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Characterization of cDNA encoding molt-inhibiting hormone of the crab, *Cancer pagurus*; expression of *MIH* in non-X-organ tissues ☆

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Abstract

Synthesis of ecdysteroids (molting hormones) by crustacean Y-organs is regulated by a neuropeptide, molt-inhibiting hormone (MIH), produced in eyestalk neural ganglia. We report here the molecular cloning of a cDNA encoding MIH of the edible crab, *Cancer pagurus*. Full-length *MIH* cDNA was obtained by using reverse transcription-polymerase chain reaction (RT-PCR) with degenerate oligonucleotides based upon the amino acid sequence of MIH, in conjunction with 5′- and 3′-RACE. Full-length clones of *MIH* cDNA were obtained that encoded a 35 amino acid putative signal peptide and the mature 78 amino acid peptide. Of various tissues examined by Northern blot analysis, the X-organ was the sole major site of expression of the *MIH* gene. However, a nested-PCR approach using non-degenerate *MIH*-specific primers indicated the presence of *MIH* transcripts in other tissues. Southern blot analysis indicated a simple gene arrangement with at least two copies of the *MIH* gene in the genome of *C. pagurus*. Additional Southern blotting experiments detected *MIH*-hybridizing bands in another *Cancer* species, *Cancer antennarius* and another crab species, *Carcinus maenas*.

Keywords: Eyestalk; Sinus gland; X-organ; Ecdysteroid; Molting hormone

1. Introduction

Development and reproduction in crustaceans is regulated by a combination of neuropeptide hormones, ecdysteroids (molting hormones) and the isoprenoid, methyl farnesoate (MF), (Chang, 1993; Laufer et al., 1993; Webster, 1998). Molting is triggered by ecdysteroids which are secreted by paired endocrine glands, the Y-organs (Skinner, 1985; Lachaise et al., 1993). Synthesis of ecdysteroids by Y-organs is negatively regulated by molt-inhibiting hormone (MIH), a neuropeptide produced by a cluster of neurosecretory cells (the X-organ) located in the medulla terminalis of the eyestalks, and released from an adjacent neurohemal tissue, the sinus gland (Skinner, 1985; Lachaise et al., 1993; Webster, 1998). MIH inhibits Y-organs during much of the molting cycle, and it has been suggested that entry into premolt, and subsequent molting is initiated when MIH secretion diminishes (Lee et al., 1998).

Based on amino acid sequence data, MIH is placed in the crustacean hyperglycemic hormone (CHH) neuropeptide family, which includes CHH, MIH and gonad-inhibiting hormone (GIH) (for reviews see Keller, 1992; De Kleijn and Van Herp, 1995; Van Herp, 1998; Webster, 1998). Since GIH and MIH are apparently more closely related to each other than to CHH, the suggestion has been made that the MIH/GIH peptides should be considered as a separate subgroup of the CHH family (Keller, 1992). The situation is complicated, since it has been reported in decapod Crustacea that MIH activity may be either exhibited by distinct peptides (Webster, 1991; Lee et al., 1995; Chung et al., 1996; Nagasawa et al., 1996; Yang et al., 1996) or CHHs may act as MIHs as well (Chang et al., 1990; Yasuda et al., 1994; Aguilar et al., 1995). Although cDNAs encoding some MIHs have been cloned and sequenced (Klein et al., 1993; Lee et al., 1995; Ohira et al., 1997; Umphrey et al., 1998), there is a paucity of information regarding the *MIH* gene (Chan et al., 1998; Lu et al., 2000).

Abbreviations: MIH, molt-inhibiting hormone; MO-IH, mandibular organ-inhibiting hormone; MF, methyl farnesoate; MO, mandibular organ; CHH, crustacean hyperglycemic hormone; GIH, gonad-inhibiting hormone; RT-PCR, reverse transcriptase-polymerase chain reaction; RACE, rapid amplification of cDNA ends

☆ MIH cDNA sequence EMBL accession no. AJ245380.

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Little is known about the expression of *MIH* and its regulation during important developmental events. In addition to the established role of ecdysteroids in regulating molting, these hormones are also present during the intermolt stage, where they appear to function in ovarian development (Lanot and Cledon, 1989; Lee et al., 1998; for review see Lachaise, 1990). In our studies on the hormonal control of development and reproduction in the edible crab, *Cancer pagurus*, we have shown that MF concentrations in haemolymph of females vary throughout gonadal maturation, exhibiting a peak at the beginning of vitellogenesis (Wainwright et al., 1996a) followed by a peak in ecdysteroid titre (Wainwright et al., unpublished). To examine the regulation of this ecdysteroid production by MIH, we now report molecular cloning and characterization of cDNA encoding MIH. Additionally, production of the CHH-family peptides has, classically, been limited to the X-organ-sinus gland complex within the eyestalk, however, recent reports have demonstrated that transcripts of such peptides are expressed in tissues other than the X-organ (Chang et al., 1999; Chung et al., 1999). Thus, questions are raised about possible alternative functions of these peptides in crustaceans. To investigate whether this non-X-organ expression of *CHH*-family transcripts is also applicable to *MIH* transcripts, a combination of Northern blotting and a sensitive nested-PCR approach were used to analyse a variety of nervous and other tissues for the presence of *MIH* transcripts. Finally, results of Southern blot analysis of the *MIH* gene are also reported.

2. Materials and methods

2.1. Amplification of MIH cDNA and cloning

2.1.1. Cloning of the coding region

From the known *C. pagurus* peptide sequences (Chung et al., 1996), two degenerate primers were designed for use in PCR. The upstream sense primer PMIHds-1 5′-ATGAATTCGTNATHAAYGAYTGYCC-3′ encoding VINDDC(P) and antisense primer PMIHdas-1 5′-TAGGATCCARYTGNSWCATYTCYTC-3′ encoding EEMSQ(L) were used (parenthesis indicate that only the first two bases of the triplets that code for these amino acids were used). Restriction sites are underlined.

