

## Molecular cloning and characterisation of a novel membrane receptor gene from the lobster *Jasus edwardsii*

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### Summary

The eyestalk of the lobster, *Jasus edwardsii*, is an important source for hormones involved in the regulation of growth and reproduction. How these hormones transfer their messages to the cell and nucleus is not known. This paper describes the cloning, characterization and expression analyses of two genes that code for two membrane-associated peptides that may be involved in signal transduction. These genes, peJK2 and peJK3, were isolated from a cDNA library derived from lobster eyestalk mRNAs. The two clones shared 96.6% sequence homology, and code for putative proteins of 110 and 113 amino acids, respectively. These were likely to be two allelic forms of the same gene. Northern blot analysis using these clones as probes detected the same mRNA from eyestalk, muscle and epithelial extracts, but with

greater intensity in the eyestalk extract. *In situ* hybridisation also indicated the predominant expression of these genes in the eyestalk. Analysis of the putative protein sequences showed that they contained two transmembrane (TM) helices, a short amino acid sequence sharing high homology with the G-protein-coupled receptor (GPCR) motif in the second TM, a signal sequence between the TMs, and a protein kinase phosphorylation site at the C termini. Sequence analyses therefore suggested that the deduced peptides may function in signal transduction.

Key words: signal transducer, G protein-coupled receptor, membrane protein, lobster, *Jasus edwardsii*, eyestalk

### Introduction

The transduction of extracellular messages across the plasma membrane to the intracellular environment is achieved by the interaction of regulatory molecules with specific membrane-spanning cell-surface receptors. In recent years, molecular cloning methods have allowed the identification of several hundred discrete G-protein-coupled receptor (GPCR) molecules and it has been established that approximately 80% of known hormones and neurotransmitters activate cellular signal transduction mechanisms by activating G-protein-coupled receptors (Birnbaumer et al., 1990; Iismaa et al., 1995; Bockaert and Pin, 1999). GPCRs are also involved in transduction of messages such as light, Ca<sup>2+</sup>, odorants, amino acid residues, nucleotides, peptides and proteins (Bockaert and Pin, 1999).

In the crustacean, the eyestalk X-organ sinus gland complex is an important site for the production of several endocrine factors, which are implicated in almost every aspect of crustacean physiology. These include the pigment concentration and dispersion hormones, and members of the CHH/MIH/VIH peptide family, which consists of the crustacean hyperglycemic hormone (CHH), a putative moult-inhibiting hormone (MIH), and the vitellogenesis-inhibiting

hormone (VIH). These are responsible for the regulation of glucose levels in the haemolymph, and inhibition of moulting and gonad development, respectively (Quackenbush, 1986; Keller, 1992). Precisely how these hormones transmit their messages to the cell is not known.

In a recent study, in order to isolate factors that are involved in the regulation of growth in the lobster, *Jasus edwardsii*, we constructed cDNA libraries using mRNAs from the eyestalk and a genomic library using DNA from gill tissues. Using a novel peptide sequence (NPS) (Khoo and Sin, 1999) isolated from the eyestalk as a probe in library screenings we isolated a number of cDNA clones. In the present study, we describe the characterisation of two novel protein genes from the lobster. We show that these genes encode proteins that are associated with membranes and share characteristics with the GPCRs of other organisms.

### Materials and methods

#### *Animals*

Live lobsters, *J. edwardsii* (Hutton 1875), were obtained from the Pacifica Kaikoura Ltd Fisheries at Kaikoura, New

Zealand. Eyestalks were ablated from live lobsters and stored in liquid nitrogen.

#### *Extraction of poly(A)<sup>+</sup> RNA*

Total RNA was extracted from 66 eyestalks (45 µg per eyestalk), and also from the epithelial tissue, gill, heart, hepatopancreas, and abdominal flexor muscle tissue of a single individual, by the acid guanidinium thiocyanate–phenol–chloroform extraction procedure (Chomczynski and Sacchi, 1987). Poly(A)<sup>+</sup> RNA was extracted from total RNA using the polyATtract® mRNA Isolation System (Promega, Madison, WI, USA).

#### *Cell-free translation*

The integrity of the mRNA isolated from the eyestalks was tested by cell-free translation using the wheat germ translation kit according to the manufacturer's instructions (Boehringer, Mannheim). The proteins were analysed by SDS-polyacrylamide gel electrophoresis on 12% gels, as described previously (Laemmli, 1970). After electrophoresis, the gels were treated in Amplify (Amersham Pharmacia Biotech, Little Chalfont, UK), dried under vacuum, and exposed to X-ray film for 3 days.

#### *cDNA library construction*

Using oligo(dT)<sub>12–18</sub> primer, cDNA was synthesized from 5 µg of eyestalk poly(A)<sup>+</sup> RNA using the TimeSaver cDNA synthesis kit (Amersham Pharmacia Biotech, Little Chalfont, UK). The cDNA, which appeared as a smear between 0.46 and 2.3 kilobases (kb) in an agarose gel, was cloned into the *Eco*RI site of λgt 10 vector, and packaged *in vitro* (Rosenberg, 1987) to give several libraries with a combined size of 5.6 × 10<sup>6</sup> plaque-forming units (p.f.u.) µg<sup>-1</sup> cDNA.

