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Catfish hepcidin gene is expressed in a wide range of tissues and exhibits tissue-specific upregulation after bacterial infection

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Abstract

Antimicrobial peptides (AMPs) are important components of the host innate immune response against microbial invasion. The cysteine-rich AMPs such as defensin and hepcidin have been extensively studied from various organisms, but their role in disease defense in catfish is unknown. As a first step, we sequenced a hepcidin cDNA from both channel catfish and blue catfish, and characterized the channel catfish hepcidin gene. The channel catfish hepcidin gene consists of two introns and three exons that encode a peptide of 96 amino acids. The amino acid sequences and gene organization were conserved between catfish and other organisms. In contrast to its almost exclusive expression in the liver in humans, the channel catfish hepcidin gene was expressed in a wide range of tissues except brain. Its expression was detected early during embryonic and larval development, and induced after bacterial infection with *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC) in a tissue-specific manner. The upregulation was observed in the spleen and head kidney, but not in the liver. The expression of hepcidin was upregulated 1–3 days after challenge, but returned to normal levels at 7 days after challenge. The expression profile of the catfish hepcidin gene during the course of bacterial infection mirrors those of inflammatory proteins such as chemokines, suggesting an important role for hepcidin during inflammatory responses.

Keywords: Antimicrobial peptide; Hepcidin; Gene expression; Infection; Fish; Catfish; Edwardsiella; Disease; Innate immunity

Abbreviations PCR, polymerase chain reaction; BAC, bacterial artificial chromosome; RT-PCR, reverse transcriptase-polymerase chain reaction; cDNA, complementary DNA; EST, expressed sequence tags; ESC, enteric septicemia of catfish; BHI, brain heart infusion; CFU, colony forming units; AMP, antimicrobial peptides; IL-6, interleukin-6; MS 222, tricaine methanesulfonate; dATP, deoxyadenosine triphosphate; dCTP, deoxycytosine triphosphate; CHORI, Children's Hospital of Oakland Research Instiutute; UV, ultraviolet; UTR, untranslated region; SNP, single nucleotide polymorphism.

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1. Introduction

Hepcidin was initially isolated from human plasma ultrafiltrate and urine and shown to possess antimicrobial activities [1,2]. Subsequent research has demonstrated that it is an iron regulatory molecule involved in hereditary hemochromatosis and anemia of chronic disease [3–5], that it is part of the acute phase response to infection and inflammation [6–8], and that its expression changes during hypoxia [7]. Significant overlaps exist between these functions, which were examined together by Nicolas et al. [7]. Hepcidin expression is now understood to increase with the body's iron levels [6] and to be induced by inflammatory cytokine IL-6 during infection and inflammation [9]. The upregulation of hepcidin results in a decrease in circulating iron, increased iron sequestration to reticuloendothelial macrophages, and decreased intestinal iron absorption. Hypoxia and anemia down-regulate hepcidin gene expression and can override the inflammatory upregulation [7]. Several recent editorials and reviews contemplate the significant implications of these findings and provide insight into the role of iron in disease [10–12].

Hepcidin complementary deoxynucleic acids (cDNAs) have been cloned and characterized from various species including human, mouse, rat, striped bass, medaka, rainbow trout, Japanese flounder, winter flounder, long-jawed mudsucker, Atlantic salmon, and zebrafish [6,13–16]. However, hepcidin genes have been characterized only from human, white bass [14], Atlantic Salmon [15], mouse [17], and zebrafish [16]. The antimicrobial activity of hepcidin may lend itself to potential applications in fighting the bacterial and viral outbreaks that plague aquaculture species such as catfish. Toward this end, and as a part of our efforts to elucidate the innate immune components in catfish, we have characterized hepcidin cDNAs from both channel catfish and blue catfish, sequenced and characterized the channel catfish hepcidin gene, and analyzed its expression in various tissues, after bacterial challenge, and during development. The catfish hepcidin gene was also mapped to specific BACs for comparative genome analysis.

2. Materials and methods

2.1. Fish rearing, bacterial challenge, and tissue sampling

Channel catfish larvae were reared at the hatchery of the Auburn University Fish Genetics Research Unit. Challenge experiments were conducted as previously described [18] with modifications. Briefly, the catfish were challenged in a rectangular tank by immersion exposure for 2 h with a freshly prepared culture of enteric septicemia of catfish (ESC) bacteria, *E. ictaluri*. One single colony of *E. ictaluri* was isolated from a natural outbreak in Alabama (outbreak number ALG-02-414), inoculated into brain heart infusion (BHI) medium, and incubated at 28 °C overnight with shaking. The bacterial culture was added to the tank to a concentration of 3×10^7 CFU/ml. This dosage of bacterial pathogen was used in our previous experiments to provide a mortality level of 50–60% [18–20]. During challenge, an oxygen tank was used to ensure a dissolved oxygen concentration above 5 mg/ml (but below 7 mg/ml). After 2 h of immersion exposure, 15 fish were randomly taken and placed into a rectangular trough containing pond water with constant water flow through. Replicates of troughs were used to provide one trough for each sampling time point. For the control (unexposed) fish, 15 fish were incubated in a separate rectangular tank at the same fish density as the challenge tanks and moved to a separate trough after 2 h as described above.

