

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

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The *rad2* mutant of *Schizosaccharomyces pombe* is sensitive to UV irradiation and deficient in the repair of UV damage. In addition, it has a very high degree of chromosome loss and/or nondisjunction. We have cloned the *rad2* gene and have shown it to be a member of the *Saccharomyces cerevisiae* *RAD2/S. pombe rad13*/human *XPG* family. Using degenerate PCR, we have cloned the human homolog of the *rad2* gene. Human cDNA has 55% amino acid sequence identity to the *rad2* gene and is able to complement the UV sensitivity of the *rad2* null mutant. We have thus isolated a novel human gene which is likely to be involved both in controlling the fidelity of chromosome segregation and in the repair of UV-induced DNA damage. Its involvement in two fundamental processes for maintaining chromosomal integrity suggests that it is likely to be an important component of cancer avoidance mechanisms.

The multistep process of carcinogenesis is now well established. The development of a malignant tumor requires a series of mutations and chromosomal rearrangements and often involves missegregation events. Alterations in the p53 tumor suppressor gene have been identified in a large proportion of human cancers. These alterations involve point mutations followed by loss of heterozygosity to permit expression of the mutant allele. Such loss of heterozygosity can be brought about by mitotic recombination, chromosome loss, or nondisjunction. Chromosome loss and nondisjunction are also responsible for a high proportion of fetal abnormalities. Maintenance of the fidelity of chromosome segregation is therefore a process of crucial importance for humans.

In model systems such as the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, genomic instability can be the result of a wide variety of defects. In both yeasts, defects in DNA replication or repair enzymes (9) or in the apparatus that physically segregates the chromosomes can increase the frequency of chromosome loss during mitosis (29), although the exact mechanism by which this occurs is often not clear.

DNA repair processes are also essential for maintaining genomic integrity. Several highly cancer-prone genetic disorders (such as xeroderma pigmentosum) are associated with defects in DNA repair. The mechanisms of DNA repair have been particularly amenable to study in the lower eukaryotes, especially *S. cerevisiae* and *S. pombe*, and these mechanisms have been shown to be highly conserved among eukaryotes.

DNA repair mutants from *S. pombe* have been mapped to approximately 30 complementation groups (2, 28, 31), and recent work has assigned some of these to specific DNA

damage response and repair pathways (1, 6, 19). DNA repair in *S. pombe* is apparently very efficient, as fission yeast can tolerate much higher levels of both UV- and ionizing radiation-induced lesions than can budding yeast. This may reflect, in part, the fact that *S. pombe* spends a large proportion of its cell cycle in G₂, where recombination-based mechanisms could potentially repair much DNA damage. However, there appear to be two excision repair processes for UV photoproducts in *S. pombe*, one of which is conserved in *S. cerevisiae* and human cells. In null alleles of *S. pombe* genes in the conserved pathway, the rate of excision of UV-induced pyrimidine dimers and 6-4 photoproducts, unlike with *S. cerevisiae* and human cells, is only slightly reduced (23). This appears to confirm previous speculation (4) that a second, novel, excision repair pathway exists in this yeast.

In addition to the conserved and the putative additional excision repair pathways, recent work has identified several genes which apparently function in a recombination repair pathway conserved in mammalian and yeast cells (24, 39). A radiation checkpoint pathway in *S. pombe* has also been defined through several mutants which are unable to arrest in G₂ prior to mitosis following DNA damage (1, 34). Many of the fission yeast DNA damage-sensitive mutants have yet to be assigned to specific repair pathways or damage responses.

We screened a selection of DNA damage-sensitive *S. pombe* mutants for an aberrant chromosome segregation phenotype by using a minichromosome loss assay (30). This screen identified the *rad2* mutant as the UV-sensitive mutant with the most severe minichromosome instability phenotype. We have cloned, sequenced, and deleted the *S. pombe rad2* gene. Using the homology between *rad2* and an *S. cerevisiae* open reading frame (ORF) (YKL510) identified by the chromosome XI sequencing project (16), we have identified from human cells a structural homolog which can functionally complement a *rad2* null mutant of *S. pombe*.

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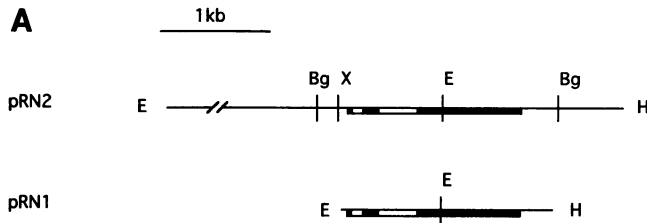


FIG. 1. Molecular analysis of the *rad2* gene. (A) Restriction map. Two clones, pRN1 (1.9-kb insert) and pRN2 (6.4-kb insert) complemented the radiation sensitivity of the *rad2-44* mutant. The restriction maps are shown together with the positions of the ORFs (solid boxes) and introns (open boxes) which comprise the *rad2* gene. Vector-derived restriction sites are shown at either end. Bg, *Bgl*II, E, *Eco*RI, H, *Hind*III, X, *Xba*I. (B) Sequence of the *S. pombe rad2* gene. The introns are shown in lowercase letters, and the predicted translation is shown below in single-letter amino acid code.

