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Mutations in Two Ku Homologs Define a DNA End-Joining Repair Pathway in *Saccharomyces cerevisiae*

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DNA double-strand break (DSB) repair in mammalian cells is dependent on the Ku DNA binding protein complex. However, the mechanism of Ku-mediated repair is not understood. We discovered a *Saccharomyces cerevisiae* gene (*KU80*) that is structurally similar to the 80-kDa mammalian Ku subunit. Ku80 associates with the product of the *HDF1* gene, forming the major DNA end-binding complex of yeast cells. DNA end binding was absent in *ku80*Δ, *hdf1*Δ, or *ku80*Δ *hdf1*Δ strains. Antisera specific for epitope tags on Ku80 and Hdf1 were used in supershift and immunodepletion experiments to show that both proteins are directly involved in DNA end binding. In vivo, the efficiency of two DNA end-joining processes were reduced >10-fold in *ku80*Δ, *hdf1*Δ, or *ku80*Δ *hdf1*Δ strains: repair of linear plasmid DNA and repair of an HO endonuclease-induced chromosomal DSB. These DNA-joining defects correlated with DNA damage sensitivity, because *ku80*Δ and *hdf1*Δ strains were also sensitive to methylmethane sulfonate (MMS). Ku-dependent repair is distinct from homologous recombination, because deletion of *KU80* and *HDF1* increased the MMS sensitivity of *rad52*Δ. Interestingly, *rad50*Δ, also shown here to be defective in end joining, was epistatic with Ku mutations for MMS repair and end joining. Therefore, Ku and Rad50 participate in an end-joining pathway that is distinct from homologous recombinational repair. Yeast DNA end joining is functionally analogous to DSB repair and V(D)J recombination in mammalian cells.

DNA repair is essential for the successful maintenance and propagation of genetic information. Chromosomal double-strand breaks (DSBs) may occur spontaneously, during DNA recombination events, or may be induced by DNA damage. In eukaryotes, two major DSB repair pathways have been identified that differ in the requirements for DNA homology. DSB repair by homologous recombination results in the precise repair of the DNA lesion but requires the presence of homologous sequences elsewhere in the genome (e.g., a homologous chromosome or a sister chromatid). This is the primary mechanism of DSB repair in yeast species and prokaryotes. In contrast, higher eukaryotes appear to favor a nonhomologous DNA end-joining pathway for DSB repair. In this case, the two ends of a DSB are joined by a process that is largely independent of terminal DNA sequence homology and that therefore produces junctions that can vary in their sequence composition. In mammalian cells, DSBs are generated as intermediates in immunoglobulin or T-cell receptor gene rearrangement [V(D)J recombination] and are the potentially lethal DNA lesions caused by ionizing radiation (IR) (44). The DNA sequence of these repaired DSBs indicates that the latter pathway is utilized.

The three subunits of the DNA-dependent protein kinase (DNA-PK) are needed for both IR repair and V(D)J recombination, as demonstrated by a series of mutational and biochemical experiments. The DNA binding subunit of DNA-PK, named Ku, is a heterodimer of 70- and 80-kDa subunits (Ku70 and Ku80, respectively). Ku avidly binds to DNA ends and distortions in the DNA helix, consistent with a direct role in DSB repair (6, 12, 26–28). Cell mutants containing Ku defects are IR sensitive, do not complete V(D)J recombination, and are deficient in Ku-mediated DNA end-binding activity (8, 31, 41, 42). A third protein that associates with Ku, the DNA-PK catalytic subunit (DNA-PKCS), is deficient in the rodent *scid* and *V-3* mutants, which are characterized by similar IR repair and V(D)J recombination defects (7, 10, 16, 21, 23, 29). Thus, it has been conjectured that Ku has a direct role in DSB repair by associating with DSBs in a complex with DNA-PKcs.

In *Saccharomyces cerevisiae*, the principal DSB repair mechanism requires a group of at least 11 genes comprising the *RAD52* epistasis group, including *RAD50* to *RAD58*, *XRS2*, and *MRE11* (1, 11, 19, 30). *RAD52* epistasis group proteins function in a recombinational repair pathway in which DSBs are precisely repaired by utilizing DNA homology between recombining sequences. Mutations in any of these genes result in sensitivity to agents that cause DSBs, such as ionizing radiation (IR) or methylmethane sulfonate (MMS), with only minor cross-sensitivity to agents that generate other types of DNA lesions. Whereas *rad52* mutant strains are deficient in mating type switching, meiotic and mitotic recombination in parallel to DNA DSB defects, other group members such as *RAD50*, *MRE11*, and *XRS2* show deficiencies in only a subset of the *RAD52*-dependent recombination events (30).

S. cerevisiae has a heterodimeric yeast DNA end-binding complex that is biochemically similar to that of mammalian Ku. One subunit, Hdf1, is a protein that is structurally related to Ku70 (13). However, the function of a Ku-like complex in yeast cells is poorly understood. Here we have identified a yeast structural homolog of the Ku80 gene. We show that the yeast Ku80 and Hdf1 together form the Ku DNA end-binding complex. Null mutations in either gene disrupt DNA end binding in vitro and are deficient in end joining in vivo. In addition, the Ku mutations confer sensitivity to MMS epistatic to *RAD50*, but not *RAD52*. We suggest that Hdf1 and Ku80 function

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference[a]
DWY83	*MAT*α *arg4-RV leu2-3,112 ura3-52 trp1-289 cyh*^{R}	R. Kolodner, Harvard (RKY1734)
DWY97	*MAT*α *rad52*Δ::*ura3 arg4-RV leu2-3,112 ura3-52 trp1-289 cyh*^{R}	25
DWY171	*MAT***a** *his3*Δ*1 ade2 ura3-52 trp1-289 cyh*^{S}	R. Kolodner, Harvard (RKY1730)
DWY206	*MAT***a** *rad52*Δ::*ura3 ade2 leu2-3,112 trp1-289 his3*Δ1 *cyh*^{S}	This study
DWY255	*MAT*α *hdf1*Δ::*TRP1 arg4-RV leu2-3,112 ura3-52 trp1-289 cyh*^{R}	This study
DWY257	*MAT***a** *hdf1*Δ::*TRP1 his3*Δ*1 ade2 ura3-52 trp1-289 cyh*^{S}	This study
DWY277	*MAT*α *hdf1*Δ::*TRP1 rad52*Δ::*URA3 arg4-RV leu2-3,112 ura3-52 trp1-289 cyh*^{R}	This study
DWY286	*MAT*α *rad50*::*hisG arg4-RV leu2-3,112 ura3-52 trp1-289 cyh*^{R}	This study
DWY290	*MAT***a** *hdf1*Δ::*TRP1 arg4-RV leu2-3,112 ura3-52 trp1-289 cyh*^{R}	This study
DWY291	*MAT*α *arg4-RV leu2-3,112 ura3-52 trp1-289 cyh*^{R}	This study
DWY292	*MAT*α *ku80*Δ::*URA3 trp1 ade2 ura3-52 arg4-RV leu2-3,112*	This study
DWY293	*MAT***a** *ku80*Δ::*URA3 hdf1*Δ::*TRP1 trp1 ade2 ura3-52 arg1-RV leu2-3,112*	This study
DWY294	*MAT*α *hdf1*Δ::*TRP1 rad52*Δ::*ura3 arg4-RV leu2-3,112 ura3-52 trp1-289 cyh*^{R}	This study
DWY298	*MAT***a**/α *ku80*Δ::*ura3*/*KU80 hdf1*Δ::*trp1*/*HDF1 ura3-52 trp1-289*	This study
DWY304	*MAT*α *hdf1*Δ::*TRP1 rad50*::*hisG-URA3-hisG arg4-RV leu2-3,112 ura3-52 trp1-289 cyh*^{R}	This study
DWY306	*MAT*α *ku80*Δ::*ura3 trp1 ade2 ura3-52 arg4-RV leu2-3,112*	This study
DWY315	*MAT*α *ku80*Δ::*ura3 hdf1*Δ::*TRP1 trp1 ade2 ura3-52 arg4-RV leu2-3,112*	This study
DWY323	*MAT*α *ku80*Δ::*ura3 rad52*Δ::*ura3 trp1 ade2 ura3-52 arg4-RV leu2-3,112*	This study
DWY325	*MAT*α *ku80*Δ::*ura3 rad50*Δ::*ura3 trp1 ade2 ura3-52 arg4-RV leu2-3,112*	This study
DWY327	*MAT***a** *hdf1*Δ::*TRP1 arg4-RV leu2-3,112 ura3-52 trp1-289 cyh*^{R} *pHDF1-FLU*(*URA3*)	This study
DWY328	*MAT*α *ku80*Δ::*ura3 trp1 ade2 ura3-52 arg4RV leu2-3,112* pKU80-MYC (*LEU2*)	This study
DWY331	*MAT*α *ku80*Δ::*ura3 hdf1*Δ::*TRP1 arg4-RV leu2-3,112 ura3-52 trp1-289* pHDF1-FLU (*URA3*) pKU80-MYC (*LEU2*)	This study

