

Extragenic Suppressors of *Schizosaccharomyces pombe rad9* Mutations Uncouple Radioresistance and Hydroxyurea Sensitivity From Cell Cycle Checkpoint Control

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ABSTRACT

Schizosaccharomyces pombe cells that contain a mutation within *rad9* are sensitive to ionizing radiation, UV light and hydroxyurea, relative to wild-type strains. In addition, the mutants are moderately hypomutable by UV and unable to delay initiation of mitosis after treatment with radiation or hydroxyurea. Three radioresistant derivatives of *rad9::ura4* cells were isolated, and each contained a single unique extragenic suppressor responsible for the acquired resistance. The suppressor loci also conferred radioresistance upon cells containing *rad9-192*, which differs from *rad9+* by a single base pair change. The suppressors additionally enhanced the radioresistance of cells containing *rad3-136*, a mutation that leads to phenotypes similar to those mediated by *rad9::ura4*. None of the derivatives of *rad9::ura4* cells recovered the ability to delay cycling in G2 after exposure to ionizing radiation or UV light. All three suppressor derivatives, relative to the parental *rad9::ura4* strain, also exhibited a moderate increase in resistance to the DNA replication inhibitor hydroxyurea without gaining the ability to stop progression into mitosis despite the inhibition of DNA synthesis. Results are discussed in terms of models to explain the putative role of *rad9* and the suppressor genes in promoting radioresistance and mediating checkpoint controls responsive to DNA damage or incomplete DNA replication.

THERE are many loci in the fission yeast *Schizosaccharomyces pombe* responsible for promoting cell survival after exposure to radiation (SCHÜPBACH 1971; NASIM and SMITH 1975; LIEBERMAN *et al.* 1989; SCHMIDT *et al.* 1989; AL-KHODAIRY and CARR 1992; for reviews, see PHIPPS *et al.* 1985; SUBRAMANI 1991; AL-KHODAIRY *et al.* 1994; FORD *et al.* 1994). Mutations within these genes cause sensitivity to ionizing radiation, UV light or both types of radiation. In addition, some also influence mutability and recombination ability.

S. pombe cells containing a mutation within the *rad9* gene are dramatically sensitive to UV light and ionizing radiation, relative to wild-type strains (SCHÜPBACH 1971), indicating that the corresponding protein plays an important role in promoting cell survival after irradiation. Furthermore, alterations within this gene can cause moderately reduced mutability by UV light (GENTNER *et al.* 1978). The *rad9+* gene has been isolated and its DNA sequence has been determined (MURRAY *et al.* 1991; LIEBERMAN *et al.* 1992). The gene or its corresponding amino acid sequence is not homologous to any other sequence or motif characteristic of defined functions or structures previously entered into data banks.

Recently, several groups demonstrated that *rad9* mutations, as well as alterations in other *S. pombe* genes [*i.e.*, *rad1*, *rad3*, *rad17*, *rad24*, *rad25*, *rad26* and *chk1(rad27)*],

prevent cells from transiently accumulating in G2 after irradiation (*i.e.*, these mutant cells are missing the G2/M molecular checkpoint control) (AL-KHODAIRY and CARR 1992; ROWLEY *et al.* 1992b; WALWORTH *et al.* 1993; AL-KHODAIRY *et al.* 1994; FORD *et al.* 1994). It is thought that this checkpoint serves to detect DNA damage and, if found, allows cells time to repair the damage before entry into mitosis and segregation of chromosomes (for review, see HARTWELL and WEINERT 1989). Therefore, the lack of this checkpoint is believed to be at least partly responsible for the radiosensitivity exhibited by *S. pombe rad9* mutant cells because they enter mitosis prematurely, with radiation-induced DNA damage, an event likely to lead to chromosome breakage.

Hydroxyurea, a potent inhibitor of DNA replication (KRAKOFF *et al.* 1968), induces mitotic delay in wild-type *S. pombe*, specifically inhibiting cell cycle progression in S phase (MITCHISON 1974; ENOCH and NURSE 1990). Cells containing a mutation within *rad9* (or *rad1*, *rad3*, *rad17*, *rad26*, *hus1* or *hus2*) are extremely sensitive to hydroxyurea (AL-KHODAIRY and CARR 1992; ROWLEY *et al.* 1992b; ENOCH *et al.* 1992; AL-KHODAIRY *et al.* 1994). Mutations in *hus3*, *hus4* or *hus5* are reported to cause a "late cut or constant cut" phenotype, whereby they promote sensitivity to hydroxyurea but have some cell cycle delay after exposure to the drug. Mutant *rad9* cells treated with this chemical stop DNA replication but do not stop mitosis, and often die as septated cells. Therefore, *rad9* participates in the checkpoint controls

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that are responsive to DNA damage and perturbations in DNA replication because alterations within this gene permit continued cell cycle progression after irradiation or treatment with hydroxyurea.

To identify gene products that could promote radioresistance in the absence of a functional *rad9* gene product, radioresistant derivatives of *S. pombe* cells containing *rad9::ura4* were sought. These suppressors of the disruption mutant would probably affect "bypass" pathways rather than interacting proteins. Thus, it is unlikely, although not impossible, that interactors would be found in this way. This article describes the isolation of three *rad9::ura4* cell derivatives that exhibit resistance to both ionizing radiation and UV light at levels between those observed for *rad9⁺* and *rad9::ura4* cell populations. Genetic analyses revealed that an alteration in a single unique locus, unlinked to *rad9* in each of the derivatives, conferred the radioresistance demonstrated. In addition, cells containing *rad9-192*, which is shown to differ from *rad9⁺* by a single base pair change, leading to the synthesis of a proline in place of leucine, also exhibited enhanced radioresistance when any of the three suppressor alleles were present. Interestingly, the three suppressors also confer radioresistance upon cells containing a mutation in *rad3* (*rad3-136*), another gene involved in checkpoint control and radioresistance. Further characterization of the radioresistant *rad9::ura4* derivatives indicated that they were unable to delay cycling in G2 after exposure to ionizing radiation or UV light. In addition, all three derivatives showed intermediate levels of resistance to hydroxyurea but did not regain the ability to delay entry into mitosis in response to drug treatment. These results are discussed in terms of putative roles for the suppressor genes, as well as *rad9*, in checkpoint control and resistance to both radiation and hydroxyurea.

MATERIALS AND METHODS

Yeast strains, media and genetic manipulations: The *S. pombe* strains used in this investigation were SP223 (*h⁻ ura4-294 leu1-32 ade6-216*), SP224 (*h⁺ ura4-294 leu1-32 ade6-216*), derivatives of SP223 that contain *rad3-136* (SCHÜPBACH 1971), *rad9::ura4* or *rad9-192* (LIEBERMAN *et al.* 1992), and derivatives of SP223 and these mutant strains that contain the extragenic suppressors, *srr-1*, *srr2-1* and *srr3-1*, which were made as part of this study. A *wee 1-50 ade5-36* strain was also used. Dr. M. McLEOD (SUNY Downstate Medical Center, NY) provided SP223 and SP224. Dr. A. NASIM (King Faisal Specialist Hospital, Riyadh, Saudi Arabia) provided the original *rad3-136* and *rad9-192* mutants. Cells were grown routinely in YEA liquid (0.5% Bacto yeast extract, supplemented with adenine at 75 μ g/ml, and 3.0% glucose) or on YEA agar (same as YEA liquid but with 2.0% agar) at 30°. Minimal medium (MM), as liquid (0.67% Bacto nitrogen base, supplemented with adenine and required amino acids at a concentration of 75 μ g/ml, and 0.5% glucose) or agar-based (liquid MM and 2.0% agar), was also used as indicated. The growth media, as well as the techniques used for genetic crosses and tetrad analyses, were described previously (MITCHISON 1970; GUTZ *et al.* 1974).

