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Fanconi Anemia FANCG Protein in Mitigating Radiation- and Enzyme-Induced DNA Double-Strand Breaks by Homologous Recombination in Vertebrate Cells

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The rare hereditary disorder Fanconi anemia (FA) is characterized by progressive bone marrow failure, congenital skeletal abnormality, elevated susceptibility to cancer, and cellular hypersensitivity to DNA cross-linking chemicals and sometimes other DNA-damaging agents. Molecular cloning identified six causative genes (*FANCA*, *-C*, *-D2*, *-E*, *-F*, and *-G*) encoding a multiprotein complex whose precise biochemical function remains elusive. Recent studies implicate this complex in DNA damage responses that are linked to the breast cancer susceptibility proteins BRCA1 and BRCA2. Mutations in BRCA2, which participates in homologous recombination (HR), are the underlying cause in some FA patients. To elucidate the roles of FA genes in HR, we disrupted the *FANCG/XRCC9* locus in the chicken B-cell line DT40. FANCG-deficient DT40 cells resemble mammalian *fancg* mutants in that they are sensitive to killing by cisplatin and mitomycin C (MMC) and exhibit increased MMC and radiation-induced chromosome breakage. We find that the repair of I-*Sce*I-induced chromosomal double-strand breaks (DSBs) by HR is decreased ~9-fold in *fancg* cells compared with the parental and *FANCG*-complemented cells. In addition, the efficiency of gene targeting is mildly decreased in *FANCG*-deficient cells, but depends on the specific locus. We conclude that *FANCG* is required for efficient HR-mediated repair of at least some types of DSBs.

Fanconi anemia (FA), a rare autosomal recessive disorder, is clinically characterized by progressive bone marrow failure, congenital skeletal abnormality, and elevated susceptibility to malignant tumors (reviewed in references 1, 19, and 24). FA is one of the genetic diseases in which the capacity to maintain genomic integrity is severely impaired (24). Cells derived from FA patients exhibit spontaneous chromosomal instability (24), which is intensified following treatment with DNA cross-linking agents such as mitomycin C (MMC) and cisplatin (42). The DNA cross-linker sensitivity is a hallmark of FA and has been used as a simple diagnostic test (2).

FA is caused by mutations in multiple genes. Somatic cell fusion studies defined at least eight FA complementation groups, and six of them were used to clone the complementing genes, which are designated *FANCA*, *-C*, *-D2*, *-E*, *-F*, and *-G* (1, 19, 24). These FA genes do not have any obvious domains or motifs that would suggest possible biochemical functions. Given the similar clinical and cellular features of the different complementation groups, the FA genes are thought to share at least one common function in a single biochemical pathway. Indeed, two-hybrid and immunoprecipitation analyses indicated that FA proteins (A, C, E, F, and G) form a multisubunit nuclear complex (reviewed in references 1, 19, 21, and 24), and the complex has recently been reported to interact with FANCD2 protein (38).

It has been suggested that the FA pathway works in a DNA damage response pathway (1, 19, 24). The occurrence of spontaneous chromosome breakage in FA cells is consistent with a potential role in repairing double-strand breaks (DSBs). In eukaryotic cells, there exist two major DSB repair pathways, nonhomologous end joining and homologous recombination (HR) (29, 57). Defects in either DSB repair pathway cause spontaneous chromosome breakage (27, 28, 39, 46, 51–53; reviewed in reference 55). Furthermore, the repair of interstrand cross-link damage, which may involve the generation of a DSB as an intermediate, requires HR in both prokaryotes and yeast (11, 55). However, FA cells generally exhibit normal levels of sister chromatid exchange (SCE) (31, 60), suggesting that gross defects in cellular HR activity are rather unlikely in FA cells (10, 24, 47).

Nevertheless, recent studies support the view that FA genes may have a link to the HR process. For example, the FANCD2 protein appears to function in association with the breast cancer susceptibility protein BRCA1 (17), mutations in which produce defects in HR (35, 44). These two proteins physically interact and colocalize in distinct nuclear foci following DNA damage. FANCD2 focus formation is dependent on intact

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BRCA1, the FA nuclear complex, and monoubiquitination of D2 protein at amino acid residue K561 (17). Most importantly, another link to recombination comes from the observation that biallelic causal mutations of *BRCA2* are present in human FANCD1 cells (23). BRCA2 is thought to be vital in HR, perhaps by helping to form the Rad51 nucleoprotein filament (6, 40, 62; reviewed in references 56 and 58), a key structure that initiates homologous pairing (5, 29, 57). Thus, the fact that *BRCA2* is actually an FA gene suggests that at least a subset of FA patients may have defective HR (49).

To date, except for *BRCA2* (which could vary depending on the specific mutant allele), formal proof is still lacking for involvement of FA proteins in HR. To evaluate HR activity of cells defective in FA genes, we established *FANCG*-deficient cells by using gene targeting technology with the chicken B-cell line DT40 (4). The *fancg* DT40 mutant has a phenotype resembling that of human and rodent *FANCG* mutants. In particular, we show that our *fancg* knockout cells have not only decreased HR capacity for repairing enzymatically induced, site-specific chromosomal DSBs but also elevated ionizing radiation (IR)-induced chromosomal aberrations in cells irradiated in late S and G_2 phases.

MATERIALS AND METHODS

Construction of targeting and expression plasmids and generation of antisera. A partial chicken cDNA sequence for *FANCG* was identified in the chicken WebBursalEST database and was subsequently used as a probe in screening a chicken intestinal mucosa cDNA library (Stratagene, La Jolla, Calif.). To isolate full-length *FANCG* cDNA, the N-terminal portion of chicken *FANCG* was PCR amplified from DT40 cDNA on the basis of the sequence of another chicken expressed sequence tag (EST) clone. Identity of the cDNA clones was confirmed by sequencing. An alignment of the predicted protein sequences from four species (human, mouse, hamster, and chicken) was performed with Genetyx-Mac version 10 (Software Development Co. Ltd., Tokyo, Japan).

