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# Evidence for Simultaneous Protein Interactions between Human Rad51 Paralogs*

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**In yeast, the Rad51-related proteins include Rad55 and Rad57, which form a heterodimer that interacts with Rad51. Five human Rad51 paralogs have been identified (XRCC2, XRCC3, Rad51B/Rad51L1, Rad51C/Rad51L2, and Rad51D/Rad51L3), and each interacts with one or more of the others. Previously we reported that HsRad51 interacts with XRCC3, and Rad51C interacts with XRCC3, Rad51B, and HsRad51. Here we report that in the yeast two-hybrid system, Rad51D interacts with XRCC2 and Rad51C. No other interactions, including self-interactions, were found, indicating that the observed interactions are specific. The yeast Rad51 interacts with human Rad51 and XRCC3, suggesting Rad51 conservation since the human yeast divergence. Data from yeast three-hybrid experiments indicate that a number of the pairs of interactions between human Rad51 paralogs can occur simultaneously. For example, Rad51B expression enhances the binding of Rad51C to XRCC3 and to HsRad51D, and Rad51C expression allows the indirect interaction of Rad51B with Rad51D. Experiments using 6xHis-tagged proteins in the baculovirus system confirm several of our yeast results, including Rad51B interaction with Rad51D only when Rad51C is simultaneously expressed and Rad51C interaction with XRCC2 only when Rad51D is present. These results suggest that these proteins may participate in one complex or multiple smaller ones.**

The Rad51 protein is a functional homolog of the bacterial RecA protein and is the major strand transfer protein in eucaryotic cells (1–3). In addition to Rad51, the yeast *Saccharomyces cerevisiae* has two proteins, Rad55 and Rad57, that share limited amino acid sequence homology with Rad51. These proteins appear to be Rad51 paralogs, probably derived by duplication of the ancestral gene encoding Rad51 but now divergent in function. The Rad55 and Rad57 proteins interact and form a tight dimer that weakly interacts with Rad51 and assists it in strand transfer, probably by helping Rad51 displace RPA from single-stranded DNA (4).

Human cells have a true Rad51 homolog (HsRad51), and five mitotically expressed Rad51 paralogs have recently been identified (XRCC2, XRCC3, Rad51B/Rad51L1/HsRec2, Rad51C/Rad51L2, and Rad51D/Rad51L3) (5–11). Like the yeast Rad51 paralogs, these proteins share limited (~20–30%) amino acid sequence homology with HsRad51 and with each other. The human *XRCC2* and *XRCC3* genes were isolated (5, 6, 12) and shown to complement the DNA repair defect and chromosome instability of the *irs1* and *irs1SF* hamster-derived cell lines (13–16). Each of the paralogs has recently been knocked out in the chicken B lymphocyte line DT40, and all of the knockouts are sensitive to DNA damage and show great chromosome instability (17).[1]

Several lines of evidence suggest that the human Rad51 paralogs play an important, but not crucial, role in recombination. Evidence from mammalian cells indicates that the repair of DNA cross-links requires recombination (18, 19). The extreme sensitivity to DNA cross-linking reagents, such as mitomycin C and cis-platin, of mammalian and chicken cell lines with mutations in the *RAD51* paralogs argues that they play an important role (17).[1] In addition, *RAD51B* and *RAD51D* knockouts in mice are embryonic lethals (20, 21), as are the *RAD51* knockouts (22, 23). However, both mammalian and chicken cell lines with standard mutations or knockouts in the paralogs are viable (5, 17),[1] whereas *RAD51* knockouts in both mammalian and chicken cell lines are lethal (22–24). The sensitivity of the paralog knockout DT40 cell lines is partially suppressed by overexpression of the human Rad51 protein, suggesting a supportive role for the paralogs (17).[1] Similarly, overexpression of the yeast Rad51 protein partially complements the yeast *rad55* and *rad57* mutations (25, 26).

By analogy with yeast Rad55 and Rad57, the ability of the human paralogs to interact with each other and with HsRad51 was tested. Previously we reported the interaction of XRCC3 with HsRad51 and with Rad51C and the interaction of Rad51C with Rad51B (5, 10). Here, we report additional interactions, using the yeast two-hybrid system and present evidence from a yeast three-hybrid system and from the baculovirus system that many of the paralogs can interact as heterotrimers.

## EXPERIMENTAL PROCEDURES

*Construction of Plasmids for Two- and Three-hybrid Experiments*—The *MmRAD51D, HsRAD51D*, and *XRCC2* (human) ORFs[2] were cloned into the Gal4 DNA-binding domain vector pGBT9 and the transcriptional activation domain vector pGAD424 (27) or in closely related vectors (28). The DNA sequences of the fusion regions of all two-hybrid plasmid constructions were determined to confirm that all plasmids encoded in-frame fusions. Details of these constructions are available from the authors. The plasmids fusing the human *RAD51* to the *GAL4* domains were kindly supplied by Z. Shen (29), and the two-hybrid

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[1] M. Takata and S. Takeda, personal communication.

[2] The abbreviations used are: ORF, open reading frame; Ni-NTA, nickel-nitrilotriacetic acid; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; Mm, Mus musculus; Hs, Homo sapiens; Sc, Saccharomyces cerevisiae; ONPG, 2-nitrophenyl-β-D-galactopyranoside.

plasmid constructs involving *XRCC3*, *RAD51B*, and *RAD51C* have already been published (5, 10). For use in the three-hybrid experiments, each of the human *RAD51* paralog cDNAs were cloned into pVT100U, a yeast expression vector containing the *ADH1* promoter and the *URA3* selectable marker (30). These plasmids were constructed to express in yeast the complete ORF of each human paralog (but not *HsRAD51* itself) in a non-fused form. Positive results from one or more three-hybrid experiments suggest that each construct was expressing the inserted cDNA, with the exception of *XRCC2*, for which we had no positive three-hybrid result.

