HDF2, the Second Subunit of the Ku Homologue from *Saccharomyces cerevisiae*<sup>*</sup>

Heidi Feldmann, Lucia Driller, Bettina Meier, Günter Mages, Josef Kellermann‡, and Ernst-L. Winnacker§

From the Institut für Biochemie der Universität München, Feodor-Lynen-Str. 25, 81377 München, Federal Republic of Germany and §Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, 82152 Martinsried, Federal Republic of Germany

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The high affinity DNA binding factor (HDF) protein of *Saccharomyces cerevisiae* is composed of two subunits and specifically binds ends of double-stranded DNA. The 70-kDa subunit, HDF1, shows significant homology with the 70-kDa subunit of the human Ku protein. Like the Ku protein, HDF1 has been shown to be involved in recombination and double stranded DNA break repair. We have purified and cloned HDF2, the second subunit of the HDF protein. The amino acid sequence of HDF2 shows a 45.6% homology with the 80-kDa subunit of the Ku protein. HDF1 by itself does not bind DNA, while HDF2 protein on its own seems to displays DNA binding activity. Targeted disruption of the HDF2 gene causes a temperature-sensitive phenotype for growth comparable to the phenotype of hdf1<sup>−</sup> strains. The human Ku protein cannot complement this temperature-sensitive phenotype. hdf2<sup>−</sup> strains are sensitive to bleomycin and methyl methanesulfonate, but this sensitivity is reduced in comparison with hdf1<sup>−</sup> strains.

As a safeguard against the occurrence of DNA damage prokaryotic and eukaryotic cells have developed at least three different DNA repair mechanisms. In *Saccharomyces cerevisiae* DNA double strand breaks are repaired mainly by the activities of the RAD52 epistasis group (1, 2). An evolutionary conservation of DNA double strand break pathways is suggested by the existence of eukaryotic homologues of *S. cerevisiae* DNA repair genes in this epistasis group (3–7).

Recently, an activity distinct from the RAD52 group, the DNA-activated protein kinase and its regulatory subunit, the Ku heterodimer, was identified as a component involved in repair of DNA double strand breaks and recombinational events in higher eukaryotes (8–15). The human Ku protein is a heterodimer composed of 70- and 80-kDa subunits (16). The *Drosophila* homologue of the human Ku protein, IRBP (17), has been shown to be involved in repair of DNA double strand breaks, too, indicating a conservation of repair functions in *Drosophila* and mammals (18).

The notion that Ku protein may participate in recombinational, replication, or DNA repair events (19, 20) is suggested by the observation that this protein binds to the ends of double-stranded DNA, nicks, and hairpins (19–26). Several lines of evidence appear to corroborate this view. Ku p80 is not detectable in x-ray-sensitive *hrs* hamster cell lines known to be defective in normal V(D)J recombination processes (12, 13). Both mutant phenotypes in these hamster cells can be complemented by the human XRCC5 gene encoding Ku p80 (11). Cells derived from mice with severe combined immunodeficiency (SCID) have been shown to be sensitive for ionizing radiation and defective in V(D)J recombination. For these SCID cells the catalytic subunit of the DNA-dependent protein kinase is a strong candidate for the afflicted gene (14, 15). A number of DNA-binding proteins, including human p53, have been identified as targets of the DNA-dependent protein kinase (27, 28). The DNA-dependent protein kinase and its regulatory subunit, Ku, may, therefore, play a key role in the signaling pathway of DNA damage (29).

It was shown recently that the Ku heterodimer and human RAD51 coelute with the largest subunit of RNA polymerase II (30). Moreover, DNA polymerase e, which is involved in DNA repair synthesis (31) and also interacts with the Ku protein (32), is a component of this RNA polymerase II complex (30).