Expression vectors were constructed by inserting the full-length chicken or human *FANCG* cDNA into either pApuro vector (50) or pCR3-loxP-IRES-EGFP-loxP vector (12). The human Rad51 expression vector (pAneo-hRad51) was previously described (34). The I-*Sce*I expression vector pcBASce was kindly provided by Maria Jasin (Sloan-Kettering Institute, New York, N.Y.).

Genomic DNA clones of *FANCG* were isolated by screening a chicken genomic library (Stratagene), and the gene disruption constructs were generated as previously described (4). Gene targeting with this construct was expected to replace the genomic region that encodes chicken FANCG amino acids 169 to 399 with selection markers.

Gene targeting and cell culture. Cells were maintained in RPMI 1640 medium supplemented with 10^{-5} M β-mercaptoethanol, 10% fetal calf serum, and 1% chicken serum (Sigma, St. Louis, Mo.) at 39.5°C. DNA transfections and selection were performed as described previously. To stably express human Rad51 or chicken FANCG in *FANCG*-deficient cells, expression vector pAneo-hRad51 or pCR3-loxP-chFANCG/IRES-EGFP-loxP was transfected by electroporation. Expressing clones were identified by Western blot analysis or by measuring enhanced green fluorescent protein (EGFP) fluorescence with a FACSCalibur (Becton Dickinson, Mountain View, Calif.), respectively.

Western blot analysis. Cells (10^6) were lysed in 20 μl of sodium dodecyl sulfate (SDS) sample buffer (25 mM Tris-HCl [pH 6.5], 1% SDS, 0.24 M β-mercaptoethanol, 0.1% bromophenol blue, 5% glycerol). Boiled aliquots were subjected to SDS–10% polyacrylamide gel electrophoresis. After transfer to a membrane, proteins were detected with rabbit anti-human Rad51 serum (kindly provided by Akira Shinohara) and horseradish peroxidase-conjugated goat anti rabbit immunoglobulin (Amersham Biosciences, Piscataway, N.J.) by using ECL Western blotting detection reagents (Amersham Biosciences).

Reverse transcription-PCR (RT-PCR) analysis. Total RNA was isolated from wild-type and *FANCG*-deficient cells by using RNAsol (Invitrogen, Carlsbad, Calif.). cDNA was synthesized from 1 μg of total RNA by using random hexamers and Superscript reverse transcriptase (Invitrogen). GoldTaq polymerase (Applied Biosystems, Inc., Foster City, Calif.) was used for amplification. Sequences of specific primers for chicken *FANCG* were 5′-GAACCTCTGGGCAGGACCCTAGCACACAAA-3′ and 5′-TGACCTCATCACACACTGTTA-3′. Primers for chicken *RAD51* were 5′-GCAGCCATGGCCATGCAGGT-3′ and 5′-AAGTCTTCATTCTTTTGCAT-3′.

Flow cytometric analysis of cell number, cell cycle, and EGFP expression. Cell growth was determined as previously described (52). Briefly, cells were stained with 5 μg of propidium iodide (PI) per ml, mixed with fixed numbers of plastic beads (Polysciences, Inc., Warrington, Pa.), and then counted with a FACSCalibur. For cell cycle analysis, cells were cultured in the presence of bromodeoxyuridine (BrdUrd) for 10 min and then fixed and stained with anti-BrdUrd antibody (PharMingen, San Diego, Calif.) and PI. EGFP expression was analyzed with a FACSCalibur, with dead cells gated out by PI staining.

Measurement of targeted integration frequencies. To analyze targeted integration events at the *KU70*, *OVALBUMIN*, *XRCC2*, and *XRCC3* loci, we used previously described disruption constructs (13, 52, 53). Some of them were modified by swapping drug resistance marker cassettes so that appropriate drugs could be used. DNAs were transfected into cells, and Southern blot analysis was performed following selection of clones resistant to the appropriate drug.

Analysis of chromosomal aberrations and SCE. Chromosome analysis was done with or without X-ray (2 Gy) or MMC (40 or 20 ng/ml, 24 h) treatment as previously described (51, 52). To analyze MMC-induced SCEs, cells were incubated in medium containing 50 ng of MMC per ml for 12 h. Colcemid was added to 0.1 μg/ml for the last 1.5 h of this incubation before harvest. Detection of SCE events was performed as previously described (51).

Measurement of HR-mediated repair of DSBs induced by I-*Sce*I expression. Transfections were done by electroporating (250 V, 975 μF) cells (10^7) suspended in complete culture medium with each of the following plasmid DNAs (30 μg) without linearization: I-*Sce*I expression vector (pcBASce) together with empty vector pApuro or human *FANCG* or chicken *FANCG* expression vector (pApuro-hFANCG or pApuro-chFANCG, respectively). pBluescript SK was also transfected as a negative control. At 24 h after electroporation, cells were transferred to 96-well cluster trays containing 2.0 mg of G418 per ml. Cells were grown for 10 to 14 days, and surviving G418-resistant colonies were counted to measure the frequency of HR-mediated repair of I-*Sce*I-induced DSBs. To determine the nature of repair events, genomic DNA was extracted from expanded G418-resistant colonies, restricted by *Kpn*I and *Sac*I double digestion, and examined by genomic Southern blotting with a *neo* fragment as a probe.

Measurement of sensitivity of cells to X rays, MMC, and cisplatin. Serially diluted cells were plated in medium containing methylcellulose and then irradiated with 4 MV of X rays (linear accelerator; Mitsubishi Electric Inc., Tokyo, Japan) at a dose of 100 cGy per min. To measure sensitivity to MMC (Kyowa-Hakkou, Tokyo, Japan), cells were incubated at 39.5°C in complete medium containing the compound for 1 h, washed three times with warm medium, and then plated in medium containing methylcellulose. Sensitivity to cisplatin (Nihon-Kayaku, Tokyo, Japan) was measured by plating cells onto the methylcellulose plates containing cisplatin.