For tissue expression studies, 11 tissues were collected from 1-year-old healthy channel catfish including brain, gill, head kidney, intestine, liver, muscle, ovary, skin, spleen, stomach, and trunk kidney. For developmental expression studies, 50 embryos or newly hatched larvae were sampled for each time point. For expression analysis after infection, at each time point of 4 h, 24 h, 3 days, and 7 days, 10 fish were randomly sacrificed for sampling from the control and experimental tanks. Major mortalities started at the fourth day and continued through the sixth day. Therefore, the samples of 7 days after challenge were collected from the survivors. The fish were euthanized with tricaine methanesulfonate (MS 222) at 100 mg/l before liver, head kidney, and spleen were collected. Tissues were kept in a −80 °C ultra-low freezer until preparation of RNA. Samples of each tissue from

10 fish were pooled. The pooled tissues were rapidly frozen with liquid nitrogen. In order to obtain samples representing the average of the 10 fish, the pooled tissue samples were ground with a mortar/pestle to fine powders and were thoroughly mixed. A fraction of the mixed tissue samples was used for RNA isolation. For gene expression studies during development, whole fish samples were used for extraction of RNA.

2.2. RNA isolation and RT-PCR

RNA was isolated following the guanidium thiocyanate method [21] using the Trizol reagents kit from Invitrogen (Carlsbad, CA) following manufacturer's instructions. Extracted RNA was stored in a −80 °C freezer until used as template for reverse transcription PCR (RT-PCR). The RT-PCR reaction was conducted using the SuperScript™ III One Step RT-PCR System (Invitrogen) according to manufacturer's instructions. The reaction also included the primers for β-actin amplification (Table 1), serving as an internal control. The reactions were completed in a thermocycler with the following thermo-profiles: 45 °C for 15 min for one cycle (reverse transcription reaction), at 94 °C for 2 min (denaturation) and 40 cycles (for the analysis of tissue expression and developmental expression), or 29 cycles (for the analysis of expression after infection) of 94 °C for 15 s, 53 °C for 30 s, 72 °C for 30 s. Forty cycles of PCR were used for expression analysis in various tissues and development to assure detection of RT-PCR products in tissues with minimal level of expression. Tissue expression analysis indicated that hepcidin gene was expressed at high levels in the liver and spleen and, therefore, 29 cycles were used to avoid the plateau phase of PCR. Upon the completion of PCR, the reaction was incubated at 72 °C for an additional 5 min. The RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel and documented with a Gel Documentation System.

2.3. cDNA cloning, plasmid preparation, sequencing and sequence analysis

The cDNA clone for blue catfish hepcidin was obtained during analysis of expressed sequence tags (EST) [22,23] and was used as a probe to obtain a BAC clone containing the channel catfish hepcidin gene (see Section 2.4 below). After sequence analysis of the channel catfish hepcidin genomic DNA, a pair of primers was designed based on the start and the end of the blue catfish cDNA for the amplification of the complete channel catfish hepcidin cDNA.

Plasmid DNA was prepared using the alkaline lysis method using the Qiagen Spin Column Mini-plasmid kits (Qiagen, Valencia, CA). Three microliters of plasmid DNA (about 0.5–1.0 μg) were used in sequencing reactions. Chain termination sequencing was performed using Thermosequenase (Amersham, Piscataway, NJ). The PCR profiles were an initial denaturation at 95 °C for 2 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 40 s, 72 °C for 45 s for 30 cycles, and a final 5 min extension at 72 °C. Sequences were analyzed on an automatic LI-COR DNA Sequencer Long ReadIR 4200.

BLAST searches [24–26] were conducted to determine gene identities. The DNASTAR software package was used for sequence analysis [27]. MegAlign program of the DNASTAR package was used for sequence alignment using CLUSTALW. For the analysis of potential cleavage site(s) of the signal peptide, SignalP (http://www.cbs.dtu.dk/services/SignalP/)

