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NK-lysin of channel catfish: Gene triplication, sequence variation, and expression analysis

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Abstract

Antimicrobial peptides (AMPs) are important components of the host innate immune response against microbial invasion. In addition to the previously known four classes of antimicrobial peptides, a fifth class of antimicrobial peptides has been recently identified to include NK-lysins that have a globular three-dimensional structure and are larger with 74–78 amino acid residues. NK-lysin has been shown to harbor antimicrobial activities against a wide spectrum of microorganisms including bacteria, fungi, protozoa, and parasites. To date, NK-lysin genes have been reported from only a limited number of organisms. We previously identified a NK-lysin cDNA in channel catfish. Here we report the identification of two novel types of NK-lysin transcripts in channel catfish. Altogether, three distinct NK-lysin transcripts exist in channel catfish. In this work, their encoding genes were identified, sequenced, and characterized. We provide strong evidence that the catfish NK-lysin gene is tripled in the same genomic neighborhood. All three catfish NK-lysin genes are present in the same genomic region and are tightly linked on the same chromosome, as the same BAC clones harbor all three copies of the NK-lysin genes. All three NK-lysin genes are expressed, but exhibit distinct expression profiles in various tissues. In spite of the existence of a single copy of NK-lysin gene in the human genome, and only a single hit from the pufferfish genome, there are two tripled clusters of NK-lysin genes on chromosome 17 of zebrafish in addition to one more copy on its chromosome 5. The similarity in the genomic arrangement of the tripled NK-lysin genes in channel catfish and zebrafish suggest similar evolution of NK-lysin genes.

Keywords: Antimicrobial peptide; Fish; Catfish; Disease; NK-lysin; Innate immunity; Gene

1. Introduction

The NK-lysin gene was initially cloned and characterized from humans, but its significance was unrealized until its orthologue was identified from porcine natural killer cells and cytotoxic T lymphocytes as an antimicrobial peptide (Manning et al., 1992; Houchins et al., 1993; Andersson et al., 1995). In recent years, extensive research has been conducted for the analysis of structure and antimicrobial activities of NK-lysin (Stenger et al., 1998; Ernst et al., 2000; Gansert et al., 2003; Jacobs et al., 2003). NK-lysin genes share sequence similarities to the pore-forming proteins of *Entamoeba histolytica*, termed amoebapores (Leippe, 1995). They have been reported to have antimicrobial activities against a wide spectrum of microorganisms including bacteria, fungi, protozoa, and parasites (Stenger et al., 1998; Ernst et al., 2000; Gansert et al., 2003; Jacobs et al., 2003), and therefore, have been widely regarded as antimicrobial peptides. In contrast to classical antibiotics, these peptides act by direct physical destabilization of the target cell membrane with a high specificity for bacteria (Schroder-Borm et al., 2003). With the emergence of antibiotic-resistant bacteria, and the scarcity of new classes of useful antibiotics, there is an increasing need to identify novel antimicrobial peptides from various species for the development of alternative therapeutants (for review, see Patrzykat and Douglas, 2003).

Antimicrobial peptides are widespread in nature as defense mechanisms of plants and animals. Over 800 AMP sequences have been deposited in the Antimicrobial Peptide Database (http://bbcm1.univ.trieste.it/~tossi/pag1.htm). Increasing numbers of antimicrobial peptides have been identified from teleost fishes in recent years (e.g., Cole et al., 1997; Douglas et al., 2001,

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2003a,b; Lauth et al., 2002; Shike et al., 2002, 2004; Noga and Silphaduang, 2003; Zhang et al., 2004; Bao et al., 2005, in press; Xu et al., 2005). As the largest vertebrate group containing over 23,000 species (Helfman et al., 1997), teleosts should be a rich source of antimicrobial peptides. Channel catfish has played an important role as a model species for the study of comparative immunology (Clem et al., 1990; Vallejo et al., 1991a,b). It is also the primary aquaculture species in the United States. Studies of its innate immune components should have both scientific and practical implications.

Antimicrobial peptides can be divided into four different classes according to their structural features. The first class comprises mainly linear, helical, and amphipathic peptides without disulphide bonds. Well-known examples of this class are cecropins (Steiner et al., 1981). The second class includes linear peptides with an over-representation of a single amino acid, such as histatin 5 (Oppenheim et al., 1988). The third class comprises peptides with loop structures connected by one disulphide bridge, such as the brevinins from frog skin (Simmaco et al., 1998). The fourth class includes peptides with two or more disulphide bonds. These peptides usually possess a defined β-sheet structure, as found in the well-characterized mammalian defensins (Lehrer and Ganz, 2002).

Recently, with the discovery of porcine NK-lysin and human granulysin (Andersson et al., 1995; Pena et al., 1997), a fifth class of antimicrobial polypeptides has been recognized in natural killer and T cells (Bruhn et al., 2003). Compared with the classical antimicrobial peptides, NK-lysin and granulysin are much larger with 78 and 74 amino acid residues and a globular three-dimensional structure. Since the initial identification of NK-lysin in porcine and granulysin in humans a few years ago, a bovine homolog was recently identified (Endsley et al., 2004).

The NK-lysin genes characterized to date are highly conserved in gene structure, organization, and sequences. All the NK-lysin genes identified to date have a structure of five exons and four introns. Their primary sequences are rich in positively charged amino acids and the sulfite bond-forming cysteines. We previously reported a NK-lysin cDNA from channel catfish (Wang et al., 2006). BLAST searches of the draft genome sequences of the closely related zebrafish species reveal multiple copies of the NK-lysin gene. Questions remain, therefore, as to how many NK-lysin genes exist in the catfish genome and how they are organized. Here we report the identification of novel forms of NK-lysin genes, provide evidence for the presence of three tandem copies of NK-lysin genes in the catfish genome, and discuss the evolutionary implications of these findings.

