Dominant negative alleles of RAD52 reveal a DNA repair/recombination complex including Rad51 and Rad52

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Saccharomyces cerevisiae rad52 mutants are characterized by severe defects in double-strand break (DSB) repair and recombination. In this study we have identified several regions of RAD52 that are required for these biological functions. We cloned and characterized a RAD52 homolog from Kluyveromyces lactis that partially complemented S. cerevisiae rad52 mutants while exhibiting negative dominance in wild-type (RAD52) strains. The dominant negative effect was suppressed by overexpression of RAD51, an additional gene known to be required for DSB repair and recombination, indicating a genetic interaction between these loci. Furthermore, GAL4 two-hybrid analysis revealed a physical interaction between Rad51 and the carboxy-terminal one-third of Rad52. Deletion alleles of rad52 [with or without the Rad51 association domain] also produced dominant negative defects, suggesting the disruption of repair through nonfunctional interactions with other DSB repair and recombination proteins. RAD51 relieved the negative dominance of each of these alleles either by competitive titration or functional activation of mutant or heterologous Rad52 proteins. These results demonstrate the importance of Rad52–Rad51 interactions and point to the formation of a higher order repair/recombination complex potentially containing other yet unidentified components.

[Key Words: RAD52; RAD51; double-strand break repair; negative dominance]

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Distinct DNA repair pathways are found in eukaryotes that differ in mechanism as well as the lesions upon which they act. These pathways are best defined in the yeast Saccharomyces cerevisiae, where three epistasis groups of DNA repair genes have been identified that control nucleotide excision [RAD3 epistasis group], error-prone [RAD6 epistasis group], and double-strand break [DSB] repair [RAD52 epistasis group] [for review, see Game 1983; Friedberg et al. 1991; Petes et al. 1991]. In particular, the repair of damage-induced DSBs appears to be resolved by a recombinational mechanism, such that mutants defective in DSB repair frequently have corresponding defects in recombination [Game 1983]. Furthermore, in homologous recombination and mating-type switching, there is significant evidence that DSBs represent intermediates in the recombination process [Petes et al. 1991].

Members of the S. cerevisiae DSB repair epistasis group [including RAD50–57, MRE11, and XRS2] have been identified from screens either for X-ray sensitivity or recombination deficiencies [Game 1983; Ajimura et al. 1992; Ivanov et al. 1992]. Although these genes are in the same epistasis group, mutants differ in repair sensitivity as well as the spectrum of affected recombination events. Mutations in RAD51, RAD54 and, particularly, RAD52, have the most severe and pleiotropic defects, suggesting that these gene products play pivotal roles in recombination and repair [Game 1983].

Little is known about the molecular role of the Rad52 protein. Genetic studies suggest that Rad52 is not required for the initiation of recombination because rad52 mutations can be bypassed by mutations affecting DSB formation and recombination initiation [Petes et al. 1991]. In addition, the rad52 repair defects and studies of DSB-stimulated transformation indicate a role for Rad52 at an intermediate stage in recombination after the formation of DSBs but before the appearance of stable recombinants [Petes et al. 1991]. This view is complicated by the physical detection of possible recombination products, meiotic crossovers and heteroduplex DNA (hDNA), in rad52 mutants [Borts et al. 1986; Nag and Petes 1993]. Thus, Rad52 may be required for the productive resolution of crossovers and hDNA rather than for their formation. Rad52 function may also be modulated by interaction with other repair proteins. For example, physical interaction between Rad52 and the RecA-like protein Rad51 occurs in vitro [Shinohara et al. 1992].

Here, we have identified regions of Rad52 that are critical for DSB repair/recombination functions. Through the characterization of a divergent RAD52 homolog and two partial deletions of the S. cerevisiae RAD52 gene.
that exhibit negative dominance, we have demonstrated the biological importance of physical interactions of Rad52 with other DSB repair proteins. Genetic tests with rad52 mutants and GAL4 two-hybrid analysis of RAD52 revealed an in vivo physical interaction between Rad52 and Rad51. Although overexpression of rad52 mutants and GAL4 two-hybrid analysis of RAD52 revealed an in vivo physical interaction between Rad52 and Rad51. Although overexpression of rad52 mutants and GAL4 two-hybrid analysis of RAD52 revealed an in vivo physical interaction between Rad52 and Rad51. Although overexpression of rad52 mutants and GAL4 two-hybrid analysis of RAD52 revealed an in vivo physical interaction between Rad52 and Rad51. Although overexpression of rad52 mutants and GAL4 two-hybrid analysis of RAD52 revealed an in vivo physical interaction between Rad52 and Rad51. 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ier et al. 1978) for domain II strongly suggest formation of an α-helix, where 8 of 9 KIRAD52 or 9 of 10 ScRAD52 aspartic acid residues are predicted to reside on the same face of the helix. The strict conservation of a charged surface may be indicative of residues involved in protein-protein interactions. Domain III, extending from amino acid 330, which removes domain III, results in a protein that confers significant, but partial, complementation of rad52 mutants (data not shown; Adzuma et al. 1984, Boundy-Mills and Livingston 1993).

