J. ENVIRON. TOXICOL.
Vol. 18, No. 4, 287~294 (2003)

Overexpressed HRD3 Protein Required for Excision Repair of *Schizosaccharomyces pombe* is Toxic to the Host Cell

In Soon Choi

Department of Life Science, Silla University, Pusan 617-736, Korea,
Marine-Biotechnology Center for Bio-Functional Material Industries

효모에서 절제회복에 관여하는 *HRD3* 유전자 과 발현이 숙주세포에 미치는 영향

최 인 순

신라대학교 생명과학과

요 약

출아형 효모 *Saccharomyces cerevisiae RAD3* 유전자는 절제회복 및 세포의 생존에 필수적이며, DNA dependent ATPase와 DNA-RNA helicase 활성을 가지고 있는 것으로 알려져 있다. 본 연구는 분열형 효모 *Schizosaccharomyces pombe*에서 절제회복과 세포의 생존에 필수적인 출아형 효모 *RAD3* 유전자와 유사한 유전자를 *S. pombe* genomic DNA library에서 분리하여 그 특성을 연구하였다. 분리한 *RAD3* 유사유전자를 *HRD3* 유전자라 명명하였다. 발현 vector pET3a를 이용하여 분리한 *HRD3* 유전자를 과 발현 하였을 때, HRD3 단백질은 숙주단백질의 합성 억제 또는 분해 촉진을 유발하여 숙주세포인 대장균에 독성효과를 나타냄이 관찰되었다. *HRD3* 유전자와 *lacZ* 유전자를 융합시킨 여러 가지 재조합 vector를 만들어 이들 융합단백질을 분리하였다. 이 결과 HRD3 단백질의 카르복실 말단 부위가 DNA 회복기능과 대장균에서의 독성효과를 나타내는 중요한 부위로 생각된다.

Key words : *HRD3* gene, *Schizosaccharomyces pombe*, toxic, overexpressed,

INTRODUCTION

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 *et al.*, 1987; Choi *et al.*, 1990; Choi, 2003), whereas several others, *RAD7, RAD16, RAD23*, and *MMS19*, affect the proficiency of excision repair. 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 complementa-

※ To whom correspondence should be addressed.
Tel: +82-51-309-5348, E-mail: ischoi@silla.ac.kr

tion 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 (Kim *et al.*, 1990; Miller et la., 1982; Naumovski and Friedberg, 1982).

The *RAD3* gene is required at an early stage in the excision repair of UV damage. 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 (Boothmann *et al.*, 1993; Choi *et al.*, 1993). 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 (Fleer *et al.*, 1987; Tompson *et al.*, 1988; Weber *et al.*, 1990).

Complementation of the radiation-sensitive phenotypes has been used to isolated DNA repair genes from yeast and mammalian cells, and this has led to the identification of members of an excision repair pathway which are conserved between *S. cerevisiae* and human. The *S. cerevisiae* genes *RAD3* and *RAD10* are homologues of the human *ERCC2* and *ERCC1* genes, respectively (Friedberg 1988; Fenech *et al.*, 1991; Jin *et al.*, 1998). 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 *Schizosaccharomyces pombe* (*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 isolated the *HRD3* gene in *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 and Plasmids

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

Construction of Plasmid for Expression of *HRD3*

To overproduce HRD3 protein in *E. coli*, the 2.0 kb fragment of *HRD3* gene was introduced into overexpression vector pET3a, which contains the Φ10 promoter for T7RNA polymerase (Rosengerb *et al.*, 1987). Tow plasmids (pHRD3-1 and pHRD3-2) were constructed to place the insert in opposite orientations relative to the Φ10 promoter. *E. coli* BL21/DE3 cells, which provide inducible T7 RNA polymerase under the control of the lac/UV5 promoter, were transformed with these recombinant plasmids.

Electrophoresis of Protein

The transformed cells were grown in M9 medium containing 100 μg/ml ampicillin at 37°C. HRD3 protein synthesis was induced in the presence of 0.5 mM IPTG. The addition of IPTG induces the lacUV5 promoter to produce T7 RNA polymerase, which in turn initiates the high-level expression of target gene in the plasmid (Studier and Moffatt, 1986). The cells were suspended in 2% sodium dodecyle sulfate (SDS) buffer by heating in the water bath at 100°C for 2 min and the products were separated on SDS-polyacrylamide gels using 6~14% discontinuous buffer system (Laemmli, 1970; O'Farrall, 1975; Fasullo *et al.*, 1998). The protein band were visualized by Coomassie blue staining.

