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Characterization of *RAD9* of *Saccharomyces cerevisiae* and Evidence that Its Function Acts Posttranslationally in Cell Cycle Arrest after DNA Damage

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In eucaryotic cells, incompletely replicated or damaged chromosomes induce cell cycle arrest in G2 before mitosis, and in the yeast *Saccharomyces cerevisiae* the *RAD9* gene is essential for the cell cycle arrest (T. A. Weinert and L. H. Hartwell, Science 241:317–322, 1988). In this report, we extend the analysis of *RAD9*-dependent cell cycle control. We found that both induction of *RAD9*-dependent arrest in G2 and recovery from arrest could occur in the presence of the protein synthesis inhibitor cycloheximide, showing that the mechanism of *RAD9*-dependent control involves a posttranslational mechanism(s). We have isolated and determined the DNA sequence of the *RAD9* gene, confirming the DNA sequence reported previously (R. H. Schiestl, P. Reynolds, S. Prakash, and L. Prakash, Mol. Cell. Biol. 9:1882–1886, 1989). The predicted protein sequence for the Rad9 protein bears no similarity to sequences of known proteins. We also found that synthesis of the *RAD9* transcript in the cell cycle was constitutive and not induced by X-irradiation. We constructed yeast cells containing a complete deletion of the *RAD9* gene; the *rad9* null mutants were viable, sensitive to X- and UV irradiation, and defective for cell cycle arrest after DNA damage. Although Rad$^+$ and *rad9*Δ cells had similar growth rates and cell cycle kinetics in unirradiated cells, the spontaneous rate of chromosome loss (in unirradiated cells) was elevated 7- to 21-fold in *rad9*Δ cells. These studies show that in the presence of induced or endogenous DNA damage, *RAD9* is a negative regulator that inhibits progression from G2 in order to preserve cell viability and to maintain the fidelity of chromosome transmission.

In eucaryotic cells, DNA replication must be complete and chromosomes must be undamaged for an interphase cell to progress into mitosis. Inhibition of DNA synthesis or induction of DNA damage leads to arrest of the cell cycle before mitosis and chromosome segregation (1, 4, 5, 18, 31, 54). Studies from several organisms suggest that cell cycle arrest after DNA damage is due to extrinsic control mechanisms rather than to a structural requirement of mitosis for intact chromosomes (reviewed in reference 18). For example, caffeine treatment of mammalian cells antagonizes the arrest induced either by DNA damage or by inhibition of DNA synthesis and causes the arrested interphase cells to enter mitosis prematurely (6, 44, 48, 52). In addition, several mutations that eliminate the inhibition of mitosis when chromosomes are incompletely replicated or damaged have been identified in several organisms, including *Saccharomyces cerevisiae* (55), *Schizosaccharomyces pombe* (11), and *Aspergillus nidulans* (38), and in a hamster cell line (35). Study of the mutants and corresponding wild-type gene products should reveal underlying molecular mechanisms that ensure that mitosis does not proceed until chromosomes are fully replicated and intact.

The *RAD9* gene in the yeast *S. cerevisiae* is required for cell cycle arrest after DNA damage induced by X-irradiation (55) or incompletely replicated chromosomes in cells deficient for DNA ligase (*cdc9*) (18). For example, X-irradiated wild-type yeast cells arrest before chromosome segregation, in the G2 phase of nuclear division, and proceed through mitosis only after repair of the DNA damage. (It is not known whether yeast cells with DNA damage are blocked in a G2 interphaselike state [seen in other eucaryotes with DNA damage] or in metaphaselike state because in budding yeast cells there are no observable cytological landmarks to distinguish G2 from metaphase. Whether the timing and nature of the G2-to-metaphase transition in *S. cerevisiae* are the same as in other eucaryotes remains a matter of controversy [36, 46]. We recognize the ambiguity and refer to the *RAD9*-dependent control as regulating the progression from G2 to anaphase.) In contrast to the arrest response of wild-type cells, X-irradiated *rad9* cells proceed without delay through G2 and chromosome segregation, and the cells die. Yeast cells with a temperature-sensitive defect in DNA ligase (*cdc9* mutants) also die more rapidly at the restrictive temperature in *rad9* cells than in *RAD9* cells (18, 47). The defect in *rad9* mutant cells appears to be solely in cell cycle control because *rad9* cells survive X-irradiation if progression from G2 is delayed by treatment with a microtubule inhibitor; the drug-imposed delay provides the cell time to complete DNA repair before chromosome segregation (55). Studies in mammalian cells also indicate that DNA repair essential for chromosome integrity occurs during the delay in G2 (6, 25, 52).

We report here that the *RAD9*-dependent arrest occurs by a posttranslational mechanism(s) and that the *RAD9* gene is nonessential for growth in unperturbed cells, though it contributes to the fidelity of chromosome transmission. After these studies had been completed, the DNA sequence of *RAD9*, confirmed in this study, was reported (47).

MATERIALS AND METHODS

Yeast and bacterial strains. The *S. cerevisiae* strains used in this study (Table 1) were congenic with A364a except for

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TABLE 1. Yeast and bacterial strains

Strain	Genotype	Reference or source
Yeast		
7815-6-4	*MATa rad9-1 ade2 leu2 ura3*	54
7815-6-4ΔB	*MATa rad9Δ::URA3 ade2 leu2 ura3*	This study
7236	*MATa/MATα his3/+ +/his7 can1/+ cyh2/+ ura3/ura3 leu2/leu2 trp1/trp1*	T. Formosa
7859	*MATa/MATα his3/+ +/his7 can1/+ rad9Δ::LEU2 cyh2/+ ura3/ura3 leu2/leu2 trp1/trp1*	This study
8200-1	*MATα ade2 ura3 trp1 can1 sap3*	L. Kadyk
8200-rad9-IURA3	*MATα rad9-IURA3 ade2 ura3 trp1 can1 sap3*	This study
5344-2a	*MATa ste9-1 ade2 ura3 lys2 SUP4 cry1*	This laboratory
7830-2-4	*MATa ura3 leu2 trp1 his3*	This laboratory
7832-2	*MATa ura3 leu2 trp1 his3 rad9Δ::LEU2*	This laboratory
7859-10-2	*MATa ura3 leu2 his7 trp1*	This laboratory
7859-7-4	*MATa ura3 leu2 his7 trp1 rad9Δ::LEU2*	This laboratory
7225	*MATa ura3 can1 spa3 hxk2::CAN1 + aro2 + + + ade6 +/+ lys5 + cy2 trp5 leu1 + ade3*	T. Formosa
7225-1	*MATa ura3 can1 spa3 hxk2::CAN1 + aro2 + + + ade6 +/+ lys5 + cy2 trp5 leu1 + ade3 rad9Δ::URA3*	This study
7860-3	*MATa/MATα ura3/ura3 leu2/leu2 trp1/+ his3/+ rad9Δ::LEU2/rad9Δ::LEU2 + +/can1 hom3*	This study
7861-3	*MATa/MATα leu2/leu2 trp1/+ his3/+ rad9Δ::LEU2/+ +/his7 + ura3 +/can1 ura3 hom3*	This study
7862-1	*MATa/MATα ura3/ura3 leu2/leu2 trp1/+ +/his7 his3/+ +/sap3 + +/can1 hom3*	This study
Bacterial		
TB1	*ara Δ(lac-proAB) rspL ϕ80 lacZ<*M15 (r_K^+ m_K^+)	T. Baldwin

7815-6-4, which was derived from a cross between A364a and an unrelated strain, X56-8D (55). Most strains were derived from the diploid 7236, provided by T. Formosa. All plasmids were propagated in the bacterium *Escherichia coli* TB-1 since the bacterial strains HB101 and DH1 could not be efficiently transformed by plasmids containing yeast DNA sequences linked to the *RAD9* gene (unpublished observations). The reason for the inhibition of transformation, or inhibition of growth, of certain *RAD9*-linked plasmids in strains HB101 or DH1 is unknown.