KIRAD52 partially complements S. cerevisiae rad52 mutations

To address the functional homology between ScRAD52 and KIRAD52 with regard to DSB repair, we examined the ability of the K. lactis gene to complement the sensitivity of rad52 mutants to the X-ray mimetic compound, methylmethane sulfonate (MMS). A multicopy plasmid [2μ origin] containing either the KIRAD52 XbaI genomic fragment [pDB[KIRAD52]] or the ScRAD52 gene [pDB[RAD52]] was transformed into the rad52-1 strain, DWY5 [Table 1]. Cells transformed with ScRAD52 were fully complemented, as an equal number of colonies appeared in the presence and in the absence of MMS (Fig. 2A). However, KIRAD52 gave only partial complementation when transformed on a 2μ plasmid (Fig. 2A) or a single-copy vector (data not shown). rad52 cells expressing KIRAD52 were reproducibly 50- to 100-fold more resistant to MMS than those containing only the corresponding vector but 5- to 10-fold more sensitive than rad52 cells fully complemented with ScRAD52. Partial complementation by KIRAD52 was also observed in the rad52A strains DWY97 and DWY98, although the relative levels of complementation were somewhat lower:

The observed partial complementation may be the result of poor utilization of K. lactis transcriptional regulatory sequences. Therefore, we cloned the KIRAD52 gene downstream of the powerful S. cerevisiae ADH1 promoter. Interestingly, ADH:KIRAD52 showed reproducibly lower levels of complementation than that observed with genomic clones [Fig. 2A]. The reduced complementation of ADH:KIRAD52 did not result from inadvertent truncation or mutation of KIRAD52, as several independently derived clones showed identical levels of complementation (data not shown).

KIRAD52 is a dominant negative allele in S. cerevisiae

The poor complementation of ADH:KIRAD52 may indicate that overexpression of a partially active allele of RAD52 has a dominant negative effect on the repair of MMS-induced damage. To test this hypothesis, we transformed ADH:KIRAD52 and ADH:ScRAD52 into the wild-type strain DWY83. Overexpression of KIRAD52 resulted in extreme MMS sensitivity, whereas overexpression of ScRAD52 had relatively little effect [Fig. 2B]. The MMS sensitivity of wild-type cells expressing ADH:KIRAD52 was comparable to the level of an isogenic rad52A strain (data not shown). Thus, the decreased complementation of ADH:KIRAD52 constructs observed in rad52 mutants is likely attributable to a balance between the complementation of the rad52 mutant phenotype and the sensitization resulting from overexpression. This effect appears to be general, being observed in all strains examined, including one diploid and three haploids [data not shown]. Furthermore, this phenotype is indicative of a general DSB repair defect, because the cells were sensitive to γ-irradiation as well as MMS (data not shown). Negative dominance was depen-

Table 1. Yeast strains used in this study

| Strain  | Genotype | Source or reference*                |
|---------|----------|-------------------------------------|
| DWY5    | MATα rad52-1::ura3-52 his4-912::(ura3b) | F. Winston, Harvard Medical School (FW638) |
| DWY83   | MATα arg4-RV leu2-3,112 cyhR ura3-52 trp1-189 | R. Kolodner, Harvard Medical School (RKY1734) |
| DWY84   | MATα/MATα leu2::lys2::lys2 ura3::ura3 leu2::hisG/leu2::hisG his4-X/leu2::his4-B | A. Nicolas, Universite Paris-Sud (ORT118-2) |
| DWY97   | MATα rad52-2::ura3 arg4-RV leu2-3,112 cyhR ura3-52 trp1-189 | this study |
| DWY98   | MATα/MATα leu2::lys2::lys2 ura3::ura3 leu2::hisG/leu2::hisG his4-X/leu2::his4-B | this study |
| DWY120  | MATα leu2::lys2 rad51A::hisG leu2::hisG ade2:::Lk his4-XB | this study |
| GGY1::171 | leu2::his3 | Chien et al. [1991] |
| NKY1826 | MATα::lys2::hisG/URA3::hisG ade2:::Lk his4-XB | N. Kleckner, Harvard University |
| RKY1525 | MATα::lys2 rad52::URA3 leu2::hisG his4-X | R. Kolodner |
| RKY1526 | MATα::lys2 rad52::URA3 leu2::hisG his4-B | R. Kolodner |

*Previous strain designations are displayed in parentheses.
dominant negative effects are not the result of general toxicity but are likely to occur by interrupting recombinational repair.

