The neuropeptide F (NPF) encoding gene from the cestode, *Moniezia expansa*

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SUMMARY

Neuropeptide F (NPF) is an abundantly expressed neuropeptide in platyhelminth nervous systems, and exhibits a moderate, myogenic effect on muscle preparations of parasitic flatworms. NPF displays structural similarities to peptides from molluscs and vertebrate members of the neuropeptide Y (NPY)-superfamily of peptides. NPY is one of the most abundant and highly conserved neuropeptides within vertebrates and similarities between the gene organization of NPY, pancreatic polypeptide (PP) and peptide tyrosine tyrosine (PYY), suggest a common evolutionary origin of this peptide family. Dual localization analyses coupled with confocal scanning laser microscopy revealed a close spatial relationship between NPF-containing nerves and muscle fibres in *M. expansa*. Molecular cloning techniques identified the *M. expansa* NPF (mxNPF) precursor and characterized the isolated transcript which encodes an open reading frame of 57 amino acids. The transcript possesses a 17 amino acid signal peptide and the mature NPF sequence (39 amino acids) followed by a carboxyterminal glycyl extension. Sequence analysis of genomic DNA identified a product which possessed a 54 base pair intron with consensus sequences for 5′ and 3′ splice sites. The *M. expansa npf* gene possesses a phase 2 intron within the penultimate arginyl residue, a characteristic feature of NPY superfamily peptide-genes. The intron–exon organization of the *npf* gene, coupled with the abundant expression of NPF within the nervous systems of flatworms, suggests an early evolutionary origin for this peptide transmitter family within the nervous systems of basal bilaterian metazoans.

Key words: *Moniezia expansa*, neuropeptide F, neuropeptide gene, gene structure, intron–exon organization.

INTRODUCTION

Neuropeptide Y (NPY), pancreatic polypeptide (PP) and peptide tyrosine tyrosine (PYY) are related peptides which play crucial roles in vertebrate central nervous and endocrine systems. NPY is exclusively expressed within the nervous system and has been implicated in the modulation of numerous physiological processes, such as appetite, blood pressure and circadian rhythm, while PP and PYY are expressed within endocrine cells of the intestine and pancreas (Colmers & Wahlestedt, 1993). The first invertebrate homologue to NPY was isolated from the intestinal sheep parasite, *Moniezia expansa* (Cyclophyllidea, Cestoda) due to its cross-reactivity with PP-antisera (Maule *et al*. 1991). As it possesses a C-terminal phenylalanylamide, as opposed to a tyrosylamide of most vertebrate peptides, this 39 amino acid peptide was designated neuropeptide F (NPF).

NPF is widely and abundantly expressed in the central and peripheral nervous systems of *M. expansa* (Maule *et al*. 1992), and NPF-immunostaining has frequently been identified within related flatworm species (Maule, Halton & Shaw, 1995). Similar peptides were subsequently isolated from invertebrates belonging to the Platyhelminthes (*Artioposthia triangulata* (Curry *et al*. 1992)) and Mollusca (*Helix aspersa* (Leung *et al*. 1992), *Aplysia californica* (Rajpara *et al*. 1992) and *Lymnaea stagnalis* (Tensen *et al*. 1998)). All 5 peptides display the C-terminal PP-family motif ([FY]-X(3)-[LIVM]-X(2)-Y-X(3)-[LIVMFY]-X-R-X-R-[YF]; single letter notation for amino acids; amino acids in square brackets are interchangeable; X indicates a variable residue; numbers indicate the number of variable residues) common to vertebrate NPY-family members (Table 1), but it is unknown whether they are able to assume a similar tertiary structure, referred to as the PP-fold (Glover *et al*. 1985), which is characteristic of vertebrate peptides.

Like NPY, the invertebrate homologues are expressed within the nervous system. They modulate physiological processes such as muscle motility in flatworms (Marks *et al*. 1996), and inhibit specific nerve cells at the onset of egg-laying in *A. californica* (Rajpara *et al*. 1992). Two NPY-like receptors, which have been recently identified in *L. stagnalis* (Tensen *et al*. 1998) and *Caenorhabdites elegans* (de Bono & Bargmann, 1998), promote alteration of cAMP-levels and affect feeding behaviour, respectively.

