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Genetics

RAD9-dependent G_1 arrest defines a second checkpoint for damaged DNA in the cell cycle of *Saccharomyces cerevisiae*

(UV radiation/DNA repair)

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ABSTRACT **Exposure of the yeast *Saccharomyces cerevisiae* to ultraviolet (UV) light, the UV-mimetic chemical 4-nitroquinoline-1-oxide (4NQO), or γ radiation after release from G_1 arrest induced by α factor results in delayed resumption of the cell cycle. As is the case with G_2 arrest following ionizing radiation damage [Weinert, T. A. & Hartwell, L. H. (1988) *Science* 241, 317–322], the normal execution of DNA damage-induced G_1 arrest depends on a functional yeast *RAD9* gene. We suggest that the *RAD9* gene product may interact with cellular components common to the G_1/S and G_2/M transition points in the cell cycle of this yeast. These observations define a checkpoint in the eukaryotic cell cycle that may facilitate the repair of lesions that are otherwise processed to lethal and/or mutagenic damage during DNA replication. This checkpoint apparently operates after the mating pheromone-induced G_1 arrest point but prior to replicative DNA synthesis, S phase-associated maximal induction of histone H2A mRNA, and bud emergence.**

The eukaryotic mitotic cell cycle involves an ordered sequence of events that results in the faithful transmission of genetic material to daughter cells. Entry into the mitotic cell cycle is regulated by specific signals, and during progression through the cycle additional regulatory mechanisms ensure the completion of one phase before passage to the next (1, 2). At present little is known about the influence of DNA damage on cell cycle progression. The cessation of DNA replication triggered by DNA damage has been demonstrated in *Escherichia coli* (3). Additionally, several studies indicate that a variety of DNA-damaging agents inhibit DNA replication in yeast (4). However, it is not known whether this response requires an active regulatory process or is exclusively the result of the passive stalling of replicative and/or transcriptional complexes at sites of base damage. Arrest of *Saccharomyces cerevisiae* cells in the G_2 phase of the mitotic cycle facilitates the repair of DNA damage produced by ionizing radiation and is dependent on functional *RAD9* and *RAD17* genes (5, 6).

Exposure of mammalian cells to ionizing irradiation can block cell cycle progression in G_2, the restriction point (G_1 arrest) (7, 8), or, during replicon, initiation (G_1/S arrest) (9). The phenotype of radio-resistant DNA synthesis following exposure to ionizing radiation in cells from the hereditary human disease ataxia telangiectasia (AT) suggests the existence of actively regulated checkpoints for damaged DNA at G_1/S (10) and possibly G_1 (8) in normal human cells. (For the sake of convenience we will use the designation "G_1 checkpoint" even if the phenomenon in question has not been unequivocally mapped in relation to the restriction point and S phase.)

Additional support for the notion of regulated checkpoints derives from several recent observations. A decrease in the fraction of S phase cells and an increase in the fraction of G_1 cells have been correlated with an increase in the level of p53 protein (11) in several mammalian cell lines following exposure to γ irradiation. Furthermore, p53 mutant cells failed to arrest in G_1 after γ irradiation (12, 13). An increase in p53 levels was not observed in irradiated AT cells (13). Hence, p53 and the AT gene(s) may participate in a signal transduction pathway that regulates cell cycle arrest after DNA damage.

In *S. cerevisiae* nutrient deprivation or exposure to mating pheromone (α factor) results in the arrest of haploid cells in G_1. This arrest is associated with a failure to activate the *CDC28*-encoded protein kinase, a homologue of the Cdc2 and p34 proteins in *Schizosaccharomyces pombe* and mammalian cells, respectively. In *S. cerevisiae* reentry to the cell cycle depends on a transition from this restriction point, termed START (14, 15).

In the present study we have investigated the effect of DNA damage on the progression of yeast cells through the cell cycle. We show that exposure of synchronized cells to UV radiation, the UV-mimetic chemical 4-nitroquinoline-1-oxide (4NQO), or γ radiation results in G_1 arrest. We additionally show that this arrest requires a functional *RAD9* gene. The dependence of G_1 arrest on a gene previously implicated in arrest in the G_2 phase (5) suggests that arrest during G_1 is a regulated phenomenon that operates as a cell cycle checkpoint in yeast cells exposed to various types of DNA damage.

