MOLECULAR AND CELLULAR BIOLOGY, May 2002, p. 3537–3548 Vol. 22, No. 10
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.10.3537–3548.2002

The *Schizosaccharomyces pombe rad60* Gene Is Essential for Repairing Double-Strand DNA Breaks Spontaneously Occurring during Replication and Induced by DNA-Damaging Agents

Takashi Morishita,[1] Yasuhiro Tsutsui,[1] Hiroshi Iwasaki,[1,2]† and Hideo Shinagawa[1]*

Department of Molecular Microbiology, Research Institute for Microbial Diseases, Osaka University,[1] and PRESTO JST,[2] Suita, Osaka 565-0871, Japan

Received 13 December 2001/Returned for modification 23 January 2002/Accepted 22 February 2002

To identify novel genes involved in DNA double-strand break (DSB) repair, we previously isolated *Schizosaccharomyces pombe* mutants which are hypersensitive to methyl methanesulfonate (MMS) and synthetic lethals with *rad2*. This study characterizes one of these mutants, *rad60-1*. The gene that complements the MMS sensitivity of this mutant was cloned and designated *rad60*. *rad60* encodes a protein with 406 amino acids which has the conserved ubiquitin-2 motif found in ubiquitin family proteins. *rad60-1* is hypersensitive to UV and γ rays, epistatic to *rhp51*, and defective in the repair of DSBs caused by γ-irradiation. The *rad60-1* mutant is also temperature sensitive for growth. At the restrictive temperature (37°C), *rad60-1* cells grow for several divisions and then arrest with 2C DNA content; the arrested cells accumulate DSBs and have a diffuse and often aberrantly shaped nuclear chromosomal domain. The *rad60-1* mutant is a synthetic lethal with *rad18-X*, and expression of wild-type *rad60* from a multicopy plasmid partially suppresses the MMS sensitivity of *rad18-X* cells. *rad18* encodes a conserved protein of the structural maintenance of chromosomes (SMC) family (A. R. Lehmann, M. Walicka, D. J. Griffiths, J. M. Murray, F. Z. Watts, S. McCready, and A. M. Carr, Mol. Cell. Biol. 15:7067–7080, 1995). These results suggest that *S. pombe* Rad60 is required to repair DSBs, which accumulate during replication, by recombination between sister chromatids. Rad60 may perform this function in concert with the SMC protein Rad18.

DNA double-strand breaks (DSBs) cause cellular lethality if not repaired and can lead to chromosomal aberrations such as deletions or translocations if repaired improperly. DSBs are repaired mainly through two mechanisms in eukaryotes: homologous recombination (HR) and nonhomologous end joining (NHEJ) (20). Studies of X-ray-sensitive mutants of the budding yeast *Saccharomyces cerevisiae* identified a group of genes involved in HR that contribute to the process of DSB repair (9). These genes include *RAD50*, *MRE11*, *XRS2*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, and *RAD57*. The first three of these genes are involved in processing DSB ends, and the other genes facilitate DNA strand exchange. The Rad51 protein is structurally and functionally homologous to the *Escherichia coli* RecA protein (44). In *S. cerevisiae*, HR is the major pathway for DSB repair and NHEJ plays a role in tolerance to ionizing radiation only when there is a deficiency in HR (45). In contrast, both HR and NHEJ play important roles in the repair of ionizing-radiation-induced DNA damage in vertebrates (20).

In the fission yeast *Schizosaccharomyces pombe*, the *rad32*, *rhp51*, *rad22*, *rhp54*, *rhp55*, and *rhp57* genes are homologous to the *S. cerevisiae* genes *MRE11*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, and *RAD57*, respectively, and strains with a mutation in any of these genes are hypersensitive to ionizing radiation (23, 33, 34, 40, 51, 53). In addition to these genes, the *rad18* and *rad21* genes have been implicated in DSB repair (25). *rad21* is essential for growth (3) and homologous to the *S. cerevisiae MCD1/SCC1/RHC21* gene. *rad18* is essential for growth and encodes a protein that belongs to the structural maintenance of chromosomes (SMC) superfamily (26). The SMC family proteins are structurally related to each other and include N- and C-terminal globular domains, which possess Walker A and B motifs for ATP binding, respectively, and two central coiled-coil segments, which are separated by a flexible hinge (for reviews, see references 12 and 48). The SMC family proteins of eukaryotes form heterodimers with several non-SMC proteins. The SMC protein complexes regulate higher-order chromosome structures involving chromosome cohesion, condensation, and dosage compensation. The Rad18 protein of *S. pombe* forms a complex with the SMC family protein Spr18 and five other unidentified proteins (8).

The *rad18-X* mutant is sensitive to UV irradiation and removes UV-induced DNA damage less efficiently than does the wild type (26). *rad18* is not epistatic to the conserved nucleotide excision repair pathway but is involved in a secondary nucleotide excision repair pathway that requires the *rad2* and *rhp51* genes. *rad2* encodes a structure-specific endonuclease homologous to mammalian Fen-1, which is required for Okazaki fragment maturation (37), and is involved in a second excision repair pathway, in which repair is initiated by UV-damage endonuclease in *S. pombe* (57). Nevertheless, *rad18-X* is not epistatic to UV-damage endonuclease, and hence, *rad18* is implicated in both a DNA damage tolerance pathway and the second excision repair pathway (26, 36). A *rad18* mutant,

* Corresponding author. Mailing address: Department of Molecular Microbiology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8317. Fax: 81-6-6879-8320. E-mail: shinagaw@biken.osaka-u.ac.jp.

† Present address: Science of Biological Supermolecular Systems, Graduate School of Integrated Science, Yokohama City University, Tsurumi-ku, Yokohama, Kanagawa, Japan.

TABLE 1. *S. pombe* strains used in this study

Strain	Genotype	Source
972	*h*$^-$	NCYC
NCYC1979	*h*$^-$ *rad18-X*	NCYC
NCYC1982	*h*$^-$ *rad21-45*	NCYC
HM248	*h*$^+$ *his2 ade6-M210* Ch16	Niwa et al. (39)
MP2	*h*$^+$	This work
MP10	*h*$^-$ *leu1-32 ura4-D18*	This work
MP11	*h*$^+$ *leu1-32 ura4-D18*	This work
MPR101	*h*$^-$ *leu1-32 ura4-D18 rad60-1*	This work
MPR104	*h*$^+$ *rad60-1*	This work
MPR105	*h*$^+$ *leu1-32 ura4-D18 rad18-X*	This work
MPR111	*h*$^+$ *leu1-32 ura4-D18 rad60-1*	This work
MPR113	*h*$^-$ *leu1-32 ura4-D18 rad60-1 rad2*::*ura4*$^+$	This work
MPR114	*h*$^+$ *leu1-32 ura4-D18 rad60-1*	This work
MPR115	*h*$^+$ *leu1-32 ura4-D18 rad2*::*ura4*$^+$	This work
MPR116	*h*$^-$ *leu1-32 ura4-D18*	This work
MPR117	*h*$^-$ *smt-0 leu1-32 ura4-D18*	This work
MPR118	*h*$^-$ *smt-0 leu1-32 ura4-D18 rad60-1*	This work
MPR119	*h*$^-$ *smt-0 leu1-32 ura4-D18 rad51*::*ura4*$^+$	This work
MPR120	*h*$^-$ *smt-0 leu1-32 ura4-D18 rad60-1 rhp51*::*ura4*$^+$	This work
MPR121	*h*$^+$ *mat1PΔ17*::*LEU2 leu1-32 ura4-D18 rad60-1 rhp51*::*ura4*$^+$	This work
MPR122	*h*$^+$ *mat1PΔ17*::*LEU2 leu1-32 ura4-D18 rad60-1*	This work
MPR123	*h*$^+$ *mat1PΔ17*::*LEU2 leu1-32 ura4-D18*	This work
MPR124	*h*$^+$ *mat1PΔ17*::*LEU2 leu1-32 ura4-D18 rhp51*::*ura4*$^+$	This work
MPR125	*h*$^+$ *leu1-32 ura4-D18 ade6-M210* Ch16	This work
MPR126	*h*$^+$ *leu1-32 ura4-D18 ade6-M210 rad60-1* Ch16	This work
MPD1	*h*$^+$/*h*$^-$ *leu1-32*/*leu1-32 ura4-D18*/*ura4-D18 his7-366*/+ *ade6-M210*/*ade6-M216*	This work
MPDR101	*h*$^+$/*h*$^-$ *leu1-32*/*leu1-32 ura4-D18*/*ura4-D18 his7-366*/+ *ade6-M210*/*ade6-M216 rad60*::*ura4*/+	This work

the *rad18-74* mutant, was isolated in a genetic screen for mutants defective in DNA damage checkpoint control; this mutant is impaired in the maintenance of checkpoint arrest (55). Therefore, *rad18* is also implicated in a DNA damage checkpoint.

This study is part of our ongoing effort to characterize novel genes involved in recombinational repair in *S. pombe*. Our strategy is based on the fact that strains with mutations in recombination genes are often synthetic lethals with *rad2* (37, 51). In an earlier study, seven methyl methanesulfonate (MMS)-sensitive mutants were isolated which were synthetical lethals with *rad2*. The *rhp57* gene, a homolog of *S. cerevisiae RAD57*, complements the MMS sensitivity of one of the previously identified mutants (53). In this study, we isolated a second gene, designated *rad60*, that complements one of the previously identified MMS-sensitive mutants. *rad60* interacts genetically with *rad18*. The *rad60-1* mutant is defective in DSB repair, and the *rad60* gene is essential for growth. Hence, *rad60* appears to play a role in a cellular process that is required for both DSB repair and normal DNA replication. *rad60* may act in concert with *rad18*.

MATERIALS AND METHODS

***S. pombe* media and methods.** *S. pombe* cells were grown in yeast extract-supplemented (YES) medium or Edinburgh minimal medium (EMM), and standard genetic and molecular procedures were employed as described previously (32). To measure the sensitivity of cells to γ rays, exponential-phase culture was harvested and irradiated with γ rays from a ^{60}Co source at a dose of 21 Gy/min. After irradiation, appropriately diluted samples were spread on YES plates and incubated at 30°C for 5 days, and the colonies were counted. To measure the sensitivity of cells to UV, exponential-phase cultures diluted to appropriate concentrations were spread on YES plates. The plates were irradiated with the indicated doses of UV light and incubated at 30°C for 5 days, and the colonies were counted.

