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The two channel catfish intelectin genes exhibit highly differential patterns of tissue expression and regulation after infection with *Edwardsiella ictaluri*

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Summary
Intelectins (IntL) are Ca^{2+}-dependent secretory glycoproteins that play a role in the innate immune response. The mammalian IntL is also known as lactoferrin receptor (LfR) that is involved in iron metabolism. The objective of this study was to characterize the intelectin genes in both channel catfish and blue catfish, to determine their genomic organization and copy numbers, to determine their patterns of tissue expression, and to establish if they are involved in defense responses of catfish after bacterial infection. Two types of IntL genes have been identified from catfish, and IntL2 was completely sequenced. The genomic structure and organization of IntL2 were similar to those of the mammalian species and of zebrafish and grass carp, but orthologies cannot be established with mammalian IntL genes. The IntL genes are highly conserved through evolution. Sequence analysis also indicated the presence of the fibrinogen-related domain in the catfish IntL genes, suggesting their structural conservations. Phylogenetic analysis suggested the presence of at least two prototypes of IntL genes in teleosts, but only one in mammals. The catfish IntL genes exhibited drastically different patterns of expression as compared to those of the mammalian species, or even with the grass carp gene. The catfish IntL1 gene is widely expressed in various tissues, whereas the channel catfish IntL2 gene was mainly expressed in the liver. While the catfish IntL1 is constitutively expressed, the catfish IntL2 was drastically induced by intraperitoneal injection of *Edwardsiella ictaluri* and/or iron dextran. Such induction was most dramatic when the fish were treated with both the

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bacteria and iron dextran. While IntL1 was expressed in all leukocyte cell lines, no expression of IntL2 was detected in any of the leukocyte cell lines, suggesting that the up-regulated channel catfish IntL2 expression after bacterial infection may be a consequence of the initial immune response, and/or a downstream immune response rather than a part of the primary immune responses.

1. Introduction

Host defense starts with the recognition of molecular patterns from pathogens. Such molecular patterns are often made up of the cell wall components of bacteria harboring bacteria-specific carbohydrate chains that do not exist in the host organisms. Upon infection, the host recognizes such pathogen-specific carbohydrate chains by its lectins. Such lectins can function as phagocytosis receptors, or as soluble opsonins and agglutinins. In the case of phagocytosis receptors, mannose receptor binds to bacterial cell wall components containing terminal mannose residue and enhances their clearance by phagocytosis [1,2]. Soluble lectins are present in the plasma such as collectins and ficolins, and they function as opsonins or agglutinins for bacteria [3]. In addition, the soluble lectins form complexes with mannose-binding lectin-associated serine proteases in plasma. Binding of these complexes to targets activates complement system, which induces opsonization of the targets by phagocytes and the target killing by formation of the membrane attack complex, leading to the so-called lectin pathway that play important roles in innate immunity [4].

In addition to the mannose-specific lectins, animals also have lectins with affinity to galactose called galectins. Galectins have been reported to have functions in cell differentiation [5], apoptosis [6,7], recognition of tumor antigens [8], uptake of aged proteins [9], and binding of galactofuranose present in the carbohydrate chains of bacterial cell wall [4].

A recently identified member of the galectins, intelectin (IntL), was first identified in mouse as a homolog of *Xenopus laevis* oocyte lectin [10]. Mammalian IntL is a Ca^{2+}-dependent enteric lectin, which plays a role in pathogen recognition [4]. Recombinant human (*Homo sapiens*) IntL1, which exists as a 120 kDa homotrimeric structure unit, has specific affinities to D-pentoses and D-galactofuranosyl residues as well as recognizes *Nocardia rubra* arabinogalactan [4]. It was reported that mice with a genetic defect of IntL2 showed increased susceptibility to nematode *Trichinella spiralis* infection [11]. BALB/c mouse, which exhibited Th2 immune response polarization during intestinal infection of *Trichuris muris*, showed up-regulation of the IntL2 gene [12].

Mammalian IntL exists in the intestinal surface [12] and the major deposition of IntL was observed at the enterocyte brush border [13]. Additionally, mouse IntL transcript was observed in the Paneth cells located in the lower region of the intestinal crypts [10] and jejunal goblet cells [14]. The up-regulation of mouse IntL genes has also been reported in the lung after helminth parasite *Nippostrongylus brasiliensis* infection [15]. Moreover, mammalian IntL is thought to be involved in the stabilization of microvillar rafts. Microvillar rafts in the intestines and lungs are rich in glycoproteins and glycolipids, which provide anchorage for microbial adhesions [13]. Mammalian IntL shield against microbial infection by serving as decoy pathogen receptors or protecting exposed epithelial binding sites [13,16]. The intelectin is also named small intestine lactoferrin receptor (SI-LfR). The human SI-LfR cDNA transfected Caco-2 cell, a human small intestinal like cell line, showed higher SI-LfR binding with human Lf than mock-transfected cell [17]. Also, it was suggested that the SI-LfR binding with human milk lactoferrin (Lf) is important in maintaining the iron status for infants [18]. These previous reports on mammalian IntLs indicate that they have an important role in the innate immune response, mucosal stabilization, and iron metabolism.

The information about teleost fish IntLs is limited. To date, complete coding sequences of an IntL gene have been reported only from grass carp [19]. Several teleost fish ESTs or genomic sequences, showing similarity with mammalian IntLs, have been submitted to GenBank, but they have not been characterized. We previously identified ESTs similar to the mammalian intelectin genes from both channel catfish *Ictalurus punctatus*, and blue catfish *I. furcatus*, and identified them among the most highly up-regulated genes after bacterial infection with *Edwardsiella ictaluri* [20,21]. Similarly, previous studies in rainbow trout also identified intelectin as one of the acute phase response genes [22,23]. Given the strong up-regulation of intelectin genes in both catfish and trout livers following bacterial infection and the paucity of data on teleost intelectins, our objectives in this study were to isolate and sequence the complete cDNAs of intelectin genes of channel catfish and blue catfish, characterize the genomic structure of catfish intelectin genes, analyze their expression patterns in healthy catfish tissues, and study their expression after both bacterial infection and iron administration. Here we report that the channel catfish genome harbors two intelectin genes. They exhibit highly differential patterns of tissue expression, and are differentially regulated after bacterial infection.

2. Materials and methods

2.1. Identification and sequencing of catfish IntL cDNAs

BLAST searches were used to identify channel catfish and blue catfish ESTs encoding partial cDNAs for IntL genes. Bioinformatic analysis utilizing ClustalW [24] and VectorNTI (Invitrogen) identified two distinct IntL transcript types from each species, referred to here as IntL1 and IntL2. Complete cDNA sequences for IntL1 and IntL2 were obtained

Table 1 PCR primers used for this study.

