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Isolation and Characterization of Brain-Derived Neurotrophic Factor Gene from Flounder (*Paralichthys olivaceus*)

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Abstract Brain-derived neurotrophic factor (BDNF) is a small secretory protein and a member of the nerve growth factor (NGF) gene family. We cloned the flounder BDNF gene from a flounder brain cDNA library. The nucleotide sequence of the cloned gene showed an open reading frame (ORF) consisting of 810 bp, corresponding to 269 amino acid residues. The tissue distribution of flounder BDNF was determined by reverse transcription-polymerase chain reaction (RT-PCR) in brain, embryo, and muscle tissues. To express fBDNF using a eukaryotic expression system, we constructed the vector mpCTV-BDNF containing the fBDNF gene and transformed this vector into *Chlorella ellipsoidea*. Stable integration of introduced DNA was confirmed by PCR analysis of genomic DNA, and mRNA expression in *C. ellipsoidae* was confirmed by RT-PCR analysis.

Key words: Brain-derived neurotrophic factor, flounder, expression, RT-PCR

Neurotrophins are growth factors that regulate neuronal cell survival, differentiation, and death. The neurotrophin family of growth factors, including NGF, BDNF, NT-3, NT-4/5, NT-6, and NT-7, regulates neuronal survival, differentiation, and synaptic plasticity [2, 4, 7, 8, 9, 16, 30, 31, 40, 42, 49]. These growth factors are structurally related to one another and have approximately 50% sequence identity. The regions of sequence similarity and variation are clustered [31, 42].

BDNF is essential for the development and differentiation of specific sets of peripheral and central neurons in mammals [1, 21, 22, 45, 47] and birds [6, 11, 14, 20, 43, 44]. Like mammals and birds, fish possess a unique BDNF gene, but the functions of the fish BDNF gene are still unclear. BDNF cDNA species and genes have been cloned from various sources, including *Homo sapiens* [23], *Sus scrofa* [34], *Mus musculus* [6], *Rattus norvegicus* [39], *Xiphophorus maculates* [17], *Canis familiaris* [41], *Gallus gallus* [38], *Felis catus* [35], and *Danio rerio* [19]. Knowledge of the molecular structure of BDNF in marine fish, however, is extremely limited.

In this study, we describe the molecular cloning and expression of the flounder (*Paralichthys olivaceus*) BDNF gene using a eukaryotic system. *E. coli* is the most widely used heterologous prokaryotic expression system, however, it has several limitations, such as poor expression of proteins that lack biological activity, production of proteins that are toxic to *E. coli*, and formation of insoluble inclusion bodies [15, 36]. We developed a eukaryotic expression system using a marine microalga that can produce biologically active proteins in large quantities [24]. *Chlorella ellipsoidea* is a unicellular green alga that has been widely used in aquaculture and the food industry. It can be inexpensively cultured on a large scale, because it requires only a limited amount of minerals and sunlight. Some species grow relatively fast, dividing two and nine times per day, depending on light intensity and temperature. *Chlorella* is a eukaryote, and therefore, it can synthesize complex proteins that require post-translational modification to become biologically active. These characteristics provide a rationale for using *C. ellipsoidea* as a system for foreign protein overexpression.

Materials and Methods

RNA Isolation and cDNA Library Construction

Mature flounders (*P. olivaceus*) were purchased from a fish market, and ten brains from each sex were collected. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, U.S.A.). The RNA pellet was washed with 70% ethanol, dried, and dissolved in DEPC-treated water. Poly(A) RNA was isolated with a Micro-FastTrack™ 2.0 Kit (Invitrogen,

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Carlsbad, U.S.A.). The quantity of RNA was determined by measuring optical density (OD) at 260 nm. A brain cDNA library was constructed using a ZAP-cDNA® Synthesis Kit (Stratagene, La Jolla, U.S.A.) and contained approximately 1×10^5 clones. The library was then amplified up to 3×10^9/ml.

