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Structure and expression of transferrin gene of channel catfish, *Ictalurus punctatus*

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ABSTRACT

Transferrin is important in iron metabolism and has been reported to be involved in disease defence responses after bacterial infection. In this study, we identified, sequenced, and characterized the transferrin gene from channel catfish, *Ictalurus punctatus*. The catfish transferrin gene was similar to those of other vertebrate species with 17 exons and 16 introns. Sequence analysis indicated the presence of the two duplicated lobes, each containing two sub-domains separated by a cleft harboring the iron-binding site, suggesting their structural conservation. The channel catfish transferrin cDNA encodes 679 amino acids with 42–56% similarity to known transferrin genes from various species. Southern blot analysis suggested the presence of two copies of the transferrin gene in the catfish genome, perhaps arranged in a tandem fashion. The catfish transferrin gene was mapped to a catfish BAC-based physical map. The catfish transferrin gene was highly expressed in the liver, but expression was low in most other tested tissues. Transferrin expression was significantly up-regulated after infection with *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish. Such induction was also found with co-injection of iron-dextran and *E. ictaluri*, while transferrin expression was not significantly induced with the injection of iron-dextran alone.

1. Introduction

Iron, the second most abundant metal on earth, plays essential roles in a wide variety of metabolic processes (oxygen transport, electron transport, DNA synthesis, etc), but in excess iron has the potential to cause deleterious effects [1]. Thus, maintaining an appropriate balance of iron in the body is important. The liver plays a central role in iron metabolism; it is the major storage site for iron and also expresses a complex range of molecules which are involved in iron transport and regulation of iron homeostasis [2]. One protein that has a critical role in iron metabolism, maintaining low levels of extracellular free iron, and transporting iron to tissues as required, is transferrin [2–4], which is found only in the phylum Chordata [3].

The transferrin super-family comprises a class of two-sited, single-chain, metal-binding proteins, widely distributed in physiological fluids and cells of vertebrates [3]. Serum transferrin, also known as serotransferrin [5], whose main function is iron transport, is the most extensively studied member of the transferrin class of proteins. Serum transferrin is expressed mainly in liver and also found in other tissues including brain, the testes, the ovary, the spleen, the mammary gland, and the kidney in mammals [6–9]. Lactoferrin, found in most milk and secretions, tears, saliva, etc., is thought to be an iron scavenger which prevents the proliferation of invading microbes [10,11]. Another family member, ovotransferrin, which is encoded by the avian serum transferrin gene, is expressed both in the liver and oviduct and is believed to play an antimicrobial role in egg white [12,13]. In addition to these transferrin family members, other transferrin-like proteins have been identified: melanotransferrin [14], inhibitor of carbonic anhydrase [15], and other proteins with homology to transferrin were recently identified from algae, crayfish and sea urchins [16–19]. The typical transferrin family members consist of a single polypeptide chain (MW ~80 kDa) which folds into two homologous lobes [10], the N-terminal half domain (N-lobe) and the C-terminal half domain (C-lobe), connected by a short hinge region, which is believed to have arisen by duplication of a precursor domain [20].

The structure, function, and mechanism of iron uptake, release and storage and genetic control of the transferrin gene of mammals has been an area of intensive research over the last few decades [1–12,14,15,20,21]. In spite of extensive studies in mammals, studies

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of these genes in teleost fish used to be limited to only a few species of fish including salmonids [22–26], medaka [27,28], and cod [29]. Recently, transferrin polymorphism has been studied in the polyploid *Carassius auratus* by cloning and sequencing analysis of cDNAs from its three subspecies *C. auratus gibelio*, *C. auratus auratus*, and *C. auratus cuvieri* [30]. The complete genomic sequence of Nile tilapia (*Oreochromis niloticus*) transferrin and functional studies in relation to saltwater resistance were reported [31]. Structure and expression of transferrin gene in red-blooded and hemoglobin-less antarctic notothenioids were also reported [32]. Very recently, Neves et al. reported the response of transferrin of sea bass to bacterial infection [33].

Channel catfish (*Ictalurus punctatus*) is the most important aquaculture species in the United States, accounting for more than 60% of all aquaculture production [34]. Catfish is also an important game fish (ranking within the top three sports fish), with a broad geographic range encompassing a variety of habitats [35]. Despite its economic and ecological importance, documentation of its main genes involved in iron metabolism has been minimal. With a lack of information about transferrin genes, the aim of the current study is to characterize the transferrin gene in channel catfish, to determine its genomic organization and copy numbers, to determine its patterns of tissue expression, and to establish if it is involved in defense responses of catfish after bacterial infection.

2. Materials and methods

2.1. Identification and sequencing of catfish transferrin cDNAs

BLAST searches were used to identify cDNAs encoding for transferrin from channel catfish expressed sequence tags (ESTs) from previous sequencing efforts [36–39] and from those available in public databases. The clone CBFA9174 (GenBank ID: FD320378) containing the putative complete cDNA as revealed by BLAST searches was completely sequenced to generate the full-length cDNA sequence. T7 and SP6 primers, as well as internal primers were used to generate the complete cDNA sequence (Table 1).

Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocols with modifications [40], and samples were sequenced on an ABI 3130XL automated DNA sequencer (Applied Biosystems).

Table 1
PCR primers used for this study.

Primers and their uses	Primer sequencing (5′–3′)
SP6 sequencing primer	ATTTAGGTGACACTATAG
T7 sequencing primer	TAATACGACTCACTATAGGG
Reverse primer for cDNA primer-walking sequencing	CCTGAAGGCTCCGCTGTAGC
Forward PCR primer for BAC library screening	GCAAAGCCAGCAACGATGAG
Reverse PCR primer for BAC library screening	GATTTTGGAACCTCCTGGAG
Forward PCR primer for Southern blot probes	AATCAGGCGGACGCTATTGC
Reverse PCR primer for Southern blot probes	ATCACGCTTCTCTGGACGAG
Forward primer for RT-PCR and qRT-PCR	AATCAGGCGGACGCTATTGC
Reverse primer for RT-PCR qRT-PCR	ATGTTCCAGCCTGCTGTACG
β-actin forward primer	AGAGAGAAATTGTCCGTGACATC
β-actin reverse primer	CTCCGATCCAGACAGAGTATTTG
18S rRNA forward primer	GAGAAACGGCTACCACATCC
18S rRNA reverse primer	GATACGCTCATTCCGATTACAG
1st intron forward primer	GCTGCATCCAAGAGTCCAGC
1st intron reverse primer	ACCTTCTCACACTGCACTGC
2nd intron forward primer	CCAAACCAACAAACCAACACTG
2nd intron reverse primer	AGTGATGGCATCCGCTTCAC
3rd intron forward primer	TGCCATCACTCTGGACGGAG
3rd intron reverse primer	AGTGCCGATGGGGATGTTCC

2.2. BAC library screening and genomic sequencing of the catfish transferrin gene

cDNA probes were used for screening the transferrin gene from the channel catfish CHORI-212 BAC library [41]. High-density BAC filters containing the whole CHORI-212 BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA, USA). The plasmid IpLvr00643 (GenBank ID: BM438281) harboring transferrin cDNA was used as template for the amplification of the cDNA insert using PCR with T7 and SP6 primers (Table 1). The PCR amplified segment of the transferrin cDNA was gel-purified and labeled by using the Random Primed DNA Labeling kit from Roche Diagnostics (Indianapolis, IN, USA) following manufacturer's protocols. Sephadex G50 spin columns were used to remove unincorporated nucleotides. Probes were denatured at 95 °C for 10 min and added into hybridization tubes containing the BAC filters that had been under pre-hybridization at 63 °C for 2 h with hybridization solution (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 sulphate, 40 μg mL^{-1} salmon sperm). The filters were hybridized at 63 °C for 16 h in 30 mL hybridization solution. The filters were washed twice at 60 °C with washing buffer (0.75 M NaCl, 75 mM sodium citrate, 0.1% sodium dodecyl sulphate), and then exposed to X-ray film (Fujifilm, Tokyo, Japan) at −80 °C for 20 h. A second screening was conducted by PCR for transferrin-positive BAC clones using catfish gene specific PCR primers (Table 1). Positive clones were identified according to the clone distribution pattern from CHORI. Positive clones were picked and cultured in 2× YT medium. After overnight culture, BAC DNA was isolated using the R.E.A.L. Prep 96 BAC DNA isolation kit (Qiagen, Valencia, CA, USA) as previously described [40,42]. BAC clone 163_I24 containing transferrin was sequenced using a primer-walking method. Targeted PCR and cloning were used when the primer-walking method failed, using primers specific for transferrin (Table 1). The PCR products were cloned for sequencing using the pGEM-T Easy vector (Promega, Madison, WI, USA). Sequencing was conducted as described in Section 2.1.

2.3. Southern blot analysis

Southern blot analysis was conducted to determine the genomic copy number of the transferrin gene in channel catfish. Genomic DNA was isolated from three individual adult channel catfish following standard protocols as previously described [43]. Genomic DNA concentration was estimated using an Ultrospec 1100 pro (Amersham Biosciences, Fairfield, CT, USA), and 10 μg was digested with 30 U of the restriction endonucleases *Eco* RI, *Hin*d III or *Pst* I (New England Biolabs, Beverly, MA, USA) in a 25 μL reaction at 37 °C. The digested DNA samples were electrophoresed on a 0.7% agarose gel. The gel was submerged in 0.25 N HCl for 15 min, and in 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) for 30 min, respectively. The DNA was transferred to an Immobilon positively-charged nylon membrane (Millipore, Bedford, MA, USA) by capillary transfer with 20× SSC buffer (3 M NaCl, 0.3 M sodium citrate) for 18 h. The DNA was fixed to the membrane using a UV cross-linker (Stratagene, La Jolla, CA, USA) and the auto-crosslink setting. The membrane was hybridized with cDNA probes amplified using primers listed in Table 1, as described for BAC-based screening.