#### *cDNA library screening*

The cDNA library was screened initially by *in situ* plaque hybridisation (Sambrook et al., 1989), using a genomic DNA clone (Khoo, 1996) containing the NPS sequence (Khoo and Sin, 1999). Phage DNA was prepared from plate lysate stocks of phage samples showing positive hybridisation to the genomic clone (Santos, 1991; Sambrook et al., 1989), and analysed by Southern hybridisation to NPS. For library screening, Hybond-N nylon filters were washed to a final stringency of 1 × SSC (0.15 mol l<sup>-1</sup> NaCl, 0.015 mol l<sup>-1</sup> sodium citrate)/0.1% SDS at 65 °C, and exposed for autoradiography for 1–5 days. The DNA probes (10–100 ng) were labelled with [α-<sup>32</sup>P]dCTP (1.8 MBq, 3000 Ci mmol) to 10<sup>8</sup>–10<sup>9</sup> c.p.m. µg<sup>-1</sup> DNA using the random priming kit (NEBlot kit, New England Biolabs, MA, USA).

For sequencing, DNA from the NPS-positive cDNA clones was extracted using the WIZARD prep kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The purified DNA was digested with *Not*I restriction endonuclease (New England Biolabs, MA, USA). The digested DNA fragments were gel-purified and cloned into the *Not*I restriction site of a cDNA cloning plasmid vector, pSPORT 1 (Gibco BRL, New York).

#### *Sequence analysis of cDNA clones*

Double-stranded plasmid DNA templates were isolated by the boiling method (Sambrook et al., 1989) and were sequenced manually from both ends by the dideoxy sequencing method (Sanger et al., 1977) using the <sup>32</sup>P Sequencing kit (Amersham Pharmacia Biotech, Little Chalfont, UK). Nucleotide sequences were analysed using DNASIS, and the NCBI BLAST tool (Altschul et al., 1997).

#### *Northern blot analysis of mRNAs*

1–3 µg of poly(A)<sup>+</sup> RNA isolated from epithelial, eyestalk, gill, heart, hepatopancreas and abdominal muscle tissue were fractionated on a 1.2% agarose gel as described previously (Khoo and Sin, 1999), and transferred by capillary action in 10 × SSC onto Hybond-N (Amersham Pharmacia Biotech, Little Chalfont, UK). Heat-denatured *Hind*III digested λ DNA was included as a molecular size marker. Following transfer, the membrane was dried and the RNA was crosslinked to the membrane by UV transillumination.

Northern hybridisation was carried out as described by Lee et al. (Lee et al., 1992), but with a final post-hybridisation wash in 0.1 × SSC/0.1% SDS at 65 °C for 15 min. The membrane was exposed to X-ray film at –80 °C with intensifying screens for 2 h, 4 h and 14 h. The membrane was probed with the two cDNA sequences isolated. Prior to hybridisation with the second probe, the membrane was stripped by washing twice in 0.1 × SSC/0.5% SDS at 95 °C for 20 min, then once in 0.1% SDS at 100 °C for 20 min. After stripping, the membrane was then exposed to an X-ray film to determine whether the stripping of the radioactive materials was efficient. When no radioactivity was detected on the X-ray film after exposure for 16 h, the membrane was used for hybridisation with the second probe.

#### *Tissue expression of cDNA sequences using in situ hybridisation*

*In situ* hybridisation was carried out on epithelial tissue dorsal to the heart, eyestalk, gill, heart and abdominal flexor muscle in the cephalothorax region as described previously (Bloch et al., 1986; Khoo and Sin, 1999).

cDNA inserts were excised from the pSPORT plasmid with *Not*I restriction endonuclease, and gel purified by electroelution. 25 ng of cDNA insert were labelled with [α-<sup>32</sup>P]dCTP (1.5 MBq, 3000 Ci mmol), to 10<sup>8</sup> c.p.m. µg<sup>-1</sup> using the random prime labelling kit (Boehringer, Mannheim). The radiolabelled probe was purified by column chromatography prior to use as probes in northern blot analysis and *in situ* hybridisation.

As controls, RNase-digested tissue sections were included in each set of experiments. Control tissue sections were incubated in 2 × SSC containing 100 µg ml<sup>-1</sup> RNase A at 37 °C for 60 min prior to *in situ* hybridization. Both control and experimental slides were developed after a 3-day exposure period, which was the time required to produce an autoradiographic image according to Bloch et al. (Bloch et al., 1985).

## Results

### Construction and screening of the cDNA libraries

The quality of the mRNA from the eyestalks was evaluated by its ability to direct protein synthesis in a cell-free translation assay. More than 20 protein bands were radioactively labelled, including two major bands of approximately 13.8 and 20.7 kDa (Fig. 1, lanes 3 and 4). The positive control,  $\beta$ -globin mRNA, produced at least four protein bands with a major band, as expected, of 16.6 kDa (lanes 1 and 6). There was no background labelling in the water control (lanes 2 and 5).

In the primary screening of the cDNA libraries using a lobster genomic clone, 29 plaques were recovered. The NPS hybridised to the DNA from 10 plaques in Southern blot analysis. In the secondary screening using NPS as a probe, five clones were isolated and sequenced. Two distinct sequences, named peJK2 (four of the five clones) and peJK3 (one clone) were identified. However, these two genes showed low homology to NPS. Thus, we carried out more detailed sequence analysis with the aim of identifying their function.

