1316–1324 *Nucleic Acids Research, 2002, Vol. 30, No. 6* © 2002 Oxford University Press

Differential expression and requirements for *Schizosaccharomyces pombe RAD52* homologs in DNA repair and recombination

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Received December 19, 2001; Revised and Accepted January 22, 2002

ABSTRACT

In fission yeast two *RAD52* homologs have been identified, *rad22A*$^+$ and *rad22B*$^+$. Two-hybrid experiments and GST pull-down assays revealed physical interaction between Rad22A and Rad22B, which is dependent on the N-terminal regions. Interaction with Rhp51 is dependent on the C-terminal parts of either protein. Both Rad22A and Rad22B also interact with RPA. The expression of *rad22B*$^+$ in mitotically dividing cells is very low in comparison with *rad22A*$^+$ but is strongly enhanced after induction of meiosis, in contrast to *rad22A*$^+$. *Rad22B* mutant cells are not hypersensitive to DNA-damaging agents (X-rays, UV and cisplatin) and display normal levels of recombination. In these respects the *Schizosaccharomyces pombe rad22B* mutant resembles the weak phenotype of vertebrate cells deficient for *RAD52*. Mutation of *rad22A*$^+$ leads to severe sensitivity to DNA-damaging agents and to defects in recombination. In a *rad22Arad22B* double mutant a further increase in sensitivity to DNA-damaging agents and additional mitotic recombination defects were observed. The data presented here indicate that Rad22A and Rad22B have overlapping roles in repair and recombination, although specialized functions for each protein cannot be excluded.

INTRODUCTION

DNA double-strand breaks (DSBs) are among the most genotoxic DNA lesions and differ from most other types of damage in that both strands of the DNA double helix are affected. If not repaired or repaired incorrectly, DSBs may lead to gross chromosomal rearrangements, i.e. inversions, translocations and deletions, which are frequently found to be associated with carcinogenesis. Two main pathways to repair DSBs have been conserved during evolution: (i) homologous recombination (HR) and (ii) non-homologous end joining (NHEJ) (1). The relative importance of each mechanism in the repair of DSBs is dependent on the organism and phase of the cell cycle and may also be dependent on the nature of the DNA ends (1,2).

Preservation of the genome by HR has been studied extensively in *Saccharomyces cerevisiae* and involves replication protein A (RPA), all three replicative DNA polymerases and the RAD52 group of genes (*RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *MRE11*, *XRS2* and *RDH54/TID1*) (1,3). Although mutation of any of the genes in the RAD52 group results in sensitivity to DSB-inducing agents (i.e. ionizing radiation), *rad52 S.cerevisiae* mutants display the most severe radiation sensitivity and defects in recombination (4). *RAD52* is essential for meiotic recombination and almost all forms of mitotic recombination and gene conversion (4–6). Repair of a DSB flanked by repeated sequences can occur by a process called single-strand annealing (SSA), which can be considered as a subpathway of HR. SSA is dependent on *RAD52* and partially on *RAD59*, especially when the DSB is flanked by short repeats (7,8). However, SSA does not require the presence of the *RAD51*, *RAD54*, *RAD55* and *RAD57* gene products (9). Telomere maintenance by a long tract gene conversion mechanism, known as break-induced replication, also relies on *RAD52* and is independent of *RAD51*, *RAD54*, *RAD55* and *RAD57* (10–12).

Consistent with the crucial role in SSA is the observation that Rad52 binds to single-stranded DNA (ssDNA) and that it mediates DNA strand annealing (13) in a reaction that is stimulated by RPA (14,15). After resection of the DSB, the second step in the conservative HR pathway is invasion of intact duplex DNA by one of the single-stranded ends and formation of D-loop DNA. This process of strand exchange requires the action of the RecA-related protein Rad51 (16). Recent investigations have shown that Rad52 and/or a heterodimer of Rad55 and Rad57 are involved in the nucleation of Rad51 onto ssDNA tails and promote strand exchange between the Rad51 nucleoprotein filament and the undamaged duplex DNA (17–20). The Rad52 protein, or the heterodimer of Rad55 and Rad57, stimulates the efficiency of these processes probably by overcoming the inhibitory effect that RPA has on DNA strand exchange. In agreement with these observations are findings that Rad52 interacts with Rad51 as well as with RPA (21–23). The biochemical activities of *S.cerevisiae* Rad52 are conserved in evolution. As in *S.cerevisiae*, Rad52 in man interacts with

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HsRad51 and HsRPA and stimulates the pairing and strand exchange activities of HsRad51 (24–27). Both Rad52 from *S.cerevisiae* and human Rad52 form multimeric ring structures that can bind to single- and double-stranded DNA ends (14,28).

The importance of HR in the repair of DSBs in mammalian cells has been demonstrated by assays based on the repair of a site-specific DSB flanked by direct repeats. Repair by HR occurred in 30–50% of the molecules recovered (29). Moreover, inactivation of *RAD54* in mouse ES cells and *Drosophila melanogaster* leads to reduced resistance to X-rays and defects in recombination (30,31). Mouse embryos homozygous for mutations in *RAD51*, *RAD50* or *MRE11* or in one of the RAD51 paralogs (*XRCC2*, *RAD51L1* or *RAD51L3*) die during early embryonic development, implying an essential role in cell proliferation (32–38).

