Nonerythroid αII spectrin is required for recruitment of FANCA and XPF to nuclear foci induced by DNA interstrand cross-links

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Summary

The events responsible for repair of DNA interstrand cross-links in mammalian cells, the proteins involved and their interactions with each other are poorly understood. The present study demonstrates that the structural protein nonerythroid α spectrin (αSpIIΣ*), present in normal human cell nuclei, plays an important role in repair of DNA interstrand cross-links. These results show that αSpIIΣ* relocalizes to nuclear foci after damage of normal human cells with the DNA interstrand cross-linking agent 8-methoxypsoralen plus ultraviolet A (UVA) light and that FANCA and the known DNA repair protein XPF localize to the same nuclear foci. That αSpIIΣ* is essential for this re-localization is demonstrated by the finding that in cells from patients with Fanconi anemia complementation group A (FA-A), which have decreased ability to repair DNA interstrand cross-links and decreased levels of αSpIIΣ*, there is a significant reduction in formation of damage-induced XPF as well as αSpIIΣ* nuclear foci, even though levels of XPF are normal in these cells. In corrected FA-A cells, in which levels of αSpIIΣ* are restored to normal, numbers of damage-induced nuclear foci are also returned to normal. Co-immunoprecipitation studies show that αSpIIΣ*, FANCA and XPF co-immunoprecipitate with each other from normal human nuclear proteins. These results demonstrate that αSpIIΣ*, FANCA and XPF interact with each other in the nucleus and indicate that there is a close functional relationship between these proteins. These studies suggest that an important role for αSpIIΣ* in the nucleus is to act as a scaffold, aiding in recruitment and alignment of repair proteins at sites of damage.

Key words: α Spectrin, FANCA, XPF, DNA interstrand cross-link, DNA repair

Introduction

Repair of DNA interstrand cross-links is a complex process because both strands of DNA need to be repaired at the site of the cross-link. Although the mechanism of repair of interstrand cross-links has been elucidated in *Escherichia coli* (Jones and Yeung, 1988; Sladek et al., 1989; Van Houten, 1990; Cheng et al., 1991) and a significant amount of information is available on repair of these lesions in yeast (reviewed in Dronkert and Kanaar, 2001; Greenberg et al., 2001; Cohen et al., 2002), the precise mechanisms and proteins involved in this repair process in mammalian cells have not yet been defined. We have isolated from the nucleus of normal human cells a complex of proteins involved in the repair of DNA interstrand cross-links (Lambert et al., 1988; Parrish and Lambert, 1990; Parrish et al., 1992; Lambert and Lambert, 1999). This complex contains proteins involved in both damage recognition and incision of cross-linked DNA (Hang et al., 1993; Kumaresan et al., 1995; Lambert and Lambert, 1999). We have recently identified a structural protein, nonerythroid α spectrin (αSpIIΣ*), as a component of this protein complex in normal human cell nuclei and have shown that it binds directly to DNA containing a 4,5′,8-trimethylpsoralen (TMP) plus UVA-light-induced interstrand cross-link and plays a role in the initial damage recognition/incision steps involved in the repair of this lesion

(McMahon et al., 1999; McMahon et al., 2001). We have also shown that the nucleotide excision repair protein XPF is involved in the repair of this interstrand cross-link and that it functions in production of the incisions made on the 5′ side and 3′ side of the cross-link (Kumaresan and Lambert, 2000; Kumaresan et al., 2002). These studies are in agreement with those of Kuraoka et al. (Kuraoka et al., 2000), who showed that ERCC1-XPF cleaves a psoralen interstrand cross-link on either side of the adduct.

Cells from patients with the autosomal recessive genetic disorder Fanconi anemia (FA) provide an excellent experimental model for examination of the mechanism of repair of DNA interstrand cross-links and the proteins involved. FA is characterized by progressive bone marrow failure, a marked predisposition to development of cancer, particularly acute myeloid leukemia, spontaneous chromosome instability and hypersensitivity to DNA interstrand cross-linking agents (Glanz and Fraser, 1982; Auerbach, 1995; Auerbach et al., 1998). Correlated with sensitivity to these agents is a defect in ability to repair DNA interstrand cross-links (Papadopoulo et al., 1987; Averbeck et al., 1988; Lambert et al., 1992; Zhen et al., 1993; Lambert et al., 1997; Lambert and Lambert, 1999). There are eight complementation groups of FA and, although the genes responsible for six of the

complementation groups have been cloned (Strathdee et al., 1992; Lo Ten Foe et al., 1996; Fanconi Anemia/Breast Cancer Consortium, 1996; de Winter et al., 1998; de Winter et al., 2000a; de Winter et al., 2000b; Timmers et al., 2001) and the interactions of their protein products studied (Kupfer et al., 1997; Garcia-Higuera et al., 1999; Waisfisz et al., 1999; de Winter et al., 2000c; Christianson et al., 2000; Reuter et al., 2000; Medhurst et al., 2001; Ahmad et al., 2002), neither their role in the etiology of the disorder nor their involvement in the repair of DNA interstrand cross-links has been elucidated. We have shown that, in all of the FA complementation groups we have examined (FA-A, FA-B, FA-C, FA-D1, and FA-G), there is a deficiency in levels of αSpIIΣ* present (Brois et al., 1999; McMahon et al., 1999). In FA-A cells, this correlates with a deficiency in ability to produce dual incisions at sites of interstrand cross-links (Kumaresan and Lambert, 2000). This incision defect is not due to decreased levels of XPF in FA-A cells because the level of this protein in these cells is similar to that of normal cells (Brois et al., 1999). We have shown that FA-A cells are deficient in a DNA binding protein that recognizes TMP interstrand cross-links (Hang et al., 1993). Because our studies demonstrate that αSpIIΣ* binds directly to DNA containing TMP interstrand cross-links (McMahon et al., 2001), it is possible that this is the deficient binding protein in FA-A cells. That FANCA is involved in this repair process is indicated by our studies, which show that it also binds to DNA containing interstrand cross-links, although it is not clear whether this binding is direct or indirect (McMahon et al., 2001). It is possible that αSpIIΣ* acts as a scaffold to aid in the recruitment of repair proteins to the site of damage and in their alignment at these sites, thus enhancing the efficiency of the repair process. In FA-A cells, where levels of αSpIIΣ* are decreased, there would be reduced recruitment of repair proteins to the sites of damage, which would in turn lead to reduced levels of DNA repair, as has been observed (Brois et al., 1999; McMahon et al., 1999). The same could hold true for the other FA complementation groups in which levels of αSpIIΣ* have also been shown to be decreased (McMahon et al., 1999).

In the present report, the relationship between αSpIIΣ* and the FANCA and XPF proteins was analyzed by examining the localization of αSpIIΣ*, in relation to that of FANCA and XPF, in the nucleus of human cells damaged with a DNA interstrand cross-linking agent, 8-methoxypsoralen (8-MOP) plus UVA light, and by assessing the interaction of these proteins by co-immunoprecipitation analysis. For these studies, damage-induced foci formation of αSpIIΣ*, FANCA and XPF was examined not only in normal human nuclei, in which αSpIIΣ* is present, but also in FA-A cell nuclei, in which there is a deficiency in αSpIIΣ*. Here, we show that, in response to 8-MOP plus UVA light, αSpIIΣ*, FANCA and XPF co-localize with each other in discrete foci in the nucleus and that αSpIIΣ* plays an important role in modulating the formation of these damage-induced foci. Co-immunoprecipitation results show that αSpIIΣ*, FANCA and XPF interact with each other. These results together support the concept of a close functional link between these proteins and an involvement for them in the repair of DNA interstrand cross-links. These studies further suggest that an important function of αSpIIΣ* in the nucleus is to act as a scaffold and aid in the recruitment of repair proteins to sites of DNA damage.

Materials and Methods

Cell lines and culture conditions

The normal human (GM 3299) lymphoblastoid cell line was obtained from the Coriell Institute for Medical Research (Camden, NJ). The FA-A (HSC 72) lymphoblastoid cell line was a gift from Manuel Buchwald (Hospital for Sick Children, Toronto, Canada). FA-A lymphoblastoid cells (HSC 72) were stably transduced with a retroviral vector expressing the FANCA cDNA (HSC 72-17) (Fu et al., 1998; Brois et al., 1999). Cell lines were grown in suspension culture in RPMI 1640 medium as previously described and routinely checked for mycoplasma using an American Type Culture Collection polymerase-chain-reaction-based mycoplasma detection kit (Lambert et al., 1992; Brois et al., 1999).