Determination of sensitivity to radiations or hydroxyurea:

Sensitivity of cells to UV light or ionizing radiation was determined qualitatively by a simple spot test and quantitatively by establishing dose-response curves, using previously published procedures (LIEBERMAN *et al.* 1989). However, for the gamma-ray source, a Gammacell 220 irradiator (Nordion International, Inc., Kanata, Ontario, Canada) containing ⁶⁰Co, with a dose rate of 1.68 Gy/min, was used.

Sensitivity to hydroxyurea (HU) was determined by growing cells in MM liquid to log phase (approximately 5×10^6 cells/ml) and then adding the drug to a final concentration of 10 mM. Immediately, and at indicated times after reincubation at 30°, aliquots of cells were removed, diluted and plated on agar medium to yield 200–400 colonies per petri dish. The number of colonies appearing after treatment relative to the number formed from cultures immediately before exposure to the drug was calculated and expressed as a percentage.

Synchronization of cells: Cells were synchronized in the late S to early G2 phase of the cell cycle using a modified version of procedures described by MITCHISON and CARTER (1975). Briefly, 100 ml of a log phase culture, grown to $\sim 2 \times 10^7$ cells/ml of MM supplemented with adenine, leucine and uracil, were pelleted and resuspended in 1 ml of supplemented MM. Cells were then layered onto an approximately 15-ml lactose nine-stage step gradient, ranging from 30% lactose at the bottom to 7% on the top. The gradient was made by adding 1.2 ml of supplemented MM devoid of glucose but containing decreasing concentrations of lactose. The stock solutions for the steps in the gradient were made by mixing 30% and 7% solutions of lactose in the following ratios: 10:0, 8.75:1.25, 7.5:2.5, 6.25:3.75, 5.0:5.0, 3.75:6.25, 2.5:7.5, 1.25:8.75 and 0:10. Gradients were spun at $500 \times g$ for 8 min, or until a 1-inch smear of cells had migrated one half to two thirds down the tube. Subsequently, 300 μ l of liquid just above the bottom of the smear were collected. Cells were pelleted in an Eppendorf tube by spinning 5 sec in a microfuge and then resuspended in supplemented MM containing 10 mM HU to test the sensitivity of cells to the drug. Immediately, and at times thereafter, cells were sampled and the septation index of the population was scored as described below.

Isolation of radioresistant strains: A derivative of *S. pombe* strain SP223, containing *rad9::ura4*, was mutagenized with ethylmethane sulfonate as described by SHERMAN *et al.* (1986). After treatment, $\sim 40\%$ of the population remained viable. Subsequently, cells were resuspended in YEA liquid medium, allowed to incubate for several hours at 30°, exposed to 475 Gy of gamma rays (from a ¹³⁷Cs source, dose rate of 1.32 Gy/min) and reincubated overnight at 30°. Cells were then diluted, reirradiated and grown overnight. After another cycle of dilution, irradiation and growth overnight, cell suspensions were diluted and plated onto YEA agar to allow formation of distinct colonies after incubation at 30° for 3–4 days. Single colonies were then picked, purified and spot tested for the acquisition of radioresistance relative to *rad9⁺* and *rad9::ura4* cell populations.

DNA manipulations, genomic library construction and screening for *rad9-192*: *S. pombe* genomic DNA was prepared using procedures described by HOFFMAN and WINSTON (1987). A library was made from the total genomic DNA of the *S. pombe rad9-192* mutant strain by digesting both genomic and plasmid pUC19 DNA with the restriction enzymes *EcoRI* and *HindIII* (New England BioLabs, Beverly, MA) because these enzymes cut the gene into two fragments convenient for cloning and subsequent DNA sequence determination. Vector-genomic DNA ligation reactions (SAMBROOK *et al.* 1989) were used to transform *Escherichia coli* HB101 (HANAHAN 1985) to generate a library consisting of $\sim 2 \times 10^5$ clones. The wild-type *rad9* gene, isolated from plasmid pHH4.2 on a 4.2 kbp *HindIII*-generated DNA fragment (LIEBERMAN *et al.*

1992) and radiolabeled with ^{32}P by random primer synthesis (FEINBERG and VOGELSTEIN 1983), was used to screen the library by *in situ* colony hybridization (SAMBROOK *et al.* 1989). Two plasmids, pUC19HR and pUC19RH, which together span the entire *rad9-192* gene region, were isolated and used for further studies.

DNA sequence determination and analysis: The dideoxy chain termination method described by SANGER *et al.* (1977) was used to determine the DNA sequence of both strands of the *rad9-192* gene. Universal M13 oligonucleotide primers, as well as synthetic 20 bp oligonucleotides (produced by the Columbia University DNA Core Facility) corresponding to regions along the gene, were used in conjunction with pUC19HR and pUC19RH. The DNA sequence of *rad9-192* has been submitted to the EMBL data bank. It has been assigned the Accession Number X65864.

PCR analysis: PCR (INNIS and GELFAND 1990; KAWASAKI 1990) was used to amplify *rad9*-containing genomic DNA from both wild-type and *rad9-192* *S. pombe* cells for subsequent DNA restriction enzyme analyses and from wild-type, *rad9::ura4* and suppressor strains for analyses of fragment size. Specifically, 169 bp of wild-type and *rad9-192* mutant genomic DNA, presumably containing the single nucleotide base pair difference between the *rad9⁺* and *rad9-192* genes, were amplified. Oligonucleotides, generated by the DNA Core Facility of Columbia University, were used. The reaction mix contained 1 μg of genomic DNA, 1 \times PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 2.5 mM MgCl_2 , and 0.1 mg/ml of bovine serum albumin), 0.2 mM of each deoxynucleotide triphosphate, and 100 pmol each of oligonucleotides #1A (5'-CGA-CCTCTTCTGTGAAACTT-3') and #5 (5'-ACCCAAACTTTA-CACGCTGT-3') (LIEBERMAN *et al.* 1992) in a final total volume of 100 μl . Samples were boiled for 2 min and then cooled on ice for 5 min. After the addition of *Taq* polymerase (2.5 units) to the mixture, the reaction tubes were incubated in a thermal cycler and subjected to the following conditions: 95°—1 min, 55°—2 min, 72°—3 min for 10 cycles; 95°—1 min, 55°—1 min, 72°—3 min for 19 cycles; 95°—1 min, 55°—1 min, 72°—10 min for 1 cycle. Amplified fragments were purified from a low melting temperature agarose gel. DNA fragments were treated with restriction enzyme *AluI* and then analyzed after electrophoresis through a 2.0% agarose gel. To amplify *rad9* regions in genomic DNA flanking the site where *ura4* was inserted, PCR was performed as described, but with oligonucleotides #12 (5'-CTGCAAATTTAAAGCAGCTA-3') and #24 (5'-CAGTCTAAGCTTCTGGATGAGTCACTTGTCTA-3') instead of #1A and #5. The reaction products were, without additional purification, run on and visualized in a 1.0% agarose gel.

