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Isolation and Characterization of a Novel Peptide Gene in the Lobster *Jasus edwardsii*

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Jenny G.I. Khoo and Frank Y.T. Sin (1999) Isolation and characterization of a novel peptide gene in the lobster *Jasus edwardsii*. *Zoological Studies* **38**(1): 95-109. Previous studies have demonstrated an immunological and functional relationship between vasopressins and the moult-inhibiting hormone (MIH) of crustaceans. Using primers derived from the rat vasopressin gene, 2 fragments were amplified from the genomic DNA of the lobster *Jasus edwardsii*. This paper describes the characterization of the 960-bp sequence. Northern blot analysis showed that the 960 bp sequence was expressed in the epithelia, eyestalk, heart, hepatopancreas, and muscle, but expression was most prominent in the eyestalk. In situ hybridization using the 960-bp sequence as a probe detected RNAs in the neurosecretory regions of the eyestalk. The expression of these genes appeared to be higher in the intermoult than the ecdysial eyestalk. Sequence analysis of the 960-bp PCR product revealed an intron/exon splice junction, a protein coding region with a stretch of repetitive sequences which is translated into a metallothionein-like protein. Furthermore, the 960-bp sequence also shared over 40% sequence homology to rat vasopressin, and MIH and CHH of some crustaceans. We called this sequence the Novel Peptide Sequence (NPS).

Key words: Lobster, Vasopressin-like gene, Moult-inhibiting hormone, Eyestalk, Metallothionein-like protein.

In crustaceans, the process of moulting is under the immediate control of the moulting hormone, 20-hydroxyecdysone (20-HE). The precursor to 20-HE is ecdysone which is synthesized and secreted by the moulting gland (Y-organs). The most widely accepted hypothesis concerning the control of moulting in crustaceans proposes that an increase in ecdysteroid synthesis, and consequently haemolymph titre, is necessary to initiate and sustain proecdysis. There also appear to be other factors, both inhibitory and stimulatory, that regulate moulting (for reviews see Aiken 1980, Chang 1985, Skinner 1985, Quackenbush 1986, Chang et al. 1993, Lachaise et al. 1993, Chang 1995). In decapod crustaceans, the synthesis and/or secretion of ecdysone by the Y-organs appears to be inhibited by the neuropeptide moult-inhibiting hormone (MIH) (Soumoff and O'Connor 1982, Mattson and Spaziani 1985a). MIH also acts by inhibiting 20-HE action in the epidermal tissue (Freeman and Bartell 1976). MIH originates in the eyestalk neuro-secretory cells ("X-organs") in the proximal ganglion of each eyestalk and is released from the sinus gland into the haemolymph (reviewed in Quackenbush 1986). Mattson and Spaziani (1985b) tested the effects of several vertebrate neuropeptides for possible MIH-like biological activity in vitro on cultured Y-organs from the crab *Cancer antennarius*. Peptides of the vasopressin-oxytocin family were shown to mimic MIH action by inhibiting Y-organ ecdysteroid production with relative potencies: lysine vasopressin (LVP) > arginine vasopressin (AVP) > vasotocin (VT) >>> oxytocin (OT). The effects of LVP and MIH activity from sinus gland extracts of the crab *C. antennarius* on cultured Y-organs were shown to be similar; i.e., having similar time courses, being reversible, and like MIH, LVP could

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induce significant increases in Y-organ cAMP content to 200%-250% of control levels (Mattson and Spaziani 1985a). A comparative study of the immunological properties of these peptides showed that MIH is most closely related to LVP (Mattson and Spaziani 1985b).

Vasopressin- and neurophysin-like peptides have been detected in the eyestalks of the prawn *Palaemon serratus* (Van Herp and Bellon-Humbert 1982) and in several invertebrates (Cruz et al. 1987, Proux et al. 1987, Reich 1992, van Kesteren et al. 1992, Oumi et al. 1994). Using PCR primers derived from the rat vasopressin gene, 2 fragments with approximate sizes of 560 bp and 960 bp were amplified from lobster DNA. This study describes the characterization of the longer fragment, called Novel Peptide Sequence (NPS). Sequence analysis of the 960-bp fragment suggested that it consisted of both coding and non-coding regions. In situ hybridization of this fragment to lobster eyestalk sections showed tissue-specific hybridization of this probe to the neurosecretory regions of the eyestalk. The expression of this gene was found to be higher during intermoult than in the ecdysial stage. Northern analysis, using the 960-bp fragment as a probe, detected 2 mRNAs, of 0.68 and 1.68 kb, expressed solely in the eyestalk.

MATERIALS AND METHODS

Isolation of lobster DNA

Live lobsters, *Jasus edwardsii*, were obtained from the Kaikoura coast in New Zealand. Animals were maintained in circulating seawater at 15 °C, on a diet of mussel, for up to several months. High molecular weight DNA (200 µg DNA/g tissue) was isolated from freshly dissected lobster gill tissue (McGinnis et al. 1983).

Southern blot analysis

The presence of vasopressin-like gene sequences in the lobster was detected by Southern hybridization to a plasmid clone containing the rat vasopressin gene (a gift from Dr. Evita Mohr of Universitätskrankenhaus Eppendorf, Hamburg, Germany).

Twenty micrograms each of lobster and rat genomic DNA were digested with 12 U and 5 U/µg, respectively, of *Bgl*II and *Eco*RI restriction enzymes (Boehringer Mannheim). The digested DNA was separated on a 1% agarose gel and then transferred by alkaline transfer (Amersham) to a nylon membrane (Zeta-Probe, Bio-Rad). The DNA was bound to the membrane by ultraviolet cross-linking.

Southern hybridization (Southern 1975) was carried out according to the manufacturer's instructions in 50% formamide, 0.25 M $NaHPO_4$ (pH 7.2), 7% (w/v) SDS, 1 mM EDTA (150 µl soln./cm^2) at 43 °C for 16-24 h, using 250 ng of nick-translated [α-^{32}P] dCTP radioactively labelled probe (10^6 cpm/µg). Probes were purified by separation on a P-60 sephadex column (Sambrook et al. 1989). After hybridization, the membrane was washed to a final stringency of 0.5 × SSC/ 0.1% SDS at 65 °C for 15 min or 0.1 × SSC/0.1% SDS at 65 °C for 15 min, and exposed to X-ray film. Southern blots were stripped of probe by washing the membranes in 0.1 × SSC/ 0.5% SDS twice for 20 min at 95 °C, and checked by overnight exposure to X-ray film.

