Korean J Biol Sci 7: 159-164, 2003

Characterization of *HRD3*, a *Schizosaccharomyces pombe* Gene Involved in DNA Repair and Cell Viability

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Key Words:

HRD3 gene
Schizosaccharomyces pombe
RAD3 homolog
Excision repair
UV-induction

The *RAD3* gene of *Saccharomyces cerevisiae* is required for excision repair and is essential for cell viability. The *RAD3* encoded protein possesses a single stranded DNA-dependent ATPase and DNA and DNA-RNA helicase activities. To examine the extent of conservation of structure and function of a *S. pombe RAD3* during eukaryotic evolution, the *RAD3* homolog gene was isolated by screening of genomic DNA library. The isolated gene was designated as *HRD3* (homolog of *RAD3* gene). Southern blot analysis confirmed that *S. pombe* chromosome contains the same DNA as *HRD3* gene and this gene exists as a single copy in *S. pombe*. The transcript of 2. 8 kb was detected by Northern blot analysis. The level of transcripts increased by ultraviolet (UV) irradiation, indicating that *HRD3* is one of the UV-inducible genes in *S. pombe*. Furthermore, the predicted partial sequence of HRD3 protein has 60% identity to *S. cerevisiae RAD3* gene. This homology was particularly striking in the regions identified as being conserved in a group of DNA helicases. Gene deletion experiments indicate that the *HRD3* gene is essential for viability and DNA repair function. These observations suggest evolutionary conservation of other protein components with which HRD3 might interact in mediating its DNA repair and viability functions.

Excision repair of ultraviolet light (UV) damaged DNA in eukaryotes is a complex process involving a large number of genes. In the yeast *Saccharomyces cerevisiae*, six genes, *RAD1, RAD2, RAD3, RAD4, RAD10,* and *RAD14*, are known to be required for the incision step in excision repair of UV damaged DNA (Reynolds and Friedberg, 1981; Choi et al., 1990), whereas several others, *RAD7, RAD16, RAD23,* and *MMS19*, affect the proficiency of excision repair (Friedberg, 1988). In human, seven xeroderma pigmentosum (XP) complementation groups, XPA through XPG, have been identified. XP cells are defective in the incision of UV damaged DNA and as a consequence, XP patients are highly sensitive to sun light and suffer from a high incidence of skin cancers. Eight complementation groups have been identified among UV-sensitive rodent cell lines and mutants from five of these groups are defective in incision. Three human excision repair genes, *ERCC1, ERCC2,* and *ERCC3,* have been cloned by complementing the UV sensitivity of rodent cell lines, and all three genes show homology to *S. cerevisiae* genes. *ERCC1* is homologous to *RAD10*, and *ERCC2* is a homolog of *RAD3.* The *ERCC3* gene complements the excision repair defect in XP-B mutant cells, and a homolog of this gene has been identified in *S. cerevisiae*. The conservation of excision repair genes between yeast and human implies that information gleaned from the yeast system would be applicable to higher eukaryotes, including humans (Lewis et al., 1998; Masson et al., 1998).

The *RAD3 gene is required at an early stage in the* excision repair of UV damage (Reynold et al., 1992). Analysis of the rad3 mutant has indicated that the gene product is required for nicking of DNA containing pyrimidine dimers. The gene encodes a single stranded DNA-dependent nucleotide triphosphatase with DNA helicase and DNA/RNA helicase activities (Murray et al., 1992). In addition to its role in excision repair, the RAD3 protein has an essential function for cell proliferation, and mutational analysis has revealed that different regions of the protein are involved in the repair and essential functions (Naumovski and Friedberg, 1988; Reynolds et al., 1992).

