

**Molecular and Genetic Analysis of the *vha16* Gene in
*Drosophila melanogaster***

A thesis submitted for the degree of
Doctor of Philosophy at the University of Glasgow

By

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Abstract

Vacuolar H⁺-ATPases (V-ATPases) are multi-subunit enzymes which pump protons across cellular membranes via hydrolysis of ATP. They are related, both in structure and function, to F₁-F₀ ATP synthases and much about the V-ATPase mode of action has been inferred from F₁-F₀ studies. V-ATPases have been isolated from a broad phylogenetic range of eukaryotes, from single celled organisms to higher plants and animals. In these higher organisms they can play both endomembrane and plasma membrane roles in a large array of cell types.

Several genes encoding subunits of the V-ATPase holoenzyme have been cloned from the model genetic organism *Drosophila melanogaster*. One of the subunits cloned from *D. melanogaster*, *vha16*, encodes the 16 kDa pore forming protein, through which the hydrogen ions pass.

Firstly, this project focuses on this proteolipid gene in *Drosophila melanogaster*, describing the isolation and characterisation of a lethal P-element insert within the originally described gene, and discussing how the insertion of this element disrupts the normal function of the gene. Also discussed is how this P-element can be used to show the spatial and temporal expression of the gene *in vivo*.

Secondly it describes the isolation of four new homologues of this gene in *D. melanogaster*. It shows their sequence relationship to the original *Drosophila* protein, and the protein isolated from other organisms and discusses if they are expressed and whether they would function as V-ATPase pores.

This study also describes the attempted rescue of the lethal P-element phenotype with a range of proteolipid sequences cloned from *Drosophila* and also proteolipids of other organisms.

Lastly, this project describes the isolation of two other *Drosophila* V-ATPase membrane associated subunits, one homologous to *PPA1* in yeast and the other homologous to *M9.7* in *Manduca sexta*.

Abbreviations

ADP	adenosine diphosphate
APS	ammonium persulphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BDGP	Berkeley <i>Drosophila</i> Genome Project
bp	base pairs
BSA	bovine serum albumin
CAP _{2b}	cardioacceleratory peptide
cDNA	complementary DNA
CIP	calf intestinal alkaline phosphatase
d	days
dATP	2' deoxyadenosine triphosphate
dCTP	2' deoxycytosine triphosphate
dGTP	2' deoxyguanosine triphosphate
dNTP	2' deoxy(nucleotide) triphosphate
dTTP	2' deoxythymidine triphosphate
dUTP	2' deoxyuridine triphosphate
DCCD	<i>N,N'</i> -dicyclohexylcarbodiimide
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DMF	dimethylformamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetra acetic acid (disodium salt)
EM	electron microscopy
EMS	ethylmethansulphonate
ER	endoplasmic reticulum
EST	expressed sequence tag
EtBr	ethidium bromide
g	gram
g	centrifugal force equal to gravitational acceleration
GFP	green fluorescent protein
h	hours

HEPES	N-([2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid])
HT	hydroxy tryptamine
I.D.	inner diameter
kb	kilobases
kDa	kiloDaltons
M	molar
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
min	minute
mRNA	messenger RNA
MOPS	3-[N-morpholino]propane-sulphonic acid
NBT	nitro-blue-tetrazolium
ng	nanograms
nm	nanometre
OD	optical density
O.D.	outer diameter
OLB	oligo-labelling buffer
OR	Oregon R
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmf	proton-motive force
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
<i>rp49</i>	<i>Drosophila</i> ribosomal protein 49
RT-PCR	reverse transcriptase polymerase chain reaction
s	second
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	unit
UAS	upstream activation sequence
UV	ultraviolet
V-ATPase	vacuolar H ⁺ adenosine triphosphatase
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
μ Ci	microCurie

μg	microgram
μl	microlitre
μM	micromolar
$^{\circ}\text{C}$	degrees Celsius

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Chapter 1
Introduction

1.1 Vacuolar H⁺-ATPases

1.1.1 Discovery and function

Vacuolar H⁺-ATPases (V-ATPases) are vital enzymes that play many roles within a large array of cell types and organisms. They are complex multi-subunit proton pumps that function in eukaryotic endomembranous organelles, such as clathrin-coated vesicles, chromaffin granules (Nelson, 1992a), synaptic vesicles (Moriyama *et al.*, 1992), endosomes, lysosomes (Schneider, 1987) and plant and fungal vacuoles (Bowman, 1986; Taiz, 1992).

