Molecular characterization and expression of mandibular organ-inhibiting hormone, a recently discovered neuropeptide involved in the regulation of growth and reproduction in the crab *Cancer pagurus*

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Methyl farnesoate, the crustacean juvenoid, is synthesized and secreted from the mandibular organs of crustaceans under the negative control of the sinus gland-derived mandibular organ-inhibiting hormone (MO-IH). Previously we isolated and sequenced two isoforms, MO-IH-1 and MO-IH-2, differing by just one amino acid, from sinus glands of the edible crab, *Cancer pagurus*. We now report the isolation of cDNAs encoding MO-IH-1 and MO-IH-2 by a combination of reverse-transcriptase-mediated PCR in conjunction with 5′ and 3′ rapid amplification of cDNA ends ('RACE'). Full-length clones of MO-IH-1 and MO-IH-2 encoded a 34-residue putative signal peptide and the mature 78-residue MO-IH sequences. Northern blot analysis of various tissues showed that MO-IH expression is confined to the X-organ (a cluster of perikarya within the eye). Southern blot analysis indicated that there are approx. 10 copies of the gene for MO-IH in *C. pagurus*. Additional Southern blotting experiments detected MO-IH-hybridizing bands in another *Cancer* species, *C. antennarius*. In support of this, an HPLC-radioimmunoassay analysis of sinus gland extracts of *C. antennarius* and *C. magister* also revealed MO-IH-like immunoreactivity.

Key words: methyl farnesoate, juvenoid, crustacean development.

INTRODUCTION

The development and reproduction of crustaceans are regulated by a combination of neuropeptide hormones, ecdysteroids (moulting hormones) and the isoprenoid methyl farnesoate (MF), the unepoxidized analogue of insect juvenile hormone III (reviewed in [1–3]). Although the physiological roles of MF have not been fully established, it has been proposed to have, in a manner analogous to that of juvenile hormone III in insects, a key role in the regulation of reproduction [3]. In support of this, MF biosynthesis and haemolymph titres have been demonstrated to be positively correlated with ovarian maturation in some crustacean species [4,5]. However, in the crab *Cancer pagurus*, an increase in haemolymph MF concentration in animals at ovary stage 0 is coincident with the beginning of vitellogenesis; the MF concentration decreases again by stage II of ovarian development [6]. Furthermore, a report that MF stimulates ecdysteroid synthesis *in vitro* [7] remains unsubstantiated and seems untenable.

MF is synthesized and excreted from the paired mandibular organs [4,8–10], the synthesis being modulated by one or more neuropeptides produced and released from the X-organ–sinus gland complex of the eyestalk [4,5,11]. We have reported the purification and determination of the primary structure of a 78-residue neuropeptide, mandibular organ-inhibiting hormone (MO-IH-1) and a variant, MO-IH-2, which inhibit MF synthesis in the edible decapod crustacean *C. pagurus* [12]. These peptides are members of the moult-inhibiting hormone (MIH) group within the crustacean hyperglycaemic hormone (CHH) neuropeptide family [12,13]. MIH itself negatively regulates ecdysteroid production in the Y-organs (a site of synthesis of ecdysteroids).

Here we report the isolation and characterization of the cDNAs encoding MO-IH-1 and MO-IH-2, derived from the cluster of perikarya comprising the X-organ, which establishes the differences between the nucleotide sequences of the respective cDNAs. Furthermore, this will permit the elucidation of the biosynthetic origin of the MO-IHs and, in particular, whether or not the mature peptides arise by post-translational processing of precursor peptides. This is particularly relevant because the CHH preprohormones contain an additional peptide preceding the hormone, called the CHH-precursor-related peptide (CPRP), whereas such a CPRP-like peptide is lacking from the precursor of MIH (reviewed in [14]). In addition, we report the tissue distribution and size of the MO-IH transcripts, established by Northern blot analysis, together with preliminary gene analysis and species distribution by Southern blotting.

EXPERIMENTAL

Amplification of MO-IH cDNA and cloning

Cloning of the coding region

From the known amino acid sequences of MO-IH-1 and MO-IH-2 of *C. pagurus* [12], two degenerate primers were designed for use in PCR, an upstream sense primer, Ps-1 (5′-ATGAATTCTGGATHTGYAARGAYTGYGC-3′), encoding the peptide WICKDC(A) (single-letter codes) and a downstream anti-sense primer, Pas-1 (5′-TAGGATCCATNGCNGCCCAYTGYTC-3′), encoding EQWAA(I) (parentheses indicate that only the first one or two bases of the triplets that code for these amino acids were used). Restriction sites are underlined.

