Cells expressing murine RAD52 splice variants favor sister chromatid repair

Citation for published version:

Digital Object Identifier (DOI):

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Molecular and Cellular Biology

Publisher Rights Statement:
© 2006, American Society for Microbiolog

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Cells Expressing Murine RAD52 Splice Variants Favor Sister Chromatid Repair†

Peter H. Thorpe,1 Vanessa A. Marrero,1 Margaret H. Savitzky,1§ Ivana Sunjevaric,1
Tom C. Freeman,2‡ and Rodney Rothstein1*

Department of Genetics and Development, Columbia University Medical Center, 701 West 168th St., New York, New York 10032,1 and Gene Expression Group, Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, United Kingdom2

Received 11 July 2005/Returned for modification 2 February 2006/Accepted 24 February 2006

The RADS52 gene is essential for homologous recombination in the yeast Saccharomyces cerevisiae. RADS52 is the archetype in an epistasis group of genes essential for DNA damage repair. By catalyzing the replacement of replication protein A with Rad51 on single-stranded DNA, Rad52 likely promotes strand invasion of a double-stranded DNA molecule by single-stranded DNA. Although the sequence and in vitro functions of mammalian RAD52 are conserved with those of yeast, one difference is the presence of introns and consequent splicing of the mammalian RADS52 pre-mRNA. We identified two novel splice variants from the RADS52 gene that are expressed in adult mouse tissues. Expression of these splice variants in tissue culture cells elevates the frequency of recombination that uses a sister chromatid template. To characterize this dominant phenotype further, the RADS52 gene from the yeast Saccharomyces cerevisiae was truncated to model the mammalian splice variants. The same dominant sister chromatid recombination phenotype seen in mammalian cells was also observed in yeast. Furthermore, repair from a homologous chromatid is reduced in yeast, implying that the choice of alternative repair pathways may be controlled by these variants. In addition, a dominant DNA repair defect induced by one of the variants in yeast is suppressed by overexpression of RADS51, suggesting that the Rad51-Rad52 interaction is impaired.

Homology-directed repair (HDR) allows cells to repair DNA double-strand breaks (DSBs) in an error-free way and is a key mechanism for maintaining genome integrity in all eukaryotes (11, 48, 52). RADS52 is an essential gene for HDR in the yeast Saccharomyces cerevisiae (20, 30, 46). Identified as one member of an epistasis group of radiation-sensitive yeast mutants, rad52 strains are defective in HDR, the major DSB repair pathway in yeast. RADS52 is largely conserved throughout eukaryotes, and both the human and yeast Rad52 proteins stimulate the replacement of replication protein A with Rad51 on single-stranded DNA (4, 29, 39, 45). Additionally, Rad52 promotes DNA annealing and strand exchange between a DNA duplex and complementary single-stranded DNA (14, 21, 27). Three-dimensional structures of the N terminus of human RAD52 (hRAD52) reveal a multimeric ring containing a positively charged surface groove that has been suggested to bind single-stranded DNA (15, 40). Relative to the N terminus of Rad52, the C terminus is poorly conserved between homologs. The C terminus of Rad52 is essential for its interaction with Rad51, a bacterial RecA homolog (26, 37). Consistent with its ability to bind both itself and other DNA repair proteins, Rad52 forms discrete nuclear foci both spontaneously and in response to DNA damage, and these DNA repair centers are the sites of DSB repair (23–25, 32).

Although yeast RADS52 plays a critical role in HDR, surprisingly, RAD52 knockout mice are resistant to DNA damage (35). However, it is likely that mammalian RAD52 is important for DNA repair, as its activities are conserved with yeast Rad52; in addition, HDR in chicken cells requires RAD52 (5, 42). Furthermore, RAD52 null mice have a defect in gene targeting (35), and loss of RAD52 rescues the tumorigenic phenotype of ATM-deficient mice (50). An alternative explanation for the phenotype of the RAD52 knockout mice is that the functions of yeast Rad52 are redundant in higher eukaryotes. Mammals have a number of other genes, such as XRCC3 and BRCA2, that may encode complementary functions with yeast RAD52 (9, 10, 38).

Another difference between the mammalian and yeast RAD52 genes is the presence of introns. The open reading frames (ORFs) of both human and mouse RAD52 are divided into 12 exons whose positions relative to the RAD52 open reading frame are conserved. Splice variants of hRAD52 have been isolated from cDNA libraries derived from both brain and testes tissues, and the encoded protein variants were found to bind to DNA but not to wild-type hRAD52 in vitro (16).

Here we report the isolation of two new splice variants from adult mouse tissues. We show that these variant transcripts act as dominant alleles in both mammalian cells and the yeast S. cerevisiae. In both mammalian cells and yeast, the variants perturb HDR, specifically by elevating the frequency of sister chromatid repair. In yeast, both variants also decrease the frequency of repair from a homologous chromatid. Further-

---

* Corresponding author. Mailing address: Department of Genetics and Development, Columbia University Medical Center, HHSC 1608, 701 West 168th St., New York, NY 10032. Phone: (212) 305-1733. Fax: (212) 923-2090. E-mail: rothstein@cancercenter.columbia.edu.
† Present address: Scottish Centre for Genomic Technology and Informatics, University of Edinburgh Medical School, Chancellor’s Building, Edinburgh EH16 4SB, United Kingdom.
‡ Present address: Thomas A. Edison High School, 165-65 84th Avenue, Jamaica, NY 11432.
more, for one variant in yeast, the defect in DNA repair is suppressed by overexpression of RAD53. These data show that the splice variants have the potential to alter the ratio of repair from the homologous to the sister chromosome, possibly by impairing the interaction of Rad52 with Rad51.

