Characterization of the Pacific Oyster *BTG1* homologue gene (*Crassostrea gigas*)

참굴(*Crassostrea gigas*)의 *BTG1* homologue 유전자의 특성

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by

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A Dissertation

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Abstract

BTG1(B-cell translocation gene 1) protein that belongs to anti-proliferative protein family(APRO) was identified through the molecular charaterization of a chromosomal translocation in lymphoid malignancy.

In the present study, the cDNA encoding the oyster(*Crassostrea gigas*) *BTG1* gene was isolated from the gill cDNA library by an expressed sequence tag(EST) analysis and its nucleotide sequence was determined. The *BTG1* gene contains an open reading frame of 182 amino acids with 57 %, 56 % identities to its zebrafish and human counterparts, and the coding region is not interrupted by introns. Maximal homologies were shown in conserved Box A and B. In addition, the predicted promoter region and the different transcription-factor binding site like an activator protein-1(AP-1) response element involved in negative regulation and serum response element(SRE) were able to be identified by the genomic DNA walking experiment.

To identify whether the expression of the oyster *BTG1* gene is tissue specificity, RT-PCR and Northern blot were performed with the different tissues(mantle, gill, heart and digestive gland).

Key words : BTG1, APRO family(anti-proliferative protein family), serum response element(SRE), genomic DNA walking, AP-1 response element

Introduction

The BTG family of anti-proliferation gene products including Pc3/Tis21/Btg2, Btg1, Tob, Tob2, Ana/Btg3, Pc3k and others are thought to play an important role in the regulation of cell cycle progression (Matsuda *et al.*, 2001 ; Tirone 2001).

The *BTG1* was identified near the breakpoint of chromosomal translocation found in a B-cell chronic lymphocytic leukemia (Rouault et al., 1992) and shared high homology with the previously identified *Tis21* (Fletcher *et al.*, 1991) and *Pc3* (Bradbury *et al.*, 1991) genes. It is expressed by several types of non-endothelial cells including fibroblasts and T-lymphocytes, and is highly regulated during cell growth and proliferation (Rouault *et al.*, 1992 ; Raburn *et al.*, 1995 ; Suk *et al.*, 1997). Its expression was maximal in the G0/G1 phases of the cell cycle and down-regulated when cells progressed throughout G1 (Rimokh *et al.*, 1991), and was also increased in response to DNA damage (Cortes *et al.*, 2000).

Their predicted peptidic sequence displays a high degree of conservation in two regions of Box A and B (Guehenneux *et al.*, 1997) which constitute the two signature motifs of this family.

The well conserved box B has been shown to mediate the interaction of BTG1 with the nucler protein CAF1 in yeast (Rouault *et al.*, 1998). In addition, the BTG1 amino-terminal domain bears an LxxLL motif favouring nuclear accumulation and another region encompassing the box A inhibiting nuclear localization (Rodier *et al.*, 2001).

The *BTG1* regulates cell-growth through interacton with different transcription factors. Recent studies using a system of two hybrid interactions demonstrated that BTG1 and BTG2(Tis21/Pc3) interact with PRMT1(Protein-Arginine N-methyltransferase) (Lin *et al.*, 1996), which asymmetrically methylates arginine residues occuring in the glycine and arginine-rich domain(RGG) of hn RNP substrates (Rajpurohit *et al.*, 1994) or histones (Ghosh *et al.*, 1988).

Other interactions have been subsequently reported with CAF1 (Rouault *et al.*, 1998 ; Bogdan *et al.*, 1998), the murine homologue of a yeast component of the CCR4 transcriptional regulatory system, and the homeoprotein Hoxb9 (Prevot *et al.*, 2000).

In addition to the anti-proliferative properties, previous studies have indicated that BTG1 is also involved in cell differention and organogenesis both *in vitro* and *in vivo* (Rodier *et al.*, 1999 ; Sakaguchi *et al.*, 2001). In a myoblast cell line, for instance, BTG1 mediates triiodothyronine- and cAMP-induced myoblast differention in myocytes (Rodier *et al.*, 1999 ; Marchal *et al.*, 1995).

More recently, Iwai *et al.*(2004) described that BTG1 may play an important role in the process of angiogenesis.

To date, more than 20 members of this family have been found from several species by a lot of laboratories. Though a biological common feature of these gene products was their ability to inhibit cell proliferation, their molecular role is poorly understood.

In the current study, we have cloned and characterized the oyster *BTG1* and analyzed its expression. The Pacific oyster(*Crassostrea gigas*) is important food source and is harvested commercially. Also,

it is the subject of considerable research activity in the U.S, Europe and Japan.

However, althouth it is one of the most commercially important bivalves, our understanding of the oyster genetics is primitive compared with our knowledge of domestic animals and plants. And then, it must be investigated further from various angles.

So we researched the oyster *BTG1* obtained by EST analysis. These results may provide a fundamental investigation to achieve an integrated understanding of the genetics basis of the oyster.

Materials and methods

The oyster tissue and gill cDNA library construction

The pacific oyster(*Crassostrea gigas*) was purchased from Ja gal chi fish market (length 4.5 ± 0.53 cm).

The cDNA library was constructed using a ZAP-cDNA synthesis kit(Stratagene) according to the manufacturer's protocol. Briefly, first strand cDNA was synthesized from 5 mg of poly(A)+ RNA with Moloney-murine leukamia virus reverse transcriptase and *Xho* I-linked oligo(dT) primers in the presence of methyl nucleotides, followed by secondary synthesis with RNase H and DNA polymerase I. The double stranded cDNA was ligated to *Eco*R I adaptors, and then digested with *Xho* I. The size-fractionated cDNA fragments were ligated into the *Eco*R I and *Xho* I sites of the Uni-ZAP XR vector. Ligated vectors were packaged using Gigapack II Gold extracts.