MATERIALS AND METHODS

Strain Construction. A *rad9* deletion mutation in the haploid yeast strain SX46A (*MATa RAD9 ade2 ura3-52 trp1-289 his3-532*) was constructed by one-step gene disruption (16). Plasmid pTW039 containing the complete *RAD9* gene was cut with *Eco*RV and *Xba* I and the *URA3* gene was cloned into the gap on a *Hin*dIII fragment (from plasmid YEp24), thereby creating a deletion from nucleotide positions 712–2922 of the *RAD9* open reading frame. A *Bgl* II–*Sal* I fragment of the resulting plasmid was used for gene transplacement. Strain SX46A was transformed by a modified lithium protocol (17). A Ura^+ derivative of this strain was constructed by integration of the *Stu* I-cut plasmid YIp5 (containing the *URA3* gene) at the chromosomal *ura3-52* locus. Plasmid manipulations were performed according to published protocols (18).

Cell Synchronization and Mutagen Treatment. The haploid yeast strain SX46A and its isogenic *rad9*Δ derivative were grown overnight to early logarithmic phase (2–5 × 10^6 cells per ml) in liquid YPD (1% yeast extract/2% dextrose/2% peptone) at 30°C. Cells were harvested and resuspended at 3

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Abbreviations: AT, ataxia telangiectasia; 4NQO, 4-nitroquinoline-1-oxide.

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$\times 10^7$ cells per ml in fresh YPD (pH 4.0) containing 5 μg of *S. cerevisiae* α factor per ml or 2.5 μg of *Saccharomyces kluyveri* α factor per ml (both from Sigma). After incubation for 1.5 hr at 30°C a second aliquot of α factor was added and incubation was continued for another 1.5 hr. Arrest of cultures was verified microscopically by the absence of budding cells and the "shmoo" appearance in virtually all cells. Cultures were centrifuged, rinsed with ice-cold water, and resuspended in ice-cold water at 2.5×10^7 cells per ml. Samples (4 ml) were stirred in 35-mm Petri dishes and irradiated with a 254-nm germicidal UV lamp at a dose rate of 1 $J/m^2 \cdot sec^{-1}$. Under these conditions UV radiation doses of 30, 60, and 100 J/m^2 resulted in 95%, 45%, and 3% survival of the wild-type strain and 30%, 10%, and 0.2% survival of the isogenic *rad9*Δ deletion mutant, respectively. The lethality of the administered UV dose was reduced by a factor of 3–4 as compared to irradiation on plates, due to shielding effects in suspension. Cells were recentrifuged, resuspended in prewarmed YPD at 1×10^7 cells per ml, and incubated at 30°C with vigorous agitation. Samples were withdrawn at different times, diluted with glycerol (10% final concentration), and immediately frozen in a dry ice/ethanol bath for microscopic examination. Buds less than one-third the size of the mother cell were defined as "small buds." Bud counts were independently performed by two individuals. During the initial increase in the fraction of budding cells inter-individual differences in budding counts were <5%. For experiments with 4NQO cells were treated similarly, except that 4NQO (Sigma) was added at 3 μg/ml to part of the culture during the last hour of incubation with α factor. Treatment with γ-rays was effected by irradiating α-factor-arrested cultures with a ^{137}Cs source at a dose rate of 1 krad/min (1 rad = 0.01 Gy). For stationary-phase experiments cells were grown in liquid YPD at 30°C for 36 hr and UV-irradiated as described above.

Northern Blot Analysis. Cells were irradiated in 150-mm Petri dishes and 5×10^7 cells were removed at the times indicated. Lysis of the cells by spinning in a Vortex with zirkonium beads and RNA extraction with hot phenol were carried out as described (19). The OD_{260} was determined for each sample. Equal amounts of RNA were separated on a 1.2% agarose gel containing formaldehyde, blotted to a GenescreenPlus nylon membrane (New England Nuclear), and hybridized according to the manufacturer's instructions. Hybridizations were carried out at 43°C in the presence of 50% formamide and 10% dextran sulfate. A 677-bp histone H2A gene probe was generated by PCR using the primers 5'-CACGAAGCCAGCCAG-3' and 5'-GGAGAAGCAATTTAA-3' labeled with ^{32}P by the random primer method (20). A *Sal* I–*Xho* I fragment of plasmid pG12 was used as a probe for *GAL4* mRNA, which served as a control. Signal intensities were evaluated on a Pharmacia/LKB Ultroscan laser densitometer.

