A Molecular Genetic Dissection of the Evolutionarily Conserved N Terminus of Yeast Rad52

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ABSTRACT

Rad52 is a DNA-binding protein that stimulates the annealing of complementary single-stranded DNA. Only the N terminus of Rad52 is evolutionarily conserved; it contains the core activity of the protein, including its DNA-binding activity. To identify amino acid residues that are important for Rad52 function(s), we systematically replaced 76 of 165 amino acid residues in the N terminus with alanine. These substitutions were examined for their effects on the repair of γ-ray-induced DNA damage and on both interchromosomal and direct repeat heteroallelic recombination. This analysis identified five regions that are required for efficient γ-ray damage repair or mitotic recombination. Two regions, I and II, also contain the classic mutations, *rad52-2* and *rad52-1*, respectively. Interestingly, four of the five regions contain mutations that impair the ability to repair γ-ray-induced DNA damage yet still allow mitotic recombinants to be produced at rates that are similar to or higher than those obtained with wild-type strains. In addition, a new class of separation-of-function mutation that is only partially deficient in the repair of γ-ray damage, but exhibits decreased mitotic recombination similar to *rad52* null strains, was identified. These results suggest that Rad52 protein acts differently on lesions that occur spontaneously during the cell cycle than on those induced by γ-irradiation.

HOMOLOGOUS recombination is involved in many biologically important processes. In meiosis, it not only generates genetic variation but also ensures proper chromosome pairing and segregation (reviewed in Roeder 1997). In mitotically growing cells, recombination is important to maintain genome integrity (reviewed in Pâques and Haber 1999). For example, it maintains the number of rDNA units in the rDNA cluster (Szostak and Wu 1980; Gangloff *et al.* 1996) and constitutes a telomerase-independent alternative to maintaining telomere length (Le *et al.* 1999). In addition, recombination plays a major role in the repair of DNA *d*ouble *s*trand *b*reaks (DSBs) that may be generated by exposure to radiation or rogue chemicals, the replication of damaged DNA, and the mechanical stress induced during transcription, replication, and chromosome segregation (reviewed in Petes *et al.* 1991).

Homologous recombination and DNA DSB repair have been studied extensively in the budding yeast, *Saccharomyces cerevisiae*. In this organism, DNA DSBs are repaired almost exclusively by pathways that involve homologous recombination and many of the genes involved in this process were identified in screens for γ-ray-sensitive mutants (Game and Cox 1971). Collectively, these genes constitute the *RAD52* epistasis group and include *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *RDH54*, *RFA1*, *MRE11*, and *XRS2* (Pâques and Haber 1999). Among these genes, disruption of *RAD52* causes the most severe recombination phenotype, including elimination of most DNA DSB repair pathways. This is evidenced by its extreme γ-ray sensitivity, defects in mating-type switching, plasmid targeting, and reduced levels of both mitotic and meiotic recombination (Resnick and Martin 1976; Game *et al.* 1980; Malone and Esposito 1980; Orr-Weaver *et al.* 1981). In addition, *rad52* strains are characterized by elevated mutation rates, increased chromosome loss, and the failure to produce viable spores (Petes *et al.* 1991).

The importance of *RAD52* is further underscored by its conservation during evolution as the gene has been identified in species ranging from yeast to humans (Bezzubova *et al.* 1993; Ostermann *et al.* 1993; Bendixen *et al.* 1994; Muris *et al.* 1994; Shen *et al.* 1995; Suto *et al.* 1999). Its biochemical properties have also been conserved as both the yeast and human Rad52 proteins bind DNA and stimulate annealing of complementary DNA molecules (Mortensen *et al.* 1996; Reddy *et al.* 1997; Shinohara *et al.* 1998; Sugiyama *et al.* 1998). Both yeast and human Rad52 can self-associate and form ring structures that bind single-stranded DNA (ssDNA) as well as double-stranded DNA (dsDNA; Milne and Weaver 1993; Shen *et al.* 1996a,b; Shinohara *et al.*

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1998; Van Dyck *et al.* 1998, 1999; Stasiak *et al.* 2000). In addition, Rad52 proteins from both yeast and humans interact directly with Rad51 and replication protein A (RP-A; Shinohara *et al.* 1992; Milne and Weaver 1993; Mortensen *et al.* 1996; Park *et al.* 1996; Shen *et al.* 1996a; Hays *et al.* 1998) and both collaborate with RP-A to facilitate Rad51-catalyzed strand invasion (Baumann and West 1997, 1999; Sung 1997; Benson *et al.* 1998; New *et al.* 1998; Shinohara and Ogawa 1998; Shinohara *et al.* 1998; Song and Sung 2000).

The region of Rad52 that interacts with Rad51 is located in the C-terminal end of the protein (Milne and Weaver 1993; Mortensen *et al.* 1996). Interestingly, a truncation allele that lacks the C-terminal third of Rad52, including the Rad51 interaction domain, can be suppressed by overexpression of Rad51, suggesting that the main function of the C-terminal domain is to establish a physical link to Rad51 (Milne and Weaver 1993; Asleson *et al.* 1999). It is therefore of interest to determine the function of the rest of the protein. A comparison of the available primary structures of Rad52 shows that the region spanning amino acids (aa) 34–198 within the N terminus is highly conserved during evolution, pointing to the existence of important functional residues in this region. This idea is further supported by the existence of a truncated *RAD52* homolog, *RAD59* (Bai and Symington 1996). This gene encodes a protein that consists of a region homologous to the conserved N terminus of Rad52 followed by a short C-terminal extension, which is not homologous to the Rad51-binding region in Rad52. In addition, the classic *rad52* mutants, *rad52-1* (A90V; Resnick 1969; Adzuma *et al.* 1984) and *rad52-2* (P64L; Game and Mortimer 1974; Boundy-Mills and Livingston 1993) as well as several conditional mutations, are located in the N terminus (Kaytor and Livingston 1994; Nguyen and Livingston 1997). *rad52-1* displays a null phenotype in most assays whereas *rad52-2*, despite its severe defects in DSB repair and sporulation, typically exhibits increased levels of mitotic recombination (Malone *et al.* 1988). None of these mutations can be suppressed by overexpression of *RAD51*, indicating that they impair a function other than the Rad51-Rad52 interaction (Kaytor and Livingston 1996). Physically, the presence of a DNA-binding domain was identified in the N terminus; it coordinates Rad52 self-association, and it interacts with the largest subunit of RP-A, Rfa1 (Mortensen *et al.* 1996; Park *et al.* 1996; Hays *et al.* 1998; Ranatunga *et al.* 2001). Finally, the N terminus of Rad52 can stimulate DNA annealing (Kagawa *et al.* 2001).

It is not surprising that *rad52* deletion mutants exhibit a pleiotropic phenotype, given that Rad52 can interact with numerous different proteins, that it binds DNA, and that it participates in mechanistically different recombination/repair pathways. The actual function(s) of the protein, however, is still unknown. Therefore, we have initiated a systematic study of the conserved N-terminal region of Rad52 by performing an alanine scan (Cunningham and Wells 1989) of this region. The main purpose of this study has been to map amino acids responsible for function and to screen for separation-of-function mutations. Accordingly, we analyzed 76 altered proteins for their effects on the repair of γ-ray-induced DNA damage and both mitotic interchromosomal and direct repeat heteroallelic recombination. Five regions were identified that are necessary for DNA DSB repair and mitotic recombination. In addition, several mutations were identified that differentially affect mitotic recombination and DNA DSB repair.

MATERIALS AND METHODS

Genetic methods and strains: All media were prepared as described previously (Sherman 1991) with minor modifications as the synthetic medium contains twice the amount of leucine (60 mg/liter). Standard genetic techniques were used to manipulate yeast strains (Sherman *et al.* 1986). All strains are derivatives of W303 (Thomas and Rothstein 1989a) except that they are *RAD5* (Fan *et al.* 1996; Zou and Rothstein 1997); strains are listed in Table 1. The other genetic markers have been described previously (Erdeniz and Rothstein 2000).

Plasmids and site-directed mutagenesis: Site-directed mutagenesis was performed using a modified version of the "altered site *in vitro* mutagenesis system" from Promega (Madison, WI). Briefly, to produce pWJ1086, an *Xma*I-*Sca*I fragment from pALTER-1 (Promega) containing a nonfunctional β-lactamase gene (due to a 4-bp deletion) was fused to an *Xma*I-*Sca*I fragment from pWJ646 (Erdeniz and Rothstein 2000) that contains pRS414, a *CEN* vector with a *TRP1* selectable marker (Sikorski and Hieter 1989), as well as the entire *RAD52*-containing *Sal*I fragment originally isolated by the Ogawa laboratory (Adzuma *et al.* 1984). Site-directed mutagenesis was performed according to the protocol supplied by Promega. The sequences of the oligonucleotides used to introduce specific mutations in *RAD52* are available on request. To facilitate confirmation of successful site-directed mutagenesis, most alterations were designed to result in an altered restriction site at or near the mutation. In most cases, the mutagenic oligonucleotides were designed to anneal to the coding strand of *RAD52*, and in these cases the Amp^r oligo (Promega) was used simultaneously to repair the 4-bp deletion in *bla*. In a few cases the mutagenic oligonucleotides were designed to anneal to the noncoding strand and here the Rev-Amp^r, which is complementary to Amp^r, was used to repair the deletion in the *bla* gene. All mutations were confirmed by DNA sequencing.