2. Materials and methods

2.1. DNA sequencing and sequence analysis

Plasmid DNA containing the NK-lysin cDNA was prepared using the alkaline lysis method (Sambrook et al., 1989) using the Qiagen Spin Column Mini-plasmid kits (Qiagen, Valencia, CA). Three microliters of plasmid DNA (about 0.5–1.0 μg) were used in sequencing reactions. Chain termination sequencing was performed using the BigDye sequencing kits (ABI, Foster City, CA). The PCR profiles were: 95 °C for 30 s, 54 °C for 15 s, 60 °C for 4 min for 60 cycles. An initial 3 min denaturation at 95 °C and a 5 min extension at 60 °C were used. Sequences were analyzed on an ABI Prism 3100 automatic sequencer.

Bioinformatic analysis of sequences was conducted by using BLAST and DNASTAR software package (Serapion et al., 2004). BLAST searches were conducted to determine gene identities, and to determine if the cDNA contained a full open reading frame. DNASTAR software package was used for sequence analysis. MegAlign program of the DNASTAR package was used for sequence alignment using CLUSTAL W.

2.2. Tissue sampling and RNA extraction

Eleven tissues were collected from healthy channel catfish including brain, gill, head kidney, intestine, liver, muscle, ovary, skin, spleen, stomach, and trunk kidney. Samples of each tissue from 10 fish were pooled. The experimental fish were euthanized with tricaine methanesulfonate (MS 222) at 100 mg/l before tissues were collected. Tissues were quick frozen in liquid nitrogen and kept in a −80 °C ultra-low freezer until preparation of RNA. In order to obtain samples representing the average of the 10 fish, the pooled tissue samples were ground with a mortar/pestle to fine powder and thoroughly mixed. A fraction of the tissue samples was used for RNA isolation. RNA was isolated following the guanidium thiocyanate method (Chomczynski and Sacchi, 1987) using the Trizol reagents kit from Invitrogen following manufacturer's instructions. Extracted RNA was stored in a −70 °C freezer until used as template for reverse transcriptase-PCR (RT-PCR).

2.3. Assigning NK-lysin gene to BAC clones

We previously reported a NK-lysin cDNA (Wang et al., 2006). The cDNA sequences were used to design overgo probes (Bao et al., 2005, in press; Xu et al., 2005) for hybridization to BAC filters. High-density filters of channel catfish BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA). Each set of filters contained 10-genome coverage of the channel catfish BAC clones from BAC library CHORI 212 (http://bacpac.chori.org/library.php?id=103). Sequences of the overgo primers are shown in Table 1 and their positions within NK-lysin cDNA are shown in Figs. 1 and 2. Overgo hybridization method was adapted from a web protocol (http://www.tree.caltech.edu/). Briefly, overgo primers were selected following a BLAST search against GenBank to screen out repeated sequences and then purchased from Sigma Genosys (Woodlands, Texas). Two hundred nanograms of overgo primers each were labeled with 40 μl of a freshly prepared master mix composed of 14.0 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.02 mM dGTP, 0.02 mM dTTP, 20 μCi [α-^{33}P]dCTP, 20 μCi [α-^{33}P]dATP (3000 Ci/mmol, Amersham, Piscataway, NJ), and five units of Klenow enzyme (Invitrogen). Labeling reactions were carried out at room temperature for 2 h. After removal of unincorporated nucleotides using a Sephadex G50 spin column, probes were denatured at 95 °C for 10 min and added

Table 1
Primers and their sequences used in this study

Primer name	Sequences (5′ to 3′)	Utilization
Overgo 1 upper	CCTGTGCAATGCACATGGAATACC	Assign gene 1 and gene 2 NK-lysin to BACs
Overgo 1 lower	GCAGAGTCAACTCTCAGGTATTCC	Assign gene 1 and gene 2 NK-lysin to BACs
Overgo 2 upper	GACAAACTCCCAGTAGTGAAGGAT	Assign gene 3 NK-lysin to BACs
Overgo 2 lower	CCATTTTCTTACACAAATCCTTCA	Assign gene 3 NK-lysin to BACs
PCR primer 1 upper	GGAACCTCCTCGTTGCTTC	Amplify Southern probes for gene 1 and gene 2
PCR primer 1 lower	CTTGCAAATACCAAGATTAC	Amplify Southern probes for gene 1 and gene 2
PCR primer 2 upper	TCAAGAAGTCACTTACCTTGTG	Amplify Southern probes for gene 3
PCR primer 2 lower	TGCCATGCTGGAGTTTTGTG	Amplify Southern probes for gene 3
Gene 1-specific PCR primer upper	GACTTGGCGATGTCTGAGAC	Gene-specific PCR and RT-PCR
Gene 1-specific PCR primer lower	CTTGCAAATACCAAGATTAC	Gene-specific PCR and RT-PCR
Gene 2-specific PCR primer upper	GATTTGCCGATGTCTGAGAT	Gene-specific PCR and RT-PCR
Gene 2-specific PCR primer lower	TATCCAAGTACTTATTAATGATTG	Gene-specific PCR and RT-PCR
Gene 3-specific PCR primer upper	GGATGAGGATTTGCTGATGC	Gene-specific PCR and RT-PCR
Gene 3-specific PCR primer lower	TGCCATGCTGGAGTTTTGTG	Gene-specific PCR and RT-PCR

For positions of overgo primers and gene-specific PCR primers, please see Figs. 1 and 2.

to the hybridization tubes containing high-density BAC filters. Hybridization was performed at 54 °C for 18 h in hybridization solution (50 ml of 1% BSA, 1 mM EDTA at pH 8.0, 7% SDS, 0.5 mM sodium phosphate, pH 7.2). BAC filters were washed with 2× SSC at room temperature for 15 min, and exposed to X-ray film at −80 °C for 2 days. Positive clones were identified according to the clone distribution instructions from CHORI, and picked out from the channel catfish BAC library.