Total RNA was isolated from 5 X-organs using TRIzol® Reagent (Life Technologies, Inc.). Using total RNA equivalent to one X-organ, the first strand cDNA synthesis and PCR were performed as described in the First Strand cDNA Kit (Roche Diagnostics) using Q_T antisense primer [5′-CCATCAGTGCTAGACAGCTAAGCTTGAGCTCG-GATCC$(T)_{17}$-3′] modified from Frohman (1993). 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 using PMIHds-1 and PMIHdas-1 primers with the following temperature profile: 94°C/1 min, 55°C/1 min, 72°C/2 min, using the step-cycle program 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 of each dNTP, and 100 pmol of each primer. Approximately one-third of the PCR product was used to clone into pBluescript II KS^+ vector using *Eco* RI and *Bam* HI restriction sites. Three isolates were sequenced and found to be identical, containing a 195 bp fragment with an open reading frame of 65 amino acids corresponding to the *C. pagurus* MIH peptide.

2.1.2. 3′-End cDNA amplification

One specific primer was designed based on the *MIH* cDNA sequence obtained (PMIHs-1) having the sequence 5′-CTTTATAAGAAAGTAGAATGGATCTGTGAG-3′.
PCR was performed using PMIHs-1 and Q_0 primers [5′-CCATCAGTGCTAGACAGCT-3′, modified from Frohman (1993)], and 2 μl of Q_T-cDNA as template. The same temperature profile and number of cycles were used as described for PMIHds-1 and PMIHdas-1 primers. The resulting PCR product (approximately 960 bp) was cloned into the pGEM®-T Easy vector and sequenced. Three isolates of the clone proved to be 3′- RACE products of MIH.

2.1.3. 5′-End cDNA amplification

5′-End cDNA amplification was performed using the 5′-RACE system (Life Technologies, Inc.). First strand cDNA synthesis was carried out using the PMIHas-1 (5′-CTAGTGTTCTCCGTTGCGTCG-3′) primer to give PMIHas-1-cDNA which was then tailed using terminal transferase and dCTP to create an abridged primer binding site on the 3′-end of the cDNA. The target cDNA was amplified by PCR with the same temperature profile and cycle number as above. The PCR was performed using the antisense primer PMIHas-2 (5′-CTCACAGATCCATTC-TACTTTCTTATAAAG-3′) and Abridged Anchor Primer (AAP, 5′-GGCCACGCGTCGACTAGTACGGGIIGG-GIIGGGIIG-3′, provided by the kit). The amplified product was cloned into pGEM®-T Easy vector and sequenced. Three isolates had 5′-end sequences corresponding to MIH.

2.2. Construction of full-length cDNAs

Thirty five cycles of PCR were carried out on the Q_T-cDNA using the upstream sense primer PMIHs-3 (5′-GGGAATTCATGATGTCACGAACGG-3′) designed from the sequence obtained from the 5′-end cDNA clones, with an *Eco* RI restriction site being included at the 5′- end. The same PCR conditions were used on 2 μl of the above Q_T-cDNA using PMIHs-3 primer and Q_1 primer, and the resulting 1094 bp PCR product was cloned into pGEM®-T Easy vector and sequenced. Three isolates had full-length sequences corresponding to MIH.

2.3. Tissue distribution of MIH transcripts

Total tissue RNA was extracted from muscle, gill, epidermis, hepatopancreas, heart, ovary, optic nerve, brain, ventral nerve cord, thoracic/abdominal ganglion, mandibular organ,

Y-organ, sub-epidermal adipose tissue, gut, eye (includes all soft tissues within the eyestalk), eye from which X-organ had been carefully dissected, and X-organ, using TRIzol® Reagent (Life Technologies, Inc.). Ten μg of each purified total RNA (or two organ equivalents for X-organ tissue) were electrophoresed on a 1% denaturing agarose gel for 3.5 h at 100 V. The RNA was blotted onto Electran® nylon membrane (BDH), using 20× SSC prepared with DEPC-treated water, and cross-linked to the membrane using ultraviolet radiation. The *MIH* cDNA probe (Probe A: nucleotides 220–1314, 1094 nt) was randomly labelled with α-[^{32}P]dCTP. Hybridization was carried out in QuikHyb® solution (Stratagene) at 68°C for 1 h. The blot was washed twice with 2× SSC containing 0.1% (w/v) SDS at room temperature for 15 min, and once with 0.1× SSC containing 0.1% (w/v) SDS at 60°C for 30 min. Autoradiographs were exposed at −70°C using Fuji medical X-ray film.

For PCR-based analysis, 2 μg of total RNA extracted from eyestalk (positive control), brain, fused thoracic/abdominal ganglion, gut, ventral nerve cord, mandibular organ, Y-organ and water (negative control) was used as a template for first-strand cDNA synthesis using SuperScript™ II RNase H$^-$ reverse transcriptase and the Q_T antisense primer (see Section 2.1.1) according to the manufacturer's instructions (Life Technologies). The resultant mixture containing first-strand cDNA was stored at −20°C prior to use. To selectively amplify MIH- coding transcripts, a nested PCR-based approach was employed. In the first round, thirty cycles of PCR were performed on approximately one quarter (5 μl) of the cDNA using 1 U Taq DNA polymerase (Roche), PMIHs-3 (Fig. 1) sense and PMIHas-3 (5′-GAAGATCTTCACTCCCTGCCTGCCCCG; Fig. 1) antisense primers with the following temperature profile: 94°C/1 min, 55°C/1 min, 72°C/1 min, using the step-cycle program on a Hybaid DNA Thermal Cycler in 50 μl of 50 mM KCl, 10 mM Tris/HCl, pH 8.3, 1.5 mM $MgCl_2$ 200 μM of each dNTP, containing 100 pmol of each primer. For the second round, thirty-three cycles of PCR were performed on one tenth (5 μl) of the first round PCR products using PMIHs-1 (Fig. 1) sense and PMIHas-1 (5′-CGTGCGTTCCGTGGCGTACAC, Fig. 1) antisense primers with the same temperature profile as the first round PCR. The PCR products were separated by eletrophoresis on a 1.5% (w/v) agarose gel in 0.5× TBE (Tris/Borate/EDTA buffer, pH 8.0), and visualized under UV after staining with ethidium brimide. Bands of the correct size (135 bp) were excised from the gel, cloned into a pGEM®-T Easy vector (Promega) and two isolates of each of the products were sequenced.