Amplification of the vasopressin-like gene sequence by polymerase chain reaction (PCR)

Primers vaso 1, 5′-AACTGCCCAAGAGGA GGC-3′, and vaso 2, 5′-AGGGCCGCAGGG-

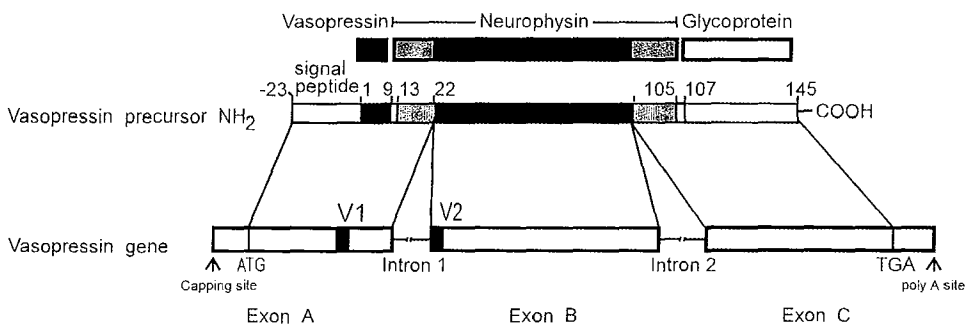

Fig. 1. Structural organization of rat arginine vasopressin precursor and its gene (Schmale et al. 1983). Locations of primers used in PCR are indicated as V1 and V2.

GAGACA-3′, were designed from the rat vasopressin gene sequence (Fig. 1; Schmale et al. 1983). Vaso 1 was based on the DNA sequence coding for amino acid residues 5 to 10; residues 5 to 9 are common to most of the peptides in the vasopressin-oxytocin family, and residue 10 is the glycine residue which is involved in the maturation process leading to the active hormone (Acher 1981, Richter 1987). Vaso 2 was the DNA sequence of the highly conserved part of exon B in the rat vasopressin gene (Fig. 1). These 2 primers amplify a 1239-bp fragment from the rat vasopressin gene.

Initial PCR was carried out on lobster DNA (0.5 μg) with varying $MgCl_2$ concentrations (0.5-5.0 mM) and annealing temperatures (45, 50, and 55 °C) in a 50-μl reaction volume containing 0.8 μM of each primer, 200 μM dNTPs, and 1 U *Taq* DNA polymerase in reaction buffer (Promega). As positive controls, 500 ng of rat liver DNA and 10 ng of the plasmid containing the rat vasopressin gene were included with each set of reactions. PCR was carried out in the Autogene II thermal cycler (Grant) for 30 cycles with the following cycling profile: 94 °C for 1 min, an annealing step at 55 °C for 1 min, and an extension step at 72 °C for 1 min, with a final extension step of 7 min. Ten microliters of PCR products was analyzed by electrophoresis on a 1.6% agarose gel, visualized by staining in ethidium bromide, and viewed over a UV transilluminator.

Re-amplification of PCR product

The 960-bp PCR product was gel purified (Koenen 1989) and re-amplified using the same primers, to obtain sufficiently pure PCR products which could be used as probes, and for sequencing and cloning. The PCR product was then gel purified using the Prep-A-Gene kit from Bio-Rad. The 960-bp PCR fragment was subsequently used as a probe for Southern and Northern blot analyses, and for in situ hybridization to the lobster eyestalk.

Between 10 to 100 ng of the 960-bp PCR product was random prime labelled with [α-^{32}P] dCTP (50 μCi, 3000 Ci/mmol) to 10^8-10^9 cpm/μg DNA using the random priming kit (NEBlot kit, Biolab). The probes were purified by separation on a P-60 sephadex column (Sambrook et al. 1989). Southern blot analysis of lobster and rat genomic DNA, previously probed with the rat vasopressin gene, was carried out using the 960-bp PCR product as a probe.

In situ hybridization of lobster eyestalk sections using NPS as a probe

The in situ hybridization methods used were modified from previous studies (Pardue 1985, Bloch et al. 1986, Zeller et al. 1989, Chesselet 1990, Leitch et al. 1994). Precautions were taken to avoid RNase contamination (Sambrook et al. 1989).

Eyestalks were removed from a lobster which had been previously chilled on ice for 40 min. The overlying cuticle and muscle were dissected from the eyestalks in ice cold crustacean saline (224 mM NaCl, 7.51 mM KCl, 12.5 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.97 mM $MgSO_4$, 4.44 mM H_3BO_3 adjusted to pH 7.5 with NaOH; Midsukami 1979). The dissected eyestalks were fixed in freshly prepared 4% paraformaldehyde at 4 °C for 50-60 min, washed twice in phosphate-buffered saline (PBS), pH 7.2, and then left overnight in 15% sucrose in PBS at 4 °C.

The tissue was mounted in O.C.T. compound (Tissue-Tek) and snap frozen in liquid nitrogen vapor. Sixteen-micron sections were cut at – 26 °C, in a Starlet *2212* Cryostat, and sections were collected on warm gelatin-chrome alum-subbed slides (0.5% gelatin [bloom 300], 0.5% chrome alum), dried for 2 min on a hot plate (60 °C), and stored at – 80 °C.

In situ hybridization of lobster eyestalks using random prime labelled 960-bp PCR product ([α-^{32}P] dCTP, 3000 Ci/mmol, to 10^8 cpm/μg of DNA) was carried out as described (Bloch et al. 1986). Approximately 500 000 cpm or 1-5 ng of probe was applied on each slide in a volume of 20 μl of hybridization buffer per 20 × 22 mm^2 area; tissue sections were overlaid with acetone-washed, siliconized (Sigmacote) glass coverslips. Hybridization was carried out for 16 h at 42 °C.

Following hybridization, the coverslips were washed off with 4 × SSC, and slides were soaked in 2 × SSC for 10 min and 45 min at room temperature. The slides were then soaked in 1 × SSC at 40 °C for 10 min, then in fresh SSC for 45 min. The slides were dehydrated in 70% ethanol and then in 2 changes of absolute ethanol. Air-dried sections were initially exposed to X-ray film for 0.5-16 h, and subsequently coated with Ilford LM-1 liquid emulsion (diluted with 0.5 volumes of distilled water) at 43 °C, air dried, and stored in the dark at 4 °C with desiccant for 1-4 d (approximately 28 times the exposure to X-ray film). The slides were developed with Kodak D-19 developer; sections

were stained in Ehrlich's haemotoxylin stain (Merck), and mounted for observation.

Extraction of poly $(A)^+$ RNA

Eyestalks were ablated from live lobsters obtained from the Pacifica Kaikoura Ltd. Fisheries at Kaikoura, and stored in liquid nitrogen (approximately 10 mo). Total RNA was extracted from eyestalks, epithelial tissue, gill, heart, hepatopancreas, and abdominal flexor muscle by the acid guanidinium thiocyanate-phenol-chloroform extraction procedure of Chomczynski and Sacchi (1987). Poly $(A)^+$ RNA was extracted from total RNA using the polyATtract mRNA isolation system from Promega.

Northern hybridization of mRNAs

One to three micrograms of poly $(A)^+$ RNA from each tissue was analyzed by Northern blotting (Sambrook et al. 1989, Liu and Chou 1990, Rosen and Villa-Komaroff 1990, Lee et al. 1992). Heat-denatured *Hin*dIII-digested λ DNA was included as molecular size markers. Fractionated poly$(A)^+$ RNA was transferred by capillary action in 10 × SSC onto a nylon membrane (Hybond-N, Amersham) for 16 h according to Sambrook et al. (1989). Post-hybridization washes were carried out as follows: 2 × SSC/0.1% SDS at 42 °C for 2, 5, and 15 min, followed by 0.2 × SSC/0.1% SDS at 60 °C for 3 × 15 min.