Flow Cytometry. About 1×10^7 cells were withdrawn at each time point, washed with water, and fixed in ice-cold 70% ethanol. Cells were washed with water, resuspended in 0.2 ml of 10 mM Tris, pH 8.0/10 mM NaCl, containing 2.5 μg of propidium iodide per ml and 250 μg of RNase per ml, and incubated for 3 hr at 37°C. Samples were diluted in saline, sonicated, and analyzed on a FACScan flow cytometer using the CellFIT cell cycle analysis program (version 2.0, Becton Dickinson).

RESULTS

Haploid exponentially growing wild-type (*RAD9*) yeast cells were synchronized in G_1 with α factor from either *S. cerevisiae* or *S. kluyveri*. When synchronized cultures were irradiated with UV light and immediately released from α-factor arrest, we observed a dose-dependent delay in the emergence of small-budded cells (Fig. 1*A*). In untreated wild-type cells, the appearance of small-budded cells is commonly used as an indicator of the progression of yeast cells from G_1 to S and the initiation of DNA replication (14). We quantitated the budding delay by subtracting the time required to reach 20% small-budded cells in untreated cultures from that required in treated cultures (Table 1). Similar results were observed when budding delay was measured in

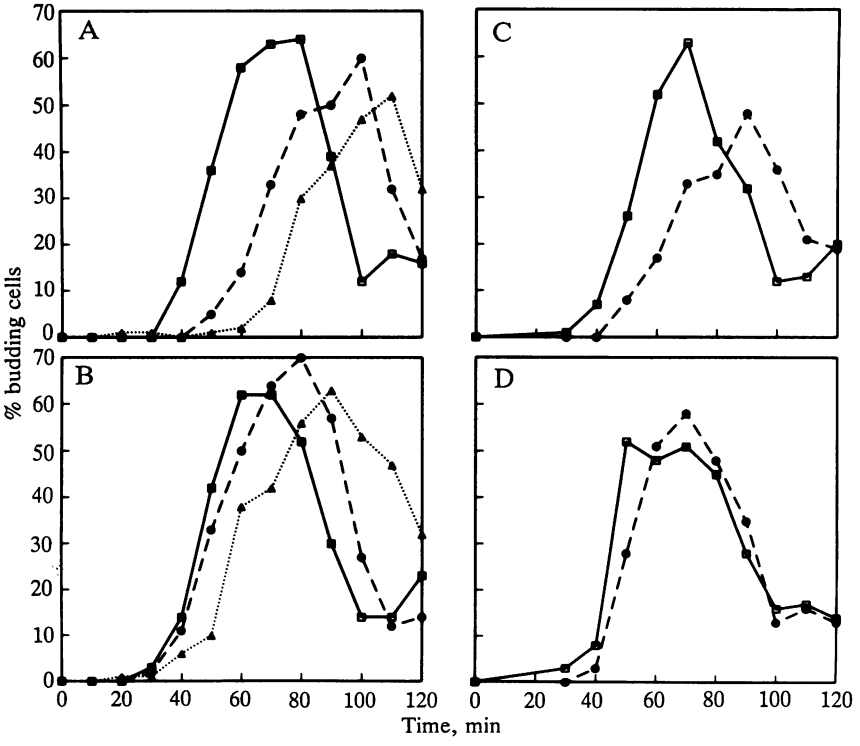

FIG. 1. Percentage of small-budded cells formed as a function of the time after release from *S. kluyveri* α-factor arrest in untreated (□), UV-irradiated (*A* and *B*) (●, 60 J/m^2; ▲, 100 J/m^2), or γ-irradiated (*C* and *D*) (●, 10 krad) cultures. In *A* and *C* the wild-type (*RAD9*) response is shown. *B* and *D* show the response in an isogenic *rad9* deletion mutant.