Visualization of subnuclear focus formation of Rad51. Cells were harvested at the indicated time points after X irradiation (8 Gy) or MMC exposure (500 ng/ml for 1 h). Cytospin slides were prepared by using Cytospin 3 (Shandon, Pittsburgh, Pa.). Visualization of subnuclear foci was performed as previously described (61) with anti-hRad51 rabbit antiserum. Images were captured by TCS-NT laser scanning confocal microscopy (Leica Microsystems, Bannockburn, Ill.), and processed with Photoshop software (version 6; Adobe Systems Inc., San Jose, Calif.). Cells with more than four brightly fluorescing foci were scored as positive. At least 100 morphologically intact cells were examined at each time point.

Nucleotide sequence accession number. The chicken *FANCG* cDNA sequence has been deposited to DDBJ under accession number AB096039.

RESULTS

Cloning of chicken *FANCG* cDNA and cross-species comparison with other FANCG homologs. To isolate chicken FANCG cDNA, we examined the WebBursalEST database (http://swallow.gsf.de/dt40Est.html) and found several EST sequences that have significant homology to human *FANCG*/*XRCC9* (8, 32). Library screening combined with RT-PCR based on these sequences led to the identification of full-length chicken *FANCG* cDNA. Of note, RT-PCR products of N-terminal chicken *FANCG* were found to contain several alternatively spliced variants (data not shown). Most of them ap-

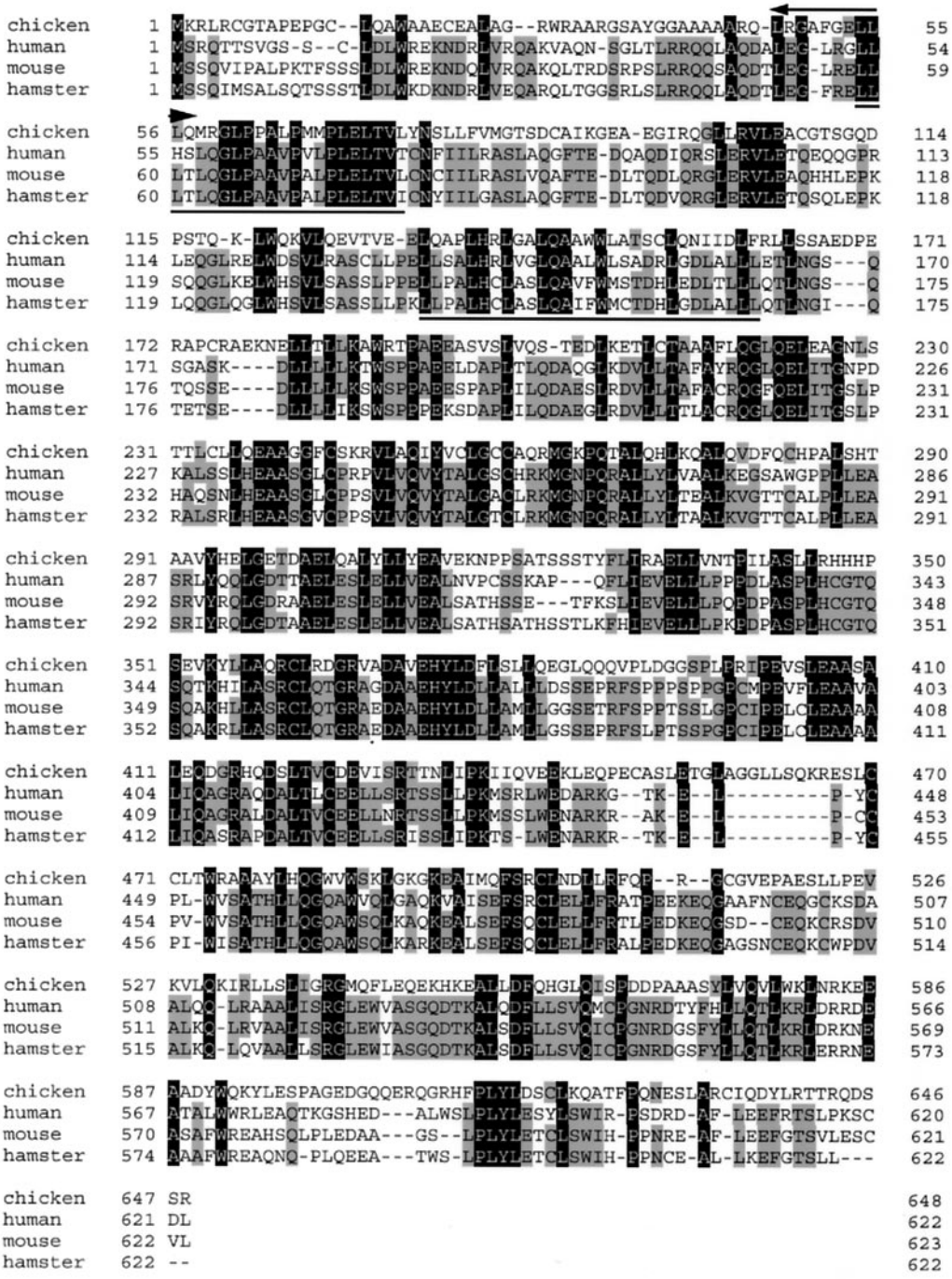


FIG. 1. Amino acid sequence comparison between chicken, human, mouse, and hamster FANCG proteins. Residues that are identical across four species or three species are indicated by black or grey shading, respectively. Locations of previously reported putative leucine zipper motifs (7, 32) are underlined. A line with arrowheads indicates amino acids that are missing in a variant transcript (see text).

peared to be out of frame, but we identified two sequences as candidate full-length chicken *FANCG*. One of them could indeed complement chicken *FANCG*-deficient cells (see below), whereas the other could not (data not shown). The latter transcript lacks nucleotides encoding 11 consecutive amino acids that overlap the putative leucine zipper (Fig. 1). These N-terminal variants were not detected during the course of human *XRCC9* cloning (L. H. Thompson, unpublished data) and were not found in silico by BLAST searching among more than 100 EST *FANCG* sequences deposited in the human EST database.

