Deficient DNA End Joining Activity in Extracts from Fanconi Anemia Fibroblasts

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Fanconi anemia (FA) is an autosomal recessive genetic disorder associated with genomic instability and cancer predisposition. Cultured cells from FA patients display a high level of spontaneous chromosome breaks and an increased frequency of intragenic deletions, suggesting that FA cells may have deficiencies in properly processing DNA double strand breaks. In this study, an in vitro plasmid DNA end joining assay was used to characterize the end joining capabilities of nuclear extracts from diploid FA fibroblasts from complementation groups A, C, and D. The Fanconi anemia extracts had 3–9-fold less DNA end joining activity and rejoined substrates with significantly less fidelity than normal extracts. Wild-type end joining activity could be reconstituted by mixing FA-D extracts with FA-A or FA-C extracts, while mixing FA-A and FA-C extracts had no effect on end joining activity. Protein expression levels of the DNA-dependent protein kinase (DNA-PK)/Ku-dependent nonhomologous DNA end-joining proteins Xrcc4, DNA ligase IV, Ku70, and Ku86 in FA and normal extracts were indistinguishable, as were DNA-dependent protein kinase and DNA end binding activities. The end joining activity as measured by the assay was not sensitive to the DNA-PK inhibitor wortmannin or dependent on the nonhomologous DNA end-joining factor Xrcc4. However, when DNA/protein ratios were lowered, the end joining activity became wortmannin-sensitive and no difference in end joining activity was observed between normal and FA extracts. Taken together, these results suggest that the FA fibroblast extracts have a deficiency in DNA end joining process that is distinct from the DNA-PK/Ku-dependent nonhomologous DNA end joining pathway.

Fanconi anemia (FA)1 is an autosomal recessive disease characterized by developmental abnormalities, progressive bone marrow failure, chromosomal instability, and predisposition to cancer (1, 2). Somatic cell fusion studies have demonstrated the existence of at least eight complementation groups (FA-A through FA-H) (3). Presently, five of the FA genes have been cloned, FANCA, FANCC, FANCE, FANCF, and FANCG (4–8). The biochemical functions of these proteins are unknown; thus, the underlying defect of this disease has not been established.

In vitro analysis of cultured cells obtained from FA patients reveals an elevated level of spontaneous chromosome breaks. The frequency of these chromosomal lesions is amplified following exposure to DNA cross-linking agents (1). FA cells also experience spontaneous and psoralen-induced DNA deletions at a higher frequency than normal cells. These DNA deletions have been detected both within the endogenous hypoxanthine-guanine phosphoribosyltransferase gene and within a target gene present on an autonomously replicating plasmid (9, 10). These cellular phenotypes suggested that FA cells may have deficiencies in processing DNA double strand breaks.

Recent reports have supported the hypothesis that FA cells have deficiencies in rejoining double strand breaks (DSBs) (11, 12). In these studies, linearized plasmid DNA was transfected into immortalized FA lymphoblasts and recovered after 48 h. Analysis of recircularized products revealed that the overall efficiency of plasmid end joining was normal in FA lymphoblasts from complementation groups B, C, and D, but error-free processing of blunt-ended substrates was significantly compromised in these cells.

To gain further insight into the process of DNA end joining in FA cells, we used an in vitro assay to examine the ability of nuclear protein extracts prepared from diploid FA fibroblasts to rejoin linear plasmid DNA substrates. Nuclear extracts from diploid fibroblasts from patients from complementation groups A, C, and D had 3–9-fold less end joining activity and rejoined linear substrates imprecisely at a higher frequency than extracts from normal donors. This end joining deficiency was not due to the presence of an inhibitor in the FA extracts or to deficiencies in proteins or activities known to be involved in the well characterized DNA-PK/Ku nonhomologous DNA end joining pathway (13). Wild-type end joining activity could be reconstituted by mixing FA-D extracts with FA-A or FA-C extracts but not by mixing FA-A with FA-C extracts. The end joining activity that was deficient in the FA extracts was not sensitive to the DNA-PK inhibitor wortmannin or dependent on Xrcc4. When a lower substrate DNA/protein ratio was used in the end joining assay, the end joining activity was wortmannin-sensitive and indistinguishable end joining levels were observed between normal and FA extracts.

