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Characterization of *RAD4* Homologous Gene from *Coprinus cinereus*

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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. The insert DNA of the *RAD4* homolog was contained 3.2 kb. Here, we report the characterization of fungus *C. cinereus* homolog of yeast *RAD4* gene. Southern blot analysis confirmed that *C. cinereus* contains the *RAD4* homolog 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 1.2 kb *Pvu*II DNA fragment of the *S. cerevisiae RAD4* gene, a 2.5 kb of transcript was detected. In order to investigation whether the increase of transcripts by DNA damaging agent, transcripts levels were examined after treating the cells. The level of transcript did not increase by untraviolet light (UV). This result indicated that the *RAD4* homologous gene is not UV inducible gene. Gene deletion experiments indicate that the *RAD4* homologous gene is essential for cell viability.

Key words – *RAD4* homolog, *C. cinereus*, cell viability, DNA repair, essential gene

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[3,7,8,9]. In humans, seven xeroderma pigmentosum (XP) complementation groups, XPA through XPG, have been identified[10,11]. 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[3,4]. 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 demon-

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strated that the *RAD4* gene contains 2190 nucleotides encoding 730 amino acids [5,14]. The *RAD4* gene is not essential for viability of the haploids under normal growth condition and that it is not UV-inducible [5,6,14].

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[23]. Fungi possess small genomes and have chromosomes that can be separated by pulse-field gel electrophoreses[23]. Most fungi contain low amount of repetitive DNA, almost all of which consist of rDNA occuring as a long tandemly repeated array of elements. 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, we report a *RAD4* homologous gene from fungus *C. cinereus* and yeast *Schizosaccharomyces pombe*. At present, however, the extent of regulation of this gene expression and functional role of its gene product are unknown. As a first step in elucidation of such, we have analyzed the RNA expression and its cell viability of *RAD4* homolog in fungus.

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[6,17,18]. Genetic techniques and media for *C. cinereus* were as described and *C. cinereus* transformations were carried out according to Zolan *et al.* [2, 15]. Yeast transformations were performed according to Ito *et al* [12].

Preparation of *C. cinereus* chromosomes

C. cinereus chromosomes were prepared by a modification of a procedure developed by Zolan *et al.* [23]. Protoplasts were prepared using NovoZyme (10 mg/m)l. Washed protoplasts were resuspended in MM (0.5 M mannitol; 0.05M maleate, pH 5.5) at a concentration of 6×10^8 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.

Southern blot analysis

Chromosomal DNA was digested for a gel blot analysis with various restriction enzymes, separated 0.8% agarose gel, and transferred onto a nylon membrane (Hybond-H^+, Amersham) using the manufacturer's protocol. The membrane was hybridized with *RAD4* DNA fragment, which was gel purified and [a-^{32}P] dCTP labeled using the random priming method with a Megaprime Labeling Kit (Amersham) and a Quick Hybridization (Stratagene) at 68^0C. After hybridization, the membrane was washed twice in 2X SSPE, 0.1% SDS and 0.2X SSC, 0.01% at 55^0C. After the final rince, the membrane was wrapped with plastic wrap, and exposed onto X-ray film (X-Omat, Kodak) for 12 hrs or more.

UV survival test

A survival test was performed as previously described [13,15]. For UV survival, 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 30^0C for 4 to 5 days, 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 of DNA damaging agent and northern blot analysis

One hundred milliliters of cells grown to mid exponential stage were harvested, washed and then resuspended in 10 ml of distilled water. The cell suspension was evenly spreaded onto 150mm petri dish and then exposed to 200 J/m^2 of ultraviolet (UV)-light from mercury germicidal lamp. The irradiated cells were inoculated into fresh YES medium, incubated at 30^0C in the dark, and collected at indicated times.

Total RNA was prepared according to Jang *et al.*[13]. 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

Expression 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[16-18]. However, it remains unclear whether a similar mechanism exists in fungus *C. cinereus*.

