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MARINE BIOTECHNOLOGY

Cloning of Two Crustacean Hyperglycemic Hormone Isoforms in Freshwater Giant Prawn (*Macrobrachium rosenbergii*): Evidence of Alternative Splicing

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Abstract: A full-length *chh* cDNA was cloned from the eyestalk of *Macrobrachium rosenbergii.* The 991-bp cDNA contains an open reading frame of 408 bp that encodes the prepro-CHH. The tissue-specific expression pattern was examined by reverse transcriptase-polymerase chain reaction. Positive signals were detected in the eyestalk, heart, gills, antennal glands, and thoracic ganglion but not in muscle and hepatopancreas. However, two types of products were observed. The nucleotide sequences revealed the existence of 2 *chh* transcripts, named *chh* and *chh-l*, respectively. Direct sequence evidence suggests that these two isoforms come from a *Chh* gene transcribed in an alternative splicing manner. The *Mar-Chh* gene consists of 4 exons. The eyestalk transcript (*chh*) contains exons I, II, and IV, whereas the *chh-l* transcript in heart, gills, antennal glands, and thoracic ganglion contains all 4 exons. The appearance of exon III in *chh-l* cDNA changes the sequence content in the latter half of the mature peptide, starting within the codon of the 40th residue, arginine. The amino acid sequence deduced from exon III matched no homologue in public protein databases, while the 2 cysteine residues in this segment preserved the positional conservation characters of CHH neuropeptide family members. The common organization of *Chh* genes between palaemonid, brachyuran, and astacus crustaceans suggests that the *Chh* gene has a 4-exon structure in these species.

Keywords: CHH, CHH-L, noneyestalk expression, secondary structure prediction, gene structure.

INTRODUCTION

The homeostasis of glucose metabolism is of great importance in multicellular organisms. Circulating glucose, as the energy fuel and building block for the maintenance of physiologic processes of growth, reproduction, and stress response, is regulated by the coordination of neural and endocrine systems. In crustaceans, an eyestalk neuropeptide, crustacean hyperglycemic hormone (CHH), was named for its diabetogenic function (Abramowitz et al., 1944). This neuropeptide was later isolated and characterized in many decapod species. Injection of the eyestalk extract or purified CHH will remedy hypoglycemia in eyestalkless individuals by regulating phosphorylase and glycogen synthase activities on the target tissues, as well as

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amylase secretion of the midgut gland (Sedlmeier, 1982; Keller and Sedlmeier, 1988). CHH is released in response to internal signals, such as the circadian rhythmicity of hemolymph glucose content (Kallen et al., 1990; Santos and Keller, 1993b), or to external stimuli, such as hypoxia (Santos and Keller, 1993a; Webster, 1996), parasite infection (Stentiford et al., 2001), thermal shock (Santos et al., 1997; Kuo and Yang, 1999), and pollutants (Reddy et al., 1994, 1996; Lorenzon et al., 1997, 2000). In addition to carbohydrate metabolism, CHH was proved to be involved in lipid metabolism in *Chasmagnathus granulata*, *Carcinus maenas,* and *Orconectes limosus* (Santos et al., 1997).

In spite of its primary role in energetic regulation, CHH has been demonstrated to be pleiotropic. Two CHH isoforms of the American lobster *Homarus americanus*, CHH A and CHH B, were markedly elevated in hemolymph soon after molting and upon the onset of vitellogenesis (de Kleijn et al., 1998). CHH-B was noted especially for its potency to stimulate oocyte growth (Tensen et al., 1989). CHH was also demonstrated to regulate both methyl farnesoate synthesis in the mandibular organs and hemolymph glucose content in the spider crab *Libinia emarginata* (Liu and Laufer, 1996), to suppress ecdysteroid synthesis on Y organ in *Procambarus clarkii* and *C. maenas* (Webster and Keller, 1986; Yasuda et al., 1994), and to display both hyperglycemic and molt-inhibiting activities in astacuran and penaeid species (Chang et al., 1990; Sefiani et al., 1996). Moreover, Pierrot et al. (2000) showed the involvement of CHH in the control of branchial ionic transport in gill perfusion experiments.

The multiplicity of CHH isoforms is also the subject of controversy. Two CHH isoforms from American lobster were reported to exert different biological potencies, as mentioned above, though they only differ in several residues. Even more CHH isoforms are being identified, purified, and characterized from penaeid shrimps. Seven CHH-related eyestalk peptides were characterized in *Marsupenaeus japonicus*, of which 6 (SGP I–III, V–VII) possessed hyperglycemic potency (Yang et al., 1996, 1997; Gu and Chan, 1998). The D/L isomerization on the third amino acid residue, phenylalanine, was observed in CHHs from several astacuran species (Yasuda et al., 1994; Soyez et al., 1994, 2000; Aguliar et al., 1995; Ollivaux and Soyez, 2000). The bioactivity assay data further suggested the different potencies of these enantimoerized isomers. Dircksen et al. (2001) purified a novel CHH isoform from the pericardial organs of *C. maenas*, named PO-CHH. They proposed that the PO-CHH would be the alternative transcribed product of multiple genes without typical CHH activities.

