

Role of Mammalian RAD51L2 (RAD51C) in Recombination and Genetic Stability*

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The highly conserved RAD51 protein has a central role in homologous recombination. Five novel RAD51-like genes have been identified in mammalian cells, but little is known about their functions. A DNA damage-sensitive hamster cell line, *irs3*, was found to have a mutation in the *RAD51L2* gene and an undetectable level of RAD51L2 protein. Resistance of *irs3* to DNA-damaging agents was significantly increased by expression of the human *RAD51L2* gene, but not by other RAD51-like genes or RAD51 itself. Consistent with a role for RAD51L2 in homologous recombination, *irs3* cells show a reduction in sister chromatid exchange, an increase in isochromatid breaks, and a decrease in damage-dependent RAD51 focus formation compared with wild type cells. As recently demonstrated for human cells, we show that RAD51L2 forms part of two separate complexes of hamster RAD51-like proteins. Strikingly, neither complex of RAD51-like proteins is formed in *irs3* cells. Our results demonstrate that RAD51L2 has a key role in mammalian RAD51-dependent processes, contingent on the formation of protein complexes involved in homologous recombination repair.

The use of cultured mammalian cell lines selected for sensitivity to DNA-damaging agents to identify genes and gene functions has had a major impact on our understanding of DNA repair pathways. In particular, components of the nucleotide excision repair and DNA end joining pathways were identified and their cellular responses characterized using DNA damage-sensitive cell lines (1, 2). Recently, the value of this approach has been reinforced by the identification of genes from the homologous recombination repair pathway, with the cloning of the *XRCC2* and *XRCC3* genes by their ability to complement sensitive mammalian cell lines (3, 4).

The repair of DNA damage by homologous recombination is important for the maintenance of genetic stability in cells. The RAD51 protein is central to the recombination process, and this

protein is highly conserved from yeast to humans (5). Using molecular recombination assays, yeast (*Saccharomyces cerevisiae*) and human RAD51 proteins have been shown to promote DNA strand exchange. In *S. cerevisiae*, two RAD51-like proteins, Rad55p and Rad57p, form a heterodimer and stimulate Rad51-mediated recombination reactions (6). Yeast mutants that lack either Rad51 or these Rad51-like recombination proteins are extremely sensitive to agents causing severe forms of damage to DNA, such as double-strand breaks and interstrand cross-links (7). Rad55p and Rad57p also have counterparts in mammalian cells, including XRCC2, XRCC3, RAD51L1¹ (hREC2, RAD51B, R51H2), RAD51L2 (RAD51C), and RAD51L3 (R51H3, RAD51D) (8). The last three proteins were identified through data base searches using partial homologies to RAD51-like proteins (9–13), and have not as yet been found to be defective in DNA damage-sensitive mammalian cell lines. At present very little is known about the functions of these mammalian RAD51-like proteins, although specific protein-protein interactions have been described which suggest that they form heterodimers and larger complexes that may help recruit RAD51 to sites of DNA damage (14–22).

A series of DNA damage-sensitive hamster cell lines, termed the *irs* mutants, were previously isolated in this laboratory (23). We and others used the *irs1* cell line to clone the *XRCC2* gene by its complementing ability for sensitivity to the potent DNA cross-linking agent, mitomycin-C (24–26). The *irs1* line is also sensitive to other DNA-damaging agents, including ionizing radiation, ultraviolet light, and alkylating agents (23). It shows spontaneous genetic instability, with increased frequencies of mutations (27), chromosomal aberrations (28), and chromosome non-disjunction (29). It has also been shown that the repair of a site-specific double-strand break by homologous recombination is severely reduced in *irs1* compared with the paternal V79 cells (30). We have recently established a functional link between XRCC2 and RAD51 by showing that the *irs1* line is defective in the formation of damage-dependent RAD51 focus formation (31).

The *irs3* cell line is another member of this series of damage-sensitive cell lines. In cell fusions, *irs3* was found to be able to complement damage sensitivity in several other radiation-sensitive mammalian cell lines, including *irs1*, showing that it has a unique genetic defect (32). However, the *irs3* cell line has a similar damage sensitivity profile to *irs1*, being sensitive to x-rays (2-fold), ethyl methanesulfonate (2.5-fold) and especially MMC² (7-fold), and shows chromosomal instability (23, 33). We

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¹ RAD51L is the recommended symbol for the RAD51-like genes/proteins (Human Gene Nomenclature Committee).

² The abbreviations used are: MMC, mitomycin-C; RT, reverse transcriptase; SCE, sister chromatid exchanges; pAb, polyclonal antibody; mAb, monoclonal antibody; IRES, internal ribosome entry site.

have therefore considered that *irs3* may have a similar functional defect to *irs1*, and have characterized the *irs3* cell line further as well as identifying the defective gene responsible for this phenotype.

EXPERIMENTAL PROCEDURES

Gene Cloning and Vector Construction—The cloning of *RAD51L1* and *RAD51L3* was described previously (11). The human cDNAs were cloned into mammalian expression vectors; *RAD51L1* in pEBS7 (34) and *RAD51L3* in pZeoSV2(−) (Invitrogen). Human *RAD51L2* (also called *RAD51C* (12)) was cloned from a human testis cDNA library (CLONTECH) into pZeoSV2(−), and was subsequently recloned into the bicistronic mammalian expression vector pIRESneo2 (CLONTECH). The human *RAD51* gene was cloned from the same cDNA library into pIRESneo2. The Chinese hamster *RAD51L2* gene was cloned from a CHO-K1 cDNA library (Invitrogen); the central portion of the gene was cloned first using primers designed to conserved regions of the human (12) and mouse genes (mouse gene data came from sequencing a mouse IMAGE clone 3598583 found by data base search to have close homology to the human cDNA; data not shown). The remainder of the hamster gene was cloned from the same library using nested primers designed to the central portion and to the library vector, pCDNA1. The accession number of the Chinese hamster *RAD51L2* sequence is AJ413202. All cloned genes were verified by sequencing.

Isolation and Chromosomal Mapping of a Genomic Clone Carrying *RAD51L2*—Gridded filters and clones from the human genomic PAC library RPC11 were supplied by the UK HGMP Resource Centre. Filters were probed with full-length human *RAD51L2* cDNA, and DNA from positive clones was isolated using a Qiagen large-construct kit. One PAC clone (259-O5) was mapped to human metaphase chromosomes using a biotinylated probe, detected with Texas Red avidin. Well spread metaphases were captured and karyotyped using the Genus Applied Imaging system.

