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The *Saccharomyces cerevisiae* Ku Autoantigen Homologue Affects Radiosensitivity Only in the Absence of Homologous Recombination

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ABSTRACT

In mammalian cells, all subunits of the DNA-dependent protein kinase (DNA-PK) have been implicated in the repair of DNA double-strand breaks and in V(D)J recombination. In the yeast *Saccharomyces cerevisiae*, we have examined the phenotype conferred by a deletion of *HDF1*, the putative homologue of the 70-kD subunit of the DNA-end binding Ku complex of DNA-PK. The yeast gene does not play a role in radiation-induced cell cycle checkpoint arrest in G_1 and G_2 or in hydroxyurea-induced checkpoint arrest in S. In cells competent for homologous recombination, we could not detect any sensitivity to ionizing radiation or to methyl methanesulfonate (MMS) conferred by a *hdf1* deletion and indeed, the repair of DNA double-strand breaks was not impaired. However, if homologous recombination was disabled (*rad52* mutant background), inactivation of *HDF1* results in additional sensitization toward ionizing radiation and MMS. These results give further support to the notion that, in contrast to higher eukaryotic cells, homologous recombination is the favored pathway of double-strand break repair in yeast whereas other competing mechanisms such as the suggested pathway of DNA-PK-dependent direct break rejoining are only of minor importance.

THERE is substantial evidence that DNA double-strand breaks in the yeast *Saccharomyces cerevisiae* are primarily repaired by homologous recombination in a pathway represented by the *RAD52* epistasis group of genes (GAME 1993; FRIEDBERG *et al.* 1995). Corresponding mutants are generally highly X-ray and γ-ray sensitive because double-strand breaks constitute the primary lethal lesion after treatment of yeast with ionizing radiation (FRANKENBERG-SCHWAGER 1990; FRANKENBERG-SCHWAGER and FRANKENBERG 1990). Mutants of the *RAD52* epistasis group are characterized by varying degrees of defectiveness in DNA strand break processing, homologous recombination, plasmid integration, mating-type switching and meiosis (HO 1975; RESNICK and MARTIN 1976; MALONE and ESPOSITO 1980; WEIFFENBACH and HABER 1981; GAME 1983, 1993; ALANI *et al.* 1990; PETES *et al.* 1991; FISHMAN-LOBELL *et al.* 1992; ESPOSITO *et al.* 1994; SCHIESTL *et al.* 1994; FRIEDBERG *et al.* 1995). Among this group of genes the *RAD51* gene product has been identified as a structural and functional homologue of bacterial RecA protein (ABOUSSEKHRA *et al.* 1992; SHINOHARA *et al.* 1992). Rad51 forms filaments with double-stranded DNA and catalyzes exchange between double-stranded circular DNA and homologous single-stranded linear DNA (OGAWA *et al.* 1993; SUNG 1994). Homologues of the yeast genes *RAD51*, *RAD52* and *RAD54* have been characterized in higher eukaryotes (BEZZUBOVA *et al.* 1993b; MORITA *et al.* 1993; SHINOHARA *et al.* 1993; BENDIXEN *et al.* 1994; HEYER 1994; MURIS *et al.* 1994; ZDZIENICKA 1995). Their tissue-specific expression hints at a role in meiosis (BEZZUBOVA *et al.* 1993a; MORITA *et al.* 1993; SHINOHARA *et al.* 1993). However, a role in double-strand break repair in mitotic cells has not been excluded. Recently, increased intrachromosomal homologous recombination and a somewhat higher γ-ray resistance have been observed in monkey cells overexpressing the human *RAD52* homologue (PARK 1995).

The detailed mechanism of double-strand break repair in mammalian cells is not known. However, circumstantial evidence suggests some type of direct strand-break rejoining rather than homologous recombination as the prevailing mechanism (ROTH and WILSON 1986, 1988; NORTH *et al.* 1990; FAIRMAN *et al.* 1992; GANESH *et al.* 1993; LUTZE *et al.* 1993; DERBYSHIRE *et al.* 1994; NICOLÁS *et al.* 1995). Such strand-break rejoining may be responsible for intrachromosomal deletions, the major class of mutations recovered from cells treated with ionizing radiation (MORRIS and THACKER 1993). Rodent mutant cell lines with enhanced sensitivity to ionizing radiation have been classified into 11 complementation groups and human cross-complementing genes (*XRCC* genes) corresponding to eight of these

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have been isolated (COLLINS 1993; THOMPSON and JEGGO 1995; ZDZIENICKA 1995). V(D)J recombination during differentiation of cellular immunity involves processing of a double-strand break at a defined site (GELLERT 1992). Thus V(D)J recombination is also affected in cell lines that are defective in double-strand-break repair (PERGOLA *et al.* 1993; TACCIOLI *et al.* 1993; THACKER *et al.* 1994).

Several cloned *XRCC* genes have been correlated with the subunits of mammalian DNA-dependent protein kinase (DNA-PK) (ROTH *et al.* 1995; ZDZIENICKA 1995). DNA-PK consists of three subunits. Polypeptides of ~70 and ~80 kD (termed autoantigen Ku) can bind to free double-stranded DNA ends as well as to partially duplex single-stranded substrates and single-strand gaps as a heterodimer (ANDERSON and LEES-MILLER 1992; ANDERSON 1994). The third (catalytic) subunit of ~350–450 kD acquires kinase activity after complex formation with DNA-bound Ku. Extracts of rodent cells from the *XRCC5* complementation group lack DNA-end-binding activity and the 80-kD Ku subunit has been shown to be encoded by the *XRCC5* gene (GETTS and STAMATO 1994; RATHMELL and CHU 1994a,; SMIDER *et al.* 1994; TACCIOLI *et al.* 1994; BOUBNOV *et al.* 1995). The results of mapping, complementation and DNA-PK activity studies suggest identity of the 70-kD subunit with the *XRCC6* gene product (with *sxi-1* being the corresponding rodent cell line) (N. V. BOUBNOV, S. E. LEE, Z. P. WILLIS, K. T. HALL, E. A. HENDRICKSON, and D. T. WEAVER, unpublished data). Finally, a defect in the 350-kD subunit confers the murine *scid* phenotype, characterized by severe immundeficiency and defective double-strand break repair (BLUNT *et al.* 1995; KIRCHGESSNER *et al.* 1995; PETERSON *et al.* 1995).

DNA-PK activities have been characterized in several eukaryotic organisms (FINNIE *et al.* 1995). Although such activity has not yet been reported in yeast, a major DNA-end-binding activity detected in crude extracts has been shown to consist of two polypeptides of 67 and 85 kD, reminiscent of the mammalian Ku heterodimer (FELDMANN and WINNACKER 1993). The yeast gene encoding the 67-kD subunit has been cloned and the predicted amino acid sequence indicates a region of 298 amino acids (positions 226–578) with 21.8% identity to a region in the human Ku70 gene (residues 213–556) (FELDMANN and WINNACKER 1993). This value increases to 37% identity over a stretch of 30 amino acids (positions 490–520 of the yeast protein). Here we report the phenotype of a yeast deletion mutant of this gene (termed *HDF1*). A role for mammalian DNA-PK in DNA strand-break induced cell cycle arrest has been suggested frequently (*e.g.*, RATHMELL and CHU 1994b), however, *HDF1* plays no detectable role in cell cycle checkpoint control. Consonant with the relative significance of homologous recombination for DNA-double-strand-break repair in yeast compared with mammalian cells we detected increased sensitivity to ionizing radiation after inactivation of *HDF1* in the absence of homologous recombination. However, such sensitivity was not observed in cells that are proficient for homologous recombination.

