Interspecific Comparison of a *Drosophila* Gene Encoding FMRFamide-Related Neuropeptides

Paul H. Taghert and Lynne E. Schneider

Department of Anatomy and Neurobiology, Washington University School of Medicine, Saint Louis, Missouri 63110

In order to identify functionally important regions of a neuropeptide gene in *Drosophila melanogaster*, we have studied its occurrence in related species and have characterized the structure of a homologous gene in *Drosophila virilis*. The *melanogaster* gene encodes a precursor that contains 13 neuropeptides related to the molluscan tetrapeptide FMRFamide (Nambu et al., 1988; Schneider and Taghert, 1988). Using the *melanogaster* gene as a probe in Southern blot analysis, related sequences were detected in DNA from each of 7 species tested. *D. virilis*, which is estimated to have diverged from *D. melanogaster* between 60 and 80 million years ago (Throckmorton, 1975), was chosen for more detailed study. Immunocytochemical staining using an antibody to authentic FMRFamide revealed a similar set of immunoreactive neurons in the CNS of larvae from the 2 *Drosophila* species.

Using a *melanogaster* gene probe, overlapping clones were isolated from a *virilis* genomic library; DNA sequence analysis indicated the presence of a homologous gene. Comparisons of the genes and deduced proteins between the 2 species revealed the following points. (1) Both genes are divided into 2 exons: in *D. melanogaster* the exons are 106 and 1352 bp long; in *D. virilis*, they are 169 and at least 1232 bp long; in both species, the intron is approximately 2.5 kb long. (2) The sequence of exon I has largely diverged, and in neither species are exon I sequences translated. In this vicinity of the gene, sequence conservation is limited to a 67 bp region that spans the TATA box and the RNA start site. (3) The deduced neuropeptide precursors have very similar sizes (347 vs 339 amino acids) and the presumed signal sequences are perfectly conserved. (4) While the *melanogaster* precursor contains 13 FMRFamide-related peptides, the *virilis* precursor contains only 10. (5) The sequences of some but not all of the FMRFamide-like peptides are perfectly conserved. (6) In the rest of the precursor, significant sequence conservation is found only in the N-terminal portion; immediately downstream of the final FMRFamide-like peptide, the protein sequences are highly

divergent. (7) 5' to the RNA start sites (1.2 kb of *melanogaster* DNA and 1.8 kb of *virilis* DNA), 17 small (9–52 base pairs) regions are evolutionarily conserved (>80% sequence conservation). We discuss neuropeptide biosynthesis, the functions and evolution of FMRFamide-like neuropeptides in insects, and the cell-specific regulation of neuropeptide gene expression in the contexts of these results.

FMRFamide was first purified as a cardioregulatory neuropeptide from the CNS of the clam (Price and Greenberg, 1977). In addition to its modulation of cardiac output (Price and Greenberg, 1977; Painter, 1982), the peptide has well-defined roles in modulating the actions of neurons (Cottrell et al., 1984; Boyd and Walker, 1985; Columbaioni et al., 1985) and in regulation of evoked muscle tension (Doble and Greenberg, 1982; Weiss et al., 1984; Muneoka and Matsuura, 1985). Using an anti-FMRFamide antiserum as the primary tool of purification, numerous biologically active peptides from a host of diverse animal genera have been purified. In the case of *Aplysia*, the endogenous peptide is the same as authentic FMRFamide (Schaefer et al., 1985). In many other cases, however, the related peptides are longer by virtue of extensions at the N-termini. In various molluscan species, FMRFamide is one of a group of structurally related peptides that include FLRFamide, SPFLRFamide, and several heptapeptides with the general sequence XDPFLRFamide [X is pGlu, Gly, Ser, or Asn (Ebberink et al., 1987; Price et al., 1985, 1987a, b)]. Similarly, SDRNFLRFamide and TNRNFLRFamide were purified from lobster CNS (Trimmer et al., 1987), and 2 immunoreactive bovine peptides with anti-analgesic properties were purified by Yang et al. (1985). In *Drosophila*, the predominant FMRFamide-like immunoreactive peptide is DPKQDFMRFamide (Nambu et al., 1988); cloning and sequencing of the *Drosophila* FMRFamide gene has indicated that this and related peptides are cosynthesized as parts of a large precursor (Nambu et al., 1988; Schneider and Taghert, 1988). Both the *Drosophila* and *Aplysia* (Taussig and Scheller, 1986) FMRFamide genes share sequence homologies with mammalian genes encoding the opioid peptides and corticotrophin-releasing factor.

Extensive physiological characterizations of FMRFamide-related peptides in molluscs have been reported, but the roles of similar peptides in insects are not well defined. Their effects have been studied at the neuromuscular junction of locust (Evans and Myers, 1986; Walther and Schiebe, 1987), where they modulate tension produced by the stimulation of an identified motoneuron. YGGFMRFamide was found to be an order of magnitude more potent than was FMRFamide in this prepa-

Received Nov. 21, 1989; revised Dec. 28, 1989; accepted Jan. 4, 1990.

We thank Bill Gelbart for providing the *D. virilis* genomic library and Jay Hirsch for communicating unpublished results. Kyoko Marayuma kindly provided animals of representative *Drosophila* species; we also thank Kyoko Marayuma and Larry Salkoff for their comments on a draft of the manuscript. This work was supported by grants from the NIH (NS-21749) and from the Washington University-Markey Center for the Molecular Biology of Human Genetics to P.H.T.

Correspondence should be addressed to Paul H. Taghert, Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110.

Copyright © 1990 Society for Neuroscience 0270-6474/90/061929-14\$02.00/0

ration. Although the endogenous FMRFamide-like peptides in the locust have not been defined, these results support the hypothesis that they are likely to include N-terminally extended forms, as are found in *Drosophila*. FMRFamide-like immunoreactivity is present in neurohemal organs of the moth CNS and can be released upon depolarization (Carroll et al., 1986). The principle neurons expressing the FMRFamide gene in the *Drosophila* CNS are a set of segmentally homologous neuroendocrine neurons (Schneider, et al., 1988, and in preparation). Together these results suggest that, in insects, a salient role for FMRFamide-like peptides may be as hormonal modulators of peripheral synapses and target organs. Because the gene is also expressed by interneurons (L. E. Schneider, M. A. O'Brien, and P. H. Taghert, unpublished observations), it is possible that the peptides may play similar modulatory roles at central synapses.

The *Drosophila* FMRFamide precursor is comparable to other neuropeptide precursors (e.g., Noda et al., 1982; Mahon et al., 1985; Schaefer et al., 1985) in that it contains diverse yet related neuropeptides: some are multiply represented, while others are uniquely present. These features of neuropeptide precursor organization raise a number of interesting questions relating to neuropeptide biosynthesis and function. For example, is the copy number of a specific neuropeptide within a precursor critical for its functions? To what extent do slightly divergent peptide sequences mediate different peptide functions? Such questions are difficult to answer using standard physiological experiments, due to both the potential diversity of neuropeptide sequences and to a lack of specific pharmacological antagonists. In this regard, a complementary molecular and genetic approach in *Drosophila* would be of value.

We have isolated and characterized a gene from *D. virilis* that is homologous to the FMRFamide neuropeptide gene of *D. melanogaster*. The goal in comparing the DNA sequences of the 2 species is to identify evolutionarily conserved features of both the protein-coding regions and potential regulatory regions. Previous studies (Blackman and Meselson, 1986; Treier et al., 1989; Johnson et al., 1989) have shown that *D. virilis* is sufficiently diverged from *D. melanogaster* to predict that sequence conservation should be restricted to functionally important regions. Based on this assumption, the comparison is informative regarding the significance of neuropeptide gene exons and of the size of the neuropeptide precursor. In addition, the 2 species demonstrate a remarkable degree of variability in peptide sequences and copy number. Finally, conserved regions in the putative promoter region are useful in defining DNA regions that may regulate the expression of the gene.

Materials and Methods

DNA extraction. A total of 50 animals from a given species was homogenized in a glass mortar and pestle in 500 μ l of 0.1 M Tris-HCl (pH 9.0), 0.1 M EDTA, 1% SDS, and 1% DEPC. Then, 70 μ l of 8 M KOAc was added and the homogenate chilled and centrifuged; 1/2 volume of isopropanol was added to the supernatant and the DNA collected by centrifugation.

