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Study on Expression and Characterization of *HRD3* Gene Related DNA Repair from Eukaryotic Cells

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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 and DNA-RNA helicase activities. To examine the extent of conservation of structure and function of *RAD3* during eukaryotic evolution, the *RAD3* homolog gene was isolated by screening of genomic DNA library. The isolated gene was designated as *HRD3* (Homologue of *RAD3* gene). The over-expressed HRD3 protein was estimated to be a 75 kDa in size which is in good agreement with the estimated by the nucleotide sequence of the cloned gene. Two-dimensional gel electrophoresis showed that a number of other protein spots dramatically disappeared when the *HRD3* protein was over-expressed. The overexpressed RAD3 protein showed a toxicity in *E. coli* host, suggesting that this protein may be involved in the inhibition of protein synthesis and/or degradation of host protein. To determine which part of *HRD3* gene contributes to the toxicity in *E. coli*, various fusion plasmids containing a partial sequence of *HRD3* and lac'Z gene were constructed. These results suggest that the C-terminal domain of *HRD3* protein may be important for both toxic effect in *E. coli* and for its role in DNA repair in *S. pombe*.

Key words – *HRD3* gene, *Schizosaccharomyces pombe*, toxicity, DNA repair

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[1,2,9,10], 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 complementation 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*[1,3,7]. 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[13,25].

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 [8,14,16,17]. 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[24-26].

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[13,19,25]. 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

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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[22]. 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[12], 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.[6].

Construction of Plasmid

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 T7 RNA polymerase[20]. Two 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[23].

Electrophoresis of Protein

The transformed cells were grown in M9 medium containing 100 μg/ml ampicillin at 37℃. 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 [20]. The cells were suspended in 2% sodium dodecyle sulfate (SDS) buffer by heating in the water bath at 100℃ for 2 min and the products were separated on SDS-polyacrylamide gels using 6-14% discontinuous buffer system[18, 22]. The protein band were visualized by Coomassie blue staining.

Dot Blot Analysis

RNA isolated from the yeast strain was denatured and applied to nitrocellulose filters prewetted with 1M ammomium acetate using a 96-well manifold apparatus. The 1.2 kb *Pvu*II fragment, which is the internal sequence of the *HRD3* gene[4,5], was labeled with [α-^{32}P]dCTP (3,000 ci/mmole [Amersham Corp]) and used as a probe in the subsequent DNA-RNA dot blot analysis[22].

Isoelectric Focusing

To prepare samples for two-dimensional (2D) gel electrophoresis, *E coli* crude extracts were added to 0.2 volume of 10% SDS and heated to 100℃. The samples were cooled and urea was added to 1 mg/μl (Ultrapure, BRL). Ampholines (LKB) and NP-40 (Sigma) were added to each final concentration of 2%. A mixture of ampholines in the ratio of 1 part of pH 3.5-10 and 4 part of pH 5-7 was used. Tow-mercaptoethanol was added to the final concentration of 5% and 2D-gel electrophoresis was performed according to the method described by O'Farrall[18]. Isoelectric focusing was carried out at a pH gradient of approximately 4.5-7.0 and the second dimension on a 10% polyacrylamide gel containing 0.1% SDS. After the electrophresis, the gels were fixed and stained with silver nitrate.

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 suggest that the *S. pombe HRD3* gene is also likely to encode an ATP-dependent DNA helicase[5,9,11]. This result suggests that *HRD3* contains DNA helicase motifs. To overproduce the HRD3 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 [20]. The two plasmids were constructed by inserting the