The early 1980s provided the first biochemical characterisations of the V-ATPase in yeast, (Kakinuma, 1981), *Neurospora*, (Bowman, 1982), chromaffin granules, (Cidon, 1983) and clathrin-coated vesicles (Forgac, 1983). Purification of the *Saccharomyces cerevisiae* enzyme followed soon after (Uchida, 1985) and now the enzyme has been purified and characterised from such varied sources as clathrin-coated vesicles (Xie, 1986), chromaffin granules (Cidon, 1983; Moriyama, 1987), Golgi membranes (Young, 1988), synaptic vesicles (Floor, 1990), kidney tubules (Gluck, 1987), insect membranes (Dow, 1995; Schweikel *et al.*, 1989) and plants (Parry *et al.*, 1989; Randall, 1986).

V-ATPases have been implicated in several clinical situations, for example, infertility, bone and kidney disease and cancer, all of which will be discussed later in this section.

Endomembrane roles

The presence of V-ATPases in intracellular membrane organelles is vital as they play important roles in many stages of membrane trafficking, from internalisation of clathrin-coated vesicles, through endosomes, lysosomes, vesicle recycling and neurotransmitter release and uptake (Mellman, 1992). The major role of the V-ATPase in these organelles is to pump protons, via hydrolysis of ATP, into the organellar lumen, thereby generating a proton-motive force (pmf) that is used to drive secondary transport processes, e.g., accumulation of both hormones, such as catecholamines into chromaffin granules, and neurotransmitters, such as glutamate, acetylcholine and GABA, into neurotransmitter vesicles via their respective transporters (Moriyama *et al.*, 1992). In plant vacuoles this pmf is aided by a vacuolar pyrophosphatase and energises transport into the vacuole of a variety of amino acids, ions and carbohydrates, this being critical for osmoregulation and homeostasis. Another function of the V-ATPase, as well as generation of a pmf, is

acidification of the organellar lumen (Mellman, 1992). In lysosomes this acidification provides the correct pH for dissociation of receptor/ligand complexes, therefore playing an important role in receptor recycling and ligand targeting, meanwhile in plant and fungal vacuoles a lower pH (pH 5-5.5) is required for function of certain enzymes such as proteases (Bowman, 1982; Taiz, 1992; Yoshimori, 1991).

Plasma membrane roles

V-ATPases do not simply operate in endomembranes as they have now been isolated from many higher eukaryotic plasma membranes. These include such diverse cell types as osteoclasts (Blair, 1989), macrophages (Lukacs, 1990; Swallow, 1990), neutrophils (Grinstein, 1992; Nanda, 1992), kidney proximal and distal tubules (Gluck and Nelson, 1992), superficial cells of bladder epithelium and certain cell types in the vas deferens and epididymis (Breton, 1996). They are also present in insect (Schweikel *et al.*, 1989; Wieczorek *et al.*, 1989) and fish epithelia (Lin, 1994; Sullivan, 1995).

Single cells

Osteoclasts are motile cells involved in the breakdown of bone during skeletal growth and remodelling. It has been observed that the chicken osteoclast V-ATPase is more sensitive to vanadate than V-ATPase from other sources (Chatterjee, 1992; Chatterjee *et al.*, 1992). This differing V-ATPase pharmacology has interested medical researchers working in the fields of osteoporosis, osteoarthritis and skeletal cancer, diseases in which the reduction of bone degradation would be of benefit to the sufferers.

Osteoclasts function by attaching to bone, and in an enclosed extracellular space excrete protons, producing an acidic environment between the cell and bone. They then excrete proteolytic enzymes that require these acidic conditions to mature and function. These enzymes aid the degradation of bone material. Research is being undertaken to try to isolate alternative compounds that will only reduce the action of the osteoclast V-ATPase (Farina, 1999), this approach would allow the treatment of the degradative disease without altering the function of the V-ATPases in other cells.

In macrophages, V-ATPases control intracellular pH which is important to these cells as they are often subjected to acidic environments (Heming, 1995; Swallow, 1990). Upon activation, neutrophils require V-ATPases to maintain cytosolic pH by excreting acid that they themselves produce as a result of their respiratory burst (Nanda, 1992).

Epithelia

In the male reproductive tract luminal fluid is acidified in the epididymis by a V-ATPase. This acidity is required to prevent the sperm from maturing before they pass through into the *vas deferens*, where they are kept immotile before ejaculation. The change in pH upon ejaculation causes activation (Brown, 1997). Failure of this sperm activation is thought to be one of the many causes of male infertility.

In the kidney two sets of specialised intercalated cells containing V-ATPases exist. In the α -intercalated cells V-ATPases are recruited to apical membranes in response to acidosis when H^+ secretion and bicarbonate resorption are at their highest. β -intercalated cells can insert V-ATPases into their basolateral membranes in response to alkalosis which requires bicarbonate secretion (Brown *et al.*, 1992). The symptom of distal renal tubular acidosis (DeFranco, 1995), in Sjogren's syndrome, an autoimmune disease, is as a result of loss of V-ATPase in the kidney collecting duct cells. This is another example of V-ATPase malfunction in a clinical situation.