Treatment of cells with 8-methoxypsoralen and UVA light

The cells in culture were treated with 3.5 μM 8-methoxypsoralen (8-MOP) (Sigma-Aldrich, St Louis, MO) in RPMI 1640 media for 20 minutes at room temperature in the dark. They were then irradiated with UVA light (principally 366 nm) (6 kJ m^{-2}), washed with fresh media and exposed to a second dose of UVA irradiation (6 kJ m^{-2}), as previously described, so as to increase the number of DNA interstrand cross-links produced (Lambert et al., 1988; Lambert et al., 1992). For those experiments in which the effect of dosage of 8-MOP plus UVA irradiation was examined, the concentration of 8-MOP was kept constant at 3.5 μM and the dosage of UVA light was increased over a range from 0 kJ m^{-2} to 10 kJ m^{-2}. At each dosage, an aliquot of cells was counted to determine the number of surviving cells using a trypan blue exclusion assay.

Indirect immunofluorescence

Cells were applied to poly-L-lysine coated chamber slides and allowed to attach for 40 minutes at 37°C. They were then fixed with 4% paraformaldehyde for 20 minutes, washed with PBS and permeabilized with 0.2% Triton-X100 in PBS for 10 minutes at room temperature. The cells were then blocked in 10% goat serum (Gibco, Grand Island, NY), 10% rabbit serum (Jackson Immuno Research, Westgrove, PA) or 10% donkey serum (Sigma-Aldrich) as appropriate, for 1-2 hours. The primary antibody was then added and allowed to bind for 2 hours at 37°C. The primary antibodies used were purified mouse monoclonal anti-αSpIIΣ* antibody, 1:100 dilution (McMahon et al., 1999), purified mouse monoclonal anti-α-spectrin antibody, 1:150 dilution (mAb 1622, Chemicon International, Temecula, CA), affinity-purified rabbit polyclonal anti-FANCA antibody, against the C-terminal region of FANCA, 1:100 dilution (Bethyl Laboratories, Montgomery, TX) (McMahon et al., 2001) and affinity-purified goat polyclonal anti-XPF antibody, 1:100 dilution (Ab sc-10161, Santa Cruz Biotech, Santa Cruz, CA). Pre-immune mouse, rabbit and goat serum (Sigma-Aldrich) was used as a negative control. After five 5-minute washes with PBS, the appropriate secondary antibody was added: Alexafluor 488 goat anti-mouse IgG conjugate, highly cross-adsorbed, 1:250 dilution (green fluorescence), when anti-α-spectrin was the primary antibody; Alexafluor 594 goat anti-rabbit IgG conjugate, highly cross-adsorbed, 1:500 dilution (red fluorescence), when anti-FANCA was the primary antibody; and Alexafluor 594 rabbit anti-goat IgG conjugate, highly cross-adsorbed, 1:500 dilution (red fluorescence) (Molecular Probes, Eugene, OR), when anti-XPF was the primary antibody. Incubation with the secondary antibodies was carried out for 20 minutes at room temperature in the dark. Primary and secondary antibodies were diluted in PBS. In double-labeling experiments, the cells were treated sequentially with the appropriate blocking agent and then the primary and secondary antibodies against each of the proteins under investigation. The secondary antibodies used were as follows: for dual staining with anti-α-spectrin and anti-FANCA, Alexa 488 goat anti-mouse IgG and Alexa 594 goat anti-rabbit IgG were used,
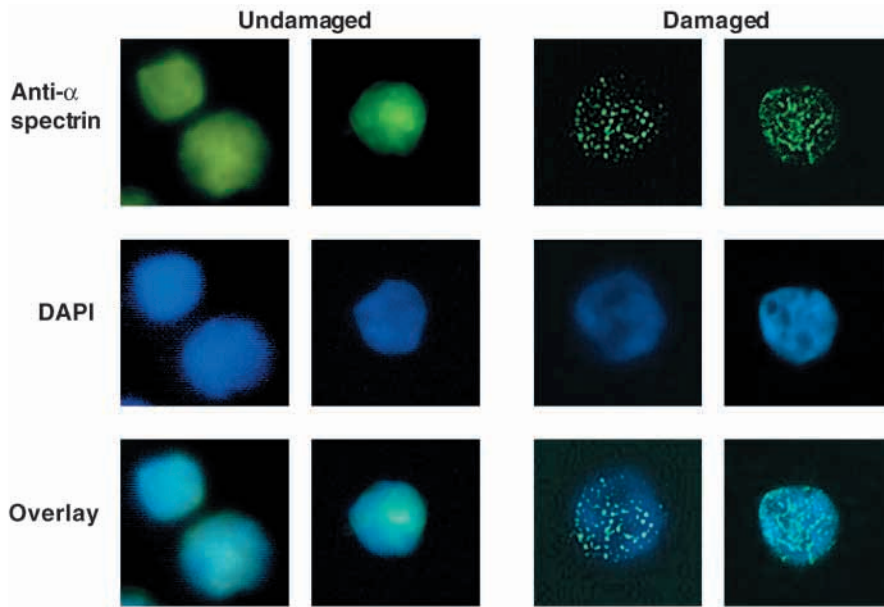
Fig. 1. Relocalization of αSpIIΣ* to nuclear foci following treatment of normal cells with 8-MOP plus UVA light. The localization of αSpIIΣ* in the nucleus of undamaged normal human lymphoblastoid cells or lymphoblastoid cells treated with 8-MOP plus UVA light (6 kJ m^{-2}) was examined by immunofluorescence with purified monoclonal anti-α-spectrin antibody. Counterstaining for the DNA-specific dye DAPI is shown in the middle panels. In the bottom panels, the fluorescent signal for DAPI was overlaid on that for anti-α-spectrin. Cells were fixed for staining 15 hours after treatment with 8-MOP plus UVA.

respectively; for dual staining with anti-α-spectrin and anti-XPF, Alexa 488 goat anti-mouse IgG and Alexa 594 rabbit anti-goat IgG were used; and for dual staining with anti-FANCA and anti-XPF, Alexa 488 donkey anti-rabbit IgG and Alexa 594 donkey anti-goat IgG were used, respectively. The slides were then mounted with cover slips using an aqueous anti-fade mounting agent (Molecular Probes). For those cells which were examined with a DNA counter stain, after the last antibody labeling step, the cells were treated with 4′-6′ diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at 100 ng ml^{-1}.

Stained cells were then viewed using a Leitz DMRB microscope (Leica, Deerfiels, IL) equipped with a 40× oil objective lens. Appropriate filter sets were used to distinguish between red and green emissions. Settings were optimized using positively stained cells. Images were captured using a cooled-head three-color high resolution DEI-750 analog camera (Optronics, Bolton, MA) using the same parameters (brightness/contrast). A fixed exposure time was used for direct comparison of the image intensity. Images were imported into a computerized imaging system, Image Pro-Plus 4.0 (Media Cybernetics, Silverspring, MD) and Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA) and similarly processed for presentation. Quantitation of foci was computerized using Image Pro-Plus. For dual staining experiments, images were merged and co-localization of foci examined. Overlapping foci appeared yellow. 100 cells were counted for each experiment and cells were scored as positive for staining based on comparison with the control pre-immune serum-treated cells.

Immunoprecipitation and western blotting

For immunoprecipitation (IP), chromatin-associated protein extracts from normal cells were utilized. For this, cell nuclei were isolated and the chromatin-associated proteins were extracted in a series of steps as described previously (Lambert et al., 1992; Hang et al., 1993). For anti-α-spectrin IPs, anti-α-spectrin or mouse IgG$_1$ (Sigma-Aldrich) was bound to protein-G-coated agarose beads (Sigma-Aldrich) and the binding reactions and IPs were carried out as previously described (McMahon et al., 1999; McMahon et al., 2001). For IP, anti-α-spectrin was used because our anti-αSpIIΣ* is of the IgM class and cannot be used effectively in IPs (McMahon et al., 1999). For anti-FANCA IPs, affinity-purified rabbit polyclonal antisera generated from the C-terminal region of the FANCA protein, or pre-immune serum, was bound to protein-A-coated agarose beads (Sigma-Aldrich) and the binding reactions and IPs carried out as previously described

(McMahon et al., 1999). For anti-XPF immunoprecipitation, anti-XPF (an affinity-purified polyclonal antibody against the XPF protein, a generous gift of Michael Thelen, Lawrence Livermore National Laboratory) was bound to protein-A-coated agarose beads as described (McMahon et al., 1999). All of the IPs were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted as previously described (McMahon et al., 1999; McMahon et al., 2001). Immunoblots were developed using Pierce Ultra chemiluminescent substrate (Pierce) and then exposed to X-ray film (McMahon et al., 1999; McMahon et al., 2001). The primary antibodies used were anti-αSpIIΣ* (Brois et al., 1999; McMahon et al., 1999), anti-FANCA (C-terminal) (McMahon et al., 2001) and anti-XPF (from M. Thelen). Images were scanned using a Hewlett-Packard ScanJet 4c/T scanner and analyzed with ImageQuant (Molecular Dynamics).

