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Isolation of HRD3 gene, a homologous RAD3 gene from fission yeast *Schizosaccharomyces pombe*

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ABSTRACT : **The RAD3 gene of *Saccharomyces cerevisiae* is required for excision repair and is essential for cell viability. RAD3 encoded protein possesses a single stranded DNA-dependent ATPase and DNA-RNA helicase activies. To examine the extent of conservation of structure and function of RAD3 during eukaryotic evolution, we have cloned the RAD3 homolog, HRD3, from the distantly related yeast *Schizosaccharomyces pombe*. Here, we report the partial cloning and characterization of HRD3 gene (Homologous of RAD3 gene) which was isolated by PCR amplification using conserved domain of *Saccharomyces cerevisiae* RAD3 gene. Chromosomal DNA isolated from *S. pombe* had similar restriction patterns to those from *S. cerevisiae*, as determined by Southern blot analysis. The 2. 8 kb transcript of mRNA was identified by Northern hybridization. The level of transcript did not increase upon UV-irradiation, suggesting that the HRD3 gene in *S. pombe* is not UV-inducible.**

Keywords : **RAD3 homolog, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, DNA-RNA helicase, UV-inducible**

Introduction

Excison repair of ultraviolet light 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).

The RAD3 gene is required at an early stage in the excision repair of ultraviolet-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 (Reynolds and Friedberg, 1981). The gene encodes a single stranded DNA-dependent nucleotide triphosphatase with DNA helicase and DNA/RNA helicase activities (Murray *et al.*, 1992). As well as 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).

Complementation of the radiation-sensitive phenotypes has been used to isolated DNA repair genes from species of yeast and from mammalian cells, and this has led to the identification of members of an excision repair pathway which are conserved between *S. cerevisiae* and man. The *S. cerevisiae* genes RAD3 and RAD10 are homologues of the human ERCC2 and ERCC1 genes respectively (Weber *et al.*, 1988; Van Duin *et al.*, 1989; Carr *et al.*, 1994). Reports to date on the cloning of DNA repair genes from *S. pombe* have not revealed homologies to any previously identified DNA repair genes. 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 RAD3 homolog, HRD3 gene from the evo-

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lutionarily divergent fission yeast *Schizosaccharomyces pombe.* *S. pombe* resembles higher eukaryotes more closely than does *S. cerevisiae*. Here, we report a new member of the family from *S. pombe* which is an important model system for the study of basic processes in eukaryotes.

Materials and Methods

Strains, cell culture, and genetic methods

E. coli strain DH5α (F- endA1, hsd17, (r-, mk-), supE44, thi-1, recA1, gyrA96, relA1, lacIqZ-M15) and yeast *S. pombe* strain JY 741(h- ade6-M210 leu1-32 ura4-D18) were used for this study. *E. coli* strains were grown on LB media (1% Tryptone, 1% Sodium chloride, 0.5% Yeast extract). Yeast strain was grown in YE (2% glucose, 0. 5% yeast extract) supplemented appropriate amino acids (Choi *et al.*, 1991). Plasmid DNA from *E. coli* was isolated by the alkaline lysis procedure of Sambrook *et al.* (1989).

Transformation of yeast was carried out by treatment of lithium acetate (Ito *et al.*, 1983), and that of *E. coli* was carried out according to the calcium chloride/rubidium chloride method (Maniatis *et al.*, 1982).

Chromosomal DNA isolation and Polymerase Chain reaction (PCR) amplification

Chromosomal DNA from *S. pombe* was prepared according to the methods of Cryer *et al.*, (1975) and used as template (30 ng) for PCR amplification. PCR was performed with 0.5 μM degenerative PCR primers. Primers as follow: B1, 5'-ACAGAATTATTTGGGGAC-3'; B2, 5'-TGGAAAACACAGTTGT-3'; B3, 5'-TTGACCGAGGAGGTGGAGAA-3'; B4, 5'-GCCATGTACATTGACACT-3'. PCR was carried out for 30 cycles with 1 min at 94°C, 2 min at 58°C, 2.5 min at 70°C. The PCR amplified products were isolated and subcloned into pCRII vector (In vitrogene).