Table 1
Primers and their sequences used in this study

Primers	Sequences (5′–3′)	Utilization
AU75078F	GAAGCAGCAGAAGAAGGAGAACCTTTGAAGG	Sequencing BAC 188B14
AU75078R	GCTAGCAAGCATAGCTTCTAGGTGAAATTCCC	Sequencing BAC 188B14
Overgo 1	CTGCTGCAGGTTCTAATAACGGAC	Overgo probes for hybridization to BAC filters
Overgo 2	TGAAAACTTGCATGTGGTCCGTTA	Overgo probes for hybridization to BAC filters and RT-PCR
AU75052	GTAAAGCTGCACTCTGAAACCGC	Sequencing BAC 188B14
AU50919	CGAAGGAGACGAGTCCTGAGGT	RT-PCR and sequencing BAC 188B14
Actin forward	AGAGAGAAATTGTCCGTGACATC	RT-PCR internal control
Actin reverse	CTCCGATCCAGACAGAGTATTTG	RT-PCR internal control

software was used [28]. Transcriptional factor binding sites were determined by searching the upstream sequences against the TRANSFAC database using TFSEARCH software (http://www.cbrc.jp/research/db/TFSEARCH.html) [29]. The 'vertebrate' database was used and the cut-off value was set at 90. After obtaining the results, the putative transcriptional factor binding sites were tabulated using the transcription start site as +1; all upstream sequences were given a negative number.

2.4. BAC library screening and genomic DNA sequencing

High-density filters of a channel catfish BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA), and screened using overgo hybridization probes [30]. Overgo probes were designed from cDNA sequences at a region where secondary structures are minimal. Two 24-base-long oligonucleotide primers were synthesized such that they had an 8-bp complementary region at their 3′ end. Upon mixing the two primers, they base pair to form a complex structure that can be filled in with radioactively labeled nucleotides. Each set of filters contained a 10-genome coverage of the channel catfish BAC clones from BAC library CHORI 212 (http://bacpac.chori.org/library.php?id=103). The overgo hybridization method was adapted from a web protocol (http://www.tree.caltech.edu/). Briefly, overgos were selected following a BLAST search against GenBank to screen out repeated sequences and then purchased from Sigma Genosys (Woodlands, TX). Overgos were labeled with ^{32}P-dATP and ^{32}P-dCTP (Amersham, Piscataway, NJ) in overgo labeling buffer [31] at room temperature for 1 h using the Klenow polymerase (Invitrogen, Carlsbad, CA). After removal of unincorporated nucleotides using a Sephadex G50 spin column, probes were denatured at 95 °C for 10 min and added to the hybridization tubes. Hybridization was performed at 54 °C for 18 h in hybridization solution (50 ml of 1% BSA, 1 mM EDTA at pH 8.0, 7% SDS, 0.5 mM sodium phosphate, pH 7.2). Filters were washed and exposed to X-ray film at −80 °C for 2 days.

Positive clones were identified according to the clone distribution instructions from CHORI and picked from the channel catfish BAC library. BAC DNA was isolated with the Perfectprep® BAC 96 BAC DNA isolation kit (Brinkmann Instruments, Inc., Westbury, NY). In order to confirm the BAC clones were indeed positive clones containing the hepcidin gene, the clones were subjected to PCR amplification using channel catfish hepcidin gene-specific primers (the RT-PCR primers, Table 1). PCR reactions were carried out in a total volume of 10 μl containing 1 μl 10× PCR buffer, 0.6 μl $MgCl_2$ (25 mM), 0.4 μl each of the RT-PCR primer (100 ng/μl), 1 μl dNTP (each at 2.5 mM), and 1 unit of JumpStart Taq polymerase (St Louis, MO). The samples were pre-denatured at 94 °C for 5 min, and then amplified for 35 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

BAC clone 188B14 was sequenced directly using the primer walking method [32]. Fourteen picomoles of each primer were used for sequencing reactions. BAC sequencing was performed in a 10 μl reaction using the BigDye Terminator v3.0 Ready Reaction kit (Applied Biosystems, Foster City, CA) following manufacturer's instructions, and sequencing reaction products were analyzed on an ABI PRISM 3100 automated sequencer (Applied Biosystems).

2.5. Southern blot

In order to determine the copy number of the hepcidin gene, all positive BAC clones were subjected to Southern analysis using *Eco*R1, *Hin*dIII, and *Pst*I, and the entire channel catfish cDNA as a probe as we previously described [32]. The probe was prepared using the random primer method [33] with a labeling kit from Roche Diagnostics (Indianapolis, IN). Hybridized membranes were washed and autoradiographed as previously described [32].

3. Results

3.1. Channel catfish and blue catfish hepcidin cDNA sequences

Both the channel catfish and blue catfish hepcidin cDNAs were sequenced and their sequences have been deposited to GenBank with accession numbers of AY834209 and AY834210, respectively.