Measuring cell cycle delay: Cell cultures in log phase and exposed to gamma rays (50 Gy in YEA liquid medium), to UV light (50 J/m^2 in sterile H_2O , and then resuspended in YEA liquid medium) or synchronized in late S or early G2 and treated with HU (10 mM in MM liquid medium), or mock-treated, were maintained as described for routine growth and sampled at intervals. Cells were placed in a hemocytometer, and cell number as well as the presence of septa were determined using either 150 \times or 600 \times magnification, respectively. A cell was considered to be undergoing septum formation and completing mitosis if a septum was clearly visible and cell division, indicated by formation of a "notch" at the union of two cells, had not started (MITCHISON 1970). The number of cells within a population having a septum, relative to the population as a whole, is referred to as the septation index and is expressed as a percentage (septation index ranges from 0 to 1.0, where, for example, 10% septation is a septation index of 0.1). Each data point illustrated for these measure-

ments represents an average of at least two trials and the scoring of a minimum of 400 cells.

Fluorescence activated cell sorter analysis: The cell cycle stage distribution of log phase *S. pombe* populations was determined by fluorescence activated cell sorter analysis, using a modification of a previously published protocol (SAZER and SHERWOOD 1990). Exponentially growing cells ($\sim 10^7$), in MM liquid medium, were pelleted and resuspended in 3 ml of ice-cold H_2O . Seven milliliters of ice-cold ethanol was added dropwise as the cells were vortexed, and the cell suspension was then stored at 4° overnight or longer. Cells were then pelleted, washed once in 5 ml of 50 mM sodium citrate, pH 7.0, and then resuspended in 1 ml of the sodium citrate solution. Fifty microliters of a 5 mg/ml preboiled RNase solution was then added, and the tube was incubated for 1 hr at 37°. One milliliter of a 50 mM sodium citrate, pH 7.0, solution, containing 2.5 μg of propidium iodide, was then added. Samples were stored for ≥ 24 hr, and as long as 1 week, before being analyzed on a Coulter Epics Elite ESP fluorescence activated cell sorter (Coulter Corporation, Miami, FL) located at Cold Spring Harbor Laboratory (Cold Spring Harbor, NY).

RESULTS

Isolation of radioresistant derivatives of *S. pombe rad9::ura4* cells: Two thousand one hundred fifty-six *S. pombe rad9::ura4* cells surviving the radioresistance enrichment scheme and forming colonies were tested for the acquisition of radioresistance by a simple spot test. Three gamma-ray resistant derivatives were identified in this manner. The names assigned to the putative non-*rad9* suppressor genes responsible for the increased radioresistance in each are *srr1-1*, *srr2-1* and *srr3-1*, indicating suppressor of *rad9* radiosensitivity. As shown in Figure 1A, they exhibit gamma-ray resistance levels between those observed for *rad9⁺* and *rad9::ura4* cells. The D_0 values (*i.e.*, dose that permits 37% survival) in Gy for *rad9⁺*, *rad9::ura4*, and the *srr1-1 rad9::ura4*, *srr2-1 rad9::ura4* and *srr3-1 rad9::ura4* strains are approximately 930, 97, 478, 361 and 369, respectively.

Figure 1B illustrates the sensitivity of the same set of strains to UV light. As for gamma rays, the derivatives exhibit intermediate levels of UV light resistance. For this type of radiation, the D_0 values in J/m^2 exhibited by *rad9⁺*, *rad9::ura4*, and the *srr1-1*, *srr2-1* and *srr3-1* strains are ~ 100 , 5, 69, 28 and 21, respectively.

The radioresistant derivatives each contain a single unique extragenic suppressor locus that also confers radioresistance upon cells containing *rad9-192* or *rad3-136* but has no detectable effect on *rad9⁺* cells: To confirm that the radioresistant derivatives retained the *ura4* disruption within *rad9*, analyses of the region of insertion was performed by using PCR. Oligonucleotide primers #1A and #5 (LIEBERMAN *et al.* 1992) (see MATERIALS AND METHODS), which flanked that site, were used to amplify the region in appropriate strains. A 1.5-kbp fragment is found when *rad9⁺* genomic DNA is used. However, a 3.4-kbp fragment, representing *rad9* and *ura4* sequences, was synthesized when DNA from either the original *rad9::ura4* parent or derivatives also containing *srr1-1*, *srr2-1* or *srr3-1* are used. These results

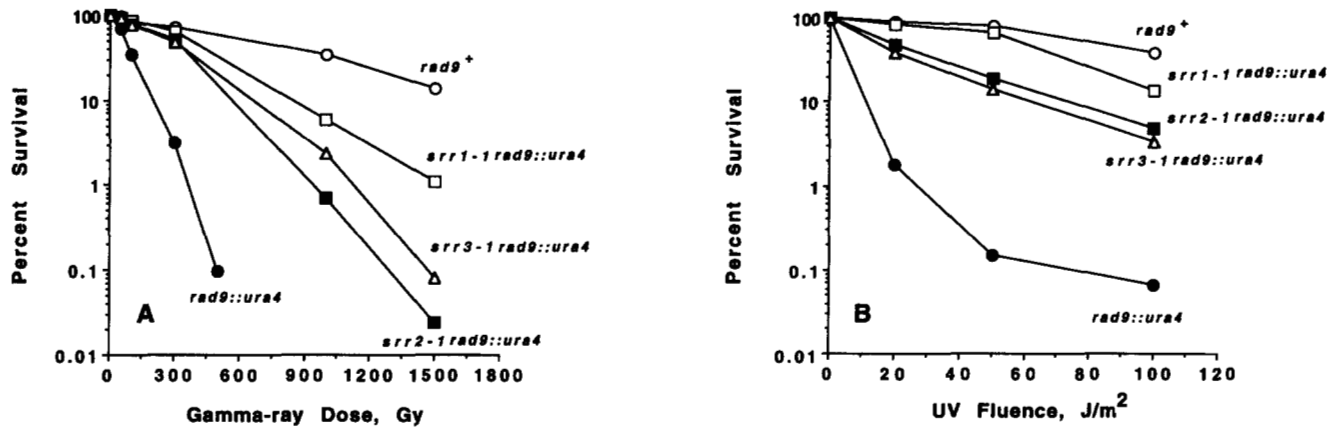


FIGURE 1.—Sensitivity of *S. pombe* cells to (A) gamma rays or (B) UV light. Strains: *rad9*⁺ (○), *rad9::ura4* (●), *srr1-1 rad9::ura4* (□), *srr2-1 rad9::ura4* (■), *srr3-1 rad9::ura4* (△).

indicate that all three derivatives are radioresistant despite the retention of *rad9::ura4*.

To determine whether *srr1-1*, *srr2-1* and *srr3-1* each represented a single locus within radioresistant derivatives, the corresponding populations were crossed with an *h*⁺ *rad9::ura4* strain and resulting tetrads were dissected. Cells within colonies grown from progeny spores were then tested for resistance to both UV light and ionizing radiation. Of 10 tetrads examined from each cross, all exhibited a 2:2 segregation of radioresistance to radiosensitivity. These results indicate that *srr1-1*, *srr2-1* and *srr3-1* most likely represent single loci.

