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Characterization of the Gene for Leucomyosuppressin and its Expression in the Brain of the Cockroach *Diploptera punctata*

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Using HPLC separation, radioimmunoassay, and subsequent bioassay, we have detected the presence of an active peptide, which co-elutes with the insect myoinhibitory peptide leucomyosuppressin, in the brain of the cockroach *Diploptera punctata*. We have isolated a cDNA encoding the precursor for this peptide from cDNA libraries representing *D. punctata* brain RNA. The cDNA sequence contains an open reading frame that upon translation would result in a prepropolypeptide of 96 amino acids. Proteolytic cleavage of the predicted precursor could result in several peptides, including a 10 amino acid C-terminal peptide that would, upon modification of the NH_2 and COOH-terminal amino acids, be identical to the insect FLRFamide, leucomyosuppressin. No other RFamide products are predicted to be processed from the precursor. Southern blot analysis indicates that the gene is present in the *D. punctata* genome in a single copy. Northern blot analysis shows that the gene is predominantly expressed as a 3.8 kb mRNA in cockroach brain. Study of the expression of the leucomyosuppressin gene in *D. punctata* brain, using *in situ* hybridization, indicates that expression occurs primarily in the pars intercerebralis of the protocerebrum, a region showing abundant FMRFamide-like immunoreactive neurosecretory cells. Immunohistochemistry and HPLC coupled to radioimmunoassay indicates that leucomyosuppressin represents a significant proportion of FMRFamide-related peptide production in the brain. However, HPLC analysis also indicates the presence of significant levels of other related peptides, demonstrating the presence of more than one FMRFamide-related gene in this insect. Crown copyright © 1996 Published by Elsevier Science Ltd

FLRFamide Cockroach Diploptera punctata Neuropeptide Gene Leucomyosuppressin

INTRODUCTION

The tetrapeptide FMRFamide (Phe-Met-Arg-Phe-NH_2), first isolated from molluscs (Price and Greenberg, 1977), is but one member of a diverse family of structurally-related peptides. These FMRFamide-related peptides (FaRPs) are characterized by their common C-terminally-amidated RF moiety, with structural diversity conferred at the N-terminus. FaRPs are known to occur across a broad range of phyla in the animal kingdom (Greenberg and Price, 1992). FaRPs include the basic tetrapeptide FMRFamide and its close structural variant FLRFamide, as well as a host of N-terminally extended variations of these basic structures. Other less related groups of peptides maintain only the RFamide terminus.

In insects, several distinct sub-families of FaRPs are recognized, two of which have been described at the molecular level; multiple FMRFamides (Nambu *et al.*, 1988; Chin *et al.*, 1990; Schneider and Taghert, 1988; Taghert and Schneider, 1990) and the sulfakinins (Nichols *et al.*, 1988). A third subfamily, the extended FLRFamides, have been sequenced at the amino acid level in six insect species. Their sequences are identical at seven of their ten amino acid residues (see Peeff *et al.*, 1994). As a

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Abbreviations: FaRP, FMRFamide-related peptide LMS, leucomyosuppressin Dip-LMS gene, *Diploptera punctata* leucomyosuppressin gene CC, corpora cardiaca CA, corpora allata NCCI, nervi corpori cardiaci I NCA, nervi corpori allati bp, base pair(s) kb, kilobase(s) RP-HPLC, reverse-phase high performance liquid chromatography.

group, these molecules all show potent myotropic activity (primarily being strong inhibitors of visceral muscle contraction), and have been collectively referred to as the myosuppressins (Nachman *et al.*, 1993). Their structures are not predicted in the genes characterized to date.

The first of the myosuppressins to be characterized was isolated from the cockroach *Leucophaea maderae* on the basis of its inhibitory activity on spontaneous contractions of isolated hindgut (Holman *et al.*, 1986). The amino acid sequence of the peptide, which was named leucomyosuppressin (LMS) based on this observed activity, was determined to be pQDVDHVFLRFamide. It shows close similarity to other decapeptides more recently isolated from two locusts, *Schistocerca gregaria* (Robb *et al.*, 1989) and *Locusta migratoria* (Schoofs *et al.*, 1993a; Peeff *et al.*, 1994), two dipterans, *Drosophila melanogaster* (Nichols, 1992) and *Neobellieria bullata* (Fonagy *et al.*, 1992), and a lepidopteran, *Manduca sexta* (Kingan *et al.*, 1990). Comparison of the structures yields the common sequence XDVXHXFLRFamide.

We set out to clone the gene for the analogous FLRFamide from the cockroach, *Diploptera punctata* (the Dip-LMS gene) for three reasons. First, we chose a myosuppressin gene as our objective since these peptides have been found in various species of the Orthoptera, Diptera, Lepidoptera, and Dichtyoptera, suggesting the likely presence of an analagous peptide in *D. punctata*. Second, molecular biological tools were available from *D. punctata* as a consequence of the recent isolation of the allatostatin gene (Donly *et al.*, 1993). Third, if the myosuppressins proved to be encoded on a separate gene from other FaRPs, the potential to study FaRP gene expression in insect systems is greatly expanded.

In contrast to previously characterized FaRP genes which have shown multiple distinct peptides in the deduced prohormone structures (as well as multiple copies for particular peptide species), the LMS gene from *D. punctata* reveals a rather simple precursor, from which LMS is the only RFamide predicted. Also, the peptide that we observed in brain tissue extracts and have subsequently predicted from the gene structure, is identical to that first characterized from the cockroach *L. maderae* (Holman *et al.*, 1986).

MATERIALS AND METHODS

Tissue extraction and HPLC analysis

Nine groups of 60 adult mated female *D. punctata* brains [including corpora cardiaca (CC) and corpora allata (CA)] were dissected in physiological saline, transferred to 1 ml of extraction medium (90% methanol; 9% glacial acetic acid; 1% water), freeze-thawed, sonicated, and centrifuged at 8800 **g** for 15 min. The supernatant was collected and pooled. The pellet was resuspended in extraction medium and processed as above. The second supernatant was pooled with the first and evaporated to dryness using a Speed-Vac concentrator (Savant, Farmingdale, NY). This was taken up in 1 ml of saline, then run through a C_{18}-containing Sep-Pak cartridge, and prepared for HPLC analysis as described by Peeff *et al.* (1994). Extracts were resuspended in HPLC buffer and chromatographed sequentially on two HPLC systems using various acetonitrile gradients.