Complementation of the radiation-sensitive phenotypes has been used to isolate DNA repair genes from yeast and mammalian cells, and this has led to the identification of members of the excision repair pathway which are conserved between *S. cerevisiae* and human. The *S. cerevisiae* genes *RAD3* and *RAD10* are homologs of the human *ERCC2* and *ERCC1* genes, respectively (Weber et al., 1988; Van Duin et al., 1989; Carr et al., 1994; Fasullo et al., 1998; Otrin et al., 1998; Schauber et al., 1998). Reports to date on the cloning

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of DNA repair genes from *Schizosaccharomyces pombe* have not revealed homologies to any previously identified DNA repair genes (Reynolds et al., 1992). It was therefore of interest to determine whether *S. pombe* does contain pathways conserved in other eukaryotes or whether the processes are different in this yeast.

To gain insight into the extent of conservation in the structure and function of *S. cerevisiae RAD3* gene, we have characterized the *HRD3* of *S. cerevisiae RAD3* homolog, *HRD3* gene from the evolutionarily divergent fission yeast *S. pombe*. *S. pombe* resembles higher eukaryotes more closely than does *S. cerevisiae*. Here, we report a new gene from *S. pombe* which is an important model system for the study of basic processes in eukaryotes.

Materials and Methods

Strains, cell culture, reagents, and genetic methods

The haploid *S. pombe* strain, JY741 (h⁻ade6-M210 leu1-32 ura4-D18) was used for this study and grown in YE (2% glucose, 0.5% yeast extract) medium supplemented with appropriate amino acids. Complete and minimal growth media for fission yeast and chemical reagents were purchased from Difco and Sigma Aldrich. Plasmids were constructed by standard techniques (Sambrook and Russell, 2001).

Plasmid DNA from *E. coli* was isolated by the alkaline lysis method using the manufacturer's protocol (Qiagen). Transformation of yeast was carried out by treatment with lithium (Ito et al., 1983), and that of *E. coli* was carried out according to the calcium chloride/rubidium chroride method. Chromosomal DNA from *S. pombe* was prepared according to the methods of Cryer et al. (1975).

Southern blot analysis

Chromosomal DNA isolated from *S. pombe* cells was digested for a gel blot analysis with various restriction enzymes, separated on a 0.8% agarose gel, and transferred onto a nylon membrane (Hybond-H^+, Amersham) using the manufacturer's protocol. The membrane was hybridized with the *HRD3* DNA fragment, which was gel purified and [α-^{32}P] dCTP labeled using the random priming method with Megaprime Labeling Kit (Amersham) in Quick Hybridization (Stratagene) at 68°C. After hybridization, the membrane was washed twice in 2X SSPE, 0.1% SDS and 0.2X SSC, 0.01% at 55°C. After the final rinse, the membrane was wrapped with plastic wrap and exposed onto X-ray film (X-Omat, Kodak) for 12 h or more.

UV survival test

A survival test was performed as previously described (Choi et al., 1991). Briefly, mid-log phase cells were serially diluted to a final density of 4×10^3 cells/ml in distilled water. Four hundred cells were plated onto YES and irradiated with various doses of UV using a Stratalinker 1800 (Stratagene). Plates were incubated at 30°C for 4 to 5 d, and colonies were counted. The relative survival of strains was calculated as the ratio of the number of colonies on UV-irradiated plates relative to the number of colonies on unirradiated plates.

Treatment with DNA damaging agent and Northern blot analysis

Cells were grown to mid exponential stage, harvested, washed and then resuspended in 10 ml of distilled water. The cell suspension was evenly spread onto a 150 mm Petri dish and exposed to 200 J/m^2 of ultraviolet UV from mercury germicidal lamp. The irradiated cells were inoculated into fresh YES medium, incubated at 30°C in the dark, and collected at indicated times. The treatment with methyl methanesulfonate (MMS) was done by adding MMS into the exponentially growing cell culture to a concentration of 0.1%.

Total RNA was prepared according to Jang et al. (1995). RNA was denatured and electrophoresed in 1.2% agarose gel containing formaldehyde and transferred onto nitrocellulose filters. The probe, filter hybridization, and washing conditions were identical to those of Southern hybridization.