Genomic Southern blots. Genomic Southern blots were performed as described by Maniatis et al. (1982). After blotting to Nytran membranes (Schleicher and Schuell, according to the manufacturer's recommendations), prehybridization and hybridizations were performed at 42°C in a solution containing 30% (vol/vol) formamide, 6 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate, pH, 7.0) 0.1% Sarkosyl, salmon sperm DNA at 100 μ g/ml, and 1% dry milk. The probe was a 962 bp R1-Pst I fragment of the *Drosophila* FMRFamide cDNA B(8) (Schneider and Taghert, 1988) that was labeled by random hexamer priming (specific activity, $\sim 5 \times 10^8$ cpm/ μ g). Filters were washed at 47°C in 2 $\times$

SSC/0.1% Sarkosyl or at 65°C in 0.1 $\times$ SSC/0.1% Sarkosyl, then dried, and autoradiographed.

Isolation and characterization of *D. virilis* FMRFamide clones. Approximately 240,000 clones from a lambda EMBL 4 *D. virilis* genomic library were screened by the technique of Benton and Davis (1977) using the same conditions as in the genome blot experiments (washed at 47°C) but with a 1 kb "upstream" Pst I fragment of *melanogaster* genomic DNA that immediately precedes the FMRFamide gene start site (Schneider and Taghert, 1990). Positive clones were rescreened with the 962 bp cDNA probe used for the genome blot experiments. Clones that were positive for both hybridization probes were further characterized by restriction endonuclease mapping and blotting procedures. Hind III and Xba I fragments that hybridized to either the cDNA or "upstream" probes were subcloned into the Bluescribe plasmid.

DNA sequencing methods and analysis. Plasmid DNA was alkali denatured and sequenced by using a commercially available kit (Sequenase, U.S. Biochemicals), α -[35 S-dATP] and buffer gradient acrylamide gels. Amino acid and nucleic acid identities were computed in the manner of Nambu and Scheller (1986) and are described in the legend to Figure 7.

Immunocytochemistry. A rabbit anti-FMRFamide antiserum was produced by conjugating the peptide (Sigma, Saint Louis, MO) to thyroglobulin with carbodiimide. Peptide conjugates containing 100 μ g peptide were emulsified in Freund's complete adjuvant and injected subdermally at multiple sites. Conjugate solutions emulsified in incomplete adjuvant were used for boost injections every other week for 6 weeks, and once a month thereafter. Blood was collected 5–7 d following each monthly boost. In radioimmunoassays, the serum contained a low titer of specific antibody (M. A. O'Brien, C. R. Conway, and P. H. Taghert, unpublished observations). However, in immunocytochemical experiments, useful staining was observed with a 1:5000 dilution of the serum. All neuronal staining with the anti-FMRFamide antiserum was blocked by preincubating the diluted antiserum solution with 100 μ g/ml authentic FMRFamide overnight at 4°C prior to tissue incubation.

Whole-mount antibody staining was performed on third instar animals of both species in the following manner. The body was opened by a mid-dorsal incision and pinned open; Bouin's fixative was applied (after removing extraneous tissues) with the nervous system still connected to the body and to the imaginal disks. Following fixation at 4°C for 24 hr, the tissues were dehydrated in an ethanol series (including the use of a saturated LiCO₃/70% EtOH solution to remove picric acid), rehydrated, then stained with the antiserum to authentic FMRFamide. The primary antiserum was used at a concentration of 1:5000, along with Triton X-100 (at 0.3%) normal goat serum (at 3%), NaN₃ (at 0.01%), in PBS; primary antiserum incubation lasted for 48 hr at 4°C, following which the tissues were repeatedly rinsed in PBS with 0.1% Triton X-100 overnight at 4°C. The tissues were then processed for HRP-based immunocytochemical detection using a commercially available kit (Vector Labs., Burlingame, CA) or for fluorescence visualization using FITC-conjugated anti-rabbit antibodies (Sigma). The gut and CNS were dissected free of the body and imaginal disks, and mounted in 90% glycerol/PBS (containing phenylenediamine at 1 mg/ml) for viewing with fluorescence or with Nomarski optics, and for photography and drawing at up to 650 $\times$.

Results

Presence of FMRFamide-related sequences in various *Drosophila* species

Genomic Southern blotting experiments revealed that each of the 7 *Drosophila* species tested contained restriction fragments that hybridized to a *D. melanogaster* FMRFamide gene probe (Fig. 1). Varying degrees of hybridization were observed among the different species. *D. simulans*, *D. mauritiana*, and *D. yakuba* (closely related species of the *melanogaster* subgroup) all gave strong signals that persisted in stringent washing conditions. In Figure 1, the *yakuba* signals appear weak due to less DNA in the digests. *D. pseudoobscura* is more distantly related to *melanogaster* than are *mauritiana*, *simulans*, and *yakuba*, but it is still a member of the same subgenus (*Sophophora*). *D. funebris*, *D. virilis*, and *Zaproinus inermis* are the most distantly related of the set. *D. funebris* and *D. virilis* are members of a different subgenus (*Drosophila*) and *Z. inermis* is a member of the genus

Figure 1. Southern blot analysis of genomic DNA from 8 *Drosophilid* species: using a *melanogaster* FMRFamide gene probe, specific hybridization signals are seen in the case of each species included. Each lane represents ~10 μ g DNA from a given species that was digested to completion with either of 3 restriction enzymes: Bam HI (B), Hind III (H), or Eco RI (R). The 3 lanes marked *D. yakuba* received only ~3 μ g DNA. A hexamer-labeled 962 bp RI-Pst I restriction fragment (representing most of the open reading frame of the *melanogaster* FMRFamide gene) was used as a probe at lowered stringency (see Materials and Methods for conditions used). DNA size markers are given at right (in kb).

Zaprionus, which is related to the genus *Drosophila*. Signals from DNA of these species were considerably weaker and, upon high-stringency washing, were lost.

FMRFamide-like immunoreactivity in *D. melanogaster* and *D. virilis*

White et al. (1986) first used anti-FMRFamide antibodies for specifically staining a stereotyped set of ~35 neurons in the CNS of *Drosophila melanogaster*. Using a newly prepared anti-FMRFamide antiserum on the *melanogaster* CNS, we have found a similar, although more extensive, set of ~50 neurons. Much of this immunoreactivity is likely due to expression of the DPKQDFMRFamide gene (Schneider and Taghert, 1988), as indicated by comparison with results from *in situ* hybridization histochemistry (Schneider et al., 1988; Schneider et al., unpublished observations). The CNS and gut from both *D. melanogaster* and *D. virilis* were stained using the anti-FMRFamide antiserum (Fig. 2), and the patterns of immunoreactive neurons were compared (Fig. 3). Few differences were found: the most prominently staining cells of the brain (MP, LP, and SP cells) and ventral nerve cord (SE and Tv cells) were equally so in *D. virilis*. Likewise, scattered groups of small neurons (these demonstrated light to moderate staining, e.g., the Td cells) were found in both species. For the case of a few identifiable cell groups, the number of cells per group was often reliably different between species. For example, the *virilis* Tv cell group typically consisted of 1 large neuron accompanied by 2–3 small immunoreactive cells; in *melanogaster*, the 1 large Tv cell was often unaccompanied, and only infrequently accompanied by a single small cell. All staining was blocked by preabsorbing the diluted antiserum in 100 μ g/ml authentic FMRFamide (data not shown).

Isolation and sequencing of FMRFamide-homologous clones

A genomic clone (λ Mt-8) that contains sequences encoding the FMRFamide gene was previously isolated from a *D. melanogaster* phage library (Schneider and Taghert, 1988); the organization of the gene was determined by DNA sequencing, primer extension, and nuclease protection assays (Schneider and Taghert, 1990). A ~1 kb Pst I restriction fragment (from the region lying immediately 5' upstream of the RNA start site) was used as a hybridization probe to screen a *D. virilis* genomic library at lowered stringency. Five positive clones were purified and rescreened with a 962 bp RI-Pst I fragment (this second probe

represented most of the protein-encoding region of the *melanogaster* gene); all 5 clones contained hybridizing regions to this second probe as well. We concluded that the *virilis* clones contained the entire gene including a substantial portion of 5' upstream regions. Phage clone V6 was chosen for further study; a partial restriction map is shown in Figure 4, along with the positions of 2 regions (v-A and v-B) chosen for subcloning and sequence analysis.