[a] Previous strain designations are displayed in parentheses.

together in a distinctive DSB repair pathway involving the Rad50 gene product.

MATERIALS AND METHODS

Propagation of yeast cells and mutant constructions. Yeast cells were grown at 30°C in YPD or SC liquid or solid (2% agar) medium (38). Diploids were sporulated in liquid medium at 30°C by overnight growth in YPA (38) followed by 3 days of growth in 1% potassium acetate, and tetrads were dissected by standard genetic methods. Yeast transformation was done as previously described (14, 18).

All strains used in this study are isogenic derivatives of DWY83 and DWY171 that are both of the MGD background (Table 1). *hdf1*Δ strains DWY255 and DWY257 were constructed by a single-step gene disruption (34) of DWY83 and DWY171 with a genomic fragment of *HDF1* (bp 573 to 2534) (13) in which the *TRP1* gene has replaced almost all of the *HDF1* open reading frame (ORF). Wild-type diploid (DWY224) and *hdf1*Δ/*hdf1*Δ (DWY259) strains were created by mating DWY83 × DWY171 and DWY255 × DWY257, respectively. Heterozygous (*hdf1*Δ/*HDF1*) diploids were formed by mating DWY83 and DWY255 prior to introduction of *ku80* mutations in case the null mutation was lethal.

Heterozygous diploid *ku80*Δ strains were formed by gene disruption methods with PCR-generated targeting DNA as described previously (5). For PCR, 60-mer oligonucleotides containing 20 bases of 5′ and 3′ pSKII (Bluescript) sequence and 40 residues complementary to specific regions 5′ and 3′ to the 9718_5 ORF were synthesized. The 5′ primer was 5′-CTAGATTACCGCATGTCCGT CAGGGCATTTGTTGTCATGCAATTAACCCTCACTAAAGGG-3′, and the 3′ primer was 5′-TTAGACCTTTTTTAATTATTGCTATTGTTTGGACTTCC CCTAATACGACTCACTATAGGG-3′; underlines denote pSKII sequences flanking the URA3 promoter and polyadenylation signal. PCR was conducted on the pSKII-URA3 plasmid containing the entire *URA3* gene, including the promoter and polyadenylation signal. The *URA3* PCR product was then transformed into DWY83 × DWY255 (HDF1/*hdf1*Δ) diploids and Ura$^+$ transformants selectively grown on SC-Ura media. The DNA integration pattern was confirmed in approximately 50% of the transformants to be at the 9718_5 ORF by restriction digestion and Southern hybridization with a *URA3* DNA probe.

*rad52*Δ derivatives DWY97 and DWY277 were constructed as described previously by integration of pTM42 (25). DWY323 was also derived by integration of pTM42 into DWY306. Ura$^-$ clones of DWY277 and DWY323 were selected on plates containing 5-fluoroorotic acid to produce DWY294 and DWY323 respectively. DWY206 is the result of mating and sporulation of strains DWY97 and DWY171. *rad50*Δ strains, DWY286, DWY304, and DWY325 were constructed by single-step gene disruptions in DWY83, DWY255, and DWY306, respectively, with a *rad50*::(*hisG-URA3-hisG*) deletion plasmid (pNKY83 [gift from E. Alani]) (2). 5-Fluoroorotic acid selection then yielded Ura$^-$ DWY325 cells.

HDF1 and *KU80* ORFs were recovered from the yeast genome by PCR with primers at N- and C-terminal positions. Epitope-tagged derivatives of *KU80* and *HDF1* were prepared by PCR by standard methods. PCR primers 500 bp upstream of the translation initiation site for *KU80* and *HDF1* were used in reactions with primers composed of the C-terminal four amino acids of *KU80* and *HDF1* followed by the 9E10 and 12CA5 epitope-encoding sequences respectively. PCR products were subcloned into the pDB20 (*URA3*) or pDBL (*LEU2*) yeast expression vectors (25).

DNA end binding. Five-milliliter late-log-phase cultures were produced by growth overnight at 30°C in YPD, SC-Ura, SC-Leu, or SC-Ura-Leu medium according to the strain genotypes and biosynthetic markers on introduced plasmids (Table 1). Protein extracts were made by glass bead-mediated cell disruption (15) in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.9]), 5 mM EDTA, 1 mM dithiothreitol, 500 mM KCl. DNA end binding was performed for 5 min at room temperature in 10-μl reaction mixtures containing 20 mM Tris (pH 7.5), 10 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1 μg of covalently closed circular DNA (pRc-CMV; Invitrogen), 0.2 to 1 μg of yeast extract, and 1 ng of a ^{32}P-labeled 159-bp *Xma*I-*Pvu*II fragment of pJH290 (8). End-binding reaction mixtures were fractionated on a 5% polyacrylamide gel (acrylamide-bisacrylamide, 30:0.8) in 50 mM Tris (pH 8.5)–380 mM glycine–10 mM EDTA. The gels were dried and exposed to X-ray film.

To assess the involvement of epitope-tagged Ku80 and Hdf1 proteins in DNA end-binding complexes, two methodologies were employed. The monoclonal antibody (MAb) 12CA5 recognizes the YPYDVPDYAR epitope from *Haemophilus influenzae* antigen and 9E10 recognizes the EQKLISEEDL epitope from c-Myc. Ascites fluid from 12CA5 (1 μl) was mixed with end-binding reaction mixtures, prepared as described above, and fractionated in a 5% polyacrylamide gel to visualize MAb-specific supershifting. Alternatively, 5-μg protein extracts were immunodepleted with 1 μl of either 12CA5 or 9E10 MAb combined with 25 μl of protein A-Sepharose at 4°C for 1 h. Immunodepleted extracts were then used in DNA end-binding assays as described above.

MMS sensitivity assays. Clonogenic cell survival after MMS treatment was determined as described previously (25). Three independent colonies per strain were picked into sterile water and serially diluted 1:5 for six cycles. Five-microliter aliquots were plated in duplicate on YPD plates. In each experiment, one plate served as a control to calculate the total viable colony number, while additional plates were subjected to experimental conditions. Cells were plated directly onto solid media containing low concentrations of MMS as indicated in the figure legends. Colonies formed on the control and MMS-containing plates were counted after 3 days of incubation at 30°C, and the surviving fraction was calculated.

For complementation of MMS sensitivity of Ku-deficient strains, we introduced epitope-tagged versions of these Ku genes by yeast transformation. SC-Ura, SC-Leu, and SC-Ura, Leu plates with or without 0.01% MMS were used to evaluate complementation of DWY306, DWY255, and DWY315 respectively.

