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CLONING AND CHARACTERISATION OF GENES ENCODING MOLECULAR RECOGNITION PROTEINS FROM INSECTS

By

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Abstract

Olfaction is one of the most important senses by which insects obtain information about their environment. In the early stages of olfactory perception in insects, odour molecules are carried across the sensillum lymph by small soluble Odorant Binding Proteins (OBPs). This is followed by activation of the appropriate olfactory receptor, resulting in an electrical impulse, and subsequent degradation of the initial signal.

OBPs have been studied in a range of insect orders including Lepidoptera, Diptera and Orthoptera, and this study reports the cloning and characterisation of cDNAs with a potential olfactory role in the vetch aphid, *Megoura viciae* (Buckton, Homoptera: Aphididiae).

Construction and sequencing of antennal cDNA libraries identified two cDNAs, MvicOBP1 and Mv164, which were approximately 0.8kb and 1kb respectively. The amino acid sequence of MvicOBP1 has the spacing pattern of six cysteine residues that is characteristic of insect OBPs, and Mv164 shows similarity to insect cytochrome P450 enzymes. RT-PCR showed that these cDNAs have specific or enhanced expression in the chemosensory tissues of *M. viciae*, and parallel expression patterns suggest a "linked" function. Related sequences are present and expressed in other aphid species, and sequencing of genomic fragments allowed the partial elucidation of the intron/exon organisation of these genes.

Subtracted antennal cDNA libraries identified two cDNAs encoding proteins with significant similarity to insect chemosensory proteins (CSPs), cDNAs encoding Juvenile Hormone Binding Proteins (JHBPs), and a tissue-specific cDNA with a potential carrier role. These, coupled with the OBPs, add evidence to the suggestion that there is an insect superfamily of binding proteins.

A PBP from *Bombyx mori* (BmorPBP1) was used as a model system for *in vitro* expression of an insect OBP and subsequent characterisation of the recombinant protein. Four forms of this protein, identified through their interaction with an anti-BmorPBP antibody, were resolved by isoelectric focusing.

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Abbreviations

A	amp(s)
AAP	abridged anchor primer
AC	adenylate cyclase
AOB	accessory olfactory bulb
ATP	adenosine triphosphate
AUAP	abridged universal anchor primer
A_{260}	absorbance at 260nm
bp	base pair(s)
cAMP	cyclic adenosine 3',5'-monophosphate
cDNA	complementary DNA
cGMP	cyclic guanosine 3',5'-monophosphate
Ci	curie(s)
cm	centimetre(s)
cps	counts per second
CSP	chemosensory protein
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine 5'-triphosphate
dITP	deoxyinosine 5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide 5'-triphosphate
ds	double stranded
DTT	dithiothreitol
dTTP	deoxythymidine 5'-triphosphate
DTW	DEPC-treated water
EAG	electroantennogram
EBF	*E*-ß-farnesene
EC	enzyme commission
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
EtOH	ethanol
g	gram(s)
gDNA	genomic DNA
GDP	guanosine diphosphate
GOBP	general odorant binding protein
GPCR	G-protein coupled receptor
GSP	gene specific primer
GTP	guanosine triphosphate
h	hour(s)
IEF	isoelectric focusing
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactoside
IP_3	inositol 1,4,5-triphosphate

JHBP	juvenile hormone binding protein
kb	kilobase(s)
kDa	kilodalton(s)
l	litre(s)
M	molar
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
MOB	main olfactory bulb
MOE	main olfactory epithelium
MOPS	3-[N-morpholino]-propanesulfonic acid
mRNA	messenger RNA
MUP	major urinary protein
ng	nanogram(s)
nm	nanometre(s)
OBP	odorant binding protein
OR	olfactory receptor
PAGE	polyacrylamide gel electrophoresis
PBP	pheromone binding protein
PCR	polymerase chain reaction
pDNA	plasmid DNA
pg	picogram(s)
pI	isoelectric point
PIP_2	phosphatidyl inositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
Poly(A+) RNA	polyadenylated RNA
RACE	rapid amplification of cDNA ends
rBmorPBP1	recombinant BmorPBP1
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse-transcription PCR
s	second(s)
SAP	sensory appendage protein
SC-PCR	single colony PCR
SCR	single cell recording
SDDS	stimulo-deterrent diversionary strategy
SDS	sodium dodecyl sulfate
SDW	sterile distilled water
SFM	serum free media
SSC	saline sodium citrate
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
TBS-T	tris-buffered saline-Tween
TdT	terminal deoxynucleotidyl transferase

Tm	melting temperature
Tween 20	polyoxyethylene-sorbitan monolaurate
U	unit(s)
UV	ultraviolet
V	volt(s)
VNO	vomeronasal organ
v/v	volume per volume
W	watt(s)
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
μCi	microcurie(s)
μg	microgram(s)
μl	microlitre(s)
°C	degrees centigrade

Chapter 1. Introduction

1.1 Opening Remarks

Chemosensory perception is highly developed in animals, and olfaction, in particular, is possibly the most powerful sense by which animals obtain information about their environment. Olfaction is key for an organism's survival and adaptation (Breer *et al*, 1990a), playing an important role in detecting food, hosts, appropriate territories, enemies and potential mates. In humans, olfaction evokes, more than any other sense, vivid memories and emotional responses resulting in strong approach or avoidance behaviour. The olfactory systems of mammals can distinguish a vast number of volatile chemicals, for example, humans can recognise over ten thousand different odour compounds, at extremely low concentrations, equivalent to being able to taste one teaspoon of salt in one million gallons of water. Odours are detected in the main olfactory epithelium (MOE), whereas pheromones, which influence subconscious behaviour and neuroendocrine changes e.g. the regulation of ovulation mediated by volatile compounds (Stern & McClintock, 1998), are detected in the vomeronasal organ (VNO) that is present in most amphibians, reptiles and non-primate mammals (Keverne, 1999). In man and higher primates, this is thought to be a vestigial organ, the function of which is still debated (Halpern, 1987; Calvi *et al*, 1998).

The mechanisms of odour recognition and discrimination are complex in mammals, and not yet fully understood. The existence of

pheromones complicates the matter still further. Invertebrates, and insects in particular, have simplified olfactory organs, and offer a model system for studies of olfaction. Research on insects and vertebrates has revealed many parallels, which have probably arisen by convergent evolution (Hildebrand & Shepherd, 1997; Strausfeld & Hildebrand 1999).

Getchell *et al* (1984), in a review of vertebrate olfaction, coined the term "perireceptor events" to denote those processes, secondary to both receptor activation and signal transduction, which "influence the entry, exit, or residence time of odorant molecules in the receptor environment". These events have proven to be an integral component of chemical sensing in organisms as diverse as bacteria, yeast, insects and mammals (Carr *et al*, 1990).

Early studies on olfaction had suggested that to elicit a response, an odorant molecule had to bind directly to a receptor protein, which resulted in the activation of a signal transduction pathway and the production of an action potential in the olfactory neuron. However, as more systems are studied, the discovery of odorant binding proteins has suggested that this might not be the case. The principles of perireceptor events, receptor activation and signal transduction are discussed below.

1.2 Perireceptor Events

1.2.1 Entry of the odorant molecule

1.2.1.1 Vertebrate Systems

Terrestrial animals detect volatile hydrophobic odorant molecules, which are carried in air, and the first step in the perireceptor pathway is inhalation, where odorant molecules enter the nasal cavity, and the mucous that protects the epithelium (Breer *et al*, 1994). This mucous, secreted by the Bowman's gland, protects the epithelium against damage by xenobiotics and invasion by microorganism (Anholt, 1993). Once in the nasal mucosa, the odorant molecule must pass through this aqueous layer *en route* to the olfactory receptor.

1.2.1.2 Insect Systems

In a similar situation to that of vertebrates, the hydrophobic volatile molecules that insects detect must first pass from the air to the antenna, the olfactory organ. Studies on pheromone interactions with antennae have shown that the molecules are adsorbed by the sensilla, the sensory hairs on the antennae, leading to an increase in the concentration of odorant molecule on the antennae (Kanaujia & Kaissling, 1985). From here, the compounds diffuse through the porous sensillum cuticle, and along pore tubules, which extend into the hair lumen and act as "wicks", dispersing odorant molecules in an inward direction into the lumen of the sensillum (Steinbrecht, 1997). Once inside, odorant molecules must pass through the aqueous sensillum lymph fluid to the olfactory receptors.

1.2.2 Odorant Binding Proteins (OBPs)

1.2.2.1 Vertebrate OBPs

The mucous of the vertebrate nasal epithelium consists of water, electrolytes and proteins (Carr *et al*, 1990), and in order to pass through this medium, the hydrophobic molecules interact with soluble OBPs (Pevsner & Snyder, 1990). It is suggested that the OBPs solubilise the odorant molecules, thereby increasing their concentration in the vicinity of the receptor. Alternatively, OBPs may remove odorant molecules from the receptor after activation, or act as a filter to protect the receptor from excessive concentrations of odorant molecules (Pevsner & Snyder, 1990). Whatever the function, OBPs have probably arisen as an adaptation to terrestrial life, as fish do not appear to possess such proteins but amphibians, insects and mammals do (Baldaccini *et al*, 1986; Vogt *et al*, 1990). The presence of OBP-type proteins has also been demonstrated in the VNO, where they are also suggested to be involved in chemical signal recognition (Miyawaki *et al*, 1994).

Early analysis of OBPs, in mainly mammalian species, suggested a role as carriers of odorant molecules based on their significant similarity to the lipocalins, a superfamily of soluble proteins that carry small, principally hydrophobic substrates such as retinol and cholesterol (Flower, 1994, 1995a). The main motif of the lipocalins, G-X-W (Flower *et al*, 1993), is present in all vertebrate OBPs, approximately fifteen to twenty amino acids form the N-terminus, as is the motif Y-X-X-X-Y-X-G (Tegoni *et al*, 2000). The lipocalin superfamily

also includes the Major Urinary Proteins (MUPs) of rodents that bind pheromones in the urine (Bishop *et al*, 1982; Cavaggioni *et al*, 1987; Bacchini *et al*, 1992; Robertson *et al*, 1996), the protein aphrodisin, which has a pheromonal role in the vaginal discharge of female hamsters (Singer *et al*, 1986; Mägert *et al*, 1995; Briand *et al*, 2000b), and the salivary proteins of the mouse (Shaw, 1983) and boar (Marchese *et al*, 1998; Loebel *et al*, 2000), where the role of saliva as a carrier of pheromones is well established. However, the presence of soluble binding proteins is not restricted to systems using volatile molecules. For example, proteins, similar in sequence to OBPs, have been found in the von Ebners gland in rat and humans (VEG proteins) (Schmale *et al*, 1990, 1993; Bläker *et al*, 1993; Kock *et al*, 1994), and it has been suggested that they are involved, in the gustatory system, in transporting molecules to taste receptors.

OBPs reported in a number of vertebrate species are shown in Table 1.1. Complete amino acid sequences have been determined for cow, rat, mouse, frog, and human, whilst N-terminal sequences have been reported for the remainder.

Paolini *et al* (1998) suggested that vertebrate OBPs could be divided into subclasses, based on amino acid sequence similarity, in the same way as insect OBPs have been (see 1.2.2.2). The first subclass would include pig OBP-I, bovine OBP, rat OBP-I, and both mouse OBP-I subunits (a and b), whilst the second sub-class would contain rat OBP-II, the two OBPs of mouse VNO, and the frog OBP, and the third would compromise mouse OBPs-III and IV, rabbit OBP-III, and porcupine

Species	Number of OBPs	Reference
Cow	1	Pelosi *et al*, 1982; Bignetti *et al*, 1985; Pevsner *et al*, 1985; Tirindelli *et al*, 1989
Rabbit	3	Dal Monte *et al*, 1991; Garibotti *et al*, 1997
Pig	3	Dal Monte *et al*, 1991; Paolini *et al*, 1998; Loebel *et al*, 2000
Rat	3	Pevsner *et al*, 1988; Dear *et al*, 1991; Briand *et al*, 2000a; Löbel *et al*, 1998
Mouse	4	Pes *et al*, 1992; 1998; Pes & Pelosi, 1995; Miyawaki *et al*, 1994
Frog	1	Lee *et al*, 1987
Old World Porcupine	8	Felicioli *et al*, 1993; Ganni *et al*, 1997
Human	2	Scalfari *et al*, 1997; Lacazette *et al*, 2000
Boar	3	Marchese *et al*, 1998
Elephant	1	Rasmussen, 2000

Table 1.1. Some vertebrate OBPs reported to date.

OBP-I. Hydropathy profiles of these OBPs lend support to such a classification. (Paolini *et al*, 1998).

The first OBP identified was from a cow, and was found to be a soluble homodimer, with two subunits of ~19kDa (Bignetti *et al*, 1985), and an acidic pI (4.7), characteristics that have since been shown to be shared by all vertebrate OBPs. The main feature of this OBP was the ability to bind 2-isobutyl-3-methoxypyrazine (pyrazine), and subsequent OBPs were identified using this binding criterion. However, other soluble proteins, e.g. from the frog *Rana pipiens* (Lee *et al*, 1987), failed to show binding to pyrazine but did show amino acid sequence similarity to the other OBPs, and these too were classed as OBPs.

Vertebrate OBP subunits consist of eight antiparallel ß-sheets and one α-helix at the C-terminus (Monaco & Zanotti, 1992; Pelosi,

1998; Tegoni *et al*, 2000), similar in structure to the lipocalins (Flower, 1995b). The binding domain was originally suggested to be at the channel formed between the two subunits of bovine OBP (Monaco & Zanotti, 1992; Pelosi & Garibotti, 1993), but subsequent studies on this OBP suggested that each monomer binds an odorant molecule in its hydrophobic core (Tegoni *et al*, 1996; Bianchet *et al*, 1996), as do the MUPs (Böcskei *et al*, 1992); Tegoni *et al* suggest that this is in addition to the site at the dimer interface. Some, but not all, OBPs undergo swapping of monomer domains (Spinelli *et al*, 1998), and this, coupled with the two binding sites, allows binding to an increased repertoire of ligands (Brownlow & Sawyer, 1996; Tegoni *et al*, 1996).

OBPs were suggested originally to have a role as receptors (Pelosi *et al*, 1982), in a manner similar to those proteins involved in bacterial chemotaxis, where periplasmic proteins (e.g. the Maltose-, Glucose/Galactose- and Ribose-binding proteins of *Escherichia coli*, Shilton *et al*, 1996) act as initiators of signal transduction as well as binders of the stimulus molecule (Koshland, 1981). However, the similarity to the lipocalins, coupled with the binding of an OBP to several ligands (Pevsner *et al*, 1986; 1990) led to the suggestion that OBPs are non-specific carriers of volatile molecules to (or from) the membrane-bound olfactory receptors (Pevsner & Snyder, 1990). Binding studies, which have been performed mainly on bovine OBP and, to a lesser extent, pig OBP (Dal Monte *et al*, 1993), support this idea of "non-specificity", with bovine OBP having a broad specificity, but only weak binding (K_D ~3μM; Bignetti, 1985). However, a recent study on rat OBPs

suggests greater ligand specificity (Löbel *et al*, 1998), and Garibotti *et al* (1997) suggest that the diversity of OBPs might indicate physiologically important discriminatory functions towards odorant molecules. Recent studies have shown that MUPs, whilst functioning as pheromone carriers (Bacchini *et al*, 1982), also possess pheromonal activity (Mucignat-Caretta *et al*, 1995), being able to induce receptor-mediated G-protein activation (Krieger *et al*, 1999a). This suggests that lipocalins have a wider role than that of general carrier proteins. Similarly, the presence of several different OBPs in the same species (see Table 1.1) suggests a specific function (Pes & Pelosi, 1995), and a possible role in molecular discrimination.

This large superfamily of proteins obviously plays a very important role in chemical communication, but the actual function(s) have yet to be completely resolved. The OBPs are part of a very complex olfactory system in vertebrates, and insects, with their simple olfactory organs, have come to be seen as better model systems of terrestrial olfaction.

1.2.2.2 Insect OBPs

The insect sensillum lymph, which bathes the receptor cell dendrites, is functionally equivalent to the vertebrate mucous (Pelosi, 1996). This fluid contains ions (sodium, potassium, magnesium, phosphorus, sulfur, chloride and calcium; Kaissling, 1998a), biotransformation enzymes (see 1.5.2), and a high concentration of OBPs (Kaissling *et al*, 1985; Kaissling, 1996). These OBPs show some

similarities to their vertebrate counterparts, namely size (15-20kDa) and an acidic isoelectric point (4.43-5.3; Pelosi & Maida, 1995b; Maïbèche-Coisné *et al*, 1998a). This suggests that insect OBPs have a similar role, i.e. as transporters of hydrophobic odorant molecules (Pelosi & Maida, 1990). The LUSH OBP of the fruit fly *Drosophila melanogaster* (Meigen, Drosophilidae) is the only OBP with functional evidence of involvement in olfaction: the absence of LUSH mediating an abnormal response to alcohols (Kim *et al*, 1998).

The amino acid sequences of insect OBPs differ markedly from those of vertebrates, suggesting that they have arisen independently, probably as a result of convergent evolution (Breer *et al*, 1994; Vogt *et al*, 1990). The main characteristic of insect OBPs is the presence of six cysteine residues in a conserved spacing profile; indeed, this characteristic is often the basis of assigning a putative OBP function when sequence similarity is lacking. Insect OBPs also possess a signal peptide (of varying length), which is a characteristic of all secreted proteins.

The first insect OBP to be identified was a Pheromone-Binding Protein (PBP) from the wild silkmoth, *Antheraea polyphemus* (Saturniidae; Vogt & Riddiford, 1981), at a similar time, and in a similar biochemical manner, to the identification of the first vertebrate OBP. This PBP, with an apparent molecular weight of 15kDa, was shown to bind (*E,Z*)-6,11-hexadecadienyl acetate, the major component of the pheromone of *A. polyphemus*. Experimental evidence suggested that this protein might exist as a dimer *in vivo* (DeKramer & Hemberger,

1987), and also that the PBP was involved in inactivation of the pheromone, this being coupled with degradation by a sensilla esterase (Vogt & Riddiford, 1981). Many OBPs from a wide range of insect species have now been identified, using a variety of molecular and biochemical techniques (see Table 1.2). The tertiary structure of these OBPs has been shown to be mainly α-helical (Breer *et al*, 1992; Sandler *et al*, 2000), which is in contrast to their vertebrate equivalents, and studies have shown that all six conserved cysteine residues are involved in forming disulphide bonds (Leal *et al*, 1999; Scaloni *et al*, 1999; Sandler *et al*, 2000).

Insect OBPs were initially thought to have a role in inactivation of odorant molecules (Vogt & Riddiford, 1981), but other possible functions for these proteins include transport to and/or from the olfactory receptor (van den Berg & Ziegelberger, 1991), and filtering of the odorant molecules. The multiplicity of OBPs often found within a species [e.g. at least ten members of the OBP family in the Cabbage army worm *Mamestra brassicae* (L., Noctuiidae; Blais *et al*, 1996)] has, by analogy to the vertebrate system, provoked the question of whether or not insect OBPs play a role in molecular discrimination (Pelosi 1994; Pelosi & Maida, 1995a). Evidence for such a role includes the binding studies demonstrating that two different PBPs of the Chinese Oak silk moth *Antheraea pernyi* (Saturniidae) have different affinities for two distinct pheromone components (Du & Prestwich, 1995). The demonstration that two different OBPs from *D. melanogaster* are localized to different areas of the sensilla (PBPRP5 being restricted to

Species	Common name	Order	Family	PBP	GOBP	Other	References
Agrotis segetum	Turnip moth	Lepidoptera	Noctuiidae	1			LaForest *et al*, 1999
Antheraea pernyi	Chinese oak silk moth	Lepidoptera	Saturniidae	3	GOBP1, GOBP2	ABPX	Raming *et al*, 1990; Breer *et al*, 1990c; Krieger *et al*, 1991; Krieger *et al*, 1997; Mameli *et al*, 1997; Maida *et al*, 2000a
Antheraea polyphemus	Wild silk moth	Lepidoptera	Saturniidae	3	GOBP2		Vogt & Riddiford 1981; Raming *et al*, 1989; Vogt *et al*, 1991b; Maida *et al*, 2000a
Argyotaenia velutinana	Red banded leafroller	Lepidoptera	Tortricidae	1			Willett, 2000b
Bombyx mori	Cultivated Silk moth	Lepidoptera	Bombycidae	1	GOBP1, GOBP2	ABPX	Krieger *et al*, 1996
Choristoneura fumiferana	Spruce budworm	Lepidoptera	Tortricidae	1			Willett, 2000b
Choristoneura murinana	European fir budworm	Lepidoptera	Tortricidae	1			Willett, 2000b
Choristoneura parallela	Spotted fireworm	Lepidoptera	Tortricidae	1			Willett, 2000b
Choristoneura pinus	Jackpine budworm	Lepidoptera	Tortricidae	1			Willett, 2000b

Species	**Common name**	**Order**	**Family**	**PBP**	**GOBP**	**Other**	**References**
Choristoneura rosaceana	Oblique banded leafroller	Lepidoptera	Tortricidae	1			Willett, 2000b
Epiphyas postvittana	Light brown apple moth	Lepidoptera	Tortricidae	2			Greenwood *et al*, 2000
Helicoverpa armigera	Cotton bollworm	Lepidoptera	Noctuiidae	1			Wang & Guo, 2000
Heliothis virescens	Bud worm	Lepidoptera	Noctuiidae	1	GOBP1, GOBP2	ABPX	Krieger *et al*, 1993; 1997
Helicoverpa zea	Corn ear worm	Lepidoptera	Noctuiidae	1			Callahan *et al*, 2000
Hyalophora cecropia	Cecropia moth	Lepidoptera	Saturniidae	1	GOBP2		Vogt *et al*, 1991b
Lymantria dispar	Gypsy moth	Lepidoptera	Lymantriidae	2	GOBP2		Vogt *et al*, 1989; Vogt *et al*, 1991b; Merritt *et al*, 1998;
Mamestra brassicae	Cabbage army worm	Lepidoptera	Noctuiidae	2	GOBP2		Nagnan-Le Meillour *et al*, 1996; Maïbèche-Coisné *et al*, 1998a; Maïbèche-Coisné *et al*, 1998b
Manduca sexta	Tobacco hawk moth	Lepidoptera	Sphingidae	3	GOBP1, GOBP2	ABPX	Györgyi *et al*, 1988; Vogt *et al*, 1991a; Robertson *et al*, 1999
Orgyia pseudosugata	Douglas-fir tussock moth	Lepidoptera	Lymantriidae	1	GOBP		Vogt *et al*, 1991b
Ostrinia furnacalis	Asian corn borer	Lepidoptera	Pyralididae	1			Willett & Harrison, 1999a

Species	Common name	Order	Family	PBP	GOBP	Other	References
Ostrinia nubalis	European corn borer	Lepidoptera	Pyralididae	1			Willett & Harrison, 1999a, b
Pectinophora gossypiella	Pink bollworm	Lepidoptera	Gelechiidae	1			Willett, 2000a
Sesamia nonagrioides	Corn stalk borer	Lepidoptera	Noctuiidae	1			Labropoulou & Mazomenos, 2000; Konstantopoulou *et al*, 2000
Spodoptera frugiperda	Fall army worm	Lepidoptera	Noctuiidae	1			Callahan *et al*, 2000
Syanthedon exitiosa	Peach tree borer	Lepidoptera	Aegeriidae	1			Willett, 2000a
Thaumetopoea pityocampa	Processionary moth	Lepidoptera	Thaumetopoeidae			SH-15 (PBP); SH-20	Feixas *et al*, 1995
Yponomeuta cagnagellus	Spindle ermine	Lepidoptera	Yponomeutidae	1			Willett, 2000a
Anomala osakana	Osaka beetle	Coleoptera	Scarabidae	1			Wojtasek *et al*, 1998
Phyllopertha diversa	Pale brown chafer	Coleoptera	Scarabidae			OBP1; OBP2	Wojtasek *et al*, 1999
Popillia japonica	Japanese beetle	Coleoptera	Scarabidae	1			Wojtasek *et al*, 1998
Rhychophorus palmarum	African palm weevil	Coleoptera	Curculionidae			OBP1, OBP2	Jacquin-Joly *et al*, 1999
Anopheles gambiae	African malaria mosquito	Diptera	Culicidae			Putative OBP	Maida & Ziegelberger, 2000

Species	Common name	Order	Family	PBP	GOBP	Other	References
Drosophila melanogaster	Fruit fly	Diptera	Drosophilidae			PBPRP1-5; OS-E, OS-F; LUSH; ~13 predicted from genome sequence	Pikielny *et al*, 1994; McKenna *et al*, 1994; Kim *et al*, 1998; Adams *et al*, 2000
Phormia regina	Blow fly	Diptera	Calliphoridae			CRLBPpr	Ozaki *et al*, 1995
Apis mellifera	Honey bee	Hymenoptera	Apidae			ASP1 (PBP); ASP2 (GOBP2)	Danty *et al*, 1997; 1999
Lygus lineolaris	Tarnished plant bug	Hemiptera	Miridae			LAP	Dickens *et al*, 1995; 1998a; 1998b; Vogt *et al*, 1999

Table 1.2. Insect OBPs (i.e. those with six cysteine residues)

the sensillum lymph and PBPRP2 being restricted to the space immediately below the antennal cuticle, Park *et al*, 2000), has led to the suggestion that OBPs can function both as a carrier of odorant molecules (PBPRP5) and as a protector of the olfactory dendrites from excess or harmful odorant molecules. Furthermore, the presence of different OBPs in different subsets of sensilla suggests an important role in chemosensory coding (Carlson, 1996). Overall, there is no evidence for a single function for insect OBPs.

Lepidopteran OBPs can be divided into four subclasses: PBPs, General Odorant Binding Proteins (GOBPs) 1 and 2, and Antennal Binding Protein Xs (ABPXs). These, and other insect OBPs and chemosensory proteins, are discussed below.

(a) Lepidopteran PBPs

PBPs have been identified in many lepidopteran species (Table 1.2), where they are localized at high concentration (10-20mM; DeKramer & Hemberger, 1987; Vogt & Riddiford, 1986b; Pelosi & Maida, 1995b), in the pheromone sensory hairs which, in males, are usually the long sensilla trichodea (Steinbrecht *et al*, 1995; Vogt & Riddiford, 1981; Vogt *et al*, 1991b; Györgyi *et al*, 1988; Raming *et al*, 1989,1990; Krieger *et al*, 1991,1996; Merritt *et al*, 1998). However the degree of sexual dimorphism in expression of PBPs varies between species. Early studies indicated that the PBPs of *A. polyphemus*, the Cecropia moth *Hyalophora cecropia* (L., Saturniidae), the Cultivated silk moth *Bombyx mori* (L., Bombycidae), the Gypsy moth *Lymantria dispar*

(L., Lymantriidae) and the Douglas-fir tussock moth *Orgyia pseudosugata* (McDunnough; Lymantriidae) were predominantly male specific with almost no detectable protein expression in females (Vogt *et al*, 1991b). However, the PBP of the Tobacco hawk moth *Manduca sexta* (L., Sphingidae) is strongly expressed in the male antennae, with low but detectable levels in the female antennae (Györgyi *et al*, 1988).

Subsequent immunological studies showed that PBPs were expressed at very low levels in the female antennae of *A. polyphemus*, *A. pernyi* and *B. mori* (Steinbrecht *et al*, 1992, 1995), and at relatively high levels (in the range of 50-100% of male expression) in the female antennae of the Corn ear worm *Helicoverpa zea* (Boddie; Noctuiidae), the Bud worm *Heliothis virescens* (Fabricius; Noctuiidae), the Fall army worm *Spodoptera frugiperda* (J. E. Smith; Noctuiidae, Callahan *et al*, 2000), and *M. brassicae* (Maïbèche-Coisné *et al*, 1997). Whilst it is the male of these species that responds to the sex pheromone, expression of PBPs in females suggests that they too are able to detect their pheromone, although whether there is a subsequent behavioural response has not yet been established (Callahan *et al*, 2000). However, if the PBP has a "protection" role, the presence in female antennae may not be unexpected.

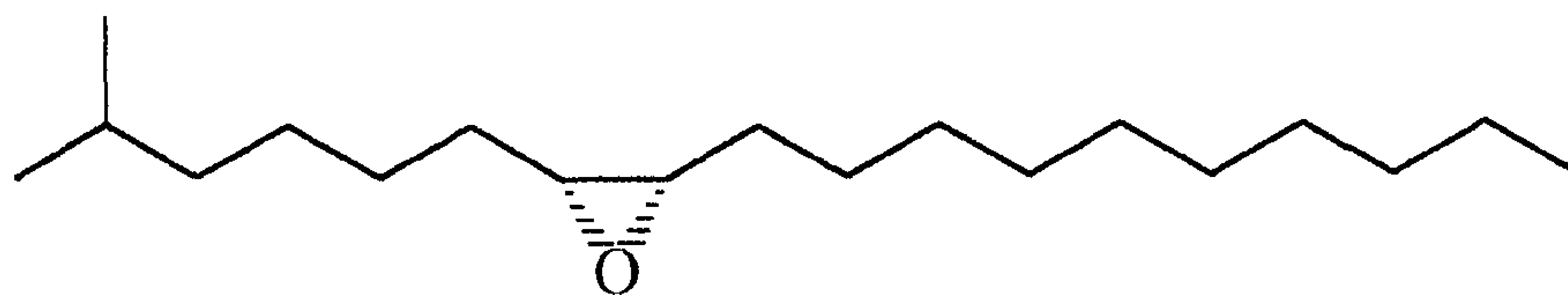
The PBPs of the Lepidoptera possess the characteristics of insect OBPs: i.e. a signal peptide, small size (<20kDa, Prestwich, 1996), and six conserved cysteine residues. They also have a major hydrophobic domain (residues 39 to 60), which is thought to be the binding pocket for the ligand, a second hydrophobic region (residues

80-90) and a highly conserved C-terminal amino acid sequence (GEVLAEV: Pelosi & Maida, 1995a). The PBP amino acid sequences are highly variable, showing 32-90% similarity across species (Vogt *et al*, 1991b; Pelosi, 1996), with the level of similarity being reflected in the similarity of the pheromone compounds (see Figure 1.1) i.e. the more similar the pheromone, the more similar the PBPs. The pheromone of *L. dispar* is (+)-disparlure [(7*R*,8S)-*cis*-2-methyl-7,8-epoxyoctadecane] (Figure 1.1 (3)), and the PBPs of this species show only 34-50% similarity to the other lepidopteran species (Pelosi & Maida, 1995b). This supports the suggestion of species-specific PBPs, and of PBP similarity being more reflective of ligand relatedness than phylogenetic relationship (Vogt *et al*, 1991b), and also lends credence to the argument of PBPs being involved in pheromone discrimination (Steinbrecht, 1996a and b). However, Willett (2000a) argues that PBPs have not converged in amino acid sequence to bind similar pheromone structures, although he does admit evidence suggesting that some PBP amino acid sequences have altered with pheromone changes (Willett, 2000b).

Multiple PBPs have been found in *A. polyphemus* and *A. pernyi* (Maida *et al*, 2000a; Krieger *et al*, 1991), *M. brassicae* (Maïbèche-Coisné *et al*, 1998a) and *L. dispar* (Vogt *et al*, 1989; Merritt *et al*, 1998), and these are thought to allow differential binding of the individual pheromone components. The hydrophobic domain shows variations between PBPs, and contains the binding site for the pheromone in *A. polyphemus* (Du *et al*, 1994). Specific binding of pheromone

1

2



3

ApolPBP1			
90	AperPBP1		
62	50	BmorPBP1	
51	51	44	LdisPBP1

Figure 1.1 (A) Structures of major pheromone components of *A. polyphemus* and A. *pernyi*, (1 = (*E,Z*)-6,11-hexadecadienyl acetate), *B.mori* (2 = (*E,Z*)-10,12-hexadecadienol) and *L. dispar* (3 = (7*R*,8*S*)-*cis*-2-methyl-7,8-epoxyoctadecane). Source: http://www.nysaes.cornell.edu/fst/faculty/acree/pheronet/genusindex.html. (B) Similarity matrix of PBPs from these species.

components has been demonstrated for the PBPs of *A. polyphemus* and *A. pernyi* (De Kramer & Hemberger, 1987; Du & Prestwich, 1995; Prestwich, 1996), *L. dispar*, (Plettner *et al*, 2000), and *M. brassicae* (Maïbèche-Coisné *et al*, 1997), with dissociation constants in the nano- or micromolar range depending upon the method used (see Table 1.3).

Pheromone	PBP				
	Apol	AperPBP 1	AperPBP 2	LdisPBP 1	LdisPBP 2
16C	0.64	1.83	11.2	-	-
14C	21.0	29.4	3.75	-	-
Disparlure	-	-	-	3.5	2.3

Table 1.3. Dissociation constants (μM) for binding studies on *A. polyphemus*, *A. pernyi* and *L. dispar* PBPs. 16C: 6*E*,11*Z*-hexadecadienylacetate,14C: 4*E*,9*Z*-tetradecadienyl acetate, Disparlure: racemic mixture of (+) and (-) enantiomers.

In addition to specific binding studies, the putative PBP of the processionary moth *Thaumetopoea pityocampa* (Denis & Schiffermüller, Thaumetopoeidae), SH-15, has been shown to have a higher affinity for its native pheromone than for the pheromonal metabolites (Feixas *et al*, 1995), the PBP of *B. mori* has been shown to bind its major pheromone component, bombykol (Maida *et al*, 1993; Sandler *et al*, 2000; Oldham *et al*, 2000), whilst pheromone binding of the recombinant PBP of *A. pernyi* has also been demonstrated (Krieger *et al*, 1992).

(b) Lepidopteran GOBPs

GOBPs have also been identified in the lepidopteran species listed in Table 1.2, and these proteins are present in the antennae of both sexes, residing in those sensilla that respond to "general", or non-pheromonal, odours, such as plant odours. However, whilst these sensilla do respond to general odours, the name GOBP implies that these proteins bind a broad range of ligands with moderate affinity (Feng & Prestwich, 1997), whereas, in fact, binding can be as specific as that shown by PBPs (Jacquin-Joly *et al*, 2000). In males and females, these "general odour" sensilla are usually the sensilla basiconica, although the presence of a particular OBP in a sensillum is related to the functionality of the sensillum rather than the morphological type (Steinbrecht, 1996a; Steinbrecht *et al*, 1996). Interestingly, until now PBPs and GOBPs were never found co-localized in the same sensillum (Laue *et al*, 1994; Steinbrecht, 1996a), but a recent study demonstrated the presence of MbraPBP1 and MbraGOBP2 in the same sensilla trichodea (Jacquin-Joly *et al*, 2000).

GOBPs also have the characteristics of the OBPs, but can be further divided into two subclasses, GOBP1 and GOBP2, based on their C-terminal amino acid sequence (MIEAVXEKY for GOBP1, EFIME for GOBP2; Krieger *et al*, 1993; Pelosi & Maida, 1995b). Whereas the PBPs show inter-specific variation, especially in the region of the ligand-binding pocket, the subclasses of the GOBPs show much higher levels of similarity across species, 70-90% intraclass, and ~50% interclass (Feng & Prestwich, 1997; Pelosi & Maida, 1995b). There is only 20-50%

similarity between the GOBPs and PBPs (Pelosi, 1994), probably reflecting their different function. The extreme similarity of amino acid sequences within the GOBP classes of different species probably reflects the detection of common odours that are of equal importance in all species, e.g. plant odours (Vogt *et al*, 1991b; Steinbrecht *et al*, 1996). The presence of multiple GOBPs may enhance the detection of a more diverse class of volatiles (Vogt *et al*, 1991b), and it has been suggested that the PBP pathway, for pheromone perception, evolved from a common ancestral GOBP pathway, and then became more specialised (Vogt & Lerner, 1989; Breer *et al*, 1992).

Limited discriminate ligand binding studies have been reported in the literature for GOBPs. Recombinant MsexGOBP2 showed modest affinity for two common leaf odorants (Feng & Prestwich, 1997), but recent reports (Bohbot *et al*, 1998; Jacquin-Joly *et al*, 2000) have suggested a more unexpected role for the GOBPs, with MbraGOBP2 binding specifically to a pheromone antagonist that plays a role in the avoidance of inter-specific mating mistakes. This suggests that, at least in this case, the GOBPs may not be involved solely in "general odour" detection.

(c) Other Insect OBPs

i. *D. melanogaster* OBPs

cDNAs encoding seven OBP-like proteins with the characteristic signal peptide and six conserved cysteine residues have been cloned

from the antennae of *D. melanogaster* (PBPRPs, Pikielny *et al*, 1994; OS-E and OS-F proteins, McKenna *et al*, 1994; LUSH, Kim *et al*, 1998), whilst the genome sequencing project has identified a further thirteen putative OBPs (Adams *et al*, 2000). Two of these OBPs have been shown to be co-expressed in specific sensilla (Hekmat-Scafe *et al*, 1998). By analogy to their vertebrate equivalents, these OBPs have been suggested to exist as homo- or hetero-dimers, potentially conveying different ligand-binding properties on the protein.

ii. ABPX

Studies on the binding proteins found in the antennae of moths led to the discovery of a fourth class of OBPs, termed Antennal Binding Protein X (ABPX), in *B. mori* (Krieger *et al*, 1996), *H. virescens*, *A. pernyi* (both Krieger *et al*, 1997) and *M. sexta* (Robertson *et al*, 1999). These proteins show only a low similarity to other PBPs and GOBPs, with the highest similarity being to the set of OBPs found in the fruit fly *D. melanogaster* (Krieger *et al*, 1996). Since they have OBP characteristics, and are antennal specific, they have been classed as another group of OBPs, although no ligands have yet been identified.

iii. Chemosensory /"OS-D-like" proteins

The studies that identified *D. melanogaster* OBPs also identified related olfactory proteins (A10, Pikielny *et al*, 1994; OS-D, McKenna *et al*, 1994) with four conserved cysteines. These proteins show high similarity to the ejaculatory bulb protein (ejbIII) of *D. melanogaster*,

present in a non-chemosensory organ (Dyanov *et al*, 1994).

Other "OS-D-like" proteins, also known as sensory appendage proteins (SAPs), and more recently termed chemosensory proteins (CSPs), have been identified in a diverse range of insect species, as shown in Table 1.4. These CSPs are expressed in a range of tissues with chemosensory capabilities i.e. antennae, legs, tarsi, labrum, head and labial palps, and suggested functions include carbon-dioxide sensing (Maleszka & Stange, 1997), contact chemoreception involving sugar molecules (Angeli *et al*, 1999), a role in the recognition of conspecific pheromones (Picimbon & Leal, 1999) and odour transport (Bohbot *et al*, 1998). Unfortunately, with the exception of *M. brassicae* AOBP2, which has been shown to bind vaccenyl acetate [an anti-aphrodisiac of *D. melanogaster* (Jallon *et al*, 1981)] *in vitro* (Bohbot *et al*, 1998), ligand-binding studies have been unsuccessful. Interestingly, hydropathy profiles of these proteins show them to be almost completely lacking in hydrophobic domains (Picimbon *et al*, 2000b), which would contradict a role in binding of hydrophobic ligands. Whatever the function, there is a high degree of conservation across the orders (~40-60%), suggesting an important common function.

iv. Miscellaneous

A number of proteins, with some of the characteristics of insect OBPs, have been identified in a range of insect species (see Table 1.5). Some of these proteins have been classed as OBPs, or OBP-like, but have only four conserved cysteines, although it should be noted that

Species	**Common name**	**Order**	**Family**	**No of proteins/ cDNAs**	**Reference**
B. mori	Silk worm	Lepidoptera	Bombycidae	2	Picimbon *et al*, 2000(b)
Cactoblastis cactorum	Moth borer	Lepidoptera	Pyralidae	1	Maleszka & Stange, 1997
M. brassicae	Cabbage army worm	Lepidoptera	Noctuiidae	8	Bohbot *et al*, 1998; Nagnan-Le Meillour *et al*, 2000
M. sexta	Tobacco hawk moth	Lepidoptera	Sphingidae	5	Robertson *et al*, 1999
Locusta migratoria	Migratory locust	Orthoptera	Acrididae	5	Picimbon *et al*, 2000(a)
Schistocerca gregaria	Desert locust	Orthoptera	Acrididae	7	Angeli *et al*, 1999
Blatella germanica	German cockroach	Dictyoptera	Blattidae	1	Picimbon & Leal, 1999
Periplaneta americana	American cockroach	Dictyoptera	Blattidae	4	Picimbon & Leal, 1999; Kitabayashi *et al*, 1998
Periplaneta fugilosum	Smoky brown cockroach	Dictyoptera	Blattidae	1	Picimbon & Leal, 1999
Carausius morosus	Indian stick insect	Phasmatodea	Heteronemiidae	1	Mameli *et al*, 1996
Eurycantha calcarta	Giant spiny stick insect	Phasmatodea	Phasmatidae	3	Mameli *et al*, 1996; Marchese *et al*, 2000
Extatosoma tiaratum	Giant prickly stick insect	Phasmatodea	Phasmatidae	1	Mameli *et al*, 1996
D. melanogaster	Fruit fly	Diptera	Drosophilidae	1	Pikielny *et al*, 1994 McKenna *et al*, 1994
Apis mellifera	Honey bee	Hymenoptera	Apidae	5	Danty *et al*, 1998

Table 1.4. Insect CSPs.

Species	Common name	Order	Family	Protein/DNA	Reference
Tenebrio molitor	Mealworm beetle	Coleoptera	Tenebrionidae	B1 & B2	Paesen & Happ, 1995
Ceratitis capitata	Mediterranean fruit fly	Diptera	Tephritidae	MSSP α1-2, β1-3, γ1-2	Thymianou *et al*, 1998; Christophides *et al*, 2000
Ceratitis rosa	Natal fruit fly	Diptera	Tephritidae	MSSP	Christophides *et al*, 2000
D. melanogaster	Fruit fly	Diptera	Drosophilidae	Putative binding proteins	Adams *et al*, 2000

Table 1.5. Miscellaneous insect OBP-like proteins.

this is not in the same pattern as in the CSPs. They have the signal peptide, acidic pI and a central hydrophobic region. Such proteins, for example those in the seminal fluid of the tubular accessory glands of the male mealworm beetle *Tenebrio molitor* (L., Tenebrionidae) and the fat bodies of the mediterranean fruit fly *Ceratitis capitata* (Weideman, Tephritidae), have been suggested as carriers of hydrophobic ligands (Rothemund *et al*, 1999), and they extend the family of OBPs beyond the olfactory system in insects. This raises the hypothesis that perhaps insects have a lipid-carrying superfamily that contains all of the binding proteins discussed in 1.2.2.2 (Thymianou *et al*, 1998; Christophides *et al*, 2000).

1. 3 Olfactory Receptors

Following the perireceptor events of olfaction, the binding of the ligands to the olfactory receptors (ORs) occurs, and ORs play a most important role in olfaction. ORs have now been identified in both vertebrate and insect species.

1.3.1 Vertebrate ORs

The accuracy of odour discrimination depends on the specificity with which odorant molecules interact with their ORs (Breer *et al*, 1994), and how the receptors discriminate between ligands. Do a limited number of receptors have overlapping activity, or is there a large family of multiple receptors, each with its own well-defined specificity?

In an attempt to answer this question, Buck and Axel cloned cDNAs encoding ORs that were shown to be expressed in the MOE of rats (Buck & Axel, 1991), and studies demonstrating ligand binding by the expressed recombinant proteins (Raming *et al*, 1993; Kiefer *et al*, 1996; Zhao *et al*, 1998) was final proof that this family was indeed an OR family. Dulac and colleagues cloned the two families of cDNAs encoding the equivalent vomeronasal receptors (VRs) of the VNO (Dulac & Axel, 1995; Herrada & Dulac, 1997; Matsunami & Buck, 1997), which are each activated by distinct ligand classes (Krieger *et al*, 1999a), although it is suggested that the V2R genes may encode pseudogenes (Keverne, 1999; Martini *et al*, 2000). Both sets of receptors are members of the G-protein coupled receptor (GPCR) superfamily, a large and diverse family of integral membrane proteins (Carr, 1998), although the low level of sequence similarity between them suggests that they may have evolved separately (Dulac & Axel, 1995).

The two populations (ORs and VRs) express different G-proteins, and project their axons to different areas of the olfactory bulb (Halpern, 2000). ORs have also been cloned from fish, mice, rat, dog, pig, frog and humans (Levy *et al*, 1991; Nef *et al*, 1992; Selbie *et al*, 1992; Strotmann *et al*, 1992; Ngai *et al*, 1993a; Raming *et al*, 1993; Hatt *et al*, 1999). Interestingly, the amino acid sequences of fish ORs are quite distinct from those of mammals, perhaps reflecting the fact that fish detect water-soluble compounds whereas terrestrial mammals smell volatile compounds. This is supported by the fact that the ORs of

amphibians are both fish-like and mammal-like (Freitag *et al*, 1995; Zhao & Firestein, 1999).

ORs have the characteristics of other GPCRs, i.e. (i) seven transmembrane domains; (ii) a glycosylation site at the extracellular N-terminus; (iii) potential phosphorylation sites at the third cytoplasmic loop; and (iv) an intracellular C-terminus. The amino acid sequences of the different ORs show variability in the central transmembrane domains, which form the ligand-binding funnel, reflecting the diversity of ligands that can be bound. This sequence variation has led to the categorisation of ORs into subfamilies, with those with similar sequences presumed to recognise similar odorant molecules, and those with divergent sequences binding structurally unrelated ligands (Buck, 1992; Ressler *et al*, 1993). Sequence similarity within subfamilies can be as high as 90% (Ronnett & Snyder, 1992).

The OR superfamily represents one of the largest multigene families identified to date, with estimates of 1000 genes for rodents, 500-700 for humans, and 100 for fish (Ngai *et al*, 1993a; Axel, 1995; Zhao *et al*, 1998; Mombaerts, 1999a). As is characteristic of other multigene families (Krumlauf, 1992), the OR genes are clustered at several loci on different chromosomes (Breer *et al*, 1994; Sullivan *et al*, 1996; Goodenough, 1998; Zhao & Firestein, 1999). A large proportion of the human OR genes are pseudogenes (Mombaerts, 1999b), in contrast to the OR genes of rodents (Mombaerts, 1999a) and this has raised the suggestion that these pseudogenes might allow for the diversity of odour perception present in the human population

(Mombaerts, 1999c) by resulting in differing olfactory perceptive capabilities. This might reflect the reduction of the selection pressure on OR genes during primate evolution, in accordance with a decreasing dependency on the sense of olfaction (Goodenough, 1998; Mombaerts, 1999b).

The mystery of how the large numbers of OR proteins handle odour recognition is also being unravelled. The question of "how does the brain know what the nose is smelling?" can be equated to "how does the brain know which ORs have been activated?" (Rouhi, 1996). Each olfactory neuron expresses only one or very few ORs (Nef *et al*, 1992; Ngai *et al*, 1993b; Chess *et al*, 1994; Malnic *et al*, 1999), and the OR genes are expressed in three or four distinct, non-overlapping zones (Ressler *et al*, 1993; Buck, 2000).

Recent studies on mammals have suggested that one OR recognises multiple odours, and one odorant molecule is recognised by multiple ORs, with the result that different odours are recognized by a combinatorial code (Malnic *et al*, 1999). Neurons bearing the receptors project to specific glomeruli in the main olfactory bulb (MOB) of the brain, which in turn contacts higher sensory centres, and thus the brain identifies the odour by identifying the pattern of glomerular activity (Sullivan *et al*, 1995; Mombaerts *et al*, 1996; Buck, 2000). The same processing appears to be the case for amphibians with, interestingly, ORs of the "water-nose" projecting to the ventral MOB, and those of the "air-nose" projecting to the dorsal MOB (Nezlin & Schild, 2000). It has been shown in rodents that the OR plays an instructive role in guiding

the axons to the glomeruli (Dreyer, 1998). In the VNO of rat and mouse, a similar situation exists with each neuron expressing one VR gene (Dulac & Axel, 1995; Matsunami & Buck, 1997), and neurons expressing the same VR being localised in spatially segregated zones (Matsunami & Buck, 1997). The VR neurons project to the accessory olfactory bulb (AOB) (Buck, 2000), which has projections to the amygdala and hypothalamus (Halpern, 1987; Goodenough, 1998), but organisation beyond the olfactory bulb, and how the signals are ultimately decoded remains elusive (Buck, 2000).

1.3.2 Insect ORs

First report of an insect OR was in *A. polyphemus* where the pheromone that bound to the PBP was also shown to bind to a 69kDa protein, suggested to be the OR (Vogt *et al*, 1988). Later, a 67kDa sensory neuron membrane protein (SNMP), was identified in olfactory neurons of *A. polyphemus*, and this showed similarity to CD36, a receptor-like membrane protein present in vertebrates, arthropods and nematodes (Rogers *et al*, 1997). Although only three transmembrane domains are present in SNMP, it has been suggested to be a novel insect pheromone receptor and, more recently, an inducer of odorant molecule off-loading in the vicinity of the OR (Vogt & Rogers, 2000).

Searches for ORs in other insects proved elusive until the recent *D. melanogaster* genome-sequencing project began to yield data. Three groups independently reported the discovery of genes encoding putative ORs in the *D. melanogaster* genome. Clyne *et al* (1999) and

Gao & Chess (1999) used a computational approach to find genes with transmembrane domains, coupled with RT-PCR, to identify seven OR genes expressed specifically in the antennae in a subpopulation of olfactory neurons, whilst Vosshall *et al* (1999) employed a difference cloning method to identify an OR cDNA present at low levels in the antenna and maxillary palp. A total of nineteen candidate OR genes were initially identified, and whilst the projected number was ~100 (Gao & Chess, 1999), the recently completed *D. melanogaster* genome sequence (Adams *et al*, 2000), gave a final estimation of 60 (*Drosophila* Odorant Receptor Nomenclature Committee, 2000). These ORs are extremely divergent from known OR- and other GPCR-families (Clyne *et al*, 1999), and show diversity within this family (0-75% identity). The identification of ORs in *D. melanogaster* should allow the elucidation of the relationship between ORs and OBPs, which, in this species, show a spatial overlapping pattern of expression (Krieger & Breer, 1999). In particular, the OBPs OS-E and OS-F of *D. melanogaster*, which have been shown to have arisen as a result of an ancient gene duplication event, possess two highly diverged regions (Hekmat-Scafe *et al*, 1998) that have been implicated in allowing these OBPs to present structurally different ligands to different ORs (Hekmat-Scafe *et al*, 2000).

The organisation and processing of the insect olfactory system is very similar to that of vertebrates (Strausfeld & Hildebrand, 1999), although there is only one structure, the antenna, to deal with both odours and pheromones. The olfactory neurons, expressing the different types of ORs, segregate to specific individual glomeruli

(Hansson *et al*, 1992; Stocker *et al*, 1993; Stocker, 1994; Hansson, 1995; Laissue *et al*, 1999), which are organised chemotopically in the antennal lobe (Hildebrand, 2000). All information regarding pheromones and intraspecific signals is processed in one large macroglomerular complex, which is male-specific in some species (Mustaparta, 1996), whilst the ordinary glomeruli process plant odour information (Hansson, 1995). The glomeruli encode the quantitative, qualitative and temporal identity of the odorant molecule by activation pattern (Carlsson & Hansson, 2000). Glomeruli in insects vary greatly in distinctness, being evident in cockroaches and lepidopterans but much less evident in flies (Laissue *et al*, 1999). These glomeruli, in turn, project to the mushroom bodies in the protocerebrum of the forebrain (Hansson, 1995; Mustaparta, 1996; Homberg *et al*, 1988). Although similar, there are fundamental differences in the organisation of OR genes between vertebrates and insects, suggesting independent origins of the two systems (Strausfeld & Hildebrand, 1999).

1.4 Signal Transduction

Once the odorant molecule has reached the receptor protein, the activation of the receptor produces an action potential in the associated olfactory neuron and a behavioural response is initiated. In both vertebrates and insects, this is thought to be achieved by interactions of G-proteins, intracellular messengers and ion channels. These interactions provide amplification of the initial signal, which is the basis of the sensitivity of the olfactory system (Breer et al, 1996).

1.4.1 Vertebrate Systems

As discussed, the ORs of vertebrates belong to the superfamily of GPCRs, with the binding of an odorant molecule to the OR resulting in activation of the coupled G-protein. G-proteins are membrane-bound guanosine triphosphate (GTP)-binding proteins, situated in the cytosolic face of the membrane (Vogt *et al*, 1990). These heterotrimeric proteins consist of an α-subunit (39-52kDa), a β-subunit (35-36kDa) and a γ-subunit (7-10kDa; Barritt, 1992), and they are the coupling system for many receptors and their effector molecules. Several types of G-protein subunits have been identified, the most important in this context being the α-subunit, G_{olf}, which has been shown to be expressed in the olfactory epithelium of rats (Jones & Reed, 1989). This subunit, and others, have also been identified in the OR neurons of the channel catfish *Ictalurus punctatus*, suggesting that the different G-proteins may mediate multiple signal transduction pathways (Abogadie *et al*, 1995). In the "off" state, guanosine-diphosphate (GDP) is bound to the α-subunit, and receptor activation induces the exchange of GDP for GTP. The α-subunit dissociates from the βγ dimer and, although it was originally thought that only the α-subunit was capable of activating target proteins, there are also examples of the βγ dimer activating channels (Logothetis *et al*, 1987) and enzymes (Rhee & Bae, 1997). GTP is then dephosphorylated to GDP, and the G-protein returns to the "off" state.

The G-protein activates enzymes, resulting in the production of effector molecules, generally termed second messengers, and these

trigger cascades of reactions that mediate the chemo-electrical transduction processes (Breer *et al*, 1994). A schematic diagram of the processes involved is presented in Figure 1.2. Evidence suggested initially that cyclic adenosine monophosphate (cAMP), which is hydrolysed from adenosine triphosphate (ATP) by the action of adenylate cyclase (AC), was the sole second messenger. However, a number of odours fail to activate AC, suggesting that there is an alternative pathway (Sklar *et al*, 1986). These odours produce an increase in inositol 1,4,5-trisphosphate (IP_3) (Boekhoff *et al*, 1990a; Raming *et al*, 1993), which results from the action of phospholipase C (PLC) on phosphatidyl inositol 4,5-bisphosphate (PIP_2). Both cAMP and IP_3 are capable of binding to ion channels, resulting in the depolarization of the receptor neuron (Nakamura & Gold, 1987; Firestein & Werblin, 1989). Therefore, there appears to be two second messenger pathways involved in olfactory signal transduction (Boekhoff *et al*, 1990a).

The presence of two second messengers, cAMP and IP_3, mediating olfactory signal transduction led to the suggestion that compounds of the same odour class might activate the same second messenger system. However, this is not so, as demonstrated by Breer & Boekhoff (1991), who showed that odorant molecules of the same class activate different, and mutually-exclusive, second messengers in rats. Both cAMP and IP_3 can coexist in the same olfactory neurons (Fadool & Ache, 1992). In the Caribbean spiny lobster (*Panulirus argus*), the two systems have opposing effects on the cell:

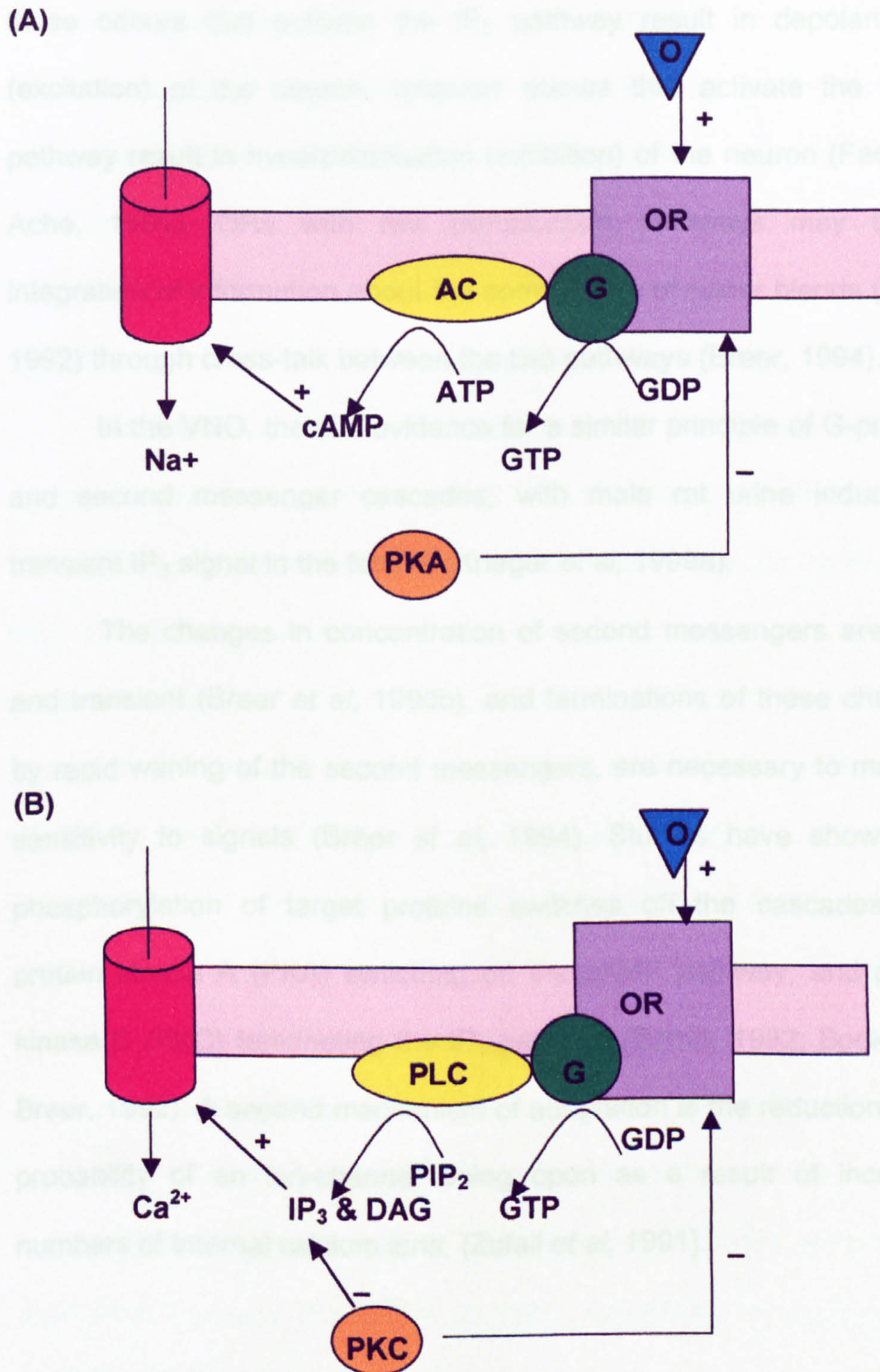


Figure 1.2. Signal transduction pathways mediated by (A) cAMP or (B) IP_3. O = odorant molecule (perhaps complexed to OBP); OR = olfactory receptor; G = G-protein; AC = adenylate cyclase; PKA = protein kinase A; PKC = protein kinase C; PLC = phospholipase C; PIP_2 = phosphatidylinositol 4,5-bisphosphate; DAG = diacylglycerol; IP_3 = inositol 1,4,5-triphosphate; + = stimulatory effect; - = inhibitory effect.

those odours that activate the IP_3 pathway result in depolarisation (excitation) of the neuron, whereas odours that activate the cAMP pathway result in hyperpolarisation (inhibition) of the neuron (Fadool & Ache, 1994). ORs with two transduction pathways may enable integration of information about the composition of odour blends (Reed, 1992) through cross-talk between the two pathways (Breer, 1994).