The KIRAD52 dominant negative phenotype can be suppressed by co-overexpression of S. cerevisiae RAD51

The heterologous KIRad52 protein may be titrating a critical component of DSB repair to generate dominant negative effects. Other members of the DSB repair epistasis group might interact with Rad52 and suppress dominant negative phenotypes. We focused on S. cerevisiae RAD51 because Escherichia coli-purified Rad51 was found to bind to a Rad52 protein column (Shinohara et al. 1992). Co-overexpression of RAD51 suppresses the dominant effects of KIRAD52 to near wild-type levels of resistance to MMS (Fig. 2B). This suppression is not the result of a general increase in MMS resistance, as wild-type strains are equally resistant to MMS both in the presence and absence of RAD51 overexpression (data not shown).

Rad52 physically interacts with Rad51

The suppression of the KIRAD52 dominant negative effect by RAD51 could be mediated by a direct protein association. We used a genetic screen, the GAL4 two-hybrid fusion system, to test for interaction between Rad52 and Rad51 in vivo (Chien et al. 1991). The DNA-binding domain of GAL4 (amino acids 1–147) was fused to RAD52 fragments to produce Gal4–Rad52 fusion proteins containing the full-length (GAL–52), amino-terminal two-thirds (GAL–52B), and carboxy-terminal one-third (GAL–52C) of S. cerevisiae RAD51 because Escherichia coli-purified Rad51 was found to bind to a Rad52 protein column (Shinohara et al. 1992). Co-overexpression of RAD51 suppresses the dominant effects of KIRAD52 to near wild-type levels of resistance to MMS (Fig. 2B). This suppression is not the result of a general increase in MMS resistance, as wild-type strains are equally resistant to MMS both in the presence and absence of RAD51 overexpression (data not shown).

Table 2. Dominant negative alleles of RAD52 affect mitotic recombination

| Recombination rate [±s.d.]* | ×10^-5 events/cell per generation |
|-----------------------------|----------------------------------|
| rad52Δ                      | RAD51                            |
| pDB20                       | 0.03 ± 0.02                      | 0.89 ± 0.29 | 0.93 ± 0.31 |
| RAD52                       | 1.32 ± 0.40                      | 0.91 ± 0.27 |
| KIRAD52                     | 0.19 ± 0.09                      | 4.12 ± 1.40 | 0.09 ± 0.04 | 1.34 ± 0.47 |
| rad52B                      | 0.18 ± 0.10                      | 1.08 ± 0.37 | 0.37 ± 0.14 | 0.83 ± 0.28 |

*Rates of spontaneous mitotic recombination were calculated as described in Materials and methods on the basis of the frequency of His+ colonies arising from DWY84 (RAD52/RAD52) or DWY98 (rad52Δ/rad52Δ) cotransformed with ADH:RAD52 alleles in the presence or absence of ADH:RAD51. Columns designated −RAD51 were transformed with pDBL such that all strains were grown in media lacking uracil and leucine. (−) Values were not evaluated for these strains.
body [data not shown]. Interaction between Rad52 and Rad51 will effectively reconstitute the two domains of the Gal4 protein to allow transcription of a β-galactosidase reporter gene (Chien et al. 1991).

β-Galactosidase activity, and thus interaction, was observed upon the cotransformation of TA-51 with either GAL-52 or GAL-KL52 (Fig. 3). Similar results were obtained when RAD51 was fused to the DNA-binding domain and RAD52 to the trans-activation domain of GAL4 [data not shown]. The interaction region was localized to the carboxy-terminal one-third of Rad52 because cotransformation of TA-51 and GAL-52C, but not TA-51 and GAL-52B, also demonstrated an interaction (Fig. 3). Clones cotransformed with TA-51, and either GAL-52 or GAL-52C gave 0.5-10 Miller units of β-galactosidase activity, 16- to 300-fold above background levels determined in strains containing the GAL4 DNA-binding fusion alone [0.03 units]. Cotransformation of TA-51 and GAL-KL52 yielded 10-80 units [300- to 2500-fold above background]. Despite being extremely consistent for duplicates of a single culture (<20% error), β-galactosidase units varied among clones transformed with the same plasmids. We attribute this variability to reduced plasmid copy number or decreased fusion protein expression. Thus, the sequences contained in GAL-52C are both necessary and sufficient for Rad52–Rad51 association. Control transformations of each of the GAL4 fusion constructs alone failed to produce any β-galactosidase activity. Thus, the dominant effects of KIRAD52 may be mediated, at least in part, by a direct interaction with Rad51. Presumably, this interaction is occurring in the conserved residues of domain III.

Other RAD52 alleles also exhibit negative dominance and can be suppressed by RAD51

On the basis of the observed Rad52–Rad51 interactions, mutations in S. cerevisiae rad52 alleles might also sensitize wild-type strains to MMS. Two other alleles of RAD52, which differ by whether or not they retain the Rad51 association domain, were examined for sensitivity to MMS (Fig. 4). rad52C was deleted for all of domains I and II but retains domain III and the Rad51 interaction region. Overexpression of rad52C may severely perturb MMS repair by interacting nonproductively with Rad51. Transformation of the wild-type strain DWY83 with ADH:rad52C resulted in extreme sensitivity to MMS [Fig. 4A]. Consistent with the proposed competition between Rad52 and Rad52C for interaction with Rad51, this effect was completely suppressed by co-overexpression of RAD51.

