CLONING, SEQUENCE ANALYSIS AND EXPRESSION OF THE HYPERGLYCEMIC NEUROHORMONE FAMILY IN CRUSTACEANS

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GENERAL INTRODUCTION

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In order to survive, organisms need the ability to adapt to changes in their internal and external environment. For this, communication between cells is essential for the regulation of the functioning of tissues, organs and the organism as a whole. Two important systems are involved in this regulation: the nervous system and the endocrine system.

The nervous system contains many neurons which are designed for quick responses to environmental changes. The neurons are able to transfer a message by an electrical signal, the action potential. This signal is transported through the axon to other neurons or target cells. The communication between neurons and their targets occurs at a specialized structure, the synapse. Such a structure can be identified morphologically by the presence of a pre-(axon) and postsynaptic (neuron or target cell) membrane separated by the small synaptic cleft. In order to send the message from the axon to the target cell, chemical messengers (neurotransmitters and neuropeptides) are released from the presynaptic site as a result of an action potential. The neurotransmitters influence the target cell by binding to postsynaptic receptors or ion channels. The response of the target cell is fast and therefore the nervous system is responsible for the short-term regulation of physiological processes. Many neuropeptides can function synaptically or act in a paracrine fashion as a "diffusible hormone" and seem to affect many neurons over long distances (Kandel & Schwarz, 1981).

The other regulatory system, the endocrine system, produces messengers like peptide hormones and steroid hormones. In contrast to neurotransmitters, hormones are released into the circulation and can be transported over longer distances. This process is slower and lasts longer than communication through neurotransmitters. The endocrine system is therefore responsible for medium- and long-term regulation of physiological processes such as growth, development and reproduction.

The finding that neuron-like cells produce peptides that are released into the blood made clear that the existence of only two systems that are responsible for regulating physiological processes is too simple and resulted in the description of the neuroendocrine system (Scharrer, 1990). Moreover, it was established that the neurohormones produced by this

system can also be colocalized with classical neurotransmitters (Hokfelt et al, 1989). Overall, the interactions between the three systems can be summarized as follows: nervous structures are mostly used for perception of distinct environmental cues, a complex neuroendocrine system is responsible for transduction of the messages to the endocrine glands which produce factors regulating the activity of organs involved in processes dealing with the life cycle of animals.

The neuroendocrine system in Crustacea

In crustaceans, short-, medium- and long-term regulatory processes are also involved in the adaptation of these animals to changes in their environment. For example, a short-term regulatory system is involved in background adaptation by controlling pigment migration in these animals. Medium-term regulation is important for metabolic processes such as the regulation of glucose levels in a diurnal cycle, whereas long-term regulation is necessary for tuning of seasonal cycles like the molting and reproduction cycles. In crustaceans, the neuroendocrine system is distributed throughout the central nervous system. Active centers for the storage and release of neurosecretory products are concentrated in three neurohemal regions: the postcommissural organs, the pericardial organs and the sinus gland (Kleinholz & Keller, 1979).

The best-studied neurohemal organ is the sinus gland in the eyestalk of decapod crustaceans. This gland is the storage- and release-site of peptides produced by a group of neuronal perikarya located in the rostro-ventral side of the medulla terminalis ganglia which is one of the four optic ganglia in the eyestalk (Bliss, 1951; Passano, 1951; Fig. 1). More than sixty years ago, this structure of clustered cell perikarya was named the medulla terminalis ganglionic X-organ (MTGX) by Hanström (1928, 1931, 1933). Later, it was found that the sinus gland is also a neurohaemal organ for neuroendocrine cells originating from all four optic ganglia (Cooke & Sullivan, 1982). Because of its relatively simple structure, the MTGX-sinus gland (MTGX-SG) is well suited as a model for research on neuroendocrine systems. Moreover, anatomically it can be divided into the region of neuroendocrine perikarya, the tractus and the neurohemal site of axon-terminals. This characteristic makes the system suitable for independent manipulation of the different

regions. As well, mRNA (perikarya) and stored neuropeptides (sinus gland) can be simultaneously monitored by biochemical techniques and their levels can be determined in individual eyestalks.

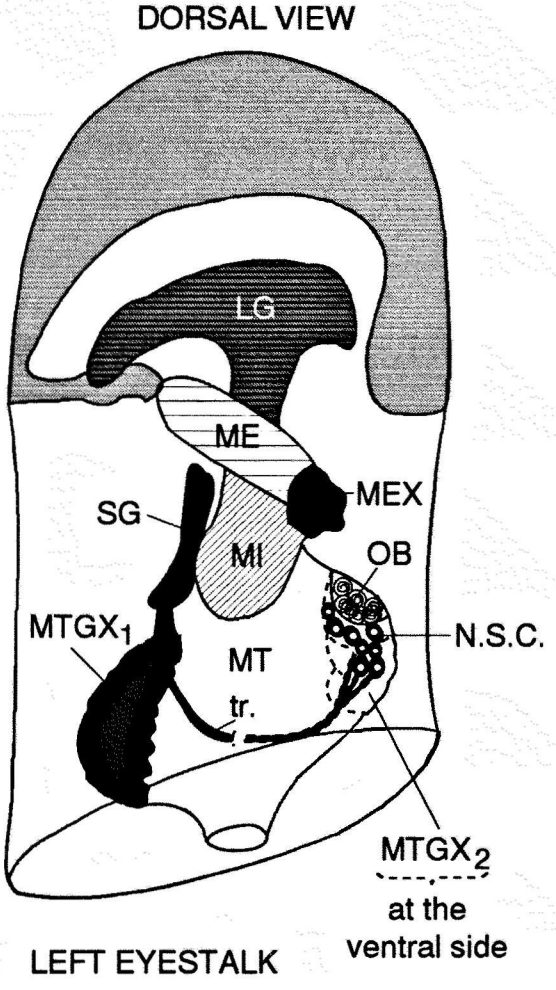

Fig. 1 Schematic representation of a longitudinal section of a crustacean eyestalk showing the four optic ganglia and the X-organ sinus gland complex.
Abbreviations: LG: lamina ganglionaris; ME: medulla externa; MEX: medulla externa X-organ; MI: medulla interna; MT: medulla terminalis; MTGX: medulla terminalis ganglionic X-organ; NSC: neuro-secretory cells; OB: organ of Bellonci; SG: sinus gland; tr: tractus

Regulation of the medulla terminalis ganglionaris X-organ sinus gland complex

The crustacean hyperglycemic hormone (CHH) is the major neuropeptide produced and released by the MTGX-SG complex. It is responsible for the increase in blood sugar levels. Injection of a number of neurotransmitters also showed an effect on glycemia in Crustacea. For instance, not only serotonin but also dopamine provokes hyperglycemia (Keller & Beyer, 1968; Strolenberg & Van Herp, 1977; Martin, 1978; Kallen, 1988), whereas Met-enkephalin decreases blood glucose levels (Kallen, 1988). Subsequently, synaptic contacts immunopositive for anti-serotonin, anti-dopamine and anti-Met-enkephalin have been demonstrated to form a junction with a presumptive CHH axon using double immunocytochemical methods at the light and electron microscopical level (Van Herp & Kallen, 1991). These studies revealed serotonergic input on CHH axon-branches and suggest that serotonin, dopamine and Met-enkephalin might well be involved in the regulation of the MTGX-SG system.

Crustacean neurohormones

The first invertebrate neurohormone for which the amino acid sequence was determined, is the red pigment concentrating hormone of crustaceans (RPCH; Fernlund, 1974a). Later, other members of the chromatophorotropin family such as the pigment dispersing hormone (PDH) were characterized. These neuropeptides are involved in short-term regulation of background adaptation (for review see: Rango Rao & Riehm,1989). Subsequently, neurohormones involved in medium- and long-term regulation were characterized. CHH of the crab *Carcinus maenas* was the first neurohormone which was sequenced (Kegel et al., 1989), followed by crayfish CHH (Kegel et al., 1991) and two lobster CHHs (CHH-A and CHH-B; Tensen et al., 1991). Next, the primary structure of the molt-inhibiting hormone (MIH) of the shore crab, which inhibits synthesis of ecdysteroids by the Y-organ, was determined (Webster, 1991). Finally, the structure of lobster vitellogenesis-inhibiting hormone (VIH), which inhibits the onset of vitellogenesis, was elucidated (Soyez et al., 1991). Sequence comparison between MIH, VIH and the CHHs, especially the positions of the Cys residues, showed that these neurohormones constitute an authentic peptide family across species bounderies (Keller, 1992). Together, this group is known as the CHH/MIH/-

VIH family (Kegel et al., 1991; Keller, 1992). A feature of this family is the existence of isoforms. CHH-isoforms have been found in crab, crayfish and lobster (Kegel et al., 1989; Kegel et al., 1991; Tensen et al., 1991a) and GIH-isoforms have been reported in the lobster (Soyez et al., 1991). The isoforms have identical amino acid sequences, iso-electric points and molecular weights, and recently it was reported that the difference in HPLC elution times of the CHH-isoforms was due to the presence of a D-amino acid instead of the normal L-amino acid at the third position (Soyez et al., 1994).

The members of the CHH family (CHH, MIH, VIH) are all involved in medium- and long-term regulatory events. The function(s) of CHH in medium- as well as in long-term regulation is remarkable. In addition to its reported medium-term hyperglycemic effect in the circadian cycle (Kallen et al., 1988), CHH may also have a function in long-term regulation, like during molting and reproduction. Using a heterologous bioassay system, it was shown that HPLC-purified lobster CHH stimulates ovarian growth in shrimp (Tensen et al., 1989) and may have a molt-inhibiting as well as a hyperglycemic effect (Chang et al., 1991). In order to grow, crustaceans have to change their hard external crust, a process which is known as the molting process. Molting and reproduction are cyclic processes, which both require optimal tuning of the three regulatory systems described above. For example, crustaceans should not molt when they are brooding otherwise they lose their progeny. In addition, the processes of molting and vitellogenesis need large amounts of energy and are probably not succesfull when they occur simultaneously. On the basis of physiological events the molting process is divided into four basic stages called postmolt, intermolt, premolt and molt or, according to morphological criteria, into five major stages A to E (Drach, 1939). Concerning crustacean reproduction most research is carried out on females and the different steps in ovary development can be divided into oogenesis, primary vitellogenesis (or previtellogenesis), secundary vitellogenesis (or vitellogenesis), oocyte maturation and ovulation (or ovarian spawning) (Charniaux-Cotton and Payen, 1988; Van Herp and Payen, 1991; Van Herp, 1992).

Molting and reproduction biology in the female lobster

A table for the determination of the stages in the molting cycle of lobsters has been

described by Aiken (1973) and Gilgan and Zinck (1975) based on the stages A to E according to Drach (1939). Ovarian development in lobster can be divided into six different stages according to morphological criteria such as ovary size, ovary color and oocyte size, or in four stages (immature, previtellogenesis, vitellogenesis and mature) according to metabolic criteria (Aiken & Waddy, 1980). The American lobster *Homarus americanus* and the European lobster *Homarus gammarus* are unique among Nephropsidae in that they require two years to produce a single brood. Moreover, their molting and reproductive cycles are differentially controlled by temperature and photoperiod (Waddy & Aiken, 1992). Female American lobsters are normally in intermolt stage C4 at the time of

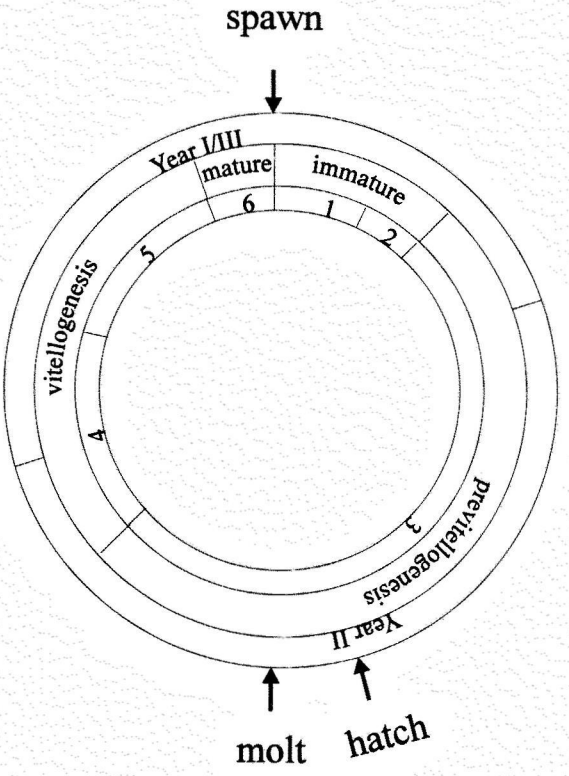

Fig. 2. Schematic representation of the reproductive cycle of a typical female American lobster. The outer circle represents the time in years, the inner circle the oocyt stages 1-6 and in between the different physiological stages of the ovary (immature, previtellogenesis, vitellogenesis and mature).

oviposition which implies complete synchronization between the molting and reproductive cycles. Since females without eyestalks may extrude their eggs in early to mid-premolt, it is likely that the MTGX-SG in the eyestalks is involved in the process of synchronization (Aiken et al., 1979).

The reproductive cycle of a typical female American lobster is summarized in Figure 2. After spawning of mature eggs in June/July of year one, the white immature ovary is in stage one and contains oocytes smaller than 0.5 mm. Stage two is also an immature stage with first a yellow, beige ovary changing to pale green and oocytes are smaller than 0.8 mm. The immature ovary changes to a developing ovary after a few months and is in stage three in the autumn of year one. In this stage the ovary is light to medium green and the oocytes are still smaller than 1.0 mm. This stage can be associated with previtellogenesis and has a duration of almost one year. In the summer of year two, the larvae hatch and in September/October the lobsters molt and mate. In the autumn of year two, the ovary becomes medium to dark green and the oocytes have a size of 0.1 - 1.6 mm. This stage four is probably associated with the start of vitellogenesis which is retarded during winter but accelerates in the spring of year three. Via stage five, with a dark green ovary and an oocyte size of 1.0 - 1.6 mm, the ovary enters mature stage six with oocytes of 1.4 to 1.6 mm and the female spawns again in June/July of year three (Aiken & Waddy, 1980a). Although molting frequency decreases with age or size, the bi-annual ovarian cycle continues and the necessity for alternating years of molting and mating is avoided by the ability of fertilizing at least two broods from a single insemination (Aiken & Waddy, 1980b).

Methodological approach, aim and outline of the thesis

At the start of this work only amino acid sequences of the stored hormones of the CHH family were available for some crustacean species, especially of the lobster *Homarus americanus* (Tensen et al, 1991; Soyez et al, 1991) and one cDNA sequence of pre-proCHH of the crab *Carcinus maenas* was reported (Weidemann et al, 1989). The latter was determined by a molecular biological approach which was also successfull for the

characterization of the CHH-encoding regions of lobster CHH cDNAs (Tensen et al, 1991a). Using this approach, Tensen (1991d) started a study of gene expression in crayfish and lobster, although at that time no specific cDNA probes for lobster CHH-A, CHH-B and GIH were isolated. This study was extended in the present thesis using molecular biological techniques in combination with micro-HPLC analysis, immunocytochemistry and ELISA. Our approach provided the tools to study, at different periods of the life cycle, not only the cellular and tissue-specific expression of the CHH gene family but also the storage and release of neurohormones belonging to this physiologically important family.

The study presented in this thesis had a number of goals. Firstly, lobster cDNAs encoding the preprohormones of the CHH-family were isolated and sequenced. The availability of the cDNAs enabled us to study the tissue-specific expression of the CHH family members preproCHH-A and -B (Chapter 1) and preproGIH (Chapter 2) in several tissues of the lobster *Homarus americanus*. Secondly, these specific tools were used in *in situ* hybridization studies combined with immunocytochemistry in order to study the cellular localization and synthetic activity of the MTGX-SG complex in adult lobsters (Chapter 3) and in lobster larvae (Chapter 4). Thirdly, expression of lobster CHHs and GIH during the reproduction cycle was studied at the level of mRNA and peptide storage in the MTGX-SG complex, and peptide levels in hemolymph (Chapter 5). These results provide information on the multifunctionality of the CHH hormones. Finally, our knowledge was extended from a sea water species to a fresh water species in Chapter 6 which reports the primary structures of CHH-family members in the crayfish *Orconectes limosus*, together with their tissue-specific expression, mRNA/peptide storage ratios and the number of CHH-genes.

CHAPTER 1

Cloning and expression of two mRNAs encoding structurally different Crustacean Hyperglycemic Hormone precursors in the lobster *Homarus americanus*

ABSTRACT

The Crustacean Hyperglycemic Hormone (CHH) of the X-organ sinus gland complex is a multifunctional neurohormone primarily involved in the regulation of blood sugar levels. HPLC analysis of lobster sinus glands revealed two CHH-immunoreactive groups, each consisting of two isoforms with identical amino acid sequences and molecular weights. In order to obtain more information concerning the number and sequences of preproCHHs, and to study their expression, we isolated two full-length cDNAs encoding two different CHH preprohormones. Both preprohormone structures consist of a signal peptide, a CHH-Precursor-Related Peptide and a highly-conserved CHH peptide. Expression studies revealed that the X-organ is not the only source of CHH mRNA because the ventral nerve system also expresses this mRNA. Based on these findings and earlier studies on the effect of eyestalk ablation, implantation of thoracic/abdominal ganglia as well as the multfunctionality of CHH, we postulate that CHH, present in the ventral nerve system is a good candidate for a supplementary role in the control of reproduction and molting.

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INTRODUCTION

The X-organ sinus gland complex, situated in the optic ganglia of the eyestalk, regulates and modulates various physiological processes in decapod Crustacea. This neuroendocrine system consists of neurosecretory cell somata whose axon endings form a neurohaemal organ, the sinus gland. The Crustacean Hyperglycemic Hormone (CHH), primarily involved in regulating blood sugar levels and glycogen metabolism, is one of the most abundant hormones produced by this complex. HPLC analysis revealed that this neurohormone is polymorphic in several Crustacea (Kegel et al., 1989; Kegel et al., 1990; Huberman & Aguilar, 1988; Huberman & Aguilar, 1989). In the HPLC pattern of *Homarus americanus* sinus gland extracts, two groups of hyperglycemic neuropeptides were found. Each group consists of two isoforms with the same amino acid sequence and molecular mass (Tensen et al., 1991b; Tensen et al., 1991c). The primary structures of the two variants were established by the cloning of partial cDNAs encoding CHH-A corresponding to the first group (CHH-I/II) and CHH-B corresponding to the second group (CHH-VI/VII) (Tensen et al., 1991b). In addition, the amino acid sequence of one CHH Precursor Related Peptide (CPRP) has been established (Tensen et al., 1991d). Up to now, however, the number and complete structures of the preproCHHs have not been determined and it is not known how many different CPRPs exist in the lobster. In recent years different physiological effects of CHH peptides were found, indicating the multifunctional role of the CHHs. Peptide isoforms of CHH-B (peak VI/VII) showed not only a hyperglycemic activity but also a stimulatory effect on oocyte growth (Tensen et al., 1989). In addition, a lobster neuropeptide with both molt inhibiting and hyperglycemic activity has been found to be highly related to the CHH-A sequence (Chang et al., 1990).

In order to study the number and structural organization of preproCHHs, we cloned and sequenced two full-length cDNAs encoding lobster preproCHH-A and -B, and used these cDNAs to study their expression in different tissues of the lobster.

MATERIALS AND METHODS

Animals

Specimens of the lobster *Homarus americanus* were obtained from a commercial importer and originated from the east coast of Canada. The animals were kept in the Department of Animal Physiology (University of Nijmegen) in artificial seawater at about 5°C for a maximum of one week after arrival.

RNA isolation

Total RNA was isolated according to the method of Chomczynski and Sacchi (1987) using acid-guanidine-thiocyanate-phenol-chloroform extraction. For the construction of a Medulla Terminalis (MT) cDNA library, tissue from 5 eyestalks were pooled and homogenized in an all-glass homogenizer containing 500 µl guanidine thiocyanate solution (4 M guanidine thiocyanate; 25 mM sodium citrate, pH 7.0; 5 % sarcosyl; 0.1 M 2-mercaptoethanol) and transferred to a microcentrifuge tube. Sequentially, 50 µl of 2 M sodium acetate (pH 4.1), 500 µl water-saturated phenol and 100 µl chloroform-isoamylalcohol (49:1) were added and the sample was vigorously mixed. RNA was, after ethanol precipitation, used for poly (A^+) RNA isolation according to the instructions of the manufacturer (Stratagene).
Tissues used for Northern blot analysis (e.g. medulla terminalis (MT), ventral nerve system, testis, ovaria, hepatopancreas, heart, green gland) were extracted in the same way.

Construction and screening of the Homarus americanus MT cDNA library

About 5 µg MT poly (A^+) RNA was used for the construction of a cDNA library in the vector λZAP-II (Stratagene). Approximately 500.000 clones of this library were screened with a partial *H. americanus* CHH-A cDNA probe (corresponding to bp 183-399 in Fig. 1) (Tensen et al., 1991b). This CHH-A encoding cDNA fragment was labeled with ^{32}P by random priming according to standard procedures (Sambrook et al., 1989). Hybridization of the replica nitrocellulose filters was performed at 42°C in 6 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1 x Denhardts solution, 25 mM sodium phosphate buffer pH 7.0, 10% dextran sulphate, 200 µg/ml yeast tRNA and 50% formamide.

Following hybridization, the filters were washed once in 2 x SSC, 0.1 % sodium dodecyl sulphate (SDS) at room temperature for 10 min and subsequently washed in 2 x SSC, 0.1% SDS, 1 x SSC, 0.1% SDS and 0.25 x SSC, 0.1% SDS at 68°C for 20 min each. Hybridization-positive phage plaques were purified and the recombinant pBluescript SK- phagemids were rescued from the bacteriophage (λZAP) clones by in vivo excision according to the instructions of the manufacturer (Stratagene). Sequencing on both strands was performed with single- and double-stranded DNA using T7 DNA polymerase and the dideoxy chain termination method (Sanger et al., 1977).

Labelling and purification of RNA probes

RNA probes were synthesized as run-off transcripts from 200 ng linearized DNA of pBluescript KS^+ containing an insert corresponding to nucleotides 1-302 of preproCHH-A mRNA (Fig. 1). Labelling was performed in a final volume of 10 µl containing 50 µCi [^{32}P] UTP, 15 units T3/T7 RNA polymerase in transcription buffer (Promega), 17,5 U RNasin (Promega) and 1 mM each of rATP, rCTP, rGTP. After 30 min at 37°C, 10 U of DNase I (Pharmacia) was added and the reaction mixture was incubated for another 10 min at 37°C. The cRNA probe was recovered after phenol extraction and ethanol precipitation using 10 µg tRNA as carrier.

Northern blotting

Poly (A^+) RNAs of different tissues (MT ~4 µg/lane, other tissues ~2 µg/lane) were separated by electrophoresis on denaturing formaldehyde agarose gels (1.0%) in MOPS buffer. Northern blot transfers to Hybond N (Amersham) were performed essentially as described by Thomas (1980). RNA was fixed by baking for 2 hr at 80°C. The CHH-A cDNA fragment corresponding to nucleotides 1-302 (Fig. 1) was cloned into pBluescript KS^+ and used for the generation of a cRNA probe hybridizing with both CHH mRNAs. Hybridization was performed at 50 °C for 16 hr and the filter was washed once with 0.25 x SSPE (1 X SSPE is 0.15 M NaCl, 0.01 M NaH_2PO_4 and 0.001 M EDTA) at 56°C for 30 min. RNA coloration according to Sambrook et al. (1989) was additionally used to control if all lanes contained undegraded RNA (results not shown).

RESULTS AND DISCUSSION

Isolation and characterization of cDNAs encoding Homarus americanus CHH prepro-hormones.