2.4. Phylogenetic analysis

Transferrin coding sequences from various species were retrieved from GenBank for multiple sequence alignment using ClustalW. A phylogenetic tree was constructed using the neighbour-joining

method within the Molecular Evolutionary Genetics Analysis (MEGA 4) package [44]. Data were analyzed using Poisson correction, and gaps were removed by complete deletion. The topological stability of the tree was evaluated by 10,000 bootstrap replications.

2.5. Bacterial challenge and iron-dextran treatment

Channel catfish, *I. punctatus*, with an average body weight of 6.1 g and average body length of 9.5 cm, was used in this study. The fish were kept at 27 °C in a flow-through system utilizing heated municipal water. Forty-five fish were treated in each of four groups: (1) control group (phosphate-buffered saline, PBS injected); (2) *Edwardsiella ictaluri* challenged group; (3) iron-dextran treated group; and (4) iron-dextran treated and *E. ictaluri* challenged group. To inoculate bacteria for the challenge, a single colony of *E. ictaluri* was isolated and cultured in BHI broth at 28 °C overnight. The bacterial culture was diluted with PBS (pH 7.4), and 1×10^5 cfu of bacteria in 100 μL PBS were injected intraperitoneally into the channel catfish. For the iron-dextran group, 100 μL of the iron-dextran solution (Sigma, St. Louis, MO, USA) diluted in sterile PBS to a final concentration of 12.5 mg mL^{-1} was injected intraperitoneally. The fish in the combination group were injected with both bacteria and iron-dextran. Injections were carried out under anesthesia using tricaine methanesulphonate (MS 222) at 100 mg L^{-1}.

2.6. Tissue sampling and RNA extraction

Fish used in this study were euthanized by MS 222 exposure at 300 mg L^{-1} before dissection. To determine transferrin gene expression in various healthy catfish tissues, samples of 13 tissues including blood, brain, gill, heart, head kidney, trunk kidney, intestine, liver, muscle, skin, spleen, stomach and ovary from 4 control channel catfish were isolated, pooled, and flash-frozen in liquid nitrogen. Tissues were homogenized under liquid nitrogen using a mortar and pestle, and stored at −80 °C until RNA extraction. Similarly, the liver tissues from 45 fish (3 pools of 15 fish each) at 4 h, 24 h, 3 d and 7 d after treatment in each group of the bacterial challenge/iron-dextran experiment were pooled and homogenized for RNA extraction. Corresponding, uninfected control samples were taken at each time interval.

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the supplied protocol. The concentration of the total RNA was quantified using spectrophotometry. The RNA samples were subsequently used for determination of gene expression.

2.7. RT-PCR analysis

For a semi-quantitative expression analysis of transferrin in 13 healthy channel catfish tissues, cDNAs were synthesized from 1 μg of total RNA using the Superscript II First-Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Concentrations of the reverse-transcribed products were adjusted using a spectrophotometer, and 100 ng used in 20 μL PCR reaction mixture. PCR primers specific to the channel catfish transferrin gene (Table 1) were used. Thermal cycling was performed using a DNA Engine Thermal Cycler (Bio-Rad, Hercules, CA, USA). The PCR reaction mixture was denatured at 95 °C for 3 min and then subjected to 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension time of 10 min at 72 °C. PCR primers for β-actin (Table 1) were also used in the RT-PCR reactions to serve as an internal control. The PCR products were electrophoresed on a 1.2% agarose gel and documented with a gel documentation system (Nucleotech Corp., San Mateo, CA, USA).

2.8. Quantitative real-time RT-PCR analysis

Quantitative real-time RT-PCR (qRT-PCR) analysis was conducted using liver tissues from the bacterial challenge and iron-dextran treatment groups. The reactions were performed on a LightCycler 1.0 (Roche Applied Science, Indianapolis, IN, USA). Concentration of total RNA was determined by spectrophotometry. One-step real time RT-PCR was carried out using the Fast Start RNA Master SYBR Green I Reagents kit (Roche Applied Science), following manufacturer's instructions with modifications previously described [45,46]. Total RNA (100 ng) was used in each reaction. The fold induction of the channel catfish transferrin gene after different treatments was quantified. The 18S rRNA gene was used as an internal control for comparative normalization of expression levels. The primers that were used in qRT-PCR are listed in Table 1. Each pool of RNA was run in triplicate to serve as biological replicates. The triplicate fluorescence measured by crossing point (Ct) values were compared and converted to fold differences by the relative quantification method using the Relative Expression Software Tool 384 v.1 (REST) [47]. PCR specificity was assessed by melting curve analysis.

