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Neuropeptides Gly-Asp-Pro-Phe-Leu-Arg-Phe-amide (GDPFLRFamide) and Ser-Asp-Pro-Phe-Leu-Arg-Phe-amide (SDPFLRFamide) Are Encoded by an Exon 3′ to Phe-Met-Arg-Phe-NH_2 (FMRFamide) in the Snail *Lymnaea stagnalis*

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Biochemical analysis has shown the pond snail *Lymnaea stagnalis* to contain 2 main classes of Phe-Met-Arg-Phe-NH_2 (FMRFamide)-like neuropeptides: the tetrapeptides FMRFamide and Phe-Leu-Arg-Phe-NH_2 (FLRFamide), and the heptapeptides Gly-Asp-Pro-Phe-Leu-Arg-Phe-NH_2 (GDPFLRFamide) and Ser-Asp-Pro-Phe-Leu-Arg-Phe-NH_2 (SDPFFRFamide).

By genomic mapping and DNA sequencing, we show here that the GDP/SDPFLRFamide coding region lies 3′ to the FMRFamide coding region. The absence of an initiating start methionine and the presence of good-concensus 3′ and 5′ splice sites suggests that the GDP/SDPFLRFamide coding region makes up 1 exon of a larger gene. In addition to 7 copies of GDPFLRFamide and 6 copies of SDPFLRFamide, the exon encoding the heptapeptides also encodes 3 novel peptides, Glu-Phe-Phe-Pro-Leu-NH_2 (EFFPLamide), Ser-Asp-Pro-Tyr-Leu-Phe-Arg-NH_2 (SDPYLFRamide), and Ser-Asp-Pro-Phe-Phe-Arg-Phe-NH_2 (SDPFFRFamide). In contrast to the tetrapeptide FMRFamide precursor protein, the GDP/SDPFLRFamide peptides are encoded contiguously, being separated only by single basic amino acids.

Phe-Met-Arg-Phe-NH_2 (FMRFamide) belongs to a well-characterized family of structurally related neuropeptides that are thought to function as neurotransmitters, neurohormones, and/or neuromodulators in the regulation of activity of central neurons and in controlling a broad range of fundamentally important physiological processes (Stone and Mayeri, 1981; Abrams et al., 1984; Cottrell et al., 1984; Boyd and Walker, 1985; Yang et al., 1985; Benjamin et al., 1988; Ichinose and McAdoo, 1988; Thompson and Ruben, 1988).

FMRFamide was first discovered as a cardioexcitatory agent in the venus clam *Macrocallista nimbosa* (Price and Greenberg, 1977), and though FMRFamide itself appears to be limited to molluscs and annelids (Price, 1986; Krajniak and Price, 1990), immunoreactivity to FMRFamide has been reported in all the major animal phyla from chordates to coelenterates (Boer et al., 1980; Dockray et al., 1983; Grimmelikhuijzen and Graff, 1985). A number of FMRFamide-like peptides have been isolated, and in most organisms, these have been shown to be N-terminally extended forms characteristically ending in Arg-Phe-NH_2 (RFamide). The N-terminal may allow interactions with different types of receptor channels (Cottrell, 1984) or may function to stabilize the peptide by preventing proteolytic breakdown (Price, 1986).

Lymnaea stagnalis has been shown by biochemical analysis and HPLC to contain 3 forms of FMRFamide-like peptides: the extended heptapeptide forms Gly-Asp-Pro-Phe-Leu-Arg-Phe-NH_2 (GDPFLRFamide), Ser-Asp-Pro-Phe-Leu-Arg-Phe-NH_2 (SDPFLRFamide) and FMRFamide itself (Price, 1986; Ebberink et al., 1987). Using immunocytochemistry, FMRFamide-related peptides have been found in a number of identified neurons and neuron clusters in *Lymnaea* (Benjamin et al., 1988; Buckett et al., 1990). Analysis of the distribution of the different FMRFamide-like peptides using these techniques, however, is difficult because most antibodies raised against these peptides show considerable cross-reactivity due to the antigenic carboxy-terminal RFamide moiety. A molecular approach is therefore needed in order to determine the distribution and expression of the different hepta- and tetrapeptide FMRFamide peptides.

