Chromosome Instability and Defective Recombinational Repair in Knockout Mutants of the Five Rad51 Paralogs

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The Rad51 protein, a eukaryotic homologue of *Escherichia coli* RecA, plays a central role in both mitotic and meiotic homologous DNA recombination (HR) in *Saccharomyces cerevisiae* and is essential for the proliferation of vertebrate cells. Five vertebrate genes, *RAD51B*, *-C*, and *-D* and *XRCC2* and *-3*, are implicated in HR on the basis of their sequence similarity to Rad51 (Rad51 paralogs). We generated mutants deficient in each of these proteins in the chicken B-lymphocyte DT40 cell line and report here the comparison of four new mutants and their complemented derivatives with our previously reported *rad51b* mutant. The Rad51 paralog mutations all impair HR, as measured by targeted integration and sister chromatid exchange. Remarkably, the mutant cell lines all exhibit very similar phenotypes: spontaneous chromosomal aberrations, high sensitivity to killing by cross-linking agents (mitomycin C and cisplatin), mild sensitivity to gamma rays, and significantly attenuated Rad51 focus formation during recombinational repair after exposure to gamma rays. Moreover, all mutants show partial correction of resistance to DNA damage by overexpression of human Rad51. We conclude that the Rad51 paralogs participate in repair as a functional unit that facilitates the action of Rad51 in HR.

Double-strand DNA breaks (DSBs) are produced by ionizing radiation (IR) and certain chemicals, and they likely occur frequently during DNA replication (21, 34). A single unrepaired DSB may stimulate cell cycle checkpoints and cause cell death (3, 25). Homologous recombination (HR) has emerged as a major DSB repair pathway in mammalian cells (29, 35, 44, 65, 66), as well as in the yeast *Saccharomyces cerevisiae*. Indeed, the analysis of radiosensitive yeast mutants has revealed a number of key genes involved in HR, which comprise the *RAD52* epistasis group (2, 32, 54), and the HR pathway is conserved from yeast to humans (4, 18, 53, 65). Although yeast is capable of proliferating at a reduced rate in the absence of functional HR, this repair pathway is essential for viability in cycling vertebrate cells for coping with DNA lesions arising during DNA replication (55, 56, 67, 73). This species difference is probably due to the several-hundred-fold difference in genome size between vertebrates and yeast.

ScRad51 is closely related to the *Escherichia coli* recombination protein RecA (5). Among the proteins of the Rad52 epistasis group, Rad51 has the highest degree of structural and functional conservation among all eukaryotes. The high degree of identity of ScRad51 with the human homolog (59% identity) and chicken homolog (59% identity) suggests that Rad51's function is conserved across eukaryotes. A central role for Rad51 in HR in vertebrates is supported by the finding that Rad51 deficiency (36, 55, 67), but not Rad52 or Rad54 defi-

ciency, is lethal to cells (4, 18, 49, 72). In vitro studies show that RecA and Rad51 form multimeric helical nucleoprotein filaments that are assembled on single-stranded DNA (ssDNA) (2). Recent work suggests that the preferred DNA substrate for ScRad51 protein is not ssDNA but rather double-stranded DNA (dsDNA) with either 5' or 3' ssDNA tails (40). The nucleoprotein filaments are most likely involved in the search for homologous sequence, strand pairing, and strand exchange. Such filaments could be the basis of IR-induced Rad51 nuclear foci in vertebrate cells (20, 38, 39, 47, 52, 62). In both yeast and mammals, meiotic cells express the Rad51 homolog called Dmc1, which shares ~50% identity with Rad51 in each case (6, 22).

Other relatives of the *RAD51* gene that probably arose by gene duplication and the evolution of new functions (paralogs) are present in yeast and higher eukaryotes (64, 65). Mitotic as well as meiotic cells express these paralogs, which consist of Rad55 and Rad57 in *S. cerevisiae* and XRCC2 (12, 37), XRCC3 (37, 63), Rad51B (or Rad51L1) (1, 11, 48), Rad51C (or Rad51L2) (17), and Rad51D (or Rad51L3) (11, 33, 46) in vertebrates. These five human Rad51 paralogs have only 20 to 30% identity with human Rad51 and show less than 30% identity to each other and to yeast Rad55 and Rad57 (reviewed by Thacker [64]). Unlike Rad51, none of the Rad51 paralogs appears to interact with itself in yeast two-hybrid assays (50, 65), which is reminiscent of yeast Rad55 and Rad57 (24, 30). Overexpression of yeast Rad51 partially suppresses the DNA repair defect of *rad55* and *rad57* mutant yeast strains, implying that Rad55 and Rad57 may functionally cooperate with Rad51. This idea is supported by physical interactions between Rad51 and Rad55 and between Rad55 and Rad57 (24, 30, 57). Similarly, physical interactions can occur between human Rad51 and XRCC3, between XRCC3 and Rad51C, between Rad51B and

