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# Cloning and expression of mRNA encoding prepro-gonad-inhibiting hormone (GIH) in the lobster *Homarus americanus*

Dominique P.V. de Kleijn*, Frank J.G.T. Sleutels, Gerard J.M. Martens, François Van Herp

*Department of Animal Physiology, Faculty of Sciences, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands*

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**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.

*Key words:* Gonad-inhibiting hormone; Neurohormone; cDNA sequence; Lobster; Medulla terminalis ganglionic X-organ

## 1. 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 [1] 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, and can also be called the gonad-inhibiting hormone (GIH) because it is produced in the eyestalk of male lobsters [2] and of lobster larvae [3], 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 [4], 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 [5]. However, this hypothesis was not supported after characterization of the CHH mRNAs of *Homarus americanus* and *Orconectes limosus* [6,7]. Until now, no amino acid or cDNA data has been 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 [6]. 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.

*Corresponding author. Fax: (31) (80) 652 714.

## 2. Materials and methods

### 2.1. 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 $\mu$g poly(A)$^+$ RNA was used to construct a cDNA library in the vector $\lambda$ZAP-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'-GGGAATTCGCNTGGTTYACNGAYA-AYGARTG-3') and 100 pmol of primer 2 (5'-CTRACRAARGTRT-GITACACCAA-3'; R = purine, Y = pyrimidine, N = any nucleotide, I = inosine). Both oligonucleotide sequences were based on the amino acid sequence of *Homarus americanus* GIH [4]. 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 s), 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 [8] and labeled with $^{32}$P by random priming according to standard procedures [9]. Hybridization of the replica nitrocellulose filters was performed at 42°C in 6 × SSC (1 × SSC contains 0.15 M NaCl and 0.015 M sodiumcitrate at pH 7.0), 1 × Denhardts solution, 25 mM sodium phosphate buffer, pH 7.0, 10% dextran sulphate, 200 $\mu$g yeast tRNA/ml and 50% formamide. After hybridization, the filters were washed in 2 × SSC, 0.1% sodium dodecyl sulphate (SDS) at 20°C for 10 min and subsequently washed in 2 × SSC, 0.1% SDS, 1 × SSC, 0.1% SDS and 0.25 × 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 ($\lambda$ZAP) 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 [10].

### 2.2. 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 [11]. 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 plasmid DNA. Labelling was performed in a final volume of 10 $\mu$l containing 50 $\mu$Ci [$\gamma$-$^{32}$P]UTP, 15 U T3/T7 RNA polymerase in transcription buffer (Promega),1 mM rATP,