*Yeast Two- and Three-hybrid Systems*—Yeast strains Y190 (31) and PJ69-4A (28) were used for most two-hybrid experiments as described previously (10). Using 5-fluoroorotic acid selection, a *ura3* derivative of Y190 (Y190-ura$^-$) was isolated and then used in three-hybrid experiments. To test three-hybrid interactions, strains containing the two-hybrid plasmids, grown in media lacking leucine and tryptophan, were transformed with the third plasmid, derived from pVT100U (containing the *URA3* selectable marker). The triply transformed strains were selected on plates lacking leucine, tryptophan, and uracil. The triply transformed PJ69-4A strains were tested for expression of the *ADE2* reporter gene by looking for growth on plates lacking adenine (28). The triply transformed Y190-ura$^-$ strains were tested for expression of the *lacZ* reporter gene. β-Galactosidase activity was tested qualitatively by the X-gal filter assay (32) and was quantified using either the chemiluminescent detection method with Galacton-Star substrate and Sapphire-II enhancer as described previously (10) or the ONPG assay (32). Generally, all testing for reporter gene activity was done on three or more individual transformants.

*Construction of Plasmids for Baculovirus Experiments*—The human *RAD51B*, *RAD51C*, and *RAD51D* ORFs were amplified by polymerase chain reaction from a human thymus cDNA library (CLONTECH) and confirmed by sequence analysis. The human *XRCC2* and *XRCC3* cDNAs were kindly provided by Dr. Nan Liu (Lawrence Livermore National Laboratory). The *RAD51C*, *RAD51D*, *XRCC2,* and *XRCC3* ORFs were cloned into pFastBac1 (BAC-TO-BAC Baculovirus Expression System, Life Technologies, Inc.), and *RAD51B* and *RAD51C* ORFs were cloned into pFastBacHTb, a 6xHis-tagged baculoviral vector. The resulting plasmids were transformed into DH10Bac *Escherichia coli* cells for site-specific transposition into bacmid bMON14272 (33). High molecular weight recombinant bacmids were isolated for transfection into insect cells. The human *RAD51* cDNA was cloned into pBlueBac4, and the recombinant baculoviruses were generated according to the Bac-N-Blue manual from Invitrogen.

*Transfection and Protein Expression*—The isolated bacmids were transfected into Sf9 cells with CellFECTIN reagent as described by the manufacturer (Life Technologies, Inc.). The virus stock from the transfected cells was harvested and used to infect fresh Sf9 cells for virus amplification. The virus titer was determined with the BacPAK baculovirus rapid titer kit (CLONTECH) as described in the manuals. For protein expression, $8 \times 10^6$ Sf9 cells were infected with the recombinant baculoviruses at a multiplicity of infection of 10. Three days post-infection, cells were collected by centrifugation at 10,000 × *g* for 10 min at 4 °C and washed twice with ice-cold phosphate-buffered saline. The cells were then frozen immediately at −70 °C.

*Ni-NTA Magnetic Agarose Beads Pull-down Experiments*—Cells with the expressed protein of interest were thawed on ice for 5 min and resuspended in 1 ml of ice-cold lysis buffer containing 50 mM $NaH_2PO_4$ (pH 8.0), 300 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM imidazole in a microfuge tube. Lysozyme (1 mg/ml) was added to the cell suspension, and the mixture was incubated on ice for 30 min. Cells were lysed by vortexing six times for 5 s each, and the lysate was clarified by centrifugation at 10,000 × *g* for 30 min at 4 °C. The supernatant (~1 ml) was transferred to a fresh tube, and 50 μl of 5% Ni-NTA magnetic agarose beads homogeneous suspension (Qiagen, Inc.) was added. The mixture was then incubated on an end-over-end shaker overnight at 4 °C. The following day, each tube was placed on a magnetic separator (Dynal, Inc.) for 5 min, and supernatant was removed with a pipette. The beads were washed with 1 ml of wash buffer containing 50 mM $NaH_2PO_4$ (pH 8.0), 300 mM NaCl, and 20 mM imidazole and were incubated on an end-over-end shaker for 30 min at 4 °C. Subsequently, each tube was placed for 5 min on a magnetic separator, and the wash buffer was removed. The wash step was repeated twice. Finally, an aliquot of 50 μl of elution buffer (50 mM $NaH_2PO_4$, pH 8.0, 300 mM NaCl, and 500 mM imidazole) was added, mixed, and incubated for 10 min on ice, and then each tube was placed for 5 min on a magnetic separator and the eluate was collected. An aliquot of each eluate was subjected to immunoblot analysis to determine its protein content.

*Antibodies and Western Blotting*—Polyclonal antiserum against human Rad51B or Rad51D was raised in rabbits using a synthesized peptide as the immunogen. The 6xHis-tagged bacterial recombinant protein of human Rad51C was purified and used for polyclonal antiserum production in rabbits. The human Rad51B, Rad51C, and Rad51D antibodies were affinity-purified from the antiserum. The human Rad51, XRCC2, and XRCC3 antibodies were kindly provided by Drs. Akira Shinohara, Nan Liu, and Patrick Sung, respectively.

The eluates from Ni-NTA bead pull-down experiments were separated in 10% SDS-polyacrylamide gels and transblotted to nitrocellulose. Western blotting analysis was performed with primary antibody α-Rad51, α-Rad51B, α-Rad51C, α-Rad51D, α-XRCC2, and α-XRCC3. Although the Rad51 paralogs and HsRad51 are members of a family of related proteins, no evidence was seen for cross-reactivity of any of the antibodies used. The secondary antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase, Bio-Rad, Inc.) was used at a 1:5000 dilution.