Sequence analysis

Plasmid DNA was purified using a plasmid preparation kit (Qiagen). Nucleotide sequences of both strands were determined for both strands by dideoxy-chain termination method (Sanger et al., 1977) using Sequenase 2.0 (US Biomedical, U.S.A.), according to the manufacturer's recommendations. The sequences were compared with the protein and the nucleotide data bases using FASTA and BLAST (Altschul et al., 1990).

Results and Discussion

Identification and homology search with other DNA repair gene of HRD3 gene

Comparison of DNA repair mechanisms between *S. cerevisiae* and human shows that a number of genes required for a nucleotide excision repair pathway are conserved between these two organisms (Choi et al., 1991; Troelstra et al., 1992; Caldecott et al., 1994; Jin et al., 1996; Park and Choi, 2002), but to date, little information has been available as to whether a similar mechanism exists in *S. pombe*. We show here that the *S. pombe HRD3* gene has a high degree of identity to the *S. cerevisiae RAD3* gene, showing that this component of the excision repair pathway is highly

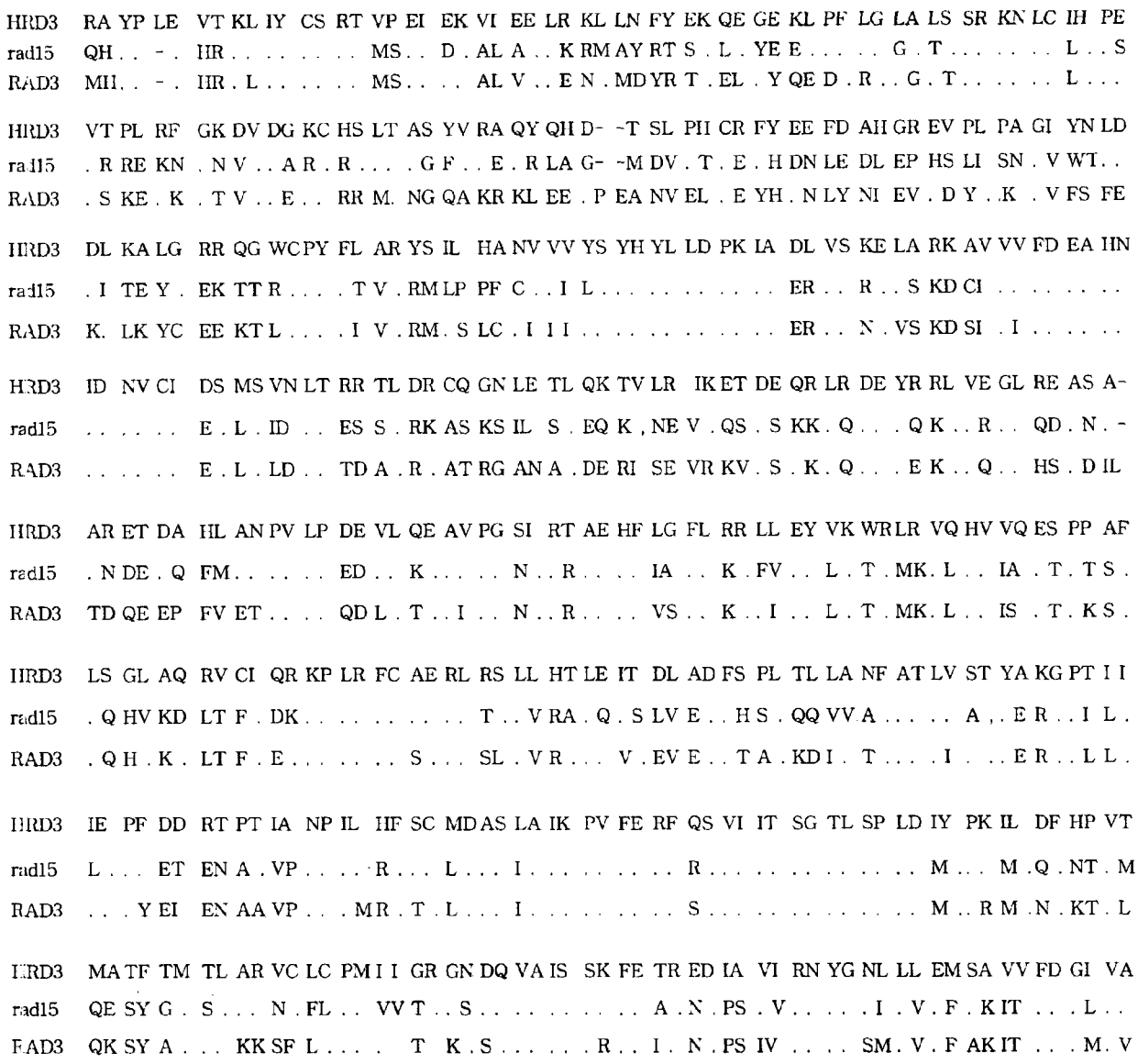

Fig. 1. Comparison of the protein sequence of the *Schizosaccharomyces pombe HRD3*, *S. pombe rad15* and *S. cerevisiae RAD3* genes. A dot indicates identity between *HRD3* and *RAD3* or *rad15* genes. Gaps have been introduced to maximize the alignment. The conserved helicase domains are underlined.

conserved between these two organisms.