To determine whether *srr1-1*, *srr2-1* and *srr3-1* are allelic, each suppressor locus was crossed, when necessary, into a related genetic background containing *rad9::ura4* and the opposite mating type. Strains containing potentially different suppressors were then mated. Random spore analysis was used to determine whether crosses could generate progeny sensitive to UV light, which would indicate that the suppressor loci in the original crosses were nonallelic. When strains containing *srr1-1* and *srr2-1* are mated, 26% of the progeny (102 of 390) are sensitive to UV light, indicating that these two loci are different and are unlinked. Similar results were obtained with the cross between *srr1-1* and *srr3-1*, where the percent of radiosensitive progeny was 28% (132 of 480). The genetic cross between *srr1-1* and *srr2-1* yielded radiosensitive colonies at a frequency of 13% (59 of 443), indicating that these loci are also not allelic but are genetically linked. In conclusion, these results indicate that *srr1-1*, *srr2-1* and *srr3-1* are three distinct loci capable of partially suppressing the radio-sensitivity promoted by *rad9::ura4*.

To determine whether the extragenic suppressors could enhance the radioresistance of cells containing *rad9-192*, another mutant allele of *rad9*, strains that contained this mutation in addition to *srr1-1*, *srr2-1* or *srr3-1* were constructed. All of these strains exhibited resistance to ionizing radiation and UV light at levels similar to those observed for the radioresistant *rad9::ura4*-con-

taining strains (data not shown). Therefore, the suppressor alleles not only have the ability to bypass loss of function of *rad9* because the disruptant is suppressed, but they can also promote radioresistance in cells containing a point mutation in *rad9* (see below).

Cells containing the *rad3-136* mutation exhibit phenotypes similar to those observed for strains harboring *rad9-192* or *rad9::ura4*. They are radiosensitive and lack the G2/M checkpoint control. Strains containing *rad3-136* and *srr1-1*, *srr2-1* or *srr3-1* were constructed and tested for radiosensitivity to determine whether the suppressor loci can enhance the resistance of *rad3-136*-containing cells to UV light or ionizing radiation. Because all double mutants could be constructed, none of the suppressor loci are allelic with *rad3*. In addition, as shown in Figure 2, all of the suppressors increase the resistance of *rad3-136* mutant cells to both ionizing radiation and UV light.

To determine whether the suppressors affect the radioresistance of *rad9*⁺ cells, *srr1-1*, *srr2-1* and *srr3-1* were crossed into a *rad9*⁺ genetic background. The sensitivity of all of these cell populations to UV light and ionizing radiation was indistinguishable from the survival levels observed for cells containing *rad9*⁺ alone (data not shown).

A single nucleotide base pair difference between *rad9*⁺ and *rad9-192*: Southern blot analysis (SOUTHERN 1975) of the *rad9* gene region in genomic DNAs from *rad9*⁺ and *rad9-192* cells cut with restriction enzymes *Dde*I, *Eco*RI, *Eco*RV, *Hind*III, *Hpa*II, *Nde*I, *Rsa*I, *Sau*3AI or *Xba*I revealed no detectable differences between the two versions of *rad9* (data not shown), suggesting that a point mutation or some other small modification within the gene is most likely responsible for the phenotypes associated with the mutant. To precisely identify the alteration at the DNA level, *rad9-192* was isolated from a genomic library made with *Hind*III/*Eco*RI-cut DNA of a strain containing the mutant allele. Approximately 15,000 clones in the library were screened, using as a probe the 4.2-kbp DNA fragment from plasmid

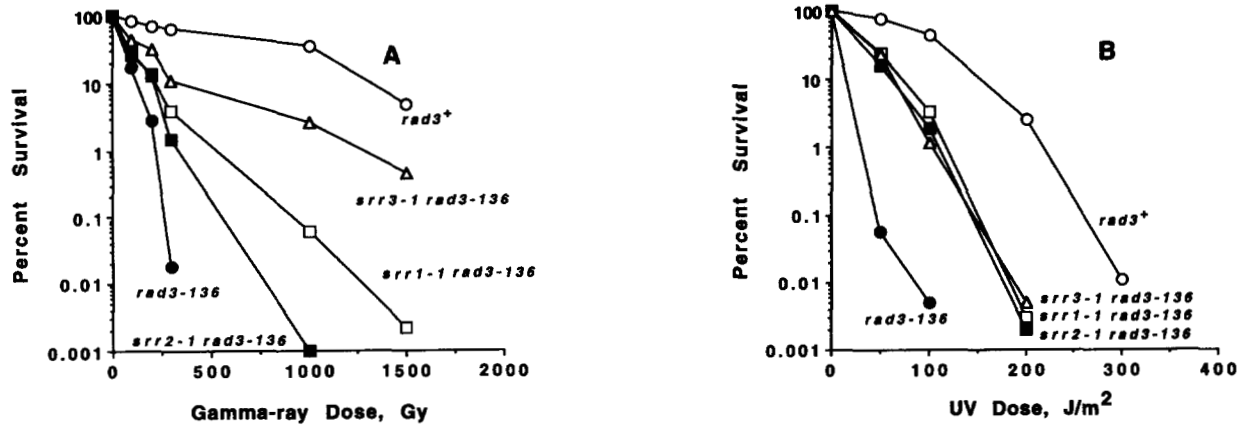


FIGURE 2.—Sensitivity of *S. pombe* cells to (A) gamma rays or (B) UV light. Strains: *rad3*⁺ (○), *rad3-136* (●), *srr1-1 rad3-136* (□), *srr2-1 rad3-136* (■), *srr3-1 rad3-136* (△).

pHH4.2 that contains the wild-type *rad9* gene and its flanking regions (LIEBERMAN *et al.* 1992). Two positive clones, designated pUC19HR and pUC19RH, were found to contain inserts that together span the entire *rad9-192* gene region.

The sequences of the *rad9-192* gene regions within pUC19HR and pUC19RH (2229 bp total) were determined and compared with the previously determined sequence of *rad9*⁺ (LIEBERMAN *et al.* 1992). These sequences include the entire coding region, three introns and 5' as well as 3' flanking regions. A single nucleotide base pair difference was detected and corresponded to codon 196 of *rad9*. As shown in Figure 3A, the mutation is a T to C transition. This change leads to the substitution of a proline for leucine in the *rad9* protein (Figure 3C).

Experiments were performed to confirm that the single base pair difference found in the cloned *rad9-192* gene is present in the genome. An *AluI* restriction site (AGCT), found in the wild-type *S. pombe* sequence, overlaps the point mutation of *rad9-192* and predictably would be missing in the mutant DNA (AGCC). PCR was used to amplify a 169-bp region, within both mutant and wild-type genomic DNA, that spans the site of non-homology. Figure 3B shows a 2.0% agarose gel of the mutant and wild-type amplified DNA untreated or treated with *AluI*. The 169-bp DNA fragment made from wild-type *S. pombe* DNA is digested with *AluI*, resulting in the production of two smaller DNA fragments, 58 and 111 bp long. A 169-bp fragment produced from the DNA of the *rad9-192* mutant is not cut by the enzyme. Therefore, the single base pair difference between *rad9*⁺ and *rad9-192* is present in the genomes of the corresponding strains.

Experiments were also performed to confirm that the point mutation was sufficient to inactivate *rad9*. First, the *rad9-192* gene region was reconstituted from plasmids pUC19HR and pUC19RH by ligating together the genomic DNA inserts. This newly formed gene was unable to confer radioresistance upon *rad9-192*-containing *S. pombe* cells. Second, a 817-bp *EcoRI/MluI*

fragment within *rad9-192*, which differed from the corresponding region of the *rad9*⁺ gene by only the point mutation, was used to replace that region within the active wild-type gene in the genomic DNA fragment of pHH4.2 (LIEBERMAN *et al.* 1992). The plasmid containing this altered *rad9* was not capable of restoring radioresistance to *rad9-192* cells. Therefore, the point mutation is present in the genome of *rad9-192*-containing cells and is sufficient to neutralize *rad9* activity.

