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EST-based Survey of Gene Expression in Seven Tissue Types from the Abalone *Haliotis discus hannai*

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The analysis of expressed sequence tags (ESTs) is an efficient approach for gene discovery, expression profiling, and the development of resources for functional genomics. To analyze the transcriptome of the abalone *Haliotis discus hannai*, we conducted EST analysis using seven cDNA libraries made from gill, gut, hepatopancreas, skin, muscle, testis, and ovary. Redundant ESTs were assembled into overlapping contiguous sequences using the assembly program ICAtools. We found that the total 1,393 ESTs formed 135 clusters and 951 singletons, indicating that the overall redundancy of the library was 22%. Of the 1,393 clones, BLAST identified 1,278 clones (91.7%) as known genes; 115 clones (8.3%) did not match any previously described gene. Based on the major functions of their encoded proteins, the identified clones were classified into 16 broad categories. Sequence analysis revealed the presence of microsatellite-containing genes that may be valuable for further gene mapping studies. This study contributes to the identification of numerous EST clones that can be applied to further clarifying the genetics and developmental biology of abalone.

Key words: Abalone, *Haliotis discus hannai*, Expressed sequence tags (ESTs), Expression profile, Marker

Introduction

The abalone *Haliotis discus hannai* is one of the most valuable commercial aquaculture shellfish species in China, Japan, and Korea due to its important nutrients. The abalone stocks currently used in aquaculture have become appreciably different from their wild counterparts, which suggests that abalone genetics will play a more important role in improving the performance of brood stocks (Lie et al., 1994). Traits amenable to genetic improvement include growth, delayed maturity, sex determination, disease resistance, and temperature tolerance. Despite this interest, relatively little information is available about abalone genes and their sequences, with less than 500 typical nuclear-encoded sequences currently deposited in international DNA sequence databases. This limited knowledge may represent one obstacle to the effective use of genetics in aiding both abalone aquaculture and conservation activities. To expand the current database of abalone genes, we identified genes and analyzed their expression using an EST-based strategy (Adams et al., 1991) commonly used for the identification of large numbers of genes in a species of interest (Marra et al., 1998).

To analyze the components of the abalone transcriptome, seven cDNA libraries were constructed from gill, gut, hepatopancreas, skin, muscle, testis, and ovary of abalone. In total, 1,393 EST clones were identified and analyzed.

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Materials and methods

Animals and tissues preparation

Three-year old abalones (*Haliotis discus hannai*) were supplied by Jeju Fisheries Research Institute

(Jeju, Korea) and maintained in 0.5-ton flow-through tanks at 12±1°C under a natural photoperiod. Seven tissue types, including gill, gut, hepatopancreas, skin, muscle, testis, and ovary, were collected and cut into small pieces. Pooled tissues were rapidly frozen in liquid nitrogen and ground with a mortar and pestle.

Construction of abalone cDNA libraries

Messenger RNA (mRNA) was isolated using a PolyA Tract mRNA isolation kit (Promega, Madison, WI). First-strand cDNA synthesis was carried out using a ZAP-cDNA synthesis kit with oligo-(dT) primers (Stratagene, La Jolla, CA). The cDNA library was constructed in Uni-ZAP XR vectors according to the manufacturer's instruction. All primary libraries were amplified and aliquots of each amplified library were stored at both 4°C and -80°C.

Plasmid preparation and sequencing

Recombinant Uni-ZAP XRs were converted into pBluescript phagemids via *in vivo* excision, according to the manufacturer's instructions (Stratagene). Plasmid DNA was prepared by the alkaline lysis method (Sambrook et al., 2000) using Qiagen Spin Column Mini-plasmid kits (Qiagen, Valencia, CA). Single-pass sequencing of the 5′-termini of selected cDNA clones in phagemid form was performed using an ABI 3130 automatic DNA sequencer (PE Applied Biosystems, Foster City, CA).

Bioinformatic analysis

Bioinformatic analysis was conducted to identify genes using GeneMaster 3.0 software (Ensoltek, Daejeon, Korea). Briefly, the vector sequence was removed and the database search was limited to ESTs >100 bp in length. ESTs were then assembled into clusters of contiguous sequences (contigs) using the ICAtools program (Parsons, 1995). Gene annota-tion procedures and homology searches for the sequenced ESTs were performed using BLASTX for amino acid similarity comparisons (Altschul et al., 1997). Matches were considered to be significant only when the probability (P) was less than 1×10^{-3} using BLASTX with all parameters at the defaults. All ESTs that were not identified as orthologs of known genes were designated as unknown EST clones. Hypothetical proteins were identified from known EST clones.