Table 1. Effect of exposure to DNA-damaging agents on the kinetics of early bud formation in wild-type and *rad9* mutant cells

DNA-damaging treatment	Method of synchronization	Strain SX46A *RAD9*		Strain SX46A *rad9Δ::URA3*	
		Time to reach 20% budding cells, min	DNA damage-induced delay, min	Time to reach 20% budding cells, min	DNA damage-induced delay, min
UV, J/m²					
0	SC α factor	26		33	
60	SC α factor	44	18	36	3
100	SC α factor	65	39	46	13
UV, J/m²					
0	SK α factor	44		42	
60	SK α factor	63	19	44	2
100	SK α factor	76	32	53	11
UV, J/m²					
0	G_0 cells	72		83	
60	G_0 cells	133	61	85	2
4NQO, μg/ml					
0	SK α factor	39		34	
3	SK α factor	70	31	46	12
γ-Rays, krad					
0	SK α factor	46		43	
10	SK α factor	63	17	47	4

SC, *S. cerevisiae*; SK, *S. kluyveri*.

synchronized cells treated with the UV-mimetic chemical 4NQO (Table 1), γ-rays (Fig. 1*C* and Table 1), or bleomycin (data not shown). Budding delay was also observed following UV irradiation of stationary-phase cultures (Table 1), indicating that the phenomenon is independent of the method used to synchronize cells.

To determine the period during cell cycle progression when yeast cells are able to respond to DNA damage by the delayed onset of budding, cells were irradiated at different times after release from α-factor arrest and budding delay was determined as before. No significant differences in the kinetics of the emergence of small-budded cells were observed when cells were exposed to UV radiation immediately after release from α-factor arrest or 10 min later (Fig. 2). When cells were exposed to UV radiation 20 min after release from α-factor arrest the delay in budding was reduced (Fig. 2). However, when cells were UV-irradiated after a significant fraction of small-budded cells was already evident—i.e., 35 min after release from α-factor arrest, further bud emergence was not significantly delayed (Fig. 3). Hence, cells retained the ability to respond to DNA damage by delayed budding for at least 20 min after release from α-factor arrest.

We also analyzed the DNA content of cells by flow cytometry. DNA profiles of unirradiated wild-type cells and cells irradiated at delivered doses of 30 and 60 J/m² were compared following release from α-factor arrest (Fig. 3 *Left*). The fraction of cells in G_1 at different times after release was consistently greater in irradiated cultures and was clearly correlated with the dose of UV radiation used. Flow cytometric analysis of DNA content was a more sensitive indicator of G_1 arrest than budding delay. For example, exposure of cells to 30 J/m² did not cause detectable budding delay (data not shown). However, a relative increase in the fraction of G_1 cells was evident at this dose of UV radiation (Fig. 3).

The abundance of H2A transcripts is known to fluctuate precisely as a function of the yeast cell cycle, with peak levels in early S phase (15, 21). When synchronized wild-type (*RAD9*) cells were UV irradiated and immediately released from α-factor arrest we observed a decrease in the steady-state level of histone H2A mRNA during the first few minutes (Fig. 4*B*). Comparable decreases in the levels of *GAL4* mRNA (a non-cell cycle-dependent control) were also observed (data not shown). These decreases likely reflect a general inhibition of transcription associated with DNA damage. For H2A mRNA, the initial decrease was followed by a progressive increase, consistent with the maximal accumulation of H2A messenger during the onset of DNA replication (Fig. 4*B*). This increase was considerably delayed in UV-irradiated cells relative to unirradiated cells (Fig. 4 *A* and *B*).