In striking contrast to the severe phenotype of mutant *rad52 S.cerevisiae* cells are the mild phenotypes of *RAD52*$^{-/-}$ mouse ES and chicken DT40 cells. Mouse and chicken cells deficient for *RAD52* are not hypersensitive to DSB-inducing agents and show only subtle defects in HR (39,40). Moreover, deletion of *MmRAD52* does not affect viability, fertility or lymphocyte development (40). An explanation for these observations could be the presence of additional genes functionally related to *RAD52*. Evidence for such a functional redundancy has been provided by the identification of the *RAD59* gene in *S.cerevisiae* and *Klyveromyces lactis* (41,42). The Rad59 proteins of both *S.cerevisiae* and *K.lactis* display sequence homology to the N-terminal domain of Rad52 protein, but lack the C-terminal part which is required for the interaction with Rad51. Deletion of *RAD59* leads in both yeast species to a moderate X-ray sensitivity (41,42). Moreover, in *S.cerevisiae* it has been shown that a *rad59* mutation can be partially complemented by overexpression of *RAD52*, suggesting that the functions of both proteins overlap (41).

A set of two *RAD52* homologs, *rad22A*$^+$ and *rad22B*$^+$, has also been isolated from the distantly related fission yeast *Schizosaccharomyces pombe* (43–45). Mutant *rad22A* cells are sensitive to DSB-inducing agents and show defects in the ability to perform homologous plasmid integration (45). Inactivation of *rad22B*$^+$ does not lead to X-ray hypersensitivity, and homologous integration of linear DNA is not affected (45). Deletion of *rad22B*$^+$ in a *rad22A*-deficient background, however, resulted in a further increase in radiation sensitivity. Overexpression of the *rad22B*$^+$ gene in *rad22A* mutant cells partially suppresses the hypersensitivity to X-rays, suggesting functional overlap between the two genes. Here we report that Rad22A and Rad22B can interact with each other. Both proteins also show physical interaction with Rhp51, the homolog of Rad51 in *S.pombe*, and RPA. In addition, we have investigated the *S.pombe rad22A*, *rad22B* and *rad22Arad22B* mutant phenotypes in more detail, using mitotic and meiotic recombination assays.

MATERIALS AND METHODS

Strains and growth conditions

The *S.pombe* strains used in this study are listed in Table 1. The media have been described elsewhere (46). Cells were grown in rich medium (YE) or YNB minimal medium [0.67% Yeast Nitrogen Base (Difco), 2% glucose]. For recombination assays Eagle's minimal medium (EMM) was used. Appropriate amino acids were added to a final concentration of 100 mg/l. Geneticin selection was performed using YE medium containing 100 mg/l G418 (Gibco).

Table 1. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype
RGL2	*smt-0 ura4-D18 leu1-32 ade6-M26*
RGL14	*smt-0 rad22A:: ura4*$^+$ *ura4-D18 leu1-32 ade6-M26*
RGL15	*smt-0 rad22B::LEU2 ura4-D18 leu1-32 ade6-M26*
RGL16	*smt-0 rad22A::ura4*$^+$ *rad22B::LEU2 ura4-D18 leu1-32 ade6-M26*
RGL17	*h*$^+$ *rad22A::ura4*$^+$ *rad22B::LEU2 ura4-D18 leu1-32 ade6-M26*
RGL20	*h*$^+$ *ura4-D18 leu1-32 ade6-52*
RGL21	*h*$^+$ *rad22A::ura4*$^+$ *ura4-D18 leu1-32 ade6-52*
RGL22	*h*$^+$ *rad22B::LEU2 ura4-D18 leu1-32 ade6-52*
RGL23	*h*$^+$ *rad22A::ura4*$^+$ *rad22B::LEU2 ura4-D18 leu1-32 ade6-52*
RGL26	*h*$^-$ *leu1-32 arg1-14*
RGL27	*h*$^+$ *rad22A::loxKANMX4lox leu1-32 arg1.14*
RGL28	*h*$^+$ *rad22B::LEU2 leu1-32 arg1-14*
RGL29	*h*$^+$ *rad22A::loxKANMX4lox rad22B::LEU2 leu1-32 arg1-14*
RGL30	*h*$^+$ *leu1-32 ura4-595*
RGL31	*smt-0 rad22A::loxKANMX4lox ura4-595*
RGL32	*smt-0 rad22B::LEU2 leu1-32 ura4-595*
RGL33	*smt-0 rad22A::loxKANMX4lox rad22B::LEU2 leu1-32 ura4-595*
RGL34	*h*$^+$ *ura4-D18 leu1-32*
RGL35	*h*$^+$ *rad22A::ura4*$^+$ *ura4-D18 leu1-32*
RGL36	*h*$^+$ *rad22B::loxKANMX4lox ura4-D18 leu1-32*
RGL37	*h*$^+$ *rad22A::ura4*$^+$ *rad22B::loxKANMX4lox ura4-D18 leu1-32*
SL1	*h*$^+$ *ura4-D18 leu1-32 ade6-M26 int::pUC8/ura4/MAT***a***/ade6-L469*
RGL38	*rad22A::loxKANMX4lox* SL1
RGL39	*rad22B::loxKANMX4lox* SL1
RGL40	*rad22A::loxKANMX4lox rad22B::LEU2* SL1
RGL41	*rhp51::KANMX4* SL1
RGL42	*rhp54::KANMX4* SL1
GP338	*h*$^-$*/h*$^-$ *ura4-294/+ +/leu1-32 ade6-M26/ade6-M210 + /arg1-2 pat1-114/pat1-114 end1-458/end1-458*

Two-hybrid analysis

The single copy two-hybrid vectors pPC97, carrying the Gal4 DNA-binding domain, and pPC86, carrying the Gal4 activation domain, have been described elsewhere (47). Gene fragments containing 5′ *Sal*I and 3′ *Bam*HI restriction sites were obtained by PCR using the Expand High Fidelity PCR System (Roche). The fragments obtained were first cloned in the Zero Blunt vector (Invitrogen) and verified by sequencing before cloning into the appropriate restriction sites of pPC97 and pPC86. Two-hybrid studies were performed using *S.cerevisiae* strain Y190 (48). Transformants were selected for tryptophan and leucine prototrophy on YNB medium containing 30 mg/l adenine. Protein–protein interactions were detected by a filter

assay for β-galactosidase activity in permeabilized cells. At least 12 individual colonies from two independent transformations were tested.

