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Characterization of growth hormone-like sequence of loach, *Misgurnus mizolepis*

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We have prepared cDNA library of loach, *M. mizolepis*, in order to isolate cDNA clone of growth hormone gene. Total RNA was isolated from pituitary of loach, and then mRNA was further purified from total RNA by oligo (dT)-coupled magnetic beads. The purified mRNA was used as substrates to prepare cDNA. The resulting cDNA was ligated into the EcoRV/SmaI site of pBlueKS+. The ligation mixture have transformed *E. coli* JM109 strain with electroporator to obtain high yield of transformation efficiency. All the transformants was screened with DIG-labeled Tilapia growth hormone gene by high density colony hybridization. After isolating 10 putative colonies showing the positive signals, secondary colony hybridization and southern hybridization could confirm it as true clones. The nucleotide sequence of one candidate, pCGH1, was compared with 312 bp DNA fragment used as DNA probe and show 52% relative homology to Tilapia growth hormone gene.

Key Words : Growth hormone gene, cDNA, DNA sequencing, Hybridization

Recently, the biological interests on fish have increased gradually because the importance of fish has been recognized as a good food source and a model system to study molecular biology (Powers *et al.*, 1991). Although the fish biology has not been well understood yet, more advanced tools of molecular biology like cloning, sequencing and amplification could realize the isolation of some useful genes and the production of transgenic fish (Zhang *et al.*, 1990). Generally the genome of fish is considered as very complex same as other all eukaryotic organisms. However the tetraodonoid fish, *Fugu rubripes*, have very small genome about 400 Mb in length (Brenner *et al.*, 1993). This pufferfish genome is 7.5 times smaller than the human genome. There are many interesting genes in fish like growth hormone gene, antifreeze protein gene and metallothionein gene. Such genes have been already cloned and revealed the gene organization and transcription machinery (Ono and Takayama, 1992). Especially growth hormone genes are very important in fish because it could control the growth of fish and produce new transgenic fish using hybrid gene fused with growth hormone gene and other eukaryotic promoter region (Liu *et al.*, 1990).

We wish to isolate growth hormone cDNA clone from loach by colony hybridization with

Tilapia growth hormone gene (Ber and Daniel, 1992) as a DNA probe. The piscine growth hormone genes including Atlantic salmon, *Salmo salar* (Johansen *et al.*, 1989), tuna (Sato *et al.*, 1989) and yellowtail (Watahiki *et al.*, 1988) show strong homology to each other. There are some conserved region in C-terminus region of growth hormone. Growth hormone (GH), prolactin and their relatives constitute a multigene family which is considered to have evolved from a common ancestor. The structural and functional domains of GH appear to be highly conserved among vertebrates (Rubin and Dores, 1994).

Therefore it could be possible to isolate growth hormone gene from loach using other piscine growth hormone gene as a DNA probe. The full-length carp growth hormone gene contains 1187 nucleotides with 210 amino acid residues as precursor form (Chao *et al.*, 1989) including a signal peptide of 22 amino acid residues (Koren *et al.*, 1989). There are some examples to isolate piscine growth hormone genes from cDNA library by colony hybridization. The sole, *Solea senegalensis*, growth hormone-encoding cDNA was cloned using flounder GH cDNA (Pendon *et al.*, 1994). The sGH cDNA sequence shows homologies of 80.9, 76.9, 73.8 and 54.2% with GH of tuna, gilthead seabream (Funkenstein *et al.*, 1991), flounder and rainbow trout. In Salmonidae fish family, growth hormone pseudogene was also found as non-allele (Kavsan *et al.*, 1994) and one of two growth hormone gene in coho salmon is sex linked (Forbes *et al.*, 1994). Therefore growth hormone genes in fish is considered as one of important gene due to showing polymorphism, structural and functional homology in all vertebrates.

Here we report the characterization of growth hormone-like sequence of loach.

Materials and methods

Fish sample : The loach used in this work was supplied from Aquaculture Center in National Fisheries University of Pusan.

Restriction enzymes, DNA modifying enzymes and chemicals : All restriction enzymes were purchased from KOSCO. cDNA synthesis kit and DNA modifying enzymes like T4 DNA ligase and Alkaline Phosphatase (CIAP) were purchase from Boehringer Mannheim. X-Gal and IPTG were provided from Jersey Lab. & Glove Supply, Inc. (New Jersey, USA). SeqPlaque Low Melting Agarose was supplied from FMC, Co. (USA).