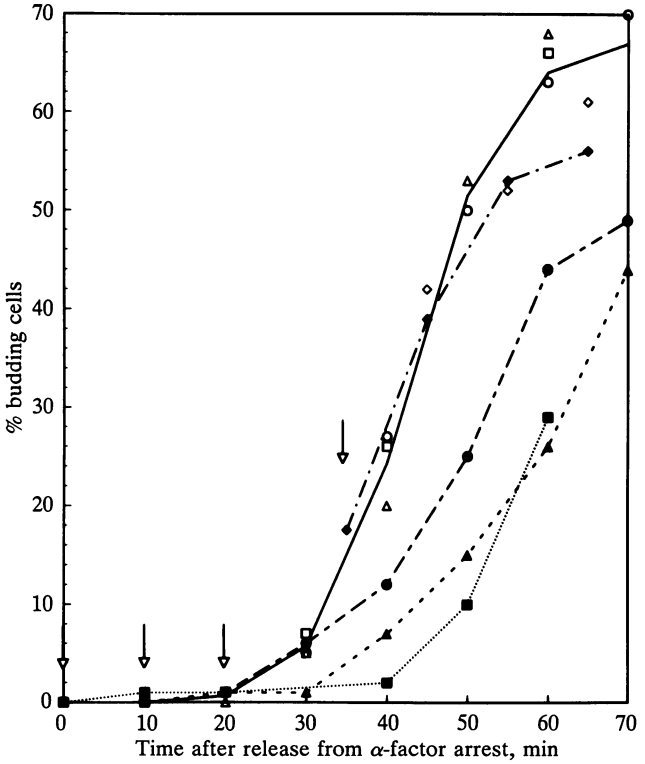

FIG. 2. Percentage of small-budded cells as a function of time after release from *S. kluyveri* α-factor arrest in untreated cultures and cultures treated with UV radiation (60 J/m²) at 0 (■), 10 (▲), 20 (●), and 35 (◆) min (indicated by the arrows) after release from α-factor arrest. The corresponding open symbols and the solid line indicate budding counts in unirradiated cultures that were otherwise treated identically.

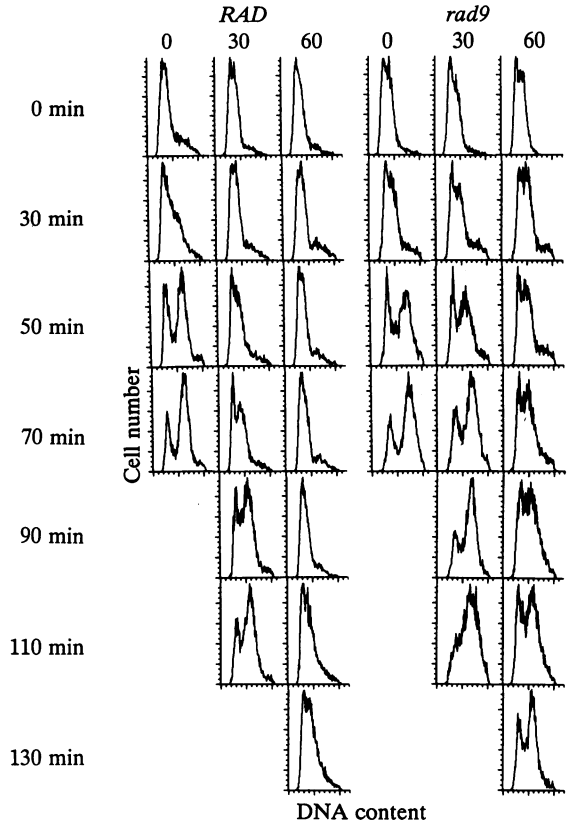

FIG. 3. Flow cytometric analysis of DNA content in the wild-type (*RAD9*) (*Left*) and *rad9* deletion mutant (*Right*) at the times indicated, after irradiation of synchronized cells with 0, 30, or 60 J/m^2 of UV radiation and release from *S. cerevisiae* α-factor arrest. The left-most peak in the figure represents the G_1 population and the right-most peak represents the G_2 population. These were independently determined by flow cytometric analysis of asynchronous cultures during synchronization in G_1 with α factor or in G_2 by treatment with the microtubule inhibitor nocodazol (20 μg/ml for 3 hr).