In order to determine whether *S. pombe* contains a homolog to the conserved *S. cerevisiae RAD3* gene, an *S. pombe* genomic library in pDB262 was constructed. *S. pombe* genomic DNA was digested with *Sau*3AI and ligated with *Sal*I-linearized pDB262 vector. To identify *S. pombe RAD3* homologous gene, the *S. cerevisiae RAD3* DNA fragment was used as a probe (Murray et al., 1992). Cloning and sequencing of this isolated gene revealed the conserved *S. cerevisiae RAD3* gene. The size of this DNA fragment was 3,400 base pairs (Choi, 2001). This isolated gene was designated *HRD3* (Homolg of *RAD3* gene).

Comparison of the deduced amino acid sequence with that of the *S. pombe HRD3, S. cerevisiae RAD3* and human ERCC2 proteins is shown in Figure 1. The putative HRD3 protein has 65% identity to the RAD3 protein and 55% identity to the ERCC2 protein. The *S. cerevisiae RAD3* gene encodes an ATP-dependent DNA helicase (Naumovski and Friedberg, 1988; Murray et al., 1992; Caldecott et al., 1994), and by comparison with other helicase it has been shown to have the seven conserved helicase domains described by Gorbalenya et al. (1989). The deduced amino acid sequences were compared with those of HRD3 and RAD3. The high level of sequence homology suggests that the *S. pombe HRD3* gene is also likely to encode an ATP-dependent DNA helicase. This result suggests that *HRD3* contains DNA helicase motifs.

Our knowledge of the extent of conservation of excision

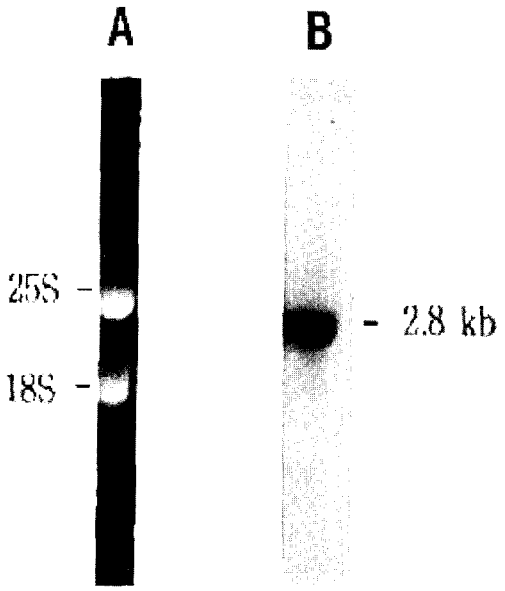

Fig. 2. Southern hybridization analysis of yeast chromosomal DNA. Chromosomal DNA was digested with various restriction enzymes, electrophoresed on a 0.7% agarose gel (A), transferred to nitrocellulose filters and then hybridized with a ^{32}P-labeled *HRD3* gene fragment (B). Lane M, phage DNA digested with *Hin*dIII; 1, genomic DNA digested with *Bam*HI; 2, *Eco*RI; 3, *Hin*dIII; 4, *Sal*I; 5, *Sac*I; 6, *Xho*I.