Reports on the noneyestalk sources of CHHs are still limited. A significant elevation of hemolymph CHH was observed in American lobsters that had been eyestalk-ablated for an extended period (Chang et al., 1998). Immunologic data further suggested that CHH was widely expressed in the central nervous system, and the CHH-secretory cells were localized in the second root of the thoracic ganglion and subesophageal ganglion (Chang et al., 1999). Similarly, the isoform CHH B of the sand shrimp *M. ensis* was expressed in the central nervous system (Gu et al., 2000). The *chh* transcript detected in the gut of C. *maenas* was identical to that expressed in the eyestalk, and the possible role of CHHs in the hydration process in postmolting stages was suggested (Chung et al., 1999). Dircksen et al. (2001) reported an unusual spliced isoform of *Chh* gene expressed in the pericardial organs in the shore crab *C. maenas.*

In this study we cloned the full-length *chh* cDNA (*Mar-chh*) of *Macrobrachium rosenbergii* from the eyestalk. A *chh*-like cDNA (*Mar-chh-l*) was observed in some tissues other than the eyestalk, such as heart, gills, antennal glands, and thoracic ganglion, with perfect positional conservation of the cysteine residues in its deduced amino acid sequence. Direct cloning data revealed the possibility that an alternative splicing event in the *Chh* gene transcription formed these two transcripts. This is the first report of gills and antennal glands expressing a *Chh* gene in the form of a *chh-l* transcript. This is also the first report of the thoracic ganglion as a site expressing *chh-l* messenger RNA. The exon and intron structures of several *Chh* genes were further analyzed. The physiologic function of the newly identified CHH isoform remains to be elucidated. Considering the expression site of the transcript, we propose that CHH-L may be involved in ionic or osmotic regulation, volume control, or acid-basic balance.

Materials and Methods

Sample Collection

Freshwater giant prawns (*M. rosenbergii*) were collected from culture farms in southern Taiwan. Eyestalks and other tissues including hepatopancreas, muscle, gills, heart, thoracic ganglion, and glands were dissected and frozen immediately in liquid nitrogen. The collected samples were all stored at −80°C until use.

RNA Preparation and RT-PCR

Total RNA was extracted from frozen samples following the method described by Sambrook and Russell (2001). The RNA pellet was redissolved in DEPC-treated water, adjusted to 1 μg/μl in concentration. Messenger RNA was purified using a Quickprep mRNA purification kit (Pharmacia) following the manufacturer's instructions.

In order to clone *chh* transcripts from *M. rosenbergii* eyestalks, degenerated nested primers were designed from the consensus analysis of previous results (Lin et al., 1998) and some known CHH sequences. First-strand cDNA was synthesized from 1 μg of total RNA or 0.1 μg mRNA in a final volume of 25 μl containing 1× reverse transcriptase (RT) reaction buffer (Life Gibco), 500 μM dNTP, 1 μg RT primer (T_1: 5′-GTTGCCGACGACGAGCCTAC-T_{12}-3′, or gene-specific primer), 200 units of SuperScript II Reverse Transcriptase (Life Gibco), and 40 units of RNAsin (Promega). Upon addition of the enzymes, the reaction mixture was incubated at 42°C for 60 minutes, terminated at 70°C for 15 minutes, and then stored at −20°C.

Polymerase chain reactions (PCRs) were performed on 1 μl of RT product as template. The reaction mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 250 μM dNTP, 1 unit of VioTaq DNA polymerase (Viogene), 0.5 μg degenerate primer 5-1 (5′-GC(C/T)AT(C/T)CT(G/C)GA(C/T)GA(C/T)CA(A/G)GC-3′), and 0.5 μg degenerate primer 3-1 (5′-AC(C/T)GA(G/C)CT(A/G)CT(A/G)GA(G/C)GA(G/C)-3′). Amplification was performed in a DNA thermal cycler (GeneAmp System 2400, PE Applied Biosystems) with 35 cycles of 94°C denaturing for 1 minute, 50°C annealing for 1 minute, and 72°C elongation for 2 minutes, ending with 7 minutes of incubation at 72°C of final extension. The PCR products were analyzed on 1.5% agarose gel in 1× TBE. Amplified fragments in the expected size range were ligated into pGEM-T easy vector (Promega), and then were transformed into *Escherichia coli* XL1-blue competent cells. Ampicillin and IPTG/X-gal double-selected colonies were picked and checked for the insertion size. The colonies containing plasmid with adequate inserts were purified with a mini-M plasmid purification kit (Viogene) for autosequencing. The standard protocols suggested by the manufacturer (PE Applied Biosystems) were followed for the DNA autosequencing reaction.

The CHH-specific primer set 5a/3a (5a, 5′-GCCATCCTCGACCAGTCC-3′; 3a, 5′-GGATTTACTTCCCGACGAT-3′) and 5b/3b (5b, 5′-AGGTATCTTCGACCGTGAG-3′; 3b, 5′-TCCTGGATGCACTGTCGGA-3′) were designed to detect the *chh* transcripts from various tissues. The RT-PCR conditions in the detection procedures were adjusted in 40 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72° C for 2 minutes, followed by 7 minutes of incubation at 72°C as a final extension.

3′ RACE and 5′ RACE

In 3′ rapid amplification of cDNA ends (RACE), primer T_1 was used for priming the RT reaction of the poly(A) tail containing total RNA molecules from the eyestalk. PCR amplification was performed on 1 μl of RT product, 0.1 μg of gene-specific primer 5a, 0.1 μg of T_1 primer, and serially diluted anchored primer T_2 (5′-GTTGCCGACGACGAGCCTAC-3′). The same PCR conditions described above were followed. The second run of PCR amplification was performed using the same PCR conditions with gene-specific primer 5b and the anchor primer T_2. The amplified fragment was cloned and sequenced as described.

The RT reaction for 5′ RACE was performed as described above except the RT primer T_1 was replaced by the primer 3ex (5′-GCAGATGAGAGGGACTGA-3′), designed from the 3′ RACE sequence data. The RT product was electroeluted and precipitated. Poly(A) tail was added onto the 3′ end of the first-strand cDNA product by terminal transferase (Stratagene) following the manufacturer's protocol. The PCR amplification was primed by 1 μg of gene-specific primer 3a, 0.01 μg T_1 primer, and 0.1 μg T_2 primer, using the same conditions described above. Secondary PCR was performed under the same PCR conditions with primer 3b and T_2. The amplified fragment was cloned and sequenced as described above.