The V-ATPase is also thought to be involved in regulating the pH of aqueous humour in ocular ciliary epithelium and therefore is a prospective target in the treatment of glaucoma, a disease treated by altering the amounts of aqueous humour in the eye (Wax, 1997; Wu, 1997; Wu, 1998).

In the lepidopteran midgut the V-ATPase is situated in the apical cell membrane of one of two principal cell types in this epithelium, the goblet cell (Klein *et al.*, 1991; Wieczorek *et al.*, 1990). The action of this V-ATPase results in alkalinisation of the outer lumen, in direct contrast to the systems previously mentioned, where acidification occurs. This is due to the presence of a K^+/H^+ exchanger that is very tightly coupled to the V-ATPase (Lepier *et al.*, 1994; Wieczorek, 1991). In the goblet cell, every two H^+ produced by the V-ATPase are exchanged for a K^+ by the exchanger, resulting in H^+ being driven back into the cell and K^+ being excreted into the lumen. This process is shown in the figure below (figure 1) along with other ions that take part in the reaction (Wieczorek, 2000).

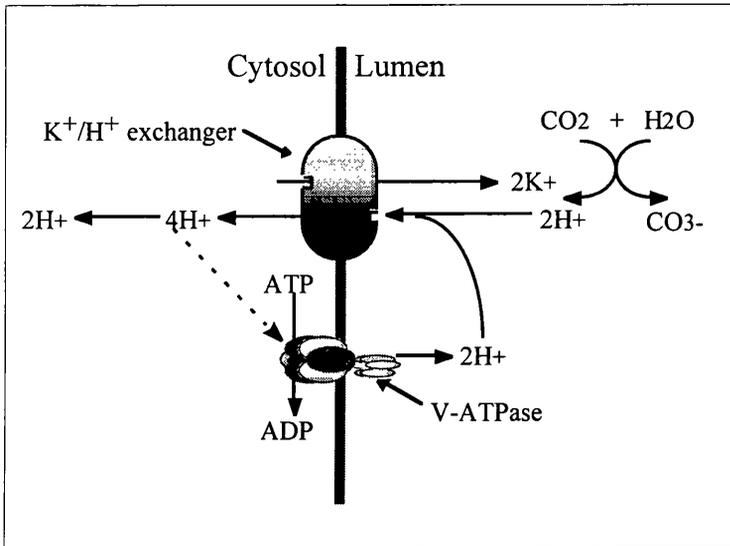


Figure 1.1 Schematic diagram showing alkalisation of the *Manduca sexta* lumen. Modified from (Wieczorek, 2000).

Some of the varied functions of the V-ATPase are summarised in table 1.1.

Plasma membrane	Function	Ref.
Kidney proximal tubule	Bicarbonate secretion, intracellular pH regulation.	(Gluck and Nelson, 1992)
Kidney collecting duct	Bicarbonate secretion, intracellular pH regulation.	(Gluck and Nelson, 1992)
Osteoclasts	Produces acidic environment for bone degradation.	(Blair, 1989)
Macrophage	Protect cells against acidic environments by regulating pH.	(Swallow, 1990)
Seminal duct	Sperm maturation and immobility.	(Brown, 1997)
Corneal epithelium	Production of aqueous humour.	(Wax, 1997)
Intrahepatic bile duct	H ⁺ -secretion into periductular fluid.	(Villanger, 1993)
Hippocampal astrocytes	Regulation of intracellular pH _i .	(Pappas, 1993)
Chick embryo chorioallantoic membrane	Intracellular pH regulation.	(Narbaitz, 1995)
Turtle bladder	Urinary acidification.	(Gluck, 1982)
Frog skin	Body fluid homeostasis.	(Ehrenfeld, 1997)
Trout gill	Acid/base homeostasis.	(Lin, 1994)
Lepidopteran midgut	Provides proton-motive force to drive secondary exchanger.	(Wieczorek, 1991)
Malpighian tubules	Provides proton-motive force to drive secondary exchanger.	(Maddrell and O'Donnell, 1992)
Insect sensory sensilla	Provides proton-motive force to drive secondary exchanger.	(Klein, 1992)
Locust hindgut	Lumen acidification.	(Phillips, 1996)
Cockroach salivary glands	Energisation of distal duct cell transport processes.	(Just, 1994)
Endomembrane		
Plant vacuole	Provides proton-motive force to drive secondary exchanger.	(Taiz, 1992)
Thyroid parafollicular cells	Drives uptake of 5-HT.	(Cidon, 1991)
Neurotransmitter vesicles	Provides pmf to drive neurotransmitter accumulation.	(Moriyama <i>et al.</i> , 1992)
Chromaffin granules	Provides energy for catecholamine uptake.	(Cidon, 1983)

Table 1.1 **Roles of Vacuolar H⁺-ATPases** in different cell types and membranes.

