Three different prohormones yield a variety of Hydra-RFamide (Arg-Phe-NH_2) neuropeptides in *Hydra magnipapillata*

Dorothea DARMER*†[1], Frank HAUSER*, Hans-Peter NOTHACKER*†[2], Thomas C. G. BOSCH‡, Michael WILLIAMSON* and Cornelis J. P. GRIMMELIKHUIJZEN*†[3]

*Department of Cell Biology and Anatomy, Zoological Institute, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen Ø, Denmark, †Center for Molecular Neurobiology (ZMNH), University of Hamburg, Martinistrasse 52, D-20246 Hamburg, Federal Republic of Germany, and ‡Zoological Institute, University of Jena, Erbertstrasse 1, D-07743 Jena, Federal Republic of Germany

The freshwater polyp *Hydra* is the most frequently used model for the study of development in cnidarians. Recently we isolated four novel Arg-Phe-NH_2 (RFamide) neuropeptides, the Hydra-RFamides I–IV, from *Hydra magnipapillata*. Here we describe the molecular cloning of three different preprohormones from *H. magnipapillata*, each of which gives rise to a variety of RFamide neuropeptides. Preprohormone A contains one copy of unprocessed Hydra-RFamide I (QWLGGRFG), II (QWFNGRFG), III/IV [(KP)HLRGRFG] and two putative neuropeptide sequences (QLMSGRFG and QLMRGRFG). Preprohormone B has the same general organization as preprohormone A, but instead of unprocessed Hydra-RFamide III/IV it contains a slightly different neuropeptide sequence [(KP)HYRGRFG]. Preprohormone C contains one copy of unprocessed Hydra-RFamide I and seven additional putative neuropeptide sequences (with the common N-terminal sequence QWF/LSGRFGL). The two Hydra-RFamide II copies (in preprohormones A and B) are preceded by Thr residues, and the single Hydra-RFamide III/IV copy (in preprohormone A) is preceded by an Asn residue, confirming that cnidarians use unconventional processing signals to generate neuropeptides from their precursor proteins. Southern blot analyses suggest that preprohormones A and B are each coded for by a single gene, whereas one or possibly two closely related genes code for preprohormone C. Northern blot analyses and *in situ* hybridizations show that the gene coding for preprohormone A is expressed in neurons of both the head and foot regions of *Hydra*, whereas the genes coding for preprohormones B and C are specifically expressed in neurons of different regions of the head. All of this shows that neuropeptide biosynthesis in the primitive metazoan *Hydra* is already rather complex.

INTRODUCTION

Cnidarians are the lowest animal group to have a nervous system and it was probably within cnidarians, or a related ancestor phylum, that nervous systems first evolved [1]. The primitive nervous systems of cnidarians are strongly peptidergic. From a single sea anemone species *Anthopleura elegantissima* we have recently isolated 16 different neuropeptides, and about 15 neuropeptides have been isolated from other cnidarian species (reviewed in [2]). The sea anemone neuropeptides are located in neuronal dense-cored vesicles and low concentrations of these peptides (1–10 nM) have excitatory or inhibitory actions on intact sea anemones, isolated muscle preparations or isolated muscle cells, suggesting that they are transmitters [2].

Cnidarians, and especially the freshwater polyp *Hydra*, have an amazing potency to regenerate. From small slices of *Hydra*, for example, or even from dissociated and subsequently reassociated cells, new, complete animals develop within a few days [3,4]. Small diffusible substances have been proposed to control regeneration and pattern formation in cnidarians [4,5] and this makes the presence of a large number of neuropeptides in cnidarians especially interesting because some of these substances might have a function in development. Of all cnidarians, *Hydra* is the most widely used model for the study of development. Recently, with a radioimmunoassay for the sequence Arg-Phe-NH_2 (RFamide), we isolated four novel neuropeptides from *Hydra magnipapillata*, Hydra-RFamide I (< Glu-Trp-Leu-Gly-Gly-Arg-Phe-NH_2), Hydra-RFamide II (< Glu-Trp-Phe-Asn-Gly-Arg-Phe-NH_2), Hydra-RFamide III (Lys-Pro-His-Leu-Arg-Gly-Arg-Phe-NH_2), and Hydra-RFamide IV (His-Leu-Arg-Gly-Arg-Phe-NH_2) [6]. By using RFamide antisera we have shown that the Hydra-RFamides are neuropeptides produced by a plexus of neurons located in the head and foot regions of *Hydra* [7]. With the use of immunoelectron microscopy we have also demonstrated that the RFamide neuropeptides in *Hydra* are located in dense-cored neurosecretory vesicles [8]. Here we describe the molecular cloning of three different preprohormones for the Hydra-RFamides and show that the biosynthesis of neuropeptides in the simple metazoan *Hydra* is already rather sophisticated.

MATERIALS AND METHODS

Radioactive labelling of DNA probes

DNA fragments were labelled with [α-^{32}P]dCTP (Amersham; specific radioactivity 110 TBq/mmol) with the Ready-To-Go DNA-labelling kit from Pharmacia [9]. Oligonucleotides were labelled with 250 μCi of [γ-^{32}P]ATP (Amersham; specific radioactivity 110 TBq/mmol) by using 10 units of T4 polynucleotide

Abbreviations used: RFamide, Arg-Phe-NH_2; SSC, 150 mM NaCl/15 mM sodium citrate (pH 7.0).

[1] Present address: Institute for Pathophysiology, Martin Luther University of Halle-Wittenberg, Magdeburger Strasse 18, D-06112 Halle, Federal Republic of Germany.

[2] Present address: Department of Pharmacology, College of Medicine, University of California, 354 Med. Surg. II, Irvine, CA 92697-4625, U.S.A.

[3] To whom correspondence should be addressed at the University of Copenhagen.

The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers Y11678, Y11679 and Y11680.

kinase (Stratagene) for 30 min at 37 °C. After being heated at 70 °C for 10 min, the unincorporated nucleotides were removed by passing the mixture through a NAP-5 column (Pharmacia).

Screening of the *Hydra* cDNA library

H. magnipapillata was cultured as described previously [10]. A λgt11 cDNA library from 1-day-starved *H. magnipapillata* was prepared as described earlier for sea anemones [11,12]. This library consisted of 2×10^7 primary plaques and was screened with a ^{32}P-labelled oligonucleotide, 5′-CCA/GAAICT/GICC/ICCIAA/GCCAT/CTG-3′, corresponding to the presumed unprocessed amino acid sequence of Hydra-RFamide I, or with a labelled cDNA fragment. Plaque lifting and hybridization procedures were performed as described in [13]. Hybridization and final washing steps were at 39 °C for the oligonucleotide probe and at 60 °C for a cDNA fragment.

Cloning of the 5′ end of preprohormone B cDNA

The 5′ end of the preprohormone B cDNA was cloned by a nested PCR approach. The first round of amplification was performed with a λgt11 reverse primer, 5′-CCAGACCAACTGGTAATG-3′, and with the oligonucleotide corresponding to the immature Hydra-RFamide I sequence described above. The reaction mixture (20 μl) consisted of 1 × *Taq* buffer (Promega), containing 2.5 mM $MgCl_2$, 0.75 unit of *Taq* polymerase (Promega) and various concentrations of *Hydra* λgt11 cDNA library (10^6 or 2.5×10^5 plaque-forming units). The PCR parameters were: 30 cycles at 96 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The resulting PCR products were diluted 1:10 in *Taq* buffer and subjected to a second round of PCR with the same conditions as described above but replacing the primer corresponding to Hydra-RFamide I with an anti-sense primer, 5′-TGGAATTCCCTGACTCTCTGTTTCG-3′, corresponding to positions 387–366 in the preprohormone B cDNA sequence (see Figure 2) and containing three additional 5′ nucleotides to give an *Eco*RI site for subcloning. The resulting PCR product of about 350 bp was digested with *Eco*RI, gel-purified, subcloned into pBluescript SK(+) (Stratagene) and sequenced.