Genomic Library Construction and Screening

The genomic DNA library of *M. rosenbergii* was constructed using Zap Express Predigested Gigapack Cloning Kit (Stratagene). Frozen hepatopancreas from a single individual was ground into powder with mortar and pestle under liquid nitrogen. After the powder was resuspended and dissolved in digestion buffer (10 mM Tris-HCl, pH 7.8, containing 5 mM EDTA, 0.5% sodium dodecylsulfate, 100 μg/ml proteinase K), it was incubated at 50°C overnight. DNA was extracted by phenol saturated with STE (10 mM

Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl) and with phenol-chloroform, twice each. Genomic DNA was precipated by an equal volume of ice-cold isopropanol, air-dried, and redissolved in TE buffer.

The procedure and instructions suggested by the manufacturer for genomic library construction were followed. Briefly, genomic DNA was partially digested by *Eco*RI. The digested DNA fragments were mixed with predigested vector arms and ligated overnight at 16°C. Phage packing product was titered and harvested. The genomic library was screened for *Chh* gene with ^{32}P-labeled probe prepared from the partial *chh* cDNA fragment cloned in this study. Positive plaques were picked and replated for secondary screening. Single phage plaques carrying a *Chh* gene were picked and transfected into XL1-Blue MRF$^-$ competent cells, and the phagemid was harvested and sequenced.

Sequence Data Processing and Bioinformatic Analysis

Simple assembly of the successive sequence data and the amino acid sequence alignment for primer design was performed on Macaw, a local alignment software provided by NCBI (Charles et al., 1993). The exon and intron organization of the *Chh* gene was determined by comparing cDNA sequences with the genomic sequence through the National Center for Biotechnology Spidey server (*http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/index.html*; Wheelan et al., 2001).

Conceptual translation was performed in Translator, an application of GCG package in Web interface (Seqweb, *http://gcg.nhri.org.tw:8001*, Wisconsin Package Version 10.1). In order to determine the open reading frame starting site, a translational initiation site (TIS) analysis was performed (*http://www.hri.co.jp/atgpr/*; Salamov et al., 1998). It compares the 5′-flanking sequence of each initiation codon ATG within the sequence to the criteria set by the Kozak rule. The signal peptide cleavage site was predicted on both SignalP server (Version 2.0, *http://www.cbs.dtu.dk/services/SignalP-2.0/*; Nielsen et al., 1999) and SPScan of GCG/SeqWeb.

The deduced amino acid sequences were used to search for homologue sequence in the public database by NCBI-BLAST (*http://www.ncbi.nlm.nih.gov/blast*; Altschul et al., 1997). The genome sequence comparison was performed using the EMBOSS package (Rice et al., 2000), and the protein secondary structure was predicted using the PHD Server (Rost and Sander, 1994).

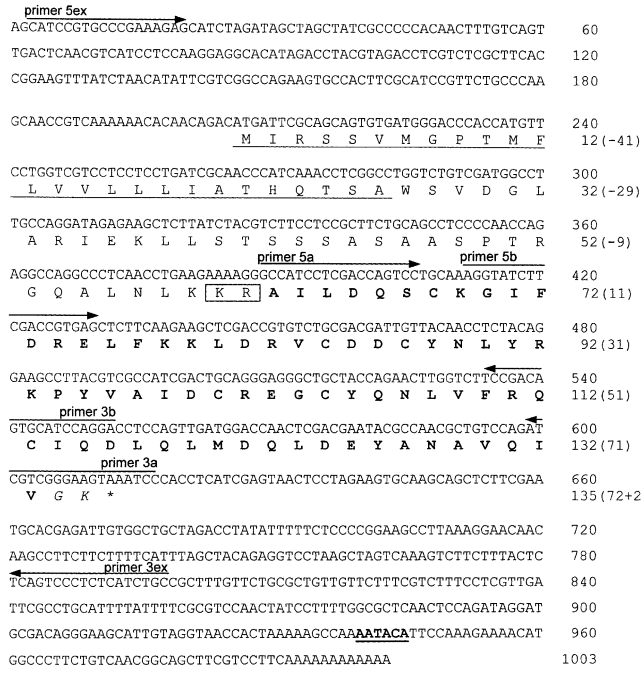

Figure 1. Nucleotide sequence of *Mar-chh* cDNA compiled from 3′ RACE, RT-PCR, and 5′ RACE. Signal peptide is underlined. Dibasic residue recognition site for prohormone convertase cleavage is boxed. Mature CHH peptide fragment is marked by bold characters, and the C-terminal dipeptide processing sequence is italicized. Poly(A) signal is bold face and underlined. The asterisk indicates stop codon. This sequence has been deposited in GenBank under accession number AF219382.

RESULTS

Cloning of *chh* cDNA from Eyestalk of *M. rosenbergii*

Nested PCR was performed on the eyestalk cDNA sample with degenerated primers, and bands in expected sizes were cloned and sequenced. The deduced amino acid sequences of the possible *chh* cDNA candidates fit the partial amino acid sequence previously reported (Lin et al., 1998; Sithigorngul et al., 1999) and the other known characters of CHH family members. Partial sequences of *chh* cDNA were obtained, and *chh*-specific primers for further experiments were then designed.