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Rad51C, between Rad51C and Rad51D, and between Rad51D and XRCC2 (8, 37, 50, 65). These observations argue that Rad51 paralogs may form a functional complex and cooperate with Rad51, analogous to the *S. cerevisiae* Rad55 and Rad57 proteins.

Chicken DT40 cells, which have much more efficient HR than mammalian cells (10), are an attractive model for mammalian systems. Murine embryonic stem cells and DT40 cells have exhibited the same phenotypes for the previously reported HR mutants, including defective proliferation of Rad51-deficient cells (55, 67) and Mre11-deficient cells (71, 73), nearly normal phenotype of Rad52-deficient cells (49, 72), and elevated radiosensitivity of Rad54-deficient cells (4, 18). To investigate the role of the five Rad51 paralogs in vertebrate cells, we generated mutants deficient in each of these proteins in DT40 cells. Here we report the properties of the *xrcc2*, *xrcc3*, *rad51c*, and *rad51d* mutants and compare them with our recently described *rad51b* mutant (59). Remarkably similar, but not identical, phenotypes of all five DT40 mutants, as well as the XRCC2 and XRCC3 hamster mutants (7, 37, 63), support the concept that the Rad51 paralogs have nonoverlapping roles and might operate as a single functional entity in HR.

MATERIALS AND METHODS

Plasmid constructs. Chicken cDNAs for *RAD51* paralog genes, except for *XRCC3*, were isolated from a chicken intestinal mucosa cDNA library (Stratagene, La Jolla, Calif.) by low-stringency cross hybridization using human or mouse cDNA as a probe. We isolated a chicken *XRCC3* partial cDNA using a degenerate PCR strategy based on human (63) and mouse (kindly provided by J. E. Lamerdin) amino acid sequences. Identity of the cDNA clones was confirmed with sequencing. We have obtained full coding sequences of chicken Rad51D and Xrcc2, which correspond to the published human cDNAs, whereas our chicken Rad51C and Xrcc3 cDNA clones lack N-terminal portions corresponding to human amino acids 1 to 91 and 1 to 147, respectively (17, 37). Nevertheless, comparison between corresponding human and chicken putative amino acid sequences revealed relatively high identities, namely, 71% (Rad51C), 65% (Rad51D), 69% (XRCC2), and 55% (XRCC3). Genomic DNA fragments of the *RAD51* paralogs were isolated from DT40 genomic DNA by long-range PCR with primers based on cDNA sequences, and the gene disruption constructs were generated as previously described (10). Strategies for the gene disruption are shown at our web site <http://www.rg.med.kyoto-u.ac.jp/homepage/publications/publications>. Gene targeting of these constructs was expected to replace amino acid sequences with selection markers as follows, corresponding to the published human genes: amino acids 196 to 235 in Rad51C (17), 138 to 153 in Rad51D (11), 47 to 89 in XRCC2 (12, 37), and 212 to 242 in XRCC3 (63). The human Rad51 cDNA and human or mouse Rad51 paralog cDNAs were cloned into an expression vector with the chicken β -actin promoter. The conditions for cell culture and DNA transfections were described previously (10).

Flow cytometric analysis. To determine the proportion of dead cells, cells were washed, resuspended in phosphate-buffered saline containing 5 μ g of propidium iodide (PI)/ml, and analyzed immediately by FACSCalibur analysis (Becton-Dickinson, Mountain View, Calif.).

Measurement of targeted integration frequencies. To analyze targeted integration events at the β -actin, *Ovalbumin* (10), and *XRCC2* loci, disruption construct DNAs were transfected into cells, and Southern blot analysis was performed following selection of clones resistant to the appropriate antibiotic.

