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Characterization of UV-damaged repair genes in cells

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Abstract The *RAD4* gene of *Saccharomyces cerevisiae* is essential for the incision step of UV-induced excision repair. A yeast *RAD4* gene has been previously isolated by functional complementation. In order to identify the *RAD4* homologous gene from fungus *Coprinus cinereus*, we have constructed cosmid libraries from electrophoretically separated chromosomes of the *C. cinereus*. The 13 *C. cinereus* chromosomes were resolved by pulse-field gel electrophoresis, hybridized with *S. cerevisiae RAD4* DNA, and then isolated homologous C. *cinereus* chromosome. Here, we report the cloning and characterization of fungus *C. cinereus* homolog of yeast *RAD4* gene. Southern blot analysis confirmed that *C. cinereus* contains the sequence homologous DNA to *RAD4* gene and this gene exists as a single copy in *C. cinereus* genome. When total RNA isolated from *C. cinereus* cells was hybridized with the 3.4 kb *Bgl*II DNA fragment of the *S. cerevisiae RAD4* gene, a 2.5 kb of transcript was detected. The isolated gene encodes a protein of 810 amino acids.

Key words: *RAD4* homolog, pulse-field gel electrophoresis, *C.. cinereus*

Introduction

Excision 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, whereas several others, such as *RAD7, RAD16, RAD23*, and *MMS19*, affect the proficiency of excision repair [1-3]. In humans, seven xeroderma pigmentosum (XP) complementation groups, XPA through XPG, have been identified [4]. 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. Five human excision repair genes, ERCC1, ERCC2, ERCC3, ERCC5, and ERCC6 have been cloned by complementing the UV sensitivity of rodent cell lines, and all genes show holmology to *S. cerevisiae* genes [5-8]. The conservation of excision repair genes between yeast and human implies that information gleaned from yeast system would be applicable to higher eukaryotes, including humans.

The *S. cerevisiae RAD4* gene is of particular interest because of its requirement in excision repair. This gene have been isolated by phenotypic complementation with *rad* mutant. In our previous report, we have demonstrated that the *RAD4* gene contains 2190 nucleotides encoding 730 amino acids [9-11]. The *RAD4* gene is not essential for viability of the haploids under normal growth condition and that it is not UV-inducible [12]. In an attempt to gain insight into the extent of conservation in structure and function of *RAD4*, we have characterized the *RAD4* homolog from the fungus *Coprinus cinereus*. The basidiomycetes *C. cinereus* is an attractive model system for studies of meiosis and DNA repair, because it is a genetically tractable organism in which the process of meiosis is naturally synchronous.

To make more efficient recovery of *C. cinereus* genes, we decided to take advantage of recent advances in electrophoresis technology. The methods of pulse field gel electrophoresis(PFGE) have enabled the separation of chromosome-sized DNA molecules from a variety of fungi [13]. Fungi posses small genomes and have chromosomes that can be separated by pulse-field gel electrophoreses [14]. Most fungi contain low amount of repetitive DNA, almost all of which consist of rDNA occuring as a long tandemly repeated array of elements [15]. The remainder of the reiterated DNA consists mainly of short, low copy, interspersed repeats. Based on these observations, we could anticipate to order existing genomic DNA libraries according to chromosome with using PFGE-isolated chromosomes by hybridization probes. Here, I report a key step toward defining this system at the molecular level by the analysis of a gene implicated in the preferential repair pathway.

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Materials and Methods

Strains, plasmids, and genetic methods

S. cerevisiae wild type strain LP2693-21B was used for transformation. Fungi MZC3 strain was used for chromosome preparation. The cosmid vector was used for construction of chromosome library. Standard genetic techniques and growth media for *S. cerevisiae* were used [12]. Genetic techniques and media for *C. cinereus* were as described [16]. Yeast transformations were performed according to Ito *et al.* [17], and *C. cinereus* transformations were carried out according to Binninger *et al.* [18].

Preparation of *C. cinereus* chromosomes

C. cinereus chromosomes were prepared by a modification of a procedure developed by Zolan *et al.* [19]. Protoplasts were prepared from oidia as described except that the enzymes used to degrade the oidial cell walls were NovoZyme at 10 mg/ml. Washed protoplasts were resuspended in MM (0.5 M mannitol; 0.05M maleate, pH 5.5) at a concentration of 6 X 10^8 cells/ml. A 2% solution of low melting point agarose was prepared in MM, and held at 50^0C. Aliquots of protoplasts were mixed with an equal volume of agarose, and the solution was immediately used to fill a BioRad CHEF plug mold.