Peptide genes often encode families of neuropeptides with similar sequences that are processed from a common precursor to liberate multiple bioactive peptide products. Recently, cDNAs encoding FMRFamide itself have been isolated and sequenced from *Lymnaea* and have been shown to encode a precursor protein that contains 9 copies of FMRFamide, 2 copies of the related peptide Phe-Leu-Arg-Phe-NH_2 (FLRFamide), and 2 putative pentapeptides Glu-Phe-Leu-Arg-Ile-NH_2 (EFLRIamide) and Glu-Phe-Tyr-Arg-Ile-NH_2 (QFYRIamide; Linacre et al., 1990). The precursor does not encode the extended heptapeptides GDPFLRFamide or SDPFLRFamide. Here, we present evidence that GDPFLRFamide and SDPFLRFamide are encoded contiguously by an exon 3′ to the FMRFamide coding region, and that the peptides are processed from the precursor by enzymes that cleave at either single arginine or lysine residues. Data from *in situ* hybridization suggest that the GDPFLRFamide/SDPFLRFamide mRNA is expressed in specific neurons.

Materials and Methods

Molecular procedures. General procedures such as growth of phage, Southern transfers, and plasmid preparations were carried out as described by Maniatis et al. (1982).

Construction of oligonucleotides. Mixed oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer according to the manufacturer's instructions. The sequences of the oligonu-

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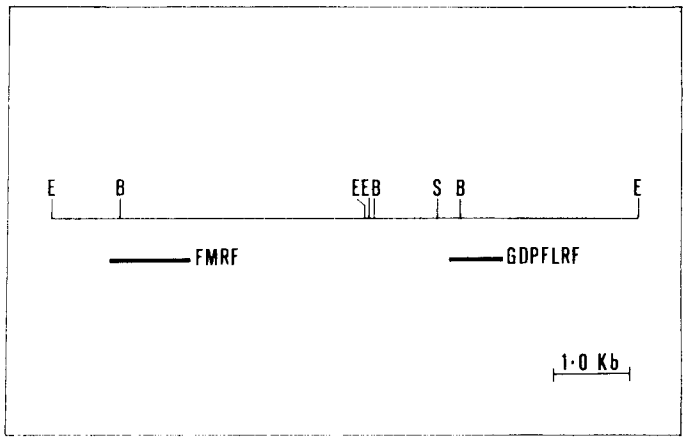

Figure 1. Relative position of the FMRFamide and GDP/SDPFLRFamide encoding regions in the λEMBL3 phage λSS4.5. Data were obtained by restriction mapping, subcloning, and Southern hybridization. *E, Eco*RI; *B, Bam*HI; *S, Sal*I. Transcription of the FMRFamide gene is from *left* to *right* (Linacre et al., 1990).

cleotides were (5′ to 3′) (A/G)AA*N*GG(A/G)TC*N*GA(SDPF) and (A/G)AA*N*GG(A/G)TC*N*CC(GDPF), where *N* represents A, G, C, or T.

Isolation of GDPFLRFamide- and SDPFLRFamide-encoding genomic clones. λEMBL3 phage containing *Lymnaea stagnalis* genomic sequences [inserts 15–20 kilobases (kb)], were hybridized with FMRFamide cDNA clones, and a single plaque was selected (λSS4.5). DNA was extracted and digested with *Eco*RI restriction enzyme, and hybridized with mixed oligonucleotides encoding GDPF and SDPF labeled with γ-^{32}P-ATP by means of T_4 polynucleotide kinase. Hybridizations were carried out at 37°C in a solution containing 6 × SSC, 5 × Denhardt's solution, and 1% SDS. Filters were washed at 23°C in 6 × SSC with 10% SDS. The oligonucleotides were found to hybridize to an *Eco*RI fragment of 3.4 kb, which was different from the *Eco*RI fragment encoding FMRFamide (Linacre et al., 1990). The 3.4-kb *Eco*RI fragment was subcloned in both orientations into pUC19 (pGDP17/18) for sequence analysis.

Nucleotide sequence analysis. Deleted subclones, in each orientation, were used for double-strand DNA sequencing (Wallace et al., 1981). The subclones were generated by *Exo*III-nuclease digests of linearized plasmids, end trimmed with Klenow fragment of DNA polymerase I, and ligated to give a set of overlapping deletion clones. Double-strand sequencing using α-^{35}S-dATP was performed using Sequenase 2.0 (U.S. Biochemicals). The samples were separated on a 7 M urea, 6% acrylamide gel.

