Copyright © 2001 by the Genetics Society of America

Multiple Interactions Among the Components of the Recombinational DNA Repair System in *Schizosaccharomyces pombe*

Yasuhiro Tsutsui,* Fuat K. Khasanov,† Hideo Shinagawa,* Hiroshi Iwasaki*,‡,1 and Vladimir I. Bashkirov†,1

**Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan,* ‡*PRESTO, JST, Suita, Osaka 565-0871, Japan and* †*Institute of Gene Biology, Russian Academy of Sciences, Moscow 117334, Russia*

Manuscript received March 8, 2001
Accepted for publication June 28, 2001

ABSTRACT

Schizosaccharomyces pombe Rhp55 and Rhp57 are RecA-like proteins involved in double-strand break (DSB) repair. Here we demonstrate that Rhp55 and Rhp57 proteins strongly interact *in vivo*, similar to *Saccharomyces cerevisiae* Rad55p and Rad57p. Mutations in the conserved ATP-binding/hydrolysis folds of both the Rhp55 and Rhp57 proteins impaired their function in DNA repair but not in cell proliferation. However, when combined, ATPase fold mutations in Rhp55p and Rhp57p resulted in severe defects of both functions, characteristic of the deletion mutants. Yeast two-hybrid analysis also revealed other multiple *in vivo* interactions among *S. pombe* proteins involved in recombinational DNA repair. Similar to *S. cerevisiae* Rad51p-Rad54p, *S. pombe* Rhp51p and Rhp54p were found to interact. Both putative Rad52 homologs in *S. pombe*, Rad22p and Rti1p, were found to interact with the C-terminal region of Rhp51 protein. Moreover, Rad22p and Rti1p exhibited mutual, as well as self-, interactions. In contrast to the *S. cerevisiae* interacting pair Rad51p-Rad55p, *S. pombe* Rhp51 protein strongly interacted with Rhp57 but not with Rhp55 protein. In addition, the Rti1 and Rad22 proteins were found to form a complex with the large subunit of *S. pombe* RPA. Our data provide compelling evidence that most, but not all, of the protein-protein interactions found in *S. cerevisiae* DSB repair are evolutionarily conserved.

DNA double-strand breaks (DSBs) are the major genotoxic lesions to cellular DNA and can be repaired in eukaryotes by several DNA repair pathways. Homologous recombination is one of the important pathways to repairing DSBs. Studies in *Saccharomyces cerevisiae* established that the genes comprising the *RAD52* epistasis group are responsible for faithful repair of DSBs by homologous recombination. At least 10 genes belong to this group: *RAD50*, *MRE11*, *XRS2*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, and *RFA1* (reviewed in Paques and Haber 1999). Mutations in these genes cause pleiotropic defects in DNA damage repair, including high sensitivity to ionizing radiation (IR) and the alkylating drug methyl methanesulfonate (MMS), and moderate UV sensitivity (Game 1993; Friedberg *et al.* 1995; Shinohara and Ogawa 1995; Bai and Symington 1996). Mitotic and/or meiotic recombination is also impaired in the mutants, although considerable heterogeneity of their phenotype exists, depending on the assay used (reviewed in Paques and Haber 1999). Rad50, Mre11, and Xrs2 proteins form a higher-order complex *in vivo*, which is believed to play a dual role as a structural component of a DSB repair complex and as a nucleolytic activity processing DNA ends (reviewed in Haber 1998; Hopfner *et al.* 2000). *RAD52* is the most important gene in the group as it is required for recombinational DSB repair and the full level of all types of homologous recombination events in the cell. *In vitro* it stimulates the Rad51p-catalyzed strand exchange reaction (Sung 1997a; New *et al.* 1998; Shinohara and Ogawa 1998; Sugiyama *et al.* 1998). Together with the structurally similar Rad59p, Rad52 protein also has a major role in single-strand annealing (SSA; Sugawara and Haber 1992; Bai *et al.* 1999; Sugawara *et al.* 2000). Consistent with this, both Rad52 and Rad59 proteins can mediate the annealing of complementary DNA strands *in vitro* (Mortensen *et al.* 1996; Petukhova *et al.* 1999a). The *RAD51* gene encodes the functional homolog of *Escherichia coli* RecA protein and was shown to form a nucleoprotein filament similar to the RecA-DNA filament (Ogawa *et al.* 1993). Rad51p is able to mediate homologous pairing and strand exchange *in vitro* (Sung 1994; Sung and Robberson 1995). Two other genes, *RAD55* and *RAD57*, also encode proteins with structural similarity to RecA. Rad55p and Rad57p form a stable heterodimer and promote Rad51p-mediated strand exchange in the presence of RPA but are not able to promote this reaction on their own (Sung 1997b). It is believed that the Rad55p:Rad57p heterodimer helps Rad51 protein to overcome the inhibitory binding of RPA to single-stranded DNA (Sung 1997b), the role hypothesized also for Rad52p (Sung 1997a;

Corresponding author: Vladimir I. Bashkirov, Institute of Gene Biology, Russian Academy of Sciences, 34/5 Vavilov St., Moscow 117334, Russia. E-mail: vibashkirov@mail.ru

[1]These authors contributed equally to this work.

New et al. 1998; Shinohara and Ogawa 1998). The product of the *RAD54* gene belongs to the Snf2p/Swi2p family of putative helicases involved in chromatin remodeling (Gorbalenya and Koonin 1993). Rad54p possesses a DNA-dependent ATPase activity (Petukhova et al. 1998) but has not been demonstrated to have helicase activity. It is suggested that Rad54 protein can facilitate Rad51p-mediated *in vitro* recombination reactions via its ability to remodel target DNA through specific interaction with Rad51-ssDNA (Petukhova et al. 1999b; Mazin et al. 2000; Van Komen et al. 2000).

Besides the stable complex of Rad55 and Rad57 proteins, other associations including Rad51-Rad52, Rad51-Rad54, Rad51-Rad55, and Rad52-RPA interacting pairs have been detected by two-hybrid, co-immunoprecipitation, and biochemical experiments (Donovan et al. 1994; Hays et al. 1995; Johnson and Symington 1995; Clever et al. 1997). Genetic and biochemical studies of these protein-protein interactions provided compelling evidence that they are important for recombinational DNA repair.

In the distantly related fission yeast *Schizosaccharomyces pombe*, the mechanisms of recombinational repair have been studied less than in *S. cerevisiae*. However, a number of genes involved in DSB repair in this microorganism have been identified in the last decade. Homologs of *RAD51* and *RAD54* have been isolated and named *rhp51*$^+$ and *rhp54*$^+$, respectively (Muris et al. 1993, 1996; Shinohara et al. 1993). *rhp55*$^+$ and *rhp57*$^+$ have been found to be putative homologs of *RAD55* and *RAD57*, respectively (Khasanov et al. 1999; Tsutsui et al. 2000). In addition, the *rad11*$^+$ and *rad32*$^+$ genes were found to be homologs of the large subunit gene of RPA (Parker et al. 1997) and *MRE11* (Tavassoli et al. 1995), respectively. Two putative homologs of *S. cerevisiae RAD52* have been identified in *S. pombe*, namely *rad22*$^+$ and *rti1*$^+$ (Ostermann et al. 1993; Suto et al. 1999). Similar to the mutations of the budding yeast *RAD52* group members, mutations in fission yeast genes, except *rti1*, confer sensitivity to IR and MMS and cause moderate defects in mitotic and/or meiotic recombination. However, there are noticeable differences in the function of *S. pombe* DSB repair proteins compared to their *S. cerevisiae* counterparts. The *S. pombe* mutants are highly sensitive to UV, implicating DSB repair mechanisms in an active UV damage response, presumably through involvement in the second UV excision repair (UVER) pathway and UV damage tolerance/recovery mechanism (reviewed in McCready et al. 2000). Moreover, the mitotic phenotypes of *rad32*, *rad22*, *rhp51*, *rhp54*, *rhp55*, and *rhp57* mutants suggest a high degree of genomic instability and proliferation defects, even in the absence of exogenous damage, which is less observed in the corresponding *S. cerevisiae* mutants (Ostermann et al. 1993; Tavassoli et al. 1995; Muris et al. 1996; Khasanov et al. 1999). Epistasis analysis placed all these genes except *rad22*$^+$ in one pathway of DSB repair (Tavassoli et al. 1995; Khasanov et al. 1999; Tsutsui et al. 2000). However, more recent characterization of the phenotypes of *rad22* and *rti1* deletion mutants (van den Bosch et al. 2001) suggests that the results of previous analysis were the consequence of the partial functional redundancy of these two Rad52p homologs and of the hypomorphic nature of the *rad22* allele used. Thus, it is likely that in *S. pombe* both *rad22*$^+$ and *rti1*$^+$ are also in the recombinational repair epistasis group. Taken together, these observations suggest that the recombinational repair pathway in *S. pombe* might be organized on the whole similarly to that of *S. cerevisiae*. The fact that the phenotypes of *Rad52*$^{-/-}$ mutants in vertebrates (Rijkers et al. 1998; Yamaguchi-Iwai et al. 1998) are more reminiscent of those found in fission yeast *rti1* mutants suggests the existence of a Rad22-like protein in higher eukaryotes and that the mechanism of DSB repair in *S. pombe* might provide a useful insight into that of higher eukaryotes. However, comparably few biochemical and molecular studies have been done with the *S. pombe* DSB repair proteins.

As a first step in the molecular characterization of the recombinational repair mechanism of *S. pombe*, we have undertaken a systematic analysis of protein-protein interactions. Here we report that Rhp55 and Rhp57 proteins strongly interact *in vivo*, suggesting that they represent an equivalent to the budding yeast Rad55p:Rad57p heterodimer. However, by mutating the conserved amino acid residues in ATP binding/hydrolysis motifs of Rhp55p and Rhp57p, we demonstrate a functional difference between the heterodimers in budding and fission yeast. Moreover, we show that certain interactions among seven *S. pombe* proteins appear to differ from those in *S. cerevisiae*, suggesting the mechanistic differences in DSB repair in these two distantly related yeasts.

MATERIALS AND METHODS

Strains, media, and growth conditions: The *S. pombe* strains used in this study were: BVY5 *h*$^-$ *smt-0 ura4-D18 leu1-32*; IBGY84 *h*$^-$ *smt-0 rhp55Δ::ura4*$^+$ *ura4-D18 leu1-32*; BVY233 *h*$^-$ *smt-0 rhp55 K57A ura4-D18 leu1-32*; BVY234 *h*$^-$ *smt-0 rhp55K57R ura4-D18 leu1-32*; BVY246 *h*$^-$ *smt-0 rhp55K57A rhp57K106A ura4-D18 leu1-32*; BVY247 *h*$^-$ *smt-0 rhp55K57R rhp57K106R ura4-D18 leu1-32*; MP11 *h*$^+$ *ura4-D18 leu1-32*; TMP761 *h*$^+$ *ura4-D18 leu1-32 rhp57K106R*; TMP762 *h*$^+$ *ura4-D18 leu1-32 rhp57K106A*; TMP-711 *h*$^+$ *rhp57Δ::ura4*$^+$ *ura4-D18 leu1-32 his3-766 ade6-M216*; and TMP754 *h*$^-$ *smt-0 rhp55Δ::arg3*$^+$ *rhp57Δ:: his3*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*. The media, yeast extract agar (YEA), minimal media (MMA and EMM), yeast extract liquid (YEL), and genetic manipulations with *S. pombe* have been described elsewhere (Gutz et al. 1974; Alfa et al. 1993). MMS was added to precooled media before pouring and the plates were used the same day. Strain DH5α was used as a host for gene manipulations in *E. coli*.

