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

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ABSTRACT

To identify *Schizosaccharomyces pombe* genes involved in recombination repair, we identified seven mutants that were hypersensitive to both methyl methanesulfonate (MMS) and γ-rays and that contained mutations that caused synthetic lethality when combined with a *rad2* mutation. One of the mutants was used to clone the corresponding gene from a genomic library by complementation of the MMS-sensitive phenotype. The gene obtained encodes a protein of 354 amino acids whose sequence is 32% identical to that of the Rad57 protein of *Saccharomyces cerevisiae*. An *rhp57* (***R****AD57* **h**omolog of *S. p**ombe*) deletion strain was more sensitive to MMS, UV, and γ-rays than the wild-type strain and showed a reduction in the frequency of mitotic homologous recombination. The MMS sensitivity was more severe at lower temperature and was suppressed by the presence of a multicopy plasmid bearing the *rhp51* gene. An *rhp51 rhp57* double mutant was as sensitive to UV and γ-rays as an *rhp51* single mutant, indicating that *rhp51* function is epistatic to that of *rhp57*. These characteristics of the *rhp57* mutants are very similar to those of *S. cerevisiae rad57* mutants. Phylogenetic analysis suggests that Rhp57 and Rad57 are evolutionarily closest to human Xrcc3 of the RecA/Rad51 family of proteins.

DOUBLE-strand breaks (DSBs) can be repaired by at least two mechanisms in eukaryotes: recombinational repair and nonhomologous end joining (NHEJ). In lower eukaryotes such as baker's yeast, recombinational repair is the major pathway for DSB repair, while in higher eukaryotes, such as mammals, NHEJ is the major pathway. The recombination repair mechanism in eukaryotes has been studied extensively in the budding yeast *Saccharomyces cerevisiae*, and many genes belonging to the *RAD52* epistasis group have been identified. Strains with a mutation in these genes are sensitive to DNA-damaging agents that cause DSBs. The genes in the *RAD52* group are divided into two subgroups according to their distinct roles in meiosis, the *MRE11* and *RAD51* subgroups. *RAD50*, *MRE11*, and *XRS2*, belonging to one group, are required for the formation and processing of DSBs (Tsubouchi and Ogawa 1998). The *RAD51*, *RAD52*, *RAD54*, *RAD55*, and *RAD57* genes belonging to *RAD51* subgroup are involved in promoting pairing and strand exchange reactions between two homologous DNA molecules, leading to formation of recombination intermediates (Shinohara and Ogawa 1995).

Studies on the functions of the *RAD51* subgroup of genes revealed the physical interactions *in vivo* between Rad51 and Rad52, Rad51 and Rad54, Rad51 and Rad55, and Rad55 and Rad57 (Hays *et al.* 1995; Johnson and Symington 1995; Jiang *et al.* 1996; Clever *et al.* 1997). The biochemical properties of these proteins have also been studied. Rad51, which is structurally homologous to *Escherichia coli* RecA, forms nucleoprotein filaments with ssDNA, like RecA (Shinohara *et al.* 1992; Ogawa *et al.* 1993), and mediates homologous pairing and strand exchange (Sung 1994; Sung and Robberson 1995). Therefore, Rad51 is a functional homolog of RecA. Rad52 stimulates this reaction when the single-stranded DNA-binding factor RPA is added prior to or simultaneously with Rad51 (Sugiyama *et al.* 1997; Sung 1997a; Shinohara and Ogawa 1998), and Rad54 is required for the promotion of D-loop formation by Rad51 (Petukhova *et al.* 1998). Rad55 and Rad57, both of which also have some sequence similarity to RecA, form a stable heterodimer and promote the Rad51-mediated strand exchange in the presence of RPA (Sung 1997b). Although no strand exchange activity of these two RecA homologs by themselves has been detected, focus formation of Rad51 in meiosis is dependent on the presence of Rad57, suggesting that Rad55 and Rad57 are required for efficient strand exchange by Rad51 (Gasior *et al.* 1998). The detection of protein-protein interactions between functionally related proteins suggests that such interactions provide further evidence in support of this view (Hays *et al.* 1995).

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As a model system to study repair systems for DSBs, we have selected *Schizosaccharomyces pombe*, in which much less work has been done than in *S. cerevisiae*. The *rhp51*, *rhp54*, *rad22*, and *rad32* genes are structurally and functionally similar to the *RAD51*, *RAD54*, *RAD52*, and *MRE11* genes of *S. cerevisiae*, respectively (Muris *et al.* 1993, 1996; Ostermann *et al.* 1993; Shinohara *et al.* 1993; Jang *et al.* 1994; Tavassoli *et al.* 1995). We have initiated extensive efforts to identify new genes of *S. pombe* involved in DNA repair by isolating repair-deficient mutants and cloning the genes that complement the mutant phenotypes. One of the strategies used to identify repair-deficient mutants is described below.

S. pombe Rad2 is a member of the human FEN-1 family. The FEN-1 protein has a flap-structure-specific endonuclease and 5′ to 3′ exonuclease activities and is implicated in the removal of RNA primers attached to the 5′ ends of Okazaki fragments in DNA replication (for review see Waga and Stillman 1998). A *rad2* mutant is sensitive to UV and exhibits elevated chromosome loss rates (Murray *et al.* 1994). The *FEN-1* gene complements a *rad2* mutant, indicating that FEN-1 is structurally and functionally similar to Rad2 (Murray *et al.* 1994). Thus, Rad2 is thought to act in a similar manner to FEN-1 in DNA replication. Strains defective in processing Okazaki fragments require recombination function for viability in *S. cerevisiae* and in *E. coli*, since they produce DSBs during replication (Cao and Kogoma 1995; Symington 1998). Indeed, *rhp51*, *rhp54*, and *rad32* mutations also cause lethality when combined with the *rad2* mutation in *S. pombe* (Tavassoli *et al.* 1995; Muris *et al.* 1996). Mutations in the genes involved in processing the Holliday recombination intermediates into mature recombinant molecules cause lethality when combined with a *polA* mutation in *E. coli* (Ishioka *et al.* 1997, 1998). None of the genes involved in the late stage of recombination have been identified in eukaryotes; thus, identifying mutations that cause synthetic lethality when combined with *rad2* might provide a means to identify late acting as well as early acting recombination functions.

To identify novel genes involved in recombination repair, in the present study we screened for mutants that were sensitive to methyl methanesulfonate (MMS) and γ-rays and that were, in addition, nonviable when combined with the *rad2* mutation. Using complementation of the MMS sensitivity of one such mutant, we cloned a novel gene that encodes a protein whose sequence is similar to *S. cerevisiae* Rad57, and we named this gene *rhp57* (***R****AD57* **h**omolog of *S. **p**ombe*). An *rhp57* deletion strain was constructed and its phenotypes were studied in relation to those of *rad57* mutants of *S. cerevisiae*.

