

Requirement for End-Joining and Checkpoint Functions, but Not *RAD52*-Mediated Recombination, after *EcoRI* Endonuclease Cleavage of *Saccharomyces cerevisiae* DNA

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***RAD52* and *RAD9* are required for the repair of double-strand breaks (DSBs) induced by physical and chemical DNA-damaging agents in *Saccharomyces cerevisiae*. Analysis of *EcoRI* endonuclease expression in vivo revealed that, in contrast to DSBs containing damaged or modified termini, chromosomal DSBs retaining complementary ends could be repaired in *rad52* mutants and in G₁-phase Rad⁺ cells. Continuous *EcoRI*-induced scission of chromosomal DNA blocked the growth of *rad52* mutants, with most cells arrested in G₂ phase. Surprisingly, *rad52* mutants were not more sensitive to *EcoRI*-induced cell killing than wild-type strains. In contrast, endonuclease expression was lethal in cells deficient in Ku-mediated end joining. Checkpoint-defective *rad9* mutants did not arrest cell cycling and lost viability rapidly when *EcoRI* was expressed. Synthesis of the endonuclease produced extensive breakage of nuclear DNA and stimulated interchromosomal recombination. These results and those of additional experiments indicate that cohesive ended DSBs in chromosomal DNA can be accurately repaired by *RAD52*-mediated recombination and by recombination-independent complementary end joining in yeast cells.**

DNA double-strand breaks (DSBs) occur spontaneously, after exposure to ionizing radiation or various chemical clastogens, and as part of normal cellular development. Developmental processes which are initiated by enzymatically induced DSBs include V(D)J recombination in mammalian cells and mating-type switching, intron homing, and meiotic recombination in yeast cells (17, 25, 37, 56, 58, 61). Repair of DSBs in eukaryotic DNA involves complex interactions between proteins associated with chromatin structure, enzymatic repair of broken DNA ends, and cell cycling. In mammalian cells, the primary mechanism of repair of DSBs is through nonhomologous end-joining processes (25, 61, 70). In *Saccharomyces cerevisiae*, the predominant pathway of DSB repair is through homologous recombination, although other, less common pathways have been described (discussed below). Models for the recombinational repair of DSBs in yeast DNA which involve pairing of broken DNA ends with complementary strands of an unbroken homolog have been proposed (63, 75, 77; see references in reference 56).

Recombinational repair of damage-induced DSBs occurring during mitotic growth of yeast cells requires genes within the *RAD52* epistasis group, which includes *RAD50-59*, *MRE11*, and *XRS2* (2, 16, 19, 56, 65). *RAD52* group mutants are hypersensitive to ionizing radiation and methyl methanesulfonate but display only a slight sensitivity to UV light. Physical analysis of DNA from *rad51*, *rad52*, and *rad54* mutants demonstrated that these strains are specifically deficient in the rejoining of radiation-induced DSBs (15, 16, 64). Recent studies have suggested that Rad51, Rad52, Rad54, Rad55, and Rad57 associate to form a multiprotein complex (2, 20, 70). Furthermore, both Rad52 and Rad51, which are conserved in lower and higher eukaryotes, display intrinsic strand-annealing activ-

ity (49, 70). Although *RAD52* group-mediated homologous recombination is the primary pathway of repair of DSBs in yeast cells, additional pathways have been described. These include nonconservative repair processes which occur in DNA containing direct repeats (e.g., single-strand annealing [30, 56]) and nonrecombinational end-joining pathways, which may be either precise or error prone (9, 13, 39, 44, 45, 47, 68, 72, 78). Genes which have been associated with end-joining pathways include *HDF1/YKU70*, *HDF2/YKU80*, *RAD50*, *XRS2*, and *MRE11*. End-joining mechanisms in yeast share several characteristics of DSB repair observed in higher eukaryotes and involve two genes (*HDF1/YKU70* and *HDF2/YKU80*) which are homologs of genes required for DSB repair and V(D)J recombination in mammalian cells (Ku70 and Ku80 [25]). Recent experiments have established that the yeast Ku70 homolog is also essential for maintenance of normal telomere lengths (59).

Eukaryotic cells possess checkpoint mechanisms which interrupt cell cycling when chromosomal DNA is damaged. This arrest ensures that DNA repair is completed before downstream cycling events are initiated. Exposure of yeast cells to ionizing radiation or methyl methanesulfonate causes growing cells to arrest in G₂. Arrested cells retain large buds and a single undivided nucleus (55, 71, 80, 82). Initial experiments established that *rad9* strains were deficient in DNA damage-induced cell cycle arrest (80), and additional genes were subsequently identified (71). Direct evidence that DSBs can initiate the arrest of yeast cells has been obtained from analysis of the consequences of HO endonuclease expression (described below). DSB-induced cell cycle arrest has also been observed in mammalian cells and is dependent upon p53 (50). Recent studies have demonstrated that additional damage-responsive checkpoints in G₁ and S phase are present in yeast cells (71).

Analysis of DSB repair in yeast has been facilitated by the development of systems for studying the genetic and cytological consequences of a single DSB induced by HO endonuclease. This enzyme stimulates mating-type switching, a gene con-

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version process, by producing a single DSB at the *MAT* locus (17, 26, 51). The ends of breaks produced by HO retain complementary 3' extensions which are 4 nucleotides in length (51). Several groups have used plasmid and chromosomal fusions containing the HO gene under the control of a galactose-regulated promoter to examine effects of DSBs on viability, chromosome metabolism, and cell division. Processing of breaks occurring at *MAT* has been studied (see, e.g., references 12, 26, and 47), as well as at synthetic HO target sites located on other chromosomes (see, e.g., references 6, 7, 43, 52, 62, and 66). In addition, the consequences of HO expression in radiation-sensitive mutants have been investigated (14, 18, 28, 40, 42, 74, 79). These experiments revealed that HO-induced strand breaks have several of the characteristics of damage produced by ionizing radiation treatment of cells. Such DSBs are recombinogenic, increase aneuploidy and mutagenesis, can arrest cell cycling in G₂, and inhibit the growth of radiation-sensitive *RAD52* group mutants.

High-level expression of two other endonucleases, the restriction enzyme *EcoRI* and mitochondrial endonuclease I-*SceI*, has also been examined in yeast cells. Expression of *EcoRI* in vivo resulted in the formation of DSBs at its target sequence (G^AAATTC) and inhibition of the growth of Rad⁺ and *rad52* cells (3). A single DSB produced by I-*SceI* efficiently induced homologous recombination in a plasmid containing its recognition sequence (58).

While investigating the genetic consequences of enzymatically induced DSBs containing cohesive ends (*EcoRI* and HO) or blunt ends (*PvuII*), we confirmed that expression of *EcoRI* inhibits the growth of *rad52* cells. However, most Rad⁺ strains which were tested grew after the induction of plasmid or chromosomal *GAL1::EcoRI* fusions. *EcoRI*-induced DSBs inhibited the growth of *rad52* strains and arrested cell division in the G₂ phase. Remarkably, *rad52* mutants were not more sensitive to killing than wild-type cells. Thus, *RAD52* was required for the growth of cells continuously expressing the endonuclease but not for precise repair of induced DSBs after *EcoRI* synthesis was repressed. In contrast, strains deficient in Ku70-mediated end joining or *RAD9*-dependent cell cycle checkpoint responses were hypersensitive to killing by *EcoRI*.

MATERIALS AND METHODS

Yeast strains and plasmids. Strain designations, genotypes, and sources of the yeast strains used are listed in Table 1. The *reg1-501* mutant strain T334, used for most of the experiments described here, is a derivative of 334 (24) in which the *TRP1* gene has been deleted. The *GAL1::EcoRI* cassette was integrated by PCR fragment-mediated gene disruption (5). Briefly, primers containing 50 bases of *HIS3* or *LYS2* gene sequence and 26 bases corresponding to the vector pRS314 (73) were used to amplify sequences within pLKL31. pLKL31 was constructed by inserting a *GAL1::EcoRI* cassette into pRS314 (*TRP1*) between the *EcoRI* and *BamHI* sites within this vector. Fragments generated with primer pairs *prslsA* and *prslsC* or *prshisA* and *prshisC* were transformed into T334 to create strains YLKL340 [Δ *lys2::(GAL1::EcoRI TRP1)*] and YLKL350 [Δ *his3::(GAL1::EcoRI TRP1)*]. *EcoRI* synthesis in these strains is regulated by *GALI*, but transcription of the associated *TRP1* gene is controlled by its natural promoter. All the oligonucleotides were obtained from BioServe Biotechnologies. The plasmid p316Gal was created by inserting a *GALI-10* promoter fragment (27) into the *EcoRI* and *BamHI* sites of pRS316. Plasmid pGALHO has been described previously (22), and YCpGal:Rib (*URA3*) was a gift from J. Rine (3). Disruption/deletion of *RAD52* was accomplished with p Δ 52Blast (*Rad52::hisG-URA3-hisG*) or p Δ 52Leu (*Rad52::LEU2*), provided by Ed Perkins. The *RAD9* gene was deleted with pRR330 (67), and Δ *hdf1::HIS3* strains were created by PCR as described previously (5).