Abbreviations used: CHH, crustacean hyperglycaemic hormone; MF, methyl farnesoate; MIH, moult-inhibiting hormone; MO-IH, mandibular organ-inhibiting hormone; ORF, open reading frame; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

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The nucleotide sequences for MO-IH-1 and MO-IH-2 reported in this paper appear in the GenBank/EBI Data Bank under the accession numbers AJ245378 and AJ245379 respectively.

mRNA was purified from 20 X-organs with a Dynabeads™ mRNA Direct kit (Dynal). By using mRNA equivalent to one X-organ, first-strand cDNA synthesis and PCR were performed as described in the instructions for the First Strand cDNA kit (Boehringer Mannheim) using the following Q_T anti-sense primer, 5′-CCATCAGTGCTAGACAGCTAAGCTTGAGCT-CGGATCC$(T)_{17}$-3′, which was modified from [15]. The reaction mixture was stored at −20 °C (Q_T-cDNA). Thirty-five cycles of PCR were performed on half (10 μl) of the Q_T-cDNA with the use of Ps-1 and Pas-1 primers with the following temperature profile: 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, with the step-cycle programme on a Hybaid DNA Thermal Cycler in 100 μl of 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM dNTPs, containing 10 μl of Q_T-cDNA and 100 pmol of each primer. After digestions, approx. one-third of the PCR product was used to clone into pBluescript II KS+ vector using *Eco*RI and *Bam*HI restriction sites. Two isolates were sequenced and found to be identical, containing a 163 bp fragment with an open reading frame (ORF) of 49 residues corresponding to the *C. pagurus* MO-IH-2 peptide.

3′-end cDNA amplification

Two specific primers were designed on the basis of the MO-IH-2 cDNA sequence obtained, each containing an *Eco*RI site at the 5′-end. Ps-2 had the sequence 5′-ACGAATTCCTGTGGTGT-ATCGACGCAAC-3′; Ps-3 had the sequence 5′-GCGAATTC ACTAGAAACAAGGAACAGCT-3′.

Nested PCR was performed with Ps-2 and Q_0 primers (5′-CCATCAGTGCTAGACAGCT-3′) (modified from [15]) and 2 μl of Q_T-cDNA as template for the first round of PCR, then with Ps-3 and Q_1 primer (5′-TAAGCTTGAGCTCGGATCC-3′) (modified from [15]) and with 1 μl of the first-round PCR product as template. The same temperature profile and number of cycles were used as described for Ps-1 and Pas-1 primers. The resulting PCR product of approx. 550 bp was cloned into the *Eco*RI and *Bam*HI sites of pBluescript II KS+ and sequenced. Two isolates proved to be 3′ rapid amplification of cDNA ends (RACE) products of MO-IH-1 and two were MO-IH-2.

5′-end cDNA amplification

5′-end cDNA amplification was performed with a Life Technologies 5′ RACE system. First-strand cDNA synthesis was performed with Pas-1 primer to give Pas-1-cDNA, which was then tailed with terminal transferase and dCTP to create an abridged primer-binding site [oligo(dC)] on the 3′ end of the cDNA. The target cDNA was amplified by nested PCR with the same temperature profile and cycle number as above. The first round PCR was performed with the anti-sense primer Pas-2 (5′-ACCACAGAAATTCAGTG-3′) and Abridged Anchor Primer (AAP; 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIG-GGIIG-3′, provided with the kit). The second round of PCR used anti-sense Pas-3 (5′-GCGAATTCAGTTACTCCTACAA-TTAT-3′) and Abridged Universal Amplification Primer (AUAP; 5′-GGCCACGCGTCGACTAGTAC-3′, provided with the kit). The amplified product was cloned between the *Sal*I and *Eco*RI sites of pBluescript II KS+ and sequenced. Six isolates had 5′-end sequences corresponding to either MO-IH-1 or MO-IH-2.

Construction of full-length cDNAs

Thirty five cycles of PCR were performed on the Q_T-cDNA with the upstream sense primer MPR (5′-ACGAATTCATATGAT-GTCACGTGCTAAC-3′) designed from the sequence obtained from the 5′-end cDNA clones, with *Eco*RI and *Nde*I restriction sites being included at the 5′ end. The same PCR conditions were used on 1 μl of the above Q_T-cDNA with MPR primer and Q_1 primer; the resulting 814 bp PCR product was cloned between the *Eco*RI and *Bam*HI sites of pBluescript II KS+ and sequenced. Two of the clones isolated were MO-IH-1 and two were MO-IH-2.

