Genomic organization and tissue-specific expression analysis of hepcidin-like genes from black porgy (*Acanthopagrus schlegelii* B.)[☆]

Ming Yang, Ke-Jian Wang[*], Jun-Hui Chen, Hai-Dong Qu, Shao-Jing Li

State Key Laboratory of Marine Environmental Science, College of Oceanography and Environmental Science, Xiamen University, Xiamen, Fujian 361005, China

Abstract

Hepcidin is an antimicrobial peptide and putative iron regulatory hormone previously described in mice and humans. Dozens of fish hepcidins have been isolated and characterized so far. Here we present seven hepcidin-like cDNA sequences named AS-hepc1−7, amplified from the normal commercially cultured fish (black porgy) by RACE−PCR. Sequence analysis reveals that these seven potential hepcidin peptides have highly conserved sequences with other known hepcidins, but they are different from each other in constitution and characteristics of predicted mature amino acids. Based on the study, it is deduced that AS-hepc1−7 represent different variants of a family of hepcidin genes in black porgy. To understand the organization of these hepcidin-like genes, we sequenced AS-hepc2 DNA, AS-hepc3 DNA, AS-hepc4 DNA, AS-hepc7 DNA and AS-hepc2 upstream region; and all of the four genomic DNAs consisted of two introns and three exons, the same organization as other reported hepcidins. The tissue-specific gene expression of hepcidins in normal black porgy was evaluated using RT−PCR and dot blot approaches. RT−PCR showed that transcripts of hepcidin-like mRNAs were present in each tested tissue of normal juvenile black porgy, including liver, spleen, kidney, heart, brain, stomach, intestine, gill, skin and blood, but abundant hepcidin-like mRNA transcripts were only detected in the liver, kidney, spleen, intestine and stomach by dot blot assay. In addition, using dot blot and Northern blot approach, a significant increase of hepcidin mRNA transcription was observed in the liver within 48 h after immersion in a suspension of live bacteria, which suggested that the expression pattern of hepcidin-like genes in black porgy might be different in the liver from the other tissues as previously reported in several hepcidin studies.
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1. Introduction

Hepcidin peptide was first identified from human [1,2] and presumed to be an antimicrobial peptide primarily expressed in the liver of mammals. In fishes, hepcidin genes have been cloned and sequenced from hybrid striped bass [3], winter flounder, Atlantic salmon [4], zebrafish [5], catfish [6], Japan sea bass [7], and others. They have been predicted also in expressed sequence tag databases from medaka, rainbow trout, Japanese flounder [8], long-jawed mudsucker [9], and by analysis of a cDNA library for red seabream [10]. These observations imply that hepcidin might be widely spread in fishes [4]. Bass hepcidin peptide was the first isolated and its gene expression was predominantly in the liver after bacterial challenge [1], which corresponded with observations on the earlier identified mammalian hepcidin from human [1,2] and mice [11]. However, arguments against hepcidin being predominantly expressed in the liver have recently occurred in a few publications, either in mammalian hepcidin or in fish hepcidin studies. In mammals, Kulaksiz and colleagues suggested that hepcidin was not liver-specific but might be expressed also in the kidney [12,13]. While in the red seabream and catfish, hepcidin mRNA was widely expressed in multiple tissues [6,10], from which the conclusion was derived that in fish hepcidin might have a non-liver-specific expression.

The purpose of this study was to analyze the new hepcidin cDNAs, the gene organizations and the tissue-specific gene expression pattern in black porgy. This is the first report on seven hepcidin variants being cloned from one fish.

2. Materials and methods

2.1. Fish rearing and sample collection

Juvenile black porgy ($\sim$ 30 g) were obtained from a fish farm in Xiamen, Fujian province, China. After being kept for 2 days in sea water tanks in the laboratory, with daily water changes, at room temperature (20—30 °C), healthy-looking fish ($n = 3$) were chosen for sampling. Fish were taken out of the tanks, measured, weighed, and euthanized. Blood was firstly collected and then liver, spleen, kidney, heart, brain, stomach, intestine, gill, and skin were collected separately from each individual fish, frozen immediately in liquid nitrogen, and stored at −80 °C.

2.2. Cloning of hepcidin cDNA from normal black porgy

RT−PCR and RACE (rapid amplification of the cDNA ends) were performed to amplify the hepcidin cDNA sequences from black porgy. Primers S1 (5′-CGA AGC AGT CAA ACC CTC CTA AGA TG-3′) and A1 (5′-GAA CCT GCA GCA GAC ACC ACA TCC G-3′) were designed based on the published cDNA sequence of white bass hepcidin [1]. Individual tissue ($\sim$ 50 mg), such as the liver, was ground quickly in liquid nitrogen using a mortar and pestle and transferred to 1 ml TRIzol (Invitrogen). Total RNA was then extracted according to the manufacturer's instructions. RT−PCR analysis was performed with primers S1 and A1 as in our previous study on Japanese sea bass [5]. 3′-RACE was performed with aliquots of RNA using the 3′-full RACE core set (TaKaRa). Briefly, first strand cDNA was synthesized from $1 \sim 3$ µg total RNA using AMV reverse transcriptase XL and oligo dT-3 sites adaptor primer, and the 3′ region of the hepcidin cDNA was amplified with a gene-specific sense primer S1 and 3 sites adaptor primer by the hot-start PCR method (which was used to minimize non-specific amplification while increasing target yield) using the RT reaction mixture as cDNA template. The PCR cycles were performed using a PTC-200 thermocycler (MJ Research) as follows: 94 °C for 3 min; 30 cycles of 30 s at 94 °C, 30 s at 57 °C, 1 min at 72 °C; and extension at 72 °C for 5 min. PCR products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide in TAE buffer (Tris/acetic acid/EDTA), purified using a QIAquick gel extraction kit (Qiagen) after being excised from the gel, subcloned into pMD$_{18}$-T vectors (TaKaRa) and sequenced (Invitrogen).

2.3. Genomic DNA determination of hepcidin

Genomic DNA was isolated using a MiniBEST Animal Tissue Genomic DNA Extraction Kit (TaKaRa) according to the manufacturer's instructions. Primers U1 (5′-AGG AGA AGA AGT GAA AGG AGC TGA CG-3′) and D1

(5′-GTG GTT GTT GTG TAA ATC CCA GGC TG-3′) were designed based on 5′-UTR (untranslated regions) and 3′-UTR of the cDNA sequences of red seabream (GenBank accession number: AY557619) and black porgy, in order to amplify coding region and introns. Inverse PCR was also used to amplify 5′- and 3′-flanking sequences of AS-hepc2 by specific nest primers SR (5′-ACA ACG GCC ACT GCA ACT GCA ACA C-3′),UP (5′-GGT GCA AGA GCT GGA GGA GCC AAT G-3′), In-U51 (5′-AGG CTT CCA CTG CTT CAA CTT GTT C-3′) and In-D549 (5′-TAC CTG CTG CAA GTT CTG AGG ATT C-3′). The PCR product was purified and its sequence analyzed as described in Section 2.2. Intron positions and transcription factors were identified by comparison with the cDNA sequence.