Yeast two-hybrid analysis and genetic methods: Pairwise combinations of DNA-binding domain and activator fusions were transformed into the reporter strain, and three colonies grown on selective medium were used for quantitative β-galac-

tosidase assays in each case (Harshman *et al.* 1988). β-Galactosidase activity was expressed in Miller units. Two-yeast two-hybrid systems were employed. The first consists of LexA fusion and activator fusion plasmids and reporter *S. cerevisiae* strain EGY48 (Gyuris *et al.* 1993; Golemis *et al.* 1994). This system was used for analysis of pairwise interactions between full-length Rad22, Rhp51, Rhp54, Rhp55, and Rhp57 proteins. The second system was based on LexA DNA-binding domain (DBD) and B42 activation domain (AD) fusions and *S. cerevisiae* reporter strain L40 (Invitrogen, San Diego). It was employed for analysis of interactions among Rhp51ΔN, Rhp51ΔC, Rad22, Rti1, and Rpa1 proteins.

The sensitivity to MMS of wild-type and mutant cells was tested by the drop assay. Sequential 10-fold dilutions of exponentially growing cells were spotted on the appropriate plates with or without MMS, and plates were incubated at the indicated temperatures.

Plasmid construction and DNA manipulations: The cDNAs of *rhp51*$^+$, *rhp57*$^+$, *rad22*$^+$, and *rad11*$^+$ were amplified by PCR from an *S. pombe* cDNA library. The cDNAs of *rhp54*$^+$, *rhp55*$^+$, and *rti1*$^+$ were synthesized by reverse transcription (RT)-PCR using 1 μg of total RNA with the RNA LA PCR kit (TaKaRa, Japan) according to manufacturer's instructions. To generate an *Nde*I site at the ATG initiation codon and a *Bam*HI site downstream from the termination codon of each cDNA, the following PCR primers were used: 5′-TCACATATGGCAGATACAGAGGTGG-3′ (TW51-1) and 5′-TCAGGATCCTTAGACAGGTGCGATAATTTCCTTGGG-3′ (TW51-2) for *rhp51*$^+$; 5′-TCACATATGTCTTTTGAGCAAAAACAG-3′ (TW22-1) and 5′-TCAGGATCCTTATCCTTTTTTGGCTTTCTTATCCAC C-3′ (TW22-2) for *rad22*$^+$; 5′-TCACATATGATTCAGCAACCAACAAC-3′ (TW54-1) and 5′-TCAGGATCCTTAATGAGATTTGTATTGG-3′ (TW54-2) for *rhp54*$^+$; 5′-TCACATATGCTTGTCTAGTCAACATC-3′ (TW55-1) and 5′-TCAGGATCCCTAGGACTCACATTCC-3′ (TW55-2) for *rhp55*$^+$; 5′-TCACATATGGATATTTCGAATTATG-3′ (TW57-1) and 5′-TCAGGATCCTAGCACGAATATATCCCAACC-3′ (TW57-2) for *rhp57*$^+$; 5′-TCACATATGGGCTCGCTACCTG-3′ (TWRTI1-1) and 5′-TCAGGATCCTTATTTCGTTGAGAACG-3′ (TWRTI1-2) for *rti1*$^+$; and 5′-TCACATATGGCTGAGCGATTATCC-3′ (TW11-1) and 5′-CCTTATTGAGCAGACTCAATG-3′ (TW11-2) for *rad11*$^+$. The PCR products were digested with *Nde*I (partially in the case of *rhp51*$^+$) and *Bam*HI and cloned into the *Nde*I-*Bam*HI site of pUC19 to result in the following plasmids: pYS201 for *rhp51*$^+$, pYS202 for *rad22*$^+$, pYS203 for *rhp54*$^+$, pYS204 for *rhp55*$^+$, pYS205 for *rhp57*$^+$, pYS206 for *rad11*$^+$(RPA1), and pYS207 for *rti1*$^+$. The primary sequences of cloned cDNAs were confirmed by sequencing. To construct the plasmids with truncated *rhp51*$^+$ alleles, the 28-mer (5′-CTGCATATGTACCATATTCGAAGAAGTG-3′) and TW51-2 primer together with pYS201 DNA as a template were used to amplify the coding region, which lacked 340 bases of the 5′ end of *rhp51*$^+$ cDNA. The PCR product was cloned into the *Nde*I-*Bam*HI of pUC19 to result in the *rhp51ΔN* plasmid named pYS208. For C-terminal truncation, the 0.7-kb region between *Nsp*V and *Sty*I of pYS201 was excised to generate the *rhp51*ΔC plasmid named pYS209.

Two sets of yeast two-hybrid plasmids were employed in this study. The first consists of LexA DBD fusion plasmid pEG202 and AD fusion plasmid pJG4-5 (Gyuris *et al.* 1993). The second consists of DBD fusion plasmid pHybLex/Zeo and AD fusion plasmid pYESTrp2 (Invitrogen).

The first set was used for construction of DBD and AD fusions of the full-length *rhp51*$^+$, *rhp54*$^+$, *rhp55*$^+$, *rhp57*$^+$, and *rad22*$^+$ genes. This was performed by in-frame cloning of coding sequences (cds) of the respective genes generated by Pfu polymerase-mediated PCR on the *S. pombe* cDNA library. The *rhp51*$^+$ cds was amplified using 5′-GGCCTCGAGATGGCAGATACAGAGGTGG-3′ and 5′-GGCCTCGAGTTAGACAGGTGCGATAATTTCC-3′ primers and cloned in the *Xho*I site to produce pEG202-*rhp51*$^+$ and pJG4-5-*rhp51*$^+$. The *rhp54*$^+$ cds was amplified with either 5′-GGCGTCGACTAATGATTCAGCAACCAACAACTG-3′ and 5′-GGCGTCGACTTAATGAGATTTGTATTGGAAAACGG-3′ or 5′-GGCGTCGACATGATTCAGCAACCAACAACTG-3′ and 5′-GGCGTCGACTTAATGAGATTTGTATTGGAAAACGG-3′ primers and cloned into the *Sal*I site of pEG202 or the *Xho*I site of pJG4-5 to generate pEG202-*rhp54*$^+$ and pJG4-5-*rhp54*$^+$, respectively. The *rhp55*$^+$ cds was amplified using either 5′-GGCGAATTCATGCTGTCTAGTCAACATC-3′ and 5′-GGCGGATCCTAGGACTCACATTCC or 5′-GGCGAATTCATGCTGTCTAGTCAACATC-3′ and 5′-GGCCTCGAGCTAGGACTCACATTCCAAAATG primers and cloned as the *Eco*RI-*Bam*HI or *Eco*RI-*Xho*I fragment, respectively, to produce pEG202-*rhp55*$^+$ or pJG4-5-*rhp55*$^+$ plasmids. The cds of the *rhp57*$^+$ gene was amplified by PCR using 5′-GGCCTCGAGATGGATATTTCGAATTATGTTG-3′ and 5′-GGCCTCGAGCTAGCACGAATATATCCCAACC-3′ primers and cloned as an *Xho*I fragment to construct pEG202-*rhp57*$^+$ and pJG4-5-*rhp57*$^+$ plasmids. The *rad22*$^+$ cds was amplified with primers 5′-GGCGAATTCATGTCTTTTGAGCAAAAACAGC-3′ and 5′-GGCCTCGAGCCAATCATCACATTTTGCCTC-3′ and cloned as an *Eco*RI-*Xho*I fragment to construct pEG202-*rad22*$^+$ and pJG4-5-*rad22*$^+$. All fusion constructions were sequenced across the fusion junctions.

The second set of plasmids was first modified by generating an *Nde*I site to allow in-frame recloning of cDNAs from pUC19-based constructs. The *Eco*RI-*Nde*I-*Sac*I adaptor (5′-AATTCCATATGGAGCT-3′/5′-CCATATGG-3′) and a *Hin*dIII-*Nde*I-*Kpn*I adaptor (5′-AGCTTCATATGGGTAC-3′/5′-CCATATGA-3′) were inserted into the *Eco*RI-*Sac*I site of pHybLex/Zeo and the *Hin*dIII-*Kpn*I site of pYESTrp2, respectively, resulting in pHybLex/ZeoN and pYESTrp2N. Then, for DBD fusion construction each *Nde*I-*Sal*I cDNA fragment excised from the pYS20X series plasmids was ligated into the *Nde*I-*Sal*I site of pHybLex/ZeoN. This resulted in plasmid pYS211 for *rhp51*$^+$, pYS212 for *rad22*$^+$, pYS213 for *rhp54*$^+$, pYS214 for *rhp55*$^+$, pYS215 for *rhp57*$^+$, pYS216 for *rad11*$^+$, pYS217 for *rti1*$^+$, pYS218 for *rhp57*ΔN, and pYS219 for *rhp51*ΔC. For the AD fusion constructs, each *Nde*I-*Bam*HI cDNA fragment excised from the pYS20X series plasmids was ligated into the *Nde*I-*Bam*HI site of pYESTrp2N. The resultant plasmids were designated pYS221 for *rhp51*$^+$, pYS222 for *rad22*$^+$, pYS223 for *rhp54*$^+$, pYS224 for *rhp55*$^+$, pYS225 for *rhp57*$^+$, pYS226 for *rad11*$^+$, pYS227 for *rti1*$^+$, pYS228 for *rhp51*ΔN, and pYS229 for *rhp51*ΔC.

The construct for overexpression of His$_6$-tagged Rhp55p in *E. coli* was made by PCR cloning of cDNA between the *Nde*I and *Bam*HI sites of pET-14b vector (Novagen). The following primers were employed for PCR of *rhp55*$^+$ cds: 5′-AGGCCATATGCTGTCTAGTCAACATCG-3′ and 5′-GGCGGATCCTAGGACTCACATTCC-3′. To produce Rhp57p in *E. coli*, the *Nde*I-*Bam*HI *rhp57*$^+$ fragment excised from pYS205 was ligated into the *Nde*I-*Bam*HI site of pET15b to result in pYS275.

Two plasmids were constructed for performing co-immunoprecipitation experiments. Recloning of the *Nde*I-*Bam*HI *rhp57*$^+$ fragment from pYS205 into the *Nde*I-*Bam*HI site of pREP1 resulted in the pYS235 plasmid. To construct the pYS255 plasmid two primers, 5′-GCTCAGTGCGGCCGCATGCTGTCTAGTCAAC-3′ and 5′-CTCTGTCGACTAGGACTCACATTCC-3′, and pYS204 template were used for PCR to generate a new *Not*I site upstream of the ATG initiation codon and a *Sal*I site downstream from the TAG termination codon of *rhp55*$^+$ cDNA, respectively. The PCR product was digested with *Not*I and *Sal*I and ligated into the *Not*I-*Sal*I site of pSLF173 (Forsburg and Sherman 1997) to give pYS255.

Site-directed mutagenesis of the *rhp55*$^+$ gene cloned into the pIRT2 vector [plasmid pIBG81 (Khasanov *et al.* 1999)]

was performed using the QuickChange system from Stratagene (La Jolla, CA). Two sets of synthetic oligonucleotide primers were used: (1) to generate the *rhp55K57R* mutant gene, 5′-ACCTGGGATGGGAAGAACAAGTTTGGC-3′ and 5′-GCCAAACTTGTTCTTCCCATCCCAGGT-3′, and (2) to generate the *rhp55K57A* mutant gene, 5′-CACCTGGGATGGGAG CAACAAGTTTGGCTTTAC-3′ and 5′-GTAAAGCCAAACTT GTTGCTCCCATCCCAGGTG-3′. Site-directed mutagenesis and construction of pREP1-*rhp57K106A* and pREP1-*rhp57K106R* were described in Tsutsui *et al.* (2000).

Generation of the *rhp55*K57A and -K57R and *rhp57*K106A and -K106R alleles in a chromosome: To construct strains with chromosomal mutations *rhp55K57A* and *rhp55K57R*, the plasmid pIBG81 with the mutated *rhp55*$^+$ gene was cut with *Sal*I-*Kpn*I and the DNA fragment containing the *rhp55*$^+$ promoter region, mutated *rhp55*$^+$ cds, and 3′ UTR was isolated. This fragment was cotransformed together with the pIRT2 vector into the IBGY84 strain, followed by selection for Leu$^+$ transformants. The chromosomal integrants with the mutated *rhp55*$^+$ gene were selected by replica plating on 5-fluoroorotic acid (5-FOA) plates as Ura$^-$ colonies, and the presence of the desired mutations was verified by genomic sequencing.