Cell Culture and DNA Transfection—Parental V79 and mutant *irs3* cells were cultured at 37 °C as monolayers in minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics. 10 µg of the *RAD51L1*, *RAD51L2*, *RAD51L3*, or *RAD51* constructs were electroporated into 10⁶ *irs3* cells (BioRad Gene Pulser set at 500 µF, 400 V) and transfected clones were selected in the appropriate drugs. Following clone isolation, resistance to mitomycin-C (MMC) was initially tested in 24-well plates by seeding 100–300 cells/well in graded concentrations of MMC (31). In control experiments we did not see any reversion of the *irs3* phenotype (*i.e.* no background clonal growth under selective conditions; data not shown). PAC 259-O5 was chosen to test complementation with genomic *RAD51L2* DNA. 15 µg of PAC DNA was co-transfected with 2 µg of the vector pEGFP-N2 (CLONTECH) into *irs3* cells as described above, followed by selection in 500 µg/ml G418. Colonies of G418-resistant cells were pooled and reseeded in 300 µg/ml G418 and 4 × 10^{−8} M MMC. The presence of the appropriate *RAD51L2* gene (cDNA or PAC) was tested in drug-resistant *irs3* clones by PCR using cellular DNA, and gene expression was checked by reverse transcriptase (RT-) PCR (Superscript II, Invitrogen) of cellular RNA with oligo(dT) as the first-strand primer.

Mitomycin-C and X-ray Survival—Subconfluent cells were harvested as a single-celled suspension. Appropriate numbers of cells were reseeded into 9-cm dishes for MMC treatment, which lasted for the duration of the experiment. Compared with acute treatment (23), chronic MMC treatment increased the survival difference between *irs3* and V79 cells to about 20-fold. X-ray treatment (250 kV) was given to 2 × 10⁵ cells in suspension, followed by reseeding appropriate numbers into 9-cm dishes. Three dishes were used for each dose point.

Immunofluorescence—Cells were seeded onto coverslips and grown for 48 h to subconfluent levels before being mock-treated or exposed to 10 Gray X-rays, a dose previously shown to induce a measurable frequency of foci (31, 35). After a further 5-h incubation at 37 °C, the cells were washed with phosphate-buffered saline and fixed in 1% paraformaldehyde for 2 h at 4 °C. Fixed cells were permeabilized for 10 min at room temperature in 0.1% Triton X-100, 0.1 × SSC and then blocked for 1 h at room temperature in phosphate-buffered saline, 5% normal horse serum. Cells were incubated overnight at 4 °C with 1:100 dilution primary antibody, rabbit polyclonal anti-RAD51 (Ab-1) antibody (Oncogene Research), then incubated with 1:100 dilution secondary antibody, Cy5-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) for 1 h at room temperature in the dark. The coverslips were mounted in Vectashield anti-fade medium and the cells analyzed by confocal microscopy at 633 nm (BioRad MRC600). Images were selected at random, and were scored from coded slides by two independent scorers,

recording the number of discrete strongly fluorescing nuclear foci present in each cell. At least 50 cells were scored for each data point, and data were compared statistically to assess homogeneity using the Mann-Whitney *U* test.

Chromosome Analysis—To measure sister chromatid exchanges (SCE), cells were incubated in the dark with bromodeoxyuridine (10 µg/ml) for 2 cell cycles to differentially label sister chromatids. MMC was added for 2 h at the start of the experiment. Colcemid (0.05 µg/ml) was added for 2 h prior to harvesting the cells. Metaphase preparations were made using standard techniques. Differential staining of chromatids was achieved with the fluorescence plus Giemsa (FPG, “harlequin”) staining method (36). Chromatid and chromosome aberrations were scored and classified (37) from coded slides. Chromatid breaks were scored where a physical displacement of the broken fragment was seen.

Hamster *RAD51L2* Gene Analysis—RNA was isolated from parental V79 and from the damage-sensitive lines *irs1*, *irs2*, and *irs3* using an RNeasy Mini kit (Qiagen). This RNA was used in an RT-PCR reaction (as above). The resulting cDNA was used as a template for PCR primers designed to amplify the full-length *RAD51L2* hamster gene. Individual PCR products were sliced out of gels and cloned into pCR2.1-TOPO (Invitrogen) before sequencing. In analyzing PCR products for splice variants, intron/exon boundaries were assumed to be the same as in human genomic sequence (9 exons found using data from accession numbers AC021455 and AC025521).

Antibodies—All antibodies were raised against human recombinant denatured protein as described (17). The monoclonal antibodies 1E11 (*RAD51L1*), 2H11 (*RAD51L2*), 5A8 (*RAD51L3*), 1G4 (*XRCC2*), and 7F12 (*XRCC3*) specifically recognized the corresponding human proteins. The anti-RAD51 mAb (14B4) was purchased from Abcam.

Immunoprecipitation Analysis—V79 or *irs3* cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.5% Nonidet P-40) containing protease inhibitors, incubated for 30 min on ice, dounced 10 times and sonicated twice for 30 s. Insoluble material was removed by high-speed centrifugation. Protein complexes in the supernatant (equivalent to ~15 mg of extract) were pulled down for 1.5 h at 4 °C using preimmune serum or pAbs raised against *RAD51L2* or *XRCC2* cross-linked to Aminolink beads (Pierce). Complexes were washed four times in lysis buffer and visualized by Western blotting using monoclonal antibodies.

RESULTS

The *RAD51L2* Gene of *irs3* has a G → T Mutation in Exon 6—To establish a link between *RAD51L2* and the *irs3* phenotype, we sought to clone the hamster *RAD51L2* gene and check its integrity in *irs3*. We used sequence homologies between mouse and human *RAD51L2* genes to design primers for the isolation of the hamster gene (see “Experimental Procedures”). Cloning of the full-length hamster cDNA showed that its predicted protein sequence is highly conserved in comparison to the human and mouse *RAD51L2* proteins. The mouse and hamster proteins show 84% identity to each other, and each shows 77% identity to the human protein with only one amino acid difference in length (Fig. 1). Reverse-transcribed RNA from the V79 parent and *irs3* lines was used to make *RAD51L2* cDNA. Using full-length gene primers in PCR, both cell lines showed a number of different sized cDNA products (Fig. 2A). To check that the altered profile of cDNA products in *irs3* was related to the *RAD51L2* gene defect, we assayed the *RAD51L2* cDNA profiles in two other radiation-sensitive mutants isolated in the same screen from mutagenized V79 cells. The *irs1* line (*XRCC2*-deficient) and the *irs2* line (shown by complementation testing to be defective in a different gene from *irs1* or *irs3* (32)) both showed *RAD51L2* profiles that were essentially the same as that for V79 (Fig. 2A).