In situ hybridizations. CNSs from *Lymnaea* were dissected, fixed, and sectioned essentially as described by Dirks et al. (1989). Sections were hybridized overnight at 37°C with α-^{35}S-dATP nick-translated insert of pSM1 encoding 4 copies of GDPFLRFamide (nucleotides 170–280; see Fig. 2). Slides were exposed for autoradiography (Amersham) for 1–3 weeks.

Results

Isolation of GDPFLRFamide/SDPFLRFamide genomic clones

The FMRFamide sequence previously reported did not encode the related heptapeptides GDPFLRFamide and SDPFLRFamide (Linacre et al., 1990). Because it seemed likely that members of a peptide gene family would be encoded in close proximity, λEMBL3 genomic clones containing FMRFamide inserts 15–20 kb were screened with a mixture of 64 different degenerate oligonucleotides, 12 residues in length, complementary to the coding sequences for SDPF and GDPF.

Restriction analysis and Southern blotting of one of the original λEMBL3 FMRFamide-containing clones indicated that an *Eco*RI fragment of 3.4 kb hybridized to the GDP/SDP oligomers. This fragment was different from the *Eco*RI fragment

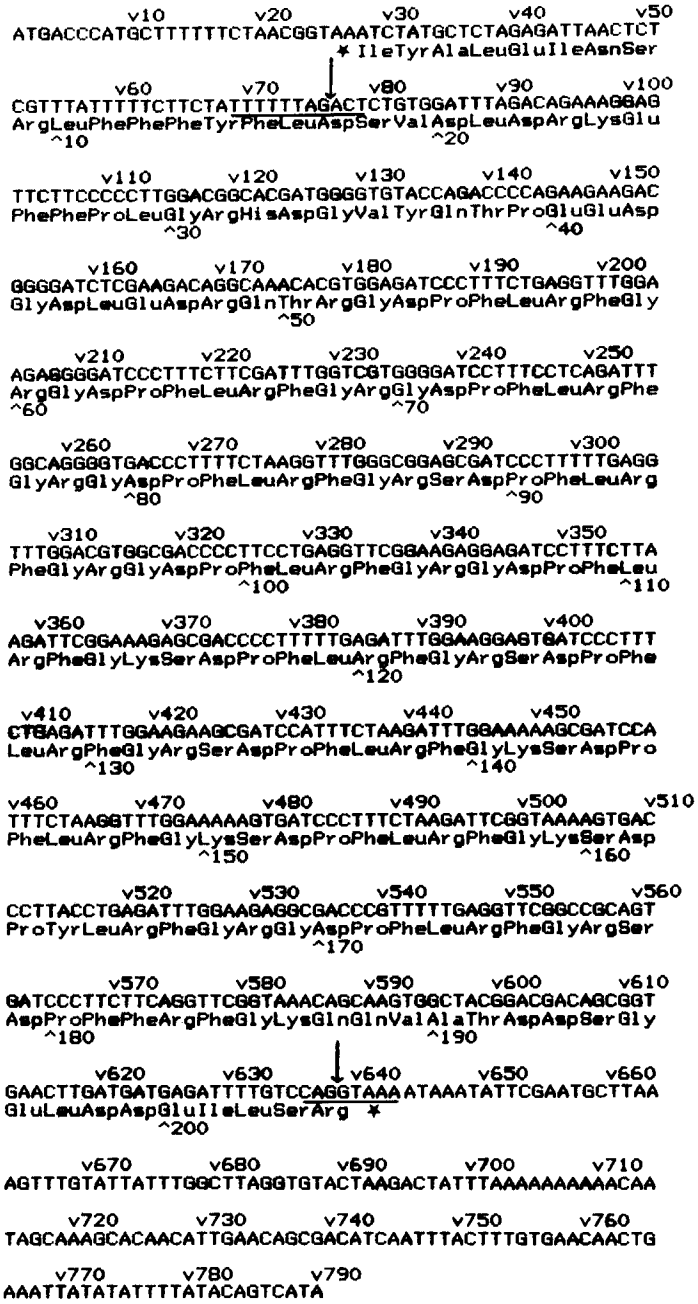

Figure 2. Partial nucleotide sequence of a contiguous region of genomic DNA encoding the GDP/SDPFLRFamide precursor. The sequence was deduced on both strands as described in Materials and Methods. The translation of the largest ORF (243 amino acids) is shown below the DNA sequence. Stop codons at the start and end (marked by *stars*) of the ORF are indicated. Potential 5′ and 3′ splice sites are *underlined.* The *arrows* show the potential splice junctions.

encoding FMRFamide (Linacre et al., 1990). Detailed restriction analysis and Southern blot analysis showed that GDP/SDPFLRFamide sequences were located 3′ to the FMRFamide sequence (Fig. 1).

