EXPRESSION OF MRNAS ENCODING FMRFAMIDE-RELATED PEPTIDES IN ADULT AND EMBRYO 'HELIX ASPERSA'

Murdo Macdonald

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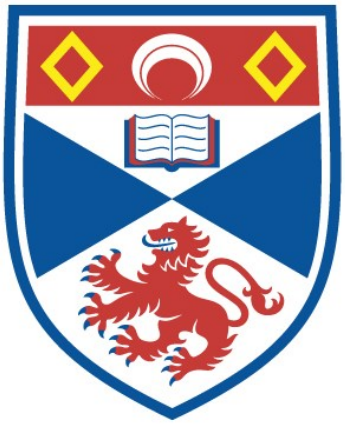

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EXPRESSION OF mRNAs ENCODING FMRFamide - RELATED PEPTIDES IN ADULT AND EMBRYO *Helix aspersa*

BY

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DECLARATION

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 on July 1st, 1988, and as a candidate for the degree of Ph. D. on July 1st, 1988.

30/9/92

DECLARATION

I, Murdo Macdonald, hereby certify that this thesis has been composed by myself, that it is a record of my own work, except where expressly stated otherwise, and that it has not been accepted in partial or complete fulfillment of any other degree or professional qualification.

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Dedication

To my parents , for all they have taught me .

" Be careful that you do not forget the LORD your God , failing to observe his commands , his laws and his decrees .....But remember the LORD your God , who gives you the ability to produce wealth , and so confirms his covenant , which he swore to your forefathers . "

Deuteronomy 8 v. 11 , 18 .

ABSTRACT

The gastropod mollusc *Helix aspersa* is known to contain at least seven FMRFamide - related peptides (FaRPs), neuropeptides which fall into two broad classes, distinguished by their primary structure and their physiological actions . We have sought to use the techniques available to us through molecular biology to study the structure and expression of the nucleic acids (RNA and DNA) which encode these peptides in this organism . The two classes of peptide, tetra- and heptapeptides, were found by us to be apparently separated by the stage of mRNA generation : the precursor polypeptides encoded by these mRNAs were also found to have differing structures .

Expression of mRNAs specific for the FaRPs were studied during embryogenesis , where there appears to be regulated expression of these mRNAs . *In situ* hybridization analyses of the central nervous system of adult *Helix* revealed expression of FaRP-specific mRNAs to be limited to a small number of discrete neurons: it was again observed that there was an apparent distinction between the tetra and extended FaRPs , no cells being identified in our studies to express both types of mRNA. Confocal scanning microscopy indicated that the distribution of the mRNA , which appeared to be limited to the cytoplasm of cell bodies expressing the peptides , was non-uniform , probably reflecting a functional characteristic of the cells concerned.

ABBREVIATIONS

5HT	5 Hydroxy Tryptamine
^{o}C	Degrees centigrade
λ	lambda
%	per cent
Amp	Ampicillin
Arg	Arginine
AVP	Avian pancreatic polypeptide
BSA	Bovine Serum Albumin
cm	centimetre
CTAB	Cetyl trimethyl ammonium bromide
DMF	Dimethyl formamide
DNA	Deoxy ribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
F	Phenylalanine
FaRP(s)	FMRFamide - related peptide(s)
FLRFa	Phenylanalyl Leucyl Arginyl Phenylanalyl amide
FMRFamide	Phenylanalyl Methionyl Arginyl Phenylanalyl amide
(μ/n/p) g	(micro / nano / pico) gram
HEPES	N - [2 - Hydroxyethyl] piperazine - N'[2 - sulphonic acid]
HPLC	High pressure liquid chromatography
HPRI	Human Placental Ribonuclease Inhibitor
IPTG	Isopropyl-B-D-thiogalactoside
l	Litre(s)
M	Methionine
Met	Methionine
MOPS	3 [N Morpholino] propanesulphonic acid polynucleotide
kinase	
(m/μ)l	(milli / micro) litre(s)
nm	nanometres
NPY	Neuropeptide Y
OD	Optical density
PCR	Polymerase chain reaction
Phe	Phenylalanine
PVP	Polyvinyl pyrolidone
R	Arginine
RIA	Radio immunoassay
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
SSC	Standard sodium citrate
uv	Ultra violet
X - gal	5-Bromo-4-Chloro-3-Indoyl-B-D-Phosphate

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CHAPTER 1

INTRODUCTION

1.1 : General Introduction

The means by which cells within the nervous system of an organism communicate with each other and with their target organs has been the subject of much intensive study . Such studies have uncovered the existence of a large number of neuroactive molecules , which act as neurotransmitters , neurohormones or neuromodulators (although the divisions between these classes has become somewhat blurred as the list of molecules considered to be neuroactive has grown). These include the amino acid transmitters , such as glutamate and aspartate , and the monoamines , a class of transmitter which includes serotonin (5 hydroxy tryptamine , or 5HT) and dopamine . Peptide transmitters , such as neuropeptide Y , proctolin and substance P , have also been found to comprise an important class of neuroactive molecule . The elucidation of a large and diverse family of neuropeptide transmitters was initiated by the discovery by Price and Greenberg of the amidated tetrapeptide , Phe - Met - Arg - Phe - NH_2 (Price and Greenberg 1977 a , b). This peptide , often referred to as FMRFamide , using the single letter abbreviations for the amino acids comprising it , was initially identified as the cardioactive peak C in ganglion extracts from the sunray clam *Macrocallista caliphora* . It was subsequently found to be one member of an extended family of related peptides , the FMRFamide - like peptides . However , significant variability in the primary structures of peptides considered to belong to this family has been found : many are N - terminally extended with respect to FMRFamide , and the majority of the peptides isolated to date also contain the sequence -FLRFamide , a leucyl

residue replacing the methionine at the third position from the carboxy terminus . These facts have led Price and Greenberg (Price and Greenberg 1989) to coin the term FaRP (from **F**MRF**a**mide - **R**elated **P**eptide) , reflecting the finding that FMRFamide , although first - isolated , may to some extent be an atypical member of the group of peptides which are defined as being related .

The relative simplicity of the nervous system of the snail *Helix aspersa* , and its maleable behavioural responses , in addition to a plentiful supply of animals and their ease of maintainance in the laboratory , mean that this organism is an excellent experimental model for studies of the nervous system, and of how neuronal and muscular actions co - ordinate to produce behaviour : it has thus been extensively used . The FaRPs have been closely studied in this species : there are at least seven FMRFamide related peptides found in this organism, which fall into two broad classes , distinguished by both their primary structure (tetra - and heptapeptides) and their actions : there is also evidence to suggest that they are differentially distributed within the organism (Lehman and Price 1987b) . The actions of the FaRPs in *Helix* have also been studied at the pharmacological and physiological levels , as have their anatomical distribution : however , little is as yet known concerning the molecular biology of these peptides in *Helix* - how these peptides are synthesised , at what levels this is regulated, and how these peptides are encoded in the genome .

The aims of this study have been to investigate the molecular biology of the FaRPs in the snail *Helix aspersa* : to ascertain the means by which the FMRFamide - related peptides are encoded and synthesised , and to determine the extent of expression of these peptides , and of the

messenger RNAs encoding them , during embryogenesis in this organism .

1.2 : FMRFamide related peptides

Following their detection of a cardioexcitatory fraction in extracts from ganglia of the sunray clam *Macrocallista caliphora*, Price and Greenberg sought to purify and identify the active molecule(s) : this they found to have the primary structure Phe - Met - Arg - Phe - amide (Price and Greenberg 1977) . Using this information it has been possible to raise antibodies specific to this sequence , and thus to partially circumvent the need for suitable bioassays which had previously presented significant problems in the identification and investigation of bioactive substances . In addition , since antibodies often show inherent cross - reactivity , the detection of related but differing molecules is facilitated , a fact which can be exploited in inter - specific studies of related peptides .

An additional advantage of antibody - based detection systems over those dependent on bioactivity is the the ability to study the location of the substances of interest within tissues , using antibodies in conjunction with appropriate tissue sections . This is particularly relevant in investigations of the nervous system , where , although the concentration of important transmitter molecules may be significant at specific sites (e.g. synapses) , these substances rarely constitute a significant fraction of the material found in any area of the nervous system , and may therefore be difficult to detect using a bioassay . (Vertebrate exceptions to this include acetylcholine in neuromuscular synapses and GABA in Purkinje cells (Batini *et al* , 1992 ; Astrow *et al* , 1992)) .

Antibodies raised against the -RFamide moiety of FMRFamide have been shown to bind substances in a variety of tissues from a wide range of organisms , including anthazoans (Carlberg *et al* , 1989 : Weber 1989) , arthropods (Callaway *et al*, 1987 ; Mercier *et al* , 1991) , molluscs (Elekes and Nassel 1990 ; Griffond and Mounzih 1990 ; Takayanagi and Takeda , 1987 , 1988a and 1987b) , insects (Copenhaver and Taghert 1989 ; Homberg *et al* 1990 ; Lundquist and Nassel 1990 ; Ohlson *et al* 1989 ; Schurmann and Erber 1990) and vertebrates (Chen *et al* 1989 ; Dockray *et al* 1983 ; Dockray 1985 ; Kivipelto *et al* 1989 ; Kivipelto and Panula 1991 ; Majane *et al* 1988 , 1989; Ostholm *et al* 1990 ; Wirsig - Weichmann 1990) . Although it is likely that some of the substances detected using these antibodies are unrelated to FMRFamide (a consequence of the previously mentioned antibody promiscuity), a number of peptides have been isolated as a result of these studies which bear structural relation to the FaRPs . These include peptides from sea anemones (Darmer *et al* 1991 ; Graff and Grimmelikhuijzen , 1988 a , b ; Grimmelikhuijzen and Graff 1986 ; Grimmelikhuijzen *et al* 1990) , molluscs (Fujimoto *et al* 1990 ; Lehman *et al* , 1984 ; Price 1982 ; Price *et al* , 1985, 1987 , 1990 ; Mat Jais *et al* , 1990) , arthropods (Marder *et al* , 1987) , insects (Matsumoto *et al*, 1989 ; Robb *et al* , 1989 ; Holman *et al* , 1986; Nachman *et al* , 1986a , b) , and vertebrates (Dockray *et al* , 1981 , 1983 ; Yang *et al* , 1985). It was initially thought that authentic FMRFamide was limited in its distribution to molluscan species , where it and its tetrapeptide homologue FLRFamide are widespread , but FMRFamide has now been isolated from both the polychaete *Nereis* (Krajniak *et al* , 1990 ; Baratte *et al* , 1991) and the leech *Hirudo* (Li and Calabrese 1987 ; Evans and Myers 1991).

Some of the FaRPs isolated and characterised to date are listed in Table 1 : many of these peptides prove to be similar to FMRFamide not only in their primary structure but also in their physiological actions , a fact which tends to confirm their putative relatedness. A number of peptides have been isolated as a result of their C - terminal structure being identical to that of the originally identified peptide , FMRFamide , and therefore reacting with antibodies raised against this peptide. In addition to FMRFamide , several other FaRPs have been isolated from *Helix* , including another tetrapeptide analogue , FLRFamide , and a number of N - terminally extended peptides . Some other molluscs are found to contain peptides in common with *Helix* (e.g. FMRFamide and FLRFamide in *Lymnaea* , and NDPFLRFamide in *Siphonaria*) , as well as some which are apparently not present in *Helix* (e.g. SGQSWRPQGRFamide in *Achatina*) .

While retention of the -RFamide structure is important in their detection using antibodies , a few peptides have been found by this means which deviate from the normal pattern . For example, three of the peptides isolated from anthazoan tissues have residues other than phenylalanine in their most C - terminal position , specifically tryptophan and asparagine (Graff and Grimmelikhuijzen , 1988a ,b ; Grimmelikhuijzen and Graff 1986 ; Grimmelikhuijzen *et al* , 1990) . These peptides were initially noted as comprising HPLC fractions which reacted weakly with anti - FMRFamide antibodies ; the constituent peptides were isolated from reactive fractions and the peptide sequences derived . Other peptides have been isolated independently of FMRFamide and the relatedness of their structure subsequently noted: the peptides leukomyosupressin and leukosulphakinin are examples which fall into this category (Nachman *et al* , 1986 a , b ; Holman *et al* , 1986) . (The fact that the tyrosine residues in the leukosulphakinins

TABLE 1 : Some FaRPs isolated to date

Organism	Peptide	Observed Effects	Refs.
Molluscs:			
Macrocallista	FMRFamide	Cardioexcitation	1
Helix	FMRFamide	Cardioexcitation	2
"	pQDPFLRFamide	Neuronal Effects	3
"	SDPFLRFamide	"	4
"	NDPFLRFamide	"	4
"	SEPYLRFamide	"	4
Achatina	SGQSWRPQGRFamide		5
Siphonaria	NDPFLRFamide		6
Insects :			
Schistocerca	PDVDHVFLRFamide	Cardio / muscle modulator	7
Leucophaea	EDVDHVFLRFamide		8
"	pQDVDHVFLRFamide		9
Mosquito	pQRPLPSLKTRFamide	N / P	10
Drosophila	DPKQDFMRFamide		11
Manduca	pEDVVHSFLRFamide		12
Other Invertebrates :			
Anthozoans	pQLLGGRFamide	Muscular contractions	13
"	pQSLRWamide	" "	14
"	pQGLRWamide	" "	15
"	EGRFamide	" "	16
"	FLRNamide	" "	17
Nereis	FMRFamide		18
Ascaris	KNEFIRFamide		19
Hirudo	FMRFamide	Muscular contractions	20
	FLRFamide		20
	YLRFamide		20
	GGKYMRFamide		20
Octopus	AFLRFamide		21
"	TFLRFamide		21
Homorus	TNRNFLRFamide		22
"	SDRNFLRFamide		22
Callinectes	GYNRSFLRFamide		23
Vertebrates :			
Bovine	FLFQPRFamide	Neural / cardiac actions	24
"	AGEGASSPFWSLAAPQRFamide	" " "	24
Chicken	LPLRFamide	" " "	25

References : (1)Price and Greenberg ,1977a,b ;(2) Price 1982; (3) Price et al , 1985 ; (4) Price et al ,1990 ; (5) Fujimoto et al , 1990 ;(6) Price et al 1987 ; (7) Robb et al ,1989 ; (8) Nachman et al , 1986a; (9) Nachman et al 1986b ; (10) Matsumoto et al 1989; (11) Nambu et al ,1988 ; (12) Kingan et al 1990 ; (14) Graff and Grimmelikhuijzen ,1988a ;(15) Graff and Grimmelikhuijzen ,1988b ; (16) Grimmelikhuijzen and Graff 1986 ; (17) Grimmelikhuijzen et al 1990 ; (18) Krajniak and Price 1990 ; (19) Cowden et al 1990 ; (20) Evans et al 1991; (21) Martin and Voight 1987 ;(22) Trimmer et al 1987 ; (23) Krajniak 1991 ; (24) Yang et al 1985 ; (25) Dockray et al 1983 .

are sulphated probably means that the secondary structure of these peptides differs significantly from the FaRPs .) It is interesting to note the corollary of this latter observation in the case of the FMRFamide - related peptide from locust , so-called " Schisto - FLRFamide " , which was isolated on the basis if its structural similarity to FMRFamide , but which proves to differ from leukomyosupressin only in the N - terminal substitution of proline for glutamic acid (Robb *et al* , 1989).

In vertebrates , only LPLRFamide from chicken brain (Dockray *et al* ,1983) and the bovine FaRPs A-18-Famide and F-8-Famide (Yang *et al* , 1985) have been isolated and identified . However , evidence from HPLC / RIA (radio immunoassay) studies by Majane *et al.* (1988) suggests that peptides bearing structural similarity to A-18-Famide and F-8-Famide are present in the central nervous systems of some other vertebrates . Two RIA peaks in extracts from rats appear to have similar mobilities to their mouse equivalents on HPLC , suggesting significant sequence homology between FaRPs from these species ; their retention times differ from authentic F-8-Famide and A-18-Famide , however. No significant peak equivalent to A-18-Famide is seen in guinea pig extracts analysed in this way , while the HPLC / RIA profile of human spinal cord extracts is more complex : two major peaks have elution times similar to authentic F-8-Famide and A-18-Famide , but a third peak between these is also apparent . This intermediate peak may be a degradation product of the A-18-Famide - equivalent in human spinal material , extraction of such material being necessarily more complex than that from laboratory animals (Majane *et al* , 1988).

Actions of FaRPs

1.3 : Cardiac effects

Although FMRFamide was originally isolated as a cardioexcitatory substance (Price and Greenberg, 1977a , b) , it has subsequently been found that this and other related peptides have effects on a variety of tissues in a number of diverse species . The cardioexcitation observed on application of the endogenous heptapeptide FaRPs , SDPFLRFamide and NDPFLRFamide , to *Helix* heart is approximately 100 fold greater than that seen with the tetrapeptides FMRFamide and FLRFamide (Price *et al* ,1990). This may not be the case in *Lymnaea* , however : there are conflicting reports regarding the relative potencies of the tetra - and heptapeptide FaRPs in *Lymnaea* heart . While one group has found them to be equipotent (Ebberink *et al* , 1987) , another set of data indicates that FMRFamide is approximately 100 times more potent than either SDPFLRFamide or GDPFLRFamide (Buckett *et al* , 1990), this latter result being a reversal of the situation observed in *Helix* . FMRFamide itself has been found to be mildly cardioinhibitory in the slug *Limax maximus* (Welsford and Prior 1991) , while studies by Robb *et al.* (1989) indicate that the locust analogue schisto - FLRFamide is strongly cardioinhibitory on the semi - isolated locust heart . A variety of FaRPs were found by Raffa (1988) to increase both heartbeat rate and mean arterial blood pressure in the rat .

It has also been observed that FaRPs are able to potentiate the osmoregulatory release of amino acids from the ventricle of the clam *Mercenaria* by up to 100 % ; this effect is found not to be *via* cAMP , although it may involve protein kinase C (Deaton 1990) .

1.4 : Muscular effects

The effects of FMRFamide - related peptides on a number of other muscle systems have also been studied . Application of FMRFamide to *Helix* tentacle retractor muscle causes it to contract , while pQDPFLRFamide relaxes it (Cottrell *et al* , 1983b ; Lehman and Greenberg , 1987a) . How FMRFamide performs this function *in vivo* is not clear : the large identified neuron C3 innervates the tentacle retractor muscle and contains FMRFamide (Cottrell *et al* , 1983a) . However , experiments by Bewick *et al* (1990) indicate that contraction of this muscle induced by C3 stimulation is due largely to acetyl choline , co - localised in C3 with FMRFamide , and not primarily to FMRFamide itself.

Also in *Helix* , muscles of the male reproductive tract are contracted on application of FaRPs , and rhythmic contractions of the isolated crop (Lehman and Greenberg , 1987a) and oesophagus are inhibited (Lloyd *et al* , 1987) . Rhythmic contractions of other molluscan muscle systems tested , excluding the heart , tend to be inhibited by FaRPs - this is true of the *Helisoma* salivary gland (Bulloch *et al* , 1988) , and of the *Limax* crop , for example (Krajniak 89) . Other observations in molluscan species include the induction of muscular contractions in *Limax* penis (Krajniak *et al* , 1989) , isolated *Aplysia* gill muscle fibres (Cawthorpe and Lukowiak , 1990) and *Busycon* radula protractor muscle. (The last of these has been extensively used as a specific bioassay for the presence of FMRFamide (Nagle and Greenberg , 1982)) . It has also been observed that the gill withdrawal reflex of *Aplysia* is potentiated , and its habituation (reduction in extent of gill withdrawal on repeated stimulus) is prevented by the presence of FMRFamide (Higgins *et al* , 1989) .

There is experimental evidence to indicate that FaRPs are able to modulate responses of certain molluscan muscles to nervous stimuli : for example , neurally induced contractions of the *Rapana* radula protractor muscle are enhanced (Kobayashi and Muneoka , 1989) , and acetylcholine - induced contractions of the *Mytilus* ABRM (anterior byssus retractor muscle) are potentiated , possibly by mobilization of a Ca^{2+} pool normally inaccessible to ACh (Raffa and Bianchi , 1986) . (Note that the " catch " contracture induced by acetylcholine in this same muscle preparation is relaxed by FMRFamide , possibly *via* serotonin (Kobayashi and Muneoka , 1989)) .

Studies using insect preparations have indicated that FaRPs also have effects on these muscle systems : contraction of locust foregut is induced by such peptides (Banner and Osborne , 1989) , and the actions of serotonin and proctolin on locust foregut are modulated by FaRPs (Wood *et al* , 1990 ; Banner and Osborne , 1989). Inhibition of neurally induced contractions in locust oviduct muscle (Lange *et al* , 1991) , and enhancement of neurally induced contractions of locust tibea extensor muscle are observed (Evans and Myers , 1986) . Analagous effects of FaRPs are observed in other invertebrates: *Palaemon* (shrimp) pyloric dilator muscle (Meyrand and Marder , 1991) and anthozoan slow muscle (Macfarlane *et al* , 1991) are contracted by FMRFamide , while various FaRPs are observed to inhibit neurally induced contractions of leech longitudinal muscle (Norris and Calabrese , 1990).

1.5 : Nervous tissue

According to data obtained from studies using FaRP - specific antibodies , the major (if not exclusive) site of synthesis of such peptides is in neuronal tissue . It is therefore not surprising to find that FMRFamide - related peptides evoke responses from nervous preparations. In some cases application of FaRPs cause excitation of a neuron , while in others it causes inhibition; neuronal responses to extended peptides in *Helix* are often observed to be inhibitory (Boyd and Walker , 1985) . (In at least one situation , the sign of the response is dependent on the membrane potential (Cottrell *et al* , 1984)) . Modulation of spike duration in cerebral neurons (Rosen *et al* , 1989) and of EPSPs (excitatory post - synaptic potentials) in sensory neurons (Small *et al* , 1989) of *Aplysia* have been observed; the mechanosensory threshold of the same organism is also found to be modulated by FMRFamide (Billy and Walters , 1989). Effects on other neuroactive molecules are observed : acetylcholine release from *Helisoma* B5 cells is inhibited by FMRFamide (Man - Son - Hing *et al* , 1989) , while its release at an *Aplysia* synapse is potentiated by FLRFamide (Fossier *et al* , 1990) .

Many of these effects involve the modulation of ion channels: for instance , increases in K^+ current are observed (Belardetti *et al* , 1987 ; Brussard *et al* , 1988 ; Cottrell , 1982; S. - Rozsa and Dynakonova , 1987 ; Boyd and Walker , 1985) . This can be compared with somatostatin , which also stimulates Ca^{2+} - activated K^+ channels, probably *via* protein dephosphorylation (White *et al* , 1990) . Reversal by FaRPs of serotonin - induced phosphorylation is observed in *Aplysia* sensory neurons (Sweatt *et al* , 1989) and modulation of Na^+ (Boyd and Walker , 1985), Ca^{2+} (Kramer *et al* , 1988 ; Yakel , 1991 ; Colombaioni *et al* , 1985) and

Cl^- currents (Thompson and Ruben , 1988) have been observed . Differences in the neuronal ionic currents affected by the tetra - and heptapeptides are also noted - e.g. while the tetrapeptides cause an increase in the Na^+ current , the heptapeptides have no effect on this current ; the opposite is true for the fast K^+ current (Cottrell *et al*, 1987) . It is also found that FMRFamide is able to activate a ligand gated ion channel - the first demonstration of such an action by a neuropeptide (Cottrell and Davis , 1990) . Intracellular responses to binding of the FaRPs involve cyclic AMP in some cases , but not in others (Greenberg *et al* , 1983 ; S. - Rosza and Dynakonova , 1987 ; Lange *et al* , 1991). In addition , Fossier *et al.* (1990) found that protein kinase C is involved in the increase in acetylcholine release at an *Aplysia* synapse induced by FLRFamide , while the mechanism by which FMRFamide inhibits protein synthesis in *Helix* dorsal body cells is as yet unclear (Griffond and Mounzih , 1989) .

The finding that the FaRPs are so widespread as to include peptides present in vertebrates has meant that their effects on higher organisms have also been studied . Experiments have largely been performed on rodents : these indicate that both FMRFamide itself and the related vertebrate peptides F-8-Famide and A-18-Famide are capable of modulating responses of these animals to opioids (Kavaliers 1987 , 1990) , and that IgG against these peptides potentiates the effects of opioids (Kavaliers and Yang , 1989). Endocrine pancreas secretions in the rat and human lymphocyte proliferation , both known to be responsive to opioid peptides , can be modulated by the bovine FaRPs F-8-Famide A-18-Famide (Fehmann *et al* , 1990 ; Lecron *et al* , 1992). Morphine withdrawal symptoms can be induced on injection of F-8-Famide into rats (Malin *et*

al , 1990a), and morphine dependent individuals are found to have , on average , a 100% increase in F-8-Famide - immunoreactive material present in their cerebrospinal fluid (Malin *et al* , 1990a , b). Raffa (1988) found that both mean arterial blood pressure and heartbeat rate are increased by a variety of FaRPs , while respiration rate is reduced (Raffa and Jacoby , 1989) . The rate of colonic bead expulsion (and presumably the rate of peristaltic contraction of the gut) is reduced by the injection of FaRPs (Raffa and Jacoby , 1989) , and injection of FMRFamide increases feeding rate in rats (Robert *et al* , 1989) - these latter two effects demonstrating the ability of FMRFamide - related peptides to display opioid agonist as well as antagonist properties . However, although FaRPs are seen to modulate the effects of the endogenous opioids , evidence from experiments by both Zhu and Raffa (1986) and Allard *et al* (1989) suggests they do not do so by directly competing for binding of specific opioid receptors - they appear rather to do so *via* serotonin (Raffa , 1988) .

The large numbers of FaRP - containing neurons apparent in areas of the central nervous system concerned with the control of nociception , the periaqueductal gray and the dorsal horn of the spinal cord (Kivipelto *et al* , 1989 ; Majane *et al* , 1989) , tends to confirm their supposed role in modulating nociception *in vivo* : recently elucidated neuroanatomical evidence from the rat also indicates that mammalian FaRPs participate in the regulation of blood pressure , feeding behaviour and endocrine functions (Kivipelto and Panula , 1991) . Various effects on mammalian nervous tissue are elicited by FMRFamide - for example , excitation of brain medullary neurons in the rat (Gayton 1982) and production of depolarization and hyperpolarization of cultured mouse spinal cord neurons (McCarthy and Cottrell , 1984) have been observed ; F - 8 -

Famide has also been shown to have similar effects on cultured mouse spinal cord neurons (Guzman *et al* , 1989) . Many of these findings (e.g. increase in heartbeat rate , inhibition of rhythmic muscle contractions , modulation of nervous tissue) are in line with the equivalent data from invertebrates , perhaps indicating a similar rolê for these peptides in diverse organisms . The increased feeding rate observed in rats following administration of FMRFamide (Robert *et al* , 1989) is somewhat anomalous , however , since FMRFamide is known to inhibit feeding in both *Aplysia* (Sossin *et al* , 1987) and *Helisoma* (Murphy *et al* , 1985) .

1.6 : FaRPs and behaviour

The apparent involvement of FaRPs in cardiac , muscular and nervous responses means that they are implicated in the production of modes of behaviour. The tissues found to be affected by FaRPs in invertebrates include the heart (Price *et al*, 1990) , reproducive organs (Lehman and Greenberg 1987a) , and other muscular systems such as tentacle retractor and gill and siphon withdrawal muscles (Small *et al* , 1992) . Exactly how these peptides integrate with each other and with other molecules involved in controlling behaviour is only beginning to be understood ; however , it appears in *Helix* that the tetrapeptide FaRPs may be involved in fast responses (such as tentacle withdrawal) and may act over relatively short distances, while the actions of the heptapeptides in *Helix* may be in slower responses (e.g. relaxation of muscles to allow emergence from the shell) and may be over longer distances . Thus the FaRPs may be involved in producing (or helping to produce) a variety of behavioural responses , in an organism . The inhibition by FaRPs of feeding in *Aplysia* and *Helisoma* (Sossin *et al* , 1987 ; Murphy *et al* , 1985) , and that implied in experiments in *Helix* (Lehman and Greenberg , 1987 ;

Lloyd *et al* , 1987) , coupled to their cardioexcitatory properties and their effects on muscles of the male reproductive tract suggest that among other things these peptides may be involved in the control of reproductive activity in invertebrates.

Among the best studied of invertebrate behaviour systems is that of the *Aplysia* gill withdrawal reflex : on tactile stimulation the *Aplysia* gill is withdrawn by the animal - the extent of this withdrawal is reduced on repeated stimulation (a phenomenon termed habituation) . This reflex action is found to be affected by perfusion of the gill with FMRFamide: the extent of gill withdrawal is potentiated , while its habituation is prevented (Higgins *et al* , 1989). Experimental evidence suggests that this action is not due to an effect of FMRFamide on the *Aplysia* CNS, but rather is *via* the peripheral nervous system or by a direct effect of the peptide on the gill muscle (Higgins *et al* , 1989 ; Cawthorpe and Lukowiak , 1990) .

The involvement of FaRPs in behaviour in vertebrates is apparently more complex : not only do behavioural patterns tend to be more complex in these organisms than in invertebrates , but there appear to be more interacting pathways involved in controlling behaviours in vertebrates . There is experimental evidence which indicates that FaRPs are involved in modulating the actions of the opioid peptides in vertebrates : in some situations they are antagonistic (Raffa, 1988) , while in others they act as agonists (Raffa and Jacoby , 1989) . The opioid peptides themselves appear to be involved in the modulation of pain thresholds in these organisms . Thus a number of neuroactive molecules , including FaRPs , appear to be interacting to control physiological systems, such as rates of heartbeat and colonic contraction .

1.7 : FaRP receptors

The mechanisms by which these peptides exert their effect have been extensively studied . In *Helix* , where a variety of FMRFamide related peptides are found , there are reckoned to be two (Cottrell *et al* , 1987 ; Payza , 1987 ; Payza *et al* , 1989), three (Greenberg *et al* , 1983) or perhaps as many as five (Cottrell , 1989) distinct types of specific receptor on which the FaRPs act directly , whereas Allard *et al.* (1989) found there to be evidence for a homogeneous population of receptors for FaRPs in the rat spinal cord . It should be noted , however , that the findings of this latter group were based on observations concerning the binding of F-8-Famide , a FaRP isolated from bovine brain (Yang *et al* , 1985) , to membranes from the rat . The FMRFamide - related peptides from the rat have not to date been isolated or sequenced : evidence from HPLC studies suggests that , although closely related to the bovine FaRPs, they differ slightly in structure (Majane *et al* , 1988) . The binding of the endogenous rat peptides to their receptors may therefore differ from that of the bovine peptides, and the observations made by Allard *et al.* (1989) may be affected by this fact . In most cases , however , it is thought that FaRPs bind to specific cell - surface receptors , as opposed to exerting their effect *via* general acceptor sites (Allard *et al* , 1989) , or interacting with receptors for other peptides or proteins . Binding studies have shown that some interaction with the receptor for the related peptide avian pancreatic polypeptide (APP) takes place at a low level (Ganeshan *et al* , 1987) , although such interactions are probably fortuitous and of little biological significance . However , a productive interaction between FaRPs and receptors for another transmitter appears to occur in the locust, where modulation of a serotonergic response by FaRPs is due at least in part

to postsynaptic interaction with receptors for this neurotransmitter (Wood *et al* , 1990) .

Cross desensitization of FMRFamide and FLRFamide in *Helix* suggests that these two endogenous tetrapeptides utilise the same receptor subtype (Boyd and Walker , 1985) , while it is supposed that the related heptapeptides use a separate receptor , since they displace a radioligand from FMRFamide receptors with an effectiveness approximately 20 times less than that of FMRFamide itself (Payza 1987 ; Payza *et al*, 1989) . Such a scenario would help to explain the differing responses of nerves and muscles often observed on exposure to the tetra - and heptapeptides (e.g. Boyd and Walker 1985) . The situation in *Lymnaea* may differ , however , where tetra - and heptapeptides may all utilise a single class of receptor (Brussard *et al* , 1989 ; Ebberink *et al* , 1987 ; Buckett *et al* , 1990).

Little is known about the molecular structure of the receptor(s) which recognise the FaRPs . Receptors generally fall into three distinct classes : the ligand gated ion channels, the GTP - binding protein coupled receptors , and those which are transmembrane regulated , such as the receptors which bind epidermal - and platelet - derived growth factors , for example (Heidaran *et al* ,1991) . The receptors for a number of other neuroactive substances have been isolated and studied : the receptors with which excitatory amino acids such as glutamate interact seem to often be ligand gated ion channels (Dingledine *et al* , 1990 ; Boulter *et al* , 1990) , while those for the monoamines 5HT and dopamine appear to belong to the G - protein coupled category (Dal Toso *et al*, 1989 ; Waeber *et al* , 1990 ; El Mestikawy *et al* , 1991) . Of the neuropeptides whose receptors have been isolated , substance P (Hershey and Krause 1990) , gastrin releasing peptide (Battey *et al*, 1991) and the opioid peptides (Offermanns *et al*, 1991) also seem to interact with

receptors which are coupled to GTP binding proteins . The recent report of the isolation of a receptor for tachykinin - like peptides in *Drosophila* , may provide pointers to the likely structure of the receptors for the FaRPs , since not only is this an invertebrate receptor , but the primary structure of the peptides which it recognises bears some similarity to the FMRFamide related peptides (Li *et al* , 1991) .

The type of receptor which a particular substance may activate is not , however , always limited to one arbitrarily defined class : for example , it has been shown that acetylcholine , 5HT , GABA and glutamate are able to activate both G - protein coupled receptors and ligand gated ion channels (Maricq *et al* , 1991) . In the case of the FaRPs , these peptides have been shown to be able to directly activate ion channels in at least some situations (Cottrell and Davis 1990) , while in others they appear to act *via* second messengers (Greenberg *et al* , 1983 ; Lange *et al*, 1991) . The structure of the receptors at which the FaRPs act in *Helix aspersa* is currently under investigation (D.A. Price , pers. comm.).

1.8 : Peptide structure

The structures of the peptides are important determinants of their FMRFamide - like receptor - binding efficiency . It appears that the presence of a C - terminal amide group is usually essential to the activity of the peptide , and that 4 amino acids is the minimal requirement for receptor binding in *Helix* (Payza 1987) and *Lymnaea* (Brussard *et al*, 1989). However , in at least some situations , responses can be elicited by non - amidated analogues (Thiemermann *et al* , 1991 ; Brussard *et al*, 1989) . (The non - amidated endogenous opioid peptides , Met - and Leu - enkephalin

(YGGFM and YGGFL respectively), are usually inactive on FaRP - responsive preparations; however , the synthetic opioid - related peptides YGGFMRFamide and YGGFLRFamide can often mimic the actions of the FaRPs (Greenberg *et al* , 1983)) . FMRFamide itself can tolerate certain amino acid substitutions without greatly reducing its efficacy , especially in the Phe^1 and Met^2 residues (Kobayshi and Muneoka , 1986) . The Arg - Phe - NH_2 C - terminal structure appears to be important , replacement of either residue reducing potency (Brussard *et al* , 1989) , while naturally occurring N - terminally extended variants may be indicative of less stringent requirements on this part of the peptides to elicit their bioactivity . It is thought that the carboxy termini of the invertebrate tetrapeptides interact most closely with the receptor binding site , while the differential binding of the tetra- and heptapeptides to receptors which is apparent in *Helix* (Payza 1987 ; Payza *et al* , 1989) suggests that the N - terminus , at least in the heptapeptide FaRPs , plays a role in receptor - peptide binding .

The repetoire of effects available to peptides within this family is significantly increased by the availability of extended variants of the FaRPs, and the differential responses effected in target tissues by these . At least in *Helix* , the FaRPs present appear to fall into two categories , the tetra and heptapeptides , often distinguished by their physiological actions as well as their primary structures . The existance in *Lymnaea* of both tetra and extended peptides , which are apparently not differentiated between by the receptor in this organism , may be indicative of their playing a subtly different role to that performed in *Helix* .

The increased biopotency often observed of the heptapeptides over the tetrapeptides (e.g. pQDPFLRFamide is approximately 100 times more potent than FMRFamide in

exciting the *Helix* heart (Price *et al* , 1985)) is sometimes attributed to their having a longer life-span *in vivo* , due to greater resistance to endogenous peptidases afforded them by their N - terminal extension . The presence of specific peptidases involved in the inactivation of FaRPs in tissues which express them , analogous to the cholinesterases found in cholinergic systems , has previously been implied in turbellarians and nemerteans (Jennings *et al* , 1987) . Recent data published by Ennis *et al.* (1991) tend to confirm this suggestion . These investigators were able to partially characterise three aminopeptidases which are found to be present in various *Helix* tissues (haemolymph levels of these peptidases were found to be very low - this may be analagous to the membrane - bound enkephalin degrading aminopeptidase recently characterised (Shimamura *et al* , 1991)) . Two of the three aminopeptidases isolated by Ennis *et al* were shown to efficiently cleave an N - terminal phenylalanyl residue from a substrate ; this action *in vivo* would have the effect of inactivating the tetrapeptides . Endogenous heptapeptides appeared to be more resistant to degradation by these aminopeptidases , though cleavage of pQDPFLRFamide at Phe^4 or Leu^5 by another enzyme found to be present in extracts of *Helix* ganglia (Irvine *et al* , 1988) would produce fragments susceptible to these recently characterised activities .

In addition to their increased resistance to specific peptidolysis , it may be that the extended peptides in *Helix* are more stable due to their lack of a methionine residue : it is known that oxidation of this residue inactivates FMRFamide (Brussard *et al* , 1989) , and it is possible that this method of inactivation is used *in vivo* (this would not , of course , apply to the tetrapeptide FLRFamide , which also contains no methionine residue) .

1.9 : Peptide synthesis and its control

The existence of a variety of small related peptides in a single organism raises the question as to how these are synthesised . Given their generally small size , and the multiplicity of "structural variants" , it seems unlikely that an individual transcription unit will be dedicated to each member of the FMRFamide - related peptide family , with all the concomitant promoter sequences and other *cis* - elements required for control of their expression. In addition, their apparent role in modulating nervous tissue responses means that it is probable that differential expression of the peptides by individual cells within the nervous system will be required, so some means of achieving this may need to be available .