Screening of BDNF cDNA and DNA Sequencing

The National Center for Biotechnology Information (NCBI) nucleotide and protein sequence databases were searched for conserved nucleotide sequences of vertebrate BDNF, which were then used for designing oligonucleotide primers. Degenerate oligonucleotide primers used to prepare probes for screening BDNF were synthesized by GenoTech (Taejeon, Korea). The probe was amplified by PCR, using the forward primer BDNF-F1 (5'-ACCATCCTG/TTTCCTTACTATGG-3') and the reverse primer BDNF-R1 (5'-TGCCGCTTGT-CTATTCCTC-3'), and labeled with a DIG (digoxigenin) oligonucleotide 3' end-labeling kit (Roche, Mannheim, Germany). The PCR program consisted of 30 cycles at 94°C for 30 sec, 65°C for 30 sec, and 72°C for 40 sec. Approximately 1×10^5 colonies from the cDNA library were screened with the above probe. Positive colonies identified by the first screening were confirmed by a second screening [10, 25, 26, 27, 28, 29]. The phagemid was excised from colonies found to be positive in the second screening, according to the manufacturer's instructions (Stratagene, La Jolla, U.S.A.). The excised phagemid was sequenced using an ABI PRISM™ DNA sequencing kit (Applied Biosystems, Foster, U.S.A.) and determined with an ABI 377 Genetic Analyzer according to the manufacturer's instructions (Applied Biosystems, Foster, U.S.A.).

Comparative Sequence Analysis of Flounder BDNF

To examine the molecular evolution of BDNF, the following vertebrate BDNF amino acid sequences were imported from the SwissProt/GenBank databases: *Homo sapiens* (human, P23560), *Sus scrofa* (pig, P14082), *Mus musculus* (house mouse, NP_031566), *Rattus norvegicus* (Norway rat, P23363), *Xiphophorus maculates* (southern platyfish, CAA42567), *Canis familiaris* (dog, Q7YRB4), *Gallus gallus* (chicken, P25429), *Felis catus* (cat, AAF03423), *Danio rerio* (zebrafish, NP_571670), and *Paralichthys olivaceus* (flounder, AAL71888). The nucleotide sequences were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). A multiple sequence alignment was conducted using Clustal W (http://www.ebi.ac.uk/clustalw), and sequence identities were calculated using GeneDoc (http://www.psc.edu/biomed/genedoc). A phylogenetic tree was constructed by the neighbor-joining (NJ) method using the Treecon program [18, 46, 48] with the amino acid sequences of BDNF from *H. sapiens*, *S. scrofa*, *M. musculus*, *R. norvegicus*, *X. maculates*, *C. familiaris*, *G. gallus*, *F. catus*, *D. rerio*, and *P. olivaceus*.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

In order to perform RT-PCR, total RNA was isolated from brain, embryo, and muscle tissues of mature flounder (N=10; size: 45±10 cm; body weight: 900±300 g; age: 3 years) using the Titan™ one tube RT-PCR system (Roche, Mannheim, Germany). Master mix 1 contained 0.2 mM dNTPs, 5 mM dithiothreitol, 50 pmol each of upstream (BDNF-F2; 5'-ACCATCCTGTTCCTTACTATGG-3') and downstream (BDNF-R1; 5'-TGCCGCTTGTCTATTCCTC-3') primers, template RNA, and 5U of RNase inhibitor. Master mix 2 consisted of 5× RT-PCR buffer and polymerase. Mix 1 and mix 2 were placed in a 0.2-ml thin-walled PCR tube on ice. The sample was incubated in a thermocycler (Applied Biosystems, GeneAmp PCR system 2400) for 1 h at 50°C for reverse transcription, and PCR was performed using a program of 94°C for 5 min followed by 30 cycles at 94°C for 40 sec, 65°C for 30 sec, and 72°C for 40 sec, and finally 7 min of extension at 72°C.

