found in the same cell as well as in different cells, which suggests distinct functions for each (*30*, *49*−*51*). The splice form of the αSpIIΣ* we have identified is not yet known and is currently under investigation. Different splice forms

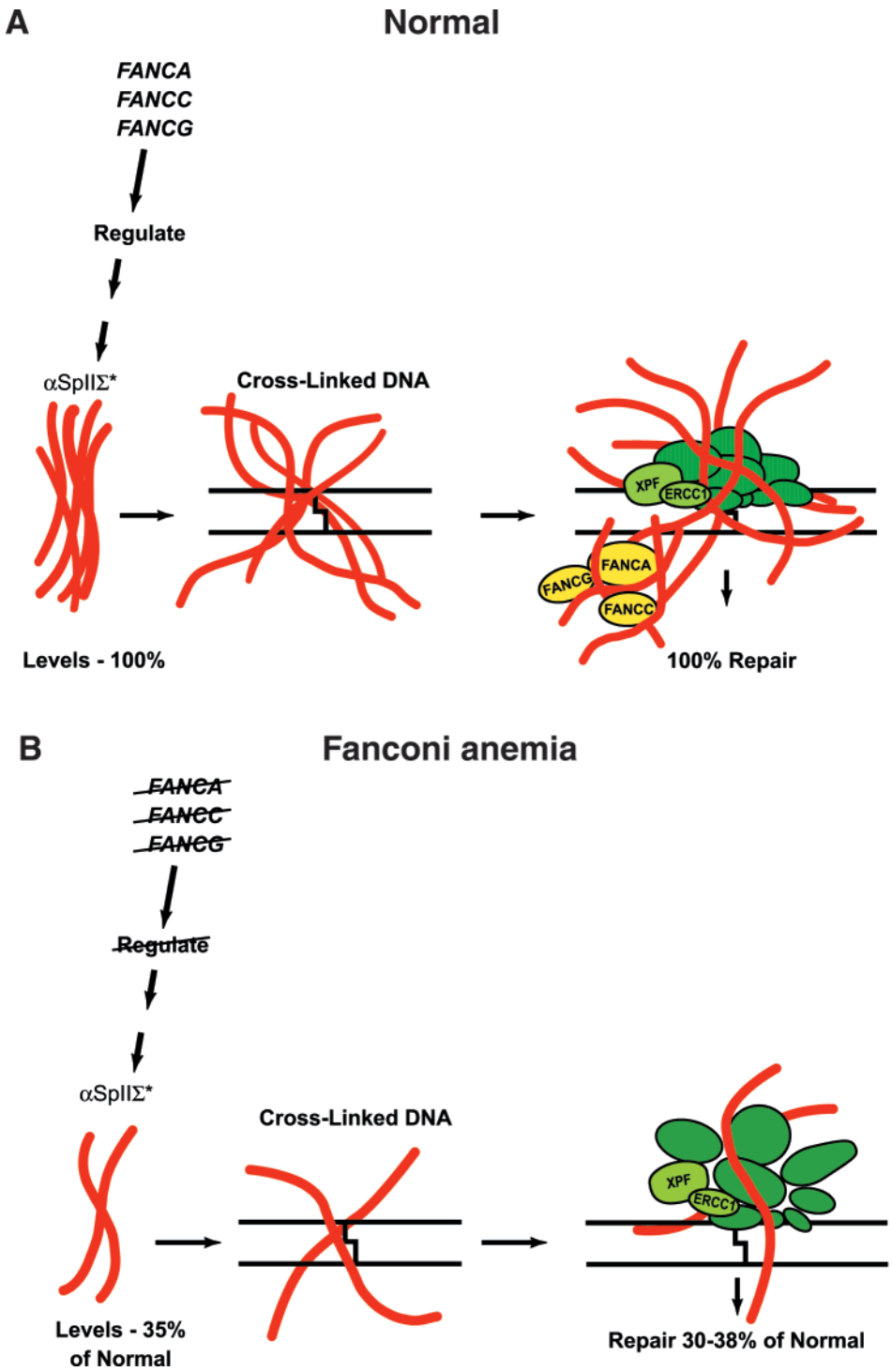

FIGURE 7: A model is presented for the role of αSpIIΣ* in the repair of DNA interstrand cross-links in normal cells and the role of αSpIIΣ* in the repair defect in FA cells. (A) In normal cells, the *FANCA*, *FANCC*, and *FANCG* genes, either directly or indirectly, regulate the expression or stability of αSpIIΣ* to produce normal cellular levels of this protein. αSpIIΣ* binds to cross-linked DNA, aids in the recruitment of repair proteins (dark green) and the FANCA, FANCC, and FANCG proteins to the site of damage, and acts as a scaffold to help align these proteins and enhance the efficiency of the repair process to give normal levels of DNA repair. (B) In FA cells, defects in the *FANCA*, *FANCC*, and *FANCG* genes lead to decreased levels of αSpIIΣ* in these FA cells (35% of normal) (*13*, Lambert et al., in preparation) due to either decreased expression or stability of αSpIIΣ*. Decreased levels of αSpIIΣ* ultimately lead to decreased efficiency of DNA repair in the FA cells (30−38% of normal) (*8, 9, 13*). This is a result of less binding of αSpIIΣ* to cross-linked DNA which in turn results in decreased efficiency in the recruitment of the repair proteins (dark green) to the site of damage and decreased aid in enhancing the efficiency of interaction of these proteins.

of each spectrin gene have been shown to occur due to alternate splicing of pre-mRNA (*30, 31, 49, 50*).