The largest cDNA product in V79 was cloned and sequenced to show that it was the full-length *RAD51L2* gene; other products were found to represent splice variants with different exons missing. In repeated amplifications of cDNA from *irs3* the full-length gene product was either of very low abundance or not present, but there were also visible levels of some splice variants (Fig. 2A). The largest *RAD51L2* product that could be seen readily from *irs3* preparations was found to lack exon 6.

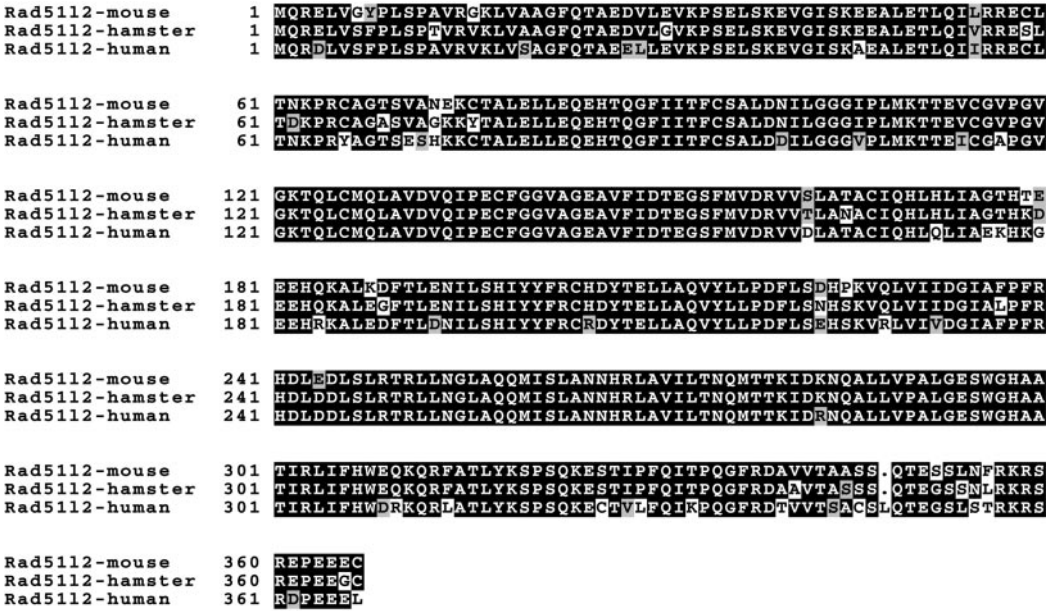
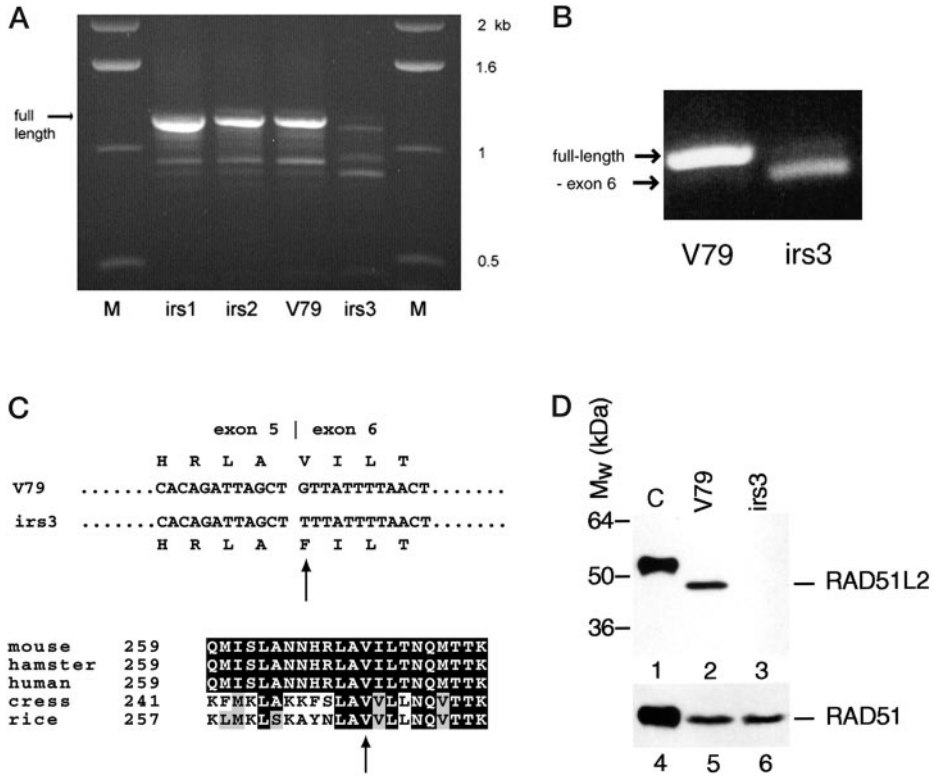


FIG. 1. Conservation of mammalian RAD51L2 protein sequences. RAD51L2 aligned from human, mouse, and hamster (human sequence from accession number O43502 and mouse data³ and accession number AAK58420 (61)). Note that the human sequence is shown starting from a different codon (9 amino acids downstream) than previously published (12), since neither the mouse nor hamster sequence show conservation of that putative start codon. Predicted alignments from PILEUP (GCG version 10), shaded using BOXSHADE (www.ch.embnet.org/).