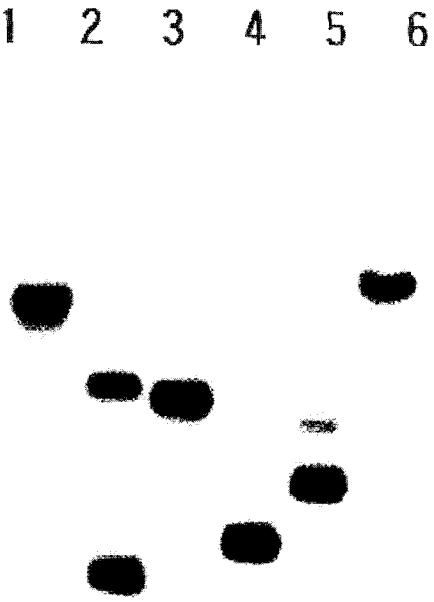

Fig. 3. Northern blot analysis of *HRD3* mRNA in *S. pombe*. Total RNA was isolated, electrophoresed, transferred onto nitrocellulose filters, and then hybridized with the radiolabeled *HRD3* DNA probe. From this the estimated size of the transcript is 2.8 kb.

repair genes between *S. cerevisiae*, *S. pombe* and human has recently been extended by the identification of additional *S. pombe* homologs to *S. cerevisiae* genes. Further evidence for the conservation of excision repair pathways is provided by novel homologs to the human excision repair gene *ERCC3*, which have been identified in both yeast strains (Koken et al., 1992). Other repair pathways conserved between the two yeasts or between yeast and man have yet to be identified. It is evident that many DNA repair genes are highly conserved in eukaryotes.

Genetic mapping of HRD3 gene

In order to confirm that *S. pombe* chromosome contains the same DNA as the *HRD3* gene, Southern analysis was performed (Fig. 2). Their restriction sites are identical to those found in *RAD3* in *S. cerevisiae* (Naumovski and Friedberg, 1987). This result indicates that *S. pombe* chromosome contained the same locus as the *HRD3* gene, and also suggests that *HRD3* locus existed as a single copy in *S. pombe* genome.

DNA damage inducibility of HRD3 gene

To determine whether a *RAD3* homologous gene is expressed in *S. pombe*, total RNA isolated from wild type *S. pombe* cells was hybridized with the *HRD3* DNA fragment. Northern blot analysis revealed 2.8 kb mRNA transcript (Fig. 3). The transcript size is consistent with the size of *HRD3* open reading frame.

Although several DNA damage inducible genes have been isolated from *S. cerevisiae*, it is not known whether *RAD* genes belong to this class (Maga et al., 1986; Reynolds et al., 1992). To examine whether this *HRD3* gene transcription in *S. pombe* is regulated by DNA damaging agents, its mRNA levels were determined after treatment with DNA damaging agents. At various time after UV irradiation (200 J/m^2) and MMS treatment (0.1%), equal amount of total RNA samples prepared from *S. pombe* were hybridized with the radiolabeled DNA fragment (Fig. 4). The UV-irradiation increased *HRD3* gene expression but the treatment of MMS did not. This result implied that the effects of damaging agents are complex and different regulatory pathways exist for the induction of this gene. This result suggested that the *HRD3* gene product might be involved in UV-specific cellular responses such as DNA repair, recombination or mutagenesis. Among the repair-related genes, the transcripts level of *S. cerevisiae CDC9* and *RAD2* genes were shown to be elevated after UV irradiation (Robinson et al., 1986).

