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Molecular cloning of a cDNA encoding a member of CHH/MIH/GIH family from *Penaeus monodon* and analysis of its gene structure

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Abstract

We report the nucleotide and deduced amino acid sequences of Pem-CMG peptide, a member of crustacean CHH/MIH/GIH peptide family, in black tiger prawn (*Penaeus monodon*). The 5' and 3' fragments of *Pem-CMG* cDNA were cloned by the method of rapid amplification of cDNA ends (RACE). The two fragments constitute a combined cDNA length of 593 bp with a 77 bp overlapping region. Sequence analysis reveals the presence of a 384 bp open reading frame which was subsequently cloned. The open reading frame encodes a precursor peptide that is comprised of 128 amino acids, with a putative processing site, KR. The mature peptide consists of 74 amino acid residues, the sequence of which is significantly homologous to those of the CHH/MIH/GIH family known from other crustaceans. Analysis of a genomic fragment of *Pem-CMG* reveals a single intron of 314 bp interrupting the coding sequence for the mature peptide. The presence of only one intron in *Pem-CMG* gene suggests that this gene is structurally different from the previously reported *MIH* gene of *Charybdis feriatius* and *CHH*-like gene of *Metapenaeus ensis* which possess two introns in their coding sequences. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Eyestalk; Neuropeptide; Penaeid shrimp; Rapid amplification of cDNA ends

1. Introduction

A number of physiological processes in decapod crustaceans are known to be regulated by diverse neuropeptides synthesized by a neurosecretory system called

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X-organ-sinus gland complex (XOSG) located in the optic ganglia of the eyestalks (De Kleijn and Van Herp, 1995; Charmantier et al., 1997). Most of the peptide hormones isolated from the XOSG complex e.g. pigment dispersing hormone (PDH) and crustacean cardioactive peptide (CCAP) have also been identified in some other arthropods such as insects. Recently, a group of related peptides from XOSG complex that constitutes a novel hormone family has been described and thus far has been found only in crustaceans (Keller, 1992). This family includes crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH) and gonad inhibiting hormone (GIH) that are involved in blood sugar regulation, inhibition of ecdysteroid synthesis, and regulation of reproduction, respectively (Cooke and Sullivan, 1982; Chang, 1993).

Direct amino acid sequencing of the hormones in this CHH/MIH/GIH family from several crustaceans has been conducted (Kegel et al., 1989; Chang et al., 1990; Webster, 1991; De Kleijn et al., 1994a,b). Comparison of the amino acid sequences of these hormones revealed significant degrees of similarity (Keller, 1992; Sun, 1994; Chang, 1997) suggesting that they constitute an authentic peptide family across species boundaries. In addition to several similarities in their structure, some hormones in the CHH/MIH/GIH family are multifunctional i.e. exhibit more than one biological activity (Chang et al., 1990; Tensen et al., 1991; Lee et al., 1995). However, the certainty concerning the primary physiological functions of different peptides still needs to be clarified.

In recent years, the cDNA coding for CHH, MIH and GIH in a number of crustaceans has been cloned based on the information of available amino acid sequences (Weideman et al., 1989; Tensen et al., 1991; Klein et al., 1993; De Kleijn et al., 1994a,b; Sun, 1994; Lee et al., 1995; Aguilar-Gaytan et al., 1997). Despite this increasing number of reports on cDNA cloning and nucleotide sequence analysis of neuropeptides in CHH/MIH/GIH family, the hormones in several crustaceans still await complete characterization, especially at the molecular level.

We report here the molecular cloning of a cDNA encoding Pem-CMG, a peptide in the family of CHH/MIH/GIH, from black tiger prawn, *Penaeus monodon*. An open reading frame encoding Pem-CMG peptide was cloned from a cDNA of a single pair of the eyestalks. A primary structure of the gene for Pem-CMG was also analyzed and revealed that *Pem-CMG* gene of *P. monodon* has different structure from the genes encoding MIH and CHH-like peptide that have been recently reported in *Charybdis feriatius* and *Metapenaeus ensis*, respectively (Chan et al., 1998; Gu and Chan, 1998)

2. Material and methods

2.1. Animals

Black tiger prawns, *P. monodon*, were purchased from Shrimp Culture Research Center, Charoen Pokphand Group, Thailand and were kept alive in aerated seawater at room temperature until used.

