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Isolation and Characterization of *hrp2+* Gene Related to SNF2 Family in Yeast

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The SNF2/SW12 family comprises proteins from a variety of species with *in vivo* functions, such as transcriptional regulation, maintenance of chromosome stability during mitosis, and various types of DNA repair. This study was shown the characterization of *hrp2+* gene which was isolated by PCR amplification using the conserved domain of SNF2 motifs. Sequence analysis of *hrp2+* gene showed striking evolutionary conservation among the SNF2 family of proteins. The transcript of *hrp2+* gene was found to be a 4.7 kb as identified by Northern hybridization. To investigate the inducibility of *hrp2+* gene, transcript levels were examined after treating the cells to various DNA damaging agents. The transcripts of *hrp2+* were induced by UV-irradiation. But the transcripts were not induced by treatment of 0.25% Methylmethane sulfonate (MMS). These results implied that the effects of damaging agents are complex and different regulatory pathways exist for the induction of this gene. Hrp2 protein was purified near homogeneity by combination of affinity chromatography. We tested the purified Hrp2 protein for the helicase activity in an oligonucleotide release assay. However we were unable to detect any helicase activity associated with the Hrp2 protein, indicating that the helicase motifs in Hrp2 are merely indicators of a broader DNA-dependent ATPase activity.

Key words – *hrp2+* gene, DNA damaging agents, Hrp2 protein, Helicase assay

The yeast SWI2/SNF2 gene was identified in genetic spectrum of differently regulated yeast genes[18]. Transcriptional dependence on SWI2/SNF2 appears to be related to the nature of the core promoter sequence, the number of binding sites for upstream activators, and the type of activator[16]. The SWI2/SNF2 polypeptide contains the characteristic seven conserved protein motifs that are present in a large and rapidly growing group of nucleoside triphosphate (NTP)-binding proteins that include DNA and RNA helicases[4,8,11].

The number of proteins assigned to the SNF2 family has increased rapidly over the last few years and continues to expand. Interestingly, members of the SNF2-like family exhibit an impressive range of biological functions. The activities include gene-specific transcriptional activation (SNF2 subfamily), transcriptional repression (MOT1), destabilization of reconstituted nucleosomes (SNF2 and SNF2L subfamilies), transcriptioncoupled repair (ERCC6 subfamily), nucleotied excision repair of nontranscribed region of the genome (RAD16), recombination repair (RAD54 subfamily), chromosome segregation (lodestar) and chromatin compaction (CHD1 subfamily). Some of the proteins in the SNF2 family contain sequence motifs, which help identify the function conserved within the subfamily. All members of the SNF2 subfamily contain a chromodomain motif. This motif is found in a variety of proteins involved in transcriptional regulation[3] and it has been suggested that it may be involved in protein-protein interactions. CHD1 subfamily contains a chromodomain motif, which is found in a few other proteins and is proposed to play a role in chromatin compaction[2]. Finally, a RING finger motif is found in all the proteins in the RAD16 subfamily. This motif is related at the sequence and structural levels to the zinc finger motif[1,17]. It is found in many proteins that interact with DNA (including the DNA repair protein RAD18, the p53-associated protein MDM2 and the protooncogene mel-18) and suggested that it is involved in DNA binding[21].

Recently, the number of proteins in this family is increasing rapidly through genome sequencing projects or homology-based cloning. This study was aimed to isolate unidentified SNF2/SWI2 family proteins and to investigate the conservation of sequence and function within eukaryotes. The fission yeast *Schizosaccharomyces pombe* was used for this study, which serves as an important model system for the study of basic processes in higher eukaryotes.

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Materials and Methods

Strains, cell culture, and genetic methods

E. coli strain DH5α (F- *end*A1, *hsd*17, (r-, mk-), *sup*E44, *thi*-1,

*rec*A1, *gyr*A96, *rel*A1, *lac*IqZ-M15) and yeast *S. pombe* strain JY741 (h- *ade*6- M210 *leu*1-32 ura4-D18) was used for this study. *E. coli* strains were grown on LB media (1% tryptone, 1% sodium chloride, 0.5% yeast extract). Yeast strain was grown in YE (2% glucose, 0.5% yeast extract) medium supplemented with appropriate amino acids. Standard molecular biology techniques were employed as described[19]. *S. pombe* chromosomal DNAs were prepared according to the methods of Cryer et al.[7].