Primers and their uses	Primer sequencing
SP6 sequencing primer	5′-ATTTAGGTGACACTATAG-3′
T7 sequencing primer	5′-TAATACGACTCACTATAGGG-3′
Forward PCR primer for screening IntL2 from BAC library	5′-GTGAGGTTTGGAGCAGGAGAGTG-3′
Reverse PCR primer for screening IntL2 from BAC library	5′-CGAAGTCTCCACACTGATCAGG-3′
Forward PCR primer for Southern probes of IntL1	5′-GTATCAGACGTACTGCGACA-3′
Reverse PCR primer for Southern probes of IntL1	5′-GTTTGACTCCCAAGCAGAT-3′
Forward PCR primer for Southern probes of IntL2	5′-TCATTTCTGGAGGGGTGGTC-3′
Reverse PCR primer for Southern probes of Int2	5′-GGGATAGAAGGTCCGCTATC-3′
Forward primer for RT-PCR of IntL1	5′-AAGCTACGGAGGAAACCTCTACA-3′
Reverse primer for RT-PCR of IntL1	5′-CTCCTCCTATACAGCTGTGTTC-3′
Forward primer for RT-PCR of IntL2	5′-GTGAGGTTTGGAGCAGGAGAGTG-3′
Reverse primer for RT-PCR of IntL2	5′-CGAAGTCTCCACACTGATCAGG-3′
β-actin forward primer	5′-AGAGAGAAATTGTCCGTGACATC-3′
β-actin reverse primer	5′-CTCCGATCCAGACAGAGTATTTG-3′
Realtime RT-PCR forward primer of IntL2	5′-TCGGAGCTGCCGGGACATCAAGGAG-3′
Realtime RT-PCR reverse primer of IntL2	5′-CCCTGCTCGCTTGACCAGCGATCAC-3′
Realtime 18S ribosomal RNA forward primer	5′-TGCGCTTAATTTGACTCAACAC-3′
Realtime 18S ribosomal RNA reverse primer	5′-CGATCGAGACTCACTAACATCG-3′

from both channel catfish and blue catfish by sequencing full-length cDNA clones using SP6 and T7 primers (Table 1). The following cDNA clones were sequenced: *Ip*IntL1 (AUF_IpHdk_44_N02); *Ip*IntL2 (IpLvr00069); *If*IntL1 (AUF_IfSpn_234_H05); and *If*IntL2 (AUB_IfLvr00167). Sequencing reactions were performed using the BigDye terminator v3.0 ready reaction kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocols, and samples were sequenced on an ABI 3130 automated DNA sequencer.

2.2. BAC library screening and genomic sequencing of catfish IntL2 gene

The complete cDNA sequences of IntL1 and IntL2 from channel catfish were amplified by polymerase chain reaction (PCR) using SP6 and T7 primer from cDNA clones. The random primed DNA labeling kit (Roche Applied Science, Indianapolis, IN) was used for the labeling of the cDNA fragment with ^{32}P-deoxycytidine triphosphate (Amersham Biosciences, Piscataway, NJ). Sephadex G50 spin columns (Amersham Biosciences) were used to remove unincorporated nucleotides. The labeled probe was denatured at 95 °C for 5 min. The probe was then used for screening the channel catfish CHORI 212 genomic BAC library [25]. The BAC filters were prehybridized at 65 °C for 1 h with 30 ml of hybridization buffer (0.75 M NaCl, 0.05 M sodium phosphate, 5 mM EDTA, 2% polyvinyl pyrrolidone, 2% Ficoll 400, 2% bovine serum albumin, 0.5% sodium dodecyl sulfate, 40 μg/ml salmon sperm). After prehybridization, the hybridization buffer was changed to 30 ml of fresh prewarmed hybridization buffer with the labeled probe. The filters were hybridized at 65 °C for 16 h. The filters were washed twice at 60 °C with washing buffer (0.75 M NaCl, 75 mM sodium citrate, and 0.1% sodium dodecyl sulfate), and then exposed to X-ray film (Fujifilm, Tokyo, Japan) at −80 °C for 20 h. No BAC clones were positive for the IntL1 probe. A second screening was conducted by PCR for IntL2-positive BAC clones using catfish IntL2 gene-specific PCR primers (Table 1). Restriction and Southern analysis was conducted to determine if the five clones were from the same genomic region. A single BAC clone (clone 16-E12) containing IntL2 was sequenced using the primer-walking method, using BAC DNA without subcloning into plasmid. Sequencing was conducted as described above.

2.3. Southern blot analysis

Southern blot analysis was conducted to determine the genomic copy numbers of channel catfish IntL1 and IntL2. Genomic DNA was isolated from three individual catfish following standard protocols as previously described [26]. The genomic DNA concentration was calculated using an Ultrospec 1100 pro (Amersham Biosciences), then 10 μg of genomic DNA was digested with 10 units of restriction endonuclease *Eco* RI, *Hin*d III, or *Pst* I (New England Biolabs, Beverly, MA) in a 25 μg reaction at 37 °C for 18 h. As a control, 1 μg of BAC DNA encoding catfish IntL2 was digested using the same endonucleases. The digested DNA samples were electrophoresed on a 0.6% agarose gel at 20 V for 24 h. The gels were submerged in 0.25 N HCl, denaturation buffer (1.5 M NaCl, 0.5 N NaOH) and neutralization buffer (1 M Tris–HCl at pH 7.5, 1.5 M NaCl) at 30 min intervals. The DNA was transferred to an Immobilon nylon membrane (Millipore, Bedford, MA) by capillary transfer with 20 × SSC buffer (3 M NaCl, 0.3 M sodium citrate) overnight. The DNA was fixed to the membrane using a UV cross-linker (Stratagene, La Jolla, CA). To synthesize the gene-specific DNA probe, cDNA fragments of catfish IntL1 and IntL2 were amplified by gene-specific PCR primers (Table 1). The labeling of the DNA probes was conducted as described above.

2.4. Phylogenetic analysis

The relevant amino acid sequences of vertebrate IntLs: frog *Xenopus tropicalis* IntL1 (AAH61445), grass carp *Ctenopharyngodon idella* IntL2 (AAY43357), human *H. sapiens* IntL1(NP_060095), human IntL2 (NP_543154), mouse *Mus musculus* IntL1 (NP_034714), mouse IntL2 (NP_001007553), zebrafish *Danio rerio* IntL2 (XP_682917), zebrafish 35 kDa serum lectin (XP_693369) were retrieved from GenBank. Additional zebrafish intelectin-like genes were identified by BLAST searches within the zebrafish genome. Furthermore, 16 rainbow trout *Oncorhynchus mykiss* ESTs (BX874212, BX881738, BX868693, BX867785, BX890433, CR943369, CR943537, CR943565, CR943544, BX910505, BX864969, BX885024, CU068041, CA360897, CA347369, CR943366) were obtained through the tblastx search using the catfish IntL genes, and two different EST contigs were generated by phrap (http://www.phrap.org/). Phylogenetic analysis was conducted using deduced amino acid sequences of these genes. The multiple alignment and bootstrap tree were constructed using ClustalW (http://www.clustalw.ddbj.nig.ac.jp/top-j.html) with p-distance correction. The topological stability of the trees was evaluated by 1000 bootstrap replications.