GDP/SDPFLRFamide genomic encoding sequences are flanked by consensus RNA splice sequences

Translation of the nucleotide sequence encoding GDP/SDPFLRFamide showed that the longest open reading frame (ORF) is 727 nucleotides in length and encodes 1 copy of a

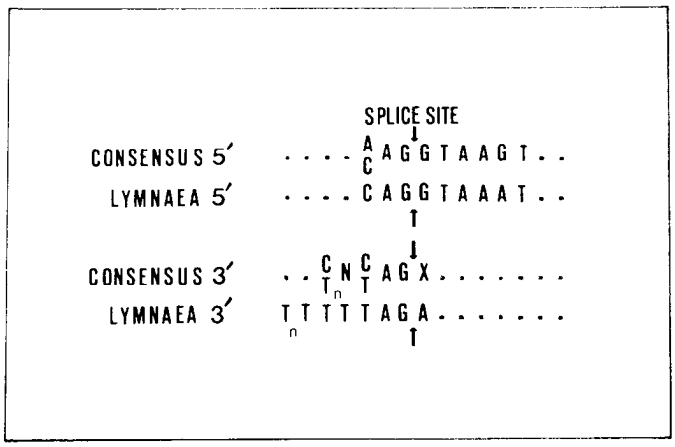

Figure 3. Comparison of the putative 5′ and 3′ splice sequences (underlined in Fig. 2) with the mammalian splice consensus sequences (Padgett et al., 1986).

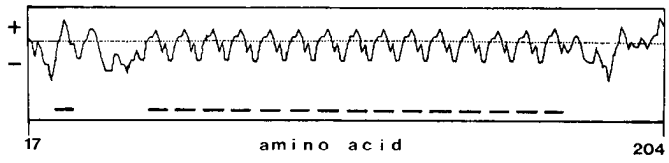

Figure 5. A Hopp hydrophobicity plot of amino acids encoded by the spliced genomic sequence (amino acids 17–204 in Fig. 2). *Solid bars* represent the putative peptides depicted in Figure 4*A*. These data were calculated using a computer program (DNA STAR) with a window size of 7 amino acids. Hydrophobic sequence is represented by $+ve$ values and hydrophilic by $-ve$ values.

novel putative peptide Glu-Phe-Phe-Pro-Leu-NH_2 (EFFPLamide), 7 copies of GDPFLRFamide, 6 copies of SDPFLRFamide, and 2 novel putative peptides, Ser-Asp-Pro-Tyr-Leu-Phe-Arg-NH_2 (SDPYLFRamide) and Ser-Asp-Pro-Phe-Phe-Arg-Phe-NH_2 (SDPFFRFamide; Fig. 2).

Analysis of sequence (nucleotides 1–99) 5′ to the putative EFFPLamide peptide shows no initiator methionine in frame with the GDP/SDPFLRFamide coding sequences, stop codons being found in all reading frames. Analysis of genomic sequences around the start of the long ORF and around the 3′ stop codon close to the last putative peptide SDPFFRFamide reveals (Fig. 3) good consensus splice sites at the 5′ end (nucleotides 69–77) and at the 3′ end (nucleotides 637–645) when these sequences are compared with splice sites in other genes (Padgett et al., 1986). The absence of a methionine start and the finding of good-consensus 5′ and 3′ splice sites strongly argue for GDP/SDPFLRFamide being encoded as an exon, making up part of a larger gene.