TABLE I
*Interaction of XRCC2 with human or mouse Rad51D in the yeast two-hybrid system*

| DNA-binding domain fusion | Activation domain fusion | β-Galactosidase activity | | |
|---|---|---|---|---|
| | | Exp. 1 | Exp. 2 | Average |
| | | *RLU*[a] | | |
| HsRad51D | XRCC2 | 4613 | 2899 | 3756 |
| XRCC2 | HsRad51D | 8203 | 8468 | 8336 |
| MmRad51D | XRCC2 | 486 | 523 | 505 |
| XRCC2 | MmRad51D | 280 | 284 | 282 |
| HsRad51D | HsRad51D | 30 | 27 | 29 |

[a] Relative light units (chemilumminescent detection; see "Experimental Procedures"). The background signal from negative control strains expressing non-interacting proteins or empty vectors is typically about 30 RLU, similar to HsRad51D/HsRad51D.

## RESULTS

*XRCC2 Interacts with Rad51D in the Yeast Two-hybrid System*—In two-hybrid experiments, the human Rad51D protein interacts strongly with the human XRCC2 protein (Table I). Prior to having the full-length human *RAD51D* cDNA, we used the mouse cDNA in some initial experiments (Fig. 1). All of the other mammalian Rad51 paralogs in our studies are the human proteins. Like the HsRad51D protein, the mouse Rad51D protein also interacts with XRCC2, although much more weakly (Table I). XRCC2 does not interact with itself (Fig. 1) nor does either HsRad51D (Table I) or MmRad51D (Fig. 1).

*Rad51C Interacts Weakly with Human Rad51D but More Strongly with Mouse Rad51D*—Human Rad51C was originally found to interact moderately with MmRad51D (Fig. 1), but to our surprise, Rad51C interacts only very weakly with the HsRad51D. The Y190 two-hybrid strain transformed with both *RAD51C* and *HsRAD51D* are pale blue on X-gal plates; similarly, the PJ69-4A strain is ade+, but very slow growing on plates lacking adenine (data not shown). A more detailed analysis of the interaction between Rad51C and Rad51D is presented below under three-hybrid results.

*The Yeast Rad51 Protein Interacts with Both the Human Rad51 and XRCC3 Proteins*—The yeast two-hybrid system was used to determine whether any of the human Rad51 paralogs could interact with the yeast (*S. cerevisiae*) Rad51 paralogs. The only strongly positive results were the interaction of XRCC3 with yeast Rad51 and the interaction of yeast Rad51 with human Rad51 (Table II). A very weak interaction was observed between yeast Rad51 and Rad51C and between yeast Rad55 and human Rad51. All of these results are consistent with the high conservation of the yeast and human Rad51 proteins (3).

*Three-hybrid Analysis Shows That the Interaction between Two Rad51 Paralogs Can Sometimes Be Enhanced by the Expression of a Third Paralog*—Expression of a third protein in a non-fused form can sometimes enhance a two-hybrid interaction, presumably by binding to one or both of the two-hybrid

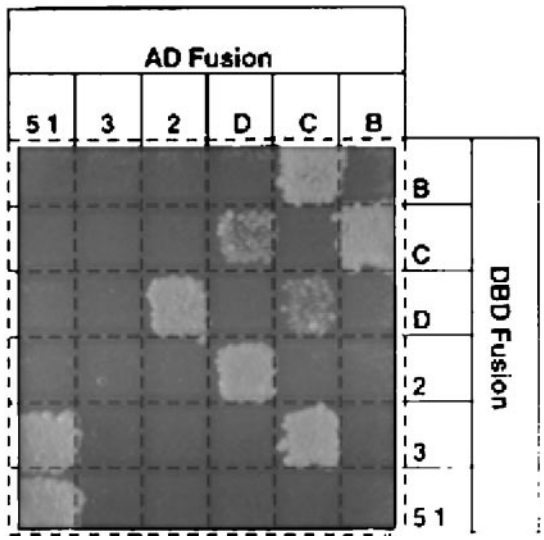


FIG. 1. **Each Rad51 paralog interacts with one or more of the other paralogs.** Thirty-six two-hybrid interactions were tested in yeast strain PJ69-4A and assayed for growth on a plate lacking adenine. *51*, HsRad51; *3*, XRCC3; *2*, XRCC2; *D*, MmRad51D; *C*, Rad51C; *B*, Rad51B; *AD*, activation domain; *DBD*, DNA-binding domain. Note that not all interactions are symmetrical in the two-hybrid system discussed in Ref. 10. PJ69-4A expressing HsRad51 and Rad51C cannot grow on plates lacking adenine, but strain Y190 expressing these two proteins is slightly blue on X-gal plates, indicating a very weak interaction (10).

TABLE II
*Interaction of yeast Rad51 with XRCC3 and human Rad51*

| DNA-binding domain fusion | Activation domain fusion | β-Galactosidase activity (ONPG assay) | | |
|---|---|---|---|---|
| | | Exp. 1 | Exp. 2 | Average |
| | | *Miller units* | | |
| XRCC3[a] | ScRad51 | 2.3 | 3.5 | 2.9 |
| XRCC3[a] | HsRad51 | 7.1 | 8.2 | 7.7 |
| XRCC3[b] | ScRad51 | 9.1 | 6.7 | 7.9 |
| XRCC3[b] | HsRad51 | 5.0 | 9.8 | 7.4 |
| HsRad51 | ScRad51 | 6.3 | 9.3 | 7.8 |
| HsRad51 | HsRad51 | 2.1 | 3.3 | 2.7 |
| HsRad51 | ScRad55 | .06 | .10 | .08 |
| HsRad52 | ScRad51 | .06 | .09 | .08 |
| Vector[c] | Vector[c] | .03 | .08 | .06 |

[a] XRCC3 in vector pGBT9.
[b] XRCC3 in vector pAS2.
[c] pGBT9 and pGAD424.

fusion proteins and stabilizing their interaction. Previously we reported a very weak interaction between Rad51C and HsRad51 (10). Using the three-hybrid system, we have now found that expression of XRCC3 greatly enhances the weak interaction between Rad51C and HsRad51 (Table III). The moderately strong interaction of XRCC3 with Rad51C was also reported previously (10), and in three-hybrid experiments, expression of Rad51B greatly enhances the interaction of XRCC3 with Rad51C (Table III). The specificity of the enhancement is seen in Fig. 2, since expression of the other proteins in their non-fused form does not influence the interaction of Rad51C with HsRad51 or of XRCC3 with Rad51C. A summary of our two- and three-hybrid results is diagrammed in Fig. 3.