The first organism in which the molecular genetics of the FaRPs was studied was the sea slug, *Aplysia californica* . Using a synthetic oligonucleotide complementary to the nucleic acid sequence encoding Phe - Met - Arg - Phe, Scheller and colleagues (Taussig and Scheller , 1986) screened a cDNA library generated from mRNA isolated from *Aplysia* ganglia . The amino acid sequence of the 597 residue precursor deduced from the genomic DNA clones which were obtained as a result of investigations using this probe showed it to encode 28 copies of the sequence - Phe - Met - Arg - Phe - Gly - , plus a single copy of the sequence - Phe - Leu - Arg - Phe - Gly - . (A glycine residue is known to be the donor of the amide group of many C - terminally amidated peptides (Bradbury and Smyth 1991) ; since some estimates suggest that up to 50% of bioactive peptides are amidated (Stoffers *et al* , 1991) , this suggests that many such peptides may be processed from such precursor polypeptides .) A representation of the precursor

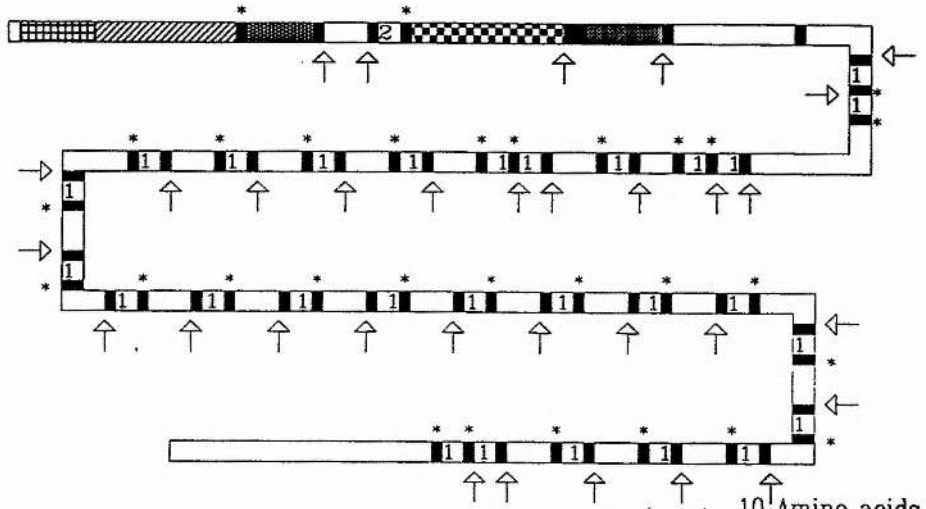
polypeptide of *Aplysia* is shown in figure 1 . As is illustrated , peptide - encoding sequences are bounded by dibasic amino acid sequences , which are thought to be involved in specifying cleavage sites to liberate the functional peptides from the precursor , and are often separated from each other in the precursor polypeptide by "spacer" regions which do not encode FaRPs and which contain a number of residues which have acidic sidechains . The homology between amino acid sequences encoded near the N - terminus of the precursor and the mammalian peptides CRF (corticotropin releasing factor) , α - MSH (α - melanocyte stimulating hormone) and CLIP (corticotropin-like intermediate lobe peptide) was also noted by Taussig and Scheller (1986) , although the reversal of the amidation states of the proposed related peptides in vertebrates and invertebrates is also perhaps noteworthy : there are no reports to date of the isolation of any of these extended peptides from *Aplysia* , nor of their having physiological effects .

Similar techniques to those used in the isolation of the *Aplysia* FaRP cDNA have been applied in the examination by us of cDNA libraries from *Helix aspersa* . Another mollusc, *Lymnaea stagnalis* (Linacre *et al* , 1990 ; Saunders *et al*, 1991) , an insect , *Drosophila melanogaster* (Schneider and Taghert 1988 ; Nambu *et al* , 1988), and the sea anenome *Calliactis parasitica* (Darmer *et al* , 1991) have also been studied in a similar fashion. Diagrammatic representations of the precursor polypeptide structures derived from these nucleic acid sequences are also shown in figure 1 . Several points of interest are noted : each sequence contains a number of different FaRPs encoded within it , some of which had not been previously identified in the organism in question. As yet , only tetrapeptide FaRPs have been

Figure 1 : Schematic representation of FaRP - containing precursor polypeptides .

Illustrated are FaRP - containing precursor polypeptides isolated to date : each key indicates the meaning of the symbols used , and the organism concerned and the source of the sequences from which they are derived is indicated in each case . In figure 1E , *Helix aspersa* , the boxed region represents the sequence derived by Dr. D. Price by PCR of *Helix pomatia* genomic DNA .

A : *Aplysia californica*
B : *Lymnaea stagnalis*
C : *Drosophila melanogaster*
D : *Calliactis parasitica*
E : *Helix aspersa*



PEPTIDE		No. Copies
1.	FMRFa	28
2.	FLRFa	1

Basic Residues

Amidation Signal

Cleavage Site

Hydrophobic Leader Sequence

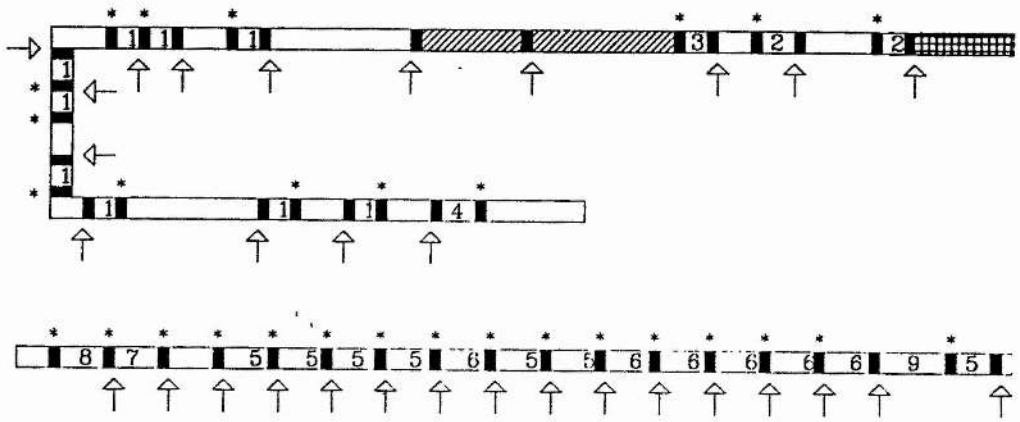
CRF –Like Peptide

–MSH Like Peptide

CLIP –Like Peptide

Aplysia californica (Adapted from Taussig and Scheller , 1986)

FIGURE 1A



10 Amino acids

Basic Residues

Amidation Signal

Cleavage Site

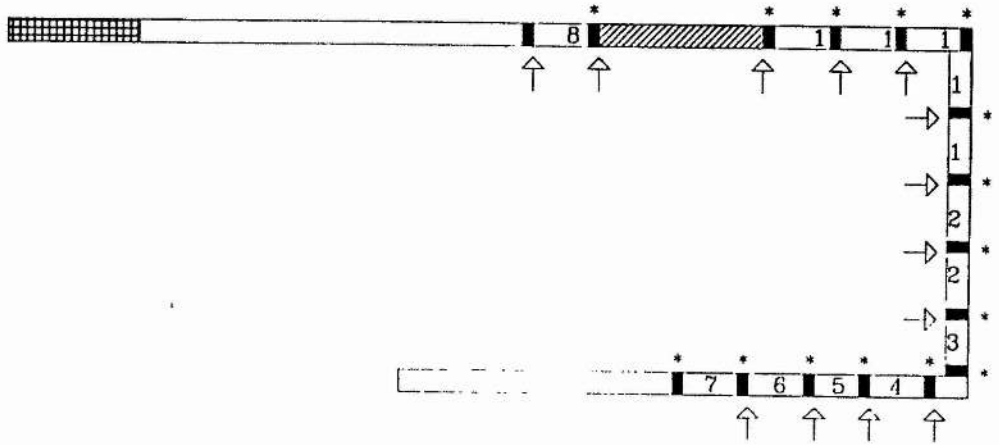
Hydrophobic Leader Sequence

CRF –Like Peptide

PEPTIDE		No. Copies
1.	FMRFa	9
2.	FLRFa	2
3.	QFYRIa	1
4.	EFLRYa	1
5.	GDPFLRFa	6
6.	SDPFLRFa	6
7.	EFFPLa	1
8.	SDPFFRFa	1
9.	SDPYLRFa	1

Lymnaea stagnalis (Adapted from Saunders *et al.* 1991)

FIGURE 1B

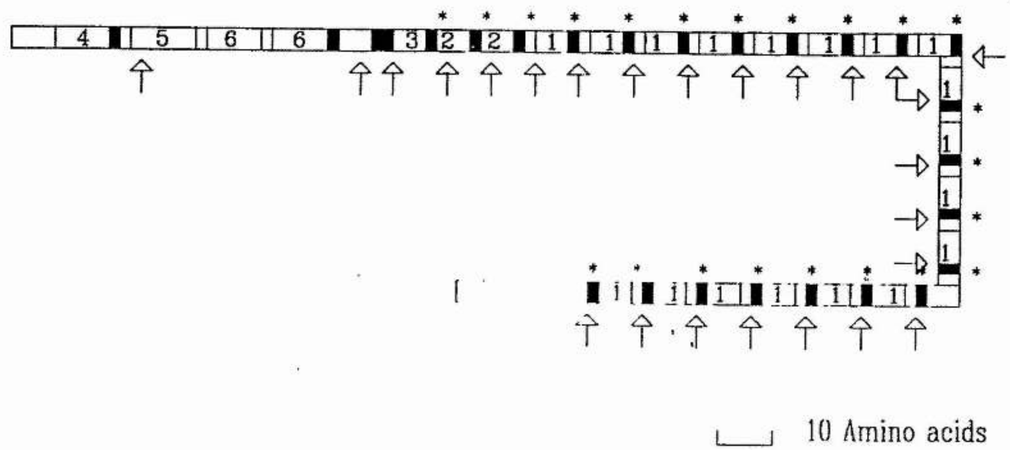


PEPTIDE		No. Copies
1.	DPKQDFMRFa	5
2.	TPAEDFMRFa	2
3.	SDNFMRFa	1
4.	PDNFMRFa	1
5.	SPKQDFMRFa	1
6.	SAPQDFVRSa	1
7.	MDSNFIRFa	1
8.	SVQDNFMHFa	1

- Basic Residues
- * Amidation Signal
- Cleavage Site
- Hydrophobic Leader Sequence
- CRF –Like Peptide

Drosophila melanogaster (Adapted from Schneider and Taghert , 1988)

FIGURE 1C



PEPTIDE		No. Copies
1.	QGRFa	19
2.	FQGRFa	2
3.	YVPGRYa	1
4.	PQYWRGRFA	1
5.	VVPQFWLGRFS	1
6.	PQFWKGRFS	2

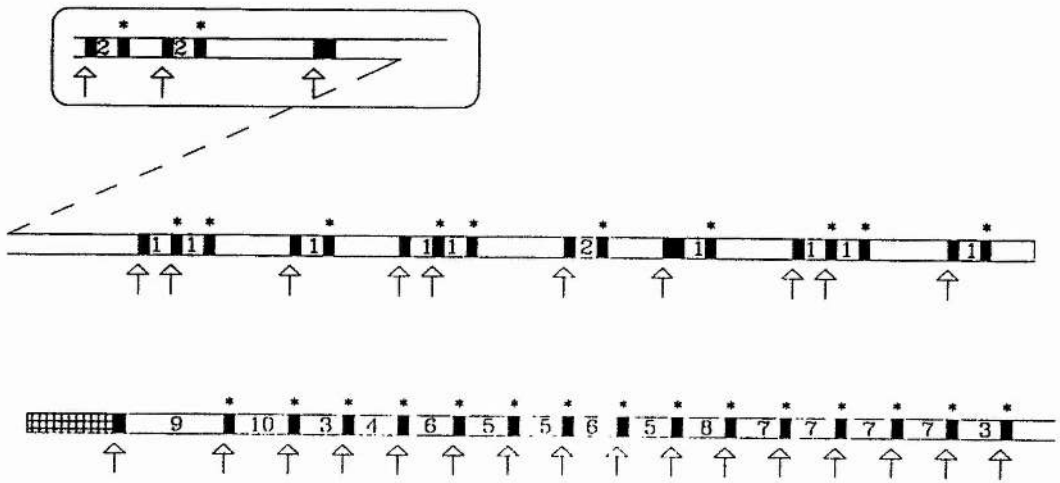
■ Basic Residues

* Amidation Signal

↑ Cleavage Site

Calliactis parasitica (Derived from Darmer *et al* , 1991)

FIGURE 1D



Helix aspersa

PEPTIDE		No. Copies
1.	FMRFa	10
2.	FLRFa	1
3.	NDPFLRFa	2
4.	SDPFLRFa	1
5.	pQDPFLRFa	3
6.	pQDPFLRIa	2
7.	NDPYLRFa	4
8.	SEPYLRFa	1
9.	SYGWAEGDTTNDEYLRFa	1
10 .	ENNNGYIRFa	1

■ Basic Residues

* Amidation Signal

↑ Cleavage Site

▩ Hydrophobic Leader Sequence

FIGURE 1E

identified in *Aplysia* ; *Drosophila* appears to contain no tetrapeptides related to FMRFamide - in fact , to date tetrapeptide FaRPs have only been isolated from molluscs, anthozoans , leech and a polychaete . Examination of the cDNA sequences derived from these *Aplysia* and *Drosophila*, and their comparison with those from *Lymnaea* and *Calliactis* , suggests a difference in the way in which tetrapeptide and N - terminally extended FaRPs are encoded. While the tetrapeptides are generally separated by "spacer" sequences in their precursors , there is an "economy" of amino acid usage observed in the polypeptides containing extended FaRPs - the peptides are often separated from one another only by a single amino acid , which in the case of *Helix* , *Lymnaea* and *Drosophila* are always basic in nature , and which are thought to be involved in directing processing of the peptides from the precursor . The cDNA sequence published by Darmer *et al.* (1991) is acknowledged by them to be somewhat problematic : although it appears to be derived from an expressed mRNA (as judged by its hybridization to a single band in an RNA blot) , the putative peptides encoded by it are seen to be bounded by acidic residues . To date there is no evidence of peptide cleavage being specified by such amino acids , causing Darmer *et al.* to postulate a novel cleavage pathway directed in this way.

Evidence for the existance of a number of peptides predicted from cDNA sequences awaits their isolation - for example , there has been only one publication to date describing the isolation of FaRPs from *Drosophila* , that of the peptide DPKQDFMRFamide (Nambu *et al* , 1988) . It is also noted that efforts to isolate the proposed " extended peptides " encoded in the *Calliactis* cDNA using antibodies raised against synthetic peptides with these sequences have not to date met with success (Darmer *et al* , 1991) . Since

these sequences are not terminated by a glycine residue, it is unlikely that the resultant peptides would be amidated , unless *Calliactis* proves to be atypical in its generation of C - terminal amide groups , as it appears it may be in its polypeptide cleavage mechanism ! An alternative , and perhaps more plausible , explanation is that these extended peptides were originally amidated , but mutation has destroyed the glycine codon , and they are therefore now non - functional , or perform a role different to the FaRPs. It can be seen that , excepting the last residue of each of the extended peptides proposed by Darmer *et al.* (1991) , these sequences conform to the " - Gly - Arg - Phe - " pattern of the anthozoan peptides previously isolated , and that two of the four codons which represent the carboxy terminal amino acids in these putative peptides are a single base different from that encoding glycine (GGX , where X = A , C , G or T). Moreover , each of the codons preceding the core " - Gly - Arg - Phe - " tripeptide sequence in these peptides is one base removed from the CAA or CAG required to specify a glutamic acid residue , which would complete the " Gln - Gly - Arg - Phe - " pattern seen in the tetrapeptides also encoded on this cDNA clone . It is possible that the amino acid sequences found encoded at the 5' ends of the cDNA clones from *Aplysia* , *Lymnaea* and , as we have found , *Helix* (Lutz *et al* , 1992) , which bear sequence similarity to vertebrate peptides are derived from these non - amidated peptides seen near the amino terminus of the *Calliactis* precursor.

The snail *Helix aspersa* is an excellent organism in which to study the molecular biology of the FaRPs : this mollusc has been found to contain at least seven peptides which bear structural relation to FMRFamide (Price *et al* , 1990) , which are known not only to have many physiological effects on *Helix* tissues , but which can be shown to be differentially located within the animal (Lehman and Price 1987b) . (It is

perhaps interesting to note that many organisms contain more than one FMRFamide - related peptide: why this should be so , and whether all FaRP - containing organisms possess a variety of such peptides , is as yet unclear - it may be that the different peptides often work either in concert or in opposition *in vivo* , so deeming several related peptides necessary in many situations.) The purpose of this project was to investigate the way in which these peptides are genetically encoded , and to obtain some indication of the levels and sites of expression of these gene(s) , especially during embryogenesis . As illustrated in figure 4, evidence from FaRP - encoding cDNA clones isolated from a *Helix* ganglionic library is consistent with that from other organisms- that is , tetrapeptide FaRPs are encoded separately from extended forms , and the way in which the different classes of peptide are arranged within the precursor polypeptide is of the form previously described (Lutz *et al* , 1992) . Since no genomic clones have to date been isolated and analysed , the manner in which these peptides are encoded in the genome is not yet clear, but there is evidence to suggest that the tetra - and heptapeptide FaRPs are encoded separately , perhaps as much as 14 Kb (kilobase pairs) apart . Results are presented here regarding the structure , expression and distribution of messenger RNAs encoding these peptides in the adult central nervous system of *Helix aspersa* , and at different stages during embryogenesis in this organism .

CHAPTER 2

MATERIALS AND METHODS

2.1 : Animals

Adult *Helix aspersa* were collected from the Kingsbarns area of Fife , Scotland . These were kept in a moist atmosphere at a constant temperature of 23^{o}C , in a 9 hr. light / 15 hr. dark cycle, and fed with green vegetable matter (usually lettuce). Individual animals were marked with a letter or a number using nail varnish; 15 individuals (e.g. snails A - O) were exchanged once every two weeks for other animals marked with the same numbers / letters - in this way , an individual snail was in the breeding colony for 6 weeks , unless it was observed to have mated , in which case this individual was retained for a further 6 weeks . Similarly , if an individual was seen to lay a batch of embryos , it was exchanged for an identically marked snail at the next cycle . Soil 20mm deep was provided : embryos were laid in this 7 - 10 days following mating (≈ 100 embryos per mating, in individual ≈ 5mm cases). The day on which these were laid in the soil was designated D1 ; hatching occured on D20. Since embryos examined on D1 can be observed to be at the 1 - , 2 - and 4 - cell stages of development , it is thought that , although there is usually a significant time lapse between mating of two individuals and laying by one of them of embryos, fertilization does not take place until immediately before the time of laying . Embryos were dissected from their cases into *Helix* saline at various developmental stages, and treated as described .

Helix Saline :

80 mM NaCl
5 mM $MgCl_2$
5 mM KCl
7 mM $CaCl_2$
20 mM HEPES
pH 7.5

2.2 : RNA extraction

LiCl precipitation :

Embryos were hand homogenised on ice in ≈ 1ml of 3M LiCl / 6M Urea in DEPC dH_2O, incubated at 4°C overnight, and centrifuged at 16,000*g* , 0°C , 20 min.. Pellets were resuspended in ≈ 5ml of 10mM Tris - HCl (pH 7.5) , 0.5% SDS in DEPC dH_2O. This was extracted with an equal volume of chloroform / isoamyl alcohol (24 : 1) by shaking by hand for 10 min. , then centrifuged at 10,000*g* , 4°C for 10 min.. Aqueous (top) layer was removed to a clean Corex tube and precipitated with 1/10th vol. 3M Na Acetate and 2.2 vol. ethanol at -20°C overnight. RNA was pelleted by centrifugation at 10,000*g* , 4°C for 20 min. , washed with cold (-20°C) ethanol , and respun as above. Pellet was dried under vaccuum and resuspended at 1mg/ml in DEPC dH_2O.

GuSCN extraction : Some early embryos (D1 - 3) were homogenised in GuSCN solution. Homogenisation was by hand on ice ; homogenate was transferred to a corex tube , then centrifuged to remove debris (4,000*g* , 4°C for 20 min.). 1g of CsCl was added per 2.5 ml retained supernatant ; this solution was carefully layered over ≈ 1.5 ml dense CsCl solution (5.7M CsCl / 0.1M EDTA) in a 6 ml polycarbonate tube , and the tube filled with sterile liquid parafin. RNA was pelleted by centrifugation at 35,000 rpm , 15°C for 16 hr. in a 3x6 titanium rotor. Following careful removal of parafin and CsCl , the bottom of the tube (containing RNA pellet) was cut off and placed on tin foil on top of ice. RNA was resuspended in DEPC dH_2O , precipitated overnight at -20°C following addition of 1/10th vol. 3M Na Acetate (pH 5.2) and 2.2 vol. cold ethanol (-20°C) , then repelleted , washed and resuspended as above.

GuSCN solution :

6M Guanudinium Thiocyanate
2% Sarkosyl (v / v)
10mM EDTA
30mM Na Citrate (pH 7.6)

Immediately before use, 25µl β - Mercaptoethanol was added per 3 ml GuSCN stock solution .

2.3 : GTC / LiCl extraction

The following method was adapted from that published by Abood *et. al.* (1990) , to facilitate the extraction of both RNA and peptides from the same embryological samples . Embryos at appropriate developmental stages were collected and hand homogenised in a small volume (200 - 400 µl) of:

5M Guanidinium Thiocyanate
50mM Tris - HCl (pH 7.5)
8% β - mercaptoethanol (v / v)

Extracts were precipitated overnight at 4°C with 7 vols. of 4M LiCl , and the RNA pelleted by centrifugation at 12,000*g* for 30 mins. . The pellet was resuspended in 1ml. 3M LiCl , spun at 12,000*g* for 30 mins. and resuspended in 400µl solubilization buffer :

10mM Tris - HCl (pH 7.5)
1mM EDTA
0.1% SDS (w / v)

RNA was then extracted with an equal volume of phenol / chloroform / isoamyl alcohol (25 : 24 : 1 (v / v / v)) , followed by extraction with an equal volume of chloroform / isoamyl alcohol (24 : 1 (v / v)) , aqueous and solvent phases being separated in each case by centrifugation (4 mins. , high speed , Microcentaur) , and aqueous (upper) phase being removed to a fresh tube . RNA was precipitated by addition of 0.1 vol. 3M Na Acetate , pH 5.5 , 2.5 vol. ethanol and incubation at - 20°C overnight . RNA was then collected by centrifugation at 12,000*g* for 30 mins. at 4°C.

2.4 : RNA solutions and glassware

DEPC dH_2O : All solutions were made up in distilled water (dH_2O) treated with diethylpyrocarbonate (DEPC). 1µl DEPC was added per 1 ml dH_2O , which was then stirred and boiled 15 minutes before being autoclaved.

Eppendorf tubes: Autoclaved eppendorf tubes were washed individually by filling with the water repellant dimethyldichlorosilane ("Repelcote" , BDH) , rinsed in a large beaker of dH_2O, then transferred individually to DEPC H_2O and stirred for 2 hours. Tubes were then rinsed in fresh distilled water and autoclaved.

Glassware : All glassware was baked overnight at 145°C before use.

2.5 : Slot blots

DNA and RNA were applied to nitrocellulose membranes (prewet in 20X SSC and air dried 5 min.) *via* a BRL (Bethesda Research Laboratories) " Hybri-slot " manifold , with pre - and post - washing of wells with 800 µl 2 X SSC. Prior to application to filters, RNA was glyoxalated (see below) ; following application of DNA to nitrocellulose , filters were floated for 1

min. on denaturation solution then 1 min. on neutralisation solution (see also below). All filters were baked 2 hrs. under vaccuum at 80°C. Prehybridization was for 2 hrs. in prehybridization solution at temperatures indicated for individual experiments ; hybridization was performed at the same temperature overnight in a similar solution , with the addition of the appropriately radiolabeled probe. Filters were sequentially washed to a stringency considered appropriate in :

2X SSC , 0.1% SDS (w / v) , 37°C , 2 x 30 min. ,
1X SSC , 0.1% SDS (w / v) , 37°C , 2 x 30 min. ,
1X SSC , 0.1% SDS (w / v) , 42°C , 2 x 30 min. ,
1X SSC , 0.1% SDS (w / v) , 60°C , 2 x 30 min. ,
0.5X SSC , 0.1% SDS (w / v) , 60°C , 2 x 30 min. ,
0.5X SSC , 0.1% SDS (w / v) , 65°C , 2 x 30 min. .

Exposure to X-ray film was at -70°C for an appropriate length of time , as indicated for individual experiments . Films were hand developed at room temp. for 4 mins., then fixed for 4 mins., room temp. .

20X SSC (Standard sodium citrate) :

3M NaCl
0.3M tri-Sodium Citrate

Denaturation solution :

1.5M NaCl
0.5M NaOH

Neutralisation solution :

1M Tris - HCl (pH 8)
1.5M NaCl

Prehybridization solution :

5X	SSC
1X	Denhardts soln.
50%	Deionised Formamide (v / v)
100mM	KPO_4 (pH 6.6)
10%	Dextran sulphate (w / v)
100µg/ml	Denatured *E. coli* DNA (boiled for 5 min. before addition)

50X Denhardts solution :

1%	Ficoll (w / v)
1%	PVP 44K (w / v)
1%	BSA (w / v)

2.6 : Glyoxalition

RNA samples were treated with a solution containing glyoxal before application to dot - blot filters in order to reduce higher - order intramolecular structures due to base pairing , which may reduce access of the probe to RNA sequences of interest . This was done by incubating RNA samples at 50°C for 1 hr. in an equal volume of :

34% deionised glyoxal (pH 5.5 - 6) (v / v)
20mM Sodium Phosphate (pH 6.5)

Glyoxalated RNA is stable for approximately 4 hrs. at room temperature . Following immobilization of RNA samples on filters glyoxal must be removed , or it may interfere with probe hybridization ; this was done by placing filters in 20mM Tris - HCl (pH 8) at 100°C for 10 mins. , the filter remaining in the solution while it is allowed to cool to room temp. .

2.7 : Glyoxal deionization

10 ml. glyoxal and 10g AG 501 - X8 ion exchange resin were stirred together in a 50ml. beaker for 30 mins. at room temp.. Supernatant was transferred to a fresh 25 ml. beaker containing 2g AG 501 - X8 resin and stirred again as above . The pH of the resultant solution was measured - if it was not in the range pH 5.5 - 6 , further incubations with 2g ion exchange resin were performed as above until this point was reached . Supernatant was then poured through a Pasteur pipette containing loosely packed glass wool , collected , and stored in 100µl aliquots at - 20^{o}C . This could be kept for many months , but pH was checked immediately before use - if pH was not in range 5.5 - 6 , a small amount of AG 501 - X8 resin was added . Tube was incubated at room temp. for 10 mins. with occasional agitation , centrifuged 2 mins. , high speed , Microcentaur, and supernatant transferred to fresh tube ready for use .

2.8 : Agarose gel electrophoresis

Nucleic acids were usually observed by electrophoresis through agarose .

A) In the case of DNA samples , these were first digested with restriction enzymes as appropriate . An equal volume of loading dye was added to the sample , which was then subjected to electrophoresis through an agarose gel at an appropriate voltage and for an appropriate length of time (usually 9V , overnight (gel length : 14 cm.) or 50V , overnight (gel length : 30 cm.)) . Gel was then incubated in a solution of ethidium bromide in dH_2O (≈1 µg / ml) for 10 mins. at room temperature, before being destained by incubation in dH_2O at room temp. for approx. 30 mins. . Nucleic acids were visualised on an ultraviolet transilluminator .

Agarose gels were usually 0.8% (w / v) agarose in 1 X TBE , and were run in 1 X TBE tank buffer .

10 X TBE :

0.9M Tris
0.9M Boric Acid
25mM EDTA

Loading dye :

10 % (w / v)	Ficoll (M.W. 400,000)
0.05 % (w / v)	Bromophenol Blue
0.05 % (w / v)	Xylene Cyanol FF
0.05 % (w / v)	Orange G

B) RNA samples were usually analysed on denaturing formaldehyde gels . Gel comprised :

1.5% Agarose (w /v)
1X MOPS Buffer
6.7% Formaldehyde (w / v)

Required amount of agarose was added to appropriate volume of DEPC dH_2O and boiled approx. 15 mins. , until agarose was dissolved . One tenth final volume of 10 X MOPS buffer was then added , and the solution incubated at 50^oC for approx. 30 mins. before addition of 0.18 vol. of 37 % (w / v) formaldehyde soln. ; gel was poured immediately . Gel was run in 1 X MOPS tank buffer .

Before loading on gel , RNA samples were denatured : equal volume of " Blue Juice " was added , and samples incubated at 65^oC for 5 mins. . Samples were immediately loaded onto formaldehyde gel and subjected to electrophoresis , usually

overnight (14 cm. gel : 20V , 12 mA) or 1.5 hrs. (14 cm. gel : 100V) . RNA was visualised using acridine orange according to Wilkinson *et. al.* (1991) : gel was gently agitated in AO stain for 3 mins , then in de - stain buffer for approx. 30 mins. , and observed on medium wave ultraviolet transilluminator .

10 X MOPS buffer :

200mM	MOPS
50mM	Na Acetate
10mM	EDTA
pH	7

Blue juice :

80µl	10 X MOPS buffer
80µl	Bromophenol Blue (0.1 mg / ml in 50% glycerol)
130µl	37% (w / v) Formaldehyde
360µl	deionised Formamide

AO STAIN :

15 µg / ml	Acridine Orange
10mM	Sodium phosphate (pH 6.5)
3%	Formaldehyde (w / v)

AO DESTAIN :

10µM	Sodium Phosphate (pH 6.5)
3%	Formaldehyde (w / v)

2.9 : Southern blots

Southern transfers were executed essentially as described by Maniatis *et. al.* (1982): following electrophoresis , gel was subjected to denaturation (denaturation soln. , see above) for 1 hr. , then incubated 1hr. in neutralisation solution. Tank / wick setup was with 10X SSC ; nitrocellulose and 3MM paper were prewet in 2X SSC . Blotting was allowed to procede overnight ; filter was removed and floated on 6X SSC , 5 min., air dried then baked 2 hrs. , 80^{o}C under vaccuum. Prehybridization was overnight in the previously described solution at a temperature as indicated in individual experiments ; hybridization was overnight at the same temperature , achieved by addition of the appropriately labeled probe to prehybridization solution. Filters were washed to an appropriate stringency , as described for dot blots , air dried and exposed to X-ray film at - 70^{o}C for times as indicated in individual experiments ; films were hand developed and fixed (4 mins. each) at room temperature.

2.10 : Northern blots

Transfer of RNA to membranes was essentially as described by Maniatis *et. al.* (1982) : following electrophoresis gel was washed 4 x 5 mins. in dH_2O , then incubated for 45 mins. each in :

50mM Na OH
10mM Na Cl

and :

100mM Tris - H Cl (pH 7.5) .

This was followed by a 60 min. incubation in 20 X SSC :

3M Na Cl
0.3M tri - Sodium Citrate

All incubations were at room temp. with gentle agitation . Transfer was overnight as described by Maniatis et. al. , transfer buffer being 10 X SSC . RNA was bound to filters as indicated by Wilkinson *et. al.* (1991) : filter was wraped in clingfilm and laid nucleic acid side down on a u. v. transilluminator for 3 mins., the clingfilm removed , and the filter baked under vaccuum at $80^{o}C$ for 2 hrs. .

2.11 : Colony hybridizations

Bacteria were plated and grown at room temperature overnight on AIX plates (see below). Precut circular nitrocellulose filters were applied to plates , orientated using an ink tipped syringe needle , then subjected successively to flotation on denaturation solution , neuralisation solution and 5X SET (5 min. , room temp. each). Filters baked for 2 hrs. at $80^{o}C$ under vaccuum were prehybridized , hybridized , washed and exposed to X-ray film in a manner identical to that for Southern blots.

20X SET :

3M NaCl
20mM EDTA
0.4M Tris - HCl (pH 7.8)

2.12 : End labeling of oligonucleotide probes

300ng of the appropriate synthetic oligonucleotide in 1µl dH_2O was incubated for 1 hr. at $37^{o}C$ in the following solution :

20 units polynucleotide kinase (PNK)	0.5µl
10X PNK buffer	5µl

50µCi ^{32}P γ ATP	5µl
dH_2O	38.5µl

Labeled oligonucleotide was separated from unincorporated label *via* a Sephadex G-25 column in 6X SSC, and was used in this form in hybridizations.

10X PNK buffer :

0.5M	ris - HCl (pH 7.6)
0.1M	gCl_2
50mM	TT
1mM	permidine
1mM	DTA

2.13 : Oligonucleotide labeling of cloned probes

DNA fragments to be labeled were isolated from the vector by electrophoresis through 0.8% low melting-point agarose. The appropriate bands were cut from the gel and boiled in an eppendorf tube for 5 min. . 32.5µl of this DNA in molten agarose was added to the following mixture , which had previously been warmed to 37°C for at least 2 min :

10µl	oligolabeling buffer
1µl	each 8.5mM dATP, dGTP, dTTP
1µl	BSA (20mg/ml)
48.5µl	dH_2O

5µl (50µCi) of ^{32}P dCTP was then added , the solution mixed and centrifuged briefly , and incubated at 37°C for 1 hr. . 2 units DNA polymerase , " Klenow " fragment , was added, the tube

vortexed , centrifuged briefly , then incubated at 37^{o}C overnight. Labeled DNA was separated from unincorporated nucleotides by means of a Sephadex G - 50 column in 6X SSC. (Alternatively , a " spun column " (see below) was used at this last stage to separate unincorporated nucleotides from labeled probe : radiolabeling solution was extracted twice with an equal volume of phenol / chloroform / isoamyl alcohol (24 : 24 : 1 (v/v/v)) , phases separated by centrifugation (4 mins . , high speed, Microcentaur) , and aqueous (upper) phase removed to a clean tube . This was then applied to a spun column as described below .)

Oligolabeling buffer :

Soln. O : 1.25M Tris - HCl (pH 8)
0.125M $MgCl_2$

Soln. A : 1µl Soln. O + 18µl β -Mercaptoethanol

Soln. B : 2M HEPES (pH 6.6)
(Titrate with 4M NaOH)

Soln. C: 90 OD units/ml Pharmacia product no. 2166 (random hexanucleotides)

Oligolabeling Buffer = 100µl Soln. A
250µl Soln. B
150µl Soln C

Store at -20^{o}C. Sufficient for 50 reactions.

2.14 : Riboprobe synthesis

Approximately 4 µg of plasmid of interest was linearised in 10µl using appropriate restriction enzyme (37^{o}C , 1 - 2 hrs) . To this was then added :

20 µl 5X Salts
20 µl 5X NTPs

5µl	BSA (0.1 mg / ml)
4µl	HPRI (1 unit / ml)
5µl	DTT (0.1 M)
33µl	dH_2O
1µl	^{32}P - CTP (20 µCi / µl)
1µl	T3 or T7 RNA Polymerase (1 unit / µl)

This was incubated 1 hr , 37^{o}C ; 2.5 µl 0.5 M EDTA (pH 7.5) added to stop reaction . Equal volume of phenol / chloroform / isoamyl alcohol (24 : 24 : 1 (v / v /v)) added and phases separated by centrifugation (4 mins . , high speed , Microcentaur) . Aqueous phase removed to fresh tube , re - extracted as above , and unincorporated nucleotide separated from intact probe by means of a " spun column " (see below) .

5X Salts :

200mM	Tris - HCl (pH 8)
40mM	$MgCl_2$
10mM	Spermidine
125mM	NaCl

5X NTPs :

4mM	ATP
0.8mM	CTP
4mM	GTP
4mM	TTP

2.15 : Spun column

Sephadex G - 50 resin was resuspended in TEN buffer (approx. 5g resin / 100 ml buffer) and autoclaved . Columns, as described by Maniatis *et al* (1982) , were constructed in 1 ml.

disposable syringes : a plug of ashless flock (boiled in sterile TEN immediately before use) was placed in the syringe, Sephadex G- 50 suspension was added to fill syringe, and resin was compacted to dryness by centrifugation (syringe was placed in centrifuge tube as illustrated , and tube spun at 3000 r.p.m for 4 mins.) . If necessary , further Sephadex G - 50 suspension was added and column recentrifuged as above until a column of approx. 0.9 ml. compacted resin was obtained. 100µl of TEN buffer was then loaded onto the top of the column , and the column spun as before , in order to ensure that total volume loaded was recovered from column . Radiolabeling mix to be separated was similarly loaded on column , unincorporated nucleotides being retained on the column , while solution containing labeled probe was collected in a 1.5 ml. screw - cap eppendorf tube placed at the bottom of the carrier centrifuge tube .

TEN buffer :

100mM	Tris - HCl (pH 8)
1mM	EDTA
100mM	NaCl

2.16 : Plasmid vector

Bluescript , a multipurpose *E. coli* vector (figure) , was purchased from Stratagene. This 2959 b.p. (base pairs) vector confers ampicillin resistance on *E. coli* DS 941 , where it is maintained at high copy number under the selective pressure of ampicillin at 50µg / ml when bacteria are grown in liquid culture in L-broth. Since it contains T3 and T7 RNA polymerase binding sites , this plasmid can be used in the synthessis of single stranded RNA probes (riboprobes) . DNA sequencing is also facilitated by the presence of a number of primer binding sites on either side of the multiple cloning site .

pBluescript® SK

The pBluescript® SK (+/-) plasmid is a 2958 basepair phagemid derived from pUC19. The vector possesses an f1 phage origin, a colE1 origin, and T3 and T7 promoters flanking a novel polylinker containing 21 unique restriction sites. These sites are clustered with 3' overhangs flanking 5' overhangs to facilitate generation of nested deletions with exonuclease III. The pBluescript SK (+/-) plasmid contains a *lacZ* promoter for blue/white color selection or fusion protein induction with IPTG. The SK designation indicates the polylinker is oriented such that β galactosidase transcription proceeds through the Sac I site first and the Kpn I site last. The pBluescript SK (+/-) plasmid carries an f1 origin of replication, allowing single strand DNA rescue, via helper phage infection, for site-specific mutagenesis or single strand sequencing. The rescued strand of SK (+) will contain the coding strand of the β galactosidase gene (top strand of the polylinker), the rescued strand of SK (-) will contain the non-coding strand (bottom strand of the polylinker).