In the VNO, there is evidence for a similar principle of G-proteins and second messenger cascades, with male rat urine inducing a transient IP_3 signal in the female (Krieger *et al*, 1999a).

The changes in concentration of second messengers are rapid and transient (Breer *et al*, 1990b), and terminations of these changes, by rapid waning of the second messengers, are necessary to maintain sensitivity to signals (Breer *et al*, 1994). Studies have shown that phosphorylation of target proteins switches off the cascades, with protein kinase A (PKA) switching off the cAMP pathway, and protein kinase C (PKC) terminating the IP_3 pathway (Barritt, 1992; Boekhoff & Breer, 1992). A second mechanism of adaptation is the reduction of the probability of an ion-channel being open as a result of increased numbers of internal calcium ions, (Zufall *et al*, 1991).

1.4.2 Insect Systems

Insect signal transduction is less well characterised than that of vertebrates, but recent studies have indicated similarities. G-proteins have been shown to be present in insect antennae (Breer *et al*, 1988; Boekhoff *et al*, 1990c; Laue *et al*, 1997), and they have been implicated

in mediating the formation of second messenger molecules in the olfactory response (Laue *et al*, 1997; Boekhoff *et al*, 1990b). The presence of cyclic nucleotide- and voltage- gated ion channels has been demonstrated in moth antennae (Krieger *et al*, 1999b), and work by Schleicher *et al* (1994) has suggested that the phosphorylation of specific antennal proteins can be caused by pheromones.

Maida *et al* (2000b) also identified a PLC enzyme and a pheromone-induced increase in PKC activity, suggesting the presence of an IP_3-mediated pathway (Figure 1.2), which supports earlier work that showed the presence of an odorant-sensitive PLC in insect antennae (Breer *et al*, 1990a; Boekhoff *et al*, 1990c). IP_3 has been shown to be present in the olfactory system of *D. melanogaster* (Carlson, 1996), to evoke an excitatory Ca^{2+}-current in moth olfactory cells (Breer *et al*, 1990a; Stengl, 1993; Wegener *et al*, 1993), and to be involved in the response pathway of *H. virescens* (Boekhoff *et al*, 1993) and cockroaches (Breer et al, 1996). However, IP_3 failed to activate a pheromone-activated ion channel in adult moths (Zufall & Hatt, 1991), suggesting that an alternative transduction pathway also exists in insects. The presence of cAMP has been demonstrated in cockroach and locust antennae (Krieger *et al*, 1997), and cGMP (cyclic guanosine monophosphate, another cyclic nucleotide second messenger) the enzyme involved in cGMP synthesis occur in moth OR neurons, although this has not been implicated in transduction cascades (Ziegelberger *et al*, 1990; Boekhoff *et al*, 1993). It has been suggested that the roles of cAMP and IP_3 in insects are the reverse of that in

vertebrates (Ache, 1994), i.e. that cAMP is activated by odours that result in neuron depolarisation whilst IP_3 is activated by odours that result in neuron hyperpolarisation.

1.5 Odour Degradation

For an animal to maintain sensitivity to the environment, rapid inactivation and clearance of odorant molecules is essential, and biotransformation enzymes have been identified in the olfactory organs of vertebrates and insects. However, in insects, whether or not enzymes alone are responsible for the rapid decline of the receptor potential is still controversial.

1.5.1 Vertebrate Biotransformation Enzymes

Many enzymes involved in the metabolism of odorant molecules are located in the nasal apparatus, and these enzymes are responsible for the chemical modification (Phase I reactions) and conjugation (Phase II reactions) of the odorant molecules, resulting in the inability of the molecule to further activate the receptor (Breer *et al*, 1994). Mammalian systems incorporate many enzymes, including cytochrome P450s, flavin-containing monooxygenases, aldehyde dehydrogenases (Phase I), glutathione transferases, UDP-glucuronyltransferases (Phase II) and carboxylesterases (Breer *et al*, 1994; Carr *et al*, 1998). These enzymes are present at activities comparable to those found in the liver, although the metabolic rates in the olfactory tissues are often higher (Dahl, 1988). Nef *et al* (1989) cloned a cDNA encoding a cytochrome

P450 from rat, and suggested that its unique expression in the olfactory neuroepithelium implied a role in olfactory reception, perhaps in processing of the odorant molecules. It is suggested that these enzymes are involved only in degradation of the odorant molecules, and not in the termination of the signal (Lazard *et al*, 1991), which results from waning of second messenger concentrations (Breer *et al*, 1994). Once degraded, the metabolites are cleared via the nasal mucous or the blood, depending on the particular compound (Dahl, 1988).

1.5.2 Insect Biotransformation Enzymes

Studies on the biotransformation proteins of insects have revealed a variety of enzymes associated with olfactory tissues. The first to be identified was a sensilla esterase (55kDa) from male *A. polyphemus* (Vogt & Riddiford, 1981), which hydrolysed pheromonal esters in only a few milliseconds *in vitro* (Vogt *et al*, 1985), although the pheromone remains on intact antennae for longer (Kanaujia & Kaissling, 1985). Esterases have also been identified in the antennae of the cabbage looper moth *Trichoplusia ni* (Hübner; Noctuiidae; Ferkovich *et al*, 1973) and the diamondback moth *Plutella xylostella* (L; Plutellidae; Prestwich *et al*, 1989). Other biotransformation enzymes with a proposed role in olfaction include an aldehyde dehydrogenase in *H. virescens* (Prestwich *et al*, 1989); aldehyde oxidases in *M. sexta* (Rybczynski *et al*, 1989) and *H. virescens* (Prestwich *et al*, 1989), *A. polyphemus* and *B. mori* (Vogt *et al*, 1990); an epoxidase in *L. dispar* (Vogt, 1987; Prestwich *et al*, 1989), and an alcohol

oxidase/dehydrogenase in *B. mori* (Kasang *et al*, 1989; Krieger *et al*, 1997). A glutathione-S-transferase has been cloned from the antennae of *B. mori* (Krieger *et al*, 1997) and *M. sexta*, which is thought to have an additional role in protecting the olfactory system from harmful xenobiotics (Rogers *et al*, 1999). Screening of an antennal cDNA library from *B. mori* also identified a cytochrome P-450 enzyme (Krieger *et al*, 1997).

Moths also have "non-antennal" enzymes, present on the insect's surface (*A. polyphemus*, Vogt & Riddford, 1986a; *T. ni*; Ferkovich *et al*, 1982; and the spruce budworm *Choristoneura fumiferana*, (Clemens; Tortricidae; Lonergan, 1986), which are thought to be involved in degrading sources of pheromone that could become uncontrolled signals (Vogt *et al*, 1990).

Biotransformation enzymes have also been reported in non-lepidopteran species including *D. melanogaster* (Hovemann *et al*, 1997), the house-fly *Musca domestica* (Muscidae; Ahmad *et al*, 1987), and the pale brown chafer, *P. diversa* (Scarabidae; Wojtasek & Leal, 1999a). In the latter two species, a role in metabolism of sex pheromones has been demonstrated for the enzymes.

1.5.3 Insect Non-Enzymatic Degradation

Kaissling has challenged the idea of enzymatic degradation of odorant molecules. The degradation of pheromone by the sensillar esterase *in situ* (Kasang *et al*, 1988) is much slower than that reported, *in vitro*, for the purified enzyme (Vogt *et al*, 1985), and Kaissling

suggests that this cannot therefore be responsible for the rapid stimulus decline reflected in the rapid receptor potential decline (Kaissling, 1986). Originally, Kaissling suggested that binding of the pheromone by a PBP would reduce the concentration of the pheromone in the vicinity of the receptor, and this would be sufficient for the rapid non-enzymatic inactivation of the odour.

An alternative method has been suggested. Ziegelberger (1995) showed that two forms of PBP could be observed for *A. polyphemus*, implying that the PBP might have a dual function of presentation and removal of odorant molecules. The pheromone, bound by a reduced PBP (with two unbridged cysteines), is transported to the receptor, and, once activation of the receptor has occurred, the PBP is oxidized (no unbridged cysteines) and the pheromone thus inactivated. It was further suggested that the receptor molecules catalyse this oxidation, as the redox shift is only observed in the presence of the olfactory neuronal dendrites.

The advantage of this mechanism, where the receptor molecules serve as deactivating enzymes, is that it would ensure that odorant molecules are not deactivated before they interact with a receptor (Kaissling, 1998b). However, the debate continues because Kaissling relates the rapid decline in receptor potential to odour degradation as opposed to the termination of intracellular signalling (Breer *et al*, 1994).

1.6 Aphids

1.6.1 Characteristics

Aphids are small sap-sucking insects, in the order Hemiptera (sub-order Homoptera), many with a parthenogenetic reproduction stage, and with short generation times (seven to ten days from birth to first reproduction) allowing rapid increases in population size (Hales *et al*, 1997). Within the order Hemiptera, aphids belong to the series Sternorrhyncha (Blackman & Eastop, 2000), and the aphid species used in this study belong to the subfamily Aphidinae (see Figure 1.3). Over 250 species of the superfamily Aphidoidea feed on agricultural and horticultural crops throughout the world (Blackman & Eastop, 2000), causing damage both directly, by draining the plants of nutrients resulting in reduced harvest yields, and indirectly, though transmission of plant pathogenic viruses (Hales *et al*, 1997; Eastop, 1995).

A feature of almost all of the Aphidoidea is amphygony, or the alternation of a sexual reproductive phase with the parthenogenetic reproductive phase in one life cycle (Blackman & Eastop, 2000), and a typical aphid life cycle is shown in Figure 1.4. The alternation between parthenogenetic and sexual morphs occurs in response to environmental factors such as the photoperiod and temperature, with the dark phase (scotoperiod) being most important (Lees, 1959). Environmental factors can have other effects on morphology, for example, the production of winged aphids in response to crowding or host deterioration (Lees, 1967; Dixon, 1985).

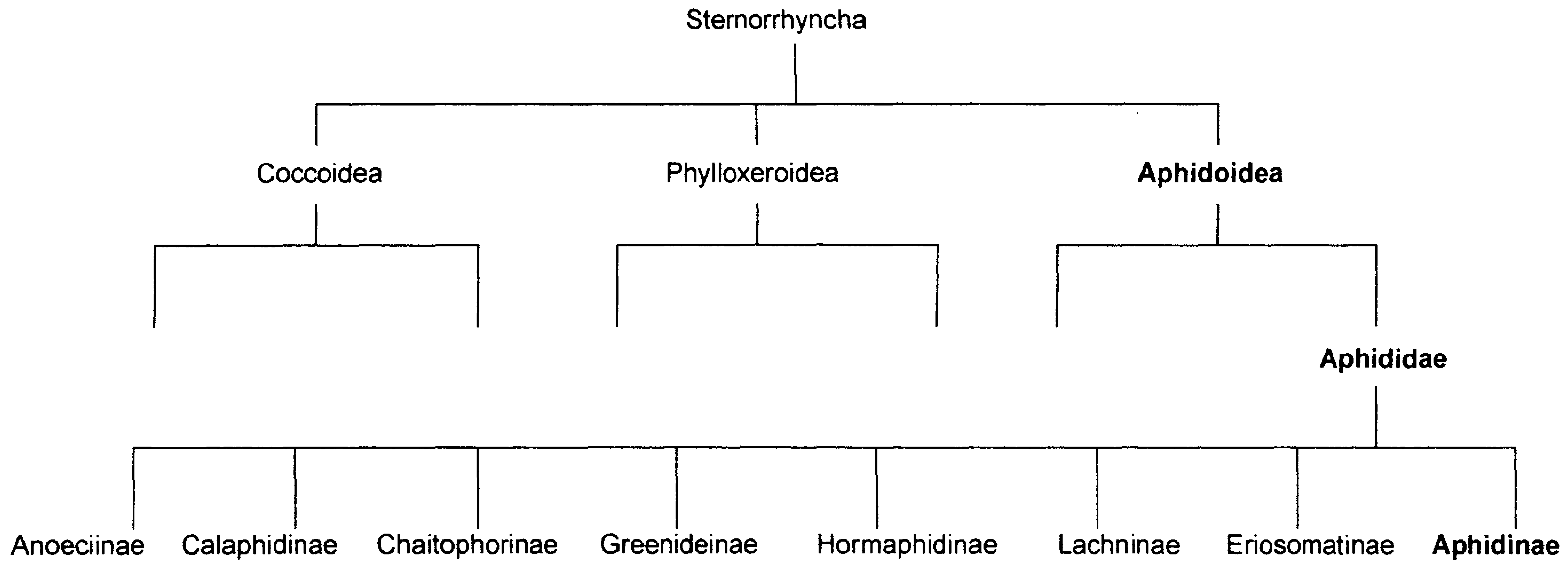


Figure 1.3. Representation of aphid taxonomical classification, including the major aphid subfamilies (as presented by Blackman & Eastop, 2000)

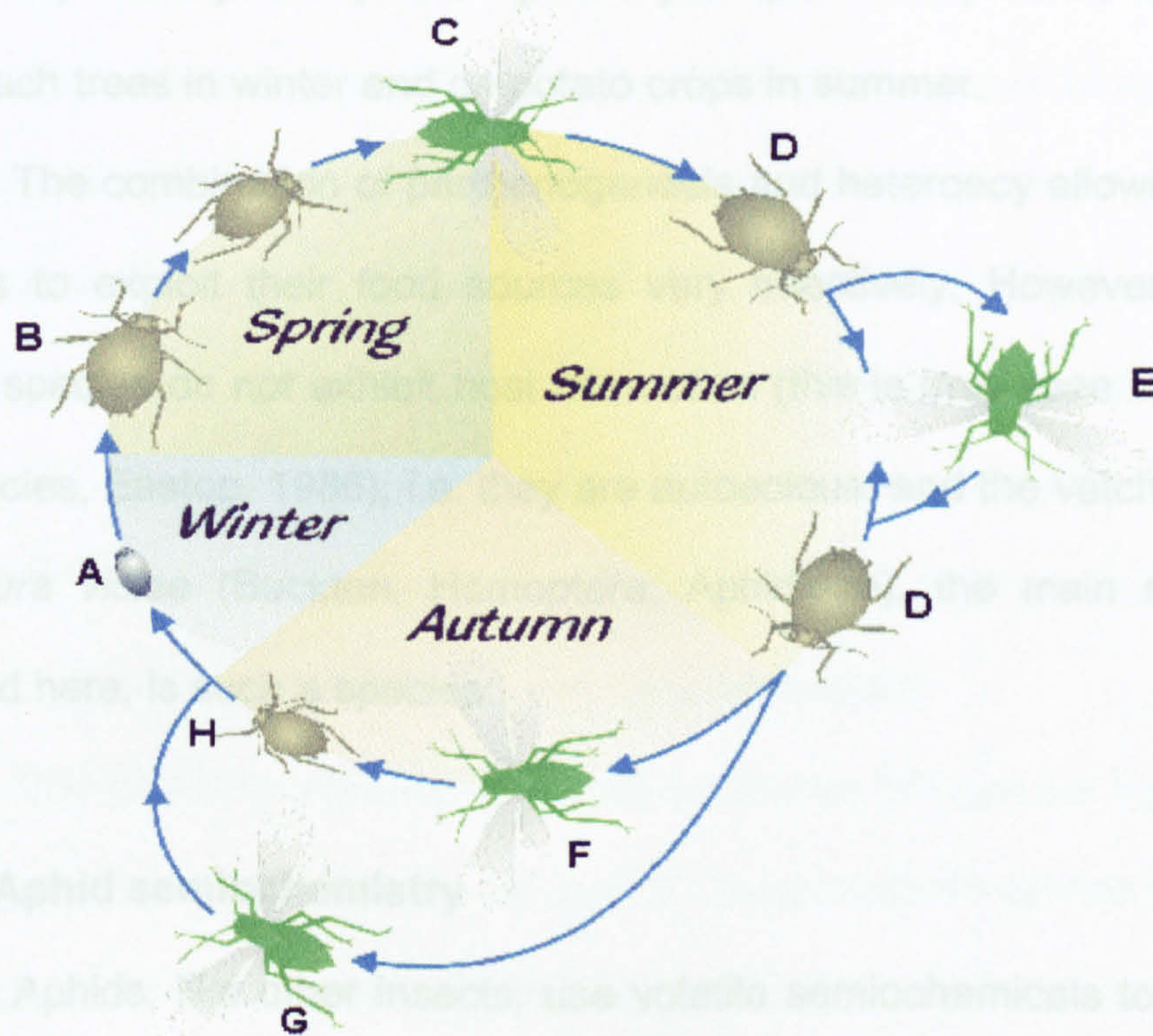


Figure 1.4. Diagrammatic representation of an aphid life cycle. A: overwintering egg; B: fundatrix; C: spring migrant; D: parthenogenetic virginoparae (apterous); E: alate virginoparae; F: autumn migrant; G: male; H: egg-laying females (oviparae)

Another important characteristic of some aphid life-cycles is the alternation of host plants (heteroecy). Many aphids have two types of host plant: the primary, or winter, host supports the sexual stages, and the secondary, or summer, host supporting the parthenogenetic phase. Most aphids tend to be associated with particular plant families, and may feed on one (monophagous), a few, or many (polyphagous) host plants, although they usually show much greater specificity for the

primary host (Blackman & Eastop, 2000), having many secondary host species. This alternation is used to give aphids their common names, for example the peach-potato aphid *Myzus persicae* (Sulzer) is found on peach trees in winter and on potato crops in summer.

The combination of parthenogenesis and heteroecy allows some aphids to exploit their food sources very effectively. However, most aphid species do not exhibit host alternation (this is only seen in ~10% of species, Eastop, 1986), i.e. they are autoecious, and the vetch aphid, *Megoura viciae* (Buckton, Homoptera; Aphididae), the main species studied here, is such a species.

1.6.2 Aphid semiochemistry

Aphids, like other insects, use volatile semiochemicals to obtain information about their environment, and to interact with other organisms. These signal chemicals, which influence the behaviour or development of the insect, are either pheromones, which are intraspecific, or allelochemicals, which are interspecific. Pheromones are commonly involved in sexual, alarm, and aggregation responses (Edwards *et al*, 1973; Blight *et al*, 1984; Dawson *et al*, 1987a). The term allelochemicals encompasses a broad range of signals i.e. kairomones, where the receiver benefits (for example plant volatiles being used by an insect to locate a host plant); allomones, where the emitter benefits (such as antifeedants); and synomones, where both the emitter and the receiver benefit (for example pollinators). These chemicals play a very important role in the natural ecology of insects, and there is a great deal

of interest in the development of such compounds for novel insect control strategies, particularly where resistance to chemical insecticides has made control difficult.

A typical example of the complex chemical ecology of aphids is in mate location. Mating females attract males by producing a sex pheromone from scent plaques on their hind tibiae. This pheromone, which is used by a number of aphid species, has been found to be a mixture of (4a*S*,7*S*,7a*R*)-nepetalactone (A) and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (B) (Dawson *et al*, 1987a; see Figure 1.5), with the ratios of A:B being very species-specific.

The olfactory cells on the male antennae (discussed further in 1.6.3) respond separately to A and B, in a manner similar to that described for *A. polyphemus* (Kaissling, 1979), thus ensuring species

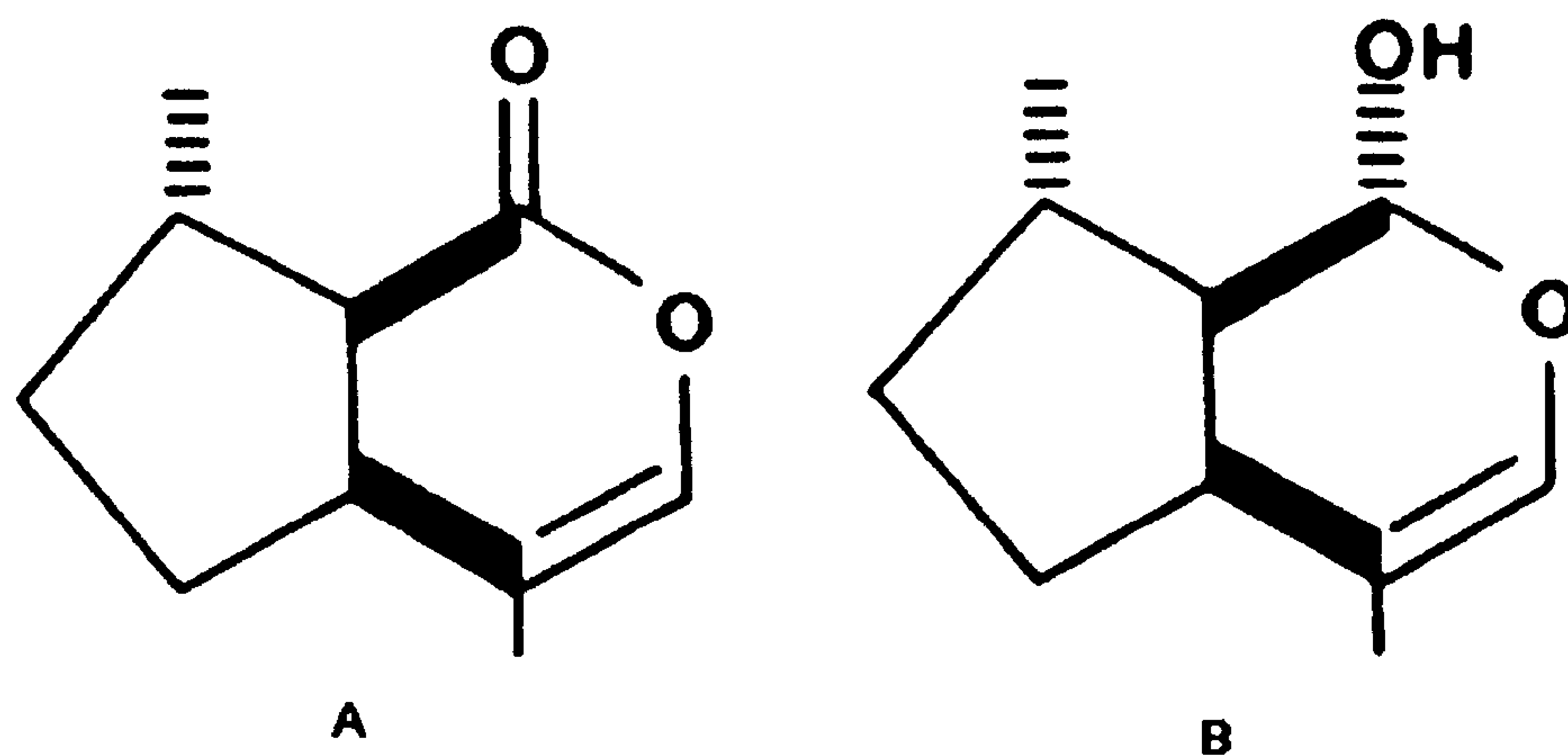


Figure 1.5. Structure of aphid sex pheromone components. A: (4a*S*,7*S*,7a*R*)-nepetalactone. B: (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (Dawson *et al*, 1987a)

discrimination. Originally, it was thought that sex pheromones were active over only short distances, and that to find a mate, a male had first to find the host plant she was on. However, recent studies have demonstrated a long-range sex-pheromone mediated attraction of male Damson-hop aphid *Phorodon humuli* (Schrank; Aphididae), with some host plant volatiles having a synergistic effect (Campbell *et al*, 1990). Unfortunately, this long-range effect can also be disadvantageous to the aphid, with the aphid sex pheromone being used as a kairomone by predators and parasitoids of aphids (Hardie *et al*, 1994; Gabryś *et al*, 1997).

Host plant selection involving semiochemicals is also highly important for aphids. Whilst early studies suggested that visual cues were most important, with olfactory cues playing only a close-range role after the aphid had landed on the plant (Müller, 1958), evidence has now demonstrated a long-range role for olfaction (Visser, 1983; Campbell *et al*, 1990; Nottingham *et al*, 1991). Selection of host plants relies on the detection of plant secondary metabolites (Klingauf, 1987), although gustation probably determines the plant's final suitability. Like the sex pheromone, volatiles released by the plant during aphid feeding are used by parasitoids to locate a host (Du *et al*, 1998).

The role of semiochemicals in the chemical ecology of aphids is very important and utilising these compounds starts with the appropriate detection machinery.

1.6.3 The aphid chemosensory system

The olfactory organs of aphids are the antennae, and a diagrammatic representation is shown in Figure 1.6 (taken from Pickett *et al*, 1992). The antennal segments bear placoid sensilla, called rhinaria, which contain the olfactory receptor cells showing a very high degree of specificity for their key compounds (Pickett *et al*, 1992). Rhinaria are surrounded by a rim, and the primary rhinaria have distinct cilia on the rim, whilst the secondary rhinaria have small, or hardly-developed cilia (Anderson & Bromley, 1987). The secondary rhinaria are more numerous in alate (winged) morphs than apterous (wingless) morphs, and as such were suggested to have a role in host plant and mate location. Indeed single cell recordings (SCR) have confirmed that the secondary rhinaria in males are involved in response to the sex pheromone (Dawson *et al*, 1990), whilst those of the alate virginoparae are involved in response to host plant volatiles (unpublished data, L.J Wadhams & C. M. Woodcock). The primary rhinaria are involved in the detection of the aphid alarm pheromone, with the distal primary rhinarium detecting (*E*)-β-farnesene (EBF), and the proximal primary rhinarium containing cells for detecting synergist compounds, e.g. α-pinene, which is a synergist for EBF in *M. viciae*, and isothiocyanates, which are synergists for the same compound in the turnip aphid *Lipaphis erysimi* (Kaltenbach, Dawson et al, 1987b; Pickett & Griffiths, 1980).

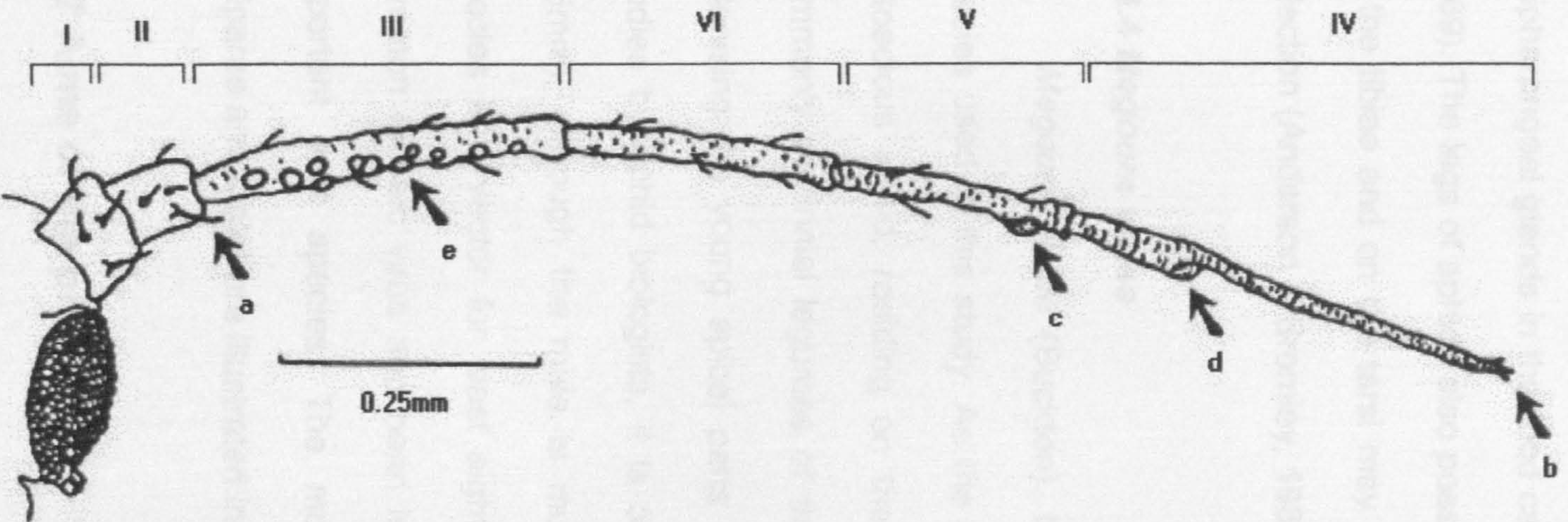


Figure 1.6. Diagrammatic representation of an aphid antenna showing olfactory sensilla (Taken from Pickett *et al*, 1992). The antenna has a maximum of six segments: the scape (I), which houses the antennal muscles; the pedicel (II), and the flagellum or antennal segments (III to VI). The segments have mechanoreceptive hairs (a), with those at the tip of the antenna (b) also having a gustatory function (Anderson & Bromley, 1987). The primary rhinaria (c and d) are present in all individuals, regardless of developmental stage (instar), whilst the additional rhinaria present on the other segments, the secondary rhinaria (e), are present only in the adult stages.

The sensilla at the tip of the antennae have a contact or gustatory function, and other gustatory organs include the epipharangeal glands in the food canal of the aphid (Wensler & Filshie, 1969). The legs of aphids also possess sensilla, and those on the apex of the tibiae and on the tarsi may have a gustatory role in host plant selection (Anderson & Bromley, 1987).

1.6.4 *Megoura viciae*

Megoura viciae (Buckton), the vetch aphid, is the main aphid species used in this study. As the common name suggests, this is an autoecious aphid, residing on the same host all year round (most commonly perennial legumes of the genera *Lathyrus* and *Vicia*), and colonising the young apical parts of the plant. The subject of many studies by aphid biologists, it is one of the larger aphid species (3-4.5mm), although the male is much smaller than the female. This species is a vector for least eight plant viruses, including the bean common mosaic virus and bean leaf roll virus, although it is not an important pest species. The morphs studied here (virginoparae, oviparae and male) are illustrated in Figures 1.7, 1.8 and 1.9.

1.7 Aims of the project

The overall aim of this research was to clone and characterise the gene(s) encoding molecular recognition proteins in the vetch aphid, *M. viciae*. To achieve this, antennal cDNA libraries and subtracted antennal cDNA libraries were constructed. The sequences of the

Figure 1.7. Apterous virginoparous (wingless parthenogenetic female) *M. viciae*.

Figure 1.8. Oviparous (egg-laying female) *M. viciae*.

Figure 1.9. Male *M. viciae*.

cDNAs and predicted encoded proteins were examined for similarity to known insect proteins involved in molecular recognition. The tissue expression patterns of such cDNAs were also determined to confirm a role in olfaction.

In a parallel piece of research, the PBP from *B. mori* was heterologously expressed and characterised biochemically, as a model system for other insect OBPs.

Chapter 2. Materials and methods

2.1 Materials

2.1.1 Chemicals

Chemicals used in this study were of either analytical or molecular biological grade and were obtained from Sigma, Gibco BRL (Life Technologies), Fisons Scientific Equipment, or BDH Laboratory Supplies.

2.1.2 Radiochemicals

Radioactive [α-^{32}P] dCTP (deoxycytidine), with a specific activity of 3000Ci/mmol, was obtained from Amersham Pharmacia Biotech.

2.1.3 Enzymes and kits

Restriction endonucleases and other DNA-modifying enzymes were purchased from Gibco BRL, MBI Fermentas, Boehringer Mannheim, Promega or Amersham Pharmacia Biotech. Kits for molecular techniques were purchased from a range of suppliers and are detailed in the methods (2.2).

2.1.4 Oligonucleotide primers

Vector-specific and gene-specific oligonucleotide primers (GSPs) were custom synthesised by Gibco BRL, reconstituted in sterile distilled water (SDW) and the absorbance measured at $\lambda = 260$nm with a Philips

PU8800 UV/VIS spectrophotometer. The primer concentration was calculated according to the formula:

$$\text{concentration } (\mu g\ ml^{-1}) = A_{260} \times 30$$

Primers were diluted to working concentrations and stored at –20°C.

2.1.5 Cloning and expression vectors

Vector	Source	Purpose
pBluescript	Stratagene	Cloning
PCR-Script™	Stratagene	Cloning
pGEM T-Easy	Promega	Construction of cDNA libraries/ cloning of PCR products
pFASTBAC	Gibco BRL	Expression in Sf9/Sf21 cells

2.1.6 *Escherichia coli* strains

Strain	Source	Purpose
JM109	Promega	Host for plasmids
XL1 Blue	Stratagene	Host for plasmids
DH10 Bac	Gibco BRL	Host for expression in Sf9/Sf21 cells

2.1.7 Insect species

Aphids used in this study came from long-term parthenogenetic cultures, maintained at IACR-Rothamsted. The following hemipteran species from the subfamily Aphidinae were used: the vetch aphid *Megoura viciae* (Buckton); the blackbean aphid *Aphis fabae* (Scopoli); the bird cherry-oat aphid *Rhopalosiphum padi* (L.); the peach-potato aphid *Myzus persicae* (Sulzer); the pea aphid *Acyrthosiphum pisum*

(Harris); the cabbage aphid *Brevicoryne brassicae* (L.); the currant-lettuce aphid *Nasanovia ribis-nigri* (Mosley); the rose-grain aphid *Metopolophium dirhodum* (Walker); the turnip aphid *L. erysimi* (Kaltenbach) and the grain aphid *Sitobium avenae* (Fabricius).

2.2 Methods

2.2.1 Insects

2.2.1.1 Insect rearing

Adult *M. viciae* were obtained from a parthenogenetic culture and reared on excised broad bean (*Vicia faba*) leaves in Blackman boxes (Blackman, 1974) at 15°C in a 12h light:12h dark regime. Oviparae and males appeared in the third generation.

Adult aphids of the other species were obtained from parthenogenetic cultures reared on appropriate host plants at 20°C in a 16h light:8h dark regime.

2.2.1.2 Harvesting of insect material

Adult aphids were anaesthetised with diethyl ether, and antennae, heads, legs and bodies manually dissected under 4x magnification. Tissues were frozen in liquid nitrogen and stored at –80°C.

2.2.2 Molecular Biology

2.2.2.1 RNA extraction

(a) Isolation of total RNA

Whole aphids, or aphid tissues, were collected in 1.5ml microfuge tubes and ground to a fine powder in liquid nitrogen. Extraction buffer [50mM sodium chloride; 50mM Tris pH 7.5; 5mM EDTA; 0.5% SDS; 200μg ml^{-1} Proteinase K (EC 3.4.21.64)] was added and the suspension incubated at 37°C for 1h. Samples were extracted twice with an equal volume of phenol:chloroform (1:1v/v) and the RNA precipitated with 0.1 volume of 3M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol at –20°C for 2h. RNA was recovered by centrifugation at 11000rpm at 4°C for 20 min, washed with 70% ethanol, air-dried, dissolved in DEPC-treated water (DTW) and stored at –80°C.

(b) Removal of gDNA from RNA preparations

RNA samples were treated with DNase I (EC 3.1.21.1 from bovine pancreas; 10U; Sigma) in 1x buffer (10mM Tris pH 8.3; 50mM potassium chloride; 1.5mM magnesium chloride) at 37°C for 1h to remove contaminating gDNA. RNA was then extracted with phenol: chloroform (1:1 v/v) and precipitated as in 2.2.2.1(a).

(c) Isolation of Poly(A)$^+$ RNA

Poly(A)$^+$ RNA was extracted from insect tissues using the Oligotex Direct mRNA Mini Kit (Qiagen) and the buffers supplied. The procedure was according to the manufacturer's instructions using the

QIAshredder method for tissue disruption and homogenization. RNA was eluted in DTW and stored at –80°C.

2.2.2.2 DNA extraction

(a) Isolation of gDNA from insect tissues

Adult aphids were collected in 1.5ml microfuge tubes and homogenized in buffer (0.1M Tris pH 9.0; 0.1M EDTA; 1% SDS). RNase A (EC 3.1.27.5 from bovine pancreas, 250μg) was added and the mixture incubated for 30 min at 37°C followed by 30 min at 70°C. Potassium acetate (8M) was added to precipitate proteins, and the mixture left on ice for 30 min. The supernatant, containing the DNA, was collected after centrifugation at 11000rpm at 4°C for 30 min, and transferred to a clean microfuge tube. The DNA was then precipitated with an equal volume of propan-2-ol at –20°C for 2h and recovered by centrifugation at 11000rpm at 4°C for 25 min. It was washed with 75% ethanol, dried under vacuum (Uniscience Univap) for 15 min, resuspended in SDW and stored at –20°C.

(b) Isolation of plasmid DNA

Small amounts of pDNA (<5μg) were prepared using either the Qiaprep® Spin Miniprep Kit (Qiagen) or the PERFECTprep™ Plasmid DNA kit (CP Laboratories). Larger amounts (5-50μg) were prepared according to the alkaline lysis method of Sambrook *et al* (1989, 1.25-1.28).

(c) Isolation of bacmid DNA

Bacmid DNA for use in the baculovirus expression system was extracted according to the user manual of the BAC-TO-BAC™ Baculovirus Expression Systems Kit (Gibco BRL). This procedure is an adaptation of the alkaline lysis method, and is optimised for high molecular weight plasmids (>100kb).

2.2.2.3 Agarose gel electrophoresis

Samples were prepared in 1x loading buffer (60% w/v glycerol; 0.1M EDTA; 0.001% (w/v) bromophenol blue) and electrophoresed through 0.8 – 1.5% agarose gels in either 1x TAE buffer (40mM Tris acetate; 1mM EDTA) or 0.5xTBE (45mM Tris borate; 1mM EDTA) following standard protocols. DNA size markers (λDNA/*HindIII* or 100bp ladder; MBI Fermentas) were used as appropriate. DNA/RNA was visualised using a UV transilluminator following staining with ethidium bromide (1μg ml^{-1}) in water for 30 min and destaining in water for 30 min.

2.2.2.4 cDNA synthesis

(a) First strand synthesis

First strand cDNA (complementary DNA) was synthesized from total RNA (200ng-2μg) by reverse transcription using an oligo(dT) primer. RNA and primer were heated at 70°C for 10 min to denature templates, then snap-frozen on ice for 2 min. 20μl reactions contained 1x buffer (supplied with enzyme: 50mM Tris-HCl pH 8.3; 75mM

potassium chloride; 3mM magnesium chloride), 1mM each dNTP (dATP, dCTP, dGTP and dTTP), 10mM DTT (Dithiothreitol; supplied with enzyme), Superscript™ II RNase H⁻ reverse transcriptase (200U; Gibco BRL) and human placental ribonuclease inhibitor (RNAguard, 10U, Amersham Pharmacia Biotech), and were incubated at 37°C for 1h. Samples were stored at –80°C.

(b) Second strand synthesis

Second strand cDNA was synthesised in a reaction volume of 150μl containing the first-strand reaction [see 2.2.2.4(a)], 1x T4 DNA ligase buffer (40mM Tris-HCl pH7.8; 10mM magnesium chloride; 10mM DTT; 0.5mM ATP), T4 DNA ligase (EC 6.5.1.1, 10U, MBI Fermentas), *E.coli* DNA Polymerase (EC 2.7.7.7, 30U, Amersham Pharmacia Biotech), RNase H (2.7U, Gibco BRL) and 0.2mM each dNTP at 16°C for 2½h. The double-stranded (ds) cDNA was precipitated with 0.05 volume 4M ammonium acetate and 3 volumes 100% ethanol at –20°C for 2h. Following centrifugation at 11000rpm at 4°C, ds cDNA was washed with ethanol (75%), air-dried, resuspended in SDW and stored at –20°C.

2.2.2.5 Homopolymeric tailing

Homopolymeric [poly(dG)] tails were added to DNAs (up to 100ng) in 40μl reactions containing 1x buffer (10mM Tris-acetate; 10mM magnesium acetate; 50mM potassium acetate), 10mM dGTP, Terminal deoxynucleotidyl transferase (TdT, from calf thymus, EC2.7.7.

31; 20U) at 37°C for 30 min. Tailing reactions were stopped by the addition of 0.1 volume of EDTA (0.5M).

2.2.2.6 Polymerase Chain Reaction (PCR)

(a) Standard PCR

PCR amplifications were done in 25µl reactions containing DNA template (100pg pDNA, 200ng cDNA or 100ng gDNA), 1x buffer (supplied with enzyme: 75mM Tris-HCl pH 8.8; 20mM ammonium sulfate; 0.01% Tween 20), magnesium chloride (1.5mM), dNTPs (0.2mM each), primers (31.25ng each of sense and antisense) and *Taq* DNA Polymerase (MBI Fermentas; 0.75U) using a programmable thermal cycler (Hybaid OmniGene, Techne Genius or Eppendorf Mastercycler Gradient). A hot start was done at 94°C for 2 min (preamplification), and then *Taq* was added at a holding temperature of 80°C. This procedure increases the specificity of the reactions by preventing any non-specific annealing between template and primers (D'Aquila *et al*, 1991). Cycling parameters were as follows: 94°C for 30s (denaturation), 45-55°C for 60s (annealing), and 72°C for 2-5 min (extension) for 25-35 cycles, followed by a final extension at 72°C for 10 min. The annealing temperature was determined by the Tm of the primers, and the extension time was 1 min per kb of template to be amplified. Amplification products were analysed by agarose gel electrophoresis [see 2.2.2.3(a)].

(b) Reverse-Transcription PCR (RT-PCR)

First strand cDNA was synthesised as in 2.2.2.4(a) using 200ng total RNA. An aliquot of cDNA (1µl) was taken into a primary PCR reaction (total volume 25µl) as detailed in 2.2.2.6(a). If necessary, a dilution (1:10 – 1:10000) of the primary PCR was used for a secondary (sometimes nested) PCR.

(c) Single colony PCR (SC-PCR)

SC-PCR was used as a rapid screening method to identify bacterial colonies containing recombinant plasmids. Candidate colonies were picked into lysis buffer (20mM Tris pH 8.5; 2mM EDTA; 1% Triton X-100), boiled for 10 min and centrifuged at 11000rpm at room temperature for 10 min. An aliquot (2µl) of the supernatant was taken into a primary PCR reaction (total volume 25µl) as detailed in 2.2.2.6(a).

(d) Rapid Amplification of cDNA Ends (RACE)

A kit for the synthesis of 5' RACE products was purchased from Gibco BRL and all procedures were carried out according to the manufacturer's instructions. Briefly, an antisense GSP was used for first strand cDNA synthesis, and a homopolymeric tail was added using TdT and dCTP. Specific cDNA was then amplified using an abridged anchor primer (AAP; complementary to the poly(dC) tail) and a second GSP. Nested PCRs were performed with a third GSP and a universal abridged anchor primer (AUAP). This protocol is shown in Figure 2.1.

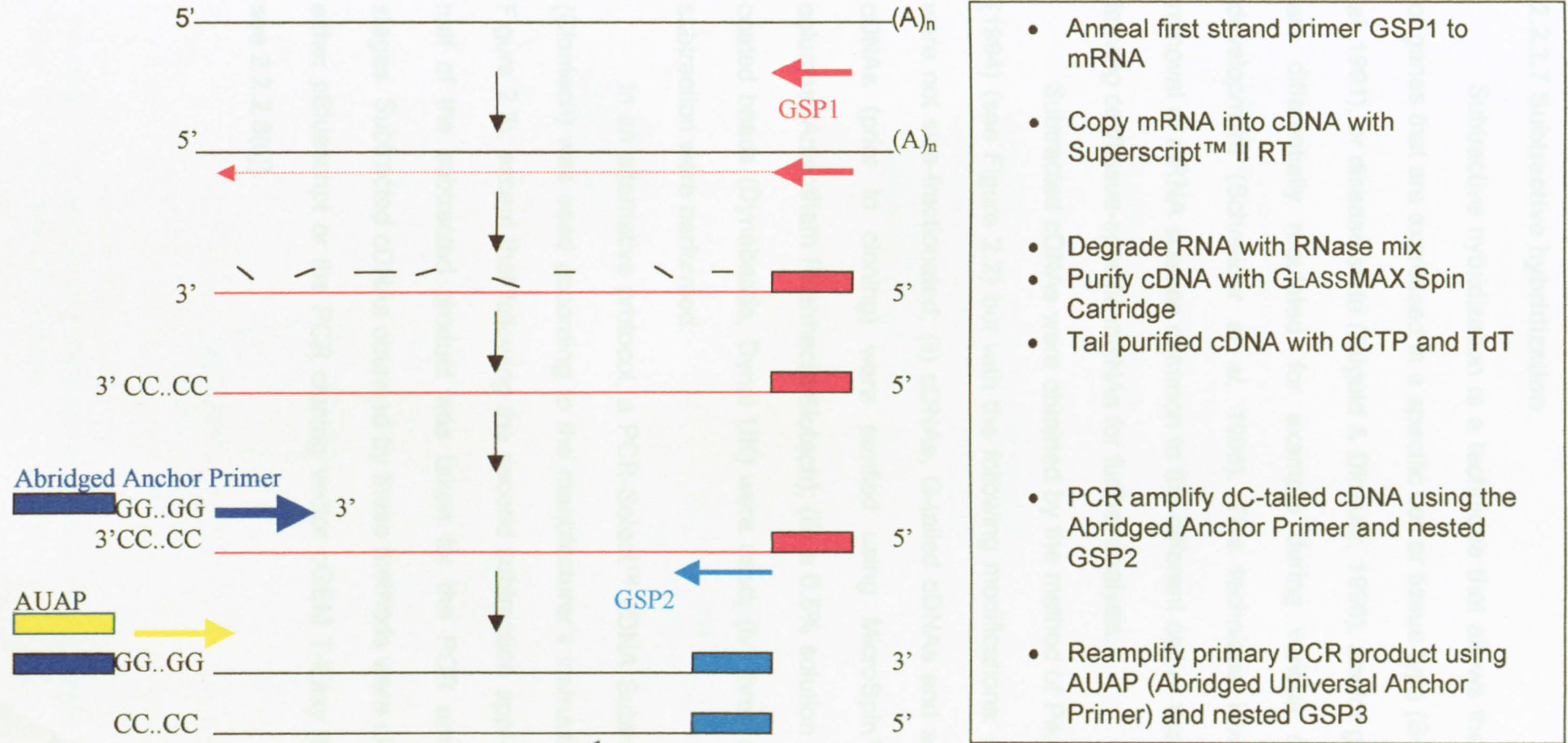


Figure 2.1 Schematic representation of the 5' RACE protocol (Gibco BRL).

2.2.2.7 Subtractive hybridization

Subtractive hybridization is a technique that allows the isolation of genes that are expressed in a specific cell or tissue type (Swaroop *et al*, 1991), or disease state (Duguid & Dinauer, 1990), and/or genes that are differentially regulated, for example during various stages of development (Schneider *et al*, 1988). This technique involves the removal of mRNA species common to the different cell or tissue types, leaving cell/tissue-specific mRNAs for further analysis.

Subtracted cDNAs were obtained by the method of Pikielny *et al* (1994) (see Figure 2.2) but with the following modifications: (i) cDNAs were not size-fractionated; (ii) cDNAs, G-tailed cDNAs and subtracted cDNAs (prior to cloning) were purified using MicroSpin™ S-400 columns (Amersham Pharmacia Biotech); (iii) a 0.5% solution of avidin-coated beads (Dynabeads, Dynal UK) were used; (iv) three rounds of subtraction were performed.

In an alternative protocol, a PCR-Select™ cDNA Subtraction Kit (Clontech) was used according to the manufacturer's instructions (see Figure 2.3), except that following the second subtraction approximately half of the subtracted product was taken for the PCR amplification stages. Subtracted cDNAs obtained by these methods were cloned into either pBluescript or the PCR cloning vector pGEM T-Easy [Promega; see 2.2.2.8(c)].

Large and small mRNAs,
10ng of total RNA.

A AAAAAAAA
B AAAAAAAA

1) Partial base hydrolysis of mRNAs.

2) First strand cDNA synthesis and size purification.

A dTdTdTdTdTdTdTdTdCdCdCdCdCdC
B dTdTdTdTdTdTdTdTdCdCdCdCdCdC

All cDNAs are of similar size.

3) G-tailing and PCR.

A
dCdCdCdCdCdC dAdAdAdAdAdAdAdAdGdGdGdGdGdG
dGdGdGdGdGdG dTdTdTdTdTdTdTdTdCdCdCdCdCdC

B
dCdCdCdCdCdC dAdAdAdAdAdAdAdAdGdGdGdGdGdG
dGdGdGdGdGdG dTdTdTdTdTdTdTdTdCdCdCdCdCdC

Amplification with "C" primer,
stringent hybridization conditions.

4) Subtractive hybridization.

TRACER

DRIVER

biotin

Repeat until tracer and driver are almost entirely different.

DENATURE
HYBRIDIZE

SA

SA BEADS

Figure 2.2 Overview of subtractive hybridization (Pikielny *et al*, 1994). (SA = streptavidin beads)

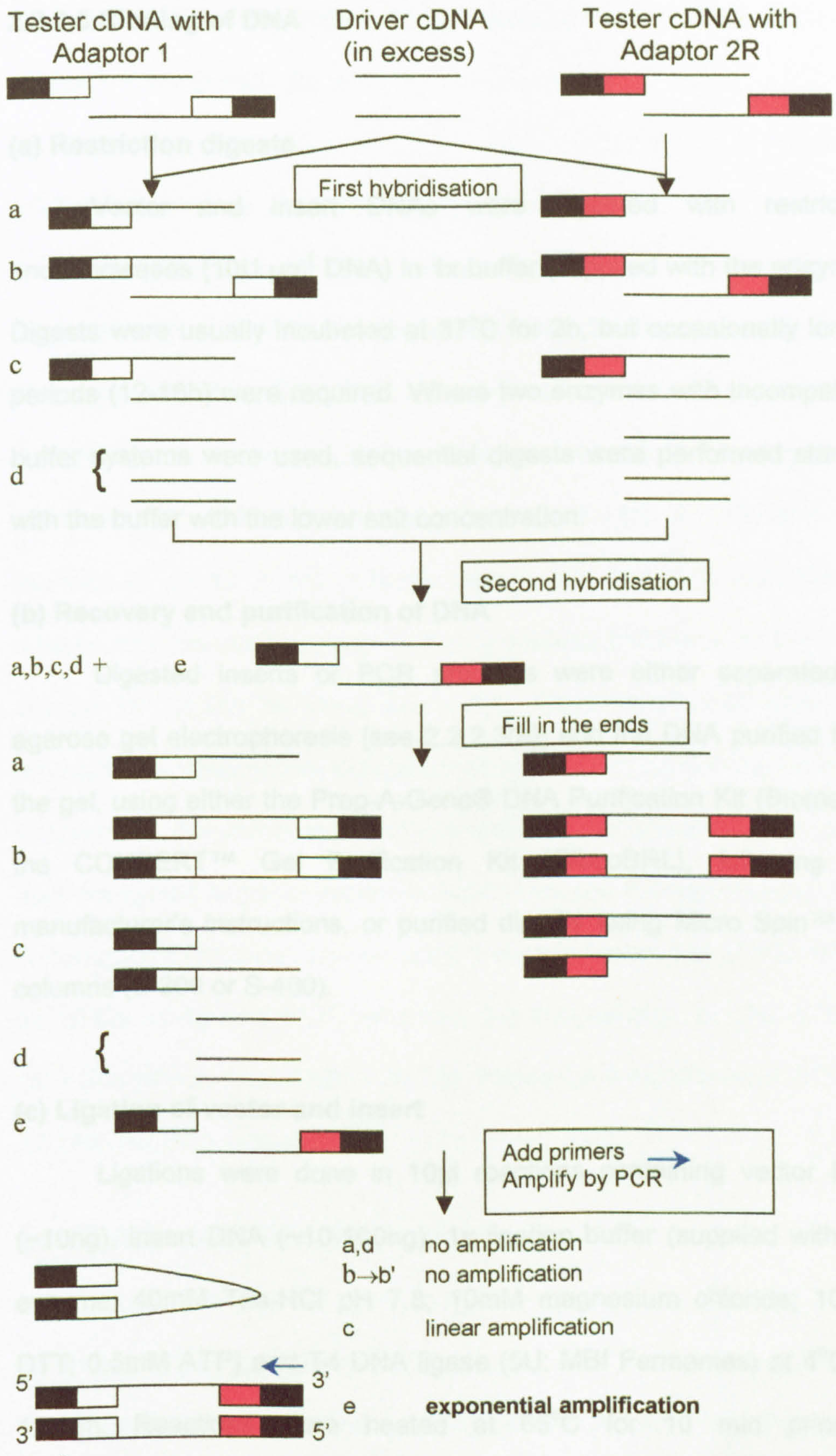


Figure 2.3 Overview of the Clontech PCR-Select procedure (taken from user manual). "Tester" contains transcript of interest; "driver" does not.

2.2.2.8 Cloning of DNA

(a) Restriction digests

Vector and insert DNAs were digested with restriction endonucleases (10U μg^{-1} DNA) in 1x buffer (supplied with the enzyme). Digests were usually incubated at 37°C for 2h, but occasionally longer periods (12-16h) were required. Where two enzymes with incompatible buffer systems were used, sequential digests were performed starting with the buffer with the lower salt concentration.

(b) Recovery and purification of DNA

Digested inserts or PCR products were either separated by agarose gel electrophoresis [see 2.2.2.3(a)] and the DNA purified from the gel, using either the Prep-A-Gene® DNA Purification Kit (Biorad) or the CONCERT™ Gel Purification Kit (GibcoBRL), following the manufacturer's instructions, or purified directly using Micro Spin™ HR columns (S-200 or S-400).

(c) Ligation of vector and insert

Ligations were done in 10µl reactions containing vector DNA (~10ng), insert DNA (~10-100ng), 1x ligation buffer (supplied with the enzyme: 40mM Tris-HCl pH 7.8; 10mM magnesium chloride; 10mM DTT; 0.5mM ATP) and T4 DNA ligase (5U; MBI Fermentas) at 4°C for 12-16h. Reactions were heated at 65°C for 10 min prior to transformation to improve the transformation efficiency. In some cases the insert and vector were co-precipitated prior to ligation.

Digested cDNAs were ligated into pBluescript, and PCR products into pGEM T-Easy or PCR-Script.

(d) Transformation of competent *Escherichia coli* cells

Competent *E.coli* cells were either prepared according to Alexander *et al* (1984) or purchased from Stratagene (XL1 Blue), Promega (JM109) or Gibco BRL (DH10 Bac). An aliquot (usually 2µl) of the ligation reaction was added to 50µl cells in a 1.5ml microfuge tube, incubated on ice for 30 min, heat-shocked at 42°C for 45s and then returned to ice for 2 min. L-Broth (950µl) was added to the cell/DNA mixture and the cells were grown in a shaking incubator at 37°C and 200rpm for 1h (4h for DH10 Bac cells). Transformed cells were then recovered by plating onto pre-prepared agar plates containing selective antibiotic(s). For identification of colonies that contained inserts, plates also contained X-gal (5-bromo-4-chloro-3-indolyl ß-D-galactoside; 40µl of 50mg ml^{-1}) {Bluo-gal [halogenated ß-D-galactoside; 5µl of 100mM] for DH10 Bac cells} and IPTG (isopropyl ß-D-thiogalactoside; 80µl of 20mg ml^{-1}) (Sambrook *et al*, 1989; 1.8-1.9). Plates were incubated at 37°C for 12-16h and then stored at 4°C.

2.2.2.9 Automated DNA Sequencing

(a) Sequencing reactions

Sequencing reactions were done with the ABI Dye Terminator and BigDye™ Terminator Cycle Sequencing Ready Reaction kits (PE Applied Biosystems). A 20µl reaction contained template DNA (30-90ng

purified PCR product or 400-600ng ds plasmid); oligonucleotide primer (40ng) and reaction mixture (A-Dye terminator; C-Dye terminator; T-Dye terminator; G-Dye terminator; dITP; dATP; dCTP; dTTP; Tris-HCl pH 9.0; magnesium chloride, pyrophosphatase, AmpliTaq® DNA Polymerase, FS). Where BigDye™ kits were used, the reaction mixture was diluted with an equal volume of dilution buffer (final concentrations: 200mM Tris pH 9.0; 5mM magnesium chloride). Cycle sequencing used the following parameters: 94°C for 30s, 50°C for 30s, and 60°C for 4 min for 25 cycles. For GC-rich templates, the following parameters were used to overcome problems of secondary structure: 96°C for 2 min (denaturation, template only) then 94°C for 30s, 66°C for 30s, and 66°C for 4 min for 25 cycles.

Following cycle sequencing, the product was precipitated with 0.1 volume 3M sodium acetate (pH 5.5) and 2 volumes ethanol (room temperature, 100%) at room temperature for 10 min followed by centrifugation at 14000rpm at room temperature for 20 min. Following an ethanol wash (70%), products were air-dried for 1h at room temperature, and then run on an ABI 373A Automated Sequencer or an ABI 310 Genetic Analyzer (PE Applied Biosystems).

(b) Sequence data analysis

Sequence data analyses and fragment assembly used the GCG (9.1 and 10; Genetics Computer Group, Wisconsin, Canada) and Staden (versions 1999.0 and 2000.0) packages. Databases were searched for sequence matches using BLAST searches (Altschul *et al*, 1997).

2.2.2.10 Nucleic acid hybridization

(a) RNA dot blot

Poly(A)$^+$ RNA in formamide (deionised, 500µl), formaldehyde (37%, 162µl) and 10x MOPS buffer (10x: 0.3M 3-[N-morpholino]propane-sulfonic acid; 0.5M sodium acetate pH 7.0; 0.01M EDTA, 100µl) was incubated at 65°C for 5 min and placed on ice for 5 min. Ice-cold 20xSSC (3M sodium chloride; 0.3M sodium citrate, 762µl) was added, and the RNA solution was transferred to a nylon membrane (Hybond N$^+$; Amersham Pharmacia Biotech), prewetted in 10xSSC, using a Minifold™ micro-sample filtration manifold (Schleicher & Schuell). The filter was air-dried and then baked at 80°C for 2h to immobilise the RNA.

(b) Preparation of probe

cDNAs were radiolabelled with [α^{32}P] dCTP using a nick translation kit (Amersham Pharmacia Biotech). A 50µl reaction containing template DNA (2ng ml^{-1} hybridisation solution); dATP, dTTP, dGTP; enzyme mix (DNA Polymerase and DNaseI), and radioisotope (50µCi), was incubated at 15°C for 3h. Prior to hybridisation, the radiolabelled DNA was denatured by boiling for 10 min and quick cooling on ice.

(c) Hybridisation conditions

Filter-bound RNA was hybridized to the DNA probe under

high stringency conditions. Filters were placed in hybridization tubes (Techne), and incubated in prehybridisation solution (PHS) (0.125ml cm^{-2} filter) at 42°C for 3h. [PHS: 50% formamide (v/v); 5x SSC; 0.05M sodium phosphate buffer pH 6.5; 0.1% (w/v) SDS; 250μg ml^{-1} herring sperm DNA; 5x Denhardts solution {0.1% (w/v) bovine serum albumin; 0.1% (w/v) ficoll; 0.1% (w/v) polyvinylpyrrolidone}]. The PHS was discarded and hybridisation solution (same composition as PHS) containing denatured probe [see 2.2.2.10(b)] was added to the tube, and hybridisation performed at 42°C overnight. The hybridization solution was then removed and the filter washed in several changes of wash buffer [2xSSC; 0.1%(w/v) SDS] at 65°C until the background radioactivity (measured with a Geiger Muller tube) was approximately 5-10 cps (counts per second). The filter was wrapped in cling film and autoradiographed.

(d) Autoradiography

^{32}P-radiolabelled filters [see 2.2.2.10(c)] were placed in X-ray cassettes, containing intensifying screens, overlayed with X-ray film (Kodak, Genetic Research Instruments) and autoradiographed at –80°C. X-ray films were processed using X-ray developer and fixer solutions (Photosol).