Yeast growth media. High-purity D-(+)-galactose from Pfanstiehl (no. G106) was used for experiments in which galactose was needed as the sole carbon source. For growth of *reg1-501* strains in glucose-plus-galactose media, 98% pure galactose (no. G105) was used. Synthetic and YP media were prepared as described previously (69).

Growth curves, plating efficiencies, and cell cycle analysis. The growth of various published haploid strains containing YCpGal:Rib or the control vector pRS316 was analyzed by aliquoting or replica plating strains grown in synthetic

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
T334	<i>MATα ura3-52 leu2-3,112 Δtrp1::hisG reg1-501 gal1 pep4-3 prb1-1122</i>	E. Perkins
T334 Δ R52T	T334, <i>Rad52::TRP1</i>	E. Perkins
YLKL292	T334, <i>Rad9::hisG</i>	This study
YLKL340	T334, <i>lys2::(GAL1::EcoRI TRP1) Δhis3::hisG</i>	This study
YLKL341	YLKL340, <i>Rad52::LEU2</i>	This study
YLKL355	YLKL340, <i>Rad9::hisG-URA3-hisG</i>	This study
YLKL350	T334, <i>his3::(GAL1::EcoRI TRP1)</i>	This study
YLKL351	YLKL350, <i>Rad52::LEU2</i>	This study
YLKL366	YLKL350, <i>Rad9::hisG-URA3-hisG</i>	This study
YLKL389	YLKL350, <i>hdf1::HIS3</i>	This study
RM10-32D	<i>MATα ura3-1 leu1-c trp5-c lys2-2 tyr1-2 his7-1 ade2-1 ade6 met13c cyh2^r</i>	41
RM26-26C	<i>MATα ura3-1 leu1-12 trp5-2 lys2-1 his1 ade2-1 ade5 can^r</i>	41
EPY214-1B	<i>MATα ura3-52 leu2-Δ1 Δtrp1::hisG his1-7 lys2</i>	32
MAR1530	<i>MATα ura3-52 leu2-Δ1 iv1-92 cha1</i>	60
VL6 α	<i>MATα ura3-52 trp1-Δ63 lys2 his3 met14 ade1</i>	33
SSL231	<i>MATα ura3-52 leu2 trp1 ade2-101 can1::BYA112 (his3-Δ200)</i>	28
LS20	<i>matΔ his3 ade2 can1 trp1 ura3 leu2 lys5 cyh^r ade3::GALHO</i>	66
GRY1060	<i>matα-inc::(trp1-089 his3-621) ura3-52 trp1-Δ1 his3-Δ200 tyr7-1 (leu2-Δ1)</i>	43
GRY1078	<i>matα-inc::(trp1-488 his3-192) ura3-52 trp1-Δ1 his3-Δ200 leu2-Δ1 ade2-101 lys2-801 cry1</i>	43
S1InsE4A	<i>MATα ura3-52 leu2-3,112 trp1-289 his7-2 ade5-1 lys2::insE-4A</i>	48
BGW1-7a	<i>MATα ura3-52 leu2-3,112 his4-519 ade1-100</i>	81
tNR85T1	<i>mat::LEU2 hmr-Δ3 ura3-52 trp1 leu2 mal2 thr4</i>	6

2% glucose medium without uracil (Glu - Ura) to 2% galactose-minus-uracil plates (Gal - Ura). The plating efficiencies of plasmid-containing T334 cells were assessed after log-phase cultures were spread to Glu - Ura plates or Glu - Ura plates supplemented with 2% galactose (Gal + Glu - Ura). Strains containing integrated (*GAL1::EcoRI TRP1*) constructions were spread to YPD and YPD+2%Gal plates. Gal⁺ colonies observed on six plates were counted after 3 days, and the results were averaged. Plating efficiency is defined here as the viable cell titer calculated from observed CFU on plates divided by the cell titer determined by hemacytometer counts.

For growth curves and viability tests, plasmid-containing T334 cells (including *Rad52* and *Rad9* derivatives) were grown to a density of approximately 7×10^6 to 10×10^6 cells per ml in Glu - Ura and shifted to 2% Gal + Glu - Ura at $\sim 5 \times 10^5$ /ml. All the cultures (5 ml) were shaken vigorously at 30°C. At each time point, aliquots of cells were sonicated, counted with a hemacytometer, diluted, and spread to glucose-complete (Glu-Com) and Glu - Ura plates. Three or four independent cultures were assayed for each strain. Plasmid loss was analyzed by comparing plating efficiency on Glu - Ura plates to that on Glu-Com plates. Strains containing integrated *GAL1::EcoRI* cassettes were grown to 1×10^7 to 5×10^7 /ml in YPD and split into separate YPD or YPD + 2% Gal cultures at 2×10^5 to 3×10^5 /ml. Aliquots were subsequently sonicated, counted, and spread to YPD plates, and the results were averaged. The fraction of cells which were unbudded, small-budded, or large-budded was analyzed with a hemacytometer after sonication. A total of 100 to 300 cells were counted at each time point, and large-budded cells were defined as those in which the bud was >50% of the size of the mother cell.

Purification and analysis of yeast genomic DNA. DNA was purified from cells containing plasmid and chromosomal *GAL1::EcoRI* fusions by three distinct methods. DNA prepared from galactose-induced cultures by alkali lysis (34), by lysis in the presence of Triton X-100, sodium dodecyl sulfate, and phenol-chloroform (23), or after vortexing with glass beads in the presence of high levels of EDTA (see below) exhibited *EcoRI*-specific chromosomal DNA repeat bands on agarose gels (described in reference 57). Using a fourth purification protocol, Barnes and Rine (3) also observed extensive cleavage of chromosomal DNA when *EcoRI* was expressed in vivo. The analysis presented in Fig. 8 used DNA prepared from wild-type and *rad52* cells by a procedure which requires only a few minutes of extraction in high-EDTA buffer. Between 25 and 200 ml of cultured cells was precipitated and resuspended in 400 μ l of 10 mM Tris (pH 8.0)-100 mM EDTA. Approximately 400 μ l of acid-washed glass beads was added, and the tube was vortexed hard for 90 s and microcentrifuged for 1 min at room temperature. The resulting supernatant was recentrifuged for 4 min and trans-

ferred, and 200 μ l of 10 mM Tris (pH 8.0)–1 mM EDTA (TE) was added. After gentle extraction with phenol, 50:50 phenol-chloroform, and chloroform, the DNA preparation was treated with RNase A, precipitated with isopropanol, washed with 70% ethanol, and resuspended in TE. The DNA concentrations were determined by fluorometry. The purified DNA was heated at 68°C for 5 min and cooled on ice before 0.8 or 1.0 μ g was loaded onto 0.6% agarose gels. Varying the initial steps of this procedure by using 50, 100, or 200 mM EDTA, by adding 40 U of commercial *EcoRI* to the cells before lysis, or by resuspending cells at 4°C rather than at room temperature did not alter the results (data not shown).

***EcoRI*-induced interchromosomal recombination rates.** Diploid strains created by crossing RM10-32D and RM26-26C (Table 1) containing p316Gal or YCpGal:Rlb were used in the assay. Cells grown in Glu – Ura were spread at low density to Glu – Ura and Gal – Ura plates. After 3 or 4 days of growth at 30°C, 11 colonies from each strain were harvested, diluted, and spread to Glu – Ura plates to determine total cells and to Glu – Leu and Glu – Trp plates to detect *LEU1* and *TRP5* recombinants.

RESULTS

Growth of *rad52* but not *Rad*⁺ cells is inhibited by *EcoRI*.