The existence of invertebrate NPY-homologues suggests an early evolutionary origin of this peptide transmitter family. The phylum Platyhelminthes is

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Table 1. Amino acid sequences of all known invertebrate and selected vertebrate members of the neuropeptide Y (NPY)-superfamily

Species	Amino acid sequence*
Moniezia expansa NPF[1]	PDKDFIVNPSDLVLDNKAALRD**Y**LRQ**I**NE**Y**FAII**G**R**P**R**F**
Artioposthia triangulata NPF[2]	KVVHLRPRSSFSSEDEYQI**Y**LRN**V**SK**Y**IQL**Y**G**R**P**R**F
Aplysia californica NPY[3]	DNSEMLAPPPRPEEFTSAQQLRQ**Y**LAA**L**NE**Y**YSI**M**G**R**P**R**F
Helix aspersa NPF[4]	STQMLSPPERPREFRHPNELRQ**Y**LKE**L**NE**Y**YAI**M**G**R**T**R**F
Lymnaea stagnalis[5]	TEAMLTPPERPEEFKNPNELRK**Y**LKA**L**NE**Y**YAI**V**G**R**P**R**F
Rana temporaria PP[6]	APSEPHHPGDQATQDQLAQ**Y**YSD**L**YQ**Y**ITF**V**T**R**P**R**F
Lampetra fluviatilis PYY[7]	FPPKPDNPGDNASPEQMAR**Y**KAA**V**RH**Y**INL**I**T**R**Q**R**Y
Homo sapiens NPY[8]	Y[8]YPSKPDNPGEDAPAEDMAR**Y**YSA**L**RH**Y**INL**I**T**R**Q**R**Y

* Single letter notation for amino acids. Amino acids which conform to the pancreatic hormone family signature are shown in bold. Conserved proline residues within the N-terminus of the NPY-family are underlined. NPF, neuropeptide F; PYY, peptide YY. P-residues are absent in analogous positions within the platyhelminth homologues, but are conserved to some degree in the molluscan peptides. The PP-fold is characterized by an N-terminal polyproline helix and an alpha-helix at the C-terminus connected through a beta-turn. [1]Maule *et al*. (1991); [2]Curry *et al*. (1992); [3]Rajpara *et al*. (1992); [4]Leung *et al*. (1992); [5]Tensen *et al*. (1998); [6]McKay *et al*. (1990); [7]Soderberg *et al*. (1994); [8]Tatemoto, Carlquist & Mutt (1982).

considered to have emerged early within the Bilateria, whereas the Mollusca are well established within the Protostomia, both phyla being evolutionarily distinct from the vertebrates, which are members of the Deuterostomia (Field *et al*. 1988). The lack of molecular sequence information for the genes encoding the invertebrate peptides, however, has prevented definite confirmation of an emergence of NPY-like peptides prior to the protostome/deuterostome divergence in early bilaterian nervous systems. To gain insight into the molecular evolution and the origin of the NPY-family of peptides, we have cloned a cDNA encoding *M. expansa* NPF and subsequently characterized the corresponding gene, and identified a remarkable case of intron-conservation between phylogenetically distinct NPY/NPF-peptides.

MATERIALS AND METHODS

Parasite material

Moniezia expansa were recovered from the small intestines of freshly slaughtered sheep at a local abattoir and transported at 37 °C in 0·9 % (w/v) NaCl and 0·1 % (w/v) glucose. Specimens were snap-frozen in liquid nitrogen and stored at −80 °C prior to use.

Reverse transcription-polymerase chain reaction (RT-PCR)

Snap-frozen tissue was ground to a fine powder under liquid nitrogen using a pestle and mortar and total RNA was isolated with TRIzol reagent (Gibco/BRL). Total RNA (30 μg in 10 ml of H_2O) was incubated at 65 °C for 3 min in the presence of an oligo d(T)-primer and subsequently cooled on ice for 5 min. After addition of dNTPs, 5 × RT-buffer and 200 units MMLV Reverse Transcriptase, the reaction was incubated at 37 °C for 90 min. First strand cDNA was used in a PCR with primers AGM36 (CCNGAYAARGAYTTYATHGTNAAYCC) and AGM37 (ATHATNGCRAARTAYTCRTTHATYTG), corresponding to amino acid sequences 1–9 (PDKDFIVNP), and 26–33 (QINEYFAII) of NPF, respectively.