***S. pombe* strains and plasmids.** The *S. pombe* strains used in this study are listed in Table 1. Strains 972, NCYC1979, and NCYC1982 were obtained from the National Collection of Yeast Cultures (NCYC; Norwich, United Kingdom). Strain HM248 (39) was kindly provided by O. Niwa. All other strains were constructed for this study. pUR19 (1) and pJK148 (21) were described previously. pU19 was constructed by deleting the *Cla*I fragment containing *ars1* from pUR19.

Chromosome segregation assay. Chromosome segregation was measured by monitoring the transmission of linear minichromosome Ch16 (39) carrying the *ade6-M216* allele, which complements the *ade6-M210* allele of chromosome III. Cells that have lost Ch16 appear pink in color on medium containing a low concentration of adenine. To determine the rate of minichromosome loss, *rad60-1* (MPR126) or *rad60*$^+$ (MPR125) cells carrying Ch16 were cultured on YES plates at 30°C for 3 days. A single colony was used to inoculate 5 ml of YES medium and cultured at 30°C until cell density reached 3×10^7 to 5×10^7/ml. The cultures were diluted and spread on YES plates containing 10 μg of adenine sulfate/ml. The plates were incubated at 30°C for 5 days, and total and pink colonies were counted. The rate of minichromosome loss (p) was determined by the equation $p = [1 - e^{(1/n \times \ln(R_n/R_0))}] \times 100$, where R_0 and R_n are the percentages of Ade$^+$ cells at generations 0 and n after transfer to nonselective medium, respectively (28). In this case, R_0 was 100, since each of the cultures was derived from a single cell.

Cloning of the *rad60* gene. *rad60-1* cells were transformed with the *S. pombe* genomic library constructed from vector pUR19 (1) and spread on EMM plates containing leucine (200 μg/ml) and MMS (0.004%). Transformants were examined for plasmid-dependent MMS resistance. Plasmids which complemented the MMS sensitivity of *rad60-1* cells were isolated, transformed into *E. coli* DH5α cells, and recovered from the transformant.

Cloning of the *rad60* cDNA. The *rad60* cDNA was constructed with RNA from the wild-type strain 972 by using the RNeasy mini kit (Qiagen) and amplified by using the RNA LA PCR kit, version 1.1 (Takara Shuzo). Primers had the sequences 5′-TCACATATGGACAACCTAGATGAAG-3′ and 5′-TCAGGATCCTTAATCCAAAACAACACTAACTTG-3′, corresponding to the 5′ and 3′ ends of the *rad60* coding region, respectively. The PCR fragment was cloned into pUC19 after cleavage with *Nde*I and *Bam*HI, whose recognition sites were engineered near the 5′ ends of the PCR primers.

Disruption of the *rad60* gene. pUC118 carrying the 2.6-kbp *Bam*HI-*Nru*I region of the *rad60* gene was used to generate a construct for gene disruption. The 0.8-kbp *Xba*I-*Sph*I fragment of the coding region was replaced with the

1.8-kbp *Hin*dIII fragment containing the *ura4* gene from pREP2 (29). The 3.6-kbp fragment for gene disruption was released from the plasmid by digestion with *Sac*I and *Sal*I and used to transform the MPD1 cells and create the strain MPDR101. Gene disruption was confirmed by Southern blot analysis, and MPDR101 cells heterozygous for the disruption were sporulated and subjected to tetrad analysis.

Recovery of the *rad60-1* mutant allele. The *rad60-1* mutant gene was recovered by the eviction method (56). pU19 carrying the 3.6-kbp *Bam*HI fragment including the wild-type *rad60* gene was linearized at the unique *Mun*I site in the *rad60* flanking region and used to transform the *rad60-1* mutant. Genomic DNA of the resulting transformant was extracted, digested with *Sal*I, ligated, and used to transform *E. coli* strain DH5α. The 3.6-kbp *Bam*HI fragment of the recovered plasmid was subcloned into pU19, and the nucleotide sequence was analyzed.

Pulsed-field gel electrophoresis. DNA plugs were prepared as described previously (46), with the exception that cells were lysed by incubation with Zymolyase 100T (Seikagaku Corporation) (0.5 mg/ml) for 30 min. Pulsed-field gel electrophoresis was carried out with 0.6% chromosomal-grade agarose (Bio-Rad) in 0.5× TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA) by using a CHEF Mapper apparatus (Bio-Rad). The settings were as follows: 2 V/cm; pulse time, 30-min; angle, 106°; 72 h.

Localization of Rad60 protein in the cell. A plasmid carrying a gene encoding an enhanced green fluorescence protein (EGFP)-Rad60 fusion protein (EGFP-*rad60* fusion gene) was constructed as follows. An *Nde*I site was engineered at the first ATG codon of the *rad60* gene with a QuickChange site-directed mutagenesis kit (Stratagene). The *Nde*I cassette of the EGFP-encoding gene from pGEM-T-EGFP (5) was inserted into the *Nde*I site to create an EGFP-*rad60* fusion. The *Nco*I-*Sal*I region of pREP42EGFPN (5) was replaced with the *Nco*I-*Sal*I fragment of the EGFP-*rad60* fusion gene. The resulting plasmid, pErad60, was transformed into wild-type *S. pombe* MP11. The transformants were recovered on an EMM plate containing leucine (200 μg/ml) and thiamine (15 μM). Expression of the EGFP-Rad60 fusion protein was induced in EMM medium containing leucine (200 μg/ml) for 20 h. Cells were fixed with 2.5% glutaraldehyde, treated with 0.1% sodium borohydride three times for 5 min each time, stained with 1 μg of 4′,6′-diamidino-2-phenylindole (DAPI)/ml, and observed under an epifluorescence microscope (38).

RESULTS

***rad60-1* cells are defective in DNA repair and temperature sensitive for growth.** This study characterizes a previously identified MMS- and γ-ray-sensitive mutant of *S. pombe* that is a synthetic lethal with *rad2* (53). This mutant, designated *rad60-1*, was repeatedly backcrossed with wild-type strains, and an MMS-sensitive segregant clone was selected. As shown in Fig. 1A, the *rad60-1* mutant is more sensitive to DNA-damaging agents, UV and γ rays, than is the wild type. The *rad60-1* mutant is temperature sensitive for growth on YES plates at 37°C but grows normally at 26°C.

A *rad60-1 rad2* double mutant was obtained by crossing *rad60-1* with *rad2* and culturing the spores on YES plates at 26°C. The growth of the double mutant was examined at different temperatures (Fig. 1B). The *rad60-1 rad2* double mutant grows at 26°C, but growth is severely impaired at 30°C and completely inhibited at 33 and 37°C. On the other hand, *rad60-1* grows at 33°C, and the *rad2* mutant grows at all temperatures tested. Thus, *rad60-1* is a synthetic lethal with *rad2* at 33°C and higher.

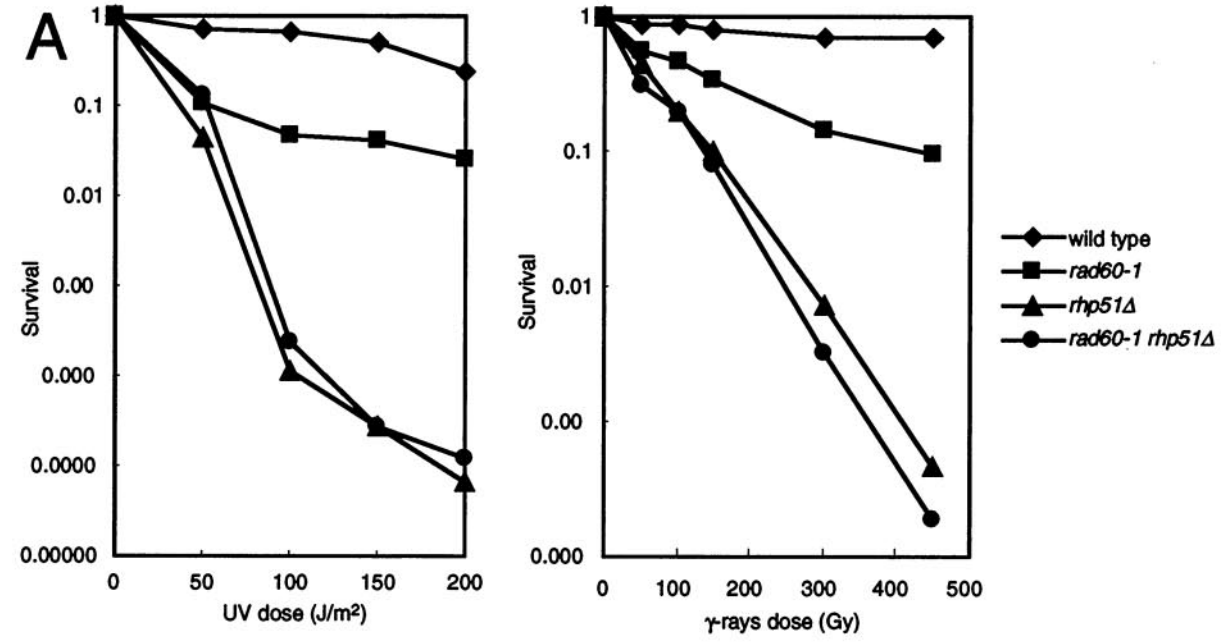
***rad60-1* is epistatic to *rhp51*.** Homologous recombination is a major pathway for the repair of radiation-induced DSBs in *S. pombe*. *rhp51*, the *S. pombe* ortholog of *S. cerevisiae RAD51*, is a key player in this process (19, 33). Therefore, the relationship between *rad60* and *rhp51* was examined. As shown in Fig. 1A, the *rhp51* mutant is more sensitive to UV and γ radiation than is the *rad60-1* mutant, but the *rad60-1 rhp51* double mutant is as sensitive as the single *rhp51* mutant. These results indicate that *rad60-1* is epistatic to *rhp51* with respect to DNA repair.