Expression of *EcoRI* in vivo was previously shown to inhibit the growth of both *Rad*⁺ and *rad52* haploid yeast cells (3). These experiments were performed with YCpGal:Rlb (*URA3*), a centromeric plasmid containing a fusion of the *EcoRI* *R* gene with the *GAL1* promoter (with no *EcoRI* sites within the plasmid). During a study of the effects of *EcoRI*-induced DSBs on chromosome stability, we observed that the growth rates of most *Rad*⁺ strains containing YCpGal:Rlb were only slightly reduced on plates containing 2% galactose (Gal – Ura plates). To investigate this discrepancy further, haploid *Rad*⁺ strains obtained from several sources were transformed with the *GAL1::EcoRI* plasmid and with a control vector (pRS316). Transformants of each strain were replica plated as patches or pronged cells from Glu – Ura plates to Gal – Ura plates and incubated at 30°C for 3 days. The strains which were tested (including A364a, S288c, and SK1 strain backgrounds) are listed and referenced in Table 1. Most *Rad*⁺ background strains tested (RM10-32D, RM26-26C, EPY214-1B, MAR1530, VL6 α , SSL231, LS20, GRY1060, S1InsE4A [CG379], and T334) Table 1) grew at near-normal rates in galactose media. Another strain, GRY1078 (43), grew slowly and only two strains, tNR85T1 and BGW1-7a, were unable to grow when the endonuclease was expressed. tNR85T1 also exhibits other strain-specific phenotypes in response to enzymatically induced DSBs (6) (see Discussion). The growth of *rad52* mutants was inhibited by *EcoRI* expression in all strains examined, although some variation in the degree of inhibition was observed. Thus, the consequences of *EcoRI*-generated DSBs on the growth of most *Rad*⁺ and *rad52* strains were comparable to those of a single HO-induced break at *MAT* (14, 28, 31, 45).

The effects of *EcoRI*-induced DSBs on cell growth have been examined quantitatively with strain T334. This strain contains the mutant alleles *reg1-501*, alleviating glucose repression of the *GAL1* and *GAL10* promoters, and *gal1*, blocking the metabolism of galactose (24, 53). Addition of galactose to T334 cells cultured in glucose media induces the *GAL1* promoter while permitting the cells to continue growth on glucose. T334 also contains mutations within the protease-encoding genes *PEP4* and *PRB1*, potentially increasing the stability of heterologous proteins. The plating efficiencies of wild-type and *rad52* cells containing YCpGal:Rlb on synthetic Gal + Glu – Ura and Glu – Ura plates were determined (Table 2). The relative plating efficiency of *rad52* strains containing the plasmid (on galactose versus glucose plates) was approximately 600-fold lower than that of wild-type cells expressing the endonuclease. T334 strains containing a chromosomal *GAL1::EcoRI* cassette at *LYS2* (YLKL340) or *HIS3* (YLKL350) were also tested. Δ *rad52::LEU2* derivatives of each strain exhibited poor plating efficiencies on YPD+2%Gal plates (Table 2).

TABLE 2. Plating efficiencies of cells expressing *EcoRI* or HO^a

Strain	PE on Gal/ PE on Glu	Fold decrease (<i>rad52</i> vs <i>RAD52</i>)	Refer- ence
<i>RAD52</i> (pRS316)	1.06		
<i>rad52</i> (pRS316)	0.89	1.2	
<i>RAD52</i> (YCpGal:Rlb)	0.32		
<i>rad52</i> (YCpGal:Rlb)	5.1×10^{-4}	627.5	
<i>RAD52</i> (Δ <i>lys2::GALEcoRI</i>)	0.71		
<i>rad52</i> (Δ <i>lys2::GALEcoRI</i>)	18×10^{-4}	394.4	
<i>RAD52</i> (Δ <i>his3::GALEcoRI</i>)	0.52		
<i>rad52</i> (Δ <i>his3::GALEcoRI</i>)	14×10^{-4}	371.4	
<i>RAD52</i> (pGALHO)	0.46		
<i>rad52</i> (pGALHO)	3.3×10^{-4}	1,394	
Previous results ^b			
<i>rad52</i> (pGALHO)	$\sim 1 \times 10^{-4}$		14
<i>rad52</i> (pGALHO)	(1–4) $\times 10^{-4}$		31
<i>rad52</i> (pGALHO)	(0.1–1) $\times 10^{-4}$		28 ^c
<i>rad52</i> (pGALHO)	16×10^{-4}		45

^a Plating efficiencies (PE) (plating CFU/hemocytometer counts) were determined at 30°C with synthetic Glu – Ura and Gal + Glu – Ura plates for plasmid-containing strains and YPD and YPD+2%Gal plates for cells containing integrated fusions. T334 (*reg1-501*) and derivatives used in the assay are described in the text.

^b Previously published plating efficiencies for *REG1 rad52* strains obtained by similar methods.

^c Gal/Glu plating efficiencies were determined at 25 and 33°C.

The plating efficiencies of cells containing pGALHO were also assessed to compare growth-inhibitory effects in T334 to results obtained previously with *REG1* strains. Several studies have indicated that *rad52* mutants containing a *GAL10::HO* fusion on a plasmid are unable to grow on selective galactose media. The relative plating efficiencies (on Gal versus Glu plates) have ranged from 10^{-3} to 10^{-4} (14, 28, 31, 45). As shown in Table 2, a similar result was obtained with T334 Δ R52T cells (Δ *rad52::TRP1*) containing pGALHO (relative plating efficiency, 3.3×10^{-4}).

Effect of plasmid-mediated *EcoRI* and HO expression on viability and cell cycling in *Rad*⁺, *rad52*, and *rad9* cells. The reduced plating efficiencies on galactose media relative to glucose media (Table 2) demonstrated that growth of *rad52* cells is inhibited by *EcoRI*. To examine changes in cell survival and cycling produced by plasmid-based synthesis of the endonuclease, a liquid culture assay system was developed (Fig. 1). pGALHO was again included as a control. Logarithmically growing *Rad*⁺ cells expressing *EcoRI* or HO progressed at near-normal growth rates to stationary phase ($\sim 4 \times 10^7$ to 8×10^7 cells/ml in synthetic Gal + Glu – Ura media). In contrast, both *rad52* and *rad9* cells halted growth at mid-logarithmic densities.

Microscopic examination of wild-type and mutant cells at various times after galactose induction revealed striking differences (Fig. 2). *Rad*⁺ cells expressing *EcoRI* or HO progressed through log phase (a mixture of unbudded, small-budded, and large-budded cells after 12 h) to early stationary phase (mostly unbudded G₁ cells [Fig. 2A to C]). This was also true for *rad52* cells containing the control vector pRS316 (Fig. 2D). However, *rad52* cells expressing *EcoRI* or HO accumulated as large-budded cells (Fig. 2E and F). Staining with 4',6-diamidino-2-phenylindole (DAPI) revealed that most of these cells contain a single nucleus located near the neck of the new bud (data not

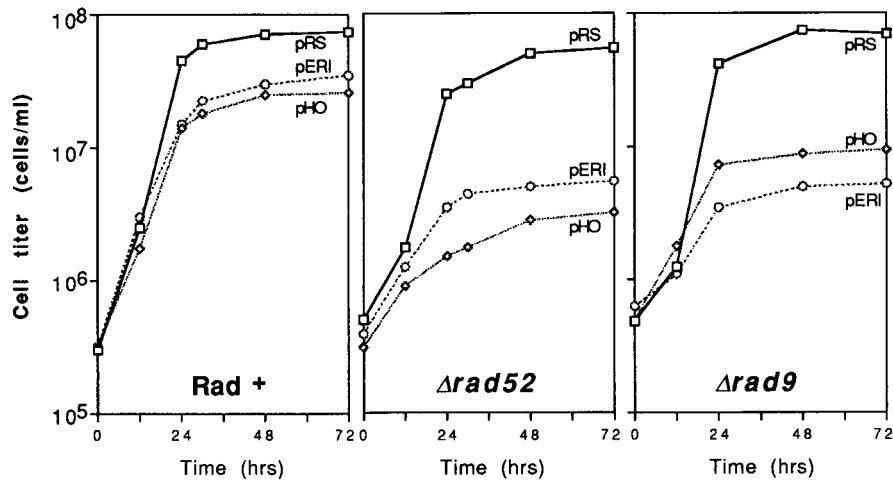


FIG. 1. Expression of *EcoRI* and HO from centromeric plasmids inhibits the growth of *rad52* and *rad9* strains. Late-log-phase cells were inoculated into Gal + Glu - Ura liquid medium at time zero and counted with a hemacytometer as described in Materials and Methods. Abbreviations: pRS, pRS316; pHO, pGALHO; pERI, YCpGal:RIb.

shown). Furthermore, the mother and daughter portions of many of the large-budded cells appeared greatly enlarged. A similar G₂ arrest phenotype has been reported for yeast cells treated with X rays and MMS (55, 71, 80). Induction of G₂

arrest by HO endonuclease has been observed previously in strains containing HO recognition sites on chromosomes or plasmids (6, 18, 66).