The reciprocal experiment was also performed in which the RAD52 sequences that do not interact with Rad51 were overexpressed. rad52B was created by carboxy-terminal truncation [BamHI, Rad52B = 330 amino acids] to remove Rad51-interacting sequences [including domain III]. This allele was predicted to have no effect

Figure 3. Rad52 and Rad51 interact physically. GGYI::171 was cotransformed with RAD52–GAL4 DNA-binding domain fusion constructs and either vector (pGAD2F) or a RAD51–GAL4 trans-activation domain fusion (TA:51). The relevant RAD52 sequences fused to GAL4 are illustrated diagramatically, with conserved domains I, II, and III indicated by bars. Interaction was quantitated as described in Materials and methods and displayed as follows: [-] 0.03 units, [+] 0.5-10 units, [++] 10-80 units.

Figure 4. S. cerevisiae rad52 deletion alleles exhibit negative dominance that is suppressed by RAD51. MMS sensitivity was determined as described in Fig. 2 and Materials and methods. The experiments depicted in A and B were performed on different days so that the relevant vector-only controls are included in each set. (A) MMS sensitivity of DWY83 (RAD52) cotransformed with vector controls (pDB20, pDBL, ○), ADH:rad52C and pDBL, ○, or ADH:rad52C and ADH:RAD51. (B) MMS sensitivity of DWY83 cotransformed with vector controls (pDB20, pDBL, ○, ADH:rad52B and pDBL, ○, or ADH:rad52B and ADH:RAD51. (C) Diagram of RAD52 sequences comprising rad52B and rad52C alleles. Conserved domains are indicated by solid lines.
owing to its inability to interact with Rad51 (Fig. 3). Interestingly, overexpression of rad52B exerted a significant, although lesser, dominant negative effect on the MMS resistance of DWY83 (Fig. 4B). Therefore, Rad52B retains the ability to interact with additional repair proteins or substrates. Paradoxically, RAD51 overexpression suppressed the negative dominance of rad52B nearly completely, despite the deletion of sequences required for Rad51 interaction.

As with KIRAD52, overexpression of rad52B or rad52C specifically affects the repair of MMS damage, as these transformants showed only slight sensitivity to UV damage [data not shown].

Dominant negative RAD52 alleles further sensitize a rad51Δ strain

To examine the hypothesis that the KIRAD52 and rad52C alleles might exert their dominant effects by titration of Rad51, we examined these alleles in a rad51Δ strain. If the dominant negative effects are solely attributable to the formation of mutant Rad52–Rad51 complexes, then one would expect these alleles to have no effect in a strain lacking Rad51. Conversely, dominant effects mediated through interactions with proteins other than Rad51 should be maintained in a rad51Δ background. A rad51Δ strain, DWY120, was transformed with ADH:KIRAD52, ADH:rad52B, and ADH:rad52C. To observe effects on recombinational repair already impaired by the absence of RAD51, MMS sensitivity was determined using a concentration of MMS that is lower than usual (0.00125%). This concentration of MMS has little or no effect on DWY120 while resulting in 20–50% survival of a related rad52Δ strain [data not shown]. No further sensitization to MMS was observed upon transformation with ADH:rad52C, indicating that its primary mechanism of action is through Rad51 [Fig. 5]. In contrast, ADH:KIRAD52 further increased the MMS sensitivity of DWY120 and thus continues to exhibit negative dominance in the absence of Rad51 [Fig. 5]. Therefore, we conclude that the defects resulting from KIRAD52 expression are not mediated solely by interaction with Rad51 but, rather, are the result of interaction with additional components of the DSB repair pathway.

Overexpression of rad52B also exerted negative dominance in the rad51Δ strain, although the magnitude of this effect, as in wild-type cells, was less than that of KIRAD52 [Fig. 5]. In this case, the MMS sensitivity of cells expressing rad52B was not significantly different from that of rad51Δ cells transformed with the ScRAD52 control, which were also slightly sensitized [Fig. 5]. We interpret the increased sensitivity of ScRAD52-expressing cells to be the result of subtle defects that are exacerbated in the absence of Rad51. These effects may be the result of imbalance, as has been described for histone sets [Meeks-Wagner and Hartwell 1986], or the channeling of DNA lesions into defective repair pathways [Aboussekhra et al. 1992].