Expression of Rad51B also greatly enhances the very weak interaction of Rad51C with HsRad51D (Table III; PJ69-4A data not shown). The enhanced three-hybrid interaction is much stronger when HsRad51D is fused to the DNA binding domain and Rad51C is fused to the activation domain. A similar orientation difference is also observed for the very weak two-hybrid result, as can be seen when no native protein is expressed (Rad51C/HsRad51D/vector is the same as background, but HsRad51D/Rad51C/vector is about 2-fold above background in

TABLE III
*Yeast three-hybrid experiments using two different strains indicate that many of the human Rad51 paralogs interact as heterotrimers*

β-Galactosidase assays were done in yeast strain Y190-ura$^-$ co-transformed with three plasmids. Growth on synthetic complete plates lacking adenine (SC-ade) was done with strain PJ69–4A co-transformed with three plasmids (data shown in Fig. 2). The term vector in the "Native protein" column refers to expression plasmid pVT100u, used (without an insert) as a control, and in the "DBD fusion" and "AD fusion" columns, vector refers to the pGBT9 and pGAD424 two-hybrid plasmids. Exp. 1: XRCC3 enhances the weak interaction of Rad51C with HsRad51, and Rad51B enhances the interaction of Rad51C with XRCC3. Exp. 2: Rad51B ehances the very weak interaction of Rad51C with human Rad51D. Rather surprisingly, in the absence of Rad51B, human Rad51C interacts better with mouse Rad51D than with human Rad51D. Exp. 3: Rad51C expression facilitates the indirect interaction of Rad51B with Rad51D, and Rad51B can bind two molecules of Rad51C. Note: although the interactions in Exp. 3 are only about 2-fold above background, they are also observed in strain PJ69-4A, using the *ADE2* reporter system (also see Fig. 2). DBD, DNA-binding domain. AD, activation domain; RLU, relative light units.

| Experiment | DBD fusion | AD fusion | Native protein | β-Galactosidase assay | Growth SC-ade |
|---|---|---|---|---|---|
| | | | | *RLU* | |
| 1 | Rad51C | HsRad51 | XRCC3 | 1618 | + |
| | Rad51C | HsRad51 | Vector | 58 | − |
| | XRCC3 | Rad51C | Rad51B | 5863 | + |
| | XRCC3 | Rad51C | Vector | 382 | + |
| 2 | HsRad51D | Rad51C | Rad51B | 3957 | + |
| | HsRad51D | Rad51C | Vector | 75 | − |
| | Rad51C | HsRad51D | Rad51B | 539 | − |
| | Rad51C | HsRad51D | Vector | 35 | − |
| | MmRad51D | Rad51C | Rad51B | 134 | + |
| | MmRad51D | Rad51C | Vector | 318 | + |
| | Rad51C | MmRad51D | Rad51B | 2280 | + |
| | Rad51C | MmRad51D | Vector | 638 | + |
| | Vector | Vector | Vector | 38 | − |
| 3 | Rad51B | MmRad51D | Rad51C | 45 | + |
| | Rad51B | MmRad51D | Vector | 25 | − |
| | Rad51C | Rad51C | Rad51B | 58 | + |
| | Rad51C | Rad51C | Vector | 28 | − |
| | Vector | Vector | Vector | 29 | − |

Table III). As discussed above, the interaction of Rad51C with MmRad51D is much higher than with HsRad51D except when Rad51B is present. The addition of Rad51B to the Rad51C-MmRad51D interacting pair gives inconsistent results, in one case increasing the interaction and in one case decreasing it. The quantification of interactions involving the MmRad51D constructs are somewhat questionable, however, because two-hybrid plasmids encoding this protein tend to give noticeable colony to colony variation (as tested by the X-gal filter assay), unlike our other two-hybrid constructs (data not shown).

*Bridging Experiments: Expression of a Third Rad51 Paralog Can Result in the Indirect Interaction of Two Paralogs*—Expression of a third protein in a non-fused form can sometimes allow the indirect interaction of two proteins that do not normally associate, presumably by binding to both of the two-hybrid fusion proteins and acting as a bridge (34–36). Our results indicate that these bridging experiments result in very weak three-hybrid results, frequently only about 2-fold above background using the β-galactosidase assay in yeast strain Y190-ura$^-$. These results are reproducible and are also seen using a different reporter system, growth of strain PJ69-4A on plates lacking adenine. In such bridging experiments, expression of Rad51C allows an interaction between Rad51B and MmRad51D (Table III and Fig. 2); in PJ69-4A, a similar three-hybrid interaction was seen with HsRad51D instead of MmRad51D (data not shown). In addition, expression of HsRad51D allowed the interaction of Rad51C with XRCC2, because the PJ69-4A triply co-transformed strain was ade$^+$,

