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Separation of Phenotypes in Mutant Alleles of the *Schizosaccharomyces pombe* Cell-Cycle Checkpoint Gene *rad1*$^+$

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The *Schizosaccharomyces pombe rad1*$^+$ gene is involved in the G2 DNA damage cell-cycle checkpoint and in coupling mitosis to completed DNA replication. It is also required for viability when the cdc17 (DNA ligase) or wee1 proteins are inactivated. We have introduced mutations into the coding regions of *rad1*$^+$ by site-directed mutagenesis. The effects of these mutations on the DNA damage and DNA replication checkpoints have been analyzed, as well as their associated phenotypes in a *cdc17*-K42 or a *wee1*-50 background. For all alleles, the resistance to radiation or hydroxyurea correlates well with the degree of functioning of checkpoint pathways activated by these treatments. One mutation, *rad1*-S3, completely abolishes the DNA replication checkpoint while partially retaining the DNA damage checkpoint. As single mutants, the *rad1*-S1, *rad1*-S2, *rad1*-S5, and *rad1*-S6 alleles have a wild-type phenotype with respect to radiation sensitivity and checkpoint functions; however, like the *rad1* null allele, the *rad1*-S1 and *rad1*-S2 alleles exhibit synthetic lethality at the restrictive temperature with the *cdc17*-K42 or the *wee1*-50 mutation. The *rad1*-S5 and *rad1*-S6 alleles allow growth at higher temperatures in a *cdc17*-K42 or *wee1*-50 background than does wild-type *rad1*$^+$, and thus behave like "superalleles." In most cases both chromosomal and multi-copy episomal mutant alleles have been investigated, and the agreement between these two states is very good. We provide evidence that the functions of rad1 can be dissociated into three groups by specific mutations. Models for the action of these *rad1* alleles are discussed. In addition, a putative negative regulatory domain of rad1 is identified.

INTRODUCTION

When cells are exposed to conditions that damage DNA or interfere with DNA replication, a signal is sent to delay cell-cycle progression, a function known as checkpoint control (Hartwell and Weinert, 1989). It is generally thought that checkpoint gene products affect components of the mitotic cell-cycle control machinery, i.e., cyclins and cyclin-dependent kinases (cdk), although direct evidence for this is sparse. It has been demonstrated that in mammalian cells, DNA damage leads to increased levels of p21, the product of the *CIP1* gene (Dulic *et al.*, 1994). This protein inhibits several cdk/cyclin complexes active in the G1 and S phases of the cell cycle (Harper *et al.*, 1993). In the S and G2 phases, the checkpoint pathway coupling mitosis to completion of DNA replication and the G2 DNA damage checkpoint pathway are operative. The exact relationship between these and the proteins controlling entry into mitosis remains obscure.

There is increasing evidence for separate genetic control of these two latter checkpoint pathways. Thus, in *Saccharomyces cerevisiae*, the *RAD9*, *RAD17*, *RAD24*, *MEC1*, *MEC2*, and *MEC3* genes are all required for the G2 DNA damage checkpoint, but only *MEC1* and *MEC2* are also necessary for the replication checkpoint

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(Weinert and Hartwell, 1988; Weinert *et al.*, 1994). In *Saccharomyces pombe*, the *rad1*$^+$, *rad3*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$, and *hus1*$^+$ genes are needed for both these checkpoint pathways (Hannan *et al.*, 1976; Al-Khodairy and Carr, 1992; Al-Khodairy *et al.*, 1994; Enoch *et al.*, 1992; Jimenez *et al.*, 1992; Rowley *et al.*, 1992). This subgroup of checkpoint genes is sometimes termed "checkpoint-rad," and the corresponding mutants share the following characteristics: 1) both the DNA damage and the replication checkpoints are affected; 2) mutants have synthetic lethality with *wee1*-50; 3) rapid death occurs with a *cdc17* mutation; 4) kinetics of entry into mitosis is unaffected by hydroxyurea (HU) ("early cut mutants") (Al-Khodairy and Carr, 1992; Al-Khodairy *et al.*, 1994; Enoch *et al.*, 1992; Rowley *et al.*, 1992; Carr and Hoekstra, 1995). There are recent indications for evolutionary conservation in eukaryotes of genes of this subgroup. Thus, the recently identified gene responsible for the human DNA damage checkpoint disorder *Ataxia Telangiectasia* bears sequence similarity to *S. pombe rad3*$^+$ and to *S. cerevisiae ESR1* (Savitsky *et al.*, 1995). Likewise, *S. pombe rad1*$^+$ bears homology to *S. cerevisiae RAD17* (Carr and Hoekstra, 1995).

In contrast, *chk1* mutants are deficient in the DNA damage checkpoint but retain the replication checkpoint (Walworth *et al.*, 1993). Further, the *rad4*$^+$/*cut5*$^+$ gene is required for coupling mitosis to DNA replication, but not for the DNA damage checkpoint (Saka and Yanagida, 1993; Saka *et al.*, 1994). The *rad24*$^+$ and *rad25*$^+$ genes are required for the DNA damage checkpoint, whereas their involvement in the replication checkpoint is as yet unclear (Al-Khodairy *et al.*, 1994; Ford *et al.*, 1994).

The *rad1* mutant was one of the first radiation-sensitive mutants in *S. pombe* to be isolated (Nasim, 1968; Schüpbach, 1971; Nasim and Smith, 1975). Later it became clear that *rad1* mutants are also sensitive to HU (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992), a consequence of the inability of this mutant to couple initiation of mitosis to completion of DNA replication. The *rad1*$^+$ gene has by now been firmly implicated in the DNA damage and DNA replication checkpoints (Hannan *et al.*, 1976; Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Rowley *et al.*, 1992). The *rad1* mutant has recently been reported to be deficient in *cis*-platinum resistance (Thiebaut *et al.*, 1994), and its genetic interactions with *swi* as well as with other *rad* genes has been re-examined (Schlake *et al.*, 1993).

The *rad1*$^+$ gene encodes a 37-kDa highly charged protein (Sunnerhagen *et al.*, 1990; Long *et al.*, 1994). The protein does not belong to any well-defined family, and its sequence is devoid of recognizable domains or motifs, although a limited stretch of similarity to *Ustilago maydis* REC1 exists (Long *et al.*, 1994). Two stretches of acidic amino acid (aa) residues are found near the carboxy-terminus. Basic residues tend to be found in small clusters closer to the amino-terminus of the otherwise acidic (pI = 4.0) rad1 protein.

In the present work, we have created a small set of mutations in the coding region of the *rad1*$^+$ gene and analyzed the associated phenotypes. The aim is to find out which of the phenotypes of the *rad1* null allele can be dissected from each other, and to lay the foundation for molecular studies of the function of the rad1 protein, as well as of its interactions with other cellular components.

MATERIALS AND METHODS

Oligonucleotide-directed Mutagenesis and Plasmid Construction

To make alleles *rad1*-S1 and S2, a full-length *rad1*$^+$ cDNA in pGSR3 (Long *et al.*, 1994) was polymerase chain reaction (PCR)

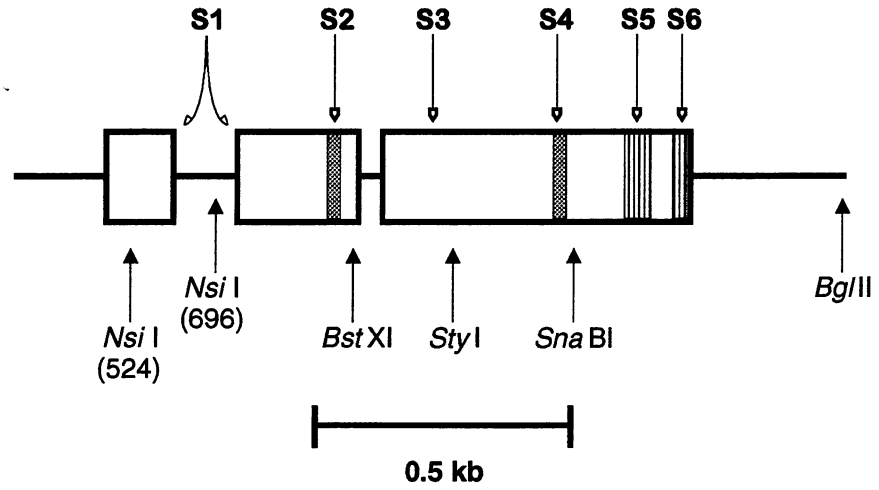

Figure 1. The *S. pombe* chromosomal *rad1*$^+$ locus and location of mutations. Coding sequences are shown as boxes. Acidic regions and basic clusters are shown as striped or shaded areas, respectively. Mutated sequences are as follows: S1, *U. maydis REC1* homology; S2, basic region 1; S3, uncharacterized conserved motif; S4, basic region 2; S5, acidic stretch 1; and S6, acidic stretch 2. Relevant restriction sites are shown.

amplified in a four-primer PCR-based mutagenesis method (Higuchi, 1990) using 5′-CGTAAATTCGCATGTTTCAA-3′ and 5′-ATCATTTAGCTATCCTCATC-3′ as outside primers. The *rad1*-S1 allele was created using the mutagenic oligonucleotides 5′-TCGCAGTCTGCGGCCGCTGCCGCCTTTCTT-3′ and 5′-AAGAAAGGCGGCAGCGGCCGCAGACTGCGA-3′. This mutation spans the first intron and substitutes aa residues L44, Q45, and H47 for A (Figure 1). The affected region (SQSLQAHAFL) bears homology to the product of *Ustilago maydis REC1* (Long *et al.*, 1994). For *rad1*-S2, 5′-GTAAACATAATGGCGGCCGCTGGCGTTATC-3′ and 5′-GATAACGCCAGCGGCCGCCAT-TATGTTTAC-3′ were the mutagenic oligonucleotides. This mutation substitutes aa H106, K107, and R108 for A. The mutated region (NIMHKRGVI) represents the first small cluster of basic aa residues in this otherwise acidic protein. After subcloning the PCR products into pCRII (Invitrogen, San Diego, CA) using its 3′ single base overhangs, the 0.4-kb fragment from *Nsi*I at nt 524 (Sunnerhagen *et al.*, 1990) to *Sty*I (Figure 1) was sequenced (Sanger *et al.*, 1977) to verify the identity of the mutations.