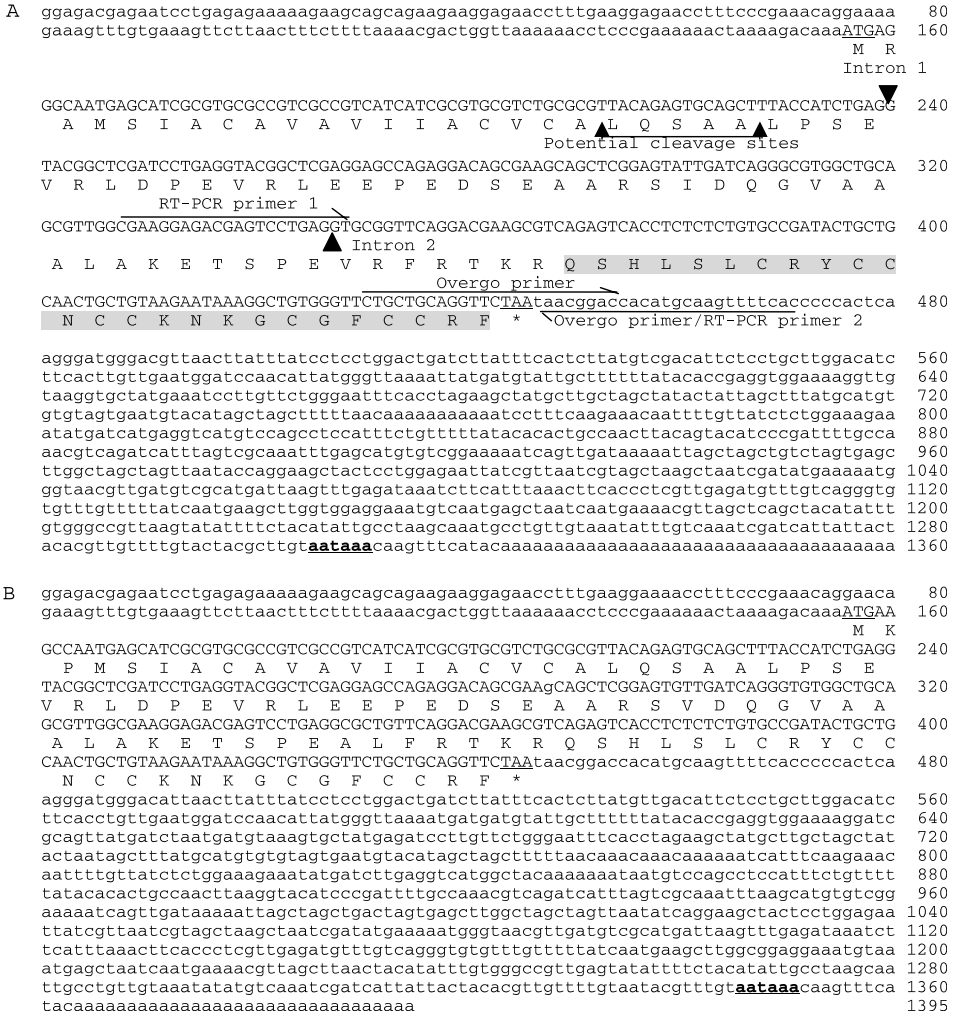

Fig. 1. (A) cDNA sequence of channel catfish hepcidin gene. The translation start codon ATG and termination codon TAA are both underlined. The asterisk indicates termination. Shaded amino acids are in the mature hepcidin. The polyadenylation signal sequences AATAAA is underlined and in bold font. The primer positions and orientations are indicated by semi-arrowed lines. Intron positions are indicated by solid triangles. (B) cDNA sequence of blue catfish hepcidin gene. The translation start codon ATG and termination codon TAA are both underlined. The asterisk indicates termination. The polyadenylation signal sequences AATAAA is underlined and in bold font.

The channel catfish hepcidin cDNA contained a 155 bp 5′-untranslated region (UTR), an open reading frame of 291-bp encoding a protein of 96 amino acids, and an 877 bp 3′-UTR (Fig. 1A). A typical AATAAA polyadenylation signal exists 12-bp upstream of the poly $(A)^+$ tail. Analysis of the cleavage site of the signal peptide indicated the presence of alternative cleavage sites. While a cleavage site was predicted between amino acids 19 and 20 using the Neural Networks model, an alternative cleavage site was predicted between amino acids 24 and 25 using the Hidden Markov model [26]. Both sites involve alanine–leucine peptide bonds (Fig. 1A).

The blue catfish hepcidin cDNA shared similar features to the channel catfish hepcidin cDNA (Fig. 1B). Three insertions in the 3′-UTR made the blue catfish cDNA longer by 30 bp. The two hepcidin cDNAs had 33 single nucleotide polymorphisms (2.5%) (Table 2). This divergence rate is almost double the average divergence rate between the channel catfish

Table 2
Similarities and differences of the channel catfish and blue catfish hepcidin cDNAs

	Channel catfish hepcidin	Blue catfish hepcidin
Accession number	AY834209	AY834210
cDNA length	1323 bp	1363 bp
5′-UTR	155 bp	155 bp
Open reading frame	291 bp	291 bp
Poly A signals	12 bp upstream of poly A tails	12 bp upstream of poly A tails
Insertions		20 bp insertion at 639–640, 3 bp insertion at 759–760, 17 bp insertion at 819–820
SNP sites	58g/a, 79a/c, 160g/a, 162g/c, 300a/g, 311c/t, 349c/t, 352g/t, 492g/a, 538c/t, 566t/c, 599t/g, 638t/a, 644g/a, 654a/g, 705t/a, 756a/c, 766c/a, 809a/t, 860c/a, 861a/g, 908g/a, 951t/a, 979c/t, 1147t/c, 1159c/a, 1188c/t, 1190g/a, 1211a/g, 1241a/t, 1258t/a, 1296c/a, 1301c/t	

genes and the blue catfish genes surveyed among ESTs (1.32%) [34], but is consistent with the fact that most immune-related genes tend to exhibit a greater level of divergence between the two species [34].

