

Complex formation in yeast double-strand break repair: Participation of Rad51, Rad52, Rad55, and Rad57 proteins

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ABSTRACT The repair of DNA double-strand breaks in *Saccharomyces cerevisiae* requires genes of the *RAD52* epistasis group, of which *RAD55* and *RAD57* are members. Here, we show that the x-ray sensitivity of *rad55* and *rad57* mutant strains is suppressible by overexpression of *RAD51* or *RAD52*. Virtually complete suppression is provided by the simultaneous overexpression of *RAD51* and *RAD52*. This suppression occurs at 23°C, where these mutants are more sensitive to x-rays, as well as at 30°C and 36°C. In addition, a recombination defect of *rad55* and *rad57* mutants is similarly suppressed. Direct *in vivo* interactions between the Rad51 and Rad55 proteins, and between Rad55 and Rad57, have also been identified by using the two-hybrid system. These results indicate that these four proteins constitute part of a complex, a “recombinosome,” to effect the recombinational repair of double-strand breaks.

The *RAD55* and *RAD57* genes of *Saccharomyces cerevisiae* belong to the *RAD52* epistasis group, a group of genes (*RAD50–57*, *MRE2*, *MRE11*, *XRS2*, and *RF1*[†]) whose products have been implicated in the recombinational repair of DNA double-strand breaks (DSBs) (for review see refs. 3–5). As a means to better understand the participants and mechanisms involved in the repair of DSBs, we devised a genetic screen designed to identify mutants unable to perform the recombinational repair of a targeted DSB and thereby identified a number of alleles of genes in the *RAD52* epistasis group (2). Further studies of some of these mutants, specifically *rad55* and *rad57* mutants, suggested that there might be interactions between the gene products of certain members of this group. We have characterized these interactions in order to gain insight into the molecular mechanisms that mediate DSB repair in yeast.

Lovett and Mortimer (6) reported that a *rad55* null mutant was more sensitive to x-rays at 23°C than at 36°C. This result was striking in that cold sensitivity is usually observed with missense alleles and not deletion alleles. Cold sensitivity is, however, a property often associated with proteins composed of multiple subunits or large multiprotein complexes (7, 8), and the authors suggested that the Rad55 protein might participate in some sort of higher-order complex responsible for the repair of x-ray-induced damage (6). Other members of the *RAD52* epistasis group would be logical candidates for participation in such a complex. Indeed, interactions between Rad51 and Rad52 and between Rad51 and itself have been identified (9–11), and a Rad52–Rad52 interaction has been inferred (10, 12). There is also genetic evidence that an interaction between the *RAD52* and *RF1* gene products is involved in the recombinational repair of DSBs (1, 2). Here we provide evidence for the existence of interactions among Rad51, Rad52, Rad55, and Rad57 that affect recombination and repair, and we show that some of these are direct physical interactions. We propose that these four proteins together with RPA constitute part of a

complex, called a “recombinosome” (2), to effect the recombinational repair of DSBs.

MATERIALS AND METHODS

Yeast Strains and Media. The parental strain from which all the *rad55* and *rad57* mutants were derived is YME2 (2), which carries a 219-base-pair (bp) deletion in the 5' region of the *ADE2* gene. To create the *rad55*, *rad57* double deletion, *hisG* insertion strain, the *hisG* sequence (13) replaced *RAD55* sequences between the *Mun* I site at position +163 and the downstream *Pst* I site. *RAD57* coding sequences between the upstream *Aat* II site and the downstream *Bst*BI site were similarly replaced. Yeast were grown according to standard techniques (14). YPD plates were routinely supplemented with adenine at 40 μg/ml (referred to as YPAD).

Plasmids. Construction of pAF35 has been described (2). *ARS/CEN* plasmids containing complementing fragments from *RAD51*, *RAD52*, *RAD55*, and *RAD57* have been described (1, 2). More detailed descriptions of plasmid constructions are available upon request.

X-Ray Survival Assays. Quantitation of x-ray survival was as described (2). Semiquantitative “spot assays” for x-ray survival determinations were performed by growing cells to midlogarithmic phase and then making dilutions so that the cell density of individual cultures fell within a ≤2-fold range of one another. Approximately 5–9 × 10⁵ cells were spotted on 10-cm YPAD plates in a predetermined array. The plates were x-irradiated immediately thereafter. After growth for 2–4 days at the appropriate temperature, the density of spots was compared.