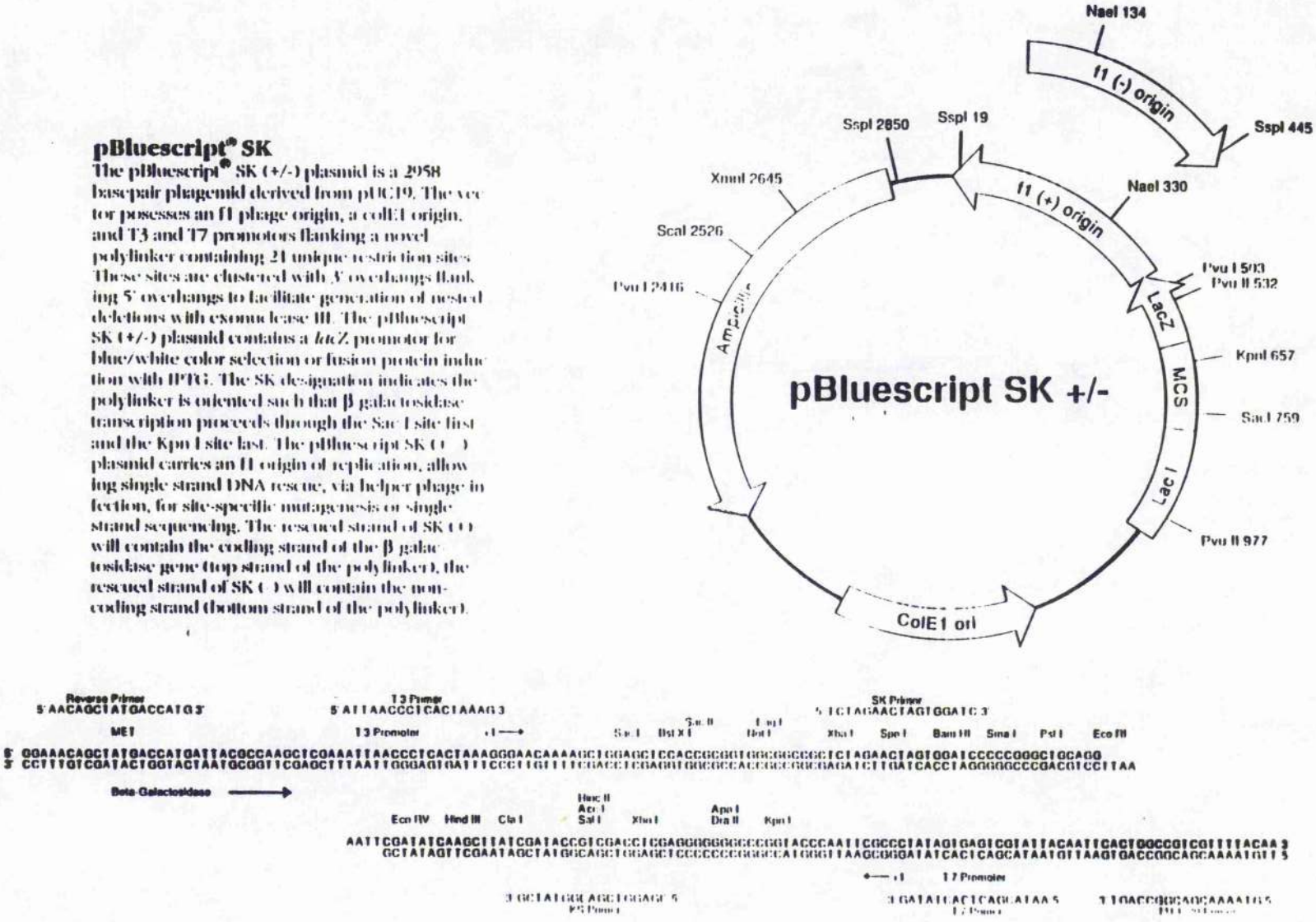


Figure 2 : Bluescript plasmid

2.17 : Transformation

Plasmids were transformed into bacteria by the standard $MgCl_2$ / $CaCl_2$ / heat shock method : a 5ml. culture of host bacteria was grown overnight in L - broth at 37°C with constant agitation . 2 ml. from this culture was added to 50 ml. of L - broth, and incubated for approx. 2 hrs. at 37°C with constant agitation, until an optical density at 650 nm of 0.2 O.D. units was achieved (1.5 - 2 hrs.) . Bacterial cells were harvested by centrifugation at 3000 r.p.m. for 10 mins. ; the supernatant was poured off , cells resuspended in 3.8 ml. ice cold 0.1M $MgCl_2$ and reharvested as above . Pelleted bacteria were resuspended in 1.9 ml. ice cold 0.1M $CaCl_2$. Centrifugation was repeated , cells resuspended in a similar volume of ice cold 0.1M $CaCl_2$ and incubated on ice for 1 - 20 hrs . Bacterial solution was thoroughly mixed immediately before the addition of 200 µl of these "competent" (i.e. able to take up DNA) cells to the appropriate amount of plasmid DNA disolved in T.E. ; this was then incubated on ice for 30 mins. , subjected to heat shock (an incubation of either 5 mins. at 37°C , or of 2 mins at 42°C) , and returned to ice for a further 30 mins. . Following the addition of 1 ml of L-broth , transformed bacteria were incubated at 37°C for 2 hrs. , pelleted by centrifugation for 4 min. on "low" setting in Microcentaur , then resuspended in 400ml L-broth . 100ml of this suspension was plated onto AIX plates , which were incubated inverted at 37°C overnight. Cells containing recombinant plasmids grew as white colonies , while those containing non-recombinants showed as blue colonies.

L-BROTH :

13g Oxoid Nutrient Broth Mix per litre in dH_2O

L-AGAR :

L-broth + 1.5g Oxoid Agar Bacteriological per 100 ml.

AIX PLATES :

L-agar + 50µg/ml Amp (Stock : 50mg/ml. in dH_2O
50µg/ml IPTG (Stock : 50mg/ml. in dH_2O)
40µg/ml X-gal (Stock : 40mg/ml. in DMF)

2.18 : Plasmid preparations

Large scale plasmid preps were performed by innoculation of a 250ml. L-broth + Amp culture with a 5ml. " starter culture " in a similar medium , grown at 37°C overnight with constant agitation. Bacteria were pelleted by centrifugation (7000*g* , 4°C , 10 min.) and resuspended in 5ml. STET buffer. Following addition of 400µl lysosyme (10mg / ml in dH_2O) and incubation on ice for 5 min. , the suspension was boiled for 90 sec. , then centrifuged 20,000*g* , 45 min , 4°C . The straw-coloured supernatant was removed , care being taken not to disturb the pelleted cell debris , extracted twice with an equal volume of phenol , twice with an equal volume of chloroform (appropriate volume of solvent added to supernatant , phases mixed by vortexing briefly , then centrifuged at 10,000 r.p.m. , 4°C , 10 mins ; aqueous (top) phase removed to clean tube) , and nucleic acid precipitated by addition of 1/10th volume 3M NaAc and 2.5 volumes cold ethanol (-20°C). Precipitate was pelleted by centrifugation (10,000*g* , 10 min. , 4°C) , washed in a small volume (1-2ml) 70% ethanol and repelleted as above , prior to being dried under vaccuum and resuspended in T.E. (10mM Tris - HCl (pH 8) ; 1mM EDTA). It was usually necessary to treat plasmid preparations with ribonuclease A : nucleic acid pellets were resuspended in 400µl dH_2O , approx. 10 µg of ribonuclease A (1 mg / ml) was added , and incubated at 37°C for 30 - 60 mins. . Ribonuclease activity was destroyed by addition of 50 µg proteinase K (10 mg / ml); successive extractions with equal volumes of phenol and chloroform (twice

with each) were performed as above , followed by DNA precipitation with 1/10th vol. 3M NaAc , 2.5 vol. ethanol . After incubating at -70°C for 20 min. , DNA was pelleted by centrifugation in Microcentaur (high speed) for 10 mins. , washed briefly in a small volume (200 µl) of 70% ethanol then recentrifuged as above . Pellets were dried under vaccuum (15 mins) then resuspended in 400 µl dH_2O . Concentration and purity of DNA in this solution was estimated by measurement of optical density at 260 and 280 nm , and plasmids were usually resuspended at a concentration of 1 µg / µl .

Small scale plasmid preps were either by the " CTAB STET " method (Del Sal *et al* , 1988) , or by alkaline lysis (Maniatis *et al* , 1982) . In the CTAB method , a single bacterial colony was picked from an AIX plate and grown overnight at 37°C with shaking in 1 ml L-broth + Amp in a 1.5ml eppendorf tube . Bacteria were pelleted (4 min., low speed in Microcentaur), resuspended in 200µl STET buffer , and incubated 5 min. at room temperature following addition of 4µl lysosyme (50µg/ µl). Samples were boiled for 45 sec. and centrifuged 10 min. , high speed (Microcentaur); supernatant was transferred to a clean tube , 8µl 5% CTAB (Cetyl trimethyl ammonium bromide) added, and samples centrifuged 5 min. at high speed . Pellet was resuspended in 300µl 1.2M NaCl , and DNA precipitated by addition of 750µl ethanol followed by centrifugation for 10 min. at high speed . Pellet was washed with 70% ethanol , dried under vaccuum and resuspended in small volume TE (e.g. 10µl).

STET buffer :

8% Sucrose (w / v)
5% Triton X-100 (v / v)
50mM EDTA
50mM Tris-HCl (pH 8)

When the alkaline lysis method was employed , 1 ml. cultures were grown overnight in L - broth + antibiotic in 1.5 ml.

eppendorf tubes at 37^{o}C with constant shaking . Bacteria were pelleted by centrifugation (4 mins. , low speed , Microcentaur) , and resuspended in 100µl ice cold :

50 mM Glucose
10 mM EDTA
25 mM Tris - HCl (pH 8)

Tubes were incubated at room temp. for 5 mins. , then 200µl freshly prepared 0.2M NaOH , 1 % SDS was added , and mixed by rapidly inverting tubes 2 - 3 times ; tubes were then incubated on ice for 5 mins. . 150 ml of ice cold :

3M Potassium acetate
2M Acetic acid

were added , tubes vortexed while inverted , then replaced on ice for 5 mins. . Cellular debris was pelleted (10 mins. , high speed , Microcentaur) , and supernatant removed to fresh tube . To this was added an equal volume of phenol / chloroform / isoamyl alcohol (24 : 24 : 1 (v / v / v)) , tubes vortexed , and phases separated by centrifugation (4 mins. , high speed , Microcentaur) . Aqueous (top) phase was removed to a clean tube , 2 vols. ethanol added ; contents of tube were mixed , incubated at room temp. for 2 mins. and nucleic acids pelleted by centrifugation (10 mins. , high speed , Microcentaur) . Pellet was briefly washed in 70 % ethanol , repelleted as above , dried under vaccuum , and resuspended in 10 µl dH_2O.

2.19 : Preparation of DNA for sequencing

All sequencing was done as double - stranded plasmid sequencing using T7 DNA polymerase (Pharmacia) . DNA was purified for sequencing in one of two ways :

A) Bacteria containing plasmid of interest were grown overnight with constant agitation in 5 ml. L - broth + relevant antibiotic , then pelleted in an eppendorf tube (1.5 ml. of culture pelleted in microcentaur , low speed ; supernatant removed , and further 1.5 ml. of culture pelleted in same tube . Repeat .) Pellet was resuspended in 180 µl ice cold 10mM EDTA (pH 8), then placed on ice and 20 µl of 10 mg / ml lysosyme (freshly prepared in 10 mM EDTA (pH 8)) added . This was gently mixed and placed at - 20^{o}C for at least 30 mins. before being thawed at room temperature . 400ml of freshly prepared 0.2 M NaOH , 1% SDS was added , and the capped tube gently inverted 5 - 10 times . This was then incubated at 37^{o}C for 30 mins. before 300 µl of 5M acetic acid , 3M KOH was added , and tube gently inverted . Following a 5 minute incubation on ice, the tube was rapidly inverted several times , then centrifuged at high speed in Microcentaur for 5 mins. . 850µl of supernatant was removed to a fresh tube , care being taken to avoid inclusion of any pelleted cellular debris , and 520 µl of isopropanol added . Tube was inverted , and nucleic acids allowed to precipitate at room temp. for at least 1 hour ; DNA was then pelleted by centrifugation (10 mins. , high speed , Microcentaur) , washed briefly in 200µl 70 % ethanol , and repelleted as before . Residual ethanol was evaporated under vaccuum (15 mins) , pellet was resuspended in 8µl of dH_2O and used directly in a sequencing reaction (see below).

B) The following protocol was adapted from Jones + Schofield (1991) . A 5 ml. culture of the plasmid of interest was grown overnight at 37^{o}C with agitation in L - broth + selective antibiotic . 1.6 ml. of this culture was decanted into an eppendorf tube , bacteria pelleted by centrifugation (4 min. , low speed , Microcentaur) then respended in 180 µl of :

50mM Glucose

10mM EDTA
25mM Tris - HCl (pH 8).

To this was added 360 µl of 0.2 M NaOH , 1% SDS ; tube was inverted several times and placed on ice for 5 mins. . 270µl of :

3M K Acetate
2M Acetic acid

was added , and tube returned to ice for a further 5 mins.. Cellular debris was pelleted by centrifugation (5 mins., high speed , Microcentaur) , and supernatant removed to a clean tube . Equal volume of absolute ethanol was added, mixed , and centrifuged immediately as above . The resulting nucleic acid pellet was washed briefly in 200 µl 70% ethanol , centrifuged as above , dried briefly under vaccuum and resuspended in 16 µl of dH_2O . Half of this solution was sufficient per sequencing reaction (see below) .

2.20 : DNA sequencing

1.5 - 2 µg plasmid DNA in 8 µl dH_2O, prepared as detailed above , was denatured by the addition of 2 µl 2M NaOH . Following incubation at room temp. for 10 mins. , 3µl of 3M sodium acetate (pH 4.5) , 7µl of dH_2O and 60µl of 100% ethanol were added . This was mixed briefly , incubated at -70^{o}C for 15 mins , then centrifuged for 10 mins. at high speed in Microcentaur . Pellet was washed briefly in 200 µl 70% ethanol , spun as above , and residual ethanol removed by 15 min. incubation under vaccuum . Dried pellet was resuspended in 10µl of dH_2O ; to this was added immediately 2µl of oligonucleotide primer (0.8µM if plasmid prepared by method A above ; 10µM if method B used in plasmid isolation) and 2µl annealing buffer :

280mM Tris - HCl (pH 7.5)
100mM $MgCl_2$
350mM NaCl

This was mixed , incubated at 37°C for 20 mins. , then at room temp. for at least a further 10 mins. . (If sequencing reactions were not to be performed the same day, samples were stored at -20°C at this point) . To this annealed primer / template mix was added :

Labelling Mix	2µl
0.3 M DTT	1µl
[α - ^{35}S] dATP (10 µCi / µl)	1µl
Diluted T7 DNA polymerase (1.5 units / µl)	2µl

Labeling mix :

2.0µM dGTP
2.0µM dCTP
2.0µM dTTP

(Amount of T7 DNA polymerase (Pharmacia) required was diluted to 1.5 units / µl in dilution buffer provided (ice cold) immediately before use .)

Components of reaction were mixed by gentle agitation , tube centrifuged briefly , then incubated at 37°C for 5 mins.. While this incubation was in progress , 4 eppendorf tubes were labelled for each reaction : A , C , G and T . To each of these tubes was added 2.5 µl of the appropriate termination mix ; these were then incubated at 37°C for at least 1 min. .

'A' mix includes 15mM ddATP
'C' mix includes 15µM ddCTP
'G' mix includes 15µM ddGTP
'T' mix includes 15µM ddTTP

Each mix also includes :

150µM dATP
150µM dCTP
150µM dGTP
150µM dTTP
10µM $MgCl_2$
40µM Tris - HCl (pH 7.5)
50µM NaCl

4.5 µl from labelling reaction was transferred to each of the pre-warmed tubes containing termination mixes and their contents mixed . Following 5 mins. incubation at 37°C , 5 µl of " stop solution " was added to each tube . Samples were either stored at - 20°C at this point or heated to 95°C for 4 mins. immediately prior to loading onto a pre - run gel .

Stop solution :

95% deionised formamide
20mM EDTA (pH 7.5)
0.05% (w/v) Xylene cyanol FF
0.05% (w/v) Bromophenol blue

Deionised formamide :

50 ml. of formamide mixed with 5g. mixed - bed , ion - exchange resin (Bio - Rad AG 501) ; stirred 30 mins. at room temp. ; filtered through Whatman No. 1 filter paper.

Sequencing gel :

42g Urea
10ml 10X TBE
15ml Acrylamide / Bis - acrylamide mix
Make up to 100 ml with water .

Sequencing gels were allowed to " age " overnight at room temperature before use . Gels were pre - run at 36 mA (constant

current) for approx. 1 hr. before samples were loaded . Samples were run 1.5 - 4 hrs. at 36 mA (constant current) . After run , gel was fixed for 1 hr. in :

10 % Methanol
10 % Acetic acid .

Gel was then dried for 2 hrs at 80^{o}C on a Savant gel dryer.

10X TBE :

0.9M Tris
0.9M Boric Acid
25mM EDTA

Acrylamide / bis - acrylamide mix :

38% (w / v) Acrylamide
2% (w / v) Bis - acrylamide

2.21 : DNA extractions from low melting point agarose

Relevant DNA digests were run through 0.8% LMP (low melting-point) agarose. Slices containing the fragments of interest were carefully cut from the gel , placed in 1.5 ml eppendorf tubes , and heated at 65^{o}C for 5 min. . Approximately 2 volumes similarly heated TE was added , and this was extracted with an equal volume phenol , also warmed to 65^{o}C. At least two further phenol extractions were performed (until aqueous / solvent interface was clear) ; three chloroform extractions were then performed, before DNA was precipitated by addition of 1/10th vol. 3M NaAc and 2.5 vol. ethanol , incubated at -70^{o}C for 20 min. (or at -20^{o}C overnight) . DNA ,

pelleted by centrifugation (10 min. , high speed , Microcentaur), was washed in 70% ethanol , similarly pelleted , then dried under vaccuum.

In an alternative process , gel slices were placed in the upper compartment of a 0.22μm " Spinex " tube (Stratagene / NBL) and centrifuged for 15 min. at high speed. Resultant solution in lower compartment of tube was phenol extracted twice , extracted twice with chloroform , then precipitated as above .

2.22 : Polymerase chain reaction

PCR mix was made up as follows :

Each primer (10μM)	10μl	
dNTP mix (2mM each)	10μl	
10X PCR buffer		10μl
DNA		10μl
Taq DNA polymerase		1.5μl
dH_2O		48.5μl

This was mixed and overlaid with 100μl mineral oil , to prevent evaporation . Reaction was incubated for 20 cycles in a Techne programmable heating block . Each cycle consisted of three sections:

DENATURATION :	1.5 min., 95^{o}C
ANNEALING :	1.5 min., 45^{o}C
EXTENSION :	3 min., 72^{o}C

Following 20 cycles as above, a " finishing extension " section of 3 min. at 72^{o}C was performed .

Mineral oil was carefully removed , aqueous phase extracted once with equal volume phenol then with equal volume chloroform , and DNA precipitated by addition of 1/10th vol. 3M NaAc , 2.5 vol. ethanol , and incubation at -20^{o}C overnight.

10X PCR BUFFER :

500mM	KCl
100mM	Tris - HCl (pH 8.3)
15mM	$MgCl_2$
0.1%	Gelatin (w / v)

2.23 : In situ hybridization

A) Sections

Whole embryos or adult *Helix* central nervous systems were fixed by incubation overnight at room temperature in ethanol / acetic acid (3 : 1 , v / v) (Dirks *et al* , 1989) prior to being mounted in paraffin wax (Paramat , BDH) and serial sections of required thickness cut (usually 5 or 10 μm) . Sections were attached to slides pretreated with 1% gelatin , 0.1% chrome alum in 30% ethanol by baking at 55°C for 2 hours (Dirks *et al* , 1989) , and postfixed for 10 mins. at 4°C in :

4%	Formaldehyde
0.1M	Sodium phosphate , pH 7.0

Slides were washed 3 x 5 mins in 2 x SSC at room temperature, then subjected to successive 10 min. washes in each of the following : xylene , 100% ethanol , 100% ethanol , 80% ethanol , 60% ethanol , 40% ethanol and 2 x SSC , in order to dewax and rehydrate the sections . To ensure binding of sections to the slides , slides were then exposed to UV - irradiation (two 30W lamps at a distance of 20 cm , total dose = 8×10^4 ergs / mm^2) (Tiedge , 1991). They were then washed for 10 mins. in 2 x SSC , and digested with 5mg / ml Proteinase K in 2 x SSC at room temp , prior to being washed in 2 x SSC (2 x 5 mins) and PBS (5 mins.) (Dirks *et al* , 1989). Prehybridization was for 1 hour under a coverslip in a moist chamber at 37°C , the humidity being produced by a solution

containing 4 x SSC , 50% formamide (Tiedge , 1991) ; 50µl of prehybridization solution was added to each slide . Probe was diluted to 5ng / µl in prehybridization solution and heated to 65°C for 5 mins. immediately before 50µl was added to each slide ; coverslips were added , and hybridization was at 37°C overnight in the moist chamber as described .

Following hybridization , slides were washed 3 x 10 mins. in 2X SSC at room temperature , once in 0.1 x SSC at room temp , and once in 0.1 x SSC at 60°C (Dirks *et al* , 1990) , then dehydrated by successive washes for 10 mins. each in 50% ethanol , 70% ethanol and 90% ethanol (each ethanol solution being 0.3M with respect to ammonium acetate) and air dried (Tiedge , 1991) . Before antibody staining slides were incubated for 1 hour at 37°C in PBS containing 0.05% Tween 20 (PBS/T) and 1% bovine serum albumin (BSA) ; antibody was diluted 1 : 100 in PBS/T plus 2% BSA , 50µl added to each slide , and a coverslip added . Incubation was for 1 hour at 37°C in the dark in a chamber humidified with PBS/T. Following 3 x 10 min. washes in PBS/T , sections were mounted in glycerol / PBS (9 : 1) containing antifade agent , and examined using a Leitz Ortholux Flourescence microscope with filter optimized for FITC. Photographs were taken using Kodak Tmax 3200 film.

Prehybridization solution (from Dirks *et al* , 1991) :

25%	Formamide
3X	SSC
0.1%	Polyvinyl pyrolidone
0.1%	Ficoll
1%	Bovine serum albumin
500µg / ml	denatured herring sperm DNA
500µg / ml	yeast tRNA

PBS :

130mM	NaCl

10mM NaPhos

B) Whole mounts

Whole ganglia or sets of ganglia were dissected from *Helix* , and as much connective tissue as possible was removed . These whole mounts were treated essentially as described by Tautz and Pfeifle (1989) , except that non - radioactive probes were used and modifications to treatments were made accordingly (e.g. samples were protected from light during washes following antibody staining) . After washing as described , mounts were incubated for 30 mins. in PBT + 1% BSA at room temp , then for 1 hr at room temp. with diluted antibody (1 : 100 in in PBT + 2% BSA) . Washes following antibody staining were 4 x 20 mins. in PBT at room temp.

2.24 : Radio immunoassays (RIA)

Following extraction of embryological material by us , amount of peptides present at each stage was estimated using radioimmunoassays . These procedures were carried out in the Whitney Marine Laboratories, University of Florida , by Dr. Wendy Lesser , essentially as described (Price $\varepsilon\tau$ $\alpha\lambda$, 1985) except that 2µl of each fraction was diluted into 48µl of RIA buffer for use in the assay . Antisera used were S253 , raised against YGGFMRFamide , and which therefore detects all the FaRPs ending in -RFamide, and Q2 , raised against the *Lymnaea* peptide EFLRIamide , which is specific for peptides with the C - terminal structure -Iamide . Dilutions of antibody Q2 were 1:250 in RIA buffer ; dilutions of S253 were 1:10,000 , also in RIA buffer . 1ml charcoal solution was added to each sample prior to counting , left for 10 mins. , then spun at 2500*g* for 10 mins. to pellet charcoal solution .

RIA Buffer :

10mM	NaH_2PO_4
1% (w / v)	BSA
0.9% (w / v)	NaCl
0.01% (w / v)	Merthiolate
25mM	EDTA
pH	7.0

Charcoal Solution :

0.25% (w / v)	Charcoal
0.025% (w / v)	Dextran
0.01% (w / v)	Merthiolate
0.1M	NaH_2PO_4
pH	7.5

CHAPTER 3

RESULTS

3.1 : Introduction

We sought to use the techniques available to us through molecular biology to elucidate the means by which FMRFamide- related peptides are encoded in the snail *Helix aspersa* , and to discover something of the means by which regulation of the production of these peptides is achieved . In addition , we investigated the expression of FaRP - specific mRNAs and FaRPs themselves during embryogenesis in this organism .

To initiate our investigations of the molecular biology of the FaRPs in *Helix aspersa* , we sought to isolate cDNA (complementary DNA) and genomic DNA clones encoding these peptides . Such an approach has been utilised in other systems , and has been invaluable in revealing in a number of these the existance of precursor polypeptide molecules , from which the production of mature , active peptides is directed . For example , the ELH (egg - laying hormone) system in *Aplysia* , which has yielded much information regarding the regulation of prohormone processing , has been ammenable to such studies largely due to the availability of DNA clones and their relative ease of manipulation (e.g. Newcomb and Scheller, 1987) .

Also in *Aplysia* , the means by which the FMRFamide - related peptides are encoded has been studied . Scheller and co - workers (Taussig and Scheller , 1986) have isolated a family of cDNA clones which encode precursor polypeptides containing the FaRPs found in *Aplysia* , FMRFa and FLRFa . Organization of these peptides in the precursor polypeptide is illustrated schematically in figure 1A . These investigators

Figure 3 : Oligonucleotides used in screening of cDNA libraries

Oligo-1

Peptide N-Phe-Met-Arg-Phe-amide-cleave

Precursor Phe-Met-Arg-Phe-Gly-Lys-

Coding 5' TTT ATG AGG TTT GGG AAA 3'
C A C

Oligo-3

Peptide pGln-Asp-Pro-Phe-Leu-Arg-Phe-amide

Precursor Gln-Asp-Pro-Phe-Leu-Arg-Phe-Gly-

Coding 5'CAA GAT CCG TTT TTG AGG TTT GG 3'
G T C C A C A C

utilised specific synthetic oligonucleotides in the screening of cDNA libraries . We have used the information gained from *Aplysia* as a basis from which to initiate similar studies of the FaRPs in *Helix aspersa* .

3.2 : Isolation of cDNA clones

Following the extraction of mRNA from *Helix* ganglia , cDNAs were synthesised and cloned according to standard techniques by Dr. E. M. Lutz . These " cDNA libraries " , containing sequences representative of all mRNAs expressed in the source tissue , were then screened in order to isolate clones which encoded FMRFamide - related peptides . Probes used in this screening process included FMRF - 1 , a cDNA clone isolated from *Aplysia* , kindly provided by Dr. R. Scheller (Taussig and Scheller , 1986) , and also synthetic oligonucleotide sequences , as detailed in figure 3 below . Initial screening of the cDNA library with oligo 1 , synthesised to be specific for the sequence encoding " - Phe - Met - Arg - Phe - Gly - Lys - " as illustrated , revealed a family of cDNAs , the most complete of which , clone HF1 , is illustrated in figure 4A .

The absence of sequences encoding N- terminally extended peptides known to be present in *Helix* (Price *et al* , 1990) , such as NDPFLRFa and SDPFLRFa , from all of these clones prompted the synthesis of several other oligonucleotides , including oligo 3 (see figure 3) . These oligonucleotides , which were synthesised , as indicated in figure 3 , taking account of the availability of several codons representing the same amino acid , were aimed at detecting cDNA clones encoding the N - terminally extended peptide pQDPFLRFa , also known to be present in *Helix* . A second , distinct , family of clones emerged from screening the cDNA library with these oligonucleotides - this is typified by the clone HF4 , illustrated in figure 4B .

Figure 4 : Sequences of FaRP - cDNA clones HF1 and HF4 isolated from *Helix aspersa* ganglia .

cDNA clones were isolated and sequenced as described : analysis of these sequences revealed that multiple copies of FMRFamide - related peptides were encoded in each clone . The most complete cDNA clone of each " family " isolated is shown here .

A : HF1 - Complete nucleotide sequence is shown , and translation of open reading frame encoding tetrapeptide

FaRPs shown . Peptides are shown underlined and in **bold** type ; single copy of FLRFamide is double underlined . Basic residues , thought to be important in directing cleavage of peptides from precursor are shown in *italics* .

B : HF4 - As in A above , peptides are shown underlined and in **bold** type , and basic residues are in *italics* .

```
          10         20         30         40         50         60
TTTTTTTTTTTTTTCTTATGCTCCTTTCATTTGATATTTTGTTTTCTTGTTTTTGACGTAA
          70         80         90        100        110        120
TTGCACTGTGCCAGTGTAAAGAAGATTATATAGATGGGTAATGTCATAAACTTTATTTCA
         130        140        150        160        170        180
AAAGACTAAGAACTGGGCACACAGGCTTGTTGAGAGAAACTAAGGTACCTGGGAATCGCC
         190        200
TTCGATAGAAACCTGTAG AAG CGA GTT GCC CGT TCA GCT GAC GCT AAC CAA
                   Lys Arg Val Ala Arg Ser Ala Asp Ala Asn Gln

        240        250        260        270
CAA TCT AAA AAT ACA CAA AGT AAC AAA TTT GGA AAG GAT TTG CAA AAG
Gln Ser Lys Asn Thr Gln Ser Asn Lys Phe Gly Lys Asp Leu Gln Lys

 280        290        300        310        320
AGG GAA ACA AAA AAG GAA AAG TTA AAT GCA AAT GAT GAT CTT GAG ATT
Arg Glu Thr Lys Lys Glu Lys Leu Asn Ala Asn Asp Asp Leu Glu Ile

        330        340        350        360
CTA TCA AAC GAG GAT GAT CTA GAA AAA AAG TTT ATG AGG TTC GGA AAA
Leu Ser Asn Glu Asp Asp Leu Glu Lys Lys Phe Met Arg Phe Gly Lys

 370        380        390        400        410
CGT TTT ATG AGG TTT GGA AGA GGA GAT GAA GAT GAA AGT TAC GAT AAA
Arg Phe Met Arg Phe Gly Arg Gly Asp Glu Asp Glu Ser Tyr Asp Lys

        420        430        440        450
AGA TTC ATG AGG TTT GGG AAA AGC CTT CGG CAT GAC CAG GAA TTT GAA
Arg Phe Met Arg Phe Gly Lys Ser Leu Gly His Asp Gln Glu Phe Glu

 460        470        480        490        500
AAG AGG TTC ATG AGA TTC GGG AAA CGA TTT ATG AGG TTC GGT AGA GGC
Lys Arg Phe Met Arg Phe Gly Lys Arg Phe Met Arg Phe Gly Arg Gly

        510        520        530        540
GAC GAG GAC GAT GCT CGC GAA GAG AAG CGG TTC TTG AGG TTC GGG AAA
Asp Glu Asp Asp Ala Arg Glu Glu Lys Arg Phe Leu Arg Phe Gly Lys

 550        560        570        580        590
AGT ACG AAT GAA GAT GAG GAT ATT AAG AAA CGA TTC ATG AGG TTC GGT
Ser Ser Asn Glu Asp Glu Asp Ile Lys Lys Arg Phe Met Arg Phe Gly

        600        610        620        630
AAA AGT GGA AAC GAA GAT GGA GAT GTT GAC AAA AGA TTC ATG AGG TTC
Lys Ser Lys Asn Glu Asp Gly Asp Val Asp Lys Arg Phe Met Arg Phe

 640        650        660        670        680
GGC AAG CGG TTT ATG AGG TTC GGG AAA AGT GAA AAA GAG GAT GGA GAT
Gly Lys Arg Phe Met Arg Phe Gly Lys Ser Glu Lys Glu Asp Gly Asp

        690        700        710        720
GTT GAC AAA AGA TTC ATG AGG TTC GGC AAG CGG TTT ATG CGA TTT GGA
Val Asp Lys Arg Phe Met Arg Phe Gly Lys Arg Phe Met Arg Phe Gly

 730        740        750        760        770
CGT GGG GAT TCA GAA ACA TCA TGA  GGTTCGTTGAGCCATTTCTGAGATTGGGAC
Arg Gly Asp Ser Glu Thr Ser Stop

        780        790        800        900        910
GTGGGGAGAGAAACACATCATGAGGTTAGGCCAGAGTTTTAGAAGGTTTAAAGTGGAAATA

   920        930        940        950        960        970
CTAAAGACAACAGTGACAACTTTGGTAAAGACTTCCAAAACATAGTTTTTTATGAACTCGA

   980        990       1000       1010       1020       1030
CAAAACAAGTACAAGATTTCCAAATTGTTTTTTTTTTCCATTATCGGTAGTAGGGTTCTAG
  1040       1050       1060       1070       1080       1090
CAATATCATCATCATGATTCATGCTTCATCATATCACGAAGCTTCTCTAGCAGTTTCCTGT
  1100       1110       1120       1130       1140       1150
GCTAGTGCTGTTGTTCCCCCCCCCCCCCCCCTATTTTCTGGCCAGTCGGCTGCTCCAACTGC
  1160       1170       1180       1190       1200       1210
GCTCTACACATAGACGTCATAGTACATTTTAAGCACTGTGTCCATATTACGGCGCTTATG
  1220       1230       1240       1250       1260       1270
CAAAGCCTTCTCGTTCTACAACTAACTAGCTACCACAGCGAATATCAGCAATTTTGTGAT
  1280       1290       1300       1310       1320       1330
CGTTTTGCAACAATGTGACGCAACGAATACCAACATCTTCTCATCTCGCAACTAACTGAT
  1340       1350       1360       1370       1380       1390
GCAGCGAATGTCAACATCTTTTCGTTCTGCAATAATTTTCTGACGCAGGCAATGCCAGCA
  1400       1410       1420       1430       1440       1450
CCATCTTTTCGTCTTGCCAACAATTTGACCTAGCGAATCGTAACATTTCTAGTCTTGCAA
  1460       1470       1480       1490       1500       1510
CTTTCTGACGCAGCGAATGCAACATCTCTTCGTCTGCAACTAACTGACTCAGCGAAAGCC
  1520       1530       1540       1550       1560       1570
AGCAACATCTTTTCATCCTGCAATTAACTATGTGACGCAGCGAATACCTGCAACAAATTC
  1580       1590       1600       1610       1620       1630
TCTGCTTGCGACTATCTTACGCTACAAATACCAGAACATCTCATGTTTCAATTAATTGAC
  1640       1650       1660       1670       1680       1690
GCAGCAAATAAAAACTAAACTAAACTATACCAACATATATTATCTTGCACCTATCTGACG
  1700       1710       1720       1730       1740       1750
CAGCGAATGCCAACATATTCTCGTCTTGCAACTATCTGACGCAGCGAATGCCAACATATT
  1760       1770       1780       1790       1800       1810
CTCGTCTTGCAACTATCTGACGCAGCGAATGCCAACATATTCTCGTATTGCAACTATCTG
  1820       1830       1840       1850       1860       1870
ACGCAGCGAATGCCAGCAACAATCGCGTAACATCAACATTGTTCTCTCCTGTACTCTAGA
  1880       1890       1900       1910       1920       1930
CAAACAAGCACAATGCAATTAATGGCACTTATTTTCAATGACATTTGGAATCTATATAAA
  1940       1950
CGCCTATGGGAATTCC
```

A

```
         10         20         30         40         50         60
CAGTAAACGTAAGGACGATAGCTTGCTAACATCACAAGTCGACAACCACTTGAGTAGAGC
         70         80         90        100        110        120
GGGACTTTGTTAGCACATTTTAGCTCACGTTTAGTTTCTAAAGAAAAACGTCTCCACTTC
        130        140        150        160        170        180
GCCACTCTTCGATTTGGAAACCAGCATAGCTCAAGGTGTACACTCAAGTCTCACAAGCAA
        190                200        210        220
CTACAGATCA  ATG ACT AGT CTG TGC CTC ACC ATC GCC CCG GCC GTG CTC
            Met Thr Ser Leu Cys Leu Thr Ile Ala Pro Ala Val Leu

230         240         250         260         270
AGT CTC ATC TGC CTG TCC TCG TAT GGG TGG GCT GAA GGT GAC ACC ACG
Ser Leu Ile Cys Leu Ser Ser Tyr Gly Trp Ala Glu Gly Asp Thr Thr

 280         290         300         310         320
GAC AAT GAG TAC TTG AGG TTC GGC CGT GAG AAT AAC AAC GGT TAC ATT
Asp Asn Glu Tyr Leu Arg Phe Gly Arg Glu Asn Asn Asn Gly Tyr Ile

    330         340         350         360         370
AGA TTT GGG AGA AAC GAT CCG TTT TTG AGA TTC GGC AAG AAG AGC GAT
Arg Phe Gly Arg Asn Asp Pro Phe Leu Arg Phe Gly Lys Lys Ser Asp

        380         390         400         410         420
CCC TTC CTA AGG TTT GGT AAA CAG GAT CCA TTC CTG AGG ATC GGC CGC
Pro Phe Leu Arg Phe Gly Lys Gln Asp Pro Phe Leu Arg Ile Gly Arg

            430         440         450         460
CAA GAT CCC TTT CTG AGG TTT GGG AAA CAG GAT CCA TTT TTG AGG TTC
Gln Asp Pro Phe Leu Arg Phe Gly Lys Gln Asp Pro Phe Leu Arg Phe

470         480         490         500         510
GGA AAA CAG GAT CCC TTC TTG AGG ATC GGA AAA CAG GAT CCG TTT TTG
Gly Lys Gln Asp Pro Phe Leu Arg Ile Gly Lys Gln Asp Pro Phe Leu

 520         530         540         550         560
AGG TTT GGA CGC AGT GAG CCT TAT CTG AGG TTT GGC AGG AAT GAT CCA
Arg Phe Gly Arg Ser Glu Pro Tyr Leu Arg Phe Gly Arg Asn Asp Pro

    570         580         590         600         610
TAT TTG AGG TTT GGC AGG AAT GAT CCC TAT TTG AGA TTT GGC AGA AAT
Tyr Leu Arg Phe Gly Arg Asn Asp Pro Tyr Leu Arg Phe Gly Arg Asn

        620         630         640         650         660
GAT CCA TAT TTG AGG TTT GGC AGG AAT GAT CCT TAT TTG AGG TTC GGA
Asp Pro Tyr Leu Arg Phe Gly Arg Asn Asp Pro Tyr Leu Arg Phe Gly

            670         680         690         700
AAG AAT GAC CCG TTT TTG AGA TTT GGC AAA AGT GTT GAC GGT GAG ATC
Lys Asn Asp Pro Phe Leu Arg Phe Gly Lys Ser Val Asp Gly Glu Ile

710         720         730         740         750
GAG GCA GGT GTT GAT GCA GTG ACT TTG TCC AGG GAA CAC GAA TTC
Glu Ala Gly Val Asp Ala Val Thr Leu Ser Arg Glu His Glu Phe
```

B

Extensive re - screening of the cDNA library , with both the oligonucleotides and clones as probes , identified further clones which were members of of both the HF1 and HF4 families , but failed to reveal any clones more complete than these .