2.4. Southern blot analysis

High molecular weight genomic DNA was isolated from crab muscle tissue using established protocols (Sambrook et al., 1989). Ten μg samples of DNA were digested to completion with either *Bam* HI, *Eco* RI, *Hin*d III, *Pvu* II, *Mbo* I or *Taq* I and the digested DNAs electrophoresed on a 0.7% agarose gel. The gel samples were partially hydrolyzed by acid depurination with 0.2 M HCl for 10 min, then denatured by soaking in 400 ml of 0.5 M NaOH, 1.5 M NaCl for 45 min at room temperature and neutralized in 400 ml of 1.0 M Tris-HCl, pH 7.4 containing 1.5 M NaCl for 45 min. The DNAs were then transferred to Electran® blotting nylon membrane (BDH) using 10× SSC and cross-linked by ultraviolet irradiation. Prehybridization was performed with QuikHyb® solution (Stratagene) for 15 min at 68°C, and hybridized with *C. pagurus MIH* cDNA probes (probe A = 1094 nt from 220–1314; probe B = 942 nt from 372–1314; probe C = 401 nt from 1–401; probe D = 194 nt from 330–523) 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 for 30 min at either 25, 35, 45, 55 or 60°C (see Section 3 for specific temperatures).

For Southern blot analysis of the distribution of *MIH* genes in other crustacean species, genomic DNA was prepared from muscle tissue and analyzed, by *Pvu* II restriction endonuclease digestion, as described above.

2.5. DNA sequencing and analysis

Double-stranded DNA sequencing was performed using the ABI PRISM dye terminator cycle sequencing kit (Perkin–Elmer) in conjunction with an automated DNA sequencer, ABI PRISM 377 (Perkin–Elmer). DNA and deduced protein sequences were compared using BLAST software and the EMBL/GenBank databases, and aligned using DNAMAN software.

3. Results

3.1. Isolation and characterization of MIH cDNA

A full-length cDNA encoding MIH of *C. pagurus* was isolated using a PCR-based approach. Fig. 1 shows the resultant full-length cDNA sequence created by combining sequence information from all of the PCR clones isolated. Excluding the poly(A) tail, the full-length *MIH* cDNA is 1314 bp. An ATG triplet at nucleotide position 222 of the *MIH* cDNA corresponds to the predicted initiation codon. The *MIH* cDNA sequence contains a 342 bp ORF encoding a putative protein of 113 amino acids, and a 750 bp 3′-UTR, followed by a poly(A) tail. The longest 5′- UTR present in the 5′ RACE clones was 221 bp. Conceptual translation of the cDNA sequence showed that the ORF encodes a 35 amino acid putative signal peptide and the 78 amino acid mature MIH peptide sequence, which corresponds exactly to that previously determined by amino acid sequencing (Chung et al., 1996). The 3′-untranslated region of the gene contains four copies of the mRNA instability consensus sequence ATTTA (Shaw and Kamen, 1986) at positions 807, 833, 1240 and 1279. Two perfect matches to the