2.2.3 Cell culture and *in vitro* expression

2.2.3.1 Cell culture conditions

Spodoptera frugiperda insect cells (Sf9 and Sf21) were purchased from Gibco BRL and maintained in Sf-900 II SFM (serum free medium) with L-glutamine supplemented with penicillin ($50Uml^{-1}$) and streptomycin ($50\mu gml^{-1}$) at 27°C either in monolayer or suspension culture according to the instructions for the BAC-TO-BAC™ baculovirus expression system user manual.

2.2.3.2 Construct preparation

Each full-length cDNA was subcloned into pFASTBAC, the donor plasmid for the BAC-TO-BAC™ baculovirus expression system. The sequence of the construct was checked, and then transformations were done according to the manufacturer's instructions. Briefly, *E.coli* DH10 Bac cells were transformed [as described in 2.2.2.7(d)], and recombinant bacmids isolated [2.2.2.2(c)] and analysed by agarose gel electrophoresis and PCR as recommended.

2.2.3.3 Transfection and infection

Transfections of insect cells with recombinant bacmid DNA, and subsequent infection of cells with recombinant virus particles was essentially as detailed in the manufacturer's protocol with the following exceptions: (i) for transfections, cells were seeded in tissue culture flasks of area $25cm^2$; (ii) 0.5ml harvested recombinant virus was used to infect cells; (iii) for infections, cells were seeded at $1x10^6$ cells ml^{-1} in a total volume of 2ml in $25cm^2$ tissue culture flasks.

2.2.4 Biochemistry and Immunochemistry

2.2.4.1 Protein preparation

Insect cell medium, containing secreted proteins, was harvested and total protein precipitated by the addition of an equal volume of ice-cold trichloroacteic acid (25%), incubation on ice for 10 min, and centrifugation at 14000rpm at room temperature for 1 min. Precipitates were washed twice with acetone, air-dried and resuspended in SDS loading buffer [62.5mM Tris-HCl pH 6.8; 10% (w/v) glycerol; 2% SDS; 10% ß-mercaptoethanol (v/v)]. Alternatively, total protein was concentrated using an Ultrafree®-MC centrifugal filter unit (PTTK 30000NMWL, Millipore) and an equal volume of 2x loading buffer added (125mM Tris-HCl pH 6.8; 20% glycerol (w/v); 4% SDS; 20% ß-mercaptoethanol for SDS PAGE; 3.2% Triton X-100; 20% sucrose for native PAGE).

2.2.4.2 Extraction of soluble proteins

Soluble proteins were extracted from aphid antennae according to the method of Dickens *et al* (1998a). Briefly, 200 antennae were homogenized in buffer (50mM Tris pH 6.8; 10% (v/v) glycerol) in a 1.5ml microfuge tube on ice. Following centrifugation at 4°C and 11000rpm for 5 min, the supernatant, which contained the soluble proteins, was transferred to a clean microfuge tube and stored at –80°C.

2.2.4.3 SDS Polyacrylamide gel electrophoresis (SDS PAGE)

Protein samples were boiled for 5 min, and then separated on the basis of size using SDS-PAGE (Laemmli, 1970), in 17% acrylamide and 1x SDS reservoir buffer [0.192M glycine; 0.025M Tris; 0.1%(w/v) SDS] at 100V for 3h. Molecular weights were determined by comparison with standard protein markers (Low Molecular Weight Markers, Promega). Following electrophoresis, gels were fixed using methanol:acetic acid:water (5:1:4 v/v), stained with Coomassie Brilliant Blue R (1.25% in fixing solution; Sigma) and destained in methanol:acetic acid:water (1:1:8 v/v) until the background was clear.

2.2.4.4 Native PAGE

Protein samples were separated on the basis of size and charge using native PAGE (Williams & Reisfeld, 1964) through 17% acrylamide and 1x barbitone reservoir buffer [0.03M barbitone; 5% Tris (w/v)] at 100V for 3h. Following electrophoresis, gels were stained with Coomassie Brilliant Blue (as 2.2.4.3), and destained using glacial acetic acid (7%).

2.2.4.5 Isoelectric Focusing (IEF)

Protein samples were separated according to their isoelectric point (pI) by isoelectric focusing (IEF) through a 1mm polyacrylamide gel (Ampholine PAGPlate; Amersham Pharmacia Biotech) pH 4.0-6.5 using an LKB Bromma 2117 Multiphor IEF electrophoresis unit. IEF was performed at 4°C and 12W constant power for 2½h using 0.1M

glutamic acid in 0.5M phosphoric acid as the anode buffer and 0.1M ß-alanine as the cathode buffer. Determination of protein pIs was made by co-focusing of IEF markers (Low pI Calibration Kit pH 2.5-6.5, Amersham Pharmacia Biotech).

Following focusing, pH measurements were taken along the distance of the gel at 0.5cm intervals to determine the pH gradient (Andrews, 1986). Gels were then fixed in 0.7M trichloroacteic acid; 0.1M sulphosalicylic acid, washed in ethanol:acetic acid:water 2.5:0.8:6.7 (v/v), stained with Coomassie Brilliant Blue [1.25% in methanol:acetic acid:water 5:1:4 (v/v)], and destained in ethanol:acetic acid:water 2.5:0.8:6.7 (v/v) until the background was clear.

2.2.4.6 Western blotting

Gels were prepared for electroblotting by washing several times in water and then equilibrating in Towbin transfer buffer [25mM Tris; 192mM glycine (20% methanol) pH8.3]. Protein samples were transferred to nitrocellulose membranes (Novex, California or Hybond ECL, Amersham Pharmacia Biotech) using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Biorad) at 10V constant voltage for 1h (4mm thick gels) or 45 min (1mm thick gels). Membranes were then incubated in blocking agent (5% low fat dried milk in TBS-T [Tris-buffered saline –Tween]: 20mM Tris; 137mM sodium chloride; 0.1% Tween 20) at 4°C overnight.

2.2.4.7 Immunodetection

Incubation of the membrane-bound proteins with antibodies, and subsequent immunodetection were performed using the ECL Western blotting analysis system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. This system results in a light-emitting reaction as shown in Figure 2.4. Briefly, the membrane was washed in three changes of TBS-T, incubated with the primary antibody (i.e. specific against the required protein; raised in rabbit) for 1h; washed as above, incubated with the secondary antibody (anti-rabbit IgG; whole molecule; peroxidase conjugate; Sigma) for 1h, and washed again as above. Equal volumes of the ECL detection reagents 1 and 2 were mixed, and the membrane incubated in the resulting solution for 1 min. The membrane was wrapped in Saran Wrap, exposed to X-ray film in a cassette and the X-ray film developed as 2.2.2.10(d).

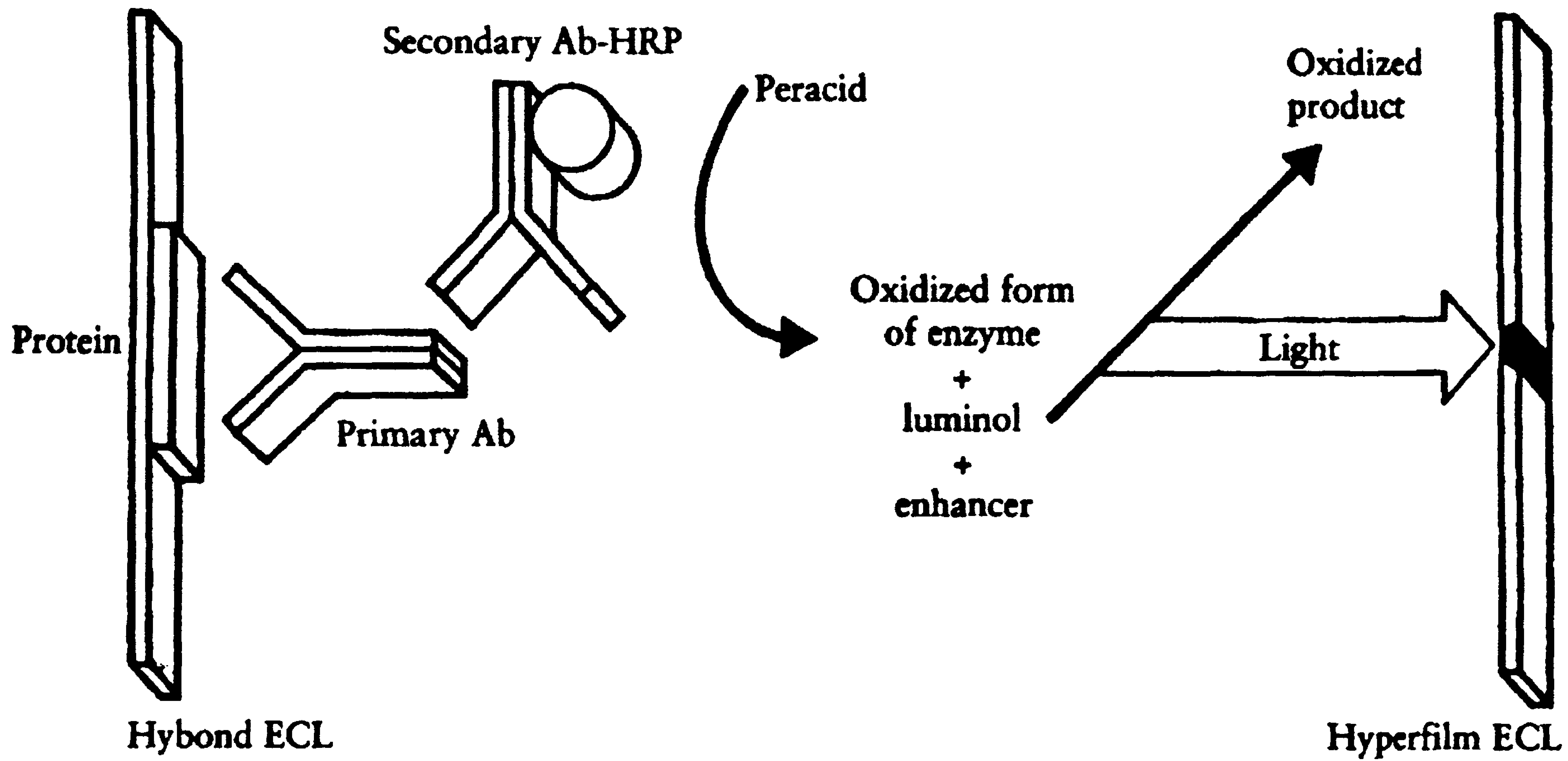


Figure 2.4 Principles of ECL Western blotting system (Amersham Pharmacia Biotech).

Chapter 3. Construction and screening of antennal cDNA libraries from *M. viciae*

3.1 Introduction

Genes and cDNAs encoding OBPs of many insect species have been cloned from chemosensory organs, primarily the antennae, where the OBPs show specific expression (Györgyi *et al*, 1988; Raming *et al*, 1989; Raming *et al*, 1990; Breer *et al*, 1990c; Vogt *et al*, 1991a; Krieger *et al*, 1991; Krieger *et al*, 1993; McKenna *et al*, 1994; Pikielny *et al*, 1994; Prestwich *et al*, 1995; Ozaki *et al*, 1995; Krieger *et al*, 1996; Danty *et al*, 1997; Maïbèche-Coisné *et al*, 1998a and b; Wojtasek *et al*, 1998; Danty *et al*, 1999; LaForest *et al*, 1999; Wojtasek *et al*, 1999; Vogt *et al*, 1999; Willett & Harrison, 1999a and b; Campanacci *et al*, 1999; Robertson *et al*, 1999; Maida *et al*, 2000a; Willett, 2000a and b; Callahan *et al*, 2000). These studies originated with lepidopteran species, and the first OBP identified was the PBP of *A. polyphemus* (Vogt & Riddiford, 1981). The first cDNA for an OBP was then cloned from the antennae of *Manduca sexta* (Györgyi *et al*, 1988) using an anti-ApolPBP antibody to screen a λ bacteriophage expression library. Subsequently, other moth OBP cDNAs were cloned either by systematic sequencing of antennal cDNA libraries (Raming *et al*, 1989), by RT-PCR using degenerate primers (Vogt *et al*, 1991a; Ozaki *et al*, 1995; Maïbèche-Coisné *et al*, 1998a and b; Wojtasek *et al*, 1998; Danty *et al*, 1999; Wojtasek *et al*, 1999; Vogt *et al* 1999; Willett & Harrison,

1999; Campanacci *et al*, 1999), or by probing of antennal libraries using degenerate oligonucleotides (Raming *et al*, 1990; Breer *et al*, 1990c; Krieger *et al*, 1991; Krieger *et al*, 1993; Krieger *et al*, 1996; Maida *et al*, 2000a; Callahan *et al*, 2000). The latter two approaches were successful because of the very high level of similarity between the amino acid sequences of OBPs within the Lepidoptera: i.e. 50-90% between PBPs, and 50-100% between GOBPs (Pelosi & Maida, 1995b).

cDNAs encoding OBPs from other insect species have also been cloned by a variety of means, most commonly by designing degenerate primers, based on the N-terminal amino acid sequence of the protein, for PCR amplification from mRNA (Ozaki *et al*, 1995; Wojtasek *et al*, 1998; Danty *et al*, 1999; Wojtasek *et al*, 1999; Vogt *et al*, 1999; Willett & Harrison, 1999a and b; Willett, 2000a and b). This has shown that the OBPs from other insect species have a much lower, some would say "barely significant" (Pelosi, 1996) similarity at the amino acid level to the moth OBPs (Table 3.1), and to each other, the only common features being a consistent size (15-20kDa), and the presence of a signal peptide and conserved cysteine residues (see Chapter 1). The lack of similarity between the OBPs of different insect species suggests that degenerate primers, designed from one species, are unlikely to detect sequences in another, unless the species are very closely related. Thus, this approach was considered to be unsuitable for the cloning of OBP cDNAs from *M. viciae*.

It has been shown, in moths, that PBPs are biosynthesized in the

OBP from insect	Shows highest % similarity to
ASP1 *A. mellifera*	28% to *A. pernyi* ABPX (1)
CRLBPpr *P. regina*	40% to *D. melanogaster* PBPRP5 (2)
OS-F *D. melanogaster*	29% to *A. pernyi* PBP1(APR1) (3)
P. japonica / A. osakana	38% to *B. mori* ABPX (4)
LAP *L. lineolaris*	37% to *H. virescens* ABPX (5)

Table 3.1 Percentage similarity (amino acid level) of OBPs from different insect orders. 1. Danty *et al*, 1999; 2. Ozaki *et al*, 1995; 3. McKenna *et al*, 1994; 4. Wojtasek *et al*, 1998; 5. Vogt *et al*, 1999

auxiliary cells at the base of the sensillum (Steinbrecht *et al*, 1992) and that the high concentration of OBPs in the sensillum lymph (10mM, Pelosi & Maida, 1995b) results from a high steady rate of turnover (Vogt *et al*, 1989). Consequently, mRNA for OBPs is abundant in antennal tissue. Assuming that aphid antennae have levels of OBPs in proportionate amounts to those of moths, and that there is an equivalent rate of turnover, the transcripts should represent a large proportion of an antennal cDNA library. Candidate OBPs could then be identified by systematic sequencing of clones, the approach used for *A. polyphemus* (Raming *et al*, 1989). Unfortunately, aphid antennae are very small in comparison to those of moths (c.5mm long), and contain flat placoid rhinaria as opposed to long hair-like sensilla (see Chapter 1), therefore the amount of mRNA obtainable, even from a large number of antennae, is very low. To overcome this limitation, the current study makes use of PCR-directed antennal cDNA libraries, which allow the study of gene expression in a specific tissue where the amount of material is very limited (Gurr & McPherson, 1991).

3.2 Aims of the study

The present study aimed to clone cDNAs encoding OBPs from the aphid *M. viciae*, using the following experimental approach:

- To construct PCR-directed antennal cDNA libraries from male and oviparous female *M. viciae*
- To sequence clones and determine if any have sequence matches in the public databases
- To use the predicted amino acid sequences to identify candidate cDNAs encoding proteins of the correct size, with a signal peptide and cysteine pattern characteristic of all insect OBPs

3.3 Materials and methods

Antennae (approximately 1000) of male and oviparous female *M. viciae* were used to construct PCR-directed cDNA libraries using a modified version of Pikielny's protocol (Pikielny *et al*, 1994), as shown in Figure 3.1. Poly (A)+ RNA was extracted from antennae [see 2.2.2.1(b)], and used for first-strand cDNA synthesis [as in 2.2.2.4(a)]. A homopolymeric [poly d(G)] tail was added [see 2.2.2.5], and the cDNA amplified using primer AA2 (see Appendix 1) as described in 2.2.2.6. Purification using MicroSpin™ S-400 columns was done after each stage in the procedure, and the purified PCR products were cloned using one of two methods. In the first method (A), products were digested with *SalI* and *HindIII*, purified using an S-400 column (Amersham Pharmacia Biotech) and ligated into pBluescript II SK (Stratagene), which had been digested to produce complementary

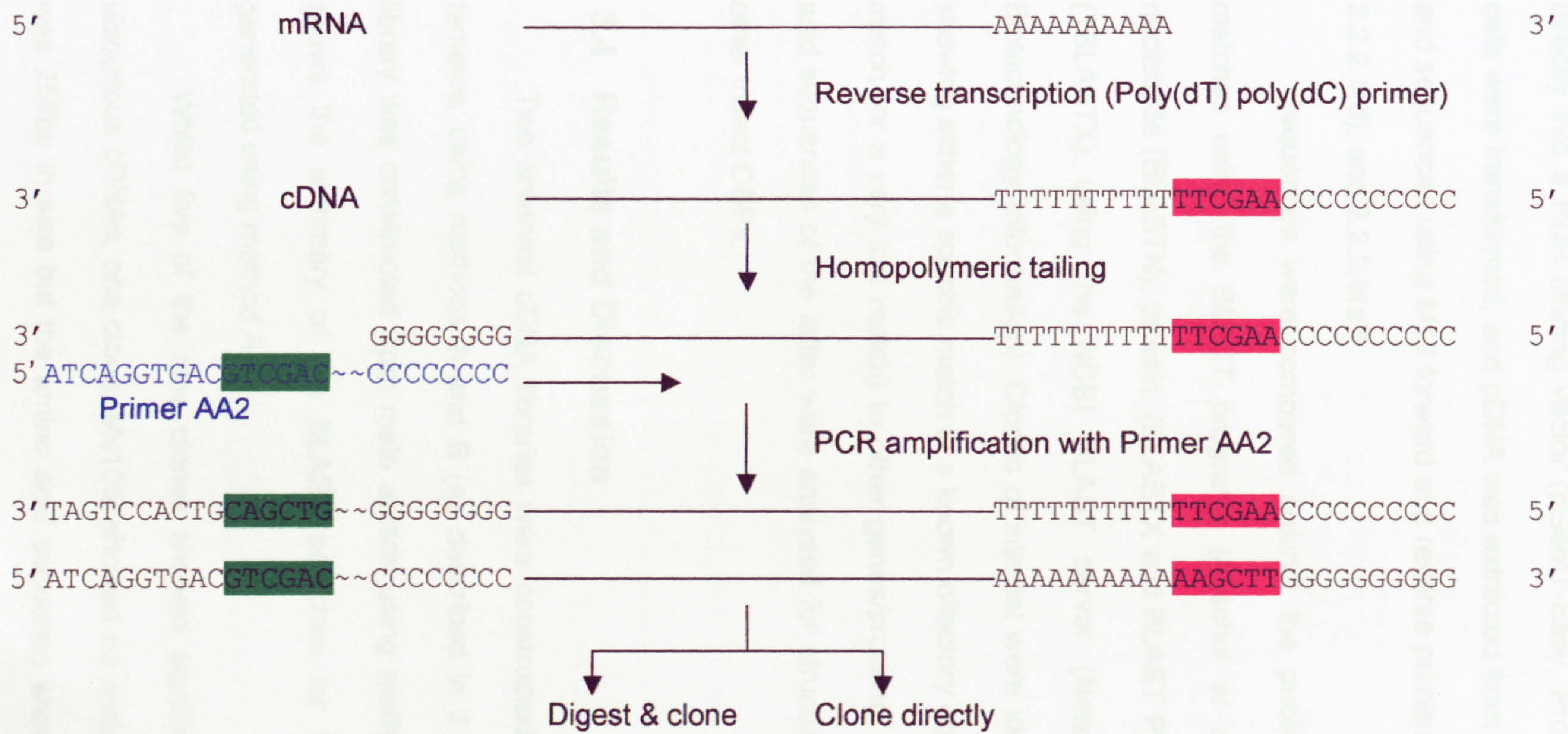


Figure 3.1 Scheme of library construction using a modification of the method of Pikielny *et al* (1994). Restriction sites built into the primers are shown highlighted in green (*SalI*) and red (*HindIII*). Use of a poly(dT)poly(dC) primer for reverse transcription coupled with the homopolymeric tail allows the use of only one primer (AA2) in the PCR steps.

sticky ends. In the second method (B), the products were cloned directly into a PCR cloning vector (pGemT-Easy, Promega). Bacterial cells were transformed, and pDNA was extracted from positive colonies and sequenced using M13 forward and reverse primers [see 2.2.2.2(b), 2.2.2.8(d), and 2.2.2.9(a)].

Sequences were compared against the public databases for matches using the BLAST programs (Altschul *et al*, 1997) at the nucleotide (BLASTN), protein, (BLASTX and BLAST P) and both levels (TBLASTX) using the NCBI BLAST server (National Centre for Biotechnology Information). Clones of interest were identified as those showing either a specific match to a known olfactory gene/protein, or no match (or a very low match) to other genes/proteins. Predicted amino acid sequences of the latter were analysed for structural similarities to other insect OBPs.

3.4 Results and Discussion

Two antennal cDNA libraries were constructed from oviparous females, using methods A and B (as described in 3.3), and a single library was constructed from male aphids using method B. Table 3.2 shows the summary of the BLAST searches for the nine clones generated using method A.

Whilst five of the nine clones showed significant matches to ubiquitous cDNAs, one clone, Mv105, showed no matches. This cDNA was 256bp in size but the amino acid sequence showed none of the features that are characteristic of insect OBPs.

Clone	Match
Mv105	No match
Mv111	100% to actin
Mv149	70-90% to bacterial transposase
Mv164	44% to insect cytochrome P450 enzymes
Mv165	61% to cuticular protein
Mv190	90% to ribonuclear protein
Mv202	Hexulose-6-phosphate synthase
Mv204	92% to actin
Mv209	93% to actin

Table 3.2 Summary of BLAST results on clones produced from female antennae using method A.

One clone of possible relevance to olfaction is Mv164, which shows significant similarity to cytochrome P450 enzymes (most similar to Types 3 and 6) from other insect species [*M. domestica* (44%), *D. melanogaster*, the Southern house mosquito *Culex pipiens quinquefasciatus* (Say; Diptera) *C. capitata*, (all 33%)]. The superfamily of cytochrome P450 enzymes are the terminal oxidase component of the electron transfer system in the endoplasmic reticulum, and are responsible for Phase I metabolism of xenobiotics and other compounds such as steroids, prostaglandins and fatty acids (Gibson & Skett, 1994). It has been reported that in *D. melanogaster*, an NADPH-cytochrome P450 oxidoreductase shows enhanced expression in the antennae and therefore may be involved in odour clearance (Hovemann *et al*, 1997). Furthermore, it has been shown that a cytochrome P450 is involved in degradation of the sex pheromone of the pale brown chafer, *P. diversa* [Wojtasek & Leal (1999a)], and the housefly, *M. domestica* (Ahmad *et al*, 1987). Thus, the P450 cloned here may also have a role in clearance of an odorant molecule. The sequence of Mv164 is shown

in Figure 3.2, and an alignment with other insect P450 enzymes is presented in Figure 3.3. This clone has been investigated further (see Chapter 5).

Unfortunately, this approach gave only nine clones, probably because the restriction sites, built into the primers, were too close to the ends for the restriction enzymes to cut effectively. It is estimated that with the sites positioned as shown in Figure 3.1, there would only have been 10% digestion, hence the low number of recombinant colonies. This, coupled with false positive colonies containing remnants of digestion products that had failed to be removed by selective precipitation of the products, and the loss of material that occurred through the purification procedures that were necessary after every stage in the protocol, led to the very low amount of digested antennal material remaining to be cloned. Even changing the restriction sites to *EcoRI* and *XbaI*, which are supposed to give >90% cleavage with only a few extra bases around the recognition site, failed to improve the number of clones. Purification using spin columns rather than selective precipitation overcame the problem of false positives, but failed to improve the number of recombinant colonies. In this technique, the low amount of starting material from the antennae was always a limiting factor. However, since the antennal cDNAs had been amplified using *Taq* DNA Polymerase, advantage could be taken of the extra adenosine at the 3' end of the products, and the cDNAs could be cloned directly into a PCR cloning vector (method B) without the need for digestion.

```
V   D   R   D   A   G   K   V   S   F   V   H   G   K   L   F
GTC GAC CGG GAC GCG GGC AAG GTG TCC TTC GTC CAC GGC AAG CTG TTC

D   H   L   V   N   L   R   G   E   Q   W   K   A   I   R   A
GAC CAC TTG GTG AAC CTG CGG GGC GAA CAG TGG AAA GCA ATT AGG GCC

K   L   S   P   T   F   S   A   A   K   L   K   S   M   L   G
AAG TTG AGT CCG ACC TTC TCG GCG GCC AAG CTC AAG TCC ATG TTG GGT

D   I   N   V   C   T   A   R   L   I   E   N   L   N   G   C
GAC ATA AAT GTG TGC ACG GCG CGT CTG ATC GAG AAC TTA AAC GGG CAG

T   T   K   N   S   G   I   V   D   V   S   E   A   S   A   Q
ATA ACG AAA AAC AGT GGA ATT GTT GAT GTA AGT GAA GCG TCG GCT CAA

F   T   T   D   T   I   G   S   C   A   F   G   L   H   C   N
TTT ACA ACT GAT ACT ATT GGC AGT TGT GCT TTT GGA CTA CAC TGC AAT

S   L   S   D   P   D   S   E   F   R   R   T   G   R   A   I
TCA TTA TCA GAT CCA GAT TCA GAA TTT CGT CGT ACT GGA CGA GCC ATA

F   T   T   S   L   R   S   T   L   L   N   L   I   R   L   V
TTT ACG ACA AGT CTT CGA TCA ACT TTA CTA AAT CTC ATC AGA TTA GTT

G   F   G   R   L   L   D   V   S   R   I   P   G   M   S   A
GGT TTT GGT CGG CTT TTA GAC GTA TCT AGG ATA CCT GGT ATG TCT GCA

N   I   Y   D   ?   F   *
AAC ATA TAT GAC NTT TTT TGAtaatctactgggagantacantgggaacaacacnag
tctgganaaaataccccttantgatttcctagcactttttagtaaagcttnaagancaaataaa
taacccaagggaaccacgggaccanaatttttttcncnaangaaccttnctcactcgggccaa
ttccnnttcggttatnccttcnggnngnccgggttcca

[                          plus ~50bp                          ]

tcaaganccacacggcagntcttcccatttgnngatgggacctaggaatatgtatcggtttgc
gattcgcgatgatagaagcaaaaacaggactagcggaaatattatcgaaattcgaagtttcac
catgtaaagaaacacaaactccgatcaaaatcaagccgaggtcaattttactcacaccaaacg
aatcgattcgtttatcatttaaaagtattgatcaatgctataacaattaaaatggtattcaag
gataagagttaattcaaaattcaaaagtattaataaattattgtgttattgcggcaaaaaaaa
aaaaaaaaaaa
```

Figure 3.2 Nucleotide and predicted amino acid sequences of Mv164, a cDNA, cloned from the antennae of female *M. viciae*. The putative polyadenylation signal is underlined.

```
      1                                                                                              90
Mdom  MDFGSFLLYA LGVLASLALY FVRWNFGYWK RRGIPHEEPH LVMGNV.KGL RSKYHIGEII ADYYRKFKGS DPLPGIFLGH KPAAVVLDKE
Dmel  MGVYSVLLAI VVVLVGYLLL KWRRALHYWQ NLDIPCEEPH ILMGSL.TGV QTSRSFSAIW MDYYNKFRGT GPFAGFYWFQ RPGILVLDIS
Cpip  MFAWIICAAA AVPLVYFLIV Y...QFSYWK RRGITQLTPS FPFGDLGPFF RQRSSLGVVY ADVYRLCKRL .PFVGIYLSL RPMLVVNDPE
Hvir  ~~~MILLLTW LVVIITAVLL YFRSVYSQLS KQGVNHLPTI PVFGNLMWMV MKQEHFVDTL GRCVKAFPD. DKIVGHYDMV SPILVVLDVD
Msex  ~~~MFVIIAI FALFLCLIHI LFNNNKKARL LKQIPGSKYN FIIGNALDFL KSPEQLFYFM REYYETWKPL NRFWAFQIAF VNVYEPHDIE
Mvic  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

      91                                                                                            180
Mdom  LRKRVLIKDF SNFANRGL.Y YNEKDDPLTG H.LVMVEGEK WRSLRTKLSP TFTAGKMKYM YN...TV.LE VGQRLLEVMY EKLEVSSELD
Dmel  LAKLILIKEF NKFTDRGF.Y HNTEDDPLSG Q.LFLLDGQK WKSMRSKLSS TFTSGKMKYM FP...TV.VK VGHEFIEVFG QAMEKSPIVE
Cpip  LIKNVLVRDF DHFHDRGL.Y VNEEKDPLSG H.LFALGGEQ WRHHRSKLTP TFTSGRLKEM FT...NL.VQ IGRVLQD..H VAKRAGEDIE
Hvir  TVKRITVKDF EHFVDR..RS FTSSFDPIFG RGLLLLHGDE WKAMRSTMSP AFTSSKMRLM VPFMEEIALE MIRVLRGKIK DSGKPYIDVE
Msex  ....VVISST KHNAKSPPYY FLKN...WLR DGLLLSKGPK WQSRRKILTP TFHFNILRQF CGILED...N SERLVQNVGK SLGKP...VN
Mvic  ~~~~~~~~~~ ~~~VDRDAGK VSFVHGKLFD H.LVNLRGEQ WKAIRAKLSP TFSAAKLKSM LG...DINVC TARLIENLNG CTTKNSGIVD

      181                                                                                           270
Mdom  MRDILARFNT DVIGSVAFGI ECNSLRNPHD RFLAMGRKSI EVP.RHNALI M.AFIDSFPE LSRKLGMRVL PEDVHQFFMS SIKETVDYRE
Dmel  VRDILARFTT DVIGTCAFGI ECSSLKDPEA EFRVMGRRAI FEQ.RHGPIG I.AFINSFQN LARRLHMKIT LEEAEHFFLR IVRETVAFRE
Cpip  IRDVMARYTT DIIASVGFGI ENDSINEKGN IFREMGTKVF SPD.LKTILR L.TSTFFTPK LNALFGFKFI AQEIEDFIMN VVRETLEYRE
Hvir  AKSMMTRYAN DVIASCAFGL KVNSQASDHE FY..VNSQAI TKFKFSAFLK V.LFFRCLPS VAQKLKMSLV PRECSDYFSN VVLTTMKDRE
Msex  IIPTISEYTL YSICETAMGS RLGEESKESQ KSYKQSICAL GRQFVYRITR IYLHSDFIYN LITFGKVKKD LDVVHNFTTK VIKDRKEYVE
Mvic  VSEASAQFTT DTIGSCAFGL HCNSLSDPDS EFRRTGRAIF TTSLRSTLIN L.IRLVGFGR LLDVSRIPGM SANIYDXF~~ ~~~~~~~~~~

      271                                                                                           360
Mdom  KNNIRRNDFL DLVLDLKNNP E.......S. .......... .ISKLG...G LTFNELAAQV FVFFLGGFET SSSTMGFALY ELAQNQQLQD
Dmel  KNNIRRNDFM DQLIDLKNSP L.......T. .......... .KSESGESVN LTIEEMAAQA FVFFGAGFET SSTTMGFALY ELAQHQDIQD
Cpip  SNKVVRKDMM QLLMQLRNSG TVSIDDRWD. .......... .IEVSTNKKK LSLEQVTAHA FVFFIAAYET SSTTISFCLF ELARNPEIQK
Hvir  KNKVVRNDLI NILMEVKKGQ LTHEKDDADA DAGFATVEES HIGRKQHNYE WTDSDLIAQA ALFLFAGFDT VSTSMSFLLY ELAVNPDVQD
Msex  KYGINMFGVS DI...DDNNV YKKNKKIAML DLLITAQKEG FIDDIG.... .....IQEEV DTFMFEGHDT IALALTYTLM LLANHRSIQH
Mvic  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
```

Figure 3.3 [illegible] *melanogaster* (Dmel; [illegible]) and *M. sexta* (Msex; Snyder *et al.*, 1995) with that cloned from *M. viciae* (Mvic). Residues conserved between all six species are highlighted in yellow. Residues in common between *M. viciae* and at least one other species are highlighted in green.

```
       361                                                                                                         430
Mdom   RLREEVNEVF DQFKEDNISY DALMNIPYLD QVLNETLRKY PVGVGSALTR QTLNDYVV.. ...PHNPKYV LPKGTLVFIP VLGIHYDPEL
Dmel   RVRKECQEVI GKYNGE.ITY ESMKDMVYLD QVISETLRLY T..VLPDLNR ECLEDYEV.. ...PGHPKYV IKKGMPVLIP CGAMHRDEKL
Cpip   KVQQEIDQVL ASHNGE.ITY DNINEMKYLE NCIDETLRKY P..AVPFLNR ECSKDYKI.. ...PGTDT.T IEKGTSLVIP VLGLHRDPDH
Hvir   RLLQEIRE.Y DEKNHGKIDY NVVQSMTYLD MVVSEGLRLW PPAAV..VDR VCVKDYNIGR PNKKATKDLI IHTGQAVAIS PWLFHRNPKF
Msex   TVIAEIDEIF GD.SERQADL DDLSKMRYLE RCIKESLRLY PP..VPAIGR LLSEDVTLSG ........YR VPEGAYCHIQ CFDLHRRGDL
Mvic   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

       431                                                                                                         500
Mdom   YPNPEEFDPE RFSPEMVKQR DSVDWLGFGD GPRNCIGMRF GKMQSRLGLA LVIRHFRFTV CSRTDIPMQI NPESLAWTPK NNLYLNVQAI
Dmel   YANPNTFNPI FFA.RTSEGS DSVEWLPFGD GPRLCIGMRF GQMQARSGLA LLINRFKFSV CEQTTIPIVY SKKTFLISSE TGIFLKVERV
Cpip   YPEPDRFIPE RFS..NFEDI STKPYLPFGA GPRNCIGLRL GKLQTKAGLV MMLSKFNVRL ADET.....Y ASKELALDAR SVVLMPVGGI
Hvir   FPEPAKFDPE RFSPENRHKI LPFTYFSFCL GPRNCIGSRF ALCEIKVILY LLIREMEVYP FEKTIYPPQL SKDRFNMHLE GGAWVRLRVR
Msex   YKDPLVFDPD RFLPENCSDR HPYAYIPFSA GPRNCIGQKF AILEMKSAIS SLLRHYELLP VTKPE.DLKF TADLVLRTTN PVYVKFVKKE
Mvic   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

       510
Mdom   RKKIK~~~~~
Dmel   ~~~~~~~~~~
Cpip   KVSISERRAS
Hvir   PEKS~~~~~~
Msex   KNK~~~~~~~
Mvic   ~~~~~~~~~~
```

Figure 3.3 Alignment of cytochrome P450 amino acid sequences from *M. domestica* (Mdom; Feyereisen *et al*, 1989), *D. melanogaster* (Dmel; Maitra *et al*, 1996), *C. pipiens quinquefasciatus* (Cpip; Kasai *et al*, 2000), *H. virescens* (Hvir; Rose *et al*, 1997) and *M. sexta* (Msex; Snyder *et al*, 1995) with that cloned from *M. viciae* (Mvic). Residues conserved between all six species are highlighted in yellow. Residues in common between *M. viciae* and at least one other species are highlighted in green.

However, with this technique, problems arose initially because of secondary structure resulting from annealing between the poly(dC) tail at the 5' end and the poly(dG) tail at the 3' end of the amplified cDNAs (see Figure 3.1) that had not been removed by digestion. This made the recombinant plasmids impossible to sequence. However, the problem was overcome by alteration of the cycle sequencing parameters [as in 2.2.2.8(a)] and using a capillary-based sequencer, which operates at a higher running temperature than a plate-based sequencer. With these modifications, 97 and 95 clones were sequenced from male and oviparous female libraries respectively. The results of the BLAST searches on the clones obtained are shown in Table 3.3.

Match	**Percentage of clones showing match**	
	Female	Male
Ribosomal RNA genes (16, 18, 23 & 28S)	23.16	61.05
Actin	18.95	-
Ribosomal proteins	3.17	-
Other	21.07	15.79
Low match (>25bp)	22.05	11.58
No match	11.60	11.58

Table 3.3 Summary of BLAST results on clones sequenced from female and male antennal cDNA libraries (Method B).

A high proportion of clones in the male library were ribosomal RNA genes, possibly because the cDNA required a secondary PCR amplification, which may have preferentially amplified the abundant messages. An alternative possibility is that the PCR amplified shorter messages, or those without the potential for secondary structure, and as such, the cDNA libraries constructed by PCR may not represent the

complete pool of mRNA molecules (Lönneborg *et al*, 1995). Thus, the female library, where there was no need for a secondary amplification, is probably a more accurate portrayal of the antennal cDNA population, and this is also reflected in the spread of cDNAs that are found in this library but not in the male one. A large percentage of the female library was actin cDNAs. Genes, such as actin, that constitute cytoskeletal elements, are very often duplicated genes (Darnell *et al*, 1990), so this high proportion is not surprising. Table 3.3 shows a large proportion of the clones in both libraries with little or no match to sequences in the database, implying that these cDNAs are either novel or specific to *M. viciae*.

Table 3.4 expands on the cDNAs that showed matches grouped in the "other" category of Table 3.3. There are a variety of cDNAs, many of which would be expected to be expressed in the antennae, e.g. the metabolic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase, as well as the more structural cDNAs, e.g. those showing matches to myosin and cuticular proteins. It is interesting, and perhaps somewhat reassuring, to note that nine of the fifteen cDNAs in this table (P25/132, P32, P73/77, P57/71, P112/128, P124, M7/M75, M43 and M80) are matches to expressed sequence tags (ESTs) from the antennae of male *M. sexta* (Robertson *et al*, 1999), showing that there can be some conservation of gene sequences across quite distantly-related insect species.

There were no clones in any of these libraries that showed a direct match to any genes known to be involved in olfaction, with the

Clone(s)	Antennal source	Shows match to
P124	F	β-tubulin
P25, P132	F	Farnesoic acid O-methyl transferase
P32	F	Fructose bisphosphate aldolase
P112, P128	F	Cuticular proteins
P48, P73, P77	F	ATP-binding protein
P57, P71	F	Glyceraldehyde-3-phosphate dehydrogenase
P89, P111	F	Myosin
P90	F	Glutaryl coenzyme A dehydrogenase
P73, P77	F	ATP synthase
M7, M75	M	UDP glucosyl transferase
M43	M	Heat shock protein
M45, M69, M77, M79, M89, M92, M93	M	Platelet glycoprotein
M64, M86	M	Cold stress protein
M80	M	Cytochrome P450
M99	M	Tyrosine phosphorylation-regulated protein kinase

Table 3.4 BLAST results of clones in "Other" category from male (M) and female (F) antennal cDNA libraries (Method B).

exception of the cytochrome P450 discussed earlier, and possibly the cytochrome P450 (M80) and the UDP-glucosyl transferases (M7/75) shown in Table 3.4. M80 is the same cytochrome P450 as Mv164 discussed earlier, and the UDP- glucosyl-transferases, which are involved in Phase II xenobiotic metabolism, may also have a role in detoxification of odorant molecules, as is the case in *D. melanogaster* (Wang *et al*, 1999).

Of the clones that showed no match, or a very low match, one

clone, P59, gave a predicted amino acid sequence with features clearly characteristic of insect OBPs, and this has been termed MvicOBP1. The sequence is presented in Figure 3.4, which shows six conserved cysteine residues (highlighted). Figure 3.5 shows the cysteine residues of MvicOBP1 in an alignment with other insect OBPs; this alignment was constructed using ClustalX (Thompson *et al*, 1997) and manually adjusted. The percentage identity of MvicOBP1 with other representative insect OBPs is 32-44% with other insect OBPs (see Figure 3.6), which is in line with that between other unrelated species. A Kyte and Doolittle hydropathy plot (Kyte and Doolittle, 1982) of the amino acid sequence of MvicOBP1 is compared with other insect OBPs in Figure 3.7, showing that MvicOBP1 possesses a major hydrophobic domain (residues 60 to 90), characteristic of insect OBPs. Interestingly, the profile shows the majority of the C-terminal half of the protein being hydrophobic, as for the non-lepidopteran insects [(b) and (c)], rather than having two distinct hydrophobic domains, which is the case with BmorPBP1 [(a)]. MvicOBP1 (P59) has also been investigated further (see Chapter 5).

3.5 Conclusions

After initial technical problems, antennal cDNA libraries were successfully produced from both male and oviparous female aphids. The direct cloning of amplified cDNAs was more successful, in terms of the number of clones generated, than the method using restriction sites built into the primers. This approach yielded two interesting clones, P59,

```
D   H   T   T   S   S   I   H   I   D   Q   V   E   V   C   R
GAT CAC ACT ACA TCA AGT ATA CAT ATC GAC CAG GTT GAA GTT TGC AGA

F   V   V   D   N   T   R   R   N   H   Q   T   R   N   G   F
TTC GTT GTA GAC AAT ACA AGA AGA AAT CAC CAA ACA CGA AAT GGC TTC

F   Q   V   S   D   I   H   H   L   G   R   V   L   R   C   R
TTT CAA GTA TCT GAT ATT CAT CAC CTT GGT CGT GTA CTC CGT TGT CGC

R   R   R   C   K   S   K   E   T   C   L   P   R   S   C   P
CGA AGA AGG TGT AAG AGC AAA GAA ACA TGC CTA CCT CGC AGC TGC CCC

S   S   G   F   G   L   F   C   L   S   P   W   F   V   L   I
AGC TCC GGT TTT GGG TTA TTC TGC CTC AGC CCC TGG TTC GTT CTC ATA

C   I   L   R   L   H   Q   I   S   V   Q   L   S   S   I   R
TGC ATA CTC CGA TTA CAC CAG ATA TCC GTA CAG CTA TCC AGT ATT AGG

S   C   S   I   P   C   T   R   S   I   P   S   S   S   V   P
AGC TGC AGC ATA CCA TGC ACC CGC AGC ATA CCC AGT AGC AGC GTA CCC

S   C   S   F   C   H   P   R   I   P   C   N   L   P   R   R
AGC TGC AGC TTT TGC CAC CCA CGC ATA CCC TGC AAT CTA CCA CGA AGA

*
TGAcggcaaatactggcccggcaaatacgaaaaggcctacatcccagcttacaaagccggata
cccgagctatcacgaagatgacggccagtattggcccggaaaatac
```

Figure 3.4 Nucleotide and predicted amino acid sequence of MvicOBP1 (P59), a cDNA cloned from the antennae of female *M. viciae*. Cysteines showing conservation in spacing pattern with other insect OBPs are highlighted in red.

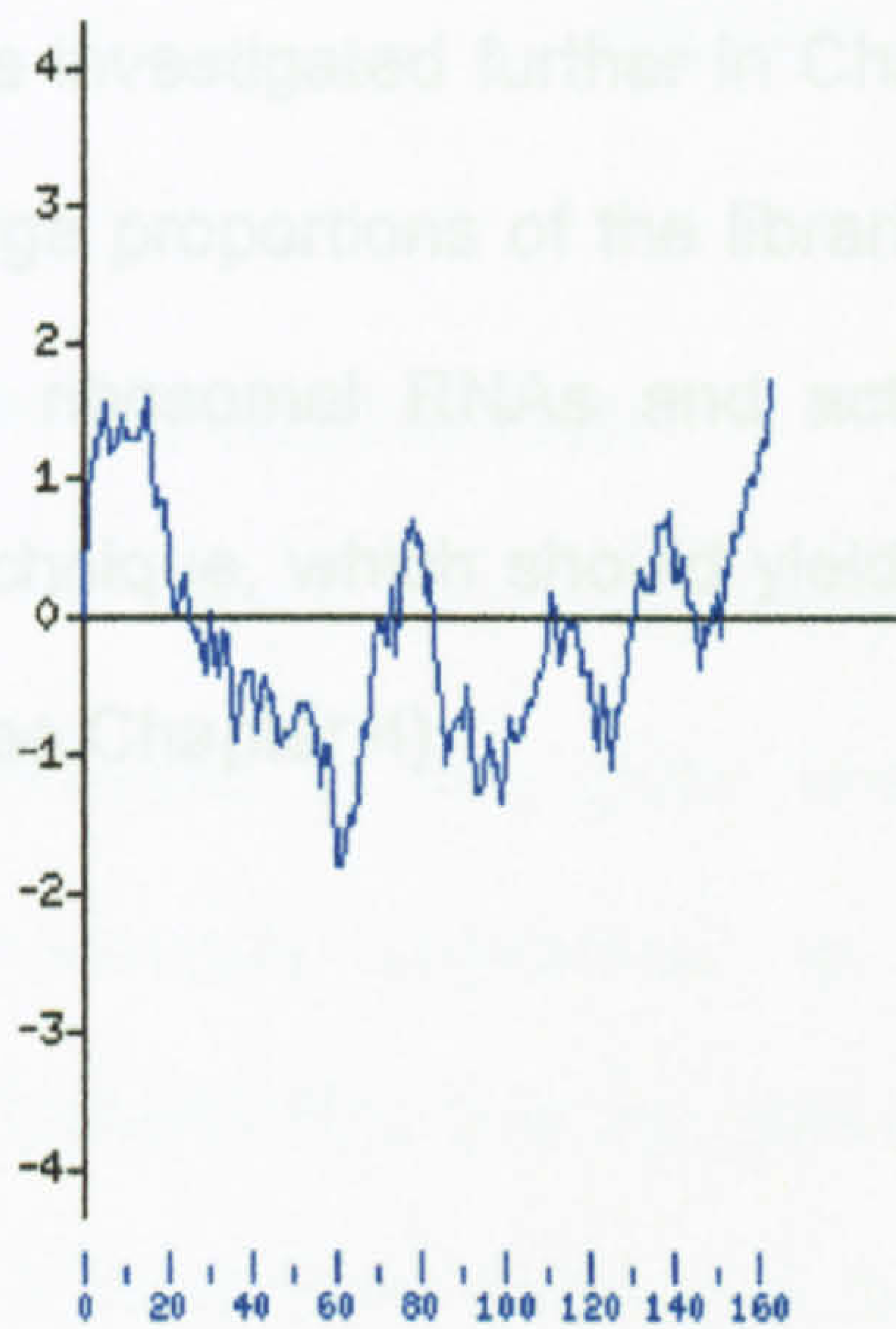
```
             1                                                                                                   90
DmelOS-E    MLKYPLI..L LLIGCAAAQE PRRDGEWPPP AILKLGKHFH DICAPKTGVT DEAIKEF.SD GQ...IHEDE ....AL.KCY MN.CLFH.EF
PjapPBP     .......... .......... .......MSE EMEELAKQLH DDCVSQTGVD EAHITTV.KD QKG.FPD.DE ....KF.KCY LK.CLMT.EM
LlinLAP     MRILVLFTAA LTCVMAG... ......ELPE EMREMAQGLH DGCVEETGVD NGLIGPCA.. .KGNFAD.DQ ....KL.KCY FK.CVFG.NL
BmorGOBP2   ......MWKL VVVLTVNLLQ GALTDVYVMK DVTLGFGQAL EQCREESQLT EEKMEEFFHF WNDDFKF.E. H...RELGCA IQ.CMS.RHF
AperPBP1    ..MLGKISL. LLLPVFVAIN LVHSSPEIIK NLSQNFCKAM DQCKQELNIP DSVIADLYNF WKDDYVMTD. ....RLAGCA IN.CMATKLD
MvicOBP1    .......... .......... .......DHT TSSIHIDQ.V EVCRFVVDNT R.RNHQTRNG F...FQVSDI HHLGRVLRCR RRRCKS.KET

             91                                                                                                  180
DmelOS-E    EVVDDNGDVH MEKVLN.AIP G....EKLRN I..MMEASKG C...IH..PE G.DTL.CHKA WWFHQCW.KK ADPVH..... .YFLV~~~~~
PjapPBP     AIVGDDGVVD VEAAVG.VLP .....DELKA K..AEPIMRK C...GF..KP G.ANP.CDNV YQTHKCY.YE TDAQ...S.. .YMIV~~~~~
LlinLAP     GVISDEGELD AEAFGS.ILP .....DNM.Q E..LLPTIRG C..AG...TT G.ADP.CELA MNFNKCL.QK VDPV...N.. .FMVI~~~~~
BmorGOBP2   NLLTDSSRMH HENTDKFIKS FPNGEI.LSQ K..MIDMIHT CEKTFDS... E.PDH.CWRI LRVAECFKDA CNKSGLAPSM ELILAEFIME
AperPBP1    .VVDPDGNLH HGNAKEFAMK HGA.DASMAQ Q..LVDIIHG CEKSAP..PN ..DDK.CMKT IDVAMCFKKE IHKLNWVPDM DVVLGEVLAE
MvicOBP1    CLPRSCPSSG FGLFCLSP.W FVLICILRLH QIVQLSSIRS CSIPCTRSIP SSSVPSCSFC HPRIPCNLPR R~~~~~~~~~ ~~~~~~~~~~

             181
DmelOS-E    ~~~~~
PjapPBP     ~~~~~
LlinLAP     ~~~~~
BmorGOBP2   SEADK
AperPBP1    V~~~~
MvicOBP1    ~~~~~
```

Figure 3.5 Multiple alignment of representative insect OBPs with MvicOBP1 (P59). Conserved cysteine residues are shown in red. DmelOS-E: *D. melanogaster* OS-E (McKenna *et al*, 1994); PjapPBP: *P. japonica* PBP (Wojtasek *et al*, 1998); LlinLAP: *L. lineolaris* LAP (Vogt *et al*, 1999); BmorGOBP2: *B. mori* GOBP2 (Krieger *et al*, 1996); AperPBP1: *A. pernyi* PBP1 (Breer *et al*, 1990).

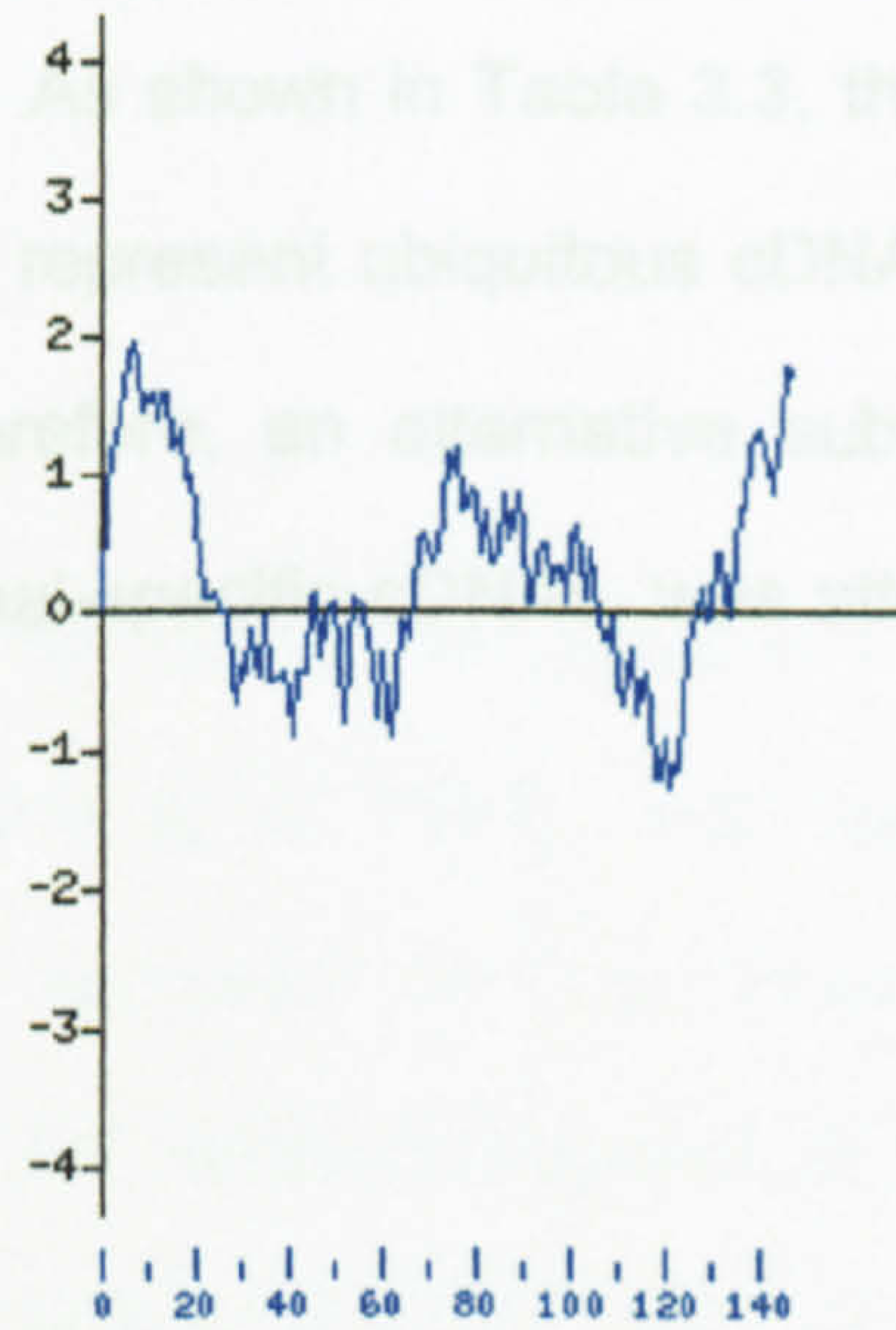
AperPBP1							
42.7	LlinLAP						
37.4	44.4	BmorGOBP2					
36.5	39.1	75.1	HvirGOBP1				
38.8	47.1	40.2	32.7	PjapPBP			
39.5	46.5	43.5	46.1	43.3	Dmel OS-E		
37.8	41.4	45.1	39.8	41.8	42.2	PregCRLBPpr	
36.7	38.4	39.2	42.8	32.0	44.1	33.7	MvicOBP1

Figure 3.6 Identity matrix of MvicOBP1 with other insect OBPs. AperPBP1 = *A. pernyi* PBP1 (Raming *et al*, 1990); LlinLAP = *L. lineolaris* LAP (Vogt *et al*, 1999); BmorGOBP2 = *B. mori* GOBP2 (Krieger *et al*, 1996); HvirGOBP1 = *H. virescens* GOBP1 (Krieger *et al*, 1993); PjapPBP = *P. japonica* PBP (Wojtasek *et al*, 1998); Dmel OS-E = *D. melanogaster* OS-E (McKenna *et al*, 1994); PregCRLBPpr = *P. regina* CLBRPpr (Ozaki *et al*, 1995). Identities are shown at the amino acid level.

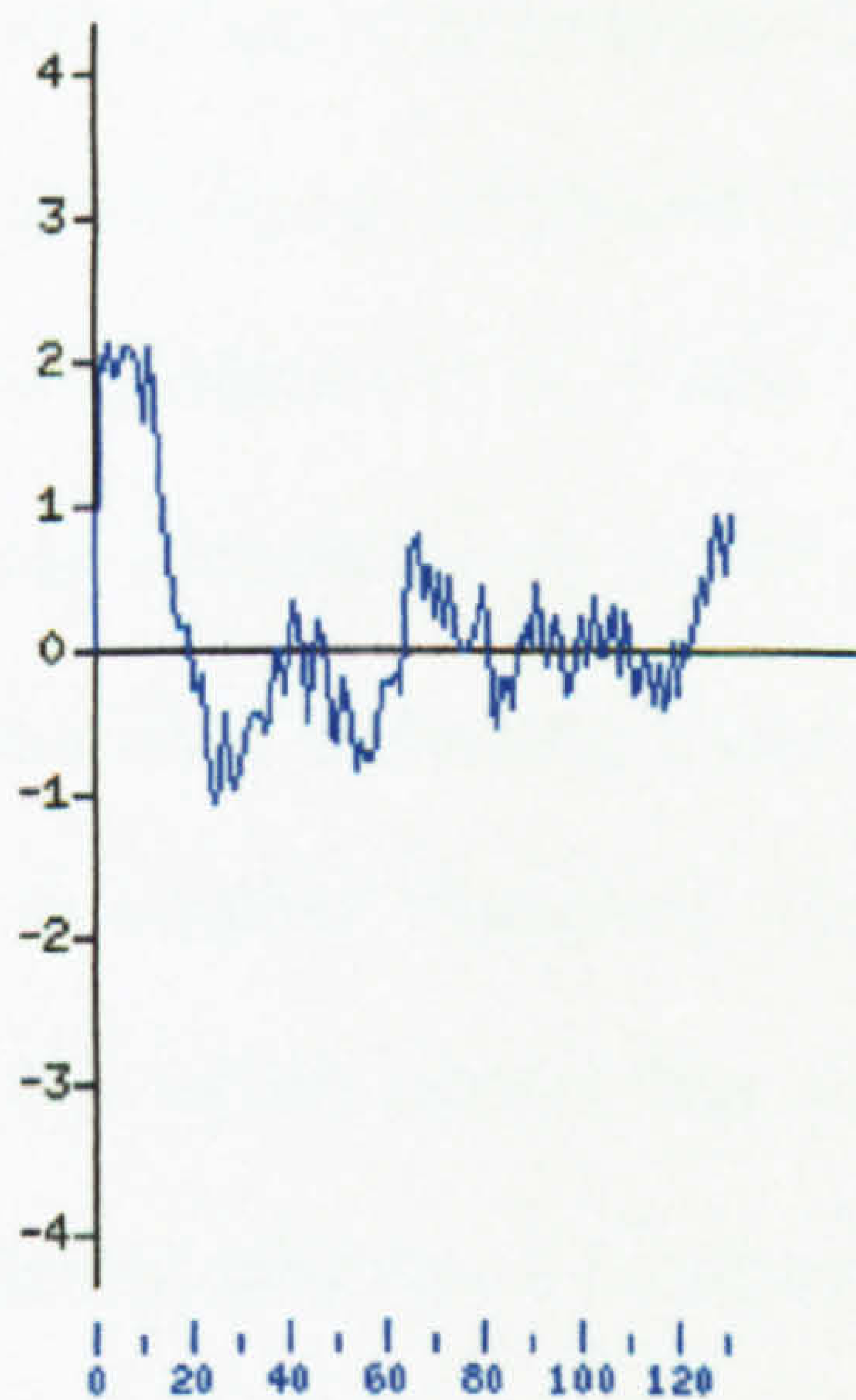
(a) BmorPBP1



(b) DmelPBPRP1



(c) LlinLAP



(d) MvicOBP1

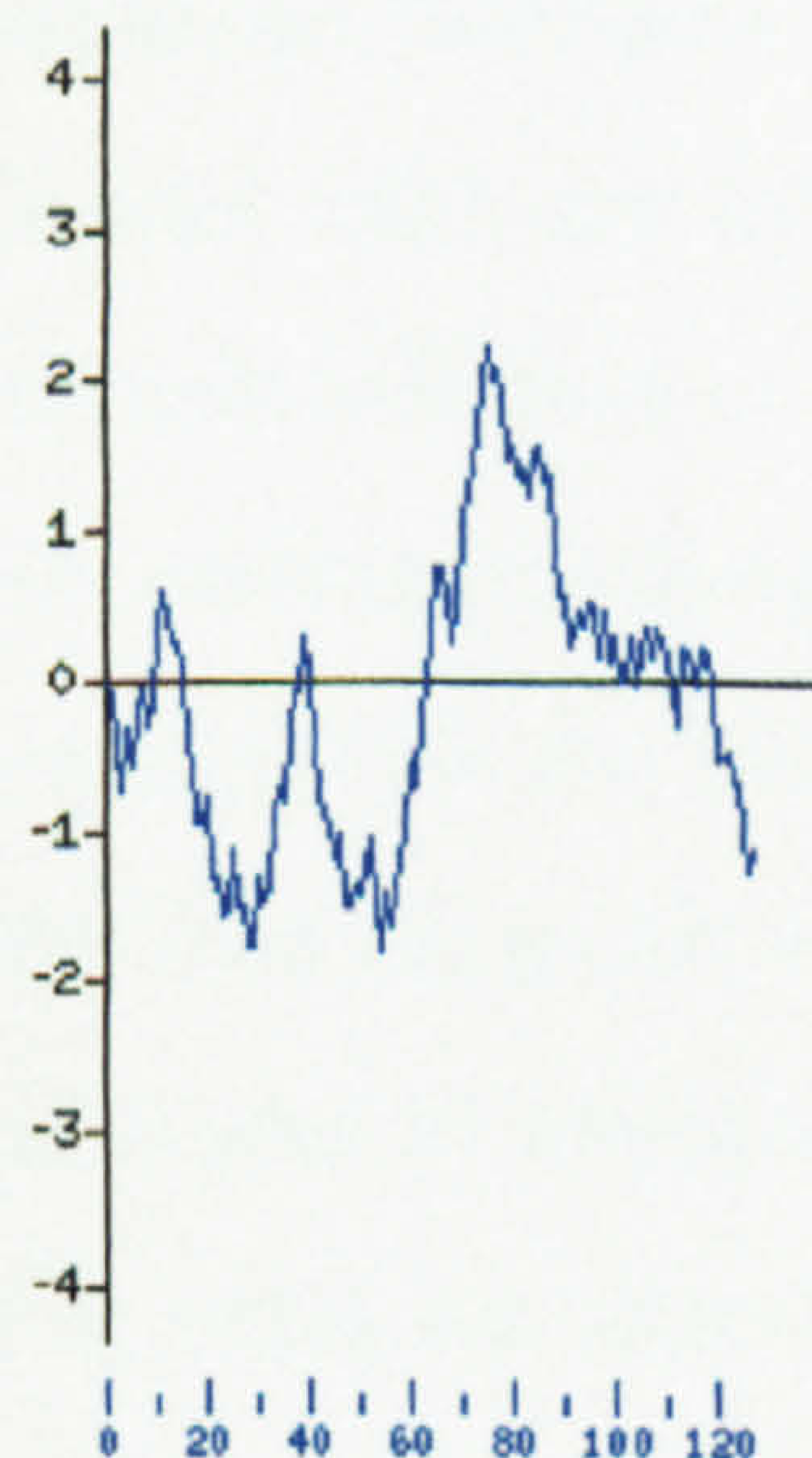


Figure 3.7 Kyte and Doolittle hydropathy plots of insect OBPs. BmorPBP1 = *B. mori* PBP1 (Krieger *et al*, 1996); DmelPBPRP1 = *D. melanogaster* PBPRP1 (Pikielny *et al*, 1994); LlinLAP = *L. lineolaris* LAP (Vogt *et al*, 1999). Positive values indicate hydrophobic amino acids. Window size = 17.

encoding a putative OBP, MvicOBP1, and Mv164, encoding a cytochrome P450 enzyme. Both have a potential role in olfaction, and are investigated further in Chapter 5. As shown in Table 3.3, there are large proportions of the libraries that represent ubiquitous cDNAs such as ribosomal RNAs and actin. Therefore, an alternative subtraction technique, which should yield antennal-specific cDNAs, was attempted (see Chapter 4).