3′ and 5′ rapid amplification of cDNA ends (RACE)

mRNA was selected from 75 μg total RNA with Dynabeads and used to synthesize double-stranded, adaptor-ligated cDNA according to the Marathon cDNA Amplification Kit instructions (Clontech). 3′ RACE was performed with primers AGM39 (TTCTCGACAATAAAGCTGCTC) and adaptor primer 1 (CCATCCTAATACGACTCACTATAGGGC). Primers AGM45 (GGTCGAGGTTCAAAGTGCCAAC) and adaptor primer 1 were used in 5′ RACE amplification. After initial denaturation at 94 °C for 2 min, 45 cycles of PCR were carried out under the following conditions: 30 sec at 94 °C, 60 sec at 55 °C and 45 sec at 72 °C. PCR products were visualized on a 1·5 % agarose gel containing 0·5 mg/ml ethidium bromide.

Amplification of genomic DNA

Genomic DNA was isolated from snap-frozen specimens as described by Price *et al*. 1996. Snap-frozen tissue (18 μg) was crushed and 90 μl of 0·1 M NaOH added and heated to 95 °C for 10 min. Following centrifugation (20000 ***g***, 20 min) the supernatant (70 μl) was stored at −80 °C. Primers AGM62 (GGTTACCATCACGACATCAACG), AGM66 (ACTCATTTTATTGCGGTTGAG) and AGM45 were used to amplify genomic regions

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of *npf*. Cycling parameters were as follows: 1 min initial denaturation at 94 °C, 35 cycles of 45 sec at 95 °C, 45 sec at 50 °C, 45 sec at 72 °C, and a final elongation step of 10 min at 72 °C.

Sequence analysis

PCR fragments were recovered from agarose gels (Boyle & Lew, 1995) and cloned into the TA cloning vector (Invitrogen). A minimum of 3 independently derived clones for each PCR reaction were sequenced using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) in the presence of 100 pmoles primer. Cycling parameters were as follows: 3 min initial denaturation at 94 °C, 50 sec of primer annealing at 50 °C and 4 min elongation at 62 °C, followed by 24 cycles of 30 sec at 94 °C, 50 sec at 50 °C and 4 min at 62 °C. Products were purified with phenol/$CHCl_3$/isoamyl alcohol (25/24/1) and precipitated with 100 % EtOH and 3 M sodium acetate containing 2 mg/ml blue dextran. Pellets were dried at RT for 20 min and resuspended in 4 μl of de-ionized formamide.

Samples were denatured at 90 °C for 3 min, and kept on ice until loaded. Bands were separated on a 12 % (w/v) *bis*-acrylamide gel (25 g urea, 0·65 g amberlite de-ionizing beads, 7·5 ml of 40 % (w/v) *bis*-acrylamide, 19·5 ml of ddH_2O, 250 ml of 10 % ammonium persulfate, 25 ml of TEMED) for 10 h using a 373A DNA Sequencer (Applied Biosystems) and analysed with the Genescan 672 software.

Northern blot analysis

An homologous, digoxygenin-labelled DNA probe was synthesized with hexanucleotides using the Boehringer Mannheim Labelling Kit. mRNA was isolated from 75 μg total RNA with Dynabeads and separated on a 2 % agarose, 2 % formaldehyde gel, and blotted by capillary transfer on to nitrocellulose membranes. Pre-hybridization and hybridization was done according to the manufacturers' recommendations. Probe detection was carried out with anti-DIG-alkaline phosphatase conjugate and incubation with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate.

