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Random Isolation of Cadmium-induced Gene by Reverse Transcriptase PCR in *Schizosaccharomyces pombe*

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The polymerase chain reaction was used to study the genes inducible under stress from the heavy metal cadmium. *Schizosaccharomyces pombe*, grown in the presence or absence of sublethal concentration of cadmium, was isolated to purify the total RNAs. The Induced RNA Random Fishing (IRRF) method in which random oligonucleotides were used as primers was applied to the identification of cadmium-induced gene expressions. A PCR-DNA product of 400-bp was cloned and sequenced. Computer analysis showed that this DNA has no homology with any known DNA sequences in GenBank or EMBL databases. The induction of this gene was confirmed by Northern blot analysis of total RNAs isolated from both cadmium-treated and untreated yeast cells.

Living organisms contain hundred thousand different genes, of which only a small fraction are expressed in any specific circumstance or cell. The determination of all life processes-development, differentiation, homeostasis, response to stress, etc. depend on which genes are chosen to be expressed. Differential gene expression may be at the center of the regulatory mechanisms that control all biological processes. Comparing the gene expression in different cells or conditions may provide the information we need to analyze the control mechanisms.

However, it is likely that the majority of regulatory genes will remain unknown. Current methods of distinguishing transcripts in differential expression rely largely on subtractive hybridization (7) or differential screening (8). mRNAs are prepared from the cells from different sources that are distinguished by some criteria such as being exposed to a particular environmental condition, or being derived from a different tissue or stage of development.

The method that we describe here is directed toward the identification of genes differentially expressed upon environmental stress, especially that applied by heavy metal cadmium, in yeast *Schizosaccharomyces pombe*. Many organisms are known to have developed defensive mechanisms to highly toxic metal. *S. pombe* (10), algae (6) and some other plants (5, 12) synthesize a small peptide called cadystin or phytochelatin in response to cadmium. However, currently, there is uncertainty about the relative importance of the various potential mechanisms for heavy metal detoxification. To explore this area further, we adopted a molecular approach to the study of heavy metal detoxification mechanisms by isolating the cadmium-induced genes of the model organism, *Schizosaccharomyces pombe*.

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Key words: polymerase chain reaction (PCR), arbitrary primer, *Schizosaccharomyces pombe*, cadmium, gene

MATERIALS AND METHODS

Strains and Culture Condition

Schizosaccharomyces pombe 732 (h-, ura4-D18) was a gift from Dr. Paul Nurse of the Dept. of Biochemistry, University of Oxford. The cells were grown at 30°C in a YPD medium (1% yeast extract, 2% polypeptone, 2% glucose) with vigorous shaking until the early stationary phase. The cells were then diluted 10 times, after which they continued to grow. Before the RNA extraction, the cells were treated with 1 mM $CaCl_2$ for 12 hr. *E. coli* strain DHα (F-φ80dlacZΔM15 Δ(*lac*ZYA argF)U 169 *end*A1 *rec*A1 *hsd*R17 (rk- mk+) deoR *thi*-1 *sup*E44 λ-*gyr*A96 *rel*A1) was used for the bacterial transformation and the plasmid preparation.

RNA Extraction and Reverse Transcription

Total RNAs were extracted using the method of Chirgwin *et al* (2). The cell homogenate was centrifuged for 10 min at 12,000 g to separate the cellular debris.

The supernatant was made into 1.3 M CsCl, layered over a 2 ml pad of 5.7 M CsCl, and centrifuged for 24 hr in a SWTi-60 rotor (Beckman) at 43,000 rpm. The resulting pellets were resuspended in 7 M urea and precipitated with ethanol. RNA concentration was determined using a spectrophotometer, prior to reverse transcription. Reverse transcription was carried out using 1 μg of total RNA in a 30 μl reaction mixture containing 1×RT buffer (10 mM Tris-HCl, pH 8.3, 15 mM KCl and 0.6 mM $MgCl_2$), 10 mM DTT, 5 mM each of the four deoxyribonucleotide triphosphates, 50 ng of random hexamers, 30 units of RNase inhibitor and 250 units of Moloney murine leukemia virus (M-MLV) reverse transcriptase. After a 60-min incubation at 37°C, reverse transcriptase was heat inactivated at 95°C for 5 min.