Measutrment of Growth Kinetics

The transformed cells containing pET3a, pHRD3-1, and pHRD3-2 plasmids were grown overnight. The cultures were diluted 1 : 200 in M9 medium and incubated in the presence or absence of 0.5 mM IPTG. Samples were removed at various time intervals, and the growth kinetics were monitored by measuring the optical density at 600 nm.

Construction of Fusion Protein

The recombinant plasmid *HRD3* was cleaved into three fragments: the N-terminal domain, the middle domain, and the C-terminal domain. Each DNA fragment was then partially digested with *Sau3*AI and inserted into the *BamHI* site of pGE374 plasmid containing the recA promoter and *lac'Z* gene to form an out-frame fusion. After transformation with these plasmids, blue colonies were isolated on X-gal plates. The transformants were cultured in LB and mitomycin C (1 μg/ml) was included to induce HRD3-β-gal fusion proteins for 1 h. Crude extracts were prepared and subjected to 10% SDS-polyacrylamide gels.

Immunoblotting

The crude extracts of *E. coli* cells transformed with various plasmids carrying HRD3-*lac'Z* fusion genes were prepared and subjected to electrophoresis on 10% SDS-PAGE. The proteins were transferred onto nitrocellulose filters, and reacted with antibodies against β-gal protein.

RESULTS AND DISCUSSION

Overexpression of *HRD3* Gene

The previously isolated *HRD3* gene reveals a high homology to the product of the yeast DNA repair *RAD3* gene (Fig. 1). The high levels of sequence homology suggests that the *S. pombe HRD3* gene is also likely to encode an ATP-dependent DNA helicase [4]. This result suggests that *HRD3* contains DNA helicase motifs. To overproduce the HRD3

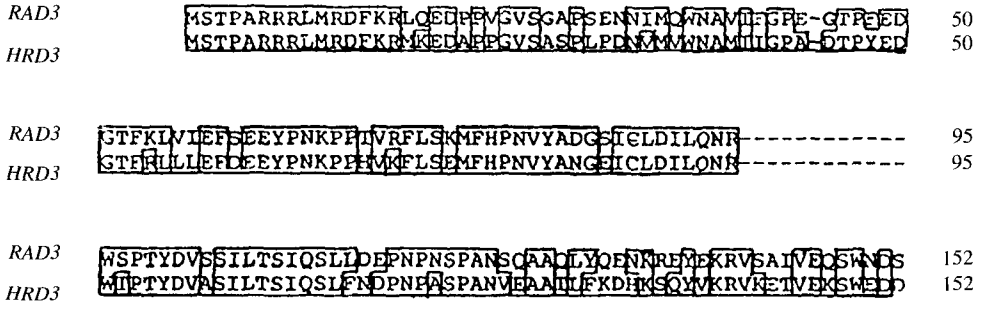

Fig. 1. Sequence homologies between yeast *RAD3* and *HRD3* gene products. The amino acid sequences are numbered on the right side. Identical amino acids occuring in two sequences at the same alignment position are boxed.