As detailed in Chapter 4 , and evinced by probing mRNA "Northern" blots (see below) , we have reason to believe that neither of these clones is a complete representation of the mRNA from which they are derived . However , it is apparent that HF1 and HF4 are representative of two distinct mRNA species , one of which encodes the tetrapeptides FMRFa and FLRFa , while the other codes for a variety of N - terminally extended peptides . We were unable to isolate a single clone which contained sequences specifying both tetra - and extended peptides , and believe that such a species of mRNA does not exist in *Helix aspersa* .

3.3 : Southern blots

Although cDNA clones are generally more easily isolated than are genomic clones , and are often useful in the initial determination of the nucleic acid sequences encoding a protein or peptide , they are of necessity lacking in other potentially useful pieces of information , such as *cis* - control sequences , intron position and structure and potential alternative splicing patterns . It is therefore of interest to isolate the relevant genomic DNA fragments containing these sequences .

To this end , genomic DNA isolated as described from *Helix aspersa* ovotestes was digested overnight with a variety of restriction enzymes . (Gonads were used as a source of DNA not only because of their obvious DNA richness , relative to

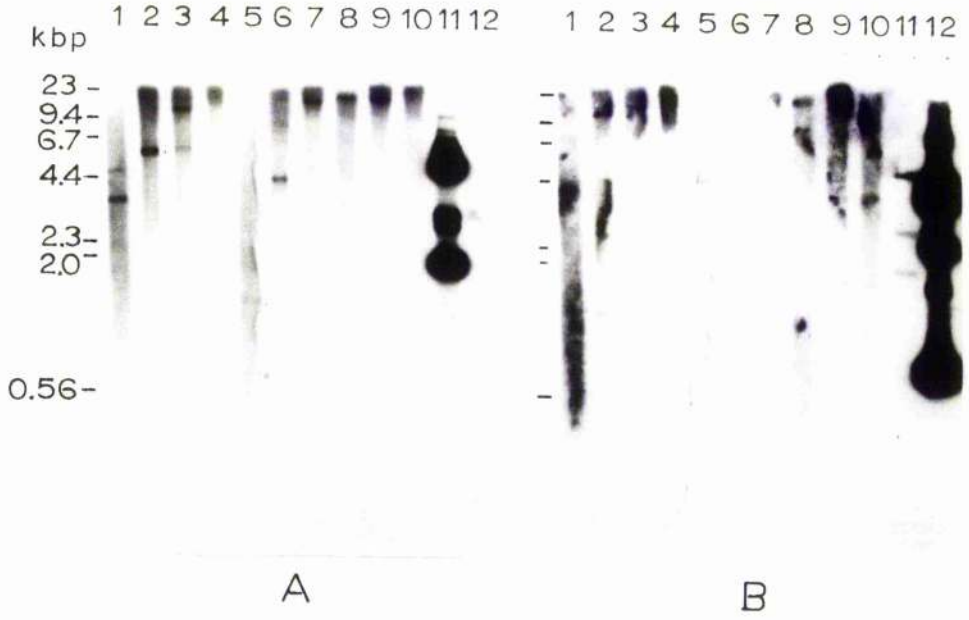
other cellular components , but also to avoid potential problems due to DNA rearrangements , known to occur in certain molluscan tissues (Chase and Tolloczkso , 1987)) . DNA was then size separated by electrophoresis through 0.8% agarose , and transferred to a nitrocellulose filter as described . This filter was sequentially hybridized with ^{32}P - radiolabeled DNA probes derived from the cDNA clones HF1 and HF4 ; the autoradiographs resulting from these hybridizations are illustrated in figure 5 A and B . A number of bands are seen - in most cases , DNA digested with a particular restriction enzyme contains two fragments of different sizes which hybridize with each of the probes (figure 5 A and B) . The approximate sizes of these DNA fragments , as estimated from a calibration curve derived from DNA markers of known size , are shown in Table 2 . It can be seen that the probes HF1 and HF4 usually bind fragments from the same restriction digest which differ significantly in size: the observation that this is true for a number of restriction enzymes indicates that the DNA sequences detected by these two probes are not contiguous in the genome , but are instead separated by a number of kilobase pairs (kb) of DNA . Other data derived from further analysis of genomic DNA by Southern blots (Lutz 92) suggests that HF1 and HF4 may detect a common 14 Kb Eco RI fragment (figure 5 , C and D) , and may therefore be separated by less than this distance in the genome . It is possible that the genomic Eco RI fragment defined by the restriction recognition sites at the 3' end of the cDNA clone HF4 and beyond the 5' end of clone HF1 (as indicated by PCR analysis (Lutz 92) comprises this putative single genomic restriction fragment : this is illustrated schematically in figure 6 .

The possibility cannot be discounted , however , that the Eco RI DNA fragments here observed as binding the HF1 and HF4 probes may in fact be two different fragments which are of the same or similar size , and are therefore indistinguishable by this technique . Until the fragment(s) concerned can be isolated and studied , the way in which the DNA sequences represented in cDNA clones HF1 and HF4 are arranged in the genome will remain unclear .

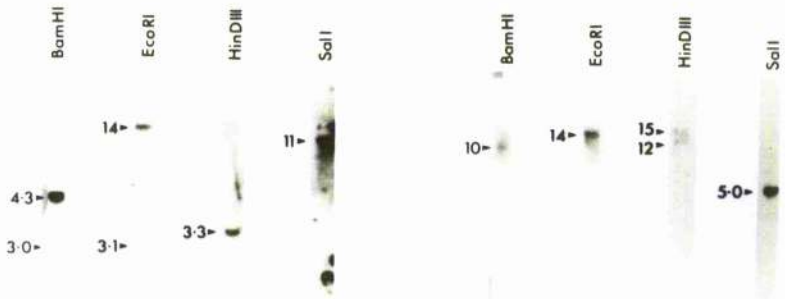
Figure 5 : Southern blots of *Helix* DNA .

A and B : 50µg of *Helix* genomic DNA was digested to completion with restriction enzymes as indicated , separated on a 0.8% agarose gel and blotted by standard techniques onto nitrocellulose . Filter was prehybridized and hybridized as described : hybridization was successively with (A) HF1 and (B) HF4 specific probes , and filter was washed to 1 X SSC , 0.1% SDS , 37^{o}C , 2 X 30 mins. in each case . Positions of λ Hind III DNA marker fragments are indicated ; calculated sizes of genomic DNA fragments hybridizing with probes are shown in Table 2 . Panels (C) and (D) show results of a similar experiment carried out by Dr. S. Hettle (see Lutz *et al*, 1992) , and illustrates the hybridization of both probes with an EcoRI fragment of similar size (approx. 14 kb) . A discrepancy between the apparent sizes of fragments observed hybridizing in the experiment shown in panels (A) and (B) and that illustrated in panels (C) and (D) is probably due to an error in the calculation of relevant fragment sizes.

Track 1 : Acc I
2 : Bam HI
3 : Bsc I
4 : Eco RI
5 : Hinc II
6 : Hind III
7 : Kpn I
8 : Pst I
9 : Sac I
10 : Sma I
11 : 1µg HF1 plasmid DNA , Eco RI digested .
12 : 1µg HF4 plasmid DNA , Eco RI digested .


kbp
1 2 3 4 5 6 7 8 9 10 11 12
23 -
9.4-
6.7-
4.4-
2.3-
2.0-
0.56-
1 2 3 4 5 6 7 8 9 10 11 12

A B


BamHI
EcoRI
HindIII
SalI
14►
11►
4·3►
3·0►
3·1►
3·3►
BamHI
EcoRI
HindIII
SalI
10►
14►
15►
12►
5·0►

C D

Table 2A : Approximate Sizes of DNA Fragments Hybridizing with HF1 Probe

ENZYME	Distance moved on gel (mm)	Size of HF 1 Hybridizing Fragment(s) (Kb)
Acc I	30 36.5	4.6 3.4
Bam HI	25 62	5.95 1.1
Bsc I	15 24.5	12.3 6.2
Eco RI *	–	14.2
Hinc II	61 79	1.18 0.58
Hind III	19 32	8.6 4.2
Kpn I	13.5 29.5 37	14.5 4.8 3.3
Pst I	12.5 43	16.2 2.55
Sac I	13 29 37	15.3 4.9 3.3
Sma I	–	–

* : Information obtained from Southern blot by Dr. S. Hettle

Table 2B : Approximate Sizes of DNA Fragments Hybridizing with HF4 Probe

ENZYME	Distance moved on gel (mm)	Size of HF 1 Hybridizing Fragment(s) (Kb)
Acc I	34.5	3.7
Bam HI	17	10.1
Bsc I	14.5	13
Eco RI *	-	14.2
Hinc II	-	-
Hind III	-	-
Kpn I	20.5 46.5	7.6 2.17
Pst I	12.5 19	16.5 8.4
Sac I	13	15.3
Sma I	35	3.6

* : Information obtained from Southern blot by Dr. S. Hettle

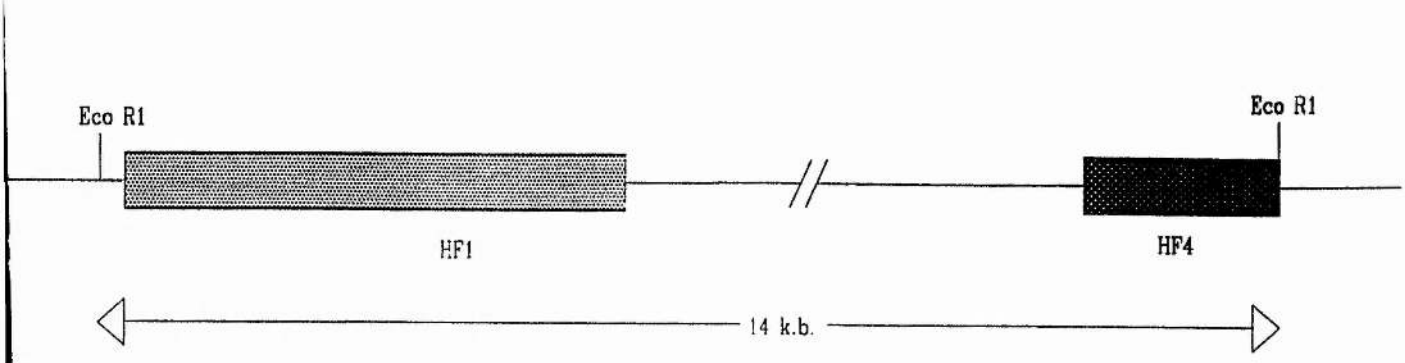


Figure 6 : Putative arrangement of tetrapeptide and heptapeptide - encoding DNA sequences in the genome of *Helix aspersa* .

The DNA regions encoding the tetra and heptapeptides are thought to lie on a common 14kb Eco RI fragment of the *Helix* genome . PCR analyses performed by Dr. D. Price indicate the existance of an Eco RI recognition site immediately 5' to the tetrapeptide - encoding sequences ; the cDNA coding for the heptapeptides terminates in a similar site , and this is proposed to define the 3' end of the common Eco RI genomic DNA fragment .

Despite various attempts to clone and isolate genomic DNA fragments containing these sequences , including the construction of *Helix* genomic libraries in λgt10 , utilisation of the "inverse PCR" technique (see figure 7) , and the isolation and subsequent attempted cloning of DNA fragments of the relevant sizes from restriction digested DNA separated on agarose , this has not proved possible to date .

PCR (polymerase chain reaction) studies carried out by Dr. D.A. Price at the University of Florida , using material supplied by us , have revealed the presence of a further 2 copies of sequences coding for FLRFamide present in the genome of *Helix pomatia* , upstream of the FMRFamide sequences found in our cDNA clones (shown boxed in figure 1E . This study also reveals a restriction site polymorphism , an Eco RI site being present in this region of *H. aspersa* genomic DNA , but which is not found in *H. pomatia* due to an A to C base change (Lutz 92).

RNA STUDIES

3.4 : Introduction

Having gained some information regarding the structure of mRNA molecules encoding the FaRPs in *Helix* , and observed the apparent dichotomy between those sequences specifying the production of tetrapeptides and those for the N- terminally extended peptides , we wished to investigate the expression of these mRNAs in this organism . This we did by specific extraction of mRNA molecules from a variety of tissue sources : the possession by mature mRNA of a poly A " tail " enables the selection of such molecules from among other material by means of its affinity for oligo dT sequences . mRNA is subsequently easily detached by alteration of the salt concentrations of washing solutions . Material thus purified can be analysed in a number of experimental systems , some of which were utilised as detailed below .

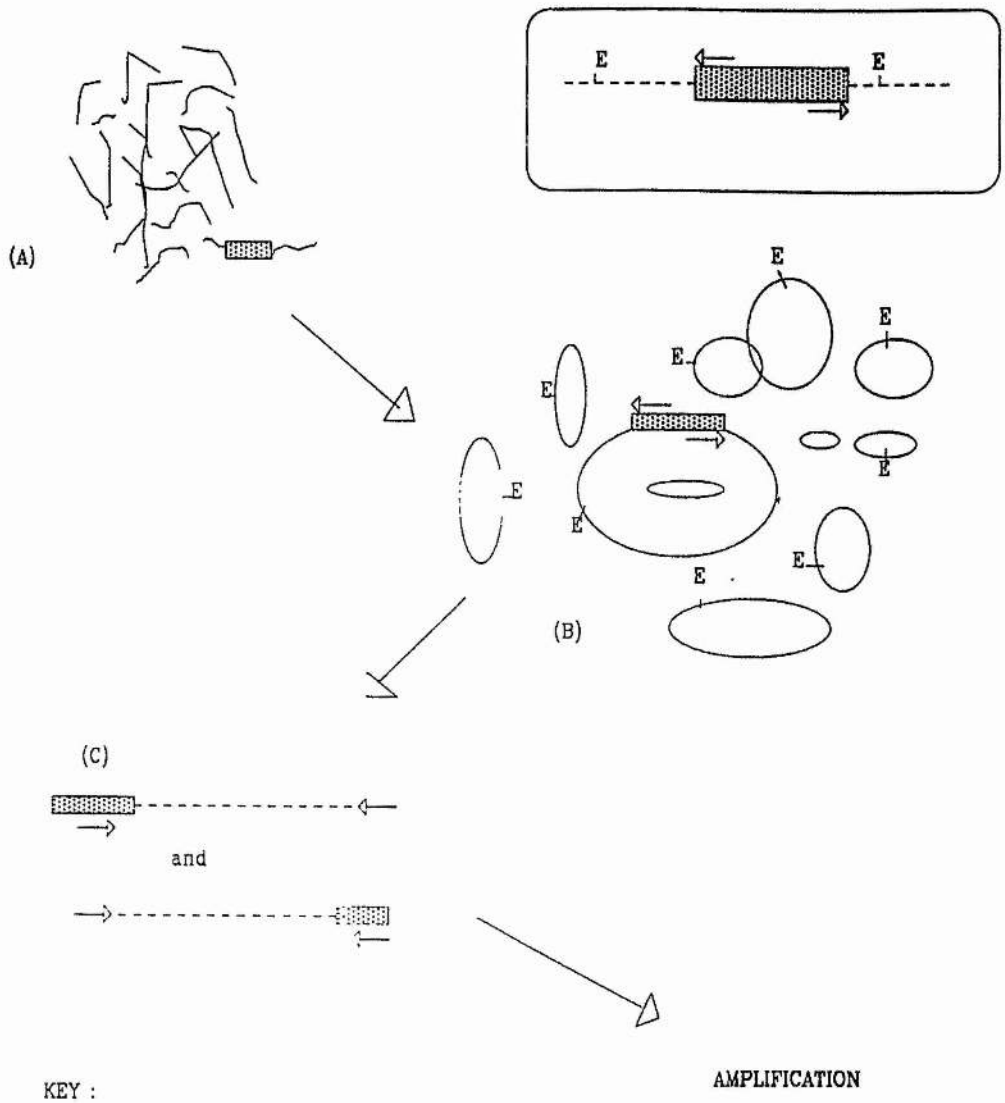


Figure 7 : Inverse PCR .

The method of design of oligonucleotide primers used in inverse PCR (polymerase chain reaction) , such that polymerisation will proceed beyond the ends of a known DNA sequence , is illustrated in the boxed section . Genomic DNA which was digested with restriction enzyme E , for which there is no recognition site within the known sequence of interest (a) is ligated to form circles (b) : this is then used in a PCR reaction with the primers described , producing DNA molecules containing unknown sequence in addition to the primer binding sites which are required for continuation of the PCR reaction (c) . Further rounds of PCR will therefore result in specific amplification of these sequences , which can then be isolated and studied .

We were also interested to elucidate the sites of synthesis of FaRP - specific mRNA within the central nervous system of *Helix* . *In situ* hybridization , by which mRNA is detected in histological preparations , was the ideal method for such investigations : for a variety of reasons we chose to detect hybridization using flourescence . This yielded some interesting results , as did analyses of mRNA isolated from specifically dissected groups of cells or ganglia using radioactive probes . (Fluorescent detection systems are inappropriate for use with filter hybridizations ; other non - radioactive methods of hybrid detection , such as alkaline phosphatase , are not conducive to analysis using densitometry . Notwithstanding their acknowledged drawbacks , radioactively labeled probes were therefore judged to be the most useful available for slot and Northern blot experiments .)

3.5 : Northern blots

In order that the nature of the mature messanger RNA molecules encoding the FaRPs be elucidated , RNA was isolated from whole juvenile (approx. 2 - 3 month old) *Helix aspersa* as described . Messenger RNA was selected from this total RNA by means of oligo dT cellulose , the possession by mRNA of a poly A tract facilitating its binding to the resin . Following size separation on denaturing formaldehyde gels and transfer to nylon filters as described , mRNA containing sequences coding for the FMRFamide related peptides was detected using specific ^{32}P labelled antisense riboprobes derived from the cDNA clones HF1 and HF4 , as illustrated in figure 8 . Probe TB1 is a subclone of HF1 , made by cloning the 750 bp Taq I fragment of HF1 into the Acc I site of the plasmid vector Bluescript (see figure 2) . Antisense riboprobes were used since such RNA probes are single - stranded : higher probe specificity and labeling is therefore achieved . As shown in figure 9 , both these probes are seen to hybridize with mRNAs of similar size - approximately 1.7 - 1.8 kb. . An mRNA

Figure 8 : Riboprobe Structures .

Specific single - stranded RNA probes were used in a number of experiments : these riboprobes (RPs) were produced from cDNA clones or subclones as illustrated , using bacteriophage RNA polymerases and appropriately labelled nucleotides . Scale at bottom of figure indicates lengths of clones and probes in kilobasepairs ; restriction enzyme sites in (A) and (B) as indicated .

R = Eco RI
D = Hind III
M = Mbo II
T = Taq I

(A) Riboprobe TF1 was produced from a Taq I subclone of HF1 - this was linearised with Acc I prior to RP synthesis , and resulted in a probe which was complementary to the tetrapeptide - encoding mRNA to the extent indicated by the arrow . Also shown is MF4 RP , which was utilised in some early experiments, but which was superceded by the more specific probe TF1 . Hatched areas indicate extent of FMRFamide - encoding regions in clone . AAUAAA indicates position of consensus polyadenylation signal .

(B) Riboprobe MF4 was synthesised following digestion of the cDNA clone HF4 with Mbo II : it too resulted in an anti - sense probe complementary to the region indicated by the arrow. Cross - hatching indicates the extent of area of clone encoding FaRPs ; AUG indicates position of putative initiation codon .

(C) Integrity and relative sizes of riboprobes used were checked by running an aliquot on a formamide - containing agarose gel : track 1 contains TF1 RP and track 2 MF4 RP . Approximate sizes of probes (in bases) are indicted to left of panel .

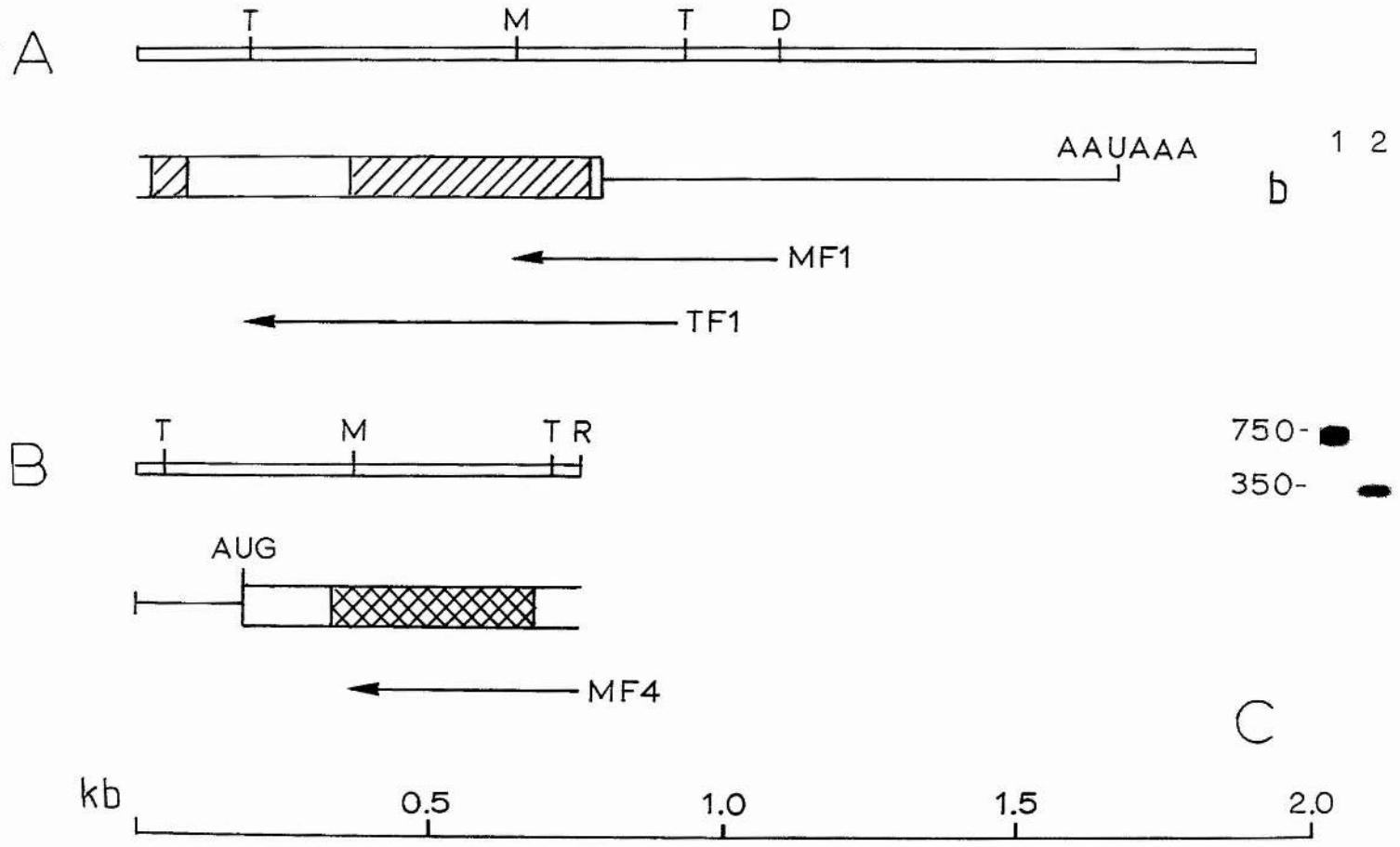
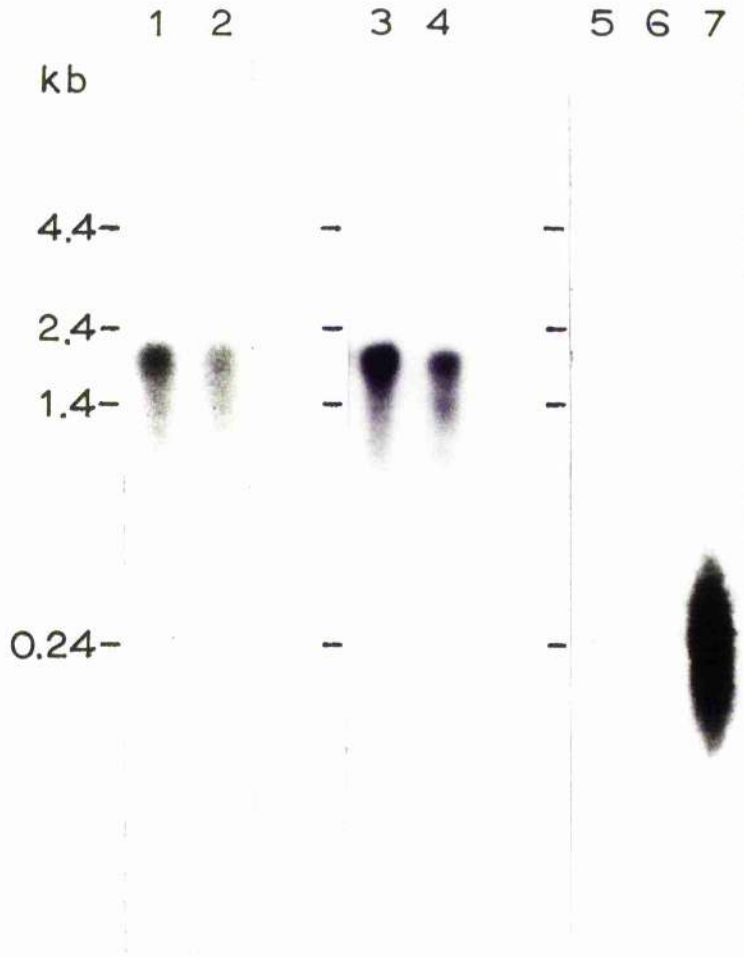

A
T
M
T
D
AAUAAA
1 2
b
MF1
TF1
B
T
M
T R
750-
350-
AUG
MF4
C
kb
0.5
1.0
1.5
2.0

Figure 9 : Northern Blot of *Helix* mRNA .

RNA was extracted from whole juvenile *Helix* as described. mRNA was separated from other nucleic acids , by means of oligo dT cellulose , and run on a gel . Identical aliquots of fractions were run on both halves of a formaldehyde - containing agarose gel , in order to produce duplicate filters on transfer of RNA to nylon . These filters were hybridised with riboprobes - Tracks 1 and 2 with TF1 ; Tracks 3 and 4 with MF4 . Approximate positions of size markers , in kilobases , are shown . Tracks 5 - 7 were probed with an TF1 sense riboprobe - i.e. which hybridizes with TF1 - specific antisense RNA . Filters were washed as described to a stringency of 0.5 X SSC , .01% SDS , 65^0C . These results indicate that antisense probes specific for tetrapeptides and extended peptides hybridize mRNAs of similar but slightly differing sizes (estimated to be 1.8kb in the case of TF1 (tracks 1 and 2) and 1.7kb in the case of MF4 (tracks 3 and 4)) . There appears to be approximately 5X as much MF4 - specific mRNA as there is TF1 - complementary mRNA present in these samples .

Tracks :

1. 10µg poly $(A)^+$ RNA
2. 2µg poly $(A)^+$ RNA
3. 10µg poly $(A)^+$ RNA
4. 2µg poly $(A)^+$ RNA
5. 10µg poly $(A)^+$ RNA
6. 2µg poly $(A)^+$ RNA
7. 2µg partially hydrolysed DIG - labelled antisense TF1 probe .


1 2 3 4 5 6 7
kb
4.4-
2.4-
1.4-
0.24-

size is not sufficient to contain both the sequences represented in HF1 and HF4 in the same molecule . In the absence of definite evidence to the the contrary , it was therefore tentatively concluded that two mRNA species of similar length but different primary structure are being detected by these probes . Also illustrated in figure 9 is the specificity of single - stranded riboprobes : no hybridization with "sense" mRNAs is observed , while antisense riboprobe (used in *in situ* experiments) hybridizes this probe sense RP strongly

Our attempts to define the distance between the 5' ends of our cDNA clones and the 5' ends of the relevant mature messenger RNA molecules using the " primer extension " technique , whereby an antisense oligonucleotide primer is annealed to the message and is extended using the enzyme reverse transcriptase and the mRNA as a template , did not meet with success . This was also true of our attempts at RNA sequencing ; we were therefore unable to draw any definite conclusions regarding the structure of FaRP - encoding mRNAs distal to that which we knew from cDNA clones .

3.6 : RNA expression during embryogenesis

Slot blots

Nucleic acids from *Helix aspersa* embryos at different times during development were isolated and analysed , in order to study the expression of mRNA representing the FMRFamide related peptides . RNA was oligo dT selected to isolate mRNA from other nucleic acids included in precipitates . Figure 10 shows the approximate amount of mRNA derived from embryos from each day during development and immediately post - hatching (hatching usually occurs on day 19) . This mRNA from embryos at different developmental stages was applied to filters as described using a BRL " Hybri - slot " slot blotting

apparatus , and hybridized with ^{32}P labelled antisense riboprobes derived from cDNA clones HF1 and HF4 . The riboprobe used in HF4 studies was obtained by first digesting the parent plasmid with the restriction enzyme Mbo II to linearise . The HF1 - specific riboprobe was derived from a subclone of the whole HF1 clone : the Taq I fragment includes the peptide - encoding region from HF1 , but little else (see figure 8). When this was subcloned into the Acc I site of Bluescript vector (figure 2) one of the Acc I sites was destroyed : linearisation of the subclone for use in riboprobe syntheses was therefore achieved by digestion with Acc I . Using these probes it was possible to estimate the level of expression of mRNAs complementary to them throughout embryogenesis. As can be seen from the resultant autoradiographs (figure 10) , the presence of the HF 4 mRNA, encoding the extended peptides is apparent from approximately day 8 , while levels of HF 1 - like mRNA are not detectable until around day 10 . The amount of mRNA in each slot is approximately equivalent to that derived from a single embryo of that age .

As is graphed in figure 11 , when the autoradiographs shown in figure 10 are analysed using densitometry , a cyclical pattern of expression of these mRNAs is apparent . Results with both probes indicate that the level of expression of these mRNAs is not constantly increasing during development , as is the case for the general message population (see figure 12) , but rather appears to undergo a number of cycles , each rising and falling taking place over a period of 4 - 5 days of development . This is true for the absolute amounts of RNA deduced to be hybridizing with the probes (figure 11) , and also for the percentages of the total mRNA population of each slot estimated to be hybridizing with the probes (figure 13) . Absolute amounts of FaRP - encoding RNA present were estimated from densitometric analysis of probe signals : in the case of HF 4 , the densitometric reading obtained from the scan of the HF 4 control slot (slot 12b , figure 10(B)) , containing 1ng of HF 4 -

Figure 10 : Slot-blots of RNA extracted from *Helix* embryos .

Helix embryos isolated during each day of development and 3 days post - hatching were used as a source of mRNA as described . An amount of mRNA equivalent to that contained in a single embryo (as detailed in figure 12 and table 4) was applied to duplicate nylon filters *via* a slot - blotter, and these were hybridized with riboprobes (A) MF1 and (B) MF4 . Following washing to a final stringency of 0.5X SSC , 0.1% SDS , 65^{o}C , these were exposed for 3 (3d) or 14 days (14d) . As can be seen , hybridization with the MF4 probe (panel B) is significantly stronger than with MF1 (panel A) , presumably reflecting the presence of a greater amount of heptapeptide - encoding mRNA (approximately equivalent amounts of radiolabelled probe was used in each hybridization) . 14 day exposure of the MF4 - probed filter (B 14d) is therefore overexposed , but such an exposure is necessary in order to detect significant amounts of hybridization with probe MF1 (A 14d) .

Slots :

1a - 12a : Embryos Day 1 - Day 12 .
1b - 10b : Embryos Day 13 - Day 22 .
11b : 1µg MF1 DNA control .
12b : 1µg MF4 DNA control .

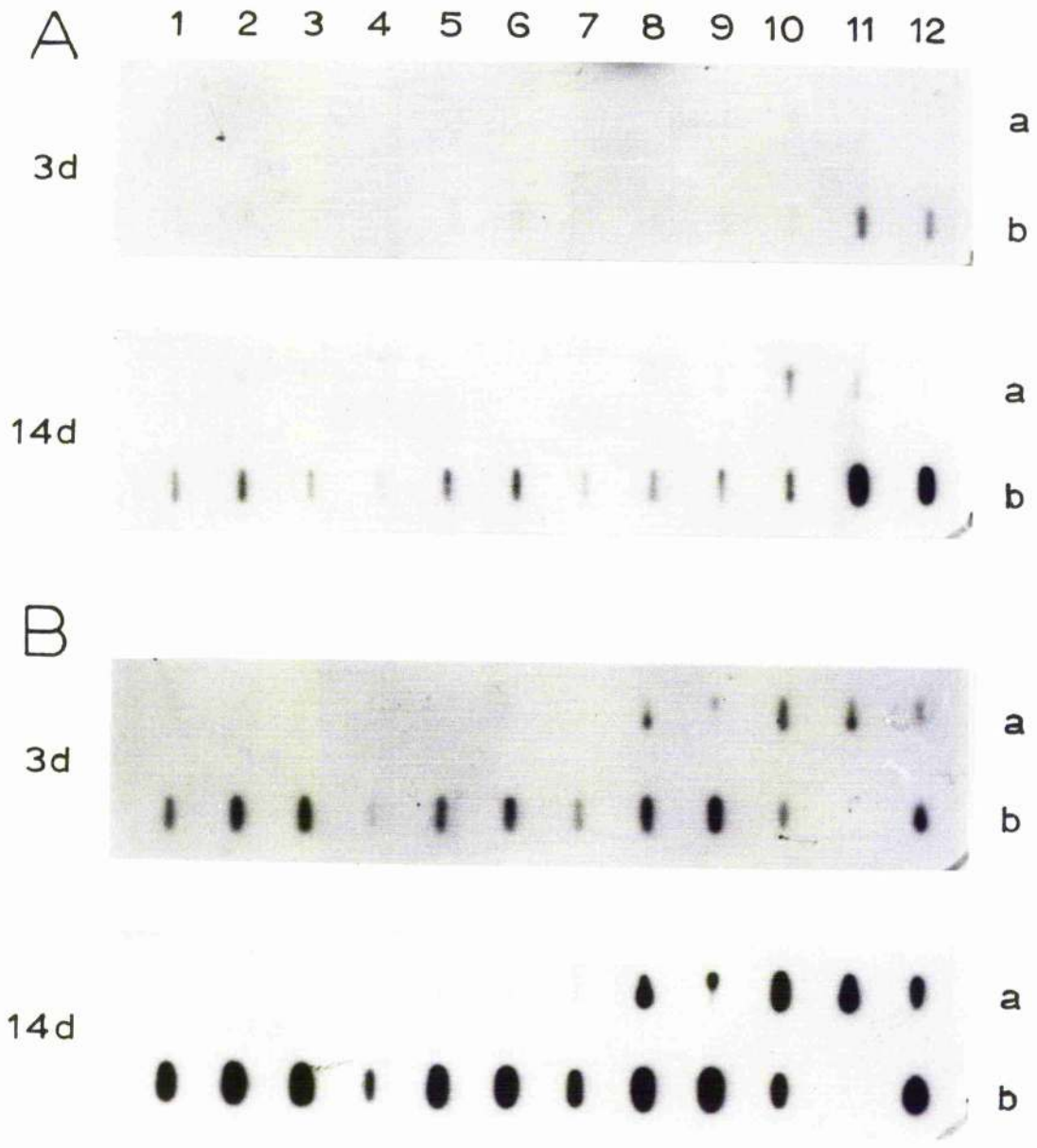

A
1 2 3 4 5 6 7 8 9 10 11 12
3d
a
b
14d
a
b
B
3d
a
b
14d
a
b

Figure 11 : Graph of pg of RNA calculated to be hybridizing with FaRP - specific antisense riboprobes .

Approximate amounts of FaRP - specific mRNA calculated by densitometric analysis of slot - blots in figure 11 to be present in *Helix* embryos during development is graphed . Densitometric reading for appropriate control slots , containing plasmid DNA equivalent to 1µg of probe DNA , was assumed to represent 1000pg : densitometric readings for experimental tracks (1a - 10b in each panel) were divided by this reading , giving the results shown in Table 3 .

These figures were used in drawing this graph , which illustrates that the amount of FaRP - specific mRNA detected in *Helix* embryos does not follow a pattern of gradual increase , as does the total mRNA isolated from these embryos (see figure 13) , but rather appears to rise and fall during embryogenesis . This apparent phenomenon is discussed further in the text .