2.2. Oligonucleotide primers

Abridged anchor primer (AAP) and abridged universal amplification primer (AUAP) were provided with the 5' RACE System for Rapid Amplification of cDNA Ends (GIBCO BRL). Other custom oligonucleotides were purchased from Bio-Synthesis, USA.

2.3. RNA isolation

A total of 100 eyestalks were collected from *P. monodon* immediately after anesthetizing the live shrimp with crushed ice. After the cuticle and non-neural tissues were removed, the dissected eyestalks were ground to fine powder in liquid nitrogen by means of mortar and pestle. Total RNA was extracted using TRIZOL[®] Reagent (GIBCO BRL). Poly (A)⁺ RNA was isolated using Dynabeads mRNA purification kit (DYNAL) and was quantified by measuring the absorbency at 260 nm.

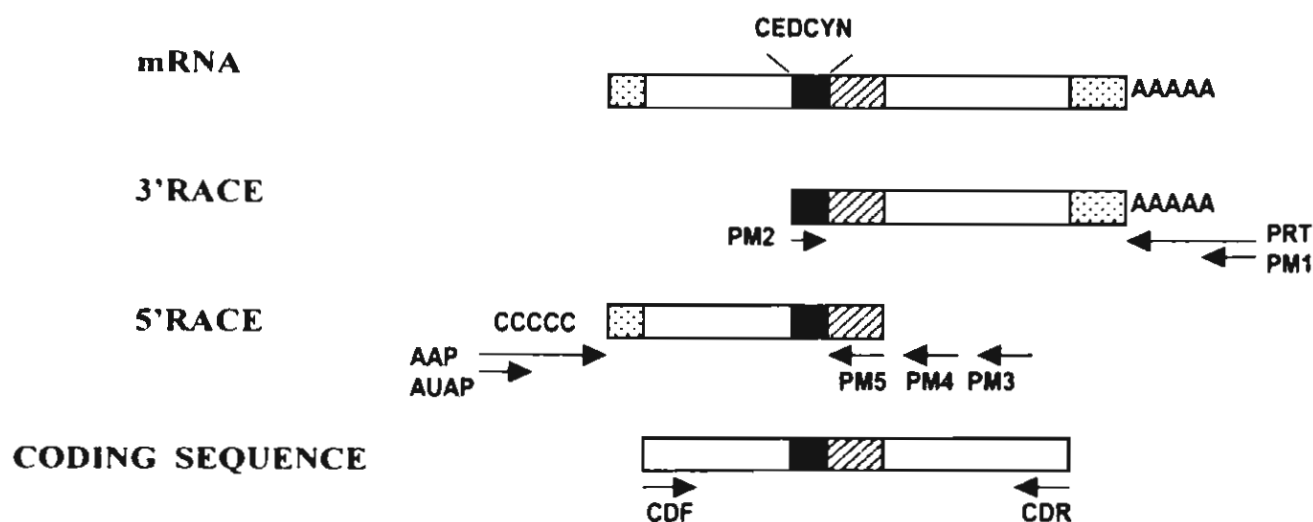
2.4. Amplification of the 3' ends of cDNA

The reaction was carried out according to the protocol of the 3' RACE System for Rapid Amplification of cDNA Ends (GIBCO BRL). First strand cDNA was synthesized from 75 ng of poly (A)⁺ RNA in a total volume of 20 μ l containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 10 mM DTT, 500 nM PRT primer, 500 μ M each dATP, dCTP, dGTP, dTTP and 200 units of SuperScript II Reverse Transcriptase. The reaction was incubated at 50°C for 50 minutes and then terminated at 70°C for 15 minutes. The RNA template was degraded with RNaseH before proceeding to amplification step. A 2 μ l aliquot of cDNA was amplified with primers PM1 and PM2 (see Fig. 1) in a reaction containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 200 nM each primer, 200 μ M each dATP, dCTP, dGTP, dTTP and 2.5 units of AmpliTaq DNA polymerase (PE Applied Biosystems). Amplification was performed in a DNA Thermal Cycler (GeneAmp System 2400, PE Applied Biosystems) with 35 cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes following with 7 minutes incubation at 72°C as a final extension.