DNA end joining. A replicating yeast plasmid, pRS316 (*ARSH4 CEN6 URA3* [40]), was modified by insertion of a 0.5-kb *Bam*HI fragment from pRc-CMV. The resulting plasmid, pRS316B, was linearized with or without *Bam*HI digestion, and the 4.9-kb pRS316 fragment was isolated by gel electrophoresis and then bound to

glass beads. This protocol enhanced the isolation of *Bam*HI-linearized DNA instead of uncut plasmid. Approximately 300 ng of linearized pRS316 was mixed with 50 or 100 ng of uncut pDBL (2 μm *LEU2* [25]). Alternatively, pRS316 was linearized with *Pvu*II and used similarly. Yeast strains were transformed as described above and plated on SC-Ura and SC-Leu plates to test for plasmid repair and control for transformation efficiency, respectively. Colonies were counted after incubation at 30°C for 3 days. Transformation efficiency was 1×10^5 to 5×10^5 transformants per μg of pDBL for all strains. To assess end-joining events, plasmids from pools of 50 to 200 transformants were recovered by yeast minipreps and transferred into *Escherichia coli* DH5α for further analysis. Restoration of an intact pRS316 plasmid was evaluated with ampicillin selection plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) (4). Blue colonies denoted the precise reconstitution of a *Bam*HI site during end joining. White colonies indicated either alteration of DNA sequence at the joined ends or the presence of contaminants from pRS316B. To distinguish between these possibilities, colonies were either screened by hybridization with ^{32}P-labeled 0.5-kb *Bam*HI pRc-CMV insert fragment (4), or we performed DNA miniprep analysis and *Bam*HI digestion. Colonies yielding plasmids that were digestible with *Bam*HI and that gave the same restriction pattern as pRS316 were scored as precise end-joining products. End-joining frequencies were then normalized to account for the level of occurrence of these products.

Mating type switching. Mating type switching, the repair of the HO endonuclease-induced DSB by gene conversion, involves Rad52 and requires sequences homologous to *MAT* at *HML* and *HMR*. Yeast *rad52*Δ strains containing additional mutations in the *KU* genes were transformed with pGAL:HO (17), containing the HO gene under the control of the *GAL10* promoter, and selected on SC-Ura plates. Three independent transformants of each strain were grown overnight at 30°C in SC-Ura medium with 1% acetate as the carbon source. Titers of cultures on SC-Ura glucose (2%) and SC-Ura sucrose-galactose (2%) plates were then determined. Sensitivity to HO-induced breaks at *MAT* results in a reduced plating efficiency on HO-inducing, sucrose-galactose plates relative to that on SC-Ura glucose plates.

Mating type switching was also assessed after a pulse of HO expression. Transformants were grown overnight in SC-Ura acetate medium, pelleted, washed, induced in SC-Ura sucrose-galactose medium for 30 min at 30°C, mixed with mating tester strains, and plated on SC plates to select for diploids. Switched cells were identified as those having a mating preference opposite to that of the parental strain.

RESULTS

Definition of Ku genes from *S. cerevisiae*. Hdf1, a Ku-related protein from the yeast *S. cerevisiae*, was previously identified by the biochemical purification of an in vitro DNA end-binding complex (13) similar to that of mammalian and *Drosophila melanogaster* Ku (reviewed in reference 44). Hdf1 was associated with another protein in this complex that was not further characterized (13). By reduced-stringency searching of the *S. cerevisiae* DNA genome database, we discovered a candidate ORF bearing limited homology to mammalian Ku80 subunit genes. ORF 9718_5 (GenBank accession number Z49702) on chromosome 13 encodes a putative 629-amino-acid protein with a predicted molecular mass of 74.5 kDa (Fig. 1). On the basis of the predicted amino acid sequence, the *S. cerevisiae* ORF 9718_5 protein (Ku80) is 21 and 20.5% homologous and 45.9 and 43.8% similar to the human and murine Ku80 proteins, respectively (Fig. 1 [murine Ku80 protein not shown]). This homology extends across the length of the ORF, and essentially all of the identified homology between *S. cerevisiae* Ku80 and human Ku80 is conserved with mouse Ku80. Since the murine and human Ku80 genes are highly homologous, it is not surprising that the degree and positioning of similarity are conserved compared with those of the yeast gene. Interestingly, an uncharacterized *Caenorhabditis elegans* ORF (GenBank number S43606; R07E5.8) also showed homology to the two mammalian Ku80 genes and ORF 9718_5 and may be yet another homolog (data not shown). On the basis of these similarities, we evaluated whether ORF 9718_5 had the properties of a Ku gene.

***HDF1* and *Ku80* are required for DNA end binding.** To examine the role of *HDF1* and ORF 9718_5 in *S. cerevisiae*, congenic strains were constructed that had either or both genes deleted. *HDF1* coding sequences were replaced with the selectable *TRP1* gene and transplaced into the genome by homologous recombination to generate *hdf1*Δ strains (Table 1 and Materials and Methods). Similarly, ORF 9718_5 coding sequences were precisely replaced with the selectable *URA3* gene by a PCR-based method and integrated into the genome of a diploid strain heterozygous for *hdf1*Δ (Materials and Methods). Null mutations created by deletion of the 9718_5 ORF were provisionally designated *ku80*Δ. Sporulation of the resulting strain heterozygous for both *hdf1*Δ and *ku80*Δ (DWY299) produced wild-type, *ku80*Δ, *hdf1*Δ, and *ku80*Δ *hdf1*Δ progeny. We examined these haploid strains for the biochemical properties previously attributed to Ku.

DNA end-binding activity was measured in protein extracts derived from wild-type (DWY83) and *hdf1*Δ (DWY257) strains as well as four haploids from DWY298 tetrads (DWY290 to DWY293). Specific binding to DNA ends is visualized by the gel electrophoretic retardation of a radiolabeled 159-bp fragment in the presence of excess supercoiled plasmid DNA as previously described (9). Wild-type (DWY83) extracts showed normal DNA end binding as demonstrated by a single, major DNA end-binding complex (Fig. 2A). Each of the *ku80*Δ (DWY292 and DWY306), *hdf1*Δ (DWY290, DWY255, and DWY257), and *hdf1*Δ *ku80*Δ (DWY293 and DWY315) extracts had no detectable DNA end-binding complexes (Fig. 2A and data not shown). Therefore, DNA end binding is strictly dependent upon *KU80* and *HDF1*. As previously documented for *HDF1*, *KU80* indeed encodes a protein functionally related to Ku.

Hdf1 and Ku80 are present in the same DNA end-binding complex. To examine whether Hdf1 and Ku80 are found in the same DNA end-binding complex, the Hdf1 gene product was tagged at the C terminus by creation of a fusion protein with the *H. influenzae* protein epitope (FLU) enabling recognition by the MAb 12CA5 (Materials and Methods). Likewise, the Ku80 protein was tagged at its C terminus by creation of a fusion protein with a protein epitope from c-Myc (MYC) recognized by the MAb 9E10. Genes encoding the Ku fusion proteins were then cloned into vectors containing the constitutively active *ADH1* promoter for expression in yeast cells. DWY255 (*hdf1*Δ) transformed with *HDF-FLU* fully restored DNA end binding (Fig. 2B, lane 6, and 2C, lane 5), whereas introduction of a vector control, pDB20, did not increase levels of DNA end binding above that of the parental mutant (Fig. 2C, lane 4). Similarly, DWY306 (*ku80*Δ) transformed with *KU80-MYC* or *KU80*, but not the vector control pDBL, reconstituted DNA end-binding activity (Fig. 2B, lane 4, and data not shown).