This isolated chicken cDNA, the first nonmammalian *FANCG* sequence reported, encodes a protein with 648 amino acids, compared with the 622-amino-acid human homolog. The percent amino acid sequence identity of chicken FANCG with the human, mouse, and hamster homologs is only 39% in each

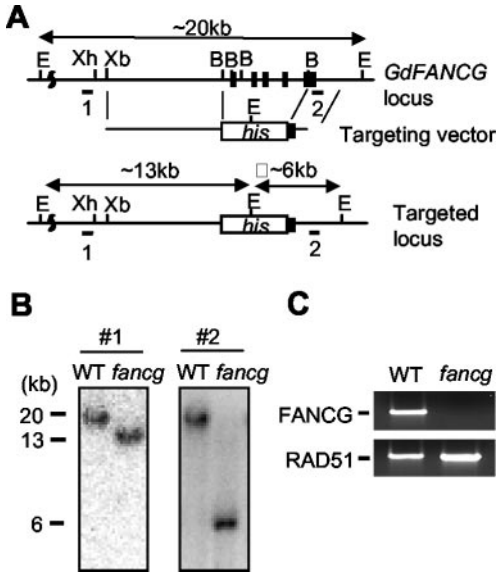


FIG. 2. Targeted disruption of the chicken *FANCG* locus. (A) Schematic representation of a partial restriction map of the chicken *FANCG* locus, the gene disruption vector, and the configuration of the targeted locus. Black boxes indicate the positions of exons that were disrupted. Relevant restriction enzymes sites: E, *Eco*RI; B, *Bam*HI; Xh, *Xho*I; Xb, *Xba*I. (B) Southern blot analysis of wild-type (WT) and *fancg* mutant cells. *Eco*RI-digested genomic DNA was hybridized with the probes indicated in panel A. (C) RT-PCR analysis of chicken *FANCG* mRNA expression in wild-type and *fancg* mutant cells. Isolation of total RNA, cDNA synthesis, and PCR were carried out as described in Materials and Methods.

case. Thus, overall conservation is rather low, whereas the identity between mouse and human FANCG is 71% and that between mouse and hamster FANCG is 83%. Similar to the case for mammalian FANCG homologs, no recognizable motifs besides a putative leucine zipper-like region are present in chicken FANCG protein (Fig. 1). Comparison of FANCG amino acid sequences revealed conserved regions or residues across the four species. It is noteworthy that all five non-disease-causing FANCG polymorphisms listed in the Fanconi anemia mutation database (http://www.rockefeller.edu/fanconi/mutate/default.html) are at residues that are not conserved. Furthermore, this database lists three bona fide missense mutations, two of which (L71P and L303P) lie at residues conserved across the four species. The third mutation is adjacent to a conserved residue, and the change is nonconservative (Arg to Pro at amino acid 22). Since human *FANCG* cDNA could not complement chicken *fancg* mutant cells (see below), some amino acid residues in the chicken sequence that are not conserved across the chicken counterparts are likely critical for chicken FANCG's function.

Generation of *fancg* mutant cells. Chicken *FANCG* genome clones were obtained by library screening with a chicken *FANCG* cDNA probe. We made a *FANCG* targeting vector by replacing ~4.5 kb of genomic segment with a histidinol resistance (*His*) (Fig. 2A) or puromycin resistance gene cassette. The upstream and downstream homology arms are ~6 and ~1 kb in length, respectively. Single transfection with this targeting vector eliminated the wild-type band in Southern blot analysis with two different genomic probes (Fig. 2B), indicating that there is only one *FANCG* allele in DT40 cells. This finding is consistent with the extensive synteny between chicken chromosome Z and human chromosome 9 (36) (the human *FANCG* locus is on chromosome 9p13 [32]) and with the fact that DT40 cells carry a single Z chromosome (46). Furthermore, we found a neighboring gene in the 5′ region of the chicken *FANCG* locus that has significant homology to the phosphatidylinositol glycan class o (*PIGO*) gene, which lies next to *FANCG* in the human genome sequence as well (data not shown). These data strongly indicate that we have deleted a phylogenetically conserved bona fide chicken *FANCG* gene.

The resulting *fancg* (*FANCG*-deficient) cells are phenotypically null, since no *FANCG* transcript was detected by RT-PCR analysis (Fig. 2C). Northern blot analysis was uninformative, perhaps because of low expression of *FANCG*. To facilitate our analysis of the role of *FANCG* in HR, we also targeted *FANCG* in wild-type cells that carry an integrated recombination substrate, SCneo (26), at the *OVALBUMIN* locus (13).

Chromosomal aberrations and SCE in *fancg* cells. We characterized the proliferative properties of wild-type and *fancg* cells by monitoring growth curves; an *xrcc3* HR-defective DT40 line (53) was monitored in parallel. DT40 *fancg* mutant cells grew at almost the same rate as wild-type cells and definitely more rapidly than *xrcc3* mutant cells (Fig. 3A). The proportion of dead cells in *fancg* mutant cultures was not increased, and the cell cycle distribution was indistinguishable from that of the wild type (Fig. 3B). The efficiencies of plating of cells in methylcellulose plates were ~100% for wild-type cells and ~60% for *fancg* cells.

We performed karyotype analysis of *fancg* cells before and after MMC treatment, since both spontaneous and MMC-induced chromosomal breakages usually characterize the FA phenotype. DT40 *fancg* cells did not have appreciably elevated levels of spontaneous chromosomal aberrations (Table 1), whereas the *xrcc3* mutant does (53). After treatment with MMC (40 ng/ml, 24 h), *fancg* cells exhibited more chromosomal aberrations than wild-type cells, and the magnitude was consistent with the survival data (see below). With a lower dose

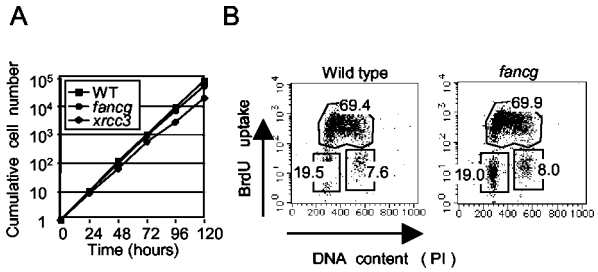


FIG. 3. Proliferation characteristics of wild-type (WT) and *FANCG*-deficient cells. (A) Cells were counted by flow cytometry, using fixed numbers of plastic beads as standards. The growth curves shown represent results from at least four independent experiments with two *fancg* clones. (B) Cell cycle distribution analysis as measured by BrdUrd incorporation and PI staining (DNA content). Cells were pulse-labeled for 10 min with BrdUrd and stained with anti-BrdUrd monoclonal antibody as described in Materials and Methods. The upper, lower left, and right lower regions identify cells in S, G_1, and G_2-M phase, respectively. Numbers are the percentages of cells in each region.