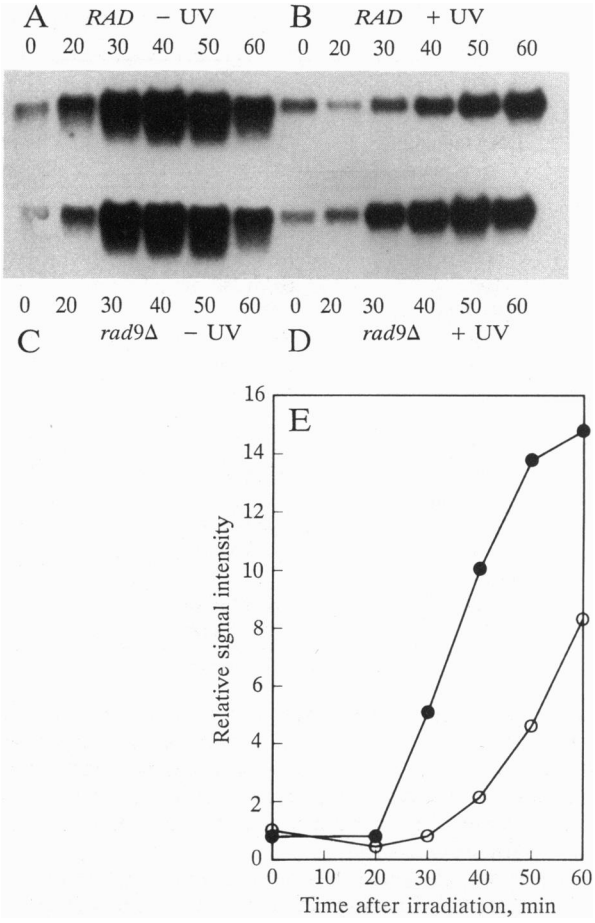

FIG. 4. Steady-state levels of histone H2A mRNA as a function of time (min) after the release of cells from *S. kluyveri* α-factor arrest. (*B* and *D*) UV-irradiated (30 J/m^2) cells. (*A* and *C*) Unirradiated cells. Wild-type (*RAD9*) cells are shown in *A* and *B* and isogenic *rad9*Δ mutant cells are shown in *C* and *D*. (*E*) H2A mRNA signals in irradiated cells (*B* and *D*) were normalized for the levels of nonfluctuating *GAL4* mRNA by densitometry, and the relative levels (*RAD9* cells at zero time = 1) in *RAD9* (○) and *rad9* cells (●) were plotted as a function of time after irradiation and release from α-factor arrest.

Collectively these results demonstrate that yeast cells exposed to a variety of DNA-damaging agents undergo transient arrest in the G_1 phase of the cell cycle. To determine whether this DNA damage-dependent arrest reflects a regulated process or results exclusively from the direct (passive) inhibition of DNA replication or transcription at sites of base damage, we examined the role of the *RAD9* gene, which is known to be involved in cell cycle control in yeast cells exposed to ionizing radiation (2, 5).

We constructed an otherwise isogenic *rad9 URA3* deletion mutant from the wild-type strain SX46A, used for the experiments described above. In contrast to the results obtained with *RAD9* cells, at 30 J/m^2 no cell cycle delay was observed by flow cytometry in the *rad9*Δ mutant (Fig. 3 *Right*). Additionally, at this dose the delay in the accumulation of H2A mRNA observed in wild-type cells was considerably reduced in the *rad9* mutant (Fig. 4 *B* and *D*). This reduced delay is not the result of a failure of *rad9* cells to experience general transcriptional inhibition associated with DNA damage, since a decrease in H2A mRNA levels comparable to that in the *RAD9* strain was observed at higher doses (data not shown). The relative delay in H2A mRNA synthesis after irradiation of *RAD9* and *rad9* mutant cells was quantitated after normalizing for steady-state levels of *GAL4* mRNA (Fig. 4*E*).

Following exposure to a higher UV radiation dose (60 J/m^2) or to 10 krad of γ irradiation, the *rad9*Δ mutant additionally showed essentially no G_1 arrest as determined by the less sensitive parameter of budding delay (Fig. 1 *B* and *D*; Table 1). At this dose of UV radiation progression through the cell cycle determined by flow cytometry was reduced, though to a lesser extent than that observed in *RAD9* cells. At even higher doses of UV radiation (100 J/m^2) (Fig. 1), or after treatment with 4NQO at a dose of 3 μg/ml, *rad9*Δ mutant cells showed delayed cell cycle progression when this was determined by bud formation. Results similar to those obtained with *rad9* mutant cells arrested with α factor were observed when the cells were arrested in stationary phase (Table 1). The results described above were also obtained when the wild-type strain SX46A carried an integrated copy of the *URA3* gene (data not shown). Hence, the differences between the *rad9*Δ::*URA3* mutant and the *RAD9 ura3* strain cannot be attributed to a trivial effect of the URA$^+$ genotype.

