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Characterization of PCR fragment of metallothionein gene from liver mRNA of channel catfish

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Metallothionein is an important and essential protein to control the intracellular concentration of heavy metals, which exist in all organisms from bacteria to vertebrates. Although the detailed functions and induction mechanisms of metallothionein gene have not been clearly characterized until yet, the structure of several metallothionein genes has been revealed. Especially, piscine metallothionein is regarded as an important protein because it is induced by several heavy metal pollutants and environmental stress and it could be determined the comparative amount of heavy metals and the extent of environmental stress by assaying the RNA transcript of metallothionein gene in the method of the quantitative RT-PCR(Reverse Transcriptase Polymerase Chain Reaction). In this study I have characterized the 450 bp PCR fragment of metallothionein gene amplified by using the mixture of internal specific primers and universal 3' end primer. The nucleotide sequence analysis of 450 bp PCR fragment amplified in cDNA library of channel catfish did not show strong homology to other piscine metallothionein genes.

Key words : Metallothionein, Channel catifish, PCR

Metallothioneins(MTs) are most abundant protein in all organisms from bacteria to vertebrates including human, control the homestasis of intracellular concentration of heavy metals, and are involved in the detoxification of heavy metal uptaken from environment. MTs are also a family of low-molecular weight, cysteine-rich proteins that have a high affinity for divalent ions(Conner and Fowler, 1994). Recently, the intracellular level of MT has become a major biomarker for monitoring metal pollution in fish, molluscs and crustaceans(Bonwick *et al.* and Chan, 1995).

For this purpose, it is necessary to develope a reliable and standard assay method for detection of MT in marine organisms, which requires several essential recombinant DNA techniques including the precise DNA probes for MT cDNA of marine organisms and the specific oligonucleotide primers of RT-PCR for detecting and quantitatively assaying of MT mRNA. Recent molecular biological and immunological studies have revealed a strong structural conservation amongst fish MTs and clear differences from their mammalian and avian MTs(Kille *et al.*, 1992). Until now, MTs

of eight species including three fish, one echinoderm, two molluscs(both bivalves), and two crustaceans(both decapods) have been characterized to the extent that the primary structure has been determined(Roesijadi, 1992). However, the occurrence of multiple forms of MTs in a single species indicates that a better understanding of MT in aquatic animals will require consideration of the regulation and function of the individual forms since it appears that specific biological functions will be attributable to the various forms. Here, we report the another form of MTs identified by RT-PCR from liver mRNA of channel catfish.

Materials and Methods

mRNA isolation from the liver of channel catfish

Total RNA was prepared from the liver of channel catfish treated with heavy metal for induction of metallothionein transcripts according to the method of Lee *et al.* (1992) and mRNA was further purified by oligo dT column chromatography.

Preparation of oligonucleotide primer for PCR amplification

Four kinds of oligonucleotides primer were determined from the data base of fish metallothionein cDNA genes. Several fish metallothionein genes show the internal conserved region and strong homologies in N-terminal region. The sequence of internal specific primer is 5'-AGGACAGCAGGGGCAGCAACTTTTCTT-3' and internal antispecific primer sequence is 5'-AAGAAAAGTTGTGCCCCTGCTGTCCT-3'.

The sequence of N-terminal region is 5'- CCCATCGATATATATATGGATCCTGTGGAATCG-3' and the sequence of 3' universal primer is 5'-GAGGCCGCTTTTTTTTTTTTTTTT-3' (dT17-NotI). All oligonucleotides were prepared by custom synthesis(Biosynthesis Co., USA)

PCR amplification

Before amplifying the metallothionein gene, cDNA library was prepared from liver mRNA of channel catfish using cDNA synthesis Kit(Amersham Co., U.K.). The PCR amplification cycle is repeated during 30 cycles at 92℃ (1 min), 55℃ (1 min), 72℃ (1 min) and followed 20min further incubation at 72℃ for preparing PCR products to blunt ends. The standard buffer including 1.5 mM of MgCl2 was used in all PCR amplification. The Taq polymerase was purchased from Promega Co. (USA).

E. coli transformation

For high yield of transformation, Electroporation methods were applied using E.coli pulser(Bio-Rad Co., U.S.A.) with the pulse condition of 1,800V in 0.1 ml cuvette.

Plasmid DNA isolation from *E. coli* transformants.

Plasmid DNA was prepared from *E. coli* cell cultured in Terrific Broth according to the method of alkaline lysis(Sambrook *et al.*, 1989).