2.4. Tissue- specific gene expression of hepcidin by RT–PCR

Expression of the hepcidin-like gene in liver, spleen, kidney, heart, brain, stomach, intestine, gill, skin, and blood was investigated by RT–PCR. RNA was separately isolated from individual tissue using TRIzol as described above. First strand cDNA was synthesized from 1 μg RNA using 10 pmol oligo $dT_{(18)}$ primer in 15 μl reaction mixture and processed using the same procedure as described in Section 2.2. Two sets of PCR were carried out with 1 μl cDNA template in separate 25 μl PCR reaction mixtures. One was performed using S1 and A1 primers to amplify hepcidin cDNA and the other using Act400F and Act400R to amplify β-actin cDNA. The latter was performed to confirm the steady-state level of expression of a housekeeping gene and provide an internal control for the hepcidin-like gene expression analyses [2]. The positive hepcidin PCR control was set using S1, A1 and the recombinant AS-hepc1–AS-7 pMD_{18}-T plasmid previously constructed in our laboratory as primers and a template, respectively. The amplification scheme was: 3 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s annealing at 57 °C for hepcidin cDNA and DNA and 52 °C for β-actin cDNA, 1 min at 72 °C; and extension at 72 °C for 5 min. PCR products were analyzed by electrophoresis in a 2% agarose gel.

2.5. Dot blot analysis of hepcidin expression

The AS-hepc2 and actin fragments were purified and labeled with digoxigenin using the Dig High Prime DNA-labeling and Detection Starter Kit I (Roche Applied Science). For hybridization, 20 μg RNA from each tissue was denatured in formaldehyde-containing buffer and incubated at 68 °C for 15 min, then dotted onto a Hybond N+ membrane (Amersham Biosciences) using vacuum-filter and UV-crosslinking. Hybridization was carried out for 20 h at 50 °C for both probes. After stringency wash, immunological detection was performed on the membrane following the kit protocol. Using S1 primer and 3 sites adaptor primer, AS-hepc1–AS-hepc7 fragments (each about 500 bp in length), as positive hepcidin blot controls, were amplified from the recombinant AS-hepc1–AS-hepc7 pMD_{18}-T plasmids, respectively.

2.6. Time course of hepcidin expression after bacterial challenge

Juvenile black porgy (50–70 g) were immersed for 15 min in a live bacterial mixture of *Staphylococcus aureus* CGMCC 1.363, *Escherichia coli* CGMCC 1.2389, *Vibrio parahaemeolyticus* CGMCC 1.1615 and *Micrococcus lysodeikticus* CGMCC 1.634 (purchased from China General Microbiological Culture Collection Center, CGMCC, Beijing, China), which were separately cultured in LB broth at 28–30 °C with shaking at 200 rpm overnight then the cultures were mixed for fish challenge. The concentration of the bacterial suspension was approximately 10^8 cfu ml^{-1} for each strain. After immersion, samples ($n = 3$) were obtained at 6 h, 24 h and 48 h, and the liver, spleen, kidney, stomach, intestine, and gill from each fish was separately collected, frozen immediately in liquid nitrogen, and stored at −80 °C. For dot blot, 50 μg RNA from each tissue was dotted onto two membranes in parallel, for analysis of hepcidin and the control actin, respectively. For Northern blot, tissues were collected from the fish 24 h and 48 h after exposure to the bacteria and 25 μg RNA from each tissue was loaded onto a denaturing 2% agarose gel and transferred onto a Hybond N+ membrane, which was then hybridized with the hepcidin-like probe to detect hepcidin-like mRNA in tissues using the same method as for dot blot analysis.

3. Results

3.1. Cloning and sequencing of hepcidin cDNAs and genomic DNAs from black porgy

3.1.1. Complete coding sequences of hepcidin

Using 3′-RACE with S1 and 3′ adaptor primer, a fragment (∼ 500 bp) was amplified from the liver, kidney, spleen, blood, gill and skin of the normal juvenile black porgy. The positive recombinants confirmed as 3′-RACE products incorporated in plasmids were sequenced. Then, seven hepcidin-like cDNA sequences were obtained from one fish and randomly denominated as AS-hepc1 to AS-hepc7 (AY669376 to AY669382). For these cDNA sequences, AS-hepc1–3 were obtained from the liver; AS-hepc 4 obtained from the gill, AS-hepc5 obtained from the gill, skin and spleen; AS-hepc6 obtained from the gill, skin, spleen, kidney and blood; and AS-hepc7 obtained from the spleen and gill. Sequence analysis reveals that all these sequences possess of a complete CDS in addition to a 3′-UTR, in which the polyadenylation signal appeared at about 200 nt downstream of the stop codon (TGA) and 10–20 nt upstream of the poly(A) tail. Unexpectedly, AS-hepc3, AS-hepc6 and AS-hepc7 possess the polyadenylation signals (AC/GTAAA) substituting for canonical polyadenylation signals (AATAAA), an "A" nucleotide substituted by "C" in AS-hepc3 or "G" in AS-hepc6 and AS-hepc7.

3.1.2. Deduced amino acid sequence

The deduced amino acid sequences of seven hepcidin cDNAs are 84–95 residues in length and all consist of three domains: signal peptide, predomain and mature peptide. Fig. 1 shows the alignments of AS-hepc1–AS-hepc7 by ClustalX 1.83 software. AS-hepc1, AS-hepc2, and AS-hepc4 are more similar to each other in a propeptide of 88 amino acids. AS-hepc3 has an additional insertion of seven amino acids near the predicted processing site, yet is highly similar to AS-hepc2 in the rest of the propeptide. However, AS-hepc5, AS-hepc6 and AS-hepc7 are more different from AS-hepc1–4 in the whole propeptide sequence. The signal peptide cleavage site of all deduced hepcidins was between Ala 24 and Gly 25 or Val 25, calculated by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP). The mature peptide regions of the AS-hepc1–7 were predicted. The RX (K/R) R motif typical of propeptide convertases [4] was recognized in AS-hepc1, -2, -4, and especially AS-hepc3, in which two procession sites (RHKR and RRRR) were recognized. A substitution of one amino acid of RHNR or RRWR for the typical K/R motif was observed in both AS-hepc5 and AS-hepc6, and a particular exception (RSKTGRRR) observed in AS-hepc7. So, the potential mature peptides contain 20–31 amino acids and are cysteine-rich. The processed AS-hepc1 and AS-hepc4 are predicted to be negatively charged at neutral pH, while the others are positively charged. AS-hepc3 mature peptide (processed by RHKR) containing seven positively charged residues has a theoretical pI of 9.34 calculated by the ProtParam tool (http://cn.expasy.org/tools/protparam.html), being the most cationic protein among the seven hepcidins.

