Deficient Regulation of DNA Double-strand Break Repair in Fanconi Anemia Fibroblasts

Received for publication, December 30, 2002, and in revised form, May 10, 2003 Published, JBC Papers in Press, May 14, 2003, DOI 10.1074/jbc.M213251200

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Fibroblasts from patients with Fanconi anemia (FA) display genomic instability, hypersensitivity to DNA cross-linking agents, and deficient DNA end joining. Fibroblasts from two FA patients of unidentified complementation group also had significantly increased cellular homologous recombination (HR) activity. Results described herein show that HR activity levels in patient-derived FA fibroblasts of groups A, C, and G were 10-fold greater than those seen in normal fibroblasts. In contrast, HR activity in group D2 fibroblasts was identical to that in normal cells. Western blot analysis revealed that the RAD51 protein was elevated 10-fold above normal levels in group A, C, and G fibroblasts, but was not altered in group D2 fibroblasts. HR activity levels in these former cells could be restored to near-normal levels by electroporation with anti-RAD51 antibody, whereas similar treatment of normal and complementation group D2 fibroblasts had no effect. These findings are consistent with a model in which FA proteins function to coordinate DNA double-strand break repair activity by regulating both recombinational and non-recombinational DNA repair. Interestingly, whereas positive regulation of DNA end joining requires the combined presence of all FA proteins thus far tested, suppression of HR, which is minimally dependent on the FANCA, FANCC, and FANCG proteins, does not require FANCD2.

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† The abbreviations used are: FA, Fanconi anemia; HR, homologous recombination; AP, apurinic/apyrimidinic.

‡ This work was supported by the Leukemia Research Fund, National Institutes of Health Grant AG12678, and Breast Cancer Research Program Grant DAMD17-99-1-9299 from the United States Department of Defense. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Fanconi anemia (FA)† is a fatal autosomal recessive disease that affects ∼1–5 million people worldwide (1). The clinical symptoms of FA are diverse and often include developmental anomalies affecting the skin, skeleton, and major organs (2, 3). However, the distinguishing features of FA, progressive pancytopenia and predisposition toward cancer, are the primary reasons for premature death in affected individuals (4–6). The genetic basis of FA is complex, and eight distinct complementation groups (A, B, C, D1, D2, E, F, and G) (7–9).

Cells cultured from FA patients display an abnormally high level of spontaneous chromosomal breaks and deletions (10–12). This effect is enhanced by exposure to bifunctional cross-linking agents such as mitomycin C and diepoxybutane (13–15). These cellular features, along with the propensity of FA patients to develop cancers, lead to the classification of FA as a DNA repair defect disorder (16). However, unlike other well defined DNA repair defect disorders such as xeroderma pigmentosum, ataxia telangiectasia, and Bloom’s syndrome, cloning of the genes defective in FA cells has not identified the molecular defect(s) responsible for the disease (17–26).

Investigation of DNA end joining activity both in intact patient-derived FA cells and in extracts prepared from these cells revealed a defect in DNA end joining activity (29–32). As this type of double-strand break repair is thought to occur in cells via a non-recombinational mechanism, these data indicate that FA cells have a deficiency in non-recombinational DNA repair. However, there is a growing body of evidence that also links FA proteins to recombinational DNA repair. First, the FANC B and FANC D1 genes were recently shown to be identical to BRCA2 (26). Although its precise function is not known, cells lacking functional BRCA2 protein are nearly completely unable to repair restriction enzyme-generated chromosomal double-strand breaks via homologous recombination (HR) (33). Second, the FANC D2 protein co-localizes with BRCA1 and RAD51 proteins to discrete nuclear foci following exposure to ionizing radiation (27, 34, 35). BRCA1-deficient cells are also unable to properly utilize HR to rejoin chromosomal double-strand breaks (36), and it is well established that the recombination protein RAD51 plays an essential catalytic role in recombinational DNA repair (37, 38). Since it is known that recombinational repair is an important mechanism in the repair of DNA cross-links (39), these results support the view that FA cells have a deficiency in HR. However, one potential difficulty with this interpretation is that direct examination of HR activity in fibroblasts from two unrelated FA patients of unidentified complementation group status failed to detect a defect in this activity (40). Instead, it was shown that the level of intraplasmid HR activity in these cells was elevated 10–20-fold above that seen in normal fibroblasts. Thus, the most consistent interpretation is that FA cells have a deficiency in the regulation of these pathways that repair DNA double-strand breaks.