MATERIALS AND METHODS

Strain construction: Deletion of *HDF1*, *RAD52* and *RAD1* was accomplished by one-step gene transplacement (ROTHSTEIN 1989) after transformation with linear fragments containing selectable markers, inserted into the gene of interest or replacing it. Plasmid manipulations were performed according to standard protocols (SAMBROOK *et al.* 1989). To construct a truncation of *HDF1* we isolated most of the open-reading frame (ORF) by polymerase chain reaction using chromosomal yeast DNA (strain SX46A) and appropriate primers. The resulting 1.8-kb fragment was cloned into the *Hin*cII site of a pBR322-derived plasmid. The *URA3* marker was inserted into the *Hin*dIII gapped gene, and a *Xmn*I digest was used to release a fragment for yeast transformation. To inactivate *RAD52* we transformed yeast cells with *Bam*HI-digested plasmid pSM21 (a gift from Dr. DAVID SCHILD), carrying a *TRP1* insertion in the *RAD52* ORF. *RAD1* was cloned previously into pUC18 on a *Pvu*II-*Hpa*I fragment. A complete replacement of the *RAD1* ORF was generated by cloning *TRP1* into the *Hin*dIII-gapped plasmid and a *Hin*cII-digest was used for transplacement. These deletions were introduced independently into two strains of opposite mating type (SX46A and WS8105-1C) and various diploid strains were constructed by mating the corresponding haploid strains (Table 1). Yeast cells were transformed by the lithium acetate method (GIETZ *et al.* 1992). Yeast media and standard methods of yeast genetics have been described (KAISER *et al.* 1994).

Electrophoretic mobility shift assays: Protein extracts from logarithmically growing yeast cells were prepared as described (SIEDE and FRIEDBERG 1992) except that the ultracentrifugation step was omitted. We used a previously described 39-bp synthetic oligonucleotide from the phosphoglycerol kinase (*PGK*) gene promoter as a probe for protein binding (FELDMANN and WINNACKER 1993). This probe was radioactively labeled by filling in the recessed ends with Klenow enzyme in the presence of ^{32}P-dCTP. Typically 1 ng of probe was incubated with 20 μg extract protein. Nonspecific competitor DNA was only added when indicated. As unlabeled competitor DNA we used the yeast/*Escherichia coli* shuttle vector plasmid YCp50, which was added uncut, cut with *Bam*HI (1 fragment) or with *Rsa*I (7 fragments). Further conditions of gel shift assays have been published (SIEDE and FRIEDBERG 1992).

Cell synchronization and determination of checkpoint arrest: Conditions for synchronization of haploid cells in G_1 with α-factor (Sigma) and for the analysis of cell cycle parameters during postirradiation incubation by flow-cytometric analysis of DNA content (FACScan, Becton-Dickinson) were as published (SIEDE *et al.* 1993, 1994). Sensitivity of α-factor synchronized cells to hydroxyurea (Sigma) was determined as described (ALLEN *et al.* 1994). Synchronization in G_2/M was accomplished by diluting a logarithmic-phase culture (2×10^7 cells/ml) 1:5 into fresh YPD medium (2% dextrose, 2% peptone, 1% yeast extract), incubating at 30° for 3 hr, adding nocodazole (Sigma) at 10 mg/ml and incubating for another 2 hr. Synchronized cells were washed free of the drug and resuspended in sterile water. An aliquot of the culture was irradiated and resuspended in fresh YPD. At various stages during incubation samples were collected and processed as described (SIEDE *et al.* 1993, 1994).

Radiation and mutagen treatments: Sensitivity of the various haploid and diploid strains to ionizing radiation was independently determined in two laboratories under different conditions. One protocol involved plating appropriate dilutions of stationary-phase cells after brief sonication on YPD

TABLE 1

Yeast Strains

Strain	Genotype
SX46A	*MATa RAD HDF ade2 his3-532 trp1-289 ura3-52*
SX46A *hdf1Δ*	*MATa RAD hdf1Δ::URA3 ade2 his3-532 trp1-289 ura3-52*
SX46A *rad52Δ*	*MATa rad52::TRP1 HDF ade2 his3-532 trp1-289 ura3-52*
SX46A *radlΔ*	*MATa rad1Δ::TRP1 HDF ade2 his3-532 trp1-289 ura3-52*
SX46A *rad52Δ hdf1Δ*	*MATa rad52::TRP1 hdf1Δ::URA3 ade2 his3-532 trp1-289 ura3-52*
SX46A *rad1Δ hdf1Δ*	*MATa rad1Δ::TRP1 hdf1Δ::URA3 ade2 his3-532 trp1-289 ura3-52*
WS8105-1C	*Matα RAD HDF ade2 arg4-17 trp1-289 ura3-52*
WS8105-1C *hdf1Δ*	*MATα RAD hdf1Δ::URA3 ade2 arg4-17 trp1-289 ura3-52*
WS8105-1C *rad52Δ*	*MATα rad52::TRP1 HDF ade2 arg4-17 trp1-289 ura3-52*
WS8105-1C *rad1Δ*	*MATα rad1Δ::TRP1 HDF ade2 arg4-17 trp1-289 ura3-52*
WS8105-1C *hdf1Δ rad52Δ*	*MATα rad52::TRP1 hdf1Δ::URA3 ade2 arg4-17 trp1-289 ura3-52*
WS8105-1C *hdf1Δ rad1Δ*	*MATα rad1Δ::TRP1 hdf1Δ::URA3 ade2 arg4-17 trp1-289 ura3-52*
WS9131	*MATa/MATα RAD/RAD HDF/HDF ade2/ade2 arg4-17/ARG his3-532/HIS ura3-52/ura3-52 trp1-289/trp1-289*
WS9134	*MATa/MATα RAD/RAD hdf1Δ::URA3/hdf1Δ::URA3 ade2/ade2 arg4-17/ARG his3-532/HIS ura3-52/ura3-52 trp1-289/trp1-289*
WS9136	*MATa/MATα rad52::TRP1/RAD hdf1Δ::URA3/HDF ade2/ade2 arg4-17/ARG his3-532/HIS ura3-52/ura3-52 trp1-289/trp1-289*
WS9137	*MATa/MATα rad52::TRP1/RAD hdf1Δ*::URA3/*hdf1Δ::URA3 ade2/ade2 arg4-17/ARG his3-532/HIS ura3-52/ura3-52 trp1-289/trp1-289*
WS9138	*MATa/MATα rad52::TRP1/rad52::TRP1 HDF/HDF ade2/ade2 arg4-17/ARG his3-532/HIS ura3-52/ura3-52 trpl-289/trp1-289*
WS9139	*MATa/MATα rad52::TRP1/rad52::TRP1 hdf1Δ::URA3/HDF ade2/ade2 arg4-17/ARG his3-532/HIS ura3-52/ura3-52 trp1-289/trp1-289*
WS9140	*MATa/MATα rad52::TRP1/rad52::TRP1 hdf1Δ::URA3/hdf1Δ::URA3 ade2/ade2 arg4-17/ARG his3-532/HIS ura3-52/ura3-52 trp1-289/trp1-289*
WS9151	*MATa/MATα rad1Δ::TRP1/rad1Δ::TRP1 HDF/HDF ade2/ade arg4-17/ARG his3-532/HIS ura3-52/ura3-52 trp1-289/trp1-289*
WS9153	*MATa/MATα rad1Δ::TRP1/rad1Δ::TRP1 hdf1Δ::URA3/hdf1Δ::URA3 ade2/ade2 arg4-17/ARG his3-532/HIS ura3-52/3-52 trp1-289/trp1-289*

plates and irradiation of plates in a ^{137}Cs γ source (J. L. Shepherd and Assoc.) at 4.4 Gy/min. Alternatively cells were resuspended in potassium phosphate buffer and aerated with N_2 or O_2 before and during irradiation. The cell suspension was exposed on ice to a ^{60}Co source (Atomic Energy of Canada, Ltd.) at 20 Gy/min. Treatment with methylmethane sulfonate (MMS) (Sigma) was as described (REAGAN *et al.* 1995). Survival of macrocolony forming cells was determined after 4–5 days of incubation at 30°, except for *rad52* mutant strains, which required incubation for up to 8 days.