MATERIALS AND METHODS

RNA isolation and reverse transcription-PCR (RT-PCR). Whole-tissue samples were homogenized first by passing approximately 15 mg tissue several times through a 27-gauge needle in RNAlater buffer (Ambion) and then through a QIAshredder column (QIAGEN). RNA was purified from the resulting suspension without delay using the RNaseasy column purification system (QIAGEN). Three to 5 μg of RNA was used for cDNA synthesis using random hexamer primers, thus ensuring that the cDNA represents all the transcripts present in the tissue. Either the Superscript first-strand synthesis system for RT-PCR (Invitrogen) or first-strand cDNA synthesis kit for RT-PCR (Roche) was used for this cDNA synthesis. Approximately 10% of the resulting cDNA was used as a template for PCR using gene specific primers.

As a control for cDNA yield, primers were designed to amplify a 200-bp product spanning exon 8 from a splice transcript of the murine glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). Each RT reaction included a matched control that omits the reverse transcriptase enzyme to confirm that the subsequent PCR products are derived from cDNA and not from DNA contamination. GAPDH primers used for PCR were 5′-TGGAGCCAAACGTCGATCACA and 5′-GTGCACTGATGGCATGGAC. RAD52 exon 1 to 7 primers were 5′-GGTTCTGCTCTACACAGGGC (all oligonucleotides were obtained from Sigma-Genosys). All PCR amplifications used the following program: 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The resulting PCR products were separated by Tris-borate-EDTA gel electrophoresis. The bands corresponding to novel transcripts were cut from the gel and purified using QIAquick gel extraction kit (Qiagen). The blots were then prehybridized with 100 ng of radiolabeled oligonucleotide probes, 1,000 U of T4 polynucleotide kinase (New England Biolabs) and then hybridized with the radiolabeled oligonucleotide probes at a temperature of 50°C for 24 h.

To sequence the RT-PCR products, the samples were reamplified with an additional 30 cycles of PCR, and the products were separated by TBE agarose gel electrophoresis. The bands corresponding to novel transcripts were cut from the gel, and the DNA was purified using QIAquick column (Qiagen). The PCR products were then purified using 30 μl of RNaseA reagents (Qiagen), and RT-PCR was performed as described above.

Polyribosome preparation. Approximately 107 mouse 3T3 cells were washed once with ice-cold phosphate-buffered saline (PBS) and then scraped from the dish in 200 μl ice-cold, diethyl pyrocarbonate (DEPC)-treated lysis buffer (10 mM HEPES, 300 mM KCl, 5 mM MgCl2, 0.5% NP-40). The lysate was passed three times through a 27-gauge needle, and the nuclei were removed by centrifugation (13,000 rpm) for 5 min at 4°C. The supernatant was applied directly to a QIAGEN mini spin column, washed twice in nocodazole-free media, and then grown in nocodazole-free media. Cells were plated in 60-cm2 dishes and harvested at various time points using trypsin. One aliquot was fixed in 100% ethanol at −20°C for at least 30 min. These fixed cells were then stained in PBS with 50 μg/ml propidium iodide and 200 μg/ml RNase A (Sigma) at 4°C for at least 30 min prior to FACs analysis. A second aliquot was placed directly into RLT buffer (Qiagen) and stored at −80°C for RNA preparation as described above.

Mammalian cell culture transfections. A plasmid, pRAD52Δ exon4 (pW13457) and pRAD52Δ+ intron8 (pW13458), were created using the Quickchange mutagenesis kit (Stratagene) to alter the RAD52 sequence. Briefly, pRAD52ΔΔ exon4 is identical to pRAD52, except for the deletion of 94 bp corresponding to RAD52 exon 4, pRAD52Δ+ intron8 is identical to pRAD52, except for the inclusion of 139 bp of mouse Rad52 intron 8 (derived from the RT-PCR of mouse tissues described above). pcDNA3 (Invitrogen), lacking the RAD52 gene, is a control vector. Chinese hamster ovary cells (CHO) were used to assay HDR. These cells contain a direct repeat of the GFP gene in which one repeat is full length but is inactivated by the inclusion of an I-SceI endonuclease target site. The second repeat lacks both the 5′ and 3′ ends of the gene. After cleavage with the I-SceI endonuclease, the resulting DSB can be repaired by gene replacement from the second GFP repeat either from the same or from a sister chromosome, resulting in a functional GFP allele (see Fig. 5A). The efficiency of repair is scored by fluorescence-activated cell sorting (FACS). The plasmid encoding the horseradish endonuclease I-SceI (pCAGGS-I-SceI) as well as the CHO and CHO-XRCC3−/− cell lines containing the DR-GFP reporter were kindly gifts from Mario Jasin and Jeremy Stark, and their use has been described elsewhere (31). Cells were grown in serum-plus medium (Dulbecco’s modified Eagle medium containing 10% newborn bovine serum, 4.5 g/liter glutamine, 4.5 g/liter glucose, 100 μ/ml penicillin-streptomycin; all from Invitrogen-GibcoBRL) at 37°C, 6% CO2. Cells were harvested from culture dishes after 2 to 10 min of incubation with trypsin-EDTA (Invitrogen) and then washed once in PBS. Cells (2×107) were plated on 60-mm tissue culture dishes and grown overnight in serum-plus medium. The following day the cells were transfected in OPTIMEM medium (Invitrogen) with 4 μg/dish plasmid DNA (2 μg of p-SceI- and 2 μg of experimental plasmid as indicated), using 4 μl/dish lipofectin (Invitrogen) following the manufacturer’s instructions (this DNA:lipofectin ratio was determined to be optimal for expression of the I-SceI enzyme). Transfection was carried out for 5 h, after which the medium was replaced with serum-plus medium. After 48 h, the cells were collected by trypsin-EDTA, washed twice in PBS, and resuspended in 200 to 600 μl PBS. FACS analysis was performed on the cells as previously described (49). Expression levels of I-SceI from the p-SceI vector were assumed to be equivalent in each case. The expression of the mammalian variants was assessed by a parallel experiment in which CHO cells were transfected with plasmid pRAD52ΔΔ exon4 and those described above but including the internal ribosomal entry site (IRES)-GFP sequence. This GFP sequence produces fluorescence signal in all cells that successfully take up and express the plasmid vector. FACS analysis of the resulting transfections allowed the measurement of the proportion of CHO cells transfected (GFP+). For each of the four vectors (pIRES-GFP, pRAD52ΔΔ exon4-IRES-GFP, pRAD52Δ Δ intron8-IRES-GFP, and pRAD52ΔΔ intron8-IRES-GFP) the number of cells transfected was equivalent (Fig. 5B in the supplemental material).