The results presented in Fig. 2B and C suggest that elonga-

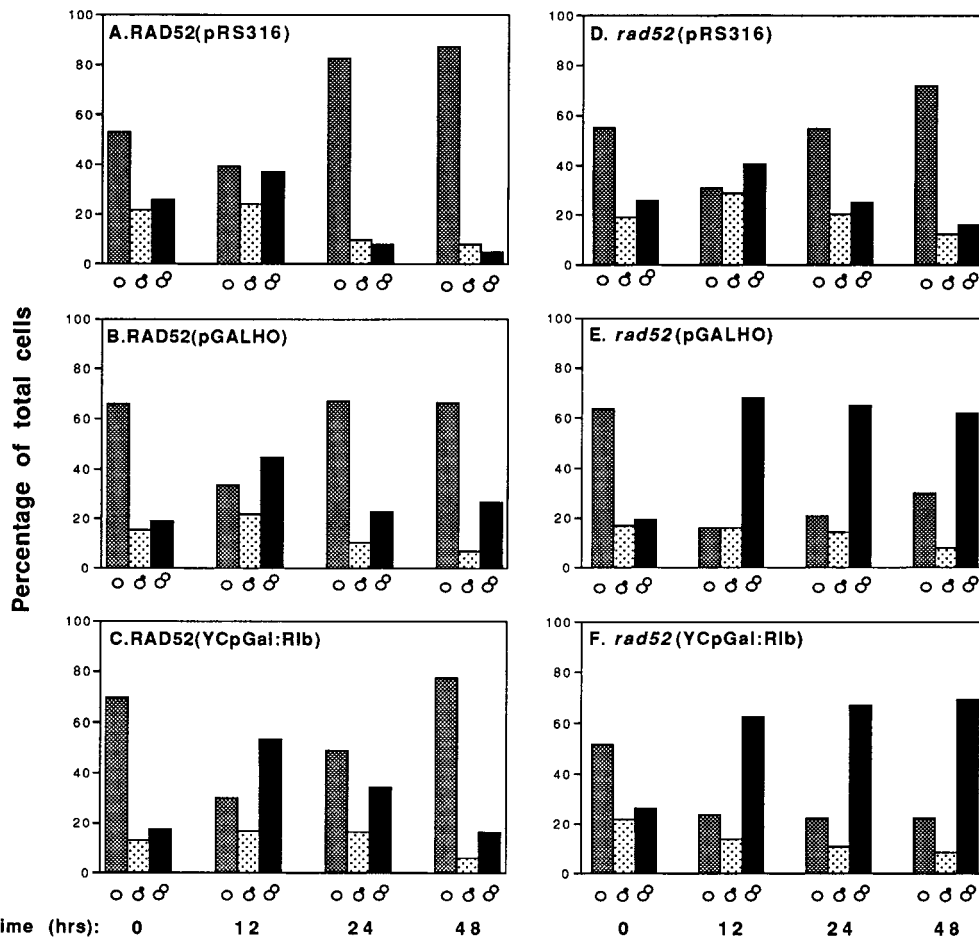


FIG. 2. *rad52* cells synthesizing *EcoRI* and HO are chronically arrested as large-budded cells. The distribution of unbudded (□), small-budded (▨), and large-budded (■) cells during growth of T334 (*Rad*⁺) and T334Δ*R52T* (*rad52*) strains in galactose media is shown (see Materials and Methods).

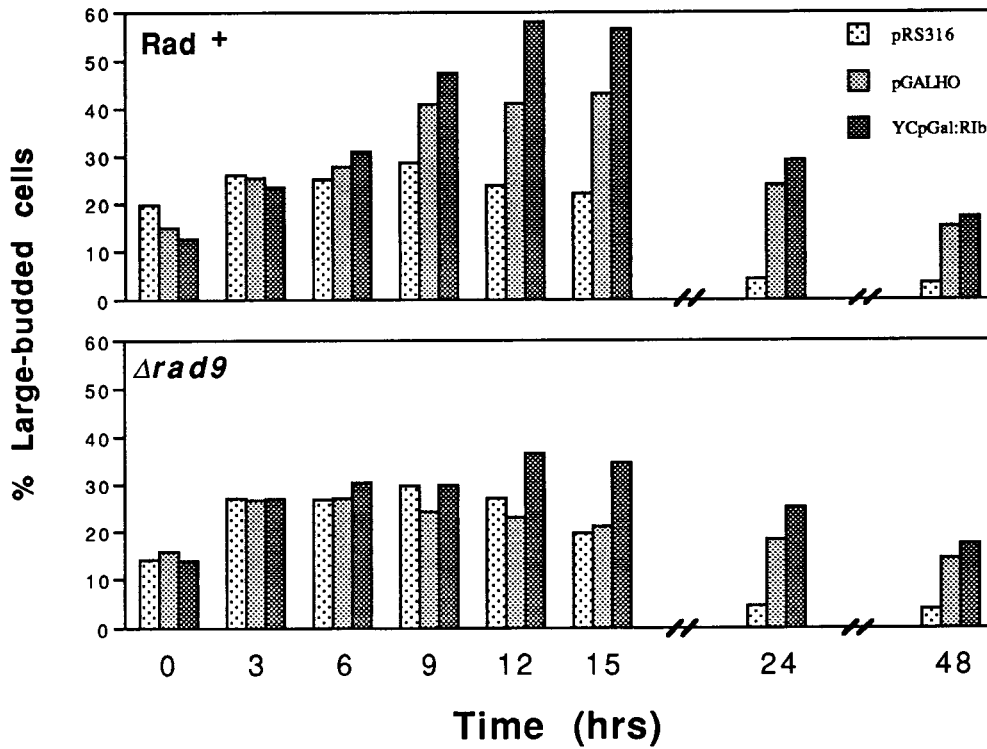


FIG. 3. Endonuclease-induced G₂ arrest is largely dependent on *rad9*. The accumulation of large-budded cells in Rad⁺ and *rad9* strains after induction of *EcoRI* and HO endonuclease expression is shown.

tion of G₂ phase occurred in the wild-type strain when *EcoRI* and HO were expressed (note the increase in the number of large-budded cells at 12 h; the cells were at mid-logarithmic-phase growth densities at this point [Fig. 1]). This transient arrest and its dependence on *RAD9* were examined by using shorter time intervals, and the results are shown in Fig. 3. Rad⁺ cells synthesizing *EcoRI* contained 50 to 60% large-budded cells at 9, 12, and 15 h after induction. The control population (pRS316) achieved a maximum of ~30% large-budded cells in this medium. The lengthening of G₂ phase generated by *EcoRI* and HO expression was largely *RAD9*

dependent; *Δrad9* strains expressing *EcoRI* or HO did not exceed 35% G₂/M cells (Fig. 3).

The effects of *EcoRI*- and HO-generated DSBs on cell viability were assessed by growing cells in liquid Gal + Glu – Ura medium (as for Fig. 1), counting them with a hemacytometer, and rescuing them onto Glu-Com plates. Cell survival at 0, 12, 24 and 48 h after induction of HO or *EcoRI* in selective medium is presented in Fig. 4. Rad⁺ cells expressing each endonuclease exhibited consistent plating efficiencies (50 to 70%) when rescued onto Glu-Com plates. Surprisingly, *rad52* cells also retained high viability throughout the time course

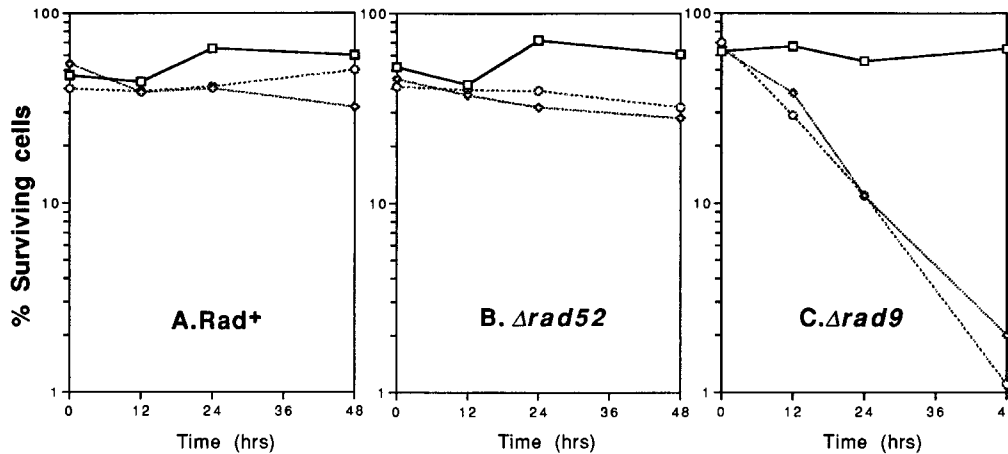


FIG. 4. Plasmid-based synthesis of *EcoRI* and HO causes cell killing in *rad9* mutants but not in Rad⁺ or *rad52* cells. Cells grown in Gal + Glu – Ura medium (maintaining selection for the plasmid) were rescued onto synthetic Glu-Com plates as described in Materials and Methods. Symbols: □, pRS316; ◇, pGALHO; ○, YCpGal:RIb.