### Sequence analysis of cDNA clones

The nucleotide and deduced amino acid (aa) sequences of the 568 bp cDNAs of clones peJK2 and peJK3 are shown in Fig. 2. peJK2 and peJK3 shared 96.6% sequence identity over a stretch of 558 bp. peJK2 contained an open reading frame encoding a 110-aa polypeptide with a deduced molecular mass of 11,177 Da, and a translation stop codon at nucleotide (nt) 401 followed by an untranslated region, which ended with a

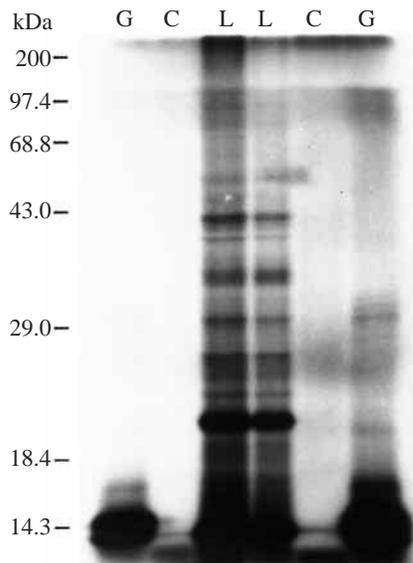


Fig. 1. Fluorogram of L-[<sup>35</sup>S]methionine-labelled proteins from a wheat germ cell-free translation assay of *Xenopus*  $\beta$ -globin mRNA (G), lobster eyestalk poly(A)<sup>+</sup> RNA (L) and water control (C). 5  $\mu$ l of translation product was loaded in lanes 1, 2 and 4, and 10  $\mu$ l in lanes 3, 5 and 6, on a 12% polyacrylamide-SDS gel. Protein molecular mass standards (kDa) are indicated on the left. The dried gel was exposed for fluorography for 3 days at  $-80^{\circ}\text{C}$ , with intensifying screens.

poly(A)<sup>+</sup> tail 146 nt downstream. The polyadenylation signal AATAAA (Birnstiel et al., 1985) was located between nucleotides 523 and 530, 26 nucleotides upstream from the poly(A)<sup>+</sup> tail, which was 19 residues long.

peJK3 contained an open reading frame encoding a 113-aa polypeptide of 11,602 Da. The 5' end of peJK3 begins 10 nt upstream from the 5' end of peJK2, and except for 10 base substitutions (at nt 131, 249, 273, 282, 313, 359, 504, 519, 550 and 564), and 9 additional bases (at nt 143-145 and 169-174), the two sequences were essentially the same. The base substitutions cause a change in the deduced aa, and the additional bases code for an extra alanine and two valine aa residues. The translational stop codon at nt 420 was followed by an untranslated region, with the AATAAA polyadenylation signal between nt 542 and 549. The poly(A)<sup>+</sup> tail was absent in peJK3 and was probably lost during the cloning process, as this feature was used to isolate the poly(A)<sup>+</sup> RNA, and also for binding of the oligo(dT)<sub>12-18</sub> primer in cDNA synthesis.

### Analysis of the deduced peJK proteins

The crustacean eyestalk neuropeptides CHH, VIH and MIH are considered to be related (reviewed in Keller, 1992; de Kleijn and Van Herp, 1995). Multiple sequence alignment of the putative peptides of peJK2 and peJK3 with these sequences, retrieved from the BLASTP searches, and published crustacean eyestalk neuropeptide sequences (reviewed in Lacombe et al., 1999) was performed using CLUSTALW (Thompson et al., 1994) on the European Bioinformatics Institute (ebi) server. Alignment of the sequences showed that the motifs present in the CHH/VIH family (Lacombe et al., 1999) were absent from the deduced protein encoded by peJK2 and peJK3. The invariant six cysteine residues, which form the three disulfide bridges, and the conserved aa residues located in the vicinity of the six cysteine residues (de Kleijn and Van Herp, 1995) were absent from the deduced proteins.

The hydrophobicity plots of peJK2 and peJK3 show that the N-terminal and central domain of these putative peptides were hydrophobic, with a high proportion of consecutive valine and alanine residues (Fig. 3). The hydrophobic domain suggests that this domain was buried in the membrane (von Heijne, 1987; Lehninger et al., 1993). The theoretical pI values of peJK2 and peJK3 were calculated to be 10.0 and 10.79 (Bjellqvist et al., 1993).

A search for signal sequences in the deduced peptides of peJK2 and peJK3 was conducted using SignalP (Nielsen et al., 1997), and by McGeoch's method (McGeoch, 1985), followed by von Heijne's method of signal recognition (von Heijne, 1992). A cleavage site was assigned between aa residues 34 and 35: VVA(34)-T(35)M, and between 37 and 38: VVA(37)-T(38)M, in peJK2 and peJK3, respectively. Based on this assignment, the extra aa encoded by peJK3 was found in the signal peptide, which would be cleaved from the mature peptide once transport was under way.

The deduced proteins were further characterised using TopPred 2 (von Heijne, 1992), which predicted two

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Met Val Tyr Val Ala Leu
pejk2  -9  ....CGAAGAACACCAAGACCCAGCCCAACATGAACCTTTCTGCGAGCTCTGCTGATGATGGCGCCCGTGATGGC ATGGTG TAC GTG GCC CTG 88
pejk3  1  CTCGTGCCAC 98

Ala Ser Gln Ser Val Ser Pro Ala Ala Asp Met Val Val Ala Ala Ser Val Val Val Val Val Val Val Ala
pejk2  89  GCG AGC CAG AGC GTG AGC CCG GCC GCA GAT ATG GTG GTG GCG GC- --T TCG GTG GTG GTG GTG GTG GTG G-- --- -CG 157
pejk3  99  ATC GCG GCT GTG GTG GCG GTG GTG GCG 176
Ile Ala Ala Val Val Ala