FIG. 2. RAD51L2 is mutated in *irs3* cells. A, RAD51L2 cDNA profiles amplified from V79 parental and the derived radiation-sensitive lines *irs1*, *irs2*, and *irs3*, using primers designed to give the full-length gene product. M, molecular size marker (Invitrogen), with sizes shown to the right. B, amplification of cDNA from V79 and *irs3* lines with primers placed either side of exon 6 of hamster RAD51L2 cDNA, to give full-length product (514 bp) or product missing exon 6 (447 bp). C, position of the mutation in the RAD51L2 gene of *irs3* (upper diagram, arrow) and conservation of protein sequence in the region of the mutation, in RAD51L2-like proteins from mammalian and plant species (lower diagram, arrow). D, Western blots of V79 and *irs3* whole cell extracts probed with human RAD51L2 mAb (lanes 2 and 3) and human RAD51 mAb (lanes 5 and 6). Lanes 1 and 4 contain purified human RAD51L2_{his10}, migrating more slowly than the endogenous hamster protein, and human RAD51, respectively.



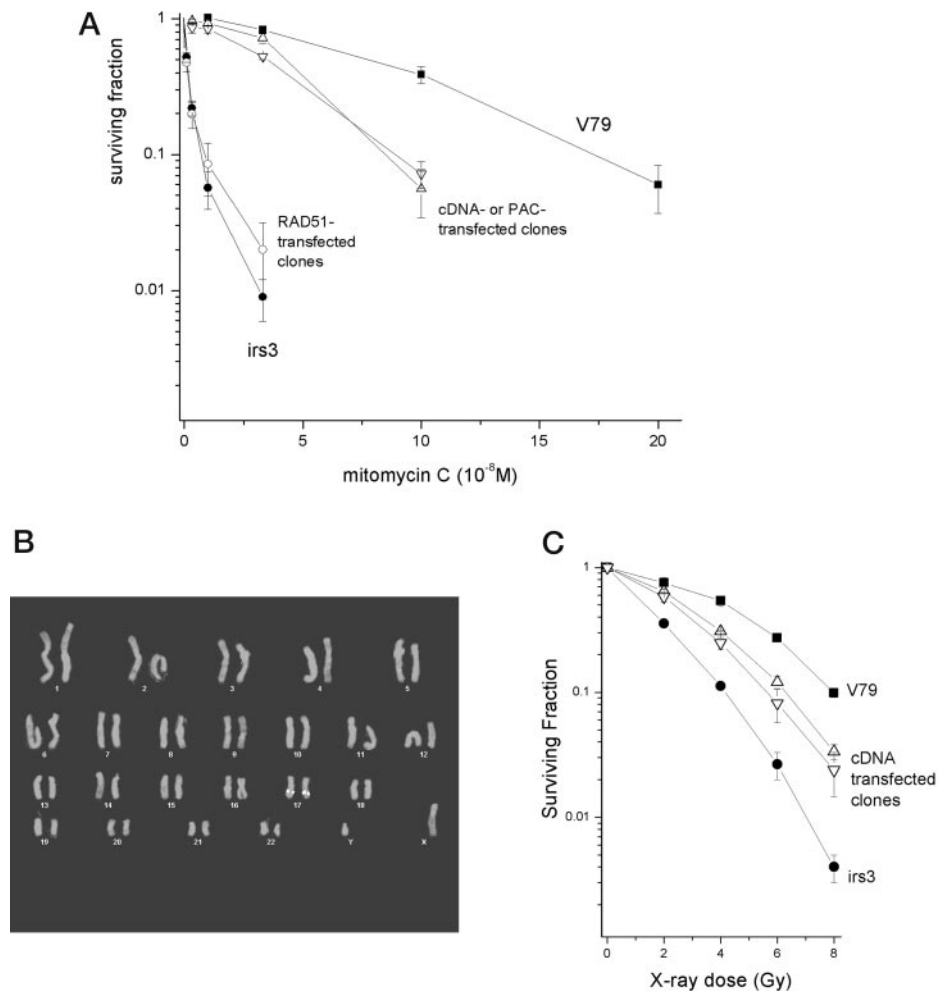
Exon 6 could not be cloned from *irs3* using primers to cDNA sequence either side of the exon (Fig. 2B). However, we were able to obtain sufficient product for cloning by “forcing” the amplification of exon 6 in two pieces, using a combination of overlapping primers internal and external to the exon. The sequence of exon 6 from *irs3* consistently showed a single nucleotide change (G → T) at position +1 (Fig. 2C). This base substitution is consistent with the mutation spectrum of ethyl nitrosourea, which was used to derive the *irs3* cell line, although G → T is one of the less common types of substitution found (38, 39). This change alters the consensus for the 3′

acceptor splice site (see “Discussion”), and gives an amino acid change (Val → Phe). While it is difficult to comment on the importance of this residue for mammalian species, since it is one of many highly conserved amino acids (Fig. 1), comparisons to putative RAD51L2 orthologues in plant species show that this valine is widely conserved (Fig. 2C, which shows comparisons to the cress *Arabidopsis thaliana* and rice *Oryza sativa* where the overall identity to the human protein is reduced to about 40%).

The effect of this mutation on RAD51L2 protein levels is seen in Fig. 2D. We found that polyclonal (not shown) or monoclonal

FIG. 3. Transfection of RAD51L2 into *irs3* increases damage resistance.

A, survival of V79, *irs3*, and *irs3*-transfected with RAD51L2 cDNA, RAD51L2 genomic DNA (PAC 259-O5), or RAD51, after chronic treatment with mitomycin C. Closed symbols, *irs3* or V79 cells; open symbols, transfected clones of *irs3* cells (upright triangle, RAD51L2 cDNA; inverted triangle, RAD51L2 PAC; circle, RAD51 cDNA). Results from two to three experiments per cell line or clone; transfected clone data shown as mean of two independent clones per transfected gene (error bars = S.E. of the mean). **B**, localization of PAC 259-O5 to human chromosome 17q (bright signals). **C**, X-ray survival of V79, *irs3* (closed symbols), and *irs3* transfected with RAD51L2 cDNA (open symbols). Results from two experiments (error bars = S.E. of the mean).



antibodies raised against human RAD51L2 recognized the hamster protein (V79 extract, lane 2), whereas RAD51L2 was not seen in the *irs3* extract (lane 3). The levels of RAD51 were the same in wild type V79 and the *irs3* cell lines (lanes 5 and 6).