GST pull-down

Protein–protein interactions were also investigated by GST pull-down experiments. DNA fragments were isolated as *Sal*I–*Not*I fragments from two-hybrid clones and inserted into *Sal*I + *Not*I-digested pGEX-6P-2 and pMV2-HA. The pMV2-HA vector contains the T7 promotor and can be used for the synthesis of HA-tagged fusion proteins *in vitro*. Glutathione *S*-transferase (GST) fusion proteins were produced in *Escherichia coli* strain BL21 and bound to glutathione–Sepharose 4B beads essentially as described by the manufacturer (Pharmacia Biotech). Sepharose beads were stored at 4°C in phosphate-buffered saline, pH 7.4, containing 1% (v/v) Triton X-100 and the amount of protein immobilized was determined by SDS–PAGE. For the generation of ^{35}S-labeled proteins, pMV2-HA clones were used as template in an *in vitro* coupled transcription/translation system (TNT System; Promega) in the presence of [^{35}S]methionine.

Binding assays were carried out for 60 min at 4°C in 500 µl of binding buffer (50 mM Tris–HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.2% Nonidet P-40) containing 15 µl of ^{35}S-labeled protein and 2.5 µg GST fusion protein loaded on Sepharose beads. The beads were washed five times with washing buffer (50 mM Tris–HCl pH 7.4, 500 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.2% Nonidet P-40). If necessary, more stringent washes were performed in buffer containing 750 mM NaCl. Bound proteins were recovered by heating in 1× Laemmli buffer for 5 min, analyzed by PAGE and visualized by autoradiography.

Protein analysis

The diploid strain GP338 (see Table 1) was used for meiotic induction experiments essentially as described by Lin *et al.* (49). Cells were grown at 25°C in liquid EMM supplemented with adenine to OD_{600} = 0.3. The temperature was raised to 34°C to induce meiosis (49). At different time points, 10 ml samples were withdrawn and cells were harvested by centrifugation, washed once in sterile water and once with 20% trichloroacetic acid (TCA) and resuspended in 200 µl of 5% TCA. An equal volume of glass beads was added and the cells were lysed by vortexing. After addition of 400 µl of 5% TCA, the bottom of the tube was punctured and inserted into another test tube. The suspension was clarified by centrifugation for 5 min at 4000 r.p.m. To each sample was added 200 µl of 1× Western Loading Buffer (1× Western Loading Buffer = 1 vol 4× sample buffer, 2 vol 250 mM Tris–HCl pH 8.5, 1 vol H_2O; 4× sample buffer = 250 mM Tris–HCl pH 6.8, 8% SDS, 20% glycerol, 20% 2-mercaptoethanol, 0.4% Bromophenol blue). Proteins were fractionated on 8% SDS–polyacrylamide gels and transferred to nitrocellulose. Anti-Rad22A polyclonal antibodies were used at 1:7000, anti-Rad22B antibodies at 1:5000 and anti-α-tubulin (Sigma) at 1:2000 dilution. Protein–antibody complexes were visualized by chemiluminescence using the Amersham ECL system.

Inactivation of genes

PCR-mediated deletion constructs were prepared in order to inactivate the *rhp51*$^+$, *rad22A*$^+$, *rad22B*$^+$ and *rhp54*$^+$ genes by replacing the ORF of these genes with the *kan*^{r} gene that confers resistance to the aminoglycoside antibiotic G418.

Synthesis of targeting fragments to delete the *rad22A*$^+$ and *rad22B*$^+$ genes was carried out using the following primer combinations and plasmid pUG6 (50) as template: *rad22A*$^+$ sense, 5′-CTGCTTCATATAAGCTAGAAGGGATGTCTTTTGAGCAAAAACAGCATGTAGCATCAGAAGACCAGGGCCATTTTAACCAGCTGAAGCTTCGTACGCTGC; *rad22A*$^+$ antisense, 5′-AATCATTAGTCATAAAACAGAAAATACTTGGTAAAAAACAAGTTGCCAATCATCACATTTTGCCTCATTACTTTTATGCATAGGCCACTAGTGGATCTG; *rad22B*$^+$ sense, 5′-GTGGCGAAAGACGCGTATAAAAAACCCATTATTCTTCTTTTAACATTCCTTTAAATTAAGATCCCCAAAACATTTTCAAACAGCTGAAGCTTCGTACGC; *rad22B*$^+$ antisense, 5′-GTTGTACAGCAATTTATTTCGCAAGCAGTTTAAAAGTTTATACCATTGAACGTTTCACTCAATTTTATTTTTTTTATGCATAGGCCACTAGTGGATCTG. The nucleotide sequences underlined overlap with the template DNA.

The following primers in combination with plasmid pFA6a-*kanMX4* (51) were used to inactivate the *rhp51*$^+$ and *rhp54*$^+$ genes: *rhp51*$^+$ sense, 5′-CGATTACAACTGGATCAAAGCAACTAGACACTTTGTTACAAGGAGGGGTTGAGACTGGAAGCATTACTGAATTATTTGGTGCGTACGCTGCAGGTCGAC; *rhp51*$^+$ antisense, 5′-TTGGTTTTTTGGGATCAGGATTAAAGGAAATGCCATCCACTTGGGCGACCACCTGATTAGTAATGACAACAGCAATACCGATCGATGAATTCGAGCTCG; *rhp54*$^+$ sense, 5′-TTTAGTGAAGAGTAACAATGGTGCGTAATTTAAAGAACGCGACACGCTATAATAGTAATAGAATTTAATTTATATATACATCGTACGCTGCAGGTCGAC; *rhp54*$^+$ antisense, 5′-TTGCCTTGAACCTAACAAACCAGGATTCGCAAAATTTAACAACGAAAAGTATTCGCTTAGATCATTTTGAATAGGGGTACATCGATGAATTCGAGCTCG. The nucleotide sequences underlined overlap with the template DNA.