Identification of microsatellite-containing cDNAs

In total, 1,393 EST sequences were screened for di-, tri-, tetra-, and pentanucleotide microsatellite repeats using the MICAS (Microsatellite Analysis Server) program (available online at http://210.212.212.7/MIC/index.html). The criteria used to identify microsatellite repeats were as follows: five repeats for dinucleotide microsatellites, four repeats for trinucleotide microsatellites, and three repeats for tetra- and pentanucleotide microsatellites. Single nucleotide repeats were not included because they are not useful as polymorphic markers. Some cDNA clones contained more than one type of repeat; in these cases, the clones were categorized according to the longest repeat.

Results

Summary of EST clones in cDNA libraries from seven tissues of abalone

We constructed cDNA libraries from seven different types of abalone tissue, including gill, gut, hepatopancreas, skin, muscle, testis, and ovary, using 1.0×10^{6} pfu/mL from polyadenylated fractions of RNA isolated from abalone tissue. The number of clones in the constructed cDNA libraries was deemed sufficient to encompass the predominantly expressed mRNAs within each tissue. The average length of the inserted cDNA fragments was 1.7 kb (range, 0.5-3 kb). In total, 1,457 clones were randomly selected from these seven cDNA libraries and sequenced using T3 primers; then the GenBank database was searched for homologous sequences. A summary of the identified genes is shown in Table 1. The ESTs were grouped into a total of 1,086 consensus sequences composed of 135 clusters (gill, 31; gut, 24; heapto-pancreas, 9; skin, 15; muscle, 19; testis, 13; ovary, 24) and 951 singletons (gill, 196; gut, 214; hepato-pancreas, 77; skin, 120; muscle, 115; testis, 119; ovary, 110), suggesting that the libraries had an overall redundancy of 22%. BLASTX comparisons established that 1,278 (91.7%) of the clones were orthologs of known genes and 157 of these genes were identified as hypothetical proteins. The remaining 115 clones were not identified via similarity comparisons ($E\pm1\times e^{-3}$). Among the 1,086 EST clones, six unique genes were identified as homologs of previously reported abalone ESTs, and 1,080 (99.5%) genes were identified as orthologs of known genes from other organisms. These results demonstrate that EST analysis is a powerful technique for the rapid discovery of large numbers of genes in abalone.

Expression profile in seven tissues of abalone

The expression profiles of the known genes identified in abalone are presented in Fig. 1. Among the 952 identified genes, 794 genes (83.4%) were