Chapter 4. Construction and analyses of subtracted cDNA libraries from *M. viciae*

4.1 Introduction

Only a small fraction, perhaps 10–15%, of the genes in a cell are expressed at any given time (Alberts *et al*, 1994), and cloning of differentially expressed genes is achieved by the isolation of mRNAs/cDNAs that are present in a test sample but absent, or present at a much lower level, in a reference sample (Coche, 1997). A number of techniques have been developed and used to identify such genes (Swaroop *et al*, 1991), and also to characterise quantitative changes in levels of gene expression during development (Schneider *et al*, 1988), in pathological situations (Duguid & Dinauer, 1990) and during inductive events (Nedivi *et al*, 1993). These techniques include (i) +/- screening of cDNA libraries, with cDNA derived from mRNAs containing the desired sequences detecting those mRNAs present (+) in one cell and absent (-) in another (Sargent, 1987); (ii) differential display RT-PCR (DDRT-PCR), which allows the simultaneous display of differences between different cell/tissue sources (Bauer *et al*, 1994), although this technique is biased towards the more abundant messages (Bertioli *et al*, 1995); (iii) representational difference analysis (RDA) which results in high levels of enrichment of sequences occurring only in the test samples (Lisitsyn *et al*, 1993); (iv) g/cDNA AFLPs (Amplified Fragment Length Polymorphism), which produces a “fingerprint” that is characteristic of a

specific cell or tissue (Vos *et al*, 1995; Bachem *et al*, 1996); and (v) constructing cDNA libraries after subtractive hybridisation.

The principle behind subtractive hybridisation is to combine the target (or "tester") cDNAs with an excess (10- to 30-fold) of reference or "driver" sequences [so-called because the excess drives the reaction (Coche, 1997)], and to allow the subsequent hybridisation in aqueous solution to "soak up" the sequences that are common to both sources. The tester sequences that have not hybridised to the driver are then isolated, either by hydroxyapatite chromatography of unhybridised tester (Stürzl & Roth, 1990), or the use of magnetic beads to remove tester-driver hybrids (Aasheim *et al*, 1998), and cloned to produce subtracted libraries.

The advantages of this technique are the enrichment of differentially expressed sequences at each round of subtraction, and the sensitivity, with mRNAs representing as little as 0.01% of the total population being identifiable (Sargent, 1987). However, there are disadvantages in that subtractive hybridisation is time-consuming, labour-intensive, and technically demanding, and most importantly, it requires a large amount of starting material [0.5μg (Sargent, 1987) to 60μg (Kriegler, 1990) of tester mRNA, and 10μg (Sargent) to 320μg (Kriegler, 1990) – at least a 10-fold excess - of driver mRNA].

The technique used for the cloning of putative OBPs from *D. melanogaster* adapted subtractive hybridisation for use with small quantities of starting material by introducing an anchored PCR amplification of the tester and driver cDNAs prior to subtraction (Pikielny

et al, 1994). The OBPs were in fact cloned from subtracted libraries generated from a subtraction between only ten antennae and a few heads. This method was therefore chosen for the present study. A commercial alternative, the Clontech PCR-Select™ cDNA Subtraction Kit, was also used. This is a suppression-subtractive method that is loosely based on the RDA protocol of Lisitsyn *et al* (1993), and couples the suppression of amplification of non-target cDNA with the exponential amplification of tester-tester hybrids, giving over 1000-fold enrichment of differentially expressed cDNAs in a model system (Diatchenko *et al*, 1996). Normalization of the cDNA populations enables the isolation of rare transcripts as well as more abundant ones (Gurskaya *et al*, 1996).

4.2 Aims of the study

Genes that are differentially expressed in the antennae of *M. viciae* were considered likely to be involved in olfaction, therefore the aims of this study were

- To construct subtracted libraries (whole aphid with antennae minus whole aphid without antennae, and antennae minus heads subtractions), which contained antennal specific cDNAs, from both male and female aphids
- To sequence clones and determine if any have sequence matches in the public databases
- To use the predicted amino acid sequences to identify candidate cDNAs encoding proteins of the correct size, with a signal

peptide and cysteine pattern that are characteristic of all insect OBPs

4.3 Materials and Methods

Subtractive hybridisations were done using two methods, A and B. In method A, subtracted cDNAs were generated by the method of Pikielny *et al* (1994) with the modifications described in 2.2.2.7, whilst in method B, the subtractions were done according to the manufacturer's instructions for the PCR-Select™ cDNA Subtraction Kit (Clontech).

Subtracted cDNAs were either purified using spin columns (MicroSpin™, Amersham Pharmacia Biotech) and digested with the appropriate restriction enzymes for ligation into pBluescript II SK vector, or cloned directly into a PCR cloning vector, pGEM T-Easy (Promega), following amplification with *Taq* DNA Polymerase. Bacterial cells were transformed, and pDNAs purified and sequenced using M13 forward and reverse primers.

Sequences were compared with the public databases as described in Chapter 3.3 (Altschul *et al*, 1997). The nucleotide sequences of candidate olfactory cDNAs (determined as those showing no match or a low match to any other gDNA/cDNA/protein) were translated, and their amino acid sequences examined for the presence of the characteristics of insect OBPs.

4.4 Results and Discussion

4.4.1 Analyses of subtracted cDNA libraries

Four subtracted libraries were generated: (1) whole female aphid with antennae minus whole female aphid without antennae, by method A; (2) the same library, by method B: (3) oviparous female antennae minus heads, by method B; and (4) male antennae minus heads, by method B.

4.4.1.1Library 1

Library 1 contained only eight clones, generated after one round of subtraction, and Table 4.1 shows the results of the BLAST searches on these sequences.

Clone	Match to
Mv12	44% to hypothetical protein
Mv16	92% to putative sulfatase
Mv18	99% to bacterial transposase
Mv25	No match
Mv28	62% to aldehyde dehydrogenase
Mv42	40% to transcriptional activator
Mv50	37% to c-abl protein
Mv77	56% to hypothetical protein

Table 4.1 Results of BLAST searches for clones in Library 1. Matches are shown as percentage of similar amino acid residues.

Seven cDNAs showed significant similarity to other genes or proteins with no obvious connection with olfaction, whilst the eighth clone, Mv25, was a cDNA of approximately 150bp, and contained the poly(A) tail, which suggests that the sequence is mainly 3' UTR (untranslated region), and possibly explains why no matches were found in the main databases.

The number of clones in this library was much lower than expected, and subsequent rounds of subtraction yielded nothing further. As with the construction of antennal cDNA libraries (Chapter 3.4), using the restriction sites within the primers to produce clonable fragments caused a major problem, with insufficient cleavage of the subtracted products, coupled with the loss of material during purification, leading to a very small amount of material remaining to be cloned.

4.4.1.2 Library 2

The construction of this library using the PCR Select™ cDNA Subtraction kit gave many more clones, of which eighty-nine were sequenced. Table 4.2 shows the results of the BLAST searches on these cDNAs.

Match	Percentage of clones showing match
Ribosomal genes (16, 18, 23 & 28S)	20.1
Buchnera aphidicola shikimate dehydrogenase gene	30.6
Other	8.2
Low match (< 25bp)	25.9
No match	15.3

Table 4.2 Summary of results of BLAST searches for clones generated for Library 2. Matches are shown in terms of percentage of similar amino acid residues.

The "other" category included ribosomal proteins (59-76% similarity to *D. melanogaster*), protein disulfide isomerase (45% similarity to *Caenorhabditis elegans*), keratin (31% similarity to *Homo sapiens*), tubulin $\gamma-$ chain (40% to *Saccharomyces cerevisiae*), and

calmodulin (81% similarity to *Achyla klebsiana*). Calmodulin is a calcium binding protein involved in stimulating a number of enzymes, such as adenylate cyclase and cAMP phosphodiesterase in second messenger pathways (Barritt, 1992), and could therefore be involved in the olfactory signal transduction pathway in the antennae. In the moth *H. virescens* it has been shown that IP_3 is involved as a second messenger in the olfactory response pathway to pheromones (Boekhoff *et al*, 1993), and calmodulin could be involved in a similar pathway in aphids. To establish this would require considerable further investigation, and since it is possible that the calmodulin came from another pathway, elsewhere in this aphid, this was not studied further.

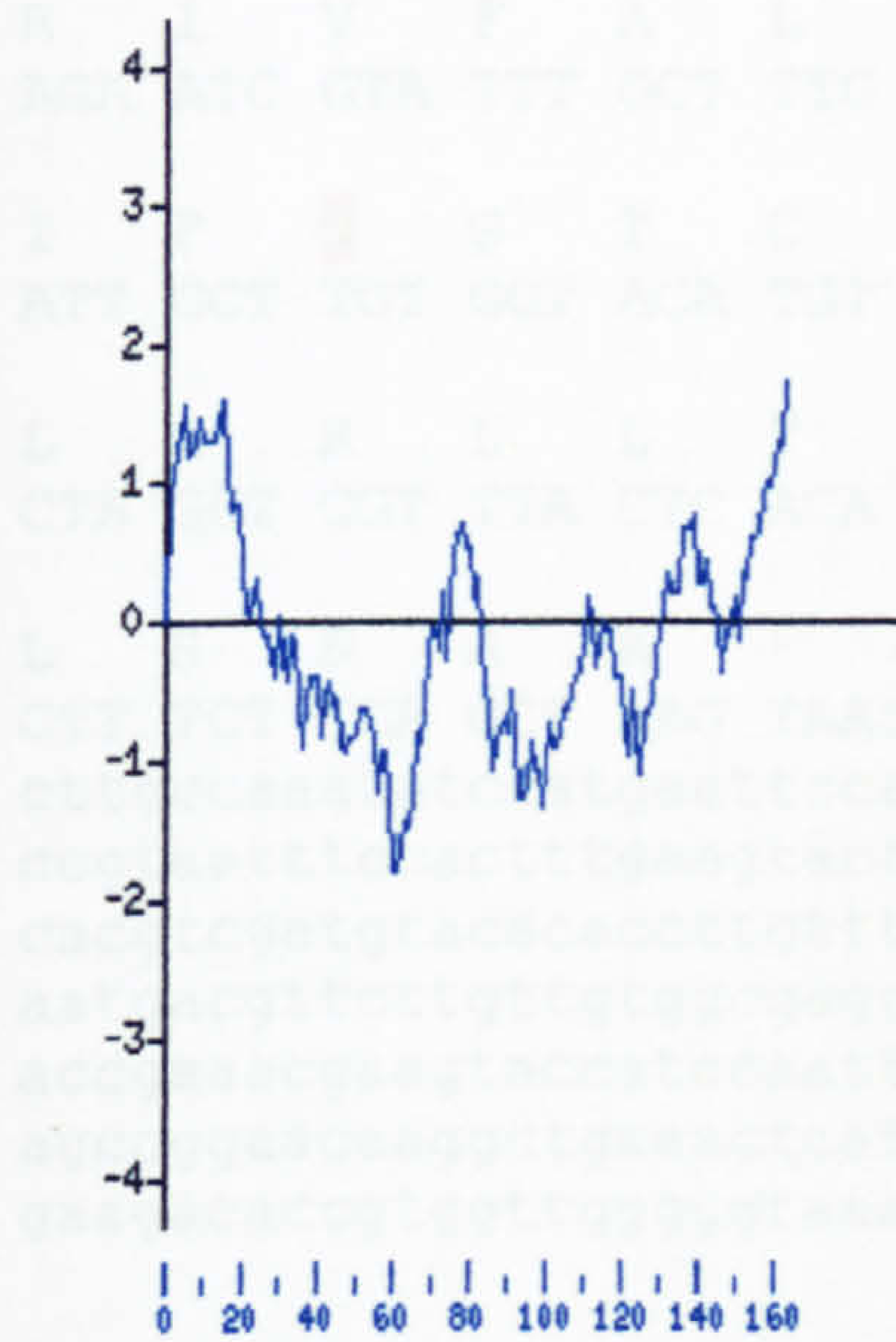
An interesting observation is that almost one third of the cDNAs in this library showed a highly significant identity (>85% at nucleotide level) to the shikimate dehydrogenase gene of *Buchnera aphidicola* (Buchnera). This bacterium is the primary symbiont of a wide range of aphid species, including *M.viciae*, and it is located in the bacteriocytes of the aphid's gut. Dixon (1998) has suggested that these symbionts, which are large in number in aphids, are important in embryonic development, and it has also been suggested that they may supplement the diet of aphids, perhaps by synthesizing essential amino acids and vitamins (Douglas, 1988). Since they are prolific in aphids, the presence of cDNAs from their genome in this library is not surprising. Additionally, with aphid and symbiont genomes being inherited as a single package during parthenogenetic reproduction, it is likely that symbiont and aphid genomes have experienced traffic in DNA (Hales *et al*, 1997). However,

even if symbiont genes are expected to be present, the large number of clones for this one cDNA seems unlikely. There is the possibility that these clones are not unique, or that there was too much material for the subtraction to completely remove the symbiont cDNAs.

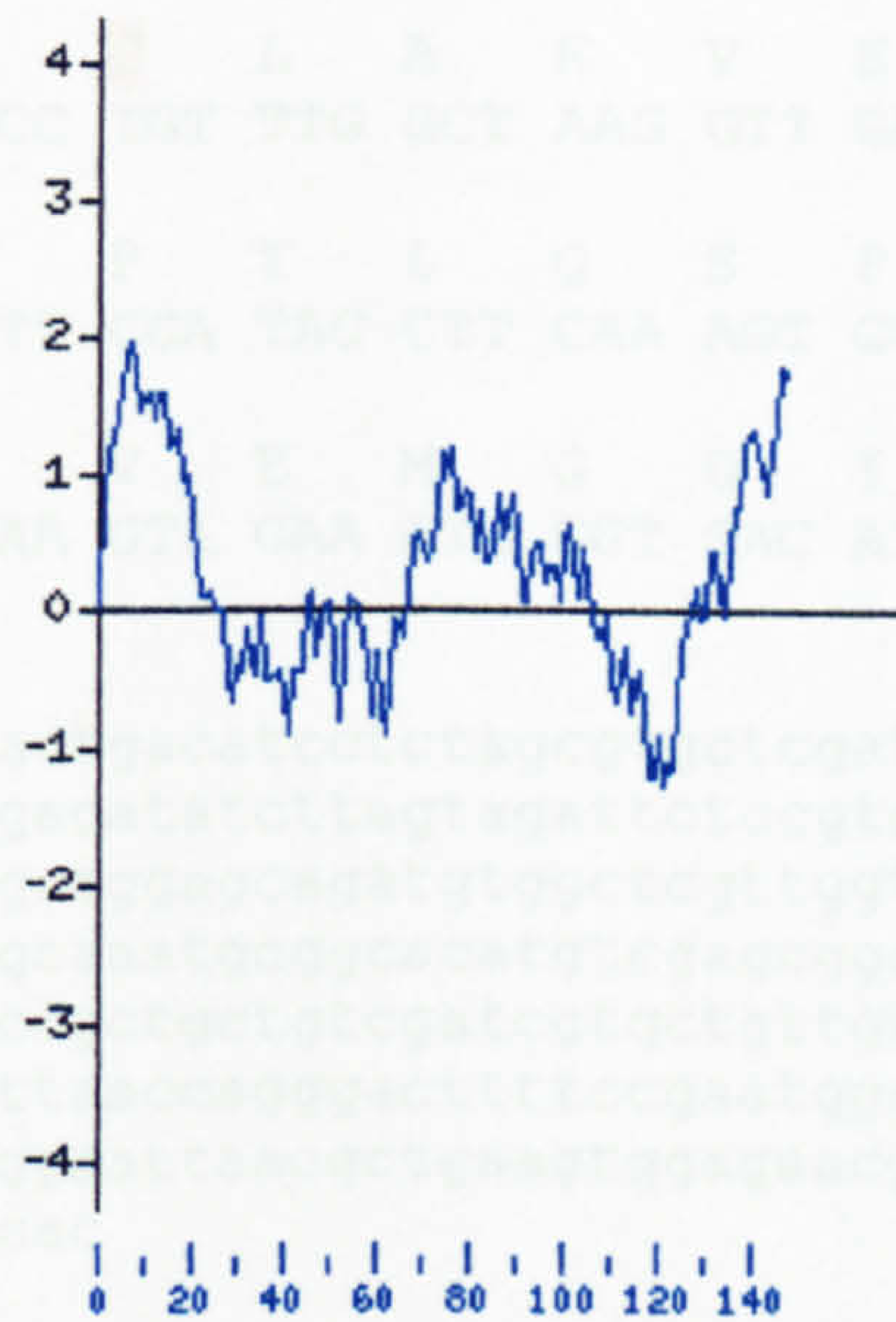
Of the clones that showed no matches in the databases, translation of the nucleotide sequences showed only one clone, U2, with features typical of insect OBPs. U2 was a 0.8kb cDNA, showing 35-40% identity to other insect OBPs at the amino acid level, with five cysteine residues and a predicted signal peptide (Nielsen *et al*, 1997; see Figure 4.1). In addition, the predicted size of the protein U2 (14.7kDa; 12.8kDa mature peptide) is in the correct range for OBPs, although the calculated pI is 8.5, much less acidic than other insect OBPs. A Kyte and Doolittle hydropathy plot of U2 (Kyte and Doolittle, 1982) alongside other insect OBPs (Figure 4.2) shows that the whole protein is fairly hydrophobic, which is in contrast to the other insect OBPs, which have distinct hydrophobic domains.

Of the 25% of cDNAs with very low similarity to other sequences, or identity across only a small number of nucleotides, three additional clones were of interest: E3, O2 and Q2, cDNAs of 0.6, 0.1 and 0.2kb respectively. The nucleotide and predicted amino acid sequences of these three cDNAs are shown in Figure 4.3. A multiple sequence alignment with some representative insect OBPs (Figure 4.4), generated using ClustalX (Thompson *et al*, 1997) and manually adjusted, shows that the amino acid sequences of U2, E3, O2 and Q2 have cysteine patterns that match the conserved patterns of insect

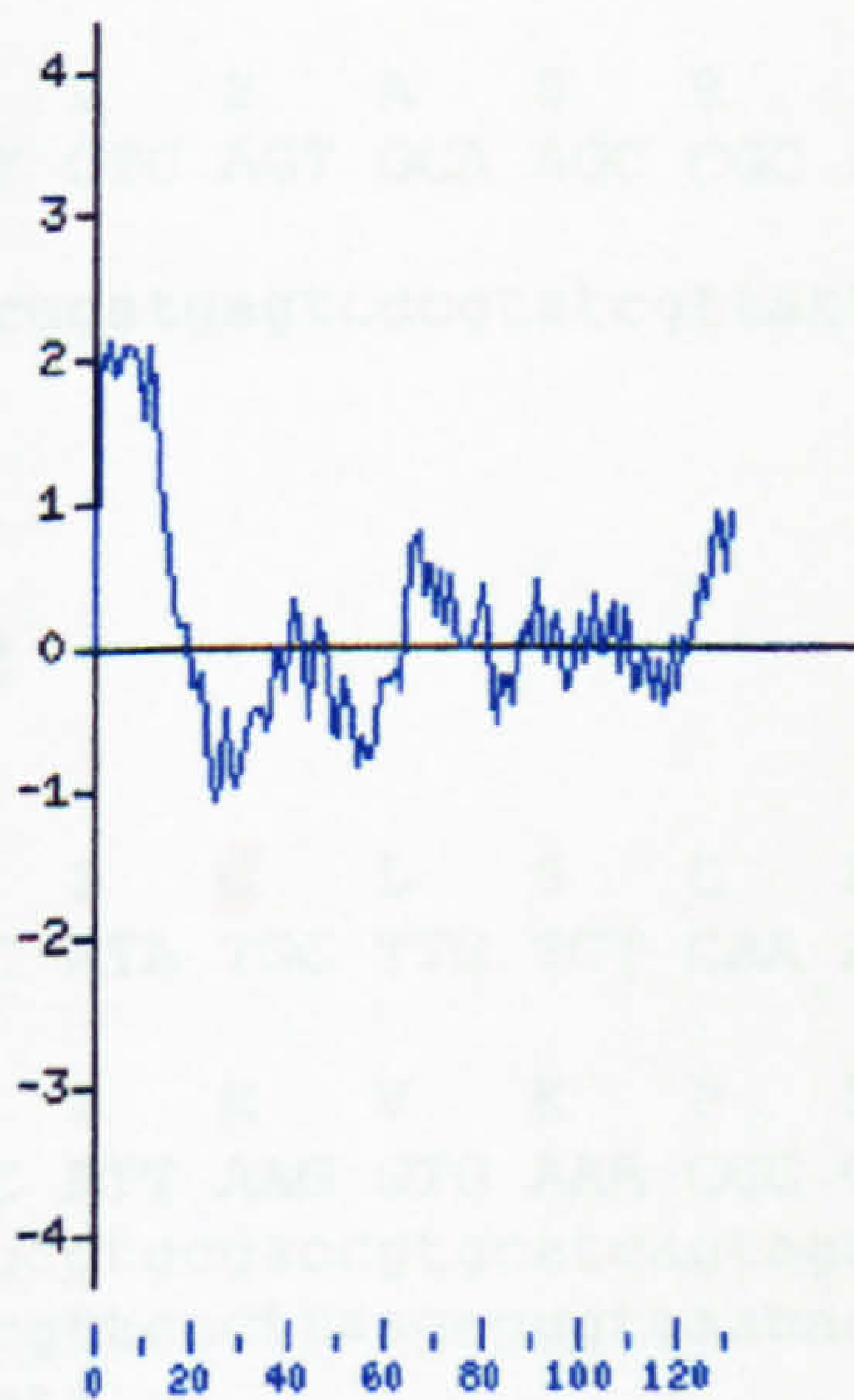
(a) BmorPBP1



(b) DmelPBPRP1



(c) LlinLAP



(d) U2

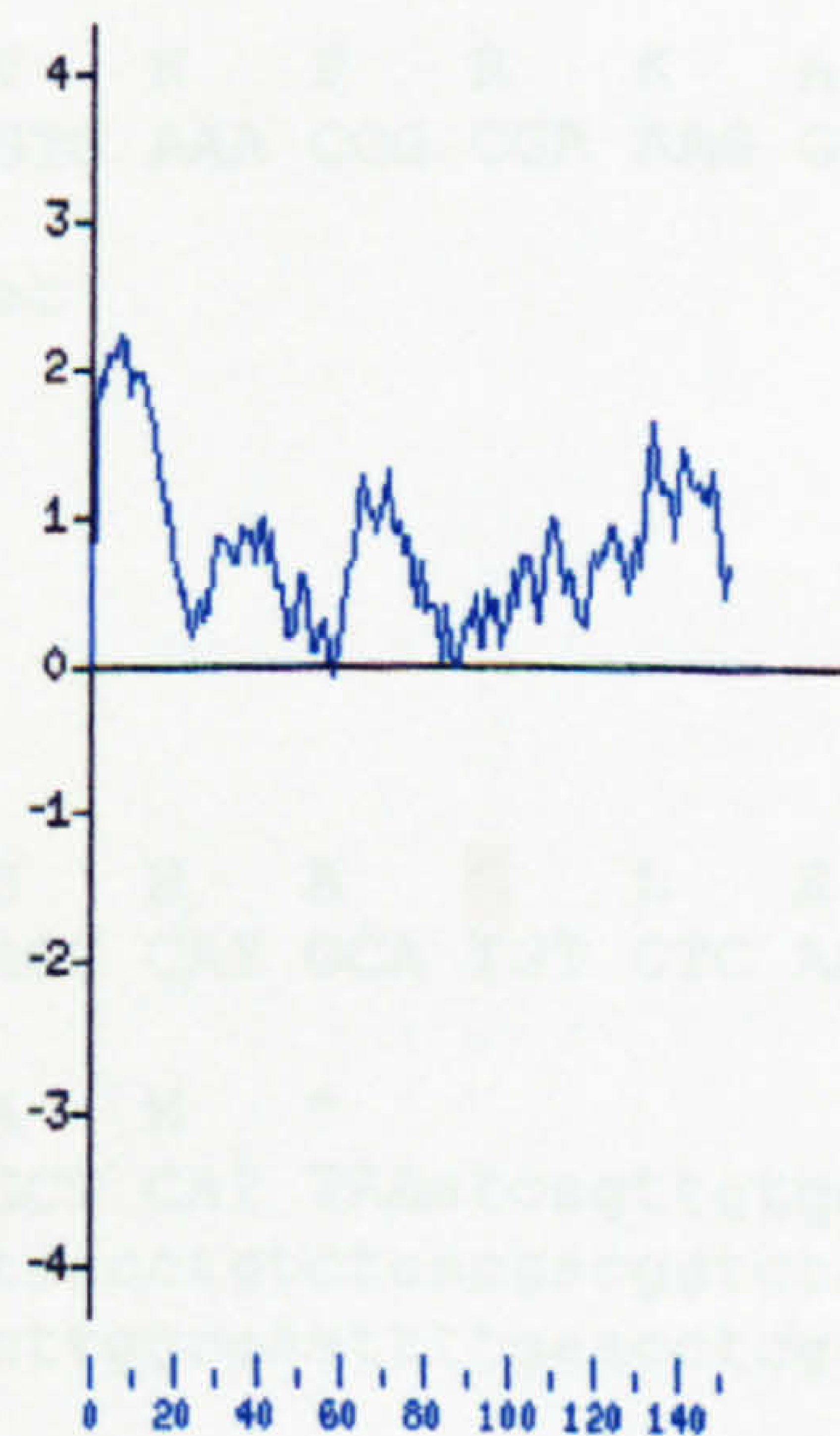


Figure 4.2 Kyte & Doolittle hydropathy plots of U2 (d) with other insect OBPs. (a) BmorPBP1 = *B. mori* PBP1 (Krieger *et al*, 1996); (b) DmelPBPRP1 = *D. melanogaster* PBPRP1 (Pikielny *et al*, 1994); (c) LlinLAP = *L. lineolaris* LAP (Vogt *et al*, 1999). Window size = 17.

E3

```
R   I   V   F   A   L   T   L   A   C   L   A   K   V   E   A
AGA ATC GTA TTT GCT TTG ACG TTG GCC TGT TTG GCT AAG GTT GAA GCA

I   P   C   G   T   C   G   G   F   P   Y   L   Q   S   P   P
ATT CCT TGT GGT ACA TGT GGC GGA TTT CCA TAC CTT CAA AGT CCT CCC

L   G   R   L   L   T   V   L   E   V   E   M   G   D   I   Y
CTA GGT CGT TTA CTC ACA GTC TTA GAA GTA GAA ATG GGT GAC ATA TAT

L   S   S   A   K   *
CTT TCT TCA GCT AAG TAAtccacaattaactgacatcctctagcgtgctcgatgtgtc
cttgccaaatatcaatgaattccatgcaagtctgacatatcttagtagattctccgtcattgc
ccgtaatttccactttgaagtactcgttgacgtgctggagcagatgtggctcgttggtcccat
cacgtcgatgtacacaccttgtttatcgttcaggcaaatgcggcacatgtcgagcggggtcaa
aatgacgttcttgttgtggcgaggcatcgctgaccgctgctgtcgatcgtgctgttgaaatcc
accgaaacgaagtaccatccaattcgagacggtttaaccagggacttttccgaatggactcac
agccggaacaaggctgaaactcatcacggcgctggaattaacgctgaagtggagaacggggca
gaagacaccgtggttggggtaaagaccgaacacac
```

O2

```
A   W   S   R   P   R   L   C   L   S   Q   R   L   S   H   A
GCG TGG TCG CGG CCG AGG TTA TGC TTG TCT CAA AGA TTA AGC CAT GCA

C   L   S   A   S   R   I   K   V   K   P   R   K   A   H   *
TGT CTC AGT GCA AGC CGC ATT AAG GTG AAA CCG CGA AAG GCT CAT TAA

atcggatgagtcccgtatcgttattttcgtcac
```

Q2

```
V   I   C   L   S   Q   R   L   S   H   A   C   L   S   A   S
GTC ATA TGC TTG TCT CAA AGA TTA AGC CAT GCA TGT CTC AGT GCA AGC

R   I   K   V   K   P   R   K   A   H   *
CGC ATT AAG GTG AAA CCG CGA AAG GCT CAT TAAatcagttgtggttccttaga
tcgcgtgcgaccgtgcatcagtagggtaaaactaacctgtctcacgacggtctaaccccagct
cacgttcccttaagcgggtgaacaatccgacgcttggcgaatttgaacctcggccgcgacca
cgcta
```

Figure 4.3 Nucleotide and predicted amino acid sequences of cDNAs E3, O2 and Q2 with amino acids written above corresponding codons. UTRs are shown in lower case. Conserved cysteine residues are highlighted in red.

```
           1                                                                                                  90
  AperPBP1 .......... .......... ....MLGKIS LLLLPVFVAI NLVHSSPEII KNLSQNFCKA MDQCKQELNI PDSVIADLYN FWKDDYVMTD
   PjapPBP .......... .......... .......... .......... ........MS EEMEELAKQL HDDCVSQTGV DEAHITTVK. DQKG..P.DD
   LlinLAP .......... .......... .......... MRILVLFTAA LTCVMAGELP EEMREMAQGL HDGCVEETGV DNGLIGPCA. ..KGN.FADD
Dmel OS-E  M......... .......... ....LKYPLI LLLIGCAAAQ EPRRDGEWPP PAILKLGKHF HDICAPKTGV TDEAIKEFS. ..DGQ.IHED
        U2 MNSFGIIAIL AVACLAKVNS TPCGGCGWAA PCLPAPCLPA PPCLPAPCLP ARP..CPP.. ...CIPSPPI SPAALASLLA TLKASAPYSP

           91                                                                                                 180
  AperPBP1 RLAG.....C AINCMATKLD VVDPDGNLHH GNAKEFAMKH GADASMAQQL VDIIHGCEKS APPNDD..KC MKTIDVAMCF KKEIHKLNWV
   PjapPBP E...KFK..C YLKCLMTEMA IVGDDG..VV D.VEAAVGVL P..DELKAKA EPIMRKC.G. FKPGANP..C DNVYQTHKCY YETDAQS...
   LlinLAP Q...KLK..C YFKCVFGNLG VISDEG..EL D.AEAFGSIL P..DNMQ.EL LPTIRGCAGT T..GADP..C ELAMNFNKCL QKVDPVN...
 Dmel OS-E E.A..LK..C YMNCLFHEFE VVDDNG..DV HMEKVLNAIP .GEKLRNIMM EAS.KGCIHP E.GDTL...C HKAWWFHQCW KKADPVH...
        U2 LTPLPVAPPC KPACGPAPT. VATTLSVPAP APQVIAPGPA PGPLIVQLPS VAG.K.CAPI VVPAVQPLIC SSN~~~~~~~ ~~~~~~~~~~
        E3 .......... .......... .......... .......... .......... .........I VFAL..TLAC LAKVEAIPCG TCGGFP..YL
        O2 .......... .......... .......... .......... .......... .......... ..AWRSPRLC LSQRLSHACL SASRIK...V
        Q2 .......... .......... .......... .......... .......... .......... .......VIC LSQRLSHACL SASRIK...V

           181                  210
  AperPBP1 PDMDVVLGEV LAEV~~~~~~ ~~~~~~~~~~
   PjapPBP .........Y MIV~~~~~~~ ~~~~~~~~~~
   LlinLAP .........F MVI~~~~~~~ ~~~~~~~~~~
 Dmel OS-E .........YF LV~~~~~~~ ~~~~~~~~~~
        U2 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
        E3 QSPPL..GRL LTVLEVEMGD IYLSSAK~~~
        O2 .KPRK.AH~~ ~~~~~~~~~~ ~~~~~~~~~~
        Q2 .KPRK.AH~~ ~~~~~~~~~~ ~~~~~~~~~~
```

Figure 4.4 Alignment of amino acid sequences from *A. pernyi* (AperPBP1; Raming *et al*, 1990), *P. japonica* (PjapPBP; Wojtasek *et al*, 1998), *L. lineolaris* (LlinLAP; Vogt *et al*, 1999) and *D. melanogaster* (Dmel OS-E; McKenna *et al*, 1994) with U2, E3, O2 and Q2 from *M. viciae*. Conserved cysteine residues are highlighted in red.

OBPs. It is also interesting to note that the sequences (both nucleotide and amino acid) of O2 and Q2 are very similar, and appear to be the same cDNA, with Q2 being the longer transcript. However, O2 showed some identity to 18S rRNA genes of other aphid species (the greenbug *Schizaphis graminum* (Rondani), *A. pisum*, and R. *padi*, 100%) whilst Q2 showed no such match (nucleotide nor amino acid) in any reading frame. U2 and E3 show matches to two sequences, only over 25 nucleotides, and no matches at the amino acid level.

Since the library was made by subtracting whole female aphid without antennae from whole female aphid with antennae, it cannot be assumed that any of the four cDNAs discussed are expressed exclusively in the antennae, as would be expected for a role in olfaction. It was therefore necessary to investigate the tissue distribution patterns of these four cDNAs, and these are presented and discussed in 4.4.2.

4.4.1.3 Libraries 3 and 4

Although Library 2 had given some promising cDNAs, trying to find antennal specific clones was a very difficult task. Therefore, subtractions with antennal material as the tester and heads as the driver, which should increase the chances of finding antennal specific messages, were undertaken, and two further libraries, 3 and 4, were constructed using the PCR-Select™ kit, for both male and oviparous females respectively; 76 clones were sequenced from the female library, and 67 from the male. A summary of the BLAST results is given in Table 4.3, showing that Libraries 3 and 4 had a very even spread of

clones throughout the categories, as such being more accurate representations of the antennal cDNA population than the previous subtracted libraries.

Match	Percentage of clones showing match	
	Male	Female
rRNA genes (16, 18, 23 & 28S)	16.42	2.56
Actin	1.49	2.56
Cuticle proteins	4.48	7.69
Low match (>25bp)	28.36	30.77
No match	25.37	28.21
Other	23.88	28.21

Table 4.3 Summary of results of BLAST searches for clones generated in Libraries 3 and 4. Matches are shown in terms of percentage of similar amino acid residues.

The clones in the "other" category (expanded in Table 4.4) included some of the more ubiquitous cDNAs such as pyruvate dehydrogenase. One would expect these to be present in head tissues and thus to have been removed by the subtraction; their presence in these libraries suggests that the subtraction was not complete. However, the percentage of actin cDNAs in Library 3 (female) is only 2.56% compared with 18.95% in the female unsubtracted antennal cDNA library (see Chapter 3.4), whilst the proportion of rRNA genes in the male library has dropped from 61.05% in the unsubtracted antennal library (see Chapter 3.4) to 16.42% here, both of which suggest that the subtraction has removed some of the ubiquitous cDNAs.

Seven of the cDNAs detailed in Table 4.4 showed similarity (35%-46%) to the juvenile hormone binding proteins (JHBP) found in the antennae of other insects (e.g. Robertson *et al*, 1999), and as such

Clone	Source	Shows match to
C31, C50, C68, C131, C151, C165, C209	M,F,F,M,F,F,M	35-46% to juvenile hormone binding protein (precursor)
C10, C130	F, F	72–81% to insect CSPs
C21	M	88% to heat shock proteins of *D. melanogaster*
C24, C76, C80	M, M, M	54-72% to glutamate synthase
C29, C47	M, F	33% to effector cell receptor
C41, C218	M, F	59%, 70% to insect Cytochrome P450
C85, C86, C105	F, F, F	30% to phosphoglycerate kinase
C53, C65	M, F	33% to NADH-oxidoreductase
C28, C43	F, F	74% to pyruvate dehydrogenase
C109	F	38% to receptors including GPCR
C110, C155, C181	F, F, F	57% to ribosomal proteins
C133	F	32% to ABC transporter
C135	F	31% to DNA polymerase
C65, C81, C166, C170	M, M, M, M	36-42% to serine/threonine protein kinase
C147	F	66% to myosin heavy chain
C194	F	95% to aphid elongation factor 1-α gene
C113	M	83% to glutamine synthase
C168	M	41% to signal recognition particle receptor α subunit
C178	M	31% to tropomyosin

Table 4.4 BLAST results of clones in the "Other" category in Table 4.3 from male (M) and female (F) subtracted antennal cDNA libraries 3 and 4.

might be expected to be present in aphids. However, this is very interesting because juvenile hormone precursors are structurally similar to *E*-(β)-farnesene (EBF), the aphid alarm pheromone (Baker *et al*, 1983), and the two groups of chemicals have been shown to have similar juvenilizing activities (Mauchamp & Pickett, 1987). Therefore, these JHBPs might actually be functioning as EBF-binding proteins.

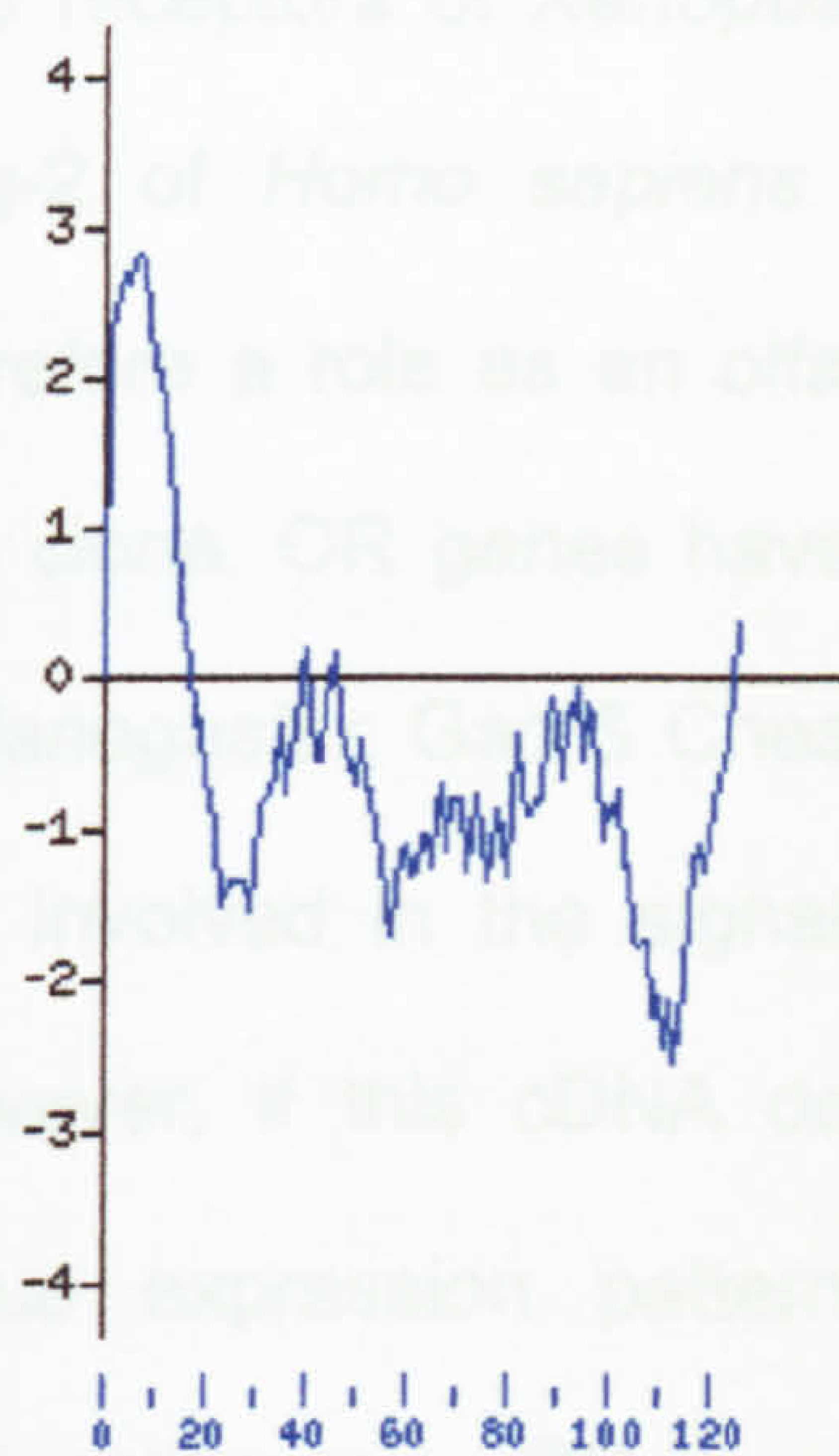
Clones C10 and C130 showed high identity (68% and 45% respectively) to insect ChemoSensory Proteins (CSPs), and are shown in a multiple sequence alignment with representative insect CSPs in Figure 4.5, where they are termed MvicCSP1 (C10) and MvicCSP2 (C130). The alignment was generated using ClustalX (Thompson *et al*, 1997), and the extent of the similarities indicates that both clones are likely to be CSPs, although neither is full-length. The amino acid sequence of MvicCSP1 has a stop codon (*) at position 14, but since the rest of the sequence is a very good match to other insect CSPs, it is likely that this stop codon results from a sequencing error, which may have arisen because *Taq* DNA polymerase, which has no proofreading capability, was used in the amplifications. Figure 4.6 shows that MvicCSP2 possesses the almost totally hydrophilic hydropathy plot (Kyte and Doolittle, 1982) that is characteristic of insect CSPs. These two clones have been characterised further and the results are presented in Chapter 5.

```
             1                                                                                     90
        OS-D MGQPGFRRAI GHFSLVVALM CTTCFQVEGL PHPPATSPSP MMERMVEQAYDDKFDNVD.LD EILNQERLLI NYIKCLEGT. .GPCTPDAKM
     CSP-ec1 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~TKYDNVNLE EVFGNERLLE SYRKCLMD.. EGLCAPDAEE
    LmigCSP1 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~KYDNIDLD DILHNDRLLK KYHECLVSSS DASCTPDGKE
    LmigCSP5 ~~~~~~~~~~ ~~~~~~~~~~ ~~MQKCTLAL LLACLVAAAA A........Y TTKYDNIDLD DVLHNDRLLK KYHECLLSDS DASCTPDGKE
     CSP-sg1 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~EEKY TTKYDNVNLD EILANDRLLN NYVKCLLEDG EANCTVDGKE
     CSP-sg5 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~EEKY TTKYDNVNLD EILANDRLLN KYVQCLLEDD ESNCTADGKE
         p10 ~~~~~~~~~~ ~~~~~~~~~~ ~~~MKCVAVF VIVAVVALAE AARFRRDDKY TTKYDNIDLD EILASDRLLA NYHKCLIE.. EGKCTPDGEE
  CSP-MbraB1 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~EEAHY TDRYDNVDLD EILGNRRLMV PYIKCILD.. QGKCAPDAKE
  CSP-MbraA6 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ MKFVLLLCVM VAAVVAEDKY TDKYDNINLD EILANKRLLV AYVNCVME.. RGKCSPEGKE
     Ccacolf ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~MMK TSLVLLCCLA AVLARPSDTY TDKYDNINIQ EILENKRLLE AYVNCVLD.. KGKCTPEGKE
    MsexSAP1 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~M KMLAVLCLFV LGALSAPERD GDLYDMFDAE MILEDDKLRS KAIDCLLD.. RGVCD.DYQP
    MsexSAP5 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~MKP VTAAILISLA CMVQCGKDMY TSRYDSMNVD DVIGNHRLLH AYIKCMLD.. EGRCTAEGRE
    BmorCSP1 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ MKVLIVLSCV LVAVLADDKY TDKYDKINLQ EILENKRLLE SYMDCVLG.. KGKCTPEGKE
    MvicCSP1 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~T QLNWTTLTWK GC*TSDRILT SYIKCLLD.. QGNC~~~~~~
    MvicCSP2 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~DNIDID QILASKRLVN NYVQCLLD.. KKPCTPEGAE
```

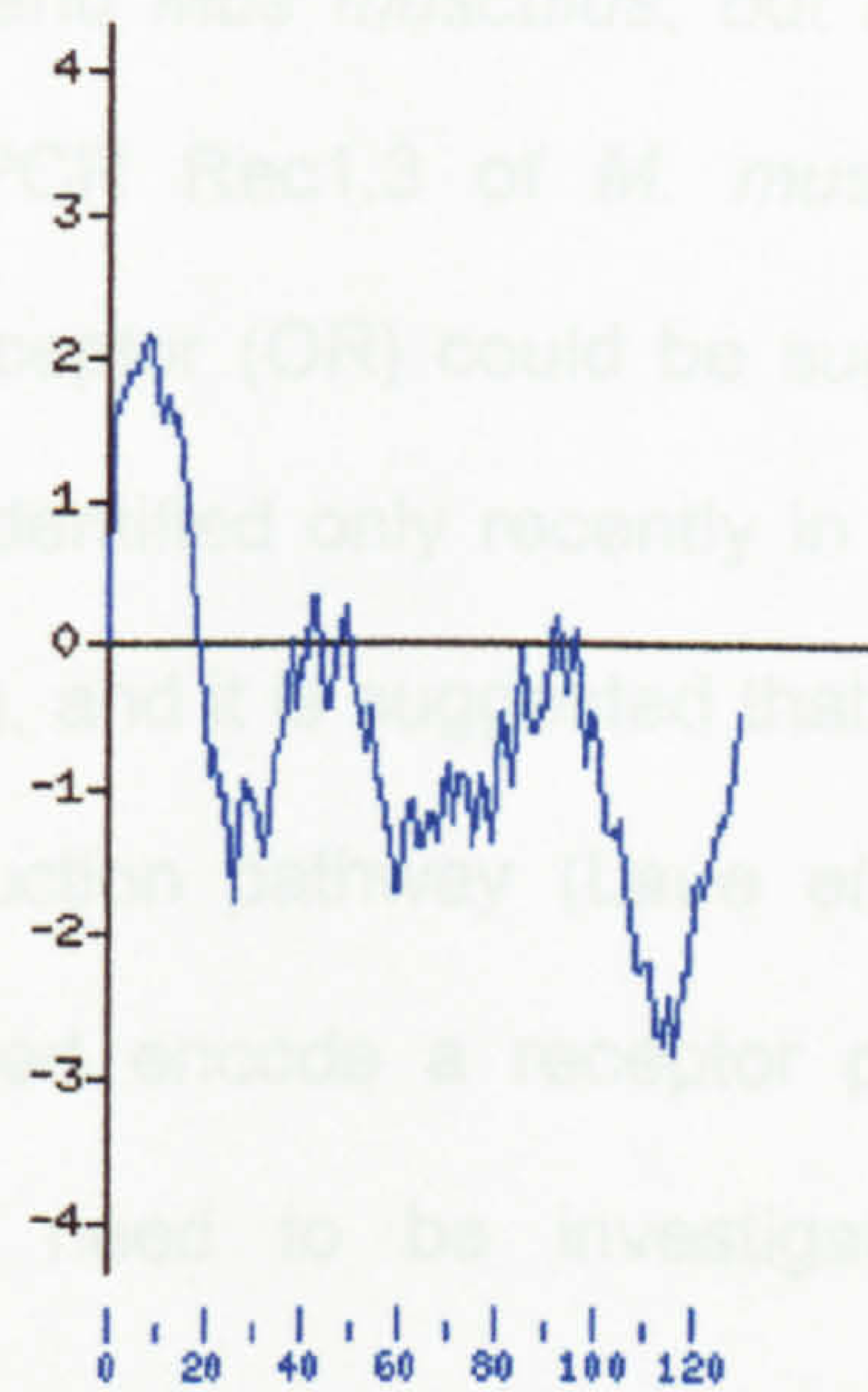
```
              91                                                                                                        180
      OS-D    LKEILPDAIQ TDCTKCTEKQ RYGAEKVTRH LIDNRPTDWE RLEKIYDPEG TYRIKYQEMK SKANEEP~~~ ~~~~~~~~~~ ~~~~~~~~~~
    CSP-ec1   LKKAIPDALE NECAKCSEKQ KAGVETTIVF LIKNKPEIWE SFKKKYDPTH KYEKIYERYI KQAEEKARKS ~~~~~~~~~~ ~~~~~~~~~~
  LmigCSP1    LKAVIPDALT NECAKCNEKQ KAGAEKVIKF LVKEKPDLWE PLEKKYDPSG SFRQKYGPEL KKVSA~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  LmigCSP5    LKAAIPDALT NECAQCNEKQ KAGAEKVIRF LIKEKPDLWT PLEKKYDPTG SFRQKYDQEL KRVSA~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
    CSP-sg1   LKKAVPDALS NECAKCNDKQ KEGTKKVLKH LINHKPDIWA QLKAKYDPDG TYSKKYEDKE KELHE~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
    CSP-sg5   LKKDIPDALS NECAKCNEKQ KEGTKKVLKH LINHKPDVWA QLKAKYDPDG TYSKKYEDRE KELHQ~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
        p10   LKSHVSDALQ NDCAKCSDKQ RAGAEKVINF LYNKKKPMWE SLQKKYDPEN TYVTKYADRL KELHD~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 CSP-MbraB1   LKEHIREALE NECGKCTETQ KNGTRRVIGH LINHEDAYWK ELTAKYDPQS KFTAKYEKEL KEIKH~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 CSP-MbraA6   LKEHLQDAIE NGCKKCTENQ EKGAYRVIEH LIKNEIEIWR ELTAKYDPTG NWRKKYEDRA KAAGIVIPEE ~~~~~~~~~~ ~~~~~~~~~~
  CcacCLP1    LKEHLQEAIE NGCEKCTEAQ EKGAYTVIEH LIKNEIEIWR QLADKFDPER KYRKKYEDRA RAKGIEIPE~ ~~~~~~~~~~ ~~~~~~~~~~
  MsexSAP1    IRDKGPRLIK TRCEDCTPEQ KAVFEESMKI LEEKFNNDFK EIIAKYA~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  MsexSAP5    LKKHITDALQ TGCSRCTDAQ KKAIRHVIKH LIEHEHDFWA LLVEKYDPHR IYTTKYEAEM KRTMRSKEQM SESGAGHEKA DMKMMGDGPG
  BmorCSP1    LKDHLQEALE TGCEKCTEAQ EKGAETSIDY LIKNELEIWK ELTAHFDPDG KWRKKYEDRA KAKGIVIPE~ ~~~~~~~~~~ ~~~~~~~~~~
  MvicCSP1    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  MvicCSP2    LRKILPDALK TQCSKCNPGQ KNAALKVVDR LQKDYDKEWK LLLDKWDPKR EQFQKFQQFL VEE~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
```

Figure 4.5 Multiple sequence alignment of representative insect CSPs and those cloned from *M. viciae* (MvicCSP1 & 2). OS-D from *D. melanogaster* (McKenna *et al*, 1994); chemosensory protein CSP-ec1 from *E. calcarta* (Marchese *et al*, 2000); CSPs 1 and 5 (LmigCSP1, 5) from *L. migratoria* (Picimbon *et al*, 2000a); CSPs 1 and 5 (CSP-sg1, 5) from *S. gregaria* (Angeli *et al*, 1999); p10 from *P. americana* (Kitabayashi *et al*, 1998); CSPs (CSP-Mbra B1/A6) from *M. brassicae* (Nagnan-Le Meillour *et al*, 2000); olfactory protein CLP1 (CcacCLP1) from *C. cactorum* (Maleszka & Stange, 1987); sensory appendage proteins 1 and 5 (MsexSAP1, 5) from *M. sexta* (Robertson *et al*, 1999); CSP1 (BmorCSP1) from *B. mori* (Picimbon *et al*, 2000b). Conserved cysteine residues are highlighted in red.

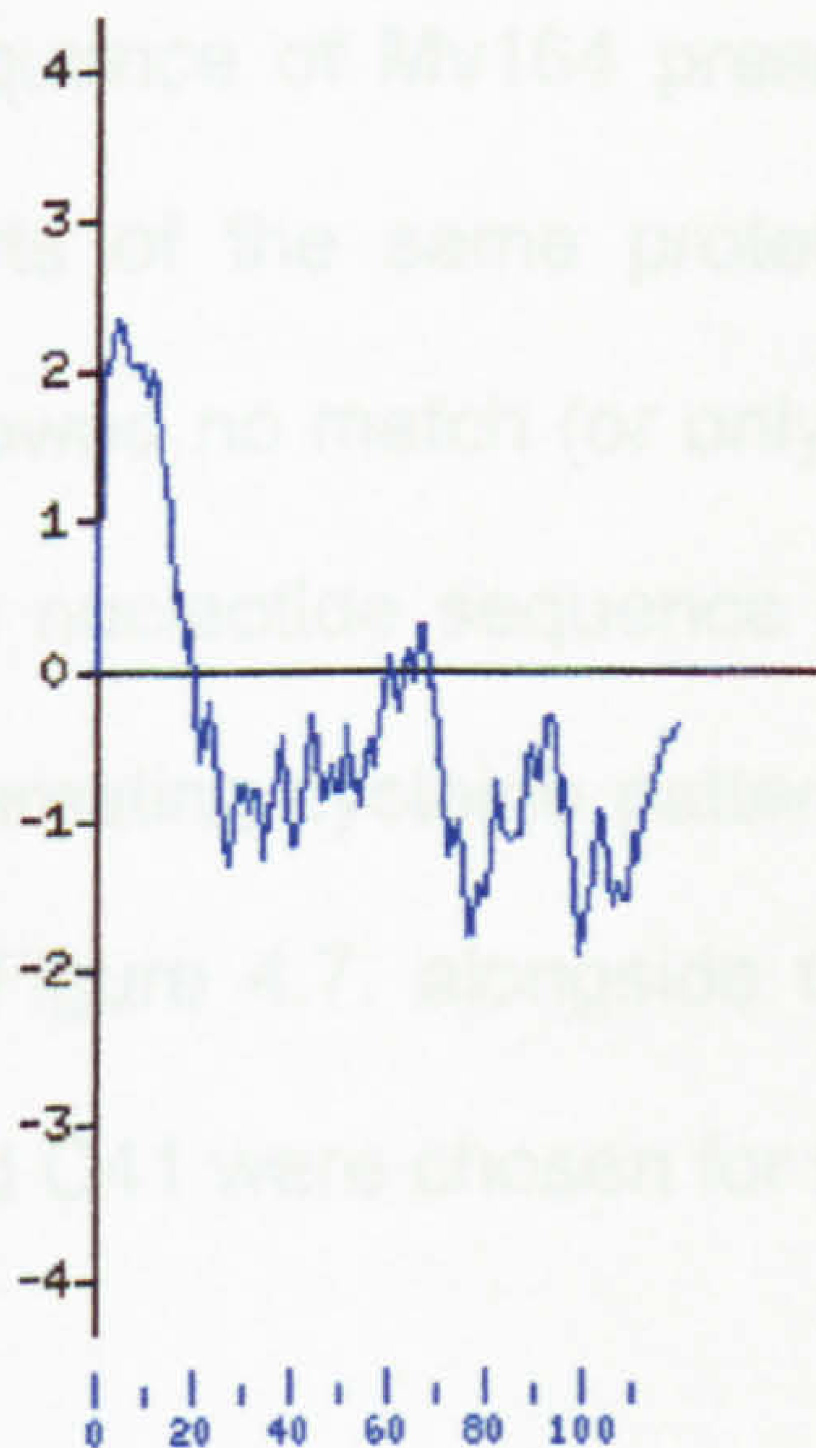
(a) BmorCSP1



(b) CcacCLP1



(c) LmigOS-D2



(d) MvicCSP2

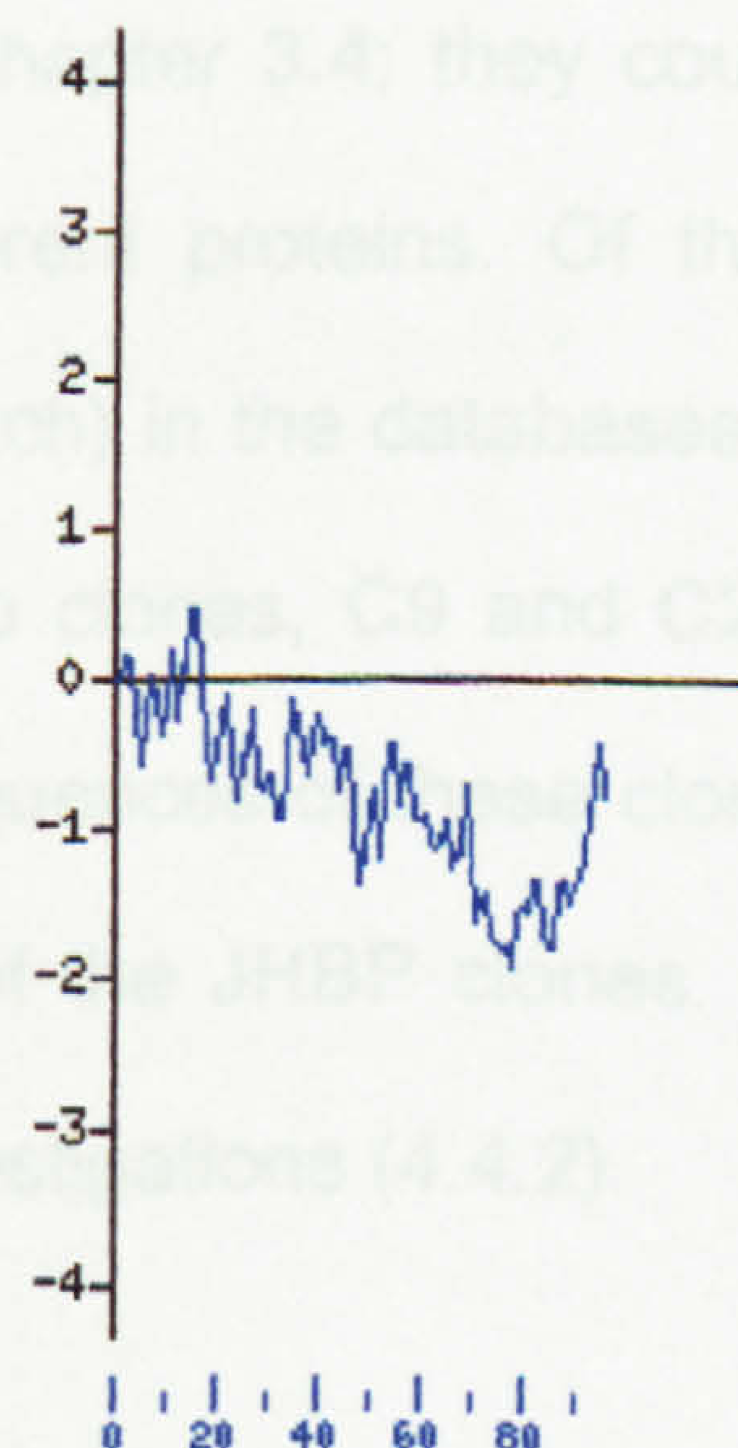


Figure 4.6 Kyte & Doolitle hydropathy plots of MvicCSP2 (d) alongside other insect CSPs. (a) BmorCSP1 = CSP1 from *B. mori* (Picimbon *et al*, 2000b); (b) CcacCLP1 = olfactory protein CLP1 from *C. cactorum* (Maleszka & Stange, 1987); (c) LmigOS-D2 = “OS-D like” protein 2 from *L. migratoria* (Picimbon *et al*, 2000a). Window size = 17.