Full-length sequence of *chh* cDNA was compiled from 5′ RACE, RT-PCR, and 3′ RACE data (991 nucle-

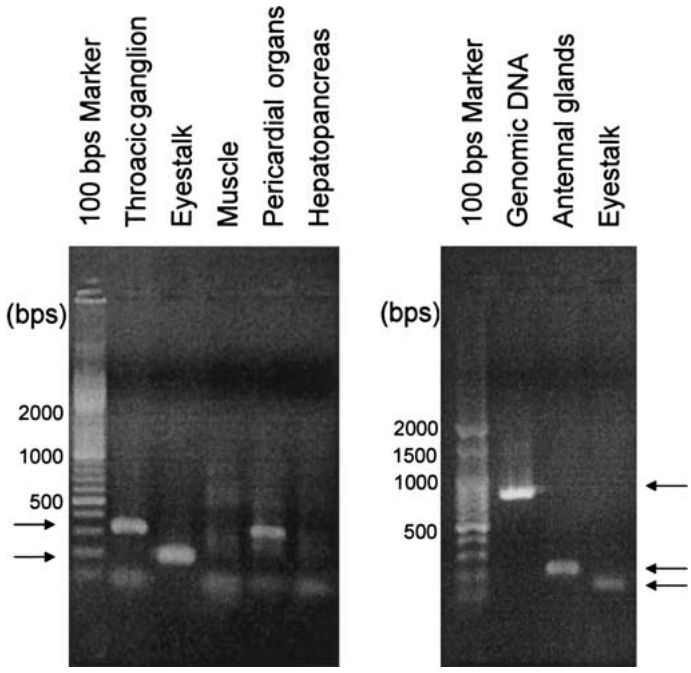

Figure 2. PCR detection of *chh* sequence by *chh*-specific primers. **A:** Tissue expression patterns of *chh* transcript are detected by RT-PCR with primer 5a,3a. cDNA template for each lane is as indicated. **B:** PCR is performed on genomic DNA with primer 5b,3b. Two cDNA samples from antennal glands and eyestalk are run in parallel to check for the size difference.

otides, poly(A) tract not included, Figure 1). Five possible translational initiation sites in sense direction were detected by the translational initiation site analysis. Reading frames of the 3-scoring predictions, 135, 129, and 125 residues, respectively, matched the reported CHH sequence from *M. rosenbergii* (Lin et al., 1998; Sithigorngul et al., 1999). The 3 possible open reading frames (ORFs) were further examined for the appearance of signal peptide on both SPScan and SignalP 2.0. The signal peptide from the longest ORF passed through the McGeoch scan successfully, whereas the other 2 failed in the scan. The dibasic residues "KR" are found in front of the reported mature CHH sequence. Thus the putative prepro-CHH is proposed to be 135 residues, consisting of a signal peptide of 26 residues, a CHH precursor-related sequence (CPRP) of 33 residues, a dibasic residue cleavage signal (KR), the mature CHH segment of 72 residues, and a C-terminal proteinase processing site (GK). It shares many known prepro-CHH characters of other species, including 6 conserved cysteine residues in mature CHH sequences.

The expression of the *Chh* gene in different tissues was detected by RT-PCR (Figure 2). A product of the expected size, 229 bp restricted by the 5a/3a primer set, or 143 bp by the 5b/3b primer set, was found in the eyestalk cDNA, but no band was amplified from the muscle and hepatopancreas samples. However, a larger band was detected in several noneyestalk tissues including heart, thoracic ganglion, antennal glands, and gills (data not shown) using the same PCR conditions. Neither new primer sets nor nested primers could eliminate the size difference of these amplified products. The consistency of repeated data implied the importance of the unknown PCR product. Furthermore, the enlarged size of the PCR products was not due to contamination of genomic DNA, which would generate a much larger product than the one observed here (about 800 bp, Figure 2, B). To clarify the nature of this phenomenon, we cloned the full-length cDNA corresponding to the unexpected PCR product and subsequently the *Chh* gene.

Identification of the Second Transcript of the *Chh* Gene

The full-length cDNA of the larger transcript was cloned from those tissues mentioned above. It revealed that within cDNA length of 1.1 kb there was similarity to and discrepancy from the one cloned from eyestalk. The discrepancy was due to an insertion of 125 bp, starting at the 508th nucleotide in the transcript. Since the 5′ sequence of this larger transcript was identical to the one we cloned and described above, we assumed that this *chh*-like mRNA would be translated at the same start codon. The 125-bp insertion does not stop the reading frame; rather, it contributes a segment of 33 residues to the ORF after the junction point. As a result, the ORF of

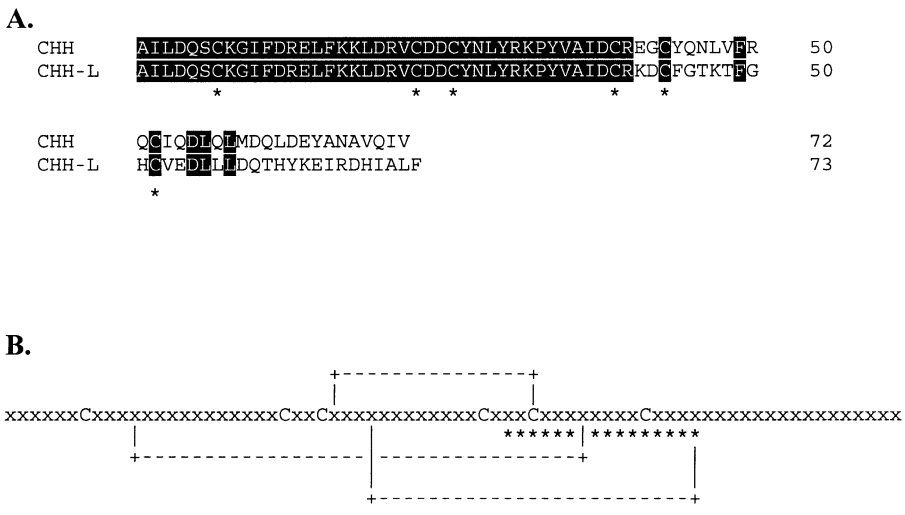

Figure 3. Primary sequence features of Mar-CHH and Mar-CHH-L. **A:** Alignment of putative mature peptide. Identical residues are highlighted. Cysteine residues are marked by asterisks. **B:** Illustration of disulfide bridge linkage of CHH family member modified from CHH family signature description of PROSITE ID:PS01250. The pairings of cysteine residues are (1,5), (2,4), and (3,6).