PCR synthesis of short flanking homology disruption cassettes was carried out using the Expand High Fidelity PCR System (Roche) according to the instructions of the manufacturer. Several PCR reactions were pooled to yield ~5 µg DNA, which was transformed into competent *S.pombe* cells according to the lithium acetate method as described by Keeney and Boeke (52). G418-resistant transformants were screened by diagnostic PCR to verify whether the disruption cassette had integrated at the correct position in the genome (data not shown). Details of the additional primers used for this purpose are available on request.

Intrachromosomal recombination

The intrachromosomal recombination substrate consists of two *ade6* heteroalleles (*ade6-M26* and *ade6-L469*) separated by plasmid sequences and a *ura4*$^+$ gene (see Fig. 4) and has been described in detail by Osman *et al.* (53). Two classes of ade6$^+$ recombinants can be obtained: Ade$^+$ Ura$^+$ conversion types and Ade$^+$ Ura$^-$ deletion types (see Fig. 4). The assays for spontaneous direct repeat recombination were performed by fluctuation tests as described previously (53). For each experiment at least six independent colonies were used and each assay was repeated at least twice. Thus a total of at least 12 colonies per strain were analyzed. For the determination of spontaneous recombination frequencies, strains were grown on EMM containing 250 mg/l adenine, 100 mg/l uracil and 100 mg/l leucine. After 4 days growth, single colonies were analyzed by differential plating. Less diluted samples were plated on EMM

lacking adenine (to select for Ade+ recombinants) but containing 100 mg/l uracil. Cell titers were determined by plating appropriate dilutions on complete medium. After 3–4 days growth, cell titer and number of Ade+ recombinants were determined. To determine the proportion of deletion versus conversion type events, Ade+ recombinants were replica-plated onto minimal medium lacking adenine and uracil, and medium lacking adenine but containing uracil.

Homologous integration of linear DNA

Recombination as measured by the integration of linear plasmid DNA at the *leu1.32* locus was carried out as described previously (45,54). Aliquots of competent wild-type and isogenic mutant strains were transformed with 5 µg *Nde*I-linearized pJK148 according to the lithium acetate method as described by Keeney and Boeke (52). To determine the uptake of DNA, an aliquot of competent cells of each strain was transformed with 5 µg pREP1. leu1+ transformants were selected on EMM containing adenine and uracil. The recombination frequency of each strain was calculated by dividing the number of leu1+ transformants of pJK148-transformed cells by the number of leu1+ transformants of pREP1-transformed cells.

Meiotic recombination

Meiotic recombination frequencies between mutant alleles of *ade6* (*ade6-M26* and *ade6-52*) and between *ade6* and *arg1* and *arg1* and *ura4*, respectively, were determined by random spore analysis. Crosses were performed on low nitrogen plates as described before (45). Dilution series of spores were plated on YE plates to determine the total number of spores. Less diluted samples were plated on YNB plates containing appropriate nutrients to select for recombinants. Recombinants were replica-plated onto sporulation medium and stained with iodine vapor after 4 days growth to detect diploid cells. The frequency of diploid cells (a diploid cell may contain two complementing chromosomes and can therefore be mistakenly counted as a recombinant) was extremely low in the experiments presented here. Therefore, the results given in Table 4 did not require correction.

RESULTS

Interactions between Rad22A, Rad22B, Rhp51 and Rhp54

In *S.cerevisiae* and mammals, physical interactions between Rad52 and Rad51 and between Rad52 and RPA have been detected (21–26). These and other studies suggested the presence of one or more protein complexes in which Rad52 resides with other members of the RAD52 group. To analyze possible interactions of Rad22A and Rad22B with other members of the RAD52 group in *S.pombe*, two-hybrid studies and GST pull-down experiments were performed. To reduce artificial interactions, single copy expression vectors were used for the two-hybrid experiments (47). When multicopy vectors are used, expression of *rhp51+* in *S.cerevisiae* is toxic. All genes and gene fragments were tested as pairwise combinations of DNA-binding domain and activator fusions. Results were confirmed by reciprocal combinations of binding domain and activator fusions and are summarized in Figure 1. Autonomous *lacZ* activation was not observed for the constructs used in the experiments mentioned here. As may be expected on the basis

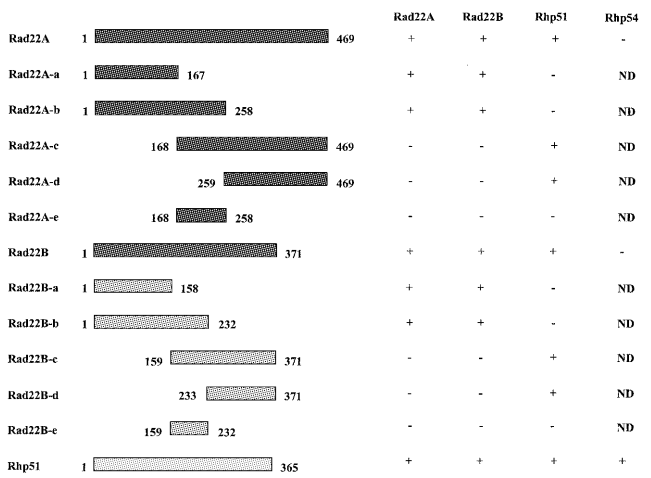

Figure 1. Schematic presentation of the protein–protein interactions investigated by two-hybrid analysis. All gene fragments were tested as DNA-binding fusions, as well as activator fusions. With the exception of the Rhp51–Rhp54 interaction, all the interactions were confirmed in the reciprocal orientation. Interactions were identified using qualitative β-galactosidase filter assays. +, positive interaction; –, no specific interaction; ND, not determined.