EXPERIMENTAL PROCEDURES

Cell Culture and Conditions—The diploid FA fibroblast cell strains PD.134.F (FA-C), PD.220.F (FA-A), and PD.20.F (FA-D) as well as the normal diploid cell strains PD.715.F, PD.19.F, PD.793.F, PD.792.F, and PD.751.F were kindly provided by Dr. Markus Grompe (Oregon Health Sciences University). These cells were maintained in minimum essential medium supplemented with 2 mM glutamine and 15% fetal bovine serum. Normal diploid strains CRL-2115, CRL-2068, and CRL-2072 were purchased from the American Type Culture Collection (Manassas,
VA) and were maintained in Eagle's minimum essential medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, and 10% fetal bovine serum. All cells were maintained at 37 °C in a humidified, 5% CO2 environment.

Nuclear Protein Extracts—Nuclear extracts were prepared as previously described (14). Briefly, cells harvested from confluent 100-mm tissue culture dishes were washed three times with ice-cold phosphate-buffered saline and resuspended in 2 ml of hypotonic buffer A (10 mM KCl, 10 mM Tris (pH 7.4), 10 mM MgCl2, and 10 mM dithiothreitol) and kept on ice for 15 min. Phenylmethylsulfonyl fluoride was added to 1 mM, and cells were disrupted using a Dounce homogenizer (20 strokes with a tight pestle). The released nuclei and cytoplasm were pelleted by centrifugation at 3,000 rpm for 10 min at 4 °C. The supernatant was then dialyzed against a buffer containing 12 mM HEPES, 5 mM MgCl2, 4 mM Tris (pH 7.9), 100 mM KCl, 0.6 nM EDTA, 0.6 mM dithiothreitol, and 6% glycerol in the presence of 200 ng of unlabeled supercoiled DNA. To specifically compete for the DNA end binding activity, 200 ng of unlabeled linear plasmid DNA was added in 2 ml of buffer A containing 350 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml leupeptin, 1.0 µg/ml aprotinin, and 0.7 µg/ml pepstatin and incubated for 1 h on ice. The nuclei were centrifuged at 70,000 rpm in a Beckman TL-100.3 rotor at 4 °C for 30 min, and the clear supernatant was adjusted to 10% glycerol and 10 mM β-mercaptoethanol. The resulting extracts were dialyzed against a buffer containing 25 mM Tris (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Protein concentrations were determined by the Bradford method (15).

End Joining Reactions—DNA end joining reactions were carried out essentially as described previously (16). Circular pCR 2.1 plasmid DNA (Invitrogen, Carlsbad, CA) was linearized by restriction digestion with KpnI, ligated to substrates with cohesive-ended ends, or EcoRV to generate blunt-ended substrates (all endonucleases were from New England Biolabs, Beverly, MA). After restriction digestion, substrates were ethanol-precipitated and resuspended in TE buffer, pH 8.0. 1 µg of linearized DNA was incubated with 5 µg of nuclear protein extract in 70 mM Tris (pH 7.5), 10 mM MgCl2, 10 mM dithiothreitol, and 1 mM ATP in a total volume of 50 µl. The reaction mixture was then treated with proteinase K at 37 °C for 30 min and electrophoretically separated on a 0.8% agarose gel in Tris borate-EDTA buffer. The gel was run at 20 V/cm for 12–15 h. After staining in ethidium bromide, gels were scanned on a Bio-Rad scanner using the Molecular Analyst program and quantified using IP Lab Gel (Signal Analytics Corp., Vienna, VA). A modification of the assay (Rathmell and Chu (18)) was used to detect DNA end joining activity following instructions provided by the manufacturer.

Wortmannin Inhibition—A 0.1 mM stock of wortmannin (Sigma) was prepared in 10% Me2SO. Wortmannin was incubated with 5 µg of nuclear extract for 30 min on ice. Plasmid DNA end joining experiments as previously described were then performed at 14 °C.

DNA-PK Assay—The SignaTECT DNA-dependent Protein Kinase Assay System (Promega, Madison, WI) was used to detect DNA-PK activity following instructions provided by the manufacturer.