To insert these two mutations, as well as *rad1*$^+$ cDNA, into a background where they would be expressed from the genomic *rad1*$^+$ promoter, a 3.4-kb *Bam*HI fragment containing the genomic *rad1*$^+$ locus (Sunnerhagen *et al.*, 1990) was inserted into the *Bam*HI site of pTZ19U, to make pR1B2. Thereafter, the 0.4-kb *Nsi* I/*Sty*I fragment was exchanged for the corresponding fragment from pR1B2. This creates *rad1* minigenes with both introns removed.

For alleles S3 through S6, pr1B2 was used as a template for mutagenesis according to the method of Kunkel (1985). The *rad1*-S3 allele was created with the oligonucleotide 5′-CATCAATAGACTTGCAAGCACAATTATGAAAAG-3′, which deleted aa 157–161 (LASLCTKIIMK) of the protein product. This corresponds to part of a short sequence motif of unknown function (LC(T)xxxxxx(N)W) found in a variety of unrelated proteins. The *rad1*-S4 allele was created with the oligonucleotide 5′-CCTACCGCTTCTCATTAATAGCATTACAAGTGG-3′, which deleted aa 235–239 (SLIRHALKALQ). This corresponds to the second weak clustering of basic residues in the protein. *rad1*-S5 was created with 5′-GTTCCTTTGGACCTTGTAAGTCCAGCAGAATCTAATC-3′, which deleted aa 285–294; this is a stretch of 10 consecutive acidic residues (LVSEDEEEDEEEEPAE). *rad1*-S6 was constructed with 5′-CCGAATTATCGAGGAGATGCGTAAATGATAATT-3′, which deleted aa 317–323 (GNAETEDEDSstop). Five of these seven carboxy-terminal residues are acidic. The identity of these mutant clones was also verified by sequencing. For each mutagenesis, the sequenced restriction fragment containing the mutated site was then exchanged for the corresponding fragment of pR1B2. For the *rad1*-S3 mutation, this was from *Bst*XI to *Sty*I; for the *rad1*-S4 mutation, from *Sty*I to *Sna*BI; and for the *rad1*-S5 and *rad1*-S6 mutations, from *Sna*BI to *Bgl*II (Figure 1).

Finally, to generate extrachromosomally replicating versions of all the mutant alleles or wild-type cDNA, the entire 3.4-kb *Bam*HI fragment was excised and inserted into the *Bam*HI site of pIRT-2 (Booher and Beach, 1986).

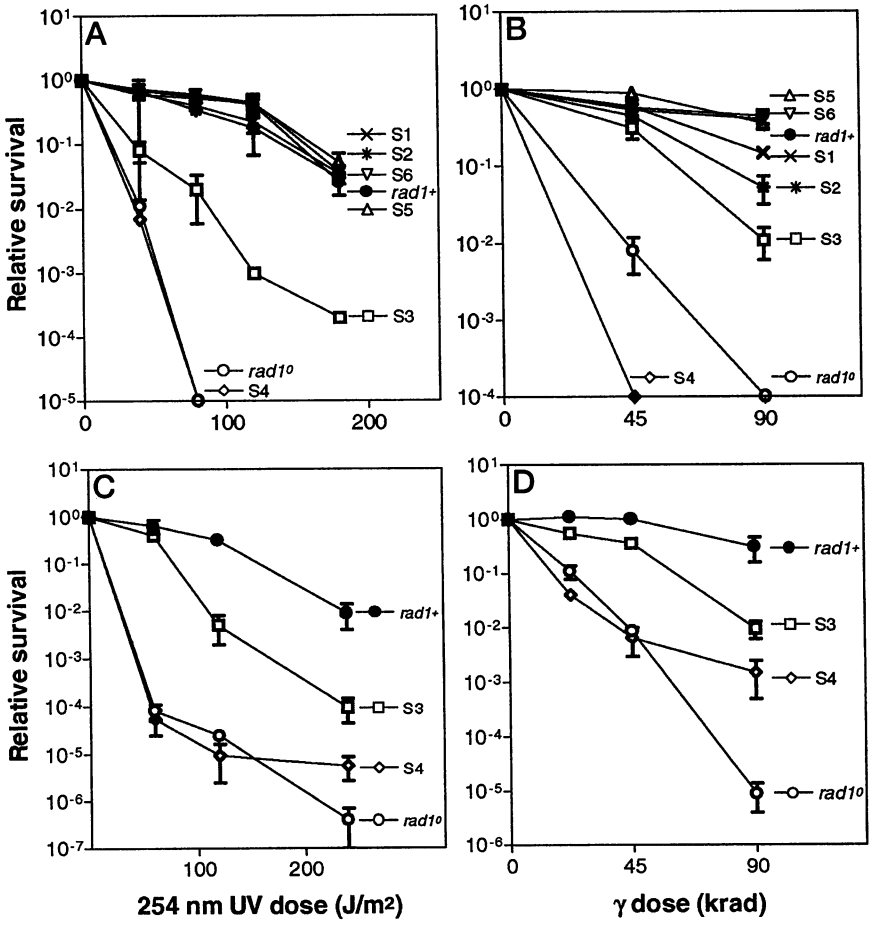

Figure 2. Radiation resistance of *S. pombe* strains (Table 1) carrying mutant or wild-type *rad1* alleles. (A and B) Chromosomal alleles. Cells were irradiated with 254 nm UV light or γ rays as described in MATERIALS AND METHODS. Survival values are expressed as fractions of the viable cell count of the respective strain unirradiated, and are the average of three different readings. Error bars denote plus or minus 1 SE. Filled circles, wild-type *rad1*$^+$ (KLP2); open circles, *rad1*^{0} (KLP1); crosses, *rad1*-S1 (KLP20); asterisks, *rad1*-S2 (KLP23); open squares, *rad1*-S3 (KLP6); open diamonds, *rad1*-S4 (KLP7); open triangles, *rad1*-S5 (KLP8); and open inverted triangles, *rad1*-S6 (KLP9). (A) UV survival; (B) γ survival; and (C and D) radiation resistance of cells carrying extrachromosomal copies of selected alleles. PS36 (*rad1*^{0}) was transformed with the indicated *rad1* alleles (*rad1*$^+$, *rad1*-S3, and *rad1*-S4) cloned into pIRT-2, or with pIRT-2 vector alone. Transformants were irradiated and survival was recorded as above. Designations and symbols for the various *rad1* alleles are as above. (C) UV survival; and (D) γ survival.

Culture Conditions and Construction of Yeast Strains

Vegetative growth of all *S. pombe* strains was at 30°C except where indicated. Nonselective medium was YES (Moreno *et al.*, 1991). Selective medium was 0.17% yeast nitrogen base (YNB) without amino acids (Difco Laboratories, Detroit, MI), 5 g/l $(NH_4)_2SO_4$, and 2% glucose with supplements as required (75 mg of adenine, 40 mg each of histidine, leucine, and uracil per liter); this was used throughout for strains transformed with plasmids.

Replacement of the genomic *rad1::ura4*$^+$ locus with various *rad1* alleles was accomplished by transformation of strain PS36 with the respective mutant plasmids restricted with *Bam*HI, which releases the pTZ portion. Homologous recombination was then selected for with 5-fluoro-orotic acid (Grimm *et al.*, 1988). Correct integration of transfected DNA was verified by Southern blot and PCR. Double mutants were made by first crossing, using established techniques (Gutz *et al.*, 1974), PS36 with a *wee1*-50 or a *cdc17*-K42 strain and then transforming the respective *rad1* double mutant with linearized plasmid containing the various mutant *rad1* alleles as above.

Measurement of Survival After Ultraviolet (UV) Irradiation

In experiments with chromosomally integrated *rad1* alleles, a single colony was picked and expanded. In experiments with alleles carried on extrachromosomal plasmids, to minimize fluctuations in average plasmid copy number, several hundred clones were pooled from a plate of fresh transformants. The cells were grown overnight in liquid medium until mid-logphase was reached (0.3–1.2 × 10^7 cells/ml). A brief centrifugation was used to collect the cells, which were then resuspended in YNB lacking adenine and uracil to a final density of 3 × 10^6 cells/ml. Irradiation was carried out on 25 ml of cell suspension in a 90-mm petri dish with continuous stirring using a UVGL-58 short wave UV lamp (UV Products, San Gabriel, CA) emitting light with a peak around 254 nm. The intensity of the light was 3 W/m^2, which was monitored with a UVX digital UV meter (UV Products). Immediately after irradiation, the cells were plated on solid medium and incubated at 30°C for 3 days.