2.5. Bacterial challenge and iron-dextran treatment

Clemson strain of channel catfish *I. punctatus* (average body weight of 193 g), were used in this study. The fish was kept at 27 °C in a flow-through system utilizing heated well water. Eighteen fish were treated in each of four groups: control group (BHI media injected); *E. ictaluri*-challenged group; iron-dextran-treated group; iron-dextran-treated and *E. ictaluri* infected group. To inoculate bacteria for the challenge, a single colony of *E. ictaluri* (isolate number S97-773) was isolated and cultured in BHI broth media at 28 °C overnight. The bacterial culture was diluted with phosphate-buffered saline (PBS) (20 mM sodium phosphate, 150 mM NaCl, pH 7.5), and then 4.8×10^5 cfu of bacteria in 200 μl PBS were injected intraperitoneally into the channel catfish. In the iron dextran group, 500 μl of the iron-dextran solution (100 mg/ml) (Sigma, St. Louis, MO) was injected intraperitoneally. The fish in the combination group were injected with the same amount of bacteria and iron-dextran. Injections were carried out under light anesthesia utilizing tricaine methanesulfonate (MS 222) at 100 mg/l.

2.6. Tissue sampling, cell lines, and RNA extraction

Fish used in this study were euthanized by prolonged MS 222 exposure at 300 mg/l before dissection. Tissues were collected at 2 h, 24 h, and 3 days after treatment. Six fish were sacrificed at each of the three time points and tissue samples from two fish were pooled, thus three pools were obtained per sampling point/tissue. For RT-PCR analysis, tissues from all six fish were pooled to determine a general pattern of gene expression after treatment. For real-time RT-PCR analysis, two fish each were pooled to allow three pools of samples at each time point to provide an assessment of individual variations in gene expression for statistical analysis. As the sizes of these fingerlings used for challenge were small, it was necessary for us to pool samples from two fish together for extraction of RNA. Head kidney (pronephros), intestine, liver, spleen, and trunk kidney (mesonephros) were collected from the *E. ictaluri* challenged group, iron-dextran-treated group and from the iron-dextran and *E. ictaluri* treated group. From the healthy, untreated group, ten tissues including gill, heart, head kidney, intestine, liver, muscle, skin, spleen, stomach, and trunk kidney were collected to determine tissue distribution of expression. Healthy tissues of blue catfish were collected separately from older 2-year fish as blue catfish of similar age were not available. The tissues were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

Several channel catfish leukocyte cell lines were used to determine expression of IntL genes including two autonomous B cell lines, 3B11 and 1G8 [27,28], a macrophage cell line, 42TA [29], a T cell line, 28.3 [30], two cytotoxic T cell lines, TS32.15 (specifically kills 3B11 B cells), and TS32.17 (kills 1G8 and 3B11 B cells) [31,32].

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA) and the concentration of the total RNA was quantified using an Ultrospec 1100 pro. The RNA samples were subsequently used for determination of gene expression.

2.7. RT-PCR analysis

The total RNA of each tissue from six fish was pooled in each time point and treated for this analysis. cDNAs were synthesized from 1 μg of total RNA using the Superscript II First-strand Synthesis Kit (Invitrogen) following the manufacturer's protocol. The reverse-transcribed products (1 μl) were used in a 50-μl PCR reaction mixture. PCR primers specific to the channel catfish IntL1 and IntL2 transcripts (Table 1) were used. The PCR reaction mixture was denatured at 95 °C for 2 min and then subjected to 25 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min. PCR primers for β-actin (Table 1) were also used in the RT-PCR reactions to serve as an internal control. Ten microliters (10 μl) of the PCR products were electrophoresed on a 0.8% agarose gel and documented with a gel documentation system (Nucleotech Corp., San Mateo, CA).

2.8. Quantitative real-time RT-PCR analysis

Quantitative real-time RT-PCR (qRT-PCR) analysis was conducted using a LightCycler (Roche) to validate the RT-PCR results. The fold induction of the channel catfish IntL2 gene after different treatments was quantified in the liver. The analysis was conducted as we previously reported [20,21]. One hundred nanograms (100 ng) of total RNA were used in one reaction. The 18S ribosomal RNA gene was used as an internal control for normalization. The sets of PCR primers that were used in this analysis are listed in Table 1. Six fish were used for each time point × treatment, and the liver tissues were harvested into three pools, each containing two fish livers. The fold induction in stimulated group was calculated from threshold cycle (Ct) using the Relative Expression Software Tool 384 v. 1 (REST).

3. Results

3.1. Identification and sequencing of intelectin cDNAs

A total of 38 partial cDNA sequences similar to intelectin genes were initially identified from channel catfish and blue catfish ESTs [33]. Cluster analysis indicated that these ESTs belong to two contigs. The EST clones containing full-length cDNAs of both channel catfish and blue catfish as compared with known intelectin genes from other species were sequenced to obtain full-length cDNA sequences. As detailed below, the two types of intelectin genes were referred to as intelectin-1 (IntL1) and intelectin-2 (IntL2). The channel catfish IntL1 cDNA contains an open reading frame (ORF) of 912 bp encoding 303 amino acids with a 90 bp 5′-untranslated region (UTR) and a 98 bp 3′-UTR. The channel catfish IntL2 cDNA contains an ORF of 951 bp encoding 318 amino acids with a 38 bp 5′-UTR and a 90 bp 3′-UTR. Similarly, the blue catfish IntL1 cDNA contains an ORF of 912 bp encoding 303 amino acids with a 97 bp 5′-UTR and a 162 bp 3′-UTR. The blue catfish IntL2 cDNA contains an ORF of 951 bp encoding 318 amino acids with a 37 bp 5′-UTR and a 113 bp 3′-UTR. All four cDNA sequences have been deposited to GenBank with the accession numbers of EU030378 (channel catfish IntL1 cDNA), EU030379 (channel catfish IntL2 cDNA), EU030380 (blue catfish IntL1 cDNA), and EU030381 (blue catfish IntL2 cDNA).

Multiple sequence alignments using amino acid sequences of all available intelectin genes suggested a high level of sequence conservation, with amino acid sequence identities ranging from 47.8% to 96.5% (Figure 1). A fibrinogen-related domain (FReD), which commonly exists in the vertebrate intelectins, was also conserved in the catfish IntL1 and IntL2 (residues 52–194). The amino acid sequence similarity of the FReD in the intelectin genes was high relative to other regions (Figure 1). Two potential *N*-glycosylation sites were reported in human IntL1 and IntL2 genes (residues 154 and 163, see Figure 1), but only the first is present in the channel catfish IntL1 and IntL2 genes (residue 170, Figure 1).

3.2. Phylogenetic analysis of intelectin genes

Phylogenetic analysis based on amino acid sequences was conducted to determine the orthology of the catfish intelectin genes. As shown in Figure 2, clear orthologies were difficult to establish between teleost intelectins and their mammalian counterparts. The mammalian intelectins (human and mouse) are more closely related to the amphibian *Xenopus* IntL1, but have undergone recent duplication events within each species as indicated by the high levels of similarity between the intelectin genes within each species. Teleost intelectins formed four distinct clades: (1) zebrafish IntL genes on chromosomes 7 and 25; (2) catfish IntL 1 genes and rainbow trout IntL genes; (3) the clades containing IntL2 genes from catfish, zebrafish, and grass carp; and (4) zebrafish IntL gene on chromosome 1. Notably, the catfish IntL1 genes were more similar to two intelectins from the phylogenetically more distant rainbow trout than to any sequence within the more closely related zebrafish genome. A grass carp IntL1 gene has not been isolated to date. The two rainbow trout IntL1 genes may have arisen during the tetraploidization event common to salmonid fishes. Additional intelectin-like zebrafish genes formed their own large clade in the center of the phylogenetic tree. The close genomic proximity of many of these zebrafish intelectin genes (as indicated on the tree) suggested that they were products of recent species-specific gene duplications (Figure 2) [34,35].