Determination of γ-ray sensitivity: The mutagenized plasmids were transformed into W2014-5C. For each transformed strain, at least two individual transformants were analyzed for their ability to repair γ-ray damage. The strains were grown overnight to midlog phase (1×10^7 cells/ml) and serial 10-fold dilutions were made. A 5-μl dilution was spotted in duplicate on plates containing synthetic medium lacking tryptophan (SC-Trp). Subsequently, one plate was irradiated with a dose corresponding to 20 krad of γ-ray using a Gammacell-220 ^{60}Co irradiator (Atomic Energy of Canada) and the other was left unirradiated. The plates were incubated for 3 days at 30° before cell survival was evaluated. As shown in Table 2, strains that are ~100-fold more γ-ray sensitive than wild type are indicated by a single asterisk (*), while those that are ~1000-fold more γ-ray sensitive are indicated by a double asterisk (**).

TABLE 1

***S. cerevisiae* strains used in this study**

Strain[a]	Genotype
W1588-4C	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W1588-4A	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W2014-5C[b]	*MAT***a** *rad52::HIS5 SUP4-***o***::CAN1-HIS3::sup4*$^+$ *leu2-*Δ*Eco*RI*::URA3::leu2-*Δ*Bst*EII
W2078[b]	*MAT***a** *rad52::HIS5 leu2-*Δ*Eco*RI
	*MAT*α *rad52::HIS5 leu2-*Δ*Bst*EII
W2686[b]	*MAT***a** *rad52::HIS5 leu2-*Δ*Bst*EII
	*MAT*α *rad52::HIS5 leu2-*Δ*Bst*EII
W2685[b]	*MAT***a** *rad52::HIS5 leu2-*Δ*Eco*RI
	*MAT*α *rad52::HIS5 leu2*Δ*Eco*RI
U1599[b]	*MAT*α *his4*Δ *HIS3 LEU2*

[a] All strains are derivatives of W1588 (Zou and Rothstein 1997), a *RAD5* derivative of W303 (Thomas and Rothstein 1989a).

[b] This study.

Quantitative survival curves were obtained as described previously (Smith and Rothstein 1995), except that all strains were pregrown as well as plated on SC-Trp. For all strains, a plot of ln(survival %) *vs.* dose yields a straight line when only data points obtained at 10 krad and higher doses are considered. Thus, for each strain, an LD_{50} was calculated as $-\ln(2)/\ln(\alpha)$, where α is the slope of the straight line.

Determination of mitotic recombination rates: Mitotic recombination between *leu2-*Δ*Eco*RI and *leu2-*Δ*Bst*EII heteroalleles was measured in diploid strains (interchromosomal) or in haploid strains (direct repeat; Figure 1, A and B). To determine interhomolog mitotic recombination rates, W2078 was transformed with each mutated plasmid. Rates and their standard deviation were determined as previously described (Lea and Coulson 1949; Smith and Rothstein 1995) with the following exception: All cultures were grown in liquid SC-Trp medium prior to plating. Furthermore, the plating efficiency and the number of recombinants were determined by plating an appropriate number of cells on SC-Trp and SC-Trp-Leu plates, respectively. For all strains, at least five different transformants were analyzed. Strains showing an ~10-fold reduction in Leu$^+$ prototrophy compared to wild type are indicated in Table 2 by a single asterisk (*), while those showing a >100-fold reduction are indicated by two asterisks (**). For those alleles presented in Table 3 displaying recombination rates similar to wild type at least nine different transformants were analyzed.

To screen for effects on direct repeat recombination, W2014-5C was transformed with each mutated plasmid. Two transformants of each were patched on one-half of an SC-Trp plate. After 3 days, it was replica plated to SC-Trp-Leu medium. The results of the screen are shown in Table 2 where a single asterisk (*) indicates an ~3-fold reduction in Leu$^+$ prototrophs compared to wild type and the double asterisks (**) indicate a >10-fold reduction. To determine accurately direct repeat recombination rates, W2014-5C was analyzed as described above for W2078, except that seven trials were used for each of the 16 mutant alleles analyzed in Table 3.

Analysis of direct repeat recombination events: As shown in Figure 1B, five kinds of events leading to Leu$^+$ can be discriminated by genetic and physical analysis: "Pop-outs," which result from deletion of the intervening *URA3*-containing sequence, score as Leu$^+$ and Ura$^-$. The remaining Leu$^+$ Ura$^+$ events were categorized as either Δ*Eco*RI or Δ*Bst*EII replacements, triplications, or disomes. Recombinants where one of the two *leu2* direct repeats is now wild type are called replacements. We have avoided calling these events "gene conversions" as we cannot examine all potential products resulting from the recombination event (Thomas and Rothstein 1989b). Triplications are Leu$^+$ recombinants that gain an additional copy of one *leu2* repeat unit and the intervening *URA3* sequence. Disomes are Leu$^+$ recombinants that contain a *LEU2* pop-out chromosome and, in addition, also maintain an unaltered assay configuration on a second chromosome. The frequencies of the different events were determined by analyzing 26–30 independent colonies for each allele.

The postrecombinational status of the Leu$^+$ Ura$^+$ recombinants was determined by PCR analysis using two pairs of primers. The first pair of primers, (A) 5′-ACATAACGAGAACACACAGG-3′ and (B) 5′-TCATAAGTGCGGCGACGATAG-3′, specifically amplifies a region of the upstream repeat and the second pair of primers, (C) 5′-ATCGTCCATTCCGACAGCATCG-3′ and (D) 5′-CGTACAAACCAAATGCGG-3′, specifically amplifies a region of the downstream repeat. The resulting PCR products contain *LEU2* sequences that encompass both the *Bst*EII and the *Eco*RI sites in a wild-type sequence, permitting the assessment of their presence or absence in a recombinant after appropriate restriction enzyme digestion. This allows the diagnosis of individual recombinants. For example, after digestion with *Eco*RI, the PCR product of the upstream repeat is digested to completion if the recombinant contains a replacement of the Δ*Eco*RI site by the wild-type *Eco*RI site. Similarly, a Δ*Bst*EII replacement can be diagnosed using the downstream PCR product. In the case of a triplication, each primer pair additionally amplifies an identically sized fragment that originates from the middle repeat. Therefore, each PCR product contains a mixture of sequences derived from the middle repeat and from one of the flanking repeats. For example, when the upstream primer pair is used, the mix will contain a wild-type middle fragment that is fully digested with *Eco*RI and the upstream *leu2-*Δ*Eco*RI fragment that is not. Finally, a disome is indicated when a Leu$^+$ Ura$^+$ recombinant fails to generate digestible PCR products with either enzyme (like the original parental nonrecombinant configuration). Since the *leu2* direct repeat assay resides on chromosome III, a simple genetic cross with a haploid strain of opposite mating type (U1599) was used to confirm this state, as the mating-type locus segregates aberrantly due to trisomy for chromosome III.

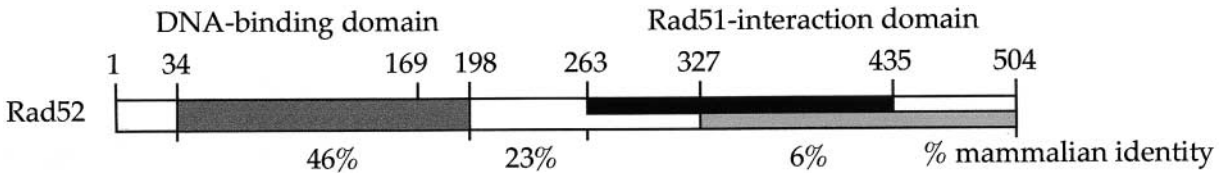
Statistical methods: A *t*-test was used to determine the significance of differences among the mutants *vs.* wild-type and *rad52*Δ strains when comparing mitotic and direct repeat recombination rates. For replacement events, the test of significance was determined using a chi-square analysis.