Results

Relocalization of αSpIIΣ* to damage-induced foci in normal cell nuclei after exposure to a cross-linking agent

Studies were undertaken to determine the localization of αSpIIΣ* in the nucleus of undamaged normal human lymphoblastoid cells and to ascertain whether this localization changes in response to exposure to a DNA interstrand cross-linking agent, 8-MOP plus UVA light. Indirect immunofluorescence of the normal cells using purified monoclonal anti-αSpIIΣ* or anti-α-spectrin antibodies showed that, in undamaged cells, the nuclear staining pattern for αSpIIΣ* was diffuse and fairly homogenous, with a slight concentration of staining along the nuclear membrane (Fig. 1, upper panel). Approximately 25-30% of the cells were stained. In ~5% of these cells, instead of a diffuse staining pattern, only one or two large foci were observed in the nucleus. 15 hours following exposure of the cells to 8-MOP plus UVA light (6 kJ m^{-2}), αSpIIΣ* redistributed to distinct foci throughout the nucleus (Fig. 1, upper panel). That the foci were present in the nucleus was confirmed by staining of these cells with DAPI, which stains nuclear DNA (Fig. 1, middle panel). Overlaying the fluorescent signal for DAPI (blue) with that of αSpIIΣ* showed that these two areas coincided and that the area that stained for αSpIIΣ* was in the nucleus (Fig. 1, bottom panel). In normal cells treated with 8-MOP but no UVA light or UVA light with no 8-MOP, no αSpIIΣ* foci were observed 15 hours following exposure (data not shown).
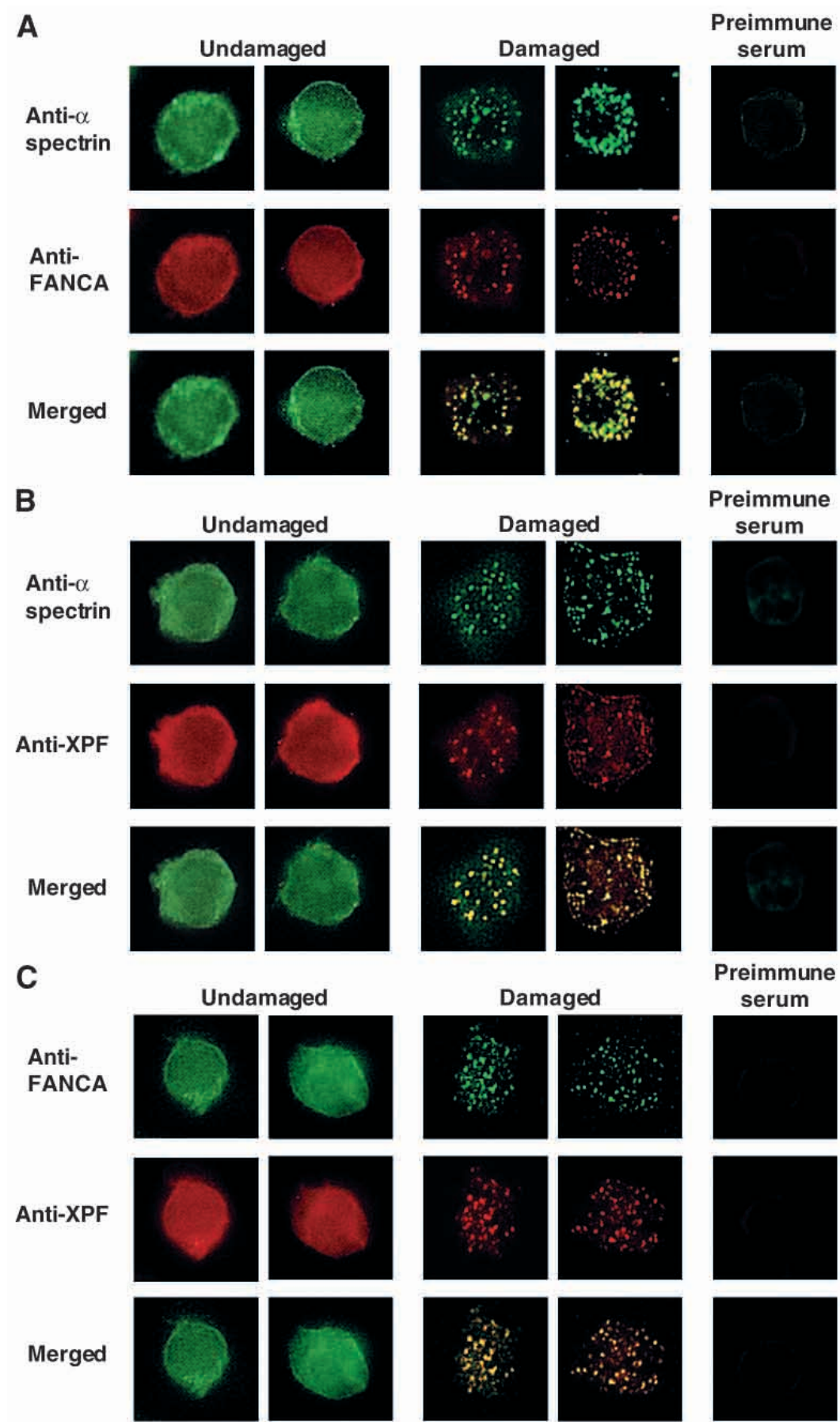
Fig. 2. αSpIIΣ*, FANCA and XPF co-localize to discrete nuclear foci after treatment of normal cells with 8-MOP plus UVA light. Normal human lymphoblastoid cells were either undamaged or treated with 8-MOP plus UVA light (6 kJ m^{-2}) and the localization of αSpIIΣ*, FANCA and XPF in the nucleus examined 15 hours after treatment with 8-MOP plus UVA. (A) Dual staining was carried out using affinity-purified monoclonal anti-α-spectrin antibody and affinity-purified polyclonal anti-FANCA antibody, and stained cells were analyzed by immunofluorescence. When the fluorescent signals for αSpIIΣ* (green) and FANCA (red) are merged, the overlapping foci are yellow, indicating co-localization of these two proteins. (B) An analysis similar to that in (A) was carried out using anti-α-spectrin antibody (green) and affinity-purified polyclonal anti-XPF antibody (red). Fluorescent signals for both proteins were merged and overlapping foci appear yellow. (C) Dual staining was also carried out using anti-FANCA (green) and anti-XPF (red) antibodies. The fluorescent signals were merged and overlapping foci are yellow. In all of the above experiments, cells were also stained with the appropriate preimmune sera.

Fig. 3. The number of αSpIIΣ* nuclear foci increases with increasing dosage of 8-MOP plus UVA light. Formation of αSpIIΣ* foci in the nuclei of normal human lymphoblastoid cells was examined 15 hours after exposure to varying dosages of 8-MOP plus UVA light. The dosage of 8-MOP plus UVA light was increased by increasing the levels of UVA light at a constant concentration of 8-MOP. Cells stained singly with anti-α-spectrin were examined. Foci were counted in 100 nuclei at each UVA dose. Nuclei were categorized as having 0-20 foci, 21-40 foci, 41-60 foci, 61-80 foci or 81-100 foci. The *y* axis indicates the percentage of nuclei in each category. Vertical lines represent s.e.m.
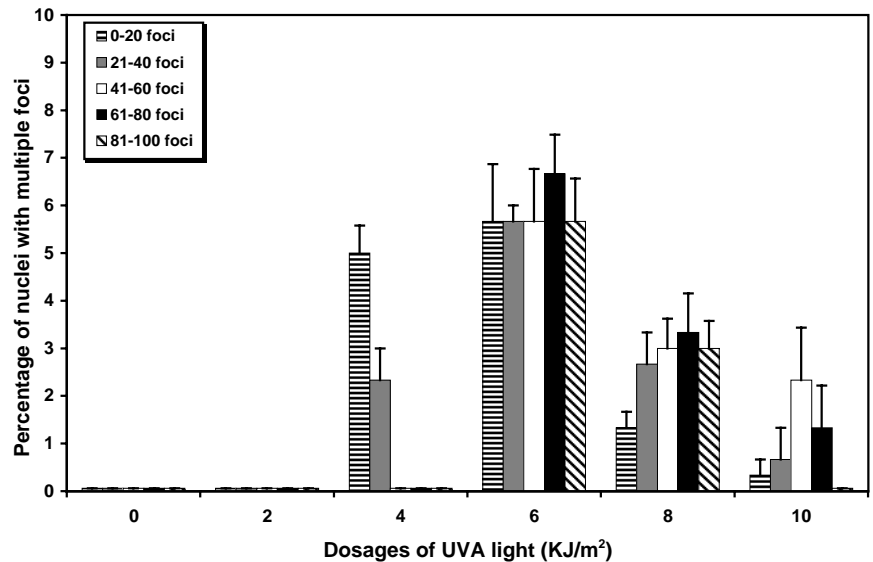
Co-localization of FANCA and XPF with αSpIIΣ* in nuclear foci in normal cells after DNA damage