Measurement of Survival After γ Irradiation

Cells, collected and pre-grown as for UV irradiation, in mid-log phase were suspended in liquid medium and irradiated with a ^{137}Cs γ ray source, at a dose rate of 2 krad/min, and were thereafter plated on solid medium. The number of colonies was recorded after 3 days of incubation.

Measurement of Survival After Transient Exposure to HU

Cells were grown in liquid medium to early log phase (10^6 cells/ml). HU was added, and cultivation was continued with shaking at 30°C. At various timepoints, samples were withdrawn and spread on solid medium. Survival was recorded after 3 days of incubation.

Assay of Cell-Cycle Arrest After γ Irradiation or Exposure to HU

Cells were maintained in exponential growth, and were either given a dose of γ rays or put in HU at the indicated concentration.

Cell-cycle progress into mitosis was monitored as described previously (Rowley *et al.*, 1992). Briefly, the proportion of cells attempting mitosis was assessed microscopically as the fraction having formed a septum, visualized by iodine staining. Cells were viewed at 400× magnification by phase contrast optics, and at least 200 cells per data point were scored for the presence or absence of a septum.

4,6-diamidino-2-phenylindole (DAPI) Staining and Fluorescence Microscopy

Cells were washed, fixed with formaldehyde, applied on poly-L-lysine–coated cover slips, and mounted in DAPI solution as described (Moreno *et al.*, 1991). Fluorescence microscopy and photography was at 630× magnification.

RESULTS

Radiation Resistance

Figure 2 shows the survival of strains carrying the investigated *rad1* alleles following UV or γ irradiation.

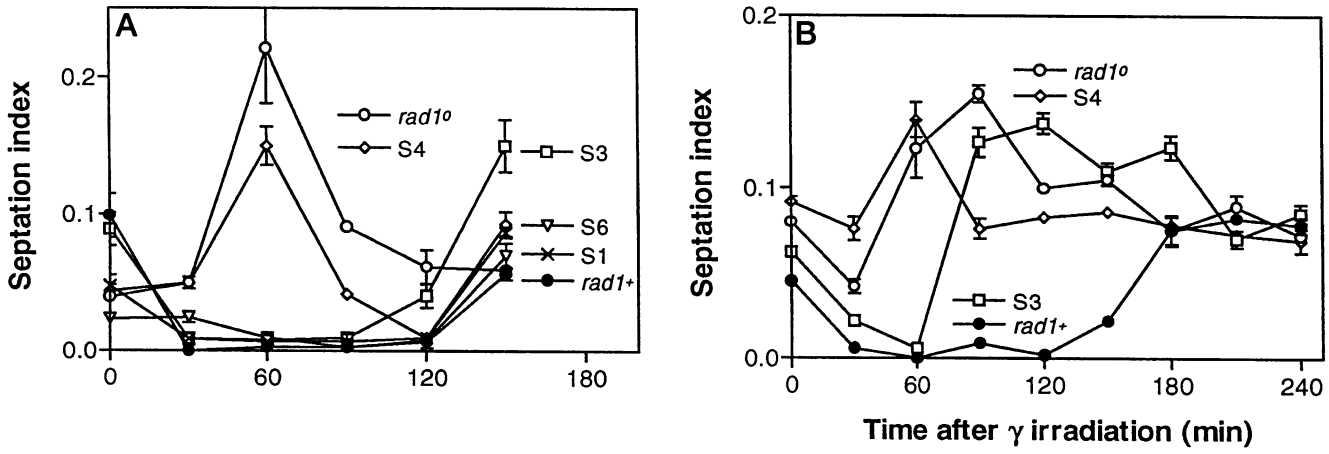

Figure 3. Cell-cycle delay of γ-irradiated cells carrying mutant or wild-type *rad1* alleles. Designations and symbols for the various *rad1* alleles are as in Figure 2. (A) Chromosomal alleles. For clarity, only selected alleles (*rad1*-S1, S3, S4, S6, *rad1*$^+$, and *rad1*^{0}) are shown. Cells were given 45 krad of γ radiation as described in MATERIALS AND METHODS and septation index was monitored for the indicated time intervals thereafter. (B) Selected plasmid-borne alleles. PS36 was transformed with the same *rad1* alleles as in Figure 2, C and D, cloned into pIRT-2. Transformants were given 60 krad of γ radiation as above; septation index was monitored for the indicated time intervals thereafter.

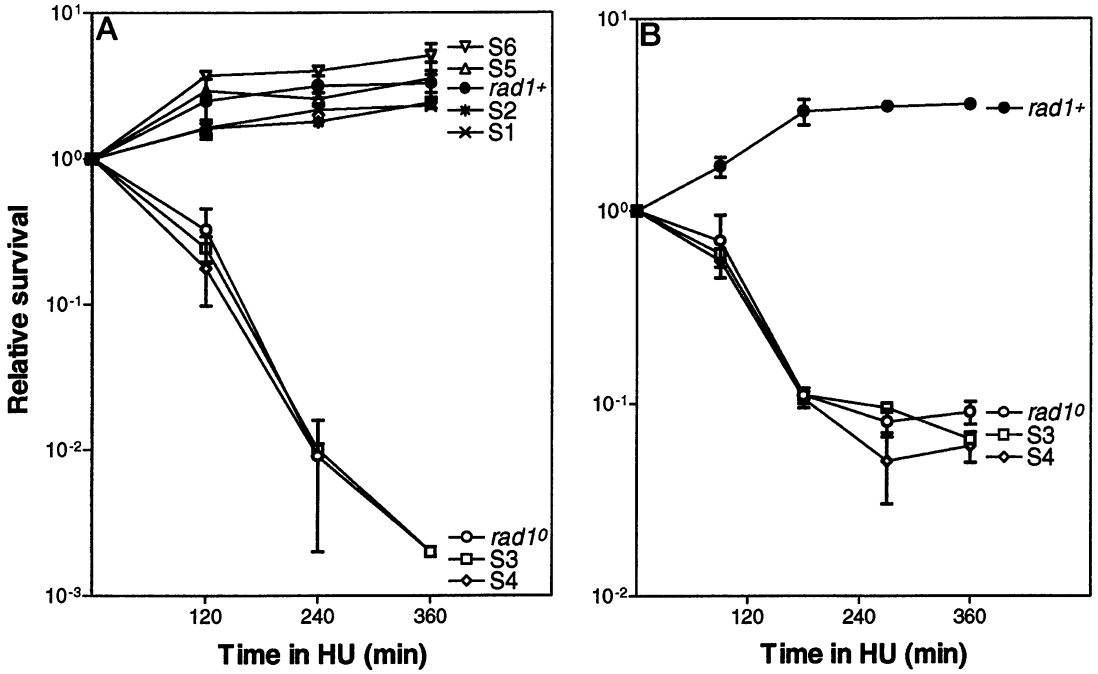

Figure 4. HU resistance. Cells were exposed to HU for the indicated time periods and survival was measured. Designations and symbols for the various *rad1* alleles are as in Figure 2. (A) Chromosomal alleles. HU concentration, 20 mM. (B) Selected plasmid-borne alleles. PS36 was transformed with the same *rad1* alleles as in Figure 2, C and D, cloned into pIRT-2. HU concentration, 10 mM.

The survival of *rad*$^+$ and *rad1*^{0} strains was in agreement with previous experience (Nasim and Smith, 1975; Sunnerhagen *et al.*, 1990) (Figure 2, A and B). The *rad1*-1 allele has been shown to be identical to the *rad1* disruption in its sensitivity to UV or γ irradiation (Sunnerhagen *et al.*, 1990). The *rad1*^{0} strain complemented with *rad1*$^+$ cDNA was also tested for radiation resistance and found to be indistinguishable from genomic *rad1*$^+$ (our unpublished data). Four of the mutant alleles, *rad1*-S1, *rad1*-S2, *rad1*-S5, and *rad1*-S6, were virtually unaffected in their resistance both to UV light and γ

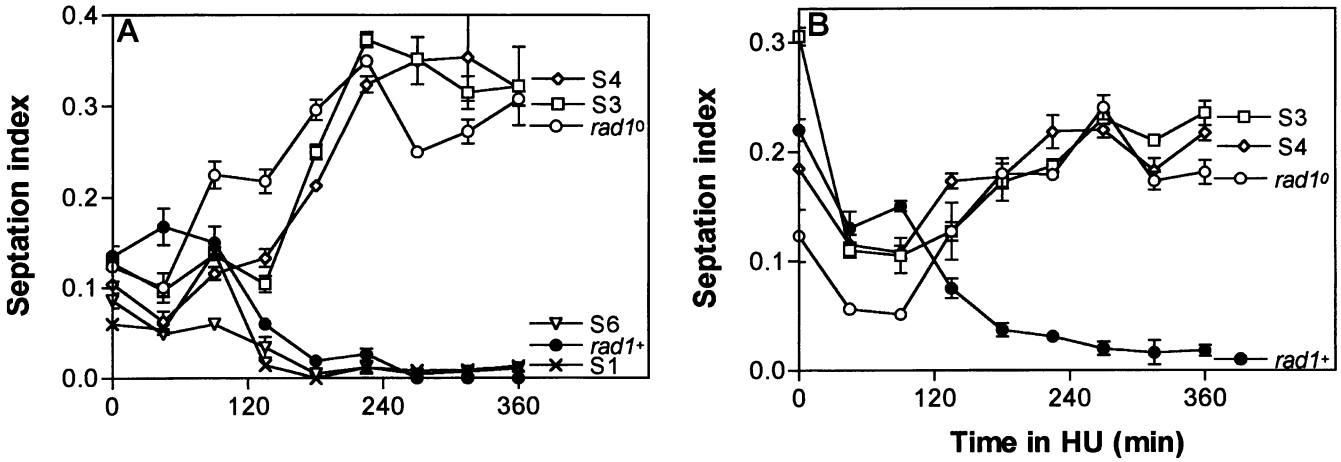

Figure 5. Cell-cycle delay of HU-treated cells carrying mutant or wild-type chromosomal *rad1* alleles. Cells were kept in medium containing HU for the indicated time periods, and samples were withdrawn, fixed, and examined microscopically for the fraction of septated cells. Designations and symbols for the various *rad1* alleles are as in Figure 2. (A) Chromosomal alleles. For clarity, only selected alleles (*rad1*-S1, S3, S4, S6, *rad1*$^+$, and *rad1*^{0}) are shown. HU concentration, 20 mM. (B) Selected plasmid-borne alleles. PS36 was transformed with the same *rad1* alleles as in Figure 2, C and D, cloned into pIRT-2. HU concentration, 10 mM.