A DNA-dependent protein kinase has not yet been described in *S. cerevisiae*. However, a yeast homologue of the human Ku heterodimer, a high affinity DNA binding factor (HDF),<sup>1</sup> has been identified (33). HDF is a heterodimeric protein binding to the ends of double-stranded DNA. The gene of the 70-kDa subunit, *HDF1*, has been cloned, and the predicted amino acid sequences share significant homology with the 70-kDa subunit of the human Ku protein (33). *hdf1* mutant yeast strains are sensitive for the radiomimetic drug bleomycin (34), an agent causing DNA double strand breaks (35–37). The disruption of the *HDF1* gene also affects mating-type switching and spontaneous mitotic recombination (34). *hdf1 rad52* double mutant strains show an increased sensitivity toward ionizing radiation (38). HDF1 has been shown also to be involved in illegitimate recombination (39). Another phenotype, which may not be related to the DNA repair activities of HDF, is the formation of substantially shorter telomeres in *hdf1* mutant strains and a synthetic interaction of the *hdf1* mutation with *tel1* mutation, resulting in strains that grow slowly and have very short telomeres (40).

Here we show the cloning of HDF2, the second subunit of the HDF heterodimer. HDF2 displays significant homology to human Ku p80, comparable to the homology of HDF1 with Ku p70. While HDF1 does not bind DNA by itself, the HDF2 protein on its own displays DNA binding activity. Disruption of the *hdf2* gene causes a temperature-sensitive phenotype for growth. This temperature sensitivity cannot be complemented by expression of the human Ku protein. *hdf2* mutant strains are also sensitive toward bleomycin and methyl methane sul-

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<sup>1</sup> The abbreviations used are: HDF, high affinity DNA binding factor; MMS, methyl methanesulfonate; bp, base pair(s).
fonate but in comparison with hdf1 mutant strain this sensitivity is reduced.

MATERIALS AND METHODS

Yeast Strains, Media, Growth Conditions, and Transformation—Strains used in this study are shown in Table I. Only relevant genotypes are listed. The hdf2-disrupted strains W303h2a and WaLh2a were generated by one-step gene disruption of the wild-type HDF2 gene in W303-1A and W303aL. Cells were grown at 30 or 37 °C in YPD liquid medium/plates containing 2% glucose, 1% yeast extract, 2% Bacto-peptone, and 2% dextrose supplemented with the appropriate nutrients (SD medium is 2% glucose, 0.67% nitrogen base without amino acids, plus nutrients) (41). Yeast transformation was performed by the lithium acetate method (42). Purification of the HDF Protein—Protein extracts for HDF purification were prepared from the protease-deficient strain ABS60. Purification was performed as described previously (33).

Gel Retardation Assay—A 39-bp long synthetic double-stranded oligonucleotide, designated PKG1.2 (33), was used for gel retardation assays. Protein extracts were incubated with DNA for 5–10 min at room temperature in a buffer containing 150–250 mM ammonium sulfate, 50 mM Tris, pH 7.5, 5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. DNA-protein complexes were analyzed by gel retardation on 10 or 12% polyacrylamide gels in 0.5 M TBE (0.09 M Tris borate, 0.002 M EDTA). All gels were prerun at 80 V for 30 min and then at 120 V for 6–12 h.

Cloning of the HDF2 Gene—Partial amino acid sequences of purified HDF2 protein were determined and used to identify an unknown open reading frame by a BLAST search. Oligonucleotides derived from the data base sequence information obtained were used for screening an EMBL3A genomic yeast library. A 2.4-kilobase SacI/SalI fragment was subcloned into pGEM4Z (Promega). To verify the sequence the fragment was sub sequenced from both ends as described previously (33).

Disruption of the HDF2 Gene—A SalI/SalI fragment spanning 175 bp upstream of the ATG codon, the open reading frame of the HDF2 gene, and 387 bp downstream of the TAG stop codon were cloned into plasmid pGEM4Z. The plasmid was cleaved with SpeI, which cuts once inside the coding region of the HDF2 gene 300 bp downstream of the ATG codon. 216 bp were removed by Bal31 endonuclease digestion and replaced by a functional Kan resistance gene (43). The resulting plasmid, pHDF2kan1, was digested with BamHI/SalI, and the DNA was used to transform the haploid yeast strains W303-1A and W303aL for the HIS3 selection marker and pAHp70 gene, and 387 bp downstream of the TAG stop codon were cloned into the plasmid pRS313 under control of the ADH promoter. The resulting plasmids, pRS313HDF2, containing the HIS3 selection marker was transformed into different yeast strains. Positive clones were selected by plating to His + SD plates. His + colonies were tested for temperature sensitivity and HDF DNA binding activity in gel retardation assays.