In order to determine the localization of FANCA and XPF in the nucleus of normal human cells and whether they co-localize with αSpIIΣ* after exposure of the cells to 8-MOP plus UVA, immunofluorescence studies were carried out using a dual-staining technique. In undamaged normal cell nuclei, staining of the cells with an affinity-purified polyclonal anti-FANCA antibody showed that FANCA, like αSpIIΣ*, is present in a diffuse and fairly homogenous pattern in the undamaged nucleus (Fig. 2A). When the cells were treated with 8-MOP plus UVA light, FANCA was observed to relocalize to prominent nuclear foci (Fig. 2A). Merging of the fluorescent signals for αSpIIΣ* (green) and FANCA (red) showed that there was clear co-localization of most of these foci (Fig. 2A). The merged foci appeared yellow. For these studies, staining was carried out using either purified monoclonal anti-αSpIIΣ* antibody or purified monoclonal anti-α-spectrin antibody. The results using either antibody were the same. We have previously shown that these two antibodies both recognize αSpIIΣ* (McMahon et al., 1999). Similarly, staining with an affinity-purified polyclonal anti-XPF antibody showed that XPF was also present in a diffuse pattern in the nucleus of undamaged normal cells and that XPF relocalized to prominent damaged induced foci after exposure of the normal cells to 8-MOP plus UVA light (Fig. 2B). Merging of the fluorescent signals for αSpIIΣ* (green) and XPF (red) showed that there was co-localization of these foci (Fig. 2B). This same pattern of relocalization of FANCA and XPF to nuclear foci that was observed after double staining of the proteins was also observed in cells singly stained for FANCA and XPF (data not shown). This result supports the above finding that both FANCA and XPF relocalize to damage-induced foci after treatment with 8-MOP plus UVA light. Cells stained with preimmune serum showed a slight signal for αSpIIΣ* under these experimental conditions (Fig. 2A,B). This correlates with our previous finding that a very low level of αSpIIΣ* antibodies are present in this preimmune serum (McMahon et al., 1999). Little signal was observed for FANCA and XPF in cell nuclei stained with preimmune serum (Fig. 2A-C).

In order to verify that FANCA and XPF are co-localizing to the same sites after exposure to 8-MOP plus UVA, experiments were carried out in which dual staining of both FANCA and XPF was examined. The results show that, in normal cell nuclei, after treatment with 8-MOP plus UVA light, FANCA foci co-localized with the XPF foci (Fig. 2C). Collectively, these results indicate that, in response to an agent that produces DNA interstrand cross-links, FANCA and XPF co-localize to the same foci as αSpIIΣ*.

Dosage of 8-MOP plus UVA light affects levels of nuclear foci formation

Formation of foci in normal cell nuclei after exposure to 8-MOP plus UVA light was quantitatively assessed to determine whether there was a relationship between the number of cells displaying foci and the number of damage-induced foci per nucleus, and the dosage of 8-MOP plus UVA light used. For these studies, cells stained singly with either anti-α-spectrin, anti-FANCA or anti-XPF antibodies were examined 15 hours after exposure to 8-MOP plus UVA. The dosage of 8-MOP plus UVA was increased by increasing the levels of UVA light at a constant concentration of 8-MOP. As shown in Fig. 3, as the dosage of UVA light was increased from 0 kJ m^{-2} to 2 kJ m^{-2}, 4 kJ m^{-2} and 6 kJ m^{-2}, the percentage of nuclei showing αSpIIΣ* foci increased from 0% to 29%. The number of foci per nucleus also increased in a UVA-dose-dependent manner, with the greatest number of foci forming at 6 kJ m^{-2} (an average of 51 foci per nucleus). For these experiments, 100 nuclei were counted for each dosage of UVA light and each experiment was repeated three times. The viability of these cells was 94% in undamaged cells and 93%, 92% and 91.5% at 2 kJ m^{-2}, 4 kJ m^{-2} and 6 kJ m^{-2}, respectively. As the dose of UVA light was increased to 8 kJ m^{-2} and 10 kJ m^{-2}, the number of αSpIIΣ* foci per nucleus and the number of cells showing nuclear foci decreased (Fig. 3). However, the viability of these cells also decreased, to 75.5% and 58%, respectively, at these higher dosages.

Similar results were obtained for the XPF nuclear foci and the FANCA nuclear foci (data not shown). In both of these instances, as the dosage of UVA light was increased stepwise
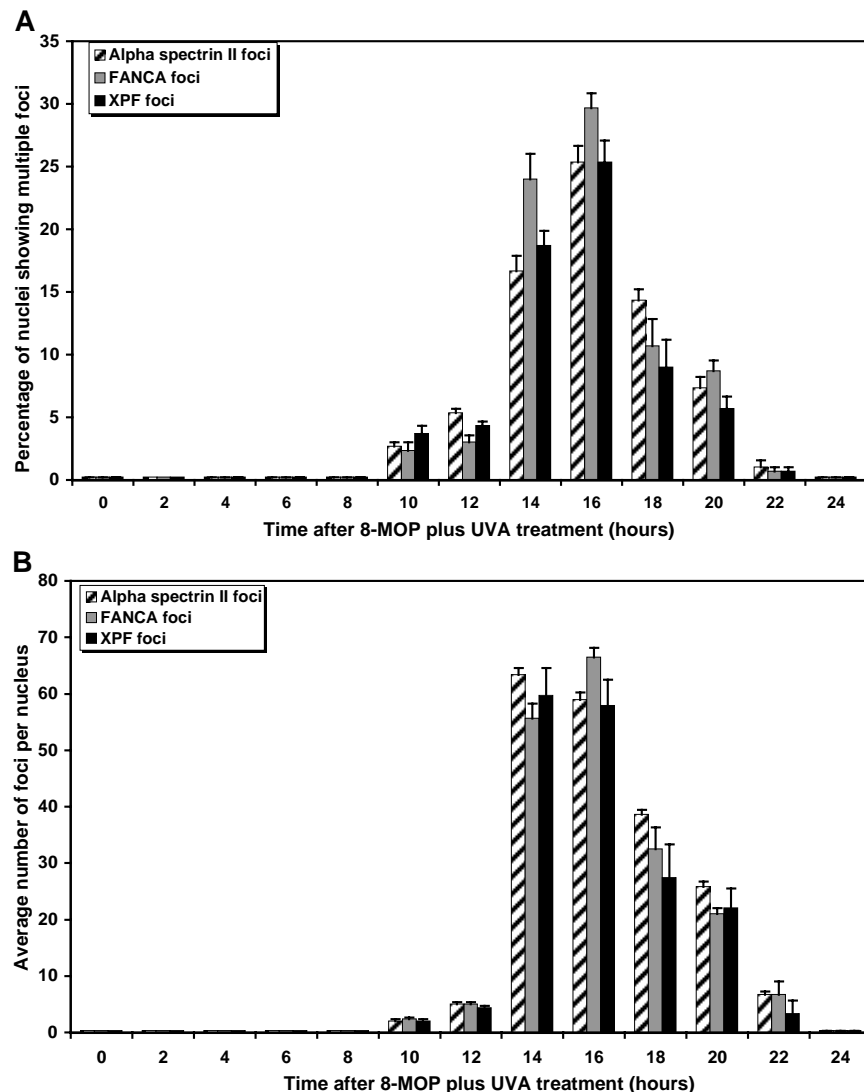
A

B

sample at each time point. Each of these experiments was repeated three times. As seen in Fig. 4A, nuclei showing αSpIIΣ* foci were first visible between 8 hours and 10 hours after exposure to 8-MOP plus UVA, and the number of nuclei showing foci increased with time and peaked at 16 hours. By 24 hours after exposure, nuclei showing αSpIIΣ* foci were no longer observed and αSpIIΣ* showed a diffuse pattern of staining in the nucleus, similar to untreated cells. FANCA and XPF nuclear foci also first appeared 8-10 hours after exposure to 8-MOP plus UVA (Fig. 4A). The number of nuclei showing FANCA and XPF foci were similar and increased up to 16 hours just as for αSpIIΣ*. By 24 hours, nuclei containing FANCA and XPF foci were also no longer visible and a diffuse pattern of staining for these proteins in the nucleus was observed as it was for αSpIIΣ*. These results show that αSpIIΣ*, FANCA and XPF foci appear in the nucleus at the same time after exposure to 8-MOP plus UVA light.

