FEBS Letters 472 (2000) 122–128 FEBS 23556

Molecular characterization of an additional shrimp hyperglycemic hormone: cDNA cloning, gene organization, expression and biological assay of recombinant proteins[1]

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Received 3 March 2000

Edited by Vladimir Skulachev

Abstract The crustacean eyestalk CHH/MIH/GIH neurohormone gene family represents a unique group of neuropeptides identified mainly in crustaceans. In this study, we report the cloning and characterization of the cDNA and the gene encoding the hyperglycemic hormone (MeCHH-B) of the shrimp *Metapenaeus ensis*. The amino acid sequence of MeCHH-B shows 85% identity to that of MeCHH-A (formerly MeCHH-like neuropeptide). Two separate but identical MeCHH-B genes were identified in the genome of shrimp by library screening and they are located on different CHH gene clusters. The organization of the MeCHH-B gene is identical to other members of the CHH/MIH/GIH neurohormone family. MeCHH-B is expressed at a constant level in the eyestalks of juveniles and mature females. Unlike the MeCHH-A gene, a low level of MeCHH-B transcripts can also be detected in the central nervous system. Interestingly, the expression pattern of MeCHH-B in the eyestalk of vitellogenic females is reversed to that of the MeCHH-A gene. At the middle stage of gonad maturation, a minimum level of MeCHH-B transcript was recorded and a maximum level of MeCHH-A transcript was detected. Recombinant proteins for MeCHH-A and MeCHH-B were produced by a bacterial expression system. The hemolymph glucose level of bilaterally eyestalk-ablated shrimp increased two-fold 1 h after the rCHH injection and then returned to normal after 2 h. The hyperglycemic effect of these fusion proteins is comparable to that of de-stalked shrimp injected with crude extract from a single sinus gland.

Key words: Crustacean hyperglycemic hormone; Gene organization; Shrimp

1. Introduction

The crustacean hyperglycemic hormone (CHH) is a member of the eyestalk CHH/MIH/GIH neuropeptide family [1,2]. The major function of CHH is to regulate glucose metabolism. CHH has been widely studied because of its abundance and the well-established biological assay for analysis. However, research in the study of CHH has been complicated by its structural similarity to other eyestalk neuropeptides, the existence of different isoforms and the multiple functions of individual neuropeptides reported in many species. For example, more than five highly similar CHH-related eyestalk peptides have been identified in the shrimp *Penaeus japonicus* [3–5] and two mRNAs encoding structurally different CHH precursors have been identified in the lobster *Homarus americanus* [6–8]. In the crayfish, two distinctive forms of neuropeptide belonging to the CHH have been purified [9,10]. Other CHH isoforms have also been reported and they are the result of a change in stereochemical structure in post-translational modification [11,12]. Although CHH's major function is to regulate glucose metabolism, recent studies indicated that it may also play a role in reproduction [13,14]. In general, the various forms of CHH reported in the same species share 96–100% amino acid homology. For example, all the MeCHH-A genes (previously MeCHH-like neuropeptide) identified in the shrimp *Metapenaeus ensis* share $>98\%$ amino acid sequence identity [15]. Here we report the identification and characterization of an additional CHH-related neuropeptide (MeCHH-B). We also demonstrate that both CHH-related neuropeptides may play an important role during the female gonad maturation cycle in shrimp.

2. Materials and methods

2.1. Reverse transcription polymerase chain reaction (RT-PCR)

Shrimp eyestalks were dissected and RNA extracted [16] within 2 min after handling to avoid stress induced to the animals. Intact RNA (1 μg) was used for the synthesis of first strand cDNA. Primers (S1 and S2) were designed based on the amino acid sequence of CHH from other crustaceans [5,15] and RT-PCR was performed to identify CHH-specific cDNA from *M. ensis*. The PCR conditions were denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min for 35 cycles. DNA sequences of amplified PCR products were determined. DNA and amino acid sequences derived from the results were compared and analyzed with the GenBank database.

2.2. cDNA and genomic library screening

For cDNA library screening, the probe was synthesized from the partial cDNA as described above. Replicate filters were hybridized at 65°C in a buffer (0.5 M sodium phosphate, 1% SDS, 2 mM EDTA, 5×Denhardt's solution and 100 μg/ml denatured salmon sperm DNA) without formamide overnight. Membranes were washed at a final concentration of 0.5×SSPE, 0.1% SDS at 65°C twice for 30 min. After the third round screening, potential positive plaques were purified and the DNA sequences of the cDNA clones were determined. For genomic DNA library screening, a shrimp genomic DNA library was constructed using a kit purchased from Promega (USA). The procedure for the genomic library screening was similar to that of

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[1] The MeCHH cDNA sequence data have been submitted to the GenBank sequence database with the accession number AF 247160.

