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Isolation of Novel Hepcidin Isoforms from the Rockbream *Oplegnathus fasciatus* (Perciformes)

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Three novel hepcidin isoforms were isolated and characterized from the perciform fish species *Oplegnathus fasciatus*. These hepcidin isoforms (designated *rbhepc5*, *rbhepc6* and *rbhepc7*) were found to share a conserved, tripartite gene structure and a considerable sequence homology one another. A comparison of their mature peptide sequences with those of other perciform hepcidin orthologs indicated that these three hepcidin isoforms as well as four other isoforms previously identified in this species, appear to belong to the HAMP2 group of hepcidin genes. Analysis of the 5′-upstream sequences showed that the proximal non-coding regions of *rbhepc5~7* do not possess canonical TATA signals; instead, they harbor several binding motifs for transcription factors involved in immune modulation. Reverse transcriptase-PCR analysis demonstrated that the *rbhepc5~7* are expressed predominantly in the liver, and that the transcription of *rbhepc5~7* is rapidly induced in the liver, but not in other tissues, by experimental challenge with any of three different bacterial species. However, transcription of *rbhepc6* appeared to be negligible under both basal and stimulated conditions, as judged by the redundancy count of randomly chosen reverse transcriptase-PCR clones.

Key words: Gene structure, Hepcidin isoforms, mRNA expression, Rockbream (*Oplegnathus fasciatus*)

Introduction

Hepcidin, also known as liver-expressed antimicrobial peptide (LEAP), is a cysteine-rich, antimicrobial, β-sheet protein that plays a central role in the homeostatic regulation of iron content (Park et al., 2001; Atanasiu et al., 2006). Although mammalian genomes contain only one hepcidin gene (with the exception of the mouse genome, which has two), many fish genomes possess multiple hepcidin gene isoforms; the number of hepcidin isoforms is variable among fish species (Shi and Camus, 2006). Species of the order Perciformes (perch-like fishes), one of the largest teleost orders, produce various hepcidin transcripts that might be decoded from each separate genomic copy (Rodrigues et al., 2006; Huang et al., 2007; Xu et al., 2008). The species with the highest number of hepcidin isoforms identified thus far is the black porgy (*Acanthopagrus schlegelii*, order Perciformes), which has seven unique isoforms (Yang et al., 2007).

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Darwinian selection driven by host-pathogen interaction (*e.g.*, adaptive evolution directed by pathogens when the host is exposed to new environments) has been suggested as being the force behind gene duplication forming the hepcidin multigene family (or cluster) in the fish genome (Padhi and Verghese, 2007; Xu et al., 2008). This hypothesis is supported by the results of several experimental studies demonstrating differential responses of hepcidin isoforms in a given species to different kinds of infectious agents (Huang et al., 2007; Cho et al., 2009). Considering this evidence, it is generally agreed that the evolutionary pathway of fish hepcidins is different from one common to mammalian hepcidins, and that isoform-specific regulation in response to environmental or physiological perturbations should be taken into account more importantly for fish hepcidins.

The rockbream *Oplegnathus fasciatus* (order Perciformes) is a commercially important and aquaculture-relevant food fish in Korea. Although its market demand has been gradually increasing, one of

the most significant hindrances to efficient production of this fish species has been frequent outbreaks of infectious diseases (Choi et al., 2006; Cho et al., 2009). For this reason, a better understanding of the immune function and defense system of this species is needed.

A potential strategy for improving the efficiency and capacity of *O. fasciatus* farming is the genetic manipulation of its hepcidin genes (see also Hsieh et al., 2010). We recently isolated four hepcidin isoforms (*rbhepc1* to *rbhepc4*) from this species and showed that, although these isoforms vary at only a few amino acid (aa) residues, they are differentially regulated in response to different types of biological stimulation (Cho et al., 2009). Because our genomic blot hybridization analyses strongly suggested that the *O. fasciatus* genome harbors other hepcidin gene copies in addition to the four identified isoforms, we carried out the present study to isolate and characterize these additional hepcidin gene copies. The ultimate aim was to gain deeper insight into the interactive orchestration or diversification of the complete set of hepcidin isoforms in rockbream.

Materials and Methods

Fish specimen and nucleic acid preparation

Rockbream juveniles (body weight 80-120 g) were purchased from a local farm and kept in 100 L laboratory tanks containing water maintained at 22±1°C throughout the experiment. Dissolved oxygen content was 5-6 ppm. A commercial pelleted flounder diet (Woosung Feed Corp., Daejeon, Korea) was fed during the 1-2 week acclimation period.

Genomic DNA was prepared from whole blood or caudal fin using the conventional SDS/proteinase K method followed by organic extraction and ethanol precipitation (Sambrook and Russell, 2001). The integrity and quantity of the prepared genomic DNA was confirmed by electrophoresis on ethidium bromide-stained 0.3%-agarose gels and spectrophotometry (Gene Quant II spectrophotometer; Pharmacia Biotech, Uppsala, Sweden). Total RNA from various tissues was purified using an RNeasy Midi Kit (Qiagen, Hilden, Germany) with DNase treatment, and the intact RNA was confirmed by determining the ratio of 28S:18S ribosomal RNA (rRNA) in a 1% MOPS/formaldehyde agarose gel.