Northern blot analysis

Total RNA was purified from muscle, gill, epidermis, hepatopancreas, heart, ovary, eyestalk ganglia, eyestalk ganglia from which X-organ had been carefully dissected, and X-organ, with the use of TRIzol® Reagent (Life Technologies). Each purified total RNA (5 μg) (or five organ equivalents for X-organ tissue) were subjected to electrophoresis on a 1 % (w/v) agarose gel for 2.5 h at 150 V. The RNA was blotted to Electran® nylon membrane (BDH) with 10 × SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate) prepared with diethyl pyrocarbonate-treated water, then cross-linked to the membrane by UV radiation. The MO-IH-1 cDNA probe (Probe A; nt 32–828) was prepared by PCR amplification with primers based on the cDNA sequence of MO-IH, and randomly labelled with [α-^{32}P]dCTP. Hybridization of the radiolabelled MO-IH probe was performed in QuickHyb™ solution (Stratagene) at 68 °C for 1 h. The blot was washed twice for 15 min with 2 × SSC containing 0.1 % SDS at room temperature and once for 30 min with 0.1 × SSC containing 0.1 % SDS at 55 °C. Autoradiographs were exposed at −70 °C.

Southern blot analysis

High-molecular-mass genomic DNA was isolated from crab muscle tissue by using established protocols [16]. Samples of DNA (10 μg) were digested to completion with *Bam*HI, *Eco*RI, *Hin*dIII, *Pvu*II, *Mbo*I or *Taq*I and the digested DNA species were subjected to electrophoresis on a 0.7 % agarose gel. The gel samples were partly hydrolysed by acid depurination with 0.2 M HCl for 10 min, then denatured by soaking in 500 ml of 0.5 M NaOH/1.5 M NaCl for 45 min at room temperature and neutralized in 500 ml of 1.0 M Tris/HCl, pH 7.4, containing 1.5 M NaCl for 45 min. The DNA species were then transferred to Electran® nylon membrane (BDH) with 10 × SSC and cross-linked by using UV. Prehybridization was performed with QuickHyb™ solution (Stratagene) for 15 min at 68 °C and hybridized with *C. pagurus* MO-IH-1 cDNA probes (probe A, 32–828, 797 nt; probe B, 329–828, 500 nt; probe C, 32–328, 297 nt) at 68 °C for 1 h. After hybridization, the blot was washed twice in 2 × SSC/0.1 % SDS for 15 min at room temperature and then once in 0.1 × SSC/0.1 % SDS at 25, 40, 55 or 60 °C (see the Results section for specific temperatures) for 30 min.

For Southern blot analysis of the distribution of gene for MO-IHs in other crustacean species, genomic DNA was prepared from muscle tissue, digested with *Pvu*II and analysed as described above.

Autoradiographs were exposed at −70 °C.

DNA sequencing and analysis

Double-stranded DNA sequencing was performed with the T7 Sequenase™ Version 2.0 DNA sequencing kit (Amersham). DNA sequences and deduced protein sequences were aligned and compared using BLAST software and the EMBL/GenBank databases.

RESULTS

Isolation and characterization of MO-IH cDNAs

To isolate full-length cDNAs encoding MO-IH-1 and MO-IH-2, a PCR-based approach was employed. The amino acid sequence from purified *C. pagurus* MO-IH [12] provided information for the design of oligonucleotide primers for the reverse transcription and PCR amplification of the gene for MO-IH as described in the Experimental section. In brief, degenerate sense and anti-sense primers (Ps-1 and Pas-1) were used on oligo(dT)-primed cDNA to amplify and clone a 163 bp internal fragment of MO-IH. On sequencing of clones, representatives of both MO-IH-1 and MO-IH-2 cDNAs were present. With the use of non-degenerate primers based on the internal MO-IH sequence, 3′- and 5′-RACE were performed to obtain PCR clones containing each end of the cDNAs. Finally, cDNA clones containing the full-length coding region and 3′-untranslated region (UTR) were produced by PCR with the primer MPR, which was designed to anneal to the ATG start codon, placing *Eco*RI and *Nde*I sites immediately upstream. Figure 1 shows the resultant full-length cDNA sequences created by combining sequence information derived from all the PCR clones isolated. Excluding the poly(A) tail, the full-length cDNAs were 828 and 810 bp long for MO-IH-1 and MO-IH-2 respectively. An ATG triplet at nucleotide position 32 of MO-IH-1 and position 14 of MO-IH-2 corresponds to the predicted initiation codon. Both the MO-IH-1 and MO-IH-2 cDNA sequences contain a 339 bp ORF encoding a putative protein of 112 residues, a 458 bp 3′ UTR and a poly(A) tail. The longest 5′ UTR present in any of the 5′ RACE clones was 31 bp for MO-IH-1 and 13 bp for MO-IH-2. The 797 bp amplicons (including the ORF and 3′ UTR) of the putative precursor of MO-IH-1 or MO-IH-2 were cloned and sequenced. Conceptual