of the results obtained with Rad52 homologs from *S.cerevisiae* and mammals, Rad22A from *S.pombe* is able to interact with Rhp51. A similar interaction was seen between Rad22B and Rhp51. The two-hybrid studies also indicated association between both Rad52 homologs from *S.pombe*, Rad22A and Rad22B. Rhp51, as well as Rad22A and Rad22B, showed self-interaction (Fig. 1). The self-interaction of Rad22A and Rad22B is compatible with the formation of ring structures, as has been reported for *S.cerevisiae* Rad52 and human Rad52 (14,28). Rad51 and its homolog in higher eukaryotes form a nucleoprotein filament on ssDNA (16,55,56). Similar properties are expected for Rhp51 and would explain the auto-interaction of this protein. The interaction seen between Rhp51 and Rhp54 is conserved in evolution, as similar observations have been made for Rad51 and Rad54 from *S.cerevisiae* and man (57–59). The two-hybrid experiments did not reveal an interaction between Rhp54 and Rad22A or Rad22B (Fig. 1). Interactions were also observed between Rad22B and Rhp57, between Rad22B and Ssb1 (the large subunit of *S.pombe* RPA) and between Rad22A and Ssb1, respectively (data not shown). Two-hybrid and GST pull-down experiments to identify possible interactions between Rad22A and Rhp57 were not conclusive.

To localize the regions that mediate the interaction of Rad22A with Rad22B and Rhp51 and of Rad22B with Rad22A and Rhp51, respectively, N- and C-terminal deletion constructs were generated by PCR and cloned in pPC97 and pPC86. As shown in Figure 1, the truncated Rad22A-a protein, containing amino acids 1–167, is sufficient for binding to Rad22B and for self-interaction. Residues 259–469 of Rad22A form a complex with Rhp51. The N-terminal part of Rad22B (containing amino acids 1–158) is also sufficient for self-interaction and for interaction with Rad22A. For association with Rhp51, residues 233–371 of Rad22B are sufficient.

The interactions as observed by two-hybrid analysis were confirmed by an *in vitro* binding assay (GST pull-down). Full-length Rad22A and Rad22B as well as C-terminal constructs were expressed in *E.coli* as GST fusion proteins. Proteins from

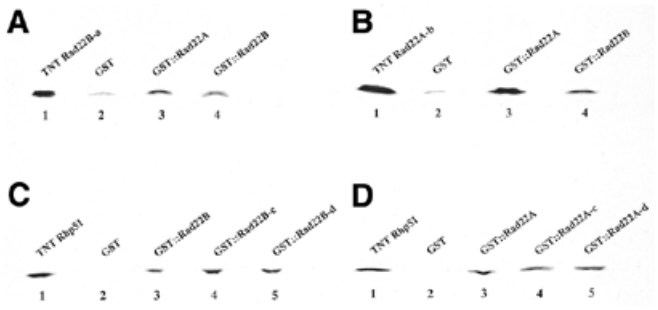

Figure 2. *In vitro* protein–protein interactions studied by GST pull-down assays. (**A**) An aliquot of 3 µl of ^{35}S-labeled Rad22B-a (nucleotides 1–158) (lane 1); 15 µl of ^{35}S-labeled Rad22B-a was mixed with glutathione–Sepharose beads loaded with 2.5 µg GST (lane 2), 2.5 µg GST::Rad22A (lane 3) or 2.5 µg GST::Rad22B (lane 4). (**B**) An aliquot of 3 µl of *in vitro* labeled Rad22A-b (nucleotides 1–258) (lane 1); 15 µl of ^{35}S-labeled Rad22A-b was mixed with glutathione–Sepharose beads loaded with 2.5 µg GST (lane 2), 2.5 µg GST::Rad22A (lane 3) or 2.5 µg GST::Rad22B (lane 4). (**C**) An aliquot of 3 µl of *in vitro* labeled Rhp51 (lane 1); 15 µl of ^{35}S-labeled Rhp51 was mixed with glutathione–Sepharose beads loaded with 2.5 µg GST (lane 2), 2.5 µg GST::Rad22B (lane 3), 2.5 µg GST::Rad22B-c (lane 4) or 2.5 µg GST::Rad22B-d (lane 5). (**D**) An aliquot of 3 µl of *in vitro* labeled Rhp51 (lane 1); 15 µl of ^{35}S-labeled Rhp51 was mixed with glutathione–Sepharose beads loaded with 2.5 µg GST (lane 2), 2.5 µg GST::Rad22A (lane 3), 2.5 µg GST::Rad22A-c (lane 4) or 2.5 µg GST::Rad22A-d (lane 5).

bacterial lysates were bound to glutathione–Sepharose beads and incubated with [^{35}S]methionine-labeled Rad22B-a, Rad22A-b or Rhp51. Glutathione–Sepharose beads containing GST protein were used as a control in all experiments. After washing, associated proteins were analyzed by gel electrophoresis. The results presented in Figure 2A and B indicate that the N-terminal domains of Rad22B and Rad22A are sufficient for interaction with Rad22A and Rad22B, respectively, and for self-interaction. Association with Rhp51 can be detected using full-length Rad22B and Rad22A and the C-terminal fragments of Rad22B and Rad22A (Fig. 2C and D).