3. Results

3.1. Identification and sequencing of transferrin cDNAs

The full-length transferrin cDNA sequence was submitted to GenBank with an accession number of FJ176740. The channel catfish transferrin cDNA contains an open reading frame (ORF) of 2037 bp encoding 679 amino acids with a 20 bp 5′-untranslated region (UTR) and a 154 bp 3′-UTR. A typical polyadenylation signal sequence AATAAA existed 15 bp upstream of the poly (A) sequences. Analysis of the deduced amino acid sequence by multiple sequence alignment indicated that the transferrin gene is moderately conserved through evolution (Fig. 1). The catfish transferrin showed 44–56% identity with those transferrin protein sequences of grass carp, goldfish, zebrafish, *Fugu*, and medaka, and 42–45% identity with mammalian (human and mouse) serum transferrin and lactoferrin. The transferrin showed a pairwise similarity of 35–71% among that of catfish, medaka, *Fugu*, grass carp, goldfish, zebrafish, human and mouse (Table 2). The catfish transferrin contains two lobes (N-lobe: residues 21–332 and C-lobe: 333–671) of approximately 330–340 amino acids each (Fig. 1). As shown in Fig. 1, of the four iron-binding residues and two anion sites in the N-lobe, Asp-82 (human coordinates including the 19 signal residues) is the only amino acid that is invariant in all the 8 transferrin members; amino acids at the other 5 sites vary in zebrafish, grass carp and goldfish. However, the four iron-binding residues and two anion sites in the C-lobe are all invariable.

3.2. Phylogenetic analysis of transferrin gene

In order to analyze the channel catfish transferrin gene in the larger context of vertebrate genomes, phylogenetic analysis was conducted based on amino acid sequences from 36 taxa. As shown in Fig. 2, the teleost and the mammalian transferrin homologues are supported by very strong bootstrap values. The mammalian (human and mouse) serum transferrin and lactoferrin are the two main members of the transferrin super-family, forming one separate clade. The teleost transferrin genes formed six distinct sub-clades: (1) zebrafish and carp transferrin genes; (2) catfish transferrin gene; (3) the Pleuronectiformes fish transferrin genes; (4) the Gadiformes fish transferrin genes; (5) the clades containing transferrin genes from Beloniformes, Tetrodontiformes, and Perciformes fish; and (6) salmonid transferrin genes.