Standard yeast genetic methods were used (50). Cells were grown either in rich medium (YM-1 liquid medium or YEPD solid medium) or in minimal medium minus uracil as described previously (16, 50). Plasmids were introduced into yeast cells by lithium acetate transformation (23) or after generation of spheroplasts (22).

Determination of radiation sensitivity. Sensitivity to radiation was determined by exposing cells on solid agar plates to radiation from a Machlett OEG 60 X-ray tube operated at 50 kV and 20 mA, delivering a dose of 106 rads/s. For qualitative tests, freshly grown patches of cells were replicated twice in succession (double replicas) and irradiated with 8 krad at the time of replica transfer and 8 to 16 h later; radiation-resistant survivors were identified after growth for 2 to 3 days at 23°C. To determine quantitatively the sensitivity to radiation, logarithmically growing cells were plated on solid medium and X-irradiated, and colonies from survivors were counted after incubation for 2 to 3 days.

Determination of nuclear and cell morphology. Nuclear morphologies were determined by fluorescence microscopy of cells fixed in 70% ethanol for 1 h at room temperature, digested for 1 h with RNase (1 mg/ml), and stained with 50 μg of propidium iodide per ml. Alternatively, cells were fixed with 10% formaldehyde for 1 h at room temperature and stained with 4,6-diamino-2-phenylindole before fluorescence microscopy (56). Cell morphologies were determined by phase-contrast microscopy. When necessary, the cell and nuclear morphology was verified by alternating between fluorescence and phase-contrast microscopic settings for each cell. At least 100 cells were scored for cell or nuclear morphology.

Tests of delay of progression from G2 to anaphase after DNA damage. Methods and rationale for determining DNA damage-induced cell cycle arrest in haploid cells were described previously (55). To determine the efficiency of cell cycle arrest, one of three assays was used. For routine determinations, the response of cells in randomly selected fields 8 to 10 h after X-irradiation was recorded. Logarithmically growing cells were sonicated to separate cell clumps, plated on solid agar medium, and X-irradiated with 2 krad. Fields of cells were examined by light microscopy after 8 to 10 h to determine colony morphologies arising from individual X-irradiated cells. After X-irradiation of wild-type strains, the haploid G2 cells (and many S-phase cells) survive X-irradiation, and in the microcolony test they generate colonies containing typically greater than 10 buds by 8 to 10 h after irradiation. The X-irradiated G1 and postanaphase cells, however, cannot repair the X-ray damage, arrest as large-budded (two-budded) and two adjacent large-budded (four-budded) cells, respectively, and never resume cell division. In arrest-proficient wild-type strains, the predicted ratio of arrested cells (microcolonies with two or four buds) to inviable cells is approximately 1.0; experimentally, the ratio is usually >0.8. In contrast, in the *rad9* arrest-deficient strains, the G1 and postanaphase cells fail to arrest as large-budded and two adjacent large-budded cells; rather, they typically stop cell division as either three-budded or five- to eight-budded microcolonies. Therefore, in *rad9* cells the ratio of arrested cells (with two or four buds) to inviable cells is typically low (<0.3). Proficiency of arrest of X-irradiated haploid cultures is expressed as a ratio of arrested cells to inviable cells after X-irradiation with 2 krad.

A second assay used time-lapse photomicroscopy to determine specifically the response of individual unbudded (G1) and budded cells to X-irradiation. Cells grown logarithmically were sonicated, plated on thin agar slabs (prepared on microscope slides), and X-irradiated with 2 krad, and the cells and agar slab were covered with a coverslip to prevent drying during the subsequent incubation. Fields of cells were photographed immediately after plating and X-irradiation and rephotographed 8 to 10 h later. The negatives from the 0-