B

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-420 gatcttttcgtagtaaaactgcgaggtgatttacatctttacagccgtgctgccacca
-360 aaccacaagaacctcaatttggttaactcttaaaatttctaatgttctctctagaaatcc
-300 ttttttaaagctataatacatgggaattttcagataaaattggctggtccctttccttg
-240 tatttacattctttggtcgatcattctttatggaaaagtaacatgtgatttactgaatcg
-180 actctttgacgtggatactactgctgtagtaagaacctttgtgtgagtgagcaaaagta
-120 aaaactgctcgattttgaggagtaataacatctagcaaaaattgaaaatttaataagaa
-60 gctctaagctagttttggagaaagcgtgttcgggatctcgtaataacataaaagta
ATGGGAATTAAGGtatgttacatcttaacgctaagagtttatcttttactctctcctt 60
M G I K G
acttaaaactaatctgggtagGTTTGGCTCAAGTACTAAGCGAGCATGCCCCAGCCAGT 120
L A Q V L S E H A P A S
GTCAACATAATGATATTA AAAATTATTTGGACGGAAAGTGGCTATAGATGCgtaagtt 180
V K H N D I K N Y F G R K V A I D A
gaaagactaaaatagaagcgaagctcagttgacttggcaaagactgtatggcttctga 240
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S M S L Y Q F L I Q V R S Q D
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G Q Q L M N E Q G E T T S H L M G M F Y
CGTACACTCCGAATTGTCGATAATGGAATAAAGCCTTGCTTTGCTTTGATGGAAAACCA 720
R T L R I V D N G I K P C F V F D G K P
CCAACATTGAAGTCAGGGGAATTAGCTAAACGTGTGGCCGACATCAAAAAGCACGAGAG 780
P T L K S G E L A K R V A R H Q K A R E
GATCAAGAGGAGACAAAAGAGGTTGGTACAGCTGAAATGGTGGATCGGTTTGCCAAACGT 840
D Q E E T K E V G T A E M V D R F A K R
ACGGTCAAAGTTACTCGGCAACATAATGACGAAGCAAAAACGTTTACTAGAGTTAATGGGA 900
T V K V T R Q H N D E A K R L L E L M G
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I P F V N A P C E A E A Q C A A L A R S
GGAAAAGTTTATGCTGCTGCTTCTGAGGACATGGACACATTGTGCTTCCAAGCTCCGTGA 1020
G K V Y A A A S E D M D T L C F Q A P V
CTTTTGAGACACCTGACATTTAGTGAGCAAAGAAAGGAACCAATTAGTGAGTATAACATA 1080
L L R H L T F S E Q R K E P I S E Y N I
GAAAAGCATTGAATGGTTTAGATATGTCGGTGGAGCAGTTTGTAGACTTGTGTATTCTT 1140
E K A L N G L D M S V E Q F V D L C I L
TTGGGTTGTGACTATTGTGAACCTATTCGGGGAGTTGGCCCCGCTAGAGCCGTCGAGTTA 1200
L G C D Y C E P I R G V G P A R A V E L
ATTAGGCAATACGGGACTCTCGATCGCTTTGTCAAAGAGGCGGATCGATCAAAAATATCCT 1260
I R Q Y G T L D R F V K E A D R S K Y P
ATTCGGAAGATTGGCCTTATGAAGATGCCAGAAGACTGTTTTTGGATGCAGAAGTACTT 1320
I P E D W P Y E D A R R L F L D A E V L
CCTGGTGAAGAGATTGAGTTGAAATGGAAAAGTCCCGATGCAGATGGCATTATACAATTT 1380
P G E E I E L K W K S P D A D G I I Q F
CTGGTTAAGGAAAAGGTTTCAATGAAGATCGTGTAAATGGGGATCAATAGACTAGAA 1440
L V K E K G F N E D R V K L G I N R L E
AAAGCCTCAAAGACAATCCACAGGGTCGTCTTGATTCCTTTTTTAAACAGTCCCTTCT 1500
K A S K T I P Q G R L D S F F K P V P S
TCTCCAAAAAACCTGTAGATACAAAGGATCAGCAAAAAGGAAAAGAGATTCA 1560
S P K K P V D T K S K G S A K R K R D S
AATAAGGGTGGTGAAGCAAGAAAAGCGTTGAAAacgaagtcattgtaattgttattaat 1620
N K G G E S K K R *
ctaaaaaaaaaacttaaaaaagcaagacgcatgtagataaaaactatactacgctataa 1680
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aaaaaactctggctgatacaactcaaaaaacgaaaagcagtggtatcggacgatggatca 1860
aagatc
    
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TABLE 1. Strains used in this study

Strain	Genotype
sp011.....	<i>ade6-704 ura4-D18 leu1-32 h⁻</i>
sp012.....	<i>ade6-704 ura4-D18 leu1-32 h⁺</i>
sp058.....	<i>rad2-44 ade6-704 ura4-D18 leu1-32 h⁺</i>
sp175.....	<i>rad2::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁺ diploid</i>
sp176.....	<i>rad2::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁺ diploid</i>
sp177.....	<i>rad2::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁺ diploid</i>
sp178.....	<i>rad2::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁺ diploid</i>
sp217.....	<i>rad2::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁻</i> (rad2-d)
sp218.....	<i>rad2::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁺</i>
sp210.....	<i>rad2::ura4⁺ rad13::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁺</i> (rad2-d rad13-d)
sp222.....	<i>rad13::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁻</i> (rad13-d)
sp227.....	<i>rad2::ura4⁺ rad9::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁺</i> (rad2-d rad9-d)
sp229.....	<i>rad2::ura4⁺ rad8::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁺</i> (rad2-d rad8-d)
sp226.....	<i>rhp51::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁺</i> (rhp51-d)
sp189.....	<i>rad8::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁻</i> (rad8-d)
sp097.....	<i>rad9::ura4⁺ ade6-704 ura4-D18 leu1-32 h⁻</i> (rad9-d)
sp1235.....	<i>Ch16(ade6-M216) ade6-M210 ura4-D18 leu1-32 h⁻</i>
sp1236.....	<i>Ch16(ade6-M216) ade6-M210 ura4-D18 leu1-32 h⁺</i>
spA1.....	<i>rad2-44 Ch16(ade6-M216) ade6-M210 ura4-D18</i>
sp1245.....	<i>rad2::ura4⁺ Ch16(ade6-M216) ade6-M210 ura4-D18</i>
spA2.....	<i>rad4-116 Ch16(ade6-M216) ade6-M210 ura4-D18</i>
sp1158.....	<i>rad8::ura4⁺ Ch16(ade6-M216) ade6-M210 ura4-D18</i>
sp1160.....	<i>rad9::ura4⁺ Ch16(ade6-M216) ade6-M210 ura4-D18</i>
spA3.....	<i>rad11-404 Ch16(ade6-M216) ade6-M210 ura4-D18</i>
spA4.....	<i>rad12-502 Ch16(ade6-M216) ade6-M210 ura4-D18</i>
sp1247.....	<i>rad13::ura4⁺ Ch16(ade6-M216) ade6-M210 ura4-D18</i>
sp1250.....	<i>rad18-X Ch16(ade6-M216) ade6-M210 ura4-D18</i>
sp1164.....	<i>rad21-45 Ch16(ade6-M216) ade6-M210 ura4-D18</i>

MATERIALS AND METHODS

***S. pombe* strains.** The genotypes of the fission yeast strains used in this work are listed in Table 1.

Chromosome segregation assay. A derivative of the Ch16 minichromosome developed by Niwa et al. (30) can be maintained by selection because of the intragenic complementation between the *ade6-M216* allele resident on the minichromosome and the *ade6-M210* allele in the genome. Loss of Ch16 from such cells gives an Ade⁻ phenotype which results in the buildup of a red metabolic intermediate. Such Ade⁻ cells, when grown in limiting concentrations of adenine, yield pink colonies, giving a simple visual screen for chromosome loss. The fidelity of chromosome transmission in nine *S. pombe* *rad* mutants was determined by introducing Ch16 into *rad⁻ ade6-M210* strains by standard genetic methods. A single *ade⁺* spore (giving rise to a single colony) was propagated for 24 generations. At 12 and 24 generations, a sample of cells was plated onto minimal medium containing 10 mg of adenine liter⁻¹ and, after incubation for 5 days, the percentage of colonies staining pink was taken as an estimate of percent chromosome loss. For all minichromosome-containing strains, four independent isolates were tested. Occasionally, one isolate produced a result more than twofold higher than the average. When this occurred, four colonies of this strain were retested. In all cases, the average for the second assay was equivalent to that of the remaining three original strains. This indicates that an early loss event had produced a "jackpot." A new average was calculated by using a representative result from the second experiment. It should be noted that this assay does not distinguish between true chromosome loss and nondisjunction.

Cloning of the *rad2* gene from *S. pombe*. The pURSP1 and pURSP2 fission yeast genomic libraries (3) were used to transform a *rad2-44 ura4-D18* strain to uracil prototrophy. For each library, two pools of approximately 10,000 independent

colonies were subjected to selection for increased survival following UV irradiation as described in our previous work (27). From each library a single complementing plasmid was identified. Restriction analysis indicated that the 1.9-kb insert from plasmid pRN1 from the pURSP1 was fully contained within the 6.4-kb insert from the pRN2 plasmid derived from the pURSP2 library (Fig. 1). Subclones were prepared and tested for complementing activity. The unique *EcoRI* site in pRN2 was found to lie in an essential region.