TABLE 1. Spontaneous and MMC-induced chromosome aberrations

Aberration type and genotype[a]	No. of aberrations/100 cells						% of cells with the following no. of aberrations[d]:		
	Chromatid		Chromosome		Chromatid exchanges	Total			
	Breaks	Gaps	Breaks	Gaps			0	1	2
Spontaneous									
Wild type[b]	0.3	1.0	0	0	0	1.3	98.7	1.3	0
fancg[b]	0.6	0.3	0	0.3	0	1.3	98.7	1.3	0
MMC induced									
Wild type[c]									
20 ng/ml	5.0	5.0	2.0	3.0	1.5	17	84	16	0.5
40 ng/ml	20	5.5	1.0	1.5	0	28	73	27	0.5
fancg[c]									
20 ng/ml	10	7.5	2.0	4.5	1.5	26	76	23	1.5
40 ng/ml	30	13	2.0	2.0	0.5	47	58	39	4

[a] For the MMC-induced aberrations, the MMC concentration is indicated.
[b] A total of 300 metaphases were scored.
[c] A total of 200 metaphases were scored.
[d] No cells contained more than two aberrations.

of MMC (20 ng/ml), the level of aberrations in *fancg* cells was comparable to that of wild-type cells treated with 40 ng of MMC per ml. Also, as for human FA cells (24), our *fancg* cells exhibited a normal level of MMC-induced SCE. The level of spontaneous SCE was 2.1 ± 1.7 and 2.7 ± 2.0 (mean ± standard deviation; $n = 50$) for wild-type and *fancg* cells, respectively. The level of MMC-induced SCE was 4.8 ± 2.7 per cell (mean ± standard deviation; $n = 50$) for both wild-type and *fancg* cells.

Sensitivity of *fancg* cells to killing by genotoxic agents. The ability of *fancg* mutant cells to form colonies following exposure to DNA-damaging agents was assessed. We define sensitivity as the dose reduction factor, based on the dose giving 10% survival, of the survival curve. Asynchronous *fancg* cells are not sensitive to X irradiation as measured by cell killing (Fig. 4A), but below we show modestly increased sensitivity based on induced chromosomal aberrations when cells are exposed in late S and G_2 phases. *fancg* cells are only slightly sensitive to MMC (~1.5-fold) but show ~3-fold sensitivity to cisplatin compared with the parental cells (Fig. 4B and C). This pattern somewhat resembles that for human FA-G cells (8), CHO UV40 and NM3 *fancg* mutants (32, 59), and mouse knockout mutants of *Fancg* (30, 63). Human *fancg* cells show 10- to 20-fold MMC sensitivity (reference 8 and our unpublished data), but there are little or no data for other agents with gene-complemented mutant cells. One study found IR sensitivity with *fancg* mouse splenocytes (63), but this was not seen with embryonic fibroblasts (30). Compared with our previously reported *rad51* paralog mutants (51, 53), the *fancg* mutant exhibits milder sensitivity toward each DNA-damaging agent tested. Indeed, *xrcc3* mutant cells were more sensitive to cisplatin than *fancg* cells, as shown in Fig. 4C.

To prove that the observed defects were indeed caused by specific gene disruption of *FANCG*, we expressed chicken or human *FANCG* cDNA in *fancg* mutant cells. *FANCG* expression was verified by EGFP fluorescence that was bicistronically expressed from the same construct. Full-length chicken *FANCG* cDNA was able to normalize cisplatin sensitivity

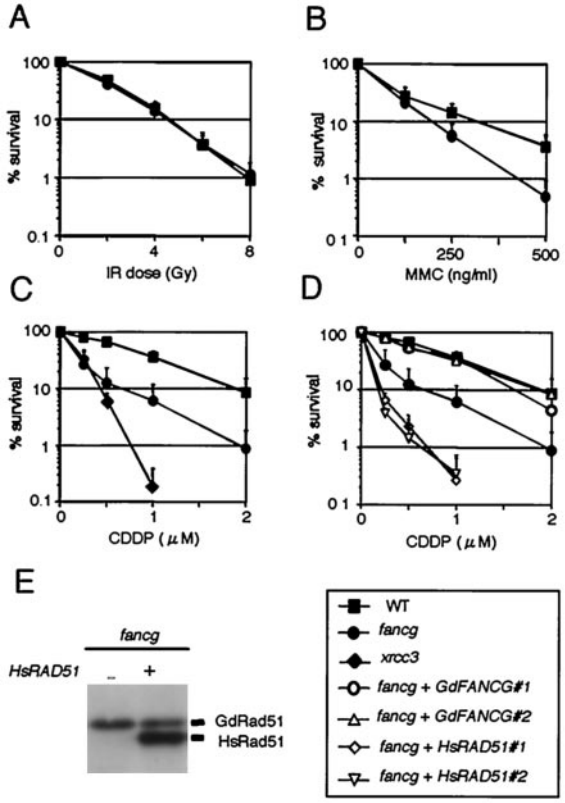


FIG. 4. Colony survival assay after genotoxic treatments. (A to D) The fractions of the surviving colonies after treatment compared to nontreated controls of the same genotype are shown as percent survivals. Error bars indicate standard deviations from at least three experiments. (A) MMC; (B) X rays; (C and D) cisplatin (CDDP). (E) Western blot analysis of Rad51 expression in *fancg* mutant cells transfected with human Rad51 expression vector. Positions of endogenous chicken Rad51 and transgene-derived human Rad51 are indicated.