The three radioresistant strains have not regained the G2/M checkpoint control: Wild-type *S. pombe* cells transiently delay cycling in G2 after irradiation, and this temporary cycling arrest is thought to allow cells time to repair their damaged DNA before entry into mitosis. At least part of the radiosensitivity exhibited by *rad9* mutant cells is believed to be due to their inability to block cell cycle progression after irradiation. To determine whether the extragenic suppressor strains are radioresistant because they are able to delay cycling in response to DNA damage, the frequency of cells blocked at the G2/M border after irradiation was monitored by determining the proportion of cells within a population bearing septum. As shown in Figure 4, *rad9*⁺ cells exhibit a drop in septation index (to 0.88%) within 30 min after exposure to 50 Gy of gamma rays. After ~3 hr, they return to a frequency of 12%. Populations of cells containing *rad9::ura4* exhibit a constant frequency of septa (~15%), even though they have also been exposed to 50 Gy of gamma rays. The *rad9::ura4* profile is also found for derivatives containing *srr1-1*, *srr2-1* and *srr3-1*. In addition, these patterns are reflected by the growth of the populations after irradiation (data not shown). All populations, except those containing *rad9*⁺, undergo at least one or two doublings immediately after treatment, with the same kinetics as the unirradiated populations. The *rad9*⁺ population delays division transiently after irradiation. Furthermore, a UV dose of 50 J/m² decreases the septation index in *rad9*⁺ cells but fails to do so in the *rad9::ura4* cell population or in suppressor-containing strains (data

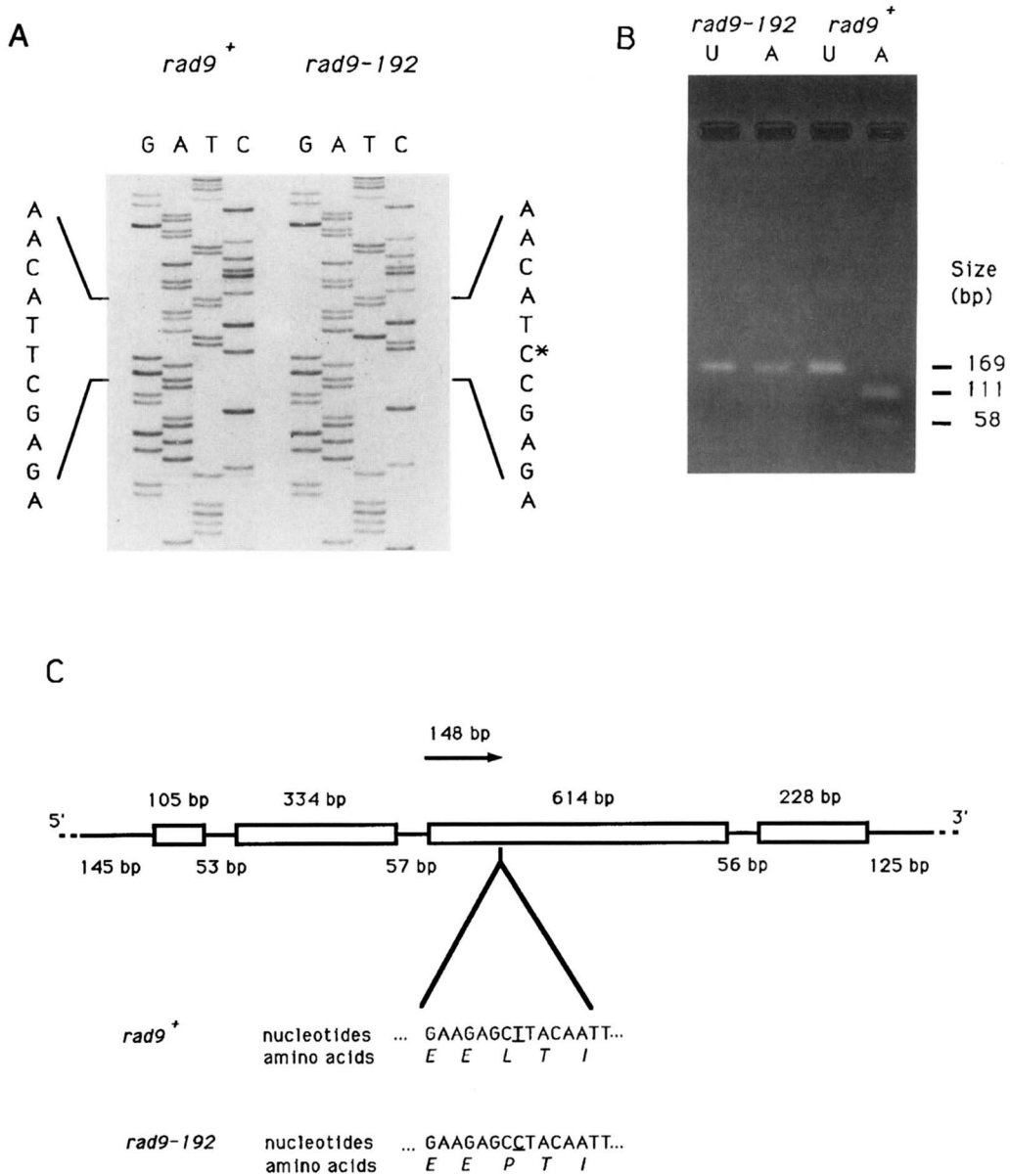


FIGURE 3.—Identification of the mutation within *rad9-192*. (A) Autoradiograph of a polyacrylamide gel showing DNA sequence of *S. pombe rad9*⁺ and *rad9-192* genes. (B) Agarose gel containing *AluI* restriction enzyme-treated (A) or untreated (U) PCR-generated DNA fragments from *S. pombe rad9*⁺ and *rad9-192* genes. (C) Schematic representation of *S. pombe rad9*⁺ and *rad9-192* genes. Indicated are protein coding regions (105, 334, 614 and 228 bp long, respectively), introns (53, 57 and 56 bp long, respectively) and 5' (145 bp) and 3' (125 bp) untranslated regions. The mutation within *rad9-192* occurs 148 bp from the start of the third exon. The nucleotide base change from thymidine to cytidine is illustrated, as is the predicted change from leucine (L) to proline (P).

not shown). In addition, all strains formed similar size colonies. All five strains, if unirradiated and grown at 30° for the same amount of time as the irradiated cultures, demonstrate a constant frequency of septa ranging from 10 to 15%. These results indicate that the suppressors do not confer radioresistance upon *rad9* mutant strains because of restoring the ability to delay cycling at the G2/M border after irradiation.