TABLE 2. Fidelity of minichromosome transmission

<i>rad</i> locus ^a	% Loss/generation ^b	Fold increase	Strain
Wild type	0.005		sp1235
<i>rad2-44</i>	0.38	76	spA1
<i>rad4</i>	0.23	46	spA2
<i>rad8-d</i>	0.004		sp1158
<i>rad9-d</i>	0.04	8	sp1160
<i>rad11</i>	0.19	38	spA3
<i>rad12</i>	0.21	42	spA4
<i>rad13-d</i>	0.1	20	sp1247
<i>rad18-X</i>	0.37	74	sp1250
<i>rad21-d</i>	0.01	2	sp1164
<i>rad2-d</i>	0.63	126	sp1245
<i>rad2-d</i> + pREP42	0.97	199	
<i>rad2-d</i> + pRAD2 ^{SP}	0.02	4	
<i>rad2-d</i> + pR2H	0.0125	2	

^a The chromosome loss frequency of *rad17-d* (null) is similar to that of *rad9-d* and has been previously reported (3). *rad2-d* + pRAD2^{SP}, *rad2* deletion strain carrying a plasmid containing the *nmt1*-promoted *rad2* gene. *rad2d* + pR2H, *rad2* deletion strain carrying the *nmt1*-promoted human gene. *rad2d* + pREP1, *rad2* deletion strain carrying the control *nmt1* promoter plasmid. Plasmid experiments were performed in the presence of thiamine, which results in modest basal level expression from the *nmt* promoter.

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

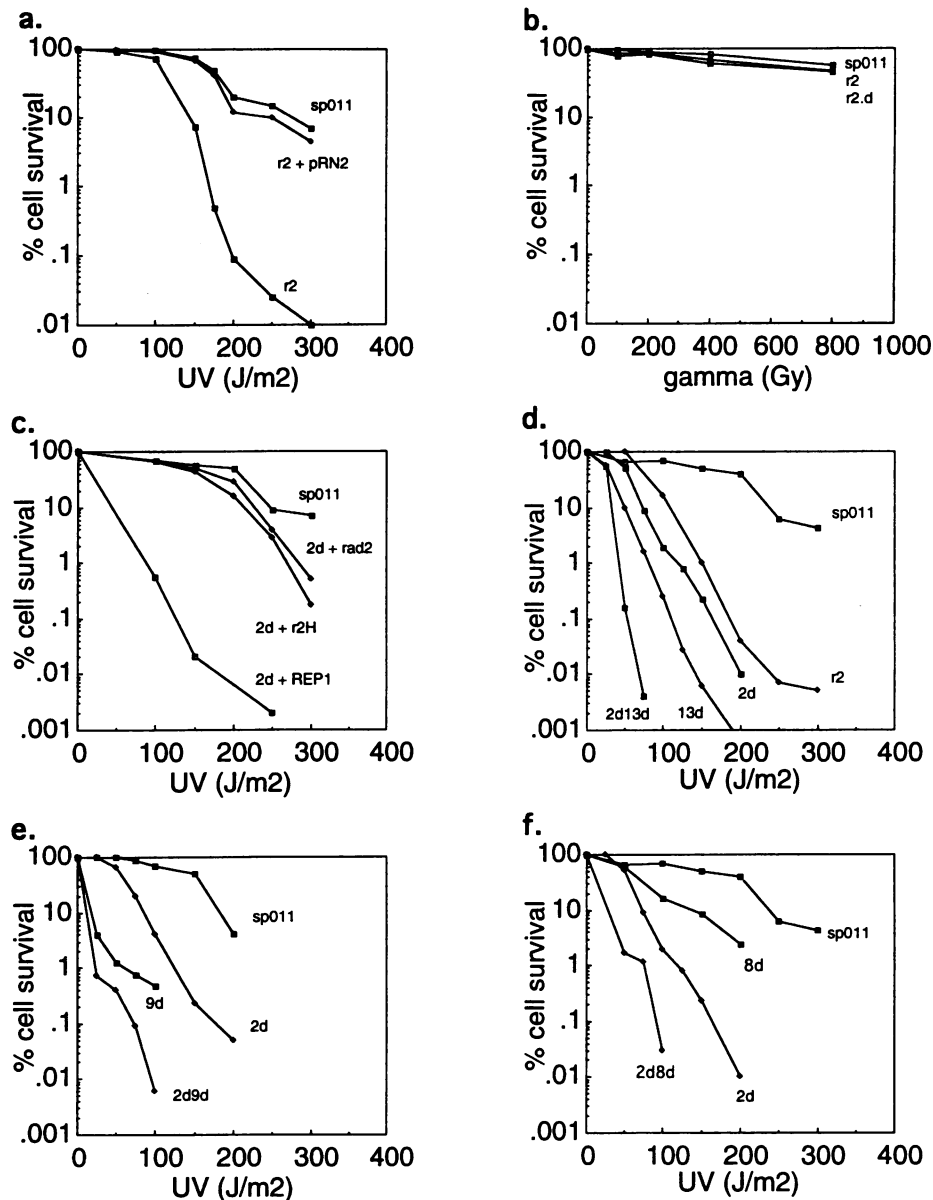


FIG. 2. Radiation survival. (a to c) Complementation of *rad2* mutants. (a) The plasmid pRN2 complements the UV sensitivity of the *rad2-44* mutant to a level approaching that of wild-type survival. (b) Gamma radiation sensitivities of the *rad2-44* and *rad2::ura4* (null) mutants are similar to the sensitivity seen in wild-type cells. (c) The human homolog complements the UV sensitivity of the *rad2::ura4* (null) mutant to approximately wild-type levels. 2d+*rad2*, *rad2* deletion strain carrying a plasmid containing the *nmt1*-promoted *rad2* gene. 2d+r2H, *rad2* deletion strain carrying the *nmt1*-promoted human gene. 2d+REP1, *rad2* deletion strain carrying the control *nmt1* promoter plasmid. Thiamine was maintained in the media throughout the experiment, ensuring moderate basal levels of transcription from the *nmt1* promoter (high levels of *rad2* expression are lethal). (d to f) Epistasis analysis. *rad2::ura4* double mutants with *rad13-d* (d), *rad9-d* (e), and *rad8-d* (f) are all more sensitive than the respective single mutants.