A number of studies suggest that the nuclear matrix is important in DNA repair. Repair analysis indicates that DNA damaged by UV irradiation is recruited to the nuclear matrix where many NER proteins are also recruited and where NER subsequently takes place (*52*−*55*). Similarly, repair of DNA double-strand breaks produced by ionizing radiation occurs in the nuclear matrix (*56*). Repair of DNA interstrand cross-links is a complex process since dual incisions need to be

made on both DNA strands. Nucleotide excision repair (NER) and recombination are thought to be involved in these repair events (*48*, *57*−*59*). It is possible that association of the repair proteins involved in these processes with a protein scaffold (i.e., αSpIIΣ*) that is associated with the nuclear matrix could aid in the alignment of these proteins and in the efficiency of repair. We have previously shown that incisions are produced on the 3′ and 5′ sides of a site-directed TMP interstrand cross-link (*12*, *27*) and that XPF is involved in the production of these incisions (*12*). The fact that we observe both 3′ and 5′ incisions on DNA containing a 5′ end label indicates that these incisions are uncoupled or occur independently of each other (*12*, *27*). Kuraoka et al. have recently reported similar results (*48*). The distance between our incisions is 9 bp while theirs is 5 bp (*48*). However, this difference could be due to the fact that their substrate was 23 bp in length compared to our 140 bp, their cross-linked substrate contained a Y junction, and they were examining incisions produced by XPF-ERCC1 alone, whereas we utilized a complex of proteins containing XPF-ERCC1. Bessho et al., on the other hand, found that on a psoralen cross-linked substrate dual incisions were produced on the 5′ side of the adduct, rather than bracketing it (*60*). It is possible that more than one pathway may be involved in repair of interstrand cross-links. That the incisions we observe are important in cross-link repair is demonstrated by studies which show that cell lines deficient in repair of DNA interstrand cross-links, FA-A, FA-B, FA-C, FA-D, and FA-G, are also deficient in ability to produce these incisions (*11*, *12*, Lambert et al., in preparation).

Based on our present findings, other work in our laboratory, and the observations described above, we have proposed a model for the role of αSpIIΣ* in DNA repair. In this model, the *FANCA*, *FANCC*, and *FANCG* genes are involved in regulating the expression or stability of αSpIIΣ* in the cell (Figure 7A). αSpIIΣ* binds to the cross-linked DNA and aids in recruiting the damaged DNA to the nuclear matrix. αSpIIΣ* would then be involved in the recruitment of proteins which play a role in the repair process and act as a scaffold upon which the repair proteins could align or spatially orient themselves, enhancing the efficiency of repair.

This model could also explain the repair defect in FA cells (Figure 7B). Defects in the *FANCA*, *FANCC*, and *FANCG* genes would lead to decreased expression or stability of αSpIIΣ* and thus to decreased levels of αSpIIΣ* in these cells. This in turn would lead to decreased binding of αSpIIΣ* to damaged DNA and decreased recruitment and alignment, at sites of damage, of the proteins involved in repair of interstrand cross-links. This alignment could be particularly important in repair of interstrand cross-links where recombination may be involved (*48*, *57*, *58*). This in turn would lead to decreased levels of repair occurring in these cells. Reduced levels of αSpIIΣ* in the nucleus would thus be expected to reduce the efficiency of the repair process in FA cells rather than inhibit it altogether, consistent with our experimental findings (*8*, *9*, *11*−*13*). The role of the FANCA, FANCC, and FANCG proteins in this repair process is not clear. They could be involved in the damage-recognition step and bind directly to the cross-linked DNA. On the other hand, their binding to damaged DNA could be indirect via their binding affinity for αSpIIΣ*. αSpIIΣ* could act as a scaffold to align or enhance interactions between the FA proteins, or between the FA proteins and proteins involved in DNA repair or in other cellular processes. FANCA, FANCC, and FANCG have been shown to interact with each other and form a complex in the nucleus (*20*−*23*); αSpIIΣ* may aid in this interaction. Preliminary studies in our laboratory show that purified brain spectrin cannot correct the decreased levels of incisions produced by FA-A and FA-C extracts on cross-linked DNA. This finding indicates that at least the FANCA and FANCC proteins are important for this initial damage recognition/incision step in the repair process.

A number of observations in our laboratory are in support of the model proposed above: (1) In FA-A, FA-B, FA-C, FA-D, and FA-G cells, levels of αSpIIΣ* are greatly reduced (35% of normal) as determined by gel electrophoretic analysis of αSpIIΣ* in these cells (*13*, Lambert et al., in preparation). This correlates with reduced levels of unscheduled DNA synthesis (UDS) or DNA repair synthesis (30−38% of normal) observed in these cells in response to DNA interstrand cross-linking agents (*8*, *9*, *13*) and with the decreased levels of incisions produced by protein extracts of these cells on cross-linked DNA (*11*−*13*). (2) Levels of αSpIIΣ* in the FA-A, FA-C, and FA-G cells appear to be regulated by the *FANCA*, *FANCC*, and *FANCG* genes since the decreased levels of αSpIIΣ* in these FA cells are corrected when these cells express the FANCA, FANCC, and FANCG proteins, respectively (*13*, *14*). (3) αSpIIΣ* preferentially binds to DNA containing a psoralen interstrand cross-link compared to DNA containing a psoralen mono-adduct. (4) Coimmunoprecipitation experiments show that αSpIIΣ* binds to XPF, a protein involved in production of dual incisions at sites of DNA interstrand cross-links. (5) αSpIIΣ* enhances the level of the dual incisions produced at the site of an interstrand cross-link, indicating a possible role for this protein in increasing the efficiency of interaction of the proteins involved in the repair process. (6) Coimmunoprecipitation studies show that αSpIIΣ* forms a complex with the FANCA, FANCC, and FANCG proteins in the nucleus (*14*). Thus, αSpIIΣ* appears not only to play a role in the repair of DNA interstrand cross-links but also to be an underlying factor in the repair defect in FA cells.

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