Isolation of novel hepcidin genes

Based on the sequences of other teleost hepcidin genes, including *rbhepc1~4*, various reverse primers containing degenerate nucleotides at varying positions were synthesized. These primers were used for 5′-directional genome walking using a GenomeWalker Universal Kit (BD Biosciences Clontech, Mountain View, CA, USA) according to the manufacturer's instructions, and 196 clones from the genome walking experiments were sequenced to survey for the presence of hepcidin gene copies differing from *rbhepc1~4* in their structural characteristics. From first-round survey primers found to be effective for the isolation of novel hepcidin copies, a new pair of PCR primers [rbHEPC-pDE (5′-AGGG GACTGTACACCATTCACA-3′) and rbHEPC cons RV1 (5′-ACAACTGTTGGAGCAGGAATCC-3′)] was designed for the isolation of a continuous fragment containing the novel hepcidin genes. PCR amplification was performed using the Expand High Fidelity Plus PCR System (Roche Applied Science, Mannheim, Germany) in a thermal cycler set for one round of initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min; and one round of final elongation at 72°C for 5 min. The PCR products were purified using PCR Purification Kit (Bioneer, Daejeon, Korea), cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA), and transformed into *Escherichia coli* cells. The resulting clones were screened using blue/white screening with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) and isopropyl β-D-thiogalactopyranoside (IPTG) according to the directions provided by Promega. Of the positive clones, 96 were randomly selected for sequencing in both directions, and their continuous sequences were analyzed using sequence-editing software (Sequencher; Gene Codes Corp., Ann Arbor, MI, USA). Of these sequences, only those showing multiple hits were considered to be the novel hepcidin gene copies; sequences showing nucleotide changes only at wobble positions (in exons) or only at a few sites in non-coding regions were discarded. A total of three novel hepcidin gene copies, designated *rbhepc5~7*, were identified by this process.

Characterization of newly identified rockbream hepcidin isoform sequences

The sequences of the newly identified hepcidin isoforms were compared with those of other perciform orthologs using a BLAST search of the National Centers for Biotechnology GenBank (NCBI) database (http://ncbi.nlm.nih.gov/Blast.cgi). A multiple sequence alignment of mature hepcidin peptides was created using the ClustalW program (http://align.genome.jp/). Ggene structures (exon-intron organization) of the rockbream hepcidin isoforms were

compared with those of other teleost hepcidin genes compiled from GenBank. Additional potential fish hepcidin isoforms were identified by a BLAST/ Text search against the Ensembl genome database (http://www.ensembl.org/index.html) and a tBLASTn search against the NCBI database of non-human, non-mouse expressed sequence tags. Putative signal peptides were identified using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/), and potential propeptide cleavage sites were predicted using the ProP 1.0 Server (http://www.cbs.dtu.dk/services/ProP/). Theoretical molecular masses and isoelectric points (pIs) of the hepcidin isoforms were estimated using the ExPASy ProtParam Tool (http://www.expasy.org/tools/protparam.html). Putative transcription factor-binding sites in the 5′-upstream regions of *rbhepc5~7* were identified using the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html) and Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess).

Basal and tissue expression of *rbhepc5~7* transcripts

To examine the basal expression patterns of the newly identified rockbream hepcidin isoforms, we examined the tissue distribution of their mRNAs in normal healthy juveniles using reverse transcriptase (RT)-PCR. However, the high sequence similarity among the isoforms precluded the use of conventional RT-PCR for isoform-specific amplification. Therefore, two oligonucleotide primers [qRB hepc 567 2F (5′-CACTCACTTGAGACACCGAAG-3′) and qRB hepc567 1R (5′-CAGCAAAAGCGACAC GTAAT-3′); amplicon size, 269 bp] designed to bind *rbhepc5~7* but not *rbhepc1~4* were used for the RT-PCR analysis. A different pair of primers [qRB hepc1234 1F (5′-AGCTGACAMGAGTCACCAAA A-3′) and qRB hepc1234 1R (5′-RCAGCAGCCG CAGCAAAATTT-3′); amplicon size, 305 bp] was designed to exclusively amplify the isoforms *rbhepc1* to *rbhepc4*. These two sets of RT-PCR primers were used to compare the basal expression and tissue distribution patterns of the newly and previously identified groups of hepcidin isoforms.

Total RNA was prepared from brain, eye, fin, gill, heart, intestine, kidney, liver, muscle and spleen tissues as described above, and 2 μg of the purified total RNA from each tissue was reverse-transcribed into cDNA using an Omniscript Reverse Transcription Kit (Qiagen) according to the manufacturer's recommendations. A reverse primer for the rockbream 18S rRNA [RB18S RV (5′-AGAATTTCACC TCTAGCGGC-3′)] was included in the reverse transcription reactions to allow for normalization as described by Lee and Nam (2009). One microliter of the resulting cDNA templates was PCR-amplified in a thermal cycler programmed for one round of initial denaturation at 94°C for 3 min and 26 cycles (for isoforms *rbhepc1~4*) or 35 cycles (for isoforms *rbhepc5~7*) of 94°C for 20 s, 58°C for 20 s, and 72°C for 20 s. A 5 μL aliquot of amplified product was electrophoresed on a 1.5% agarose gel, visualized by ethidium bromide staining, and assessed using Quantity-One image analysis software implemented on a VersaDoc 4000 imaging system (both from Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amount of input cDNA in each RT-PCR reaction was normalized to the 407 bp control rockbream 18S rRNA fragment amplified from each tissue sample using the primers RB18S 1F (5′-TACCACATCCAA GGAAGGCA-3′) and RB18S 1R (5′-TTCCTAGC TGCGGTATTCAG-3′). The thermal cycling conditions for amplification of the 18S rRNA gene were the same as used for the hepcidin isoforms, except that 20 cycles were used and the cDNA template was diluted 1:5 before use in the reaction.

Bacterial challenge and induced expression assay

To examine the induction of the newly identified hepcidin isoforms in response to inflammatory stimuli, we performed experimental challenges with non-pathogenic and pathogenic bacteria. Healthy juvenile fish (n=8 per group) were injected intraperitoneally with a suspension of 1×10^5 *E. coli* (Gram-negative; strain XL1 Blue MRF′; Stratagene), *Edwardsiella tarda* (Gram-negative; FSW910410), or *Streptococcus iniae* (Gram-positive; JSL0108) cells suspended in phosphate-buffered saline (pH 6.8). The bacterial cell count was estimated from the optical density (OD) at 600 nm of the bacterial culture in log phase. As a control, one group was injected with phosphate-buffered saline alone. At 48 h post-injection, the liver, intestine, kidney and spleen were surgically removed from six randomly chosen individuals per group for RT-PCR analysis using the same methods for total RNA and cDNA template preparation, thermal cycling parameters, and oligonucleotide primers as those used in the tissue distribution assay. Expression of hepcidin iso-forms in response to bacterial challenge was assessed using Quantity-One software (Bio-Rad Laboratories, Inc.).