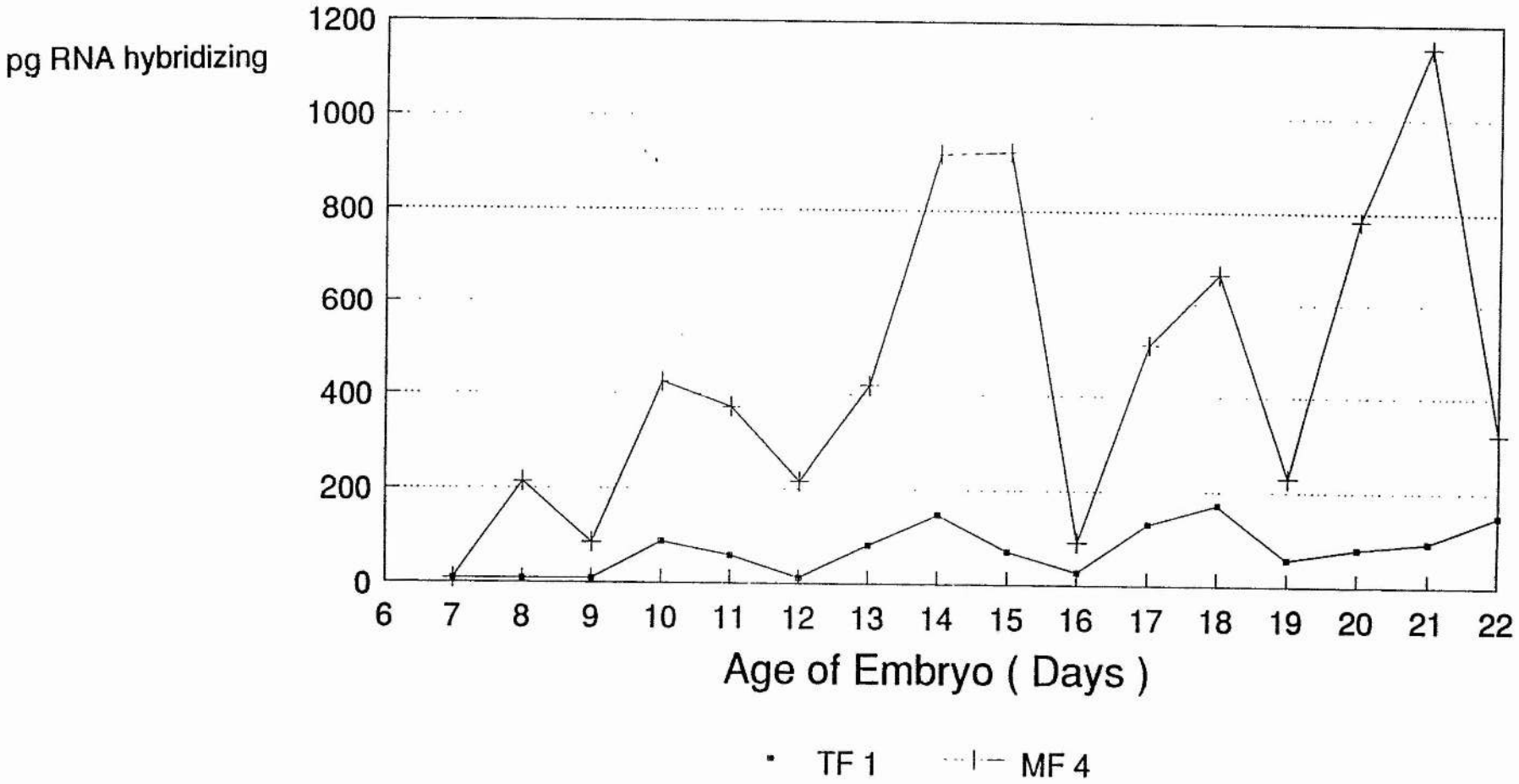

pg RNA hybridizing
1200
1000
800
600
400
200
0
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Age of Embryo (Days)
TF 1
MF 4

Table 3A : Picograms calculated to hybridize with HF1 probe in slot-blot experiment

Age of Embryo (Days)	RNA Isolated per Embryo (ug)	Area under Densitometric Curve	pg RNA Hybridizing HF1 Probe (Uncorrected)	pg RNA Hybridizing HF1 Probe (Corrected) ❶
1	0.92			
2	0.23			
3	0.42			
4	0.94			
5	0.99			
6	1.19			
7	1.32	942.508	9.5	2.7
8	1.45	916.617	9.3	2.6
9	1.56	972.357	9.8	2.8
10	1.84	8821.713	89	25.2
11	1.86	5961.879	60	17.1
12	2.10	1283.836	13	3.7
13	2.18	8207.963	83	23.5
14	3.40	14676.740	148	42.0
15	3.52	7054.050	71	20.2
16	4.27	2783.371	28	8.0
17	4.80	12934.010	131	37.0
18	4.00	17000.890	172	48.6
19	2.90	5643.215	57	16.1
20	2.97	7881.506	80	22.5
21	4.27	9236.456	94	26.4
22	4.00	14868.890	151	42.5
HF1 Control	*	98815.380	1000	–
HF4 Control	*	70883.690	717	–

* Plasmid DNA equivalent to 1ng probe used

❶ Since significant hybridization with HF4 control was observed , attempts to correct for this in estimations of amount of HF1 - specific mRNA present were made .

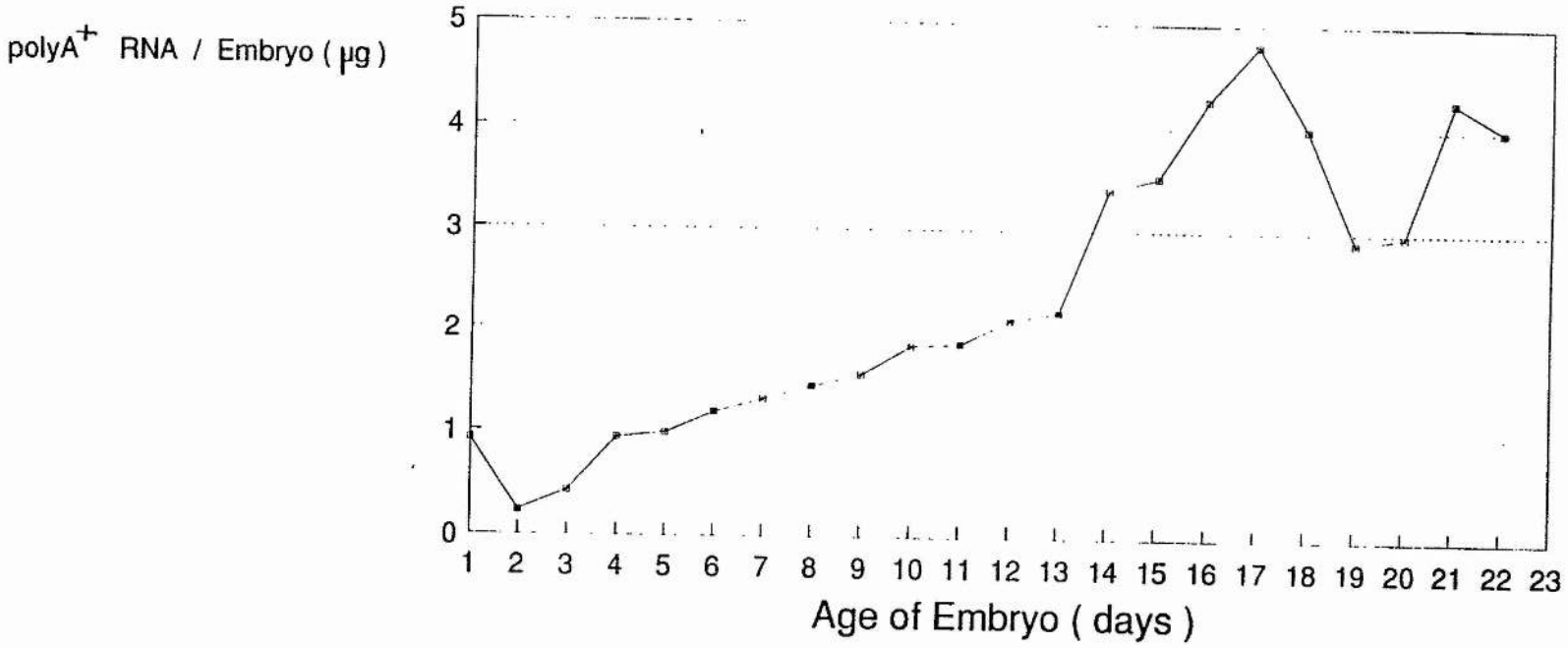
Table 3B : Picograms calculated to hybridize with HF4 probe in slot-blot experiment

Age of Embryo (Days)	RNA Isolated per Embryo (ug)	Area under Densitometric Curve	pg Hybridizing HF4 Probe
1	0.92		
2	0.23		
3	0.42		
4	0.94		
5	0.99		
6	1.19		
7	1.32		9.7
8	1.45	7245.463	214
9	1.56	3360.215	86.5
10	1.84	16021.710	425.5
11	1.86	13322.130	373.5
12	2.10	6879.913	216
13	2.18	14880.730	420
14	3.40	32578.270	920
15	3.52	32774.940	926
16	4.27	3243.836	92
17	4.80	18036.260	510
18	4.00	23589.030	666
19	2.90	8110.319	229
20	2.97	27714.640	783
21	4.27	40718.990	1150
22	4.00	11383.630	322
HF1 Control	*	–	
HF4 Control	*	35397.980	1000

* Plasmid DNA equivalent to 1ng probe used

Figure 12 : Graph of mRNA isolated from *Helix* embryos during development .

mRNA was isolated from *Helix* embryos as described , and the amount of mRNA recovered per embryo estimated by means of optical density readings at 260nm . As is illustrated , there is a general increase in the amount of mRNA found to be present per embryo as development proceeds , with the exception of day 2 , where there appears to be a reduction in the amount of mRNA extracted relative to day 1 . As is discussed in the text , this may be due to the utilization at that stage by the embryo of stored maternal RNA .


polyA+ RNA / Embryo (µg)
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Age of Embryo (days)

Age of Embryo (Days)	RNA Isolated per Embryo (µg)
1	0.92
2	0.23
3	0.42
4	0.94
5	0.99
6	1.19
7	1.32
8	1.45
9	1.56
10	1.84
11	1.86
12	2.10
13	2.18
14	3.40
15	3.52
16	4.27
17	4.80
18	4.00
19	2.90
20	2.97
21	4.27
22	4.00

Table 4 : mRNA isolated per embryo

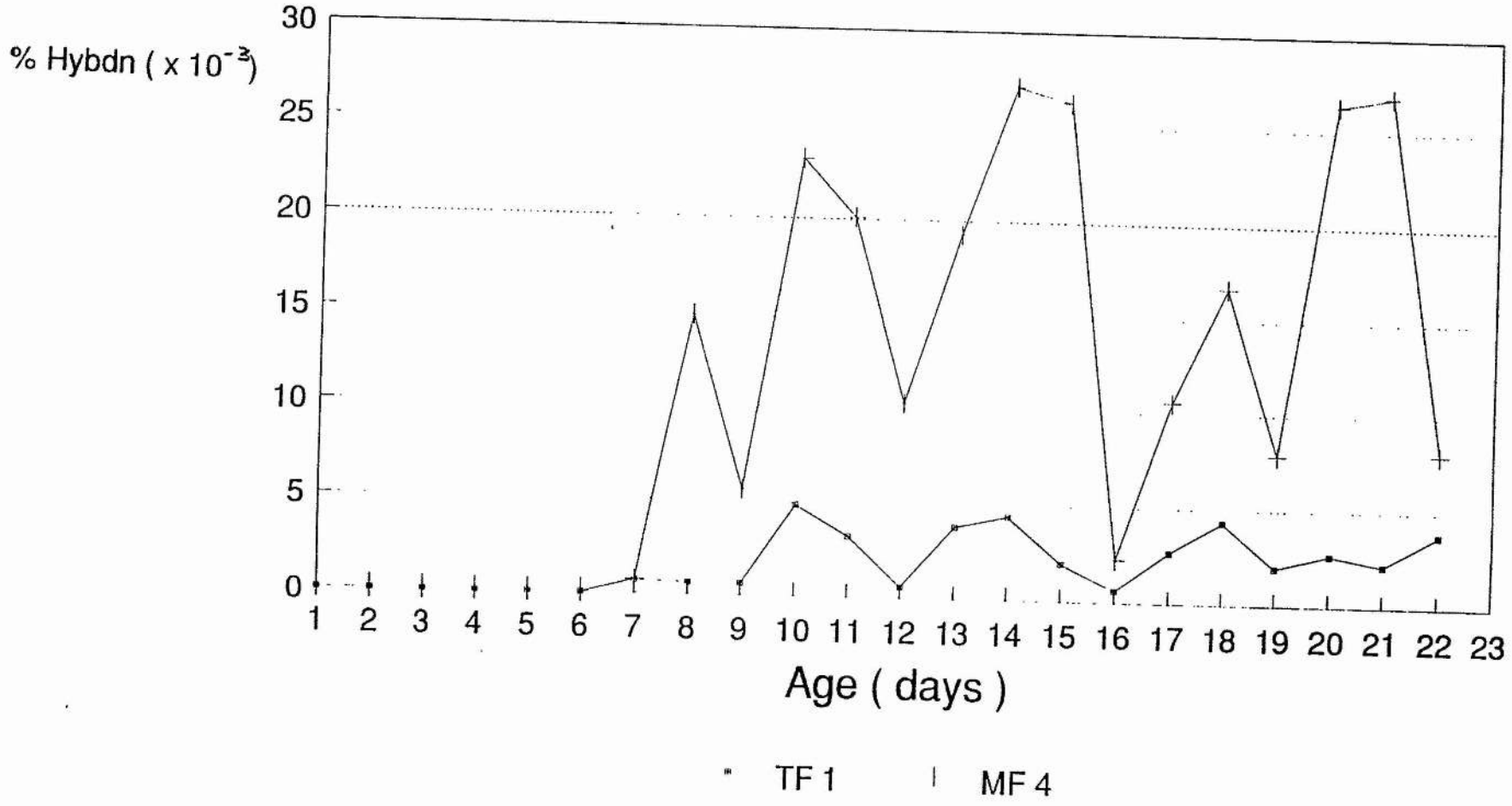
representing that amount of RNA . Amounts of RNA in other slots were estimated by dividing the densitometric reading for that slot by that for the control , then multiplying by 1000 , in order to give an estimation , in picograms , of the amount of FaRP - encoding mRNA present in that extract . For example , the area under the curve for the day 13 slot is 14880.73 ; this is divided by the densitometric reading for the control slot (containing plasmid DNA equivalent to 1ng , or 1000pg) , which is 35397.98 . This gives a figure of 0.42 , which approximates to the relative amount of RNA present in this slot which is homologous to the probe , in this case 420pg .

This calculation was complicated in the case of the HF1 results by the fact that a large percentage of the signal detected was apparently due to hybridization by the HF1 probe to HF4-specific sequences (slot 12b in figure 10(A) contains only 1ng of HF4 sequences : densitometric analysis of this slot reveals it to bind over two thirds as much probe as does the HF1 - specific slot (slot 11b in figure 10(A)) . Adjustments to measurements of HF 1 binding were therefore attempted , but estimates of the amounts of HF1 - specific mRNA present in these slots obtained from these experiments are not likely to be accurate . (Other factors in these experiments also mean that accurate estimations of absolute amounts of RNA present in the slots are unlikely to derived : these include such factors as possible non - linear response of the X - ray film to radioactive decays and errors in estimation of the extent of the densitometric peaks. However , these factors should be constant within the experiment , and thus should not affect the observed values relative to each other)

It was also found that when percentages of FaRP - hybridizing mRNA present in each slot , as a proportion of total mRNA applied to each slot , was calculated , this pattern of " cycling " values was repeated . Percentages of RNA hybridizing were calculated by dividing the amount of RNA previously calculated to be hybridizing with the probes by that known to have been applied to each slot , equivalent to the amount of mRNA derived from a single embryo

Figure 13 : Graph of percentage of RNA present calculated to be hybridizing with FaRP - specific anti sense riboprobes .

Using the information illustrated in figures 12 and 13 , the percentage of mRNA present in each slot found to be hybridizing to the FaRP - specific probes was estimated : this was done by dividing in each case the calculated amount of RNA hybridizing by the amount of RNA known to have been applied to that slot . As can be seen , a pattern similar to that illustrated in figure 12 results from such calculations - i.e. a " cycling " of values calculated is observed .


% Hybdn (x 10⁻³)
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Age (days)
TF 1
MF 4

Age of Embryo (Days)	Percentage Hybridization with HF1 probe (x 10^{-3})	Percentage Hybridization with HF4 probe (x 10^{-3})
1		
2		
3		
4		
5		
6		
7	0.72	0.74
8	0.64	14.1
9	0.63	6.1
10	4.8	24.6
11	3.2	20.2
12	0.62	9.2
13	3.8	19.3
14	4.4	27.1
15	2.0	26.3
16	0.66	2.1
17	2.7	10.6
18	4.3	16.7
19	2.0	7.9
20	2.7	26.4
21	2.2	26.9
22	3.8	8.05
HF1 Control	100	–
HF4 Control	–	100

Table 5 : Percentage hybridization with probes estimated from slot-blots

. For example , the slot for day 12 contains 2.1μg ; 216pg of HF4 - specific RNA is calculated to be present - representing ≈ 0.01% of the mRNA extracted from embryos of this stage of development . These results , which indicate that FaRP - specific mRNAs never constitute more than 0.03% of the mRNA population in developing *Helix* embryos , are graphed in figure 13 . The dependance of these calculations on the previously estimated absolute amounts of mRNA present , which are themselves unlikely to be wholly accurate , also renders these values innaccurate - though the values calculated should reflect changes in mRNA percentages relative to other stages of development

3.7 : Differential expression of mRNAs within the C.N.S

It is known from immunohistochemical and radioimmunoassay (R. I. A.) studies that the tetra - and heptapeptide FMRFamide related peptides are expressed differentially within the central nervous system (C. N. S.) of the snail *Helix aspersa* (Lehman and Price , 1987) , some cells being enriched with respect to the tetrapeptides , while other cells appear to produce the heptaptides preferentially . It was therefore of interest to study the distribution of mRNA molecules which encoded these two classes of FaRPs . To this end , two experimental approaches were utilised :

3.7 : A) Slot blots / Northern blots

Bunches of cells in the C.N.S which were known to be enriched in these peptides were dissected out and their RNA populations probed for the presence of such mRNAs . Cells of interest were dissected from the central nervous system by Prof. G. A. Cottrell and Mr. B. Powell and were transferred on glass scoops (made from crushed pasteur pipettes) into small volumes of guanidinium thiocyanate solution . Nucleic acids

were isolated from this chaotropic solution and were applied to nitrocellulose membranes *via* a slot blotting apparatus . These nitrocellulose filters , when hybridized with ^{32}P labelled antisense riboprobes , yielded the results illustrated in figure 14. Hybridization with a probe specific for the heptapeptide FaRPs in the nucleic acids extracted from clusters of cells in the parietal ganglia which express such peptides is apparent , while this is much reduced in the slot containing nucleic acid from the cerebral ganglion . The reciprocal is true for HF1 , the probe for the tetrapeptides : cerebral ganglion RNA reacts more strongly than does parietal material (see figure 14 A , B) .

Similarly , when material isolated from different ganglia was separated on agarose , Northern analysis (figure 14 C) reveals an enrichment of heptapeptide - specific mRNA in the parietal ganglia : track 3 , containing parietal - ganglial derived RNA , hybridizes more strongly with the probe specific for heptapeptides than does RNA from either cerebral (track 1) or pleural (track 2) ganglia .

3.7 : B) *In situ* hybridization of sections and whole mounts

Whole central nervous systems were dissected from adult *Helix aspersa* , appropriately fixed and sectioned , and *in situ* hybridization was used as a means of examining the distribution of mRNAs encoding FaRPs within them . Detection of nucleic acid hybrids was by means of fluorosein labelled antibodies (Boehringer) : some photographs and confocal images illustrating the detection by this method of mRNAs specific for FMRFamide - related peptides are shown in figures 15 to 24 . Fluoresence is usually observed within the cytoplasm of a number of cell bodies in the case of both probes : neither probe produced any significant hybridization in the nucleus of these cells . (Successive sections often showed the size of this nuclear "hole" apparent in the hybridization pattern to be reduced , the series terminating with the appearance of a filled

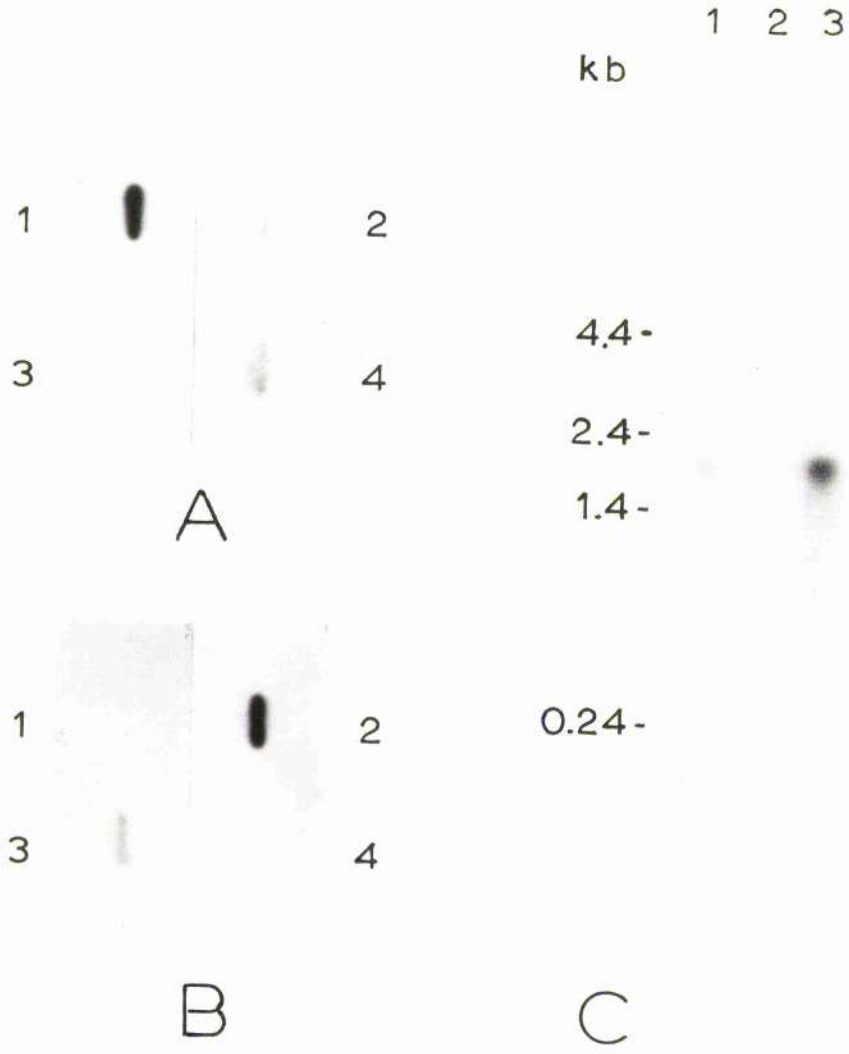
Figure 14 : mRNAs present in different ganglia hydridize predominantly with a single probe .

Different ganglia as detailed were dissected from adult *Helix aspersa* : the poly(A)$^+$ mRNAs present in these were isolated as described and applied to nylon filters *via* a slot - blotter . The resulting filters were probed with (A) an HF1 or (B) an HF4 specific probe . By this analysis it appears that there is significantly more tetrapeptide - encoding mRNA (HF1) in the cerebral ganglion (slot A4) than in the suboesophageal ganglia (slot A3) ; conversely , there appears to be more heptapeptide - encoding (HF4) mRNA in the suboesphageal ganglia (slot B3) than in the cerebral ganglion (slot B4) . A similar pattern is also illustrated in panel (C) , which shows a Northern blot of mRNA derived in a similar manner to that used in the slot blots , probed with an HF4 specific probe . Although some hybridization with the probe is observed in the track containing pleural ganglion mRNA (track 2) , it appears that HF4 - complementary mRNA is largely located in that derived from the parietal ganglion (track 3) .

(A) and (B) :
Slot 1 : 25ng HF1 plasmid DNA
Slot 2 : 25ng HF4 plasmid DNA
Slot 3 : 8µg poly(A)$^+$ RNA from suboesophageal ganglia .
Slot 4 : 8µg poly(A)$^+$ RNA from cerebral ganglion .

(C) Northern blot of poly(A)$^+$ RNA (6µg per track) from ganglia indicated hybridized with HF4 - specific probe .
Track 1 : Cerebral ganglion RNA
Track 2 : Pleural ganglion RNA
Track 3 : Parietal ganglion RNA

Approximate positions of RNA size markers are shown to left of panel C .


1 2 3
kb
1
2
4.4-
3
4
2.4-
1.4-
A
1
2
0.24-
3
4
B
C

thought the section no longer transects the nucleus. This pattern was also observed when examining whole mount *in situ* hybridizations using the confocal microscope .)

In figure 15 is shown the result of an experiment where whole right cerebral ganglia were hybridized with a DIG - labeled TF1 probe . As can be seen , there is strong hybridization with the probe in a single medium - sized cell (figure 15 , A - C) , thought to be the identified C3 neuron , which is known to contain FMRFamide (Bewick *et al* , 1990) . No other hybridization with this probe , nor with the MF4 probe , is observed in this ganglion . Also shown in figure 15 is an *in situ* hybridization with a section through the cerebral ganglion (figure 15 D) : the positively - reacting cell is thought to be C3 , although definite identification of it as such is difficult .

Similar experiments using MF4 probe and suboesophageal ganglia reveals two clusters of small - to medium - sized cells reacting with the probe (figure 16) - these are located in the left and right parietal ganglia , and apparently correspond with cells in these regions which are immunoreactive for FaRPs (Elekes and Nassel , 1990) . A photo - montage in figure 16 illustrates the positions of these clusters , and figure 17 shows a digramatic representation of the information contained in figures 15 and 16 , illustrating the location of such reactive cells within the ganglia .

Some sets of serial sections of ganglia were divided , such that sets of three serial sections were placed on alternate slides (i.e. if sections 1 - 12 denote 12 serial sections , sections 1 - 3 were placed on slide A , 4 - 6 on slide B , 7 - 9 on slide A , and 10 - 12 on slide B) . This arrangement allowed the exposure of adjacent sections on these two sets of slides , A and B , to different probes , since , for example , sections 3 and 4 , although adjacent when cut , were on different slides ; the same is also true of sections 6 and 7 , etc. . Examination of these slides indicated that cells which hybridized with HF1 did not appear to bind HF4 , and *vice versa* : in no case examined

Figure 15 : *In situ* hybridization of whole mount cerebral ganglion and sectioned ganglion with DIG - labelled TF1 riboprobe .

The identified neuron C3 (arrowed in A) reacts positively when the cerebral ganglion is hybridized *in situ* with a riboprobe derived from clone TF1 (see figure 9) .

A : Phase contrast photograph of region of right cerebral ganglion . C3 neuron is arrowed .

B : Fluoresence photograph of identical region to that in A , illustrating positively-reacting C3 neuron , known to contain FMRFamide .

C : Higher - power magnification of C3 neuron seen in B . Note that distribution of fluoresence within cell body appears to be non - uniform (see text) .

D : Section through cerebral ganglion , hybridized with riboprobe TF1 in a manner similar to those illustrated in A - C . The positively - reacting cell is thought to be C3 .

Bar = 50μm

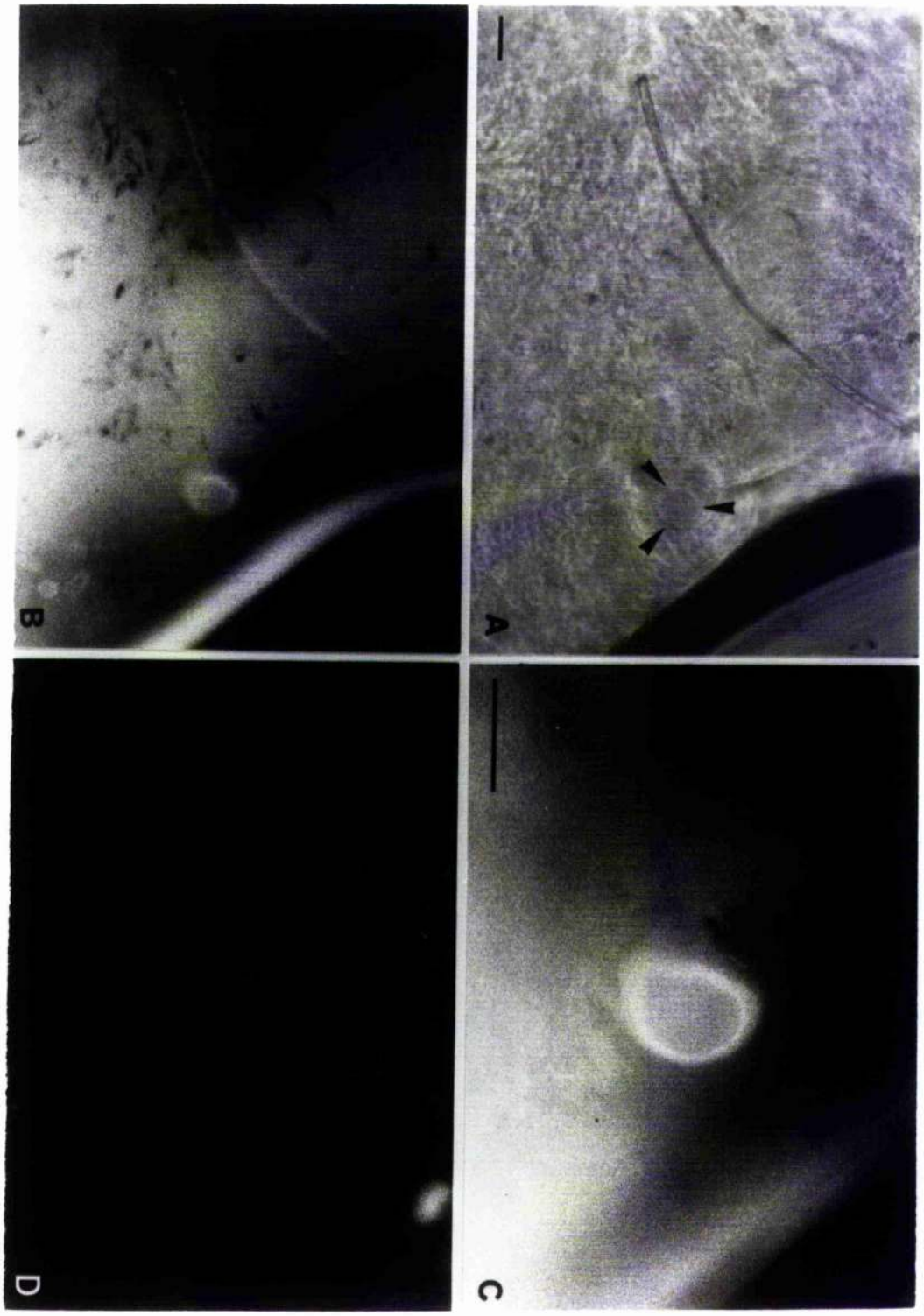

B
A
D
C

Figure 16 : Photo - montage indicating positions of clusters of cells in parietal ganglia reacting with MF4 probe .

A whole - mount preparation of *Helix* suboesophageal ganglia , comprising those illustrated in figure 16 , was used in an *in situ* hybridization experiment . Shown here is a photo - montage constructed using photographs taken of such a preparation : this indictes the positions of the clusters of cells in the left and right parietal ganglia which react with this probe .

LPl : Left pleural ganglion
RPl : Right pleural ganglion
LPa : Left parietal ganglion
RPa : Right parietal ganglion
V : Visceral ganglion

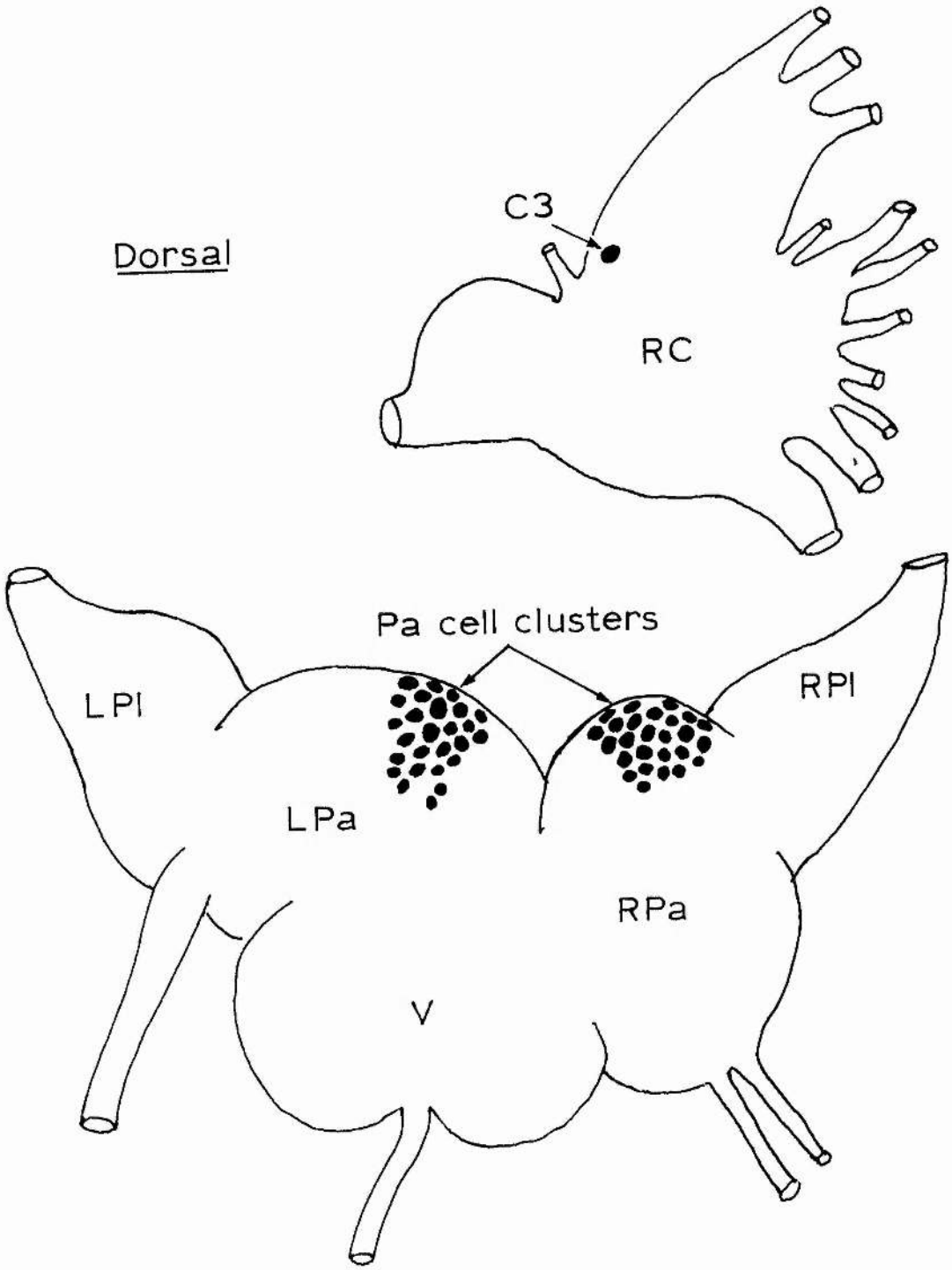
Bar = 50μm

V
LPl
LPa
RPa
RPl

Figure 17 : Schematic drawing illustrating relative positions of neurons which are reactive with FaRP specific probes in our *in situ* hybridization experiments .

Cell which are found to contain FaRP - encoding mRNAs are illustrated in a *camera lucida* drawing of *Helix* ganglia : the C3 cell in the right cerebral ganglion , and the clusters of cells in the parietal ganglia , which hybridize with tetrapeptide and heptapeptide - specific *in situ* probes , respectively , are shown shaded .

R C : Right Cerebral Ganglion
L Pl : Left Pleural Ganglion
R Pl : Right Pleural Ganglion
L Pa : Left Parietal Ganglion
R Pa : Right Parietal Ganglion
V : Visceral Ganglion


Dorsal
C3
RC
Pa cell clusters
LPl
RPl
LPa
RPa
V

binding of both probes by the same cell. Some photographs illustrating this result are shown in figures 18 and 19 . Figure 18 shows that cells expressing different mRNAs can be closely juxtaposed , suggesting that the type of peptide expressed by a neuron is not entirely dependent on its position within the ganglia ; in figure 19 , cells which hybridize the MF4 RP do not appear to bind the TF1 RP .

The expression of FaRPs is also observed to be apparently independent of cell morphology : figure 20 indicates that cells of a wide variety of appearances are seen to hybridize FaRP - specific riboprobes .

In situ hybridization was also used in conjunction with confocal microscopy in order to study the expression of FaRP encoding mRNAs in whole - mount preparations of *Helix* suboesophageal and cerebral ganglia . Such preparations have the advantage , previously alluded to , of allowing the cells to be observed in their context , and are also especially suitable for analysis using a confocal laser scanning microscope . Illustrated in figures 15 and 16 are whole mount preparations of suboesophageal and cerebral ganglia probed with TF1 and MF4 RPs , respectively : as can be seen , the tetrapeptide probe , TF1 , hybridizes with an identified medium - sized cell , the C3 cell (figure 15) . The suboesophageal ganglia probed with HF4 , the extended peptide clone , reveal two clusters of small cells in the left and right parietal ganglia which flouresce following detection of hybrids (figure 16) .

Several of the cells reacting positively with these riboprobes were analysed by means of a confocal laser scanning microscope . This instrument allows the observation of fluorescence in a particular optical section of the preparation without the interference of fluorescence at other levels of focus. (The presence of which means the analysis of whole mount preparations using conventional light microscopy is often difficult,; for example , although the sections used in figure 20C were only 5µm thick , it can be seen that there are cells present outwith the plane of focus which

Figure 18 : Adjacent sections can be hybridized with different riboprobes .

As is discussed in the text , it is possible to hybridize adjacent sections with different probes , in order to study the juxtaposition of cells displaying different FMRFadergic status. In the example illustrated , two cells reacting with the TF1 RP (lower cells arrowed in A) are found to be located close to one which reacts with MF4 RP (upper cells arrowed in A). This indicates that the type of peptide expressed by a particular cell is not dependent on its location within the ganglion , but rather appears to be defined at the level of the individual cell .

A : Phase contrast photograph of section of suboesophageal ganglion .

B : Fluoresence photograph of section in A above hybridized with MF4 RP . Positively - reacting cell is upper cell arrowed in A .