the larger cDNA encodes a peptide of 134 residues. In the presumption of a common posttranslational processing fate, it encodes a mature peptide of 73 residues, of which the first 40 residues and several in the latter half are identical to those we cloned from eyestalk (Figure 3). Two cysteine residues in the latter half were well preserved. We named the peptide deduced from the *chh* cDNA (cloned from the eyestalk) as CHH, and the peptide from the *chh*-like cDNA (*chh-l*, the larger transcript in the noneyestalk tissues) as CHH-like peptide, and we denoted them as Mar-CHH and Mar-CHH-L, respectively.

Briefly, Mar-CHH is 72 residues in length, 8.5 kDa, with theoretical pI of 4.6, and Mar-CHH-L is 73 residues in length, 8.6 kDa, pI of 6.3. The Kyte-Doolittle hydrophobicity profiles on these two peptides were similar; deviation between the plots was most obvious in the last 10 residues (data not shown). For the first time, gills and antennal glands, known as organs for osmoregulation, pH balance, and volume control, are identified as *chh*-expressing tissues and the thoracic ganglion is reported as a expressing site *chh-l* transcript. In addition, the pericardial organs have been recognized as neuroendocrine organs (Fingerman, 1992), from which a CHH spliced isoform was reported (Dircksen et al., 2001).

Chh Gene Structure

The genomic library was constructed and screened for the *Chh* gene. Two positive phage plaques with 2.5 kb insertion were obtained. They were identical except for the orientation of insertion. Comparing the genome sequence with those from the two cDNAs, we found that it did not cover the gene transcripts completely. Thus the primer set 5ex/3c (5ex, 5′-CATCCGTGCCCGAAAAG-3′; 3c, 5′-CGGAAGGAATAG TAGTGT-3′) was designed to amplify the 5′ part of the *Chh* gene from the genomic DNA, and a 1.5-kb band was obtained. The nucleotide sequences were compiled into a contig in 3.8 kb (Figure 4). Two transcripts matched the genomic sequence perfectly and defined 4 exons and 3 introns out of a 3052-bp segment. *Mar-chh* cDNA contains exons I, II, and IV, while *Mar-chh-l* cDNA contains all 4 exons (Figure 5, A). Exon III is 125 bp in length and only appears in *chh-l* form, which is consonant with the size difference between the PCR products described above. These observations also indicate that the 2 forms of *chh* transcripts originated from the gene but were alternatively spliced.

Dircksen et al. (2001) reported the putative alternative splicing products of the *Chh* genes from *C. maenas*, the PO-CHH and SG-CHH with variants, and the 4-exon structure of *Chh* genes. A further examination of Mar-CHH and Mar-CHH-L elucidated some patterns that can also be found in SG-CHH and PO-CHH of *C. maenas*. The first 40 residues of amino acid sequences are identical, but in the latter half, these peptides share low homology to each other, while the 2 cysteine residues in the latter segment, Cys^{43} and Cys^{52}, are well conserved. In both cases, the 40^{th} residue, arginine, is located in the exon II/exon III or exon II/exon IV junction. Moreover, the trinucleotide code of Arg^{40} in these splicing partners is split; the first 2 nucleotides in the codon are included in exon II, while the wobbled one is in the start of exon III or exon IV. Since we did not remove the pericardial organs from heart in the sampling procedure, the *chh-l* transcript