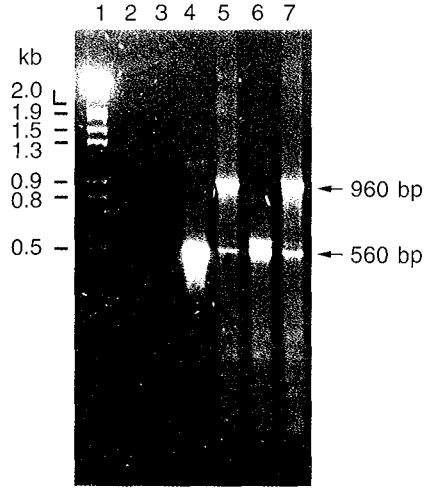

Fig. 2. PCR re-amplification of the 560-bp band (lanes 4 and 6) and 960-bp band (lanes 5 and 7). These samples were re-amplified using DNA eluted from the 560-bp PCR product and 960-bp PCR product isolated from separate PCR reactions. Re-amplification of the 560-bp PCR fragment sometimes produced 2 bands (lane 6), whereas re-amplification of the 960-bp PCR fragment produced 2 bands at 960 bp and 0.56 kb (lanes 5 and 7). Lane 2 contains no sample, and lane 3 is the negative control for PCR.

Sequencing of the NPS fragment

PCR products were partially sequenced from both ends using the ds cycle sequencing kit (BRL), in a Perkin Elmer DNA thermal cycler 480. Based on the partial sequences, internal primers were constructed to amplify an inner fragment of NPS to generate a sequence of 880 bp length. PCR products were subcloned into the *Sma* I restriction site of pBS m13 + using T-A cloning (Sambrook et al. 1989, Finney 1994). The entire sequence was then obtained by a combination of ds sequencing using T7 DNA polymerase sequencing (Pharmacia LKB) with [α-^{32}P] dCTP (3000 Ci/mmol), and automated sequencing. The final sequence was confirmed by automated sequencing using an internal primer.

The DNA sequence was analyzed using the HIBIO DNASIS software system (Pharmacia LKB Biotechnology AB), to examine for open reading frames, predicted protein coding regions based on the theory developed by Fickett (1982), and to look for DNA sequence similarity to other sequences present in the EMBL and GenBank nucleotide databases. The DNA sequence was also translated into putative proteins in 6 reading frames, and these were tested for similarities to known protein and DNA sequences using the BLAST algorithm (Altschul et al. 1990).

RESULTS

Amplification of a vasopressin-like sequence

PCR amplification of lobster genomic DNA was performed using 2 primers derived from the rat vasopressin gene at different $MgCl_2$ concentrations and annealing temperatures (45, 50, and 55 °C). Two predominant bands were produced which showed electrophoretic mobilities similar to the 947-bp and 564-bp bands of the λ molecular weight markers on agarose gel. These fragments were re-amplified and gel purified (Fig. 2).

PCR amplification of the rat genomic DNA and the rat vasopressin gene clone produced a single band, 1.2 kb long, which, when cleaved with restriction enzyme *Bgl*II, gave 2 bands, 0.69 and 0.58 kb, as predicted by the sequence (Schmale et al. 1983). However, sequence analysis of the

lobster NPS fragment revealed no *Bgl*II sites.

Southern blot analysis to detect vasopressin-like sequences

In Southern blot analysis of lobster and rat genomic DNA, the NPS fragment hybridized to a 6.5-kb band, and 2 other bands which were faint on the autoradiogram, at 4.8 kb and 3.6 kb in size, in the *Bgl*II digestion of lobster DNA (Fig. 3B). The NPS fragment also hybridized to undigested lobster DNA, observed as high molecular weight DNA near the gel wells, whereas no hybridization to rat genomic DNA was detected (Fig. 3B). The rat vasopressin gene however, hybridized to 2 bands at 4.5 and 2.3 kb in the *Eco*RI digestion of lobster genomic DNA in that particular membrane (Fig. 3A), and these bands remained when the stringency was increased (results not shown). The rat vasopressin gene also hybridized to several bands in the *Bgl*II digestion (6.1, 4.8, 1.6, and 0.5 kb) and *Eco*RI digestion (9.4 and 2.8 kb) of rat genomic DNA (Fig. 3A).

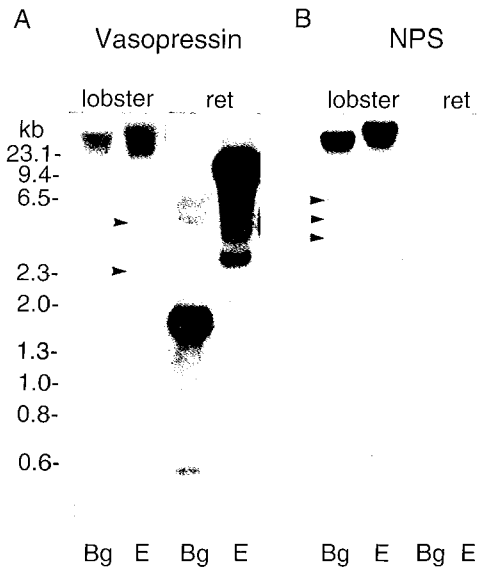

Fig. 3. Southern blot analysis of genomic DNA from the lobster and rat. Twenty micrograms of genomic DNA was digested with *Bgl*II (B) or *Eco*RI (E). (A) Hybridization to the rat vasopressin gene DNA probe, pBS 13^{+}, using a final post-hybridization wash in 0.5 x SSC/ 0.1% SDS at 65 °C for 15 min was used. The autoradiogram was exposed for approximately 9 d, at -70 °C. ▶, locations of the 2.3-kb and 4.5-kb bands. (B) Southern blot shown in panel a was stripped and hybridized to the NPS probe, using a higher final wash stringency (0.1 × SSC/ 0.1% SDS at 68 °C for 30 min). The autoradiogram was exposed for 10 d at -70 °C. Arrows denote the positions of the hybridization bands.

In situ hybridization of lobster eyestalk

To determine whether the NPS fragment contained gene sequences which were expressed in the eyestalk, the site of production of crustacean MIH, the NPS fragment was used as a probe for in situ hybridization to lobster eyestalk sections. The NPS fragment was found to hybridize to specific regions in the eyestalks of the intermoult lobster. These regions included the neurosecretory cells of the medulla terminalis, medulla interna, medulla externa, and lamina ganglionaris (Table 1). The NPS fragment was localized over the nuclei of densely packed, small neurosecretory cells (X-organs) above the medulla externa, at the junction between the medulla interna and terminalis, and the neurosecretory cells of the medulla terminalis (MTXO) and medulla terminalis ganglionaris X-organs (MTGXO) (Fig. 4A-C, E, F). Probe localization was also seen in the nuclei of some cells above the lamina ganglionaris (results not shown), and over the nuclei of the larger neurosecretory cells adjacent to the X-organs of the medulla terminalis (Fig. 4B, C).