Analysis of DNA double-strand break frequency in pulsed-field gels: Stationary phase cells were resuspended in phosphate buffer at 5×10^8 cells/ml and irradiated on ice with 300 Gy (^{60}Co source). To monitor double-strand break repair cells were resuspended at 5×10^6 cells/ml in phosphate buffer containing 100 mM glucose and incubated at 30°. Details of DNA preparation, conditions for pulsed-field gel electrophoresis and quantitation of double-strand break frequency have been published (FRIEDL *et al.* 1995).

RESULTS

Construction of isogenic haploid and diploid strains deleted for *HDF1*, *RAD52* and *RAD1*: We used a one-step transplacement strategy (ROTHSTEIN 1989) (see MATERIALS AND METHODS) to create deletions of the genes *HDF1*, *RAD52* and *RAD1* in two strains of opposite mating type (SX46A *MATα* and WS8105-1C *MATα*, Table 1). Correct transplacement was verified in each case by Southern hybridization. Additional deletions of *RAD52* and *RAD1* were constructed in *hdf1Δ* mutants and mating of single and double deletion mutant strains of opposite mating types yielded isogenic diploid strains deleted for *HDF1* and/or *RAD52* or *RAD1* in homozygous or heterozygous configurations (Table 1).

HDF1 deletion strains were also characterized by a functional assay. A 39-bp radiolabeled probe derived from the phosphoglycerol kinase (*PGK*) gene promoter was used to detect DNA-end-binding activity in yeast crude extracts (FELDMANN and WINNACKER 1993). Using electrophoretic mobility shift assays, we detected a single retarded band that was absent when extracts from haploid or diploid cells deleted for the *HDF1* gene were probed (Figure 1A). We confirmed the DNA-end specificity of the observed protein/DNA complex by competition with an excess of unlabeled double-stranded circular DNA and with the identical DNA linearized with *Bam*HI or cut into multiple fragments with *Rsa*I. In accord with previously published results (FELDMANN and WINNACKER 1993), we observed that the extent of competition by identical concentrations of competitor DNA increased with the amount of free ends, with no competition observed with closed circular DNA (Figure 1B).

An inability to grow at elevated temperatures (37°) has been described as a phenotype of *HDF1* deletion mutants (FELDMANN and WINNACKER 1993). We observed a notably slower growth at 37° in haploid and diploid *hdf1Δ* strains as compared with the isogenic wild type (Figure 2). However, a substantial fraction of the plated cells deleted for *HDF1* ultimately formed macrocolonies on YPD plates at 37°.

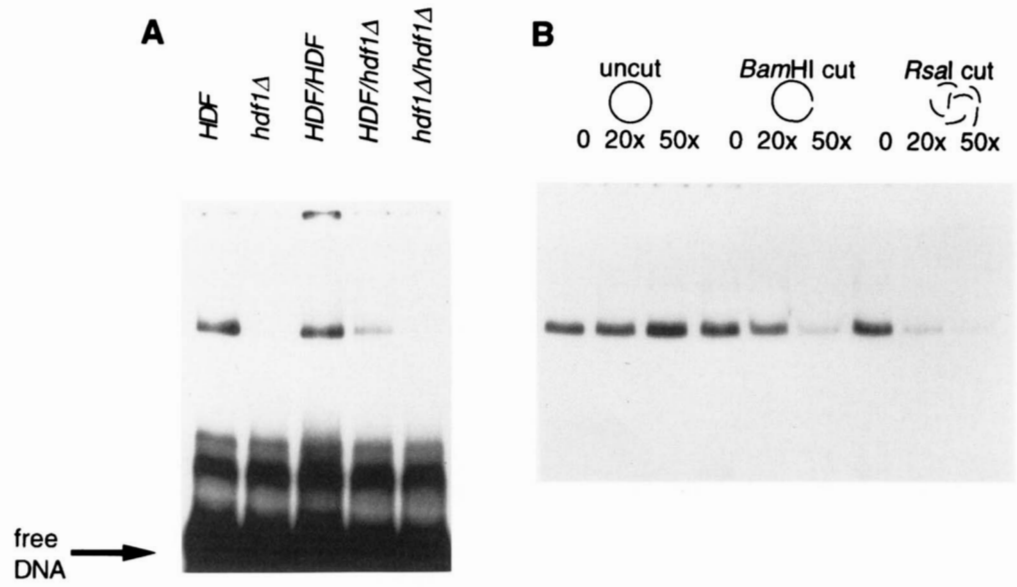

FIGURE 1.—Detection of a DNA end-specific Hdf1-DNA complex in gel retardation assays. A radioactively labeled 39-bp probe derived from the *PGK* promoter (1 ng) was incubated with protein extracts (20 μg protein per reaction) and DNA-protein complexes were separated from unbound DNA in native polyacrylamide gels. Extracts of the following strains were used: SX46A (*HDF*), SX46A (*hdf1Δ*), WS9131 (*HDF/HDF*), WS9132 (*HDF/hdf1Δ*), WS9134 (*hdf1Δ/hdf1Δ*). (A) The absence of the major retarded band in haploid and homozygous diploid strains carrying a truncation of *HDF1* is demonstrated. The heterozygous *hdf1Δ/HDF* diploid strain shows a reduced binding activity. (B) The DNA-end-specifity of the detected binding activity is verified. Before adding protein extract of the haploid wild-type strain (SX46 A *HDF*), the probe (1 ng) was mixed with 20 or 50 ng unlabeled competitor DNA (plasmid YCp50). As indicated, the plasmid was used uncut or after digestion with *Bam*HI (one fragment) or *Rsa*I (seven fragments).

Inactivation of *HDF1* does not affect checkpoint control in G_1, G_2 or S: We examined a possible role of the *HFD1* gene in arrest at cell cycle checkpoints. To study radiation-induced G_1 arrest, haploid cells were synchronized in early G_1 (at "START") with α-factor. Cells were UV-irradiated or not and incubated in fresh medium. During postirradiation incubation the DNA content of propidium-iodide stained cells was analyzed by flow-cytometry. The pronounced G_1 arrest observed in the irradiated culture was unaltered in the *hdf1Δ* strain (Figure 3A). Similar results were found with γ-irradiated cells (data not shown). To study G_2 arrest, cells were synchronized at the G_2/M boundary by treatment with the antitubulin drug nocodazole, irradiated and immediately released from the nocodazole block into fresh medium. DNA profiles indicated arrest in G_2 in γ-irradiated wild-type cultures, which was comparable with that observed in cells deleted for *HDF1* (Figure 3B). Similar results were observed in UV-treated cells (data not shown).