To determine whether bacterial challenge would induce the expression of every hepcidin isoform, we prepared and sequenced RT-PCR clones from the

livers of *S. iniae*-challenged fish. Three independent RT-PCRs were carried out using separate cDNA samples from the injected fish, and the triplicate amplification products were pooled, cloned into the pGEM-T Easy Vector (Promega), and transformed into *E. coli* cells. Of the resulting recombinant clones, 96 were randomly selected for sequencing using SP6/T7 sequencing primers (Promega), and the incidence profile was assessed by counting the redundant clones for each isoform.

Results

Structure of newly identified rockbream hepcidin isoform genes

Our analysis showed that in addition to the four hepcidin isoforms we previously described (*rbhepc1~4*), the rockbream genome contains three other hepcidin isoform genes, designated *rbhepc5*, *rbhepc6*, and *rbhepc7* (Fig. 1). These three novel hepcidin genes have identical, tripartite genomic structures (three exons separated by two introns) in which exons I, II, and III are 87, 78, and 93 bp in length, respectively, and introns I and II are 94 and 160 bp in length, respectively. The isoforms encoded by *rbhepc5~7* are highly similar in sequence and, at 86 aa in length, are 2 aa shorter than the isoforms encoded by *rbhepc1~4* (88 aa). A multiple sequence alignment revealed that the peptide region corresponding to exon II of *rbhepc5~7* lacks a Gln-Gln sequence found at the beginning of the peptide region corresponding to exon II of *rbhepc1~4*.

Comparison of the *rbhepc5*, *rbhepc6*, and *rbhepc7* sequences revealed a single, silent variation in the nucleotide sequence of exon I. The exon II sequence was found to vary at two positions causing changes in amino acid residues 43 and 44; whereas *rbhepc5* and *rbhepc7* encode Ala^{43}-Val^{44}, and the *rbhepc6* sequence encodes Val^{43}-Ala^{44}. Finally, exon III of the three isoforms encodes variant amino acids at positions 67 (Met↔Thr), 76 (Gly↔Arg), 78 (Ser↔Asp↔Glu), 79 (Ile↔Val), and 82 (Leu↔Pro).

Multiple sequence alignment of mature hepcidin isoforms

Based on ProP 1.0 Server predictions of arginine and lysine propeptide cleavage sites in Perciformes hepcidin sequences, we generated the putative mature hepcidin peptide sequences of perciform hepcidin orthologs and aligned them with the putative mature peptides encoded by *rbhepc5~7* (Table 1; Fig. 2). Redundant mature sequences from a given species were eliminated, as were sequences that did not receive a sufficient probability score for the pro-protein cleavage site, leaving 48 peptide sequences from 24 species. According to the classification described by Hilton and Lambert (2008), the matured perciform hepcidin peptides could be divided into two large clusters [hepcidin antimicrobial peptide (HAMP) clusters 1 and 2], and the HAMP2 cluster could be divided again into two groups (Groups I and II) based on the number of fully conserved cysteine residues (eight Cys residues for Group I and four for Group II). Finally HAMP2 Group I could be further divided into three subgroups (Groups Ia, Ib, and Ic) based on their overall sequence homology. All of the rockbream hepcidin isoforms (*rbhepc1~7*) were shown to belong to Group Ia in HAMP2.

Without exception, the mature HAMP1 perciform hepcidins (n=16) are identical in length (26 aa) and are positively charged, with isoelectric points (pI) ranging from 8.50 to 8.94. Furthermore, all are characterized by the presence of four highly conserved N-terminal amino acids (Gln-Ser-His-Leu/Ile). On the other hand, the lengths and pI values of the mature HAMP2 perciform hepcidins (n=32) vary considerably among and within species. The HAMP2 Group Ia peptides are all 22 aa in length and positively charged, with pI values ranging from 7.70 (ACD13024 isoform from *Micropterus salmoides*) to 8.97 (OFA4; *rbhepc4* from *O. fasciatus*). The Group 1b subgroup (n=8) varies widely in pI, ranging from 4.21 (AAU00801 from *A. schlegelii*) to 8.53 (AAZ-95008 from *A. schlegelii*). With the exception of those from the bass species *Dicentrarchus labrax* and *Morone chrysops*, all of the isoforms in Group Ib are 24 aa in length. For the *D. labrax* and *M. chrysops* active sequences, the ProP Server predicted a length of 26 aa, but a hepcidin peptide actually isolated from *M. chrysops* gills has been reported as only 21 aa in length (Shike et al., 2002). The third subgroup, Group Ic (n=5) contains peptides of 22-24 aa with cationic pI values ranging from 7.71 to 8.97, and all are from the suborder Notothenioidei. With the exception of one isoform (ABY84831 from *Notothenia angustata*), all Group Ic peptides have two positively charged amino acids (Arg-Lys) at their mature N-termini. Finally, all of the HAMP2 Group II peptides also belong to the suborder Notothenioidei. They have only four conserved cysteines, are 22 aa in length, and have notably high pI values (ranging from 10.36 to 11.63) relative to other groups.

