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The Myomodulin-related Neuropeptides: Characterization of a Gene Encoding a Family of Peptide Cotransmitters in *Aplysia*

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The myomodulin-related peptides comprise a family of cotransmitters that modulate neuromuscular signaling in the feeding system of *Aplysia*. In this study, cDNA clones encoding a myomodulin precursor polypeptide were isolated and characterized. This precursor contains seven different myomodulin-related peptides, one of which, myomodulin A, is present in 10 contiguous copies. The sequence of a myomodulin genomic clone indicates that all of these myomodulin-related peptides are encoded on a single exon. The myomodulin gene is expressed in a tissue-specific manner and myomodulin mRNA is localized to specific neurons in the *Aplysia* CNS. The presence of multiple related neuropeptides can greatly increase the range and precision of signaling at synapses where they act as modulator cotransmitters.

[Key words: neuropeptide, cotransmitter, precursor,* Aplysia californica, *myomodulin, neuromodulator, peptide family, polypeptide, mollusk, invertebrate]

The process of neuropeptide biosynthesis, in which multiple structurally related or unrelated peptides are generated from a single precursor polyprotein, serves to expand the spatial and temporal range of neuronal signaling (Herbert et al., 1981; Cropper et al., 1987b, 1991; Gainer, 1988; Sossin et al., 1989). One function for which such an enhanced range of action appears particularly well suited is the modulation of synaptic transmission (Kupfermann, 1979; Kaczmarek and Levitan, 1987). Studies with a number of simplified neuronal and neuromuscular systems from vertebrates and invertebrates have been instrumental in characterizing physiological properties of peptidergic synaptic modulation (Jan and Jan, 1982; O'Shea et al., 1985; Bishop et al., 1987; Marder, 1988).

Several neuropeptides act as modulators of neurally evoked contractions in muscles that participate in feeding-related behaviors in *Aplysia* (Lloyd et al., 1984; Richmond et al., 1986; Cropper et al., 1987a,b, 1988, 1990a, 1991; Church et al., 1991). One such peptide, myomodulin A (Pro-Met-Ser-Met-Leu-Arg-Leu-NH_2), was originally purified from neural elements in the accessory radula closer (ARC) muscle (Cropper et al., 1987b), a muscle utilized in the biting phase of feeding (Cohen et al., 1978; Cropper et al., 1990b). Exogenous application of myomodulin A produces an enhancement of evoked ARC contractions via postsynaptic actions on the muscle (Cropper et al., 1987b, 1991; Brezina et al., 1991; Probst et al., 1992). The demonstrated synthesis of myomodulin A by an identified motor neuron (B16) innervating the ARC (Cropper et al., 1987b), its rapid axonal transport to the ARC (Lloyd, 1988), and the presence of myomodulin-like immunoreactivity in nerve fibers and varicosities on the ARC (Miller et al., 1991a) further support a role of this peptide in neuromuscular signaling. It has been proposed that the motor neuron B16, in which ACh acts as the principal neurotransmitter substance (Cohen et al., 1978), utilizes myomodulin A as a modulatory cotransmitter (Cropper et al., 1987b).

Myomodulin A appears to exert multiple actions in diverse neural circuits in *Aplysia*. Within the feeding circuit, additional motor neurons synthesize myomodulin A (Church and Lloyd, 1991) and it has been shown that the peptide has modulatory effects on a class of cerebral mechanosensory neurons (CM-S_B cells; Rosen et al., 1989). A high level of transport from the cerebral to the buccal ganglion further suggests that myomodulin A is involved in the central regulation of feeding-related behaviors (Lloyd, 1988). Myomodulin-like immunoreactive material is widespread throughout the *Aplysia* nervous system, occurring in specific cell bodies and clusters in each of the central ganglia and in fibers in each of the connectives (Miller et al., 1991a). Exogenous application of myomodulin A produces a hyperpolarization of cells of the left upper quadrant (LUQ) of the abdominal ganglion (Alevizos et al., 1987) and can reverse increases in excitability and spike duration produced by 5-HT in tail sensory neurons of the pleural ganglion (Critz et al., 1989). Myomodulin-immunoreactive fibers and varicosities are present on peripheral tissues associated with the reproductive, digestive, and circulatory systems (Miller et al., 1991a).

Recent findings indicate that myomodulin A belongs to a family of related neuropeptides that modulate contraction of feeding muscles in a variety of mollusks. A structurally similar octapeptide, termed myomodulin B (Gly-Ser-Tyr-Arg-Met-Met-Arg-Leu-NH_2), was purified from the ARC of *Aplysia* and found

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to modulate evoked ARC contractions (Cropper et al., 1991). Myomodulin A and a related heptapeptide, termed *Fusinus* myomodulin (Pro-Met-Asn-Met-Leu-Arg-Leu-NH_2), have been purified from the central ganglia of the prosobranch *Fusinus ferrigineus* and were found to modulate radula muscle contractions (Kobayashi and Muneoka, 1990). Finally, the catch-relaxing peptide (CARP; Ala-Met-Leu-Met-Leu-Arg-Leu-NH_2), isolated from the pedal ganglia of the bivalve *Mytilus edulis* (Hirata et al., 1987), also modulates evoked contractions of feeding-related muscles of gastropods (Hirata et al., 1989).

In this study, a molecular cloning approach was used in order to provide a more complete characterization of the myomodulin-related peptides in *Aplysia*.

Some of these findings have been reported previously in abstract form (Miller et al., 1991b).

Materials and Methods

Specimens of *Aplysia californica* were obtained commercially (Marinus, Inc., Long Beach, CA). Standard molecular cloning protocols were followed for most of the experiments (Sambrook et al., 1989).