Expression of Flounder BDNF in *Chlorella*

Chlorella ellipsoidea was cultured as described by Kim *et al.* [24]. The flounder BDNF gene and the mpCTV vector described by Kim *et al.* [24] were digested with *Bam*H1 and *Xho*1, ligated, and transformed into *E. coli* XL1-Blue, resulting in the formation of an mpCTV-BDNF plasmid. Protoplasts of *C. ellipsoidea* cells were prepared as described by Kim *et al.* [24]. The mpCTV-BDNF plasmid was transformed into the protoplast as described by Kim *et al.* [24]. Transformants were transferred into f/2 medium containing phleomycin (1 μg/ml) and cultured under a fluorescent lamp at 25°C with a photoperiod of 18L:6D. Genomic DNA from *Chlorella* was isolated as described by Dawson *et al.* [12]. PCR was performed using *Chlorella* genomic DNA from transformants. The primers used for PCR were BDNF-F2 and BDNF-R1. Total RNA was isolated from *Chlorella* and used for RT-PCR.

RESULTS AND DISCUSSION

Fish are the largest and most diverse group of vertebrates. Their evolutionary position relative to other vertebrates and their ability to adapt to a wide variety of environments make them ideal for studying molecular evolution. In addition, fish are the most primitive vertebrates, and genetic information obtained from them can reveal the origin of genes with a similar function in other organisms.

Brain-derived neurotrophic factor (BDNF) is a member of the NGF family of neurotrophic factors (also called neurotrophins) that are required for the differentiation and survival of specific neuronal subpopulations in both the central and the peripheral nervous systems. BDNF is

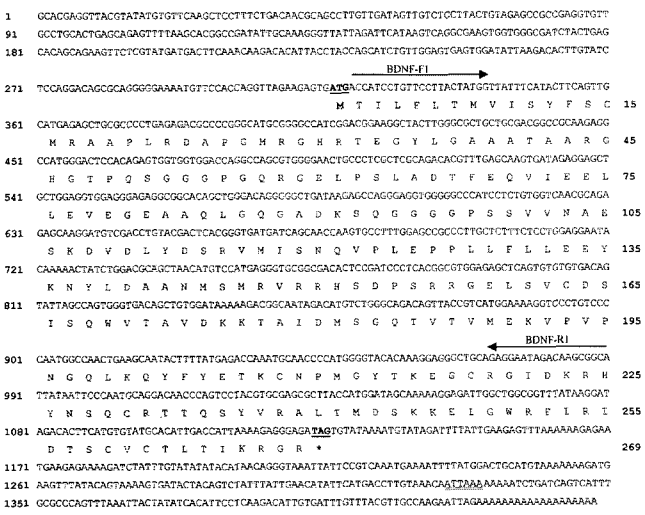

Fig. 1. Nucleotide and deduced amino acid sequences of fBDNF cDNA.
Numbers in the left and right margins correspond to the first nucleotide and last amino acid in the line, respectively. The presumptive ATG start codon and TAG stop codon (*) are bolded and underlined. The consensus polyadenylation signal (ATTAAA) is double underlined. The arrows and names indicate primer sites used in screening for the BDNF gene.

synthesized as pre-pro-peptides that are proteolytically processed to generate the mature proteins [5, 13].