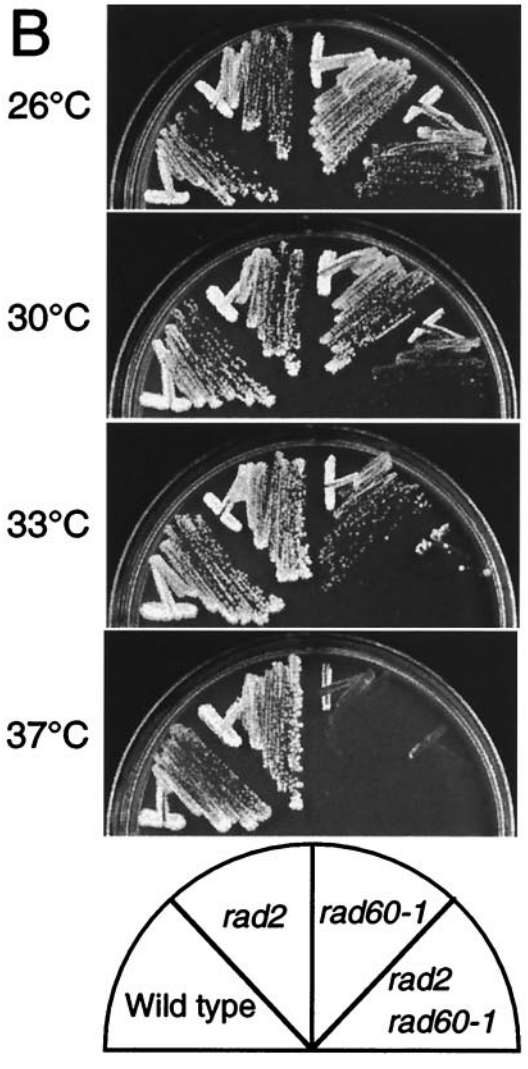
The *rad60-1* mutant is defective in repairing DSBs. *rad60-1* is hypersensitive to MMS and γ rays, suggesting that the *rad60* gene is involved in repairing DSBs. This possibility was tested by directly monitoring the efficiency of DSB repair in this mutant. DSBs were induced by γ-irradiation at 500 Gy, and DNA isolated at various times after irradiation was analyzed by pulsed-field gel electrophoresis. In wild-type cells, fragmented chromosomes were repaired and undamaged chromosomes were detected within 3 h after irradiation; in contrast, fragmented chromosomes were repaired very inefficiently in *rad60-1* cells (Fig. 1C). This result indicates that *rad60-1* cells are defective in repairing DSBs.

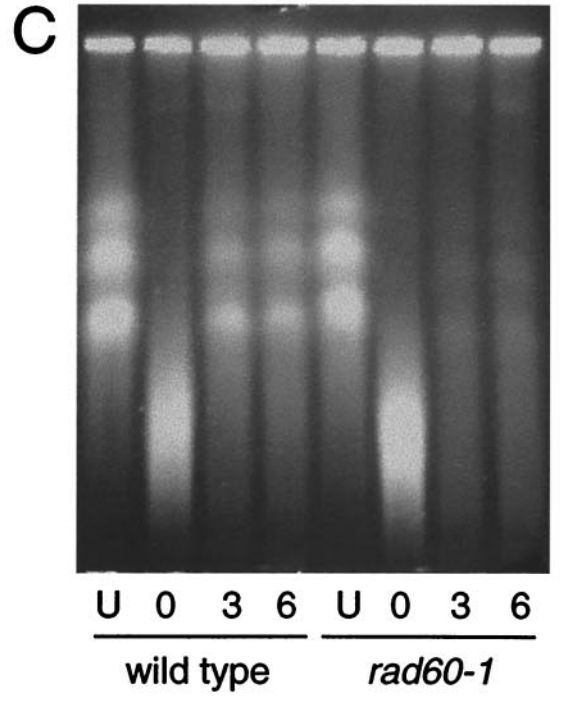
The *rad60-1* mutant is defective in maintaining chromosome structure. Experiments were carried out to determine the cause of the growth defect in the *rad60-1* mutant. *rad60-1* cells were grown logarithmically at 26°C and shifted to the restrictive temperature of 37°C, and cell growth and viability (measured in CFU) were monitored (Fig. 2A). The *rad60-1* mutant stopped growing 8 h after the temperature shift, at which time the cell number had increased 4.7-fold. The number of CFU did not increase appreciably after the temperature shift and started to decrease after approximately 6 h at 37°C. Consequently, after 8 h at the restrictive temperature, more than 80% of the cells lost their ability to form colonies. *rad60-1* cells growing at 26°C or for 8 h at 37°C were fixed, stained with DAPI to visualize chromosomal DNA, and observed under an epifluorescence microscope. Hemisphere morphology of the nuclear chromosomal domain (i.e., interphase morphology) was observed in most of the *rad60-1* cells at 26°C (Fig. 3A). The nuclear chromosomal domain of most of the *rad60-1* cells grown at 37°C was more diffuse than that of cells grown at 26°C (compare Fig. 3A and B), and nearly half of the cells had aberrant morphology (Fig. 3B). In these cells, the chromosomal domain was extended and often dappled. This result suggests that *rad60* is required to maintain proper chromosome structure. In *rad60-1* cells grown for 8 h at 37°C, about 5% had a "cut" phenotype (Fig. 3B), in which a septum forms without complete chromosomal segregation (13), and about 12% had a biased nuclear position which would occur when cut cells detached. Therefore, *rad60-1* appears to be deficient in preventing septum formation when DNA replication and/or chromosomal segregation is incomplete.

The DNA content of *rad60-1* cells was measured in cells incubated for different lengths of time at 37°C by using a fluorescence-activated cell sorter (FACS) (Fig. 2B). The DNA content of most cells was approximately 2C until at least 6 h at the restrictive temperature. At later time points, DNA content increased slightly and the peak of the FACS analysis broadened. This change was probably due to an increase in mitochondrial DNA or in cell size. Most *rad60-1* cells divided several times before arresting growth at 37°C (Fig. 2A), so the 2C DNA content at arrest indicates that DNA synthesis proceeds relatively normally in these cells.

The *rad60-1* mutant accumulates DSBs at the restrictive temperature. Chromosomal DNA in wild-type and *rad60-1* cells was examined by pulsed-field gel electrophoresis after incubation at 37°C (Fig. 2C). In wild-type cells, three chromosomes were detected at 37°C. In contrast, the three chromosomes in *rad60-1* cells decreased in size and became a smeared band of DNA after 6 or 8 h at 37°C. This result indicates that


A
Survival
1
0.1
0.01
0.00
0.000
0.0000
0.00000
0
50
100
150
200
UV dose (J/m²)
Survival
1
0.1
0.01
0.00
0.000
0
100
200
300
400
500
γ-rays dose (Gy)
wild type
rad60-1
rhp51Δ
rad60-1 rhp51Δ


B
26°C
30°C
33°C
37°C
rad2
rad60-1
Wild type
rad2
rad60-1


C
U 0 3 6 U 0 3 6
wild type
rad60-1

DSBs accumulate in *rad60-1* cells at 37°C and that chromosomes become fragmented.

Elevated rate of minichromosome loss in the *rad60-1* mutant. The results presented above suggest that the *rad60-1* mutant is defective in maintaining proper chromosome structure at the restrictive temperature. The role played by *rad60* in chromosome stability was examined further by measuring the rate of loss of the nonessential minichromosome Ch16 in *rad60-1* cells. Ch16 was lost at a much higher rate in *rad60-1* cells (0.17% per generation) than in wild-type cells (<0.005% per generation) at 30°C. Incubation of *rad60-1* cells at 37°C for 6 h caused a fourfold increase in the fraction of cells without Ch16 (data not shown). This result indicates that chromosome loss occurs more frequently in *rad60-1* cells than in wild-type cells and that chromosome loss in *rad60-1* cells is elevated at the restrictive temperature.

Cloning and sequencing of the *rad60* gene. The *rad60* gene was cloned by complementing the MMS sensitivity of the *rad60-1* mutant. *rad60-1* cells were transformed with an *S. pombe* genomic library, and plasmids were recovered from MMS-resistant transformants and characterized in *E. coli*. Three complementing plasmids carried an overlapping genomic region from *S. pombe* (Fig. 4A). The nucleotide sequence of a 2.2-kbp region encompassing an *Mun*I site and an *Nru*I site was determined (Fig. 4D). Putative exons and introns were identified and confirmed by amplifying and analyzing *rad60* cDNA by PCR (Fig. 4D). The cloned region includes a sequence coding for a protein of 406 amino acids with a calculated molecular weight of 46,077. This nucleotide sequence is identical to a portion of cosmid c1920 (accession number AL122033 in EMBL, GenBank, and DDBJ), and the predicted amino acid sequence matches that of SPBC1921.02 (EMBL accession number T397868). No homologous proteins were found by the SSEARCH program of DDBJ (http://www.ddbj.nig.ac.jp/). However, a search for amino acid sequence motifs in the PROSITE profile library (http://motif.genome.ad.jp/) (14) revealed a significant match between amino acids 336 and 406 of Rad60 and the ubiquitin-2 motif (PROSITE ID PS50053) (Fig. 4E).

The *rad60-1* allele and its flanking genomic region were recovered from the *rad60-1* mutant by the eviction method, and the nucleotide sequence was determined. *rad60-1* has a single A-to-G nucleotide change altering the AAA codon for lysine 263 to a GAA codon for glutamic acid. This nucleotide substitution was reconstructed in a chimeric *rad60* gene in which the 0.6-kbp *Not*I-*Sph*I region of the wild-type gene was replaced with the corresponding region of the *rad60-1* gene. The chimeric construct was cloned into the *S. pombe* integrating vector pJK148 and linearized at the unique *Nde*I site in the *leu1* gene. The linear plasmid was transformed into the diploid strain MPDR101, which is heterozygous for *rad60*::*ura4*$^+$, such that the plasmid was integrated into the *leu1* gene. The diploid transformant was sporulated and subjected to tetrad analysis. Leu$^+$ Ura$^+$ segregants were obtained which carry the *rad60*::*ura4*$^+$ allele and the chimeric *rad60-1* construct described above. These cells do not grow at 37°C or in the presence of 0.005% MMS at 26°C. This result demonstrates that the lysine-to-glutamic acid substitution at codon 263 of *rad60* is sufficient to cause the abnormal phenotype of *rad60-1* cells.