A phylogenetic analysis of the seven hepcidin-like precursor peptides of black porgy and some other known and predicted hepcidin peptides was performed at the amino acid level using ClustalX 1.83 and Treeview software and the neighbor-joining method. The alignment showed that all listed hepcidins, including fish and mammalian, were characterized by eight cysteine residues conserved at identical positions in the mature peptide region, and fish hepcidins were much more conserved in the signal peptide region (Fig. 2a). Based on the phylogenetic tree generated from the aligned sequences (Fig. 2b), the hepcidin family was divided into two distinct groups: mammalian hepcidins and fish


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                       Cleavage site for signal peptide              Processing site for mature peptide
                 ***************************:***** ..****: ****..:.****:* .  :**** * .*        ↓        *:*** **    ** ** :
As_hepc1  MKTFSVAVAVAVVLTFICLQESSAGSFTEVQEPEEPMNNESPVAAHEEKSEESWKMPYNNRHKRSPKD-------CQFCCGCCPDMSGCGICCTY  88
As_hepc4  MKTFSVAVAVAVVLTFICLQESSAGSFTEVQEPEEPMNNESPVAAHEEKSEESWKMPYNNRHKRSPKD------|CQFCCGCCPDMSGCGICCRF  88
As_hepc2  MKTFSVAVAVAVVLTFICLQESSAGSFTEVQEPEEPMNNESPVAAHEEKSEESWKMPYNNRHKRSPAG------↓CRFCCGCCPNMRGCGVCCRF  88
As_hepc3  MKTFSVAVAVAVVLTFICLQESSAGSFTEVQELEEPMNNESPVAAHEEKSEESWKMPYNNRHKRSPAGRNSRRRRCRFCCGCCPDMIGCGTCCKF  95
As_hepc6  MKTFSVAVAVAVVLTFICLQESSAGSFTEVQKLEEPMNSDGPVAAYKEMPEDSWKMGYGSR----RWR|------CRFCCRCCPRMRGCGLCCRF  84
As_hepc7  MKTFSVAVAVAVVLTFICLQESSAGSFTEVQELEEPMNNESPVAAYKEMPEDSWKMGYASRSKTGRRR↓------CKFCCRCCPNMIGCGTCCKF  88
As_hepc5  MKTFSVAVAVAVVLTFICIQESSAVPVTEVQELEEPMSNDNPVAAHEETSVDSWKMPYDSRHNRAIK--------CKFCCGCCIPGV-CGLCCRF  86
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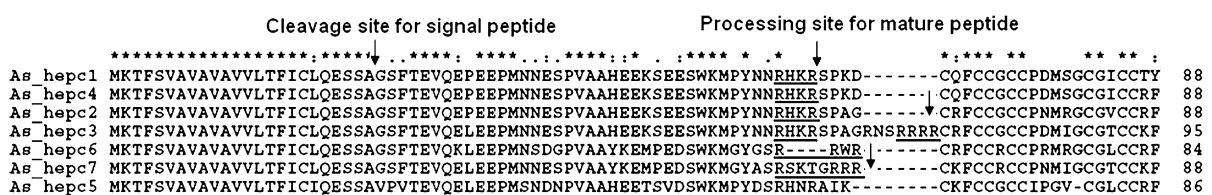
Fig. 1. ClustalX alignment of amino acid sequences of AS-hepc1–7 from black porgy. '*' Indicates positions which have a single, fully conserved residue; ':' and '.' indicate similar amino acid residues. Arrows show the predicted positions of cleavage sites for signal peptide and mature peptide.

(a)

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                          ..   :   :        ::  .                    :
   Chl_catfish   MRAMSIACAVAVIIACVCALQSAALPSEVRLDPEVRLEEPEDSEAARSIDQGVAA   55
  Blue_catfish   MKPMSIACAVAVIIACVCALQSAALPSEVRLDPEVRLEEPEDSEAARSVDQGVAA   55
     Zebrafish   MKLSNVFLAAVVILTCVCVFQITAVPFIQQVQDEHHVESEELQE-----NQHLTE   50
      As_hepc1   MKTFSVAVAVAVVLTFICLQESSAGS----FTEVQEPEEPMNNESPVA-AHEEKS   50
      As_hepc4   MKTFSVAVAVAVVLTFICLQESSAGS----FTEVQEPEEPMNNESPVA-AHEEKS   50
      As_hepc2   MKTFSVAVAVAVVLTFICLQESSAGS----FTEVQEPEEPMNNESPVA-AHEEKS   50
      As_hepc3   MKTFSVAVAVAVVLTFICLQESSAGS----FTEVQELEEPMNNESPVA-AHEEKS   50
          Pm_L   MKTFSVAVAVAVVLTFICLQESSAAS----VTEVQELEEPMSNGSPVA-AHEEMP   50
      Lj_Hepc1   MKTFSVTVAVAVVLTFICLQESSAAS----FTEVQELEEPMSNGSPVA-AHEEML   50
      As_hepc6   MKTFSVAVAVAVVLTFICLQESSAGS----FTEVQKLEEPMNSDGPVA-AYKEMP   50
      As_hepc7   MKTFSVAVAVAVVLTFICLQESSAGS----FTEVQELEEPMNNESPVA-AYKEMP   50
          Pm_G   MKTFSVAVAVAVVLTFICLQESSAVS----FTEVQELEEPMSNGSPVA-AYEEMS   50
          Pm_B   MKTFSVAVAVAVVLTFICLQESSAAS----FTEVQELEEPMSNGSPVA-ADEEMS   50
    White_bass   MKTFSVAVAVAVVLAFICLQESSAVP----VTEVQELEEPMSN------EYQEMP   45
        Turbot   MKTFSVAVAVAVVLTFICIQESSAVP----VTEVQELEEPMSNDNPVA-AHEETS   50
      Lj_Hepc2   MKTFSVAVAVAVVLTFICIQESSAVP----VTEVQELEEPMSNDNPVA-AHEETS   50
      As_hepc5   MKTFSVAVAVAVVLTFICIQESSAVP----VTEVQELEEPMSNDNPVA-AHEETS   50
    Mud_sucker   MKTVRVAAAVAVLFAFIWIQESSAKP----HSQMAEIEEHEDVDIPVEPKAEEVS   51
      Flounder   MKAFSIAVAVTLVLAFVCIQCSSAVP----FQGVQELEEAGGNDTPVA-EHQVMS   50
        Medaka   MKAFSIAVAVTLVLAFICILQSSAIP----VNGVKELEEAASNDTPVA-ARHEMS   50
        Salmon   MKAFS--VAVVLVIACMFILESTAVP----FSEVR-TEEVGSFDSPVG-EHQQPG   47
           Pig   -MALSVQIRAAC-LLLLLLVSLTAGS----VLPSQTRQLTDLRTQDTA-GAT-AG   47
           Dog   -MALSTRIQAAC-LLLLLLASVASVS----VLPHQTGQLTDLRAQDTA-GAE-AG   47
         Human   -MALSSQIWAACLLLLLLLASLTSGS----VFPQQTGQLAELQPQDRA-GAR-AS   48
      Mm_hepc1   -MALSTRTQAAC-LLLLLLASLSSTT----YLHQQMRQTTELQPLHGE-ESR-AD   47
      Mm_hepc2   -MALSTRTQAAC-LLLLLLASLSSTT----YLQQQMRQTTELQPLHGE-ESR-AD   47
           Rat   -MALSTRIQAAC-LLLLLLASLSSGA----YLRQQTRQTTALQPWHGA-ESKTDD   48