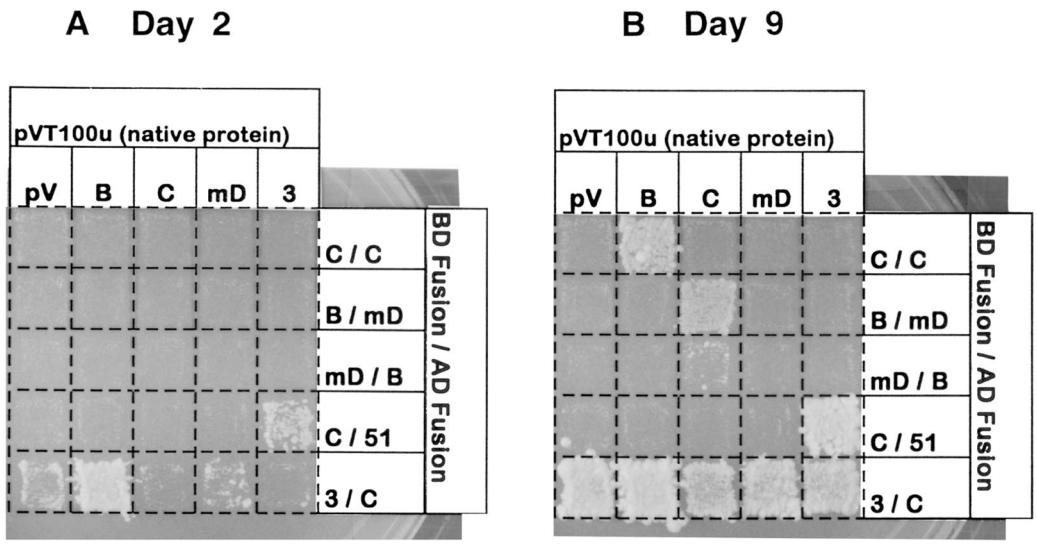


FIG. 2. **The yeast three-hybrid results for expression of a third protein are very specific.** Twenty-five PJ69-4A strains were co-transformed with the indicated combinations of three different plasmids and tested for the ability to grow on a plate lacking adenine. Labels across the *top* indicate the unfused protein expressed from vector pVT100U or empty vector (*pV*); see Fig. 1 for other abbreviations. Labels on the *right* identify the pair of proteins expressed as two-hybrid fusions of the DNA-binding (*BD*) and activation domains (*AD*), respectively. *A*, growth after 2 days at 30 °C. *B*, growth after 9 days at 30 °C. All three-hybrid strains with XRCC3 and Rad51C (*bottom row*) grow on this plate lacking adenine, but they grow much faster when Rad51B is expressed. *mD*, mouse Rad51D protein.

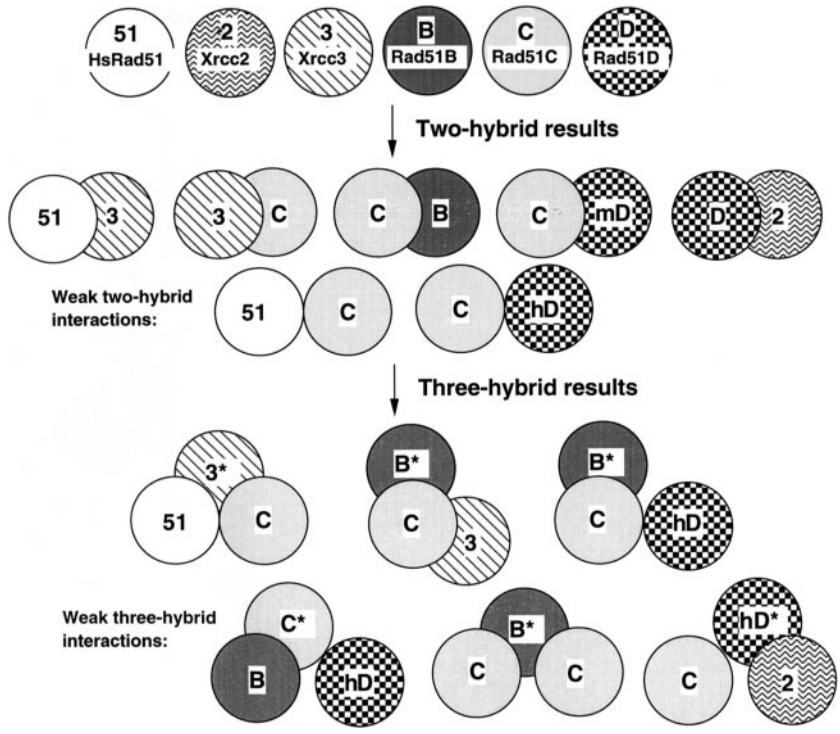


FIG. 3. **Summary of our two- and three-hybrid results.** Interactions that are moderately strong in the two-hybrid analysis are shown as *overlapping circles*, whereas interactions that are weak in the two-hybrid system are shown as *touching circles*. In the three-hybrid diagram, circles do not touch if the interaction is presumed to be indirect. Proteins expressed in a non-fused form in our reporter yeast strains are designated by "*". *hD* and *mD* refer to the human and mouse Rad51D proteins, respectively, and *D* refers to either.

albeit a very weak ade$^+$, and the triply co-infected Y190-ura$^-$ strain on X-gal plates was a pale blue (data not shown).

We also found evidence in both Y190-ura$^-$ and PJ69-4A for a very weak bridging in which Rad51C allows an interaction between HsRad51D and HsRad51 (data not shown). It is also possible that this result is more of an enhancement, because we have preliminary evidence indicating that HsRad51D may interact very weakly with HsRad51 in two-hybrid experiments. In Y190-ura$^-$ there was no evidence for an interaction, as assayed on X-gal plates, but in PJ69-4A, the transformants eventually grew up on plates lacking adenine but this growth required a month of incubation at 23 °C.

Expression of unfused Rad51B enables the interaction of Rad51C with itself (Table III), suggesting that Rad51B can bind to two molecules of Rad51C simultaneously. This interaction is very specific, because no other paralog tested could bind two molecules of Rad51C (Fig. 2). XRCC2 was not tested because it does not bind Rad51C in two-hybrid tests. For all other cases of interacting paralogs (*e.g.* XRCC2 and Rad51D, and Rad51D and Rad51C), we tested whether each one could bind to two molecules of the other. In each case, no other evidence was found of one paralog binding to two molecules of a second.