PCR Amplification

PCR was carried out in a total volume of 75 μl containing: 2.5 μl of cDNA, 40 pmoles of random primers, 250 μM dNTPs, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 15 mM $MgCl_2$, 0.5 unit of AmpliTag DNA polymerase (Perkin-Elmer Roche, USA). Amplification was done with a Perkin-Elmer DNA Thermal cycler 480 in the following conditions: 94°C for 1 min, 35°C for 1 min and 72°C for 1 min. 40 amplification cycles were preceded by a primary denaturation step (95°C for 3 min) and the last cycle was followed by a final extension step (72°C for 5 min).

PCR Products Analysis

The PCR products were analyzed by 1.5% agarose gel electrophoresis for 2 hr at 5 V/cm in the TAE buffer system and stained with ethidium bromide. A quarter of the PCR reaction was loaded onto the gel.

Cloning, Sequencing and Northern Blot Analysis

After electrophoresis, the DNA bands were visualized by UV illumination. The PCR product unique to the stress-induced RNA source was excised from the gel using a sterile razor blade and was chopped into fine pieces. The DNA fragment was electroeluted and ethanol-precipitated. The eluated DNA was cloned into plasmid pT7Blue (Novagen, USA), which has 3' unpaired deoxythimidine residues at both ends. DNA sequencing was done by the dideoxy chain termination method of Sanger *et al* (11).

Total RNA (10 μg) was loaded onto formaldehyde/1% agarose gel and blotted onto a Nylon membrane. 360-bp PCR product was labeled with Digoxygenin (Boehringer Mannheim, Germany) by the random hexamer method and it was used to probe the Northern hybridization for 17 hours.

RESULTS AND DISCUSSION

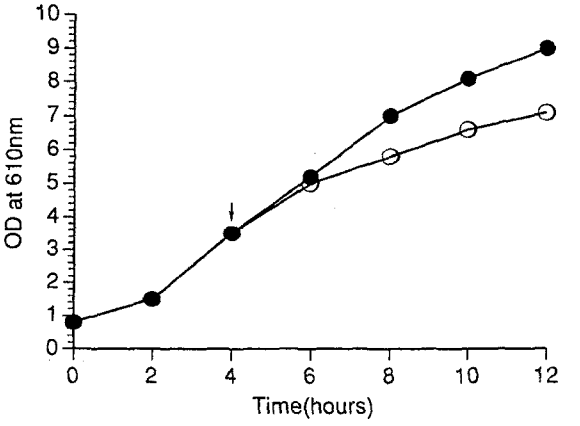

Fig. 1. Growth of *Schizosaccharomyces pombe* 732 with (○) or without (●) cadmium.
The culture was diluted 10 times when it reached the early stationary phase and was grown at 30°C in a YPD medium. Cadmium was added at the time indicated by the arrow.

Growth Inhibition of *S. pombe* by Cadmium

Schizosaccharomyces pombe is known to be relatively resistant to cadmium and to grow in any medium containing 2 mM $CdCl_2$ (10). Fig. 1 shows the growth curves for *S. pombe* 732 with and without $CdCl_2$. Strain 732 showed a slight inhibition of growth in the presence of 1 mM $CdCl_2$. Possible mechanisms of such a resistance include extracellular binding or chelation and intracellular sequestration or chelation of the heavy metal (13). *S. pombe* synthesizes cadmium-binding peptides called "cadystins", with the moleocular structure (γ-Glu-Cys)n-Gly (n=2=11), in the medium containing cadmium (9). The compounds are believed to be responsible for the cadmium-resistance in *S. pombe*. The new enzyme, cysteine dipeptidyl transpeptidase, was discovered from the *Silene cucubalus* cell suspension cultures that catalyze the transfer of the γ-glutamylcysteine dipeptide moiety of glutathione (γ-Glu-Cys-Gly) to a growing chain of (γ-Glu-Cys)n-Gly oligomers (4). Although such reports support the suggestion of a detoxification function for cadystins, it is necessary to define exactly which proteins contribute to and are responsible for intracellular cadmium detoxification.