Mammalian cell synchronization. To obtain a synchronous culture of mouse 3T3 cells, the cells were grown to near confluency in 150-cm2 tissue culture flasks and then arrested with 400 ng/ml (1.3 μM) nocodazole for 6 h. The mitotic cells were then collected by mitotic shake-off, which involves mechanical shaking of the cultures for 5 min. The mitotic cells were collected by centrifugation, washed twice in nocodazole-free media, and then grown in nocodazole-free media. Cells were plated in 60-cm2 dishes and harvested at various time points using trypsin. One aliquot was fixed in 100% ethanol at −20°C for at least 30 min. These fixed cells were then stained in PBS with 50 μg/ml propidium iodide and 200 μg/ml RNase A (Sigma) at 4°C for at least 30 min prior to FACs analysis. A second aliquot was placed directly into RLT buffer (Qiagen) and stored at −80°C for RNA preparation as described above.

Yeast strains and methods. Standard genetic methods were used for the growth and manipulation of yeast (36). The yeast strains used in this study are listed in Table 1. The strains encoding Rad52 tagged with yellow fluorescent protein (YFP) and cytoplasmic fluorescent protein (CFP) have been reported previously (23). We modified W3749-14C (Rad52-YFP) using our previously published allele replacement method (6). Briefly, PCR primers were designed to generate targeting fragments that eliminate either the 3′ 1,261 bp of RAD52 for rad52ΔΔ 3′ YFP or the 3′ 660 bp of RAD52 for rad52ΔΔ 28′. The final five amino acids of yeast Rad52 encoded by rad52ΔΔ 28′ and rad52ΔΔ 28′ are -FGTSR and -DTDLK, respectively. The two resulting RAD52 deletion strains (W5001-9C and W5002-5A) maintain the truncated yeast RAD52 ORF in frame with the downstream YFP gene. The sensitivity of yeast strains to 0-γ-irradiation was determined by growth sequences in YPD to log phase and then plating on YPD plates and incubating them at 30°C in a humidified chamber (Gammacell-220, Atomic Energy, Ottawa, Canada). The proportion of S-g phase cells in log-phase culture containing Rad52ΔΔ 3′ YFP and -CFP foci were determined using fluorescence microscopy as previously described (23, 25). The fluorescence of tagged Rad52 protein in individual cells was measured by reconstructing cells in three dimensions from fluorescent images.
and quantifying the relative fluorescence using Volocity software (Improvision, Lexington, MA).

The strains encoding the two heteroalleles of *ADE2* were a kind gift from Lorraine Symington, and their use has been described elsewhere (12). The *ade2-n* allele contains a fill-in mutation of the 3′ NdeI restriction endonuclease target site of the yeast *ADE2* gene, and the *ade2-a* allele contains a fill-in mutation of the 5′ AatII site (2). The frequency of heteroallelic recombination was measured for at least nine independent isolates of each diploid strain. Cells were grown overnight in synthetic complete (SC) medium and washed once in H₂O before being briefly sonicated. Serial 10-fold dilutions were plated both on uracil-deficient medium confirmed their genotype. The colonies are indicative of their ability to lose the single *URA3* gene. In contrast, the *ade2-a* allele contains a fill-in mutation of the *URA3* gene. AatII site (2). The frequency of heteroallelic recombination was measured for at least nine independent isolates of each diploid strain.

### RESULTS

**Identification of mouse splice variants.** Preliminary data derived from our sequencing of the mouse *RAD52* gene (unpublished observations) and from RT-PCR from an array of different mouse tissues (see Fig. 5A in the supplemental material) suggested that at least two splice variants of mouse *RAD52* are commonly expressed. To investigate these findings further, tissue samples of lung and liver were dissected from two C57BL/6J mice and used for RT-PCR. A series of overlapping PCRs were performed covering the length of the mouse *RAD52* cDNA, as illustrated in Fig. 1A. The murine *RAD52* gene contains 12 exons. Three PCRs amplify cDNA from exons 1 to 7, 6 to 11, and 10 to 12. These PCRs were designed to detect most alterations in splicing that are pre-

![Image](340x286 to 520x620)

