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Isolation of the *Schizosaccharomyces pombe RAD54* homologue, *rhp54*$^+$, a gene involved in the repair of radiation damage and replication fidelity

Daan F. R. Muris[1], Kees Vreeken[1], Antony M. Carr[2], Johanne M. Murray[3], Claudia Smit[1], Paul H. M. Lohman[1] and Albert Pastink[1,*]

[1]MGC, Department of Radiation Genetics and Chemical Mutagenesis, State University of Leiden, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands
[2]MRC Cell Mutation Unit, Falmer, Brighton, East Sussex BN1 9RR, UK
[3]Department of Biochemistry, Sussex University, Falmer, Brighton, East Sussex BN1 9QG, UK

*Author for correspondence

SUMMARY

The *RAD54* gene of *Saccharomyces cerevisiae* encodes a putative helicase, which is involved in the recombinational repair of DNA damage. The *RAD54* homologue of the fission yeast *Schizosaccharomyces pombe*, *rhp54*$^+$, was isolated by using the *RAD54* gene as a heterologous probe. The gene is predicted to encode a protein of 852 amino acids. The overall homology between the mutual proteins of the two species is 67% with 51% identical amino acids and 16% similar amino acids. A *rhp54* deletion mutant is very sensitive to both ionizing radiation and UV. Fluorescence microscopy of the *rhp54* mutant cells revealed that a large portion of the cells are elongated and occasionally contain aberrant nuclei. In addition, FACS analysis showed an increased DNA content in comparison with wild-type cells. Through a minichromosome-loss assay it was shown that the *rhp54* deletion mutant has a very high level of chromosome loss. Furthermore, the *rhp54* mutation in either a *rad17* or a *cdc2.3w* mutant background (where the S-phase/mitosis checkpoint is absent) shows a significant reduction in viability. It is hypothesized that the *rhp54*$^+$ gene is involved in the recombinational repair of UV and X-ray damage and plays a role in the processing of replication-specific lesions.

Key words: DNA repair, DNA replication, *RAD54*, *rhp54*$^+$, Fission yeast

INTRODUCTION

For every organism it is essential to maintain the integrity of the genetic information. The genome of a cell is, however, continually threatened by a broad range of chemical and physical agents. To overcome the deleterious effects of structural changes in the DNA induced by these agents, repair mechanisms have evolved in both prokaryotes and eukaryotes.

The budding yeast *Saccharomyces cerevisiae* has been used extensively to delineate DNA-repair processes. In the past a large number of UV- and X-ray-sensitive mutants have been isolated and characterized. Analysis of the radiation sensitivity of double mutants resulted in the identification of three so-called epistasis groups, which are thought to reflect three major DNA repair pathways. The *RAD3*, *RAD6* and *RAD52* epistasis group genes are involved in nucleotide excision repair, error-prone repair and recombinational repair, respectively (Friedberg, 1988; Game, 1993; Haynes and Kunz, 1981).

Besides *S. cerevisiae*, the fission yeast *Schizosaccharomyces pombe* is also used as a model organism to study the mechanisms underlying DNA repair in eukaryotes. *S. pombe* is much more resistant to both UV and ionizing radiation than *S. cerevisiae*. Unlike *S. cerevisiae*, *S. pombe* has a long G_2 and a short G_1 phase. Proliferating *S. pombe* cells spend about 75% of the cell cycle in G_2, and during this prolonged phase of the cell cycle they may use recombinational repair, to repair damaged DNA (Phipps et al., 1985). This may explain the difference in sensitivity between *S. pombe* and *S. cerevisiae*. Many mutants of *S. pombe*, sensitive to UV and/or gamma radiation have been identified but as yet no rigorous assignment into epistasis groups has been made (Phipps et al., 1985; Lehmann et al., 1991; Subramani, 1991).

A number of DNA repair genes of *S. pombe* have been isolated by phenotypic complementation or by hybridization procedures with heterologous probes from *S. cerevisiae*. On the basis of sequence homology with known genes from *S. cerevisiae* and the properties of the mutants, *S. pombe* genes can be assigned to three groups, which ultimately may be consistent with epistasis groups for *S. pombe*. The *rhp6*$^+$ gene from *S. pombe*, for instance, is presumably involved in an error-prone repair pathway, since it partially corrects the *S. cerevisiae rad6* mutant (Reynolds et al., 1990). Furthermore, the *ERCC3*^{SP}, *rad13*, *rad15* and *rad16* genes from *S. pombe* are homologous to nucleotide excision repair genes *RAD25* (unpublished results of M.H.M. Koken, G. Weeda and J.H.J. Hoeijmakers), *RAD2* (Carr et al., 1993), *RAD3* (Murray et al., 1992) and *RAD1* (Carr et al., 1994), respectively, from the *S. cerevisiae RAD3* epistasis group.

To date, only two homologues of the *RAD52* epistasis group

in *S. cerevisiae* have been isolated from *S. pombe*. Firstly, the *RAD51* homologue, *rhp51*$^+$, was isolated by heterologous hybridization (Muris et al., 1993; Shinohara et al., 1993; Jang et al., 1994). The mutual proteins share 69% identical amino acids. Secondly, the *rad22* gene is the *S. pombe* equivalent of the *S. cerevisiae RAD52* gene (Ostermann et al., 1993).

In this paper the isolation of a third gene, the *RAD54* homologue of *S. pombe*, *rhp54*$^+$, is described. In *S. cerevisiae*, the *RAD54* gene is involved in both DNA repair and mitotic recombination, but its protein does not seem to be required for meiotic recombination. *rad54* mutants are extremely sensitive to ionizing radiation and are unable to repair double-strand DNA breaks (Haynes and Kunz, 1981; Petes et al., 1991). On the basis of the presence of seven domains, conserved between a large subfamily of putative helicases, Rad54 is thought to be a DNA helicase (Troelstra et al., 1992). The extensive amino acid sequence homology between Rhp54 and Rad54 indicates that certain steps in the recombinational repair pathway are conserved in evolution.

MATERIALS AND METHODS

Strains and growth conditions

The *S. pombe* diploid strain sp.101 (*leu1.32/leu1.32, ade6.704/ade6.704, ura4.D18/ura4.D18*, h$^+$/h$^+$ (Murray et al., 1991) was used for the gene deletion/disruption experiments. The *rad17* allele used is a deletion of the majority of the *rad17* coding region marked with a *ura4*$^+$ gene (unpublished). The *chk1* null mutant has been previously described (Al-Khodairy et al., 1994) and the *cdc2.3w* mutant used has been extensively analysed in our previous work (Al-Khodairy and Carr, 1992; Al-Khodairy et al., 1994). All strains were grown under standard conditions (Gutz et al., 1974).

General procedures

Genetics of *S. pombe* were performed as described by Gutz et al. (1974). Nucleic acids were isolated from *S. pombe* cells according to Sherman et al. (1986) and Käufer et al. (1985). Poly(A)$^+$ RNA was prepared by oligo(dT) selection using the PolyATtract method (Promega). Standard manipulations of nucleic acids were performed according to established procedures (Maniatis et al., 1982), and X-ray and UV survival experiments were performed as described previously (Muris et al., 1993).