3.2. Channel catfish hepcidin gene

The channel catfish hepcidin gene was sequenced from BAC clone 188B14. The segment (3114 bp) spanning the entire three exons as well as the 760 bp upstream region and the 289 bp downstream region was sequenced and deposited in GenBank with the accession number of AY834211.

The genomic organization of the channel catfish hepcidin gene is similar to that of mammalian species such as human, mouse, and rat (Fig. 2). It has three exons and two introns as reported for white bass [14], Atlantic salmon [15], and zebrafish [16], but the intron and exon sizes are more similar to mammalian hepcidin genes. Two introns have been found in all hepcidin genes characterized. In the mammalian hepcidin genes, the first intron is large while the second intron is very small. However, both introns of the white bass and Atlantic salmon hepcidin genes were small in size [14]; both introns were large in the zebrafish genes [16].

A search of transcriptional regulatory sequences within the 760-bp upstream sequences against the TRANSFAC database indicated the presence of various regulatory elements within the upstream sequences of the channel catfish hepcidin gene (not shown). Several of the transcriptional factor binding sites appeared to be conserved between the zebrafish and channel catfish genes including C/EBPα (−450), C/EBPβ (−450, −150, and −136), HNF3β (−371, −212, −202, and −194) and NF-κβ (−131). However, the TATA box was not present in the catfish gene.

3.3. Copy number of channel catfish hepcidin gene

In order to determine the copy number of the hepcidin gene in the channel catfish genome, we conducted Southern blot analysis using a channel catfish BAC library. BAC clones positive to the hepcidin probe were first identified using a BAC filter with 10× genome coverage. If the hepcidin gene is present in multiple copies in different genome locations, then the number of positively hybridizing clones should be proportional to the genome copies. Alternatively, if only a single copy exists in the channel catfish genome, then the expected positive clones should approximate the genome coverage on the BAC filters. If multiple copies exist in the genome in a tandem arrangement in the same BAC clones, then the hybridization signal should be proportional to the number of copies. In this experiment, a total of nine positively hybridizing clones were identified which approximated the genome coverage, indicating the presence of a single copy. In order to confirm this, BAC DNA of the hepcidin-containing BAC clones, 10F22, 47D17, 49N2, 90P14, 95M9, 102L1, 132G16, 176E12, and 188B14, was isolated (of which 95M9 and 102L1 failed to grow) and was subjected to Southern blot analysis after digest with three restriction enzymes. As shown in Fig. 3, a single restriction