DNA sequencing

cDNA insertions from positive λgt11 clones were excised with *Eco*RI and subcloned into pUC19 for sequencing. Subclones containing the largest cDNA insertion coding for preprohormone C were sequenced after the generation of *Exo*III/mung bean nuclease deletion clones. These clones were obtained after cutting with *Pst*I and *Bam*HI and subsequent digestion with exonuclease III (Stratagene), in accordance with a protocol from the manufacturer (digestion was at 30 °C with six deletion time points of 90 s each). After this, blunt ends were produced with mung bean nuclease and re-ligation was performed with T4 DNA ligase (both from New England Biolabs). The deleted plasmids were subsequently transformed into *Escherichia coli* JM109 and prepared for sequence analyses. Sequencing was performed by the dideoxynucleotide chain termination method [14] with a T7 sequencing kit from Pharmacia and [α-^{35}S]dATP (Amersham). All subclones were sequenced in both directions. DNA sequence compilation, nucleotide and amino acid sequence comparisons, and database searches were performed with the DNASTAR program (DNASTAR).

Northern blots

Total RNA from *H. magnipapillata* and other species was prepared as described in [15]. Poly(A)$^+$ RNA was prepared by affinity chromatography with two passages through oligo(dT)-cellulose columns. The RNA samples and RNA marker III (Boehringer Mannheim) were denatured with glyoxal and DMSO and subjected to electrophoresis on 1.2% (w/v) agarose gels as described in [13]. The RNA was then blotted on a nylon membrane (Hybond N; Amersham) by capillary transfer and fixed to the membranes by UV irradiation as recommended by the manufacturer. Hybridization with labelled cDNA fragments was performed at 60 °C in a solution containing 6 × SSC [SSC is 150 mM NaCl/15 mM sodium citrate (pH 7.0)], 2 × Denhardt's solution [Denhardt's solution is 0.02% BSA/0.02% poly(vinylpyrrolidone)/0.02% Ficoll], 0.1% SDS, 0.01% herring sperm DNA and 50 mM sodium phosphate, pH 7.0. For hybridization, various ^{32}P-labelled cDNA fragments were used at a concentration of 5×10^5 c.p.m./ml. Washing was at 60 °C in 5 × SSC containing 0.1% SDS, with one final wash at 60 °C in 1 × SSC containing 0.1% SDS. Hybridization with oligonucleotide probes was performed at 42 °C in the same hybridization medium as for the cDNA fragments, except that 5 × Denhardt's solution was used in place of 2 × Denhardt's. With these probes, washing was at room temperature with 6 × SSC containing 0.1% SDS, with one final wash in the same washing solution at 40 °C. We used 5′-TGTTTCTAAGTAATCATTTACG-3′ as a specific oligonucleotide probe for preprohormone A, and 5′-AGCGTCTAAAATATCTTTTATA-3′ as a specific oligonucleotide probe for preprohormone B.

Southern blots

Genomic DNA from *H. magnipapillata* was prepared as described in [13], digested with various restriction enzymes and then separated on 0.8% agarose gels. Transfer to a Hybond-N membrane, hybridization with cDNA probes and washing were done as recommended by the supplier (Amersham). A final washing of the blots was performed with 0.1% SSC/0.1% SDS at 65 °C for 1 h.

In situ hybridization

Whole-mount *in situ* hybridization with digoxigenin-labelled RNA probes was performed as described in [16], with the following slight modifications. pBluescript vector (Stratagene) containing the required cDNA insertion was linearized by cleavage at a suitable position. Sense or anti-sense transcripts were obtained *in vitro* by using T3 or T7 RNA polymerase and the DIG RNA-labelling kit (Boehringer Mannheim). Hybridization was performed for 3 days at 55 °C in 50% formamide/5 × SSC/0.1% (v/v) Tween 20/0.1% CHAPS (Sigma)/1 × Denhardt's solution/0.01% heparin/0.2 μg/ml labelled RNA probe. Staining was performed in the dark with BM-Purple (Boehringer Mannheim) as a substrate. Whole mounts were investigated and photographed with a Leitz DMRD/Leica DMRXA microscope.

RESULTS

Three different Hydra-RFamide preprohormones

We screened 1.3×10^6 phages of our *Hydra* λgt11 cDNA library with a degenerate oligonucleotide probe corresponding to the presumed immature sequence of Hydra-RFamide I (Gln-Trp-Leu-Gly-Gly-Arg-Phe-Gly). This yielded 28 positive clones, of which the cDNA insertions were subcloned and sequenced. Three different types of cDNA could be identified that each coded for a preprohormone containing one copy of immature Hydra-RFamide I and a variety of other neuropeptide sequences: 16 clones contained a cDNA coding for a preprohormone of type

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                                                                TCGGGCCGCTAAAG   14

ATG TTG AGC AAT AAA AAA GTC GAA TTG TTA TTT GCT TTG GTG TTC GTT GTT GTA GCT GTT   74
Met Leu Ser Asn Lys Lys Val Glu Leu Leu Phe Ala Leu Val Phe Val Val Val Ala Val   20

GTA AGA TCT GAA GAT AAA AAA CTT TCA TTA GAA GAT AAC AAG GAT GTA AAA AGA ATC GTA  134
Val Arg Ser Glu Asp Lys Lys Leu Ser Leu Glu Asp Asn Lys Asp Val Lys Arg Ile Val   40

AAT GAT TAC TTA GAA ACA AAA AAT GGA GAA CAG CTT ATG TCA GGA AGG TTT GGA AAA AGA  194
Asn Asp Tyr Leu Glu Thr Lys Asn Gly Glu Gln Leu Met Ser Gly Arg Phe Gly Lys Arg   60

GAA ACT GAT GAG GCA GAC AGT GAT GAT GAA GAT AGC AGT GAA TAT GAA AAT GAA TAC GAT  254
Glu Thr Asp Glu Ala Asp Ser Asp Asp Glu Asp Ser Ser Glu Tyr Glu Asn Glu Tyr Asp   80

GAT GAA TTA GAA AAC CAA GGA CTT GCG AAT GCG AGA TAT GAA AGA CAA CTA ATG CGA GGA  314
Asp Glu Leu Glu Asn Gln Gly Leu Ala Asn Ala Arg Tyr Glu Arg Gln Leu Met Arg Gly  100

CGA TTT GGA AGA GAA AAA AAT GCA GCA AGT AAC GAG GAT CAG TGG CTT GGA GGA CGT TTT  374
Arg Phe Gly Arg Glu Lys Asn Ala Ala Ser Asn Glu Asp Gln Trp Leu Gly Gly Arg Phe  120

GGC AGA GAA GCT GCT ACT CAA TGG TTT AAC GGA CGG TTT GGA AGA GAT ATC GAA GGA CGA  434
Gly Arg Glu Ala Ala Thr Gln Trp Phe Asn Gly Arg Phe Gly Arg Asp Ile Glu Gly Arg  140

TTC CTC CCT CGT TTT GCA AAA GAA TCT AAT AAA CCA CAT CTC AGA GGA AGA TTT GGT AGA  494
Phe Leu Pro Arg Phe Ala Lys Glu Ser Asn Lys Pro His Leu Arg Gly Arg Phe Gly Arg  160

GCT GTG AAG TTG TAA AGAATTGTTCCTTTTATAAAAACATTTTAAAGAACTCATATAAATTATT           558
Ala Val Lys Leu  .                                                              164
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Figure 1 cDNA insertion of clone 23 from *H. magnipapillata* and deduced amino acid sequence for preprohormone A

Nucleotide residues are numbered from the 5′ end to the 3′ end, and amino acid residues are numbered starting with the Met residue encoded by the first ATG in the open reading frame. Hydra-RFamide I–IV sequences are underlined and printed in bold. Highly likely, but putative, neuropeptide sequences are underlined only. A stop codon is indicated by a dot.