C109 shows 38% identity to receptors, notably lysophosphatidic acid receptors of *Xenopus laevis* and *Mus musculus*, but also GPCR Edg-2 of *Homo sapiens* and GPCR Rec1.3 of *M. musculus*, and therefore a role as an olfactory receptor (OR) could be suggested for this clone. OR genes have been identified only recently in insects (*D. melanogaster*, Gao & Chess, 1999), and it is suggested that G-proteins are involved in the signal transduction pathway (Laue *et al*, 1997). However, if this cDNA does indeed encode a receptor protein, the tissue expression pattern would need to be investigated before assignation as an OR.

Finally, C41 and C218 showed significant similarity to cytochrome P450 enzymes of insects. Neither of these matched the sequence of Mv164 presented in Chapter 3.4; they could be different parts of the same protein, or different proteins. Of the cDNAs that showed no match (or only a low match) in the databases, translation of the nucleotide sequence of only two clones, C9 and C29, showed an interesting cysteine pattern. The sequences of these clones are shown in Figure 4.7, alongside C31, one of the JHBP clones. C9, C29, C31 and C41 were chosen for further investigations (4.4.2).

4.4.2 Analyses of candidate antennal-specific cDNAs.

Apart from the two CSP cDNAs, MvicCSP1 and MvicCSP2, discussed further in Chapter 5, other potentially interesting cDNAs identified in the subtracted libraries were U2, E3, O2, Q2, C9 and C29 as putative OBPs, C31 as a JHBP, and C41 as a cytochrome P450.

C9 (X_{19}-C-X_8-C-X_{11})

```
T   P   L   S   A   E   P   R   S   K   S   Q   K   T   R   L
ACT CCG TTG TCC GCT GAA CCA AGG AGT AAG AGC CAA AAA ACA CGC CTA

R   R   R   C   P   S   S   D   F   G   L   L   C   P   S   P
CGT CGC CGC TGC CCC AGC TCC GAT TTT GGG TTA CTC TGT CCC AGC CCC

?   I   ?   L   V   W   I   P   *
ANG ATC NTT CTC GTA TGG ATA CCC TGAttacnccncctanccngcagccatacct
cngcnnnttacccacccatcntncccaacaccnttncccancantcncctttntgtccccccn
ctnaaaagcatttacngcccantcacttttcc
```

C29 (X_{16}-C-X_{27}-C-X_5)

```
A   R   T   G   R   L   E   R   S   K   P   K   L   G   Q   R
GCG CGT ACG GGT AGA CTG GAG AGG AGT AAG CCA AAG CTG GGG CAG CGA

C   K   R   V   S   L   L   W   R   L   Q   R   W   L   V   L
TGT AAG CGT GTT TCT TTA CTC TGG CGG CTT CAG CGC TGG CTT GTT CTT

R   V   L   V   L   L   R   R   Q   R   S   T   C   P   G   G
CGG GTT TTG GTG CTT CTT CGG CGA CAG CGG AGT ACC TGC CCG GGC GGC

R   S
CGC TCG A
```

C31 (35% similarity to JHBP precursor, *M. sexta*)

```
H   F   E   K   P   I   T   L   D   G   N   Y   D   I   K   G
ACA CTT TGA AAA ACC AAT TAC GCT CGA TGG AAA TTA CGA TAT CAA AGG

K   V   I   I   L   P   I   T   G   D   G   L   C   K   I   S
CAA AGT TAT TAT CCT ACC AAT TAC CGG TGA CGG TCT CTG CAA AAT TAG

L   D   N   L   K   A   K   I   G   V   Y   L   K   P   V   V
CCT TGA TAA TCT CAA AGC AAA AAT AGG CGT ATA TTT AAA GCC CGT TGT

R   N   G   N   T   Y   A   D   V   K   D   I   K   L   T   F
AAG AAA CGG AAA CAC CTA CGC GGA TGT TAA GGA TAT TAA ATT AAC GTT

T   T   T   K   M   H   L   K   I   G   Q   L   I   Q   R   R
TAC CAC CAC CAA AAT GCA TTT GAA AAT TGG ACA ACT TAT TCA AAG GAG

P   A   L   G   N   N   M   N   V   F   L   N   E
ACC AGC TCT TGG AAA TAA TAT GAA TGT GTT CCT AAA TGA AA
```

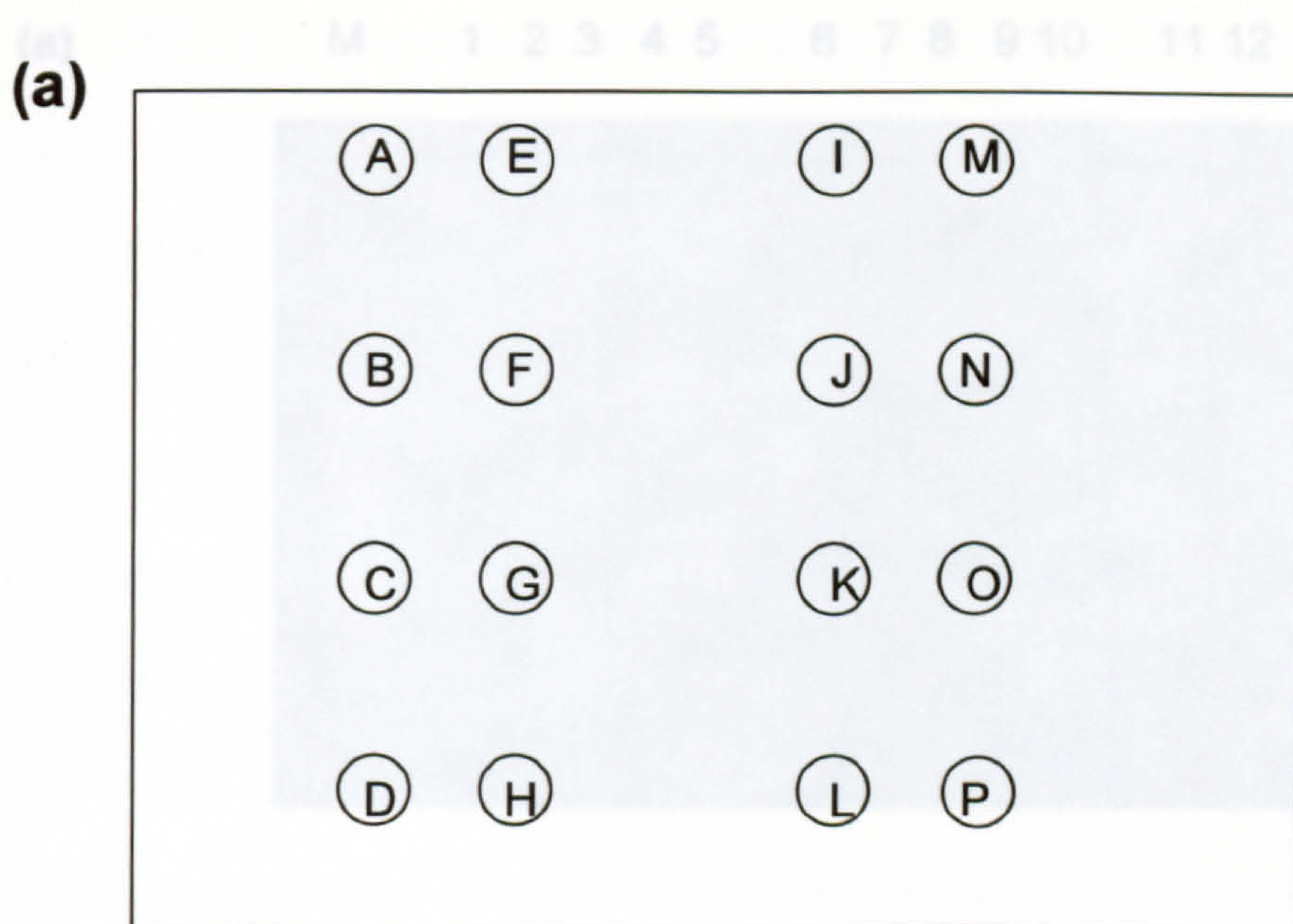
Figure 4.7 Nucleotide and predicted amino acid sequences of cDNAs C9, C29 and C31 with amino acids written above corresponding codons. UTRs are shown in lower case.

The tissue distributions of the eight were therefore investigated.

For U2, the cDNA insert was purified from the plasmid, radiolabelled, and used to probe dot blots of RNA from different aphid tissues (as described in 2.2.2.10). Figure 4.8 shows the autoradiograph produced, which clearly shows the labelled cDNA binding to the female tissues where the abdomen is included (Samples B, C, D, G and H). The only exception is the whole female sample (A, Figure 4.8), which showed no binding, perhaps due to low levels of mRNA on the filter because of incomplete mRNA transfer.

RT-PCR was used to try to confirm this result. cDNAs from antennae, legs, heads (without antennae) and abdomens (without legs) from both males and oviparous females were tested as described in 2.2.2.6(b). Figure 4.9(a) shows that a band of the same size as the positive control (Lane 11) is amplified in oviparous female abdomens (Lane 4) and whole oviparous females (Lane 5), but not in antennae or legs. There is no band in any of the male tissues, and Figure 4.9(b) shows that it is not expressed in any tissue of parthenogenetic females. This confirms the finding of the dot blot that U2 is restricted to the abdomens of oviparous females. Since it is not expressed in any of the chemosensory tissues, it is highly unlikely that U2 is an aphid OBP. However, it shares characteristics with OBPs, and it is possible that it encodes a type of binding protein, perhaps a carrier protein, with a role in the production or development of the eggs or sexual organs of the oviparous female.

(a)



(b)

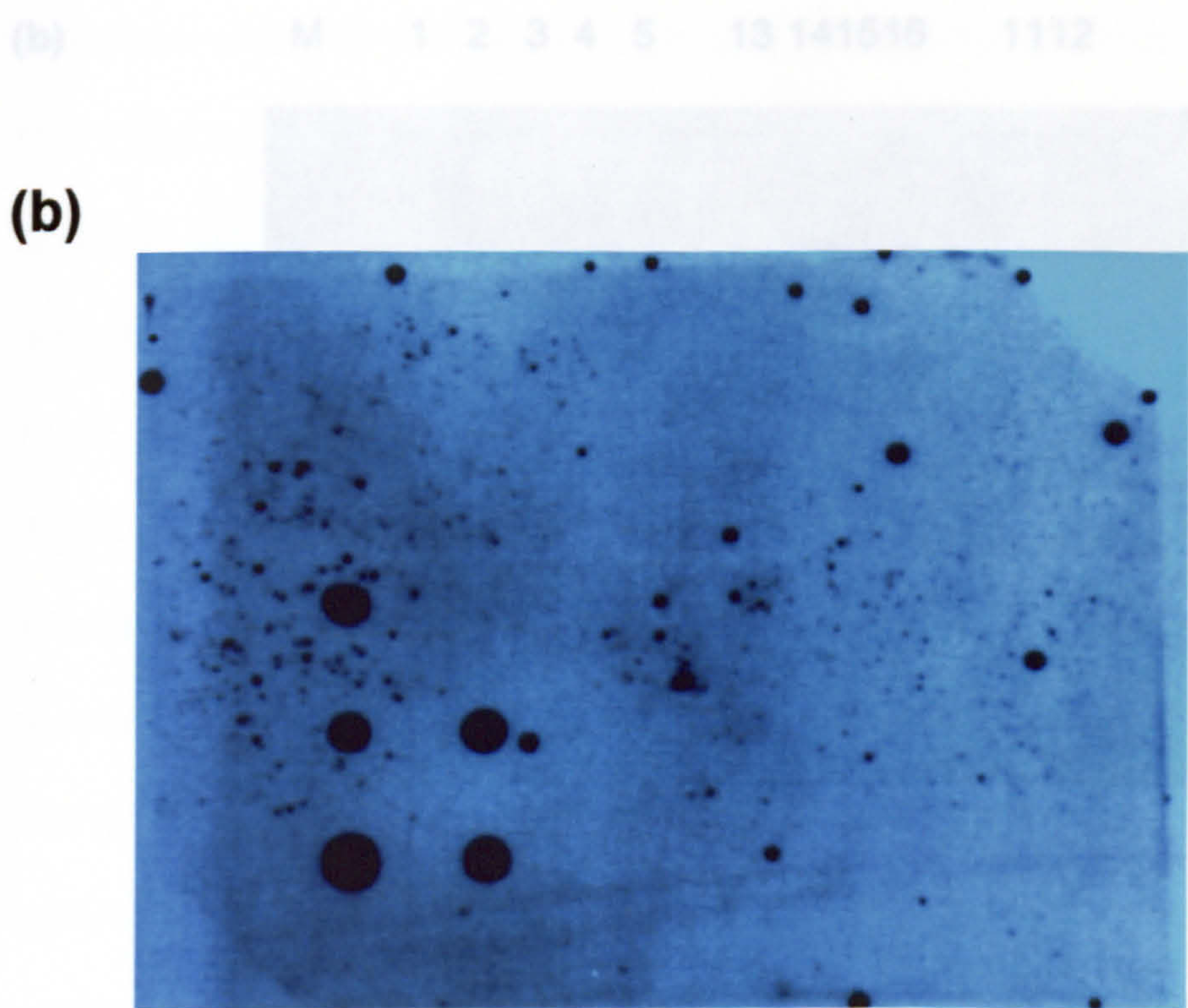
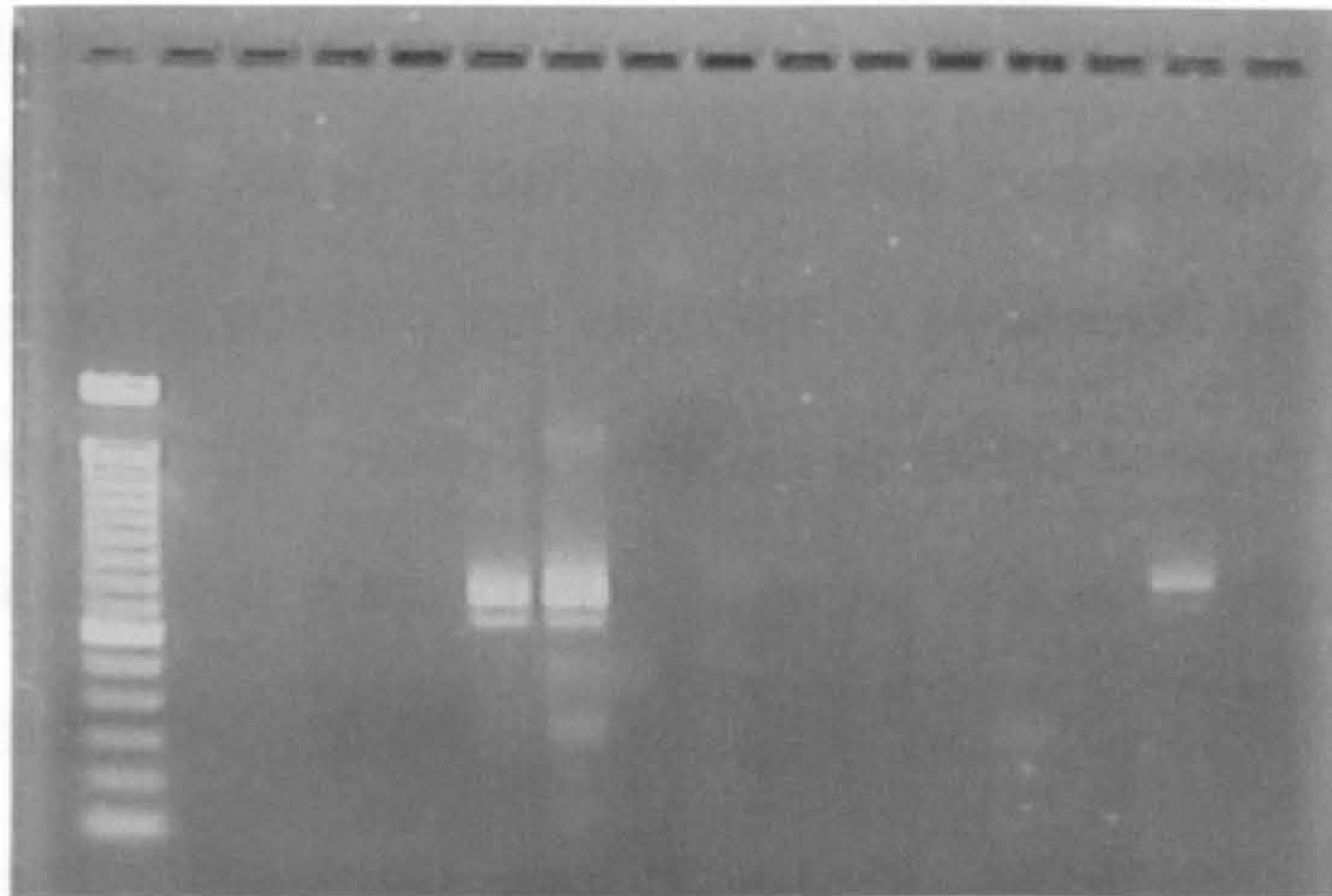


Figure 4.8 RNA dot blots. **(a)** Schematic diagram of sample position on filter. A= whole female; B = whole female minus legs; C= whole female minus antennae; D = female heads and abdomens (minus antennae and legs); E = female heads; F = female heads minus antennae; G = female abdomens; H = female abdomens minus legs; I = whole male; J = whole male minus legs; K = whole male minus antennae; L = male heads and abdomens (minus antennae and legs) M = male heads; N = male heads minus antennae; O =male abdomens; P = male abdomens (minus legs). **(b)** Autoradiograph of membrane probed with radiolabelled U2 cDNA.



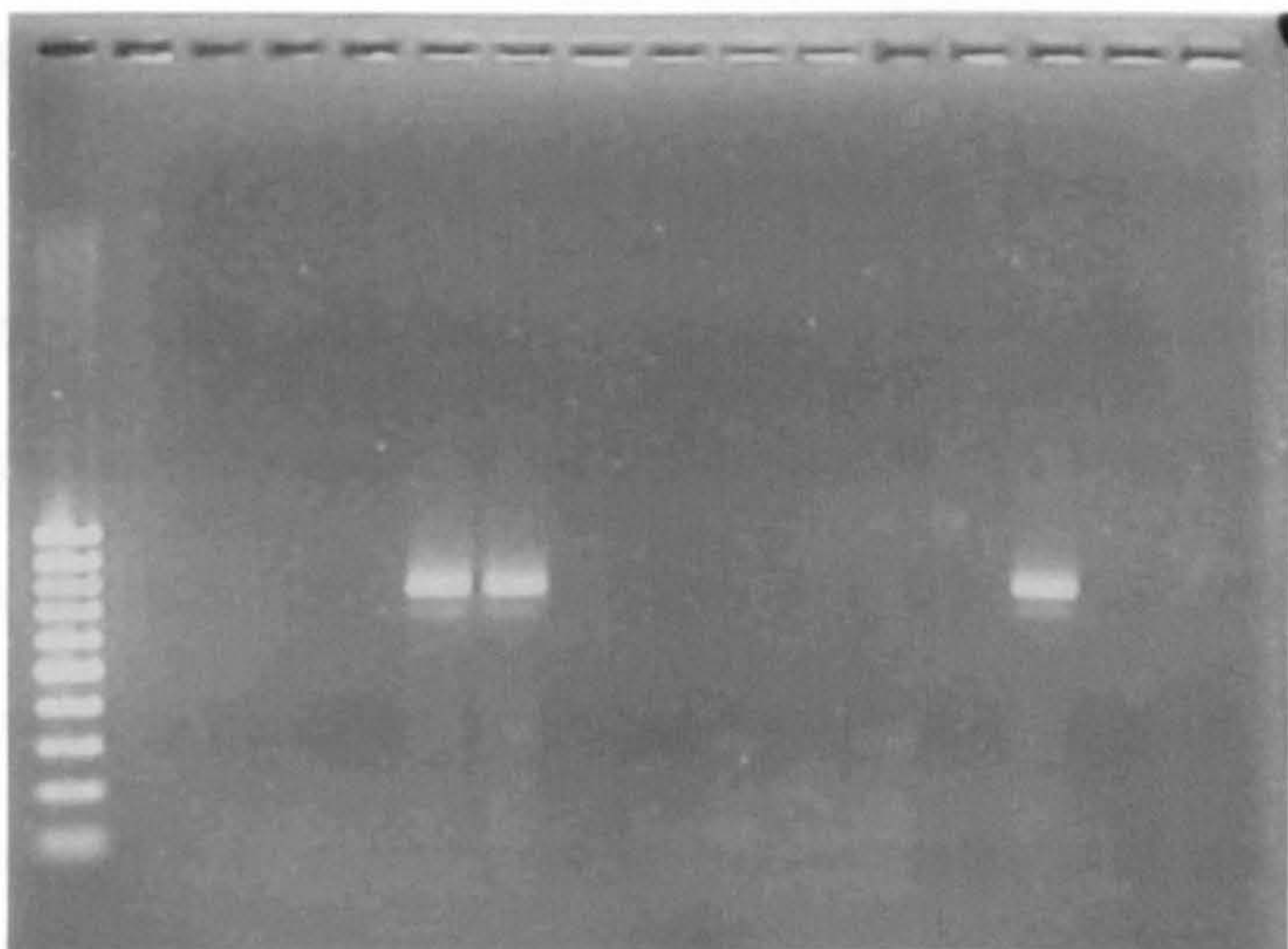


Figure 4.9 Results of RT-PCR on *M. viciae* tissues [**(a)** oviparae and male, **(b)** oviparae and virginoparae] using primers AL25 (U2 GSP) and oligo(dT). Fragments were separated on a 1.5% agarose gel. M = 100bp DNA ladder; 1 = oviparae antennae; 2 = oviparae legs; 3 = oviparae heads; 4 = oviparae abdomens; 5 = whole oviparae; 6 = male antennae; 7 = male legs; 8 = male heads; 9 = male abdomens; 10 = whole males; 11 = positive control (U2 pDNA); 12 = negative control (SDW); 13 = virginoparae antennae; 14 = virginoparae legs; 15 = virginoparae heads; 16 = virginoparae abdomens.

RT-PCR was also used to determine the tissue expression of E3, O2 and Q2, the partial length putative OBPs, and Figure 4.10 shows the results for all three clones. It can be seen that fragments of the same size as the positive controls are amplified in all tissues for all clones. Since they are not specifically expressed in chemosensory tissues, it can be concluded that these cDNAs have no role in olfaction, and as such were not studied further.

The same approach was taken to determine the tissue expression patterns of some of the promising clones produced in the antennae minus head libraries (Libraries 3 and 4): C9, C29, C31 and C41. The results are presented in Figure 4.11, and show that C9, C29 and C41 are ubiquitous cDNAs, with fragments amplified in every tissue sample, although C29 expression appears to be fainter in abdomens. From these preliminary expression patterns, C9, C29 and C41 can be discounted in terms of having an olfactory role. C31, the JHBP cDNA, shows enhanced expression in the antennae and legs of males and females, although the expression in male heads and abdomens is higher than for the same tissues in females (Figure 4.11). Since this cDNA might encode a binding protein for EBF, enhanced expression in chemosensory tissues (antennae and legs) might be expected.

A major problem encountered with these subtracted libraries was that the majority of cDNAs cloned were very small, presumably because there is an abundance of *RsaI* restriction sites in the coding DNA of antennae. As a result, searching for OBP characteristics, such as conserved cysteine patterns, became very difficult, and a small cDNA

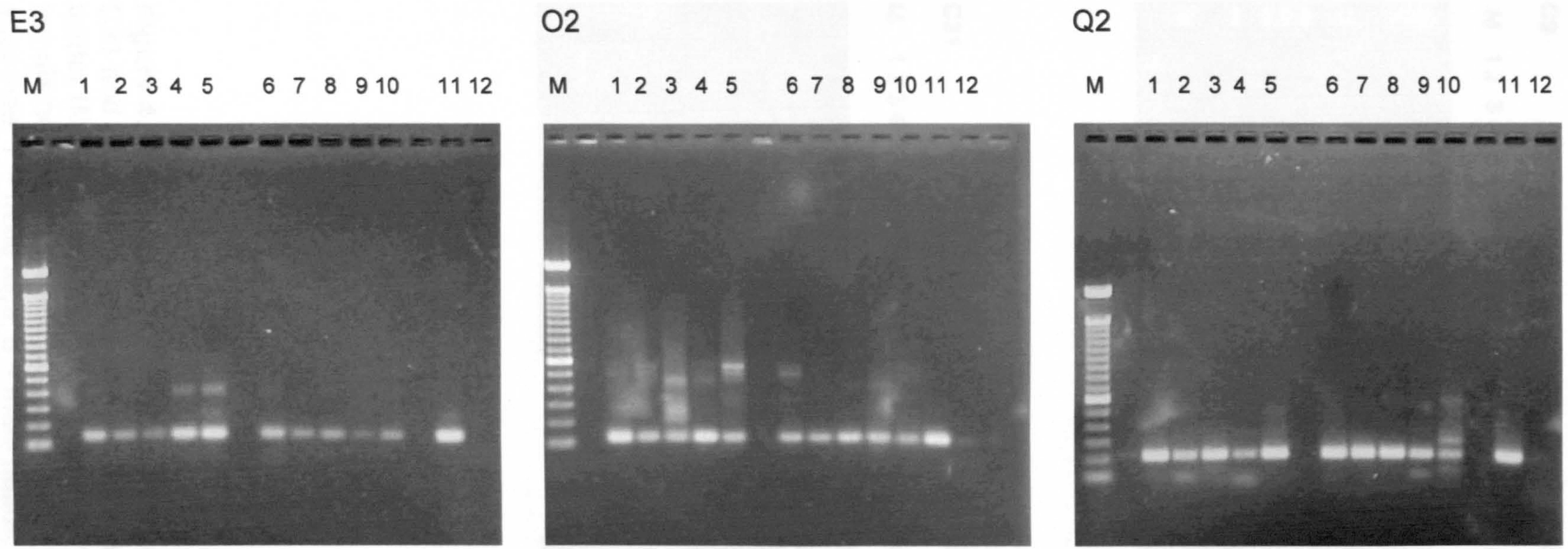
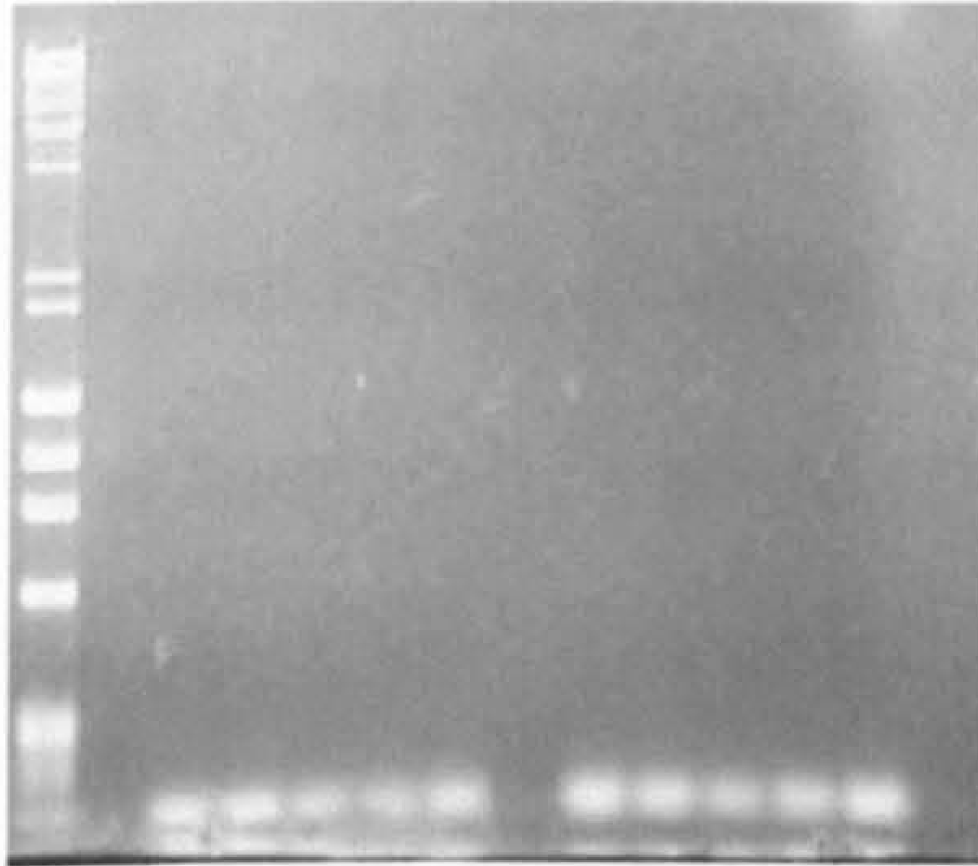


Figure 4.10 Results of RT-PCR on *M. viciae* cDNAs using E3, O2 and Q2 GSPs, separated on 1.5% agarose gels. M = 100bp GeneRuler™ Plus DNA ladder (MBI Fermentas); 1 = female antennae; 2 = female legs; 3 = female heads; 4 = female abdomens; 5 = whole females; 6 = male antennae; 7 = male legs; 8 = male heads; 9 = male abdomens; 10 = whole males; 11 = positive controls (E3, O2 or Q2 pDNAs); 12 = negative control (SDW).

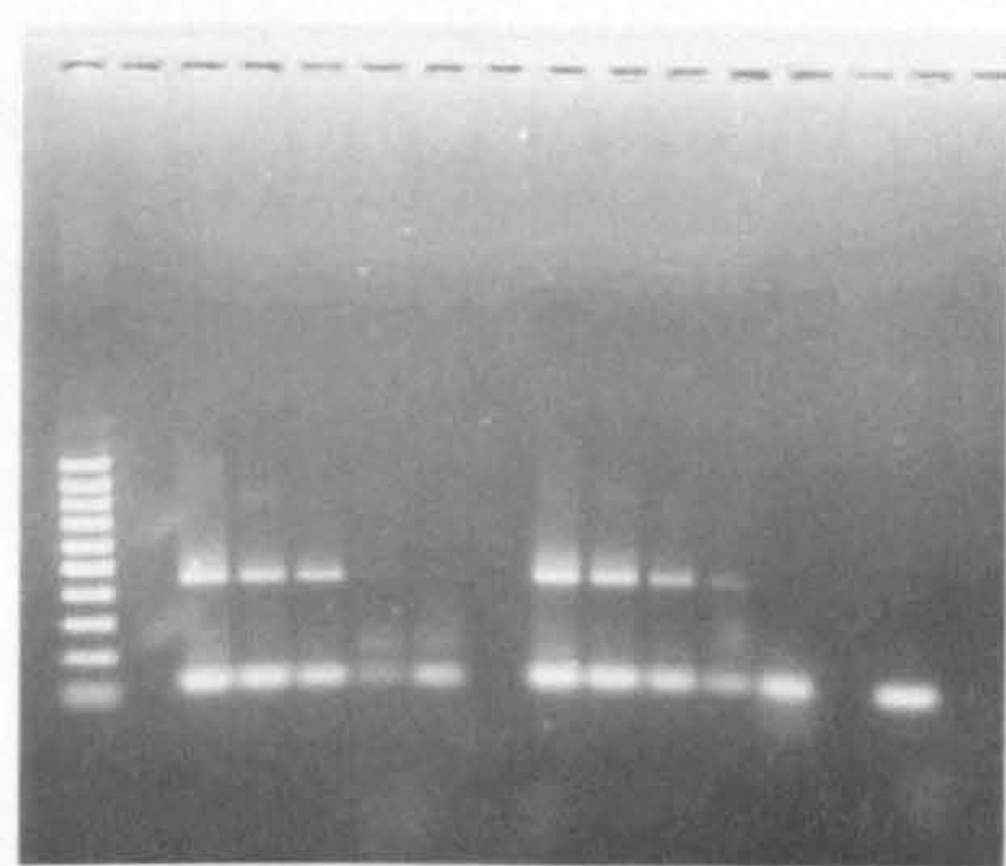
C9

M 1 2 3 4 5 6 7 8 9 10



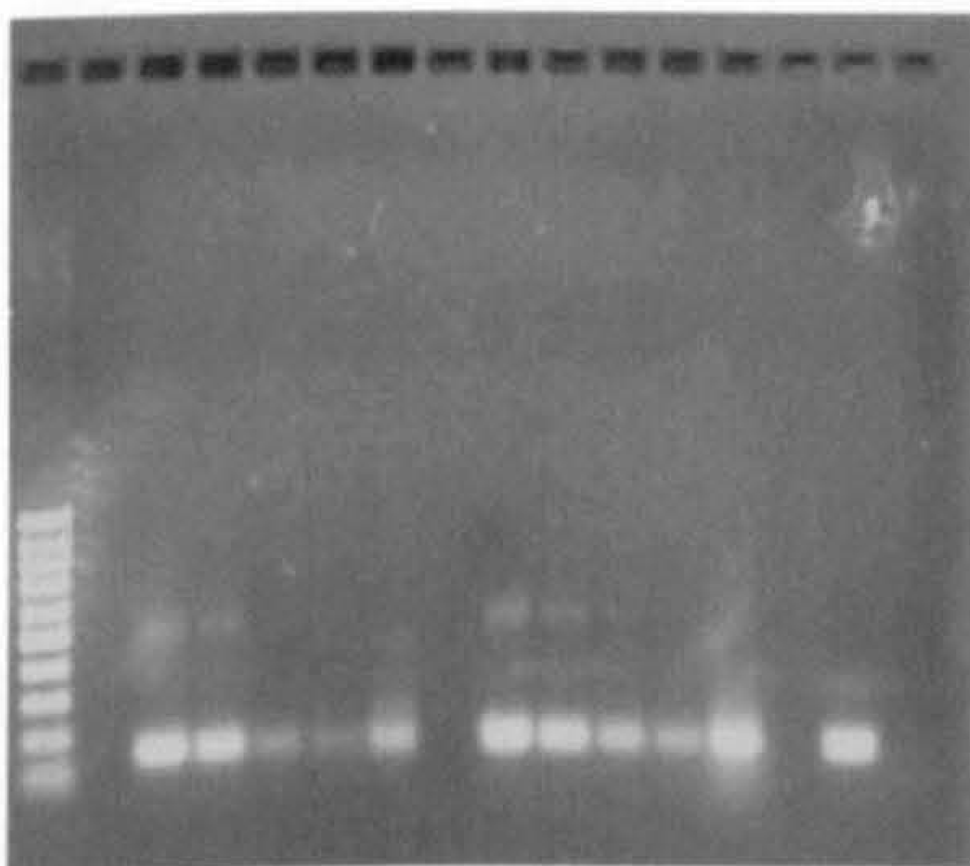
C29

M 1 2 3 4 5 6 7 8 9 10 11 12



C31

M 1 2 3 4 5 6 7 8 9 10 11 12



C41

M 1 2 3 4 5 6 7 8 9 10 11 12

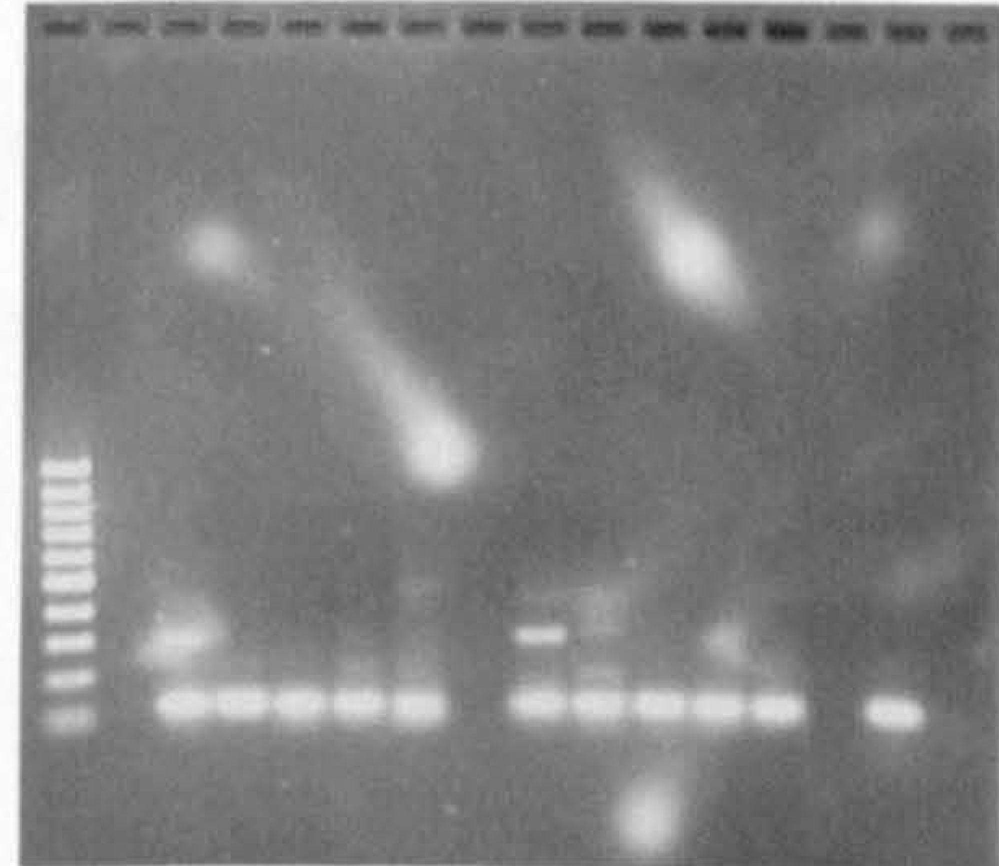


Figure 4.11 Results of RT-PCR on *M. viciae* cDNAs using C9, C29, C31 and C41 GSPs, separated on 1.5% agarose gels. M = 100bp DNA ladder (MBI Fermentas); 1 = female antennae; 2 = female legs; 3 = female heads; 4 = female abdomens; 5 = whole females; 6 = male antennae; 7 = male legs; 8 = male heads; 9 = male abdomens; 10 = whole males; 11 = positive controls (C9, C29, C31, C41 pDNAs); 12 = negative controls (SDW).

with no cysteine residues could have easily been overlooked when the sequence may have been spanning the C-$X_{(26-30)}$-C or the C-$X_{(32-43)}$-C regions of the OBP. This problem seemed to be confined to Libraries 3 and 4, as the cDNAs cloned in Library 2 were usually >600bp. A solution to this problem would be to find a restriction enzyme that cuts less frequently (perhaps a six-cutter) to prepare the cDNAs for adaptor ligation.

The RT-PCR analysis confirms the suggestions made in 4.4.1.3 that the subtractions were not 100% efficient: of the eight clones tested for their tissue distribution patterns, only two show some sort of tissue-specific expression, whilst the remaining six are ubiquitous. Consensus opinion is that no subtraction is 100% effective (Clontechniques, 1997). Indeed Hara *et al* (1991) found that only 3% of their libraries contained cDNAs specific to their tester, even after four rounds of subtraction, compared to the two rounds used here. Also in this subtraction, the amount of driver may not have been sufficiently in excess, due to the small size of aphid heads, a common problem that results in subtractions not going to completion (Anderson, 1995).

4.5 Conclusions

Four subtracted cDNA libraries were generated. The first, from whole female with antennae minus whole female without antennae, using the method of Pikielny *et al* (1994), was very limited, and yielded no cDNAs which were of interest from an olfactory perspective. A similar library, constructed using a PCR-Select™ cDNA Subtraction kit

(method B), proved to be more successful. Eighty-nine clones were sequenced, four of which were analysed using RT-PCR to determine tissue distribution patterns. Three of the clones were found to be ubiquitous, and the fourth, U2, was determined to be a morphological- and tissue- specific transcript, expressed only in the abdomens of oviparous females. A suggested role for this cDNA is that of a carrier protein, perhaps involved in the production or development of the eggs or sexual organs.

The PCR-Select™ approach was also used to construct subtracted antennal cDNA libraries from male and oviparous female aphids, using antennae as tester and head as driver, with 76 clones being sequenced from the female library, and 67 from the male library. Three cDNAs, C9, C29 and C41, initially showed promise in terms of having an olfactory role but were subsequently found to be expressed in all tissues. C31, a cDNA for a JHBP-like protein, showed enhanced expression in the legs and antennae of both sexes, and as such may be an EBF binding protein, and worthy of further studies. By far the most relevant clones were C10 (MvicCSP1) and C130 (MvicCSP2), which showed significant similarity to other insect CSPs and are studied further in Chapter 5.

Chapter 5 Investigation of aphid genes with a putative chemosensory function.

5.1 Introduction

Chapter 3 presented evidence for the cloning of two cDNAs (MvicOBP1 and Mv164), from the antennae of female *M. viciae*, with a potential role in olfaction. The amino acid sequence of MvicOBP1 has characteristics similar to those of other insect OBPs, and that of Mv164 shows significant similarity to cytochrome P450 enzymes of other insect species. Chapter 4 presented evidence for the cloning of two more cDNAs, MvicCSP1 and MvicCSP2, also from female antennae, with amino acid sequences significantly similarity to the "OS-D-like" proteins (also known as CSPs) found in other insect species. Further characterisations of these four cDNAs are presented and discussed here.

5.2 Aims of the study

In order to characterise MvicOBP1, Mv164, MvicCSP1 and MvicCSP2, both the cDNAs and the corresponding gDNAs were analysed, and the main aim was:

- To determine the tissue expression patterns of the genes in the aphid

Further studies attempted

- To obtain full-length cDNA sequences using 5' RACE
- To elucidate the genomic arrangement of MvicOBP1 and Mv164

- To look for related genes in other aphid species
- To investigate electrophysiological responses of *M. viciae* to the alarm and sex pheromones in an attempt to determine what type of OBP MvicOBP1 might be

5.3 Material and methods

The cDNA sequences of MvicOBP1, Mv164, MvicCSP1 and MvicCSP2 are repeated in Figures 5.1 to 5.3 respectively, showing the positions of primers used for RT-PCR, RACE and/or sequencing.

(a) Tissue distribution

RT-PCR [see 2.2.2.6(b)] was used to determine the tissue distribution of the mRNAs. cDNA was synthesised [2.2.2.4(a)] from a range of male and female (oviparous) aphid tissues i.e. antennae (approximately 1000); heads (without antennae; approx. 50); legs (approx. 600); and abdomens (without legs; approx. 2 females, 40 males). RT-PCR was performed using equal amounts of cDNA from each tissue and gene-specific primers (AL34 and AL33 for MvicOBP1; AL40 and AL41 for Mv164; AL46 and AL47 for MvicCSP1; AL77 and AL78 for MvicCSP2), and products were analysed by agarose gel electrophoresis. The suitability of the cDNAs as PCR templates was assessed with GSPs for an actin gene likely to be expressed in all tissues of this aphid.

```
D  H  T  T  S  S  I  H  I  D  Q  V  E  V  C  R  F  V  V  D
GATCACACTACATCAAGTATACATATCGACCAGGTTGAAGTTTGCAGATTCGTTGTAGAC

N  T  R  R  N  H  Q  T  R  N  G  F  F  Q  V  S  D  I  H  H
AATACAAGAAGAAATCACCAAACACGAAATGGCTTCTTTCAAGTATCTGATATTCATCAC
           AL34

L  G  R  V  L  R  C  R  R  R  R  C  K  S  K  E  T  C  L  P
CTTGGTCGTGTACTCCGTTGTCGCCGAAGAAGGTGTAAGAGCAAAGAAACATGCCTACCT
AL61                AL58
                                                     AL43

R  S  C  P  S  S  G  F  G  L  F  C  L  S  P  W  F  V  L  I
CGCAGCTGCCCCAGCTCCGGTTTTGGGTTATTCTGCCTCAGCCCCTGGTTCGTTCTCATA
                                              AL42

C  I  L  R  L  H  Q  I  S  V  Q  L  S  S  I  R  S  C  S  I
TGCATACTCCGATTACACCAGATATCCGTACAGCTATCCAGTATTAGGAGCTGCAGCATA

P  C  T  R  S  I  P  S  S  S  V  P  S  C  S  F  C  H  P  R
CCATGCACCCGCAGCATACCCAGTAGCAGCGTACCCAGCTGCAGCTTTTGCCACCCACGC
                                                         AL62

I  P  C  N  L  P  R  R  *
ATACCCTGCAATCTACCACGAAGATGA
        AL33
```

Figure 5.1 cDNA sequence of MvicOBP1 showing primers (red arrows) used for RT-PCR, sequencing and RACE.

```
V  D  R  D  A  G  K  V  S  F  V  H  G  K  L  F  D  H  L  V
GTCGACCGGGACGCGGGCAAGGTGTCCTTCGTCCACGGCAAGCTGTTCGACCACTTGGTG
                                       AL40

N  L  R  G  E  Q  W  K  A  I  R  A  K  L  S  P  T  F  S  A
AACCTGCGGGGCGAACAGTGGAAAGCAATTAGGGCCAAGTTGAGTCCGACCTTCTCGGCG

A  K  L  K  S  M  L  G  D  I  N  V  C  T  A  R  L  I  E  N
GCCAAGCTCAAGTCCATGTTGGGTGACATAAATGTGTGCACGGCGCGTCTGATCGAGAAC

L  N  G  C  T  T  K  N  S  G  I  V  D  V  S  E  A  S  A  Q
TTAAACGGGCAGATAACGAAAAACAGTGGAATTGTTGATGTAAGTGAAGCGTCGGCTCAA
                                             AL55

F  T  T  D  T  I  G  S  C  A  F  G  L  H  C  N  S  L  S  D
TTTACAACTGATACTATTGGCAGTTGTGCTTTTGGACTACACTGCAATTCATTATCAGAT
                                         AL54

P  D  S  E  F  R  R  T  G  R  A  I  F  T  T  S  L  R  S  T
CCAGATTCAGAATTTCGTCGTACTGGACGAGCCATATTTACGACAAGTCTTCGATCAACT

L  L  N  L  I  R  L  V  G  F  G  R  L  L  D  V  S  R  I  P
TTACTAAATCTCATCAGATTAGTTGGTTTTGGTCGGCTTTTAGACGTATCTAGGATACCT
                                         AL41
                               AL63

G  M  S  A  N  I  Y  D  ?  F  *
GGTATGTCTGCAAACATATATGACNTTTTTTGA
```

Figure 5.2 Sequence of Mv164 showing primers (red arrows) used for sequencing and RT-PCR

MvicCSP1

```
A  A  R  A  G  T  Q  L  N  W  T  T  L  T  W  K  G  C  *  T
GCCGCCCGGGCAGGTACACAACTAAATTGGACAACTTTGACGTGGAAAGGGTGTTGAACA
         AL46

S  D  R  I  L  T  S  Y  I  K  C  L  L  D  Q  G  N  C
AGCGACAGAATTTTAACCAGCTACATCAAGTGTTTGCTAGACCAGGGAAATTGC
                                                AL47
```

MvicCSP2

```
Y  D  N  I  D  I  D  Q  I  L  A  S  K  R  L  V  N  N  Y  V
TACGACAATATTGATATCGACCAAATTTTGGCTTCCAAAAGATTAGTCAATAACTATGTC
                AL77

Q  C  L  L  D  K  K  P  C  T  P  E  G  A  E  L  R  K  I  L
CAATGTCTGTTGGACAAGAAACCGTGCACACCCGAAGGAGCTGAACTTAGAAAAATTTTA

P  D  A  L  K  T  Q  C  S  K  C  N  P  G  Q  K  N  A  A  L
CCCGATGCTTTAAAAACACAATGTTCGAAATGCAATCCCGGGCAAAAAAATGCTGCTCTG

K  V  V  D  R  L  Q  K  D  Y  D  K  E  W  K  L  L  L  D  K
AAGGTAGTTGACCGACTCCAGAAAGATTATGATAAAGAGTGGAAACTGCTTCTTGACAAA
                                                        AL78

W  D  P  K  R  E  Q  F  Q  K  F  Q  Q  F  L  V  E  E
TGGGATCCTAAACGTGAACAATTCCAAAAATTCCAACAATTTTTGGTTGAAGAGAA
```

Figure 5.3 Sequences of MvicCSP1 and MvicCSP2 showing primers (red arrows) used for RT-PCR

(b) Extension of cDNA sequence

Determination of the 5' sequence of MvicOBP1 was attempted using a 5' RACE kit (GibcoBRL; see Figure 2.1). Products generated were either sequenced directly, or cloned and sequenced using M13 forward and reverse-, and gene-specific- primers.

(c) Genomic structure

Genomic DNA was prepared from oviparous females [as in 2.2.2.2(a)] and used as a template for PCR to amplify regions of the genes from *M.viciae*. The products were analysed by agarose gel electrophoresis, gel-purified, cloned, and sequenced using M13 forward and reverse, and gene-specific primers.

(d) Studies in other aphid species

gDNA and cDNA were prepared from ten aphid species: *M. persicae*; *R. padi*; *M.viciae*; *A. fabae*; *L. erysimi*; *A. pisum*; *B. brassicae*; *M. dirhodum*; *S. avenae* and *N. ribis-nigri*. gDNA was used in PCRs with gene-specific primers (AL34 and AL33 for MvicOBP1; AL40 and AL41 for Mv164) to establish whether related genes are present in these aphids; RT-PCR was used to investigate whether the genes are transcribed.

(e) Electrophysiological studies

Electrophysiological studies were performed by Christine M. Woodcock, IACR-Rothamsted. Electroantennogram (EAG) recordings

from whole antennae of males, virginoparae, and oviparae were made against *(E)*-β-farnesene (EBF) and the two major components of the sex pheromone i.e. (4a*S*,7*S*,7a*R*)-nepetalactol and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactone, as described by Wadhams *et al* (1982). For each morph, four individual aphids were tested.

5.4 Results and Discussion

5.4.1 Characterisation of MvicOBP1

(a) Tissue distribution.

RT-PCR was used to determine the tissue expression pattern of this cDNA, as shown in Figure 5.4. Figure 5.4(a) shows that actin GSPs amplified a fragment of the expected size (260bp) in cDNAs from all tissues, confirming that the cDNAs were suitable templates for RT-PCR. Figure 5.4(b) shows the results of RT-PCR with MvicOBP1 GSPs (AL34 and AL33). There are bands of ~300bp in the antennae of males (Lane 6) and females (Lane 1), and also in the legs of males (Lane 7). The bands are of the size expected for AL34 and AL33 (see Figure 5.1), and the same as the positive control, i.e. the MvicOBP1 pDNA (Lane 11); there is no band in the negative control (Lane 12). Thus, these bands are highly likely to be primed from an MvicOBP1 mRNA. There is also a faint band in whole males (Lane 10), probably resulting from the presence of antennal and leg tissues in this sample, but it is much fainter because these tissues are smaller proportions of the total cDNA.

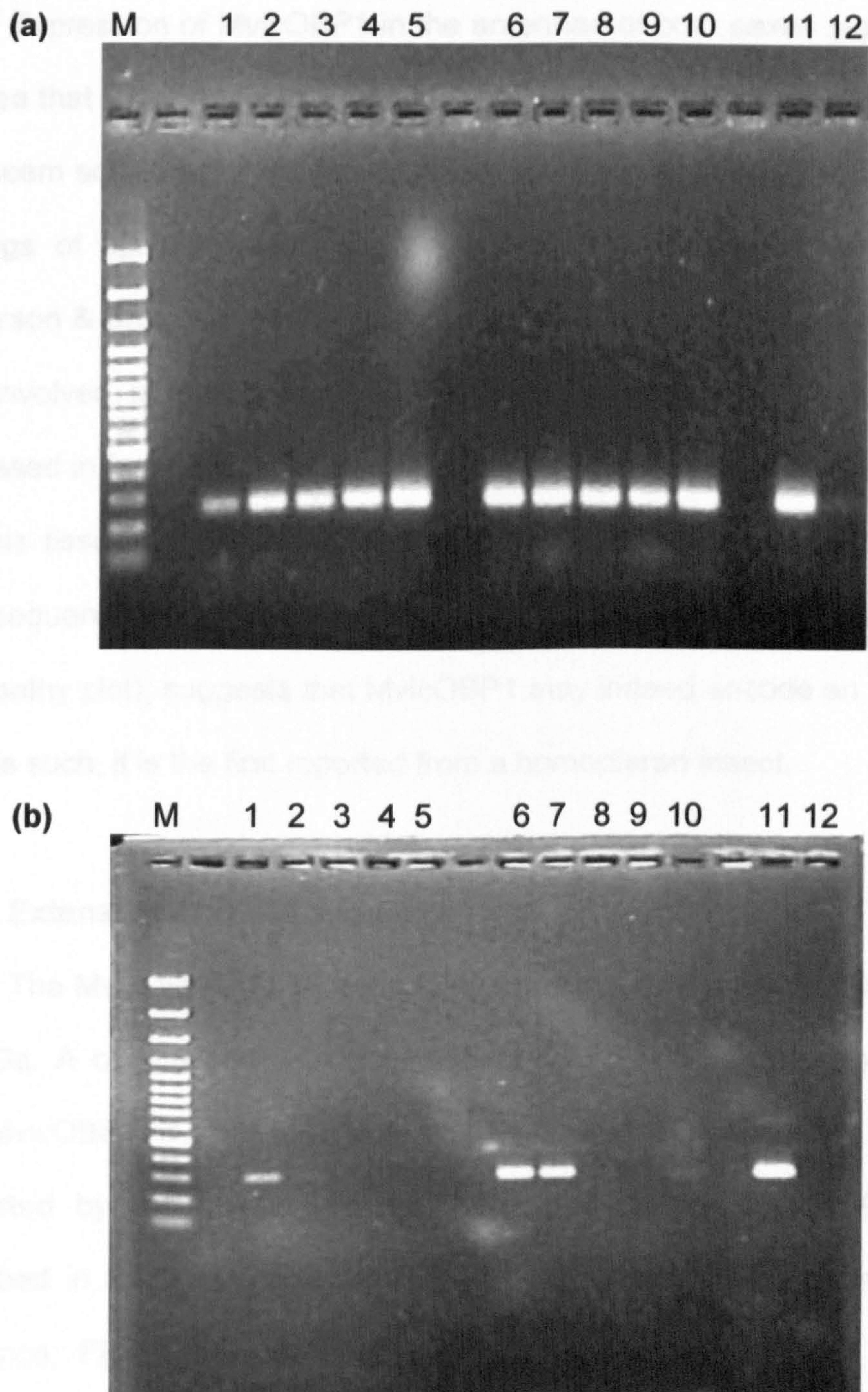


Figure 5.4 Results of RT-PCR on aphid tissue cDNAs using (a) actin GSPs AL38 and AL39 (see Appendix 1) and (b) MvicOBP1 GSPs AL34 and AL33. Fragments were separated on a 1.5% agarose gel. M = 100bp GeneRuler™ Plus DNA ladder (a), 100bp DNA ladder (b); 1 = female antennae; 2 = female legs; 3 = female heads; 4 = female abdomens; 5 = whole females; 6 = male antennae; 7 = male legs; 8 = male heads; 9 = male abdomens; 10 = whole males; 11 = positive control (MvicOBP1 / C35 pDNA); 12 = negative control (SDW as template).

Expression of MvicOBP1 in the antennae of both sexes supports the idea that it has a role in olfaction. The presence in the legs of males may seem somewhat anomalous, however, it has been suggested that the legs of aphids have gustatory functions in host plant selection (Anderson & Bromley, 1987), and thus MvicOBP1 may encode an OBP also involved in gustation. If so, it might be expected to also be expressed in female legs, which do indeed have a very faint band (Lane 2). This tissue expression pattern, coupled with the predicted amino acid sequence (i.e. conserved cysteine residues and characteristic hydropathy plot), suggests that MvicOBP1 may indeed encode an OBP, and, as such, it is the first reported from a homopteran insect.