To identify the BDNF gene from flounder, degenerate oligonucleotide primers encoding parts of the conserved domains were prepared and used to produce a probe using a flounder brain cDNA library, previously constructed using a λ ZAP-II cDNA Synthesis Kit [33]. Conserved nucleotide sequences of BDNF were determined using the NCBI nucleotide and protein sequence databases, and degenerate primers were synthesized for the preparation of a probe for flounder BDNF gene screening. Using BDNF-F1 and BDNF-R1 primers, we amplified a PCR product using a flounder brain cDNA library as a template. The ~672-bp PCR product was sequenced and shown to represent a BDNF gene sequence. Using this PCR product as a probe, approximately 1×10^5 colonies were screened and several positive clones were obtained. After DNA sequence analysis, we identified full flounder BDNF cDNA. As shown in Fig. 1, the cDNA sequence of the flounder BDNF gene was determined as shown in Fig. 1 and deposited with GenBank (AY074888). The deduced amino acid sequence for the cDNA is also shown in Fig. 1. The flounder BDNF gene is 1,438 bp long, including an open reading frame and 5'- and 3'-untranslated regions (UTR), encoding 269 amino acid residues. The cDNA consists of 319 bp of 5'-untranslated region, 810 bp of coding region, 312 bp of 3'-UTR, followed by a poly(A) sequence. The 3'-UTR contains polyadenylation signals (ATTAAA) and gave a signal peptide of 18 amino acid residues (1–18 aa), followed by a mature peptide of 119 amino acid residues (151–269 aa).

The amino acid sequence identity was calculated using the Genedoc program [32]. A comparison of the flounder BDNF amino acid sequence to that of other species is shown in Fig. 2. The flounder BDNF has greater than 50% amino acid sequence similarity to that of other species. By this analysis, flounder BDNF shows 94.4%, 85.5%,

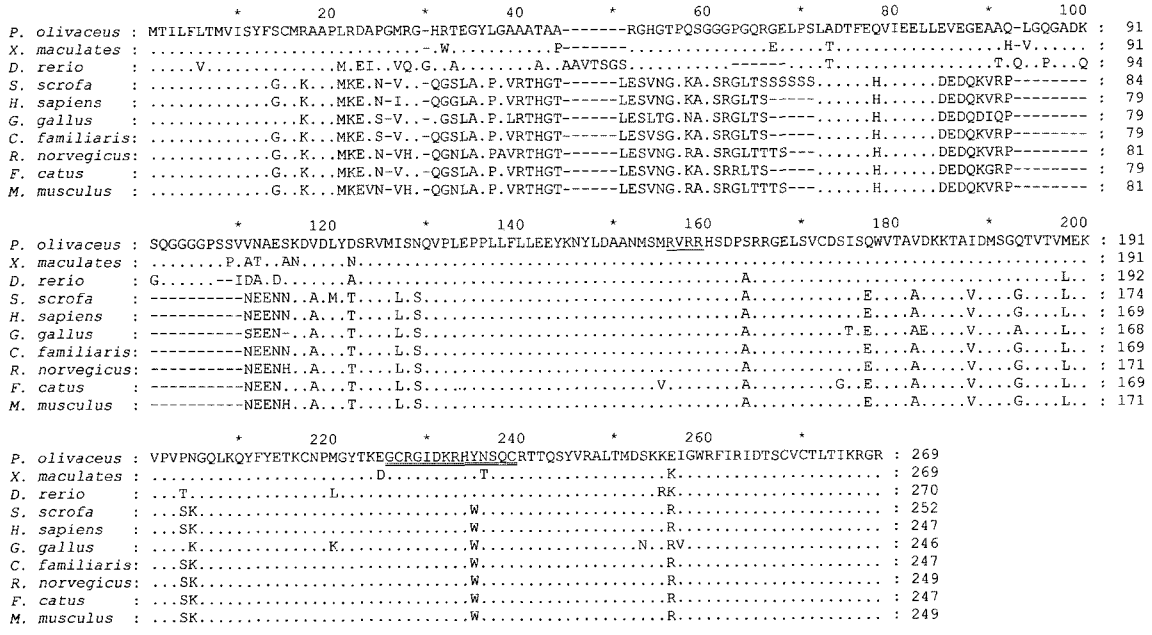

Fig. 2. Alignment of the deduced amino acid sequence of fBDNF with other vertebrate BDNFs.
Identical amino acid residues are marked with periods. Dashes represent amino acid deletions. The RVRR cleavage signal is underlined and the NGF motif is double underlined.