3.3. Structural analysis of the channel catfish IntL2 gene

Screening of a channel catfish BAC library [25] led to the identification of five BAC clones containing the channel catfish IntL2 gene, but failed to obtain any BAC clone containing the IntL1 gene. The five BAC clones containing IntL2 were 16_E12, 49_G2, 165_J16, 172_K19, and 179_D15. Restriction and Southern blot analysis revealed that all the five BAC clones were from the same genomic region, and the clone 16_E12 was used for sequencing. A genomic sequence of 5231 bp of the channel catfish IntL2 gene was obtained by primer walking sequencing, and the nucleotide sequences have been deposited to GenBank with the accession number of EU030382.

Alignment of the genomic sequence with the cDNA sequence revealed the presence of seven exon and six introns in the catfish IntL2 gene. Gene organization was highly conserved through evolution. While mammalian IntL genes are encoded by eight exons, the exon sizes were strikingly well-conserved (Figure 3), suggesting evolutionary constraints have preserved their structure and function.

The transcription factor binding sites of the catfish IntL2 promoter region, 1000 bp from putative transcription start point, were analyzed by TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html). As shown in Figure 4, a number of transcription factor binding sites, especially those involved in immune response and liver gene expression, were identified including five octamer factor 1 (Oct-1) binding sites (−879, −788, −280, −268, and −238), four CCAAT/enhancer binding protein β (C/EBPβ) binding sites (−942, −229, −130, and −67), and a hepatic nuclear factor 3β (HNF-3β, a liver-enriched transcription factor) binding site (−472). Additionally, binding elements for cyclic AMP response element-binding protein 1 (CRE-BP1) (−786, −717), GATA-1 (−819, −406), heat shock factor 2 (HSF2) (−253) and upstream stimulatory factor (−947) were also found (Figure 4).

3.4. Determination of genomic copies for the IntL genes in channel catfish

As the IntL genes are highly duplicated in the zebrafish genome, Southern blot analysis was conducted to determine the copy number of IntL genes in the channel catfish genome. A single copy of IntL2 was found to exist in the channel catfish genome. As shown in Figure 5, a single band was observed with restriction enzyme digestion using *Eco*R1 and *Hin*d III, suggesting the presence of only a single copy gene of IntL2. Digestion using *Pst*I generated two bands (Figure 5), but an internal *Pst*I site was identified in the IntL2 gene sequence, confirming the presence of a single

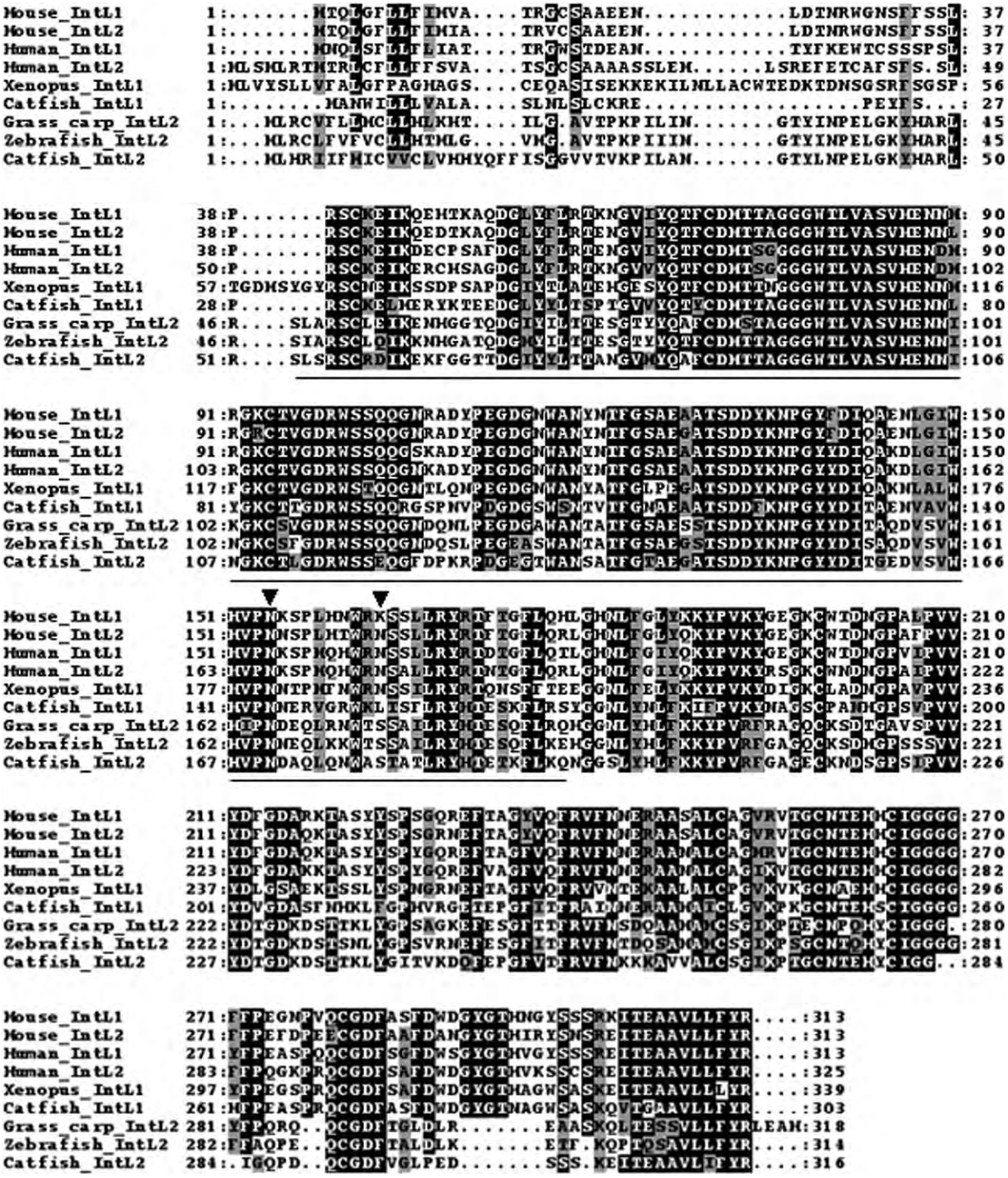

Figure 1 Alignment of amino acid sequences of the vertebrate IntLs. The conserved and identical residues are represented by black shading, and conservative substitutions are represented by gray shading. The region of fibrinogen-related domain (FreD), which was predicted by SMART program (http://www.smart.embl-heidelberg.de/), is indicated by underline. Two *N*-glycosylation sites of human IntL1 are indicated by solid triangles.

copy of the IntL2 gene in the genome. We also included one BAC clone in the Southern blot analysis, and the same restriction pattern was observed. In addition, we also determined the location of the five BAC clones positive for the IntL2 probes on the physical map of the catfish genome [36]. Only two of the five clones identified from the 10 × genome BAC filter was physically mapped, and they both fall into the same contig, Contig 1121, providing additional evidence for the presence of only a single copy gene of IntL2 in the channel catfish genome.