RESULTS

To test the end joining activity of FA fibroblasts, an in vitro DNA end joining assay that has been previously described was employed (16). Nuclear protein extracts were prepared from diploid fibroblasts from normal donors and diploid fibroblasts from FA patients of complementation groups A (FA-A), C (FA-C), and D (FA-D). 5 µg of extract was incubated for 12 h at 14 °C with 1 µg of plasmid pCR2.1 DNA that had been linearized by restriction digestion to have either blunt or cohesive ends. End joining activity was detected by the presence of circular, linear substrate, and high molecular weight products when the reaction mixture was analyzed by agarose gel electrophoresis. In end joining experiments performed with blunt-ended substrate, closed circular was the predominant product formed. When cohesive-ended substrates were used, closed circular, linear substrate, and high molecular weight products were detected on the ethidium bromide-stained gels. Fig. 1a shows the results of an end joining experiment performed with blunt-ended substrate. Scanning laser densitometry was used to quantitate the bands, and the percentage of linear substrate that had been rejoined was determined. This analysis revealed that the normal extract rejoined 34% of the linearized substrate as compared with 3, 7, and 7%, respectively, by the FA-A, FA-C, and FA-D extracts.

To confirm that the FA-A, FA-C, and FA-D extracts were deficient in DNA end joining, two or three independent extracts were prepared from each cell line and tested multiple times in end joining experiments with blunt and cohesive-ended substrates. Also, nuclear extracts were prepared from seven normal fibroblast strains in addition to the normal fibroblast strain used in Fig. 1a (PD.715.F) and tested for end joining activity. Fig. 1b depicts the percentage rejoined activity (per centage rejoin + S.E.) in the normal, FA-A, FA-C, and FA-D extracts. The mean end joining activity of blunt-ended substrate in extracts from the normal strains ranged from 19 to 45% with a cumulative mean of 31 ± 4%. Rejoining of cohesive-ended substrate by the normal extracts ranged from 16 to 37% (cumulative mean, 26 ± 3%). The normal strain, PD.715.F,
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End joining activity of DNA substrates with blunt and cohesive ends is decreased in nuclear extracts prepared from FA-A, FA-C, and FA-D fibroblasts. A, end joining of DNA substrates with blunt ends was performed as described under “Experimental Procedures.” Samples were loaded on a 0.8% agarose gel, electrophoretically resolved, and stained with ethidium bromide—no extract, N; 5 μg of nuclear protein extract from the normal cell strain PD.715.F; A, 5 μg of nuclear extract from FA-A cell strain PD.220.F; C, 5 μg of nuclear extract from FA-C cell strain PD.134.F; D, 5 μg of nuclear extract from FA-D cell strain PD.20.F. L represents the mobility of the linear substrate DNA, and CC indicates the mobility of a product that migrates with form II of uncut substrate DNA and we call “closed circular.” B, DNA end joining reactions were performed with blunt and cohesive-ended substrates with extracts from eight normal diploid fibroblast strains (N), FA-A cell strain PD.220.F (A), FA-C cell strain PD.134.F (C), and FA-D cell strain PD.20.F (D). Scanning laser densitometry was performed to determine the percentage of plasmid substrate that had been converted to product. The mean end joining activity for the normal fibroblast extracts was calculated from the mean end joining activities in eight normal fibroblast strains. The mean end joining activities in the extracts from the FA-A, FA-C, and FA-D cell strains were determined from multiple end joining experiments (FA-A, n = 4; FA-C, n = 5; FA-D, n = 3) performed with a minimum of two independently prepared extracts. The bars represent the mean percentage end joining ± S.E. Filled bars, blunt-ended substrate; open bars, 5’-cohesive-ended substrate; *, p < 0.001; #, p < 0.05.

In the above experiments, the cohesive-ended substrate had a 5’-overhang. To determine whether the FA extracts were also deficient at rejoining substrates with 3’-cohesive ends, end joining experiments were performed with a substrate that was linearized by restriction digest with KpnI. Nuclear extracts from the eight normal fibroblast strains rejoined a mean of 48 ± 4% of the substrate, with a range from 35 to 64%. The FA-A, FA-C, and FA-D extracts rejoined 3, 2, and 12%, respectively, of the 3’-cohesive-ended substrate (data not shown). We concluded that FA-A, FA-C, and FA-D extracts were deficient in rejoining plasmid DNA substrates with blunt-, 5’-cohesive, and 3’-cohesive ends.