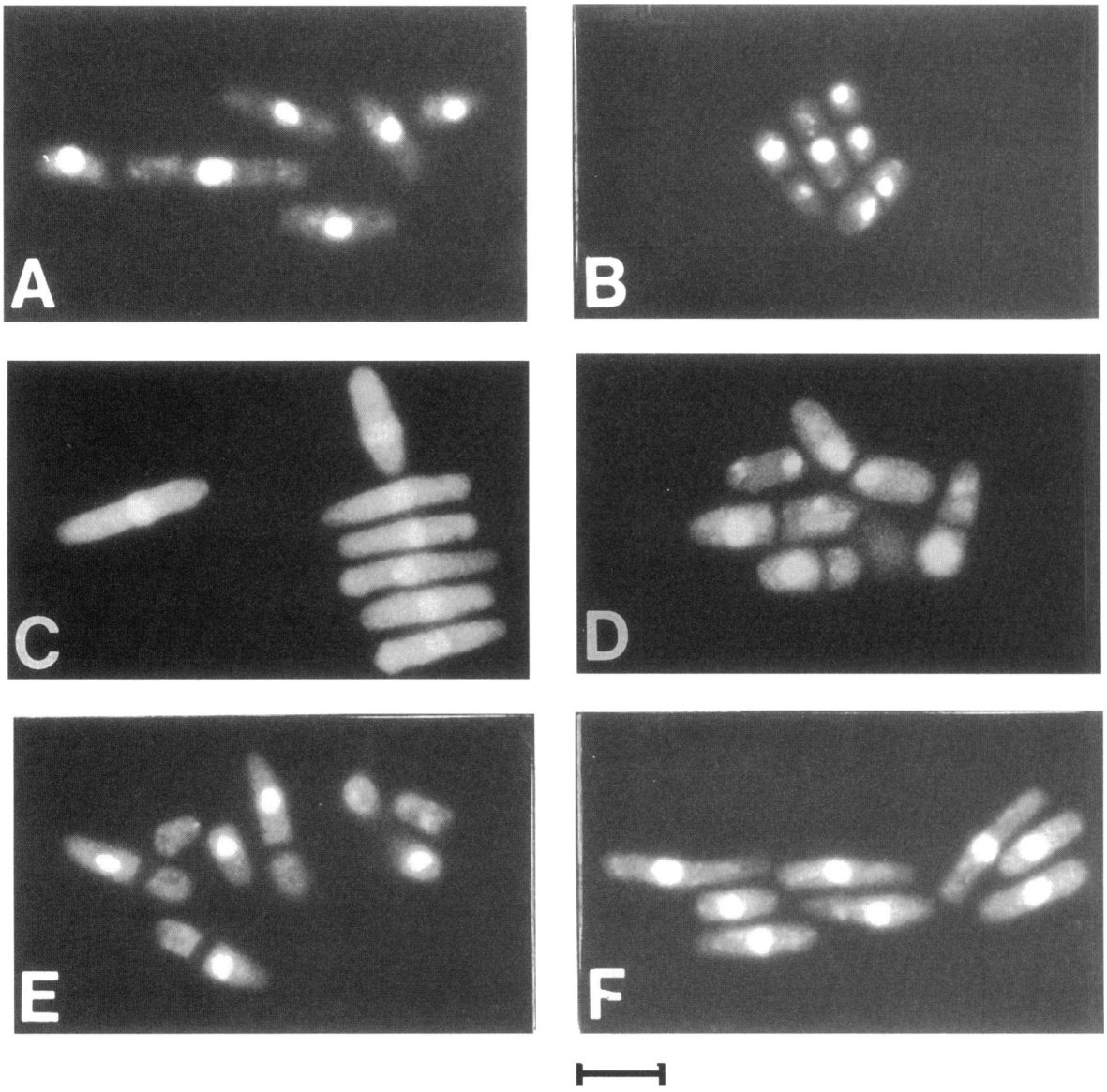

Figure 6. Morphology of *rad1* mutant and wild-type cells after treatment with 20 mM HU for 4.5 h. Cells were stained with DAPI and photographed at 630× magnification. (A) 972h^- (*rad*$^+$); (B) PS36 (*rad1*^{0}); (C) KLP20 (*rad1*-S1); (D) KLP6 (*rad1*-S3); (E) KLP7 (*rad1*-S4); and (F) KLP9 (*rad1*-S6)

radiation (Figure 2, A and B). Of the remaining two alleles, *rad1*-S3 was only marginally affected in its survival after γ irradiation and had a resistance to UV light intermediate between that of wild-type and *rad1*^{0} (Figure 2, A and B). *rad1*-S4 displayed a survival after either UV or γ irradiation as low as, or for some doses even lower than, *rad1*^{0} cells.

Because two of the mutant alleles, *rad1*-S3 and S4, were deficient to different degrees in radiation resistance, we wanted to examine whether this deficiency could be overcome by expressing these alleles from a multi-copy plasmid. These alleles, along with the *rad1*$^+$ wild-type gene, cloned into pIRT-2, were transformed into strain PS36 (*rad1*^{0}) and their radiation resistance was assayed. As seen in Figure 2, C and D, the result for both UV and γ radiation was in close agreement with that of the chromosomal alleles, and so the copy number of these alleles did not affect radiation resistance appreciably.

Cell-Cycle Arrest After Irradiation

Figure 3, A and B, shows the fraction of cells attempting mitosis after γ irradiation in strains with different

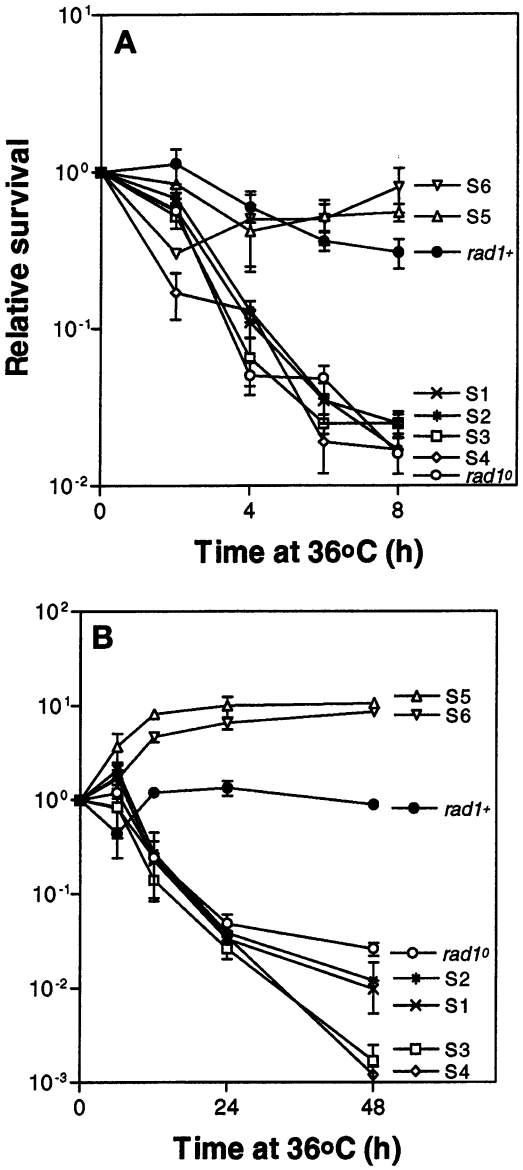

Figure 7. (A) Survival of *rad1*-x *cdc17*-K42 double mutants at the restrictive temperature for *wee1*-50, 36°C. Cells were kept in liquid culture at 36°C for the indicated time periods and were thereafter plated on solid medium and incubated at the permissive temperature, 25°C. After 3 days, the number of colonies was counted. Designations and symbols for the different *rad1* alleles are as in Figure 2. The following strains were used: h^+ *cdc17*-K42, GK13 (*rad1*::*ura4* *cdc17*-K42), GK14 (*rad1*-S1 *cdc17*-K42), GK15 (*rad1*-S2 *cdc17*-K42), GK16 (*rad1*-S3 *cdc17*-K42), GK17 (*rad1*-S4 *cdc17*-K42), GK18 (*rad1*-S5 *cdc17*-K42), and GK19 (*rad1*-S6 *cdc17*-K42). (B) As in panel A but *rad1*-x *wee1*-50 double mutants. The following strains were used: h^+ *wee1*-50 *leu1*-32 *ura4*-D18, GK6 (*rad1*::*ura4* *wee1*-50), GK7 (*rad1*-S1 *wee1*-50), GK8 (*rad1*-S2 *wee1*-50), GK9 (*rad1*-S3 *wee1*-50), GK10 (*rad1*-S4 *wee1*-50), GK11 (*rad1*-S5 *wee1*-50), and GK12 (*rad1*-S6 *wee1*-50)