*Negative Three-hybrid Results*—Many additional three-hybrid combinations were tested and yielded negative results. It is hard to know whether these negative results are significant, and we place much less importance on them than on positive two- and three-hybrid results or even negative two-hybrid results. Even if three proteins interact, it seems likely that some

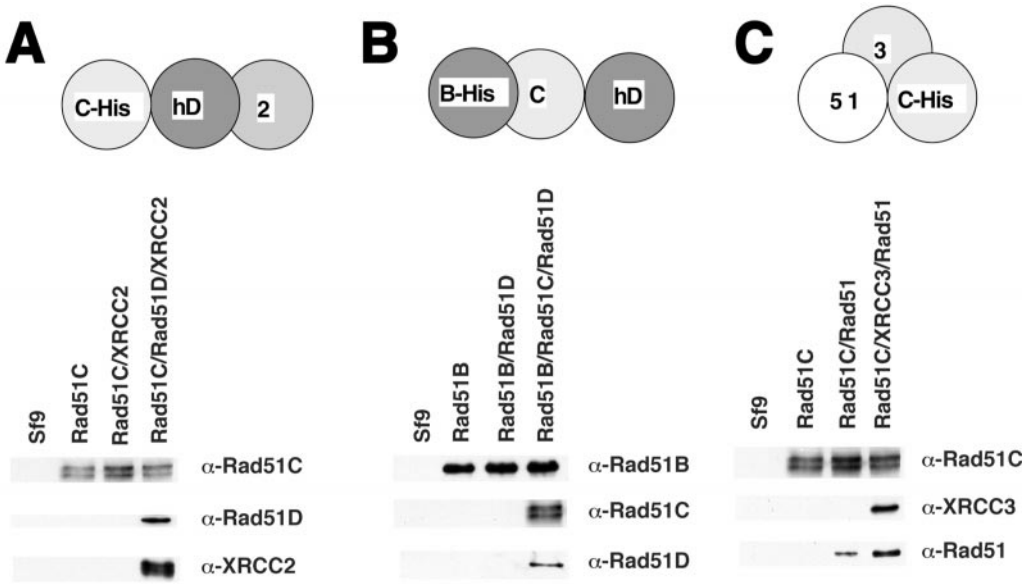


FIG. 4. **Baculovirus experiments indicate that Rad51C/Rad51D/XRCC2 and Rad51B/Rad51C/Rad51D can form heterotrimers and that Rad51C interacts directly with HsRad51.** The diagram at the *top* of each *panel* shows which protein contained a 6xHis tag and which three-hybrid result was being tested. In *each panel*: *lane 1*, uninfected Sf9 cell extract; *lane 2*, Sf9 singly infected with the 6xHis-tagged protein; *lane 3*, Sf9 doubly co-infected; and *lane 4*, Sf9 triply co-infected. Each gel was run in triplicate, and three Western blots were done, probing with three different antibodies; *each panel* shows the composite of these three Westerns. *A*, Rad51C pulls down XRCC2 but only in the presence of Rad51D. *B*, Rad51B pulls down Rad51D but only in the presence of Rad51C. *C*, Rad51C interacts with HsRad51 with or without XRCC3 but more strongly with XRCC3.

combinations would mask the DNA binding site or the transcriptional activation site or would result in the heterotrimer interacting with RNA polymerase in such a way that it was not positioned to transcribe the reporter gene. For thoroughness, however, a couple of the three-hybrid results that we might have expected to be positive are mentioned. No evidence has been found that Rad51C can interact simultaneously with both XRCC3 and Rad51D or with both HsRad51 and Rad51B. Actually, most of our negative three-hybrid results were expected to be negative based on our two-hybrid results. For example, XRCC3 would not be expected to act as a bridge between Rad51B and Rad51D (Fig. 2) because it does not interact with either in the two-hybrid system (Fig. 1).

*Baculovirus Experiments to Investigate the Simultaneous Interactions between Three Proteins*—The baculovirus system (37) and pull-down experiments with Ni-NTA beads were used to provide biochemical evidence for protein interactions. Baculoviral vectors for expressing each of the human Rad51 paralogs were constructed, as were similar constructs for expressing 6xHis-tagged versions of Rad51B and of Rad51C. Each recombinant vector was tested individually to ensure that all were suitable for expressing the appropriate recombinant protein (data not shown). Each construct expressed a single protein in insect cells except for the two Rad51C constructs (one with and one without a 6xHis tag), which each produced a doublet band. The presence of two Rad51C bands of similar size in the baculovirus system has also been observed frequently by Joanna Albala[3] using a different construct and a different polyclonal antibody (made against a Rad51C peptide). It is not known whether these two bands in the insect cells differ in a protein modification or in some type of protein truncation. In the case of the 6xHis-tagged Rad51C, both bands appear to contain the tagged N-terminal region, because both proteins bind to the Ni-NTA beads (Fig. 4, *A* and *C*).

For investigating three-protein interactions, Sf9 insect cells were infected with one, two, or three recombinant baculoviruses, only one of which contained a 6xHis tag (either Rad51B or Rad51C). Following gentle lysis of the infected cells, the 6xHis-tagged protein was isolated under non-denaturing conditions using its affinity for Ni-NTA magnetic agarose beads. The bound 6xHis protein, as well as any associated proteins, were eluted, denatured, and run on an SDS-polyacrylamide gel for Western blotting. Using Sf9 cells triply co-expressing Rad51C-6xHis and untagged XRCC2 and Rad51D, the results show that when Rad51C is isolated by binding to the Ni-NTA beads, both Rad51D and XRCC2 are also bound (Fig. 4*A*). In cells infected only with Rad51C-6xHis and XRCC2 (*i.e.* in the absence of Rad51D), Rad51C does not have XRCC2 bound. This finding confirms our very weak three-hybrid bridging result that Rad51C, Rad51D, and XRCC2 can simultaneously interact and that Rad51C can only indirectly interact with XRCC2, through an interaction with Rad51D. Similarly, the Rad51B-6xHis protein interacts with Rad51D only when Rad51C is also present (Fig. 4*B*), showing that these three proteins can interact simultaneously and that the interaction of Rad51B with Rad51D is indirect. Experiments with cells doubly co-infected with either Rad51B-6xHis and Rad51C, or with Rad51B and Rad51C-6xHis both showed that Rad51B and Rad51C interact with each other (data not shown), confirming our previously reported two-hybrid result (10).