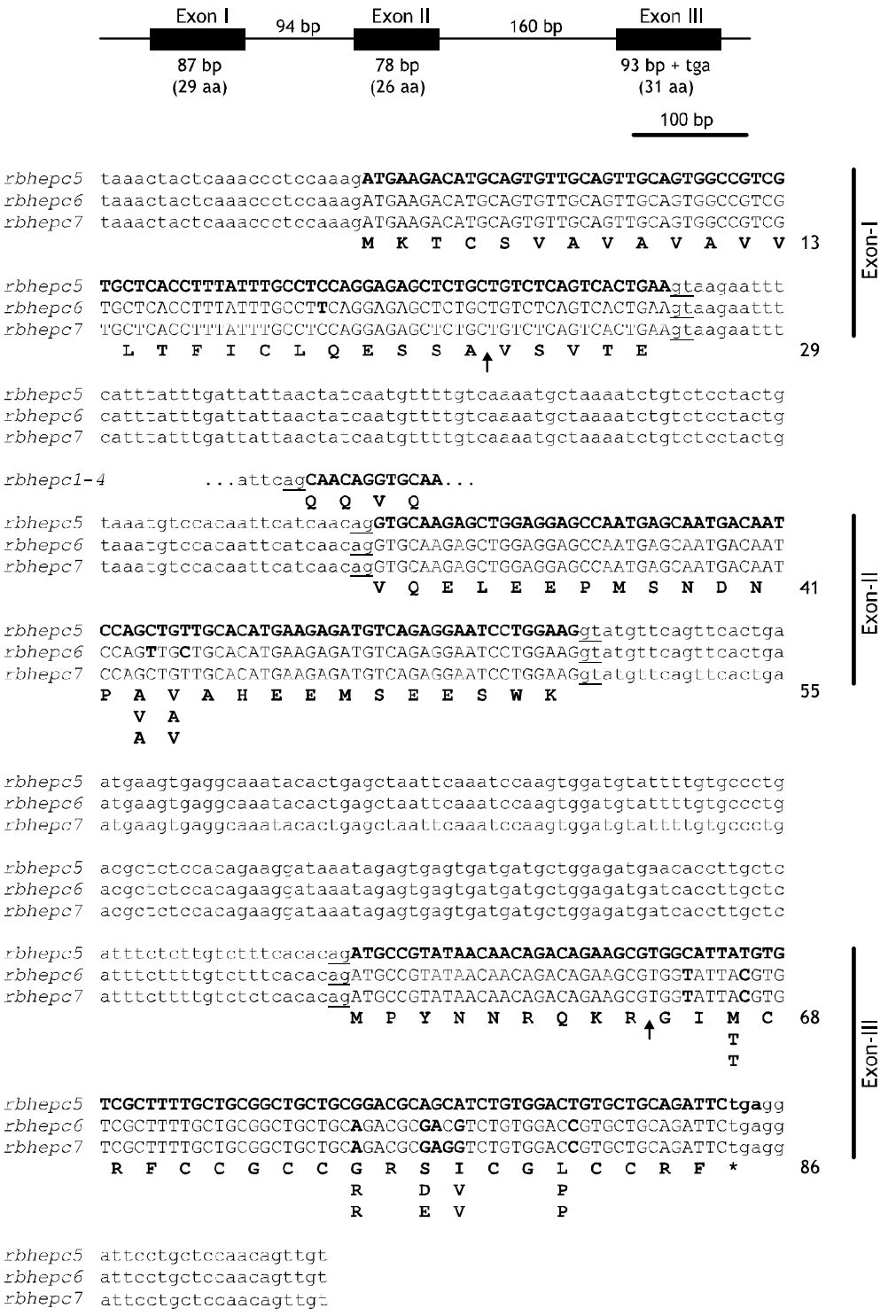

Fig. 1. Gene structure of three hepcidin isoforms (*rbhepc5~7*) newly identified from the *Oplegnathus fasciatus*. Coding sequences are noted by uppercase letters while non-coding region by lower case letters. Stop codon (tga) was noted by an asterisk. Exon-intron splicing sites (gt/ag) were underlined. Numbers of nucleotides and amino acids are indicated in the upper schematic diagram. Potential sites for preproprotein and proprotein cleavages are noted by vertical arrows. The two glutamines (Q-Q) missed in the exon-II of *rbhepc5~7* were noted in the partially aligned *rbhepc1~4* sequence. The sequences of *rbhepc5~7* are also available at GenBank under the accession numbers, JF419520 to JF419522, respectively.

Table 1. Abbreviations and taxonomic positions for perciform species used in the multiple sequence alignment of predicted mature hepcidin sequences

Suborder	Family	Species	Abbreviations
Percoidei	Centrarchidae	*Micropterus dolomieu*	MDO
		Micropterus salmoides	MSA
	Moronidae	*Dicentrarchus labrax*	DLA
		Lateolabrax japonicus	LJA
		Morone chrysops	MCH
	Oplegnathidae	*Oplegnathus fasciatus*	OFA
	Percidae	*Perca fluviatilis*	PFL
	Sciaenidae	*Larimichthys crocea*	LCR
	Sinipercidae	*Siniperca chuatsi*	SCH
	Sparidae	*Acanthopagrus schlegelii*	ASC
		Lithognathus mormyrus	LMO
		Pagrus auriga	PAU
		Pagrus major	PMA
		Sparus aurata	SAU
Notothenioidei	Artedidraconidae	*Pogonophryne scotti*	PSC
	Bathydraconidae	*Gymnodraco acuticeps*	GAC
	Channichthyidae	*Chaenocephalus aceratus*	CAC
	Eleginopidae	*Eleginops maclovinus*	EMA
	Nototheniidae	*Dissostichus mawsoni*	DMA
		Notothenia angustata	NAN
Carangoidei	Carangidae	*Seriola quinqueradiata*	SQU
Gobioidei	Gobiidae	*Gillichthys mirabilis*	GMI
Labroidei	Cichlidae	*Oreochromis niloticus*	ONI
Zoarcoidei	Zoarcidae	*Lycodichthys dearborni*	LDE

Characteristics of 5′-upstream sequences

The sequences of the 5′-upstream regions of the newly isolated *rbhepc5~7* isoforms are nearly identical (Fig. 3). Nucleotide variations were detected at only eight positions in the 683-bp sequence. Bioinformatics analysis identified binding sites or motifs for various transcription factors that might be involved in the modulation of these hepcidin isoforms. Most of these transcription factors, such as signal transduction and activation of transcription (STAT), nuclear factor kappa B (NF-κB/c-Rel), GATA factor, hepatocyte nuclear factor (HNF), and CCAAT/ enhancer-binding protein (C/EBP), have previously been shown to play important roles in vertebrate inflammatory responses or innate immunity. However, none of the 5′-upstream regions of *rbhepc5~7* were found to contain the canonical TATA box (TATAAA).

