Repair of Endonuclease-Induced Double-Strand Breaks in *Saccharomyces cerevisiae*: Essential Role for Genes Associated with Nonhomologous End-Joining

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ABSTRACT

Repair of double-strand breaks (DSBs) in chromosomal DNA by nonhomologous end-joining (NHEJ) is not well characterized in the yeast *Saccharomyces cerevisiae*. Here we demonstrate that several genes associated with NHEJ perform essential functions in the repair of endonuclease-induced DSBs *in vivo*. Galactose-induced expression of *Eco*RI endonuclease in *rad50*, *mre11*, or *xrs2* mutants, which are deficient in plasmid DSB end-joining and some forms of recombination, resulted in G2 arrest and rapid cell killing. Endonuclease synthesis also produced moderate cell killing in *sir4* strains. In contrast, *Eco*RI caused prolonged cell-cycle arrest of recombination-defective *rad51*, *rad52*, *rad54*, *rad55*, and *rad57* mutants, but cells remained viable. Cell-cycle progression was inhibited in excision repair-defective *rad1* mutants, but not in *rad2* cells, indicating a role for Rad1 processing of the DSB ends. Phenotypic responses of additional mutants, including *exo1*, *srs2*, *rad5*, and *rdh54* strains, suggest roles in recombinational repair, but not in NHEJ. Interestingly, the rapid cell killing in haploid *rad50* and *mre11* strains was largely eliminated in diploids, suggesting that the cohesive-ended DSBs could be efficiently repaired by homologous recombination throughout the cell cycle in the diploid mutants. These results demonstrate essential but separable roles for NHEJ pathway genes in the repair of chromosomal DSBs that are structurally similar to those occurring during cellular development.

CHROMOSOMAL DNA double-strand breaks (DSBs) are formed after exposure of cells to various physical and chemical DNA-damaging agents such as ionizing radiation, bleomycin, and methylmethane sulfonate (MMS). DSBs are also produced endogenously through the action of intracellular enzymes and chemicals such as the highly reactive free radicals derived from oxygen metabolism. Recent studies have suggested that DSBs occur frequently in DNA containing specific at-risk sequence motifs (ARMs; *e.g.*, in DNA containing trinucleotide repeats or large inverted repeats; Nag and Kurst 1997; Freudenreich *et al.* 1998; Gordenin and Resnick 1998) and may also form after induction of replication arrest (Seigneur *et al.* 1998). Most clastogenic agents (*e.g.*, radiation, MMS, bleomycin, etc.) produce multiple types of DNA damage, but cause cell killing primarily through induction of unrepaired DSBs (Resnick and Martin 1976; Frankenberg-Schwager and Frankenberg 1990; Obe *et al.* 1992). For example, X-irradiation generates DNA-protein crosslinks, single-strand breaks, and damaged bases and sugars, but only DSBs are strongly correlated with loss of cell viability. DSBs are also generated during cell development. Such enzymatically induced DSBs typically retain undamaged complementary overhangs (*e.g.*, at the ends of DSBs associated with meiotic recombination, mating type switching, and intron homing in yeast; Haber 1992; Mueller *et al.* 1993; Liu *et al.* 1995; Belfort and Roberts 1997). More complex termini have been observed at the ends of the defined DSBs that initiate V(D)J recombination in immunoglobulin and T-cell receptor genes during differentiation of cellular immunity in humans (Jeggo *et al.* 1995; Ramsden and Gellert 1995; Chu 1997). Despite differences in structure and cleavage mechanism, all types of DSBs appear to exert similar effects on chromosome and cellular metabolism, including reduction of cell viability and induction of mutation, recombination, and aneuploidy (Obe *et al.* 1992; Bryant and Johnston 1993; Strathern *et al.* 1995; Lewis *et al.* 1998). In addition, DSBs have been shown to arrest growth at DNA damage-responsive cell-cycle checkpoints and induce transformation of human and animal cells (Weinert and Hartwell 1988; Obe *et al.* 1992; Nelson and Kastan 1994; Siede 1995).

The repair of DSBs is primarily a function of genes associated with two discrete pathways that are conserved in all eukaryotic organisms from yeast to humans. One pathway, involving repair by homologous recombination, was first described for the repair of radiation-induced DSBs over 20 years ago (Resnick 1976; Resnick and Martin 1976). This model for recombination in

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mitotic cells remains largely as originally presented (Szostak *et al.* 1983; Thaler and Stahl 1988; Petes *et al.* 1991), although additional classes of DSB repair events have been identified (Klein 1995; Malkova *et al.* 1996). The second major pathway of DSB repair, nonhomologous end-joining (NHEJ), provides for the direct rejoining of broken molecules and may be either precise or error prone (Jeggo *et al.* 1995; Boulton and Jackson 1996; Milne *et al.* 1996; Chu 1997; Hendrickson 1997).

Genes specifically associated with recombinational repair of DSBs in mitotic yeast cells include *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, and the *RFA* (single-stranded DNA binding protein) complex genes (Haynes and Kunz 1981; Game 1993; Firmenich *et al.* 1995; Hays *et al.* 1995; Bai and Symington 1996). *rad52* mutants are profoundly deficient in recombination in many assays (Resnick 1976; Rattray and Symington 1995; Sugawara *et al.* 1995; Zou and Rothstein 1997), while mutations in *RAD51* (a structural and functional homologue of the *recA* gene of *Escherichia coli*), *RAD54*, *RAD55*, *RAD57*, or *RAD59* often produce less severe deficiencies. In addition, Rad51, Rad52, Rad54, Rad55, and Rad57 (involved in strand annealing and exchange) appear to form distinct protein:protein associations *in vivo* (Bai and Symington 1996; Clever *et al.* 1997; Golub *et al.* 1997; Sung 1997a,b; Benson *et al.* 1998; New *et al.* 1998; Shinohara and Ogawa 1998).

The efficiency of homologous recombination is influenced by several additional genes associated with DSB repair. For example, *RAD50*, *MRE11*, and *XRS2* participate in NHEJ (described below) and are not required for mating type switching or interchromosomal recombination; however, each of these genes is required for resistance to ionizing radiation (Saeki *et al.* 1980; Haynes and Kunz 1981; Game 1993; Hendrickson 1997) and a functional *RAD50* gene is necessary for efficient homologous intrachromosomal recombination in some assays (Rattray and Symington 1995; Tran *et al.* 1995; Elias-Arnanz *et al.* 1996). In addition, mutations within the genes encoding DNA helicases Hpr5(Srs2), Rad5, and Rdh54 (Lawrence 1994; Milne *et al.* 1995; Schild 1995; Chanet *et al.* 1996; Ahne *et al.* 1997; Klein 1997; Shinohara *et al.* 1997), the exonuclease Exo1 (Fiorentini *et al.* 1997), excision repair endonuclease Rad1/Rad10 (Habraken *et al.* 1994; Rodriguez *et al.* 1996; Paques and Haber 1997), mismatch repair proteins Msh2 and Msh3 (Paques and Haber 1997), or Cdc1 (Halbrook and Hoekstra 1994) can exert strong effects on spontaneous and/or damage-induced recombination.

NHEJ repair has been detected in yeast by assessing the efficiency of recircularization of linear plasmid DNA after cellular transformation and through study of infrequent, homology-independent DSB repair events (Schiestl *et al.* 1994; Boulton and Jackson 1996; Milne *et al.* 1996; Moore and Haber 1996; Tsukamoto *et al.* 1997b). Genes involved in recombination-independent NHEJ repair include *HDF1*(*YKU70*), *YKU80*(*HDF2*), *RAD50*, *MRE11*, *XRS2*, *DNL4* (the yeast homologue of human DNA ligase IV), *SIR2*, *SIR3*, and *SIR4* (Boulton and Jackson 1996; Milne *et al.* 1996; Hendrickson 1997; Schar *et al.* 1997; Teo and Jackson 1997; Tsukamoto *et al.* 1997a; Wilson *et al.* 1997). The proteins encoded by most genes involved in NHEJ bind to the ends of linear double-stranded DNA. Past studies (including two-hybrid analyses and immunoprecipitation experiments) have identified Ku70:Ku80, Rad50:Mre11:Xrs2, and Sir2:Sir3:Sir4 complexes within cells (Johzuka and Ogawa 1995; Dolganov *et al.* 1996; Chu 1997; Hendrickson 1997; Sherman and Pillus 1997; Tsukamoto *et al.* 1997a). More recent reports have suggested that these complexes have different functions in NHEJ; *e.g.*, Ku heterodimers are associated with bridging of the ends of broken DNA molecules while Rad50 and Mre11 appear to be involved in nucleolytic processing of DNA ends (Sharples and Leach 1995; Moore and Haber 1996; Cary *et al.* 1997; Hendrickson 1997; Pang *et al.* 1997).

Most yeast genes primarily involved in recombination (*RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*) or NHEJ (*HDF1*, *YKU80*, *RAD50*, *MRE11*, *SIR2*, *DNL4*) have structural homologues in human cells. Protein:protein interactions have also been conserved, *e.g.*, Rad51:Rad52, Rad51:Rad54, Ku70:Ku80, and Rad50:Mre11 associations have been identified in both yeast and human cells (Johzuka and Ogawa 1995; Shen *et al.* 1996a; Clever *et al.* 1997; Golub *et al.* 1997; Hendrickson 1997). Conservation of the function(s) of members of each pathway is suggested by biochemical and genetic studies and by reports that expression of human Ku70 and Rad54 can complement repair defects in yeast *hdf1*(*yku70*) and *rad54* mutants, respectively (*e.g.*, Kanaar *et al.* 1996; Barnes and Rio 1997; Bezzubova *et al.* 1997). In addition to their roles in DSB repair, genes involved in recombination and NHEJ have also been implicated in normal cell development and in processes leading to human pathology. For example, human Ku70 and Ku80, in conjunction with an associated DNA-dependent protein kinase (DNA-PK), are required for V(D)J recombination events that occur in immunoglobulin and T-cell receptor genes during development of immunity (Jeggo *et al.* 1995; Chu 1997). In addition, deletion of the mouse RAD51 recombinase gene produces an embryonic lethal phenotype (Lim and Hasty 1996; Tsuzuki *et al.* 1996). Furthermore, recent experiments have established that human Rad51 interacts with the tumor suppressor proteins encoded by p53, *BRCA1*, and *BRCA2 in vivo* (Shen *et al.* 1996a,b; Sturzbecher *et al.* 1996; Buchhop *et al.* 1997; Mizuta *et al.* 1997) and that sequence polymorphisms in human KU80 (XRCC5) and XRCC3 (a *RAD51* homologue) are associated with tumorigenesis (Price *et al.* 1997).