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                                        TTTCAACCATGTGTTGTTTCCGTTCGGTTAGTTTAAATACTAC   43

TTCTTTGTAAAAATAAAAACATAAGTTTAAAGAAGACCATAGAGCAGGCAATCAATTAAGGTCCAGCTAAAAAGGTAAAA  123

ATG TTG AGC TAT AAA AAA TTC GAA TTA TTA TTT GCT TTG GTG TTA ATT GTT GTA GAA GTT  183
Met Leu Ser Tyr Lys Lys Phe Glu Leu Leu Phe Ala Leu Val Leu Ile Val Val Glu Val   20

GTA AAA TCT GAC GAT AAA AAC TTT TCA TTA GAA GTT AAC AAG GAT GTA AAA AGA TTT ATA  243
Val Lys Ser Asp Asp Lys Asn Phe Ser Leu Glu Val Asn Lys Asp Val Lys Arg Phe Ile   40

AAA GAT ATT TTA GAC GCT AAA AGT GAA GAA CAA CTT ATG TCT GGA CGT TTT GGA AAA AGC  303
Lys Asp Ile Leu Asp Ala Lys Ser Glu Glu Gln Leu Met Ser Gly Arg Phe Gly Lys Ser   60

TTA CCC GAT GAA GAA GAC ATT GAT AAT GAA GTA GAA AAT GAA TAT GAT AAT GAA TAT GAT  363
Leu Pro Asp Glu Glu Asp Ile Asp Asn Glu Val Glu Asn Glu Tyr Asp Asn Glu Tyr Asp   80

GAC GAA ACA GAG AGT CAG GGA ATT ATT AAT GGA AGA TAT GGA AGA CAA CTA CTT CGA GGA  423
Asp Glu Thr Glu Ser Gln Gly Ile Ile Asn Gly Arg Tyr Gly Arg Gln Leu Leu Arg Gly  100

CGA TTT GGA AGA CAA AAT GAC AAT AAT GCA GCA AGC AAA GAA AAC CAG TGG CTC GGG GGA  483
Arg Phe Gly Arg Gln Asn Asp Asn Asn Ala Ala Ser Lys Glu Asn Gln Trp Leu Gly Gly  120

CGT TTT GGC AAA GAA GTT GCT ACT CAG TGG TTT AAC GGA CGG TTC GGA AGA GAA ATT GGA  543
Arg Phe Gly Lys Glu Val Ala Thr Gln Trp Phe Asn Gly Arg Phe Gly Arg Glu Ile Gly  140

GGA CGA TTT CTC CCA CGG TTT GCA AGA GAA TTT AAT AAG CCC CAT TAT AGA GGT AGA TTT  603
Gly Arg Phe Leu Pro Arg Phe Ala Arg Glu Phe Asn Lys Pro His Tyr Arg Gly Arg Phe  160

GGT AGA ATA GCA AAG TTG TAA AAAT                                                628
Gly Arg Ile Ala Lys Leu  .                                                      166
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Figure 2 Composite cDNA coding for preprohormone B

This cDNA is composed of the cDNA insertion of λgt11 cDNA library clone 32 (nt 137–628) and of a 5′ PCR clone (nt 1–365). Hydra-RFamide I and II sequences are underlined and printed in bold. Highly likely, but putative, neuropeptide sequences are underlined only. Another possible putative neuropeptide sequence is underlined by a broken line. This sequence is less certain because it is preceded by a Ser residue. Stop codons are indicated by a dot.

A, 4 coded for a preprohormone of type B and 8 for a preprohormone of type C. Below we describe the three precursor types.

Preprohormone A

The longest cDNA insertion coding for preprohormone A was 558 bp long. It was sequenced on both strands in one sequencing reaction each; its structure is shown in Figure 1. The cDNA contains an open reading frame of 492 bp and has an upstream untranslated sequence of 14 bp and an untranslated trailer of 52 bp. All other clones had untranslated regions that were shorter.

The open reading frame of the cDNA shown in Figure 1 codes for a preprohormone that is 164 amino acid residues long. The protein has an N-terminal hydrophobic signal sequence that is probably cleaved off at Ala^{19} or Ser^{23} [17]. The C-terminal region of the prohormone contains the unprocessed sequences of Hydra-RFamide I (amino acid positions 114–121 of Figure 1), Hydra-RFamide II (amino acid positions 127–134) and Hydra-RFamides III and IV (amino acid positions 151–159 and 153–159). All these neuropeptide sequences are followed by an Arg residue, which is an established cleavage site [18], but are preceded by Asp (Hydra-RFamide I), Thr (Hydra-RFamide II), or Asn (Hydra-RFamide III/IV) residues, which must therefore represent novel cleavage sites.

The preprohormone contains, in addition to the Hydra-RFamide I–IV sequences (printed in bold type and underlined in Figure 1), two putative neuropeptide sequences (Figure 1, underlined only). These putative neuropeptide sequences are followed by one or two basic amino acid residues, suggesting that they will be released from the precursor and converted into mature neuropeptides (see also the Discussion section).

Preprohormone B

All four cDNA insertions coding for preprohormone B failed to contain the start codon of the open reading frame. A second round of screening of 10^6 phages with a cDNA insertion coding for preprohormone B (nt 196–615 of Figure 2) yielded three