Characterization of the *D. virilis* gene and deduced FMRFamide precursor

A long open reading frame of 1029 bp was found in *virilis* genomic clone V6 that encodes a deduced protein that is highly similar to the DPKQDFMRFamide precursor of *melanogaster* (Fig. 5). In the case of *melanogaster*, the initiator ATG codon has been assigned to the first nucleotide of exon II (Schneider and Taghert, 1990). In the case of *virilis*, the initiator codon is found at position +12 of exon II: this assignment relied on the positions of conserved sequences preceding and defining the 3' end of the intron. We defined the acceptor splice junction of the *virilis* intron based on the following sequence features. First, 2 sequences that matched previously described 3' intron splice signals (Keller and Noon, 1984) were found at positions –85 and at –51; both of these regions were highly conserved between the 2 *Drosophila* species (Fig. 5). Further, the second such “signal” was followed in each species by a CT-rich region lacking in AG dinucleotides. These are typical features preceding intron splice sites (Padgett et al., 1986). In the case of *virilis*, the first and only AG di-nucleotide of this region was found 12 base pairs upstream of the initiator ATG.

The deduced open reading frames generated proteins with very similar sizes: *melanogaster*, 347 amino acids; *virilis*, 339 amino acids. Each begins with a hydrophobic signal peptide region that is precisely conserved for a length of 23 amino acids (AA). FMRFamide-like peptides are found in each precursor, their organization is the same in each, and all are N-terminally extended. However, the *virilis* precursor contains 3 fewer peptides than does that of *melanogaster* (Fig. 6). The *melanogaster* precursor contains 13 FMRFamide-containing peptides, the first (m1) is followed by a 27 AA spacer region, then a group of 8 FMRFamide-like peptides (m2a–m4), a second short spacer region of 6 AA, and then a final group of four FMRFamide-like peptides (m5–m8); the *virilis* precursor contains a total of

300 μ m

Drosophila melanogaster**Drosophila virilis**

Figure 3. Schematic diagrams that indicate the similar number and positions of FMRFamide-immunoreactive cell bodies in the central nervous systems of *D. melanogaster* and *D. virilis*. Filled symbols represent strongly staining neurons and open symbols represent lightly staining neurons. Abbreviations: MP, medial protocerebrum; LP, lateral protocerebrum; SP, superior protocerebrum; SE, subesophageal; S1-3 and T1-3, subesophageal and thoracic segments 1-3 respectively; A1-7, abdominal segments 1-7 respectively. Lowercase *d* and *v* indicate dorsal versus ventral cell body positions; lowercase *m* indicates a medial cell body position.

Figure 4. Restriction maps and sequencing strategies. *A*, Partial restriction map of the phage lambda EMBL 4 clone V6 that encompasses approximately 13 kb of *D. virilis* DNA. *B*, Two regions of the V6 clone (*v-A* and *v-B*) chosen for subcloning are highlighted; the sequencing strategy is indicated. For the case of the *v-B* subclone, only those regions that were sequenced on both strands are described. The structure of the *virilis* FMRFamide gene, based on the sequence similarities to the FMRFamide gene of *D. melanogaster*, is indicated at the top. The 3' end of exon II cannot be accurately surmised from sequence analysis. The dashed line indicates the presumed splicing strategy. *C*, Partial restriction map and sequencing strategy used to study the portion of the *D. melanogaster* gene (from a lambda Charon 4A phage clone) that contains the first exon and regions upstream. Restriction enzyme abbreviations are as follows: C, Hinc II; H, Hind III; P, Pst I; R, Eco RI; S, Sma I; X, Xba I.

Figure 2. Immunocytochemical staining of the central and enteric nervous systems of *D. melanogaster* and of *D. virilis* using an anti-FMRFamide antibody. Immunoreactive neurons were visualized within the CNS and gut of third instar larvae in wholemount. *A*, The MP1 (triangles) and MP2 (curved arrows) neurons in the dorsal protocerebrum of the CNS. The unmarked cells in the anterior regions of the brain are SP cells. *B*, The LP1 (thin arrows) and SP (fat arrows) neurons in the ventral and anterior protocerebrum, respectively. Note the greater amount of stained processes within the protocerebrum of *virilis*. *C*, Cells within the oesophageal ganglion of the gut (open arrows). *D*, The Tv1-3 and SE2 neurons (arrowheads) within the ventral region of the thoracic and subesophageal neuromeres of the ventral nerve cord. *E*, The At1 neurons within the terminal abdominal ganglion (solid arrow). Note the immunoreactive connectives within the ventral nerve cord. Abbreviations based on nomenclature of White et al., (1986); *mel*, *melanogaster*; *vir*, *virilis*.

Figure 5. DNA sequences encoding the FMRFamide gene compared between *D. melanogaster* and *D. virilis*. The sequences are aligned at the initiator methionine and the numbering begins at this residue. To the *left* is indicated the bp numbering; to the *right*, amino acid numbering. Lowercase *m* and *v* stand for *melanogaster* and *virilis*, respectively. Within the open reading frames, deduced protein sequences are presented above (in the case of *melanogaster*) or below the DNA sequence (in the case of *virilis*). Asterisks indicate conserved amino acid residues; dashes indicate gaps introduced so as to align the sequences at clear regions of homology. In *boldface*, peptide names (e.g., *M1* and *V1*) indicate the positions of

is illustrated in Figure 6. The sequence of the first FMRFamide-like peptide (m1 and v1) is highly conserved: these peptides have a single amino acid difference in the N-terminal extension sequence, and in both peptides, *His* is substituted for *Arg* at the