We have observed that the *RAD4* gene in *S. cerevisiae* encodes a single 2.3 kb mRNA without any intervening sequences. In addition, we have also identified the *RAD4* homologous gene from fission yeast *Schizosaccharomyces pombe*, and determined the sizes of the gene transcripts to be 3.1 and 1.8 kb[6,14].

In order to determine whether *C. cinereus* contains a homolog of *S. cerevisiae RAD4* gene, total RNA isolated from *C. cinereus* cells were hybridized with the *RAD4* clone (pPC100). Results from Northern hybridization analysis showed that *C. cinereus* RNA strongly cross-hybridized with the 1.2 kb *Pvu*II DNA fragment of pPC100 (Fig. 1A), indicating the presence of a *RAD4* homologous transcript. From this result, we estimated that the size of transcript was 2.5 kb (Fig. 1B) and subsequently examined the genome of *C. cinereus* for the presence of *RAD4* homolog sequence by Southern blot analysis.

Genetic mapping of *RAD4* homologous gene

In order to confirm that *C. cinereus* chromosome contains the same DNA as the *RAD4* gene, southern analysis was performed (Fig. 2). These restriction sites are identical to those found in *RAD4* homolog in *S. cerevisiae*[6]. This result indicate that *C. cinereus* chromosome contained the same locus as the *RAD4* gene, and also suggesting that *RAD4* homologous gene existed as a single copy in *C. cinereus* genome. This result indicated that the *C. cinereus* chromosome contained the *RAD4* homologous gene and also suggest that two organisms have highly homologous *RAD4* gene and that 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 [19-21]. In addition, antisera raised against Rad3 protein appeared to react with two unidentified polypeptides extracted from human HeLa cells. In view of such reports, it is not surprising that *RAD4* gene in *S. cerevisiae* is conserved in the fungus *C. cinereus*.