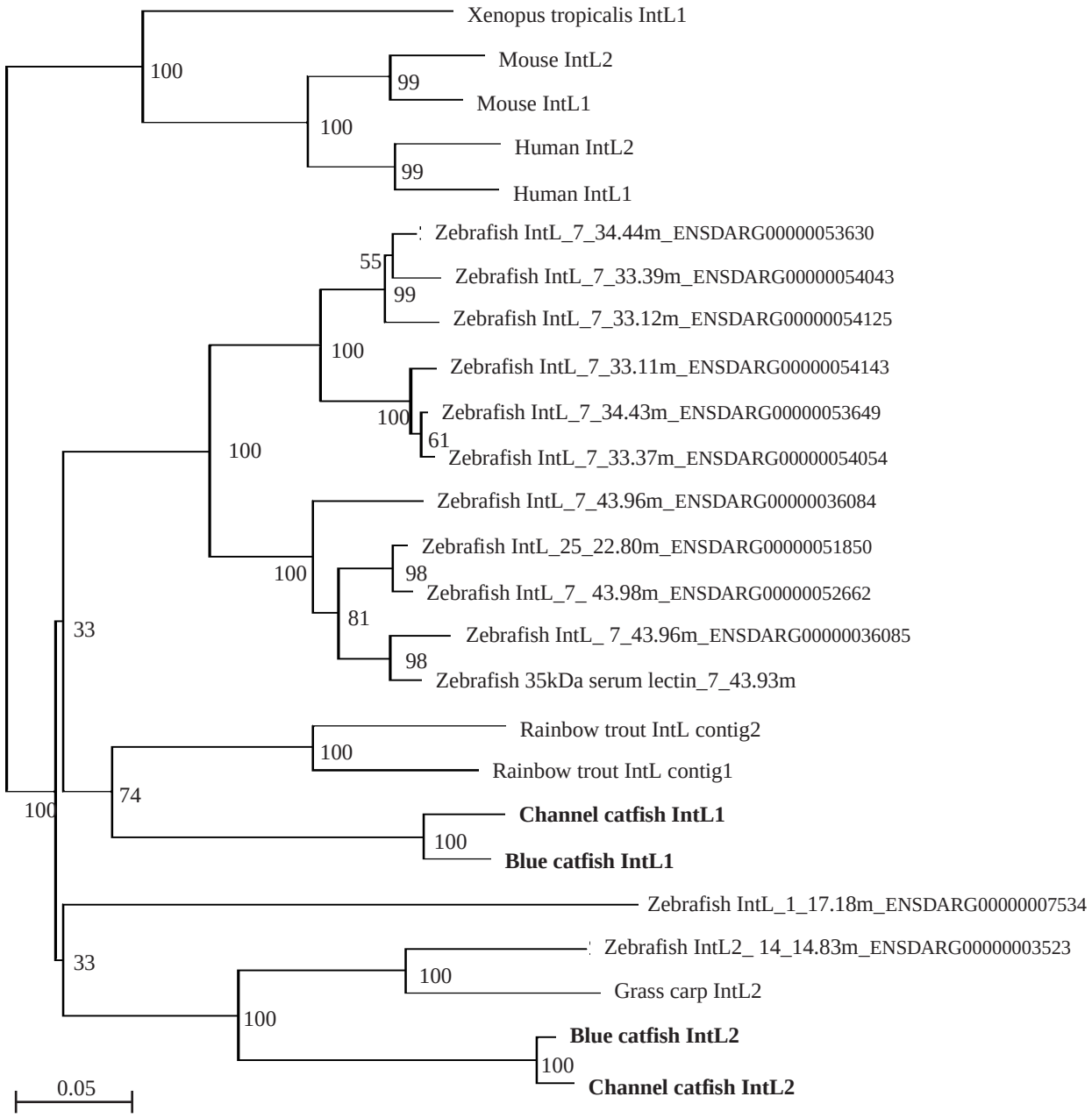

Figure 2 Phylogenetic analysis of vertebrate IntLs. The phylogenetic tree was constructed based on multiple sequence alignment of amino acid sequences using the neighbor-joining method which ignores gaps. The bootstrapping values are indicated at the nodes. As multiple copies of zebrafish intelectin genes were identified from its genome, chromosome number, and the gene locations are indicated. For instance, Zebrafish IntL_7_34.44m_ENSDARG00000053630 indicated that this intelectin gene was identified from zebrafish chromosome 7, on the location of 34.44 million bp, followed by the genome sequence ID ENSDARG00000053630.

The copy number of IntL1 could not be conclusively determined at present as its genomic sequence is not yet available (and therefore the information of restriction sites are not available), but we believe that the catfish genome may contain more than one copy of the IntL1 gene. This assessment is based on the Southern blot (Figure 6). Three–five bands were detected with *Eco*R1, *Hin*d III, and *Pst* I, with many bands of large sizes. In order to eliminate the possibility of cross-hybridization, Southern blot analysis was conducted a second time at very high stringency, the same hybridization patterns were observed. This suggested that the catfish genome may actually contain more than one copy of the IntL1 gene, with some polymorphism among individuals that generated slightly different patterns of Southern blot hybridization using *Hin*d III (Figure 6).

3.5. Tissue expression of the catfish intelectin genes

RT-PCR was used to determine tissue distribution of IntL gene expression. As shown in Figure 7, the two catfish IntL