Regulated S-phase arrest is observed in normal yeast cells treated with the ribonucleotide reductase-inhibiting drug hydroxyurea (HU). Mutants such as *rad53* that are defective in this checkpoint response quickly lose viability in the presence of HU because entry into mitosis is attempted with an unreplicated genome (ALLEN *et al.* 1994; HUMPHREY and ENOCH 1995). When α-factor synchronized cells were released into medium containing HU, the fraction of colony-forming cells remained unaltered for ≥90 min in the wild-type strain and in the corresponding *hdf1Δ* mutant, whereas the survival of a *rad53* mutant strain was reduced to 10% after 45 min of incubation (Figure 4) (ALLEN *et al.* 1994). Collectively these data do not indicate any role of *HDF1* in checkpoint control in G_1, G_2 or S phases of the cell cycle.

Inactivation of *HDF1* per se does not alter radiosensitivity or double-strand break repair: Diploid yeast cells repair double-strand breaks primarily by homologous recombination, even under nongrowth conditions (FRANKENBERG-SCHWAGER *et al.* 1980). Isogenic diploid strains proficient and deficient in the *HDF1* function were tested in stationary phase for γ-ray sensitvity under different irradiation conditions. Cells were either irradiated on plates with a ^{137}Cs γ source at a low dose-rate (4 Gy/min) (Figure 5A), or in suspension with a ^{60}Co source at a high dose-rate (20 Gy/min) (Figure 5, B and C). Because the availibility of O_2 significantly influences the spectrum of γ-ray-induced lesions (FRANKENBERG-SCHWAGER *et al.* 1979; WARD 1988; FRIEDBERG *et al.* 1995), suspension irradiation was performed both under O_2 aeration (Figure 5B) and with exclusion of O_2 (N_2 conditions) (Figure 5C). In all cases, the homozygous *hdf1Δ* deletion strain was no more sensitive than the homozygous wild-type strain (Figure 5, A–C), or the heterozygous *HDF/hdf1Δ* strain (Figure 5A). This result was not influenced by elevating the postirradiation temperature from 30 to 37° (data not shown).

In the diploid wild-type and the homozygous *hdf1Δ* strains irradiated with 300 Gy under O_2 conditions, the kinetics of double-strand break repair were determined during incubation at 30° under nongrowth conditions

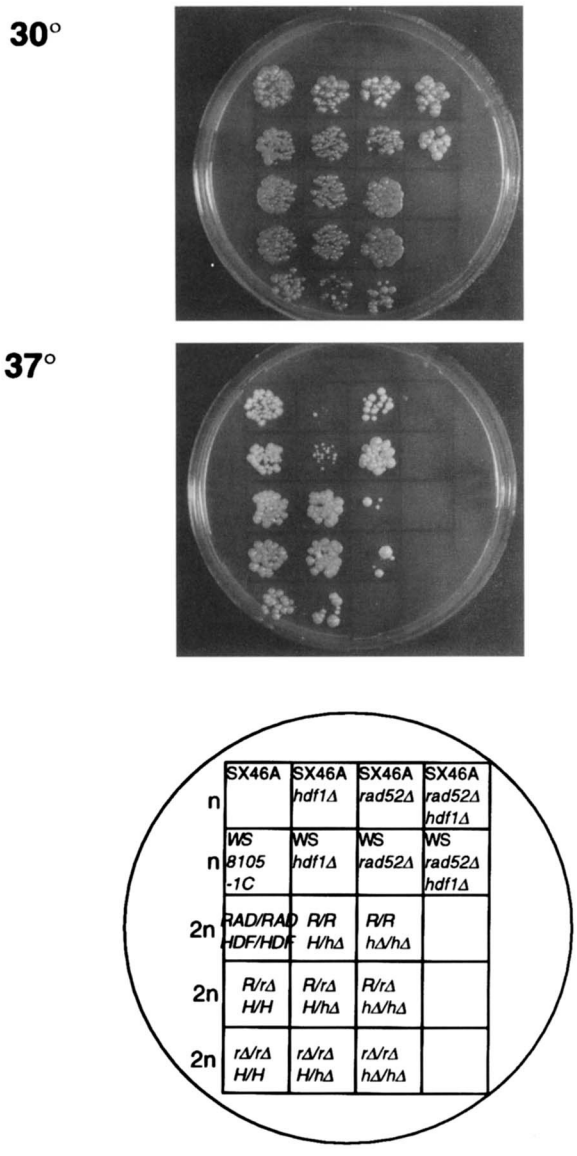

FIGURE 2.—Colony formation of *hdf1* mutant strains at elevated temperatures. Equal amounts of stationary-phase cells of the various haploid and diploid strains indicated were plated on YPD plates and incubated at 30 or 37° for 4 days. Growth inhibition of the haploid and diploid homozygous *hdf1*Δ mutant strain at elevated temperature is evident. A complete failure of macrocolony formation at 37° is observed following additional inactivation of *RAD52*.

("liquid holding"), using the highly sensitive method of pulsed-field gel electrophoresis to analyze the recovery of the chromosome-size molecules (FRIEDL *et al.* 1995). In confirmation of the survival data discussed above, no difference in the repair of double-strand breaks was observed (Figure 6).

Double-strand breaks subject to repair by homologous recombination are also observed after treatment of yeast cells with the alkylating agent MMS (CHLEBOWICZ and JACHYMCZYK 1979). Once again the homozygous *hdf1*Δ strain did not show an increase in MMS sensitivity (Figure 7). Additionally, a homozygous *hdf1*Δ mutation did not notably influence sporulation efficiency or spore survival (data not shown).

Inactivation of *HDF1* affects radiosensitivity, thermosensitivity and plating efficiency if homologous recombination is disabled: We asked whether sensitization to irradiation after inactivation of *HDF1* can be observed if the pathway for homologous recombination, which depends on the *RAD52* group of genes, is excluded. We compared the γ-ray sensitivity of a diploid homozygous *rad52* deletion mutant with that of the corresponding *rad52*Δ *hdf1*Δ double mutant, both treated in stationary phase. As expected, a homozygous *rad52* deletion rendered the strain highly sensitive to γ radiation (Figure 5, A–C). This strain was further sensitized by the additional inactivation of *HDF1* (Figure 5, A–C). This effect was highly reproducible under the irradiation conditions tested. The expected increase in γ-ray sensitivity in the presence of O_2 was the same in *rad52*Δ and *rad52*Δ *hdf1*Δ strains. Sensitization of a diploid *rad52*Δ strain by homozygous deletion of *HDF1* was also evident after MMS treatment (Figure 7).

Homozygous *rad52* deletion strains required an incubation period of 8 days before macrocolony counts could be reliably determined, because such strains predominantly form heterogeneous slow-growing colonies that manifest lethal sectoring even without radiation treatment. However, the additional sensitization to ionizing radiation or MMS conferred by the inactivation of *HDF1* was evident during all stages of incubation (data not shown). Not surprisingly, unirradiated stationary-phase cultures of a *rad52/rad52* strain showed reduced plating efficiency when microscopic cell counts and colony counts were compared (Table 2). This effect was significantly more pronounced in the *rad52*Δ *hdf1*Δ double mutant (Table 2). In addition, the growth inhibition at 37° conferred by inactivation of *HDF1* was enhanced in a *rad52*Δ mutant strain and complete inactivation of macrocolony formation was observed in diploid and haploid strains (Figure 2).