Screening of 500.000 clones of the Medulla Terminalis (MT) cDNA library resulted in the isolation of nine hybridization-positive clones. Two clones (largest insert 1181 bp) enco ded the CHH-A preprohormone consisting of a signal peptide of 24 amino acids followed by a CPRP of 34 amino acids and the CHH-A peptide of 72 amino acids with a potential C-terminal amidation site (Fig.1). In addition to the open reading frame, this cDNA contained 776 bp of 3'-untranslated region. The remaining seven clones (largest insert 1863 bp) encoded the CHH-B preprohormone with a 3'-untranslated region of 1362 bp. They contained an open-reading frame of 399

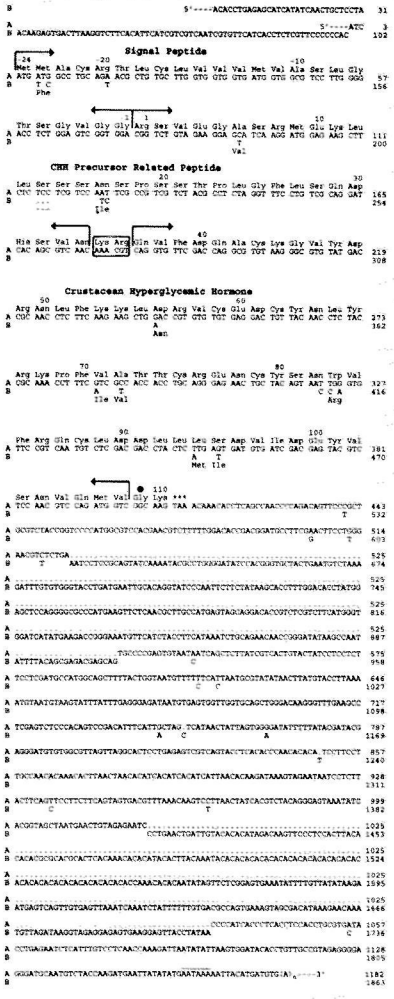

Fig. 1 Nucleotide sequences and deduced amino acid sequences of mRNAs encoding preproCHH-A and -B of the lobster *Homarus americanus*. Amino acid numbering starts at the N-terminal residue of the CHH prohormones with the presumptive signal peptide sequences being indicated by negative numbering. The deduced amino acid sequence of CHH-A preprohormone is shown above the nucleotide sequence. For CHH-B the differences in nucleotide and amino acid sequence are shown below the CHH-A sequences. The dibasic processing site between CHH and CPRP is boxed. The dot above Gly-109 indicates a potential amidation site. The signal for polyadenylation (Birnstiel et al., 1985) is overlined. Alignment was done using the method of Smith and Waterman (1981).

bp coding for a preprohormone with the same structural organization as the aforementioned preproCHH-A. In addition, this preproCHH-B-encoding cDNA differs from preproCHH-A cDNA in 17 positions resulting in 10 different amino acids: one substitution in the signal peptide, two substitutions and one deletion in the CPRP, and six substitutions in the CHH peptide (Fig.1). Between the CPRPs and CHHs of both preprohormones a potential dibasic processing site is found. Both CHHs have a potentialamidation site at the C-terminal end (Fig.1). Generation of specific probes for the two transcripts will now allow us to perform RNase protection assays in order to discriminate between CHH-A and -B mRNAs. Furthermore, it will give us the possibility to study the cellular (co)localization of CHH-A and -B mRNAs, thus extending the previous *in situ* hybridization studies on CHH mRNA in *Homarus americanus* (De Kleijn et al., 1992; Rotllant et al., 1993).

The occurrence of the two groups of CHHs, each consisting of two isoforms in the HPLC pattern of lobster sinus gland extracts, combined with the existence of only two lobster preproCHH-encoding cDNAs suggests that the origin of the different isoforms is due to a post-translational modification event. This modification could not be detected by FABS-MS or automated Edman degration (Soyez et al., 1990; Tensen, 1991e) and its nature is unknown. However, HPLC and FABS MS analyses of CHH fragments comprising the first eight amino acids suggested that the modification could involve the conversion of an L-amino acid to a D-amino acid (Tensen, 1991e), a modification previously found in two *Phyllomedusa sauvagei* skin peptides (Richter et al., 1987; Kreil et al., 1989).

Comparison of lobster preproCHH-A and -B with crab preproCHH

In the lobster *Homarus americanus* the preprohormone structures of CHH-A and -B consist of a signal peptide, a CPRP followed by the CHH peptide (Fig. 2), structural organizations similar to that of the previously cloned preproCHH of the crab *Carcinus maenas* (Weidemann et al., 1989). The overall amino acid sequence identity between the two lobster and one crab preproCHHs is only 44%, due to the low degree of identity in the signal peptide sequence (23%) as well as the CPRP sequence (23%). Our findings confirm that the CHH peptide itself is highly conserved (61%) (Tensen et al., 1991b; Keller, 1992).

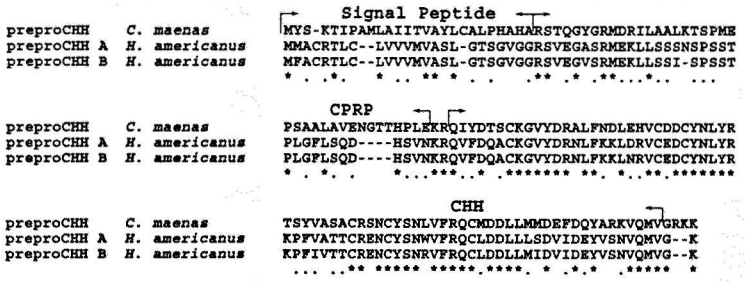

Fig. 2 Comparison between *Homarus americanus* CHH-A preprohormone, *Homarus americanus* CHH-B preprohormone and *Carcinus maenas* CHH preprohormone (Weidemannn et al., 1989). Sets of identical amino acid residues in the three preprohormones are indicated by an asterisk and conservative subsitutions are indicated by dots.

Expression of CHH-A and CHH-B mRNAs

The sizes and level of expression of CHH-A and -B mRNAs in a number of tissues from intermolt animals was determined by Northern blot analysis under very stringent conditions (Fig. 3). The sizes of the two MT CHH mRNAs (~1.2 kb and ~1.9 kb; Fig. 3A) are in agreement with the sizes of the full-length cDNAs encoding preproCHH-A (1181 bp) and preproCHH-B (1863 bp). Both mRNAs are expressed in approximately equal amounts in the MT. Longer exposure of the blot (Fig. 3B) showed that low levels of the two CHH mRNAs are also present in other parts of the nervous system. This statement is recently confirmed by preliminary RNAse protection assay experiments using CHH-A and -B specific probes (results not shown). In addition, the nervous tissue seems to contain slightly more of the smaller CHH mRNA than of the larger form. Pilot studies indicated that the slight difference in the migration distance of the CHH mRNAs in the nervous tissue, as showed in Fig. 3B, is caused by the higher amount of RNA present in the MT lane. The expression of CHH mRNA in nervous tissue shows that although the MT is an important neuroendocrine center in crustaceans, it is certainly not the only one. Previous indications for the presence of CHH in other parts of the nervous tissue than the eyestalk were given in an immunochemical study by Keller et al. (1985) and in a study using an

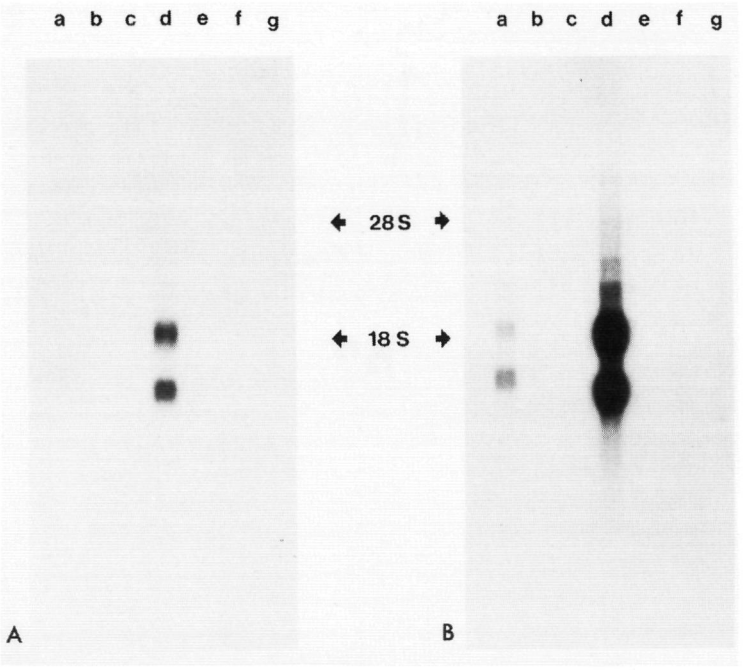

Fig. 3 Northern blot analysis of poly (A^+) RNA from the ventral nervus system (lane a), testis (lane b), ovaria (lane c), medulla terminalis (lane d), hepatopancreas (lane e), heart (lane f) and green gland (lane g) of the lobster *Homarus americanus*. The blot was hybridized with a cRNA probe (corresponding to nucleotides 1-302 of CHH-A mRNA in Fig. 1). The autoradiograph was exposed for 16 h (A) or 72 h (B) at -70°C with two intensifying screens. The positions of the 28S and 18S RNA size markers are indicated by arrows.

RNAse protection assay with a CHH probe which could not discriminate between CHH-A and -B mRNA (Tensen, 1991e). The physiological function of the CHH preprohormones in parts of the nervous tissue other than the optic ganglia is still unclear. Eyestalk ablation is routinely used for induction of vitellogenesis in female animals. Such an operation eliminates not only the Gonad-Inhibiting Hormone (GIH), a hormone controlling oocyte maturation, but also the Molt-Inhibiting Hormone (MIH). Suprisingly, lobster females mostly react on such an operation by the induction of vitellogenesis and not of molting, indicating that induction of the molt is suppressed by a factor from the nervous tissue (Aiken & Waddy, 1980). In addition, it was found that thoracic and supraoesophageal ganglion implants induce ovarian growth in the crabs *Potamon* and in *Paratelphusa,* respectively (Otsu, 1963; Gomez et al., 1965), indicating the existence of a gonad-stimula-

ting factor in the nervous system. In this context, it is interesting to point to a gonad-stimulating (Tensen et al., 1989) and a molt-inhibiting effect (Chang et al., 1990) in addition to the hyperglycemic activities of the CHH isoforms in *Homarus americanus*. Our findings that CHH-A and -B mRNAs are present in ganglia of the ventral nervous system, makes that the CHH isoforms are good candidates for the aforementioned physiological reactions. However, results from Laufer et al. (1994) that the multifunctionality of CHH also includes a possible gonad inhibiting activity, ask for a detailed study of the role of the CHHs in the ventral nervous system of crustaceans.

In this view, the cloning of two cDNAs encoding preproCHH-A and -B allows now to study the expression of the two CHH mRNAs in eyestalk as well as in nervous tissue at different stages of the reproductive cycle and the molting cycle of *Homarus americanus*.

CHAPTER 2

Cloning and expression of mRNA encoding prepro-Gonad-Inhibiting Hormone (GIH) in the lobster *Homarus americanus*

ABSTRACT

The Gonad-Inhibiting Hormone (GIH) is produced in the eyestalk X-organ sinus gland complex of male and female lobsters, and plays a prominent role in the regulation of reproduction, e.g. inhibition of vitellogenesis in female animals. To study this neurohormone at the mRNA level, we cloned and sequenced a cDNA which encodes GIH in the lobster *Homarus americanus*. The structure of preproGIH consists of a signal peptide and the GIH peptide itself. A comparative analysis revealed that lobster GIH together with crab Molt-Inhibiting Hormone belongs to a separate group of the Crustacean Hyperglycemic Hormone (CHH) peptide family which seems to be unique for crustaceans. Expression studies showed that GIH mRNA is expressed in the eyestalk, indicating that the neuroendocrine center in this optic structure is the only source of GIH. As this center modulates the other (neuro)endocrine organs in crustaceans, it is postulated that GIH regulates production and release of hormones involved in reproduction/molting processes.

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Cloning and expression of mRNA encoding prepro-Gonad-Inhibiting Hormone (GIH) in the lobster *Homarus americanus*. FEBS Lett. 353: 255-258 (1994).
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INTRODUCTION

The neurosecretory perikarya in the eyestalks of crustaceans are clustered in the so-called Medulla Terminalis Ganglionic X-organ (MTGX) and their axons form a tractus connecting them with a neurohaemal organ, the sinus gland. This gland secretes a variety of neurohormones involved in the control of several physiological processes including a novel family of large peptides: the CHH/MIH/VIH family (see Keller, 1992 for review). The Crustacean Hyperglycemic Hormones (CHH), are mainly involved in the regulation of carbohydrate metabolism, and the Molt-Inhibiting Hormone (MIH) inhibits the molting process. The other member, the Vitellogenic-Inhibiting Hormone (VIH) inhibits vitellogenesis in females can also be recalled the Gonad-Inhibiting Hormone (GIH) because it is produced in the eyestalk of male lobsters (De Kleijn et al., 1992) and of lobster larvae (Rotllant et al., 1993) too. In analogy with lobster CHHs, GIH is present in the sinus gland as two isoforms with identical amino acid sequences and molecular masses but with a different elution pattern in HPLC analysis (Soyez et al., 1991), possibly due to a different folding of the peptide. For CHH, it was suggested that the CHH Precursor-Related Peptide (CPRP) could be responsible for the different folding or processing of the CHH isoforms (Tensen et al., 1991d). However, this hypothesis was not supported after characterization of the CHH mRNAs of *Homarus americanus* and *Orconectes limosus* (De Kleijn et al., 1994a; De Kleijn et al., 1995). Until now, no amino acid or cDNA data are available concerning preproGIH. For this, information on the preprohormone of GIH would give supplemental information concerning the significance of a precursor-related peptide in folding or processing. In addition, expression studies revealed that the CHH mRNAs are also expressed in the ventral nervous system of the lobster (De Kleijn et al., 1995). In this view, it would be interesting to see if GIH is also expressed in nervous tissue regions other than the optic ganglia. For further analysis of GIH, we cloned a full-length cDNA encoding preproGIH and this cDNA was used to study GIH mRNA expression in different tissues of the lobster.

MATERIALS AND METHODS

Isolation and characterization of GIH-encoding cDNA

Poly A^+ RNA was isolated with guanidine-isothiocyanate and oligo(dT) cellulose from Medulla Terminalis (MT) tissue of the eyestalk of *Homarus americanus*. About 5 µg poly A^+ RNA was used for constructing a cDNA library in the vector λZAP-II (Stratagene). About 500.000 clones of this library were screened with an *H. americanus* GIH cDNA probe, generated by PCR on MT cDNA library fractions using 100 pmol of primer 1 (5'-GGGAATTCGCNTGGTTYACNGAYAAYGARTG-3') and 100 pmol of primer 2 (5'-CTRACRAARGTRTGITACACCAA-3'). Both oligonucleotide sequences were based on the amino acid sequence of *Homarus americanus* GIH (Soyez et al., 1994). Amplification between primers 1 and 2 was performed in 5 cycles with an annealling temperature of 68°C (2 min), followed by 5 cycles at 64°C (2 min) and 40 cycles at 58°C (2 min). The denaturation step in each cycle was at 93°C (40 sec), while the extension step was at 72°C (3 min). The 180-bp PCR product was isolated from a 1.5% agarose gel by the freeze-squeeze method (Tautz & Renz, 1983) and labeled with ^{32}P by random priming according to standard procedures (Sambrook et al., 1989). Hybridization of the replica nitrocellulose filters was performed at 42°C in 6 x SSC (1 x SSC contains 0,15 M NaCl and 0,015 M sodiumcitrate at pH 7.0), 1 x Denhardts solution, 25 mM sodium phosphate buffer pH 7.0, 10% dextran sulphate, 200 µg yeast tRNA/ml and 50% formamide. After hybridization, the filters were washed in 2 x SSC, 0.1 % sodium dodecyl sulphate (SDS) at 20°C for 10 min and subsequently washed in 2 x SSC, 0.1 % SDS, 1 x SSC, 0.1 % SDS and 0.25 x SSC, 0.1 % SDS at 68°C for 20 min each. Hybridization-positive phage plaques were purified and the recombinant pBluescript SK-phagemids were rescued from the bacteriophage (λZAP) clones by in vivo excision, according to the instructions of the manufacturer (Stratagene). Sequencing on both strands was performed with single and double-stranded DNA using T7 DNA polymerase and the dideoxy chain termination method (Sanger et al., 1977).

Northern blot analysis

Polyadenylated RNAs isolated from a number of tissues were separated by electrophoresis on denaturing formaldehyde agarose gels (1.0%) in MOPS buffer (Amersham). Northern blot transfers to Hybond N (Amersham) were performed essentially as described by Thomas (1980). RNA was fixed by baking for 2 h at 80°C. The 180-bp GIH PCR fragment corresponding to amino acids residues 3-53 of lobster GIH was cloned into pBluescript KS^+ and used for the generation of a cRNA probe. RNA probes were synthesized as run-off transcripts from 200 ng linearized DNA of pBluescript KS^+. Labelling was performed in a final volume of 10 µl containing 50 µCi $UT^{32}P$, 15 units T3/T7 RNA polymerase in transcription buffer (Promega), 1 mM rATP, rCTP, rGTP and 17,5 U RNasin (Promega). After 30 min at 37°C, 10 U of DNase I (Pharmacia) was added to the reaction mixture and incubated for another 10 min at 37°C. The cRNA probe was recovered after phenol extraction and ethanol precipitation using 10 µg tRNA as carrier. Hybridization was performed at 50°C for 16 hours and the filter was washed until 0.25 x SSPE at 56°C. Total RNA of heart and hepatopancreas, and a PstI digest of λ DNA were used as size markers.

RESULTS AND DISCUSSION

Isolation and characterization of MT cDNA encoding preproGIH

Screening 500.000 clones of the lobster MT cDNA library with a 180-bp GIH PCR fragment, corresponding to amino acid residues 3-54 of the *Homarus americanus* GIH peptide, resulted in the isolation of one hybridization-positive clone (H1B.1). The nucleotide sequence of this clone comprises 2165 bp with 336 bp in the coding region, 265 bp in the 5'-untranslated region and 1564 bp in the 3'-untranslated region (Fig. 1). The open-reading frame codes for a protein of 112 amino acids consisting of a signal peptide of 31 amino acids and a peptide of 81 amino acids with 98% amino acid sequence identity with the previously described lobster VIH (GIH) sequence (Soyez et al., 1991). We therefore conclude that cDNA clone H1B.1 encodes *Homarus americanus* preproGIH. The GIH sequence deduced from the cDNA data differs from the neuropeptide amino acid sequence of *Homarus americanus* GIH, obtained by microsequencing, in one substitution (Trp ^{52} by Asp ^{52}) and an extension of four amino acids (Ala ^{78}, Gly ^{79}, Arg ^{80} and Lys ^{81}), which indicates that the GIH sequence contains a potential amidation site at its C-terminal end. Assuming the formation of three disulfide bridges and amidation of the peptide, the molecular mass of the deduced GIH peptide is 9135 Da, which is in agreement with the value for GIH obtained by FAB/MS analysis (9135 Da; Soyez et al., 1991).

The preprohormones for CHHs contain an additional peptide preceding the hormone, the CHH Precursor Related Peptide (CPRP). Alignment with the preprohormones for the CHH/MIH/VIH peptide family (Fig. 2) reveals that preproGIH, does not contain such a CPRP-like peptide. Based on these findings, the proposed regulatory function of the precursor-related peptide (CPRP) in the synthesis of different CHH isoforms (Tensen et al., 1991d) should be reconsidered.

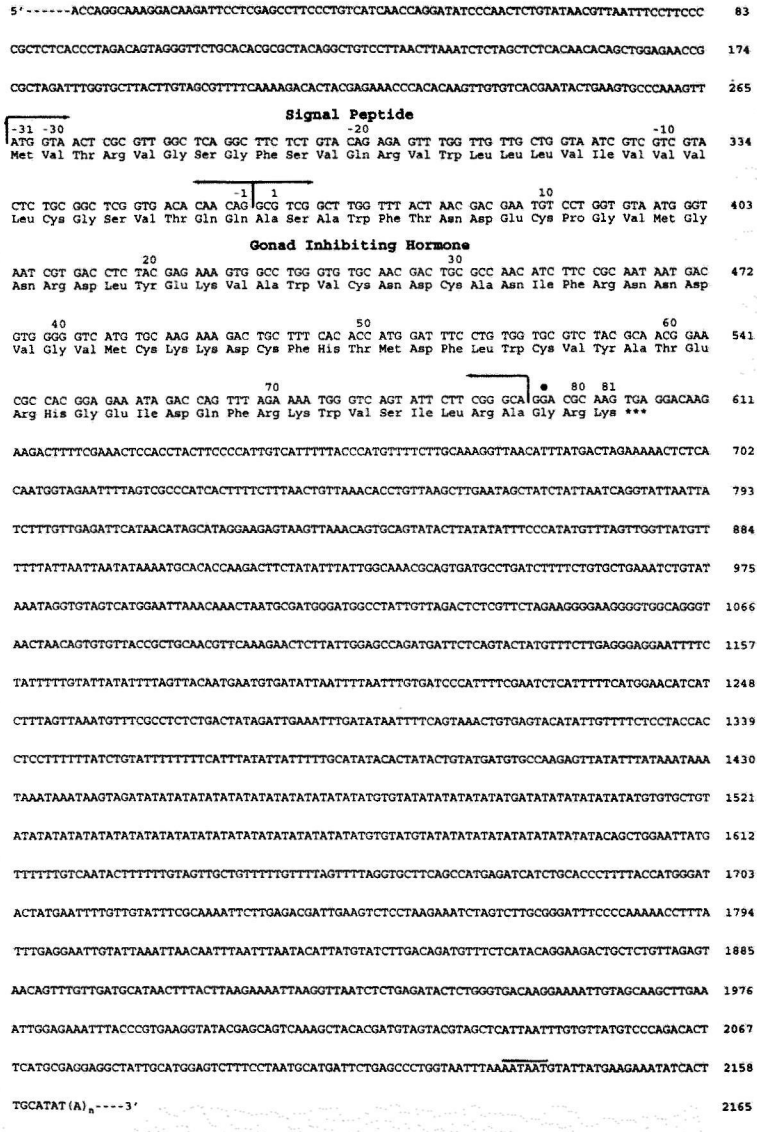

Fig. 1 Nucleotide sequence and deduced amino acid sequence of cDNA encoding preproGIH of the lobster *Homarus americanus*. Amino acid numbering starts at the N-terminal residue of the GIH hormone with the presumptive signal peptide sequence being indicated by negative numbering. The deduced amino acid sequence is shown below the nucleotide sequence. The dot above Gly-79 indicates a potential amidation site (Eipper et al., 1992). The putative signal for polyadenylation (Birnstiel et al., 1985) is overlined.

Alignment of the seven hormones shows a low degree of identity (19%) but the six cysteine residues are present in all hormones. Comparison of GIH with MIH showed a 53% amino acid sequence identity, similar to the identity between the different CHHs (55%, De Kleijn et al., 1994a). This high degree of identity in amino acid sequence between preproGIH and preproMIH, and their structural relationship indicates that these preprohormones belong to a distinct group of the CHH/MIH/VIH family. The low degree of identity between both peptide groups (GIH/MIH versus CHH) and the lack of a CPRP-like peptide in the GIH/MIH precursors suggests an early separation between the two groups, possibly by a deletion in the ancestral CHH/MIH/VIH gene.

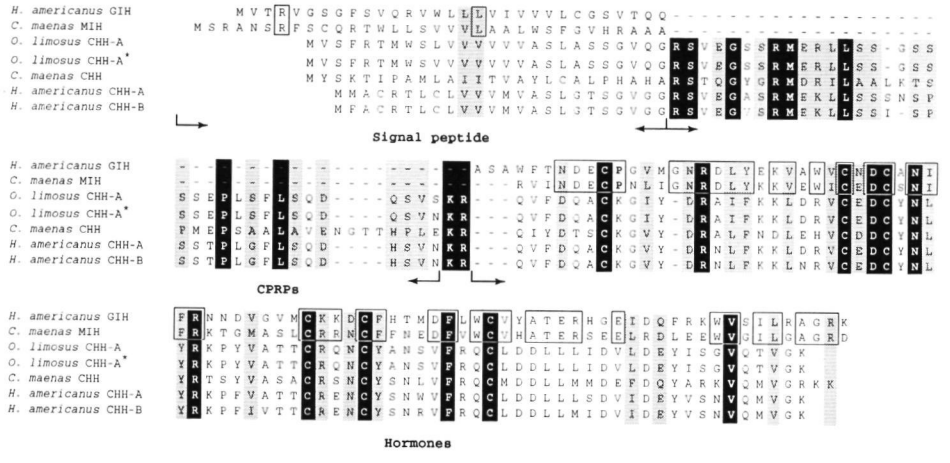

Fig. 2 Alignment of the preprohormones of *Homarus americanus* GIH, *Carcinus maenas* MIH (Klein et al., 1993), *Orconectes limosus* CHH-A, *Orconectes limosus* CHH-A* (De Kleijn et al., 1994a), *Carcinus maenas* CHH (Weidemann et al., 1989), *Homarus americanus* CHH-A and *Homarus americanus* CHH-B (De Kleijn et al., 1995). Sets of identical amino acid residues and conservative substitutions between all preprohormones are indicated in black and grey, respectively. Sets of identical amino acid residues between preproGIH and preproMIH are boxed.