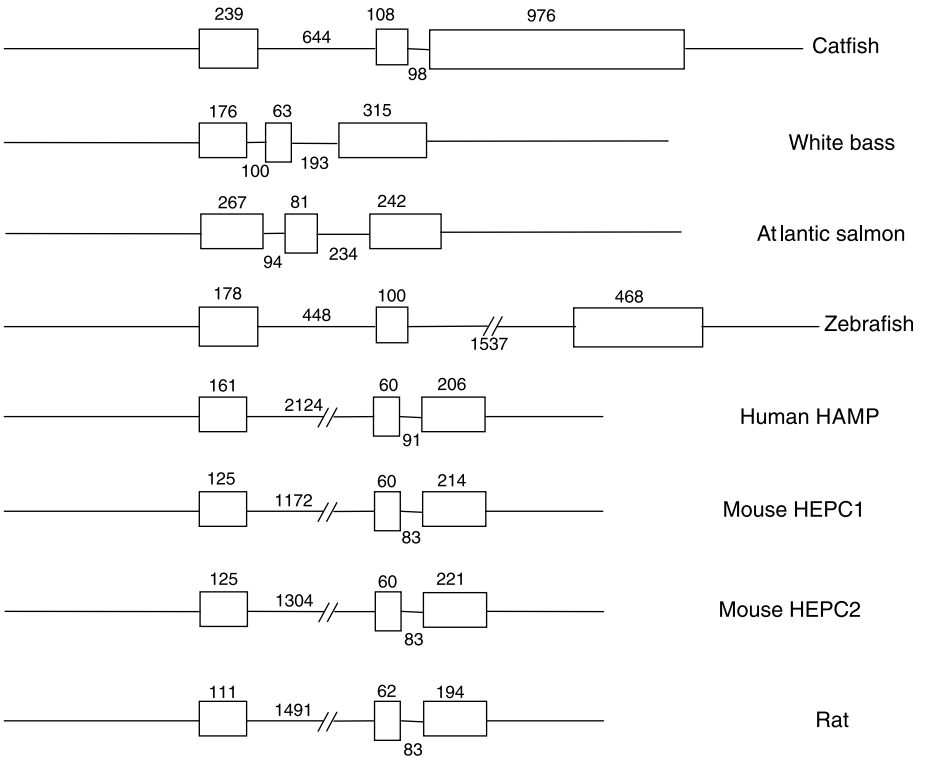

Fig. 2. Genomic structure and organization of the channel catfish hepcidin gene compared to that of white bass, Atlantic salmon, zebrafish, human, mouse, and rat. The exons are shown as rectangles and introns are shown as lines. Large intron sizes are indicated by broken lines reflecting non-proportion of the line length to the involved intron sizes.

pattern was observed with all seven BAC clones using three restriction enzymes, *Eco*R1, *Hin*dIII, and *Pst*I, confirming the existence of a single copy hepcidin gene in the channel catfish genome.

3.4. Channel catfish hepcidin gene expression

Analysis of gene expression using RT-PCR indicated that the channel catfish hepcidin gene is expressed in a wide range of tissues (Fig. 4). Its expression was found in all tested tissues but brain. The highest expression was observed in the liver and spleen, followed by gill and intestine; its expression was very low in the muscle, stomach, and skin (Fig. 4).

The channel catfish hepcidin gene is expressed early during development. During the course of study beginning 8 h after fertilization until 17 days after hatching, the hepcidin gene was found to be expressed at high levels. The only notable exception was that lower expression was found immediately after hatching (Fig. 5).

The channel catfish hepcidin gene was upregulated after infection with *Edwardsiella ictaluri* in a tissue-specific manner. Its expression was significantly upregulated in head kidney and spleen, but was not induced after infection in the liver (Fig. 6). Similar patterns of gene induction were observed in the head kidney and spleen with expression highly induced at 24 h after challenge. The high level of hepcidin gene expression continued at 3 days after challenge. Expression of hepcidin in surviving fish 7 days after challenge returned to normal levels, but the surviving fish may not represent the entire group of the challenged fish due to mortalities. Hepcidin gene expression was not induced in the liver at any time of sampling after challenge (Fig. 6), despite strong induction in the head kidney and spleen, both of which are highly related to immune functions.

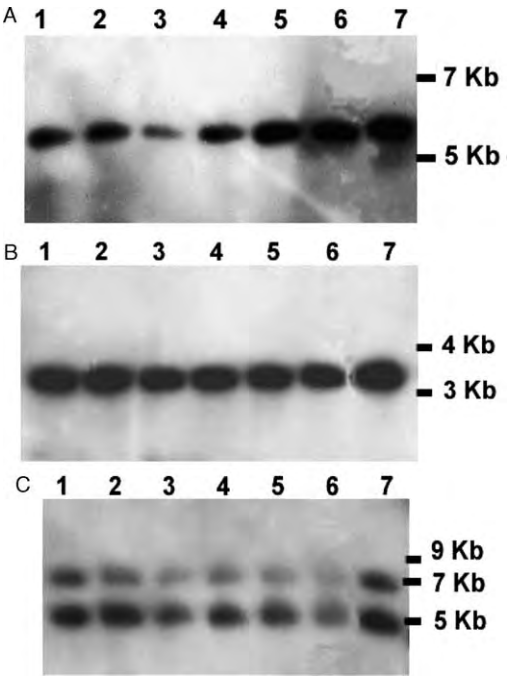

Fig. 3. Southern blot analysis using BAC clones. BAC DNAs were digested with *Eco*R1 (A), *Hin*dIII (B), and *Pst*I (C), electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized to hepcidin cDNA probes. Lanes 1–7 contained BAC DNA from seven different BAC clones. Molecular weight is indicated on the right margin.

4. Discussion

Intensive aquaculture continues to be plagued by periodic, severe outbreaks of microbial infection resulting in devastating losses. The acute nature of infections, such as those caused by the catfish pathogen *Edwardsiella ictaluri* has drawn attention to the importance of the innate immune response. Often lacking time to mount an effective adaptive response [35,36], fish rely heavily on the innate components of immunity to combat microbial invasions. Research into these components [19,20,36–38] has included the identification and analysis of a number of antimicrobial peptides and other upregulated genes during infection [39–44]. Hepcidin, as an important antimicrobial peptide and an iron-regulatory molecule stimulated by inflammation and hypoxia, holds great potential in aquaculture species for remediative therapies, marker-assisted selection and genetic engineering, as well as a greater understanding of host–pathogen interactions in the early stages of disease. As a first step, we report here molecular cloning, sequencing, and characterization of channel catfish and blue catfish hepcidin cDNAs, and the channel catfish hepcidin gene. The hepcidin gene is expressed in a wide range of tissues and its expression is regulated by infection in a tissue-specific manner.