Gel Electrophoresis, transfer, and hybridization

CHEF gels were made in molds sold by the manufacturer. Gels were run at 60V, for about 6 days with a 22 minute pulse time, in 0.5X TBE, which is maintained at about 14^0C by circulation through a 4^0C water bath. We change the buffer 2-6 times during a 6 day run. CHEF gels were blotted to membranes as described [20]. Hybridizations were carried out for 18 hr at 68^0C in 0.5 M NaCl/0.1 M Na-phosphate, pH7.0/6 mM Na-EDTA, pH8.0/1% SDS/denatured salmon sperm DNA at a probe concentration of 1 x 10^6 dpm/ml. Blots were washed at 68^0C twice for 20 min in 2X SSC/1% SDS, twice for 20 min in 0.5X SSC, and subjected to autoradiography.

Construction of cosmid libraries

The cosmid vector was cut with *Bgl*II and dephosphorylated. The chromosomal DNAs of *C. cinereus* were electroeluted in 0.5X TBE for 13 hours at 70 volts. We used about 200 ng of chromosomal DNA with an average size of 40 kb. The DNA was ligated to 2 *u*g of cosmid vector, packaged *in vitro* (Stratagene), and the phage was used to infect bacterial strain NM554. We followed the manufacturer's instructions for packaging, bacterial preparation, and infection. We estimated that we recovered about 3 X 10^5 clones/*u*g insert DNA. Individual colonies were picked into 96-well microtiter dishes, each of which contained 200 *u*l freezing medium. Plates were incubated at 37^0C overnight, and stored at -80^0C.

Southern blot analysis

Chromosomal DNA from *C. cinereus* was digested to completion by various restriction enzymes, electrophoresed on a 0.7% agarose gel and transferred onto S&S Nytran membrane. The membrane was hybridized with ^{32}P-labelled *RAD4* DNA at 42^0C for 16 hours under 50% formamide condition.

Northern blot analysis

Total RNA was prepared according to Zolan *et al.* (19). RNA was denatured, fractionated on 1.2% agarose gel containing 0.66 M formaldehyde, and transferred onto S&S Nytran membrane. The probe and the filter hybridization/washing conditions were as described in the instruction manual [20].

Nucleotide sequence analysis

All sequencing reactions were performed by the double-stranded dideoxy method (Sanger et al., 21) using kits from Pharmacia LKB or US Biochemicals. Homology searches comparing the amino acid sequence were performed using FASTA [22].

Results and Discussion

Mapping of *RAD4* homologous gene in *C. cinereus*

Comparision of DNA repair mechanisms among the eukaryotic cells shows that a number of genes required for a nucleotide excision repair pathway are highly conserved among organisms [23,24]. However, it remains unclear whether a similar mechanism exists in fungus *C. cinereus*.

In order to confirm that *C. cinereus* chromosome contains a *RAD4* gene, Southern analysis was performed (Fig. 1). This results indicate that the *C. cinereus* chromosome contained the *RAD4* homologous gene and also suggest that two organisms have highly homologous *RAD4* gene and that

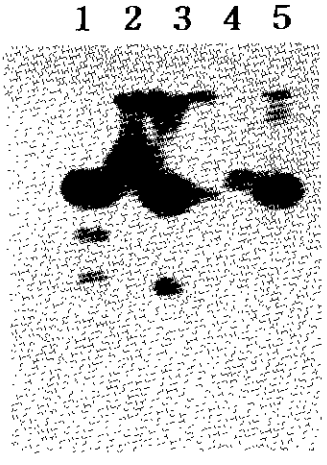

Fig. 1. Southern blot analysis of *C. cinereus* chromosomal DNA. Chromosomal DNA was isolated and digested with various restriction enzymes, electrophoresed, transferred to nitrocellulose filter and subsequently hybridized with the radiolabelled DNA probe. Lane 1, *Bam*HI; 2, *Bgl*II; 3, *Eco*RI; 4, *Hin*dIII; 5, *Pvu*II.

these genes are conserved during evolution. As a corollary, human *ERCC1* and *ERCC2* genes have been shown to share considerable structural similarity with *S. cerevisiae RAD10* and *RAD3* genes, respectively [25]. In addition, antisera raised against RAD3 protein appeared to react with two unidentified polypeptides extracted from human HeLa cells [26]. In view of such reports, it is not surprising that *RAD4* gene in *S. cerevisiae* is conserved in the fungus *C. cinereus*..