Expression of preproGIH mRNA in the lobster

The size and expression of preproGIH mRNA in different tissues of intermolt animals was determined by Northern blot analysis of poly (A^+) RNA isolated from the ventral nervous

system, testis, ovary, medulla terminalis (MT), hepatopancreas, heart and green gland. This analysis showed only in the MT lane a band with an approximate size of ~2.3 kb (Fig. 3). According to this size, the sequence showed in Fig. 1 therefore likely represents the complete lobster preproGIH mRNA, allowing for a poly (A) tail of ~100 nucleotides. Limitation of GIH expression to the MT, is in contrast with lobster CHH-A and -B encoding mRNAs which are expressed in the eyestalks but also in other parts of the nervous system (De Kleijn et al., 1995).

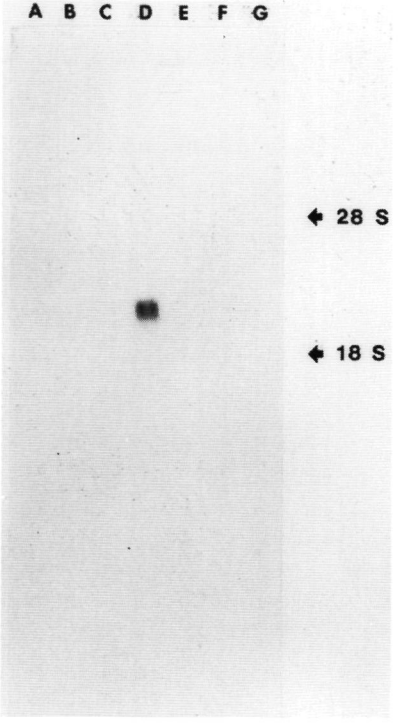

Fig. 3 Northern blot analysis of poly (A^+) RNA from the ventral nervous system (lane a), testis (b), ovaria (c), medulla terminalis (d), hepatopancreas (e), heart (f) and green gland (g) of the lobster *Homarus americanus*. The blot was hybridized with a cRNA probe (corresponding to nucleotides 364 - 516 in Fig. 1), and exposed for 72 h at -70 °C with two intensifying screens. The positions of the 28S and 18S RNA size markers are indicated by arrows.

This finding brings us to the hypothesis that GIH may be the important modulator in the synthesis or release of hormones involved in the reproduction/molting processes. For example, GIH might inhibit the activity of the androgenic gland in males and of the ovary

in females at one side and the production of presumptive gonad- stimulating/molt-inhibiting factors, for which CHH in the central nervous system is a good candidate. When the inhibitory effect of GIH is low, the activity of androgenic gland, ovary and release of a gonad-stimulating/molt-inhibiting factor(s) will be stimulated, resulting in sexual maturation in males and spawning of females and possible retardation of molting. Previous studies, describing the effect of eyestalk ablation on the androgenic gland in male crabs and isopods, support this hypothesis (Demeusy, 1960; Payen et al., 1971; Reidenbach, 1966; Le Grand et al., 1968). Furthermore, the striking similarity between crab preproMIH and lobster preproGIH, combined with the possibility that GIH is involved in molt inhibition indicates that, similar to lobster GIH, crab MIH may have a role in gonad growth. Expression studies of GIH in different stages of gonad growth and molting, together with homologous bioassays will show if GIH has indeed the postulated function. Our cloning and characterization study of preproGIH mRNA provides a specific tool to investigate in more detail whether and how GIH is involved in the regulation of gonad growth and molting in the lobster *Homarus americanus*.

CHAPTER 3

Localization of mRNAs encoding Crustacean Hyperglycemic Hormone and Gonad-Inhibiting Hormone in the X-organ sinus gland complex of the lobster *Homarus americanus*

ABSTRACT

The localization of the mRNAs encoding the crustacean hyperglycemic hormone (CHH), involved in the regulation of the carbohydrate metabolism and the gonad inhibiting hormone (GIH), which inhibits the vitellogenesis, is studied in the eyestalk of the lobster *Homarus americanus* using cRNA probes for *in situ* hybridization. For the detection of GIH mRNA we cloned and sequenced a partial cDNA encoding lobster GIH and for CHH mRNA detection an already available cDNA was used. This approach reveals that there is a frequently but not consistent cellular colocalization of the two neurohormones. Furthermore, our data show that male lobsters contain an equal number of neuroendocrine GIH cells as female lobsters. An additional study, involving the use of *in situ* hybridization in combination with immunocytochemistry, shows that the synthetic activity of the CHH- and GIH-producing cells can be followed at the mRNA as well as the protein level. This reveals that when a strong immuno staining is present, the mRNA staining is usually weak or absent and vice versa. In conclusion, the presence of cells, containing only GIH mRNA or only CHH mRNA, indicates that lobster CHH and GIH originate from two different precursors. Colocalization of the two neurohormone mRNAs confirms the colocalization at the peptidergic level found by immunocytochemistry and thus these findings were not due to cross reactions between the two antisera. The presence of GIH mRNA in the male lobster suggests an important role for this hormone in the reproduction of male Crustacea; in line with role of the gonadotrophins in vertebrates. Finally, the investigations on the synthetic activity of the neurohormone producing cells suggest an intracellular feedback mechanism which may inhibit the transcription and/or stability of CHH and GIH mRNA when high levels of the neurohormone proteins are present.

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Localization of mRNAs encoding Crustacean Hyperglycemic Hormone and Gonad Inhibiting Hormone in the X-organ sinus gland complex of the lobster *Homarus americanus.* Neuroscience 51: 121-128 (1992).
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INTRODUCTION

The X-organ sinus gland complex in the eyestalks of decapod Crustacea is a major neuroendocrine regulation center which is responsible for the synthesis of a number of neurohormones such as chromatotropins, moult inhibiting hormone (MIH), crustacean hyperglycemic hormone (CHH) and gonad (vitellogenesis) inhibiting hormone (GIH or VIH; for review see Kleinholz, 1985; Webster and Keller, 1987). Amino acid sequence analyses of CHH (Kegel et al., 1989, 1991; Tensen et al., 1989, 1991b), MIH (Chang et al., 1990; Webster, 1991) and GIH (Soyez et al, 1991) revealed the existence of a novel neurohormone family. The precise location of the cells, producing these crustacean neurohormones, has thus far been established by immunochemistry using antisera against certain purified crustacean neurohormones. CHH, involved in the regulation of the carbohydrate metabolism, was the first factor that could be visualized on light microscopical level in several crustaceans (Gorgels-Kallen & Van Herp, 1981; Gorgels-Kallen et al, 1982; Jaros & Keller, 1979; Van Herp & Van Buggenum, 1979) The cellular dynamics of the CHH-producing system in the crayfish *Astacus leptodactylus* have been studied by combined immunocytochemical, electron microscopical and physiological investigations (for review see Van Herp & Kallen, 1991). These results indicate an endogenous circadian rhythm entrained by a light/dark schedule and suggest that serotonin, dopamine and Met-enkephalin are good candidates for regulatory factors for the activity of the system. In recent years, polyclonal antisera became also available for the identification of neuroendocrine cells producing the red pigment concentrating hormone (RPCH Bellon-Hubert et al., 1986; Mangerich et al., 1986; Schooneveld et al., 1987), MIH (Dircksen et al., 1988) and the pigment dispersing hormone (PDH, Mangerich & Keller, 1988).

A polyclonal antiserum against purified GIH was produced and characterized by Meusy et al. (1987). This antiserum has been used in combination with an antiserum against CHH in order to study the localization of both neurohormones in the eyestalk of Homarus americanus (Kallen & Meusy, 1989). This study showed not only the localization of GIH-producing cells in the X-organ sinus gland complex but also demonstrated frequent but not consistent colocalization of CHH and GIH in the perikarya of this complex. Based on

these results the authors postulated that CHH and GIH might be encoded by one primary transcript or might be produced by proteolytic cleavage of the same precursor although immunochemical crossreactions were not ruled out.

Recently, degenerate synthetic oligonucleotides based on the previously determined amino acid sequence, were used in cDNA library screening and polymerase chain reaction (PCR) analysis to obtain cDNA sequences encoding CHH (Tensen et al., 1991b; Weidemann et al., 1989). We used these cDNA sequences to synthesize cRNA probes for *in situ* hybridization experiments, and succesfully localized CHH mRNA in the crayfish *Orconectes limosus* (Tensen et al., 1991a). Such an approach allowed us to study the crustacean neurohormones at the mRNA level.

In this study we present the detection of lobster CHH and GIH mRNAs in the X-organ sinus gland complex of the lobster *Homarus americanus* and describe the mRNA localization of both members of this novel neurohormone family in combination with immunocytochemical staining of the neuropeptides. In order to obtain a probe for the localization of GIH mRNA, we cloned and sequenced a partial cDNA encoding lobster GIH. This cDNA, as well as the already available CHH cDNA (Tensen et al., 1991b) was used to generate highly specific non-radioactive cRNA probes for *in situ* hybridization.

MATERIALS AND METHODS

Animals

Specimens of the lobster *Homarus americanus* were obtained from a commercial importer and originated from the east coast of Canada. The animals were kept in our laboratory in artificial seawater at about 5°C for a maximum of one week after arrival.

RNA isolation

Total RNA was isolated according to the method of Chomczynski and Sacchi (1987), using acid-guanidinium-thiocyanate-phenol-chloroform extraction. Medulla Terminalis (MT) tissue of two eyestalks was homogenized in an all glass homogenizer containing 500 µl guanidinium thiocyanate solution (4 M guanidinium thiocyanate; 25 mM sodium citrate

pH 7.0; 5 % sarcosyl; 0,1 M 2-mercaptoethanol) and transferred to a microcentrifuge tube. Sequentially, 50 µl of 2 M sodium acetate (pH 4.1), 500 µl water saturated phenol and 100 µl chloroform-isoamylalcohol (49:1) were added and the sample was mixed vigorously. RNA was recovered after ethanol precipitation and used for cDNA synthesis.

cDNA synthesis

Five µg of total MT RNA was dissolved in 7 µl water. The mRNA was reverse transcribed with 100 ng oligo dT (PL Biochemicals) using 200 Units cloned Moloney murine reverse transcriptase (Gibco-BRL) in a final reaction volume of 20 µl containing 50 mM Tris-HCl pH 8.4 at 22°C, 50 mM KCl, 8.75 mM $MgCl_2$, 1 mM dNTP and 35 U RNasin (Promega). After incubation for 10 min at 22°C, 60 min at 42°C and 10 min at 95°C, 6.6 µl was used in a polymerase chain reaction (PCR).

Oligonucleotides

Mixed oligonucleotides based on the amino acid sequence of lobster GIH (Soyez et al., 1991) were synthesized on an Applied Biosystems Model 381A DNA synthesizer and purified on Waters C18 cartridges (Sambrook et al., 1989). The nucleotide sequences of these degenerated primers are presented in Table 1.

Table 1. Sequences of synthesized oligonucleotides based on two selected parts of the GIH amino acid sequence.

Protein sequence A			3 Ala	Trp	Phe	Thr	Asp	Asn	10 Glu Cys
(primer 1)	5'GGGAATTC (EcoRI)	GCN	TGG	TTY	ACN	GAY	AAY	GAR	TG 3'
Protein sequence B			46 Asp	Cys	Phe	His	Thr	Met	53 Trp Phe
(primer 2)		3'CTR	ACR	AAR	GTR	TGI	TAC	ACC	AA 5'

The nucleotide sequences are presented according to the IUPAC-IUB standard nomenclature (Cornish-Bowden, 1985).

Amplifications and cloning

Amplification of the GIH cDNA was done in a final reaction volume of 100 µl containing 50 mM Tris-HCl pH 8.4 (at 22°C), 50 mM KCl, 2 mM $MgCl_2$, 5 µg BSA, 200 µM of

each dNTP, 100 pmol of each primer, 6.6 μl of the cDNA pool, 10^{-6} M tetra-methyl-ammonium-chloride and 1 U of Taq polymerase (Perkin Elmer/Cetus). The mixture was overlaid with 50 μl mineral oil and forty cycles were carried out in a Perkin Elmer/Cetus DNA cycler: denaturation at 93°C, 40 s; annealing at 58°C, 2 min; extension at 72°C, 3 min; the last cycle was followed by a final extension of 10 min at 72°C. The amplification mixture was phenol extracted, digested with EcoRI and size selected on a 2% agarose gel. The expected 160 bp product was subcloned into the vector Bluescript II KS$^+$ (BSHaGIH) and transformed to and propagated in *E. coli* DH5α.

The CHH cDNA was subcloned from a M13mp18 clone containing the *Homarus americanus* CHH A encoding cDNA (Tensen et al., 1991b) into Bluescript II KS$^+$ (BSHaCHH). The nucleotide sequences and orientations of all cloned products were determined with the dideoxy-sequencing method (Sanger et al., 1977) using T7 DNA polymerase (Pharmacia).

cRNA probe synthesis

RNA probes were synthesized as run off transcripts from 0.5 μg linearized and Geneclean II (BIO 101) purified recombinant Bluescript II KS$^+$ vectors. One vector (BSHaCHH) contained the *Homarus americanus* CHH cDNA encoding amino acids 1-72 (Tensen et al., 1991b). The second vector (BSHaGIH) contained *Homarus americanus* GIH cDNA encoding amino acids 3-53. Labelling was performed in a final volume containing 15 Units T3 or T7 RNA polymerase (Promega), transcription buffer (Promega), 1 mM of rATP, rCTP, rGTP, 650 μM rUTP, 350 μM dioxigenine-11-UTP and 17.5 U RNasin (Promega). After two hours incubation at 37°C, 2 μl was electrophoresed on a 4% polyacrylamide gel (Miller, 1989) to check the integrity and to estimate the amount of the synthesized RNA probes.

Tissue processing

Eyestalks of *Homarus americanus* were ablated, and immediately fixed *en bloc* in Bouin's fixative (71% picric acid, 24% formaldehyde, 5% acetic acid) for 16 h. After the classical (de)hydration treatment, fixed eyestalks were embedded in paraffin and 5 μm sections were alternately collected on slides coated with 0.01 % poly-L-lysine (Sigma).

To improve adherence of the sections to the glass surface, slides were baked for 1 h at 50°C. Mounted sections were deparaffinized in xylene, hydrated and used for the *in situ* hybridization of the specific mRNAs and visualization of the peptidergic material by immunocytochemical staining.

Southern blot analysis

The CHH and GIH cDNA inserts were cut out of the vectors BSHaGIH and BSHaCHH. Insert and vector DNA were electrophoresed on a 1% agarose gel and transferred to nitrocellulose (Sambrook et al., 1989). The blot was prehybridized for two hours at 42 °C in prehybridization buffer containing 6 x SSC, 5% blocking agent (Boehringer), 50% deionized formamide, 25 mM sodiumphosphate pH 7.0 and 200 μg/ml yeast tRNA. The digoxigenine labelled RNA probes were dissolved at a concentration of 5 ng/μl in hybridization mix (comprising prehybridization buffer plus 10% dextran sulphate). Hybridization was performed at 42 °C for 16 h. After hybridization the blot was washed in 2 x SSC, 0.1% SDS at 20 °C for 10 min and subsequently in 2 x SSC - 0.1% SDS, 1 x SSC - 0.1% SDS and 0.25 x SSC - 0.1% SDS at 68 °C for 30 min each. Visualization of the probes was performed using an alkaline phosphatase conjugated anti-digoxigenin antiserum (1:5000).

In situ hybridization

Before hybridization at 50°C for 16 h, the sections were pretreated according to Tensen et al. (1991a) including pepsine incubation steps. Washing of the sections and visualization of the probes was also carried out according to Tensen et al. (1991a).

Immunocytochemistry

The sections for immunocytochemistry were treated according the aforementioned method but without the incubation step. Detection of CHH occured by incubation with a polyclonal rabbit antiserum raised against CHH of the crayfish *Astacus leptodactylus* (for details see Kallen et al., 1982). GIH was visualized after incubation with a polyclonal rabbit

antibody raised against GIH (for details see Meusy et al., 1987). Immunochemical staining was performed according to the method of Kallen & Meusy (1989).

RESULTS

Generation of cDNA clones encoding GIH

The availability of the amino acid sequence for GIH in combination with polymerase chain reaction (PCR) analysis, allowed the generation of a partial GIH cDNA sequence by using mixed oligonucleotides. Ten individual recombinant clones were sequenced and found to contain, between the mixed primers, identical sequences encoding the partial amino acid sequence of *Homarus americanus* GIH. This sequence, deduced from the cDNA confirmed the sequence previously determined by amino acid sequencing methods (Soyez et al., 1992). One of these clones was linearized and used as a template for the synthesis of anti-sense or sense GIH RNA probes.

Specificity of the cRNA probes for CHH and GIH

In a first experiment the specificity of the cRNA probes was tested in alternate sections. *In situ* hybridization with the anti-sense cRNA probe for CHH (Fig. 1A) and the

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Fig. 1 Specifity of the cRNA probes for CHH and GIH.

Fig.1A. *In situ* hybridization of mRNA encoding CHH with an anti-sense cRNA probe against CHH mRNA. Positive reactions are only present in the cytoplasm of the cell perikarya in the MTGX.

Fig.1B. Adjacent section to section in Fig.1A (both 5 µm), showing no hybridization after using the control CHH sense cRNA probe.

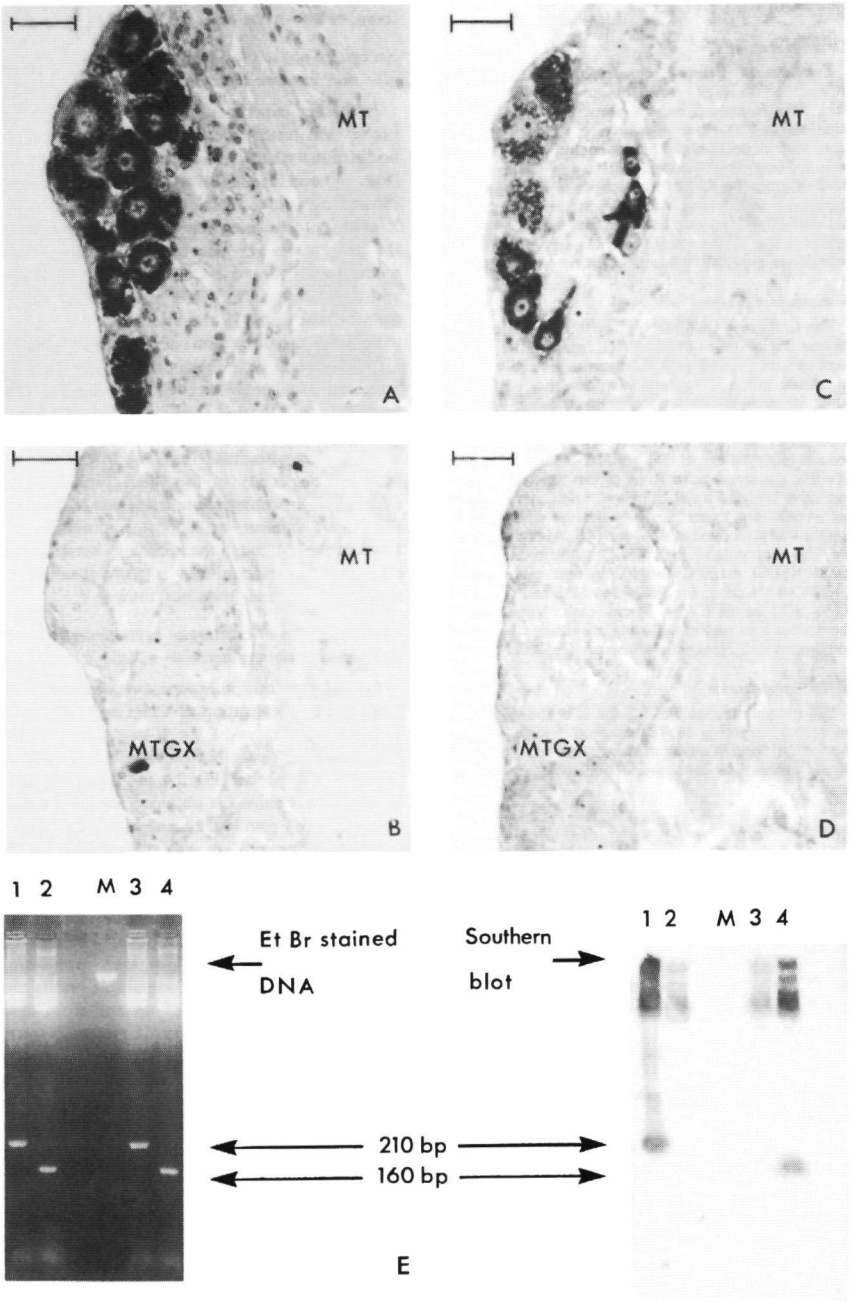
Fig.1C. *In situ* hybridization of mRNA encoding GIH with an anti-sense cRNA probe against GIH mRNA. Comparable to CHH, positive signals are only found in the cell somata of the MTGX.

Fig.1D. Adjacent section to section in Fig.1C (both 5 µm), demonstrating no hybridization after using the control GIH sense cRNA probe.

All cRNA probes are digoxigenine labelled and used at a concentration of appr. 2 ng/µl. Staining is performed overnight at 4 ^{oC}.

Abbreviations in Figs. 1A - D: MT: medulla terminalis; MTGX: medulla terminalis ganglionic x-organ. Bars: 50 µm.

Fig.1E. EtBr stained gel and Southern blot of GIH (lane 2 and 4) or CHH (lane 1 and 3) encoding cDNA digested out of Bluescript II KS$^+$. M is marker lane containing Hind III digested lambda DNA. The left part of the Southern blot (lane 1 and 2) is hybridized with an anti-sense cRNA probe (5 ng/µl) against CHH. The right part is hybridized with an anti-sense cRNA probe against GIH. Hybridization mixture and cRNA probes are the same as used in the *in situ* hybridization.


MT
A
MT
C
MT
MTGX
B
MT
MTGX
D
1 2 M 3 4
Et Br stained
DNA
Southern
blot
1 2 M 3 4
210 bp
160 bp
E

anti-sense probe for GIH (Fig. 1C) showed a positive reaction in cell perikarya in the X-organ of the medulla terminalis. Specifity of the probes was revealed after incubating alternate sections with the sense CHH RNA probe (Fig. 1B) and the sense GIH RNA probe (Fig. 1D). No staining was found in these sections.

Possible crossreaction of the CHH and GIH cRNA probes was excluded by hybridizing a Southern blot of lobster CHH and GIH cDNA fragments with the digoxygenin labelled cRNA probes (Fig. 1E). It is clear that the CHH cRNA probe does not hybridize with the GIH cDNA fragment and vice versa. The specificity of the RNA probes was also shown by hybridizing eyestalk sections of the crayfish *Orconectes limosus* with lobster anti-sense CHH RNA probe. Although the homology between lobster and crayfish CHH mRNA is more then 70%, no staining was found (data not shown).

Colocalization of CHH and GIH mRNA

In situ hybridization experiments demonstrate that there is a colocalization of CHH and GIH mRNA in several perikarya of the X-organ of the lobster *Homarus americanus*, but other cells react only with one of the two probes (Figs 2A and B and Figs 2C and D respectivily show the obtained results in consecutive sections). From Figs 2A and B, it is clear that 2 neuroendocrine cells are only stained with the CHH probe, 1 cell reacts strongly with both riboprobes and that the mRNA from another cell hybridized only with the GIH riboprobe. The results in Figs 2C and D confirm the aforementioned statements but also indicate that the intensity of the signal for each probe can be different in the same cells. Comparable results can be found by the application of an antiserum against CHH (Fig. 2E) and GIH (Fig. 2F) respectively in an immunocytochemical staining procedure.