Cloning of the human homolog of *rad2*. Four primers derived from conserved regions of the *S. pombe rad2* and *S. cerevisiae YKL510* gene pair (marked on Fig. 4A) were used to PCR amplify human cDNA made by priming HeLa cell polyA⁺ RNA with oligo(dT) and by extending synthesis with reverse transcriptase in the presence of 1 mM concentrations of each of the four deoxynucleoside triphosphates (dNTPs). Primers R2-1 (TTYGGIAGRAARGTIGCIATHGAYGC) and R2-4 (CCYCHTATIGGYTCRCARTARTCRCA) were used in 20 cycles of 1 min at 94°C, 2 min at 53°C, and 3 min at

70°C. Two separate 1- μ l samples of this reaction mixture were then used in 20 cycles under the same conditions either with primers R2-3 (CATRTCYTICISWIGCIGCIGCRTA) and R2-1 or with primers R2-2 (AARCCITGYTTYGTYYGAYGG) and R2-4. Bands of the expected size were seen after amplification with both these sets of semi-internal primers (420 bp with R2-1 and R2-3 and 450 bp with R2-2 and R2-4). A third round of amplification was then carried out by using 1- μ l samples of these two reaction mixtures in 20 cycles under the same conditions with the internal primers R2-2 and R2-3. A

band of the expected size (270 bp) was seen after amplification of the 450-bp R2-2/R2-4 sample. DNA from this third PCR reaction mixture was purified and cloned into a pGEM5+-based T vector (18) and was subjected to DNA sequence analysis. From the R2-2/R2-3 fragment a single ORF with 64% amino acid identity with the *S. pombe rad2* gene was identified. This fragment was used to isolate six full-length cDNA clones from a pDR2 human cDNA library (25, 41) by standard high-stringency colony hybridization techniques (35).

DNA sequence analysis. The *rad2* insert from pRN1 was cloned into M13 in both orientations, and one clone was subjected to exonuclease III deletions as described by Henikoff (13). Overlapping deletions were sequenced and aligned by computer analysis. The reverse strand was sequenced by using synthetic oligonucleotide primers. A further 400 bp of sequence 5' of the ORF was obtained from sequence analysis of the *Bgl*II fragment from pRN2. The *rad2* gene was identified as a 380-amino-acid coding sequence generated by splicing together three exons. The two introns were confirmed by sequence analysis of a PCR product generated from *S. pombe* cDNA by using primer pairs R2A (GATCTCGTAATATACATAAATAAGTATATT) and R2B (GCATCTATAGCCACTTTCCGTCCAA) and R2A and R2C (ACGTTTAGCTAATCCCCTGACTTCA).

The sequence of the human homolog was determined as follows: two independently isolated full-length human cDNAs were excised from the respective pDR2 clones with *Xba*I and *Bam*HI, and the resulting fragments (two from each clone) were subcloned into M13. Overlapping deletions were constructed and sequenced as described above. Sequence data for each strand were derived from separate isolates. The sequence across the internal *Bam*HI site was confirmed by subcloning a *Hind*III-*Xba*I fragment into M13. A single ORF which could encode a product with 55% identity with the *S. pombe rad2* gene was identified. There were no differences in sequence within the coding region between the two independent isolates. The two cDNA clones started at the same base pair, but contained different-length poly(G·C) tails, indicating that they were derived from separate cloning events (as opposed to library amplification). The remaining four clones were shown by restriction mapping to be of similar lengths.

Gene deletion of *rad2*. In order to create a null allele of *rad2*, a DNA construct was generated whereby the entire coding region of the *rad2* gene, which is flanked by two *Bgl*II sites, was replaced by the *ura4*⁺ gene by the method described by Barbet et al. (3). A linear *Bam*HI-*Pst*I fragment was used in a one-step gene deletion experiment with diploid cells. Four stable integrants were shown, by Southern blot hybridization, to contain a single *ura4*⁺ insert at one of the two chromosomal *rad2* loci. Sporulation of these diploid strains followed by tetrad analysis demonstrated that each tetrad yielded four haploid colonies, two *rad2*⁻ *ura*⁺ and two *rad2*⁺ *ura*⁻. Genetic analysis mapped the site of the deletion to the same locus as that of the original *rad2-44* mutation (38), demonstrating that the *rad2* gene had been cloned. The *rad2* gene has also been physically mapped to chromosome I by hybridization to an ordered array of cosmid clones (15).

Subcloning into expression vectors. The *rad2* cDNA was amplified by PCR using an *S. pombe* cDNA library with the primers R2-Nde (TAACATATGGGAATTAAAGGTTTGGCT) and R2-Sal (TTAGTCGACTTCGTTTTCAACGCTTTT). The amplified product was cloned into a T vector and subcloned into the pREP1 expression vector (22). The human homolog of the *rad2* gene was subcloned, by using the flanking *Nco*I and *Sal*I sites, into pREP1N, a derivative of pREP1 containing an *Nco*I site in place of the published *Nde*I site.

Radiation survival experiments. For survival analysis, cells were grown to mid-log phase in the appropriate medium and were plated at a cell density of 10³ per plate on the same medium plus agar. Plates were irradiated by using a Stratagene Stratalinker, and colonies were counted and compared with unirradiated controls after 3 to 4 days. Plasmid-containing strains were kept under the appropriate selection. Thiamine was maintained in the medium (resulting in a low level of transcription) during complementation experiments with the human gene in the pREP1 expression vector.

RNA analysis. The *Bgl*II fragment of pRN2 was used as a probe for Northern (RNA) blot analysis of total *S. pombe* RNA, and the *Nco*I-*Sal*I fragment of human cDNA was used to probe a Northern blot prepared by using total human RNA. Probes were prepared by random priming in the presence of [³²P]dCTP (8).

Protein analysis. A synthetic peptide (Alta Bioscience, Birmingham, United Kingdom) corresponding to amino acids 338 to 446 (QGRDLSFFK) in a conserved region of the *rad2* protein (see Fig. 4A) was coupled to thyroglobulin with glutaraldehyde (12) and was used to raise polyclonal antibodies. Antiserum was affinity purified on columns containing peptide coupled to Affinity gel 10 (Biorad).

Immunofluorescence microscopy using the anti-Rad2p antibody on formaldehyde-fixed cells was as described by Hagan and Hyams (11). DAPI (4',6-diamidino-2-phenylindole) staining was as described by Al-Khodairy and Carr (1). Procedures for Western blotting (immunoblotting) were as described by Harlow and Lane (12). The antigen was detected by using horseradish peroxidase-conjugated secondary antibody and the ECL detection system (Amersham).

RESULTS

Chromosome stability in *rad* mutants. We have tested nine *S. pombe rad* mutants for the fidelity of chromosome segregation by using a minichromosome-loss assay (30). In this assay, loss of the minichromosome results in adenine auxotrophy and the subsequent development of pink colonies on plates containing limiting quantities of adenine. Of the mutants tested (Table 2) the *rad2-44* mutant showed the highest level of chromosome loss, at approximately 76 times the loss rate of an isogenic wild-type strain. A deletion strain of *rad2* (see below) had an even higher rate of minichromosome loss, some 126-fold greater than in the wild-type strain.