Rad22A and Rad22B protein levels

In order to understand the phenotype of *rad22B* mutant cells and the mitotic and meiotic properties of *rad22A* cells, we investigated the transcript and protein levels of Rad22A and Rad22B. Whole cell extracts from mitotically growing cells and cells induced to undergo meiosis were prepared as described in Materials and Methods. As shown in Figure 3, expression of Rad22B protein was barely detectable in mitotically growing cells (0 h) and at 1–3 h after meiosis induction. At later stages in meiosis Rad22B protein can be detected. Rad22B protein is most abundant at 5 and 6 h after induction of meiosis. At later time points the level of Rad22B protein is reduced (Fig. 3). In *rad22A* mutant cells, expression of Rad22B is not increased relative to wild-type cells (data not shown). After probing with anti-Rad22B, the membrane was stripped and reprobed with anti-Rad22A. In contrast to Rad22B, Rad22A protein can be detected in both vegetatively growing cells and in cells induced to undergo meiosis. The level of Rad22A protein is not up-regulated after induction of meiosis (Fig. 3). Analysis of the transcript levels of *rad22B*$^+$ by northern blot analysis also showed a strong increase in the RNA level of *rad22B*$^+$ 5 h after induction of meiosis (data not shown). Transcripts of *rad22A*$^+$ were easily detected in vegetative

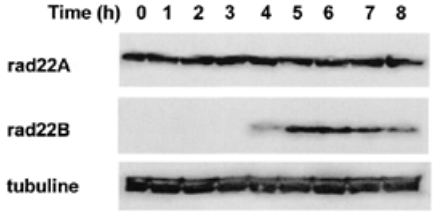

Figure 3. Relative expression of the Rad22A and Rad22B proteins in mitotically dividing cells ($t = 0$) and in cells induced to undergo meiosis ($t = 1$–8). To serve as a control for equivalent loading of proteins, blots were re-probed with an antibody raised against β-tubulin.

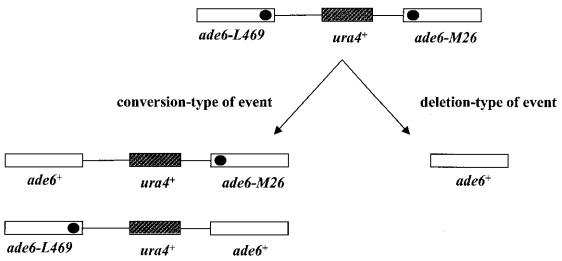

Figure 4. Schematic representation of the intrachromosomal recombination substrate and the two classes of Ade$^+$ recombinants, conversion types and deletion types that can be distinguished.

cells and a slight up-regulation in the RNA level was observed after induction of meiosis (data not shown). These observations indicate that Rad22A is more abundant than Rad22B in mitotically growing cells. Although not detectable by northern blot hybridization, the presence of *rad22B*$^+$ RNA in mitotically growing cells was shown previously by RT–PCR (45).

Recombination in vegetative cells

Strains bearing direct repeats of *ade6* heteroalleles, separated by plasmid sequences and a functional *ura4*$^+$ gene (Fig. 4), were used to study spontaneous intrachromosomal recombination in mitotic cells as measured by the recovery of Ade$^+$ recombinants. As indicated in Figure 4, two main classes of Ade$^+$ recombinants can be distinguished: Ade$^+$ Ura$^+$ conversion types and Ade$^+$ Ura$^-$ deletion types. Both types of events can occur within a single chromatid (intrachromosomal), but may also be due to recombination between sister chromatids after replication of the DNA. A gene conversion event not associated with crossing-over will result in an intact *ade6*$^+$ gene and retention of the *ura4*$^+$ gene. A similar outcome results by conservative one-sided invasion. On the other hand, possible mechanisms resulting in deletion type events are SSA, non-conservative one-sided invasion, gene conversion associated with crossing-over and unequal crossing-over between sister chromatids. In wild-type SL1 cells, spontaneous Ade$^+$ recombinants arose at an average frequency of 3 per 10^4 viable cells. As indicated in Table 2, 52% of the recombinants were conversion type and the remainder deletion type. Next we determined the effects of several RAD52 group mutations on spontaneous direct repeat recombination. For *rad22A*, *rad22B* and *rad22Arad22B* mutant cells, no significant difference ($P > 0.05$) in the frequency of Ade$^+$ recombinants (Table 2) was observed. However, a highly significant shift to deletion type events was

Table 2. Spontaneous intrachromosomal mitotic recombination frequencies

Strain	Total Ade+	*P*	Deletion types (%)	Conversion types (%)	*P*
Wild type	2.96 ± 1.16	–	48	52	–
rad22A	2.84 ± 1.35	>0.05	84	16	<0.01
rad22B	5.30 ± 3.18	>0.05	49	51	>0.5
rad22Arad22B	1.55 ± 0.51	>0.05	89	11	<0.01
rhp51	10.93 ± 4.36	<0.05	99	1	<0.01
rhp54	20.50 ± 6.66	<0.01	97	3	<0.01

Recombination frequencies (per 10^4 viable cells ± SD) and percentages of deletion type versus conversion type were obtained as described in Materials and Methods. *P* values are from two-sample *t*-tests in which individual recombination frequencies and percentage deletion types of all colonies assayed were analyzed. $P < 0.05$ indicates a significant difference in recombination frequency (and percentage deletion type) for a given mutant strain, compared with the wild-type strain, at the 95% confidence level. $P < 0.01$ indicates a very significant (99% confidence interval) difference.

Table 3. Recombination as measured by the integration of plasmid DNA

Strain	Leu+ transformants[a]	
	pJK148/pREP1 ratio	Fold reduction
Wild-type	0.450	1
rad22A	0.036	13
rad22B	0.440	1
rad22Arad22B	0.003	>100

[a]The reduction in the frequency of Leu+ transformants was calculated as described in Materials and Methods.

observed in *rad22A* and *rad22Arad22B* mutant cells. About 84% of the Ade+ recombinants arose by a deletion type event in *rad22A* mutant cells. A preference for deletion type events in direct repeat recombination was also observed in *rad22Arad22B* mutant cells, as 89% of the recombinants arose by such a mechanism (Table 2). In *rad22B* mutant cells the distribution of recombination events was not different from that in wild-type cells (Table 2). The distribution of recombination events differs significantly between *rad22A* and *rad22B* mutant cells and also differs between *rad22Arad22B* and *rad22B* mutant cells ($P < 0.01$). No significant shift in conversion versus deletion types of Ade+ recovery is observed between *rad22A* and *rad22Arad22B* mutant cells. As indicated in Table 2, both *rhp51* and *rhp54* mutant strains exhibit a strong hyper-recombination phenotype. In both mutants recombination occurred primarily via a deletion type event. These latter observations are in agreement with previous *S.pombe* and *S.cerevisiae* studies using direct repeat recombination assays (60,61).