Isolation of RNA and Northern blot analysis

Total RNA was prepared according to Jang et al.[12]. RNA was denatured, fractionated on 1.2% agarose gel containing 0.66 M formaldehyde, and transferred onto S&S Nytran membrane. The probe and filter hybridization and washing conditions were identical to those of Southern blot analysis.

Preparation of DNA probe by random priming

The DNA fragment was labeled with [α-^{32}P] dCTP(3,000 Ci/mmole) by random primed DNA labeling method[10]. The labeling reaction was carried out in 20 μl of the standard random priming buffer containing 50 ng of DNA, 30 uCi of [α-^{32}P] dCTP, dATP, dGTP, dTTP and 2 unit of Kenow enzyme for 1 hr at 37℃.

Treatment Of various DNA damaging agents

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 spread onto 150 mm-petridish and exposed to 200 J/m^2 of ultraviolet (UV)-light or 0.25% MMS treatment. The treated cells were inoculated into fresh YES medium, incubated at 30℃ in the dark, and collected at indicated times.

DNA Sequencing

Plasmid DNA that was to be sequenced was purified by a plasmid preparation kit (Qiagen). An ABI PRISM 377 DNA sequencer (Perkin-Elmer) analyzed the nucleotide sequence. The nucleotide sequence and inferred amino acid sequences were aligned by GenBank database.

Preparation of whole cell extract (WCE)

The JYK672 cells grown in one liter of TMM (MM with 1 mM of thiamine) to OD_{595}=2.0 were harvested and washed three times with fresh MM. After dilution to OD_{595}=0.1 into 15 liters of fresh MM, cells were further cultured at 30℃ for 12 hr, collected and washed with extraction buffer (20 mM Tris-HCl, 1 mM DTT, 10% glycerol, pH 8.0). Then, cells were quickly frozen in liquid nitrogen and stored at -70℃until use. The stored cells were washed once in extraction buffer and lysed in 150 ml of extraction buffer by glass beads with 6×30 sec pulses on a BioSpec Products beadbeater, and clarified by centrifugation for 2 hr at 43,000×g in a Beckman Ti70 rotor. The supernatant was dialyzed against the same buffer and referred to as whole cell extract (WCE)

DNA helicase assay

DNA helicase activity was assayed according to Lee et al.[15]. Unless otherwise indicated, the measurement of helicase activity was carried out in a reaction mixture (20 μl) containing 20 mM HEPES-KOH, pH 7.4, 2 mM DTT, 5 mM $MgCl_2$, 1 mM ATP, 0.2 mg/ml BSA, 50 fmol of dsDNA substrate and the indicated amount of the hap2 protein. Mixtures were incubated for 30 min at 30℃, and then stopped by the addition of 5 μl of a mixture (5X) containing 0.1 M Tris-HCl, pH 7.5, 20 mM EDTA, 0.5% SDS, 0.1% Nonidet P-40, 0.1% bromophenol blue, 0.1% xylene cyanol, and 50% glycerol. Aliquots (10 μl) of each reaction were loaded onto a 8% polyacrylamide (30:1) gel and electophoresed at 15 mA for 1.5 hr. The gel was vacuum-dried on Whatman DE81 paper, and the dsDNA substrate and the displaced ssDNA product were visualized by autoradiography.

Gel electrophoresis mobility shift assay (EMSA)

Complexes formed with the hap2 protein and dsDNA were measures in reaction mixtures (20 μl) containing 20 mM HEPES-KOH, pH 7.4, 2 mM DTT, 0.2 mg/ml BSA, 100 mM NaCl, 5% glycerol, 50 fmol of substrate and the indicated amount of hap2 protein in the absence or presence of 4 mM $MgCl_2$ and 1 mM ATP. After 30 min of incubation at 30℃, complexes formed were analyzed by 4% polyacrylamide (30:1) gel in 0.5 X TBE.