Transcriptional regulation by treatment of DNA damaging agent

Although several DNA damage inducible genes have

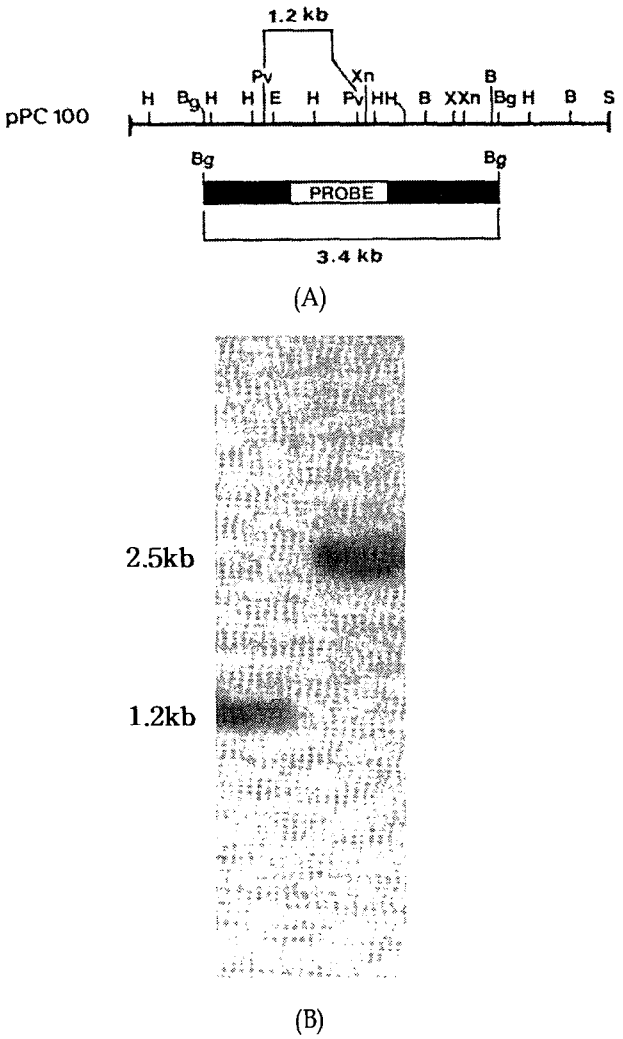

Fig. 1. Northern blot analysis of *RAD4* homologous mRNA in *C. cinereus*. (A) Total RNA was isolated, electrophoresed, transferred onto nitrocellulose filters, and then hybridized with the radiolabelled *RAD4* probe. The 2.5 kb transcript is indicated. (B) The 1.2 kb *Pvu*II DNA fragment of the internal sequence of the cloned *RAD4* gene was used as the DNA probe. Various restriction enzyme sites of *RAD4* gene are indicated.

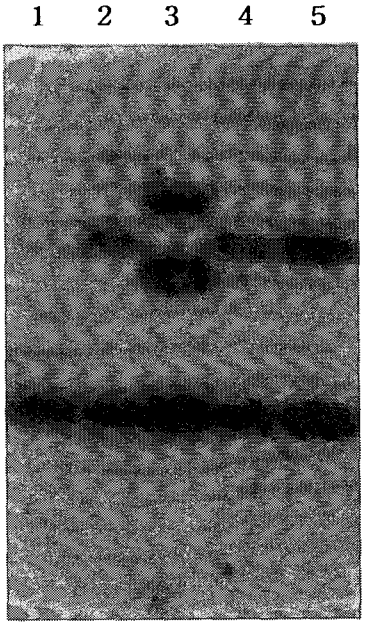

Fig. 2. Southern blot analysis of *C. cinereus* chromosomal DNA. Chromosomal DNA was isolated and digested with various restriction enzymes, electrophoresed, transferred to nitrocellulose filter and subsequently hybridized with the radiolabelled DNA probe. Lane 1, *Bam*HI; 2, *Bgl*II; 3, *Eco*RI; 4,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

been identified from *S. cerevisiae*, it is not known whether *RAD* genes belong to this class. To determine whether the *RAD4* gene transcription in *C. cinereus* is regulated by DNA damaging agents, its mRNA levels were measured after UV irradiation (200J/m^2). At various times after UV irradiation, equal amount of total RNA samples prepared from *C. cinereus* was hybridized with the radiolabeled DNA fragment (Fig 3). This result showed that the amount of *RAD4* homologous mRNA in *C. cinereus* did not change upon UV-irradiation. Among the repair-related gens, the transcripts level of *S. cerevisiae CDC9* and *RAD2* gene were elevated after UV irradiation [18,22]. This result indicate that the *RAD4* homologous gene is not UV inducible as the other genes in *S. cerevisiae* RAD3 epistasis group except for *RAD2* gene.

To determine the steady-state level of *RAD4* homologous transcripts during growth, *C. cinereus* cells were cultured in rich medium and collected at every 2 hours to prepare total RNAs. The Northern blot analysis showed that the level of transcripts reached its maxim after the cells entered the exponential growth phase and then decreased gradually (Fig. 4). This expression pattern

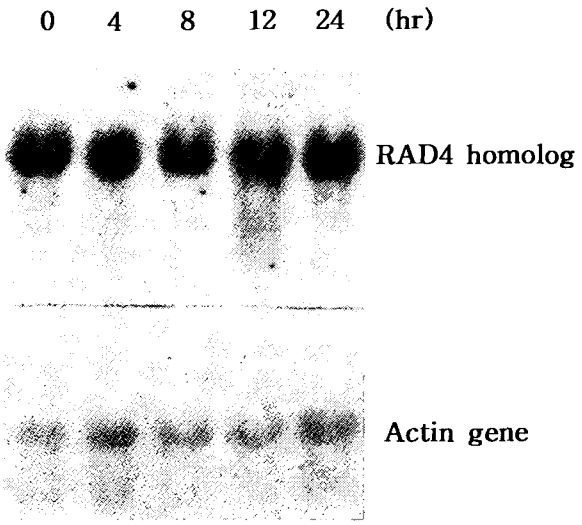

Fig. 3. Determination of UV-inducibility of the *RAD4* homologous gene. Total RNA was isolated from *C. cinereus* cells at various postincubation times after UV irradiation and hybridized with the radiolabelled *RAD4* DNA probe (Fig. 1B). The actin gene was used as a internal control.