TABLE 2. Targeted integration frequency in wild-type and *fancg* mutant cells

Targeting construct	Targeted integration frequency[a]				
	Wild type	*fancg*			Complemented with *GdFANCG*
		Clone 1	Clone 2	Clone 3	
KU70-*His*	14/34 (41) [2.1×10^{-6}]	10/46 (22) [2.0×10^{-6}]	ND[b]	ND	22/36 (61) [3.8×10^{-6}]
XRCC2-*Bsr*	16/37 (43) [2.0×10^{-6}]	ND	6/24 (25) [7.9×10^{-6}]	6/24 (25) [3.8×10^{-6}]	18/28 (64) [4.8×10^{-6}]
XRCC3-*His*	8/25 (32) [8.3×10^{-7}]	1/22 (4.5) [7.3×10^{-7}]	ND	ND	9/20 (45) [6.6×10^{-7}]
Ovalbumin-*Bsr*	24/26 (92) [3.9×10^{-5}]	24/28 (86) [3.8×10^{-5}]	ND	ND	ND

[a] Data are numbers of targeted clones at each locus per number of drug resistant clones analyzed by Southern blotting. The percentage of targeted integration events is given in parentheses. Numbers in brackets indicate transfection efficiency. Results for three different *fancg* mutant clones and one clone complemented with chicken *FANCG* cDNA are shown.
[b] ND, not determined.

(Fig. 4D), while human *FANCG* cDNA gave no correction (data not shown).

Mildly decreased targeted integration in *fancg* cells. We evaluated one aspect of HR capacity of *fancg* cells by measuring the efficiency of integration of transfected gene-targeting vectors. Transformants were selected with appropriate drugs, and targeting events were examined in each clone by Southern blot analysis. As summarized in Table 2, depending on the targeting vector or locus, we observed modest reductions (ranging from ~2- to 9-fold) in the ratio of targeted to total (targeted plus random) integration events in *fancg* cells at three loci, but not for *OVALBUMIN*. The extremely high targeting efficiency at *OVALBUMIN* may have a bearing on this result. In *fancg* cells complemented with chicken *FANCG* cDNA, the efficiency of targeting at these three loci was restored, confirming that defective gene targeting was caused by the *FANCG* gene disruption. These data suggest that FANCG might participate in the HR process that mediates targeted integration.

Defective HR-mediated repair of induced DSBs in *fancg* cells. To directly test whether *fancg* cells are defective for HR-mediated repair of DSBs, we used a transformant that carries the artificial recombination substrate SCneo (26) at the *OVALBUMIN* locus (13). The growth properties and sensitivities to genotoxic agents were indistinguishable between *fancg* cells with or without the integrated SCneo (data not shown). By using transient transfection of the plasmid encoding the rare restriction enzyme I-*Sce*I, a DSB is produced in one of two tandem nonfunctional *neo* genes and can be repaired by HR, resulting in expression of a functional *neo* gene. Thus, the frequency of G418-resistant colonies represents the HR-directed DSB repair capacity.

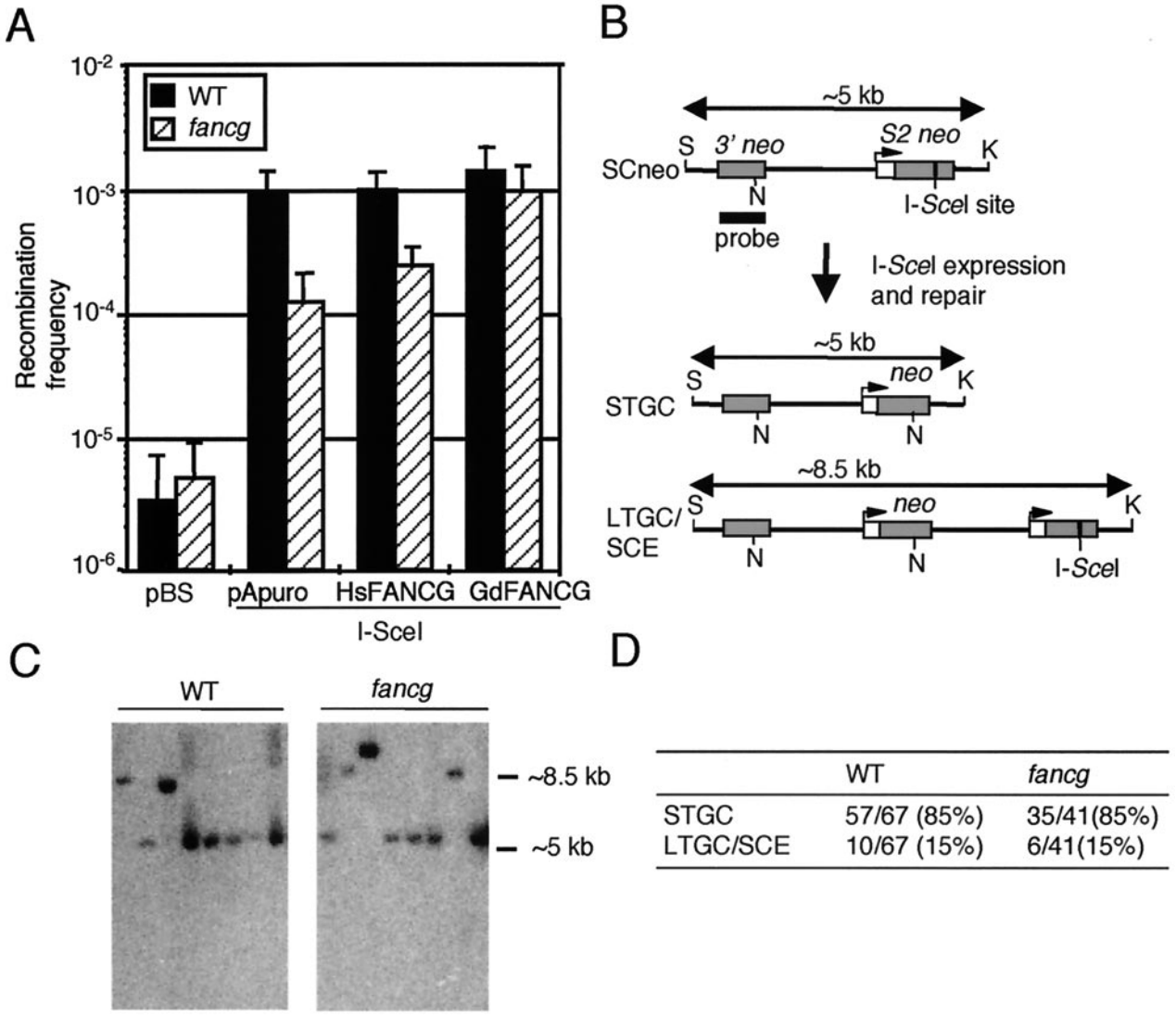
In parallel cultures, a negative control plasmid, pBluescript, was used; <100 colonies from 10^7 cells arose from spontaneous recombination. However, after transfection of the I-*Sce*I plasmid pcBASce into wild-type cells, a >100-fold induction occurred versus cells transfected with pBluescript. In contrast, we detected ~9-fold fewer G418-resistant colonies in *fancg* cultures, even after prolonged incubation following I-*Sce*I expression (Fig. 5). Importantly, this defect was corrected nearly to the wild-type level by transient transfection with a plasmid expressing chicken *FANCG* but not with plasmid carrying human *FANCG*. These data indicate that FANCG is required for efficient HR repair of I-*Sce*I-induced DSBs.