Preparation of T-vector

Because Taq DNA polymerase shows strong affinity to dATP resulting in short(two or three nucleotide) dA tailing of PCR product, it cause the low efficiency of blunt end ligation(Marchuk

et al., 1991). T-vector was prepared according to the method of Marchuk *et al.* (1991) and used in general ligation procedure of PCR product into the linearized cloning vector.

DNA sequencing

Dideoxy chain termination method was used to determine the nucleotide sequence using Sequenase Version 2.0 Kit(United States Biochemical Co., U.S.A.) and 35S-dATP was purchased from Amersham Co. (U.K).

Results and Discussion

We designed four kinds of different oligonucleotide primers for PCR amplification from the consereved sequences of metallothionein gene of channel catfish. The sequences of several fish metallothionein genes have been reported in rainbowtrout (metallothionein A and metallothionein B), pike, stone loach, flounder, and plaice(Kille, *et al.*, 1991). There are two highly conserved regions in N-terminus and internal region in piscine metallothionein genes. Four kinds of PCR primers as shown in Fig. 1 were synthesized and the nucleotide sequences of each PCR primers were described in Materials and Methods. As shown in Fig. 2, several combinations of PCR reactions were perfomed as described in Materials and Methods. From lane 1 to lane 5, 50 ng of total DNA pool of cDNA library prepared from liver mRNA of channel catfish was used as template DNA for PCR reactions while, from lane 6 to lane 10, 150 ng of cDNA pool was used as DNA template. There are no differences in PCR reaction resulting from the different concentration of template DNA. When 5' MT primer was only added in PCR reaction, prime dimer was identified in the case of lane 1 and lane 6. The formation of primer dimer was also identified when oligo(dT)17 adapter primer and MT specific primer were added. Formation of primer dimer was not observed in oligonucleotide primers. It is suggested that our primers designed for this study are proper to amplify the metallothionein gene in channel catfish.

PCR reaction in lane 4 and 9 shows three distinct bands above 500 bp and in lane 5 and 10 shows a distinct band about 450 bp, named as pMT450. We have analyzed this PCR product by subcloning and nucleotide sequencing.

For subcloning pMT450, we prepared T-vector using the linearized pUC19 at SmaI site and ligated the pMT450 DNA fragment with T-vector electroeluted from agarose gel. After the ligated mixture transformed to *E. coli* strain JM109, the recombinant carrying pMT450 was screened on selective media containing X-Gal and IPTG. White colonies were picked up and plasmid DNAs were prepared. As shown in Fig. 3, we could identifythe recombinant plasmids carrying 450bp fragment.

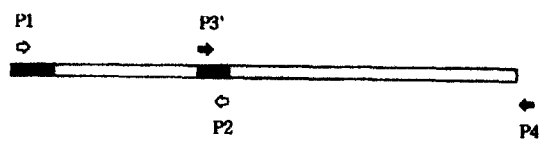

Fig. 1. Design of PCR primer in metallothionein gene.

The symbol, ▬, is the conserved region of metallothionein gene in N-terminal and internal conserved region. P1 is a N-terminal specific primer, P2 is an antispecific primer of internal conserved region, P3 is a specific primer of internal conserved region and P4 is an unversal oligo dT primer. In this work we designed the NotI-dT primer as P4.

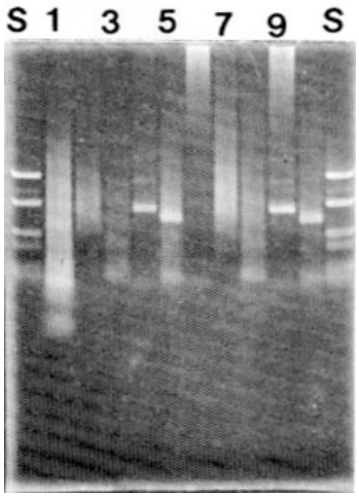

Fig. 2. The patterns of PCR amplified fragment in 1.5% agarose gel electrophoresis. The pUC19 fragments digested with Sau3A1 were used as size marker (960 bp, 585 bp, 341 bp, 285 bp, 141 bp and 105 bp are visualized). Reaction mixture(100 μℓ) for PCR contained 200 μM of dNTPs, 1 μℓ of cDNA(50 ng of cDNA pool ; lane 1-5) or 3 μℓ of cDNA(150 ng of cDNA pool ; lane 6-10), and 2.5 unit of Taq DNA polymerase. 5' MT primer(66 pmole), oligo(dT)17 adapter primer(80 pmole) and MT specific primer(60 pmole) were added reaction mixtures. Denaturation, annealing, and chain termination were performed in an Ericomp Easy Cycler. The cycle condition was described in Materials and Methods.
lane 1, 6 : only 5' MT primer was added in PCR reaction. lane 2, 7 : only oligo(dT)17 adapter primer was added in reaction. lane 3, 8 : MT specific primer was added in reaction. lane 4, 9 : 5' MT primer and oligo(dT)17 adapter primer wereadded. lane 5, 10 : MT specific primer and oligo(dT) 17 adapter primer were added.