Results and Discussion

Isolation and nucleotide sequence of *hrp2+* gene from *S. pombe*

To isolate new members of SNF2 helicase related genes from *S. pombe*, this study took advantage of the highly conserved sequence elements within the *ERCC6* gene and generated the degenerative oligo primers[1,21]. Sequence homologies in domain I and III allowed the design of PCR

primers[5,6,9]. This work was focused on hrp2 PCR product containing 438 bp DNA. The nucleotide sequence of hrp2 PCR product was determined[13,14,17] and amino acid sequences of the amplified regions of hap2 and hrp2 were aligned with the corresponding regions of ERCC6 and MOT1 proteins using Multalin software. Examination of the alignment revealed that the four regions of amino acid identity were clustered.

Based on these results, hrp2 PCR product can be used as a DNA probe for isolation of helicase related genes. The helicase related gene (designated as *hrp2+)* in *S. pombe* was isolated by screening genomic libraries constructed in pIRT5. The screening of library was initiated with the 438 bp PCR product as a probe. Among 40,000 colonies screened, a clone with 6.5 kb insert DNA was isolated, and then sequenced [5,14]. From these nucleotide sequence, amino acid sequence were analyzed. This result showed that Hrp2 has a chromodomain, ATPase/7helicase domain, and one DNA binding domain (Fig. 1).

Identification of *hrp2+* transcripts

In order to identify the transctipt size of *hrp2+* gene, northern analysis was performed. Total yeast RNA from *S. pombe* was hybridized with the denatured cloned DNA. The size of transcript was found to be a 4.7 kb as identified by northern blot (Fig. 2). To determine the transcription initiation site of the *hrp2+* gene start, primer extension analysis was employed using the 20-mer oligonucleotide. This result showed a extended band of 64bp[6,15]. The transcriptional start point was mapped to a position of 47 base pair from the first ATG codon of translational initiation codon.

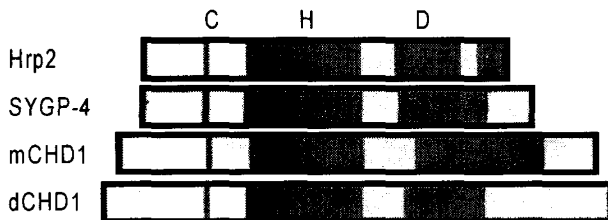

Fig. 1. Comparison of Hrp2 with CHD1 subfamily members. Schematic representation of Hrp2, SYGP-4, mCHD1 and dCHD1 to indicate the location of the homologous segment in the proteins. Chromodomain (C) is lightly shaded: ATPase/7 helicase domain is indicated by the closed box: the region of DNA binding domain (D) is darkly shaded. The numbers at the ends of diagrams denote the length of each polypeptide. Schematic drawing of the *hrp2*[+] gene shows that the protein product encodes 1548 amino acids.

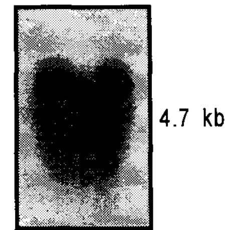

Fig. 2. Northern blot analysis of *hrp2+* mRNA in *S. pombe*. Total RNA was isolated, electrophoresed, transferred onto nitrocellulose filter, and then hybridized with the radiolabelled *hrp2+* DNA probe. The size of transcript is 4.7 kb.

To examine the transcriptional regulation of *hrp2+* gene by treatment of DNA damagng agents, total RNA from the cells was treated with UV and MMS. The UV-irradiation (200 J/m^2) increased *hrp2+* gene expression in time dependent manners (Fig. 3). But MMS (0.25%) treatment did not increase (Fig. 3). These results implied that the effects of damaging agents are complex and different regulatory pathways exist for the induction of this gene. Also, the expression of the