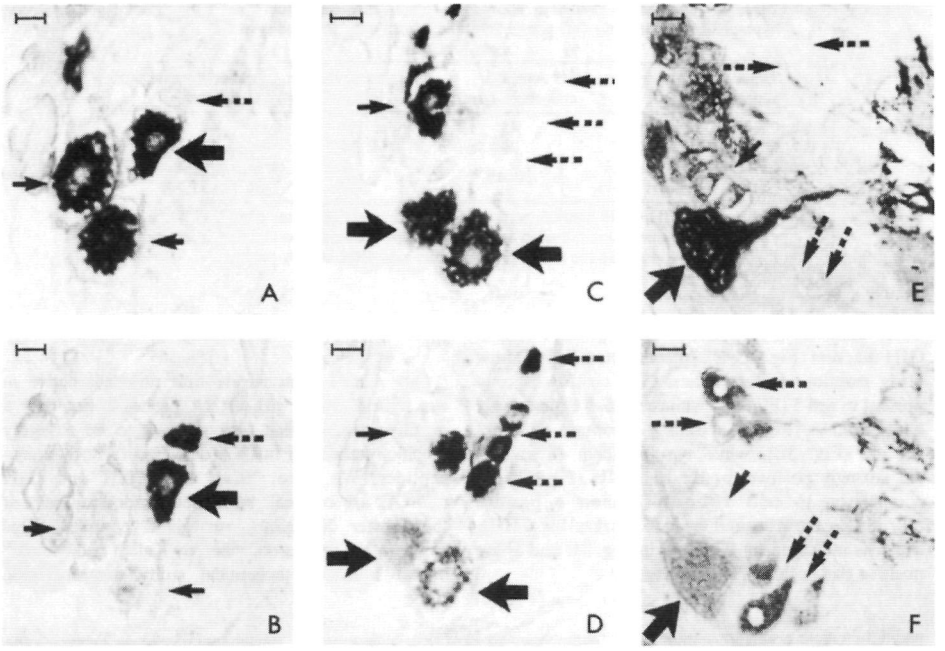


Fig. 2 Colocalization of CHH and GIH.
Figs 2A to D. *In situ* hybridization of serial sections (5 µm) demonstrating mRNA encoding CHH and GIH in the cell somata of the MTGX. Figs 2A, B and 2C, D illustrate colocalization and single cell labelling of the transcription products.
Figs 2E and F. Immunocytochemical staining of serial sections (5 µm) showing CHH and GIH peptidergic material in the cell somata of the MTGX. Complement to the results of the *in situ* hybridization (Figs 2A to D), colocalization and single cell labelling is also demonstrated at the protein level.
Abbreviations in Figs 2A to F: Arrows indicate the cells expressing mRNA encoding only CHH (——▸) only GIH (----▸) and both (➞) in Figs 2A to D and the CHH neuropeptide (——▸), GIH neuropeptide (----▸) and both (➞) in Figs 2E and F. Bars: 25 µm. Labelling, concentration and staining of the cRNA probes is the same as in Fig. 1.

Some cells are only stained with the CHH antiserum, others react only with anti GIH, while a third group of cells shows a strong or weak immunoreaction for both.

To obtain an impression of the number of stained perikarya and to see if GIH mRNA is also detectable in male lobster, we counted the number of positive cells in both sexes and divided them according to their intensity of staining in the *in situ* hybridization technique. The results are given in Table 2 and can be summarized as follow.

Table 2. Number of perikarya showing a positive reaction for the CHH and GIH riboprobes in female and male lobster.

Types of perikarya	Average of celldiam. (μm)	Number in female lobster	Number in male lobster
CHH +	45	50	44
GIH +	20	34	32
CHH +++/GIH +	}30 to 45	7	9
CHH +++/GIH +++		-	3

Number of GIH/CHH stained perikarya in a complete X-organ complex of the lobster *H. americanus*, divided into four groups according staining intensity (+ or +++) with the CHH cRNA probe and GIH cRNA probe.

It is evident that there are no striking differences in the neuroendocrine cells of the X-organ of female and male lobster capable to express mRNA encoding CHH and/or GIH. About half of the counted cells (50% to 55%) are positive for the CHH riboprobe, a lower number (36% to 37%) react with the GIH riboprobe and only a few cells (8%-14%) express both messengers .

Synthetic activity in CHH- and GIH-producing cells

In order to get an idea of the relation between the expression of CHH and GIH mRNA and the synthesis of both neuropeptides, the neuroendocrine cells of the X-organ in lobster were studied on alternated sections. These sections were hybridized with the CHH (Fig. 3A) or GIH riboprobe (Figs 3C and E) or were immunocytochemically stained with the CHH antiserum (Fig. 3B) respectively GIH antiserum (Figs 3D and F). The examined neuroendocrine cells demonstrate a variety of staining patterns for the used markers, reflecting differences in the level of their synthetic activity. The cells indicated by 1-4, are positive for the CHH riboprobe (Fig. 3A) and the CHH antibody (Fig. 3B) but are negative for both GIH markers (Figs 3C and E). In contrast, cells 5 to 8 show other reaction characteristics. GIH mRNA is only present in cell 5 (Fig. 3C) while cells 6-8 express only CHH mRNA (Fig. 3A). Cells 6-8 contain no CHH peptide (Fig. 3B) while two of them (6 and 7) are strongly positive for the anti-GIH (Fig. 15). In comparison to

cells 1-4, showing a positive reaction to the ribo and peptide marker for CHH, the cells indicated by 9 and 10 in Figs 3E and F are positive for both GIH markers.

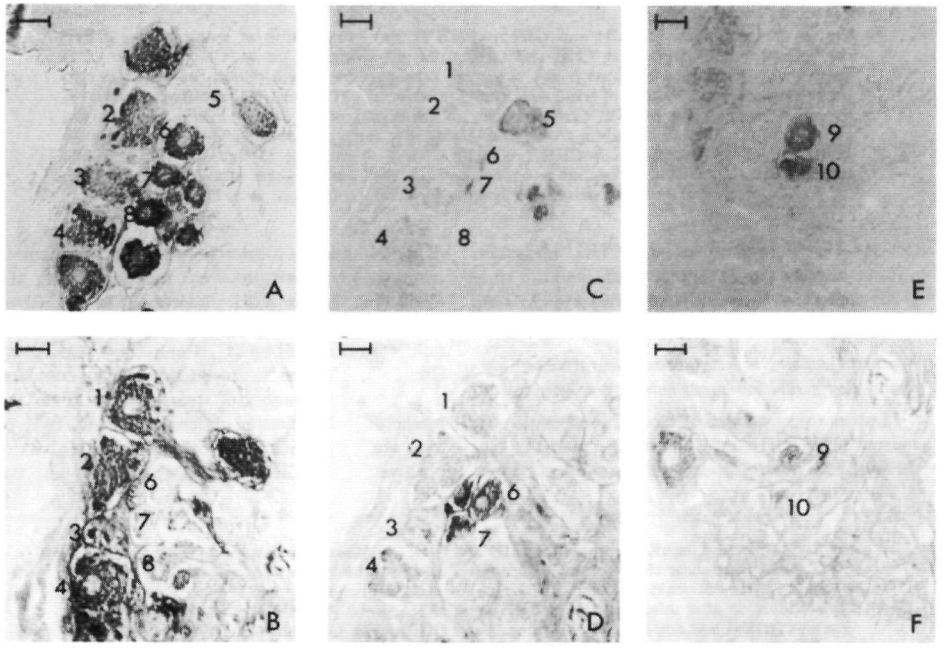


Fig. 3 Synthetic activity in CHH- and GIH-producing cells.
Figs 3A to F. Two series of consecutive sections (5 μm) illustrate different stages in the synthetic activity of the cell somata in the MTGX, producing CHH and GIH. Figs 3A and C show the hybridizing material using the riboprobe for CHH (Fig. 2A) or GIH (Fig. 3C). In Figs 3B and D the peptidergic material is visualized applying the antiserum for CHH (Fig. 3B) or GIH (Fig. 3D). Fig. 3E and F demonstrate two cells in which mRNA encoding GIH (Fig. 3E) and the neuropeptide GIH (Fig. 3F) are present at the same moment.
Abbreviations: the numbers 1 upto 10 in the figures indicate cell-somata from which the characteristics are described in the results. Bars: 25 μm. Labelling, concentration and staining of the cRNA probes is the same as in Fig. 1.

DISCUSSION

Our results demonstrate that non-radioactive *in situ* hybridization with cRNA probes is not only a sensitive method but also appears to be a highly specific method for mRNA detection of both neuropeptides. We have therefore proven that CHH and GIH mRNAs are colocalized in neuroendocrine cells of the lobster X-organ sinus gland complex. This confirms the results described by Kallen and Meusy (1989) in an immunocytochemical study and shows that their data are not due to cross reactions of the used antisera. Colocalization of CHH and GIH demonstrated on the mRNA and protein levels in the same neuroendocrine cells is frequently but not always present. In addition, the presence of cells synthesizing only one of the two neuropeptide mRNAs establish that both neuropeptides do not have a common precursor and suggests they originate from two different primary transcripts, although alternative splicing pathways cannot be ruled out.

Based on the results of this study, we postulate that CHH and GIH may be synthesized in one cell group of the MTGX and that the variations in staining characteristics reflect differences in the synthetic activity of the individual cells in this group. Nevertheless, we can not exclude the possibility that the cell types mentioned in Table 2 belong to three different groups; two showing production of either CHH or GIH, and a third group which synthesizes both.

Until now most physiological research on GIH was only carried out in female animals. Most of these studies indicated that GIH inhibits vitellogenesis (for review see Meusy & Payen, 1988). Due to this inhibitory effect on vitellogenesis Charniaux-Cotton and Touir (1973) proposed to change the name gonad inhibiting hormone (GIH) to vitellogenesis inhibiting hormone (VIH) in order to describe its function more precisely. *In situ* hybridization carried out in male lobsters reveal that GIH mRNA is present in an equal number of neuroendocrine cells as in female animals. This finding opens perspectives for investigations on its regulatory role in reproduction of male Crustacea in analogy with the gonadotropic releasing factors in vertebrates. Finally, the presence of GIH in males puts the name VIH again in discussion as it might be better to use the name GIH in order to generalize the role of the same neurohormone in reproduction of both sexes.

Furthermore, our data on the synthetic activity of the CHH- and GIH-producing cells using the combination of *in situ* hybridization and immunocytochemical studies reveal that this approach can have benefits in studies concerning the regulatory mechanism controlling the cellular dynamics of the neuroendocrine cells in the medulla terminalis ganglionic X-organ (MTGX). But also the relations with several biological cycles such as a seasonal, tidal or diurnal rhytmicity. Although the amounts of neurohormone protein and mRNA cannot be estimated accurately, it seems that when strong immunostaining is present the mRNA staining is mostly weak or absent and vice versa. This may suggest an intracellular feedback mechanism which inhibits GIH/CHH gene transcription and/or decreases mRNA stability at elevated neurohormone protein levels.

CHAPTER 4

Localization of crustacean hyperglycemic hormone (CHH) and gonad-inhibiting hormone (GIH) in the eyestalk of *Homarus gammarus* larvae by immunocytochemistry and *in situ* hybridization

ABSTRACT

This study deals with the localization of crustacean hyperglycemic hormone (CHH) and gonad-inhibiting hormone (GIH) in the eyestalk of larvae and postlarvae of *Homarus gammarus*, by immunocytochemistry and *in situ* hybridization. The CHH and GIH neuropeptides are located in the perikarya of neuroendocrine cells belonging to the X-organ of the medulla terminalis, in their tract joining the sinus gland, and in the neurohemal organ itself, at larval stages I, II and III and at the first postlarval stage (stage IV). In all the investigated stages, the mRNA encoding the aforementioned neuropeptides could only be detected in the perikarya of these neuroendocrine cells. In stage I, approximately 19 CHH-immunopositive and 20 GIH-immunopositive cells are present, both with a mean diameter of 7 ± 1 μm. GIH cells are preferably localized at the periphery of the X-organ surrounding the CHH cells that are centrally situated. Colocalization of CHH and GIH immunoreactions can be observed in some cells. The cell system producing CHH and GIH in the larval and postlarval eyestalk is thus functional and is morphologically comparable to the corresponding neuroendocrine center in the adult lobster.

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INTRODUCTION

The X-organ sinus gland complex, located in the eyestalk, is a major neuroendocrine system in which several neuropeptides are synthesized, in crustaceans. The chromatophorotropins such as the red pigment concentrating hormone (RPCH) and the distal retinal pigment hormone (DRPH), regulate pigment migration in the chromatophores of the epidermis, the distal pigment cells and some of the internal organs (Rao, 1985). The molting inhibiting hormone (MIH) represses ecdysteroid synthesis responsible for the initiation and sustaining of the molt (see Skinner, 1985 for review). Gonad-inhibiting hormone (GIH) controls reproduction by delaying secondary vitellogenesis and gonad maturation (Adiyodi, 1985; Charniaux-Cotton and Payen, 1988), and is also called vitellogenesis-inhibiting hormone (VIH). The main role of crustacean hyperglycemic hormone (CHH) is to increase the sugar level in the blood (Keller et al., 1985), but recently it was also found to be involved in the neuroendocrine control of the liberation of enzymes from the digestive gland (Keller & Sedlmeier, 1988), to stimulate oocyte development (Tensen et al., 1989) and to inhibit ecdysone release from the Y-organs (Webster & Keller, 1987).

In contrast to the extensive information on the cytology of the eyestalk structures in adult crustaceans, little is known about the neuroendocrine system in larvae. Cells, corresponding to the neuroendocrine cells in the X-organ, have been found in the first larval stages of some crustaceans, such as crabs (Pyle, 1943; Matsumoto, 1958; Webster & Dircksen, 1991), lobsters (Pyle, 1943; Le Roux, 1989; Rotllant et al., 1991), crayfish (Zielhorst & Van Herp, 1976; Gorgels-Kallen & Meij, 1985), prawns and shrimps (Dahl, 1957; Hubschman, 1963; Little, 1969; Elofsson, 1969; Bellon-Humbert et al., 1978; Le Roux, 1989), anomures (Orlamünder, 1942; Le Roux, 1989) and some stomatopods (Jacques, 1969, 1975).

In previous light microscopic studies, the sinus gland of freshwater crustaceans, which hatch at an advanced developmental stage, was observed to arise in the first juvenile stage of *Potamon dehaani* (Matsumoto, 1958) and in the first juvenile stage of *Astacus leptodactylus* (Zielhorst & Van Herp, 1976). However, in marine crustaceans, which hatch at an early developmental stage, the sinus gland appears to develop later in the larval life cycle at about the time of metamorphosis: in stage I for *Palaemon macrodactylus* (Little, 1969), in stage III

for *Pinnotheres maculatus* and *Homarus americanus* (Pyle, 1943), and in stage V for *Palaemonetes* sp. (Hubschman, 1963) and *Palaemon serratus* (Bellon-Humbert et al., 1978). In 1975, Jacques described the presence of the sinus gland in the larval stage I of *Squilla mantis* using an electron microscopic approach.

With the introduction of immunocytochemical techniques, it became possible to identify the neuroendocrine perikarya in larvae. Gorgels-Kallen and Meij (1985) described the CHH-producing system in juvenile *Astacus leptodactylus*; Beltz and Kravitz (1987) and Beltz et al. (1990) studied the proctolin-like substances in the larvae of *Homarus americanus*, and Webster and Dircksen (1991) investigated the MIH-producing neurosecretory system in the first zoeal stage of *Carcinus maenas*.

Recently, cRNA probes became available enabling the study of mRNA synthesis by in situ hybridization. Tensen et al. (1991a) were able to detect mRNA encoding CHH in the eyestalk of adult crayfish *Orconectes limosus*, using non-radioactive *in situ* hybridization. By a comparable approach, Laverdure et al. (1992) visualized mRNA encoding GIH (or VIH) in neuroendocrine cells of the eyestalk of the adult lobster *Homarus americanus*. De Kleijn et al. (1992) studied the synthetic activity of the cells producing CHH and GIH in the eyestalk of the adult lobster, at the mRNA and at the peptide level using immunocytochemistry in combination with *in situ* hybridization. In a previous ultrastructural study of the neuroendocrine system in the larval eyestalk of *Homarus gammarus*, we have demonstrated that the sinus gland is present and that it contains neurosecretory granules as early as larval stage I, suggesting that this neurohemal organ is functional (Rotllant et al., 1991). The present study deals with the localization of CHH and GIH in the larval eyestalk of *Homarus gammarus* by immunocytochemistry and *in situ* hybridization techniques.

MATERIALS AND METHODS

Laboratory rearing of larvae, tissue sampling, and processing

Egg-carrying female lobsters, caught on the coast of Brittany, were maintained in the laboratory in recirculated filtered sea water (salinity: 36‰-37‰) at 15°C and at a photoperiod

of 12L/12D. They were fed with mussels *ad libitum*. After hatching, the larvae were cultured in "planktonkreisels" at 20°C according to the method of Hughes et al., (1974) and fed with frozen *Artemia*.

Larvae were collected at the following larval (Charmantier and Charmantier-Daures, 1991) and molting stages (Drach and Tchernigovtzeff, 1967): I A and B, I C, I D_{1-2}, II A and B, II C, II D_{1-2}, III A and B, III C, III D_{1-2}, IV A and B, IV C and IV D_{1-2}. After removal of the abdomen, the specimens were immediately fixed (approximately 24 h) in Bouin-Hollande for the immunocytochemical study or in Bouin, treated with 0.1% diethyl pyrocarbonate (Sigma), for the *in situ* hybridization and the combined immuno-*in situ* study. After dehydration, they were embedded in Paraplast (57°C).

Immunocytochemistry

For the preparation of antisera, CHH and GIH purified by high pressure liquid chromatography (HPLC) from *Homarus americanus* were isolated according to Soyez et al. (1991). The polyclonal antiserum against lobster CHH was produced in guinea pig, that against lobster GIH in rabbit; both are a gift by Dr. D. Soyez. Production and characterization of the guinea pig antiserum against CHH (peak 1) are described by Meusy and Soyez (1991). They demonstrated by ELISA experiments that this antiserum does not recognize GIH. The rabbit antiserum against GIH (peak 3; see Meusy & Soyez, 1991) was firstly absorbed with HPLC-purified lobster CHH to eliminate cross reactivity before adding to the slides. The specificity of the applied immunocytochemical staining methods was tested successively substituting each of the incubation reagents in the normal procedure by buffer and was carried out on serial sections of eyestalks from adult animals. In those incubation experiments, staining of the neuroendocrine centers was always absent.

Serial or alternating sections (4 ± 1 μm) were treated according to Kallen and Meusy (1989). The sections were deparaffinized, rinsed in tap water and equilibrated in Tris-HCl buffered saline (0.05 M; pH 7.6). After blocking with a 1:5 dilution of normal rabbit serum (NRS) or normal goat serum (NGS), the sections were incubated with the primary antiserum. For visualization of CHH, a dilution of 1:10.000 of the CHH antiserum was applied; for GIH, pre-absorbed anti-GIH was added to the slides also at a dilution of 1:10.000. The slides were

incubated overnight at 4°C. After rinsing in Tris-HCl buffer, rabbit anti-guinea pig peroxidase (RAGp-PO, Nordic; dilution 1:150) for CHH staining, and goat anti-rabbit (GAR, Nordic; dilution 1:150) for GIH detection were applied to the sections; incubation was carried out for 1 h. The slides for GIH staining were further treated with peroxidase anti-peroxidase (PAP, Nordic; 1:500). Finally, a solution of 3,3-diamino-benzidine tetrahydrochloride and nickel (DAB+Ni, Sigma) was used as substrate for both immunocytochemical detections.

In situ hybridization

For *in situ* hybridization, cRNA probes prepared according to De Kleijn et al., (1992) were used. They were synthesized as run-off transcripts from 0.5 µg linearized, Geneclean-II (BIO 101)-purified recombinant Bluescript II KS^+ vectors (Stratagene). One vector (BSHa CHH) contained the *Homarus americanus* CHH cDNA encoding amino acids 1-72 (Tensen et al., 1991b). The second vector (BSHa GIH) contained *Homarus americanus* GIH cDNA encoding amino acids 3-53 (Soyez et al., 1991).

For this approach, we used the non-radioactive *in situ* hybridization method described by Tensen et al., (1991a). The sections were collected on slides that had been coated with 0.01% poly-L-lysine (Sigma) and previously baked for 1 h at 50°C to improve adherence of the sections to the glass surface. After deparaffinization, the sections were treated with 0.1% pepsin (Boehringer) in 0.2 N HCl at 37°C for 20 min and washed in twice distilled water. They were then post-fixed in freshly prepared 2% paraformaldehyde dissolved in PBS (PBS = 10 mM sodium phosphate pH 7.4, 0.1% KCl, 0.8% NaCl) for 4 min, rinsed in PBS containing 1% hydroxylammonium chloride for 15 min, and finally washed for 5 min in PBS and dehydrated. Each cRNA probe was dissolved in a hybridization mix, composed as follows: 50% deionized formamide (Fluka), 4 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate pH 7.0), 10% dextran sulfate (Pharmacia), 0.1% polyvinylpyrrolidone (Fluka), 0.1% bovine serum albumin (Boehringer), 0.1% Ficoll 400 (Pharmacia), 200 µg/ml yeast tRNA (Boehringer) and 25 mM sodium phosphate buffer pH 7.0. The concentration of the CHH and GIH cRNA probes was 2 ng/µl. After heating the hybridization solution for 10 min at 80°C, 150 µl was added to each slide supporting 20 sections. The slides were overlaid with a coverslip, sealed with melted Paraplast and incubated for 16 h at 50°C in a moist chamber

for hybridization. They were then rinsed sequentially for 30 min in 2 x SSC, in 1 x SSC, and in 0.5 x SSC at room temperature, and in 0.5 x SSC at 37°C. Visualization of the mRNA was performed using the alkaline phosphatase anti-digoxigenin (DIG; Boehringer; dilution 1:500). Solutions used for the hybridization were first treated with 0.1% diethyl pyrocarbonate. All glassware was baked for at least 16 h at 250°C.

RESULTS

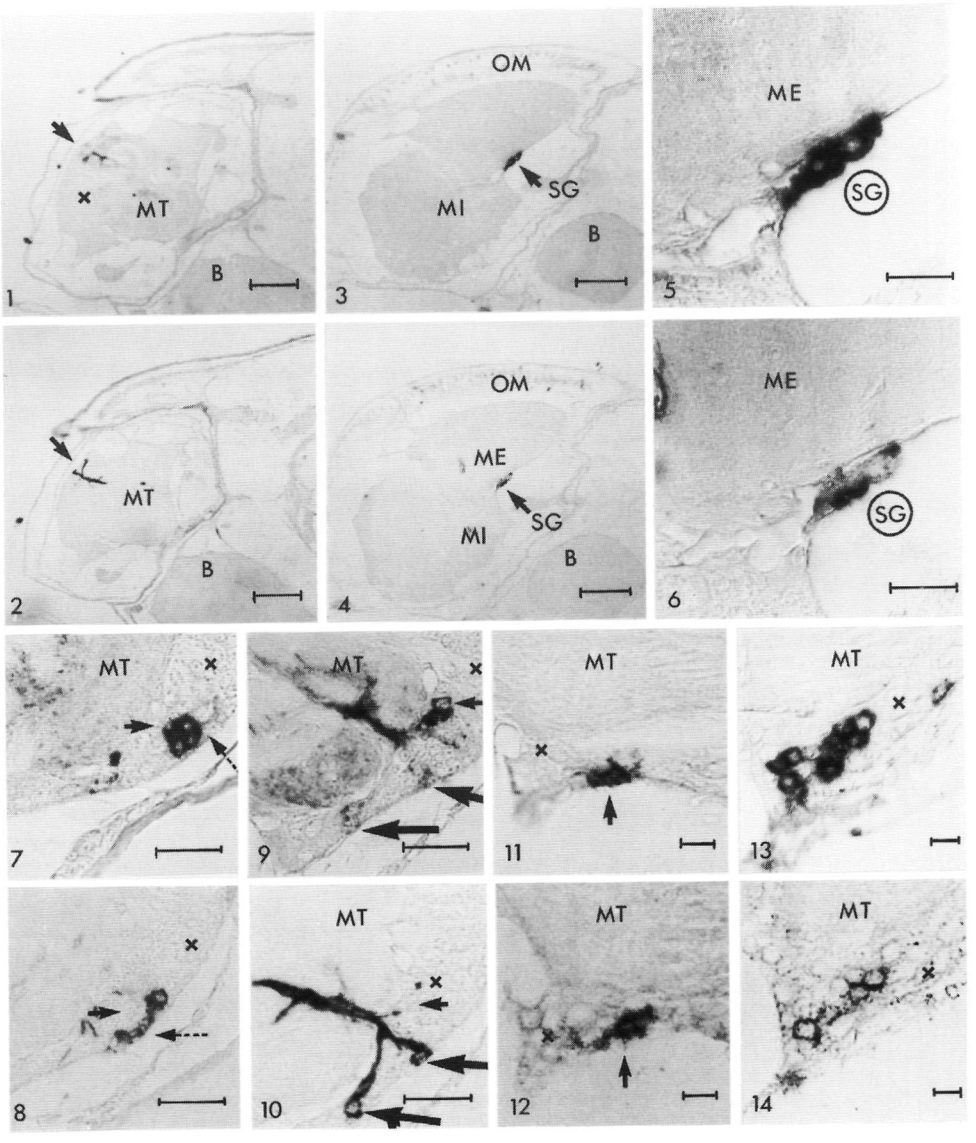
For optimizing the immunocytochemical investigations in the larvae of *Homarus gammarus*, the antisera against CHH and GIH of *Homarus americanus* were screened in a dilution series and compared with the results obtained for the adults of both species. As shown in Table 1, we found that the antisera could be diluted about ten times more for larvae than for adults. The immunoreaction in the sinus gland of larvae at stage I was positive up to a dilution of 1:100.000. However, at that dilution the corresponding perikarya remained negative. In contrast, the optimal dilution for the adults was 1:1.000.