(b) Extension of cDNA sequence

The MvicOBP1 cDNA encodes a protein with a predicted size of 14.6kDa. A comparison with other insect OBPs (14-20kDa) suggests that MvicOBP1 is not complete at the 5' end of the cDNA, also supported by the absence of an ATG start codon. 5' RACE [as described in 2.2.2.6(d)] was used to attempt to obtain the remaining sequence. Figure 5.5 shows the range of products obtained from tertiary PCRs using primers AL42 and AUAP (A) and AL43 and AUAP (B) (see Figures 5.1 and 2.1) (the primary and secondary reactions did not yield any visible products). The smallest discernible band produced with AL42 and AUAP (a) is ~300bp, and there appears to be a corresponding band (b) of ~250bp, produced with AL43. This difference (approximately 50bp) is consistent with the distance between the two

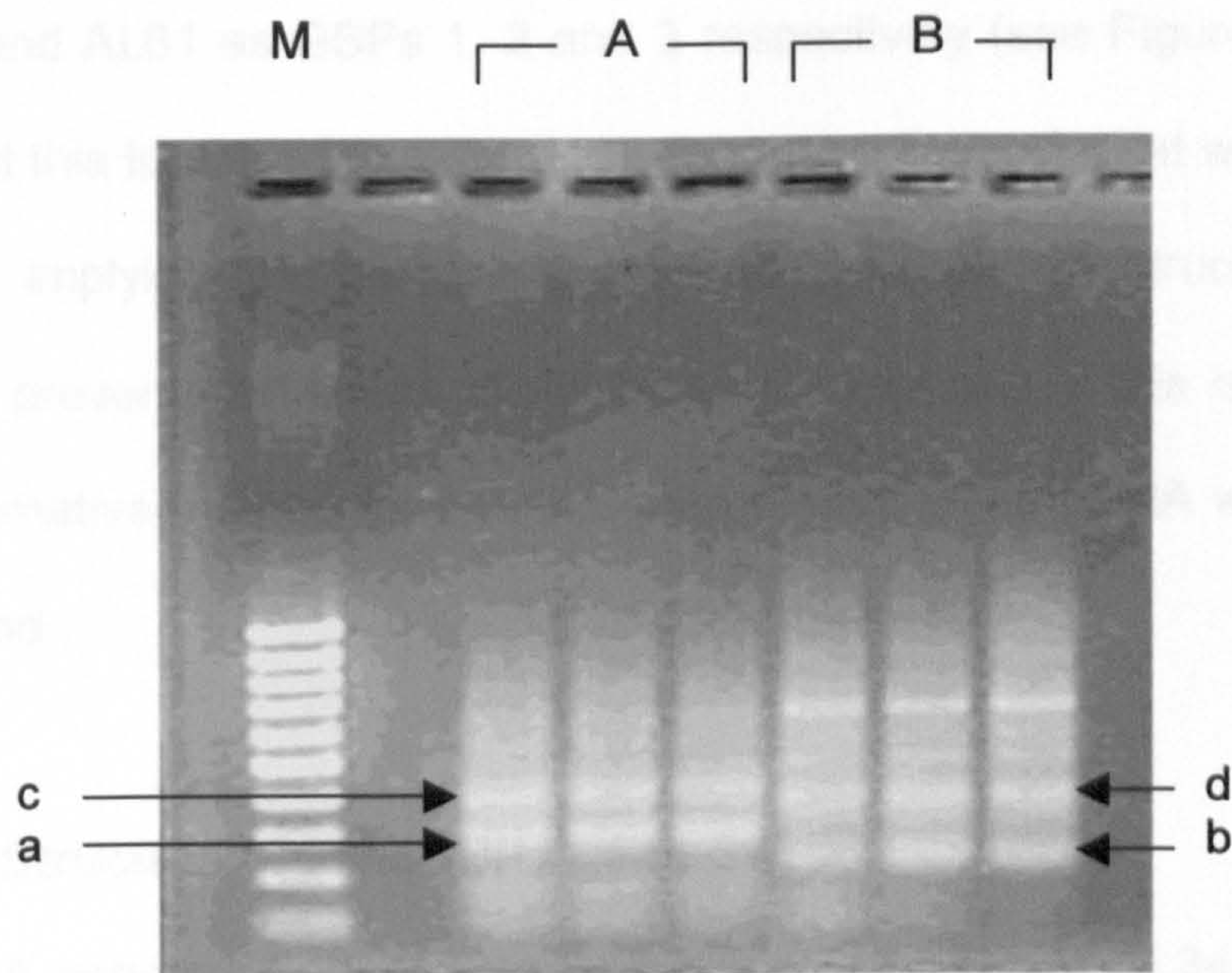


Figure 5.5 Results of 5' RACE tertiary PCR reactions, separated on a 1.5% agarose gel. M = 100bp DNA ladder; A = products generated with AL42 and AUAP; B = products generated with primers AL43 and AUAP. Arrows indicate bands discussed in the text.

primers used (see Figure 5.1). This suggests that the smaller fragments may be primed from the cDNA.

Cloning and sequencing of these fragments gave the already known sequence, but failed to extend it. The two larger fragments, (c) and (d), were also cloned and sequenced, but unfortunately these were found to be PCR artefacts. 5' RACE was repeated using primers AL42, AL43 and AL61 as GSPs 1, 2 and 3 respectively (see Figures 2.1 and 5.1) but this too failed to extend the sequence beyond what was already known, implying that perhaps some kind of secondary structure in the mRNA prevents full length cDNA being synthesised. If this is the case, an alternative method for obtaining the 5' end of the cDNA will need to be found.

(c) Structure of MvicOBP1 gene

A genomic fragment was amplified using GSPs AL34 and AL33 from oviparous female *M. viciae* gDNA, as shown in Figure 5.6. The fragment generated from *M.viciae* gDNA is ~1600bp, compared to 300bp from cDNA, showing that ~1300bp of intron sequences are present. The primers are both internal to the cDNA, so this fragment is only a partial gDNA, but probably covers a large proportion of the gene. The sequence is given in Figure 5.7 and a diagrammatic alignment of the gDNA and cDNA nucleotide sequences (see Figure 5.8) allows the designation of two introns of ~1160bp and 142bp, but neither the pattern nor the sizes of the introns resemble other insect OBP genes (Krieger *et al*, 1991; LaForest *et al*, 1999; Willett & Harrison, 1999a and

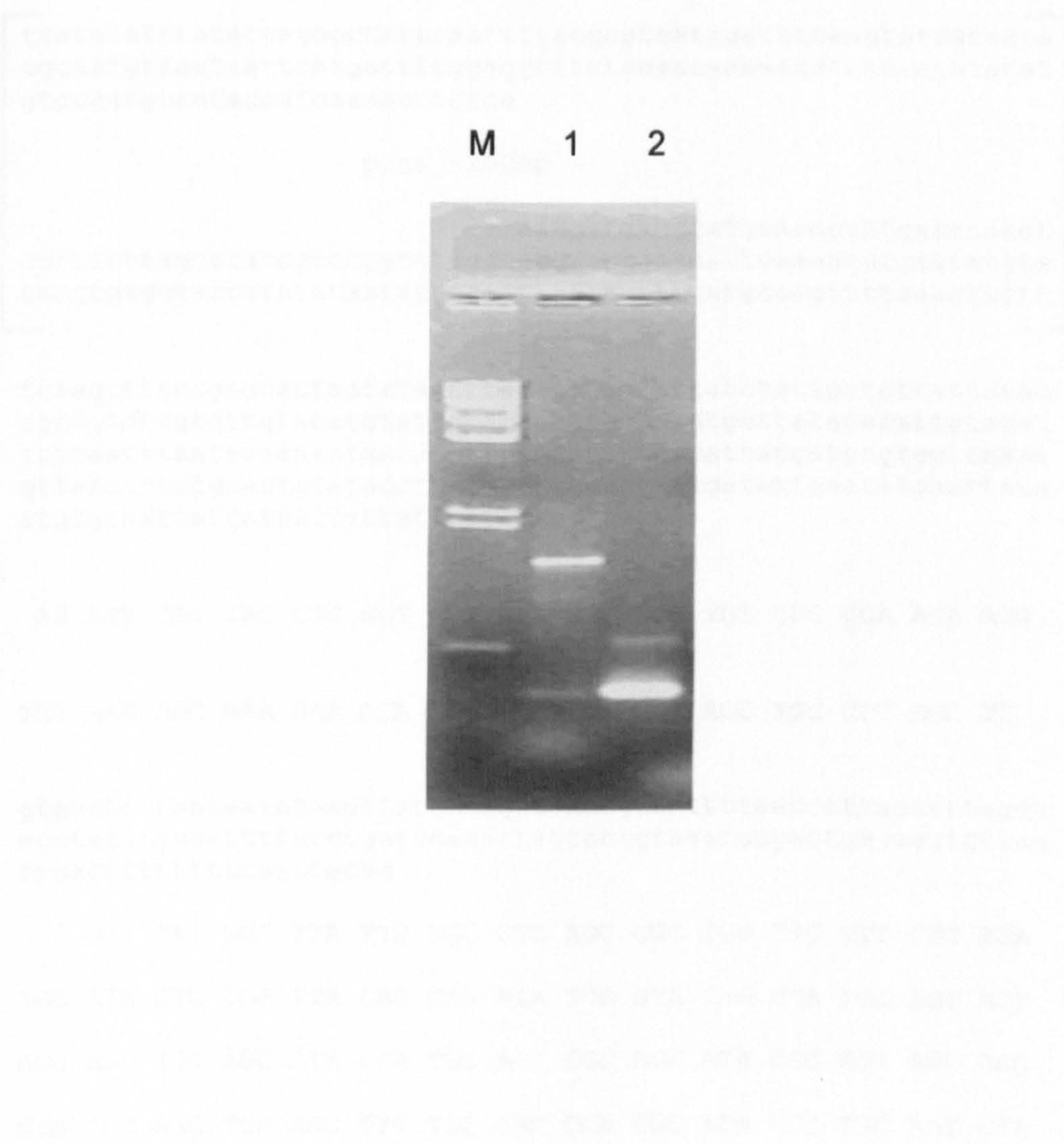


Figure 5.6 Products of PCR on *M. viciae* gDNA (1) and cDNA (2) using GSPs AL34 and AL33, separated on a 1.2% agarose gel. M = λ DNA/*HindIII* marker.

```
CAC CAA ACA CGA AAT GGC TTC TTT CAA GTA TCT G  gtgagtgacttta

acaagtttcatcgcctcatgaatattaaattgtttaagaccgataaatttttttgtgta
gctatacacacaataatatgtacctacgacttaatagttttcgatcgattattataaatt
atcttataaatacaaattcacacgtaaaatactcaattatacgccaatgtataatatttt
tcgaaaaatgtttgactcttaaaaaagaaacaactataattagtataccgatttatactg
taacatgatttgccttaataatataattgccaacacttttatcgagcacccgataaagct
cagatgtataacaacagtcggaggctttaatacattacttattatacatcttacttgata

[ttatatatttatattatcgctattcaattttatgcgtcatagatttcaagtataataata
cgcaatgtcaataattcatgatttcggaggtttctaaaacaaaaaaatcatcatatatat
gtccaatgcaacaccatcaaaaattctca

                    - plus ~200bp -

                                aatgttaacccatgaacactttgaaacaact
cgcttcttaggataccgtccggtttaggtgtgaacggaaaattcaaaatatcaatatgta
tacgtgtgctactatatataatatgaaagttataactttcatgcacgttttaaacttgtt]

tcaagctttccgagcactaatataatttacatttaatattatctattgatcttattctac
tgtcgtattgtattgtacatgtattgtacttattgctctgtgcttatacagattgtagat
tttcaatttaatagaaaaataacatattgtatattataacattatgatttgtggttaaaa
gttaaaactctgcactgtatacctattaaccgcgagttatgatattaaatatgaattata
atatgcaattattattactattattgttattatttacag

 AT ATT CAT CAC CTT GGT CGT GTA CTC CGT TGT CGC CGA AGA AGG

TGT AAG AGC AAA GAA ACA TGC CTA CCT CGC AGC TGC CCC AGC TC

gtgagtaccaataatataagttataatagcttaacgcacttttaacctttaaatttaggt
acctatatgaaattttacctgattcaaattattcctgtaaatccgactgagaggtgttaa
ttgattttttttttaattacag

  C GGT TTT GGG TTA TTC TGC CTC AGC CCC TGG TTC GTT CTC ATA

TGC ATA CTG CGA TTA CAC CAG ATA TCC GTA CAG CTA TCC AGT ATT

AGG AGC TGC AGC ATA CCA TGC ACC CGC AGC ATA CCC AGT AGC AGC

GTA CCC AGC TGC AGC TTT TGC CAC CCA CGC ATA CCC TGC AAT CTA
```

Figure 5.7 Nucleotide sequence of MvicOBP1 gDNA between GSPs AL34 and AL33. Coding regions are in capital letters, and introns are in lower case. Nucleotides in brackets are ambiguous.

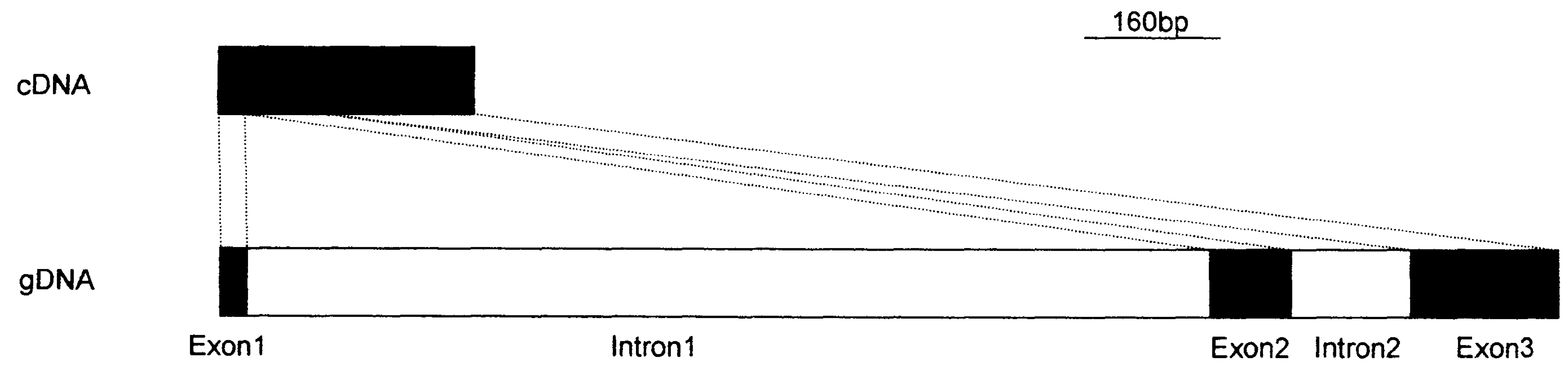


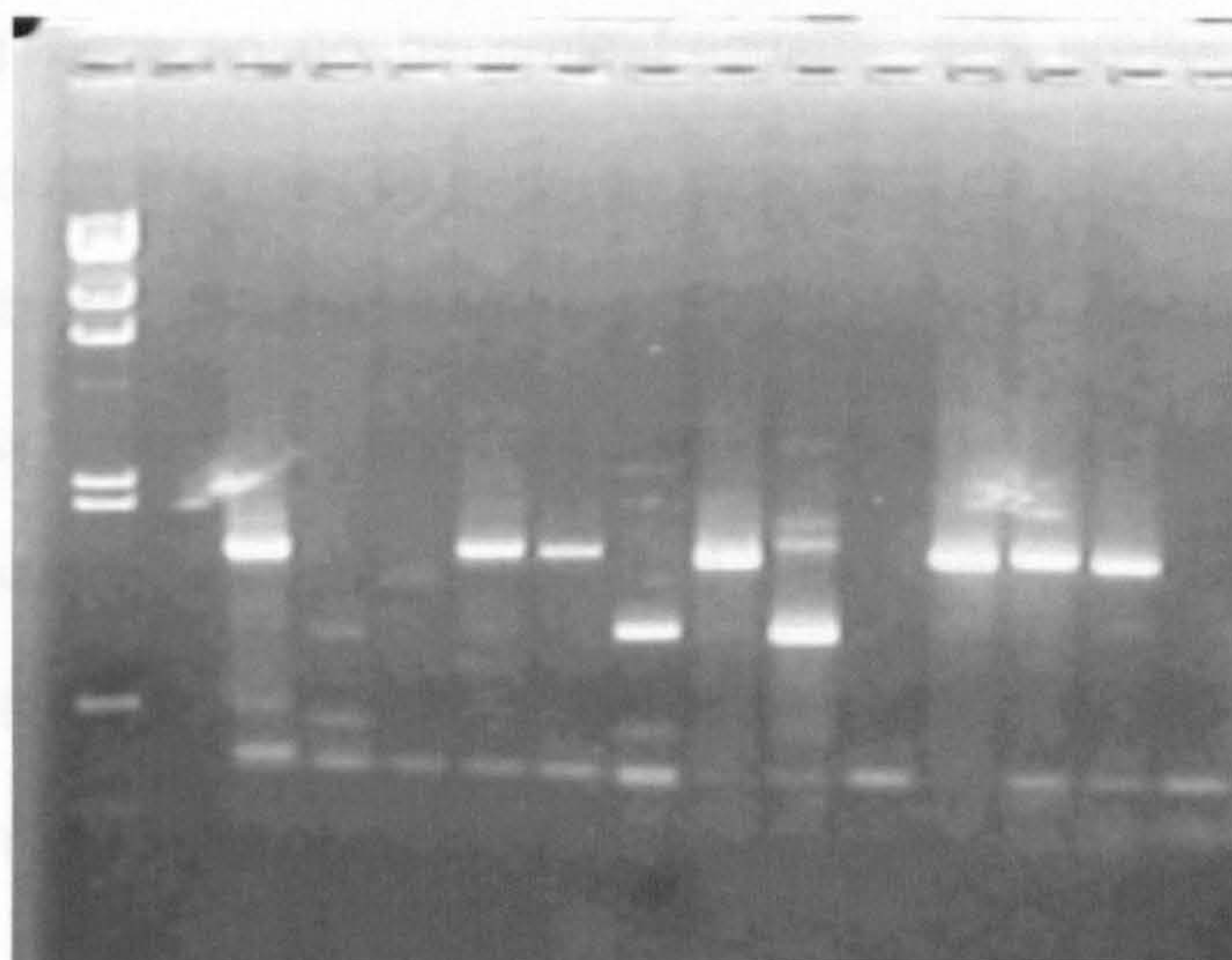
Figure 5.8 Structure of the MvicOBP1 cDNA (AL34-AL33) and the corresponding gDNA fragment. ■ = exons; □ = introns.

b; Willett, 2000a and b).

(d) Presence of genes related to MvicOBP1 in other aphid species

gDNA and cDNA were prepared from the aphid species listed in 5.2(d), and used for PCR using GSPs AL34 and AL33. The results are shown in Figure 5.9. Fragments produced from the gDNA of *M. dirhodum* (Lane 1), *N. ribis-nigri* (Lane 4), *A. fabae* (Lane 5), and *M.persicae* (Lane 11) were ~1.6kb, the same size as the fragment from *M. viciae* (Lanes 7 and 10) [Figure 5.9(a)]. *L. erysimi* (Lane 6) and *S. avenae* (Lane 8) gave strong bands of ~1.0kb. The gDNA for *L. erysimi* also gave a fragment of ~1.6kb, and although this is fainter than the main product, it may suggest the presence of two related genes in this species. The gDNA from *R. padi* failed to give a product, and *B. brassicae* and *A. pisum* gave very faint bands (Lanes 2 and 3 respectively). This may suggest that there is a gene that is related to MvicOBP1 in these species, but the sequence similarity may be less than for the other species. Thus, it is likely that at least nine other aphid species have a gene related to MvicOBP1. The smaller size of the fragments from *L. erysimi* and *S. avenae* probably reflects the presence of smaller introns in this gene of these species.

RT-PCR was used to determine whether the MvicOBP1-related genes are expressed in these other aphids [see Figure 5.9(b)]. Fragments of the same size as the positive control (Lane 12) were amplified from all species except *L. erysimi* (Lane 6), indicating that



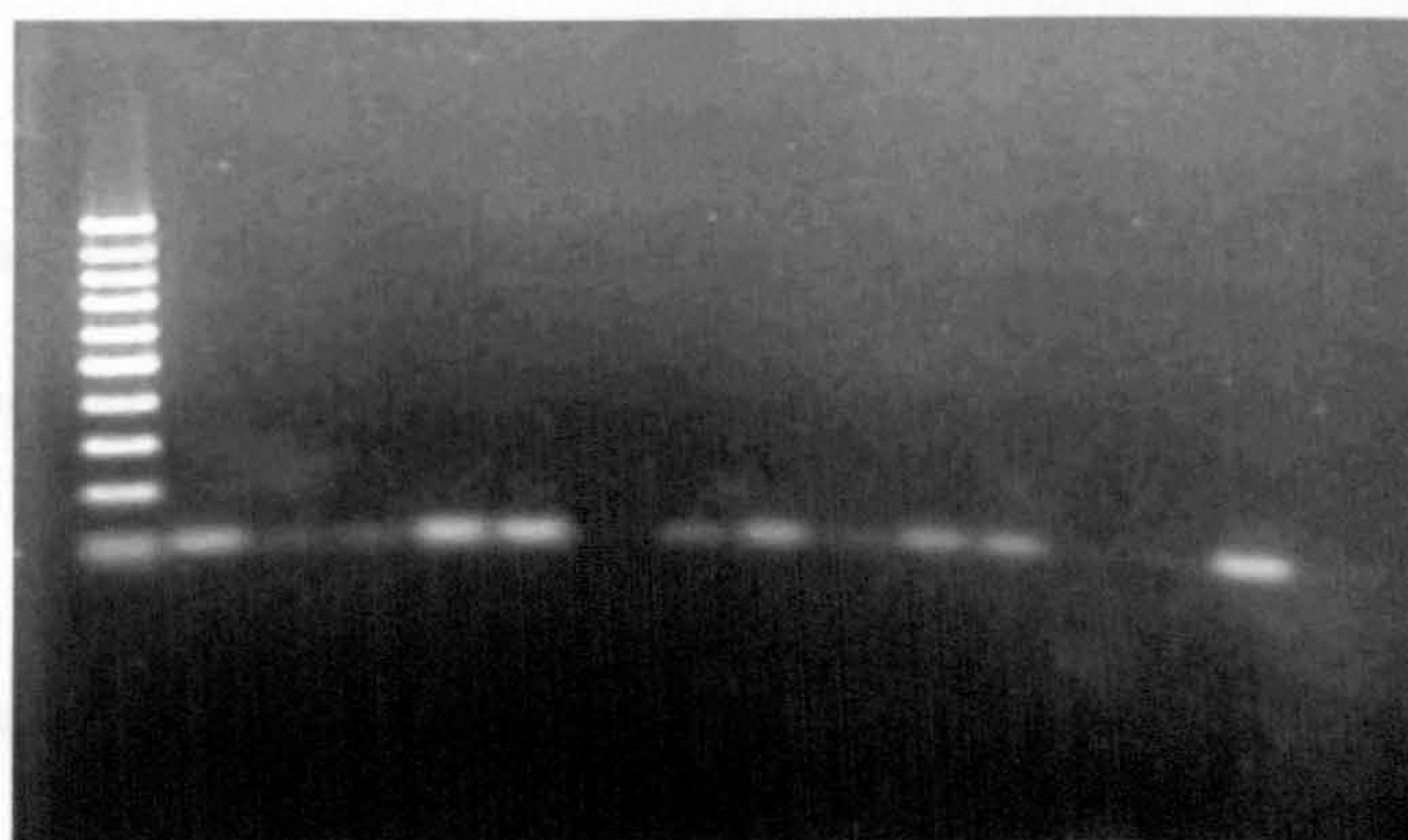


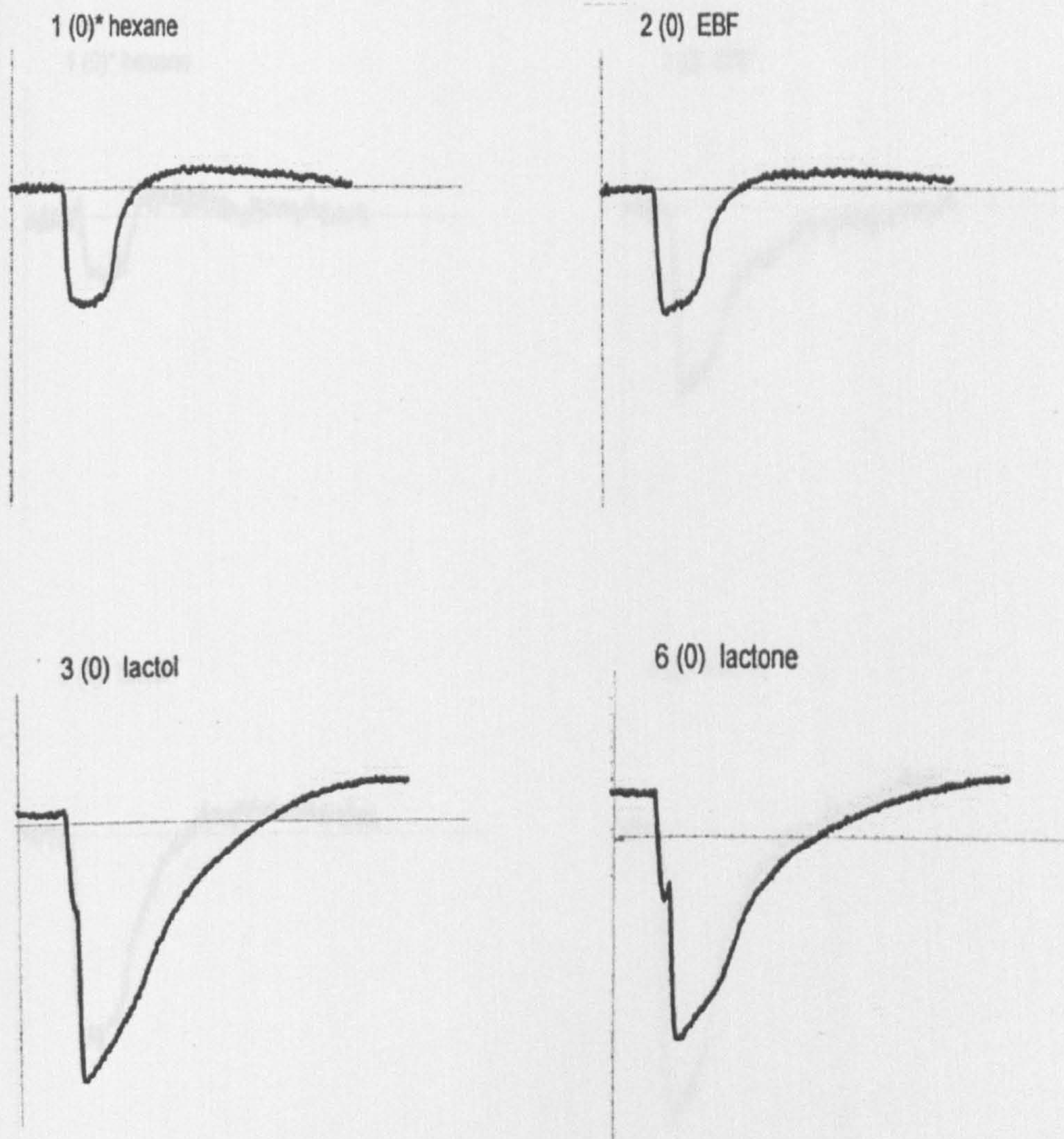
Figure 5.9 Fragments generated by PCR on gDNA (a) and cDNA (b) from ten aphid species using primers AL34 and AL33, separated on 1.2% (a) and 1.5% (b) agarose gels. M = λDNA/*HindIII* marker (a), 100bp ladder (b); 1 = *M. dirhodum*; 2 = *B. brassicae*; 3 = *A. pisum*; 4 = *N. ribis-nigri*; 5 = *A. fabae*; 6 = *L. erysimi*; 7 = *M.viciae* (virginoparae); 8 = *S. avenae*; 9 = *R. padi*; 10 = *M. viciae* (oviparae); 11 = *M. persicae*; 12 = positive control [*M. viciae* gDNA(a), MvicOBP1 pDNA (b)]; 13 = negative control (SDW as template).

species are expressing the MvicOBP1-related genes. The *R. padi* result is interesting because no genomic fragment was produced for this species, although this may be because the extension time was too short to amplify a larger genomic fragment. RT-PCR on *B. brassicae* and *A. pisum* cDNAs produced very faint bands, as was the case with the gDNA, supporting the suggestion that this gene is perhaps more distantly-related than those from the other species. *L. erysimi*, which has a genomic fragment, (Figure 5.9 (a); Lane 6) does not have a corresponding cDNA (Figure 5.9(b); Lane 6), possibly because the gene is redundant. How similar these genes are to MvicOBP1, and whether or not they have a role in olfaction in these other species remains to be established; cloning, sequencing and tissue expression patterns would need to be investigated.

(e) Electrophysiological studies

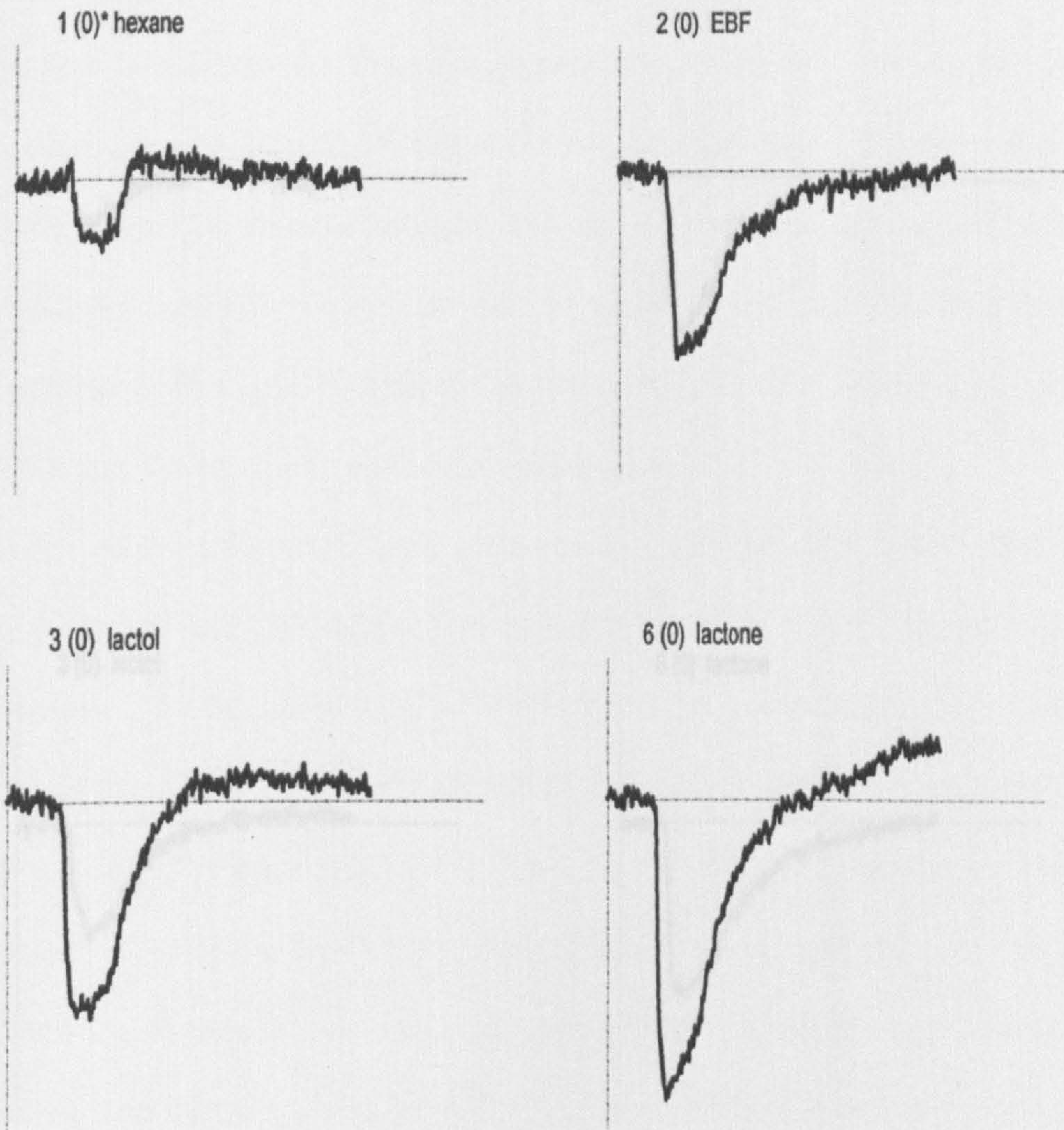
The antennal responses of the three *M. viciae* morphs (male, virginoparae and oviparae) to the test compounds (EBF, the nepetalactol and the nepetalactone) are shown in Figure 5.10 – 5.12. EAG recordings for each of the three stimuli are shown alongside the response generated for hexane, the solvent control. The compounds were chosen in an attempt to determine what type of OBP MvicOBP1 might be. The table in each figure shows the responses as a percentage of the control response.

The results show that all three morphs are similarly responsive to the nepetalactone. The two female morphs are equally responsive to



Stimulus	% Response
(*E*)-β-farnesene (EBF)	119 ± 6.87
Nepetalactol	424 ± 102.37
Nepetalactone	485 ± 103.34

Figure 5.10. Responses of male *M. viciae* to the alarm pheromone (EBF) and to both components of the sex pheromone (nepetalactol and nepetalactone). The table shows the EAG responses expressed as a percentage of the response to the solvent control (hexane). Results from Woodcock, unpublished.



Stimulus	% Response
(*E*)-β-farnesene (EBF)	318 ± 32.15
Nepetalactol	246 ± 30.49
Nepetalactone	400 ±68.44

Figure 5.11. Responses of virginoparous *M. viciae* to the alarm pheromone (EBF) and to both components of the sex pheromone (nepetalacol and nepetalactone). The table shows the EAG responses expressed as a percentage of the response to the solvent control (hexane). Results from Woodcock, unpublished.

1 (0)* hexane

2 (0) EBF

3 (0) lactol

6 (0) lactone

Stimulus	% Response
(*E*)-β-farnesene	351 ± 50.72
Nepetalactol	238 ± 46.15
Nepetalactone	469 ± 48.47

Figure 5.12. Responses of oviparous *M. viciae* to the alarm pheromone (EBF) and to both components of the sex pheromone (nepetalactol and nepetalactone). The table shows the EAG responses expressed as a percentage of the response to the solvent control (hexane). Results from Woodcock, unpublished.

the nepetalactol (Figure 5.11 & 5.12), but the male shows a much greater sensitivity to this compound (Figure 5.10), although these figures are not significant because of the high standard error for the male. The two female morphs are equally responsive to the alarm pheromone (EBF), whilst the male is significantly less sensitive to this compound ($P < 0.001$). This is the first indication of oviparous *M. viciae* detecting the sex pheromone components.

Since both male and oviparous *M. viciae* can detect the sex pheromone, and MvicOBP1 is present in both [Figure 5.4(b)], it is possible that MvicOBP1 is a PBP-type OBP, responsible for binding one of the sex pheromone components. Male *M. viciae* is less sensitive to the alarm pheromone (EBF) than the female morphs, so one might expect there to be less OBP in male antennae to bind this compound. Since the expression in antennae of MvicOBP1 appears to be equal in males and females [Figure 5.4(b)], a role of EBF-binding protein for MvicOBP1 seems unlikely.

5.4.2 Characterisation of Mv164

(a) Tissue distribution

The finding of a cytochrome P450 cDNA in an antennal library from *M. viciae* suggested a role in the clearance of an odour signal, as seen in Ahmad *et al*, 1987; Hovemann *et al*, 1997; and Wojtasek & Leal, 1999a. However, insect cytochromes P450s, of one subfamily or another, are present in practically all insect tissues (Feyereisen, 1999;

Scott *et al*, 1998), so it was important to investigate whether Mv164 had a specific association with chemosensory tissues in *M.viciae*. Figure 5.13 shows the results of RT-PCR on *M. viciae* tissues using Mv164 GSPs (AL40 and AL41; see Figure 5.2). There are fragments of the expected size (363bp) amplified in all tissues. However, the intensity of the band in the antennae of males and females, and the legs of males, is much greater than for the other tissues. Since identical amounts of RNA were used in RT-PCR, this higher intensity should reflect a higher level of expression, which results in higher levels of mRNA. This implies that the gene is over-expressed in these tissues, and therefore may have a role in olfaction, perhaps in clearance or degradation of the signal. This suggestion is further strengthened by the similarity in the tissue expression pattern of this cDNA to that of MvicOBP1, the putative OBP, which may suggest a "link" or association between expressions of these two genes.

(b) Structure of Mv164 gene

Since insect cytochrome P450s are ~500-700 amino acids long (Hovemann *et al*, 1997), and Mv164 cDNA encodes only 151 amino acids, this cDNA represents approximately one third of the gene, at the 3' end. However, the genomic arrangement of Mv164 was studied using the same method as for MvicOBP1. A genomic fragment of ~820bp was amplified using GSPs (AL40 and AL41; see Figure 5.2), compared to 363bp for the cDNA, as shown in Figure 5.14, showing that ~460bp of intron sequences are present. This fragment was cloned into a PCR

M 1 2 3 4 5 6 7 8 9 10 11 12

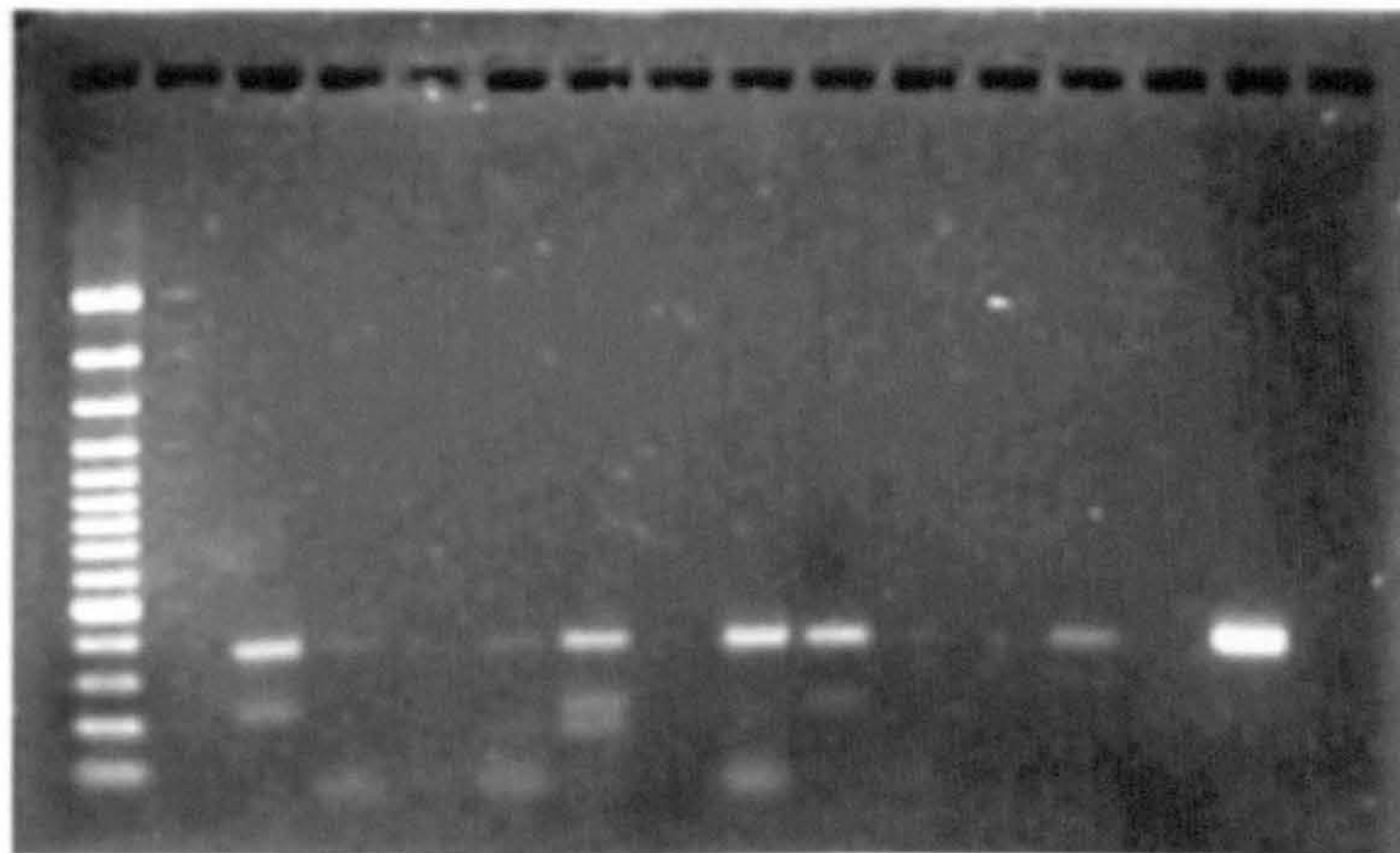


Figure 5.13 Results of RT-PCR on *M. viciae* tissue cDNAs using Mv164 GSPs AL40 and AL41, separated on a 1.5% agarose gel. M = 100bp GeneRuler™ Plus ladder (MBI Fermentas); 1 = female antennae; 2 = female legs; 3 = female heads; 4 = female abdomens; 5 = whole females; 6 = male antennae; 7 = male legs; 8 = male heads; 9 = male abdomens; 10 = whole males; 11 = positive control (Mv164 pDNA); 12 = negative control (SDW as template).

Figure 5.14 Products of PCR on M. viciae gDNA (1) and cDNA (2) using AL40 and AL41, separated on a 1.2% agarose gel. M = 100bp DNA ladder.

cloning vector, sequenced, and the sequence aligned with the cDNA to show that this region of the P450 gene covers two exons and one intron. The genomic sequence and intron/exon structure are shown in Figure 5.15.

(c) Presence of genes related to Mv164 in other aphid species

Genomic fragments from genes related to Mv164 were amplified from other aphid species using Mv164 GSPs AL40 and AL41 (see Figure 5.1). Figure 5.16(a) shows that fragments were amplified in five of the ten species: *B. brassicae* (Lane 2), *L. erysimi* (Lane 6), *S. avenae* (Lane 8), *M. viciae* (Lanes 7 & 10), and *M. persicae* (Lane 11), and ranged in size from 0.9kb to 1.2kb compared to the ~0.9kb fragment generated for *M. viciae*. Again, the size variation probably results from different sizes of introns in the different species. The gDNA of *M. dirhodum* produced a very faint band, suggesting that this species may have a more distantly-related gene. *A. pisum*, *N. ribis-nigri*, *A. fabae*, and *R. padi* did not produce fragments, suggesting that Mv164-related genes do not exist in these species.

Using RT-PCR, fragments were amplified from the same species [Figure 5.16(b)], with the exception of *A. pisum*. Here a fragment was produced from cDNA but not from gDNA. A possible explanation for this is that the genomic fragment is too large to be amplified with the extension time used in the PCRs. These fragments are of the same size as the positive control (Lane 12), and there is no band in the negative control (Lane 13). Therefore, there does not appear to be any Mv164-

TC GAC CAC TTG GTG AAC CTG CGG GGC GAA CAG TGG AAA GCA ATT

AGG GCC AAG TTG AGT CCG ACC TTC TCG GCG GCC AAG CTC AAG TCC

ATG TTG GGT GAC ATA AAT GTG TGC ACG GCG CGT CTG ATC GAG AAC

TTA AAC GGG CAG ATA ACG AAA AAC AGT G gtaatgataaaatt

tacctaaatgtagctatgttatgcatgtaaatatatacaggttaggtatcggcgaaattc
atcatttgctgtactcgcgtcgaaatgtgtaatttggtcacaaatttaatcattgttat
acggtatatacaatagtacagagacaataaaaaaaattccgtagccgcgcgatgaccaa
aaatagtgataggacgtgatatttggatatgtatgcctaatgcctatacgtaatcggatt
actgtaagcacccgcatcctttattagacgctaatatattttatacattacaaatattc
aatattttaatagtacatatatttattgttatgtgaccaaaatacaaaatgcataaatta
attcctaggtacttattatttatctattatgagtattataataaatgtacctaaaaata
aagacaaaaatataaatgttttacag

GA ATT GTT GAT GTA AGT

GAA GCG TCG GCT CAA TTT ACA ACT GAT ACT ATT GGC AGT TGT GCT

TTT GGA CTA CAC TGC AAT TCA TTA TCA GAT CCA GAT TCA GAA TTT

CGT CGT ACT GGA CGA GCC ATA TTT ACG ACA AGT CTT CGA TCA ACT

TTA CTA AAT CTC ATC AGA TTA GTT GGT TTT GGT CGG CTT TTA GA

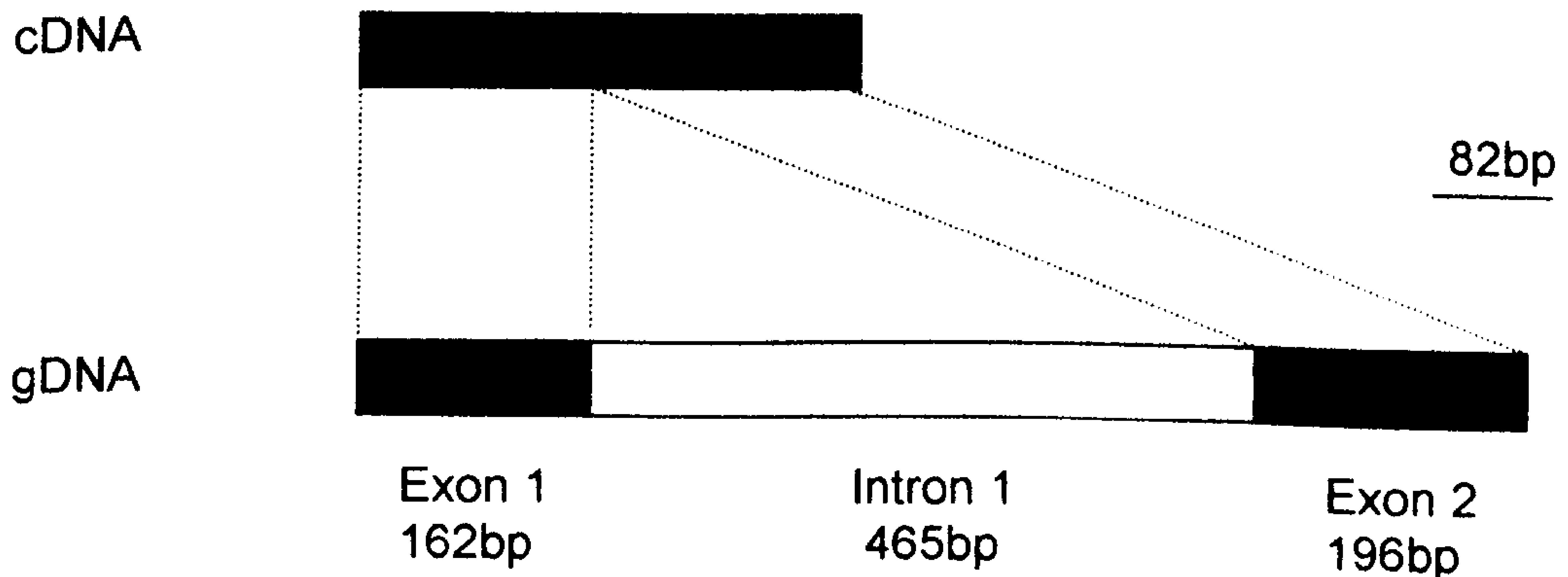
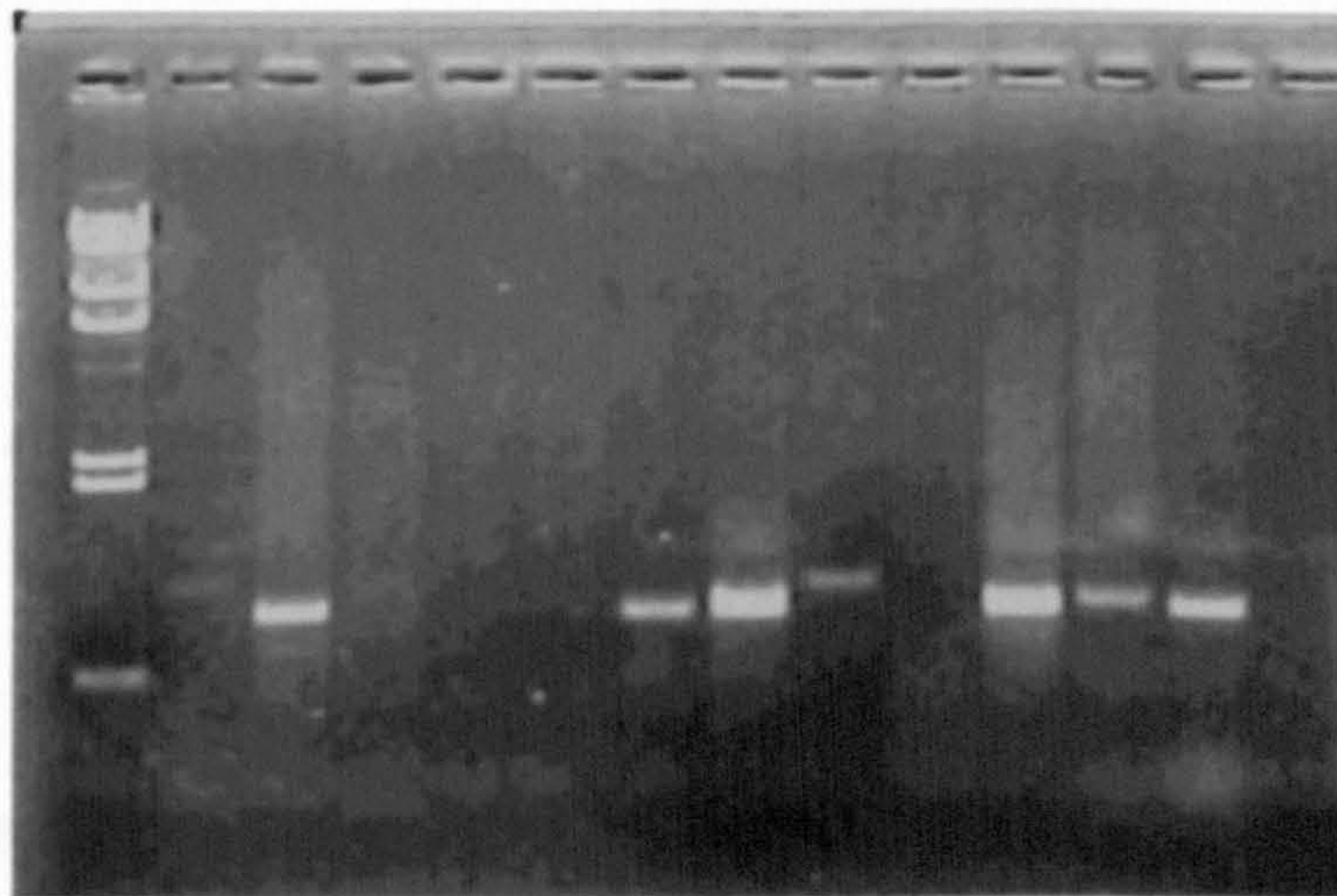


Figure 5.15 Top: Nucleotide sequence of Mv164 gDNA. Coding regions are presented in capital letters, and introns are presented in lower case. Bottom: Structure of the Mv164 cDNA (AL40-AL41) and the corresponding gDNA fragment. ■ = exons; □ = introns.



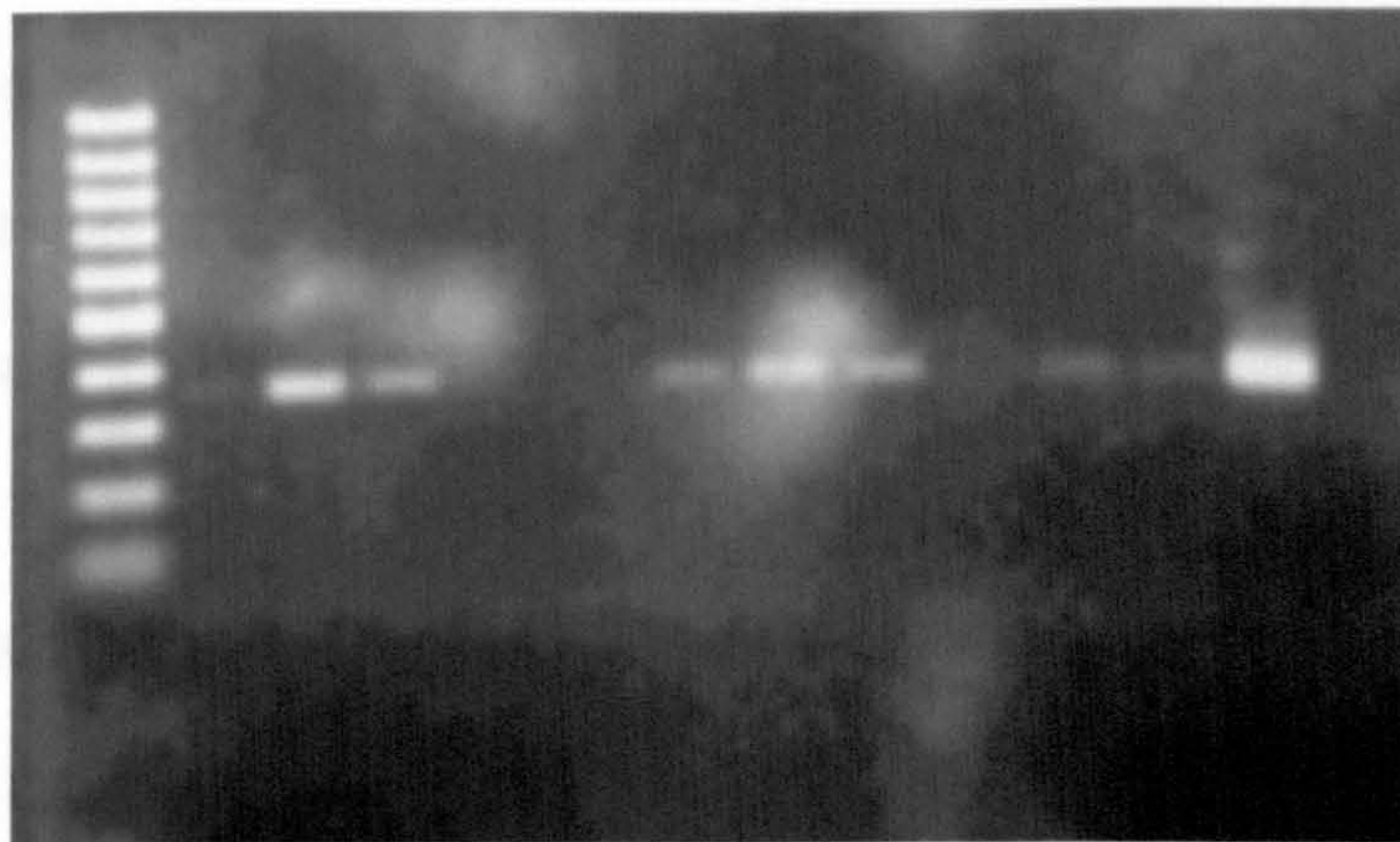


Figure 5.16 Fragments, generated by PCR using gDNA (a) and cDNA (b) from ten aphid species with GSPs AL40 and AL41, separated on 1.2% (a) and 1.5% (b) agarose gels. M = λDNA/*HindIII* (a), 100bp DNA ladder (b); 1 = *M. dirhodum*; 2 = *B. brassicae*; 3 = *A. pisum*; 4 = *N. ribis-nigri*; 5 = *A. fabae*; 6 = *L. erysimi*; 7 = *M. viciae* (virginoparae); 8 = *S. avenae*; 9 = *R. padi*; 10 = *M. viciae* (oviparae); 11 = *M. persicae*; 12 = positive control (Mv164 pDNA); 13 = negative control (SDW as template).

related genes that are redundant. As with MvicOBP1, determination of tissue expression patterns and sequencing of these genes in other aphids might provide further evidence of a potential role in olfaction.

(d) Association between MvicOBP1 and Mv164

The similar tissue distribution patterns of MvicOBP1 and Mv164 in *M. viciae* suggests that the expression of these genes may be "linked", or associated in some way, and it may be that MvicOBP1 is an OBP and Mv164 the cytochrome P450 that is involved in degradation of the signal odour it binds. The question then arises: does this "association" between these two genes occur in the other aphid species studied. Table 5.1 summarises the presence and expression of these genes in the ten different species, showing that the presence and expression of both genes occurs only in *M. viciae, S. avenae* and *M. persicae*. This suggests that these three species may share a common odour that is not detected by the others. Clearly, further studies of both MvicOBP1 and Mv164 are necessary to establish the role of these genes.

5.4.3 Characterisation of aphid MvicCSP1

(a) Tissue distribution

The finding of two aphid CSPs in a subtracted antennal cDNA library potentially broadens the range of proteins involved in the detection of odours. However, although two subtractions were used to produce the libraries, the tissue distribution patterns of these two

Pattern	Aphid species									
	M. dirhodum	*B. brassicae*	*A. pisum*	*N. ribis-nigri*	*A. fabae*	*L. erysimi*	*M. viciae*	*S. avenae*	*R. padi*	*M. persicae*
MvicOBP1 gDNA present	++	+	+	++	++	++	++	++	-	++
MvicOBP1 mRNA present	++	+	+	++	++	-	++	++	+	++
Mv164 gDNA present	+	++	-	-	-	++	++	+	-	++
Mv164 mRNA present	+	++	++	-	-	+	++	++	-	+

Table 5.1 Table showing patterns of presence and expression of MvicOBP1 and Mv164 in various aphid species. + and ++ indicate levels of gDNA/mRNA presence; - = gDNA/mRNA absent.

cDNAs were investigated in order to determine whether they were antennal specific. Figure 5.17 shows the preliminary result of RT-PCR on *M. viciae* tissues using MvicCSP1 GSPs (AL46 and AL47; see Figure 5.3). Fragments are produced in all tissues with the exception of whole females (Lane 5), which is unexpected since all other female tissues have given a positive result. Female legs, heads and abdomens have given fainter bands than the other tissues, which may reflect different levels of expression, or this may be an erroneous result of this PCR. Also unexpectedly, the positive control (Lane 11) has failed to produce a result. However, this is the first RT-PCR to test this cDNA, and as such is a very preliminary result.