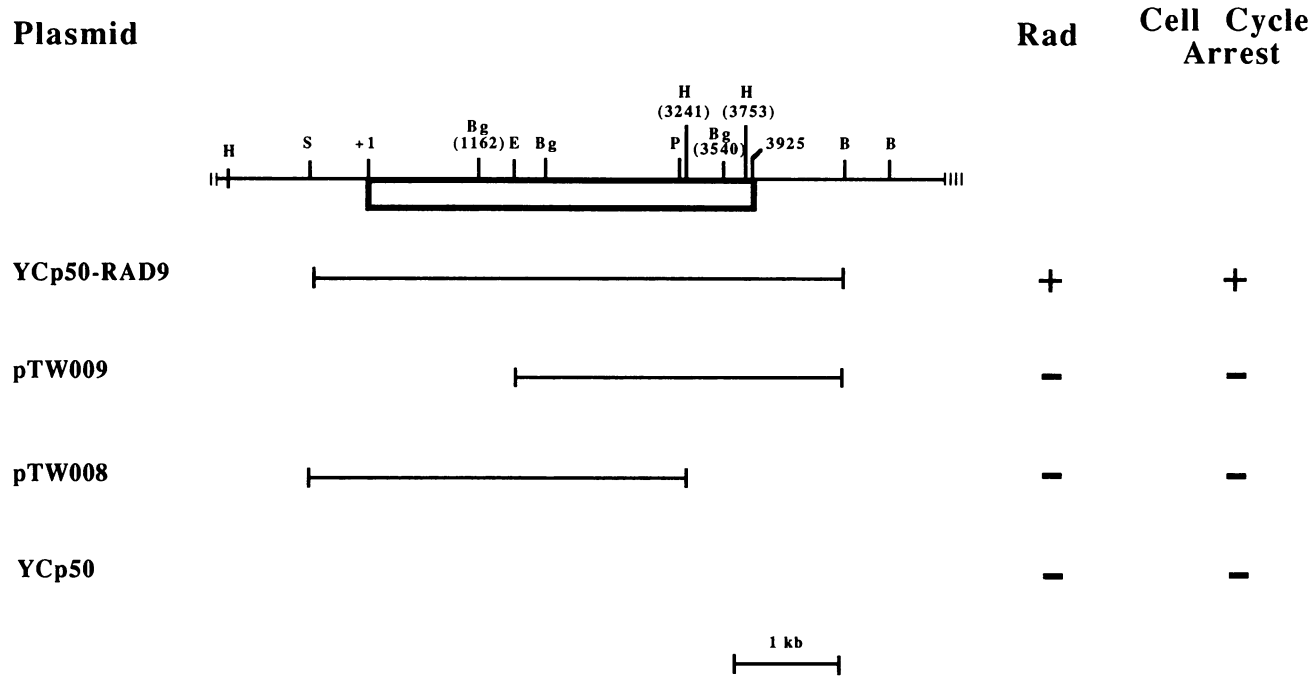


FIG. 1. DNA fragments complementing radiation sensitivity and the cell cycle arrest defect of *rad9-1*. Plasmids are derivatives of the low-copy-number vector YCp50 and contain the DNA fragments indicated. The coding region of the *RAD9* protein, predicted from the DNA sequence (not shown; 47), is indicated by the open box. Radiation sensitivity was tested qualitatively by X-irradiating patches of cells on solid agar medium. Cell cycle arrest was calculated by determining the ratio of percent cells arrested/percent cells inviable after cells were treated with 2 krad; the ratios for *rad9-1* cells, containing the plasmids indicated, were as follows; YCp50, 0.37; YCp50-*RAD9*, 0.79; pTW009, 0.39; and pTW008, 0.21. Restriction sites: H, *Hin*dIII; E, *Eco*RI; Bg, *Bgl*II; B, *Bam*HI; P, *Pst*I; S, *Sau*3A.

and 8- to 10-h photographs were projected, and fields of cells were aligned to determine the fates of individual cells.

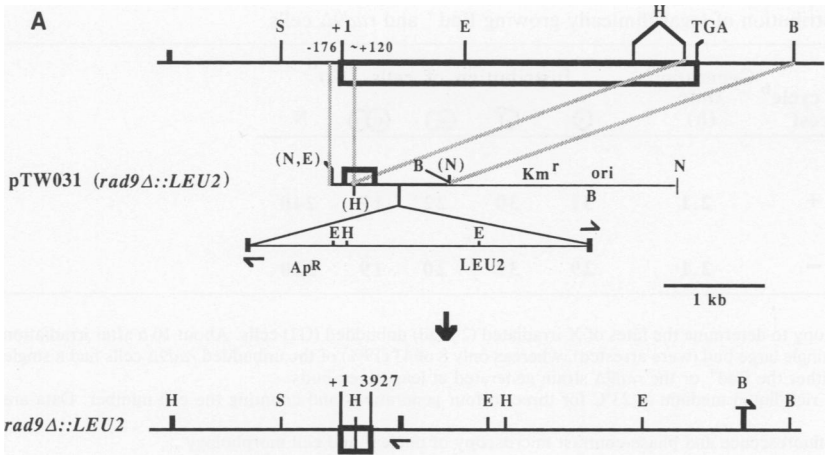
In a third assay, we determined directly how long G2 cells delay progression through anaphase after DNA damage. Cells can be synchronized in G2 by treatment with the microtubule inhibitor methyl benzimadazole-2yl-carbamate (MBC) (41), and we have shown previously that MBC-arrested cells are sensitive to *RAD9*-dependent delay after X-irradiation (55). To quantify the delay in G2 after X-irradiation, logarithmically growing haploid cells were synchronized in G2 by incubation for 3 h at 23°C with MBC (100 μg/ml in rich medium diluted from a 10-mg/ml stock in dimethyl sulfoxide). After 3 h in MBC, more that 80% of cells were arrested with a large bud and contained an undivided nucleus with G2 DNA content (data not shown). In A364a strains, 5 to 10% of the MBC-arrested cells had a third bud but undivided nucleus, as noted previously (24); these cells were included as G2 cells. The MBC-arrested G2 cells were plated on MBC-containing agar plates and X-irradiated; the cells were washed twice with medium to remove MBC and to recover cells into liquid medium. During the subsequent incubation in fresh medium, cell survival and cell and nuclear morphology were determined. Cell survival was determined by plating cells on solid medium and after 24 h determining microscopically the percentage of cells that generated microcolonies of about 50 buds or more; X-ray-resistant cells generate large microcolonies, whereas X-ray-sensitive cells typically generate fewer than eight buds. Survival frequencies determined microscopically agreed with survival frequencies determined by colony formation (not shown). The nuclear morphology of cells was determined as described above.

After release from the MBC-induced G2 block, the decrease in G2 cells (cells with the nucleus at the neck of a large-budded cell) was accompanied by an increase in cells that had completed anaphase (cells containing a divided nucleus, one half in the mother and one half in daughter bud). At later times after release from the MBC-induced G2 block, the percentage of unbudded and small-budded cells increased, which indicated progression of cells into the next cell cycle.

Inhibition of protein synthesis. The role of protein synthesis in cell cycle arrest by DNA damage was determined by treating MBC-arrested G2 cells (see above) with cycloheximide (10 μg/ml; Sigma) for the 15-min period immediately prior to X-irradiation, during X-irradiation, and during the subsequent incubation. The inhibition of protein synthesis in the MBC-arrested G2 cells was >95%, determined by incorporation of ^{35}S-labeled methionine into acid-precipitable material (not shown).

Isolation and analysis of the *RAD9* gene. A genomic library of yeast sequences inserted into the low-copy-number vector YCp50 (generated by M. Rose; 42) was transformed into a *ura3 rad9-1* strain (7815-6-4), and the ~6,000 Ura$^+$ transformants were screened for radiation resistance. A single transformant containing a plasmid conferring suppression of radiation sensitivity was isolated, and a 5.5-kb fragment containing the *RAD9* gene was localized in the ~20-kb yeast insert (Fig. 1).

The DNA sequence of the *RAD9* gene was determined by using a series of deletions generated by exonuclease III digestion (19) and specific primers (U.S. Biochemicals) homologous to vector sequences to prime sequencing reactions of yeast sequences. The DNA sequence that was



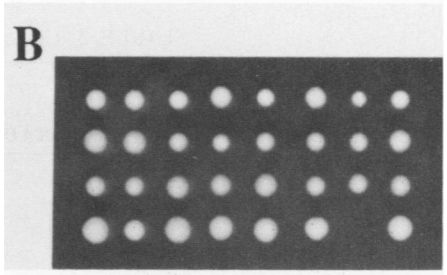


FIG. 2. Construction and analysis of strains with a deletion of *RAD9*. (A) Deletion removing 92% of the open reading frame of *RAD9* gene. Restriction fragments flanking the *RAD9* open reading frame were joined in vitro, and a transposon encoding the yeast *LEU2* gene was inserted by transposition in bacteria in vivo, using m*Tn3* transposons (49). The structure of pTW031 includes 2 kb of vector sequence, pHSS6. Restriction sites are as noted in the legend to Fig. 1; N, *Not*I. (B) Meiotic segregants from tetrads from the *RAD9* hemizygous strain 7859 (see Table 1), grown for 3 days at 23°C.

determined (not shown) revealed an open reading frame of 3,927 nucleotides encoding a protein 1,309 amino acids in length, identical to the DNA sequence published by Schiestl et al. (47).

The *RAD9* gene in plasmid pTW007 (Fig. 2) contains a *Sau*3A and a *Bam*HI site at the junctions of bacterial and yeast sequences. A *Sal*I site was inserted adjacent to the *Sau*3A site to facilitate cloning of the *Sau*3A-*Bam*HI 5.5-kb fragment (to generate YCp50-*RAD9* and YEP24-*RAD9*). Plasmid pTW007 was used as a probe of Southern blots to determine the genomic structure of the *RAD9* locus. Plasmid pTW039, containing the sequence from +31 to +3927 in an M13-based vector, was used to detect the *RAD9* transcript in Northern (RNA) analysis.