The Human RAD51L2 Gene Restores DNA Damage Resistance to *irs3*—To show that the phenotype of *irs3* is related to the observed RAD51L2 defect, we transfected the human RAD51L2 cDNA into *irs3* cells. To ensure that any responses found were specific to this gene, we also transfected separately the human RAD51L1 and RAD51L3 cDNAs into *irs3*. Cell clones carrying stably integrated cDNAs were selected using dominant drug-resistant markers present in the vectors, and were subsequently tested for resistance to MMC. None of the clones transfected with RAD51L1 or RAD51L3 showed an increase in MMC resistance, despite the presence of transcripts from the full-length gene as seen by RT-PCR (data not shown). However, with RAD51L2, a fraction of the selected clones showed significant MMC resistance. To verify this result under conditions where the RAD51L2 cDNA is expressed coordinately with the dominant selectable marker, we recloned RAD51L2 into an expression vector with an internal ribosome entry (IRES) site. Following transfection of this construct, every clone showing the presence of transcripts from the RAD51L2 gene product was found to be resistant to MMC (Fig. 3A).

In addition to complementation tests with the human RAD51L2 cDNA, we identified human genomic (PAC) DNA carrying the RAD51L2 gene (see "Experimental Procedures"). In Fig. 3B we show that the PAC DNA mapped to the expected chromosomal site of the gene, chromosome 17q (12) (now known to be at 17q23; see genome.ucsc.edu). Stable PAC-

transfected clones of *irs3* cells gave a similar level of MMC resistance to the cDNA carrying clones (Fig. 3A). We conclude that there is a direct correlation between the presence of the RAD51L2 gene and the restoration of MMC resistance to *irs3* cells.

Cells deficient in homologous recombination genes show sensitivity to a number of DNA-damaging agents, in addition to MMC. We have previously shown an ~2-fold increase in sensitivity to x-rays in *irs3* (23). In the present series of experiments, we confirmed this radiation response and in agreement with the MMC data showed that the RAD51L2 cDNA was able to partially complement the *irs3* X-ray sensitivity (Fig. 3C).

Elevated Isochromatid Breaks and Reduced Sister Chromatid Exchange in *irs3*—Importantly, recombination-deficient cells show spontaneous genetic instability, and *irs3* is no exception to this finding. The frequency of chromosome damage in *irs3* cells was substantially higher than in the V79 parent line, and we found again that the presence of RAD51L2 largely complemented this defect (Table I). It is notable that the frequency of isochromatid breaks was especially high in *irs3* (77% of total breaks; see "Discussion").

SCE are thought to result from molecular crossing over of DNA strands, especially arising during replication when recombination may rescue forks stalled at sites of damage. A small reduction in spontaneous SCE frequency was found for *irs3* (Table II). However, using the same MMC concentration for both V79 and *irs3* cells (4×10^{-7} M; i.e. not allowing for their survival difference) *irs3* cells gave a highly significant 30% reduction in SCE frequency. If differences in the MMC sensitivity of V79 and *irs3* are taken into account, using approxi-

TABLE I
High levels of spontaneous chromosome damage in *irs3*

Cell line	Cells scored	Chromatid breaks	Isochromatid breaks	Exchanges	Total aberrations (per 100 cells)
V79	204	0	2	0	2 (1.0)
<i>irs3</i>	249	5	17	11	33 (13.2)
<i>irs3</i> + <i>RAD51L2</i> clone 1	205	1	3	2	6 (2.9)
<i>irs3</i> + <i>RAD51L2</i> clone 2	206	4	4	0	8 (3.9)

TABLE II
Reduced frequencies of spontaneous and mitomycin C-induced sister chromatid exchanges in *irs3*

Cell line	Cells scored	SCE/cell (\pm se)	p Value ^a
V79	101	5.5 \pm 0.2	0.0002
<i>irs3</i>	103	4.4 \pm 0.2	
V79 + MMC (4×10^{-7} M) ^b	153	48.0 \pm 1.1	<10 ⁻⁶
<i>irs3</i> + MMC (4×10^{-7} M) ^b	127	33.2 \pm 1.1	
<i>irs3</i> + <i>RAD51L2</i> clone 1 (4×10^{-7} M)	98	51.0 \pm 1.2	<10 ⁻⁶
<i>irs3</i> + <i>RAD51L2</i> clone 2 (4×10^{-7} M)	100	42.4 \pm 1.2	<10 ⁻⁶
V79 + MMC (4×10^{-6} M) ^c	76	88.2 \pm 2.4	
<i>irs3</i> + MMC (4×10^{-8} M) ^d	105	8.1 \pm 0.3	

^a Based on Mann-Whitney U-tests, comparing either V79 with *irs3* or the *RAD51L2*-transfected clones with *irs3* at the same dose of MMC.
^b Data summed from two independent experiments, where the frequencies for V79 were 47.5 and 48.9 SCE/cell, and for *irs3* were 33.2 and 33.2 SCE/cell, respectively.
^c Equivalent survival level to 4×10^{-7} M for *irs3*.
^d Equivalent survival level to 4×10^{-7} M for V79.

mately equitoxic MMC concentrations, much larger differences in SCE frequency were found. Again, stable clones transfected with the *RAD51L2* cDNA showed a substantial increase in SCE frequencies compared with the *irs3* line (Table II).

Defective Formation of RAD51-like Protein Complexes in *irs3*—It has recently been shown that two distinct complexes of RAD51-like proteins occur in human cells; a heterodimer of RAD51L2 with XRCC3 (16, 17), and a heterotetramer of RAD51L1, RAD51L2, RAD51L3, and XRCC2 (18, 20, 22). To show that hamster V79 cells also contain these complexes, we immunoprecipitated the RAD51-like proteins from extracts using appropriate polyclonal antibodies. As seen in Fig. 4A, anti-RAD51L2 antibodies pulled down XRCC3 from V79, representing the heterodimer (Fig. 4B). Use of anti-XRCC2 antibodies pulled down RAD51L1, RAD51L2, and RAD51L3, but not XRCC3 (Fig. 4C), demonstrating the presence of the heterotetramer in V79 cells (Fig. 4D). In *irs3* extracts, neither of these complexes was formed (Fig. 4, A and C), although RAD51L3 was pulled down by anti-XRCC2 antibodies indicating that the RAD51L3/XRCC2 heterodimer is formed (Fig. 4C).