**Fig. 1.** (A) Twelve exons of the mouse *RAD52* gene are shown as numbered boxes approximately to scale; the position of each intron is shown as a short gap, but the introns are not shown to scale. A gray box above the gene indicates the open reading frame of the full-length *RAD52* peptide, which extends from the start of exon 2 to the start of exon 12. The three primer pairs for RT-PCR across the *RAD52* transcript are indicated by converging arrows (exons 1 to 7, 6 to 11, and 10 to 12). (B, C, and D) Autoradiographs of the blots derived from the mouse RT-PCR are shown. A limited number of PCR cycles (30) was used, followed by genomic blotting, to ensure that all products detected are specific for the mouse *RAD52* gene. Each autoradiograph has 18 lanes, with samples derived from the lung and 8 with samples derived from liver, plus 2 control lanes (H₂O, no template added). Two separate tissue samples were prepared from each of the two mice, and in each case the cDNA was prepared with and without reverse transcriptase (rt, indicated by + or − above the lanes) to ensure that all products are derived from RNA. The sizes of the bands are indicated based upon the position of size markers from the original TBE-agarose gels. The top autoradiograph (B) shows the results using primers designed to amplify *RAD52* exons 1 to 7, and the middle autoradiograph (C) shows the results using primers designed to amplify *RAD52* exons 6 to 11. The bottom autoradiograph (D) shows the results using primers designed to amplify *GAPDH* exons 5 to 8, which serves as a control for RNA preparation.

### TABLE 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>W5933 rad52::HIS5/RAD52-YFP</td>
<td></td>
</tr>
<tr>
<td>W5934 rad52::HIS5/rad52Δ77-YFP</td>
<td></td>
</tr>
<tr>
<td>W5935 rad52::HIS5/rad52Δ284-YFP</td>
<td></td>
</tr>
<tr>
<td>W5936 rad52::HIS5/rad52Δ77-YFP</td>
<td></td>
</tr>
<tr>
<td>W5937 RAD52-CFP/RAD52-YFP</td>
<td></td>
</tr>
<tr>
<td>W5938 RAD52-CFP/rad52Δ77-YFP</td>
<td></td>
</tr>
<tr>
<td>W5939 RAD52-CFP/rad52Δ284-YFP</td>
<td></td>
</tr>
<tr>
<td>W5957 ade2-n/ade2-a RAD52/RAD52</td>
<td></td>
</tr>
<tr>
<td>W5958 ade2-n/ade2-a RAD52/rad52Δ77-YFP</td>
<td></td>
</tr>
<tr>
<td>W5959 ade2-n/ade2-a RAD52/rad52Δ284-YFP</td>
<td></td>
</tr>
<tr>
<td>W5977 {ura3ΔecoRI::URA3/ura3ΔBstEII/ura3ΔecoRI::URA3/ura3ΔBstEII/ura3ΔecoRI::URA3/ura3ΔBstEII}</td>
<td></td>
</tr>
<tr>
<td>W5978 {ura3ΔecoRI::URA3/ura3ΔBstEII/ura3ΔecoRI::URA3/ura3ΔBstEII}</td>
<td></td>
</tr>
<tr>
<td>W5979 {URA3/URA3}</td>
<td></td>
</tr>
<tr>
<td>W5980 {URA3/URA3}</td>
<td></td>
</tr>
</tbody>
</table>

* All strains in this study are RADS4 derivatives of W303 (47, 54). In addition to the genotype listed above, all strains are *MATA/MATA his3Δ1-101 ura3Δ-52 his3Δ-111 BAR1Δ3-112, except where indicated. Strains W5933 through W5939 are also homozygous for bar1-1; LEU2; the other strains are BAR1Δ. The rad53::HIS3 allele is rad53Δ.
RAD52 encoded by exon 5. The RAD52/H11001 RAD52 active splicing in addition to the expected template RNA, as the RT-PCRs have not saturated. Subsequent gel blotting also allows a semiquantitative assessment of the gene. Using a relatively low number of PCR cycles and subsequent gel blotting also allows a semiquantitative assessment of the template RNA, as the RT-PCRs have not saturated.

We observed two variant products consistent with alternative splicing in addition to the expected RAD52 transcript products corresponding to the annotated RAD52 sequence (Fig. 1B and C). The expected product from exons 1 to 7 is 591 bp; however, a smaller, ~500-bp product was also identified (Fig. 1B). As depicted in Fig. 1C, products amplified from exons 6 to 11 give rise to a larger product of 750 to 800 bp in addition to the expected 631-bp product. No alternatively spliced products were observed from exons 10 to 12 (data not shown). Figure 1D shows the GAPDH control.

Both of these variant products are predicted to encode truncated RAD52 peptides (Fig. 2). DNA sequencing of the novel products revealed the alternative splicing. The small-variant product from exons 1 to 7 corresponds to a deletion of 591 bp; however, a smaller, ~500-bp product was also identified (Fig. 1B). As depicted in Fig. 1C, products amplified from exons 6 to 11 give rise to a larger product of 750 to 800 bp in addition to the expected 631-bp product. No alternatively spliced products were observed from exons 10 to 12 (data not shown). Figure 1D shows the GAPDH control.

Both of these variant products are predicted to encode truncated RAD52 peptides (Fig. 2). DNA sequencing of the novel products revealed the alternative splicing. The small-variant product from exons 1 to 7 corresponds to a deletion of 591 bp; however, a smaller, ~500-bp product was also identified (Fig. 1B). As depicted in Fig. 1C, products amplified from exons 6 to 11 give rise to a larger product of 750 to 800 bp in addition to the expected 631-bp product. No alternatively spliced products were observed from exons 10 to 12 (data not shown). Figure 1D shows the GAPDH control.

Both of these variant products are predicted to encode truncated RAD52 peptides (Fig. 2). DNA sequencing of the novel products revealed the alternative splicing. The small-variant product from exons 1 to 7 corresponds to a deletion of 591 bp; however, a smaller, ~500-bp product was also identified (Fig. 1B). As depicted in Fig. 1C, products amplified from exons 6 to 11 give rise to a larger product of 750 to 800 bp in addition to the expected 631-bp product. No alternatively spliced products were observed from exons 10 to 12 (data not shown). Figure 1D shows the GAPDH control.