2.4. Preparation of BAC DNA and Southern blot analysis

After assigning of NK-lysin to BACs, the putative NK-lysin-positive BAC DNA was isolated with the Perfectprep® BAC 96 BAC DNA isolation kit (Brinkmann Instruments Inc., Westbury, NY). The isolated BAC DNA was subjected to Southern blot analysis to determine genomic copy numbers as we previously described (Bao et al., 2005, in press; Xu et al., 2005). Briefly, BAC DNA was first digested with restriction endonuclease *Rsa*I, *Eco*RI, and *Dra*I, separately, and electrophoresed on a 1% agarose gel. The DNA was transferred to a piece of Immobilon nylon membrane (Millipore, Bedford, MA) by capillary transfer with 0.4 M NaOH overnight. DNA was fixed to the membrane by UV cross-linking using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) with the auto cross-link function. The membrane was washed in 0.5% SDS (w/v) at 65 °C for 15 min and then pre-hybridized in 50% formamide, 5× SSC, 0.1% SDS (w/v), 5× Denhardt's, and 100 μg/ml sonicated and denatured Atlantic salmon sperm DNA (100 μg/ml) overnight. Hybridization was conducted overnight at 42 °C in the same solution with probes added. The NK-lysin cDNA was used as the probe. The probe was prepared using the random primer labeling method with a labeling kit from Roche Diagnostics

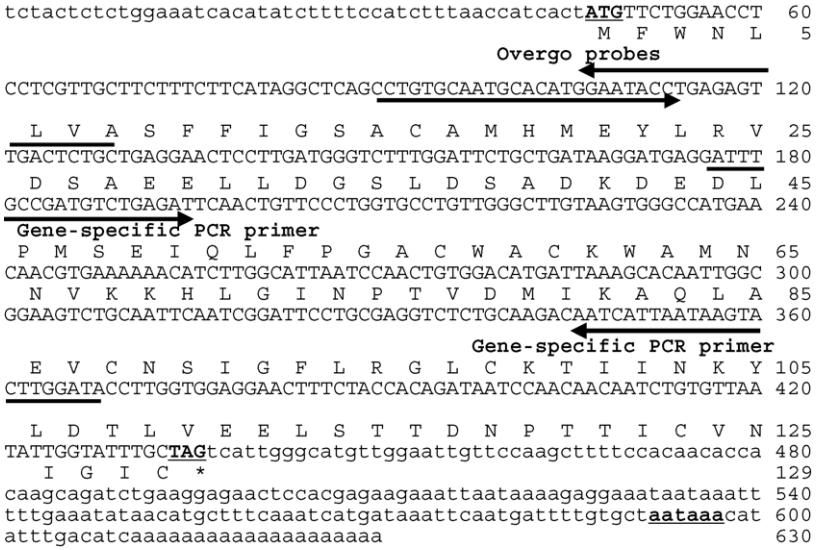

Fig. 1. The nucleotide and deduced amino acid sequences of channel catfish NK-lysin cDNA encoded by gene 2. 5′- and 3′-untranslated regions are shown as small letters. The translation start codon ATG, the termination codon, and the poly $(A)^+$ signal sequence AATAAA are both bold and underlined. The terminal codon is labeled by asterisk. The locations of the primers are indicated by underline arrows. The coordinates of the cDNA and the amino acids are indicated on the right margin.

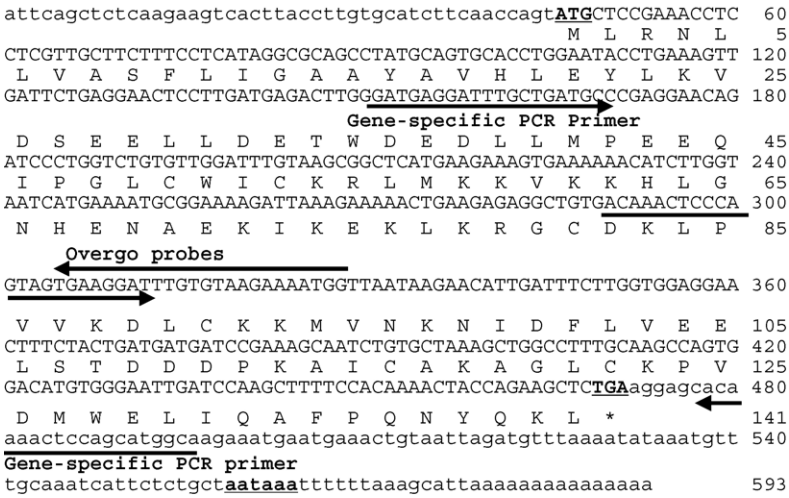

Fig. 2. The nucleotide and deduced amino acid sequences of channel catfish NK-lysin cDNA encoded by gene 3. 5′- and 3′-untranslated regions are shown as small letters. The translation start codon ATG, the termination codon, and the poly $(A)^+$ signal sequence AATAAA are both bold and underlined. The terminal codon is labeled by asterisk. The locations of the primers are indicated by underline arrows. The coordinates of the cDNA and the amino acids are indicated on the right margin.

(Indianapolis, Indiana). The nylon membranes were washed first in 500 ml of 2× SSC for 10 min, followed by three washes in 0.2× SSC with SDS at 0.2% (w/v) at 65 °C for 15 min each. The membranes were then wrapped in Saran wrap and exposed to Kodak BioMax MS film for autoradiography.

2.5. Gene-specific PCR

Gene-specific PCR primers were designed by alignment of three types of NK-lysin cDNA. Each gene-specific primer was designed from regions with specific sequence at the 3′ of the PCR primer. PCR conditions were tested using plasmids containing a specific type of NK-lysin as template such that the gene-specific PCR primers work only for its own template, but not for the other two types of NK-lysin templates. The following PCR profiles were used: 94 °C for 3 min, followed by 94 °C for 30 s, 55 °C for 15 s, 72 °C for 1 min for 30 cycles. The reaction was extended for 5 min at 72 °C. To determine which specific NK-lysin gene(s) were included in the BACs, bacterial clones harboring BACs with NK-lysin genes were first cultured overnight. The BAC-containing bacteria were directly used as templates for gene-specific PCR analysis.