Plasmid Complementation—To complement the hdf2 deletion in the yeast strain W303h2a a 2.450-bp SalI/SacI fragment was cloned into the multiple cloning site of plasmid pRS313 (45). The fragment contains 175 bp upstream of the ATG start codon, the entire HDF2 coding region, and 387 bp downstream of the stop codon. The resulting plasmid, pRS313HDF2, containing the HIS3 selection marker was transformed into the hdf2 deletion strain W303h2a. Positive clones were selected by plating to His + SD plates. His + colonies were tested for temperature sensitivity and HDF DNA binding activity in gel retardation assays.

Expression of Human Ku p70 and Ku p80 in Yeast Cells—For expression of human Ku p80 protein the cDNA was cloned into the plasmid pRS316 (45) under control of the GAL1-10 promoter. For expression of the Ku p70 protein the cDNA was cloned into the plasmid pRS313 under control of the ADH promoter. The resulting plasmids pRS316GALp80 containing the URA3 selection marker and pAHp70 containing the HIS3 selection marker were transformed into different yeast strains. Positive clones were selected by plating to Ura + , His + , or Ura + /His + SD plates.

Drop Titer Test—A single colony from a YPD or SD plate grown for 3 days was suspended in 500 μl of YPD medium. This cell suspension was diluted from 10−1 to 10−5. 10 μl of each dilution were dropped onto a YPD or SD plate. The plates were incubated at 30 or 37 °C for 3–6 days.

If strains transformed with a galactose-inducible promoter were used, a single colony was resuspended in 10–20 ml of YP-Gal liquid medium (1% yeast extract, 2% Bacto-peptone, 2% galactose) and incubated at 30 °C for 4 h. Cells were collected and resuspended in 500 μl of YP-Gal. This cell suspension was diluted as described above and spread on YP-Gal or SD-Gal plates.

RESULTS

Cloning of the HDF2 Gene—HDF exists as a stable heterodimer. The purified protein displays two bands in SDS-gel electrophoresis of 70 (HDF1) and about 85 kDa (HDF2) (data not shown). To clone the HDF2 gene we purified the protein to homogeneity. The purification procedure included four column chromatography steps, phenyl-Sepharose, DEAE-cellulose, phosphocellulose, as well as DNA affinity chromatography on a column-bound oligonucleotide (33). Starting with crude extract prepared from 500 g of wet yeast cells we obtained about 20 μg of a highly purified protein preparation. The amino acid sequence of four HDF2 peptides were obtained by microsequencing of proteolytic cleavage products. These sequences were used for comparison with sequences in the protein data base. All four peptides matched to one sequence of an unknown protein. The sequence of this unknown protein was identified in connection with the yeast genome project (accession no. SC9718.5). The open reading frame contained 1.890 bp coding for 629 amino acids. The molecular mass predicted from this DNA sequence was 71.25 kDa. This is not in agreement with the molecular mass of about 85 kDa determined by SDS-polyacrylamide gel electrophoreses but may be due to unknown posttranslational modification or the result of an artifact of SDS-gel electrophoresis.

Comparison of the amino acid sequence of the SC9718.5 open reading frame sequence with sequences in the protein data base revealed a significant homology with the p80 subunit of the human Ku autoantigen of 45.6%. This is comparable with the homology of HDF1 with the p70 subunit of the Ku protein of about 46.5%. The amino acid sequence lacks a leucine zipper region shown to be present in the Ku p80 sequence. The SC9718.5 open reading frame sequence also lacks any other known protein domains. An EMBL3A genomic yeast library was screened using oligonucleotides derived from the data base sequence information. Four positive clones were isolated and a 2450-bp SalI/SacI fragment was subcloned. The sequence was verified by sequencing from the 5′- and 3′-end.