		M2C																		M2D																	
		Phe	Gly	Arg	Asp	Pro	Lys	Gln	Asp	Phe	Met	Arg	Phe	Gly	Arg	Asp	Pro	Lys	Gln	Asp	Phe	Met	Arg	189,m													
505,m		TTT	GGC	AGG	GAT	CCA	AAG	CAG	GAC	TTC	ATG	AGA	TTC	GGT	CGG	GAT	CCC	AAG	CAG	GAT	TTC	ATG	AGA														
525,v		TTC	GGA	CGT	GCA	CCC	TCG	---	GAC	TTT	ATG	CGT	TTC	GGA	CGC	GAT	CCC	AGC	CAG	GAT	TTC	ATG	CGC														
		*	*	*	Ala	*	Ser	---	*	*	*	*	*	*	*	*	*	Ser	*	*	*	*	*	193,v													
		V2C																		V2D																	
		M2E																		M3A																	
		Phe	Gly	Arg	Asp	Pro	Lys	Gln	Asp	Phe	Met	Arg	Phe	Gly	Arg	Thr	Pro	Ala	Glu	Asp	Phe	Met	Arg	211,m													
571,m		TTC	GGT	CGA	GAT	CCC	AAG	CAG	GAT	TTC	ATG	AGG	TTT	GGA	CGC	ACT	CCG	GCT	GAG	GAT	TTC	ATG	AGG														
588,v		TTC	GGG	CGC	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---														
		*	*	*	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	196,v													
		M3B																		M4																	
		Phe	Gly	Arg	Thr	Pro	Ala	Glu	Asp	Phe	Met	Arg	Phe	Gly	Arg	Ser	Asp	Asn	Phe	Met	Arg	Phe	Gly	233,m													
637,m		TTC	GGA	CGC	ACT	CCG	GCG	GAG	GAC	TTC	ATG	AGG	TTC	GGA	CGC	TCC	GAC	AAT	TTC	ATG	CGC	TTC	GGA														
597,v		---	---	---	---	---	---	---	---	---	---	---	---	---	---	TCC	GAT	AAC	TTT	ATG	CGC	TTT	GGC														
		---	---	---	---	---	---	---	---	---	---	---	---	---	---	*	*	*	*	*	*	*	*	204,v													
		M5																		M6																	
		Arg	Ser	---	---	Pro	His	Glu	Glu	Leu	Arg	Ser	Pro	Lys	Gln	Asp	Phe	Met	Arg	Phe	Gly	Arg	Pro	253,m													
697,m		CGC	AGT	---	---	CCC	CAC	GAG	GAG	CTT	CGC	AGT	CCC	AAA	CAG	GAT	TTC	ATG	CGA	TTC	GGT	CGC	CCG														
621,v		AGG	AAC	CTG	AAC	TTT	CAC	GAG	GAG	CTG	CGC	AGT	CCC	AAA	CAG	GAT	TTC	ATG	CGT	TTC	GGT	CGC	CCG														
		*	Asn	Leu	Asn	Phe	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	226,v													
		V5																		V6																	
		M7																		M8																	
		Asp	Asn	Phe	Met	Arg	Phe	Gly	Arg	Ser	Ala	Pro	Gln	Asp	Phe	Val	Arg	Ser	Gly	Lys	Met	Asp	Ser	275,m													
763,m		GAC	AAC	TTC	ATG	CGC	TTC	GGG	CGT	TCC	GCT	CCG	CAG	GAT	TTT	GTG	CGC	TCC	GGG	AAG	ATG	GAC	TCA														
687,v		GAC	AAT	TTC	ATG	CGC	TTC	GGC	CGA	TCC	GCG	CCC	ACA	GAA	TTC	GAG	CGT	AAT	GGC	AAA	ATG	GAC	TCG														
		*	*	*	*	*	*	*	*	*	*	*	Thr	Glu	*	Glu	*	Asn	*	*	*	*	*	248,v													
		V7																		V8																	
		Asn	Phe	Ile	Arg	Phe	Gly	Lys	---	Ser	Leu	Lys	Pro	Ala	Ala	Pro	Glu	Ser	Lys	Pro	Val	Lys	Ser	296,m													
826,m		AAC	TTC	ATT	CGA	TTC	GGT	AAG	---	AGC	TTA	AAG	CCG	GCG	GCT	CCC	GAG	TCC	AAG	CCA	GTC	AAG	TCC														
753,v		AAC	TTC	ATG	CGC	TTT	GGC	AAG	AGA	TCA	GGT	GTA	ATG	GCC	AAA	TTG	ACC	AAA	TCC	CAG	CTG	CAG	CAA														
		*	*	Met	*	*	*	*	Arg	*	Gly	Val	Met	*	Lys	Leu	Thr	Lys	Ser	Gln	Leu	Gln	Gln	270,v													
		V9																		V10																	
		Asn	Gln	Gly	Asn	Pro	Gly	Glu	Arg	Ser	Pro	Val	Asp	Lys	Ala	Met	Thr	Glu	Leu	Phe	Lys	Lys	Gln	318,m													
892,m		AAT	CAA	GGC	AAC	CCA	GGC	GAA	CGC	AGT	CCA	GTG	GAC	AAG	GCC	ATG	ACG	GAG	CTG	TTC	AAG	AAA	CAG														
819,v		AAC	AAA	TTG	ACA	ACT	GCC	GAT	GGC	AAG	CAG	CAG	CCA	GCC	GAG	GAG	GGC	AAT	CCC	ACG	GAC	AAG	GCC														
		*	Lys	Leu	Thr	Thr	Ala	Asp	Gly	Lys	Gln	Gln	Pro	Ala	Glu	Glu	Gly	Asn	Pro	Thr	Asp	*	Ala	292,v													
		Glu	Leu	Gln	Asp	Gln	Gln	Val	Lys	Asn	Gly	Ala	Gln	Ala	Thr	Thr	Thr	Gln	Asp	Gly	Ser	Val	Glu	340,m													
958,m		GAG	CTG	CAG	GAT	CAG	CAG	GTG	AAG	AAC	GGC	GCA	CAG	GCG	ACC	ACC	ACG	CAG	GAT	GGG	AGT	GTG	GAA														
885,v		ATC	TCG	ATG	CTA	TTC	AAT	AAG	CAC	CAG	CAG	CAG	CAG	CAG	CAG	CAG	CAG	GGG	CAG	AGG	CTG	CAG	CAG														
		Ile	Ser	Met	Leu	Phe	Asn	Lys	His	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gly	Gln	Arg	Leu	Gln	Gln	314,v													
		347																																			
		Gln	Asp	Gln	Phe	Phe	Gly	Gln	STOP																												
1026,m		CAG	GAC	CAG	TTC	TTC	GGC	CAG	TGA	GGTAGTCCTGCGGGACGCGCTCCTTGTAAATAGATATGGACAAATGTACGCAAGG																											
951,v		GAA	GAC	CGA	CAG	CAG	ATG	AAG	AGC	AGC	GCA	GAG	CAG	AAC	AAC	TTG	GAG	GAA	GCC	AGC	GTG	GAG	CAG														
		Glu	*	Arg	Gln	Gln	Met	Lys	Ser	Ser	Ala	Glu	Gln	Asn	Asn	Leu	Glu	Glu	Ala	Ser	Val	Glu	Gln	336,v													
1100,m		ATCTAAATTGATATACGTATATAACCCACTCCTCACACGAACCTCTGACTTATGCCTGAACATATGAATTTTAAATGAATGGGCTGGA																																			
1017,v		TTC TAT GAG CCA TAG ATTCTACCGCGCAGCAGCAGCTGTAAATATAAATTGGCCAAAAAACCAGAACTATGATAATTG																																			
		Phe Tyr Glu Pro STOP																																			
		341																																			
1185,m		TAAAAATTCCACCGTGCTTTGAAGTCTTATCTATAAAATATATCTAGTGTAATATTGAAGAAATTGAAATTGGCGTGATAAAATCCTT																																			
1103,v		TACTTAACACTATGTATGTATGTACTTAAACGCATTATAGACATTGCAGAAGAAATTTAAAAAAGGAAAAAAAAAAGTAGAAAACTCT																																			
1276,m		GTGGCAACATTTTAAATAAAGATTGCTTTACTGTAAATTATGGCGC																																			
1190,v		ATTTTAAATGGGGCTGGATTAAATAATTACCACGCTTTGATCTGATCATAGAAAACAAAAGAAAAAAGTAGAAAACTCT																																			

the FMRFamide-like neuropeptide-coding regions. In the intronic region, 2 sequences that display homology between species, and that resemble consensus 3' intron splice signals, are indicated by *boldface* and *underlining*. In the 3' untranslated regions, potential polyadenylation signals are indicated by *underlines*. The 3' untranslated region of *melanogaster* ends at a point where, in the cDNA, a poly A tract begins (Schneider and Taghert, 1988). These sequences have been submitted to Genbank: accession numbers M32640, *melanogaster*, and M32643, *virilis*.

penultimate residue. In *virilis*, the first spacer region is shorter and conserved only at its ends. The second set of FMRFamide-like peptides in *melanogaster* contains 8 peptides: 5 are tandem repeats of DPKQDFMRFG (m2a-m2e). The following 2 have

the extension sequence TPAED- (m3a, m3b), and the last (m4) has the extension SDN-. In the case of *virilis*, there are only 5 peptides in this set; the first (v2a) and last (v4) are precisely conserved. The intervening 3 appear similar to DPKQDFMRFG

Figure 6. A comparison of the structures of the deduced precursor proteins encoded by the FMRFamide genes of *D. virilis* and *D. melanogaster*. **Top**, Schematic diagrams of the proteins placed with the initiator methionines in register. The presumed signal sequences are indicated by *lightly filled boxes* at the extreme left-hand ends. **Dark boxes** within each protein indicate positions of deduced FMRFamide-containing peptides, v1–8 and m1–8 respectively. **Bottom**, Direct comparison of the peptides according to the registration of the proteins indicated above. The peptide names are *boxed* and are located at the amino-terminus of each peptide; note that in this imposed registration, peptides m2e, m3a, and m3b lack counterparts in the *virilis* precursor. The peptide sequences are given in *single-letter code* and in *boldface* and are followed in roman text by glycine and basic residues, which, although these are not likely to be present in the mature processed peptides, are included here for the purpose of direct comparison.

but differ at 1–3 residues. The final set of FMRFamide-containing peptides in the *melanogaster* precursor (m5–m8) consists of 4 peptides each with a unique extension sequence. A very comparable set of peptides exists in the *virilis* precursor (v5–v8), but with 2 notable sequence differences: the third peptide of this set in the *melanogaster* precursor (m7 in Fig. 6) is the most degenerate FMRF-like sequence, FVRS. Likewise, in the *virilis* precursor peptide, v7 is similarly degenerate at the same residues, but with different amino acid substitutions, FERN. In addition, peptide m8 in *melanogaster*, which ended in -FIRF, was an -FMRF peptide in *virilis* (v8).