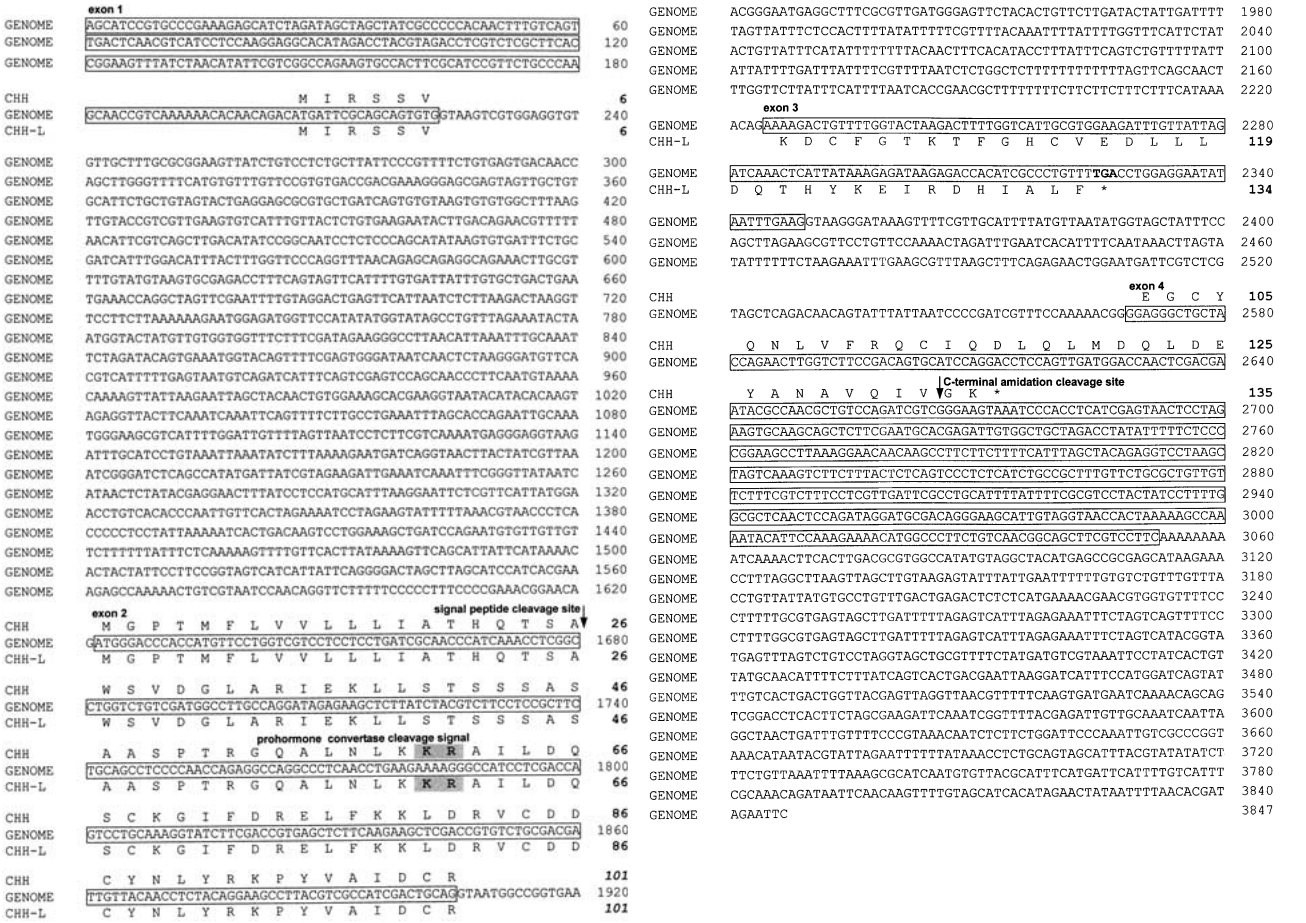

Figure 4. Nucleotide sequence of the genome contig A130968, in which the *Mar-chh* structure gene is included. The amino acid sequences of 2 transcripts are represented above and beneath the coding regions, and the stop codons are marked with asterisks. Exon regions are boxed, and arrows mark several peptide processing sites.

detected in heart may come from these organs and act as the functional homologue to PO-CHH of *C. maenas.* Besides, a *Chh* gene of the crayfish *P. clarkii* (GenBank accession no. AF474409) was annotated to have 2 alternative splicing transcripts. Two peptide sequences are translated from its transcripts conceptually; one is identical to the precursor sequence of the CHH from the species (abbreviated as Prc-CHH in the later description), and the other is described as a thoracic ganglia–specific CHH-like protein precursor sequence (Prc-CHH-L, GenBank accession no. AF474408). We found that the switch-on coding sequences, the split pattern of the codon at the exon-exon junction, and the conservation of cysteine residues on the 2 splicing pairs were exactly same as those observed in this study. Although the cDNA sequences of *Prc-chh* and *Prc-chh-l* are still incomplete, the genome-cDNA alignment suggested that the *Prc-Chh* gene is in a 4-exon structure.

Comparative Genomics

Mar-Chh gene was compared to the most related sequence, the *Chh* gene of *M. lanchesteri (Mal-Chh*, GenBank accession no. AF088854), which is characterized as a 3-exon gene in the sequence annotation. Each exon and intron from *M. rosenbergii* was compared to its counterpart on *M. lanchesteri Chh* gene sequence. The identity scores of exon I, intron I, exon II, intron II, exon III, intron III, and exon IV and their counterparts were 95.5%, 93.4%, 98.6%, 88.8%, 96.0%, 96.3%, and 98.4%, respectively.

A segment of *Mal-Chh* with 96.0% identity to exon III of *M. rosenbergii* was identified. Several single-base deletions were observed in this region. The deduced amino acid sequence on this putative exon contains 2 cysteine residues, as we found in *M. rosenbergii* exon III, but one single-base deletion in the presumed ORF caused the reading frame to shift and a stop codon to appear precociously. Thus the

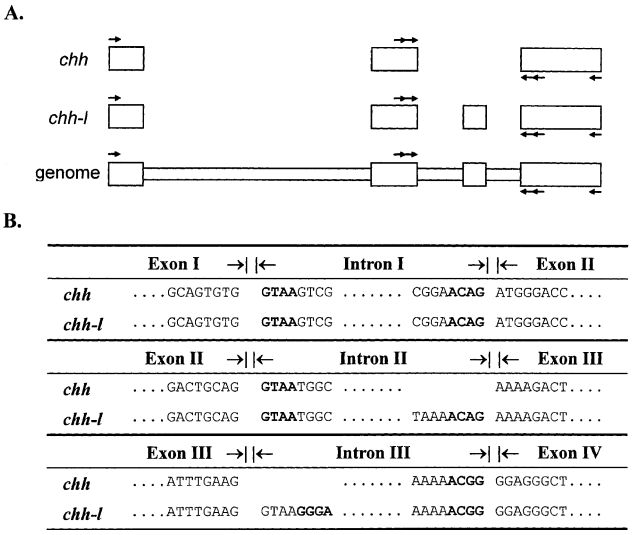

Figure 5. The structure of *Macrobrachium rosenbergii chh* gene. **A:** Alignment of *chh* gene, *chh* cDNA and *chh-l* cDNA. Exons are boxed, left arrows and right arrows mark the locations of gene-specific primers 5ex, 5a, 5b, 3b, 3a, and 3ex, respectively (from left to right). **B:** Nucleotide sequence at the exon-intron splicing boundaries. In *chh* cDNA, exon III is spliced out.