2.5. Amplification of the 5' ends of cDNA

The detailed protocol of the 5' RACE System for Rapid Amplification of cDNA Ends Version 2.0 (GIBCO BRL) was as follows. First strand cDNA synthesis was carried out as described in Section 2.4, except that 100 nM of primer PM3 was substituted for primer PRT. The cDNA synthesized was then purified using a GlassMax DNA isolation spin cartridge (GIBCO BRL).

A 10 μ l aliquot of purified cDNA was tailed with dCTP in 25 μ l of 10 mM Tris-HCl (pH 8.4), 25 mM KCl, 1.5 mM MgCl₂, 200 μ M dCTP and 1 μ l of terminal deoxynucleotidyl transferase (TdT). The reaction was incubated at 37°C for 10 minutes and TdT was heat inactivated at 65°C for 10 minutes. The PCR amplification of 5 μ l of the dC-tailed cDNA was performed as described for the 3' RACE using 200 μ M of



Oligonucleotide primers

3'RACE primers

PRT : 5' – CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTTTTTTTTT – 3'
 PM1 : 5' – CCGGAATTCAAGCTTCTAGAGGATCC – 3'
 PM2 : 5' – CCGGAATTCTGYGAAGAYTGYTACAAC – 3'

5'RACE primers

AAP : 5' – GGCCACGCGTCGACTAGTACGGGGIIGGGIIGGGIIG – 3'
 AUAP : 5' – GGCCACGCGTCGACTAGTAC – 3'
 PM3 : 5' – CCGGAATTCCCTTTGACGAGGCCGGAAC – 3'
 PM4 : 5' – CCGAAGCTTGTCCACGCAGTAGAG – 3'
 PM5 : 5' – CCGGAATTCACCTCGTTGTGGAACAG – 3'

coding sequence

CDF : 5' – CCGAAGCTTCATATGGTTGCCGTTGGAC – 3'
 CDR : 5' – CGGGATCCCTACTTGCCGAGCCTCTA – 3'

Fig. 1. Schematic diagram showing the 3' and 5' RACE products and the primers used in cDNA synthesis and PCR. Filled boxes represent the CEDCYN conserved amino acid sequence and shaded areas represent the overlapping region between the products of 3' and 5' RACE. Dotted boxes represent 5' and 3' untranslated regions. Detail of the primers used in 3' RACE, 5' RACE and amplification of a complete coding region is given below. 3'RACE: PRT primer contains oligo(dT)₁₆ linked to adapter sequences. PM1 is adapter sequences homologous to that of PRT containing restriction sites for *EcoRI*, *HindIII*, *Xba I* and *BamH I*. PM2 is a degenerated primer derived from the CEDCYN conserved amino acid sequence. 5'RACE: Abridged anchor primer (AAP) contains 3' sequence complementary to the dC-tail and additional 5' sequence that is composed of restriction sites for *Not I*, *Mlu I*, *Sal I* and *Spe I*. Abridged universal amplification primer (AUAP) contains a restriction site sequence homologous to that of AAP. PM3, PM4 and PM5 are gene specific primers derived from the nucleotide sequences of the 3'RACE product. Coding sequence: Gene specific primers derived from the nucleotide sequences extended from the start codon (CDF) and the stop codon (CDR) of *Pem-CMG* sequences. CDF and CDR contain restriction sites for *Hind III* and *BamH I* at their 5' termini, respectively.

primer PM4 and abridged anchor primer (AAP). The second round PCR was performed using primer PM5 and abridged universal amplification primer (AUAP) to obtain specific amplified product.