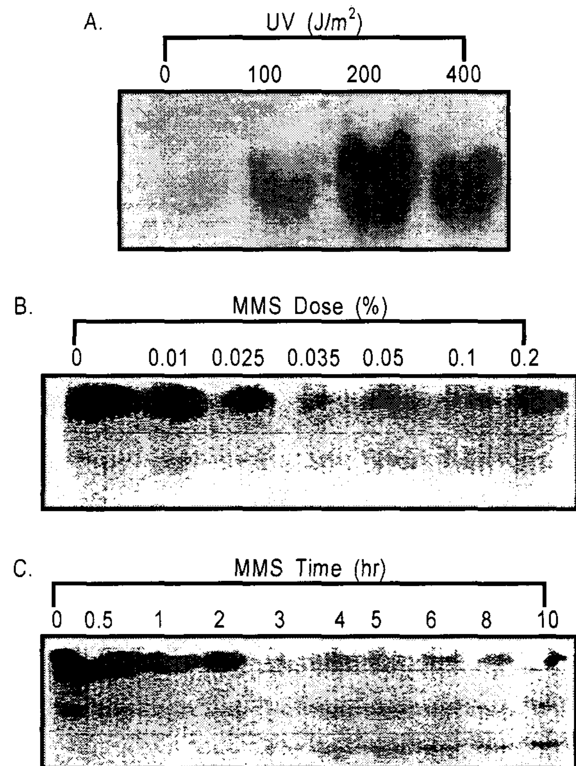

Fig. 3. The transcript levels of *hrp2+* gene in UV and MMS treated cells. Cells in log phase were treated with 200 J/m^2 UV and 0.25% MMS, and then incubated at 30°C. After incubating, RNA was isolation and northern blot analysis was performed. The numbers at the top of each lane indicate the cell-collection times (in hours) after the treatment. ACT1 was used as an internal control.

gene increased after UV-irradiation, indicating that the *hrp2+* is a novel UV-inducible gene in *S. pombe.* To determine the level of *hrp2+* gene during cellular growth, JY741 cells were cultured in rich medium and collected at every one or two hours to prepare total RNA. The Northern blot analysis showed that the level of *hrp2+* transcript reached its maxim before the cells entered the exponential growth phase and then decreased gradually. This suggests that *hrp2+* gene is expressed mainly at the early stage of cell growth[18].

Purification of hap2 from JYK672

Hrp2 protein was purified near homogeneity by combination of affinity chromatography on Ni^{2+}-NTA agarose, DEAE-Sepharose ion exchange and Sephacryl S-200 gel filtration chromatographies (Fig. 4). Whole cell extract (WCE) made from 15-liter culture of JY741 (3.8g protein, Materials and Methods) was bound batchwise with 20 ml of Ni^{2+}-NTA agarose (Qiagen) for 2.5 hr at 4℃. The resin was poured into a column and washed sequentially with 300 ml of extraction buffer, 100 ml of extraction buffer with 10 mM Imidazole. Then, protein were eluted with 100 ml of extraction buffer with 500 mM Imidazole. Peak fraction (fraction numbers 6 to 10, 20 ml) were pooled, dialyzed against equilibration buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1mM DTT, 10% glycerol, pH 8.0) and loaded onto an DEAE Sepharose column (2×15 cm). After washing the column, bound proteins were eluted with 50 ml of linear salt gradient (100 mM to 400 mM NaCl) in equilibration buffer. A single peak of ATPase activity appeared at about 250 mM NaCl. Peak fraction were pooled (30 ml), concentrated to 2 ml in a Amicon concentrator (Amicon, USA), and applied at onto a Sephacryl S-200 column (4×60 cm) 0.2 ml/min. Peak fractions containing full sized hap2 were pooled fraction fraction numbers 14 to 17, 16 ml), dialyzed against equilibration buffer and re-concentrated. The protein was used in the further experiments.