In mammalian cells, an induced DSB in SCneo undergoes HR-mediated repair through either short tract gene conversion (STGC) or long tract gene conversion (LTGC)/SCE events with the sister chromatid as the repair template (25). To examine how DT40 cells repair induced DSBs, we analyzed genomic DNA from G418-resistant clones by Southern blotting. We found that most repair events occurred by STGC in both parental (85%) and *fancg* (85%) cells, and relatively few LTGC/SCE events were detected (Fig. 5C and D), indicating that the HR repair proceeds in a normal manner in *fancg* cells even though the efficiency is reduced.

Given result described above, we investigated IR-induced chromosome aberrations in X-irradiated (2 Gy) cells at 3-h intervals after exposure. *fancg* cells showed significantly elevated chromosomal aberrations only in the 0- to 3-h interval (Fig. 6). Since the irradiated cells are asynchronous and evenly distributed throughout the cell cycle, cells entering M phase during this time period likely received irradiation in late S and G_2 phases. Thus, the result suggests a modest role for FANCG in HR repair between sister chromatids in cells that have replicated their DNA (52). Chromosomal radiation sensitivity was previously reported for FA lymphoblasts and fibroblasts before complementation analysis was developed (3, 20).

Normal Rad51 focus formation in *fancg* cells. The recent report that mutations of *BRCA2* can cause FA (23) suggests that other FA proteins might also influence Rad51's activity. DSBs induce subnuclear Rad51 foci that are thought to represent sites of HR. We found that both X-ray- and MMC-induced Rad51 focus formation (Fig. 7) (IR data not shown) were indistinguishable between *fancg* and wild-type cells. Since BRCA2 is essential for Rad51 focus formation (64), our results suggest that any direct function of FANCG in HR is distinct from that of BRCA2 or Rad51 paralogs.

For all five DT40 *rad51* paralog mutants, sensitivity to DNA damage was substantially suppressed by transfecting human *RAD51* (51, 53). This finding is probably consistent with the proposed roles of Rad51 paralogs being mediators of Rad51's function (33, 43). To obtain further insights regarding any relationship of FANCG to Rad51, we stably introduced human *RAD51* into our *fancg* cells and measured cisplatin sensitivity. Despite high human Rad51 expression that was comparable to the levels achieved in previous studies (Fig. 4E), cisplatin sensitivity was actually potentiated in *fancg* mutant cells (Fig. 4D). This might be due to overexpressed Rad51 acting in a dominant negative fashion by titrating away an essential factor in



	WT	*fancg*
STGC	57/67 (85%)	35/41(85%)
LTGC/SCE	10/67 (15%)	6/41(15%)

FIG. 5. Analysis of HR-mediated repair of I-*Sce*I-induced DSBs. (A) Recombination frequencies in integrated SCneo substrate in wild-type (WT) (black bars) and *fancg* (hatched bars) cells. Means ± standard deviations from at least six separate experiments are shown. Cells carrying the recombination substrate were transiently transfected with the indicated plasmids and then selected in the presence of G418. (B) Structures of the SCneo construct and expected HR repair products. K, *Kpn*I; N, *Nco*I; S, *Sac*I. STGC or LTGC/SCE events could be identified by Southern blotting with *Kpn*I- and *Sac*I-restricted genomic DNA and the indicated *neo* probe. (C) Representative Southern blot analyses of G418-resistant clones from wild-type (eight clones) and *fancg* (eight clones) cells. The expected sizes of STGC (~5 kb) and LTGC/SCE (~8.5 kb) events are indicated. (D) Number of G418-resistant clones with STGC or LTGC/SCE events. One clone from *fancg* cells exhibited a larger band than expected (shown in panel C), but this was counted as LTGC events.

the cisplatin damage response pathway and thus further reducing damage tolerance. Together, these results lead us to conclude that FANCG plays a different role than BRCA2 and Rad51 paralogs in the HR repair pathway.

DISCUSSION

In this study, we provide genetic evidence for a contributory role of *FANCG* in HR as measured by three different assays. First, HR-mediated repair of I-*Sce*I-induced chromosomal DSBs was reduced in *fang* mutant cells. Second, the overall efficiency of gene targeting was mildly decreased at three of four loci examined. Third, the levels of chromosome aberrations were elevated in X-irradiated cells in late S to G_2 phase compared with cells irradiated in other phases. Given a primary role of HR in repairing IR-induced DSBs in late S and G_2 phases in DT40 cells, compared with the secondary role of nonhomologous end joining (52), these findings are consistent with a role of FANCG in HR-mediated repair.

