Environmental Mutagens & Carcinogens 17-1 : 1-6 (1996)

Characterization of Excision Repair Genes Related to Damaged DNA Repair from Eukaryotic Cells

In Soon Choi*, Yong Hwan Jin[1] and Sang Dai Park[1]

Department of Biology, Pusan Women's University, Pusan 616-736, Korea

[1]Department of Molecular Biology, Seoul National University, Seoul 151-742, Korea

(Received, january 14, 1997)

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 partial 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 1.2 kb PvuII DNA fragment of the *S. cerevisiae RAD4* gene, a 2.5 kb of transcript was detected. The level of the transcript did not increase upon UV-irradiation, suggesting that the *RAD4* homologous gene in *C. cinereus* is not UV-inducible.**

Keywords : UV inducibility, RAD4 homolog, pulse-field gel electrophoresis, *S. cerevisiae*

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 (French *et al.*, 1991; Miller *et al.*, 1982; Reynolds et al., 1992). In humans, seven xeroderma pigmentosum (XP) complementation groups, XPA through XPG, have been identified (Bootsma *et al.*, 1989). 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 incisidence 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 (Reynolds and Friedberg, 1981; van Duin *et al.*, 1986; Weber *et al.*, 1990; Weeda *et al.*, 1990). 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 previously report we have demonstrated that the *RAD4* gene contains 2190 nucleotides encoding 730 amino acids (Choi *et al.*, 1990; Choi *et al.*, 1993; Kim *et al.*, 1994). The *RAD4* gene is not essential for viability of the haploids under normal growth condition and that it is not UV-inducible (Choi *et al.*, 1991).

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 *C. cinereus*. The basidiomycetes *Coprinus 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 more efficiently, we decided to take advantage of recent advances in

*To whom correspondence should be addressed.

electrophoresis technology. The methods of pulse field gel electrophoresis have enabled the separation of chromosome-sized DNA molecules from a variety of fungi (Skinner *et al.*, 1991). Fungi posses small genomes and have chromosomes that can be separated by pulse-field gel electrophoreses (PFGE) (Baker *et al.*, 1992). Most fungi contain low amounts of repetitive DNA, almost all of which consist of rDNA occuring as a long tandemly repeated array of elements (Brody *et al.*, 1990). The remainder of the reiterated DNA consists mainly of short, low copy, interspersed repeateds. 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 UV inducibility of *RAD4* homolog in fungus and yeast.

Materials and Methods

Strains, plasmids, and genetic methods

S. cerevisiae wild type strain LP2693-21B was used for transformatiom. 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 (Choi *et al.*, 1991). Genetic techniques and media for *C. cinereus* were as described (Guha and Guschlbauer, 1992). Yeast transformations were performed according to Ito *et al.* (1983), and *C. cinereus* transformations were carried out according to Binninger *et al.* (1991).

Preparation of *C. cinereus* chromosomes

C. cinereus chromosomes were prepared by a modification of a procedure developed by Zolan *et al.* (1992). 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; 0.05 M 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°C. 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°C by circulation through a 4°C water bath. We change the buffer 2-6 times during a 6 day run. CHEF gels were blotted to membranes as described (Sambrook *et al.*, 1989). Hybridizations were carried out for 18 hr at 68°C 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 106 dpm/ml. Blots were washed at 68°C 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 BglII and dephosphorylated. The chromosomeal DNAs of *C. cinereus* were electroeluted in 0.5X TBE for 13 hours at 70 volts. We used about 200 ng chromosomal DNA with an average size of 40 kb. The DNA was ligated to 2 ug 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 3×10^5 clones/ug insert DNA. Individual colonies were picked into wells of 96-well microtiter dishes, each of which contained 200 ul freezing medium. Plates were incubated at 37°C overnight, and stored at -80°C.

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°C for 16 hours under 50% formamide condition.

Treatment of DNA damaging agent

The *C. cinereus* cells were spread onto plates at appropriate concentrations and irradiated on the plates before incubation. The UV dose rate was 1.6 J/m^2/s (using a GE germicidal 30W lamp). Plates were rotated for even exposure.