1.1.2 Structure

V-ATPases are holoenzymes consisting of at least ten separate proteins with unique subunits (table 1.2) thought to exist in various organisms and organelles, for instance V-ATPases isolated from different kidney substructures show different properties (Gluck *et al.*, 1992) and compositions. However these holoenzymes always have the same overall structure in the form of two distinct structural domains, similar in structure to those of the F_1-F_0 ATP synthases, called V_1 and V_0 . V_1 is the soluble head group facing the cytoplasm and V_0 the membrane bound sector conducting the flow of ions. The similarity between V- and F-ATPases is very pronounced with many F-subunits having homologues in the V-ATPase that are thought to have similar functions (Hilario, 1998). F_1-F_0 -ATPases have been studied in much greater depth than V-ATPases, but much can be inferred about V-ATPases from them.

Cloning of many V-ATPase subunits has been facilitated by the *Saccharomyces cerevisiae vma⁻* model (Nelson and Nelson, 1990). Yeast, when mutant for V-ATPase can only survive under certain conditions, namely they cannot grow at a pH above 7. Therefore replica-plating colonies on plates at pH 5.5 and 7.5 has provided an ideal screen for mutations in V-ATPase function and has led to the identification of most V-ATPase subunits and accessory proteins. Additionally, gene replacement experiments can be performed whereby DNA sequences can be introduced back into the yeast mutant to see if the wild-type phenotype can be rescued.

Subunit	Subunit/ enzyme	Mr (kDa)	Gene	Function	Ref.
V₁					
A	3	69	<i>VMA1</i>	ATP-binding, catalytic	(Hirata, 1990)
B	3	60	<i>VMA2</i>	ATP-binding, regulatory	(Nelson <i>et al.</i> , 1989)
C	1	42	<i>VMA5</i>	Assembly, activity	(Beltran <i>et al.</i> , 1992)
D	1	32	<i>VMA8</i>	Assembly, activity	(Graham <i>et al.</i> , 1995)
E	1	27	<i>VMA4</i>	Assembly, activity	(Ho, 1993a)
F		14	<i>VMA7</i>	Assembly, activity	(Nelson <i>et al.</i> , 1994)
G		16	<i>VMA10</i>	ATPase activity	(Supekova <i>et al.</i> , 1995)
H (SFD)		54	<i>VMA13</i>	Activity	(Ho, 1993b)
V_o					
100 kDa	1	100	<i>VPH1</i>	Proton transport, assembly, targeting	(Manolson, 1992)
36 kDa		36	<i>VMA6</i>	Assembly, activity	(Bauerle, 1993)
c	4	17	<i>VMA3</i>	Proton translocation	(Umemoto <i>et al.</i> , 1990)
c'	1	17	<i>VMA11</i>	Proton translocation	(Umemoto <i>et al.</i> , 1991)
c''	1	23	<i>PPA1</i> (<i>VMA16</i>)	Proton translocation	(Hirata, 1997)
Associated					
vph2p		25	<i>VPH2</i>		(Bachhawat, 1993)
vma21p			<i>VMA21</i>	Assembly in ER	(Hill, 1994)
vma22p			<i>VMA22</i>	Assembly in ER	(Hill, 1995)
vma12p			<i>VMA12</i>	Assembly in ER	(Hirata, 1993)

Table 1.2 Subunits of the *Saccharomyces cerevisiae* V-ATPase.

V₁ subunits

Subunits in the V₁ head group include subunit A which contains the ATP-binding catalytic domain, which was discovered using agents such as *N*-ethylmaleimide which binds to a conserved cysteine in the A subunit and inhibits ATP hydrolysis (Arai, 1987; Bowman *et al.*, 1988; Feng, 1992; Zimniak *et al.*, 1988). This subunit shares highly conserved residues with the catalytic β subunit of the F₁-F_o-ATPase, particularly at the catalytic site (Omote, 1992; Senior, 1992).