Analysis of chromosomal aberrations and SCE. Chromosome and sister chromatid exchange (SCE) analyses were done as previously described (55, 56). To analyze mitomycin C (MMC)-induced SCEs, cells were incubated in medium containing 0.05 μ g of MMC/ml for 12 h. The length of the DT40 cell cycle is $\sim$ 8 h. Colcemid, to a concentration of 0.1 μ g/ml, was added for the last 1.5 h of this incubation before harvest. For the statistical evaluation of SCE, we performed an analysis of variance with the Bonferroni/Dunn multiple comparison test for intergroup comparison using StatView software (version 5; Abacus Concepts, Inc., Berkeley, Calif.).

Measurement of sensitivity of cells to gamma rays, MMC, and cisplatin. Serially diluted cells were plated in medium containing methylcellulose and irradiated with a ^{137}Cs gamma-ray source. To measure sensitivities 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. Plating efficiencies of wild-type and Rad51 paralog mutants in methylcellulose plates are $\sim$ 100% and $\sim$ 50%, respectively.

Western blot analysis. A total of 10^6 cells were washed with phosphate-buffered saline and lysed in 20 μ l of sodium dodecyl sulfate (SDS) lysis buffer (25 mM Tris-HCl [pH 6.5], 1% SDS, 0.24 M β -mercaptoethanol, 0.1% bromophenol blue, 5% glycerol). Following sonication and boiling, aliquots (routinely 50%) were subjected to SDS-10% polyacrylamide gel electrophoresis (PAGE). After transfer to nylon membrane, proteins were detected by polyclonal rabbit anti-human Rad51 polyclonal serum (61) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, Calif.) using a Super Signal CL-HRP substrate system (Pierce, Rockford, Ill.).

Rad51 focus formation assay. Cells were harvested at various time points after gamma irradiation. Cytospin slides were prepared using Cytospin 3 (Shandon, Pittsburgh, Pa.). Staining and visualization of Rad51 foci were performed as previously described (72) using the same anti-Rad51 rabbit antiserum as in Western blotting experiments. Cells with more than four brightly fluorescing foci were counted as positive. At least 100 morphologically intact cells were counted at each time point.

RESULTS

Construction and growth properties of Rad51 paralog mutants. In chicken DT40 cells, we generated mutant clones deficient in each Rad51 paralog. Strategies for each gene disruption are shown in the supplementary material at our web site (<http://www.rg.med.kyoto-u.ac.jp/homepage/publications/publications>). As previously observed in our *rad51b* (represents *RAD51B*^{-/-}) mutant clones (59), the growth rates of *rad51c*, *rad51d*, *xrcc2*, and *xrcc3* mutants were significantly lower than that of wild-type cells. While the length of the cell cycle is comparable between wild-type and mutant clones (data not shown), higher proportions of dead cells (20 to 30%) were seen in these mutant cultures (Fig. 1), which can explain their lower growth rates. The absence of overt accumulation of mutant cells in either G₁ or G₂ phase (data not shown) might be explained by the defective p53 status in parental DT40 cells (58).

An increased occurrence of spontaneous chromosomal aberrations was observed in *rad51b* DT40 cells (59) and in XRCC2- and XRCC3-deficient hamster cells (12, 37, 63). In close agreement with results for those mutants, the four new mutant clones had significantly increased levels of spontaneous chromosomal breaks (Table 1), which are likely to be responsible for the reduced viability. However, the number of breaks varied among mutants; the reason for this is currently unclear. In particular, the *rad51d* cells had $\sim$ 3-fold more breaks than *rad51c* and *xrcc2* and *-3* mutants, whereas *rad51b* cells had a low number (59). These differences suggest that there may be functional differences in the roles of the paralogs in HR repair.