Cloning and sequencing of *rad2*. The *S. pombe rad2-44* mutant is moderately sensitive to UV radiation (Fig. 2a) but is not significantly sensitive to gamma radiation (Fig. 2b). In order to further investigate the properties of the *rad2* mutant, we have cloned the corresponding gene by complementation of the UV sensitivity of the *rad2-44* mutant. Two overlapping clones were isolated, the smaller one (1.9 kb) being contained entirely within the larger clone (6.4 kb) (Fig. 1A). A unique *Eco*RI site was found by functional analysis to be in a region essential for the complementing activity. The DNA fragment from the small clone and a 400-bp region from the larger clone were sequenced in both directions (Fig. 1B). Computer analysis identified a single ORF which spanned the *Eco*RI site. A screen against the Swiss Prot data base identified this ORF as 55% identical to an *S. cerevisiae* ORF identified in the Chromosome XI sequencing project (16). The identity could be extended further by postulating two introns (69 and 380 bp) which linked two further ORFs to the ORF spanning the *Eco*RI site. The existence of these introns has been confirmed by sequence analysis of a PCR product generated from cDNA. The exon-intron structure is unusual in that the first exon

A

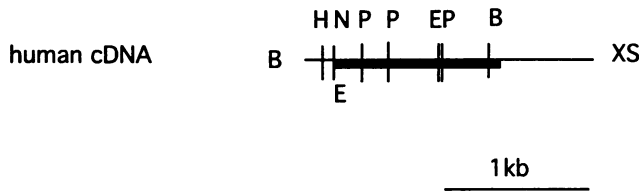


FIG. 3. Molecular analysis of the human homolog of *rad2*. (A) Restriction map. Two independent cDNA clones were isolated by colony hybridization. The ORF is indicated as a solid bar. Vector-derived restriction sites are shown at either end. B, *Bam*HI, E, *Eco*RI, H, *Hind*III, P, *Pst*I, S, *Sal*I, X, *Xba*I, N, *Nco*I. (B) Sequence of the human homolog to *rad2*. The predicted translation product is shown below in single-letter amino acid code. A polyA⁺ tail is seen at the end of the sequence (data not shown). The sequence context of the ATG is consistent with the mammalian consensus.

B

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-367                                     agtcctg
-360 cgatttcggtgtagaggagcaggggctgcgggacctgggtggtgggtggagtgggacaagcg
-300 gtggagaagggtacgccagggtcgtgagagactctgttctccctggagggtggttgc
-240 catgagagcagccgtctgaggggacgcagcctgcactacgcgcccccaagaggctgtgctg
-180 ggcgagcaggtcacgtgacgggagcgcgggctttggaagggcggtgaacgtcaggccacc
-120 cgccgctaagctgagaaggagagcgagcttaggaccgctgccccggggcaacccccgaac
-60 caagcttttagccgagggccgctgtcccaaaggccagtcacccctcctctgtgtgtgcc
ATGGGAATTC AAGGCCTGGCCAAACTAATGTCTGATGTGGCCCCAGTGCCATCCGGGAG 60
M G I Q G L A K L I A D V A P S A I R E
AATGACATCAAGAGCTACTTTGGCCGTAAGTGGCCATTGATGCCTCTATGACATTTAT 120
N D I K S Y F G R K V A I D A S M S I Y
CAGTTCCTGATGTCTGTTCCGAGGGTGGGGATGTGCTGCAGAATGAGGAGGGTGAGACC 180
Q F L I A V R Q G G D V L Q N E E G E T
ACCAGCCACTGATGGGCATGTTCTACCGCACCATTTCGCATGATGGAGAACGGCATCAAG 240
T S H L M G M F Y R T I R M M E N G I K
CCCGTGTATGCTTTGATGGCAAGCCGCCACAGCTCAAGTCAGGCGAGCTGGCCAAACGC 300
P V Y V F D G K P P Q L K S G E L A K R
AGTYAGCGCGGGCTGAGGCAGAGAAGCAGCTGCAGCAGGCTCAGGCTGCTGGGCGCGAG 360
S E R R A E A E K Q L Q Q A Q A A G A E
CAGGAGGTGGAATACTAAGCGGCTGGTGAAGGTCACTAAGCAGCACAAATGATGAG 420
Q E V E K F T K R L V K V T K Q H N D E
TGCAAATCATCTGCTGAGCCTCATGGGCATCCCTTATCTTGATGCACCCAGTGAGCAGAG 480
C K H L L S L M G I P Y L D A P S E A E
GCCAGCTGTGCTGCCCTGGTGAAGGCTGGCAAAGTCTATGCTGCGGCTACCGAGGACATG 540
A S C A A L V K A G K V Y A A A T E D M
GACTCCCTCACCTTCGGCAGCCCTGTGCTAATGCGACACCTGACTGCCAGTGAAGCCAAA 600
D C L T F G S P V L M R H L T A S E A K
AAGCTGCCAATCCAGGAATCCACCTGAGCCGGATTCTGCAGGAGCTGGGCCGTAACCAG 660
K L P I Q E F H L S R I L Q E L G L N Q
GAACAGTTTGTGGATCTGTGCACTCTGCTAGGTCAGTACTACTGTGAGAGTATCCGGGT 720
E Q F V D L C I L L G S D Y C E S I R G
ATTGGGCCAAGCGGGCTGTGGACCTCATCCAGAAGCACAAGAGCATCGAGGAGATCGTG 780
I G P K R A V D L I Q K H K S I E E I V
CGGCACTTGACCCCAACAAGTACCCTGTGCCAGAAAATTGGCTCCACAAGGAGCTCAC 840
R R L D P N K Y P V P E N W L H K E A H
CAGCTCTTCTTGAACCTGAGGTGCTGGACCCAGAGTCTGTGGAGCTGAAGTGGAGCGAG 900
Q L F L E P E V L D P E S V E L K W S E
CCAAATGAAGAAGAGCTGATCAAGTTCATGTGTGGTGAAGAAGCAGTTCTCTGAGGAGCGA 960
P N E E E L I K F M C G E K Q F S E E R
ATCCGAGTGGGGTCAAGAGGCTGAGTAAGAGCCGCCAAGGCAGCACCCAGGGCCGCTG 1020
I R S G V K R L S K S R Q G S T Q G R L
GATGATTTCTCAAGGTGACCGGCTCACTCTTTCAGCTAAGCGCAAGGAGCCAGAACCC 1080
D D F F K V T G S L S S A K R K E P E P
AAGGGATCCACTAAGAAGAAGGCAAAGACTGGGGCAGCAGGGAAGTTTAAAAGGGGAAA 1140
K G S T K K K A K T G A A G K F K R G K
TAAatgtgtttccccattatacctccttcacccagaatatttgccgtctttagaccctta 1200
*
agagctacagctagagaaaccttcacggggtggagagaggattctaaggcttttctagcg 1260
tgacccttttcagtagtgtagtccctttttacttgatcttaatggcaagaaggccaca 1320
gaggtactttccttttttagctcaggaataatgtcagggtcaaaccttctcagcg 1380
agtttaatggacactaagtccattgtttacatgaaagtgatagatagcaacaagttttggg 1440
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caactggtggccagtgagggtgatggtggacctagactgtgcttttctgtctgtttcag 1560
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tgagagaagatgggagaaagctggagccctggagttggctgtgtctgtgttctgtgact 1680
gattactggctgtgtcttgggtgggagaaactcgaacttgctatgtaatttgtgtctag 1740
ttattcagaggagtaagaatggtgatgttcacctggcaatcagctgagttgagactttgga 1800
ataagacactggttttcatgctgtgtttttgttttaagttatgaagaaaaaagtcaata 1860
aaattctaaaagtaacc 1877
    