Both of these variant products are predicted to encode truncated RAD52 peptides (Fig. 2). DNA sequencing of the novel products revealed the alternative splicing. The small-variant product from exons 1 to 7 corresponds to a deletion of 591 bp; however, a smaller, ~500-bp product was also identified (Fig. 1B). As depicted in Fig. 1C, products amplified from exons 6 to 11 give rise to a larger product of 750 to 800 bp in addition to the expected 631-bp product. No alternatively spliced products were observed from exons 10 to 12 (data not shown). Figure 1D shows the GAPDH control.

Both of these variant products are predicted to encode truncated RAD52 peptides (Fig. 2). DNA sequencing of the novel products revealed the alternative splicing. The small-variant product from exons 1 to 7 corresponds to a deletion of 591 bp; however, a smaller, ~500-bp product was also identified (Fig. 1B). As depicted in Fig. 1C, products amplified from exons 6 to 11 give rise to a larger product of 750 to 800 bp in addition to the expected 631-bp product. No alternatively spliced products were observed from exons 10 to 12 (data not shown). Figure 1D shows the GAPDH control.
and D) for the RT-PCR products used to identify

expression of the splice variant constructs elevates the frequency of repair compared to that of full-length RAD52 (Fig. 5C).

Since there is evidence from chicken cells to suggest that RAD52 acts in parallel with XRCC3 in DNA damage repair (10), the mammalian HDR experiments were repeated with a CHO cell line that lacks XRCC3 (CHO-XRCC3<sup>-/-</sup>) to see how the splice variants act in this mutant. Consistent with previous observations (31), the overall frequency of HDR in this cell line is more than 10-fold lower than in wild-type cells. However, overexpression of the RAD52 splice variants did not change the levels of HDR (Fig. 5D).

Finally, we attempted to knock down the level of the RAD52<sup>+intron8</sup> variant in CHO cells by designing small interfering RNA against the hamster intron 8 sequence. However, transfection of this small interfering RNA had no detectable effect on the level of the intron 8 variant (as judged by RT-PCR) or the frequency of recombination of the GFP targets in the presence of I-Sce1 (data not shown).

**Splice variants modeled in yeast cause dominant recombination effects.** The ability to conduct functional recombination studies in mammalian cells is currently hindered by the limited number of functional assays that distinguish between recombination products; for example, it is difficult to differentiate repair from a homolog as opposed to a sister chromosome. Consequently, we employed the budding yeast Saccharomyces cerevisiae to assay for dominant-negative behavior of the splice variants. To mimic the C-terminal truncations that are predicted to be encoded by the two mouse splice variants, RAD52<sup>Δexon4</sup> and RAD52<sup>Δintron8</sup>, the RAD52-YFP (encoding Rad52 fused to yellow fluorescent protein) gene from Saccharomyces cerevisiae (23) was modified at the yeast chromosomal locus. The RAD52<sup>Δexon4</sup> and RAD52<sup>Δintron8</sup> mouse splice variants were recreated in the yeast RAD52-YFP gene as rad52<sup>Δ77</sup> and rad52ΔΔ284, respectively (the numbers correspond to the final amino acid in the deleted Rad52 sequences). It is important to note that the mouse sequences were not transferred into yeast; rather, the yeast sequence was used to model the mouse transcripts by creating truncations of the endogenous yeast RAD52 gene. We refer to these two yeast alleles as “splice variant equivalents.”

As the mouse variants showed increased HDR when expressed in mammalian cells (Fig. 5), a similar repair assay was performed in yeast. Recombination between direct repeats was assayed using a reporter system containing two defective alleles of LEU2 (leu2<sup><small>ΔEcoRI</small></sup> and leu2<sup><small>ΔBstEI</small></sup>) (Fig. 6A). Similar to the HDR assay used in CHO cells, the leu2 direct-repeat reporter assays primarily intrachromatid and sister chromatid recombination (17, 41). Commonly, two kinds of LEU2<sup>+</sup> products result: replacements, where one of the leu2 alleles is repaired without loss of the intervening URA3 sequence; or pop-out events, where the URA3 is lost by deletion of the sequence between the two LEU2 alleles (Fig. 6A). As described in Materials and Methods, these two events are distinguished by a sequence to restore a functional GFP open reading frame. CHO cells were transfected with vectors expressing the normal RAD52 cDNA, the two variant cDNAs, RAD52<sup>Δexon4</sup> and RAD52<sup>Δintron8</sup>, or the control (summarized in Fig. 5B). Expression of RAD52 has no significant effect on HDR in this assay (Fig. 5C), similar to the expression of RAD52 in a cell line mutated in rad52 (43). Interestingly, expression of either of the splice variant constructs elevates the frequency of repair compared to that of full-length RAD52 (Fig. 5C).

**Splice variants modeled in mammalian cell culture cause dominant recombination effects.** To test whether the mammalian splice variants cause functional changes in HDR, an established assay for gene repair in Chinese hamster ovary (CHO) cells was used. These cells contain the HDR reporter system outlined in Materials and Methods (also see Fig. 5A). Briefly, an I-SceI-induced DSB, within a nonfunctional GFP gene, can be repaired by gene conversion from a neighboring formed on the RNA as described above. Figure 4A shows the FACS profile of the cells following release from nocodazole arrest, and Fig. 4B shows the results of RT-PCR. We could detect no significant difference in the ratio of splice variant to normal transcript for either RAD52<sup>Δexon4</sup> or RAD52<sup>Δintron8</sup> at any stage in the cell cycle. The RT-PCR products appear the same as asynchronously dividing cells (as in Fig. 3).