To confirm the initial finding that FA cells have altered HR activity, as well as to gain insight into the nature of the regulation of DNA double-strand break repair pathways in FA cells, we first examined extrachromosomal HR activity in intact patient-derived FA cells of several known complementation groups. We found a 10-fold elevation in HR activity in FA cells belonging to complementation groups A, C, and G, which was reversed by genetic complementation via retroviral transduction. Furthermore, extracts from these cells also had an ∼10-fold increase in the level of RAD51 protein. Inhibition of RAD51
by co-electroporation of anti-RAD51 antibody into these cells restored HR activity to wild-type levels. Interestingly, FA fibroblasts of complementation group D2 did not display a similar elevation in either HR or RAD51 protein. Antibody-mediated inhibition of FANCDD2 in intact normal cells showed that only DNA end joining activity was affected, whereas similar treatment of normal cells with anti-FANCC antibody altered both DNA end joining and RAD51 antibody activities. These data support the conclusion that the FA proteins deficient in the cells studied herein are involved in the regulation of both recombination and non-combination DNA repair.

EXPERIMENTAL PROCEDURES

Cell Culture and Plasmid Constructs—Cells were maintained in a humidified 5% CO₂-containing atmosphere at 37 °C. All cell strains were obtained from the Oregon Health Sciences University Fanconi Anemia Cell Repository unless otherwise noted. Cell strains PD.715.F, PD.751.F, PD.792.F, PD.793.F, CCD-1059Sk, CCD-1056Sk, and CCD-1108Sk (American Type Culture Collection Cell Repository) are human diploid fibroblasts derived from normal subjects. The immortalized cell line HT1080 (American Type Culture Collection Cell Repository) was derived from a spontaneous human fibrosarcoma. Cell strains PD.720.F, PD.551.F, PD.145.F, and PD.352.F (referred to as A, C, D2, and D3, respectively) are human diploid FA fibroblasts from patients of complementation groups A, C, D2, and G, respectively. The diploid cell strain PD.20.F, derived from an unrelated FA patient of complementation group D2, was used to prepare the nuclear extract used for Western blot analysis. The former plasmid contains an intact NEO gene, which drives replication of plasmids containing an SV40 origin of replication (43). The latter plasmid encodes the SV40 large T-antigen, expression of which prevents replication of the pSV2neo plasmid (44). Different nonfunctional allele of the neomycin phosphotransferase gene (referred to as DR and DL), which are tandem repeat of two defective heteroalleles of the neomycin phosphotransferase gene, renders the plasmid nonfunctional. HR occurring between these heteroalleles can regenerate an intact and functional NEO gene, which confers resistance to the antibiotic ampicillin, as well as a tandem repeat of two defective heteroalleles of the neomycin phosphotransferase gene (NEO). Each allele contains a different, non-overlapping deletion within the NEO gene (referred to as DR and DL), which renders it nonfunctional. HR occurring between these heteroalleles can regenerate an intact and functional NEO gene, which confers resistance to the antibiotic kanamycin. Following plasmid electroporation, mammalian cells were incubated for 48 h. Plasmid DNA was then recovered from the cells (44) and incubated with the restriction enzyme DpnI to digest plasmids that did not replicate in mammalian cells. This recovered plasmid DNA was then introduced into DH10B electrocompetent reporter bacteria and plated onto LB agar-containing Petri dishes. Percent end joining was determined by dividing the number of bacterial colonies obtained from mammalian cells electroporated with linearized pSV2neo by the number of bacterial colonies obtained from mammalian cells electroporated with circular pSV2neo in a parallel experiment.

Preparation of Protein Extracts—Nuclear protein extracts were prepared as previously described (31) and were used for Western blot analysis. Whole cell protein extracts were prepared by washing cells once with RIPA buffer and scraping cells into culture media to homogenize the cell pellet. The homogenate was then sonicated and centrifuged at 7000 × g for 15 min. Subsequently, the lysed cell solution was collected and centrifuged at 7000 × g for 5 min at 4 °C. Protein concentration of the supernatant was determined using the Bradford assay (45), which is a colorimetric assay and was used to quantitate the amount of protein present in each sample. Each sample contained 100 μg of protein for Western blot analysis. Western blot analysis was performed as previously described (31). Briefly, antibody (0.4 μg) was co-electroporated into murine cells along with plasmid substrates for the indicated experiments.