Deduced structure of GDPFLRFamide and SDPFLRFamide precursor

The organization of the putative peptides encoded by the sequence in Figure 2 is represented schematically in Figure 4*A*. The GDP/SDPFLRFamide peptides are encoded contiguously, being separated at their carboxyl termini by the residues Gly Arg (10/15) or Gly Lys (5/15), glycine being required for C-terminal amidation and the single basic amino acid probably serving as a proteolytic cleavage signal. A Hopp hydrophobicity plot (Hopp and Woods, 1981) of the precursor protein encoding GDP/SDPFLRFamide (Fig. 5) shows how the arginines (and lysines) flanking the consecutively encoded peptides can be distinguished from the arginines in the peptides. Arginines that are thought to be processed are always found to reside in a hydrophilic environment, whereas those arginines that form part of the neuropeptides are found in a hydrophobic environment.

The nucleotide sequences specifying the common FLRFamide amino acids in individual GDPFLRFamides and SDPFLRFamides are not identical, and there is no common

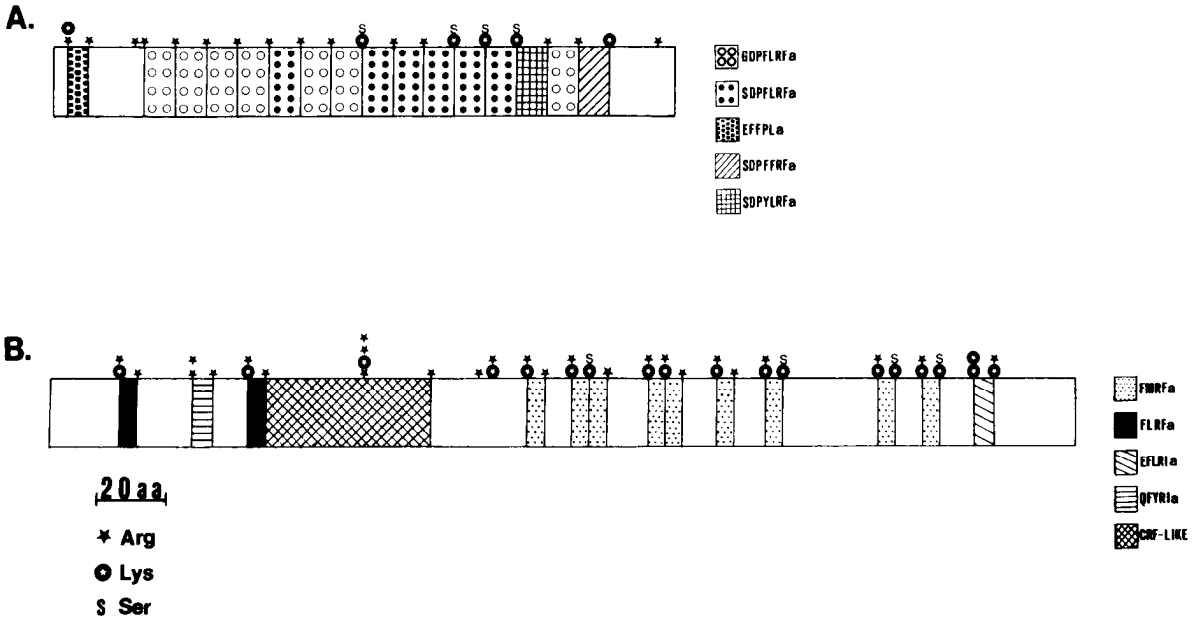

Figure 4. *A*, Representation of the peptides encoded by the genomic sequences within the spliced GDP/SDPFLRFamide exon (amino acids 17–204 in Fig. 2). *B*, Peptides encoded by the FMRFamide precursor (Linacre et al., 1990) are shown for comparison. The first 18 amino acids make up a hydrophobic leader sequence.