DNA end binding of a *hdf1*Δ *ku80*Δ double mutant strain (DWY315) was fully restored by coexpression of *HDF-FLU* and *KU80-MYC* (Fig. 2B, lane 2). In contrast, the double mutant strain transformed with either *HDF-FLU* or *KU80-MYC* separately or the pDB20 and pDBL vectors only did not increase the level of DNA end binding relative to that of the parental mutant background (Fig. 2B, lanes 10 and 11). Thus, coexpression of Ku80 and Hdf1 is necessary to reconstitute DNA end binding. We have previously found that the mobility of DNA end-binding complexes containing Ku is dictated by the combination of Ku subunits and particularly the larger subunit of Ku (8). Interestingly, we observed that introduction of either the *HDF1-FLU* or *KU80-MYC* genes triggered an alteration in the mobility of the DNA end-binding complex (Fig. 2B). The Ku80-MYC complex had the greatest reduction in mobility relative to that of the wild-type or KU80-complemented cells (lane 4), although Hdf1-FLU was also found to retard the DNA end-binding complex (lane 6). Complexes containing both protein tags had approximately the same mo-

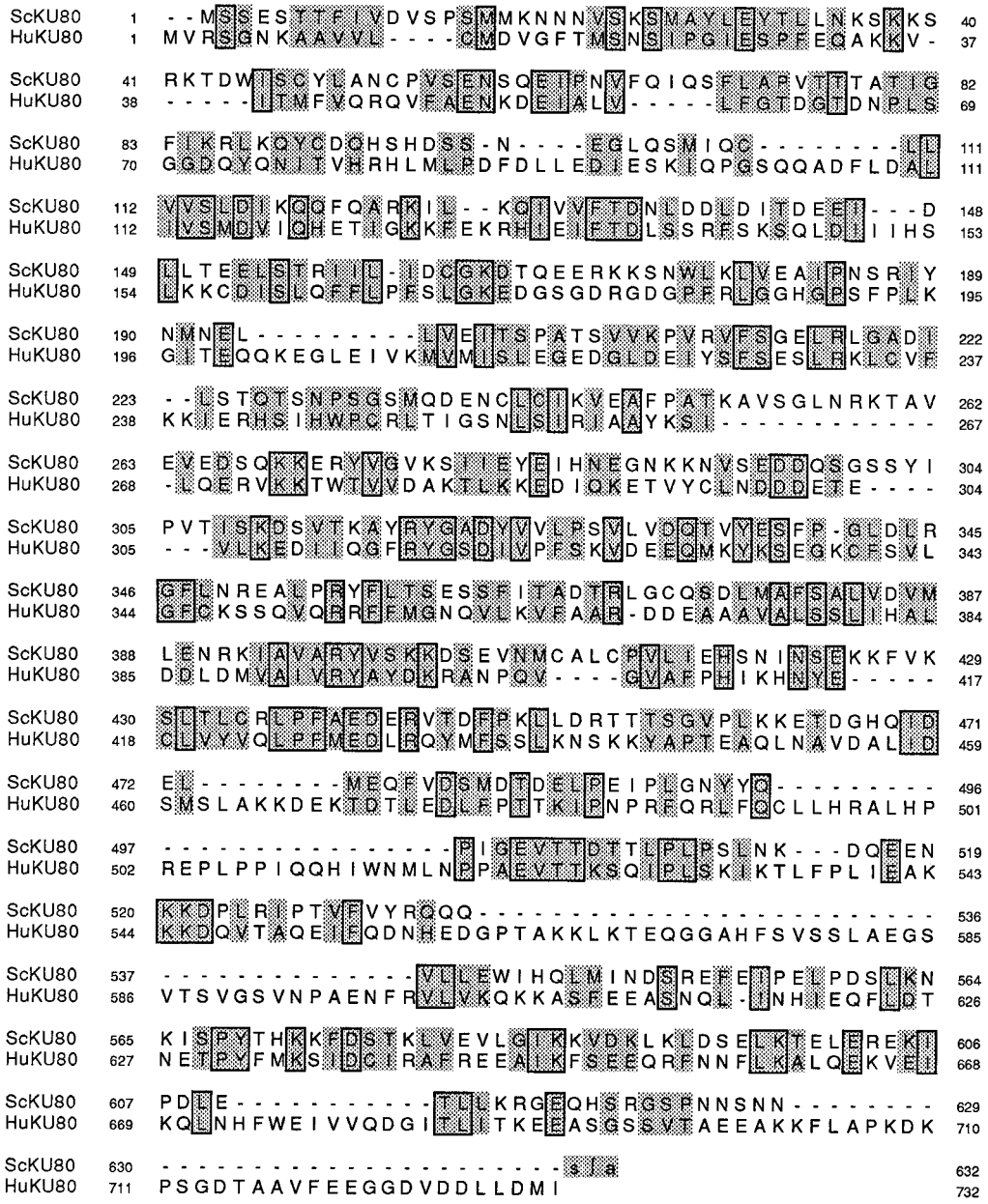

FIG. 1. Ku80 homolog gene of *S. cerevisiae*. Human (Hu) and *S. cerevisiae* (Sc) ORF 9718_5 were aligned by Pileup with amino acid similarities specified according to a GSE program within Pileup. Amino acid identities are displayed in boxes, and similarities are highlighted with shading.

bility as Ku80-MYC complexes (lane 2). Therefore, a slower mobility is indicative of the Ku80-MYC and Hdf1-FLU proteins in the specific DNA end-binding complexes. Since no other complexes of wild-type mobility were found in DWY315 extracts, we conclude that all of the DNA end-binding capacity of these extracts is contributed by the epitope-tagged Ku proteins in association with each other.

We previously observed with human Ku that MAbs against Ku added to the DNA end-binding reaction mixtures significantly reduce the mobility of the end-binding complex without influencing DNA binding itself (9). Therefore, the compositions of yeast DNA end-binding complexes were also assessed by antibody supershift experiments. Wild-type DNA binding complexes were unaffected by the presence of the antibody (Fig. 2C, lanes 1 and 2). In contrast, we found that Hdf1-FLU-containing complexes were supershifted with added 12CA5 MAb (lane 6). Supershifts with 12CA5 were also demonstrated with Hdf1-FLU/Ku80-MYC complexes (lanes 7 and 8). However, MAb 9E10 failed to cause supershifting of Ku80-MYC complexes under the conditions tested.

Specific antigenic epitopes can also be exploited to selectively remove or deplete proteins from an extract by immunoprecipitation with the cognate antisera. Immunodepletion of DNA end-binding complexes was performed with either 9E10 or 12CA5. We found that treatment of Ku80-MYC and/or Hdf1-FLU extracts with 9E10 or 12CA5, respectively, was able to significantly diminish the number of DNA end-binding complexes (Fig. 2D). Neither antibody had an effect on the DNA binding activity of untagged Ku proteins in DWY83 (wild-type) extracts (Fig. 2D). Importantly, essentially all of the detectable