Northern blot analysis

Total RNA was prepared according to Zolan *et al.* (1992). 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 (Sambrook *et al.*, 1989).

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 (Thompson *et al.*, 1988; Weeda *et al.*, 1990; Murray *et al.*, 1992). 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 (Choi *et al.*, 1991).

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. 1B), indicating the presence of a *RAD4* homologous transcript. From this result, we estimated that the size of transcript was 2.5 kb (Fig. 1A) and subsequently examined the genome of *C. cinereus* for the presense of *RAD4* homolog sequence by Southern blot analysis.

As described in the legend to Fig. 2, chromosomal DNA from *C. cinereus* was digested with various restriction enzymes. The 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 these genes are conserved during evolution. As a corollary, human *ERCC1* and *ERCC2* genes have been shown to share considerable structural similarity with *S. cerevise RAD10* and *RAD3* genes, respectively (McCready *et al.*, 1989; Hoeijmakers and Bootsma, 1990; Weber *et al.*, 1990; Friedberg, 1991). In addition, antisera raised against *RAD3* protein appeared to react with two unidentified polypeptides extracted from human HeLa cells (Bailly *et al.*, 1991). In view of such reports, it is not surprising that RAD4 gene in *S. cerevisiae* is conserved in the fungus *C. cinereus.*

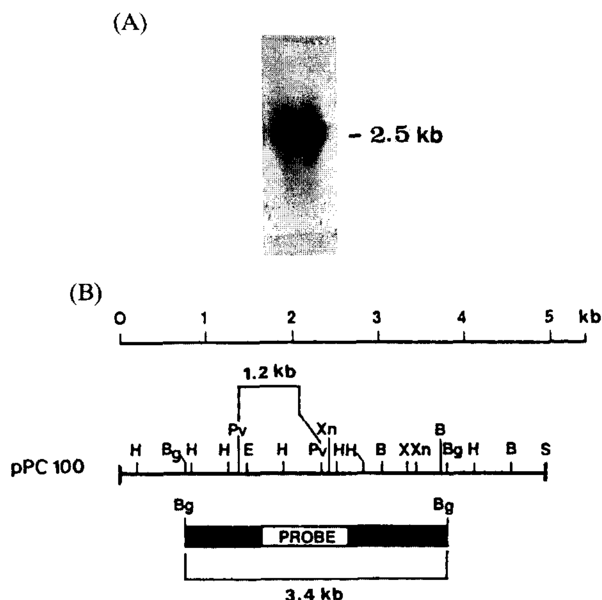

Fig. 1. Northern blot analysis of *RAD4* homolog mRNA in *C. cinereus*. (A) Total RNA was isolated, electrophoresed, transferred onto nitrocellulose filters, and then hybridized with the radiolabelled *RAD4* probe. The 2.5 kb transcript is indicated. (B) The 1.2 kb PvuII DNA fragment of the internal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

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 from about

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 *RAD4* DNA probe as indicated in Fig 1B. Lane 1, *Bam*HI; 2, *Bgl*II; 3, *Eco*RI; 4, *Hin*dIII; 5, *Pvu*II.