Standard recombinant and nucleic acid procedures were used (29).

Construction of yeast strains containing disruptions or deletions of *RAD9*. To determine the genomic origin of DNA in pTW007, the *Eco*RI-*Hin*dIII fragment (Fig. 2) was inserted into a yeast integrating plasmid (YIP5), which was digested with *Bgl*II to target its integration (43) in transformation of a Rad$^+$ *ura3* auxotroph (strain 8200). A Ura$^+$ Rad$^-$ transformant (8200:1 *rad9-URA3*) with the predicted genomic structure was identified and used for meiotic mapping of the insert.

Two deletions of *RAD9* were constructed. A disruption-partial deletion was generated by replacing the *Bgl*II-*Bgl*II fragment (1162 to 3540) with a DNA fragment containing the *URA3* gene (flanked by *Bam*HI sites). The disruption-deletion is similar to that previously reported (47) and leaves 1.1 kb of open reading frame at the N terminus of the *RAD9* gene. Therefore, a second deletion removing 92% of the *RAD9* coding region was generated as shown in Fig. 2: a DNA fragment extending from −176 to +~120 was joined to the 3′ *Hin*dIII-*Bam*HI fragment vector pHSS6. m*Tn3* transposons (49) were used to insert yeast selectable markers, by in vivo transposition, into the DNA fragment containing the deletion of *RAD9*. Genomic deletions of *RAD9* in yeast cells were generated by transforming an appropriate haploid or diploid auxotroph with *Not*I-digested plasmid DNA. Deletions in haploids were identified routinely by first screening prototrophs for radiation sensitivity and then confirming the genomic structure of the deletion of *RAD9* by Southern hybridization. Most (seven of eight) radiation-sensitive, haploid transformants contained the predicted genome structure for a deletion of *RAD9*. Only about one-fourth of prototrophic haploid transformants are sensitive to radiation, however, so many transformants are presumably gene conversions of the yeast selectable marker. Prototrophic diploid transformants were screened by Southern hybridization to identify strains hemizygous for *RAD9*. Haploid strains deleted for *RAD9* and the plasmids used in their construction included (by strain, selectable marker used, and plasmid used) 7831-1, *URA3*, pTW030; 7832-2, *LEU2*, pTW031; 7833-1, *TRP1*, pTW032; and 7834-1, *HIS3*, pTW033. Strains 7831 to 7834 were generated by transformation of strain 7830-2-4 (Table 1).

Chromosome loss and recombination. Two different chromosome loss and recombination assays used in this study were previously developed in this laboratory (7, 17; M. Carson, Ph.D. thesis, University of Washington, Seattle, 1986). Both systems use parental strains that are heterozygous for the locus encoding arginine permease (*CAN1*) and are sensitive to canavanine. Cells that have lost the *CAN1* gene become resistant to canavanine and can be selected from the parental heterozygotes. Canavanine-resistant cells arise either from mitotic recombination or from chromosome loss events, which are distinguished by the use of auxotrophic loci on the chromosome arms opposite the *CAN1* gene. In strains disomic for chromosome VII (7225 and 7225Δ1; see Table 3), *ade3* and *ade6* are on the right arm and *CAN1* is on the left arm; chromosome losses are Can^r Ade$^-$, and mitotic recombinants are Can^r Ade$^+$. For diploids (7860-3,7861-3, and 7862-1; see Table 3), events on chromosome V are monitored and *HOM3*/*hom3* is on the right arm opposite *CAN1*/*can1* on the left arm; chromosome losses are Can^r Met$^-$, and mitotic recombinants are Can^r Met$^+$. Strain 7225Δ1 contains a disruption of *RAD9*, generated by transformation as described above. All homozygous and heterozygous *rad9* diploid strains contain complete deletions of *RAD9*.

The rates of chromosome loss and recombination were determined by using the Lea-Coulson method of the median (26). Fifteen colonies were analyzed for each strain. The mutation rates were 0.3×10^{-6} and 0.8×10^{-6} in wild-type and Δ*rad9* strains, respectively, as measured by the rate of canavanine resistance in *CAN1* haploid strains; therefore,

TABLE 2. Cell cycle distribution of logarithmically growing Rad$^+$ and *rad9*Δ cells

Strain[a]	RAD9	Cell cycle[b] arrest	Generation[c] time (h)	Distribution of cells (%)[d]				
				⊙	♂	⊙⊙	⊙⊙	N
7859-10-2	+	+	2.1	31	30	22	17	240
7859-7-4	−	−	2.1	29	32	20	19	220

[a] Rad$^+$ (7859-10-2) and *rad9*Δ (7859-7-4) (see Table 1).
[b] Proficiency of arrest in G2 was analyzed by photomicroscopy to determine the fates of X-irradiated (2 krad) unbudded (G1) cells. About 10 h after irradiation with 2 krad, 35 of 38 (92%) of the unbudded Rad$^+$ cells had a single large bud (were arrested), whereas only 8 of 43 (19%) of the unbudded *rad9*Δ cells had a single large bud. The remainder of the initially unbudded cells of either the Rad$^+$ or the *rad9*Δ strain generated at least three buds.
[c] The generation time was determined by growing cells in rich liquid medium at 23°C for three to four generations and counting the cell number. Data are averages of triplicate cultures.
[d] Distribution of cells in the cell cycle was determined by fluorescence and phase-contrast microscopy of nuclear and cell morphology.

most Can^r cells result from mitotic recombination or chromosome loss events and not from mutation.

An unexpected class of mitotic recombinants was observed in analysis of strains disomic for chromosome VII (not shown). A minority of Can^r Ade$^+$ colonies on solid medium that contained canavanine in minimal medium minus adenine grew very poorly and appeared highly sectored (nibbled). Genetic analysis of the colonies to determine the region of recombination along the arm of the chromosome VII (see reference 7 for details) revealed that the poor-growing, sectoring recombinants had apparently arisen by recombination events that had occurred preferentially near the *CYH* locus (114 of 115 of the poor-growing Can^r Ade$^+$ colonies were Lys$^-$ Cyh^r Trp$^+$) as opposed to the random sites of recombination present in round, normal-size colonies (data not shown; 7). The abnormal Can^r Ade$^+$ recombinants occurred at a 12-fold-higher rate in *rad9*Δ than in wild-type strains (rates of events were 1.2×10^{-4} and 0.9×10^{-5} in *rad9*Δ and wild-type strains, respectively). The data for rates of chromosome loss and recombination shown in Table 3 include only recombinants with normal growth and colony morphology.