Expression of the NPS fragment in the ecdysial and intermoult stages

To compare the expression of the NPS fragment between the intermoult (C_4) and ecdysial stages (D_4; Lachaise et al. 1993), in situ hybridization of the PCR product to eyestalk sections, prepared from a lobster at the intermoult stage and from a lobster which was preparing to moult, was carried out (ecdysial-presence of a new exoskeleton underneath the outer cuticle). The fixation and hybridization of eyestalk sections from intermoult and ecdysial lobsters were carried out simultaneously; eyestalk sections from ecdysial and intermoult lobsters were placed on either half of the glass slide. Following exposure of the slides to liquid emulsion, the slides were developed simultaneously.

In the ecdysial lobster eyestalk section, the NPS fragment probe hybridized to similar regions as previously observed in the intermoult eyestalk section. However, significantly lower densities of silver grains were seen over the neurosecretory regions of the ecdysial lobster eyestalk as compared to the intermoult eyestalk, as well as in the medulla terminalis (Fig. 4C, D) and sinus gland (Fig. 4F, G) (paired *t*-test; $p < 0.0001$). Estimates of the average number of silver grains over similar positions within the neurosecretory regions of the

intermoult and ecdysial lobster eyestalk section are summarized in Table 1.

Northern blot analysis using the NPS fragment as a probe

Northern hybridization using the NPS fragment as a probe detected 2 bands (1.68 and 0.68 kb) in poly $(A)^+$ RNA purified from the eyestalk (Fig. 5, lane 2). The NPS fragment also hybridized to 2 bands (1.8 and 0.3 kb) in poly $(A)^+$ RNA from the heart, and 3 bands from the hepatopancreas (6.0, 2.2, and 0.90 kb) and muscle (5.3, 1.75, and 0.4 kb) (Fig. 5, lanes 4-6). The presence of all these different-sized mRNA bands suggests that the NPS fragment could be detecting different mRNAs. The intensity of the signal was darker in the eyestalk compared to the other tissues, despite the fact that the amount of poly $(A)^+$ RNA loaded was approximately half in the case of the eyestalk (eyestalk: 1.44 μg < muscle: 2.43 μg < hepatopancreas: 2.7 μg). The size of the RNA bands was estimated by comparison to the sizes of single-stranded λ DNA markers run on the gel; hence the sizes (kb) given are only estimates.

Hybridization bands on the autoradiogram were analysed semi-quantitatively by measuring the optical density of each band with a scanner (AAB, Advanced American Biotechnology, Fullerton, California). The autoradiogram was scanned using 3 different sensitivity settings to establish a linear relationship between the band intensity and scanner sensitivity. Readings were normalized to 1-μg quantities of poly $(A)^+$ RNA, and expressed as a percentage of the 0.68-kb eyestalk band for that particular sensitivity (Table 2). The relative intensities of the darkest bands in the other tissues as compared to the 0.68-kb eyestalk band were: heart, 77%; muscle, 43%; hepatopancreas, 41%; and to the 1.68-kb eyestalk band, 33% of the 0.68-kb eyestalk band (Table 2).

Sequence analysis of the NPS fragment

The nucleotide sequence and deduced amino acid sequence of the cloned NPS fragment are shown in Fig. 6. By sequencing, the length of NPS was found to be 960 bp. The DNA sequence of NPS showed several interesting features. (a) There was a possible splice junction, with the consensus sequence (GT-AG) (Mount 1982) at possible exon-intron boundaries in reading frame 3+ (highlighted in Fig. 6). This corresponded to the protein coding region prediction, conducted on DNASIS, which suggested nucleotides 3-134 and 240-533 could include a protein coding region (containing a stop codon, TAA), whereas nucleotides 534-960 was non-coding (algorithm calculations based on the theory developed by Fickett 1982).

(b) There was a stretch of repetitive sequences from nucleotides 249 to 440; the sequence AGTTGTAGTGTAGATGTATGCTGT which coded for amino acids Ser-Cys-Ser-Val-Asp-Val-Cys-Cys was repeated 8 times. A sequence similarity search of the nucleotide and protein sequence databases on the NCBI Blast E-Mail Server (National Center for Biotechnology Information, USA) was done. The repetitive sequence, when translated in reading frame 3+, showed similarity to a whole range of metal-lothionein protein sequences (62% of the 100 homologous entries).

Table 1. Comparison of the average number of silver grains in a 64-μm^2 quadrat, taken from the neurosecretory regions of the medulla terminalis, medulla externa, and tissue designated as sinus gland. Silver grains were counted at a 1000 × magnification, in 10 random quadrats within a 10 × 10 eyepiece micrometer grid, placed over the neurosecretory region of the eyestalk. The background level of silver grains was counted from tissue adjacent to the neurosecretory region

Probe	Tissue region	Intermoult eyestalk		Ecdysial eyestalk		Paired *t*-test[a]
		n.s region	background	n.s region	background	
NPS	Medulla terminalis	96.6 ± 22.7	10 ± 4.5	65.6 ± 12.6	4.9 ± 4.3	***
	Medulla externa	89.8 ± 17.6	16.7 ± 7.6	38.6 ± 13	3.2 ± 3.5	***
	Sinus gland	104.7 ± 12.5	22.2 ± 6.8	44.5 ± 8.3	2.8 ± 1.2	***

[a]Paired *t*-test comparison of the number of silver grains in the neurosecretory regions (n.s.) of the intermoult and ecdysial lobster eyestalk sections.

*** $p < 0.0001$.

Sequence homology search

When compared to the rat vasopressin gene, the NPS fragment shared 42.6% sequence identity over a 954-bp overlap, with the region flanked by the 2 PCR primers on the rat vasopressin gene. This was not unexpected as this region within the rat vasopressin gene contained mostly intron sequences. This would account for the absence of hybridization signals when a post hybridization wash with high stringency was used (Fig. 3B). Comparison of coding sequences of the NPS fragment and the rat vasopressin gene showed 46.5% sequence identity in a 303-bp overlap (Fig. 7).