RESULTS

Experimental strategy: Several lines of evidence suggest that a fundamental activity of Rad52 is located in the conserved N-terminal region of the protein (Milne and Weaver 1993; Mortensen *et al.* 1996; Asleson *et al.* 1999; Ranatunga *et al.* 2001). Using yeast aa numbers, this region of Rad52 stretches from aa 34 to 198 (Table 2). The first 33 aa in *S. cerevisiae* Rad52 are not evolutionarily conserved and an S1 nuclease protection experiment with the mRNA suggests that this region may not even be expressed (Adzuma *et al.* 1984). Indeed, the existence of five putative start codons within

TABLE 2

Alanine scan of the N terminus of Rad52



Mutant allele	Type of aa	γ-Ray sensitive	Recombination		Mutant allele	Type of aa	γ-Ray sensitive	Recombination	
			Mitotic	DR				Mitotic	DR
None	—	—	—	—	K117A	B	—	—	—
Δ	—	**	**	**	F118A	A	—	—	—
N35A	H	—	—	—	S119A	H	—	—	—
K43A	B	—	—	—	C122A		—	—	—
K44A	B	—	—	—	T123A	H	—	—	—
F47A	A	—	—	—	**R127A**	**B**	—	*	—
N49A	H	—	—	—	T129A	H	—	—	—
H50A	B	—	—	—	T131A	H	—	—	—
S51A	H	—	—	—	S132A	H	—	—	—
Q55A	H	—	—	—	T134A	H	—	—	—
T56A	H	—	—	—	Y135A	A	—	—	—
K57A	B	—	—	—	**R136A**	**B**	**	**	**
K60A	B	—	—	—	Y141A	A	—	—	—
K61A	B	—	—	—	T143A	H	—	—	—
Y66A	**A**	*	—	—	N146A	H	—	—	—
S68A	H	—	—	—	R148A	B	—	—	—
K69A	B	—	—	—	R149A	B	—	—	—
R70A	**B**	*	—	—	**K150A**	**B**	—	*	—
F73A	A	—	—	—	F154A	B	—	—	—
T75A	H	—	—	—	**R156A**	**B**	*	—	—
S76A	H	—	—	—	K158A	B	—	—	—
R77A	B	—	—	—	**K159A**	**B**	*	**	*
Y80A	A	—	—	—	S160A	H	—	—	—
W84A	**A**	*	—	—	**T163A**	**H**	*	—	—
R85A	**B**	*	—	—	K167A	B	—	—	—
N88A	H	—	—	—	R168A	B	—	—	—
N91A	**H**	**	**	**	S169A	H	—	—	—
Q92A	H	—	—	—	R171A	B	—	—	—
F94A	**A**	**	**	**	**F173A**	**A**	**	**	**
Y96A	**A**	*	—	—	N175A	H	—	—	—
N97A	H	—	—	—	N179A	H	—	—	—
W99A	A	—	—	—	**C180A**		*	—	—
S100A	H	—	—	—	Y182A	A	—	—	—
T101A	H	—	—	—	K184A	B	—	—	—
K104A	B	—	—	—	**F186A**	**A**	*	—	—
S105A	H	—	—	—	K189A	B	—	—	—
F110A	A	—	—	—	K192A	B	—	—	—
R114A	B	—	—	—	K194A	B	—	—	—
Q115A	H	—	—	—	F195A	A	—	—	—

An outline of the functional domains of Rad52 is shown at the top. The dark-shaded region spanning aa 34–198 corresponds to the evolutionarily conserved region in Rad52 that has been subjected to an alanine scan in this study. The percentage identity between yeast and mouse Rad52 is indicated below the bar. The region from aa 34 to 169 contains a DNA-binding domain (Mortensen *et al.* 1996). The Rad51-binding domain described in Mortensen *et al.* (1996) is shown as solid and the one described by Milne and Weaver (1993) is lightly shaded. The type of amino acid that was replaced is abbreviated as "A" for aromatic (F, Y, and W), "B" for basic (H, K, and R), and "H" for polar, neutral, nonaromatic (N, Q, S, and T). The results of preliminary screens using the assays described in materials and methods are shown. Alleles set in boldface type and underlined differ from wild type for at least one of the assays. DR, direct repeat recombination. —, no change from wild type. The meaning of * and ** for each assay is described in materials and methods.

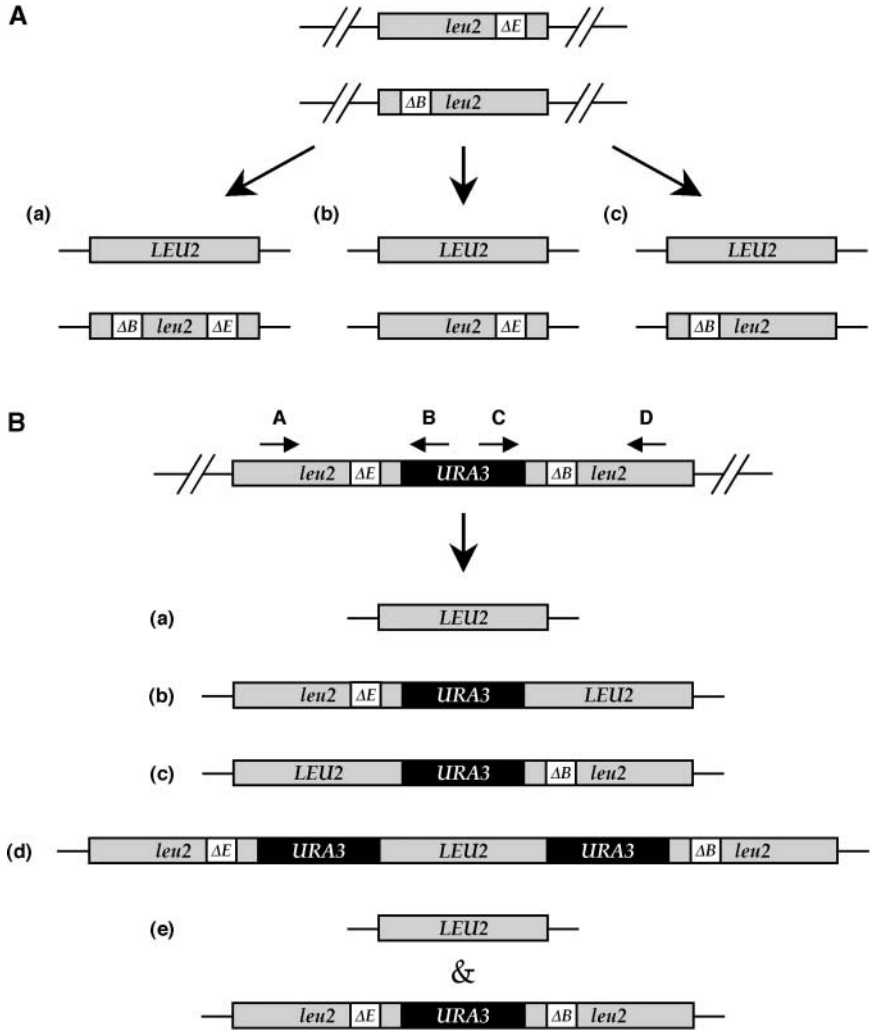


FIGURE 1.—Possible *LEU2* recombination in *leu2* heteroallelic diploids and between directly repeated *leu2* heteroalleles flanking a *URA3*-containing plasmid insert. (A) A heteroallelic diploid is represented by two mutant *leu2* genes with Δ*E* and Δ*B* denoting the Δ*Eco*RI and Δ*Bst*EII mutated site, respectively. Three possible Leu$^+$ prototroph outcomes are illustrated: (a) reciprocal exchange, (b) conversion of the Δ*Bst*EII allele, and (c) conversion of the Δ*Eco*RI allele. (B) A *leu2* direct repeat heteroallelic recombination assay is depicted. The alleles are as in A. Five possible Leu$^+$ prototroph outcomes are shown. The first results in Leu$^+$, Ura$^-$ colonies while the latter four give rise to Leu$^+$, Ura$^+$ colonies. The five are: (a) "pop-out" recombination, (b) Δ*Bst*EII replacement, (c) Δ*Eco*RI replacement, (d) triplication, and (e) disome with pop-out on one chromosome and the parental construct on the other. Small arrows above the assay indicate the annealing positions of the four primers, A, B, C, and D, which are used to diagnose the physical status of the *LEU2* locus in Leu+ prototrophs (see MATERIALS AND METHODS).

the first 40 aa prompted us to insert a stop codon mutation between the second (aa 14) and third (aa 34) start codon. This mutation did not produce an altered phenotype nor did it change the size and cellular concentration of the Rad52 protein (A. ANTÚNEZ DE MAYOLO, N. ERDENIZ, U. H. MORTENSEN and R. ROTHSTEIN, unpublished results). To identify functionally important amino acids within the conserved N terminus, an alanine scan was performed. All basic (H, K, and R), aromatic (F, Y, and W), polar, neutral, nonaromatic (N, Q, S, and T), and cysteine (C) residues were systematically substituted, one by one, for alanine. In total, 76 amino acid residues corresponding to 46% of the region were altered. To determine the consequence of each alteration, single-copy plasmids carrying individual alanine substitutions were introduced into appropriate *rad52*Δ strains and tested for complementation of different aspects of the *rad52*Δ phenotype. Accordingly, the effects of each substitution on γ-ray damage repair and on mitotic heteroallelic and direct repeat recombination were investigated.