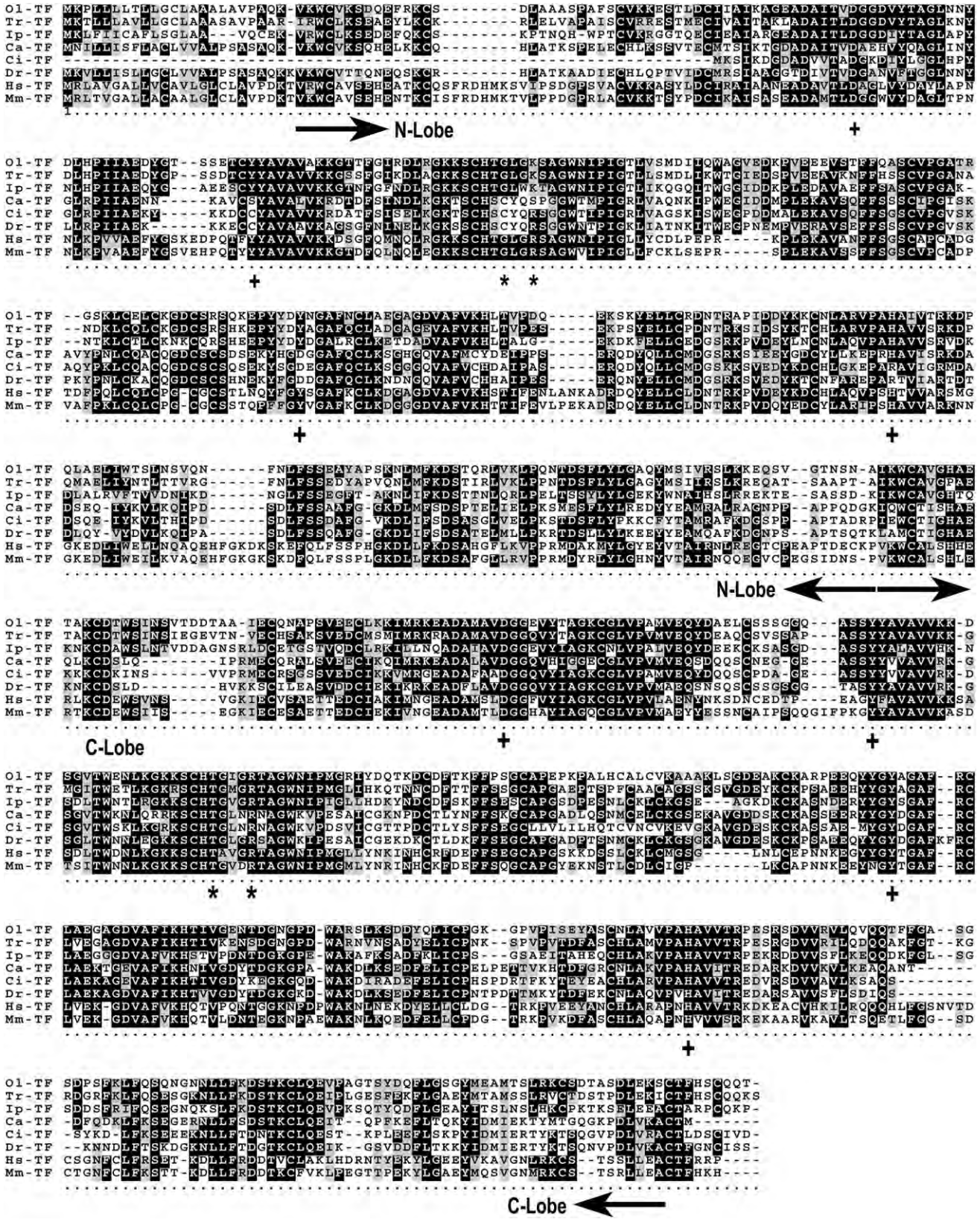

Fig. 1. Alignment of amino acid sequences of the vertebrate transferrin genes. The conserved and identical residues are represented by black shading, and conservative substitutions are represented by grey shading. The region of N-lobe and C-lobe, which was predicted by SMART program (http://www.smart.embl-heidelberg.de/), are indicted by arrows. Four iron-binding sites of each lobe of transferrin are indicated by plus signs and two sites of anion of each lobe are indicated by stars. Ol: *Oryzias latipes*, BAF81983; Tr: *Takifugu rubripes*, SINFRUP00000140191; Ip: *Ictalurus punctatus*, FJ176740; Ca: *Carassius auratus*, AAK92216; Ci: *Ctenopharyngodon idella*, AAR20997; Dr: *Danio rerio*, DAA01798; Hs: *Homo sapiens* P02787; Mm: *Mus musculus*, Q92111.

Table 2
Pairwise similarities of selected transferrin proteins. Unless otherwise specified, all genes were serum transferrins, or in the cases of fish, they are transferrins.

Fugu	Catfish	Goldfish	Grass carp	Zebrafish	Human	Mouse	Human lactoferrin	Mouse lactoferrin	
71	56	49	49	48	44	47	44	42	Medaka
	55	48	48	49	46	47	47	45	*Fugu*
		45	44	46	45	43	45	42	Catfish
			68	66	38	39	35	37	Goldfish
				64	37	35	38	37	Grass carp
					38	37	38	37	Zebrafish
						73	61	57	Human
							59	55	Mouse
								70	Human lactoferrin

3.3. Structural analysis of the channel catfish transferrin gene

Screening of a channel catfish BAC library [41] led to the identification of six BAC clones containing the transferrin gene. The six BAC clones from the CHORI-212 library positive for transferrin gene were 27_A7, 42_L13, 75_F24, 154_F7, 163_I24 and 167_A14. The clone 163_I24 was used for sequencing of the channel catfish transferrin gene. A genomic sequence of 11,874 bp of the channel