high identity of nucleotide sequence (98% in the coding region of exon III) did not result in high identity in amino acid sequence (84%). Despite the G deletion in the putative intron II/exon III boundary, which caused a violation of the splicing acceptor consensus sequence, and several other mismatched bases that were found, we still propose the existence of the exon III in *M. lanchesteri.* Reexamination of the gene sequence corresponding to this segment and screening of gene products in noneyestalk tissues, as in this study, will validate the hypothesis. Furthermore, we expected to find a sequence homologue of *Chh* exon III via BLAST search strategies. The output of the TBlastX query included 2 sequences we submitted, the *Chh* gene of *M. lanchesteri*, and several other short, meaningless nucleotide sequences. The deduced amino acid sequence of the exon III did not match any homologue other than itself.

Secondary Structure Prediction on Splicing Isoforms of CHH

Although we expected the relationship of Mar-CHH-L, PO-CHH of *C. maenas*, and Prc-CHH-L, neither the nucleotide sequence nor the deduced amino acid of exon III in these sequences could match to each other in the BLAST search. If there is any evolutionary relationship between these exon III sequences, the simple conclusion that it exists over a great distance will be drawn from the null BLAST result. Therefore, we changed the search to detect a far-distant relationship in the conservation of protein structure.

Predictions of the secondary structures of Mar-CHH, Mar-CHH-L, Cam-SG-CHH, Cam-PO-CHH, Prc-CHH, and Prc-CHH-L were attempted. Mar-CHH was predicted as 3 major helix structures (residues 13–27, 47–55, 61–70) with high confidence (score above 7), separated with several coiled coils, scored from high to low, and a short helix structure (36–40) with median score (scored from 4 to 6); no β-sheet structures were found (Figure 6). Three high-scoring predicted helices were also found in Mar-CHH-L (12–26, 46–54, and 60–70). The results for these 6 sequences were very similar to each other in terms of the location of predicted secondary structures and the confidence score assigned to each residue. This suggests that the ORF switch starting within Arg^{40} does not introduce great differences into the predicted secondary structures, which may guide them to fold to their tertiary structure. However, the replacement in the latter half of the amino acid sequence causes the PO-CHH to being lost in the hyperglycemic activity. These observations suggest the importance of a C-terminal fragment of CHH family peptides in their functional specificity (Sonobe et al., 2001).

Discussion

CHH is defined by its biological function—the dramatic decrease in the hemolymph glucose content after eyestalk excision and restoration of the physiologic defect in eyestalk-ablated individuals by the injection of tissue lysate. Two *Chh* gene transcripts, *Mar-chh* and *Mar-chh-l*, from eyestalk and from 4 noneyestalk tissues, were identified in this study. Direct sequence evidence suggests that these 2 isoforms come from a *Chh* gene transcribed in an alternative splicing manner. Six cysteine residues in the presumed ORFs and their predicted secondary structures are well aligned, leading to the hypothesis of preservation on the tertiary structure. The hyperglycemic activity of Mar-CHH was demonstrated (Lin et al., 1998), while the function of CHH-L remains to be elucidated. Similarly, the *Chh* gene spliced product, the PO-CHH cloned from pericardial organs of *C. maenas*, was also reported (Dircksen et al., 2001). PO-CHH did not induce hemolymph hyperglycemia in eyestalk-ablated individuals. It did not act in equal potency to SG-CHH in elevating [cGMP] in

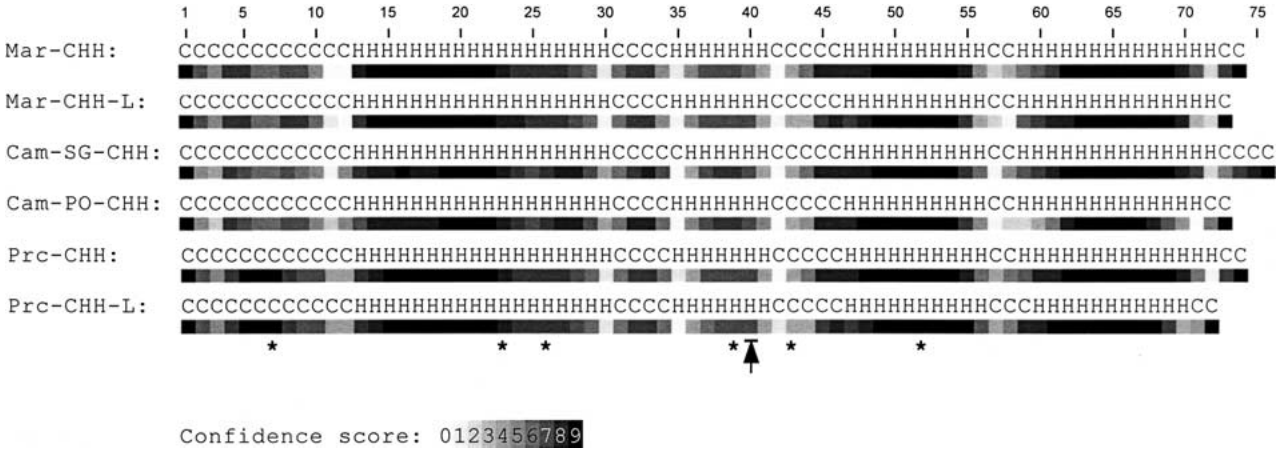

Figure 6. Secondary structure prediction of the alternative spliced CHH isoforms by PHD. H represents helix structure; C, coiled-coil. Confidence in the prediction result is translated into a gray scale expression. The darkest shading is equivalent to the highest score. ORF switching position (residue 40) is marked by the arrow. Six cysteine residues are marked by asterisks.

cultivated cardiac muscle and Y organ, and did not inhibit ecdysteroid synthesis in Y organ. The authors described the low expression of PO-CHH and proposed that it may function in autocrine or paracrine manner. The expression of the *chh-l* transcript in noneyestalk tissues is much lower than that of the *chh* transcript in eyestalk. The protein product of *Mar-chh-l* is barely detectable in tissue lysates. These observations would probably support the conclusion.