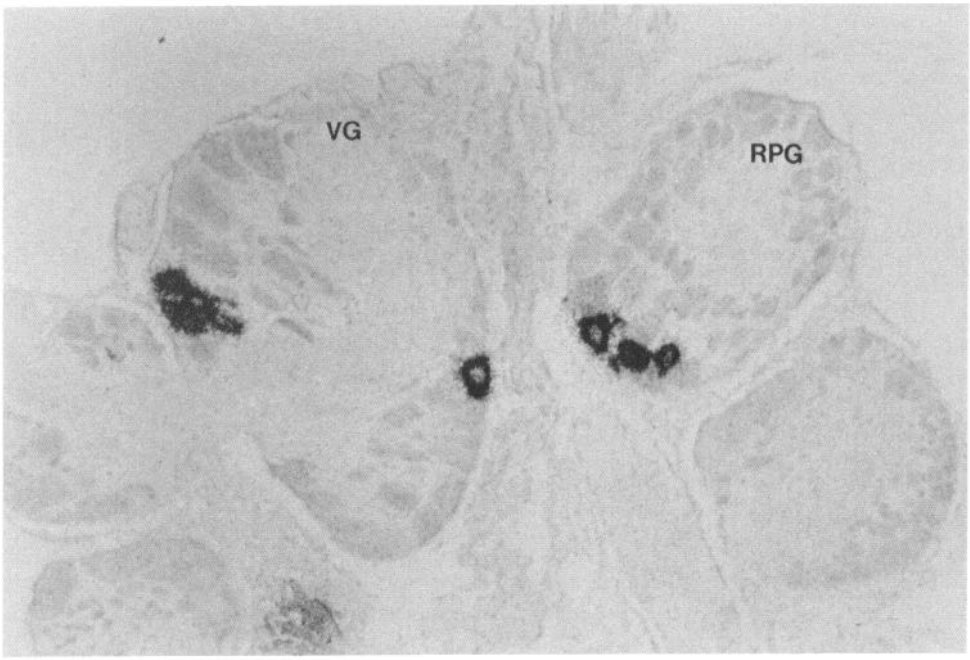

Figure 6. Localized expression of GDPFLRFamide encoding mRNA in the visceral (*VG*) and right parietal ganglia (*RPG*) of the CNS of *Lymnaea stagnalis* as shown by *in situ* hybridization. ^{35}S-labeled probe was prepared by nick translation of gel-purified insert (nucleotides 170–280, Fig. 2) from the plasmid pSM1.

codon usage. Analysis of the sequence specifying the novel putative peptides SDPFFRFamide and SDPYLRFamide indicates that they have probably arisen as a result of a single nucleotide change converting one codon into another. For example, leucine (tt*g*) to phenylananine (tt*c*) in SDPFFRFamide and phenylalanine (t*t*c) to tyrosine (t*a*c) in SDPYLRFamide. It is not known if these substituted heptapeptides are biologically active.

The other putative peptide, EFFPLamide, found at the N-terminal of the GDP/SDPFLRFamide precursor, is separated from the GDP/SDPFLRFamide coding region by an acidic spacer region of 19 amino acids. This peptide is bounded by Arg Lys at the amino terminal and Gly Arg at the carboxyl terminal, indicating that this peptide would be amidated if processed from the precursor. The distance of this peptide from the heptapeptides and cleavage of this putative peptide more closely resemble the organization and cleavage of peptides from the FMRFamide precursor (Linacre et al., 1990) rather than those of the GDP/SDPFLRFamide peptides from their precursor (see Fig. 4).

The GDP/SDPFLRFamide exon is expressed in the Lymnaea *CNS*

In order to determine whether the GDP/SDPFLRFamide coding exon is expressed in the *Lymnaea* brain, *in situ* hybridization was performed (Fig. 6). Preliminary analysis shows that GDP/SDPFLRFamide mRNA is expressed in discrete neurons in the CNS.

Discussion

Organization of the locus encoding the heptapeptides and tetrapeptides

We have characterized a genomic clone that contains an exon that lies 3′ to the previously described FMRFamide sequence and encodes 7 copies of GDPFLRFamide, 6 copies of SDPFLRFamide, and 3 putative peptides, SDPYLRFamide, SDPFFRFamide, and EFFPLamide. This exon lies approximately 3.4 kb 3′ to the loci encoding FMRFamide. From *in situ* hybridization experiments, the exon appears to be expressed in a limited number of neurons in the CNS of *Lymnaea* (K. Bright et al., unpublished observations). Sequence analysis failed to reveal a methionine start codon at the start of the ORF encoding the GDP/SDPFLRFamide peptides. However, the presence of good-concensus 5′ and 3′ splice sites and the absence of an obvious polyA addition site indicates that the sequences described here represent an exon that is likely to be subjected to sophisticated regulation, probably at the level of RNA splicing.