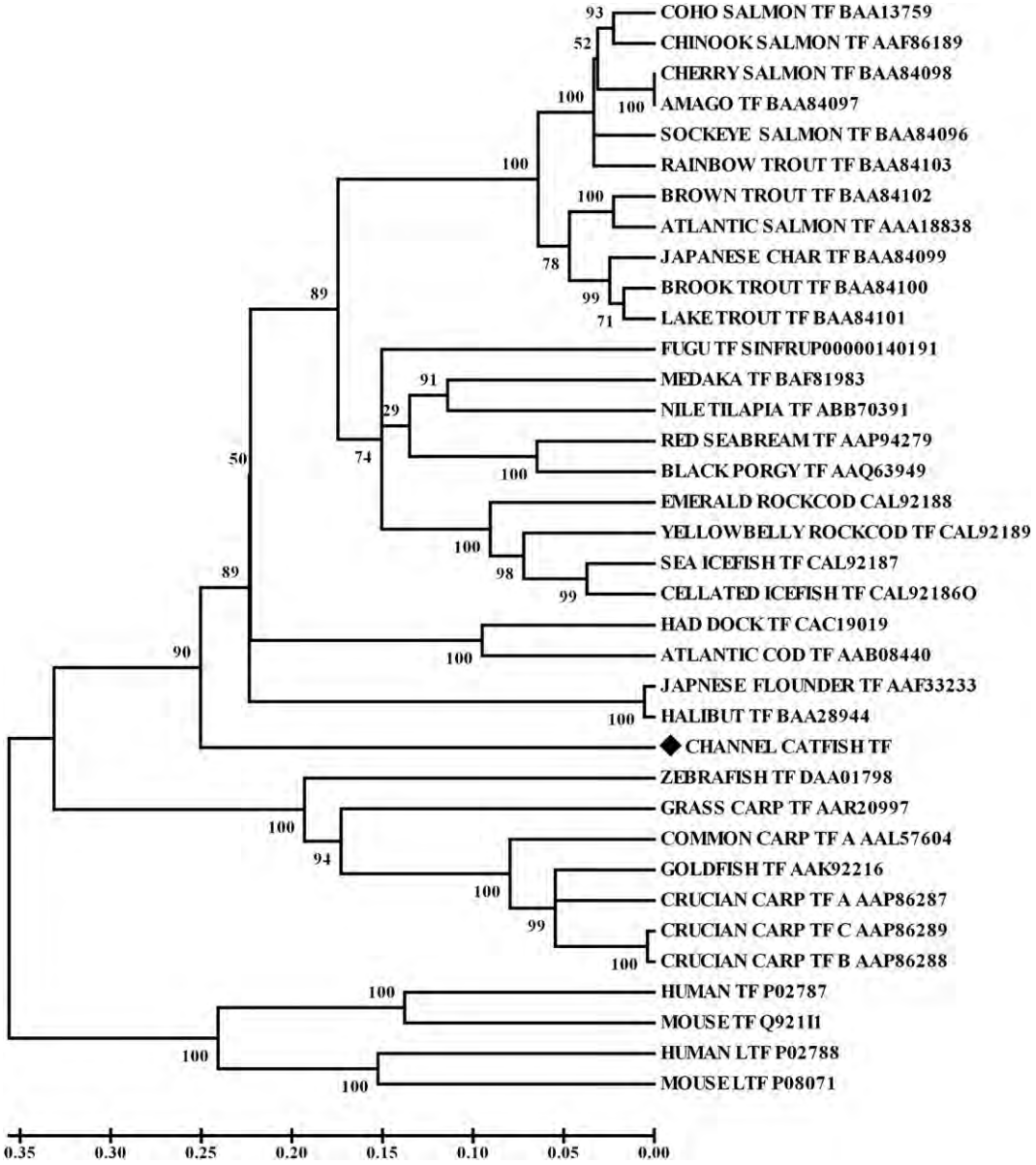

Fig. 2. Phylogenetic analysis of catfish and other vertebrate transferrin genes. The phylogenetic tree was constructed based on ClustalW-generated multiple sequence alignment of amino acid sequences using the neighbour-joining method within the MEGA 4 package. The topological stability of the neighbour-joining trees was evaluated by 10,000 bootstrapping replications, and the bootstrapping percentage values are indicated by numbers at the nodes.

catfish transferrin gene was obtained; the complete genomic sequence of channel catfish transferrin gene has been deposited to GenBank with the accession number of FJ176741.

Alignment of the genomic sequence with the cDNA sequence revealed the presence of 17 exons and 16 introns in the channel catfish transferrin gene, similar to that of known genes from three other fish species (zebrafish, medaka and *Fugu*), as well as to that of the serum transferrin gene of mammalian species (Fig. 3). The number of exons is completely conserved and the sizes of exons are similar among all the vertebrate transferrin genes. However, the gene size is highly divergent, with *Fugu* having the smallest gene size of approximately 5.4 kb and human having the largest gene size of 32.4 kb.

3.4. Determination of genomic copy number of transferrin

Southern blot analysis was conducted to determine the copy number of the transferrin gene in the channel catfish genome. As shown in Fig. 4, two bands were observed with restriction enzyme digestion using *Eco* R1 and *Hin*d III, and five bands were observed using *Pst* I. An examination of restriction sites within the genomic sequence indicated the presence of one *Hin*d III site, three *Pst* I sites (two of the three fall into the same intron), but no *Eco* R1 site within the genomic sequence. Therefore, if the genome contains a single copy gene, a single band was expected from *Eco* R1 digestion; two bands were expected from *Hin*d III digestion; and three bands were expected from *Pst* I digestion. As shown in Fig. 4, *Eco* R1 digestion produced two bands; *Hin*d III digestion produced two bands; and *Pst* I digestion produced five bands. Such restriction patterns highly suggested the presence of two copies of transferrin gene in the catfish genome. Of the six BAC clones positive for the transferrin gene probes, three (27_A7, 42_L13 and 75_F24) were mapped to the physical map of the catfish genome [42], and they all fell into the same contig, Contig 1311 [42], suggesting that both copies may reside in the same genomic region.

3.5. Tissue expression of the catfish transferrin gene

RT-PCR was used to determine tissue distribution of transferrin gene expression. As shown in Fig. 5, transferrin was expressed most abundantly in the liver, and also at a relatively high level in the stomach. It is expressed at low levels in the blood, brain, gill, head kidney, trunk kidney, heart, intestine, spleen, and ovary, and its expression was not detected in the muscle and skin tissues.