MATERIALS AND METHODS

Strains, plasmids, media, and mutagenesis: The *S. pombe* strains used in this study are shown in Table 1. *S. pombe* cells were grown and maintained in YES or EMM media supplemented with appropriate nutrients as described in Moreno *et al.* (1991). Mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was carried out as described in Moreno *et al.* (1991). pUR19 is described in Barbet *et al.* (1992). The plasmid pAUR2 carries the *Bgl*II fragment of pRN2 (Murray *et al.* 1994; a gift from A. M. Carr), which contains the *rad2* gene, on pUR19. pSP102 was a gift from H. Masukata, and it contains the *S. cerevisiae LEU2* gene and an autonomous replicating sequence (*ars2004*) of *S. pombe* (Okuno *et al.* 1997). The *Bgl*II-*Bam*HI fragment of the *S. pombe* chromosomal DNA carried on the plasmid p51-410 (a gift from A. Pastink) containing the *rhp51* gene (Muris *et al.* 1993) was cloned into the *Bam*HI site of pSP102 to generate pYT101. pYT102 carries the *LEU2*, *rhp57* genes and the *ars* sequence. The *S. pombe* cDNA library was a gift from H. Nojima (Chen and Okayama 1987). The *leu1*$^+$-containing pJK148 plasmid used for the homologous integrations at the *leu1-32* locus is described in Keeney and Boeke (1994). The autonomously replicating pREP1 plasmid carries the *S. cerevisiae LEU2* marker gene and was used as a control for DNA uptake (Maundrell 1993).

Cloning of the gene that complements the MMS sensitivity of a repair-deficient mutant: One of the mutant strains defective in DNA repair was transformed with 5–10 μg of *S. pombe* genomic library as described by Okazaki *et al.* (1990), and the transformants were plated directly onto selective agar plates containing 0.004% MMS. Cells were grown at 30° for 3–4 days. More than 20,000 individual transformants were screened for MMS resistance and the candidate transformants were examined for plasmid dependency of MMS resistance. Plasmids were prepared from the plasmid-dependent resistant colonies and recovered in *E. coli* after transformation as described by Barbet *et al.* (1992). The physical map of the isolated plasmids was constructed and the DNA sequence of the minimal complementing region was analyzed.

Construction of the deletion mutants: The *rhp57* deletion mutant was constructed essentially as described in Grimm *et al.* (1988). The *Dra*I-*Kpn*I fragment of pSLR2-2 carrying the *rhp57* gene was cloned into the *Sma*I-*Kpn*I site of pBluescript SK(+) to generate pYT103. The *Eco*RI-*Hin*dIII fragment of pYT103, which covers most of the *rhp57* coding region, was replaced with the 1.8-kb *Hin*dIII fragment containing the *S. pombe ura4* gene or the 2.2-kb *Hin*dIII fragment containing *S. cerevisiae LEU2* to generate pYT103U and pYT103L. pYT103U and pYT103L were linearized by *Bam*HI digestion and transformed into the haploid *S. pombe* strain TMP701. The Ura$^+$ or Leu$^+$ transformants were checked for the *rhp57* deletion by genomic Southern analysis. The plasmid for the *rhp51* gene disruption was as described in Muris *et al.* (1993), and the linearized plasmid was transformed into the haploid strain TMP701. The *rhp51* disruption was confirmed by genomic Southern analysis.

γ-ray, UV light, and MMS sensitivity tests: Cells were grown to midlog phase (OD_{595} = 0.3–0.5) in YES, suspended in water, and irradiated with γ-rays from a ^{60}Co source at a dose rate of 184 Gy/hr. After irradiation, appropriately diluted samples were plated on YES plates and incubated at 30° for 4–7 days, and the resulting colonies were counted.

Midlog phase cultures diluted to appropriate concentrations were plated onto YES plates and the plates were irradiated with UV light at the indicated doses. They were incubated in the dark at 30° for 4–7 days, and the colonies were counted.

MMS was added to the concentration of 0.1% to midlog phase cells suspended in 5 ml of 0.05 m K_2HPO_4 in a 50-ml plastic tube, and the mixture was incubated at 30° for the indicated periods. Samples (500 μl) were taken from the cultures and mixed with 500 μl of 20% $Na_2S_2O_3$ to inactivate MMS. The cells were then washed with water, resuspended in 1 ml of water, appropriately diluted, plated onto YES plates,

TABLE 1

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

Strain	Genotype	Source
*rad2*Δ	*h*⁻ *rad2::ura4*⁺ *ura4-D18 leu1-32 ade6-704*	A. M. Carr
IBGY5-1	*h*⁻ *smt-0 ura4-D18 leu1-32*	V. I. Bashkirov
IBGY10-1	*h*⁺ *mat1PΔ17::LEU2 ura4-D18 leu1-32 his3-D1 arg6-1*	V. I. Bashkirov
TMPR2C[a]	*h*⁻ *rad2::ura4*⁻ *ura4-D18 leu1-32 ade6-704* / pAUR2[b]	This study
TMPR2-2 (*slr2*)	*h*⁺ *rhp57-1 ura4-D18 leu1-32*	This study
TMP701[c]	*h*⁺ *ura4-D18 leu1-32 his7-366 ade6-M216*	This study
TMP702	*h*⁺ *ura4-D18 leu1-32*	This study
TMP703	*h*⁻ *ura4-D18 leu1-32*	This study
TMP711 (*rhp57*Δ)	*h*⁺ *rhp57::ura4*⁺ *ura4-D18 leu1-32 his7-366 ade6-M216*	This study
TMP712 (*rhp51*Δ)	*h*⁺ *rhp51::ura4*⁺ *ura4-D18 leu1-32 his7-366 ade6-M216*	This study
TMP713 (*rhp51*Δ *rhp57*Δ)	*h*⁺ *rhp51::ura4*⁺ *rhp57::LEU2 ura4-D18 leu1-32 his7-366 ade6-M216*	This study
TMP714	*h*⁺ *rhp57::LEU2 ura4-D18 leu1-32 his7-366 ade6-M216*	This study
TMP715	*h*⁻ *rhp57::LEU2 ura4-D18 leu1-32 his7-366*	This study
TMP721	*h*⁺ *rad2::ura4*⁺ *ura4-D18 leu1-32*	This study
TMP751	*h*⁺ *mat1PΔ17::LEU2 ura4-D18 leu1-32 ade6-M216*	This study
TMP752	*h*⁺ *mat1PΔ17::LEU2 rhp57::ura4*⁺ *ura4-D18 leu1-32 ade6-M216*	This study
TMP753	*h*⁻ *smt-0 rhp57::ura4*⁺ *ura4-D18 leu1-32*	This study

[a] The *rad2::ura4*⁻ strain was isolated from the *rad2*Δ cells by selecting a colony formed on an EMM plate containing 5-FOA, uracil, leucine, and adenine. The UV sensitivity of the resulting Ura⁻ colony was the same as that of the parental *rad2*Δ strain.

[b] The plasmid pAUR2 carries the *S. pombe ura4* and *rad2* genes and the *ars1* sequence.

[c] TMP701 was used as a wild-type haploid strain for MMS and γ-ray sensitivity tests and for homologous integration assays.

and incubated at 22° or 30° for 4–7 days before counting the colonies.

For the spot assay of MMS sensitivity, sequential 10-fold dilutions of late log cultures were spotted on EMM plates with or without MMS 0.004%. Plates were incubated at 30° for 4 days.