Disruption of the HDF2 Gene—The HDF2 gene was disrupted by employing the one-step disruption procedure of Rothstein (44) as described under "Materials and Methods." As shown in Fig. 1, lane 4, crude extract of the HDF2-deficient

| Strain   | Relevant genotype | Source |
|----------|-------------------|--------|
| W303-1A  | Mata, ade2-1, his3-11, leu2-3, 112, ura3-1, trp1 | Ref. 34 |
| W303aL   | Mata, hdf1::LEU2, ade2-1, his3-11, ura3-1, trp1 | Ref. 33 |
| W303h2   | Mata, hdf2::KAN, ade2-1, his3-11, ura3-1, leu2-3, 112, trp1 | This study |
| W303a    | Mata, hdf1::LEU2, hdf2::KAN, ade2-1, his3-11, ura3-1, trp1 | This study |
| ABYS60   | Mata, ade2, prr1-1, pbr1-1, pcl1-1, cph1-3 | Ref. 33 |
strain did not show any HDF-specific DNA binding activity. As a positive control the hdf2-deficient strain W303h2 was transformed with yeast expression plasmid pRS313 containing a 2,450-bp long DNA insert with the entire open reading frame as well as upstream and downstream regions of the HDF2 gene. Crude extract from the transformed strain W303h2 displayed DNA binding activity in gel retardation assays which was indistinguishable from that observed with control strains (Fig. 1, lanes 1 and 5).

**HDF2 Can Bind to DNA on Its Own—**Comparison of the DNA-protein complexes detectable in the crude extracts of hdf1- and hdf2-deficient strains showed an additional band formed by the crude extract of hdf1-deficient strain (Fig. 1, lane 2).

These results suggest that HDF2 possesses a DNA binding activity of its own. This DNA binding activity of HDF2 was much weaker than the DNA binding activity of the HDF heterodimer in crude extract of wild-type cells (Fig. 1, lanes 1 and 2). To verify that the observed DNA protein complex was formed by the HDF2 protein the hdf1 hdf2 double mutant strain WaLh2 was used. In crude extracts of the double mutant strain the HDF2-DNA complex was not detectable (Fig. 1, lane 6). This complex was also absent if this strain was transformed with the HDF1 expression plasmid, pRS316HDF1 (33) (Fig. 1, lane 7). The complex reappeared in crude extracts of the double mutant strain transformed by the HDF2 expression plasmid, pRS313HDF2 (Fig. 1, lane 8). Transformation of the double mutant strain with both the HDF1 and HDF2 expression plasmid led to HDF heterodimer DNA binding activity indistinguishable from that observed in the wild type (Fig. 1, lanes 1 and 9).

**HDF2 Strains Are Temperature-sensitive for Growth—**Since the HDF1-deficient strain W303aL showed a temperature-sensitive phenotype for growth (33), this growth phenotype was also studied in the hdf2-deficient strain W303h2. When haploid wild-type and hdf2-deficient strains were kept at 30 °C for 3 days on YPD plates, suspended in liquid medium, and spot-plated onto YPD plates at different dilutions, the wild-type strain grew normally at 37 °C, whereas the hdf2-deficient strain did not grow at this temperature. When kept at the permissive temperature (30 °C) both wild-type and hdf2-deficient strain grew normally (data not shown). This phenotype of the hdf2-deficient strain is similar to that observed for the hdf1-deficient strain. Growth of hdf2-deficient cells in liquid medium at 37 °C for 10–12 h resulted in the development of enlarged single budded cells (data not shown, but see Feldmann and Winnacker (33)). This is in agreement with the phenotype of hdf1- cells. Growth phenotypes of the hdf1 hdf2 double mutant strains were identical with the phenotype of the single mutant strains.

**Human Ku Protein Cannot Complement Temperature Sensitivity of HDF-deficient Cells—**HDF is the homologue of the human Ku protein. The proteins share biochemical properties and structural homology. We therefore tested the ability of the human Ku subunits to complement the temperature-sensitive phenotypes caused by HDF2 and HDF1 deficiency.