The three radioresistant strains exhibit normal cell division kinetics: Growth kinetics were examined to determine whether a protracted cell cycle time was

responsible for the increased radioresistance exhibited by the *rad9::ura4* strains bearing either *srr1-1*, *srr2-1* or *srr3-1*. Strains containing *rad9*⁺, *rad9::ura4* or the latter mutation in combination with either *srr1-1*, *srr2-1* or *srr3-1* exhibited comparable growth kinetics (data not shown). All populations had a doubling time, during logarithmic growth, of ~3 hr. These results indicate that a prolonged cell cycle is not the manner in which the suppressors increase the radioresistance of strains containing a mutation within the *rad9* gene. In addition, there was no gross difference

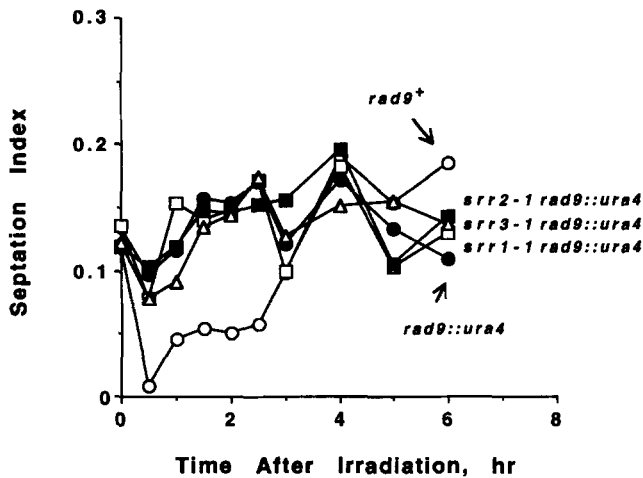


FIGURE 4.—Effects of exposure to gamma rays on septation index. Cells were exposed to 50 Gy of gamma rays and immediately as well as at indicated times, cells were sampled and the presence of septa within the population was determined, as described in MATERIALS AND METHODS. Strains: *rad9*⁺ (○), *rad9::ura4* (●), *srr1-1 rad9::ura4* (□), *srr2-1 rad9::ura4* (■), *srr3-1 rad9::ura4* (△).

in the cell size or morphology of all the populations examined.

Fluorescence activated cell sorter analysis was performed on the *rad9*⁺, *rad9::ura4* and *rad9::ura4*/suppressor double mutant strains to determine whether the *srr* alleles alter the distribution of *rad9::ura4* cell populations within the cell cycle. As illustrated in Figure 5, the *wee1-50* control population contains prominent G1 and G2 peaks and serves as a marker for these phases of the cell cycle. All other strain profiles were essentially identical, with the exception of the *rad9::ura4 srr2-1* strain, which showed a detectable percent of cells in G1, in addition to the usual large population in G2. Because, in general, cells in G1 are more radiosensitive

than cells in G2, the small shift in cell cycle phase observed cannot account for the radioresistance demonstrated by the *rad9::ura4 srr2-1* double mutants.

The three radioresistant strains exhibit intermediate levels of hydroxyurea resistance and no hydroxyurea-induced cell cycle delay: *S. pombe* strains containing a mutation within *rad9* attempt mitosis despite the presence of unreplicated DNA after treatment with hydroxyurea and are sensitive to the drug relative to *rad9*⁺ cells. These drug-treated mutant cells are small and most have a septum. In addition, they arrest cell cycle progression with this morphology and die (Figure 6). The response of suppressor gene-containing *rad9::ura4* strains to hydroxyurea treatment was examined to test whether *srr1-1*, *srr2-1* or *srr3-1* can complement the altered response of *rad9* mutant cells to the drug. As shown in Figure 6, the suppressor strains exhibit somewhat higher levels of resistance to hydroxyurea, relative to that observed for the *rad9::ura4* strain. The *srr1-1* and *srr2-1* derivatives are the most drug resistant, whereas *srr3-1 rad9::ura4* cells are repeatedly but not dramatically more resistant than *rad9::ura4* cells (*i.e.*, especially after 6 and 7 hr of treatment). In addition, *rad9*⁺ strains containing *srr1-1*, *srr2-1* or *srr3-1* exhibit hydroxyurea resistance levels comparable with the level observed for the *rad9*⁺ *srr*⁺ strain (SP223; data not shown). Therefore, the ability of *srr1-1*, *srr2-1* and *srr3-1* to increase the resistance of cells to hydroxyurea is *rad9::ura4*-dependent. Cell survival (Figure 6) and cell cycle checkpoint (Figure 7) experiments are presented for cells cultured no longer than 6 or 7 hr with hydroxyurea because the drug only prevents bulk DNA synthesis in fission yeast for a short period of time. In a synchronous culture, fluorescence activated cell sorter analysis demonstrates that, in the presence of 10 mM hydroxyurea, bulk DNA synthesis is complete in 6–8 hr. There-

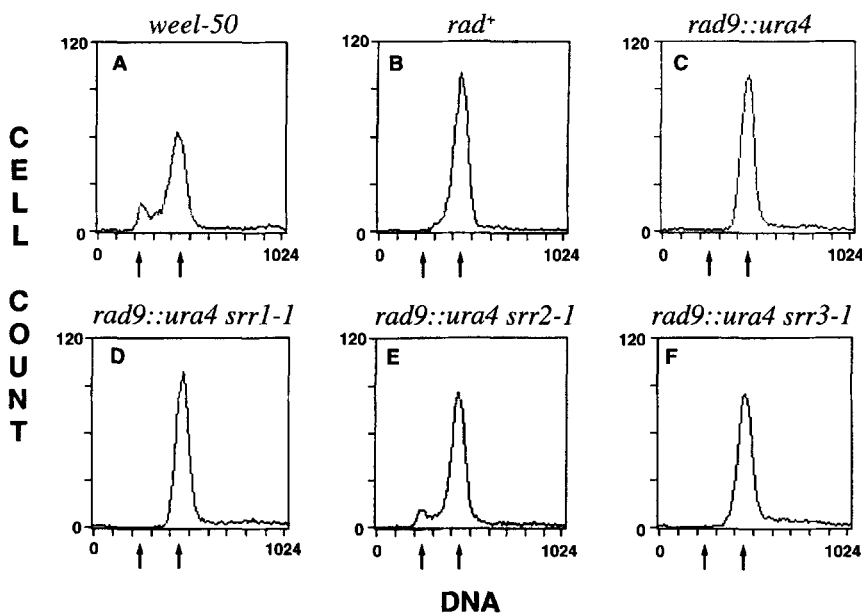


FIGURE 5.—Fluorescence activated cell sorter analyses. See MATERIALS AND METHODS for experimental details. Left arrows at bottom of graphs indicate G1 amount of DNA; right arrows indicate G2 levels. Strains: (A) *wee1-50*, (B) *rad*⁺, (C) *rad9::ura4*, (D) *rad9::ura4 srr1-1*, (E) *rad9::ura4 srr2-1*, (F) *rad9::ura4 srr3-1*.

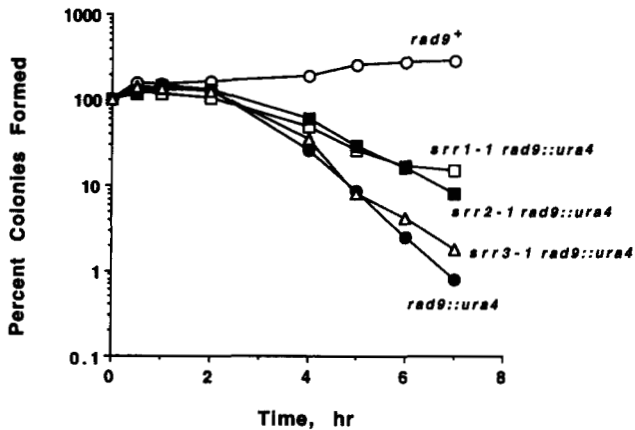


FIGURE 6.—Sensitivity of *S. pombe* cells to hydroxyurea. Strains: *rad9*⁺ (○), *rad9::ura4* (●), *srr1-1 rad9::ura4* (□), *srr2-1 rad9::ura4* (■), *srr3-1 rad9::ura4* (△).

fore, it is difficult to interpret survival and septation data, in terms of checkpoint control, when drug treatment goes beyond the time when bulk DNA replication is occurring.