Determination of HO Endonuclease-Mediated Gene Conversion Levels. Gene conversion frequencies were determined as described (2).

Two-Hybrid Analysis. Pairwise combinations of fusion expression plasmids were transformed into reporter strain Y190 (15) and colonies from these transformations were picked after growth on selective medium at 30°C for 2–5 days. Cultures (10 ml) from individual colonies were grown under selection for both plasmids, and extracts from these cells were prepared and assayed as described (16). Plasmids constructed for the two-hybrid experiments were derivatives of pAS2-CYH2 (15), which is designed to produce gene fusions to the Gal4 DNA-binding domain (amino acids 1–147), or pGAD GH (constructed by Greg Hannon, Cold Spring Harbor Laboratory), in which fusions to the Gal4 transcriptional activation domain (amino acids 768–881) are created. A second plasmid, pBG4D-1 (provided by Robert Brazas, University of California, San Francisco), was used to create a fusion of the C terminus of the Rad52 protein to the N terminus of the Gal4 DNA-binding domain. All fusion constructions were se-

Abbreviations: DSB, double-strand break; RPA, replication protein A; SSB, single-strand-binding protein.

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[†]The *RF1* gene encodes the large subunit of the yeast single-strand-binding protein (SSB), a heterotrimer called replication protein A (RPA). A mutation in *RF1* which leads to defective recombination and repair phenotypes is epistatic to *RAD52* (1, 2).

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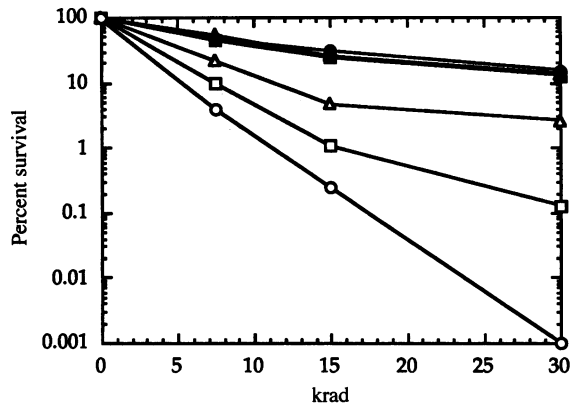


Fig. 1. X-ray survival curves of YME2 (*RAD*⁺) at 36°C (▲), 30°C (■), and 23°C (●) and YME22 (*rad57::LEU2*) at 36°C (△), 30°C (□), and 23°C (○).

quenced across the fusion junction to ensure that the junctions were in frame.

RESULTS

Fig. 1 shows the comparative x-ray sensitivity of a *rad57::LEU2* disruption mutant, YME22 (provided by Montserrat Elias-Arnanz, Stanford University), at three temperatures. This mutant is most sensitive to x-irradiation at 23°C and least sensitive at 36°C; the respective sensitivities differ by >3 orders of magnitude. By contrast, isogenic wild-type cells are equally resistant to x-rays at these three temperatures (Fig. 1). All other *rad55* and *rad57* mutants tested show similar cold sensitivities (6, 17). Comparable temperature sensitivities were found with 10 mutants isolated in a previous screen (2): 4 *rad55* mutants and 6 *rad57* mutants, and with a *rad55::LEU2* insertion allele. Thus, this cold-sensitive phenotype appears to be a general property of *rad55* and *rad57* mutants.

While sorting the mutants obtained previously (2) into complementation groups, we noted what appeared to be partial suppression of the x-ray sensitivity of *rad55* mutants by the presence of *RAD51* and *RAD52* expression plasmids (1). Following this lead, we measured the x-ray sensitivity of strain YAF37 (*rad55-37*) carrying centromeric plasmids which expressed either *RAD51*, *RAD52*, *RAD55*, or *RAD51* and *RAD52* together (Fig. 2). Note that the plasmid-borne genes are in addition to the copy already present in the chromosome. As the overexpressed genes are under the control of their endogenous promoters, we presume that the increase in gene product is approximately double.