Comparison of the NPS sequence with published cDNA sequences of other crustacean eyestalk neuropeptides, putative MIH, and crustacean

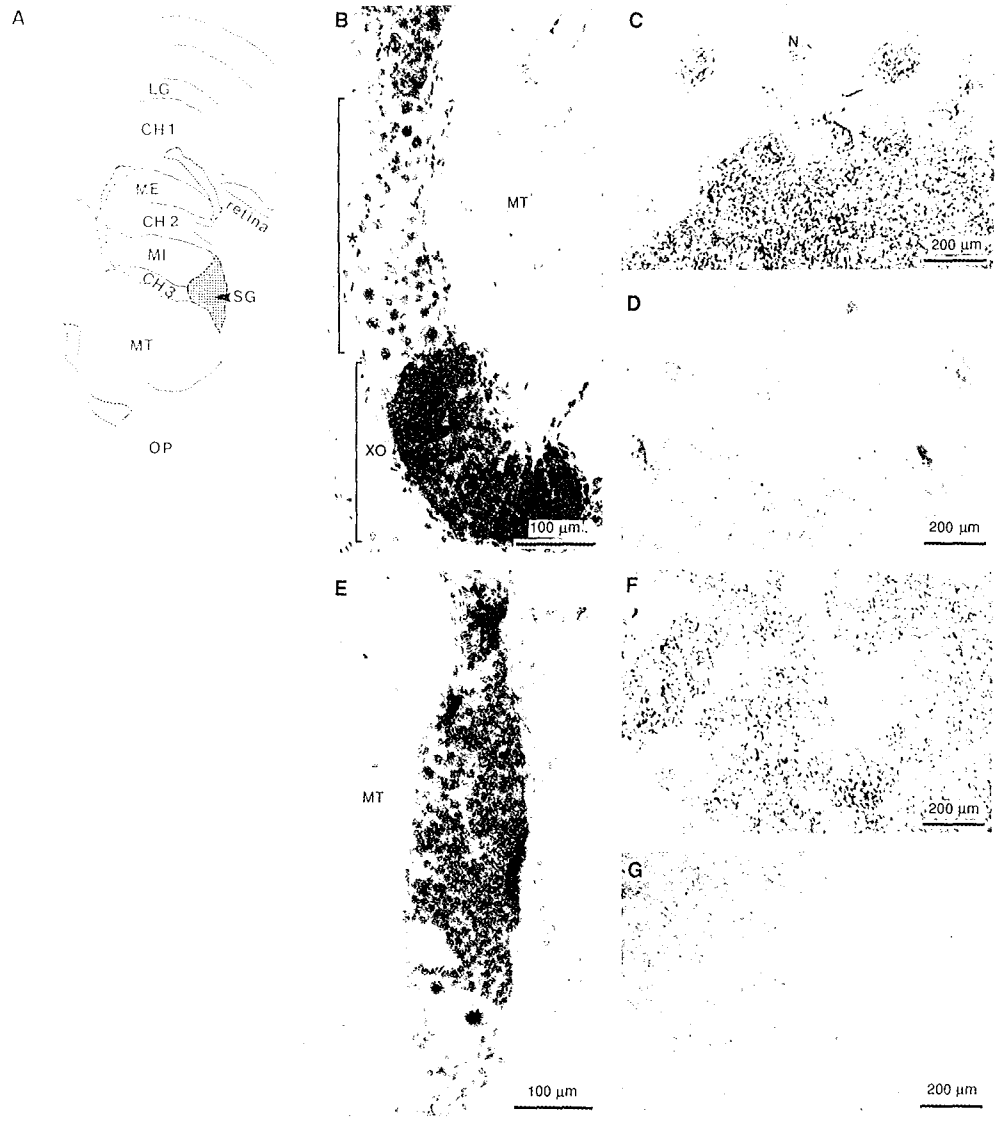

Fig. 4. In situ hybridization of NPS to the eyestalk of *Jasus edwardsii*. (A) Diagram of the structures identified in a longitudinal section of the lobster eyestalk showing the neurosecretory regions where probe localization was observed (shaded areas). LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; CH 1,2,3, chiasma 1,2,3; SG, sinus gland; OP, optic peduncle. (B, C--enlarged detail) In the medulla terminalis of the lobster at the intermoult stage, the NPS probe hybridized to the nucleus and cytoplasm of the small, densely packed cells of the MT ganglionaris X–organs (XO), and mainly to the nucleus (N) of the large neurosecretory cells (*). (E, F--enlarged detail) Probe hybridization is also clearly visible in the region between the MT and MI, possibly the sinus gland. In a lobster at the premoult stage, probe hybridization was significantly reduced in the neurosecretory region of the MT (D), and in the sinus gland (G). These areas correspond to the ones shown in C and F (intermoult stage).

hyperglycemic hormone (CHH), suggested that they may be related. The NPS fragment shared 46%-48% homology over a > 338-bp overlap with the MIH cDNA sequences isolated from the shrimp, *Penaeus vannamei* (Sun 1994), and shore crab, *Carcinus maenas* (Klein et al. 1993b), respectively, whereas the complementary sequence of the NPS fragment shared 44% homology over a 917-bp overlap to the CHH A cDNA sequence from the crayfish, *Orconectes limosus* (de Kleijn et al. 1994).

A comparison of the NPS coding sequence (428 bp) with the coding sequences of the crustacean eyestalk neuropeptides showed 45%-48% homology over a > 350-bp overlap with MIH from *P. vannamei* and *C. sapidus*, and 49% homology over a 297-bp overlap with MIH from *C. maenas*. The deduced amino acid sequence encoded by the repetitive segment of the NPS sequence was also compared with the amino acid sequences of other crustacean eyestalk neuropeptides, the CHH/MIH/VIH peptide family (reviewed in Keller 1992). In this family, the 6 invariant cysteine residues are located in the same positions, and conserved amino acid residues are located in the vicinity of the 6 cysteine residues (Sun 1994, de Kleijn and Van Herp 1995 [review]). Alignment of these sequences showed that the NPS-deduced amino acid sequence contained 4 of the 6 invariant cysteine residues (Fig. 8A).

There are, however, differences in the deduced amino acid sequences of the putative MIH peptides isolated from the 4 different species. The *P. vannamei*-deduced MIH sequence has 49% and 29% homology to *H. americanus* MIH, and *C. maenas* MIH, respectively, whereas both crabs, *Callinectes sapidus* (Lee et al. 1995) and *C. maenas*, share 78% sequence homology.

When translated into a putative protein, the NPS also showed consistent similarity to the metallothionein amino acid sequence isolated from several vertebrates. The homologous protein region was encoded by the repetitive sequence of the NPS. In this region, the putative amino acid sequence showed 46% sequence identity (19/41 amino acids) to the rat metallothionein amino acid sequence, and 41% and 39% sequence identities to the mouse and human metallothionein amino acid sequences, respectively (Fig. 8B). Comparisons of the nucleotide sequences showed 44%-46% sequence identity over a > 900-bp overlap for the MT-1 gene from the rat (Andersen et al. 1986) and mouse (Glanville et al. 1981) and metallothionein-1F gene from humans (Schmidt et al. 1985).

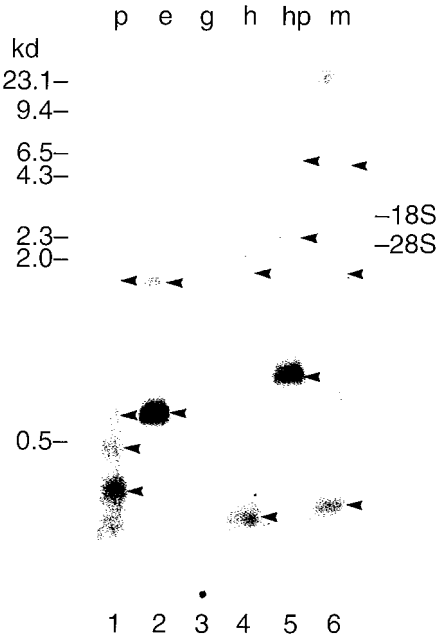

Fig. 5. Northern blot analysis of poly(A)$^+$ RNA isolated from several tissue types of the lobster, using the NPS probe. Poly(A)$^+$ RNA was isolated from the following tissue: **p**, epithelial; **e**, eyestalk; **g**, gill; **h**, heart; **hp**, hepatopancreas; and **m**, muscle. The Northern blot was washed in 0.2 × SSC/0.1% SDS at 60 °C for 3 × 15 min, and exposed for autoradiography for 7 d at –80 °C, with intensifying screens. ◀, indicates the locations of the bands.