                          *                  *   **  **     **  **
   Chl_catfish   ALAKETSPEVRFRTKRQSHLS-----LCRYCCNCC-KNKGCGFCCRF-          96
  Blue_catfish   ALAKETSPEALFRTKRQSHLS-----LCRYCCNCC-KNKGCGFCCRF-          96
     Zebrafish   AEHRQTDPLVLFRTKRQSHLS-----LCRFCCKCC-RNKGCGYCCKF-          91
      As_hepc1   EESWKMP--YNNRHKRSPKD-------CQFCCGCCPDMSGCGICCTY-          88
      As_hepc4   EESWKMP--YNNRHKRSPKD-------CQFCCGCCPDMSGCGICCTY-          88
      As_hepc2   EESWKMP--YNNRHKRSPAG-------CRFCCGCCPNMRGCGVCCRF-          88
      As_hepc3   EESWKMP--YNNRHKRSPAGRNSRRRRCRFCCGCCPDMIGCGTCCKF-          95
          Pm_L   EESWKMP--YNNRHKRSPAG-------CRFCCGCCPNMIGCGVCCRF-          88
      Lj_Hepc1   EESWKMP--YNNRHKRSPAD-------CRFCCGCCTDVSGCGVCCRF-          88
      As_hepc6   EDSWKMG--YGSR----RWR-------CRFCCRCCPRMRGCGLCCRF-          84
      As_hepc7   EDSWKMG--YASRSKTGRRR-------CKFCCRCCPNMIGCGTCCKF-          88
          Pm_G   EESWKMP--YASR----RWR-------CRFCCRCCPRMRGCGLCCQRR          85
          Pm_B   EESWKMP--YASR----RWR-------CRFCCRCCPRMRGCGLCCQRR          85
    White_bass   VESWKMP--YNNRHKRHSSPGG-----CRFCCNCCPNMSGCGVCCRF-          85
        Turbot   VDSWKMP--YNSRHKRAIK-------CKFCCGCCTPGV-CGVCCRF-           86
      Lj_Hepc2   VDSWKMP--YNSRHKRAIK-------CKFCCGCCTPGV-CGVCCRF-           86
      As_hepc5   VDSWKMP--YDSRHNRAIK-------CKFCCGCCIPGV-CGLCCRF-           86
    Mud_sucker   EDAMTSPY-YRSREKRGIK-------CKFCCGCCTGV--CGICCRF-           88
      Flounder   MESWME---NPTRQKRHISHISL----CRWCCNCCKANKGCGFCCKF-          90
        Medaka   MQPWMLP--NHIREKRQ-SHISM----CTMCCNCCKNYKGCGFCCRF-          90
        Salmon   GESMHRTLPEPFRFKRQIHLS-----LCGLCCNCC-HNIGCGFCCKF-          88
           Pig   LTPVAQ----RLRR-DTHFP------ICIFCCGCCRKAI-CGMCCKT-          82
           Dog   LQPTLQL--RRLRRRDTHFP------ICIFCCGCCKTPK-CGFCCKT-          85
         Human   WMPMFQ----RRRRRDTHFP------ICIFCCGCCHRSK-CGMCCKT-          84
      Mm_hepc1   IAIPMQ----KRRKRDINFP------ICIFCCKCCNNSQ-CGICCKT-          83
      Mm_hepc2   IAIPMQ----KRRKRDINFP------ICRFCCQCCNKPS-CGICCEE-          83
           Rat   SALLML----KRRKRDTNFP------ICLFCCKCCKNSS-CGLCCIT-          84
```
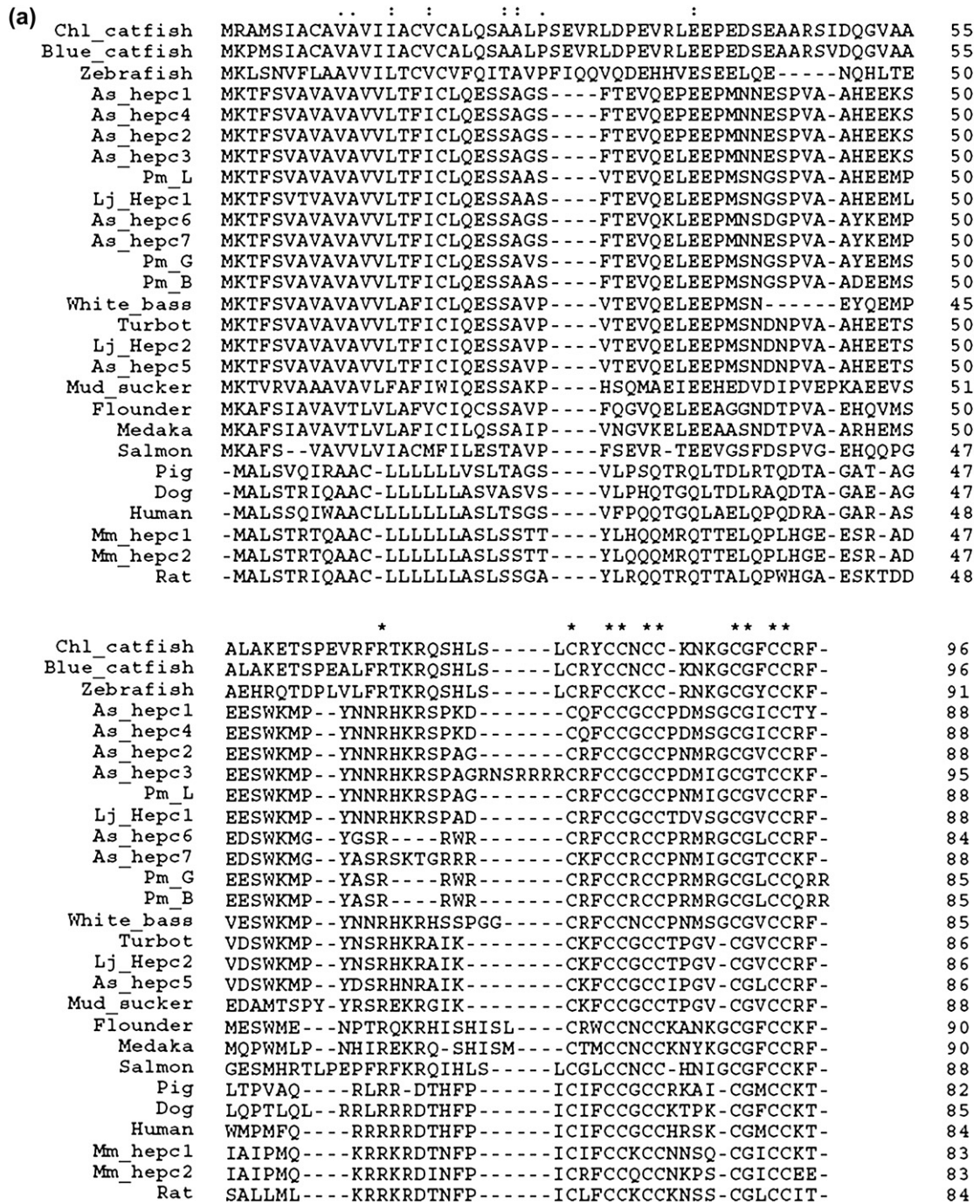
Fig. 2. Phylogenetic analysis of a family of hepcidin peptides. (a) ClustalX alignment of black porgy hepcidin-like amino acid sequences with some other known and predicted hepcidins. '*' Indicates positions which have a single, fully conserved residue; ':' indicates that one of the "strong" groups is fully conserved; '.' indicates that one of the "weaker" groups is fully conserved. SwissProt and GenBank accession numbers are as follows: pig (AAM77745), dog (AAV40979), human (NP066998), rat (NP_445921), mouse hepcidin1 (Mm Hepc1: NP_115930), mouse hepcidin2 (Mm Hepc2: P_899080), channel catfish (chl_catfish: AAX39713), blue catfish (AAX39714), zebrafish (AY363454), Altantic salmon (BI468191), winter flounder (AW783824), medaka (AU178966), mud sucker (AW013026), turbot (AM113708), white bass (P82951), red seabream (Pm G: AAU10849; Pm S: AAR28076; Pm L: AAS66305), black porgy hepcidin (AS hepc1–7: AAU00794 to AAU007910), Japan sea bass (LjHepc1: AAS547281; LjHepc2: AAT09138). (b) Molecular phylogenetic tree, constructed using the NJ method with ClustalX1.83, showing the relationships among the hepcidin family. The scale bar refers to percentage of divergence. Numbers next to the branches indicate bootstrap value from 1000 replicates.
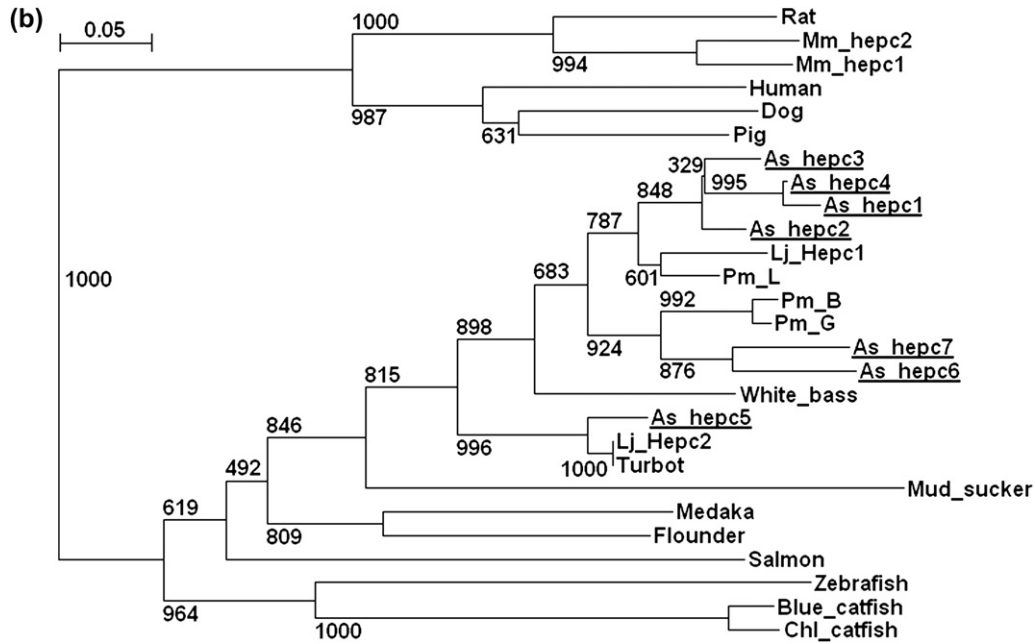
Fig. 2 (*continued*).

hepcidins. Six mammalian hepcidins formed a single well-supported clade, independent of fish hepcidins, implying a relatively distant evolutionary relationship between fish and mammalian hepcidin peptides.

3.1.3. Genomic DNA sequence analysis of hepcidin