Identification of γ-ray-sensitive *rad52* mutants: To identify amino acids in Rad52 that are important for repair of DNA DSBs, a spot assay was employed to screen for γ-ray-sensitive mutants. For each strain, two dilution series ranging from ~5 to 5×10^4 cells were spotted on solid medium and exposed to 0 and 20 krad, respectively (Figure 2). These conditions result in maximal sensitivity since 20 krad produces sufficient damage to kill all *rad52*Δ cells, but not enough to significantly affect survival of wild-type cells. To avoid identifying amino acid substitutions that result in a weak phenotype, only those changes that consistently reduced viability at least 100-fold after 20 krad were analyzed in more detail (for an example, see Figure 2). Among the 76 alterations tested, 14 γ-ray-sensitive mutants were identified (Table 2).

Next, the γ-ray sensitivity of these 14 alleles was quantitated by producing γ-ray survival curves (Figure 3 and Table 3). In each case, the curve exhibited a logarithmic decline with increasing doses from 10 krad and the corresponding LD_{50} for each strain was calculated (see MATERIALS AND METHODS). The LD_{50} allows a direct comparison of survival curves between strains. As expected, a *RAD52*-containing plasmid fully complements the *rad52*-null strain in the entire dose range investigated, while the 14 mutants exhibited differential effects

TABLE 3

Effects of *rad52* mutations on γ-ray damage repair and mitotic *leu2* heteroallelic and direct repeat recombination

Allele	Region[a]	Class[a]	γ-Ray sensitivity (LD_{50})[b]		Mitotic recombination		Direct repeat recombination			
			Haploid	Diploid	Rate[d] × 10^{-4}	Fold reduction[e]	Rate[d] × 10^{-4}	Fold reduction[e]	% deletion events (*N*)[f]	Replacements *Eco*RI:*Bst*EII[g]
RAD52			47 ± 9.9	38 ± 7.9	190 ± 30**	1	66 ± 15**	1	30 (30)**	19:11**
pRS414			1.6 ± 0.06	1.3 ± 0.21	1.6 ± 0.67*	160	2.4 ± 0.3*	27.5	73 (30)*	8:14*
Y66A	I	C	6.2 ± 0.21	5.6 ± 0.29	290 ± 52**	0.65	40 ± 7**	1.7	37 (30)**	16:12**
R70A	I	C	4.8 ± 0.23	ND[c]	680 ± 180*,**	0.28	84 ± 20**	0.8	40 (30)**	14:13**
W84A	II	C	4.3 ± 0.16	3.6 ± 0.20	290 ± 57**	0.66	77 ± 15**	0.9	29 (28)**	16:13**
R85A	II	C	5.2 ± 0.20	3.9 ± 0.14	210 ± 64**	0.90	41 ± 8**	1.6	40 (30)**	18:11**
N91A	II	A	1.7 ± 0.18	ND	1.3 ± 0.76*	150	3 ± 1*	22.0	73 (30)*	7:17*
F94A	II	A	1.6 ± 0.17	ND	1.0 ± 0.63*	190	3 ± 1*	19.4	67 (27)*	9:15*
Y96A	II	C	4.0 ± 0.15	ND	120 ± 32**	1.6	26 ± 5*,**	2.5	21 (29)**	15:14**
R127A	III	D	20 ± 1.6	12 ± 1.7	3.4 ± 1.7*	56	20 ± 4*,**	3.3	47 (30)**	15:13**
R136A	III	A	1.7 ± 0.11	ND	2.5 ± 1.4*	76	2.1 ± 0.3*	31.4	77 (26)*	7:16*
K150A	III	D	12 ± 1.8	7.1 ± 0.41	8.5 ± 3.2*	42	34 ± 7**	1.9	50 (30)	12:17*
R156A	IV	C	5.8 ± 0.29	ND	370 ± 80*,**	0.53	55 ± 9**	1.2	27 (30)**	18:12**
K159A	IV	B	3.7 ± 0.15	3.4 ± 1.5	0.9 ± 0.45*	210	9 ± 2*,**	7.2	27 (30)**	15:15**
T163A	IV	C	4.2 ± 0.18	3.5 ± 0.21	150 ± 32**	1.3	55 ± 13**	1.2	30 (30)**	19:10**
F173A	V	A	1.6 ± 0.10	ND	1.6 ± 1.4*	120	6 ± 2*	15.3	70 (30)*	15:14**
C180A	V	C	4.8 ± 0.29	4.0 ± 0.37	120 ± 28**	1.6	31 ± 8**	1.9	40 (30)**	18:11**
F186A	V	C	4.2 ± 0.085	ND	170 ± 35**	1.1	59 ± 13**	1.1	30 (30)**	13:15*

*$P < 0.05$ compared to wild type (*RAD52*). **$P < 0.05$ compared to *rad52*Δ (pRS414).
[a] Refers to the regions and classes as defined in RESULTS and DISCUSSION.
[b] LD_{50} in kiloradian as described in MATERIALS AND METHODS.
[c] Not determined.
[d] Recombination rate (events per cell per generation) is presented as the mean ± SD as described in MATERIALS AND METHODS.
[e] Relative to wild type.
[f] Percentage of deletion events among *LEU2* recombinants (number tested shown in parentheses).
[g] The ratio of *leu2*-Δ*Eco*RI *vs. leu2*-Δ*Bst*EII replacements among Leu+ Ura+ recombinants (see Figure 1B,b and c).

ranging from an intermediate ~8-fold increase to a severe, ~30-fold increased, null-like sensitivity. In addition, it is likely that we identified most of the significant mutants in our collection in the initial screen since the spot assay overestimated rather than underestimated their actual γ-ray sensitivity.

On the basis of the mutations identified in this study as well as previously identified *rad52* mutations, *RAD52* homolog sequence comparisons, and secondary structure analysis, we subdivided the N terminus of Rad52 into five regions (see DISCUSSION). Most of the mutations identified in this study that cause γ-ray sensitivity map into the following four small regions: I (aa 61–70), II (aa 84–97), IV (aa 156–163), and V (aa 173–186). The large region, III (aa 127–150), contains only one γ-ray-sensitive mutant, *rad52-R136A* (Table 2). Analysis of the five regions reveals 4 mutations that completely fail to complement *rad52*Δ: 2 mutations (*rad52-N91A* and *rad52-F94A*) are located in region II, 1 mutation (*rad52-R136A*) in region III, and 1 mutation (*rad52-F173A*) in region V. The remaining 10 mutations cause intermediate sensitivities ranging from the weakest located in region I (*rad52-Y66A*; LD_{50} is 6.2 krad) to the strongest in region IV (*rad52-K159A*; LD_{50} is 3.7 krad).

Analysis of mutants for effects on heteroallelic mitotic recombination: Next, the complete *rad52* mutant collection was screened for those that affect interchromosomal heteroallelic recombination (Figure 1A). This was investigated by introducing plasmids carrying individual *rad52* alleles into a homozygous *rad52*-null diploid strain that contains two different nonfunctional alleles, *leu2*-Δ*Bst*EII and *leu2*-Δ*Eco*RI (SMITH and ROTH-

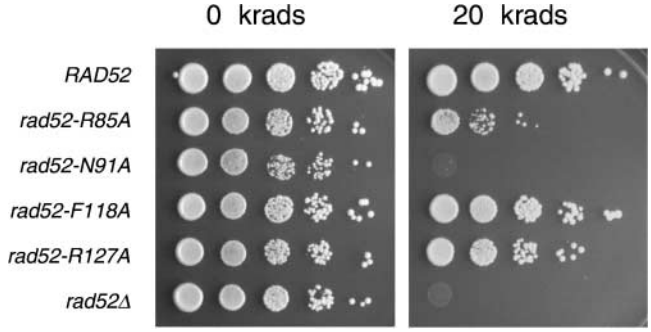


FIGURE 2.—Identification of γ-ray-sensitive mutants. A γ-ray spot assay was performed to evaluate the ability of mutant Rad52 expressed from a single-copy plasmid to complement the γ-ray sensitivity of a *rad52*Δ haploid strain. Serial 10-fold dilution series of each transformed strain were spotted on solid media and irradiated with the indicated dose.