To determine whether the observed end joining deficiency in the FA extracts was due to reduced kinetics of activity, a time course experiment was performed with normal and FA-C extracts. The plasmid end joining assay was performed using a blunt-ended substrate, and aliquots were removed for analysis at 4, 8, and 12 h. As shown in Fig. 2a, the normal cell extract rejoined the blunt-ended substrate in a linear fashion between 4 h (25% rejoined) and 8 h (44% rejoined) before reaching saturation at 12 h (52% rejoined). On the other hand, no activity was detected in samples incubated with the FA fibroblast extract for up to 8 h. Following a 12-h incubation period, 4% rejoicing was detected. Similarly, we tested the end joining activity of the normal and the FA-C fibroblast extracts as a function of protein concentration. Plasmid DNA end joining with blunt-ended substrate was performed using 2.5, 5, 7.5, 10, and 12.5 μg of protein. As seen in Fig. 2b, there was a noticeable difference in end joining activity between the two extracts at all protein concentrations tested. Similar observations were made when cohesive-ended substrates or FA-A and FA-D extracts were used (data not shown).

Deficient end joining capabilities of the FA extracts can be explained by two alternate hypotheses; either the extracts derived from the FA fibroblasts contain an inhibitor of the end joining reaction, or, conversely, a factor or factors essential for the end joining reaction may be absent from the FA extracts. To distinguish between these two possibilities, an end joining experiment with blunt-ended substrate was performed using a mixture of equal amounts of nuclear protein extract from normal and FA-C cells. To keep the total protein present in the reaction at 5 μg; 2.5 μg of the normal extract was mixed with 2.5 μg of the FA extract. As seen in Fig. 3, 5 μg of normal extract alone yielded 42% product formation. When a mixture of normal and FA extracts was tested, 34% product formation was detected. This level of end joining is consistent with the percentage of end joining obtained when 2.5 μg of normal extract is used in end joining reactions (see Fig. 2b). The same results were obtained when FA-A or FA-D extracts were mixed with normal extracts or when cohesive-ended substrates were used (data not shown). The wild-type level of plasmid end joining activity present in the mixed sample is inconsistent with the notion that an inhibitor of end joining is present in the FA nuclear extract. This finding indicates that nuclear extracts from FA fibroblasts lack a factor or factors essential for the end joining reaction.

The reduced DNA end joining activity in extracts prepared from FA-A, FA-C, and FA-D fibroblast strains raised the possibility that wild-type end joining activity could be reconsti-
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**Fig. 3.** Deficient end joining in FA extracts is not due to the presence of an inhibitor. End joining of blunt end DNA substrates was performed with no extract (N), 5 µg of fibroblast nuclear extract from normal donor PD.715.F (N), 5 µg of fibroblast nuclear extract from FA-C patient PD.134.F (FA), or a mixture of 2.5 µg of PD.715.F and 2.5 µg of PD.134.F (N + FA) nuclear extracts.

We therefore examined the nature of DNA end joining in normal and FA-C fibroblasts in combination with one another and performed DNA end joining experiments. As seen in Fig. 4, 5 µg of normal extract alone rejoined 33% of the linearized DNA substrate. Combining 2.5 µg of FA-A or FA-C extracts with 2.5 µg of FA-D extract resulted in 29 and 32%, respectively, of DNA end joining activity, whereas a mixture of the FA-A and FA-C extracts had no effect on end joining levels (7% end joining activity).

It has been previously established that immortalized FA lymphoblasts rejoin blunt-ended DNA substrates in an error-prone manner in vitro relative to control lymphoblasts (12, 13). We therefore examined the nature of DNA end joining in nuclear extracts from FA-C fibroblasts and nuclear extracts from normal donors. A bacterial transformation assay was employed to analyze the fidelity of end joining in the extracts (see “Experimental Procedures”). Using blunt-ended substrate, a significantly higher number of white colonies were recovered from rejoining by the FA-C extract as compared with the normal extract, 27% (217 of 780) versus 18% (171 of 780) (p < 0.005, χ² = 36.67) (Fig. 5). Similarly, a significantly higher number of white colonies were recovered from the FA-C extract when cohesive-ended substrates were used. 4% (33 of 899) of the colonies resulting from the rejoining by the FA-C extract were white compared with 0.5% (15 of 2917) from the normal fibroblast extract. Statistical analysis again revealed that the difference was significant (p < 0.005, χ² = 52.8).