The AS-hepc2, -3, -4 and -7 genomic DNA sequences were found and sequenced. Within the coding sequence, two introns were inserted into hepcidin cDNA, thus separating the cDNA into three exons. The organizations of introns and extrons of the four hepcidin genomic DNA are indicated in Fig. 3, which were identical with most reported hepcidins. The upstream and downstream regions of AS-hepc2 gene were sequenced and shown in Fig. 4. A TATA box and "Cap" were found 80−120 nt upstream of the start codon so that the transcriptional start site was verified. Some important transcription factors such as C/EBPβ, NF-κB, HNF-3b and GATA were also indicated on their binding sites upstream from the transcriptional site, as predicted by TFSEARCH software (http://www.cbrc.jp/research/db/TFSEARCH.html).

3.2. Expression analysis of hepcidin in normal juvenile fish

The result of positive PCR control is shown in Fig. 5. It was proved that AS-hepc1, -2, -3, -4, -6 and -7 cDNAs could be positively amplified using S1 and A1 primers under the test conditions. RT−PCR analyses of tissue-specific expression of the hepcidin genes in black porgy are shown in Fig. 6. The signal of the housekeeping gene, β-actin, was strong in each tissue. The signal of the hepcidin genes was detected in each tested tissue, but was strong in the liver, spleen, kidney, stomach, intestine and gill, and weak in the heart, brain, skin and blood.

To analyze mRNA abundance in multiple tissues of black porgy, a dot blot method was used. Using AS-hepc2 probe to hybridize with each of seven hepcidin-like cDNAs, an obvious signal could be detected in all cDNAs by Southern blot (Fig. 7), indicating that the AS-hepc2 probe can not distinguish the AS-hepc2 from the other six hepcidin-like cDNA sequences. Thus, the AS-hepc2 probe could be used as a universal probe to hybridize with all the seven hepcidin mRNAs in black porgy. The dot blot analysis is shown in Fig. 8. The signal of β-actin was strong in each tissue as shown by RT−PCR analysis, but the hepcidin signal was strong only in the liver, kidney, stomach and intestine, and weak in the spleen, gill, heart, brain, skin and blood.
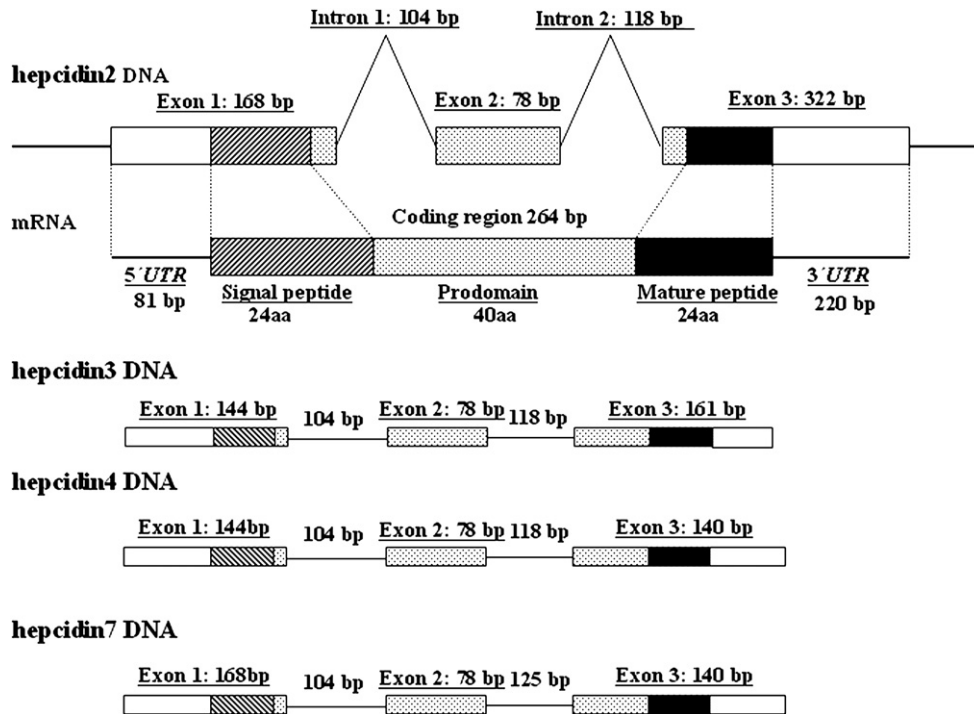
Fig. 3. The genomic organizations of AS-hepc2, -3, -4 and -7 DNA and mRNA in black porgy.

3.3. Time course of hepcidin expression after bacterial challenge

The hepcidin-like mRNA abundance after bacterial stimulation was also evaluated by dot blot. By measuring the optical density ratio of the hepcidin dot to the actin dot using Scion Image software, the relative hepcidin-like mRNA expression level was determined. A significant increase ($P \leq 0.05$, one-way ANOVA) in mRNA expression was observed in the liver of juvenile black porgy when the infection time was within 48 h (Fig. 9). Meanwhile, a higher hepcidin expression was also observed in the kidney but, unlike the liver, there was no obvious change of expression over time. Also, no obvious signal changes were detectable in the spleen, stomach or intestine. Northern blot was performed to confirm the hepcidin expression level in several inoculated tissues (Fig. 10). The positive band was 600–800 bp in length according to the RNA marker, which was consistent with the cDNA length of hepcidin analyzed above. The result obtained using Northern blot analysis showed that hepcidin was highly expressed in the liver and kidney and more weakly expressed in the spleen.

4. Discussion

As reported previously, mice have two hepcidin genes clustered on the genome [14,15] while in humans and hybrid striped bass only one hepcidin gene has been identified [1]. Using the 3′-RACE method with one gene-specific primer that was designed according to the highly conserved sequences in the signal peptide region and a universal adaptor primer, we were able to amplify seven homologous hepcidin-like cDNA sequences from one single normal juvenile black porgy. This result was repeated in several juvenile and mature fish afterwards and further confirmed the existence of the seven hepcidin-like cDNAs. Similarly, four matched genomic DNA sequences of the AS-hepc2, -3, -4 and -7 were obtained using PCR method with universal primers. Although the seven hepcidin genes were not fully amplified, it could be concluded that at least four hepcidin genes existed in black porgy. This is the first report to identify a family of hepcidin-like peptides in black porgy.