Genomic DNA isolation of the oyster gill

The genomic DNA of the oyster gill was prepared by the method of Blin and Stafford (1976). 0.1 mg of the gill was homogenized using a glass homogenizer in extraction buffer (0.01 M Tris, 0.1 M

EDTA, 0.5 % SDS), RNase (final concentration : 20 ㎍/㎖) was added, incubated at 37 ℃ for 1 hr. Proteinase K (final concentration : 10 ㎍/㎖) was added to homogenate and incubated at 50 ℃ overnight. An equal volume of phenol was added, mixed by inverting of the tube several times and centrifuged at 12,000 rpm for 8 min at 4 ℃. This procedure was repeated. The supernatant was transferred to a fresh tube, and mixed with equal volume of chloroform : isoamyl alcohol (24:1) and organic phase was removed. The DNA was precipitated by adding 2 volumes of absolute ethanol. The DNA pellet was transferred to a fresh tube containing 70 % ethanol by using a glass pippette, dried, and resuspended in TE buffer. The isolated DNA was stored at 4 ℃ until use.

Digestion of the genomic DNA and Purification

For each reaction, 25 ㎕ genomic DNA(0.1 ㎍/㎕) , 8 ㎕ restriction enzymes such as *Dra* I, *Eco*R V, *Pvu* II, *Stu* I(all of blunt-end digestion), 10 ㎕ restriction enzyme buffer, 57 ㎕ deionized H_2O was combined and mixed gently by inverting, incubated at 37 ℃ for 2 hr. And then briefly vortexed, returned to 37 ℃ overnight. From each reaction tube, 5 ㎕ of the DNA fragments was loaded in a 0.5 % agarose/EtBr gel to determine whether digestion is complete.

After this, the purification of them was performed. An equal volume of phenol was added to each reaction tube and vortexed

briefly, centrifuged at 8,000 rpm for 1 min to separate the aqueous and organic phases. The aqueous layer was transferred into a fresh 1.5 ml tube and added an equal volume of chloroform. It was briefly vortexed, centrifuged at 8,000 rpm for 1 min and the aqueous layer transferred into a fresh 1.5 ml tube. Two volume of ice cold 95 % ethanol, 1/10 volume of 3 M sodium acetate, and 20 μg of glycogen were added to each tube. It was vortexed, centrifuged at 15,000 rpm for 10 min. Then, the supernatant was decanted and the pellet was washed in 100 $\mu\ell$ of ice cold 80 % ethanol. The pellet was air dried and dissolved in TE buffer(10 mM Tris, 0.1mM EDTA , pH 7.5)

The DNA fragment was electrophoresed in a 0.5% agarose/EtBr gel to determine the approximate quantity of DNA after purification.

Ligation of genomic DNA to GenomeWalker Adaptor (Clontech)

4 $\mu\ell$ of digested, purified DNA was transferred to a fresh 0.5 ml tube and 1.9 $\mu\ell$ GenomeWalker Adaptor(Clontech), 1.6 $\mu\ell$ 10X ligation buffer, 0.5 $\mu\ell$ T4 DNA ligase(6units/$\mu\ell$) were added to each tube. And the mixture was incubated at 16 ℃ overnight and the reaction was stopped by incubating at 70 ℃ for 5 min. Then, 72 $\mu\ell$ of TE buffer(10 mM Tris, 1 mM EDTA, pH 7.4) was added to each tube.

Primer design

To the design of gene specific primers for genomic DNA walking, we needed to search introns in the ORF(open reading frame). It was determined by PCR of genomic DNA and cDNA clone using SI primers designed outside the ORF : SI-forward (5′ GAA ACT CGA TCG TTC TAT GAA C 3′), SI-reverse (5′ CAA ACA CGA CGG CTC TTC AA 3′) And then, for walking upstream from cDNA sequence, two gene-specific primers -one for primary PCR(GSP1) and the other for secondary PCR(GSP2)- were designed from cDNA sequence. The primer was designed as close to the 5′ end as possible.

GSP1 (5′ AGG AAT GAT GGA AAA TAT GAC GGT CTC 3′)

GSP2 (5′ CCT GTT GTT CAC TTA CAT GTT TAG AAG 3′)

Primary- and Secondary PCR

Before the primary PCR, the master mix was prepared. It was composed of 37.8 $\mu\ell$ of deionized H_2O, 5 $\mu\ell$ of 10 X Tth PCR reaction buffer, 1 $\mu\ell$ of dNTP(10 mM each), 2.2 $\mu\ell$ of $Mg(OAc)_2$(25 mM), 1 $\mu\ell$ of AP1(10 uM)*, 1 $\mu\ell$ of 50 X Advantage Genomic Polymerase mix per reaction. 48 $\mu\ell$ of the primary PCR master mix was aliquted and 1 $\mu\ell$ of each adaptor ligated DNA, 1 $\mu\ell$ of GSP1 was added to each tube.

The PCR was performed using the following two-step cycle

parameters : 7 cycles at 94 ℃ for 25 sec, 72 ℃ for 3 min, and 32 cycles at 94 ℃ for 25 sec, 67 ℃ for 3 min and 67 ℃ for an additional 7 min after the final cycle. The primary PCR product was analyzed on a 1.5 % agarose/EtBr gel, along with DNA 100bp size marker(Bioneer).