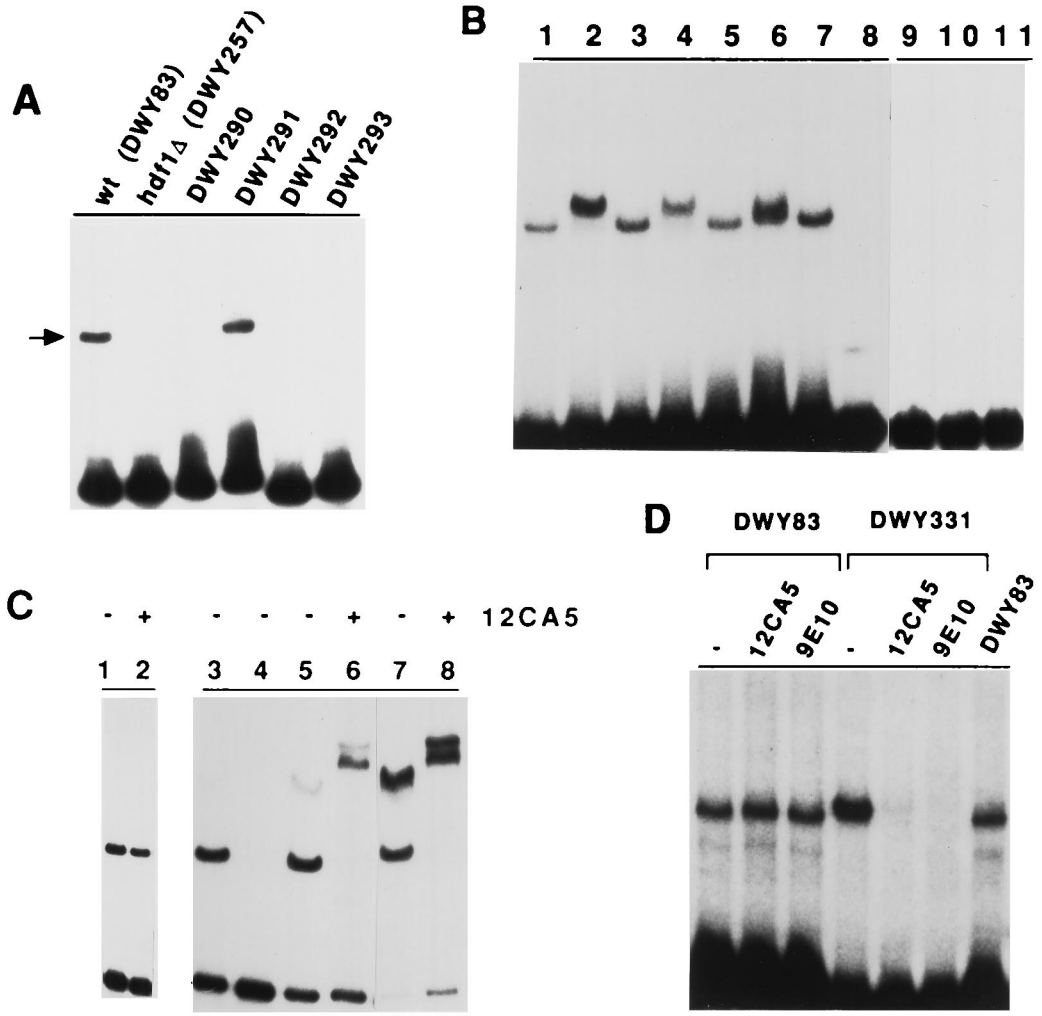

FIG. 2. DNA end binding in yeast cells is formed by the products of the two Ku gene homologs, *KU80* and *HDF1*. DNA end-binding assays were performed to evaluate Ku in the yeast protein extracts of various mutant strains according to Materials and Methods. (A) DNA end binding for Ku-deficient strains as described in Materials and Methods. DWY290 to DWY293 are haploids from sporulation of a *ku80*Δ/*KU80 hdf1*Δ/*HDF1* diploid (Table 1): DWY83, wild type (wt); DWY257, *hdf1*Δ; DWY290, *hdf1*Δ; DWY291, wild type; DWY292, *ku80*Δ; DWY293, *ku80*Δ *hdf1*Δ. The arrow denotes the Ku end-binding complex. (B) Restoration of DNA end binding and relative electrophoretic mobility by epitope-tagged Ku subunits. Lanes 1, 3, 5, and 7, DWY83 (wild type) loaded in alternating lanes to show the wild-type Ku DNA end-binding complex mobility; lane 2, *ku80*Δ *hdf1*Δ (DWY315) transformed with KU80-MYC and HDF1-FLU; lane 4, *ku80*Δ (DWY306) transformed with KU80-MYC; lane 6, *hdf1*Δ (DWY255) transformed with HDF1-FLU; lane 8, no protein added to reaction mixture. In a second gel shift experiment, DWY315 samples transformed with the pDB20 vector (lane 9), KU80-MYC (lane 10), or HDF1-FLU (lane 11) were compared. (C) Supershifting of DNA end-binding complexes from *hdf1*Δ and *ku80*Δ mutants with anti-FLU antibody. Lanes 1 and 2, wild type (DWY83) with or without MAb 12CA5 (anti-FLU); lane 3, wild type (DWY83); lane 4, *hdf1*Δ (DWY255) plus pDB20 vector; lanes 5 and 6, *hdf1*Δ (DWY255) plus HDF1-FLU without or with MAb 12CA5; lanes 7 and 8, *ku80*Δ *hdf1*Δ plus HDF1-FLU plus KU80-MYC (DWY331) without and with 12CA5. (D) DNA end binding after immunodepletion of epitope-tagged proteins. MAb 12CA5 or anti-Myc MAb 9E10 was mixed with extracts according to Materials and Methods. After removal of immunoprecipitation complexes, DNA end-binding reaction mixtures were processed as described above. Lanes 4 to 6 are the *ku80*Δ *hdf1*Δ plus HDF1-FLU plus KU80-MYC strain (DWY331). Lanes 1 to 3 and 8 are the wild-type strain (DWY83). Additions of volumes of 12CA5 or 9E10 are as noted.

DNA end-binding complexes were immunodepleted and/or supershifted. These observations indicate that Ku80p and Hdf1p are associated components of the major DNA end-binding complex.

Ku is required for the repair of MMS-induced DNA lesions. Since Ku is considered to be important for mammalian DSB repair mechanisms, we examined whether DNA damage repaired through DSB intermediates in yeast cells might also be Ku dependent. We examined the wild-type and mutant haploid products from DWY298 for MMS hypersensitivity phenotypes. Wild-type (DWY291), *ku80*Δ (DWY292), *hdf1*Δ (DWY290), and *ku80*Δ *hdf1*Δ (DWY293) haploids were plated on YPD plates with and without 0.01% MMS, and the number of colonies appearing after 3 days on MMS plates was normalized to the total number of colonies on control plates as previously described (25). We found that deletion of *KU80* or *HDF1* sensitizes strains to MMS-induced DNA damage (Fig. 3A), implicating the products of these genes in MMS repair. Importantly, the *ku80*Δ *hdf1*Δ double mutant strain (DWY293) had a sensitivity to MMS equal to that of the single mutants. Thus, mutations in *KU80* and *HDF1* are epistatic with regard to MMS repair. The hypersensitivity of *hdf1*Δ strains to MMS was additionally demonstrated with a liquid assay in which cells are exposed to a fixed concentration of MMS for various periods of time (data not shown). Although the repair of MMS-induced lesions is significantly impaired in the absence of Ku80

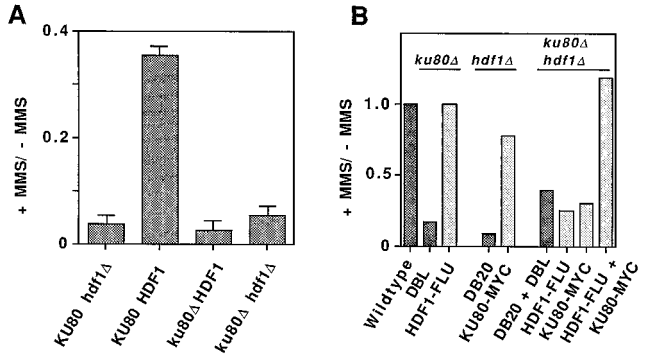

FIG. 3. MMS repair defects of Ku single and double mutant strains and complementation by Ku genes. *ku80*Δ/*KU80 hdf1*Δ/*HDF1* diploids were sporulated, and haploids were examined for MMS sensitivity. (A) MMS repair was measured in a clonogenic cell survival assay by plating of cells on YPD or 0.01% MMS plates (Materials and Methods). The fraction of day 3 surviving cells is plotted, and standard deviations are shown with error bars. The strain genotypes shown are as follows: DWY290, *hdf1*Δ *KU80*; DWY291, *KU80 HDF1*; DWY292, *ku80*Δ *HDF1*; and DWY293, *ku80*Δ *hdf1*Δ. (B) MMS repair after introduction of epitope-tagged Ku subunits. Ku fusion genes containing epitope tags (Fig. 2) were introduced into Ku mutant strains. MMS sensitivity (0.01%) was compared for *ku80*Δ, *ku80*Δ plus KU80-MYC, *hdf1*Δ, *hdf1*Δ plus HDF1-FLU, *ku80*Δ *hdf1*Δ, *ku80*Δ *hdf1*Δ plus KU80-MYC alone, *ku80*Δ *hdf1*Δ plus HDF1-FLU alone, and *ku80*Δ *hdf1*Δ plus KU80-MYC plus HDF1-FLU as shown. DBL and DB20 refer to introduction of either of these two vectors only into each strain for comparison. Values were normalized to the MMS repair compared with that for a wild-type strain (DWY83). Light shading refers to strains in which epitope-tagged proteins were introduced.

and Hdf1, the defects are less profound than those of mutants defective in homologous recombination. For instance, an isogenic *rad52*Δ strain exhibits much greater sensitivity to MMS by either assay (25).