Damage-dependent RAD51 Focus Formation Is Reduced in *irs3*—The *RAD51L2* gene has been suggested to be involved in RAD51-dependent repair processes in mammalian cells (15), although definitive proof of this is lacking. An important indicator of RAD51 response is the formation of nuclear foci following x-ray damage (35, 40, 41). Measurement of RAD51 foci in *irs3* cells showed that this response was significantly reduced when compared with V79 cells. In *irs3* clones transfected with *RAD51L2* cDNA the RAD51 focus formation was partially restored, in agreement with the other responses measured (Fig. 5, A and B). A low frequency of RAD51 foci forms in unirradiated cells, primarily in S-phase (42), suggesting that differences in cell-cycle distribution may influence the ability to detect foci in *irs3* after irradiation. However, cell-cycle profiles for irradiated *irs3* and V79 cells were very similar, with most cells blocked in G₂ phase at 5 h after a dose of 10 Gray (data not shown). The formation of RAD51 foci may also be affected by the levels of RAD51 protein in *irs3* relative to V79, but we found that RAD51 levels were similar in *irs3*, in *irs3* lines transfected with *RAD51L2*, and in V79, both before and after

irradiation (Fig. 2D and data not shown).

Finally we wished to know whether, as in both yeast (43) and chick (44) cells, the *RAD51* gene itself can partially correct DNA-damage sensitivity when expressed in cell lines defective in RAD51-like genes. To check this possibility, we cloned the human *RAD51* cDNA into the IRES vector and transfected it into *irs3*. However, clones expressing *RAD51* transcript were found to survive MMC treatment no better than *irs3* itself (Fig. 3A).

DISCUSSION

Our data show a direct correlation between the presence of the *RAD51L2* cDNA and resistance to MMC in the *irs3* cells, while the *RAD51L1* and *RAD51L3* cDNAs showed no ability to complement. In support of these results, we also found that human genomic DNA carrying the *RAD51L2* gene significantly complemented the MMC sensitivity of *irs3*. *RAD51L2* cDNA or genomic DNA did not fully complement this phenotype, suggesting that the human *RAD51L2* gene is not fully functional in hamster cells. In addition to the complementation of sensitivity to MMC, we found a similar degree of complementation for x-ray sensitivity, spontaneous chromosomal damage, SCE formation, and damage-dependent RAD51 focus formation. These data reinforce the conclusion that mutation of the *RAD51L2* gene causes the *irs3* phenotype.

The mutation detected in exon 6 of *RAD51L2* in *irs3* (G \rightarrow T at position +1) is not typical of splicing defects, which commonly alter intronic consensus sites. However, the first and last base of exons do form part of the consensus splice junction sequences, where the base G is generally conserved and the base T is found in less than 10% of splice sites (45). Loss-of-function mutants arising from changes at these sites have been documented in well studied genes, such as human *HPRT* and *TP53* (46, 47). This mutation may reduce "exon definition"; exon 6 is relatively short (67 bp) for mammalian exons (average size 137 bp) and it is known that short exons have an increased chance of differential splicing (48). The loss of exon 6 (Fig. 2) from the full-length transcript provides a possible explanation for the *irs3* phenotype. While other splice variants occur in both *irs3* and wild-type cells, these may not contribute to the *irs3* phenotype (e.g. sequencing of different sized transcripts shows

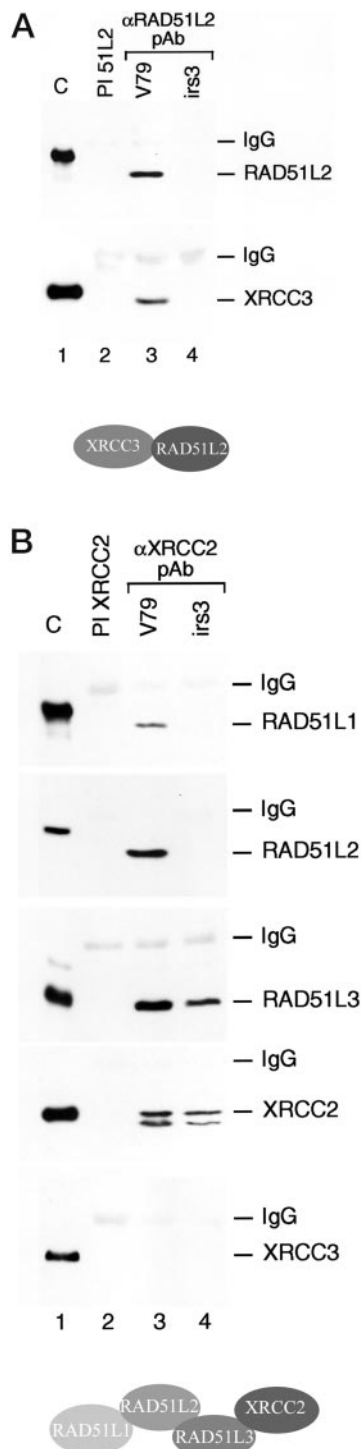


FIG. 4. Lack of RAD51-like protein complex formation in *irs3*. A, complex formation between endogenous RAD51L2 and XRCC3 hamster proteins. Protein complexes from V79 or *irs3* were precipitated using preimmune serum (PI; lanes 2) or anti-RAD51L2 pAbs (lanes 3–4), and revealed using anti-RAD51L2 or anti-XRCC3 mAbs as indicated. Lanes 1, marker proteins (human RAD51L2^{his10}, human XRCC3^{his6}). Lower diagram illustrates the complex between RAD51L2 and XRCC3 in V79 cells. B, interactions between the RAD51-like proteins in V79 and *irs3* hamster cells. Lanes 1, marker proteins (human RAD51L1^{his6}, RAD51L2^{his10}, RAD51L3^{his6}, XRCC2, XRCC3^{his6}); lanes 2, preimmune serum; lanes 3, co-immunoprecipitation of endogenous RAD51L1, RAD51L2, RAD51L3, and XRCC2 from hamster wild type extract; lanes 4, co-immunoprecipitation of RAD51L3 and XRCC2 from *irs3*. The origin of the doublet detected by XRCC2 antibodies (lanes 3 and 4) remains to be determined. Lower part illustrates the complex between RAD51L1, RAD51L2, RAD51L3, and XRCC2 in V79 cells. In A and B the His-tagged controls migrate more slowly than the endogenous hamster protein.