RESULTS

FA Fibroblasts of Complementation Groups A, C, and G Have Elevated Extrachromosomal HR Activity—It was previously shown that cells derived from two unrelated FA patients of unknown complementation groups had HR levels that were significantly greater than those seen in fibroblasts derived from normal subjects (40). To confirm and extend this observation, an intraplasmid HR assay (40) was used to determine the
HR activity present in intact fibroblasts from FA patients from multiple known complementation groups. As shown in Fig. 1, FA fibroblasts from patients belonging to complementation groups A, C, and G all had significantly increased extrachromosomal HR frequency. The HR frequency observed was between 90 and 100 × 10⁻⁸. In contrast, the diploid cell strain PD.792.F, derived from a normal subject, had an HR frequency of only 9 × 10⁻⁸ (Fig. 1). Similar examination of three other cell strains from normal subjects revealed HR frequencies of 9 × 10⁻⁵, 10 × 10⁻⁵, and 10 × 10⁻⁵, respectively. These values were essentially identical to the HR frequencies of 9.5 × 10⁻⁵ and 9.9 × 10⁻⁵ previously reported in two different strains of diploid fibroblasts from unrelated normal donors (40). Thus, HR frequencies of complementation groups A, C, and G had a 9–10-fold increase in HR frequency. Interestingly, examination of HR in fibroblasts of complementation group D2 revealed that they did not have elevated HR activity (Fig. 1). Instead, these cells displayed HR activity of ~9 × 10⁻⁵, similar to that in all normal cells examined. The frequency with which the reporter bacteria catalyzed HR was determined to be <1 × 10⁻⁵, indicating that the HR frequency results obtained reflect recombination events occurring in the mammalian somatic cells.

Because concerns over the influence of the SV40 large T-antigen in DNA repair processes have been raised (46), a second set of HR experiments were performed in which plasmid pRSVEdl884 was not co-electroporated into cells along with the recombination plasmid substrate. In these experiments, in which the SV40 large T-antigen was absent, the recombination plasmid substrate did not replicate. Nevertheless, we observed that HR frequency in diploid FA fibroblasts of complementation groups A, C, and G was elevated 8-, 9-, and 8-fold, respectively, above that observed in diploid fibroblasts from normal donors (data not shown).

Retrovirus-mediated gene transfer with the appropriate cDNAs has been shown to render FA cells resistant to bifunctional cross-linking agents (18, 22–26). Furthermore, this treatment has been shown to eliminate the sensitivity of FA cells to the cytotoxic effects of restriction enzyme-induced chromosomal DNA double-strand breaks (32). Therefore, we examined HR frequency in the retrovirally corrected counterparts of FA cells that displayed elevated HR activity in Fig. 1. As shown in Table I, retrovirally corrected FA fibroblasts had HR frequencies that were not statistically different from those seen in normal diploid fibroblast cells. These data therefore show that the elevation of HR activity in FA fibroblasts of complementation groups A, C, and G is reversible and is not likely to be the consequence of spontaneous immortalization or secondary mutations affecting HR.

**Table I**

| Cells                                | HR frequency¹ |
|--------------------------------------|---------------|
| Normal fibroblasts                   | 9             |
| PD.792.F                             | 9             |
| FA fibroblasts                       |               |
| PD.720.F (A)                         | 99           |
| PD.551.F (C)                         | 94           |
| PD.352.F (G)                         | 81           |
| Retrovirally corrected FA            |               |
| 720-FAA (A<sub>cor</sub>)           | 15           |
| 551-FAC (C<sub>cor</sub>)           | 12           |
| 352-FAG (G<sub>cor</sub>)           | 14           |

¹ Expressed as the number of recombinant colonies/10⁸ non-recombinant colonies; average of six independent experiments.
² p < 0.05 compared with normal diploid fibroblasts.