At 30°C and at an x-ray dose of 30 krad, YAF37 carrying the *RAD52* plasmid is ≈7-fold more resistant than YAF37 with only a control plasmid. With a single extra copy of *RAD51*, YAF37 is nearly 170-fold more resistant to 30 krad of x-rays. Providing additional *RAD51* and *RAD52* together results in virtually wild-type levels of resistance—i.e., a >400-fold increase in survival from the basal level (Fig. 2B). At 23°C, where the sensitivity of YAF37 to radiation is greatest, only the combined expression of *RAD51* and *RAD52* causes a substantial increase in resistance to x-rays (Fig. 2A). Expression of *RAD52* alone in YAF37 at 36°C (Fig. 2C), the temperature at which this mutant is least sensitive to x-rays, has only a small effect. However, at this temperature, both *RAD51* by itself and the *RAD51/RAD52* combination confer wild-type levels of resistance (Fig. 2C). Interestingly, the presence of either *RAD51* or *RAD52* on a multicopy 2- μ m plasmid does not result in increased x-ray resistance above that seen when these genes are present on centromere-based plasmids (A.A.F., unpublished data). Note that the x-ray sensitivity of our wild-type strain, YME2, is unaffected when transformed with both *RAD51* and *RAD52* (Fig. 2B).

Because *rad55* and *rad57* mutants have very similar phenotypes, we examined the suppressive effects of overexpression of *RAD51*, *RAD52*, or *RAD51* and *RAD52* on two different *rad57* point mutants. Survival curves indicate that the extent to which *RAD51* and/or *RAD52* suppresses the x-ray sensitivity of these *rad57* mutants closely resembles that found with the *rad55* mutant. Table 1 shows the influence of *RAD51* and/or *RAD52* overexpression on the x-ray survival of one of these *rad57* mutants, YAF12 (*rad57-32*), at different temperatures and at an x-ray dose of 30 krad. A second *rad57* mutant, YAF51 (*rad57-37*), behaved virtually identically to YAF37 in its response to the overexpression of *RAD51* and/or *RAD52* (S.L.H., unpublished data).

Semiquantitative “spot assays” (see *Materials and Methods*) indicated that the x-ray sensitivity of both *rad55* and *rad57* disruption mutants was also suppressed by *RAD51* and/or *RAD52*. This suppressive effect was quantitated with YME22 (*rad57::LEU2*) at 23°C, the temperature at which it is most sensitive to x-rays. The disruption mutant is suppressed by single-copy overexpression of *RAD51* and/or *RAD52* in very much the same way as the other *rad55* and *rad57* mutants tested (Fig. 3).

Because both *rad55* and *rad57* disruption mutants, which presumably fail to make the corresponding proteins, are still suppressed by *RAD51* and *RAD52*, it is unlikely that Rad51 and/or Rad52 exert their suppressive effects by stabilizing a mutant Rad55 or Rad57 protein. However, there was still the possibility that the disruption mutants we tested yielded some truncated and partially active Rad55 or Rad57 protein. To rule out this possibility, and to determine whether a *rad55* Δ , *rad57* Δ

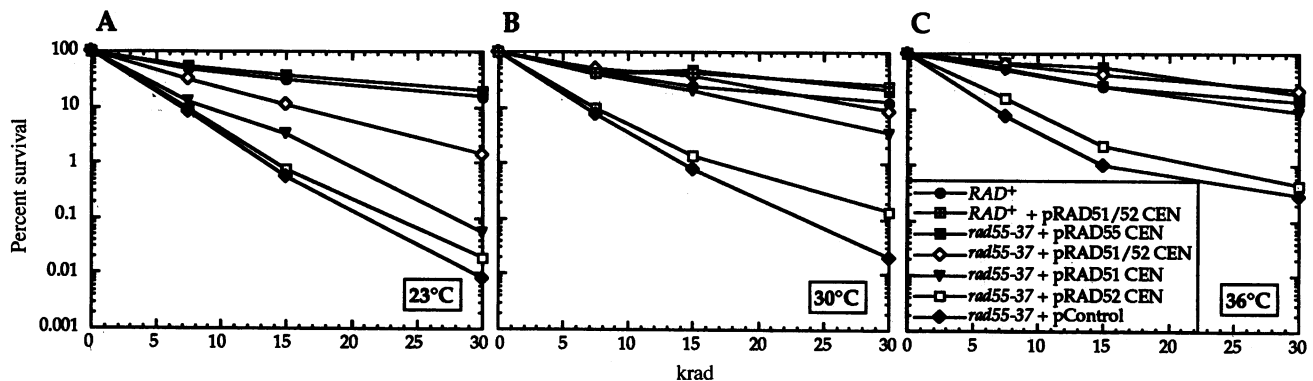


Fig. 2. Effect of overexpression of *RAD51* and/or *RAD52* on the x-ray survival of YAF37 (*rad55-37*) at 23°C (A), 30°C (B), and 36°C (C). ●, YME2 (*RAD*⁺); ▨, YME2 with both *RAD51* and *RAD52* expression plasmids; ■, YAF37 with *RAD55* plasmid; ◆, YAF37 with *RAD51* and *RAD52* plasmids; ▼, YAF37 with *RAD51* plasmid; □, YAF37 with *RAD52* plasmid; ◆, YAF37 with control vector.