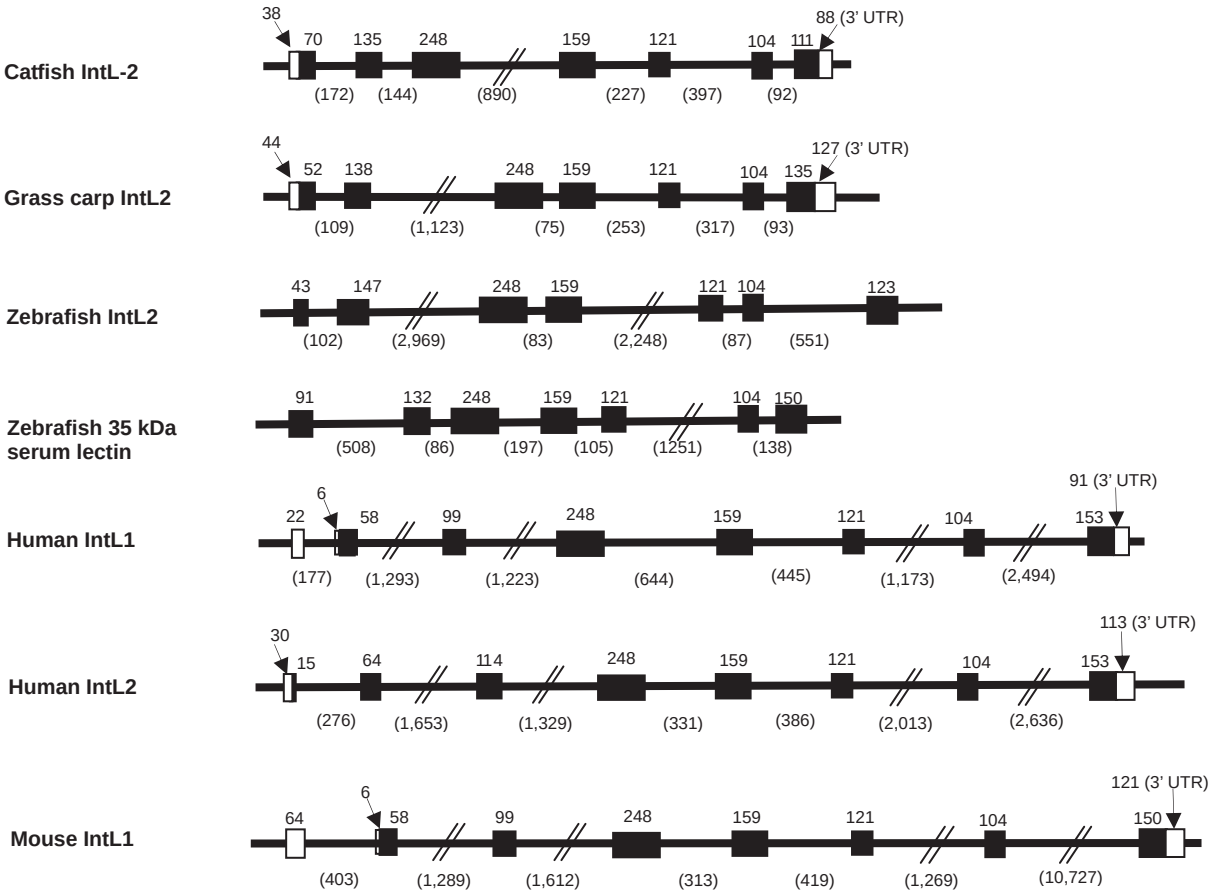

Figure 3 Schematic diagram of vertebrate intelectin gene structure. Exons are represented by boxes. Solid boxes indicate the coding region of the gene while open boxes indicate untranslated regions. The numbers that are indicated on the top of boxes are the length of the region in base pairs. The length of introns is represented in parentheses below each gene structure. Double slashes indicate non-proportional representation of the introns as specified.

genes exhibited different patterns of tissue expression. IntL1 is expressed in all tissues tested including gill, intestine, head kidney, heart, trunk kidney, liver, skin, spleen, stomach, and muscle. The expression patterns were similar in channel catfish and blue catfish (Figure 7A). In contrast, IntL2 exhibited tissue preferences in its expression. In channel catfish, IntL2 was mainly expressed in the liver, with weak expression in the intestine and trunk kidney, while its expression was not detected in any other tissues tested (Figure 7B). In blue catfish, some differences were noted: in addition to strong expression in the liver, IntL2 was also highly expressed in the blue catfish heart, head kidney, and at lower levels in trunk kidney, intestine, and gill.

3.6. Intelectin expression after bacterial infection and administration of iron dextran

RT-PCR was initially used to assess expression of the channel catfish IntL genes after infection of channel catfish with *E. ictaluri*. In the RT-PCR analysis, the IntL1 gene was only slightly induced after infection in the head kidney, and spleen, but not induced in the intestine, trunk kidney and liver, while IntL2 was highly induced in the liver (data not shown). Real-time quantitative RT-PCR was then conducted to provide a better assessment of gene induction. In contrast with the insignificant expression changes observed for channel catfish IntL1 (data not shown), the expression of channel catfish IntL2 was drastically up-regulated by *E. ictaluri* infection, and mildly induced by treatment with iron dextran (Figure 8). At 24 h after infection with the bacteria, IntL2 expression was already noticeably up-regulated, although the difference was not statistically significant due to variation between pools. Three days after infection, the expression of IntL2 was induced over 153-fold. A similar temporal pattern was observed in the group injected with both *E. ictaluri* and iron dextran, but the extent of induction was even greater, over 353-fold up-regulation (Figure 8).

3.7. Expression of IntL genes in leukocytes

In order to determine if the two catfish IntL genes are expressed in various leukocytes, six leukocyte cell lines were used including two autonomous B cell lines, one macrophage cell line, one T cell line, and two cytotoxic T cell lines. As shown in Figure 9, IntL1 was expressed in all the leukocyte cell lines, but IntL2 was not expressed in any of the six leukocyte cell lines.

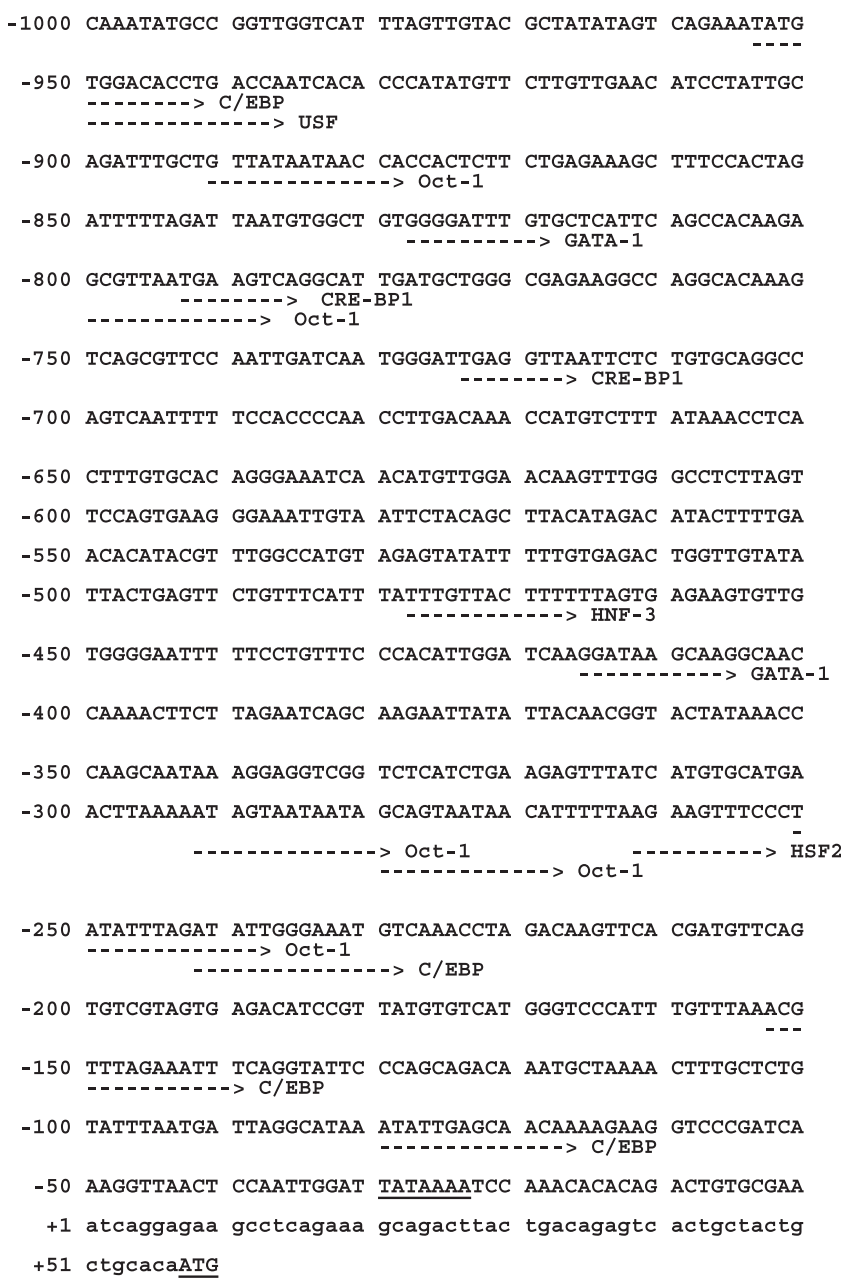

Figure 4 Nucleotide sequences of the 5′-flanking region of catfish IntL2. The nucleotide sequence of 5′ untranslated region is indicated by small letters. The position of transcription factors that are involved in stress and immune responses are indicated. Oct-1, octamer factor 1; C/EBPβ, CCAAT/enhancer binding protein β; HNF-3β, hepatic nuclear factor 3β; CRE-BP1, cyclic AMP response element-binding protein 1; HSF2, heat shock factor 2; GATA-1, GATA binding protein 1.