Preparation of DNA probe by random priming

The amplified PCR product was labeled with [α-^{32}P] dCTP (3,000 Ci/mmole) by random primed DNA labeling method (Feinberg and Vogelstein, 1984). The labeling reaction was carried out in 20 μl of the standard random priming buffer containing 50 ng of PCR product, 30 μCi of [α-^{32}P] dCTP, dATP, dGTP, dTTP and 2 unit of Klenow enzyme for 1 hr at 37°C.

Southern blot analysis

Chromosomal DNA isolated from *S. pombe* cells were digested with various restriction enzymes, electophoresed in 0. 7% agarose, and transferred onto nitrocellulose filters. After the membrane was washed in 2X SSPE, prehybridization was performed for 1 hr at 42°C in prehybridization buffer (1% SDS, 2X SSPE, 10% dextran sulphate, 50% deionized formamide), followed by hybridization using [α-^{32}P] dCTP labeled probe. The membrane was briefly washed with 2X SSPE, washed twice with 2X SSPE, 1% SDS for 15 min at 55°C. After the final rince, the membrane was wrapped with plastic wrap, and exposed onto X-ray film (X-Omat, Kodak) for 12 hr or more.

Treatment of DNA damaging agent

Cells were grown to mid exponential stage, and harvested, washed and then resuspended in 10 ml of distilled water. The cell suspension was evenly spreaded onto 150mm petri dish and irradiated with 200 J/m^2 by UV light, and further incubated for the indicated period of time.

Northern blot analysis

Total RNA was prepared according to Jang *et al.* (1995). RNA was denatured and electrophoresed in 1. 2% agarose containing formaldehyde and transferred onto nitrocellulose filters. The probe, filter hybridization, and washing conditions were identical to those of Southern hybridization.

Results and Discussion

Identification of a homologue to the *S. cerevisiae* RAD3 gene

Comparision of DNA repair mechanisms between *S. cerevisiae* and man 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; Cal-

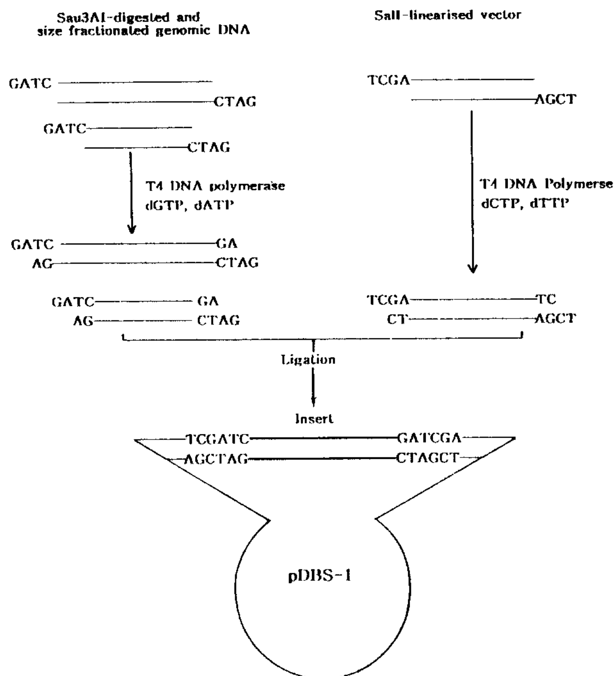

Fig. 1. Construction of *S. pombe* genomic library. The chromosomal DNA of *S. pombe* was treated with Sau3AI and then T4 DNA polymerase dor gap filling. This DNA was then ligated with SalI digested of pDB262 vector, thus generating pDBS-1, which contain the *S. pombe* chromosomal DNA. This recombinant DNA was screened on tetracycline plates.

decott *et al.*, 1994), 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 conserved between these two organisms.