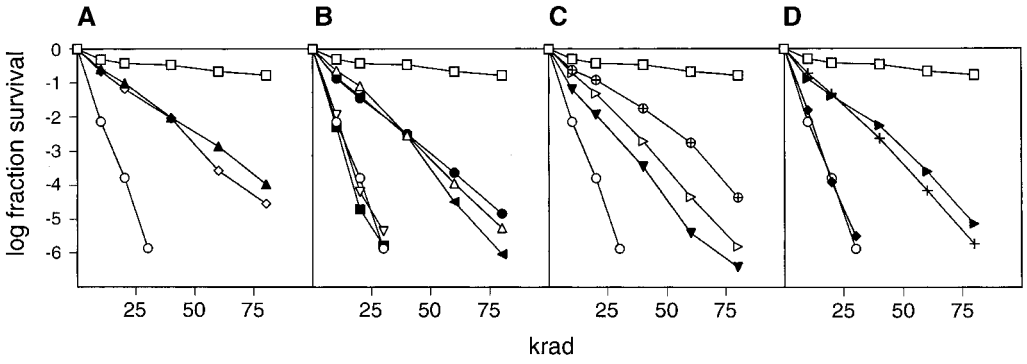


FIGURE 3.—The ability of mutant Rad52 expressed from a single-copy plasmid to complement the γ-ray sensitivity of a *rad52*Δ haploid strain. Mutants that map in regions I, II, IV, and V are represented in A, B, C, and D, respectively. (A–D) □, *RAD52*; ○, pRS414. (A) ▲, *rad52-Y66A*; ◇, *rad52-R70A*. (B) △, *rad52-W84A*; ●, *rad52-R85A*; ■, *rad52-N91A*; ▽, *rad52-F94A*; ◀, *rad52-Y96A*. (C) ⊕, *rad52-R156A*; ▼, *rad52-K159A*; ▷, *rad52-T163A*. (D) ◆, *rad52-F173A*; ▶, *rad52-C180A*; +, *rad52-F186A*.

STEIN 1995). In the absence of Rad52, the rate of prototroph formation is 160-fold lower than that of wild type. Since *rad52* is a known mutator (VON BORSTEL *et al.* 1971), we measured the reversion rates in the two homoallelic *leu2* diploids. In both cases, the reversion rates in *rad52*-null diploids are $<2 \times 10^{-9}$. This rate is more than six-fold lower compared to that found for heteroalleles in the *rad52*-null background. Thus, prototrophs are most likely true heteroallelic recombinants and not *LEU2* revertants.

Each Rad52 alteration in the collection was subjected to five independent trials. For each trial, $\sim 10^7$ cells were analyzed for the presence of prototrophic recombinants. Most alterations produced recombinants at rates that deviate <3-fold from that obtained with the wild type. However, seven mutants were identified that rarely formed any prototrophs under these conditions and therefore 10-fold more cells were plated for each strain to measure the rate of prototroph formation (Table 3). These seven mutants exhibited recombination rates similar to those measured for a *rad52*-null. Not surprisingly, this group includes all four mutations that fail to complement γ-ray sensitivity in a *rad52*Δ background. The remaining three mutations include two mutations in region III, *rad52-R127A* and *rad52-K150A*, that were not identified in the initial screen for γ-ray sensitivity and one in region IV, *rad52-K159A*, which causes only partial γ-ray sensitivity.

It was surprising that only 1 mutation, *rad52-K159A*, out of the 10 described above that cause an intermediate sensitivity to γ-irradiation was accompanied by reduced recombination. We increased the number of trials to accurately measure the recombination rates for the other 9 mutant strains and confirmed the results of the initial screen (Table 3). None of them display recombination rates that are >2-fold lower than the rate obtained with the wild-type strains. Thus, mutations located in the N terminus of Rad52 that cause intermediate sensitivity to γ-ray only rarely decrease the mitotic recombination rate. In fact, two of these mutations, *rad52-R70A* and *rad52-R156A*, cause a hyperrecombination phenotype with the highest rate observed for the *rad52-R70A* mutant, which forms prototrophs with a rate 3.6-fold higher than that of wild type.

The two mutations in region III, *rad52-R127A* and *rad52-K150A*, which affect mitotic recombination but not the repair of γ-ray-induced DNA damage, were also analyzed in more detail by quantitating γ-ray survival. Examination of γ-ray survival curves at 20 krad shows that survival is reduced by only 2- and 6-fold, respectively (Figure 4). These results explain why both mutations were not identified in the initial screen, since only those with 100-fold reduction in survival at 20 krad were chosen. Furthermore, the mild γ-ray sensitivities of *rad52-R127A* and *rad52-K150A* are also reflected in modest decreases in LD_{50}: 2.4- and 3.9-fold, respectively (Table 3).

Thus far, the repair of γ-ray damage was measured in haploid strains while mitotic recombination was analyzed in diploid strains. Therefore, in those cases where separation of function was detected, it could have been related to a difference in ploidy between the two experiments. Thus, a set of relevant mutations was analyzed for γ-ray damage repair in a *rad52*Δ homozygous diploid strain (Figure 4 and Table 3). All alleles investigated, including the wild type, appear slightly more sensitive in the diploid than in the haploid strain, but, overall, the differences between haploids and diploids were not dramatic.

Effect of *rad52* mutants on *leu2* direct repeat recombination: In the heteroallelic recombination assay described in the previous section, recombinants arise from an exchange of information between homologous chromosomes. For direct repeat recombination, information can be exchanged between sequences that are situated close together on the same chromosome (Figure 1B). This allows recombinants to be generated by different recombination pathways, for example, single-strand annealing, replication slippage, or gene conversion. Since direct repeat recombination may require different Rad52 functions compared to homologous recombina-

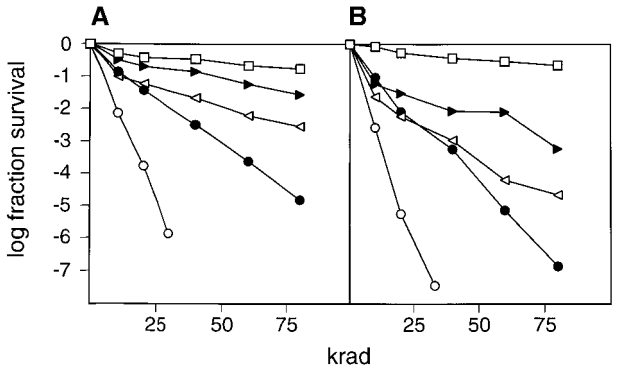


FIGURE 4.—The ability of mutant Rad52 expressed from a single-copy plasmid to complement the γ-ray sensitivity of a haploid *rad52*Δ (A) and diploid homozygous *rad52*Δ strains (B). (A and B) □, *RAD52*; ○, pRS414; ▶, *rad52-R127A*; ◁, *rad52-K150A*; ●, *rad52-R85A*.

tion between chromosomes (KLEIN 1995; SMITH and ROTHSTEIN 1999), we examined a direct repeat assay consisting of a *URA3* marker flanked by *leu2*-Δ*Eco*RI *and leu2*-Δ*Bst*EII. In this assay, the rate of Leu$^+$ prototroph formation is 20- to 30-fold lower in the absence of Rad52 (SMITH and ROTHSTEIN 1999 and results presented below).

Initially, all 76 substitutions were screened for their effect on Leu$^+$ prototroph formation. Approximately 5–8 × 10^6 cells were patched on solid medium lacking leucine. In wild-type strains, 400–700 papillae are typically observed whereas *rad52*-null strains form 10–30 papillae. Among the 60 substitutions that affect neither γ-ray-induced damage repair nor the rate of interchromosomal heteroallelic recombination, no new mutations were uncovered in this screen. Therefore we focused on the remaining 16 mutant alleles presented in Table 3.

First, the rates of Leu$^+$ prototroph formation in the direct repeat assay were determined. With the mutants that exhibit intermediate sensitivity to γ-irradiation yet are proficient for heteroallelic recombination (*rad52-Y66A*, *-R70A*, *-W84A*, *-R85A*, *-Y96A*, *-R156A*, *-T163A*, *-C180A*, and *-F186A*), Leu$^+$ prototrophs are formed at the *RAD52* rate. The only exception in this group is *rad52-Y96A*, which results in an intermediate phenotype that is significantly different from both wild type and *rad52*Δ. An intermediate phenotype is also observed for the *rad52-K159A* mutant, which exhibits intermediate γ-ray sensitivity, but a *rad52*-null-like rate of interchromosomal heteroallelic recombination. Among the two mutants that are deficient in heteroallelic recombination, but are rather tolerant to γ-rays, *rad52-R127A* results in an intermediate phenotype for direct repeat recombination while *rad52-K150A* displays a wild-type rate. The *rad52*-null-like mutants (*rad52-N91A*, *-F94A*, *-R136A*, and *-F173A*) are not significantly different from *rad52*Δ.