The baculovirus system was also used to examine the interactions between the HsRad51, Rad51C, and XRCC3 proteins. In Sf9 cells doubly co-infected with Rad51C (with a 6xHis tag) and HsRad51, using Ni-NTA beads to pull down Rad51C also pulls down HsRad51 (Fig. 4*C*). In cells triply co-infected (*i.e.* also infected with XRCC3), pulling down Rad51C also pulls down both HsRad51 and XRCC3. Although both the doubly and triply co-infected Sf9 cells expressed HsRad51 and Rad51C at approximately the same level (data not shown), the amount of HsRad51 that was bound to Rad51C was quantified as about 2.5-fold higher in the cells expressing XRCC3 as compared with the cells not expressing XRCC3 (Fig. 4*C*). The baculovirus system confirms our two-hybrid result that Rad51C interacts with HsRad51 (10), and the higher level of HsRad51 bound to

[3] J. Albala, personal communication.

Rad51C in triply co-infected cells is consistent with our three-hybrid result that this interaction appears to be enhanced by the presence of XRCC3 (Table III).

## DISCUSSION

In the yeast *S. cerevisiae*, the two Rad51 paralogs Rad55 and Rad57 form a heterodimer that interacts with Rad51 through an interaction with Rad55 (4, 25, 26). The Rad55/Rad57 dimer appears to function by assisting Rad51 in strand transfer (4). Results from our yeast two-hybrid experiments indicate that each of the five human Rad51 paralogs can similarly interact with one or more of the other paralogs. In addition, XRCC3 and Rad51C can interact with HsRad51 as well (Refs. 5 and 10, and data presented here). Like Rad55 and Rad57, none of the human paralogs can interact with itself (Fig. 1). The fact that each human paralog has a unique set of interactions with the other paralogs suggests that these proteins are not functionally redundant and that each must therefore play a different role in recombinational repair. This conclusion is consistent with the fact that the *irs1* (defective for only XRCC2) and *irs1SF* (defective for only XRCC3) cell lines both exhibit a mutant phenotype, being sensitive to DNA damage and defective in recombinational repair of double-stranded breaks (38–40). In addition, the Rad51 paralogs have been individually knocked out in the DT40 chicken B lymphocyte line, and all of these cell-lines show increased sensitivity to DNA damage and increased spontaneous chromosomal instability (17).[1] Although each paralog is necessary for normal recombinational repair, the phenotypes of these mutant hamster and chicken cell lines are virtually identical, which suggests that these proteins are either involved in a single pathway or are part of a protein complex.

Both the two- and three-hybrid yeast systems and the baculovirus system are excellent for determining which proteins have an affinity for each other. The yeast two-hybrid system was used to show that each human Rad51 paralog has a specific set of interactions with other members of this family (Fig. 3). If these interactions occur simultaneously *in vivo* in human cells, then one would expect a single complex of proteins with the relationship shown in Fig. 5*A*. Because Rad51B appears able to bind two molecules of Rad51C in the yeast three-hybrid system, one might expect a larger complex such as that shown in Fig. 5*B*. For such complexes to occur, multiple individual interactions must occur simultaneously. This idea was initially tested using the yeast three-hybrid system, and many of these interactions were found to occur simultaneously (Fig. 3). Data from the yeast system was then used to decide which triple protein interactions to test in the baculovirus system. We intentionally tested two bridging experiments that gave weak results in the three-hybrid system (Rad51C bridging Rad51B and Rad51D, and Rad51D bridging Rad51C and XRCC2) and also tested both the direct interaction of Rad51C with HsRad51 (a very weak interaction in the yeast system) and its enhancement by XRCC3. All three sets of baculovirus experiments (Fig. 4) yielded results confirming the interactions seen in the yeast system. In addition, experiments with doubly co-infected insect cells confirmed a number of our other two-hybrid results, in particular that Rad51C can interact with Rad51B, but not with XRCC2, and that Rad51B cannot interact directly with Rad51D. The interaction of Rad51C with Rad51B in the baculovirus system has been shown independently.[3] The interaction of Rad51D with XRCC2 that we observed in the yeast two-hybrid system has also been seen using proteins purified from *E. coli*.[4] The combination of positive yeast two- and three-hybrid results and results from baculovirus and *E. coli* expressed proteins strongly suggests, but does not prove, that these interactions will also be found to occur *in vivo* in human cells. Co-immunoprecipitation of HsRad51 and XRCC3 from Hela cells (5) has shown that this interaction occurs in human cells. Since it has also been demonstrated that the related Rad55 and Rad57 proteins form a stable dimer *in vivo* in yeast (4), it would not be surprising to find that all of the observed interactions between human paralogs occur *in vivo* in human cells. Experiments with epitope-tagged paralogs overexpressed in human cell lines have been initiated to determine whether this is indeed the case.