After the reaction, the nested PCR was performed with AP2* and GSP2 primers. To prepare nested PCR master mix, instead of AP1 primer, AP2 primer was mixed. The secondary(or nested) PCR was performed using the following two-step cycle parameters : 5 cycles at 94 ℃ for 25 sec, 72 ℃ for 3 min, and 20 cycles at 94 ℃ for 25 sec, 67 ℃ for 3 min, and then 67 ℃ for an additional 7 min. The nested PCR product was analyzed on a 1.5 % agarose/EtBr gel, along with DNA 100bp size marker(Bioneer).

Finally, the longest PCR product was cloned into pGEM T-vector(Promega) and was sequenced with T7, Sp6 primers.

The structure of the GenomeWalker adaptor and adaptor primers(Clontech) was shown Fig. 1.

* The AP primer indicates adaptor primer of Clontech

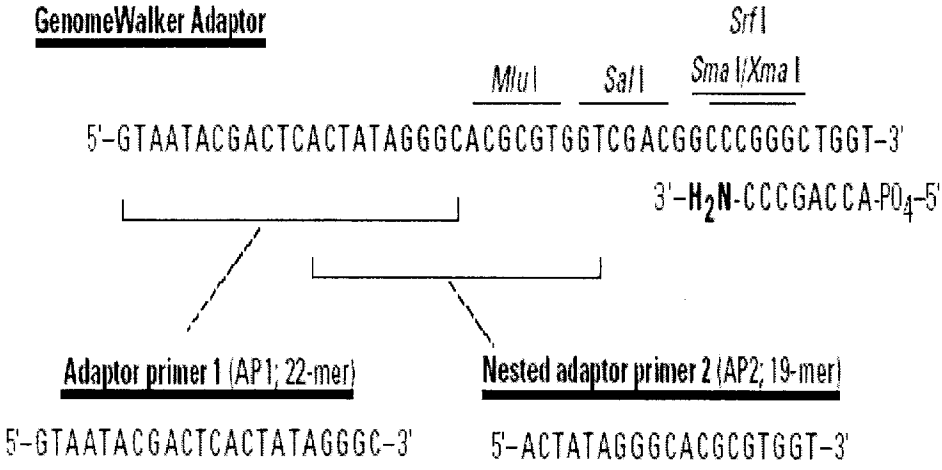


Figure 1. The structure of the GenomeWalker adaptor and adaptor primers. The amine group on the lower strand of the adaptor blocks extension of the 3′ end of the adaptor-ligated genomic fragment, and thus prevents formation of an AP1 binding site on the general population of fragments.

Total RNA isolation

Total RNA isolation was extracted from the oyster mantle, gill, heart and digestive gland using TRIzol reagent(Difco).

Each tissue was homogenized in 1 ml of TRIzol reagent per 50 mg of tissue using a power homogenizer and the homogenized sample was incubated at room temperature for 5 min. 0.2 $\mu\ell$ chloroform was added, the tube was mixed vigorously by vortexing for 15 sec and incubated at room temperature for 2~3 min. Then, the sample was centrifuged at 12,000 rpm for 15 min at 4 ℃ and the aqueous phase was transferred to a fresh tube. 0.5 ml of isopropyl alcohol was added, mixed by inverting and incubated at -20 ℃ for 10 min. The sample was centrifuged at 12,000 rpm for 10 min at 4 ℃ and the supernatant was discarded. The RNA pellet was washed with 1 ml of 75 % ethanol and centrifuged at 12,000 rpm for 5 min at 4 ℃. Finally, the pellet was dried briefly for 5 min and dissolved in DEPC-treated water. The quality of RNA was estimated by electorphoresis on 1 % formaldehyde-agarose gel. The isolated RNA was used for RT-PCR and Northern blot analysis.

RT-PCR(Reverse transcription-polymerase chain reaction)

Performing RT-PCR, total RNA was isolated from different tissues.

First-strand cDNA was synthesized using SuperScript^{TM}II reverse transcriptase(Invitrogen) following the manufacturer's protocol.

50 ng of random primer, 3 ㎍ of total RNA, 1 ㎕ of dNTP mix (10 mM each) were added and sterile, distilled water was added up 12 ㎕ to a nuclease-free microcentrifuge tube. The mixture was heated at 65 ℃ for 5 min and quickly chilled on ice. After briefly spin-down, 4 ㎕ of 5 X First-strand buffer, 2 ㎕ of 0.1 M DTT were added and mixed gently, incubated at 42 ℃ for 2 min. Then, 1㎕(200 units) of SuperScript™ II Reverse transcriptase was added and mixed gently by pipetting. The mixture was incubated at 25 ℃ for 10 min and at 42 ℃ for 50 min.

After the reaction was inactivated by heating at 70 ℃ for 15 min, the cDNA was used as template for amplification in PCR. The PCR amplification was performed with SI primer sets using the following parameters : 94 ℃ for 5 min and 35 cycles at 94 ℃ for 1 min, 55 ℃ for 30 sec, 72 ℃ for 1 min and 72 ℃ for an additional 7 min. The PCR product was analyzed on a 1.5 % agarose gel by electrophoresis.

The preparation of probe and Northern blot analysis

According to the manufacturer's protocol, the probe was prepared using PCR Dig Probe Synthesis Kit(Roche).

Isolated total RNA(10 ㎍ per sample) was electrophoresed in a 1.0 % agarose gel containing 0.7 % formaldehyde, and 20 mM 3-(N-morpholino)-propanesulphonic acid(MOPS), 5 mM sodium acetate, and 1 mM ethylene diamine tetra-acetic acid(EDTA), and transferred onto nylon membrane(Hybond, Amersham) for overnight.

After ultraviolet cross-linking, the membrane was hybridized with the DNA fragments of BTG1 coding region that were labeled with Digoxigenin-11-dUTP by PCR priming using PCR Dig Probe Synthesis Kit(Roche). And then, the membrane was washed twice at room temperature for 5 min with shaking under Low Stringency Buffer(2X SSC containing 0.1 % SDS) and twice at 50 ℃ in High Stringency Buffer(0.1X SSC containing 0.1 % SDS).