Leu$^+$ prototrophs can be generated by four major types of events (Figure 1B). One, pop-out recombination, results in loss of the intervening *URA3*-containing sequences as well as one *leu2* repeat and is detected as Leu$^+$ Ura$^-$ colonies. In wild-type cells, ~30% of the Leu$^+$ recombinants fall into this group while they comprise >70% of the events in *rad52*Δ strains. The remaining three events are replacements, triplications, and disomes, which all result in a Leu$^+$ Ura$^+$ phenotype. As described in MATERIALS AND METHODS, all three events can be distinguished. Both triplications and disomes rarely occur in *RAD52* and *rad52*Δ strains and this is also the case for all of the mutants analyzed (data not shown). Accordingly, replacements constitute ~70 and 30% of the events in wild-type and *rad52*Δ strains, respectively. In wild-type strains, twice as many replacements involve the Δ*Eco*RI allele compared to Δ*Bst*EII. The opposite is found in *rad52*Δ, where most replacements involve Δ*Bst*EII.

We examined recombinants generated by all of the mutant alleles for their percentage of *URA3* deletions and for their type of replacement. Most *rad52*Δ-like mutants (*rad52-N91A*, *-F94A*, *-R136A*, and *-F173A*) are also null-like for deletion percentage and replacement distribution, except *rad52-F173A*, which exhibits a wild-type-like replacement distribution. The very γ-ray-sensitive *rad52-K159A* strains are *rad52*Δ-like for interchromosomal recombination, but they produce a wild-type percentage of intrachromosomal deletion events. Furthermore, the distribution of replacement events in *rad52-K159A* and *rad52-F173A* strains is significantly different from that obtained with *rad52*Δ strains. Interestingly, *rad52-R127A* and *rad52-K150A*, which exhibit *rad52*Δ levels of interchromosomal recombination, display a more wild-type-like percentage of deletion events. However, for *rad52-K150A*, the distribution of replacements is comparable to that obtained in the absence of Rad52. Finally, most mutations that exhibit intermediate sensitivity for γ-rays and are interchromosomal recombination proficient (*rad52-Y66A*, *-R70A*, *-W84A*, *-R85A*, *-Y96A*, *-R156A*, *-T163A*, *-C180A*, and *-F186A*) are similar to wild type. The one exception is *rad52-F186A*, which is more *rad52*Δ-like with respect to the distribution of replacement events.

DISCUSSION

The complex biology of *rad52* mutants suggests that the Rad52 protein is multifunctional. This is supported by the emerging picture of its biochemical properties. Rad52 binds both ssDNA and dsDNA and stimulates DNA annealing (MORTENSEN *et al.* 1996; REDDY *et al.* 1997; SHINOHARA *et al.* 1998; SUGIYAMA *et al.* 1998). It also collaborates with RP-A to enhance Rad51-catalyzed strand invasion (BAUMANN and WEST 1997, 1999; SUNG 1997; BENSON *et al.* 1998; NEW *et al.* 1998; SHINOHARA and OGAWA 1998; SHINOHARA *et al.* 1998; SONG and SUNG 2000). In addition, Rad52 has been shown to self-associate as well as form a heptameric ring structure

with a preference for ssDNA ends (Milne and Weaver 1993; Shen *et al.* 1996a,b; Shinohara *et al.* 1998; Van Dyck *et al.* 1998, 1999; Stasiak *et al.* 2000). Furthermore, Rad52 interacts with Rad51 (Shinohara *et al.* 1992; Milne and Weaver 1993; Mortensen *et al.* 1996) and RP-A (Park *et al.* 1996; Hays *et al.* 1998). Both two-hybrid and genetic analyses suggest that the role of the C-terminal region is to recruit Rad51 protein to the site of DNA repair. Interestingly, the Rad51-binding domain in yeast Rad52 is highly diverged from the corresponding sequences found in higher organisms even though the physical interaction between Rad51 and Rad52 is evolutionarily conserved (Shen *et al.* 1996a). In fact, only the N terminus is highly conserved among all Rad52 homologs. A genetic analysis has shown that this region contains the core activity of Rad52 (Asleson *et al.* 1999), a view that is supported by the existence of a truncated Rad52 homolog, Rad59, that completely lacks the C-terminal domain of Rad52 (Bai and Symington 1996). Importantly, biochemical analyses of truncated Rad52 species that consist of the conserved N-terminal region show that it can bind DNA, stimulate DNA annealing, and form higher-order ring structures. (Mortensen *et al.* 1996; Kagawa *et al.* 2001; Ranatunga *et al.* 2001). Together these observations inspired a more detailed exploration of this region.

Mutational analysis of the N terminus of Rad52: We performed an extensive alanine scan from aa 34 to aa 198 of a broad variety of amino acid residues that are typically involved in protein-protein and protein-DNA interactions. There are several advantages to mutating a given amino acid residue into alanine (Cunningham and Wells 1989). First, it does not cause extreme electrostatic or steric effects on the protein. Second, it rarely changes the secondary structure of the protein and alanine residues are found in both buried and exposed positions. Finally, it reduces the side chain of the mutated residue to a rather inert methyl group. Thus, the effect of the mutation is expected to be exerted locally and often directly reflects the function of the "missing" side chain. In contrast, random mutations frequently result in dramatic changes that are likely to cause global alterations of the protein. Accordingly, 46% of the amino acid residues present in the N terminus were systematically replaced by alanine.

Phenotypic classification of the mutants: Analysis of the entire mutant collection revealed individual mutants that exhibit different phenotypes, which we have divided into four classes, each representing a unique phenotype with respect to γ-ray-induced DNA repair and spontaneous inter- and intrachromosomal heteroallelic recombination.

Class A: The first class of mutants (class A) is characterized by a phenotype similar to that obtained in the absence of Rad52. Therefore these mutations either destroy an essential Rad52 function or destabilize the resulting protein. Phenotypically, they fail to repair γ-ray-induced damage and exhibit severely reduced mitotic heteroallelic interchromosomal and direct repeat recombination rates. The missense mutation *rad52-1* (A90V) that originally defined *RAD52* (Resnick 1969) falls into this class. In this study we have identified four new mutations, *rad52-N91A*, *-F94A*, *-R136A*, and *-F173A*, which result in a class A phenotype. In the direct repeat recombination analysis, class A mutants, like *rad52*Δ strains, preferentially produce pop-out recombinants.

Class B: Class B is defined by *rad52-K159A*, a mutant that, like class A, does not perform interchromosomal heteroallelic recombination, but is slightly less γ-ray sensitive compared to class A mutant strains. Furthermore, there are two important differences between class A and class B mutants. First, the rate of direct repeat recombination for *rad52-K159A* is significantly higher than that obtained with class A mutants. Second, the direct repeat recombinants obtained in *rad52-K159A* are mostly replacements.

Class C: Class C mutants show an intermediate sensitivity to γ-irradiation but exhibit wild-type or higher heteroallelic recombination rates. The first class C mutant described was *rad52-2* (P64L), which is sensitive to DNA-damaging agents, but capable of supporting spontaneous intragenic heteroallelic recombination and inverted repeat recombination (Malone *et al.* 1988; Boundy-Mills and Livingston 1993; Kaytor and Livingston 1994). In this study, we have identified several new class C mutants (*rad52-Y66A*, *-R70A*, *-W84A*, *-R85A*, *-Y96A*, *-R156A*, *-T163A*, *-C180A*, and *-F186A*). Indeed, all mutants that exhibit intermediate sensitivities to γ-irradiation, with the exception of *rad52-K159A*, displayed this phenotype.

Class D: The last class of mutants, class D, is only mildly γ-ray sensitive but exhibits very low interchromosomal recombination rates. Two mutants, *rad52-R127A* and *rad52-K150A*, fall into this class. In contrast to their effect on interchromosomal recombination, these mutants exhibit direct repeat recombination rates that are slightly reduced or close to those obtained with wild-type strains. Among the direct repeat recombinants, the percentage of deletion events falls between that obtained with *rad52*Δ and wild-type strains. Similarly, an intermediate phenotype is also observed when the ratio of Δ*Eco*RI *vs.* Δ*Bst*EII replacements is considered. In the case of *rad52-R127A*, this ratio differs from that obtained in *rad52*Δ strains, whereas in the case of *rad52-K150A*, the ratio is different from wild-type strains.