Restriction digest analysis of imprecisely rejoined plasmids from the FA-C (101 plasmids) and normal (69 plasmids) end joining experiments revealed that all imprecise joining events resulted in deletions (data not shown). Sequence data of the region flanking the rejoined site obtained from 26 plasmids that resulted from imprecise rejoining (13 from FA and 13 from normal extracts) showed no difference in the range of the sizes of the deletions (2–47 base pairs). It also showed that all deletions, irrespective of whether obtained from the FA-C or normal extract, were flanked by direct repeat sequences of between 1 and 7 base pairs in length (data not shown). From this analysis, we concluded that there was no difference in the nature of the imprecise end joining events between the normal and FA-C extracts.

Currently, two independent pathways are known to repair DSBs in cells: homologous recombination and nonhomologous DNA end joining (NHEJ). In contrast to genetic screens of x-ray-sensitive yeast cells, which identified genes involved in homologous recombination (HR) (19, 20), mutant screens of x-ray-hypersensitive mammalian cells have led to the identification of factors involved in NHEJ (21). This led to the suggestion that DSBs are preferentially repaired by NHEJ in mammalian cells (13).

A NHEJ pathway, generally referred to as the DNA-PK- or Ku-dependent pathway, has been well characterized in recent years. It has been demonstrated to be minimally dependent upon five proteins: Xrcc4, DNA ligase IV, Ku70, Ku86, and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (22). To test if any of these proteins were absent from FA-C extracts, Western blot analysis was performed using antisera specific for the Ku70, Ku86, DNA ligase IV, and Xrcc4 proteins. The levels of these four proteins present in nuclear extracts from FA-C fibroblasts were essentially identical to those seen in the normal fibroblast extract (Fig. 6a), suggesting that low expression of any of these four proteins was not the cause of the deficient end joining in the FA extracts.

The presence of wild-type levels of these proteins does not prove that they are functional. However, the amount of DNA end binding activity, which is dependent upon the functional Ku70/Ku86 heterodimer as determined by the ability of Ku70 and Ku86 antibodies to supershift the end binding band (23), was identical in control and FA-C extracts (Fig. 6b). Similarly, DNA-PK activities in FA-C extracts were indistinguishable from those present in extracts from control fibroblasts (not shown). Western blot analysis, DNA end binding, and DNA-PK assays performed with FA-A and FA-D extracts revealed no differences from normal fibroblasts in these extracts as well (data not shown). Previous studies performed with FA lymphoblasts also found no deficiencies in these factors or activities (11, 12).

We next wished to determine whether the assay used in this...
study was measuring NHEJ activity. To do this, we determined if the end joining activity was dependent on DNA-PK or Xrcc4, two components of the DNA-PK/Ku-dependent NHEJ pathway (22). Wortmannin is a potent inhibitor of phosphatidylinositol 3-kinases (24) and has been demonstrated to inhibit DNA-PK activity (25). 5.0 μg of nuclear extract prepared from normal diploid fibroblasts was incubated for 30 min on ice with 5.0 μM wortmannin. This concentration of wortmannin abolished DNA-PK activity in the extract as measured by an in vitro DNA-dependent protein assay system (Promega, Madison, WI) as specified by the manufacturer. DNA end joining experiments with 5'-cohesive-ended substrates were performed with 5 μg of nuclear protein extract from normal cell line PD.715.F (B) or with 5 μg of nuclear protein extract from the FA-C cell line PD.134.F that had been incubated with 10% Me2SO (−) or 5 μM wortmannin (+) for 30 min on ice. DNA-PK activity was determined using the SignaTECT DNA-dependent protein assay system (Promega, Madison, WI) as specified by the manufacturer. DNA end joining experiments with 5'-cohesive-ended substrates were performed with 5 μg of nuclear protein extract from normal cell line PD.715.F (B) or with 5 μg of nuclear protein extract from the FA-C cell line PD.134.F that had been incubated with 10% Me2SO (−) or 5 μM wortmannin (+) for 30 min on ice (C). The samples were loaded on a 0.8% agarose gel, electrophoretically resolved, and stained with ethidium bromide. L represents the mobility of the linear substrate DNA, while CC and D indicate the mobility of closed circular and linear dimer products, respectively.