Transformation of *E. coli* : *E. coli* gene pulser (Bio-Rad, USA) was used to obtain maximum yield of transformation efficiency in order to prepare cDNA library. Preparation of competent cell and electro-transformation were performed according to manufacturer's protocols. In other subcloning procedure, the fresh or frozen competent cells were prepared by Hanahan's method (Hanahan, 1985).

Recombinant techniques : General recombinant techniques were followed by the basic protocols (Sambrook, *et al.*, 1989). High quality plasmid DNA was purified by the differential ammonium

acetate precipitation [Lee and Rasheed, 1990] from *E. coli* cells grown in Terrific Broth including an appropriate antibiotics. DNA fragments from SeaPlaque GTG agarose gel were recovered by LiCl method [Favre, 1992] after adding glycogen to prevent the loss of DNA.

mRNA isolation and cDNA synthesis : Total RNA was extracted from the pituitary of loach by thoroughly homogenizing in Dounce homogenizer. After incubation at 56℃ for 30 min, the NaCl concentration of lysis buffer [Hengerer, 1993] was increased to 500 mM, and then 2 mg oligo (dT)-coupled magnetic beads (Dynabead oligo (dT), Dynal, Norway) resuspended in 50 μl lysis buffer was added. The supernatants were recovered by aspiration, the beads were resuspended in 100 μl wash buffer (100 mM HEPES-KOH, pH 7.5, 180 mM NaCl, 10 mM EDTA, 1% SDS), collected with the magnet and the supernatant was collected. The resulting mRNA was used in cDNA synthesis as following the protocol described in cDNA synthesis kit.

Colony hybridization : All the recombinants in cDNA library were screened by directly plating transformants on nitrocellulose membrane covered agar plate [Hanahan and Meselson, 1989]. High density colony hybridization reduce the number of plates to be screened. Secondary screening of transformants showing positive signal in high density colony hybridization was done by conventional colony hybridization (Grunstein and Walls, 1979).

Hybridization and probe preparation : DNA probe (1 ~ 2 μg) was prepared with digoxigenin-11-dUTP [Martin *et al.*, 1990] by adding 2 units of Klenow fragment with hexanucleotide mixtures as random primers. DNA fragments separated on agarose gel electrophoresis were capillarity transferred or vacuo-blotted [Kadokami and Lweis, 1992] to the capillarily nylon membrane. Prehybridization and hybridization was performed using Enprotech hybridization bottles at 68℃ in hybridization oven of Integrated Separation System. Detection of probe-target DNA hybrids and color developing were done in vinyl bag within a few minutes.

DNA sequencing and DNA sequence analysis : Recombinant DNA carrying growth-hormone like sequence was analyzed the nucleotide sequence by the modified dideoxy chain termination method [Schuurman and Keulen, 1991] using Sequenase V2.0 Kit. PC genes. DNA sequence analysis program, was used to analyze DNA sequence including sequence alignment and homology search.

Results

Screening of cDNA library with high density colony hybridization : All the ligation mixture was transformed into *E. coli* strain, JM109, with electroporation. We have adjusted the number of transformants to be appeared upon nitrocellulose paper laid on LB medium containing ampicillin to about 10^4 colonies by the appropriate dilution of transformed cells. The transformants were incubated at 30℃ for 15 hours until the size of colonies was about 1 mm in diameter. As shown in Fig. 1, we could

identify the positive colonies showing strong dark signal in secondary colony hybridization.

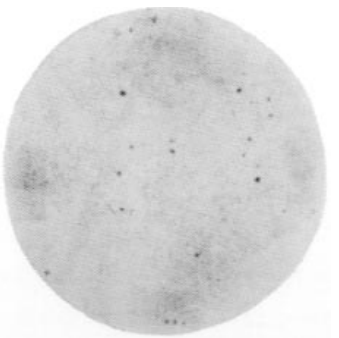

Fig. 1. High density colony hybridization of cDNA library : The preparation of the cDNA library from the pituitary of *M. mizolepis* was described in material and methods. About 20,000 colonies were screened on 85 mm Petri-dish laid nitrocellulose paper. The 312 bp of DNA fragment obtained from Tilapia growth hormone gene were used as DNA probe.

Because DIG-labeling and detection system is highly sensitive compared with the radioisotopic labelling method, the color developing time was limited within 5 minutes. Some false positive signals could also appear in DIG detection system (Martin *et al.*, 1990), further confirming experiment should have be performed like colony hybridization and southern hybridization.