By using a dilution of 1:10.000, CHH and GIH can be detected in the perikarya of neuroendocrine cells belonging to the X-organ, in their tract joining the sinus gland, and in

Table 1. Comparison of the immunoreaction in *Homarus gammarus* larvae and *Homarus americanus* adults to dilution series of anti-CHH and anti-GIH sera

Diluted Anti-sera	1/100		1/200		1/400		1/800		1/1000		1/5000		1/10000		1/30000		1/60000		1/100000	
	C	SG	C	SG	C	SG	C	SG	C	SG	C	SG	C	SG	C	SG	C	SG	C	SG
Larvae	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	+	±	+	-	+
Adults	+	+	+	+	+	+	+	+	+	+	-	+	*	*	*	*	*	*	-	-

+, positive; -, negative; *, no data; ±, weak reaction; C, MTGX cell-perikarya; SG, sinus gland


1
MT
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the neurohemal organ itself. The immunoreactions obtained in larval stage I are illustrated in Figs 1-4. These results are representative for the investigated larval stages I-III and for postlarval stage IV. In addition, a comparable immunocytochemical reaction has been observed in all studied (AB, C & D_{1-2}) moulting stages. We have thus mainly investigated the intermolt stage C. A detailed observation in larval stage I revealed the following results. The immunoreactions for both antisera were partially overlapping in the sinus gland and the amount of immunoreactive CHH material was much higher than the amount of immunopositive GIH material (Figs 5, 6). Futhermore, at this stage, the X-organ contained a similar number of perikarya immunopositive to CHH and GIH: 19 ± 4 for CHH and 20 ± 2 for GIH, both cell types having a diameter of approximately 7 ± 1 µm, as summarized in Table 2. In all the investigated specimens, the immunoreaction demonstrated that the GIH-immunoreactive cells are preferentially localized at the periphery of the X-organ (Fig. 8) surrounding the CHH-immunopositive cells (Fig. 7). Although, most cells are only stained with the CHH antiserum or with anti-GIH, 3 to 4
cells showed an immunoreaction to both antisera (Figs. 9, 10).
In situ hybridization revealed only a reaction at the level of the perikarya of the neuroendocrine cells for all the investigated stages (Figs. 12, 14).

Figs. 1-6. Immunocytochemical staining of CHH and GIH in the X-organ sinus gland complex of *Homarus gammarus* larvae (intermolt stage C). Figs. 1, 3, 5. Immunopositive reaction to CHH. Figs. 2, 4, 6. Immunopositive reaction for GIH. The detection of both antisera was only observed in some perikarya of the MTGX cells and in part of their tract joining them to the sinus gland (Figs. 1, 2), and in the neurohemal organ itself (Figs. 3, 4). Figs. 5, 6. The difference in staining patterns for the antisera at the level of the sinus gland. B brain; ME medulla externa; MI medulla interna; MT medulla terminalis; OM ommatidia; SG sinus gland; x X-organ. Bars represent 100 µm

Figs. 7-10. Details of the immunocytochemical staining of CHH (Figs. 7, 9) and GIH (Figs. 8, 10) at the level of perikarya in alternating sections. From Figs. 7 and 8, it is evident that the CHH immunoreactive cells are preferentially localized at the inner size of the X-organ (full arrows) whereas the GIH positive cells (dotted arrows) are found at the periphery surrounding the CHH perikarya. Double staining is regulary detected in some perikarya (Figs. 9 and 10 ; large arrows). MT medulla terminalis; x X-organ. Bars represent 50 µm

Figs. 11-14. Alternating sections comparing immunocytochemical staining with in situ hybridization. As examples, Figs. 11, 12 show (arrows) that immunopositive CHH perikarya (Fig. 11) are also positive for the cRNA probe encoding CHH (Fig. 12). Figs. 13, 14 demonstrate that some cells are only reactive for the anti-GIH serum (Fig. 13) or for the cRNA probe encoding GIH (Fig. 14). MT medulla terminalis; x X-organ. Bars represent 10 µm

Table 2. Characteristics of CHH and GIH cells in *Homarus gammarus* larval stage I

Antisera	Number	Mean number	Mean diameter (μm ± sd)	Maximum diameter (μm)	Minimum diameter (μm)
Anti-CHH	20	19 ± 4	7.16 ± 1.87	13.3	4.0
	17		7.14 ± 1.48	9.5	4.8
	15		5.93 ± 1.45	8.3	3.9
	21		6.48 ± 1.16	9.1	4.5
	25		6.94 ± 1.23	9.6	4.5
Anti-GIH	22	20 ± 2	6.22 ± 1.33	6.9	5.5
	19		7.46 ± 1.29	10.0	4.8
	19		6.74 ± 1.52	9.6	4.0
	21		6.67 ± 1.92	12.8	3.8

Furthermore, colocalization of mRNA encoding CHH and GIH was found in some of the positive neuroendocrine cells (not shown).

Finally, from series of alternating sections in which the neuroendocrine cells of the X-organ were studied using in situ hybridization or immunocytochemistry, we could deduce that the examined neuroendocrine cells showed a difference in staining pattern that may depend on differences in their synthetic activity. For example, as shown in Figs 11 and 12, the same perikarya were marked by the CHH antiserum (Fig. 11) and the CHH cRNA probe (Fig. 12). On the other hand, with respect to GIH, some cells were only reactive for either the cRNA probe or the antiserum, whereas others were positive at the mRNA and the protein levels (Figs 13 and 14).

DISCUSSION

Although the antisera and mRNA probes have been prepared from *Homarus americanus* material, the immunocytochemical and *in situ* hybridization results obtained in this study reveal that the neuroendocrine system in the eyestalk of *Homarus gammarus* larvae actively synthesizes the CHH and GIH neuropeptides. This confirms the results of our previous ultrastructural studies (Rotllant et al., 1991) demonstrating that the sinus gland is present and active in stage I larvae of *Homarus gammarus*. The present approach thus permits us to study

the synthetic activity of the neuroendocrine cells at the mRNA and protein level in both adult (De Kleijn et al., 1992) and larval lobsters.

As in adult lobster, CHH and GIH are synthesized in a group of neuroendocrine cells of the X-organ, transported along the X-organ sinus gland tract and accumulated in and released from the neurohemal organ, i.e., the sinus gland, of larval lobster. Colocalization of both neurohormones, demonstrated in some perikarya at the peptidergic level by immunocytochemistry and at the mRNA level by *in situ* hybridization, is in complete agreement with the results of Kallen and Meusy (1989) and De Kleijn et al., (1992) obtained when studying the same neuroendocrine system in adult *Homarus americanus*.

In addition, the distributions of peptidergic material in the sinus gland and in the perikarya of the X-organ cells are comparable. CHH is present in larger amounts in the sinus gland than GIH, and the GIH cells are more peripherally localized in the medulla terminalis ganglionaris X-organ (MTGX) than the CHH cells. The statement that the immunoreactions for CHH and GIH partially overlap each other in the sinus gland may reflect colocalization of both neuropeptides in the same axon terminals. Although, this postulation must be proven by an ultrastructural investigation.

Taking into account the statement of Gorgels-Kallen et al. (1982) that the cytological characteristics of the CHH-producing cells in adult lobster of the species *Homarus americanus* and *Homarus gammarus* are comparable, we can discuss the homologies and differences between the larval and adult systems in some detail. In larval stage I, for example, the cells immunopositive to CHH and GIH are 7 ± 1 μm in diameter, whereas in adults, CHH cells have a larger diameter with a maximum of 78 μm and a minimum of 55 μm (Gorgels-Kallen et al., 1982), the GIH cells having a diameter of approximately 20 μm (De Kleijn et al., 1992). Indeed, the much smaller larval cells are still in a growing state, although they are active in synthesis. Concerning the number of cells per eyestalk, approximately 19 CHH and 20 GIH cells have been counted during larval life at stage I, whereas adults possess approximately 40 CHH cells (Gorgels-Kallen et al., 1982) and 15-25 GIH cells (Kallen & Meusy, 1989). The reason for the increasing number of CHH cells and the constant number of GIH cells during growth is difficult to explain. Tensen et al. (1989) have found four isoforms of CHH in *Homarus americanus*, two of them having a stimulating

effect on oocyte growth in addition to their hyperglycemic activity. In this respect, there may be no synthesis of the latter two isoforms during larval life, thus accounting for the smaller number of CHH cells in larvae. More information concerning the relationship between the isoforms of CHH and cell type is therefore necessary, as the aforementioned hypothesis depends on the presence of subgroups of CHH-producing cells.

A difference in staining potential between larvae and adults has also been observed, the optimal dilution of antisera being about ten times higher in larvae than in adults. These results are difficult to explain. However we propose that the smaller-sized larval CHH and GIH cells have a higher concentration to volume ratio than the adults.

In conclusion, this study, based on immunocytochemistry and *in situ* hybridization techniques, confirms that the X-organ sinus gland neuroendocrine complex is present and functional in early larvae (stage I), subsequent larval stages (II, III) and postlarvae (IV) of *Homarus gammarus*. At least two neuropeptides, CHH and GIH, are synthesized in, and released from, this system as early as stage I. Moreover, as in adults, some neuroendocrine cells in the X-organ demonstrate colocalization of both neuropeptides at the mRNA and protein level. As the eyestalk neuropeptides are involved in many aspects of crustacean adaptative physiology and play a major coordinating role in the adaptation to variations in the external and internal conditions, detailed knowledge of neuroendocrine regulation within lobster larvae may therefore be useful for improving the breeding of these animals.

CHAPTER 5

Expression, storage and release of Crustacean Hyperglycemic Hormones and Gonad-Inhibiting Hormone during the reproductive cycle of the female American lobster *Homarus americanus*

ABSTRACT

The crustacean hyperglycemic hormones (CHHs) regulate carbohydrate metabolism and are probably also involved in stimulation of oocyte growth as well as molting, while the gonad-inhibiting hormone (GIH) inhibits the onset of vitellogenesis. These hormones are both produced in the same neuroendocrine perikarya of the medulla terminalis X-organ in the eyestalks of the American lobster *Homarus americanus*. In order to get more information on the synthesis, storage, release and possible function(s) of CHH-A and -B, and GIH during the reproductive cycle, we measured the levels of their mRNAs in the X-organ, their peptide storage in the neurohemal organ and their hemolymph peptide levels at different stages of the female reproductive cycle. For CHH, a high CHH-A mRNA level was found at the previtellogenic stage and higher mRNA levels were estimated for CHH-B in the mature as well as in the previtellogenesis stages compared to the other stages. During previtellogenesis, high storage levels for both CHHs were found in the sinus gland. In the hemolymph, the total amount of CHH (CHH-A plus -B) was high only during maturation. For GIH, a low level of mRNA in the X-organ and a low amount of the GIH I isoform in the sinus gland were found only in the immature stage. In contrast, GIH hemolymph levels were high during the immature and previtellogenic stages. Together, we conclude that CHH-A and -B are probably involved in triggering the onset of vitellogenesis and especially CHH-B seems to be responsible for stimulating oocyte maturation before spawning, while GIH prevents the start of vitellogenesis in the ovary. Moreover, the balance between the hemolymph levels of CHHs and GIH may regulate the synchronization of reproduction and molting during the bi-annual reproductive cycle of the American lobster.

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Expression, storage and release of Crustacean Hyperglycemic Hormones and Gonad-Inhibiting Hormone during the reproductive cycle of the female American lobster *Homarus americanus*. Neth. J. of Zoology. in press, (1995). Dominique De Kleijn[1], Karel P.C. Janssen[1], Susan L. Waddy[2], René Hegeman[1], Wilfred Y. Lai[2], Gerard J.M. Martens[1] and François Van Herp[1].

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INTRODUCTION

The crustacean hyperglycemic hormones (CHHs) are primary involved in the regulation of carbohydrate metabolism while the gonad-inhibiting hormone (GIH), also termed vitellogenesis-inhibiting hormone (VIH), inhibits vitellogenesis as reflected by its second name. Together with the molt-inhibiting hormone (MIH), these neurohormones belong to the CHH\MIH\VIH neuropeptide family (Keller, 1992). All these neurohormones are produced in the neuroendocrine cells of the medulla terminalis X-organ in the eyestalks of crustaceans and are transported to their clustered axon endings which form a neurohemal organ, the sinus gland. Detailed *in situ* hybridization and immunocytochemical studies revealed a frequent colocalization of both neurohormones (Kallen et al., 1989; De Kleijn et al., 1992). HPLC analysis of sinus gland extracts showed that CHH and GIH are present in different isoforms. For example, in lobster, CHH is eluted as two immunoreactive groups (CHH-A and CHH-B) and GIH as one immunoreactive group (Tensen et al., 1991b; Meusy et al., 1991). Each group consists of two isoforms with identical amino acid sequences and molecular masses (Tensen et al., 1991a; Soyez et al., 1991). It was recently established that the presence of a D-phenylalanine residue in one of the CHH isoforms, belonging to both CHH-immunoreactive groups, is responsible for the difference in HPLC elution time (Soyez et al., 1994). The CHH isoforms are therefore characterized as L- and D-Phe^{3}CHH-A and L- and D-Phe^{3}CHH-B.

The function of GIH in females may concern the inhibition of the onset of vitellogenesis (Soyez et al., 1987). However, as GIH is also present in male lobsters (De Kleijn et al., 1992), we have to consider another role for GIH. After characterization of preproGIH and its alignment with crab preproMIH, we found that not only the mature hormones have a high degree of amino acid identity but that also the primary structures of their preprohormones are nearly identical (De Kleijn et al., 1994c). Together with the fact that, in contrast to CHH mRNAs, GIH mRNA is present only in the eyestalk, we suggested that GIH may be an important modulator of synthesis or release of hormones involved in the reproduction/molting processes (De Kleijn et al., 1994c).

The existence of several groups and isoforms of the aforementioned neuropeptides suggests

that they may have several physiological functions and different target organs. The multifunctionality of CHH has been demonstrated already a number of times. While all CHH isoforms have a hyperglycemic effect, it is reported that CHH-B can also stimulate oocyte growth (Tensen et al., 1989) and CHH-A may be a peptide with hyperglycemic and molt-inhibiting activity (Chang et al., 1991). The effects of eyestalk ablation and implantation of thoracic/abdominal ganglia indicate the presence of a vitellogenesis-stimulating hormone (VSH; Aiken & Waddy, 1980; Otsu, 1963; Gomez & Nayar, 1965). These results, in combination with recent studies showing that CHH-A and -B mRNAs are present in parts of the nervous system other than the optic ganglia, indicate that CHH may have an additional role in the control of reproduction and molting (De Kleijn et al., 1994b).

In order to get more information about the synthesis, release and the function(s) of CHH-A and -B, and GIH in reproduction, we determined the levels of the three neurohormones during different stages of the female reproductive cycle in the lobster *Homarus americanus*.

MATERIALS AND METHODS

Animals

Specimens of the American lobster *Homarus americanus* were obtained commercially at Miminegash, Prins Edward Island, Canada. Experimental female animals were selected in September 1993 on the basis of their molting stage (stages C1-C4), the females should have molt recently and their carapax length must be 81-85 mm. As the lobster has a bi-annual reproductive cycle, these females would be mature and would spawn normally in the summer of 1995 but by induction of spawning according to the method by Waddy & Aiken (1992), the different reproductive stages could be obtained earlier. Therefore, the reproductive condition of each female was confirmed by examining the pleopodal cement glands in early spring 1994. All selected animals were kept in vertically stacked tanks with plexiglass front panels so that spawning could be observed without disturbing the animals.

In March 1994, a first group of animals were put into the artificially changed environmental conditions (12°C, LD 8:16) to induce spawning at the beginning of April 1994. These animals were used to study the different parameters after spawning. At the beginning of April 1994, a second group was put into the same changed environmental conditions in order to study the different parameters during maturation until spawning.
The reproductive stages were determined according to the method of Aiken & Waddy (1980).

Isolation of RNA

Total RNA was isolated according to the method of Chomczynski and Sacchi (1987), using acid-guanidine-thiocyanate-phenol-chloroform extraction. Medulla terminalis (MT) tissue from 10 eyestalks was pooled and homogenized in an all glass homogenizer containing 500 µl guanidine thiocyanate solution (4 M guanidine thiocyanate; 25 mM sodium citrate pH 7.0; 5 % sarcosyl; 0.1 M 2-mercaptoethanol). Sequentially, 50 µl of 2 M sodium acetate (pH 4.1), 500 µl water-saturated phenol and 100 µl chloroform-isoamylacohol (49:1) were added, the sample was mixed vigorously and the RNA was recovered after ethanol precipitation. RNA preparations were used in an RNase protection assay.

Labelling and purification of RNA probes

RNA probes were synthesized as run-off transcripts from 200 ng linearized plasmid DNA of pBluescript KS^+ plus insert. Labelling was performed in a final volume of 10 µl containing 50 µCi $UT^{32}P$, 15 units T3/T7 RNA polymerase in transcription buffer (Promega), 17.5 U RNasin (Promega) and 1 mM each of rATP, rCTP, and rGTP. After a incubation of 30 min at 37°C, the probe was separated from the template by electrophoresis on a 4% polyacrylamide gel. RNA was eluted from the gel in 500 µl 2.5 M ammonium acetate, pH 4.6 during 2 h at 55°C and then precipitated with ethanol using 20 µg tRNA as carrier.

Quantification of CHH-A, CHH-B and GIH mRNA levels in the eyestalk by RNase protection assay

For CHH-A, CHH-B and GIH cDNA fragments (CHH-A, nucleotides: 183 to 395 and CHH-B nucleotides: 675 to 865, De Kleijn et al., 1994b; GIH, nucleotides: 527 to 850, De Kleijn et al., 1994c) were cloned into pBluescript and used as templates for specific cRNA probes. After synthesis and purification, each cRNA probe was dissolved in the protection-assay hybridization mix (80% formamide, 400 mM NaCl, 40 mM PIPES, pH 6.4; 1 mM EDTA). Separately, total RNA samples obtained from the collected eyestalks were dissolved in 23 µl protection-assay hybridization mix. After addition of 2 µl RNA probe and incubation for 10 min at 80°C, hybridization was performed for 16 h at 55°C. Next, 300 µl RNase digestion buffer (10 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 300 mM NaCl, 25 µg RNase A/ml, 500 U RNase T1/ml) was added, the mixture was incubated for 30 min at 37°C, 8 µg/ml proteinase K and 0.1% SDS were added, and the incubation was continued for another 30 min at 37°C. Samples were phenol extracted, ethanol precipitated, dissolved in 5 µl formamide loading mix (80% formamide, 1 mM EDTA pH 8.0, 1 mg/ml xylene cyanol, 1 mg/ml bromo-phenol blue) and loaded onto a 4% polyacrylamide gel. After electrophoresis, the gel was fixed by drying and exposed to X-ray film using an intensifying screen at -70°C.

The radioactivity present in the protected band was estimated by scintillation counting of the corresponding gel fragment. The amount of CHH mRNA was calculated from a dilution series of sense cRNA.

Micro HPLC analysis

Both sinus glands of one animal were dissected and collected in a dry ice-cooled glass-glass homogenizer, immediately homogenized in 350 µl 0.1 N HCl, heated at 80°C for 5 min and then freeze-dried. Before chromatography, the dried samples were dissolved in 45 µl solvent A (0.1% trifluoroacetate in water) and centrifuged at 12.000 g for 30 min. The supernatant was then injected onto a micro HPLC system (SMART™, Pharmacia LKB) using a reversed phase column (type µRPC C_2/C_{18}; 2.1x100 mm; particle size 3 µm; gel volume 350 µl). The peptides were eluted by a gradient using solvent A and solvent B

(80% acetonitrile/20% water/0.1% trifluoroacetate): 100% solvent A, 5 min; 0% B to 35% B, 5 min; 35% B to 65% B, 30 min; 65% B to 100% B, 15 min. The flow rate was 200 µl/min. UV detection was performed at 214 nm using a UV-MII µ Peak Monitor (Pharmacia LKB).

The chromatographic data from the individual animals were evaluated with the SMART Manager 1.31 software enabling comparison and integration by area and peak heights. The relative amounts of the different CHH isoforms in both sinus glands of individual lobsters were calculated.

Determination of CHH and GIH hemolymph levels by a double sandwich ELISA

Hemolymph sampling

Hemolymph samples were taken from the tail with a 1-ml seringe. The hemolymph was divided into five fractions of 200 µl each diluted 1:1 with hemolymph buffer (PBS = 10 mM sodium phosphate pH 7.2, 0.1% KCl, 0.8% NaCl; 0.03% EDTA pH 8.0, 2% BSA and 0.02% NaAzide). After centrifugation (1 min, 12.000 g) the supernatants were stored at -20°C.

ELISA

Based on earlier findings (Klein, 1989), a double sandwich ELISA was developed in order to measure CHH and GIH hemolymph levels during the reproductive cycle. For this purpose three antisera were used: a polyclonal rabbit anti-*Astacus leptodactylus* CHH serum (Gorgels-Kallen & Van Herp, 1981), a polyclonal guinea pig anti-*Homarus americanus* CHH serum against HPLC-purified CHH (Meusy & Soyez, 1991) and a polyclonal rabbit anti-*Homarus americanus* GIH serum against HPLC-purified GIH.

The antibodies were isolated from the serum with a Protein A Sepharose CL-4B column buffered with PBS, pH 7.3. In order to be used as second antibody, anti-*Homarus* CHH and anti-*Homarus* GIH were labelled with biotine using a biotinylation kit (Sigma).

The ELISA method was carried out as follows. After pretreatment with 1% glutaraldehyde, microtiter plates (Nunc) were coated for 16 h at 4°C with 100 µl anti-*Astacus* CHH-

IgG (20 µg/ml in 0.1 M sodiumphosphate buffer, pH 8.0) for the quantification of CHH or with 100 µl anti-*Homarus* GIH (20 µg/ml in 0.1 M sodiumphosphate buffer, pH 8.0) for the determination of GIH. After coating of the first antibody, the wells were washed five times with 0.1 M sodium phosphate buffer, pH 8.0 and successively blocked with 400 µl 2% BSA in PBS, pH 7.2 for 8 h at 4°C. After blocking, 100 µl of each collected hemolymph sample was incubated for 16 h at 4°C and washed seven times with 400 µl 0.1 % Tween 20 in PBS, pH 7.2 (PBS-T). Addition of the respective biotinylated second antibody (anti-*Homarus* CHH for CHH and anti-*Homarus* GIH for GIH detection both 5 µg/ml in PBS, pH 7.2 containing 2 % BSA), the plates were incubated for 6 h at 37°C and washed seven times with PBS-T. Finally, 100 µl streptavidin-peroxydase conjugate (1:5 dilution of the Histomark KPL solution) was added and the sample was incubated for 1 h at 37°C and washed seven times with 400 µl PBS-T. The enzymatic reaction was then initiated by the addition of 200 µl 1,2 benzenediamine (1 mg/ml 0.1 M Mac-Ilvaine buffer, pH 5.2, containing 0.8 µl/ml 40% H_2O_2) and stopped by addition of 100 µl 4 N H_2SO_4. The optical density was measured at 492 nm with an EAR-400 ELISA reader.

Because the antibodies could not dicriminate between CHH-A and -B, and between the different isoforms of CHH and GIH, with this method the total amount of CHH and GIH in the hemolymph was measured.

Cross reactivity was determined using HPLC purified GIH as antigen for the CHH ELISA and vice versa. Hemolymph samples from *Carcinus maenas* were used as antigen to screen for possible cross reactivity with hemolymph proteins.