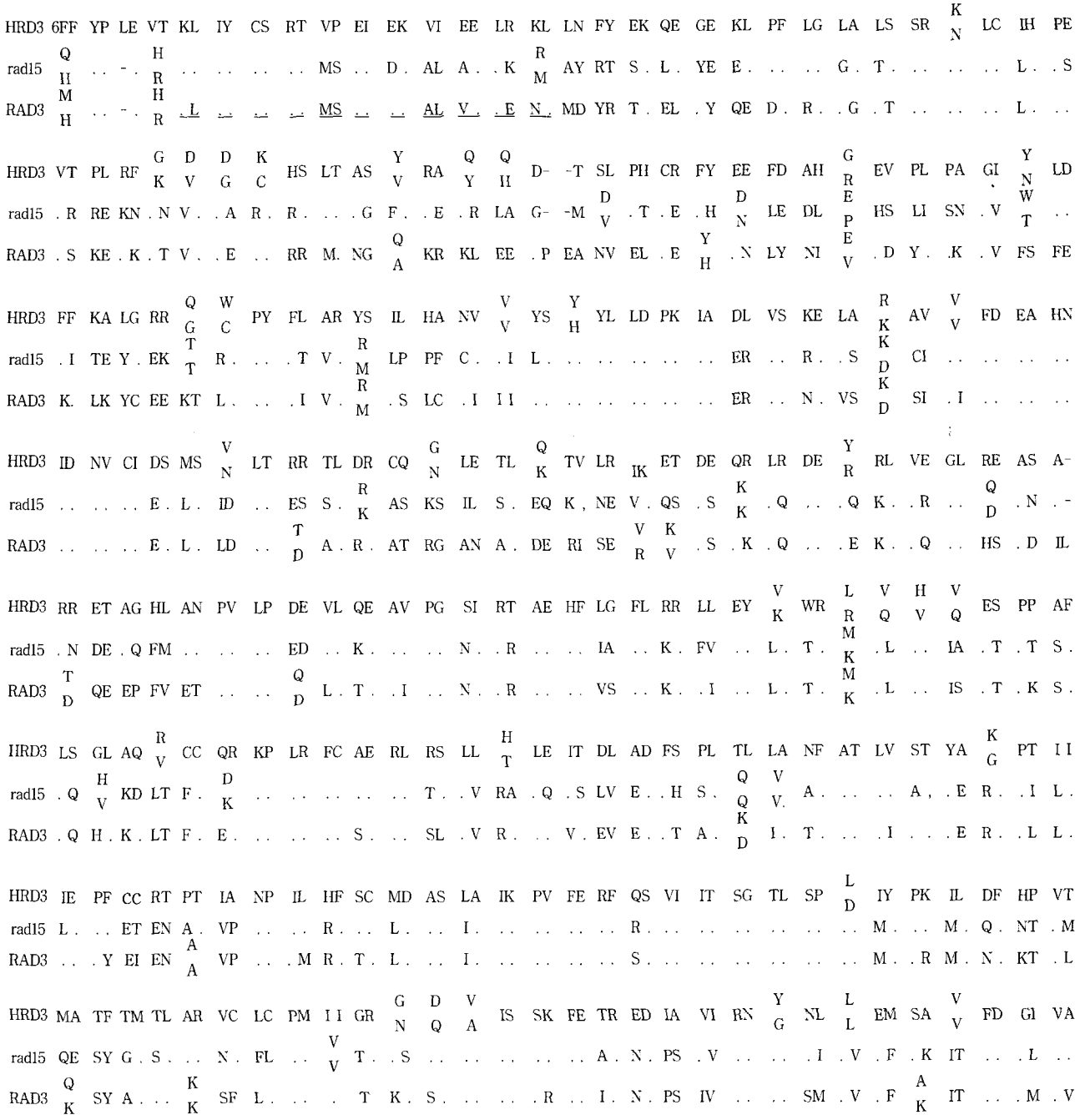

Fig. 1. Sequence homologies between yeast *RAD3, Rad 15,* and *HRD3* gene products. The amino acid sequences are numbered on the right side.

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 (Fig. 3). As expected, the cells carrying pHRD3-1 expressed a high-level of transcripts. The extracts prepared from the *E. coli* cells carrying with pHRD3-1 showed a unique band of 75 kDa protein (Fig. 4), which is good agreement with the calculated HRD3 protein size of 76.5 kDa from its DNA sequence[5].