Isolation of cDNA clones. Six 64-fold degenerate 17-mer oligodeoxynucleotide probes, designed from the amino acid sequence of myomodulin A, were synthesized by the DNA Core Facility, Mount Sinai School of Medicine. The probes differed only in the codon for serine (italicized): CCN ATG *TCA* ATG YTN MG, CCN ATG *TCG* ATG YTN MG, CCN ATG *TCC* ATG YTN MG, CCN ATG *TCT* ATG YTN MG, CCN ATG *AGC* ATG YTN MG, and CCN ATG *AGT* ATG YTN MG (Y = C or T; M = C or A; N = A, C, G, or T; orientation 5′ to 3′). The oligonucleotides were each end-labeled with γ-^{32}P-ATP using T4 polynucleotide kinase. The six probes were pooled for screening duplicate replica filters of 5×10^5 plaque-forming units (pfus) from a λgt10 *Aplysia* buccal ganglion cDNA library (provided by R. Scheller, Stanford University). Hybridization solution consisted of 5× SSC (0.75 M NaCl, 0.075 M Na citrate), 50 mM NaP_i (pH 6.8), 1 mM sodium pyrophosphate, 5× Denhardt's solution [Denhardt's solution: 0.2% Ficoll, 0.2% bovine serum albumin (BSA), 0.2% polyvinylpyrrolidone], 20% formamide, and 100 μg/ml denatured salmon sperm DNA. Filters were hybridized overnight at 40°C, washed [1× SSC, 1% sodium dodecyl sulfate (SDS); 44°C, 4 × 20 min], and autoradiographed overnight (−80°C; with Spectroline L Plus intensifying screen). Candidate clones were purified through a tertiary screen.

Nucleotide sequence analysis. Phage inserts were subcloned into the plasmid vector pBluescript SK$^+$ (Stratagene, La Jolla, CA), and the nucleotide sequences of both strands were determined using the chain-termination method (Sanger et al., 1977) on double-stranded template DNA (Sequenase system, U.S. Biochemical, Cleveland, OH). Samples were electrophoretically separated on denaturing gradient polyacrylamide gels. Ambiguities resulting from compressions were resolved using 7-deaza dGTP or dITP according to the directions supplied with the kit.

Polymerase chain reaction (PCR). The order and orientation of clone fragments (see Results) were confirmed by generating a PCR product spanning two internal *Eco*R I sites. Oligonucleotide primers (positions denoted by arrows in Fig. 1) PMS009 (Fig. 2, nucleotide positions 157–173) and PMS004 (Fig. 2, nucleotide positions 843–859) were used to amplify template DNA from 1 μl of phage stock. PCR was performed in a 50 μl reaction mixture: 16.6 mM $(NH_4)_2SO_4$; 200 μM deoxynucleoside triphosphates (dNTPs; Pharmacia LKB); 67 mM Tris HCl, pH 8.8; 1.5 mM $MgCl_2$; 170 μg/ml bovine serum albumin; 1 U of AmpliTak (Perkin-Elmer); and 200 nM concentration of each primer. Thirty cycles were used: 30 sec denaturation (94°C), 1 min annealing (50°C), and 2 min extension (72°C). Products were isolated (Geneclean kit, Bio 101) from a 1% agarose gel, digested with *Hind* III and *Rsa* I, and subcloned between *Eco*R V and *Hind* III sites of pBluescript (Fig. 1, pMM21), and the sequences of both strands were determined (see above).

DNA blot hybridizations. For Southern blot analyses, genomic DNA was isolated from the ovotestis of a single *Aplysia* specimen, digested to completion (overnight at 37°C) with selected restriction enzymes, size-fractionated on a 0.8% agarose gel (10 μg/lane), denatured, and transferred onto nylon membrane (MSI, Westboro, MA). The filter was prehybridized at 42°C for 3 hr in 6× SSC, 2.5× Denhardt's solution (see above), 1.0% SDS, 10% dextran sulfate, 35% formamide, and 100 μg/ml heat-denatured salmon sperm DNA. Insert DNA [~550 base pair (bp)] from a myomodulin clone (Fig. 1, pMM13) was labeled by random hexamer priming (U.S. Biochemical), heat denatured (95°C, 5 min), and hybridized overnight at 42°C. Blots were washed (final conditions: 0.2× SSC, 0.2% SDS, 65°C) prior to autoradiography.

RNA blot hybridizations. PolyA$^+$-selected RNA was isolated from the central nervous system or the ovotestis, denatured and size fractionated (5 μg/lane) on a 1.2% agarose gel, transferred to nylon membrane, and hybridized with insert DNA from pMM13 ^{32}P-labeled by random hexamer priming. The hybridization solution contained 5× SSC, 2.5× Denhardt's solution, and 100 μg/ml heat-denatured salmon sperm DNA. Blots were washed (final conditions: 0.2× SSC, 0.2% SDS, 42°C) prior to autoradiography.

In situ *hybridization.* Complementary RNA (cRNA) probes were generated from a cloned 390 bp fragment of the coding region of the myomodulin cDNA (corresponding to bases 294–683 of Fig. 2). The probes were labeled with digoxigenin-11-dUTP according to manufacturer's specifications (Genius Kit, Boehringer Mannheim Biochemicals, Indianapolis, IN). Sections (10–15 μm) of the ring ganglia were collected on silanated slides (Clayton and Alvarez-Buylla, 1989), cross-linked using UV light (Tiedge, 1991), treated with proteinase K (10 μg/ml, 5 min), and subjected to postfixation (4% paraformaldehyde, 5 min). Prehybridization (1–2 hr) and hybridization were carried out at 50°C. Following washes (60°C, 2× SSC) and RNAse treatment (RNase A, 25 μg/liter, 37°C), 1 hr), hybridizing probe was detected using an antidigoxigenin alkaline phosphatase–conjugated antibody (Boehringer Mannheim).

Results

Characterization of a cDNA clone encoding a myomodulin precursor

Screening a buccal ganglion cDNA library with six pooled oligonucleotides designed from the sequence of myomodulin A (see Materials and Methods) resulted in the isolation of four candidate clones. Restriction enzyme and sequence analyses indicated that all four clones contained related, overlapping inserts. An *Eco*R I digestion of the largest insert produced three fragments ~750, ~550, and ~120 bp in length (designated pMM12, pMM13, and pMM22, respectively, in Fig. 1). The order and orientation of these fragments within the λ clone were deduced using the linker positions, polyA tail, and open reading frame (ORF) as cues. This arrangement was confirmed by cloning and sequencing a PCR product (Fig. 1, pMM21) generated using primers (Fig. 1, PMS009 and PMS004) flanking the two internal *Eco*R I sites and the λ phage stock isolate (1 μl) as template.

The complete phage insert (1432 bp) corresponds to an mRNA that encodes a myomodulin precursor protein 370 amino acids in length (Fig. 2). The assigned translation initiation methionine (Fig. 2, MET) is preceded by 223 nucleotides of 5′ untranslated sequence in which two in-frame stop codons are present. The assigned initiator codon is located within a sequence (CAACCAUG) that agrees closely with the consensus for eukaryotic initiation sites (Kozak, 1983). However, the possibility that the initiation of translation occurs at a second methionine residue (position 5) cannot be ruled out. The ORF is terminated by a UAA stop codon (Fig. 2, asterisk) that is followed by 31 nucleotides exclusive of the polyA tail of 3′ untranslated sequence including a consensus signal (AAUAAA) for polyadenylation (Setzer et al., 1980). Twelve nucleotides separate the polyadenylation signal from the polyA tail. Short 3′ untranslated sequences have been noted in several genes encoding neuropeptides in molluscs (Schaefer et al., 1985; Smit et al., 1988).