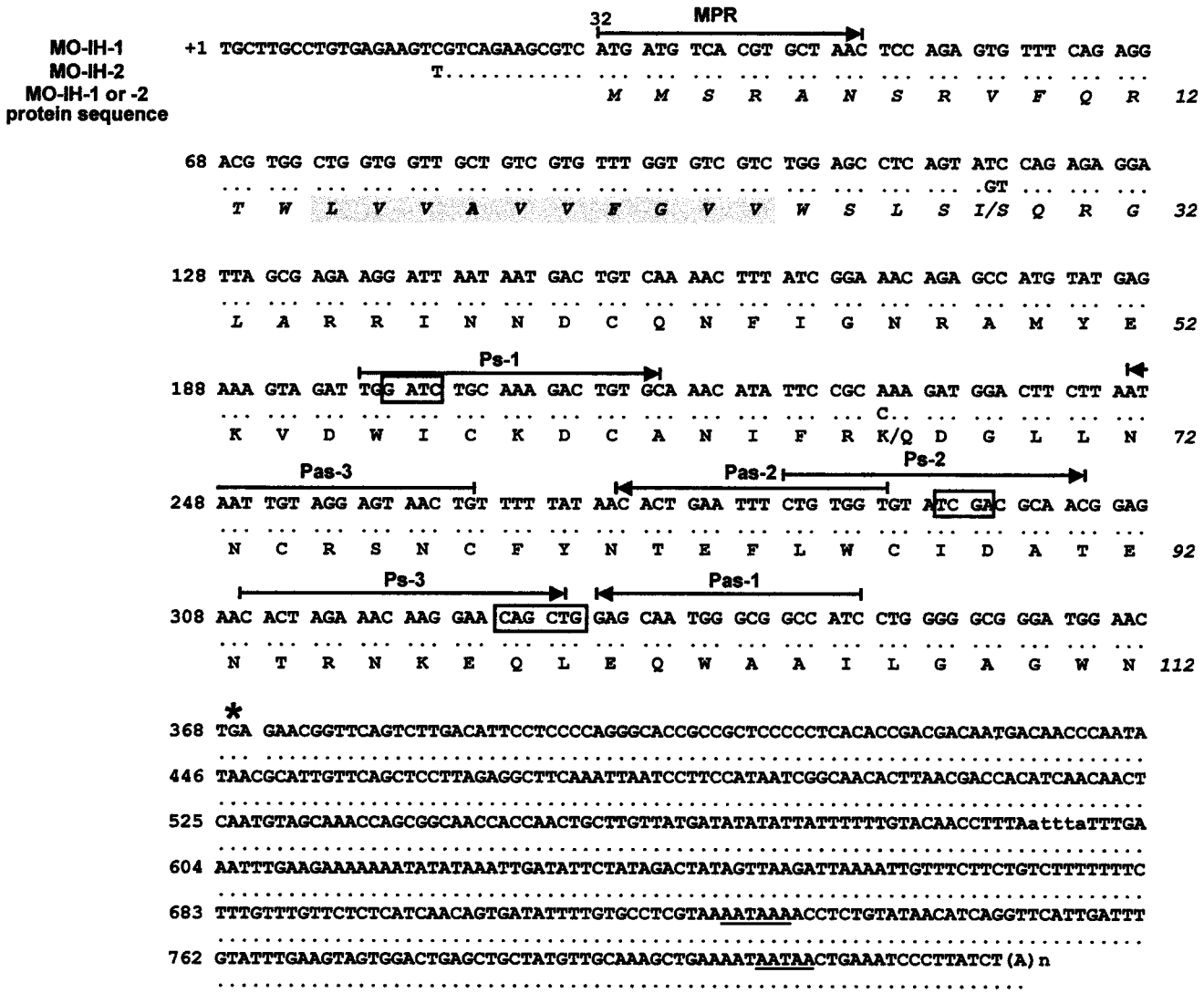

Figure 1 Sequences of cDNAs encoding MO-IH-1 and MO-IH-2 of *Cancer pagurus*

Full-length cDNAs encoding MO-IH-1 and MO-IH-2 were isolated using a PCR-based approach and the nucleotide sequence was determined as described in the Experimental section. Dots indicate residues in MO-IH-2 that are identical with those in MO-IH-1. Amino acid sequences (single capital letters) begin at nt 32 and 14 for MO-IH-1 and MO-IH-2 respectively. The precursor peptides are indicated in italics, the mature peptides in normal letters, and a core of hydrophobic amino acids in a shaded box. The stop codon is indicated by an asterisk. Putative polyadenylation signals [AATAA(A)] are underlined. Arrows denote the position and direction of primers used for PCR reactions. Boxed regions indicate *Mbo*I (199–202 bp), *Taq*I (294–297 bp) and *Pvu*II (326–331 bp) restriction sites within MO-IH-1 and MO-IH-2 coding regions.