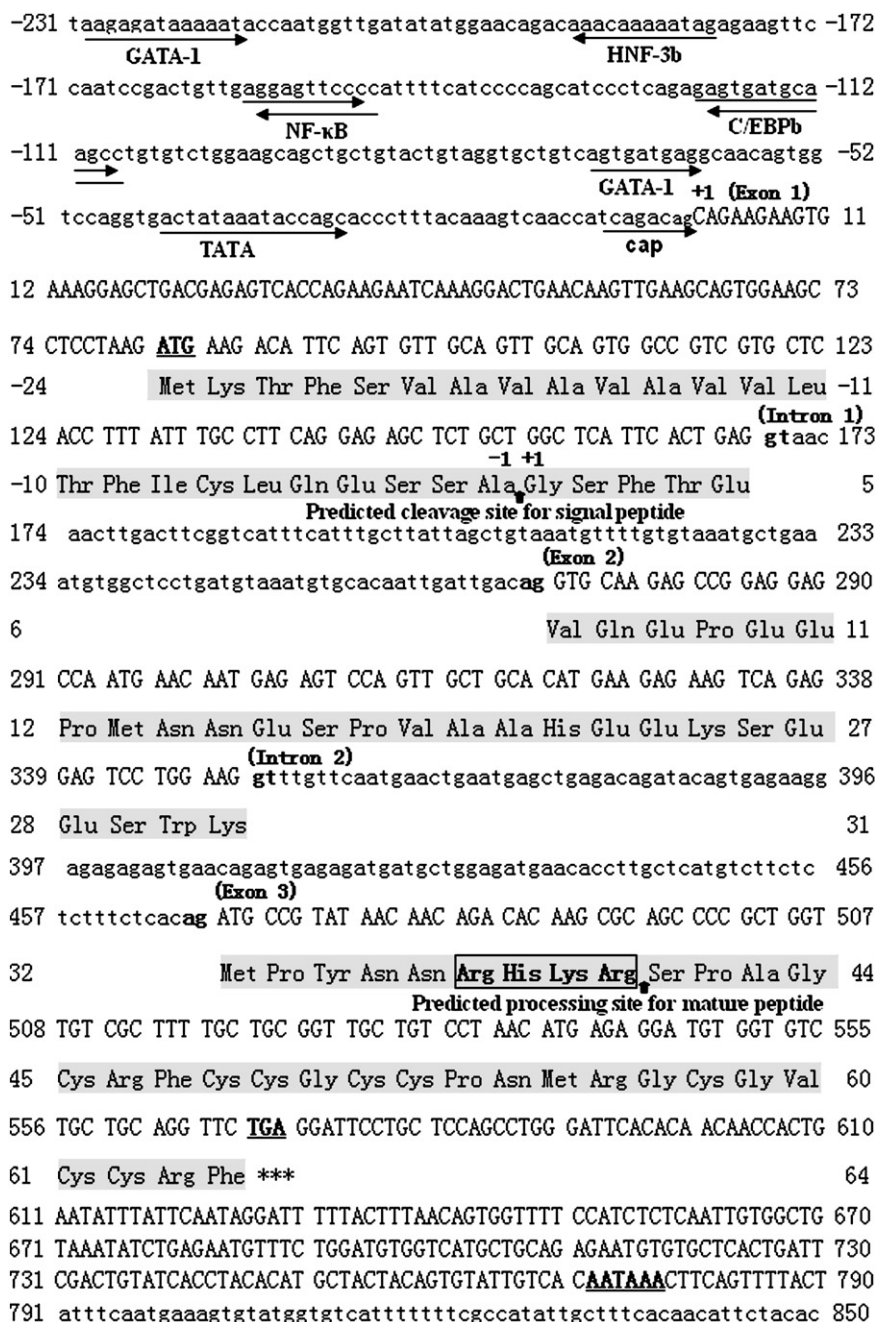
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-231 taagagataaaaataccaatggttgatatatggaacagacaaacaaaaatagagaagttc -172
             GATA-1                                    HNF-3b
-171 caatccgactgttgaggagttccccattttcatccccagcatccctcagagagtgatgca -112
                      NF-κB                                C/EBPb
-111 agcctgtgtctggaagcagctgctgtactgtaggtgctgtcagtgatgaggcaacagtgg -52
                                                 GATA-1   +1 (Exon 1)
-51 tccaggtgactataaataccagcacccttttacaaagtcaaccatcagacagCAGAAGAAGTG 11
              TATA                                    cap
12 AAAGGAGCTGACGAGAGTCACCAGAAGAATCAAAGGACTGAACAAGTTGAAGCAGTGGAAGC 73

74 CTCCTAAG ATG AAG ACA TTC AGT GTT GCA GTT GCA GTG GCC GTC GTG CTC 123
-24          Met Lys Thr Phe Ser Val Ala Val Ala Val Ala Val Val Leu -11
                                                      (Intron 1)
124 ACC TTT ATT TGC CTT CAG GAG AGC TCT GCT GGC TCA TTC ACT GAG gtaac 173
                                               -1 +1
-10 Thr Phe Ile Cys Leu Gln Glu Ser Ser Ala Gly Ser Phe Thr Glu    5
                      Predicted cleavage site for signal peptide
174 aacttgacttcggtcatttcatttgcttattagctgtaaatgttttgtgtaaatgctgaa 233
                                                    (Exon 2)
234 atgtggctcctgatgtaaatgtgcacaattgattgacag GTG CAA GAG CCG GAG GAG 290
6                                           Val Gln Glu Pro Glu Glu 11

291 CCA ATG AAC AAT GAG AGT CCA GTT GCT GCA CAT GAA GAG AAG TCA GAG 338
12  Pro Met Asn Asn Glu Ser Pro Val Ala Ala His Glu Glu Lys Ser Glu 27
                                      (Intron 2)
339 GAG TCC TGG AAG gtttgttcaatgaactgaatgagctgagacagatacagtgagaagg 396
28  Glu Ser Trp Lys                                              31
397 agagagagtgaacagagtgagagatgatgctggagatgaacaccttgctcatgtcttctc 456
                        (Exon 3)
457 tctttctcacag ATG CCG TAT AAC AAC AGA CAC AAG CGC AGC CCC GCT GGT 507
32           Met Pro Tyr Asn Asn Arg His Lys Arg Ser Pro Ala Gly 44
                            Predicted processing site for mature peptide
508 TGT CGC TTT TGC TGC GGT TGC TGT CCT AAC ATG AGA GGA TGT GGT GTC 555
45  Cys Arg Phe Cys Cys Gly Cys Cys Pro Asn Met Arg Gly Cys Gly Val 60

556 TGC TGC AGG TTC TGA GGATTCCTGC TCCAGCCTGG GATTCACACA ACAACCACTG 610
61  Cys Cys Arg Phe ***                                          64
611 AATATTTATTCAATAGGATT TTTACTTTAACAGTGGTTTT CCATCTCTCAATTGTGGCTG 670
671 TAAATATCTGAGAATGTTTC TGGATGTGGTCATGCTGCAG AGAATGTGTGCTCACTGATT 730
731 CGACTGTATCACCTACACAT GCTACTACAGTGTATTGTCA CAATAAACTTCAGTTTTACT 790
791 atttcaatgaaagtgtatggtgtcattttttttcgccatattgctttcacaacattctacac 850
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Fig. 4. AS-hepc2 genomic sequence of black porgy. The Exon sequence is bold. The deduced amino acid sequences are translated and shadowed. Vertical arrows show the predicted positions of cleavage sites for signal peptide and mature peptide. Horizontal arrows indicate the location of putative transcription factor binding sites. The box indicates RX (K/R) R motif. The start codon (ATG), stop codon (TGA) and polyadenylation signal (AATAAA) are underlined.

During our cloning of hepcidin cDNA, at least one form of hepcidin transcript was amplified and sequenced from each tested tissue of black porgy, even from the blood. This result was consistent with the observations of the RT−PCR analysis, which indicated that hepcidin genes were expressed in multiple tissues, a similar expression pattern for hepcidin gene to those previously reported in Atlantic salmon [4], catfish [6] and red seabream [10].
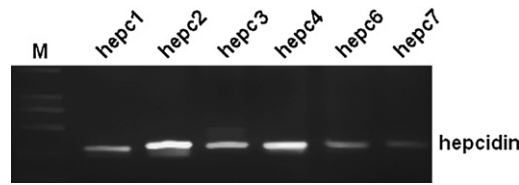
Fig. 5. PCR analysis of the specialty of S1 and A1 primers. The amplification product from the recombinant AS-hepc1−7 plasmid was resolved by electrophoresis in a 2.0% agarose gel. Markers (M) are DL2000 (TaKaRa).
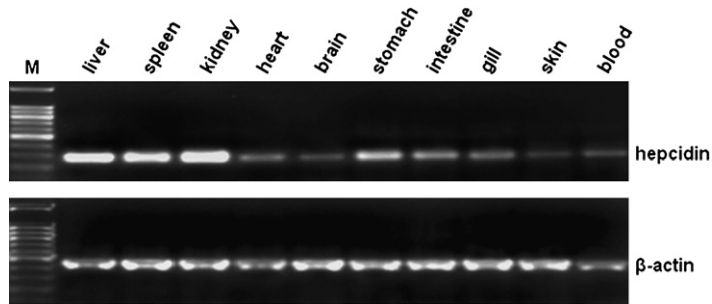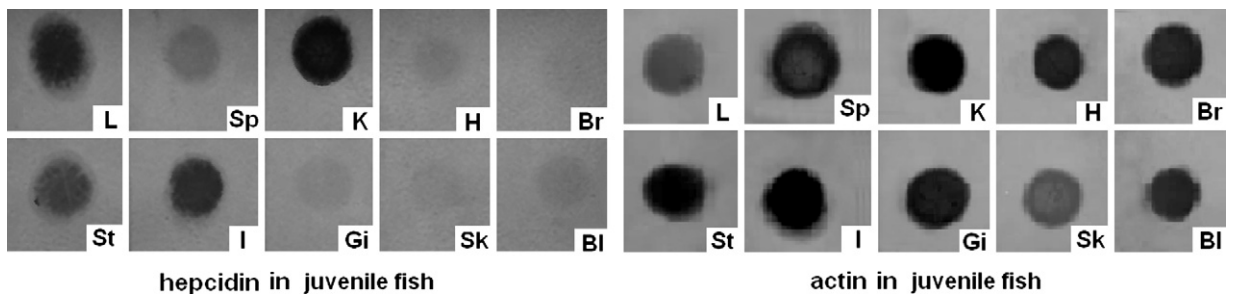