DISCUSSION

Vasopressin-like gene sequences were initially

Table 2. Relative intensities of Northern hybridization bands. The autoradiogram was scanned using 3 different sensitivity settings. The optical density measurement of each band was normalized to 1 μg, and expressed as a percentage of the 0.68-kb eyestalk band (n = 3)

Tissue	Band size (kb)	Mean % ± s.d.
Eyestalk	1.68	32.7 ± 6.8
	0.68	100
Heart	1.80	24.5 ± 9.7
	0.30	76.9 ± 7.3
Hepatopancreas	6.0	12.6 ± 6.0
	2.2	14.5 ± 7.1
	0.9	41.1 ± 3.6
Muscle	5.3	16.3 ± 7.2
	1.75	12.6 ± 6.0
	0.40	43.0 ± 7.4

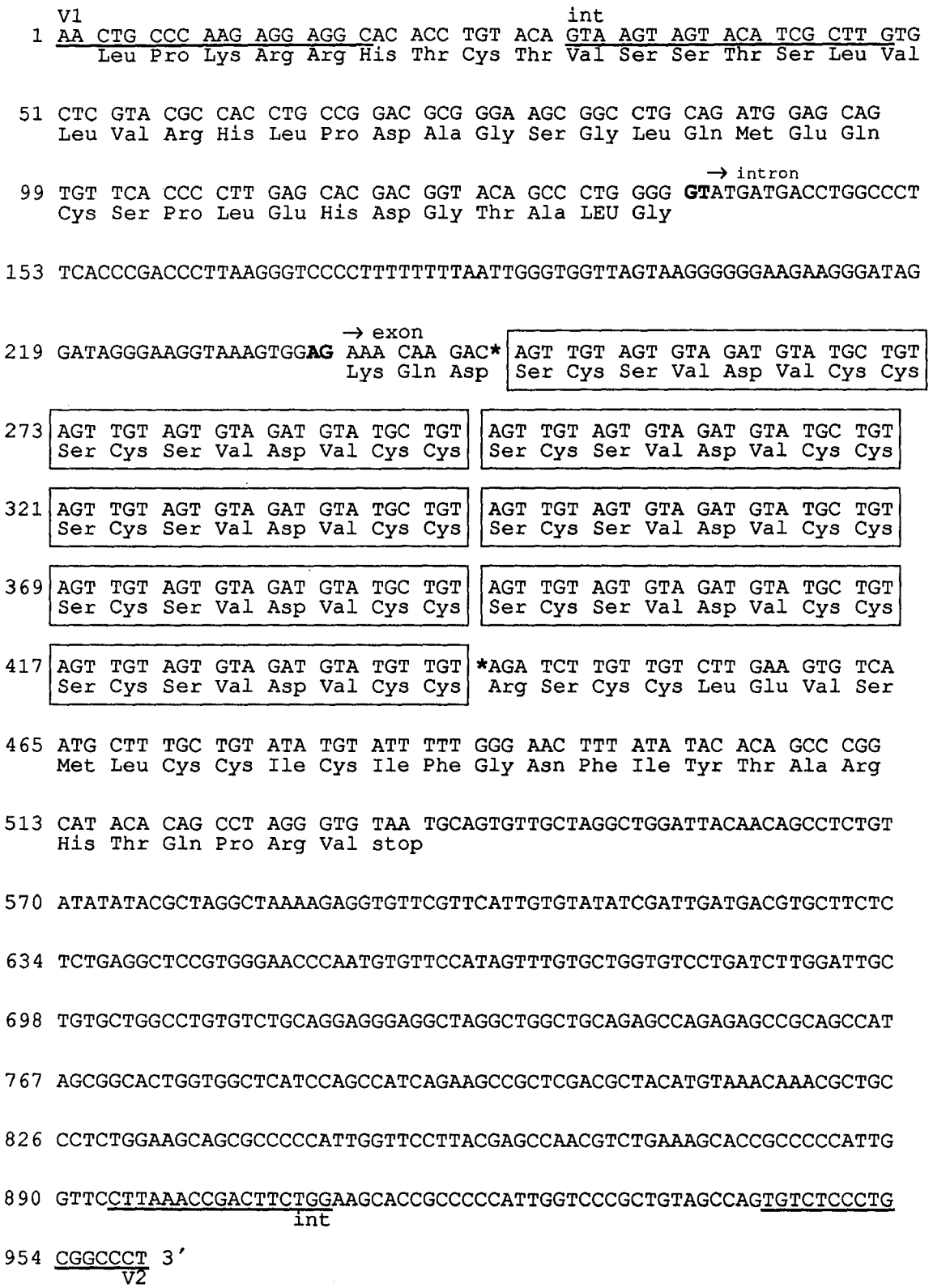

Fig. 6. Nucleotide sequence and deduced amino acid sequence of the NPS fragment. Positions of the PCR primers, V1 and V2, and internal primers, int, are underlined. Complementary sequences of the primers are indicated at the 3′ end. The beginning and end of the DNA repeats are indicated by *, with the repeat units boxed. Possible exon/intron boundaries are indicated by the GT-AG consensus sequences which are in boldface type. The sequence data has been deposited in the GenBank database (accession no. AF091369)

detected in lobster DNA by Southern blot analysis. Using PCR primers derived from the rat vasopressin gene, a 960-bp PCR product (NPS fragment) was amplified from lobster DNA. Sequence analysis of the NPS fragment revealed the presence of putative protein coding regions and an intron/exon boundary which suggested that this sequence was part of a structural gene.

Vasopressin, a nonapeptide, is a product of post-translational modifications of a larger precursor molecule (preprohormone), which consists of the peptide hormone, its carrier protein, neurophysin, and a glycopeptide of as yet unknown function (Richter 1983, Richter 1987). The principal functional domains of the hormone precursor are encoded by 3 distinct exons separated by 2 intervening introns (Fig. 1). The 1st exon (A) comprises the 5´-untranslated region, the putative signal peptide, the hormone vasopressin, and the N-terminus of neurophysin. The 2nd exon (B) encodes the highly conserved center part of neurophysin; and the 3rd exon (C) encodes the remaining C-terminus of neurophysin, the glycoprotein, and the untranslated 3´ region with the polyadenylation site (Richter 1987). Comparison of the NPS sequence and the coding region alone with the rat vasopressin gene sequence revealed no obvious sequence similarity.