Table 1. Effect of overexpression of *RAD51* and/or *RAD52* on the survival of YAF12 (*rad57-32*) at various temperatures after exposure to 30 krad of x-rays

| Strain + plasmid(s) | 23°C | | 30°C | | 36°C | |
|---------------------|----------------------|-------------------------------------|----------------------|-------------------------------------|----------------------|-------------------------------------|
| | Relative survival | Fold suppression by <i>RAD</i> gene | Relative survival | Fold suppression by <i>RAD</i> gene | Relative survival | Fold suppression by <i>RAD</i> gene |
| YME2 | 1 | — | 1 | — | 1 | — |
| YAF12 + pControl | 8.8×10^{-4} | — | 6.0×10^{-3} | — | 2.5×10^{-2} | — |
| YAF12 + pRAD52 | 2.0×10^{-3} | 2 | 6.1×10^{-2} | 10 | 1.3×10^{-1} | 5 |
| YAF12 + pRAD51 | 1.4×10^{-2} | 16 | 3.3×10^{-1} | 55 | 3.0×10^{-1} | 12 |
| YAF12 + pRAD51/52 | 1.3×10^{-1} | 148 | 6.4×10^{-1} | 107 | 4.0×10^{-1} | 16 |
| YAF12 + pRAD57 | 1.2 | 1364 | 1.1 | 183 | 1.3 | 52 |

double deletion mutant was also suppressed by *RAD51* and *RAD52* overexpression, a strain with a complete deletion of the *RAD57* coding sequence as well as a nearly complete deletion of the *RAD55* coding sequence was constructed (see *Materials and Methods*). The results of overexpression of *RAD51* and/or *RAD52* on the x-ray sensitivity of this strain, YSH14, at 23°C are shown in Table 2. The extent of suppression provided by *RAD51* and *RAD52* is less than that seen for the single mutants but follows the same general pattern in that *RAD52* suppresses less well than *RAD51*; the two together suppress to a greater extent.

Spot assays also revealed that the suppressive effects of *RAD51* and/or *RAD52* applied to all seven of the other *rad55* and *rad57* mutants tested. Thus, suppression of the x-ray sensitivity of *rad55* and *rad57* mutants by overexpression of *RAD51* and *RAD52* appears to be a general property of *rad55* and *rad57* mutants. This effect seems to be specific for *RAD51* and *RAD52* inasmuch as expression of *RAD54* or *RFA1* failed to relieve the x-ray sensitivity of any of the *rad55* or *rad57* mutants tested. Moreover, overexpression of *RAD55* had no effect on the x-ray sensitivity of *rad57* mutants and overexpression of *RAD57* failed to alter the x-ray sensitivity of *rad55* mutants.

A relevant question is whether overexpression of *RAD51* and *RAD52* alters the defective recombination phenotype of our *rad55* and *rad57* mutants. To answer this question, we used a modified version of the papillation assay (2) to measure the recombination defect of some of our *rad55* and *rad57* mutants. This assay scores the frequency of *ADE2*⁺ recombinants in strains carrying two copies of a mutant *ade2* gene, one of which is carried on a single-copy plasmid, and the other of which is at its normal locus in the chromosome. Recombination is induced by the introduction of a targeted DSB in the plasmid-borne *ade2* allele. Cells that undergo recombinational repair of

the plasmid-borne *ade2* allele give rise to colonies on medium lacking adenine.

Using this assay, we examined the effect of the expression of *RAD51*, *RAD52*, *RAD55*, *RAD57*, or both *RAD51* and *RAD52* on the frequency of gene conversion of *rad55* and *rad57* mutants. Representative results for one *rad55* and one *rad57* mutant are shown in Table 3. In each case, the recombination defect of the mutants was partially suppressed by *RAD51* or *RAD52* and almost completely suppressed by the combination of *RAD51* and *RAD52*. The extent of suppression of the recombination defect of these strains mirrors closely the extent of suppression of their x-ray sensitivity. Here too, multicopy 2- μ m plasmids for expression of *RAD51* or *RAD52* are no more effective in suppressing the recombination defect than when these proteins are expressed from single-copy plasmids (A.A.F., unpublished data).