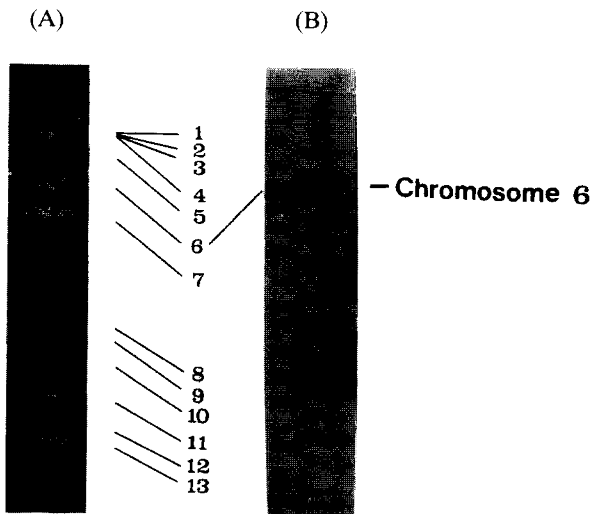

Fig. 3. Seperation of intact chromosomes of *C. cinereus*. (A) Gel was run at 60V for 6 days. The position of each chromosome was observed between marker fragment size and distance traveled. (B) The seperated chromosomes were hybridized with *RAD4* DNA probe (Fig. 1B). The *RAD4* DNA was hybridized with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

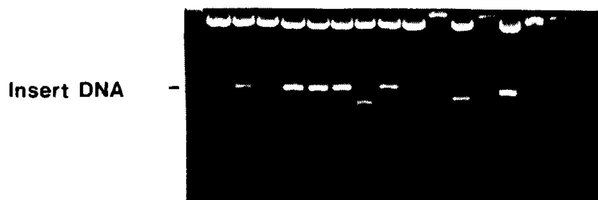

Fig. 4. Determination of insert DNA for putative clone. The chromosome 6 was cut from a CHEF gel, digested with *Mbo*I, and ligated with cosmid vector. Samples were packed *in vitro*, and used to transform bacterial host strains. Ampicillin-resistant colonies were picked for DNA isolation. The isolated DNA was digested with *Hin*dIII enzyme, and then electrophoresed.

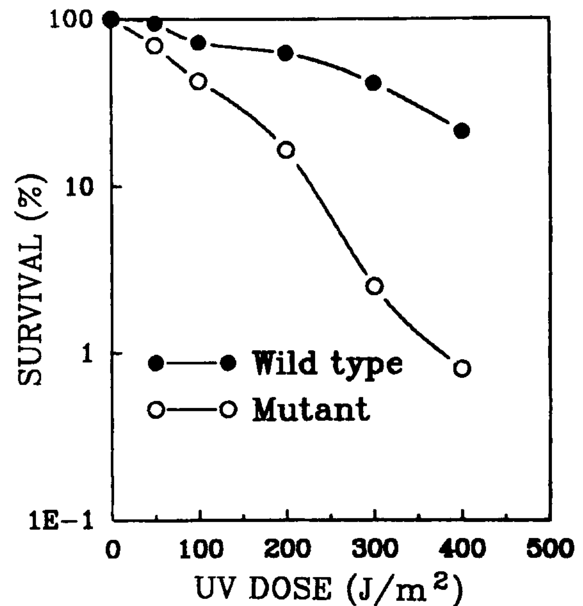

Fig. 5. Comparative UV susceptibility of *C. cinereus*. Cells were grown, spread onto agar plate, and exposed to various UV doses. Symbols; ●, wild type; ○, mutant.

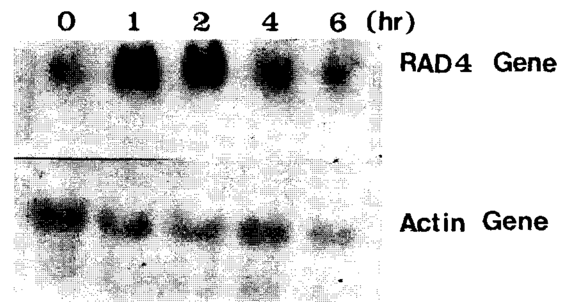

Fig. 6. Determination of UV-inducibility of the *RAD4* homolog gene. Total RNA was isolated from *C. cinereus* cells at various postincubation times after UV irradiation and hybridized with the radiolabelled *RAD4* DNA probe (Fig. 1B). The actin gene was used as a internal control.

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 electroeluted, digested with MboI, and ligated with *Bgl*II-cut cosmid vectors. We estimated that we had recovered 3×10^5 colonies per microgram of ligated DNA. To isolate the homologous DNA, the chromosome specific libraries were hybridized with *S. cerevisiae RAD4* DNA (Fig. 1B). 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 (Fig. 4). 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.