RESULTS

Isolation, genetic mapping, and DNA sequence of *RAD9*. The *RAD9* gene was isolated by transforming a single-copy YCp50 library containing random yeast fragments (42) into 7815-6-4 (*rad9-1 ura3*) and screening approximately 6,000 Ura$^+$ transformants for resistance to X-irradiation. A single plasmid-linked Ura$^+$ Rad$^+$ transformant was identified. The plasmid also restored cell cycle arrest after X-irradiation (not shown). A 5.5-kb fragment was identified that fully complemented the radiation sensitivity and cell cycle defect of *rad9-1* cells (Fig. 1). The genomic origin of the complementing DNA fragment was determined by constructing a strain (8200:1 Ura$^+$ Rad$^-$) containing the *URA3* gene integrated at a site linked to a disruption of the putative *RAD9* gene (see Materials and Methods). Meiotic analysis showed that *URA3* was tightly linked to *rad9-1*; the meiotic segregants from a *rad9-IURA3/rad9-1 ura3/ura3* strain segregated 4:0 for radiation sensitivity in all 27 tetrads analyzed in which Ura$^+$ segregated 2:2. The integrant was also tightly linked to *ste9*; meiotic segregants of a *ste9/+ +/rad9-IURA3* diploid yielded 26 parental ditypes, no nonparental ditypes, and one tetratype. *ste9* and *rad9* map to adjacent intervals on IVR, adjacent to *adr1* (32). During a computer search for sequence similarities, we found that the putative carboxyl terminus of the *RAD9* open reading frame is 550 bp from the putative carboxyl terminus of the *ADR1* open reading frame (15). The DNA fragment shown in Fig. 1 therefore contains the *RAD9* gene.

DNA sequence analysis confirmed the presence of a single long open reading frame encoding a protein of predicted length of 1,309 amino acids and M_r of 148,412 (not shown). The DNA sequence that we determined agreed with the sequence previously published (47) to the nucleotide. A computer search of several data bases (including GenBank and a private collection of M. Goebl) failed to identify any known proteins with a similar sequence.

***RAD9* is nonessential for cell growth.** A complete deletion of the *RAD9* gene was made to test whether *RAD9* has an essential role in cell division (the *rad9-1* mutation used in previous studies has not been molecularly characterized). DNA fragments flanking the *RAD9* open reading frame (removing 92% of the *RAD9* open reading frame) were joined, marked with the *LEU2* gene, and transformed into a *leu2/leu2* strain (strain 7236). A Leu$^+$ transformant (strain 7859) with the correct genomic structure was identified (not shown). Sporulation and meiotic analysis of the tetrads from the *RAD9/rad9*Δ hemizygote showed that cell viability segregated 4:0 in 33 of 40 tetrads (94% spore viability), Rad$^-$Leu$^+$:Rad$^+$Leu$^-$ segregated 2:2 as expected, and the genomic structures of wild-type and *rad9*Δ strains were as expected (not shown). The growth rates determined by colony size of Rad$^+$ and *rad9*Δ cells were virtually indistinguishable (Fig. 2B). In the remaining seven tetrads, viability segregated 3:1 or 2:2 and lethality was not linked to *rad9*Δ.

We examined more closely the growth properties of congenic Rad$^+$ and *rad9*Δ strains during exponential growth in liquid culture. The growth rates and distributions of cells in the cell cycle in wild-type and *rad9*Δ strains were indistinguishable (Table 2). Furthermore, the kinetics of the transition from G2 (after MBC treatment) to completion of anaphase (after release from MBC block) were very similar (see below; compare Fig. 5A and C). We conclude that in unperturbed (e.g., unirradiated) cells, *RAD9* is not essential and does not have a constitutive role in regulating progression of the cell cycle.

The *RAD9* gene is transcribed constitutively. We determined whether expression of *RAD9* is transcriptionally regulated by DNA damage. A 4.2-kb *RAD9*-specific transcript was present in wild-type cells and absent in *rad9*Δ cells (Fig. 3A). After X irradiation, the levels of *RAD9* transcript were reduced about fivefold by 10 min after

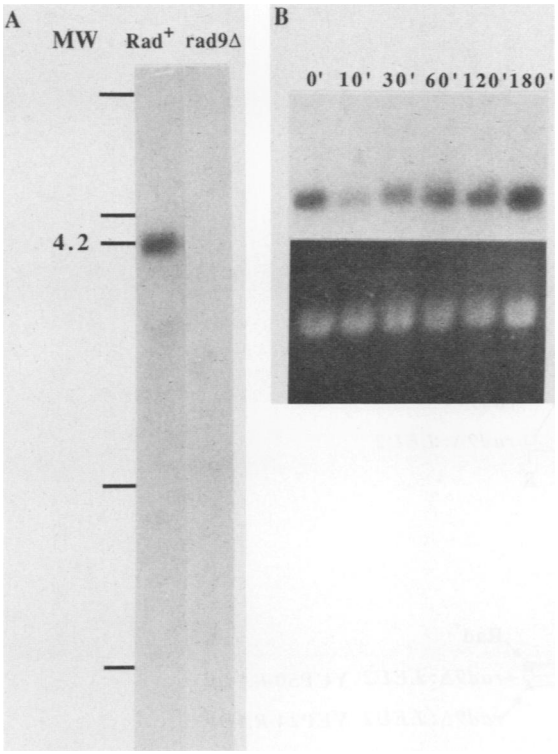


FIG. 3. Analysis of the *RAD9* transcript. Total RNA was prepared from logarithmically growing cells and size separated by agarose formaldehyde-gel electrophoresis, and the *RAD9* transcript was detected by using the *RAD9*-specific probe pTW039. The migration of RNA molecular size markers is shown (7.5, 4.2, 2.4, and 1.3 kb; Bethesda Research Laboratories). (A) The 4.2-kb *RAD9* transcript is present in *Rad*$^+$ (7830-2-4) and absent in *rad9*Δ (7832-2) strains. (B) Synthesis of the *RAD9* transcript is not induced by DNA damage. Total RNA was isolated from *RAD9* cells after X-irradiation (8 krad) at the times indicated. About 5 μg of RNA was loaded on each lane; the quantity of RNA was confirmed by ethidium-stained rRNA (shown below) and determined by densitometry of background hybridization to rRNA and by specific hybridization to a probe to a second control transcript encoding protein 1 (not shown; 20). The lane for the 180-min (180') time point contains about two times more RNA than do lanes for the other time points. MW, Molecular weight.

irradiation, and the levels of transcript then returned to levels similar (less than twofold difference) to those of unirradiated cells by 30 min after irradiation (Fig. 3B). The levels of a protein 1 control transcript also decreased transiently and modestly (about two- or threefold decrease) after X-irradiation before returning to unirradiated levels (data not shown). A transient decrease in levels of two transcripts (encoded by *URA3* and *POL1*) after treatment with the DNA-damaging agent 4-nitroquinoline 1-oxide has been reported (10); therefore, the transient decrease observed for the *RAD9* transcript could be due to a general DNA-damage-induced inhibition of transcription or decrease in mRNA stability. Treatment of cells with a low dose of X rays (2 krad) also did not significantly change the steady-state levels of *RAD9* transcript, nor did the level of *RAD9* transcript vary in different phases of the cell cycle, as judged by the level of transcript in temperature-sensitive *cdc* mutants arrested at their restrictive temperatures (including *cdc28*, *cdc13*, *cdc16*, and *cdc15*; data not shown).