The average number of αSpIIΣ*, FANCA and XPF foci per nucleus also increased up to a period between 14 hours and 16 hours after exposure to 8-MOP plus UVA (Fig. 4B). These numbers were similar for αSpIIΣ*, FANCA and XPF. After 16 hours, the number of foci per nucleus decreased for each of these proteins. No foci were observed by 24 hours.

Failure of localization of αSpIIΣ*, FANCA and XPF to nuclear foci in FA-A cells after damage

In FA-A cells, there is a deficiency in levels of αSpIIΣ* that correlates with a defect in ability to repair DNA interstrand cross-links produced by either TMP or 8-MOP plus UVA light (Brois et al., 1999; Lambert and Lambert, 1999; McMahon et al., 1999; Kumaresan and Lambert, 2000). This makes these cells an excellent source for studying the influence of αSpIIΣ* on formation of damage-induced nuclear foci, particularly because levels of the DNA repair protein XPF are normal in these cells (Brois et al., 1999). Studies were undertaken to determine the localization of αSpIIΣ* in the nuclei of

from 0 kJ m^{-2} to 6 kJ m^{-2}, both the number of nuclei showing foci and the average number of foci per nucleus increased to levels comparable to those of the αSpIIΣ* foci. At 6 kJ m^{-2}, the percentage of nuclei showing FANCA and XPF foci was 25% and 27%, respectively, and the average number of foci per nucleus was 57 for FANCA and 49 for XPF. At 8 kJ m^{-2} and 10 kJ m^{-2}, the average number of FANCA and XPF foci decreased as it did for αSpIIΣ*. These results show that there is a relationship between the number of αSpIIΣ*, FANCA and XPF nuclear foci formed per cell and the percentage of cells showing nuclear foci with the dose of 8-MOP plus UVA light the cells are exposed to.

Time course of the formation of nuclear foci

To further examine the characteristics of nuclear foci formation, the time course for formation of αSpIIΣ*, FANCA and XPF nuclear foci following exposure of normal cells to 8-MOP plus UVA (6 kJ m^{-2}) was investigated. For these studies, cells were fixed at various periods of time after treatment and stained independently for either αSpIIΣ*, FANCA or XPF. The number of foci per nucleus was counted in 100 cells for each

undamaged FA-A cells and FA-A cells exposed to 8-MOP plus UVA. In addition, the localization of XPF and FANCA was examined in these cells. It was found that, in undamaged FA-A cells, a diffuse pattern of staining was observed with αSpIIΣ*, but that this staining was much fainter than in normal cells (Fig. 5A). This correlates with the reduced levels of αSpIIΣ* in FA-A cells (Brois et al., 1999; McMahon et al., 1999). When the FA-A cells were damaged with 8-MOP plus UVA, αSpIIΣ* was shown to relocalize to a few foci in the nucleus (Fig. 5A), but there were far fewer foci than in damaged normal cell nuclei. The mean number of αSpIIΣ* foci per nucleus 15 hours after treatment was 12 (20% of normal). No staining for FANCA was observed above background levels in the nuclei of FA-A cells, either undamaged or damaged with 8-MOP plus UVA (Fig. 5A). This correlates with reports of lack of detectable levels of this protein in this FA-A cell line (HSC 72) owing to the mutation in the *FANCA* gene (Kupfer et al., 1997; de Winter et al., 2000c).

Examination of the XPF protein in FA-A cell nuclei showed that a diffuse staining pattern of XPF was present at levels similar to normal (Fig. 5B), which is consistent with our finding that levels of XPF are similar in FA-A cells and normal cells (Brois et al., 1999). However, in FA-A cells damaged with 8-MOP plus UVA, XPF only relocalized to a few foci in the nucleus and mainly showed a more diffuse pattern of staining (Fig. 5B). The average number of foci per nucleus 15 hours after treatment was 11 (18% of normal). Merging of the fluorescent signals for αSpIIΣ* and XPF showed co-localization of only a few foci (Fig. 5B). To verify this deficiency in localization of XPF to damage-induced foci after treatment with 8-MOP plus UVA, experiments were carried out in which dual staining for XPF and FANCA was examined. The results show that XPF again relocalized to only a few damage-induced nuclear foci (Fig. 5C). No staining for FANCA was observed above background levels. Thus in these FA-A cells, in which there is a deficiency in αSpIIΣ*, XPF was not able to relocalize to many discrete nuclear foci after damage with a DNA interstrand cross-linking agent.

αSpIIΣ*, FANCA and XPF co-localize to nuclear foci in corrected FA-A cells after DNA cross-link damage

When FA-A cells express the *FANCA* cDNA, the DNA repair defect and the deficiency in levels of αSpIIΣ* and FANCA are corrected; in our corrected FA-A cells, we have also found that these levels are actually slightly greater than normal (Brois et al., 1999). Studies were therefore undertaken to determine whether, in the corrected FA-A cells, the defect in ability of αSpIIΣ*, FANCA and XPF to localize to nuclear foci after DNA damage had been corrected. In the nuclei of corrected undamaged FA-A cells (HSC72) transduced with a retroviral vector expressing the *FANCA* cDNA, a diffuse staining pattern was observed for αSpIIΣ* and FANCA just as in normal cell nuclei (Fig. 6A). In corrected FA-A cells exposed to 8-MOP plus UVA, αSpIIΣ* relocalized to discrete nuclear foci as in normal cells (Fig. 6A). The average number of αSpIIΣ* foci per nucleus at 15 hours after treatment with 8-MOP plus UVA light was 66 (108% of normal). Similarly, FANCA relocalized to foci in the nucleus after damage (Fig. 6A). At 15 hours after damage, the average number of FANCA foci per nucleus was 68 (111% of normal). Merging the fluorescent signals for αSpIIΣ* and FANCA showed that these proteins localized to the same discrete foci in the nucleus of corrected FA-A cells exposed to 8-MOP plus UVA light (Fig. 6A).

Similar results were obtained for XPF in the corrected FA-A cells. In the nuclei of untreated FA-A cells, XPF was present in a diffuse pattern (Fig. 6B), as it was in the uncorrected FA-A cells. However, in the corrected FA-A cells, XPF relocalized to discrete nuclear foci after treatment with 8-MOP plus UVA (Fig. 6B), as it did in damaged normal cells. At 15 hours after damage, the average number of XPF foci per nucleus was 62 (105% of normal). XPF also co-localized with αSpIIΣ* to the same nuclear foci (Fig. 6B). Dual-staining experiments for both XPF and FANCA showed that these two proteins co-localized to the same damage-induced nuclear foci (Fig. 6C). These results indicate that correcting the deficiency in levels of αSpIIΣ* in FA-A cells restores the ability of XPF to relocalize to nuclear foci after the cells are damaged with 8-MOP plus UVA light.

αSpIIΣ*, FANCA and XPF bind to each other

The above immunofluorescence studies show that αSpIIΣ*, FANCA and XPF localize to the same nuclear foci in normal cells after exposure to 8-MOP plus UVA, and suggest that these proteins interact with each other in the nucleus. Studies were undertaken to provide additional evidence for the existence of this interaction using immunoprecipitation. A series of IPs were carried out using chromatin-associated-protein extracts from normal human lymphoblastoid cells. Anti-α-spectrin IP and immunoblotting with anti-αSpIIΣ*, anti-FANCA or anti-XPF demonstrated that FANCA and XPF co-immunoprecipitated with αSpIIΣ* (Fig. 7A). IP using anti-FANCA and immunoblotting with anti-αSpIIΣ*, anti-FANCA or anti-XPF showed that XPF and αSpIIΣ* co-immunoprecipitated with FANCA (Fig. 7B). Anti-XPF immunoprecipitation and immunoblotting with anti-αSpIIΣ*, anti-FANCA or anti-XPF demonstrated that FANCA and αSpIIΣ* co-immunoprecipitated with XPF from the normal extracts (Fig. 7C). These IP studies thus further confirm that αSpIIΣ*, FANCA and XPF interact with each other in the nucleus, although whether this interaction is direct or indirect is not yet clear.