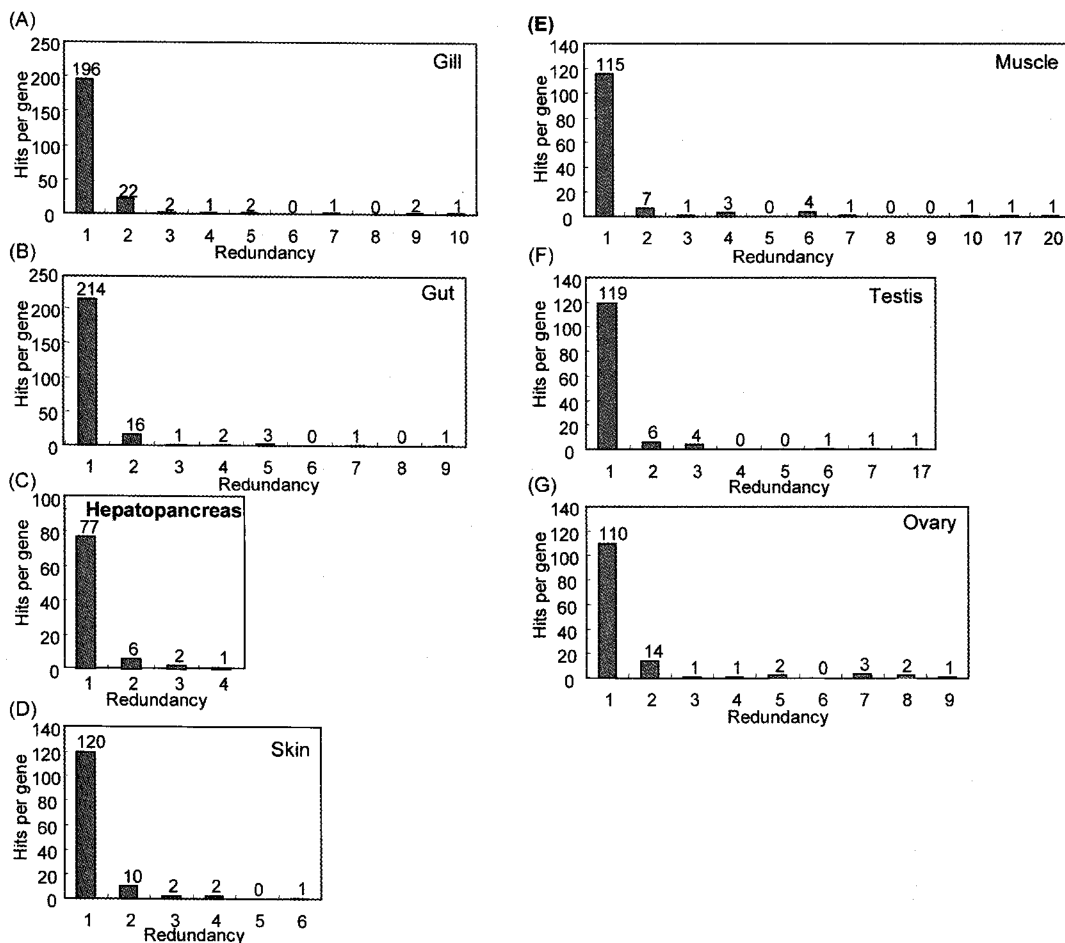

Fig. 1. Expression profiles and sequencing redundancy in the analysis of ESTs from the seven tissues of abalone.

sequenced only once, 115 genes (12.1%) were sequenced two to four times, and 43 (4.5%) were sequenced five times or more. The percentage of singletons was acceptable, although redundancy tended to increase with increasing numbers of sequenced clones. The most abundantly expressed genes in each tissue type were as follows: in the gill, peroxidasin (4.4%), actin A3 (4.0%), actin (4.0%), and guanine nucleotide-binding protein 7 (3.1%); in the gut, serine protease (3.8%), cytochrome *c* oxidase subunit I (2.9%), translation elongation factor 1α (2.1%), and actin α3 (2.1%); in the hepatopancreas, ferritin-like protein 4 (4.7%) and elongation factor 1α3 (3.5%); in the skin, actin α3 (4.4%), cytochrome *c* oxidase subunit I (3.0%), and laminin receptor precursor (3.0%); in the muscle, cytoskeletal β-actin (14.9%), actin α1 (12.7%), myosin essential light chain (7.5%), and aminodeoxychorismate lyase (5.2%); in the testis, arginine kinase (12.9%), putative α-tubulin (5.3%), and elongation factor 1α (4.5%); in the ovary, vitellin coat protein 41 (6.7%) and 42 (6.0%), vitellogenin structural genes (yolk protein genes) family member (vit-1) (6.0%), and α-tubulin (5.2%) (Fig. 1).

Distribution of the identified clones in seven tissues of abalone

Based on the major functions of their encoded proteins, the identified clones were classified into 16 broad categories as follows: biogenesis of cellular components, cell cycle/DNA processing, cell fate, cell rescue/defense/virulence, cell differentiation, cellular communication/signal transduction, cellular transport/transport facilitation/transport routes, development (systemic), energy, interaction with the cellular environment, metabolism, protein activity regulation, protein fate (folding, modification, destination), protein synthesis, proteins with binding functions/cofactor requirement (structural or catalytic), and transcription. The distribution of the identified clones across these categories is shown in Table 2.

Libraries derived from abalone tissues show some differences in overall gene expression patterns that

Table 1. General characteristics of seven tissues ESTs taken from abalone

	Gill	Gut	Hepato-pancreas	Skin	Muscle	Testis	Ovary	Sum
Number of clones sequenced	288	288	101	171	204	199	206	1,457
Number of clones analysed	287	284	99	158	196	173	196	1,393
Average insert size (kb)	1.6±0.6	1.7±0.4	2.0±0.5	1.3±0.6	1.6±0.4	1.8±0.4	1.7±0.3	1.7±0.5
EST clusters	31	24	9	15	19	13	24	135
Unigene	227	238	86	135	134	132	134	1086
Redundancy (%)	20.9%	16.2%	15.2%	14.6%	31.6%	23.7%	31.6%	22%
ESTs with E value < $1\times e^{-3}$ (matched) (%)	268 (90.9%)	263 (92.6%)	90 (90.9%)	141 (89.2%)	173 (88.3%)	158 (91.3%)	185 (94.4%)	1278 (91.7%)
ESTs with E value ≥ $1\times e^{-3}$ (unknown) (%)	19 (6.6%)	21 (7.4%)	9 (9.1%)	17 (10.8%)	23 (11.7%)	15 (8.7%)	11 (5.6%)	115 (8.3%)