As expected, the cells carrying pHRD3-1 expressed a

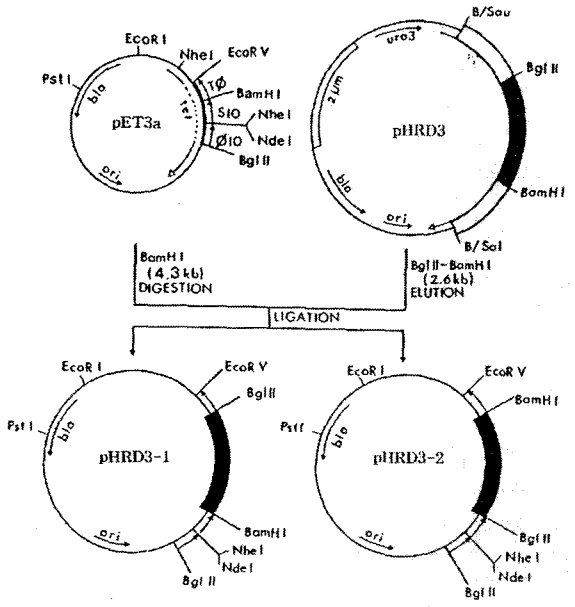

Fig. 2. Vector construction for the determination of the *HRD3* gene function. A 2.0 kb *Bgl*II fragment from the pHRD3 was introduced into *BamH*I site of the high expression vector pET3a. Two different constructions of insert DNA were designated as pHRD3-1 (forward orientation) and pHRD3-2 (reverse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

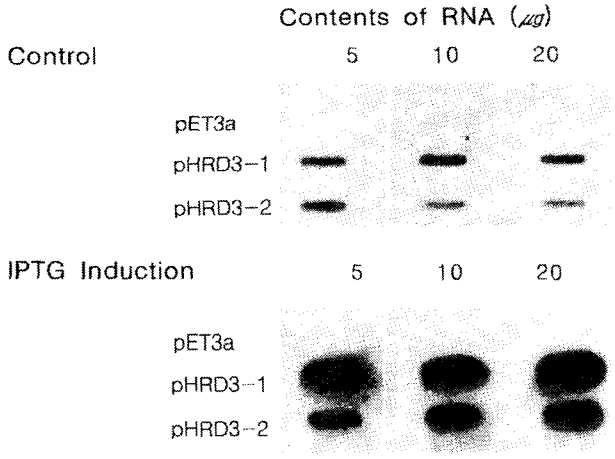

Fig. 3. Confirmation of the constructed plasmids by dot blot analysis. The host *E. coli* BL21/DE3 cells transformed with pET3a, pHRD3-1, or pHRD3-2 were grown to mid log phase; IPTG(-), a portion of which was induced with 0.5 mM IPTG for 3 hrs; formaldehyde, and blotted onto nitrocellulose filters. The filters were hybridized with the ^{32}p-labeled 1.2 kb *Pvu*II DNA fragment of the cloned *HRD3* gene.

high-level of transcripts. The extract of the bacterial transformants were subjected to 2D-gel eletrophoresis to

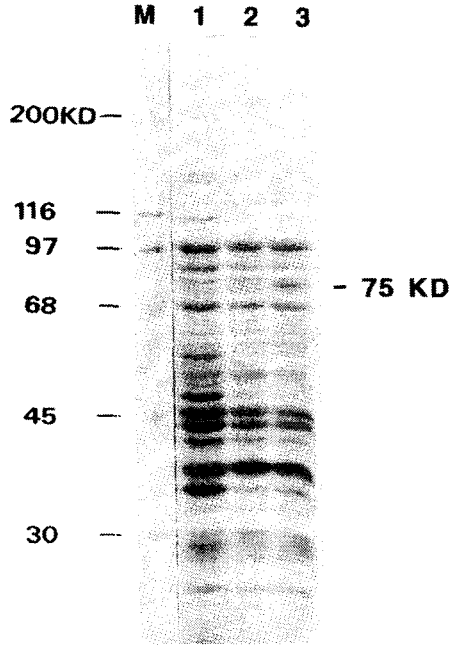

Fig. 4. Determination of the HRD3 protein size by SDS-polyacrylamide gel electrophoresis. Host *E. coli* cells (BL21/DE3) transformed with pET3a, pHRD3-1 and pHRD3-2 plasmids were grown to a mid log phase and induced by IPTG of a 0.5 mM. The cells were resuspended in a sample buffer and electrophoresed in a 6 to 14% acrylamide gradient. The numbers indicate molecular weight markers (116, 97, 68, 45, 30 kDa). lane M; standard parker, 1; pET3a, 2; pHRD3-2, 3; pHRD3-1.

enhance the resolution of the HRD3 protein band (Fig. 5). The crude extracts prepared from cells containing pHRD3-1 showed a unique spot of 75kDa representing the HRD3 protein, the numbers of approximately one-third in the cells containing pHRD3-1 compared to the pHRD3-2 containing cells. this suggests that the overexpressed HRD3 protein may inhibit synthesis of cause degradation of other proteins and thus result in toxicity to the host *E. coli* cells.