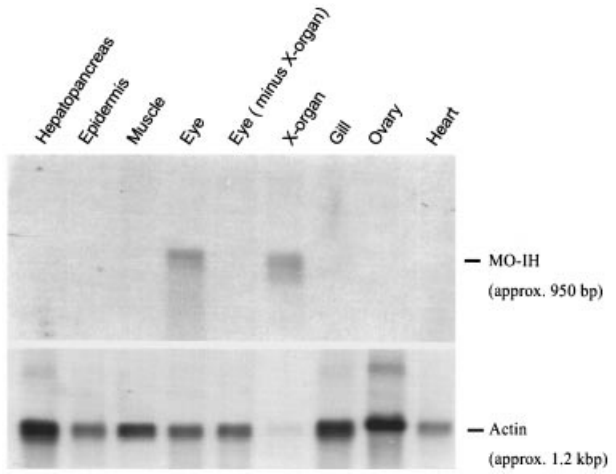

Figure 2 Tissue distribution of MO-IH mRNA

Northern blot showing the tissue distribution and size of the *C. pagurus* MO-IH transcript. Total RNA (approx. 5 μg) from a variety of *C. pagurus* tissues (five organ equivalents of X-organ RNA) was subjected to electrophoresis, blotted to a nylon membrane, hybridized at 68 °C with a ^{32}P-labelled MO-IH-1 probe (nt 32–829) and washed at 60 °C (upper panel). The blot was rehybridized under the same conditions with a β-actin cDNA probe from crayfish (lower panel) [22].

translation of the cDNA sequences showed the ORF to encode a 34-residue putative signal peptide and the 78-residue mature MO-IH-1 or MO-IH-2 peptide sequence. Comparison of the cDNA sequences of MO-IH-1 and MO-IH-2 shows that within the ORF there were three base differences. The first two differences were at positions 86 and 87 of the MO-IH-1 cDNA sequence and resulted in the substitution of an isoleucine residue in MO-IH-1 for serine in MO-IH-2. The third base difference was at position 230 of the MO-IH-1 cDNA sequence, which resulted in the substitution of a lysine residue in MO-IH-1 for glutamine in MO-IH-2. This latter difference is consistent with our previous reports of the difference in the amino acid sequences of the MO-IH-1 and MO-IH-2 peptides. Both MO-IH-1 and MO-IH-2 cDNAs encode putative peptides of 112 residues with calculated molecular masses (reduced) of 13123 and 13062 Da, and predicted pI values of 8.88 and 8.64 respectively. The mature MO-IH-1 and MO-IH-2 peptides have calculated molecular masses (reduced) of 9241 Da and predicted pI values of 6.37 and 5.25 respectively. The 3′ UTR contains one copy of the mRNA instability consensus sequence ATTTA at position 594 of the MO-IH-1 cDNA sequence [17]. The polyadenylation signal AATAAA [18] was 97 bp upstream of the poly(A) tail in all MO-IH-1 and MO-IH-2 clones, except for one clone in which this polyadenylation signal was 86 bp upstream. The variant polyadenylation signal AATAA, used *in vivo*, began 21 bp upstream of the poly(A) tail in all the MO-IH-1 and MO-IH-2 cDNA clones.

Alignment of MO-IH sequence and database searches

To determine the sequence similarity of *C. pagurus* MO-IH-1 and MO-IH-2 to other peptides, a search of the current non-redundant combination of all nucleic acid and protein databases with MO-IH nucleotide and amino acid sequences was performed. The sequence of *C. pagurus* MO-IH 5′ UTR and coding region showed a high degree of similarity (70 %) to the cDNA sequence of the shore crab, *Carcinus maenas* MIH [19] and the blue crab, *Callinectes sapidus* MIH [20]. The putative MO-IH