Abbreviations: CHH, crustacean hyperglycemic hormone; MIH, molt inhibiting hormone; GIH, gonad inhibiting hormone; RT-PCR, reverse transcription polymerase chain reaction

PII: S0014-5793(00)01420-4

the cDNA library screening. Lambda clones that contained the CHH gene were subcloned into a plasmid vector and were subjected to DNA sequence determination to confirm the presence of the MeCHH-B-specific sequence.

2.3. Northern blot analysis and RT-PCR detection of MeCHH-B

Both Northern blot analysis and RT-PCR were used to study the expression of the shrimp CHH gene. RNAs were separated on 1.5% formaldehyde agarose gel, transferred onto a nitrocellulose membrane and hybridized in a formamide hybridization buffer contained a MeCHH-B-specific probe at 42°C overnight. High stringency (0.1×SSC and 0.1% SDS wash at 65°C) washes were performed to eliminate cross-hybridization of the probe to other neuropeptide genes. For RT-PCR, gene-specific primers for MeCHH-A (CHH-F: TCTGTT(CT)GACCCGTCGTGCA and CF22: CTTGGATCCGTCTGCTTGTGAGA) and MeCHH-B were used (forward CHH-F: and reverse CM22: CGAGGATCCGGGAGTGTACTGGAGTGTA). PCR products were analyzed by agarose gel electrophoresis and/or Southern blot to confirm the presence of MeCHH-B. For genomic PCR, the same gene-specific primers for the CHH-A and CHH-B genes were used as described before and PCR was performed under similar conditions with DNA purified from a single shrimp.

2.4. Protein expression and purification

The nucleotide sequences for CHH-A and CHH-B were amplified by PCR using primers CHH-F and CM22, digested with restriction enzymes and ligated to the expression vector pProEX™HTb (Life Technologies, USA) previously digested with the same enzymes. To ensure in-frame insertion, the DNA sequence of the construct was also determined. The constructs were transformed into the bacterial strain BL21(DE3)pLysS. Bacterial cells from a single colony were grown at 37°C in LB agar containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml). Expression of fusion protein was confirmed by Western blot analysis using an anti-histidine antibody. For protein purification, 25 ml culture of the LB medium seeded with the bacteria containing the construct was incubated at 37°C overnight. It was used to inoculate 1 l of LB medium containing both antibiotics and IPTG (0.4 mM final) was added. The culture was allowed to grow further for another 2–4 h. Bacteria cells were harvested and suspended in a buffer (50 mM Tris–HCl pH 7.9, 2 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol) and sonicated with a microtip to lyse the cells. The cell pellet was collected by centrifugation at 12 000×*g* for 20 min at 4°C and re-suspended in the same buffer containing 0.1% Triton X-100. The solution was mixed and the soluble protein fraction containing rCHH was collected by centrifugation at 12 000×*g* for 30 min at 4°C. The supernatant was dialyzed in Ni^{2+} starting buffer (20 mM Tris–HCl pH 7.9, 5 mM imidazole and 0.5 M NaCl). The dialyzed solution was purified using a HiTrap chelating column (Pharmacia, Uppsala, Sweden). The protein was eluted from the column in elution buffer (20 mM Tris–HCl pH 7.9, 1 M imidazole and 0.5 M NaCl) and stored at −80°C.

2.5. Biological assay of rCHH hyperglycemic activity

To obtain crude eyestalk extract, shrimp eyestalks were dissected for sinus gland in cold saline. Extracts from 100 sinus glands were pooled and combined in 1 ml of phosphate buffered saline (PBS). For CHH bioassay, juvenile shrimp (8–10 g) were bilaterally eyestalk-ablated with a pair of sharp scissors and the wounds were immediately sealed with a hot soldering iron and returned to culture conditions. Shrimp were returned to culture tanks for 2 days for recovery. Before the injection experiment, 100 μl of hemolymph was removed for baseline measurement of glucose. Individual shrimp were injected with 10 μl (2 μg) of rCHH-A, rCHH-B, PBS or 1 eyestalk equivalent crude extract through the arthrodial membrane of the fifth periopod by a syringe. Hemolymph (100 μl) was removed at 0, 0.5, 1 and 2 h for the measurement of glucose. Hemolymph glucose concentrations were determined by a glucose oxidase diagnostic kit (Sigma, USA). For the hyperglycemic effect of the rCHH, eyestalk sinus gland extract was compared with a control with only saline injection and a treatment group injected with a total of a single sinus gland extract. Student's *t*-test and analysis of variance (ANOVA) were used to analyze the statistical difference of different treatments.