Because traditionally employed clastogens (X rays,

MMS, bleomycin, etc.) produce a variety of chromosomal DNA lesions in addition to DSBs, systems for studying the genetic and cytological consequences of expression of site-specific endonucleases (which produce complementary or blunt-ended DSBs only) have been developed in yeast and mammalian cells (*e.g.*, Obe *et al.* 1992; Bryant and Johnston 1993; Godwin *et al.* 1994; Bennett *et al.* 1996; Sargent *et al.* 1997; Liang *et al.* 1998). Studies in yeast have analyzed the repair of DSBs induced by HO, *Eco*RI, and I-*Sce*I (Barnes and Rine 1985; Haber 1992; Plessis *et al.* 1992; Barnes and Rio 1997; and see references in Lewis *et al.* 1998).

We have recently employed yeast strains that permit modulation of *GAL* promoter induction kinetics to show that *Eco*RI-induced DSBs arrest cell growth at the G2/M transition and stimulate interchromosomal recombination, but do not cause more cell killing in *rad52* mutants than in wild-type cells (Lewis *et al.* 1998). However, expression of *Eco*RI was lethal in *hdf1* and *rad9* mutants, suggesting separable roles for end-joining, *RAD52*-mediated recombination, and DNA damage-responsive checkpoint pathways in the repair of endonuclease-induced DSBs.

In this study we demonstrate that many genes associated with NHEJ are essential for repair of chromosomal DSBs *in vivo.* Although several recombinational repair genes were required for progression of cells past the G2 checkpoint, only NHEJ genes were required for survival after *Eco*RI-induced cleavage of chromosomal DNA. In addition, analysis of cellular responses suggested that the DSB repair functions of several end-joining pathway genes, *e.g.*, *HDF1*/*YKU80 vs. RAD50*/*MRE11*/*XRS2* or *SIR2*/*SIR3*/*SIR4*, are genetically separable. The complementary-ended DSBs produced by the endonuclease are structurally similar to DSBs generated during normal cell development (*i.e.*, during meiosis, mating type switching, and site-specific homing of intron DNA), which suggests that such DSBs might also be repairable by both recombination and NHEJ pathways.

TABLE 1

Description of yeast strains

Strain	Genotype	Source
T334	*MATα ura3-52 leu2-3,112 Δtrp1::hisG reg1-501 gal1 pep4-3 prb1-1122*	Hovland *et al.* (1989)
YLKL313	T334, *MAT***a** *lys2::insE*	D. Gordenin
YLKL350	T334, Δ*his3::[GAL1::EcoRI, TRP1]*	Lewis *et al.* (1998)
YLKL351	YLKL350, Δ*rad52::LEU2*	Lewis *et al.* (1998)
YLKL371	YLKL350, Δ*rad1::hisG-URA3-hisG*	This study
YLKL372	YLKL350, Δ*rad50::hisG-URA3-hisG*	This study
YLKL374	YLKL350, Δ*rad54::hisG-URA3-hisG*	This study
YLKL381	YLKL350, Δ*rad51::LEU2*	This study
YLKL382	YLKL350, Δ*rad6::URA3*	This study
YLKL386	YLKL371, Δ*rad52::LEU2*	This study
YLKL387	YLKL350, Δ*rad5::URA3*	This study
YLKL389	YLKL350, Δ*hdf1::HIS3*	Lewis *et al.* (1998)
YLKL393	YLKL389, Δ*rad50::hisG-URA3-hisG*	This study
YLKL397	YLKL313, Δ*rad50::G418*	This study
YLKL399	YLKL350, Δ*rad2::G418*	This study
YLKL400	YLKL351, Δ*rad50::G418*	This study
YLKL401	YLKL351, Δ*xrs2::G418*	This study
YLKL402	YLKL350, Δ*rad55::LEU2*	This study
YLKL403	YLKL350, Δ*rad57::LEU2*	This study
YLKL407	YLKL350, Δ*mre11:G418*	This study
YLKL423	YLKL313, Δ*rad22::LEU2*	This study
YLKL425	YLKL350, Δ*sir4::LEU2*	This study
YLKL436	YLKL350, Δ*exo1::G418*	This study
YLKL437	YLKL350, Δ*rdh54::G418*	This study
YLKL438	YLKL350, Δ*hpr5::G418*	This study
YLKL447	YLKL372, Δ*rad2::G418*	This study
YLKL448	YLKL351, Δ*rad2::G418*	This study
YLKL449	YLKL372, Δ*exo1::G418*	This study
YLKL450	YLKL371, Δ*rad50::G418*	This study

MATERIALS AND METHODS

Genetic methods and media: Yeast growth media, including YPD, YPG, synthetic dropout, and sporulation plates were prepared as described (Sherman 1991). Transformation of yeast cells was accomplished using a modification of the lithium acetate procedure (Gietz *et al.* 1995). For selection for G418 resistance, YPD plates containing 0.2 mg/ml G418 (Life Technologies) were prepared as described (Wach *et al.* 1994). 5-fluoroorotic acid (5-FOA)-resistant colonies were isolated using synthetic plate media containing 0.1% 5-FOA (American Biorganics, Niagara Falls, NY). All strains were propagated and assayed at 30° except Δ*hdf1* mutants, which were propagated at 25° and assayed for DNA repair at 30°. A ^{137}cesium source was employed at a dose rate of 2.75 krads/min for analyses of cellular growth responses to ionizing radiation.

Strain construction: Yeast strains constructed for this study are listed in Table 1. All strains are derivatives of the A364-based strain 334 (Hovland *et al.* 1989). This strain contains the mutant allele *reg1-501*. *GAL1* and *GAL10* promoter activity in *reg1-501* cells is low in glucose media, but addition of galactose induces the *GAL1/10* promoters while cells continue using glucose as carbon source. We note that although *GAL* transcription is apparently quite low in *reg1-501* strains in glucose media (Hovland *et al.* 1989; Niederacher and Entian 1991; Lewis *et al.* 1998), basal levels of expression in Δ*reg1* host strains remain to be established. Strain 334 (and T334, the Δ*trp1* derivative used here) also contains a mutation in the *GAL1* gene that blocks metabolism of exogenous galactose. Plasmids used for gene disruption included p52LEU (Δ*rad52::LEU2*) and pΔ*rad1*Blast (Δ*rad1::hisG-URA3-hisG*) obtained from E. Perkins, pNKY83 (Δ*rad50::hisG-URA3-hisG*) obtained from N. Kleckner, pSL101 (Δ*rad54::hisG-URA3-hisG*) and pSTL11 (Δ*rad55::LEU2*) from L. Symington, pAM50 (Δ*rad51::LEU2*) and pSM51 (Δ*rad57::LEU2*) from D. Schild, and pΔ21 (Δ*rad6::hisG-URA3-hisG*) and pDM610.23 (Δ*sir4::LEU2*) obtained from C. Bennett.

Deletions of *rad2*, *rad5*, *rad27*, *rdh54*, *exo1*, *hpr5(srs2)*, *xrs2*, and *mre11* were accomplished using PCR-mediated disruption as described (Baudin *et al.* 1993; Wach *et al.* 1994; Lewis *et al.*

1998). *rad50 rad52* and *xrs2 rad52* double mutants were created by deleting *RAD50* or *XRS2* in YLKL351 (Δ*rad52::LEU2*, Δ*his3::GAL1::EcoRI*) cells containing the plasmid YcpRAD52 (*RAD52*, *URA3*). Gene disruption/deletions were confirmed by PCR analysis of genomic DNA and/or by genetic crosses except for *rad1* and *rad2* strains, which were identified by their sensitivity to ultraviolet light. Two or three independent isolates of each gene disruption were analyzed in the experiments described below. Nucleotide sequences of primers used for deletion and PCR confirmation are available upon request.

Endonuclease-induced changes in cell growth and viability: Growth of cell cultures was monitored by counting cells using a hemacytometer. Cell survival after induction of endonuclease expression in galactose was calculated as the number of viable cells per milliliter observed on YPD plates divided by the number of cells per milliliter in the culture determined by hemacytometer. YPD broth containing 3% glucose was used for overnight cultures and for control time-course experiments. Earlier studies using the *reg1-501* strain employed YPD containing 2% glucose (Hovland *et al.* 1989; Lewis *et al.* 1998), but this was modified after determination that prolonged growth of *rad9* strains containing integrated *GAL1::EcoRI* in standard YPD media (for 12–24 hr) led to glucose depletion and apparent modest induction of the *GAL1* promoter (Lewis *et al.* 1998). Inducing media consisted of YPD broth with 3% glucose and 2% galactose (YPD + Gal). Haploid cell assays were performed by shifting logarithmically growing cell cultures from YPD to YPD + Gal media at a density of 2–4 × 10^5 cells per milliliter. For the analysis of wild-type and mutant diploid cells depicted in Table 2, eight independent cultures were used to calculate cell viabilities and standard deviations. Standard deviations were ≤15% of the mean for all other cell survival assays. Diploid strains used for the assays in Table 2 included YLKL350 × YLKL313 (Rad^+), YLKL372 × YLKL397 (*rad50*), YLKL381 × YLKL439 (*rad51*), YLKL351 × YLKL423 (*rad52*), and YLKL407 × YLKL433 (*mre11*). Each of the diploid strains contains a single integrated Δ*his3::GAL1::EcoRI* fusion.