System 1. A Brownlee RP-C_{18} Spheri-5 column (46 mm × 25 cm) was used with a linear acetonitrile gradient (containing 0.1% TFA) of 9–60%, run at 1 ml/min. This gradient was run over 34 min, 2 min after injecting the sample. Elution times of fractions positive for FaRPs were compared with elution times of synthetic SchistoFLRFamide, ManducaFLRFamide and LMS (Peninsula Laboratories, Belmont, CA). Fractions co-eluting with LMS were pooled, evaporated to dryness and redissolved in HPLC solvent to be run on system 2.

System 2. A Brownlee Phenyl Spheri-5 column (46 mm × 25 cm) was used with two different acetonitrile gradients (containing 0.1% TFA). The first gradient was increased from 18–60% acetonitrile, over 60 min, 2 min after injecting the sample. Fractions were compared with synthetic LMS and those co-eluting were pooled and processed, as described for system 1, and rerun on the Phenyl column with a slower acetonitrile gradient. The slower gradient ran from 20–29% acetonitrile, over 60 min, 2 min after injection. Elution times were again compared with the elution time of synthetic LMS and the fraction co-eluting with LMS was evaporated to dryness for bioassay.

The radioimmunoassay for FMRFamide-like peptides has been described previously (Peeff *et al.*, 1993). LMS is able to compete with FMRFamide in a linear fashion, although with less avidity than FMRFamide itself (Tsang and Orchard, 1991). Thus, the quantification of LMS using this radioimmunoassay is an underestimate of the actual amount of LMS present.

Bioassays

Oviducts were dissected from mature adult female locusts and isotonic contractions measured as described (Lange *et al.*, 1986; Peeff *et al.*, 1993). Appropriate reverse-phase HPLC (RP-HPLC) fractions (34–38) from the brain extracts run on the slow gradient of system 2 were dried using Speed-Vac, redissolved in 50 μl saline, and 10 μl assayed for bioactivity. The fractions were added directly to the oviducts to examine their ability to inhibit spontaneous contractions (Lange *et al.*, 1991). Synthetic LMS (Peninsula Laboratories) was also analyzed for bioactivity on the locust oviduct as described above.

DNA synthesis and sequencing

Oligonucleotides coding for the amino acid sequence of the predicted non-modified form of LMS (QDVDHVFLRFG) were synthesized on a Biosearch model 8750 DNA synthesizer at the Queen's Core Facility for Protein/DNA Chemistry (Kingston, Ontario). The amino acid sequence begins with glutamine, the

unmodified form of pyroglutamate, and also includes a C-terminal glycine required for processing into the mature, amidated peptide structure. Two overlapping oligonucleotides, (5′-GGAATTCCARGAYGTNGAYCAYGTNTT-3′ and 5′-GGAATTCAYCAYGTNTTYCTNMGNTTYGG-3′; Y=C or T, R=A or G, M=A or C, N=A, C, G or T), were created for use in nested PCR in combination with a vector specific primer from the T7 promoter region of the Bluescript plasmid (Stratagene).

DNA sequencing was carried out on both strands of the DNA by the dideoxynucleotide chain termination method using the Taq DyeDeoxy Terminator Cycle sequencing kit from Applied Biosystems (Queen's Core Facility for Protein/DNA Chemistry).

RNA isolation and library construction

Brains were dissected from day 2–3 mated female cockroaches and frozen immediately in liquid nitrogen. Poly(A)$^+$ RNA was directly extracted from 1000 brains using the Pharmacia QuickPrep mRNA purification system and stored under ethanol at −20°C.

A cDNA library was constructed from the poly(A)-enriched RNA using a Zap-cDNA synthesis kit (Stratagene) and 1 million plaques were immediately amplified. Nucleic acid screening was performed on aliquots of the primary library before amplification. Aliquots of the amplified library (at a titre of 10^{10} plaque forming units per ml), were prepared for PCR analysis by extraction with phenol/chloroform and ethanol precipitation. Final resuspension was in TE at 1/10 the original volume.

PCR methods

Standard PCR was done in 50 μl volumes containing 2.5 μl cDNA (above), 1× PCR buffer (20 mM Tris–HCl [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$), 200 μM dNTPs, 2.5 units of Amplitaq DNA polymerase (Perkin-Elmer), and, when using degenerate primers, one Ampliwax PCR Gem (Perkin-Elmer). Unique oligonucleotide primers were added to 0.25 μM concentration, whereas degenerate ones were used at 2.5 μM. Temperature cycling profiles varied according to the primers being used, but were generally based on the following model: an initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 45 s, 50–65°C for 45 s, and 72°C for 1 min per kilobase (kb) of expected product. A final 10 min extension at 72°C was included to complete all products.

Southern blotting

High-molecular-weight DNA was purified from adult *D. punctata* as outlined by Ding *et al.*, 1995. Aliquots containing 10 μg of extracted DNA were digested with selected restriction enzymes and then separated on a 0.9% agarose gel. The DNA was denatured and transferred to Hybond N$^+$ nylon membrane, on which it was alkali fixed before hybridization.

Northern blotting

Poly(A)$^+$ RNA was isolated from brains of day 2–3 mated female cockroaches as described above. An aliquot containing 4 μg was denatured with glyoxal (McMaster and Carmichael, 1977) for 60 min at 50°C and then fractionated on a 1.1% agarose gel. The RNA was transferred to Hybond N$^+$ nylon membrane and alkali fixed before hybridization.

Nucleic acid hybridizations

For Southern and Northern blots, membranes were prehybridized and hybridized at 65°C in Quikhyb solution (Stratagene) as directed. The hybridization probe in each case was a random-primed ^{32}P-labeled fragment of the Dip-LMS gene corresponding to bases 348–719 in Fig. 4. The membranes were washed once in 2× standard saline citrate (SSC)/0.5% SDS at room temperature, followed by two washes in 1× SSC/0.1% SDS at 60°C (Sambrook *et al.*, 1989).

Hybridizations of plaque lifts for library screening were carried out as follows: Hybond N membrane circles (Amersham) were prehybridized in 5× standard saline phosphate/EDTA (SSPE), 10× Denhardt's solution, 0.1% SDS, and 250 μg/ml denatured salmon sperm DNA at 65°C for 4 h (Sambrook *et al.*, 1989). Hybridization was done in fresh solution including the same ^{32}P-labeled fragment as above, for 18 h at 65°C. The first wash contained 5× SSPE/0.5% SDS at 60°C, followed by two more washes in 1× SSPE/0.1% SDS at 60°C.