Deduced structure of the myomodulin precursor

The proposed initiator methionine is followed by a sequence of 12 amino acids that could act as a signal peptide, directing

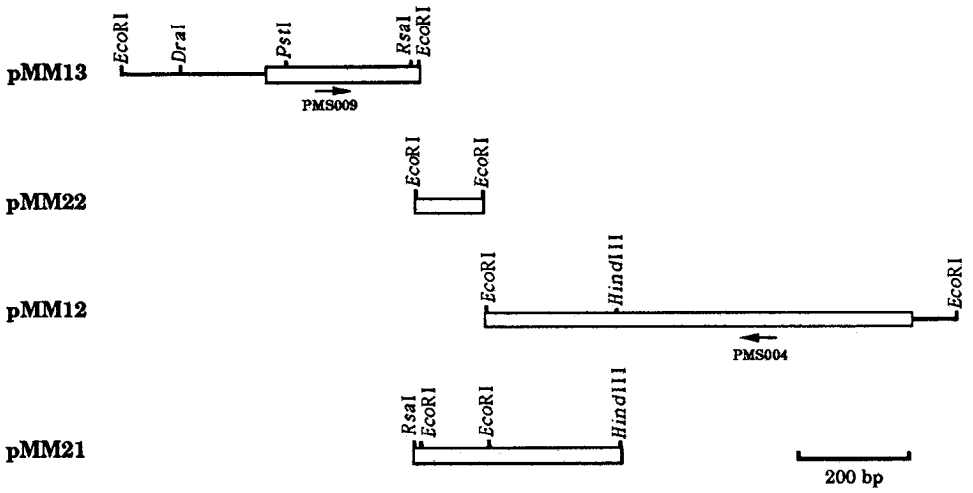

Figure 1. Restriction maps of fragments derived from a single recombinant buccal ganglion cDNA clone that encodes a myomodulin precursor polyprotein. *Open bars* denote proposed ORF and *thinner lines* represent regions corresponding to 5′ and 3′ untranslated sequence. Clones *pMM13, pMM22,* and *pMM12* were derived from an *Eco*R I digestion of a λ clone insert ~1.3 kb in length. Two 17-mer PCR primers (denoted *PMS009* and *PMS004*) were used to generate *pMM21* (see Materials and Methods) in order to confirm the position and orientation of two internal *Eco*R I restriction sites.

translocation of the nascent myomodulin precursor protein to the endoplasmic reticulum (Walter and Blobel, 1981). This sequence contains several well-conserved features of signal peptide structure (von Heijne, 1988), for example, a hydrophobic core rich in apolar residues (3 leucines, 2 alanines, 1 phenylalanine). Cleavage of this sequence from the prohormone may occur following the alanine residue in position 13 (Fig. 2, downward arrowhead). This proposed site of signal peptidase cleavage is based upon amino acid patterns observed in eukaryotic signal peptides (Perlman and Halvorson, 1983; von Heijne, 1986): (1) it conforms to the "(−3, −1) rule" since the amino acids in these positions, valine and alanine (denoted −3 and −1, respectively, in Fig. 2) are both apolar; (2) the −2 position is occupied by the bulkier residue phenylalanine; and (3) a proline is present in the −6 position. The predicted signal peptide is very short (13 residues) and further experiments will be required to clarify whether signal peptidase cleavage actually occurs at this position or at a site more distal from the amino terminus (e.g., Ala^{25} Ala^{26} Ala^{27}).

The remaining prohormone, 357 residues in length, contains three separate regions from which myomodulin-related peptides may be cleaved (Figs. 2, 3). Two of these peptides, myomodulin B and myomodulin H, are located in the amino-terminal half of the precursor. Dibasic endoproteolytic cleavage sites flank this region and a single arginine residue is present between the two peptides (position 62). The single octapeptide present on the precursor, myomodulin B, has been purified from neural elements in the ARC muscle (Cropper et al., 1991), indicating that cleavage takes place at this site. The sequence in the vicinity of this arginine conforms to each of four rules and five tendencies that were compiled from comparisons of known monobasic processing sites in peptide precursors (Devi, 1991). Processing at specific monobasic sites occurs in several precursors for molluscan regulatory peptides (Mahon et al., 1985; Newcomb and Scheller, 1987; Linacre et al., 1990).

The amino-terminal peptide-coding portion of the myomodulin precursor is followed by a sequence of 118 amino acids (positions 73–190) that does not contain myomodulin-related peptides. This region is very hydrophilic, containing 39 charged residues. Acidic amino acids predominate, accounting for 34 of the residues (20 glutamic acid and 14 aspartic acid) in this part of the precursor. A pair of arginine residues (positions 126 and 127) provides an additional potential site of endoproteolytic cleavage.

The carboxyl-terminal half of the precursor consists primarily of myomodulin-related peptides, all of which are flanked by dibasic Lys-Arg cleavage sites. Two such peptides, myomodulin I and myomodulin D (Figs. 2, 3), occur in tandem (Fig. 3*B*), separated from the remainder of the carboxyl-terminal portion of the precursor by a 25 residue acidic region that contains six glutamic acid and six aspartic acid residues. A pair of lysines (positions 222 and 223) occurs within this sequence. This region is followed by a single copy of myomodulin G and 10 contiguous copies of myomodulin A. As is the case for all of the myomodulin-related peptides, a glycine residue precedes each dibasic cleavage site. This glycine is likely to contribute to the carboxyl-terminal amide group during the posttranslational processing of these peptides (Loh and Gainer, 1983). Carboxyl-terminal amidation is required for bioactivity of myomodulin-related peptides (Cropper et al., 1988, 1991).

The 10 copies of myomodulin A are followed by a short (six residues) acidic region and a single copy of myomodulin F. The carboxyl-terminal Lys-Arg of myomodulin F is followed by three amino acids. An extension of at least three amino acids on the carboxyl-terminal side of the Lys-Arg doublet may be required for efficient endoprotease substrate recognition (Brakch et al., 1989).