The nucleotide sequence of pMT450 was determined by dideoxy chain termination method as shown in Fig. 4. In the previous studies, we have

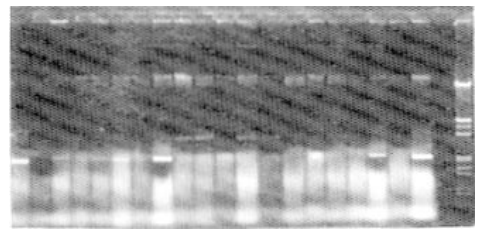

Fig. 3. Identification of subclones carrying 450 bp of PCR fragment shown in Fig. 2 (lane4).

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        10         20         30         40         50         60
AAGCTTGATA TCGCGAAACA GCTCG.ACAG GCGCGTTACA GATACCGTCC AGATAACCGG

        70         80         90        100        110        120
TTTGTCGTCA TTAAGCCGTG GTGGATGTGC CATAGCGACC GCAAAGTTAA GAAACCGAAT

       130        140        150        160        170        180
ATTGGGTTTA GTCTTGTTTC ATAATTGTTG CAATGAAATG CGGTGAAACA TTGCCTGAAA

       190        200        210        220        230        240
CGTTAACTGA AAGTGTATAT TTCGCGGATT AGTTCATGAC TTTATCTCTA ACAAATTGAA

       250        260        270        280        290        300
ATTAAACATT TAATTTTATT AAGGCCAATT TGTGGCACAC CCCTTGCTTT GTCTTTATCA

       310        320        330        340        350        360
ACGCAAATAA CAAGTTGATA ACAAAGGATG GGCTATGTCT CTACACTCTC CAGGTAAAGG

       370        380        390        4'         410        420
CTTTCGCGCT GCACTGACTA AAGAAAATCC ATTGCAGATT GTTGGCACCA TCAACGCTAA

       430        440        450        460
TCAGGCTCTG TTGGCGCAGC GTGCCGGATA TCGAATTC
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Fig. 4. Nucleotide sequence of pMT450

already characterized the induction of metallothionein-like protein and 150 bp of PCR fragment obtained from liver mRNA of channel catfish(Lee *et al.*, 1992 ; Lee and Song, 1992). That fragment showed strong homology to other piscine metallothionein genes(Kille, *et al.*, 1991). However, 450 bp fragment does not show the relative homologies to other piscine metallothionein genes. It suggests that metallothionein gene of channel catfish consists of at least two kinds of different genes. For clearly identification of gene organization of metallothionein gene of channel catfish, it requires further research including the genomic clones of

metallothionein and purification of metallothionein. We would like to develope the in situ biomonitoring system using metallothonein DNA probe.

Acknowledgment

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챠넬메기의 간 mRNA로부터 분리한 metallothionein 유전자의 PCR 절편의 특성

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Metallothionein은 세균에서 척추동물에 이르기 까지 모든 생명체에 존재하며, 중금속의 세포내농도를 조절하는 중요한 단백질이다. 현재까지 metallothionein의 기능 및 유도기작에 관한 연구는 많이 진척되지는 않았으나, 여러 metallothionein 유전자의 구조가 밝혀져 있는 실정이다. 특히 어류의 metallothionein은 여러종류의 중금속과 환경적인 자극에 의하여 유도되고 정량적인 RT-PCR의 방법으로 metallothionein 유전자의 RNA transcript를 측정함으로써 환경적인 자극의 정도와 중금 속의 상대적인 양을 측정할 수 있기 때문에 중요한 단백질로 인식되고 있다. 본 연구에서는 유전자내부의 특이적 primer와 통상적인 3'말단의 primer를 이용하여 PCR에 의해 450 bp에 해당하는 metallothionein 유전자의 일부의 특성을 조사하였다. 챠넬메기의 cDNA library로부터 PCR에 의해 증폭된 450 bp의 PCR 절편은 다른 어류의 metallothionein 유전자와는 유사성을 보이지 않았다.

Key words : Metallothionein, Channel catfish, PCR