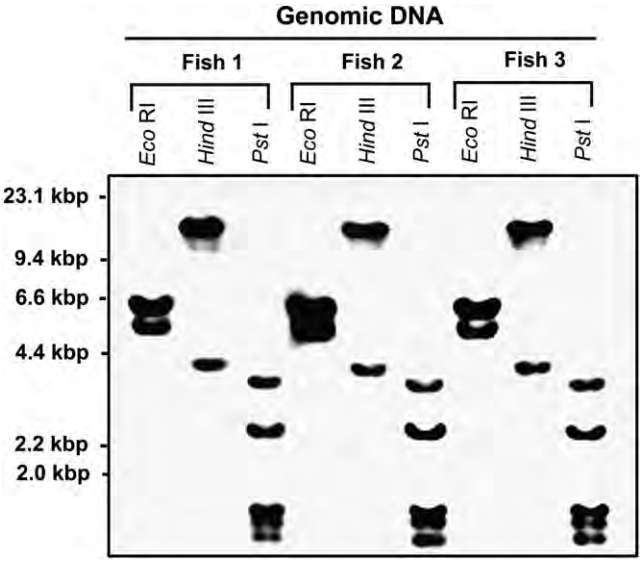

Fig. 4. Southern blot analysis of catfish transferrin gene. Southern blot analysis procedures are detailed in the Materials and Methods section. Genomic DNA of three individual fish was used as marked. Molecular weight standards (Kb) are indicated on the left margin.

3.6. Transferrin expression after bacterial infection and administration of iron-dextran

In the catfish group challenged with *E. ictaluri* injection, transferrin was up-regulated approximately 4.2 fold at 3 d and 1.7 fold at 7 d after infection in the liver. The expression of channel catfish transferrin was not significantly regulated by treatment with iron-dextran alone. In the group injected with both *E. ictaluri* and

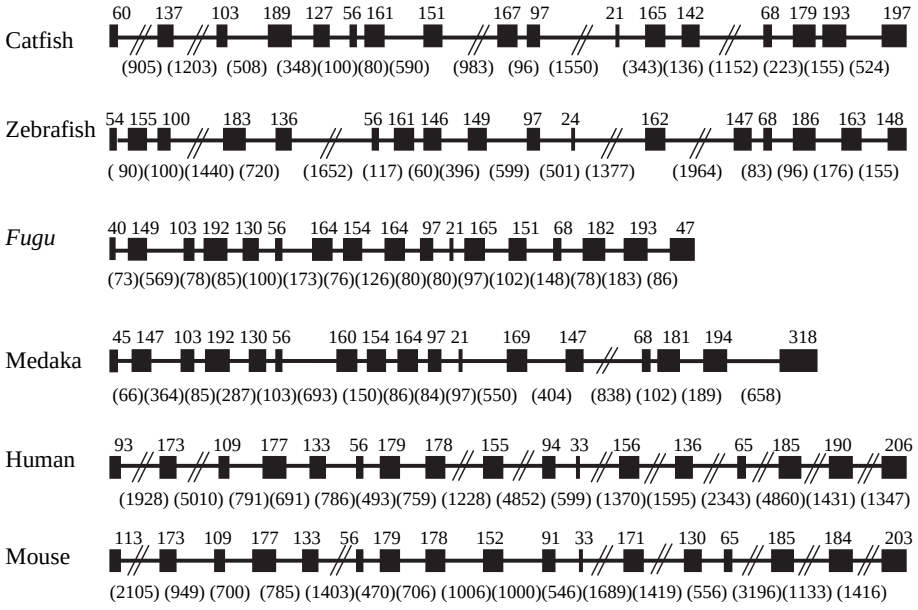

Fig. 3. Schematic diagram of the transferrin gene structure. Exons are represented by boxes. The numbers that are indicated on the top of boxes are the length of the exons in base pairs. The length of introns is indicated in the parentheses below each gene structure. Double slashes indicate non-proportional representation of the introns.

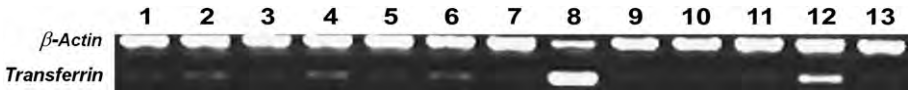

Fig. 5. RT-PCR analysis of catfish transferrin expression in various healthy tissues. RT-PCR products were analyzed on an agarose gel. The positions of the RT-PCR amplified bands of β-actin and the transferrin gene are indicated on the left margin. The tissues are as the following: 1, blood; 2, brain; 3, gill; 4, head kidney; 5, trunk kidney; 6, heart; 7, intestine; 8, liver; 9, muscle; 10, skin; 11, spleen; 12, stomach; 13, ovary.

iron-dextran, the transferrin gene was significantly up-regulated post-administration, and was more dramatically up-regulated than with *E. ictaluri* injection alone. Transferrin gene expression was up-regulated by 3.8 fold at 24 h, 4.7 fold at 3 d and 2.8 fold at 7 d after co-injection with bacteria and iron-dextran (Fig. 6).

4. Discussion

In this work, the channel catfish transferrin gene was identified, cloned, sequenced, and characterized. The catfish transferrin gene is highly conserved with those from other species with 17 exons and the typical iron-binding domains, suggesting that it has the necessary structural properties to serve as an iron transport protein. The catfish transferrin gene was highly expressed in the liver, and was significantly up-regulated after bacterial infection with *E. ictaluri* and after treatment with both of iron-dextran and *E. ictaluri*.