Complementation of *rad9-1* and *rad9*Δ strains by low- and high-copy-number plasmids containing the *RAD9* gene. Previously we reported that *rad9-1* mutants were defective for cell cycle arrest after DNA damage and that their radiation sensitivity was suppressed by blocking irradiated cells in G2 with MBC (55). To determine whether the *rad9*Δ and *rad9-1* mutants have similar phenotypes with respect to radiation sensitivity, cell cycle arrest, and DNA repair, a complete deletion of *RAD9* was introduced by transformation into a *rad9-1* strain (7815-6-4). A Ura$^+$ transformant (7815-6-4ΔB) with the expected genomic structure (not shown) was identified and analyzed. The radiation sensitivities of congenic *rad9-1* and *rad9*Δ strains were similar (Fig. 4A). In addition, *rad9-1* and *rad9*Δ strains showed quantitatively similar defects for cell cycle arrest, and a G2 block imposed by MBC suppressed the radiation sensitivity of both strains similarly (not shown). We conclude that *rad9-1* and *rad9*Δ mutants have similar phenotypes and that by several criteria the *rad9-1* mutation is a complete loss-of-function mutation.

A single-copy plasmid (YCp50-*RAD9*) or multicopy 2μm plasmid (YEP24-*RAD9*) containing the 5.5-kb *Sau*3A-*Bam*HI fragment fully complemented the X-ray and UV sensitivity of a *rad9*Δ strain (Fig. 4B and data not shown). We did not observe greater resistance to X-irradiation in strains containing the *RAD9* gene on a multicopy plasmid than in strains containing a single copy of *RAD9* as reported by Shiestl et al. (compare Fig. 4B with Fig. 2B in reference 47).

Rate of chromosome loss is elevated in *rad9*-defective strains. We tested whether *RAD9*-dependent arrest contributes to genomic stability by analyzing the spontaneous rates of chromosome loss and mitotic recombination in wild-type and *rad9*Δ strains. Chromosomal events were monitored on two different chromosomes, chromosome VII in disomic strains and chromosome V in diploids (Table 3). *rad9*Δ strains showed a 7- to 21-fold-higher rate of chromosome loss than did wild-type cells, with little to no effect on mitotic recombination, and the chromosome loss phenotype was recessive to wild type. This result shows that *RAD9* contributes to the fidelity of mitotic chromosome transmission.

The mechanism of arrest of nuclear division by DNA damage involves posttranslational control. The protein synthesis inhibitor cycloheximide was used to test whether *RAD9*-dependent arrest of progression from G2 to anaphase requires the induced synthesis of new proteins in response to DNA damage. Previously D. Burke (personal communication) and Fantes (12) have shown that yeast cells (budding and fission, respectively) that had completed DNA replication but had not undergone chromosome segregation (G2 cells) are capable of completing chromosome segregation (anaphase) in the presence of cycloheximide. This finding shows that the G2-to-anaphase transition in yeast cells does not require protein synthesis. We could therefore determine whether X-irradiated G2 cells treated with cycloheximide delayed in G2 or progressed to anaphase without delay despite the DNA damage. Wild-type and *rad9*Δ cells were synchronized in G2 by treatment with MBC, and cycloheximide was then added to inhibit protein synthesis. DNA damage was induced by X-irradiation, and the MBC was then washed out (using medium containing cycloheximide) to allow chromosome segregation to proceed. The kinetics of progression from G2 to anaphase was determined directly by examining the nuclear morphology of cells at times after release from the MBC block. X-irradiated wild-type G2 cells delayed nuclear division in the absence or presence of cycloheximide (Fig. 5A and B); protein synthesis is not required for the *RAD9*-dependent arrest induced by DNA

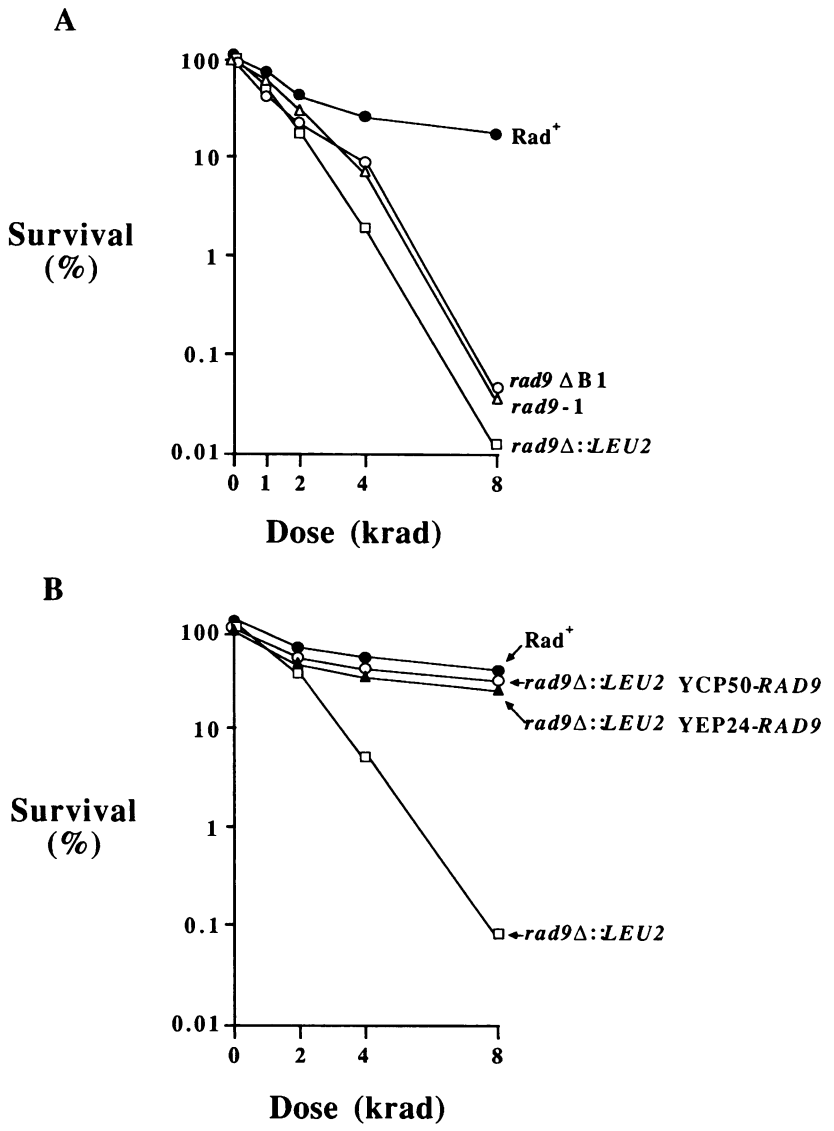


FIG. 4 Radiation sensitivity and complementation of *rad9Δ* strains by low- and high-copy-number plasmids containing the *RAD9* gene. (A) Sensitivity to X- irradiation of Rad$^+$ (7830-2-4), *rad9Δ* (7832-2), *rad9-1* (7815-6-4), and *rad9ΔB* (7815-6-4ΔB) strains. Data are averages of duplicate cultures from the X-irradiated logarithmically growing cells compared with unirradiated control cultures. (B) Suppression of the radiation sensitivity of *rad9Δ* by low- and high-copy-number plasmids containing the *RAD9* gene. Ura$^+$ transformants of a *rad9Δ ura3* strain (7832-2), containing either YCp50, YCp50-*RAD9*, or YEP24-*RAD9*, were grown logarithmically, plated on uracil-deficient solid agar medium, and X-irradiated. Data are averages of duplicate cultures from X-irradiated cells compared with unirradiated control cultures.

damage. In X-irradiated wild-type G2 cells, the duration of the delay was longer in the presence than in the absence of cycloheximide (compare Fig. 5A and B), a result that is addressed further in Discussion.