Haploid G_1- or G_0-phase cells are considered to be incapable of homologous recombination because no homologous chromosome or chromatid is available. It was therefore of interest to determine whether deletion of *HDF1* affects γ-ray sensitivity without deletion of *RAD52* in stationary-phase haploid cells. When such cells were treated with <200 Gy, a small but reproducible sensitization by inactivation of *HDF1* was observed (Figure 8A). No difference was detectable at higher doses (Figure 8B), but in this situation the sensitivity of the culture is predominantly determined by the small γ-ray resistant fraction of G_2-phase cells commonly present in stationary-phase yeast cultures. As expected this resistant fraction was absent in the isogenic haploid *rad52*Δ strain and a purely exponential survival curve was observed (Figure 8B). Additional deletion of *HDF1* resulted in a modest increase in sensitivity, even at higher doses (Figure 8B). It is unclear why this effect is less pronounced compared with that observed in diploid cells.

In yeast, an alternative mechanism of the repair of

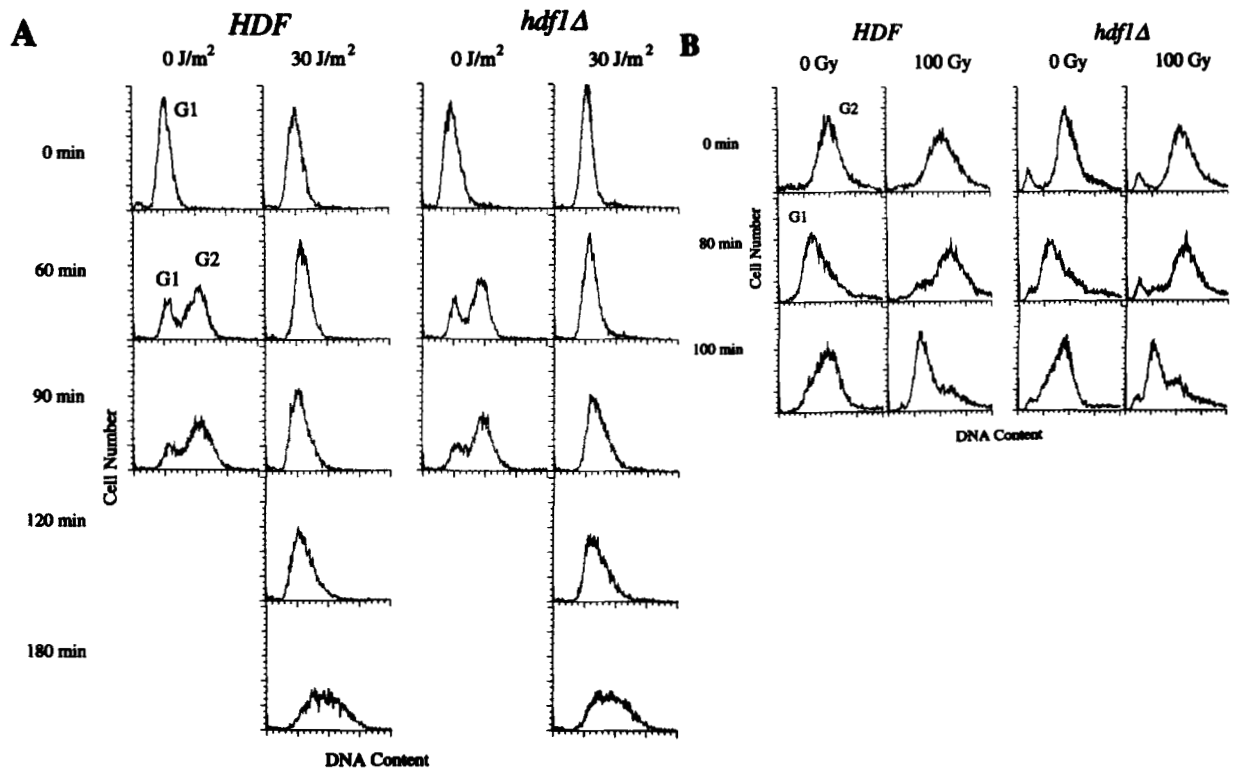

FIGURE 3.—Detection of normal radiation-induced cell cycle arrest in *hdf1* mutant strains. Cells of a haploid wild-type strain (SX 46A) and of a corresponding *hdf1* mutant (SX 46A *hdf1*Δ) were synchronized with α-factor in G_1 (A) or with nocodazole in G_2/M (B), irradiated with UV light (254 nm) (20 J/m^2; A), γ rays (^{137}Cs) (100 Gy; B) or mock-treated and immediately released into fresh YPD medium. At the times indicated, samples were withdrawn and cellular DNA content was analyzed by laser flow-cytometry.

localized double-strand breaks generated during mating-type switching is effected by a Rad1/Rad10-dependent single-strand annealing process with concomitant intrachromosomal deletion (FISHMAN-LOBELL and HABER 1992; FISHMAN-LOBELL *et al.* 1992). We explored the possibility that *HDF1* participates in such a pathway. Because *rad1* mutants are primarily involved in nucleotide excision repair, they are not known to be abnormally sensitive to γ-ray (HAYNES and KUNZ 1981). However, detailed comparisons between deletion mutants and isogenic wild-type strains have not been reported. At higher radiation doses, we detected a very moderate sensitization to γ-rays by deletion of the *RAD1* gene in diploid wild-type strains that appeared to be further increased in the *rad1*Δ *hdf1*Δ double-mutant strains (Figure 9).

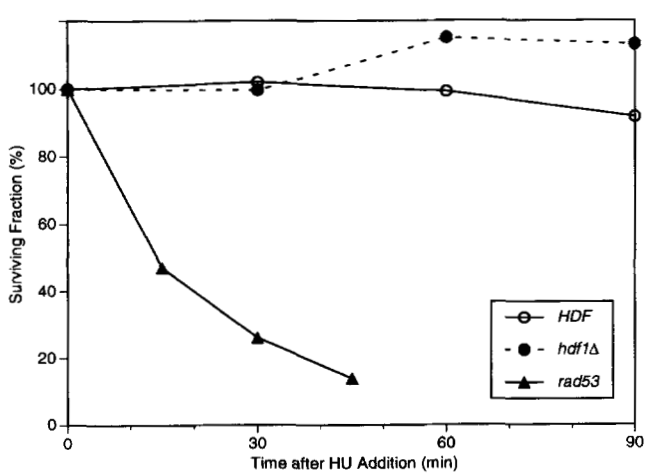

FIGURE 4.—Sensitivity of *hdf1* mutant cells towards inhibition of S-phase by hydroxyurea (HU). Haploid wild-type cells (strain SX46A) and isogenic *hdf1*Δ cells were synchronized in G_1 with α-factor and released into fresh medium containing 200 mM HU. Cells were plated at the times indicated and the surviving fraction of macrocolony forming cells was determined. The data for the nonisogenic HU-sensitive *rad53* mutant (taken from ALLEN *et al.* 1994) have been obtained under otherwise identical conditions and are included for comparison.