4. Discussion

In this work, we identified two types of intelectin transcripts from both channel catfish and blue catfish. Their complete cDNAs were sequenced and characterized. The gene of channel catfish IntL2 was cloned, sequenced, and analyzed. These genes exhibit highly differential patterns of tissue expression and are highly differentially regulated after bacterial infection. Sequence analysis of the deduced amino acid sequences of the channel catfish IntL genes indicated possession of the typical FreD at the N-terminus, suggesting that catfish IntL genes have the necessary structural properties to serve as galactose-binding lectins. The genomic structure of channel catfish IntL2 was strikingly well conserved with mammalian IntL genes, suggesting their common ancestry.

Phylogenetic analysis suggested that teleost fish likely have variable numbers of intelectin genes while mammalian species appear to have inherited a single copy of intelectin, which has been duplicated since species divergence. Preliminary research suggests, however, that important functional differences may exist between different mammalian IntL genes. For instance, human intelectin-1 is a disulfide-linked trimer, whereas mouse homolog is a

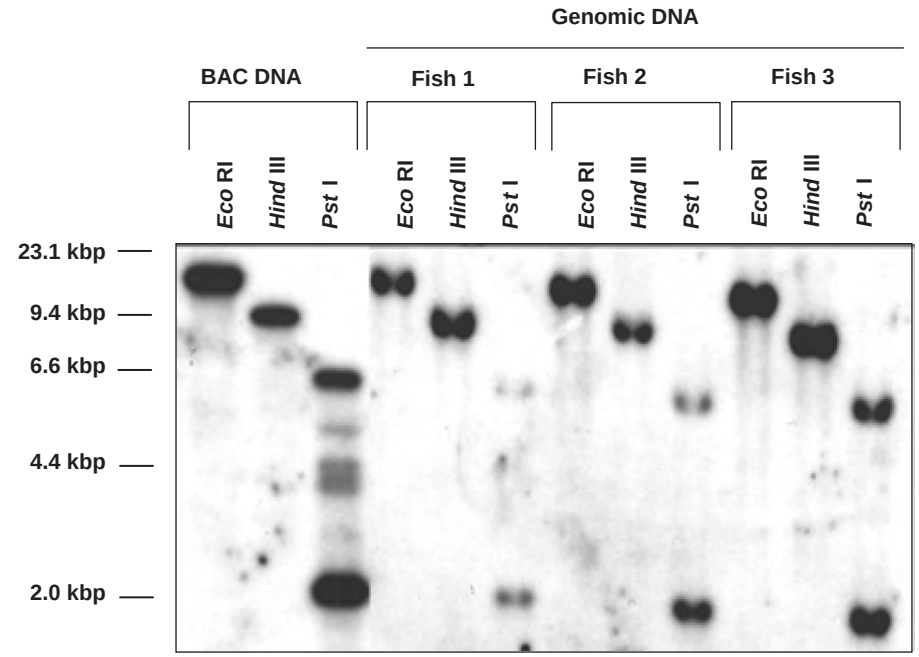

Figure 5 Southern blot analysis of channel catfish IntL2 using BAC DNA and genomic DNAs. Southern blot analysis procedures are detailed in the Materials and Methods section. Kb markers are indicated on the left margin.

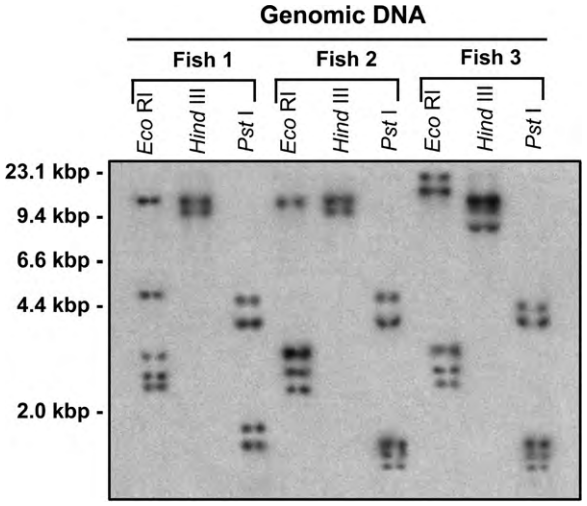

Figure 6 Southern blot analysis of channel catfish IntL1 using genomic DNA. Southern blot analysis procedures are detailed in the Materials and Methods section. Kb markers are indicated on the left margin.

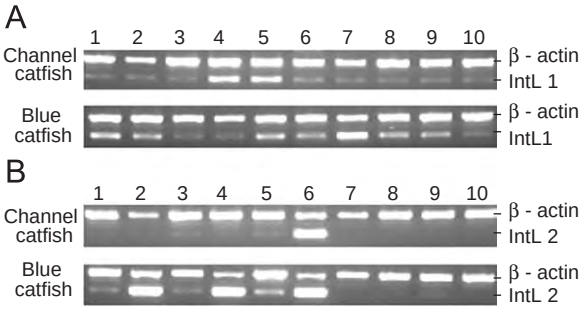

Figure 7 RT-PCR analysis of catfish IntL1 (A) and IntL2 (B) expression in various healthy tissues of channel catfish and blue catfish as specified on the left margin of the gel. RT-PCR products were analyzed on an agarose gel. The positions of the RT-PCR amplified bands of β-actin and IntL genes are indicated on the right margin. The tissues are as the following: (1) gill; (2) heart; (3) intestine; (4) head kidney; (5) trunk kidney; (6) liver; (7) muscle; (8) skin; (9) spleen; and (10) stomach.

monomer [37,38]. While genomic structure appears to be well conserved in teleost IntL genes, IntL copy numbers appear to vary widely in teleost fish. We report here two IntL genes from catfish. While catfish IntL2 has clear equivalents (orthologue) in zebrafish and grass carp, catfish IntL1 is only similar to IntL genes from rainbow trout. We sought a more comprehensive picture from the sequenced fish genomes, searching the genomes of zebrafish and two pufferfish species *Tetraodon nigroviridis* and *Takifugu rubripes* for IntL-like genes. Interestingly, while 12 IntL-related genes were identified from the zebrafish genome in a pattern suggesting extensive intrachromosomal gene duplication [34,35], no sequences similar to IntL genes were identified from either species of pufferfish. Further studies are clearly warranted to examine the evolutionary pressures that have selected for variable genome copy numbers of IntL genes among various teleost species, as well as the functional implications of different IntL gene repertoires.