To generate chromosomal *rhp57* point mutant genes with K106A or K106R alterations in Walker A motif, the pop-in/pop-out method was used (Scherer and Davis 1979). Briefly, pYS298 (*rhp57K106A*) or pYS299 (*rhp57 K106R*), both of which carry the *ura4*$^+$ gene and the *Dra*I-*Kpn*I fragment of the *rhp57* mutant gene, was digested with *Mun*I, and the wild-type strain MP11 was transformed with the linearized plasmid. Ura$^+$ transformants were plated onto EMM plates containing 5-FOA, uracil, and leucine to select the "pop-out" of the *ura4*$^+$ marker. The Ura$^-$ clones were confirmed by genomic Southern analysis. Then the *rhp57* mutants with the *K106R* (TMP761) or *K106A* (TMP762) mutation were identified by genomic sequencing.

Protein methods: Polyclonal antibodies against Rhp55p (generated in rabbits) and against Rhp57p (generated in rabbits and rats) were produced using the immunization protocol described previously (Santos Rosa *et al.* 1996). His_6-tagged Rhp55 protein was purified on Ni-NTA agarose (QIAGEN, Valencia, CA) under denaturing conditions after overexpression in *E. coli*. The crude extract of *E. coli* cells overexpressing His_6-tagged Rhp57 protein was fractionated by ammonium sulfate precipitation (50% saturation), and the protein was further purified by preparative SDS-PAGE and electroeluted from the gel. The antibodies were affinity purified from the immune serum as described (Pringle *et al.* 1991).

For immunoprecipitation experiments, strain TMP754 was transformed with plasmid pHA-Rhp55 (pYS255) and/or pRhp57 (pYS235). The cells were grown in EMM medium containing 10 μg/ml thiamine and selected for the presence of plasmids. Eighteen hours after the depletion of thiamine, cells were harvested and washed with water. Fifty OD_{595} units of cells were resuspended in 300 μl of lysis buffer (25 mm Tris-HCl pH 7.5, 1% Triton X-100, 50 mm NaCl, 2 mm EDTA, 1 mm dithiothreitol (DTT), 1 mm phenylmethylsulfonyl fluoride, 5 μl/ml leupeptin, and 5 μl/ml aprotinin) and extracted using acid-washed glass beads (0.45 μm). After lysate was clarified by centrifugation, the protein extract was diluted 1:10 with T buffer (50 mm NaCl, 20 mm Tris-HCl pH 7.5, and 10% glycerol) containing 0.1% BSA, 0.5% NP-40, and 1 mm DTT. Anti-hemagglutinin (HA) antibody (12CA5; Roche Molecular Biochemicals, Indianapolis), or anti-Rhp57 rabbit antibody was added, and extracts were rocked for 1 hr at 4°. Ten microliters of 50% slurry of protein A-Sepharose 4FF (Amersham Pharmacia Biotech) prewashed in T buffer was added, and incubation was continued for 1 hr at 4°. Immunoprecipitates were washed three times with T buffer, resuspended in 40 μl of 5% SDS, and incubated for 10 min at 37°. After centrifugation, the supernatant was mixed with 10 μl of SDS-PAGE sample buffer, boiled for 5 min, separated by SDS-PAGE, and subjected to Western blotting. Immunodetection was performed using ECL substrate (Amersham Pharmacia Biotech).

RESULTS

Rhp55p and Rhp57p interact *in vivo*: We previously reported the identification and cloning of two *S. pombe* genes, *rhp55*$^+$ and *rhp57*$^+$, with similarity to *E. coli* RecA (Khasanov *et al.* 1999; Tsutsui *et al.* 2000). Genetic characterization of the corresponding mutations and analysis of amino acid (aa) sequences suggested that Rhp55p and Rhp57p are likely to represent the *S. pombe* homologs of *S. cerevisiae* Rad55p and Rad57p, respectively. As Rad55p and Rad57p were shown to form a stable heterodimer *in vivo* (Sung 1997b), we tested the interaction between their *S. pombe* counterparts using a yeast two-hybrid system. Figure 1A shows that the AD-Rhp55 construct activated transcription of the *lacZ* reporter 24-fold when present in the cell together with DBD-Rhp57. Reciprocally, an even higher increase in β-galactosidase activity (147-fold) was observed when the AD-Rhp57 fusion was combined with the DBD-Rhp55 construct. No evidence of Rhp55p-Rhp55p or Rhp57p-Rhp57p interaction was found. Thus, Rhp55p appears to interact with Rhp57p, regardless of whether Rhp55p or Rhp57p is fused to AD or DBD.

To corroborate this result, we demonstrated the existence of the Rhp55p:Rhp57p complex in the cell by immunoprecipitation. We used anti-Rhp57p antibodies for immunoprecipitation and showed that Rhp55 and Rhp57 proteins can be coprecipitated (Figure 1B, lanes 6). Detection of Rhp55p was performed using anti-HA antibodies, as the Rhp55 protein was tagged with the HA epitope. In a control strain lacking Rhp57p, no Rhp55p was precipitated (Figure 1B, lanes 4), indicating that the HA-tagged Rhp55p is not simply precipitated by itself. In a strain lacking HA-Rhp55p, Rhp57 protein was precipitated (Figure 1B, lane 5, left), but HA-specific signal was not detectable (Figure 1B, lane 5, right). The specificity of the antibodies used was demonstrated by direct probing of crude cell extracts with anti-Rhp57 antibodies (Figure 1B, lanes 1–3, left) or anti-HA antibodies (Figure 1B, lanes 1–3, right).

Rhp55p:Rhp57 complexes were also identified in reciprocal immunoprecipitation experiments using anti-HA antibodies to precipitate Rhp55 protein (Figure 1C, lanes 6). Again, the control experiments demonstrated the specificity of interaction (Figure 1C, lanes 4 and 5) and the specificity of antibodies (Figure 1C, lanes 1–3).

Mutations in Walker motif A of Rhp55 and Rhp57 proteins result in impaired DNA repair: The *in vivo* interaction between Rhp55p and Rhp57p together with previously published genetic analyses of the *rhp55*Δ and *rhp57*Δ mutants (Khasanov *et al.* 1999; Tsutsui *et al.*

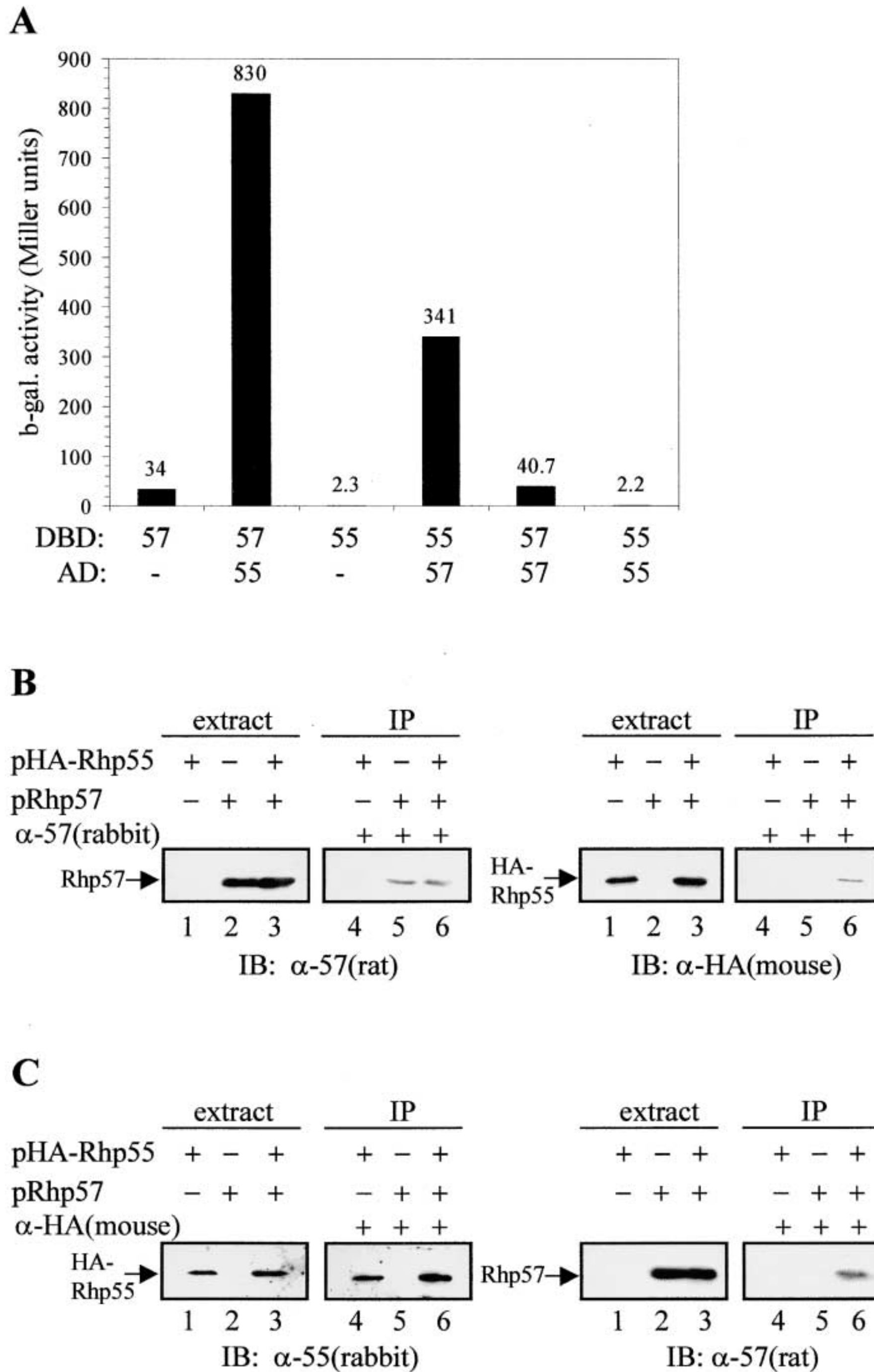

Figure 1.—Rhp55 and Rhp57 proteins strongly interact and form a complex *in vivo*. (A) Interaction in the yeast two-hybrid system. (B) Co-immunoprecipitation of Rhp55 and Rhp57 proteins using anti-Rhp57 antibodies. (C) Co-immunoprecipitation of Rhp55 and Rhp57 proteins with anti-HA antibodies.