RESULTS

Experimental set up

Female American lobsters, in different stages of ovary development, were obtained by inducing the reproduction cycle in 70 animals. The animals were divided into ten groups and at each time point seven animals were sacrificed. Hemolymph samples of each animal were taken at the same time of the day in order to exclude the influence of diurnal

rhythms. Then, the eyestalks were ablated for extirpation of the MTGX and the sinus gland, and finally the ovaries were dissected and the exact developmental stage was determined using weight, color and ovary factor as parameters. The first samples were taken just before the experimental animals were put at the environmental conditions inducing reproduction which is 28 days before spawning. The time points for sampling were 16 days, 7 days and 1 day before spawning, and 1 day, 3 days, 10 days, 21 days, 31 days and 40 days after spawning. The different time points represented characteristic physiological stages in the female reproduction cycle and correspond to groups of animals in the following four stages of ovaria development: immature (spawned ovaries; 1, 3 and 10 days after spawning), previtellogenesis (21, 31 and 40 days after spawning), vitellogenesis (28, 16 and 7 days before spawning) and mature (1 day before spawning).

Expression, storage and release of CHH-A during the reproductive cycle

Fig. 1 shows that CHH-A mRNA levels change during the different stages of the reproductive cycle and especially the increase during previtellogenesis is remarkable and significantly different from the other stages. This increase of mRNA is also reflected in the significantly higher amount of CHH-A peptide stored in the sinus gland. For all stages, no differences were found

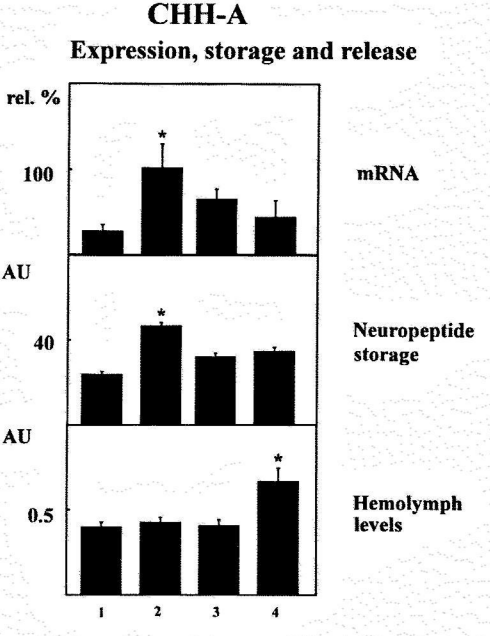

Fig. 1 Levels of mRNA expression in the medulla terminalis ganglionaris X-organs, neuropeptide storage in the sinus glands and hemolymph levels of CHH-A during the reproductive cycle of female American lobsters. Levels of mRNA are in percentages of the maximum value. Total neuropeptide storage of the two CHH-A isoforms and hemolymph levels are in arbitrary units (AU). * indicates a significant difference between CHH-A mRNA levels in the previtellogenic stage and the immature and vitellogenic stages ($p<0.02$), CHH-A neuropeptide levels in the previtellogenic stages and the other three stages ($p<0.001$) and between CHH hemolymph levels (CHH-A plus -B) in the mature stage and the three other stages ($p<0.005$). 1 = immature, 2 = previtellogenesis, 3 = vitellogenesis, 4 = mature.

between the amounts of L- and D-Phe^{3}CHH-A (results not shown). In contrast to the mRNA and peptide storage levels, the highest amounts of total CHH (CHH-A and CHH-B) in hemolymph were found in the mature stage that significantly differed from the three other stages.

Expression, storage and release of CHH-B during the reproductive cycle

When compared to the level in the immature stage, a significant increase in CHH-B mRNA levels was found in the previtellogenesis and mature stages (Fig. 2). A significant increase of the storage level of CHH-B was found during previtellogenesis, while the total CHH level in hemolymph was high during the mature stage. No difference was found between the amounts of neuropeptide isoforms L- and D-Phe^{3}CHH-B (results not shown).

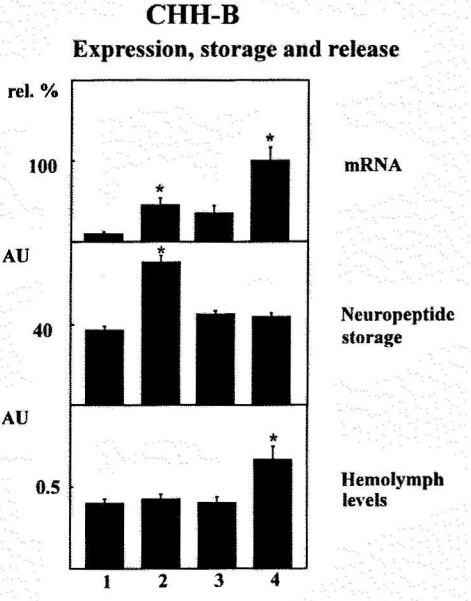

Fig. 2 Levels of mRNA expression in the medulla terminalis ganglionaris X-organs, neuropeptide storage in the sinus glands and hemolymph levels of CHH-B during the reproductive cycle of female American lobsters. Levels of mRNA are in percentages of the maximum value. Total neuropeptide storage of the two CHH-B isoforms and hemolymph levels are in arbitrary units (AU). * indicates a significant difference between CHH-B mRNA levels in the previtellogenic and mature stages, and the immature and vitellogenic stages ($p<0.02$), CHH-B neuropeptide levels in the previtellogenic stages and the other three stages ($p< 0.001$) and between CHH hemolymph levels (CHH-A plus -B) in the mature stage and the three other stages ($p<0.005$).1 = immature, 2 = previtellogenesis, 3 = vitellogenesis, 4 = mature.

Expression, storage and release of GIH during the reproductive cycle

Fig. 3 illustrates that the level of GIH mRNA was low in the immature stage and differs significantly from the levels during previtellogenesis and vitellogenesis. No significant differences were found in total GIH neuropeptide storage levels in the sinus gland.

However, the amount of the GIH I isoform changed dramatically in the immature stage (results not shown). During vitellogenesis, the total GIH level in the hemolymph was significantly lower then in the other stages.

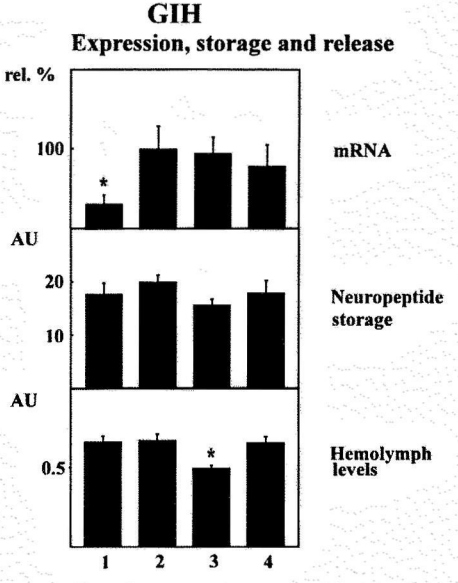

Fig. 3 Levels of mRNA expression in the medulla terminalis ganglionaris X-organs, neuropeptide storage in the sinus glands and hemolymph levels of GIH during the reproductive cycle of female american lobsters. Levels of mRNA are in percentages of the maximum value. Total neuropeptide storage of the two GIH isoforms and hemolymph levels are in arbitrary units (AU). * indicates a significant difference between GIH mRNA levels in the immature stage and the pre- and vitellogenic stages ($p<0.02$), and between GIH hemolymph levels in the vitellogenic stage and the three other stages ($p<0.001$). 1 = immature, 2 = previtellogenesis, 3 = vitellogenesis, 4 = mature.

DISCUSSION

In this study we found that the changes in the levels of CHH-A and -B mRNAs and their respective amounts of stored peptide during the previtellogenic stage are not related to changes in the level of total CHH in the hemolymph. This finding indicates that there is no relation between intracellular mRNA and peptide levels, and the release of the CHH peptides in the hemolymph at that stage. While the CHH-B mRNA level and the total hemolymph CHH peptide level are high in the mature stage, no differences are found in peptide storage, illustrating that synthesis and release are not reflected at the storage level. This may be due to the fact that the sinus gland can store high amounts of peptides and that only a small portion is released, as already postulated by Stuenkel (1983) on the basis of pulse-chase studies on crab sinus glands (Stuenkel, 1983) which suggested a low turn-

over of sinus gland peptides. However, the increased CHH-B mRNA levels combined with the elevated total CHH hemolymph levels may suggest that in particular CHH-B is involved in oocyte maturation. The CHH-A and -B neuropeptide storage levels in the sinus gland were found to be higher during previtellogenesis than during vitellogenesis and this difference was not reflected in the total CHH content of the hemolymph. These findings indicate that the peptides are released during late previtellogenesis or at the onset of vitellogenesis and that more precise time points of sampling are necessary for a definitive conclusion. If this is the case, release of CHH-A and -B would trigger vitellogenesis during autumn. This hypothesis is in agreement with Waddy & Aiken (1992) who found that before winter, females only rarely respond to environmental changes. However, in early November most females respond to artificially changed day length and temperature by spawning three months later. Finally, a circadian rhythmicity in the release of the neuropeptides during the reproductive cycle is not evident because no day/night changes were found in glucose, total CHH and total GIH hemolymph levels (data not shown). In order to prove our hypothesis, hemolymph samples should be taken during the onset of vitellogenesis.

The higher hemolymph levels of GIH at the immature and previtellogenic stages are in agreement with the proposed function for GIH: inhibition of the onset of vitellogenesis. The higher level at maturation may reflect the restart of inhibition after vitellogenesis. However, GIH levels are mostly high when CHH levels decrease with exception of the mature stage where both CHH and GIH level are high. We can not exclude the possibility that the high amounts of GIH at maturation are due to cross reactivity in the ELISA because the control experiments demonstrate cross reactivity with CHH (Meusy & Soyez, 1991).

The intracellular regulation of biosynthesis is still unclear because peptide storage in the sinus gland and mRNA levels in the X-organ do not show any relationship. Using the combined *in situ* and immunocytochemical techniques, as described by De Kleijn et al. (1992), during a reproductive cycle may demonstrate if the changes in mRNA levels are reflected by changes in the number of perikarya producing the CHHs and GIH. When the presumed molt-inhibiting function of GIH (De Kleijn et al., 1994c) and the described MIH

effects of the hyperglycemic hormone (Chang et al., 1990) is taken into account, the lower blood levels of both peptides at late previtellogenesis may be responsible for induction of the molting process. Such a combined effect would be ideal for synchronization of the molt and the reproduction cycles because molting is then inhibited in the immature ovary stages by GIH and in mature stages by the CHHs.

Our results and interpretation are summarized in Fig. 4 showing the CHH-A, CHH-B and GIH hemolymph levels during a molt/reproduction cycle. High levels of CHH-A and -B

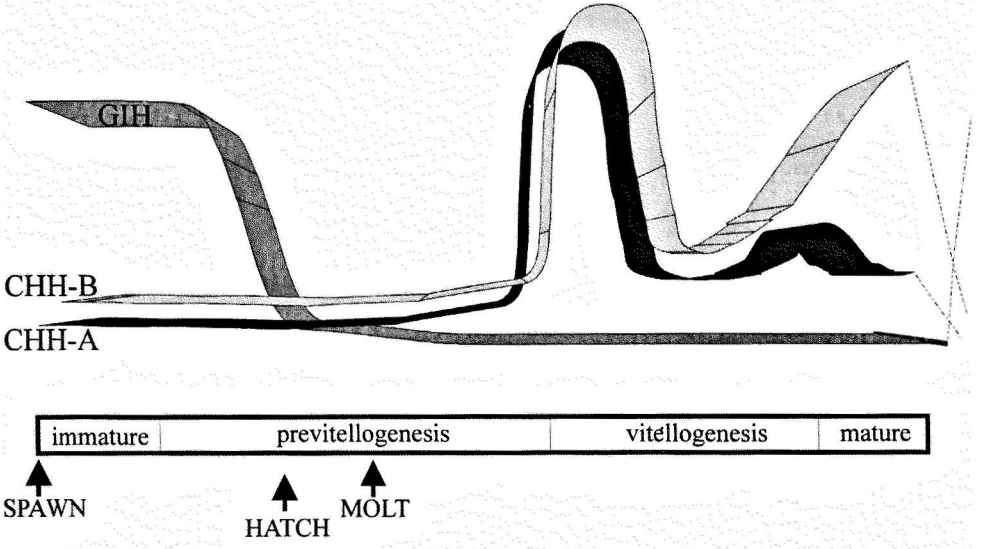

Fig. 4 Diagram of CHH-A, CHH-B and GIH hemolymph levels during the bi-annual reproductive cycle in female lobsters *Homarus americanus* based on our results and their interpretation. The new cycle starts when the female has spawned and incubates her eggs of the last cycle. Spawning, hatching of the "last cycle" larvae and the consecutive molt of the adult animal is indicated by arrows. Division of the cycle in 4 stages (immature, previtellogenesis, vitellogenesis and mature) is based on Aiken & Waddy (1980).

after molting and at the onset of vitellogenesis are followed by a lower CHH level in winter after which especially the CHH-B level increases during the last stages of vitellogenesis. The hemolymph level of GIH is mostly high when CHH is low and vice versa. Levels increase after spawning and remain high until they decrease before molting,

possibly during hatching of the larvae.

In conclusion, our study suggests that CHHs, like GIH, are involved in the regulation of reproduction and that the balance between CHHs and GIH hemolymph levels probably can regulate the synchronization of the reproductive and molt cycles. Nevertheless, monitoring of complete reproduction/molting cycles, applying specific antisera against the different forms of the neuropeptides, is necessary to provide the evidence for the role of CHHs and GIH in these cycles.

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CHAPTER 6

Cloning and expression of two crustacean hyperglycemic hormone (CHH) mRNAs in the eyestalk of the crayfish *Orconectes limosus*

ABSTRACT

Crustacean Hyperglycemic Hormone (CHH) is a multifunctional neurohormone produced in the eyestalk of crustaceans and primarily involved in the regulation of carbohydrate metabolism. In several crustacean species CHH HPLC isoforms with identical amino acid sequences and molecular weights are synthesized. In order to obtain sequence information on the CHH preprohormone in the crayfish *Orconectes limosus,* we isolated two full-length cDNAs encoding two structurally different preproCHHs. Their sequences slightly differ in the signal peptide, the CHH-Precursor-Related Peptide (CPRP)-coding sequences, and in the non-coding regions but are identical in the CHH peptide-coding sequence. Determination of the levels of preproCHH mRNAs and the amount of CHH peptide in the eyestalks of individual animals revealed that the ratio between the two preproCHH mRNAs varies individually while the ratio between the two CHH peptide isoforms does not differ among animals. Our results suggest that the existence of two CHH isoforms in the crayfish *Orconectes limosus* is due to a post-translational modification event. Northern blot analysis showed only one band in eyestalk tissue with a size of ~2.4 kb, similar to the sizes of the cDNA sequences. Southern blot analysis revealed the presence of at least two preproCHH genes in the crayfish suggesting a gene duplication event. Slight modifications in the duplicated genes could then be responsible for the existence of the two preproCHH-encoding mRNAs.

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INTRODUCTION

An important part of the neuroendocrine system in decapod Crustacea is situated in the optic ganglia of the eyestalk and consists of neurosecretory cells whose clustered axon endings form a neurohaemal organ, the sinus gland. One of the most abundant neurohormones produced by these neuroendocrine cells is the Crustacean Hyperglycemic Hormone (CHH). This neuropeptide is primarily involved in the regulation of blood sugar levels and glycogen metabolism, and belongs to a novel family of crustacean neuropeptides (for review see Keller, 1992). HPLC analysis revealed polymorphism of the CHH neuropeptides in several decapod crustacean species. The major CHH peak in the HPLC pattern of sinus gland extracts of the shore crab *Carcinus maenas* was the first CHH peptide for which the amino acid and mRNA sequences have been elucidated (Kegel et al., 1989; Weidemann et al., 1989). CHH isoforms were also found in the crayfish *Orconectes limosus* and the primary amino acid sequence of the most abundant form has been determined (Kegel et al., 1991). CHH polymorphism was studied in more detail in the lobster *Homarus americanus* (Tensen et al., 1989; Soyez et al., 1990) and in the crayfish *Procambarus bouvieri* (Huberman & Aguilar, 1988, 1989). Two CHH groups, each consisting of two isoforms with the same amino acid sequence and molecular mass, were found in the HPLC pattern of sinus gland extracts from the lobster *Homarus americanus* (Soyez et al., 1990; Tensen et al., 1991b; Tensen et al., 1991c). Further studies indicated that the difference in the elution patterns of the isoforms is due to a difference in the N-terminal octapeptide (Tensen et al., 1991e). Similar results were obtained in tryptic mapping studies with sinus gland material from the crayfish *Procambarus bouvieri* (Huberman & Aquilar, 1988; Huberman & Aquilar, 1989). Moreover, circular dichroism analysis demonstrated a difference in secondary structure between the two CHH isoforms of this crayfish species (Huberman et al., 1989).

In this study, we report on the cloning and expression of two crustacean hyperglycemic hormone (CHH) mRNAs in the eyestalk of the crayfish *Orconectes limosus*. A CHH-encoding cDNA fragment obtained by the polymerase chain reaction was used to screen an Medulla Terminalis (MT) cDNA library. An RNase protection assay has been developed to study the level of CHH mRNAs relative to the amounts of CHH neuropeptides stored in the sinus gland.

MATERIALS AND METHODS

Animals

Specimen of the crayfish *Orconectes limosus* were obtained from a local fisherman fishing in the river Meuse (The Netherlands). The animals were kept in the laboratory in running tap water (13-15°C) on a 12 h light/12 h dark cycle. They were fed with fish once a week.

RNA isolation

Total RNA was isolated according to the method of Chomczynski and Sacchi (1987), using acid-guanidine-thiocyanate-phenol-chloroform extraction. Medulla terminalis (MT) tissue from 10 eyestalks was pooled and homogenized in an all glass homogenizer containing 500 µl guanidine thiocyanate solution (4 M guanidine thiocyanate; 25 mM sodium citrate pH 7.0; 5 % sarcosyl; 0,1 M 2-mercaptoethanol). Sequentially, 50 µl of 2 M sodium acetate (pH 4.1), 500 µl water-saturated phenol and 100 µl chloroform-isoamylacohol (49:1) were added, the sample was mixed vigorously and the RNA was recovered after ethanol precipitation. RNA preparations were used for cDNA synthesis or poly (A^+) isolation according to the instructions of the manufacturer (Stratagene) as well as for an RNase protection assay.

cDNA synthesis

Twenty µg of total MT-RNA was reverse transcribed with 100 ng oligo dT (PL Biochemicals) using 200 Units cloned Moloney murine reverse transcriptase (Gibco-BRL) for 1 h at 37°C in a final reaction volume of 20 µl containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 500 µM dNTP, 10 mM DTT and 35 U RNasin (Promega). The cDNA was diluted to 500 µl and used for the PCR amplification between mixed oligonucleotide primers.

For the 5' amplification, cDNA synthesis was performed in the same way, except that 20 µCi $d^{32}ATP$ was added to the reaction. After incubation for 1 h at 37°C, the radioactive cDNA was passed through a Biogel A 50 M 1.5 ml column equilibrated with 0.05 x TE (1 x TE= 10 mM Tris pH 8.0, 1 mM EDTA) and one-drop fractions were collected. The fractions -3 to +4 (relative to the first drop containing radioactivity) were pooled and the volume was reduced to 23 µl using a centrifugal dryer (Speed Vac). Six µl of 5 x Tailing buffer (BRL),

1 µl of 6 mM dATP and 10 Units Terminal Deoxynucleotidyl Transferase (BRL) were added and the sample was incubated for 5 min at 37°C, 5 min at 65°C and diluted to 200 µl.

PCR analysis

Amplification between mixed oligonucleotides

The cDNA sequence encoding amino acid residues 1-29 of crayfish CHH (Kegel et al., 1991) was specifically amplified in a polymerase chain reaction (PCR) using two mixed oligonucleotide primers: M1 (sense to amino acids 1-8; 5'-GGGAATTCARGTNTTYGAY CARGCNTGYAA-3') and M2 (anti-sense to amino acids 22-29; 5'-CCGGATCCARRTTRTA RCARTCYTCRCANAC-3'). Forty PCR cycles were carried out in a Perkin-Elmer/Cetus DNA Thermal cycler: denaturation at 94°C, 40 sec; annealing at 60°C, 2 min; extension at 72°C, 3 min; final extension at 72°C for 10 min. The amplified cDNA was phenol extracted, digested with EcoRI and BamHI, and size selected on low melting point agarose (LMP, Gibco-BRL). The expected 100-bp product was recovered (Tautz & Renz, 1983), cloned into pBluescript KS^+ (Stratagene), transformed to and propagated in DH5α, and sequenced.

Amplification of 5' CHH cDNA ends

A first round of amplifications was performed using 10 µl of the diluted cDNA pool, 100 pmol of primer M1, 10 pmol of primer dtRiRo (5'-ATCGATGGTCGACGCAATGC GGATCCAAAGCTTGAATTCGAGCTCTTTTTTTTTTTTTTTT-3'), and 25 pmol of primer Ro (5'-ATCGATGGTCGACGCAATGCGGATCC-3'). Forty PCR cycles were carried out in a Perkin-Elmer/Cetus DNA Thermal cycler under the conditions described above. 2.5 µl of the obtained PCR product was reamplified using 25 pmol of primer Ri (5'-GGATCCAA AGCTTGAATTCGAGCTCT-3') and S1 (5'-GTCAAGCTTCTTGAAGATGGCTCTG-3') for 32 cycles and an annealing temperature of 70°C. The S1 primer was designed on the basis of the sequences obtained by amplification between M1 and M2. The reamplified PCR products were size selected on LMP agarose, digested with EcoRI and HindIII, and cloned into pBluescript KS^+ for sequence analysis. All amplifications were performed in 100 µl of Taq-buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 3 mM $MgCl_2$, 200 µM of each dNTP, 10 µg BSA and 2.5 U amplitaq (Perkin-Elmer/Cetus), overlaid with 50 µl mineral oil.

Construction and screening of the Orconectes limosus MT cDNA library

Using 1 µg poly (A^+) RNA, a MT cDNA library was constructed in the vector λZAP-II according to the instructions of the manufacturer (Stratagene). The library was screened with the CHH 5' cDNA end obtained from the 5' cDNA-end amplification. The ~350 bp fragment was labeled with ^{32}P by random priming according to standard procedures (Sambrook et al., 1989). Hybridization of the replica nitrocellulose filters was performed at 42°C in 6 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1 x Denhardts solution, 25 mM sodium phosphate buffer pH 7.0, 10% dextran sulphate, 200 µg yeast tRNA/ml and 50% formamide. Hybridization-positive phage plaques were purified, and the recombinant pBluescript SK-phagemids were rescued from the bacteriophage (λZAP) clones by *in vivo* excision, according to the instructions of the manufacturer (Stratagene).

DNA sequencing

Sequencing on both strands was performed with single-stranded DNA using T7 DNA polymerase and the dideoxy chain termination method (Sanger et al., 1977). For preparation of single-stranded templates, *E.coli* strain Xl1-Blue, harboring recombinant plasmids based on phagemid pBluescript KS^+ containing HindIII, PstI or ApaI CHH cDNA fragments in both orientations, were used according to the instructions of the manufacturer (Stratagene).

Labelling and purification of RNA probes

RNA probes were synthesized as run-off transcripts from 200 ng linearized DNA of pBluescript KS^+. Labelling was performed in a final volume of 10 µl containing 50 µCi $UT^{32}P$, 15 units T3/T7 RNA polymerase in transcription buffer (Promega), 17.5 U RNasin (Promega) and 1 mM each of rATP, rCTP, and rGTP. After a 30-min incubation at 37°C, the probe was separated from the template by electrophoresis on a 4% polyacrylamide gel, the RNA material was cut out and eluted from the gel in 500 µl 2.5 M ammonium acetate pH 4.6 for 2 h at 55°C followed by ethanol precipitation using 20 µg tRNA as carrier.

Northern blotting

RNA (total or polyadenylated) was separated by electrophoresis on denaturing formaldehyde agarose gels (1.0%) in MOPS buffer. Northern blot transfers to Hybond N (Amersham) were performed essentially as described by Thomas (1980). RNA was fixed by baking for 2 h at 80°C. A XbaI/HindIII fragment (corresponding to nucleotides 41-344 of CHH-A cDNA in Fig. 1) was derived from a recombinant containing the 5'-CHH cDNA end, cloned into pBluescript KS^+ and used for the generation of a cRNA probe. Hybridization was performed at 50°C for 16 h and the filter was washed until 0.25 x SSPE at 56°C. A λ PstI digest and total RNA of heart and hepatopancreas were used as size markers.