Distribution of cells in G1, S, and G2/M: Cell-cycle progression was monitored as described (Lewis *et al.* 1998). Large-budded cells were defined as cells in which the bud was >50% of the size of the mother cell. For analysis of unbudded, small-budded, and large-budded cells after 12 hr of induction in galactose media, 300–400 cells in each culture were counted.

RESULTS

Survival of cells expressing *Eco*RI requires genes associated with NHEJ, but not recombinational repair: We previously demonstrated that expression of *Eco*RI endonuclease inhibits growth and induces G2 arrest in *rad52* strains, but does not produce significantly more killing than in wild-type cells (Lewis *et al.* 1998). This work, like the previous study, has employed the *reg1-501* strain T334 (Hovland *et al.* 1989; Niederacher and Entian 1991). *GAL1-GAL10* promoter activity is repressed in glucose media in this strain, but is induced in media containing glucose plus galactose. The ability to induce *GAL* promoter activity without changing carbon source is advantageous because cell growth rates and the relative lengths of each cell-cycle phase are different in cells metabolizing glucose *vs.* galactose (Barford and Hall 1976).

The observation that *RAD52* was required for progression of cells past the checkpoint at G2, but not for survival, indicated that repair of *Eco*RI-induced DSBs involves additional genes. The effects of *Eco*RI expression on cell viability in strains containing deletions in several additional RAD52 group genes are described in Figure 1, A–C. All cells used in the experiments contain a chromosomal Δ*his3::GAL1::EcoRI* fusion. Assays were performed using logarithmically growing cells as previously described (Lewis *et al.* 1998) with slight modification (see materials and methods). The *rad52* strain YLKL351 (Lewis *et al.* 1998) is included in Figure 1 for comparison. Strains containing deletions in genes specifically associated with strand annealing and exchange in mitotic cells (*RAD51*, *RAD52*, *RAD54*, *RAD55*, and *RAD57*) displayed high survival throughout the time course (Figure 1A).

rad50, *mre11*, and *xrs2* mutants previously have been shown to be deficient in NHEJ repair using plasmid recircularization assays, but are not defective in homologous interchromosomal recombination or mating type switching (gene conversion) in mitotic cells (Boulton and Jackson 1996; Milne *et al.* 1996; Moore and Haber 1996; Hendrickson 1997). As shown in Figure 1B, continuous *Eco*RI-induced scission of DNA in these mutants caused extensive cell killing. Plating efficiency was reduced to 2–3% after 12 hr of endonuclease expression.

The *SIR2*, *SIR3*, and *SIR4* genes, which are required for transcriptional silencing at *MAT* and in the telomeric regions of yeast chromosomes, are also involved in plasmid DSB repair (Tsukamoto *et al.* 1997a). The ability of cells containing a deletion of *SIR4* to repair chromosomal DSBs induced by *Eco*RI is depicted in Figure 1C. The mean surviving fraction was reduced to 8% in *sir4* mutants, indicating that repair of the cohesive-ended DSBs is impaired in these strains. Also presented in the figure is a comparative analysis of cell survival and cell cycling in the Δ*hdf1* strain YLKL389. As noted earlier, we previously observed (using slightly different media; see materials and methods) that expression of *Eco*RI is lethal in *hdf1* cells in the T334 strain background. The kinetics of cell killing in Ku-deficient cells was similar to that of *rad50*, *mre11*, and *xrs2* mutants and plating efficiency was reduced 30-fold after 12 hr (Figure 1C).

The effects of *Eco*RI-induced breakage of chromosomal DNA on cell-cycle progression are shown in Figure 2, A–C. Recombination-defective mutants arrested growth as large-budded cells (Figure 2A), indicating that they were unable to progress past the G2/M boundary when *Eco*RI was continuously expressed. Most arrested cells were much larger in size than normal G2 cells. We previously demonstrated that this DSB-induced arrest corresponds to a *RAD9*-dependent, DNA damage-responsive checkpoint that is phenotypically similar to the damage-induced arrest described by Weinert and Hartwell (1988). Wild-type cultures displayed a modest increase in large-budded cells during the time course. This prolongation of the G2 phase is transient

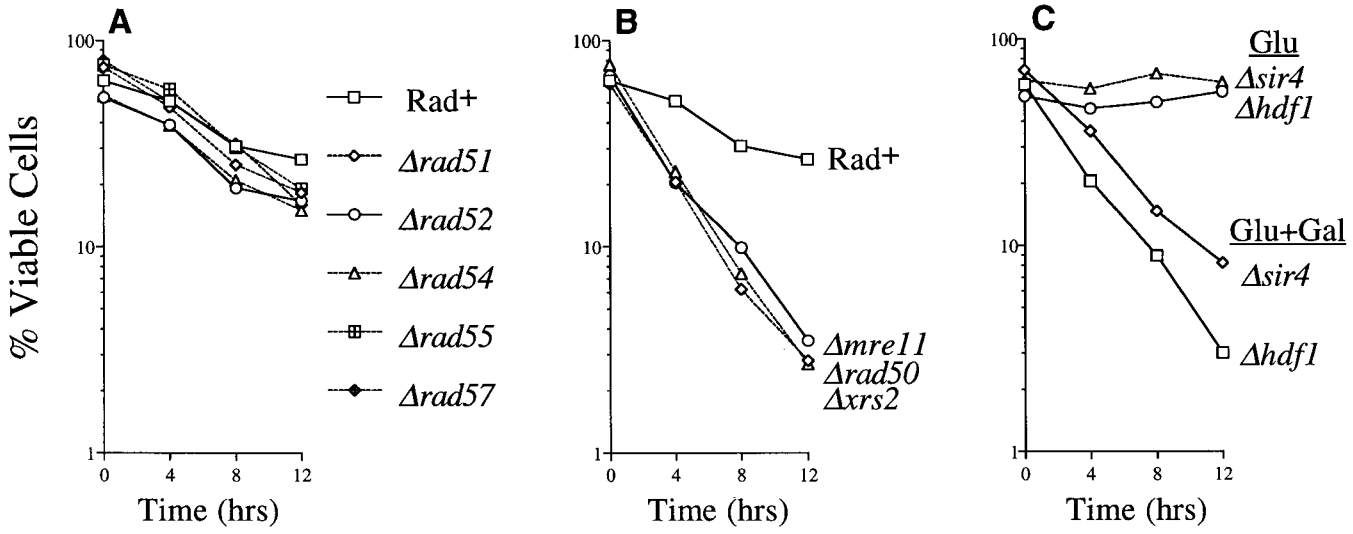

Figure 1.—Effects of endonuclease-induced DSBs on survival of wild-type cells and strains deficient in homologous recombination and/or end-joining pathways of repair. (A and B) Logarithmically growing cells were transferred to YPD + galactose media and cell viability was assessed at the indicated times after induction of *Eco*RI expression. (C) Survival of end-joining-defective *hdf1* and *sir4* strains propagated in noninducing (Glu) and galactose-containing media (Glu + Gal). Standard deviation (SD) values were ≤15% of the mean for all cell survival assays for which SD is not shown (see materials and methods).

and wild-type cells advance to early stationary phase at 24 and 48 hr (Lewis *et al.* 1998; data not shown). All of the strains studied in Figures 1 and 2 exhibited high survival and normal cell cycling when grown in YPD (noninducing) media (Figure 1C and data not shown).

Interestingly, the growth of *rad50*, *mre11*, and *xrs2* mutants was blocked, with most cells arrested at the G2 checkpoint (Figure 2B; ~70% large-budded cells after 12 hr), but this pause was not sufficient for cells to repair the induced strand breaks after synthesis of the endonuclease was repressed. Most *sir4* cells eventually arrested growth in G2 phase, but Δ*hdf1* cells did not (Figure 2C). Growth of Ku-defective cells was blocked, but these cultures consisted primarily of a mixture of enlarged unbudded and large-budded cells, suggesting that arrest occurred in both G1 and G2 (discussed below).

Requirement for RAD3 and RAD6 group genes after *Eco*RI-induced scission of chromosomal DNA: The potential involvement of *RAD1* and *RAD2* (nucleotide excision repair genes in the RAD3 epistasis group; Haynes and Kunz 1981; Habraken *et al.* 1994) and *RAD5* and *RAD6* (RAD6 epistasis group genes involved in postreplication repair and mutagenesis; Haynes and Kunz 1981; Lawrence 1994) in the repair of *Eco*RI-induced DSBs has also been examined (Figure 3, A–D). *rad5* and *rad6* mutants are moderately sensitive to ionizing radiation and ultraviolet light, but their functions in the repair of single- or double-strand breaks remains obscure. Survival curves for Δ*rad5* and Δ*rad6* strains were comparable to those of wild-type cells (Figure 3A). However, growth of *rad5* cells was strongly inhibited and these mutants displayed a strong cell-cycle arrest response that was similar to that of recombination-deficient RAD52 group mutants (Figure 3B). This result suggests a role for *RAD5* in the recombinational repair pathway and is consistent with a recent report indicating that recombinational repair of gapped plasmid DNA is greatly reduced in cells lacking the Rad5 DNA helicase (Ahne *et al.* 1997). The response of *rad6* cells was complex. These mutants grew slowly when *Eco*RI was expressed and accumulated enlarged G2 phase cells only at late time points (12 and 24 hr; Figure 3B and data not shown). These results suggest that repair of the induced DSBs was relatively efficient at earlier time points (4 and 8 hr) when fewer breaks are detectable by gel analysis (Lewis *et al.* 1998 and data not shown), but that a subset of DSBs required processing by Rad6 at later time points.