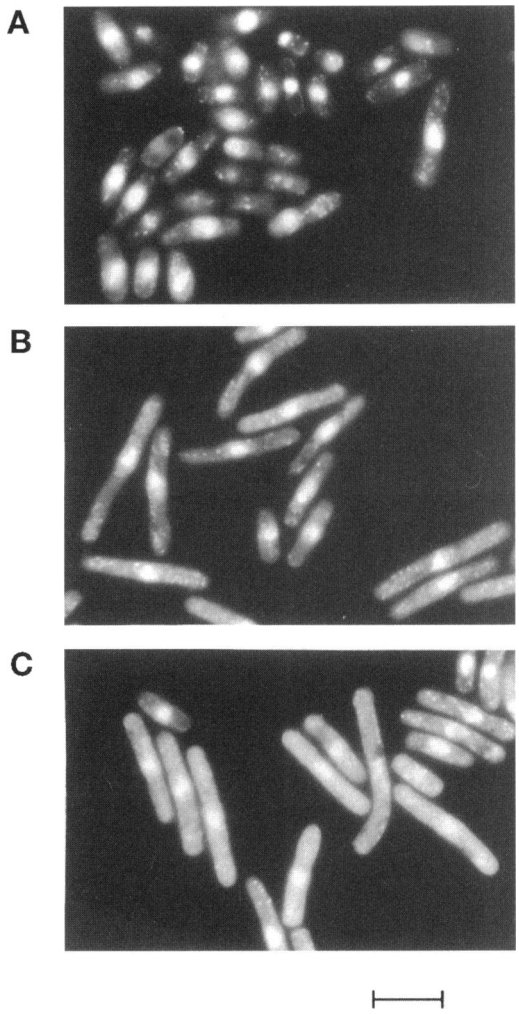

Figure 8. Morphology of selected *rad1*-x *cdc17* double mutants. Cells were grown at the restrictive temperature (36°C) for 8 h, fixed, stained with DAPI and photographed as above. Scale bar, 10 μm. (A) $rad1^0$ *cdc17*-K42 (GK13); (B) *rad1*-S1 *cdc17*-K42 (GK14); and (C) *rad1*-S2 *cdc17*-K42 (GK15)

rad1 alleles carried in a chromosome or an episome. For $rad1^+$ cells, there was a depression in the number of mitotic cells at 60–120 min post-irradiation, as a consequence of the DNA damage checkpoint (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992). After about 2.5 h, the cell division cycle resumed. This pattern was paralleled by all other *rad1* alleles with a wild-type radiation resistance, namely *rad1*-S1 and S6 (Figure 3A), and S2 and S5 (our unpublished data). In contrast, in $rad1^0$ cells, where the damage checkpoint is eliminated, the fraction of mitotic cells did not decrease, in agreement with earlier reports (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992). The only clearly radiosensitive allele, *rad1*-S4, was similar to $rad1^0$ in this respect also, and *rad1*-S4 cells were clearly unable to delay mitotic entry following irradiation (Figure 3, A and B). The remaining allele, *rad1*-S3, which was only slightly γ-sensitive (Figure

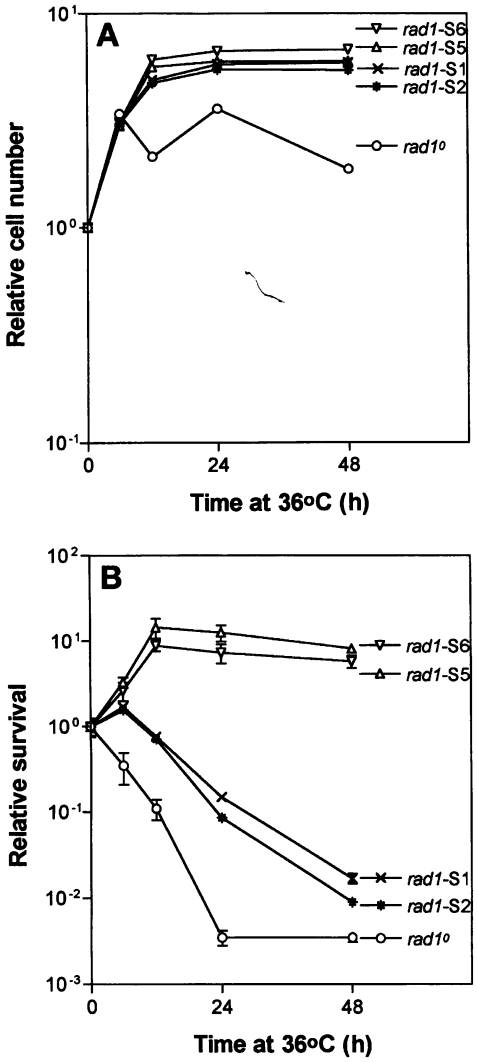

Figure 9. Comparison of total cell number and number of viable cells at 36°C for selected *rad1*-x *wee1*-50 double mutants. Cells were incubated at 36°C in liquid medium for the indicated time periods. Total cell numbers were determined by direct microscopy of aliquots of fixed cells and by measurement of optical density at 595 nm; values shown are from optical density measurements. For determination of the number of viable cells, aliquots were plated and grown at 25°C as described in Figure 7. (A) Total cell number. Designations and symbols for the different *rad1* alleles are as in Figure 2. The following strains were used: GK6 (*rad1*::*ura4* *wee1*-50), GK7 (*rad1*-S1 *wee1*-50), GK8 (*rad1*-S2 *wee1*-50), GK11 (*rad1*-S5 *wee1*-50), and GK12 (*rad1*-S6 *wee1*-50). (B) Number of viable cells. Strains, designations, and symbols are as in panel A.

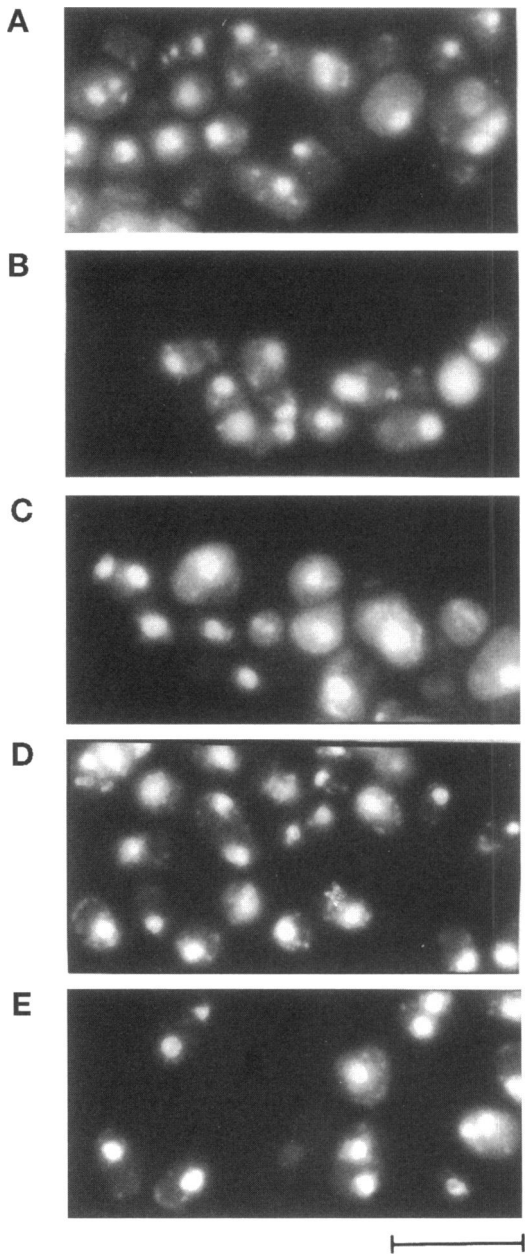

Figure 10. Morphology of selected *rad1*-x *wee1* double mutants. Cells were grown at 36°C for 24 h, fixed, stained with DAPI and photographed as above. (A) *rad1*^{0} *wee1*-50 (GK6); (B) *rad1*-S1 *wee1*-50 (GK7); (C) *rad1*-S2 *wee1*-50 (GK8); (D) *rad1*-S5 *wee1*-50 (GK11); and (E) *rad1*-S6 *wee1*-50 (GK12). Scale bar, 10 μm.

2, B and D), appeared to mainly retain the damage checkpoint, in that its septation index was depressed as deeply as those of the radioresistant alleles (Figure 3, A and B). The checkpoint was not completely intact in this mutant, however, as it showed a somewhat shorter G2 delay than *rad*$^+$ cells (Figure 3B and to some extent 3A).