***rad60* is an essential gene.** The 0.8-kbp *Xba*I-*Sph*I region containing about two-thirds of the *rad60* coding region was replaced with the DNA fragment containing the *ura4* gene (Fig. 4A), and the resulting DNA fragment was used to disrupt one of the chromosomal *rad60* genes in a diploid strain. The heterozygous diploid cells obtained were sporulated and subjected to tetrad analysis. Twelve asci were dissected and allowed to germinate on YES medium at 30°C, and only two viable segregants arose from each of the 12 asci (Fig. 4B). All 24 viable segregants required uracil for growth, indicating that these segregants did not carry the *rad60*::*ura4*$^+$ allele. These results indicate that *rad60*::*ura4*$^+$ cells are lethal and that the *rad60* gene is essential for growth. Of the 24 nonviable putative *rad60*::*ura4*$^+$ segregants, 21 formed microcolonies of more than 10 cells and then stopped growing (Fig. 4C); the remaining 3 segregants stopped growing at the two-cell stage. One possible explanation for the limited and variable amount of growth of these cells is that residual Rad60 in the mutant spores supports several cycles of cell division. In most of the *rad60*::*ura4*$^+$ cells, the morphology of the nuclear chromosomal domain was aberrant. The following morphologies were observed: amorphously extended (37%; Fig. 5Aa and b), shrunk (17%; Fig. 5Ac), fragmented (9%; Fig. 5Ad), and biased in position (27%; Fig. 5Ae). The latter morphology could result from the detachment of cut cells. This nuclear morphology appears to be similar to but more extreme than the morphology of *rad60-1* cells at 37°C.

Rad60 protein is localized to the nucleus. Cellular localization of the Rad60 protein was studied by examining *S. pombe* expressing a fusion protein of Rad60 and EGFP. When the EGFP-*rad60* fusion gene was expressed from the *rad60* promoter, no fluorescent signal was detected (data not shown). However, fluorescence was detected when the EGFP-*rad60* fusion gene was expressed from the modified *nmt1* promoter on pREP42 (2, 29). In the absence of thiamine, the EGFP signal colocalized with DAPI-stained chromosomal DNA (Fig. 5B). The EGFP-*rad60* fusion gene produces a functional protein that complements the MMS sensitivity and temperature sensitivity of *rad60-1* cells (data not shown). Thus, these results indicate that Rad60 protein is localized to the nucleus and suggest that Rad60 carries out its biological function in the nucleus.

Evidence that the *rad60* gene is functionally related to the

FIG. 1. The *rad60-1* mutant is defective in DSB repair. (A) Epistasis between *rad60-1* and *rhp51*Δ. Midexponential phase cells were irradiated with UV or γ rays at the indicated doses, and relative plating efficiencies were determined. (B) Synthetic lethality of *rad60-1* and *rad2*Δ. Cells of wild-type (MPR116), *rad2* (MPR115), *rad60-1* (MPR114), and *rad60-1 rad2* (MPR113) strains were streaked on YES plates. The plates were incubated at the indicated temperature for 6 days and photographed. (C) Repair of DSBs in wild-type and *rad60-1* cells. Genomic DNA of wild-type (MP2) and *rad60-1* (MPR104) strains was subjected to pulsed-field gel electrophoresis. Samples were taken from unirradiated cells (U) or cells incubated in YES medium at 30°C for the indicated number of hours after irradiation with 500 Gy of γ rays.

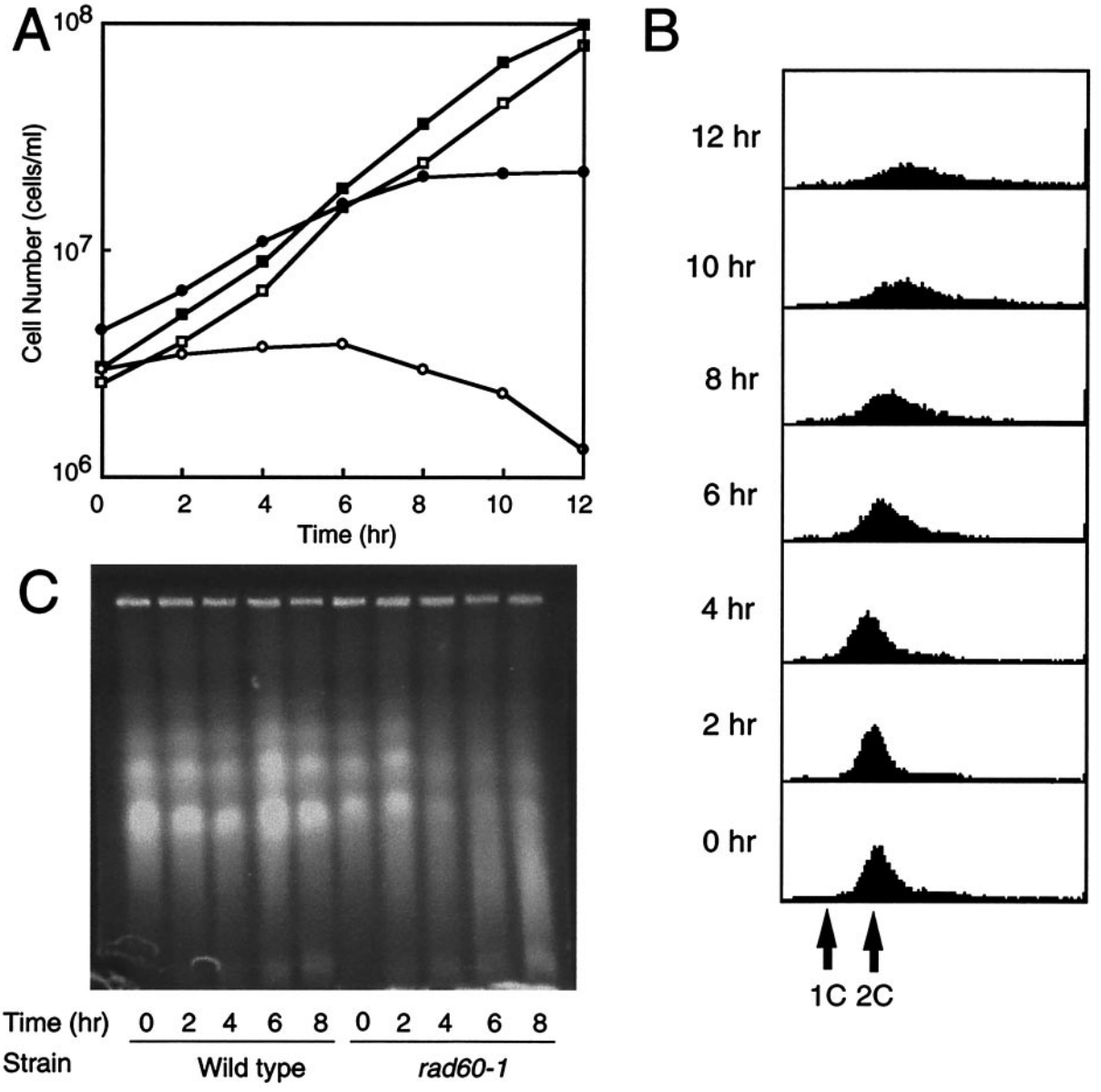


FIG. 2. Growth arrest of *rad60-1* cells at 37°C. Wild-type (MP2) or *rad60-1* (MPR104) cells growing exponentially at 26°C in YES medium were shifted to 37°C. (A) Cell number (filled symbols) and CFU (open symbols) were measured for wild-type (squares) or *rad60-1* (circles) cells at the indicated time points. (B) Cells of the *rad60-1* strain were fixed at the indicated time points, stained with propidium iodide, and processed by FACS to determine DNA content. (C) Genomic DNA of wild-type and *rad60-1* strains was subjected to pulsed-field gel electrophoresis. Samples were taken at the indicated time points.

***rad18* gene.** Two previously identified *S. pombe rad* genes, *rad18* and *rad21*, and *rad60* are required for DSB repair and are essential for growth. The relationship between *rad60*, *rad18*, and *rad21* was examined by constructing double mutants between *rad60* and these two genes. *rad60-1* was crossed with *rad18-X* or *rad21-45*, and the spores were subjected to tetrad analysis at 26°C. In the cross between *rad60-1* and *rad18-X*, the numbers of viable colonies were 4, 3, 2, and 1 for 1, 8, 3, and 0 tetrads, respectively. Each clone with a four-viable-tetrad showed parental ditype segregation, and all four segregants were hypersensitive to MMS. Each of the three-viable-colony tetrads had one wild-type segregant. One of the two-viable-colony tetrads had two wild-type segregants and is likely to be a nonparental ditype with two nonviable *rad60-1 rad18-X* segregants. These results strongly support the conclusion that the *rad60-1 rad18-X* double mutant is nonviable. In contrast, the *rad60-1 rad21-45* double mutant is viable.

When the *rad60* gene was expressed in a *rad18-X* background from a multicopy plasmid, the MMS hypersensitivity of *rad18-X* was partially suppressed (Fig. 6). This result suggests that the *rad60* and *rad18* genes are functionally related. Expression of *rad60* from a multicopy plasmid did not suppress the leaky growth defect of the *rad18-X* mutant at 37°C (data not shown).

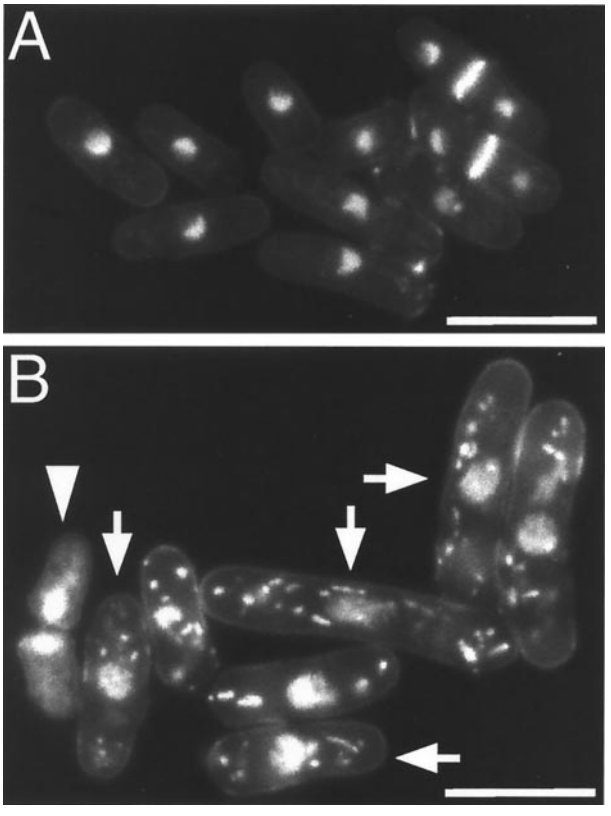


FIG. 3. Nuclear morphology of the *rad60-1* cells at 37°C. *rad60-1* cells growing at 26°C (A) or after 8 h at 37°C (B) were fixed with 2.5% glutaraldehyde and stained with 1 μg of DAPI/ml and 20 μg of calcofluor white/ml. Fluorescence images obtained with an epifluorescence microscope are shown. In panel B, arrows indicate cells with disorganized nuclear morphology and an arrowhead indicates a cut cell. Scale bars, 10μ m.