Table 2. Functional categorization of the seven tissues cDNA libraries taken from abalone

Function	Percentages in each functional category						
	Gill	Gut	hepato-pancreas	Skin	Muscle	Testis	Ovary
Biogenesis of cellular components	6	7	4.3	9.3	6.3	7.3	12
Cell cycle and DNA processing	8.2	9.9	6.4	15	8.4	13	5.3
Cell fate	3.7	2.1	4.3	4.7	6.3	3.7	2.1
Cell rescue, defense and virulence	4.5	7.7	8.5	4.7	4.2	3.7	4.2
Cell type differentiation	7.5	4.9	2.1	4.7	7.4	2.4	2.1
Cellular communication/signal transduction mechanism	0.75	0	4.3	0	0	2.4	0
Cellular transport, transport facilitation and transport routes	9	9.2	8.5	7	15	7.3	12
Development (Systemic)	1.5	1.4	0	4.7	0	3.7	2.1
Energy	4.5	7	2.1	2.3	3.2	7.3	8.4
Interaction with the cellular environment	4.5	2.8	6.4	2.3	4.2	1.2	2.1
Metabolism	9	11	13	1.2	3.2	4.9	11
Protein activity regulation	0	0	2.1	0	1.1	1.2	0
Protein fate (folding, modification, destination)	13	9.2	8.5	13	12	6.1	11
Protein synthesis	10	9.9	11	14	12	9.8	16
Protein with binding function or cofactor requirement (structural or catalytic)	11	11	8.5	9.3	11	15	9.5
Transcription	5.2	6.3	11	8.1	7.4	9.8	3.2
Unclassified protein	1.5	0.7	0	0	0	1.2	1.1

reflect differences in gene function between abalone and other organisms. However, notable differences were not detected. Clones associated with cell cycle/DNA processing, cellular transport/transport facilitation/transport routes, metabolism, and protein synthesis were all represented at high percentages. In contrast, clones associated with cellular communication/signal transduction, development (systemic), and protein activity regulation were represented at relatively low percentages.

Discussion

Genes potentially involved in defense mechanisms

Group I: Immune and stress-related genes

A secondary objective of this study was to identify cDNA clones in abalone tissues that may be involved in the immune or stress-related responses. Database searches identified several putative immune and stress response-related genes from the abalone cDNA libraries. The putative amino acid sequence deduced from the cDNA clone DGT207 was identified as macrophage migration inhibitory factor (MIF). MIF was initially described as an immunoreactive factor isolated from the supernatants of T lymphocytes that inhibited the random migration of macrophages (Bacher et al., 1997). MIF expression is associated with physiological stress and systemic inflammatory conditions, such as endotoxemia (Calandra and Roger, 2003). The putative amino acid sequence deduced from the cDNA clone GIL71 was identified as the B cell lymphoma leukemia 2 (Bcl2)-associated athanogene, a cell death inhibitory protein. During apoptosis, mitochondrial swelling allows cytochrome *c* to leak into the cytosol, which results in subsequent DNA

fragmentation. Bcl2 interacts with the mitochondrial outer membrane and prevents mitochondrial swelling (Cosulich et al., 1999).

Several ESTs (DGT236, DGT152, GIL143, GIL 165, and SKN117) with high homology to various heat shock proteins (HSPs) were identified in our abalone libraries. HSPs are stress proteins that show enhanced transcriptional activation and biosynthesis in stressed organisms, ranging from bacteria to humans (Lindquist, 1986). In recent years, cDNAs encoding HSP70 and HSP90 have been described in abalone (HSP70, GenBank accession no. DQ324856; Cheng et al., 2007) and tube abalone, *Haliotis tuberculata* (HSP70, EMBL accession no. AM-283516.1; HSP90, EMBL accession no. AM-283515.1; Farcy et al., 2007). Our libraries also contained clones that showed a high degree of similarity to HSP70 (GIL165) and HSP90 (DGT152).

Group II: Recognition proteins

In the gut cDNA library, the clone DGT151 showed a high degree of similarity to the immune recognition molecule β-1,3 glucan-binding protein (β-GBP). β-GBP is also known as lipoprotein 1 (LP1) and is associated with lipid transport to the ovary (Yepis-Plascencia et al., 1995, 1998; Vargas-Albores et al., 1996, 1997; Lubzens et al., 1997; Ruiz-Verdugo et al., 1997; Ravid et al., 1999).