Then, after localizing probe-target with hybrids with Anti-Dig (anti-digoxigenin-alkaline phosphatase), the membrane was incubated in the dark with a freshly prepared color substrate solution containing NBT/BCIP stock solution overnight.

Sequence analysis of the oyster *BTG1*

The DNA samples were sequenced by using ABI PRISM BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) and ABI 310 Genetic Analyzer automatic DNA sequencer (Perkin Elmer) according to the manufacturer's protocol. The resulting sequences were analyzed with BLAST N and BLAST X at the NCBI site (http://www.ncbi.nlm.nih.gov) and multiple alignment of the BTG1 was performed with Clustal W(1.82).

Also, to define the molecular evolution of the oyster BTG1, several BTG1 sequences were imported from the GenBank. The phylogenetic tree was obtained using MEGA 3 and the predicted promoter region was searched using the PROSCAN 1.7 at BIMAS website.

Results

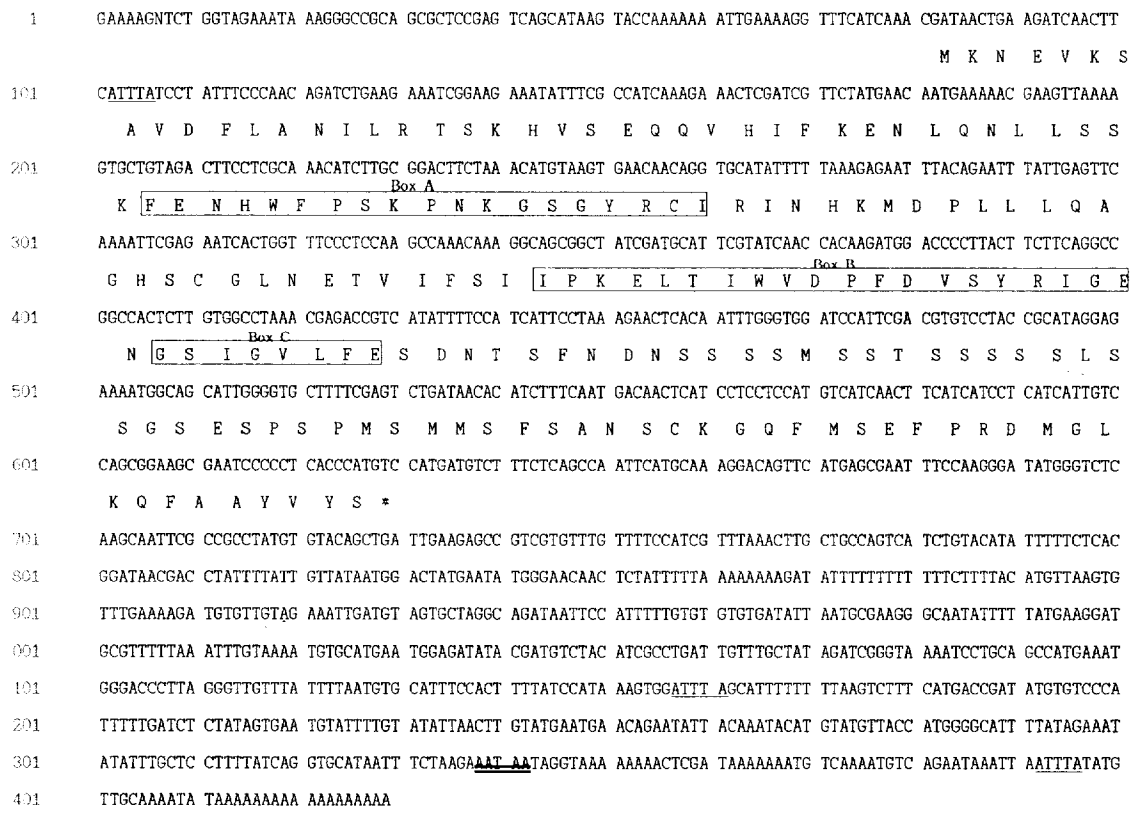
Analysis of the oyster *BTG1* cDNA

We obtained the oyster *BTG1* clone by random sequencing of gill cDNA library and EST analysis. The nucleotide sequece of oyster *BTG1* and deduced amino acid sequence are shown in Fig. 2.

The oyster *BTG1* cDNA exhibits 1,429 bp and contains an open reading frame(ORF) consisting of 182 amino acids. Moreover, The *BTG1* cDNA contains the conserved Box A, B and some copies of ATTTA motif that is known as the most common determinant of the RNA stability in mammalian cells, and we found that the oyster BTG1 displays an LxxLL-related sequence(LQNLL, amino acids). Such sequences, previously identified in transcriptional coactivators, mediate their interaction with members of the nuclear receptor superfamily (Heery *et al.*, 1997). And Box C(GSIGVLFE, amino acids) known as PRMT1(protein-arginine methyltransferase of type 1) binding site was found (Berthet *et al.*, 2002).

Analysis of the oyster BTG1 genomic sequence revealed the coding region of oyster *BTG1* gene is intronless (Fig. 3). Genes with no introns in their coding regions are common in yeast, fungi and plants, but they account for perhaps only 4 % of mammlian genes (Ajima *et al.*, 2000). Also, similar to the human and mouse sequence, oyster *BTG1* cDNA contained a long AT-rich 3′ untranslated region (Shaw *et al.*, 1986 ; Rouault *et al.*, 1993).