![FIG. 4.](image-url)

FIG. 4. (A) The FACS profile shows cells stained with propidium iodide at seven different time points after release from nocodazole arrest and mitotic shake off. At time zero the cells are arrested in G2, by 6 h most of the cells have entered G1, and by 12 h many of the cells have entered S phase. The percentages for 2n and 4n indicate the percentage of the cell population that fall within the limits of the black bars shown above the FACS profile. (B) Autoradiographs (as in Fig. 1B, C, and D) for the RT-PCR products used to identify RAD52<sup>Δexon4</sup> (top), RAD52<sup>Δintron8</sup> (middle), and GAPDH control (bottom) from the time points shown above.
Since the mouse splice variants detected in our screen are expressed together with the normal transcript (Fig. 1B and C, 3, and 4B), we next created diploid yeast strains that contain a full-length \textit{RAD52} allele in addition to a splice variant-equivalent allele to screen for dominant effects on direct-repeat recombination. Both of the \textit{RAD52} splice variant equivalents elevate the frequency of \textit{LEU2} recombinants (Fig. 6B). While the effects upon recombination tested in the same way (D). The difference between the wild-type \textit{RAD52} plasmid and the pRAD52\textsubscript{Δexon4} plasmid is not significant (one-tailed \(t\) test, \(P = 0.094\)). The difference between the wild-type \textit{RAD52} plasmid and the pRAD52\textsubscript{intron8} plasmid is significant (one-tailed \(t\) test, \(P = 0.044\)).
are modest, they are significantly different from those of a heterozygous strain (one-tailed t tests, \( P = 8.5 \times 10^{-3} \) and \( 4.4 \times 10^{-6} \) for rad52Δ77 and rad52Δ284, respectively). These effects are similar to those seen in mammalian cell culture (Fig. 5). Interestingly, in yeast, the rad52Δ277 allele increases replacement events, while the rad52Δ284 allele increases the frequency of URA3 pop-outs (Table 2).

The yeast system has the advantage that the variants are easily assayed for effects on other types of DNA repair. Therefore, we analyzed the splice variant equivalents for their effects on heteroallelic recombination, focus formation, and DNA damage sensitivity. First, spontaneous heteroallelic recombination was assayed in diploid strains with two defective ade2 heteroalleles (ade2-\( n \) and ade2-\( a \)). Recombination between these alleles, which reside at each end of the ADE2 gene on homologous chromosomes, restores a functional ADE2 gene (Fig. 7A). Surprisingly, in contrast to the elevated frequency of direct-repeat recombination seen in both mammalian cells (Fig. 5) and yeast (Fig. 6B), both of the splice variant equivalents lowered the frequency of yeast heteroallelic recombination (Fig. 7B). These disparate phenotypes suggest that the variants may be promoting sister chromatid repair while at the same time suppressing repair from a homologous chromosome.

In yeast, Rad52 and other DNA repair proteins form distinct nuclear foci at the sites of double-strand break repair (23–25). These DNA repair centers appear predominantly in S and G2 phases of the cell cycle. Altered levels of foci within yeast and mammalian cells correlate with DNA repair deficiencies (25). Next, we analyzed the splice variant equivalents for their effects on other types of DNA repair. Therefore, DNA damage sensitivity is a standard assay for the function of yeast Rad52. First, we estimated protein levels in diploid strains containing differentially tagged Rad52 peptides (CFP and YFP) to ensure that the splice variant equivalent alleles do not alter the expression of RAD52 from either the wild-type or mutant allele. Table 3 shows the amount of the CFP and YFP detected in the nucleus of the different strains, and there is no significant difference between the two mutant strains and the wild-type diploid. Next, the repair capacity of the splice variant equivalent strains was assayed by measuring viability with increasing doses of \( \gamma \)-irradiation. Both rad52Δ277 and rad52Δ284 are as sensitive to \( \gamma \)-irradiation as rad52 null strains, either as haploid strains (data not shown) or as heterozygous diploids containing a null allele (Fig. 9A, first four rows). The diploid strains containing both a mutant and wild-type RAD52 allele were tested for sensitivity to \( \gamma \)-irradiation, and rad52Δ277 (Fig. 9A, sixth row) is as sensitive as a wild type/null (Fig. 9A, top row). In contrast, rad52Δ284 confers a dominant-negative phenotype for \( \gamma \)-irradiation sensitivity (Fig. 9A, last row, indicated by the white arrow).

<table>
<thead>
<tr>
<th>Strain*</th>
<th>RAD52 genotype</th>
<th>( n^b )</th>
<th>Proportion of LEU2 (^+ ) colonies that are rad3 (^ - )/URA3 (^ + ) (10^{-3} )</th>
<th>Frequency of replacement events (10^{-3} )</th>
<th>Frequency of pop-out events (10^{-3} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>W5977</td>
<td>wt/wt</td>
<td>933</td>
<td>0.17</td>
<td>2.1</td>
<td>0.43</td>
</tr>
<tr>
<td>W5978</td>
<td>rad52::HIS5</td>
<td>532</td>
<td>0.32</td>
<td>2.2</td>
<td>1.0</td>
</tr>
<tr>
<td>W5979</td>
<td>rad52Δ277-YFP</td>
<td>1291</td>
<td>0.30</td>
<td>2.7</td>
<td>1.2</td>
</tr>
<tr>
<td>W5980</td>
<td>rad52Δ284-YFP</td>
<td>1132</td>
<td>0.53</td>
<td>2.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

\( ^a \) wt, wild-type RAD52 genotype. The full genotypes are listed in Table 1. 
\( ^b \) \( n \) is the number of LEU2 \(^+ \) colonies screened on 5-FOA-containing medium.