Homologous recombination in the *rad22Arad22B* mutant was also studied by measuring the efficiency of integration of linear pJK148DNA at the *leu1.32* locus (Table 3). The number of colonies obtained after transformation with linear pJK148 plasmid DNA was related to the number of pREP1 transformants. As reported previously, no reduction was seen in the pJK148/pREP1 ratio in the *rad22B* mutant and a 13-fold reduction was observed in the *rad22A* mutant (45). Inactivation of both *rad22A*+ and *rad22B*+ affected genomic integration of linear DNA at least 100-fold (Table 3). These results indicate that *rad22B*+ has the ability to contribute to HR, especially in the absence of *rad22A*+.

Table 4. Meiotic recombination frequencies

Strain	Interval			Spore viability[d]
	ade6-M26–ade6-52[a]	*arg1-14–ade6-M26*[b]	*arg1-14–ura4-595*[c]	
Wild-type	75.1 ± 12.9	12.4 ± 2.1	15.9	92
rad22A	64.5 ± 10.8	7.9 ± 0.2	2.7	23
rad22B	70.6 ± 10.3	21.9 ± 5.3	23.2	88
rad22Arad22B	60.3 ± 2.1	20.0 ± 5.3	23.1	3

[a]Average recombination frequency per 10^4 cells of at least four independent crosses (± SD).
[b]Average recombination frequency per 10^2 cells of at least four independent crosses (± SD).
[c]Average recombination frequency per 10^2 cells of two independent crosses.
[d]Results obtained previously (45).

Meiotic recombination

In a previous study we showed that intragenic meiotic recombination at the *ade6* locus, between *ade6-M216* and *ade6-469*, was slightly affected in *rad22A* and *rad22B* mutant strains (45). The analysis of meiotic recombination was extended by measuring recombination at the intragenic interval between *ade6-M26* and *ade6-52* and by determining recombination at the intergenic intervals *arg1–ade6* and *arg1–ura4*. As shown in Table 4, meiotic recombination in the *rad22A*, *rad22B* and *rad22Arad22B* mutant strains was only slightly affected at the *ade6* locus. Recombination at *ade6* was also studied in *rhp51* and *rhp54* mutant strains. Meiotic recombination was decreased ~2-fold in the *rhp51* mutant, whereas no reduction in intragenic meiotic recombination at *ade6* was observed in the *rhp54* mutant strain (data not shown).

Intergenic meiotic recombination was determined at different genetic intervals on chromosome III between mutant alleles of *arg1* and *ade6* and between mutant alleles of *arg1* and *ura4*. At both intervals the *rad22B* mutant showed a reproducible 1.5-fold increase in recombination (Table 4). Intergenic recombination at both intervals was reduced in the *rad22A* mutant strain (Table 4). Surprisingly, inactivation of *rad22B*+ increased the intergenic recombination frequencies of a *rad22A* mutant strain to the level of the *rad22B* single mutant (Table 4).

DISCUSSION

The role of Rad22A and Rad22B in vegetative cells

The *RAD52* homologs *rad22A*+ and *rad22B*+ from the fission yeast *S.pombe* show strong conservation at both the nucleotide and amino acid levels. Despite the similarities in sequence, *S.pombe* cells inactivated for *rad22A*+ show severe hypersensitivity to DNA-damaging agents (X-rays, bleomycin and UV light) and display defects in the ability to perform HR as measured by the integration of plasmid DNA (45). On the other hand, cells inactivated for *rad22B*+ are not hypersensitive to these

DNA-damaging agents and are proficient in the ability to perform HR (44,45). Similar observations were made using cisplatin, which induces interstrand DNA crosslinks. In general, repair of these lesions also depends on HR. The *rad22A S.pombe* mutant displays increased sensitivity to cisplatin, however, the *rad22B* mutant is not hypersensitive to this compound (data not shown). In the *rad22Arad22B* double mutant the hypersensitivity is further increased (data not shown). Apparently, Rad22B is not required for the repair of a wide variety of DNA lesions if Rad22A is present. One explanation might be the low level of Rad22B in mitotically dividing cells (Fig. 3). The enhanced hypersensitivity of *rad22Arad22B* double mutant cells indicates that Rad22B functionally overlaps with Rad22A. The partial alleviation of the severe *rad22A* mutant phenotype by overexpression of *rad22B*$^+$ is in agreement with this assumption (45). In addition, Rad22B might be required for the repair of specific types of DNA lesions.

Very similar observations were made when HR was studied by integration of plasmid DNA. In the *rad22Arad22B* double mutant the level of recombination is further reduced in comparison with the *rad22A* single mutant (at least 7-fold; see Table 3). As for the repair of exogenously inflicted DNA damage, Rad22B functionally overlaps with Rad22A in this type of recombination. HR was also studied using a substrate containing direct repeats of two *ade6* heteroalleles (Fig. 4). In the *rad22* single and double mutant cells, recovery of Ade$^+$ cells was not significantly different from that in wild-type cells (Table 2). However, mostly deletion type events were recovered in *rad22A* and *rad22Arad22B* mutant cells. A similar system has been used by McDonald and Rothstein (61) to study HR in *S.cerevisiae* cells deficient for *RAD52*. A slight reduction in the recovery of Ade$^+$ colonies was observed in the *S.cerevisiae rad52* mutant (61). As observed for *S.pombe rad22A* and *rad22Arad22B* mutant cells, in *rad52* mutant cells recombinants arose mainly by deletion type events. Apparently, *rad22A* and *rad22Arad22B* mutant cells can survive spontaneous DSBs by Rad22A/Rad22B-independent subpathways of recombination, which lead to deletions.