In situ *hybridization*

Wholemount *in situ* hybridization was performed using a modification of the protocol of Tautz and Pfeifle, 1989. All incubation steps were performed at room temperature unless stated otherwise. Cockroach brains, with CC and CA intact, were dissected from adult mated female *D. punctata* under physiological saline (Elia and Gardiner, 1990), and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 10 mM phosphate buffer, pH 7.2, containing 0.9% NaCl) for 2 h. After fixation, tissues were washed in PBS followed by PBS with 0.1% Tween 20 (PBT). Tissues were incubated for 15 min with 1 mg/ml collagenase in PBS to remove the brain sheath, washed again in PBT, and then refixed in 4% paraformaldehyde prior to hybridization.

Hybridization solution (HS) consisted of 50% formamide, 5× SSC, 1× Denhardt's solution, 0.1% Tween 20 and 100 μg/ml denatured salmon sperm DNA. Fixed tissues were initially washed in equal volumes PBT/HS, then incubated for 1 h in HS at room temperature, followed by another hour at 45°C. Hybridization was carried out for 18 h at 45°C in HS with 0.5 μg/ml heat-denatured DIG-labeled DNA. The hybridization probe was a fragment of the Dip-LMS gene (corresponding to bp 348–719 in Fig. 4), labeled using the digoxigenin (DIG)-labeling kit from Boehringer Mannheim.

After hybridization, the tissues were put through a series of PBT:HS washes (0:1, 1:4, 2:3, 3:2, 4:1, 1:0;

vol:vol) and then incubated with a 1/5000 dilution of anti-digoxigenin–alkaline phosphatase antibody conjugate (Boehringer Mannheim) in PBT. After another wash, tissues were incubated in 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris (pH 9.5), 0.1% Tween 20, and 1 mM Levamisole (a potent inhibitor of lysosomal phosphatase). Brains were then processed for colorimetric detection using nitroblue tetrazolium salts and X-Phosphate. Color development was controlled under the dissection microscope and stopped in PBT. The brains were dehydrated in an ethanol series (30%, 60%, 80%, 100% for 10 min each) and mounted in 66% permount, 34% xylene. Test DNA provided in the labeling kit (Boehringer Mannheim) was DIG labeled and hybridized to prepared brains as a control for non-specific binding in the protocol.

Immunohistochemistry

Cockroach brains were dissected as described above and fixed in 2% paraformaldehyde in Millonigs buffer (0.13M $NaH_2PO_4 \cdot H_2O$; 0.1M NaOH; 1.2% glucose; 0.3mM $CaCl_2$; pH 7.4) for 1.5 h. The tissues were washed in PBS for 4–5 h and then incubated in 4% Triton X-100 in PBS with 2% normal goat serum (NGS) for 1 h at room temperature to enhance penetration of the antibody and to pre-absorb non-specific antigenic sites. Tissues were incubated for 48 h at 8°C in a 1:1000 dilution of polyclonal rabbit anti-FMRFamide antiserum (Incstar Corporation, Stillwater, MN) in 0.4% Triton X-100 with 2% BSA and 2% NGS. This was followed by a 5–8 h wash in cold PBS prior to incubation in the secondary antibody for 18 h at 8°C. The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (Daymar Laboratories, Toronto), diluted 1:200 in PBS containing 10% NGS. Tissues were washed in PBS and cleared and mounted on depression slides in 5% *n*-propyl gallate in 80% glycerol (pH 7.3).

RESULTS

Identification of LMS in D. punctata *brain*

To determine if FaRPs were present in *D. punctata* brain, we performed RP-HPLC separations on an extract from 540 isolated brains, and analyzed fractions for the presence of FMRFamide-like material using radioimmunoassay. Prepared brain extract was separated using two sequential HPLC systems through three acetonitrile gradients (Materials and Methods). Elution times of fractions that were positive for FaRPs using radioimmunoassay were compared with elution times of SchistoFLRFamide, ManducaFLRFamide and LMS. Those co-eluting with synthetic LMS were pooled after each run and further purified on the subsequent system (Figs 1–2). System 1 resolved two broad peaks of immunoreactive material (Fig. 1A), one of which eluted over the time range of synthetic LMS, covering a 5 min period. These fractions were pooled, evaporated to dryness and separated on system 2. One major immunoreactive peak was observed (Fig. 1B), which was collected (solid fractions), and run again on system 2 using a shallower acetonitrile gradient (Fig. 2A). This produced a single peak of immunoreactivity eluting with the same retention time as synthetic LMS. The fractions encompassing the peak, plus three more before and after it, were tested for activity on isolated locust oviduct (Lange *et al.*, 1991). Myographs of oviduct contractile activity after treatment with relevant fractions are shown in Fig. 2B. Fractions 35–37 (co-migrating with LMS) produced an effect similar to an LMS standard, inhibiting spontaneous contractions and reducing basal tonus. Fraction 36 from the final chromatographic separation showed the strongest effect, producing inhibition similar to 10^{-6} M synthetic LMS. Fractions 32–34 and 38–40 had little or no activity (examples of fractions 34 and 38 are shown in Fig. 2B).

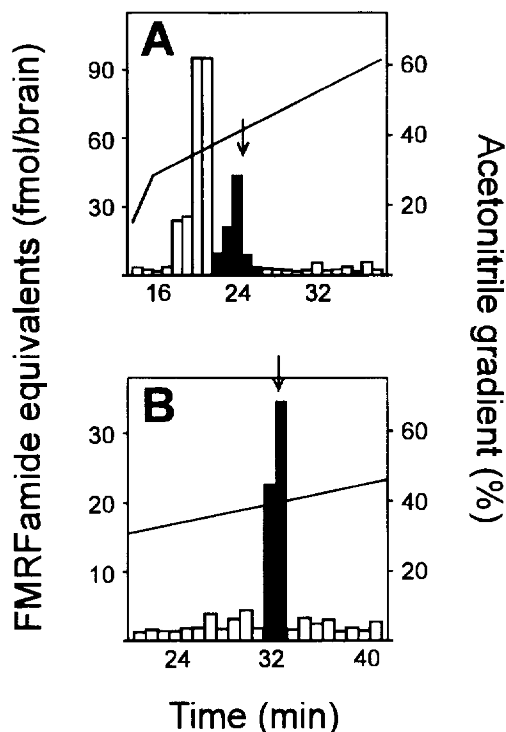

FIGURE 1. RP-HPLC fractionation of FMRFamide-like material from extracts of 540 brains, (including corpora cardiaca and corpora allata), of adult mated female *D. punctata*. The values have been converted to single brain equivalents of FMRFamide. Arrows indicate the elution times of synthetic LMS. The solid line is the acetonitrile gradient. (A) RP-HPLC separation on a C_{18} column (system 1) revealed at least two broad peaks of FMRFamide-like immunoreactive material. FMRFamide-like immunoreactive fractions eluting close to synthetic LMS (arrow) were pooled (dark bars) and run on system 2. (B) RP-HPLC separation of pooled fractions (22–26) from system 1 through a Phenyl column with the first acetonitrile gradient (system 2). One broad peak of immunoreactivity was detected and fractions were pooled (dark bars).