DISCUSSION

DNA-dependent protein kinase activity has been observed in several eukaryotic organisms. Although a similar activity has not yet been reported in yeast, functional homologs of the DNA-end-binding Ku subunits have been detected and a gene encoding the putative homologue of the mammalian 70-kD Ku subunit has been isolated (FELDMANN and WINNACKER 1993). In mammalian cells, all three subunits of DNA-dependent protein kinase have been implicated in double-strand break repair and V(D)J recombination (GETTS and STAMATO 1994; RATHMELL and CHU 1994a,b; SMIDER *et al.* 1994; TACCIOLI *et al.* 1994; BLUNT *et al.* 1995; BOUBNOV *et al.*

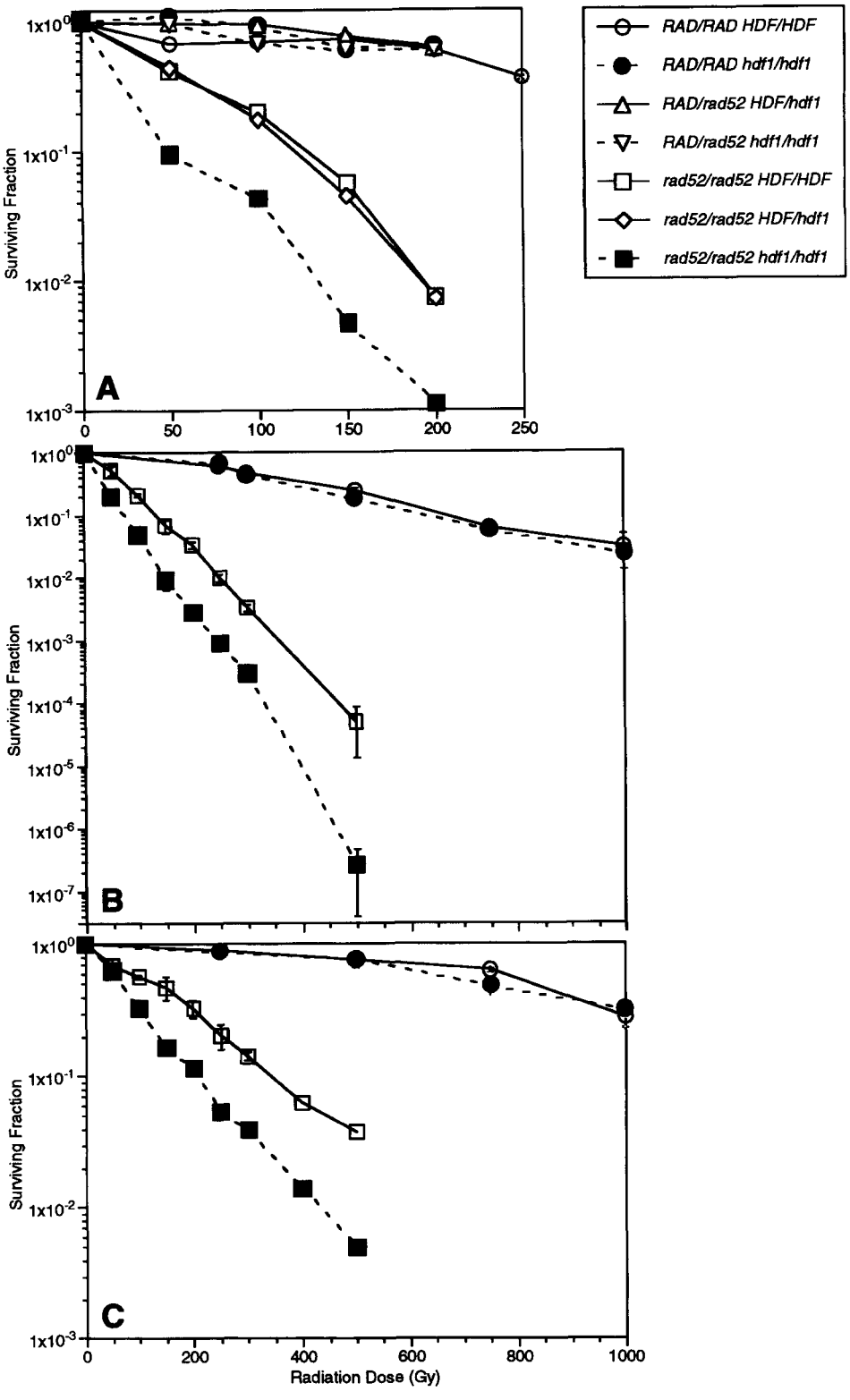

FIGURE 5.—Survival of diploid strains following γ-ray treatment of stationary-phase cells under various conditions. Cells were irradiated with at ^{137}Cs source at 4.4 Gy/min on plates (A), with a ^{60}Co source at 20 Gy/min in suspension under O_2 aeration (B) or with the same ^{60}Co source under exclusion of oxygen (N_2 conditions) (C). The following strains were used: WS9131 (*RAD/RAD HDF/HDF*), WS9134 (*RAD/RAD hdf1Δ/hdf1Δ*), WS9136 (*RAD/rad52Δ HDF/hdf1Δ*), WS9137 (*RAD/rad52Δ hdf1Δ/hdf1Δ*), WS9138 (*rad52Δ/rad52Δ HDF/HDF*), WS9139 (*rad52Δ/rad52Δ HDF/hdf1Δ*) and WS9140 (*rad52Δ/rad52Δ hdf1Δ/hdf1Δ*). When no error bars are shown, data points were derived from a single representative experiment.

1995; KIRCHGESSNER *et al.* 1995; PETERSON *et al.* 1995; THOMPSON and JEGGO 1995; ZDZIENICKA 1995; N . V. BOUBNOV, S. E. LEE, Z. P. WILLIS, K. T. HALL, E. A. HENDRICKSON, and D. T. WEAVER, unpublished data), and a model for their direct involvement in nonrecombinational DNA strand-break rejoining has been proposed (ROTH *et al.* 1995).

The properties of the Ku subunits and DNA-PK are also compatible with models assuming an indirect role in DNA repair. DNA-PK may be required for transcriptional activation of repair genes, or it may be involved in cell cycle arrest (checkpoint arrest) (HARTWELL and WEINERT 1989), thereby allowing increased time for repair. Indeed, protein kinases have been clearly impli-

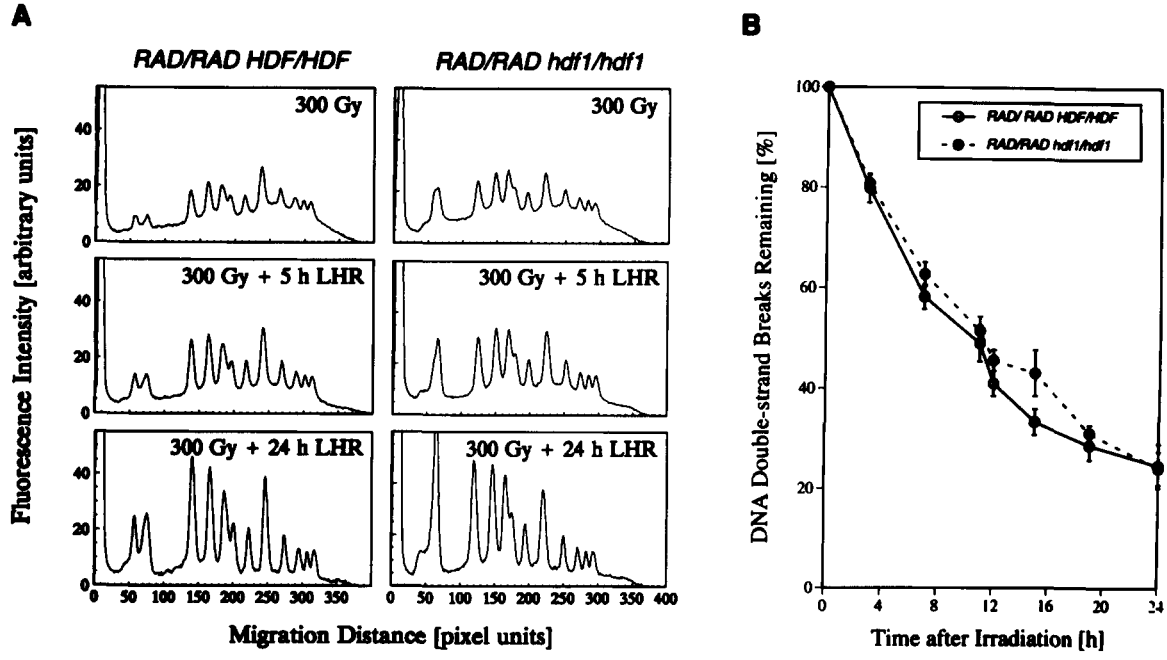

FIGURE 6.—Repair of double-strand breaks in diploid strains (*HDF/HDF vs. hdf1Δ/hdf1Δ*) as measured by pulsed-field gel electrophoresis. (A) Examples of the scanned ethidium-bromide stained agarose gels are shown. Recovery of chromosome-size signals during post-irradiation incubation (LHR = liquid holding recovery) is observed. (B) These data have been converted into fractions of broken chromosomes. Initial double-strand break frequencies (set to 100%) were 0.79 per Mb for *HDF/HDF* and 0.67 for *hdf1Δ/hdf1Δ*.