The results of the protein–protein interaction studies are in agreement with a functional overlap between Rad22A and Rad22B. Both proteins show interaction with Rhp51 and with the large subunit of RPA. Also, in *S.cerevisiae* and higher eukaryotes Rad52 protein is able to interact with Rad51 and with RPA (21–26). As biochemical studies have indicated, the ability of Rad52 to interact with RPA and with Rad51 is essential for Rad51-dependent pairing and strand exchange reactions, which are the first steps in HR (17,19,20). The Rad52 protein from *S.cerevisiae* and man is able to form multimeric ring structures (14,28). In *S.pombe*, similar structures may consist of either Rad22A or Rad22B, or both proteins. Biochemical studies using purified Rad22A and Rad22B may provide more detailed information on the similarities and possible differences between both proteins.

Meiotic properties of Rad22A and Rad22B

It has been shown that the formation of DSBs during early meiosis is essential to promote meiotic recombination in *S.cerevisiae* and *S.pombe* (62–64). In both organisms, DSB formation during meiosis requires the function of multiple gene products. For example, inactivation of the meiosis-specific endonuclease *SPO11* in *S.cerevisiae* and its corresponding homolog in *S.pombe* (*rec12*$^+$) affects DSB formation during meiosis and results in drastically reduced meiotic recombination frequencies (>100-fold reduction) (64,65). In the *rad22A* mutant, meiotic intra- and intergenic recombination is slightly reduced at the *ade6* locus and the *arg1–ade6* interval, respectively, but is more strongly reduced at the larger *arg1–ura4* interval. Spore viability is 4-fold reduced in the *rad22A S.pombe* mutant (45). In the fraction of surviving spores DSBs are probably repaired by *rad22A*$^+$-independent mechanisms which may depend on *rad22B*$^+$. The meiotic phenotype of *S.cerevisiae rad52* mutant cells is much more severe than that of *rad22A S.pombe* cells. Meiotic recombination is at least 100-fold reduced and the production of viable spores is >200-fold decreased in the *S.cerevisiae rad52* mutant (5). In contrast to *rad22A*$^+$, expression of the *rad22B*$^+$ gene is strongly regulated during meiosis. However, inactivation of *rad22B*$^+$ does not affect spore viability and results in a very small increase in meiotic recombination at both intergenic intervals tested (Table 4).

Surprisingly, the meiotic recombination frequency in the *rad22Arad22B* double mutant is comparable to the level of recombination in the *rad22B* single mutant. However, the viability of spores is dramatically reduced in the double mutant (45). The results show that residual repair of meiosis-specific DSBs is possible in the absence of Rad22A and Rad22B in the surviving fraction of spores. The level of recombination is hardly influenced in the surviving cells. In agreement with the meiotic recombination data and reduced spore viability are observations that meiotic DSBs are formed and accumulate in the *rad22A* and *rad22Arad22B* mutant strains (J.Young, C.Rubio and G.R.Smith, Fred Hutchinson Cancer Center, Seattle, WA, USA, personal communication). A similar phenotype has been observed for *rhp51* mutants. Spore viability is drastically reduced, but the level of meiotic recombination is only slightly affected (54). Moreover, meiotic DSBs also accumulate in the *rhp51* mutant strain (66).

Rad52 mutant phenotypes

The *RAD52* gene from *S.cerevisiae* is required for nearly all (sub)pathways of recombination, as reviewed by Paques and Haber (3). The results presented here suggest that in *S.pombe* two proteins, Rad22A and Rad22B, perform the essential role that Rad52 has in most types of recombination in *S.cerevisiae*. However, minor Rad22A/Rad22B-independent mitotic and meiotic recombination pathways exist in *S.pombe*, explaining the less dramatic recombination defects of the double mutant in comparison with the *S.cerevisiae rad52* mutant.

Inactivation of *rad22B*$^+$ hardly affects recombination and cell survival after exposure to DNA-damaging agents. In these respects, the *rad22B S.pombe* mutant resembles *RAD52*-deficient mouse ES and chicken DT40 cells. A second *RAD52* homolog, which could be functionally related to *rad22A*$^+$ in *S.pombe*, has not yet been identified in vertebrate cells. It cannot be excluded, however, that a second homolog is only functionally conserved and not structurally. Another possibility to explain the weak phenotype of *RAD52*-deficient mouse and chicken cells is functional complementation by other members of the RAD52 epistasis group. Rad51-dependent pairing and strand exchange reactions are stimulated by the addition of Rad52, and also by the addition of Rad55/Rad57, which are Rad51

paralogs (18). Recently, Fujimori *et al*. (67) reported that *XRCC3*$^{-/-}$*RAD52*$^{-/-}$ double mutant DT40 cells could not be isolated, whereas either single mutant is viable. Possibly, the major contribution to assisting Rad51 in the formation of a nucleoprotein filament has shifted from Rad52 in *S.cerevisiae* towards the Rad51 paralogs in vertebrate cells. Therefore, it cannot be excluded that vertebrates have only one *RAD52* homolog and that mutation of this gene is complemented by one (or more) of the *RAD51* paralogs in higher eukaryotes.

ACKNOWLEDGEMENTS

The authors thank F. Osman for kindly providing the *S.pombe* SL1 strain, G. Smith for sharing unpublished data and sending the GP338 strain, J. Hegemann for providing the pUG6 vector, P. Philippsen for sending plasmid pFA6a-KANMX4, J. Dorsman for discussions and N. de Wind for critical reading of the manuscript. This work was supported by the Netherlands Organization for Scientific Research (NWO), grant 901-01-97.

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