HRD3 gene deletion

To determine if the *HRD3* gene is required for cell viability and DNA repair, a *HRD3* deleted strain was constructed. The construction was made in which a 2.5 kb *Bgl*II fragment, containing the majority of the ORF including the ATG, was replaced by the *Leu2* gene. The generation of the genomic *HRD3* mutations was verified

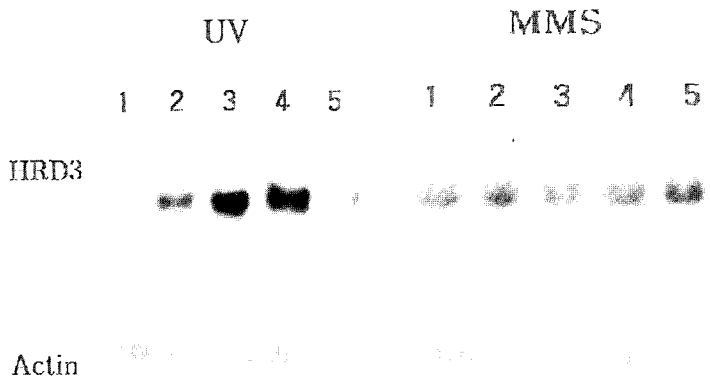

Fig. 4. Northern blot analysis of *HRD3* gene transcript after the treatment of DNA damaging agents. Total RNA was isolated from *S. pombe* cells at various postincubation times after UV irradiation and MMS treatments. RNA was hybridized with the radiolabeled *HRD3* DNA probe. The numbers at the top of each lane indicate the cell collection times after UV irradiation and MMS treatments.

by Southern blot analysis. The *HRD3* gene deleted strain was not well grown compared with *HRD3* gene (Fig. 5A). This result indicates that *HRD3* gene is essential for cell viability. Furthermore, *HRD3* deleted strain did not complement the UV sensitivity (Fig. 5B). These observations indicate that the *HRD3* gene is required for the DNA repair function and cell viability.

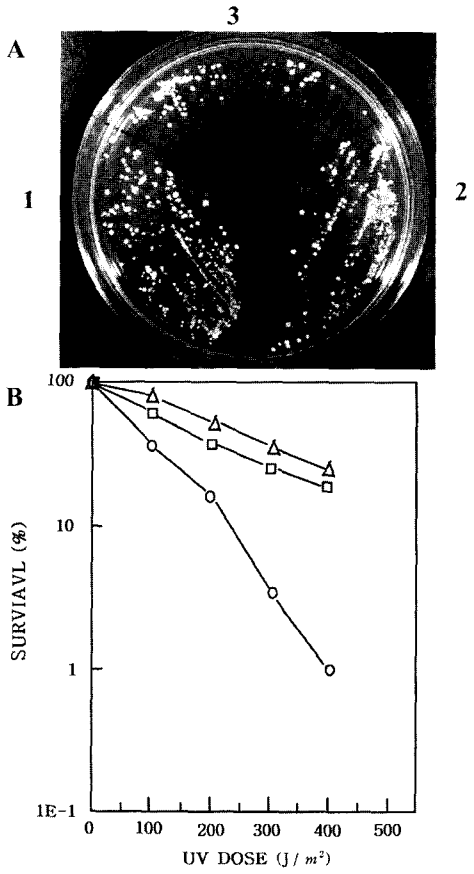

Fig. 5. *HRD3* gene restores cell viability and DNA repair. A, *HRD3* gene is an essential gene for cell growth. (1), wild type cell; (2), *HRD3* gene; (3), *HRD3* gene deleted strain. Cells from a single colony were streaked onto YES medium and incubated at 30°C for 3 d. B, *HRD3* restores wild type levels of UV resistance in *S. pombe*. Symbols, △, wild type cell; □, *HRD3* gene; ○, *HRD3* gene deleted strain.

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[Received April 26, 2003; accepted May 11, 2003]