The excision repair endonuclease encoded by *RAD1*/*RAD10* plays a role in cleavage of recombination intermediates and in the processing of nonhomologous DSB ends (Habraken *et al.* 1994; Rodriguez *et al.* 1996; Paques and Haber 1997). Furthermore, 5′ single-stranded overhangs similar to those produced by *Eco*RI have previously been shown to be substrates for cleavage by the Rad2 endonuclease *in vitro* (Habraken *et al.* 1995). The possibility that Rad1/Rad10 or Rad2 might be involved in processing of the ends of *Eco*RI-induced DSBs has been assessed in Figure 3, C and D. Survival of Δ*rad2* mutants was not affected by *Eco*RI, but Δ*rad1* strains displayed a modest, linear decrease in viability during the time course (Figure 3C). Cell-cycle progression in *rad2* mutants was essentially identical to that of wild-type cells. In contrast, *rad1* mutants were moderately growth-inhibited and slowly accumulated a high proportion of G2 cells (≥70%) at 12 and 24 hr after induction (Figure 3D and data not shown). These re-

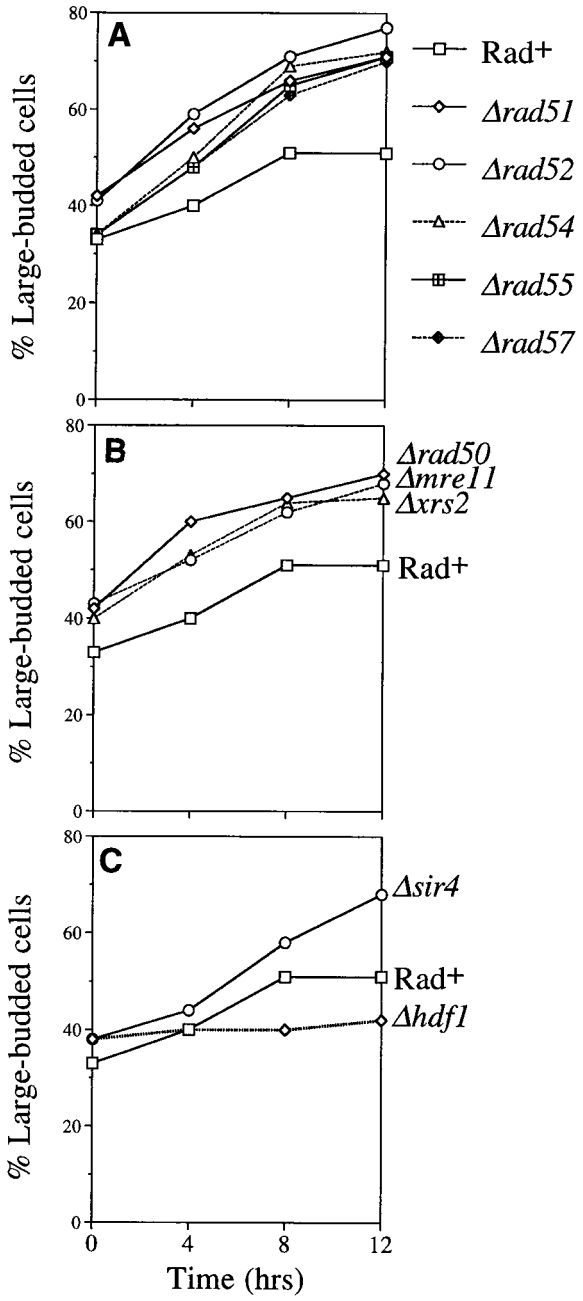

Figure 2.—Analysis of cell-cycle progression after induction of *Eco*RI expression in wild-type cells and in mutants deficient in recombination and/or NHEJ. Cells were transferred to galactose-containing media and analyzed microscopically at the indicated times after induction. Most large-budded cells observed in endonuclease-producing strains corresponded to enlarged G2 phase cells (see text).

sults suggest that a subset of DSBs produced by *Eco*RI are processed by the Rad1/Rad10 endonuclease, but not by Rad2.

To test the possibility that *RAD1/RAD10* or *RAD2* nuclease processing of cohesive-ended DSBs becomes more significant in the absence of other repair pathways, *i.e.*, recombination or end-joining, cell survival was also monitored in double mutants. The viability of Δ*rad1* Δ*rad52* and Δ*rad2* Δ*rad52* cells was reduced ~7-fold during the time course (Figure 3C). This effect was slightly greater than that seen in Δ*rad1* and Δ*rad2* single mutants, but cell killing did not approach levels observed in *hdf1*, *rad50*, *mre11*, or *xrs2* mutants (~20–40-fold killing after 12 hr). The kinetics and magnitude of growth arrest as large-budded cells in each double mutant cell culture appeared similar to that of *rad52* cells (Figure 3D).

The data presented in Figures 1–3 and in past studies (see Lewis *et al.* 1998 and references within) support the hypothesis that DSB termini containing single-stranded complementary overhangs can be repaired by multiple pathways *in vivo*. In contrast, repair of damaged ends produced by ionizing radiation is largely dependent on recombinational repair. The negative effects of *Eco*RI- and radiation-induced DSBs in wild-type and repair-deficient cells have been compared directly in Figure 4. Growth of cells containing a deletion of *RAD52* (recombination deficient), *RAD50* or *MRE11* (partially defective in recombination and NHEJ), and *SIR4* (NHEJ deficient) is inhibited by *Eco*RI. However, gamma-irradiation (30 krads) blocked growth of the Rec$^-$ strains (*rad52*, *rad50*, *mre11*), but not Δ*sir4* cells (Figure 4). Growth of excision-repair-defective *rad1* and *rad2* strains on plates was largely unaffected by either clastogen.

Viability and checkpoint responses in end-joining and recombination-defective double mutant strains: The experiments described above indicated that cells experiencing *Eco*RI-induced DSBs require several recombinational repair genes for progression past the G2 checkpoint, but not for survival. In contrast, Ku-deficient strains and RAD52 group mutants involved in end-joining repair (*rad50*, *xrs2*, and *mre11*) displayed extensive cell killing, but had dissimilar arrest phenotypes (compare Figure 2B *vs.* Figure 2C). The effects of *Eco*RI-induced cleavage of DNA in strains deficient in both end-joining and recombinational repair are presented in Figure 5. Surprisingly, growth arrest and cell killing kinetics in *rad50 rad52* and *xrs2 rad52* double mutants were similar to those of *rad50* and *xrs2* single mutants, respectively (~2–3% viable cells after 12 hr; Figure 5A and Figures 1B and 2B). Thus, combination of a deficiency in NHEJ with elimination of almost all homologous recombination did not produce additional killing in this assay.

Interestingly, *rad51 hdf1* and *rad52 hdf1* double mutants could be constructed in the T334-derived strains used here, but the cells grew poorly and displayed several stress phenotypes in both glucose and galactose media. Characteristics included slow growth, reduced plating efficiency, elevated levels of petite mutants, and an increased fraction of dark (presumably lysed) cells upon microscopic examination (data not shown). These effects were not observed in any other single or double mutant created for these studies and their source remains unclear. We note, however, that expression of

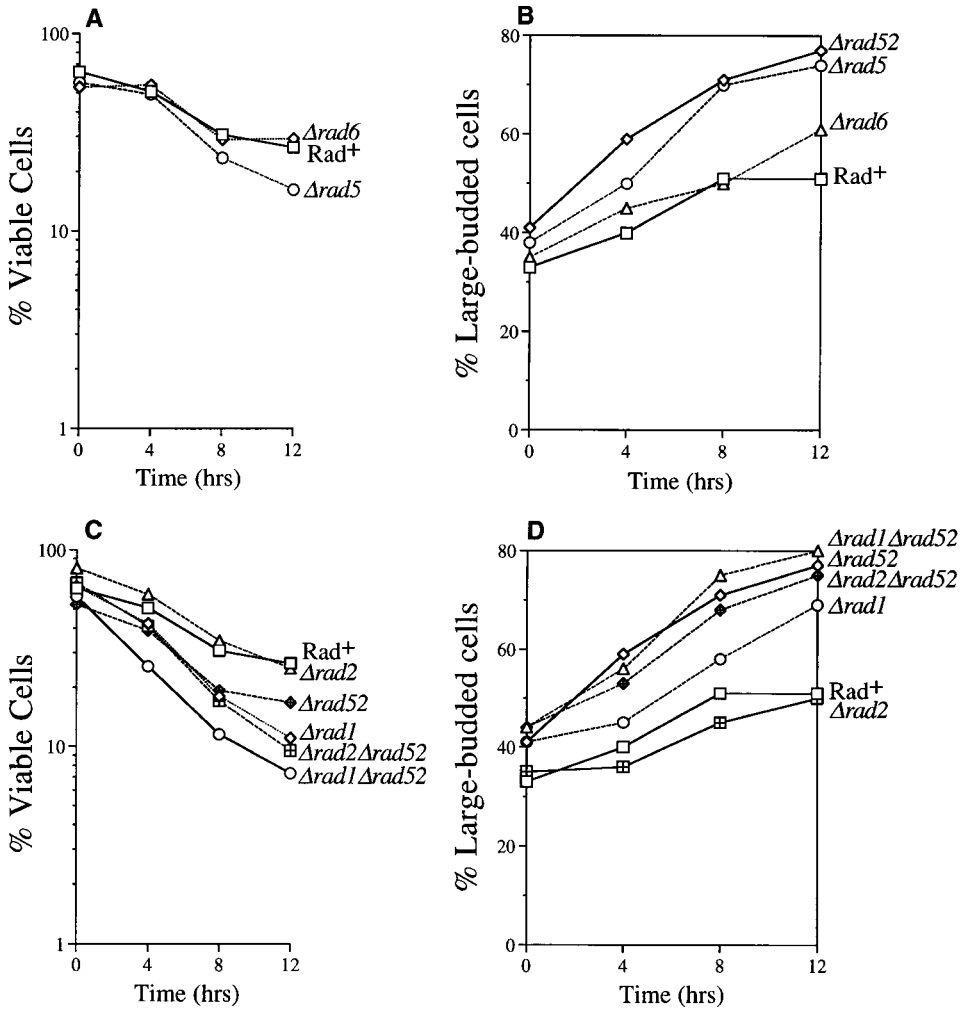

Figure 3.—Effects of endonuclease-induced DSBs on survival and cell cycling in mutants deficient in nucleotide excision repair (*RAD3* group) or mutagenesis and postreplication repair (*RAD6* group). Quantitation of cell viability and formation of large-budded cells (mostly G2 cells) was performed as for Figures 1 and 2.