Helicase activity

Many DNA-dependent ATPase have an associated DNA helicase activity. The presence of conserved helicase domains in hrp2 suggested that it might also have DNA helicase activity. We tested the purified hrp2 protein for the helicase activity in an oligonucleotide release assay (see Materials and Methods) using two types of substrates: an annealed oligonucleotide with flushed-ends and standard duplex dsDNA (see materials and methods). However, we were unable to detect any helicase activity associated with the hap2 protein (Fig. 5), indicating that the helicase motifs in hap2 are merely indicators of a broader DNA-dependent ATPase activity,

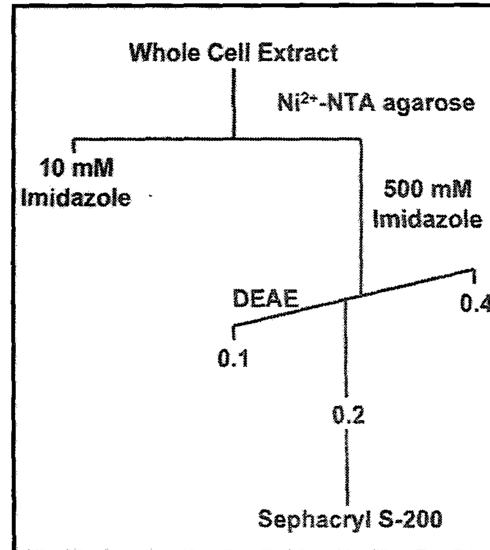

Fig. 4. Purification schem. Whole cell extract made from 15 liter culture of JKY672 was bound batchwise with 20 ml of Ni2+-NTA agarose for 2.5 hr at 40°C. The resin was poured into a column and washed sequentially with 300 ml of extraction buffer, 100 ml of extraction buffer with 10 mM imidazole (see materials and methods).

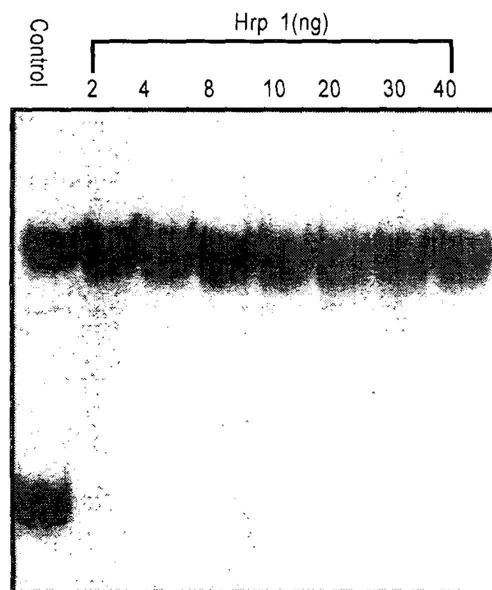

Fig. 5. DNA helecase activity assay. The standard helicase assay was performed as described in materials and methods with indicated amount of Hrp2 protein. However, no helicase activity was detected. The substract only and boiled enzyme control is indicated above the appropriate lane.

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초록 : *Schizosaccharomyces pombe*에서 SNF2에 속하는 *hrp2+* 유전자의 특성 연구

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본 연구는 분열형 효모 *Schizosaccharomyces pombe*에서 여러 가지 DNA 절제회복 및 유전자 발현에 관여하는 SNF2/SW12 유전자의 기능을 연구하기 위하여 이에 관련되는 유전자를 분리하고 그 특성을 연구하였다. SNF2 motif의 conserved sequence를 primer로 하여 중합효소 연쇄반응 (PCR) 방법으로 480 bp 크기의 DNA fragment를 분리하여, 이를 probe로 하여 효모에서 *hrp2+* 유전자를 분리하였다. 분리한 *hrp2+* 유전자의 sequence homology를 비교한 결과 3개의 SNF2 motif를 포함하고 있었다. *hrp2+* 유전자의 전사체 크기는 4.7kb임을 Northern hybridization 으로 확인하였다. 분리한 유전자의 특성 연구를 위하여 Northern hybridization 으로 *hrp2+* 유전자의 UV와 MMS 에 대한 유도성을 조사한 결과 자외선에 대해서만 유전자의 발현이 유도되었다. 이 결과 분리한 *hrp2+*는 UV-inducible 유전자임을 확인하였다. 또한 분리한 유전자의 특성연구 중 하나로 hrp2 단백질을 분리하여 helivase activity를 측정하였다. 이 결과 분리한 *hrp2+* 유전자는 전혀 helicase activity를 나타내지 않았다.