The degree of sequence conservation outside of the regions containing FMRFamide-like peptides varied greatly; the precursors have approximately 59% amino acid identity. Plots of the percentage identity between precursors at both the amino acid and nucleic acid levels are shown in Figure 7. The region from the end of the hydrophobic signal peptide to the first FMRFamide-like peptide (peptide 1, Fig. 6) is highly divergent for the first ~50 AAs, then displays increasing identity in the ~40 AAs preceding the first FMRFamide-like peptide. The highest conservation (besides the region of the hydrophobic leader sequence) is found in the 3 regions encoding FMRFamide-like peptides; the degrees of identity fall precipitously immediately following peptide 8. The C-terminal region of the *virilis* precursor is 18 AAs longer than is that of *melanogaster*. It is noteworthy that all single, di-, and tribasic AA residues, the pre-

sumed cleavage sites for the liberation of the FMRFamide-like peptides, are preserved between the 2 species. The 2 exceptions include a conservative change *Arg* to *Lys* (immediately preceding the v1 peptide) and the addition of an *Arg* to form *Lys-Arg* immediately following peptide v8.

In summary, 5 regions of sequence conservation within the neuropeptide precursor are evident: the putative signal peptide region, a ~40 AA region in the N-terminal portion of the precursor that immediately precedes the first FMRFamide-like peptide, and finally the 3 groups of FMRFamide-containing neuropeptides.

Homologies in the 5' exon and upstream regions

We compared the DNA sequences of regions that are 5' to the mRNA start sites of the 2 species. A total of ~1.2 kb of *D. melanogaster* DNA and 1.8 kb of *D. virilis* DNA was analyzed (Fig. 8). Whereas the start site in *melanogaster* has been defined by Northern blot analysis, primer extension, and nuclease protection assays (Schneider and Taghert, unpublished observations), the interpretation of the *virilis* neuropeptide gene structure is based solely on sequence comparisons. In *virilis*, the sequence of exon I is largely divergent: sequence conservation is limited to the first 34 bases, of which 26 are conserved. This conserved region is part of a larger 68 bp region of homology (#18 in Fig. 9) that begins with a sequence resembling the canonical TATA box. In total this region is conserved at 56 of 68

Figure 7. Polyprotein precursors containing FMRFamide-like peptides from *D. virilis* and *D. melanogaster* presented schematically along with amino acid and nucleic acid identity plots. The signal sequences are *lightly shaded* and aligned at the extreme amino-terminus. The FMRFamide-like peptides are *shaded darkly*. The *virilis* precursor is interrupted so as to align C-terminal regions more precisely with homologous regions of the *melanogaster* precursor. Y-axis, percentage identity; x-axis, amino acid position along the precursors. The plots were generated as follows: for the amino acid identity plot, beginning at position #5, a run of 10 consecutive (nos. 1–10) was compared between the 2 precursors and the percentage (the number of conserved residues $\times 10$) was determined. The determination was then repeated for the next 10 consecutive residues (from nos. 2–11) to define the identity value at residue 6. This procedure was repeated through all residues up to the fifth from the C-terminal end of the precursor, and the percentages were graphed with the percentage identity plotted along the y-axis and the position along the precursor serving as the x-axis. For the nucleotide identity plot, a similar strategy was employed: for each amino acid position, the corresponding 30 nucleotides (encoding the 10 respective amino acids) were compared. The percentage nucleic acid identity was determined for each amino acid position and plotted as for the amino acid identity. Apparent deletions and/or insertions were identified by eye, and therefore the following residues were not considered in the homology analysis: *melanogaster*, nos. 133–136, 174, and 193–224 (shown as a gap in the figure); *virilis*, 60–66, 242–243, and 295.

residues. Following this region, no significant homology is observed through the rest of exon I (72 bp). The *melanogaster* exon I ends at +106 bp, as mapped by genomic DNA sequencing and a nuclease protection assay; this splice junction is: A-G-/G-T-G-A-G-T (where the slash denotes the splice site) and it matches the consensus sequence (A-G-/G-T-A-A-G-T, derived by Padgett et al., 1986). A conserved sequence occurs in *virilis* at +170 bp (Fig. 8), because of which, we propose that exon I is slightly larger in this species. In neither species does exon I contain an ATG codon to initiate an open reading frame; hence

in both species, the protein is encoded completely within exon II. Upstream of the TATA box region, DNA sequences were markedly divergent, but 17 small regions of sequence conservation were found. In all the regions that are noted in Figure 8, the sequences are at least 80% conserved. The longest such region (#9) is 51 bp long with 2 mismatches. The relative order of the homologous regions between the 2 species (Fig. 9) is roughly comparable, although in *virilis* they spanned a region that is $\sim 1/3$ longer and 2 regions are displaced (#8 and 17). The ~ 2 kb of upstream DNA in *virilis* was marked by the inclusion