In order to determine whether *S. pombe* contains a homologue to the conserved *S. cerevisiae* RAD3 gene, an *S. pombe* genomic library in pDB262 was constructed (Fig. 1). *S. pombe* genomic DNA was digested with Sau3AI and ligated with SalI-linearised pDB262 vector. To identify *S. pombe* RAD3 homologous gene, we took advantage of the highly conserved sequence elements within RAD3 gene and generated the degeneration oligo primers (see materials and methods). PCR reactions using these primers with *S. pombe* genomic DNA yielded 4 products (Fig. 2). Cloning and sequencing of these products revealed that only one DNA fragment contain the conserved *S. cerevisiae* RAD3 gene. The size of this DNA fragment showed

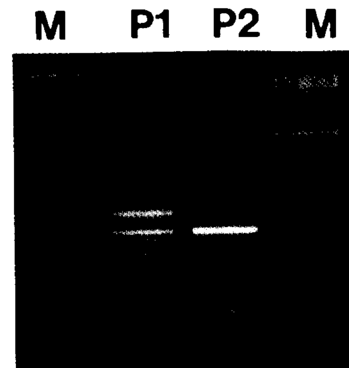

Fig. 2. Electrophoretic pattern of the PCR products. *S. pombe* genomic DNA was used as template for PCR amplification. After PCR, the product was analyzed on a 1.5% agarose gel. Lane M, 1 kb ladder DNA standard marker; P1, the product of B1 and B3 primer; P2, the product of B2 and B4 primer.

680 bp. This product was designated HRD3 (**H**omologous of **RAD3** gene). The nucleotide sequence of HRD3 product was determined.

The *S. cerevisiae* RAD3 gene encodes an ATP-dependent DNA helicase (Naumovski and Friedberg, 1988; Murray *et al.*, 1992), and by comparision with other helicase it has been shown to have the seven conserved helicase domains described by Gorbalenya *et al.* (1989). So, comparision of the deduced amino acid sequence with that of the *S. cerevisiae* and PCR product has 65% identity(data not shown). The high level of sequence homology suggests that the *S. pombe* HRD3 gene is also likely to encode an ATP-dependent DNA helicase. From this result, HRD3 contains similar sequence of RAD3 gene. To do overall these experiment, this PCR product directly used as a probe for identify of RAD3 homologous gene.

Genetic mapping of HRD3 gene

In order to confirm that *S. pombe* chromosome contains the same DNA as the PCR product, southern analysis was performed (Fig. 3). These restriction sites are identical to those found in RAD3 in *S. cerevisiae* (Naumovski and Friedberg, 1987). This result indicate that *S. pombe* chromosome contained the same locus as the HRD3 gene, and also suggesting that HRD3 locus existed as a single copy in *S. pombe* genome.

Transcriptional regulation

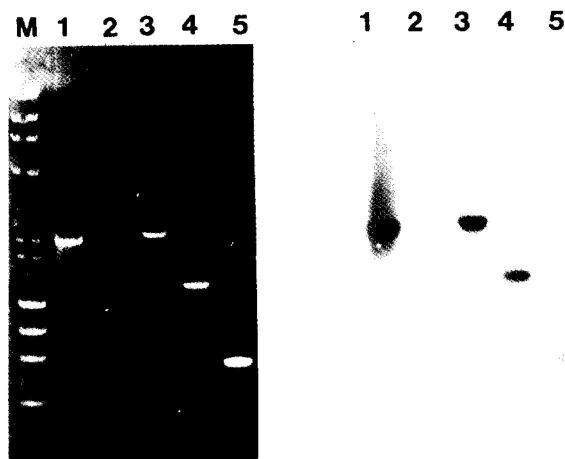

Fig. 3. Southern hybridization analysis of yeast chromosomal DNA and PCR product. Chromosomal DNA was digested with various restriction enzymes, electrophoresed on a 0.7% agarose gel (A), transferred to nitrocellulose filters and then hybridized with a ^{32}P-labelled 680 bp PCR product (B). Lane M, phage DNA digested with HindIII; 1, genomic DNA digested with BamHI ; 2, HindIII ; 3, SalI ; 4, SacI ; 5, XhoI.