Subunit B is also ATP-binding, but in this case the ATP is not hydrolysed. Binding of the ATP is thought to be regulatory, this being inferred from experiments where blockage of and mutations in this site caused inhibition of the clathrin vesicle enzyme and yeast enzyme respectively (Vasilyeva, 2000; Vasilyeva and Forgac, 1996). The hydrolysis of ATP occurs in a co-operative manner, with catalysis being much quicker when all 3 ATP-binding sites are occupied in subunit A. Three of each of subunits A and B are present in V₁, arranged tandemly in a circular fashion around a central core (figures 1.2 and 1.3a), where subunit D is thought to sit acting as a spindle. Subunit D acting as the spindle is purely speculative as it only has a low degree of homology to the spindle subunit (γ) in F₁-F_o ATPases but is the only likely candidate proteins so far discovered, although other ideas are that the spindle could also be subunit E, or a combination of both. Other V₁ subunits, C, E, F, and G are also required for function and assembly of V₁ as a mutation in any of them will both abolish ATPase activity and assembly of the subunits onto the V_o subunits in the membrane. The function of these subunits is unclear but experiments have shown that subunit F mutations also affect assembly of V_o with V₁ and so may be involved in contact between the two domains and stabilisation of the complex.

V_o subunits

V_o components include vph1p, the largest of the V-ATPase subunits (100 kDa). This has a large hydrophilic domain as well as a hydrophobic domain that spans the membrane several times. In *Saccharomyces cerevisiae* this protein is encoded by two genes, *VPH1* (Manolson, 1992) and *STV1* (Manolson *et al.*, 1994). *STV1* has a Golgi retention signal and is transcribed at a much lower rate than *VPH1*, thus providing us with an example of alternate subunit composition at an organellar level.

Other V_o subunits include the hexamer of subunit c that forms the pore for proton translocation. This subunit will be discussed in section 1.1.5.

In yeast subunits c' (vma11p) (Umemoto *et al.*, 1991) and c'' (vma16p or ppa1p) (Hirata, 1997) are also implicated in formation of the proton pore. These three proteins are similar, with subunit c and c' sharing 56% protein identity. Subunit c'' shares less identity and has an extended N-terminus that may form a fifth membrane

spanning domain. These subunits all contain a critical glutamic acid residue known to be critical for proton translocation (Hirata, 1997).

The final V_o subunit *vma6p* (Bauerle, 1993), is a hydrophilic protein that does not appear to have membrane spanning domains and is peripherally associated. A further associated subunit, so far not discovered in yeast but present in *Manduca sexta*, humans, mice and *Arabidopsis thaliana* is a very small highly glycosylated subunit called M9.7 in *Manduca* (Merzendorfer, 1999) or M9.2 (Ludwig, 1998) in other organisms. The function of this protein is unknown.

Electron microscopy has shown that the V-ATPases look very similar to the ball and stalk structure of F_1 - F_o ATPases, with a few differences in overall dimensions. Microscopy of the V-ATPase from *Clostridium fervidus* (Boekema, 1997) clearly shows a side-arm structure connecting V_1 and V_o sectors (figure 1.3b). This is thought to be a stator structure that prevents rotation of the head group relative to the membrane sector. It is thought that there may be more than one of these structures per enzyme.