Previously, we had shown that the deduced protein sequence of the FMRFamide precursor contained an N-terminal hydrophobic leader sequence (Linacre et al., 1990). However, in addition to this, the genomic sequences also showed a 3′ consensus splice sequence 5′ to the first encoded peptide, which was utilized by at least 1 of the 2 sequenced cDNAs (D1). Furthermore, no polyA addition site was observed 3′ of the coding sequences. There is, however, a putative 5′ consensus splice sequence (data not shown). These observations suggest that the FMRFamide "gene" previously described could also be considered as a single potential exon making up part of a larger gene in which both FMRFamide and GDP/SDPFLRFamide sequences are found in the primary transcript.

Recent evidence also suggests that the *Drosophila* FMRFamide-like gene is made up of different exons, the first exon consisting of a 5′ untranslated region that is spliced onto the second exon, which encodes the prohormone (Chin et al., 1990; Schneider and Taghert, 1990).

Processing of the precursor

The organization of the *Lymnaea* heptapeptides within the precursor resembles closely the organization of the *Drosophila* gene that encodes multiple related N-terminally extended FMRFamides, the extended peptides being separated by only 2 amino acids: Gly, which is required for amidation of the peptides, and a single basic amino acid, Arg (Schneider and Taghert, 1988).

The hydrophobicity profile of the *Lymnaea* heptapeptide precursor protein indicates that the potentially cleaved arginines and lysines are in an environment that would allow cleavage if

the appropriate enzymes were present. Cleavage at a single arginine has been well documented (reviewed by Loh and Gainer, 1983), and though there is no direct evidence for cleavage at a single lysine residue, in *Aplysia,* all but 7 out of 28, and 4 out of 9 FMRFamide peptides in *Lymnaea,* are also flanked at their carboxyl termini by a single lysine (Schaefer et al., 1985; Linacre et al., 1990). In each case, the lysine is always followed by a serine, and it has therefore been proposed that the serine may have arisen from a single nucleotide change converting an arginine to a serine (Schaefer et al., 1985).

In the GDP/SDPFLRFamide precursor, 4 out of 5 potentially cleaved lysines are also followed by a serine; the other, by a glutamine. In each case, the serine is the first amino acid of the heptapeptide SDPFLRFamide. This raises the possibility that either those peptides flanked by lysines are not processed, or that there is a novel enzyme that recognizes either a single lysine or a single lysine followed by a serine. We favor the latter explanation because, if the serine had arisen from a point mutation converting an arginine to a serine as proposed, then only the 2 SDPFLRFamides bounded by single arginines would be processed, which is unlikely.

Evolution of the exon encoding GDP/SDPFLRFamide

It had previously been suggested that the sequences encoding the tetrapeptide and heptapeptide FMRFamide-related peptides were likely to be encoded by 2 separate genes that arose as a result of a gene duplication event. Following this event, it was proposed that the 2 genes would evolve independent control mechanisms so that the tetra- and heptapeptide genes could be expressed in separate neurons and the peptides used in different systems (Price, 1986). With the data presented here, we can address these possibilities directly. One obvious difference between the FMRFamide sequences and GDP/SDPFLRFamide sequences is the presence of a pair of basic amino acids at the N-terminals of the former and just single basic amino acids flanking the latter. In addition, the evenly spaced heptapeptides contrast with the arrangement of the tetrapeptide encoding sequences (Fig. 4*B*). These findings, together with the observation that the heptapeptide precursor also contains a pentapeptide sequence with a pair of basic amino acids at the N-terminal and is separated from the next peptide by a long spacer (which is similar to the organization of the tetrapeptide precursor), make models of gene duplication complex.

One possibility is that the *Lymnaea* FMRFamide gene was originally a single uninterrupted gene that encoded a heptapeptide (GDPFLRFamide?) at the C-terminus. This heptapeptide sequence may then have been duplicated [in a similar manner to the reiteration of peptide 7 in the *Aplysia* FMRFamide precursor to give 19 identical FMRFamide copies (Price et al., 1987)], and then, single base changes could have occurred over time to give rise to GDPFLRFamide variants such as SDPFLRFamide. The separation of the tetrapeptide precursor and pentapeptide plus heptapeptide sequences into 2 exons could have been a later event caused by the insertion of an intron sequence (reviewed in Rogers, 1989). This separation could allow independent regulation of tetrapeptide and heptapeptide expression, not by differential transcription of the 2 genes, but possibly by alternative splicing. A detailed investigation of the transcripts from the 2 potential exons will provide evidence for this model.

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