We also evaluated whether the restoration of DNA end binding by epitope-tagged Ku proteins paralleled the reconstitution of MMS repair in these strains. The *ku80*Δ strain, DWY306, and the *hdf1*Δ strain, DWY255, were transformed with *KU80-MYC* (*URA3*) and *HDF1-FLU* (*LEU2*) plasmids respectively. Ura^+ and Leu^+ colonies were tested for MMS sensitivity as described above, except the cells were plated on SC-Ura or SC-Leu plates. We observed that MMS repair was fully restored by an epitope-tagged Ku80 or Hdf1 protein (Fig. 3B). Likewise, the double mutant, DWY315, was cotransformed with *KU80-MYC* and *HDF1-FLU*, together or separately, and tested for MMS sensitivity. Expression of both proteins was necessary to restore MMS repair to the double mutant (Fig. 3B), consistent with a functional role of the DNA end-binding Ku complexes.

DNA end joining is deficient in Ku mutant yeast cells. In mammalian cells, Ku is required for V(D)J recombination as well as IR repair (44). Although the molecular basis of the IR repair mechanism is poorly understood, V(D)J recombination products form with the use of little or no DNA homology at the recombination junctions. Therefore, Ku may also be expected to play a role in other forms of illegitimate recombination in other organisms that utilize little or no DNA homology. Nonhomologous DNA end joining occurs in *S. cerevisiae* (24, 35, 36, 43). To prepare a substrate for end joining, pRS316B (*URA3*) was linearized in the polylinker region by *Bam*HI digestion. We ensured linearization at the two *Bam*HI sites by excision of a 0.5-kb *Bam*HI fragment from this vector and gel purification of the pRS316-Bam vector backbone. Singly cut or undigested substrates will yield apparent products containing the 0.5-kb *Bam*HI insert and can be identified by restriction enzyme digestion analysis or colony hybridization (Materials and Methods). The site of the *Bam*HI-induced DSB does not have homology with the yeast genome and should be repaired by homology-independent mechanisms. *Bam*HI-digested pRS316 was cotransformed into wild-type and mutant strains with pDBL (*LEU2* [25]) to serve as a control for transformation efficiency. Yeast transformations were plated on SC-Ura plates to select for pRS316 recircularization and onto SC-Leu plates to control for transformation efficiency. Plasmid integration events are not expected to be recovered, as they would generate dicentric chromosomes resulting in mitotic catastrophe and cell death. We found that strains with one or both Ku genes deleted had approximately the same transformation efficiency as that of the wild type (1×10^4 to 3×10^4 Leu^+ colonies per μg of pDBL DNA). Double mutant strains of Ku deletions with either *rad52*Δ or *rad50*Δ caused an additional 10-fold decrease in transformation efficiency, and thus all end-joining results were normalized to the transformation efficiency of circular DNA in the same experiments.

TABLE 2. DNA end-joining defects of Ku mutants

Strain	Genotype	Relative end-joining efficiency (*n*)[a]	Fold decrease from wild type
DWY83	Wild type	1.000 (5)	1
DWY255	*hdf1*Δ	0.073 ± 0.005 (3)	14
DWY306	*ku80*Δ	0.100 ± 0.03 (3)	10
DWY293	*hdf1*Δ *ku80*Δ	0.093 ± 0.05 (3)	11
DWY97	*rad52*Δ	0.358 ± 0.099 (4)	3
DWY294	*hdf1*Δ *rad52*Δ	0.069 ± 0.026 (3)	14
DWY323	*ku80*Δ *rad52*Δ	0.090 ± 0.04 (3)	11
DWY286	*rad50*Δ	0.081 ± 0.058 (5)	12
DWY304	*hdf1*Δ *rad50*Δ	0.076 ± 0.012 (3)	13
DWY325	*ku80*Δ *rad50*Δ	0.12	8

[a] Relative end-joining efficiency was calculated on the basis of the ratio of pDBL (uncut) to pRS316 (*Bam*HI-linearized) transformants. Values are expressed relative to that of the wild type (DWY83). Values represent the mean (± standard deviation) of *n* independent transformations.

pRS316 end joining was efficient in wild-type cells, yielding a transformation efficiency of 40 to 80% relative to that of the uncut plasmid. This value was normalized to 1.0 for comparison with isogenic mutant strains (Table 2). We found that *hdf1*Δ, *ku80*Δ, or *ku80*Δ *hdf1*Δ strains exhibited a 10- to 14-fold decrease in the transformation efficiency with *Bam*HI-digested pRS316. The *ku80*Δ *hdf1*Δ strain was no more defective in end joining than either single mutant, indicating an epistatic relationship for DNA end joining. Also, the DNA end-joining deficiencies of Ku mutants were not restricted to particular types of DNA ends, because *Pvu*II-digested pRS316 DNAs (CAGĈTG blunt ends) were also poorly repaired relative to those of a wild-type strain (data not shown). Deletion of *RAD52* (DWY97) decreased the recovery of Ura^+ clones by only two- to threefold, consistent with the design of this assay to examine nonhomologous repair events that should be *RAD52* independent. Similarly, double mutants of *rad52*Δ with either *ku80*Δ (DWY323) or *hdf1*Δ (DWY294) did not exhibit additional DNA end-joining defects beyond those conferred by the Ku mutations.

We also studied whether the distribution of DNA end-joining products was influenced by Ku mutations. Rejoining of restriction enzyme-digested ends can occur by precise ligation to regenerate a *Bam*HI site or can occur imprecisely, leading to loss of a *Bam*HI site and formation of novel joints. In wild-type, *hdf1*Δ, and *ku80*Δ strains, we examined clonal events by transformation of recovered plasmid products into *E. coli*. pRS316 encodes the α fragment of β-galactosidase such that precisely rejoined products will complement *E. coli lacZ* mu-

TABLE 3. Repair of HO-induced chromosomal DSBs[a]

Strain	Relevant genotype	HO-resistant survival (no. of colonies [10^{-3}])	Fold decrease[b]
DWY97	*rad52*Δ	1.60 ± 0.100	1
DWY323	*ku80*Δ *rad52*Δ	0.19 ± 0.120	8.4
DWY294	*hdf1*Δ *rad52*Δ	0.12 ± 0.075	13.3
DWY292	*ku80*Δ	900	NA
DWY255	*hdf1*Δ	1,080	NA
DWY83	Wild type	910	NA

[a] Yeast strains were transformed with pGAL:HO and selected on SC-Ura plates. Three independent transformants of each strain were grown in SC-Ura acetate medium and plated on SC-Ura glucose (HO repressed) and on SC-Ura sucrose-galactose (HO induced) media. Colony numbers were scored in terms of comparison between the two plating conditions.

[b] The effects of *ku80*Δ and *hdf1*Δ were assessed by comparison of the fold decrease in cell survival of the double mutants relative to the cell survival of the *rad52*Δ strain. NA, not applicable.