The channel catfish hepcidin gene is well conserved in its genomic structure and organization compared to other species, containing three exons and two introns.

However, the hepcidin genes appeared to have a rapid rate of sequence divergence. The channel catfish hepcidin gene shared 95% amino acid identity with the blue catfish hepcidin gene. Their entire cDNA exhibited a high level of SNPs (2.5%), in addition to three major insertions at the 3′-UTR of the blue catfish gene. This divergence rate is almost twice the rate between average channel catfish and blue catfish genes, as revealed by EST analysis [22,23,45]. However, it is compatible with the divergence rate of immune-related genes between the two species, which was found to be higher than the average divergence rate [37]. The three deletions in the 3′-UTR of channel catfish gene as compared to the blue

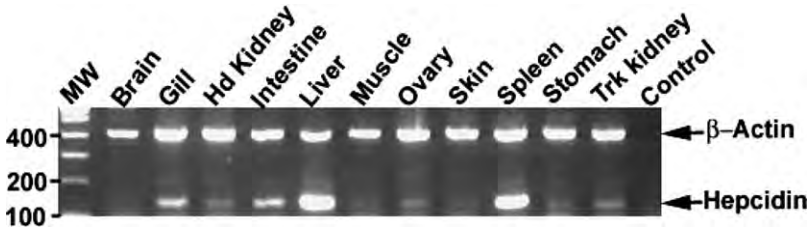

Fig. 4. RT-PCR analysis of hepcidin gene expression in various tissues. RT-PCR products were analyzed on an agarose gel. Tissues were specified on the top of the gel, with molecular weight (MW), head kidney (Hd Kidney), trunk kidney (Trk kidney), and control (no RNA).

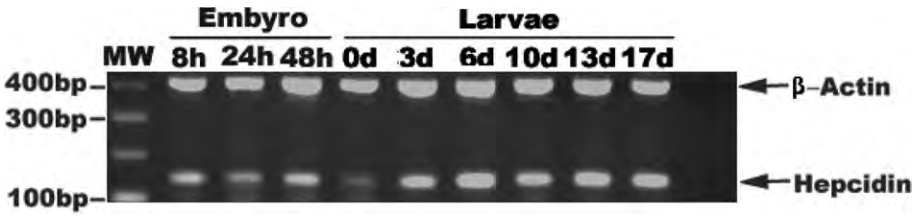

Fig. 5. RT-PCR analysis of hepcidin gene expression during development. Fifty embryos or larvae were collected and whole fish samples were used for the extraction of RNA. Total RNA was used for RT-PCR reactions. RNA amount was determined using a spectrophotometer and confirmed by visual inspection of RNA gel electrophoresis. An equal amount of RNA was used for RT-PCR reactions. RT-PCR products were analyzed on an agarose gel. Developmental stages were specified on the top of the gel, molecular weight (MW) on the left margin, and the bands corresponding to β-actin and hepcidin on the right margin.

catfish gene should be useful for mapping the hepcidin genes to the catfish genome using interspecific hybrid mapping families [45,46].

The genomic copies of the hepcidin gene vary greatly depending on the organism. In contrast to the unique copy of the hepcidin gene in the human genome, the hepcidin locus has undergone duplication in mice leading to two hepcidin genes, HEPC1 and HEPC2. Both of the mouse hepcidin genes are on chromosome 7, but are located very distantly. In fish, various copy numbers have been found. While only a single copy was identified from striped bass [14] and from catfish (this communication), two copies have been found from Atlantic salmon [15] and zebrafish [16]. It is worth noting that Atlantic salmon is believed to have undergone entire genome duplication and, therefore, is generally regarded as a tetraploid fish [47]. In winter flounder, Douglas et al. [15] suggested the presence of at least five copies of hepcidin genes arranged in clusters in the genome. Therefore, it appears that the gene duplication events of hepcidin genes in various lineages were quite recent events.

We used a BAC-based Southern blot procedure [41,44] rather than the traditional genomic DNA-based Southern blot for the assessment of genomic copy numbers. The accuracy of the BAC-based Southern blot for the analysis of genomic copy numbers depends on inclusion of all copies of the gene of interest in the BAC library; the quality of the channel catfish BAC library, CHORI-212 (http://bacpac.chori.org/library.php?id=103), is very good with a 10.7× genome coverage with an average insert size of 161 kb. Using BAC-based Southern blot, the restriction patterns defines the copy numbers, not the number of bands as used in the traditional Southern blot. This characteristic is of particular advantage when the size of the target gene is extremely large and the genomic sequences for the gene are not yet available [44].