2000) suggests that the Rhp55p:Rhp57 pair may be functionally similar to the budding yeast Rad55:Rad57 heterodimer. It has been shown that mutations affecting highly conserved residues in the ATP-binding/hydrolysis Walker A box (GxxxxGKT/S) of *S. cerevisiae* Rad55p, but not Rad57p, completely abolished its function in DNA damage repair (Johnson and Symington 1995). We asked whether equivalent mutations in box A of Rhp55p and Rhp57p would affect the repair of MMS-induced DNA damage. The conserved lysine residue within the GKT motif of Rhp55p box A was changed by site-directed mutagenesis to alanine or arginine, generating two mutant alleles, *rhp55K57A* and *rhp55K57R*. The generation of Walker A box mutations *K106A* and *K106R* in Rhp57p was described previously (Tsutsui *et al.* 2000). The effect of mutations of Walker A box on DNA repair in *S. cerevisiae* was examined in complementation experiments with mutant *rad55* and *rad57* genes expressed from a centromeric vector in the corresponding mutant strain, thus approximating the situation with the single-copy genes expressed from their chromosomal loci. As there is no centromeric vector available in *S. pombe*, we generated strains with chromosomal mutations in Walker A box of $rhp55^+$ and $rhp57^+$ genes by

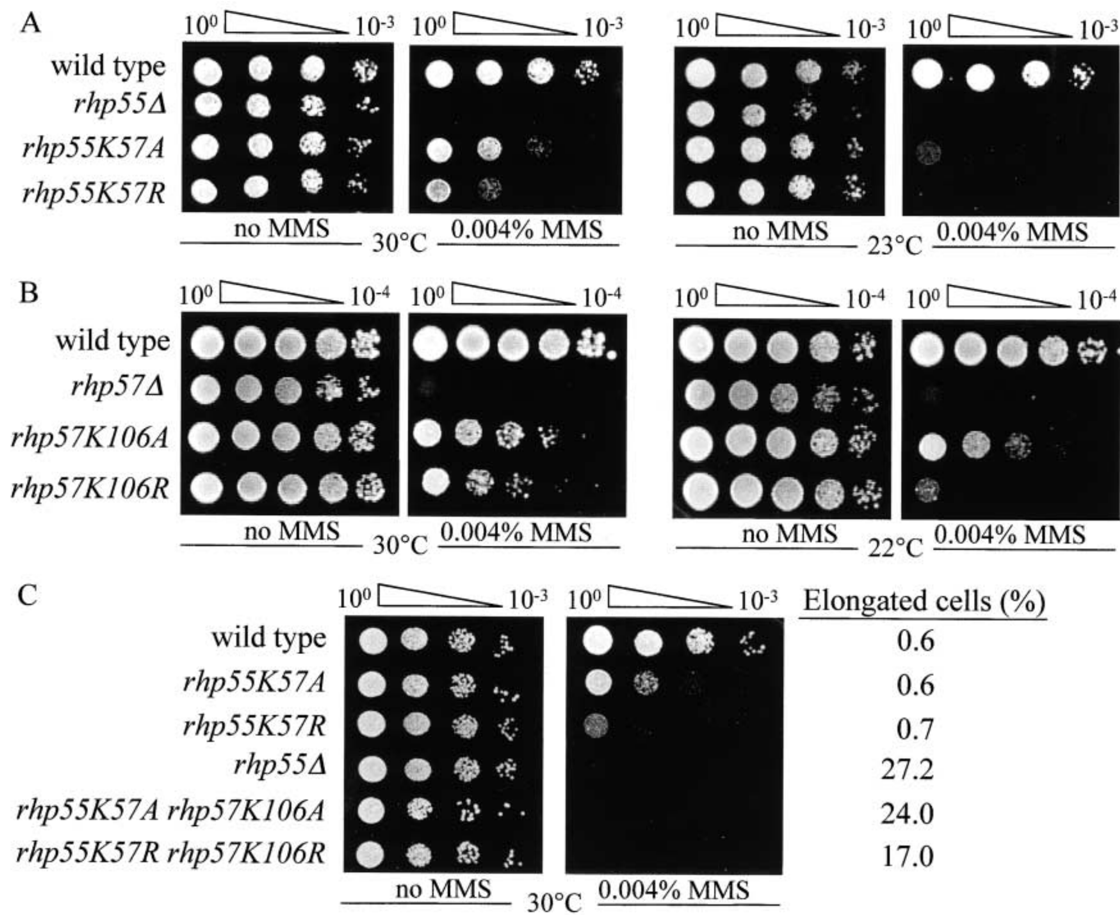

Figure 2.—Mutations in Walker A box of Rhp55p and Rhp57p cause a defect in repair of MMS-induced DNA damage. Serial dilutions of late-log phase cultures were spotted on YEA plates with or without MMS. Plates were incubated at 30° for 4 days or at 22°–23° for 7 days. (A) Drop assay of wild-type (BVY5), *rhp55*Δ (IBGY84), *rhp55K57A* (BVY233), and *rhp55K57R* (BVY234) strains. (B) Drop assay of wild-type (MP11), *rhp57*Δ (TMP711), *rhp57K106R* (TMP761), and *rhp57K106A* (TMP762) strains. (C) Drop assay and quantification of the fraction of elongated cells of wild-type (BVY5), *rhp55K57A* (BVY233), *rhp55K57R* (BVY234), *rhp55*Δ (IBGY84), *rhp55K57A rhp57K106A* (BVY246), and *rhp55K57R rhp57K106R* (BVY247) strains. The fraction of elongated cells was counted in the exponentially growing cultures at 23°. At least 1000 cells were examined microscopically.

transplacement of the wild-type alleles, as described in materials and methods. The sensitivity of the resultant mutants to MMS-induced damage was tested in a drop assay along with that of wild-type and *rhp55*Δ and *rhp57*Δ cells (Figure 2, A and B). The *K57A* and *K57R* mutations in *rhp55*$^+$ resulted in sensitization of cells to MMS in comparison with the isogenic wild-type cells, and this effect was more pronounced at low-incubation temperature (Figure 2A). However, the mutant strains were significantly more resistant to MMS than the strain with a complete deletion of the *rhp55*$^+$ gene. Likewise, *rhp57K106A* and *rhp57K106R* mutants were more sensitive to MMS than the isogenic wild-type strain, and the deficiency was more severe at lower temperature (Figure 2B). Again, as with *rhp55* mutants, the *rhp57* mutants were not as sensitive to MMS as the *rhp57*Δ mutant at either 30° or 22°. However, when combined, *KA* mutations in both subunits of the heterodimer sensitized cells to the level of the *rhp55* deletion mutant (Figure 2C). Likewise, *KR* double mutants also showed a synergistic increase in sensitivity to MMS (Figure 2C). This indicates that the ATP binding/hydrolysis folds of both proteins are the major determinants of functionality of the Rhp55p:Rhp57p complex in the repair of MMS-induced DNA damage.

Remarkably, in both *rhp55* and *rhp57* mutants the substitution of lysine by arginine had a stronger effect on cell viability in the presence of MMS than the change of lysine to alanine (Figure 2, A and B). However, when overexpressed from high-copy-number plasmids, the mutant Rhp55K57A or Rhp55K57R and Rhp57K106A or Rhp57K106R proteins were all able to restore the deficiency in repair of MMS-induced damage of the corresponding *rhp55* and *rhp57* deletion mutants (Figure 3, A and B). This indicates that the requirement for the ATP binding/hydrolysis function of Rhp55p and Rhp57p in the repair of MMS damage can be overcome by simple mass action of the mutated protein. This also demonstrates that the level of the mutant protein is crucial for the resultant cellular phenotype and suggests

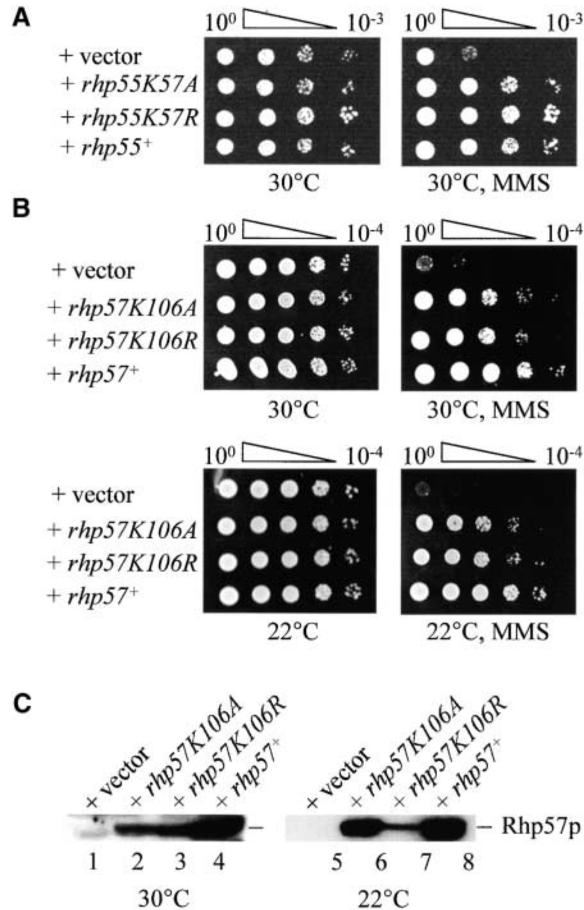

Figure 3.—Overexpression of mutant Rhp55 and Rhp57 proteins suppresses the sensitivity of the corresponding *rhp55* and *rhp57* deletion mutants. (A) Drop assay of *rhp55*Δ strain (IBGY84) transformed with pIRT2 empty vector, pIRT2-*rhp55*$^+$ (pIBG81), pIRT2-*rhp55K57A*, and pIRT2-*rhp55K57R*. (B) Drop assay of *rhp57*Δ strain (TMP711) carrying pREP1, pREP1-*rhp57*$^+$ (pYS235), pREP1-*rhp57K106A*, and pREP1-*rhp57K106R*. Serial dilutions of late-log phase cells were spotted on the selective minimal media with or without MMS (0.004%). Plates were incubated at 30° for 5 days or at 22° for 10 days. (C) Cellular levels of the wild-type and mutant Rhp57 proteins under the overexpression conditions. Strain and plasmids used for the overexpression of wild-type and mutated Rhp57 proteins are the same as in B. Protein expression was induced in the EMM media without thiamine for 18 hr at 30° or for 48 hr at 22°. Protein extracts were prepared as described in materials and methods. The equal amount of protein extract from each strain was separated by SDS-PAGE. Wild-type and mutated Rhp57p were visualized by Western blotting using anti-Rhp57 antibodies.

that the difference in repair defects between chromosomal *KA* and *KR* mutations could be the consequence of altered protein stability. As Rhp55 and Rhp57 are proteins of very low abundance and cannot be directly detected in the total cellular protein extract using anti-Rhp55 or anti-Rhp57 antibodies (data not shown), we decided to evaluate the levels of wild-type and mutant Rhp57 proteins under the overexpression conditions. The same strains as in the drop assay (Figure 3B) were used to induce the protein expression at two temperatures, 22° and 30°, and Rhp57 protein was directly immunodetected in the cellular extracts. Figure 3C shows that Rhp57K106A and Rhp57K106R mutant proteins are less stable (lanes 2–3 and 6–7) than the wild-type protein (lanes 4 and 8) at both temperatures. Lanes 1 and 5 represent a control for the specificity of the antibodies used. Moreover, while at 30° both mutant proteins exhibit similar expression levels, the Rhp57K106R was much less stable at 22° than Rad57K106A (lanes 6 and 7). Nevertheless, the produced amount of Rad57K106R protein was sufficient to complement the repair deficiency of *rhp57*Δ cells at 22° (Figure 3B). These data strongly correlate with the small difference in MMS sensitivity between *K106A* and *K106R* mutants at 30° and with the more severe repair defect of *K106R* than K106A cells at 22° (see Figure 2B).

Taken together, our results show that the nucleotide-binding motifs of Rhp55 and Rhp57 proteins are critically important for the heterodimer function in DNA damage repair. Also, our data suggest that the difference between the repair phenotypes conferred by the Walker box A *KR* and *KA* mutations in *rhp57*$^+$ and, by inference, in *rhp55*$^+$ genes is due to the different stability of the corresponding mutant proteins.