Fig. 6. RT−PCR analysis of the hepcidin-like and actin gene expressions in multiple tissues of the naturally raised juvenile black porgy. Tissues assayed were liver, spleen, kidney, heart, brain, stomach, intestine, gill, skin, and blood. Amplification products from the reactions using gene-specific primers for hepcidin (∼300 bp) and for β-actin (∼400 bp) were resolved by electrophoresis in a 1.5% agarose gel. Markers (M) are 100 bp ladder (TaKaRa).
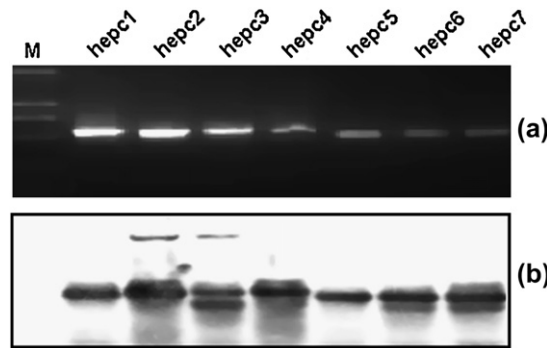
Fig. 7. Southern-blot analysis of the specialty of hepcidin probe. (a) The cDNA fragments amplified from the recombinant AS-hepc1−7 plasmids were resolved by electrophoresis in a 2.0% agarose gel. Markers (M) are DL2000 (TaKaRa). (b) Southern blot analysis of the cDNA fragments amplified from the recombinant AS-hepc1−7 plasmid using the hepcidin probe.

Fig. 8. Dot blot analysis of the hepcidin-like and actin gene expressions in multiple tissues of the naturally raised juvenile black porgy. Tissues assayed were liver (L), spleen (Sp), kidney (K), heart (H), brain (Br), stomach (St), intestine (I), gill (Gi), skin (Sk), and blood (Bl).
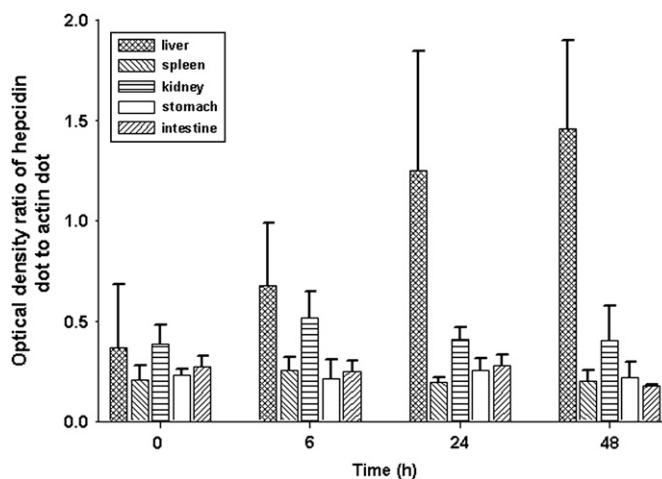
Fig. 9. The relative hepcidin-like mRNA expression in several tissues of the bacterially challenged juvenile black porgy by dot blot analysis ($n = 3$). Tissues assayed were liver, spleen, kidney, stomach and intestine. In the liver, a significant increase in hepcidin-like mRNA expression was observed, $P \leq 0.05$ (one-way ANOVA).

In addition, among seven cDNA sequences, AS-hepc3, -6 and -7 take on another two polyadenylation signals (AC TAAA, AGTAAA) in the 3′-UTR instead of a canonical one (AATAAA). It has been found that single-base variants of the AATAAA sequence occur with a high rate, and correlate with processing efficiencies in polyadenylation of the pre-mRNA (a coupled reaction involving endonucleolytic cleavage followed by poly(A) synthesis), that is, variant signals are processed less efficiently than the canonical signal, and could therefore be selected for regulatory purposes [16,17]. Thus, we presume that variant polyadenylation signals in hepcidin cDNA sequences might affect the stability, translation efficiency of hepcidin, or as regulatory elements govern the temporal and tissue- specific expression of hepcidin gene in black porgy.

The seven deduced amino acid sequences of AS-hepc1−7 have the same gene organization of three domains: signal peptide, predomain, and mature peptide. In comparison of deduced amino acid sequences of AS-hepc1−7, differences in amino acids existed in mature peptides. Thus, each of AS-hepc1−7 has distinct characteristics. At neutral pH, AS-hepc1 and AS-hepc4 mature peptides will be negatively charged (pI 4.21 and 5.66, respectively), while the other
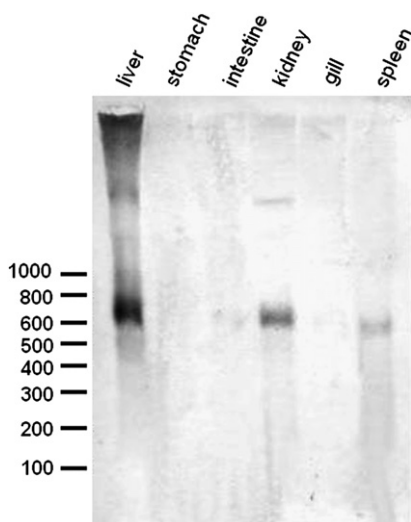
Fig. 10. Northern blot analysis of hepcidin-like mRNA expression in bacterially challenged juvenile black porgy. Tissues assayed were liver, stomach, intestine, kidney, gill and spleen. RNA marker RL1000 (TaKaRa) is indicated on the left side.

five will be positively charged (pI > 7). In particular, AS-hepc3 (processed by ''RHKR'' site) and AS-hepc6 have especially high charges (pI 9.19 and 9.01, respectively), indicating a greater potential for anti-microbial function *in vitro*. Cationic peptides are widely distributed in animals and comprise the largest group of antimicrobial peptides, while anionic peptides are a novel group of antimicrobial molecules that are usually isolated from mammalian epithelia [18]. So far, only one predicted peptide from winter flounder [4] has an acidic pI (5.54) among all known hepcidins. In this study, two anionic predicted hepcidin-like peptides, AS-hepc1 and AS-hepc4, with acidic pI, are presumed to be involved in different activities from the other five cationic ones. Based on the result of the alignment of whole pre-propeptides, it was found that fish and mammalian hepcidins were highly similar within respective species, and they were sorted into two different groups by phylogenetic analysis, suggesting recent duplication of ancestral genes and a relatively distant evolutionary relationship between fish and mammalian hepcidin peptides. Comparison of black porgy hepcidin with other fish hepcidins revealed that they all had a highly conserved signal peptide region, which suggested that fish might adopt the same mechanism for proteolytic cleavage of the hepcidin signal peptide [5].