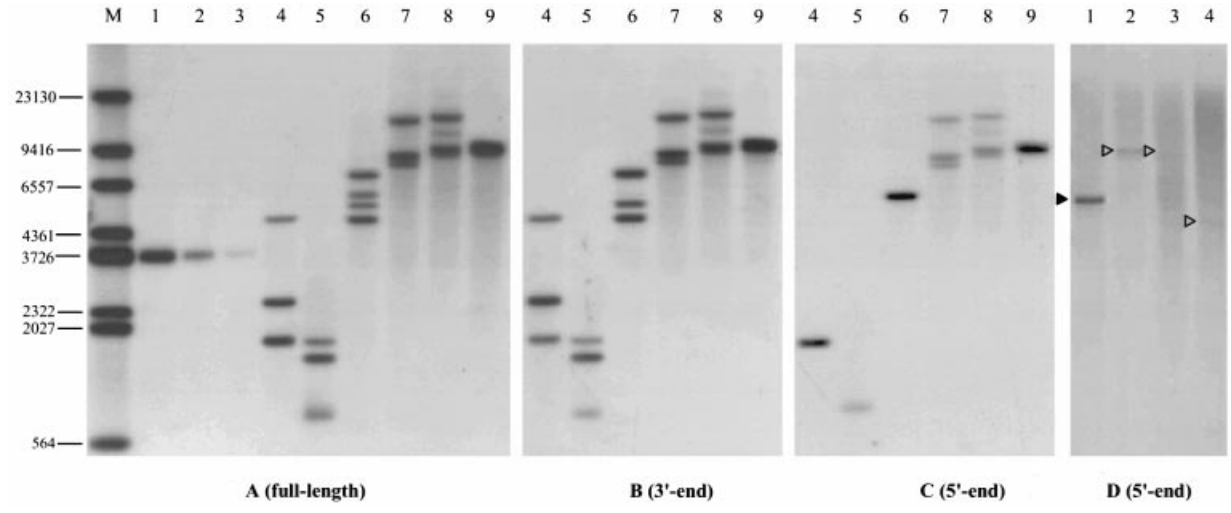

Figure 3 Southern blot analysis of genes for MO-IH

(**A**–**C**) *C. pagurus*. *C. pagurus* genomic DNA (10 μg) was digested with a variety of restriction enzymes (lanes 4–9), separated by agarose gel electrophoresis and blotted to a nylon membrane. Hybridization of radiolabelled MO-IH cDNA probes was performed at 68 °C followed by high-stringency washing at 60 °C. Three different probes were used for these experiments: (**A**) full-length MO-IH-1 cDNA probe (nt 32–828); (**B**) 3′-end MO-IH-1 fragment (nt 329–828) generated by digestion of full-length MO-IH-1 cDNA with *Pvu*II; (**C**) 5′-end MO-IH-1 fragment (nt 32–328) generated by *Pvu*II digestion of full-length MO-IH-1 cDNA. Lane M, λ DNA digested with *Hin*dIII containing 500 pg of linearized MO-IH-1 cDNA (sizes of fragments in bp are given on the left); lane 1, 100 pg of linear MO-IH-1 cDNA; lane 2, 20 pg of linear MO-IH-1; lane 3, 4 pg of linear MO-IH-1 cDNA; lanes 4–9 respectively, *C. pagurus* genomic DNA digested with *Taq*I, *Mbo*I, *Pvu*II, *Hin*dIII, *Eco*RI and *Bam*HI. (**D**) Distribution in other crustacean species. Genomic DNA (10 μg) from a variety of crustaceans (lanes 1–4) was separated by agarose-gel electrophoresis and blotted to a nylon membrane. Hybridization of MO-IH-1 probe (nt 32–328) was performed at 60 °C followed by washing at 25 °C (**D**), 40 or 55 °C (results not shown). Lanes 1–4 are genomic DNA isolated from *C. pagurus* (lane 1), *C. antennarius* (lane 2), *Car. maenas* (lane 3) and *P. bernhardus* (lane 4). Filled and open arrowheads indicate hybridization of the MO-IH probe to genomic DNA from *C. pagurus* (lane 1) and other species (lanes 2–4) respectively.

signal peptide amino acid sequence (Figure 1) exhibited 56%, 44% and 32% sequence identity with *Car. maenas* MIH, *C. sapidus* MIH and the lobster *Homarus americanus* gonad-inhibiting hormone putative signal peptides respectively [19–21]. In contrast, it exhibited a lower degree of sequence similarity to any of the CHH precursor peptides. The mature *C. pagurus* MO-IH sequence revealed significant similarity to *Car. maenas* and *C. sapidus* MIHs.

Northern blot analysis of MO-IH

To determine the tissue distribution of MO-IH mRNA, a 797 bp (nt 32–828) insert of a clone of MO-IH-1 was used as probe for the Northern blot analysis of several *C. pagurus* tissues. As shown in Figure 2, a single band of approx. 950 nt was detected in eye and X-organ RNA samples, which is consistent both with the predicted length based on the cDNA clones obtained and with the expected tissue localization of the mRNA. In contrast with the strong expression in the X-organ, there was no detectable MO-IH mRNA expression in other tissues or in eyestalk tissue from which the X-organ had been removed by dissection. To confirm that equivalent amounts of RNA from each tissue type were analysed, the blot was reprobed with an actin probe from the crayfish, *Procambarus clarkii* [22]. This confirmed that similar quantities of RNA had been loaded for all tissue samples except the X-organ (Figure 2). The reason for the poor actin hybridization signal in X-organ tissue is that the X-organ is very small and five X-organs do not contain sufficient mass to generate a strong actin signal. Long exposures of Northern blots did not show the presence of pre-mRNA species or alternatively spliced products of different sizes. The 950 nt mRNA detected for MO-IH was consistent with the sizes of similar hormone mRNA species in the other crustacean systems studied so far [18].