A related EST (RM162) from the muscle library showed similarity to a scavenger receptor expressed by human endothelial cells that mediates the binding and degradation of acetylated low-density lipoprotein (LDL; Adachi et al., 1997). The EST isolated from abalone contained a cysteine-rich region homologous to the scavenger receptor cysteine-rich (SRCR) domains that characterize the SRCR superfamily. In addition to binding modified LDL, these receptors can also bind to bacterial endotoxins and intact Gram-negative bacteria (Dunne et al., 1994; Resnick et al., 1994).

Group III: Proteinases and proteinase inhibitors

With respect to invertebrate immune function, the roles of proteinases and proteinase inhibitors have been well characterized in the regulation of melanization, which is mediated by the prophenoloxidase system, and hemolymph coagulation (Soderhall and Cerenius, 1998; Kanost, 1999). The protective role of these proteins in molluskan immunity has been demonstrated in several previous studies. Pipe (1990) used antibodies against various cathepsins and cysteine proteinases to demonstrate that these proteins are present in the granules of hemocytes from *Mytilus edulis*. Proteinase inhibitors also support host defense mechanisms by inhibiting a variety of proteinases associated with pathogenic virulence (Kanost, 1999). A comparative study demonstrated that proteinase inhibitors are present in the hemolymph of *Crassostrea* spp. (Faisal et al., 1998). In addition to inhibiting a variety of activities attributable to the mechanistic classes of proteinases, plasma from *Crassostrea* spp. inhibited extracellular proteinases produced by *Perkinsus marinus* and *Vibrio vulnificus* (Faisal et al., 1998). The production of extracellular serine proteinases in *P. marinus* is an important factor in the virulence of this parasite. Interestingly, *Crassostrea gigas*, which is resistant to *P. marinus*, appears to possess higher proteinase inhibitor activity than *Crassostrea virginica*, a nonresistant species (Faisal et al., 1998, 1999).

Consistent with these previous observations, we also identified two cysteine proteinases in our abalone cDNA libraries. A single EST (GIL257) isolated from the gill showed similarity to cathepsin B. Two clones (VHP215 and GIL269) that showed similarity to cathepsin L were identified from the hepatopancreas and gill libraries, respectively.

A clone (VHP242) with a high degree of similarity to multicystatin, another cysteine proteinase inhibitor, was identified from the hepatopancreas library. Cystatins play a role in coagulation and complement activation (Vasta et al., 1984; Iwanaga et al., 1998; Smith et al., 1999). Interestingly, these molecules were expressed in the gill and hepatopancreas of abalone. It is possible that the gill and hepatopancreas are designated organs for the production of immune-related molecules.

Group IV: Other immune effectors

Several other immune and potential immune transcripts were identified in our abalone cDNA libraries. Among them, transcripts for metal-binding proteins were sequenced. Two major metal-binding proteins, ferritin (VHP227) and metallothionein 1 (GOF031), were identified in the hepatopancreas and ovary, respectively. Homologs for ferritin were quite common in the hepatopancreas cDNA library. Ferritin is a major intracellular iron binding protein that protects cells from oxidative stress (Balla et al., 1992; Epsztejn et al., 1999) and sequesters free iron from bacterial pathogens. Metallothioneins, scavengers of reactive oxygen intermediates, bind heavy metals and are involved in detoxification processes and up-regulated during the immune response in both vertebrates and invertebrates. An abalone cyclophilin was also identified (RM084). Cyclophilins have diverse regulatory functions in mammalian cells, but they have been specifically implicated in viral attach-

Table 3. Putative defense mechanism-related genes isolated from the seven tissues cDNA libraries of abalone