Effect of mutations in ATP binding/hydrolysis fold on the function of Rhp55p:Rhp57p complex in cell proliferation: A cell proliferation defect in *rhp55*Δ and *rhp57*Δ mutants is manifested in vegetatively growing cultures by the accumulation of highly elongated cells with aberrant nuclear contents (Khasanov *et al.* 1999). We tested whether *K57A*- and *K57R*-mutated *rhp55* and *K106A*- and *K106R*-mutated *rhp57* strains retain the cell proliferation defect of the corresponding deletion strains. In exponentially growing *rhp55*Δ and *rhp57*Δ cultures at 30°, ∼17 and 18%, respectively, of cells were elongated at least twofold, a much larger fraction than in the wild-type cells (<1.2%). The mutant *rhp55K57A* and *rhp55K57R*, as well as *rhp57K106A* and *rhp57K106R*, cells did not show the elevated level of highly elongated cells (<1.1%). The cell elongation phenotype of gene deletion mutations was enhanced by growing cells at 23°, as shown in Figure 2C for the *rhp55*Δ strain (27.2% of elongated cells). There was no increase in the fraction of elongated cells in both *KA* and *KR rhp55* mutants at low temperature (see Figure 2C). Thus, mutations in the nucleotide-binding motif of the Rhp55, or Rhp57, subunit alone do not affect the function of the heterodimer in cell proliferation. However, the double *rhp55K57A rhp57 K106A* and *rhp55K57R rhp57K106R* mutants manifested the same severe defect in cell proliferation (24 and 17%, respectively), as the *rhp55*Δ mutant (27%; Figure 2C). From these data we conclude that ATPase function of both subunits of the heterodimer is required not only for the repair of induced DNA damage but also for faithful replication. However, in contrast to the repair defect, one functional ATP binding/hydrolysis domain

TABLE 1

Two-hybrid assay with full-length *rhp51*$^+$, *rhp54*$^+$, *rhp55*$^+$, *rhp57*$^+$, and *rad22*$^+$

DBD fusion	AD fusion	β-Gal activity[a]	Fold increase[b]
Rad22	—	3.4	1
	Rad22	1.4	0.4
	Rhp51	495	146
	Rhp54	3.2	0.9
	Rhp55	8.4	2.5
	Rhp57	5.9	1.7
Rhp51	—	1.3	1
	Rad22	0.7	0.6
	Rhp51	0.8	0.6
	Rhp54	1	0.7
	Rhp55	4	3.1
	Rhp57	1.3	1
Rhp54	—	0.9	1
	Rad22	0.9	1
	Rhp51	148.5	168.8
	Rhp54	1.5	1.7
	Rhp55	1.6	1.8
	Rhp57	1.3	1.5
Rhp55	—	2.3	1
	Rad22	5.2	2.2
	Rhp51	5.8	2.5
	Rhp54	2.3	1
Rhp57	—	34	1
	Rad22	70	2
	Rhp51	280	8.1
	Rhp54	70	2

The DBD fusion plasmids pEG202:*rhp51*$^+$, pEG202:*rhp54*$^+$, pEG202:*rhp55*$^+$, pEG202:*rhp57*$^+$, and pEG202:*rad22*$^+$ and AD fusion plasmids pJG4-5:*rhp51*$^+$, pJG4-5:*rhp54*$^+$, pJG4-5:*rhp55*$^+$, pJG4-5:*rhp57*$^+$, and pJG4-5:*rad22*$^+$ were used.

[a] β-Gal activity was determined as described in MATERIALS AND METHODS.

[b] Fold increase was determined by expressing the specific activity of strains bearing both DBD and AD fusion plasmids relative to the specific activity of strains with DBD fusion plasmid only and AD empty vector.

per heterodimer is sufficient to support normal cell proliferation.

***S. pombe* DSB repair proteins are involved in mutual interactions:** In *S. cerevisiae*, the Rad55p:Rad57p heterodimer has been shown to interact with strand-exchange protein Rad51 via the Rad55p moiety (Hays *et al.* 1995; Johnson and Symington 1995). Moreover, multiple interactions between other recombinational repair proteins of the *RAD52* group have been demonstrated (reviewed in Paques and Haber 1999). Using the yeast two-hybrid system, we performed a systematic analysis of interactions between *S. pombe* proteins involved in DSB repair. The N-terminal fusions of the full-length Rad22, Rhp51, Rhp54, Rhp55, and Rhp57 proteins with DBD (bait) and AD (prey) of yeast two-hybrid vectors were constructed and pairwise interactions were tested. As shown in Table 1, no detectable interaction between Rhp55p and Rhp51p was found, in contrast to the Rad55p-Rad51p interaction observed in budding yeast. However, significant interaction (8-fold increase in β-galactosidase activity) of Rhp57 bait with Rhp51 prey was detected. Similar to the equivalent proteins in *S. cerevisiae*, Rhp54p strongly interacted with Rhp51p (169-fold increase). Moreover, Rad22 protein, the putative homolog of *S. cerevisiae* Rad52p, showed a strong interaction with Rhp51p prey (146-fold increase). These data show that Rhp51p-Rhp54p/Rad51p-Rad54p and Rhp51p-Rad22p/Rad51p-Rad52p interactions are evolutionarily conserved, while the interaction between Rhp51p and the putative Rhp55p:Rhp57p heterodimer occurs through the Rhp57p moiety in contrast to *S. cerevisiae*, where Rad51p forms a complex with the Rad55p:Rad57p heterodimer via the Rad55 protein.

Surprisingly, we did not find interaction between bait and prey constructs bearing full-length Rhp51p (see Table 1). However, as Rad51p was shown to form a nucleoprotein filament (Ogawa *et al.* 1993) and interaction between Rad51 monomers was seen in the two-hybrid system (Donovan *et al.* 1994), we had expected to observe self-self interaction for Rhp51p. We examined the intracellular level of DBD-Rhp51 fusion by Western blotting using anti-LexA antibodies and found the fusion protein to be very unstable (data not shown). Therefore, we extended our analysis of interactions involving Rhp51p by using truncated versions of the protein. The N-terminal domain of Rhp51p (aa 1-117) was fused with DBD and AD to generate Rhp51ΔC bait and prey plasmids (Figure 4). Likewise, the core and C-terminal domains of Rhp51p (114–365) were used to construct Rhp51ΔN bait and prey. The results of two-hybrid analysis with these constructs are shown in Table 2. Both Rhp51ΔN and Rhp51ΔC were found to interact strongly with the full-length Rhp51p (763-fold and 2300-fold increases, respectively), which is consistent with the ability of RecA/Rad51-family members to polymerize on DNA via monomer-monomer interaction. Moreover, both bait and prey Rhp51ΔC constructs showed more visible interaction with Rhp57p (13-fold and 73-fold, respectively) than did full-length Rhp51p (see Table 1). This indicates that the Rhp57-binding domain of Rhp51p is located in the N-terminal region of the protein (aa residues 1–117). However, like full-length Rhp51p (Table 1), neither Rhp51ΔNp nor Rhp51ΔCp interacted with Rhp55p. In addition, both bait and prey Rhp51ΔN fusions showed strong interaction with Rad22p (78- and 1125-fold, respectively), corroborating the results with the full-length Rhp51 prey (Table 1).

Interactions involving Rad22, Rti1, and Rpa1 proteins: Two homologs of Rad52 protein have been identified in fission yeast: Rad22p and Rti1p (Ostermann *et al.* 1993; Suto *et al.* 1999; van den Bosch *et al.* 2001). As Rad52p has been shown to interact with RPA (Hays *et al.* 1998; Shinohara *et al.* 1998), we tested in the yeast two-hybrid system the possible interaction of *S. pombe* Rad22p and Rti1p with Rad11p, which encodes the large

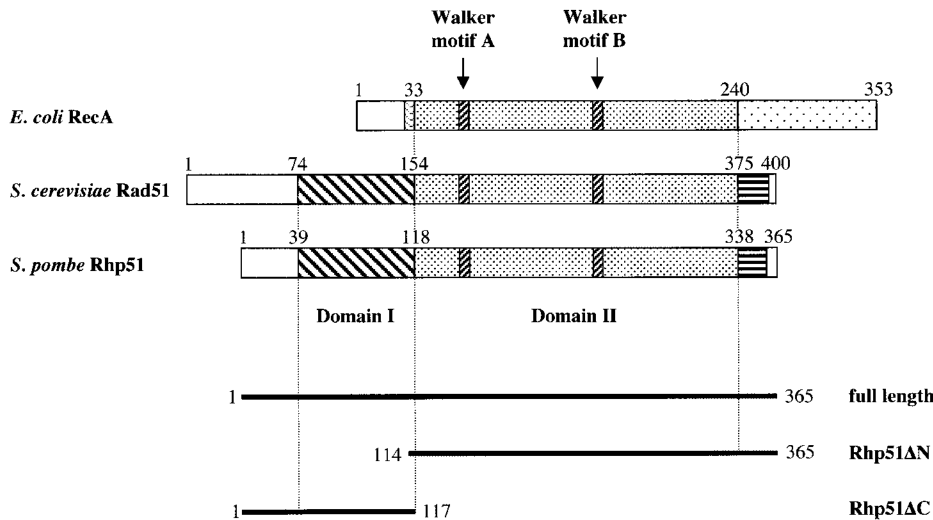

Figure 4.—Diagrammatic representation of the truncated variants of Rhp51 protein used in the two-hybrid analysis and their structural relations to *E. coli* RecA and *S. cerevisiae* Rad51 proteins. Adopted from Heyer (1994).

subunit of RPA (Parker *et al.* 1997). Rti1p was also tested for interaction with Rhp51p, as the high homology of Rti1p to Rad22p suggests that it could potentially also cross talk with Rhp51p. Indeed, strong interaction between Rti1p and Rhp51ΔNp was detected with both bait and prey Rti1p constructs (49-fold and 539-fold, respectively), which implicates the region of Rhp51 protein between amino acids 114 and 365 in binding with Rti1p (Table 3). Thus, not only Rad22p (see Tables 1 and 2) but also its homolog, Rti1p, is able to interact with Rhp51 protein.

When Rad22p was used as the DBD and Rti1p as the AD fusion, their interaction was manifested by a 111-fold increase in β-galactosidase activity compared to the control (Table 3). This suggests that the two *S. pombe* homologs of Rad52 protein may form a complex *in vivo.* Moreover, moderate but significant self-interactions of Rad22p-Rad22p (38-fold increase) and Rti1p-Rti1p (10-fold increase) were observed, suggesting that *in vivo* these proteins may exist as oligomers. Finally, we found that Rad11p (= Rpa1p) bait could interact strongly with Rti1p (73-fold increase) and less strongly with Rad22p (12-fold increase). No interaction of Rad11p with Rhp55p or Rhp57p was detected.

DISCUSSION

Our previous work established that the two fission yeast proteins, Rhp55p and Rhp57p, with structural similarity to RecA are important for recombinational repair of DNA damage and required for genomic stability and faithful meiosis (Khasanov *et al.* 1999; Tsutsui *et al.* 2000). In this study, we extended our examination of these two *S. pombe* proteins by analyzing their interaction *in vivo* and the role of their putative nucleotide-binding motifs for their functions in repair and mitosis. Yeast two-hybrid analysis and co-immunoprecipitation experiments demonstrated that Rhp55p and Rhp57p interact *in vivo* (Figure 1) and are likely to form a stable heterodimer similar to that formed by *S. cerevisiae* Rad55 and Rad57 proteins. Thus, the heterodimeric complex of two RecA-like proteins is evolutionarily conserved, as it is found in two distantly related yeasts. However, in vertebrates, besides Rad51, five additional RecA-like proteins, often referred to as Rad51 paralogs (Rad51B, Rad51C, Rad51D, Xrcc2, and Xrcc3) have been identified and their roles in DNA damage repair and genome stability have been demonstrated (reviewed in Morrison and Takeda 2000). Unlike $RAD51^{-/-}$, knockouts of paralogs in a chicken cell line are viable, suggesting that they may play roles as accessory factors to Rad51 protein, similar to yeast Rad55 and Rad57 (Takata *et al.* 2001). Each human Rad51 paralog has been shown to be involved in a unique set of interactions with other paralogs in yeast two- and three-hybrid systems (reviewed in Thompson and Schild 1999; Schild *et al.* 2000). It seems that two or three component complexes of Rad51 paralogs may exist in higher eukaryotes. Among them, Rad51p-Xrcc3p-Rad51Cp is the only complex that contains Rad51 strand exchange protein (Schild *et al.* 2000). Xrcc3p is phylogenetically related to Rhp57p (Tsutsui *et al.* 2000) and interacts with both Rad51Cp and Rad51p. Therefore, the Rad51C:Xrcc3 complex is a possible candidate for the functional homolog of the Rhp55:Rhp57 heterodimer.