As is the case with the suppression of the radiation sensitivity, the effect of *RAD51* and *RAD52* appears to be specific and not shared with other members of the *RAD52* epistasis group; thus, overexpression of *RAD54* and *RFA1* has no effect on the recombination ability of *rad55* or *rad57* mutants. Likewise, *RAD57* does not suppress the recombination defect of any of the *rad55* mutants and *RAD55* has no significant effect on the *rad57* mutant's recombination defect (Table 3). Additionally, the overexpression of *RAD51* and *RAD52* together does not stimulate the recombination frequency of wild-type cells above the level seen when only plasmid vectors are used (S.L.H., unpublished data).

A possible explanation for the ability of *RAD51* and *RAD52* to partially overcome the defects of *rad55* and *rad57* mutants is that addition of Rad51 and/or Rad52 stimulates an alternative pathway from that of Rad55 and/or Rad57. While epistasis analysis suggests that the gene products of the *RAD52* epistasis group all act in the same pathway (17–20), more persuasive evidence that all four of these proteins act in the same pathway, or even as components of a complex, requires the demonstration of physical interactions between these proteins.

To determine whether there are physical interactions of Rad55 and Rad57 with Rad51 and Rad52, we used the *GAL4* two-hybrid fusion system (21). In this assay, interaction be-

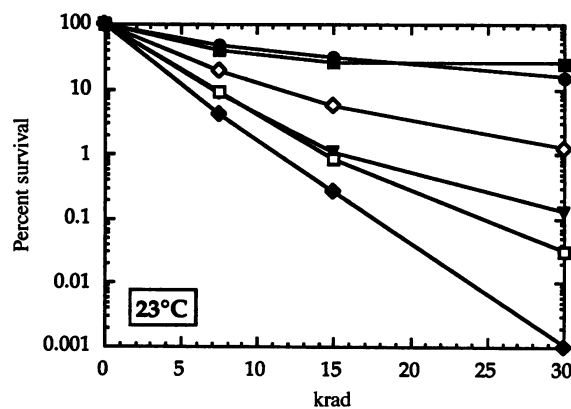


FIG. 3. Effect of overexpression of *RAD51* and/or *RAD52* on the x-ray survival of YME2 (*rad57::LEU2*) at 23°C. ●, YME2 (*RAD*⁺); ■, YME2 with *RAD57* plasmid; ◇, YME2 with *RAD51* and *RAD52* plasmids; ▼, YME2 with *RAD51* plasmid; □, YME2 with *RAD52* plasmid; ◆, YME2 with control vector.

Table 2. Effect of overexpression of *RAD51* and/or *RAD52* on the survival of YSH14 (*rad55Δ::hisG*, *rad57Δ::hisG*) at 23°C after exposure to 30 krad of x-rays

| Strain + plasmid(s) | Relative survival | Fold suppression by <i>RAD</i> gene |
|---------------------|----------------------|-------------------------------------|
| YME2 | 1 | — |
| YSH14 + pControl | 1.2×10^{-3} | — |
| YSH14 + pRAD52 | 6.3×10^{-3} | 5 |
| YSH14 + pRAD51 | 1.3×10^{-2} | 11 |
| YSH14 + pRAD51/52 | 2.3×10^{-2} | 19 |
| YSH14 + pRAD55 | 1.6×10^{-3} | — |
| YSH14 + pRAD57 | 1.6×10^{-3} | — |
| YSH14 + pRAD55/57 | 0.8 | 667 |

Table 3. Effect of overexpression of *RAD51* and/or *RAD52* on the gene conversion defect of YAF37 (*rad55-37*) and YAF51 (*rad57-37*)