Identification and Purification of Gene Induced by Cadmium Stress

The methods used currently in comparative studies to distinguish mRNAs, rely on the subtractive hybridization technique (7) or the cloning method based on DNA insertions (3). For our study, we approached the identification of cadmium-stress induced genes by using the induced RNA Random Fishing (IRRF) method. This method (Fig. 2) was used to amplify the partial cDNA sequences from the subsets of total RNAs by reverse

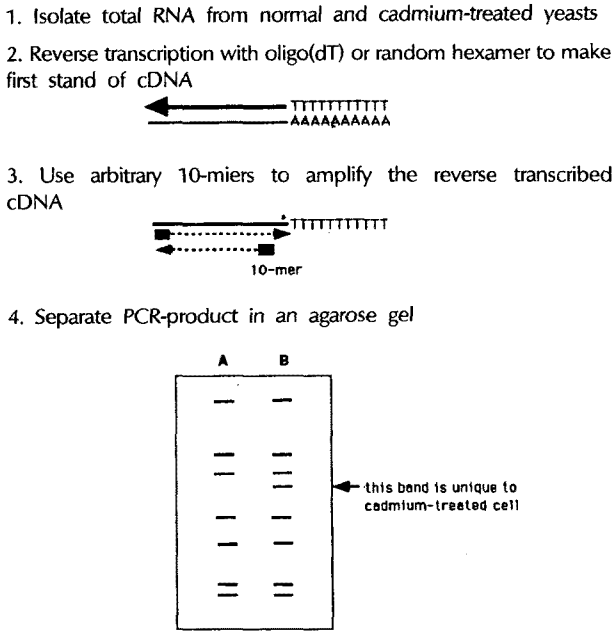

Fig. 2. Cloning strategy using the Induced RNA Random Fishing (IRRF) method

transcription and the polymerase chain reaction (PCR) with arbitrary primers. Comparison of reaction products from the two sources of total RNAs on gel revealed the differences in gene expression between the cell conditions.

Total RNAs from normal and cadmium-treated yeast cells were reverse transcribed with random hexamer or oligo (dT) primer followed by PCR in the presence of an arbitrary 10-mer as PCR primer. The PCR products from both RNAs were compared on a 1.5% agarose gel. When normal and cadmium-treated cells were compared with one of the arbitrary 10-mers (5′-CTGGCAAGGA-3′), most bands were found to be the same, but a few bands such as those 400-bp in size were seen only in cadmium-stressed cells (Fig. 3). This result suggested that a DNA band of 400-bp was produced when the yeast cells were under the stress caused by cadmium. Repeated experiments produced reproducible patterns of PCR products. In order to analyze the induced DNA, a 400-bp PCR fragment was isolated by excising the band from an agarose gel and was cloned into pT7Blue plasmid.

cDNA Synthesis with Different Primers

In a normal Reverse Transcriptase-PCR experiment, there are two ways of priming the mRNA for cDNA synthesis. Total RNAs are usually converted into cDNA by priming with either oligo (dT) or random hexamers. In most cases, it does not matter which priming method

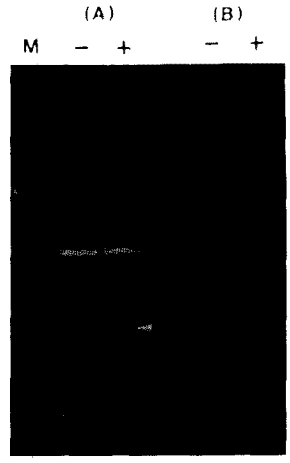

Fig. 3. 1.5% agarose gel analysis of PCR products of cDNA reverse-transcribed with random hexamer (A) or oligo (dT) primer (B).
The arrowhead indicates a band unique to cadmium-treated cells (+). The markers (M) are pBR322/*Hinf*1 fragments.