Northern blot analysis, and in situ hybridization of lobster eyestalk sections confirmed that the NPS fragment contained protein coding sequences which were expressed in the epithelia, eyestalk, heart, hepatopancreas, and muscle tissue. However, expression of the gene was most prominent in the eyestalk.

Detection of mRNA in the eyestalk suggests that it could contain sequences coding for an eyestalk neuropeptide. Other neuropeptides known to be produced in crustacean eyestalks include the putative MIH in *P. vannamei* (Sun 1994), *C. maenas* (Dircksen et al. 1988, Klein et al. 1993a), and *H. americanus* (Chang et al. 1990); and CHH in *C. maenas* (Kegel et al. 1989), *O. limosus* (Kegel et al. 1991, de Kleijn et al. 1994), *H. americanus* (Tensen et al. 1991, de Kleijn et al. 1995) and the Mexican crayfish *Procambarus bouvieri* (Huberman et al. 1993). However, it has been demonstrated that MIH is also detected in the brain of *P. vannamei* (Sun 1995), and CHH has been detected in the ventral nervous system of *H. americanus* (de Kleijn et al. 1995). In the lobster *H. americanus*, the putative MIH, Hoa-MIH, has significant CHH activity (Chang et al. 1990). There is 90% identity between Hoa-MIH and

```
                  10         20         30         40         50         60
NPSCDS.SEQ     AACTGCCCAAGAGGAGGCACACCTGTACAGTAAGTAGTACATCGCTTGTGCTCGTACGCC
               X:::::::::::::::::: :   :  ::  :      :    :::    :  ::::  :
RATVPCDS.SEQ   AACTGCCCAAGAGGAGGCA-AGAGGGCCA-CATCCGACATGGAGCTGAGACAGGTACCAC
                     90        100        110        120        130
                      70         80         90        100        110
NPSCDS.SEQ     ACCTGCCGGACGCGGGAAGCGGCCTGCAGATGGAGCAGTGTTCACCCCTTGAGCA-CGAC
                  :: :   :  :^:v  : : : ::: : :: :: :  :::   ::  ::::: :  :
RATVPCDS.SEQ   TGTTGTCTCCCCTGCGGCCCTGGCGGCAAAGGGCGCTG-CTTCGGGCC--GAGCATCTGC
             140        150        160        170        180         190
             120        130        140        150        160        170
NPSCDS.SEQ     GGTACAGCCCTGGGGAAACAAGACAGTTGTAGTGTAGATGTATGCTGTAGTTGTA-GTGT
                :  : : :  :  :          :    : :  :  ::     ::::   :   : : :
RATVPCDS.SEQ   TGCGCGGACGAGCTGGGCTGCTTCCTGGGCACCGCCGA--GGCGCTGCGCTGCCAGGAGG
                200        210        220        230         240        250
              180        190        200        210        220        230
NPSCDS.SEQ     AGATGTATGCTGTAGTTGTAGTGTAGATGTATGCTGTAGTTGTAGTGTAGATGTATGCTG
               :::  ::  :::   : :   ::    : :   : : ::   : : : :   : :: : :
RATVPCDS.SEQ   AGAACTA-CCTGCCCTCGCCCTGCCAGTCTGGCCAGAAG-CCTTGCGGAAGCGGAGGCCG
                  260        270        280        290        300        310
               240        250        260        270        280        290
NPSCDS.SEQ     TAGTTGTAGTGTAGATGTATGCTGTAGTTGTAGTGTAGATGTATGCTGTAGTTGTAGTGT
                :   :   :  :   : ::::: ::  :  : : :: ::  ::    ::    :::::
RATVPCDS.SEQ   CTGCGCTGCCGCGGGCATCTGCTGCAG-CGATGAG-AGCTGCGTGGCCGAGCCCGAGTGT
                    320        330        340        350        360        370
               300        310
NPSCDS.SEQ     AGATGTATGCT
                ::
RATVPCDS.SEQ   CGAGAGGGTTT
                       380
```

Fig. 7. Comparison of the coding sequence of NPS (428 bp) with the rat vasopressin coding sequence (Schmale et al. 1983). Both sequences share 46.5% identity over a 303-bp overlap. The position of the PCR primer V1 is underlined.

Hoa-CHH (CHH B, Tensen et al. 1991). CHH is involved in the regulation of blood sugar levels and glycogen metabolism (reviewed in Santos and Keller 1993).

In situ hybridization of intermoult lobster eyestalk sections using the NPS fragment as a

A Alignment with crustacean eyestalk neuropeptides

```
                            1                               2   3                               5
NPS          49  C S V D V - C C S C   S V D V C C S C S V   D V C S - C S V D V   C C S C S V D V C C
Pev-MIH-like     D T F D H S C K G I   Y - D R E L F R K L   D R C E D C Y - N V   F R E P K V A T E C
Hoa-MIH       1 pE V F D Q A C K G V   Y - D R N L F K K L   D R C E D C Y - N L   Y R K P F V A T T C
Cam-MIH       1  R V I N D E C P N L   I G N R D L Y K K V   E W C E D C S - N I   F R K T G M A S L C
Cam-CHH       1 pE I Y D T S C K G V   Y - D R A L F N D L   E H C D D C Y - N L   Y R T S Y V A S A C
Hoa-CHH-B     1 pE V F D Q A C K G V   Y - D R N L F K K L   N R C E D C Y - N L   Y R K P F I V T T C
Orl-CHH       1 pE V F D Q A C K G I   Y - D R A I F K K L   D R C E D C Y - N L   Y R K P Y V A T T C

                       5                   6
NPS              S C S V D V C C S C   S V D V C C S C S V   D V C C R S C C L E   V S M L C C I C I F
Pev-MIH-like     K S N C F V N K R F   N V C V A D L R H -   D V S R F L K M A N   - S A L S
Hoa-MIH          R E N C Y S N W V F   R Q C L D D L L L S   N V I D E Y V S N -   V Q M V -NH2
Cam-MIH          R R N C F F N E D F   V W C V H A T E R S   E E L R D L E E W -   V G I L G A G R D
Cam-CHH          R S N C Y S N L V F   R Q C M D D L L M M   D E F D Q Y A R K -   V Q M V -NH2
Hoa-CHH-B        R E N C Y S N R V F   R Q C L D D L L M I   D V I D E Y V S N -   V Q M V -NH2
Orl-CHH          R Q N C Y A N S V F   R Q C L D D L L L I   D V L D E Y I S G -   V Q T V -NH2

NPS              G N F I Y T A R H T   Q P R V
```

B Alignment with metallothionein amino acid sequences

```
NPS          48  S C S V D V  C S C S V D V C C S C S V  D V C C S C S V D V
rat MT-I      5               C S C S T G G S C T C S S  S C G C K N C K - -
mouse MT-I    5               C S C S T G G S C T C T S  S C A C K N C K - -
human MT-IF   5               C S C A A G V S C T C A G  S C K C K E C K - -

NPS              C C S C S V D V C C S C S V D V C C S -  C S  98
rat MT-I         C T S C K K - S C C S C C P - V G C S K  C A  45
mouse MT-I       C T S C K K - G C C S C C P - V G C S K  C A  45
human MT-IF      C T S C K K - S C C S C C P - V G C S K  C A  45
```