Immunocytochemistry

Worms recovered from intestinal lavage were cut into 2 cm pieces and flat-fixed between microscope slides with 4 % paraformaldehyde for 1 h, transferred to fresh fixative for 3 h and washed in antibody diluent (AbD; PBS pH 7·4, 0·1 % Triton X–100, 1 % BSA, 0·1 % NaN_3). Specimens were incubated for 48 h with antiserum 792(3) (Maule *et al*. 1992) raised against the C-terminal decapeptide of NPF. To visualize the muscle system, specimens were subsequently washed with AbD, and incubated overnight with TRITC-conjugated phalloidin (Sigma Chemical Company) (200 ng/ml). Worms were finally mounted in PBS/glycerol (1/9) and analysed using a Leica TCS-NT confocal scanning laser microscope.

RESULTS

Characterization of the NPF precursor

A 101 base pair (bp) cDNA fragment was amplified from whole tissue RNA using degenerate primers (AGM36 and AGM37) based on the peptide sequence of *Moniezia expansa* NPF in an RT–PCR approach. Specific primers (AGM 39 and AGM45) were then used to identify the full-length transcript by 3′ and 5′ RACE (rapid amplification of cDNA ends). Sequence analysis revealed a 445 bp cDNA containing 2 open reading frames (ORF) of 18 and 57 amino acids, respectively (Fig. 1A). The *M. expansa npf* gene sequence was deposited in the EMBL Nucleotide Sequence database (accession number AJ242779).

Northern blot

Northern blot analysis of *M. expansa* mRNA revealed the presence of a single transcript of approximately 590 nucleotides (nt; Fig. 1B), which suggests the presence of a poly$(A)^+$ tail of 140–150 nt.

Characterization of the NPF gene

To determine the gene organization of *M. expansa* NPF, specific primer pairs based on the cloned cDNA sequence for NPF, and located within the 5′ and 3′ untranslated regions, were used in conventional PCR methodology on genomic DNA templates. Amplification of genomic DNA resulted in the detection of a single band, approximately 50 bp longer than the band obtained by PCR using double-stranded cDNA. DNA sequence analysis of this PCR-fragment resulted in the characterization of a 54 bp phase-2 intron within *npf* (Fig. 1A). No other intervening sequences were identified within the analysed fragment. The intron is located within the coding region of the penultimate R-residue of *npf* and displays the consensus sequences for the 5′ and 3′ splice sites (SS), GT and AG, respectively. An 18 bp polypyrimidine tract (80 % C and T) was identified close to the 3′ SS, but no branch site consensus sequence was evident.

Interrelationship of NPF-immunoreactive nerves and muscle

NPF-immunopositive nerves were particularly evident in the innervation of muscular structures

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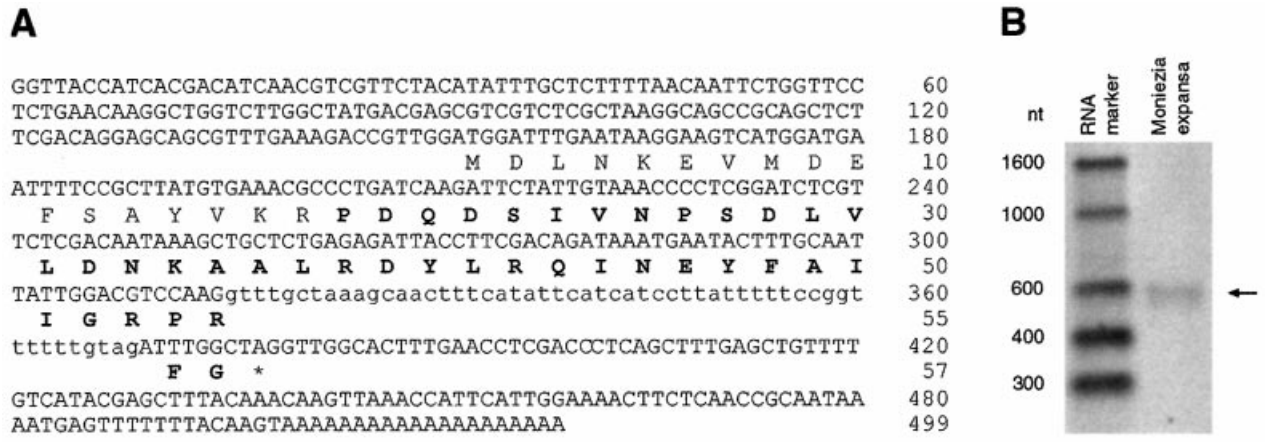