| Strain + plasmid(s)* | Gene conversion to <i>ADE2</i> ⁺ | | |
|----------------------|---|------------------------------|-------------------------------------|
| | Relative level† | Fold decrease from wild type | Fold suppression by <i>RAD</i> gene |
| YME2 | 1 | — | — |
| YAF37 + pControl | 1.2×10^{-3} | 855 | — |
| YAF37 + pRAD52 | 3.8×10^{-3} | 260 | 3 |
| YAF37 + pRAD51 | 2.9×10^{-2} | 34 | 25 |
| YAF37 + pRAD51/52 | 2.5×10^{-1} | 4 | 214 |
| YAF37 + pRAD55 | 7.0×10^{-1} | 1.5 | 570 |
| YAF37 + pRAD57 | 1.2×10^{-3} | 866 | — |
| YAF51 + pControl | 9.3×10^{-5} | 10,720 | — |
| YAF51 + pRAD52 | 2.9×10^{-3} | 350 | 31 |
| YAF51 + pRAD51 | 3.6×10^{-2} | 29 | 370 |
| YAF51 + pRAD51/52 | 3.0×10^{-1} | 3 | 3,248 |
| YAF51 + pRAD57 | 1.4 | 0.7 | 15,314 |
| YAF51 + pRAD55 | 1.9×10^{-4} | 5,214 | 2 |

*All strains carried pAF35 in addition to the plasmids shown.

†Levels of gene conversion were 20–25% for wild-type cells.

tween a Rad protein which is fused to the Gal4 DNA-binding domain and another Rad protein fused to the Gal4 transcriptional activation domain leads to activation of a β -galactosidase reporter gene which is under the control of the *GAL1/10* promoter region. Accordingly, the sequence encoding the Gal4 transcriptional activation domain (amino acids 768–881) was fused in frame to full-length *RAD51*, *RAD55*, or *RAD57*. Similarly, full-length *RAD52*, *RAD55*, or *RAD57* was fused to the sequence encoding the Gal4 DNA-binding domain (amino acids 1–147).

Strains carrying different combinations of these fusion protein expression vectors were tested for their β -galactosidase activity (Fig. 4). Our data confirm earlier reports of an interaction between Rad51 and Rad52 (10, 11). Moreover, an interaction between Rad51 and Rad55 can be inferred from the fact that the β -galactosidase activity is substantially above the levels seen when vectors lacking the fusion genes are introduced. Similarly, Rad55 appears to interact with Rad57 regardless of whether Rad55 or Rad57 is fused to the DNA-binding domain or to the transcriptional activation domain of Gal4. Conversely, no interactions were detected of Rad51 with Rad57, Rad55 with itself, Rad57 with itself, or Rad52 with either Rad55 or Rad57 (Fig. 4).

DISCUSSION

The effect of single-copy overexpression of *RAD51* and/or *RAD52* on the x-ray sensitivity of YAF37 (*rad55-37*) (Fig. 2),

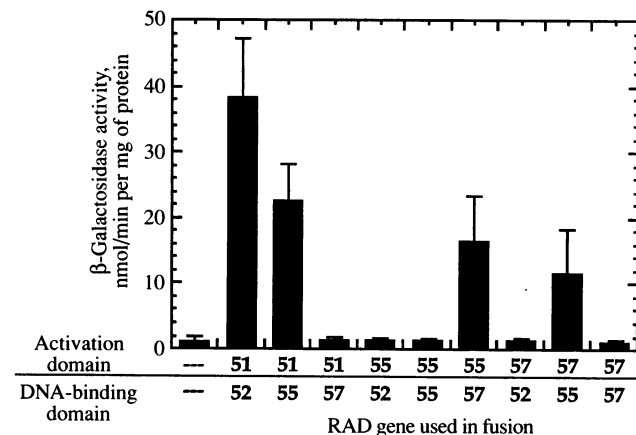


FIG. 4. Two-hybrid assay results.

YAF12 (*rad57-32*) (Table 1), YME2 (*rad57::LEU2*) (Fig. 3), and YSH14 (*rad55 Δ ::hisG*, *rad57 Δ ::hisG*) (Table 2) follows a basic theme: *RAD52* can suppress these mutants' sensitivity to a small extent, *RAD51* suppresses to a greater extent, and *RAD51* and *RAD52* together generally suppress to give levels of resistance close to those of wild-type at certain temperatures. These suppressive effects of *RAD51* and *RAD52* were confirmed for every one of the other *rad55* and *rad57* mutants tested with the semiquantitative spot assay. Furthermore, expression of *RAD51* and/or *RAD52* suppresses the inability of *rad55* and *rad57* mutants to repair a targeted DSB. The *RAD51*- and/or *RAD52*-mediated suppression of the recombination defect of *rad55* and *rad57* mutants paralleled the extent of suppression of their x-ray sensitivity. Finally, the interactions suggested by genetic experiments are supported by two-hybrid analysis demonstrating molecular interactions between Rad51 and Rad52, between Rad51 and Rad55, and between Rad55 and Rad57.