Heterologous hybridizations

PCR primers for the preparation of two flanking probes of the *S. cerevisiae RAD54* gene were designated as follows: the 5′-end probe was constructed with sense primer: 5′-GGCAAGACGCAGATTACCAG-3′ (nucleotides 411 to 430) and anti-sense primer: 5′-CAGTAG-CATCAGCATCACGC-3′ (nucleotides 1996 to 1977). The 3′-end probe was constructed with sense primer: 5′-ATAAAGAAATCA-CAAAGGGC-3′ (nucleotides 1997 to 2016) and anti-sense primer: 5′-GATGCTCGTTTTTTAGTAGATG-3′ (nucleotides 3061 to 3040). PCR was performed using plasmid YEp13-RAD54-216A as template (Calderon et al., 1983). This plasmid contains the coding region of the *RAD54* gene, located between nucleotides 409 and 3102 (Kans and Mortimer, 1991). PCR reactions and heterologous hybridizations were performed as described previously (Muris et al., 1993).

Sequence analysis

Genomic fragments were subcloned in pUC119 and 120 or in M13mp18 and 19 vectors (Maniatis et al., 1982; Yanisch-Perron et al., 1985). If necessary, unidirectional deletions were generated using the Erase-a-Base method (Promega). The nucleotide sequence was determined on both strands by the dideoxy chain termination method using T7 polymerase (Sanger et al., 1977). In some cases sequencing reactions were carried out using the Auto-Read sequencing kit (Pharmacia/LKB) and analyzed on an ALF automatic sequencer (Pharmacia/LKB).

Amplification of 5′ cDNA ends

Rapid amplification of 5′ cDNA ends (5′ RACE) was essentially done according to the manufacturer's instruction manual (Gibco BRL), except for the use of a third biotinylated anti-sense primer, used for an additional purification step. The third anti-sense primer (primer 13, see Fig. 2) was located between the two other anti-sense primers. First strand synthesis was carried out using primer 14 (see Fig. 2). After dC-tailing of the cDNA, the biotinylated primer 13 and the anchor primer were used for PCR. Amplified DNA was bound to Streptavidin Magne-Sphere Particles (Promega) according to Rossi et al. (1992). After removal of the nonbiotinylated strand, the Universal Anchor primer and nested primer 12 (see Fig. 2) were used for amplification of the Streptavidin-bound strand. The PCR products obtained were purified with Glassmax (BRL), cloned into pT7Blue (Novagen) and sequenced.

Gene disruption/replacement

The construction of a null allele was carried out as previously reported (Barbet et al., 1992). The 1450 bp *Xba*I-*Kpn*I fragment (see Fig. 1) of the *rhp54*$^+$ gene was deleted and replaced with a 1.7 kb fragment containing the *S. pombe ura4*$^+$ gene. The *Bgl*II-*Eco*RI fragment was isolated and used to transform the diploid *S. pombe* strain sp.101. Two *ura*$^+$ transformants were identified and h^{90}/h$^+$ derivative isolated and sporulated. *Ura*$^+$ haploid cells were all radiation sensitive. The disruption was verified in the diploid and in the haploid derivatives by Southern blotting.

Cytometry and cell staining

For DAPI and Calcofluor staining, cells were harvested, washed once in sterile water and resuspended in 90% methanol. Samples were spotted onto microscope slides, stained with DAPI and Calcofluor, and photographed. For cytometry, cells were harvested, washed with sterile water and ethanol was added (while agitating the tube) to a final concentration of 70%. Cells were stored at −70°C. Before analysis a sample of the cells was removed, washed once with 50 mM sodium citrate (pH 7.0) and resuspended in 0.5 ml of the same solution. After addition of RNase A to a concentration of 1 mg/ml, the cells were incubated for 3 hours at 37°C and stored on ice. The sample was diluted into PBS, and after addition of propidium iodide the cells were analyzed using a Coulter Elite cell sorter.

Chromosome segregation assay and plating efficiency

The fidelity of chromosome segregation was determined essentially as described by Niwa et al. (1986). For the determination of the plating efficiency, cells from exponentially growing cultures were plated onto YES plates and allowed to grow for 12 hours. The plates were inspected microscopically and a hundred individual cells were scored for microcolony formation (4 cells or greater), cells that had not divided or cells that had divided only once. The last two categories were assumed to be unable to form colonies. Plates were rescored after a further 12 hours with no significant change in the results. Numbers given are an average of three separate observations.

Nucleotide accession number

The nucleotide sequence of the *rhp54*$^+$ gene is listed in the GenBank/EMBL under accession number: Z29640.

RESULTS

Isolation of the *Schizosaccharomyces pombe rhp54*$^+$ gene

To identify a possible *RAD54* homologue in *S. pombe*, the

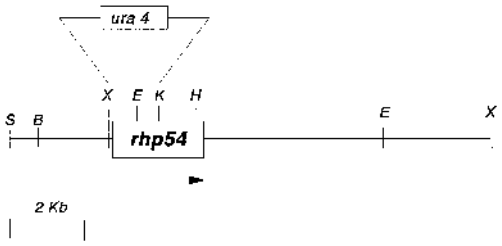


Fig. 1. Restriction enzyme map of the *rhp54*$^+$ region. The position of the open reading frame of *rhp54*$^+$ and the direction of translation are indicated. The region replaced by the *ura4*$^+$ fragment in the gene disruption/replacement experiment is also indicated. B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; X, *Xba*I.

junction probe strategy was applied (Muris et al., 1993; Koken et al., 1992). Junction fragments could be identified if Southern blots containing *S. pombe* genomic DNA were probed with two flanking PCR fragments, derived from the 5′-end and the 3′-end of the coding region of the *S. cerevisiae RAD54* gene. Subsequently, a genomic lambda library was screened using both probes. Approximately 200 double-positive plaques could be identified among approximately 45,000 plaques screened. Five of the double-positive recombinant lambda phages were purified and studied in detail. Southern blot hybridizations of the recombinant phages treated with different restriction enzymes showed very similar restriction enzyme patterns. A 10 kb *Xba*I fragment and a partly overlapping 5.3 kb *Sal*I-*Hin*dIII fragment were subcloned in pUC119 (see Fig. 1).

The 1.5 kb *Eco*RI-*Hin*dIII fragment located within the *rhp54*$^+$ ORF was used for the chromosomal mapping of the *rhp54*$^+$ gene. *Not*I-digested genomic *S. pombe* DNA was separated by pulse-field gel electrophoresis. Hybridization was observed with the *Not*I restriction fragment K, which is located on chromosome I. A more exact localization could be made when filters containing DNAs from cosmid, phage P1 and YAC contigs were probed. In this way, the *rhp54*$^+$ gene was found to map on the long arm of chromosome I, close to the centromere (Hoheisel et al., 1993).

DNA sequence analysis of the *rhp54*$^+$ gene

The nucleotide sequence (nt) of a region of 5 kb encompassing the *Xba*I-*Hin*dIII fragment was determined (see Fig. 2). The 10 kb *Xba*I fragment contains a large open reading frame of 2556 nt, located between nt 1905 and 4462, which can be translated into a putative protein of 852 amino acids with a predicted molecular mass of 96 kDa. In the upstream region of the *rhp54*$^+$ gene, at position 1739, a putative TATA-box (TATAAT) is found. Except for two other TATA-like motifs at position 1762 (TATATATA) and position 1801 (TATAAA), no other putative upstream regulatory sequences were evident. In contrast to the *RAD54* promoter region, no *Mlu*I-site nor a damage-responsive element was detected in the *rhp54*$^+$ upstream region. In the 3′-untranslated region putative polyadenylation signals were found at positions 4636, 4730 and 4888 (see Fig. 2).