74.2%, 72.0%, 71.6%, 71.6%, 71.3%, 71.2%, and 70.6% sequence identity with southern platyfish, zebra fish, pig, human, chicken, dog, cat, Norway rat, and house mouse, respectively. A putative N-linked glycosylation site (N-X-S/T) is located at position 143 (Fig. 2), in common with other species. Several characteristic domains within the BDNF pre-pro-protein have been described [23]. The amino-terminus encodes a secretory signal sequence (1–18 aa), which appears to be strongly conserved in all BDNF sequences. Sequences between 21 and 120 aa reveal large differences, especially when the peptide sequences of mammals are included in this comparison. The RVRR motif sequences (147–150 aa) are conserved compared with other BDNF species. The RVRR motif is the cleavage signal necessary for formation of the mature BDNF molecule. The 'NGF' motif between 217 and 230 aa, a typical feature of all neurotrophin peptide sequences analyzed to date, is highly conserved in all BDNF sequences [3, 37]. Flounder BDNF has six conserved cysteine residues, at 169, 208, 218, 230, 259, and 260 aa, that are involved in the formation of three disulfide bonds.

Figure 3 illustrates a molecular phylogenetic tree of vertebrate BDNF, which shows the evolutionary divergence of the BDNF genes of southern platyfish, zebra fish, pig, human, chicken, dog, cat, Norway rat, house mouse, and flounder. The results of this study provide evidence for the relatedness of BDNF, which may be essential to understand the molecular evolution of this gene in vertebrates.

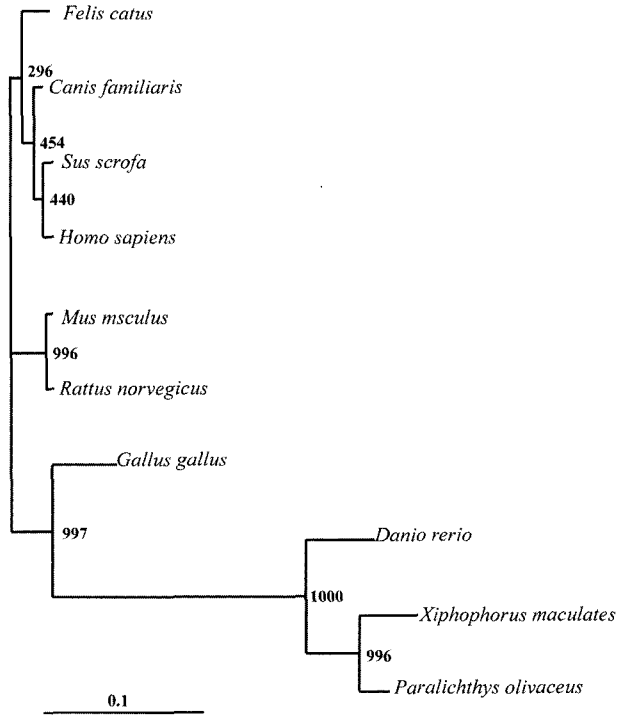

Fig. 3. Phylogenetic tree for BDNF from vertebrates, drawn by the neighbor-joining method using the amino acid sequence of BDNF from *Homo sapiens*, *Sus scrofa*, *Mus musculus*, *Rattus norvegicus*, *Xiphophorus maculates*, *Canis familiaris*, *Gallus gallus*, *Felis catus*, *Danio rerio*, and *Paralichthys olivaceus*. The numbers at the nodes are the bootstrap values based on 1,000 trials.

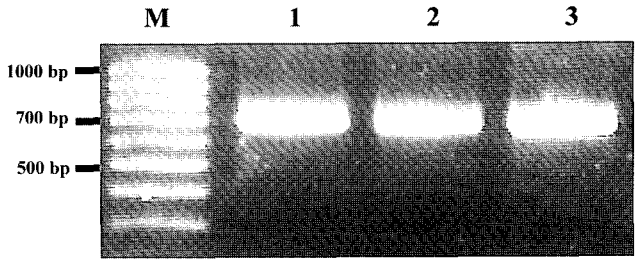

Fig. 4. Patterns of fBDNF expression detected by RT-PCR. Lane M contains a molecular marker. Lanes 1–3 contain brain, embryo, and muscle tissue, respectively.