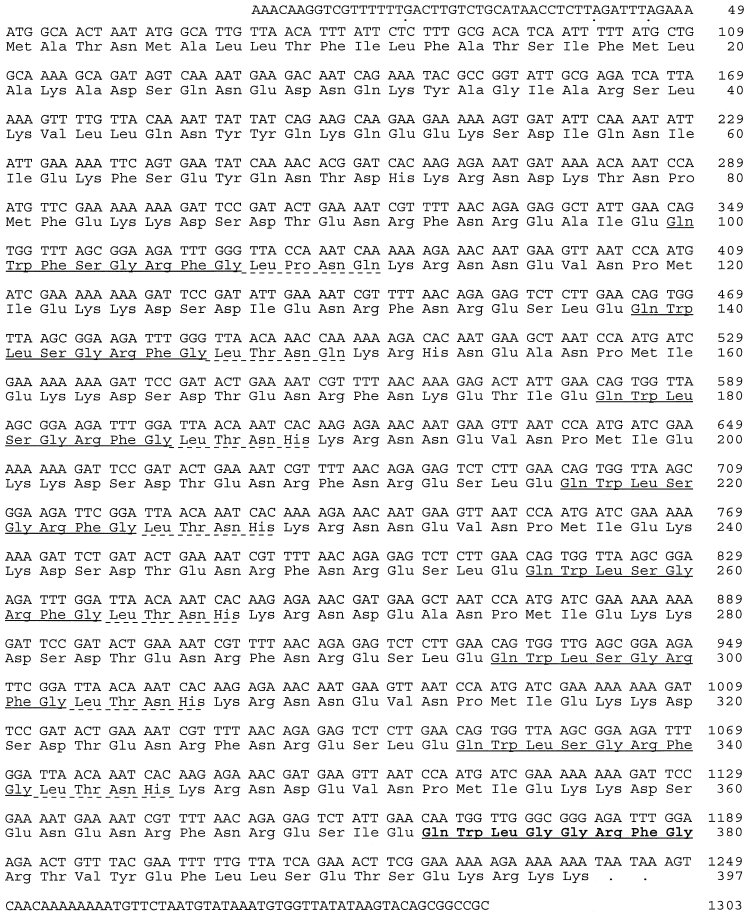

Figure 3 cDNA insertion of clone 42 coding for preprohormone C

A single copy of Hydra-RFamide I is underlined and printed in bold. Seven putative neuropeptide sequences are underlined only. It is difficult to give a prediction on the C-terminal regions of these seven putative neuropeptides; the last amino acid residues might be Gln or His, but if processing occurs at Asn or Thr residues the C-terminal regions might be shorter. Therefore the C-terminal regions of these peptide sequences are underlined by a broken line to indicate this uncertainty. Stop codons are indicated by a dot.

additional clones with insertions coding for preprohormone B, which again, however, did not contain the start codon. Therefore we used an anchored PCR approach, which finally yielded the 5′ end of the precursor protein cDNA. Figure 2 shows the composite cDNA coding for the whole preprohormone. The cDNA is 628 bp long and contains a non-coding 5′ sequence of 123 bp and a non-coding 3′ sequence of 7 bp. The non-coding 5′ sequence contains two stop codons, of which one is located shortly before the start codon (Figure 2).

The open reading frame of the cDNA codes for a protein of 166 amino acid residues. This protein has a signal sequence that is probably cleaved off at Ser^{23} [17]. Preprohormone B has an organization very similar to that of preprohormone A: the C-terminal region of the protein contains unprocessed Hydra-RFamide I (at amino acid positions 116–123 of Figure 2) and Hydra-RFamide II (at amino acid positions 129–136). These two sequences are followed by basic amino acid residues and preceded by Asn or Thr residues that, as we have seen in preprohormone A, can be cleavage sites.

There is an amino acid sequence (at amino acid positions 153–161 of Figure 2) nearly identical with that of Hydra-RFamide III/IV except that a Leu residue has been replaced by Tyr. Because this peptide sequence has not yet been isolated, it must be regarded as putative. Furthermore similar putative neuropeptide sequences to those that we found earlier in preprohormone A are also present, at similar positions, in preprohormone B.

Preprohormone C

The clone containing the largest cDNA insertion coding for preprohormone C is 1303 bp long and contains an untranslated 5′ region of 49 bp and a 63 bp trailer (Figure 3). The untranslated 5′ region contains three stop codons located shortly before the start codon.

The open reading frame is 1191 bp long and codes for a protein of 397 amino acid residues. The preprohormone has a signal sequence that is probably cleaved off at Ala^{21} or Ala^{23} [17]. There is an unprocessed Hydra-RFamide I sequence near the C-terminus of the precursor protein (at amino acid positions 373–380; printed in bold type and underlined in Figure 3). This sequence is followed by a basic amino acid residue (Arg) and preceded by an acidic residue (Glu), showing that it can be released from the precursor protein. In addition to the single copy of Hydra-RFamide I, the precursor contains seven putative neuropeptide sequences that are very regularly distributed over the central part of the precursor protein (at amino acid positions 100–111, 139–150, 178–189, 217–228, 256–267, 295–306 and

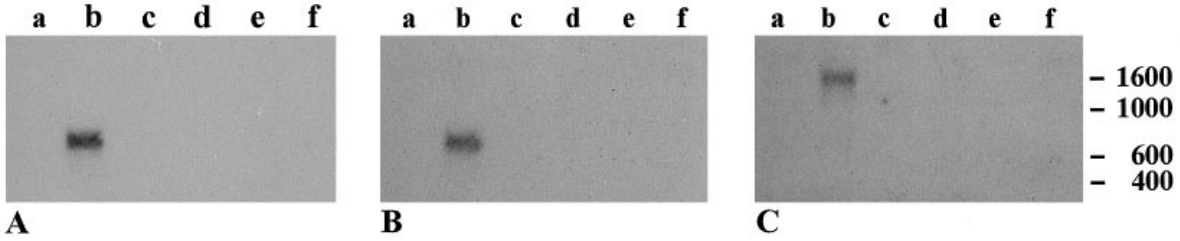

Figure 4 Northern blot analysis of poly(A)$^+$ RNA (5 μg per lane) from several cnidarian species and rat

mRNA is from whole *Cyanea lamarckii* (lanes a), *H. magnipapillata* (lanes b), rat brain (lanes c), *Polyorchis penicillatus* (lanes d), *Calliactis parasitica* (lanes e) and *Anthopleura elegantissima* (lanes f). (**A**) Hybridization with a cDNA fragment (nt 24–537 of Figure 1) coding for preprohormone A. (**B**) Hybridization with a cDNA fragment (nt 196–615 of Figure 2) coding for preprohormone B. (**C**) Hybridization with a cDNA fragment (nt 1–1303 of Figure 3) coding for preprohormone C. Hybridization was observed only with mRNA from *H. magnipapillata* (lanes b). This mRNA had a size of approx. 800 bases (**A**, **B**) or 1600 bases (**C**).

334–345 in Figure 3). These sequences are followed by pairs of basic residues (Lys-Arg) and preceded by acidic residues (Glu), suggesting that they will be released from the precursor.

Northern blot analyses

mRNA was isolated from whole *H. magnipapillata* and analysed in a Northern blot. Both a cDNA probe coding for preprohormone A (nt 24–537 of Figure 1) and a probe coding for preprohormone B (nt 196–615 of Figure 2) hybridized with mRNA of approx. 800 bases (Figure 4). The cDNA coding for preprohormone C (nt 1–1303 of Figure 3) hybridized with a mRNA population of approx. 1600 bases (Figure 4). These findings show that we have cloned almost the full lengths of the cDNA species coding for the three preprohormones, if one assumes that the three mRNA species coding for the three precursors have a poly(A)$^+$ tail of approx. 250 bases, which is normally true in eukaryotes.

The three types of cDNA were also used as probes in Northern blots containing, in addition to mRNA from *H. magnipapillata* (Hydrozoa, Cnidaria), whole-animal mRNA species from the hydromedusa *Polyorchis penicillatus* (Hydrozoa, Cnidaria), the scyphomedusa *Cyanea lamarckii* (Scyphozoa, Cnidaria), the sea anemones *Anthopleura elegantissima* and *Calliactis parasitica* (Anthozoa, Cnidaria), and mRNA from rat brain. Only mRNA from *H. magnipapillata* gave hybridization signals (Figure 4).