Analysis of the amino acid sequence of the Rhp54 protein showed the presence of putative nuclear location signals (NLS) at amino acid positions 178, 665 and 796, respectively. The NLS all match the consensus sequence, K/R K/R X K/R (Fenech et al., 1991). Further examination of the amino acid sequence revealed seven domains (domains I, IA-VI in Fig. 3) that are highly conserved among a subfamily of DNA and RNA helicases (Gorbalenya and Koonin, 1993).

The alignment of the amino acid sequences of the *S. cerevisiae* Rad54 and *S. pombe* Rhp54 proteins is shown in Fig. 3. The Rad54 protein is 46 amino acids longer than the Rhp54 protein. The overall homology between the two proteins is 67% with 51% identical amino acids and 16% similar amino acids. The homology is, however, most extensive in the conserved helicase domain region, up to the C terminus of the proteins. In this region 65% identical and 14% similar residues were found. Most pronounced differences are found in the region upstream of helicase domain I. The Rad54 protein also has an additional stretch of 34 amino acids in this part of the protein in comparison with Rhp54.

To exclude the presence of possible introns in the less conserved 5′-end of the gene, 5′-RACE experiments were performed (see Materials and Methods). PCR products of three different lengths were obtained. The 5′-ends of these cDNA sequences map at positions 1793, 1832 and 2067, respectively (see Fig. 2). The 5′-end of the longest cDNA maps 113 nucleotides upstream of the ATG. Sequence analysis of cDNA products revealed no discrepancies with the genomic DNA, indicating that no introns are present in this region.

Northern blots showed hybridization with a transcript of approximately 3 kb (results not shown). The amount of RNA loaded and the exposure times suggest that the *rhp54*$^+$ gene is expressed at a low level, which is in agreement with both a low Codon Bias Index (0.12) and a low G/C content (38.7%) (Russell, 1989).

Construction of the *rhp54* disruption mutant

A *rhp54* disruption mutant was made by replacing the 1.4 kb *Xba*I-*Kpn*I restriction fragment located at the 5′-end of the gene with the *ura4*$^+$ marker gene (see Fig. 1). In consequence of this, part of the ORF, including helicase domains I, IA, II and part of helicase domain III, were deleted. Subsequently, the mutagenized *Bgl*II-*Eco*RI fragment (see Fig. 1) was isolated and used to transform the diploid *S. pombe* strain sp.101. Two *ura*$^+$ h^{90}/h$^+$ transformants that segregated approximately 50% radiation-sensitive *ura*$^+$ haploids were verified by blot analysis (see Fig. 4). Random spore analysis showed that, in both integrants, the *ura*$^+$ and radiation-sensitive phenotypes always cosegregated. Tetrad analysis of the two integrants further supported correct integration at the *rhp54*$^+$ locus. For the first integrant, 7 out of 8 tetrads segregated 2:2 (*ura*$^-$ *rad*$^+$:*ura*$^+$ *rad*$^-$) and 1 segregated 2:1, one spore not germinating. For the second integrant, 5 spores segregated 2:2, 2 segregated 2:1 and 1 segregated 2:0. The first integrant was used for further experiments.

Analysis of the *rhp54* disruption mutant

The isolation of viable *ura4*$^+$ haploid cells indicated that the *rhp54*$^+$ gene is not essential. The mutant, however, is characterized by a slow growth. Examination of DAPI-stained *rhp54* mutant cells by fluorescence microscopy revealed that a large proportion of the cells are elongated and occasionally contain aberrant nuclei. FACS analysis of *rhp54* mutants (see Fig. 5A,B) shows that the majority of these cells have a DNA content of 2*n* or more. X-ray and UV survival curves of the

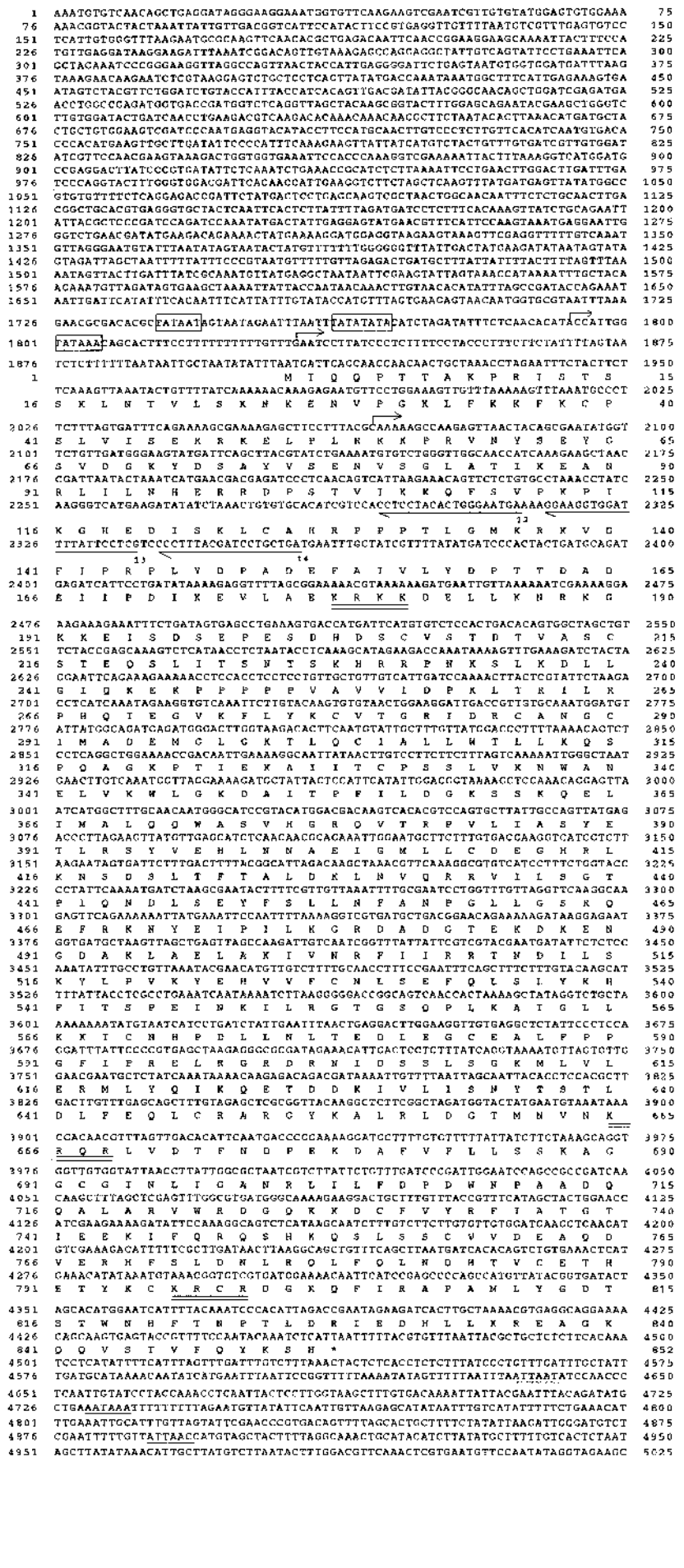


Fig. 2. Nucleotide sequence of the *rhp54*$^{+}$ gene and the predicted amino acid sequence of the protein product. Putative TATA motifs are boxed and putative polyadenylation sequences (A A/T TAA T/A/C) are underlined. The position of the primers 12, 13 and 14, used for the 5′-RACE experiments are indicated by arrows directed to the left. The 5′-ends (which were found by sequence analysis) of the 5′- RACE cDNAs are indicated by arrows pointing to the right. Nuclear location signals (K/R K/R X K/R) are double underlined.