Isolation of cDNA clones

A PCR-based approach was used to isolate a fragment of the *D. punctata* LMS gene. One-sided PCR was done with degenerate oligonucleotides designed from the LMS

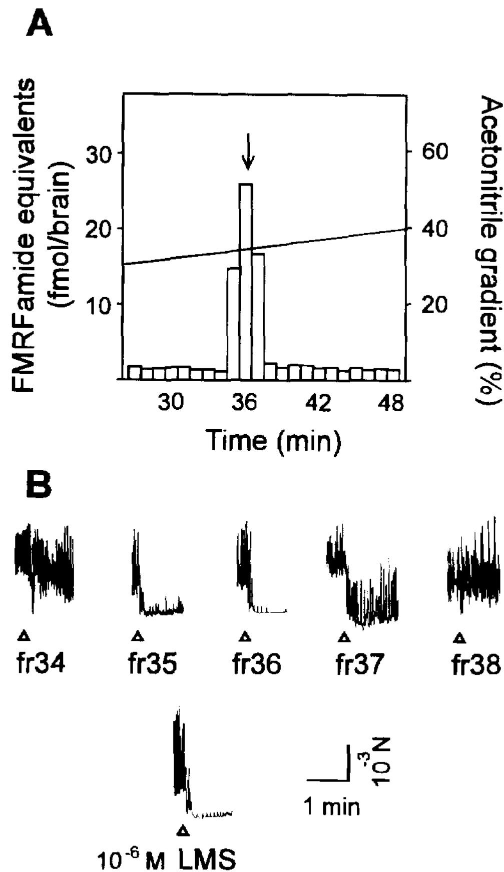

FIGURE 2. Further RP-HPLC purification of fractions, and bioactivity using the isolated locust oviduct bioassay. (A) The FMRFamide-like immunoreactive fractions separated on the Phenyl column in Fig. 1 were separated using a slower acetonitrile gradient (system 2). The arrow indicates the elution time of synthetic LMS. The solid line is the acetonitrile gradient. (B) Fractions 34–38 from A and 10^{-6} M LMS were applied to locust oviduct preparations and the effects on contractile activity measured. The open triangles indicate application of the various sample fractions; fr–fraction.

amino acid sequence (Materials and Methods), in combination with a T7 promoter primer (vector specific anchor sequence), using DNA extracted from λ LZap cockroach brain cDNA library as the template. The two degenerate gene-specific primers were used sequentially in two nested PCR reactions (see Materials and Methods), to produce a 400 bp DNA fragment representing a 3′ portion of the Dip-LMS gene.

Using this PCR-generated fragment of the gene, aliquots of unamplified cockroach brain cDNA library were screened for homologous clones (Materials and Methods). From approximately 500 000 plaques screened, 68 positively hybridizing clones were isolated. Numbers of positive clones were found to be much higher using primary library versus aliquots of amplified library, suggesting that these clones do not propagate well during amplification. All but one of the isolated phages were confirmed as positive LMS clones by PCR amplification from each of a small DNA fragment encompassing the region encoding the 10 amino acids of the LMS peptide (primers A and B in Fig. 3). The 5′ termini of the majority of the clones were found to fall within approximately 50 bp of one another by PCR analysis using an internal primer (primer C in Fig. 3) and a site in the λ LZap vector. Similar characterization of the 3′ ends of the clones (primer D in Fig. 3) revealed the clones fall into three overall size groups, consisting of approximately 1600, 1900, or 3500 bp (Fig. 3). PCR using the FLRFamide specific primers described in the Materials and Methods section confirmed that the FLRFamide encoding portion of the gene was located in the 5′ region. The deduced relative alignments of the various structures are shown schematically in Fig. 3.

DNA sequence analysis

The DNA sequence of a cDNA clone selected from one of the three insert sizes observed (1.9 kb) was determined, and is presented in Fig. 4. Upon translation of the sequence, an open reading frame was revealed in the 5′ region (Fig. 3) which encodes a FLRFamide peptide identical to LMS, the first characterized myosuppressin (Holman *et al.*, 1986). The deduced amino acid sequence includes potential proteolytic processing sites before (KR), and after (RRR), the putative FLRFamide. Bounded by these sites, the encoded peptide begins with glutamine, which can undergo conversion to pyroglutamate in the mature peptide (Smyth *et al.*, 1963), and ends with a glycine, which is necessary for subsequent amidation of the C-terminus of the processed product (Bradbury *et al.*, 1982).

The complete open reading frame encoding this FLRFamide extends as far as the methionine at nucleotide 93, which represents the first possible initiation site for translation (an in-frame stop codon [TGA] is found near the 5′ terminus of the clone). The ATG which encodes this methionine is preceded by a C and three A residues, which conforms to the consensus found among *Drosophila* start codons (Cavener, 1987). Analysis of the amino acids immediately following reveals a bias toward hydrophobic residues, suggestive of the presence of a hydrophobic core for a secretory signal sequence. Using the method of von Heijne (1986), the most likely sites for cleavage of the signal sequence are found after amino acid 17 or amino acid 25 (Fig. 4). Therefore, the clone structure would suggest the occurrence of a preprohormone precursor containing 96 amino acids, terminating at a stop codon immediately following the FLRFamide and its processing signals. To ensure this deduced prohormone structure is representative of all three clone types, the 5′ regions of independent clones representative of the 1.6 kb and 3.5 kb types were also sequenced and found to be identical to the 1.9 kb clone through the entire open reading frame.