The polyadenylation signal AATAA was detected 69 bp upstream of poly(A) tail.



igure 2. Sequence analysis of the oyster *BTG1* cDNA and its protein product. lumber on the left indicates nucleotide positions. The "ATTTA"motif is nderlined and the polyadenylation signal is double-underlined. An asterisk idicates the stop codon. The conserved Box A, B and C are contained.

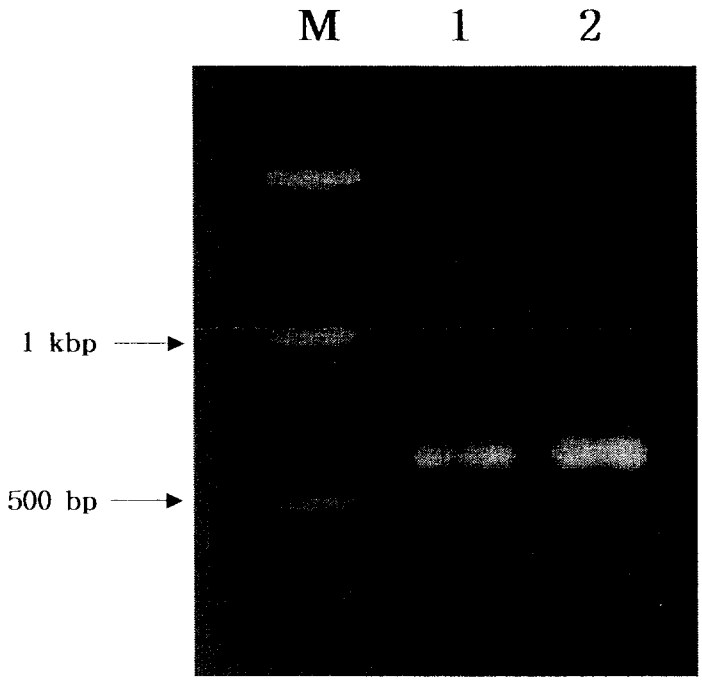


Figure 3. The PCR amplification to search introns in the ORF.
M : 100bp size marker(Bioneer), Lane 1 : Oyster genomic DNA as template, Lane 2 : *BTG1* cDNA as template

Comparison the sequence of the oyster *BTG1* with those of other vertebrates

The oyster *BTG1* exhibits an ORF consisting of 182 amino acids. Fig. 4 shows comparison of the deduced amino acid sequence of the oyster *BTG1* with those of other vertebrates. The sequence was shown 57 %, 56 %, 54 % identities to that of zebrafish, human and chicken, respectively, and shown high homology in an LxxLL motif favouring nuclear accumulation and conseved Box A, B and C. Interestingly, the amino terminal region of the oyster BTG1 is different from others (Fig. 4).

The evolutionary relationship of the BTG1 protein was analyzed using a molecular phylogenetic tree (Fig. 5).

```
M. musculus      MHPFYTRAATMIGEIAAAVSFISKFLRTKGLTSERQLQTFSQSLQELLAEHYKHHWFPEK 60
H. sapiens       MHPFYTRAATMIGEIAAAVSFISKFLRTKGLTSERQLQTFSQSLQELLAEHYKHHWFPEK 60
B. taurus        MHPFYSRAATMIGEIAAAVSFISKFLRTKGLTSERQLQTFSQSLQELLAEHYKHHWFPEK 60
R. norvegicus    MHPFYTRAATMIGEIAAAVSFISKFLRTKGLTSERQLQTFSQSLQELLAEHYKHHWFPEK 60
G. gallus        MHPALYTRASMIREIAAAVAFISKFLRTKGLMNERQLQTFSQSLQELLAEHYKHHWFPEK 60
X. laevis        MHTHYP-WANMKPEIMAAVSFITKFLRTKGLMNDLDLQTFNQSLQDLLADHYKHHWFPEK 59
D. rerio         MHTLCA-RGTMKPEINAAVGFLSRFLRIKGHVNDRQLQTFSQTLQDILAEQYKHHWFPDR 59
C. gigas         ----------MKNEVKSAVDLAN-ILRTSKHVSEQQVHIFKENLQNLLSSKFENHWFPSK 49
                   .      ..*  *: :** : . :** .   .: ::: *.:.**::*:.::::****.:

M. musculus      PCKGSGYRCIRINHKMDPLIGQAAQRIGLSSQELFRLLPSELTLWVDPYEVSYRIGEDGS 120
H. sapiens       PCKGSGYRCIRINHKMDPLIGQAAQRIGLSSQELFRLLPSELTLWVDPYEVSYRIGEDGS 120
B. taurus        PCKGSGYRCIRINHKMDPLIGQAAQRIGLSSQELFRLLPSELTLWVDPYEVSYRIGEDGS 120
R. norvegicus    PCKGSGYRCIRINHKMDPLIGQAAQRIGLSSQELFRLLPSELTLWVDPYEVSYRIGEDGS 120
G. gallus        PCKGSGYRCIRINHKMDPLIGQAAQRIGLSSQELFQLLPSELTLWVDPYEVSYRIGEDGS 120
X. laevis        PTKGSAYRCIRINHKMDPLIGQAADRIGLNSQQMFKLLPSELTLWVDPYEVSYRIGEDGS 119
D. rerio         PNKGSGYRCIRINHKMDPLVGQAGQRIGLSIQQLYLLLPSELTLWVDPFEVSYRIGEDGS 119
C. gigas         PNKGSGYRCIRINHKMDPLLLQAGHSCGLNETVIFSIIPKELTIWVDPFDVSYRIGENGS 109
                 * ***.**************: **..  **.   :: ::*.***:****::********:**
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Figure 4. The multiple alignment of the deduced amino acid sequence of the oyster BTG1 with other vertebrates. An asterisk indicates the identical amino acids.