Others have suggested that the repair of DSBs in *S. cerevisiae* may be performed by a multiprotein complex (6, 10). Our data suggest that Rad55 and Rad57 may be members of such a complex, termed a "recombinosome" (2). Because Rad55 and Rad57 appear to be partially dispensable for DSB repair and recombination under certain conditions (refs. 6 and 17; this paper), it is possible that Rad55 and Rad57 are needed in an auxiliary way, perhaps to stabilize a complex which is intrinsically unstable at low temperatures. Excess Rad51 and Rad52 might overcome a lack of functional Rad55 or Rad57 by driving or stabilizing complex formation by "mass action." This type of effect is exemplified by the suppression of the repair defect of an *Escherichia coli umuC* mutant (*umuC-36*) by overexpression of *umuD'* (22). That Rad51, but not Rad52, interacts directly with Rad55, which in turn interacts with Rad57, may account for the greater suppressive effect of *RAD51* overexpression in the x-ray survival and recombination experiments. Thus, the suppressive effects of Rad52 may be indirect, mediated by its interaction with Rad51. After completion of these experiments we learned that R. D. Johnson and L. S. Symington have also obtained data indicating interactions of Rad55 with Rad51 and Rad57 and have suggested their participation in a complex involved in DSB repair (Roger D. Johnson and Lorraine S. Symington, personal communication).

In considering possible functions for Rad55 and Rad57, it should be borne in mind that Rad55 and Rad57, like Rad51, have some sequence similarity to the bacterial RecA protein (23). Rad51 can promote low levels of ATP-dependent strand-exchange activity, an activity which is enhanced by RPA, the yeast heterotrimeric SSB (24, 25). Quite possibly, maximal strand-exchange activity requires, in addition to Rad51 and RPA, Rad55 or Rad57 or the two together.

In this model, Rad55, Rad57, and RPA mimic the activities of RuvA, RuvB, and SSB in increasing the overall efficiency of strand transfer by RecA *in vitro* (26, 27). Similarly, the finding that the phage-encoded gene 32 protein enhances UvsX-mediated strand exchange in the bacteriophage T4 recombination system (28) provides another comparable example. In the latter case, the UvsY protein mediates the UvsX–gene 32 protein interaction by interacting directly with each of these proteins (29, 30), a situation similar to Rad55's interactions with Rad51 and Rad57. In the T4 system, however, the gene 32 protein, a SSB, serves as a helix-destabilizing factor (31), a property which has not been detected with either Rad55 or Rad57. However, the involvement of RPA in strand exchange and the indications that RPA's large subunit interacts with Rad52 implicate RPA as a participant of the recombinosome. Fig. 5 provides a diagram of this putative complex and of the protein–protein interactions that have been implicated in this and other studies. The recombinosome is shown assembled at the site of a DSB; however, the role of DNA, if any, in complex

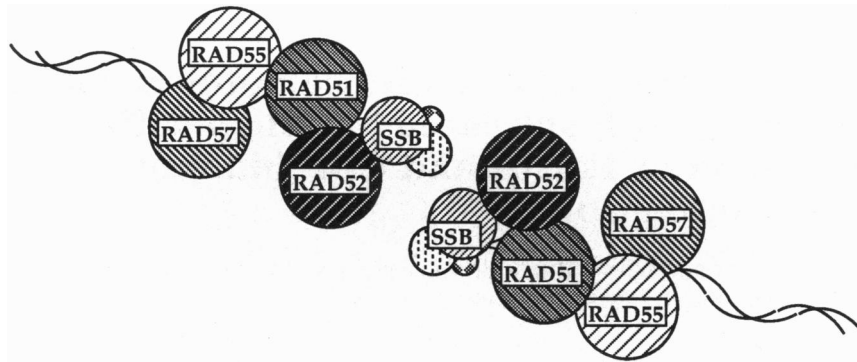


FIG. 5. Schematic diagram of the putative recombinosome.

formation is not known. Some of the members of the recombinosome may be present in multimeric forms.

Perhaps, unlike the situation in *E. coli*, in which the multi-step process of recombination is mediated by smaller complexes or individual peptides, the yeast recombinosome incorporates many proteins to carry out these biochemical functions. Identification of the individual components of the recombinosome and their associated activities awaits further genetic as well as biochemical characterization.

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