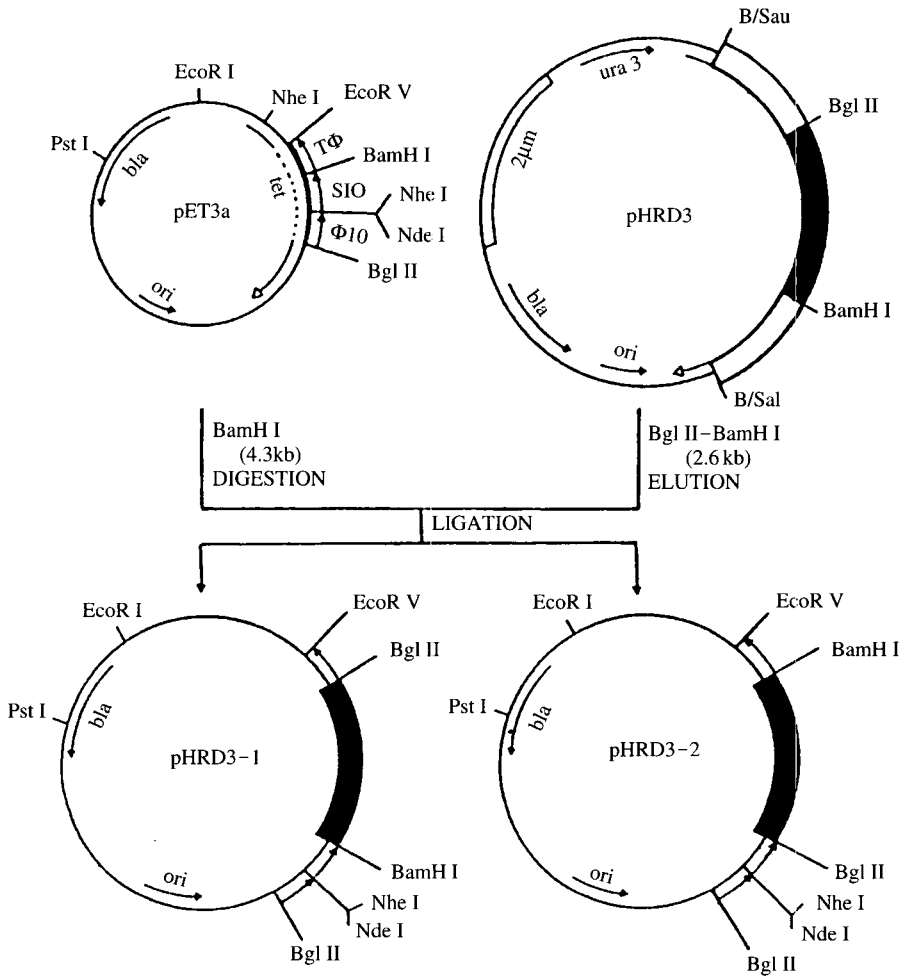

Fig. 2. Vector construction for the determination of the *HRD3* gene function. A 2.0 kb *Bgl*II fragment from the pHRD3 was introduced into *BamH*I site of the high expression vector pET3a. Two different constructions of insert DNA were designated as pHRD3-1 (forward orientation) and pHRD3-2 (reverse orientation).

protein in *E. coli*, DNA fragment containing the entire *HRD3* gene was introduced into the overexpression vector pET3a, which contains a strong Φ10 promoter and the TΦ transcription terminator for T7 RNA polymerase (Rosenberg *et al.*, 1987). The two plasmids were constructed by inserting the fragment in opposite orientation, and designated as pHRD3-1 (*HRD3* gene in the same orientation as the promoter). These recombinant plasmids (Fig. 2) were used to transform *E. coli* BL21/DE3 cells carrying the T7 RNA polymerase gene whose expression is controlled under inducible lac/UV5 promoter to produce T7 RNA polymerase, which in turn initiates the high-level expression of the *HRD3* gene in the plasmid. The *HRD3* gene transcript in *E. coli* cells was examined by RNA dot blot analysis. As expected, the cells carrying pHRD3-1 expressed a high-level of transcripts.

Growth Kinetics of *E. coli* Containing HRD3 Protein

To investigate the effect of high expression of

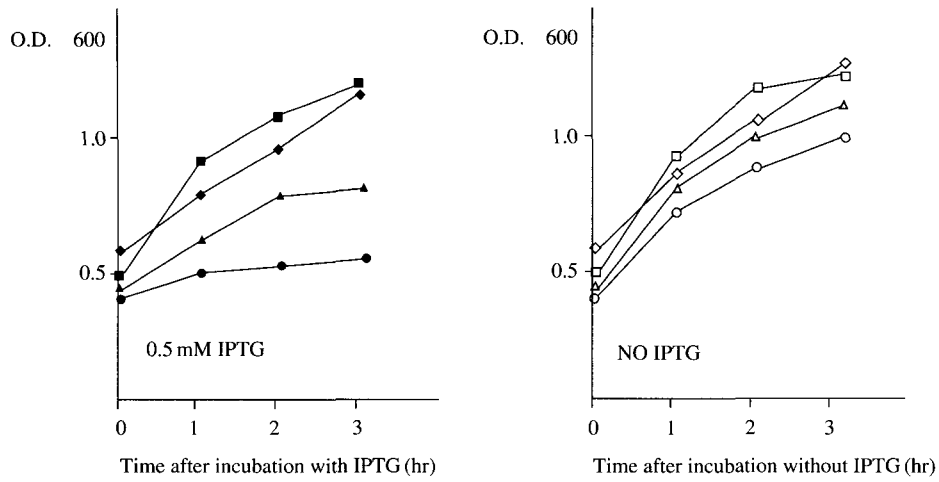

Fig. 3. Growth kinetic of the host *E. coli* cells transformed with pET3a, pHRD3-1, or pHRD3-2 plasmids. The cells were grown, induced by a 0.5 mM IPTG. These samples were incubated for various time interval and their optical densities determined at 600 nm. Symbols: □, BL21/DE3; ◇, pET3a; ○, pHRD3-1; △, pHRD3-2.