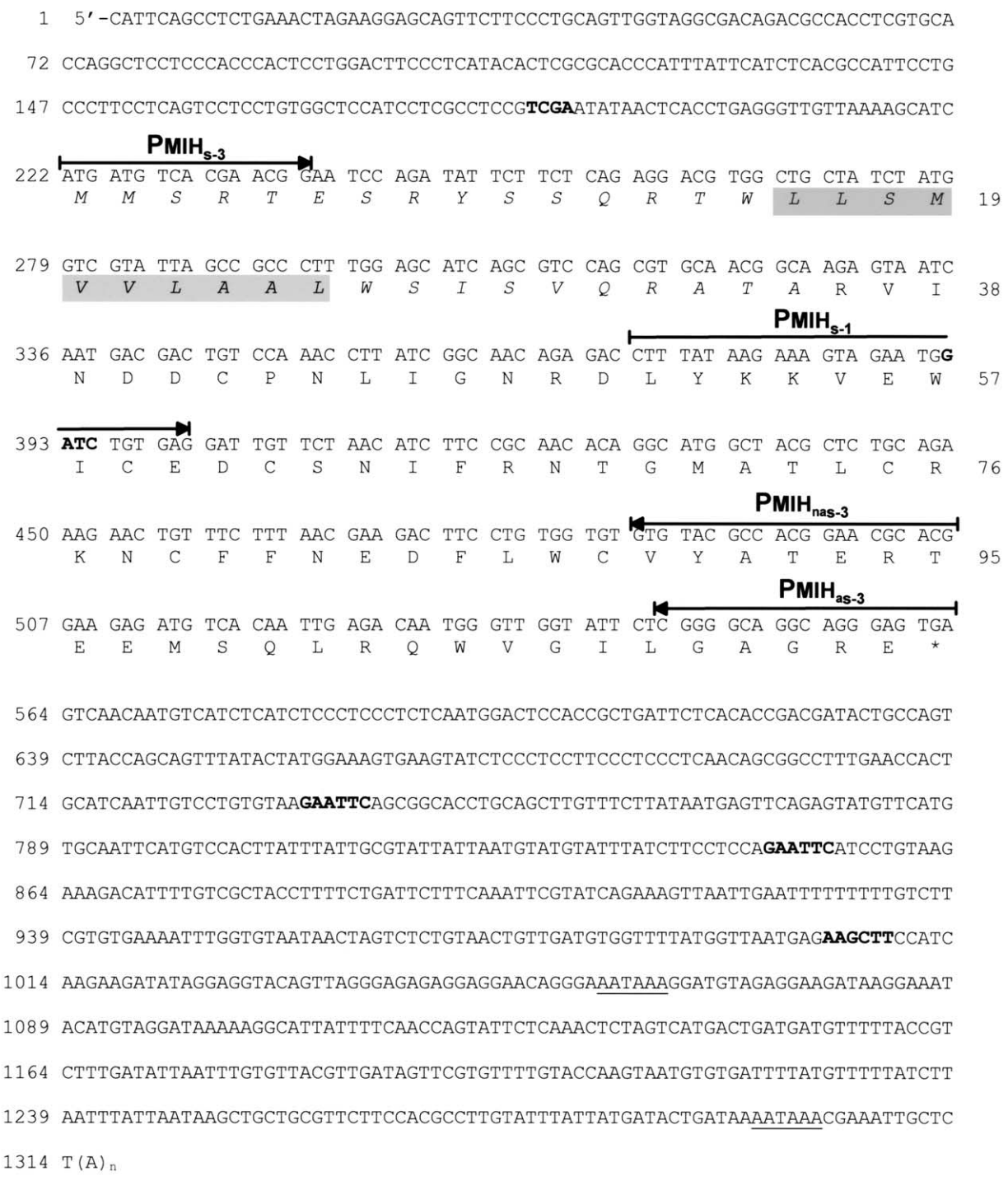

Fig. 1. Sequence of cDNA encoding MIH of *Cancer pagurus*. Full-length cDNA encoding MIH was isolated using a PCR-based approach and nucleotide sequence determined as described in Section 2. Deduced amino acid sequence (single capital letters) begins at nucleotide 222. The precursor peptide is indicated in italics, the mature peptide in normal letters, and a core of hydrophobic amino acids in a shaded box. The stop codon is indicated by an asterisk. Two polyadenylation signals (AATAAA) are underlined. *Eco* RI (positions 734 and 848 bp), *Hind* III (1003 bp), *Mbo* I (392 bp), and *Taq* I (186 bp) restriction sites are in bold. Arrows indicate positions of primers.

consensus polyadenylation signal AATAAA (Proudfoot and Brownlee, 1976) are present at positions 1060 and 1297, however all clones isolated used position 1297 where the poly(A) tail is added 13 nucleotides downstream. Fig. 1 also shows that the *MIH* cDNA sequence contains two *Eco* RI sites (positions 734 and 848), and one *Hin*d III (position 1003), *Mbo* I (position 392) and *Taq* I (position 186) site, but no *Bam* HI or *Pvu* II sites were found. These enzymes have been used in the Southern blot analysis described in Section 3.4.

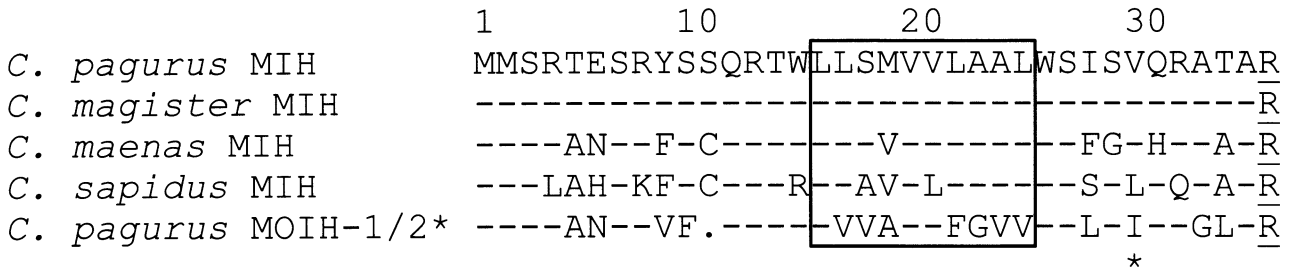

Fig. 2. Comparison of signal peptides of CHH family members. Direct alignment of amino acid sequences of signal peptides from *Cancer pagurus* MIH, *Cancer magister* MIH (Umphrey et al., 1998), *Carcinus maenas* MIH (Klein et al., 1993), *Callinectes sapidus* MIH (Lee et al., 1995) and *C. pagurus* MO-IH-1 and -2 (Tang et al., 1999) is given. A gap (dot) has been introduced into *C. pagurus* MO-IH-1/2 to maximise alignment. The hyphens denote identical amino acids to *C. pagurus* MIH. The boxed area outlines a core of hydrophobic amino acids common to signal peptides. The asterisk indicates a substitution of I in MO-IH-1 for S in MO-IH-2. The first amino acid of each mature peptide is underlined.

3.2. Alignment of the MIH sequence and database searches

To determine the sequence similarity of *C. pagurus* MIH to other peptides, a search of the current non-redundant combination of all nucleic acid and protein databases with MIH nucleotide and amino acid sequences was carried out. The putative MIH signal peptide amino acid sequence (Fig. 2) exhibits 100, 74, 60, and 54% identity to *Cancer magister* MIH, the shore crab, *Carcinus maenas* MIH, the blue crab, *Callinectes sapidus* MIH, and *C. pagurus* MO-IH (mandibular organ-inhibiting hormone), respectively (Klein et al., 1993; Lee et al., 1995; Umphrey et al., 1998; Tang et al., 1999). In contrast, it has a lower degree of similarity to any of the crustacean hyperglycaemic hormone (CHH) precursor peptides and the lobster, *Homarus americanus,* gonad-inhibiting hormone (GIH) putative signal peptides (De Kleijn et al., 1994a).

The mature *C. pagurus* MIH amino acid sequence reveals 98.7, 83, 83, and 60% identity to *C. magister* MIH, *C. maenas* MIH, *C. sapidus* MIH, and *C. pagurus* MO-IH, respectively. Fig. 3 shows a cDNA alignment of the coding region of the *C. pagurus MIH* with sequences from *C. maenas MIH*, *C. sapidus MIH*, *Penaeus japonicus MIH*, *C. magister MIH* and *C. pagurus MO-IH* (Klein et al., 1993; Lee et al., 1995; Ohira et al., 1997; Umphrey et al., 1998; Tang et al., 1999). Table 1 lists the percent identities of pairwise comparisons of these nucleotide sequences.

3.3. Tissue distribution of MIH transcripts

To determine the tissue distribution of *MIH* mRNA, the 1094 bp insert of the full-length MIH clone, Probe A (see Section 2.3) was used as a probe for the Northern blot analysis of several *C. pagurus* tissues. As shown in Fig.

Fig. 3. cDNA sequence comparison of the coding regions of CHH family members. Direct alignment of nucleotide sequences of putative peptides is given: *C. pagurus MIH*, *C. magister MIH* (Umphrey et al., 1998), *C. maenas MIH* (Klein et al., 1993), *C. sapidus MIH* (Lee et al., 1995), *C. pagurus MO-IH*-1 (Tang et al., 1999) and *P. japonicus MIH* (Ohira et al., 1997). Gaps (.) have been introduced into *C. pagurus MO-IH*-1 and *P. japonicus MIH* to maximise alignment. The hyphens denote identical nucleotides to *C. pagurus MIH*. The sequence alignment was performed using DNAMAN software.