Figure 7 illustrates the percentage of each initially late S to early G₂-synchronized population bearing a septum after various times of culturing in the absence or presence of 10 mM hydroxyurea. Unlike for the experiments performed to monitor radiation-induced checkpoint control, synchronized cultures were required for the study of hydroxyurea-induced changes in cell cycle progression to yield unambiguous data. As shown in Figure 7A, all untreated cultures were well synchronized in late S to early G₂ and maintained that synchrony at least through one cell division cycle. As illustrated in Figure 7B, the septation index for *rad9*⁺ cells drops to zero during continued incubation in drug-containing medium, reflecting a cycling delay and, presumably, the accumulation of cells in S phase. The septation index for drug-treated cell populations containing *rad9::ura4*, and others additionally bearing

srr1-1, *srr2-1* or *srr3-1*, initially drops. However, the septation index does not level off but instead rises and cells accumulate in a morphological state characterized by the presence of a prominent septum. Thus, these cells appear to attempt mitosis despite being cultured in the presence of hydroxyurea.

DISCUSSION

Three radioresistant derivatives of *S. pombe rad9::ura4* cells have been isolated. Genetic analyses revealed that each contains a single unique non-*rad9* locus capable of mediating the radioresistance observed. The names *srr1*, *srr2* and *srr3* (suppressor of *rad9* radiosensitivity) have been assigned to the genes, and *srr1-1*, *srr2-1* and *srr3-1* correspond, respectively, to their active alleles. All three extragenic suppressors of *rad9::ura4* only partially restore wild-type levels of resistance to both gamma rays and UV light. Furthermore, the suppressors also increase the radioresistance of strains containing *rad9-192*, another mutant allele of *rad9*.

To gain insight into the function of *rad9* and better understand the activity of the suppressors, the *rad9-192* gene was isolated. DNA sequence analyses revealed that it differs from *rad9*⁺ by a single nucleotide base pair. The *S. pombe* mutant containing *rad9-192* was originally isolated as a radiosensitive derivative from a population of cells mutagenized with 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) (SCHÜPBACH 1971). The single base pair change found in the mutant is a transition from T to C. Interestingly, MNNG typically causes G to A transitions, but the transition observed still falls within the range of alterations induced by the drug (HAMPSEY 1991). The change in the mutant leads to the replacement of a leucine by a proline. Although a single amino acid alteration is consistent with the change being located in a critical place in the protein, the addition of proline has the potential to induce gross modifications in protein structure that could occur at sites distal to

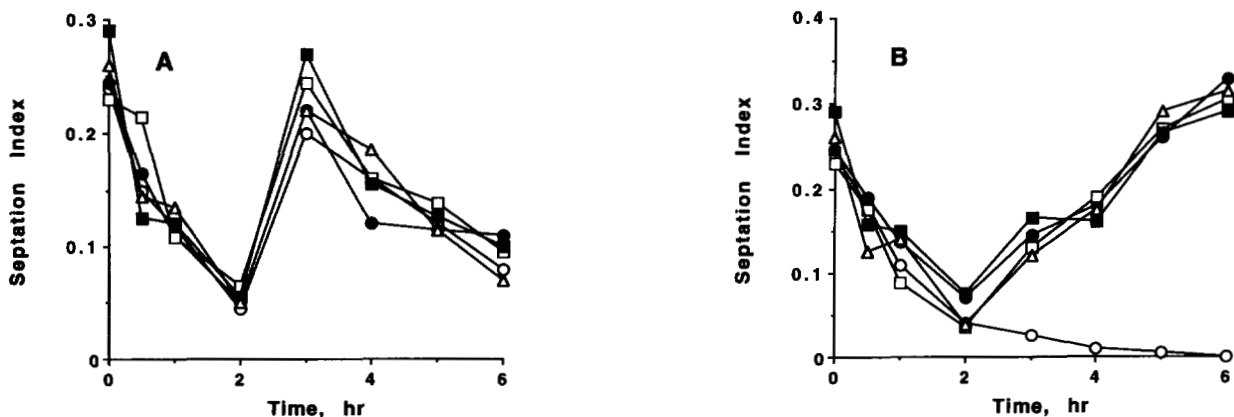


FIGURE 7.—Effects of incubation in hydroxyurea on septation index. Synchronized cells were incubated in supplemented MM liquid medium either devoid of hydroxyurea (A) or containing the drug at a concentration of 10 mM (B). Immediately, as well as at indicated times thereafter, cells were sampled and the presence of septa within the population was determined, as described in MATERIALS AND METHODS. Strains: *rad9*⁺ (○), *rad9::ura4* (●), *srr1-1 rad9::ura4* (□), *srr2-1 rad9::ura4* (■), *srr3-1 rad9::ura4* (△).

the effected region. This marked change in protein structure makes the strain containing *rad9-192* as radio-sensitive and defective in the G2 delay phenotype as cells containing the *rad9* gene disruption, *rad9::ura4* (LIEBERMAN *et al.* 1992; AL-KHODAIRY and CARR 1992; ROWLEY *et al.* 1992b; Figure 3, this study). Because the suppressors were isolated by virtue of their ability to complement a defect mediated by the disruption mutation (*rad9::ura4*) and there is a lack of specificity in relation to the rescued allele (*i.e.*, *srr1-1*, *srr2-1*, and *srr3-1* enhance the radioresistance of strains containing *rad9::ura4* or *rad9-192*), it is unlikely that the suppressor gene products interact directly with the *rad9* protein. This is supported further by the nature of the *rad9::ura4* disruption. The *ura4* gene was inserted 67 bp downstream from the start of translation, at a *Clal* site initially cleaved with the restriction enzyme and then partially digested with an exonuclease, thereby essentially eliminating *rad9* production (LIEBERMAN *et al.* 1992). Therefore, because the suppressors can function in a *rad9* null genetic background, it is likely that they act in a survival-promoting mechanism that does not directly involve *rad9*, making this separate pathway more efficient and therefore able to at least partly compensate for a defect in *rad9*. Furthermore, at least two suppressor alleles, *srr1-1* and *srr2-1*, act dominantly in relation to *srr1*⁺ and *srr2*⁺, respectively, when present in a diploid *rad9::ura4* genetic background heterozygous for the suppressor loci (*srr1*⁺/*srr1-1*; *srr2*⁺/*srr2-1*) and tested for radioresistance (*i.e.*, the diploids are relatively radioresistant; HBL, data not shown). These results suggest that *srr1-1* and *srr2-1* could represent gain of function alleles. In addition, the radioresistance or morphology of *rad9*⁺ cells is not affected by *srr1-1* or *srr2-1* (or *srr3-1*), indicating that a suppressor-related phenotype, as tested thus far, is dependent on the absence of *rad9* function. Because the screen identified three mutants in three different complementation groups, it is likely that alterations in additional loci could produce similar phenotypes.