Ala Ser Val Val Ala Thr Met Glu Ala Ala Ser Val Val Ala Ala Ser Val Val Ala Val Ser Val Val Ala Val Val
pejk2  158 GCT TCG GTG GTG GCC ACC ATG GAG GCA GCT TCG GTG GTG GCA GCT TCG GTG GTG GCA GTT TCG GTG GTG GCC GTC GTG 235
pejk3  177 ATC 254
Ile

Val Ala Met Ala Ala Thr Val Ala Arg Gly Asn Lys Leu Asp Val Arg Gln Asp Gly Gln Thr Asn Lys Ile Tyr Pro
pejk2  236 GTG GCT ATG GCG GCT ACC GTG GCT AGA GGT AAC AAA CTG GAT GTG CGG CAG GAC GGA CAG ACG AAC AAG ATC TAC CCA 313
pejk3  255 ATG CGT CCG 332
Met Arg Arg

Arg Glu Pro Ala Arg Arg Arg Ala Cys Gly Ser Asp Val Thr Pro Ala Arg Gln Arg Pro Gly Val Ser Ser Phe Gln
pejk2  314 CGA GAA CCA GCA AGA CGA CGA GCG TGT GGC AGT GAC GTC ACT CCT GCA CGA CAA CGT CCA GGC GTC TCC AGT TTT CAA 391
pejk3  333 TGC 410

Leu Val Pro stop

pejk2  392 CTC GTC CCT TAA CTACAACCTTAAAGGAATCTCCACACATTAAGATGTAATTCGAAGCGATCTTTTGCATTGTGCTTAATGTTTCTCCTTAAGTGAATATT 490
pejk3  411 G 509

pejk2  491 TGTTTTTATAAGATTGTAAATATCTCAAAATCAAATAAACAAGAACTGGAAGATTTTCGAAAAAAAAAAAAAAAAAAAAA 3' 568
pejk3  510 G C A ..... 3' 568

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Fig. 2. Sequence comparison of the nucleotide sequences and deduced aa sequences of cDNA clones peJK2 and peJK3 (568 bp long). The two clones were isolated from a cDNA library of the lobster eyestalk, and share 96% sequence identity. The deduced aa sequence is shown above the nucleotide sequence; both the initiation and stop codons are underlined, and the polyadenylation signal **AATAAA** is indicated in bold-face type. The sequence data has been deposited in the GenBank database (accession no. AF112985 and AF112986).

transmembrane alpha helices present in the deduced proteins encoded by peJK 2 and peJK3 (Fig. 4). In peJK2 protein, helix 1 is between aa 17 and 37, and helix 2 between aa 43 to 63. Similar helices were found in peJK3, between aa 17 and 37, and aa 46 and 66. Motifs were detected using PROSITE (Hofman et al., 1999). peJK2 had one GPCR motif II, VAVVVAMAATVARGN, located in the deduced aa sequence in the second transmembrane helix, but a similar sequence (VAIVVAMAATMARAN) was identified in peJK3 in a similar region of the deduced peptide. A protein kinase C phosphorylation site (TNK) (Kishimoto et al., 1985) was also detected in position 76 in peJK2 and position 79 in peJK3.

#### Tissue expression analysis by northern blot

Northern blot analysis using peJK2 and peJK3 as probes detected strong hybridisation to a 0.70 kb band in eyestalk mRNA after 2 h exposure (Fig. 5A). A faint band (0.70 kb) was seen in the epithelial tissue after 4 h exposure (Fig. 5B). After 14 h exposure, several other bands were detected in the epithelial tissue (1.7, 0.40, 0.30 kb) and eyestalk (1.7, 0.43 kb).

peJK2 also hybridised to at least three additional bands (2.16, 1.8 and 0.34 kb) in the heart and in the hepatopancreas (6.0, 2.2 and 0.90 kb), and at least four bands (5.35, 1.7, 0.70 and 0.36 kb) in the muscle after 14 h exposure (Fig. 5C).

Hybridisation bands on the autoradiograms were analysed by measuring the optical density of each band with a scanner. The autoradiogram was scanned using three different sensitivity settings to establish a linear relationship between the band intensity and scanner sensitivity. Readings were normalized to 1- $\mu$ g quantities of poly(A)<sup>+</sup> RNA, and expressed as a percentage of the eyestalk band for that particular sensitivity (Khoo and Sin, 1999). The intensities of the bands detected by peJK2 and peJK3, expressed relative to the major eyestalk band, showed that peJK2 and peJK3 mRNA transcripts were produced primarily in the lobster eyestalk, and to a lesser extent, in other tissues (Table 1). After 14 h (Fig. 5C), the intensity of the 0.7 kb epithelial band was approximately one third the intensity of the major eyestalk band, and twofold to fivefold greater than the heart, hepatopancreas and muscle bands (Table 1).