Other insect CSPs have been shown to be expressed mainly in the legs and antennae of the insect (Nomura *et al*, 1992; Pikielny *et al*, 1994; McKenna *et al*, 1994; Mameli *et al*, 1996; Tuccini *et al*, 1996; Bohbot *et al*, 1998; Picimbon & Leal, 1999; Picimbon *et al*, 2000a), although there are examples of CSPs being expressed in other tissues (Maleszka & Stange, 1997; Thymianou *et al*, 1998; Kitabayashi *et al*, 1998; Angeli *et al*, 1999; Picimbon *et al*, 2000b; Nagnan-Le Meillour *et al*, 2000). It is possible that MvicCSP1 is expressed in all aphid tissues, and is not therefore associated with a particular tissue in the way OBPs are. Aphids are covered in tiny hairs (Anderson & Bromley, 1987), which may have a sensory function, and the CSPs may be associated with these hairs. A preliminary RT-PCR using MvicCSP2 (result not presented) gave the same result as for MvicCSP1, with the cDNA being expressed in all tissues, again suggesting a non-olfactory

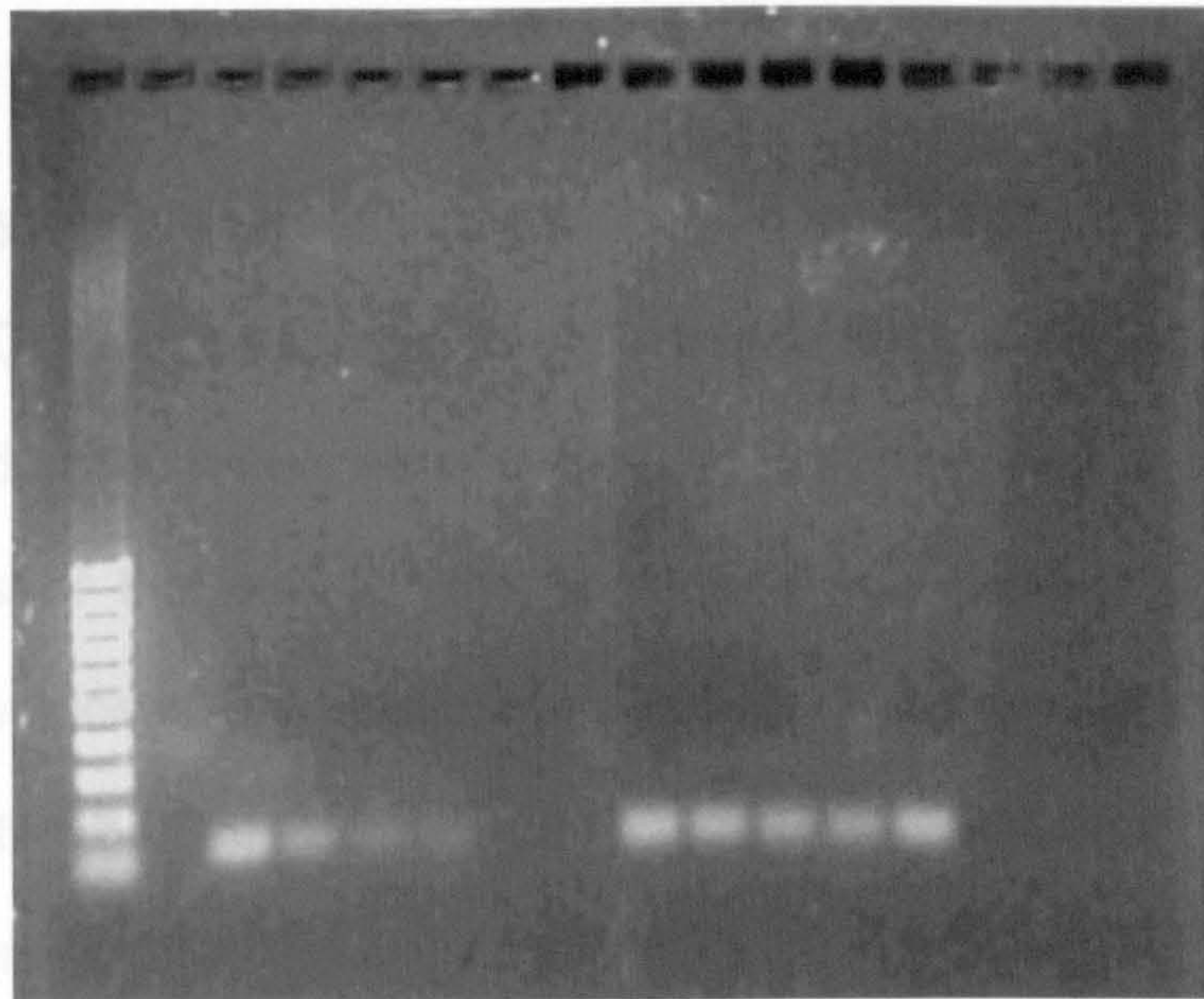


Figure 5.17. Results of RT-PCR on *M. viciae* tissue cDNAs using MvicCSP1 GSPs AL46 and AL47, separated on a 1.5% agarose gel. M = 100bp ladder (MBI Fermentas); 1 = female antennae; 2 = female legs; 3 = female heads; 4 = female abdomens; 5 = whole females; 6 = male antennae; 7 = male legs; 8 = male heads; 9 = male abdomens; 10 = whole males; 11 = positive control (MvicCSP1 pDNA); 12 = negative control (SDW as template).

chemosensory role for these CSPs.

5.5 Conclusions

Antennal cDNA libraries had been used to isolate two clones, MvicOBP1 and Mv164, which encoded proteins with characteristics for involvement in olfaction. The amino acid sequence of MvicOBP1 showed six cysteine residues in a spacing pattern similar to that seen in other insect OBPs. (see Chapter 3.4) Further investigation showed it to be expressed in the antennae of male and oviparous female *M. viciae*, and the legs of the male of the species, which have a known or suggested chemosensory function respectively. Therefore MvicOBP1 is the first putative OBP to be cloned and characterised from a homopteran insect.

Electrophysiological studies on oviparous, virginoparous and male *M. viciae* showed that all three morphs were capable of detecting both components of the sex pheromone, and the alarm pheromone. The male showed greater sensitivity to the nepetalactol component of the sex pheromone compared with the females, and showed significantly less sensitivity to the alarm pheromone. This is the first indication of female *M. viciae* detecting the sex pheromone. On the basis of this report, and the equal levels of expression of MvicOBP1 in the antennae of both sexes, a putative role as a PBP-type OBP has been suggested for MvicOBP1.

The genomic arrangement of MvicOBP1 was established using PCR and sequence analysis, showing that the coding region between

AL34 and AL33, which spans 1.6kb of the genome, comprises 3 exons and 2 introns. Closely related gene sequences were detected in *M. dirhodum, N. ribis-nigri, A. fabae, L. erysimi, S. avenae,* and *M. persicae,* and all but *L. erysimi* transcribe these genes into mRNA.Mv164 showed 44% similarity to other insect cytochrome P450s (see Chapter 3.4), and has enhanced expression in the antennae of males and females, and in the legs of males. This is a similar pattern to MvicOBP1, suggesting that it may have an olfactory role, and that it may be associated with MvicOBP1 in some way. A partial genomic sequence was determined, showing that the region of the DNA between AL40 and AL41 has one intron. Other aphid species, *B. brassicae*, *L. erysimi*, *S. avenae*, and *M. persicae,* were shown to have related genes, and to express them, whilst *A. pisum* appears to express a gene for which no corresponding genomic fragment could be produced. In *S. avenae*, *M. persicae*, and *M. viciae* there appears to be a correlation in the expression of MvicOBP1 and Mv164, again suggesting a "link" between these two genes, and adding further evidence to the suggestion of Mv164 having a specific role in olfaction.

Subtracted antennal cDNA libraries had been used to isolate two cDNAs, MvicCSP1 and MvicCSP2, which encoded proteins with characteristics of insect CSPs, and which showed 81-64% and 72-52% similarity to these CSPs at the amino acid level respectively. Preliminary investigations showed these two cDNAs to be expressed in all aphid tissues, which suggest that they may not be restricted to an olfactory role.

Chapter 6. Expression and biochemical studies of a *Bombyx mori* PBP (BmorPBP1) and aphid antennal proteins

6.1 Introduction

The silk moth *B. mori* has been widely used in molecular, biochemical and chemical studies of OBPs (Maida & Pelosi, 1989; Steinbrecht *et al*, 1992; Maida *et al*, 1993; Steinbrecht *et al*, 1994; 1995; Krieger *et al*, 1996; Maida *et al*, 1997; Campanacci *et al*, 1999; Leal *et al*, 1999; Scaloni *et al*, 1999; Wojtasek & Leal, 1999b; Sandler *et al*, 2000; Leal, 2000; Damberger *et al*, 2000, and Oldham *et al*, 2000). Several cDNAs encoding OBPs have been cloned from the antennae of this insect, including a PBP (BmorPBP1), and two GOBPs (BmorGOBP1 and BmorGOBP2) (Krieger *et al*, 1996). A fourth cDNA encoding an antennal binding protein X (BmorABPX) shows higher homology to the putative OBPs of *D. melanogaster* (26.3% at the amino acid level) than to the other reported *B.mori* OBPs (11-17%; Krieger *et al* 1996). Although the ABPX protein is smaller than other insect OBPs (12.8kDa), it has the characteristic conserved cysteine profile and signal peptide. These factors, coupled with specific expression in the antennae, support a function as an OBP. Most recently, cDNAs encoding CSPs, BmorCSP1 and BmorCSP2, have been cloned and shown to be expressed in the antennae, legs, thorax, abdomen and head of *B. mori* (Picimbon *et al*, 2000b).

Immunocytochemical studies have demonstrated the presence of four PBPs in the antennae of male *B.mori*, and two in females, as well as three GOBP2 proteins in males and five in females (Maida *et al*, 1997), and this diversity supports the hypothesis that OBPs may play a major role in molecular discrimination.

The sequence of the cDNA encoding BmorPBP1 has been reported by Krieger *et al* (1996), and the nucleotide and predicted amino acid sequences are shown in Figure 6.1. J. Krieger and H. Breer kindly supplied the cDNA, cloned in the plasmid vector pBluescript II SK, for the current expression studies.

6.2 Aims of the study

This part of the study used the BmorPBP1 cDNA as a "model" gene to test the feasibility of *in vitro* expression and characterisation of a recombinant OBP. The aims were:

- To subclone the BmorPBP1 cDNA into the pFASTBAC vector
- To express BmorPBP1 using a baculovirus expression system
- To confirm the production of recombinant protein using an anti-BmorPBP1 antibody
- To characterise the recombinant protein
- To test for any cross-reactivity between the anti-PBP1 antibody and soluble proteins from the antennae of the aphid *M. viciae*.

```
 M   S   I   Q   G   Q   I   A   L   A   L   M   V   N   M   A
ATG TCT ATC CAA GGA CAG ATC GCT TTG GCG CTC ATG GTC AAC ATG GCT

 V   G   S   V   D   A   S   Q   E   V   M   K   N   L   S   L
GTG GGC TCA GTG GAT GCG TCT CAA GAA GTC ATG AAG AAC TTA TCC TTA

 N   F   G   K   A   L   D   E   C   K   K   E   M   T   L   T
AAT TTC GGT AAA GCA TTG GAC GAA TGC AAA AAA GAG ATG ACG CTA ACA

 D   A   I   N   E   D   F   Y   N   F   W   K   E   G   Y   E
GAT GCG ATC AAT GAA GAC TTC TAC AAT TTC TGG AAA GAG GGA TAT GAA

 I   K   N   R   E   T   G   C   A   I   M   C   L   S   T   K
ATT AAA AAC CGG GAG ACT GGA TGC GCT ATA ATG TGC CTT TCC ACC AAG

 L   N   M   L   D   P   E   G   N   L   H   H   G   N   A   M
CTA AAC ATG CTC GAC CCT GAA GGA AAC CTT CAT CAC GGA AAC GCT ATG

 E   F   A   K   K   H   G   A   D   E   T   M   A   Q   Q   L
GAG TTT GCC AAG AAA CAT GGA GCC GAT GAG ACG ATG GCC CAA CAA CTG

 I   D   I   V   H   G   C   E   K   S   T   P   A   N   D   D
ATC GAC ATA GTG CAT GGC TGC GAA AAG TCC ACT CCA GCT AAC GAT GAC

 K   C   I   W   T   L   G   V   A   T   C   F   K   A   E   I
AAG TGT ATC TGG ACC CTC GGT GTC GCG ACC TGC TTC AAA GCT GAA ATC

 H   K   L   N   W   A   P   S   M   D   V   A   V   G   E   I
CAC AAA TTG AAC TGG GCT CCG AGC ATG GAC GTA GCA GTG GGA GAA ATT

 L   A   E   V   *
TTA GCT GAA GTT TGA
```

Figure 6.1 Nucleotide and predicted amino acid sequences of BmorPBP1 (from Krieger *et al*, 1996). The signal peptide is underlined, the translation termination codon is marked with an asterisk, and cysteine residues are highlighted in red.

6.3 Materials and methods

The cDNA for BmorPBP1, cloned into pBluescript II SK (Krieger *et al*, 1996) at the *EcoRI* site, was excised using *PstI* and *SalI*, and ligated into pFASTBAC. The construct was sequenced to confirm the orientation of the insert with respect to the polyhedrin promoter in the vector, and the cDNA was then expressed as described in 2.2.3. Briefly, the construct was transformed into *E. coli* DH10Bac cells, and bacmid DNAs were isolated, and analysed by agarose gel electrophoresis to assess the integrity of the bacmid DNAs. PCR across the multiple cloning site, using M13 forward and reverse primers, was used to confirm the recombinant/non-recombinant status of the bacmid (as suggested by the white or blue bacterial colonies). Following viral infection of Sf9 cells, total secreted protein was harvested, and precipitated or concentrated from both recombinant and non-recombinant (negative control) viral infections. This was analysed by SDS and native PAGE (SDS-PAGE used reducing conditions), IEF, and immunodetection using a polyclonal antibody raised against BmorPBP1, a gift from G. Ziegelberger (Steinbrecht *et al*, 1992). The same antibody was used to check for cross-reactivity with soluble antennal proteins from the aphid *M. viciae*.

6.4 Results and Discussion

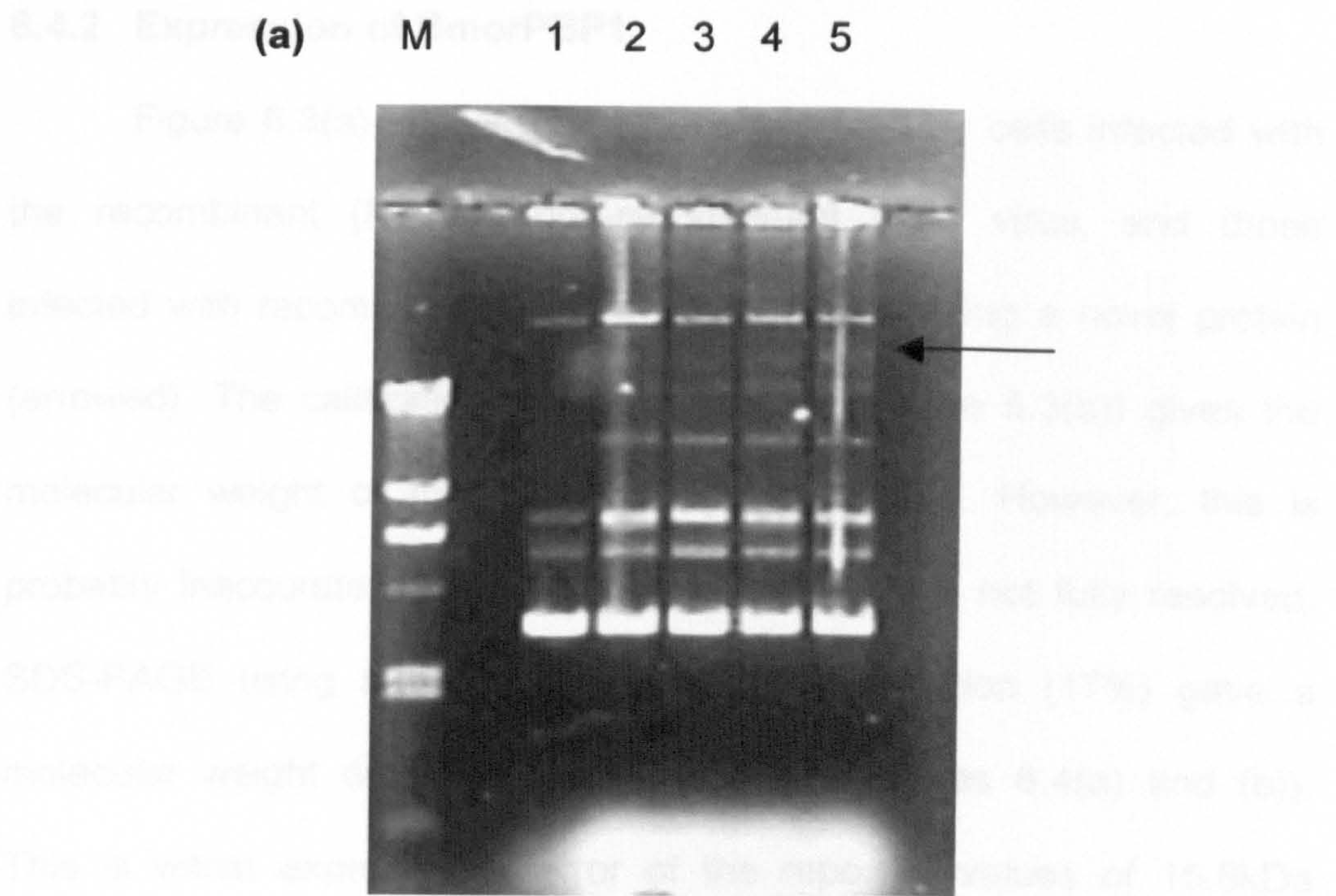
6.4.1 Bacmid DNA analyses

Bacmid DNAs were isolated from blue (non-recombinant) and

white (recombinant) bacterial colonies [2.2.2.2(c)], and analysed using agarose gel electrophoresis, and PCR with primers spanning the cloning site (universal M13 forward and reverse primers). Figure 6.2(a) shows the DNA isolated from 1 blue and 4 white colonies, and Figure 6.2(b) shows the result of the PCR on the bacmid templates.

The bacmid DNA is >135kb, and therefore agarose gel electrophoresis is unable to resolve it (the upper resolution limit is ~50kb Sambrook *et al*, 1989, 6.2). However, the bacmid DNA can be seen to run behind the 23kb band of the λDNA/*HindIII* marker, and is arrowed in Figure 6.2(a). The bands are faint but show no smearing, suggesting that the bacmid DNA is intact.

PCR analysis of the bacmid DNA was used to confirm the presence of an insert at the cloning site. PCR amplification of the bacmid alone (no transposition) should produce a product of ~300bp [see user manual for this baculovirus expression system (BAC-TO-BAC™, Gibco BRL)], whilst a bacmid transposed with pFASTBAC should give a PCR product of ~2.3kb. Insertion of a foreign gene will increase the size of the product, and since the size of the BmorPBP1 cDNA (plus some flanking pBluescript II) is 730bp, a fragment of ~3.0kb would be expected for a recombinant bacmid containing the BmorPBP1 cDNA. The bands in Figure 6.2(b) are ~280bp for bacmid 1 (non-recombinant), and ~3.3kb for bacmids 2, 3, 4 & 5 (recombinant). These bacmids were used to produce virus for infection of the insect cells.



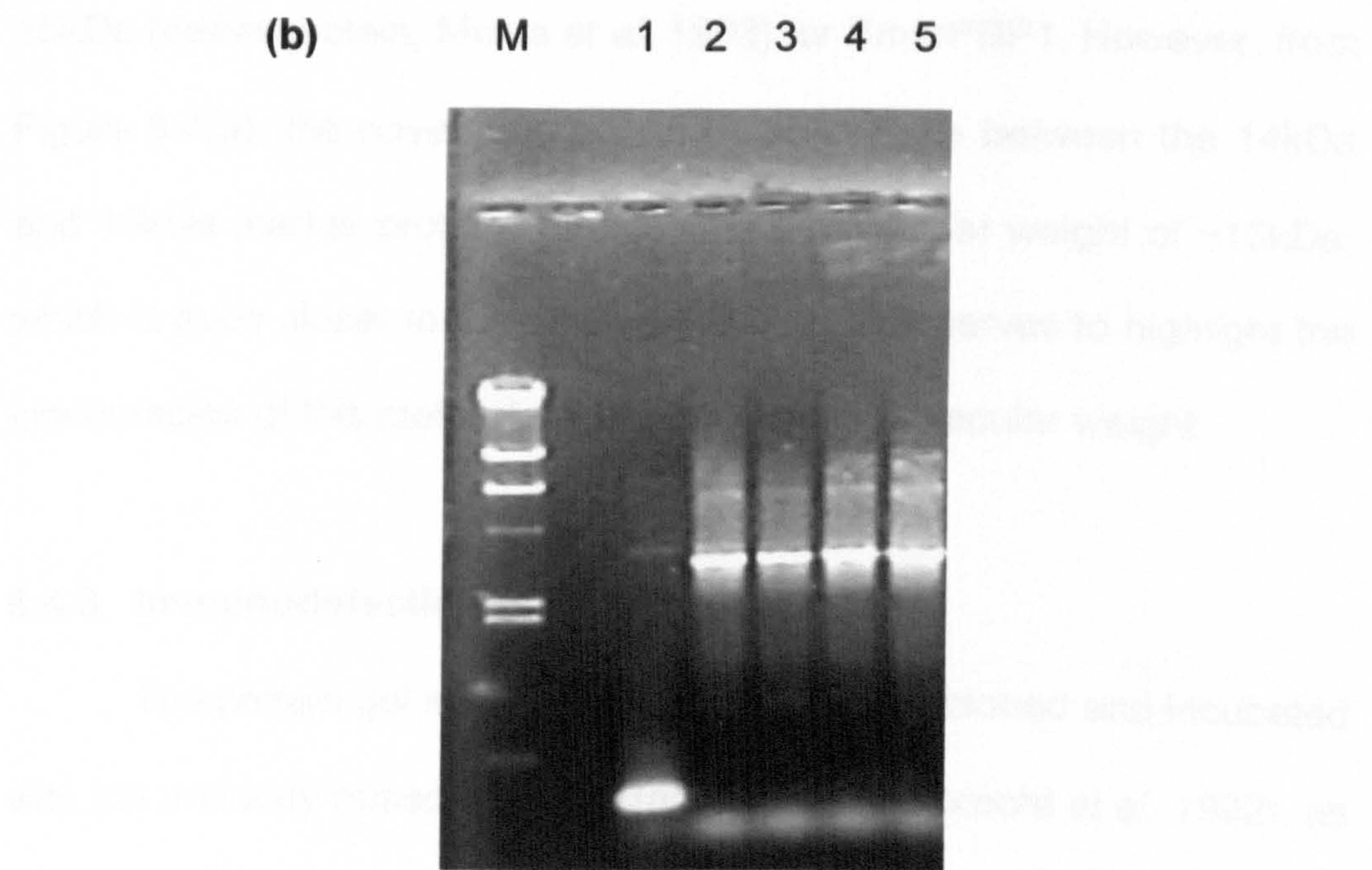


Figure 6.2 (a) DNAs isolated from blue (1) and white (2, 3, 4 & 5) colonies; bacmid DNA is arrowed. (b) Results of PCR amplification of bacmid DNA. M = λ DNA/*HindIII* marker.

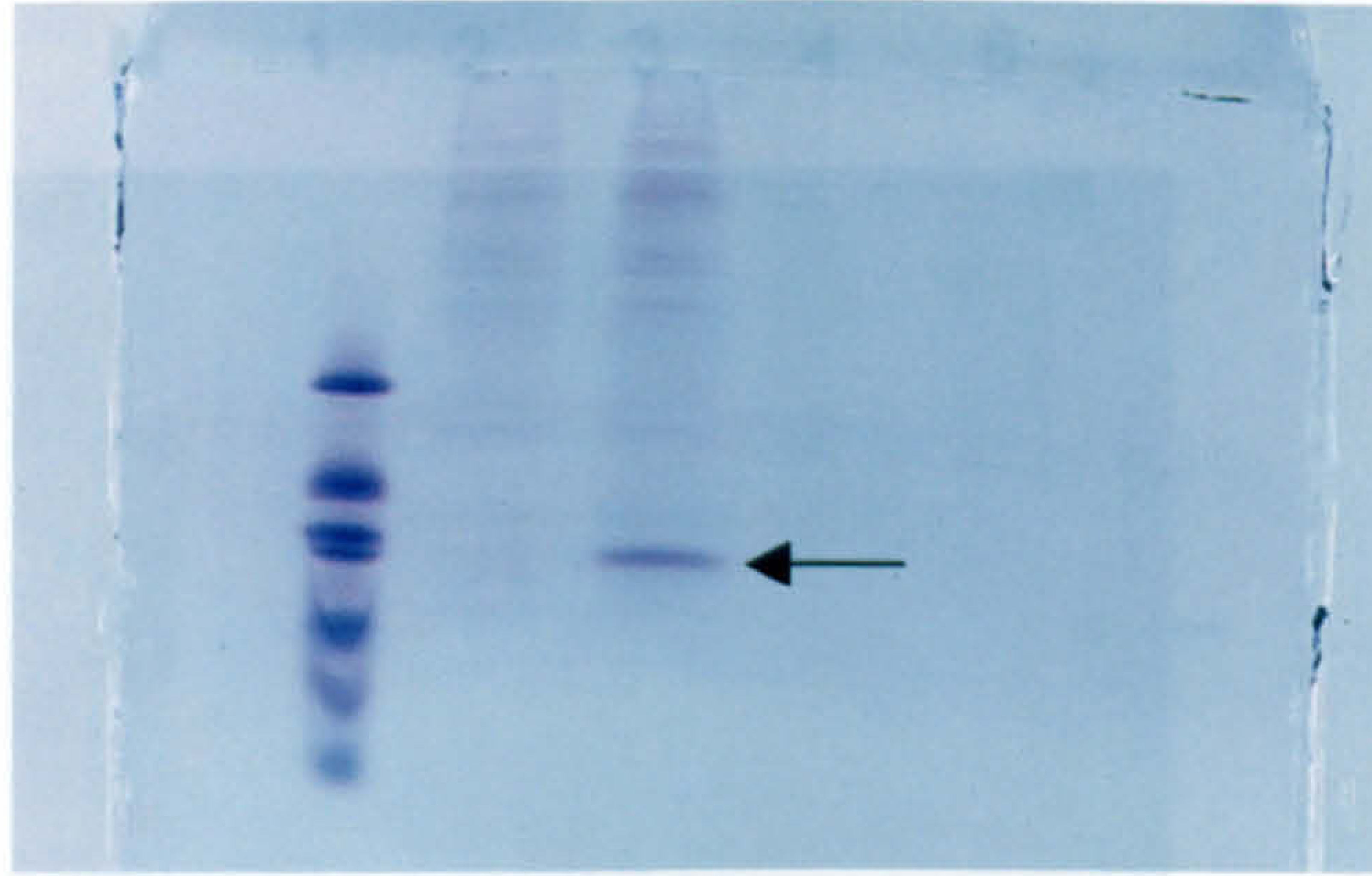
6.4.2 Expression of BmorPBP1

Figure 6.3(a) shows the proteins secreted by cells infected with the recombinant (R) and non-recombinant (NR) virus, and those infected with recombinant virus are clearly expressing a novel protein (arrowed). The calibration curve for this gel (Figure 6.3(b)) gives the molecular weight of this novel protein as 10kDa. However, this is probably inaccurate as the markers in this gel are not fully resolved. SDS-PAGE using a higher acrylamide concentration (17%) gave a molecular weight determination of 13.5kDa (Figures 6.4(a) and (b)). This is within experimental error of the reported values of 15.6kDa (predicted from the amino acid sequence; Krieger *et al*, 1996) and 15kDa (native protein; Maida *et al*, 1993) for BmorPBP1. However, from Figure 6.4(a), the novel protein can be seen to lie between the 14kDa and 16kDa marker proteins, suggesting a molecular weight of ~15kDa, which is even closer to the reported values. This serves to highlight the inaccuracies of this method used to determine molecular weight.

6.4.3 Immunodetection

The protein gel shown in Figure 6.4(a) was blotted and incubated with the antibody raised against BmorPBP1 (Steinbrecht *et al*, 1992), as described in 2.2.4.7. The results are shown in Figure 6.5. The antibody reacts with the novel protein produced by the recombinant virus, confirming that this novel protein is BmorPBP1.

(a) M NR R



(b)

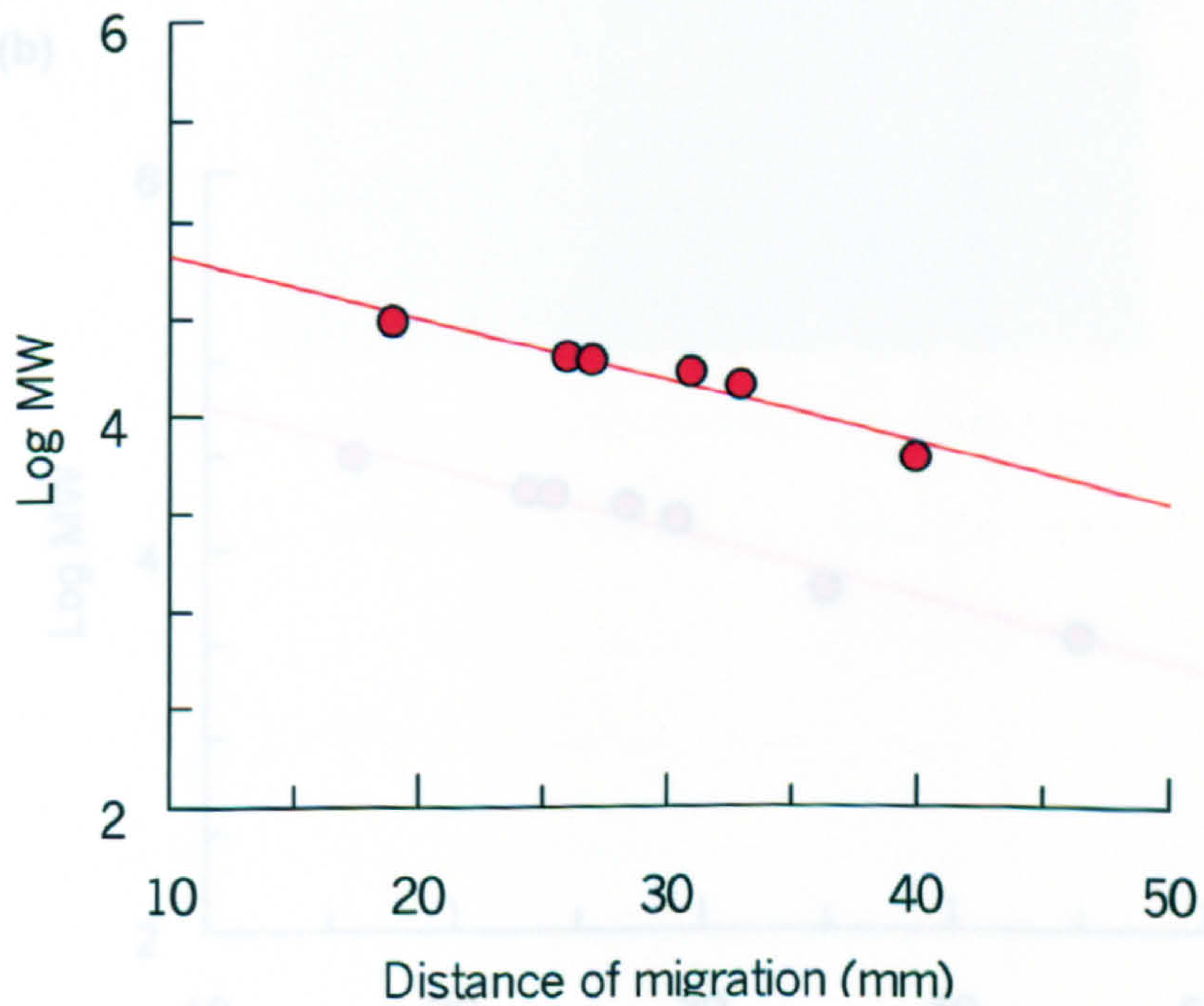


Figure 6.3 (a) Proteins secreted by insect cells infected with virus run on SDS-PAGE using 15% acrylamide (with 2% cross linker) and stained with Coomassie Brilliant Blue R. M = low molecular weight markers; NR = non-recombinant viral infection; R = recombinant viral infection. Arrow indicates novel protein produced by recombinant virus. (b) Calibration curve of log_{10} molecular weight versus distance of migration for marker proteins.

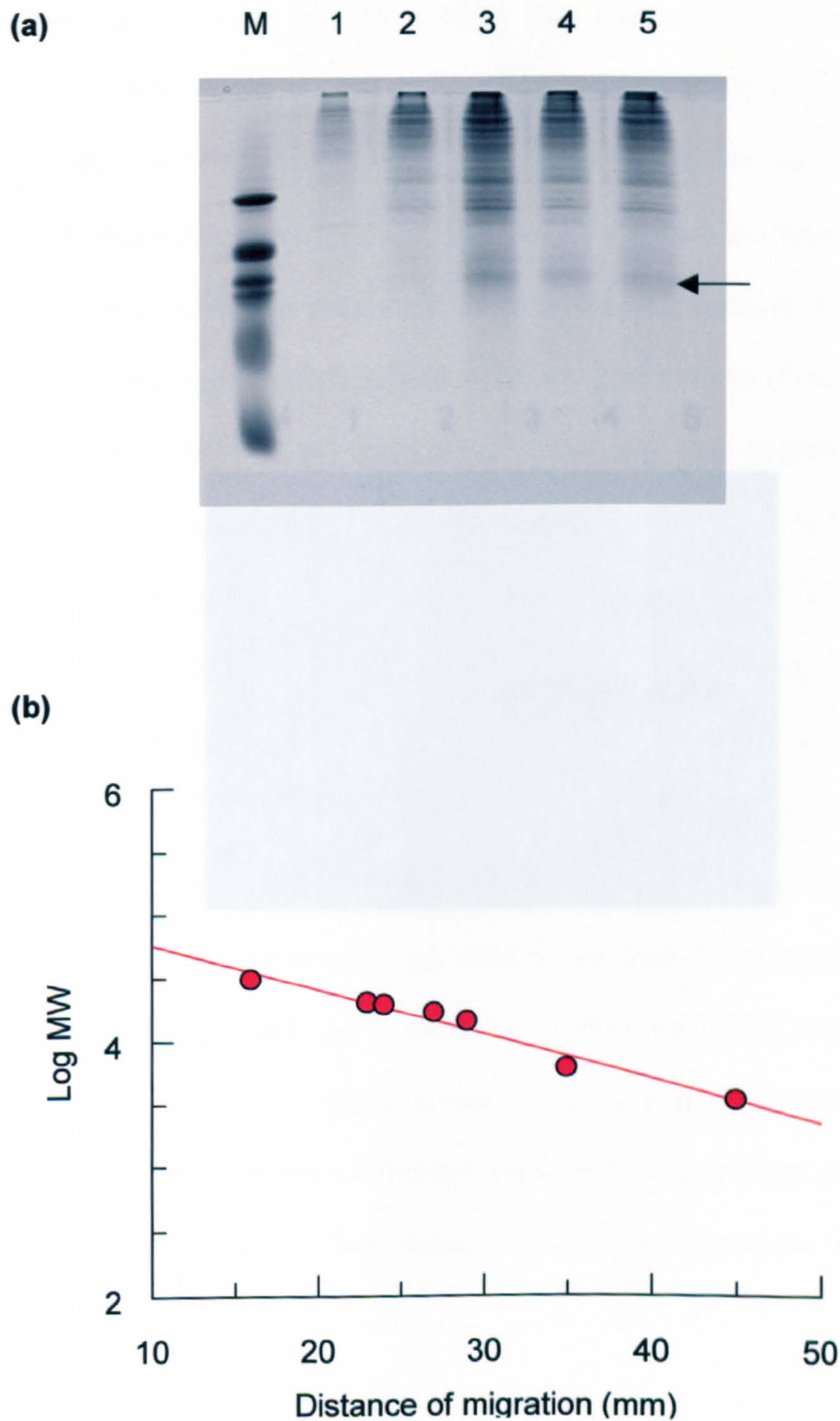


Figure 6.4 (a) Proteins secreted by insect cells infected with virus run on SDS-PAGE using 17% acrylamide (with 2% cross linker) and stained with Coomassie Brilliant Blue R. M = low molecular weight markers; 1 = cell supernatant (no viral infection); 2 = non-recombinant viral infection; 3,4 & 5 = replicate recombinant viral infections. (b) Calibration curve of $\log_{10}$ molecular weight versus distance of migration for marker proteins.

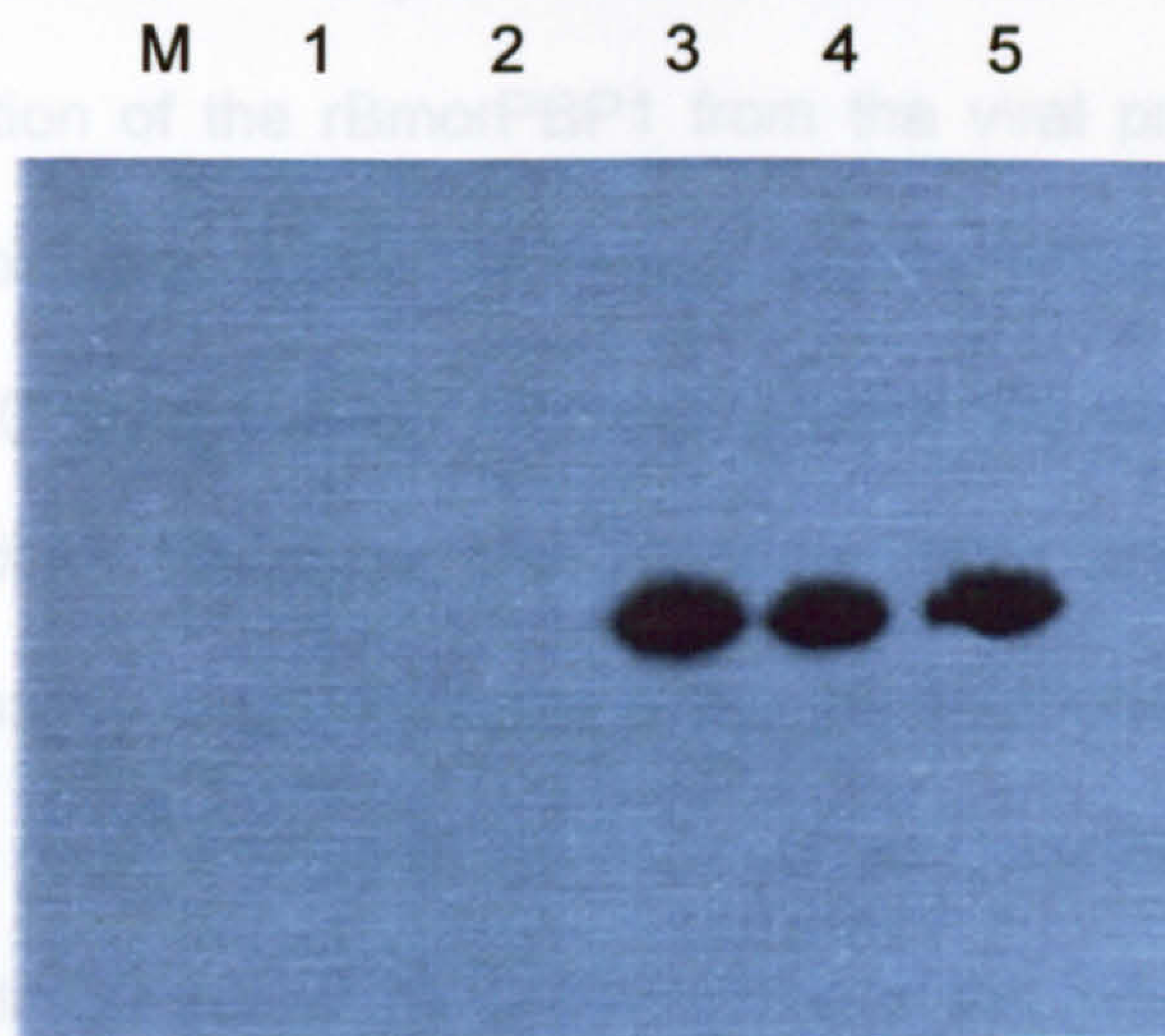


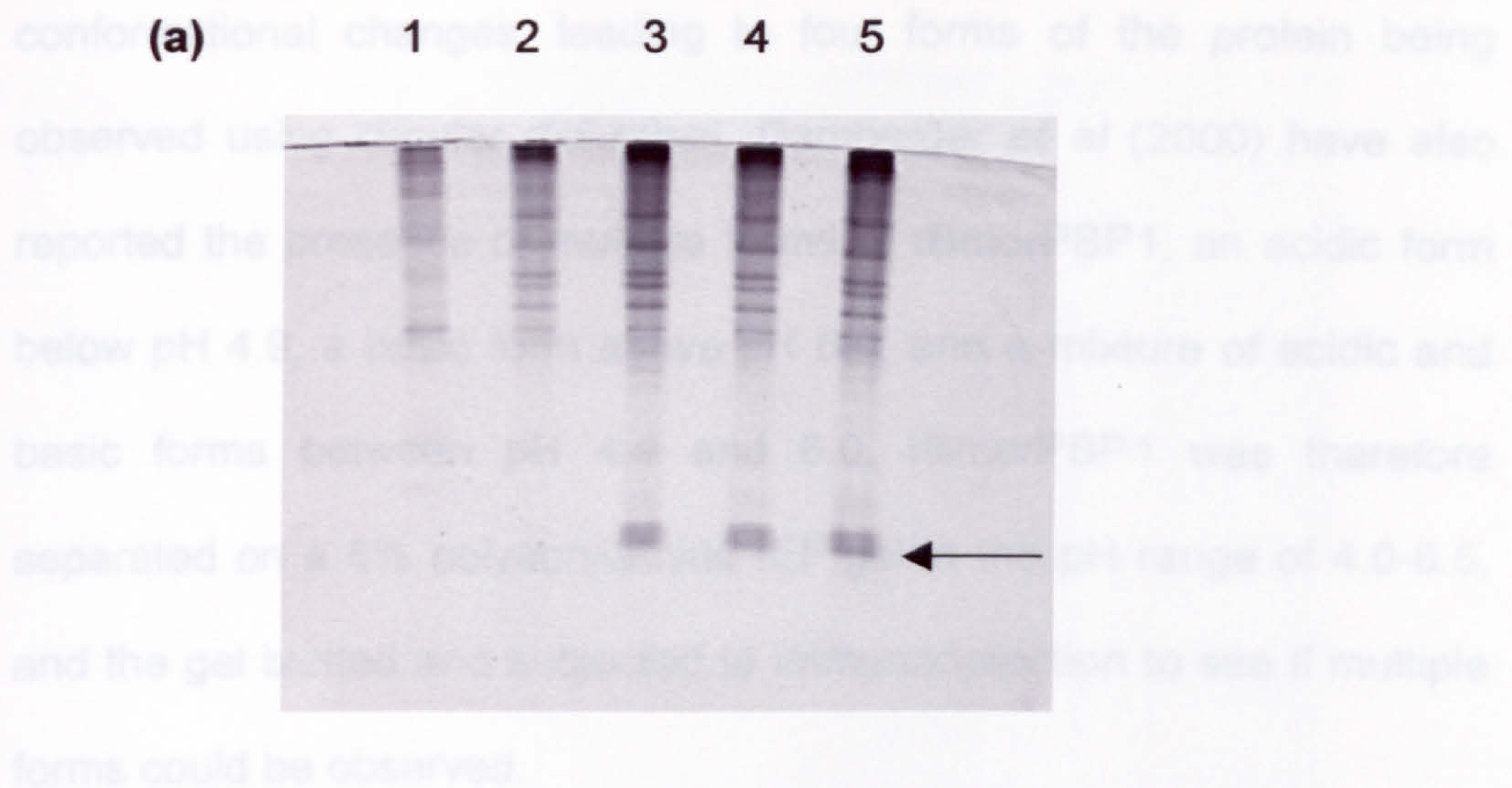
Figure 6.5 Western blot of the gel shown in 6.4(a) following incubation with a polyclonal antibody to BmorPBP1 and ECL detection. Gel loadings as for Figure 6.4.

6.4.4 Native PAGE

There are reports that BmorPBP1 can exist as a (homo)dimer (Maida *et al*, 1997; Campanacci *et al*, 1999; and Leal, 2000), with the dimeric form being resolvable from the monomer by PAGE. Protein samples of recombinant BmorPBP1 (rBmorPBP1) were analysed in an attempt to determine the presence or absence of multiple forms of BmorPBP1, using native PAGE (see 2.2.4.4). The results (Figure 6.6) show good separation of the rBmorPBP1 from the viral proteins, and the stained gel appears to show two protein bands, which both react with the antibody (Figure 6.6(b)). However, these do not show the resolution expected for the dimer and monomer from previous work (Leal, 2000). A possible explanation for these observations is that the larger weaker band could be due to the detection of Coomassie stained protein which has diffused towards each surface of the 4mm thick gel, or that the upper band represents (a) trailing residue(s) that lie(s) in the epitopic region recognised by the antibody. A third possibility is that the upper band is a viral or cellular protein that is partly immunogenic, resulting in the fainter signal, although there is no equivalent signal in the control lanes (1 and 2). Thus there is no good evidence for multiple forms.

6.4.5 Isoelectric focusing (IEF)

Wojtasek and Leal (1999b) reported the appearance of multiple forms of recombinant BmorPBP1 protein using ion exchange chromatography, and showed that between pH 5-6, there were dramatic



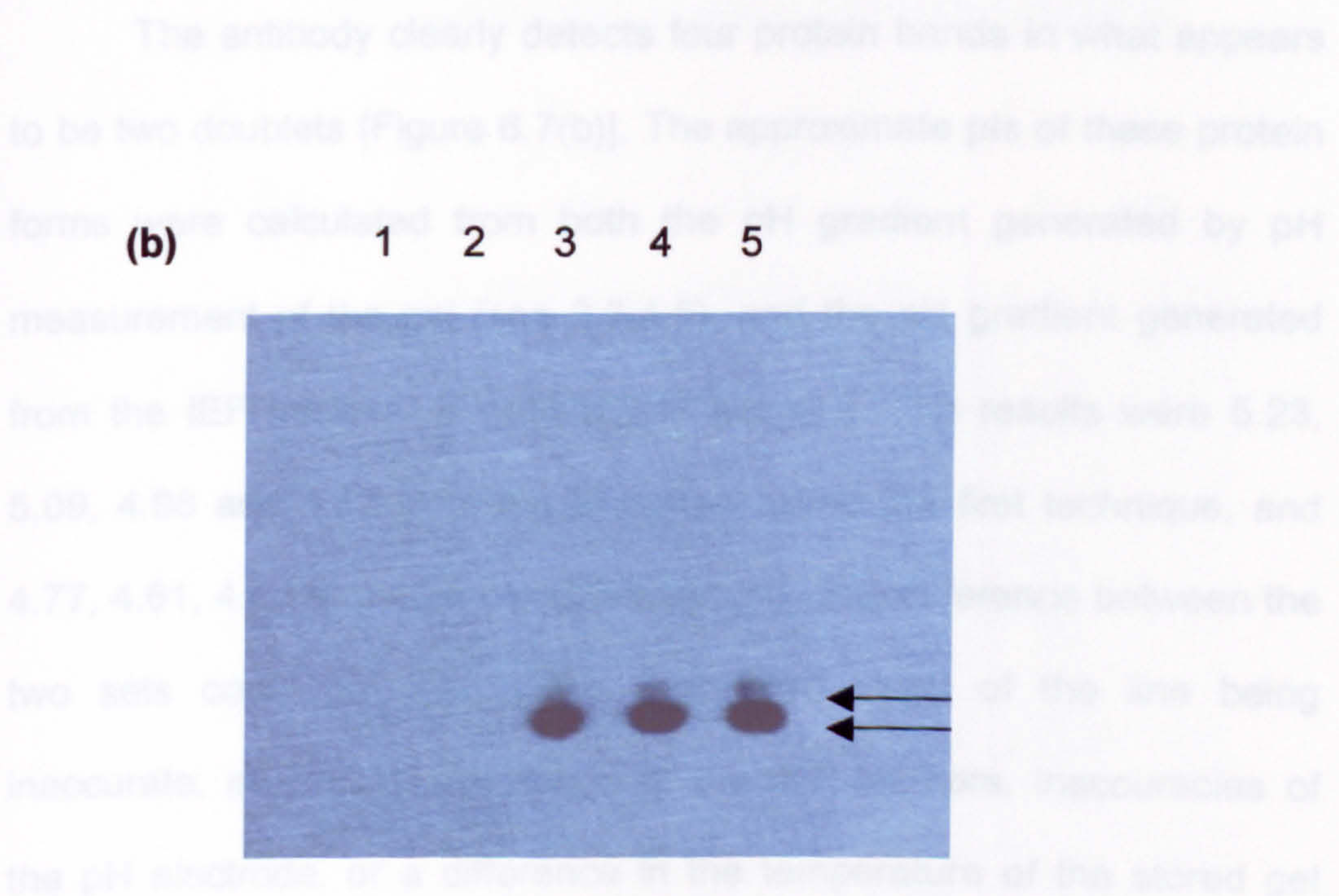
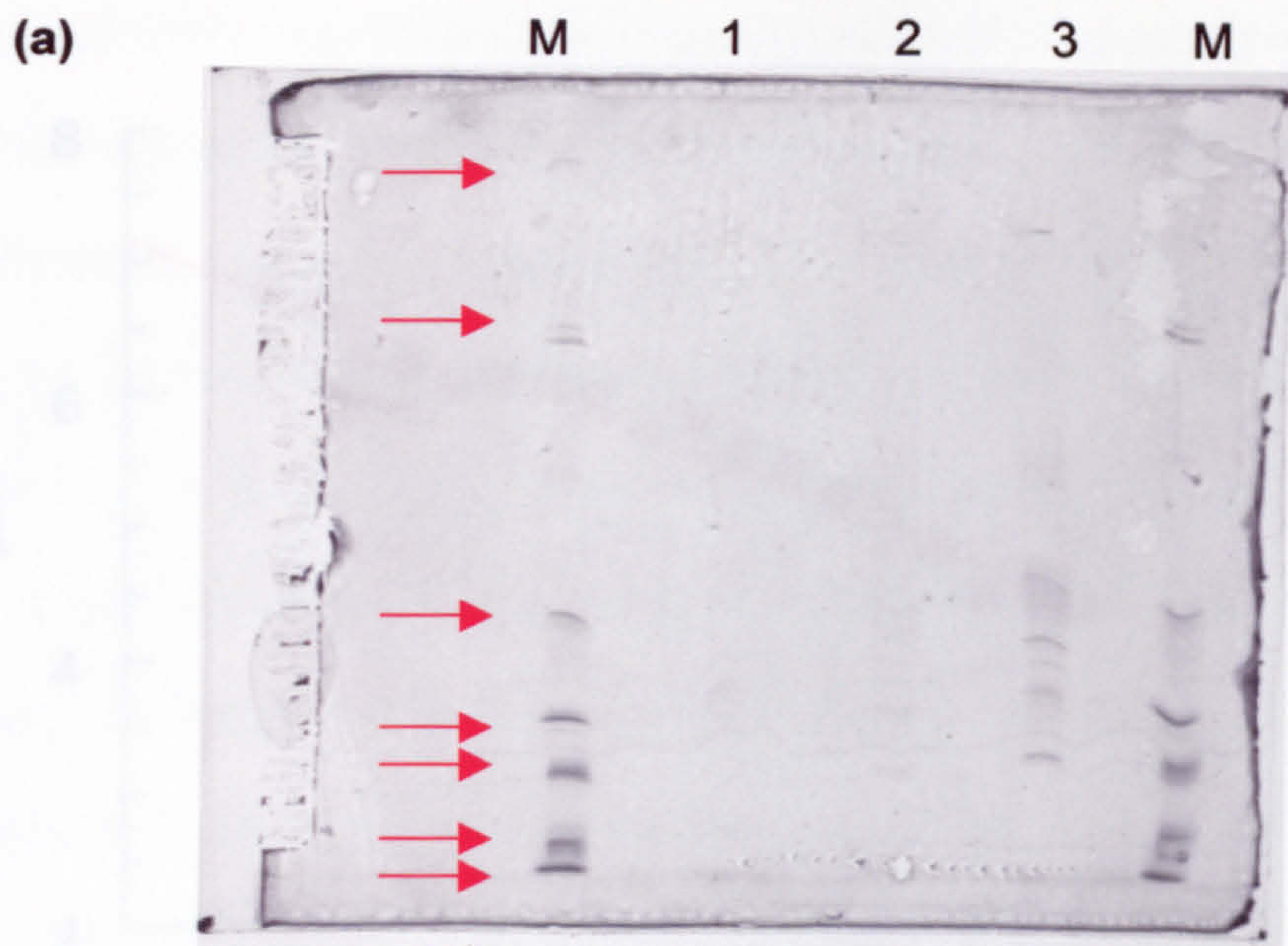


Figure 6.6 (a) Native PAGE gel showing proteins produced by insect cells following viral infection, stained with Coomassie Brilliant Blue R. (a) and incubated with BmorPBP1 antibody (b). 1 = cell supernatant (no infection); 2 = non-recombinant viral infection; 3,4 & 5 = replicate recombinant viral infections. Arrows indicate bands discussed in text.

conformational changes, leading to four forms of the protein being observed using circular dichroism. Damberger *et al* (2000) have also reported the presence of multiple forms of rBmorPBP1, an acidic form below pH 4.9, a basic form above pH 6.0, and a mixture of acidic and basic forms between pH 4.9 and 6.0. rBmorPBP1 was therefore separated on a 5% polyacrylamide IEF gel in the pH range of 4.0-6.5, and the gel blotted and subjected to immunodetection to see if multiple forms could be observed.

The antibody clearly detects four protein bands in what appears to be two doublets [Figure 6.7(b)]. The approximate pIs of these protein forms were calculated from both the pH gradient generated by pH measurement of the gel (see 2.2.4.5), and the pH gradient generated from the IEF markers [Figure 6.8(a) and (b)]. The results were 5.23, 5.09, 4.96 and 4.87 from top to bottom using the first technique, and 4.77, 4.61, 4.45 and 4.35 using the second. The difference between the two sets could be due to the calculated slope of the line being inaccurate, incomplete resolution of the IEF markers, inaccuracies of the pH electrode, or a difference in the temperature of the stored gel slices compared to the gel when running. Unfortunately, a surface probe to measure the pH gradient during focusing was unavailable.

The four detected forms possibly represent charge isomers of the protein with the different charges resulting from the exposure of internal amino acids, resulting in different pIs. Indeed these four bands may represent the four forms reported by Wojtasek & Leal, which Leal (2000) suggested represents the dissociation of the dimeric form



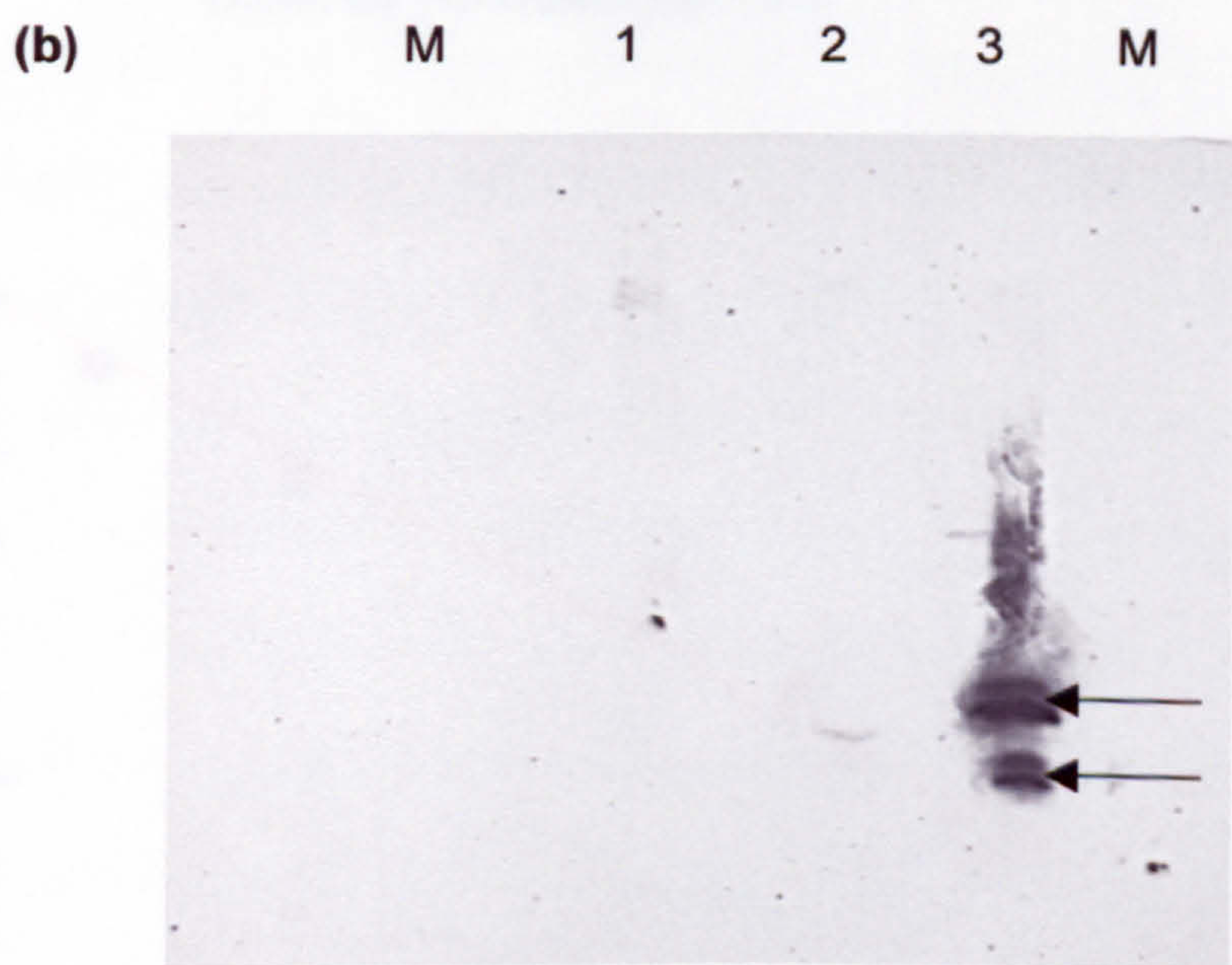
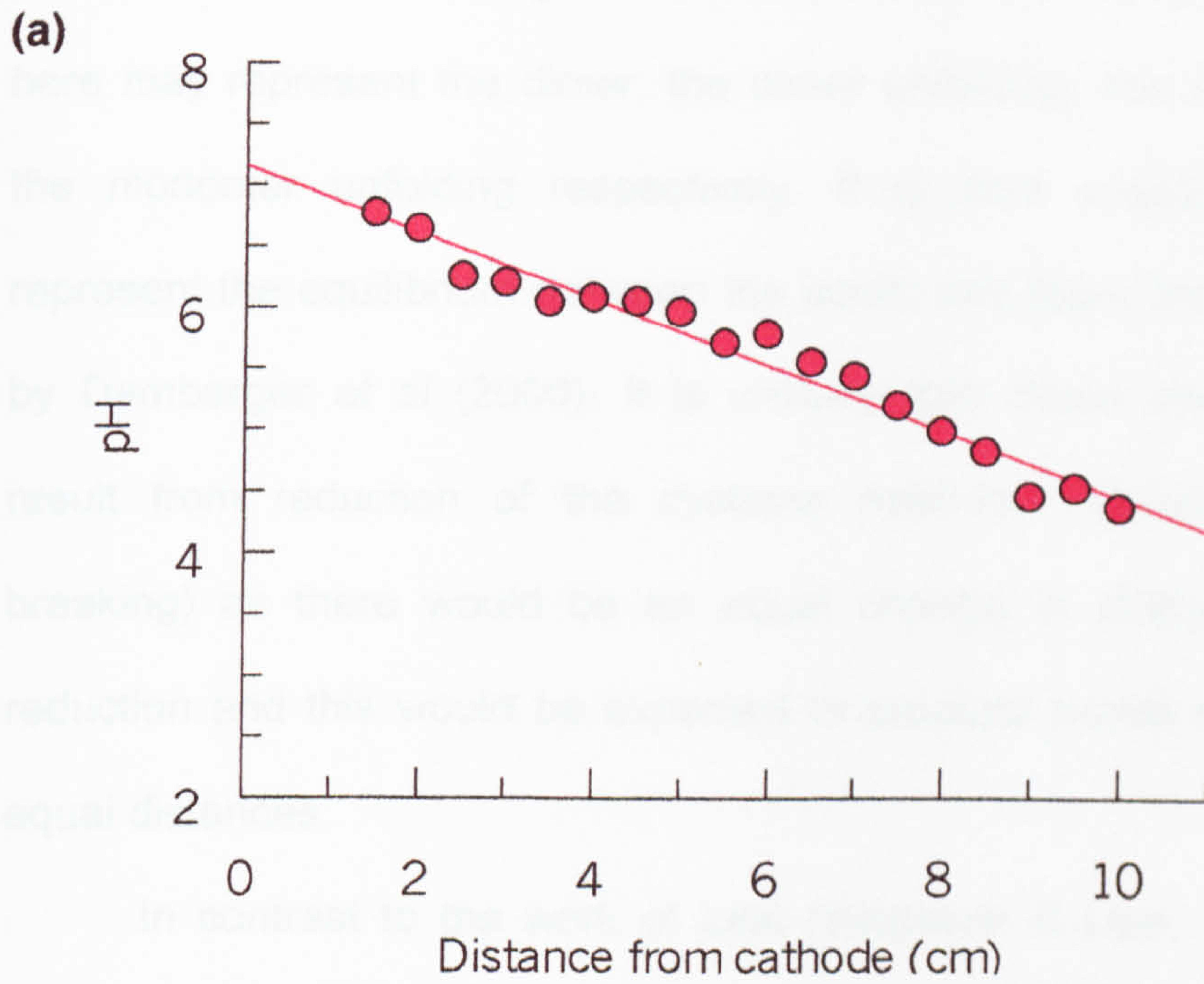


Figure 6.7 IEF gel of proteins produced by insect cells infected with virus stained with Coomassie Brilliant Blue R. (a) and immunodetected with antibody to BmorPBP1 (b). M = IEF molecular weight markers; 1 = protein from cell supernatant (no viral infection); (2) = non-recombinant viral infection; (3) = recombinant viral infection. Red arrows show position of marker bands, whilst black arrows show the two protein doublets discussed in 6.4.5.