2.6. Reverse transcriptase-PCR

RT-PCR reactions were conducted using SuperScript™ III One Step RT-PCR System (Invitrogen). The system contained a mixture of SuperScript™ III reverse transcriptase and the Platinum *Taq* DNA polymerase in an optimized buffer. Detailed procedures followed the instructions of the manufacturer. Briefly, the following was added to a reaction of 50 μl: 25 μl 2× reaction mix, 1 μl total RNA (∼100 ng), 1 μl (100 ng) each of the upper and lower primer (Table 1), 2 μl SuperScript III RT/Platinum *Taq* polymerase mix, and water to bring the reaction volume to 50 μl. The reaction also included the primers of β-actin (Table 1), serving as an internal control. The reactions were completed in a thermocycler with the following thermo-profiles: 45 °C for 15 min for 1 cycle (reverse transcription reaction), the samples were pre-denatured at 94 °C for 2 min, then the samples were amplified for 30 cycles with 94 °C for 15 s, 45 °C for 30 s, 68 °C for 1 min. Upon the completion of PCR, the reaction was incubated at 68 °C for an additional 5 min. The RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel and documented with a Gel Documentation System. The RT-PCR products were quantified by using GelExpert software package, Version 3.5 (Nucleotech Corp., San Mateo, CA).

3. Results

3.1. Identification of novel NK-lysin transcripts

A search of dbEST using the previously identified NK-lysin cDNA (Wang et al., 2006) identified 166 catfish ESTs with significant sequence similarities ($<10^{-8}$) to the query sequence. These sequences were downloaded from the GenBank in Vector NTI Contig Express and subjected to cluster analysis. Initial cluster analysis indicated that three distinct clusters existed. These cDNAs were then analyzed by using DNASTAR software package. DNA sequence alignments and analysis of the deduced amino acid sequences indicated the presence of three distinct types of NK-lysin, herein referred to as type 1, type 2, and type 3 NK-lysin.

The cDNA sequence of the type 1 NK-lysin was previously reported (Wang et al., 2006, Accession number AY934592), while the type 2 and type 3 NK-lysin are novel. The entire cDNAs of type 2 and type 3 NK-lysin were sequenced and their sequences have been deposited to GenBank with Accession numbers of DQ153186 and DQ153187, respectively. As shown in Figs. 1 and 2, type 2 NK-lysin encodes 129 amino acids whereas type 3 NK-lysin encodes a peptide of 141 amino acids. Both of these NK-lysin types were shorter than the type 1 NK-lysin that encodes a peptide of 148 amino acids. The comparison

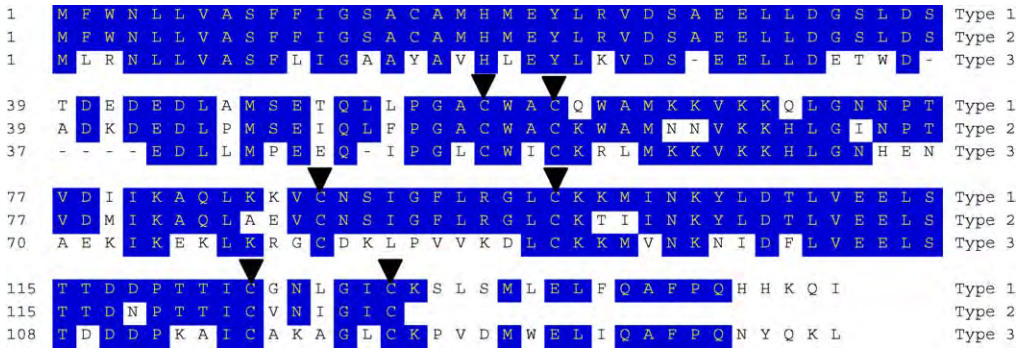

Fig. 3. Alignment of amino acid sequences encoded by the three channel catfish NK-lysin genes. Arrows indicated the six conserved cysteine residues.

of the amino acid sequences of the three types of NK-lysin is shown in Fig. 3. Type 1 NK-lysin is highly similar to type 2 NK-lysin with 86% amino acid sequence identity. The main difference between type 1 and type 2 NK-lysin is at the C-terminus where the type 2 NK-lysin was truncated by acquisition of a termination codon. Sequence variation from AAG in type 1 NK-lysin to TAG in type 2 NK-lysin made the open reading frame shorter by 19 amino acids. Type 3 NK-lysin is more divergent, sharing 54.6% and 49.6% amino acid identities to type 1 and type 2 NK-lysin, respectively (Fig. 3).

Similar to many antimicrobial peptides, such as defensins, hepcidins, and LEAP-2 (Bao et al., 2005, in press; for a review, see Douglas et al., 2003a,b), NK-lysin is rich in cysteine residues. Sequence alignment indicated that, for the most part, six cysteine residues were well conserved in all three types of the catfish NK-lysin (Fig. 3).

3.2. Assigning NK-lysin to BACs

Bacterial artificial chromosome libraries serve as the basis for BAC-based physical maps. In channel catfish, a physical mapping project has been initiated. Therefore, mapping genes to linkage maps and assigning the same genes to BACs is of interest for the alignment of physical and linkage maps. In addition, assigning genes to BACs would allow comparative genome analysis in the genome neighborhood. As part of comparative genomics efforts in channel catfish, we are interested in assigning known genes to BACs. Initially, a pair of overgo probes was designed based on the previously reported cDNA sequences. The overgo hybridization resulted in the identification of 29 positive BACs. As we later realized the sequence divergence of type 3 NK-lysin, a new pair of overgo probes was designed (Table 1). A second round of overgo hybridization using type 3 overgo probes resulted in the identification of additional five NK-lysin-positive BACs. Altogether, hybridization of two pairs of NK-lysin overgo probes to high-density 10× genome coverage BAC filters resulted in identification of 34 positive BAC clones, and subsequent Southern blot analysis confirmed that all 34 BACs contained NK-lysin genes (Table 2).