DISCUSSION

Based on multiple criteria of cell cycle progression (cell budding, DNA content, and histone H2A mRNA levels) we have characterized *RAD9*-dependent G_1/S delay in yeast cells as a response to various DNA-damaging agents. The dependence on a gene function previously implicated in progression through the G_2 phase when cells are exposed to UV or ionizing radiation (5, 6, 22), or after induction of DNA

damage by the inactivation of enzymes involved in DNA replication (6, 23, 24), suggests that arrested cell cycle progression in G_1 is an actively regulated process.

The biological significance of the *RAD9*-dependent G_1 arrest for protection against lethality and/or mutability caused by DNA-damaging agents remains to be determined. Cells carrying mutations in the *RAD9* gene are abnormally sensitive to killing by UV and γ radiation (5, 23) and are also characterized by chromosome instability during growth (25). A recent study has indicated a similar checkpoint function for *RAD9* in meiosis (26). The *rad17-1* mutant and several UV-sensitive mutants of *S. pombe* appear to be affected in a similar way (6, 27–29).

A dose of 30 J/m^2 that results in significant cell cycle delay in *RAD9* but not *rad9* deletion mutant cells is associated with >90% survival of the wild-type cells. Hence, it is improbable that a generalized nonspecific inhibition of cellular functions is a *major* determinant of the cell cycle differences observed. However, G_1 arrest was observed to a limited extent in *rad9* mutant cells exposed to higher levels of DNA damage. Similar observations have been made during γ-ray-induced G_2 arrest in yeast (5). It is possible that under these conditions other regulated checkpoint determinants are operative in yeast cells. Additionally or alternatively, nonspecific metabolic parameters such as a generalized inhibition of transcription may interfere with cell cycle progression at these higher doses.

Our studies suggest that DNA damage-dependent G_1 arrest occurs at or subsequent to the α-factor arrest point, but prior to the time of bud emergence, DNA synthesis, and the peak of H2A messenger accumulation. This conclusion stems from the observations that (*i*) bud emergence, DNA synthesis, and the accumulation of maximal levels of H2A mRNA all are delayed in cells exposed to DNA damage and (*ii*) cells released from pheromone-induced arrest continue to manifest damage-dependent budding delay for at least 10 min after release from α factor. Due to technical limitations with respect to flow cytometry analysis on yeast cells it cannot be unequivocally determined at this time whether *RAD9*-dependent arrest occurs in G_1 or in very early S phase (or in both). Additionally, the utility of bud emergence as an accurate indicator of the initiation of DNA synthesis in irradiated cells is questionable, since we observed bud emergence under conditions in which the G_1 DNA profile was virtually unchanged as determined by DNA analysis. Clearly, the threshold for DNA damage that results in detectable budding delay is higher than for DNA synthesis delay.

Preliminary data suggest that at least one *RAD9*-dependent DNA damage checkpoint in G_1 is not equivalent to START and maps downstream of the step that is defective in the temperature-sensitive cell division cycle mutant *cdc4* (W.S. and E.C.F., unpublished observations). *cdc4* mutants are known to arrest subsequent to G_1/S-related induction of replication-associated enzymes (e.g., ribonucleotide reductase and DNA ligase) but prior to the induction of histone mRNA (15).

If the damage-dependent G_1 checkpoint is equivalent to START one might expect that prolonging the G_1 phase by extended α-factor arrest after exposure of cells to UV radiation would increase the UV resistance of G_1-arrested *rad9* mutant cells. However, these experimental conditions rendered wild-type and *rad9* cells more UV sensitive (data not shown). These results suggest that pheromone-induced arrest precedes the *RAD9* checkpoint(s) in G_1 and that enhanced survival due to cell cycle arrest following UV irradiation is dependent on a process(es) that operates between the pheromone arrest point and the *RAD9*-dependent arrest point. Alternatively, survival after UV may not be enhanced by G_1 arrest, and other genetic endpoints (e.g., induced mutation, recombination, gene amplification) may be more relevant.

We propose that the *RAD9* gene functions in a signal transduction pathway(s) that operates in the G_1 and G_2 phases of the cell cycle. The protein kinase that is encoded by the yeast *CDC28* gene and that is required for progression to the S and M phases of the cycle is a possible target for *RAD9* control.

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