RAD51 increases the complementation activity of some RAD52 alleles

We then examined the effects of coexpression of RAD51 and various RAD52 alleles in a rad52Δ strain. If RAD51 suppresses the MMS sensitivity of cells expressing dominant negative RAD52 alleles by competetively removing mutant proteins from repair complexes, then RAD51 overexpression should have no effect on the biological activity [and thus complementation] of these mutant alleles in the absence of wild-type Rad52. DWY97, an isogenic rad52Δ derivative of DWY83, was cotransformed with ADH:KIRAD52, ADH:rad52B, and ADH:rad52C with and without ADH:RAD51. The MMS sensitivity of the transformants defines two classes of dominant negative alleles [Fig. 6]. The first class consists of the rad52C allele, which exhibited no significant complementation activity in the presence or absence of RAD51 overexpression [Fig. 6D]. This is consistent with a model in which RAD51 relieves the dominant effects of rad52C in RAD52 strains by direct titration of the mutant protein. The second class of alleles is comprised of the partially complementing KIRAD52 and rad52B alleles. In these cases, co-overexpression of RAD51 dramatically increased the MMS resistance of the rad52Δ strain near wild-type levels [Fig. 6B,C]. This increase is dependent on the RAD52 activity of the cotransformed gene, because no such increase was observed in cells expressing RAD51 alone [Fig. 6A]. Thus, RAD51 overexpression appears to stimulate the repair activity of both rad52B and KIRAD52. Therefore, the suppression of negative dominance by RAD51 does not necessitate a direct physical association between Rad51 and the mutant or heterologous Rad52 protein. The apparently paradoxical dominant negative effects of rad52B [which lacks a Rad51 association domain] may be mediated through interactions with other proteins. RAD51 may suppress these defects by stimulating partially active Rad52 protein.

Figure 5. MMS sensitivity of dominant negative alleles of RAD52 in a rad51Δ strain. DWY120 (rad51Δ) was transformed with vector (pDB20, □), ADH:RAD52 (■), ADH:KIRAD52 (○), ADH:rad52B (●), or ADH:rad52C (△). MMS sensitivity was determined on plates in the presence or absence of 0.00125% MMS as described in Fig. 2 and in Materials and methods.
complexes rather than titrating away the mutant protein.

Effect of RAD52 alleles on mitotic recombination

rad52 mutants are not only defective in DSB repair but also in many types of recombination, including mating-type switching and mitotic and meiotic recombination (Petes et al. 1991). The dominant negative effects described in this study may also be manifested as deficiencies in recombination if we have identified a functionally significant property of RAD52. Thus, we examined the effects of KIRAD52 and rad52B on the rates of spontaneous mitotic recombination using isogenic wild-type (DWY84) and rad52Δ/rad52Δ [DWY98] diploid strains containing two heteroalleles of his4. The mitotic recombination rate is calculated on the basis of the median frequency of His+ colonies. Rates of mitotic recombination in a rad52Δ background were decreased by ~40-fold relative to wild type (Table 2). Expression of rad52B or KIRAD52 in a rad52Δ strain resulted in a fivefold increase in recombination rate and thus a partial complementation of the rad52 defect. This partial complementation was increased to wild-type levels upon coexpression of RAD51. In wild-type strains expressing rad52B or KIRAD52, recombination rates were depressed 2- to 10-fold below vector-only controls, respectively. These dominant negative effects on recombination were entirely suppressed by increased RAD51 expression. Expression of RAD51 alone in a wild-type strain did not change the rates of mitotic recombination significantly. Thus, the dominant effects demonstrated in the previous sections for MMS sensitivity correlate well with rates of recombination. Together, these data indicate that dominant negative alleles of RAD52 induce pleiotropic defects in both DNA repair and recombination. Therefore, RAD52 plays an essential role in both processes, and Rad52–Rad51 association is important to Rad52 function.

Discussion

We have described two types of dominant negative alleles of RAD52 that severely impair DSB repair and recombination in S. cerevisiae. One of these alleles appears to mediate a dominant effect by direct interaction with RAD51 and thus can be suppressed by overexpression of RAD51. However, the direct titration of RAD51 is insufficient to explain all of our results. Although the second type of dominant negative RAD52 allele can also be suppressed by RAD51, this effect is attributable to an activation of the mutant allele rather than competitive titration. Together, these data support a model in which RAD52 acts with RAD51 and other effectors in a greater multiprotein array to effect the repair of DSBs and recombination.

Interactions of RAD52 with other repair proteins

Overexpression of each of the RAD52 dominant negative alleles in a wild-type strain effectively competes with the endogenous Rad52 protein for its functions. Subtle KIRAD52 and ScRAD52 sequence divergence in conserved regions may result in an inability to interact with some but not all S. cerevisiae repair proteins. Similarly, the rad52B and rad52C alleles are defective in specific interactions (or catalytic activity) that, in this case, are caused by deletion of critical residues. Overexpression of these defective alleles results in negative dominance that may be analogous to the phenomena described in other repair and recombination systems involving multiprotein associations (Battista et al. 1990; Prudhomme et al. 1991; Aboussekhra et al. 1992). For instance, E. coli recA truncation mutations and mutants defective in ATP hydrolysis disrupt endogenous RecA protein function by forming inactive RecA filaments (Sedgwick and Yarranton 1982; Yarranton and Sedgwick 1982; Yancey and Porter 1984).