Quantification of CHH mRNA levels in the eyestalk by an RNase protection assay

A HindIII fragment (corresponding to nucleotides 2164-2325 of CHH-A cDNA and nucleotides 2312-2473 of CHH-A^* cDNA in Fig. 1) were cloned into pBluescript KS^+ and used as templates for specific cRNA probes. After synthesis, the cRNA probe was dissolved in the protection-assay hybridization mix (80% formamide, 400 mM NaCl, 40 mM PIPES, pH 6.4; 1 mM EDTA). Total RNA samples obtained from the collected eyestalks were dissolved in 23 µl protection-assay hybridization mix. After addition of the RNA probe and incubation for 10 min at 80°C, the hybridization was performed for 16 h at 55°C. Next, 300 µl RNase digestion buffer (10 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 300 mM NaCl, 25 µg RNase A/ml, 500 U RNase T1/ml) was added, the mixture was incubated for 30 min at 37°C, 8 µg/ml proteinase K and 0.1% SDS were added, and the incubation was continued for another 30 min at 37°C. Samples were phenol extracted, ethanol precipitated, dissolved in 5 µl formamide loading mix (80% formamide, 1 mM EDTA pH 8.0, 1 mg/ml xylene cyanol, 1 mg/ml broom phenol blue) and loaded onto a 4% polyacrylamide gel. After electrophoresis, the gel was fixed by drying and exposed to X-ray film using an intensifying screen at -70°C. The radioactivity present in the protected band was estimated by scintillation counting of the corresponding gel fragment. The amount of CHH mRNA was calculated from a dilution series of sense CHH cRNA. Further control experiments with sense RNA revealed that the detection limit of the RNAse protection assay was 0.005 pg CHH mRNA and the error in the measurement 20%.

Micro HPLC analysis

Both sinus glands of one animal were dissected and collected in a dry ice-cooled glass-glass homogenizer, immediately homogenized in 350 µl 0.1 N HCl, heated at 80°C for 5 min and then freeze-dried. Before chromatography, the dried samples were dissolved in 45 µl solvent A (0.1% trifluoroacetate in water) and centrifuged at 12.000 rpm for 30 min. The supernatant was then injected onto a micro HPLC system (SMART^{tm}, Pharmacia LKB) using a reversed phase column (type µRPC C_2/C_{18}; 2.1x100 mm (SC 2.1/10; particle size 3 µm; gel volume 350 µl). The peptides were eluted by a gradient using solvent A and solvent B (80% acetonitrile/20% water/0.1% trifluoroacetate): 100% solvent A, 5 min; 0% B to 35% B, 5 min; 35% B to 65% B, 30 min; 65% B to 100% B, 15 min. The flow rate was 200 µl/min. UV detection was performed at 214 nm using a UV-MII µ Peak Monitor (Pharmacia LKB).
The chromatographic data from the individual animals were evaluated with the SMART Manager 1.31 software enabling comparison and integration by area and peak height determination of the relative amounts of the different CHH isoforms in both sinus glands of individual crayfish.

Southern blot analysis

Genomic DNA was isolated from *Orconectes limosus* tail muscle tissue as described by Sambrook et al. (1989) and 15 µg DNA was restriction digested for 16 h and loaded on a 0.7% agarose gel. Blotting and hybridization of the Southern blot was performed according to the instructions of the manufacturer (Amersham).

RESULTS AND DISCUSSION

Cloning and characterization of two preproCHH mRNAs in the crayfish Orconectes limosus.

After cloning the amplified 5'-ends of crayfish CHH mRNAs, eleven clones containing the expected insert size were sequenced. Eight clones had an open-reading frame encoding a signal peptide of 26 amino acids, followed by a CPRP of 33 amino acids with a C-terminal Ser, a Lys-Arg pair and the first 20 amino acids of CHH. The remaining three clones differed from these clones at four positions including a C-terminal Asn in the CPRP (Fig. 1). Screening of the *Orconectes limosus* MT cDNA library with the specific 5'-CHH cDNA fragment obtained by PCR yielded 28 hybridization-positive clones. Sequence analysis of eight clones revealed a C-terminal Asn in the CPRP region of six clones (corresponding to CHH-A*) and a C-terminal Ser in the same region of the two other clones (CHH-A). Complete sequencing of the 5'-CHH cDNA PCR fragments and of the two different groups of cDNA clones isolated from the library, resulted in the sequences of two full-length CHH-encoding cDNAs (Fig. 1). The deduced amino acid sequences of both CPRPs and CHHs are identical to the respective amino acid sequences previously determined by the Edman degradation method (Kegel et al., 1991; Tensen et al., 1991d). The deduced Gly^{108} residue at the C-terminal end of the preproCHH sequence is a potential amidation signal (Eipper et al., 1992), confirming the previously found Val-NH_2 at the C-terminal end of the CHH peptide (Kegel et al., 1991).

The 5'-untranslated regions showed 99% identity between CHH-A and -A* mRNAs, while their long 3'-untranslated regions displayed 96% identity. The signal for polyadenylation is found 8 bp upstream of the poly-A tail (Fig. 1). The structural organizations of the crayfish preproCHHs are similar to the organization of crab preproCHH. Comparison of the deduced amino acid sequences of *Orconectes limosus* CHH preprohormones with the *Carcinus maenas* CHH

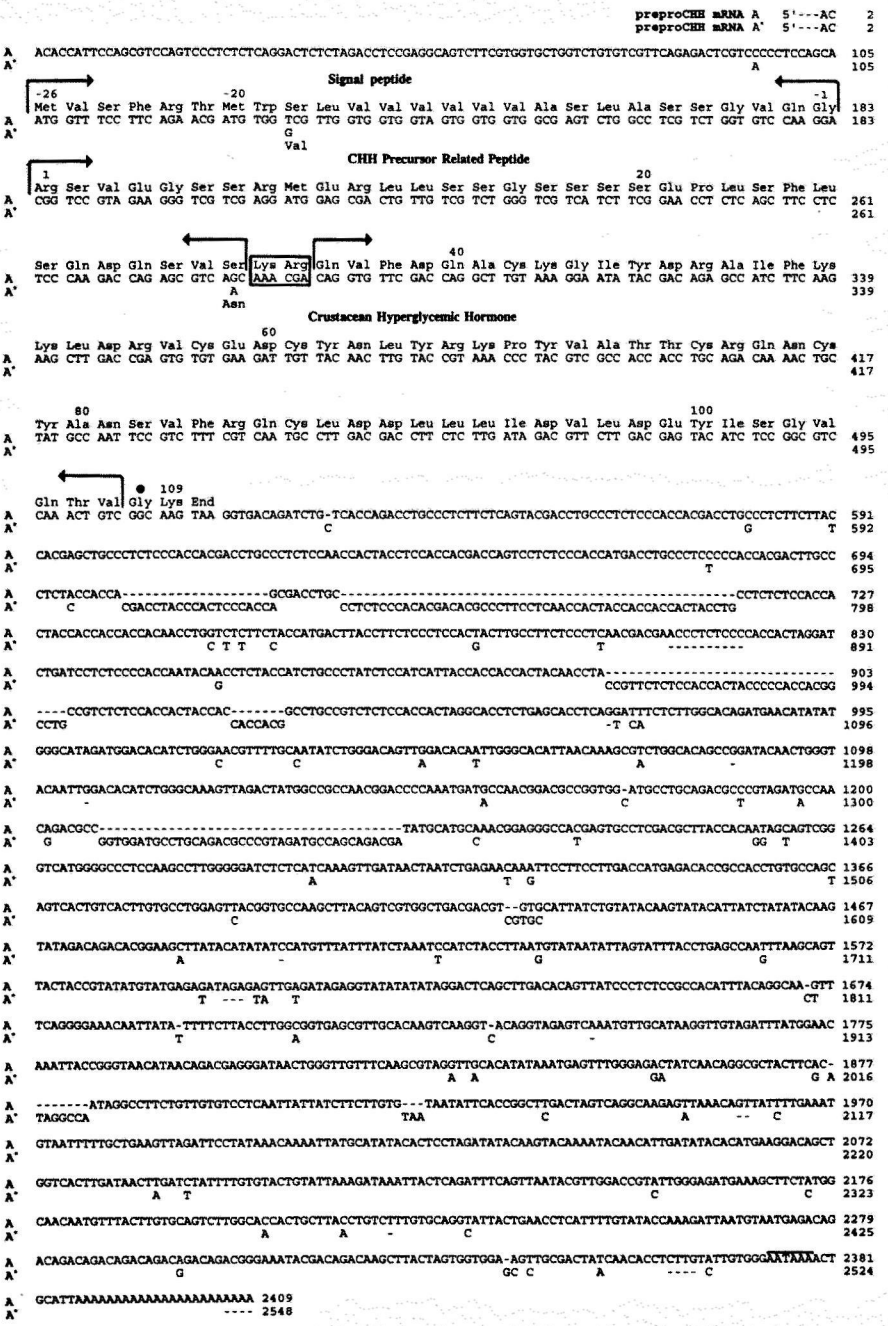
preproCHH mRNA A 5'---AC 2
preproCHH mRNA A' 5'---AC 2
Signal peptide
CHH Precursor Related Peptide
Crustacean Hyperglycemic Hormone
Met Val Ser Phe Arg Thr Met Trp Ser Leu Val Val Val Val Val Val Ala Ser Leu Ala Ser Ser Gly Val Gln Gly
Arg Ser Val Glu Gly Ser Ser Arg Met Glu Arg Leu Leu Ser Ser Gly Ser Ser Ser Ser Glu Pro Leu Ser Phe Leu
Ser Gln Asp Gln Ser Val Ser Lys Arg Gln Val Phe Asp Gln Ala Cys Lys Gly Ile Tyr Asp Arg Ala Ile Phe Lys
Lys Leu Asp Arg Val Cys Glu Asp Cys Tyr Asn Leu Tyr Arg Lys Pro Tyr Val Ala Thr Thr Cys Arg Gln Asn Cys
Tyr Ala Asn Ser Val Phe Arg Gln Cys Leu Asp Asp Leu Leu Leu Ile Asp Val Leu Asp Glu Tyr Ile Ser Gly Val
Gln Thr Val Gly Lys End

Fig. 1 Nucleotide sequences and deduced amino acid sequences of mRNAs encoding prepro CHH-A and A* of the crayfish *Orconectes limosus*. Amino acid numbering starts at the N-terminal residue of the CHH prohormones with the presumptive signal peptide sequences being indicated by negative numbering. The deduced amino acid sequence of CHH-A preprohormone is shown above the nucleotide sequence. For CHH-A* the differences in nucleotide and amino acid sequence are shown below the CHH-A sequences. The dibasic processing site between CHH and CPRP is boxed. The dot above Gly-108 indicates a potential amidation site (Eipper et al., 1992). The signal for polyadenylation (Birnstiel et al., 1985) is overlined.

preprohormone (Weidemann et al., 1989; Fig. 2) shows that all CHHs are preceeded by a CHH-Precursor-Related Peptide (CPRP). The identities between CPRPs and the signal peptides of the two species are low (33% and 24%, respectively) when compared to the identity between the CHH hormones (61%).

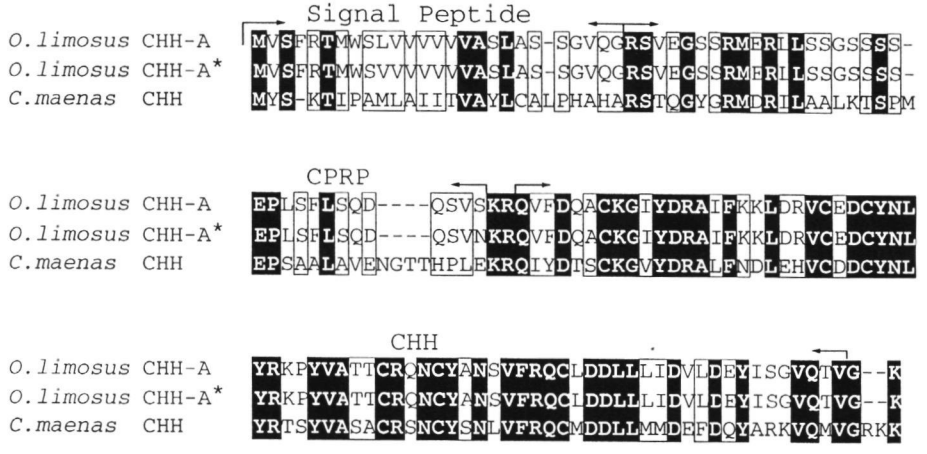

Fig. 2 Comparison between *Orconectes limosus* preproCHH-A, *Orconectes limosus* preproCHH-A* and *Carcinus maenas* preproCHH. Sets of identical amino acid residues in the three preprohormones are indicated in black and conservative subsitutions are boxed.

The size and expression of CHH mRNA in different tissues of intermoult animals was determined by Northern analysis of poly (A^+) RNA isolated from gonad, green gland, heart, hepatopancreas, MT, tail muscle and ventral nervous system. This analysis (Fig. 3) showed only one band in the MT with an approximate size of 2.4 kb, in accordance with the sizes of the CHH-A and -A* encoding cDNAs (2409 bp and 2548 bp, respectively).

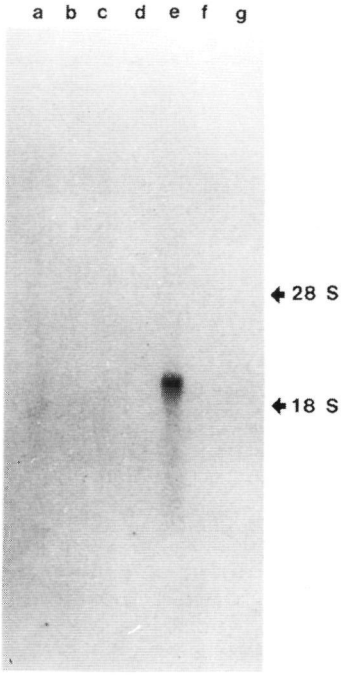

Fig. 3 Northern blot analysis of poly (A^+) RNA of gonads (a), green gland (b), heart (c), hepatopancreas (d), MT (e), tail muscle (f) and nerve system (g) from the crayfish *Orconectes limosus*. The blot was hybridized with a cRNA probe (corresponding to nucleotides 41-344 of preproCHH-A mRNA in Fig. 1). The autoradiogram was exposed for 16 h at -70°C with two intensifying screens. The positions of the 28S and 18S RNA bands are indicated.

Quantification of CHH-A and -A* encoding mRNAs and CHH neuropeptide-isoforms in the X-organ sinus gland system of individual animals

To examine at which level preproCHH gene expression is regulated, we compared the levels of the two preproCHH mRNAs (coding for identical CHH peptides) in the MT of individual animals with the levels of CHH peptide isoforms in the sinus gland of the same animal. The respective organ structures were separately dissected from each of the two eyestalks of an individual animal and used in an RNase protection assay and micro HPLC analysis.

By the use of specific CHH-A and CHH-A^* cRNA probes in the RNAse protection assay, a

Table 1. Quantification of the levels of CHH-A and -A* encoding mRNA and CHH neuropeptide isoforms in the eyestalks of individual crayfish.

Quantification of CHH mRNAs (encoding prepro CHH-A and -A*) and CHH peptide isoforms (CHH I and II) in an individual animal by an RNase protection assay and micro HPLC analyses, respectively. Data of the eight animals are shown. Peak areas of CHH I and II are given in Arbritary Units (A.U.)

Animal	CHH-A mRNA	CHH-A* mRNA	A/A* mRNA	CHH I peptide	CHH II peptide	I/II peptide
	pg	pg		AU	AU	
1	0.06	0.37	0.16	30.2	8.9	3.4
2	0.12	0.38	0.32	28.5	9.1	3.1
3	0.02	0.29	0.07	23.2	6.3	3.7
4	0.02	0.28	0.07	16.7	5.8	2.9
5	0.09	0.47	0.19	29.6	9.0	3.3
6	0.06	0.50	0.12	25.6	9.4	2.7
7	0.07	0.60	0.11	25.3	7.2	3.5
8	0.03	0.19	0.15	38.9	11.4	3.4

protected fragment of the expected size was found for both mRNAs in the eyestalks of all individual animals (Table 1). CHH-A*-encoding mRNA was found to be the most abundant mRNA in these animals. The micro-analytical SMART system made it possible to separate the different CHH HPLC isoforms in individual crayfish These isoforms are termed CHH-I and -II as shown in a typical microchromatogram of crayfish sinus glands extracts (Fig. 4). Quantitative evaluation of the chromatographic analyses showed that the CHH-I neuropeptide is the most abundant CHH isoform (Table 1). The levels of both mRNAs and peptides are very different between animals. In contrast, the ratio between the CHH-I and -II peptides is constant while the ratio between CHH-A and A* mRNAs differs, indicating the existence of a regulatory mechanism at the translational or post-translational level.

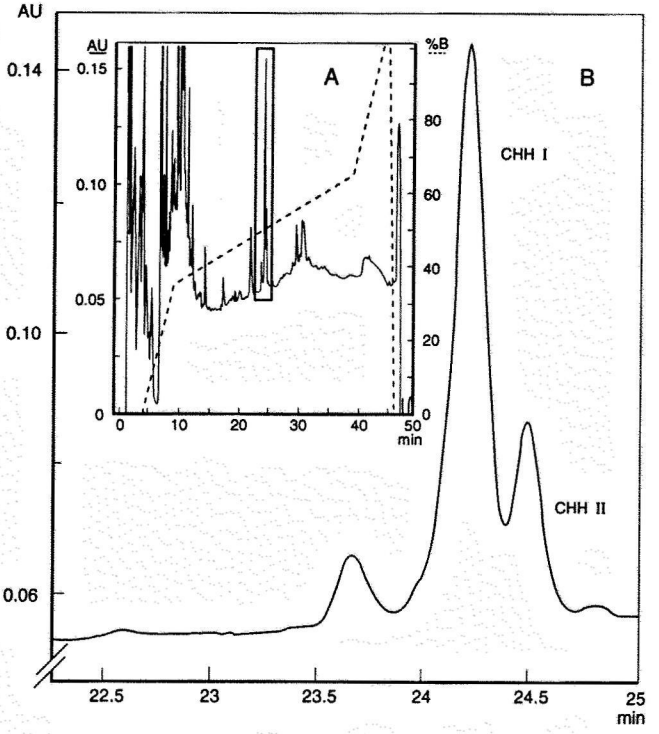

Fig. 4 HPLC microchromatogram of sinus gland extracts from the crayfish *Orconectes limosus*. A: chromatogram showing the elution pattern of the neuropeptides from two sinus glands; B: chromatogram of the magnified elution pattern corresponding to the framed region in Fig. 4A by application of the SMART system, Pharmacia/LKB, equipped with the μ RPC C_2/C_{18} reversed phase column; elution buffer: A = 0.1% TFA in water; B = 0.1% TFA in 80% acetonitril/20% water.

Southern blot analysis

Genomic DNA (15 μg) was isolated from three tail muscles and digested with a number of restriction enzymes (Fig. 5). The genomic blot was hybridized with a cRNA probe corresponding to nucleotides 41-344 of CHH-A mRNA (Fig. 1) which according to PCR

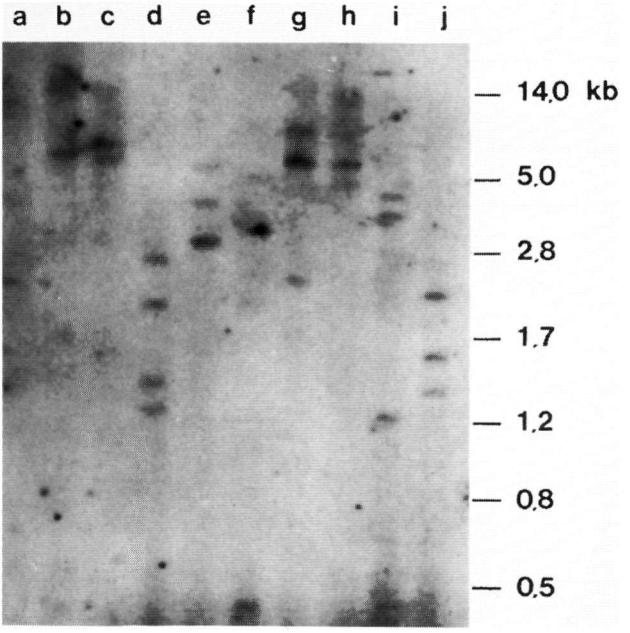

Fig. 5 Southern blot analysis of genomic DNA from the crayfish *Orconectes limosus*. The genomic DNA (15 μg) was digested with ApaI (a), BamHI (b), EcoRI (c), HindIII (d), PstI (e), PvuII (f), XbaI (g), XhoI (h), XbaI/ApaI (i) and XbaI/HindIII (j). The blot was hybridized with a cRNA probe (corresponding to nucleotides 41-344 of preproCHH-A mRNA in Fig. 1). The autoradiogram was exposed for two weeks at -70°C with two intensifying screens.

experiments on genomic DNA does not span an intronic sequence (data not shown). The restriction enzymes used do not cut in this cDNA fragment. Several bands, varying in size from 0.4 to 8.0 kb, are detectable in each of the lanes revealing the presence of at least two preproCHH genes in the crayfish. Furthermore, Table 1 shows that both CHH-encoding mRNAs are expressed in an individual animal. This finding, combined with the results of the Southern blot analysis (Fig. 5), indicates that the characterized CHH- encoding mRNAs are the result of the expression of two genes originating from a duplication of an ancestral preproCHH gene. Expression of the duplicated genes results in the production of two slightly different preproCHH encoding mRNAs each coding for an identical CHH, which is then modified to the other CHH isoform by post-translational modification.

In conclusion, we cloned two full-length cDNAs encoding two preprohormones for slightly different CPRPs but identical CHHs. Both mRNAs are highly expressed in the MTs of

individual animals. Quantification of both mRNAs and neuropeptide isoforms in individual animals together with Southern blot analysis indicates that the existence of the two isoforms of the CHH neuropeptide is due to a post-translational modification event of identical CHHs originating from two genes.

GENERAL DISCUSSION

GENERAL DISCUSSION

Molting and reproduction are processes which dominate most of the lifetime of nearly all crustaceans. Especially female crustaceans should molt only when the environmental conditions are favorable for growth and should reproduce only at times favorable for egg release and larval survival. For this, the animals have a rather complex neuroendocrine system and several regulatory pathways intervening during molting and reproduction.

At the start of this work, the paradigm of decapod molt regulation is that a molt-inhibiting hormone (MIH) from the lobster X-organ sinus gland complex in the eystalk suppresses the biosynthetic activity of the molting glands (the Y-organs). Once this inhibition is stopped, the Y-organs synthesize and secrete ecdysteroids, initiating the biochemical and physiological changes that result in ecdysis. Environmental factors influencing molting act probably through the central nervous system to control the synthesis and release of MIH. Thus, molting in crustaceans is often considered a relatively straightforward process involving two hormones, one a molt-inhibiting neuropeptide, the other a molt-promoting steroid (Aiken & Waddy, 1980). However, crustacean molting physiology is more complicated and requires optimal tuning of the endocrine mechanisms regulating reproduction.

For the reproduction process it was described that, up to the end of previtellogenesis, the regulation of oogenesis in female crustaceans is only partially under neurohormonal control (for review see Van Herp, 1992) but details on the mechanisms were still missing. Studies have shown that vitellogenesis is mainly controlled by neurohormones like the gonad-inhibiting hormone (GIH), inhibiting the onset of vitellogenesis (Soyez et al., 1987). Furthermore, indications for the existence of a vitellogenesis-stimulating hormone (VSH), probably orginating from the thoracic ganglia, have been reported (Otsu, 1960; Takayanagi et al., 1986). Crustacean hyperglycemic hormone (CHH), produced in the X-organ, was found to exert a stimulating effect on oocyte growth (Tensen et al., 1989). In addition, vitellogenin-stimulating ovarian hormone (VSOH), produced by the ovary (Meusy & Charniaux-Cotton, 1984), and methyl-farnesoate (MF) are apparently also involved in reproduction (Laufer et al., 1986).

As described in this thesis, several studies on lobster CHH-A and -B suggest that the

CHHs are multifunctional hormones which, besides their hyperglycemic effect, are also involved in reproduction and molting. Moreover, besides its function in reproduction, GIH may also be involved in molting. The postulated involvement of CHH and GIH in the reproduction and molting of female American lobsters is illustrated in Figure 1. Molting inhibition in these animals may be regulated not by one, but by three peptides, namely CHH-A, CHH-B and GIH. Several studies point to this view. For instance, Chang et al.