C : Fluoresence photograph of section adjacent to that in A and B , hybridized with TF1 RP . Positively - reacting cells are lower cells arrowed in A

Bar : 50µm

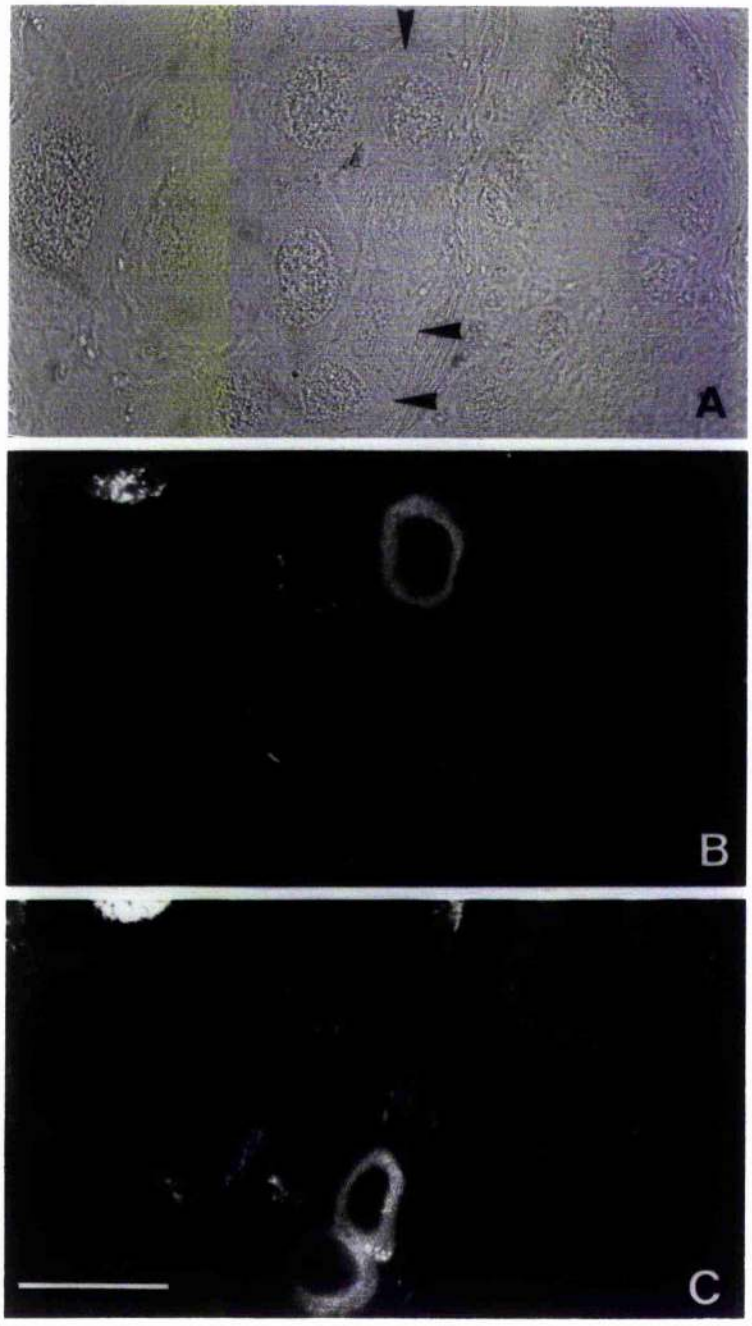

A
B
C

Figure 19 : Cells in parietal ganglion which hybridize MF4 probe do not hybridize TF1 probe .

Adjacent sections through parietal ganglion were hybridized with riboprobes for TF1 (B) and MF4 (C) ; cells indicated by arrows in (A) are those which react positively with MF4 RP , but not with TF1 . Panel (D) illustrates a higher magnification of those cells seen in (C) .

A : Phase - contrast photograph of part of section through parietal ganglion .

B : Fluoresence photograph of part of section shown in (A) , hybridized with TF1 RP .

C : Fluoresence photograph of part of section adjacent to that shown in (A) , hybridized with MF4 RP . Arrows in (A) indicate cells which react positively with this probe .

D : Higher power magnification of positively - reacting cells in (C) .

Bar = 50μm

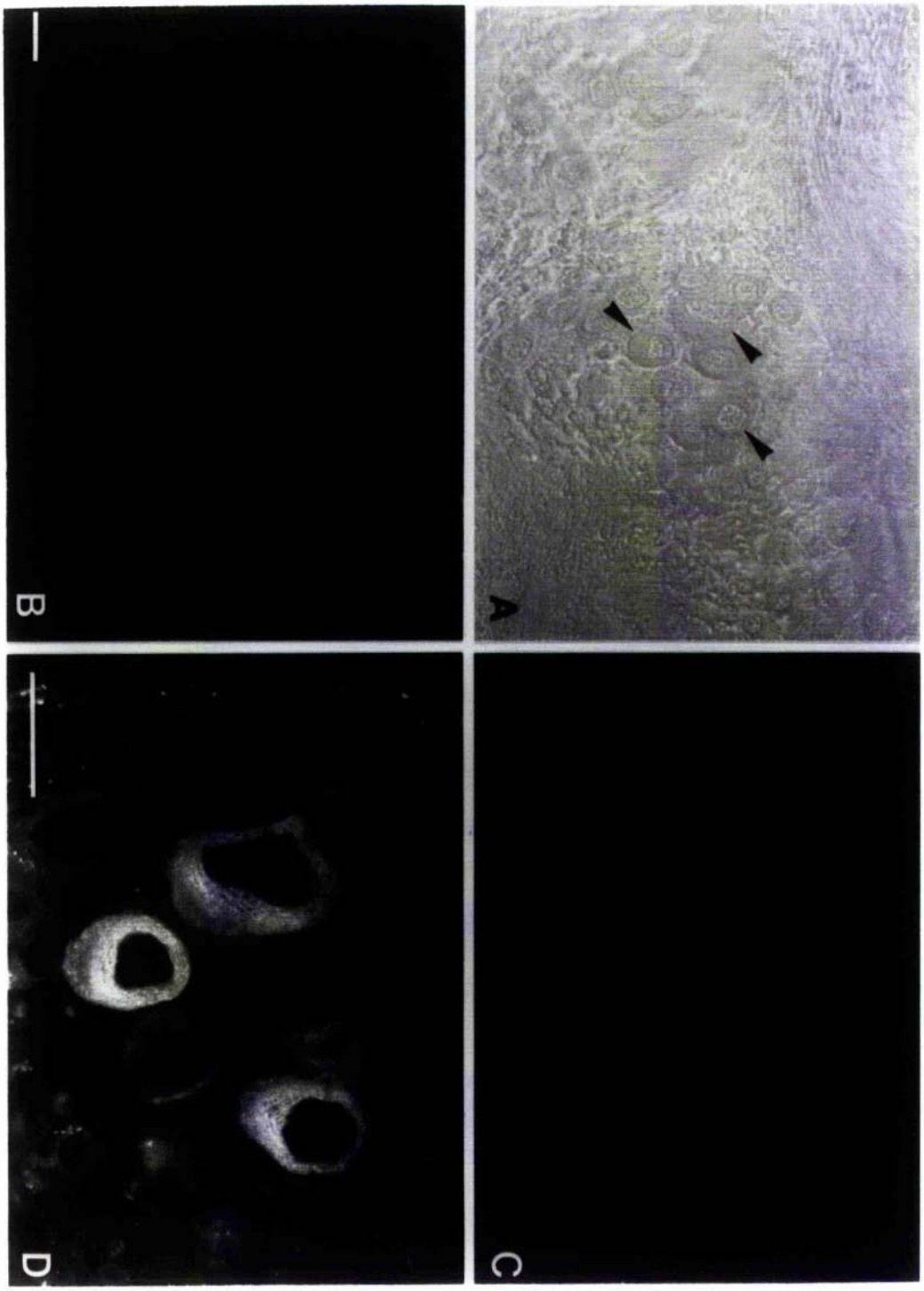

A
B
C
D

Figure 20 : A wide variety of cells in parietal ganglia react with MF4 RP .

Sectioned parietal ganglia , when hybridized with MF4 RP , reveal a variety of cell sizes with no obvious common morphology reacting with this probe . Reactive cells are found to be located among non - reacting cells ; variation in the intensity of fluoresence observed in these cells is also seen - some larger cells to the left of (A) (large arrows) apparently react more strongly than smaller cells to the right (small arrows) .

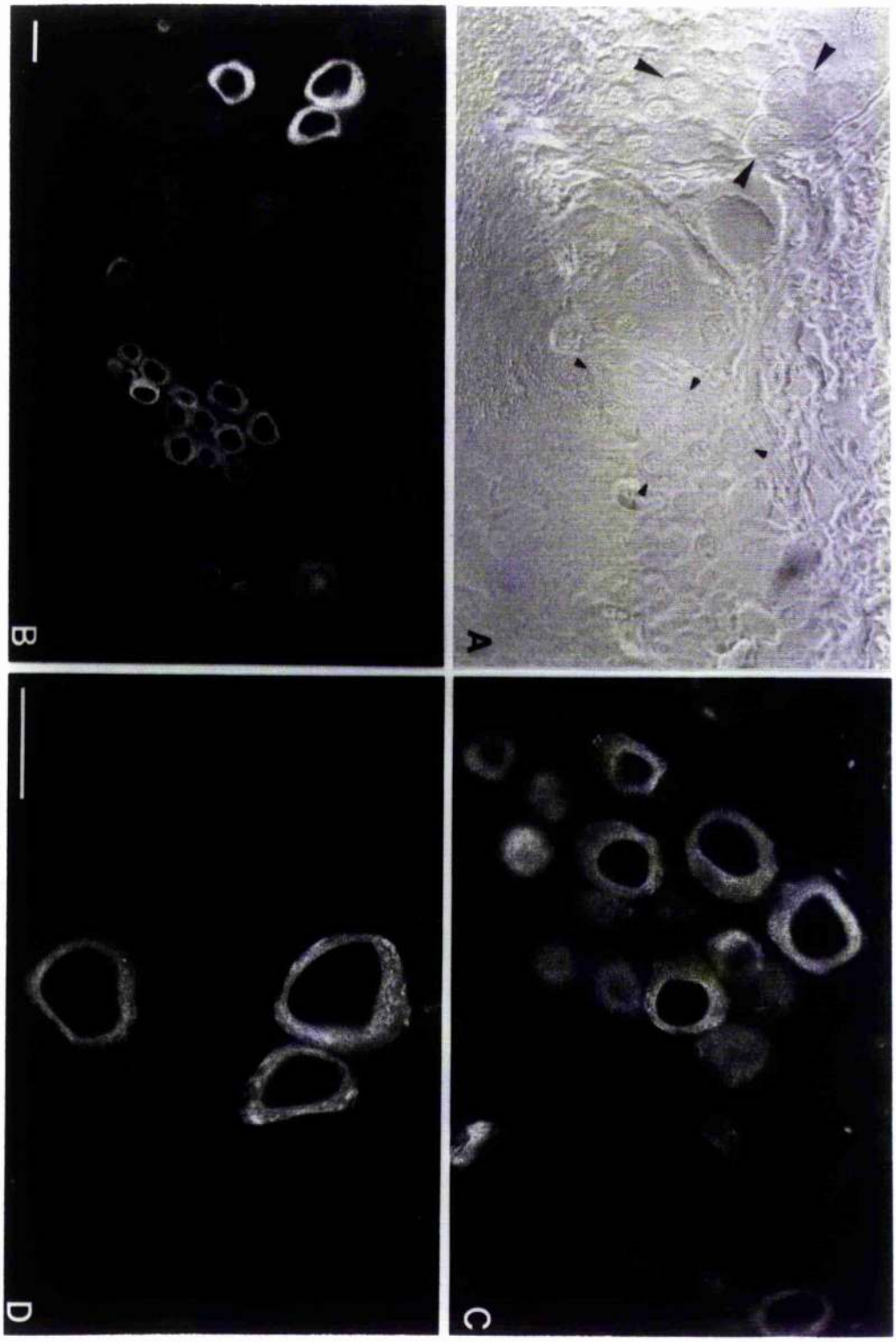
A : Phase - contrast photograph of part of section through parietal ganglion .

B : Fluoresence photograph of (A) hybridized with MF4 RP . Note variation in cell size and in intensity of staining .

C : Higher - power magnification of smaller cells to right of (A) (small arrows) . Some cells within section are positive for the probe but are outwith the plane of focus in this case .

D : Higher - power magnification of larger cells to left of (A) (large arrows) . Distribution of fluoresence within cytoplasm of cell body can be seen to be non - uniform .

Bar = 50μm


A
B
C
D

react with the probe . Problems due to the presence of such cell are multiplied as the thickness of the specimen increases .) The confocal microscope can also be programmed to reconstruct a 3 - dimensional image of an area of a preparation by merging fluorescent images taken from successive optical levels in the specimen . Shown in figure 21 is such a reconstruction of two cells in the left parietal ganglion which hybridize with the HF4 probe (centre panel) , surrounded by a selection of optical sections from the 40 used to construct this image . More intense staining (yellow) is observed in the "deeper" confocal sections , perhaps indicative of higher concentrations of mRNA being present in these areas of the cells . The approximately spherical shape of the bodies of these nerve cells is also apparent , although some elongation of them towards the axons may be indicated by the confocal sections . Little hybridization with the nuclei of these cells is apparent .

A single optical section through the cluster of cells in the parietal ganglion reacting with the probe MF4 is shown in figure 22 : again , nuclear loclisation of the signal appears to be limited . Non - - random distribution of the fluorescence produced by the detection of mRNA is indicated by the areas of more and less intense fluoresence (yellow and red , respectively) . Figure 23 suggests that this may be a real localization phenomenon , and may not be wholly attributable to bleaching of sections or penetration of probe : it may be that what is being observed here is the concentration of mRNAs within the cell , perhaps on polysomes . Shown in figure 24 is another demonstration of the uneven distribution of the fluoresence within the positively reacting cells .

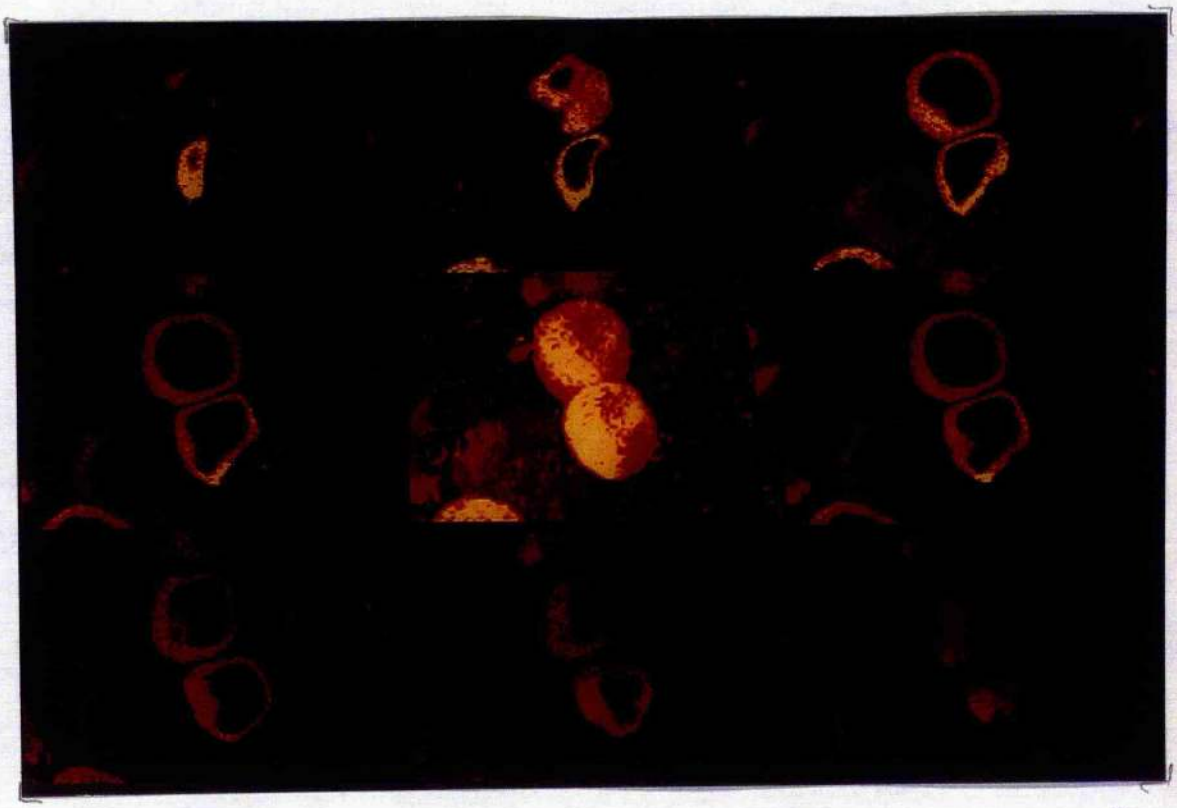
3.8 : Peptide expression during embryogenesis

Figure 21 : Serial optical sections through two cells in the parietal ganglion , and the composite produced by super - imposing them

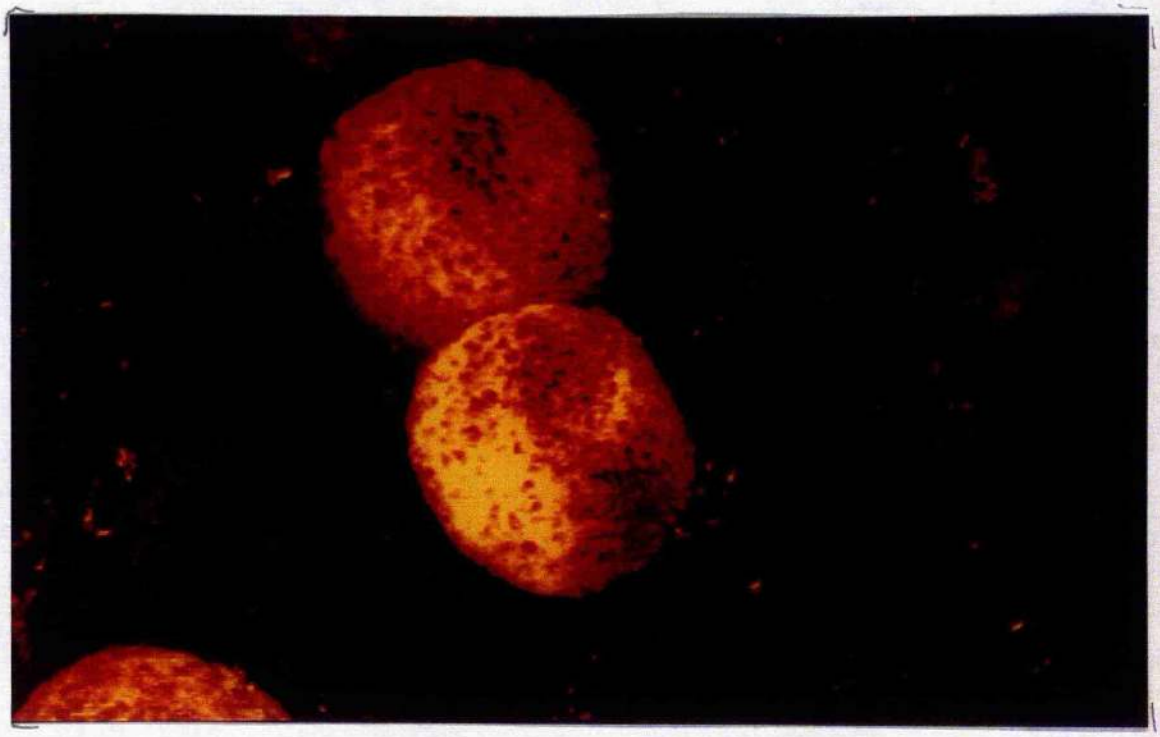
Two adjacent cells in the parietal ganglion , chosen at random from those reacting with the MF4 RP , were further analysed using the confocal microscope . A series of 40 optical sections encompassed the whole of these cell bodies; illustrated in (A) are a selection of such sections (top left - lower right : sections 5 , 8 , 12 , 16 , 18 , B , 22 , 26 and 31 , where B = composite of all 40 sections superimposed , shown on larger scale in (B) .) . Non - random distribution of fluoresence is again observed in these sections : as is detailed in the Results section of the text , there appears to be a higher concentration of FaRP - specific mRNA in the cytoplasm of positvely - reacting cell bodies nearer the axon.

Yellow = more intense staining .

Red = less intense staining .



A



B

Figure 22 : Confocal microscopical image of MF4 RP - reacting cells in left parietal ganglion .

A single optical section through the MF4 - hybridized left parietal ganglion is shown . (" Tail " of reactive cells to bottom of cluster is indicative of this being the left parietal cluster (see figure 16) .) Very little hybridization with nuclei is detected. Yellow = more intense staining ; red = less intense staining : this would suggest that FaRP - specific mRNAs are distributed non - randomly in the cytoplasm of these cells .

Bar = 50µm

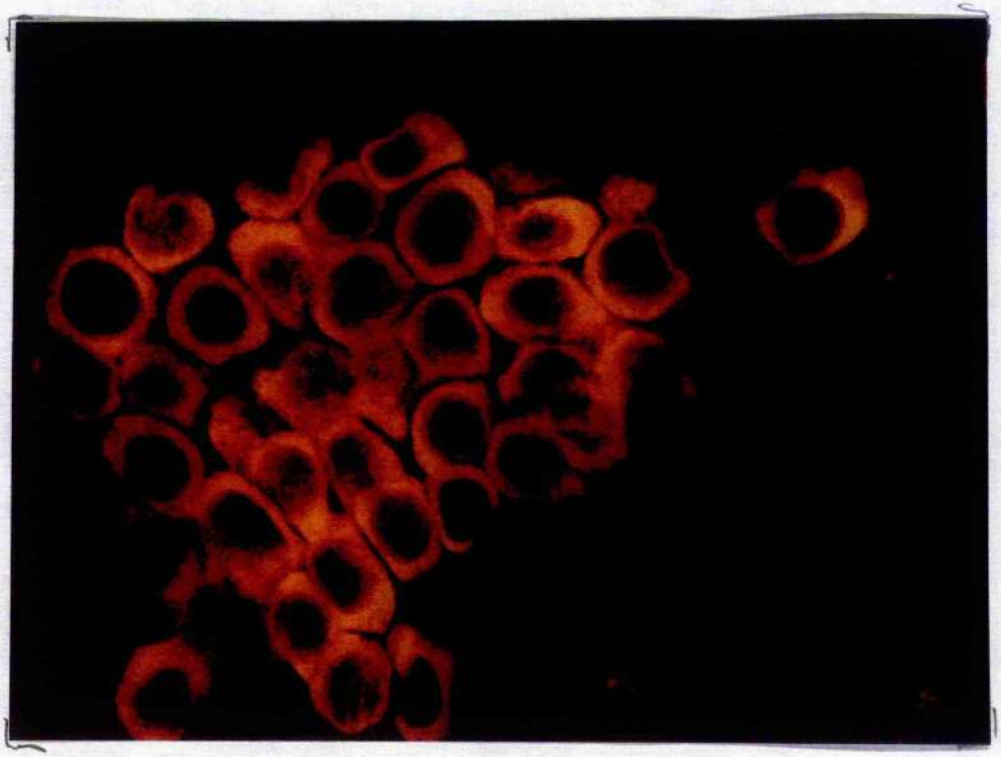


Figure 22 : Confocal microscopical image of MF4 RP - reacting cells in left parietal ganglion .

A single optical section through the MF4 - hybridized left parietal ganglion is shown . (" Tail " of reactive cells to bottom of cluster is indicative of this being the left parietal cluster (see figure 16) .) Very little hybridization with nuclei is detected. Yellow = more intense staining ; red = less intense staining : this would suggest that FaRP - specific mRNAs are distributed non - randomly in the cytoplasm of these cells .

Figure 23 : Serial optical sections through three adjacent cells indicates that intensity of staining observed is not due solely to position in ganglion .

Some of the variability in intensity of staining observed between deeper and more superficial optical section may be attributable to bleaching of whole mount : the cells shown here indicate that this is not a complete explanation of the phenomenon . When these serial sections are compared , it is seen that the most intense staining of the two cells to the left is observed in section located more deeply in the whole - mount than those where most intense staining of the adjacent cell to the right is seen . Sections shown are indicated by numbers in top left of each panel . Most intense staining of cells to left (large arrows in 3) is seen in sections 9 and 11 , while that in cell to right (small arrow in 3) is in 13 and 15 .

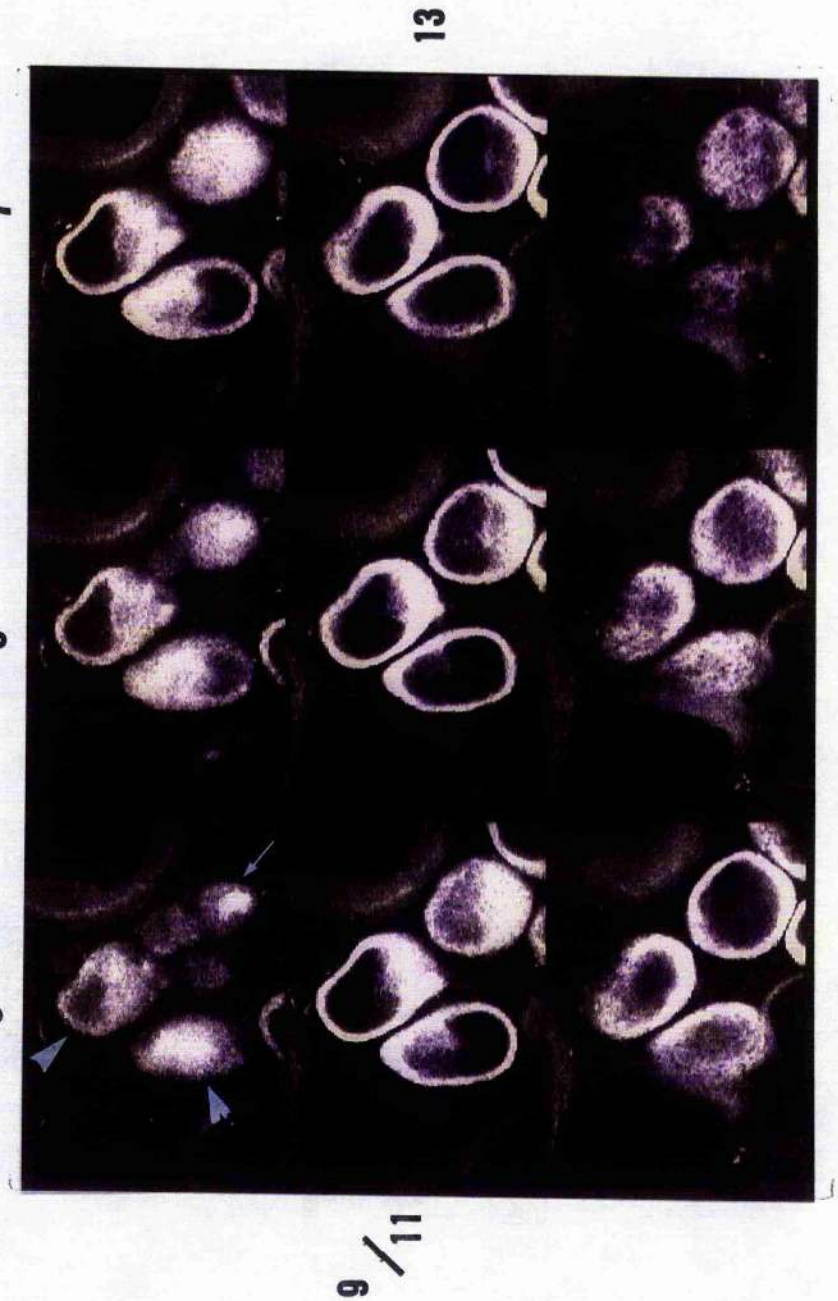
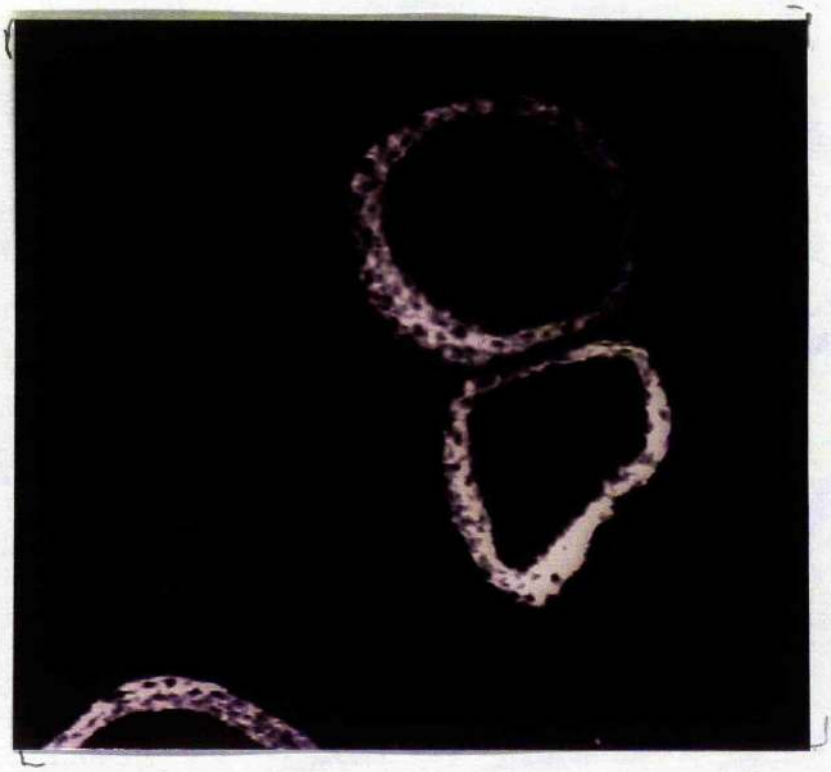

3
5
7
9/11
13
15
17
19

Figure 24 : Distribution of fluoresence within positively - reacting cells appears to be uneven .

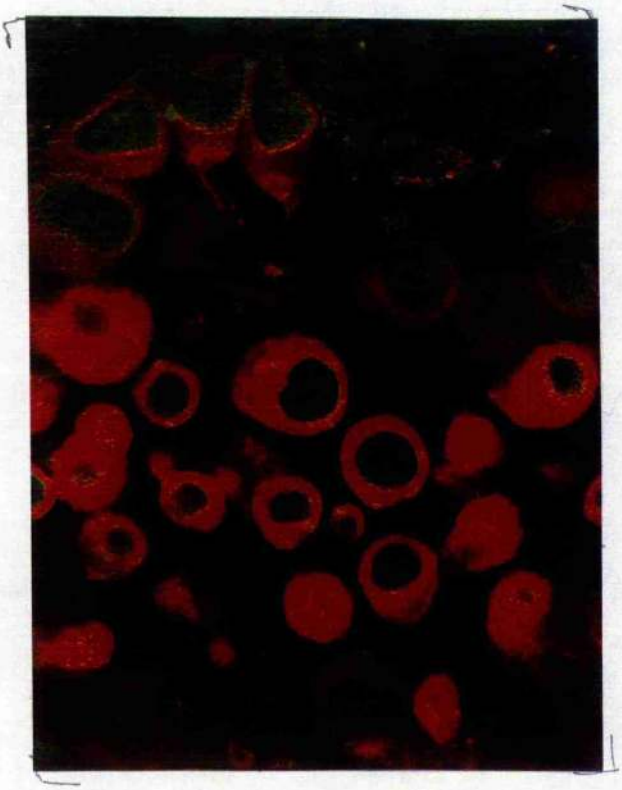
It is noted that , in many cases , the fluoresent staining in those cells which react with riboprobes is uneven . Illustrated here are two sections stained with MF4 RP which indicate this to be the case .

In panel A , a single optical section through two cells in the parietal ganglion are shown : the " particulate " appearance of the staining of the cytoplasms of both cell is obvious .

Panel B : a group of cells are stained with MF4 RP . Unusually , some low - level (green) nuclear staining is apparent in many of these cells ; most intense staining (yellow) is seen at the nuclear membrane , while the cytoplasms of many cells are stained at an intermediate intensity (red) .



A



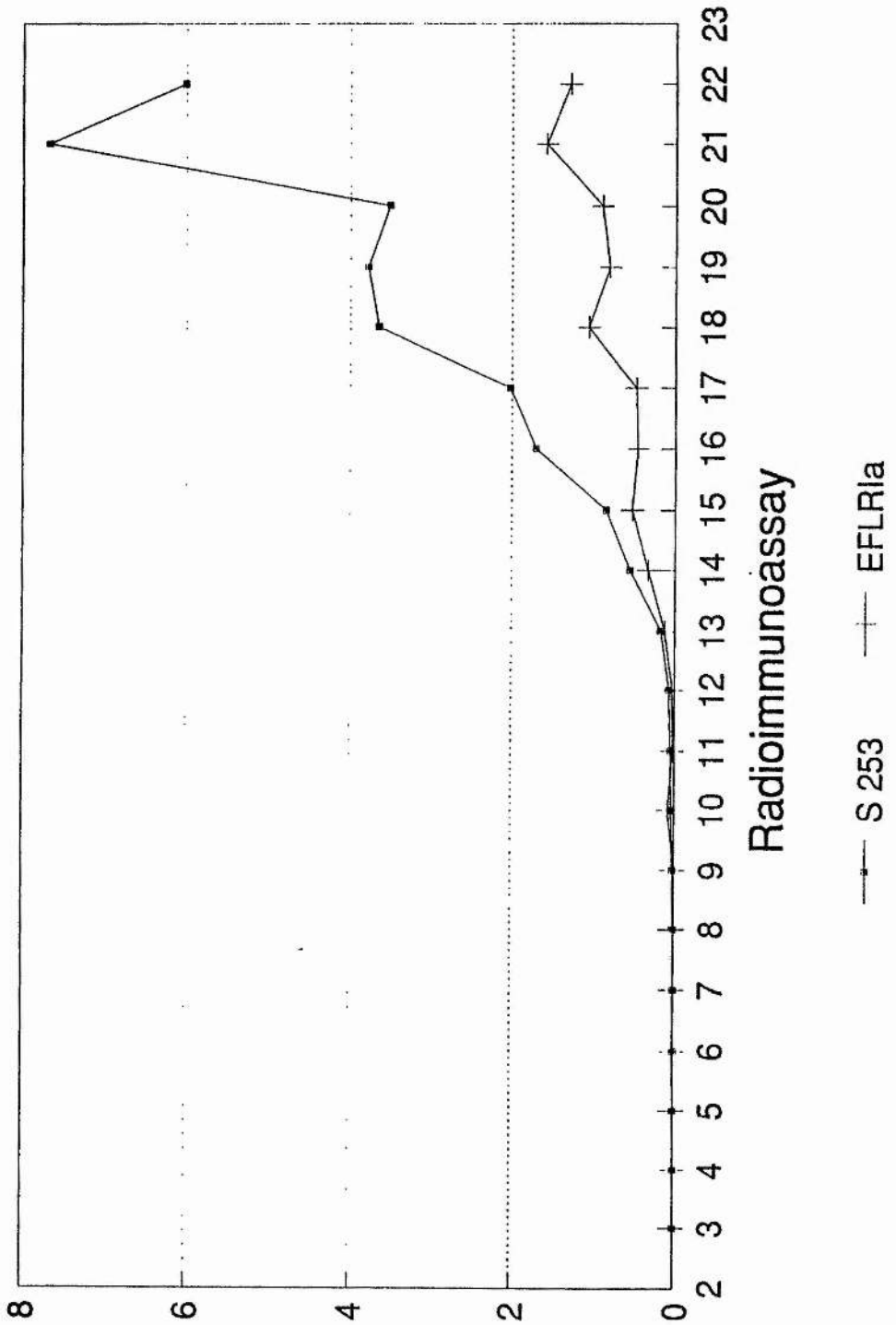
B

In order to detect the expression of the FaRPs during the development of the embryos of *Helix aspersa* we utilised a modification (as detailed in Chapter 2) of the method published by Abood *et al* (1990) , whereby RNA and peptides can be isolated from the same samples . The resulting fractions were used in separate studies , the RNA being used in slot blots as previously described , while the peptide extracts were assayed for the presence of FaRPs by means of HPLC coupled with RNA (radio immunoassay) using specific antibodies raised against various FaRPs . These HPLC / RIA studies were carried out on samples isolated by Dr. Wendy Lesser at the Whitney Marine Laboratories , University of Florida . An analysis of the results obtained from these experiments , and an estimation of the levels of peptide in embryos at different stages during development is shown in figure 25 and table .

It is apparent from the results of RIA studies that the onset of detection of FMRFamide - related peptides lags 4 - 5 days behind that of mRNAs encoding them (c.f. figs. 11 and 25). This observation may be due to a real delay between mRNA synthesis and peptide production , a control exerted during development over the expression of FaRPs ; alternatively , it may be a reflection of differing sensitivities of the assays used in these experiments (or perhaps a combination of these factors) .

Figure 25 : Graph of results of radio - immunoassay of extracts from *Helix* embryos to detect FaRPs .

Radio - immunassays , performed by Dr. W. Lesser as described , were carried out in order to analyse the level of FaRP production in *Helix* embryos during development . The values obtained using antibodies which differentiate between the two classes of FaRPs are shown in table . There is a general rise in the level of FaRPs detected as embryogenesis progresses : the values obtained approximate to an exponential rise in the FaRPs .


Radioimmunoassay
0
2
4
6
8
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
S 253
EFLRIa

Age of Embryo (Days)	Immunoreactivity with S253 (f mol)	Immunoreactivity with EFLRIa antibody (fmol)
2		
3	0.004	0.004
4	0.003	0.001
5	0.005	0.004
6	0.006	0.009
7	0.008	0.007
8	0.011	0.012
9	0.020	0.008
10	0.038	0.081
11	0.050	0.026
12	0.072	0.031
13	0.169	0.124
14	0.545	0.382
15	0.838	0.517
16	1.701	0.457
17	2.019	0.473
18	3.649	1.055
19	3.780	0.799
20	3.515	0.892
21	7.680	1.577
22	6.014	1.286

TABLE 6 : f mol of FaPRs estimated to be present per embryo by RIA.