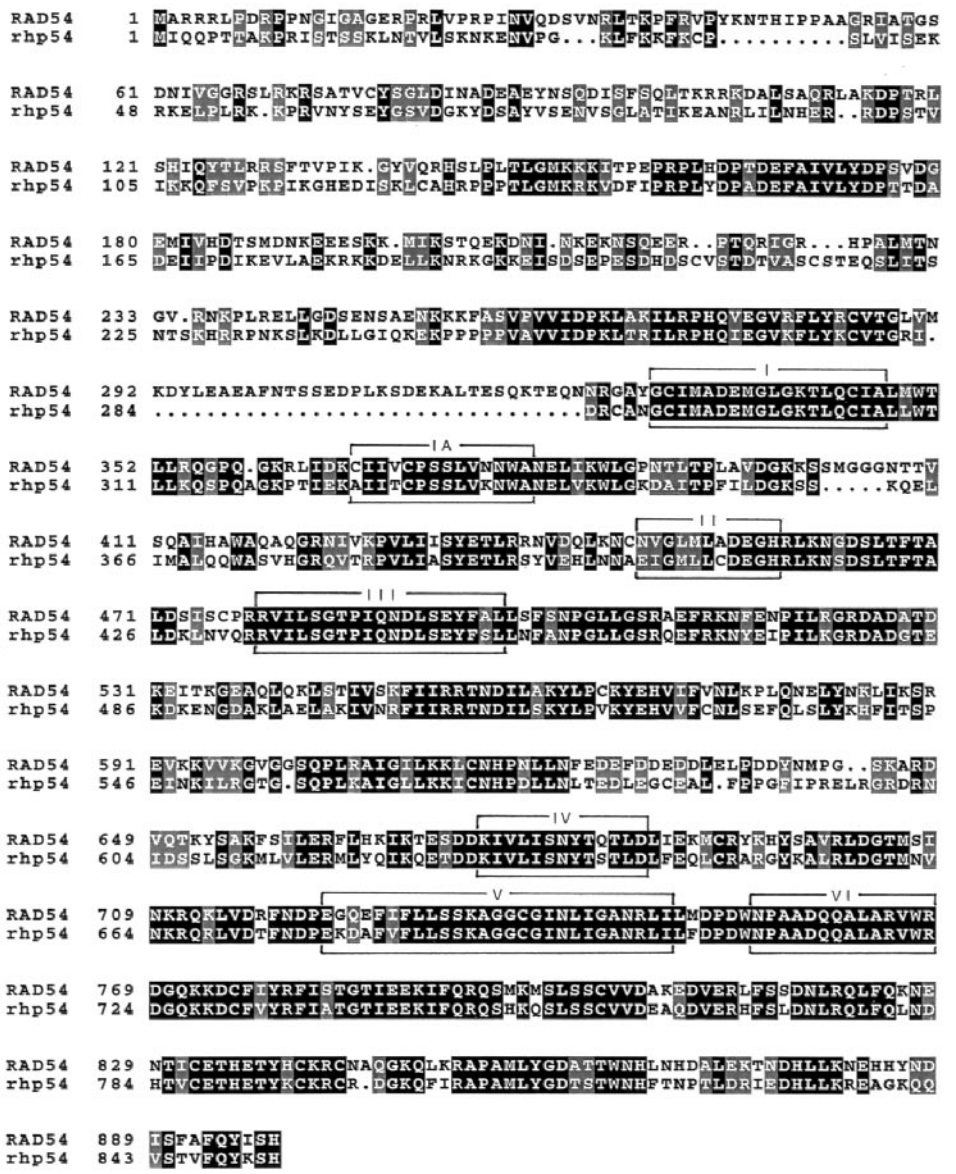


Fig. 3. Amino acid alignment of *S. cerevisiae* Rad54 and *S. pombe* Rhp54. Alignments were determined using the Boxshade programme (Corpet, 1988). Amino acids that are identical are highlighted in black. Highlighting in gray indicates functional conserved amino acid residues. Conserved amino acids were taken as follows: V, I, L and M; D, E, Q and N; F, Y and W; G, S, T, P and A; K, R and H. Conserved helicase domains are indicated (I, IA-VI).

rhp54 mutant are shown in Fig. 6. The *rhp54* mutant is very sensitive to both ionizing radiation and UV. The LD_{50} for ionizing radiation is 7 Gy for the mutant compared with 215 Gy for the wild type.

The fidelity of chromosome segregation in the *rhp54* deletion mutant was determined by using a minichromosome-loss assay (Niwa et al., 1986; Murray et al., 1994). Loss of the minichromosome results in adenine auxotrophy and the subsequent development of pink colonies on plates containing limiting quantities of adenine. The chromosome loss in the *rhp54* deletion mutant was found to be 480-fold greater than in the wild-type strain at approximately 2% per generation. This is 4-5 times higher than any other *S. pombe* repair or checkpoint mutant. In order to determine if this is due to loss of a recombination function, and not an unrelated second site mutation in the *rhp54* disruption strain, we have also examined chromosome loss in *rhp51* null mutant cells. The *rhp51* mutant strain (Muris et al., 1993) belongs to the same epistasis group as *rhp54*, and has an identical slow growth, cell elongation and