Table 1
The percent identity of nucleotide sequences encoding crustacean neuropeptides

Name	Percent identity[a]					
	C. pagurus MIH	*C. magister MIH*	*C. maenas MIH*	*C. sapidus MIH*	*C. pagurus MOIH*	*P. japonicus MIH*
C. pagurus MIH	100					
C. magister MIH	96.5	100				
C. maenas MIH	82.9	82.6	100			
C. sapidus MIH	76.7	77.3	82.6	100		
C. pagurus MOIH	67.3	68.8	68.2	68.2	100	
P. japonicus MIH	57.5	57.1	56.8	55.6	50.8	100

[a] The sequence alignment is shown in Fig. 3. Percent identity (%) was obtained using DNAMAN software.

4A, a single band of approximately 1380 nt was detected for eye and X-organ RNA samples, which is consistent with the predicted length of the mRNA, based on the cDNA clones obtained. In contrast to the strong expression in the X-organ detected by Northern blotting, there is no detectable *MIH*-specific mRNA expression in non-X-organ tissues. To confirm that equivalent amounts of RNA from each tissue were loaded on the gel, the blots were re-probed with either a mouse 18S rRNA probe (Chen et al., 1999) or an actin probe from *Procambarus clarkii* (crayfish) (Kang and Naya, 1993), which shows similar levels of hybridization in all lanes except X-organ (Fig. 4A). The reason for the poor actin hybridization signal in X-organ tissue is that X-organ is very small and two X-organs do not contain sufficient mass to generate a strong actin signal. Long exposures of Northern blots do not show the presence of pre-mRNA species or alternatively spliced products of different size. The 1380 nt mRNA detected for *MIH* is consistent with the sizes of *MIH* mRNAs in other crustacean species (Lee et al., 1998; Umphrey et al., 1998).

Given recent reports showing that transcripts of the crustacean hyperglycemic hormone are expressed in gut (Chung et al., 1999) and that transcripts for *MIH* are present in the brains of *Charybdis feriatus* (Chan et al., 1998) and *Penaeus vannamei* (Sun, 1995), a more sensitive nested PCR-based analysis of a variety of tissues for the presence of *MIH* transcripts was undertaken. The results of the nested PCR analysis (Fig. 4B) clearly show the presence of PCR products of the expected size (135 bp) in other nervous tissues (optic nerve, ventral nerve cord and thoracic/abdominal ganglion). Subsequent cloning and sequencing of two isolates of each of these fragments confirmed the sequence as that of *MIH*. In all experiments, processing of eyestalk and eyestalk-derived cDNA samples was carried out last to prevent the possibility of cross-contamination of PCR reactions with eyestalk-derived material. Inclusion of the water control demonstrated that the nested PCR products were not artefacts of the PCR process. The primers used in the nested PCR were designed so that if any contaminating genomic DNA were present in the cDNA samples, an amplified longer product (520 bp) would be produced due to the presence of an intron (*MIH* gene intron 2; Lu et al., 2000) in the template.

3.4. Southern blot analysis of genomic DNA

A preliminary study of the genomic organisation of *MIH* genes in *C. pagurus* was determined by Southern blot hybridization of restriction endonuclease-digested genomic DNA, using *Eco* RI, *Hin*d III, *Mbo* I and *Taq* I, which cut *MIH* cDNA clones, and *Bam* HI and *Pvu* II which do not (Fig. 1). Using full-length probe A (see Section 2) (Fig. 5A), two bands were detected in *Eco* RI-(lane 8), *Hin*d III-(lane 7), *Mbo* I-(lane 5) and *Taq* I-digested DNA (lane 4). A single band was detected in *Bam* HI-(lane 9) and *Pvu* II-(lane 6) digested DNA. The intensities of the two bands in lane 4 were similar to the band in lane 3 which corresponds to a single copy of *MIH* and, hence, there must be approximately two copies of the *MIH* gene sequence in *C. pagurus* (which could include pseudogenes). The Southern blot membrane was washed and re-analyzed with a 3′-end probe B (Fig. 5B) and a 5′-end probe C (Fig. 5C). Using these probes, the differences observed were for *Eco* RI-(lane 8), *Hin*d III-(lane 7), *Mbo* I-(lane 5) and *Taq* I-(lane 4) digested DNA.

Southern blot analysis of *MIH* hybridization to genomic DNA from a variety of crustacean species was carried out, using *Pvu* II which does not cut *MIH* cDNA clones. Using coding region probe D (see Section 2), hybridization was carried out at 60°C followed by washing at 25°C (Fig. 6A), 35°C (Fig. 6B), 45°C (Fig. 6C) and 55°C (Fig. 6D). Following increase in washing temperature, the differences in band intensity and hybridization patterns observed were for *C. antennarius* (Fig. 6; lane 2) and *C. maenas* (Fig. 6; lane 3).

4. Discussion

Functionally defined MIH has been purified and sequenced from the edible crab, *C. pagurus* (Chung et al., 1996) and the shore crab, *C. maenas* (Webster, 1991). The MIH purified from crab eyestalks directly inhibits ecdysteroidogenesis by Y-organs *in vitro* (Webster and Keller, 1986; Chung et al., 1996), but does not possess CHH activity (Webster and Keller, 1986). The amino acid sequence of MIH and bioassay, revealed it to be a member of the CHH/MIH/VIH family of crustacean neuropeptides (Keller,