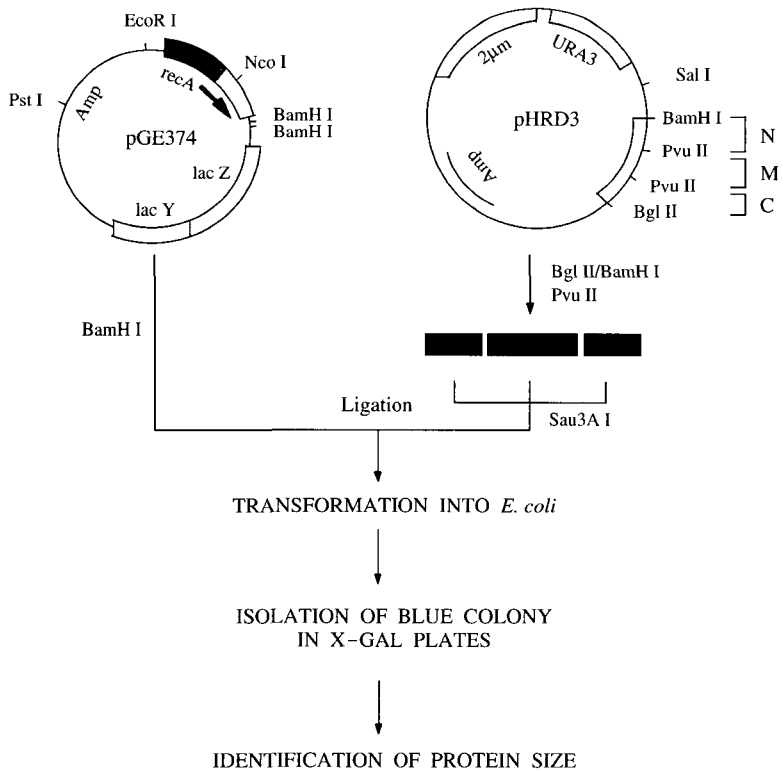

Fig. 4. Construction of plasmid for the HRD3-*lac'Z* fusion gene containing various deleted HRD3 DNA fragments. The HRD3 gene was divided into three parts and each DNA fragment partially digested with *Sau3A*I. The fragments were inserted into *BamH*I site of pGE374 containing *lac'Z* gene. The vectors were then transformed into *E. coli* and screened on X-gal plates. the fusion plasmids were induced with mitomycin C. The closed box indicated *recA* promoter.

HRD3 gene in *E. coli*, the growth rates of *E. coli* host cells BL21/DE3 transformed with pHRD3-1, pHRD3-2 or pET3a were determined by measuring optical density of the cultures. Under uninduced conditions, the cells transformed with pHRD3-1 or pHRD3-2 grew at a somewhat slower rate than the cells with pET3a or without any plasmid, but no significant delays were detected. In contrast, following the induction with IPTG, *E. coli* BL21/DE3 cells containing pHRD3-1 showed a remarkable delay in their growth rate (Fig. 3). However, such reduction was not found in cells with pET3a or host cells even in the presence of IPTG. These results suggest that overproduction of HRD3 protein may suppress the normal growth of *E. coli* cells. This suggests that the overexpressed HRD3 protein may inhibit synthesis or cause degradation of other proteins and thus result in toxicity to the host *E. coli* cells. These are consistent with the intolerance of *HRD3* gene in *E. coli* might be the result of HRD3 protein toxicity. The molecular basis for such toxicity remains to be elucidated.