(-1686) 1
G TTTTCTTGAG G

-1141 AAGCTTCTGG GGTGAGTCCA TAGATTACAG AGCAGCGCAG TTTTCTTGAG GAGATGATGG TCTAGCTGAA

2 (-1668)
T TTCAACTTGA

-1071 CTATTTTTTAA CTGGGCGATT GGCACACGAC TTGGCCTGGT CATCAAACCTC AAGCCAAGAT TTCAACTTGA

-1001 ACTAGTCTGC CCTTATCACA TTGCCTTCTA ATTCGTTTTC ATTTTCGATT TTCGCATCTA CTTATGCTAA

- 931 TGAAGCCATC TGCAGACGTG GTTTTCGAAC GTATTATATAT TGATTATGGG TGATCGTCAA CAAGAGCAGT

3 (-1494) * * *
GA GACATTCTT GCAAGT

- 861 GGACACCCAA TAAACCTGTC CAAAACCCGA CACATTCTG CCGAGTCATG CGTGGTGGAC AATAGCCAAA

4 (-1403) AAAACTT -GGCCTT
5 (-1380) GGCCGTA ATTACAGAGT TCCG

- 791 TGCCATTGAT GAGACTCGTC TCCAAAAGTT TGGCCTTTTG CCGGGCCGTA ATTACAGAGT TCCGTCTTTT

- 721 GAACAGTTTT TTCAGCCCCA CCCAAGAGTC GAGTCTTGAA AAGCTGGCTG GGATGGGGTG GTTTCGGGTG

6 (-1249) *
AGGC GCCAATGT ATCCCGTTAC AGGC----G GCCAT

- 651 CTGGACGAGA TGCCAGAGGC GCCAATGT ATCCTGTTAC AGGTTACAGG GCCATAAAGC GCCATAAAGC

7 (-1199) * * *
GGCCAAT TATACCGCAT ACGCACACGT AG

- 581 CCGCGACGGC AATGGCAAAT TATAACGCAT ACGGACACGT AGTCGATCCA CTGGCTAGAA GGCTAATTGG

8 (-1455) *
CCGG CCAAGATGT
9 (-1082) **
GTTAAAAAGTG GCGCGCACAT AACAAATTGTG

- 511 ACGTGCCCGG CCAGGATGTC CCTGCTCATC CCACAGTCGA GTTAAAAAGTG GCGGTAACAT AACAAATTGTG

10 (-1004) * *
GTCAA AATGTTGCTG CGTCAT

- 411 GGCGATTTCG ATACAATTTC G GTGCTCGAGT GGCCACCTAA CTGGCGTGGA AATGTTGCTA CGTCATTGTT

- 371 GCTGCCCTC GTGCTTCTTG GCTAATAAGT CAGCATGAAT GCGGTCTGTG TTTTGGCCTT TTGTCCCGCT

11 (-775) A ACAACATAT CA
12 (-748) GCGGACA TTGTCAAGG

- 301 GACAACCGCT GACAAGCAAA ACAACATAT CAGCGGCTGA CCAGCGACCA TTTGCCGACA TTGTCAAGGT

- 231 TTTTTTTTCA GCCCCGAATA CACCTGCTAA TCTGTGGTGG TGA TAGCGC GCTCGGAAGG CAGAAAAAAC

13 (-248) GCAAC- TGTGCGCGCA ACACTTGTTG C
14 (-217)*
TGTGCGCGCA ACACTTGTTG C

- 161 TGCAGCGAAA ATCAGTTCCA TTGAATGTCC TCTGGCAACA TGTGCGCGCA ACACTTGTTG CCGCGGTCCA

15 (-140) 16 (-201) * *
CGCAGAGG C GGTCAACA ACACCC
17 (-850) GCCAG CAGAA
18 (-34) *
ATA TAAAACCGGA

- 91 CTGGCAGAGG CACCTCAACA TCAGGTCCT TGGGCGCCAG CACAAAGGAC GCAACGCATA TAAAACAGGA

*** * * * *
V TGTTCGCCA AACATTGCA GAGAAGTGT TACATTGCG ACGGTCAACA AGTGTCTGA CCAAAATCAG

- 21, M TGTTCGCCA AACATCTCA GAGAAGTGT CCAGCTTGA GCGGCAACA AGTGTGTGTG CCGCCCAAAA

V GTAGAAGAAG AACCACACGA GTGGCCAGTG AAAAGTGT TAAAATTTC CATTTCCTCA CGGAATGTCC

+ 50 M GGATCCCCAG ACCTTCGAAT TCACTTAGT TCCCTAGTAA GGGGACAGGT TTCAGAGGTG AGTGCCATGC

MEL, EXON I: SPLICE ▲

V ATTTGATTGC ATTGCAATAT TCGTATAATT TTAAATATTT GAAAAAAG GTGAGT

+ 120 M GAAAAGTGTG VIR, EXON I: SPLICE ▲

Figure 9. Map of the conserved regions that are 5' to the RNA start site (dotted line) of the FMRFamide gene in *D. melanogaster* and *D. virilis*. These regions are 9–68 bp in length and in *D. melanogaster* are consecutively numbered 1–18 beginning with the region most distal to the start site. Region 18 begins with a canonical TATA box sequence and ends just after the start site. Note that exon I (light shading) is larger in *virilis* and does not show significant sequence similarity except in the vicinity of the RNA start site.

of numerous repetitive sequences: tracts consisting of poly A and dinucleotide (TA or CA) repeats.

Discussion

We have adopted the strategy of interspecific comparison of DNA sequences in hopes of identifying conserved regions of a neuropeptide gene and its regulatory elements. Sequence conservation may imply a correspondent functional conservation. This assumption has been borne out in the cases both of promoter elements [e.g., the *Dopa decarboxylase* gene promoter of *Drosophila* (Johnson et al., 1989)] and of protein-coding regions [e.g., the homeodomains (Bodner et al., 1988; Ingraham et al., 1988; Way and Chalfie, 1988)]. The conserved regions of the *Drosophila* FMRFamide neuropeptide gene, and its upstream regions, provide a framework on which to create testable hypotheses concerning neuropeptide biosynthesis, function, and gene regulation.

Gene organization, transcription, and translation

Each species of *Drosophila* examined contained a single gene that hybridized to the *melanogaster* neuropeptide gene (Fig. 1). This result suggests that the gene has retained functional importance for this group of animals. Direct comparisons of this neuropeptide gene revealed that its organization in *D. virilis* is similar to that of *D. melanogaster*. A single large intron separates a small exon I from the larger exon II; in both species, the latter completely encodes the deduced neuropeptide precursor protein. Neither the 5' nor the 3' boundary of the neuropeptide gene intron is perfectly conserved, although sequence conservation can be noted at the 3' end of the intron (Fig. 5). The available evidence from the study of FMRFamide gene expression in *D. melanogaster* indicates that there is only one predominant transcript throughout all stages of postembryonic development (Schneider and Taghert, 1988, and unpublished observations; Schneider et al., unpublished observations) and thus the potential for alternative splicing appears slight.

Regulation of neuropeptide gene expression may also occur at the translational level. In a great majority of genes analyzed, the 5' untranslated regions of mRNAs are short (i.e., <160 bases in length), contain no AUGs upstream of the translation initiation site, and show little conservation between species (Kozak, 1984, 1986). The *Drosophila* FMRFamide neuropeptide gene conforms to this generalization, with the only conserved sequences of the first exon existing in a short region that includes the transcription start site. Certain *Drosophila* segmentation genes (Mueller and Hinnebusch, 1986; Wilde and Akam, 1987; Tautz, 1988) and heat shock genes (McGarry and Lindquist, 1985) display translational regulation, in part controlled by sequences in long untranslated leaders, that are evolutionarily conserved. Because the untranslated leader sequence of the neuropeptide mRNA does not display these properties, we think it unlikely that it is subject to similar forms of translational control.

The neuropeptide precursors

Although the *virilis* precursor contains 3 fewer FMRFamide-like peptides, it is nevertheless of the same approximate size as that of *melanogaster*, 339 vs 347 amino acids, respectively. This conservation of length is achieved by an increased distance between the last FMRFamide-like peptide and the stop codon, a region that is composed almost exclusively of divergent sequence. This observation suggests that precursor size (independent of sequence) may be a critical feature of the protein for aspects of its posttranslational processing and/or intragranular packaging. We propose that signal cleavage occurs after residue #17 (Ala): this assignment is based on the similarity of this region to a consensus previously proposed for signal splice sites (von Heijne, 1983), the fact that it is immediately followed by charged residues (Haeutle et al., 1989), and that it shows a high degree of conservation between species (Fig. 5; for comparison to other insect signal sequences, see Lidholm et al., 1987). An evolutionary pressure to conserve the entire signal sequence is implied from the precursor structures: the first 23

Figure 8. Nucleotide sequences of exon I and regions of homology in the area immediately 5' to the FMRFamide gene. The *melanogaster* sequence is presented in its entirety from –1200 to +200 bp relative to the mRNA start site (+1, numbers at left; M, *melanogaster*; V, *virilis*). The sequences of exon I are underlined for both species. Arrowheads mark the 3' end of each exon I (note their different sizes), at which a conserved splice site sequence is displayed in bold. Boldface sequences within gray backgrounds indicate regions of significant nucleotide conservation between *melanogaster* and *virilis*. The regions are numbered 1–18 consecutively, and their relative positions in the 2 species are diagrammed in Figure 9. The *D. virilis* sequences are aligned above the corresponding *D. melanogaster* sequences with mismatches marked by asterisks and gaps by dashes. Numbers in parentheses preceding the *D. virilis* sequences indicate their positions relative to the RNA start site of that species. Complete sequences have been submitted to Genbank: accession numbers M32641, *melanogaster*; M32642, *virilis*.

amino acids of the 2 proteins are identical. Interspecific comparisons of signal sequences between genes encoding homologous proteins have tended to indicate conservation of overall hydrophobicity but a lack of sequence conservation except at the cleavage site. For example, the signal sequences of rat and human pre-proenkephalin are only 70% identical (Yoshikawa et al., 1984). The significance of the perfect conservation between the signal sequences in 2 *Drosophila* species (60–80 million years divergent; Throckmorton, 1975) is uncertain; it may imply a necessity for this region to interact with other specific molecules during neuropeptide biosynthesis, rather than simply to provide a hydrophobic core by which to enter the lumen of the endoplasmic reticulum.

The FMRFamide-like peptides

The FMRFamide precursor of *Drosophila melanogaster* contains 13 copies of FMRFamide-like peptides, which present 7 different peptide sequences and which vary in their representation (from unique copies to 5 tandem repeats). To what extent are the differing sequences, copy numbers, and/or positions within the precursor important for neuropeptide biosynthesis or neuropeptide function? Interspecific comparisons of the pre-proenkephalin gene of 3 different mammals have shown a strict conservation in the sequence, number, and order of the enkephalin and enkephalin-extended peptide forms within the precursors (Rosen et al., 1984; Yoshikawa et al., 1984). The comparison of neuropeptide precursors between the 2 *Drosophila* species indicates (1) a conservation of the basic organization of peptides within the precursor, (2) variability in peptide copy number, and (3) a limited degree of conservation in peptide sequence.