Mutations within *S. pombe rad9* eliminate the classical delay in the G2 phase of the cell cycle observed after exposure to radiation (AL-KHODAIRY and CARR 1992; ROWLEY *et al.* 1992b). The delay, which is expressed as part of a G2/M molecular checkpoint control (WEINERT and HARTWELL 1988; for review, see HARTWELL and WEINERT 1989), is thought to provide time for cells to repair damaged DNA before entry into mitosis and segregation of chromosomes. Therefore, at least part of the radiosensitivity demonstrated by *rad9::ura4* or *rad9-192* cells is most likely due to the lack of G2 delay and premature entry into mitosis after irradiation, an event that could lead to chromosome breaks if DNA damage is present and be lethal if pursued. Interestingly, all three extragenic suppressors increase the radioresistance of *rad9* mutant cells without restoring the ability to delay cycling. Furthermore, suppressor strains

do not have an unusually long overall cell cycle time, another mechanism that potentially could have compensated for the lack of a radiation-inducible G2 delay response. A redistribution of cell cycle phase length, for example, a longer G2 in combination with a shorter G1 phase, has been ruled out for the function of *srr1-1* and *srr3-1* because fluorescence activated cell sorter analyses show essentially no difference in the cell cycle phase profiles of wild-type, *rad9::ura4*, or *srr1-1* and *srr3-1* derivatives of the latter. However, relative to these strains, the *rad9::ura4 srr2-1* double mutant has a somewhat protracted G1 phase. Nevertheless, cells in G1, relative to those in other phases of the cell cycle, are, in general, highly radiosensitive (FABRE 1973). Therefore, this minimal cell cycle phase redistribution cannot account for the increased radioresistance promoted by *srr2-1* in a *rad9::ura4* mutant genetic background.

The lack of the G2/M checkpoint control in the suppressor strains is also consistent with the possibility that *rad9* not only improves survival after irradiation by controlling the cell cycle, perhaps by acting via the *cdc2*, *wee1* and/or *chk1* gene products (AL-KHODAIRY and CARR 1992; ROWLEY *et al.* 1992a,b; BARBET and CARR 1993; WALWORTH *et al.* 1993), but may in addition have some other role in promoting radioresistance. For example, *rad9* could directly or, via the regulation of some other protein(s), indirectly participate in repair of damaged DNA. JIMENEZ *et al.* (1992) suggested that *S. pombe rad3*, another G2/M checkpoint control gene, may also have a role in DNA repair because an artificial block in G2 by treatment with benomyl did not increase radioresistance of *rad3-136* cells to approximate wild-type levels. Interestingly, *srr1-1*, *srr2-1* and *srr3-1* were able to partially suppress the radiosensitivity of cells containing *rad3-136*. One possibility to explain this seemingly dual role for *rad9* (and perhaps also for *rad3*) is that the *rad9* protein can detect DNA damage and/or contribute to a signal that alerts the cell cycle machinery and repair enzymes to the presence of DNA damage. Because the suppressors do not restore the checkpoint control function missing in cells containing a defective *rad9*, they may increase or optimize the repair capability of these mutant strains. However, it is plausible that *rad9* may not participate in DNA repair at all, and increased or optimized repair, which is perhaps related to the function of the suppressors, could still enhance the radioresistance of mutants containing *rad9::ura4* or *rad9-192*.

Exposure of *rad9*⁺ cells to hydroxyurea, a chemical that disrupts DNA replication (KRAKOFF *et al.* 1968), causes a delay in the S phase of the cell cycle (MITCHISON 1974; ENOCH and NURSE 1990). *S. pombe* cells containing *rad9::ura4* or *rad9-192* lack this checkpoint control that is responsive to perturbations of DNA replication (AL-KHODAIRY and CARR 1992; ROWLEY *et al.* 1992b; Figure 7, this study). These drug-treated mutants continue cycling into mitosis, where they prematurely form septa and die. Hence, the inability to delay

cycling when DNA replication is incomplete contributes to the killing of *rad9* mutant cells by hydroxyurea. Cells containing a mutation within *rad9* in combination with *srr1-1*, *srr2-1* or *srr3-1* demonstrate moderately increased, though less than wild-type, levels of drug resistance. However, cells continue into mitosis despite exposure to hydroxyurea.

The results of this investigation indicate that the suppressor genes, in a *rad9* mutant genetic background, participate in promoting increased survival after radiation exposure or treatment with hydroxyurea. The role of the suppressor genes in promoting resistance to hydroxyurea does not include the ability of *srr1-1*, *srr2-1* and *srr3-1* to restore the delay of mitosis in response to incomplete DNA replication. In a similar manner, the suppressors can uncouple the G2/M delay response from radioresistance because they increase the ability of *rad9::ura4* cells to survive exposure to gamma rays or UV light without restoring the irradiation-associated checkpoint control. No detectable effects on resistance to hydroxyurea or radiation are observed in a *rad⁺* genetic background. The molecular processes that mediate the increased drug and radiation resistance of *rad9::ura4* cells in the absence of appropriate checkpoint controls remain to be defined.

The *S. pombe* genes *rad1*, *rad3*, *rad17*, *rad24*, *rad25*, *rad26* and *chk1* also control cell cycle delay after irradiation and/or hydroxyurea treatment (AL-KHODAIRY and CARR 1992; ROWLEY *et al.* 1992b; WALWORTH *et al.* 1993; AL-KHODAIRY *et al.* 1994; FORD *et al.* 1994). Interestingly, *srr1-1*, *srr2-1* and *srr3-1* can improve the radioresistance of *rad3-136*-containing cells, as well as those containing a mutation in *rad9*, indicating a common as of yet to be defined mechanistic link between at least *rad9*, *rad3* and the *srr* genes. FORD *et al.* (1994) determined that *rad24* and *rad25* share homology with 14-3-3 proteins, thus implicating this class of protein in cell cycle checkpoint control. *chk1* links at least *rad1* to *cdc2*, the major mediator of the transition of cells from G2 to M. It was identified by WALWORTH *et al.* (1993) as a muticopy suppressor of the cold-sensitive *S. pombe cdc.r4* mutant and was subsequently shown to increase the radioresistance of *rad1* mutant cells when overexpressed. Independently, AL-KHODAIRY *et al.* (1994) identified *chk1* (*rad27*) in a screen for mutants that lack radiation-induced checkpoint control. ENOCH *et al.* (1992) recently identified a series of loci, named *hus1*, *hus2*, *hus3*, *hus4* and *hus5*, that regulate the hydroxyurea induced cell cycle block. Mutations in these *hus* loci also increase the sensitivity of cells to UV light, indicating that these genetic elements also participate in both the cellular response to irradiation and to the disruption of DNA replication. Of note is that *hus3*, *hus4* and *hus5* mutants have some delay after hydroxyurea treatment and, thus, like the *srr* genes, can uncouple sensitivity from cell cycle arrest. However, further analysis of *hus5* indicates that it is not directly involved in checkpoint control

(AL-KHODAIRY *et al.* 1995). An understanding of the functional relationships among the *hus*, checkpoint control-related *rad* and *srr* loci, however, must await additional investigation. The molecular analysis of the suppressor genes, and the biochemical characterization of the proteins they encode, should help elucidate their roles in regulating the cell cycle and/or promoting cell survival after irradiation or treatment with agents that block DNA replication. A complex network of gene interactions and interrelationships is beginning to emerge.

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