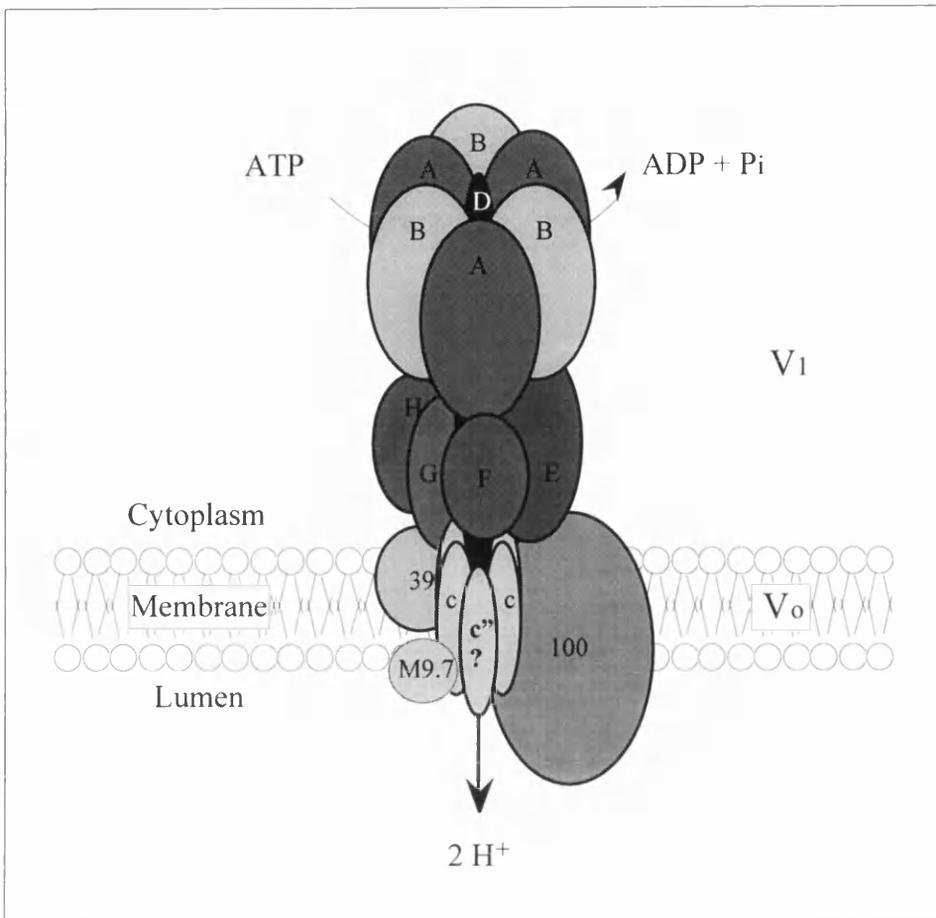


Figure 1.2 **Schematic diagram of the *Drosophila melanogaster* V-ATPase holoenzyme.** The enzyme is composed of two structural domains, V₁ and V₀. The cytoplasmic V₁ domain contains subunits A-H. Three copies of subunits A and B are ATP-binding, with subunit A being responsible for catalysis. Subunit D is thought to act as the spindle for enzyme rotation and the remaining subunits act to connect the two sectors and are required for stability and/or assembly of the complex. The V₀ domain consists of subunit c, the DCCD reactive, proton pore forming subunit (which may interact with subunit c'' (and c' in yeast)), and three proteins of unknown function, a 100 kDa transmembrane glycoprotein, a 39 kDa protein and a small 9.7 kDa protein which exists in higher eukaryotes but not in yeast.

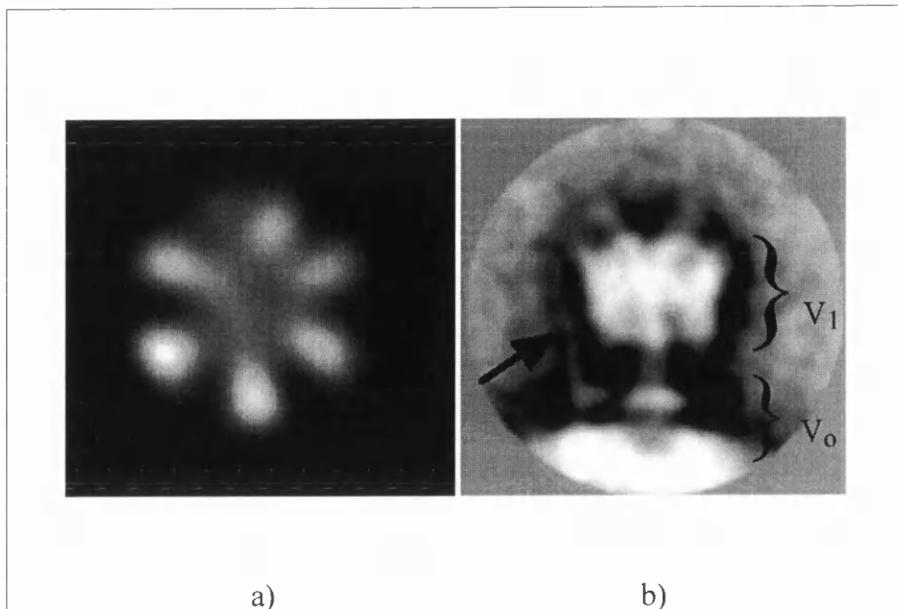


Figure 1.3 **Electron micrographs** a) showing top view of *Manduca sexta* V-ATPase showing pseudo-hexagonal arrangement of V₁ subunits (taken from (Rademacher, 1999)), and b) showing side view of *Clostridium feravidus* V-ATPase with the V₁ and V₀ groups indicated by brackets and an arrow showing the side-arm structure thought to act as a stator (taken from (Boekema, 1997)).

Little is known about how the V-ATPase functions and how protons are transported through the enzyme. Studies on the F₁-F₀-ATPase can, however, lead us in the right direction as the enzymes are so similar. Experiments using a basic F₁ headgroup (subunits α , β and γ) (Kinosita, 1999; Masaike, 2000) have shown that F₁-F₀-ATPases act as 'molecular motors'. The γ subunit was attached, via a biotin/streptavidin linker, to an actin molecule with a fluorophore on the end and the α and β subunits stuck down (therefore immobilised) onto a polystyrene bead via a His-tag. The actin molecule was observed to rotate (upon the addition of ATP), anticlockwise through 360 degrees in 120 degree steps, showing that it was the γ subunit that rotated within the headgroup. This rotation can be observed directly using an imaging camera and the resulting pictures can be viewed at the following web site, <http://www.res.titech.ac.jp/seibutu/nature/flrotate.html>. This rotation may serve as the driving force for passage of protons, with the stator structure(s) serving to prevent the other subunits from moving against the rotation. This is likely to be a similar mechanism to the action of the V-ATPase. This experiment could not, however be repeated for the V-ATPase, as the spindle subunit(s) are unknown and the V₁ headgroup does not catalyse ATP when uncoupled from V₀ (Puopolo *et al.*, 1992b).