X-irradiated *rad9Δ* G2 cells failed to delay in G2, either in the presence or in the absence of cycloheximide (Fig. 5C and D). The lack of a delay of *rad9Δ* was anticipated because we showed previously that *rad9-1* G2 cells fail to delay chromosome segregation after X-irradiation (55).

X-irradiated wild-type G2 cells did complete anaphase after a delay of 4 to 8 h in the presence of cycloheximide (Fig. 5B). We deduce that the X-irradiated cells that progressed through anaphase after a delay had repaired lethal DNA damage because the cell viability in MBC-arrested G2 *RAD9* cells was not significantly affected by X-irradiation in the presence of cycloheximide (Fig. 5B, inset). Therefore, wild-type G2 cells in the presence of cycloheximide can repair DNA damage, recover from the *RAD9*-dependent arrest, and progress from G2 to anaphase. This finding shows that both induction of and recovery from *RAD9*-dependent arrest occur by a posttranslational mechanism.

DISCUSSION

We previously reported that arrest of nuclear division by DNA damage (after either X-irradiation or inactivation of DNA ligase) is dependent on the *RAD9* gene (18, 55). Genetic and physiological studies reported here reveal two key aspects of cell cycle control by *RAD9*. First, *RAD9* is a nonessential gene in unirradiated cells, and therefore the normal progression from G2 to anaphase and the order of DNA synthesis and mitosis must be controlled by separate

TABLE 3. Rates of spontaneous chromosome loss and recombination in wild-type and Δ*rad9* strains

Strain[a]	*RAD9*	Rate of chromosome event (10^{-6})[b]	
		Loss	Recombination
Disomes (chromosome VII)			
7225	+	29	27
7225Δ1	−	603	22
Dipoids (chromosome V)			
7862-1	+/+	1.6	14
7861-3	+/−	1.8	13
7860-3	−/−	13.5	23

[a] Genotypes are listed in Table 1.

[b] Mitotic recombinants were Can^r Ade$^+$ in disomes and Can^r Met$^+$ in diploids. Chromosome loss events were Can^r Ade$^-$ in the disomes and Can^r Met$^-$ in diploids. Rates were calculated by using the Lea-Coulson method of the median (26) from analysis of 15 independent cultures. Qualitatively similar results were obtained from one additional congenic strain from *rad9Δ* disomes and from one additional diploid strain from a homozygous *rad9Δ/rad9Δ*, heterozygous *RAD9/rad9Δ*, or homozygous *RAD9/RAD9* strain (not shown).

mechanisms that can be negatively regulated by *RAD9* in the presence of DNA damage. *RAD9* defines a separate control distinct from but likely interacting with gene products whose functions are essential for mitosis. Essential genes which when mutated disrupt the coupling of mitosis to completion of DNA synthesis have been described in *Schizosaccharomyces pombe*, *A. nidulans*, and BHK tissue culture cells (11, 35, 38). The relationship between the essential genes and a nonessential, *RAD9*-like control mechanism in any organism is unknown.

Second, though *RAD9* is a nonessential gene in unirradiated cells, the fidelity of chromosome transmission is lower in *rad9Δ* cells than in wild-type cells (the rate of chromosome loss is 7- to 21-fold higher in *rad9Δ* cells; Table 3). We suggest that wild-type cells occasionally incur spontaneous DNA damage, perhaps as a result of stochastic errors in DNA replication, which necessitate a delay of the cell cycle in order to repair the damaged chromosomes or complete replication before chromosome segregation. If damaged cells fail to delay (e.g., in *rad9* cells), some aspect of chromosome segregation fails or some aspect of chromosome mechanics in the next cell cycle fails, and a chromosome can be lost.

rad9 mutants and isogenic wild-type strains are similar with respect to levels of mitotic recombination (Table 3; 47), spontaneous and UV-induced mutation rates (47; this report), sporulation and germination efficiency (9, unpublished observations), rates of gene conversion and postmeiotic segregation in meiosis (9), and proficiency for DNA repair in noncycling cells (55). Therefore, *RAD9* has no apparent role in the cell other than to control cell cycle progression.

Because *RAD9* is nonessential, the Rad9 protein is not likely a structural component required for mitosis but rather acts as an extrinsic control mechanism, or checkpoint (18), that determines whether chromosomes are fully replicated and intact. Mitosis in yeast cells is normally dependent on the completion of DNA replication, since all known temperature-sensitive mutants defective in DNA replication fail to undergo mitosis at the restrictive temperature (40). We have found that the *rad9* mutation allows many of the mutants defective for DNA replication to undergo mitosis at the restrictive temperature (18; unpublished observations). Therefore, the dependence of mitosis on completion of DNA synthesis (and on intact chromosomes) is due in part to the *RAD9* checkpoint. In multicellular organisms, the importance of an analogous checkpoint that ensures the order of mitosis and DNA replication and the fidelity of chromosome segregation may be important in prevention of aneuploidy and the resulting abnormal, uncontrolled or cancerous cell growth (14).

The extent of cell cycle delay in X-irradiated G2 yeast cells is longer in the absence than in the presence of protein synthesis (compare Fig. 5A and B). The elongated delay in G2 in the absence of protein synthesis may be due to slower DNA repair than in the presence of protein synthesis. It has been shown that the inhibition of protein synthesis slows but does not completely inhibit DNA repair (3). Furthermore, DNA damage does induce synthesis of some DNA repair functions (8) as well as synthesis of specific transcripts that may encode DNA repair functions (30, 45, 53).

Protein synthesis is not required for the *RAD9*-dependent arrest or for recovery from arrest after DNA damage (Fig. 5B), a result which has two implications for the mechanism of *RAD9*-dependent control. First, gene products required for cell cycle arrest must be present at sufficient levels in G2 cells to induce cell cycle arrest after DNA damage; DNA-damage-induced synthesis of gene products is not required for *RAD9*-dependent cell cycle control. It is therefore not surprising that we found no significant increase in the level of the *RAD9* transcript after X-irradiation or after arrest of *cdc* mutants in different parts of the cell cycle (Fig. 3B and data not shown). We also have found that *RAD9* is not required for the DNA-damage-induced increase in levels of transcripts encoding *UBI4* (53), *DIN2* (45), and *DDR48* (30), nor is the low level of constitutive expression of these genes in wild-type cells significantly altered in *rad9* mutants (data not shown). *RAD9*-dependent cell cycle arrest after DNA damage in yeast cells therefore differs from the SOS-dependent cell cycle arrest in bacteria whereby DNA damage leads to the induced synthesis of SulA, a specific cell septation inhibitor (27).