2.6. Amplification of an open reading frame of *Pem*-CMG peptide

Total RNA extracted from a single pair of the eyestalks was used to synthesize a cDNA template for amplification of the open reading frame of *Pem*-CMG peptide. The condition for PCR was the same as described earlier in Sections 2.4 and 2.5. The primers used were CDF and CDR, the sequences of which are given in Fig. 1.

2.7. Amplification of a genomic fragment of *Pem*-CMG gene

Genomic DNA was prepared from abdominal muscle tissues of *P. monodon* by phenol/chloroform extraction method (Sambrook et al., 1989). About 150 ng of genomic DNA was used as a template for PCR amplification of the *Pem*-CMG sequence with the primers CMG-F and CMG-R (Fig. 2) designed from the most 5' and 3' ends of the *Pem*-CMG cDNA. The PCR reaction mixture (150 ng of genomic DNA template, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 200 nM each primer, 200 μM each dATP, dCTP, dGTP, dTTP) was heated to 95°C for 5 minutes, then 2.5 units of AmpliTaq was added. Amplification was achieved by 35 successive cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 2 minutes, followed by a 10 minute final extension at 72°C.

2.8. Cloning and sequencing

PCR Amplification products were purified using GeneClean II kit (Bio101). The



Oligonucleotide primers

CMG-F : 5' – CGGAATTCTCAGTGCAGAGGGAGAGCC – 3'

CMG-R : 5' – CGGAATTCATTCTCTTATTTTATTAGACAGG – 3'

Fig. 2. Schematic diagram showing the strategy used for amplification of *Pem*-CMG genomic fragment and the primers used. Dotted area represents 5' and 3' untranslated regions. Exons are presented in white boxes and intron is presented in black box. Detail of the primers used is given below. CMG-F primer: contains 19 nucleotides at the most 5' region of *Pem*-CMG cDNA linked to restriction site for *EcoR* I at the 5' end. CMG-R primer: contains 25 nucleotides complementary to the most 3' region of *Pem*-CMG cDNA linked to restriction site for *EcoR* I at the 5' end.

purified DNA fragments were digested with appropriate restriction before being ligated to pUC18 vector predigested with corresponding enzymes. The ligation products were used to transform *E. coli* JM109 and the recombinants were screened by restriction enzyme digestion. The nucleotide sequences of the recombinants were determined by the method of ABI PRISM™ Dye Terminator Cycle using ABI PRISM Model 377 automated DNA Sequencer (PE Applied Biosystems).

3. Results

3.1. 3'RACE cloning of the cDNA encoding Pem-CMG peptide

Nine individual recombinants, obtained by cloning of 3' RACE products into pUC18, that contain cDNA insert of different sizes ranging from 163 bp to 450 bp were selected for nucleotide sequence analysis. One of the clones harbors a 324 bp cDNA insert (including poly (A) tail), the nucleotide sequence of which exhibits more than 60% identity to those of mRNA encoding CHH and MIH from other crustaceans (data not shown).

3.2. 5' RACE cloning of the cDNA encoding Pem-CMG peptide

Using three specific primers generated from the nucleotide sequence of the 3' RACE fragment, a single band of amplification product of 370 bp (excluding G-rich tail) was obtained from 5' RACE and was subsequently cloned into pUC18. The nucleotide sequence analysis of three individual clones revealed that they contain cDNA insert homologous to the mRNA of CHH/MIH/GIH peptide family (data not shown). No variation in the nucleotide sequences was detected among the three clones.

3.3. Nucleotide sequence of Pem-CMG cDNA and its deduced amino acid sequence

The combination of nucleotide sequences of the 3' and 5' fragments of the *Pem-CMG* cDNA revealed a 593 bp cDNA containing a 384 bp open reading frame, 65 bp 5' untranslated region and 141 bp 3' untranslated region, with a potential polyadenylation signal AATAAA located 14 bp upstream from the poly (A) sequence (Fig. 3). The two fragments shared a 77 bp overlapping region (nucleotides 294 to 370) that exhibited identical nucleotide sequences. Using a cDNA that was synthesized from the total RNA of a single pair of the eyestalks, a fragment of 384 bp starting from a start codon to a stop codon of the open reading frame was cloned by PCR.