To determine whether Xrcc4 was required for the end joining activity, antiserum raised against the human Xrcc4 protein was used to immunodeplete Xrcc4 from a nuclear extract prepared from fibroblasts from a normal donor. Western blot analysis confirmed that the extract was depleted of this protein (Fig. 8A). DNA end joining experiments were performed at 14 °C for 4 h with the Xrcc4-immunodepleted extract. As shown in Fig. 8B, the Xrcc4-immunodepleted extract had wild-type end joining activity. We concluded that the end joining assay as used in this study was measuring an activity that was not dependent on the NHEJ factors DNA-PK or Xrcc4.

Baumann and West described an in vitro end joining assay that was dependent on the NHEJ factors Ku70, Ku86, Xrcc4, DNA ligase IV, and DNA-PKcs (22). Under the conditions of this assay, 10–100 ng of substrate DNA was incubated with 20–60 μg of cellular extract (0.001 μg of DNA/μg of protein as compared with 0.2 μg of DNA/μg of protein in the current study). To more closely resemble the conditions described by Baumann and West, we performed end joining experiments for 2 h at 14 °C using 50 ng of linearized DNA and 5 μg of nuclear extracts (0.05 μg of DNA/μg of protein) from normal and FA fibroblasts. As shown in Fig. 9, similar to what was reported by Baumann and West, the end joining activity was sensitive to wortmannin under these conditions. The end joining activity observed in the normal and FA extract were identical under these conditions. The substrate DNA concentration was the only variable changed under the wortmannin-sensitive condi-
and linear dimer products, respectively. 

CC ethidium bromide-stained gel. 

resolved on a 0.8% agarose gel. Shown is an inverted image of the 

way is fully functional in the FA extracts. 

out similar to those previously reported for measuring NHEJ 

by the assay. Finally, when end joining reactions are carried 

completed of Xrcc4 have wild-type end joining activity as measured 

DNA-PK inhibitor wortmannin. Third, extracts immunode- 

ity in which the FA extracts are deficient is not inhibited by the 

and DNA end binding activities. Second, the end joining activ- 

ligase IV, Ku70, and Ku86 as well as normal levels of DNA-PK 

ects are deficient in a DNA end joining pathway that is 

suggest that more than one NHEJ pathways exist. 

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FIG. 9. Wild-type end joining activity in FA extracts in a wort- 

mannin-sensitive end joining assay. DNA end joining experiments 

with 50 ng of 5’-cohesive-ended substrate were performed for 2 h at 

14 °C with 5 μg of nuclear protein extract from normal cell line 

PD.715.F (N) or with 5 μg of nuclear protein extract from the FA-C 

cell line PD.134.F (FA) that had been preincubated with 10% MeSO (−) or 

5.0 μM wortmannin (+) for 30 min on ice. The products were then 

resolved on a 0.8% agarose gel. Shown is an inverted image of the 

DNA end joining experiments 

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studies carried out in the yeast 

con- 

in vivo 

studies strongly 

DNA end joining pathways exist. Genetic inactivation 

studies carried out in the yeast Saccharomyces cerevisiae 

convincingly demonstrate that this organism repairs DNA double 

strand breaks through precise (Ku-dependent) and imprecise 

(Ku-independent) pathways (28, 29). The same appears to be 

true in mammalian cells. Both wild-type and xrs6 hamster 

cells, which lack a functional Ku86 protein, were able to rejoin 

transfected linear plasmids. However, rejoining occurred in a 

more error prone manner in the xrs6 cells, and the deletions 

formed were on average 3–4-fold larger than in control cells 

(30). Taken together, these in vitro and in vivo studies strongly 

suggest that more than one NHEJ pathways exist. 