Proteins belonging to RecA/Rad51 family have two motifs, Walker A and B boxes, which are the signature of the nucleotide cofactor binding/hydrolysis function. The purified *S. cerevisiae* Rad55:Rad57 heterodimer has the ability to hydrolyze ATP (Sung 1997b), but this activity is not stimulated by ss- or dsDNA, unlike the activity of Rad51 ATPase. The biological significance of ATPase function was demonstrated by generating Walker box A mutations in the Rad55 protein (Johnson and Symington 1995). However, the equivalent mutations in the Rad57 subunit did not affect repair and meiosis. We show here that the nucleotide-binding motifs of both subunits of the *S. pombe* Rhp55:Rhp57 com-

TABLE 2

Two-hybrid assay with truncated *rhp51*⁺

DBD fusion	AD fusion	β-Gal activity[a]	Fold increase[b]
Rhp51ΔN	—	0.1	1
	Rhp51	76.33	763
	Rhp51ΔN	0.43	4.3
	Rhp51ΔC	260.66	2606
	Rhp54	0.09	0.9
	Rhp55	0.17	1.7
	Rhp57	0.29	2.9
	Rad22	7.84	78
Rhp51ΔC	—	0.08	1
	Rhp51	183.97	2299
	Rhp51ΔN	142.45	1780
	Rhp51ΔC	0.08	1
	Rhp54	0.1	1.3
	Rhp55	0.05	0.6
	Rhp57	1.07	13.4
	Rad22	0.03	0.4
Rhp54	—	0.06	1
	Rhp51	20	333
	Rhp51ΔN	0.02	0.3
	Rhp51ΔC	0.03	0.5
Rhp55	—	0.018	1
	Rhp51	0.004	0.2
	Rhp51ΔN	0.013	0.8
	Rhp51ΔC	0.017	0.9
Rhp57	—	0.02	1
	Rhp51	0.11	5.5
	Rhp51ΔN	0.01	0.5
	Rhp51ΔC	1.45	73
Rad22	—	0.06	1
	Rhp51	5.29	88
	Rhp51ΔN	67.5	1125
	Rhp51ΔC	0.06	1

pHybLex/ZeoN derivatives pYS212, pYS213, pYS214, pYS215, pYS218, and pYS219 and pYESTrp2N derivatives pYS221, pYS222, pYS223, pYS224, pYS225, pYS228, and pYS229 were used in the assay.

[a] β-Gal activity was determined as described in MATERIALS AND METHODS.

[b] Fold increase was determined by expressing the specific activity of strains bearing both DBD and AD fusion plasmids relative to the specific activity of strains with DBD fusion plasmid only and AD empty vector.

TABLE 3

Interactions involving Rad22, Rti1, and Rpa1 proteins

DBD fusion	AD fusion	β-Gal activity[a]	Fold increase[b]
Rti1	—	2.23	1
	Rhp51	0.41	0.2
	Rhp51ΔN	108.7	49
	Rhp51ΔC	0.3	0.1
	Rad11	0.67	0.3
	Rad22	8.13	3.7
	Rti1	21.25	9.5
Rhp51	—	0.006	1
	Rti1	0.002	0.3
	Rad11	0.024	4
Rhp51ΔN	—	0.1	1
	Rti1	53.92	539
	Rad11	0.26	2.6
Rhp51ΔC	—	0.08	1
	Rti1	0.02	0.3
	Rad11	0.07	0.9
Rhp55	—	0.018	1
	Rti1	0.008	0.4
	Rad11	0.015	0.8
Rhp57	—	0.02	1
	Rti1	0.004	0.2
	Rad11	0.038	1.9
Rad11	—	0.05	1
	Rti1	3.64	73
	Rad22	0.6	12
Rad22	—	0.06	1
	Rti1	6.67	111
	Rad11	0.05	0.8
	Rad22	2.28	38

pHybLex/ZeoN derivatives pYS211, pYS212, pYS214, pYS215, pYS216, pYS217, pYS218, and pYS219 and pYESTrp2N derivatives pYS221, pYS222, pYS226, pYS227, pYS228, and pYS229 were used in the assay.

[a] β-Gal activity was determined as described in MATERIALS AND METHODS.

[b] Fold increase was determined by expressing the specific activity of strains bearing both DBD and AD fusion plasmids relative to the specific activity of strains with DBD fusion plasmid only and AD empty vector.

plex are important for its function in repair (see Figure 2), which is different from the results obtained with *S. cerevisiae*. However, it has not been excluded that the observed difference between the two yeasts is a consequence of different expression levels of the mutant proteins rather than a real difference. Indeed, in *S. cerevisiae*, Rad57 mutant proteins have been expressed from a centromere-containing plasmid, which may result in a higher protein level as compared to expression of Rhp57 mutant proteins in *S. pombe* from the endogenous chromosomal locus. As we have demonstrated for the overexpression in *S. pombe* of mutant Rhp55 and Rhp57 proteins, the elevated protein level has the potential to suppress the repair phenotype of the corresponding deletion mutant (see Figure 3, A and B), which could explain the "no phenotype" of Walker box A mutation in the Rad57 protein.

Studies of bacterial RecA and yeast Rad3 and Rad51 DNA-dependent ATPases established that changing the invariant lysine residue to arginine in Walker A box abolished their nucleotide hydrolytic activity without affecting the ability to bind ATP, while changing it to alanine diminished their affinity for ATP (Sung *et al.* 1988; Rehrauer and Kowalczykowski 1993; Sung and Stratton 1996). As anticipated, the equivalent *KR* mutation in yeast RecA homologs Rad51p and Rad55p was more proficient than the *KA* mutation in complementing the repair and recombination defects of *rad51* and *rad55* null alleles (Shinohara *et al.* 1992; Johnson and

Symington 1995). Consistent with this, Rad51K191R protein was able to carry out extensive strand exchange *in vitro* in the absence of ATP hydrolysis (Sung and Stratton 1996). Surprisingly, in contrast to *S. cerevisiae rad51* and *rad55*, *S. pombe rhp55* and *rhp57 KR* mutants consistently showed more severe impairment of DNA repair than corresponding *KA* mutants (see Figure 2). The difference was most striking for the *rhp57* mutants at 22° (see Figure 2B). One explanation could be that ATP binding without subsequent hydrolysis might give rise to some kind of unproductive intermediate and, thus, affect the recombination repair reaction in *S. pombe* more severely than the absence of ATP binding caused by the *KA* mutation. However, this explanation seems to be unlikely, as one would expect the *KR* mutation to be dominant negative, which is not the case (see Figure 3, A and B). Another more reasonable explanation is the stability of the mutant proteins, as we have shown in the overexpression experiment that the Rhp57K106R mutant protein is much less stable than Rhp57K106A protein at 22° (see Figure 3C). Thus, it is possible that under normal expression conditions KR mutant protein is also less stable than KA mutant protein, which would explain the repair phenotypes of the corresponding mutants.

Interestingly, we found that the mutations in the ATP binding fold of either Rhp55p or Rhp57p did not affect cell proliferation, unlike the corresponding gene deletions, which had significant impact on the DNA repair capability of the cell (Figure 2). However, when the ATPase domains of both subunits were mutated, the cell proliferation defect was as strong as in the *rhp55Δ* mutant (see Figure 2C). Recombination is important for the repair of spontaneous replication-associated DNA damage (Michel *et al.* 1997; Sonoda *et al.* 1999) and one would expect DSB repair proteins, including Rhp55 and Rhp57, to work during replication restart. However, spontaneous DNA damage during replication is a rare event, as compared to massive MMS-induced damage. Our data suggest that the cellular levels of proteins with mutated Walker box A could be lower compared to the wild type (Figure 3C). Perhaps even the low intracellular level of the mutated subunit of the heterodimer, complexed with the functional partner subunit, is capable of supporting the replication, but it is not sufficient to cope with the extensive drug-induced DNA damage. When the ATPase function of both subunits is destroyed, the Rhp55p:Rhp57p heterodimer is no more functional and both cell proliferation and DNA damage repair are severely impaired.

We found that both *KA* and *KR* mutations in either of the two subunits resulted in Rhp55p:Rhp57p heterodimers with some residual activity in DNA repair and replication. In contrast, concurrent mutations in both subunits completely abolished this activity, similar to the deletion of the gene (see Figure 2). This may indicate that one functional ATPase domain per heterodimer is still sufficient to carry on the function of the Rhp55p:Rhp57p complex, albeit less efficiently, and the observed differences in the phenotypes of single mutants may be due partially to the altered protein stability. This also suggests that ATPase function is crucial for heterodimer function in DSB repair. It is likely that the heterodimer acts in DSB repair via binding to DNA and interaction with the Rhp51-DNA filament (Tables 1 and 2). If the binding of the heterodimer to DNA is provided by ATP binding/hydrolysis function of both subunits, it probably has two DNA-binding sites. When the *KA* or *KR* mutation abrogates the DNA binding of one subunit, the other subunit can still provide this function, which would result in partial activity of the complex in DSB repair. When the ATP binding/hydrolysis domains of both subunits are mutated, the Rhp55p:Rhp57p complex cannot bind the DNA, and thus the damaged DNA will remain unrepaired, as in the absence of the complex.

Taken together, our data suggest that *S. cerevisiae* Rad55p:Rad57p and *S. pombe* Rhp55p:Rhp57p complexes might be similar in the requirement for ATP binding/hydrolysis for their function in DNA repair, and the observed experimental differences are likely due to different expression levels of the mutant proteins. However, biochemical studies of wild-type and mutant heterodimers from both yeast species are necessary to finally clarify the issue.

At present, several lines of evidence suggest that recombinational repair proteins function as protein complexes both in budding yeast and human cells. Our two-hybrid analysis of protein-protein interactions shows that this is also the case in fission yeast, indicating that some of these complexes are highly conserved from yeast to mammals. As with *S. cerevisiae* (Jiang *et al.* 1996; Clever *et al.* 1997), mouse (Tan *et al.* 1999), and human (Golub *et al.* 1997; Tanaka *et al.* 2000) Rad54p and Rad51p, we found that *S. pombe* Rhp54p exhibits strong interaction with Rhp51 protein (Table 1). This interaction was detected only if the full-length Rhp51 AD fusion was used. As the truncated fusion proteins were stably expressed in the cells (data not shown), this may suggest that the amino acid residues in both the N-terminal and C-terminal halves of Rhp51p are important for the interaction with Rhp54p. This is consistent with the rather complex Rad51p interface involved in the association with Rad54 in *S. cerevisiae* (Krejci *et al.* 2001). Alternatively, the functional Rad51-DNA filament may be required for interaction with Rad54 protein (Mazin *et al.* 2000).

Like the self-association of RecA protein, the self-association of Rad51p monomers is thought to be essential for the formation of nucleoprotein filament, which performs the homology search and strand exchange. Self-association of *S. cerevisiae* and human Rad51 protein is supported by two-hybrid data with full-length proteins (Donovan *et al.* 1994; Tanaka *et al.* 2000). Our data

(Table 2) also show that *S. pombe* Rhp51 interacts with itself. Remarkably, very strong interaction between the amino-terminal one-third and carboxy-terminal two-thirds of Rhp51p, which have only three overlapping amino acid residues, was detected. This suggests that the Rhp51p monomers interact in a head-to-tail manner, as was found for RecA based on the crystal structure (Story *et al.* 1992). NMR analysis of the structure of human Rad51p also implies that there is little possibility that the N-terminal regions interact with each other (Aihara *et al.* 1999). In contrast, two-hybrid studies in budding yeast led to a suggestion of head-to-head association of Rad51p monomers (Donovan *et al.* 1994). However, taking into consideration the high structural conservation of Rad51 homologs from the two types of yeast, it is unlikely that there is a fundamental difference in their manner of polymerization on DNA.