\( ^c \) The estimated frequencies of replacements and pop-outs are calculated using the total recombination frequencies, shown in Fig. 6B, adjusted by the proportions shown in this table.

In yeast, Rad52 and other DNA repair proteins form distinct nuclear foci at the sites of double-strand break repair (23–25). These DNA repair centers appear predominantly in S and G2 phases of the cell cycle. Altered levels of foci within yeast and mammalian cells correlate with DNA repair deficiencies (25). Next, we analyzed the splice variant equivalents for their effects on other types of DNA repair. Therefore, DNA damage sensitivity is a standard assay for the function of yeast Rad52. First, we estimated protein levels in diploid strains containing differentially tagged Rad52 peptides (CFP and YFP) to ensure that the splice variant equivalent alleles do not alter the expression of RAD52 from either the wild-type or mutant allele. Table 3 shows the amount of the CFP and YFP detected in the nucleus of the different strains, and there is no significant difference between the two mutant strains and the wild-type diploid. Next, the repair capacity of the splice variant equivalent strains was assayed by measuring viability with increasing doses of \( \gamma \)-irradiation. Both rad52Δ277 and rad52Δ284 are as sensitive to \( \gamma \)-irradiation as rad52 null strains, either as haploid strains (data not shown) or as heterozygous diploids containing a null allele (Fig. 9A, first four rows). The diploid strains containing both a mutant and wild-type RAD52 allele were tested for sensitivity to \( \gamma \)-irradiation, and rad52Δ277 (Fig. 9A, sixth row) is as sensitive as a wild type/null (Fig. 9A, top row). In contrast, rad52Δ284 confers a dominant-negative phenotype for \( \gamma \)-irradiation sensitivity (Fig. 9A, last row, indicated by the white arrow).
Rad52 recruits Rad51 to sites of DNA damage and stimulates Rad51-mediated DNA strand exchange in vitro. Furthermore, overexpression of RAD51 suppresses the phenotype of a Rad52 C-terminal truncation (1, 26). Therefore, we tested whether overexpression of the RAD51 gene could rescue the dominant-negative γ-irradiation phenotype seen for rad52Δ284. The seven yeast strains shown in Fig. 9A were transformed with a plasmid containing the yeast RAD51 gene under the control of a galactose-inducible promoter (pYES-S10-51; a kind gift from Thomas Kodadek). This plasmid complements a rad51 null strain in the presence of galactose (data not shown). Figure 9B shows that an overexpression of rad52Δ284 is partially rescued after induction of RAD51 expression (compare the bottom rows of Fig. 9B, as indicated with a white arrow). This suggests that the radiation-sensitive phenotype of rad52Δ284 is dependent on its disruption of Rad51 function(s).

**DISCUSSION**

Identification of novel RAD52 transcripts from adult mouse tissue. Here we identify two novel RAD52 transcripts from adult mouse tissue which are the major alternatively spliced mRNAs from this gene (Fig. 1). One variant aberrantly splices the RAD52 mRNA from exon 3 directly to exon 5, skipping exon 4 (RAD52Δexon4), while the other variant fails to remove the short intron 8 (RAD52+intron8). These two variant mRNAs are predicted to encode truncations of the RAD52 peptide and are likely translated in vivo, since both are found on polyribosomes in the cytoplasm of mouse cells (Fig. 3). Furthermore, a RAD52 IMAGE clone sequence was identified that lacks exon 4 (locus id:BC010673, gi:14715025), and sequences including RAD52 intron 8 were identified from the National Center for Biotechnology Information Mouse UniGene Clusters Map (data not shown). However, definitive identification of these variants using database screening fails, because many cDNA clone sequences are derived from partially spliced transcripts. In addition, examination of the sequences at the intron-exon boundaries of murine RAD52 does not identify deviations from the canonical splice sites to account for the alternate splicing. Thus, a solely bioinformatics approach is inadequate for identifying these novel splice variants, which requires the rigor of the RT-PCR analysis described here.

Testing the recombination phenotype of variant RAD52 transcripts in cell culture. RAD52Δexon4 and RAD52+intron8 are predicted to encode truncated mouse RAD52 peptides of 80 and 290 amino acids, respectively (Fig. 2). We next tested the effects of the mouse variants on HDR in mammalian cells using direct repeats of GFP (Fig. 5A). Expression of the murine RAD52 variants increase the frequency of gene replacement compared to wild-type mouse RAD52 (Fig. 5), establishing that these variants are capable of altering the DNA repair phenotype of mammalian cells. Although the increase in HDR is small, it is consistent with the relatively modest effect on this type of assay seen when complementing rad52 null cells (43). Several years ago, Kito et al. speculated that human RAD52 splice variants similar in size to the mouse RAD52Δexon4 transcript might confer a dominant-negative phenotype (16). Here we demonstrate that C-terminal truncations of RAD52 do in

**TABLE 3. Quantification of Rad52 protein levels**

<table>
<thead>
<tr>
<th>Strain</th>
<th>RAD52 genotype</th>
<th>n</th>
<th>Quantification of GFP signal mean fluorescence</th>
<th>Quantification of GFP signal mean fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>W5937</td>
<td>RAD52-CFP/RAD52-YFP</td>
<td>54</td>
<td>477.5 (±71)</td>
<td>257.2 (±12.5)</td>
</tr>
<tr>
<td>W5938</td>
<td>RAD52-CFP/rad52Δ77-YFP</td>
<td>47</td>
<td>504.6 (±40.7)</td>
<td>239.04 (±2.9)</td>
</tr>
<tr>
<td>W5939</td>
<td>RAD52-CFP/rad52Δ284-YFP</td>
<td>62</td>
<td>454.4 (±39.9)</td>
<td>264 (±12.5)</td>
</tr>
</tbody>
</table>