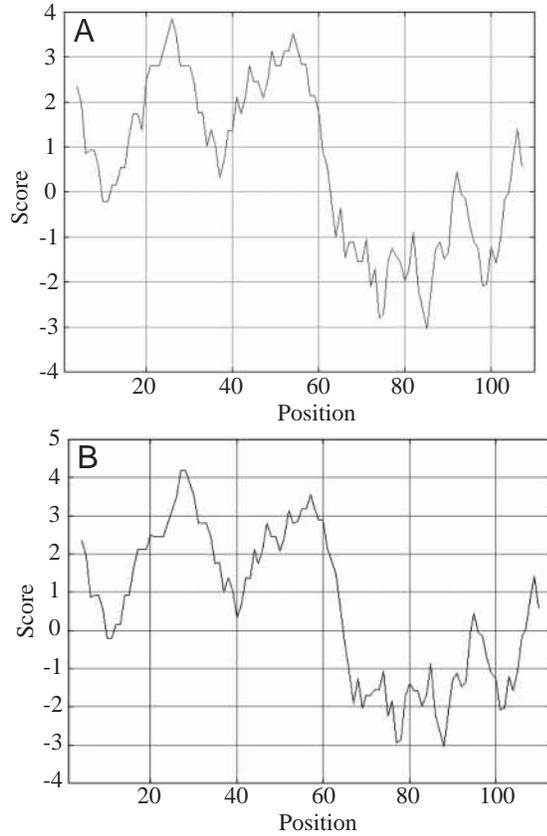


Fig. 3. Hydrophobicity plots of the deduced aa sequences of peJK2 (A) and peJK3 (B) (based on Kyte and Doolittle, 1982).

Table 1. Relative intensities of northern hybridization bands detected by peJK2 and peJK3, after 14 h exposure

Tissue	Band size (kb)	Mean % $\pm$ s.d. <sup>a</sup>
Epithelia	1.7	3.1 $\pm$ 0.6 <sup>b</sup>
	0.7	29.0 $\pm$ 3.0
Eyestalk	0.40, 0.30 <sup>c</sup>	
	1.7	3.0 $\pm$ 0.5 <sup>d</sup>
	0.7	100
Heart	0.43 <sup>c</sup>	
	0.34	14.8 $\pm$ 2.3
Hepatopancreas	2.16, 1.8 <sup>c</sup>	
	6.0	2.0 $\pm$ 0.6
	0.9	4.9 $\pm$ 0.6
Muscle	2.4, 2.2 <sup>c</sup>	
	0.7	4.7 $\pm$ 0.9
	5.35, 1.7, 0.36 <sup>c</sup>	

Optical densitometry measurements of the more obvious bands were normalized to 1  $\mu$ g, and expressed as a percentage of the major eyestalk band.

<sup>a</sup>The standard deviation reflects the amount of variation obtained when both autoradiograms were each scanned using three different sensitivity settings. Unless indicated below, *N*=6.

<sup>b</sup>Mean % based on three readings.

<sup>c</sup>Minor bands detected at 14 h.

<sup>d</sup>Mean % based on four readings.

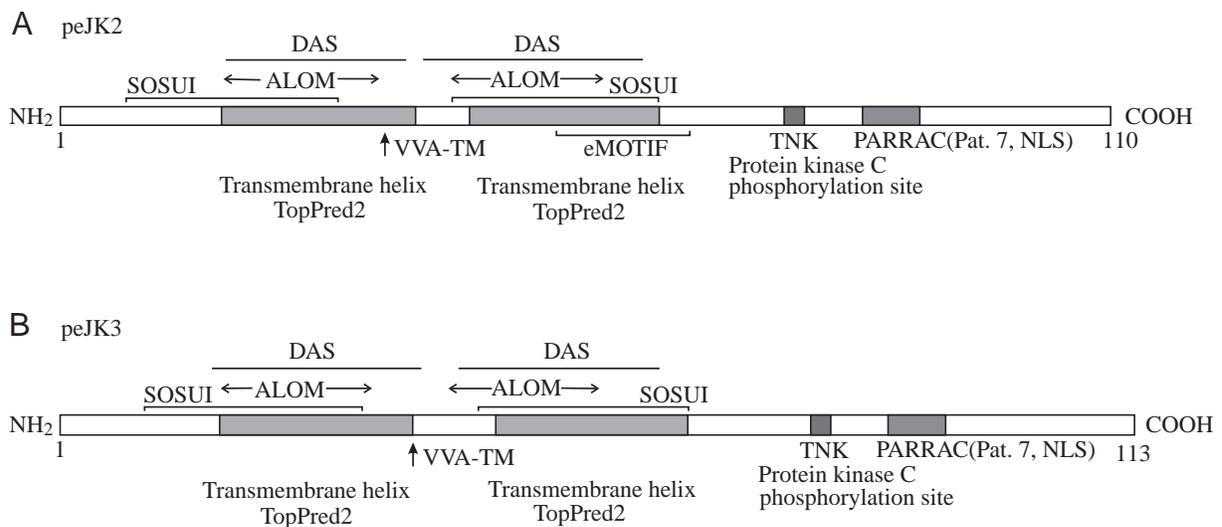


Fig. 4. Deduced features of peptides coded by peJK2 (A) and peJK3 (B). Transmembrane helices were predicted and analysed using TopPred 2 (von Heijne, 1992) (light grey boxes), SOSUI (Hirokawa et al., 1998), DAS (Cserzo et al., 1997), and ALOM (Klein et al., 1985; Nakai and Kanehisa, 1992) through the PSORT server. The GPCR motif II was detected in peJK2 only using eMOTIF (Attwood et al., 1997). The positions of the predicted signal peptide cleavage sites (VVA-TM), protein kinase C phosphorylation sites (TNK) and nuclear localization signal, pattern 7 sites (PARRAC) (Hicks and Raikhel, 1995) are also indicated.