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CTGGCAAGGA GAATTATCAC TAAATAAAGT AGGGAACCAT TCATTCTATC CTAATCAATT    60
TGTCATTCTA AAAGATGCGC TTGGTTCATC AGCTTCTGCA CTTGGCATTG TTGATCAAAC   120
GAAAGGGAAG TTGAAAAAAC TTATTTTTGA TTATGATCAT ATTTGGGGAA TTAAACCAAG   180
AAATGTACAA CAAACGATGG CATTAGAACT GCTTTTACGC AGGGACTTGC CTCTTGTGAC   240
TTTAATTGGT AAAGCAGGAA CTGGAAAGAC ATTACTTGCG CTAGCTGCAG GCCGAATGCA   300
GACTGAGGAT TTAGGAGATT TCAAAAAGTT ACTTGTCGCT AGACCAATTG TCCCGGTTGG   360
TAAAGATTTA GGGTCCTTGC CAG
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Fig. 4. Nucleotide sequence of 400-bp PCR product.
Flanking arbitrary PCR primers are underlined.

is used (14). The cDNAs with different priming method were compared in this Random PCR, and the DNA band patterns revealed that the random hexamer reactions were more consistent and resulted in clearer amplifications (Fig. 3). Because of the extremely high sensitivity of RT-PCR, amplification with cDNA made by random hexamer could have been contaminated with genomic DNAs. This potential problem was eliminated by the treatment of RNase-free DNase in the substrate total RNAs.

DNA Sequence of PCR Product

The 400-bp PCR product cloned into pT7 Blue plasmid was sequenced. The nucleotide sequence of 383 bp is shown in Fig. 4. It indicates that the 400-bp DNA fragment is flanked by an arbitrary primer (5′-CTGGCAAGGA-3′) sequence at both 5′ and 3′ end. This sequence shows a translational open reading frame and has no considerable homologies with the protein sequences in PC/GENE (USA) protein databases. Searching the GenBank and EMBL DNA databases revealed that the 400-bp DNA fragment has no homologies in nucleotide

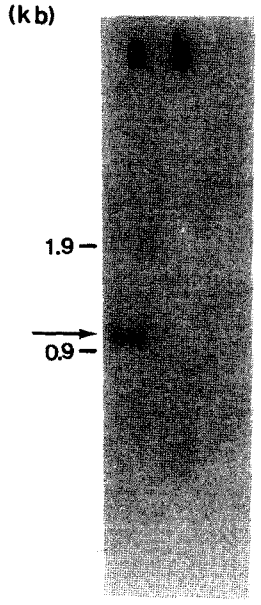

Fig. 5. Induction of transcription by cadmium stress.
Northern blots of total RNAs (10 μg) isolated from both cadmium-treated (a) and normal (b) *S. pombe* was hybridized to the 400-bp PCR product probe. The arrow indicates a 1-kb transcript specific to cadmium-treated cells.

sequence to any known DNA.

Northern blot analysis with the 400-bp fragment probe identified a single mRNA species of about. 1.0 kb. The blot indicates that the "400-bp" mRNA was expressed only in the cadmium-treated cells and not in the normal cells (Fig. 5).

We believe that the Induced RNA Random Fishing (IRRF) method described here could be an alternative to subtractive or differential hybridization techniques. Differential gene expression of cells can be detected by PCR reaction with short arbitrary primers. This is also useful in sequencing the induced cDNAs quickly; thereby the tags for environmentally stress-induced genes can be obtained and compared with the sequences in databanks. The PCR products can be used as probes for the isolation of cDNA and genomic libraries for the screening of an intact gene. This fast approach to the characterization of specifically expressed RNA would be more effective than the Expressed Sequence Tags (ESTs) method (1) in discovering other various gene expressions.

Acknowledgement

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