1.1.3 Assembly

Assembly studies in yeast have shown that the V_1 and V_o subcomplexes are assembled separately then brought together to form the final holoenzyme. The V_o complex assembles in the endoplasmic reticulum (ER) with the aid of accessory subunits, which, while not forming part of the final V-ATPase complex, are absolutely required for function of the enzyme. Mutations in these proteins result in the typical *vma*⁻ phenotype. These proteins, vma12p, (Hirata, 1993; Jackson, 1997), vma21p, (Hill, 1994) and vma22p (Hill, 1995) are only present in the ER. The model for assembly in the ER (Graham, 1998) involves a vma12p/vma22p subcomplex associating with the 100 kDa subunit in the ER membrane, followed by the association of the other subunits before the complex exits the ER and dissociates from the vma12p/vma22p subcomplex. The function of vma21p is essential but unknown. The assembled V_o complex can then be transported to the appropriate part of the cell. If any of these smaller subunits are missing then the 100 kDa subunit is rapidly degraded. These small accessory subunits, however, have not been identified in *Drosophila* or any other organism so far.

V_1 assembly occurs in the cytoplasm, with partial subcomplexes forming before assembly of the V_1 complex onto the V_o . Presence of V_o is required before the entire V_1 complex can assemble onto the membrane. Many of the assembly studies were performed using the various *vma*⁻ mutants and observing which subcomplexes did or did not form and whether the complex with V_o formed. These studies are summarised in (Nelson, 1999).

1.1.4 Regulation

Factors such as cold shock of the Golgi (Moriyama, 1989), or addition of chaotropic agents to plant tonoplasts and bovine coated vesicles (Lai, 1988; Puopolo, 1990), can remove the V_1 subunits from the V_0 subunits in these membranes, with the V_1 subunits remaining as a complex in the cytoplasm. When this separation of V_1 and V_0 occurs the V_0 can no longer allow the passage of protons and the V_1 can no longer hydrolyse ATP. This therefore seems one likely way of regulating the holoenzyme. Regulation of this type has indeed been observed, for example, in the larval midgut of *Manduca sexta* where, during each larval moult the V_1 subunits dissociate from V_0 and K^+ is no longer transported by the K^+/H^+ antiporter (Sumner *et al.*, 1995). Once the moult is over the holoenzyme reassembles without synthesis of new protein. This type of regulation also occurs in yeast when substituting the usual carbon source, glucose, for less favourable sources. Re-association occurs when glucose is reintroduced, again without the synthesis of new protein (Parra, 1998). How exactly this disassembly and reassembly occurs and what signals are involved is still unknown.

Another method of regulation is thought to occur via disulphide bond formation between cysteines in ATP-binding site of the A subunit. This reversible reaction leads to inactivity of the enzyme when the bond is formed, due to the closure of the catalytic site (Feng, 1992; Feng, 1994; Stevens, 1997). In clathrin-coated vesicles up to 50% of the holoenzymes are in this state. A recent study by (Gruber, 2000) using x-ray scattering shows that the redox modulation of the A subunit leads to a conformational change in the whole enzyme.

Enzyme shuttling is another way in which the enzyme is controlled, for example in the kidney β -intercalated cells where the enzyme can be inserted into either the apical or basal membrane depending on the cells requirements.

Little is known about how the individual V-ATPase genes are regulated, but some evidence shows that there is control at the level of gene expression. Several plant V-ATPase subunits have been studied in order to understand how these genes are regulated. It has been shown that under salt-stress (Low *et al.*, 1996), subunit c (for which there are several genes) and subunit A transcript levels (and protein levels) in sugar beet and *Mesembryanthemum crystallinum* are upregulated (Kirsch, 1996; Tsiantis *et al.*, 1996). Studies in *Arabidopsis* and cotton revealed that the several genes encoding the 16 kDa subunit show altered expression in particular cells, depending on factors such as the tissue, stage of development or presence or absence of light (Hasenfratz *et al.*, 1995; Perera *et al.*, 1995).

In *Drosophila melanogaster* it has been observed that some V-ATPase transcripts encoding subunits A and E are downregulated in the pupa (Guo, 1996b; Guo *et al.*, 1996b), but others are not, e.g., F (Guo *et al.*, 1996a).