Phylogenetic analysis suggested that teleost fish likely have only the transferrin gene while mammalian species have serum transferrin and lactoferrin, which is believed to be duplicated from one ancestral gene [20]. Catfish transferrin was placed on the phylogenetic tree in a position reflecting some ambiguity regarding its ancestral origin. Additional transferrin sequences from more closely related Siluriform catfish in the future may help to more accurately assess the phylogenetic position of catfish transferrin. Placement of transferrin sequences into tight clades closely mirroring the evolutionary relationships of the fish species indicated rapid divergence of transferrin sequences following speciation.

In mammals, serum transferrin transports iron in physiological fluids and delivers it to cells, while lactoferrin scavenges iron, limiting its availability to invading microbes [20,21]. In oviparous vertebrates there is only one transferrin gene, expressed either in the liver as serum transferrin, or in the oviduct with a final localization in egg white as ovotransferrin [48,49]. For fish, transferrin seems to serve functionally as serum transferrin and lactoferrin, but the presence of melanotransferrin-like genes have also been reported in teleost species such as *Fugu* and tetraodon [50,51]. Melanotransferrins are much older, and the serum transferrin/melanotransferrin split may have occurred not long after lobe duplication (N-lobe and C-lobe) and all subsequent duplication events diverged from the serum transferrin gene [51]. A tBLASTn search against the channel catfish ESTs suggested the presence of melanotransferrin-like sequences in channel catfish as well. However, sequence alignments between these catfish ESTs and catfish transferrin revealed low levels (<39%) of sequence identity, making it unlikely that melanotransferrin was detected by the probe used in Southern blot analysis.

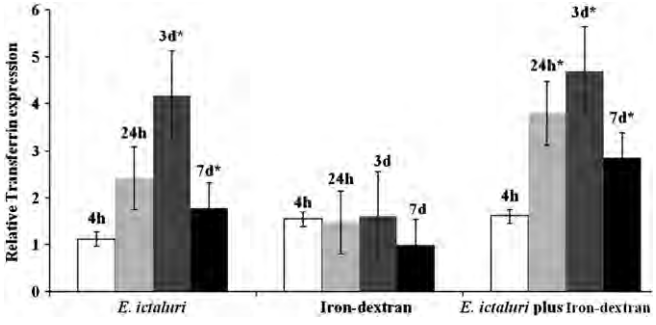

Fig. 6. Analysis of expression of the catfish transferrin after *E. ictaluri* infection and/or iron-dextran treatment in the liver using real time quantitative PCR. The samples were analyzed at 4 h, 24 h, 3 d, and 7 d post-treatment. Relative transferrin expression was expressed as fold change over control samples taken at the same time interval as normalized to change in expression in the 18S control. Asterisks indicate statistical significance at the level of $p < 0.05$.

Transferrin genes have been characterized by two lobes (N- and C-lobes) with four iron-binding residues and two anion sites within each lobe. The iron-binding residues at the C-lobe are more conserved than those in the N-lobe. Of the four iron-binding residues in the N-lobe, Asp-63 (human numbering without the 19 signal sequence) is the only amino acid that is invariant in all the 8 transferrin members (Fig. 1). At the other three positions, the catfish transferrin gene harbors the same amino acids as in the mammalian species with Tyr-95, Tyr-188, and His-249. However, amino acids in these positions are variable in grass carp [48,52], zebrafish, and goldfish. In humans, mutation at Tyr-95 and Tyr-188 caused a dramatic reduction and complete loss of iron-binding [53]. Base substitutions at the iron-binding sites in the N-lobe have also been reported in chicken and pufferfish melanotransferrins, and rat lactoferrin [50], suggesting the flexibility of these residues for iron binding in species other than human. In contrast, the amino acid residues at the iron-binding and anion sites in the C-lobe were all conserved (Fig. 1).

The channel catfish transferrin was found to be induced in the liver after bacterial infection, confirming earlier observations using microarrays [45,46]. Such infection-induced up-regulation was slightly magnified by the simultaneous treatment with iron-dextran. Similar results were recently reported [54] with a study on catfish intelectins. Transferrin appears to act as a positive acute phase protein in catfish in order to increase iron storage to make it unavailable for bacterial growth. When iron is deficient in blood serum, the iron is released from storage and thus an increase in transferrin expression is expected. In contrast, with an influx of iron, transferrin expression is expected to decrease [21]. However, in this study, transferrin expression was not significantly different in the iron-dextran treatment alone from the control, suggesting the influx of iron did not negatively regulate the expression of the transferrin gene as could be expected from other vertebrates. Similar results were also found in a recent study with sea bass, suggesting that basal levels of transferrin are sufficient to deal with the excess iron introduced in the system [33]. Further studies are need to better understand the crucial interplay between bacterial stimulation and iron level-dependent signaling in the expression and function of teleost iron homeostasis genes.

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