*Eco*RI in wild-type, *rad52*, or *hdf1* strains does not produce elevated levels of petite mutants (Barnes and Rine 1985 and data not shown), suggesting that the phenomena are not due to altered *GAL1::EcoRI* expression. Furthermore, *rad52 hdf1* double mutants have previously been reported to have significantly lower plating efficiency than either single mutant (Siede *et al.* 1996). Finally, past reports have demonstrated that DNA damage-inducible genes are constitutively activated in *rad52* and *hdf1* null mutants, presumably because of elevated levels of unrepaired DNA damage (Maga *et al.* 1986; Barnes and Rio 1997). This effect may be exacerbated in *hdf1 rad52* double mutants, which are defective in both major pathways of DSB repair. The results presented in Figure 5A also demonstrated extensive *Eco*RI-induced cell killing in *rad50 hdf1* mutants that was similar in extent to that observed in *rad50* and *hdf1* single mutants (~30-fold killing). *rad50 hdf1* cell cultures consisted primarily of unbudded and large-budded cells, suggesting that checkpoint activation occurred in both G1 and G2, as observed earlier in *hdf1* cultures (Figure 1C and see below).

Past studies have suggested that some or all DNA end-binding proteins that function in NHEJ repair might affect degradation of DSB termini (*e.g.*, Getts and Stamato 1994; Milne *et al.* 1996; Moore and Haber 1996). The absence of one or more such proteins might lead to cell killing because the DNA ends become susceptible to cleavage by specific cellular endo- or exonucleases. As an initial test of this hypothesis we constructed double mutant strains that are deficient in Rad50-mediated end-joining and that contain a deletion of an endonuclease or exonuclease gene previously implicated in DSB repair. Changes in cell viability and cell cycling in *rad1 rad50*, *rad2 rad50*, and *exo1 rad50* mutants upon induction of *Eco*RI are presented in Figure 5, C and D. *EXO1* was included because this exonuclease has been shown to be involved in the processing of DNA ends and in intrachromosomal recombination (Fiorentini *et al.* 1997). Cell survival was reduced 20- to 40-fold in each double mutant, which is comparable to that seen in *rad50* strains (Figure 5C). All double mutants displayed a strong growth arrest response that was analogous to that observed previously in *rad50* single mutants. These data suggest that aberrant processing of the ends of DSBs by Rad1/Rad10, Rad2, or Exo1 is not responsible

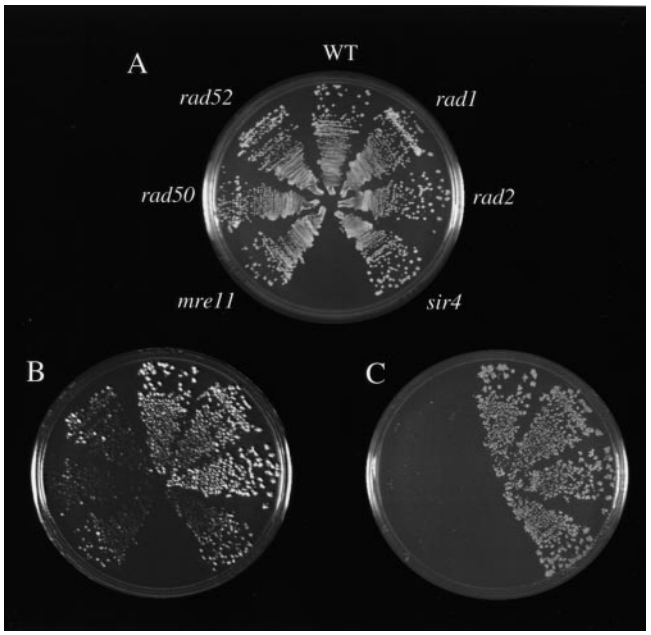

Figure 4.—Growth-inhibitory effects of DNA damage produced by *Eco*RI *vs.* ionizing radiation in wild-type, Rec$^-$ (*rad52*), NHEJ$^-$/partial Rec$^-$ (*rad50*, *mre11*), NHEJ$^-$ (*sir4*), and excision repair-deficient (*rad1*, *rad2*) strains. T334 cells (Δ*his3::GAL1::EcoRI*) were replica plated to media containing (A) YPD (B) YPD + 2% galactose, or (C) YPD exposed to gamma irradiation (30 krads).

for the killing observed in end-joining defective *rad50* mutants.

Distinct cell cycle checkpoint responses in recombination- and NHEJ-deficient mutants: A detailed examination of the distribution of wild-type and mutant cells within the cell cycle in response to *Eco*RI-induced scission of chromosomal DNA is presented in Figure 6. The proportion of unbudded (G1 phase), small-budded (an approximation of cells undergoing S phase), and large-budded cells (G2/M phase) was quantitated after 12 hr of *Eco*RI expression. Cultures of all *RAD52* group mutants tested, including recombination-defective *rad52* strains and end-joining-deficient *rad50* cells, consisted primarily of S and G2 phase cells with most cells enlarged and arrested in G2 (Figure 6 and data not shown). *rad50 rad52* double mutants (severely deficient in both recombination and NHEJ) also arrested predominantly at G2 with almost all cells in S or G2. Cultures of *hdf1* and *hdf1 rad50* mutants consisted of approximately equal numbers of G1 and G2 cells (Figure 6). Thus, the unusual biphasic DNA damage response of Ku-defective strains is dominant to that of *rad50* mutants (and by extension to *mre11* and *xrs2* mutants).

Effects of *Eco*RI expression on cell viability and checkpoint responses in wild-type and repair-deficient diploid cells: Although both haploid and homozygous diploid RAD52 group mutants are hypersensitive to ionizing radiation-induced DSBs, differences in resistance due to ploidy have been observed. For example, diploid *rad50* strains are slightly more radio resistant than their haploid counterparts (presumably due to increased recombinational repair of some induced DSBs), but *rad51* and *rad52* diploids are not (Saeki *et al.* 1980; Game 1993). The possibility that *Eco*RI-induced growth inhibition and/or cell killing observed in repair-deficient haploid strains might be altered in diploids (which can undergo recombinational repair throughout the cell cycle) was tested in wild-type cells and in homozygous *rad50*, *rad51*, *rad52*, and *mre11* mutants. Analysis of the growth characteristics of diploid cells containing a single *GAL1::EcoRI* chromosomal fusion revealed significant differences from haploid cells (Figure 7). Wild-type diploid cells containing either Δ*his3::GAL1::EcoRI* or Δ*lys2::GAL1::EcoRI* fusions (Lewis *et al.* 1998 and data not shown) did not exhibit a transient G2 arrest response as previously observed in haploid cells. This result suggests that *Eco*RI-induced DSBs in the diploids were repaired efficiently and did not activate the checkpoint response system. All homozygous mutant diploids arrested growth in G2 phase after induction of *Eco*RI expression (70–80% of cells after 8–12 hr) with most of the G2 cells greatly enlarged.

Interestingly, uninduced, logarithmically growing *rad50*, *rad51*, *rad52*, and *mre11* diploid cells in glucose media contained ~50% large-budded cells. This is apparent at the zero time point in Figure 7 and in control time-course experiments in which cells were grown in standard glucose media (data not shown). Haploid RAD52 group mutants displayed only a slight increase in G2 cells in glucose media. A previous study has reported that logarithmically growing *rad51* diploid cell cultures, but not *rad2*, *rad6*, or *rad9* diploids, contain elevated levels (52%) of G2 phase cells (Fingerhut *et al.* 1984). The extended G2 phase suggests that spontaneously occurring DNA lesions are elevated in these cells and/or that the additional chromosomes in diploids increase the likelihood of a lesion triggering the checkpoint monitoring system.

The effects of *Eco*RI-induced DNA cleavage on cell survival in wild-type and mutant diploid strains are presented in Table 2. Survival of recombination-deficient *rad51* and *rad52* mutants was similar in both haploid and diploid cells. However, the approximately twofold decrease in viability observed in wild-type haploid strains was abolished in the diploids. The greatest change was observed in *rad50* and *mre11* cells. Although these strains displayed a strong G2 arrest response, the cell killing in haploid mutants was largely eliminated in diploids. This suggests that, in contrast to ionizing radiation-induced DSBs, the cohesive-ended DSBs could be efficiently repaired by recombination between homologous chromosomes throughout the cell cycle.