**RAD51 Protein Is Overexpressed in HA Fibroblasts Displaying Elevated HR Activity—**RAD51 is the human homolog of the *Escherichia coli* recombination protein RecA (47), which functions in DNA double-strand break repair by searching for homologous sequences at sites of DNA double-strand breaks and facilitating strand pairing and exchange (37, 38, 48). Immortalized cells have been shown to have both elevated HR (49, 50) and elevated RAD51 mRNA expression (51). These findings suggest that the elevated HR is due to the higher levels of expression of RAD51. This interpretation is supported by the finding that overexpression of RAD51 increases spontaneous plasmid HR activity in Chinese hamster ovary cells (52). We therefore investigated FA fibroblasts displaying elevated HR activity for a similar increase in RAD51 protein. Nuclear protein extracts were prepared from diploid fibroblasts from FA patients and from normal subjects. Protein concentration was determined, and equivalent amounts of total nuclear protein from these extracts were resolved by SDS gel electrophoresis and Western blot analysis performed using human polyclonal anti-RAD51 antibody (a generous gift from Dr. Charles M. Radding). Fig. 2A shows that RAD51 protein levels were elevated in extracts from group A, C, and G cells compared with an extract prepared from the normal cell strain PD.792.F. Fig. 2A also reveals that RAD51 levels were not elevated in an extract prepared from diploid FA fibroblasts of complementation group D2. It is noteworthy that normal and complementation group D2 fibroblasts both had relatively low levels of HR, whereas fibroblasts belonging to complementation groups A, C, and G had elevated levels of HR.

To confirm that the level of RAD51 present in the normal fibroblast cell extract is representative of that present in other normal diploid fibroblasts, we analyzed protein levels in nuclear extracts from seven other cell strains obtained from unrelated normal subjects. Fig. 2B shows that RAD51 protein levels in these extracts were similar, ranging from 0.9 to 1.3-fold relative to that seen in the normal strain depicted in Fig. 2A. As a control, a nuclear extract from immortalized HT1080 cells was examined. Fig. 2B also shows that, as predicted, it had elevated RAD51 protein levels. To ensure that the elevated levels of RAD51 expression detected in Fig. 2A were not due to unequal protein loading, we performed a control Western blot analysis using an antibody that specifically recognizes the AP endonuclease-1 protein. As shown in Fig. 2C, whereas there were slight well-to-well variations, each extract contained approximately the same amount of immunoreactive protein, consistent with our interpretation that the elevated levels of RAD51 detected in Fig. 2A are due to elevated protein expression in FA fibroblast cells.
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Fig. 2. RAD51 protein levels are elevated in protein extracts from FA fibroblasts of complementation groups A, C, and G. A, RAD51 protein levels were examined by Western blot analysis of equal amounts of nuclear protein extracts prepared from normal diploid fibroblasts (N) and patient-derived FA fibroblasts of complementation groups A, C, G, and D2. B, RAD51 Western blot analysis was performed on equal amounts of nuclear extracts prepared from normal diploid fibroblasts (PD.792.F (lane 1), PD.751.F (lane 2), PD.715.F (lane 3), PD.793.F (lane 4), CCD-1059Sk (lane 5), CCD-1056Sk (lane 6), and CCD-1108Sk (lane 7)) and H1T180 cells (H). C, the same amounts of the same nuclear protein extracts from A were examined by Western blot analysis for AP endonuclease-1 protein expression. D, RAD51 Western blot analysis was performed on equal amounts of whole cell protein extracts prepared from normal diploid fibroblasts; patient-derived FA fibroblasts of complementation groups A, C, and G and their retrovirally corrected counterparts (Acor, Ccor, and Gcor, respectively); and patient-derived FA fibroblasts of complementation group D2. E, the same amounts of the same whole cell protein extracts from D were examined by Western blot analysis for KU86 protein expression.