Colony hybridization of putative positive colonies : All the positive colonies selected in high density colony hybridization were carefully isolated with toothpick and carried out the secondary colony hybridization. The results of secondary colony hybridization was shown in Fig. 2. All the colonies to be selected in high density colony hybridization did not show the positive signals in colony hybridization. It might be resulted from picking up the neighboring colonies around the positive signals or the false positive colonies. Therefore, the colonies showing the positive signals have to be characterized in southern hybridization.

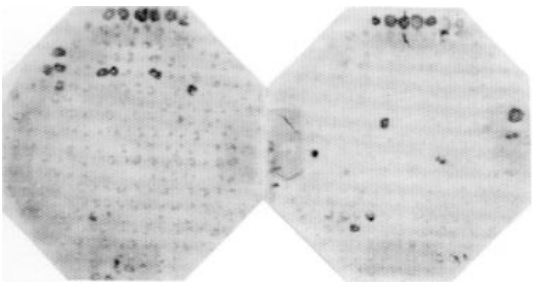

Fig. 2. Secondary screening of GH cDNA clone candidates : All colonies appeared around positive signal as shown in Fig. 1. were picked on new agar plate. The nitrocellulose paper overlaid on agar plates were peeled off and performed the conventional colony hybridization procedures.

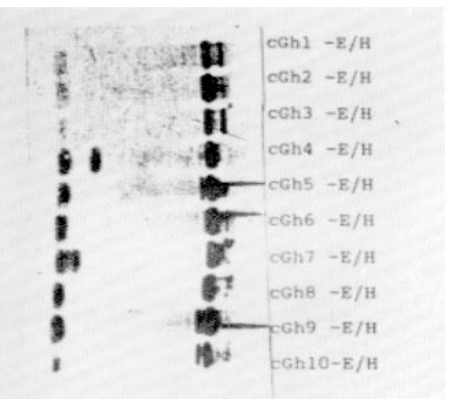

Fig. 3. Southern hybridization of cDNA clones : The plasmid DNA of 10 candidates of growth hormone cDNA clones were digested with EcoR1 and HindIII and run on 1.0% agarose gel. The small DNA fragment originated from cDNA inserts showed the positive signals.

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CLONE79    - GG-GAGTTTCCC---AGTCGCTCTCTGTCTGGA--GGTTCCTCTCTGAGG - 44
pCGH1REVE - TAAGAGATTAGATAAAGATGGTGTCTATTTTCAAAGGTTTTTACCAAACC - 50

CLONE79    - AACCAGATTTCACCAAGGCTGTCTGAGCTTAAAACGGGAATCTTGCTGCT - 94
pCGH1REVE - TTCGTCGAAAGAATAAAACTCGCTCTACTTTATCCCGGAATGGTTAGGAA -100

CLONE79    - GATCAGGGCCAATCAGGAT---GAAGCAGAGAATTATCCTG-ACAC-CGA -139
pCGH1REVE - AGTGGTCGTTTTCCCGGGTATGGTAACAATGAATTCTAGTTTACAAACTA -150

CLONE79    - CACCCTCCAG-CACGCTCCTTACGGAAACTATT-----ATCAA-AGTCTG -182
pCGH1REVE - TAAGTGCCGTACACGTTACATTAGAAGATCATTGAGCTAACAAGAATCGG -200

CLONE79    - GGAGGC--AA--------CGAATCGCT-GAGACAAACTTATGAATTGCTG -221
pCGH1REVE - GTTTGCGTAATAGTCTTTCCAAGTGTTAGAGGTTCACTCCTGTAAAGTTG -250

CLONE79    - GCTTG--CTTCAA----GAAGG-----ACATGCACAAG--GTGGA---GA -255
pCGH1REVE - TCTTGTCCGTTATTTTTGAAGTTGCTTAGTTGCTGAAGCAGTTGGTTGGA -300

CLONE79    - CCTAC-CTGA--CGGTAGCT--AAATG----TCGACTCT------CTCCA -290
pCGH1REVE - GCTTCACATATTCAGTCGTTTTAAATGAAGTTGGTTTCTAAGGGACTGGA -350

CLONE79    - GAAGCAAACTGCACTCTGTAGC        -312
pCGH1REVE - CACATAAAAGTCGCTACGTAAATCGATT -378
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Identity : 166 (53.2%)
Number of gaps inserted in CLONE79 : 22
Number of gaps inserted in pCGH1EVE : 0

Fig. 4. Alignment of two nucleotide sequences with pCGH1 and DNA probe sequence (clone 79) growth hormone gene. The character to show that two aligned residues are identical is ' : '.