Discussion

The precise events that take place during the repair of DNA interstrand cross-links in mammalian cells, the proteins involved and their interactions with each other are still poorly understood. The present study demonstrates that three proteins, αSpIIΣ*, FANCA and XPF, localize to the same nuclear foci after damage of normal human cells with the DNA interstrand cross-linking agent 8-MOP plus UVA light, and that αSpIIΣ* is important for this re-localization to damage-induced nuclear foci. In addition, these studies provide evidence that αSpIIΣ*, FANCA and XPF interact with each other in the nucleus. These results support a model in which αSpIIΣ* binds to cross-linked DNA and aids in the recruitment of repair proteins to the site of damage, where it acts as a scaffold upon which these repair proteins align or spatially orient themselves, thus increasing the efficiency of the repair process (McMahon et al., 2001). According to this model, when there is a deficiency in αSpIIΣ*,
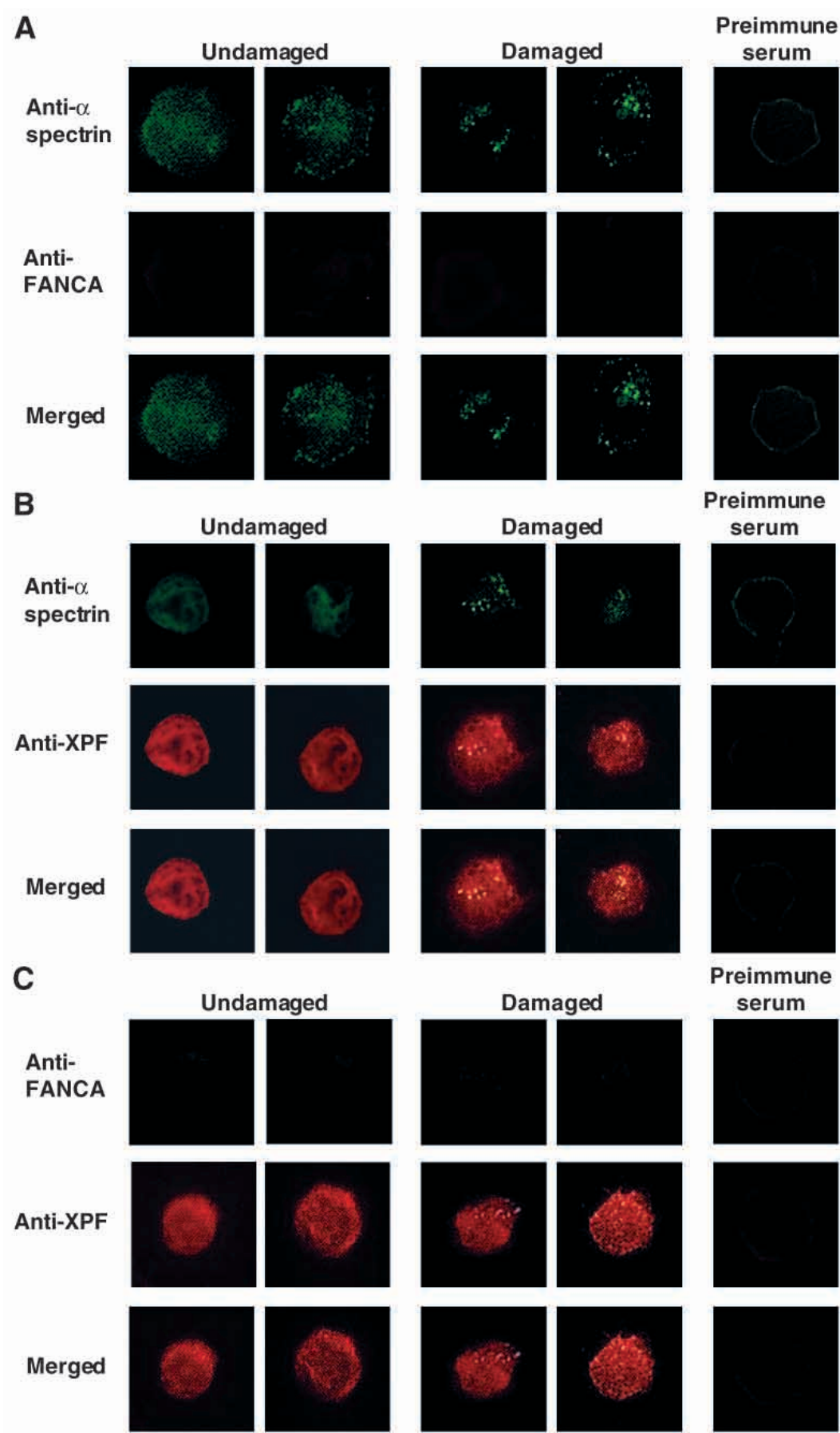
Fig. 5. Failure of localization of αSpIIΣ*, FANCA and XPF to nuclear foci in FA-A cells after treatment with 8-MOP plus UVA light. FA-A (HSC 72) lymphoblastoid cells were either undamaged or treated with 8-MOP plus UVA light (6 kJ m^{-2}) and localization of αSpIIΣ*, FANCA and XPF in the nuclei examined 15 hours after treatment using immunofluorescence. Cells were in addition stained with the appropriate preimmune serum. (A) Dual staining was carried out using anti-α-spectrin (green) and anti-FANCA (red) antibodies. Fluorescent signals were not observed for FANCA. Only low levels of αSpIIΣ* were observed in undamaged nuclei and a small number of αSpIIΣ* foci were observed in the damaged nuclei. Merged signals show only staining for αSpIIΣ*. (B) Similar analysis as in (A) was carried out using anti-α-spectrin (green) and anti-XPF (red) antibodies. Fluorescent signals for both proteins were merged. The yellow dots in the merged images indicate co-localization of both proteins. (C) Dual staining using anti-FANCA (green) and anti-XPF (red) was carried out. Only a few damage-induced foci were observed for XPF in the treated cells and none for FANCA. Merged signals show only staining for XPF.

as is observed in FA-A cells, there is decreased binding of αSpIIΣ* to damaged DNA and decreased recruitment and alignment of repair proteins at sites of damage. This alignment would be particularly important in the repair of DNA interstrand cross-links where recombination might be involved. Reduced levels of αSpIIΣ* in the nucleus would thus be expected to reduce the efficiency of the repair process in FA cells rather than to inhibit it altogether, consistent with our experimental findings (Brois et al., 1999; McMahon et al., 2001).

There is evidence that repair of DNA interstrand cross-links in mammalian cells involves elements of both nucleotide excision repair (NER) and recombination (Calsou et al., 1996; Thompson, 1996; Li et al., 1999; De Silva et al., 2000; Kuraoka at al., 2000; Wang et al., 2001; Dronkert and Kanaar, 2001; Kumaresan and Lambert, 2000; Kumaresan et al., 2002) and that double-strand breaks might form as intermediates in this repair process (De Silva et al., 2000; Zhang et al., 2002). There might be more than one pathway for the repair of DNA interstrand cross-links (Bessho et al., 1997; Li et al., 1999; De Silva et al., 2000; Wang et al., 2001; Kumaresan and Lambert, 2000; Dronkert and Kanaar, 2001). Our studies have previously shown that αSpIIΣ*, FANCA and XPF are involved in the initial damage recognition and incision steps of the repair process (McMahon et al., 1999; Kumaresan and Lambert, 2000; McMahon et al., 2001; Kumaresan et al.,