The myomodulin-related peptides

A total of seven different myomodulin-related peptides are present on the myomodulin precursor polypeptide (Fig. 3*A*). Each peptide occurs in a single copy except myomodulin A, which is found in 10 contiguous copies. Each of the myomodulin-related peptides shares a common carboxyl-terminal Met-Leu-Arg-Leu-NH_2 sequence with the exception of myomodulin B, in which methionine substitutes for leucine in the −3 position. A serine residue predominates in the −5 position; however, the two peptides located in the amino-terminal peptide-coding region of the precursor (myomodulin B and myomodulin H) have basic residues (arginine and histidine, respectively) in this position. The −6 position tends to be hydrophobic, with leucine predominating (five of seven peptides), and the amino acids in the −7 position tend to be small and polar.

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                                                                  AGAGAAGTTGTTCA
                                                                -223

CTTTTCAAACTTTTGGGAACGGGGCAGCCAGCAGCGAGGAACTTCTTGCGTACGTCAAAGGACGAGGAACTTTTAAAGA
                                                                                -138

CTTTCAGCAGCCGCCGACTTCAAGACAGCAAAGTCTCTCACCCCCGGGACAAGGAAACGAAACGAAATTGTTACCGAGA
                                                                                -59

                                                  MET Gln Val Tyr Met Leu Leu
ACCAAGATAAAAGAGATTTATTGATAGGTAAAACTTGTTACAGGAACAACC ATG CAG GTG TAT ATG CTC CTG
                                                                                21
8           -3      -1 ▼
Pro Leu Ala Val Phe Ala Ser Leu Thr Tyr Gln Gly Ala Cys Glu Glu Thr Ala Ala Ala
CCG CTT GCT GTC TTT GCC TCT CTG ACC TAC CAG GGT GCC TGT GAA GAA ACT GCT GCA GCT
                                          ▲                                     81
28
Gln Thr Ser Ser Asp Ala Ser Thr Ser Ser Ala Ser Ser Glu His Ala Glu Asn Glu Leu
CAA ACC AGC AGC GAT GCA TCA ACT TCT TCT GCT TCT TCG GAG CAC GCC GAG AAC GAG CTG
                                                                               141
48                      B                                   H
Ser Arg Ala Lys Arg Gly Ser Tyr Arg Met Met Arg Leu Gly Arg Gly Leu His Met Leu
TCA CGA GCA AAA CGA GGC AGT TAC AGA ATG ATG AGA CTT GGC AGA GGT TTG CAC ATG CTC
                                                                               201
68
Arg Leu Gly Lys Arg Gly Gly Pro Val Glu Pro Glu Ser Glu Glu Asn Leu Glu Thr Leu
AGA CTG GGG AAA AGG GGA GGG CCC GTC GAG CCT GAG AGC GAG GAA AAC CTG GAA ACC CTT
                                                                               261
88
Leu Asn Leu Leu Gln Gly Tyr Tyr Ser Asp Val Pro Glu Tyr Pro Ser Glu Phe Asp Asp
TTG AAC TTA TTG CAA GGT TAC TAC AGT GAT GTA CCG GAG TAT CCA TCT GAA TTC GAC GAC
                                                                               321
108
Thr Asp Leu Ala Tyr Pro Tyr Glu Glu Tyr Asp Ala Pro Ala His Pro Arg Tyr Arg Arg
ACT GAT TTG GCC TAC CCA TAC GAA GAA TAC GAC GCC CCC GCC CAC CCG AGA TAC CGG AGA
                                                                               381
128
Ser Thr Pro Pro Thr Asp Gly Val Val Ala Pro Asp Val Leu Gln Lys Gly Ser Ser Glu
TCT ACA CCT CCT ACA GAC GGC GTG GTG GCA CCA GAC GTG CTC CAG AAG GGA AGT TCT GAA
                                                                               441
148
Phe Glu Asp Phe Gly Asp Ser Gln Leu Asp Glu Ser Asp Glu Gly Tyr Tyr Gly Tyr Asp
TTC GAA GAC TTT GGT GAT TCC CAG TTG GAC GAA AGT GAT GAA GGT TAC TAT GGT TAC GAC
                                                                               501
168
Pro Glu Asn Tyr Leu Tyr Gly Asp Phe Glu Asp Tyr Leu Glu Pro Glu Glu Gly Gly Leu
CCT GAA AAC TAT TTG TAT GGC GAT TTT GAA GAT TAC TTG GAA CCA GAA GAA GGA GGA CTT
                                                                               561
188                     I                                   D
Gly Glu Glu Lys Arg Ser Leu Ser Met Leu Arg Leu Gly Lys Arg Gly Leu Ser Met Leu
GGA GAA GAG AAA AGA AGT CTG TCT ATG CTG CGA TTG GGA AAA CGA GGA CTG TCT ATG CTG
                                                                               621
208
Arg Leu Gly Lys Arg Glu Gly Glu Glu Gly Asp Glu Met Asp Lys Lys Gln Asp Glu Ser
AGA CTG GGA AAG AGA GAG GGG GAA GAA GGG GAT GAA ATG GAC AAG AAA CAA GAC GAA AGC
                                                                               681
228                                                 G
Leu Asn Asp Ala Phe Glu Asn Asp Asp Ile Lys Arg Thr Leu Ser Met Leu Arg Leu Gly
TTG AAC GAC GCT TTC GAA AAT GAT GAT ATC AAG AGA ACC CTC TCA ATG CTT CGT CTC GGC
                                                                               741
248     A                                           A
Lys Arg Pro Met Ser Met Leu Arg Leu Gly Lys Arg Pro Met Ser Met Leu Arg Leu Gly
AAA CGA CCA ATG AGC ATG CTT AGA TTA GGA AAA CGG CCA ATG AGC ATG CTG CGT CTT GGA
                                                                               801
268     A                                           A
Lys Arg Pro Met Ser Met Leu Arg Leu Gly Lys Arg Pro Met Ser Met Leu Arg Leu Gly
AAG CGA CCA ATG AGT ATG TTG CGT CTC GGA AAG CGA CCA ATG AGT ATG CTC CGA CTT GGA
                                                                               861
288     A                                           A
Lys Arg Pro Met Ser Met Leu Arg Leu Gly Lys Arg Pro Met Ser Met Leu Arg Leu Gly
AAG CGA CCA ATG AGC ATG CTT CGC CTC GGA AAA CGA CCA ATG AGC ATG CTG ACA CTG CGA
                                                                               921
308     A                                           A
Lys Arg Pro Met Ser Met Leu Arg Leu Gly Lys Arg Pro Met Ser Met Leu Arg Leu Gly
AAG CGG CCA ATG AGC ATG TTG CGC CTT GGA AAA CGA CCA ATG AGC ATG CTG CGT TTG GGG
                                                                               981
328     A                                           A
Lys Arg Pro Met Ser Met Leu Arg Leu Gly Lys Arg Pro Met Ser Met Leu Arg Leu Gly
AAA CGA CCA ATG AGC ATG CTC CGT CTG GGA AAG CGG CCA ATG AGT ATG TTG CGC CTA GGC
                                                                              1041
348                                                 F
Lys Arg Asp Asp Asp Glu Lys Glu Lys Lys Ser Leu Asn Met Leu Arg Leu Gly Lys Arg
AAA CGT GAT GAC GAT GAA AAG GAA AAG AAA TCT TTG AAC ATG TTA CGG CTC GGC AAA CGG
                                                                              1101
        370
Ser Thr Gln  *
TCG ACA CAG TAA TTTCTTAGCAAGTAATAAAGTATAATACAAGA(n)
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Figure 2. Nucleotide sequence of a cDNA clone encoding a myomodulin precursor protein. The predicted amino acid sequence of a myomodulin precursor polypeptide is shown above the nucleic acid sequence. *Numbers* below the nucleotide sequence and above the amino acid sequence denote positions relative to the initiator methionine (*MET*) residue. Two in-frame stop codons in the 5′ untranslated region are *overlined.* Mono- and dibasic potential sites of endopeptidase cleavage are *underlined. Boldface letters* above the amino acid sequence refer to myomodulin-related peptides that could potentially be processed from the precursor (tabulated in Fig. 3). The amino acids proposed to constitute the signal sequence are *underlined. Downward arrowhead* indicates proposed site of signal peptidase cleavage. *Upward arrowhead* denotes site of intron/exon boundary. The stop codon (TAA) is indicated by an *asterisk* and the consensus polyadenylation signal is shown in *boldface.*