TABLE 3. Stabilities of HO and *EcoRI* expression plasmids^a

Vector	Strain	% of plasmid-containing cells at time (h):		
		0	12	24
pRS316	Rad+	67.5	60.5	88.9
	$\Delta rad52$	47.2	66.7	67.8
	$\Delta rad9$	52.9	45.7	81.0
pGALHO	Rad+	55.4	48.2	52.1
	$\Delta rad52$	45.7	27.0	3.8
	$\Delta rad9$	61.9	60.2	51.2
YCpGal:RIb	Rad+	72.9	45.7	18.1
	$\Delta rad52$	55.5	41.5	7.6
	$\Delta rad9$	75.0	45.3	15.8

^a The mean percentage of cells retaining the plasmid is shown. Aliquots of cells grown in Gal + Glu - Ura liquid medium were removed and spread directly to synthetic Glu-Com and Glu - Ura plates.

(Fig. 4B). Thus, *rad52* cells displayed growth inhibition and G₂ arrest when *EcoRI* and HO were synthesized but did not exhibit appreciable killing. In contrast, both *EcoRI* and HO induced extensive cell killing in *rad9* mutants. The percentage of viable cells decreased from 60 to 1–2% after *EcoRI* and HO were induced (Fig. 4C).

Although selection was maintained for each plasmid throughout the time course (Gal + Glu - Ura medium), the possible effects of plasmid instability were addressed by determining the percentage of cells retaining each plasmid at 0, 12, and 24 h after galactose induction. A comparison of colonies arising on Glu-Com and Glu - Ura plates is presented in Table 3. pRS316 was stably maintained in wild-type and mutant cells. Loss of the *GAL1::EcoRI* plasmid was similar in all three strains (ca. five- to sevenfold reduction after 24 h). pGALHO was stably propagated in Rad⁺ and *rad9* cells but was very unstable in *rad52* cells expressing the endonuclease (~13-fold loss). The uniform instability of the *GAL1::EcoRI* plasmid in all backgrounds and the nearly identical survival results obtained with integrated fusions (described below) suggest that the higher survival of Rad⁺ and *rad52* strains than of *rad9*

mutants is not attributable to plasmid loss. It is not possible to assess the extent of HO-induced cell killing from these experiments because of the instability of pGALHO in induced *rad52* cells. However, the observation that *rad52* mutants were chronically arrested as large-budded cells (Fig. 1 and 2E) suggests that cleavage at *MAT* occurred in most cells (also see Discussion).

Consequences of chromosome-based *GAL1::EcoRI* synthesis on growth, cell cycling, and viability. To measure the effects of *EcoRI*-induced cleavage under conditions which ensured that endonuclease expression occurred in all cells, *GAL1::EcoRI* cassettes were integrated into T334 strains at *LYS2* and *HIS3*, yielding strains YLKL340 [$\Delta lys2::(GAL1::EcoRI TRP1)$] and YLKL350 [$\Delta his3::(GAL1::EcoRI TRP1)$] (Table 1). The growth of Rad⁺ and *rad52*-derived strains with and without inducer is shown in Fig. 5. Cells were grown in YPD without galactose and then shifted to YPD or YPD+2%Gal. The timescale is shortened in Fig. 5 compared to Fig. 1 because of the faster induction of the *GAL1* promoter and the shorter doubling time of cells in YPD+2%Gal than in synthetic Gal + Glu medium (24). As observed with the plasmid-based expression systems, the growth of Rad⁺ cells was only modestly affected by *EcoRI* synthesis. The growth of *rad52* mutants was impaired, especially in cells containing *EcoRI* integrated at *HIS3*; these cells achieved only one doubling between 4 and 24 h after induction (Fig. 5).

EcoRI-generated DSBs caused a modest increase in the number of large-budded cells in wild-type yeast and a much larger increase (typically 75 to 85% of total cells) in *rad52* mutants (Fig. 6A and B). Data obtained with the $\Delta lys2::(GAL1::EcoRI)$ construct is shown in the figure. Similar results were obtained with strains containing *GAL1::EcoRI* integrated at *HIS3* (data not shown). Arrest of cell cycling was not observed in *rad9* mutants (Fig. 6C). In Fig. 6, data are presented for cells cultured in YPD+0.5%Gal and YPD+2.0%Gal. The kinetics of induction of the *GAL1* promoter in *reg1-501* cells is dependent upon the concentration of the inducer (24). As shown in Fig. 6, 0.5% galactose was nearly as effective as 2.0% galactose at inducing cell cycle arrest.

Analysis of the effects of endonuclease expression on cell survival is presented in Fig. 6D to F. While the viability of

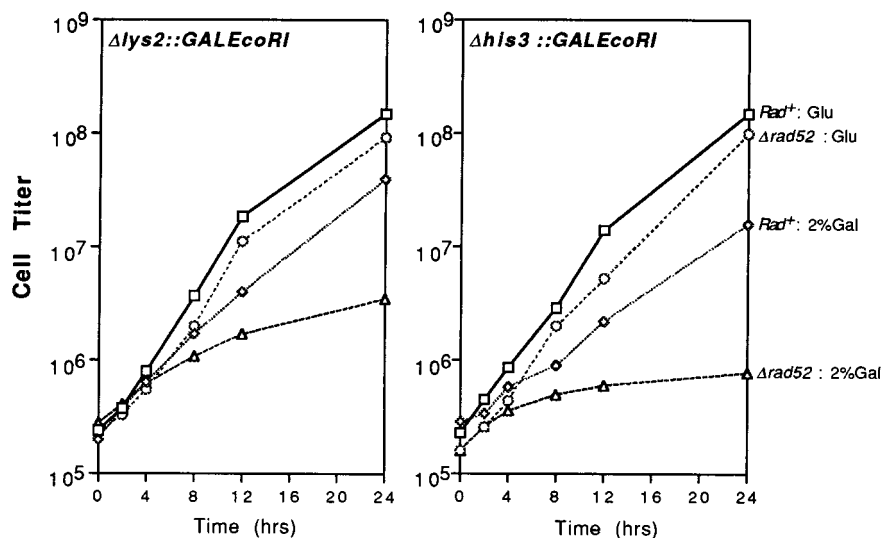


FIG. 5. Growth of *rad52* cells containing integrated *GAL1::EcoRI* is inhibited in 2% galactose medium. Growth of cells containing *GAL1::EcoRI* integrated at *LYS2* or *HIS3* in glucose (YPD) or Glu + Gal (YPD+Gal) medium.

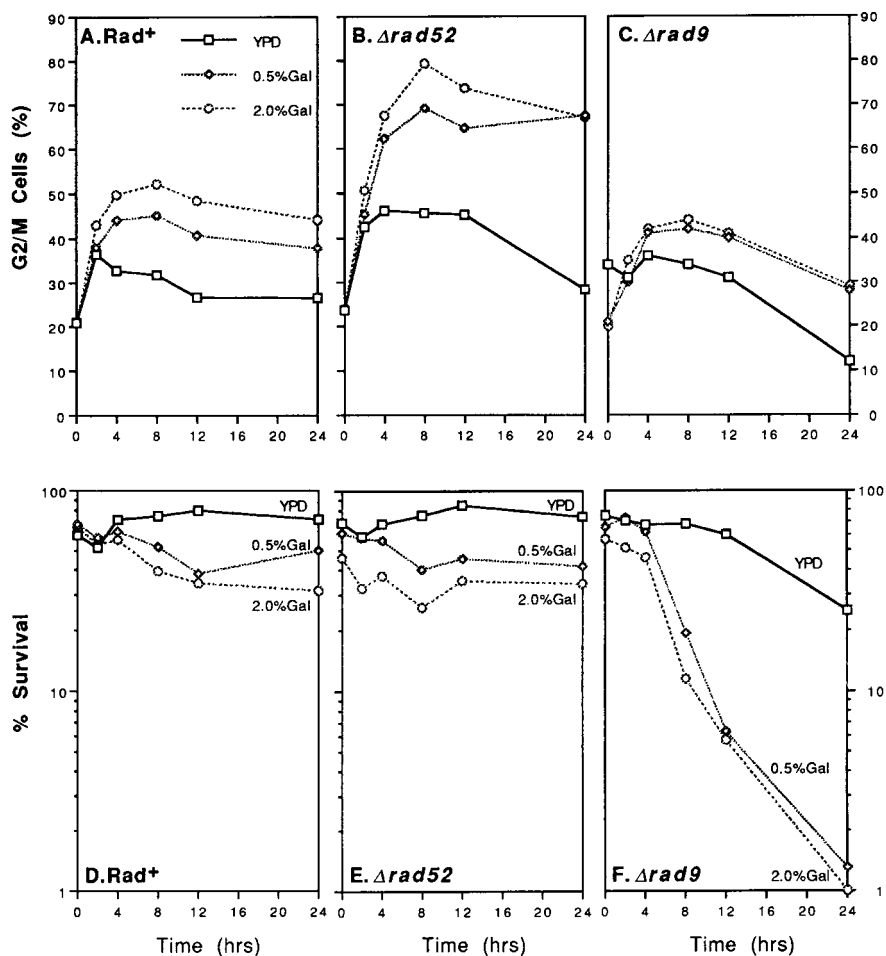


FIG. 6. Effects of chromosome-based expression of *EcoRI* on cell cycling and survival. (A to C) Analysis of cell cycling after induction of endonuclease synthesis in $\Delta lys2::(GAL1::EcoRI)$ fusion strains. (D to F) Survival of *Rad*⁺, *rad52*, and *rad9* strains containing the $\Delta lys2::(GAL1::EcoRI)$ construction after growth in YPD or YPD + Gal medium.