In addition to the cleavage sites in the prohormone for LMS itself, there are two other potential dibasic cleavage sites within the precursor (at amino acids 21 and 44). Processing at these points would produce two further internal fragments (Ala23-Phe43 and Ile46-Val80), in addition to the signal peptide. Only the first of these shows the characteristic C-terminal RF, but the resulting

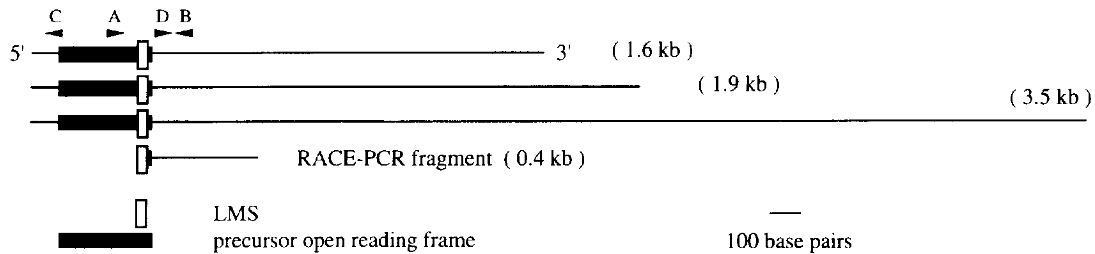

FIGURE 3. Schematic representation of isolated Dip-LMS gene cDNA clones. From 68 clones isolated, three general cDNA size groups were identified using PCR analysis. The positions of various primers (A–D) are shown. The position of the precursor coding region is represented by a filled bar. The region encoding the final mature peptide is shown by an open box. The relative position of the PCR fragment used for library screening is shown for comparison. Scale bar represents 100 bp.

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                                                                   5' GGTGATCGGGTCC      13
ACCACACACATACACGTTCACATCCGGGACCGACCCCGCGACGTATCGACAACTTCCCTTCACCGCCACAACTAACAAA      92
                                                                *
ATG AAA CAT TTG TGC ATC GTT CTG ATC GGC GTG CTG ACT GTT CTC CTG GCA TGT GCG CCG     152
MET Lys His Leu Cys Ile Val Leu Ile Gly Val Leu Thr Val Leu Leu Ala Cys Ala Pro      20
                   *                                               *
CGA CGA GCA GCA GCC GTC CCT CCA CCC CAG TGC AGT TCC AAT ATG CTG GAG GAC ATC TCT     212
Arg Arg Ala Ala Ala Val Pro Pro Pro Gln Cys Ser Ser Asn MET Leu Glu Asp Ile Ser      40
                   *
CCA AGA TTT CGC AAA ATA TGC GCA GCA CTC TCT TCA ATC TAC GAT CTA TCA AGT GCG ATG     272
Pro Arg Phe Arg Lys Ile Cys Ala Ala Leu Ser Ser Ile Tyr Asp Leu Ser Ser Ala MET      60

GAA GCT TAT CTA GAA GAC AAA GTG GTT CGG GAG AAC ACG CCA CTG ATG GAC AAC GGT GTG     332
Glu Ala Tyr Leu Glu Asp Lys Val Val Arg Glu Asn Thr Pro Leu MET Asp Asn Gly Val      80

AAG CGA CAG GAT GTG GAC CAC GTA TTT CTC AGG TTC GGT CGG AGA CGT TAA TCACATGGCGA     392
Lys Arg Gln Asp Val Asp His Val Phe Leu Arg Phe Gly Arg Arg Arg ---                  96

TATCATTGTCAAGACTGCAACATGGGACTGAATACATTGAGTGTTTCTGCATCTCTATTCCTTTCCTCCAACACAAAAC     473
TCTACGTCCATCAACATTATCAAATCAACTCCAGGCAACAACCCAAAACGATTTTCACAATAAAATAAATACGCTTTTA     552
TGTTTTGTTATTTCACAGTTTGCAGTGTTTTGTGGAATAGTTACTACGAAGAGAAATTGGAAAAGTATAATAGTCATCC     631
GTCGTCAATTTCACCGACTGACCAGCGTGTATATAAATAGTATATTAAACAAGTGTTTTCAATTAAGTTACTCATAAAT     710
AAATGCCATATGTCATCTTTCAAAAGTAAAATATCAAAACAGATTAAAATAAATTAACATTTATTGTTAATATGACCTA     789
ACATTCTAAGATTCCTAAACTCTATGACACAAATTCCAGTACTAACAGACGAAACACTGTCATCAAAATAAATGCTTCT     868
GTTTCTTCAAATATCTGGGATAGTATTTTCAAATTACCATAAAATATTTCGGCAGGGATTAACATTTAGAAAATAATTG     947
AGAGTATAATTTAATATAAATAACTTTAATTCTCATCAGCGTTTCCTGTGTATGAACGTTGTAAAGAATTTTTAATCAA    1026
CAGGACGAAGGAAAGGCGTGTGCGACGATACTGTTACGAAAACTGGAATGACCTATTTAAAACTTAAATTGACTTTTGA    1105
ATAAAGCAGCATGACTTGATTTCAAAGCAAGTTCTTATACAAGTATTTATTCTTAATTTAAATTCAAAATTATAAATGC    1184
TGAACTGCATAATATACCGTATTATTCATACATACAGCAGCTCTATGGAATAAAGTTGTTTCGCATAATCAATTTTACG    1263
TAATCGCTATTGTATTATCTCAAAATTTATAAACGGAAAAGGCATTTTGTTTGGGCTATTCAACACTGCACACTAGGTT    1342
TTAAAACACGGCCCCATTCTATTACTGAACTCTATGTCGGACAGACTGACAAGTTAAGGTATTGATACAGTTGTGTTGC    1421
TTCGTTGCGAGTGCACCTGCATGTGGATGTGTTAAATTAACAACCAGTGATGACTTCTACTTCGGCTGACTCGACAAAA    1500
TAGATCTTCAACAGTGAATGGGAACATAAATATAGTAATACCTATCAAAACAAAAATAAATAAATAAATATTGAAAAAC    1579
TTTCAAAACAAATAAAAAAAAATCGCTATTTATTAAATAAAATTATCAAATTTTTATATGATTTTCATAAATATTTATTG    1658
TATTTAACGTTCGTAACACTGTTTCAATATACTGTACCTTCATCAGACGAATGTCATACATCTAATTACAATAAATATC    1737
ATCGTCTAATGAAAATACAGTGAACTGAAACAGCTGTTACGAATATTAAATAAAATATACATTTATGGAAATGCTATAA    1816
AACTTTGATAGTTTATTTATTAAATAAATCATTCATTTTCATCAAGAAAAAACAAAAAAAAAAAAAAAAAAAAA 3'     1891
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FIGURE 4. Nucleotide sequence of a *D. punctata* LMS cDNA and deduced amino acid sequence of the peptide precursor. The numbering for each sequence is shown on the right. The sequence is derived from an example cDNA clone representative of the 1.9 kb group. The amino acid sequence of the processed peptide is shown in bold type. Dibasic (and tribasic) amino acids for potential cleavage sites are shaded, and a glycine residue available for amidation is shown in italics. Potential polyadenylation signals near the 3′ end of the sequence are underlined. The two most probable sites for signal sequence cleavage are indicated by stars. This sequence has been deposited in the GenBank data base (accession no. U50341).

peptide would not be expected to be amidated after cleavage due to the absence of a glycine residue. Using these peptide sequences for homology searches of the GenBank and SwissProt databases, only this first sequence produced a significant result, showing weak homology to the FMRFamide gene from the snail *Cepia nemoralis*.