A Myomodulin-related Peptides

	Amino Acid Sequence	Copies
	-8 -7 -6 -5 -4 -3 -2 -1	
A	Pro-Met-Ser-Met-Leu-Arg-Leu-NH_2	10
B	Gly-Ser-Tyr-Arg-Met-Met-Arg-Leu-NH_2	1
D	Gly-Leu-Ser-Met-Leu-Arg-Leu-NH_2	1
F	Ser-Leu-Asn-Met-Leu-Arg-Leu-NH_2	1
G	Thr-Leu-Ser-Met-Leu-Arg-Leu-NH_2	1
H	Gly-Leu-His-Met-Leu-Arg-Leu-NH_2	1
I	Ser-Leu-Ser-Met-Leu-Arg-Leu-NH_2	1

B Myomodulin Precursor Organization

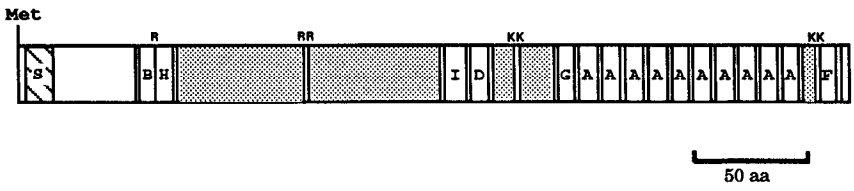

Figure 3. The myomodulin-related peptides and precursor organization. *A,* Tabulation of sequences and copy numbers of peptides potentially processed from the myomodulin precursor protein. A glycine residue present at the carboxyl terminal of each peptide in the precursor is postulated to act as a donor of an amide group. Designation of peptides corresponds to that in Figure 2. Note that endoproteolytic cleavage at a monobasic (Arg) residue would be required to generate the octapeptide, myomodulin B. *B,* Schematic structure of the predicted myomodulin precursor polypeptide. The proposed signal peptide (*S*) region is *cross-hatched. Letters* denoting myomodulin-related peptides correspond to designations in Figures 2 and 3*A*. *Shaded regions* represent charged spacer regions. Certain potential cleavage sites referred to in the text are indicated (*R,* Arg^{62}; *RR,* Arg^{126}-Arg^{127}; *KK,* Lys^{222}-Lys^{223} and Lys^{356}-Lys^{357}). All other paired vertical lines denote Lys-Arg cleavage sites.

Organization of the myomodulin gene

Myomodulin gene organization was initially examined with genomic blotting experiments (Fig. 4). Genomic DNA was isolated from the ovotestis of a single *Aplysia* specimen, digested to completion with various restriction endonucleases, and probed with insert DNA from pMM13 (Fig. 1; ~550 bp). This probe corresponds to the entire 5′ untranslated region and encodes the 104 amino-terminal residues of the precursor peptide. With the exception of *Pst* I, there are no restriction sites for the enzymes tested within the sequence of the probe (see Fig. 1). This probe hybridized to multiple bands in each lane, indicating that the *Aplysia* genome may contain additional genes with substantial homology to this myomodulin clone. The presence of an intervening sequence within this gene could also contribute to such a pattern of hybridization.

In order to examine the myomodulin gene directly, a genomic clone was isolated from a size-selected [~2.4 kilobase (kb)] library constructed from a *Sac* I digest of genomic DNA. Sequence and restriction analyses of this clone showed it to be colinear with much of the cDNA clone, including the 3′ untranslated region and the region encoding the myomodulin-related peptides (Fig. 5). However, the sequence of the genomic clone diverges from that of the cDNA at a point within the coding region, interrupting the codon for glycine in position 19 of the precursor (Fig. 5; see also Fig. 2, upward arrowhead). The sequence of the genomic clone in this region (TTTGCAGG) corresponds to a consensus 3′ intron/exon splice sequence (Fig. 5; see Padgett et al., 1986). It appears, therefore, that the myomodulin gene contains at least one intron that separates an exon encoding the myomodulin-related peptides from more 5′ portions of the gene.

Restriction mapping indicates that the genomic clone extends approximately 1 kb in the 5′ direction from the proposed intron/exon boundary (Fig. 5). Probes homologous to cDNA sequences 5′ from this point of divergence failed to hybridize to the genomic clone, indicating that the intervening sequence in the gene is at least 1 kb in length. The organization of the 5′ region of the gene has not been further studied. Large intervening sequences are present in several genes encoding invertebrate regulatory peptides (Nambu et al., 1983; Schneider and Taghert, 1990; Wickham and DesGroseillers, 1991), where they often occur between regions encoding specific functional domains of the precursor (Taussig et al., 1984; Taussig and Scheller, 1986). In the case of the myomodulin gene, an intervening sequence appears to lie between the region that encodes the signal sequence and the neuropeptide-encoding exon (see also Saunders et al., 1992).