It is also noted that , while FaRP -specific mRNA levels are observed to rise and fall regularly during embryogenesis (figure 11) , this does not appear to be the case as regards FaRPs themselves , as judged by RIA (fig 25) ; while some variation in levels of peptides detected thruogh embryogenesis is seen , it is possible that the data derived represents an essentially exponential curve . The reasons for this apparent dichotomy are not entirely clear : this is looked at in greater detail in the relevant Discussion section , but possible reasons are thought to include detection by antibodies of amidated peptides or proteins which are not related to FMRFamide , or to cyclic storage and usage of mRNAs during specific periods of *Helix* development , such that mRNAs are synthesised but not immediately translated .

CONCLUSION

It appears , therefore , that not only are the mRNAs specifying the production of the FaRPs expressed in a regulated fashion in the CNS of the adult snail *Helix aspersa* , but that there is also specific expression of these mRNAs during embryogenesis in this organism . While there are undoubtedly other levels at which regulation can be exerted , these results may be indicative of the peptides encoded by these mRNAs playing a functional role during embryogenesis , as well as their being produced in a regulated fashion in the adult CNS in order to fulfill their physiological functions in modulating behaviours of this organism . While the results presented here do not prove this to be the case , the apparently regulated expression of these specific mRNAs during development may be interpreted as being evidence in support of such a postulation .

CHAPTER 4

DISCUSSION

4.1 : Introduction

We were interested to study the molecular biology of the FMRFamide - related peptides in *Helix aspersa* . Results of experiments detailed elsewhere (Lehman and Greenberg 1987a; Payza 1987 ; Payza *et al* , 1989) have indicated that there are two classes of such peptide in this organism : the tetrapeptides FMRFamide and FLRFamide , and a variety of N - terminally extended variants , including such as NDPFLRFamide and SDPFLRFamide . Although we were unable to isolate genomic clones encoding the FaRPs , and thus to more closely study the organisation of FaRP - specific DNA and sequences relating to regulation of their gene expression , it is apparent that synthesis of these two types of peptide are separated by the stage of mRNA generation . We were able to isolate numerous cDNA (complimentary DNA) clones encoding either the tetra - or extended peptides , but none encoding both . Probing of DNA and RNA blots with probes for both types of cDNA clone revealed patterns which differed from each other sufficiently to support the belief that differential regulation of mRNAs specifying these types of peptide is taking place in *Helix* . In addition , preliminary results using *in situ* hybridization to locate mRNA expression within the nervous system of *Helix aspersa* are indicative of their being mutually exclusive at the cellular level - i.e. cells expressing the tetrapeptide - encoding mRNA do not appear to express the extended peptide specific mRNA , and *vice versa* .

Although FaRPs are found to be present in a wide variety of organisms , from hydrozoans to mammals (including humans) , the occurrence of tetrapeptide analogues appears to be much more restricted : it was thought until recently that tetrapeptide FaRPs (specifically FMRFamide and FLRFamide) were found only in molluscs . Their recent isolation from an annelid and a

polychæte has extend this slightly ; none the less , the co - existence of both tetra - and extended peptides in the same organism has to date only been shown in molluscs such as *Helix aspersa* (Price *et al* , 1990) , *Helisoma triviolus* (Bulloch *et al* , 1988) , *Limax maximus* (Krajniak *et al* , 1989) and *Lymnaea stagnalis* (Price 1986; Ebberink *et al* , 1987) . The use of *Helix* as a model therefore affords an excellent situation in which to study the relationship between these closely related peptides ; extensive information regarding the physiology and pharmacology of the FaRPs in this organism having previously been gained from experimental studies , *Helix* also lends itself to integration of results from these disciplines with results from studies of the molecular biology of the peptides.

4.2 : Precursor polypeptide structure :

The application of molecular biological techniques has proved to be especially apposite in the study of neuropeptides: such techniques have indicated the existence of a number of precursor polypeptide molecules which , due to their extreme rapidity of processing are very difficult to isolate in their intact form . Such precursors are often found to contain a variety of functionally or structurally related molecules , and must be precisely cleaved in order to release the constituent peptides , so that they can fulfil their biological role . For example , the insulin precursor polypeptide contains two bioactive peptides , A and B , separated by peptide C , the latter of which is of as yet unknown biological function , but which may be involved in correctly juxtaposing peptides A and B during their processing from the precursor molecule (Ulrich *et al*, 1977) . It is also found that many neuropeptides are first produced as part of a larger precursor molecule : egg laying hormone and the bag cell peptides in *Aplysia* (Scheller *et al* , 1983 ; Buck *et al* , 1983) , and vertebrate peptides such as the tachykinins (Macdonald *et al* , 1989) , enkephalins (Comb *et al* , 1982) and the

proopiomelanocortin peptides (Mains *et al* , 1977) being examples of this phenomenon . It is probable that in many cases there is functional significance in these arrangements , the different components being required to be synthesized in concert with one another .

We have isolated cDNA clones derived from FaRP - specific mRNA in *Helix aspersa* which indicate that these peptides are initially synthesized as part of a such a precursor molecule in this organism , from which the mature peptides are subsequently specifically cleaved . A schematic representation of this and other FaRP - containing polypeptides studied to date is shown in figure 1 .

It is apparent from analyses of cDNA clones obtained from *Helix aspersa* that the precursor polypeptides which encode the FMRFamide - related peptides bear structural similarity to those found in other organisms in which the molecular genetics of the FaRPs have been studied . Genomic DNA sequences encoding FaRPs in *Aplysia californica* (Taussig and Scheller , 1986 ; Schaefer et al , 1985) , *Lymnaea stagnalis* (Linacre *et al* , 1990 ; Saunders *et al* , 1991) and *Drosophila melanogaster* (Schneider *et al* , 1988) are available , as well as the sequence of a cDNA clone from *Calliactis parasitica* (Darmer *et al* , 1991) which encodes several putative FaRPs found in this organism: from each of these can be derived primary structures for the polypeptide precursors which contain the FaRPs found in the organisms concerned . All of these precursor polypeptides apparently contain a variety of peptides, together with sequences important in directing cleavage of the precursor molecules and directing resultant peptides to the appropriate cellular locations , in order that they may fulfil their biological roles .

4.3 : Signal sequence

All known secreted proteins are first synthesized with a hydrophobic " signal " sequence , which is usually located at their extreme amino terminus . The primary function of this signal peptide is evidently an involvement in the sequestration of the protein to the endoplasmic reticulum (High and Dobberstein , 1991) , where it is cleaved from the nascent polypeptide . Consensus sequences for such peptides have been derived by von Heijne (1986) from analyses of large numbers of eukaryotic proteins containing them . Comparison of the precursor polypeptides derived from the cDNA clones isolated by us with these consensus sequences indicate that the first 19 amino acids of that encoded by clone HF4 comprises such a sequence , containing as it does a number of residues of a hydrophobic nature (figure 26) . According to the criteria of von Heijne (1986) , the most likely cleavage point of this peptide from the precursor is between amino acids Ser^{19} and Ser^{20} . The apparent incomplete nature of clone HF1 is the probable explanation for the absence of a signal sequence at the amino terminus of this predicted precursor, although the extant molecule is likely to contain such a sequence if , as we believe is the case , HF1 and HF4 represent two different mRNA species, the products of both of which will require to be secreted .

FMRFamide - related peptides have been detected as being located in secretory vesicles (Kreiner *et al* , 1986; Nagle 1981); it is thought that these peptides are often released from the cells in such vesicles . The signals involved in directing propeptides to secretory vesicles are not as yet so well understood as are signal sequences : Kizer and Trophsa (1991) have proposed that a helical structure in the propeptide containing a serine residue succeeded by two leucyl residues which are separated by three other amino acids is of importance in this process . Precursors containing FaRPs from *Lymnaea* (Linacre *et al* , 1990) , *Calliactis* (Darmer *et al* , 1991) and *Drosophila* (Nambu *et al* ,

Figure 26 : A putative signal sequence is located at the amino terminus of the precursor polypeptide encoded by cDNA clone HF4 .

A. A hydrophobic signal sequence is often found at the amino terminus of secreted proteins : it is thought that the first 19 amino acids of the polypeptide encoded by cDNA HF4 comprise such a signal sequence . Hydrophobic amino acids are shown boxed.

B. A comparison between signal sequences from *Helix* and other related polypeptides is shown : these are scored according to von Heijne (1986).

A: Putative signal sequence encoded on cDNA clone HF4

Met Thr Ser [Leu Cis Leu] Thr [Ile Ala Pro Ala Val Leu] Ser [Leu Ile Cys Leu] Ser
1 19

B

	Amino acid position with respect to signal sequence cleavage site , and score according to von Heijne (1986)															TOTAL SCORE
	−13	−12	−11	−10	−9	−8	−7	−6	−5	−4	−3	−2	−1	+1	+2	
Helix aspersa	Thr 2	Ile 15	Ala 14	Pro 0	Ala 20	Val 15	Leu 64	Ser 16	Leu 10	Ile 1	Cys 19	Leu 20	Ser 20	Ser 15	Tyr 7	238
Aplysia californica (see ref. (1))	Pro 2	Trp 3	Cys 9	Gln 1	Leu 78	Ala 18	Leu 64	Leu 49	Ala 25	Cys 6	Leu 8	Ser 17	Leu 1	Lys 11	Trp 0	292
Lymnaea stagnalis (see ref. (2))	Gly 4	Ile 15	Val 15	Cys 7	Leu 78	Ser 10	Phe 18	Phe 13	His 5	Ser 11	Ala 47	Val 3	Thr 6	Lys 11	Arg 9	252
Drosophila melanogaster (see ref. (3))	Ile 15	His 0	Ser 8	Glu 1	Ile 11	Ile 5	Asp 0	Thr 7	Pro 20	Asn 3	Tyr 0	Ala 6	Gly 39	Asn 4	Ser 10	129
Calliactis parasitica (see ref. (4)	Lys 71	Val 25	Thr 5	Leu 79	Leu 78	Phe 7	His 1	Ile 8	Leu 10	Thr 6	Ile 10	Asn 10	Ala 80	Lys 11	Thr 10	411

References: (1) Taussig and Scheller, 1986; (2) Linacre et al, 1990; (3) Nambu et al, 1988; (4) Darmer et al, 1991.

1988) contain such motifs ; sequences near the amino terminus of the *Aplysia* propeptide (Taussig and Scheller , 1986) and of the precursors derived by us from *Helix* cDNA sequences (figure 4) bear similarity to these suggested motifs although none concur exactly with those described by Kizer and Trophsa (1991) (e.g. - Ser^3 - Leu^4 - Cys^5 - Leu^6 - in HF4 , as opposed to the consensus sequence - Ser - X - Leu - Leu - derived by Kizer and Trophsa) . Again , the apparent incompleteness of cDNA clones HF1 and HF4 may explain the absence of such a sequence from the predicted precursors derived from them , although the lack of such a motif in the (apparently complete) *Aplysia* sequence may indicate that some more flexibility than Kizer and Trophsa have allowed is possible in the sequences directing such molecules to secretory vesicles .

4.4 : Precursor organisation

Comparisons between the FaRP polypeptide structures found in other organisms and those derived from cDNA clones isolated from *Helix aspersa* reveal a similarity in the arrangement of the constituent peptides within the different precursors . Although of the organisms whose molecular biology has been studied to date only *Lymnaea* and *Calliactis* are wholly analogous with *Helix* in their possession of both tetra - and N - terminally extended FaRPs , a difference in both the context and structure in which these short and extended peptides are encoded in these different organisms is apparent : there is a separation of tetrapeptides from those which are N - terminally extended , which in the case of *Lymnaea* means their being represented on separate exons within the same transcription unit (Saunders *et al* , 1991) . There also appears to be a difference in the way in which the two classes of peptide are arranged within the precursor molecule : while the N - terminally extended peptides are encoded in an "economical" fashion , being separated from each other only by the one or two basic residues which are important in directing cleavage from the precursor , the tetrapeptides are often

separated from each other by a short sequence of " spacer " residues which do not seem to form biologically functional peptides , and which contain a number of residues which have acidic side chains . It is possible that the propensity of acidic residues in these "spacer" regions is necessary in order to counteract the effects of the positive charges of the many basic amino acids involved in defining cleavage sites within the precursor polypeptide . In the case of the precursor molecules containing the N - terminally extended peptides in *Helix* , exemplified by the cDNA clone HF4 , these " acidic spacers " are absent : however , the peptides contained within these precursors themselves contain the negatively charged amino acid asparstate (D) . The number of basic residues on either side of each peptide is also generally reduced in the extended peptide precursor , relative to the tetrapeptide precursor , from two to one : these two facts in combination may obviate the need for the inclusion of " acidic spacer " regions in the precursor polypeptide containing extended peptides .

A similar situation appears to exist in *Lymnaea* : the tetrapeptide FaRPs are encoded in a manner which means the derived precursor will contain " acidic spacer regions " between peptides , while the extended FaRPs immediately abut one another , separated only by one , or sometimes two, basic amino acids (see figure 1). While the cDNA sequences from *Calliactis* published by Darmer *et al* (1991) do not provide an ideal comparison , in that the peptides there encoded do not appear directly analogous , there seems to be a similar separation of tetra - from extended peptides , with the overall arrangement of the peptides in the precursor appearing similar to those pertaining in *Helix* and *Lymnaea* , i.e. the short peptides are separated from the long forms , and there are no " spacer " sequences found between the extended peptides (figure 1).

When a comparison is made between the " spacer " regions encoded by the three organisms for which information is presently available (*Aplysia* , *Helix* and *Lymnaea*) , it is apparent that some constraints may be

Table 7

Amino acid sequences of "spacer " regions

A . *Helix aspersa*

S	–.	. N .	. E	D	–	D	L	E	
G	D	E	D	E	S	–	Y	D	
S	L	R	H	D	Q	E	F	E	
G	D	E	D	D	A	R	E	E	
S	S	N	E	D	E	D	I		
S	G	N	E	D	G	D	V	D	
S	E	K	E	D	G	D	V	D	
G	D	S	E	T	S				

B. *Lymnaea stagnalis*

A	L	D	T	T	D	P	I	R	L
G	G	Y	Q	P	Y	Q	D		
S	E	Q	P	D	V	D	D	Y	P R
G	D	E	E	A	E				
D	M	S	D	V	D				
E	P	G	T	D					
E	P	G	A	D					
S	F	D	G	E	E	E	. .	.	
S	A	E	E						
S	Q	D	A	S	R	D			

S	V	D	–	G	D	V	D
S	V	D	–	G	D	V	D
S	V	D	–	G	D	V	D
S	V	D	–	G	D	V	D
S	V	D	–	G	D	V	D
S	V	D		D	D	V	D
S	V	D	–	G	D	V	D
S	V	D	–	D	A	V	D
S	V	D	S	–	D	L	D
S	V	G	S	D	E	V	D
S	V	G	S	D	E	V	D
S	L	G	T	D	D	V	N
S	L	G	T	D	D	V	N
S	L	G	T	E	D	V	N
S	L	G	T	D	D	V	N
S	L	G	T	D	D	V	N
S	L	G	T	E	D	V	N
S	L	G	T	D	D	V	N
S	L	G	T	E	D	V	N
S	L	G	T	D	D	V	N
S	L	G	T	E	D	V	N

placed on these regions , perhaps indicating that while they do not form functional peptides , they may play a structural role in the precursor polypeptides of which they are part . As can be seen from Tables 6A-C, the residues at -1 and -3 relative to the succeeding peptide are usually acidic (36 and 33 out of 43 , respectively) , while there is also a tendency for that at -5 to be acidic . In *Helix* , all residues at -5 are acidic , while this is true for only approximately half of those in the other organisms : whether there is any significance in this difference in unclear . Residues at -2 are almost never acidic (4 of 43) , and are often valine (23 of 43) , though considerable variability is also seen. When the other end of the "spacer" region is considered , it is found that the residue at +1 relative to the preceding peptide is most often serine (31 of 42) , with the majority of the remainder being glycine (6 of 11) or glutamic acid (3 of 11) . The highly related nature of many of the spacer regions within each precursor is also obvious from such analyses , perhaps indicative of their being derived from an internal reduplication of an ancestral gene .

The differing numbers of peptides contained in each precursor polypeptide is very noticeable : while full processing of a single precursor molecule encoded by HF1 would release (at least) 10 copies of the FMRFamide peptide , much smaller numbers of each individual extended peptide (e.g. 3 of pQDPFLRFa) are produced per HF4 - encoded precursor . This may , to some extent , be a reflection of the longer half - life thought to be possessed by the extended peptides , thus requiring fewer molecules to be produced in order that a physiological effect be achieved by them . It may also indicate a difference of the types of behavioural responses for which the different types of peptide are required : while the tetrapeptide responses are often seen to be fast - acting (e.g. muscular contractions , nervous excitation) , those of the extended peptides tend to be slower acting over longer distances , such as muscle relaxation . It may be that , in order to achieve a fast response , the ability of a cell to produce large numbers of bioactive peptides requires that they be produced

from highly reiterative precursors , such as is seen in that encoded by cDNA clone HF1 , and that this is less critical in the case of the heptapeptides , which often seem to act over longer times and distances than do the tetrapeptide FaRPs . It should also be noted that our investigations (see figures 9 and 10) often indicated that mRNA molecules encoding heptapeptides were significantly more abundant than those specifying the tetrapeptide FaRPs : such a discrepancy may have a bearing on the relative abundances of the two classes of FaRPs in *Helix* .

It is not , of course , necessary that each precursor molecule contains the full gamut of peptides available to it : we have evidence which suggests that alternative splicing of pre - mRNAs takes place in *Helix* , resulting in some cases in fewer peptides , or different combinations of peptides being encoded. For example HF7 , a variant of the HF4 family, was isolated : this clone appears to encode a precursor lacking both copies of the peptide pQDPFLRIa (peptide 6 in figure 1E) . This peptide is the only one encoded in the *Helix* cDNAs which we have isolated which is cardioinhibitory (Lutz *et al* , 1990) , and probably represents the peptide isolated from *Helix* and identified by Price (Price 1982) as pQDFIRFa on the basis of amino acid sequencing (although why it should react so strongly with an antiserum directed against YGGFMRFa , and should appear to be the most abundant *Helix* FaRP in these studies is not immediately obvious).

The precise functional significance of the arrangement of the peptides within their precursor polypeptides is as yet unclear . There is evidence that different members within a class of FaRPs are expressed together by individual cells or groups of cells within the nervous system of *Helix aspersa* , while the two classes are usually expressed separately (Lehman and Price , 1987b) ; the effects of the two classes of peptide on muscles and neurones often differ (Payza *et al* , 1989) , so separate expression may often be required . Although gene expression

can be controlled at levels other than transcription (e.g. splicing (Black , 1991) or posttranslational attenuation (Rehfeld 1990 ; Ryzanov *et al* , 1991)) , the arrangement of short and extended FaRPs into separate regions of a transcription unit may facilitate their differential expression by splicing of pre - mRNAs in order to produce alternative mature mRNA molecules , and may also be to assist the differential sequestering of peptides from the precursor polypeptide into separate secretory vesicles for release from the cell at different times and / or sites , as required .

4.5 Processing from precursor

Many peptides are , as has previously been observed , first synthesized as part of a large precursor polypeptide; their excision from this often seems to be catalysed by a furin - like endoprotease (Nakayama *et al* , 1991) , and is directed in part by the presence of basic residues bounding the peptide .

A sequence of two consecutive basic residues , usually - Lys - Arg - , is the most commonly observed signal directing the processing of a peptide from its precursor , although processing also occurs at some monobasic sites . (This latter observation indicates that it is not only the primary amino acid sequence which is involved in directing cleavage at a particular site , but that the context in which the sequence is found is also important : for example, Gomez *et. al.* (1989) have observed that the secondary structure of the precursor , specifically the presence of a β turn in the vicinity of the processing site, is also involved in defining propeptide cleavage sites .) Most of the proposed processing sites in the amino acid sequences derived by us from the *Helix* ganglial cDNA clones HF1 and HF4 are at dibasic sites , although a number appear to be defined by monobasic residues ; when the sequences surrounding the latter group are compared to the "rules" and "tendencies" derived by Devi (1991) from studies of a number of monobasic cleavage sites , they are found to follow the "rules" exactly , but there is some deviation from the

"tendencies" suggested . Some of these " failures " are likely to be due in part to constraints on the precursor amino acid sequence because of the peptides encoded (e.g. the +1 position relative to the basic residue is often part of the following peptide , therefore the propensity towards Ser , Ala or Gly could not be observed here without altering the structures of functional peptides) , but it is probable that processing occurs at these sites none the less .

Rapid initial processing of the egg laying hormone (ELH) precursor of *Aplysia* is observed to occur at a tetrabasic site (Newcomb and Scheller , 1987 ; Kreiner *et al* , 1989) , a fact which concurs with the suggestion that longer sequences of basic residues are cleaved in precedence to shorter ones . The presence of tri - or tetrabasic sequences in the FaRP precursors in *Aplysia* (Taussig and Scheller , 1986) , *Lymnaea* (Linacre *et al* , 1990) and *Calliactis* (Darmer *et al* , 1991) , and their positions separating different types of peptide is also noted ; the single tribasic sequence in the *Drosophila* precursor is located after a section of the propeptide not apparently encoding any FaRPs and immediately preceding the contained FMRFamide - related peptides (Nambu *et al* , 1988) . Neither of the amino acid sequences derived by us from *Helix* cDNAs contains a tetrabasic sequence , but the tribasic sequence " - Lys^{132} - Lys^{133} - Arg^{134} - " does occur once in the HF1 tetrapeptide precursor (figure 4) ; its location , following as it does the single copy of the " - Phe^{118} - Leu^{119} - Arg^{120} - Phe^{121} - " sequence in the precursor , makes it possible that an initial rapid cleavage of the polypeptide takes place at this site , allowing the sequestration of the precursor fragment containing both the FMRFamide and FLRFamide peptides to different parts of the cell from that containing only the FMRFamide peptides , or ultimately their packaging into separate secretory vesicles , a division which may be of functional importance in terms of the actions of the peptides . A system similar to this appears to operate in *Aplysia* , with the neuropeptides derived from the ELH precursor being separated by an initial cleavage prior to their being sequestered into

distinct vesicle classes and localised to separate processes of the cell (Sossin *et al*, 1987) .

PCR analysis of *Helix aspersa* genomic DNA has indicated that there are two additional copies of the sequence encoding FLRFamide upstream of those encoding FMRFamide found in HF1 . This analysis also revealed the presence of a tetrabasic amino acid sequence within the putative precursor molecule containing the tetrapeptides : this " RKRR " sequence conforms well with the consensus cleavage sequence for the golgi - resident enzyme furin (Lindberg , 1991) , and is also found to be present , along with a number of other residues surrounding it , in the *Aplysia* , *Lymnaea* and *Helix pomatia* FaRP precursors (see figure 27) . Were rapid cleavages to occur at this tetrabasic sequence and also at the tribasic sequence previously mentioned , 3 sections of the precursor would be produced : the first would contain only copies of FLRFamide , the second both FLRFamide and FMRFamide , and the third only FMRFamide . While there is obviously no direct evidence to support it , it is interesting to speculate that the organism may indeed use this arrangement of peptides to aid differential sequestering of combinations of peptides to separate vesicles or sites for independently regulated release .

Computer analyses of the primary structures of HF1 and HF4 reveal striking differences between the two putative precursors from *Helix* (see figure 28) . For example , while HF1 is predicted to be entirely hydrophilic (though with relatively hydrophobic regions in it , which appear to coincide with the FMRFamide - like peptides in the precursor) , HF4 is indicated to have regions of hydrophobicity at either end , one of which corresponds to the predicted signal sequence at residues 1 - 19 . The nature of the predicted secondary structures is also very different : a single β sheet is predicted for HF1 (which is coincident with the " - Lys - Lys - Arg - " tribasic sequence) , while numerous α helices are thought to be formed . In contrast to this , depending on the predictive method applied , at least 3 , or up to 16 , β sheets are indicated as occurring in

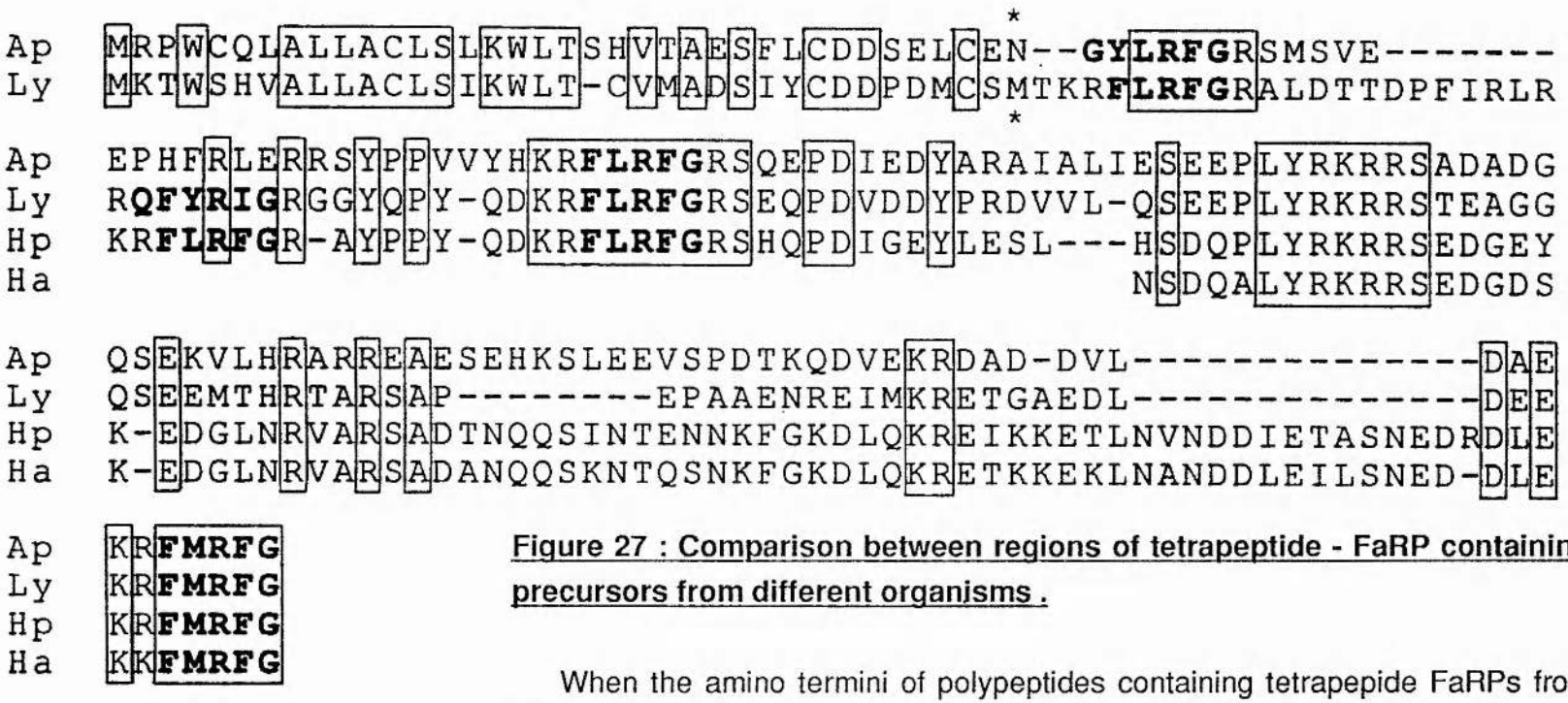


Figure 27 : Comparison between regions of tetrapeptide - FaRP containing precursors from different organisms .

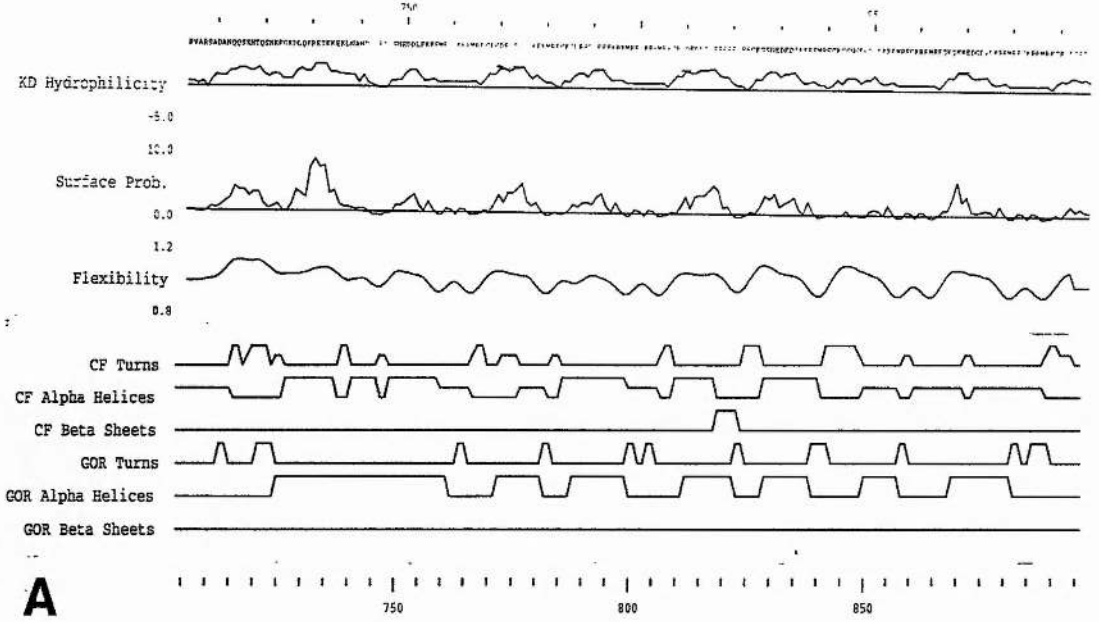
When the amino termini of polypeptides containing tetrapepide FaRPs from different organisms are compared significant homology between species is observed . Shown above are sequences derived from *Aplysia californica* (Ap) , *Lymnaea stagnalis* (Ly) , *Helix pomatia* (Hp) and *Helix aspersa* (Ha) : conserved residues are shown boxed . Greatest conservation is seen in the vicinity of peptides (bold type) and of the tetrabasic " RKRR " sequence , thought to be important in initial cleavage of polypeptide . Dashes indicate gaps introduced to facilitate alignment of sequences .

Figure 28: Computer predictions indicate secondary structures of polypeptides encoded by HF1 and HF4 to differ significantly.

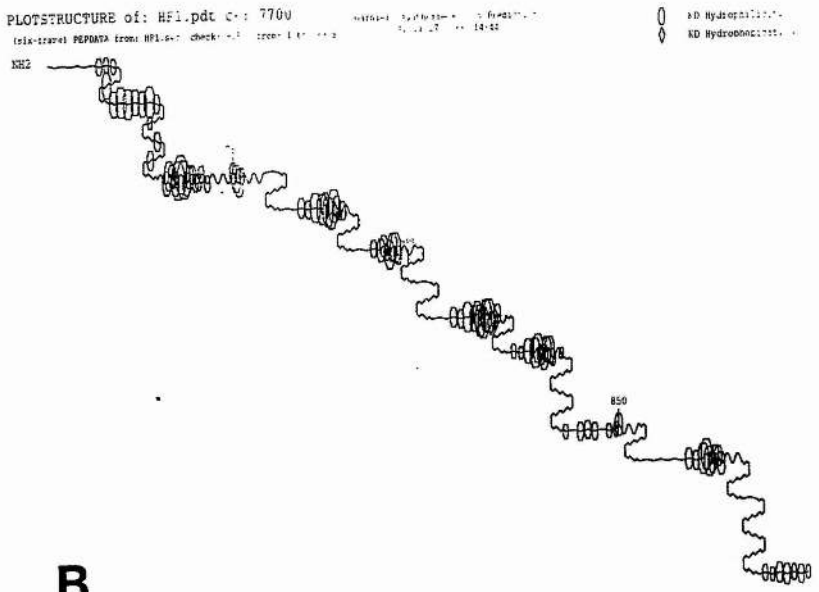
When computer analyses of precursor polypeptides containing FaRPs isolated by us from *Helix aspersa* were carried out, these indicate that the secondary structures adopted by these polypeptides will differ significantly. This may have an appreciable effect on the rate of liberation of the tetra and heptapeptides from their respective precursors, and may be used by the organism as another means of differentially regulating peptide expression. Since these are, however, computer **predictions**, care should be taken in extrapolating from them.

A and B : Predictions regarding HF1 polypeptide.

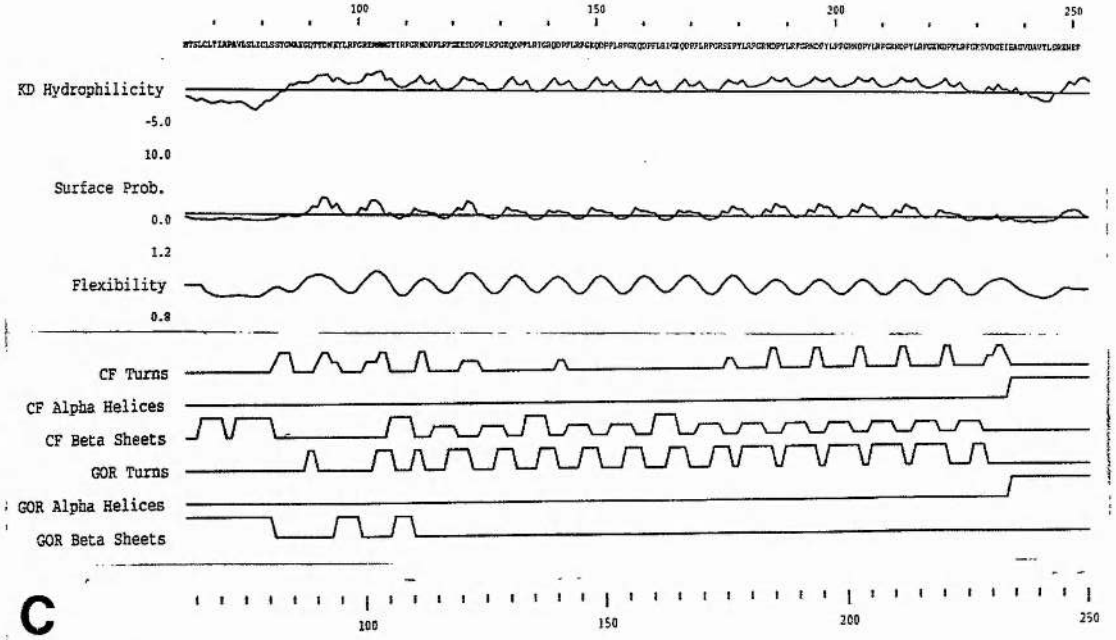
C and D : Predictions regarding HF4 polypeptide.


KD Hydrophilicity
-5.0
10.0
Surface Prob.
0.0
1.2
Flexibility
0.8
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
750
800
850

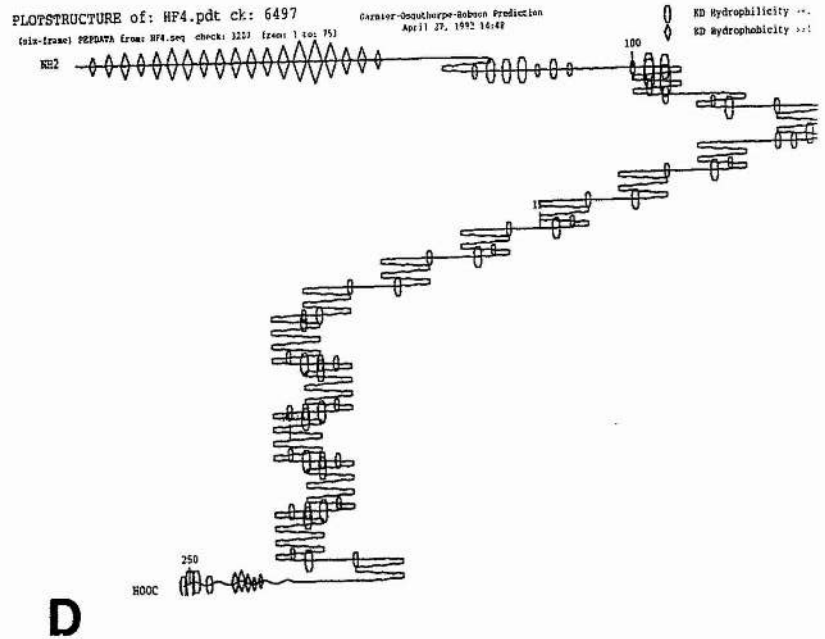
A


PLOTSTRUCTURE of: HF1.pdt
NH2
850

B


100
150
200
250
KD Hydrophilicity
-5.0
10.0
Surface Prob.
0.0
1.2
Flexibility
0.8
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
100
150
200
250

C


PLOTSTRUCTURE of: HF4.pdt ck: 6497
Garnier-Osguthorpe-Robson Prediction
April 27, 1992 14:48
KD Hydrophilicity
KD Hydrophobicity
NH2
100
250
HOOC

D

HF4 , while no α helices at all are apparent . The flexibility of both precursor polypeptides is predicted to vary significantly along their lengths : that of HF4 appears more regular and is also more frequently repeated along the precursor than that in HF1 .

4.6 : Amidation

Carboxy terminal amidation is a common modification of biologically active molecules (Stoffers *et al* , 1991) ; it has been observed that amidation appears to occur primarily among neural and hormonal peptides (Tatemoto *et al* , 1982). The C - terminal amidation of a peptide , a process which occurs almost exclusively *via* the modification of a glycine residue (Mains *et al* , 1983) and which is catalysed by two enzymes , peptidylglycine α - amidating monooxygenase and peptidyl - α - hydroglycine α - amidating lyase (which are themselves both derived from a common precursor (Stoffers *et al* , 1991 ; Kato *et al* , 1990) , is usually essential to the function of such peptides (Vale *et al* , 1981) . This certainly appears to be true for many of the FaRPs , where removal of the amide group from the carboxy terminus virtually abolishes a peptides bioactivity (Kobayashi and Muneoka , 1986 ; Payza, 1987) .

Each of the putative FaRPs in the polypeptides encoded in *Helix* by HF1 and HF4 is followed immediately by a glycine residue , which would facilitate its amidation in the presence of the appropriate processing enzymes . Thus the amino acid sequences coded for in the mRNAs from which HF1 and HF4 were generated appear to contain much of the information required for the sequestration of the precursor polypeptide to the appropriate site in the cell (i.e. the endoplasmic reticulum), and to secretory vesicles , and also to direct the precise cleavage and amidation of the constituent peptides , in order to release mature bioactive molecules .