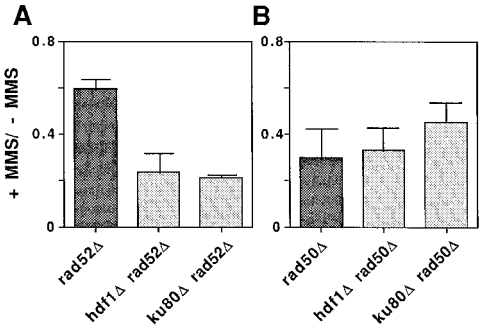

FIG. 4. Epistasis of Ku deletion mutations with *rad52*Δ and *rad50*Δ alleles for MMS repair. MMS repair was monitored as described in Materials and Methods for comparison of strains that were isogenic except for *KU*, *RAD52*, or *RAD50* null mutations (Table 1). (A) Cell survival frequencies on 0.00125% MMS-containing plates versus control plates for *rad52*Δ (DWY97), *hdf1*Δ *rad52*Δ (DWY294), and *ku80*Δ *rad52*Δ (DWY323) strains. (B) Cell survival frequencies on 0.0025% MMS-containing plates versus control plates for *rad50*Δ (DWY286), *hdf1*Δ *rad50*Δ (DWY304), and *ku80*Δ *rad50*Δ (DWY325) strains. Standard deviations are shown for two to four experiments.

tations, while imprecise junctions disrupt *lacZ* expression. Thus, the frequency of precise end joints was estimated on the basis of the fraction of blue ($LacZ^+$) colonies on plates containing the chromogenic substrate X-Gal. Plasmid DNAs from blue colonies were observed to be digested to linear DNA by *Bam*HI. Interestingly, *hdf1*Δ and *ku80*Δ strains yielded precise rejoining at lower frequencies than normal (10 to 30% relative to >90% for the wild type). These greater levels of imprecise rejoining were confirmed by demonstration of a high frequency of insensitivity of recovered plasmid DNAs to *Bam*HI. Therefore, Ku is necessary for plasmid end joining in yeast cells, and absence of Ku leads to increased levels of imprecise joining.

Ku is required for the nonhomologous repair of chromosomal DSBs. Chromosomal breaks initiated by HO endonuclease cleavage at the *MAT* locus are ordinarily repaired by homologous recombination in a *RAD52*-dependent gene conversion mechanism termed "mating type switching." In the absence of *RAD52*, these breaks are repaired by illegitimate recombination mechanisms that repair the chromosome but do not proceed to gene conversion products (22). Because many DSB repair mutants in yeast cells have concomitant defects in homologous recombination and mating type switching, the Ku mutants were examined for proficiency in these processes. *ku80*Δ and *hdf1*Δ mutants and wild-type strains (DWY306, DWY255, and DWY83) were transformed with pGAL:HO (*URA3*) carrying the structural gene for the HO endonuclease under the transcriptional control of the *GAL10* promoter. Exposure of the transformed cells to galactose induces HO expression, a DSB at *MAT*, and mating type switching. Plating of pGAL:HO transformants of wild-type, *ku80*Δ, and *hdf1*Δ strains on SC-Ura plates containing galactose gave wild-type viability (Table 3). To assess the efficiency of mating type switching, wild-type and *hdf1*Δ pGAL:HO transformants were transferred to galactose media for 30 min, mixed with both *MAT***a** and *MAT*α mating tester strains, and plated on SC glucose plates to select for mated diploids. *hdf1*Δ cells switched mating type from *MAT*α to *MAT***a** or from *MAT***a** to *MAT*α at wild-type levels. In comparison, a *rad52*Δ strain yielded plating efficiencies of less than 0.1% on SC-Ura sucrose-galactose, and no switched products were found. Therefore, deletion of *HDF1* does not impair mitotic gene conversion as assessed by mating type switching.

Ku may be needed for chromosomal end joining in the absence of homologous recombination. Therefore, we examined the ability of *ku80*Δ *rad52*Δ (DWY323) and *hdf1*Δ *rad52*Δ (DWY294) strains to repair an HO-induced DSB at *MAT*. Three independent pGAL:HO transformants of each strain were grown overnight in SC-Ura acetate medium and plated on SC-Ura glucose (HO repressed) and SC-Ura sucrose-galactose (HO induced). Deletion of *HDF1* or *KU80* reduced the frequency of HO-resistant survivors by 8- to 13-fold relative to that of a *HDF1 KU80 rad52*Δ strain (Table 3). Therefore, in the absence of homologous recombinational repair, yeast cells require a Ku-dependent pathway to repair chromosomal DSBs. We conclude that Ku is necessary for end-joining repair of both episomal and chromosomal DSBs.

Ku epistasis with other DNA repair genes. The DNA end-joining defects of *ku80*Δ and *hdf1*Δ mutants implicate Ku in a nonrecombinational DSB repair pathway that is distinct from that defined by previously characterized DNA repair mutants. However, it is possible that the yeast Ku proteins also play a role in the recombinational repair of DSBs. Several phenotypes were examined to distinguish Ku mutants from strains defective in homologous recombination. Unlike *RAD52* epistasis group mutants, *ku80*Δ, *hdf1*Δ, and *ku80*Δ *hdf1*Δ strains exhibited wild-type resistance to IR-induced damage. Similarly, deletion of HDF1 did not affect the frequency of spontaneous chromosome loss as is observed in several recombinational repair mutants (11a). Furthermore, diploid *hdf1*Δ/*hdf1*Δ, *ku80*Δ/*ku80*Δ, and *hdf1*Δ *ku80*Δ/*hdf1*Δ *ku80*Δ strains were characterized by wild-type levels of resistance to IR damage and full proficiency in sporulation and meiotic viability. Therefore, Ku does not have an essential function in IR repair, meiosis, or sporulation, in contrast to genes essential for homologous recombination. Also, *ku80*Δ, *hdf1*Δ, and *ku80*Δ *hdf1*Δ mutants were not hypersensitive to UV irradiation damage, and *hdf1*Δ strains had normal levels of UV-induced mutagenesis, indicating that UV repair and error-prone or replicative bypass mechanisms do not require Ku.

To confirm that Ku-mediated repair is genetically distinct from that defined by *RAD52*, the epistasis between these genes was explored by construction of a set of double mutants. *ku80*Δ *rad52*Δ and *hdf1*Δ *rad52*Δ double mutants (DWY323 and DWY294) were more sensitive to MMS than a *rad52*Δ single mutant strain (DWY97) (Fig. 4A). These double mutant strains were also characterized for defects in the repair of UV- and IR-induced DNA damage. Deletion of either HDF1 or KU80 had no effect on the IR and UV sensitivity of *rad52*Δ strains (data not shown). Therefore, the Ku heterodimer functions in a separable repair pathway from that of Rad52.

Mutation of *RAD50* disrupts some forms of illegitimate recombination (43), even though there is also evidence that *RAD50* is relevant to *RAD52*-dependent events such as meiotic homologous recombination (30). We found that deletion of *RAD50* resulted in a 12-fold decrease in pRS316 end joining (Table 2). Interestingly, the end-joining defects of the *hdf1*Δ *rad50*Δ and *ku80*Δ *rad50*Δ double mutants (DWY304 and DWY325) were no more severe than those of either of the single mutants, indicating that *hdf1*Δ and *ku80*Δ are epistatic with *rad50*Δ for this function. Likewise, the MMS sensitivity of double mutant strains was evaluated. We found that *ku80*Δ *rad50*Δ (DWY325) and *hdf1*Δ *rad50*Δ (DWY304) strains exhibited the same MMS sensitivity as an isogenic *rad50*Δ strain (DWY286) (Fig. 4B). These observations are consistent with the involvement of Ku in end-joining steps with Rad50 but not Rad52. Perhaps the biochemical activity of Rad50 is shared by both illegitimate and homologous recombination pathways.