Cloning of *rhp57* cDNA: *rhp57* cDNA was amplified with sense primer 5′ primer-1 5′-GAAAAAGTGGGTTAGAAGTTC GTC-3′ and antisense primer 3′ primer-1 5′-GGGCAATTCA TAAACCGG-3′ (see Figure 2) using the *S. pombe* cDNA library as template, and the PCR products were cloned into pT7-Blue-2 (Novagen) and sequenced.

Site-directed mutagenesis: A synthetic 25-mer TCACATAT GGATATTTCGAATTATG and 31-mer TCAGGATCCCTAG CACGAATATATCCCAACC were used for PCR to generate a new *Nde*I site at the ATG initiation codon and a *Bam*HI site downstream from the TAG termination codon of *rhp57* cDNA, respectively. The PCR product containing the *rhp57* gene was digested with *Nde*I and *Bam*HI, cloned into the *Nde*I-*Bam*HI site of pUC19 to yield pYS102, and sequenced. Using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), site-directed mutagenesis was carried out with two sets of the synthetic oligonucleotide primers as follows. To generate *rhp57* (K106A) mutant gene, 106A-1, AGCGGTTCA GGGGCATCACAGTTTTGTATG and K106A-2, CATACAA AACTGTGATGCC-CCTGAACCGC were used. To generate *rhp57* (K106R) mutant gene, K106R-1, AGCGGTTCAGGGCG ATCA-CAGTTTTGTATG and K106R-2, CATACAAAACTGTG ATCGCCCTGAACCG-C were used. The mutant cDNA was digested with *Nde*I and *Bam*HI and cloned into the *Nde*I-*Bam*HI site of pREP1. The resultant plasmids, pREP1-R57K106A and pREP1-R57K106R, were introduced into the *rhp57*Δ strain by transformation and MMS sensitivity was tested by the spot assay.

Sequencing of the *rhp57-1* mutant gene: The *rhp57* gene region covering 470 bp upstream of the first codon and 60 bp downstream of the stop codon was amplified using genomic DNA isolated from the *rhp57-1* mutant strain (TMPR2-2) as template with several sets of appropriate synthetic oligonucleotide primers. The PCR product was purified and directly sequenced for both strands with overlaps using synthetic primers.

Mitotic homologous recombination at the *leu1-32* locus: The efficiency of integration by homologous recombination was assayed as described in Muris *et al.* (1997). Briefly, the *rhp57*Δ (TMP711), *rhp51*Δ (TMP712), and isogenic wild-type (TMP-701) strains with the *leu1-32* mutation were transformed with 0.25 or 5 μg of linearized pJK148 plasmid DNA carrying the *S. pombe leu1*⁺ gene. Corresponding amounts of the uncut pREP1 plasmid carrying the *S. cerevisiae LEU2* gene and the *S. pombe ars* sequence were used as controls to measure the transformation efficiency. Leu⁺ transformants were selected on EMM plates supplemented with histidine, adenine, and uracil after incubation for 10 days at 30°. No Leu⁺ revertants were detected among mock-transformed cells. For each strain, the recombination frequency was calculated by dividing the number of Leu⁺ transformants obtained with linearized pJK148 by that obtained with pREP1 (pJK148/pREP1 ratio). The recombination frequencies of the *rhp57*Δ and *rhp51*Δ mutants were determined by relating the pJK148/pREP1 ratio of the mutant to the pJK148/pREP1 ratio of the wild-type strain.

Phylogenetic analysis: A multiple sequence alignment of the RecA/Rad51 homologs of humans (Hs), *S. pombe* (Sp), and *S. cerevisiae* (Sc) was constructed by using the CLUSTAL W program (Thompson *et al.* 1994). The references for 13 sequences used in this study are as follows: HsRad51 (Shinohara *et al.* 1993), HsDmc1 (Habu *et al.* 1996), Rad51B/hREC2 (Albala *et al.* 1997; Rice *et al.* 1997), Rad51C (Dosanjh *et al.* 1998), Rad51D (Pittman *et al.* 1998), Xrcc2 (Cartwright *et al.* 1998; Liu *et al.* 1998), Xrcc3 (Liu *et al.* 1998), ScRad51 (Basile *et al.* 1992; Shinohara *et al.* 1992), ScDmc1 (Bishop

et al. 1992), Rad57 (Kans and Mortimer 1991), Rhp51 (Shinohara *et al.* 1993), SpDmc1 (SWISS-PROT accession no. 042634), and Rhp57 (this work). Likewise, Rad51 and Dmc1 proteins were aligned by using the same program. It was difficult, however, to automatically align the sequences of Rad51s/Dmc1s together with those of the other homologs due to the high sequence divergence among the homologs, except for Rad51s/Dmc1s. Therefore, multiple alignment of Rad51s/Dmc1s and the other homologs was done manually based on the alignment shown in Heyer (1994), which was modified by visual inspection to increase the similarity. On the basis of this alignment, the genetic distance for every pair of aligned sequences was calculated as a maximum likelihood estimate (Felsenstein 1996). The overlapping regions used for phylogenetic analysis are from positions 82 to 354 of Rhp57. The aligned regions containing gaps were excluded from the calculation. Then, an unrooted molecular phylogenetic tree of the RecA/Rad51 homologs was constructed by the neighbor-joining method (Saitou and Nei 1987) using the genetic distances. The statistical significance of the obtained tree topology was evaluated by bootstrap analysis (Felsenstein 1985) with 1000 iterations. A program package, PHYLIP (version 3.5c; Felsenstein 1993), was used for the molecular phylogenetic analysis.

RESULTS

Isolation of mutants defective in repairing DNA DSBs: Mutations in recombination repair genes, such as *rhp51*, *rhp54*, and *rad32*, are lethal in combination with *rad2* mutations. As a strategy to isolate recombination-defective mutants, we identified mutations that caused lethality in a *rad2* background. For this purpose, we used the strain TMPR2C, which has a *rad2* null mutation, and a plasmid carrying the *rad2* and *ura4* genes. We expected that recombination repair mutants derived from TMPR2C would die in the presence of a *rad2* mutation, and therefore they would not be able to lose the $rad2^+ \cdot ura4^+$ plasmid on EMM plates and would not grow on a medium containing 5-fluoroorotic acid (5-FOA). The cells of strain TMPR2C were mutagenized with MNNG and allowed to form colonies on EMM plates lacking uracil. About 7000 colonies were examined for MMS sensitivity by replica plating on EMM plates containing 0.004% MMS, and 282 MMS-sensitive colonies were identified initially. These candidates were spotted onto YES plates and irradiated with γ-rays at 800 Gy. A total of 27 isolates that did not grow after γ-ray irradiation were selected. Of them, 17 were defective in growth on EMM plates containing 5-FOA, so they were expected to carry mutations that cause defective growth when combined with *rad2*. These candidates were examined microscopically after irradiation with γ-rays at 800 Gy. A total of 7 mutants septated without elongation, which suggested that they were defective in radiation-checkpoint control and hence they were not analyzed further. Of the remaining 10 mutant strains, 7 were successively backcrossed three times with the wild-type strain. We attempted to clone the corresponding genes of all of the 7 mutants and succeeded in isolating three genes (our unpublished results). Cloning by complementation using a mutant tentatively named *slr2* (**s**ynthetic **l**ethal with *rad2*) is described below.