Myomodulin gene expression

RNA blot hybridization was initially used to assess expression of the myomodulin gene. PolyA$^+$ RNA was isolated from *Aply-*

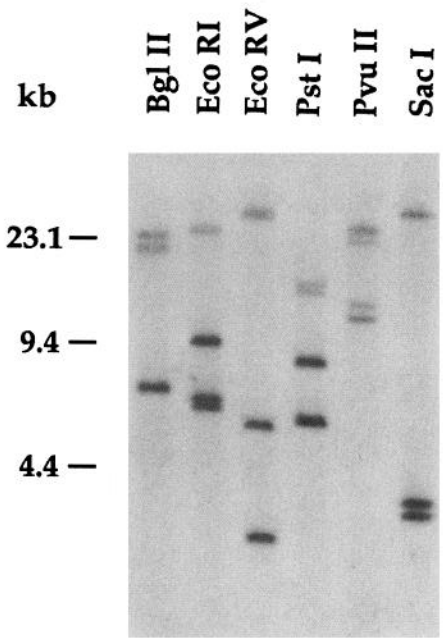

Figure 4. Genomic DNA blot analysis of myomodulin clone: Southern blot of DNA isolated from ovotestis of a single *Aplysia* specimen. DNA (10 μg/lane) was digested to completion with the restriction enzymes shown for each lane, fractionated on a 0.8% agarose gel, denatured, blotted onto nylon membrane, and hybridized to the *Eco*R I insert from pMM13 (see Fig. 1) ^{32}P-labeled by random priming. Multiple hybridizing bands were observed within each digest. The size standard was a *Hind* III digest of wild-type λ.

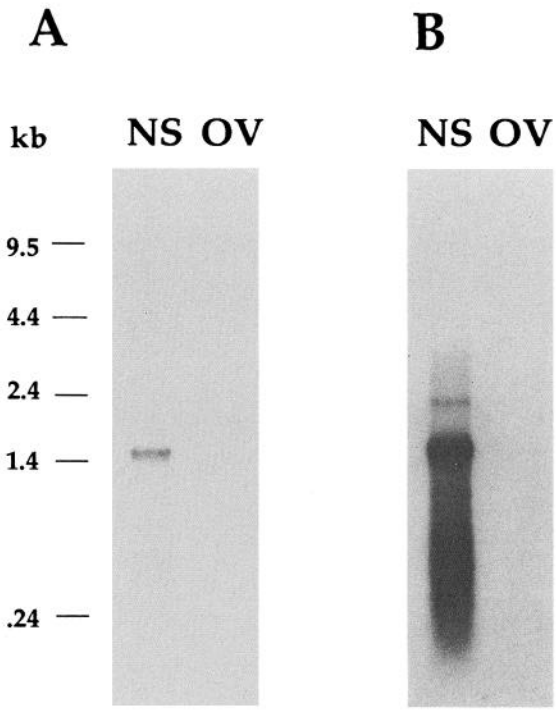

Figure 6. RNA gel blot analysis of myomodulin gene expression. PolyA$^+$ RNAs (5 μg) extracted from the nervous system (*NS*; pooled central ganglia) and the ovotestis (*OV*) were fractionated on a denaturing gel, transferred to nylon membrane, and probed with the *Eco*R I insert from clone pMM13 (see Fig. 1) ^{32}P-labeled by random priming. *A,* Overnight exposure of the blot revealed hybridization to an ~1.4 kb transcript in the nervous system, but not in the ovotestis. *B,* With longer exposures of the blot (7 d), numerous additional bands were noted in the nervous system, including one approximately 2.4 kb in length. RNA molecular weight markers (Bethesda Research Labs) were used as size standards.

sia tissues, size fractionated, transferred to nylon membrane, and probed with labeled insert from pMM13. Exposure of blots (overnight) showed that myomodulin expression occurs in a tissue-specific manner (Fig. 6*A*). While no hybridization was detected with ovotestis RNA, a single dominant hybridizing band was present in neural RNA. The length of this transcript (~1.4 kb) indicates that the cDNA clone isolated in this study (Fig. 2) is a nearly complete copy of the most prominent myomodulin mRNA species in the nervous system. Longer autoradiographic exposure (7 d) of Northern blots revealed additional hybridizing bands, including one approximately 2.4 kb

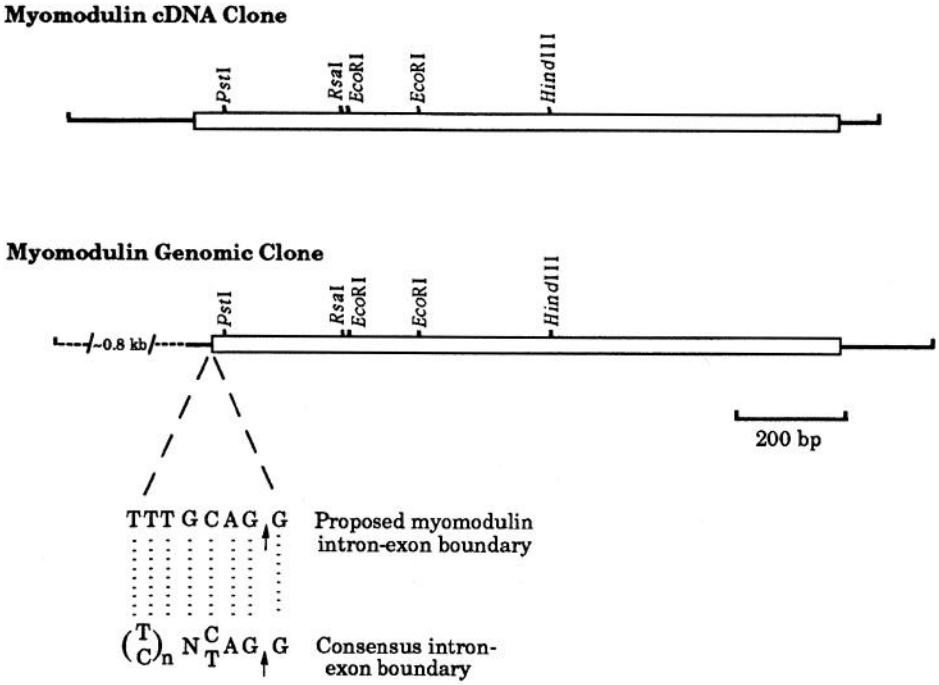

Figure 5. Comparison of restriction maps of myomodulin cDNA and genomic clones. *Open bar* indicates ORF; *dark lines* correspond to untranslated regions. *Dashed line* in genomic clone denotes portion of the clone that has not been completely characterized. The cDNA and genomic sequences are colinear throughout much of their extent, but they diverge at a point (5′ from the *Pst* I site; see Fig. 2, upward arrowhead) that is within the ORF. The nucleotide sequence found in the genomic clone at this point is shown *below* and compared to a consensus 3′ intron-exon boundary (Padgett et al., 1986).