3.3. Initial determination of copy number of the NK-lysin gene

In order to determine the gene copy numbers, BAC DNA from the positive clones were isolated and subjected to Southern blot analysis. Initially, we used a 4-bp cutter *Rsa*I with the consideration that the 4-bp cutter should provide a high resolution of fingerprinting. However, all the BACs exhibited identical Southern blot patterns using *Rsa*I (data not shown). Afterwards, two 6-bp cutters, *Eco*RI and *Dra*I, were used for the BAC-based Southern blot analysis. As we previously described, determination of gene copy numbers using BAC-based Southern blot depends on the number of restriction patterns, not the number of bands (Bao et al., 2005, in press; Xu et al., 2005). As shown in Fig. 4, three patterns of restriction fingerprint were obtained using either *Eco*R1 or *Dra*I. Initially, this may indicate the presence of three copies of NK-lysin genes in the catfish genome. However, examination of the BAC-based Southern blot suggest that, in the case of *Eco*R1, the majority of BAC clones had two bands with sizes of approximately 8.0 kb and 4.0 kb, whereas the remaining BAC clones had either the 4.0 kb band or the 8.0 kb band. The same pattern was found with *Dra*I restriction enzyme (Fig. 4). This result suggested a scenario of tandem arrangement of the NK-lysin genes. In most of the BAC clones, all copies of NK-lysin are included, while in some BAC clones, only one or two copies of NK-lysin gene(s) were included, thereby missing part of the complete Southern blot bands. The Southern blot data alone, however, could not differentiate if two or three copies of NK-lysin genes exist in the catfish genome, considering that three types of cDNA have been identified.

Table 2
NK-lysin-positive BAC clones as determined by screening of BAC filters using overgo probes

NK-lysin BAC clones including both copies of the duplicated NK-lysin genes	004_i06, 076_L03, 004_k22, 084_k10, 006_L23, 108_p08, 010_i19, 110_L23, 013_L18, 111_n11, 015_a16, 115_m14, 018_p10, 136_L07, 021_n08, 138_d21, 025_L03, 141_c09, 043_j23, 145_p03, 044_f01, 168_k21, 050_i21, 169_k18, 053_p04, 174_e24, 057_i19, 178_j24, 064_c03, 044_g1, 028_L03, 016_L18, 046_j02, 080_L10

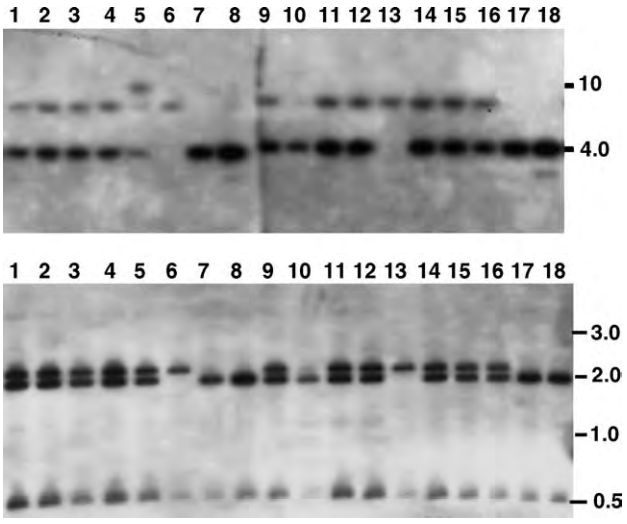

Fig. 4. Southern blot analysis using BAC clones. BAC DNAs were digested with *Eco*RI (A) and *Dra*I (B), electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized to NK-lysin cDNA probes. Lanes 1–18 contained BAC DNA from 18 different BAC clones. Molecular weight is indicated on the right margin.

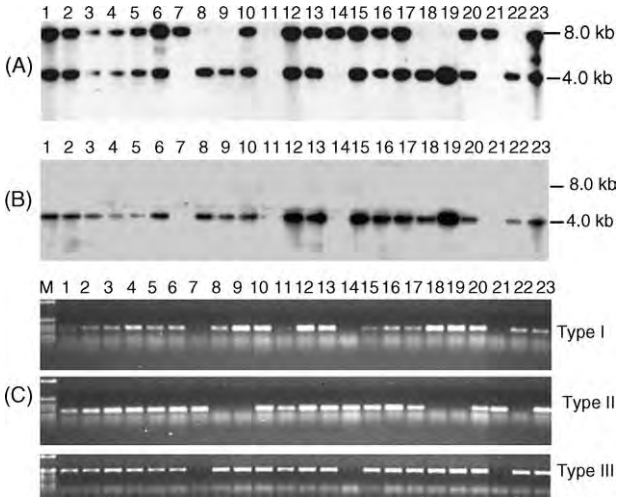

Fig. 5. Demonstration for the presence of three tandem NK-lysin genes in channel catfish: (A and B) Southern blot using gene-specific probes, and (C), gene-specific PCR analysis. In (A) and (B), all BACs were digested with restriction endonuclease *Eco*R1, electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized to NK-lysin gene-specific probes: (A) probes specific for NK-lysin gene 1 and gene 2 and (B) probes specific for NK-lysin gene 3. Note that hybridization pattern with gene 3-specific probes (B) superimposed to the lower band in (A). (C) Gene-specific PCR analysis using specific PCR primers for gene 1 (upper panel), gene 2 (middle panel), and gene 3 (lower panel). Lanes 1–23 contained BAC DNA from 23 different BAC clones. Molecular weight is indicated on the right margin.