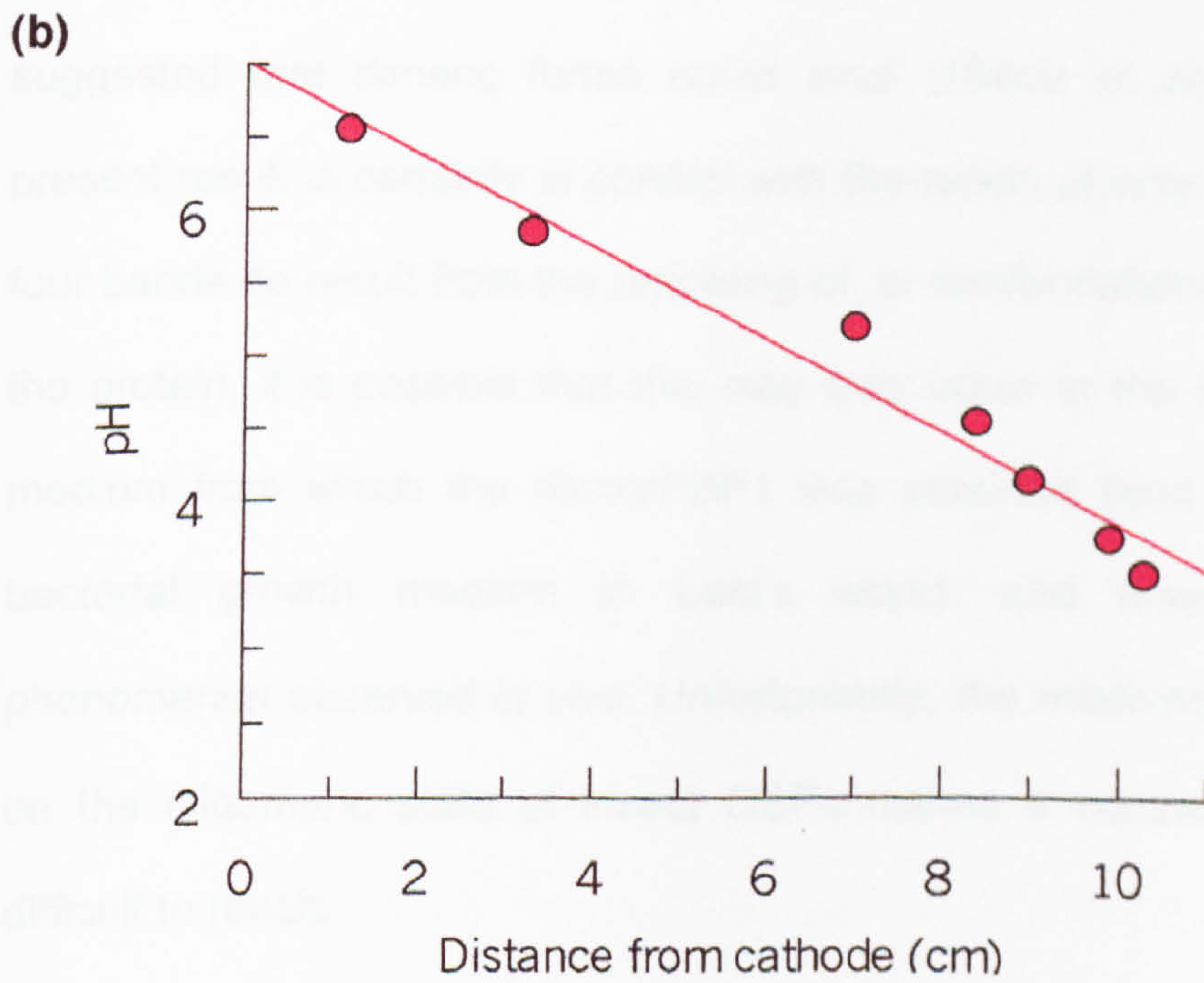


Figure 6.8 Determination of pH gradient using IEF gel slices (A) and IEF markers (Low pI calibration kit) (B)

coupled with the unfolding of the monomer; i.e. the four bands reported here may represent the dimer, the dimer unfolding, the monomer and the monomer unfolding respectively. It is also possible that they represent the equilibrium between the acidic and basic forms observed by Damberger *et al* (2000). It is unlikely that these charge isomers result from reduction of the cysteine residues (disulphide bridges breaking) as there would be an equal change in charge with each reduction and this would be expected to produce bands separated by equal distances.

In contrast to the work of Leal (Wojtasek & Leal, 1999b; Leal, 2000) and the current work, Maida *et al* (1993) reported that the native PBP was a monomer, with a single pI of 4.9, although they too later suggested that dimeric forms could exist (Maida *et al*, 1997). The present result is certainly in conflict with the report of only one pI. If the four bands do result from the unfolding of, or conformational changes in the protein, it is possible that this may only occur in the insect growth medium from which the rBmorPBP1 was obtained (and perhaps the bacterial growth medium in Leal's work), and may not be a phenomenon observed *in vivo*. Unfortunately, the relatively few reports on the oligomeric state of insect OBPs makes a consensus opinion difficult to reach.

6.4.6 Aphid antennal proteins

Soluble proteins were extracted from the antennae of male and oviparous female *M. viciae* (see 2.2.4.2), and separated on SDS-PAGE

(17% acrylamide with 2% cross linker) alongside rBmorPBP1. The gel was then blotted and subjected to immunodetection with the anti-rBmorPBP1 antibody, and the results are shown in Figure 6.9. The antibody showed no interaction with the aphid proteins, although the positive control of the rBmorPBP1 protein did react. However, despite the lack of immunoreactive proteins, both the male and female antennae do have protein bands between 10 and 20kDa [arrowed bands are 13.4kDa and 19.3kDa; Figure 6.9(a)], which is in the correct range for OBPs. These proteins may indeed be OBPs, but they must be too dissimilar, in the region of the antibody epitope, to the moth PBPs to cross-react with the antibody.

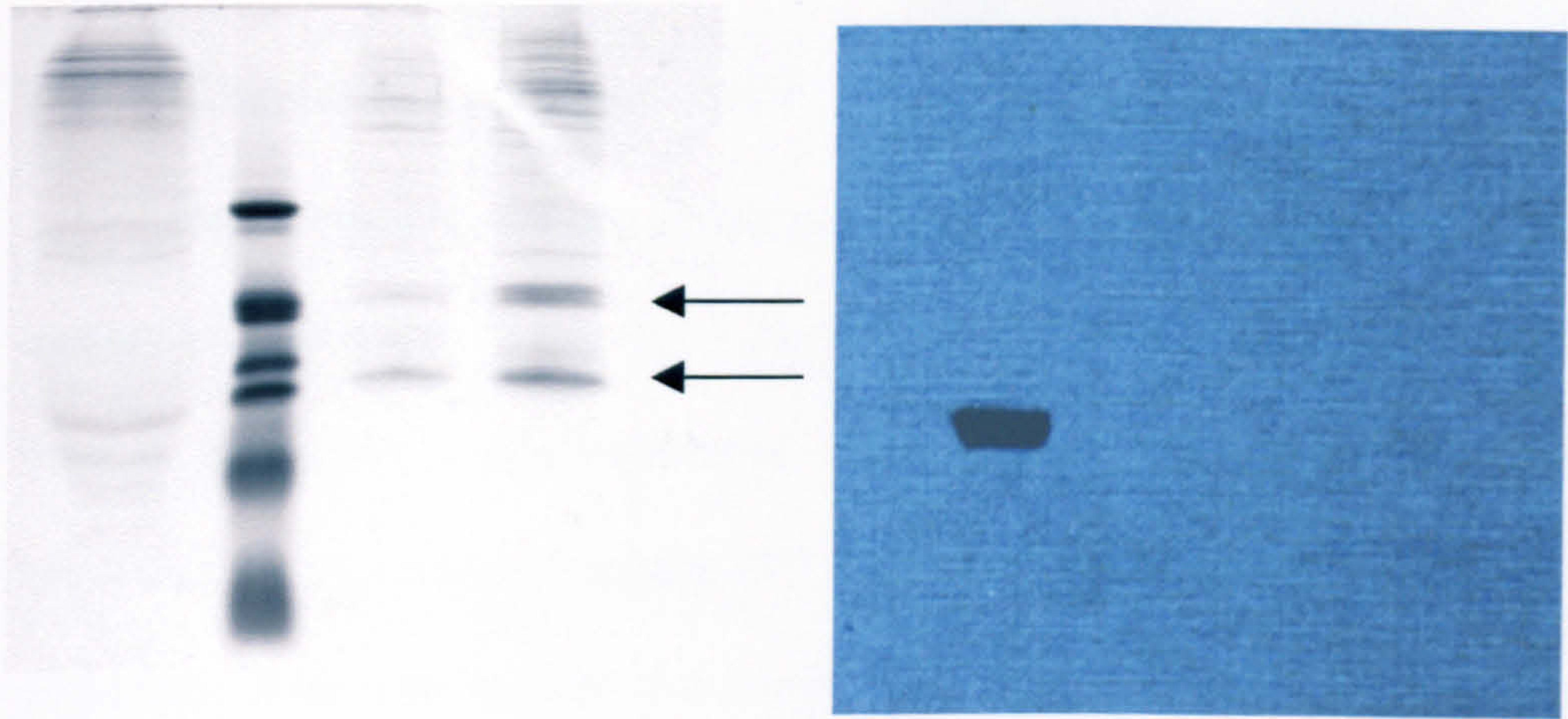
6.5 Conclusions

Recombinant BmorPBP1 was successfully expressed using the baculovirus system, giving a molecular weight of ~13.5kDa, in good agreement with reported values. The authenticity of the expressed protein was confirmed using an antibody raised against the protein.

SDS-PAGE and native PAGE showed only one form of the recombinant protein, but IEF between pH 4.0-6.5 yielded four forms that reacted with the antibody. These could represent charge isomers of the protein, possibly arising due to dissociation of the dimeric form coupled with the unfolding of the protein, or the equilibrium between acidic and basic forms of the protein.

Aphid antennal proteins of a similar molecular weight to insect OBPs could be observed with SDS-PAGE, but they did not cross-react

(a) Bm M FA MA **(b)** Bm M FA MA



(c)

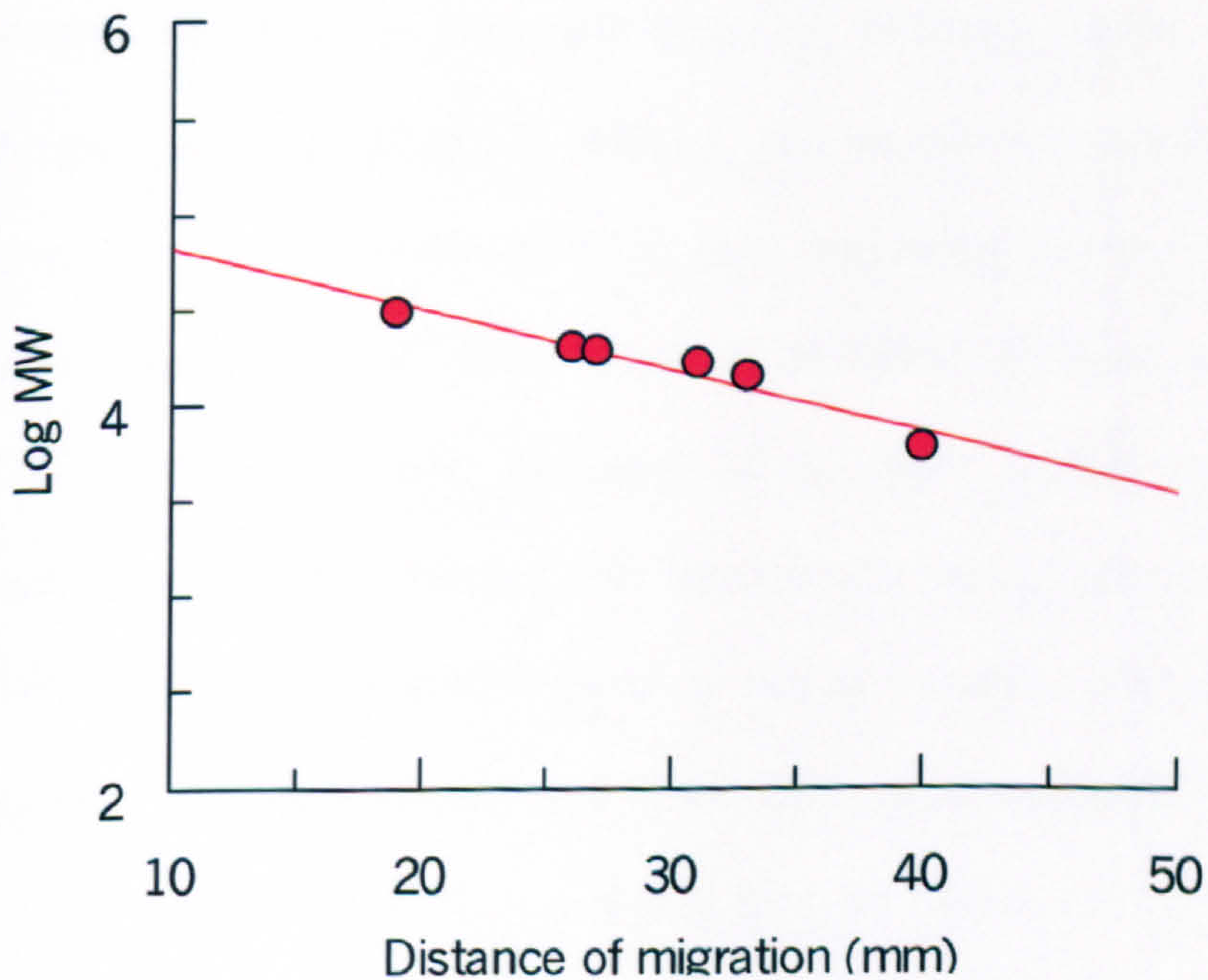


Figure 6.9 SDS-PAGE gel stained with Coomassie Brilliant Blue R. (a) and following immunodetection with anti-rBmorPBP1 antibody (b). Bm = rBmorPBP1; M = low molecular weight markers; FA = soluble proteins from the antennae of female aphids; MA = soluble proteins from the antennae of male aphids. (c) Calibration curve of $\log_{10}$ molecular weight versus distance of migration for marker proteins shown in (a).

with the rBmorPBP1 antibody. This is probably because there is not sufficient similarity between the proteins of the two insects.

Chapter 7. General Discussion.

7.1 Choosing an approach

OBPs have been identified in a number of insect species using a variety of methods. Once the initial cDNA for a PBP had been cloned from *A. polyphemus* (Györgyi *et al*, 1988), subsequent moth OBPs were identified by homology cloning (RT-PCR or screening of libraries), an approach that was successful because of the similarity, at the amino acid level, of the moth OBPs. However, the conservation of amino acid sequences of lepidopteran OBPs does not extend to other insect species, which show very little similarity to either moths or each other (in the range of 30-40%). Indeed, the non-lepidopteran OBPs have been identified by methods other than homology cloning (Pikielny *et al*, 1994; McKenna *et al*, 1994; Ozaki *et al*, 1995; Danty *et al*, 1997, 1998, 1999; Kim *et al*, 1998; Wojtasek *et al*, 1998, 1999), and most non-lepidopteran OBPs have been functionally assigned on the basis of their size, and the presence of a signal peptide, and six conserved cysteine residues, along with their specific association with olfactory organs. Vogt *et al* (1999) suggest that all insect OBPs have evolved from a common ancestor, but if this is so, the divergence that has occurred since, as each OBP became specialised for a species-specific ligand, has resulted in no remaining detectable homology.

Divergence from a common ancestral gene often occurs beyond the point at which any sequence similarity remains (Murzin & Chothia, 1992), and since selection acts on the phenotype and not the genotype,

protein function is often better conserved than amino acid sequence (Flower *et al*, 1993). Homology cloning of insect ORs failed for many years, and only proved successful following the *Drosophila* genome project, when computational approaches could be used to identify cDNAs with protein structures resembling those of other ORs, i.e. seven-transmembrane domain proteins (Gao & Chess, 1999; Clyne *et al*, 1999). Whilst these computational approaches could also be used to identify aphid OBPs or ORs, sequencing of the aphid genome is not likely in the near future.

In contrast to OBPs, insect CSPs do have a high degree of conservation of amino acid sequence, and homology cloning might have been expected to be successful in identifying aphid CSPs. However, cloning of CSPs was not an initial aim of this study, with CSPs only coming to the fore of the literature during the course of this research.

The unsuitability of homology cloning made the construction of antennal cDNA libraries a more suitable approach for cloning aphid OBPs. cDNAs could, in theory, be sequenced and their predicted amino acid sequences examined for insect OBP hallmarks. Thus identifying an aphid OBP, if indeed OBPs are present in aphids, would become a matter of sequencing systematically through the clones produced. Whilst it was hoped that aphids, like moths, had a high concentration of OBP mRNAs in their rhinaria, the use of subtractive hybridisation to enrich antennal-specific cDNAs, should have increased the chances of identifying an aphid OBP, even if the mRNAs were low copy transcripts

(Sagerström *et al*, 1997).

7.2 Major results of this study

The major success of the present study was the cloning of a number of cDNAs, encoding proteins with a potential role in olfaction, from the vetch aphid *M. viciae*: (i) the first homopteran putative OBP; (ii) a cytochrome P450; (iii) two insect CSPs; (iv) several JHBPs, and (v) a putative carrier protein (U2). In addition the BmorPBP1 cDNA was successfully expressed in insect cells, and four forms of BmorPBP1 were detected following IEF.

7.3 Characteristics of MvicOBP1.

MvicOBP1 was identified as a putative OBP from oviparous females. Six cysteine residues are present in a spacing pattern of X_{14+}-C-X_{31}-C-X_4-C-X_{45}-C-X_{15}-C-X_8-C-X_5 which is similar to the model profile of the other insect OBPs: X_{18+}-C-$X_{(26-30)}$-C-X_3-C-$X_{(32-43)}$-C-$X_{(8-12)}$-C-X_8-C-$X_{(11-213)}$. This model profile takes all insect OBPs into account, and is therefore different to that discussed by Christophides *et al* (2000) and Field *et al* (2000). Across all other insect OBPs there are two regions of completely conserved spacing, i.e. C-X_3-C and C-X_8-C, and whilst MvicOBP1 possesses the C-X_8-C region, the C-X_3-C region has been replaced with C-X_4-C. Since the six conserved cysteines of OBPs are involved in forming disulfide bonds [as has been shown for *B. mori* PBP1 (Leal *et al*, 1999; Sandler *et al*, 2000) and GOBP2 (Scaloni *et al*, 1999)], the exact conservation of spacing may not be vital for function,

and the overall tertiary structure of MvicOBP1 may be very similar to other OBPs. Unfortunately, the MvicOBP1 cDNA is incomplete at the 5' end so the presence of a signal peptide, another characteristic of insect OBPs, has not been established. However, the fact that MvicOBP1 is expressed in the olfactory tissues, the antennae, lends support to the assignment as an OBP. In addition, a major hydrophobic domain is present in MvicOBP1, between residues 60 and 90, which may function as a ligand-binding pocket, in a similar manner to that reported for the lepidopteran PBPs (Du *et al*, 1994; Du & Prestwich, 1995; Prestwich *et al*, 1995).

The amino acid sequence of MvicOBP1 shows an average identity of 30-40% with other insect OBPs, with the highest match being 44% to OS-E from *D. melanogaster* (McKenna *et al*, 1994). Since *L. lineolaris*, is more closely related to *M. viciae*, LlinLAP might be expected to show the highest similarity. However, if ligand similarity determines OBP similarity (Vogt *et al*, 1991b; Steinbrecht, 1996a and b), then major differences between the OBPs of aphids and moths, or beetles, or bees, would be expected, since the semiochemicals used often have very different structures, as is certainly the case for the sex pheromones for these taxa. Overall, the aphid OBP shows as much identity to the lepidopteran OBPs as do other insect OBPs, which supports the status of MvicOBP1 as an OBP.

7.4 Is MvicOBP1 a GOBP?

MvicOBP1 has been assigned the rather generic term "OBP" as opposed to either of the more specific terms "PBP" or "GOBP" because the nature of the ligand to which it binds is unknown. However, analysis of the expression pattern of MvicOBP1 within the aphid may provide clues to the ligand. RT-PCR showed that this cDNA is expressed equally in the antennae of male and oviparous female aphids, which, on the basis of the equal expression of GOBPs in both sexes in lepidopterans (Pelosi & Maida, 1995a), suggests a role as a "GOBP"-type protein, possibly being involved in the detection of plant odours. The GOBPs usually show an extremely high level of similarity across related species, and MvicOBP1-related genes have been found, in this study, in a number of other aphid species: *M. dirhodum*; *B. brassicae*; *A. pisum*; *N. ribis-nigri*; *A. fabae; S. avenae; R. padi*; and *M. persicae*. However, the cDNAs from these species have not yet been sequenced to determine their exact similarity. Whilst it is known that *M. viciae* can detect a wide range of plant volatiles with differential sensitivity (Visser & Piron, 1995), it is not known which of these compounds might also be detected by all of the other aphid species (Woodcock, personal communication).

MvicOBP1 is also expressed in the legs of male *M. viciae*. The legs of aphids have been suggested to have a gustatory function in detecting plant components, which is consistent with a "GOBP"-type function; indeed, lepidopteran GOBPs are expressed in those sensilla that detect plant odours (Steinbrecht *et al*, 1992; 1995; 1996). However,

if this were so, then MvicOBP1 would also be expected to be expressed in the legs of females. RT-PCR did reveal a very faint band, but at a level of expression substantially lower than in males. Therefore, the role of MvicOBP1 as a GOBP is equivocal, and the alternative hypothesis that MvicOBP1 is a "PBP"-type protein must be considered.

7.5 Is MvicOBP1 a PBP?

EAG studies showed that male and female oviparous *M. viciae* can detect the sex pheromone components, which are specific isomers of nepetalactol and nepetalactone, and although the female response is less in magnitude than that of the male, this might suggest that a PBP should be present in the antennae of both male and oviparae, as was found for MvicOBP1. The expression in female antennae of a "PBP"-type protein is in line with other studies showing the presence of "male-specific" PBPs in the antennae of females (Nagnan-Le Meillour *et al*, 1996; Maïbèche-Coisné *et al*, 1997, 1998b; Callahan *et al*, 2000), even though most lepidopteran females do not respond to their own pheromone (Hansson, 1995). If MvicOBP1 is a PBP, then why the expression in male legs? Female *M. viciae* release the sex pheromone from scent plaques on their hind tibiae, and the males detect and respond to this olfactory cue, which can be effective over long distances (Visser, 1986; Campbell *et al*, 1990). The males fly towards the female, and during mating, mount the female, and it is possible that the legs of the male contain olfactory receptors that are involved in this very close-range detection of the sex pheromone, as reassurance or confirmation

that he is mating with a conspecific female. This could explain the presence of MvicOBP1 in the legs of the male, and is analogous to the presence of ORs in *D. melanogaster* legs (Gao & Chess, 1999), possibly reflecting the presence of olfactory neurons in the legs of both species. An alternative hypothesis is that *M. viciae* may possess cuticular pheromones, as in cockroaches (Picimbon & Leal, 1999), where the male detects the female, using contact receptors rather than olfaction, and this contact reception may occur in the legs in male aphids. However, currently there is no clear evidence as to whether *M. viciae*, or aphids more generally, possess such cuticular pheromones.

Since females release the sex pheromone from their hind tibiae, and OBPs bind their ligands reversibly (Pelosi, 1998; Plettner *et al*, 2000), MvicOBP1 might play a role in releasing the sex pheromone, and this could explain the low levels of MvicOBP1 in female legs.

Does the presence of MvicOBP1-related genes in other aphids shed any light on this question? It is known that components of the sex pheromone of *M. viciae* are also present in the sex pheromones of *A. fabae*, *A. pisum*, *M. persicae*, *S. avenae*, *B. brassicae*, and *R. padi* (Hardie *et al*, 1999), whilst the pheromone of *M. dirhodum*, *N. ribis-nigri* and *L. erysimi* remains unidentified. RT-PCR indicated the expression of MvicOBP1 (or a very closely-related gene) in all of these species, supporting a "PBP"-type role. Only functional expression of MvicOBP1, which will enable ligand-binding studies, will really begin to answer the question of GOBP versus PBP for MvicOBP1.

7.6 Mv164

Mv164 cDNA was identified from the same antennal library as MvicOBP1, and encodes a protein with 45% similarity to insect cytochrome P450 enzymes (most similar to Types 3 and 6). RT-PCR showed that Mv164 is expressed in the same tissues of *M. viciae* (male and female antennae, and male legs) as MvicOBP1, suggesting that Mv164 could be the cytochrome P450 that degrades the odorant molecule that MvicOBP1 binds. If MvicOBP1 is a PBP, then Mv164 is involved in sex pheromone degradation, which is supported by the finding that cytochrome P450 enzymes are involved in sex pheromone metabolism in the housefly, *M. domestica* (Ahmad *et al*, 1987), and the pale brown chafer, *P. diversa* (Wojtasek & Leal, 1999a). The majority of moth sex pheromones are esters, aldehydes or alcohols, and the associated esterases, dehydrogenases and oxidases (Vogt & Riddiford, 1981; Vogt *et al*, 1985; Prestwich *et al*, 1989; Rybczynski *et al*, 1989), which are involved in their degradation, have been shown to be antennal–specific for a number of species.

Isoprenoids are the major components of the aphid sex pheromones, and these have no obvious biotransformation enzymes, so the more powerful oxidative cytochrome P450 system could fulfil this role. Notably, this is a similar situation to that of the scarab beetle, *P. diversa*, which uses an alkaloid compound (1,3-dimethyl-2,4-quinazolinedione) as its sex pheromone (Leal *et al*, 1997), and where a cytochrome P450 is responsible for degradation of this pheromone (Wojtasek & Leal, 1999a). This might support the idea that MvicOBP1

is a "PBP-type" protein, although the cytochrome P450 may be involved in degrading other non-pheromonal odorant molecules.

The expression of Mv164-related genes in *B. brassicae, A. pisum, S. avenae*, and *M. persicae*, which are known to use the same components as *M. viciae* for their sex pheromone (Hardie *et al*, 1994), supports the view that Mv164 and MvicOBP1 might be "linked" and involved in pheromone reception. However, Mv164, but not MvicOBP1, is expressed in *L. erysimi*, and the opposite is true for *M. dirhodum*, whilst only *S. avenae, M. persicae*, and *M. viciae* express both MvicOBP1 and Mv164 although other aphid species use the same sex pheromone components. This argues against a pheromonal link between these two proteins.

As with MvicOBP1, only functional expression of Mv164 would begin to answer the questions, as would studies of the sequences and tissue expression patterns of Mv164 or its equivalent in the other aphid species, alongside further investigations into the semiochemistry of the other species.

7.7 MvicCSP1 and MvicCSP2

cDNAs MvicCSP1 and MvicCSP2 were identified from female antennal subtracted cDNA libraries (antennae minus heads), and showed a high similarity to other insect CSPs. MvicCSP1 only represents ~25% of the cDNA, but MvicCSP2 covers probably ~70%. Both have conserved cysteine residues: MvicCSP1 possesses two of the cysteines common to CSPs, whilst MvicCSP2 possesses all four. It

has been shown that these cysteine residues are involved in forming disulfide bridges, in the same way as in OBPs (Angeli *et al*, 1999). In contrast to MvicOBP1, the spacing profile of the cysteine residues for MvicCSP2 shows a direct match to the model profile generated from the other insect CSPs (model: $X_{(44\text{-}74)}$-C-X_6-C-X_{18}-C-X_2-C-$X_{(31\text{-}154)}$; MvicCSP2: X_{20+}-C-X_6-C-X_{18}-C-X_2-C-X_{47+}). Whilst MvicCSP1 was too short for relevant hydropathy analysis, MvicCSP2 was shown to be almost completely lacking in hydrophobic domains (except near residue 15), which is also characteristic of insect CSPs. This hydrophilicity would confer solubility in an aqueous medium such as the sensillum lymph, but the lack of a hydrophobic domain, to bind a hydrophobic ligand, implies that these proteins may not have a role in binding volatile molecules. However, this could occur by particular folding, or dimerisation of the protein (Picimbon *et al*, 2000a), and a role for odour binding has been suggested based on *in vitro* binding of vaccenyl acetate by a CSP from *M. brassicae* (MbraAOBP2, Bohbot *et al*, 1998). In this case, vaccenyl acetate was used because of the high similarity (80%) between MbraAOBP2 and ejbIII, a protein expressed specifically in the ejaculatory bulb of *D. melanogaster* (Dyanov *et al*, 1994). This gland contains vaccenyl acetate, which acts as a sex pheromone in *D. melanogaster*. The presence of CSPs (or "OS-D-like" proteins) in other non-chemosensory tissues (Nomura *et al*, 1992; Kitabayashi *et al*, 1998; Picimbon *et al*, 2000b; present study,) begins to extend the role of putative chemosensory proteins beyond that of olfaction.

Whatever the function of CSPs, they are members of a large protein family, present in a wide range of insects, suggesting that they might be a common principle (Picimbon *et al*, 2000a). Interestingly, they are expressed alongside OBPs, but show much more sequence similarity across genera than do the OBPs. This suggests a common evolutionary ancestor for CSPs, with little subsequent divergence, although the similarity may have occurred completely by chance (Doolittle, 1981). If these CSPs have evolved from a common ancestor, then conservation of sequence does not seem to reflect a conservation of function, although at present, the knowledge of CSPs is limited.

7.8 Cloning of U2 and JHBPs

A cDNA, U2, was identified from an oviparous female subtracted cDNA library (whole female with antennae minus whole female without antennae), and encoded a protein with characteristics of insect OBPs i.e. the correct size (mature peptide: 12.8kDa), the presence of a predicted signal peptide, and the presence of five cysteine residues that match the expected pattern. U2 also showed 35-41% similarity to other insect OBPs, but was almost completely hydrophobic. RT-PCR showed U2 to be expressed solely in the abdomen of oviparous *M. viciae*, suggesting that if U2 is a binding protein, it is not an OBP. The specific expression suggests a possible role in binding of a compound involved in the sexual system, perhaps in egg production or maintenance.

Seven cDNAs for JHBP-like proteins were also cloned. JH plays an important role in the development and growth of insects (Wojtasek &

Prestwich, 1995), and is involved in reproductive maturation and regulation of pheromone biosynthesis in adults (Gadenne, 1998; Tillman *et al*, 1999). In aphids, the main alarm pheromone component, EBF, demonstrates JH-like activity (Mauchamp & Pickett, 1987), and it is possible that the JHBPs cloned in this study are actually binding proteins for EBF. The proteins may bind EBF prior to release from the cornicle droplets that are themselves released from the abdomen via the cornicles (Hardie *et al*, 1999), in addition to being involved in the detection of EBF by the olfactory system. One of the JHBP-like cDNAs was shown to have expression in abdomen tissues as well as antennae, which might support this hypothesis

As with MvicCSP1 and 2, the presence of potential binding proteins in tissues other than those with a chemosensory role would extend binding proteins beyond the olfactory system. A similar situation occurs in the medfly *C. capitata* (Thymianou *et al*, 1998; Christophides *et al*, 2000), where proteins sharing significant similarity to the insect OBPs, although only possessing four of the six conserved cysteine residues, were found to be specifically expressed in the haemolymph of males. Paesen and Happ (1995) presented evidence for the presence of B-proteins, which also showed similarity to insect OBPs, in the tubular accessory sex glands of the male mealworm beetle *T. molitor*, again outside the olfactory system, but still having a role in chemical communication. Thymianou *et al* (1998) have suggested that insects have a large superfamily of carrier proteins, equivalent to the vertebrate lipocalin superfamily, incorporating all of the insect binding proteins,

such as MSSPs, B-proteins, CSP and OBPs, and the JHBPs and U2 could well be subgroups of this superfamily.

7.9 Biochemical studies of insect OBPs

7.9.1 Expression and characterisation of BmorPBP1

BmorPBP1, a cDNA of ~0.6kb (Krieger *et al*, 1996), was expressed successfully using a baculovirus in insect cells, which should allow the production of properly folded and modified protein. The authenticity of the protein was confirmed by reaction with a polyclonal antibody raised elsewhere against the recombinant protein (Steinbrecht *et al*, 1992). SDS-PAGE determined the molecular weight to be 13.5kDa, in fairly good agreement with the reported values of 15.6kDa (Krieger *et al*, 1996) and 15kDa (Maida *et al*, 1993). Since this weight was determined under denaturing conditions, it is for a single subunit, and it is possible that BmorPBP1 exists as a dimer. Debate currently continues in the literature over the oligomeric states of OBPs, and BmorPBP1 has been studied extensively. Wojtasek & Leal (1999b) reported multiple forms of rBmorPBP1 with ion exchange chromatography, and Leal (2000) later suggested that these represented unfolding of the dimer and monomer.

Recently, Damberger *et al* (2000) reported that BmorPBP1 exists in a single "acidic" form below pH 4.9, a single "basic" form above pH 6.0, and as a mixture of both between pH 4.9-6.0. This suggested that the changes observed are consistent with a model whereby the pheromone-bound BmorPBP1 undergoes a transition, presumably to

the acidic form, in the presence of the receptor to release the ligand. This is consistent with the finding that ApolPBP1 bound the corresponding pheromone only between pH 6.0-9.0 (Du *et al*, 1994) i.e. as the basic form.

IEF studies of rBmorPBP1 in the present study confirm the previous findings of four protein bands (here present between pH 5.2 and 4.8) reacting with the antibody. Although the pH measurements may be slightly inaccurate, the presence of four forms, perhaps representing charge isomers, independently supports the work of Wojtasek & Leal (1999b) and Damberger *et al* (2000). It was suggested in Chapter 6 that this might be a phenomenon observed only with recombinant proteins, but some native OBPs have been reported as having a degree of heterogeneity, i.e. *A. polyphemus* (Ziegelberger, 1995; 1996); *M. brassicae* (Nagnan-Le Meillour *et al*, 1996; Maïbèche-Coisné *et al*, 1998b); and *A. mellifera* (Danty *et al*, 1998), as well as the original studies on *B. mori* (Maida *et al*, 1997), perhaps implying that the phenomenon of interconversion may be common to insect OBPs.

7.9.2 Aphid antennal proteins

The antibody raised against BmorPBP1 was incubated with soluble proteins extracted from the antennae of *M. viciae* (male and oviparae), but there was no cross-reactivity, suggesting that the epitopic region of the *B. mori* protein recognised by the antibody is not present in any soluble aphid antennal proteins. Since antisera raised against a lepidopteran OBP can be used to identify other lepidopteran OBPs

(Steinbrecht *et al*, 1992, 1994, 1995), this result is consistent with the finding that OBPs differ between unrelated species. This is in line with the differences in amino acid sequences between moth and non-lepidopteran OBPs (8-40%, McKenna *et al*, 1994; Ozaki *et al*, 1995; Danty *et al*, 1997; Wojtasek *et al*, 1998) and is supported by the percentage similarity between MvicOBP1 and other OBPs being around 30-40%.

7.10 Significance of these results

Whilst OBPs have been cloned from over thirty species of insect, the cloning of MvicOBP1 is the first report of such from an aphid, a homopteran insect, thereby extending the range of insect orders with OBPs. Only one aphid OBP was identified so this work does not contribute to the discussion of whether these proteins are involved in molecular discrimination.

It has been suggested that the cloning of an OBP homologue from within the "Hemipteroid Assemblage" would indicate that OBP-related genes were present in the species ancestral to both the Endopterygota and the Hemipteroids (Vogt *et al*, 1999). The PBP and GOBP classes appear to be Lepidoptera-specific, whilst OS-E and OS-F are specific to *D. melanogaster*, with ABPX extending over the order boundaries (Vogt *et al*, 1999; Robertson *et al*, 1999; Hekmat-Scafe *et al*, 2000). If all OBPs do belong to a single homologous group, derived from a common ancestor, MvicOBP1 extends this gene family further, with the difference reflecting the adaptation of chemosensory function

for aphid ecology.

The cloning of MvicCSP1 and MvicCSP2 is also the first report of CSPs in an aphid. Here, two different CSPs have been cloned, in accordance with the multiplicity of CSPs observed in other insects (Danty *et al*, 1998; Angeli *et al*, 1999; Marchese *et al*, 1999; Picimbon & Leal, 1999; Robertson *et al*, 1999; Picimbon *et al*, 2000a; Picimbon *et al*, 2000b). MvicCSP1 and 2 show a much higher level of amino acid similarity to other insect OBPs, in line with the average similarity of 40-50% seen across species (Vogt *et al*, 1999). Whilst OBPs have diverged and become very species-specific to cope with individual species' ligands, the CSPs have diverged much less, which may suggest a common function, and the presence of more than one type of CSP may reflect some kind of molecular discrimination.

This study also reported the cloning of U2, a tissue- and morphologically-specific transcript, and a number of cDNAs whose amino acid sequences show similarity to the JHBPs of *M. sexta* (Robertson *et al*, 1999). The JHBPs are potentially very interesting because of their possible role as EBF binding proteins, and their presence in aphids supports the idea that insects, like vertebrates, have a superfamily of binding proteins (Thymianou *et al*, 1998).

Whilst vertebrate and insect OBPs are thought to have arisen by convergent evolution, and therefore are very different at the amino acid level, "lipocalin-type" proteins have also been identified in insects. Insecticyanin, a protein that maintains biliverdin levels in the haemolymph of insects, has been identified in *M. sexta* (Riley *et al*,

1984), ASP1a, a protein identified in *A. mellifera*, showed similarity to insecticyanin (Danty *et al*, 1998), and more recently, a cuticular surface protein specific to tergal gland secretion, was cloned from the Madeira cockroach *Leucophaea maderae* (Fabricius; Blattidae; Korchi *et al*, 1999). All three of these proteins show similarity (15-20%) to vertebrate lipocalins such as human apolipoprotein D, and share the function of binding small, hydrophobic molecules, this similarity potentially bridging the gap between vertebrates and insects. Since the tergal gland secretion has an aphrodisiac influence on male *L. maderae* (Birch, 1974), this protein is still involved in chemical communication. Perhaps the vertebrate and insect OBPs are not so different after all, and maybe they have evolved from a common ancestor of both vertebrates and arthropods.

7.11 Classification of insect binding proteins.

The study of insect OBPs, and the finding of other proteins involved in binding small hydrophobic molecules, may eventually lead to a better classification of proteins involved in chemical communication, and a clearer distinction between those proteins involved in the perception of odours and those thought to release or bind other chemical messages (Felicioli *et al*, 1993). Currently, OBPs are thought to bind odour molecules and, in insects, the phrase is specifically applied to those proteins with six cysteine residues. This may turn out to be a misleading classification since other proteins, with less or more cysteines, may also be found to bind odour molecules, and thus be

"odorant binding proteins". Future studies may also show that GOBPs can be as specific in binding their ligands as the PBPs are, and lead to a better understanding of insect-environment interactions.

7.12 Exploitation of olfactory proteins

Our understanding of the mechanisms of olfactory perception has been greatly enhanced in recent years, with the essential role of OBPs emerging relatively recently, mainly as a consequence of developments in molecular biology, and in the application of chemical techniques, such as NMR (nuclear magnetic resonance), to biological molecules. Such knowledge forms the basis for a range of commercial applications for OBPs, and olfactory proteins in general.

One possible commercial opportunity is in the development of biosensors. A biosensor is "an analytical tool or system, consisting of an immobilised biological material....in intimate contact with a suitable transducer device which will convert the biochemical signal into a quantifiable electronic signal" (Gronow, 1984). Biosensors are already used to measure changes in temperature (thermistors) and conductance (conductimeters) (Gronow *et al*, 1988), and probably the best-developed biosensor allows diabetic patients to monitor their own blood glucose levels. The olfactory systems of animals, and of insects in particular, where a large array of proteins exhibits high levels of specificity towards ligands, would be an ideal basis of biosensor systems.

Whole insect biosensor systems are already being tested.

Colorado beetles detecting compounds released when potato crops are damaged can warn farmers of pest attack in potato crops (Charles, 1997). Antennae of insects are also being tested as sensors to warn when food is about to go rotten (Pickett *et al*, 1998). However, this type of biosensor lasts only as long as the insect or the isolated antenna remain active, and the individual biological components of the olfactory pathways might be more stable.

Use of ORs or OBPs in such systems could increase the specificity of the biosensor, with the protein being able to detect a single odorant molecule out of a mixture. However, problems with using recombinant ORs in biosensors may arise because they are generally difficult to express heterologously (Zhao & Firestein, 1999; Hatt *et al*, 1999; Malnic *et al*, 1999), and even if expression is successful, a functional biosensor may require part or the whole of the signal transduction pathway. However, an electronic nose has successfully been generated, using a mixture of olfactory receptor proteins from bullfrogs and piezoelectric crystal electrodes, which responds to a number of odours (Wu, 1999). OBPs, and their potential redox shift, or conformational change, may overcome the problem of unsuccessful OR expression, and bring even greater specificity to the system, whilst synthetic peptides representing only the binding site of the OBP could epitomize the future of biosensors (Felicioli *et al*, 1994). But still, the ultimate question is will these isolated proteins function as they do *in vivo* when in a synthetic biosensor system?

Long-term applications of biosensors include the identification of

illnesses (Service, 1998); the detection of pollutants or toxicants (e.g. cork taints in wine); use in chemical and quality control analyses (e.g. manufacturing processes); and the detection of dangerous and/or prohibited substances (e.g. drugs at airport customs), whilst OBPs themselves could also be used for enhancing fragrances and/or flavours, and the impregnation of fabrics to deodorize against unwanted odours (Bell, 1996).

Perhaps more immediately, research into OBPs can play a role in pest management programs. Many of the OBPs studied are from insect species that threaten world crops or pose risks to human health (Metcalfe & Metcalfe, 1993). An understanding of these insects, and their semiochemistry and ecology, could allow manipulation of the chemicals used in host location, to either divert insects from a vulnerable crop, or attract them to a trap crop. This idea is the basis of the “push-pull” or stimulo-deterrent diversionary strategies of crop protection (Pickett & Wadhams, 2000).

If the expression of OBPs in insect pests could be prevented or “knocked out” in some way (e.g. by antisense technology), the response to a particular signal compound might be negated, and the crop or host potentially protected. The technology for such gene targeting is only now coming to the fore, and whilst baculoviruses, which have been used for control of some lepidopteran pests (Moscardi, 1999), have potential as vectors for introducing antisense fragments into the Lepidoptera (Lee *et al*, 1997), research into transgene vectors for other insects, such as aphids, is moving more slowly (O’Brochta & Atkinson,

1997). The main problem with such control strategies is that they require the engineered genotype to become the stable population, which may be difficult to achieve, although recent research into mosquito transgenes suggests that this is a distinct possibility (Olson *et al*, 1996; James *et al*, 1999; Coates *et al*, 2000). Nonetheless, there are commercial opportunities for OBPs within integrated pest management schemes, and in future years, as the technology progresses, these may assist in overcoming the problems of insecticide resistance and the hazards that arise from the use of insecticides.

7.13 Future work

A number of exciting results have been reported in this study, many of which need to be extended. In order to establish the role of the putative OBP, MvicOBP1, the sequence of this cDNA must be completed to allow functional expression and ligand binding studies, perhaps in a similar approach to that used for the BmorPBP1-bombykol complex (Oldham *et al*, 2000). A similar approach should be adopted for the two aphid CSPs to determine whether they are involved in binding odour molecules, or proteins involved in other types of chemical communication, and whether they show any molecular discrimination. Other aphid OBPs should also be investigated, ideally using the rhinarium lymph rather than whole antennae, perhaps by adapting the electrode apparatus currently used for single cell recording (SCR) to isolate the rhinaria lymph. However, the small size of aphid antennae may always mean that the amount of material is a limiting factor.

The sequences of MvicOBP1 and the CSPs could also be used to search for related genes and proteins in other aphid species, and this could enhance our understanding of the relationship between these species, their ecology and their evolution. The JHBPs could be an area of great interest in aphid ecology, especially if they are involved in binding the aphid alarm pheromone, EBF, and the putative GPCR should also be studied further, as this could be the first homopteran OR to be cloned. The cytochrome P450, Mv164, and its potential metabolism of odorant molecules is also an exciting result, and could complete the perireceptor pathway in aphids: functional expression and observation of its metabolism may answer many questions.

Research into the heterogeneity of BmorPBP1 continues elsewhere, and other insect OBPs may well be found to exhibit similar properties, which will enhance understanding of the perireceptor events of olfaction.

7.14 Final thoughts

Although evolutionarily and phylogenetically distinct, studies on the olfactory perception of both insects and vertebrates reveal striking parallels as well as marked differences. Similarities include the presence of olfactory machinery such as OBPs, ORs, G-proteins and signal transduction proteins, as well as the organisation of systems beyond the receptor. Differences can be seen at the amino acid sequence level: OBPs and ORs are highly different, so much so that attempts to identify ORs in insects using homology cloning approaches

failed. However, the conservation in form and function of the olfactory system in phylogenetically diverse animals would suggest the presence of a fundamental strategy for detecting and discriminating odours (Ache, 1994), and the parallels between the systems reinforces the importance of insects as models for olfaction.

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Appendix 1. Primer sequences

Primer sequences and applications are detailed in the Tables on the following pages.

Primer name	Sequence 5'⟶3'	Orientation	Application
AA1	$(C)_{13}AAGC(T)_{17}$	Antisense	cDNA synthesis (*HindIII* site; Subtractive hybridisation)
AA2	$ATCAGGTGACGTCGA(C)_{13}$	Sense	Tester amplification (*SalI* site; Subtractive hybridisation)
AA3	Bio-$ATCAGGTGACGTCGA(C)_{13}$	Sense	Driver amplification (*SalI* site; Subtractive hybridisation)
AL22	$(C)_{13}AGATC(T)_{18}$	Antisense	cDNA synthesis (*XbaI* site; Subtractive hybridisation)
AL23	$CCGGAATT(C)_{14}$	Sense	Tester amplification (*EcoRI* site; Subtractive hybridisation)
AL24	Bio-$CCGGAATT(C)_{14}$	Sense	Driver amplification (*EcoRI* site; Subtractive hybridisation)
AL25	TGTAAACCAGCCTGTGGAC	Sense	U2 GSP for RT-PCR, sequencing
AL28	CGAGGTTATGCTTGTCTCAAAG	Sense	O2 GSP for RT-PCR
AL29	GTGACGAAAAATAACGATACGG	Antisense	O2 GSP for RT-PCR
AL30	TGCTTGTCTCAAAGATTAAGCC	Sense	Q2 GSP for RT-PCR
AL31	CGAGGTTCAAAATTCGCCAAG	Antisense	Q2 GSP for RT-PCR
AL33	AGATTGCAGGTATGCGTGG	Antisense	MvicOBP1 GSP for RT-PCR, sequencing, 5' RACE
AL34	TCACCAAACACGAAATGGC	Sense	MvicOBP1 GSP for RT-PCR, sequencing
AL36	AGTAGATTCTCCGTCATTGCCC	Sense	E3 GSP for RT-PCR
AL37	TGTGCCGCATTAGCCTGAAC	Antisense	E3 GSP for RT-PCR
AL38	CGGTTCAAAAACCCAAACCAG	Sense	Actin GSP for RT-PCR
AL39	TGGTGATGATTCCCGTGTTC	Antisense	Actin GSP for RT-PCR

Primer name	Sequence 5'→3'	Orientation	Application
AL40	TCGACCACTTGGTGAACCTG	Sense	Mv164 GSP for RT-PCR, sequencing
AL41	CTAAAAGCCGACCAAAACCAAC	Antisense	Mv164 GSP for RT-PCR, sequencing, 5'RACE (GSP1)
AL42	AGGGGCTGAGGCAGAATAACC	Antisense	MvicOBP1 GSP for 5' RACE (GSP2); sequencing
AL43	TCTTTGCTCTTACACCTTC	Antisense	MvicOBP1 GSP for 5'race (GSP3), sequencing
AL44	TGAACCAAGGAGTAAGAGCC	Sense	C9 GSP for RT-PCR
AL45	GAGTAACCCAAAATCGGAGC	Antisense	C9 GSP for RT-PCR
AL46	ATTTCCCTGGTCTAGCAAAC	Sense	C10 (MvicCSP1) GSP for RT-PCR
AL47	GGGCAGGTACACAACTAAA	Antisense	C10 (MvicCSP1) GSP for RT-PCR
AL48	AGACTGGAGAGGAGTAAGCC	Sense	C29 GSP for RT-PCR
AL49	GCCGAAGAAGCACCAAAAC	Antisense	C29 GSP for RT-PCR
AL50	GTGACGGTCTCTGCAAAATTAG	Sense	C31 GSP for RT-PCR
AL51	GCATTTTGGTGGTGGTAAAC	Antisense	C31 GSP for RT-PCR
AL52	ATGGTTTTAGCAGGAACACTC	Sense	C41 GSP for RT-PCR
AL53	ACTAGTGATTTCGAGCGGC	Antisense	C41 GSP for RT-PCR
AL54	AGTGTAGTCCAAAAGCACAAC	Antisense	Mv164 GSP for sequencing, 5' RACE (GSP2)
AL55	CGACGCTTCACTTACATCAAT	Antisense	Mv164 GSP for sequencing, 5' RACE (GSP 3)
AL58	GTCGCCGAAGAAGGTGTAAG	Sense	MvicOBP1 GSP for sequencing
AL59	GTAACATGATTTGCCTTAAT	Sense	MvicOBP1 genomic GSP (intron)
AL60	TAGTAGCACACGTATACAT	Antisense	MvicOBP1 genomic GSP (intron)
AL62	CCACGCATACCTGCAATCT	Sense	MvicOBP1 GSP for sequencing

Primer name	Sequence 5'→3'	Orientation	Application
AL61	AAGGTGATGAATATCAGATA	Antisense	MvicOBP1 GSP for sequencing, 5' RACE (GSP3)
AL63	GGTCGGCTTTTAGACGTATC	Sense	Mv164 GSP for sequencing
AL77	TCGACCAAATTTTGGCTTCC	Sense	C130 (MvicCSP2) GSP for RT-PCR
AL78	TGTCAAGAAGCAGTTTCCAC	Antisense	C130 (MvicCSP2) GSP for RT-PCR
M13 FP	GTAAAACGACGGCCAGT	Sense	Sequencing
M13 RP	GGAAACAGCTATGACCATG	Antisense	Sequencing
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	Sense	5' RACE primer for amplification
AUAP	GGCCACGCGTCGACTAGTAC	Sense	5' RACE primer for amplification
CONTROL GSP1	TTGTAATTCATTAAGCATTCTGCC	Antisense	5' RACE control reagent primer
CONTROL GSP2	GACATGGAAGCCATCACAGAC	Antisense	5' RACE control reagent primer
CONTROL GSP3	CGACCGTTCAGCTGGATATTAC	Antisense	5' RACE control reagent primer
cDNA synthesis primer	TTTTGTACAAGCT$(T)_{30}$	Antisense	Clontech PCR-Select kit cDNA synthesis primer
PCR Primer1	CTAATACGACTCACTATAGGGC	Sense	Clontech PCR-Select Kit PCR primer
Nested PCR Primer 1	TCGAGCGGCCGCCCGGGCAGGT	Sense	Clontech PCR-Select Kit PCR primer
Nested PCR Primer 2R	AGCGTGGTCGCGGCCCAGGT	Antisense	Clontech PCR-Select Kit PCR primer
G3PDH 5'	ACCACAGTCCATCGCCATCAC	Sense	Clontech PCR-Select Kit PCR control primer
G3PDH 3'	TCCACCACCCTGTTGCTGTA	Antisense	Clontech PCR-Select Kit PCR control primer

Appendix 2. Plasmid vector maps

Maps of cloning vectors used in this study are presented on the following pages.

pBluescript II SK (+/-)
phagemid vector
2961 bp

Nae I 131
f1 (-) origin
Ssp I 442
Ssp I 2850
Ssp I 19
f1 (+) origin
Nae I 330
Xmn I 2645
Sca I 2526
Pvu I 2416
Amp
Pvu I 500
Pvu II 529
lacZ
BssH II 619
Kpn I 657
MCS
Sac I 759
BssH II 792
T7
T3
Pvu II 977
ColE1 origin
Afl III 1153

Reverse primer
5' GGAAACAGCTATGACCATG 3'

T3 primer
5' AATTAACCCTCACTAAAGGG 3'

SK primer
5' CGCTCTAGAACTAGTGGATC 3'

KS Primer
3' CTATGGCAGCTGGAGCT 5'

T7 Primer
3' CGGGATATCACTCAGCATAATG 5'

M13 -20 Primer
3' TGACCGGCAGCAAAATG 5'

β-Galactosidase
T3 promoter
+1 T7 promoter

Figure 1. pBluescript II SK vector circle map

pFASTBAC1 MCS and promoter region:

```
                                 3942     → +1
5' [polyhedrin promoter] - - -AAATAAGTAT TTTACTGTTT TCGTAACAGT TTTGTAATAA AAAAACCTAT

                                            BamH I     Rsr II      BssH II   EcoR I      Stu I      Sal I   Sst I
AAATATTCCG GATTATTCAT ACCGTCCCAC CATCGGGCGC GGATCCCGGT CCGAAGCGCG CGGAATTCAA AGGCCTACGT CGACGAGCTC

 Spe I      Not I      Nsp V Xba I   Pst I     Xho I   Sph I   Kpn I   Hind III                          4171
ACTAGTCGCG GCCGCTTTCG AATCTAGAGC CTGCAGTCTC GAGGCATGCG GTACCAAGCT TGTCGAGAAG TACTAGAGGA TCATAATCAG 3'
                                                                                    stop codons
```

+1 corresponds to the transcriptional start for the polyhedrin promoter.

ATT—site of original translational start. The ATG was mutated to an ATT.

Figure 2. pFASTBAC donor plasmid map

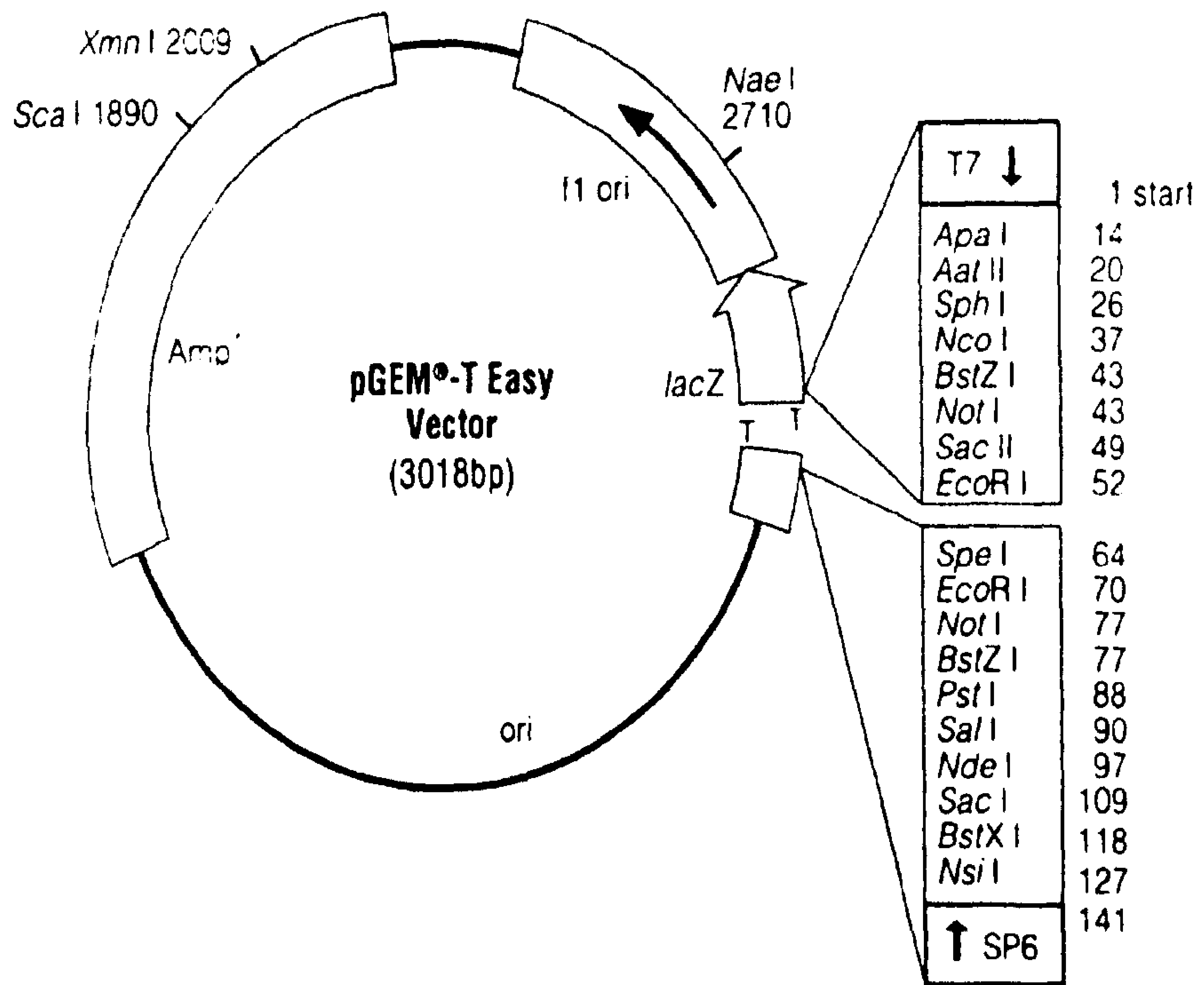

Figure 3. pGEM®-T Easy vector circle map

Appendix 3. Genetic code and amino acid symbols

Position 1	Position 2				Position 3
	U	C	A	G	
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Stop	UGA Stop	A
	UUG Leu	UCG Ser	UAG Stop	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

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