Defective HR in Rad51 paralog mutants. To assess the HR capacity of each mutant, we measured both the efficiency of targeted integration of transfected genomic DNA fragments and the distribution of SCE. As shown in Table 2, the frequencies of targeted integration events were reduced $\sim$ 8-fold in the *rad51c* clone and at least 30-fold in the *rad51d*, *xrcc2*, and *xrcc3* clones. Furthermore, the targeting frequencies were measured in clones transfected with the corresponding human or mouse cDNAs. The complemented clones of *rad51d*, *xrcc2*, and *xrcc3* mutants showed increased targeting efficiency, up to normal

FIG. 1. Level of spontaneous death in cells defective in Rad51 paralogs. (A) Cell viability was assessed by flow cytometric analysis using PI uptake (y axis) and forward scatter, representing cell size (x axis). Numbers show the percentage of dead cells (PI bright and PI dim or small), and the solid line separates live from dead cells. (B) Bars represent the level of spontaneous cell death in indicated genotypes. Complemented mutant clones were transfected with the corresponding human (*rad51c*, *xrcc2*, and *xrcc3*) or mouse (*rad51d*) cDNA. The means and standard deviations for three independent experiments are shown. WT, wild type.

levels in *rad51d* and *xrcc3* mutants. Only transfected *rad51c* clones showed an absence of complementation for targeting efficiency by human Rad51C expression, possibly due to divergence between human and chicken Rad51C. These observations demonstrate that Rad51C, Rad51D, XRCC2, and XRCC3, as well as Rad51B (59), are indeed involved in gene targeting mediated by HR.

TABLE 2. Targeted integration frequencies

Genotype	Frequency (%) ^a for targeting construct			
	<i>XRCC2-puro</i>	<i>Ov-puro</i> ^b	<i>Ov-neo</i> ^b	<i>KU70-hyg</i>
Wild type	12/20 (60)	24/37 (65)	18/36 (50)	18/37 (49)
<i>rad51c</i>	2/48 (4.2)	5/46 (11)	ND	ND
<i>rad51c</i> + <i>hRad51C</i>		2/24 (8.3)		
<i>rad51d</i>	0/38	0/22	ND	ND
<i>rad51d</i> + <i>mRad51D</i>		23/27 (85)		
<i>xrcc2</i>	ND	ND	0/34	0/43
<i>xrcc2</i> + <i>hXRCC2</i>				3/40 (7.5)
<i>xrcc3</i>	0/42	0/36	ND	ND
<i>xrcc3</i> + <i>hXRCC3</i>		11/22 (50)		

^a Data are numbers of targeted clones at each locus per number of drug resistant clones analyzed. The percentage of targeted integration events is given in parentheses. The frequencies of transformation are essentially identical in wild-type and all mutant lines (~40 clones per 10^7 transfected cells). ND, not determined.

^b Two targeting constructs of the ovalbumin locus contain either puromycin or neomycin resistance genes.

We previously showed that SCE events likely reflect post-replicative repair by HR that is associated with crossing-over between sister duplexes (56, 69). All the Rad51 paralog mutants exhibited reduced levels of both spontaneous SCE and SCE induced by MMC, an interstrand cross-linking agent (Fig. 2). These mutants showed statistically significant ($P < 0.001$) reduction in the SCE frequency compared with wild-type cells. These collective findings demonstrate that each paralog is indeed involved in HR.

Sensitivity to killing by gamma rays and cross-linking agents. The biologically relevant DNA repair capacity of each mutant was assessed in colony survival assays following exposure to DNA-damaging agents. Notably, *rad51c*, *rad51d*, *xrcc2*, and *xrcc3* mutants all showed a very similar patterns of sensitivity, which also agreed with those of *rad51b* cells. The gamma-ray sensitivity of each mutant was rather mild (≤ 2 -fold), and they were all ~3-fold sensitive to MMC based on estimated D_{10} values i.e., doses that reduce survival to 10% (Fig. 3A). However, each mutant was approximately eightfold more sensitive than normal cells to killing by cisplatin (*cis*-diaminedichloroplatinum-II), a DNA cross-linking agent that is widely used in chemotherapy (Fig. 3B). The complementation of each mutant with the corresponding human cDNA restored its cisplatin resistance partially or completely (*xrcc3* mutant) (Fig. 3B). These results for the complemented clones confirm that each specific gene disruption was responsible for the increased

TABLE 1. Spontaneous chromosomal aberrations

Genotype	No. of aberrations ^a				Total % of cells with ^b :	
	Chromosome type		Chromatid type		Breaks + gaps	Breaks
	Breaks	Gaps	Breaks	Gaps		
Wild type	0.25	0.25	0.25	1.25	2 ± 0.7	0.5 ± 0.5
<i>rad51c</i>	0	6	6.7	3.3	16 ± 3	6.7 ± 2.1
<i>rad51d</i>	14	8.7	10	6	39 ± 5	24 ± 4.0
<i>xrcc2</i>	0	9.3	6.7	5.3	21 ± 4	6.7 ± 2.1
<i>xrcc3</i>	2.7	10.7	6.7	6	26 ± 4	9.4 ± 2.5

^a Data are the numbers of aberrations per 100 cells. At least 150 mitotic cells were analyzed for each genotype. Total aberrations per cell and standard errors were calculated as described previously (60).