Rad⁺ and *rad52* cells was only modestly reduced (ca. two- to threefold for both the *lys2* and *his3* constructs [Fig. 6 and data not shown]), *rad9* strains lost viability rapidly after galactose induction (from 60% viable cells to ~1% at 24 h [Fig. 6F]). These results are similar to the survival curves obtained with the *GAL1::EcoRI* plasmid (Fig. 4). One difference observed with strains containing integrated fusions was that *rad9* mutants had reduced viability after approximately 24 h of growth in YPD medium without galactose. This result suggests that glucose depletion in stationary-phase YPD cultures resulted in derepression of *GAL1* promoter activity. In *reg1-501* strains, *GAL* promoters are repressed in 2% Glu, are derepressed in the absence of Glu and Gal (resulting in a modest elevation of transcription), and are induced in Gal or Gal + Glu media (increasing transcription 1,000 to 2,000-fold relative to repressed levels) (24, 53). The decrease in survival demonstrates that even low levels of *EcoRI* expression are lethal to *rad9* strains.

A possible explanation for the high viability of *rad52* strains containing integrated *GAL1::EcoRI* is that they rapidly produce *EcoRI*-defective and/or *EcoRI*-insensitive mutants during galactose induction. Continual production and growth of such mutants might then contribute to the observed slow growth and apparent high survival of the total population. To address this possibility, colonies formed on YPD plates as part

of the survival tests for *lys2::(GAL1::EcoRI)* and *his3::(GAL1::EcoRI)* strains were replica plated to YPD+2%Gal plates. *rad52* strains containing a functional *GAL1::EcoRI* cassette cannot grow on these plates. *rad52* cells grown in YPD or YPD+2%Gal did not accumulate a significant percentage of mutants capable of growing on galactose ($\leq 1\%$) after 4, 8, or 12 h of growth (data not shown). After 24 h of continuous *EcoRI* expression, however, *rad52* cells capable of growing on galactose were observed [average, 14.6% for *lys2::(GAL1::EcoRI)* and 34.4% for *his3::(GAL1::EcoRI)* strains]. Growth inhibition, G₂ arrest, and *EcoRI*-specific DSBs were detectable approximately 4 h after induction. These results indicate that the high viability of *rad52* cells during the first 12 h (Fig. 6E) is not due to the appearance of mutants.

Previous studies have demonstrated that linearized plasmid DNA containing complementary overhangs is efficiently recircularized after transformation into wild-type and *rad52* cells but not in strains lacking components of the DNA end-binding Ku complex (Hdf1/Yku70 and Hdf2/Yku80 [9, 44, 45]). A recent report by Barnes and Rio (4) indicated that *hdf1* cells containing a *GAL1::EcoRI* expression plasmid could not grow on selective galactose plates. We have confirmed that plasmid- and chromosome-based expression of the endonuclease blocks the growth of $\Delta hdf1$ strains (Fig. 7A and data not shown). Analysis of cell killing after induction of a chromosomal fusion

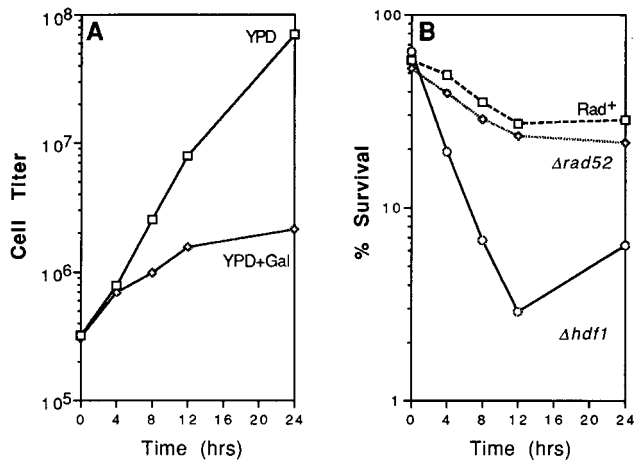


FIG. 7. Expression of *EcoRI* causes growth inhibition and cell killing in *hdf1* mutants. Strains YLKL350, YLKL351, and YLKL389 ($\Delta his3::GAL EcoRI$) were used for the assays. (A) Growth of YLKL389 ($\Delta hdf1::HIS3$) in YPD and YPD+2%Gal. (B) Survival of *Rad*⁺, $\Delta rad52$, and $\Delta hdf1$ strains in YPD+2%Gal.

[$\Delta his3::(GAL1::EcoRI)$] demonstrated that Ku70 is required for survival (Fig. 7B). The percentage of viable cells decreased from 70% to 2–3% after 12 h. The slight increase in the percentage of surviving *hdf1* cells after 24 h correlates with an increase in the number of mutant cells able to grow on galactose plates (data not shown). Accumulation of such mutants was also observed after prolonged expression of *EcoRI* in *rad52* cells (described above).

***EcoRI* expression produces extensive breakage of chromosomal DNA in *Rad*⁺ and *rad52*.** Barnes and Rine (3) previously used ethidium bromide-stained agarose gels and Southern blot experiments to show that *EcoRI* expressed in yeast cuts specifically at its 6-bp recognition site (G^AAATTC). To investigate the extent of *EcoRI*-induced DNA cleavage in T334 cells under the conditions used in these experiments, three distinct DNA purification methods were used (see Materials and Methods for descriptions of the methods and control experiments). Galactose induction of plasmid-based and integrated *GAL1::EcoRI* fusions resulted in extensive digestion of chromosomal DNA in both *Rad*⁺ and *rad52*-deleted T334 cells (Fig. 8). Genomic DNA prepared from T334 cells and cut with commercial *EcoRI* is included for comparison. *EcoRI* treatment of purified yeast DNA produces several characteristic bands (57). These bands are derived from ribosomal DNA repeats, *Ty* elements, and telomeric Y' repeats. As shown in Fig. 8, many repeat bands produced after complete digestion in vitro were present after 12 or 24 h of digestion in vivo. Differences in band intensities between DNA cleaved in vitro and in vivo may be due to differential access of the enzyme to sites in cellular DNA. The single low-molecular-weight band present at time zero in Fig. 8 may represent double-stranded RNA (57). The kinetics of break formation in wild-type and mutant cells appeared similar, although increased relative amounts of low-molecular-weight DNA were clearly visible in *rad52* cells. In particular, DNA purified from *rad52* cells after 24 h of plasmid-based expression was consistently very low in molecular weight (compare Fig. 8A and B). DNA breakage was not as extensive after 24 h of expression in *rad52* strains containing the *GAL1::EcoRI* fusion integrated at *HIS3* or *LYS2* (Fig. 8D and data not shown). The gel electrophoresis data suggest that a larger number of unrepaired breaks were present in *rad52* cells. However, characterization of differences in

TABLE 4. Recombination rates in cells expressing *EcoRI*

Alleles	Plasmid	Recombination rate ^a in cells grown on:		Fold increase
		Glucose	Galactose	
<i>leu1-12/leu1-c</i>	p316Gal	4.92×10^{-6}	5.18×10^{-6}	22.6
	YCpGal:Rlb	5.36×10^{-6}	121.00×10^{-6}	
<i>trp5-2/trp5-c</i>	p316Gal	2.70×10^{-6}	1.75×10^{-6}	33.3
	YCpGal:Rlb	2.39×10^{-6}	79.50×10^{-6}	

^a Number of events per cell per generation.

the efficiency of rejoining of DNA ends will require further studies.