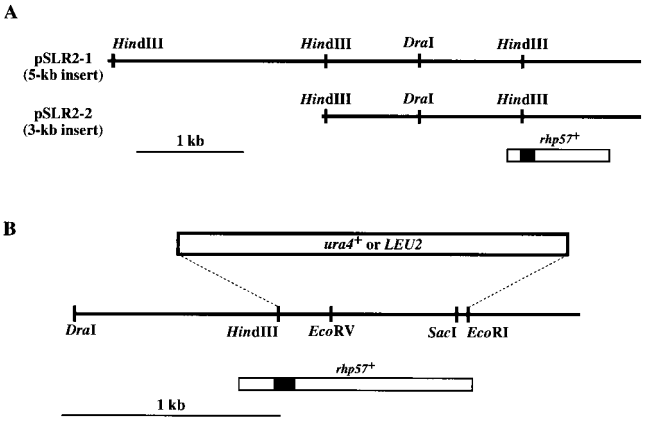

Figure 1.—Physical map of the *rhp57* region and construction of an *rhp57* deletion mutant. (A) Restriction maps of the DNA fragments that complemented the *slr2* mutant. Two kinds of plasmid clones were isolated from the *S. pombe* genomic library by complementation of the MMS sensitivity of the mutant. Sequence analysis of pSLR2-2 revealed an open reading frame, *rhp57*, which encodes a protein of 354 amino acids. The open boxes represent exons and the closed box represents an intron. (B) Diagram of construction of an *rhp57* deletion strain. Plasmids containing the *rhp57* deletion were constructed by replacing the 0.8-kb *Eco*RI-*Hin*dIII fragment with the *S. pombe ura4*$^+$ or *S. cerevisiae LEU2* gene. The chromosomal DNA fragment containing the disrupted *rhp57* region was used to transform the wild-type haploid strain TMP701 as described in materials and methods.

Cloning and sequencing of the *S. pombe* genomic DNA fragment that complemented a repair-deficient mutation: Two kinds of plasmid clones that conferred MMS resistance on the *slr2* mutant were isolated from the genomic library and these plasmids contained the overlapping chromosomal region (Figure 1). Both of them restored the wild-type level of the γ-ray sensitivity to the *slr2* mutant (data not shown). Sequence analysis of the overlapping region revealed a large open reading frame (*orf*) of 340 amino acids (Figure 2). This *orf* could be extended further by splicing a putative 96-bp intron with the consensus sequences for the 5′ splice, branch, and 3′ splice sites, which are GTA(A/T)GT, CTAA(C/T), and (T/C)AG (Figure 2), respectively, observed in many introns in *S. pombe* (Prabhala *et al.* 1992). The presence of this intron and the absence of any other introns in this *orf* were confirmed by amplifying and sequencing the cDNA corresponding to this *orf* of 354 amino acids. The nucleotide sequence data are available from the DDBJ/EMBL/GenBank nucleotide databases (accession no. AB024744). Upstream of this gene, two putative TATA-box motifs were found, one at position 754 (TATATAAAA), and one at position 794 (TATAAT; Figure 2). In the 3′ untranslated region, a putative polyadenylation signal was found at position 2066 (AATAAA).

The database search for the predicted 354 amino acid

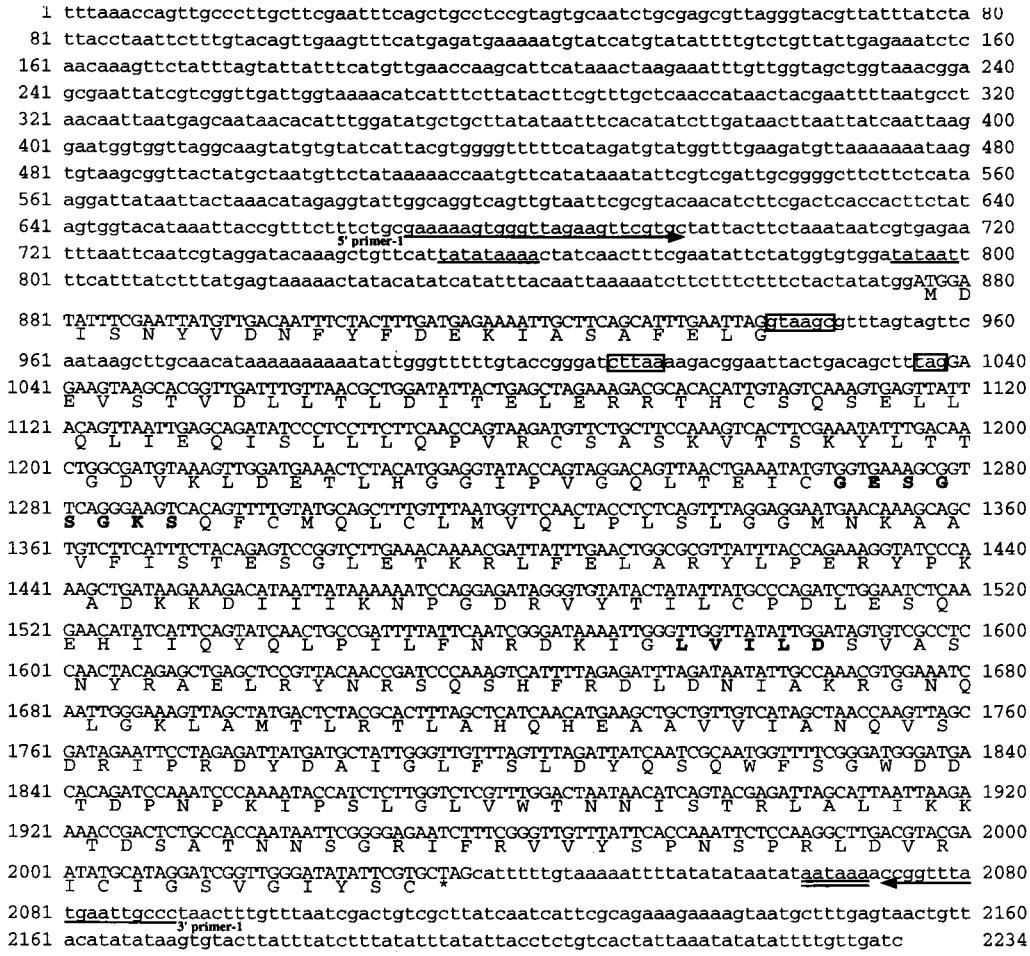

Figure 2.—Nucleotide sequence of the *rhp57* gene. The intron is shown in lowercase letters and the deduced amino acid sequence is shown with the single-letter code. The consensus sequences for the 5′ splice, branch, and 3′ splice sites are boxed and shown in that order. Putative TATA-box motifs are underlined, and a putative polyadenylation sequence is shown by a double underline. The nucleoside-triphosphate-binding motifs (Walker motifs A and B) are shown by boldface letters. The primers used in the cloning of *rhp57* cDNA are shown by arrows.

sequence encoded by the complete gene revealed that it is homologous to proteins related to *S. cerevisiae* Rad51. Among those proteins, the highest homology was found with *S. cerevisiae* Rad57, with 32% identity and 51% similarity (Figure 3). Therefore, we named this gene *rhp57*, standing for ***R****AD57* **h**omolog of *S. pombe*. Rhp57 also has some homology with *S. cerevisiae* Rad51 (24% identity and 44% similarity) and human Xrcc3 (26% identity and 49% similarity; Figure 3).