^b Data are means ± standard errors.

FIG. 2. Levels of SCE per cell before and after MMC treatment. For each preparation, 150 cells were analyzed. The mean number of SCEs per cell is indicated in each panel. The comparison of each Rad51 paralog mutant with a wild-type (WT) control cell is statistically significant ($P < 0.001$, Bonferroni/Dunn test).

sensitivity to cisplatin. It should be noted that previous findings with *xrcc2* and *xrcc3* hamster mutants (37, 63) are in close agreement with our new corresponding DT40 mutants for nearly all properties examined (mild radiosensitivity, high sen-

sitivity to cross-linking agents, chromosomal instability, and defective HR). However, the mutant hamster cells showed more pronounced sensitivity to MMC, i.e., 60- to 70-fold based on estimated D_{10} values (65). Although the reason for this

FIG. 3. Sensitivity of knockout cell lines to DNA-damaging agents. (A) Survival curves after treatments with gamma radiation and MMC. Data shown are representative of at least three independent experiments. Sensitivity data of *rad51b* cells was taken from our previous study (59). (B) Partial correction of cisplatin sensitivity in knockout mutants by overexpression of human Rad51. Data shown are representative of at least three independent experiments. (C) Western blot analysis of human Rad51 transformants derived from knockout mutants. Transformants have much higher steady-state levels of cDNA-derived human Rad51 than endogenous Rad51. Although highly conserved (95.6% identity), human Rad51 migrates slightly faster than the chicken counterpart in SDS-PAGE analysis (55). Given this high degree of conservation, it is likely that the antibody used, which was made against human Rad51, efficiently recognizes the chicken homolog. WT, wild type.

FIG. 4. Immunofluorescence visualization of Rad51 subnuclear foci after irradiation (8 Gy). A, wild-type; B and F, *rad51c*; C and G, *rad51d*; D and H, *xrcc2*; E and I, *xrcc3*. F to I, mutant cells complemented with the corresponding human (*rad51c*, *xrcc2*, and *xrcc3*) or mouse (*rad51d*) cDNA.

difference in MMC sensitivity is unclear, overall our results suggest that the biochemical roles of the Rad51 paralogs in HR are likely conserved between DT40 and mammalian cells.

Role of Rad51 paralogs in Rad51 focus formation. To further assess the role of the paralogs in HR, we analyzed nuclear Rad51 focus formation. Foci that are microscopically visible are believed to represent sites of recombinational DNA repair (20, 39, 47, 62). We exposed cycling wild-type and mutant cultures to gamma rays and then immunostained the cells with anti-Rad51 antiserum. The formation of Rad51 foci was severely impaired in each mutant cell line following IR treatments (Fig. 4 and 5), as previously observed in *xrcc3* hamster cells (7) and *rad51b* DT40 cells (59). At 5 h after IR treatment, less than 15% of the mutant cells contained a threshold number of distinct Rad51 foci (more than four per cell), whereas more than 60% of wild-type cells showed robust focus formation (Fig. 5A). Also, among focus-positive cells the average number of foci per cell at 8 h after IR was lower than the number in wild-type cells (Fig. 5B). The mutant cells transfected with the corresponding human or mouse cDNAs were able to efficiently form IR-induced Rad51 foci (Fig. 4). Since Rad51 protein levels did not change following genotoxic treatments in any mutant clone (data not shown), these results show that all Rad51 paralogs are involved in damage-induced redistribution of Rad51 within the nucleus.