HU Resistance

HU inhibits DNA synthesis, and sensitivity to transient HU exposure is found only in the subset of *S. pombe rad* mutants that have a defect in the ability to couple mitosis to the completion of DNA replication (Al-Khodairy and Carr, 1992; Al-Khodairy *et al.*, 1994; Rowley *et al.*, 1992). In Figure 4, the survival after

exposure to HU for various time periods is shown. For five of the six mutant alleles, the degree of HU resistance paralleled radiation resistance. Thus, *rad1*-S1, *rad1*-S2, *rad1*-S5, and *rad1*-S6, like *rad*$^+$ cells, all maintained viability during HU exposure. On the other hand, *rad1*^{0} cells rapidly lost viability (Figure 4), in agreement with previous reports (Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Rowley *et al.*, 1992). The *rad1*-S4 allele again behaved like the null allele (Figure 4A). The notable deviation from this pattern was *rad1*-S3. Cells carrying this mutant allele were as sensitive to HU as *rad1*^{0} cells (Figure 4A), in contrast to their behavior after irradiation (Figure 2, A and B). The outcome with *rad1*-S3 and S4 carried on multicopy plasmids (Figure 4B) was the same as for the integrated versions.

Cell-Cycle Arrest After Exposure to HU

Figure 5A shows the behavior of cells harboring the *rad1* alleles in HU. Cells carrying the HU-resistant alleles, *rad1*-S1, S2, S5, S6, and *rad*$^+$, elongated (Figure 6 and our unpublished data), and the septation index dropped to values near zero in about 2 h (Figure 5A). This was expected for cells sensing the replication inhibition and arresting cell-cycle progression (Enoch *et al.*, 1992; Rowley *et al.*, 1992; Al-Khodairy *et al.*, 1994). By contrast, in strains carrying alleles conferring HU sensitivity, *rad1*-S3, S4, and *rad1*^{0}, septation index did not fall but rather increased (Figure 5A). Such cells did not elongate; rather, in these populations, cells with abnormal nuclei or cells lacking the nucleus entirely were frequently seen (Figure 6, B, D, and E). This pattern is consistent with cells entering into mitosis without inhibition, as found previously for mutants lacking the replication checkpoint (Enoch *et al.*, 1992; Rowley *et al.*, 1992; Al-Khodairy *et al.*, 1994). The fact that the fraction of septated cells in these strains increased during HU exposure could be explained by inability to complete and productively exit from mitosis. Again, the pattern seen with the multi-copy versions of *rad1*-S3 and S4 (Figure 5B) was very close to that seen with the chromosomal alleles.

Table 1. *S. pombe* strains

Designation	Genotype	Source or reference
	h$^+$ *wee1*-50 *leu1*-32 *ura4*-D18	P. Russell
	h$^+$ *cdc17*-K42	P. Fantes
GK6	*rad1::ura4 wee1*-50 *leu1*-32 *ura4*-D18	This work
GK7	*rad1*-S1 *wee1*-50 *leu1*-32 *ura4*-D18	This work
GK8	*rad1*-S2 *wee1*-50 *leu1*-32 *ura4*-D18	This work
GK9	*rad1*-S3 *wee1*-50 *leu1*-32 *ura4*-D18	This work
GK10	*rad1*-S4 *wee1*-50 *leu1*-32 *ura4*-D18	This work
GK11	*rad1*-S5 *wee1*-50 *leu1*-32 *ura4*-D18	This work
GK12	*rad1*-S6 *wee1*-50 *leu1*-32 *ura4*-D18	This work
GK13	*rad1::ura4 cdc17*-K42 *leu1*-32 *ura4*-D18	This work
GK14	*rad1*-S1 *cdc17*-K42 *leu1*-32 *ura4*-D18	This work
GK15	*rad1*-S2 *cdc17*-K42 *leu1*-32 *ura4*-D18	This work
GK16	*rad1*-S3 *cdc17*-K42 *leu1*-32 *ura4*-D18	This work
GK17	*rad1*-S4 *cdc17*-K42 *leu1*-32 *ura4*-D18	This work
GK18	*rad1*-S5 *cdc17*-K42 *leu1*-32 *ura4*-D18	This work
GK19	*rad1*-S6 *cdc17*-K42 *leu1*-32 *ura4*-D18	This work
KLP1	*h*$^-$ *rad1*-1 *leu1*-32 *ura4*-D18	This work
KLP2	*h*$^-$ *leu1*-32 *ura4*-D18	This work
KLP6	*h*$^-$ *rad1*-S3 *his3 leu1*-32 *ura4*-D18	This work
KLP7	*h*$^-$ *rad1*-S4 *his3 leu1*-32 *ura4*-D18	This work
KLP8	*h*$^-$ *rad1*-S5 *his3 leu1*-32 *ura4*-D18	This work
KLP9	*h*$^-$ *rad1*-S6 *his3 leu1*-32 *ura4*-D18	This work
KLP20	*h*$^-$ *rad1*-S1 *his3 leu1*-32 *ura4*-D18	This work
KLP23	*h*$^-$ *rad1*-S2 *his3 leu1*-32 *ura4*-D18	This work
PS36	*h*$^-$ *rad1::ura4 his3 leu1*-32	Dahlkvist *et al.* (1995)

Rapid Death in rad1 cdc17 Double Mutants

It has been shown previously that *rad1*^{0} cells, as well as cells with other checkpoint mutations, such as *rad3*,

Table 2. Summary of phenotypes of *rad1* alleles

Mutant	DNA damage checkpoint			Replication checkpoint		*cdc17* double mutant	*wee1* double mutant
	UV res.	γ res.	γ-arrest	HU res.	HU-arrest		
rad1-S1	+++	+++	+++	+++	+++	−	−
rad1-S2	+++	+++	+++	+++	+++	−	−
rad1-S3	++	++	++	−	−	−	−
rad1-S4	−	−	−	−	−	−	−
rad1-S5	+++	+++	+++	+++	+++	++++[a]	++++[a]
rad1-S6	+++	+++	+++	+++	+++	++++[a]	++++[a]

In each column, (+++) denotes behavior of the *rad1*$^+$ wild-type allele and (−) denotes that of the null allele. UV res. and γ res., resistance to UV or γ radiation, respectively; HU res., survival after transient exposure to HU; γ arrest and HU arrest, ability to arrest the cell cycle in response to γ irradiation or HU exposure, respectively; *cdc17* double mutant and *wee1* double mutant, viability of the *rad1*-x *cdc17*-K42 or *rad1*-x *wee1*-50 double mutant, respectively, at the restrictive temperature for *cdc17*-K42 or *wee1*-50 (36°C).

[a] This denotes the fact that cells carrying these *rad1* alleles have a higher permissive temperature for growth than *rad1*$^+$ in a *cdc17*-K42 or *wee1*-50 background.

rad9, *rad17*, *rad34*, *chk1*, and *hus1*, rapidly lose viability on inactivation of DNA ligase (cdc17) (Al-Khodairy and Carr, 1992; Al-Khodairy *et al.*, 1994; Jimenez *et al.*, 1992). We wanted to investigate this property of individual *rad1* mutant alleles. To this end, all mutant *rad1* alleles were integrated into a *cdc17*-K42 background. The *rad1*-x *cdc17*-K42 double mutants, as well as rad^+ *cdc17*-K42 and $rad1^0$ *cdc17*-K42 cells, were incubated at 36°C, the restrictive temperature for the *cdc17*-K42 mutation for various times and their viability was measured (Figure 7A). The two alleles *rad1*-S5 and *rad1*-S6 conferred wild-type survival. *cdc17*-K42 double mutants carrying the same two alleles that abolished HU resistance, *rad1*-S3 and *rad1*-S4, died off as rapidly as the null allele; however, the remaining two mutations, *rad1*-S1 and *rad1*-S2, which in $cdc17^+$ cells did not disturb checkpoint functions (Figures 2–6), when combined with *cdc17*-K42 caused rapid death at the restrictive temperature. These results from *rad1*-S1 *cdc17*-K42 and *rad1*-S2 *cdc17*-K42 cells have been confirmed with the *rad1* allele on a multi-copy plasmid in a *rad1::ura4 cdc17*-K42 background; the $rad1^+$ cDNA in this background confers no cell death (our unpublished results).

Inviability of rad1 wee1 Double Mutants

The inviability of $rad1^0$ *wee1* double mutants has been demonstrated (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992), as well as the inviability of several other double mutants involving *wee1* and a checkpoint mutant (Al-Khodairy and Carr, 1992; Al-Khodairy *et al.*, 1994; Enoch *et al.*, 1992), e.g. *rad3*, *rad9*, *rad17*, *rad24*, *rad26*, *chk1*, and *hus1*. This is interpreted as the result of a "mitotic catastrophe," where deficient inhibition of the cdc2 protein kinase leads to premature mitosis with ensuing genomic aberrations. The viability of individual *rad1* alleles in *wee1*-50 cells is shown in Figure 7B. For *rad1*-S3 and *rad1*-S4, cells carrying these alleles in combination with the *wee1*-50 mutation behaved like $rad1^0$ *wee1*-50 cells and lost viability at 36°C. In keeping with the results from *rad1*-x *cdc17*-K42 double mutants, *rad1*-S1 and *rad1*-S2 cells also lost viability on inactivation of wee1 as extensively as $rad1^0$ cells. *rad1*-S5, and *rad1*-S6, which in all previous assays behaved like the wild-type $rad1^+$ gene, in a *wee1*-50 background allowed the cells to increase the viable cell count about 10 times, whereas rad^+ *wee1*-50 cells maintained an approximately constant cell number under these conditions. These results likewise have been confirmed using plasmid-borne *rad1* alleles in a *rad1::ura4 wee1*-50 background; additionally $rad1^+$ cDNA behaved like genomic $rad1^+$ (our unpublished results).