The nucleotide and deduced amino acid sequences of a 384 bp open reading frame of *Pem-CMG* cDNA from nine individual recombinant clones are shown in Fig. 3. The change in amino acids at position 37 (Arginine to Cysteine) and position 115 (Glutamine to Arginine) was observed, each was due to a single nucleotide change in two different clones. The other seven clones showed identical nucleotide and amino acid sequences. The mature peptide was composed of 74 amino acids and was separated from a 52 amino acid propeptide region by a potential processing site, KR. The propeptide region

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-tcagtgcagagggagagcctggaagttgctgaccgtcgctcccgatctgcctctacactaaagat : 65
66 : ATGGTTGCCGTTGGACCGATGCGGACAGCTGTCCTGGTGTCCCTGCTGTTGGCAATCCCGGCCTCT :131
    M V A V G P M R T A V L V S L L L A I P A S
132: GCCACCACCTTCGGAGACGGAATGACATTCCAACGTTCTCCGTTCTTCCCCAGAAGCCTCTCCT :197
    A T T F G D G N D I P T F L R S S P E A S P
198: GTGACTTCCCTTCACACCTCAGACAAACGCAGCCTATCCTTCAGGTCTTGACGGGCGCCTACGAC :263
    V T S L H T S D K R S L S F R S C T G A Y D
264: CGCGAACTCCTTGTAAGGCTCGACCGCGTGTGCGAAGACTGCTACAACGTGTACCGCGACGTCGGA :329
    R E L L V R L D R V C E D C Y N V Y R D V G
330: GTGGCAGCCGAATGCAGGAGTAACTGTTTCCACAACGAGGTGTTTCCTCTACTGTGTGGACTACATG :395
    V A A E C R S N C F H N E V F L Y C V D Y M
396: TTCCGGCCTCGTCAAAGGAACAGTACCGGGCCGCCCTACAGAGGCTCGGCAAGTAGgtggttctct :461
    F R P R Q R N Q Y R A A L Q R L G K *
462: cttcagccagacctcgccatgcgactcccaagacgaccagactcttattaggttgcttttcgtatcc :527
528: tgatttaaaaggtaaaataagagcctgtacaacaagcctcctgtcttaataaaaataagagaaatat :593
(a)n

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Fig. 3. Nucleotide sequence of *Pem*-CMG cDNA derived from the sequences of 5' and 3' RACE fragments. The 77 bp overlapping sequence is shaded. The 384 bp open reading frame is depicted by capital letters. The potential processing site, KR, is boxed. The deduced amino acids are written in one-letter symbols. The putative polyadenylation site is underlined.

contains several hydrophobic amino acid residues suggesting a possible role as a signal sequence.

3.4. Comparison of the amino acid sequence of *Pem*-CMG peptide with those of the peptides in CHH/MIH/GIH family from other crustaceans

The deduced amino acid sequence of *Pem*-CMG peptide of *P. monodon* was compared with those in the CHH/MIH/GIH family from several crustaceans. The result of this comparison (Fig. 4) revealed that *Pem*-CMG possessed a significant degree of homology to the peptides in this family (30%–50% identity). The *Pem*-CMG peptide contains six conserved cysteine residues which were found located at identical positions in all other members of CHH/MIH/GIH family. Most of the conserved amino acid residues were found in the N-terminal region whereas less homology was found in the C-terminal area.

3.5. Primary structure of *Pem*-CMG gene

A specific DNA fragment, about 900 bp, was amplified from genomic DNA of *P. monodon* with primers CMG-F and CMG-R. This fragment, designated as *g*-CMG, contained nucleotide sequence from the first start codon (ATG) to the stop codon (TAG) of *Pem*-CMG gene. Analysis of the nucleotide sequence of *g*-CMG fragment cloned in pUC18 revealed that this fragment contains one intron. An exon–intron boundary was detected using Gene Finder program of Baylor College of Medicine (BCM) on the WWW. The splice junction conformed to the splice donor and acceptor consensus sequence (Mounts, 1986).