*The full genotypes are listed in Table 1. n indicates the number of cells measured for fluorescence. The amount of GFP is greater than YFP in all cases, as GFP gives a brighter fluorescence signal. It should be noted that nuclear fluorescence was measured in all cases, as Rad52 is localized within the nucleus. However, Rad52Δ77-YFP is localized throughout the cell.*

*The mean fluorescence intensity (±standard deviation of the mean) is given in relative units.*
fact confer a dominant phenotype in mammalian cells by increasing recombination.

**Testing the recombination phenotype of variant RAD52 transcripts in yeast.** The recombination assays available in mammalian cells have a number of limitations. We therefore modeled the splice variants in yeast to allow a further assessment of their role in DNA repair and recombination. A key advantage of modeling the mouse splice variants in yeast is that a range of well-characterized assays are available against which other defined alleles of ScRad52 have previously been tested. Furthermore, the HDR assays available in yeast are more diverse and informative than the current mammalian assays.

The two mouse variants, RAD52/exon4 and RAD52/intron8, were modeled in the ScRad52 gene (rad52Δ77 and rad52Δ284, respectively). These alleles are referred to as “splice variant equivalents,” since they truncate the yeast Rad52 protein at po-
sitions homologous to the mouse variants. The mammalian assay suggests that expression of the mouse variants elevates repair from the same or a sister chromosome (Fig. 5), therefore an equivalent yeast assay was performed. Direct-repeat recombination in yeast (Fig. 6) shows results similar to those seen in mammalian cells (Fig. 5), suggesting that the yeast splice variant equivalents do indeed model their mammalian counterparts. The splice variant equivalents show additional functional differences, which were revealed by further analysis of the LEU2 recombination products from the leu2 direct-repeat assay. For rad52ΔΔ77, the increased recombination is due to a modest increase in replacement events, whereas for rad52Δ284 the increase is due to pop-out events (Table 2). In contrast to the increase in leu2 direct-repeat recombination, the splice variant equivalents dominantly decrease the frequency of recombination between ade2 heteroalleles (Fig. 7). These opposing recombination phenotypes suggest that the variants may bias the HDR pathway away from a homologous template toward a sister or intrachromatid template.

To probe further the dominant effects of the two splice variant equivalents, DNA damage sensitivity was tested. While mammalian rad52 mutant cells exhibit limited DNA damage sensitivity, yeast rad52 strains are exquisitely sensitive to DNA damage. Thus, in yeast, sensitivity to DNA damage provides a simple assay for RAD52 function. As haploid cells, both rad52ΔΔ77 and rad52Δ284 are sensitive to γ-irradiation (data not shown). These results were not surprising, since previous studies showed that truncations removing more than ~200 C-terminal amino acids of ScRad52 render strains as sensitive as a rad52 null strain to methyl methanesulfonate (MMS), a DNA-damaging agent (1, 6). In addition, a deletion of the last 175 codons of ScRAD52 confers a defective DSB repair phenotype (51).

Since the mammalian variants are coexpressed with full-length transcripts (Fig. 1B, C, 3, and 4B), diploid strains containing a splice variant equivalent allele and a wild-type ScRAD52 allele were tested for γ-ray sensitivity. The Rad52ΔΔ284 allele is dominant negative, while the Rad52ΔΔ77 allele is not (Fig. 9A). Previous work has shown that overexpression of two ScRad52 C-terminal truncations confers a dominant-negative phenotype for sensitivity to MMS (26), suggesting that N-terminal Rad52 peptides are capable of disrupting the DNA repair ability of the full-length protein. Interestingly, structural analysis of both the full-length human RAD52 protein and an N-terminal fragment reveals a multimeric complex with RAD52 monomers arranged in a ring configuration (15, 40, 44). Therefore, it is likely that the RAD52+introns8 variant is incorporated into the ring and disrupts its function. This view is supported by our finding that Rad52ΔΔ284 and wild-type Rad52 colocalize in foci (Fig. 8A). In addition, increased levels of wild-type Rad52 foci caused by the splice variant equivalents may indicate increased DNA damage. However, it is unclear how increased damage could cause a specific preference for repair from the same or a sister chromosome. Indeed, DNA damage elevates repair from both sister and homologous chromosomes (13). In addition, elevated Rad52 foci levels in yeast mutants typically indicate delayed or impaired repair processes.

Disruption of the Rad52-Rad51 interaction and its effects on recombination. C-terminal truncations of Rad52 are known to disrupt its interaction with Rad51, thus impairing the formation of Rad51 filaments (19, 26, 37, 39). Such Rad52 C-termi-
colleagues for providing the mammalian cell reporter system. We also thank David Bentley for help with the preliminary data outlined in Fig. 5A of the supplemental material, Bill Friedewald and Shing Lee for advice on binomial statistics, Christine Richardson and members of the Rothstein lab for critical comments on the manuscript, and Larry Chasin for helping us to attempt to define alternative splicing from raw sequence data.

This work was supported by grants from the Eric Greenberg Foundation, Women-at-Risk Breast Cancer Program, the Berrie Diabetes Foundation, and the NIH (NIGMS and NCI). M.S. was supported by an award from the New York Times Company Foundation to the Columbia University summer research program for science teachers.

REFERENCES