Basal and induced expression of hepcidin isoforms

Our RT-PCR analyses showed that *rbhepc5~7* transcripts are expressed predominantly in the liver and are expressed in barely detectable amounts in fin, gill, and muscle tissue (Fig. 4). We were unable to detect any *rbhepc5~7* transcripts in other tissues, including brain, eye, heart, intestine, kidney, and spleen. This expression pattern is clearly different from that of *rbhepc1~4* transcripts, which were found at high levels in the liver but were also abundant in the spleen and kidney and were readily detectable in all tissues tested except brain.

Isoforms *rbhepc5~7* also differed from isoforms *rbhepc1~4* in their transcriptional response to bacterial challenge (Fig. 5). Our semi-quantitative RT-PCR analyses showed strong transcriptional activation of *rbhepc1~4* in the four tissue types tested (intestine, kidney, liver and spleen), although the degree of activation appeared to depend on the specific tissue type and bacterial species used. In contrast, transcriptional activation of *rbhepc5~7* in response to bacterial challenge was observed only in the liver. When the incidence profile of the *rbhepc5~7* isoforms was assessed by sequencing 96 RT-PCR clones from livers of *S. iniae*-challenged fish, most of the redundant clones were either *rbhpec5* (39%) or *rbhepc7* (60%), whereas *rbhepc6* clones were scarce (1%).

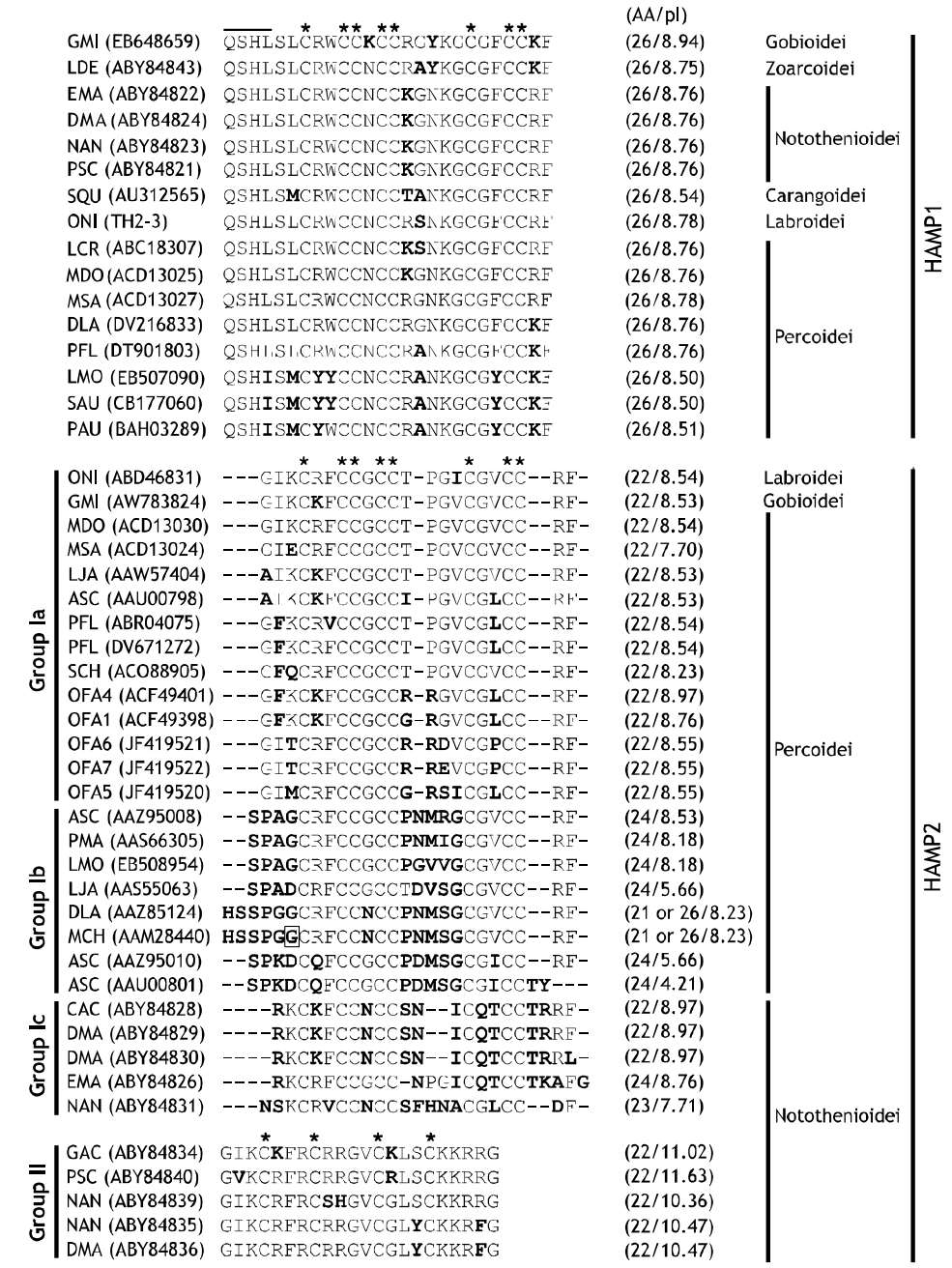

Fig. 2. Multiple sequence alignment of mature hepcidin peptides from representative perciform species. Potential propeptide cleavage sites were predicted using the ProP 1.0 Server (http://www.cbs.dtu.dk/services/ProP/), in which the perciform sequences showing no clear cleavage site were not included in this alignment. Abbreviations for species are referred to Table 1, and the accession code for each sequence was noted in parenthesis. The accession code for *Oreochromis niloticus* (ONI; TH2-3) was the label for one of tilapia hepcidin isoforms named by Huang et al. (2007). Perciform hepcidins were divided into two large clusters (HAMP1 and HAMP2; Hilton and Lambert, 2008), and the HAMP2 cluster was then further divided into several subgroups based on the number of conserved Cys residues (indicated by asterisks) and sequence homology. The conserved motif (QSHL/I) found in amino terminal of HAMP1 peptides was upperlined. In the HAMP2-Group Ib, it should be noted that the hepcidin peptide empirically isolated from the gill of *Morone chrysops* (Shike et al., 2002) was proven to have the Gly (boxed) as the first N-terminal amino acid, which was different from the prediction. The number of amino acid and theoretical pI value for each predicted peptide sequence were noted in parenthesis at the end of each sequence.