The hdf2- strain W303h2 was transformed with a yeast expression plasmid containing the Ku p80 cDNA under the control of a GAL1-10 promoter. The resulting strain W303h2-pRS316Galp80 was tested for the ability to grow at 30 and 37 °C on Ura− SD plates containing 2% galactose. As shown in Fig. 2A, human Ku p80 could not grow at 37 °C. When kept at permissive temperature (30 °C) this strain grew normally. This result suggested that Ku p80 cannot complement the temperature sensitivity of hdf2- strains. We also tested whether Ku p70 could complement the hdf1- phenotype. The HDF1-deficient strain W303aL transformed with a yeast expression plasmid containing the cDNA of Ku p70 under control of an ADH promoter could not grow at 37 °C (data not shown), indicating that Ku p70 cannot complement HDF1 deficiency. Finally we tested the hdf2 mutant strain W303h2 transformed with both plasmids, pRS316Galp80 and pAHp70, for growth at 37 °C. As shown in Fig. 2B, expression of both subunits of the Ku heterodimer cannot complement temperature sensitivity of the HDF-deficient strain.

To verify that the Ku subunits were expressed in yeast cells, we tested DNA binding activity in crude extracts of the transformed strains using gel retardation assays. We could not detect an HDF1/Ku p80 corresponding DNA protein complex with
Concentrations of 1–4 \( \text{mg/ml} \) of bleomycin were plated on solid YPD media containing bleomycin in concentrations of 1–4 \( \mu \text{g/ml} \). Colonies were counted after 5 days of incubation at 30 °C.

In crude extracts of the strain W303h2-pRS316Galp80 (Fig. 3, lanes 3 and 4), Extracts from strain W303aL-pAHp70 displayed no HDF2/Ku70 corresponding DNA binding activity (data not shown). In crude extract of the \( hdf2 \) mutant strain transformed with the Ku70 and Ku p80 expression plasmids, a new DNA protein complex corresponding to the Ku p70/p80 heterodimer was detectable (Fig. 3, lanes 5 and 6).

\( hdf^2 \) Mutants Are Sensitive to Bleomycin and MMS—Bleomycin is known to cause the introduction of double strand breaks into DNA molecules. We have shown previously that the \( hdf1 \) mutant strain W303aL is strongly sensitive to bleomycin (34). Therefore, we studied the level of sensitivity for bleomycin of \( hdf^2 \) and \( hdf1 hdf^2 \) double mutant strains. Survival assays were carried out on solid medium in the presence or absence of varying concentrations of bleomycin. A marked reduction by 1.2 orders of magnitude in the survival rates of the haploid \( hdf^2 \) mutant strain was observed at a bleomycin concentration of 4 \( \mu \text{g/ml} \). This decrease is not as prominent as the decrease of survival rates in \( hdf1 \) or \( hdf1 hdf^2 \) double mutant strains, which showed a reduction by 1.8–2.0 orders of magnitude at the same bleomycin concentration (Fig. 4).

Transformation of a \( hdf^2 \) mutant strain with a yeast single copy plasmid carrying a functional copy of the HDF2 gene restored the response of bleomycin to wild-type levels (data not shown).

Another agent known to induce strand breaks in DNA is MMS. Accordingly, \( hdf1 \) and \( hdf2 \) mutant strains and the double mutant strains were also tested for MMS sensitivity.

We observed a significant sensitivity of all three strains to MMS compared with the wild-type strain (Fig. 5A). The decrease in survival rate of the \( hdf^2 \) mutant strain in response to MMS treatment was not as pronounced as the decrease of the survival rates of the \( hdf1 \) and \( hdf1 hdf^2 \) double mutant strains. Survival rates of the \( hdf^2 \) mutant strain were reduced by 1 order of magnitude while the decrease in survival rates of the double mutant strains was by 2–2.2 orders of magnitude. Sensitivity of the \( hdf1 \) and \( hdf^2 \) mutant strains to MMS could be restored to wild-type level by expression of a functional copy of the HDF1 or HDF2 gene, respectively, from a yeast single copy plasmid (Fig. 5B).