The *g*-CMG fragment was subcloned by restriction enzyme *Hinc* II (Fig. 2), the recognition site of which lies within the intron sequence. The entire sequence of the intron was then determined from this subcloned fragment. The result showed that the

		*	20	*	40	
Pem-CMG	:	---	SLSFRSCTGAY	-	DRELLVRLDRVCEDCYNVYRDVGVA	: 36
Hoa-CHH-a	:	---	QVFDQACKGVY	-	DRNLFKKLDRVCEDCYNLYRKPFVA	: 36
Hoa-CHH-b	:	---	QVFDQACKGVY	-	DRNLFKKLNVRVCEDCYNLYRKPFIV	: 36
Cam-CHH	:	---	QIYDTSCCKGVY	-	DRALFNDLEHVCDDCYNLYRTSYVA	: 36
Prb-CHH	:	---	QVFDQACKGIY	-	DRAIFKKLDRVCEDCYNLYRKPYVA	: 36
Pej-CHH	:	---	SLFDPACTGIY	-	DRQLLRKLGRICDDCYNVFERPKVA	: 36
Cam-MIH	:	---	VINDECPNLIGNRDLYKKVEWICEDCSNIFRKTGMA			: 36
Prb-MIH	:	---	QVFDQACKGIY	-	DRAIFKKLELVCDDCYNLYRKPKVA	: 36
Pej-MIH	:	---	SFIDNTCRGVMGNRDYNNKKVVRVCEDCTNIFRLPGLD			: 37
Hoa-GIH	:		ASAWFTNDECPGVMGNRDLYEKVAWVCNDCANIFRNNDVG			: 40

		*	60	*	80	
Pem-CMG	:	AE	CRSNCFPHNEVFLYCVDYMF	PRQRNQYRAALQRLGK	---	: 74
Hoa-CHH-a	:	TT	CRENCYSNWVFRQCLDDLL	LSDDVIDEYVSNVQMVGK	---	: 74
Hoa-CHH-b	:	TT	CRENCYSNRVFRQCLDDLL	LSDDVIDEYVSNVQMVGK	---	: 74
Cam-CHH	:	S	CRSNCYSNLVFRQCMDDLL	MMDEFDQYARKVQMVG	GRKK-	: 76
Prb-CHH	:	TT	CRQNCYANSVFRQCLDDLL	LIDVVDEYISGVQTV	----	: 72
Pej-CHH	:	TG	CRSNCYHNLIFLDCLEYL	IPSHLQEEHMAAMQTVGK	---	: 74
Cam-MIH	:	SL	CRRNCFNEDFVWCVHATER	SEELRDLEEWVGILG	AGRD	: 77
Prb-MIH	:	TT	CRENCYANSVFRQCLDDLL	LINVVDEYISGVQIVGK	---	: 74
Pej-MIH	:	GM	CRNRCFYNEWFLICLKAAN	REDEIEKFRVWISILN	AGQ-	: 77
Hoa-GIH	:	VM	CKKDCFHTMDFLWCVYATER	HGEIDQFRKWVSILR	AGRK	: 81

Fig. 4. Comparison of the amino acid sequence of Pem-CMG peptide with those of *H. americanus* CHH-A and CHH-B (Hoa-CHH-A and Hoa-CHH-B, Tensen et al., 1991), *C. maenas* CHH (Cam-CHH, Kegel et al., 1989), *P. bouvieri* CHH (Prb-CHH, Huberman et al., 1993), *P. japonicus* CHH (Pej-CHH, Yang et al., 1995), *C. maenas* MIH (Cam-MIH, Webster, 1991), *P. bouvieri* MIH (Prb-MIH, Aguilar-Gaytan et al., 1997), *P. japonicus* MIH (Pej-MIH, Ohira et al., 1997) and *H. americanus* GIH (Hoa-GIH, De Kleijn et al., 1994b). The amino acid residues that are identical to Pem-CMG peptide are highlighted.

intron is 314 bp long. This intron separates the two exons in the coding region for the mature Pem-CMG peptide between the second and the third codons of ⁹⁴Arg (Fig. 5).