Determination of Toxic Region

In order to identify the region of HRD3 protein responsible for its toxicity in *E. coli*, several combinations of HRD3-*lac'Z* fusion plasmids were constructed (Fig. 4). The *HRD3* gene was cleaved into three fragments; N-terminal domain, central domain, and C-terminal domain. Each DNA fragment was then partially digested with *Sau3A*I and the resulting fragments were inserted into *BamH*I site of the plasmid pGE374 containing *recA* promoter and *lac'Z* gene. Thus three groups of fusion genes were constructed; HRD3-N-*lac'Z* containing the N-terminal domain, HRD3-M-*lac'Z* containing the central domain, and HRD3-C-*lac'Z* containing the C-terminal part of *HRD3* gene.

After induction with mitomycin C for 1 h, the overexpressed HRD3-β-gal fusion proteins were detected only in the transformants containing N-terminal HRD3-*lac'Z* DNA (Fig. 5A). However, the extracts prepared from the central and C-terminal DNA fused

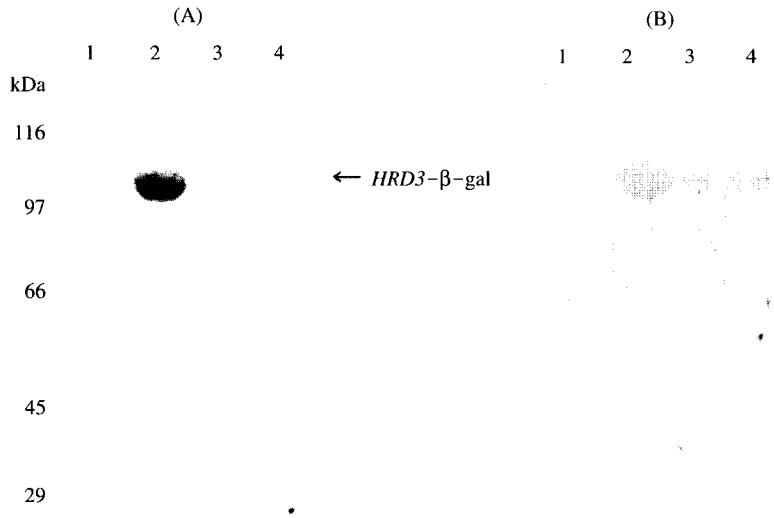

Fig. 5. SDS-PAGE (A) and immunoblotting (B) of the HRD3 proteins using antibodies to β-galactosidase. Blue colored colonies from X-gal plates were cultured and mitomycin C was added to induce HRD3/β-gal fusion protein. *E. coli* transformed with pGE374 and HRD3 genes was grown in the presence of mitomycin C. Crude extracts were prepared and subjected to 10% SDS-PAGE. The proteins were transferred onto nitrocellulose filters, reacted with antibodies against β-gal protein. Lanes 1, pGE372 plasmid whose *reA* and *lac'Z* sequences are in frame; lane 2, HRD3-N-*lac'Z*; lane 3, HRD3-C-*lac'Z*; lane 4, HRD3-M-*lac'Z*.

with *lac'Z* gene did not show any increase in size of the fusion protein. These results were confirmed by immunoblotting using antibodies against β-galactosidase protein (Fig. 5B). The immunoblot analysis showed that the only N-terminal HRD3 DNA fused *lac'Z* gene normally expressed HRD3-β-gal fusion protein in *E. coli*. To know whether the *HRD3* gene was expressed in the HRD3-M-*lac'Z* and *HRD3*-C-*lac'Z* containing cells, RNA blotting was performed. The HRD3-M-*lac'Z* and HRD3-C-*lac'Z* series produced a large amount of HRD3 mRNA but little β-gal mRNA. In contrast, RNA isolated from the HRD3-N-*lac'Z* showed weak signals against the HRD3 probe, but strong signals against the β-gal probe. From these results, it is predicted that the C-terminal part of HRD3 protein may play an important role in causing the toxic effect on *E. coli* cells. This result indicates that the protein overexpressed the C-terminal part of the *HRD3* gene might inhibit the expression of other genes in *E. coli* by taking over the protein synthesis machinary or reducing the stability of their transcripts.

The specific role of the HRD3 protein in nucleotide excision repair in *S. pombe* as well as toxicity in *E. coli* cells awaits purification and characterization of this protein from *E. coli* or yeast cells in which the cloned gene is overexpressed. The transcriptional and translational analysis in the present study may provide opportunities for informative structure-function correlations between *HRD3* gene and its gene product.

REFERENCES

Boothmann DA, Meyers M, Fukunaga N and Lee SW. Isolation of X-ray-inducible transcripts from radioresistant human melanoma cells, Proc. Nat'l. Acid Sci. USA 1993; 90: 7200-7204.