Transcriptional regulation by treatment of DNA damaging agent

Although several DNA damage inducible genes have 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 UV damage, its mRNA level was measured after UV irradiation.

The survival of *C. cinereus* cells after exposure to UV was measured. As shown in Fig. 5, cell viability in mutant cells was reduced to 10% by the exposure to 200J UV light. Under these conditions, the viability of wild type cells was 80%. From this result, we used 200J UV light to examine the UV inducibility.

At various times after UV irradiation, equal amounts of total RNA samples prepared from *C. cinereus* were hybridized with the *RAD4* DNA probe (Fig. 1B). This result showed that the amount of *RAD4* mRNA in C. cinereus did not change upon UV-irradiation (Fig. 6). Therefore, *RAD4* homolog gene is not inducible by DNA damage caused by UV-light.

The functional role of the presently identified *RAD4* homolog gene in *C. cinereus* is under investigation in our laboratory.

Acknowledgments

The authors are very grateful to Dr. N. Heyler (NIH, NCI, U.S.A.) for providing the Fungi *C. cinereus* strain and chromosome preparation. This work was supported by a Genetic Enginnering Research grant (1996) from the Minisrty of Korea (In Soon Choi), and in part by grants from the Korea Science and Engineering Foundation (53-0400-06-02-3).

References

Baker, S.M., Margison, G.P., and Striker, P., (1992): Inducible alkytransferase DNA repair proteins in the filamentous fungus nidulans. *Nucleic Acids. Res.* **20**(4), 645-651.

Brody, H. Griffith, J. Cuticchia, A.J. Arnold, J. and Timberlake, W.E., (1991): Chromosome specific recombinant DNA libraries from the fungus Aspergillus nidulans. *Nucleic Acids. Res.* **19**(11), 3105-3109.

Choi, I.S., Kim, J.B. and Park, S.D., (1990): Nucleotide sequence of RAD4 gene of Saccharomyces cerevisiae that can be propagated in Escherichia coli without inactivation. *Nucl. eic Acids Res.* **18**, 7137.

Choi, I.S., Kim, J.B., Hong, S.H. and Park, S.D., (1991): A gene in Schizosaccharomyces pombe analogous to the RAD4 gene of Saccharomyces cerevisiae. *FEMS Microbiol. Lett.* **77**, 97-100.

Choi, I.S., Kim, J.B., Jeon, S.H. and park, S.D., (1993): Expression of RAD4 gene of Saccharomyces cerevisiae that can be propagated in Escherichia coli without inactivation. *Biochem. Biophy. Res. Commu.* **193**(1), 91-197.

Fenech, M., Carr, A.M., Murray, J., Watts, F.Z., and Lehmann., A.R., (1991): Cloning and characterization of the *RAD4* gene of Schizosaccharomyces pombe; a gene showing short regions of sequence similarity to the human XRCC1 gene. *Nucleic Acids Res.* **19-24**, 6737-6741.

Friedberg, E. C., (1988): Deoxyribonucleic acid repair in the yeast Saccharomyces cerevisiae. *Microbiol. Rev.* **52**, 70-102.

Guha, S., and Guschlbauer, w., (1992): Expression of Escherichia coli dam gene in Bacillus subtilis provokes DNA damage response: N6-methyadenine is removed by two repair pathways. *Nucleic Acids Res.* **20**(14), 3607-3615.

Hoeijmakers, J.H.J. and Bootsma, D., (1990): Molecular genetics of eukaryotic DNA excision repair. *Cancer Cells* **2**, 311-320.

Ito, H. Fukuda, Y., Murata, K., and Kimmura, A., (1983): Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**, 163-168.

Kim, J.B., Jeon, S.H., Choi, I.S. and Park, S.D., (1994): Overexpressed RAD4 protein required for excision repair of Saccharomyces cerevisiae is toxic to the host *Escherichia coli. In Vitro Toxicology* **7**(3), 269-275.