4. Discussion

The 3' and 5' RACE strategy (Frohman et al., 1988) was employed in order to clone a Pem-CMG cDNA due to the lack of information on amino acid sequences of the peptides in CHH/MIH/GIH family in *P. monodon*. This technique requires only a single oligonucleotide primer which, in our case, was generated from the conserved amino acid sequence CEDCYN that is found conserved among the peptides in CHH/MIH/GIH family from a number of crustaceans (De Kleijn and Van Herp, 1995).

The open reading frame of Pem-CMG peptide from *P. monodon* was successfully cloned. The deduced amino acid sequence reveals a putative mature peptide comprising 74 amino acid residues. Amino acid change at two positions was observed when the deduced amino acid sequences from nine individual clones were compared. Since these sequences were cloned from the sinus gland of a single shrimp, the differences due to

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-TCAGTGCAGAGGGAGAGCCTGGAAGTTGCTGACCGTCGCTCCCGATCTGCCTCTACTCTAAAGAT : 65
66 : ATGGTTGCCGTTGGACCGATGCGGGCAGCTGTCTGGTGTCCCTGCTGTTGGCAATCCCGGCTCT : 131
    M V A V G P M R A A V L V S L L L A I P A S
132: GCCACCACCTTCGGAGACGGAAATGACATTCCAACGTTCTCCGTTCTTCCCCAGAAGCCTCTCCT : 197
    A T T F G D G N D I P T F L R S S P E A S P
198: GTGACTTCCCTTCACACCTCAGACAAACGCAGCCTATCCTTCAGGTCTTGACGGGCGCCTACGAC : 263
    V T S L H T S D K R S L S F R S C T G A Y D
264: CGCGAACTCCTTGTAAGGCTCGACCGGTGTGCGAAGACTGCTACAACGTGTACCGCGACGTCGGA : 329
    R E L L V R L D R V C E D C Y N V Y R D V G
330: GTGGCAGCCGAATGCAGGTAACCTTATTACTTTGCAGTAACCCACCCAGTTGTGTTGTGATTAAAGA : 395
    V A A E C R-----INTRON-----
396: CTATTGTAGAAGCGTATTAGTATACCATCTATTACTTTAGTATATCATCTATTACTATCATCTATC : 461
    -----INTRON-----
462: GGACAATGATTTCATACTGAGTTTGTATCCATGGAGCTTTCGTTAACATCGCCTGTTGCCTAAAA : 527
    -----INTRON-----
528: AGCCTCAATAATTATAAAATGTATAAACAGAATATACTTTATTCCTTATATCTCTTTATTACATA : 593
    -----INTRON-----
594: GTTATTTTTTTCACATTTCTCCTGTACGACCTACCAGTGGAACCTACATAACATTATTCTTCCTTCA : 659
    -----INTRON-----
660: GGAGTAACTGTTTCCACAACGAGGTGTTCTCTACTGTGTGGACTACATGTTCCGGCCTCGTCAAA : 725
    - S N C F H N E V F L Y C V D Y M F R P R Q
726: GGAACCAGTACCGGGCCGCCCTACAGAGGCTCGGCAAGTAGGTGGTTCCTCTTCAGCCAGACCTCG : 791
    R N Q Y R A A L Q R L G K *
792: CCATGCGACTCCCAAGACGACCAGACTCTTATTAGGTGCTTTTCGTATCCTGATTTAAAAGGTAAA : 857
858: ATAAGAGCCTGTACAACAAGCCTCCTGTCTAATAAAAATAAGAGAAATAT : 907

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Fig. 5. Nucleotide sequence and predicted amino acid sequence of *Pem-CMG* gene. The gene consists of two exons that are separated by an intron of 314 bp (dashed line). Exon one harbors coding sequence for signal peptide and part of the coding sequence for the first 40 amino acids of Pem-CMG peptide. The second exon consists of the coding sequence for the rest 34 amino acids of the peptide. The deduced amino acids are written in one-letter symbols. The nucleotide G at position 90 is substituted for an A in the cDNA which changes the corresponding amino acid from threonine (in Fig. 3) to alanine in the genomic sequences (highlighted).