Fig. 8. Alignment of the predicted amino acid sequence of the NPS repetitive sequence, with (A) other crustacean eyestalk neuropeptides, and (B) metallothionein amino acid sequences from other organisms. (A) Amino acid sequence of crustacean hyperglycemic hormone from *Carcinus maenas* (Cam-CHH, Kegel et al. 1989); *Homarus americanus* (Hoa-CHH-B, Tensen et al. 1991); and *Orconectes limosus* (Orl-CHH, Kegel et al. 1991). Amino acid sequences are of moult-inhibiting hormone from *C. maenas* (Cam-MIH, Webster 1991); *H. americanus* (Hoa-MIH; Chang et al. 1990, Chang 1995); and *Penaeus vannamei* MIH-like peptide (Pev-MIH-like, Sun 1994). The positions of the 6 invariant cysteine residues are indicated above the sequences. (B) Comparison with the amino acid sequence of metallothioneins: rat, Andersen et al. 1986; mouse, Glanville et al. 1981; and human, Schmidt et al. 1985. Identical amino acids are boxed.

probe showed that this PCR product contained sequences which were expressed in the neurosecretory cells of the medulla externa, interna, and terminalis, in retinal cells, and above the lamina ganglionaris. By testing various sections of the eyestalk neurosecretory system for MIH-like activity, Webster (1986) demonstrated that, in addition to the medulla terminalis, the medulla externa and medulla interna had a small effect on the inhibition of ecdysteroid synthesis by *Carcinus* Y-organs, suggesting that MIH may be produced in other parts of the eyestalk as well as in the medulla terminalis and the sinus gland. However, these results differed from the expression of putative MIH in the shore crab, *C. maenas*, in which putative MIH was detected at the protein and mRNA levels, in a subset of cells of the medulla terminalis and in the sinus gland (protein) using immunocytoche-mical and in situ hybridization techniques (Dircksen et al. 1988, Klein et al. 1993a).

The expression of genes detected by the NPS fragment was shown to be related to the moulting cycle; hybridization of this PCR fragment was significantly reduced in eyestalk sections taken from a lobster in the ecdysial stage of the moulting cycle, compared to an intermoult lobster. Durand (1956) found that the neurosecretory activity of type 2 cells in X-organs of *O. limosus* changed in relation to the moulting cycle, which led him to conclude that this cell type produces MIH. Dircksen et al. (1988) also found that MIH immunopositive perikarya consistently exhibited faint immunostaining in the eyestalk of premoult *C. maenas*. However, no striking moult cycle-related changes in the immunostainability of the *C. maenas* sinus gland for MIH and CHH were observed. It is possible that no moult cycle changes were detectable as the synthesized proteins were released into the haemolymph, whereas mRNA accumulated in the cytoplasm. Therefore, there might be no build-up of MIH in the eyestalk, before or after the moulting event.

The predicted amino acids of the coding region of the NPS fragment were compared with the amino acid sequences of known protein sequences in Genbank and of other crustacean eyestalk neuropeptides, the CHH/MIH/VIH peptide family (reviewed in Keller 1992). Low similarity was found with the CHH/MIH/VIH peptide family, but homology to several metallothionein sequences isolated from both vertebrate and invertebrate sources was more convincing. Particularly suggestive is the repetitive nature of the nucleotides 249-440 in the NPS fragment which predicts the amino acid sequence of Ser Cys Ser Val Asp Val Cys Cys repeated 8 times. This putative amino acid sequence contains a high proportion of cysteine residues which had a fixed distribution along the sequence (24 out of 63 amino acid residues), and it lacks the aromatic amino acids, Phe, Tyr, and Trp; these 2 features are characteristic of metallothionein proteins (Haurowitz 1963, Rainbow 1988).

Metallothioneins are metal-binding proteins which protect cells against excess concentrations of heavy metals. The gene is expressed at basal levels, but is induced to greater levels of expression by heavy metal ions (Bonwick et al. 1991, Lewin 1994, Radtke et al. 1995). Copper, bound in metallothioneins, is transferable to the respiratory pigment haemocyanin. In decapod crustaceans, haemocyanin levels vary during the moult cycle (Zuckerkandl 1960, Djangmah and Grove 1970 as cited in Rainbow 1988). Copper appears to be stored as a metallothionein complex in the hepatopancreas during the moult, and is subsequently released when new haemocyanin is synthesized (Engel et al. 1985, Brouwer et al. 1986 as cited in Rainbow 1988). In the blue crab *C. sapidus*, the expression of the metallothionein gene was estimated to be highest during intermoult and premoult, and lowest during the papershell stage (Engel 1987). Expression of the major eyestalk mRNA detected by the NPS sequence, however, was higher in the intermoult than in the ecdysial eyestalk.

The NPS fragment also detected a number of mRNA transcripts of varying sizes in the different tissues, which suggests that NPS could be detecting either products of tissue-specific alternate splicing of a common precursor, or related sequences. Most nuclear mRNA precursors (pre-mRNAs) in higher eukaryotes contain multiple introns which must be precisely excised by RNA splicing. Some pre-mRNAs are alternatively spliced in different cell types or at different times during development. Hence, regulated alternative splicing can lead to the production of different proteins from a single pre-mRNA, or can function as an on-off switch during development (Amara 1985, Maniatis 1991, McKeown 1992, López 1995).

In conclusion the nucleotide sequence of NPS suggests it could be evolved from the same ancestral gene as for the metallothionein protein, MIH, CHH, and vasopressin. Expression of this gene appears to be related to moulting in *J. edwardsii*. However, the precise physiological

function of this gene has yet to be elucidated.

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分離及描述一個龍蝦 *(Jasus edwardsii)* 新胜肽類基因

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過去的研究顯示甲殼類中的後葉加壓素與脫皮抑制激素之間有著免疫系統及功能上的相關性。利用由大鼠後葉加壓素基因序列所設計的引子，兩個片段可由龍蝦 (*Jasus edwardsii*) 基因組的脫氧核醣核酸被增殖到。這篇文章在描述對 960 bp 序列的分析。北方氏點漬法結果顯示此 960 bp 序列表現在上皮、眼柄、心臟、肝胰臟及肌肉，但是表現主要是在眼柄。利用 960 bp 序列為探針所進行的原位雜合反應可偵測到核醣核酸分布在眼柄之神經分泌的區域。這些基因的表現在已蛻皮的眼柄比蛻皮中的眼柄要高。對 960 bp 聚合酶連鎖反應產物序列分析的結果顯示有一表現序列 / 內子接合點，一蛋白質編碼區域具有一連串重覆序列並可轉譯出一金屬硫蛋白類似蛋白質。同時，此 960 bp 序列亦與大鼠後葉加壓素及一些甲殼類的 MIH ， CHH 基因有大於百分之四十序列上相似性。我們稱呼此序列為新胜肽序列 (NPS)。

關鍵詞：龍蝦，後葉加壓素類似基因，蛻皮抑制激素，眼柄，金屬硫蛋白類似蛋白質。

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