Choi IS, Kim JB, Lee KN and Park SD. Characterization of RAD4 gene required for ultraviolet-induced excision repair of *Saccharomyces cerevisiae* propagated in *Escherichia coli* without inactivation, Photochem. Photobiol. 1990; 52: 392-400.

Choi IS, Kim JB, Jeon SH and Park SD. Expression of *RAD4* gene of *Saccharomyces cerevisiae* that can be propagated in *Escherichia coli* without inactivation, Biochem. Biophy. Res. Commun. 1993; 193: 91-197.

Choi IS. Characterization of *HRD3*, a *Schizosaccharomyces pombe* gene involved in DNA repair and cell viability, Korean J. Biol. Sci. 2003; 7: 159-164.

Cryer DR, Eccleshal R and Marmut J. Isolation of yeast DNA. In: Methods in Cell Biology. 1975; XII: 39-44.

Fasullo M, Bennett T, Ahching P and Koudelik J. The *Saccharomyces cerevisiae RAD9* checkpoint reduces the DNA damage-associated stimulation of directed translocation, Mol. Cell Biol. 1998; 18-3: 1190-1200.

Fenech M, Car AM, Murray J, Watts FZ and Lehmann AR. Cloning and characterization of the *RAD4* gene of *Schizosaccharomyces pombe*; a gene showing short regions of sequence similarity to the human *ERCC1* gene, Nucleic Acids Res. 1991; 19-24: 6737-6741.

Fleer R, Siede W and Friedberg EC. Mutational inactivation of *Saccharomyces cerevisiae RAD4* gene in *Escherichia coli*, J. Bacteriol. 1987; 169: 4884-4892.

Friedberg EC. Deoxyribonucleic acid repair in the yeast *Saccharomyces cerevisiae*, Microbiol Rev. 1988; 52: 70-102.

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

Jin YH, Yoo EJ, Jang YK, Kim SH, Lee CG, Seong RH, Hong SH and Park SD. Purification and characterization of Hrp1, a homolog of mouse *CHD1* from fission yeast *Schizosaccharomyces pombe*, Kor. J. Biol. Sci. 1998; 2: 539-543.

Kim JB, Choi IS, Kim DS and PARK SD. *RAD4* gene required for nucleotide excision repair is not UV inducible and not essential for cell viability in *Saccharomyces cerevisiae*, Mol. Cells. 1990; 1: 87-92.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 1970; 227: 680-685.

Miller RD, Prakash L and Prakash S. Genetic control of excision of *Saccharomyces cerevisiae* interstrand DNA cross-links induced by psoralen plus near-UV light, Mol. Cell Biol. 1982; 2: 939-948.

Naumovski L and Friedberg EC. Molecular cloning of eucaryotic genes required for excision repair of UV-irradiated DNA: isolation and partial characterization of the *RAD3* gene of *Saccharomyces cerevisiae*, J. Bacteriol. 1982; 152: 323-331.

O'Farrall PH. High resolution two-dimensional electrophoresis of protein, Electrophoresis 1975; 250: 4007-4021.

Reynolds P, Prakash L and Prakash S. Nucleotide sequence and functional analysis of the RAD1 gene of *Saccharomyces cerevisiae*, Mol. Cell. Bio. 1987; 7: 1012-1020.

Rosenberg A, Lade BN, Chui D, Lin S, Dunn JJ and Studier W. Vectors for selective expression of cloned DNAs by T7 RNA polymerase, Gene 1987; 56: 125-135.

Sambrook J and Russell DW. Molecular Cloning: A Laboratory Mannual (3rd). 2001; Cold Spring Harbor Laboratory Press, New york.

Studier FW and Moffatt BA. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes, J. Mol. Biol. 1986; 98: 113-130.

Thompson LH, Mitchell DL, Regan JD, Bouffler SD, Stewart SA, Carrier WL, Nairn RS and Johnson RT. CHO mutant UV61 removes photoproducts but not cyclobutane dimers, Mutagenesis 1988; 4: 140-146.

Weber CA, Salazar EP, Stewart SA and Thompson LH. *ERCC2*: cDNA cloning and molecular characterization of a human nucleotide excision repair gene with high homology to yeast *RAD3*, EMBO J. 1990; 9: 1437-1447.