The distribution of N-terminal *rad52* mutations defines five regions: Thirteen *rad52* mutations that strongly reduce the ability to survive γ-ray exposure are located in two large clusters spanning residues 66–97 and 156–186 (Table 3 and Figure 5A). In addition, one class A mutation (*rad52-R136A*) and two class D mutations (*rad52-R127A* and *rad52-K150A*) are found in the region that separates these two clusters. When Rad52 homologs from different species are compared, it appears that

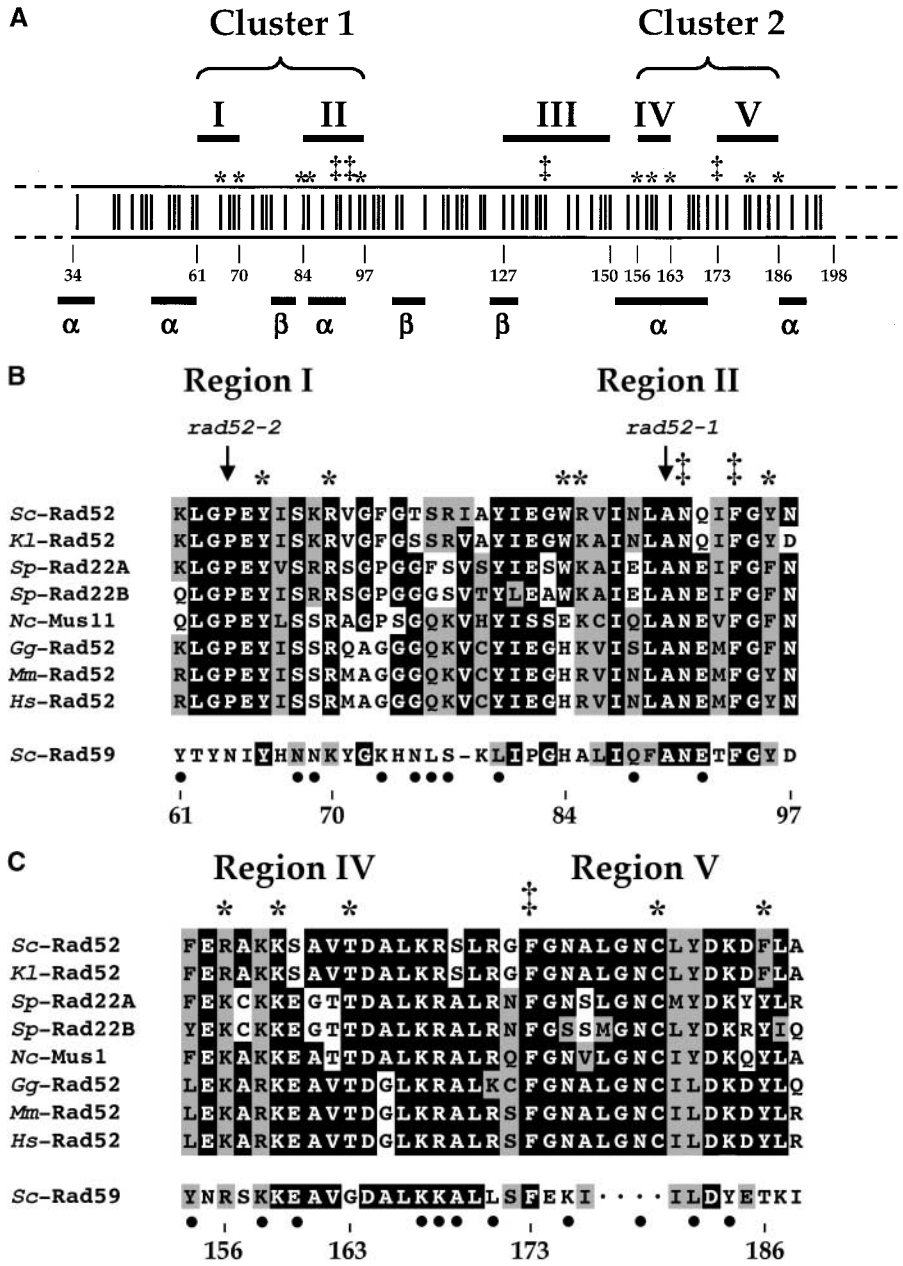


FIGURE 5.—Two aa clusters in the N terminus of Rad52 that are important for repair of γ-ray-induced DNA damage. (A) A graphic representation of aa 34–198 of Rad52. The positions of all aa substitutions performed in this study are indicated by small vertical lines. γ-ray-sensitive mutations (Table 2) defined two aa clusters: (1) aa 61–97 and (2) aa 156–186. Single asterisks and double-daggers above aa alterations mark the location of mutations that cause an intermediate and *rad52*Δ-like γ-ray-sensitive phenotype, respectively. Further analysis of the mutant collection defined five regions (I–V), which are important for γ-ray damage repair and homologous interchromosomal mitotic recombination (see below and text). Secondary structure predictions from the Predator algorithm (FRISHMAN and ARGOS 1996) are shown as thick bars below. Predicted α-helices and β-sheets are shown. The remaining regions are predicted to form random coils. (B) The alignment of cluster 1 to the corresponding known Rad52 primary structures from different species (*Sc*, budding yeast; *Kl*, *Kluveromyces lactis*; *Sp*, fission yeast; *Nc*, *Neurospora crassa*; *Gg*, chicken; *Mm*, mouse; and *Hs*, human). Identical and functionally conserved amino acids are boxed in solid and shaded backgrounds, respectively. Single asterisks and double daggers are as described in A. Dots below the alignment indicate amino acid residues that do not result in significant γ-ray sensitivity when changed to alanine. This first cluster is divided into two regions, I and II, on the basis of the alignment to Sc Rad59 (see text). (C) Cluster 2 is divided into two regions, IV and V, on the basis of the alignment to Rad59. All symbols and alignment are as described in B. The original alignment of Rad59 and Rad52 suggested that region V does not exist in Rad59 (BAI and SYMINGTON 1996). However, considering that this region might be functionally important, we reexamined the alignment. It is clear that the very conserved FGNALGNC sequence (aa 173–180) is not present in Rad59. However, we note that the downstream sequences in region V may exist in Rad59 [ILD(Y)E in Rad59] as other eukaryotes contain a similar sequence and we have changed the alignment accordingly.

both clusters are highly conserved (Figure 5, B and C). In fact, all mutations, with the exception of *rad52-W84A*, affect residues that are identical or are structurally or functionally conserved. Upon closer inspection (Figure 5, B and C), we found that each cluster could be subdivided on the basis of its sequence alignment with Rad59. The first cluster was split into region I, which is not conserved in Rad59, and region II, which is. Similarly, the second cluster was subdivided into regions IV and V, where region V is absent in Rad59. The span between regions II and IV is less conserved among the Rad52 homologs and is designated region III. Interestingly, secondary structure predictions by the Predator algorithm (FRISHMAN and ARGOS 1996) suggest that regions II and IV are likely to form α-helices whereas regions I and V are more likely to form random coils.

Features of region I (aa 61–70): In our study, two class C mutations were identified that map to region I, *rad52-Y66A* and *rad52-R70A*. Moreover, four previously described mutations also fall in this region: *rad52-2*, *rad52-K61N*, *rad52-K69A*, and *rad52-R70K* (NGUYEN and LIVINGSTON 1997; BAI *et al.* 1999). The positions of these mutations prompted us to define the borders of region I to span aa 61–70. Of the four previously described alleles, the best defined is *rad52-2* (GAME and MORTIMER 1974), a class C mutation that results in a

proline-to-leucine substitution at residue 64 (Boundy-Mills and Livingston 1993). All three class C mutants in this region are somewhat hyper-rec (Table 3; Malone *et al.* 1988; Boundy-Mills and Livingston 1993). Furthermore, another amino acid residue substitution that maps to position 70, *rad52-R70K*, was identified in a screen for mutations that cause a decrease in inverted repeat recombination in a *rad51* background (Bai *et al.* 1999). This conservative substitution causes much less γ-ray sensitivity compared to *rad52-R70A*, but heteroallelic recombination is increased as in the three class C mutants in this region. The fact that *rad52-R70K* shows synergistic defects in γ-ray damage repair, mating-type switching, and sporulation in the absence of Rad59 protein suggests that a partially defective region I mutation can be suppressed by the presence of Rad59 activity. Finally, two additional mutations, *rad52-K61N* and *rad52-K69D*, cause cold-sensitive phenotypes (Nguyen and Livingston 1997). The K-to-N change at position 61 is predicted to transform the unstructured coil around position K69 to a more rigid β-sheet (Frishman and Argos 1996), suggesting that the functionality of region I requires physical flexibility.

Features of region II (aa 84–97): Region II contains the mutation *rad52-1*, which originally defined *RAD52* (Resnick 1969). This mutation changes an alanine residue to valine at amino acid position 90 (Adzuma *et al.* 1984) and maps right in the middle of the region. The phenotype of *rad52-1* is very severe and in most assays is indistinguishable from that observed in *rad52*Δ strains. Interestingly, theoretical secondary structure analysis of the Rad52-1 mutant protein predicts that this alteration disrupts the α-helical nature of region II. In contrast, a similar analysis suggests that none of the alanine mutations identified in this study change the secondary structure of this region. The functional importance of region II is underscored by the presence of two null-like class A mutations, *rad52-N91A* and *rad52-F94A*, and three class C alleles, *rad52-84A*, *rad52-R85A*, and *rad52-Y96A*, which we identified in this study. This suggests that some alterations (class A) may result in complete loss of an essential function and some (class C) in only partial loss of the essential function. Finally, region II may be important for the integrity of Rad52 since a previously identified temperature-sensitive mutant, *rad52-N97T*, maps near the downstream end of this region (Kaytor and Livingston 1994).