3. Results

3.1. Characterization of cDNA

RT-PCR with primers S1 and S2 resulted in the amplification of two groups of partial cDNA. One group contained the MeCHH-A-specific sequence [15]. The other group consisted of cDNA encoding a protein homologous to the MeCHH-A gene. This partial cDNA neuropeptide was used as a probe to screen the shrimp eyestalk cDNA library (100 000 plaques). A cDNA clone was isolated and DNA sequence determination confirmed that it carried a coding sequence identical to the original partial cDNA PCR clone (data not shown, see appendix 1). The deduced prepro-peptide consists of 112 amino acids with a hydrophobic N-terminal end representing the signal peptide and a relatively short CHH precursor-related peptide (CPRP). The mature peptide consists of 74 amino acid residues. It lacks an amino acid Gly at position 10 of the mature peptide that is the characteristic of most CHH-related neuropeptides. It shows the highest overall amino acid identity to MeCHH-A (formerly MeCHH-like) [15], followed by CHHs of other shrimp (Fig. 1). Furthermore, MeCHH-B shows 42% amino acid identity to the ion transport protein (ITP) of the locust [17]. Compared to the MeMIH [24], it shares only 37% amino acid sequence identity (data not shown). Phylogenetic analysis of MeCHH-B with other CHH-related peptides shows that both MeCHH-A and MeCHH-B clustered together in a sub-group of the shrimp sequence (data not shown, see appendix 2).

3.2. Organization of the MeCHH-B gene in the CHH-like gene cluster

Since fewer DNA fragments hybridized to the MeCHH-B-specific probe in Southern blot analysis and several different DNA fragments amplified by CHH-A and CHH-B gene-specific primers (data not shown), we estimated that there were fewer copies of the MeCHH-B gene in the shrimp genome (Fig. 2a). Screening of an unamplified genomic DNA library with the MeCHH-B-specific probe produced two positive lambda clones (λ55 and λ69). One of the clones carries a MeCHH-B gene and is identical to λ52 that we have previously isolated from the screening of the CHH-A gene. The other lambda clone (λ69) also carries one MeCHH-B gene and two MeCHH-A genes. DNA sequence determination (Fig. 1a) of MeCHH-B in these two lambda clones indicated that they had an identical amino acid sequence in the coding region as the cDNA isolated from the eyestalk library. Unlike the MeCHH-B gene located in the λ52 clone, an *Eco*RI restriction enzyme site is located in intron 2 of the gene located in the λ69 clone. The MeCHH-B genes consist of three exons and two introns (Fig. 2b). The first intron separates the signal peptide and the second intron separates the mature peptide in the coding region. The intron and exon boundaries and the splice site donor and acceptor consensus sequence (GT-AG) for the MeCHH-B genes are conserved (data not shown). Further analysis of the gene indicates that the first 100 bp 5′ upstream region shows a high degree of similarity to the core promoter region of the MeCHH-A gene [15]. Several potential transcription factor sites identified in the MeCHH-A gene can also be identified in the MeCHH-B gene. For example, putative recognition sites for TATA, CAAT, and SP1 can be found in the MeCHH-B gene. The nucleotide se-