DISCUSSION

The experiments described here, in combination with our prior study (Lewis *et al.* 1998), have established that many genes previously implicated in recombina-

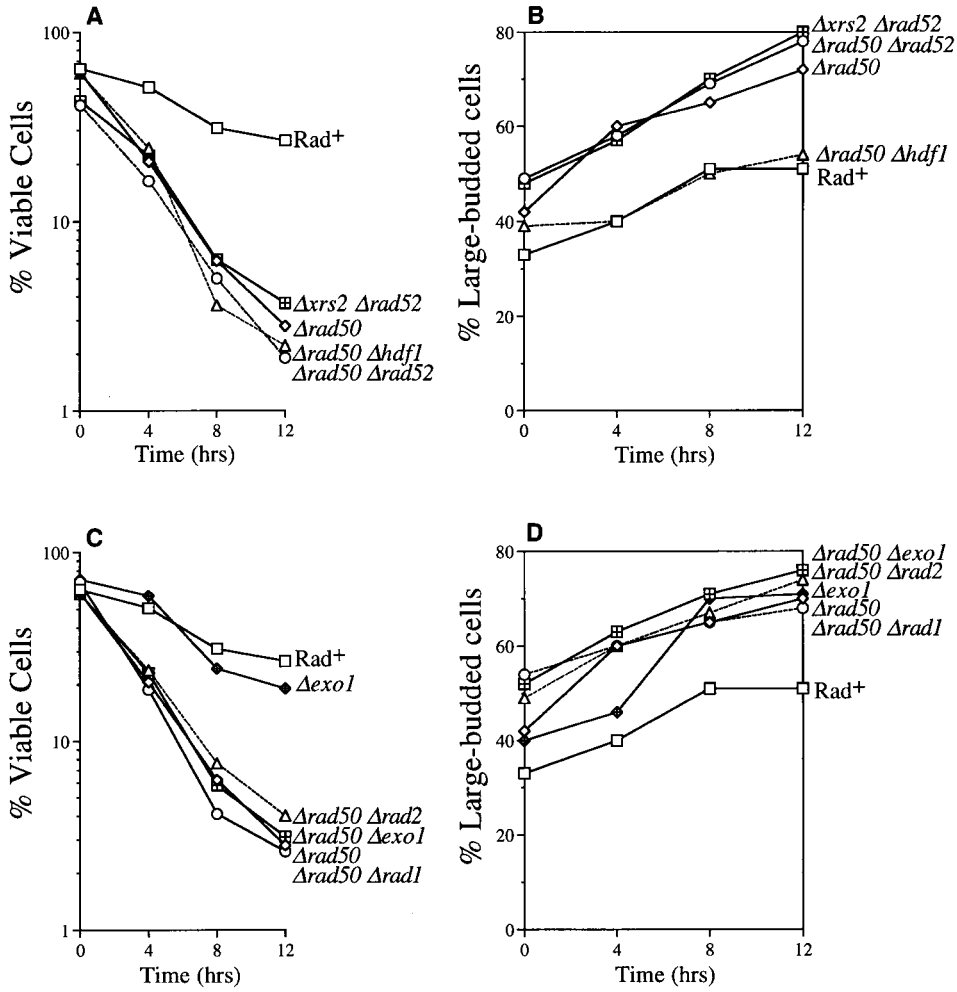

Figure 5.—Responses of single and double mutant strains deficient in homologous recombination and end-joining pathways to the induction of DSBs. (A and B) Effects of endonuclease expression on survival and cell-cycle progression in end-joining-defective double mutant cells (*rad50 hdf1*) or in strains deficient in both recombination and end-joining (*rad50 rad52*, *xrs2 rad52*). (C and D) Effects of deletion of cellular nuclease genes (*RAD1*, *RAD2*, or *EXO1*) on endonuclease-induced killing of end-joining-deficient *rad50* mutants.

tion-independent end-joining in yeast cells have an essential role in precise repair of DSBs *in vivo*. The approach presented here has permitted assessment of the repair of DSBs produced in nuclear, chromatin-associated DNA and the effects of such breaks on progression through the cell cycle. Although the NHEJ pathway was not identified in past analyses of cellular sensitivity to X rays and chemical clastogens, the current study has established that recombination and end-joining repair are both efficient processes in yeast and that this efficiency is critically dependent upon the nature of the DSB ends. Specifically, these results indicate that complementary-ended DSBs that are similar in structure to those produced by endonucleases during cellular development are rejoined by both major pathways of DSB repair. Furthermore, the combined analysis of DSB-induced effects on growth, cell cycling, and survival has led to the demonstration of separable roles for genes

TABLE 2

***Eco*RI-induced killing in diploid and haploid cells**

Haploid or homozygous diploid strain	% Viable diploid cells		Fold decrease in viable cells	
	0 hr	12 hr[a]	Diploid cells	Haploid cells[b]
Rad$^+$	76.3 ± 10.8	72.9 ± 12.9	1.0	2.4
Δ*rad52*	64.5 ± 10.9	29.3 ± 3.7	2.2	2.8
Δ*rad51*	73.0 ± 12.2	39.7 ± 4.7	1.9	3.5
Δ*rad50*	74.3 ± 12.8	20.5 ± 2.1	3.6	22.0
Δ*mre11*	66.1 ± 11.8	21.0 ± 3.4	3.1	19.3

[a] *Eco*RI expression was induced with 2% galactose and plating efficiency was assessed at $t = 0$ and $t = 12$ hr.
[b] Data for the corresponding haploid mutants are derived from experiments performed for Figure 1. The difference in mean surviving cell fraction at 0 hr and 12 hr is shown.

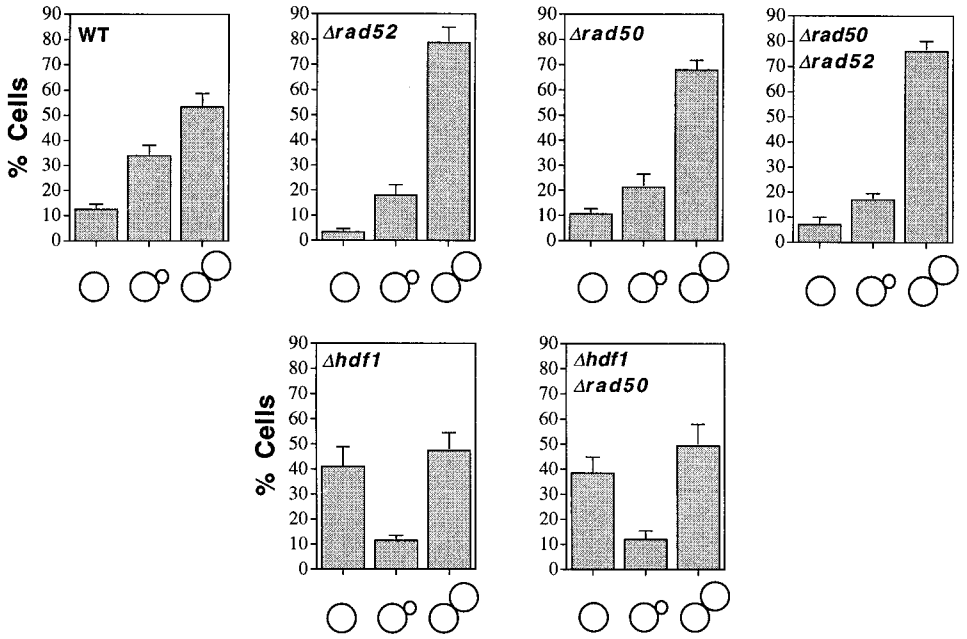

Figure 6.—Changes in the distribution of G1, S, and G2/M cells in wild-type strains and in single and double mutants after *Eco*RI-induced cleavage of chromosomal DNA. Cells were analyzed at $t = 12$ hr (see materials and methods).

involved in the NHEJ pathway of repair. A summary of the results described here and in the previous study (Lewis *et al.* 1998) is presented in Table 3.

Homologous recombination pathway genes are required for efficient progression past the G2/M checkpoint, but not for survival: We have previously demonstrated that expression of *Eco*RI in yeast cells results in extensive breakage of cellular DNA and stimulates homologous recombination (Lewis *et al.* 1998). These experiments included the surprising observation that continuous synthesis of *Eco*RI arrested the growth of *rad52* mutants, but did not produce significantly greater killing than in wild-type cells. This result is in direct contrast to previous studies demonstrating that *RAD52* is essential for repair of DSBs induced by ionizing radiation and various DNA-damaging chemicals (Resnick and Martin 1976; Saeki *et al.* 1980; Moore 1982; Game 1993). In this study we have also observed high survival in cells containing a deletion of the gene encoding the Rad51 recombinase (a structural and functional homologue of *E. coli* RecA) and in cells deficient in *RAD54*, *RAD55*, and *RAD57* (summarized in Table 3). The similar responses of these mutants are consistent with recent models suggesting that Rad54 and the Rad55/Rad57 heterodimer work in conjunction with the strand annealing and strand exchange activities of Rad51:Rad52 to effect recombination (Clever *et al.* 1997; Golub *et al.* 1997; Sung 1997a,b; Benson *et al.* 1998; New *et al.* 1998; Shinohara and Ogawa 1998). Each of these mutants exhibits deficiency in most assays of spontaneous and induced recombination (recombination between some direct repeat sequences is an exception), though *rad52* mutants are often more deficient than other single or double mutant strains (Rattray and Symington 1995; Sugawara *et al.* 1995; Zou and Rothstein 1997).

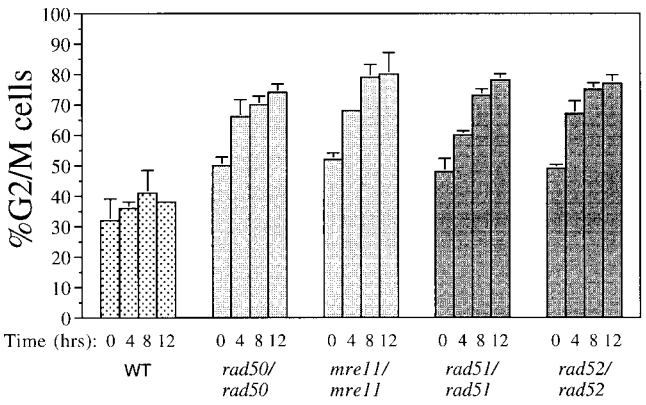

Figure 7.—Analysis of endonuclease-induced effects on cell cycling in diploid cells. Recombination-deficient (*rad51* or *rad52*) and end-joining-defective mutants (*rad50* or *mre11*) displayed a strong G2 arrest response, but the transient G2 arrest observed in Rad$^+$ haploid cells was abolished in repair-proficient diploids.