Southern blot analysis of genomic DNA

To gain some insight into the genomic organization of the *C. pagurus* genes for MO-IH, Southern blot analysis of restriction endonuclease-digested genomic DNA was performed with *Mbo*I, *Taq*I and *Pvu*II, which cut both MO-IH-1 and MO-IH-2 cDNA clones, and *Hin*dIII, *Eco*RI and *Bam*HI, which do not. Using the full-length probe A (Figure 3A; see the Experimental section), three bands were detected in DNA digested with *Taq*I (lane 4), *Mbo*I (lane 5) and *Hin*dIII (lane 7). Two bands of similar size were detected in *Bam*HI-digested DNA (lane 9), four bands in *Eco*RI-digested DNA (lane 8) and four bands (named 1 to 4 in descending order of size) were detected in *Pvu*II-digested DNA (lane 6). The weakest of these bands was of similar intensity to the band in lane 2, which corresponds to a single copy of MO-IH; therefore there must be approx. six to ten copies of the gene for MO-IH in *C. pagurus*. The Southern blot membrane was washed and re-analysed with the 3′-end probe B (Figure 3B) and the 5′-end probe C (Figure 3C). With these probes, the major difference observed was for the *Pvu*II-digested DNA (lane 6). Three bands hybridized to probe B and a single band hybridized to probe C. *Pvu*II band 2 must contain only the 5′ ends of the genes for MO-IH, suggesting that there might be more sequence conservation between individual genes for MO-IH upstream of the coding region than downstream, where three *Pvu*II bands were seen (Figure 3B, lane 6). A zooblot (Figure 3D) shows that MO-IH-like sequences could be detected in other crustaceans.

DISCUSSION

MO-IH was first isolated and characterized from the edible crab, *C. pagurus*, with a bioassay that measured the inhibition of MF synthesis in mandibular organs cultured *in vitro*. The amino acid sequence of the two isoforms of MO-IH-1 and MO-IH-2 [12] revealed them to be members of the CHH/MIH/VIH (vitellogenesis-inhibiting hormone) family of crustacean neuropeptides [13], with significant sequence identity with the MIH peptides. In common with all of these peptides, MO-IH-1 and MO-IH-2 were isolated from the sinus glands (part of the X-organ–sinus gland neurosecretory complex of the eyestalk). Considering the relatedness of the MO-IHs to these other crustacean neuropeptides, we isolated the cDNAs encoding MO-IH-1 and MO-IH-2 with the following aims: (1) to establish the molecular basis of the single amino acid difference between the peptide sequences of MO-IH-1 and MO-IH-2, (2) to elucidate the biosynthetic origin of the MO-IHs, (3) to localize the site of expression of mRNA that encodes the MO-IH peptides, and (4) to provide novel information on the structure of the gene for MO-IH.

Using a PCR-based approach, we isolated and sequenced cDNAs encoding both isoforms of MO-IH present in *C. pagurus* X-organs (Figure 1). Both cDNAs were of a similar size, comprising a 5′ UTR, an ORF encoding a 112-residue peptide and a 460 bp 3′ UTR. The 112-residue peptide consists of the mature 78-residue MO-IH peptide and a 34-residue putative signal peptide. Unlike the CHH preprohormones (see [14]), the precursors of the MO-IHs do not contain additional peptides, other than signal peptides, preceding the hormones. The deduced amino acid sequences of MO-IH-1 and MO-IH-2 highlight two differences, the latter confirming our earlier report on the difference between the amino acid sequences of the two isoforms [12].

Northern blot analysis of RNA from *C. pagurus* tissues demonstrated that transcripts of MO-IH-1 and MO-IH-2 (size indistinguishable) are approx. 950 nt in length and localized to X-organ tissue (Figure 2), which is in complete agreement with the expected site of synthesis, the X-organ–sinus gland complex. The transcript size is consistent with the isolated cDNA clones, allowing for the poly(A) tail. The 950 nt MO-IH band is smaller than the 1400 nt MIH mRNA from the blue crab, *Callinectes sapidus* [23], but analysis to determine whether there is any cross-hybridization between the MO-IHs and MIH of *C. pagurus* revealed that a full-length *C. pagurus* MIH probe hybridized to the same *C. pagurus* membrane, giving a band of 1400 nt in length (W. Lu, S. G. Webster, P. C. Turner and H. H. Rees, unpublished work). This hybridization of the MIH probe confirmed that the bands that hybridized to the MO-IH probe at the stringencies used did not contain MIH, a potential problem given that there is 61% amino acid sequence identity between MIH and the MO-IHs of *C. pagurus*.