DISCUSSION

S. pombe radiation-sensitive mutants have mutations in genes involved in nucleotide excision repair, checkpoint control, and recombinational repair (for a review, see reference 25). Earlier studies identified five DNA repair mutants that are encoded by genes that are essential for growth in *S. pombe*: these essential genes are *rad4*/*cut5*, *rad11*, *rad15*, *rad18*, and *rad21*. *rad4*/*cut5* is homologous to *S. cerevisiae DPB11* and is required for DNA replication and checkpoint control (7, 43). *rad11* encodes the largest subunit of replication protein A, which is essential for DNA replication (41). *rad15* is an ortholog of *S. cerevisiae RAD3*, encoding a component of general transcription factor TFIIH which is required for nucleotide excision repair (35, 42). *rad21* is an ortholog of *S. cerevisiae MCD1*/*SCC1*/*RHC21* which encodes a subunit of the cohesin complex (11, 30, 50). *rad18* encodes an SMC family protein (26) which forms a complex with another SMC family protein, Spr18, and five other unidentified proteins (8). This study describes *rad60*, another essential *S. pombe* gene that plays a role in DNA repair.

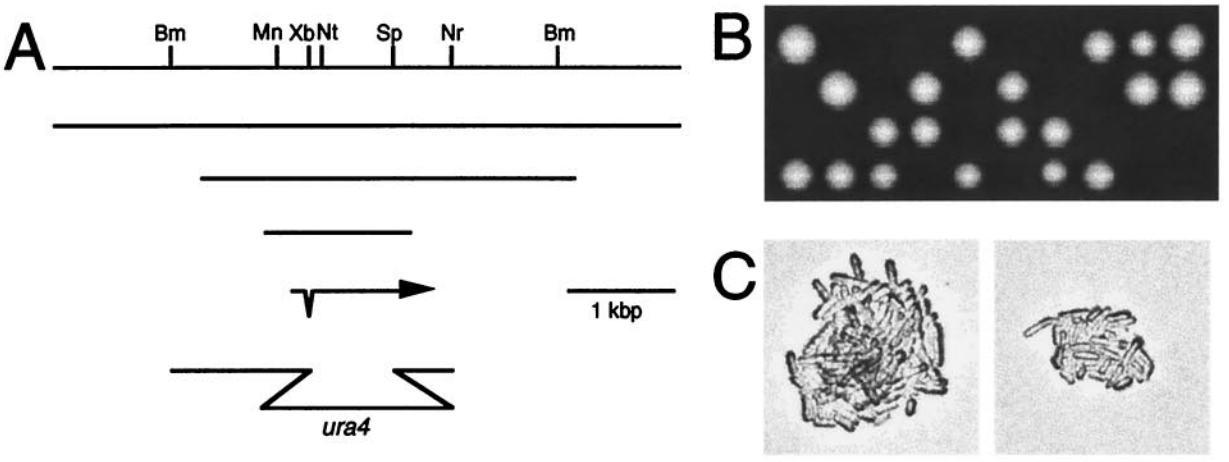
The *rad60-1* mutation changes lysine 263 of the Rad60 protein to a glutamic acid residue. The *rad60-1* mutant is sensitive to MMS and γ rays (Fig. 1A) and synergistically impaired in growth when combined with the *rad2* mutation (Fig. 1B). These phenotypes suggest that *rad60-1* is defective in DSB repair, because γ rays and MMS cause DSBs (4, 16). The *rad2* gene is the *S. pombe* ortholog of the mammalian FEN-1 gene (37). The FEN-1 protein is thought to play a role in Okazaki fragment maturation by removing RNA from the RNA-DNA hybrid (27). In *E. coli* cells, DNA polymerase I encoded by *polA* performs an analogous role, and a *polA recA* double mutant is lethal (10, 31).

It has been postulated that a DSB forms in a *polA* mutant cell when a replication fork reaches a nick or gap in the template DNA, because this mutant has a defect in maturation of Okazaki fragments; it is possible that DNA recombination is required to repair the DSB and to restart replication in the *polA* mutant (24). Consistent with this idea, *recB*, *recG*, and *ruv* mutations are also synthetic lethals with *polA* (15, 17, 18, 31).

An analogous situation might occur in eukaryotic cells. In *S. cerevisiae*, mutations that disrupt the FEN-1 ortholog *RAD27* are synthetically lethal with mutations in genes of the *RAD52* epistasis group (6, 49, 52). In *S. pombe*, the *rad2 rhp51*, *rad2 rhp54*, *rad2 rad32*, and *rad2 rhp57* double mutants are lethal (37, 51, 53). Therefore, the synergistic growth defect of *rad60-1* and *rad2* supports the hypothesis that *rad60* is required for the repair of DSBs caused by the collapse of the replication fork in *S. pombe*.

DSBs in γ-irradiated *rad60-1* cells are not repaired after further incubation (Fig. 1C), and this observation directly shows that *rad60-1* is defective in DSB repair. The phenotype of *rad60-1* also involves an elevated rate of minichromosome loss at 30°C (see "Elevated rate of minichromosome loss in the *rad60-1* mutant" above); this defect may result from the failure to repair spontaneous DSBs in the minichromosome. An increased rate of minichromosome loss has been observed for other *S. pombe* DNA repair-deficient mutants (34, 37, 51, 55).

The fact that *rad60* is essential for growth suggests that *rad60* may play a role in some cellular process other than the repair of DNA lesions caused by exogenous DNA-damaging treatments. *rad60-1* cells stop growing after 8 h at the restrictive temperature of 37°C (Fig. 2A). After 8 h at 37°C, chromosomes become highly fragmented (Fig. 2C) and cells arrest with 2C DNA content (Fig. 2B). These results suggest that in the absence of functional Rad60 protein, spontaneous DSBs occur during replication, remain unrepaired, and cause DNA fragmentation. Nevertheless, DNA replication proceeds relatively normally, with doubling of DNA content to 2C. The nuclear chromosomal domain appears diffuse and has aberrant morphology in the *rad60-1* cells grown at 37°C (Fig. 3B), which may be the consequence of accumulating DSBs. DSBs may interfere with the compaction of interphase chromosomes and cause a diffuse and apparently aberrantly shaped nuclear chromosomal domain to exist. Extensive minichromosome loss (see "Elevated rate of minichromosome loss in the *rad60-1* mutant" above) could also be the consequence of DSBs. Many *rad60-1* cells may undergo checkpoint arrest, because approximately 70% of the cells did not form a septum after 8 h at 37°C, and a small portion of the cells (17%) seem to escape checkpoint arrest, because they develop the morphology of an aberrant mitosis (i.e., cut or biased nuclear position). Spores carrying a deletion of the *rad60* gene germinate and in most cases cease to grow after forming a microcolony of 10 cells or more. DAPI