The *EXO1* gene encodes a 5′-3′ exonuclease that has been implicated in both recombination and mismatch repair. A specific role for *EXO1* in homologous recombination, but not in NHEJ repair pathways, is supported by a recent study of recombination between direct repeats (Fiorentini *et al.* 1997) and by two additional outcomes described here: the phenotypic responses of *exo1* mutants to *Eco*RI cleavage of chromosomal DNA are similar to those of *rad52* mutants and the rapid cell killing observed in *rad50* mutants is not altered in *rad50 exo1* double mutants (Figure 5). In addition to *EXO1*, we have recently observed that strains containing a deletion of the putative DNA helicases encoded by *SRS2* (*HPR5*) and *RDH54* also exhibit strong DNA-damage responses

TABLE 3

Summary of *Eco*RI-induced effects in mutants deficient in one or more DNA repair pathways

	Proposed DSB repair pathway		Growth arrest		
Gene	Rec[a]	NHEJ	G2/M	G1+ G2/M	Survival[b]
rad51	+		+		+++
rad52	+		+		+++
rad54	+		+		+++
rad55	+		+		+++
rad57	+		+		+++
hdf1		+		+[c]	—
sir4		+	+ (delayed)		–
rad50	+	+	+		—
mre11	+	+	+		—
xrs2	+	+	+		—
rad1	+[d]		+ (delayed)		–
rad2					+++
rad5	+[d]		+		+++
rad6			+ (delayed)		+++
exo1	+[d]		+		+++
rad59	+[d,e]		+		+++
rdh54	+[d]		+		+++
srs2	+[d]		+		+++

[a] Mitotic recombination.
[b] Definitions: +++, high survival; –, moderate loss of viability; —, extensive cell killing.
[c] Note: DNA damage-induced arrest in Ku mutants varies among strains and may occur in G2 only or in both G1 and G2.
[d] Genes were assigned to the recombination pathway based on phenotypic responses to *Eco*RI.
[e] L. K. Lewis and M. A. Resnick (unpublished results).

when *Eco*RI is expressed, but do not display extensive cell killing (Table 3; K. Lewis and M. Resnick, unpublished results). This suggests that *RDH54* (a homologue of *RAD54* and a suggested member of the *SWI2/SNF2* family of DNA helicases; Klein 1997; Shinohara *et al.* 1997) and *SRS2* (previously implicated in recombinational repair events mediated by *RAD51*, *RAD52*, and *RAD54* and involved in the processing of DSB termini; Milne *et al.* 1995; Schild 1995; Chanet *et al.* 1996; Paques and Haber 1997) participate in the recombinational pathway of repair of *Eco*RI-induced DSBs.

Although the two principal pathways of DSB repair, NHEJ and homologous recombination, appear to be common to all eukaryotic organisms, the relative importance of each pathway varies across phylogeny. Past studies have suggested that recombinational mechanisms are dominant in *S. cerevisiae* and that homology-independent pathways account for most DSB repair in higher eukaryotes (*e.g.*, Roth and Wilson 1986; Godwin *et al.* 1994; Chu 1997). However, a number of recent experiments have revealed that human and animal cells possess a substantial potential for recombinational repair of DSBs (*e.g.*, Carroll *et al.* 1994; Bezzubova *et al.* 1997; Sargent *et al.* 1997; Liang *et al.* 1998; Sonoda *et al.* 1998). Furthermore, recombinational repair pathways in higher eukaryotes are dependent on many of the same genes as yeast. For example, *RAD51*, *RAD52*, and *RAD54* homologues have been identified in human and animal cells and shown to affect repair of DSBs induced by DNA-damaging agents (Bezzubova *et al.* 1997; Essers *et al.* 1997; Sonoda *et al.* 1998).

Essential, but separable, roles for genes associated with NHEJ: In yeast cells the prominant role of *RAD52*-mediated recombination in repair of DSBs induced by radiation and by chemical DNA-damaging agents is well established (Haynes and Kunz 1981; Game 1993). A number of past multigenic analyses of NHEJ in yeast have analyzed predominantly nonconservative rejoining events *in vivo* (*i.e.*, restriction enzyme-mediated integration of DNA fragments, deletion formation in dicentric plasmids, and formation of insertions and deletions at MAT after HO endonuclease-induced cleavage; Schiestl *et al.* 1994; Moore and Haber 1996; Tsukamoto *et al.* 1997b). Other recent studies have suggested that conservative (precise) repair of chromosomal DSBs produced by HO and *Eco*RI is defective in cells lacking Ku70 or Ku80 (Barnes and Rio 1997; Lee *et al.* 1998; Lewis *et al.* 1998). Finally, linear, cohesive-ended plasmid DNA is accurately and efficiently recircularized after transformation into wild-type and *rad52* cells, but not in *hdf1*, *yku80*, *rad50*, *dnl4*, *sir2*, *sir3*, or *sir4* mutants (Boulton and Jackson 1996; Milne *et al.* 1996; Schar *et al.* 1997; Teo and Jackson 1997; Tsukamoto *et al.* 1997a; Wilson *et al.* 1997).

The multigenic analysis performed here revealed that all mutants previously implicated in NHEJ repair pathways (using the above assays) were hypersensitive to killing by *Eco*RI, but responses to the enzyme were not identical (see Table 3). Cell viability was reduced 20- to 40-fold in *rad50*, *mre11*, *xrs2*, and *hdf1* mutants and was reduced ~10-fold in *sir4* strains. Interestingly, growth of all end-joining mutants was blocked by *Eco*RI expression, but cell cycle arrest responses varied. *rad50*, *mre11*, and *xrs2* mutants displayed a rapid, *rad52*-like increase in enlarged G2 cells after induction of *Eco*RI synthesis. *sir4* mutants also accumulated G2 cells, but did so more slowly. In contrast, *hdf1* mutants accumulated as distended G1 and G2 phase cells. This unusual phenotype has been described recently for Ku70-deficient cells after exposure to high temperature or endonuclease-induced DSBs (Barnes and Rio 1997). Temperature-sensitive mutants deficient in *CDC28* (encoding a cyclin-dependent protein kinase), *TOP2* (topoisomerase II), and *CKA2* (coding for part of the catalytic subunit of casein kinase II) have also exhibited arrest at both G1/S and G2/M at their restrictive temperatures (Holm *et al.* 1985; Hanna *et al.* 1995; Zarsov *et al.* 1997). Potential correlations between the mechanisms responsible for biphasic arrest in Ku strains and in the other mutants

await further studies. We also note that the similar kinetics of growth and cell-cycle arrest in recombination mutants (which remain viable) and NHEJ mutants (displaying progressive loss of viability) suggests that galactose induction of the endonuclease is comparable in the different mutant strains. This conclusion is also supported by the past demonstration of similar induction kinetics for GAL::HO-induced cleavage at *MAT* in recombination and end-joining mutants (*e.g.*, see references in Haber 1992; Sugawara *et al.* 1995).

The functions of the Ku70:Ku80 heterodimer and Rad50:Mre11:Xrs2 proteins in NHEJ appear to be distinct. The Ku complex has been implicated in the joining reaction for broken DNA ends, but Rad50 and Mre11, which have significant sequence similarity to exoendonucleases encoded by the *Escherichia coli* SbcC and SbcD proteins, appear to be involved in nucleolytic processing of the ends (Sharples and Leach 1995; Moore and Haber 1996; Cary *et al.* 1997; Hendrickson 1997; Pang *et al.* 1997). Several phenotypes observed in Ku mutants are also found in *rad50*, *mre11*, and *xrs2* mutants, but differences that may reflect their disparate roles in NHEJ have also been described. For example, *hdf1* and *rad50* mutants show equivalent deficiencies in plasmid end-joining, participate in the same pathway of MMS repair based on analysis of epistatic interactions, display unusual processing of dicentric plasmids, exhibit telomere-shortening, and are hypersensitive to killing by *Eco*RI endonuclease (this work; Boulton and Jackson 1996; Milne *et al.* 1996; Porter *et al.* 1996; Siede *et al.* 1996; Kironmai and Muniyappa 1997; Tsukamoto *et al.* 1997b). In addition, Ku and Rad50 proteins have been found associated with the ends of broken DNA in yeast and in human cells (Feldmann and Winnacker 1993; Getts and Stamato 1994; Milne *et al.* 1996; Nelms *et al.* 1998). However, a number of phenotypes are unique to Ku-deficient cells. Thus, *hdf1* mutants have been reported to be temperature-sensitive for growth, have increased chromosome ploidy, exhibit constitutively elevated transcription of the damage-inducible *RNR2* and *RNR3* genes, are found associated with replication origin-binding complexes, and have distinct damage-induced growth arrest phenotypes in yeast (this work; Feldmann and Winnacker 1993; Shakibai *et al.* 1996; Barnes and Rio 1997). Furthermore, results obtained in this study have indicated that the repair deficiency of Ku mutants is dominant to that of *rad50* mutants, as *rad50 hdf1* double mutants responded similarly to *hdf1* strains (Figure 5). Finally, it is worth noting that several phenotypes of Ku mutants are remarkably strain specific. For example, temperature-sensitive growth at 37°, MMS sensitivity, damage-induced arrest in G1 and G2, and increased radiation sensitivity in *rad52 hdf1* double mutants have been described for some strains, but not for others (cited above). Although reports that Hdf1 associates with Sir4 and with replication origin complex proteins *in vivo* suggest that the phenotypic variability of *hdf1* mutants may be due to epigenetic effects, this possibility requires further study (Shakibai *et al.* 1996; Sherman and Pillus 1997; Tsukamoto *et al.* 1997a).

Involvement of RAD3 and RAD6 epistasis group genes in repair of *Eco*RI-induced DNA damage: Expression of *Eco*RI in *rad1* and *rad1 rad52* strains caused an accumulation of cells in G2 and a modest reduction in viability (Figure 3), but cell killing did not approach the levels observed in *hdf1* or *rad50* mutants. The Rad1/Rad10 excision repair endonuclease cleaves 3′ single-strand overhangs and synthetic Holliday junctions (Habraken *et al.* 1994; Rodriguez *et al.* 1996) and affects processing of nonhomologous DSB ends after cleavage of DNA by HO (Paques and Haber 1997). Unpaired 3′ extensions might be produced *in vivo* by exonuclease processing of the 5′ overhangs generated by *Eco*RI. Although 5′ single-stranded overhangs have previously been shown to be substrates for cleavage by the Rad2 endonuclease *in vitro* (Habraken *et al.* 1995), cell cycling and cell survival were not significantly affected by *Eco*RI expression in *rad2* mutants. These results suggest a role for the Rad1/Rad10 endonuclease, but not Rad2, in the processing of *Eco*RI-induced DSBs *in vivo*. We note, however, that the cell killing observed in *rad50* mutants was not influenced by subsequent deletion of *RAD1* or *RAD2*. Specifically, we found that growth arrest and lethality in *rad50 rad1*, *rad50 rad2*, and *rad50 exo1* double mutants were not different from that of *rad50* single mutants (Figure 5). This indicates that the extensive killing observed when Rad50 is absent is not due to aberrant processing of DSB ends by these three nucleases.