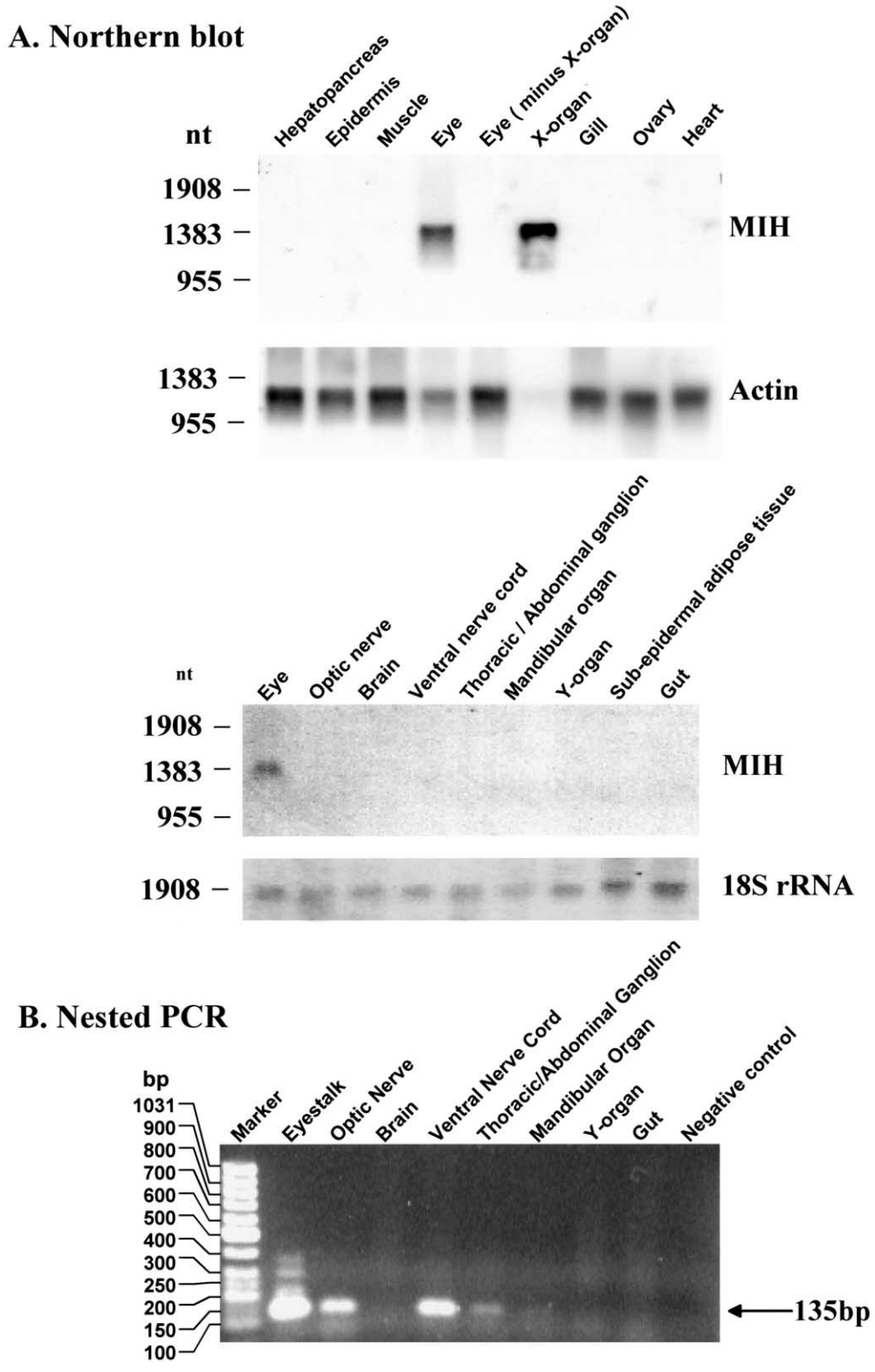

Fig. 4. Tissue distribution of *MIH* mRNA. (A) Northern blot showing tissue distribution and size of the *C. pagurus MIH* transcript. Approximately 10 μg of total RNA from a variety of *C. pagurus* tissues and two organ equivalents of X-organ RNA was electrophoresed, blotted onto a nylon membrane, hybridised at 68°C with a ^{32}P-labelled-*MIH* probe A (nucleotides 220–1314) and washed at 60°C. Blots were re-hybridised under the same conditions using a β-actin cDNA probe from crayfish (Kang and Naya, 1993) or a mouse 18S rRNA probe (Chen et al., 1999). (B) Nested PCR analysis of *C. pagurus* tissues for the presence of *MIH* transcripts. Two μg of total RNA from a variety of tissues was used as template for synthesis of first-strand cDNA. The cDNA was used, in turn, as template for nested PCR using *MIH* sequence-specific primers to amplify a 135 bp *MIH* fragment.

1992), with significant sequence identity to the MO-IH peptides from *C. pagurus* (Wainwright et al., 1996b).

Utilizing a PCR-based approach, we isolated and sequenced cDNA encoding MIH from *C. pagurus* X-organs (Fig. 1). The ORF encodes a 113 amino acid proMIH peptide consisting of the mature 78 amino acid MIH peptide and a 35 amino acid putative signal peptide. This deduced MIH amino acid sequence confirms the earlier report on the sequence of MIH determined by peptide sequencing (Chung et al., 1996), and shows only one residue difference from the deduced amino acid sequence of MIH from the Dungeness crab, *C. magister* (Umphrey et al., 1998).

The alignment of the mature MIH peptide sequence with other members of the CHH/MIH/VIH family of crustacean neuropeptides has been discussed elsewhere (Chung et al., 1996; Umphrey et al., 1998). However, isolation of cDNAs encoding MIHs has allowed alignment of their putative signal peptide sequence and nucleotide sequence (Figs. 2 and 3). It is clear that the putative signal peptide of *C. pagurus* MIH, including a core of hydrophobic amino acids, has a high degree of similarity to the putative signal peptide of other MIH neuropeptides. This includes a cleavage site predicted by A^{-3} and A^{-1}, which is not shared with the MO-IHs (G^{-3} and A^{-1}). Furthermore, each proMIH lacks the CHH precursor-related peptide present in proCHH (Weidemann et al., 1989; De Kleijn et al., 1994b, 1995). These features further strengthen the link between all MIH-like members (78 amino acids in length with free N- and C-termini) of the CHH family of peptides, suggesting a common evolutionary origin. Amongst the four brachyurans, *C. pagurus*, *C. magister*, *C. maenas*, and *C. sapidus*, there is greater than 76% identity of nucleotide sequences in the full-length cDNAs encoding MIH between any pair of species (Table 1). By contrast, the cDNA sequence encoding the *P. japonicus* (prawn) MIH showed only 55–58% identity with the *MIH*s from any of the above species, suggesting an evolutionary dichotomy in this case. The latter identity is even lower than that of the *C. pagurus* *MO-IH* cDNA (67–69%) with any of the brachyuran *MIH* cDNAs.