Hydra consists of a tube-like body column (gastric region) that is connected at one end to a head (with mouth and tentacles) and at the other end to a foot. We cut *Hydra* into three portions of equal length containing head, foot or gastric regions, isolated RNAs from these fragments and analysed them in Northern blots (Figure 5). The complete cDNA species coding for preprohormones A and B show 80 % nucleotide identity and might cross-react. For the experiments of Figure 5 we therefore used anti-sense oligonucleotide probes that were specific for mRNA coding for either preprohormone A (a probe corresponding to nt 131–152 of Figure 1) or preprohormone B (a probe corresponding to nt 240–261 of Figure 2). We used the whole cDNA insertion of Figure 3 as a probe specific for preprohormone C mRNA. The specificities of these probes were confirmed by using dot-blot analyses. The specific oligonucleotide probe for preprohormone A hybridized with head and foot mRNA species but not with mRNA from the gastric region. Both the oligonucleotide probe specific for preprohormone B and the cDNA probe specific for preprohormone C hybridized with mRNA from the head, but not with mRNA species from the foot or gastric regions (Figure 5). The mRNA coding for preprohormone B seemed to be less abundant in *Hydra* than the mRNA species coding for preprohormones A and C.

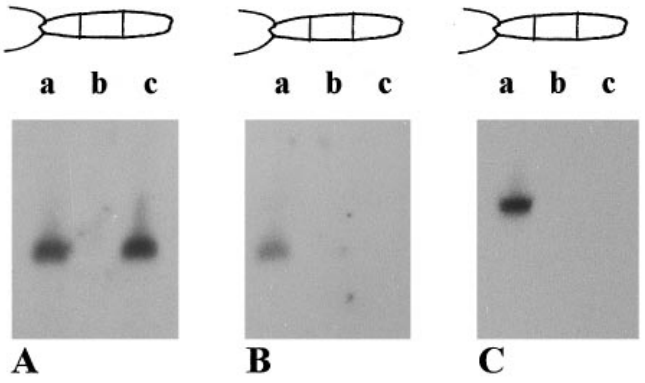

Figure 5 Northern blot analyses of total RNA (15 μg per lane) isolated from the upper one-third portion of *Hydra*, containing the head and tentacles (lanes a), the middle one-third portion, containing the gastric region (lanes b) or the lower one-third portion, containing the foot (lanes c)

(**A**) Hybridization with an anti-sense oligonucleotide probe specific for preprohormone A mRNA (corresponding to nt 131–152 of Figure 1). (**B**) Hybridization with an anti-sense oligonucleotide probe specific for preprohormone B (corresponding to nt 240–261 of Figure 2). (**C**) Hybridization with the whole cDNA sequence of Figure 3 specific for preprohormone C. The gene for preprohormone A is expressed in both head and foot regions of *Hydra*, whereas the genes for preprohormones B and C are expressed exclusively in the head region.

Whole-mount *in situ* hybridization

We obtained no good *in situ* hybridization signals in whole-mounted *Hydra* by using the single-stranded anti-sense oligonucleotide DNA probes specific for either preprohormone A mRNA or preprohormone B mRNA (see Northern blots, Figure 5). We therefore used anti-sense RNA probes produced by the transcription *in vitro* of cDNA insertions coding for each of the three preprohormones. This gave excellent *in situ* hybridization signals but the expected disadvantage was that the probes coding for preprohormones A and B cross-reacted slightly with the same mRNA species (again, the probe coding for preprohormone C was specific). By using an RNA probe directed against the mRNA of preprohormone A (corresponding to nt 2–537 of Figure 1), we found strong hybridization signals in neurons located in the upper gastric region (just below the tentacles and

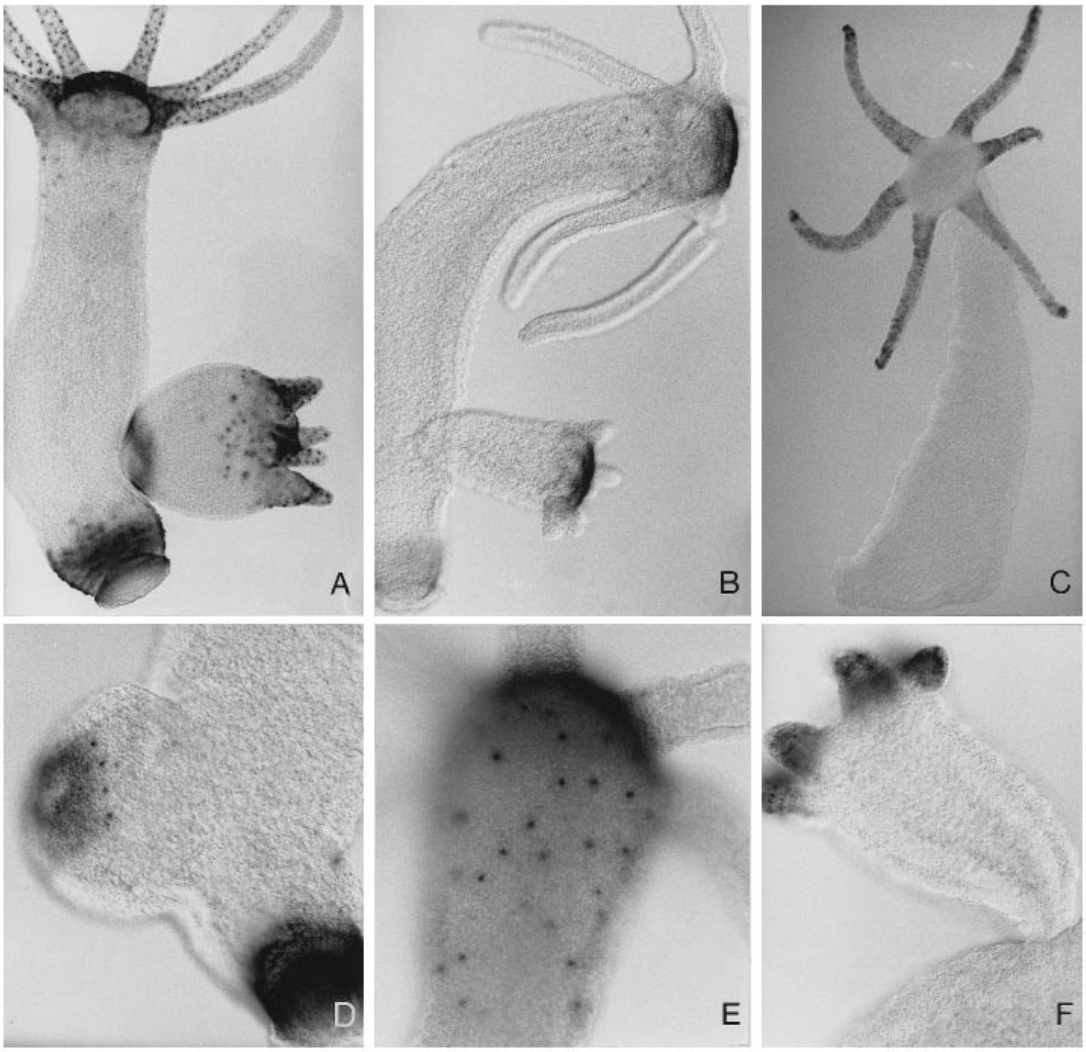

Figure 6 ***In situ*** **Hybridization of whole-mount** ***H. magnipapillata*** **with anti-sense RNA probes directed against the mRNA species of preprohormones A (corresponding to nt 2–537 of Figure 1), B (corresponding to nt 146–614 of Figure 2) or C (corresponding to nt 1–1303 of Figure 3)**