```
M. musculus     ICVLYEA--------SPAGGSTQNSTN----------VQMVDSRISCKEELL--LGRT 158
H. sapiens      ICVLYEA--------SPAGGSTQNSTN----------VQMVDSRISCKEELL--LGRT 158
B. taurus       ICVLYEA--------SPAGGSTQNSTN----------VQMVDSRISCKEELL--LGRT 158
R. norvegicus   ICVLYEA--------SPAGGSSQNSTN----------VQMVDSRISCKEELL--LGRT 158
G. gallus       ICVLYEA--------APAGGS-QNNTN----------MQMVDSRISCKEELL--LGRT 157
X. laevis       ICVLYES--------VPGSGISPNSS----------GSLVESRISCKNELL--LGRT 156
D. rerio        ICVLYES--------HPGTNGNPSTTTGNSIPASSVTQVSAMVESHISCKEELLV-LGRT 170
C. gigas        IGVLFESDNTSFNDNSSSSMSSTSSSSSLSSGSESPSPMSMMSFSANSCKGQFMSEFPRD 169
                * **:*:          ..    . ..:.. :  :.: :    :  *  *** :::  : *

M. musculus     SPSKNYNMMTVSG 171
H. sapiens      SPSKNYNMMTVSG 171
B. taurus       SPSKNYNMMTVSG 171
R. norvegicus   SPSKNYNMMTVSG 171
G. gallus       SPSKSYNMMTVSG 170
X. laevis       SPSKRYNMMTVSG 169
D. rerio        SPAKPY-MMTVSS 182
C. gigas        MGLKQFAAYVYS- 181
                   * :    . *.
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Figure 4. -continued

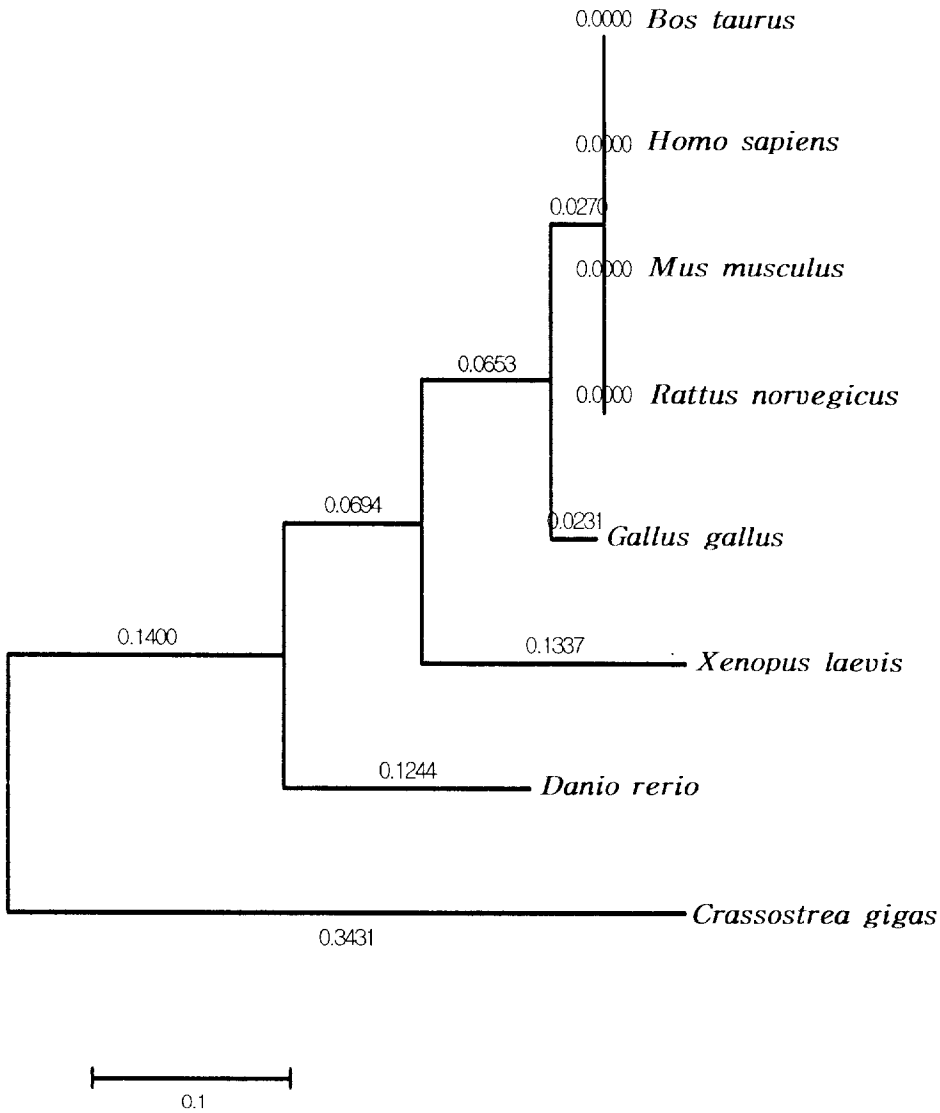

Figure 5. Phylogenetic relationship in the BTG1 protein. An evolutionary tree was calculated analyzing the sequences by the nearest neighbour algorithm(a scale of "0.1" means 0.1 nucleotide substitutions per site).

Genomic DNA walking upstream from the *BTG1* cDNA

To search transcription factors and the promoter region, we performed genome walking experiment(PCR-based) using genome walking kit(Clontech) (Fig. 6). The walking sequence contains the predicted promoter, TATA box and cAMP reponse element(CRE) and AP-1 like sequence (Fig. 7).