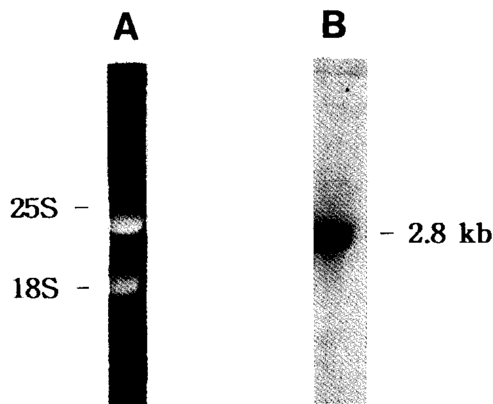

Fig. 4. Northern blot analysis of HRD3 mRNA in *S. pombe.* Total RNA was isolated, electrophoresed (A), transferred onto nitrocellulose filters, and then hybridized with the radiolabelled HRD3 DNA probe (B). 25S and 18S RNA was used as size markers. From this the estimated size of the transcript is 2. 8 kb.

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 PCR product. Northern blot analysis revealed 2. 8 kb mRNA transcript (Fig. 4). Also, *S. pombe* RNA strongly cross-hybridized with the 680 bp PCR fragment.

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 UV-damage, its mRNA level was determined after UV irradiation (200 J/m^2). At various times after UV irradiation, equal amount of total RNA samples prepared from *S. pombe* were hybridized with the radiolabeled PCR DNA fragment (Fig. 5). The result showed that the amount of HRD3 mRNA in *S. pombe* did not change upon UV-irradiation. Among the repair-related genes, the transcripts level of *S. cerevisiae* CDC9 and RAD2 gene were elevated after UV irradiation (Robinson *et al.,* 1986). Our present results indicate that the HRD3 gene is not UV inducible as the other genes in *S. cerevisiae* RAD3 epistatic group except for RAD2 gene.

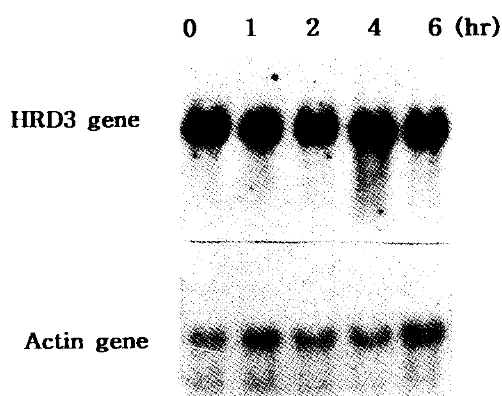

Fig. 5. Determination of UV-inducibility of the HRD3 gene. Total RNA was isolated from *S. pombe* cells at various postincubation times after UV irradiation. RNA was hybridized with the radiolabelled HRD3 DNA probe. The numbers at the top of each lane indicate the cell collection times after the UV irradiation.

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분열형 효모 *Schizosaccharomyces pombe*에서 RAD3 유사 유전자인 HRD3의 분리와 특성 연구

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적 요

본 연구는 출아형 효모 ***Saccharomyces cerevisiae***의 절제회복 유전자인 **RAD3**를 이용하여, 이와 유사한 유전자가 분열형 효모 ***Schizosaccharomyces pombe***에서도 존재하는지를 알기 위하여 **RAD3** 유전자의 **conserved sequence**를 **primer**로하여 중합효소 연쇄반응 **(PCR)** 방법으로 그 와 유사한 **680 bp** 크기의 **DNA fragment**를 분리하여, 이를 **HRD3**라 명명하였다. **RAD3** 유전자는 **DNA**의 절제회복 기작 중 **helicase activity**를 갖는 것으로 알려져있다. 따라서 본 연구에서 분리한 **HRD3** 유전자가 **helicase** 기능을 갖고 있는 **RAD3**유전자와의 **sequence homolology**를 비교한 결과 **65%**의 동일성을 나타내었다. 이 결과 **HRD3**도 이와 유사한 기능을 갖을 것으로 추측된다. 또한 **HRD3** 유전자가 ***S. pombe***의 염색체내에 존재함을 **Southern blot**으로 확인하였다. **Northern hybridization**으로 **HRD3** 유전자의 전사체 크기는 **2. 8 kb**임을 확인하였다. **Northern hybridization**으로 **HRD3** 유전자의 **UV inducibility**를 조사한 결과 자외선에 대하여 전혀 **inducibility**가 없었다.