In order to confirm that *C. cinereus.* chromosome contains the *S. cerevisiae RAD4* gene, total RNA isolated from *C. cinereus* cells were hybridized with the *RAD4* clone (pPC100). Northern hybridization analysis showed that *C. cinereus* RNA strongly cross-hybridized with the 3.4kb BglII DNA fragment of pPC100 (Fig. 2B). This result indicates the presence of a *RAD4* homologous transcript in *C. cinereus*. From this result, we estimated that the size of transcript was 2.5 kb (Fig. 2A).

Construction of chromosome specific libraries

To construct chromosome specific libraries of *C. cinereus,* we achieved separation of a specific chromosome (Fig. 3A). The *C. cinereus* has 13 chromosomes, whose sizes vary from about 1 to 5 megabases. These chromosomes fractionated were hybridized with *RAD4* DNA. This result indicated that the *RAD4* homologous gene is located on chromosome 6 of *C. cinereus* (Fig. 3B). The chromosome 6 was electro-

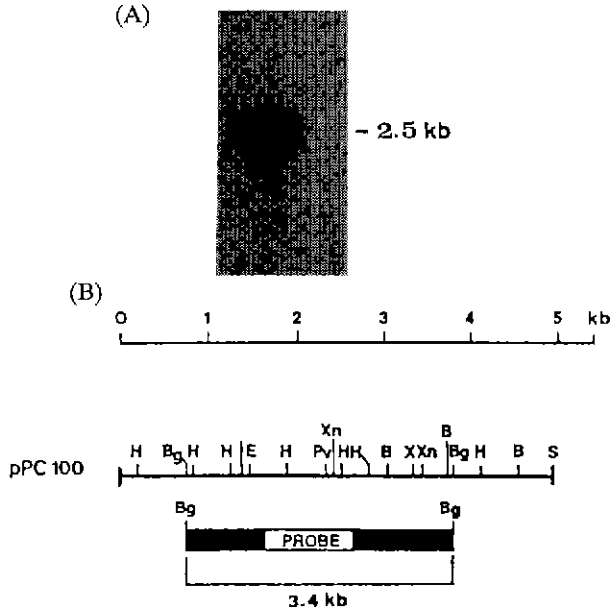

Fig. 2. Northern blot analysis of *RAD4* homolog mRNA in *C. cinereus*. (A) Total RNA was isolated, electrophoresed, transferred onto nitrocellulose filters, and then hybridized with the radiolabelled *RAD4* probe. The 2.5 kb transcript is indicated. (B) The 3.4 kb *Bgl*II DNA fragment of the internal sequence of the cloned *RAD4* gene was used as the DNA probe. Various restriction enzyme sites of *RAD4* gene are indicated.

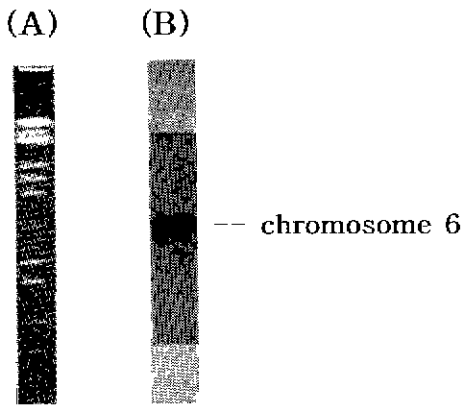

Fig. 3. Seperation of intact chromosomes of *C. cinereus*. (A) Gel was run at 60V for 6 days. The position of each chromosome was observed between marker fragment size and distance traveled. (B) The seperated chromosomes were hybridized with *RAD4* DNA probe (Fig. 1B). The *RAD4* DNA was hybridized with *C. cinereus* chromosome 6 and the hybridized chromosome was electroeluted from gels.

eluted, digested with *Mbo*I, and ligated with *Bgl*II-cut cosmid vectors. We estimated that we had recovered 3 X 10^5 colonies per microgram of ligated DNA. To isolate the homologous DNA, the chromosome specific libraries were hybridized with *S. cerevisiae RAD4* DNA (Fig. 2B). Approximately 10,000 colonies were screened, and seven positive clones were selected, all of which contained the identical 3.2 kb *Hin*dIII DNA insert. These colonies were subcultured and their DNAs were isolated. To confirm that these DNAs contained the homologous DNA with *S. cerevisiae RAD4*, we will determined the nucleotide sequence of the inserts.