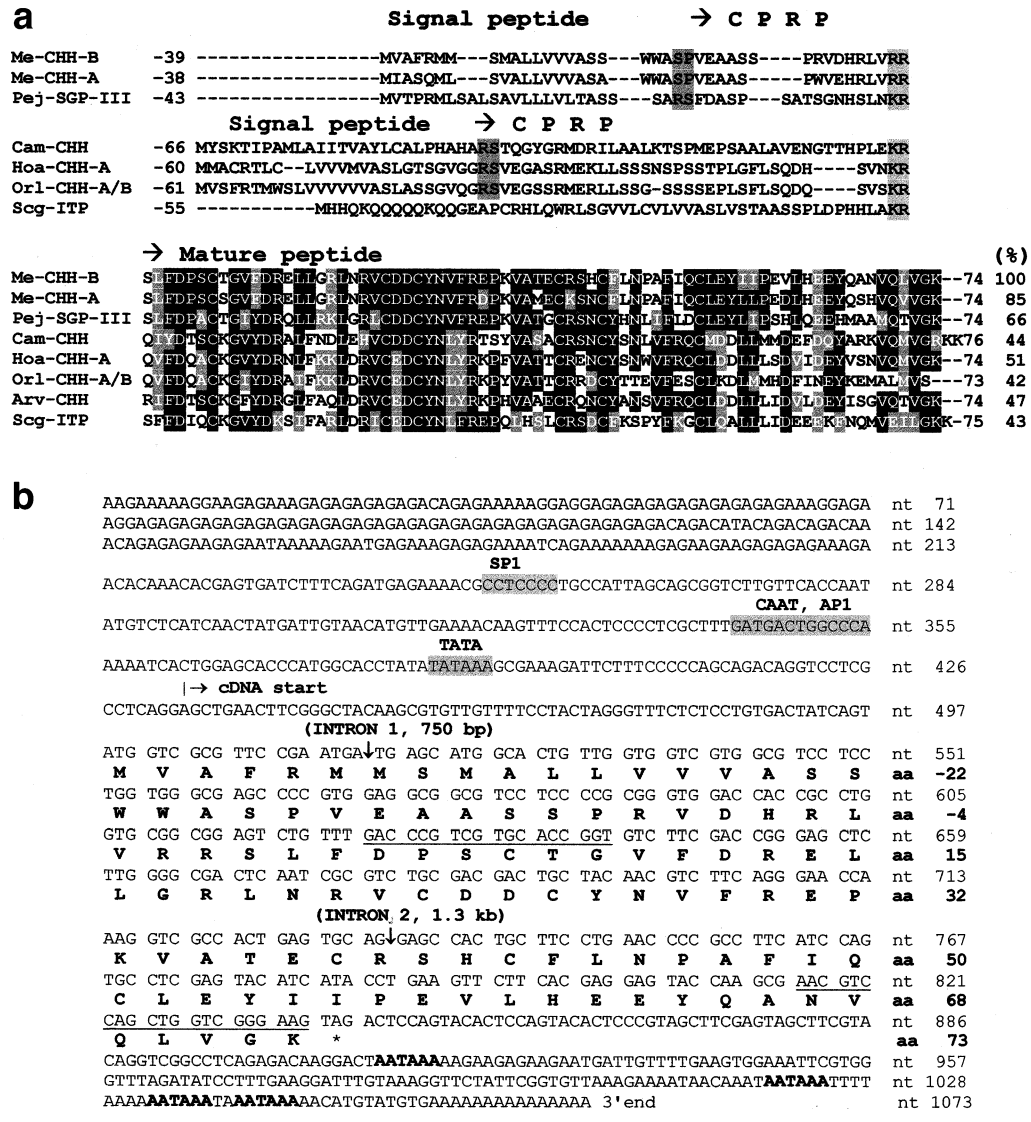

Fig. 1. a: Top: Alignment of the signal peptide and CPRP for different CHH-related neuropeptides. The cleavage site for the mature peptide is shaded in light gray box. Bottom: Alignment of the amino acid sequence of the mature MeCHH-B with CHHs of other decapods and the insect ion transport protein (ITP). The amino acid sequences included the CHH-A neuropeptide of the shrimp *M. ensis*, the lobster *Homarus americanus*, the crab *Carcinus maenas*, the crayfish *Orconectes limonus*, the woodlouse *Armadillidium vulgare* and the ITP of the locust *Schistocerca gregaria*. Identical amino acid residues are shaded in darker color and similar amino acid is shaded in light gray color. Dashes are inserted to allow maximum alignments. The percentage indicates the overall amino acid identity for the mature peptide. b: Nucleotide and deduced amino acid sequence of the shrimp MeCHH-B gene. The putative polyadenylation signal sites are in bold letters. The positions corresponding to the sequences of the primers (S1: forward and S2: reverse) used in the initial RT-PCR are underlined. The downward arrow indicates the intron insertion position. Numbers on the right margin represent amino acid and nucleotide positions. The horizontal arrow indicates the start of the cDNA. Putative elements for AP1, CAAT-BF and TATA are indicated above and below the nucleotide sequences.

quence beyond 100 bp shows little homology between the MeCHH-B and MeCHH-A genes. Together with the lambda clones isolated from the CHH-A genomic library screening, the arrangement of MeCHH-B in the CHH gene clusters was reconstructed (Fig. 2c). We have analyzed 16 MeCHH-A and two MeCHH-B genes in six lambda clones and could not establish any link between the two CHH-A/CHH-B gene clusters.

3.3. Expression of CHH in shrimp

By Northern blot analysis, CHH-B transcripts can be detected in the eyestalks but not in the hepatopancreas, muscle, ovary, testis, nerve cord, epidermis and the heart (Fig. 3a). However, by RT-PCR, MeCHH-B transcripts can also be detected in different regions of the ventral nerve cord (Fig. 3b). Furthermore, MeCHH-B is expressed in the eyestalks of female shrimp undergoing vitellogenesis. However, the level of MeCHH-B transcript detected in the eyestalk of mature females is much lower than that of the MeCHH-A gene (Fig. 3c). To investigate the potential role of MeCHH-B during the vitellogenic cycle, eyestalk total RNAs from females of different gonad developmental stages were analyzed by Northern blot (data not shown). The result indicates that the MeCHH-B transcript level is high during the early vitellogenic stage, but drops to the minimum level at the mid-vitellogenic stage and increases to the maximum level in the pre-spawning female (Fig. 3d). This expression pattern is in contrast to that of the MeCHH-A gene [15].