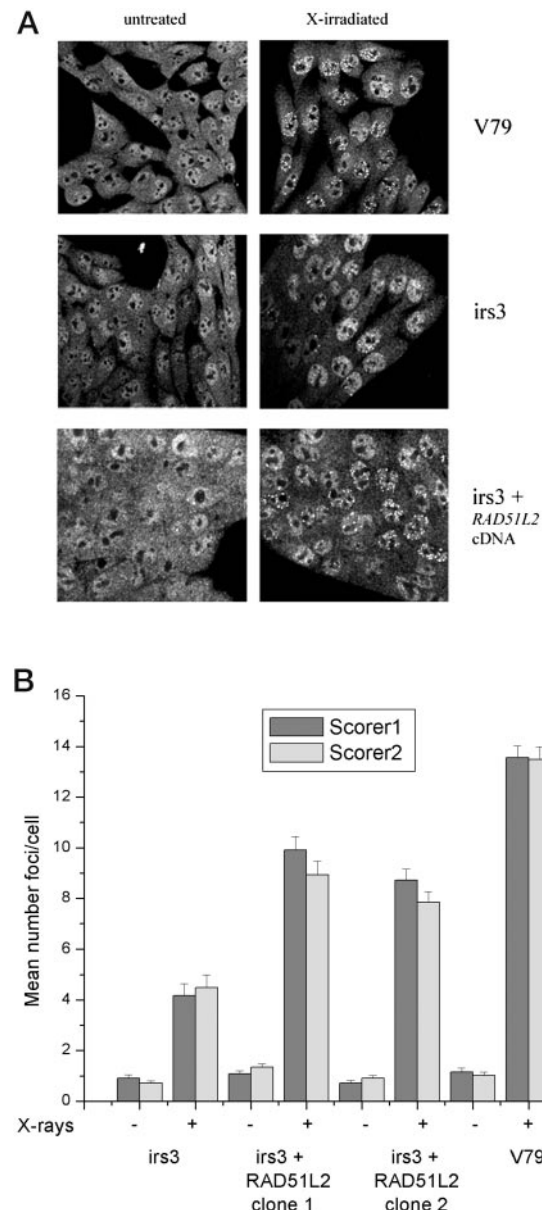


FIG. 5. Damage-dependent RAD51 focus formation is reduced in *irs3*. A, foci in wild type V79, *irs3*, and *irs3* cells transfected with RAD51L2 cDNA, with and without X-ray exposure (measured at 5 h after 10 Gray). Representative fields are shown. B, quantitation of focus formation at 5 h following 10 Gray X-rays. Data shown for two independent scorers, with standard errors calculated from individual cell counts. All differences between irradiated cell lines were significant ($p < 10^{-6}$) except for the two clones transfected with RAD51L2 ($p = 0.63$).

that all have loss of exon 6; data not shown). We detected no other type of RAD51L2 transcript in *irs3*, consistent with the hemizygous nature of many genes in established hamster lines (49). We presume that these exon-deleted transcripts are not acting as dominant negatives, since the phenotype of *irs3* is recessive in hybrids with wild-type cells (32) and can be complemented by introduction of the wild type RAD51L2 gene. The exon 6 mutation in *irs3* also gives rise to an amino acid change at position 271 (Val → Phe) which may play a part in the loss of gene function. However, the mutation has clear consequences for the level of RAD51L2 protein; none could be measured by Western blot analysis of *irs3* extracts (Fig. 2D).

Our RAD51-like protein interaction data support inferences initially developed from two-hybrid systems (15) suggesting that the association of RAD51L2 in complexes with

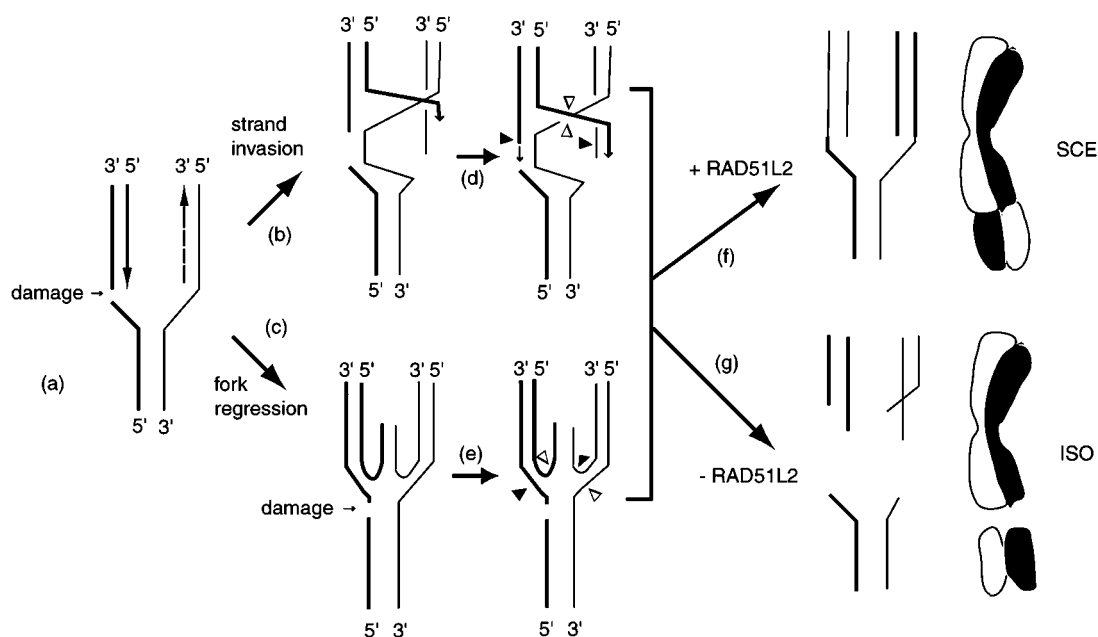


FIG. 6. Scheme for resolution of DNA damage on a replicating chromosome in the presence and absence of RAD51L2, either by strand invasion (a, b, and d) or fork regression (a, c, and e). a, stalled replicating DNA molecule with a strand break (thick and thin lines with large arrows: newly synthesized leading and lagging strands, respectively). b and d, strand exchange to form a recombination intermediate, followed by repair synthesis (small arrows). c and e, regression so that damage now behind fork, while newly synthesized strands can anneal to form a 4-way (Holliday) junction. At d and e the junctions may be resolved to give no crossover (cut at open arrowheads) or crossover (cut at closed arrowheads); however, the intermediate is very vulnerable, having 3 strands with "free" ends, and the fourth strand is broken by cutting (junction resolution) at the open arrowheads. f, RAD51L2 present, SCE can occur in a proportion of chromosomes if correct repair of damage is followed by crossing over; g, RAD51L2 absent, attempted repair in d or lack of repair (e) of the damage can lead to free ends in all strands and separation to give isochromatid breaks (ISO). Note that there would not necessarily be a direct numerical relationship between SCE and ISO formation.