Hybridization analysis of the D. punctata *LMS gene*

The copy number of the Dip-LMS gene was examined by Southern analysis of *D. punctata* genomic DNA. Samples of cockroach genomic DNA were digested with a panel of restriction enzymes, separated by agarose gel electrophoresis, blotted to membrane, and then probed with a radio-labeled fragment from the cDNA sequence. The result, shown in Fig. 5, reveals hybridization by the labeled fragment to a single band in each of the six digested samples. This suggests that the gene encoding LMS is present in the haploid *D. punctata* genome in a single copy.

Gene expression in cockroach brain was studied by Northern blot analysis. Poly(A)$^+$ RNA purified from isolated brain tissue was glyoxylated, separated by agarose gel electrophoresis, and then blotted and probed as above for Southern analysis. Fig. 6 shows the resulting strong

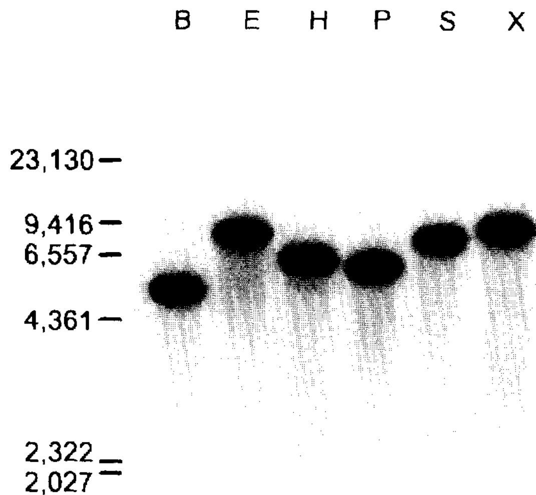

FIGURE 5. Southern hybridization analysis of total genomic DNA (10 µg per lane) using a ^{32}P-labeled fragment of the Dip-LMS gene. Genomic DNA samples were digested with various restriction enzymes (B, *Bgl*II; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sst*I; X, *Xba*I). DNA size markers (bp) are shown on the left.

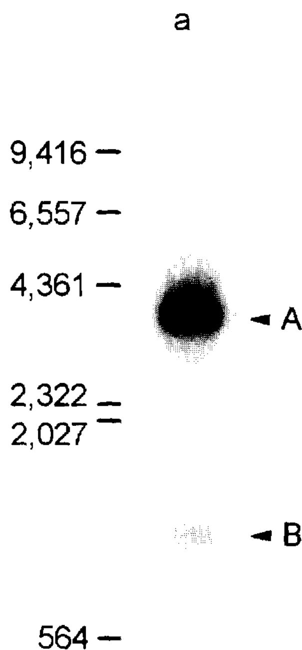

FIGURE 6. Northern hybridization analysis of poly(A)$^+$ RNA extracted from day 2/3 mated female cockroach brains. RNA separated by denaturing electrophoresis was blotted and hybridized with a ^{32}P-labeled fragment of the Dip-LMS gene. (A) Denotes the predominant RNA species of approximately 3.8 kb. (B) Marks a faint band observed at approximately 1.1 kb. Size markers (denatured lambda DNA digested with *Hind*III) are shown on the left.

band (arrow A) of approximately 3.8 kb that was observed after autoradiography. This result indicates the LMS gene is expressed primarily as a 3.8 kb mRNA in *D. punctata* brain. Another much less intense band of approximately 1.1 kb was also observed on longer film exposures (arrow B). Although other FaRP genes which have been characterized utilize alternative RNA splicing as a regulatory tool (Kellett *et al.*, 1994; Rosoff *et al.*, 1992), we have no evidence to suggest this second RNA species is the result of such activity. It could also represent a degradation product of the abundant 3.8 kb species. It is noteworthy, however, that the smaller product was apparent in comparable quantities in two separately extracted lots of brain tissue from different staged groups of animals (data not shown).

Analysis of Dip-LMS mRNA and peptide expression in D. punctata

Expression of the Dip-LMS gene was examined *in situ* using both DNA hybridization and immunological approaches. Production of mRNA was studied using a DIG-labeled fragment of the cloned cDNA as a hybridization probe (Figs 7A and 8A). *In situ* analysis of wholemount preparations of *D. punctata* brains showed hybridization to numerous cells, the majority of which were located in the pars intercerebralis of the protocerebrum. Approximately 15 to 20 positively hybridizing cells were observed here, whereas two lateral cells and only one cell near the optic lobe were also positive (Fig. 8A). A number of cells in the deutocerebrum and tritocerebrum appeared in *in situ* preparations only after prolonged development in the color reaction, and were very faint in appearance (Fig. 8A; clear cells). No hybridization was found in axonal processes or in the CA or CC.