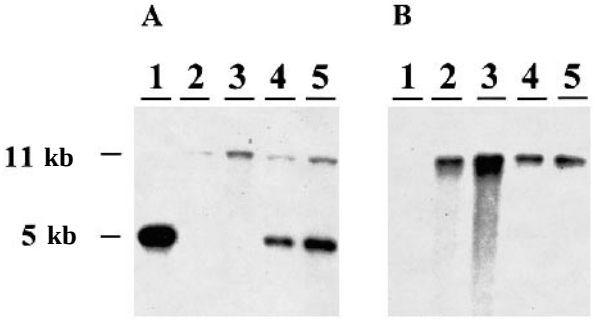
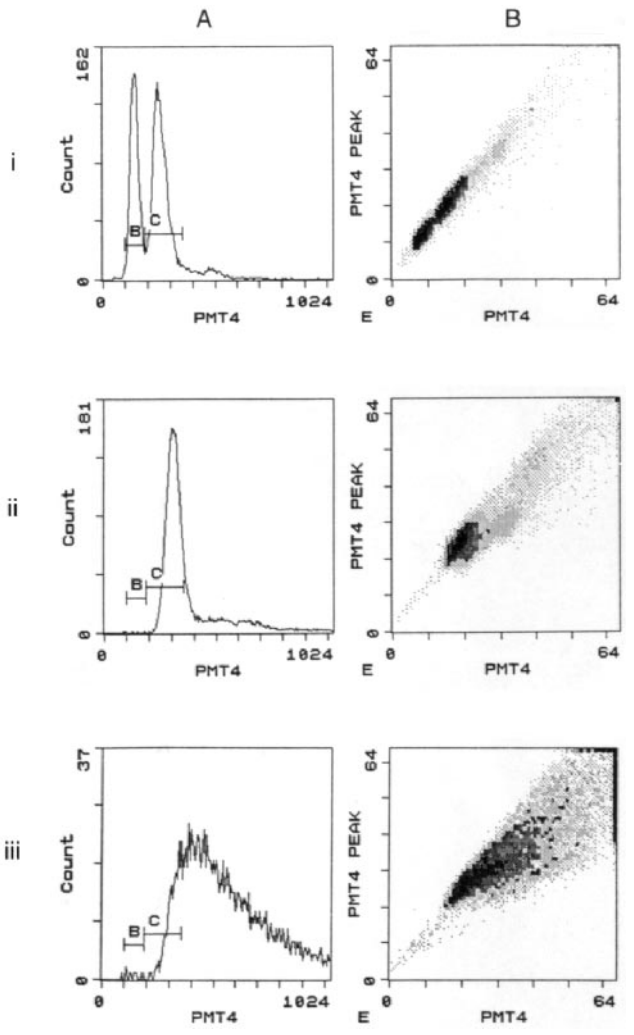
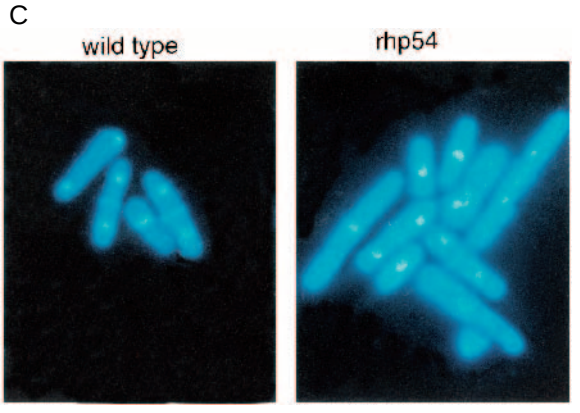


Fig. 4. Southern analysis of the *rhp54* disruption mutant. The DNAs were digested with *Eco*RI. Lane 1 contains wild-type genomic DNA; lanes 2 and 3 contain genomic DNA of two independent isolated *rhp54* mutants; and lanes 4 and 5 contain genomic DNA of their heterozygous diploids, respectively. (A) Probed with a 3 kb *rhp54*$^+$ *Sal*I-*Xba*I fragment. (B) Probed with a 1.7 kb *ura4*$^+$ fragment.





DNA repair phenotype as the *rhp54* strain. Cells deleted for *rhp51* show an equivalent increase in the rate of minichromosome loss (550-fold), consistent with the chromosome loss phenotype being a direct result of the loss of a recombination related function.

Cell elongation in *S. pombe* usually reflects a checkpoint-dependent cell cycle delay caused by loss of a specific cell cycle function, which then activates one of the checkpoint pathways that establish the dependency relationships of the cell

Fig. 5. FACS analysis and fluorescence microscopy of *rhp54* mutants. (A) Plot of cell count for intensity of fluorescence by propidium iodide staining. (B**)** Distribution of cells plotting the peak of intensity of PI fluorescence and the total intensity. This gives an indication of the heterogeneous nature of the cells and the nuclear organization. (i) Wild-type control grown in limiting concentration of nitrogen (minimal medium with 0.05 g/l NH_4Cl). These cells accumulate in G_1 as nitrogen is exhausted. Two peaks, corresponding to 1*n* (G_1-gate B) and 2*n* (G_2-gate C) DNA content are clearly discernable. (ii) Wild-type cells in exponential growth (YES medium). Only cells of 2*n* or greater DNA content are discernable as S phase is completed before the daughter cells separate. (iii) *rhp54* mutants in exponential growth (YES medium). All cells are seen to contain a 2*n* or greater DNA content. (C) DAPI and Calcofluor staining of exponentially growing wild-type cells and *rhp54* mutants.

Table 1. Fidelity of minichromosome transmission, plating efficiency and abortive mitosis

Rad locus	% Loss/ generation*	Fold increase	Plating efficiency (%)	Abortive mitosis (%)†
Wild type	0.005	-	98	-
rhp51	2.75	550	83	-
rhp54	2.4	480	80	3
chk1	0.035	7	98	-
rad17	0.04	8	92	<1
cdc2.3w	-	-	97	<1
rhp54-chk1	-	-	71	3
rhp54-rad17	-	-	9	19
rhp54-cdc2.3w	-	-	24	23

*Loss rates per generation are calculated from the total percentage of cells auxotrophic for adenine divided by the number of generations in culture.

†The percentage of cells, pooled from multiple minicolonies that are not capable of propagation, showing evidence of abortive mitosis.

cycle (Sheldrick and Carr, 1993). Double mutants between *rhp54* and *rad17*, *chk1* or *cdc2.3w* mutants have been constructed and tested for cell morphology, evidence of the "cut" phenotype and cell viability. The results, summarized in Table 1, demonstrate that the elongation and viability of the *rhp54* disruption mutant is dependent on an intact replication checkpoint system.

DISCUSSION

This paper describes the isolation of *rhp54*$^+$, a *RAD54* homologue of *Schizosaccharomyces pombe*. The *rhp54*$^+$ gene was isolated using two flanking PCR fragments, derived from the 5′-end and the 3′-end of the coding region of the *S. cerevisiae RAD54* gene. In this way, the isolation of sequences with homology to other putative DNA/RNA helicases or other related sequences was avoided.

Analysis of the *rhp54*$^+$ sequence reveals a large uninterrupted ORF of 2556 nt, which predicts a protein of 852 amino acids. The alignment of the Rhp54 amino acid sequence with the sequence of the *S. cerevisiae* Rad54 protein shows that the two proteins are highly conserved in the putative helicase domains and the C terminus (Fig. 3). The overall homology between Rad54 and Rhp54 is 67%. The N terminus of the Rhp54 and Rad54 proteins is less conserved, as was also

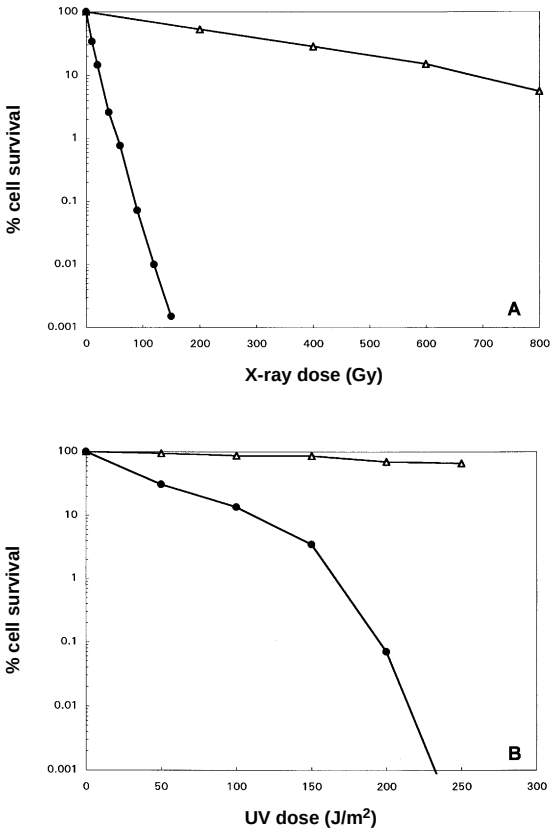


Fig. 6. Survival following irradiation with X-rays (A) and UV light (B) of cells from a haploid *rhp54* mutant (●) and from a haploid wild-type *S. pombe* strain (△; sp.011).

observed for Rhp51 and Rad51 (Muris et al., 1993). Despite the great sequence homology, the *rhp54*$^+$ gene does not functionally substitute for *RAD54*. When the *rhp54*$^+$ gene is introduced into a *rad54* mutant strain on a high copy expression vector, no correction of the radiation sensitivity was observed (details to be published elsewhere).