Northern blot analysis of RNA from *C. pagurus* tissues demonstrated that transcripts of *MIH* are approximately 1380 nt in length and localized to X-organ tissue (Fig. 4A), 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. There is no evidence in the Northern blot that the AATAAA at position 1060 is used in some mRNA molecules and it would seem to be non-functional. The 1380 nt *MIH* band is similar to the 1400 nt and 1264 nt *MIH* mRNA from the blue crab, *C. sapidus* (Lee et al., 1998) and the crab, *C. magister* (Umphrey et al., 1998). The X-organ is also the site of synthesis of *MO-IH*, which in *C. pagurus* is 67.3% identical to *MIH* and under the hybridization conditions used, there was no cross-hybridization of the *MIH* probe to the 950 nt *MO-IH* mRNA (Tang et al.,

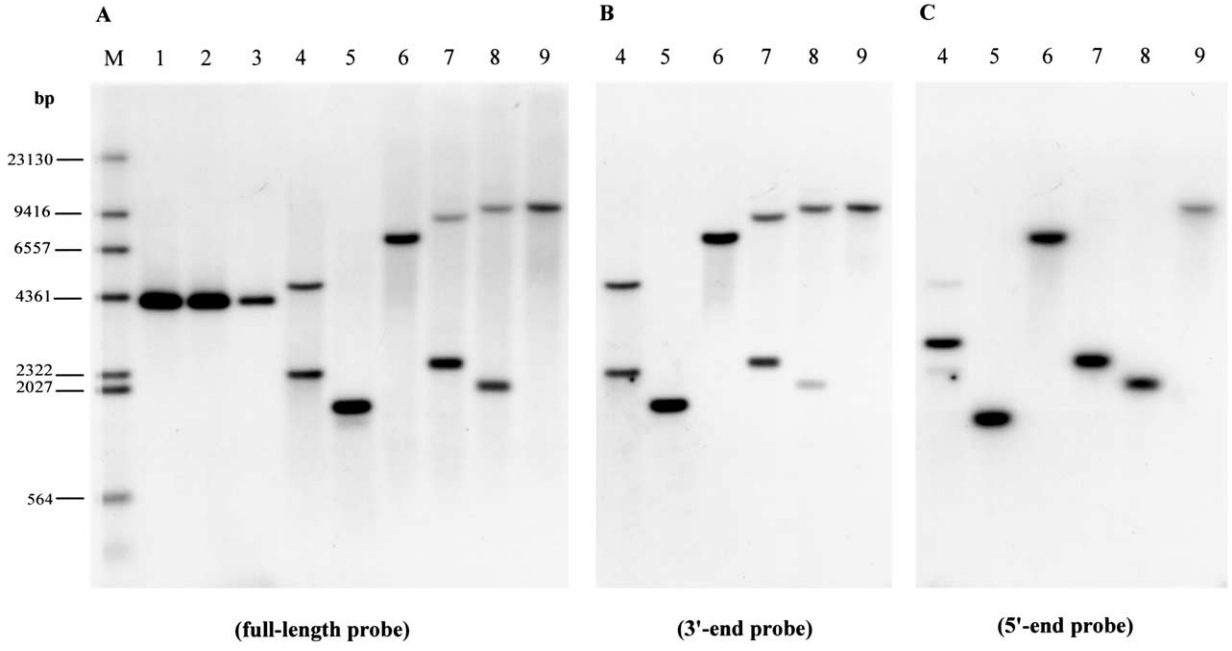

Fig. 5. Southern blot analysis of the *MIH* gene of *C. pagurus*. Ten μg of *C. pagurus* genomic DNA was digested with a variety of restriction enzymes (lanes 4–9), separated by agarose gel electrophoresis and blotted onto a nylon membrane. Hybridisation of radiolabelled *MIH* cDNA probes was carried out at 68°C followed by high stringency washing at 60°C. For these experiments, three different probes were used. Panel A, full-length *MIH* cDNA probe (nucleotides 220–1314); Panel B, 3′-end *MIH* fragment (nucleotides 372–1314); Panel C, 5′-end *MIH* fragment (nucleotides 1–401). Lane M, λ DNA digested with *Hin*d III; Lane 1, 100 pg linear *MIH* cDNA; Lane 2, 50 pg linear *MIH* cDNA; Lane 3, 10 pg linear *MIH* cDNA; Lanes 4–9, respectively, *C. pagurus* genomic DNA digested with *Taq* I, *Mbo* I, *Pvu* II, *Hin*d III, *Eco*R I and *Bam* HI.