Notable features of the channel catfish IntL genes were their strikingly different patterns of tissue expression and their differential regulation after bacterial infection. While the channel catfish IntL1 gene was widely expressed in all tissues, the IntL2 gene was predominantly expressed in the liver, with low expression in trunk kidney and

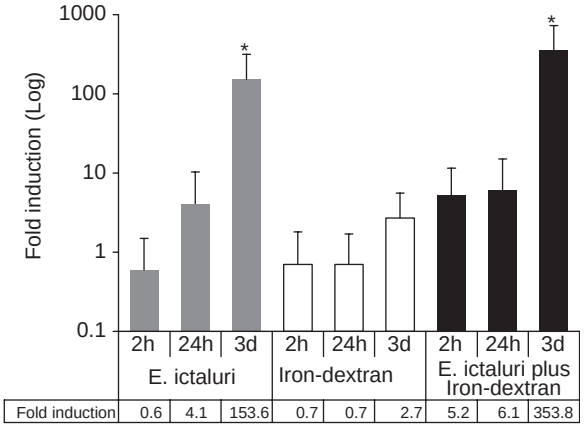

Figure 8 Fold induction of channel catfish IntL2 after *E. ictaluri* and/or iron-dextran treatment in the liver. The RNA samples were collected at 2 h, 24 h and 3 days post-treatment. The *Y*-axis is displayed logarithmically. Asterisks indicate statistical significance at the level of $p < 0.05$.

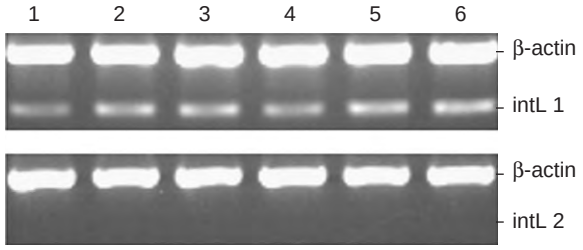

Figure 9 RT-PCR analysis of IntL1 (upper) and IntL2 (lower) gene expression in leukocyte cell lines. The positions of beta-actin and IntL PCR amplification products are specified on the right margin of the gel. Cell lines used in the study were: (1) cytotoxic cell line TS32.15; (2) cytotoxic cell line TS32.17; (3) T cell line 28.3; (4) macrophage cell line 42TA; (5) B cell line 3B11; and (6) B cell line 1G8.

intestine. This pattern of tissue expression suggests that the two genes likely play different roles or have partitioned their functions in different tissues. IntL1 expression did not appear to be induced after bacterial infection, but might be induced with iron dextran in the head kidney and spleen although the induction was not statistically significant. In contrast, IntL2 was highly induced in the liver with bacterial infection, and such induction was most dramatic when also administered with iron dextran. The induced up-regulation of IntL2 expression after bacterial infection was rapid as elevated expression was observed beginning at 24 h after infection, similar to the situation of many other innate immune response genes such as chemokines [41–43], Toll-like receptors [44–46], and antimicrobial peptides [40,47–51], providing suggestive evidence that IntL genes may be involved as host immune responses in teleost species [39,40]. However, functional analysis is needed in future studies to determine if these proteins actually bind pathogens, function as opsonins or agglutinins for bacteria (or parasites), and/or activate the complement system as circulating soluble lectins. Since the ORFs of these genes have been determined, recombinant proteins can be produced in future studies to examine the functions of IntL with a series of binding/agglutination experiments. Recombinant IntL could also be administered in conjunction with IP injection with pathogens to see if any protective effects are observed.

The expression patterns of the catfish IntL genes were also notable in comparison to known expression profiles from other intelectin genes. While IntL2 gene of channel catfish was most highly expressed in the liver, IntL2 gene of grass carp was expressed in all tissues but liver [19]. This difference may be the result of the two species inhabiting different environments and encountering different pathogens. However, expression in mammalian intelectins also has not been found in liver [11]. Such strong constitutive and induced expression of IntL2 in channel catfish liver is therefore striking. However, this report confirms our earlier observations of dramatic inductions of catfish IntL2 expression in both channel catfish and blue catfish liver 3 days after *E. ictaluri* infection by microarray and QRT-PCR analysis [20,21]. Considering the great variation of IntL2 genes in various organisms, we also analyzed its expression in blue catfish, a closely related member of the same genus with channel catfish. Even in blue catfish, expression patterns were quite different from those in channel catfish. In particular, expression was also high in the heart and head kidney in addition to the liver in blue catfish. However, the blue catfish used in this analysis was older (2-year-old as compared with fingerlings for channel catfish) because blue catfish with similar age were unfortunately unavailable. Nonetheless, it is clear that IntL2 expression could be highly different with different organisms, development, physiology, and the environment. Additionally, a rainbow trout IntL gene contig (rainbow trout contig 2 in Figure 2) has been reported to be similarly induced in the liver following bacterial infection [23]. However, further complicating matters, the rainbow trout gene shares highest similarity with catfish IntL1 rather than the highly induced catfish IntL2 (Figure 2). Additional research is warranted to investigate these differences in tissue expression and pathogen response among various teleost species.

The channel catfish IntL2 was strongly induced in the liver (Figure 8). Heavy mortality was observed in *E. ictaluri* infected group but not in the group of combination treatment with *E. ictaluri* and iron dextran. Hence, iron-dextran treatment seems to confer protective ability. Furthermore, in spite of extremely low expression in the spleen of healthy tissues (Figure 7B), IntL2 was expressed in the spleen 3 days after *E. ictaluri* challenge (data not shown). This suggested that IntL2 was expressed in correlation with immune functions. However, further studies are required to elucidate the expression and function of IntL2 in disease responses. The expression of catfish IntL genes in the liver suggests their unique physiological functions. In mammals, the liver is a major organ of acute phase plasma protein synthesis, which involves iron uptake, such as ferritin, haptoglobin, hemopexin, and transferrin [51]. The genes that are involved in the iron metabolism were up-regulated after *E. ictaluri* infection in catfish [20,21]. Mammalian IntLs are known as receptors of lactoferrin that are involved in iron uptake [18,52]. The expression of IntL2 gene was modestly induced when administered with

iron-dextran 3 days post-iron-dextran injection (Figure 8). Also, the co-injection of *E. ictaluri* and iron dextran seemed to boost expression of the catfish IntL2 gene (Figure 8). Hence, we surmised that the channel catfish IntL2 may have a role in iron regulation similar to mammalian LfR, although no lactoferrin gene has been identified from teleost fish.

Expression of IntL genes in leukocyte cell lines further confirms a wide range of expression with IntL1, but highly tissue-specific expression with IntL2. IntL2 was identified as the most up-regulated gene 3 days after bacterial infection with *E. ictaluri* using microarrays [20,21], and its induced expression after infection was further confirmed in this study using RT-PCR and real-time quantitative PCR, but here we also demonstrated that it was not expressed in any of the leukocyte cell lines. Taken together, these results may suggest that IntL2 may not be directly involved as a factor in the first line of immune defenses, but may be a downstream (consequence) player of immune responses.

Acknowledgments

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