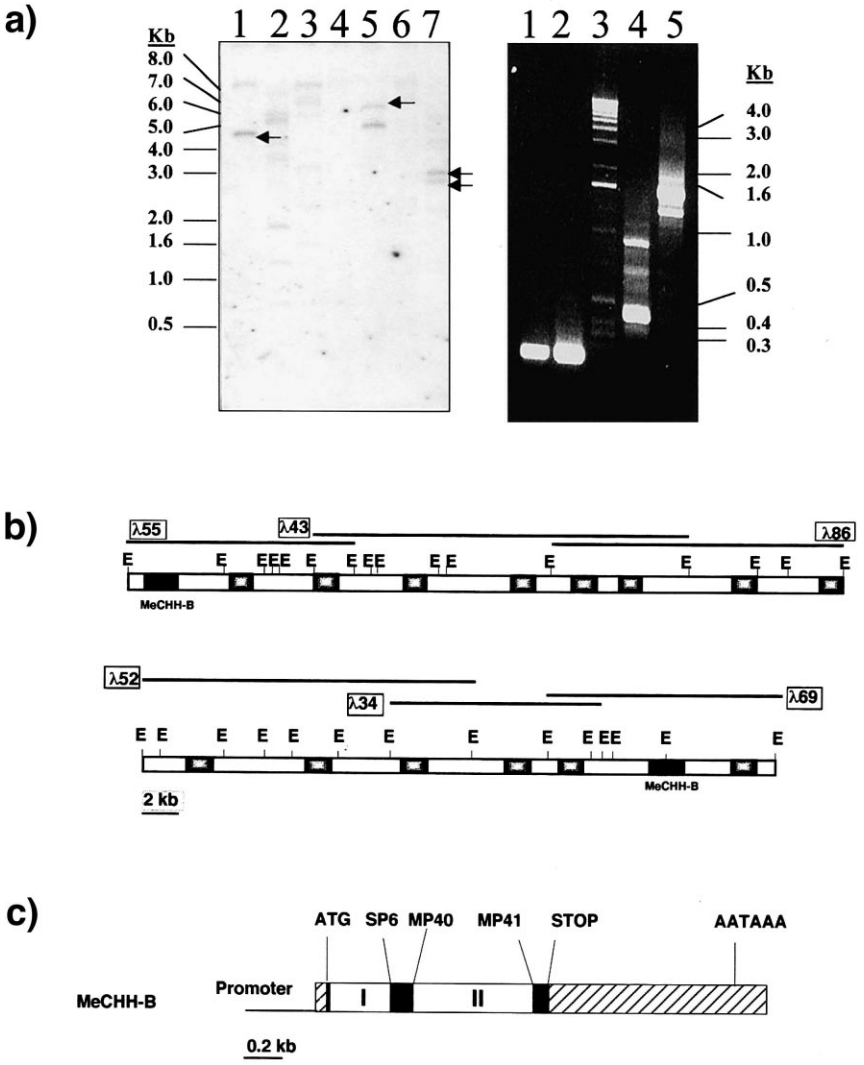

Fig. 2. a: Genomic Southern blot analysis of MeCHH-B. Left: Genomic DNA prepared from a single shrimp was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hin*dIII (lane 3), *Pst*I (lane 4), *Xba*I (lane 5), *Xho*I (lane 6) and *Sst*I (lane 7). The CHH-B-specific probe was derived from CHH cDNA. Numbers on the left indicate the size of the DNA molecular weight markers. Arrows indicate DNA fragments also hybridizing to the MeCHH-A probe [15]. Right: RT-PCR and genomic PCR detection of MeCHH-B RNA and the MeCHH-B gene. A common forward primer for the CHH-B and CHH-A genes and a specific reverse primer were used in PCR with genomic DNA template derived from a single individual. Lanes 1–2 are RT-PCR results for CHH-B (lane 1) and CHH-A (lane 2), lane 3 is the DNA size marker, lane 4 is genomic PCR with a gene-specific reverse primer for CHH-A and lane 5 shows major DNAs amplified by the CHH-B-specific primer. The sizes of the major DNA bands amplified are 1.6 kb and 1.2 kb. b: Reconstruction of the two gene clusters containing MeCHH-A and MeCHH-B. E indicates the position of the *Eco*RI restriction enzyme on the lambda clone. c: Schematic of the MeCHH-B gene. Coding region, intron and untranslated region are indicated by black, white, and diagonally hatched boxes respectively.