Fig. 1. (A) *npf* and deduced amino acid sequence of the NPF precursor from *Moniezia expansa*. Nucleotides are numbered from the 5′ to the 3′ end, and amino acids starting with the ATG of the second open reading frame. Note that the bases derived from the cDNA are in upper case whereas the intron sequence is presented in lower case. The NPF sequence, including the C-terminal amide donor (G) is in bold-face. (B) Northern blot analysis of *M. expansa* mRNA. nt, nucleotides.

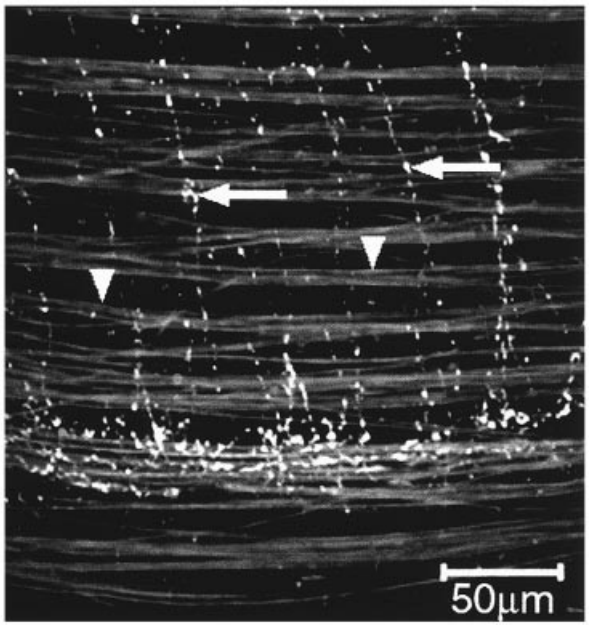


Fig. 2. Confocal scanning laser microscope image showing neuropeptide F (NPF)-expression within the nervous system of *Moniezia expansa* and its association with the musculature. Positive immunostaining was obtained using an antiserum to the C-terminal decapeptide of NPF (Maule *et al*. 1992). Co-visualization of the musculature using the TRITC-labelled F-actin marker, phalloidin, reveals a close spatial relationship of NPF-expressing neurons (arrows) and parenchymal longitudinal muscle fibres (arrowheads).

including the suckers. They were particularly prominent in close spatial proximity to prominent longitudinal muscle fibres of the parenchyme (Fig. 2). There was no obvious relationship between the muscle fibres of mature genitalia or the body wall musculature and NPF-immunopositive nerves.

DISCUSSION

In this study, the NPF-encoding gene from the cestode, *Moniezia expansa* was characterized. Excluding the poly(A)$^+$ tail, the entire *npf* transcript was found to contain 445 nucleotides (nts) and 2 open reading frames (ORFs). None of the 2 translation initiation codons (ORF1: TGGCTAUGA, ORF2: GTTGGAUGG) conforms to the Kozak consensus sequence, CC(A/G)CCAUGG, for translation initiation (Kozak, 1987). However, the second open reading frame codes for a 15 amino acid peptide (which may function as a signal peptide but lacks a typical hydrophobic core region (von Heijne, 1990)), followed by a dibasic cleavage recognition site (KR), the mature NPF peptide sequence and a C-terminal G-extension. The G-extension presumably facilitates the amidation of the C-terminal F-residue (Eipper, Stoffers & Mains, 1992), which is present in mature NPF.

Northern blot analysis revealed the occurrence of a single transcript for NPF of approximately 590 nt. The transcript size (445 nts excluding the poly(A)$^+$ tail) of *M. expansa* NPF is similar to transcript sizes observed for members of the vertebrate NPY-superfamily (human *npy*: 552 nt (Minth *et al*. 1984), human *pyy*: 509 nt (Kohri *et al*. 1993), human *pp*: 468 nt (Leiter *et al*. 1984, 1985)), but differs significantly from the only other known invertebrate NPF-transcript isolated from *A. californica* which has a size of approximately 3400 nt as determined by Northern blot analysis (Rajpara *et al*. 1992).