Phenotypic suppression of Rad51 paralog mutants by human Rad51 overexpression. In *S. cerevisiae*, the overexpression of Rad51 partially suppresses the IR sensitivity of *rad55* and *rad57* mutant strains, but not vice versa (24, 30). Furthermore, we previously showed that the overexpression of human Rad51 (hRad51) cDNA in *rad51b* cells also restored the sensitivity to gamma rays and MMC to wild-type levels (59). Similarly, hRad51 overexpression partially corrected the sensitivity of *rad51c*, *rad51d*, *xrcc2*, and *xrcc3* mutants to cisplatin (Fig. 3B) and almost fully corrected gamma-ray resistance (data not shown). Thus, hRad51 can at least partially compensate for each of these paralogs under conditions where the amount of

hRad51 protein is highly overexpressed (~10-fold) compared with the endogenous Rad51 level (Fig. 3C). However, hRad51 overexpression in the Rad51 paralog mutants did not fully restore their capacity for HR, since gene targeting was still defective in *xrcc2* or *xrcc3* mutant clones highly expressing hRad51 (data not shown). We were unable to examine the reconstitution of Rad51 focus formation because of the high background of immunostaining in the presence of overexpressed hRad51 protein. These overexpression data, combined with the defective Rad51 focus formation in each Rad51 paralog mutant, imply that the paralog proteins are involved in the

FIG. 5. Induction of Rad51 foci by IR treatments. Cells were analyzed at the indicated time points after gamma irradiation (8 Gy). (A) A cell containing more than four distinct foci was scored as positive. Each bar represents the results of scoring at least 100 cells. (B) Average number of Rad51 foci per cell in cells scored as positive at 8 h after 8 Gy of IR.

recruitment of Rad51 into subnuclear assemblies that mediate homologous pairing and strand exchange.

DISCUSSION

Role of Rad51 paralogs in promoting the activity of Rad51 during HR. Our results shown here and other studies (29, 44, 59) indicate that all five Rad51 paralogs are important for HR in vertebrate cells. Mutant clones of each Rad51 paralog are quite similar in phenotype although quantitative differences were seen for several end points. Two-hybrid and coimmunoprecipitation analyses have suggested that each Rad51 paralog appears to have different interacting partners within the family, and together they might form a single complex (8, 17, 37, 50; reviewed by Thompson and Schild [65]). These results combined with our genetic data are consistent with the idea that Rad51 paralogs may act as a single functional unit during HR.

The following data have suggested that *S. cerevisiae* Rad51 paralogs, Rad55 and Rad57, participate in the formation of nucleoprotein filaments involving Rad51. First, stable protein interaction between Rad55 and Rad57 and transient interaction between Rad51 and Rad55 suggest that these molecules act in multiprotein complexes. Second, the repair defects of *rad55-rad57* mutants are partially suppressed by the overexpression of Rad51 protein (24, 30). Third, biochemical analysis points toward the Rad55-Rad57 heterodimer acting as a cofactor to promote the assembly of Rad51-ssDNA nucleoprotein filaments in the presence of replication protein A (57). These results support the notion that Rad55 and Rad57 are involved in HR by forming a stable complex that transiently interacts with Rad51 to promote the formation of Rad51 nucleoprotein filaments.

We investigated functional interactions between Rad51 and Rad51 paralogs in our genetic system. The overexpression of hRad51 at least partially normalized the defects of each Rad51 paralog mutant in repairing genomic damage by gamma rays or cisplatin. This observation might imply that each vertebrate Rad51 paralog participates in HR by facilitating the function of Rad51, analogous to Rad55-Rad57 in *S. cerevisiae*. Additionally, defective Rad51 focus formation in Rad51 paralog mutants suggests that the Rad51 paralogs promote the assembly of Rad51 nucleoprotein filaments at DNA lesions. A similar situation applies in *S. cerevisiae*, where mutations in *RAD55* and *RAD57* prevent the appearance of Rad51 foci during meiosis (19).

In mammalian *xrcc2* and *xrcc3* mutant cells, the repair of site-specific DSBs by HR was reduced 25- to 250-fold, and these defects were not restored by transient cotransfection with human *Rad51* cDNA (9, 29, 44). These dramatic reductions in intragenic HR efficiency were in marked contrast with only a fewfold reduction in late-S-phase radioresistance to gamma rays (15), where induced DSBs on one chromatid should be repaired by HR with the other intact sister chromatid (60). These observations suggest that some Rad51 paralogs might be involved in an HR subpathway that does not require Rad51. Given that the Rad51 paralogs, but not Rad51, are expressed in some nondividing cells (e.g., all except Rad51B are expressed in brain) (17, 37, 46, 48, 53), the paralogs might conceivably play a role in HR in resting cells in vertebrates.