The *rhp57-1* mutation site: The *rhp57* gene of the original mutant was amplified and sequenced. A single transition of G to A was found at the second base of

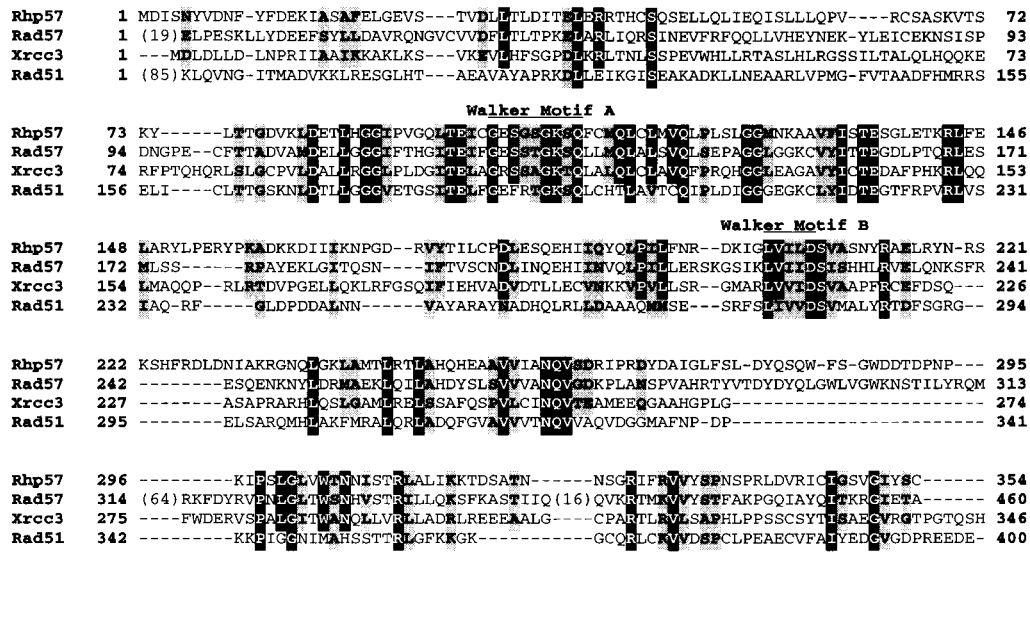

Figure 3.—Sequence alignment of Rhp57, *S. cerevisiae* Rad57, human Xrcc3 and *S. cerevisiae* Rad51. The invariant and similar amino acid residues conserved among Rhp57, Rad57, and Xrcc3 are shaded in black and gray, respectively. When these are conserved also in Rad51, they are shaded in the same manner. Here, amino acid residues are classified into six groups based on physicochemical similarity: LIMV, SPTAG, YWF, DEQN, KRH, and C. The Walker motifs A and B for nucleotide binding are overlined.

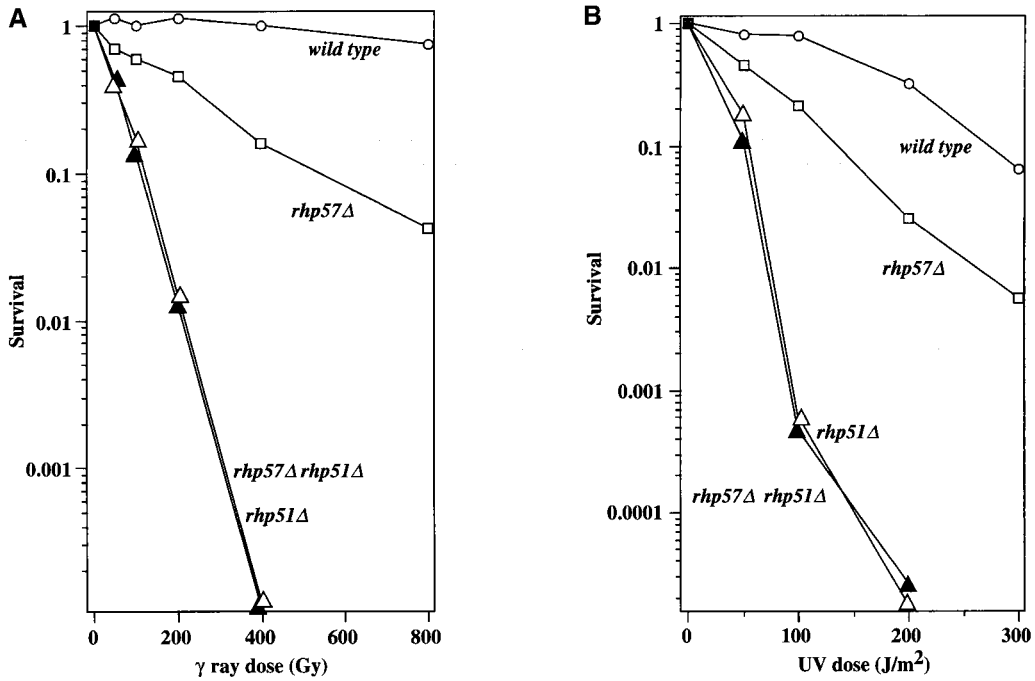

Figure 4.—Sensitivities of the *rhp57*Δ mutants to UV (A) and γ-rays (B). Midlog phase cells were irradiated with UV or γ-rays at the indicated doses, and the relative plating efficiencies were determined. Wild-type strain, TMP701 (open circles); *rhp57*Δ strain, TMP711 (open squares); *rhp51*Δ strain, TMP712 (open triangles); and *rhp51*Δ *rhp57*Δ strain, TMP713 (solid triangles). The data represent the average of three experiments.

the 304th codon, causing a change from the TGG codon for tryptophan to a TAG amber nonsense codon. Therefore, we concluded that this mutation is responsible for the phenotype of the original mutant and named this mutant allele *rhp57-1*.

Phenotypes of the *rhp57* deletion mutant: The *rhp57* gene was disrupted by replacing most of the coding region with the *ura4* gene (Figure 1). The resultant strain was more sensitive to MMS, UV, and γ-rays than the wild-type strain (Figures 4 and 5), and the sensitivities were comparable to those of the *rhp57-1* mutant, suggesting that *rhp57-1* is a null mutation (data not shown).

S. cerevisiae rad57 mutants were more sensitive to ionizing radiation at a lower temperature (22°) than at 30° (Hays *et al.* 1995; Johnson and Symington 1995). Therefore, the effect of growth temperature on MMS sensitivity of the *rhp57*Δ mutant was examined. As shown in Figure 5, the *rhp57*Δ mutant was more sensitive to MMS at 22° than at 30°, while the sensitivities of the wild-type strain and the *rhp51*Δ mutant were not increased at the lower temperature.