Rabbit polyclonal anti-FMRFamide antibodies were used to detect the presence of FaRPs in similar wholemounts of *D. punctata* brains (Figs 7B and 8B). This antibody crossreacts with LMS due to the structural similarity of the FLRFamide moiety to FMRFamide, although with less avidity (Tsang and Orchard, 1991). It is also general enough to detect the presence of many other FaRPs, some of which were suggested to be present in brains by HPLC analysis. Staining patterns were similar to expression patterns of LMS mRNA, in that the majority of FMRFamide-like immunoreactivity was concentrated in the protocerebrum (Fig. 7B). In the protocerebrum, 30 to 35 cells were found to stain positively in each preparation. Of these cells, most were located in the pars intercerebralis, whereas two lateral cells and three cells near the optic lobes also possessed FMRFamide-like immunoreactivity (Fig. 8B). A few cells in the deutocerebrum that were immunoreactive, were not positive for the mRNA probe. Some cells in the deutocerebra and tritocerebra that appeared to contain Dip-LMS mRNA, but were not immunoreactive to anti-FMRFamide did, however, appear to possess FMRFamide-like immunoreactive processes (compare Fig. 8A and 8B). In fact, staining of many of the cell bodies in the brain was

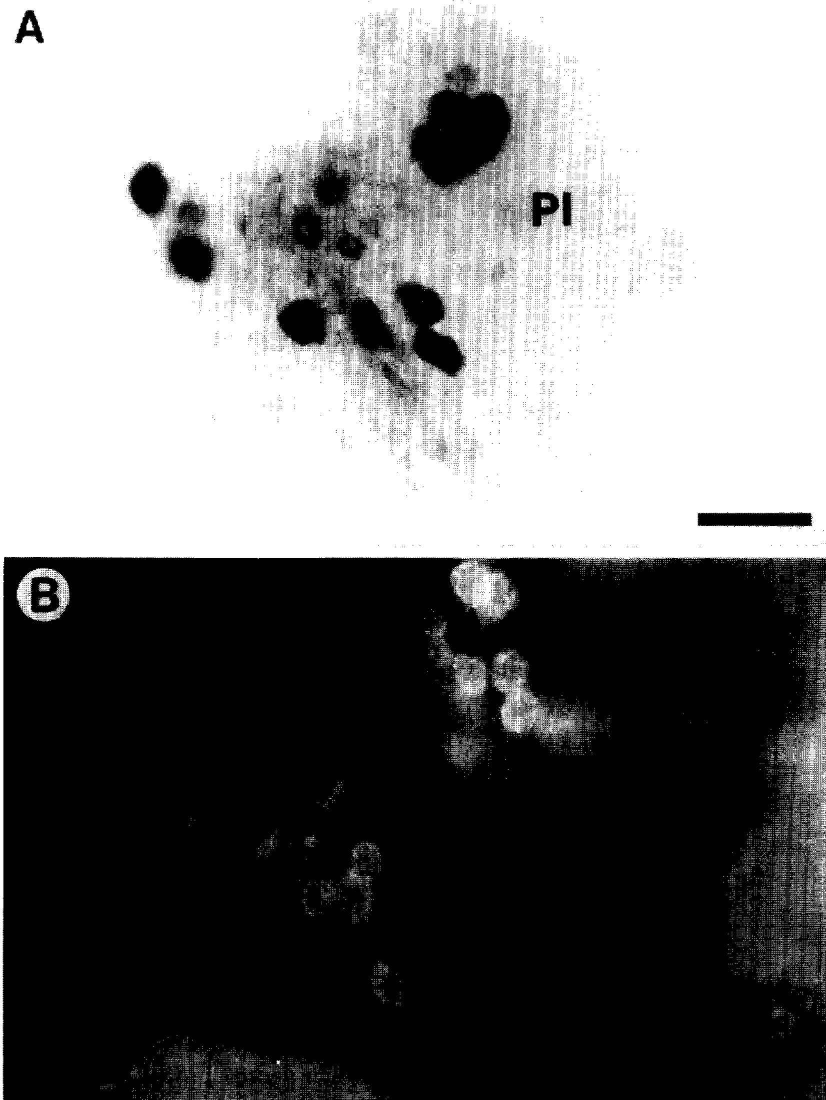

FIGURE 7. *In situ* hybridization (panel A) and immunohistochemistry (panel B) of wholemount preparations of *D. punctata* brain. The pars intercerebralis (PI) of the protocerebrum is depicted (see Fig. 8 for the entire brain and neurohaemal tissues). (A) Positive cell bodies observed in the PI after hybridization with DIG-labeled Dip-LMS cDNA. Nuclei do not show positive hybridization. (B) FMRFamide-like immunoreactivity observed in the same region of the brain. Immunoreactive axons (arrows) are extensive, some of which lead to the corpora cardiaca (not shown). Scale bar equals 60 μm.

accompanied by an elaborate plexus of immunoreactive processes (Fig. 7B; arrows), many of which appeared to run through the nervi corpori cardiaci I (NCCI) and nervi corpori allati (NCA). The immunoreactive axons of the NCCI leading to the corpora cardiaci (CC) often produced large neurohaemal-like blebs, resembling storage and release sites of neurosecretory material.

DISCUSSION

The myosuppressin family of peptides in insects is of particular interest because of the potent inhibitory myotropic activity which these molecules exhibit. Although many FaRP genes have now been characterized, including one encoding sulfakinins (Nichols *et al.*, 1988) and others encoding short FMRFamides (Taghert and Schneider, 1990) from *Drosophila*, no gene encoding a FLRFamide from the myosuppressin group has yet been found. We analyzed the FaRPs present in the brain of the cockroach *D. punctata* by HPLC separation using radioimmunoassay detection of immunoreactive fractions. Crude separation of brain extracts revealed multiple peaks of FMRFamide-like immunoreactive material (Fig. 1A), one of which co-eluted with authentic LMS (Fig. 1B). Further purification resulted in the isolation of a single peak which was demonstrated to have biological activity on the locust oviduct assay similar to that of synthetic LMS.

Having established the presence of a FaRP in the brain of this cockroach which co-elutes with authentic LMS, we made use of a PCR-based approach (using the known amino acid sequence of LMS) to isolate a cDNA encoding the precursor for the corresponding peptide from *D. punctata*. Sixty eight positive clones of varying sizes

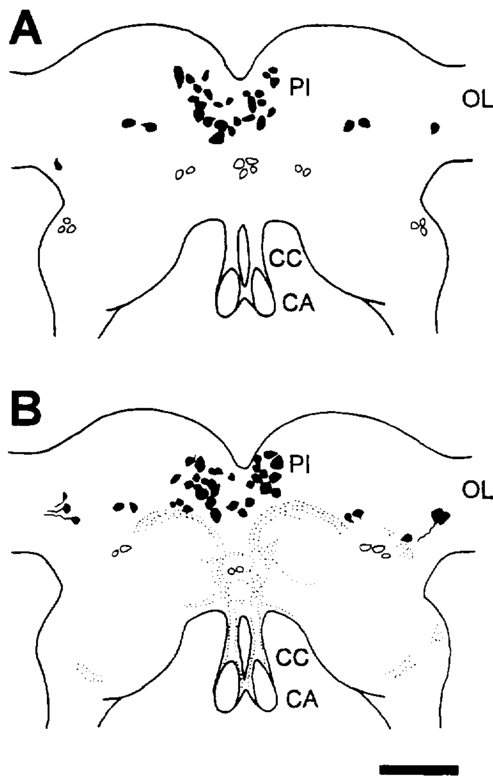

FIGURE 8. Composite camera lucida drawings of cells detected by *in situ* hybridization using a Dip-LMS cDNA (panel A) or immunohistochemistry using anti-FMRFamide antiserum (panel B) in wholemount preparations of *D. punctata* brains. In (A) open cell bodies denote very faint color reaction, only apparent after prolonged color development. In (B) both cell bodies (filled cells) and axons (stippling) immunoreactive to anti-FMRFamide antiserum are shown. Open cell bodies denote weakly reactive cells. Scale bar equals 150 μm. (PI) pars intercerebralis; (OL) optic lobes; (CC) corpora cardiaca; (CA) corpora allata.