The mutant defects of dominant negative RAD52 al-
Milles and Weaver

alleles appear to be mediated through both RAD51-dependent and RAD51-independent interactions. We have provided several lines of genetic evidence that RAD52 and RAD51 association may be functionally significant. RAD51 suppressed the negative dominance of each of the RAD52 mutant alleles. Additionally, the in vivo association of Rad51 and Rad52 was shown by GAL4 two-hybrid fusion analysis and localized to the carboxy-terminal one-third of Rad52 (Fig. 3). These findings are consistent with the association between purified Rad52 and Rad51 proteins in vitro (Shinohara et al. 1992). Thus, the rad52C allele provides the simplest interpretation of the requirement for Rad52–Rad51 association because it contains the Rad51-binding domain. We propose that the negative dominance exhibited by rad52C is the product of direct titration of endogenous Rad51. This model is supported by the observation that overexpression of rad52C further sensitizes wild-type strains to DSB repair defects while having no effect on a strain deleted for RAD51 (Figs. 4 and 5). Furthermore, the rad52C negative dominance is completely relieved by overexpression of RAD51 (Fig. 4).

In contrast, rad52B is likely to exhibit negative dominance through a RAD51-independent mechanism. Because rad52B is deleted for the Rad51 interaction domain (Fig. 3), the rad52B dominant negative effects must occur by competition with endogenous Rad52 for interaction with other proteins or recombination structures. We still cannot eliminate the possibility that Rad51 can associate with Rad52B through a weak, unidentified binding site. However, the activation of Rad52B function by Rad51 is indicative of a more complicated situation than simple competitive titration of Rad51 (discussed below).

Analysis of KIRAD52 provides further support for the functional interaction of Rad52 with additional proteins. The partial complementation of a rad52Δ strain by KIRAD52 probably results from a reduced ability to interact with selected components of the S. cerevisiae DSB repair pathway. KIRAD52 physically associates with Rad51 as scored by GAL4 two-hybrid analysis (Fig. 3). Although we cannot assess the functionality of KIRad52–Rad51 binding, it seems unlikely that this is the source of the defect of KIRad52. KIRAD52 expression was observed to further sensitize a rad51Δ strain to DSB repair (Fig. 5). Thus, KIRad52 probably competes with endogenous Rad52 for interaction with Rad51 but then fails to assemble all of the other proteins required for DSB repair/recombination. Therefore, the dominant negative effects of KIRAD52 are in part RAD51-independent and must include the nonproductive association with other proteins.

Although KIRad52 and Rad52B differ in their ability to associate with Rad51, they appear to share the RAD52 domain required for a second function. One possibility is that another protein–protein interaction of Rad52 is necessary for repair and recombination. Alternatively, a specific DNA intermediate in the repair/recombination process may represent the other Rad52 interaction function. We favor the hypothesis of another protein–protein interaction for Rad52 as the KIRAD52 allele was cloned from a wild-type strain and thus presumably retains all catalytic activities associated with Rad52 function. Also, differences between the functioning of RAD52 or KIRAD52 in S. cerevisiae based on species-specific DNA intermediates seem unlikely.

Role of RAD51 in modulating the effects of RAD52 alleles

Paradoxically, the dominant negative effects of rad52C, rad52B, and KIRAD52 were all suppressed by the co-overexpression of RAD51 despite the absence of a Rad51 association domain for Rad52B. However, the functioning of these alleles is easier to understand by analysis of the effects of RAD51 co-overexpression in a rad52Δ background. In the case of rad52C, we found no significant changes in the MMS sensitivity of a rad52a strain whether rad52C was coexpressed with RAD51 or not (Fig. 6). These findings are consistent with a model in which rad52C acts solely by associating with Rad51 and competing for Rad52–Rad51 interactions.

In contrast to rad52C, rad52B and KIRAD52 show a dramatic increase in complementation activity as a result of RAD51 co-overexpression in a rad52a strain (Fig. 6). The increased MMS resistance is in parallel to the RAD51 suppression of the dominant negative rad52B and KIRAD52 alleles in wild-type strains (Fig. 4). Therefore, we interpret the suppression of the dominant negative effects of rad52B and KIRAD52 alleles to result primarily from the activation of partially complementing alleles. A similar phenomenon is observed in E. coli mutagenic DNA repair, which involves a multiprotein complex containing RecA*, UmuC, and UmuD'. In this system, suppression of a non-null umuC allele by UmuD' overexpression is thought to occur by mass action (Bates et al. 1991). Similarly, overexpression of RAD51 may recreate a wild-type repair complex despite the deletion of a Rad51 association domain from rad52B. This could result from the strengthening of a very weak Rad52–Rad51 interaction site retained in rad52B or by indirect coassociation with Rad52B via secondary associations with other proteins in a repair complex. However, neither explanation fully accounts for the effects of RAD51 on the KIRAD52 allele, which appears to interact well with Rad51.