The data presented in this study suggest that the FA ex- 

tracts are deficient in a DNA end joining pathway that is 

distinct from the well characterized NHEJ pathway. First, FA 

extracts have wild type levels of the NHEJ factors Xrcc4, DNA 

ligase IV, Ku70, and Ku86 as well as normal levels of DNA-PK 

and DNA end binding activities. Second, the end joining activ- 

ity in which the FA extracts are deficient is not inhibited by the 

DNA-PK inhibitor wortmannin. Third, extracts immunode- 

pleted of Xrcc4 have wild-type end joining activity as measured 

by the assay. Finally, when end joining reactions are carried 

out similar to those previously reported for measuring NHEJ 

(22), there is no difference in end joining between normal and 

FA extracts, indicating that the well characterized NHEJ path- 

way is fully functional in the FA extracts. 

The possibility cannot be ruled out that Ku is involved in this 

alternate end joining process. However, given that FA extracts 

have wild-type DNA end binding activity, it seems unlikely 

that the defect in FA cells is the result of defective Ku proteins. 

In addition, cells with Ku defects are sensitive to ionizing 

radiation (13, 21), a phenotype not associated with FA cells 

(31). Regardless of the involvement of Ku in this pathway, the 

data indicates that the end joining activity that is deficient in 

the FA extracts is independent of DNA-PK and Xrcc4. Thus, 

this end joining pathway appears to be distinct from that 

classically referred to as NHEJ. 

It is tempting to speculate that the DNA end joining defi- 

ciency observed in FA extracts is representative of a cellular 

defect in rejoining double strand breaks. This alternate end 

joining pathway may represent an additional end joining path- 

way in cells. This could explain why end joining activity is still 

detected in Ku-deficient cells (28–30). This could also explain 

why Escarceller et al. (11, 12) found no deficiency in the overall 

end joining efficiency of linearized plasmid substrates but did 

see error-prone rejoining of blunt-ended substrates in FA lym- 

phoblasts. In vivo, a defect in this additional end joining pathway 

would be masked by other DNA end joining pathways. 

An end joining deficiency in FA cells could account for many 

of the cellular phenotypes associated with this disorder such as 

high levels of spontaneous and DNA cross-link-induced chromo- 

somal breaks and high frequencies of spontaneous and psor- 

alen-induced deletions. An end joining deficiency could also 

explain the predisposition to cancer associated with these pa-

tients, since unrepaired or misrepaired DNA lesions could ul-

timately lead to loss of function of genes essential for proper 

cellular maintenance and growth. 

In addition, a DNA end joining defect in FA cells could 

potentially explain a previous result observed by our labora-

tory. HR activity was found to be elevated in Fanconi anemia 

fibroblasts and in nuclear extracts prepared from FA cells as 

compared with HR activity in fibroblasts from normal donors 

(32). We have also observed that Rad51, the mammalian ho-

mologue of the bacterial recombination protein RecA, is sub-

stantially elevated in extracts prepared from FA fibroblasts.2 

Recently, it was demonstrated that the human Rad52 protein, 

a protein involved in mammalian HR, binds double strand 

breaks (33). We speculate that HR and Rad51 may be elevated 

in FA cells in response to an increased number of unrepaired 

DSBs that result from the described end joining deficiency. 

Finally, the data indicate that mixing FA-A or FA-C extracts 

with FA-D extracts is able to reconstitute wild-type end joining 

activity levels, while mixing FA-A and FA-C extracts has no 

effect on end joining levels. While FA-D patients have the same 

clinical symptoms as FA patients from the other complemen-

tation groups, there are reports of FA-D cells having unique 

biochemical characteristics (34–36). In particular, a multipro- 

tein complex of four cloned FA gene products (FANCA, FANCC, 

FANCG, and FANCF) is detected only in wild-type and FA-D 

cells, indicating that all of the FA proteins, with the exception 

of FANCD, are required for the proper formation of this com-

plex (36). One could imagine that a preassembled “FA complex” 

is required for wild-type end joining activity in the extracts. 

This could only be provided by extracts from FA-D cells. Thus, 

when FA-A and FA-C extracts are mixed, neither would pro-

vide the “FA complex,” and wild-type end joining activity would 

not be reconstituted. 

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