Through a conserved cAMP reponse element(CRE), cyclic AMP is believed to mediate its regulatory effect on gene expression. Elevation of cAMP activates cAMP-dependent protein kinase(PKA) and this activated kinase post-translationally modifies the CRE-binding protein or the CRE modulator that binds to the CRE to either enhance or suppress gene transcription (Montminy *et al.*, 1990 ; Foulkes *et al.*, 1991). Thinking over previous studies (Suk *et al.*, 1997), this result suggests that the oyster *BTG1* espression related to intracellular cAMP levels. Unlike the human, AP-1 like sequence is near the TATA box.

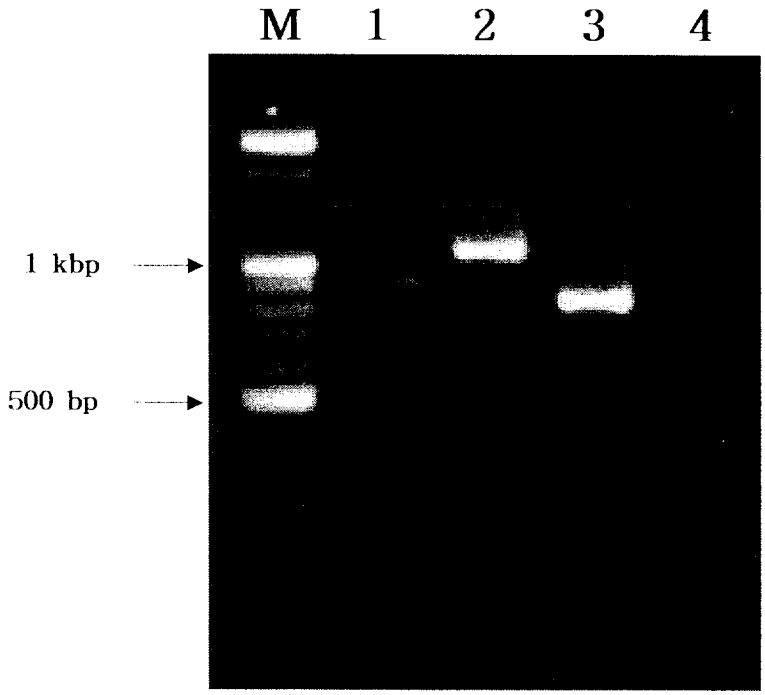

Figure 6. The patterns of nested PCR amplication. (the primary PCR product was unconfirmed on a 1.5 % agarose gel ; not shown) M : 100 bp size marker(Bioneer), Lane 1 : the fragment cut with *Dra* I, Lane 2 : the fragment cut with *Eco*R V, Lane 3 : the fragment cut with *Pvu* II, Lane 4 : the fragment cut with *Stu* I. The Lane 2 product was cloned into pGEM-T vector(Promega) and sequenced.

```
1    ATCGCAATAA TCATGTTTTG TTTGTTTAAT TCTTCATAAT CAGATAATAA
51   TCTGATAATT TTGTCCATAT CTTTACATCT TTGATCCTTT TTACGCTGCA
101  CAGTGATGAT AACTAATTCA CGCAAAGAAG TTTTCATTAC TGCCAACATT
151  TTTTAAATGA TCAAATAGCT CAATTCTAAA TTATATAGGG TCATCGCAGG
201  ATACTCCCAA ATTGTCTGAC ACTTGTCACG TCACCTGCCA TACCCCCTTC
251  GGTGGCCTCA TTCTGACGCG ATGTTTATTG TGTGTGTATG TGTTTTGCGT
301  ATCGTGTGTA AAATCACCCG ATGTCATGTC ATGCAGAGCC ATTTAATTTA
351  ATTTAGTATA AATTTTGACA AATTTCAACA TAAAAAAGAA GATACCATTG
401  GTCAAGACGT TCATCGCATT TCATAATTCT CATCTGTACA TTTCACAAAA
451  ATTACCTAAT ATACGATCTA GCATATTCCA GTGTTTTTAT CGTACGTGAA
501  AATAGGTCAA ACAGAGGATA TAGCGGTCAC AGTCGAATAA TAAACAAAGC
                c-fos/ATF          TATA box                SRE
551  ATGTTGTGAC GTCTCATTCT ATAATTAGAG GTCTCCATAT ATGGAAGACG
601  AATAATTATA TCCTCTGTTT TCATTGGTCG ACGGTCTATC GATTGTGATG
                    CRE
651  TCACAGAGTC TGACGTCACG AATCTAATAA ACCAATGTAC ACAGAAGGTT
701  GGTCGCCATA CTGGAAGAGA AGAACGAACA AAGAGAAAGG CGTAGAAAAA
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Figure 7. Genome-walked sequences. These contain the predicted promoter, TATA box, cAMP reponse element(CRE) and AP-1 element(c-fos/ATF). The predicted promoter is underlined.

Analysis of transcriptional level of the *BTG1* mRNA

Expression of the oyster *BTG1* mRNA was assessed by RT-PCR and Northern blot analysis with different adult tissues. As shown in Fig. 8, 591 bp DNA fragment was amplified by RT-PCR, the expression of oyster *BTG1* mRNA could be identified in mantle, gill, heart and digestive gland. Northern blot analysis showed that the oyster *BTG1* mRNA was widely expressed. However, the transcription level in heart was higher than that of others (Fig. 9).

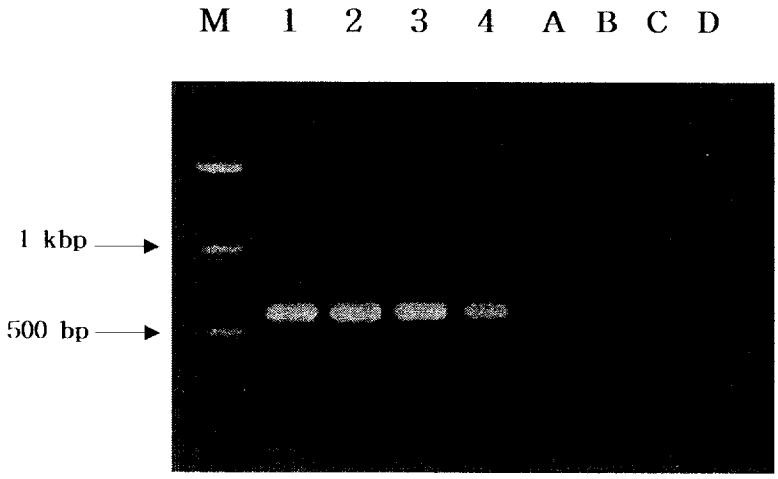

Figure 8. RT-PCR analysis. M : 100 bp size marker(Bioneer), lane 1 : mantle, lane 2 : gill, lane 3 : heart, lane 4 : digestive gland, lane A~D : negative control not containing RTase to confirm DNA contamination

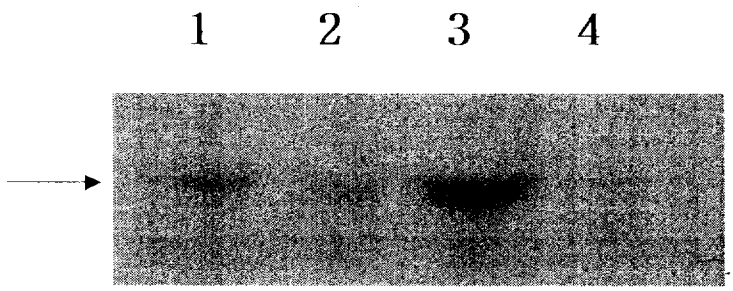