```

A

Human	MGIQGLAKLIADVAPSAIRENDIKSYFGRKVAIDASMSIYQFLIAVR-QGGDVLQNEEGE
rad2	...K...QVLSEH..ASVKH...N.....L.....Q...S.D.QQ.M..Q..
YKL510	...K..NAI..SEHV.....KS...F.....L.....Q.D.GQ.T..A..
	R2-1->
Human	TTSHLMGMFYRTIRMMENGIKPVYVFDGKPPQLKSGELAKRSERRAEAEKQLQQAAGA
rad2L.IVD.....CF.....T.....VA.HQK.REDQEETKEV.T
YKL510L..ID.....C.....D...H..T...S..V.T..K.---AE.TT
	R2-2->
Human	EQEVEKFTRKRLVKVTKQHNDCKHLLSLMGIPYLDAPSEAEASCAALVKAGKVYAAATED
rad2	AEM.DR.A..T...R....A.R..E.....FVN..C....Q....ARS.....S..
YKL510	.L.KM.QER.....S.E..E.AQK..G.....II..T....Q..E.A.K.....S..
	<-R2-3
Human	MDCLTFGSPVLMRHLTASEAKKLP IQEFHLSRILQELGLNQEQFVDLCILLGSDYCESIR
rad2	..T.C.QA...L....F..QR.E..S.YNIEKA.NG.DMSV.....C...P..
YKL510	..T.CYRT.F.L....F.....E..H.IDTELV.RG.D.TI.....M..C.....
	<-R2-4
Human	GIGPKRAVDLIQKHKSIEEIVRRL-----DPNKYPVPENWLHKEAHQLFLEPEVLDPEVS
RAD2	.V..A...E..RQYGTLDRF..KEA-----RS...I..D.PYED.RR...DA...PG.EI
YKL510	.V..VT.LK..KT.G...K..EFIESGESNNT.WKI..D.PY.Q.RM...D...I.GNEI
Human	ELKWSEPNEEELIKFMCGEKQFSEERIRSGVKRLSKSRQGSTQGRLLDDFFKVTGSLSSAK
rad2	...KS.DADGI.Q.LVK..G.N.D.VKL.IN..E.ASKTIP.....S...PVP.-.PK.
YKL510	N...P.K.K...EYL.DD.K....VK..IS..K.GLKSGI.....G..Q.VPK-TKEQ
	Antibody
Human	RKEPEPKGSTKKKAKTGAAGFKRKG.
rad2	PVDTKS...A.R.RDSNKG.ES.KKR.
YKL510	LAAAAKRAQEN..LNKNKNKVT.GRR.

B

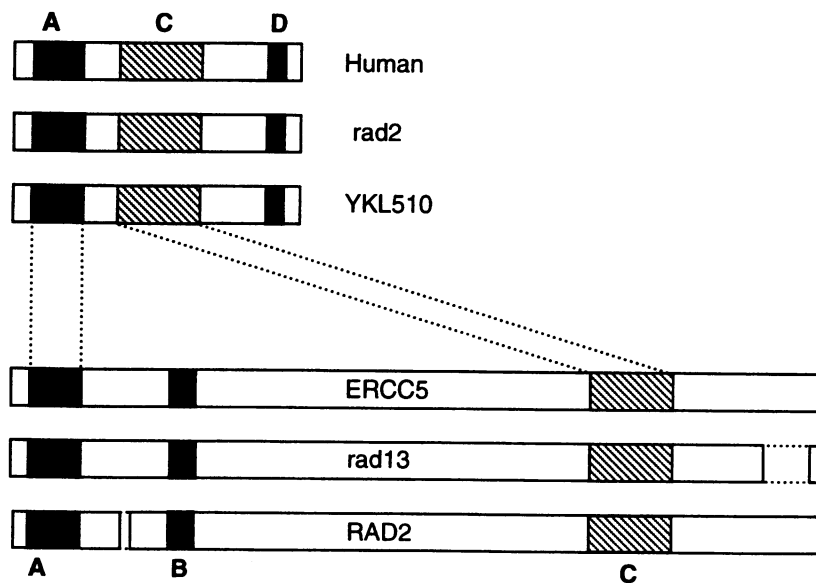


FIG. 4. Sequence comparisons. (A) Alignment of the predicted products of *rad2*, *YKL510*, and the human homolog. The *S. cerevisiae* *YKL510* ORF and *S. pombe* *rad2* are shown aligned to the human homolog. Dots indicate residues identical to the human gene product. The identities are *rad2*/*YKL510*, 56%; human/*rad2*, 55%; and human/*YKL510*, 58%. The regions corresponding to the primers used to identify the human gene are marked, and directionality is indicated by an arrowhead. The peptide used to raise antibodies is also indicated. The conserved regions corresponding to domains A, C, and D in panel B are highlighted in boldface on the sequence alignment. (B) The structural comparison of the *rad2*/*YKL510*/human gene products with the *rad13*/*RAD2*/*ERCC5* subfamily are shown, with shaded blocks representing conserved domains.

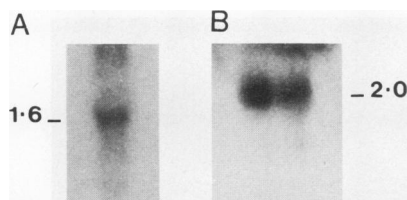


FIG. 5. Northern blot analysis (A) *S. pombe rad2*. The *Bgl*III fragment from pRN2 (Fig. 1A) was used to probe a Northern blot of 5 μ g of total mRNA. A single band was detected at 1.6 kb. (B) The *Bam*HI fragment from human cDNA was probed against a Northern blot of 10 μ g and 5 μ g of total mRNA. A single transcript was detected at 2.0 kb.

contains only 5 amino acids and the second intron contains 380 bases and has a structure related to large introns in *S. pombe* (32). The predicted product of the complete gene is a 380-amino acid protein with a molecular mass of 42.8 kDa, a pI of 8.68, and 56.6% identity with the *S. cerevisiae* YKL510 ORF (see Fig. 4). In addition to the identity with the YKL510 ORF, a less pronounced but significant homology is observed with the *S. pombe rad13*, *S. cerevisiae* RAD2, and human XPG (ERCC5) genes. This sequence similarity is concentrated in two domains and has been previously reported (6).

Gene deletion of *rad2*. In order to determine whether the *rad2* gene is essential for cell viability, the entire *rad2* gene was deleted from diploid cells and was replaced by a *ura4*⁺ marker. Tetrad analysis of spores derived from four such diploids (strains sp175 to sp178) demonstrated that the *rad2* gene from *S. pombe* is inessential for cell proliferation and results in sensitivity to UV radiation (Fig. 2a) but not to gamma radiation (Fig. 2b). The *rad2* null mutant was tested in the chromosome loss assay. A 126-fold increase in loss of the minichromosome, approximately one and a half times the loss rate of the *rad2-44* mutant, was reproducibly seen.

Epistasis analysis. We have previously reported that in the *rad2-44* and *rad2-d* strains the rate of removal of cyclobutane and 6-4 photodimers is reduced, as is the case for mutants of the *rad13*, *rad15*, and *rad16* genes, which are homologs of the *S. cerevisiae* excision repair genes RAD2, RAD3, and RAD1, respectively (5, 6, 27). In order to determine whether the *S. pombe rad2* gene functions in a discrete DNA repair pathway, we undertook a limited epistasis analysis by constructing double mutants with other *S. pombe rad* mutants. In contrast to a double excision repair mutant (for example, *rad13 rad16*), the *rad2* double mutant with *rad13* was more radiation sensitive than the respective single mutants (Fig. 2d). This indicates that *rad2* functions in a pathway other than the conserved excision repair pathway. Furthermore, double mutants of *rad2* with *rad8* (7) and *rad9* (26) were also more UV sensitive than the single mutants, indicating that *rad2* is involved in a pathway distinct from the G₂ checkpoint pathway (defined by *rad9*) and from the as yet uncharacterized pathway involving *rad8* (Fig. 2e and f). The double mutant with *rhp51*, the *S. pombe* homolog of the *S. cerevisiae* RAD51 recombination repair gene (24), was not viable. This indicates that the *rad2* protein has a role in a complementary pathway. However, as *rad2* null mutants are not significantly sensitive to gamma irradiation (Fig. 2b) (whereas the *rhp51* null mutant is highly sensitive) it seems unlikely that the *rad2* gene acts in a recombination repair pathway.