DISCUSSION

Ku complexes in *S. cerevisiae*. Ku80 and Hdf1 are likely to form the heterodimeric Ku complex that was previously identified in *S. cerevisiae* (13) and that has been defined in other eukaryotic systems. Our experiments show that the major DNA end-binding complex contains both Hdf1 and Ku80 as determined by several means. Mutants of either or both genes disrupt the DNA end-binding complex (Fig. 2). The DNA end-binding and MMS repair defects of the *ku80*Δ *hdf1*Δ double mutant are complemented only when both Ku80 and Hdf1 are coexpressed (Table 2). In addition, antibody supershifting and immunodepletions show that Ku80 and Hdf1 are subunits of the same complex, because all of the DNA end-binding activity can be altered or removed with epitope-specific antibodies. Also, double mutant strains expressing both tagged Ku proteins achieve a significantly higher level of both Ku subunits than when only one of the proteins is present and overexpressed (data not shown). Therefore, Ku80 and Hdf1 are not likely to be stable in the absence of the other subunit. These observations are consistent with mammalian Ku80 mutant cell lines containing low levels of Ku70 and essentially no DNA end-binding activity (8, 32, 42). We cannot exclude the possibility that some of the Ku80 or Hdf1 is involved in other protein and/or DNA associations without the other subunit. However, these other complexes are not expected to be relevant to the end-joining and MMS repair processes described here, which are equally affected by loss of one Ku subunit or the other. Therefore, we argue that Ku80 and Hdf1 are partners in the same functionally active protein complexes.

DNA end joining. This study is the first to show that Ku controls joining of DNA ends in the different contexts of episomal plasmids and chromosomal DSBs. Mutations in either *KU80* or *HDF1* block end joining of a linearized plasmid (Table 2). Likewise, the *ku80*Δ *hdf1*Δ double mutants had the same defect as single mutants, suggesting a common biochemical pathway for the two proteins. An interesting feature of end joining studied here is that it can occur by precise restoration of the DNA sequence. In fact, *Bam*HI-cut ends are usually repaired by precise joining (*Bam*HI cleavable) in the wild type, although the *ku80*Δ and *hdf1*Δ backgrounds lead to both reduced efficiency of joining and an increase in the frequency of imprecisely joined products (*Bam*HI resistant) (Table 2). In addition, both Ku gene products are necessary for the *RAD52*-independent repair of a chromosomal DSB at the HO endonuclease site in *MAT* (Table 3). It was previously shown that in the absence of Rad52, DSBs at *MAT* are repaired by nonhomologous end-joining mechanisms that recruit short stretches of homology at the break termini (22). Therefore, the nonhomologous repairs of linearized plasmids and DSBs at *MAT* are likely to be manifestations of the same biochemical process. These assays suggest a mechanism of end joining that is similar to that characterized in other eukaryotes, in which a direct role for DNA-PK components at the DNA ends has been speculated but never proven.

The epistatic relationship of *ku80*Δ and *hdf1*Δ suggests that the biologically significant roles of these proteins in DNA repair events are linked to each other. Two models can explain the role of Ku in DNA end joining. First, Ku may bind to the ends of the transformed DNA as it enters the nucleus and merely protect against exonucleolytic digestion. A protective function would facilitate end joining by preventing the loss of cohesive ends. In an alternative model, Ku may play an active role in the rejoining process, such as distorting or aligning DNA ends by binding to direct the activity of DNA-modifying enzymes and/or DNA ligase. To date, the association of Ku (Hdf1/Ku80 dimers) with other DNA-modifying factors has not been described, although it is likely that a yeast DNA-PKcs-like protein would be important if identified.

Epistasis analysis with *rad50*Δ and the Ku mutations indicates that *RAD50* is also likely to be relevant to the same DNA end-joining mechanisms. Other studies of nonhomologous integration and plasmid end joining recently identified *RAD50* as an important component (36, 43). We found that *hdf1*Δ *rad50*Δ and *ku80*Δ *rad50*Δ strains are equally deficient in plasmid DNA end joining to single mutants of any of the three genes (Table 2). These observations strongly support the model in which Ku plays a direct role in the end joining rather than a protective function. If the role of Ku in the recircularization assays described here is merely to protect the DNA ends and facilitate the ligation of annealed cohesive ends, mutations in *KU80* or *HDF1* would be predicted to impair both *RAD50*-dependent and *RAD50*-independent events. Instead, the observed epistasis suggests a direct role for Ku in the nonhomologous repair pathway in conjunction with Rad50.

Ku DNA repair pathways in *S. cerevisiae*. Our data indicate that Ku-dependent repair is phenotypically and genetically distinct from *RAD52*-mediated homologous recombination. The phenotypes of *ku80*Δ, *hdf1*Δ, and *ku80*Δ *hdf1*Δ mutants differ dramatically from that of *rad52*Δ strains. For instance, repair of linearized plasmid is highly dependent on *HDF1* and *KU80* while only slightly impaired in the absence of *RAD52* (Table 2). Also, while mutations in RAD52 result in pleiotropic defects in mating type switching, chromosome stability, meiotic viability, and the repair of IR-induced damage, *ku80*Δ and *hdf1*Δ strains appear proficient in each of these processes. The only area of phenotypic overlap is in the MMS sensitivity of *rad52*Δ and *ku* mutants (Fig. 3). However, double mutant analysis reveals that the effects of these mutations are additive with regard to MMS repair, and thus the pathways are genetically distinct (Fig. 4A). A recent report suggests that *hdf1*Δ mutants are weakly IR sensitive, but only in the absence of *RAD52* (39). By comparison, our double mutant strains had the same IR sensitivity as *rad52*Δ mutants. These subtle differences are most likely attributable to strain differences or assay conditions and do not affect the conclusion that *KU* is required for a *RAD52*-independent mode of repair.

It is interesting that these different repair pathways are not entirely distinct, because *RAD50* is required for both types of DSB healing. Although Rad50 may play different roles in Ku- and Rad52-mediated processes, it is more likely that it supplies the same function for both. One possible model is that Rad50 is involved in the processing of intermediates common to both pathways. Rad50 is an ATP-dependent DNA binding protein

(33) and has been noted to have amino acid motifs common to a class of phosphoesterases (37). Indeed, *rad50S* mutants fail to process the ends of meiosis-specific DSBs formed at recombination hot spots (30). Therefore, Rad50 may process DNA ends to form substrates for subsequent Ku- or Rad52-dependent steps along separable repair mechanisms. It is noteworthy that *MRE11* and *XRS2* share *RAD50*-type phenotypes for MMS repair and recombination and have demonstrated interactions with Rad50 (20). These additional proteins may be significant for Ku-dependent DNA repair pathways as well.

Ku associates with the DNA-PKcs in other eukaryotes in a manner such that the kinase is activated by Ku-dependent DNA binding (3). The fact that the *scid* mutational group (DNA-PKcs$^-$) shares DSB repair and V(D)J recombination defects that are similar to those of the rodent Ku mutants strongly suggests that these proteins operate as an associated complex in DNA repair events. The broad utilization of the three subunits in invertebrate and vertebrate systems supports this hypothesis (3). Our demonstration that Ku impacts DNA end-joining pathways in yeast cells in ways that closely resemble V(D)J recombination product formation and illegitimate recombination may mean that there is a well-conserved evolutionary function for the DNA-PK complex.

Yeast Ku80 and Hdf1 also provide important insights into fundamental differences between yeasts and mammals with regard to DNA metabolism. The relatively minor DNA repair defects of *ku80*Δ and *hdf1*Δ mutants highlight the relative importance of nonhomologous repair in mammalian systems compared with homology-dependent recombination favored in yeasts and many prokaryotes. The preference for one type of repair or the other may be a consequence of genomic organization and/or complexity. For organisms containing few introns and closely spaced genes, the fidelity of repair associated with homologous recombination may be essential for DSB repair. In contrast, in organisms with large genomes containing significant amounts of repetitive and noncoding sequences, end joining may be preferentially used in order to alleviate the difficulties of DNA homology searching and ectopic recombination, which can result in translocations.

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