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[4,5]. After

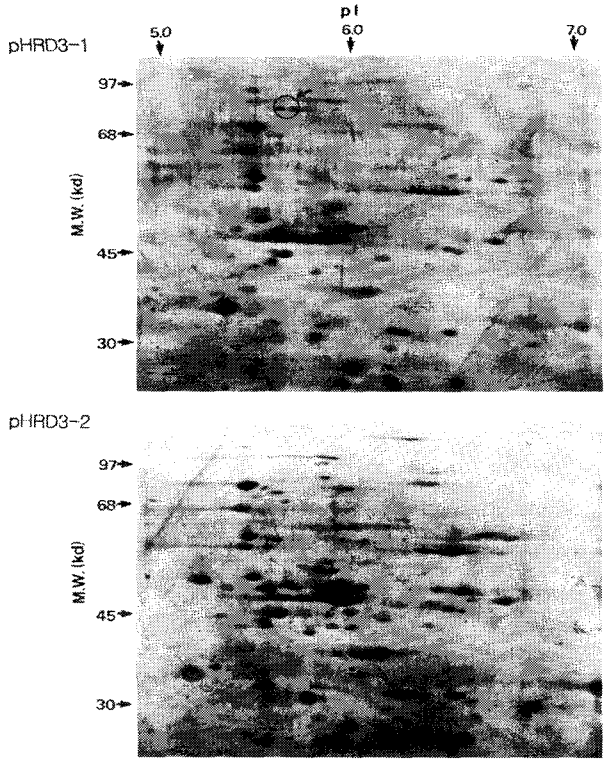

Fig. 5. Size determination of the HRD3 protein by 2D-gel eletrophoresis. Host cells transformed with phrd3-1 and pHRD3-2 were grown to mid log phase and induced with 0.5 mM IPTG for 3 h. The cells were lysed by sonication in the lysis buffer. Approximately 10 μg proteins were loaded in the gel, Isoelectric focusing was carried out using a pH gradient of 4.5-7.0 followed by 10% polyacrylamide gel electrophoresis containing 0.1% SDS. The circle indicates the newly synthesized HRD3 protein spot.

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. However, the extracts prepared from the central and C-terminal DNA fused 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. 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.

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초록 : 진핵세포에서 DNA 회복에 관련된 *HRD3* 유전자의 분리, 발현 및 특성 연구

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효모에 있어 자외선에 의한 절제회복 관여 DNA 회복유전자가 많이 알려져 있으나, 이들이 어떤 기능을 하는지는 아직 잘 알려져 있지 않다. 본 연구에서는 자외선 조사 시 절제회복의 초기 단계에 절대적으로 필요한 *RAD3* 유전자와 유사한 유전자인 *HRD3* 유전자를 분열형 효모인 *Schizosaccharomyces pombe*에서 분리하여 그 특성을 연구하였다. 이 결과 분리한 유전자는 효모 *RAD3* 유전자와 염기서열에서 약 70%이상의 유사성을 보였다. 이 유전자의 염기서열 결과 유전자 산물의 분자량은 75 kDa였다. 2-D gel 결과 과잉발현 시 HRD3 단백질은 숙주 단백질의 합성 억제 또는 분해 촉진을 유발하여 숙주세포인 대장균에 독성효과를 나타내었다. *HRD3* 유전자와 *lacZ* 유전자를 융합시킨 여러 가지 재조합 vector를 만들어 이들 융합단백질을 분리, 연구 한 결과 HRD3 단백질의 카르복실 말단부분이 효모에 있어서 DNA 회복기능과 대장균에서의 독성효과를 나타내는 중요부위임이 확인되었다.