D

```
   1  atctctaatagtacaacaatttggtttatctgaactaaataaaatttatttagcgaatgaagaacaaattatgaatggcagtaatggacggacgcaatg

 101  tagccgtcgtaaggtgagccaatgtcattgaaattcatcaaacctaatgtctcagtacaagaagcaaagagttttcaaattacttaacctaaaaaatgaa

 201  attctatagacttaaatctgtttgctaagtggacaagatcgggttttgcgtaagtaaacaaacttcatttcgaagatgagttggaggaatgttcattgaa

 301  ctttttcaattgaattataaaattattatataactttcaaaatcgatttgtaataaacttgagtgaaaacaaaactaagctaaaaattacctagtttaag

 401  gtgtgtcgctacaaattctcgttaaatccataagaagctttgtggaaccaccgtaattgctaaaactcgccaaATGGACAACCTAGATGAAGATGACTTA
   1                                                                          M  D  N  L  D  E  D  D  L

 501  GCATTCTTCTCAAAGCCTATTAAAAAGCCTCCATTAAACTATGCCAAGCAATTAATAGCCTCTTCCTCAGATTCTGgtaagttttaattccgattgcaaa
  10  A  F  P  S  K  P  I  K  K  P  P  L  N  Y  A  K  Q  L  I  A  S  S  S  D  S  E

 601  gtgtcagcttgcttacaacattctagAAGAGGAAAGTGAACTTGATACCAATAAACAAGCATTAGAACACATAAATGCACAGAAAAATATAACTCATAAT
  36                                E  E  S  E  L  D  T  N  K  Q  A  L  E  H  I  N  A  Q  K  N  I  T  H  N

 701  GAAAACAAAAGTGCCGAACCTTTATCTCGTCAATCCACTATTTTAGATGCTGATGAAGGCAACCAAGACGTTTCAGACACTACTCCTAATGCGTGTTTGA
  60  E  N  K  S  A  E  P  L  S  R  Q  S  T  I  L  D  A  D  E  G  N  Q  D  V  S  D  T  T  P  N  A  C  L  N

 801  ATGAAGGTAGGCACTCACCCAAAAGTGCTATCTCCTGTGTAACACAACCGGTTTCACCAGTTTATAATACTAGGGCGGCCGCTAACTTGCGAAACAACAG
  94    E  G  R  H  S  P  K  S  A  I  S  C  V  T  Q  P  V  S  P  V  Y  N  T  R  A  A  A  N  L  R  N  N  S

 901  CATTAATAGTGAGGCTGCGCTTTCTACGACTTCATCACTGCTGGACGATGACTTTGCTCGTAGGTTAGAGGAAATTGATCGGCAAGTTCAGGAGTTTGAA
 127   I  N  S  E  A  A  L  S  T  T  S  S  L  L  D  D  D  F  A  R  R  L  E  E  I  D  R  Q  V  Q  E  F  E

1001  AAATCATCAAGTGATATGGATGTACAAATTCACACGCATAAGAGAGAAATTGAAGAAGACGATGACAATACTTCGGCTGATGTGCCCCTTTTAAAGCATT
 160  K  S  S  S  D  M  D  V  Q  I  H  T  H  K  R  E  I  E  E  D  D  D  N  T  S  A  D  V  P  L  L  K  H  S

1101  CAAAAAGTGACCACTCAACGCTGTATCACTCCAAATCGGAATTCTCAACAAATGAACCAGTTATTTCCGTCGTTCTCCAGTTAGCCGTAATTGGACAGAG
 194    K  S  D  H  S  T  L  Y  H  S  K  S  E  F  S  T  N  E  P  V  I  S  V  V  L  Q  L  A  V  I  G  Q  R

1201  AATTCCAAACAGCAATATCTCTCTTCCCCGGGACTGGGAGGCACCGTTATTTTTCAAAGTTAAGTCGAACCAACAGTTTCGTCGTGTCAGAATAGCGTAC
 227   I  P  N  S  N  I  S  L  P  R  D  W  E  A  P  L  F  F  K  V  K  S  N  Q  Q  F  R  R  V  R  I  A  Y
                 G(rad60-1)
1301  TCTGAAAGAAAAAAAGTAGATAACGTTGTTCTTGTATTTCAAAATCAAAGATTATGGGATTATGGAACCCCAAAGGGCGCTGGTATGCTTAAAGTCGATA
 260  S  E  R  K  K  V  D  N  V  V  L  V  F  Q  N  Q  R  L  W  D  Y  G  T  P  K  G  A  G  M  L  K  V  D  T

1401  CCCGTTTGGTTGTGCATGCCTACTGTCACTCGGATTTTATATCACTCAAAAGAATAAAAGAATTGGAAGTGGAAAAGTTATCCTCGGTAACTGAAGATTC
 294    R  L  V  V  H  A  Y  C  H  S  D  F  I  S  L  K  R  I  K  E  L  E  V  E  K  L  S  S  V  T  E  D  S

1501  AACTGCTCAAACGTGTAAACTTATAACGTTGCTTTTGCGTTCGAGTAAGAGTGAGGATCTTCGTCTCTCAATACCCGTCGATTTCACTGTTAAAGATTTG
 327   T  A  Q  T  C  K  L  I  T  L  L  L  R  S  S  K  S  E  D  L  R  L  S  I  P  V  D  F  T  V  K  D  L

1601  ATTAAGAGATATTGTACTGAAGTAAAGATTAGTTTTCATGAACGCATTAGATTAGAATTTGAAGGTGAATGGTTAGATCCCAACGATCAAGTGCAAAGCA
 360  I  K  R  Y  C  T  E  V  K  I  S  F  H  E  R  I  R  L  E  F  E  G  E  W  L  D  P  N  D  Q  V  Q  S  T

1701  CGGAACTTGAAGATGAAGATCAAGTTAGTGTTGTTTTGGATtaaaaggatatttggctcttacttttaccattgttctttcttatatttaattaccgcca
 394    E  L  E  D  E  D  Q  V  S  V  V  L  D

1801  taatacttttatagaatgatcacgaacgactatgtataaatctaacttattcatttcatcaatatgtaccattattaaataaaagtttccatttccccag

1901  tttgtaatttttaaccatatctatctatacacgtcactagaagttcatgaaaatgcttcggctggtataacatttcgcgactttaattccaaaaaagat

2001  gctagagcgctgttgtaatcccaattattttgctcacaacacatgtgagcatagtaatcatttaaacctgtctccgccttaatttttaatacaatttgtt

2101  gtctagtgtctaacattgcttgttccggtcttggctgtacaatatcactaggctttgaagcagacgcagacatggcattattgcttgttcgtacactgct
```

E

```
Rad60          334 ITLLLRSSKSEDLRLSIPVDFTVKDLIKRYCTEVKISFHERIRLEFEGEWLDPNDQVQSTELEDEDQVSVVLD
                   . . ... ..... ...  . ::..: : . .: .   ... :: :::. :. .. . . ..::.. : ..:
Ubiquitin_2      1 MQITVKTLQGQTIEIEVEPSETVQEL-KEHIEEREGVPPDQQRLIFEGKVLEDDQTLSDYGIEDGBTVHLMLRQRGG
```

staining of the *rad60*::*ura4*$^+$ cells revealed the presence of cells with aberrant nuclear morphology, some of which were cut cells or cells with biased nuclear position (Fig. 5A). These arrest phenotypes largely coincide with those of the *rad60-1* cells at 37°C. These observations suggest that *rad60* is required for replication to proceed normally and to prevent the accumulation of spontaneous DSBs.

rad60-1 is a synthetic lethal with *rad18-X*, and overexpression of *rad60* suppresses the MMS sensitivity of *rad18-X* (Fig. 6); these results suggest that there may be a functional link between *rad60* and *rad18*. Both *rad60* and *rad18* are essential for growth, and some *rad* mutants (e.g., *rad60-1*, *rad18-X*, and *rad18-74* mutants) are defective in the repair of DSBs (26, 55). Cells that overexpress dominant-negative *rad18* alleles or have a disruption in *spr18*, an SMC partner of Rad18 (8), and *rad60-1* cells at 37°C often contain aberrant chromosomes or have a cut phenotype. In addition, some *rad18*-disrupted cells show aberrant mitosis (55). *rad60-1*, *rad18-X* (37), and *rad18-74* (55) mutants have a higher rate of minichromosome loss than wild-type *S. pombe*. Both *rad60-1* and *rad18-X* (26) are epistatic to *rhp51*Δ. Therefore, *rad60* and *rad18* appear to act in a cellular process that is required for cell proliferation and DNA repair.

A protein complex has been purified which includes Rad18, Spr18, and five other unidentified proteins (8). Rad60 may be one of the components of this Rad18 complex. We examined the direct interaction between Rad60 and Rad18 proteins by immunoprecipitation experiments. Epitope-tagged Rad60 and Rad18 proteins were overexpressed in wild-type cells, but no coprecipitaion of Rad60 and Rad18 by either antibody was observed even though each protein was efficiently precipitated (data not shown). Moreover, no interaction was detected between Rad60 and Rad18 by two-hybrid analysis. Therefore, Rad60 and Rad18 are not likely to be directly associated. It is still possible that Rad60 is included in the Rad18-Spr18 complex described by Fousteri and Lehmann (8).

The *brc1* gene has been isolated as a multicopy suppressor of *rad18-74* (55). Cells with a deletion of *brc1* have a partial defect in chromosome segregation, and double *brc1*Δ and *rad18-X* or *rad18-74* mutants are lethal, suggesting a functional relationship between *rad18* and *brc1* (55). It has been proposed that *brc1* plays a role downstream of *rad18* and is needed for a subfunction of *rad18*. However, the functional relationship between *brc1* and *rad60* remains to be elucidated.

As discussed above, *rad60* and *rad18* appear to participate in the same cellular function, but the exact nature of that function is not clear. *rad18* encodes an SMC family protein, and proteins in this family form protein complexes that may regulate higher-order chromosome structure. Examples of these protein complexes are the cohesin, condensin, and dosage compensation complexes (12, 48). Fousteri and Lehmann propose that the Rad18/Spr18 complex may be analogous to these complexes. They suggest that the Rad18/Spr18 complex may facilitate recombinational repair by holding and/or bringing together damaged DNA and allowing the Rhp51 protein to initiate strand exchange (8). This model explains the defect of *rad18* mutants in DNA repair.

In addition to their role in DNA repair, both *rad60* and *rad18* are essential for growth. The *rad60-1* mutant causes DSBs at the restrictive temperature in cells not exposed to exogenous DNA-damaging agents. Therefore, *rad60* appears to play a role in repairing spontaneous DSBs that occur during DNA replication. By analogy to SMC complexes, Rad60 and Rad18 may be required to hold newly replicated DNA duplexes together at a collapsed replication fork to facilitate efficient recombinational repair. In *E. coli*, when a DSB occurs at a stalled replication fork, homologous recombination is required to repair the DSB and assemble an alternative primosome (24). In vertebrate cells, *RAD51* is essential for growth (54), and chicken DT40 cells with a conditional *RAD51* transgene accumulate chromosomal breaks when *RAD51* expression is suppressed (47). This result suggests that DSBs occur during normal cell cycle progression and that the recombination machinery is needed to repair them to avoid their lethality. The *S. pombe RAD51* homolog *rhp51* is not essential for growth; however, *S. pombe rhp51*Δ cells grow slowly even in the absence of exogenous DNA-damaging agents (34). Therefore, a similar situation is likely to exist for *S. pombe*. In particular, a stalled replication fork could lead to DSBs in dividing *S. pombe* cells, and *rhp51*Δ cells may be partially impaired in repairing DSBs and reassembling the replication fork. In contrast to *rhp51*Δ cells, which are viable, *rad60*Δ and *rad18*Δ cells are lethal. It is possible that an inefficient *rhp51*-independent recombination pathway repairs the breaks at the stalled replication fork in the absence of *rhp51*. In contrast, *rad60*Δ and *rad18*Δ cells might be defective in *rhp51*-dependent and *rhp51*-independent recombination pathways, and these cells might not survive the spontaneous DSBs that occur during normal DNA replication.

The above model does not explain the fact that a small fraction of cut cells are observed in the *rad60-1* mutant at 37°C. Verkade et al. (55) isolated a *rad18* mutant, *rad18-74*, which is defective in the maintenance of checkpoint arrest

FIG. 4. Physical map of the *rad60* genomic region and disruption and nucleotide sequence of the *rad60* gene. (A) Restriction map of the *rad60* region. Abbreviations for restriction enzymes are as follows: Bm, *Bam*HI; Mn, *Mun*I; Xb, *Xba*I; Nt, *Not*I; Sp, *Sph*I; Nr, *Nru*I. Lines represent three overlapping genomic fragments which complement *rad60-1*. The arrow represents the *rad60* coding region. The single intron is represented by a protrusion downward within the arrow. The 2.6-kbp *Bam*HI-*Nru*I region of the *rad60* gene was used to disrupt the chromosomal *rad60* gene. To prepare this construct, the 0.8-kbp *Xba*I-*Sph*I segment of the coding region was replaced with a 1.8-kbp *Hin*dIII fragment containing the *ura4* gene. (B) The diploid strain MPDR101 (heterozygous for *rad60* disruption) was sporulated and subjected to tetrad analysis. The segregants from the dissected spores were grown on a YES plate at 30°C for 7 days and photographed. (C) Procedures were carried out as described for panel B, with the exception that the segregants from the dissected spores were grown for 2 days. Images of microcolonies of two putative *rad60*::*ura4*$^+$ segregants are shown. (D) Nucleotide and predicted amino acid sequences of the *rad60* gene are shown. The exon sequence is shown in uppercase, and the intron sequence is shown in lowercase. The amino acid sequence with homology to the ubiquitin-2 motif is underlined. (E) The sequence of the C-terminal 73 amino acids of the Rad60 protein was aligned with the ubiquitin-2 motif. Identical amino acids are shown with a double dot, and similar amino acids are shown with a single dot.