Second, because recovery from *RAD9*-dependent arrest (after DNA repair is completed) does not require new protein synthesis, the *RAD9*-dependent mechanism cannot degrade a protein essential for the G2-to-anaphase transition. We suggest the Rad9 protein, directly or indirectly, modifies posttranslationally a protein essential for the G2-to-anaphase transition, a modification that is reversed once DNA repair is complete.

The identity of the gene product(s) essential for cell division that is the target of *RAD9*-dependent inactivation is unknown. Proteins essential for the G2-to-mitosis transition in all eucaryotes have been identified and are highly conserved in structure, in function, and possibly in modes of regulation (34, 37, 46). A central regulator controlling mitosis is the *cdc2*$^+$ gene, first identified in *Schizosaccharomyces pombe*, which encodes a protein kinase that is a component of mitosis-promoting factor (MPF) (37). *CDC28* from *S. cerevisiae* is functionally and molecularly homologous to *cdc2*$^+$ from *Schizosaccharomyces pombe* (2, 21, 28), and *CDC28* appears to have an essential function in the G2-to-anaphase transition (39). An attractive target of negative regulation after DNA damage is *cdc2/CDC28*. Experiments of cell cycle arrest after DNA damage in *Schizosaccharomyces pombe* and in mammalian cells are consistent with *cdc2*$^+$ and MPF inactivation by a posttranslational mechanism induced by DNA damage. In mammalian cells, it has been shown that MPF activity, which is low in interphase cells and high in metaphase cells (34, 37), is low in cells arrested in G2 by DNA damage (51). In mammalian cells, X-irradiation did not result in degradation of factors required for mitosis (presumably MPF) (44), consistent with a post-

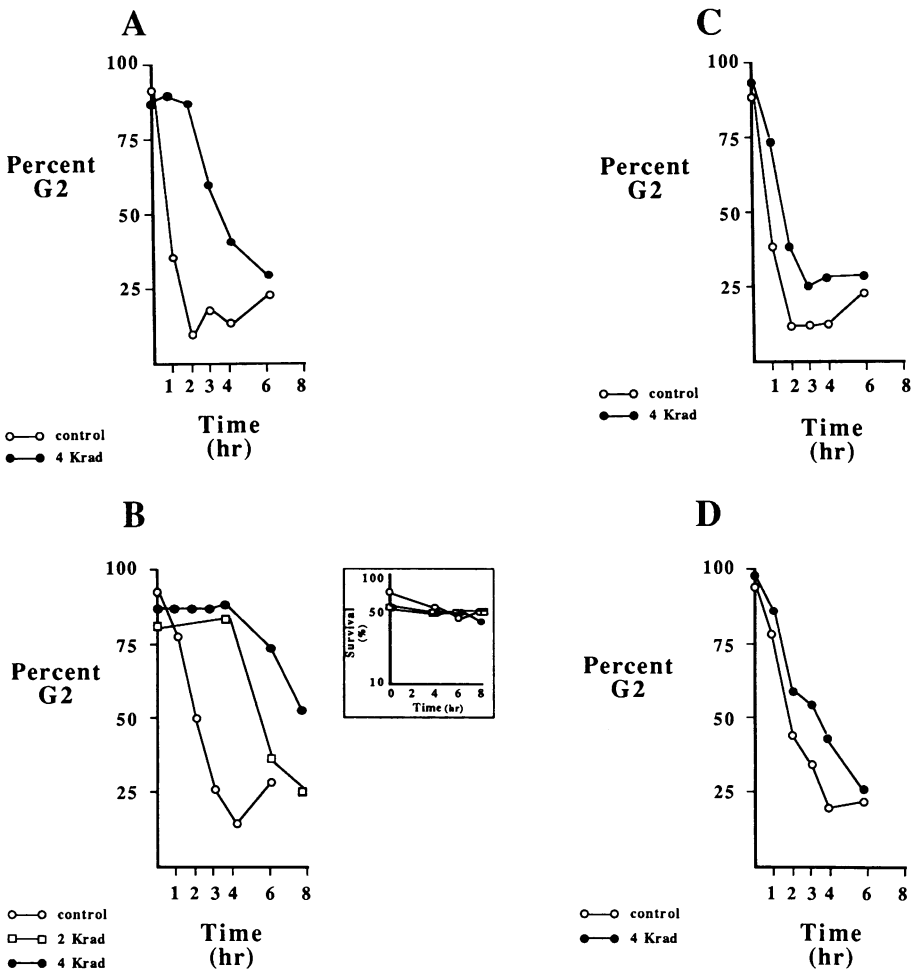


FIG. 5. Demonstration that protein synthesis is not required for DNA damage-induced arrest or recovery from arrest. The kinetics of exit from G2 was determined as described in Materials and Methods. Cells were synchronized in G2 by treatment with MBC, incubated with cycloheximide, and X-irradiated, and the MBC was washed out. Exit from G2 (completion of anaphase) after removal of MBC was monitored by fixing and staining cells and determining their nuclear morphology. (A [Rad$^+$] and C [*rad9*Δ]) Kinetics of exit from G2 in the absence of cycloheximide; (B [Rad$^+$] and D [*rad9*Δ]) kinetics of exit from G2 in the presence of cycloheximide. Strains were Rad$^+$ (7859-10-2) and *rad9*Δ (7859-7-4). (Inset) Viability of Rad$^+$ cells after exposure to radiation and after different times of incubation in cycloheximide. In addition, the viability of MBC-arrested cells either with or without cycloheximide and with or without X-irradiation did not vary significantly (absolute cell viability ranged from 59 to 74%; not shown).

translational inactivation of MPF rather than its degradation. (The same study [44] shows that, as we have shown in *S. cerevisiae*, induction of arrest in G2 after DNA damage does not require protein synthesis.) Recently, genetic studies of cell cycle arrest in *Schizosaccharomyces pombe* showed that a specific *cdc2*$^+$ mutant (*cdc2-3w*) no longer arrests when DNA replication is blocked, indicating that in wild-type cells *cdc2*$^+$ may be the target of negative regulation. A specific *cdc2*$^+$ mutant (*cdc2 PHE15*), carrying a mutation that removes a site of phosphorylation, behaves genetically like the *cdc2-3w* mutant (11, 13). Phosphorylation of *cdc2*$^+$ may be involved in inhibition of mitosis when DNA is incompletely replicated or damaged. Inhibition of *cdc2*$^+$ and MPF by negative-feedback controls acting by a posttranslational mechanism has been suggested (34). Also essential for *cdc2*$^+$ activity is a second highly conserved protein called cyclin, which may also be a target of regulation by negative-feedback controls (34). Whether the *RAD9*-dependent negative control inactivates or prevents the activation of *CDC28* or other cell cycle essential proteins to block cell cycle progression is currently unknown and under investigation.

Cell cycle arrest after DNA damage is genetically complex, requiring recognition of DNA damage, generation and amplification of a signal to arrest, and inhibition of the function of gene product(s) essential for progression from G2 to anaphase. The molecular role of the Rad9 protein is unknown; however, since the *RAD9* gene is nonessential, the Rad9 protein must either recognize DNA damage directly or be involved in signal transduction. Recently we have found five genes in addition to *RAD9* that are also essential for arrest in G2 after DNA damage (unpublished observations). Their roles in cell cycle control are under investigation.

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