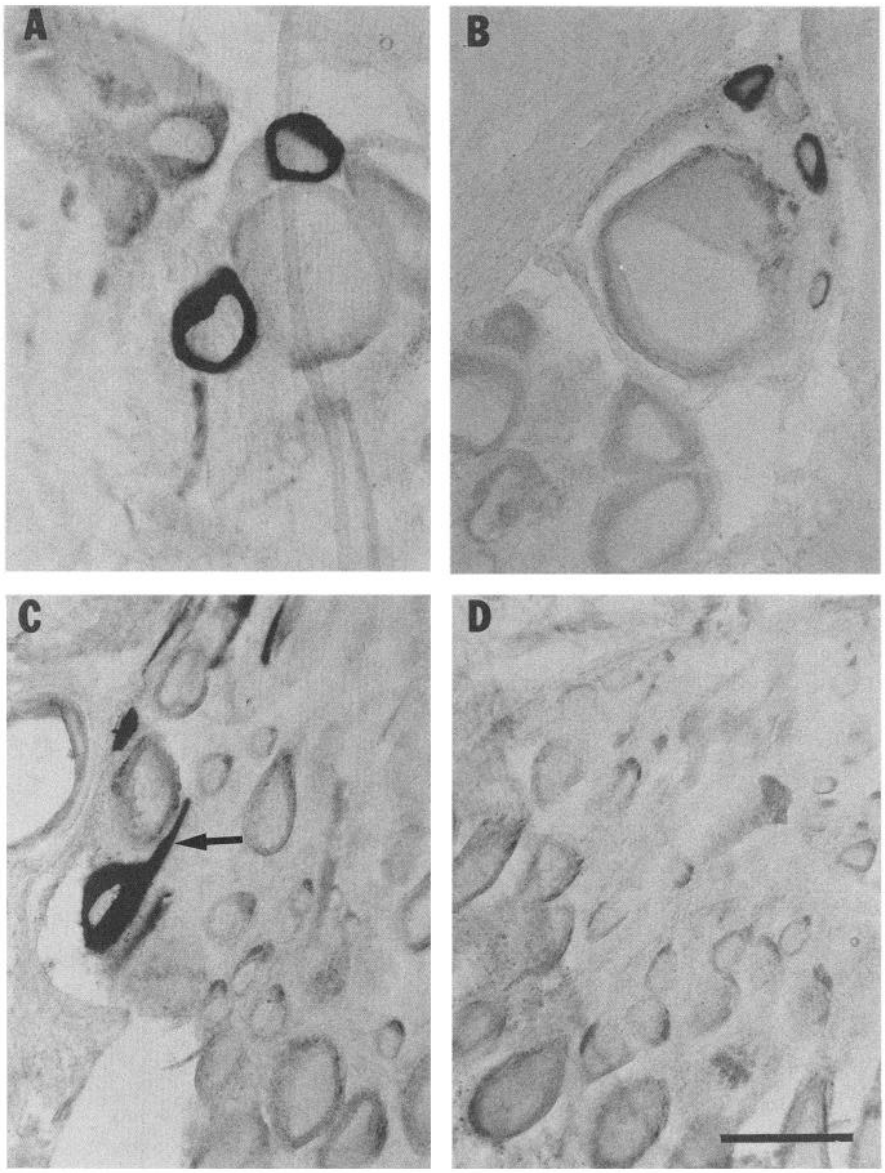

Figure 7. Expression of the myomodulin precursor mRNA occurs in specific cells in the *Aplysia* nervous system: *in situ* hybridization of a digoxigenin-UTP–labeled cRNA probe generated from a 386 bp *Rsa* I *Hind* III fragment from the coding region of the myomodulin cDNA (corresponding to the bases 294–683 of Fig. 2). Antisense probe hybridized to the cytoplasmic region of specific neuronal somata in the anterior portion of the pedal ganglion (*A*), the anterior portion of the pleural ganglion (*B*), and lateral cells in the cerebral ganglion (*C*). Note signal present in initial segment of neuron in cerebral ganglion (*arrow*). *D*, No specific hybridization was observed in the same region of the cerebral ganglion as *C* with a sense-strand cRNA probe. Scale bar, 100 μm.

in length (Fig. 6*B*). These bands could result either from less abundant forms of this myomodulin transcript including incompletely processed precursors or from transcripts of related genes.

Myomodulin gene expression was also investigated using *in situ* hybridization to histological sections of *Aplysia* central ganglia. Hybridization of a digoxigenin-labeled antisense probe occurred to specific neurons and cell clusters in each of the ganglia (Fig. 7*A–C*). The myomodulin message was localized to the cytoplasmic region of cell somata and was also present in the initial segment of a few cells (Fig. 7*C*, arrow). Probes generated from the complementary (sense) strand failed to hybridize, indicating specificity of transcript detection (Fig. 7*D*). These findings are in general agreement with a recent mapping of myomodulin-like immunoreactivity in the *Aplysia* CNS (Miller et al., 1991a).

Discussion

In this study, we have isolated and characterized cDNA and genomic clones that encode a precursor for myomodulin-related peptides in *Aplysia californica*. The organization of the myomodulin gene and the precursor that it encodes presents several potential mechanisms for generating peptide diversity and specificity in neurons.

Myomodulin precursor organization

The cDNA and genomic clones characterized in this study encode a myomodulin precursor from which multiple distinct but structurally related peptides are likely to be cleaved during post-translational processing (Loh et al., 1984; Sossin et al., 1989). Two acidic "spacer" domains serve to define three distinct regions in which the myomodulin-related peptides tend to occur in tandem, that is, separated only by Gly-Lys-Arg processing sites (Figs. 2, 3*B*). Acidic sequences often occur in precursors for neuropeptides, where they are thought to affect processing enzymes or to facilitate packaging into secretory granules via ionic interactions with the basic peptide–containing regions (Noda et al., 1982; Schaefer et al., 1985; Taussig and Scheller, 1986).