Because these experiments with double mutants were necessarily carried out at 36°C, the restrictive temperature for *cdc17*-K42 and *wee1*-50, we were concerned that the inviability of strains with *rad1*-S1 or *rad1*-S2 combined with the above mutations could be caused by a temperature sensitivity of the *rad1*-S1 and *rad1*-S2 alleles themselves. This possibility was ruled out by examining their behavior as single mutants at 36°C. Under such conditions, the radiation-induced cell-cycle arrest was intact and the cells were fully radiation-resistant (our unpublished observations).

Because of the aberrant behavior of *rad1*-S5 and S6 in a *wee1*-50 background (Figure 7B), we wanted to quantitate the capacity of these alleles to sustain growth in a *cdc17*-K42 or *wee1*-50 background. As measured by the colony-forming ability of the double mutants during continuous exposure to different temperatures, the maximum permissive temperature for both *rad1*-S5 and S6 was higher than that of $rad1^+$, both in a *cdc17*-K42 background (35°C vs. 34°C) and in a *wee1*-50 background (37°C vs. 36°C).

Morphology and Cell Cycle Characteristics of Double Mutants

To explore the reason for the inviability of double mutants with a combination of *rad1*-S1 or *rad1*-S2 with *cdc17*-K42, we examined microscopically such strains at the restrictive temperature. The arrest of the mitotic cycle with ensuing cell elongation of *cdc17*-K42 mutants at 36°C is abolished by a checkpoint mutation such as $rad1^0$ (Al-Khodairy and Carr, 1992). As predicted, *rad1::ura4 cdc17*-K42 did not elongate at the restrictive temperature (Figure 8A). In contrast, *rad1*-S1 *cdc17*-K42 and *rad1*-S2 *cdc17*-K42 cells clearly did elongate (Figure 8, B and C). We further assayed mitotic index of such mutant cells, and found that *rad1*-S1 *cdc17*-K42 and *rad1*-S2 *cdc17*-K42 cells indeed arrested upon temperature shift to 36°C with the same kinetics as rad^+ *cdc17*-K42 cells, whereas *rad1::ura4 cdc17*-K42 did not arrest (our unpublished observations).

We also further examined double mutants with a combination of *wee1*-50 with the *rad1*-S1, S2, S5, and S6 alleles. As is evident from Figure 9A, at 36°C all these strains, as well as *rad1::ura4 wee1*-50, continued to divide and increase their cell number during the first 12 h after temperature up-shift. During this period the number of viable cells increased at approximately the same rate as the total cell number for *rad1*-S5 *wee1*-50 and *rad1*-S6 *wee1*-50; by contrast, for the remaining mutants the fraction of viable cells decreased rapidly throughout the length of incubation (Figure 9B; see also Figure 7B). A morphological examination of these strains at 36°C revealed highly wee cells with aberrantly shaped nuclei, in agreement with what has previously been reported for $rad1^0$ *wee1*-50 cells (Al-Khodairy and Carr, 1992) (Figure 10).

DISCUSSION

From the results obtained from the mutant *rad1* alleles in this work, it is possible to dissect the *rad1* phenotypes into three groups (Table 2). The first is the G2 DNA damage checkpoint (as witnessed by resistance to UV and γ radiation and the ability to delay mitosis after irradiation), the second is the DNA replication checkpoint (as witnessed by HU resistance and the ability to delay mitosis after HU treatment), and the third is viability in a *cdc17* or *wee1* background. *rad1*-S3 retains the DNA damage checkpoint, but has lost both the replication checkpoint and viability when combined with *cdc17*-K42 or *wee1*-50. *rad1*-S1 and *rad1*-S2 have both checkpoints intact, but are inviable as *cdc17* or *wee1* double mutants. *rad1*-S4 was deficient in all functions tested.

Both for exposure to radiation and inhibition of DNA replication, the degree of resistance for all alleles correlated perfectly with the ability to arrest in the cell cycle (Figure 2 and Table 2). By contrast, this is not the case for *rad26* mutants, where the *rad26*-T12 mutant retains G2 arrest capability, yet has a significant residual radiation sensitivity (Al-Khodairy *et al.*, 1994). From these studies, there is thus no evidence for a direct role of rad1 in DNA repair distinct from G2 arrest although such a possible role has not been disproved. This issue has previously been interpreted somewhat differently (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992). There was also close agreement between UV and γ resistance (Figure 2, A vs. B and C vs. D; Table 2). This is not surprising because these agents cause overlapping sets of DNA lesions, and resistance to these types of radiation correlates closely in different experimental settings for many checkpoint mutants (Al-Khodairy *et al.*, 1994). Further, the extent of genetic interaction between a certain *rad1* allele with *cdc17* correlated entirely with the extent of interaction of the same allele with *wee1*-50 (Figure 7 and Table 2). Genetic interaction with these two mutations do not always go together; for *rad21* there is interaction with *cdc17* but not with *wee1*-50 (Al-Khodairy and Carr, 1992), whereas the converse is true for *rad30* (Al-Khodairy *et al.*, 1994). Finally, in all cases examined there was close agreement between the results from chromosomal versus multi-copy alleles.

The *rad1*-S3 allele is distinguished by partially retaining one mitotic checkpoint function but having lost the other (Table 2). As mentioned in the INTRODUCTION, several genes in both *S. cerevisiae* and *S. pombe* are required for just one of these functions. Another mutant allele of a gene required for both mitotic checkpoints that is similar in this respect is *rad26*-T12 (Al-Khodairy *et al.*, 1994); however, *rad26*-T12 cells are different from *rad1*-S3 in having a G2 DNA damage checkpoint response identical to wild-type but still being significantly radiation sensitive, as mentioned above. In *rad1*-S3 cells there is a partial defect both in radiation resistance and in radiation-induced cell-cycle delay (Figures 2 and 3).

Our finding that two alleles, *rad1*-S1 and *rad1*-S2, retain full checkpoint functions as well as radiation and HU resistance as single mutants, yet confer lethality when combined with either the *cdc17*-K42 or the *wee1*-50 mutation (Table 2), was unexpected but not unprecedented. The recently isolated *S. pombe huw*-T17 mutant has intact radiation checkpoint and replication feedback control, yet this mutant has enhanced lethality when combined with *cdc17*-K42 or *wee1*-50 (Al-Khodairy *et al.*, 1994).

We see two alternative models for the function of the rad1 protein that are consistent with the separation of phenotypes and their distribution among the mutant alleles observed in this work. The first model, favored by us, assumes that rad1 fulfills qualitatively different functions. There would be at least three functions, and they would be defined by the three categories of phenotypes discerned in this paper: 1) the G2 DNA damage checkpoint; 2) the replication checkpoint; and 3) a function related to retention of viability in *wee1*-50 and *cdc17*-K42 mutant backgrounds at the nonpermissive temperature. It is possible to envisage rad1 as a docking protein, a member of one or more multi-protein complexes, where interaction with some components is required for one function, and interaction with a separate set of protein components for another function. It could further be speculated that one or several checkpoint proteins in concert bind to replication complexes or DNA repair complexes, as the need may be (Sheldrick and Carr, 1993). This paradigm has gained credibility from the demonstration that DNA polymerases are directly involved in the coupling of mitosis to DNA replication (Francesconi *et al.*, 1995; Navas *et al.*, 1995).

Under this model, the separation of the G2 DNA damage checkpoint from the replication checkpoint in *rad1*-S3 would be because the function of this allele in the replication checkpoint is selectively inactivated. From a mechanistic perspective, the idea has been put forward that the G2 DNA damage and DNA replication checkpoints act in quite distinct manners. Specifically, although there is evidence that the DNA replication checkpoint operates through phosphorylation of Y15 of cdc2 in *S. pombe* (Enoch and Nurse, 1990; Enoch *et al.*, 1992; Sheldrick and Carr, 1993), several mutants deficient in this phosphorylation control (and in the replication checkpoint) are proficient in G2 DNA damage checkpoint function (Sheldrick and Carr, 1993). It is thus possible that the G2 DNA-damage checkpoint has a different effector mechanism than phosphorylation of cdc2 Y15.

The phenotypes of *rad1*-S1 and S2 can be interpreted by assuming that rad1 performs another function, unrelated to checkpoints. The existence of this other

function would be revealed by synthetic lethality with *wee1* or *cdc17*, and it would be impaired in the *rad1*-S1 and *rad1*-S2 mutants. In line with this is the observation that some extragenic suppressors of lethality of *rad1 wee1* double mutants do not restore checkpoint function (Jimenez and Subramani, unpublished observations). Also, mutations in several *rad* genes such as $rad1^+$ confer considerably more HU sensitivity than mutations affecting cell-cycle control genes directly, e.g. *cdc2*-3w (Enoch *et al.*, 1992). This suggests that the HU sensitivity of such mutants is partly due to something other than the cell-cycle control defect. Finally, our observation that *rad1*-S1 *cdc17*-K42 and *rad1*-S2 *cdc17*-K42 double mutants do arrest and elongate at 36°C, in contrast to $rad1^0$ *cdc17*-K42, suggests that such cells die for a reason unrelated to the G2 DNA damage and replication checkpoints. Possibly this undefined function of rad1 is in a pathway distinct from checkpoints, which are by definition nonessential.