(**A**) Overview of a whole *Hydra* bearing a young bud hybridized with a probe directed against preprohormone A mRNA. Hybridization occurs in neurons of the basal two-thirds portion of the tentacles, in the hypostome, in neurons of the upper part of the gastric region just below the tentacles and in the foot (in an area called the peduncle, just above the basal disk). Staining is absent from most of the gastric region. In the young bud the same staining pattern is observed. Magnification $\times 45$. (**B**) Overview of a whole *Hydra* bearing a young bud, hybridized with a probe directed against preprohormone B mRNA. Hybridization can be seen in the hypostome and in neurons of the upper gastric region just below the tentacles. There is a very weak hybridization signal in the peduncle and no hybridization in the tentacles and most of the gastric region. A similar staining pattern is observed in the young bud: note that staining is absent from foot and tentacles. Magnification $\times 45$. (**C**) Overview of a whole *Hydra* hybridized with a probe directed against preprohormone C mRNA. There is staining exclusively in neurons of the tentacles. Note that the hybridization signals seem to be stronger in the tips of the tentacles, where clusters of strongly hybridizing neurons occur. Note also the clear border between tentacles and hypostome. Staining is completely absent from the hypostome, the whole gastric region and the foot. Magnification $\times 45$. (**D**) A very early bud, before the emergence of its tentacles, hybridized with a probe directed against preprohormone A. Note that a region at the tip of the bud is stained in a ring-like fashion. At its borders, four regularly distributed neurons can be seen. In the right corner is the strongly hybridizing foot of the adult *Hydra*. Magnification $\times 110$. (**E**) Detail of the upper gastric region just below the tentacles hybridized with a probe directed against preprohormone B mRNA. Strong hybridization signals can be seen in neurons, whereas hybridization is absent from the tentacles. Magnification $\times 110$. (**F**) A young bud hybridized with a probe directed against preprohormone C mRNA. Note the very prominent staining of neurons in the early tentacles and the absence of staining from the rest of the body. Magnification $\times 100$.

mouth), in the mouth area (hypostome), in neurons of the basal two-thirds portion of the tentacles and in neurons of the foot (in an area called the peduncle, just above the basal disk) (Figures 6A and 6D). Hybridization was absent from the tentacle tips (of adult animals), the mid-gastric region and basal disk. The staining of the hypostome area could be seen already in very early buds, where it had a ring-like or doughnut-like shape (Figure 6D). Using an RNA probe directed against preprohormone B mRNA (corresponding to nt 146–614 of Figure 2), we found strong staining in the hypostome and upper gastric region (just below the head) but little or virtually no staining in the tentacles and foot (Figures 6B and 6E). This suggested that the slight staining in the tentacles and foot was due to cross-reaction with preprohormone A mRNA and that the preprohormone B gene was expressed only in neurons of the mouth area (hypostome) and upper gastric region, which is in agreement with the data from our Northern blots (Figure 5B). By using an RNA probe specific for pre-prohormone C mRNA (corresponding to nt 1–1303 of Figure 3), we found that staining exclusively occurred in neurons of the tentacles. This staining was especially strong in the apical parts

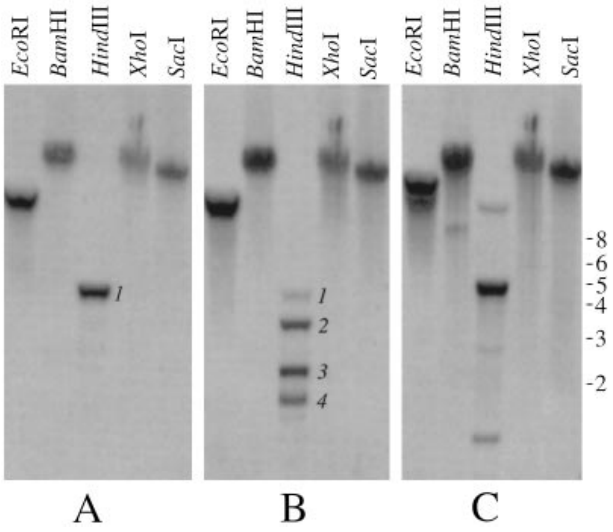

Figure 7 Southern blot analyses

Genomic DNA from *H. magnipapillata* was digested with several restriction enzymes and hybridized with cDNA probes coding for preprohormones A, B or C. The restriction enzymes are given at the top of each lane. The sizes of DNA markers (in kbp) are indicated at the right. (**A**) Southern blot analysis with a cDNA probe (nt 2–537 of Figure 1) coding for preprohormone A. Single bands appear. (**B**) Southern blot analysis with a cDNA probe (nt 146–614 of Figure 2) coding for preprohormone B. Single bands appear, except where genomic DNA has been digested by *Hin*dIII, where one weakly hybridizing fragment (band 1) and three strongly hybridizing fragments (bands 2–4) can be seen. (**C**) Southern blot analysis with a cDNA probe (nt 1–1303 of Figure 3) coding for preprohormone C. Single hybridizing fragments appear in the lanes where genomic DNA has been digested with *Xho*I or *Sac*I, whereas two or more hybridizing fragments appear in the lanes where the genomic DNA has been digested by *Eco*RI, *Bam*HI or *Hin*dIII.

of the tentacles, with a cluster of positive neurons frequently being located in the tentacle tip (Figure 6C). The production of preprohormone C mRNA occurred very early in tentacle development, as could be seen after staining of early developing buds (Figure 6F). No staining was observed when we used sense instead of anti-sense RNA probes corresponding to preprohormone A, B or C mRNA. The distribution of mRNA species coding for preprohormones A, B and C (Figure 6) corresponds well to the distribution of neurons stained with an antibody against RFamide peptides [7].

Southern blot analyses

The cDNA probe coding for preprohormone A (nt 2–537 of Figure 1) hybridized with single DNA fragments obtained after digestion of genomic *Hydra* DNA with *Eco*RI, *Bam*HI, *Hin*dIII, *Xho*I, *Sac*I (Figure 7A) or *Pst*I (results not shown), suggesting the presence of one single gene coding for preprohormone A.

When a cDNA fragment coding for preprohormone B (nt 146–614 of Figure 2) was used as a probe in our Southern blot analysis, single hybridizing bands were obtained after digestion of genomic DNA with *Eco*RI, *Bam*HI, *Xho*I, *Sac*I (Figure 7B) or *Pst*I (results not shown) that had about the same size as those hybridizing with the cDNA probe coding for preprohormone A (Figure 7A). However, four hybridizing bands appeared in genomic DNA cleaved by *Hin*dIII. One of them was a weakly hybridizing band (band 1 in Figure 7B) with the same size as the strongly hybridizing band (band 1) in Figure 7(A). Its presence could be explained by cross-reaction of the preprohormone B probe with the *Hin*dIII fragment of the DNA coding for preprohormone A (band 1 in Figure 7A). The other three bands are strongly hybridizing (bands 2–4 in Figure 7B) and their presence could be explained by two *Hin*dIII restriction sites in the gene coding for preprohormone B: one located inside the region corresponding to the DNA shown in Figure 2 (there is a *Hin*dIII restriction site at nt 299–305 of Figure 2) and another located within a presumed intron between positions 146 and 614 of the DNA shown in Figure 2. The hybridizing bands 1–4 in Figure 7(B) could therefore be explained on the basis of one single gene coding for preprohormone B.