Numerous studies on decapod crustaceans have shown that the neuroendocrine system, such as pericardial organs, thoracic ganglion, supraesophageal ganglion, and ventral nerve cord, is involved in osmotic and ionic regulations. Extracts of pericardial organs increased the uptake of Na^+ in perfused posterior gills of the crab *Callinectes sapidus* (Kamemoto, 1991). Injection of supraesophageal ganglion homogenates induced an increase in Na^+ influx and hemolymph Cl^- concentration in the crayfish *P. clarkii* (Kamemoto and Tullis, 1972). In addition, the water influx in crab gills perfused with thoracic ganglion extracts was decreased. Although the ionic regulating capability of the tissue homogenates was attributed to various types of biogenic amines such as dopamine, 5-HT, and octopamine, or to peptide hormones such as enkephalin, proctolin, and cardioactive peptide, the involvement of CHH-related molecules in osmoregulation should not be neglected. Pierrot et al. (2000) demonstrated the potential of CHH to regulate gill function in *Pachygrapsus marmoratus.* Both sinus gland extract and CHH fractions purified by high-performance liquid chromatography significantly increased the transepithelial potential difference and Na^+ influx by about 50%. Chung et al. (1999) reported an unusual endocrine function of the gut of *C. maenas.* The immunopositive secretory ''hydroid cell'' in foregut and hindgut was similar in shape to the paraneuron. Studies on these secretory cells revealed precisely timed correlation of CHH release and ecdysis. These results suggested the possible role of CHH in the hydration process during the postmolting stage. In this study the 4 noneyestalk *chh-l*-expressing tissues, directly or indirectly, are involved in osmoregulation. Gills and antennal glands are well-characterized excreting organs in both morphologic and biochemical studies. The pericardial organs and thoracic ganglion were reported to be involved in osmotic regulation (Muramoto, 1988; Morris, 2001).

The thoracic ganglion was identified as one of the *chh-l*-expressing sites by RT-PCR. This is supported by the discovery of the thoracic ganglia–specific CHH-like sequence in the crayfish. Chang et al. (1998) detected a significant amount of CHH in the hemolymph of American lobster that had been eyestalk-ablated for an extended period. Immunological data suggested that *Chh* gene might be widely expressed in central nervous system, and the CHH-secretory cells were detected in the thoracic ganglion second root and subesophageal ganglion (Chang et al., 1999). We speculate the possibility of cross-recognition on CHH-L by CHH antiserum, for the common antigenicity from the first half peptide encoded by the common exon.

In the phylogenetic analysis of CHH family members, CHHs and insect ITPs are clustered together in CHH subfamily I (Lacombe et al., 1999). This suggests that CHHs

might share many characteristics with ITPs. ITP and ITP-L were identified and proposed as alternative splicing products (Macins et al., 1999). The pattern of ORF switching in the ITP/ITP-L of 2 locust species, *Schistocerca gregaria* and *Locusta migratoria*, is the same as we found in CHH/CHH-L of *M. rosenbergii* (this study) and in SG-CHH/PO-CHH of *C. maenas* (Dircksen et al., 2001). A role in osmotic regulation was proposed for CHH, while the closely related homologue sequences, ITP and ITP-L from insects, were not shown to evoke hyperglycemia *in vivo*. The divergence or preservation of functions among the CHHs and ITPs in view of molecular evolution remains to be clarified.

The common gene organization among palaemonid, brachyuran, and astacuran crustaceans suggests that the *Chh* gene as a 4-exon structure and the transcriptional control of tissue-specific exon III exist in a variety of crustacean species. In contrast, a 3-exon pattern was reported for the *Chh* genes of *M. ensis* (Gu and Chan, 1998). This discrepancy might be due to the uncertainity of the evolutionary relationship between penaeid shrimps and other decapods (Barnes and Harrison, 1992), and the existence of tissue-specific expression of exon III in these species remains unclear. The copy number of *Chh* gene is especially high in penaeid shrimps, which may arise from a different evolutionary route of speciation and adaptation. Although the exon and intron organizations are different in these *Chh* genes, the exon-exon junctions within the mature peptides are in the same mode. The trinucleotide codon of the residue at the junction site is split in a 2-to-1 mode in both the 3-exon *Chh* gene and 4-exon *Chh* genes. The first 2 nucleotide codes are attached at the end of the former exon, and the third one at the start of the successive one. In fact, the junction patterns are conserved among all *Chh*-related genes (unpublished data).

Considering the expression site of the transcript, we propose that the alternative splicing *chh* may be involved in ionic and osmotic regulation, volume control, or acid-basic balance. Yet the low homology among the exon III sequences implies a distant relation and possible diversity in the physiological roles. What cell type expresses the *chh*-like transcript in these noneyestalk tissues remains to be elucidated. The biological activities of the CHH family members rely on accurate posttranslational modification. The prohormone processing machinery, such as the prohormone convertase identified by Toullec et al. (2002), should be well equipped in these secretory cells. Investigations of details including the targets of these splicing isoforms and the peptide-releasing controls are needed to describe the functions.

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