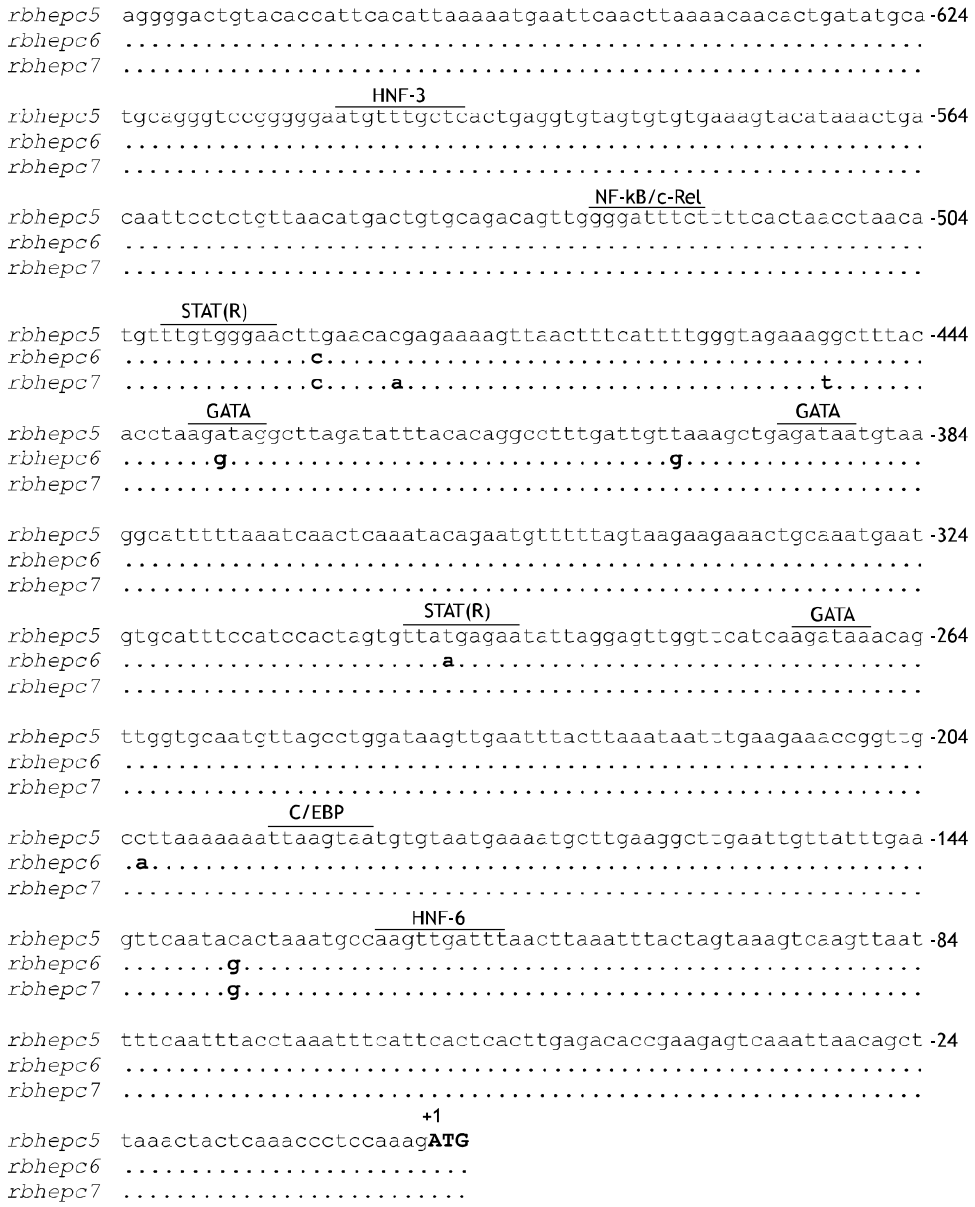

Fig. 3. Alignment of 5′-upstream sequences from the three rockbream hepcidin isoforms (*rbhepc5~7*) to show putative transcription factor binding motifs (upperlined). The binding sequences in a reverse orientation are noted by (R).

Discussion

The genomes of perciform fish species have been shown to harbor multiple hepcidin gene isoforms, many of which exhibit different structural and functional characteristics (Huang et al., 2007; Cuesta et al., 2008; Cho et al., 2009). We recently identified four hepcidin isoforms (designated *rbhepc1~4*) from the perciform species *O. fasciatus* (rockbream). In the present study, we isolated and characterized three additional hepcidin isoforms (designated *rbhepc5~7*) from the same species. This result suggests that *O. fasciatus* has at least seven genomic copies of hepcidin, similar to *A. schlegelii*, a species belonging to the same suborder Percoidei (Yang et al., 2007). Although the seven genomic isoforms were consistently detectable in both wild and farmed rockbream (data not shown), the possibility of creating allelic differences in various stocks through breeding programs, including artificial parthenogenesis, should be further examined in the future.