Figure 9. Northern blot analysis in different tissues. Lane 1 : mantle, lane 2 : gill, lane 3 : heart, lane 4 : digestive gland

Discussion

In the present study, the cDNA clone of the oyster *BTG1* was obtained from the gill cDNA library and its characterization was researched. The full-length sequence of the cDNA clone was determined, and the *BTG1* mRNA expression analyzed by RT-PCR and Northern blot analysis.

The involvement of member of the BTG family for development and cell differentiation is clearly appearing. A negative correlation between BTG1 expression and cell proliferation has been observed in various cell types (Rouault *et al.*, 1992 ; Raburn *et al.*, 1992 ; Suk *et al.*, 1997). In addition to its anti-prolifeation properties, BTG1 helps in differentiation process such an embryogenesis, spermatogenesis, and angiogenesis (Raburn *et al.*, 1995 ; Sakaguchi *et al.*, 2001 ; Iwai *et al.*, 2004).

In the present experiments, the full-length of the cDNA clone exhits 1,429 bp and contains an open reading frame of 549 bp that corresponds to a protein of 182 amino acids.

Interestingly, unlike the human and mouse, we found that the coding region of the oyster *BTG1* is not interrupted by introns, through the analysis of its genomic sequence. Moreover, like the human and mouse sequences, the oyster *BTG1* cDNA contained a long AT-rich 3′ untranslated region which may be important in post-transcriptional regulation of the genes (Shaw *et al.*, 1986 ; Rouault *et al.*, 1993). And some copies of ATTTA motif related RNA stability were found.

Through genomic DNA walking of the oyster *BTG1*, a potential promoter was identified and contained cAMP reponse element(CRE), AP-1 element. Also, according to RT-PCR and Northern blot, the wide tissue distribution of *BTG1* mRNA was confirmed. These results suggest that the BTG1 may have a role in a fundamental cellular process.

In conclusion, the coding region of the oyster *BTG1* is intronless and serum response element, AP-1 element exist in the potential promoter region. Further studies are required to clarify the intracellular mechanism for BTG1 actions in oyster.

국문초록

BTG1(B cell translocation gene 1)은 APRO family(antiproliferative protein family)에 속하며, 이들의 발견을 위한 일련의 경로가 다름에도 불구하고 공통적 생물학적 기능은 세포증식을 억제하는 것으로 알려져 있다.

본 연구에서는, 굴의 gill cDNA library를 random sequencing을 통한 EST analysis 과정에서 *BTG1* clone을 확보하였으며, 이의 분자생물학적 특성을 조사하였다.

굴의 BTG1은 182개의 아미노산으로 구성되며, zebrafish와 57%, human과 56%의 상동성을 보였으며, human이나 mouse와 달리 ORF(open reading frame) 내에 intron이 존재하지 않았다. 또한, 5'-, 3'-flanking region에 RNA stability에 관련된 "ATTTA"motif가 존재하였으며, 특히 3'-flanking region은 A(adenine), T(tyrosine)이 높은 비율로 존재함을 확인할 수 있었다.

genomic DNA walking을 통해 굴의 *BTG1*의 predicted promoter을 확인하였으며, PROSCAN 1.7을 통한 분석 결과 AP-1 element와 SRE(serum response element)가 존재함을 볼 수 있었다. 뿐만 아니라, 5'-flanking region에 CREB binding site로 알려진 cAMP response element(CRE)가 확인되었다.

나아가, 굴의 *BTG1*의 조직에(mantle, gill, heart and digestive gland) 따른 transcriptional level을 보기 위해 RT-PCR과 Northern blot을 실행하였으며, 그들 조직에서 모두 expression이 이루어지나 특히 heart에서 보다 높은 수준의 transcription이 이루어짐을 확인하였다.

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