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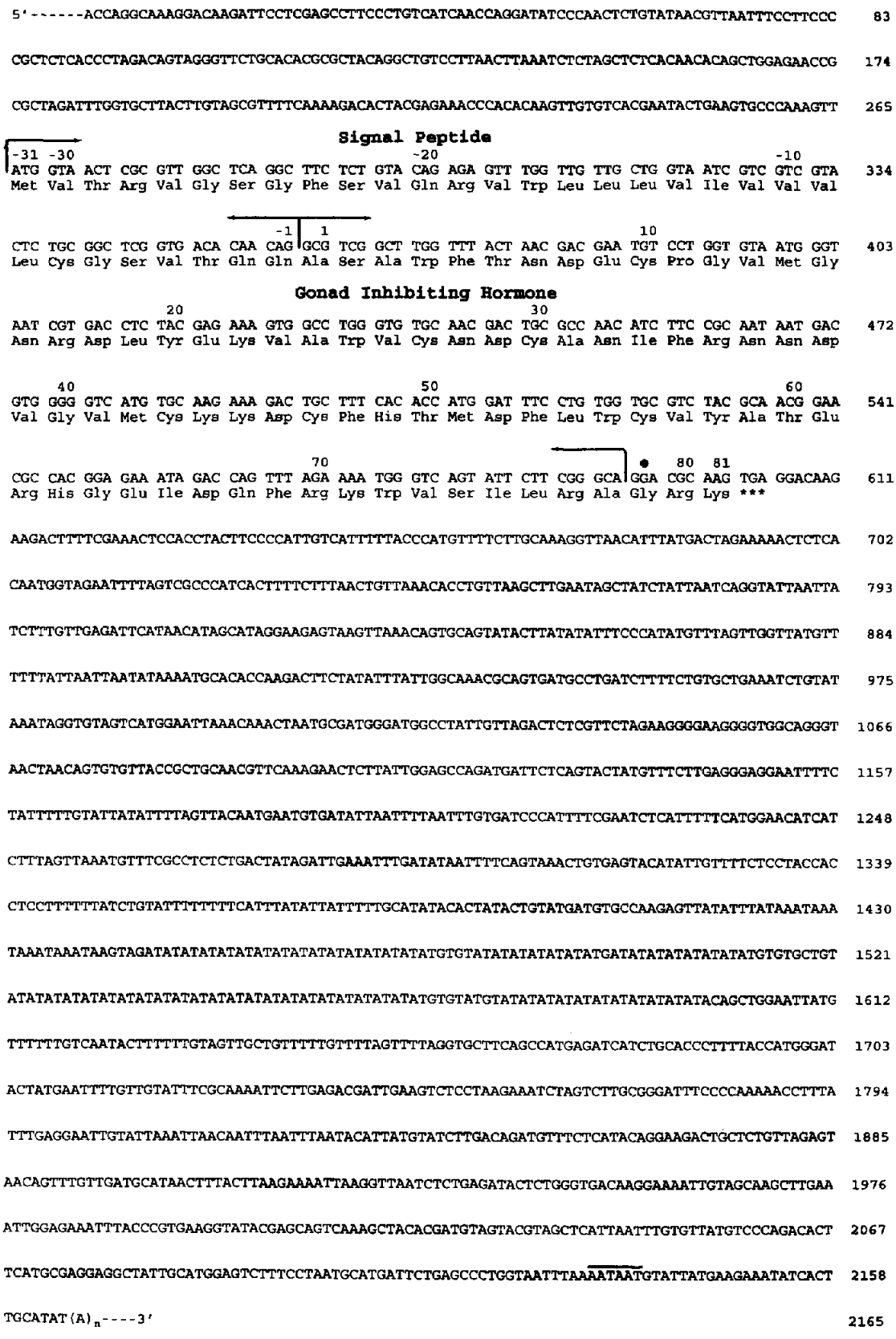


Fig. 1. Nucleotide sequence and deduced amino acid sequence of cDNA encoding preproGIH of the lobster *Homarus americanus*. Amino acid numbering starts at the N-terminal residue of the GIH hormone with the presumptive signal peptide sequence being indicated by negative numbering. The deduced amino acid sequence is shown below the nucleotide sequence. The dot above $Gly^{79}$ indicates a potential amidation site [16]. The putative signal for polyadenylation [17] is overlined.

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 h and the filter was washed until 0.25 × SSPE at 56°C. Total RNA of heart and hepatopancreas, and a *PstI* digest of λ DNA were used as size markers.

## 3. Results and discussion

### 3.1. 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 [4]. 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 [4]).

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 [5] should be reconsidered.

Aligment 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% [7]). 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 vs. 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.

### 3.2. 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 shown in Fig. 1 therefore most 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 [7]. This finding