Fig. 5. Northern blot analysis of poly(A)<sup>+</sup> RNA from epithelial tissue (p), eyestalk (e), gill (g), heart (h), hepatopancreas (hp) and muscle (m). The blot was hybridised to cDNA probe peJK2, and a final high stringency wash of 0.1× SSC/0.1% SDS at 65 °C was included. The blot was exposed for 2 h (A), 4 h (B) and 14 h (C). The positions of the 28S and 18S rRNA bands are indicated on the right, and single-stranded *Hind*III-digested  $\lambda$  DNA size markers (kb) are indicated on the left. The positions of the bands are indicated by arrowheads; large arrowheads indicate the major eyestalk band (0.7 kb).

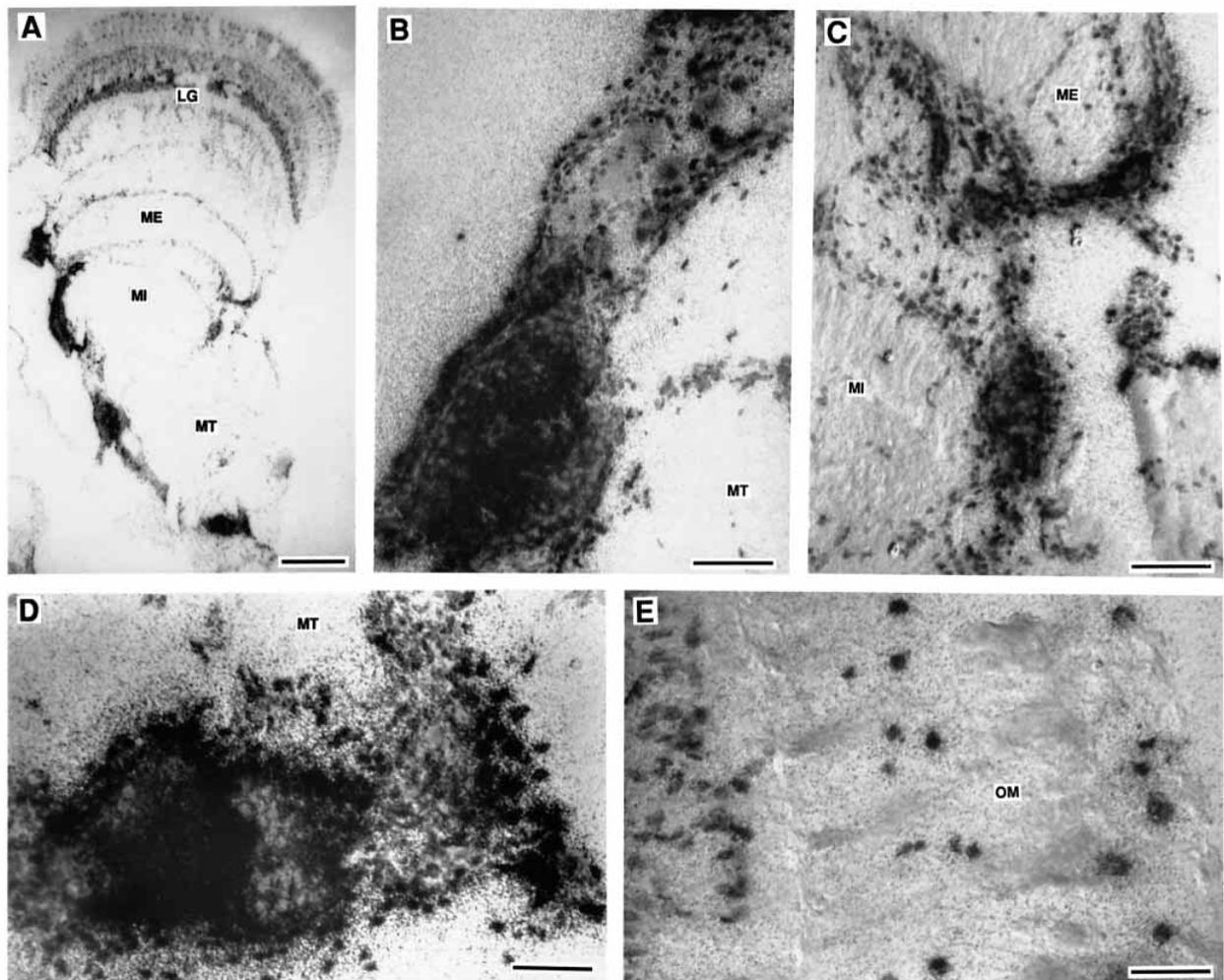
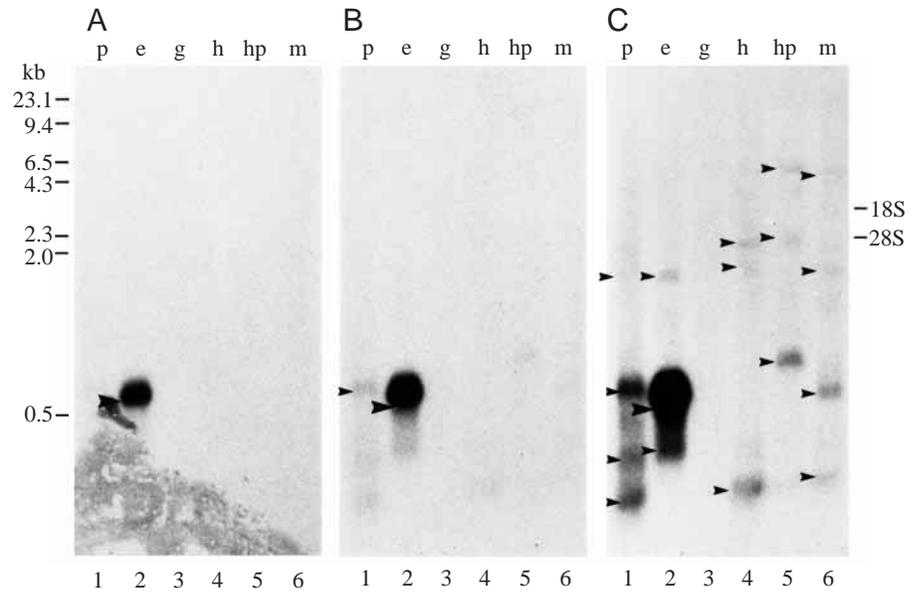