The presence of introns in the less conserved 5′ part of the gene was excluded by 5′-RACE experiments. Sequence analysis of the products, revealed no discrepancies with the genomic sequence (see Fig. 2).

Upstream of the ATG start codon three putative TATA boxes can be observed at positions 1739 (TATAAT), 1762 (TATATATA) and 1801 (TATAAA). Further studies are needed to determine whether these motifs are involved in specifying the transcription of the *rhp54*$^+$ gene.

A *rhp54* disruption mutant was constructed to characterize the Rhp54 gene function. In *S. cerevisiae*, the *rad54* mutant is sensitive to X-ray radiation and only has a marginal sensitivity to UV radiation (Glassner and Mortimer, 1994). The *rhp54* disruption mutant is, like its homologue in *S. cerevisiae*, extremely sensitive to X-ray radiation but is, in addition, also sensitive to UV. The properties of the *rhp54* disruption mutant are very comparable with the characteristics of the *rhp51* disruption mutant described previously: for example, the same levels of X-ray and UV sensitivities were observed in both mutants (Muris et al., 1993). The survival experiments indicate that in *S. pombe* the recombinational repair pathway is also involved in the repair of UV damage. *S. pombe* strains carrying a mutation in one of the genes required for nucleotide excision repair still show a significant residual capacity to repair UV damage, suggesting a second excision repair pathway. Recently the presence of an endonuclease was shown in *S. pombe* that catalyses a single ATP-independent incision immediately 5′ to the UV photoproduct and generates termini containing 3′ hydroxyl and 5′ phosphoryl groups (Bowman et al., 1994). The adducted bases may than be removed by exonucleolytic activity and the gap filled in by DNA polymerase, as in the base excision repair pathway. Another possibility is that the presence of single-strand breaks initiates recombinational repair.

A functional *rhp54*$^+$ gene is not essential for viability of *S. pombe*. The slow growth and the decreased plating efficiency of the haploid mutant suggest, however, that proliferation in this mutant is affected. This is also evident from microscopic examination of the cells and FACS analysis; exponentially growing cultures contain a large fraction of elongated cells, which are about twice as long as the parental wild-type cells. In some cells aberrant nuclei can also be seen (see Fig. 5C). Most wild-type cells in exponential growth have a DNA content of 2*n* as S-phase is completed before the daughter cells separate. Analysis of the *rhp54* mutant showed differences in DNA content of the cells. The mutant culture contains a mixture of cells having 2*n* DNA and cells with a DNA content exceeding 2*n* (see Fig. 5B). The increase in DNA content may be due to additional DNA synthesis. Another possibility is that the culture contains a mixture of haploid and diploid cells, and that the elongation of the *rhp54* cells is causing a skewing of the peak to the right. Diploidization has previously been observed in *S. pombe* mutants with cell cycle defects (Broek et al., 1991).

To verify if the chromosomes in the *rhp54* mutant are segregated properly during mitosis, chromosome segregation was tested using a minichromosome-loss assay. Chromosome loss in the *rhp54* mutant is 480-fold greater than in the isogenic wild-type strain (Table 1). The *rhp51* mutant, which acts in the same pathway and has an identical phenotype, also has a high degree of chromosome loss and/or non-disjunction: 550-fold greater than the background. In *S. cerevisiae* it has been shown that mutations in genes required for DNA replication also cause increased rates of chromosome loss (Newlon, 1988). The cell elongation in the *rhp54* mutant, also seen in the case of the *rhp51* mutant, further indicates that there may be a mitotic delay due to the activation of a checkpoint responding to a defect in a cell cycle event, possibly some aspect of DNA replication.

To further investigate the basis of the high level of chromosome loss and the mitotic delay, *rhp54-rad17*, *rhp54-chk1* and *rhp54-cdc2.3w* double mutants were constructed. The Rad17 gene product has multiple functions and is required for the S-phase checkpoint that delays mitosis until replication is complete, for the DNA damage checkpoint that delays mitosis in the presence of DNA damage and for an unknown repair pathway required for maximal survival of DNA damage during S phase (Al-Khodairy Carr, 1992; Sheldrick and Carr., 1993; Al-Khodairy et al., 1994). In contrast, Chk1 is required only for the DNA damage checkpoint (Walworth et al., 1993; Al-Khodairy et al., 1994) and the *cdc2.3w* mutant is only defective for the checkpoint that delays mitosis until DNA replication is

complete (Sheldrick and Carr, 1993). The low plating efficiency of the *rhp54-rad17* and *rhp54-cdc2.3w* double mutants when compared to the *rhp54-chk1* double and *rhp54* single mutants (9% and 24% vs 71% and 80%, respectively, see Table 1) indicates that the viability of the *rhp54* mutant is dependent largely upon an intact S-phase checkpoint.

The decreased cell viability of the *rhp54-cdc2.3w* double mutant, compared to the *rhp54-chk1* double mutant confirms that the inviability is a reflection of the loss of the replication checkpoint rather than a cumulative defect in DNA repair or loss of the DNA damage checkpoint. As anticipated, both *rhp54-rad17* and *rhp54-cdc2.3w* mutant cells show increased levels of abortive mitosis ("cut" phenotype) compared with the *rhp54, rad17* and *cdc2.3w* single mutants and the *rhp54-chk1* double mutant, suggesting that the *rhp54-rad17* and *rhp54-cdc2.3w* double mutant cells die when an attempt is made to segregate incompletely replicated DNA, possibly implicating Rhp54 in late S phase events. Such a defect in the processing of replication-specific lesions in *rhp54* mutants is also consistent with the chromosome instability and mitotic delay phenotypes. Thus, taken together, our data could suggest that *rhp54*$^+$ (and, by virtue of the identical phenotype, also *rhp51*$^+$) has a role in DNA replication.