Interestingly, *rad5* mutants displayed a rapid, *rad52*-like G2 arrest phenotype and near-wild-type survival when *Eco*RI was expressed. A recent report has demonstrated that recombination-mediated gap repair in plasmids transformed into yeast cells is greatly reduced in *rad5* mutants (Ahne *et al.* 1997). This result and the data presented in Figure 3 support a role for the Rad5 DNA helicase in the *RAD52* pathway of DSB repair. Expression of *Eco*RI in *rad6* mutants produced a complex phenotype. These strains grew slowly with an approximately wild-type distribution of cells in the cell cycle during the first 8 hr, but with elevated levels of enlarged G2 phase cells 12 and 24 hr after induction. We previously demonstrated that DNA purified from induced Rad$^+$ and *rad52* cells contains progressively more unrepaired DSBs after 4, 8, 12, and 24 hr of expression of *Eco*RI (Lewis *et al.* 1998). The delayed cell-cycle arrest observed in *rad6* mutants suggests that at 4 and 8 hr, when low levels of breaks are present, *RAD6*-independent pathways are able to repair the damage efficiently. However, at later time points a fraction of the induced DSBs are processed by Rad6.

Endonuclease-induced DSBs produce different effects on cell cycling and viability in haploid and diploid

cells: Analysis of the effects of endonuclease expression in diploid cells revealed striking differences between wild-type, recombination-deficient, and NHEJ-defective strains (Table 2). The transient G2 arrest response and small reduction in viability observed during the time course of *Eco*RI induction in haploid cells were abolished in diploid wild-type cells, but not in *rad51* and *rad52* diploids. The fact that the loss in viability consistently observed in haploid Rad$^+$ cells was eliminated in diploids suggests that the modest killing effect is due to unrepaired DSBs in haploid G1 cells. Such cells lack sister chromatids for recombinational repair and previously have been shown to be hypersensitive to DSBs (Brunborg *et al.* 1980; Game 1993).

The rapid *Eco*RI-induced lethality observed in NHEJ-deficient *rad50* and *mre11* mutants was largely eliminated in *rad50*/*rad50* and *mre11*/*mre11* diploid cells (Table 2). Small and even substantial increases in the ionizing radiation resistance of diploid cells relative to haploids have been observed previously in *rad50* mutants, but not in *rad51* or *rad52* strains (McKee and Lawrence 1980; Saeki *et al.* 1980; Rao and Reddy 1982; Ivanov *et al.* 1992; J. L. Nitiss and M. A. Resnick, unpublished results). This increased resistance is generally interpreted to be primarily due to increased recombination capability and we suggest that this is true for repair of *Eco*RI-induced DSBs in diploids *vs.* haploids. However, the presence of mating type heterozygosity in the diploids may also contribute to the increased radioresistance of the mutants (Heude and Fabre 1993).

A recent study of plasmid recircularization by Astrom *et al.* (1999) has suggested that end-joining repair is reduced in **a**/α diploids relative to ***a***(Δ**p**)/α diploids (containing a deletion of the *MAT***a** promoter). It was also demonstrated that the plasmid end-joining deficiency of *sir2*, *sir3*, and *sir4* haploids could be rescued by blocking expression of the **a** and α genes from *HML*, *MAT*, and *HMR*. This result implies that *SIR* genes function indirectly in NHEJ by derepressing expression of the **a1**/α2 repressor complex. In this study *Eco*RI-induced DSBs were repaired more efficiently in wild-type **a**/α diploids than in haploid cells, suggesting that potential defects in NHEJ due to mating type heterozygosity were compensated by the increased capacity for recombinational repair. In addition, cell survival was similar in *rad51* and *rad52* haploids and diploids, though the latter strains would be predicted to be deficient in both recombination and NHEJ if mating type heterozygosity inhibits end-joining. The simplest interpretation of these data is that the **a1**/α2 repressor regulates one or more genes involved in rejoining of plasmid DNA ends, but does not play a critical role in end-joining of DSBs in chromatin-associated chromosomal DNA. We are currently investigating this idea.

A schematic representation of the results obtained in this study and in our earlier report (Lewis *et al.* 1998), based on previously identified DNA repair pathways and protein:protein interactions, is presented in Figure 8. Cell survival after induction of DSBs by *Eco*RI required gene products implicated in end-joining (Ku70 and Sir4), both end-joining and recombination (Rad50, Mre11, and Xrs2), and DNA damage-responsive checkpoints (Rad9 and Rad17). Our data are consistent with a role for several additional genes (*EXO1*, *HPR5*/*SRS2*, *RDH54*, and *RAD5*) in the recombinational pathway of DSB repair. Also shown is *DNL4*, the yeast homologue

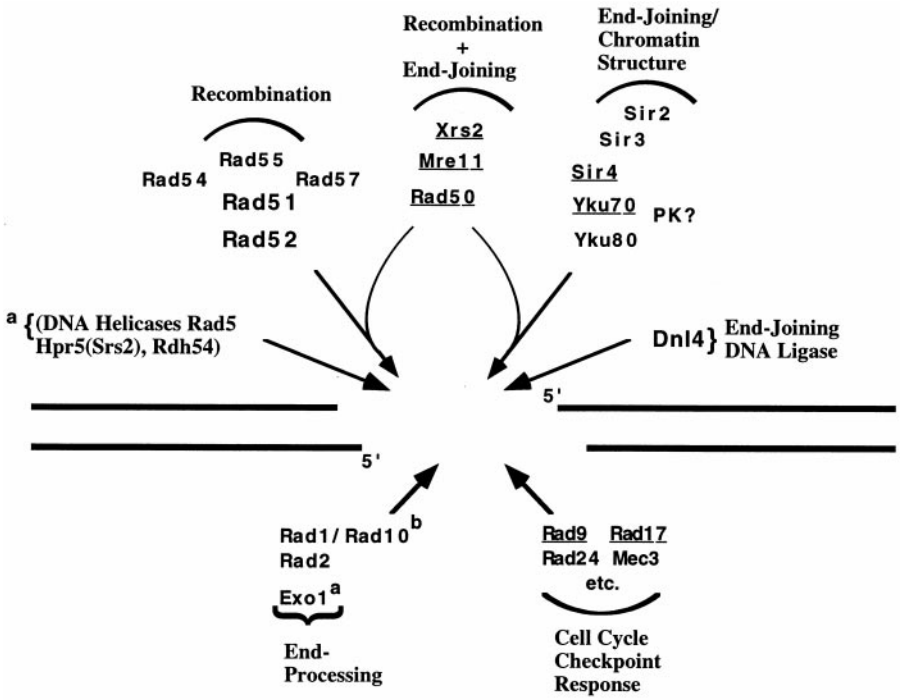

Figure 8.—Repair of DSBs in yeast involves multiple pathways. Proteins active in homologous recombination, end-joining, damage-responsive checkpoints, and in processing of the ends of DSBs affect repair of endonuclease-induced DSBs. Components that are underlined are essential for cell survival when *Eco*RI is expressed *in vivo.* (a) DSB repair phenotypes in mutant strains suggest that these proteins participate in the recombination pathway of repair. (b) Growth and cell-cycling responses of *rad1* mutants are consistent with a role for the Rad1/Rad10 endonuclease in repair of endonuclease-induced DSBs.

of human DNA ligase IV. Cells containing mutations within this gene are not sensitive to commonly used DNA-damaging agents but do display a reduced efficiency of recircularization of linearized plasmid DNA after transformation into yeast cells (Schar *et al.* 1997; Teo and Jackson 1997; Wilson *et al.* 1997). Recent experiments have revealed that expression of *Eco*RI in T334 strains containing a *dnl4::G418* insertion induces a strong G2 arrest response (data not shown), which supports the proposition that this ligase is involved in repair of at least some classes of chromosomal DSBs in nuclear DNA.

Many previous studies have suggested that the efficiency of repair of DSBs is affected by structural differences at the ends of the broken DNA. For example, DSB termini may be damaged (*i.e.*, containing missing or altered bases and sugars as observed at the ends of DSBs produced by ionizing radiation), covalently modified (such as DSBs induced by bleomycin, which can retain phosphoglycolate ester moieties after cleavage), or the ends may be blunt or contain complementary overhangs. DSB ends induced by ionizing radiation have a requirement for *RAD52*-mediated recombinational repair (Resnick and Martin 1976; Moore 1982; Game 1993).

The experiments described here and in a previous study (Lewis *et al.* 1998) have now demonstrated that *Eco*RI-induced chromosomal DSBs, which retain complementary four-base overhangs, can be processed by both the homologous recombination and NHEJ pathways inside cells. This finding is significant because many DSBs that are produced during the course of normal development in eukaryotic cells contain similar end structures. Both HO-induced mating type switching and intron homing (the process by which an intron-containing DNA sequence is inserted into an intronless allele in nuclear and mitochondrial genes) are initiated by formation of a DSB containing four-base complementary ends (Haber 1992; Mueller *et al.* 1993; Belfort and Roberts 1997). Interestingly, past studies have revealed that recombination occurring in meiosis is initiated by DSBs containing two-base overhangs (Liu and Lichten 1995). Repair of meiotic DSBs has the added complexity that the endonuclease involved in DSB formation, Spo11, forms a transient covalent attachment with one end of the DNA (Keeney *et al.* 1997). The results described here suggest that such complementary-ended DSBs may be accurately repaired by distinct groups of proteins active in either homologous recombination or the restitutional mechanism of precise end-joining.

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