3.4. The presence of triplicate NK-lysin genes in channel catfish

The restriction fragment patterns of the BAC-based Southern blot strongly suggest the presence of tandem copies of NK-lysin in the same genomic neighborhood, but failed to reveal the copy number of NK-lysin genes in channel catfish. Thus, the determination of copy numbers required additional tests. The sequence divergence of type 3 NK-lysin provided the possibility of gene-specific Southern blot hybridization analysis, but type 1 and type 2 NK-lysin were too similar to be differentiated by hybridization. We therefore conducted two stringent Southern blots using probes from highly divergent sequence regions. As shown in Fig. 5, probes specific to type 1 and type 2 NK-lysin genes produced a very similar Southern blot band pattern as shown in Fig. 4; both the 4.0 kb and the 8.0 kb *Eco*R1 bands hybridized to the probe (Fig. 5A). However, only the 4.0 kb fragment hybridized to the type 3 NK-lysin specific probe. This suggested that type 3 NK-lysin gene was included in the 4.0 kb *Eco*R1 fragment, whereas type 1 and type 2 NK-lysin genes were included in 4.0 kb and 8.0 kb fragments. It is noteworthy that it is possible that two 4.0 kb fragments could co-locate within the 4.0 kb band. In order to demonstrate the presence of three copies of NK-lysin genes in channel catfish, gene-specific PCR was conducted using BAC clones as templates. PCR primers were designed at regions that would specifically amplify a specific type of NK-lysin. As shown in Fig. 5C, in correspondence with the Southern blot patterns, the BAC clones with both the 4.0 kb and 8.0 kb fragments hybridizing to the NK-lysin probes supported PCR amplification of all three types of NK-lysin; the BAC clones with only the 8.0 kb fragment hybridizing to the NK-lysin probes supported amplification of only type 2 NK-lysin, whereas the BAC clones with only the 4.0 kb fragment hybridizing to the NK-lysin probes supported amplification of both the type 1 and type 3 NK-lysin. Taken together, these data strongly suggested the presence of three copies of NK-lysin genes in the catfish genome: type 1 and type 3 NK-lysin genes were included in the doublet 4.0 kb *Eco*R1 fragment, and type 2 NK-lysin was included in the 8.0 kb fragment.

3.5. Genomic sequencing of the three types of NK-lysin genes

Initially, genomic sequencing was attempted by direct sequencing of BAC clones by using primers designed from NK-lysin sequences. Considering the complication of tandem NK-lysin genes contained in the BAC clones, we chose BAC clones with only one positive band (in the Southern blot using *Eco*R1) for sequencing. Direct sequencing was readily achieved with BAC clones with only 8.0 kb band hybridizing to the NK-lysin probes. However, it was nearly impossible to directly sequence BACs with 4.0 kb hybridizing to NK-lysin or both 4.0 kb and 8.0 kb hybridizing to the NK-lysin probes, also suggesting the inclusion of more than one NK-lysin genes in these BACs. The 4.0 kb and 8.0 kb fragments were then subcloned into a pBluescript SK^+ vector for sequencing. As expected, sequencing analysis revealed three different genomic sequences corresponding to the three types of NK-lysin genes. The complete nucleotide sequences of the three NK-lysin genes have been deposited to GenBank with Accession numbers of DQ153188, DQ153189, and DQ153190. The sequence information obtained from a single BAC clone provided the ultimate proof for the presence of three tandem copies of NK-lysin genes on the same chromosome in the channel catfish genome.

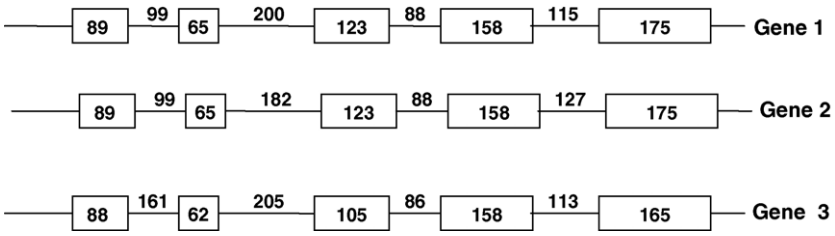

Fig. 6. Comparison of gene structure of the three catfish NK-lysin genes. Exons are indicated by rectangles and introns by lines. The sizes of the exons and introns are indicated by the numbers in their respective positions.

The channel catfish NK-lysin gene has five exons and four introns, a feature that appears to be conserved throughout NK-lysin gene organization (Fig. 6). Only a few NK-lysin genes have been reported, but the five exons/four introns structural feature is conserved between the catfish gene and the human gene (Houchins et al., 1993). However, in humans, alternative splicing of granulysin gene led to the identification of two isoforms of granulysin referred to as NKG5 and 519. The two transcripts are encoded by the single copy human granulysin gene located on chromosome 2 (Manning et al., 1992), but in one of the isoforms, a part of intron 1 was used as exon in the other isoform (Manning et al., 1992). The three catfish NK-lysin genes share the conserved gene organization, although the sizes of exons and introns vary (Fig. 6). Overall, type 1 NK-lysin is highly similar to type 2 NK-lysin with all five exons having identical sizes. Even the introns of the type 1 and type 2 NK-lysin genes have conserved sizes, as well as conserved sequences. Type 3 NK-lysin, however, was highly divergent from the other two types of NK-lysin. Among the five exons, only exon 4 had the identical sizes among all three types of NK-lysin. Type 3 NK-lysin differs from the other two types of NK-lysin in size of all other exons and introns. In terms of sequence identity, the type 3 NK-lysin was only conserved in exon sequences whereas the intron sequences were very different.