Fig. 6. *In situ* hybridization of peJK2 to the longitudinal section of the lobster eyestalk. peJK2 hybridised to the neurosecretory regions of the eyestalk (A), as shown by the dense accumulation of silver grains in the eyestalk section whole mount. Nuclear localization of peJK2 was shown in the neurosecretory cell groups of the medulla externa and interna (A,C), the medulla terminalis (B), the X-organs of the medulla terminalis (D), and above the lamina ganglionaris, in the retina (E). LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; OM ommatidia. Bars, 500  $\mu$ m (A); 100  $\mu$ m (B,C), 50  $\mu$ m (D,E).

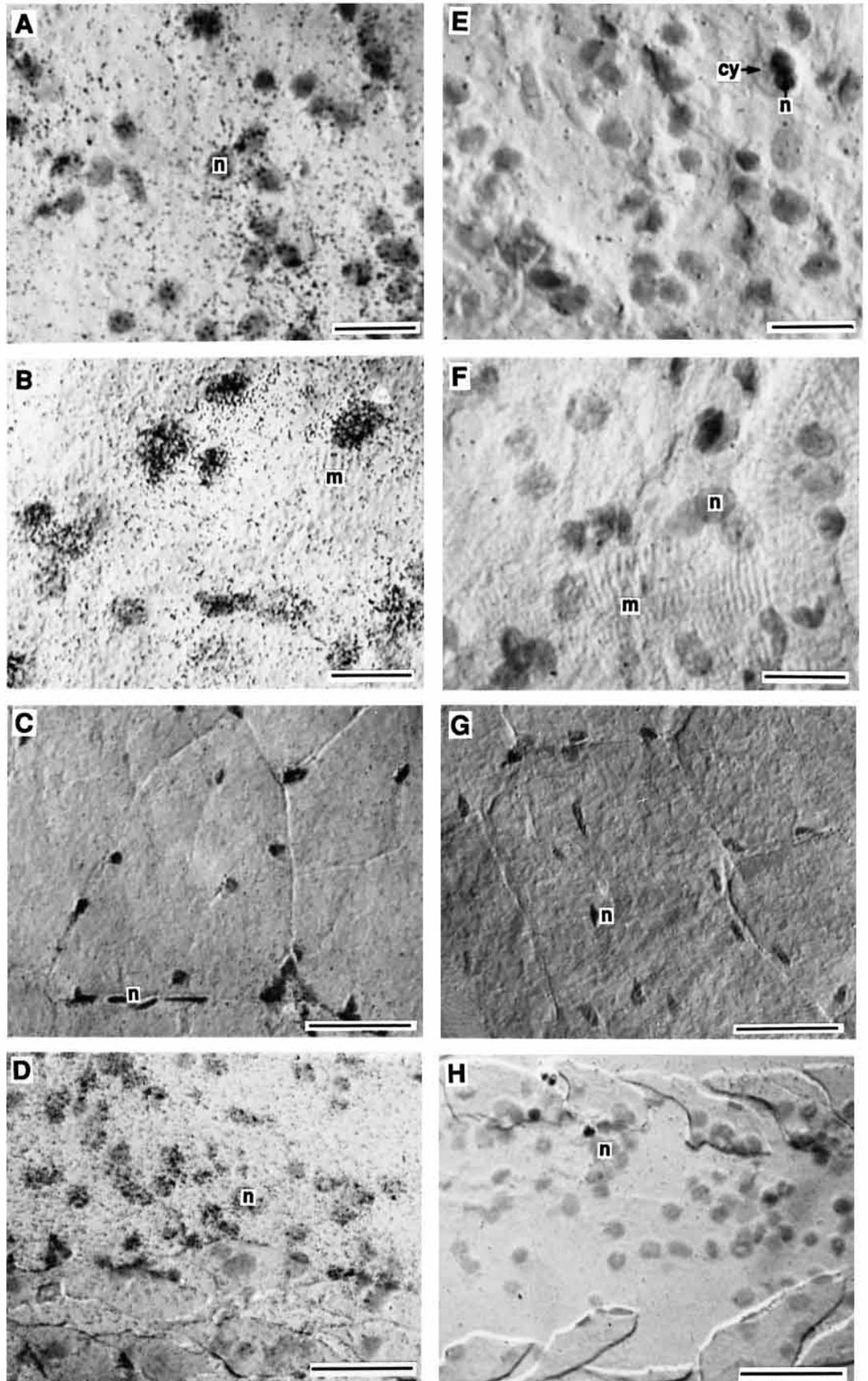


Fig. 7. *In situ* hybridization of peJK2 to lobster body tissue sections. Nuclear and cytoplasmic hybridization of peJK2 is evident as silver grains accumulated over the nuclei and tissue of the epithelium (A) and heart (B). peJK2 hybridization is confined to the nuclei of the muscle fibres (C) and the nuclei and cytoplasm of the cells within the gill lamellae (D). RNase treatment of the controls reduced probe binding to background levels (E–H). cy, cytoplasm; n, nucleus; m, muscle. Bars, 20  $\mu$ m (A,B,E,F); 50  $\mu$ m (C,D,G,H).