S. cerevisiae Rad52p plays a key role in recombinational repair (reviewed in Paques and Haber 1999). Interaction between Rad52 and Rad51 proteins has been demonstrated in yeast (Shinohara *et al.* 1992; Donovan *et al.* 1994; Hays *et al.* 1995) and humans (Kurumizaka *et al.* 1999). We showed in this study that Rad22p and Rti1p, the two *S. pombe* homologs of Rad52p, are involved in interactions with other DNA repair proteins. Rad22p strongly interacts with full-length Rhp51 (Table 1) and with Rhp51ΔN truncated protein (Table 2). Likewise, Rti1p strongly associates with Rhp51ΔN (Table 3). These findings indicate that the Rad22- and Rti1-binding sites of Rhp51p are in the C-terminal region. It is not clear if both proteins compete for the same interaction site or use different sites. Interestingly, in *S. cerevisiae* the Rad52-binding domain of Rad51p was mapped to the N-terminal domain by two-hybrid analysis (Donovan *et al.* 1994) and the C-terminal half of Rad51p (amino acid residues 152–400) did not show interaction with Rad52p. However, the corresponding region of Rhp51p (114–365; see Figure 4) strongly interacted with both Rti1 and Rad22 proteins (Tables 2 and 3). Our data are in agreement with the finding by Kurumizaka *et al.* (1999) that the N-terminal region of human Rad51p does not bind strongly to human Rad52p. Moreover, no randomly generated mutations in *S. cerevisiae* Rad51p disrupting its association with Rad52p have been identified in the N-terminal region of Rad51p (Krejci *et al.* 2001).

Both human and *S. cerevisiae* Rad52p are able to self-associate and to form a complex with the heterotrimeric single-stranded DNA-binding protein RPA through multiple contacts (Park *et al.* 1996; Hays *et al.* 1998). Here we show (Table 3) that *S. pombe* Rad52 homologs Rad22p and Rti1p can also self-interact and form a complex with Rpa1 protein. This is in agreement with genetic interactions found between *rad22*$^+$, *rti1*$^+$, and *rpa1*$^+$ (Suto *et al.* 1999). Moreover, Rti1p and Rad22p can associate with each other (Table 3). The potentially

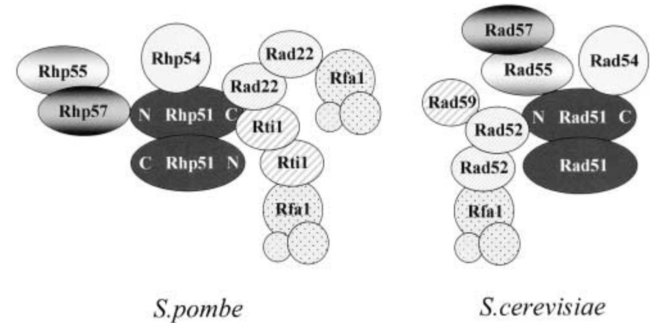

Figure 5.—Schematic diagram of mutual interactions between the yeast proteins involved in recombinational repair. Interactions may not occur *in vivo* simultaneously, as depicted, but more likely happen in a sequential manner. Strong interactions are shown by overlapping ellipsoids and weaker interactions by touching ellipsoids.

equivalent association of *S. cerevisiae* Rad52p and Rad59p was also recently identified (A. P. Davis and L. S. Symington, personal communication); moreover, they interact genetically (Bai and Symington 1996; Bai *et al.* 1999). It is possible that Rad22p and Rti1p form homo- or heteromultimers *in vivo*. Notably, yeast and human Rad52p were visualized as ring-like oligomers on DNA (Shinohara *et al.* 1998; Van Dyck *et al.* 1998).

The Rad55p:Rad57p heterodimer in *S. cerevisiae* interacts in a two-hybrid system with Rad51p (Hays *et al.* 1995; Johnson and Symington 1995). Accordingly, the heterodimer stimulates the *in vitro* strand exchange reaction promoted by Rad51protein (Sung 1997b). This interaction occurs via contact of Rad51p with the Rad55p subunit of the complex. However, in *S. pombe*, Rhp57p is the subunit, which interacts with Rhp51p (Tables 1 and 2). The Rhp57-binding site is located within the nonconserved N-terminal domain of 117 amino acid residues of Rhp51 protein (Table 2). This indicates that the Rhp55:Rhp57 heterodimer uses a binding site different from that of Rad22p and Rti1p and does not compete with them for binding to Rhp51 protein. It may be that one large complex Rhp55p: Rhp57p:Rhp51p:(Rad22p:Rti1p) assembles during DNA repair, at least temporarily.

The different mode of interaction with the strand-exchange protein of two heterodimers may raise the question of how accurate the assignments of Rhp55p and Rhp57p are to be the homologs of Rad55 and Rad57p, respectively. As with the *S. cerevisiae rad55*Δ and *rad57*Δ, the phenotypes of *rhp55*Δ and *rhp57*Δ mutants are almost indistinguishable. Several lines of evidences of the structural character suggest the validity of the original assignments. First, pairwise global alignment of protein sequences (Needleman and Wunsch 1970) shows that Rhp55p has higher overall homology to Rad55p (20.2% identity and 42.1% similarity) than to Rad57p (18.2% identity and 31.5% similarity). Likewise, Rhp57p has higher homology to Rad57p (30% identity

and 42.8% similarity) than to Rad55p (16.8% identity and 31.3% similarity). Second, multiple sequence alignments and molecular phylogenetic analysis also show the closer similarity of Rhp55p to Rad55p and Rhp57p to Rad57p (Khasanov *et al.* 1999; Tsutsui *et al.* 2000). Finally, the N-terminal regions of Rhp55p (32 aa) and Rad55p (25 aa) are shorter than N termini of Rhp57p (82 aa) and Rad57p (107 aa) when the conserved D33, D26, D83, and D108, respectively, are taken as the first residue of the core domain of RecA-like proteins (see the protein alignments in Khasanov *et al.* 1999 and Tsutsui *et al.* 2000). Thus, our data indicate that the mode of association of the strand exchange protein with the heterodimer of RecA-like accessory proteins is not conserved between two yeasts. It could be that the parallel evolution of the two yeasts has resulted in the acquisition of similar function by nonhomologous subunits of the heterodimer complex.

Our two-hybrid analysis demonstrated multiple interactions among *S. pombe* proteins with a role in recombinational repair. These interactions are diagrammed in Figure 5 along with those known in *S. cerevisiae*. Depicted interactions do not necessarily occur simultaneously but more likely occur in a temporal and sequential manner. Among them, such associations as Rhp51p-Rhp51p, Rhp55-Rhp57p, and Rhp51p-Rhp54p are evolutionarily conserved, as similar associations have also been found for their homologs in *S. cerevisiae*. Both fission yeast Rad52 homologs Rad22p and Rti1p may multimerize and form a complex with RPA. Likewise, in budding yeast, Rad52p interacts with itself and associates with RPA. Moreover, Rad22 protein can associate with and act in concert with Rti1 protein in recombinational repair. Similarly, budding yeast Rad52p and Rad59p physically interact. Finally, the Rad52 homologs of both yeasts, except Rad59p, strongly interact with strand exchange factor, Rhp51p, or Rad51p. Importantly, despite the general similarity of the interaction scheme, the use of the particular protein domain or the subunit of a protein complex differs between the two yeasts, as we demonstrated for Rhp51p-(Rad22p/Rti1p) and Rhp51p-(Rhp57p:Rhp55p) interactions, respectively. Therefore, the mode of interaction between some components of the putative "recombinosome" is not conserved between distantly related yeasts.

We thank W.-D. Heyer, E. Haghnazari, M. Rolfsmeier, and J. Solinger for critically reading the manuscript; O. Parshenkova for technical assistance; H. Okayama for *rti1*$^{+}$ cDNA plasmid; and H. Nojima for the *S. pombe* cDNA library. We appreciate the communication of unpublished results by L. S. Symington. This work was supported by International Research Scholar's grants HHMI 75195544401 and HHMI 55000299 from the Howard Hughes Medical Institute and Research Grant 9604-49121 from the Russian Fund for Basic Research to V.I.B; and Grants-in-Aid for Scientific Research on Priority Areas (08280102 and 08280103) and a Grant-in-Aid from the Monbusho International Scientific Research Program (10044206) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to H.S. Y.T. was supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

LITERATURE CITED

Aihara, H., Y. Ito, H. Kurumizaka, S. Yokoyama and T. Shibata, 1999 The N-terminal domain of the human Rad51 protein binds DNA: structure and a DNA binding surface as revealed by NMR. J. Mol. Biol. **290:** 495–504.

Alfa, C., P. Fantes, J. Hyams, M. McLeod and E. Warbrick, 1993 *Experiments with Fission Yeast.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Bai, Y., and L. S. Symington, 1996 A Rad52 homolog is required for *RAD51*-independent mitotic recombination in *Saccharomyces cerevisiae*. Genes Dev. **10:** 2025–2037.

Bai, Y., A. P. Davis and L. S. Symington, 1999 A novel allele of *RAD52* that causes severe DNA repair and recombination deficiencies only in the absence of *RAD51* or *RAD59*. Genetics **153:** 1117–1130.

Clever, B., H. Interthal, J. Schmuckli-Maurer, J. King, M. Sigrist *et al.*, 1997 Recombinational repair in yeast: functional interactions between Rad51 and Rad54 proteins. EMBO J. **16:** 2535–2544.

Donovan, J. W., G. T. Milne and D. T. Weaver, 1994 Homotypic and heterotypic protein associations control Rad51 function in double-strand break repair. Genes Dev. **8:** 2552–2562.

Forsburg, S. L., and D. A. Sherman, 1997 General purpose tagging vectors for fission yeast. Gene **191:** 191–195.

Friedberg, E. C., G. C. Walker and W. Siede, 1995 *DNA Repair and Mutagenesis*. ASM Press, Washington, DC.

Game, J. C., 1993 DNA double-strand breaks and the *RAD50-RAD57* genes in *Saccharomyces*. Semin. Cancer Biol. **4:** 73–83.

Golemis, E. A., J. Gyuris and R. Brent, 1994 Interaction trap/two hybrid system to identify interacting proteins, pp. 13.14.1–13.14.17 in *Current Protocols in Molecular Biology*, edited by F. M. Ausubel, R. Brent, R. Kingston, D. Moore, J. J. Seidman *et al.* John Wiley & Sons, New York.

Golub, E. I., O. V. Kovalenko, R. C. Gupta, D. C. Ward and C. M. Radding, 1997 Interaction of human recombination proteins Rad51 and Rad54. Nucleic Acids Res. **25:** 4106–4110.

Gorbalenya, A. E., and E. V. Koonin, 1993 Helicases—amino acid sequence comparisons and structure function relationships. Curr. Opin. Struct. Biol. **3:** 419–429.

Gutz, H., H. Heslot, U. Leupold and N. Loprieno, 1974 *Schizosaccharomyces pombe*, pp. 395–446 in *Handbook of Genetics*, edited by R. C. King. Plenum Press, New York.

Gyuris, J., E. Golemis, H. Chertkov and R. Brent, 1993 Cdi1, a human G1-phase and S-phase protein phosphatase that associates with Cdk2. Cell **75:** 791–803.

Haber, J. E., 1998 The many interfaces of Mre11. Cell **95:** 583–586.

Harshman, K. D., W. S. Moye-Rowley and C. S. Parker, 1988 Transcriptional activation by the SV-40 AP-1 recognition element in yeast is mediated by a factor similar to AP-1 that is distinct from GCN4. Cell **53:** 321–330.

Hays, S. L., A. A. Firmenich and P. Berg, 1995 Complex formation in yeast double-strand break repair: participation of Rad51, Rad52, Rad55, and Rad57 proteins. Proc. Natl. Acad. Sci. USA **92:** 6925–6929.

Hays, S. L., A. A. Firmenich, P. Massey, R. Banerjee and P. Berg, 1998 Studies of the interaction between Rad52 protein and the yeast single-stranded DNA binding protein RPA. Mol. Cell. Biol. **18:** 4400–4406.

Heyer, W. D., 1994 The search for the right partner—homologous pairing and DNA strand exchange proteins in eukaryotes. Experientia **50:** 223–233.

Hopfner, K. P., A. Karcher, D. S. Shin, L. Craig, L. M. Arthur *et al.*, 2000 Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. Cell **101:** 789–800.