In both *Drosophila* precursors, 3 groups of FMRFamide-like peptides are found: peptide 1, peptides 2–4, and peptides 5–8 (Fig. 5). These groups are separated by short protein regions that may themselves represent biologically active molecules, although for both spacer regions there has occurred at least partial sequence divergence. Based on the conservation of sequences between the 2 *Drosophila* species, we suspect that these FMRFamide peptide groups may represent 3 functionally distinct sets. In the case of *melanogaster*, 3 of the 13 FMRFamide-like peptides differ from the sequence -FMRF, i.e., -FMHF (m1), -FVRS (m7), and -FIRF (m8). In the case of *virilis*, only one of these variations has been conserved: peptide v1 is also an -FMHFamide. Because its extension (N-terminal extension of -FMRFamide) is also conserved, we suggest that this neuropeptide may represent a functionally distinct molecule. Likewise, the final peptide group (m5–m8 and v5–v8) is highly conserved between the 2 species in both number and extension sequence.

In contrast to the others, the middle group of peptides varies in the number of peptide repeat units and in their sequences. We make 2 inferences from this observation. First, because the extension sequences vary, the physiological roles of these individual peptides may not be intimately coupled to the specific extension sequences that are found. Second, the variation in copy number of peptides in this group (total of 5 vs 8) suggests that, while multiple copies of specific peptides may be advantageous, their absolute number is not a critical feature of the precursor. Perhaps only a minimum number is required, and having “extra” copies of peptides m2 or m3 may provide the latitude to experiment with new peptide sequences that are free from strong negative selection. Payza (1987) has argued that

FMRFamide and related heptapeptide forms of the snail *Helix* interact with distinct receptors. Whether the various forms of FMRFamide found in *Drosophila* also have distinct receptors, as we suggest here, must await experimental analysis. Alternatively, variations in the extension sequences of the FMRFamide-like peptides may represent evolutionary attempts to change degradation rates following release. In the cases of other genes that contain repetitive sequence elements, changes in the copy number are thought to have arisen by unequal crossover events (e.g., Enea et al., 1984; Wiborg et al., 1985; Lee et al., 1988). However, the mechanisms leading to such changes are unknown.

The promoter regions of the neuropeptide gene

In situ hybridization studies to localize FMRFamide gene transcripts (Schneider et al., 1988; Schneider et al., unpublished observations) suggest large differences in the amount of specific neuropeptide gene expression among different neurons of the CNS. Hence, as is thought the case with a variety of other neuropeptide genes and neurotransmitter-related genes (Rosenfeld et al., 1984; Scheller et al., 1984; Beall and Hirsch, 1987), FMRFamide gene expression in *Drosophila* appears to be regulated in a precise, cell-specific manner. Conserved sequence elements in the 5' upstream regions of the FMRFamide gene (Fig. 8) are therefore of interest in that they may represent candidate regulatory elements for the control of gene transcription. Regulatory elements of the neuropeptide gene promoter may control the activation of transcription during development (Bodner et al., 1988; Ingraham et al., 1988), as well as by physiologically induced changes in gene expression (Comb et al., 1986). It is unlikely that the sequence conservation that we observed upstream of the FMRFamide gene represents a contiguous transcription unit because this region lacks a significant open reading frame (data not shown). This possibility can be further discounted because of the irregular spacing of the conserved regions displayed by the 2 species (Fig. 9).

The *Dopa decarboxylase* gene of *Drosophila* exhibits a comparable cell-specific pattern (~150 neurons) of expression. Using germ line transformations, Hirsch and colleagues have identified sequences within the first ~200 bp 5' to the gene that are necessary for nervous system-specific expression and sequences in a distal enhancer region, ~1600 bp upstream that are necessary for cell type-specific expression (Beall and Hirsch, 1987). These upstream regions contain sequences that bind specific proteins from embryonic nuclear extracts (i.e., bind putative regulators of transcription) and these functionally important DNA sequences are highly conserved between the species *melanogaster* and *virilis* (Scholnick et al., 1986; Johnson et al., 1989). Likewise, functionally defined promoter elements upstream of the *Drosophila hunchback* gene [these confer sensitivity to regulation by the *bicoid* gene (Tautz et al., 1987; Schroeder et al., 1988)] show significant sequence homology between *melanogaster* and *virilis* species (Treier et al., 1989). We think it likely, therefore, that the present interspecific comparison provides a reasonable first approximation of the functionally important regions of the FMRFamide neuropeptide gene promoter. Although their exact sequences do not correspond to previously defined *cis*-acting elements, their sizes and their evolutionary conservation are consistent with possible roles in gene regulation. We hope to test some of the hypotheses derived from this interspecific comparison of neuropeptide genes through the

use of classical genetic methods and the use of germ line transformation techniques.