Alternatively, Rad52 may function as part of a complex with Rad51 in more than one step in a repair/recombination pathway. Overexpression of RAD51 might stabilize a substrate on which Rad52 acts and thus facilitate the action of partially defective alleles of RAD52 in an allele-nonspecific fashion. This is consistent with the observation that RAD51 overexpression increases the level of complementation of all partially active alleles of RAD52 examined including KIRAD52, rad52B, GAL–52, and GAL–52B which differ in size, ability to interact with Rad51, and fusion with other proteins (Fig. 6; data not shown). However, no combination of RAD52 and RAD51 alleles increased resistance to MMS above the
wild-type level, suggesting that other repair components or structures are limiting.

**Biological role of Rad52–Rad51 interactions**

Rad52 could possibly function by modulating Rad51 activity. Rad51 has striking amino acid and protein structural similarity to *E. coli* RecA protein (Shinohara et al. 1992). Both proteins form filaments on DNA, although *recA* prefers single-stranded DNA and Rad51 only oligomerizes on double-stranded DNA in vitro (West 1992, Ogawa et al. 1993). The well-characterized *recA* strand exchange activity has not been observed for Rad51 or structures are limiting. Moreover, Rad52-Rad52B complexes should retain wild-type activity until rad52B overexpression causes the formation of mostly Rad52B–Rad52B complexes of significantly reduced activity (based on the partial complementation of rad52B in a rad52A). In fact, rad52B fails to exhibit negative dominance unless its expression is driven by the ADH1 promoter (data not shown).

Consistent with the conservation of functionally important domains between *S. cerevisiae* and *K. lactis*, we would predict that the residues of domains I and II are important for RAD52 biological activity. Two point mutations, rad52-1 and rad52-2, map to residues that are invariant between the two species, and these residues lie within the highly conserved domain I (Fig. 1; Adzuma et al. 1984, Boundy-Mills and Livingston 1993). This region probably forms contacts in Rad52 critical for protein–protein or protein–DNA interactions.

**Materials and methods**

*K. lactis* [ATCC 8585] genomic DNA was digested with *XbaI* and fractionated on a 1% TAE gel, and DNA fragments of 3–5 kb were glass bead/Nal purified and ligated into the *XbaI* site of pSKII+. Four thousand *E. coli* DH5α ampicillin-resistant colonies were hybridized (30% formamide, 0.75 M NaCl, 50 mM NaH2PO4, 5 mM EDTA, 1% SDS, 1× Denhardt’s solution, 100 μg/ml of sheared salmon sperm DNA at 42°C) with the amino-terminal RAD52 BamHI fragment (1.97 kb). Three 3.5-kb hybridizing clones shared a common restriction map. Sequencing of KIRAD52 was performed using Sequenase 2.0 (U.S. Biochemical) by standard procedures.

*Yeast strains, media, and genetic methods*

The strains used in this study are displayed in Table 1. An isogenic derivative of DWY83 was constructed by a single-step gene disruption using a RAD52 genomic EcoRI–SalI fragment in which the RAD52-coding sequence (Hpall–XmnI) had been deleted and marked by the insertion of *URA3*-coding sequences at the Murl site. Ura– clones were selected with 5-fluoro-orotic acid (5-FOA) to generate DWY97. DWY98, a rad52Δ strain isogenic with DWY84, was constructed by mating 5-FOA-selected Ura– clones of RKY1525 and RKY1526. DWY120 is a 5-FOA-selected clone of NKY1826. Strains used for low-stringency Southern blot analysis were obtained from ATCC and propagated as recommended.

*S. cerevisiae* strains were propagated at 30°C in YPD, SC media lacking the amino acids required for plasmid selection, or on SC plates containing 1.5% agar. Transformation was performed according to the methods of Geitz et al., with the modification that DMSO was added to 10% immediately before heat shock (Geitz et al. 1992; Hill et al. 1992).

**Plasmids**

A RAD52-coding sequence subclone (pTM10) was created by *Hpall* and *XmnI* digestion of a *SalI* genomic clone, fill-in, and ligation to EcoRV-digested pSKII+. KIRAD52-coding sequence was amplified from a genomic clone by PCR using primers KL-A (5’-GGAAATCCGACTCGCATCAATTCCC-3’) and KL-C (5’-CCATCGATTAGACACACCAATTCTTGTC-3’) and cloned into the EcoRI and *ClaI* sites of pSKII+ (pTM13C). All PCR-amplified sequences were either sequenced or fragment ex-
changed with the genomic clone of KIRAD52. A precise carboxy-terminal truncation at ScRad52 codon 330 was formed by ligating annealed oligonucleotides [5'-GATCTATGAGCTT-3' and 5'-GATCATAGCTTCTACA-3'] into the BamHI site of pTM10 [pTM14]. To delete RAD52 domains I and II [while retaining all six potential sites of translation initiation], pTM10 was digested with BstEII and BamHI, filled in, and religated to generate pTM10C. pSK[RAD52] was constructed by cloning RAD51 (Stul–PstI) into the BamHI (filled in) and PstI sites of pSKII+ [J. Donovan and D. Weaver].