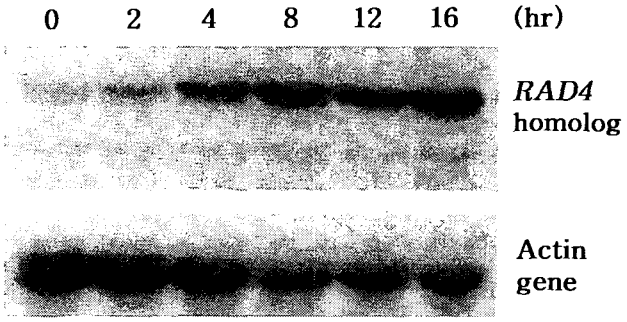

Fig. 4. The change of *RAD4* homologous transcript level during growth stage. Cells in log phase were diluted to a final concentration of 1×10^5 cells/ml and were grown. RNA was extracted and then northern blot analysis was performed using DNA fragment indicated in Fig. 1 as a probe.

was similar with that of *RAD4* transcripts, implying that *RAD4* homologous gene may be required at late stages of cell growth.

RAD4 homologous gene deletion

To determine the *RAD4* homologous gene is required for cell viability and DNA repair, *RAD4* homologous gene 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 *RAD4* homologous gene mutations was verified by Southern blot analysis. The *RAD4* homologous gene deleted strain was not well grown compared with *RAD4* homologous gene (Fig. 5). This result indicated that *RAD4* homologous gene is essential for cell viability.

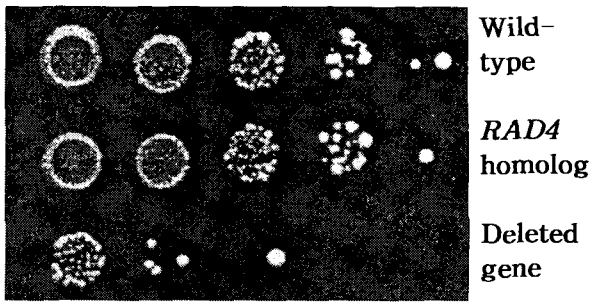

Fig. 5. *RAD4* homologous gene restores cell viability and DNA repair. *RAD4* homologous gene is an essential gene for cell growth. Cells from a single colony were streaked onto YES medium and incubated at 30°C for 3 days.

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초록 : 균류 *Coprinus cinereus*에서 DNA 회복에 관여하는 *RAD4* 유사유전자의 분리와 특성

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본 연구는 출아형 효모 *Saccharomyces cerevisiae*에서 자외선의 상해 시 이를 정상으로 회복시키는 절제회복 (excision repair) 유전자로 알려진 *RAD4*의 특성 규명을 위하여 균류 *Coprinus cinereus*에서 이와 유사한 유전자를 분리하였다. *RAD4* 유사 유전자를 분리하기 위하여 균류 *C. cinereus*의 염색체 DNA를 전기영동하여 분리한 다음 효모 *RAD4* DNA를 probe로하여 이와 hybridization하였다. 이 결과 *RAD4* 유사 유전자는 3.2 kb의 insert DNA를 갖고 있었다. 또한 Southern hybridization으로 이 유사 유전자는 fungus *C. cinereus*의 염색체에 존재함을 확인하였다. 분리한 *RAD4* 유사 유전자의 전사체 크기는 2.5 kb 였으며, 자외선의 상해 시 전혀 'inducibility가 없음을 Northern hybridization으로 확인하였다. 또한 유사유전자 부분을 삭제하였을 때 이 부분이 없는 세포는 전혀 생존을 못하였다. 이 결과 분리한 RAD4 유사유전자는 세포의 생존에 관여함을 알 수 있었다.