Role of Walker motif A of Rhp57 for recombination repair: In *S. cerevisiae*, the nucleoside-binding motif in Rad55 (GxxxxGKS/T) is important for the function, but the corresponding one in Rad57 is not (Johnson and Symington 1995). We constructed two *rhp57* mutant genes by site-directed mutagenesis, altering the highly conserved lysine residue of Rhp57 to either an alanine or arginine residue. These mutant cDNAs were cloned into pREP1, introduced into the *rhp57*Δ mutant by transformation, and their abilities to complement the MMS sensitivity were tested by spot assay (Figure 6). The strains containing K106A and K106R were as resistant to MMS as the wild type, indicating that the nucleoside-binding motif of Rhp57 is not important for recombinational repair as in the case of Rad57 in *S. cerevisiae*.

***rhp51* is epistatic to *rhp57*:** To examine the possible epistasis between *rhp57* and *rhp51*, an *rhp51*Δ *rhp57*Δ double mutant was constructed and the sensitivities of the mutant to UV and γ-rays were assayed. As shown in Figure 4, the *rhp51*Δ mutant was more sensitive to UV and γ-rays than the *rhp57*Δ mutant and comparable to the *rhp51*Δ *rhp57*Δ double mutant in sensitivity, indicating that *rhp51* is epistatic to *rhp57*.

Evidence for functional interactions between *rhp57* and *rhp51*: Overexpression of either the Rad51 or Rad52 proteins in *S. cerevisiae* partially suppresses the sensitivity of *rad55* and *rad57* mutants to ionizing radiation (Hays *et al.* 1995; Johnson and Symington 1995). To investigate the functional interaction between the Rhp57 and Rhp51 proteins in *S. pombe*, the MMS sensitivity of the *rhp57*Δ mutant carrying a multicopy plasmid bearing the *rhp51* gene was measured (Figure 7). The overexpressed Rhp51 protein suppressed the MMS sensitivity of the *rhp57* mutant, indicating a functional interaction between the Rhp57 and Rhp51 proteins.

Mitotic homologous recombination in the *rhp57*Δ mutant: The capability of the *rhp57* mutant to carry out homologous recombination was studied by measuring the efficiency of integration of homologous linear DNA into the *leu1-32* locus (Table 2). The efficiency of homologous integration in the *rhp57*Δ strain was reduced 3.6-fold when 0.25 μg DNA, which is in the linear range of dose response, was used. When the efficiencies were

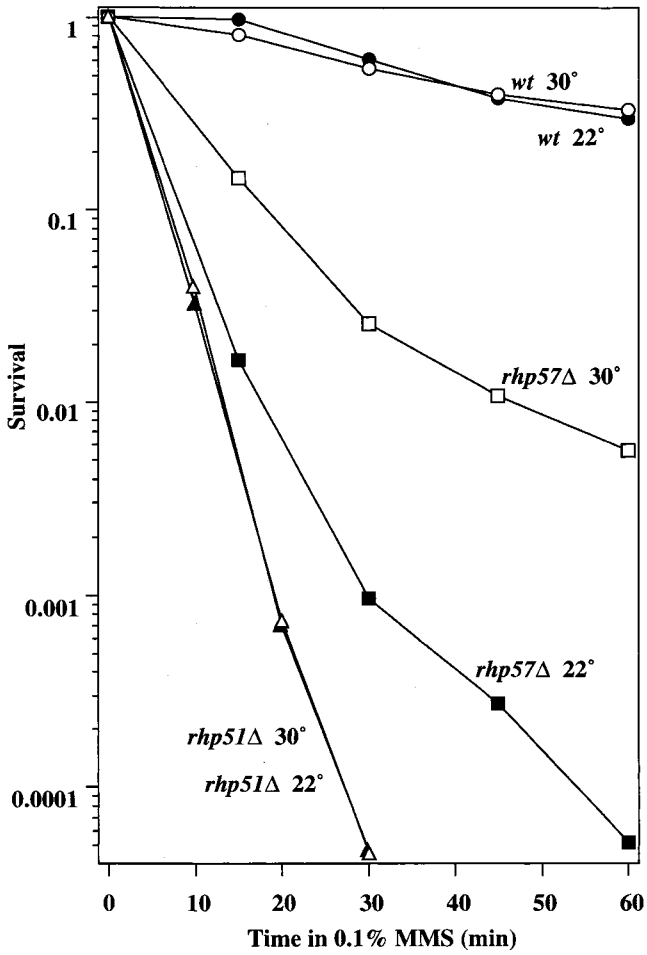

Figure 5.—Sensitivities of *rhp57*Δ mutant to MMS at 30° and 22°. MMS sensitivities of the *rhp57*Δ mutant grown at 30° and 22° were assayed as described in materials and methods. Wild-type strain (TMP701) at 30° (open circles), wild-type strain (TMP701) at 22° (solid circles), *rhp57*Δ strain (TMP711) at 30° (open squares), *rhp57*Δ strain (TMP711) at 22° (solid squares), *rhp51*Δ strain (TMP712) at 30° (open triangles), and *rhp51*Δ strain (TMP712) at 22° (solid triangles). The data represent the average of three experiments.

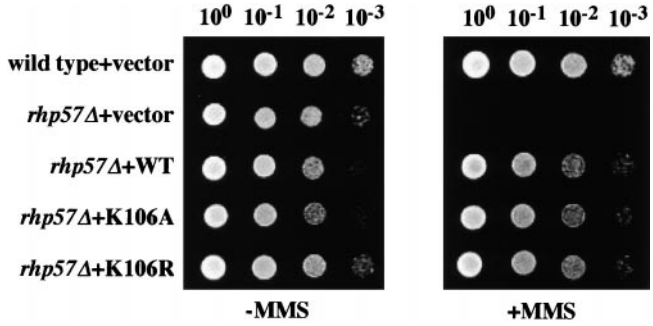

Figure 6.—Effect of the *rhp57* mutations altering the Walker motif A on the sensitivity to MMS. Late-log phase cultures of the wild-type strain (TMP701) carrying pREP1 and *rhp57*Δ (TMP711) strains carrying pREP1, pREP1-Rhp57, pREP1-R57K106A, or pREP1-R57K106R were spotted at different dilutions on EMM plates with or without MMS (0.004%) as described in materials and methods.

compared by using 5 μg DNA, which is above the saturating dose of the plasmid transformation, it was reduced 5-fold in the *rhp57*Δ strain and 30-fold in the *rhp51*Δ strain compared to that of the wild-type strain. These results indicate that Rhp57 protein is involved in homologous recombination in vegetative cells.

Synthetic lethality with the *rad2* mutation: Synthetic lethality of the *rhp57* mutation with the *rad2* mutation was confirmed by crossing a *rhp57::LEU2* strain, TMP715, with a *rad2::ura4*$^+$ mutant, TMP721, and analyzing the resulting segregants. Among the 13 tetrads dissected, the ratios of the segregants of Leu$^-$Ura$^-$, Leu$^+$Ura$^-$, Leu$^-$Ura$^+$, and Leu$^+$Ura$^+$ were 7, 13, 14, and 0, respectively. By random spore analysis, the ratios of the segregants of Leu$^-$Ura$^-$, Leu$^+$Ura$^-$, Leu$^-$Ura$^+$, and Leu$^+$Ura$^+$ were 570, 435, 626, and 0, respectively. These results show that the *rhp57::LEU2 rad2::ura4*$^+$ double mutant is nonviable, as is the *rhp57-1 rad2::ura4*$^-$ double mutant.