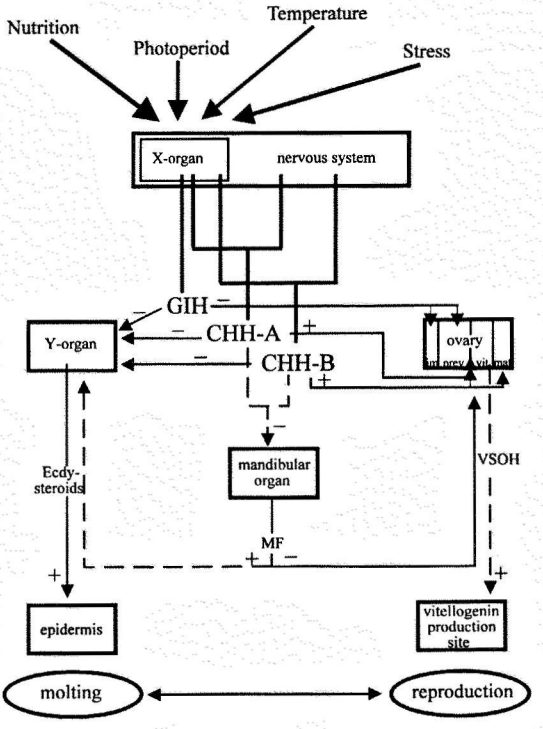

Fig. 1. Illustration of the proposed role of the CHH-family (CHH, GIH) in the regulation of molting and reproduction in the female lobster *Homarus americanus*. + indicates a stimulating effect of the hormone on the target organ whereas - indicates inhibition.
⟶ represents effects found in *Homarus americanus* ⇢ represents effects found in other crustacean species; im, immature; prev, previtellogenesis; vit, vitellogenesis; mat, mature.

(1989) described a peptide with both molt-inhibiting and hyperglycemic activity in the lobster and it turned out to be CHH-A (Tensen et al., 1991e). Furthermore, besides in the eyestalks, both CHHs are also expressed in other parts of the nervous system (Chapter 1). Eyestalk ablation eliminates not only GIH but also MIH. Suprisingly, lobster females mostly react on such an operation by the induction of vitellogenesis and not of molting. This indicates that molting induction is suppressed by an additional factor from the nervous system (Aiken & Waddy, 1980) which points to a molt-inhibiting role for CHH expressed in the thoracic ganglia of the lobster. The putative molt-inhibiting function of GIH is based on the high degree of amino acid sequence identity between mature lobster GIH and crab MIH, and the similar structural organization of their preprohormones (Chapter 2). Moreover, in female lobsters molting probably occurs only when CHH and GIH hemolymph levels are low (Chapter 5). Together, this indicates that GIH is the molt-inhibiting factor during the immature stages of reproduction and CHHs during the mature stages of reproduction. The presence of CHH-A and -B in the eyestalks and in other parts of the nervous system already points to a stimulatory function for CHH during oocyte growth (Chapter 1). Moreover, in a heterologous bioassay for oocyte growth, lobster CHH-B has a stimulating effect (Tensen et al., 1989). Furthermore, Chapter 5 shows high CHH hemolymph levels in animals with ovaries in the mature stage and indications were found that CHH-A and -B are responsible for the initiation of vitellogenesis. Thus, the two CHHs and GIH are probably three neuropeptides involved in the regulation and synchronization of the molting and reproduction cycles.

MF, produced in the mandibular organ (MO), seems to be involved in the regulation of reproduction in both sexes (Laufer et al., 1993a) but its effect varied among crustaceans. In the hemolymph of the American lobster, it was found that MF levels were high when vitellogenin was low while MF dropped to undetectable levels when vitellogenin levels were high (Tsukimura et al., 1992). *In vitro*, MF stimulates ecdysteroid secretion from the Y-organ of the crab *Cancer magister* (Tamone & Chang, 1993). However, this is in conflict with studies showing that high MO activity has been found in the reproductive stages, in which MF levels in the blood of vitellogenic females of other crustacean species are high (Laufer et al., 1986; Sagi et al., 1993; Vogel & Borst, 1989). Interestingly, it was

recently reported that CHH of the crayfish *Procambarus clarkii* inhibits the release of MF from the mandibular organ (Laufer et al., 1993b) which suggests another, thus far unknown function for CHH.

In conclusion, results in this thesis combined with earlier experiments (Otsu, 1960; Aiken & Waddy, 1980; Takayanagi et al., 1986) point to a central role for CHHs and GIH in the regulation of molting and reproduction. However, our studies dealt mainly with female American lobsters although members of the CHH-family are also present in male lobsters (Chapter 3) and larvae (Chapter 4), and many other crustacean species (Chapter 6; Keller, 1992). Our findings can therefore be a basis for future research on the role of the CHH-family in the regulation of molting and reproduction in male and female crustaceans. Moreover, as wild catches of American lobsters have declined rapidly during the last two years, lobster farming becomes again interesting not only from an ecological but also from an economical point of view. For this, our knowledge on the regulation of growth and reproduction is essential to allow rearing of lobsters in aquaculture systems.

In lobster, we have establised the occurrence of polymorphism and the functional significance of the CHH-family (Tensen et al., 1991c; Soyez et al., 1991; this thesis). In other crustaceans, such as the fresh water crayfish and the sea water crab *Carcinus maenas*, this neuropeptide family is also present but the pattern of polymorphism is somewhat different. In the crayfish *Orconectes limosus*, two CHH-immunoreactive groups occur (Tensen, 1991e). The first group consists of two isoforms for which the amino acid sequences of the stored neurohormones (Kegel et al., 1991) and the nucleotide sequences of the preprohormones were elucidated (Chapter 6). These two isoforms are the most potent hyperglycemic hormones (Tensen, 1991e) while the second immunoreactive group seems to be involved in inhibition of molting (Von Glyzinsky, 1994). Comparable results were obtained for the crayfish *Procambarus bouvieri* (Huberman & Aquilar, 1988; Huberman & Aquilar, 1989). In this species, two prominent isoforms with hyperglycemic activity were found. In addition, two minor peptides were detected, one having a molt- inhibiting and the other a gonad-inhibiting effect (Huberman et al., 1993). The amino acid sequences of these peptides are very similar to those of the prominent isoforms. Thus, fresh water crayfishes appear to have two potent hyperglycemic isoforms and two additional peptides

probably involved in molting and reproduction, and highly related to the isoforms. For the shore crab *Carcinus maenas*, the existence of only one CHH-immunoreactive group has been reported. This group consists of a major and a minor product for which no differences in amino acid compositions could be demonstrated (Kegel et al., 1989). Furthermore, for the crab, the amino acid sequence of only one CHH preprohormone has been described and one MIH peptide have been found (Weidemann et al., 1989; Webster, 1991). MIH displayed a low degree of amino acid sequence identity to CHH. Finally, a CHH-like peptide involved in the regulation of ion and fluid absorption in the insect digestive system was recently reported (Audsley et al., 1994), indicating that the CHH-family members are not restricted to crustaceans but probably are typical for Arthropoda, the phylum containing the largests number of species in the animal kingdom.

SUMMARY

SUMMARY

In general, animals use distinct environmental cues for timing of their reproduction, development and growth. They have a wide range of nervous structures for perception of the signals and employ a complex (neuro)endocrine system for transduction of the messages to endocrine glands. These glands produce factors regulating the activity of organs involved in the three aforementioned processes. Survival depends on optimal regulation of each step in the life cycle of the animal.

Arthropoda form a group of animals (phylum) which is divided into smaller groups (subphyla and classes) and includes the groups of insects (*Insecta* or *Hexapoda*), spiders (*Arachnida*) and crustaceans (*Crustacea*). Instead of bones (like in vertebrates), all animals belonging to this phylum contain a hard external crust as exoskeleton. In order to grow, these animals have to change this external crust by a process called molting. In crustaceans molting and reproduction are cyclic processes, occurring in the same life period. Therefore, crustaceans have developed their own strategies and have adapted their molting, reproduction and development cycles to the environment using appropiated control mechanisms for optimal tuning. As such they avoid molting when they are brooding otherwise they lose their progeny. In addition, the processes of molting and reproduction need large amounts of energy and are probably not succesfull when they occur simultaneously.

Knowledge of the regulation of growth and reproduction is essential to allow rearing of crustaceans in aquaculture systems. Since the number of wild catches of American lobsters has declined rapidly in the last two years, lobster farming becomes interesting again, not only from an ecological but also from an economical point of view. Therefore, study of the regulation of molting (growth) and reproduction is not only interesting for fundamental science but can also be used in applied research, e.g. in aquaculture.

The study described in this thesis concerns the crustacean hyperglycemic hormone (CHH)-family in crustaceans and is mainly focussed on the CHH-family in the American lobster *Homarus americanus*. The neurohormones of this family are involved in the regulation of physiological processes like glucose metabolism, reproduction and growth, and are synthesized in the neuroendocrine system located in the eyestalks of these animals. They

are released from the neurohemal region, the sinus gland, into the blood through which they can reach their target organs and regulate the aforementioned physiological processes. At the start of this study, information was available concerning the amino acid sequences of a number of mature CHH peptides and one CHH-preprohormone. Determination of their physiological effects was mostly studied by heterologous bioassays which showed not only the hyperglycemic effect of CHH (increase of glucose levels in the blood) but also a possible stimulating effect on gonad growth and an inhibiting effect on molting. These results indicate not only the multifunctionality of CHH but also the complexity of the regulation of molting and reproduction. The elucidation of the amino acid sequences and the physiological characterization of two other members of this novel peptide family, namely the gonad-inhibiting hormone (GIH) and the molt-inhibiting hormone (MIH), did not show multifunctionality for these hormones as found for CHH. In a heterologous bioassay, it was found that GIH inhibits the onset of yolk protein uptake in a stage of the reproductive cycle called vitellogenesis, resulting in another name, the vitellogenesis-inhibiting hormone (VIH), for the same hormone. MIH is involved in the inhibition of the molting process.

Using molecular biological techniques, Tensen (1991e) started a study of CHH gene expression in crayfish and lobster, but at that time no specific cDNA probes for the different neuropeptides were available. This study was extended in the present thesis using molecular biological techniques in combination with micro-HPLC analysis, immunocyto-chemistry and ELISA.

The first two chapters of this thesis deal with the elucidation of the preprohormone structures of CHH-A and CHH-B (Chapter 1), and GIH (Chapter 2) in lobster. Both preproCHH-A and -B consist of a signal peptide, a CHH-precursor-related peptide and a highly-conserved CHH-peptide. Expression studies in the lobster revealed that the eyestalk is not the only source of CHH mRNA because the ventral nervous system also expresses this mRNA. These findings, together with earlier studies on the effect of eyestalk ablation, implantation of thoracic/abdominal ganglia as well as the multifunctionality of CHH, indicate that this hormone may be a good candidate for a supplementary role in the control of molting and reproduction. The elucidation of the preproGIH structure revealed that

lobster GIH together with crab molt-inhibiting hormone (MIH) belong to a separate group of the CHH neurohormone family. Expression studies showed that GIH mRNA is only expressed in the eyestalk. GIH may therefore regulate synthesis and release of hormones involved in molting and reproduction. Moreover, the high degree in amino acid sequence identity between preproGIH and preproMIH suggests that GIH itself could also be directly involved in molt inhibition.

The localization of cells producing lobster CHHs and GIH was studied in adult lobsters (Chapter 3) and larvae (Chapter 4) by immunocytochemistry and *in situ* hybridization. Both studies showed a colocalization of CHHs and GIH in some of the neurohormone-producing cells. The data on adults reveal that male lobsters contain an equal number of neuroendocrine GIH cells when compared to female lobsters, pointing to an important but unknown function for this hormone in males. In larvae, GIH/CHH-producing cells are already present and it was found that about 20 CHH-producing cells are present in larval stage I, whereas in adults the number is about 40 cells. This indicates that the importance of the CHH-producing system increases during the life cycle of adult lobsters.

Chapter 5 reports an introductory study dealing with the expression of CHH-A, CHH-B and GIH during the reproductive cycle of female lobsters. This study indicates a possible role for CHH-A and -B in the triggering of the onset of vitellogenesis and suggests a role for especially CHH-B during the last stages of oocyte maturation. The primary role of GIH was confirmed and, moreover, evidence for the function of GIH as a modulator synchronizing the molting and reproduction cycles in conjunction with CHH was strenghtened.

To compare the lobster data with a related species, two cDNAs encoding crayfish preproCHHs were isolated and sequenced as described in Chapter 6. This study demonstrates, besides the expected similarity with other CHHs, a possible duplication of the CHH gene. Northern blot analysis revealed that, in contrast to lobster CHH mRNA, crayfish CHH mRNA is detectable only in the eyestalk. Determination of the levels of preproCHH mRNAs and the amount of CHH peptide in the eyestalks of individual animals reveals that the ratio between the two preproCHH mRNAs varies among individuals while the ratio between the two CHH peptide isoforms does not differ among animals.

In the general discussion section, the possibility that CHH-A and -B, and GIH are involved

in molting and reproduction of a female American lobster is discussed. In female lobsters molt inhibition is regulated not by one, but by three peptides. Also, besides its gonad-inhibiting function, the molt-inhibiting function of GIH is discussed. Combined with other findings in crustaceans, the central role of CHHs and GIH in molting and reproduction is summarized in a model, showing the presumed regulation and synchronization of the molting and reproductive cycles in the female American lobster *Homarus americanus*.

SAMENVATTING

SAMENVATTING

Dieren gebruiken specifieke signalen uit de omgeving om te bepalen wanneer de omgeving geschikt is voor reproductie, ontwikkeling en groei. Ze hebben een grote verscheidenheid aan structuren in het zenuwstelsel om de signalen te ontvangen en maken gebruik van een complex (neuro)endocrien systeem om de boodschappen door te geven aan de endocriene klieren. Deze klieren geven factoren af die de activiteit reguleren van de organen die betrokken zijn bij de drie hiervoor genoemde processen. Overleving hangt af van een optimale regulatie van elke stap in de biologische cycli van de dieren.

De Artropoda (geleedpotigen) vormen een groep van dieren (phylum) die onderverdeeld is in verschillende kleinere groepen (subphyla en classes) waaronder de groep van insecten (*Insecta*), spinachtigen (*Arachnida*) en schaaldieren (Crustacea). In plaats van beenderen (zoals bij gewervelde dieren) hebben de geleedpotigen, een harde uitwendige korst als skelet. Om te groeien moeten deze dieren dit uitwendige skelet vervangen door een proces dat vervelling wordt genoemd. Bij schaaldieren zijn vervelling en voortplanting cyclische processen die zich in dezelfde levensperiode afspelen. Daarom hebben schaaldieren hun eigen strategieën ontwikkeld en hun vervelling-, reproductie- en ontwikkelingscyclus aan de omgeving aangepast, hierbij gebruikmakend van de juiste controle-mechanismen voor optimale afstelling. Hiermee stellen ze de vervelling uit als ze broeden omdat ze anders hun broedsel zouden verliezen. Tevens zijn vervelling en reproductie, processen die grote hoeveelheden energie kosten en zijn daarom waarschijnlijk minder succesvol wanneer ze tegelijkertijd optreden.

Kennis van de regulatie van groei en vervelling is essentiëel voor het kweken van deze schaaldieren in aquacultuur-systemen. Omdat de wildvangst van de Amerikaanse zeekreeft *Homarus americanus* in de afgelopen twee jaar sterk is gedaald, wordt het kweken van zeekreeft weer interessant, niet alleen vanuit een ecologisch maar ook vanuit een economisch gezichtspunt. Daardoor is de studie van de regulatie van de vervelling en reproductie niet alleen interessant voor fundamentele wetenschap maar de resultaten kunnen ook gebruikt worden voor toegepast onderzoek, b.v. in de aquacultuur.

De studie beschreven in dit proefschrift betreft de crustaceeën hyperglycemisch hormoon

(CHH) familie in kreeften en heeft zich vooral gericht op de CHH-familie in *Homarus americanus*. De hormonen behorende tot deze familie zijn betrokken bij een aantal fysiologische processen zoals suiker-metabolisme, voortplanting en groei, en worden geproduceerd in een neuroendocrien systeem dat bij deze dieren gelegen is in de oogstelen. Ze worden afgegeven aan het bloed door de sinusklier, het opslag- en afgifte-orgaan, via welke ze hun doelwit-organen kunnen bereiken en zo de hiervoor genoemde fysiologische processen kunnen reguleren.

Bij de start van deze studie waren aminozuurvolgordes bekend van een aantal "rijpe" CHH eiwitten en één CHH preprohormoon. De fysiologische effecten werden veelal bestudeerd met bioassays die niet alleen het hyperglycemisch effect van CHH lieten zien, toename van de glucose niveau's in het bloed, maar ook indicaties gaven voor een stimulerend effect op de gonade-groei en een remmend effect op de vervelling. Deze resultaten geven niet alleen het multi-functionele karakter van CHH aan, maar wijzen ook op de complexiteit van de regulatie van vervelling en voortplanting. Het ophelderen van de aminozuursequenties en fysiologische karakterisatie van twee andere leden van deze nieuwe eiwit-familie, namelijk het gonade-inhiberend hormoon (GIH) en het vervellings-inhiberend hormoon (MIH) liet zien dat deze hormonen in tegenstelling tot CHH niet zo multi-functioneel zijn. In een heterologe assay werd gevonden dat GIH de start van de dooiereiwit-opname remt in een stadium van de voortplantingscyclus dat vitellogenese wordt genoemd. Dit resulteerde in een andere naam voor hetzelfde hormoon, namelijk het vitellogenese-inhiberende hormoon (VIH). Het MIH is betrokken bij de remming van het vervellingsproces.

Met behulp van moleculair-biologische technieken startte Tensen (1991e) een onderzoek naar de expressie van het CHH-gen in de zeekreeft en zoetwaterkreeft. Op dat moment waren er echter nog geen specifieke cDNA probes voor de verschillende neuropeptiden beschikbaar. Dit onderzoek is voortgezet in het huidige proefschrift, gebruikmakend van moleculair-biologische technieken in combinatie met micro-HPLC analyse, immuuncyto-chemie en ELISA (enzyme-linked immunosorbent assay).

De eerste twee hoofdstukken van het voorliggende proefschrift hebben betrekking op de opheldering van de preprohormoon structuren van CHH-A en CHH-B (Hoofdstuk 1) en GIH (Hoofdstuk 2) in de zeekreeft. PreproCHH-A en -B zijn ieder samengesteld uit een

signaalpeptide, een CHH-precursor-verwant eiwit en een sterk geconserveerd CHH eiwit. Expressie-studies hebben laten zien dat de oogsteel niet de enige bron van CHH mRNA is, maar dat dit ook in het ventrale zenuwstelsel aanwezig is. Gecombineerd met de resultaten van eerdere studies betreffende het effect van de verwijdering van de oogsteel, implantatie van thoracale en abdominale ganglia als ook de multi-functionaliteit van CHH, lijken deze bevindingen erop te wijzen dat dit hormoon een goede kandidaat kan zijn voor een supplementaire rol in de controle van de vervelling en voortplanting. De opheldering van de preproGIH structuur maakte duidelijk dat GIH van de zeekreeft samen met het MIH van de strandkrab tot een aparte groep in de CHH-familie behoort. Expressie studies lieten zien dat GIH alleen tot expressie komt in de oogsteel, het centrale controle-centrum. GIH zou daarom de primaire factor kunnen zijn die de synthese en afgifte reguleert van hormonen betrokken bij de vervelling en voortplanting. Verder suggereert de grote overeenkomst in aminozuursequenties tussen preproGIH en preproMIH dat beide dezelfde factoren zouden kunnen zijn.

De lokalisatie van CHH- en GIH-producerende cellen in de zeekreeft werd bestudeerd in volwassen kreeften (Hoofdstuk 3) en in larven (Hoofdstuk 4) met behulp van immuuncytochemie en *in situ* hybridisatie. Beide studies lieten een colokalisatie van CHH en GIH zien in een aantal neurohormoon-producerende cellen. Uit de gegevens bleek tevens dat mannelijke kreeften een even groot aantal GIH-producerende cellen bevatten als vrouwelijke kreeften wat wijst op een belangrijke, maar tot nu toe onbekende, functie van dit hormoon in mannelijke kreeften. In larven zijn GIH/CHH-producerende cellen reeds aanwezig, waarbij gevonden werd dat er ongeveer 20 CHH-producerende cellen aanwezig zijn in larve stadium I terwijl in volwassen dieren dit aantal ongeveer 40 is. Dit wijst erop dat het CHH-producerende systeem belangrijker wordt gedurende het volwassen worden van de kreeften.

Hoofdstuk 5 rapporteert over een inleidende studie die betrekking heeft op de expressie van CHH-A, CHH-B en GIH in vrouwelijke kreeften gedurende een voortplantingscyclus. De studie wijst op een mogelijke rol voor CHH-A en CHH-B in het opstarten van het begin van de vitellogenese en suggereert een rol voor CHH-B in de laatste stadia van eicel-rijping. De fysiologische rol van GIH werd bevestigd. Bovendien werden aanwijzin-

gen gevonden dat GIH ook een functie heeft als modulator die de vervellings- en voortplantingscyclus synchroniseert in samenwerking met CHH.

Uit fylogenetisch oogpunt werd het onderzoek uitgebreid naar een verwante soort. Hiervoor zijn twee cDNAs coderend voor zoetwaterkreeft preproCHHs geisoleerd en gekarakteriseerd (Hoofdstuk 6). Deze studie liet, behalve de verwachte overeenkomst met andere CHHs, een mogelijke duplicatie van het CHH-gen zien. Northern blot analyse toonde aan dat, in tegenstelling tot de zeekreeft, CHH mRNA alleen detecteerbaar is in de oogsteel van de zoetwaterkreeft. Bepaling van de preproCHH mRNA niveaus en de hoeveelheid CHH-peptide in de oogsteel van individuele dieren lieten zien dat de verhouding tussen de twee preproCHH mRNAs varieert tusen de verschillende dieren, terwijl de verhouding tussen de twee CHH-peptide isovormen niet varieert.

In de algemene discussie wordt de mogelijkheid besproken dat CHH-A, CHH-B en GIH betrokken zijn bij de regulatie van vervelling en voortplanting in de vrouwelijke Amerikaanse zeekreeft. Remming van de vervelling in de vrouwelijke zeekreeft gebeurt niet door één hormoon maar door drie hormonen. Ook wordt naast de gonade-inhiberende functie, de vervellings-remmende functie van GIH besproken. Dit is tesamen met andere bevindingen in schaaldieren, samengevat in een model waarin de CHHs en GIH een centrale rol spelen in de regulatie en synchronisatie van de vervellings- en voortplantingscyclus in de vrouwelijke zeekreeft *Homarus americanus*.

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Nicky

CURRICULUM VITAE

Dominique de Kleijn werd geboren op 23 april 1962 in Wijchen. Na in 1980 zijn eindexamen HAVO behaald te hebben, begon hij aan de Nieuwe Lerarenopleiding te Tilburg. De tweede graads onderwijsbevoegdheid voor biologie en scheikunde werd behaald in 1986. Na de Nieuwe Lerarenopleiding werd de studie biologie voortgezet aan de Katholieke Universiteit van Nijmegen. In 1990 werd het doctoraalexamen afgelegd met als hoofdvak Dierfysiologie (bij Drs. T.A.Y. Ayoubi, Dr. G.J.M. Martens en Prof. Dr. E.W. Roubos) en als bijvak Ontwikkelingsbiologie der Dieren (bij Drs. C.P. Tensen, Dr. F. Van Herp en Prof. Dr. J.M. Denucé). Tevens werd een bijvak in Bonn, Duitsland gevolgd in het kader van het EG-uitwisselingsproject ERASMUS (bij Dr. F. Van Herp en Prof. Dr. R. Keller). Vanaf november 1990 tot november 1994 was hij werkzaam als AIO op de Afdeling Moleculaire Dierfysiologie (voorheen Zoologisch Laboratorium I) van de vakgroep Experimentele Dierkunde. In deze periode werd het onderzoek verricht dat in dit proefschrift is beschreven. Tijdens het promotie-onderzoek werden werkbezoeken gebracht aan laboratoria in Bonn, Duitsland (Prof. Dr. R. Keller), in Pensacola, Florida, USA (Prof. Dr. R. Rao) en in St. Andrews, New Brunswick, Canada (Dr. S. L. Waddy). Naast wetenschappelijk onderzoek werd een bijdrage geleverd aan het biologie onderwijs als assistent bij de cursussen Ontwikkelingsbiologie der Dieren en Zoölogie (deel: Evertebraten). Verder werden studenten uit Montpellier en Bonn begeleid die deelnamen aan de EG-uitwisselingsprogrammas (ERASMUS, COMETT) en doctoraalstudenten uit Nijmegen. Vanaf 1 november 1994 is hij werkzaam als post-doc op het Nederlands Instituut voor Hersenonderzoek te Amsterdam.