Fig. 6. In corrected FA-A cells, αSpIIΣ*, FANCA and XPF co-localize to discrete nuclear foci after DNA cross-link damage. FA-A (HSC 72) cells expressing the *FANCA* cDNA were either undamaged or treated with 8-MOP plus UVA (6 kJ m^{-2}) and examined for the presence of αSpIIΣ*, FANCA or XPF in the nuclei. Cells were fixed 15 hours after treatment. (A) Immunofluorescence with anti-α-spectrin (green) and anti-FANCA (red) antibodies was examined. When the green and red signals were merged, the overlapping foci appeared yellow, indicating co-localization of αSpIIΣ* and FANCA. (B) An analysis similar to that in (A) was carried out using anti-α-spectrin (green) and anti-XPF (red) antibodies. The fluorescent signals were merged and analyzed as above. (C) Dual staining with anti-FANCA (green) and anti-XPF (red) antibodies was also examined. Again, the fluorescent signals were merged and overlapping foci appear yellow.
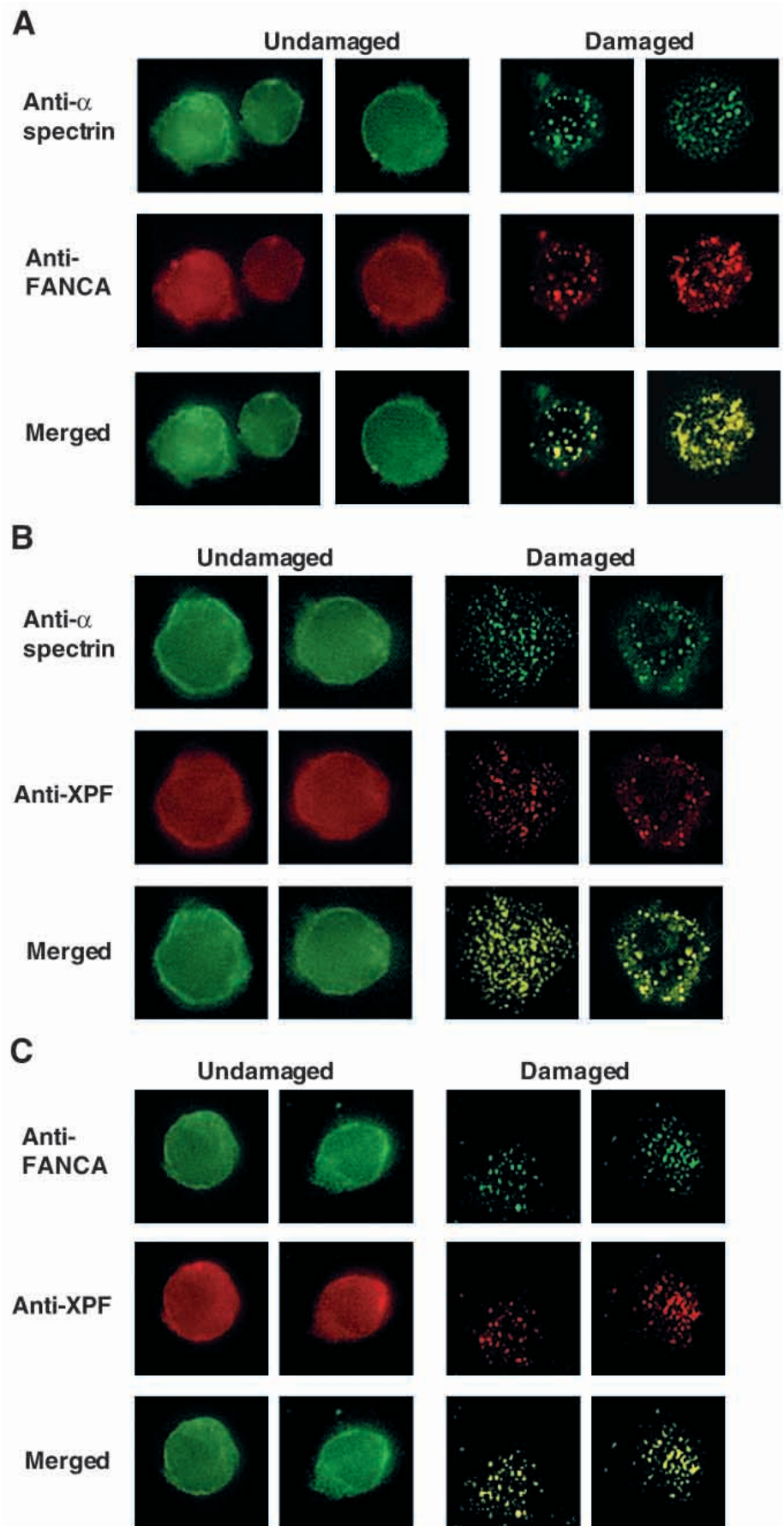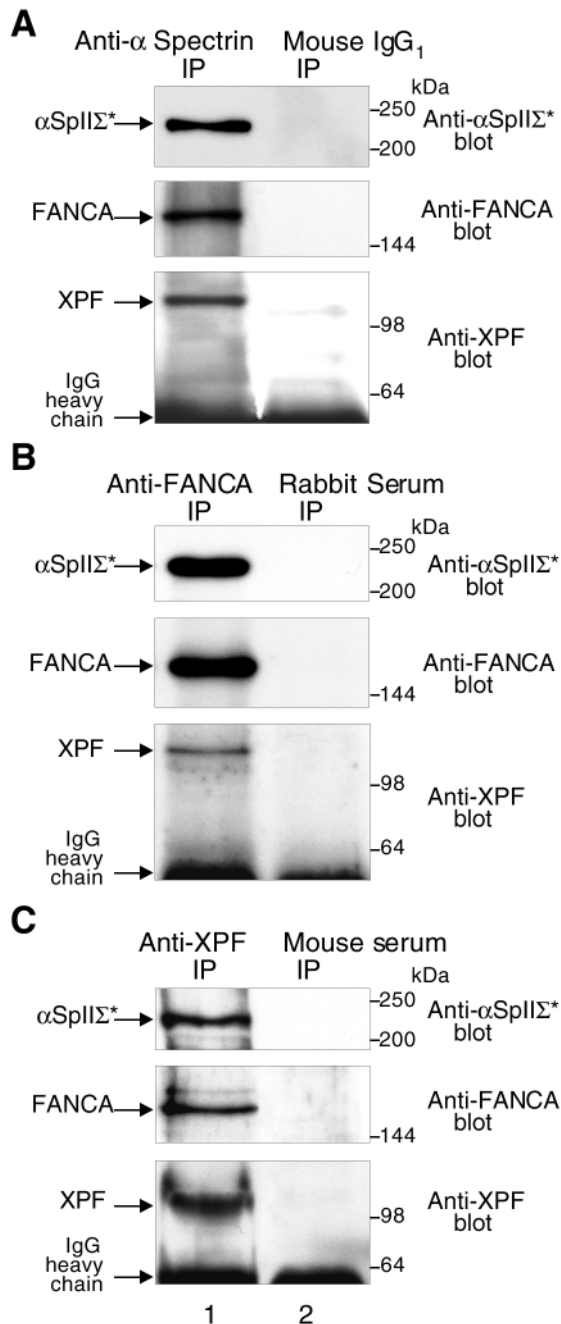
Fig. 7. Binding of αSpIIΣ*, FANCA and XPF to each other in the nucleus. Chromatin-associated proteins extracts from normal human lymphoblastoid cells were immunoprecipitated with anti-α-spectrin, anti-FANCA and anti-XPF antibodies. (A) Immunoprecipitation was carried out using anti-α-spectrin antibody or mouse IgG₁ (as a control). (B) Proteins were immunoprecipitated using anti-FANCA antibody or rabbit serum. (C) Immunoprecipitation was done using anti-XPF antibody and mouse serum. In all of the above immunoprecipitations (A-C), immunoblot analysis was carried out using anti-αSpIIΣ* (top panel), anti-FANCA (second panel) or anti-XPF (third panel).

2002), though this does not preclude their involvement in subsequent steps as well. The evidence for this is that α spectrin binds directly to DNA containing a TMP interstrand

cross-link and FANCA also binds to this cross-linked DNA, although whether this binding is direct or indirect is not clear (McMahon et al., 2001). A mAb against αSpIIΣ* (McMahon et al., 2001) and a polyclonal antibody against FANCA (M.W.L., L.W.M. and K. Kumaresan, unpublished) inhibit the dual incisions we observe at sites of a TMP interstrand cross-link. A mAb against XPF also specifically inhibits the 5′ and 3′ incisions we observe at the site of a cross-link (Kumaresan and Lambert, 2000), and XPF cells, deficient in the XPF protein, are defective in ability to produce dual incisions at sites of cross-links (Kumaresan and Lambert, 2000). In addition, Kuraoka et al. (Kuraoka et al., 2000) have also shown that XPF-ERCC1 is involved in production of the 5′ and 3′ incisions at the sites of DNA interstrand cross-links. However, although our studies indicate that these three proteins play a role in repair of DNA interstrand cross-links, the exact relationship between them has not yet been elucidated.