cated in checkpoint arrest in yeast (ALLEN *et al.* 1994; SIEDE 1995). The latter possibility is also suggested by studies that indicate a role of DNA strand breaks for radiation-induced cell cycle arrest in yeast (WEINERT and LYDALL 1993; SIEDE *et al.* 1994) and for the post-translational stabilization of p53 in irradiated mammalian cells (LU and LANE 1993; NELSON and KASTAN 1994; ALMASAN *et al.* 1995). p53 is a determinant of G_1 checkpoint arrest in mammalian cells (KASTAN *et al.* 1992; KUERBITZ *et al.* 1992) and is among the many transcriptional activators that are substrates for DNA-PK *in vitro* (LEES-MILLER *et al.* 1992). We did not detect a role of the *HDF1* gene in cell cycle arrest in G_1 or G_2 triggered by ionizing radiation or by UV radiation, or in S-phase arrest after treatment with the nucleotide-depleting drug hydroxyurea. We predict a similar lack of dependence of Ku and DNA-PK in checkpoint arrest in mammalian cells, because normal delay of S-phase following ionizing radiation treatment has been demonstrated in mutant rodent cells from the *XRCC5* (Ku 80) complementation group (JEGGO 1985).

In examining a possible direct role of *HDF1* in DNA

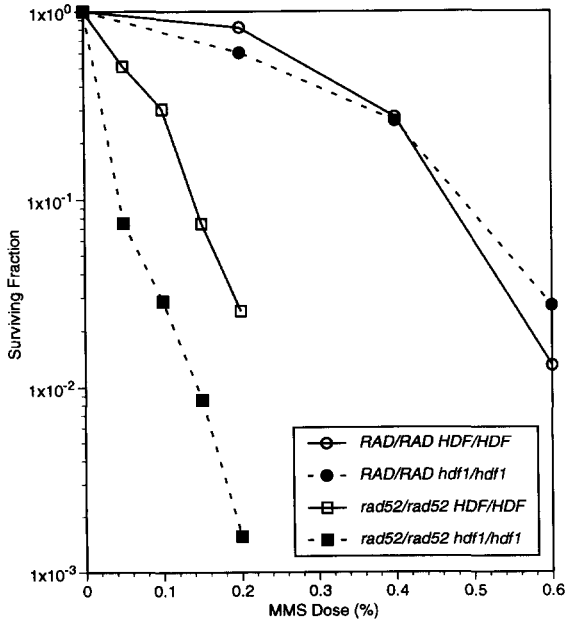

FIGURE 7.—Survival of diploid strains after treatment of stationary-phase cells with MMS. The following strains were used: WS9131 (*RAD/RAD HDF/HDF*), WS9134 (*RAD/RAD hdf1Δ/hdf1Δ*), WS9138 (*rad52Δ/rad52Δ HDF/HDF*), and WS9140 (*rad52Δ/rad52Δ hdf1Δ/hdf1Δ*).

TABLE 2

Influence of *HDF1* on plating efficiency

Strain and relevant genotype	Fraction of colony-forming cells (%)
WS9131 (*RAD/RAD HDF/HDF*)	93/105
WS9134 (*RAD/RAD hdf1Δ/hdf1Δ*)	101/93
WS9138 (*rad52Δ/rad52Δ HDF/HDF*)	53/68
WS9140 (*rad52Δ/rad52Δ hdf1Δ/hdf1Δ*)	34/33

Cells of the diploid strains listed were grown to stationary phase, the titers were determined by microscopic examination in a hemacytometer and the fraction of macrocolony forming cells determined following plating on YPD. Data of two independent experiments are shown.

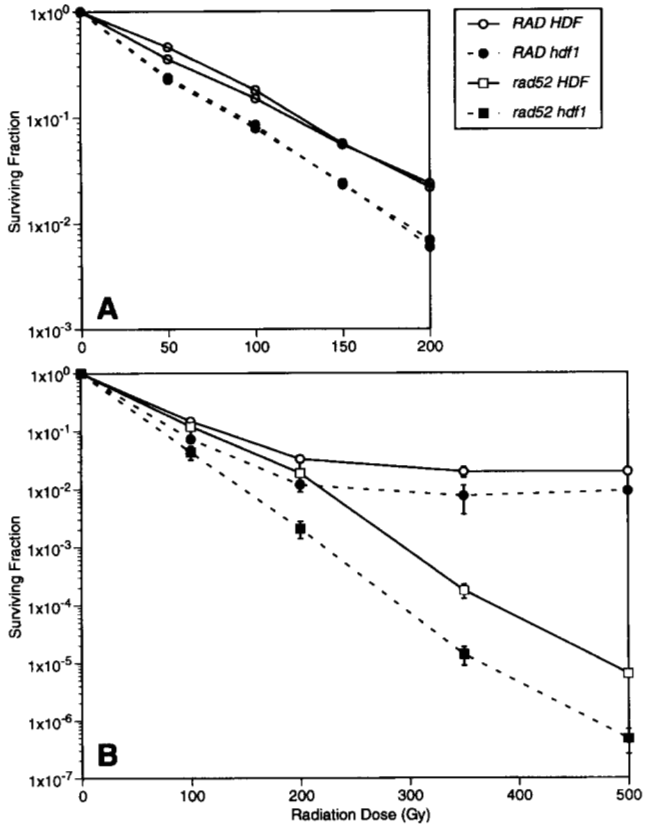

FIGURE 8.—Survival of haploid strains (SX46A and isogenic derivatives) after treatment of stationary-phase cells on plate with a ^{137}Cs source (A) or in suspension under oxygen conditions with a ^{60}Co source (B). Note the difference in dose range. (A) Data of two independent experiments are shown.