Two differences were observed between the published mxNPF sequence (Maule *et al*. 1991) and the cDNA sequence translation. Both substitutions (K to Q at position 3 and F to S at position 5 of NPF) may result from single base changes (AAR (peptide sequence) to CAA (cDNA translation) and TTY to

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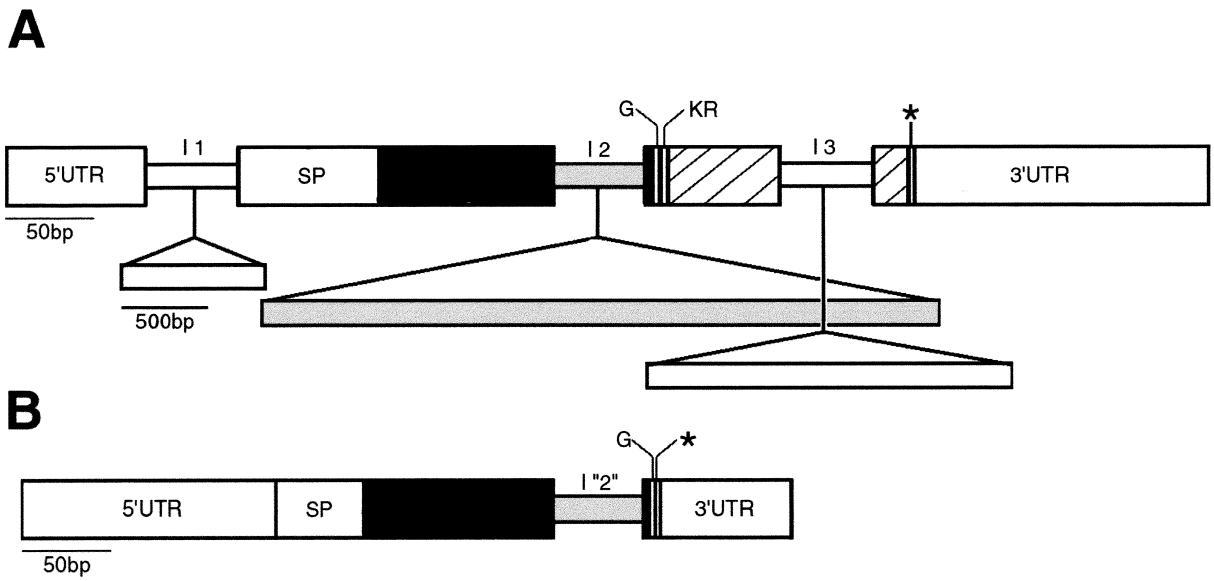


Fig. 3. Linear comparison of human *npy* and *Moniezia expansa npf*. (A) Human *npy* spans approximately 8 kilobase pairs, comprising 4 exons (broad bars) and 3 introns (narrow bars) of 965 bp (intron [I] 1, phase-0), 4300 bp (I 2, phase-2) and 2300 bp (I 3, phase-2). The phase correlations of the corresponding introns are also maintained within human *pyy* and *pp*, the sizes, however, vary significantly, with introns 2 of *pyy* and *pp* being 109 and 245 bp in length, respectively. Exon 1 of *npy* encodes the entire 5′ untranslated region (UTR), exon 2 encodes the signal peptide (SP) and most of the parent peptide (black shading) through to the penultimate R residue. Exon 3 contains the C-terminal Y (black shading), the G-residue, the KR cleavage signal site and the first 23 amino acids of the C-terminal peptide of NPY (CPON, diagonal shading). Exon 4 encodes 7 C-terminal amino acids of CPON and the 3′ UTR. The stop codon is indicated by an asterisk. (B) *M. expansa npf* contains a single intron within the coding region. *npf* and *npy* are aligned at intron 2 (gray shading). Note that *npf* lacks introns 1 and 3, but does possess the characteristic phase-2 intron within the C-terminal R-residue (gray shading). PreNPF also lacks a C-terminal extension peptide. Homologous regions within *npf* and *npy* are shown in identical shading.