The *rbhepc5~7* and *rbhepc1~4* isoforms share the

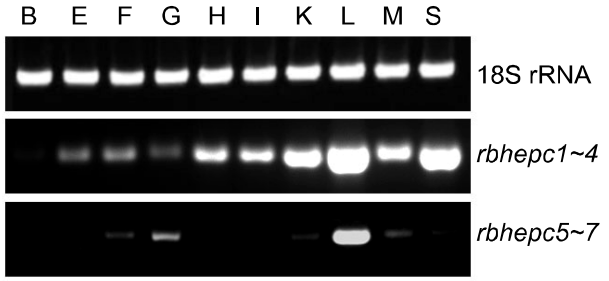

Fig. 4. Representative RT-PCR gels showing the tissue distribution of rockbream hepcidin isoform transcripts (*rbhepc5~7*) in comparison with that of *rbhepc1~4* isoforms. Abbreviations for tissues are brain (B), eye (E), fin (F), gill (G), heart (H), intestine (I), kidney (K), liver (L), muscle (M) and spleen (S). Note that transcripts of *rbhepc5~7* isoforms were vastly predominant in liver only whereas *rbhepc1~4* isoforms were detectable in a wide array of tissue types. A gel showing the normalization control (18S rRNA) to validate no notable variation of the input cDNA amount across samples is also shown at top.

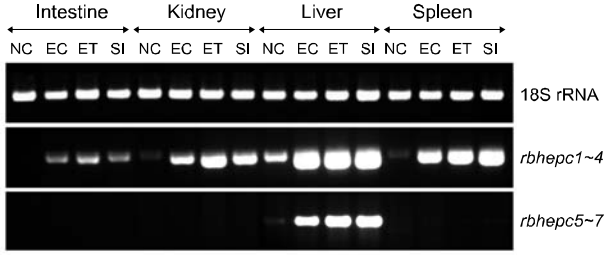

Fig. 5. Transcriptional activation of rockbream hepcidin isoforms in different tissues resulting from the experimental challenge with *Escherichia coli* (EC), *Edwardsiella tarda* (ET) or *Streptococcus iniae* (SI). A negative control group (non-challenged; NC) was prepared by injecting only the medium for bacterial suspension, phosphate buffered saline (PBS). Based on the preliminary experiments, RT-PCR conditions for the semi-quantitative amplification of *rbhepc1~4* and *rbhepc5~7* isoforms were optimized. Note that the transcriptional response of *rbhepc1~4* was readily observable in all the tissues, while that of *rbhepc5~7* was liver-exclusive.

tripartite genomic structure that is a conserved feature of all known vertebrate hepcidin genes (Douglas et al., 2003; Shike et al., 2004; Ren et al., 2006). However, in contrast to the *rbhepc1~4*-encoded peptides, which are 88 aa in length, the *rbhepc5~7*-encoded peptides are 86 aa in length, lacking two glutamines encoded in exon II of *rbhepc1~4* (Fig. 1). Whereas the nucleotide sequence at the intron I/exon II junction in *rbhepc1~4* is -att-cag-CAA-CAG-GTG-CAA- (-Gln-Gln-Val-Gln-), it is -att-cat-caa-cag-GTG-CAA- (-Val-Gln-) in *rbhepc5~7*. A single nucleotide change from guanine in *rbhepc1~4* to thymine (boxed above) in *rbhepc5~7* might shift the ag intron/exon splicing site so that the CAA-CAG codons encoding Gln-Gln in *rbhepc1~4* are recognized as an intronic sequence in *rbhepc5~7*. However, it should be noted that Cho et al. (2009) have reported the occurrence of *rbhepc1~4*-like transcripts encoding 86 aa peptides lacking the two glutamines, suggesting that misrecognition of the codon for the second Gln (CAA-CAG) in *rbhepc1~4* as an ag splicing site might result in alternative splicing of at least a certain portion of *rbhepc1~4* transcripts. The importance of the Gln-Gln sequence of uncharged polar amino acids in the function of preproproteins or proproteins is not yet understood.

A comparison of the sequences of mature perciform hepcidin peptides allowed us to categorize each peptide into either the HAMP1 or HAMP2 cluster (for nomenclature, see Hilton and Lambert, 2008). Within these clusters, the amino acid sequence homology among the mature peptides is broadly in accordance with the known taxonomic appraisal of the various species at the suborder level. Perciform hepcidins in the HAMP1 cluster share a predicted 24-aa signal peptide and a conserved Arg/Lys propeptide cleavage recognition site (RXRR or RXKR), consequently generating mature peptides of identical lengths (26 aa). Without exception, all of the HAMP1 peptides contain eight conserved Cys residues and have a conserved N-terminal motif (Gln-Ser-His-Leu/Ile-Ser).

The perciform HAMP2 cluster can be divided into two groups (I and II) based on the number of conserved Cys residues, and Group I can be further divided into three subgroups (a, b, and c) based on sequence homology and the amino acids N-terminal to the first conserved Cys residue. The predicted mature peptides of both the *rbhepc5~7* and *rbhepc1~4* isoforms belong to the same subgroup (Group Ia). Whereas most mature HAMP1 peptides possess six amino acids (including the QSHL/I signature) prior to the first conserved Cys residue, most mature HAMP2 peptides are predicted to possess only two, three, or four aa in this region (although for most HAMP2 hepcidins, these predictions need to be confirmed by typing of the actual active peptides) (Shike et al., 2002; Lauth et al., 2005; Valore and Ganz, 2008).

It is widely agreed that the net positive charge of

antimicrobial peptides is functionally important in their facilitated interactions with negatively charged microbial surfaces, and that charge-altering amino acid substitutions are expected to be important in their evolution (Tossi et al., 2000; Tennessen, 2005). Experiments with mammalian HAMP1 peptides have indicated that the overall charge of the first five N-terminal amino acids of a mature hepcidin peptide is important for its primary function in iron regulation (Nemeth et al., 2004). As in mammalian HAMP peptides, the five N-terminal amino acids of all mature perciform HAMP1 peptides have an overall negative charge (pI of 6.74). However, the majority of HAMP2 peptides, including the rockbream hepcidins, have an overall positive charge in this region (pI of 8.25-10.86). For this reason, the overall negative charge of the N-terminus of a mature HAMP1 peptide has been proposed to be an essential requirement for its interaction with ferroportin molecules, a crucial step in the hepcidin-mediated regulation of iron homeostasis (Nemeth et al., 2006). Moreover, the results of a recent molecular phylogeny study led to the hypothesis that every fish species has a HAMP1 ortholog of mammalian hepcidin that carries out dual functions (*i.e.,* iron regulation and immune response), while the HAMP2 genes also present in certain fish species may serve only to support innate immunity but not iron regulation, since the mature HAMP2 isoforms have cationic N-terminal regions (Hilton and Lambert, 2008).