3.4. Bacterial expression of the shrimp rCHH-A and rCHH-B and bioassay

Since the expression vector contains an additional sequence of 27 amino acids, the expected size of the fusion proteins should be 11.8 kDa. Western blot analysis using anti-histidine polyclonal antibody detected a protein of the expected size in the purified recombinant protein (both rCHH-A and rCHH-B). Thus a large quantity of the recombinant protein was prepared. For bioassay of CHH activity, shrimp were bilaterally eyestalk-ablated. Over 80% of the shrimp survived the ablation treatment. During the bioassay for rCHH, shrimp were handled with great care to reduce stress which may cause a rise of hemolymph glucose level. Shrimp were injected with 2 μg (approximately 150 pmol) of rCHH. The average glucose level of unablated shrimp was 7 mg/dl. This level was reduced significantly to 5 mg/dl. A preliminary experiment was performed to study the hyperglycemic effects of the fusion protein. An initial time course experiment was performed to determine the optimal time for taking glucose measurement after hormonal treatment. The result indicates that the hemolymph glucose level increases to a maximum at 1 h after injection of the eyestalk sinus gland extract. The concentration used in the injection study could elicit a similar response in terms of the time and effect on the glucose increase. For shrimp injected with PBS, no change in hemolymph glucose level was recorded. However, we noticed that about 20% of shrimp did not respond to hormone or eyestalk extract treatment. Hemolymph glucose levels of these shrimp usually dropped as the sampling time progressed during the time course experiment. The results show that rCHH could produce a significant in-

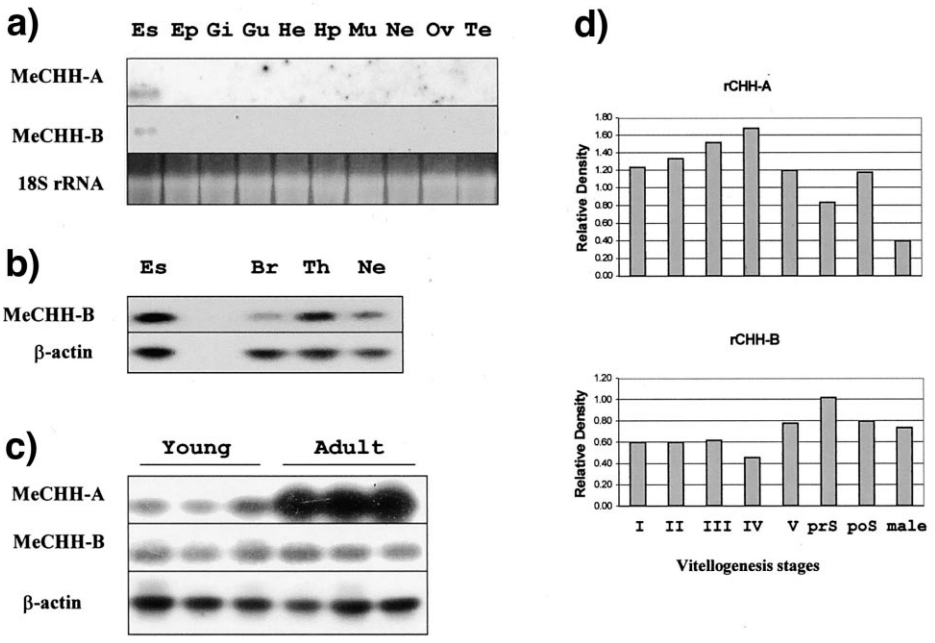

Fig. 3. Expression of the MeCHH-B gene. a: Northern blot analysis to show tissue distribution of MeCHH-B. Lanes are RNA samples from eyestalk (Es), epidermis (Ep), gill (Gi), gut (Gu), heart (He), hepatopancreas (Hp), muscle (M), nerve cord (Ne), ovary (Ov) and testis (Te). The bottom panel shows ethidium bromide staining of the 18S rRNA of the corresponding samples. b: RT-PCR detection of MeCHH-B transcripts in the CNS of shrimp. The lanes are eyestalk (Es), brain (Br), thoracic ganglion (Th) and nerve cord (Ne). The lower panel indicated RT-PCR detection of the shrimp β-actin gene using gene-specific primers [29]. c: Expression of MeCHH-A and MeCHH-B in juveniles and adult females. d: Expression of MeCHH-B and MeCHH-A during the gonad maturation cycle of *M. ensis*. The histogram indicates the estimation of the relative RNA level from a scan of relative intensity of the autoradiograph. The RT-PCR of the shrimp β-actin gene [30] was used to normalize CHH-A and CHH-B expression.

crease in hemolymph glucose level. In each of these studies, more than 20 individuals were sampled. Hemolymph glucose level increased one-fold 30 min after injection of rCHH and increased to two-fold after 1 h. The glucose level of the shrimp increased two-fold after injection of rCHH. The hyperglycemic effect of rCHH was comparable to injection of one sinus gland extract (Fig. 4b).