other RAD51-like proteins may be functionally important. The interaction of human RAD51L2 with both XRCC3 (16, 17) and RAD51L1 (19, 21) has recently been confirmed in biochemical studies. Additionally it has been shown that there are two protein complexes containing RAD51L2 in human cells: one containing RAD51L2 and XRCC3, and the other consisting of four proteins, RAD51L1, RAD51L2, RAD51L3, and XRCC2 (18, 20–22). In the present study we have shown that these two RAD51-like protein complexes exist similarly in hamster cells, generalizing previous data. Strikingly, neither of these complexes is formed in *irs3*; the only complex remaining is the RAD51L3-XRCC2 heterodimer (14). Even this heterodimer may be less stable in the absence of RAD51L2, since slightly lower levels of these two proteins were consistently found in *irs3* (Fig. 4C). This is the first demonstration that the native proteins require RAD51L2 for complex formation.

The RAD51-like protein complexes bind preferentially to single-stranded DNA, suggesting that they may assist in the recruitment of RAD51 to sites of damaged DNA. For example, RAD51 filament formation occurs close to the RAD51L1-L2-L3-XRCC2 complex when it is bound to gapped duplex DNA (18). In chronic myeloid leukemia cells, lower levels of the RAD51L2-XRCC3 complex and higher levels of RAD51L1, RAD51L2, and XRCC2 (as well as RAD51) are expressed compared with normal cells (50). Furthermore, in brain tissue, where the key recombination protein RAD51 is in low abundance, it is suggested that the RAD51L2-XRCC3 complex carries out some of the functions of RAD51 (16). These studies suggest that the RAD51L2-XRCC3 complex may have a different role in recombination repair than the RAD51L1-L2-L3-XRCC2 complex. It is clear from our studies that RAD51L2 is a key component of both complexes, and therefore has a major role in homologous recombination processes in mammalian cells.

In eukaryotes there are several different pathways involving homologous recombination that can lead to the repair of DNA damage, and only some of these depend on RAD51 (51). In a number of different cell types it has been shown that RAD51 protein accumulates at nuclear foci following DNA damage, but we found that this process was much reduced in *irs3* cells. This finding links RAD51L2 to RAD51-dependent recombination pathways, joining a growing list of mammalian proteins, including XRCC2 (31), XRCC3 (35), BRCA1 (52), and BRCA2 (53) implicated by lack of RAD51 focus formation.

The *irs3* cells have a phenotype consistent with loss of homologous recombination repair, including their profile of sensitivity to DNA-damaging agents, spontaneous genetic instability, and reduction in SCE frequency. Very recently chick DT40 cells with a disruption of a homologue of the RAD51L2 gene have also been shown to have these defects, although to different degrees (44). For example, chick RAD51L2 homologue-deficient cells show similar survival differences to *irs3*, compared with wild type, in response to MMC or X-rays. However, the chick mutant cells have a larger relative reduction in SCE frequency than *irs3*, possibly because of a greater reliance of chick lymphoblastoid cells on homologous recombination for repair (54). The validity of these comparisons will depend on whether the chick gene is an orthologue of the mammalian RAD51L2 genes (the full coding sequence of the chick gene has not been identified). Significantly, we found that the human RAD51 cDNA could not complement the defect in *irs3*, while human RAD51 can partially complement the RAD51L2-homologue gene knockout in chick cells (44). Other differences between the functioning of homologous recombination proteins in chick and mammalian cells have previously been observed: for example, RAD54 is required for RAD51 focus formation in mouse cells (55), but not in chick cells (56). Taken together these data emphasize the need for caution in extrapolating functional

data for homologous recombination from one species to another.

A further feature of the present data that supports the proposed defect in homologous recombination in *irs3* is the reduction in SCE frequency together with an increase in chromosome aberrations (in particular, isochromatid breaks). While SCE can arise from crossing-over during homologous recombination repair, isochromatid breaks (Table I) can be viewed as one outcome of failure to properly carry out homologous recombination during replication. When a replication fork stalls due to a break on one DNA strand, the break may be resolved by RAD51-dependent recombination, including strand transfer and Holliday junction formation (Fig. 6, *a*, *b*, and *d*). Alternatively the fork may regress (57, 58) to allow damage repair against an intact strand, but this allows the annealing of newly synthesized strands and the formation of a Holliday junction at the fork (Fig. 6, *a*, *c*, and *e*). In either case the replicating DNA is in a highly vulnerable state, where incomplete resolution of the junctions could lead to disintegration of the intermediate structure (as shown in Fig. 6*g*). We suggest that in *irs3*, lack of the RAD51L2 protein increases the probability of disintegration at this stage, yielding isochromatid breaks. Such a failure would also lead to a reduction in the frequency of SCE. Support for these models is seen when a transfected human *RAD51* cDNA is down-regulated in chick *RAD51* knockout cells: a massive increase in chromosomal damage occurs, especially isochromatid breaks (59), associated with an ~50% reduction in SCE frequency (60). The first model (Fig. 6, *a*, *b*, and *d*), however, implies that RAD51-dependent processes are still (at least partially) active in *irs3*. This is supported by our finding that some damage-dependent RAD51 focus formation occurs in *irs3*. RAD51 in these circumstances may not form stable recombination intermediates, leaving these vulnerable to disintegration. In the second model (Fig. 6, *a*, *c*, and *e*) RAD51 is not required for isochromatid formation, consistent with the findings in RAD51-deficient chick cells (see above); in the absence of RAD51L2 the ability of RAD51-dependent recombination to rescue the fork is severely compromised, and isochromatid breaks may result. While the RAD51-like proteins are thought to be involved primarily in early stages of homologous recombination repair, to promote strand exchange by RAD51 (8), the first model suggests that RAD51L2 may normally have an additional role in later stages of recombination repair. This finding is not general for other RAD51-like proteins; for example, the XRCC2-deficient cell line *irs1* does not show a striking increase in isochromatid breaks (31).³

The high degree of conservation of RAD51L2 between mammalian species suggests that the gene has an important function in mammals, although this has yet to be clearly defined. We believe that the availability of a mammalian cell line lacking RAD51L2 activity will be of considerable value in identifying this function.

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