We suggest that this hypothesis merits careful revision or challenge. First, HAMP1 genes may not be present in all perciform species. Although the complete genome sequences of *O. fasciatus* and *A. schlegelii* are not yet available, none of the *A. schlegelii* or *O. fasciatus* hepcidin isoforms we examined proved to be a HAMP1 ortholog; despite extensive searching against rockbream transcriptomes obtained from various stimulated tissue samples, no HAMP1 isoforms were recovered (data not shown). Second, some mature perciform HAMP2 peptides (*e.g.*, belonging to Group Ib defined in this study) might have negatively charged N-terminal regions like those of HAMP1 peptides, although this speculation is based on sequence-based prediction of the mature sequences, rather than physical analyses. Third, at least some perciform HAMP2 isoforms predicted to have positive N-terminal charges in their mature forms might be involved not only in innate immunity but also in iron regulation, as evidenced by the rapid activation of their transcription by iron overload (Huang et al., 2007; Cho et al., 2009). Thus, the diversification pattern of perciform hepcidins may belie the suggestion that duplicated fish HAMP2 genes function only in innate immunity.

Unlike *rbhepc1~4* transcripts, which exhibit nearly ubiquitous tissue distribution, the vast majority of the *rbhepc5~7* gene transcripts were detected only in the liver, although a very low amount of transcription was observed in several non-liver tissue (see also Shike et al., 2002; Douglas et al., 2003). However, even in the liver, the basal expression level of *rbhepc5~7* seemed to be significantly lower than that of *rbhepc1~4*. Although we did not perform a quantitative comparison of the two isoform groups, RT-PCR detection of *rbhepc5~7* transcripts required many more amplification cycles than the number needed to detect *rbhepc1~4* transcripts. This low level of basal expression for *rbhepc5~7* might be related to our finding that the proximal 5′-upstream regions of these isoforms do not contain canonical TATA boxes (see also Bao et al., 2005), although other possible factors affecting their transcriptional activity should also be explored (see also Truksa et al., 2007). In addition, genome walking into the distal 5′-upstream regions and actual typing of the transcription initiation sites for the *rbhepc5~7* isoforms might provide valuable insights into the structural and functional features of their regulatory mechanism. Meanwhile, the liver-predominant expression of the *rbhepc5~7* transcripts is consistent with the presence of several predicted binding motifs for liver-enriched transcription factors, such as HNF-3, HNF-6, and C/EBP, in their 5′-upstream regions (Courselaud et al., 2002). Other transcription factors, particularly STAT and NF-κB, are important in the Toll signaling pathways involved in innate immunity, consistent with the inflammation-mediated modulation of hepcidin genes (Anderson, 2000; Wrighting and Andrews, 2006).

Transcriptional response of the *rbhepc5~7* isoforms to bacterial challenge was clearly liver-specific; no induction was observed in any tissue except liver. In contrast, bacterial challenge induced transcription of the *rbhepc1~4* isoforms in all tissues tested, and the degree of induction in non-liver tissues, was comparable to that found in the liver. Our experimental set-up did not allow direct comparison of the degrees of induction of *rbhepc1~4* vs. *rbhepc5~7* in the liver because similar amplification conditions could not be used for the two groups owing to the great difference in their basal expression levels. Nevertheless, as judged solely by the end-point PCR results, the *rbhepc5~7* group appeared to be induced to a degree comparable to that of

rbhepc1~4.

Differential inducibility of hepcidin isoforms across tissue types under stimulated conditions has been reported in many fish species of the Perciformes superorder Acanthopterygii, which is known for its many species exhibiting multiple hepcidin genes (for review, see Hilton and Lambert, 2008). However, the mechanism responsible for the tissue-specific modulation of hepcidin isoform(s) has not yet been com-prehensively studied. In our experiments, redundancy counting of RT-PCR clones of hepatic *rbhepc5~7* transcripts from *S. iniae*-challenged rockbream re-vealed that *rbhepc7* or *rbhepc5* account for most of the transcripts, whereas the *rbhepc6* transcript was barely detectable. We were unable to detect *rbhepc6* transcripts in the liver of normal, non-challenged rockbream individuals (data not shown), suggesting that *rbhepc6* is a non-expressed gene copy. Similarly, one of the five hepcidin gene copies in the genome of the winter flounder (*Pseudopleuronectes americanus*; Pleuronectiformes) was previously reported to pro-duce no detectable transcripts in developing embryos or adult tissues (Douglas et al., 2003). However, it is possible that *rbhepc6* transcription is activated under stimulatory conditions other than bacterial infection, as a dynamic change in hepcidin profiles, depending on different stimulatory treatments, has also been reported in this species (Cho et al., 2009). For this reason, more extensive analysis using various experimental forms of stimulation, including iron overload and viral infection, would be a valuable future research subject. In addition, genome walking to a more distal 5′-region might be useful in exploring the potential factors responsible for the difference in the transcriptional activity of *rbhepc6* vs. *rbhepc5/7*.

In summary, we have isolated three novel hepcidin isoform genes (*rbhepc5~7*) from *O. fasciatus* (Perciformes). These genes share a conserved gene structure and predicted mature peptide form with other perciform HAMP2 orthologs. Unlike the previously identified rockbream hepcidin isoforms, *rbhepc5* and *rbhepc7* are expressed predominantly in the liver and are transcriptionally induced exclusively in the liver in response to bacterial challenge. However, *rbhepc6* might be minimally expressed under stimulated as well as basal conditions.

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