Certain unique pairs of basic residues that occur only within the acidic spacer regions (see Fig. 3*B*) could provide posttransla-

tional flexibility of myomodulin precursor processing. The egg-laying hormone (ELH) precursor of *Aplysia* is processed via a series of specific endoproteolytic cleavages, the first of which serves to divide the protein into two polypeptides (Newcomb and Scheller, 1987; Newcomb et al., 1988; see also Linacre et al., 1990). Endopeptidases targeting specific loci (e.g., Arg^{126}-Arg^{127}, Lys^{222}-Lys^{223}) could similarly partition the myomodulin precursor. Immunohistochemical and autoradiographic data indicate that the peptides derived from the two initial ELH precursor polypeptides are differentially packaged and sorted by the neurosecretory bag cells (Fisher et al., 1988), and that they are targeted to different release sites (Sossin et al., 1990). If such sorting were to occur in cells that express the myomodulins, it could provide a means of segregating certain peptides, for example, the peptides (myomodulins B and H) present in the amino-terminal half of the precursor. These possibilities could be tested using identified neurons, for example, the widely acting abdominal ganglion interneuron L10, which synthesizes myomodulins A and B (Alevizos et al., 1987; Cropper et al., 1991; Miller et al., 1991a) and exerts numerous central and peripheral actions (Frazier et al., 1967; Koester and Alevizos, 1989).

Myomodulin gene organization

Genomic blotting experiments (Fig. 4) indicated that the myomodulin gene comprises at least two exons, but left unresolved the possibility that additional related genes could be present. Interpretation of these experiments is rendered difficult by the high degree of polymorphism that appears to be present in regions encoding peptide precursors in *Aplysia* (Scheller et al., 1982; Nambu et al., 1983; Miller et al., 1993). Furthermore, the pattern of "doublet" bands in genomic blots (see Fig. 4) has been observed to vary among individual specimens (not shown), indicating the presence of allelic variation in the vicinity of the myomodulin gene.

An intron appears to partition the myomodulin gene into at least two domains (Figs. 2, upward arrowhead; 5). All of the cDNA clones examined were colinear with the genomic clone in the region corresponding to the myomodulin-related peptides (Fig. 5), indicating that alternative RNA splicing mechanisms are not used to produce differential expression of these peptides (cf. Nawa et al., 1984; Buck et al., 1987). The position of the intron, separating the region encoding the proposed signal peptide from the region encoding the remainder of the precursor, is strikingly similar to the arrangement of the genes encoding Phe-Met-Arg-Phe-amide (FMRFamide) in *Aplysia* (Taussig and Scheller, 1986) and *Lymnaea* (Saunders et al., 1991, 1992). This organization is consistent with Gilbert's (1978) hypothesis, in which it is proposed that exons correspond to functional domains of proteins.

Five of the myomodulin-related peptides (myomodulins A, B, D, F, and G) encoded by the clones isolated in this study have been purified from neural elements in the ARC muscle (Cropper et al., 1987b, 1991; Miller et al., 1990). In purification studies, myomodulin A is typically found at approximately six-fold higher concentrations than myomodulin B (Cropper et al., 1991). Such deviations from the precursor stoichiometry (10:1) may simply reflect differences in peptide recovery or they may be due to specificities of peptide processing, sorting, or degradation. The purification of two additional related peptides (myomodulins C and E; see Miller et al., 1990) that are not encoded by the clones indicates the presence of additional myomodulin-encoding transcripts. These peptides could be encoded by related genes (see Scheller et al., 1982; Douglass et al., 1984) or by a separate exon within a single gene (see Amara et al., 1982; Saunders et al., 1991, 1992). One hypothetical scenario, that is, that the exon encoding the signal sequence could be spliced to an exon encoding a distinct set of myomodulin-related peptides, was tested by isolating 19 nervous system cDNA clones with a probe homologous to the sequence encoding the signal peptide. All of these clones hybridized to a second probe based upon the most 5′ sequence of the exon encoding the myomodulin-related peptides (not shown). These results indicate that alternative splicing of an exon encoding this signal sequence to a second peptide-encoding exon does not occur in the nervous system (cf. Saunders et al., 1992); however, a complete characterization of the gene(s) encoding myomodulin-related peptides will require further study.

The myomodulin peptide family

Members of a peptide family typically exhibit considerable homology in their carboxyl-terminal sequences (e.g., Mahon et al., 1985; Price, 1986). The myomodulin-related peptides encoded by the clones isolated in this study (with the exception of myomodulin B) share the common Met-Leu-Arg-Leu-NH_2 carboxyl motif (Fig. 3*A*). This sequence is also present in the myomodulin-related peptides isolated from *Fusinus* (Kobayashi and Muneoka, 1990) and *Mytilus* (Hirata et al., 1987; see introductory remarks), indicating that it is a conserved feature of peptides that constitute this intraphyletic family (see Price et al., 1987).

Differences in the amino-terminal extensions of peptides are thought to confer variation in susceptibility to enzymatic degradation and/or receptor specificity (see Greenberg, 1983; Cottrell and Davies, 1987). The specific substitution of leucine for methionine, commonly observed within peptide families (Hughes et al., 1975; Price, 1986), occurs at two positions (−3 and −6) in the myomodulin-related peptides. This substitution may serve to expand the spatial or temporal range of peptide action by lessening the likelihood of oxidation (Price, 1986). Alternatively, or in addition, multiple myomodulin receptors with highly specific sensitivities to individual peptides may occur in certain tissues (cf. Brussaard et al., 1991; Kemenes et al., 1992). This possibility is supported by the observation of differences between the effects of myomodulin A and those of myomodulin B on evoked contractions of the ARC muscle (Cropper et al., 1991). It would be of interest to compare further the effects of the myomodulin-related peptides in this system and to extend such comparisons to the myomodulin receptors that are present on neurons (see Rosen et al., 1989; Critz et al., 1989).

The myomodulin-related peptides exhibit a highly specific and widespread pattern of expression in identifiable neurons throughout the CNS of *Aplysia* (Church and Lloyd, 1991; Miller et al., 1991a). This characterization of the myomodulin gene should facilitate further studies aimed toward determining ontogenetic, physiological, and environmental factors that influence this pattern of expression. It may also contribute to our understanding of the evolutionary and functional relationships that exist among the members of this neuropeptide family.

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