Cloning of a human homolog to *S. pombe rad2*. The extensive sequence identity between the *S. pombe rad2* gene and the *S. cerevisiae* YKL510 ORF indicates that the *rad2* sequence is

highly conserved through evolution. In order to identify the equivalent human gene, we used a PCR-based strategy utilizing degenerate primers designed to correspond to four of the most highly conserved regions. By using oligo(dT)-primed human cDNA as a template, a novel DNA fragment which was capable of encoding 92 amino acids with 64% identity with the *S. pombe rad2* protein was generated (see Materials and Methods). This fragment was used to identify a full-length cDNA by hybridization to a human cDNA library. Sequence analysis of the entire cDNA (Fig. 3B) revealed a single ORF with a product identical in size (380 amino acids) to the *S. pombe rad2* protein, with 55% amino acid sequence identity with it (Fig. 4A), and a similar pI (8.62). The sequence identity is most pronounced in two domains also found in the related proteins *S. cerevisiae* Rad2p (21), *S. pombe* Rad13p (6), and human ERCC5/XPG (20, 37) (Fig. 4B). Sequence comparison between Rad2p, YKL510, and the human protein (Fig. 4A) reveals a 68-amino-acid domain (corresponding to domain A in Rad13p) from amino acids 29 to 97, with 76% identity (90% conservation) between all three proteins. A second domain of 117 amino acids (equivalent to domain C in Rad13p), from amino acids 127 to 244, shows a 57% identity (79% conservation). A third short domain (D) of nine amino acids (with no obvious equivalent in Rad13p) is also evident. The C-terminal 30% of the proteins is less well conserved, but the extreme C terminus is highly basic and contains consensus sequences for a bipartite nuclear localization signal (33).

Complementation of the UV sensitivity and chromosome stability phenotypes of the *S. pombe rad2* null allele. Both the human cDNA (cloned into an *S. pombe* expression vector) and the *S. pombe* gene were used to complement the *rad2* null mutant. Radiation survival analysis following UV irradiation showed that both genes could restore the radiation sensitivity to approximately wild-type levels (Fig. 2c). Following transformation of both the human cDNA and the *S. pombe* gene into two independent *rad2::ura4* minichromosome-containing strains, the stability of the minichromosome was restored to

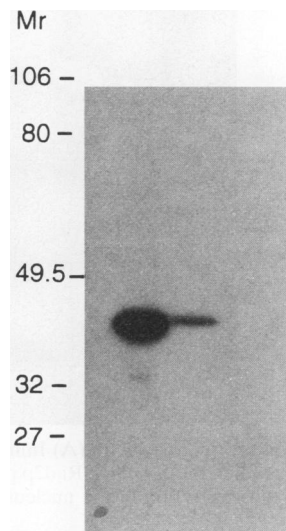


FIG. 6. Overexpression and protein analysis. An immunoblot with anti-Rad2p antibodies shows that the Rad2p protein migrates at 44 kDa following overexpression from the *nmf1* promoter in *S. pombe* cells grown in the absence of thiamine. Whole-cell lysates were separated by electrophoresis on PAGE gels, electroblotted onto nitrocellulose, and detected by anti-Rad2p antibody. Lanes 1 and 2 represent 10⁷ and 10⁶ *S. pombe* cells, respectively.

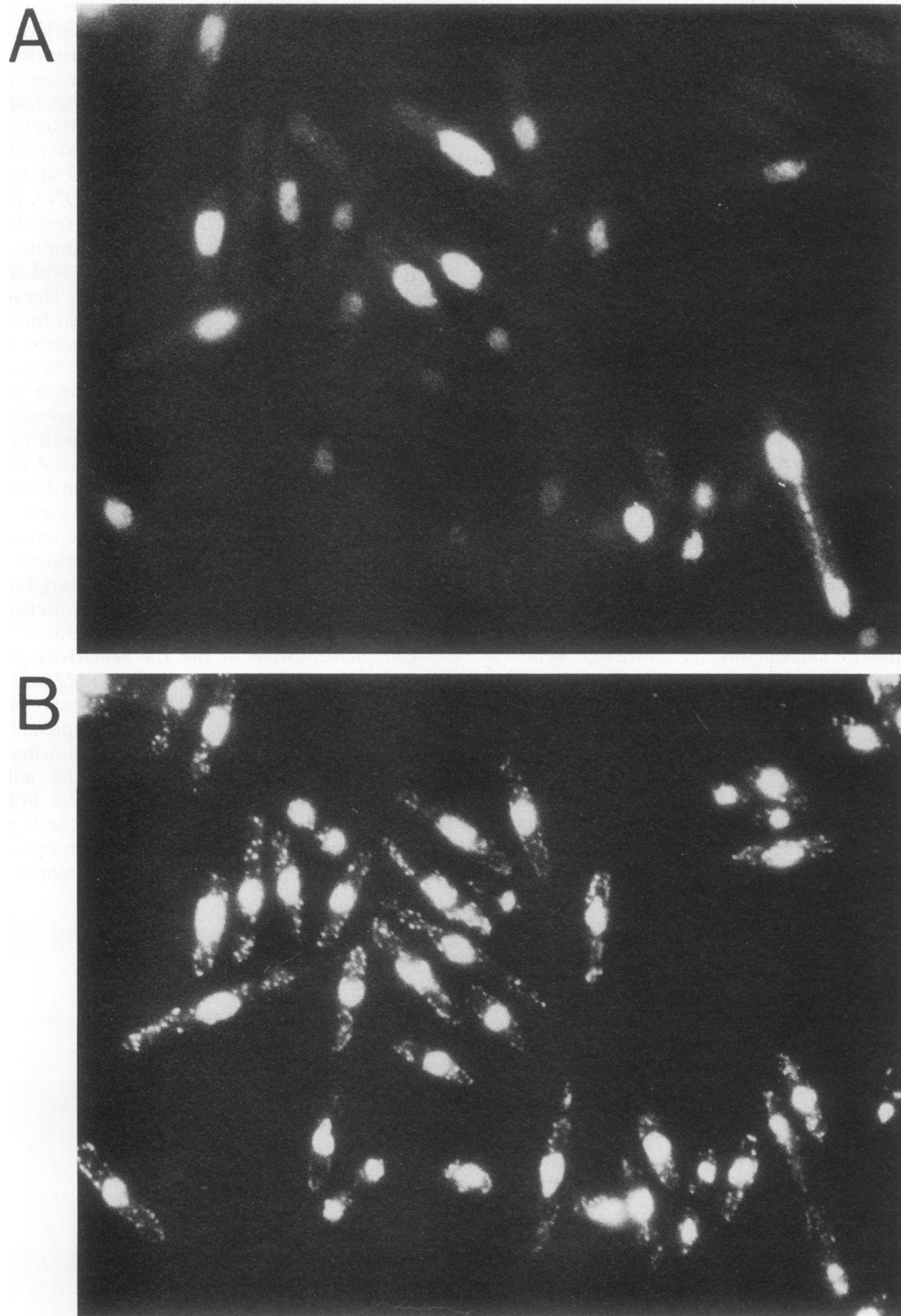


FIG. 7. Localization of Rad2p protein. (A) Immunofluorescence staining using anti-Rad2p antibodies demonstrates that the Rad2p protein is localized to the nucleus when the *S. pombe* Rad2p protein is overexpressed from the *nmt1* promoter in the absence of thiamine. (B) DAPI staining of the same cells shows the position of the nucleus.

approximately wild-type levels. Similar restoration was not seen with vector-only controls (Table 2).

mRNA and protein analysis. Northern blot analyses of total yeast and human RNA indicate that both genes are expressed at moderate levels. The *S. pombe rad2* gene, contained in the

*Bgl*II fragment from pRN2, hybridized to a single transcript from *S. pombe* of approximately 1.6 kb (Fig. 5A) with an intensity similar to that of the *cdc2* gene and considerably higher than that seen with the *rad13* gene. A single transcript of 2.0 kb was observed following hybridization when human

cDNA was probed against a similar quantity of human mRNA (Fig. 5B). This transcript size is consistent with the cDNA sequence reported here.

Anti-Rad2p antibodies were generated by using a peptide corresponding to a potential antigenic site conserved between the three *rad2* homologs (Fig. 4A). The antibody was able to detect the yeast and human proteins in whole-cell extracts, but only when they were overexpressed behind the *nmt1* promoter in fission yeast. Overproduced Rad2p protein in *S. pombe* migrates in denaturing polyacrylamide gel electrophoresis (PAGE) as a protein with a relative molecular mass (Mr) of 44 kDa (Fig. 6), which is consistent with the size predicted from the DNA sequence (43 kDa).