Southern hybridization : Plasmid DNA was isolated from 10 clone candidates selected in secondary colony hybridization. Because the cDNA was inserted into the SmaI/EcoRV site, all plasmid DNA was digested with HindIII and BamHI, run on 1.0% agarose gel and capillarily transferred to nitrocellulose membrane. Fig. 3 illustrated the result of southern hybridization when EocRI/BamHI fragment of clone 79 (including 312 bp fragment) was used as DNA probe. The "clone 79" is carrying 312 bp of Tilapia growth hormone cDNA fragment corresponding to sequences in exon IV to VI (nucleotide 1009 to 1471) in growth hormone gene [Ber and Daniel, 1992]. Thus the 312 bp fragment was isolated and labeled with DIG for preparing as DNA probe. There are small fragments showing positive signals. One of them, pCGH1, was further characterized with nucleotide sequence analysis.

Nucleotide sequence of pCGH1 and alignment with probe DNA : The 300 bp fragment of inserted DNA in pCGH1 was eluted and subcloned into SmaI site of pUC19 for DNA sequencing with 16 mer reverse sequencing primer and 17 mer sequencing primer in pUC19 sequence. The nucleotide sequence of pCGH1 was aligned with probe DNA in Tilapia growth hormone gene using PCgene program as shown in Fig. 4. It show olny 53.2% homology with probe DNA. Because the pCGH1 does not contain the full length growth hormone gene in loach, we could not determine the coding region in pCGH1. We have also prepared the total genomic DNA library of loach. If we will isolate the genomic DNA clone by hybridization, the precise organization of growth hormone gene could be revealed.

Discussion

The amino acid sequence of growth hormone of vertebrate including fish and human show some homology in C- terminus. The growth hormone genes in fish show strong homology to each other. We chose the Tilapia growth hormone gene as DNA probe to isolate growth hormone gene in loach because growth hormone gene are conserved in fish. We have tried the genomic southern blotting to identify the homologous fragment with Tilapia growth hormone gene after digesting the genomic DNA of loach with several restriction enzymes. Unfortunately the results were not sufficient to reveal the homology. In recent, the nucleotide sequence of growth hormone gene from the silver carp, *Hypophthalmichthys molitrix*, has been revealed and compared with that of other vertebrates [Hong and Schartl, 1993]. The arrangement of exons and introns is identical to the GH genes of common carp, grass carp and very similar to mammals and birds, but quite different from that for the GH genes of tilapia and salmonids. The silver carp GH gene shares a high homology at the nucleotide and amino acids levels with those of grass carp (93.5% in nucleotides, 99.5% in amino acids) and of common carp (81% in nucleotides, 95.7% in amino acids). In recent, two growth hormone genes resulting from their polyploid ancestry have been characterized in salmonid fish, coho

salmon (Forbes *et al.*, 1994). One of two growth hormone genes in coho salmon is sex linked. Therefore, the growth hormone gene as well as other genes in fish is very interesting and valuable for studying gene evolution, heterologous expression in fish and the production of trasngenic fish.

Acknowledgement

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미꾸라지 성장 호르몬 염기 서열의 특성에 대하여

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미꾸라지의 성장호르몬 유전자를 분리하기 위하여 미꾸라지의 cDNA library 를 준비하였다. total RNA 는 미꾸라지의 뇌하수체로부터 얻었으며 oligo (dT)-coupled magnetic bead 를 이용하여 total RNA 로부터 mRNA 를 순수분리하였다. 정제된 mRNA 는 cDNA 를 합성하기 위한 기질로 사용하였으며, 합성된 cDNA 는 EcoRV/SmaI 으로 절단된 pBlueKS+ plasmid vector 에 삽입하였다. 모든 ligation 반응용액을 *E. coli*, JM109 균주에 형질전환을 유도하였으며 형질전환 효율을 최대화시키기 위하여 전기천공법을 이용하였다. 얻어진 모든 형질전환주들을 DIG 로 표지된 Tilapia 의 성장호르몬 유전자를 이용하여 고밀도 colony hybridization 에 의하여 검색하였다. 양성반응을 나타내는 10 개의 형질전환주를 분리하여 2 차 colony hybridization 및 southern hybridization 에 의하여 성장호르몬 유전자가 cloning 되었음을 확인하였다. 10 개의 형질전환주 중 하나인 pCGH1 을 probe 로 사용한 Tilapia 성장호르몬 유전자의 염기서열과 비교분석하였으며 53.2% 의 유사성을 나타냄을 확인하였다.

Key Words : Growth hormone gene, cDNA, DNA sequencing, Hybridization