#### In situ hybridisation analysis of *peJK2* and *peJK3*

In the eyestalk, *peJK2* hybridised to the neurosecretory cell groups of the medulla externa and interna, the X-organs of the medulla terminalis, and to the region above the lamina ganglionaris including the retinal and ommatidia cells (Fig. 6A–E). Some of these regions (for example Fig. 6D) were so saturated with silver grains that the nuclei were obscured. Fig. 7 shows, at higher magnification, that *peJK2* hybridised to both the nuclei and cytoplasm of cells in the epithelial and heart tissue (Fig. 7A,B). In the muscle tissue, nuclear hybridisation of *peJK2* was observed (Fig. 7C); approximately 40% of the myocardial cell nuclei showed probe hybridisation. RNase treatment of the controls reduced the silver grain density to background levels (Fig. 7E–H) (paired *t*-test;  $P < 0.0001$ ). *peJK2* hybridised to the cell nuclei and cytoplasm within the gill lamellae of the experimental slide, but not in RNase-treated controls (Fig. 7D,H).

When expression of *peJK2* in the different tissues were compared, the number of nuclei showing nuclear hybridisation was significantly different between the tissue types. Nuclear hybridisation of *peJK2* was highest in the heart tissue (paired *t*-test;  $P < 0.005$ ), and lowest in the epithelial tissue (paired *t*-test;  $P < 0.0005$ ). *peJK3* showed similar results to *peJK2* (not shown).

#### Discussion

In this study, our aim was to isolate genes from an eyestalk cDNA library of the lobster, *Jasus edwardsii*, that may be related to a novel peptide sequence (NPS) that was identified in a previous study (Khoo and Sin, 1999). Using this NPS sequence as a probe we have isolated a number of cDNA clones, but sequence comparison revealed low homology between NPS and these novel genes. This paper reports more detailed studies carried out on two of these clones, *peJK2* and *peJK3*. These two clones appear to be coding for proteins, but are unrelated to NPS. Expression of the two cloned genes was detected in a wide range of tissues, but predominantly in the eyestalk. These two clones share 96.6% sequence identity, suggesting that they are possibly allelic forms of the same gene, or that *peJK3* may be a different splicing product of the same pre-mRNA. Both these clones detected mRNAs of a similar size (0.7 kb) in extracts of tissues such as the eyestalk, epithelia and muscle, suggesting that the same mRNA transcript was being produced in all these tissues, but at different levels. In addition to this RNA band, *peJK2* and *peJK3* also detected several mRNA transcripts of varying sizes in different tissues such as heart and hepatopancreas. However, no detectable level of hybridisation was observed in the gill extracts under the conditions used. These mRNA bands could be either related sequences or products of tissue-specific alternate splicing of a common precursor (Amara, 1985; McKeown, 1992).

Sequence analyses suggest that the deduced proteins encoded by these two cDNA clones have two transmembrane alpha helices. Analyses of the aa sequences using four different

programmes, TopPred 2 (von Heijne, 1992), SOSUI (Hirokawa et al., 1998), DAS (Cserzo et al., 1997) and ALOM (Klein et al., 1985; Nakai and Kanehisa, 1992), all indicate the presence of two transmembrane helices (Fig. 4). Analysis of the aa sequence of the deduced proteins further suggests the presence of a signal peptide in these peptides. The signal peptide itself is a transient N-terminal signal sequence found in most secretory proteins, and serves to initiate export across the endoplasmic reticulum (von Heijne, 1986; Lehninger et al., 1993). Thus, it is highly likely that these peptides are synthesised and transported to the membrane where the signal peptide is cleaved and the remaining peptide forms part of the membrane structure.

Further, *peJK2* has a GPCR motif in the second transmembrane helix, while *peJK3* had a sequence in the same region showing high homology to the GPCR motif. The presence of a GPCR motif strongly indicates that these peptides may function as signal transducers. This, however, is in contrast to the rhodopsin-like GPCRs, a widespread protein family that includes hormones, neurotransmitter and light receptors, which have seven GPCR motifs (Lameh et al., 1990; Attwood and Findlay, 1993; Bockaert and Pin, 1999). In order for the peptides encoded by *peJK2* and *peJK3* to function as a signal transducer, they would have to form a complex structure consisting of multiple units of the peptides. The variation of the GPCR motif-like sequence in *peJK3* may suggest that this is an error resulting from sequencing or cloning, or it might truly represent an allelic form of the GPCR motif in the lobster.

The presence of a kinase C phosphorylation site in these deduced proteins strongly suggests that they are phosphoproteins and their function may be mediated by protein kinase C (Pitcher et al., 1998). Phosphorylation of the receptor molecules after interaction with ligands is a known mechanism in GPCR regulation (Daaka et al., 1997; Carman et al., 1998).

At present, there is no explanation for the presence of a nuclear localisation signal in the carboxyl terminal of both deduced proteins. Detailed functional analyses are required to determine the functional significance and the mechanism of function of these proteins.

From the evolutionary perspective, the identified features of these deduced peptides suggest that the proteins encoded by the *peJK* genes may be related to, or derived from, the same ancestral gene(s) for the GPCR family of proteins. Their evolutionary relationship to other GPCR proteins will await the elucidation of the function and mechanism of action of these proteins.

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