TCT; R = A or G; Y = T or C), and were consistently observed; on at least 6 occasions they were independently identified in both cDNA and genomic DNA sequences. The basis for this discrepancy is unclear, but possible explanations include allelic variation or the occurrence of unidentified *M. expansa* subspecies.

NPF-expression is widely distributed within the nervous system of free-living and parasitic platyhelminths (Maule *et al.* 1992). A possible role for NPF includes modulation of body wall muscle activity. Application of the C-terminal nonapeptide of NPF (NPF9) to muscle-strip preparations of *M. expansa* results in an inhibition of spontaneous contraction frequency (N. J. Marks, personal communication), and appears moderately excitatory on muscle-strip preparations of *Fasciola hepatica* (Marks *et al.* 1996). Previous immunocytochemical studies localized NPF-immunostaining to central and peripheral components of the nervous system of *M. expansa*. Furthermore, NPF-immunoreactive nerves were localized to muscular structures, including the suckers. However, the spatial interrelationships of *M. expansa* nerve and muscle structures have not been previously reported. Co-localization studies using NPF-antisera and fluorescently-labelled phalloidin as a marker for F-actin facilitated investigation of the spatial organization of NPF-positive nerve fibres and their relationship to particular muscle fibre systems within *M. expansa*. NPF-expressing nerve fibres were particularly evident in the innervation of the suckers, and appeared in close spatial proximity to the large, centrally localized, longitudinal muscle fibres of the strobila, but showed no obvious relationship to the muscle fibres of the genitalia or the body wall musculature.

Neuropeptide Y (NPY), pancreatic polypeptide (PP) and peptide tyrosine tyrosine (PYY) not only share primary structural homologies, but also show a highly-conserved gene organization, which implies a common evolutionary origin of this peptide family. It is assumed that an ancestral gene gave rise to an NPY-gene and a PYY-gene, which then in a second gene duplication event gave rise to *pp* (Conlon, Chartrel & Vaudry, 1992; Larhammar, 1996). The close spatial proximity of *pp* and *pyy* on human chromosome 17q21.1 supports the second gene duplication event (Hort *et al.* 1995).

Examination of the genomic organization of the *M. expansa npf* gene identified a 54 bp phase-2 intron. The intron is located within the coding region of the penultimate arginyl residue of NPF. The specific location, but not the size, of this particular intron in *M. expansa npf* is conserved in all other NPY-family genes characterized to date (Fig. 3). Size variation is considerable within the cor-

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responding introns of human *npy*, *pyy* and *pp*, and varies from 4300 bp in *npy* (Minth, Andrews & Dixon, 1986) to 105 bp in *pyy* (Kohri *et al*. 1993) and 245 bp in *pp* (Leiter *et al*. 1985). The similar sizes of intervening sequences of *pp* and *pyy* indicate a closer relationship between these two genes to each other than to *npy*. The absence of additional introns in *M. expansa npf*, which are present in vertebrates at highly, but not absolutely conserved positions, may be due to the loss of introns from the flatworm gene, or the insertion of introns within the vertebrate genes. The specific localization of a characteristic phase-2 intron in *M. expansa npf*, as well as the neuronal localization of *M. expansa* NPF, and its sequence similarity to the vertebrate NPY superfamily of peptides provide strong evidence for an early evolutionary origin of this peptide family.

The Platyhelminthes are considered an early clade within the Bilateria, possibly the sister group of all other bilaterian phyla (Adoutte & Philippe, 1993). Alternatively, they represent an early branch within the protostomes (Carranza, Baguna & Riutort, 1997). In either case, the unique intron position in vertebrate *npy* and platyhelminth *npf* can be expected to have been present in a common ancestor of flatworms and vertebrates prior to the divergence of the protostome and deuterostome (of which the vertebrates are a member) lineages. Since protostomes and deuterostomes are estimated to have diverged between 600 and 1000 million years ago (Wray, Levinton & Shapiro, 1996; Ayala, Rzhetsky & Ayala, 1998), NPY-like peptides must have appeared before this divergence and, therefore, represent one of the most ancient neuropeptides within the Bilateria.

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