4.7 : Context of translation start site

It is noted that the nucleotide sequence surrounding a putative translation start site in an mRNA has a significant influence on the efficiency with which such a site is used *in vivo* (Kozak , 1986) . Studies have indicated that it is possible to " score " a given sequence against a consensus sequence in order to predict the likelihood of a specific AUG sequence being used as a translation start site . As is illustrated in figure 29 , the context within which the proposed start site of translation of the mRNA represented by cDNA clone HF4 is located is found by such analyses to be quite favourable , indicating that this may be a translation start site used *in vivo* .

4.8 : Intracellular mRNA localization

The occurrence of peptide transmitters in invertebrate nervous systems is known to be very widespread - in a study of certain identified *Aplysia* motor neurones by Church and Lloyd (1991) it was found that all cells studied contained at least one such transmitter , and many cells synthesized a number of different peptides ; FMRFamide - like immunoreactivity , for example , has been co - localised with a number of neurotransmitter substances (e.g. ACh (Ping *et al* , 1989) , 5HT (Takayanagi and Takeda , 1988b) , and GABA (Homberg *et al* , 1990)) as well as various other neuropeptides (e.g. buccalin (Church and LLoyd , 1991) substance P (Lundquist and Nassel , 1990) and Met - enkephalin (Takayanagi and Takeda , 1988b) . In neural cells , the site of production of mRNA , the nucleus , which is located in the cell body , is often very distant from the sites at which a peptide may be required to be released , such as at synapses . Since the production of many biologically active amidated peptides , including the FaRPs , requires not only the means of translating the mRNA encoding them , but also the cellular "

Figure 29 : Context of putative translation start site in HF4.

When the proposed translation start sites from various FaRP-encoding cDNAs are analysed according to G.D. Stromo, it appears that the ATG at 191-193 proposed by us to be the site of initiation of translation is found to be located within a context favourable for its utilization as such *in vivo*.

	NUCLEOTIDE POSITION										TOTAL SCORE
	−4	−3	−2	−1	0	1	2	3	4	5	
Helix	A	T	C	A	A	T	G	A	C	T	
	−1	−32	3	0	14	14	14	−1	5	5	21
Lymnaea	G	T	T	T	A	T	G	T	A	T	
	−8	−32	−2	−7	14	14	14	−8	1	5	−9
Drosophila	A	G	A	G	A	T	G	T	A	T	
	−1	−7	3	−6	14	14	14	9	−4	−5	31
Calliactis	T	C	G	T	A	T	G	C	T	G	
	−8	−18	−11	−7	14	14	14	−18	−6	1	−25
Aplysia	T	G	C	C	A	T	G	A	G	G	
	−8	−7	3	7	14	14	14	−1	−4	1	33

machinery " to bring about their excision from their precursor polypeptide and their effective amidation , it might be anticipated that all production and processing of peptides take place in the cell body , with the peptides subsequently being transported to their site of release. This appears in fact to often be the case : mature neuropeptides including FMRFamide (Kreiner *et al* , 1986 ; Nagle , 1981) , are detected in dense cored secretory vesicles in cell bodies (Zimmerman , 1990 ; Nagle , 1981 ; Kreiner *et al* , 1986) , by which means peptides are transported to their sites of action or release (Lloyd , 1988) , or sometimes between ganglia (Lloyd , 1989) . In some *in situ* hybridization studies , however , the somewhat surprising observation that mRNAs encoding a number of neuropeptides , such as arginine vasopressin , oxytocin and the ELH peptides , are detectable outwith nerve cell bodies , in dendrites and possibly at synapses (Bloch *et al* , 1990 ; Mohr *et al* , 1991 ; Dirks *et al* , 1989 , 1990) has been made . Our studies did not reveal any such localization of FaRP - specific mRNA in *Helix* : such mRNA appeared to be limited to nerve cell bodies (see sections below on *in situ* hybridization experiments) . However, as is explained in detail elsewhere , this may be due at least in part to the detection system used in these studies : our studies using non - radioactive probes may not be sufficiently sensitive to detect small amounts of mRNA , such as may be present in sites distant from the nucleus .

4.9 : Genomic DNA sequences

It is not yet clear whether in *Helix aspersa* the extended FMRFamide - related peptides are encoded in a separate transcription unit from the tetrapeptide FaRPs , or whether , as is the case in *Lymnaea* (Saunders *et al* , 1991), the genomic sequences representing them are separated by a large intron but are under the influence of the same *cis* regulatory sequences . Evidence from Southern blots of *Helix* genomic DNA suggests that the sequences complementary to the cloned cDNA probes HF1 and HF4 (encoding the tetrapeptide and extended FaRPs , respectively) are a number of kilobase pairs

apart , since these probes hybridize , in most cases , different sized genomic DNA fragments (figure 5 ; Table 2) . This being true for DNA digested with several restriction enzymes means it is statistically unlikely that the sequences being detected are contiguous in the genome but are fortuitously separated on Southern blots by virtue of a restriction enzyme site ; it is more likely that they are separated in the genome by several kilobase pairs of intervening sequences , containing the restriction enzyme recognition sites which result in the differing patterns seen in Southern blots (figure 5) .

Probing of DNA digested with a variety of enzymes often reveals two bands hybridizing with the HF1 and HF4 probes , with one of these bands often appearing to bind the probe more strongly . Since these patterns with HF1 and HF4 probes are not reciprocal (i.e. the weak band hybridizing with HF1 does not strongly hybridize with HF4) , it is not thought likely that this represents simple cross - hybridization between the probes . It is not , however , clear what these results mean : possibly the bands being detected weakly represent pseudogenes which have diverged somewhat in sequence , or perhaps some other genomic sequence related to FaRPs . (If these putative genes were expressed in nervous tissue , it would be expected that cDNA clones representing them may be picked out from libraries using FaRP specific probes : we have no evidence for this having occurred . Perhaps less stringent re - screening of the cDNA library may lead to such a selection .)

It is apparent that the probes HF1 and HF4 hybridize DNA fragments produced by digestion with the enzyme Eco RI of approximately the same size (Lutz *et al* , 1992 ; figure 5C and D) : it unclear , however , whether these hybridizations are detecting the same DNA fragment , or whether the DNA fragments concerned are of differing sequence but of approximately equal length . If it were assumed that HF1 and HF4 bound an identical Eco RI fragment of 14.2 Kb , this would indicate that the sequences encoding the tetrapeptide and extended FaRPs are less than this distance apart in the genome

of *Helix aspersa* . (Clone HF4 terminates at its 3' end in an Eco RI recognition site ; a similar site is found by PCR to be present in the genome of *Helix aspersa* (though not , interestingly , of *H. pomatia* , due to a point mutation) immediately 5' to the sequences found in HF1 , as judged by PCR analysis (Lutz *et al*, 1992) . It is possible that the Eco RI fragment defined by these two sites is the same fragment being detected by us in DNA blots - i.e. that HF1 and HF4 are at the extreme 5' and 3' ends , respectively , of a 14.2 kb fragment resulting from Eco RI digestion of *Helix aspersa* genomic DNA (see figure 6). That the cDNA clone HF4 ends in an Eco RI recognition site (see below) tends to confirm this suggestion) .

It has been found to be the case in *Aplysia* (Taussig and Scheller , 1986) , *Lymnaea* (Saunders *et al* , 1991) and *Drosophila* (Chin *et al* , 1990) that the FMRFamide - related peptides are encoded by a single gene interrupted by a large intron (intron sizes 3.4 Kb and 2.8 Kb , respectively , in the cases of *Lymnaea* and *Drosophila* ; the exact size of the *Aplysia* intron is as yet undetermined) ; whether this is also the case in *Helix* has not yet become apparent - although such a possibility is not contradictory of current evidence , the precise structure of the relevant genomic regions remains to be elucidated .

A variety of approaches have been utilised in our attempts to clone the genomic DNA fragment(s) concerned from *Helix*, and so to gain access to their structure , to date without success. These have included the construction and probing of genomic DNA libraries , the isolation of fragments of the appropriate size (as judged by results from Southern blots) from genomic DNA separated on low - melting point agarose , and the utilisation of the " inverse PCR " technique . This latter approach is one which allows use of the polymerase chain reaction (PCR) in order to gain access to previously unknown sequences which are adjacent to sequences which are already known (see figure 7) . It involves the digestion of genomic DNA with a restriction enzyme which does not cut within the known sequence , and the ligation of the resultant fragments to form DNA circles . These

are then subjected to PCR , using primers derived from the known sequences which are located towards the ends of the sequence, and which prime DNA synthesis away from the centre of the known fragment and therefore into the regions of unknown sequence at either end of the known sequence . Since the molecule is circular , polymerisation should be able to proceed round the molecule until the opposite end of the known sequence is reached ; synthesis into this will allow the production of the binding site for the opposite primer which is essential for the effectiveness of the PCR process . Utilization by us of this approach failed to yield success in our attempts to isolate FaRP - specific genomic DNA , however .

Taken in conjunction with evidence from other organisms , it may be supposed that the sequences being detected by us in Southern blots using the probes HF1 and HF4 represent (parts of) two exons of a single transcription unit, or alternative splicing products thereof . The cDNA sequences shown (figure 4 A and B) represent the most complete data available at present , but in the light of evidence from various sources , including our RNA blots which indicate that probes generated from both cDNAs hybridize mRNA species in the size range 1.7 - 1.8 Kb (figure 9), and the absence of a methionine start site in the tetrapeptide precursor and of a 3' end in the heptapeptide precursor , we conclude that neither cDNA is a complete representation of the mRNA from which it is derived . This problem is often encountered with cDNA clones , due to their method of generation : it is not uncommon for mRNA used as a template for cDNA synthesis to be , to a greater or lesser extent , degraded . Since first strand cDNA synthesis is initiated from the 3' end of the molecule , using the poly A tail of the mRNA as target for the annealing of a poly T primer , and extension from this proceeds in 3' to 5' direction using reverse transcriptase , the extreme 5' end of an mRNA molecule is often not copied - due either to degradation of the template mRNA before or during first strand synthesis , or perhaps to the reverse transcriptase molecule detaching from the mRNA before the 5' end is reached. It would appear that the cDNA product HF1 has been

truncated by one of these means , lacking as it does the ubiquitous methionine translation start signal . cDNA clone HF4 , however , appears to be missing its extreme 3' end , probably as a result of the failure of the modification methylase enzyme to adequately protect the Eco RI recognition site which terminates HF4 . Following completion of synthesis of both strands , cDNAs are treated with modification methylase , an enzyme which methylates guanine residues : this has the effect of rendering any Eco RI sites within the cDNA refractory to digestion with this enzyme . Such protection is necessary since , following ligation of Eco RI linkers to the cDNAs , linker concatamers are eliminated by digestion with Eco RI , while Eco RI sites within the cDNA , and consequently the cDNA itself , remain intact . Clone HF4 , however , terminates at its 3' end in what appears to be an internal Eco RI site , indicating that it was inadequately protected from digestion at this site during its generation . Thus the 3' end of the mRNA from which HF4 was derived , including the poly A tail which must originally have been present to allow priming of first strand synthesis , has been lost .

Although numerous cDNA clones of each class were identified by us , we have been unable to isolate a single *Helix* cDNA encoding both tetrapeptide and extended peptide FaRPs . The onset of the expression of HF1 and HF4 during embryogenesis seems to occur at similar (though possibly slightly different) times (see figure 10) ; the messages detected by the two probes are of slightly different size (figure 9) , and their expression appears to be mutually exclusive at the cellular level, as judged by *in situ* hybridization studies (figure 18) . It therefore appears that the sequences encoding the tetra- and extended peptide FaRPs in *Helix* are separate by the stage of mRNA generation : whether this separation is due to differential splicing of a common mRNA precursor , or is as a result of their being derived from different transcription units will remain unclear until access to the appropriate DNA fragment(s) is obtained , and the precise genetic arrangement and sequences governing control of the expression of the FMRFamide - like peptides in *Helix aspersa* elucidated .

4.10 : Gene expression during embryogenesis

It is possible to study expression of the gene(s) encoding the FMRFamide - related peptides as the embryo develops by isolating poly A^+ mRNA from embryos at different stages during development . This was done as described , and the mRNA populations probed in order to determine whether or not the messages representing the FaRPs were there represented . As is shown in figure 10 , it is possible to detect mRNA for the tetrapeptides as early as day 10 in development (53 %) , while the antisense riboprobe to the HF4 extended peptide cDNA clone hybridizes with RNA isolated from embryos as young as day 8 (42 %) . However , in contrast to the general increase in the amount of mRNA obtained from embryos as they progress through development (figure 12) , the amount of FaRP - specific mRNA detected appears to rise and fall in three successive cycles during development , and perhaps a fourth after hatching (see figure 11) . Analysis of the slot blots obtained on probing with both HF1 - and HF4 - specific probes appears to indicate this to be the case for both the tetra - and extended peptides . In addition , the presence of these specific mRNAs in *Helix* embryos seems to rise and fall in phase with one another , each cycle taking 3 - 4 days . Peptides were also extracted from *Helix* embryos according to the method of Abood *et al* (1990) : these were analysed by means of radio immuno assay (RIA) by Dr. W. Lesser at the University of Florida . It is apparent from these RIA studies that , while some rising and falling of peptide levels as development proceeds may be occurring , it is not nearly so dramatic as that seen for mRNA (see figures 25 and 11) : indeed , it is possible that an exponential curve may be drawn which approximates to this data , indicating a general rising in levels of FaRPs during development in *Helix* .

Given that mRNA molecules are unlikely to have any significant direct bioactivity , but that this function resides mainly in the mature peptides , is it therefore likely that the " cycling " of mRNA levels observed by us is of biological significance , or indeed is a real phenomenon at all , as opposed to being an experimental artefact ? We have apparently observed large variations in FaRP - specific mRNA levels through embryogenesis , but this does not seem to be reflected in peptide levels detected at similar stages : if no changes in peptide levels result , why is there an apparent regulation of mRNA expression ? There are a number of possible reasons why this may be the case . Although peptide production must , of necessity , succeed mRNA synthesis , it is not necessary for it to do so immediately . For example , mRNA may be transcribed at some stages during development , but not immediately translated . Alternatively , mRNAs may be transcribed and translated , but processing of the precursor in some way prevented : since only mature , amidated peptides will be detected by the antibodies used in these studies , such a possibility cannot be eliminated . Thus mRNA synthesis may be temporally disconnected from peptide synthesis by exertion of control over a combination of a number of steps - e.g. translation, precursor polypeptide cleavage , or peptide amidation . (VIP (vasoactive intestinal peptide) mRNA expression in developing mouse brain appears to precede peptide expression by a few days (Gozes *et al* , 1987): FaRP expression may be similarly delayed . Certainly , according to our studies , detectable levels of FMRFamide - related peptides lag 4 - 5 days behind mRNA , although this apparent delay may be due in part to differential sensitivities in the assays being utilised .)

It is also possible that the promiscuity of antibody binding in RIA studies is masking real changes in FaRP peptide levels through development : the RNA probes used in detection of mRNA are likely to be much more specific in their binding than are antibodies . It is therefore possible that amidated proteins / peptides unrelated to FMRFamide present in the extracts of the

embryos are being detected by these antibodies , in addition to the FaRPs themselves , thus skewing the experimental observations . It should also be remembered that , although it is possible to isolate a virtually pure sample of mRNA from a tissue source , almost entirely free of any other cellular component , this was not the case for the samples used in the RIA studies . While it is believed that lithium chloride precipitation of the sort used here favours the isolation of peptides from a sample , it does not do so so selectively as ethanol precipitation and oligo dT selection can do for mRNA molecules : HPLC separation will render the sample more pure , but it is probable that embryological material other than peptides is also present in the samples , some of which may interfere in some way with the assay used .

Thirdly , while it has been possible to estimate the percentages of mRNA molecules present in slot blots which are binding the FaRP - specific probe , no such analysis has been possible with regard to the RIA data , since no information is available regarding the amount of protein present in each sample , nor indeed what percentage of that was peptide , of any sort . Thus changes in FaRP levels may go unobserved in RIA studies , due to interference from some other factor(s) present in the extracts .

It may also be that the peaks of FaRP - specific mRNA observed by us reflect a situation in which , due to a need to avoid gene expression during periods of rapid cell division during development of the nervous system , mRNA is synthesized in advance and stored for translation at an appropriate time , in a way similar to that known to occur during oocyte maturation and early embryogenesis in many organisms (Davidson , 1989) . Were this the case , the troughs in FaRP - specific mRNA levels observed by us would correspond to periods between such syntheses , where RNA stores are being used up without being replenished . No data is available regarding the development of the nervous system in *Helix* : this being the major (and perhaps

exclusive) site of FaRP synthesis , development of this tissue is most likely to be relevant to control of mRNAs encoding them .

Each of the samples in the slot - blot as shown contains the mRNA equivalent to a single embryo . As the size of the embryo increases dramatically during development , so does the amount of mRNA obtained , on average , from an individual embryo . (The exception to this pattern is at the very beginning of development : the amount of mRNA isolated per embryo on day 1 is found to be greater than that from either of the two successive days (figure 12) . This is perhaps attributable to the phenomenon previously alluded to , whereby oocytes of many organisms are found to contain a store of maternal mRNA , accumulated as they mature , which is used during early rapid cleavages : this is thought to be in order to obviate the need for mRNA synthesis during this period (Davidson 1989)) . This being so , it is possible that detection of specific messages is facilitated at later stages in development : any errors in estimations of RNA concentrations or in pipetting are magnified when amounts of material are small . It is also likely that , as development proceeds , larger numbers of cells in an individual embryo will be expressing a particular mRNA ; this effect , however , is probably (at least in part) offset by a concomitant increase in the complexity of the mRNA population of the embryo as more organs and tissue types become extant , and a wider variety of genes expressed . It is therefore possible that mRNAs encoding the FaRPs are present in embryos at stages earlier than is detected in slot - blots studying " embryo equivalents " .

It may be that the presence of mRNA encoding the FaRPs and therefore , potentially , functional peptides during early embryogenesis is suggestive of a role for these peptides during development . Indeed , there is evidence to suggest that this is the case with regard to at least some peptide systems (Broadie *et al* , 1990) . It has previously been shown that a neurotransmitter , 5HT or serotonin , is involved in directing , to at least some extent , the development of certain cells in the

nervous system of the pond snail *Helisoma triviolis* (Lipton and Kater ,1989)) , and that this same neurotransmitter is capable of inhibiting neuronal sprouting and synaptogenesis in culture (Haydon *et al* , 1984 ; Goldberg and Kater , 1989) : a developmental role for 5HT in lobster has also been suggested (Beltz *et al* , 1990) . (Experiments performed by McCobb and Kater suggest that these effects of 5HT can be over - ridden by membrane hyperpolarization (McCobb and Kater , 1988)) That another neuroactive substance , the neurotransmitter acetylcholine , may possess neurotrophic properties has long been suggested (Welsh , 1948) , and stimulation *via* ACh receptors of DNA synthesis in brain derived cells (Ashkenazi *et al* , 1989) tends to support this . The ability of neurotransmitters to exert such effects appears to be limited to a subset of nerve cells , and does not seem to represent a general growth - regulating property attributable to these substances , in the way that , for example , nerve growth factor can affect neuronal cells (Cho *et al* , 1989) . Rather , it is probable that the phenomenon being observed represents the utilization by the organism of neurotransmitters to perform a function in development in addition to their role in the adult , that of directing the formation of interneuronal connections as the nervous system is laid down in development .

Although such a function has not yet been attributed to FMRFamide - related peptides , their expression is known to be closely related to nervous system development in *Manduca* (Copenhaver and Taghert , 1989) , and there is evidence that some other neuropeptides are implicated in neuronal development : results of experiments by Zagon and McLaughlin (Zagon and McLauchlin , 1983 , 1991 ; Lauder ,1990) suggest that the opioid peptides inhibit neuronal cell proliferation during development , while Nillson *et al.* (1985) suggest that the observed mitogenic effects of the tachykinins may indicate a role for them in development as well as their known transmitter function . It is therefore at least possible that the various endogenous FaRPs found to be present in many adult organisms may also play a role during neuronal development.

To test this possibility , interruption of the function of these peptides would be required ; as there are at present no effective antagonists of the FMRFamide - related peptides , the disruption of their expression at the level of the mRNA is the most accessible means of achieving such interruption . Since FaRPS tend to be expressed at a high level in a small number of cells , the saturation of the FaRP mRNAs by anti - sense RNA might prove difficult . However , with the advent of such techniques as the targeted destruction of mRNAs by so called " ribozymes " (Haseloff and Gerlach , 1988 ; Cotten and Birnstiel , 1989 ; Cotten , 1990) , RNA molecules which can be designed to catalyse the sciscion of messenger molecules containing defined sequences , the effective expression of a gene can be specifically perturbed at the level of mRNA synthesis . If the ribozyme were encoded on a suitable plasmid vector , and its expression linked to an inducable promoter , such as the metallothionine promoter , which can be induced by Cd SO_4 (Piccioli *et al* , 1991)) , it should be possible to destroy a target message at specific times during development of an organism ; in this way , and with suitable control experiments , the specific effects of a gene product in embryogenesis could be evaluated .

This is potentially useful approach ; however , its application to the system in which we work , *Helix aspersa* , is limited by a number of factors . Firstly , no plasmid vectors are available which are known to be maintained in the cells of this species , or indeed of any mollusc : such a vehicle would be essential for the effective delivery of the "ribozyme" to all cells . In addition , even were such vectors available , the biology of the snail *Helix aspersa* does not lend itself readily to this technique : in order that all cells in an individual possess the plasmid in question , it must be injected into all cells of an early embryo . Although 1 , 2 and 4 cell embryos have been observed by us immediately after laying , the maintenance of *Helix* embryos outwith their normal environment has to date proved impossible . The possibility of introducing a ribozyme - containing vector into germ cells , and thus producing " transgenic " *Helix* , remains. However , a third

difficulty , the long maturation time of this organism , is then encountered : it is thought that sexual maturity may take up to two years to achieve . It is therefore concluded that , although the function of the FMRFamide - related peptides during *Helix* embryogenesis is of interest , investigation of such , at least *via* the "ribozyme" technique , may be more easily achieved in other organisms : *Drosophila* , for example is advantageous in that there are a variety of plasmid and other vectors readily available for use in this organism which could be utilized in " ribozyme " delivery , and that understanding of the molecular biology of the *Drosophila* nervous system is relatively well advanced (Thomas and Crews , 1990) . However , although *Drosophila* possesses a variety of FaRPs , all appear to be of the extended type : studies in this organism would yield little information regarding the potentially interesting interplay between this type of FaRP and their tetrapeptide analogues .

We have been able , therefore , using the techniques described earlier , to estimate the level of production of both mRNA encoding the FaRPs and the peptides themselves during the embryological development of *Helix* . We were unable, however , to ascertain the distribution in the embryo of the cells expressing these peptides , since our *in situ* hybridization studies of embryos , both sectioned and whole mount , failed to reveal any significant hybridization of either probe . (The possible exception to this observation was the radula , which showed fluorescence in most sectioned embryos examined ; this is not , however , thought to represent the presence of FaRPs in this structure , but rather to be as a result of autofluorescence of this tissue , or of its binding the antibody used in *in situ* studies in a non - specific manner). *In situ* studies in *Drosophila* , however , indicate that expression of the FaRPs in this organism is consistently restricted to a subset of neurons , amounting to approximately 60 cells in all (Schneider *et al* , 1991) . These cells corresponded in position with FMRFamide immunoreactive neurons previously observed in adult *Drosophila* , although a number of cells which reacted with antibodies against FaRPs did not hybridize with the *in situ* probes . These studies did not

detect expression of FaRP - encoding message until approximately 70% of embryogenesis (which appears to be slightly later than the onset of gene expression detected by us in *Helix* , where expression detected at day 8 of 19 represents 42% of development) ; the distribution and variety of cell types observed in *Drosophila* (including motoneurones , interneurones and neuroendocrine cells) is taken as an indication that the cells expressing FaRPs are not obviously related cell lineages or clones , but instead represent specific regulation of gene expression at the level of individual cells in the developing nervous system . We observed cells with a variety of morphologies (figure 20) which reacted with the riboprobes specific for FaRPs in *in situ* hybridization experiments ; we also found that cells closely juxtaposed with each other could express different FaRPs (figure 18) . Both these observations support the suggestion that control of gene expression is , to at least some extent , at the level of the individual cell .

Further studies by O'Brien *et al.* (1991) indicate that , in contrast to 5HT and dopamine , the expression of FaRPs continues through metamorphosis in *Drosophila* , and that the number of FaRP - positive cells increases to approximately 100 in the adult . This latter finding , which indicates that many FaRP-expressing cells are born embryonically , is especially interesting since up to 90% of all *Drosophila* neurons are born post - embryonically : the presence of so many cells expressing the FaRPs in the embryo may be indicative of these peptides playing a role during development - at least in this organism . Our apparent observation of the regulated expression of FaRP - specific mRNAs during embryogenesis (figure 11) may be suggestive of these peptides playing a role during the development of *Helix* , and close regulation of their expression therefore being necessary .

A large degree of plasticity in the transmitter phenotype of cells in invertebrate nervous systems both during and after development has been observed : for example , it has been

observed that cells which express CAP (cardioacceleratory peptide) in *Manduca* (hawk moth) larvæ change to expressing bursicon , a peptide hormone , in pupæ and adults (Tublitz and Sylwester , 1990) , and that changes in the number of FaRP - IR cells in the central nervous system of *Achatina* can be induced with incubation of the CNS in media containing other transmitters such as dopamine , oxytocin and 5HT (Takayanagi and Takeda , 1988a) . The dynamics of FMRFamide - related peptide expression during development in molluscs (which obviously lack the pupal stage , and consequent nervous system rearrangement , seen during insect development) remains to be fully elucidated : care should be taken in attempting to extrapolate between two such different kinds of organism as insects and molluscs . Some studies of neurotransmitter expression in embryogenesis in molluscs have been made , however (5HT expression in *Lymnaea* , for example (Croll and Chaison , 1989)) , but it is probable that the pattern of expression of different neurotransmitters will vary .

The means by which such differential expression of transmitters in the nervous system is achieved also appears to vary - known examples include alternative processing of pre - mRNA molecules (Adema and Baas , 1991 ; Nawa *et al* , 1984), selective processing of a precursor polypeptide (Thorne *et al* , 1991) and cell - type specific promoter structure (Oberdick *et al*, 1990 ; Des Groselliers *et al* , 1987) . It has been noted that the final pattern of expression of FaRPs in leech appears to be determined by competition between paired cellular homologues (Martindale and Shankland , 1990) , concurring with a model which implies a large degree of plasticity in the control of nervous system development .

4.11 : *In situ* hybridization :

As has been previously pointed out , many FMRFamide - related peptides were originally detected and isolated through their being bound by antibodies directed against the " - Arg -

Phe- NH_2 " moiety . This technique , although useful in many situations , is disadvantageous not only in that is it often difficult to detect by this method peptides which are genuinely related to FMRFamide but which have amino acid substitutions in this region , but also because the antibodies used in these studies often detect material which is not related to FMRFamide (e.g. APP (avian pancreatic polypeptide) and neuropeptide Y in rats (Moore *et al* , 1984).) The technique of *in situ* hybridization is one which allows the detection of mRNA molecules encoding the substance of interest in the context in which it is normally produced , and is especially useful in studies of the nervous system , where the expression of many substances is confined to a discrete number of cells ; it has the additional advantage that the problem of cross - reaction which is often encountered in studies with antibodies does not often arise , since the hybridization is usually between RNAs containing significant homology .

In order to study the location of the expression of the RNAs encoded in the cDNAs HF1 and HF4 within the nervous system of *Helix aspersa* we chose to use *in situ* hybridization with non - radioactive probes , specifically RNA probes incorporating digoxygenin - labelled UTP (DIG - UTP) . Non - radioactive detection systems in *in situ* hybridization allow all the advantages such systems generally have over radioactive detection (e.g. no loss of probe activity with time ; reduction of hazard in handling probes) while not generally suffering as a result of the acknowledged disadvantage of non - radioactive detection , the approximately 10 - fold reduction of sensitivity of detection . Non - radioactive probes also have the advantage that more than one type of probe can be detected and distinguished simultaneously : although we have not utilised this advantage to date , it should be possible , for example , to label HF1 antisense RNA with digoxygenin , while using biotin - labelled ribonucleotides during the synthesis of an HF4 probe . If these probes were detected with antibodies with different fluorescent conjugates , e.g. fluoriscine and rhodamine , both probes could be used on the same preparations and their

different specificities distinguished ; in addition , the use of a confocal laser scanning microscope would allow their simultaneous detection . Such flexibility is not available using radioactive detection techniques .

Our studies using *in situ* hybridization indicate that the mRNAs represented by HF1 and HF4 , encoding the tetrapeptide and extended FaRPs , respectively , are expressed by a small number of cells in the nervous system of *Helix aspersa* (figures 15 , 16 and 17) . Specifically , hybridization is detected in the cell bodies of the identified cerebral neuron C3 and of a number of smaller cells in that ganglion using the tetrapeptide TF1 clone (figure 15) , while MF4 detects expression of the mRNA encoding the extended peptides in clusters of cells in the left and right parietal ganglia (figure 16) . These results appear to be reciprocal - i.e. no hybridization of TF1 with the parietal cells or of MF4 with the cells in the cerebral ganglion is observed . Interestingly , we do not usually detect any significant labelling in the nuclei of cells which are positive for these probes , resulting in an " open circle " of fluorescence usually being observed (similar to that often observed in immunohistochemical studies (Elekes and Nässel , 1990 ; Takayanagi and Takeda , 1987) .) This is in contrast to Saunders *et al.* (1991) and Dirks *et al.* (1989) , who report detection by *in situ* of FaRP - and ELH - encoding RNAs , respectively , in the nucleus of some cells in the central nervous system of *Lymnaea* . Both of these investigators utilized radioactive probes in their *in situ* hybridizations ; the long exposure times required for such probes (up to 3 weeks (Saunders *et al* , 1991)) may in part explain the differences in mRNA distribution observed . Any low level or background hybridization present may not be observed using non - radioactive probes , since photographic exposures in our experiments never exceeded 1 minute . It also possible that some of the sections in which nuclear staining is apparent glance across the nucleus - staining is often most intense at the nuclear membrane (Saunders *et al* , 1991 , our observations (see figure 24)) , so inclusion of this area in sections may create

the impression of positive hybridization . It is also noted that distribution of fluorescence in the cytoplasm of the cell body does not seem to be uniform ; rather , there is a " flecked " appearance to much of the fluorescence , perhaps due to the detection by our probes of FaRP - specific mRNA located on the endoplasmic reticulum .

Perhaps surprisingly , some *in situ* hybridization studies suggest that mRNA can be located in dendrites and even at synapses (Dirks *et al* , 1989 ; Bloch *et al* , 1990) . Diffusion of messenger molecules away from the cell body could be anticipated , but it appears that in at least some cases mRNA is actively transported large distances in nerve cells (Mohr *et al* , 1991 ; Davis *et al* , 1987) before it is translated . This implies that the many enzymes and nucleic acids required in the synthesis of active peptides , including in the case of the FaRPs those involved in processing peptides from their precursor and generating the C - terminal amide group , are also transported to these sites ; there is experimental evidence that this is true for at least some of these components (Steward and Levey , 1982) .

Messenger RNAs encoding the FaRPs (Saunders *et al* , 1991) and other invertebrate peptides such as ELH and MIP (Dirks *et al* , 1989) have also been reported to have been located in sites distant from cell bodies . Our observations following *in situ* hybridization in the central nervous system of *Helix* are that fluorescence due to the presence of specific mRNAs is limited to the cytoplasm of a small number of cells - we do not detect FaRP - encoding mRNAs in sites distant from the bodies of these cells . Although this could be attributed in part to the loss of sensitivity of detection experienced when using non - radioactive probes, it is noteworthy that hybridization also appears to be limited to certain cell bodies when whole mount preparations are used . One advantage of such preparations is that cells remain intact , allowing sub - cellular localization of fluorescence to be more easily observed : it might be expected that the presence of significant amounts of mRNA at sites other than the cell body would be detected in such

preparations , but no such observations were made be us . Distribution of mRNA within the cell body appeared to be uneven, an observation which may be of functional significance (see below).

The use of a confocal laser scanning microscope in the analysis of fluorescence in certain of these cells appears to indicate that subcellular distribution of mRNas being detected by this technique is not even . A " patch like " pattern of *in situ* hybridization within invertebrate nerve cells has previously been noted by other investigators (Dirks *et al* , 1989) . We too found distribution of fluorescence indicative of the presence of mRNA to be unevenly distributed in the cytoplasm of cells which reacted with the TF1 and MF4 probes (see figures 21 - 24). It is also worthy of note that higher concentrations of message , as judged by levels of fluorescence , tend to be found towards one end of the cells in whole mount preparations (see figure 21) . The arrangement of cells in the central nervous system of *Helix* is usually such that cell bodies are located on or near the surface of the ganglia , with most axons consequently projecting into the neuropil , where they diversify and make contacts with other cells . This being the case , the apparent concentration of mRNA at one end of the cell may be of significance . In interpreting our experimental results , however , it should be remembered that it is probable that " bleaching " of the fluorescence near the surface of the preparation occurs during handling of the specimen , positioning it on the microscope , etc. : such an occurrence may result in a stronger fluorescent signal being apparent in the parts of cells located further from the surface of the preparation , simply by virtue of the fact that they are " protected " from the effects of bleaching . The suggestion that the differential distribution observed by us is due to a real biological phenomenon , rather than to an experimental artefact , is , however , supported by recent observations of Mr. Brian Powell . It is possible to isolate and grow as primary cultures *Helix* neurons : when neurons treated in such a way are examined by *in situ* hybridization , a higher concentration of fluorescence is found to be present in the vicinity of the axon ,

indicating an accumulation of FaRP - specific mRNA in this area of the cell body (B. Powell , pers. comm.) . We also have evidence from our observations of *in situ* hybridizations (e.g. figures 22 and 23) to support this suggestion that a real biological phenomenon is being observed

Our results using the very powerful techniques of *in situ* hybridization and confocal microscopy are largely in accord with those using anti "RFamide " specific antibodies (Elekes and Nässel , 1990) , although , as discussed earlier , *in situ* hybridization is a more selective technique : as a result , fewer positive cells are observed using *in situ* than with antibodies. Those which are seen to be positive with *in situ* hybridization are essentially a subset of those which are stained with FaRP - specific antibodies , and are probably a more accurate reflection of the FMRFadergic status of these cells than are those defined as being positive for FaRPs with antibodies , some of which may contain unrelated peptides which cross - react with the antibodies used in the studies . Studies conducted by Prof G. Cottrell and Dr. D. Price (pers. comm.) indicate this to in fact be the case - individual cells were identified by Elekes and Nässel (1990) to stain positively with an anti - FMRFamide antibody , but when these were dissected out and RIA used in detection of peptides extracted from them , a number of the cells were found not to contain detectable levels of FaRPs .

We were unable to detect FaRP - encoding mRNAs in *Helix* embryos using *in situ* hybridization techniques . This is almost certainly due to a lack of probe sensitivity rather than to an absence of FaRP - specific mRNAs , since it is apparent from our studies using mRNA isolated from these embryos (see figures 10 and 11) , and also from RIA studies of peptides similarly extracted (figure 25) , that such molecules are present from partway through development ; it should be remembered that , while our *in situ* studies of adult ganglia involved detection of FaRP - containing cells within a single tissue system , similar studies of embryos required that a small number of cells in one tissue type among many , in individual embryos of small size

(most of them smaller than the adult ganglia used in the parallel studies). While it is probable that many cells producing FaRPs contain large amounts of FaRP - specific mRNA , detection of these cells under the circumstances described would be extremely difficult . FaRP - specific mRNAs have been detected during development in other organisms (Schneider *et al* , 1991 ; O'Brien *et al* , 1991) using radioactive *in situ* hybridization techniques : perhaps such an approach , with its increased sensitivity over non - radioactive detection systems, may be necessary here .

4.12 : Future prospects

It would be of great interest to identify the specific sites of synthesis of FaRPs during embryogenesis in *Helix* , and the times during development at which such gene expression is initiated . Further , it may be possible in the future to ascertain to some extent the means by which the expression of the FaRPs is controlled and directed : this control is likely to be *via* a variety of mechanisms , including control of gene expression , differential mRNA splicing , and control of precursor polypeptide synthesis and of its processing . An additional level at which control is likely to be exerted is that of receptors : although very little is known as yet about receptors for FaRPs , neuronal receptors generally appear to be regulated in a very specific manner (Klein *et al* , 1989) , and are thus probably used as a further level of regulation in neuronal response . The additional fact of many organisms synthesizing multiple related members of the FaRP group of peptides in parallel , and the consequent interplay between such molecules in modulating behaviours also promises fruitful and interesting investigation .

The ability of the nervous system to respond to a wide range of stimuli , and to produce an appropriate behavioural response to such stimuli , will almost certainly mean that the production of many neuroactive substances , including such as the FMRFamide - related peptides , is governed closely by a complex series of controls , in order to prevent aberrant

production and release of such substances . In many ways our understanding of such controls is only beginning : studies using invertebrate models have been invaluable in aiding elucidation of some of the basic principles involved , and will in all probability continue to play a vital role in the future . The involvement of FaRPs in at least one well studied behavioural response , that of the *Aplysia* gill withdrawal reflex , means that investigations relating to this family of peptides are likely to be of interest to the wider scientific community .

Amino acid	*Three-letter abbreviation*	*One-letter symbol*
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

APPENDIX A

Amino acid codes: single and 3-letter codes.

Standard 1- and 3-letter abbreviations for amino acids which, are used in this thesis. Table was taken from Biochemistry (L. Stryer , 3rd edition, Freeman 1988) , Table 2.2.

First position (5' end)	*Second position*				*Third position (3' end)*
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	**Stop**	**Stop**	A
	Leu	Ser	**Stop**	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

APPENDIX B

Genetic code: triplet assignment.

Table showing genetic code : each amino acid is represented by some combination of three of the four bases , the designation of which are shown here. Specific triplets also signal translation starts (AUG) and terminations (UAA, UAG and UGA). Table was taken from Biochemistry (L. Stryer , 3rd edition, Freeman 1988) , Table 5.5.

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