Expression of *EcoRI* induces recombination between homologous chromosomes. As discussed above, a single DSB produced in vivo by HO or I-*SceI* endonuclease is recombinogenic in yeast cells. The effects of *EcoRI*-induced DSBs on mitotic interchromosomal recombination were assayed in the diploid strain RM10-32D × RM26-26C (Table 1) containing YCpGal:Rlb. This strain contains heteroalleles of *LEU1* and *TRP5* whose recombination has been studied previously (41). There are two recognition sites for *EcoRI* in the coding sequence of *LEU1* and three sites within *TRP5*. Recombination rates in

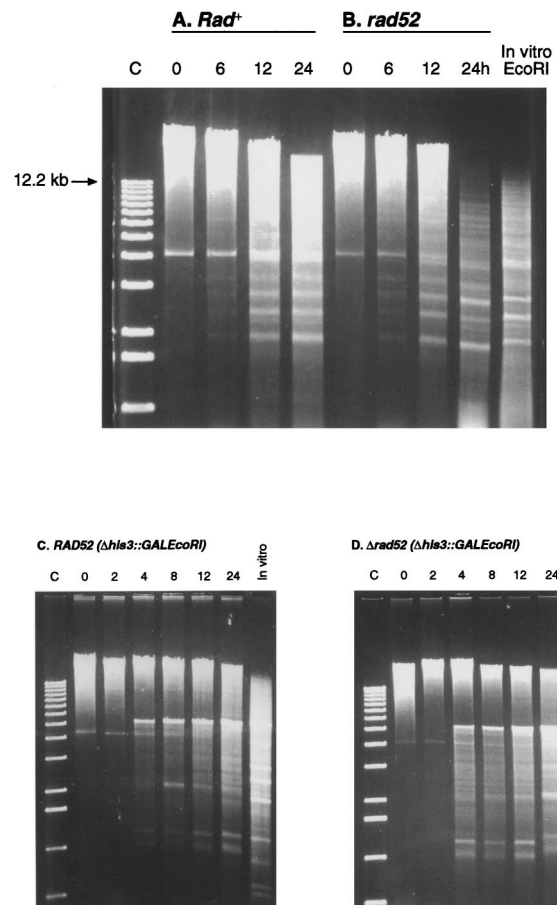


FIG. 8. Expression of *EcoRI* in *Rad*⁺ and *rad52* cells produces extensive breakage of chromosomal DNA. (A and B) DNA was purified from cells containing YCpGal:Rlb at the indicated times after induction in galactose. (C and D) Analysis of DNA purified from cells containing chromosomal $\Delta his3::(GAL1::EcoRI)$ fusions (YLKL350 and YLKL351).

cells grown under inducing conditions (on Gal – Ura plates) were 23 times (*leu1*) and 33 times (*trp5*) higher than in cells grown on glucose plates (Table 4). Thus, as previously observed for DSBs produced by treatment with ionizing radiation or expression of HO and I-SceI (see the introduction), *EcoRI*-induced DSBs stimulated homologous interchromosomal recombination.

DISCUSSION

We have examined the effects of *EcoRI*-induced DSBs on viability and cell cycle progression in wild-type, repair-deficient, and checkpoint-defective yeast cells. Several of the responses observed in cells expressing *EcoRI* have also been found in cells containing DSBs generated by ionizing radiation or after induction of a single DSB by HO endonuclease. Expression of *EcoRI* in logarithmically growing Rad⁺ cells caused slightly decreased growth rates and elongation of G₂ phase, but cells were able to progress to stationary phase. This result suggests that most breaks were efficiently repaired in all stages of the cell cycle. Previous assays of *GAL* promoter activity have demonstrated that galactose-induced transcription occurs throughout the cell cycle (12, 26). Synthesis of *EcoRI* or HO in *rad52* mutants inhibited cell growth and caused cells to arrest in G₂. Induction of G₂ arrest by HO has been observed previously in strains containing either chromosomal or plasmid recognition sites for the enzyme (6, 18, 66). Other examples of DSB-induced cell cycle arrest have been reported for cells containing a dicentric chromosome (10) and for cells expressing a mutant topoisomerase I enzyme (34).

***EcoRI*-induced DSBs can be repaired in *rad52* mutants.** Surprisingly, Rad⁺ and *rad52* cells expressing *EcoRI* in liquid galactose media could be rescued onto glucose plates with approximately equal efficiency (Fig. 4 and 6). This result indicates that there is an alternative pathway for the repair of enzymatically induced DSBs in chromosomal DNA which is *RAD52* independent. Expression of *EcoRI* in *rad52* derivatives of several other strains (EPY214, MAR1530, VL6 α , and SSL231 [Table 1]) also did not result in cell killing (data not shown). Although the protein was not essential for the maintenance of cell viability, some function(s) of Rad52 was clearly required for efficient progression of cells past the checkpoint at G₂. This implies that a significant fraction of broken DNA ends in logarithmically growing Rad⁺ cells were repaired through a *RAD52*-dependent repair process, allowing passage beyond G₂, but that this pathway did not provide a survival advantage over *rad52* cells. Furthermore, growing and stationary-phase Rad⁺ cells in G₁ lacked sister chromatids and were unable to participate in *RAD52*-mediated recombination but remained viable when *EcoRI* was expressed. The high viability of wild-type and *rad52* cells was observed even though continuous expression of *EcoRI* produced numerous DSBs within cellular DNA (Fig. 8) (3). This result suggests that the broken ends produced after endonuclease cleavage remain in proximity, probably due to constraints imposed by chromatin structure (including components of the nucleosome and possibly end-joining proteins such as Hdf1 and Hdf2). It is possible that the 4-bp overhangs produced by *EcoRI* are efficiently reannealed in vivo, creating DNA segments containing two staggered nicks which are repairable by *RAD52*-independent mechanisms.

Several previous studies have established that *rad52* cells are highly susceptible to killing by ionizing radiation and are specifically unable to rejoin radiation-induced DSBs (15, 16, 64). The broken ends of irradiated DNA usually have associated base or sugar damage and are often missing one nucleotide base (54). It is therefore likely that such ends require extensive

processing and/or recombinational repair to restore the intact, unmutagenized strand. *RAD52* is also required for the repair of DSBs containing modified termini such as those generated by various chemical clastogens, e.g., bleomycin (29, 46). Furthermore, we have recently observed that high-level expression of *PvuII*, which generates blunt termini, produces much greater killing in *rad52* cells than in Rad⁺ strains (36). These results suggest that repair of DSBs containing complementary overhangs can be *RAD52* independent but that repair of DSBs with damaged or blunt termini requires homologous recombination.

The possibility that *EcoRI*-induced DSBs in yeast chromosomes can be repaired by nonrecombination-independent pathways has gained considerable additional support from previous studies: (i) recircularization of linear, cohesive-ended plasmid DNA after transformation into yeast cells is not greatly reduced in *rad52* mutants (9, 45) (see below); (ii) Schiestl et al. (68) observed that restriction enzyme-mediated integration of linear DNA into yeast chromosomes in vivo is *RAD52* independent, but *RAD50*, which has been associated with end-joining pathways of plasmid DSB repair (described below), was required for this process; (iii) a study by Heitman et al. (21) found that *Escherichia coli* cells deficient in the recombinase enzyme RecA, which plays a central role in radiation resistance, recombination, and mutagenesis (76), were not more sensitive to *EcoRI*-induced killing than wild-type cells; and (iiii) expression of restriction endonucleases in mammalian cells produces many of the same effects as treatment with X rays, e.g., decreased viability and an increase chromosomal aberrations, mutations and cellular transformation. However, several studies have indicated that DSBs with complementary overhangs produce such effects less efficiently than do DSBs with blunt termini (11, 54). These data are consistent with the hypothesis that prokaryotic and eukaryotic cells have repair mechanisms for cohesive-ended DSBs which are not available for damaged or blunt termini.

Plasmid-based expression of HO endonuclease, which produces a single cohesive-ended DSB at the *MAT* locus, caused prolonged G₂ arrest of *rad52* mutants but did not produce detectable killing (although *rad9* mutants were killed [Fig. 4 and see below]). However, the centromeric plasmid used in these studies (pGALHO) was unstable in *rad52* cells in galactose medium despite selection for the vector (Table 3). Under the same conditions, pGALHO was stably maintained in Rad⁺ and *rad9* cells. Thus, the effects of high-level expression of HO on viability could not be determined from these experiments. The instability of pGALHO in *rad52* mutants may be due to the chronic disruption of the chromosome and cell cycles in these cells.