References

- Beall CJ, Hirsch J (1987) Regulation of the *Drosophila Dopa decarboxylase* gene in neuronal and non-neuronal cells. *Genes Dev* 1:510–520.
- Benton WD, Davis RW (1977) Screening lambda gt recombinant clones by hybridization to single plaques *in situ*. *Science* 196:180–182.
- Blackman RK, Meselson M (1986) Inter-specific nucleotide sequence comparisons used to identify regulatory and structural features of the *Drosophila hsp82* gene. *J Mol Biol* 188:499–515.
- Bodner M, Castrillo J-L, Theill LE, Deernick T, Ellisman M, Karin M (1988) The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell* 55:505–518.
- Boyd PJ, Walker RJ (1985) Actions of the molluscan neuropeptide FMRFamide on neurones in the subesophageal ganglia of the snail *Helix aspersa*. *Comp Biochem Physiol* 81C:379–386.
- Carroll LS, Carrow GM, Calabrese RL (1986) Localization and release of FMRFamide-like immunoreactivity in the cerebral neuroendocrine system of *Manduca sexta*. *J Exp Biol* 126:1–14.
- Columbaioni L, Paupardin-Tritsch D, Vidal PP, Gershenfeld HM (1985) The neuropeptide FMRFamide decreases both the Ca^{2+} conductance and a cyclic 3'5'-adenosine monophosphate-dependent K^{+} conductance in identified molluscan neurons. *J Neurosci* 5:2533–2538.
- Comb M, Birnberg NC, Seaholtz A, Herbert E, Goodman HM (1986) A cyclic AMP- and phorbol ester-inducible DNA element. *Nature* 323:353–356.
- Cottrell GA, Davies NW, Green KA (1984) Multiple actions of a molluscan cardioexcitatory neuropeptide and related peptides on identified *Helix* neurons. *J Physiol (Lond)* 365:315–333.
- Doble KE, Greenberg MJ (1982) The rectum is sensitive to FMRFamide, the enkephalins and their common analogs. *Neuropeptides* 2:157–167.
- Ebberink RHM, Price DA, van Loenhout H, Doble KE, Riehm JP, Geraerts WPM, Greenberg MJ (1987) The brain of *Lymnaea* contains a family of FMRFamide-related peptides. *Peptides* 8:515–522.
- Enea V, Arnot D, Schmidt EC, Cochran A, Gwadz R, Nussenzweig R (1984) Circumsporozoite gene of *Plasmodium cynomolgi* (Gombak): cDNA cloning and expression of the repetitive circumsporozoite epitope. *Proc Natl Acad Sci USA* 81:7520–7524.
- Evans PD, Myers C (1986) The modulatory actions of FMRFamide and related peptides on locust skeletal muscle. *J Exp Biol* 126:403–422.
- Haeuptle M-T, Flint N, Gough NM, Dobberstein B (1989) A tripartite structure of the signals that determine protein insertion into the endoplasmic reticulum membrane. *J Cell Biol* 108:1227–1236.
- Ingraham HA, Chen R, Mangalam HJ, Elshotz HP, Flynn SE, Lin CR, Simmons DM, Swanson L, Rosenfeld MG (1988) A tissue-specific transcription factor containing a homeo-domain specifies a pituitary phenotype. *Cell* 55:519–529.
- Johnson WA, McCormick CA, Bray SJ, Hirsch J (1989) A neuron-specific enhancer of the *Drosophila Dopa decarboxylase* gene. *Genes Dev* (in press).
- Keller EB, Noon WA (1985) Intron splicing: a conserved internal signal in introns of *Drosophila* pre-mRNAs. *Nucl Acids Res* 13:4971–4981.
- Kozak M (1984) Compilation and analysis of sequences upstream from the translation initiation start site in eukaryotic mRNAs. *Nucleic Acids Res* 12:857–872.
- Kozak M (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 44:283–292.
- Lee H, Simon JA, Lis JT (1988) Structure and expression of ubiquitin genes of *Drosophila melanogaster*. *Mol Cell Biol* 8:4727–4735.
- Lidholm D-A, Gudmundsson GH, Xanthopoulos KG, Bowman HG (1987) Insect immunity: cDNA clones coding for the precursor forms of cecropins A and D, antibacterial proteins from *Hyalophora cecropia*. *FEBS Lett* 226:8–12.
- Mahon AC, Nambu JR, Taussig R, Shyamala M, Roach A, Scheller RH (1985) Structure and expression of the egg-laying hormone gene family in *Aplysia*. *J Neurosci* 5:1872–1880.
- Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning. Cold Spring Harbor, NY: Cold Spring Harbor Press.
- McGarry TJ, Lindquist S (1985) The preferential translation of *Drosophila hsp70* mRNA requires sequences in the untranslated leader. *Cell* 42:903–911.
- Mueller PP, Hinnebusch A (1986) Multiple upstream AUG codons mediate translational control of GCN4. *Cell* 45:201–207.
- Muneoka Y, Matsuura M (1985) Effects of the molluscan neuropeptide FMRFamide and the related opioid peptide YGGFMRFamide on *Mytilus* muscle. *Comp Biochem Physiol* 81:61–70.
- Nambu JR, Scheller RH (1986) Egg-laying hormone genes of *Aplysia*: evolution of the ELH gene family. *J Neurosci* 6:2026–2036.
- Nambu JR, Murphy-Erdosh C, Andrews PC, Feistner G, Scheller RH (1988) Isolation and characterization of a *Drosophila* neuropeptide gene. *Neuron* 1:55–61.
- Noda M, Furutani Y, Takahashi H, Toyosato M, Hirose T, Inayama S, Nakanishi S, Numa S (1982) Cloning and sequence analysis of cDNA for bovine adrenal preproenkephalin. *Nature* 295:202–206.
- Padgett RA, Grabowski PJ, Konarska MM, Seiler S, Sharp PA (1986) Splicing of messenger precursors. *Annu Rev Biochem* 55:1119–1150.
- Painter SD (1982) FMRFamide catch contractures of a molluscan smooth muscle: pharmacology, ionic dependence and cyclic nucleotides. *J Comp Physiol* 148:491–501.
- Payza K (1987) FMRFamide receptors in *Helix aspersa*. *Peptides* 8:1065–1074.
- Price DA, Greenberg MJ (1977) Structure of a molluscan cardioacceleratory peptide. *Science* 197:670–671.
- Price DA, Cottrell GA, Doble KE, Greenberg MJ, Jöhrenby W, Lehman HK, Riehm JP (1985) A novel FMRFamide-related peptide in *Helix*: pQDPLRFamide. *Biol Bull* 169:256–266.
- Price DA, Davies NW, Doble KE, Greenberg MJ (1987a) The variety and distribution of FMRFamide-related peptides in molluscs. *Zool Sci* 4:395–410.
- Price DA, Cobb CG, Doble KE, Kline JK, Greenberg MJ (1987b) Evidence for a novel FMRFamide-related heptapeptide in the pulmonate snail *Siphonaria pectinata*. *Peptides* 8:533–538.
- Rosen H, Douglass J, Herbert E (1984) Isolation and characterization of the rat proenkephalin gene. *J Biol Chem* 259:14309–14313.
- Rosenfeld MG, Amara SG, Evans RM (1984) Alternative RNA processing: determining neuronal phenotype. *Science* 225:1315–1320.
- Schaefer M, Picciotto MR, Kreiner T, Khaldany RR, Taussig R, Scheller RH (1985) *Aplysia* neurons express a gene encoding FMRFamide neuropeptides. *Cell* 41:457–467.
- Scheller RH, Khaldany RR, Kreiner T, Mahon AC, Nambu JR, Schaefer M, Taussig R (1984) Neuropeptides: mediators of behavior in *Aplysia*. *Science* 225:1300–1308.
- Schneider LE, Taghert PH (1988) Isolation and characterization of a *Drosophila* gene encoding multiple neuropeptide related to FMRFamide (Phe-Met-Arg-Phe-NH₂). *Proc Natl Acad Sci USA* 85:1993–1997.
- Schneider LE, Taghert PH (1990) Organization and expression of the *Drosophila* FMRFamide neuropeptide gene. *J Biol Chem* 265:6890–6895.
- Schneider LE, Sun E, Taghert PH (1988) Cellular analysis of a neuropeptide gene in *Drosophila melanogaster*. *Soc Neurosci Abstr* 14:29.
- Scholnick SB, Bray SJ, Morgan BA, McCormack CA, Hirsch J (1986) CNS and hypoderm regulatory elements of the *Drosophila melanogaster Dopa decarboxylase* gene. *Science* 234:998–1002.
- Schroeder C, Tautz D, Seifert E, Jackle H (1988) Differential regulation of the two transcripts from the *Drosophila* gap segmentation gene *hunchback*. *EMBO J* 7:2881–2887.
- Taussig R, Scheller RH (1986) The *Aplysia* FMRFamide gene encodes sequences related to mammalian brain peptides. *DNA* 5:453–461.
- Tautz D (1988) Regulation of the *Drosophila* segmentation gene *hunchback* by two maternal morphogenetic centers. *Nature* 332:281–284.
- Tautz D, Lehmann R, Schnurch H, Schuh R, Seifert E, Keinlin A, Jones K, Jackle H (1987) Finger protein of novel structure encoded by *hunchback*, a second member of the gap class of *Drosophila* segmentation genes. *Nature* 327:383–389.
- Throckmorton, LH (1975) In: Handbook of genetics, Vol 3 (King RC, ed), pp 421–469. New York: Plenum.
- Treier M, Pfeifle C, Tautz D (1989) Comparison of the gap segmentation gene *hunchback* between *Drosophila melanogaster* and *Drosophila virilis* reveals novel modes of evolutionary change. *EMBO J* 8:1517–1525.
- Trimmer BA, Kobierski LA, Kravitz EA (1987) Purification and char-

- acterization of FMRFamide-like immunoreactive substances from the lobster nervous system: isolation and sequence analysis of two closely related peptides. *J Comp Neurol* 266:16–26.
- von Heijne G (1983) Patterns of amino acids near signal-sequence cleavage sites. *EMBO J* 5:3021–3027.
- Walther C, Scheibe M (1987) FMRF-NH₂-like factor from neurohaemal organ modulates neuromuscular transmission in the locust. *Neurosci Lett* 77:209–214.
- Way JC, Chalfie M (1988) *mec-3*, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in *C. elegans*. *Cell* 54:5–16.
- Weiss S, Goldberg JJ, Chohan KS, Stell WK, Drummond GI, Lukowiak K (1984) Evidence for FMRFamide as a neurotransmitter in the gill of *Aplysia californica*. *J Neurosci* 4:1994–2000.
- White K, Hurteau T, Punsal P (1986) Neuropeptide FMRFamide-like immunoreactivity in *Drosophila*: development and distribution. *J Comp Neurol* 247:430–438.
- Wiborg O, Pederson MS, Wind A, Berglund LE, Marcker KA, Vuust I (1985) The human ubiquitin multigene family: some genes contain multiply repeated ubiquitin coding sequences. *EMBO J* 4:755–759.
- Wilde CD, Akam M (1987) Conserved sequence elements in the 5' region of the *Ultrabithorax* transcription unit. *EMBO J* 6:1393–1401.
- Yang H-Y, Frata W, Majane EA, Costa E (1985) Isolation, sequencing, synthesis and pharmacological characterization of two brain neuropeptides that modulate the action of morphine. *Proc Natl Acad Sci USA* 82:7757–7761.
- Yoshikawa K, Williams C, Sabol SL (1984) Rat brain preproenkephalin mRNA: cloning, primary structure and distribution in the central nervous system. *J Biol Chem* 259:14301–14308.