Two potential cleavage sites for the removal of the signal peptide were identified; the first between amino acids 19 and 20 (A–L) was predicted by using the Neural Network Model, while the second between amino acids 24 and 25 (also A–L) was predicted using Hidden Markov Model. Based on sequence homology analysis, it is likely that the cleavage site is between amino acids 24 and 25, as the same position was predicted for the zebrafish hepcidin and the white bass hepcidin [14,16], although additional research is needed to confirm this speculation.

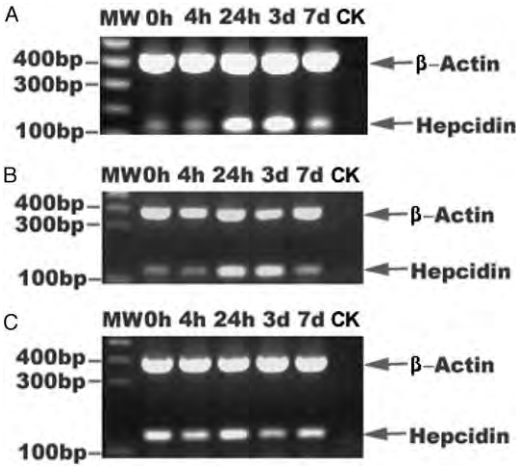

Fig. 6. RT-PCR analysis of hepcidin gene expression after bacterial challenge with *Edwardsiella ictaluri*. Samples of head kidney (A), spleen (B), and liver (C) were collected at 0 h, 4 h, 24 h, 3 days, and 7 days after challenge. RT-PCR products were analyzed on agarose gels along with no RNA control (CK).

This research provides evidence that hepcidin is expressed in a wide range of tissues with the highest expression in the liver and spleen in catfish. Highly sensitive RT-PCR assays showed that the hepcidin gene was expressed in all tested tissues except brain: liver, spleen, intestine, gill, head kidney (anterior kidney), trunk kidney (posterior kidney), stomach, skin, ovary, and muscle. In human, hepcidin RNA was detected only in the liver, but not in any other tested tissues using Northern blot analysis [2]. However, it appears that hepcidin expression may vary greatly depending on the organism. For instance, no expression of hepcidin was found in the spleen of white bass without bacterial challenge [14], but its expression in catfish spleen was very strong. Different hepcidin genes also exhibit great variation in their patterns of tissue expression. For instance, in winter flounder, type I hepcidin was expressed in the liver and cardiac stomach; type III hepcidin was expressed in the liver, cardiac stomach, and esophagus, whereas type II hepcidin was not expressed in any of the 12 tissues tested including liver, cardiac stomach, esophagus, pyloric stomach, pyloric caecae, spleen, intestine, rectum, gill, brain, skin, and muscle [15]. Similarly, the mouse HEPC2 was preferentially expressed in the liver and pancreas, while HEPC1 was expressed predominantly in the liver [17].

Hepcidin gene regulation by infection could be both tissue-specific and infection-specific. In white bass, the hepcidin gene was induced over 4500-fold by *Streptococus iniae* challenge in the liver [14], while in this study, we did not observe any induction of hepcidin gene expression in the liver of channel catfish following *E. ictaluri* challenge. However, a significant induction was observed in both the spleen and head kidney following *E. ictaluri* challenge in catfish, suggesting that gene induction is tissue-specific. The lack of induction in the liver of channel catfish could be from the differences in the pathogen, progression of infection, and the nature of pathogenesis between *E. ictaluri* and *S. iniae*. The significant gene induction in the spleen and head kidney of channel catfish following bacterial challenge indicated that hepcidin could be an important part of innate immune response of catfish. Tissue-specific pathogen-induced gene expression of hepcidin was previously observed in Atlantic salmon where the Sal2 transcript (transcript from the second copy of the salmon hepcidin gene) of hepcidin was strongly induced in the liver, spleen, stomach, pyloric caecae, and intestine, but not in other tissues tested [15]. It is likely that the tissue-specific induction is related to the status of inflammation as hepcidin is known to be regulated by anemia, hypoxia, and inflammation [7]. The strong developmental expression, seen as early as 8 h after fertilization, supports the idea that hepcidin is critical to the innate immune system of fish. Hepcidin expression prior to organogenesis further supports the notion of its wide range of expression beyond the liver tissue. In winter flounder, type I and type III hepcidin were expressed during early development, whereas type II hepcidin was found not to be expressed in pre-metamorphic larvae [15].

In summary, we reported the sequencing, expression analysis, and characterization of the channel catfish hepcidin gene. Hepcidin, in its overlapping roles as an iron-regulatory molecule and an antimicrobial peptide, may prove to be a crucial part of our efforts to understand teleost innate immunity and to apply that knowledge towards eventual control of large-scale microbial infections in aquaculture species. Recent research progress with striped bass hepcidin against Gram-negative pathogens demonstrates the potential for the application of antimicrobial peptides for the control of fish diseases [48].

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