Jiang, H., Y. Q. Xie, P. Houston, K. Stemke-Hale, U. H. Mortensen *et al.*, 1996 Direct association between the yeast Rad51 and Rad54 recombination proteins. J. Biol. Chem. **271:** 33181–33186.

Johnson, R. D., and L. S. Symington, 1995 Functional differences and interactions among the putative RecA homologs *RAD51*, *RAD55*, and *RAD57*. Mol. Cell. Biol. **15:** 4843–4850.

Khasanov, F. K., G. V. Savchenko, E. V. Bashkirova, V. G. Korolev, W.-D. Heyer *et al.*, 1999 A new recombinational DNA repair gene from *Schizosaccharomyces pombe* with homology to *E. coli* RecA. Genetics **152:** 1557–1572.

Krejci, L., J. Damborsky, B. Thomsen, M. Duno and C. Bendixen, 2001 Molecular dissection of interactions between Rad51 and members of the recombination-repair group. Mol. Cell. Biol. **21:** 966–976.

Kurumizaka, H., H. Aihara, W. Kagawa, T. Shibata and S. Yokoyama, 1999 Human Rad51 amino acid residues required for Rad52 binding. J. Mol. Biol. **291:** 537–548.

Mazin, A. V., C. J. Bornarth, J. A. Solinger, W. D. Heyer and S. C. Kowalczykowski, 2000 Rad54 protein is targeted to pairing loci by the Rad51 nucleoprotein filament. Mol. Cell **6:** 583–592.

McCready, S. J., F. Osman and A. Yasui, 2000 Repair of UV damage in the fission yeast *Schizosaccharomyces pombe*. Mutat. Res. **451:** 197–210.

Michel, B., S. D. Ehrlich and M. Uzest, 1997 DNA double-strand breaks caused by replication arrest. EMBO J. **16:** 430–438.

Morrison, C., and S. Takeda, 2000 Genetic analysis of homologous DNA recombination in vertebrate somatic cells. Int. J. Biochem. Cell Biol. **32:** 817–831.

Mortensen, U. H., C. Bendixen, I. Sunjevaric and R. Rothstein, 1996 DNA strand annealing is promoted by the yeast Rad52 protein. Proc. Natl. Acad. Sci. USA **93:** 10729–10734.

Muris, D. F. R., K. Vreeken, A. M. Carr, B. C. Broughton, A. R. Lehmann *et al.*, 1993 Cloning the *RAD51* homologue of *Schizosaccharomyces pombe*. Nucleic Acids Res. **21:** 4586–4591.

Muris, D. F. R., K. Vreeken, A. M. Carr, J. M. Murray, C. Smit *et al.*, 1996 Isolation of the *Schizosaccharomyces pombe RAD54* homologue, *rhp54*$^{(+)}$, a gene involved in the repair of radiation damage and replication fidelity. J. Cell Sci. **109:** 73–81.

Needleman, S. B., and C. D. Wunsch, 1970 A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Mol. Biol. **48:** 443–453.

New, J. H., T. Sugiyama, E. Zaitseva and S. C. Kowalczykowski, 1998 Rad52 protein stimulates DNA strand exchange by Rad51 and replication protein A. Nature **391:** 407–410.

Ogawa, T., X. Yu, A. Shinohara and E. H. Egelman, 1993 Similarity of the yeast RAD51 filament to the bacterial RecA filament. Science **259:** 1896–1899.

Ostermann, K., A. Lorentz and H. Schmidt, 1993 The fission yeast *Rad22* gene, having a function in mating-type switching and repair of DNA damages, encodes a protein homolog to Rad52 of *Saccharomyces cerevisiae*. Nucleic Acids Res. **21:** 5940–5944.

Paques, F., and J. E. Haber, 1999 Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **63:** 349–404.

Park, M. S., D. L. Ludwig, E. Stigger and S. H. Lee, 1996 Physical interaction between human RAD52 and RPA is required for homologous recombination in mammalian cells. J. Biol. Chem. **271:** 18996–19000.

Parker, A. E., R. K. Clyne, A. M. Carr and T. J. Kelly, 1997 The *Schizosaccharomyces pombe rad11*$^{+}$ gene encodes the large subunit of replication protein A. Mol. Cell. Biol. **17:** 2381–2390.

Petukhova, G., S. Stratton and P. Sung, 1998 Catalysis of homologous pairing by yeast Rad51 and Rad54 proteins. Nature **393:** 91–94.

Petukhova, G., S. A. Stratton and P. Sung, 1999a Single strand DNA binding and annealing activities in the yeast recombination factor Rad59. J. Biol. Chem. **274:** 33839–33842.

Petukhova, G., S. Van Komen, S. Vergano, H. Klein and P. Sung, 1999b Yeast Rad54 promotes Rad51-dependent homologous DNA pairing via ATP hydrolysis-driven change in DNA double helix conformation. J. Biol. Chem. **274:** 29453–29462.

Pringle, J. R., A. E. M. Adams, D. G. Drubin and B. K. Haarer, 1991 Immunofluorescence methods for yeast. Methods Enzymol. **194:** 565–602.

Rehrauer, W. M., and S. C. Kowalczykowski, 1993 Alteration of the nucleoside triphosphate (NTP) catalytic domain within *Escherichia coli* RecA protein attenuates NTP hydrolysis but not joint molecule formation. J. Biol. Chem. **268:** 1292–1297.

Rijkers, T., J. VandenOuweland, B. Morolli, A. G. Rolink, W. M. Baarends *et al.*, 1998 Targeted inactivation of mouse RAD52 reduces homologous recombination but not resistance to ionizing radiation. Mol. Cell. Biol. **18:** 6423–6429.

Santos Rosa, H., B. Clever, W. D. Heyer and A. Aguilera, 1996 The yeast *HRS1* gene encodes a polyglutamine-rich nuclear protein required for spontaneous and hpr1-induced deletions between direct repeats. Genetics **142:** 705–716.

Scherer, S., and R. W. Davis, 1979 Replacement of chromosomal segments with altered DNA sequences constructed *in vivo*. Proc. Natl. Acad. Sci. USA **76:** 4951–4955.

Schild, D., Y. Lio, D. W. Collins, T. Tsomondo and D. J. Chen, 2000 Evidence for simultaneous protein interactions between human Rad51 paralogs. J. Biol. Chem. **275:** 16443–16449.

Shinohara, A., and T. Ogawa, 1995 Homologous recombination and the roles of double-strand breaks. Trends Biochem. Sci. **20:** 387–391.

Shinohara, A., and T. Ogawa, 1998 Stimulation by Rad52 of yeast Rad51-mediated recombination. Nature **391:** 404–407.

Shinohara, A., H. Ogawa and T. Ogawa, 1992 Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. Cell **69:** 457–470.

Shinohara, A., H. Ogawa, Y. Matsuda, N. Ushio, K. Ikea *et al.*, 1993 Cloning of human, mouse and fission yeast recombination genes homologous to RAD51 and recA. Nat. Genet. **4:** 239–243.

Shinohara, A., M. Shinohara, T. Ohta, S. Matsuda and T. Ogawa, 1998 Rad52 forms ring structures and co-operates with RPA in single-strand DNA annealing. Genes Cells **3:** 145–156.

Sonoda, E., M. S. Sasaki, C. Morrison, Y. Yamaguchi-Iwai, M. Takata *et al.*, 1999 Sister chromatid exchanges are mediated by homologous recombination in vertebrate cells. Mol. Cell. Biol. **19:** 5166–5169.

Story, R. M., I. T. Weber and T. A. Steitz, 1992 The structure of the *E. coli* recA protein monomer and polymer. Nature **355:** 318–325.

Sugawara, N., and J. E. Haber, 1992 Characterization of double strand break-induced recombination: homology requirements and single-stranded DNA formation. Mol. Cell. Biol. **12:** 563–575.

Sugawara, N., G. Ira and J. Haber, 2000 DNA length dependence of the single-strand annealing pathway and the role of *Saccharomyces cerevisiae RAD59* in double-strand break repair. Mol. Cell. Biol. **20:** 5300–5309.

Sugiyama, T., J. H. New and S. C. Kowalczykowski, 1998 DNA annealing by Rad52 protein is stimulated by specific interaction with the complex of replication protein A and single-stranded DNA. Proc. Natl. Acad. Sci. USA **95:** 6049–6054.

Sung, P., 1994 Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast RAD51 protein. Science **265:** 1241–1243.

Sung, P., 1997a Function of yeast Rad52 protein as a mediator between replication protein A and the Rad51 recombinase. J. Biol. Chem. **272:** 28194–28197.

Sung, P., 1997b Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. Genes Dev. **11:** 1111–1121.

Sung, P., and D. L. Robberson, 1995 DNA strand exchange mediated by a RAD51-ssDNA nucleoprotein filament with polarity opposite to that of RecA. Cell **82:** 453–461.

Sung, P., and S. A. Stratton, 1996 Yeast Rad51 recombinase mediates polar DNA strand exchange in the absence of ATP hydrolysis. J. Biol. Chem. **271:** 27983–27986.

Sung, P., D. Higgins, L. Prakash and S. Prakash, 1988 Mutation of lysine-48 to arginine in the yeast RAD3 protein abolishes its ATPase and DNA helicase activities but not the ability to bind ATP. EMBO J. **7:** 3263–3269.

Suto, K., A. Nagata, H. Murakami and H. Okayama, 1999 A double-strand break repair component is essential for S phase completion in fission yeast cell cycling. Mol. Biol. Cell **10:** 3331–3343.

Takata, M., M. S. Sasaki, S. Tachiiri, T. Fukushima, E. Sonoda *et al.*, 2001 Chromosome instability and defective recombinational repair in knockout mutants of the five Rad51 paralogs. Mol. Cell. Biol. **21:** 2858–2866.

Tan, T. L. R., J. Essers, E. Citterio, S. M. A. Swagemakers, J. de Wit *et al.*, 1999 Mouse Rad54 affects DNA conformation and DNA-damage-induced Rad51 foci formation. Curr. Biol. **9:** 325–328.

Tanaka, K., T. Hiramoto, T. Fukuda and K. Miyagawa, 2000 A novel human Rad54 homologue, Rad54B, associates with Rad51. J. Biol. Chem. **275:** 26316–26321.

Tavassoli, M., M. Shayeghi, A. Nasim and F. Z. Watts, 1995 Cloning and characterization of the *Schizosaccharomyces pombe rad32* gene: a gene required for repair of double strand breaks and recombination. Nucleic Acids Res. **23:** 383–388.

Thompson, L. H., and D. Schild, 1999 The contribution of homolo-

gous recombination in preserving genome integrity in mammalian cells. Biochimie **81:** 87–105.

Tsutsui, Y., T. Morishita, H. Iwasaki, H. Toh and H. Shinagawa, 2000 A recombination repair gene of *Schizosaccharomyces pombe*, *rhp57*, is a functional homolog of the *Saccharomyces cerevisiae RAD57* gene and is phylogenetically related to the human XRCC3 gene. Genetics **154:** 1451–1461.

van den Bosch, M., K. Vreeken, J. B. M. Zonneveld, J. A. Brandsma, M. Lombaerts *et al.*, 2001 Characterization of *RAD52* homologs in the fission yeast *Schizosaccharomyces pombe*. Mutat. Res. **461:** 311–323.

Van Dyck, E., N. M. A. Hajibagheri, A. Stasiak and S. C. West, 1998 Visualization of human Rad52 protein and its complexes with hRad51 and DNA. J. Mol. Biol. **284:** 1027–1038.

Van Komen, S., G. Petukhova, S. Sigurdsson, S. Stratton and P. Sung, 2000 Superhelicity-driven homologous DNA pairing by yeast recombination factors Rad51 and Rad54. Mol. Cell **6:** 563–572.

Yamaguchi-Iwai, Y., E. Sonoda, J. M. Buerstedde, O. Bezzubova, C. Morrison *et al.*, 1998 Homologous recombination, but not DNA repair, is reduced in vertebrate cells deficient in RAD52. Mol. Cell. Biol. **18:** 6430–6435.

Communicating editor: L. S. Symington