A number of studies have shown that the expression of HO from its natural promoter or from a *GAL* promoter blocks the growth of *rad52* mutants (see the introduction). This effect has generally been interpreted as evidence of lethality, but this conclusion has not been tested rigorously. Using *reg1-501* strains containing integrated *GAL10::HO* cassettes, we have recently observed that high levels of expression of HO (in 0.2 to 2% galactose) produced greater killing in *rad52* cells than in Rad⁺ cells. However, synthesis of low levels of HO (with 0.005% galactose) resulted in high viabilities for both strains (36). The growth of *rad52* cells was inhibited at this lower galactose concentration, with >70% of cells arrested in G₂, indicating that breakage occurred in most cells. Thus, when the level of endonuclease activity was reduced (presumably corresponding to a reduction in repeated cleavage, repair, and re-cleavage at *MAT*), *RAD52* was required for cell growth but not for survival. These results are analogous to the effects of *EcoRI*

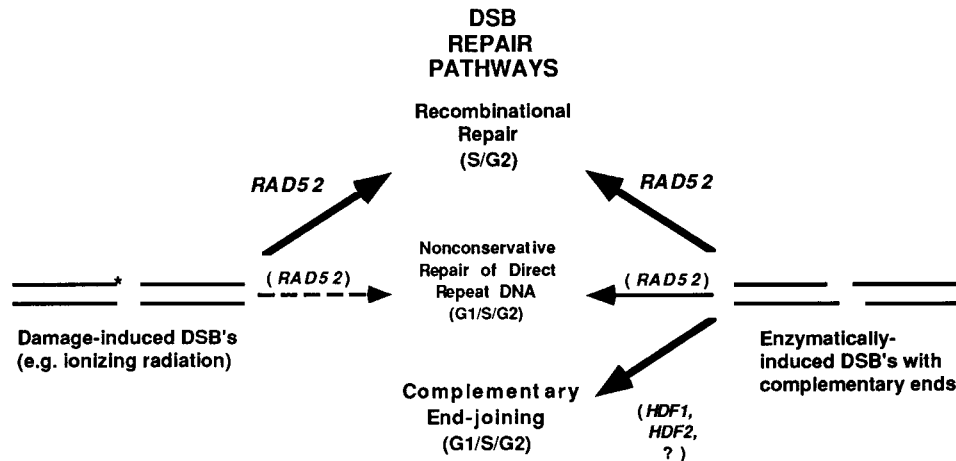


FIG. 9. Alternative pathways of repair for DSBs containing damaged ends (*) or ends with complementary overhangs. *RAD52*-mediated homologous recombination is required for repair of DSBs with damaged termini produced by physical and chemical DNA-damaging agents (e.g., ionizing radiation) but is not essential for repair of DSBs containing complementary ends (see the text for details).

expression in *rad52* mutants and suggest that DSBs induced by both HO and *EcoRI* can be repaired by recombination-dependent and -independent mechanisms.

How are DSBs repaired in *rad52* mutants? The growth and survival of haploid cells synthesizing the endonuclease required a functional *HDF1* (*YKU70*) gene (Fig. 7). Past studies have demonstrated that repair of damage-induced DSBs in *S. cerevisiae* occurs primarily by conservative, homologous recombination mechanisms (16, 56, 70). However, recombination-independent end-joining processes, which may be either precise or error prone (discussed below), and nonconservative forms of repair within long direct repeat sequences have been described. Repair of DSBs in DNA with direct repeats often results in formation of deletions and may be *RAD52* dependent or independent (30).

Genes which have been associated with end-joining pathways include *HDF1/YKU70*, *HDF2/YKU80*, *RAD50*, *XRS2* and *MRE11* (9, 13, 39, 44, 45, 47, 68, 72, 78). *HDF1* and *HDF2* encode subunits of a DNA end-binding complex which is found in yeast and in higher eukaryotes (referred to as Ku70 and Ku80, respectively). This heterodimeric complex participates in the repair of radiation-induced DSBs and V(D)J recombination in mammalian cells (25). Recent experiments have demonstrated that the repair of plasmid DNA containing a DSB with complementary overhangs is highly precise in yeast cells (9, 45). In these studies, linearized plasmids containing cohesive ends (produced by a single restriction enzyme) were efficiently recircularized after transformation into *Rad*⁺ cells. This end-joining process was accurate and was reduced only two- to threefold in *rad52* mutants. However, in yeast Ku70- and/or Ku80-deficient strains, transformation efficiencies were reduced 10- to 14-fold (45) and 25- to 400-fold (9). In addition, the proportion of recircularized plasmids that contained precisely joined termini was greatly reduced in Ku-defective mutants. In contrast to the results obtained for DNA with cohesive ends, DNA molecules containing blunt ends could not be repaired by Ku-dependent end joining (9).

The results described above suggest that pathways of repair are operative for DSBs containing enzymatically induced complementary overhangs that are not available for DSBs produced by physical and chemical DNA-damaging agents (Fig. 9). It is likely that wild-type cells are able to cycle and survive when *EcoRI* is expressed because at least three principal path-

ways of DSB repair are available: (i) *RAD52*-dependent homologous recombination; (ii) Ku-mediated complementary end joining; and (iii) nonconservative repair of breaks occurring in dispensable direct-repeat DNA (e.g., within ribosomal DNA) which has variable dependency on *RAD52* (30). In *rad52* strains, only pathways (ii) and (iii) are operative. These repair systems are insufficient to permit cells to progress past the G₂ checkpoint when *EcoRI* is continuously expressed. However, after transfer to glucose medium (inhibiting the synthesis of *EcoRI*), these two pathways are sufficient for repair of the broken chromosomes, and cycling resumes without cell death.

Requirement for a functional cell cycle checkpoint. Induction of G₂ arrest by *EcoRI* was largely dependent on *RAD9*. The growth rate of *rad9* cells was strongly reduced by plasmid-based expression of *EcoRI* and was modestly inhibited in cells containing *GALI::EcoRI* fusions integrated at *LYS2* and *HIS3*. In contrast to results obtained with *rad52* strains, *rad9* mutants died rapidly after galactose induction of the endonuclease (Fig. 4 and 6). Killing was also seen in *rad9* strains after HO endonuclease-induced breakage at *MAT* (using pGALHO). In addition, we have recently observed that expression of *EcoRI* and HO produces growth inhibition and cell killing in *rad17* mutants, which are also defective in damage-induced cell cycle arrest (36).

RAD9 plays a critical role in the cell-cycling response of yeast cells to DNA damage and is required for cell cycle arrest responses in G₁ and G₂ but not S (1, 67, 71, 80). Damage-induced G₂ arrest requires *RAD17*, *RAD24*, and *MEC3* in addition to *RAD9*. Interestingly, analysis of UV light and methyl methanesulfonate sensitivity in single and double mutants has demonstrated that *RAD9* acts in a different repair pathway(s) from the other three genes (38). Recent experiments have indicated that *RAD9* is also required for DNA damage-induced transcription of several repair genes, including *RAD51* and *RAD54* (1). *EcoRI*-induced DSBs caused transient arrest in *Rad*⁺ cells and prolonged G₂ arrest in *rad52* mutants, but most *rad9* cells continued cycling. This suggests that endonuclease-induced killing of *rad9* mutants was a result of defective arrest mechanisms. However, impaired transcriptional induction of genes required for DSB repair may also be involved (1).

Rad⁺ strains continue cycling when *EcoRI* is expressed. Most previously published Rad⁺ strains containing the *GALI::EcoRI* plasmid formed colonies on selective galactose medium at near normal growth rates (10 of 12 strain backgrounds tested, including A364a, S288c, and SK1 strains). Two strains, tNR85T1 (6) and BGW1-7a (81), were unable to grow when *EcoRI* was expressed. These strains are radiation resistant and it remains unclear whether the strain disparities are attributable to differences in galactose induction kinetics, *EcoRI* stability and/or transport, chromatin structure, or some other factor. We note, however, that induction of *GALI-10* promoters and growth in galactose are much faster in tNR85T1 and BGW1-7a than in T334 and several other strains we have tested (Table 1 and data not shown). Growth of tNR85T1 was previously reported to be hypersensitive to induction of a single DSB on a plasmid by HO endonuclease (6). It seems likely that HO-mediated killing and *EcoRI*-induced blockage of growth of tNR85T1 (and BGW1-7a) are related phenomena. Recent experiments with diploid strains created by mating BGW1-7a (*EcoRI*⁺) with MAR1530 and VL6 α (*EcoRI*⁻) (Table 1) or with a *MAT α* version of BGW1-7a have established that the sensitivity phenotype is recessive (8).

Summary. We have shown that *EcoRI* endonuclease expression is lethal in Ku-deficient end-joining mutants and checkpoint-defective *rad9* mutants but not in Rad⁺ or *rad52* cells. These results suggest that DSBs containing complementary ends produced by *EcoRI* (or by low-level synthesis of HO) can be repaired via recombination-dependent and -independent mechanisms. The data also suggests that developmentally programmed DSBs, e.g., endonuclease-induced DSBs initiating meiotic recombination, intron homing, or mating-type switching, might in principle be accurately repaired by two separate pathways. In the first pathway, processing of the ends (e.g., 5'→3' exonuclease digestion of one strand [17]) precedes strand exchange and subsequent resolution of recombination intermediates. The second pathway may serve as a conservative restitution mechanism which rejoins the broken DNA molecules without deletion or strand exchange.

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