The organization of introns and exons of the hepcidin genomic DNA was identical within several reported fishes [3–7,10]. However, the lengths of the two introns of hepcidin gene were unequal in fishes. The lengths of the two introns of the four black porgy hepcidin-like genes (104 bp and 118 bp or 125 bp, respectively) corresponded more with those of white bass [3], Atlantic salmon [4], red seabream [10] and Japan sea bass [7], but were much shorter than those of zebrafish (hepcidin1: 624 bp, 1553 bp; hepcidin2: 446 bp, 1556 bp) [5] and more different from that of catfish [6], in which the first intron is longer (644 bp) while the second intron is shorter (99 bp).

We examined the whole hepcidin expression (except for AS-hepc5) in multiple tissues using RT–PCR with a pair of universal primers and observed that hepcidin genes were widely expressed in the tissues of the normal juvenile black porgy. To shed light on the abundance of hepcidin mRNA, a dot blot or Northern blot approach was also carried out to quantify the hepcidin gene expression in tissues. Considering that the AS-hepc2 probe hybridized with all the seven hepcidin cDNAs, it can be a universal probe for analysis of all potential hepcidin-like mRNA transcripts in tissues. According to the result of dot blot assay, the liver, kidney, intestine and stomach were highly abundant in hepcidin mRNA, but in spleen, gill, heart, brain, skin and blood less or nearly no hepcidin mRNA signal was detected although an obvious PCR product was detected in those tissues.

It is well known that hepcidin gene expression can increase to a high level in response to iron metabolism [11,14,15,19], inflammation [20,21] and bacterial infection [3–6,10]. However, in our study, the observations suggested that the hepcidin genes in black porgy might be of a high-level expression in several tissues under normal culture conditions. This is a very interesting observation on hepcidin expression in the tissues of the various organisms investigated to date. In particular, the observation of high expression in the kidney of juvenile fish corresponds to the recent study from Kulaksiz and colleagues [13], who presented a detailed analysis of hepcidin expression in the kidney at RNA and protein level, and found that hepcidin was not liver specific but also might be expressed in the kidney of three mammalian species (human, rat and mouse) [12, 13]. Using dot blot method, we found that the expression of hepcidin genes was significantly induced in the liver of black porgy after exposure to bacteria, but not obviously changed in other tested tissues. Our observation on the hepcidin expression pattern in the liver completely matched the report in white bass [3], in which hepcidin gene was induced over 4500-fold in the liver following challenge with a bacterial fish pathogen. However, we found that the level of hepcidin expression in the kidney remained high before and after challenge, and Northern blot analysis also confirmed the result. Thus, it appears that in black porgy, kidney might be involved in hepcidin metabolism, although in a different expression pattern from the liver.

In conclusion, we have presented seven new hepcidin-like cDNAs and four matched DNAs cloned from black porgy. The seven variants belong to a family of hepcidins according to the sequence analysis. Hepcidin-like mRNAs existed in multiple tissues of normal cultured black porgy but the high expression of hepcidin-like mRNAs was mainly in the liver, kidney, spleen, stomach and intestine. In addition, the expression of hepcidin-like mRNAs was significantly induced in the liver of black porgy after exposure to live bacteria. The existence of several hepcidin variants may be a strategy for fish to survive in the complicated aquatic environment.

Acknowledgments

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