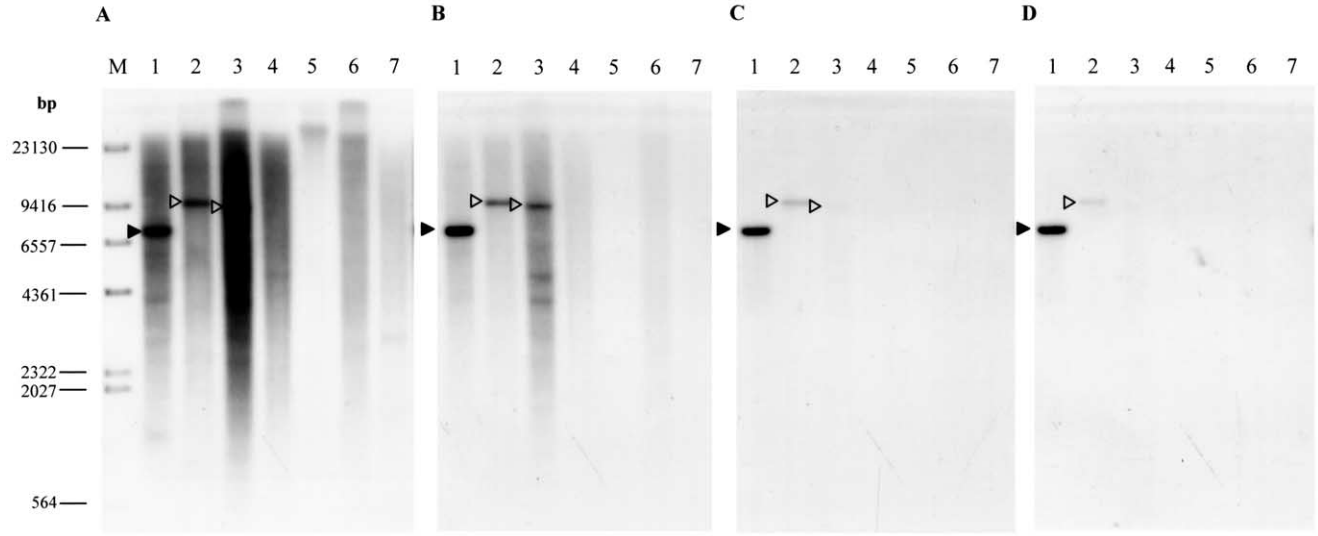

Fig. 6. Southern blot analysis of the distribution of *MIH* genes in crustaceans. Ten μg of genomic DNA from a variety of crustaceans (lanes 1–7) was digested with *Pvu* II and separated by agarose gel electrophoresis and blotted onto a nylon membrane. Hybridisation of *MIH* probe D (nucleotides 330–523) was carried out at 60°C followed by washing at either 25°C (Panel A), 35°C (Panel B), 45°C (Panel C) or 55°C (Panel D). Lane M, λ DNA digested with *Hin*d III. Lanes 1–7 are genomic DNA isolated from *C. pagurus* (lane 1), *Cancer antennarius* (lane 2), *Carcinus maenas* (lane 3), *Pagurus bernhardus* (lane 4), *Artemia salina* (lane 5), *Homarus americanus* (lane 6), *Macrobrachium rosenbergii* (lane 7). Filled and open arrow heads indicate hybridization of the *MIH* probe to genomic DNA from *C. pagurus* (lane 1) and other species (lanes 2–3), respectively.

1999). In the case of CHH, it has been reported that tissues other then the X-organ are capable of producing *CHH* transcripts (Chang et al., 1999; Chung et al., 1999). In our case, we have been unable to detect *MIH*-like transcripts in non-X-organ-containing nervous tissues by Northern blotting. However, given the comparative lack of sensitivity of Northern blotting compared with techniques that involve some form of amplification process (e.g. PCR), a nested PCR-based assay was set up to qualitatively determine whether tissues other than X-organ were producing *MIH* transcripts, but at a level below the detection limits of Northern blotting. The results (Fig. 4B), confirmed by sequencing of the PCR products, demonstrate that tissues other than X-organ-sinus gland contain *MIH* transcripts. It is noteworthy that these other tissues do indeed produce transcripts for *MIH*, but the corresponding peptide could not be detected in these tissues by radioimmunoassay (limit of detection of 1 fmol per assay tube per tissue). Also, it is unclear whether or not these other tissues represent sites of synthesis and release of MIH into the circulating hemolymph or are tissues in which the peptides are released and act locally. This raises the questions as to the function of MIH in these other tissues and whether, as previously found with CHHs, MIH also has multiple biological activities dependent upon the site of synthesis and release.

Southern blot analysis of restriction endonuclease-digested *C. pagurus* genomic DNA detected the presence of multiple bands which hybridized to *MIH* probes (Fig. 5). These could be due to the existence of multiple genes, the presence of introns containing sites for the restriction enzymes used, cross-hybridization to related sequences, or incomplete methylation of restriction enzyme sites. Because *Eco* RI, *Hin*d III and *Mbo* I all cut within the *MIH* cDNA at least two bands should hybridize in genomic blots. This is clearly the case for *Eco* RI (lane 8) and *Hin*d III (lane 7). It is also true for *Mbo* I (lane 5) where the two bands are very similar in size, which is seen when the 5′ and 3′ probes are used independently (Fig. 5C,B). *Pvu* II (lane 6) and *Bam* HI (lane 9) do not cut the cDNA and only one band is detected in these lanes (Fig. 5). These observations are consistent with there being only one *MIH* gene per haploid genome, or a number of very similar genes with considerable sequence conservation extending well outside the coding region. The observations using *Taq* I (lane 4) are not so easy to explain. There is a single *Taq* I site at position 186 in the 5′-UTR and the 3′ and full-length probes cannot hybridize to the upstream fragment. As expected, these probes fail to detect the band seen using the 5′ probe (Fig. 5C), although the 5′ probe does detect, albeit weakly, the two *Taq* I bands seen when using the full-length or 3′ probes. Hence the two bands detected by the full-length and 3′ probes in *Taq* I digests must arise from different genes, or be due to allelic variation of a single copy gene.

Given that for *C. pagurus*, the DNA content has been determined to be 4.5 pg/nucleus (E.M. Rasch, personal communication), the band in lane 3 (Fig. 5A) is equivalent to approximately one copy of the *MIH* gene in 10 μg of genomic DNA. Since this band is similar in intensity to each band in lane 4 (*Taq* I) and half that in lane 5 (*Mbo* I) and lane 6 (*Pvu* II), this together with the results of digestion by *Taq* I suggests that there are no less than two copies of the *MIH* gene per haploid *C. pagurus* genome.

Southern blot analysis of *MIH* hybridization to genomic DNA from a variety of crustacean species has revealed strong cross-hybridization of the *MIH* probe to DNA of another *Cancer* species, *C. antennarius* (Fig. 6; lane 2) and slightly weaker hybridization to *C. maenas* (Fig. 6; lane 3). For these experiments, hybridization was carried out at 60°C followed by washing at 25, 35, 45 and 55°C. After washing at 55°C, the bands for the *Cancer* species (Fig. 6; lanes 1 and 2) were still detected, whilst the bands for *C. maenas* were removed. This suggests that although the putative *MIH* gene in *C. antennarius* is similar to *MIH* in *C. pagurus*, they are by no means identical. Since the *C. pagurus MIH* cDNA sequence is 96.5 and 82.9% identical to *C. magister MIH* and *C. maenas*, respectively, one would expect that the *C. pagurus MIH* cDNA and amino acid sequences should be at least 82.9% identical to *Cancer antennarius MIH*.

The cloned *C. pagurus MIH* cDNA will provide a useful experimental tool to *further* resolve the structure of the *MIH* gene, to elucidate the relationship between *MIH* and *MO-IH* expression, and to investigate the role of *MIH* gene expression and MIH secretory cycles in the nervous system of *C. pagurus*.

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