To further confirm this conclusion, we examined the relative expression levels of RAD51 in whole cell lysates from control and FA fibroblasts. Equivalent amounts of protein from these extracts were resolved by electrophoresis, and RAD51 protein levels were determined. In these experiments, Western blot analysis was performed using a second anti-RAD51 antibody (Novus Biologicals Inc.). The results revealed that extracts from retrovirally corrected FA fibroblasts had RAD51 protein levels that were similar to those detected in extracts from normal and FA group D2 fibroblasts (Fig. 2D). Quantitation of RAD51 protein bands from multiple Western blots revealed that, whereas RAD51 levels were 8–11-fold higher in extracts from FA fibroblasts from complementation groups A, C, and G, RAD51 protein levels in retrovirally corrected cells were not statistically different from those in normal or group D2 cell extracts. Additionally, Western blot analysis of these same extracts with a third anti-RAD51 antibody (Santa Cruz Biotechnology Inc.) revealed similar results, showing that RAD51 protein levels were elevated ~10-fold in whole cell protein lysates from FA fibroblasts of groups A, C, and G, whereas protein levels were relatively low in their retrovirally corrected counterparts and in normal and group D2 extracts (data not shown). Finally, to demonstrate that the different levels of RAD51 protein expression detected in Fig. 2D were not due to unequal protein loading, we performed Western blot analysis using anti-KU86 antibody. We (31) and others (30) have shown that KU86 protein levels are not elevated in FA cells compared with control cells. As shown in Fig. 2E, we observed that there was no significant difference in the relative expression levels of KU86 among these extracts, confirming that unequal protein loading is not responsible for the elevated levels of RAD51 detected in FA fibroblasts.

Subsequent to having observed that FA cells express elevated levels of RAD51, we became aware of a study by Digweed et al. (34), who reported that RAD51 expression levels are not elevated in FA fibroblasts from complementation groups A and G relative to those in normal or retrovirally corrected FA fibroblasts. The underlying source of this discrepancy was unclear especially since the Western blot analysis depicted in Fig. 2A was performed using the same antibody that Digweed et al. reported using (Novus Biologicals Inc.). However, due to the consistency with which we observed elevated RAD51 protein levels using three different anti-RAD51 antibodies, we were confident that our conclusion that FA cells of complementation groups A, C, and G possess elevated levels of RAD51 protein was correct. We nevertheless wished to uncover the source of this apparent discrepancy.

Upon careful examination, we noted that the conclusion of Digweed et al. (34) that RAD51 expression levels are not altered in FA cells was based upon comparing relative expression levels of RAD51 and MRE11. We therefore considered the possibility that MRE11 levels could conceivably differ between normal and FA fibroblasts. To test this hypothesis, we performed Western blot analysis on whole cell extracts from normal, FA, and retrovirally corrected FA fibroblasts using antibodies specific for RAD51 and MRE11 (both from Novus Biologicals Inc.). As shown in Fig. 3A, both MRE11 and RAD51 proteins were overexpressed in FA fibroblasts from complementation groups A, C, and G. Interestingly, MRE11 protein expression levels in retrovirally corrected FA cells were indistinguishable from those in normal cells. Similar results were obtained when Western blot analysis was performed on these same extracts with a second anti-MRE11 antibody (obtained from BD Biosciences) (data not shown). Again, Western blot
The finding that overexpression of a patient-derived mutant FANCC gene (referred to as pL554P) rendered normal cells sensitive to both DNA cross-linking agents and induced chromosomal DNA double-strand breaks (32, 42) prompted us to hypothesize that normal cells expressing this dominant-negative gene would have elevated RAD51 protein and HR activity. To test this hypothesis, Western blot analysis was performed on whole cell protein extracts from transgenic normal diploid fibroblasts overexpressing the pL554P FANCC gene and on extracts from unmodified normal cells. As shown in Fig. 4, RAD51 protein levels were 8–9-fold higher in transgenic cell extracts compared with normal cell extracts. This increase in RAD51 protein was similar to that seen in FA fibroblast group C cell extracts (10–11-fold) compared with their retrovirally corrected counterparts (Fig. 4B). HR frequency in transgenic normal diploid fibroblasts was also elevated compared with unmodified normal diploid cells (Fig. 4B). HR frequency in transgenic cells was increased 8-fold compared with unmodified normal cells. Control experiments showed that overexpression of the wild-type FANCC gene in normal diploid fibroblasts had no effect on RAD51 protein levels or HR frequency (data not shown).