4. Discussion

So far only two CHH-related peptides have been described in lobster, crayfish and crabs. The CHH isoforms described in these crustaceans usually share $>95\%$ amino acid identity [5,6,18,19]. This study provides proof that an additional CHH-related neuropeptide with low homology exists in the shrimp genome. When we performed genomic PCR using specific primers, several genomic DNAs were amplified (Fig. 4). Since only the major amplified fragments were characterized, other amplified fragments may represent other CHH-related genes. It appears that additional CHH-related peptides also exist in shrimp. In *Penaeus vannamei*, two different CHH-related cDNAs have been cloned [20,21]. In *Penaeus japonicus* and *Penaeus monodon*, several CHH neuropeptides have been isolated from the sinus gland [5]. It appears that more CHH-related neuropeptide occur in shrimp than other crustaceans.

The MeCHH-A/B gene cluster in shrimp is most likely the result of gene duplication from an ancestral gene during evolution. In our analysis of the gene cluster, a higher degree of homology was found between neighboring MeCHH-A genes. Although different isoforms of CHH have been identified in lobster, crayfish and crab, the organization of these genes in the genome is unknown. However, it is well known that many closely related genes have evolved from gene duplication and/or amplification. For example, in insects, multiple PTTH genes have been reported [22] and in some vertebrates, five copies of growth hormone genes have been identified [23]. Most of these genes are also arranged in a cluster on the same chromosome. Although we have isolated several CHH-containing lambda clones, only two clones (λ52 and λ69) contained the MeCHH-B gene. Based on these results and the genomic information, we estimate that the shrimp has at least two MeCHH-B genes. We have analyzed seven different lambda clones containing the CHH gene cluster, but we have not established any physical link between the two gene clusters. Moreover, other eyestalk neuropeptide genes (viz. MIH and GIH) have not been identified. The absence of MIH genes in the CHH gene cluster suggests that the CHH subfamily and MIH subfamily genes might be distributed in different chromosome loci. Alternatively, MIH and CHH genes may be located on a different chromosome. Therefore, it appears that the gene duplication/amplification event for the CHH and other eyestalk-related neuropeptide is highly complicated. Further studies using chromosome walking and/or fluorescent in situ hybridization may be able to resolve the issue.

From our study of the gene structure of the shrimp MeCHH-B, MeCHH-A neuropeptides [15], partial MeMIH-like neuropeptide [24], and the crab ChfMIH [25], we could deduce that the crustacean CHH/MIH/GIH family of neuropeptides share a conserved basic gene structure (Fig. 2c). The gene consists of three exons interrupted by two introns. Furthermore, the intron/exon boundary site is highly conserved [15]. The first intron is always inserted behind the ATG en-

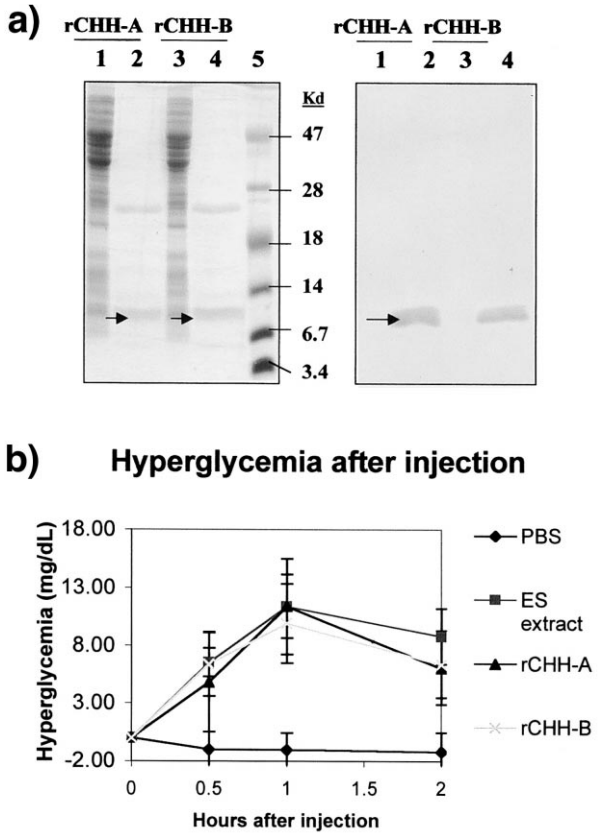

Fig. 4. a: Detection of MeCHH-A and MeCHH-B fusion protein. Left: SDS–PAGE of recombinant MeCHH-A (rCHH-A) and MeCHH-B (rCHH-B) stained by Coomassie blue. Lane 1: bacterial whole lysate, lane 2: Ni^{2+}–agarose-purified rCHH-A; lane 3: bacteria whole cell extract; lane 4: Ni^{2+}–agarose-purified rCHH-B. Right: Analysis of fusion proteins by Western blot using anti-histamine polyclonal antibody. The sizes of the protein standard are indicated (in kDa). b: Biological assay of shrimp rCHH-A and rCHH-B. Effect of rCHH-A and rCHH-B on the hemolymph hyperglycemic effect in bilaterally eyestalk-ablated shrimp. The *y*-axis indicates the increase of hemolymph glucose (mg/dl) and the *x*-axis represents the time of hemolymph sampling after 0, 0.5, 1.0 and 2.0 h.