An alternative view would be that the function performed by rad1 is one and the same, and that the differing effects on the various phenotypes is a reflection of different quantitative requirements of rad1 function. Under this model, viability in a *cdc17* or *wee1*-50 background would require the highest level, the replication checkpoint would require a lower level, and the G2 DNA damage checkpoint would require the lowest level of rad1 activity. We consider this alternative less likely than the first, for the following reasons: 1) in no case examined were any significant differences found between expression from a chromosomal allele or from a multi-copy episomal allele; 2) blocking of replication is likely to produce at least as extensive DNA damage as radiation, yet *rad1*-S3 cells clearly arrest following γ irradiation but their rate of mitotic entry is unaffected by HU; and 3) *rad1*-S1 *cdc17*-K42 and *rad1*-S2 *cdc17*-K42 cells elongate and arrest on temperature shift like rad^+ *cdc17*-K42 cells (indicating intact checkpoint function), yet die. In the light of these facts, we favor the first model.

In the course of our mutation analysis, we were also able to identify the carboxy-terminal acidic tail as a potential negative regulatory region of the rad1 protein. The *rad1*-S5 and *rad1*-S6 alleles sustain growth at a higher temperature than wild-type $rad1^+$ in both a *cdc17*-K42 and a *wee1*-50 background (Figures 7B and 9). In other words, these two *rad1* mutations behave as "superalleles" in these genetic backgrounds. This is suggestive of the stretches of acidic aa deleted in *rad1*-S5 and S6, constituting negative signals of some type. They could be, for example, part of a protein degradation signal or a negative regulatory domain.

A cdc17 (DNA ligase) deficiency during replication results in an accumulation of single-strand breaks and other replication intermediates. These could serve as the signal for inhibition of mitosis as well as of DNA replication. In both these cases, the protein encoded by *rad1*-S5 or S6 could transmit this signal more strongly than wild-type rad1. Inactivation of $wee1^+$ leads to premature mitosis (Russell and Nurse, 1987). The products of *rad1*-S5 and S6 may improve survival in this situation by acting as inhibitors of mitosis. Chromosome loss would then be decreased by allowing more time for the G2/M transition. This view is reinforced by our finding that all *rad1 wee1*-50 double mutants at 36°C increase their total cell number (Figure 9A), although the viable cell count for the $rad1^0$, *rad1*-S1, and *rad1*-S2 alleles in this background decreases rapidly (Figure 9B). We interpret this as the result of unrestrained mitoses, producing genetically dead cells. In contrast, *rad1*-S5 *wee1*-50 and *rad1*-S6 *wee1*-50 cells go through a higher proportion of balanced mitoses, leading to net growth. If these differences are reflected in morphological variations between the mutants, they are too subtle to be detected with certainty in our present examination. The number of mutations studied in this work is too small to allow definitive conclusions concerning structure/function relationships in the rad1 protein, nor has this been the aim of this work. Further studies involving larger sets of mutations created in a systematic manner are required to determine whether the functions of the rad1 protein can be assigned to specific domains, as for example, the polyacidic tail of the *S. cerevisiae* Rad6 protein, which is required for ubiquitination of histones and sporulation, but not for radiation resistance (Morrison *et al.*, 1988; Sung *et al.*, 1988). The well-characterized mutations described in this work will be valuable tools when dissecting checkpoint pathways in eukaryotes, as well as when defining the biochemical function(s) of the rad1 protein. With this perspective, an attractive path to follow would be to analyze genetic or biochemical interactions of the *rad1* alleles described in this work with the products of other genes.

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REFERENCES

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

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

Booher, R., and Beach, D. (1986). Site-specific mutagenesis of $cdc2^+$, a cell-cycle control gene of the fission yeast *Schizosaccharomyces pombe*. Mol. Cell. Biol. *6*, 3523–3530.

Carr, A.M., and Hoekstra, M.F. (1995). The cellular responses to DNA damage. Trends Cell Biol. *5*, 32–40.

Dahlkvist, A., Kanter-Smoler, G., and Sunnerhagen, P. (1995). The *RCK1* and *RCK2* protein kinase genes from *Saccharomyces cerevisiae* suppress cell cycle checkpoint mutations in *Schizosaccharomyces pombe*. Mol. Gen. Genet. *246*, 316–326.

Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T.D., Lees, E., Harper, J.W., Elledge, S.J., and Reed, S.I. (1994). p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell *76*, 1013–1023.

Enoch, T., Carr, A.M., and Nurse, P. (1992). Fission yeast genes involved in coupling mitosis to completion of DNA replication. Genes Dev. *6*, 2035–2046.

Enoch, T., and Nurse, P. (1990). Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. Cell *60*, 665–673.

Ford, J.C., Al-Khodairy, F., Fotou, E., Sheldrick, K.S., Griffiths, D.J.F., and Carr, A.M. (1994). 14–3-3 protein homologs required for the DNA damage checkpoint in fission yeast. Science *265*, 533–535.

Francesconi, S., De Recondo, A.M., and Baldacci, G. (1995). DNA polymerase delta is required for the replication feedback control of cell cycle progression in *Schizosaccharomyces pombe*. Mol. Gen. Genet. *246*, 561–569.

Grimm, C., Kohli, J., Murray, J., and Maundrell, K. (1988). Genetic engineering of *Schizosaccharomyces pombe*: a system for gene disruption and replacement using the *ura4* gene as a selectable marker. Mol. Gen. Genet. *215*, 81–86.

Gutz, H., Heslot, H., Leupold, U., and Loprieno, N. (1974). *Schizosaccharomyces pombe*. In: Handbook of Genetics, vol. 2, ed. R.C. King, New York: Plenum Press, 395–446.

Hannan, M.A., Miller, D.R., and Nasim, A. (1976). Changes in UV-inactivation kinetics and division delay in *Schizosaccharomyces pombe* strains during different growth phases. Radiat. Res. *68*, 469–479.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 CDK-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805–816.

Hartwell, L.H., and Weinert, T.A. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science *246*, 629–634.

Higuchi, R. (1990). Recombinant PCR. In: PCR protocols: methods and applications, ed. M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, San Diego, CA: Academic Press, 177–183.

Jimenez, G., Yucel, J., Rowley, R., and Subramani, S. (1992). The *rad3*$^+$ gene of *Schizosaccharomyces pombe* is involved in multiple checkpoint functions and in DNA repair. Proc. Natl. Acad. Sci. USA *89*, 4952–4956.

Kunkel, T.A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA *82*, 488–492.

Long, K.E., Sunnerhagen, P., and Subramani, S. (1994). The *Schizosaccharomyces pombe rad1* gene consists of three exons and the cDNA sequence is partially homologous to the *Ustilago maydis REC1* cDNA. Gene *148*, 155–159.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of the fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. *194*, 795–823.

Morrison, A., Miller, E.J., and Prakash, L. (1988). Domain structure and functional analysis of the carboxy-terminal polyacidic sequence of the RAD6 protein of *Saccharomyces cerevisiae*. Mol. Cell. Biol. *8*, 1179–1185.

Nasim, A. (1968). Repair-mechanisms and radiation-induced mutations in fission yeast. Genetics *59*, 327–333.

Nasim, A., and Smith, B.P. (1975). Genetic control of radiation sensitivity in *Schizosaccharomyces pombe*. Genetics *79*, 573–582.

Navas, T.A., Zhou, Z., and Elledge, S.J. (1995). DNA polymerase ϵ links the DNA replication machinery to the S phase checkpoint. Cell *80*, 29–39.

Rowley, R., Subramani, S., and Young, P.G. (1992). Checkpoint controls in *Schizosaccharomyces pombe-rad1*. EMBO J. *11*, 1335–1342.

Russell, P., and Nurse, P. (1987). Negative regulation of mitosis by *wee1*$^+$, a gene encoding a protein kinase homolog. Cell *49*, 559–567.

Saka, Y., Fantes, P., Sutani, T., Mcinerny, C., Creanor, J., and Yanagida, M. (1994). Fission yeast *cut5* links nuclear chromatin and M phase regulator in the replication checkpoint control. EMBO J. *13*, 5319–5329.

Saka, Y., and Yanagida, M. (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.

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

Savitsky, K., *et al.* (1995). A single *Ataxia Telangiectasia* gene with a product similar to PI-3 kinase. Science *268*, 1749–1753.

Schlake, C., Ostermann, K., Schmidt, H., and Gutz, H. (1993). Analysis of DNA repair pathways of *Schizosaccharomyces pombe* by means of *swi-rad* double mutants. Mutat. Res. *294*, 59–67.

Schüpbach, M. (1971). The isolation and genetic classification of UV-sensitive mutants of *Schizosaccharomyces pombe*. Mutat. Res. *11*, 361–371.

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

Sung, P., Prakash, S., and Prakash, L. (1988). The RAD6 protein of *Saccharomyces cerevisiae* polyubiquitinates histones, and its acidic domain mediates this activity. Genes Dev. *2*, 1476–1485.

Sunnerhagen, P., Seaton, B.L., Nasim, A., and Subramani, S. (1990). Cloning and analysis of a gene involved in DNA repair and recombination, the *rad1* gene of *Schizosaccharomyces pombe*. Mol. Cell. Biol. *10*, 3750–3760.

Thiebaut, F., Enns, R., and Howell, S.B. (1994). Cisplatin sensitivity correlates with its ability to cause cell cycle arrest via a Wee1 kinase-dependent pathway in *Schizosaccharomyces pombe*. J. Cell. Physiol. *159*, 506–514.

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

Weinert, T.A., and Hartwell, L.H. (1988). The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. Science *241*, 317–322.

Weinert, T.A., Kiser, G.L., and Hartwell, L.H. (1994). Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. *8*, 652–665.