Features of region III (aa 127–150): Region III is the least defined of the five that we describe and it is somewhat expanded in Rad59. Originally, we considered this region simply as a spacer between regions II and IV as it contains only one mutation, *rad52-R136A*, which strongly affects γ-ray sensitivity. However, the region was further refined when *rad52-R127A* and *rad52-K150A* were shown to lower mitotic heteroallelic recombination. Secondary structure predictions suggest a mixed structure for region III where Rad52-R127A is in a β-sheet and Rad52-R136A and Rad52-K150A are located in a random coil.

Features of region IV (aa 156–163): Region IV shares several features with region II. Both exist in Rad59 and both contain temperature-sensitive mutations (*rad52-N97T* and *rad52-V162A*; Kaytor and Livingston 1994). Furthermore, both regions are predicted to form α-helices where the most severe mutation(s) is located near the center. The three important amino acid residues identified by the alanine scan in region IV are hydrophilic (R, K, and T), suggesting that they are exposed on the surface. Furthermore, if they are indeed organized in an α-helix, their spacing is such that their side chains would protrude from the same face. Although none of the three mutations that we identified in region IV display a *rad52*-null phenotype, the sole class B allele, *rad52-K159A*, displays a very severe phenotype.

Features of region V (aa 173–186): Region V, like region I, is predicted to form an unstructured coil and neither region is present in Rad59. However, in contrast to region I where no null-like class A mutants have been uncovered, one class A mutant was identified in region V (*rad52-F173A*). To our knowledge, no other *rad52* mutations have previously been identified in this region.

Finally, in the overall Rad52 primary structure, we note the symmetrical arrangement of the five regions: a coiled region not present in Rad59—13-aa spacer—an α-helix present in Rad59—58-aa spacer (that includes region III)—another α-helix present in Rad59—9-aa spacer—a coiled region absent from Rad59. It is important to note that the subdivision of the Rad52 sequence into five regions is based on the linear map of the protein. However, although the close proximity of mutants within individual regions suggests that they impair the same function, regions separated by a significant number of amino acid residues in the primary structure may indeed be physically close in the tertiary structure. For this reason, the five regions identified do not necessarily have to have different functions. Furthermore, it is apparent that none of the individual classes of mutants map in any single region.

Separation-of-function alleles: The mutational analysis presented in this study identified two important types of separation-of-function alleles. The first, class C mutants, display wild-type or even higher levels of spontaneous mitotic interhomologous recombination, but are very sensitive to γ-ray-induced damage. The other type, class D, is rather insensitive to γ-irradiation yet displays very low levels of spontaneous mitotic interhomologous recombination. Two possible explanations for the class C mutants have been proposed previously (Malone *et al.* 1988; Boundy-Mills and Livingston 1993). In the first, the *rad52* mutant is postulated to be "leaky," producing sufficient Rad52 activity to support an occasional rare mitotic recombination event, but not enough to ensure the efficient repair of the numerous lesions pro-

duced by DNA-damaging agents. In the second explanation, mitotic recombination and γ-ray damage repair by Rad52 may require separate functional units on the protein. For *rad52-2*, the "leaky" allele explanation is unlikely since it is partially dominant when combined with *rad52-1* or *RAD52* (Malone *et al.* 1988) and since overexpression of *rad52-2* does not rescue its methyl methanesulfonate sensitivity (Boundy-Mills and Livingston 1993). Similarly, we tested five of our *rad52-2*-like class C mutants, which map in four different regions (I, II, IV, and V), and in no case did overexpression of these alleles on multicopy plasmids significantly improve γ-ray survival (our unpublished data). These results strongly support the separation-of-function model for these mutants.

The *rad52-2* mutation and two new class C alleles described here (*rad52-R70A* and *rad52-R156A*) cause a hyper-rec mitotic recombination phenotype. There are several possible explanations for this seemingly paradoxical result. First, the mutant proteins may channel lesions that would otherwise be repaired differently (*e.g.*, nucleotide excision repair or base excision repair) into a recombinogenic pathway. Second, the mutations, by virtue of their defect, may cause an increased number of lesions, resulting in a higher rate of recombination. Finally, the mutant proteins may shunt repair events, which normally use a newly replicated sister chromatid as a template, to the homologous chromosome. This shuttling will increase the apparent recombination rate because repair from only the homolog, and not from the identical sister, will generate a genetically measurable recombination event.

Class D mutants, which are only mildly sensitive to γ-ray damage but exhibit dramatically reduced rates of heteroallelic recombination, may, like class C mutants, be explained by either a true separation-of-function mutation or a leaky allele. For *rad52-R127A*, we favor the leaky allele hypothesis, since expression of this mutant allele from a multicopy plasmid fully complements a *rad52*Δ with respect to mitotic recombination and repair of γ-ray-induced damage (our unpublished data). Furthermore, the leaky allele explanation is supported by the observation that neither *rad52-R127A* nor *rad52-K150A* dramatically influence direct repeat recombination, a reaction that occurs more efficiently than interchromosomal recombination in the absence of Rad52 (Table 3). Thus, a simple explanation of the class D mutant phenotype is that it results from a reduced concentration of functional Rad52 activity. If this is true, it suggests that a higher concentration of Rad52 is required to efficiently support mitotic recombination compared to the repair of γ-ray damage.

The existence of two classes of *rad52* mutations that separate its function in mitotic recombination and γ-ray damage repair raises the possibility that the lesion(s) that provokes most mitotic recombination in yeast is not the same as that induced by γ-rays. For example, replication of a nicked template may create lesions that are different from those produced by γ-irradiation since proteins present at the stalled replication fork may remain bound at such a break (Rothstein *et al.* 2000). These proteins could either facilitate or hamper access of Rad52 to the break. Similar spontaneous lesions could be induced as accidental by-products of other aspects of DNA metabolism. For example, induced transcription from the *GAL1* and *GAL10* promoters increases recombination (Thomas and Rothstein 1989a; Bratty *et al.* 1996) and, in fact, proteins involved in homologous recombination have been identified in such RNA polymerase II complexes (Maldonado *et al.* 1996).

If the association of Rad52 with DNA metabolic protein complexes is responsible for its role in spontaneous mitotic recombination, it may provide a framework to understand the different classes of *rad52* mutations. Class C mutations may impair Rad52 function, but not its presence in the complexes. Thus, if such complexes stall at a lesion, then the impaired mutant Rad52 protein is delivered to and accumulates at the lesion. This may provide a sufficiently high local concentration of mutant protein to carry out a repair reaction. In contrast, when a DNA DSB is introduced randomly by γ-irradiation, a high local concentration of Rad52 does not exist at the lesion, and therefore the damage will remain unrepaired. By this reasoning, a small change in the dissociation constant for the binding of Rad52 to a lesion may block γ-ray repair but not spontaneous mitotic recombination.

For the leaky class D mutations, a lower overall concentration of the mutant protein will reduce its chance to be present in DNA metabolic complexes when they stall at a lesion. Thus, if the lesion needs to be repaired by recombination, mutant Rad52 protein needs to be recruited from the overall pool of mutant protein present elsewhere. This may be an inefficient process for several reasons: First, it may be difficult for the mutant Rad52 protein outside the complex even to recognize the complex as being stalled at a lesion, and thus it will not act on it. Second, the stalled Rad52-less complex itself may prevent the mutant Rad52 protein from accessing the lesion if the mutant protein were not built into the complex before it stalled. Finally, other repair proteins present in the complex may start to act on the lesion (because Rad52 is not there), thereby converting it into a substrate that mutant Rad52 protein will not recognize or cannot repair. In each case, the lesion will either remain unrepaired and ultimately cause cell death or will be repaired by an alternative pathway that does not involve recombination.

In summary, we have used an alanine scan to identify amino acid residues in the evolutionarily conserved N terminus of Rad52 that are important for its function. This region of the molecule has been suggested to contain its core activity (Asleson *et al.* 1999; Kagawa *et al.* 2001), including interactions to DNA (Mortensen *et*

al. 1996), to RP-A (Hays *et al.* 1998), and to itself (Shen *et al.* 1996b; Ranatunga *et al.* 2001). The identification of four conserved regions that are required for efficient repair of γ-ray-induced damage described here strongly supports the multifunctional nature of the Rad52 N terminus. Furthermore, this mutant collection will be highly valuable to dissect the complex biochemistry of this important recombination protein.

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