polymorphism can be excluded. Therefore it is likely that these differences represent gene isoforms or could be an error introduced during PCR amplification. The Pem-CMG peptide of *P. monodon* shared the highest degree of identity with CHH of *P. japonicus* (50%). Significant degrees of identity were also observed between Pem-CMG and CHH of *Homarus americanus* (45% for CHHa and 41% for CHHb), *Carcinus maenas* (42%) and *Procambarus bouvieri* (41%). These values were close to that between Pem-CMG and the MIH reported for *P. bouvieri* (39%), whereas lower degrees of identity are found between Pem-CMG sequence and MIH of *C. maenas* and *P. japonicus* (32% and 34%, respectively). The GIH of *H. americanus* also shows comparable degree of identity (30%) with Pem-CMG of *P. monodon*.

Since the peptides in the CHH/MIH/GIH family are structurally related, it is not surprising that some of them also possess overlapping activities. Chang et al. (1990) reported that MIH from *H. americanus* also exhibits hyperglycemic activity. This is consistent with the fact that *H. americanus* MIH is nearly identical to CHH peptide from the same species (Tensen et al., 1991). However, MIH peptide of *P. bouvieri* that share 90% identity in the amino acid sequence with its own CHH does not present any hyperglycemic activity (Huberman et al., 1993; Aguilar-Gaytan et al., 1997). *C. maenas* CHH, on the other hand, has been demonstrated to possess MIH activity i.e. inhibition of

ecdysteroid secretion by the Y-organ in vitro, albeit at low level, (Webster and Keller, 1986) despite the low degree of identity (28%) it shares with MIH peptide from the same species. In addition, the MIH of *H. americanus* also seems to be involved in the regulation of methylfarnesoate synthesis by mandibular organ in this species (Chang et al., 1990). Methylfarnesoate has been proposed to play a role in controlling vitellogenesis in crustaceans (Laufer et al., 1987), therefore, *H. americanus* MIH is probably involved in the control of vitellogenesis, the function previously assigned to GIH, as well. Thus, it is unlikely to distinguish crustacean peptides in CHH/MIH/GIH family from each other by homology in their amino acid sequences.

The amino acid sequence deduced from the genomic copy of *Pem-CMG* differs from the sequence deduced from cDNA at one position in the signal peptide region (Fig. 5). This difference may reflect polymorphism as the genomic DNA and the cDNA used for amplification of *Pem-CMG* gene and cDNA were obtained from different individual shrimp. The gene for *Pem-CMG* of *P. monodon* contains only one intron that separates the coding sequence for the mature peptide. This is in contrast to the primary structure of the genes encoding MIH in *Charybdis feriatatus* and CHH-like peptide in *Metapenaeus ensis* that have recently been characterized (Chan et al., 1998; Gu and Chan, 1998). The *MIH* gene of *C. feriatatus* contains two introns, intron 1 interrupts the sequence coding for signal peptide and the second intron separates the mature peptide sequence. Two introns were also found in *CHH*-like gene of *M. ensis*. They interrupt homologous regions to those of *C. feriatatus* *MIH*, one in the signal peptide sequence and the other in the mature peptide sequence. The differences in the structure of genes in the same family among different species is not uncommon and may reflect their evolutionary pathway. It is, however, too early to draw any conclusion concerning gene structure of the member of CHH/MIH/GIH family because only little information has been obtained from a few species so far. The structure of the genes coding for neuropeptides in CHH/MIH/GIH family from other crustaceans needs to be explored. This would give a clue to answer the question of how these structurally different genes have evolved from related gene families.

The sequence information from several members of CHH/MIH/GIH family together with their biological activities suggest that the neuroendocrine regulation of related physiological activities in crustaceans is more complicated than previously thought. A complete understanding of these regulatory processes requires further information from molecular studies as well as physiological studies to define more clearly the roles of individual peptides in this family.

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