However, other HR-related processes examined were unaffected in the *fancg* cells. Specifically, the frequency of spontaneous or MMC-induced SCE appears to be normal. SCEs may be considered a surrogate marker of recombination efficiency, in contrast to chromosomal aberrations, which reflect biologically relevant HR repair. Moreover, the IR sensitivity of asynchronous *fancg* cells was normal, which differs from the case

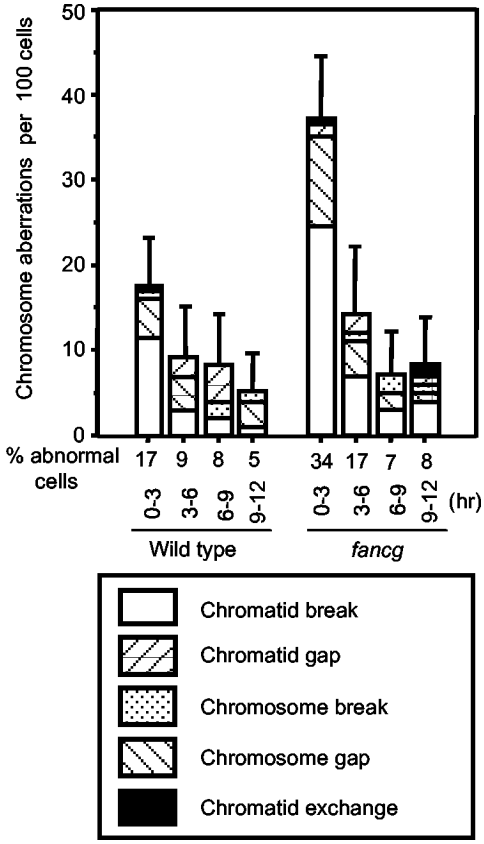


FIG. 6. IR-induced chromosome aberrations in wild-type and *fancg* mutant cells. Cells were X irradiated (2 Gy) and sampled at 3-h intervals. One hundred cells were scored at each time point except for the 0- to 3-h interval, where 200 cells were scored. Error bars represent 95% confidence intervals. The percentage of abnormal cells at each time point is also shown. Cells harboring more than two aberrations were not detected after IR in both wild-type and *fancg* cells.

for *rad51* paralog mutants. Thus, *FANCG* in DT40 cells seems to be important for optimal activity of only selected processes that depend on HR.

It has been shown that the FA multiprotein complex's assembly and nuclear localization is impaired in human *fancg* cells (15, 16). However, at present it is not clear whether defective complex localization underlies the entire phenotype of *fancg* cells. For example, human FANCG has been reported to interact directly with cytoplasmic CYP2E1 and appears to down-regulate the level of CYP2E1, a potential generator of reactive oxidative species (ROS) (14).

The FANCG protein likely has a function different from that of BRCA2 or RAD51 paralogs in HR for several reasons. First, we found no change in Rad51 focus formation in *fancg* cells, in keeping with one recent report (18) but not with another (9). Second, overexpression of human Rad51 did not increase cisplatin resistance of *fancg* mutant cells, whereas the defects in Rad51 paralog mutants were compensated for by elevating Rad51 protein (51, 53). (The effect of Rad51 overexpression in *brca2* DT40 cells remains to be clarified.) We suggest that FANCG may function in parallel with BRCA2 by leading to common phenotypic consequences. It will be interesting to test this hypothesis by making mutant cells deficient in both *BRCA2* and *FANCG*. Given the possibility that ATM regulates BRCA2 (58), this relationship may be relevant to the idea that FANCD2, which is also a phosphorylation target of ATM (54), may represent a convergence of two separate pathways, e.g., ATM signaling and the function of the FA nuclear complex.

So far, with the exception of BRCA2, no direct interactions between FA proteins and repair enzymes such as Rad51 and Mre11 have been identified. The defects in *fancg* cells are consistently milder than those observed in other HR-defective mutants such as the Rad51 paralogs (45, 48). These facts seem to be consistent with the view that FANCG plays an indirect, facilitating role in HR. For example, the FA proteins may function in part through chromatin remodeling (41). FANCA was reported to interact with Brg1 (37), a component of the human SWI/SNF complex, and FANCC interacts with the transcriptional repressor protein FAZF (22). It is possible that defective chromatin remodeling could influence cellular HR capacity (such as gene targeting efficiency) by modulating transcription, or temporal expression, of genes directly involved in HR.

Accumulating evidence supports a role for the FA proteins in determining the level of cytoplasmic ROS (reviewed in reference 1). Specifically, ROS may be elevated in FA group G human lymphoblasts, since they are reported to have elevated oxidative DNA damage, which is reversed in *FANCG*-complemented cells (14). Importantly, DNA damage by ROS appears to be a major cause of spontaneous chromosome breaks (28). Thus, the extensive chromosome aberrations often seen in FA cells (but not *fancg* DT40 cells) may be due to increased DNA damage by excessive generation of ROS along with compromised damage response processes linked to HR.

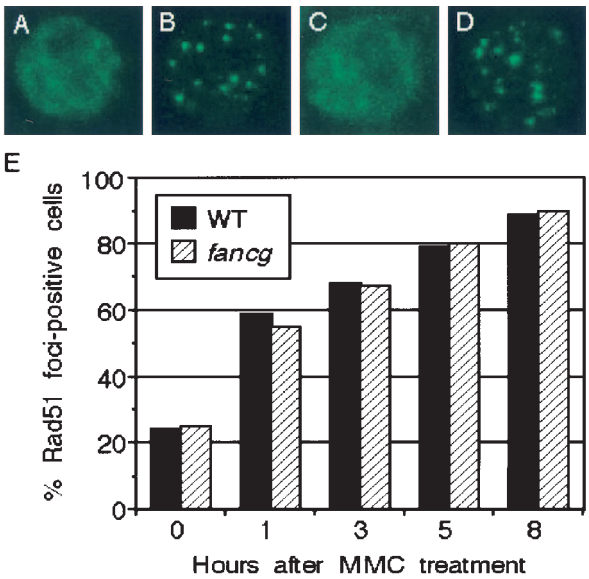


FIG. 7. Subnuclear focus formation of Rad51 in response to MMC treatment. (A to D) Wild-type (A and B) and *fancg* (C and D) cells were analyzed 5 h after treatment with MMC (500 ng/ml for 1 h) (B and D) or were untreated (A and C). (E) Kinetics of Rad51 focus formation after the same MMC exposure. Cells with more than four distinct and bright foci were counted as positive. WT, wild type.

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