Spore viability was reduced in the *rhp57*Δ strain: Since it was reported that the spore viabilities of recombination mutants such as *rhp51*, *rad22*, *rhp54*, and *rhp55* were reduced in *S. pombe* (Muris *et al.* 1997; Khasanov *et al.* 1999), spore viability was examined in the *rhp57* mutant. To avoid the synergistic effect of spontaneous DSB at *mat1* locus on spore viability, the strains carrying *cis* mutations (*smt-0* or *mat1p*Δ*17::LEU2*), which eliminate DSB formation at *mat1*, were used (Khasanov *et al.* 1999). The viability of spores formed by a cross of *mat1p*-Δ*17::LEU2 rhp57::ura4*$^+$ × *smt-0 rhp57::ura4*$^+$ (TMP752 × TMP753) was reduced to 58%, while the spore viability *mat1p*Δ*17::LEU2 rhp57*$^+$ × *smt-0 rhp57*$^+$ (TMP751 × IBGY5-1) was 98%.

Phylogenetic analysis: Three RecA/Rad51 protein homologs, Rhp51, Rhp55, and SpDmc1, have been identified previously in *S. pombe*. Rhp57, which we isolated in this work, is the fourth RecA/Rad51 homolog found in *S. pombe*. *S. cerevisiae* has counterparts of these four proteins, namely ScRad51, Rad55, ScDmc1, and Rad57, respectively. In humans, 7 RecA/Rad51 homologs have been identified: HsRad51, Rad51B/hREC2, Rad51C, Rad51D, Xrcc2, Xrcc3, and HsDmc1. The evolutionary relationships between these seven human proteins and four fungal proteins have not yet been extensively investigated. Therefore, we performed molecular phylogenetic analysis using amino acid sequence data of 13 RecA/Rad51 homologs to reveal the evolutionary relationships among them. However, RecA, Rad55, and Rhp55 were not included in the 13 sequences analyzed in the present phylogenetic study since the distinctly greater divergence of these 3 sequences from the other 13 sequences hindered calculation of the phylogenetic relationship.

The unrooted phylogenetic tree obtained as described in materials and methods is shown in Figure

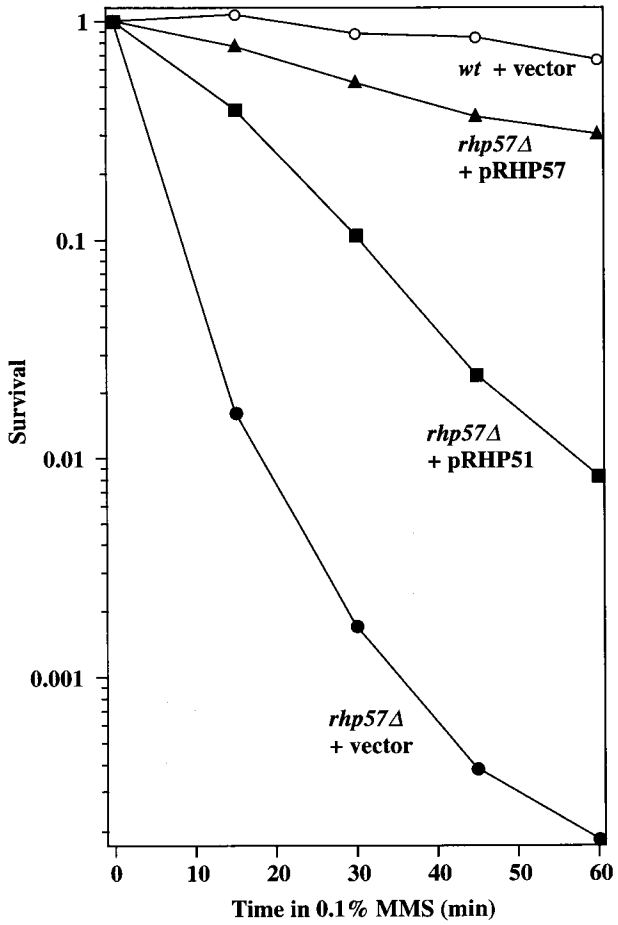

Figure 7.—The effect of overexpression of Rhp51 on the MMS sensitivity of the *rhp57*Δ strain. The MMS sensitivity of the *rhp57*Δ strain carrying either the *rhp57* or *rhp51* gene on a multicopy plasmid was determined. Wild-type strain (TMP701) with the vector pSP102 (open circles), *rhp57*Δ strain (TMP711) with the vector pSP102 (solid circles), *rhp57*Δ strain (TMP711) with the *rhp51*$^+$ plasmid pYT101 (solid squares), and *rhp57*Δ strain (TMP711) with the *rhp57*$^+$ plasmid pYT102 (solid triangles). The data represent the average of three experiments.

8. The number associated with each node indicates the bootstrap probability. When the number was ≥90.0%, the clustering at the node was regarded as statistically significant. As shown in Figure 8, ScRad51, Rhp51, and HsRad51 are closely related to each other, and the bootstrap probabilities of the clustering suggest that clustering is highly significant. Node X is regarded as the species divergence point between fungi and vertebrates. Likewise, ScDmc1, SpDmc1, and HsDmc1 are closely related to each other, and the clustering is highly significant. Node Y is also regarded as the species divergence point between fungi and vertebrates. The cluster of Dmc1 and that of Rad51/Rhp51 gather to form a larger cluster, which is also highly significant. Considering the position of node Z relative to nodes X and Y, the functional divergence between Rad51/Rhp51 and Dmc1 is considered to have occurred before the divergence between fungi and vertebrates. Rhp57, Rad57, and Xrcc3 also form a cluster, and comparison of their sequences is shown in Figure 3. Rhp57 and Rad57 are more similar to Xrcc3 than ScRad51 is. The identities/similarities between Rhp57 and Xrcc3, between Rad57 and Xrcc3, and between ScRad51 and Xrcc3 are 26/49%, 25/46%, and 24/45%, respectively. The bootstrap probabilities associated with the nodes within the cluster did not satisfy the statistical criterion, 90.0%, but were quite close to 90.0%. Therefore, the clustering at node A is considered to be meaningful.

TABLE 2

Efficiency of integration by homologous recombination at the *leu1-32* locus in the *rhp57*Δ strain

	pJK148/pREP1 ratio[a]		Fold reduction	
Strain	0.25 μg	5 μg	0.25 μg	5 μg
TMP701 (wildtype)	0.26	0.72	1.0	1.0
TMP711 (*rhp57*Δ)	0.071	0.14	3.6	5.1
TMP712 (*rhp51*Δ)	ND[b]	0.024	ND[b]	31

[a] The efficiency of homologous recombination was assayed by measuring the ratio of Leu$^+$ transformants by linearized pJK148 to those by intact pREP1 plasmid as described in materials and methods. Data represent the average of three experiments.
[b] Integration was not detected.