A mouse model of FA with a mutation affecting the carboxyl terminus of the FANCC protein has been generated (41). Embryonic fibroblasts from these mice are hypersensitive to the cytotoxic effect of DNA cross-linking agents (41). We have found that these cells are also sensitive to DNA double-strand breaks induced by electroporation of bacterial restriction enzymes (32). Given these similarities to human FA cells, it seemed likely that murine FA cells would also have elevated RAD51 protein and HR activity. Western blot analysis of extracts from murine homozygous mutant FancC cells showed that the RAD51 protein was elevated compared with murine homozygous wild-type cell extracts (Fig. 5A). Quantitation revealed that the RAD51 protein was elevated 10–11-fold. Fig. 5B shows that HR frequency in murine mutant FancC cells was also elevated ~9-fold compared with wild-type cells.

Protein Expression of RAD51 Paralogs Is Not Altered in FA Fibroblasts—Several RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) have been identified and shown to function in cellular HR (53–57). These paralogs have also been suggested to play roles in DNA cross-link repair and maintenance of genomic stability (58–60). Given the finding that FA cells of complementation groups A, C, and G have both elevated RAD51 protein and elevated HR activity and the inherent genomic instability and sensitivity FA cells display to DNA cross-linking agents, we hypothesized that FA fibroblasts may also have altered protein expression of one or more of these paralogs. Western blot analysis of whole cell protein extracts from FA and retrovirally corrected FA fibroblasts was performed with antibodies specific for the RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3 proteins. As shown in Fig. 6, no discernible difference in the levels of any of these proteins was seen in extracts from FANCC or retrovirally corrected FANCC fibroblasts. Similar results were obtained from extracts from other FA complementation groups (data not shown). These results show that, contrary to the expression of RAD51 and MRE11 proteins, there is no evident change in expression of
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Fig. 6. Protein levels of the RAD51 paralogs are not altered in FA fibroblast extracts. Equal amounts of whole cell protein extracts prepared from FANCC fibroblasts (C) and retrovirally corrected FANCC fibroblasts (Ccor) were examined by Western blot analysis using polyclonal anti-RAD51B, anti-RAD51C, anti-RAD51D, anti-XRCC2, and anti-XRCC3 antibodies.

Introduction of Antibodies Specific for RAD51 Results in Altered HR Activity in FA Fibroblasts of Complementation Groups A, C, and G—It has previously been shown that overexpression of the RAD51 protein in mammalian cells results in a stimulation of chromosomal HR (52). This finding, along with the data presented above, suggests that the elevated HR activity seen in FA fibroblast of complementation groups A, C, and G is due to overexpressed RAD51 protein. To test this hypothesis, we examined the ability of an antibody specific for the RAD51 protein to attenuate HR activity in these FA cells. Antibodies have previously been shown to be effective at inhibiting protein function when introduced directly into cells by electroporation (61). Therefore, we examined the effect of co-introduced polyclonal anti-RAD51 primary antibody (Novus Biologicals Inc.) on intracellular HR activity in diploid FA fibroblasts. As shown in Table II, the presence of anti-RAD51 antibody reduced HR frequencies in FA fibroblasts of complementation groups A, C, and G to levels that were not statistically different from those seen in normal fibroblasts not treated with antibody. In contrast, the presence of anti-RAD51 antibody had no effect on HR frequencies in normal diploid or complementation group D2 fibroblasts (Table II). As a control, goat anti-rabbit IgG was co-introduced into normal and FA fibroblasts along with recombination plasmid substrates. As expected, no effect on HR was observed following this treatment (Table II). Similar to human FA cells, treatment of murine homozygous mutant FA cells with anti-RAD51 antibody reduced HR frequency to wild-type levels, whereas antibody treatment of murine wild-type cells had no effect (data not shown).

Effects of Introduction of Anti-FA Antibodies on Cellular Double-strand Break Repair—The data presented above indicate that the absence of functional FANCC protein results in elevated expression of the RAD51 protein, thereby leading to elevated intraplasmid HR activity. To confirm this result, we performed an HR experiment in which anti-FANCC antibody was co-electroporated into intact normal diploid fibroblast cells along with the recombination plasmid substrate. As shown in Fig. 7, normal fibroblasts treated with anti-FANCC antibody had significantly increased HR activity. A >8-fold increase in HR frequency was observed in these cells versus normal cells not treated with antibody. Additionally, anti-FANCC antibody introduction into both FA fibroblasts of complementation group D2 and retrovirally corrected FA fibroblasts of complementation group C resulted in a similar increase in HR frequency compared with cells not treated with antibody (Fig. 7). As expected, anti-FANCC antibody did not influence HR frequency in FA fibroblasts of complementation groups A, C, and G.