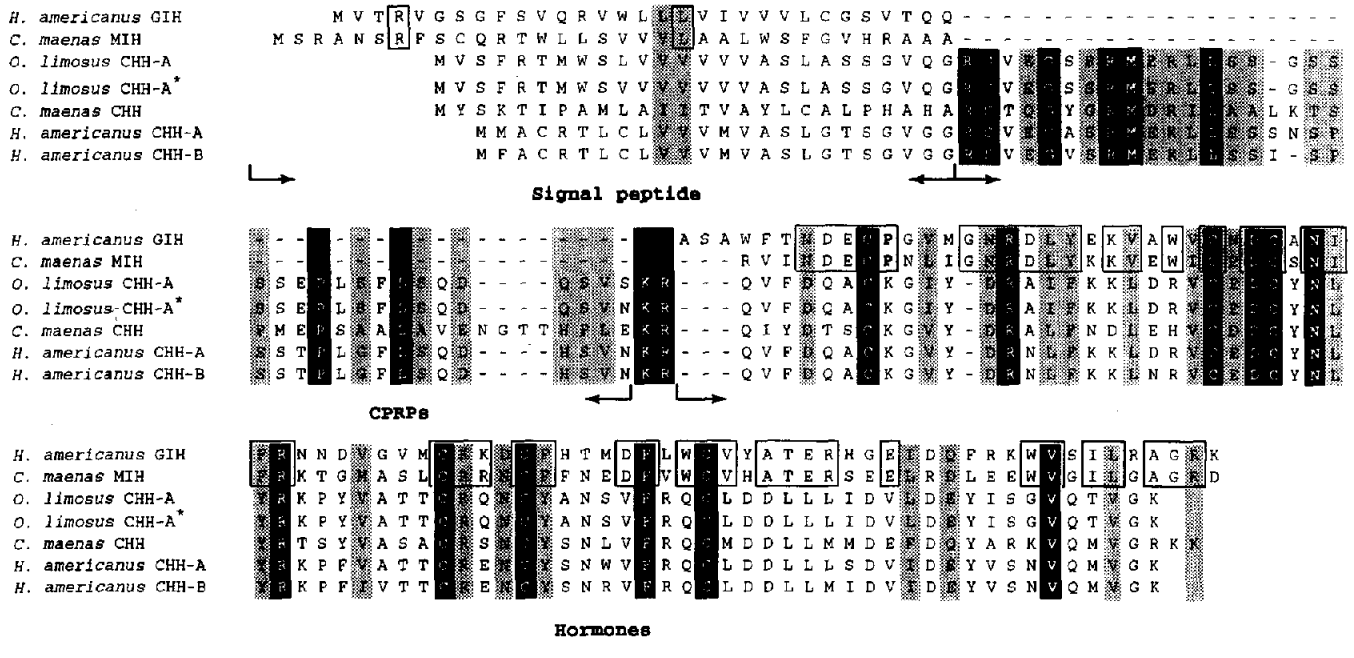


Fig. 2. Alignment of the preprohormones of *Homarus americanus* GIH, *Carcinus maenas* MIH [18], *Orconectes limosus* CHH-A, *Orconectes limosus* CHH-A* [7], *Carcinus maenas* CHH [19], *Homarus americanus* CHH-A, and *Homarus americanus* CHH-B [6]. Sets of identical amino acid residues and conservative substitutions between all preprohormones are indicated in black and grey, respectively. Sets of identical amino acid residues between preproGIH and preproMIH are boxed.

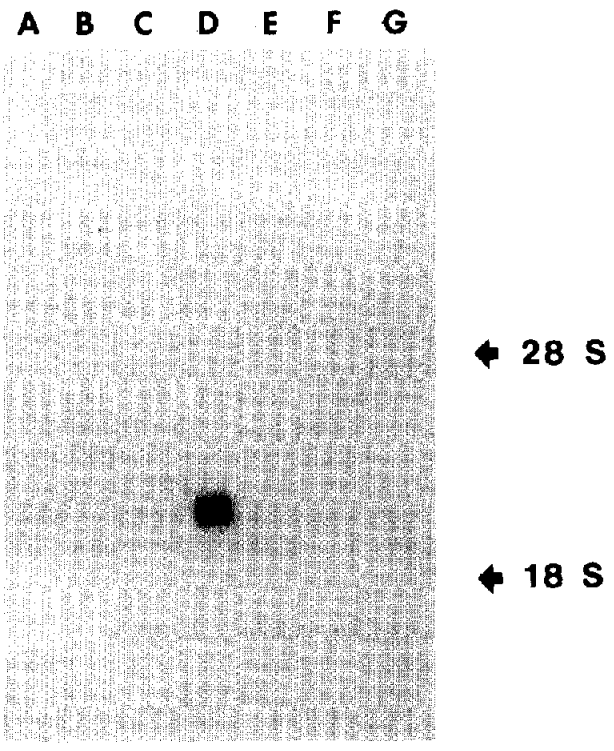


Fig. 3. Northern blot analysis of poly(A)$^+$ RNA from the ventral nervous system (lane A), testis (B), ovaria (C), medulla terminalis (D), hepatopancreas (E), heart (F) and green gland (G) of the lobster *Homarus americanus*. The blot was hybridized with a cRNA probe (corresponding to nucleotides 364–516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

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 the 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 [12–15]. 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 play 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*.

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