Clone name	Identified gene	Closest species	aa Identity (%)
Immune and stress related genes			
DGT207	MIF	*Branchiostoma belcheri tsingtaunese*	52
GIL71	Bcl2-associated athanogene	*Strongylocentrotus purpuratus*	33
DGT152	heat shock protein 90	*Haliotis tuberculata*	99
DGT236	heat shock protein HtpG	*Fusobacterium nucleatum* subsp. *polymorphum ATCC 10953*	32
GIL143	heat shock protein 108	*Xenopus laevis*	68
GIL165	heat shock protein 70	*Crassostrea virginica*	52
SKN117	heat shock protein 108	*Xenopus laevis*	68
Recognition proteins			
DGT151	β-1,3 glucan binding protein	*Chlamys farreri*	63
RM162	scavenger receptor class F	*Canis familiaris*	32
Proteinases and proteinase inhibitors			
GIL257	cathepsin B	*Triatoma sordida*	56
VHP215	cathepsin L	*Strongylocentrotus purpuratus*	60
GIL269	cathepsin L	*Strongylocentrotus purpuratus*	64
VHP242	multicystatin	*Vigna unguiculata*	33
Other immune effectors			
VHP227	ferritin	*Pinctada fucata*	70
GOF031	metallothionein 1	*Megathura crenulata*	70
RM084	cyclophilin	*Apis mellifera*	71

Table 4. EST-containing microsatellite cDNA clones from the seven tissues cDNA libraries of abalone

Clone name	Nucleotide repeat unit	Microsatellite repeats	Identified gene
GIL99	Dinucleotide	(GT)5	ENSANGP00000010305
SKN011	Dinucleotide	(AT)5/(TG)4	AMP-dependent synthetase and ligase
GIL219	Pentanucleotide	(GTGAC)3	hypothetical protein XP_780435
DGT230	Dinucleotide	(GT)5	agrin isoform 1
VHP286	Tetranucleotide	(ACAA)4	putative transposase b homolog
SKN101	Trinucleotide	(TCA)4	hypothetical protein AN4541.2
VHP209	Dinucleotide	(CT)5	hypothetical protein
GIL74	Trinucleotide	(GCA)4	ENSANGP00000003404
DGT11	Trinucleotide	(GAT)6	LOC400590 protein
GIL140	Trinucleotide	(GAA)4	AFR490Cp
DGT112	Dinucleotide	(AG)5	unnamed protein product
GOF091	Tetranucleotide	(ATTG)3	unknown
GIL108	Tetranucleotide	(CTCA)4	unknown
GOF086	Dinucleotide	(GA)6	vitelline envelope sperm lysin receptor
GIL82	Trinucleotide	(CAA)4	unknown
DGT213	Trinucleotide	(CCA)4	ependymin-related protein
DGT152	Trinucleotide	(GAA)4	heat shock protein 90
RM091	Tetranucleotide	(TGAG)4	unknown
RM042	Tetranucleotide	(AGTG)3	hypothetical protein
GIL210	Dinucleotide	(TC)5	MGC81140 protein
GOM067	Trinucleotide	(CAG)4	ENSANGP00000003404
DGT183	Tetranucleotide	(ACCA)3	GA17076-PA
GIL5	Tetranucleotide	(GTGC)3/(GAGT)3	ferrisiderophore, outer membrane receptor
DGT20	Dinucleotide	(GA)5	Rho-related BTB domain-containing protein 1
SKN202	Trinucleotide	(CAC)4	unknown
SKN008	Dinucleotide	(AT)5	Serpentine Receptor, class T family member
GOF049	Trinucleotide	(TAT)4	keratin associated protein 10-2
VHP-221	Dinucleotide	(GA)5	hypothetical protein MGG_ch7g1038
GIL230	Dinucleotide	(TA)7/(TA)13/ (TA)7	unknown

ment to cells (Saphire et al., 1999) and the stress response to oxygen depletion (Santos et al., 2000).

Isolation of EST clones containing microsatellite sequences

Several sequenced cDNA clones harbored micro-satellite sequences (Table 4). They were located in the 5′- or 3′-untranslated regions (UTRs) at a high frequency (data not shown). Microsatellites are generally thought to occur in noncoding DNA. However, surveys of other cDNA libraries have revealed that up to 8% of clones may harbor micro-satellites (Depeiges et al., 1995). When polymorphic, these microsatellites may prove useful for genomic mapping. We have determined that the targeting of microsatellite regions within cDNA is an efficient way to develop type I polymorphic markers that represent genes of known function. Due to evolutionary conservation, the mutation rates within coding sequences are lower than those detected in noncoding genomic sequences. As a result, type I polymorphic markers are usually more difficult to identify. Therefore, by tagging highly polymorphic microsatellites to known genes, the efficiency of the development of type I markers can be greatly enhanced. Because the primary objective of this study was to develop EST resources, we did not attempt to characterize the polymorphisms of these micro-satellite clones.

The EST clones isolated in this study can be used as probes to develop molecular markers for tissue-specific or cell-specific expression to determine full-length sequences for cDNA or genes of interest and to perform functional analysis for abalone genes.

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