DISCUSSION

S. cerevisiae RAD51, *RAD52*, and *RAD54* are homologous to *S. pombe rhp51*, *rad22*, and *rhp54*, respectively, and these *S. pombe* genes have been shown to be involved in DNA recombination and repair. Here we identified and characterized the *S. pombe rhp57* gene, which is structurally similar to *S. cerevisiae RAD57*. The *rhp57* null mutant was viable and more sensitive to DNA-damaging agents than the wild-type strain. Interestingly, this sensitivity was more severe at 22° than at 30°. The cold sensitivity of the *rhp57* mutant is similar to that of the *S. cerevisiae rad57* null mutant, which has increased sensitivity to X rays at a lower temperature (Hays *et al.* 1995; Johnson and Symington 1995). Moreover, *S. pombe rhp57* and *rhp51* interact functionally in a manner very similar to the *S. cerevisiae RAD57* and *RAD51* genes (see below). On the basis of these common properties, in conjunction with the structural resemblance, we propose that *S. pombe rhp57* is a functional homolog of *S. cerevisiae RAD57*. Most recently, an *S. pombe* gene structurally and functionally similar to *S. cerevisiae RAD55* was identified and designated *rhp55* (Khasanov *et al.* 1999). Therefore, *RAD51*, *RAD52*, *RAD54*, *RAD55*, and *RAD57* genes are conserved in *S. cerevisiae* and *S. pombe*. Consistent with the *in vitro* data, most of the currently available data from *in vivo* experiments suggest that Rad55 and Rad57 act as accessory factors in recombination by stimulating the Rad51 function rather than by playing a role

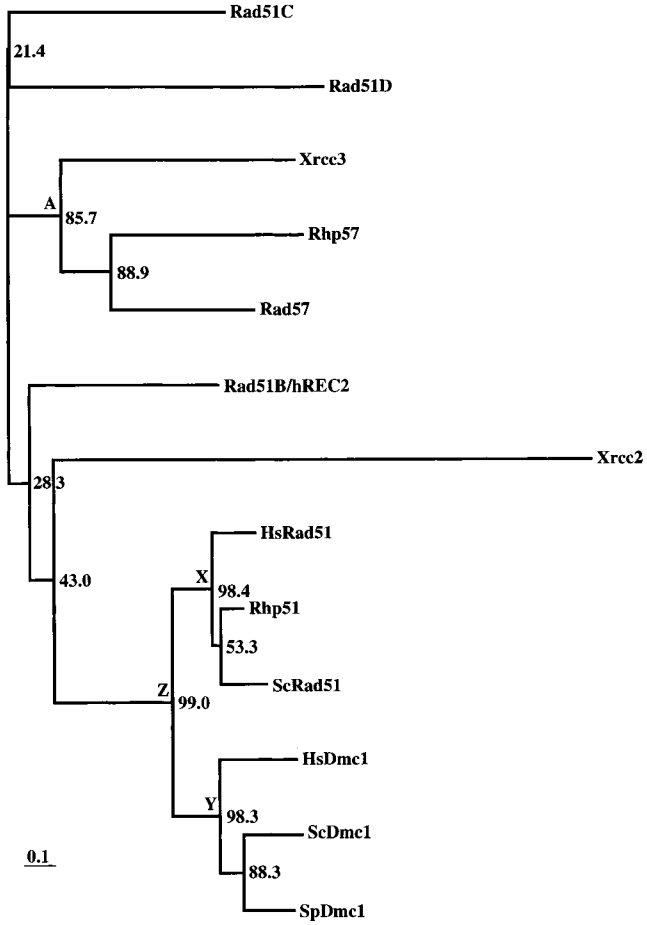

Figure 8.—The unrooted phylogenetic tree of 13 RecA/Rad51 homologs including Rhp57. A multiple alignment of 13 RecA/Rad51 homologs was done by the procedure described in materials and methods. On the basis of the alignment, the tree was constructed. The number at each node indicates the bootstrap probability of the clustering at the node. The references are listed in materials and methods.

redundant with that of Rad51. The *rad51* mutation is epistatic to the *rad55* and *rad57* mutations, suggesting that Rad55 and Rad57 play roles distinct from that of Rad51 in a pathway also involving Rad51. Moreover, overexpression of Rad51 partially suppresses the radiation-sensitive phenotype of the *rad55* and *rad57* mutants, suggesting that even in the absence of Rad55 or Rad57, Rad51 can promote recombination when present at a higher concentration. These phenotypes were also seen in the *S. pombe rhp57* mutant (this work) and *rhp55* mutant (Khasanov *et al.* 1999). Therefore, Rhp57 and Rhp55 also appear to be accessory factors that form a complex and stimulate Rhp51 function.

As noted above, five RecA/RAD51 homologs in mammals, in addition to human HsRad51 and HsDmc1, have been isolated recently. They probably also play accessory roles for HsRad51 (Bishop *et al.* 1998; Cartwright *et al.* 1998). Our phylogenetical analysis shows that these five proteins are much diverged from Rad51s and Dmc1s. The phylogenetical relationships among Rhp57, Rad57, and Xrcc3 are relatively close, suggesting that Xrcc3 may be most similar in function to Rhp57 and Rad57. Consistent with this notion, interaction between Rhp57 and Rhp51 was suggested by two-hybrid analysis in *S. cerevisiae* (our unpublished data), which would correspond to the interaction between Xrcc3 and HsRad51 suggested also by two-hybrid analysis (Liu *et al.* 1998). Further genetic and biochemical studies are needed to reveal the functional relationships between Rhp57/Rad57 and Xrcc3 in recombination. Unfortunately, we cannot draw any clear conclusion about the evolutionary relationships among the remaining RecA/Rad51 homologs due to the low bootstrap probabilities. At this stage we can only state that the seven human RecA/Rad51 homologs diverged before fungi and vertebrates diverged, because neither the clusters of ScDmc1/SpDmc1/HsDmc1 and HsRad51/Rhp51/ScRad51, nor the cluster of Xrcc3/Rhp57/Rad57 includes any other homolog within the cluster. The analysis also suggests that three copies of the seven duplicated genes have been deleted in the fungal lineage to *S. cerevisiae*, while they have been kept in the lineage to vertebrates. We must wait for further accumulation of sequence data of RecA/Rad51 homologs to clarify the evolutionary relationships among the RecA/Rad51 homologs.

In this study, we screened *S. pombe* mutants for MMS sensitivity, γ-ray sensitivity, and *rad2* synthetic lethality. We expected to isolate mutants defective in DSB repair, since MMS, γ-rays, and *rad2* mutations all cause DSBs in DNA. Two mechanisms that can repair DSBs are known in eukaryotes: recombination repair and NHEJ. Mutants defective in either mechanism may become hypersensitive to MMS and γ-rays, but a mutation in *HDF1* (NHEJ pathway) did not cause synthetic lethality when combined with a *rad27* mutation in *S. cerevisiae* (Symington 1998). Thus, the synthetic lethality with the *rad2* mutation is specific to homologous recombination defects and enabled us to isolate a mutant defective in a novel gene, *rhp57*, involved in recombination repair. This result promises further isolation of novel recombination genes by the present strategy, which will lead to extension of our knowledge of the regulatory mechanisms of recombination repair in *S. pombe*.

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