were isolated from a primary cDNA library using a PCR-generated fragment of the gene. One of these clones, approximately 1.9 kb in size, was sequenced and found to encode the complete open reading frame for the desired prohormone. However, based on the appearance of a 3.8 kb primary product on Northern analysis of brain mRNA, it is apparent that this clone does not represent the complete transcript of this gene. It is likely that the dominant mRNA transcript is represented by the 3.5 kb group of cDNA clones isolated, and the additional sequence not contained in the characterized clone represents further 3′ untranslated structure (Fig. 3). The functional significance of 3′ untranslated regions of mRNAs remains poorly understood, however the study of some mRNAs indicates that this region can play critical roles in determining mRNA stability and translation efficiency (Jackson and Standart, 1990).

Differences between the various size groups of clones isolated were determined to be the result of variability observed at their 3′ ends. Also, the 3′ RACE-PCR product intitially derived using degenerate oligonucleotide primers, exhibited its own unique 3′ end point as well. Normally, these data might suggest the possibility that alternate polyadenylation signals are being used to terminate the mature RNAs. However, Northern blotting reveals a single dominant 3.8 kb mRNA species in the brain, with only a very low level of another smaller 1.1 kb species. Therefore, it seems more likely that most of these clones (with the exception of the 3.5 kb group), are actually the result of mispriming in A/U rich regions of the mRNA during cDNA synthesis.

The sequence of the characterized cDNA was found to encode a peptide which when fully processed would be identical to LMS. Translation of the cDNA sequence predicts a prohormone of 96 amino acids, which includes within it the extended FLRFamide at its C-terminus. Necessary processing sites for cleavage and amidation into mature peptide are present. Two further potential dibasic cleavage signals are present in the precursor, which could result in additional peptide products. One of these potential products shows weak homology to a peptide predicted from the FaRP gene of a snail, *Cepia nemoralis*. However, the predicted cockroach peptide would not be amidated and it may simply be an evolutionary remnant from an ancestral multi-peptide molecule. Therefore, unlike the multi-peptide FaRP precursors which have been deduced in other organisms, the cockroach FLRFamide gene appears to encode a precursor containing only a single FLRFamide, LMS.

Expression of the Dip-LMS gene in *D. punctata* brain was studied *in situ* at two levels (Figs 7 and 8). *In situ* hybridization with a cDNA probe showed that the gene is transcribed in many cells in the brain, with the majority located in the pars intercerebralis of the protocerebrum, a region containing an abundance of neurosecretory cells (Figs 7A and 8A). Immunohistochemical analysis using a general anti-FMRFamide antibody revealed the presence of FaRPs in many cell bodies and processes in the brain (Figs 7B and 8B), again located predominantly in the pars intercerebralis. However, this antibody was able to reveal the presence of other FaRPs as well, whose presence had been indicated previously by HPLC analysis. The apparent similarity of many of the cells detected by both methods, and the identification of a FaRP co-eluting with LMS by HPLC purification of brain extracts, suggests that this peptide represents a significant proportion of FaRP expression in the cockroach brain. Meola *et al.* (1991a,b) have shown a similar abundance of LMS-like immunoreactivity in the pars intercerebralis of the cockroach *L. maderae* and the stable fly *Stomoxys calcitrans*.

FMRFamide-related peptides have also been observed in the brain of the oviparous cockroach *Periplaneta americana* (Verhaert *et al.*, 1985) as well as a multitude of other insects including locusts (Myers and Evans, 1987; Remy *et al.*, 1988; Peeff *et al.*, 1994), moths (Carroll *et al.*, 1986), beetles (Veenstra and Schooneveld, 1984) and fruitflies (Schneider *et al.*, 1993). Within the protocerebra, the majority of immunoreactive cells were concentrated in the pars intercerebralis in all cases. Our studies indicate this is true for *D. punctata* as well.

Weak hybridization by the cDNA probe, to a number of cells in the deutocerebrum and tritocerebrum which

were not seen in the immunohistochemical preparations (Fig. 8A and B), suggests that in some cells, either LMS is not processed immediately, is processed and then transported rapidly to other areas, or is only processed in limited amounts. The appearance of immunoreactive processes in these same regions of the brain (Fig. 8B) favors the possibility that LMS is produced in the cell bodies and then transported rapidly through axonal processes (or is produced at low levels which then accumulate in the processes).

The FMRFamide-like immunoreactivity observed in the NCCI and NCA (Fig. 8B) was often present in large neurohaemal-like blebs that resemble storage and release sites of neurosecretory material. A neurohormonal role for these FaRPs is thus implied in *D. punctata*, as has been suggested for FaRPs in various other insects (Verhaert *et al.*, 1985; Carroll *et al.*, 1986; Schoofs *et al.*, 1993b). The pattern is similar to that observed by Meola *et al.* (1991a) who used an anti-LMS antibody to study sections of brain from the cockroach *L. maderae*.

As mentioned previously, because the antibody used in this study was directed against FMRFamide, immunohistochemical detection in these brains was able to reveal the presence of multiple FaRPs. The appearance of some immunopositive cells which were not detected by the cDNA probe indicates that other FaRPs are expressed in addition to LMS. This is supported by the appearance of multiple immunoreactive fractions after initial HPLC separation of brain extract using a C_{18} column. Analysis of the complete complement of FaRPs produced in the brain of this cockroach awaits the isolation and sequencing of each of the peptides present, and this work is currently under way. Since LMS has been shown here to be expressed from a precursor independent of other such FaRPs, any coordination which exists between the expression of the different members of this peptide family must occur through less direct mechanisms. Cloning of the genes involved provides the necessary groundwork to address such questions.

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