Two different approaches were used in the present study to examine the relationship between αSpIIΣ*, FANCA and XPF. One was to investigate whether any of these proteins co-localize in the nucleus after damage with a DNA interstrand cross-linking agent; the other was to determine whether any of these proteins interact with each other as ascertained by immunoprecipitation. Immunofluorescence studies using dual-staining techniques showed that, after normal cells were treated with 8-MOP plus UVA, αSpIIΣ*, FANCA and XPF changed their localization in the nucleus and co-localized to the same discrete nuclear foci. Time course experiments on foci formation showed that the appearance of FANCA and XPF foci coincide with that of αSpIIΣ* foci. The co-localization of these three proteins to the same foci after damaging the DNA with 8-MOP plus UVA indicates that they might act in concert and play a role together in the repair of DNA interstrand cross-links. The average number of αSpIIΣ*, FANCA and XPF foci per nucleus as well as the number of nuclei showing foci increased with increasing dosage of 8-MOP plus UVA light, thus indicating that the foci assembled in response to DNA damage and that the number of foci depended on the levels of DNA damage. Presumably, these foci are forming at sites of damage. Other studies have shown that proteins involved in DNA repair and checkpoint signaling pathways are relocalized to nuclear foci after DNA damage. These include XPG, RPA, FANCD2, BRCA1, Rad51, H2AX, 53BP1, BLM, hMLH1 and the Mre11-Rad50-Nbs1 complex (Park et al., 1996; Scully et al., 1997; Wang et al., 2000; Paull et al., 2000; Cantor et al., 2001; Anderson et al., 2001; Pedrazzi et al., 2001; Garcia-Higuera et al., 2001; Choudhary and Li, 2002). The increased local concentration of these proteins has been proposed to facilitate their various enzymatic activities and their functioning in processes such as signal transduction (Anderson et al., 2001). In the present study, the number of foci formed peaked between 14-16 hours, then started to decrease and was back to background levels at 24 hours. Presumably, this reflected the repair of cross-links and dispersal of the proteins in the foci at the completion of the repair process.

Immunoprecipitation studies showed that αSpIIΣ*, FANCA and XPF have binding affinity for each other, although whether this binding is direct or indirect is not yet clear. We have previously reported, in separate experiments, that FANCA co-immunoprecipitates with αSpIIΣ* (McMahon et al., 1999) and that XPF co-immunoprecipitates

with αSpIIΣ* (McMahon et al., 2001). In the present study, co-immunoprecipitation of all three proteins in the same experiment was examined. When immunoprecipitation was carried out using anti-α-spectrin, FANCA and XPF were shown to co-immunoprecipitate with αSpIIΣ* from normal chromatin-associated proteins. The binding of these proteins to each other was confirmed by anti-FANCA and anti-XPF immunoprecipitation. This demonstrated association between these three proteins again indicates that they might be involved in a common biochemical pathway such as repair of DNA interstrand cross-links. Whether they form one complex at the site of damage or are part of at least two interacting complexes is not yet clear.

The use of FA cells in the present study has enabled us to get a much better understanding of the importance of αSpIIΣ* in the repair of DNA interstrand cross-links and its relationship to other proteins involved in the repair process. Because the deletion of α spectrin from a cell has been shown to be lethal (e.g. in *Drosophila melanogaster* and *Caenorhabditis elegans*) (Lee et al., 1993; Lee et al., 1997; Norman and Moerman, 2002), the FA-A cell line examined in the present study provides an excellent model for examination of the effects on the repair process of decreased levels of αSpIIΣ* (reduced to 30-35% of normal) in the nucleus. When FA-A cells were treated with 8-MOP plus UVA light, the reduction in number (but not elimination) of αSpIIΣ* nuclear foci correlated with decreased levels of αSpIIΣ* in the FA-A nuclei, which was quantitatively assessed by examination of the αSpIIΣ* band on an SDS gel that was electroblotted onto a nitrocellulose membrane and stained with colloidal gold (Brois et al., 1999; McMahon et al., 1999). This decrease in αSpIIΣ* levels in FA-A cells correlated in turn with a decreased number of endonucleolytic incisions produced at the site of a TMP interstrand cross-link by chromatin-associated-protein extracts from FA-A cells and with the observed reduction of DNA repair levels, measured as unscheduled DNA synthesis, in these cells (Brois et al., 1999; Kumaresan and Lambert, 2000). All these values were approximately 25-35% of those of normal cells (Brois et al., 1999; Kumaresan and Lambert, 2000). Preliminary studies using a FA-C cell line (HSC 536) indicate that there is a similar correlation between decreased levels of αSpIIΣ*, a decreased number of αSpIIΣ* nuclear foci after damage with 8-MOP plus UVA light and decreased repair of interstrand cross-links in these cells. In addition, the present demonstration that, in FA-A cells, there is a markedly reduced recruitment of XPF to nuclear foci after damage with 8-MOP plus UVA light, compared with normal cell nuclei, further supports our model that αSpIIΣ* is involved in targeting repair proteins to sites of damage and that, when levels of αSpIIΣ* are significantly reduced, so is the recruitment of repair proteins to these damage sites. Because the FANCA protein is absent in this FA-A cell line, it is also possible that it is needed for formation of XPF foci. However, the present study shows that, in the absence of FANCA, there is still a low level of αSpIIΣ* and XPF focus formation and that the number of foci per nucleus correlates with the levels of αSpIIΣ* present in FA-A cell nuclei (McMahon et al., 1999). If FANCA were essential for the formation of these foci, no foci should be observed in this FA-A cell line.

The present studies also show that a functional *FANCA* gene is essential for the re-localization of αSpIIΣ*, FANCA and XPF to damage-induced nuclear foci. In corrected FA-A cells, which express the *FANCA* cDNA, αSpIIΣ*, FANCA and XPF again co-localize to nuclear foci and both the number of foci per nucleus and the number of nuclei showing foci are restored to normal. This correlates with return to levels of αSpIIΣ* and FANCA in the nucleus that are slightly greater than normal and of levels of DNA repair (unscheduled DNA synthesis) that are slightly higher than normal (McMahon et al., 1999; Brois et al., 1999). It is possible that the FANCA protein is needed for stability of αSpIIΣ* in the nucleus or that the *FANCA* gene is involved in regulating the expression of αSpIIΣ*. Our studies show that levels of *αSpIIΣ** mRNA are the same in FA-A cells as in normal cells, which would indicate that reduced levels of αSpIIΣ* in the FA-A nucleus are not due to decreased expression of αSpIIΣ* (J. Lefferts and M.W.L., unpublished). It is possible that FANCA and other FANC proteins are involved in the stability of αSpIIΣ*. Studies indicate that FANCA, FANCC, FANCE, FANCF and FANCG form a complex in the nucleus; they further suggest that the presence of each of these proteins is important for the stability of the complex and that, in the absence of any of these proteins, this complex is disrupted (Kupfer et al., 1997; Yamashita et al., 1998; Garcia-Higuera et al., 1999; Waisfisz et al., 1999; de Winter et al., 2000c; Reuter et al., 2000; Garcia-Higuera et al., 2000; Medhurst et al., 2001; Siddique et al., 2001; Pace et al., 2002; Taniguchi and D'Andrea, 2002). In the FA-A cells used in the present work, in addition to undetectable levels of FANCA, there are reduced levels of this protein complex (Yamashita et al., 1998; Garcia-Higuera et al., 1999; Garcia-Higuera et al., 2000). In the present work, the reduced levels of αSpIIΣ* observed in the FA-A cells could thus possibly be due not only to the reduced levels of FANCA in these cells but also to the reduced levels of this FA protein complex.

These studies show that there is localization of at least three different proteins in the nucleus of normal human cells to common sites after damage with a DNA interstrand cross-linking agent: one is a structural protein, αSpIIΣ*, which we hypothesize acts as a scaffolding protein; one an FA protein, FANCA; and one a DNA repair protein, XPF, which has been shown to be involved in repair of DNA interstrand cross-links (de Laat et al., 1998; Kumaresan and Lambert, 2000; Kuraoka et al., 2000; Kumaresan et al., 2002). The use of FA-A cells, which contain normal levels of XPF, in these studies enabled us to demonstrate that αSpIIΣ* is needed for the localization of XPF to these nuclear foci after damage with a DNA interstrand cross-linking agent and emphasizes the importance of αSpIIΣ* in the recruitment of repair proteins to sites of DNA damage. These results, combined with the present demonstration that these three proteins interact with each other and with our previous studies on the involvement of these proteins in the initial damage-recognition and incision steps of the repair process, greatly strengthen the concept that αSpIIΣ*, FANCA and XPF play an important role in the repair of DNA interstrand cross-links. In addition, they emphasize the importance of a structural protein, αSpIIΣ*, in aiding in the targeting and interaction of specific proteins in the nucleus, a role that could extend beyond an involvement in DNA repair to an involvement in other processes as well.

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