The E. coli–S. cerevisiae shuttle vector pDB20 contains URA3, 2μ origin, and the ADH1 promoter and terminator flanking a unique HindIII site (Becker et al. 1991). pDBL was constructed by replacing URA3 of pDB20 (digested with NdeI, filled in, and digested with SalI) with LEU2 (Smal–SalI) from pGAD10 [obtained from S. Fields, State University of New York, Stony Brook]. The KIRAD52 genomic Xbal fragment was filled in and ligated into the filled-in HindIII site of pDB20 to create pDB[KIR52]. K. lactis promoters sequences, rather than those of ADH1, are likely to be used in expressing KIRAD52 as both orientations of the subcloned KIRAD52 Xbal fragment gave equal MMS complementation of a rad52A-1 strain [data not shown]. pDB[RAD52] was created by ligating the filled-in SalI ScRad52 genomic fragment into the filled-in HindIII site of pDB20. Constructs pTM22 [ADVH–RAD52], pTM25 [ADVH–rad52B], and pTM27 [ADVH–rad52C] were created by subcloning the filled-in Smal–HindIII fragment of pTM10, pTM14, and pTM10C, respectively, into the filled-in HindIII site of pDB20. pTM24 [ADVH–KIRAD52] was constructed by filling in the ends of the EcoRI–ClaI fragment of pTM13C and ligating into the filled-in HindIII site of pDB20. A RAD51 overexpression construct was created by ligating the filled-in Xbal–EcoRV fragment of pSK[RAD51] into the filled-in HindIII site of pDBL to create pDBL[S1] [ADVH–RADS1]. Constructs for the two-hybrid system are derivatives of pMA424 (containing amino acids 1–147 of GAL4, DNA-binding region) and pGAD2F (containing amino acids 768–881, transactivation region) [Chien et al. 1991]. pMA52 [GAL–52] [GAL–52B] [GAL–52B, and pMA52C] [GAL–52C] were constructed by filling in the HpaII–XmnI, HpaII–BamHI, and BamHI–XmnI fragments of RAD52 from pTM10, respectively, and ligating into the filled-in BamHI site of pMA424. pMA[KLS2] [GAL–KL52] was created by ligating the filled-in EcoRI–ClaI fragment of pTM13C into the filled-in BamHI site of pMA424. pGAD51 [TA–S1] contains full-length RAD51 ligated to the BamHI site of pGAD2F [J. Donovan and D. Weaver].

MMS sensitivity measurements
Analysis of MMS sensitivity was performed with three independent transformants of each strain. Representative colonies were picked into sterile water and titrated in six fivefold serial dilutions. Ten-microliter aliquots of each dilution were plated in duplicate on SC plates (selecting for the appropriate plasmids) in the presence and absence of MMS (Aldrich, 0.0025% unless otherwise indicated). Plates were incubated at 30°C, and colonies were counted daily for 6 days. Data are displayed as the ratio of colonies visible in the presence of MMS relative to the non-MMS control platings and averaged among the three transformants.

GAL4 two-hybrid fusion analysis
GAL4 fusion constructs were created as described above and cotransformed into GGY1::171. Transformants were incubated at 30°C for 2–7 days until colonies were relatively large. B-Ga lactosidase production was visualized by replica plating onto SSX plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-Gal, Gibco) or quantitated as described (Chien et al. 1991). Alternatively, to accommodate different strain growth rates, the latter procedure was modified by using suspensions of cells scraped from selective plates instead of log-phase cultures.

Measurement of mitotic recombination rates
Rates of spontaneous mitotic recombination were determined for transformants of DWY83 [RAD52/RAD52] and DWY98 [rad52A/rad52A]. These His+ diploid strains contain two heteroalleles of his4 such that either a crossover between the his4 mutations or a gene conversion of one allele will result in the formation of a His+ colony. Rates of recombination were then determined using fluctuation analysis [Lea and Coulson 1948], with the following modifications. Overnight cultures were sonicated, plated to single colonies, and grown overnight at 30°C on selective plates [SC–Ura, Leu]. Twenty colonies from each strain were used to inoculate 3-ml cultures and grown overnight to OD600 of 0.5–1.0 in SC–Ura, Leu. Seven cultures of the same optical density were then serially diluted and plated in the presence and absence of histidine. Rates of spontaneous mitotic recombination were calculated based on the median frequency of His+ colony appearance according to the method of Lea and Coulson [1948].

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Note added in proof
The KIRAD52 sequence data have been submitted to the GenBank database.

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