coding Met in the prepro-peptide region and the second intron splits a basic amino acid codon Arg-40 (in MeCHH-B) or Lys-40 (in MeCHH-A, i.e. 43-1 to 43-4) or Arg-41 (in MeMIH) into two parts (Fig. 1b). The sites of the first and second intron insertion are also identical to those in the MIH of the crab *Charybdis feriatus* [25]. Although the crustacean eyestalk CHH/MIH/GIH neurohormone gene family has not been described in vertebrates, an ITP gene identified in the locust *Schistocerca gregaria* shows a significant 40% similarity to the CHHs. Interestingly, an addition of 40 amino acid residues was inserted at the site of Arg-40 to form a long form of the ITP [17]. The relative position of this site is identical to the intron 2 position in the genes of the MeCHH-B, MeCHH-A neuropeptide, MeMIH and ChfMIH. Therefore, it appears that both the insect ITP and crustacean CHH may have a similar gene structure. We speculate that a similar gene organization occurs for other crustacean neuropeptides belonging to the CHH/MIH/GIH family. Since both MeCHH-A and MeCHH-B neuropeptides clustered together with the insect ITP as a group (data not shown), it appears that the shrimp CHH is derived from a more ancient primordial ancestral gene common to the crustacean and insect.

At present, the sources for the eyestalk CHH/MIH/GIH neuropeptides have expanded in other neuronal tissues. At the RNA level, MeCHH-B can be detected in the eyestalk and the CNS. This study demonstrates that MeCHH-B is also expressed in other nervous tissues of the shrimp. In the crab and other crustaceans without eyestalks, additional sources for CHH-related neuropeptides have also been identified [26,27]. Although CHH-like peptide has also been detected in the subesophageal ganglion and thoracic root of the lobster [28], the antibody used cannot distinguish between CHH-A and CHH-B.

During female reproduction, there is a drop in MeCHH-B transcript level during the middle stage of vitellogenesis. During this drop of MeCHH-B RNA level, a maximum level of MeCHH-A was recorded. The antagonistic expression pattern of MeCHH-A and MeCHH-B suggests that the ratio of MeCHH-A and MeCHH-B may be important for the regulation of gonad maturation. Because both rMeCHH-A and rMeCHH-B produce similar hyperglycemic effects as shown in the bioassay, a continuous high level of MeCHH-A or B is needed for the maintenance of glucose/energy metabolism. A high level of MeCHH-A may be needed for the initial gonadal stage (stage I–II) and a high level of MeCHH-B is required for gonad maturation during the middle and late vitellogenic stages. Thus the ratio of MeCHH-A and MeCHH-B may be important for the regulation of vitellogenesis in female shrimp. In the lobster *H. americanus*, the amounts of CHH-A form and CHH-B form are more or less equal [9] during the pre-vitellogenic stage of the adult and the ratio changes as gonad maturation proceeds. In the crayfish, *Orconectes limosus*, CHH-I neuropeptide appears to be the major expressed form [18,29].

Although the recombinant protein consists of 20 additional amino acids at the N-terminal end, it has no effect on the hyperglycemic property of the rCHH. The hyperglycemic effect of the recombinant protein is comparable to that of the crude eyestalk extract. Although the concentration of recombinant protein used in the injection study is high, we reason that various possible combinations of disulfide bonding occur during the renaturation of the fusion protein and only a small fraction of the renatured protein contributes to the bioactivity.

In conclusion, MeCHH-B shows characteristics of the crustacean hyperglycemic hormone and the gene encoding MeCHH-B has a similar gene organization as the members of the CHH/MIH/GIH family. Furthermore, the genes for MeCHH-B are located in the MeCHH-A neuropeptide gene cluster. The close physical relationship of these two genes provides significant information for the evolutionary history of the CHH/MIH/GIH neuropeptide gene family. Although both MeCHH-A and MeCHH-B genes are expressed in the eyestalk of reproductive females, their antagonistic pattern of expression and the hyperglycemic effect of the recombinant proteins suggest an important role of these two neuropeptides in the regulation of glucose metabolism and the regulation of vitellogenesis in the females. Finally, this study also provides an implication for similar approaches in the study of other important eyestalk hormones such as MIH and GIH in crustaceans.

Acknowledgements: This work was supported by a HKU institutional grant (CRCG) awarded to S.M.C.

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