3.6. Expression of NK-lysin genes

RT-PCR analysis using total RNA from various normal tissues of channel catfish indicated that the NK-lysin gene exhibited tissue-specific expression (Fig. 7). Several general statements can be made concerning expression of the three types of NK-lysin genes: (1) not all tissues express NK-lysin genes, (2) expression levels differ in various tissues or organs, and (3) the three types of NK-lysin exhibit different expression profiles. It is clear from the RT-PCR data presented in Fig. 7 that the expression patterns of type 1 and type 2 are similar but the level of expression varies, while expression pattern of type 3 is quite different. Type 1 and type 2 were expressed highly in the gill, head kidney, intestine, spleen, and trunk kidney, but expressed at lower levels in the liver, skin, stomach, and muscle. No expression of type 1 and type 2 NK-lysin was detected from the ovary. For type 3 NK-lysin, a different expression pattern was observed. Particularly, no expression was detected from ovary, skin, and stomach, while expression in the muscle was extremely low. Since the organs under study may contain various proportions of different types of leukocytes, we speculate that the NK-lysin

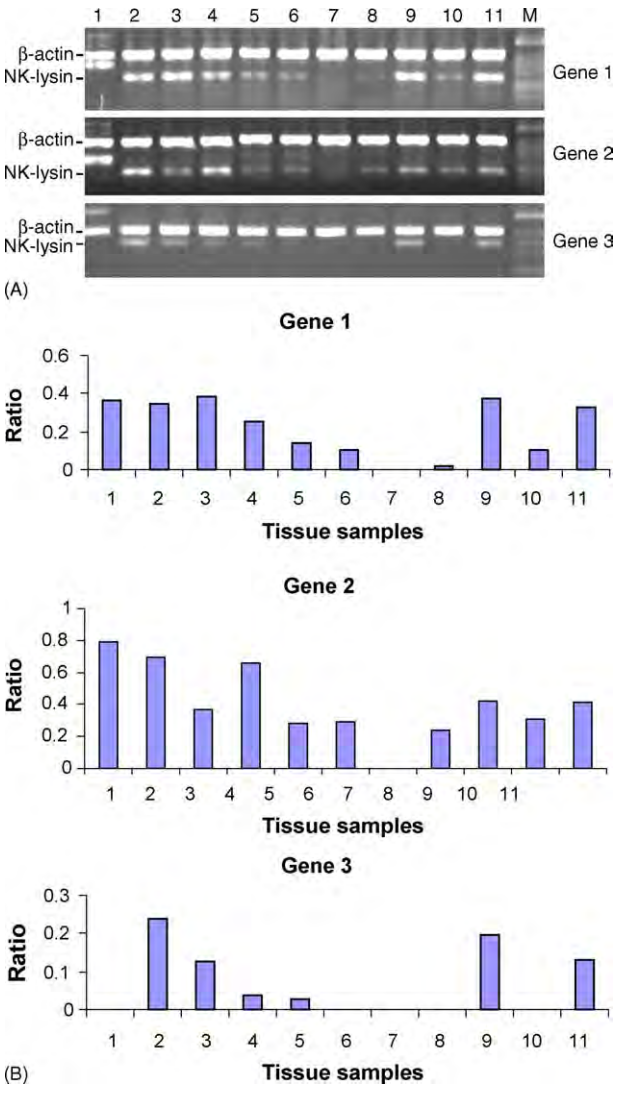

Fig. 7. (A) RT-PCR analysis of NK-lysin gene expression in various tissues. Gene-specific PCR was conducted using gene-specific primers to analyze expression of the three NK-lysin genes in various tissues. RT-PCR products were analyzed on agarose gels. Tissues were specified on the top of the gel, with 1 being brain; 2, gill; 3, head kidney; 4, intestine; 5, liver; 6, muscle; 7, ovary; 8, skin; 9, spleen; 10 stomach; 11, trunk kidney, and molecular weight (MW). The positions of the RT-PCR amplified bands of β-actin and NK-lysin are indicated on the left margin. (B) Quantitative analysis of RT-PCR results as presented in (A) using GelExpert software. Tissue samples are the same as under (A). Ratio refers to the RT-PCR product of NK-lysin genes as compared to that of β-actin.

Table 3
The numbers of NK-lysin-related ESTs identified from dbEST using BLAST searches

	Jx13 library (mixed leukocytes)	Jx14 library (macrophage)	Spleen library	Total
Type 1 NK-lysin ESTs	8	0	3	11
Type 2 NK-lysin ESTs	10	3	0	13
Type 3 NK-lysin ESTs	87	54	1	142
Total number of NK-lysin like ESTs				166

Jx13 was made from a week-old catfish mixed leukocyte culture, designated MLC52-1, that presumably contains NK-like cells (Stuge et al., 2000). Jx14 was made from the catfish autonomous (immortal) macrophage cell line 42TA (Miller et al., 1994b). The spleen cDNA library was previously reported (Kocabas et al., 2002).

genes may exhibit strong tissue or cell type specificity. This speculation is supported by the BLAST search results of the NCBI's dbEST database. Searches using NK-lysin as queries produced 166 ESTs with a significant level of identities to NK-lysin. Interestingly, eight type 1 NK-lysin ESTs were identified from Jx13 cDNA library made from a week-old catfish mixed leukocyte culture, designated MLC52-1, that presumably contains NK-like cells (Stuge et al., 2000), but none was identified from the Jx14 cDNA library made from the catfish autonomous (immortal) macrophage cell line 42TA (Miller et al., 1994). This may suggest that type 1 NK-lysin is not expressed from macrophage cells, but expressed highly in NK cells. In contrast, type 2 and type 3 NK-lysin were identified from both libraries, though a larger number of ESTs were identified from the mixed leukocyte library Jx13 (Table 3).

4. Discussion

In this work, we report three catfish NK-lysin genes. The NK-lysin gene cDNAs were identified, sequenced, and characterized. The catfish NK-lysin genes were found to be tripled in tandem and contained in the same BAC clones. The catfish NK-lysin genes exhibited tissue-specific and different profiles of expression, suggesting spatial partitioning of the tripled NK-lysin genes.

The characterization of the channel catfish NK-lysin genes is a part of the larger effort to elucidate the molecular components of the catfish innate immune system. Much has been learned in recent years concerning genes involved in both the innate and adaptive immunity (e.g., Wilson et al., 1997; Miller et al., 1998; Antao et al., 2001; Hawke et al., 2001; Zou et al., 2003). Recently, we have characterized a set of 27 CC chemokines (He et al., 2004; Peatman et al., 2005), 7 CXC chemokines (Baoprasertkul et al., 2004, 2005; Chen et al., 2005), and 4 antimicrobial peptides (Bao et al., 2005, in press; Xu et al., 2005; Wang et al., 2006). In this work, we further characterized genomic structure and organization of three NK-lysin genes. Such information will facilitate understanding of gene evolution and genome evolution as more information will soon become available from genome sequences along the evolutionary spectrum.