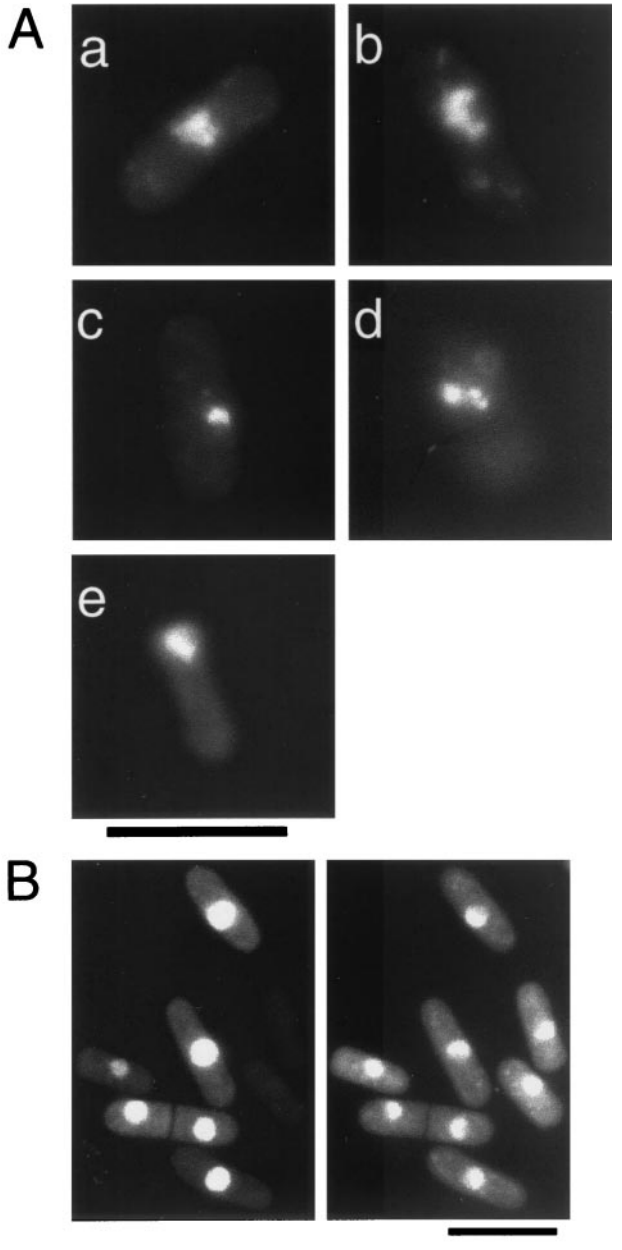


FIG. 5. Nuclear morphology of the cells from the *rad60*Δ microcolonies and cellular localization of the EGFP-Rad60 fusion protein. (A) The diploid strain MPDR101 was sporulated and subjected to tetrad analysis, and segregants from the dissected spores were grown on a YES plate at 30°C for 2 days. Cells of putative *rad60*Δ microcolonies were collected, fixed with 70% ethanol, and stained with DAPI (1 μg/ml). Panels a and b show fluorescence images of representative cells with extended, aberrantly shaped nuclear chromosomal domains. Other aberrant morphologies shown are as follows: shrunk (panel c), fragmented (panel d), and biased in position (panel e). (B) Cells of the wild type (MP11) carrying pErad60 were fixed, stained with DAPI (1 μg/ml), and observed under an epifluorescence microscope. Fluorescence images of EGFP (left) and DAPI (right) are shown. Scale bars, 10 μm.

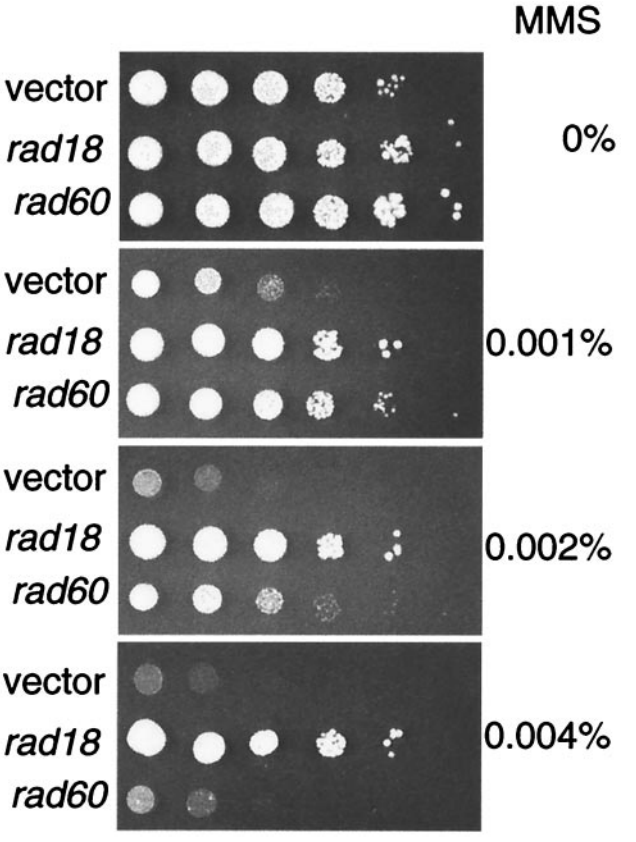


FIG. 6. Suppression of *rad18-X* by a multicopy plasmid expressing *rad60*. *rad18-X* cells (MPR105) carrying the pUR19 vector or pUR19 with the *rad18* or the *rad60* gene were grown to saturation on EMM medium with leucine (200 μg/ml). Serial 10-fold dilutions were spotted on YES plates with the indicated concentrations of MMS. The plates were incubated at 30°C for 3 days and photographed.

after DNA damage. Therefore, in addition to their roles in repairing DNA damage, *rad18* and *rad60* may play roles in maintaining checkpoint arrest until damage is repaired, and the cut cells in *rad60-1* might result from failure to maintain the checkpoint arrest. Several proteins play roles in DNA replication and replication checkpoint response, including replication factor C, Dpb11/Cut5, *S. pombe* DNA polymerase α, and *S. cerevisiae* Orc1 and DNA polymerase ε (22). Therefore, Rad60 and Rad18 could play roles in DNA repair, DNA replication, and checkpoint arrest in response to DNA damage.

Functional and physical interactions between Rad60 and Rad18 should be further explored to better understand their roles in DNA recombination and replication. These proteins may contribute to unique functional features of eukaryotic chromosomes, including maintenance of their complex higher-order structure and organization. It is possible that novel results could emerge from these studies, because these enzymes may help maintain chromosome stability and integrity by mechanisms that are unique to eukaryotic cells.

ACKNOWLEDGMENTS

We thank Osami Niwa and Antony Carr for providing materials and Toshiji Ikeda for γ-irradiation.

This work was supported by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan and by a grant from the Human Frontier Science Program Organizaion (HFSPO) to H.S. Y.T. was supported by a Re-

search Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

REFERENCES

1. **Barbet, N., W. J. Muriel, and A. M. Carr.** 1992. Versatile shuttle vectors and genomic libraries for use with *Schizosaccharomyces pombe.* Gene **114:**59–66.
2. **Basi, G., E. Schmid, and K. Maundrell.** 1993. TATA box mutations in the *Schizosaccharomyces pombe nmt1* promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. Gene **123:**131–136.
3. **Birkenbihl, R. P., and S. Subramani.** 1992. Cloning and characterization of *rad21*, an essential gene of *Schizosaccharomyces pombe* involved in DNA double-strand-break repair. Nucleic Acids Res. **20:**6605–6611.
4. **Chlebowicz, E., and W. J. Jachymczyk.** 1979. Repair of MMS-induced DNA double-strand breaks in haploid cells of *Saccharomyces cerevisiae*, which requires the presence of a duplicate genome. Mol. Gen. Genet. **167:**279–286.
5. **Craven, R. A., D. J. Griffiths, K. S. Sheldrick, R. E. Randall, I. M. Hagan, and A. M. Carr.** 1998. Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe.* Gene **221:**59–68.
6. **Debrauwere, H., S. Loeillet, W. Lin, J. Lopes, and A. Nicolas.** 2001. Links between replication and recombination in *Saccharomyces cerevisiae*: a hypersensitive requirement for homologous recombination in the absence of Rad27 activity. Proc. Natl. Acad. Sci. USA **98:**8263–8269.
7. **Fenech, M., A. M. Carr, J. Murray, F. Z. Watts, and A. R. Lehmann.** 1991. Cloning and characterization of the *rad4* gene of *Schizosaccharomyces pombe*; a gene showing short regions of sequence similarity to the human *XRCC1* gene. Nucleic Acids Res. **19:**6737–6741.
8. **Fousteri, M. I., and A. R. Lehmann.** 2000. A novel SMC protein complex in *Schizosaccharomyces pombe* contains the Rad18 DNA repair protein. EMBO J. **19:**1691–1702.
9. **Game, J. C.** 2000. The *Saccharomyces* repair genes at the end of the century. Mutat. Res. **451:**277–293.
10. **Gross, J. D., J. Grunstein, and E. M. Witkin.** 1971. Inviability of *recA*$^-$ derivatives of the DNA polymerase mutant of De Lucia and Cairns. J. Mol. Biol. **58:**631–634.
11. **Guacci, V., D. Koshland, and A. Strunnikov.** 1997. A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of *MCD1* in *S. cerevisiae*. Cell **91:**47–57.
12. **Hirano, T.** 1999. SMC-mediated chromosome mechanics: a conserved scheme from bacteria to vertebrates? Genes Dev. **13:**11–19.
13. **Hirano, T., S. Funahashi, T. Uemura, and M. Yanagida.** 1986. Isolation and characterization of *Schizosaccharomyces pombe cut* mutants that block nuclear division but not cytokinesis. EMBO J. **5:**2973–2979.
14. **Hofmann, K., P. Bucher, L. Falquet, and A. Bairoch.** 1999. The PROSITE database, its status in 1999. Nucleic Acids Res. **27:**215–219.
15. **Hong, X., G. W. Cadwell, and T. Kogoma.** 1995. Escherichia coli RecG and RecA proteins in R-loop formation. EMBO J. **14:**2385–2392.
16. **Iliakis, G.** 1991. The role of DNA double strand breaks in ionizing radiation-induced killing of eukaryotic cells. Bioessays **13:**641–648.
17. **Ishioka, K., A. Fukuoh, H. Iwasaki, A. Nakata, and H. Shinagawa.** 1998. Abortive recombination in *Escherichia coli ruv* mutants blocks chromosome partitioning. Genes Cells **3:**209–220.
18. **Ishioka, K., H. Iwasaki, and H. Shinagawa.** 1997. Roles of the *recG* gene product of *Escherichia coli* in recombination repair: effects of the delta *recG* mutation on cell division and chromosome partition. Genes Genet. Syst. **72:**91–99.
19. **Jang, Y. K., Y. H. Jin, E. M. Kim, F. Fabre, S. H. Hong, and S. D. Park.** 1994. Cloning and sequence analysis of *rhp51*$^+$, a *Schizosaccharomyces pombe* homolog of the *Saccharomyces cerevisiae RAD51* gene. Gene **142:** 207–211.
20. **Kanaar, R., J. H. Hoeijmakers, and D. C. van Gent.** 1998. Molecular mechanisms of DNA double strand break repair. Trends Cell Biol. **8:**483–489.
21. **Keeney, J. B., and J. D. Boeke.** 1994. Efficient targeted integration at *leu1-32* and *ura4-294* in *Schizosaccharomyces pombe.* Genetics **136:**849–856.
22. **Kelly, T. J., and G. W. Brown.** 2000. Regulation of chromosome replication. Annu. Rev. Biochem. **69:**829–880.
23. **Khasanov, F. K., G. V. Savchenko, E. V. Bashkirova, V. G. Korolev, W. D. Heyer, and V. I. Bashkirov.** 1999. A new recombinational DNA repair gene from *Schizosaccharomyces pombe* with homology to *Escherichia coli* RecA. Genetics **152:**1557–1572.
24. **Kogoma, T.** 1997. Stable DNA replication: interplay between DNA replication, homologous recombination, and transcription. Microbiol. Mol. Biol. Rev. **61:**212–238.
25. **Lehmann, A. R.** 1996. Molecular biology of DNA repair in the fission yeast *Schizosaccharomyces pombe.* Mutat. Res. **363:**147–161.
26. **Lehmann, A. R., M. Walicka, D. J. Griffiths, J. M. Murray, F. Z. Watts, S. McCready, and A. M. Carr.** 1995. The *rad18* gene of *Schizosaccharomyces pombe* defines a new subgroup of the SMC superfamily involved in DNA repair. Mol. Cell. Biol. **15:**7067–7080.
27. **Lieber, M. R.** 1997. The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. Bioessays **19:**233–240.
28. **Matsumoto, T., S. Murakami, O. Niwa, and M. Yanagida.** 1990. Construction and characterization of centric circular and acentric linear chromosomes in fission yeast. Curr. Genet. **18:**323–330.
29. **Maundrell, K.** 1993. Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene **123:**127–130.
30. **Michaelis, C., R. Ciosk, and K. Nasmyth.** 1997. Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell **91:**35–45.
31. **Monk, M., and J. Kinross.** 1972. Conditional lethality of *recA* and *recB* derivatives of a strain of *Escherichia coli* K-12 with a temperature-sensitive deoxyribonucleic acid polymerase I. J. Bacteriol. **109:**971–978.
32. **Moreno, S., A. Klar, and P. Nurse.** 1991. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe.* Methods Enzymol. **194:**795–823.
33. **Muris, D. F., K. Vreeken, A. M. Carr, B. C. Broughton, A. R. Lehmann, P. H. Lohman, and A. Pastink.** 1993. Cloning the *RAD51* homologue of *Schizosaccharomyces pombe.* Nucleic Acids Res. **21:**4586–4591.
34. **Muris, D. F., K. Vreeken, A. M. Carr, J. M. Murray, C. Smit, P. H. Lohman, and A. Pastink.** 1996. Isolation of the *Schizosaccharomyces pombe RAD54* homologue, *rhp54*$^+$, a gene involved in the repair of radiation damage and replication fidelity. J. Cell Sci. **109:**73–81.
35. **Murray, J. M., C. L. Doe, P. Schenk, A. M. Carr, A. R. Lehmann, and F. Z. Watts.** 1992. Cloning and characterisation of the *S. pombe rad15* gene, a homologue to the *S. cerevisiae RAD3* and human *ERCC2* genes. Nucleic Acids Res. **20:**2673–2678.
36. **Murray, J. M., H. D. Lindsay, C. A. Munday, and A. M. Carr.** 1997. Role of *Schizosaccharomyces pombe* RecQ homolog, recombination, and checkpoint genes in UV damage tolerance. Mol. Cell. Biol. **17:**6868–6875.
37. **Murray, J. M., M. Tavassoli, R. al-Harithy, K. S. Sheldrick, A. R. Lehmann, A. M. Carr, and F. Z. Watts.** 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.
38. **Nabeshima, K., S. Saitoh, and M. Yanagida.** 1997. Use of green fluorescent protein for intracellular protein localization in living fission yeast cells. Methods Enzymol. **283:**459–471.
39. **Niwa, O., T. Matsumoto, and M. Yanagida.** 1986. Construction of a mini-chromosome by deletion and its mitotic and meiotic behaviour in fission yeast. Mol. Gen. Genet. **203:**397–405.
40. **Ostermann, K., A. Lorentz, and H. Schmidt.** 1993. The fission yeast *rad22* gene, having a function in mating-type switching and repair of DNA damages, encodes a protein homolog to Rad52 of *Saccharomyces cerevisiae*. Nucleic Acids Res. **21:**5940–5944.
41. **Parker, A. E., R. K. Clyne, A. M. Carr, and T. J. Kelly.** 1997. The *Schizosaccharomyces pombe rad11*$^+$ gene encodes the large subunit of replication protein A. Mol. Cell. Biol. **17:**2381–2390.
42. **Reynolds, P. R., S. Biggar, L. Prakash, and S. Prakash.** 1992. The *Schizosaccharomyces pombe rhp3*$^+$ gene required for DNA repair and cell viability is functionally interchangeable with the *RAD3* gene of *Saccharomyces cerevisiae.* Nucleic Acids Res. **20:**2327–2334.
43. **Saka, Y., and M. Yanagida.** 1993. Fission yeast *cut5*$^+$, required for S phase onset and M phase restraint, is identical to the radiation-damage repair gene *rad4*$^+$. Cell **74:**383–393.
44. **Shinohara, A., and T. Ogawa.** 1995. Homologous recombination and the roles of double-strand breaks. Trends Biochem. Sci. **20:**387–391.
45. **Siede, W., A. A. Friedl, I. Dianova, F. Eckardt-Schupp, and E. C. Friedberg.** 1996. The *Saccharomyces cerevisiae* Ku autoantigen homologue affects radiosensitivity only in the absence of homologous recombination. Genetics **142:**91–102.
46. **Smith, C. L., T. Matsumoto, O. Niwa, S. Klco, J. B. Fan, M. Yanagida, and C. R. Cantor.** 1987. An electrophoretic karyotype for *Schizosaccharomyces pombe* by pulsed field gel electrophoresis. Nucleic Acids Res. **15:**4481–4489.
47. **Sonoda, E., M. S. Sasaki, J. M. Buerstedde, O. Bezzubova, A. Shinohara, H. Ogawa, M. Takata, Y. Yamaguchi-Iwai, and S. Takeda.** 1998. Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. EMBO J. **17:**598–608.
48. **Strunnikov, A. V., and R. Jessberger.** 1999. Structural maintenance of chromosomes (SMC) proteins: conserved molecular properties for multiple biological functions. Eur. J. Biochem. **263:**6–13.
49. **Symington, L. S.** 1998. Homologous recombination is required for the viability of *rad27* mutants. Nucleic Acids Res. **26:**5589–5595.
50. **Tatebayashi, K., J. Kato, and H. Ikeda.** 1998. Isolation of a *Schizosaccharomyces pombe rad21*ts mutant that is aberrant in chromosome segregation, microtubule function, DNA repair and sensitive to hydroxyurea: possible involvement of Rad21 in ubiquitin-mediated proteolysis. Genetics **148:**49–57.
51. **Tavassoli, M., M. Shayeghi, A. Nasim, and F. Z. Watts.** 1995. Cloning and characterisation of the *Schizosaccharomyces pombe rad32* gene: a gene re-

quired for repair of double strand breaks and recombination. Nucleic Acids Res. **23:**383–388.
52. **Tishkoff, D. X., N. Filosi, G. M. Gaida, and R. D. Kolodner.** 1997. A novel mutation avoidance mechanism dependent on *S. cerevisiae RAD27* is distinct from DNA mismatch repair. Cell **88:**253–263.
53. **Tsutsui, Y., T. Morishita, H. Iwasaki, H. Toh, and H. Shinagawa.** 2000. A recombination repair gene of *Schizosaccharomyces pombe*, *rhp57*, is afunctional homolog of the *Saccharomyces cerevisiae RAD57* gene and is phylogenetically related to the human *XRCC3* gene. Genetics **154:**1451–1461.
54. **Tsuzuki, T., Y. Fujii, K. Sakumi, Y. Tominaga, K. Nakao, M. Sekiguchi, A. Matsushiro, Y. Yoshimura, and T. Morita.** 1996. Targeted disruption of the *Rad51* gene leads to lethality in embryonic mice. Proc. Natl. Acad. Sci. USA **93:**6236–6240.
55. **Verkade, H. M., S. J. Bugg, H. D. Lindsay, A. M. Carr, and M. J. O'Connell.** 1999. Rad18 is required for DNA repair and checkpoint responses in fission yeast. Mol. Biol. Cell **10:**2905–2918.
56. **Winston, F., F. Chumley, and G. R. Fink.** 1983. Eviction and transplacement of mutant genes in yeast. Methods Enzymol. **101:**211–228.
57. **Yonemasu, R., S. J. McCready, J. M. Murray, F. Osman, M. Takao, K. Yamamoto, A. R. Lehmann, and A. Yasui.** 1997. Characterization of the alternative excision repair pathway of UV-damaged DNA in *Schizosaccharomyces pombe.* Nucleic Acids Res. **25:**1553–1558.