FA fibroblasts of complementation groups A, C, D2 and G have all been shown to have significantly decreased DNA end joining activity (32), whereas the results presented thus far indicate that only FA fibroblasts of complementation groups A, C, and G have elevated HR activity. To confirm this finding and to gain insight into the relationship between FA proteins and DNA double-strand break repair pathways, we examined both recombinational and non-recombinational DNA repair in the presence of either anti-FANCC or anti-FANCD2 antibody (generous gifts from Dr. Alan D. D’Andrea). We hypothesized that if FANCC and FANCD2 function in different capacities in the regulation of these DNA double-strand break repair pathways, specific inhibition of either of these proteins in normal cells would result in different DNA repair phenotypes. Intact normal diploid cells were therefore co-electroporated with anti-FANCC or anti-FANCD2 antibody along with plasmid substrates for either HR repair or DNA end joining. Table III

| Cells | Antibody | HR frequency* |
|-------|----------|---------------|
| PD.792.F (N)<sup>a</sup> | 9 |
| PD.720.F (A) | 93<sup>b</sup> |
| PD.551.F (C) | 96<sup>b</sup> |
| PD.352.F (G) | 89<sup>b</sup> |
| PD.145.F (D2) | 10 |
| PD.792.F (N)<sup>a</sup> | RAD51 | 10 |
| PD.720.F (A) | RAD51 | 17 |
| PD.551.F (C) | RAD51 | 21 |
| PD.352.F (G) | RAD51 | 16 |
| PD.145.F (D2) | RAD51 | 11 |
| PD.792.F (N)<sup>a</sup> | IgG | 11 |
| PD.720.F (A) | IgG | 91<sup>b</sup> |
| PD.551.F (C) | IgG | 96<sup>b</sup> |
| PD.352.F (G) | IgG | 83<sup>b</sup> |
| PD.145.F (D2) | IgG | 10 |

<sup>a</sup> Expressed as the number of recombinant colonies/10<sup>5</sup> non-recombinant colonies; average of six independent experiments.

<sup>b</sup> N, normal fibroblasts.

*<sup>p</sup> < 0.05 compared with normal diploid fibroblasts.

Fig. 7. Introduction of anti-FANCC antibody into cells alters HR activity. HR frequencies in normal diploid fibroblasts (N), patient-derived FA fibroblasts from patients of complementation groups A, C, G, and D2; and retrovirally corrected FA group C fibroblasts (Ccor) were determined in the absence (black bars) or presence (white bars) of anti-FANCC antibody. Results are an average of three independent experiments in each cell strain. Error bars represent S.E. *<sup>p</sup> < 0.05 compared with normal diploid fibroblasts in the absence of antibody.
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Table III

| Antibody      | HR frequency | End joining |
|---------------|--------------|-------------|
| None          | 9            | 27.1 ± 2.4  |
| IgG           | 10           | 26.6 ± 4.0  |
| FANCC 95%     | 10           | 5.5 ± 2.7†  |
| FANCD2 10%    |              | 4.6 ± 2.9†  |

a Expressed as the number of recombinant colonies/10⁶ non-recombinant colonies.

b Expressed as the number of colonies obtained from electroporated linearized plasmids divided by the number of colonies obtained from electroporated circular plasmids.

†p < 0.05 compared with HR frequency in normal diploid fibroblasts with no antibody.

†p < 0.05 compared with end joining activity in normal diploid fibroblasts with no antibody.

The results presented above indicate that human fibroblasts belonging to FA complementation groups A, C, and G have dramatically elevated levels of intraplasmid HR activity. These cells also display elevated levels of RAD51 protein, and results obtained from electroporation of anti-RAD51 antibody into these cells indicate that the elevated HR activity is RAD51-dependent. This elevated RAD51-dependent HR activity clearly results from the absence of the respective FA proteins since retrovirus-mediated gene correction restored HR activity to wild-type levels. Murine embryo fibroblasts from a FancC knockout mouse model also expressed elevated levels of RAD51 protein as well as elevated levels of interplasmid HR activity. In contrast, human FA fibroblasts belonging to complementation group D2 had neither elevated RAD51 protein expression nor elevated HR activity. We observed that anti-FANCC antibody introduced into normal fibroblasts led to elevated levels of HR activity, whereas similar treatment with anti-FANCD2 antibody was without effect on intracellular HR activity. Further analysis of these cells revealed that, in both cases, antibody treatment sensitized the cells to the cytotoxic effects of bifunctional cross-linking agents, proving that introduction of the antibodies inhibited the FANCC and FANCD2 proteins, respectively. Thus, we can conclude that a subset of FA proteins, minimally including the FANCA, FANCC, and FANCG proteins, but exclusive of the FANCD2 protein, function to suppress HR in fibroblasts.