Analysis of cells containing overexpression constructs of the *S. pombe rad2* gene by in situ fluorescence microscopy with anti-Rad2p antibody indicates that Rad2p is localized to the nucleus (Fig. 7). This is consistent with the predicted nuclear localization signal at the C terminus of the protein. Overexpression of the fission yeast gene from the *nmt1* promoter resulted in large elongated cells which were inviable (Fig. 7). This suggests interference with progression through the mitotic cycle. *S. pombe* cells overexpressing the human gene also show nuclear localization of the protein when there is modest overexpression. High levels of overexpression lead to the protein being distributed throughout the cell. This is associated with inviability, fragmentation of the nucleus (as judged by DAPI staining), and an elongated cell phenotype (data not shown).

DISCUSSION

We have isolated the *rad2* gene of *S. pombe* and shown that it encodes a highly conserved protein which is required for the fidelity of chromosome separation at mitosis and which is also involved in the response to DNA damage. As we reported previously (6), the *S. pombe rad2* gene shows limited sequence similarity to the *S. cerevisiae RAD2*, *S. pombe rad13*, and human *XPG (ERCC5)* genes. This sequence similarity is confined to two domains close to the amino and carboxy termini of the proteins (Fig. 4B), suggesting a possible limited functional similarity between these two sets of proteins. It has recently been reported that the product of the *S. cerevisiae RAD2* gene has single-stranded nuclease activity (10). It is possible, therefore, that the conserved domains in the *RAD2* family (Fig. 4B) might be involved in this nuclease activity and that the much smaller *S. pombe rad2* gene product and its homologs might also be nucleases with roles different from those of the larger homologs. At present this suggestion remains totally speculative.

The observation that the *rad2* mutant is defective in the excision of UV-induced photoproducts (23) suggests a role in excision repair for the *rad2* gene product. However, our epistasis analysis (Fig. 2) demonstrates that the role of the *rad2* gene product in the response to UV is unlikely to be exclusively within the conserved excision repair pathway defined by the *rad13* (5), *rad15* (27), and *rad16* (6) mutants (homologs of the *S. cerevisiae RAD2*, *RAD3*, and *RAD1* excision repair genes, respectively). The exact nature of the defect(s) responsible for the UV sensitivity of the *rad2* null mutant are thus as yet uncertain.

Several human genes involved in the response to UV damage have been isolated so far. The *ERCC1*, *XPA*, *XPB (ERCC3)*, *XPC*, *XPD (ERCC2)*, *XPG (ERCC5)*, and *CSB (ERCC6)* genes have been cloned through their ability to complement UV-sensitive rodent or human mutants (14). In addition, two human homologs of the *S. cerevisiae RAD6* gene

have been isolated by low-stringency hybridization techniques (17). In this current work we have used an alternative approach, degenerate primer PCR, and we have succeeded in cloning the human homolog of the *S. pombe rad2* gene.

The high structural conservation of the human gene and its ability to complement the repair defect in the *S. pombe rad2* null mutant make it highly probable that the *S. pombe* and human genes have similar functions. This functional conservation is significant, as the human *XPD (ERCC2)* gene, which shows a level of structural homology similar to that of the *S. cerevisiae RAD3* gene, does not complement the excision repair defect of *S. cerevisiae RAD3* mutants, although it does complement the defect in the essential function of the *RAD3* gene (40). The identification of human genes through their homology to repair genes from model eukaryotes provides a method of identifying repair-related genes which also display other phenotypes (such as loss of the fidelity of chromosome transmission) and which may potentially exhibit a more severe phenotype in higher eukaryotic cells.

There are several possible explanations for the involvement of the *rad2* gene, and by inference its human homolog, in both DNA repair and chromosome segregation. It seems unlikely that this is simply a result of incomplete repair, as the levels of chromosome loss in other repair-deficient cells is much lower than that seen in *rad2-d* mutants. The dual repair and chromosome loss phenotypes of *rad2* mutants may be the result of the same enzymatic function being involved in two different pathways. Alternatively, they may provide another example of DNA repair proteins having two apparently quite different functions. It has recently been shown that the *XPB (ERCC3)* (36) and *XPD (ERCC2)* (42) genes have two distinct roles, in excision repair and as part of the basal transcription factor BTF2/TFIIH.

Our current and future experiments characterizing the *S. pombe rad2* protein and constructing null mutants of the conserved gene in rodent cells using targeted mutagenesis should help to elucidate the precise role of Rad2p and its human homolog in DNA repair and chromosome segregation.

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