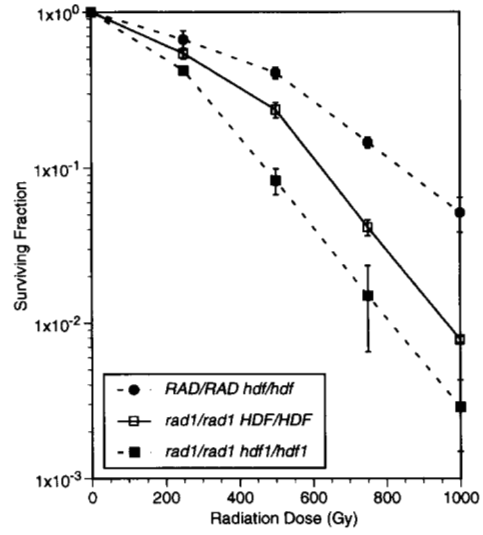

FIGURE 9.—Survival of diploid strains after γ-ray treatment of stationary-phase cells with a ^{60}Co source at 20 Gy/min in suspension under O_2 aeration. The following strains were used: WS9134 (*RAD/RAD hdf1Δ/hdf1Δ*), WS9151 (*rad1Δ/rad1Δ HDF/HDF*), and WS9153 (*rad1Δ/rad1Δ hdf1Δ/hdf1Δ*).

repair in yeast, we observed that a homozygous deletion of *HDF1* in a diploid yeast strain does not render cells sensitive to ionizing radiation or to MMS. Sporulation and spore survival was also unaffected. However, when homologous recombination was inactivated, the additional loss of *HDF1* resulted in increased γ-ray and MMS sensitivity of the (already highly sensitive) mutant cells. Experiments with haploid cells confirmed these data. Because haploid stationary phase (G_1) cells are deprived of the capability for recombination between homologous chromosomes or chromatids, a slight sensitization was observed even without additional inactivation of *RAD52*. Diploid cells lacking *RAD52* are characterized by reduced plating efficiency and the formation of heterogeneous slow-growing colonies, most likely due to aneuploidy resulting from unrepaired spontaneous strand breakage (MORTIMER *et al.* 1981). These effects were all significantly enhanced by additional deletion of *HDF1*. Our data suggest that *HDF1* plays a role in a non-recombinational repair process that can only be observed when homologous recombination is disabled. By inference from the situation in mammalian cells, we suggest a role of the yeast Ku homologue and a potential DNA-PK activity in a mechanism of double-strand break repair that involves direct DNA end-joining.

The utilization of alternative processes for breakage fusion in yeast when homologous recombination is inactivated is not a novel observation. In a recent study, different systems for the conditional induction of localized double-strand breaks were used to investigate their fate in the absence of a functional *RAD52* gene (KRAMER *et al.* 1994). The recovered fusion products were mostly deletions whose origin is compatible with 5′ → 3′ exonucleolytic degradation and single-strand annealing at limited regions of homology. Events involving blunt-end ligation without homology were also found at a lower frequency. Thus, the preferred mechanism of double-strand break repair in a *rad52* mutant background is apparently analogous to that observed in nuclear extracts of normal human cells and *in vivo* (NICOLÁS *et al.* 1995), and in Xenopus oocyte extracts (PFEIFFER and VIELMETTER 1988). Processing of the localized double-strand breaks introduced by the HO endonuclease during mating-type switching in yeast can involve a deletion/fusion mechanism that relies on 5′ → 3′ single-strand degradation and single-strand annealing (FISHMAN-LOBELL and HABER 1992; FISHMAN-LOBELL *et al.* 1992). The Rad1/Rad10 endonuclease is apparently essential for this process. We did detect a moderately increased γ-ray sensitivity at higher irradiation doses conferred by *rad1* deletions that was further increased by additional deletion of *HDF1*. Hence, we have no indication that *HFD1* plays a role in a single-strand annealing mechanism that depends on the Rad1/Rad10 endonuclease. However, firm conclusions cannot be based on radiation sensitivity data alone, because the sensitivity of the *rad1* mutant strains may not be caused by a defect in strand-break repair but by a failure to remove certain types of γ-ray-induced base damage by nucleotide excision repair.

The precise molecular function of the yeast Ku homologue remains to be determined. We perfomed repair assays in the absence of homologous recombination by introducing double strand breaks in the plasmid-borne bacterial *Tet* gene before transformation of yeast with autonomously replicating plasmids (data not shown). Using restriction enzyme digestion breaks were created that could be readily repaired by direct ligation (*e.g.*, following a *Bam*H1 cut) (SUZUKI *et al.* 1983), as well as breaks that would require additional processing before ligation (*e.g.*, a blunt end to be joined to a nonhomologous 5′ overhang). However, we failed to detect a role for the *HDF1* gene product in either situation (data not shown). This suggests that a repair defect in a *hdf1* mutant might only be detectable in the chromosomal context. Such a situation is not without precedence for yeast mutants involved in double-strand break repair (SUGAWARA *et al.* 1995).

Our results contradict the notion that, in the absence of the *RAD52*-dependent pathway of homologous recombination, repair of double-strand breaks is completely inactivated and hence the introduction of a single DNA double-strand break per cell constitutes a lethal event. This assumption is inferred from sedimentation studies (HO 1975; RESNICK and MARTIN 1976), from the lack of liquid-holding recovery and dose-rate effects (DARDALHON *et al.* 1994) and from the lethality conferred by attempted mating-type switching (MALONE and ESPOSITO 1980; WEIFFENBACH and HABER 1981) in *rad52* mutants. However, the HO-endonuclease-induced site-specific double-strand break at the *MAT* locus may create a special situation. Based on neutral sucrose sedimentation data, a value of 2.1 γ-ray-induced double-strand breaks per cell was estimated to constitute a lethal event in a *rad52* mutant (RESNICK and MARTIN 1976). Experiments are in progress, using the highly sensitive method of pulsed-field gel electrophoresis, to measure a low level of double-strand break repair that is potentially functional even in a *rad52* mutant background and dependent on *HDF1*. Alternatively, we cannot exclude that *HDF1* plays a role in the repair of radiation damage other than double-strand breaks, such as single-strand breaks or gaps, single-base damage or clustered base damage resulting in S1 endonuclease-sensitive sites (GEIGL and ECKARDT-SCHUPP 1991).

HDF1 is clearly required for normal growth of unirradiated cells at higher temperatures. Possibly due to differences in genetic backgrounds, we did not observe complete cessation of growth at 37° as described previously (FELDMANN and WINNACKER 1993). Nonetheless growth retardation was evident in *hdf1*Δ strains. Interestingly, this effect was also enhanced in a *rad52* background, resulting in the complete failure of haploid and diploid *rad52*Δ *hdf1*Δ strains to form macrocolonies at 37°. We are currently investigating whether this phenotype can be explained by a role of Hdf1 in DNA replication. Recent data indicate that Rad52 has an additional function in replication (TRAN *et al.* 1995). On the other hand, the Rfa1 subunit of replication factor A plays also a role in recombination (SMITH and ROTHSTEIN 1995) and could very well interact with Hdf1. Additionally, a similar lethality at higher temperature has been observed for deletion mutants of *RAD27*, a cell-cycle regulated gene with dual function in repair and replication (REAGAN *et al.* 1995).

In summary, we have shown that the yeast Ku homologue does not play a detectable role in cell cycle checkpoint control and does not have a major role in the repair of DNA damage induced by ionizing radiation. An influence on radiation sensitivity is only revealed if homologous recombination is disabled. These results lend further support to the often-cited notion that, in contrast to mammalian cells, homologous recombination is the favored pathway of double-strand break repair in yeast and that direct break-fusion processes are of minor importance. The latter pathway may have gained significance during the evolution of higher eukaryotes and was also recruited for V(D)J recombination. The reason for this reversed relative preference in yeast and mammalian cells may have a kinetic imperative associated with the diminished accessibility of the homologous chromosome in higher eukaryotic cells. One could also argue that haploid yeast cells resemble mammalian germ line cells more than somatic cells and the most efficient mechanism in preventing genetic alteration (that is also found in mammalian meiotic cells) should be the preferred one here. Additionally, the increased fraction of noncoding DNA in mammalian cells as compared with yeast results in a reduced probability of a potentially fatal intrachromosomal deletion of coding sequence during direct break fusion.

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