Nucleotide and amino acid sequence of *RAD4* homology gene

The nucleotide sequence of the *RAD4* homology gene in *C. cinereus* was shown in Fig. 4. The first ATG codon starts an open reading frame (ORF) of +44 bp. The purine found at the important -3 position before a start codon is present. All other ORFs present encode much smaller polypeptides. Therefore, we conclude that the ORF of 3345 bp, deficted in figure 4, defines the RAD4 homologous protein. The deduced amino acid sequence specifies a protein of 1115 amino acids. Although a systematic computer search did not identify any protein with overall homology to *RAD4* homologous gene.

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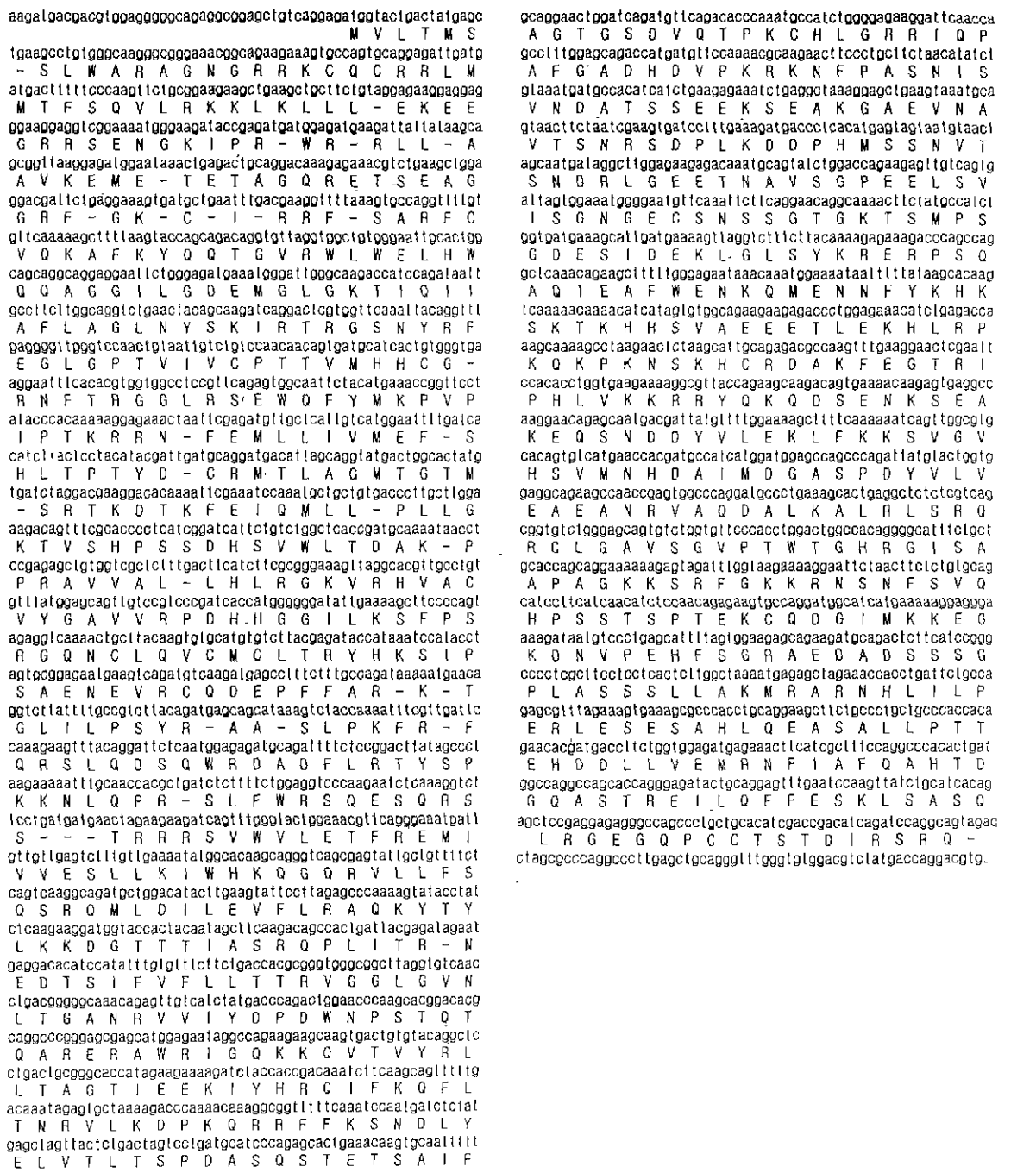

Fig. 4. Nucleotide sequence of *RAD4* homolog gene and the deduced sequence of protein.

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