When the cDNA coding for preprohormone C (nt 1–1303 of Figure 3) was used as a probe, single hybridizing bands appeared in a Southern blot using genomic DNA digested with *Xho*I, *Sac*I (Figure 7C) and *Pst*I (results not shown). However, when genomic DNA digested with *Eco*RI, *Bam*HI or *Hin*dIII was used, two (with *Eco*RI or *Bam*HI) or four (with *Hin*dIII) hybridizing bands appeared (Figure 7C). The presence of two or four hybridizing bands can be explained in two ways. First, there could be one gene coding for preprohormone C that, in the region corresponding to the cDNA of Figure 3, contains at least three introns. Each of these three introns has a *Hin*dIII restriction site giving rise to four hybridizing fragments, whereas only one of the introns has an *Eco*RI and/or *Bam*HI restriction site, giving rise to two hybridizing fragments. The other possibility is that there are two or more closely related genes hybridizing with the preprohormone C probe.

DISCUSSION

Novel prohormone-processing sites

From earlier work on the biosynthesis of cnidarian neuropeptides, we have seen that cnidarians use a variety of unconventional cleavage signals for the generation of their neuropeptides. These novel cleavage sites are Asp and Glu residues [11,12,19,20], Asn and Ser residues [10] and N-terminal Xaa-Pro or Xaa-Ala sequences [21]. These unconventional cleavage signals are always located at the N-terminal sides of the unprocessed neuropeptide sequences, whereas at the C-terminal flanking regions conventional monobasic or dibasic processing sites occur. The enzyme responsible for the removal of N-terminal Xaa-Ala or Xaa-Pro sequences is most probably a dipeptidyl aminopeptidase [21], whereas the other enzymes are hitherto unknown aminopeptidases or endoproteinases [2].

Because we have recently isolated and sequenced four Hydra-RFamide peptides from *H. magnipapillata* [6], we are now able to draw conclusions on the maturation of their precursors. Each single copy of immature Hydra-RFamide I located on preprohormones A, B and C is followed by a monobasic processing site and preceded by Asp, Glu or Asn (Table 1), confirming our earlier findings that processing at these residues is possible in cnidarian prohormones. Each single copy of immature Hydra-RFamide II located on preprohormones A and B is followed by a monobasic processing site but is preceded by a Thr residue (Table 1). This means that Thr residues also must function as a cleavage signal. Cleavage at Thr residues, however, is not surprising because Thr closely resembles Ser, which, as mentioned above, has already been recognized as a cleavage site for cnidarian prohormone processing [10]. The single copy of Hydra-RFamide III in preprohormone A is followed by a single basic processing site and preceded by an Asn residue (Table 1), in agreement with our earlier conclusions that Asn can be a cleavage signal in cnidarian precursor proteins [10]. The single copy of immature Hydra-RFamide IV (HLRGRFG) on preprohormone A can be generated from Hydra-RFamide III (KPHLRGRFG) by the removal of an N-terminally located Lys-Pro sequence which, as

Table 1 N- and C-terminal extensions of Hydra-RFamides I–IV and related putative neuropeptide sequences

Sites of initial clevage at dibasic or monobasic residues are indicated by arrows. The immature copies of Hydra-RFamides I-IV are underlined and printed in bold. Highly likely, but putative, peptide sequences are underlined only. Uncertain mature sequences are underlined by a broken line. The underlined neuropeptide sequences can be found in Figures 1–3 at the following amino acid sequences: a, 113 (Figure 1); b, 116 (Figure 2); c, 373 (Figure 3); d, 127 (Figure 1); e, 129 (Figure 2); f, 151 (Figure 1); g, 91 (Figure 1); h, 51 (Figure 2); i, 96 (Figure 1); j, 96 (Figure 2); k, 153 (Figure 2); l, 100 (Figure 3); m, 139 (Figure 3); n, 178 (Figure 3); o, 217, 256, 295, 334 (Figure 3); p, 86 (Figure 2).

N- and C-terminal extensions and neuropeptide sequence	Copy no.	Name
Ala-Ser-Asn-Glu-Asp-**Gln-Trp-Leu-Gly-Gly-Arg-Phe-Gly**-Arg↓[a]	1	Hydra-RFamide I
Lys↓Glu-Asn-**Gln-Trp-Leu-Gly-Gly-Arg-Phe-Gly**-Arg↓[b]	1	Hydra-RFamide I
Arg↓Glu-Ser-Ile-Glu-**Gln-Trp-Leu-Gly-Gly-Arg-Phe-Gly**-Arg↓[c]	1	Hydra-RFamide I
Arg↓Glu-Ala-Ala-Thr-**Gln-Trp-Phe-Asn-Gly-Arg-Phe-Gly**-Arg↓[d]	1	Hydra-RFamide II
Lys↓Glu-Val-Ala-Thr-**Gln-Trp-Phe-Asn-Gly-Arg-Phe-Gly**-Arg↓[e]	1	Hydra-RFamide II
Lys↓Glu-Ser-Asn-**Lys-Pro-His-Leu-Arg-Gly-Arg-Phe-Gly**-Arg↓[f]	1	Hydra-RFamides III/IV
Lys↓Asn-Gly-Glu-Gln-Leu-Met-Ser-Gly-Arg-Phe-Gly-Lys-Arg↓[g]	1	Hydra-RFamide V
Lys↓Ser-Glu-Glu-Gln-Leu-Met-Ser-Gly-Arg-Phe-Gly-Lys↓[h]	1	Hydra-RFamide V
Arg↓Gln-Leu-Met-Arg-Gly-Arg-Phe-Gly-Arg↓[i]	1	Hydra-RFamide VI
Arg↓Gln-Leu-Leu-Arg-Gly-Arg-Phe-Gly-Arg↓[j]	1	Hydra-RFamide VII
Arg↓Glu-Phe-Asn-Lys-Pro-His-Tyr-Arg-Gly-Arg-Phe-Gly-Arg↓[k]	1	Hydra-RFamides VIII/IX
Arg↓Glu-Ala-Ile-Glu-Gln-Trp-Phe-Ser-Gly-Arg-Phe-Gly-Leu-Pro-Asn-Gln-Lys-Arg↓[l]	1	Hydra-RF-elongated II
Arg↓Glu-Ser-Leu-Glu-Gln-Trp-Leu-Ser-Gly-Arg-Phe-Gly-Leu-Thr-Asn-Gln-Lys-Arg↓[m]	1	Hydra-RF-elongated I
Lys↓Glu-Thr-Ile-Glu-Gln-Trp-Leu-Ser-Gly-Arg-Phe-Gly-Leu-Thr-Asn-His-Lys-Arg↓[n]	1	Hydra-RF-elongated I
Arg↓Glu-Ser-Leu-Glu-Gln-Trp-Leu-Ser-Gly-Arg-Phe-Gly-Leu-Thr-Asn-His-Lys-Arg↓[o]	4	Hydra-RF-elongated I
Asp-Glu-Thr-Glu-Ser-Gln-Ile-Ile-Asn-Gly-Arg-Tyr-Gly-Arg↓[p]		

proposed earlier [21], can be catalysed by a dipeptidyl aminopeptidase.

It is very possible that the enzymes responsible for the above-mentioned cleavages at Asp/Glu, Thr and Asn residues are aminopeptidases that exert their actions after the elongated Hydra-RFamide I–III sequences have been excised at their flanking basic amino acid residues (Table 1 and Figures 1–3) by conventional endoproteolytic cleavage [18]. However, the structure of another cnidarian preprohormone, the sea anemone prepro-Antho-RPamide I (where there is only one copy of the established, unprocessed Antho-RPamide I sequence, which is preceded by acidic amino acid residues without the presence of basic residues), suggests that cleavage at Asp/Glu residues is catalysed by an endoproteinase [2]. The removal of the N-terminal extensions of the immature Hydra-RFamides I–IV sequences might therefore occur by the concerted actions of both endo- and exo-peptidases.