Clinical implications of the role of Rad51 paralogs. Our experiments show that DNA damage induces Rad51 foci in a Rad51 paralog-dependent manner, and all Rad51 paralog mutants are highly sensitive to cisplatin. Thus, each Rad51 paralog plays a critical role in the response to this clinically important drug. Most DNA adducts produced by cisplatin are intrastrand cross-links and therefore would be repaired by nucleotide excision repair (NER) pathway (76). However, a small proportion of interstrand cross-links also occurs, and this type of lesion likely requires an HR repair mechanism besides NER. Since *xrcc2* hamster cells were shown to exhibit dramatic increases in chromosomal breaks following exposure to MMC (68), the formation of unrepaired DSBs during abortive cross-link repair most likely explains the extremely high sensitivity of *xrcc2* cells to MMC. Similarly, the high cisplatin sensitivity of DT40 clones deficient in Rad51 paralogs can be explained by defective HR-mediated repair of these DSBs. Consistent with a role of HR in cisplatin cross-link repair, HR-defective *S. cerevisiae* strains are equally sensitive to cisplatin as NER-defective strains (23). The low sensitivity of DT40 paralog mutants to MMC, compared with the hamster mutants, might be explained by less efficient formation of cross-links during activation of MMC in DT40 cells (16).

The occurrence of HR during the normal mitotic cell cycle is indicated by the appearance of Rad51 foci in S phase and by spontaneous SCE (61). SCEs are mediated at least partially by HR and occur at a frequency of approximately three exchanges per cell cycle in vertebrate cells (45, 56). However, the frequency of DSB repair events during S phase may be much higher than this, since crossing over during DSB repair seems to occur rarely in mammalian cells (28). Additionally, the presence of excessive chromosomal breaks in *rad51* and *mre11* chicken cell mutants indicates that HR plays an essential role in repairing potentially lethal chromosomal breaks, which likely occur during DNA replication (21, 55, 73). Thus, defective HR results in a phenotype of chromosomal instability analogous to that of the human syndromes showing unstable chromosomes, which include Bloom syndrome, Fanconi anemia, and ataxia telangiectasia. These are all associated with an increased incidence of cancer (reviewed in reference 42). Given that *RAD51* paralogs can be expected to function as tumor suppressor genes by maintaining the integrity of chromosomes, it will be desirable to screen for mutations in these loci in various tumors. Indeed, chromosomal translocation breakpoints within *RAD51B* at position 14q23-24 are common in uterine leiomyomas (26, 51).

The BRCA2 cancer susceptibility protein is associated with Rad51 in mitotic and meiotic cells (13, 14), suggesting a direct role of BRCA2 in HR. It is noteworthy that human and murine mutant cells in which BRCA2 is truncated exhibit phenotypes remarkably similar to those of our Rad51 paralog mutants: elevated spontaneous chromosomal aberrations (43), sensitivity to MMC (74), and defective Rad51 focus formation (75). Thus, BRCA2 might participate in the formation of a complex involving the Rad51 paralogs which acts as a cofactor of Rad51 during HR. In addition to the presence of BRCA2 homologs in vertebrates (41, 70) but not in yeast, the presence of five Rad51 paralogs in vertebrates, instead of only two as in yeast (Rad55 and Rad57), implies that the assembly of Rad51 during HR is regulated in a more complex manner in vertebrate cells. In-

deed, although HR occurs efficiently in the G₁ phase in diploid yeast (31), there was no detectable induction of Rad51 focus formation by IR in the G₁ phase in CHO hamster cells (7). This finding implies that the assembly of Rad51 might be actively suppressed in the G₁ phase to prevent gene conversion between homologous chromosomes, which would lead to loss of heterozygosity (27). In order to investigate the regulation of HR of vertebrate cells, our mutant clones could be quite useful. Furthermore, using these mutant clones, functional interactions between BRCA2 and Rad51 paralogs should be examined by generating double or triple mutants.

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