In accordance with this theory is the observation that we have not been able to generate *rhp51-rad2* and *rhp54-rad2* double mutants, suggesting that a mutation in *rhp51*$^+$ or *rhp54*$^+$ is lethal in a *rad2*^{sp} mutant. *S. pombe rad2*^{sp} mutant strains are sensitive to UV irradiation and also show a high degree of chromosome loss (Murray et al., 1994). The Rad2^{sp} gene product is the *S. pombe* homologue of the mammalian MF-1/FEN-1 protein, which is absolutely required for lagging strand synthesis in vitro (Waga et al., 1994; Harrington and Lieber, 1994), but is inessential in *S. pombe*. The reason for this inessential phenotype may be one of several possibilities: (1) Rad2 is functionally redundant. No evidence is available to support this possibility, and genetic analysis suggests that it is not the case. (2) The requirements for replication are fundamentally different in vitro and in vivo. (3) Recombination-based mechanisms can participate in processing the replication-specific lesions, which are alternatively processed by the Rad2^{sp}/MF-1 pathway identified in vitro by Waga et al. (1994). On the basis of our data we believe that this latter explanation (that the *rhp54*$^+$ gene has a role in the processing of replication structures during late replication that is separate from its role in the repair of radiation damage) warrants serious consideration. This is supported by the fact that the equivalent genetic interactions between the Rad2/MF-1 homologue and Rad50/Rad52 have also been observed in *S. cerevisiae* (the deletion of the *S. cerevisiae* Rad2/MF-1 homologue, *RAD27*, is inviable in combination with *rad50* and *rad52* mutations; Reagan et al., 1995).

rhp54$^+$ is the third gene of the *RAD52* epistasis group that is conserved between budding and fission yeast. The recent identification of *RAD51*, *RAD52* and *RAD54* homologues in man, mouse and chicken (Bendixen et al., 1994; Bezzubova et al., 1993; Bezzubova and Buerstedde, 1994; Muris et al., 1994; Morita et al., 1993; Shinohara et al., 1993; Troelstra et al., unpublished results) is clear evidence that the recombinational repair pathway is conserved through evolution. The identification of these genes in fission yeast has lead to novel insights into their role in normal cell cycle progression (in addition to the anticipated role in double-strand break repair), and opens up the possibility of examining the role of the Rad52 group of proteins in the context of the well defined and experimentally amenable cell cycle pathways in this organism.

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REFERENCES

Al-Khodairy, F. and Carr, A. M. (1992). DNA repair mutants defining G2 checkpoint pathways in *Schizosaccharomyces pombe. EMBO J.* **11**, 1343-1350.

Al-Khodairy, F., Fotou, E., Sheldrick, K. S., Griffiths, D. J. F., Lehmann, A. R. and Carr, A. M. (1994). Identification and characterization of new elements involved in checkpoint and feedback controls in fission yeast. *Mol. Biol. Cell.* **5**, 147-160.

Barbet, N., Muriel, W. J. and Carr, A. M. (1992). Versatile shuttle vectors and genomic libraries for use with *Schizosaccharomyces pombe*. *Gene* **114**, 59-66.

Bendixen, C., Sunjevaric, I., Bauchwitz, R. and Rothstein, R. (1994). Identification of a mouse homologue of the *Saccharomyces cerevisiae* recombination and repair gene, *RAD52*. *Genomics* **23**, 300-303.

Bezzubova, O., Shinohara, A., Mueller, R. G., Ogawa, H. and Buerstedde, J.-M. (1993). A chicken *RAD51* homologue is expressed at high levels in lymphoid and reproductive organs. *Nucl. Acids Res.* **21**, 1577-1580.

Bezzubova, O. Y. and Buerstedde, J.-M. (1994). Gene conversion in the chicken immunoglobulin locus: a paradigm of homologous recombination in higher eukaryotes. *Experientia* **50**, 270-276.

Bowman, K. K., Sidik, K., Smith, C. A., Taylor, J.-S., Doetsch, P. W. and Freyer, G. A. (1994). A new ATP-independent DNA endonuclease from *Schizosaccharomyces pombe* that recognizes cyclobutane pyrimidine dimers and 6-4 photoproducts. *Nucl. Acids Res.* **22**, 3026-3032.

Broek, D., Bartlett, R., Crawford, K. and Nurse, P. (1991). Involvement of p34^{cdc2} in establishing the dependency of S phase on mitosis. *Nature* **349**, 388-393.

Calderon, I. L., Contopoulou, C. R. and Mortimer, R. K. (1983). Isolation and characterization of yeast DNA repair genes. II. Isolation of plasmids that complement the mutations *rad50-1, rad51-1, rad54-3,* and *rad55-3*. *Curr. Genet.* **7**, 93-100.

Carr, A. M., Sheldrick, K. S., Murray, J. M., Al-Harithy, R., Watts, F. Z. and Lehmann, A. R. (1993). Evolutionary conservation of the excision repair in *Schizosaccharomyces pombe*: evidence for a family of sequences related to the *Saccharomyces cerevisiae RAD2* gene. *Nucl. Acids Res.* **21**, 1345-1349.

Carr, A. M., Schmidt, H., Kirchhoff, S., Muriel, W. J., Sheldrick, K. S., Griffiths, D. J., Basmacioglu, C. N., Subramani, S., Clegg, M., Nasim, A. and Lehmann, A. R. (1994). The *rad16* gene of *Schizosaccharomyces pombe*: a homolog of the *RAD1* gene of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14**, 2029-20240.

Cole, G. M., Schild, D., Lovett, S. T. and Mortimer, R. K. (1987). Regulation of *RAD54- RAD52-lacZ* gene fusions in *Saccharomyces cerevisiae* in response to DNA damage. *Mol. Cell. Biol.* **7**, 1087-1084.

Cole, G. M. and Mortimer, R. K. (1989). Failure to induce a DNA repair gene, *RAD54*, in *Saccharomyces cerevisiae* does not affect DNA repair or recombination phenotypes. *Mol. Cell. Biol.* **9**, 3314-3322.

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucl. Acids Res.* **16**, 10881-10890.

Emery, H. S., Schild, D., Kellogg, D. E. and Mortimer, R. K. (1991). Sequence of *RAD54*, a *Saccharomyces cerevisiae* gene involved in recombinational repair. *Gene* **104**, 103-106.

Fenech, M., Carr, A. M., Murray, J., Watts, F. Z. and Lehmann, A. R. (1991). Cloning and characterization of the *rad4* gene of *Schizosaccharomyces pombe*; a gene showing short regions of sequence similarity to the human *XRCC1* gene. *Nucl. Acids Res.* **19**, 6737-6741.

Friedberg, E. C. (1988). Deoxyribonucleic acid repair in the yeast *Saccharomyces cerevisiae*. *Microbiol. Rev.* **52**, 70-102.

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

Glassner, B. J. and Mortimer, R. K. (1994). Synergistic interactions between *RAD5, RAD16* and *RAD54*, three partially homologous yeast DNA repair genes each in a different repair pathway. *Radiat. Res.* **139**, 24-33.

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

Gutz, H., Heslot, H., Leupold, U. and Loprieno, N. (1974). In *Handbook of Genetics*, vol. 1 (ed. R. C. King), pp. 395-446. Plenum Press, New York.

Harrington, J. J. and Lieber, M. R. (1994). Functional domains within FEN-1 and RAD2 define a family of structure specific endonucleases: implications for nucleotide excision repair. *Genes Dev.* **8**, 1344-1355.

Haynes, R. H. and Kunz, B. A. (1981). In *The Molecular Biology of the yeast* Saccharomyces cerevisiae: *Life Cycle and Inheritance* (ed. J. Strathern, E. W. Jones, and J. R. Broach), pp. 371-414. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Hoheisel, J. D., Maier, E., Mott, R., McCarthy, L., Grigoriev, A. V., Schalkwyk, L. C., Nizetic, D., Francis, F. and Lehrach, H. (1993). High resolution cosmid and P1 maps spanning the 14 Mb genome of the fission yeast *S. pombe*. *Cell* **73**, 109-120.