McCready, S.J., Burkill, H., Evans, S., and Cox, B.S., (1989): The Saccharomyces cerevisiae RAD2 gene complements a Schizosaccharomyces pombe repair mutation. *Curr. Genet.* **15**, 27-30.

Miller, R.D., Prakash, L. and Prakash, S., (1982): Genetic control of excision of Saccharomyces cerevisiae interstrand DNA cross-links induced by psoralen plus near-UV light. *Mol. Gen. Genet.* **188**, 235-239.

Murray, J.M., Carr, A.M., Lehmann, A.R. and Watts, F. Z., (1991): Cloning and characterization of the rad15 gene, a homologue to the S. cerevisiae RAD3 and human ERCC2 gene. *Nucleic Acids Res.* **19**, 3525-3531.

Reynolds, R.J. and Friedgerg, E. C., (1981): Molecular mechanisms of pyrimidine dimer excision of ultraviolet-irradiated deoxyribonucleic acid. *J. Bacteriol.* **146**, 692-704.

Reynolds, P.R., Biggar, S., Prakash, L., and Prakash, S., (1992): The Shizosaccharomyces pombe rhp3+ gene required for DNA repair and cell viability is functionally interchangeable with the RAD3 gene of Saccharomyces cerevisiae. *Nucleic Acids Res.* **20**(9), 2327-2334.

Sambrook, J., Fritsch, E.F. and Maniatis, T., (1989): *Molecular Cloning: A Laboratory Mannual (2nd). Cold Spring Harbor Laboratory Press, New york.*

Thompson, L.H., Mitchell, D.L., Regan, J.D., Bouffler, S.D., Stewart, S.A., Carrier, W.L., Nairn, R.S., and Johnson, R. T., (1988): CHO mutant UV61 removes photoproducts but not cyclobutane dimers. *Mutagenesis* **4**, 140-146.

Van Duin, M., De Wit, J., Odijk, H., Westerveld, A., Yasui, A., Koken, M.H.M., Hoeijmakers, J. H. J. and Bootsma, D., (1986): Molecular characterization of the human excision repair gene ERCC1: cDNA cloning and amino acid homology with the yeast DNA repair gene RAD10. *Cell* **44**, 913-923.

Weber, C.A., Salazar, E.P., Stewart, S.A., and Thompson, L. H., (1990): ERCC2: cDNA cloning and molecular characterization of a human nucleotide excision repair gene with high homology to yeast RAD3. *EMBO J.* **9**, 1437-1447.

Weeda, G., Van Ham, R. C. A., Vermeulen, W., Bootsma, D., Van Der Eb, A. J., and Hoeijmakers, J.H.J., (1990): Molecular cloning and biological characterization of the human excision repair gene *ERCC3*. *Cell* **62**, 6160-6171.

Zolan, M.E., Crittenden, J., Heyler, N.K.., and Seitz, L.C. (1992): Efficient isolation and mapping of rad genes of the fungus Coprinus cinereus using chromosome specific libraries. *Nucleic Acids Res.* **20**(15), 3993-3999.

진핵세포에서 DNA 회복에 관여하는 유전자의 분리와 발현 연구

최인순 · 진용환[1] · 박상대[1]
부산여자대학교 생물학과, [1]서울대학교 분자생물학과

적 요

본 연구는 출아형 효모 ***Saccharomyces cerevisiae***에서 자외선의 상해시 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 **DNA**를 전기영동하여 분리한 다음 효모 ***RAD4*** **DNA**를 **probe**로하여 이와 **hybridization**하였다. 이 결과 **RAD4** 유사 유전자는 **3.2 kb**의 **insert DNA**를 갖고 있었다. 또한 **Southern hybridization**으로 이 유사 유전자는 **fungus *C. cinereus***의 염색체에 존재함을 확인 하였다. 분리한 ***RAD4*** 유사 유전자의 전사체 크기는 **2.5 kb**였으며, 자외선의 상해시 전혀 **inducibility**가 없음을 **Northern hybridization**으로 확인하였다.