DISCUSSION

We have cloned HDF2 from \( S. cerevisiae \), the gene encoding the second subunit of the HDF heterodimer, which is the homologue of the human Ku protein. HDF2 displays a homology of 45.6% with Ku p80. The molecular mass predicted from the sequence of the HDF2 gene is 71.25 kDa. This is substantially smaller than the mass of the Ku p80 subunit of 82.5 kDa. The significance, if any, of the observed differences in the molecular masses of the two proteins remains unresolved as homology comparisons do not provide any indications for the presence of regional or local decreases in the homology of the two proteins.

No DNA binding activity is detectable for HDF1 on its own. The analysis of extracts of \( hdf1 \)-deficient cells reveals the presence of a weak DNA binding activity which is not detectable in extracts of \( hdf^2 \)-deficient cell. This DNA binding activity is also absent in cells of a double mutant strain but it can be restored by expression of a HDF2 gene from a single copy plasmid. These experiments indicate that HDF2 protein possesses a weak DNA binding activity of its own. Since the DNA binding activity of HDF2 alone is weak compared with the DNA binding activity of the heterodimer it may well be that HDF2 is the DNA binding subunit of the HDF heterodimer, while HDF1 is increasing the affinity of the heterodimer to DNA.

\( hdf^2 \) mutant strains are temperature-sensitive for growth. Cells grown at 37 °C display the same phenotype as observed...
for hdf1 mutant cells, arresting as enlarged single-budded cells. hdf1 hdf2 double mutant strains display no additional growth defects. These results indicate that this phenotype depends on the missing HDF heterodimer activity.

The temperature-sensitive phenotype for growth caused by HDF deficiency cannot be complemented by the expression of either the single subunits or the heterodimeric human Ku protein. A HDF/Ku corresponding DNA binding activity is not detectable in hdf1- or hdf2-deficient strains transformed with plasmids expressing human Ku p70 or Ku p80, respectively. These results indicate that HDF1 and Ku p80 or HDF2 and Ku p70 cannot form functional heterodimers. Functional expression of the Ku heterodimer can be shown by detection of the DNA binding activity of the Ku protein in crude yeast extract. We suggest therefore that loss of HDF DNA binding is not responsible for temperature sensitivity but loss of protein-protein interactions. It appears that human Ku cannot take over the function of HDF in protein-protein complexes. One candidate for protein interaction with the HDF heterodimer is the catalytic subunit of a postulated DNA-dependent protein kinase. Until now it was not possible to show the existence of a DNA-dependent protein kinase in yeast. But the functional and structural homology of HDF and Ku heterodimers leads to the assumption that a DNA-dependent protein kinase activity exists in S. cerevisiae, as well.

We have shown recently that hdf1 mutant strains are sensitive for the radiomimetic agent bleomycin and, in addition, show a reduced rate of mating-type switching and mitotic recombination (34). These experiments indicate that the HDF heterodimer is involved in DNA repair and recombination events. In this communication we show that hdf2-deficient strains are also sensitive to bleomycin and that hdf1- or hdf2-deficient strains are sensitive to MMS, an agent inducing DNA breaks. Surprisingly hdf1 mutant strains are about 10 times more sensitive toward both agents than hdf2 mutant strains. Deletion of both subunits in hdf1 hdf2 double mutant strains only slightly increases sensitivities. This observation indicates that HDF1 is the critical component of HDF heterodimers functioning in DNA repair. In conjunction with the data reported for the DNA binding activity of the HDF2 subunit, these results suggest a mechanistic model of the function of the HDF heterodimer. It may be that HDF2 is the DNA-binding component of the HDF heterodimer, while HDF1 is stabilizing the protein-DNA complex and is the active component in forming protein-protein complexes. In this case the HDF1 subunit alone could function in DNA repair but in a reduced manner. It may be that binding of the HDF heterodimer to DNA localizes the protein toward the position it is needed, and in hdf2-deficient cells the HDF1 subunit reaches this position only with a strongly reduced affinity.

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