Southern blot analysis of restriction endonuclease-digested *C. pagurus* genomic DNA detected the presence of multiple bands that hybridized to MO-IH probes. The most likely explanations for the presence of multiple bands in Southern blots are (1) the existence of multiple genes, (2) the presence of introns containing sites for the restriction enzymes used, (3) cross-hybridization to related sequences, or (4) incomplete methylation of restriction enzyme sites. The first explanation best explains the present results. Given that for *C. pagurus* the DNA content has been determined as 4.5 pg per nucleus (E. M. Rasch, personal communication), the band in lane 2 (Figure 3A) is equivalent to approximately one copy of the gene for MO-IH in 10 μg of genomic DNA. This value, taken together with the intensities of the bands in lanes 4–9, suggests that there could be as many as ten copies of the genes for MO-IH in the *C. pagurus* genome. One problem with the interpretation of copy number from these results is the apparent lack of correspondence between the

number and intensity of 5′-end and 3′-end fragments as seen in the *Pvu*II, *Mbo*I and *Taq*I lanes. There are three *Pvu*II bands (1, 3 and 4), two of which hybridize most strongly with full-length and 3′-end probes, whereas only band 2 contains 5′ ends. A possible explanation might be that some sequence in the 3′ UTR of the MO-IH probe cross-hybridizes with non-MO-IH sequences or even MO-IH pseudogenes that lack 5′ ends. In view of this observation, the copy number estimate of approximately ten genes for MO-IH should be considered a maximum. The presence of small bands in Figure 3 (lanes 4 and 5) that hybridize to both 5′-end and 3′-end probes, even though the clones' cDNA contains *Mbo*I and *Taq*I sites, can readily be explained by polymorphism in some copies of the genes for MO-IH, or by each small band representing separate bands of very similar sizes.

Southern blot analysis of MO-IH hybridization to genomic DNA from a variety of crustacean species has revealed strong cross-hybridization of the MO-IH probe to DNA of another *Cancer* species, *C. antennarius* (Figure 3D, lane 2) and weaker hybridization to *Car. maenas* (Figure 3D, lane 3) and *Pagurus bernhardus* (hermit crab) genomic DNA (Figure 3D, lane 4), but only under low-stringency washing conditions. This suggests that although the putative gene for MO-IH in *C. antennarius* is similar to MO-IH in *C. pagurus*, they are by no means identical and the gene(s) in *P. bernhardus* are more distant. Indeed, preliminary investigation of sinus gland extracts of *C. antennarius* and *C. magister* clearly show the presence of MO-IH-like immunoreactive material in both species. Radioimmunoassay of sinus gland extracts of *C. pagurus*, *C. magister* and *C. antennarius* demonstrated that sinus glands of the three species contained approx. 10–15, 10 and 1 pmol of MO-IH immunoreactivity respectively, whereas in other species examined, unequivocal MO-IH immunoreactivity could not be detected (S. G. Webster, unpublished work).

Although we have shown that MO-IH-like molecules exist in *Cancer* species (by using either antibody or cDNA probes), the existence of a discrete MO-IH-like peptide in cancrid crabs is still somewhat enigmatic. In the relatively few other species examined so far (i.e. an oxyrhynchan decapod, *Libinia emarginata* [24], and a brachyrhynchan decapod, *Car. maenas* [25]), available evidence suggests that peptides that are structurally identical with CHH inhibit MF synthesis (and could have the role of MO-IHs in these species). However, in *C. pagurus*, CHH definitely does not fulfil such a role, because incubation of the mandibular organ with high concentrations (100 nM) of CHH, which profoundly inhibited MF synthesis in *Car. maenas*, entirely failed to elicit any significant decrease in MF synthesis in *C. pagurus* MOs [25]. Thus there is a dichotomy with regard to the structure and function of related neuropeptides between and within related organisms (brachyurans). Nevertheless, although it is tempting to use terms such as 'unique' or 'unprecedented' with regard to *Cancer* MO-IHs, it is becoming ever more apparent that various members of the CHH neuropeptide family are pleiotropic with respect to function. For instance, isoform A of CHH seems to act as a functional MIH in lobsters (*H. americanus*) [26], although in other astacurans functionally distinct MIHs have been identified (reviewed in [2]) and in penaeids many different CHH-like molecules have been reported to possess CHH and/or MIH activities ([27–30]; reviewed in [2]). Thus our finding that MO-IHs, which are structurally distinct from CHHs, seem to be limited to the Cancridae might have a hitherto unknown additional significance. Further systematic and comparative studies on the organization of CHH-like genes, their products and their physiological roles are now timely and should shed new light on integrative mechanisms related to moulting, reproduction and energy metabolism in decapod crustaceans. The availability of the cDNAs encoding the MO-IHs presented in this paper will significantly aid these studies.

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