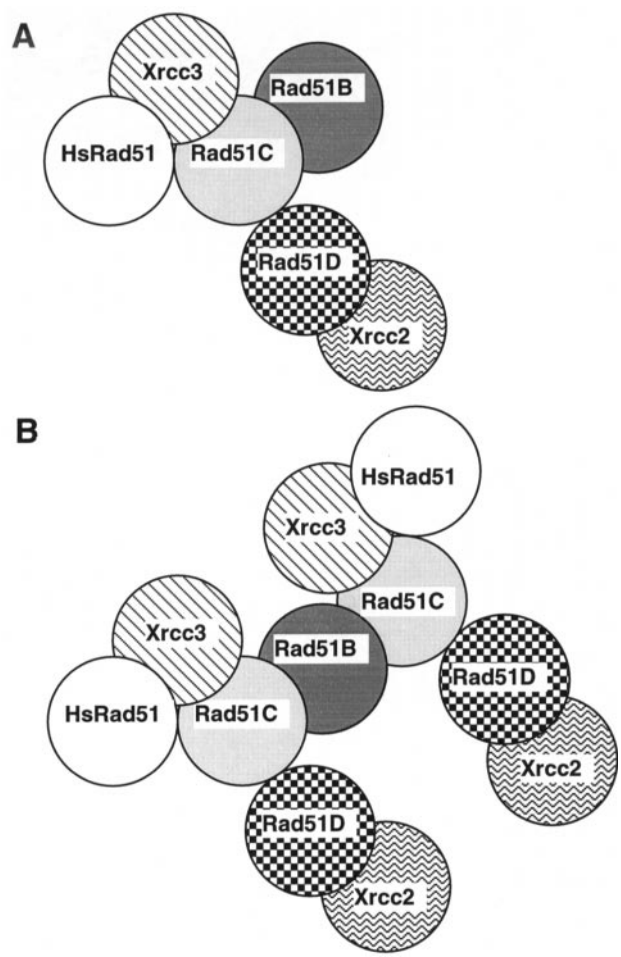


FIG. 5. **Hypothetical architecture of a complex of human Rad51 paralogs interacting with HsRad51.** *A*, diagram showing what the complex might look like if all of our observed interactions occurred simultaneously *in vivo*. *B*, the interaction of Rad51B with two molecules of Rad51C could result in this type of structure if this interaction did not interfere with any of the other interactions.

A cautionary note about the yeast two-hybrid system relates to the strength of the observed interactions. The relative strengths of the interactions in this system do not always correlate with the strengths *in vivo*. For example, the two-hybrid interaction of Rad55 with Rad51 is slightly stronger than the interaction of Rad55 with Rad57 (25, 26). However, experiments suggest the opposite occurs *in vivo*; the interaction of Rad55 with Rad57 is very stable, whereas the interaction of the Rad55/Rad57 dimer with Rad51 is much less stable (4). In our experiments with the baculovirus system, the strengths of the interactions were generally not tested. In one case, we did find evidence that the relative strength of the interaction of HsRad51 with Rad51C was increased by the presence of XRCC3 (Fig. 4*C*).

[4] J. P. Braybrooke, K. G. Spink, J. Thacker, and I. D. Hickson, submitted for publication.

Our three-hybrid and baculovirus analyses strongly suggest that the human Rad51 paralogs are not just interacting as separate heterodimers but at the least as heterotrimers. Many of the predictions, based on two-hybrid results, about which protein(s) might enhance or bridge other pairs were confirmed by three-hybrid analysis. In addition, experiments with the baculovirus system have been used to confirm a number of the results from the yeast system. In general, our results support the idea that all of the human Rad51 paralogs might be involved in one large complex, but our results are not inconsistent with a few smaller complexes. Some of our negative three-hybrid results, if confirmed, would lend support for more than one complex. For example, our experiments indicate that Rad51C can interact with four different partners individually and that it can interact with some sets of two different partners at once (*e.g.* with Rad51B and Rad51D, and with XRCC3 and HsRad51). Some of our negative three-hybrid results indicate, however, that Rad51C may not be able to simultaneously interact with some other pairs of partners (*e.g.* with XRCC3 and Rad51D, or with HsRad51 and Rad51B). As is often the case, however, negative results are usually less reliable than positive results.

Several lines of evidence suggest that the human Rad51-paralogs play a similar role to that played by the yeast Rad55 and Rad57 proteins. As previously discussed, the pattern of protein interactions involving the human and yeast Rad51-paralogs is similar. In addition, in chicken DT40 cells it has also been shown that mutations in the paralogs are partially complemented by overexpression of HsRad51 (17),[1] and this has also been observed in yeast (25, 26). Together, these results suggest that the human and yeast Rad51 paralogs probably share related functions, although there is currently no explanation as to why there are more human than yeast paralogs. In yeast, the Rad55/Rad57 dimer plays a role in assisting ScRad51 in forming a DNA filament on ssDNA, probably by helping ScRad51 displace the single-strand binding protein RPA (4). The formation of a Rad51 filament on ssDNA appears to be an early step necessary for the formation of paired DNA molecules and eventual strand exchange and recombination (41). It seems likely that the human Rad51-paralogs play a role in the formation of HsRad51/ssDNA filaments, although even if they do, they may have additional functions. For example, it has recently been reported that Rad51B (HsRec2/Rad51L1) has protein kinase activity (42).

If the human Rad51 paralogs are involved in one or more large complexes with HsRad51, as our results suggest, it is not clear what other proteins might participate. The human Rad51 protein has been shown to bind directly or indirectly to p53, c-Abl, BRCA1, BRCA2, BART1, Rad52, and Rad54 (reviewed in Refs. 19 and 43). Some or all of these proteins might also be involved in such a complex. It has been known for many years that many of the proteins involved in nucleotide excision repair are part of one or more complexes, but the details of these complexes are still not understood (44). In the area of recombinational repair of DNA damage, much work remains to be done to determine whether there really is a "recombinosome" and, if so, what proteins it contains.

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DNA: REPLICATION REPAIR AND RECOMBINATION:

# Evidence for Simultaneous Protein Interactions between Human Rad51 Paralogs

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