Novel neuropeptide sequences

In addition to the Hydra-RFamide I–IV sequences in preprohormone A, there are two putative neuropeptide sequences (at amino acid positions 51–58 and 96–103 of Figure 1) that are followed by basic processing sites and preceded by either a Glu or an Arg residue. Thus it is very likely that these sequences also are released from the precursor protein. Their mature structures are probably < Glu-Leu-Met-Ser-Gly-Arg-Phe-NH_2 (Hydra-RFamide V) and < Glu-Leu-Met-Arg-Gly-Arg-Phe-NH_2 (Hydra-RFamide VI). These peptides are closely related to the other Hydra-RFamides (Table 2).

Preprohormone B contains, in addition to the established Hydra-RFamide I and II sequences, the same putative Hydra-RFamide V sequence (at amino acid positions 51–58 of Figure 2) as that found in preprohormone A, flanked by similar processing sites. Furthermore there is a neuropeptide sequence (at amino acid positions 96–103 of Figure 2) that is nearly identical with the Hydra-RFamide VI sequence of preprohormone A. It is flanked at both sides by basic amino acid residues and its mature structure is probably < Glu-Leu-Leu-Arg-Gly-Arg-Phe-NH_2 (Hydra-RFamide VII; Tables 1 and 2). Finally, there is an amino acid sequence that closely resembles the Hydra-RFamide III/IV sequence of preprohormone A (at amino acid positions 153–161 of Figure 2). This sequence has the same processing signals as the immature Hydra-RFamide III/IV sequence and is therefore likely to be released and converted into Lys-Pro-His-Tyr-Arg-Gly-Arg-Phe-NH_2 (Hydra-RFamide VIII) and His-Tyr-Arg-Gly-Arg-Phe-NH_2 (Hydra-RFamide IX; Tables 1 and 2).

Preprohormone C is remarkable because it contains, in addition to the single copy of Hydra-RFamide I, seven totally novel neuropeptide sequences (Figure 3). Each of these putative neuropeptide copies is followed by a dibasic processing site and preceded by a Glu residue (Table 1), which makes it likely that they are released from the precursor protein. The N-terminal regions of the seven peptides show a striking similarity to members of the Hydra-RFamide family, especially Hydra-RFamides I and II (Table 2). The mature neuropeptides, however, probably do not carry an amidated C terminus, because a C-terminal Gly residue, which is necessary for amidation [22,23] is lacking at the appropriate positions (positions 111, 150, 189, 228, 267, 306 and 345 of Figure 3) in the unprocessed sequences (see also Table 1). Instead, the mature neuropeptides are likely to be C-terminally elongated compared with the Hydra-RFamides (Tables 1 and 2). It is difficult to give a prediction of the C-terminal regions of these seven putative neuropeptides. The last amino acid residues might be Gln or His; however, if processing occurs at Asn or Thr residues the C-terminal regions might be shorter. Because the biological action of most amidated neuropeptides is dependent on their C-terminal amidation, one can expect that the putative C-terminally elongated, non-amidated *Hydra* peptides have an action different from that of the Hydra-RFamides.

Flanking the putative and established neuropeptide sequences of preprohormone C (Figure 3) are eight highly conserved

Table 2 Established and putative neuropeptides that could be released from prohormones A, B and C

Amino acid residues identical with those of Hydra-RFamide I are boxed. Hydra-RFamides I-IV are established neuropeptides, the others are putative. Hydra-RFamide IV can be formed from Hydra-RFamide III by removal of its N-terminal Lys-Pro sequence (probably catalysed by dipeptidyl aminopeptidase). The same holds for Hydra-RFamide IX, which can be formed from Hydra-RFamide VIII. The precise C-terminal extensions of the two elongated neuropeptides I and II are not known.

Copy no.	Structure	Name
2	<Glu-Trp-Phe-Asn-Gly-Arg-Phe-NH_2	Hydra-RFamide II
3	<Glu-Trp-Leu-Gly-Gly-Arg-Phe-NH_2	Hydra-RFamide I
6	<Glu-Trp-Leu-Ser-Gly-Arg-Phe-Leu-...	Hydra-RF-elongated I
1	<Glu-Trp-Phe-Ser-Gly-Arg-Phe-Leu-...	Hydra-RF-elongated II
2	<Glu-Leu-Met-Ser-Gly-Arg-Phe-NH_2	Hydra-RFamide V
1	<Glu-Leu-Met-Arg-Gly-Arg-Phe-NH_2	Hydra-RFamide VI
1	<Glu-Leu-Leu-Arg-Gly-Arg-Phe-NH_2	Hydra-RFamide VII
1	Lys-Pro-His-Leu-Arg-Gly-Arg-Phe-NH_2	Hydra-RFamides III/IV
1	Lys-Pro-His-Tyr-Arg-Gly-Arg-Phe-NH_2	Hydra-RFamides VIII/IX
18		

sequences, of which three have the sequence KRNNEVNPM-IEKKDSDTENRFNRESLE (at amino acid positions 190–216, 229–255 and 307–333 of Figure 3), whereas the others have very similar sequences (at amino acid positions 73–99, 112–138, 151–177, 268–294 and 346–372). Database searches (SwissProt) did not reveal any significant similarity of any of these sequences with known proteins or peptides. It could be that these sequences represent novel, biologically active peptides. However, because these sequences are not protected by a < Glu group and also contain many of the processing sites discussed earlier (Lys, Arg, Asn, Glu, Asp, Ser and Thr), it could be that they merely have a spacing function and that they are degraded during processing of the prohormone.

Comparison between the three preprohormones

It is clear that the overall organizations of the preprohormones A and B are much more similar to each other than to that of preprohormone C. This is confirmed by sequence alignments, where we found 70% amino acid residue identity between preprohormones A and B and only 20–56% identity when we compared whole preprohormone A (or B) with different portions of preprohormone C. The same conclusion was reached when we aligned the cDNA sequences of the three preprohormones. All of this suggests that the genes coding for preprohormones A and B have originated from the same gene duplication event, whereas the gene coding for preprohormone C has been involved in another (perhaps earlier) gene duplication.

Preprohormone gene expression

Our Southern blot analyses (Figure 7) suggest that preprohormones A and B are each coded for by a single gene, whereas preprohormone C might be coded for by one or possibly two or more closely related genes. The three (or more) preprohormone genes are differentially expressed. The preprohormone A gene is expressed in neurons of both the head and foot regions, whereas the preprohormone B and C genes are specifically expressed in neurons of the head (Figures 5 and 6). This means that the putative, elongated *Hydra* peptides (Hydra-RF-elongated I and II), but also Hydra-RFamides V, VII, VIII and IX (Table 2) are produced only in the head region. Head-specific or foot-specific neurohormones are interesting because they are good candidates for factors that control morphogenesis and cell differentiation in *Hydra* [4,5,24]. Recently it has been found that a novel group of *Hydra* neuropeptides with the C-terminal sequence Leu-Trp-NH_2 (LWamide) influences gene expression in *Hydra* [25]; in the marine hydroid *Hydractina*, LWamide neuropeptides induce metamorphosis in planula larvae to become primary polyps [26,27]. These findings show that it is very possible that cnidarian neuropeptides have a role in development. Our work on the cloning of the three Hydra-RFamide preprohormones could therefore be the basis for future studies on the involvement of these proteins and their derived neuropeptides in *Hydra* development.

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