In addition to the effects summarized above, electroporation with either the anti-FANCC or anti-FANCD2 antibody inhibited cellular DNA end joining activity, which was previously shown to be deficient in FA fibroblasts belonging to complementation groups A, C, D, and G (32). The finding that both end joining and HR are aberrant in FA fibroblasts raises the possibility that the two pathways are coordinately regulated by the FA proteins. It is clear that both end joining and HR pathways can, in principle, be utilized to repair similar types of DNA damage, including double-strand breaks and interstrand cross-links. Clearly, however, the ramifications for the cell of choosing one particular pathway over the other are significant. Although DNA end joining is frequently imprecise, it is less likely to generate chromosome translocation events compared with HR (62). On the other hand, HR is more likely to precisely repair a lesion, particularly if the lesion involves an interstrand cross-link (63, 64). However, the existence of repetitive DNA provides a greater opportunity for potentially oncogenic translocation events to occur during HR. It is thus reasonable to propose that mammalian cells coordinately regulate these distinct DNA repair pathways, in effect “deciding” which pathway is utilized to repair a specific lesion. A number of observations are consistent with the idea that the FA proteins perform this role. First, although it has long been believed that FA cells are defective in DNA repair, the absence of recognizable sequence motifs within the FA proteins has cast doubt on the idea that they participate directly in DNA repair. Second, it has been difficult to understand why, if the FA proteins are essential players in DNA repair, they are not present in lower eukaryotes. Third, the FANCD2 protein interacts with a number of DNA repair and regulatory proteins and relocates within the nucleus of cells following induced DNA damage (27, 35).

Based on the finding that FA group A, C, and G fibroblasts, but not D2 cells, have elevated HR, we propose that a signal transduced by the FANCA, FANCC, and FANCG proteins suppresses HR. These FA proteins are known to exist within the cell in a complex that also contains the FANCE and FANCF proteins (65–67), and the complex is absent from cells lacking any of these proteins. We therefore propose that this intact complex of FA proteins generates a signal that suppresses HR activity. The FANCD2 protein cannot play an essential role in this suppression since HR levels are not aberrantly elevated in FANCD2 cells. However, the FANCD2 protein nevertheless does play a critical role in regulating cellular DNA double-strand break repair. We propose that monoubiquitination of this protein, which requires the presence of the intact FA protein complex and which occurs in response to a variety of triggers including induced DNA damage and cell cycle progression (9, 27), generates a separate signal that overcomes the suppression of HR mediated by the FA protein complex, thereby activating cellular HR. A role in activating cellular HR is consistent with the fact that the FANCD2 protein localizes to discrete loci within the nucleus in response to DNA damage.
and during S phase of the cell cycle (68). The recombination protein RAD51 also localizes to these loci under these conditions, as does the BRCA1 protein, which is thought to play an important role in regulating cellular HR (27, 68). It is somewhat paradoxical that elevated levels of the recombinational repair protein RAD51 are associated with enhanced sensitivity to DNA cross-linking agents in FA cells. It is unclear at this time whether the enhanced sensitivity of these cells results from the elevated HR activity or is instead due to the deficient DNA end joining activity within these cells. We do not favor the former explanation, particularly in light of reports demonstrating increased radiosensitivity in RAD51-overexpressing cell lines (52). Rather, the latter explanation is supported by a recent report showing that overexpression of the human RAD51 protein is associated with reduced levels of double-strand break repair protein RAD51 are associated with enhanced sensitivity for the signal transduction events we have described above. It is presently not clear precisely which enzymatic machin-

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J. Biol. Chem. 2003, 278:29487-29495.
doi: 10.1074/jbc.M213251200 originally published online May 14, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M213251200

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