In spite of unavailability of sequences in many of the intermediate species, the conservation of five exons and four introns in channel catfish and humans suggest that the gene structure and organization was well conserved through evolution. Other conserved features of NK-lysin genes include six well-conserved cysteine residues and the relative size and presence of a high proportion of charged amino acids in the protein. The human granulysin was found to possess a structure of the five-helical bundles resembling other "saposin folds" (such as NK-lysin) (Anderson et al., 2003). Well-conserved features involved in secondary structure formation, such as the sulfite bonds in the catfish NK-lysin, could imply its conserved structure and thereby its function as an antimicrobial peptide. While the antimicrobial activities of catfish NK-lysin remain to be determined, its gene features, such as the conservation of the cysteine residues, size and sequence similarities to the mammalian NK-lysin genes, suggest their potency against microbial organisms.

An interesting finding of this work is that the catfish NK-lysin gene was present in a tandem triple, in contrast to the single copy gene in humans. Questions remain as to when during evolution gene duplication occurred. This question can soon be answered as more genome information is available from various species in the evolutionary spectrum. As the genomes of bovine, swine, and chickens are to be finished in the near future, this question may soon be answered accordingly. In the humans, NK-lysin gene is present as a single copy gene located on chromosome 2. In *Fugu rubripes*, a TBLASTN search using the channel catfish NK-lysin as a query revealed a single hit of NK-lysin in the *Fugu* genome within the whole genome shotgun assembly SCAFFOLD_6758, suggesting the presence of a single copy of NK-lysin gene in the pufferfish. BLAST searches against the zebrafish genome draft sequence (Zv4) indicated the presence of multiple NK-lysin genes (Table 4). Altogether, there appear to be seven copies of NK-lysin genes in the zebrafish genome with a similarity *p*-value smaller than 10^{-9} as compared to the catfish NK-lysin genes. Six copies exist on chromosome 17, while one additional copy exists on chromosome 5. Interestingly, on chromosome 17, the zebrafish NK-lysin genes are organized in triple tandems as three copies of the zebrafish NK-lysin genes are located within contig Dr17_WGA1317_1 with approximately a distance of 10 kb and 11 kb separating the three genes (Table 4). Very similarly, three more copies are located within contig Dr17_WGA1316_1 with approximately 15.7 kb and 10 kb separating the three genes.

The genome context neighboring NK-lysin gene(s) of humans and zebrafish does not have conserved syntenies. In the human chromosome region containing NK-lysin gene, there are SFTPB (surfactant, pulmonary-associated protein B, approximately 35 kb away), ubiquitin specific protease 39 (approximately 65 kb away), hypothetical LOC388969 (approximately 85 kb away), fasting-inducible integral membrane protein TM6P1 (approximately 95 kb away), hypothetical protein

Table 4
BLAST search results of the draft zebrafish genome sequence using the channel catfish NK-lysin deduced amino acid sequences as a query

Contig	Chromosome	Sequence position in the contig	Distance between the neighboring NK-lysin gene (kb)	Homologous region (amino acids)	*p*-Value of similarities
Dr17_WGA1317_1	17	30946–30767		78–130	1e−14
	17	40770–40615	10	78–129	6e−11
	17	51628–51479	11	80–129	3e−11
Dr17_WGA1316_1	17	518908–519057		80–129	3e−11
	17	534602–534757	15.7	78–129	6e−11
	17	544587–544745	10	Aa78–130	2e−13
Dr5_WGA336_1	5	2260288–2260443		78–129	6e−11

The TBLASTN program was used to search against the genome sequence of zebrafish (Zv4). Hits with *p*-values smaller than 1×10^{-9} were recorded.

LOC51255 (approximately 100 kb away) vesicle-associated membrane protein 5 (approximately 110 kb away), and vesicle-associated membrane protein 8 (approximately 120 kb away); and on the other side of the chromosome, the human NK-lysin gene is neighboring a gene homologous to the Drosophila atonal homolog 8 (approximately 70 kb away). In the zebrafish genome containing the NK-lysins, either on chromosome 17 or chromosome 5, a totally different set of genes exists in the genome context, suggesting that major reorganizations could have occurred during evolution.

In channel catfish, type 1 and type 2 NK-lysin appeared to be recently duplicated from each other since not only the exon numbers were identical, their sizes, as well as sequences were well conserved. Even the intron sequences were highly conserved between the two genes. The type 3 NK-lysin, however, was highly divergent, suggesting a long history after gene duplication. Although we did not directly analyze the physical distances between the NK-lysin genes, type 1 and type 3 NK-lysin genes appear to be closely linked together as they were always identified together in the positive BAC clones. Type 2 NK-lysin was more physically distant. All 23 BAC clones tested harbor type 1 and type 3 NK-lysin, of which five BACs (21.7%) lack type 2 NK-lysin, suggesting that type 2 NK-lysin could be statistically over 40 kb away from the other two NK-lysin genes, given that the average sizes of the catfish BACs are 161 kb (http://bacpac.chori.org/library.php?id=103). However, the exact distances between the tandem NK-lysin genes were not determined in this work.

In this work, we examined expression profiles of NK-lysin genes using gene-specific primers. Differences in expression were found between the NK-lysin genes. In particular, type 1 and type 2 NK-lysin appeared to have a highly similar expression pattern, whereas type 3 NK-lysin exhibited slightly different expression pattern. It is interesting that all three types of NK-lysin appeared to be transcribed in the brain, but were not properly spliced (Fig. 6). It should be interesting to investigate how the processing of NK-lysin gene RNA is controlled at the molecular level in the brain. The spatial differences in expression could indicate spatially partitioned subfunctions, but that does not exclude the possibilities of neofunctions or temporally partitioned subfunctions (Torgerson and Singh, 2004; Lynch and Katju, 2004; Postlethwait et al., 2004; He and Zhang, 2005).

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