The tissue distribution of the flounder BDNF gene was investigated by RT-PCR with total RNA isolated from flounder tissues as a template. As shown in Fig. 4, a DNA fragment of approximately 672 bp was amplified from all total RNAs extracted from the brain, embryo, and muscle tissues. RT-PCR provided evidence for the expression of the BDNF gene. The DNA banding patterns suggest that BDNF mRNA may have a wide tissue distribution in flounder.

In order to functionally express the flounder BDNF gene, we used a *Chlorella* expression system as described by Kim *et al.* [24]. Flounder BDNF gene subcloned into mpCTV was transformed into *C. ellipsoidea* protoplasts. The transformed protoplasts were transferred from regeneration medium into f/2 medium containing 1 μl/ml of phleomycin. The incorporation of the fBDNF gene into the chromosomal DNA of *C. ellipsoidea* was confirmed by PCR amplification using chromosomal DNA isolated from the transformant after 5 days of transformation (Fig. 5). The transcription of BDNF gene in *C. ellipsoidea* was confirmed by RT-PCR amplification using total RNA isolated from the transformant (Fig. 6).

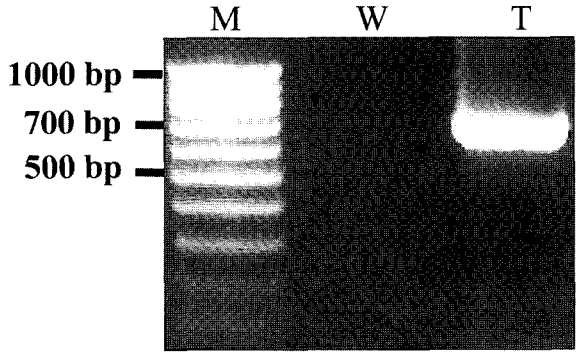

Fig. 5. Confirmation of transformed fBDNF gene by PCR. Lane M contains a molecular marker, Lane W contains wild-type *C. ellipsoidea*, and Lane T contains transformed *C. ellipsoidea*.

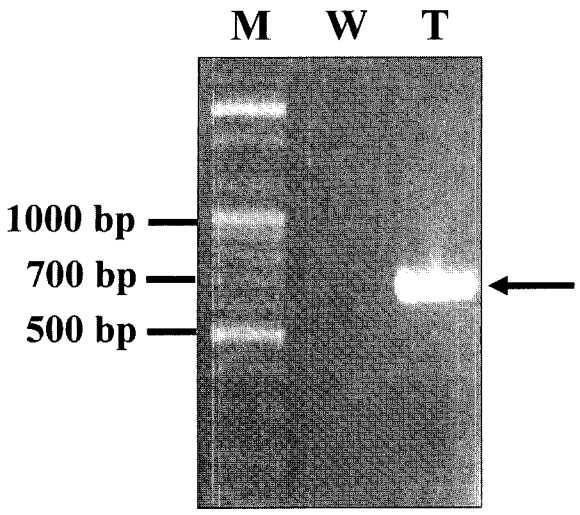

Fig. 6. Confirmation of transformed fBDNF gene by RT-PCR. Lane M contains a molecular marker, Lane W contains wild-type *C. ellipsoidea*, and Lane T contains transformed *C. ellipsoidea*.

Herein, we have reported the flounder BDNF gene sequence for the first time. The 10 six-cystines and the one potential N-linked glycosylation site are completely conserved. Observations and genetic manipulations of fBDNF make this species a very useful model for studying the mechanism of BDNF participation in the differentiation and survival of specific neuronal subpopulations in the central as well as the peripheral nervous systems.

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