Jang, Y. K., Jin, Y. H., Kim, E. M., Fabre, F., Hong, S. H. and Park, S. D. (1994). Cloning and sequence analysis of *rhp51*$^+$, a *Schizosaccharomyces pombe* homolog of the *Saccharomyces cerevisiae RAD51* gene. *Gene* **142**, 207-211.

Kans, J. A. and Mortimer, R. K. (1991). Nucleotide sequence of the *RAD57* gene of *Saccharomyces cerevisiae*. *Gene* **105**, 139-140.

Käufer, N. F., Simanis, V. and Nurse, P. (1985). Fission yeast *Schizosaccharomyces pombe* correctly excises a mammalian RNA transcript intervening sequence. *Nature* **318**, 78-80.

Koken, M. H. M., Vreeken, C., Bol, S. A. M., Cheng, N. C., Jaspers-Dekker, I., Hoeijmakers, J. H. J., Eeken, J. C. J., Weeda, G. and Pastink, A. (1992). Cloning and characterization of the *Drosophila* homolog of the xeroderma pigmentosum complementation-group B correcting gene, *ERCC3*. *Nucl. Acids Res.* **20**, 5541-5548.

Lehmann, A. R., Carr, A. M., Watts, F. Z. and Murray, J. M. (1991). DNA repair in the fission yeast, *Schizosaccharomyces pombe*. *Mutat. Res.* **250**, 205-210.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor University Press, Cold Spring Harbor.

Morita, T., Yoshimura, Y., Yamamoto, A., Murata, K., Mori, M., Yamamoto, H. and Matsushiro, A. (1993). A mouse homolog of the *Escherichia coli recA* and *Saccharomyces cerevisiae RAD51* genes. *Proc. Nat. Acad. Sci. USA* **90**, 6577-6580.

Muris, D. F. R., Vreeken, K., Carr, A. M., Broughton, B. C., Lehmann, A. R., Lohman, P. H. M. and Pastink, A. (1993). Cloning the *RAD51* homologue of *Schizosaccharomyces pombe*. *Nucl. Acids Res.* **21**, 4586-4591.

Muris, D. F. R., Bezzubova, O., Buerstedde, J.-M., Vreeken, K., Balajee, A. S., Osgood, C. J., Troelstra, C., Hoeijmakers, J. H. J., Ostermann, K., Schmidt, H., Natarajan, A. T., Eeken, J. C. J., Lohman, P. H. M. and Pastink, A. (1994). Cloning of the human and mouse genes homologous to *RAD52*, a yeast gene involved in DNA repair and recombination. *Mutat. Res.* **315**, 295-305.

Murray, J. M., Carr, A. M., Lehmann, A. R. and Watts, F. Z. (1991). Cloning and characterization of the *rad9* DNA repair gene from *Schizosaccharomyces pombe*. *Nucl. Acids Res.* **19**, 3525-3531.

Murray, J. M., Doe, C. L., Schenk, P., Carr, A. M., Lehmann, A. R. and Watts, F. Z. (1992). Cloning and characterization of the *S. pombe rad15* gene, a homologue to the *S. cerevisiae RAD3* and human *ERCC2* genes. *Nucl. Acids Res.* **20**, 2673-2678.

Murray, J. M., Tavassoli, M., Al-Harithy, R., Sheldrick, K. S., Lehmann, A. R., Carr, A. M. and Watts, F. Z. (1994). Structural and functional conservation of the human homolog of the *Schizosaccharomyces pombe rad2* gene, which is required for chromosome segregation and recovery from DNA damage. *Mol. Cell. Biol.* **14**, 4878-4888.

Newlon, C.S. (1988). Yeast chromosome replication and segregation. *Microb. Rev.* **52**, 568-601.

Niwa, O., Matsumoto, T. and Yanagida, M. (1986). Construction of a minichromosome by deletion and its mitotic and meiotic behaviour in fission yeast. *Mol. Gen. Genet.* **203**, 397-405.

Ostermann, K., Lorentz, A. and Schmidt, H. (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*. *Nucl. Acids Res.* **21**, 5940-5944.

Petes, T. D., Malone, R. E. and Symington, L. S. (1991). Recombination in yeast. In *The Molecular and Cellular Biology of the Yeast* Saccharomyces: *Genome Dynamics, Protein Synthesis, and Energetics* (ed. J. Broach, J. Pringle and E. Jones), pp. 407-521. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Phipps, J., Nasim, A. and Miller, D. R. (1985). Recovery, repair, and mutagenesis in *Schizosaccharomyces pombe*. *Advan. Genet.* **23**, 1-72.

Reagan, M. S., Pittenger, C., Siede, W. and Friedberg, E. C. (1995). Characterization of a yeast strain deleted for the *RAD27* gene, a structural homolog of the *RAD2* nucleotide excision repair gene. *J. Bacteriol.* **177**, 364-371.

Reynolds, P., Koken, M. H. M., Hoeijmakers, J. H. J., Prakash, S. and Prakash, L. (1990). The *rhp6*$^+$ gene of *Schizosaccharomyces pombe*: a structural and functional homolog of the *RAD6* gene from the distantly related yeast *Saccharomyces cerevisiae*. *EMBO J.* **9**, 1423-1430.

Rossi, A. M., Tates, A. D., Van Zeeland, A. A. and Vrieling, H. (1992). Molecular analysis of mutations affecting hprt mRNA splicing in human T-lymphocytes in vivo. *Environ. Mol. Mutagen.* **19**, 7-13.

Russell, P. R. (1989). Gene cloning and expression in fission yeast. In *Molecular Biology of the Fission Yeast* (ed. A. Nasim, P. Young and B. Johnson), pp. 243-271. Academic Press Inc., London.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-termination inhibitors. *Proc. Nat. Acad. Sci. USA* **74**, 5463-5467.

Sheldrick, K. S. and Carr, A. M. (1993). Feedback controls and G2 checkpoints, fission yeast as a model system. *BioEssays* **15**, 775-782.

Sherman, F., Fink, G. R. and Hicks, J. B. (1986). *Methods in Yeast Genetics*. 125 Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Shinohara, A., Ogawa, H., Matsuda, Y., Ushio, N., Ikeo, K. and Ogawa, T. (1993). Cloning of human, mouse and fission yeast recombination genes homologues to *RAD51* and *recA*. *Nature Genet.* **4**, 239-243.

Subramani, S. (1991). Radiation resistance in *Schizosaccharomyces pombe*. *Mol. Microbiol.* **5**, 2311-2314.

Troelstra, C., van Gool, A., de Wit, J., Vermeulen, W., Bootsma, D. and Hoeijmakers, J. H. J. (1992). *ERCC6*, a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes. *Cell* **71**, 939-953.

Waga, S. Bauer, G and Stillman, B. (1994). Reconstitution of complete SV40 DNA replication with purified replication factors. *J. Biol. Chem.* **14**, 10923-10934.

Walworth, N., Davey, S. and Beach, D. (1993). Fission yeast *chk1* protein kinase links the rad checkpoint pathway to *cdc2*. *Nature* **363**, 368-371.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.

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