There may also be control at the level of transcription as antisense molecules to the *Manduca* M40 gene transcript have been found in *Manduca sexta* (Merzendorfer, 1997).

As V-ATPases serve different roles in the various membranes in which they occur, it would be sensible to suggest that different isoforms of subunits must exist and these isoforms could be used in producing slightly differing enzymes. Evidence for this exists in cow and rat (Nelson, 1992b; Puopolo *et al.*, 1992a) where alternate B subunits are used in different tissues such as kidney and brain. Also the many plant isoforms of various subunits show tissue specific expression (Hasenfratz *et al.*, 1995; Low *et al.*, 1996; Perera *et al.*, 1995).

1.1.5 Subunit c

One of the most widely studied subunits of the vacuolar H⁺-ATPase is the 16-17 kDa proteolipid, which, as a hexamer, forms the pore for proton translocation with each monomer of the 16 kDa protein containing four transmembrane helices. Figure 1.4 is a schematic diagram of how the subunits in each monomer are arranged and how six of these come together to form the proton pore. The arrangement of the helices within each monomer has been elucidated by cysteine replacement studies which determine whether a series of introduced cysteines can be cross-linked together, therefore are near each other within the protein. It can also be determined by the use of lipid soluble probes whether these cysteines are exposed to a lipid environment (Harrison 1999).

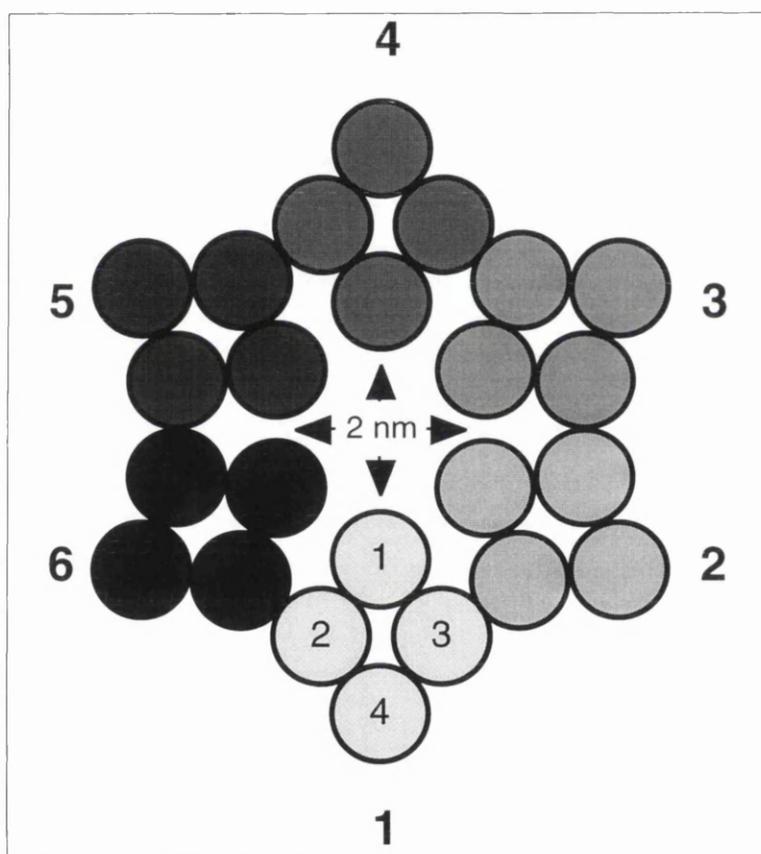


Figure 1.4 **Schematic diagram showing the arrangement of subunits within the V-ATPase 16 kDa proteolipid pore.** The larger numbers beside the shaded circles correspond to individual proteolipid subunits and the smaller numbers to each of the four transmembrane helices in each subunit, helix four containing the highly conserved DCCD reactive residue (Harrison 1999). The central pore of the hexamer is 2 nm in diameter which prospectively allows the passage of larger ions and small molecules. However one of the monomers may be the c" subunit as it is in yeast but this has yet to be elucidated in *Drosophila*.

Since its initial cloning in 1988 from bovine chromaffin granules (Mandel, 1988), subunit c has been cloned and characterised in a wide variety of organisms, from yeast and fungi through to arthropods, plants and mammals. The proteolipid subunit is extremely well conserved in all species studied so far with there being greater than 65% identity across a wide phylogenetic range. In fact it has been shown in a *Saccharomyces cerevisiae* V-ATPase model that mutants for the proteolipid gene (*VMA3*) can be fully rescued by the addition of the gene from *Nephrops norvegicus* (Harrison *et al.*, 1994). Table 1.3 shows the wide range of organisms from which subunit c has been cloned.

Organism/Organelle	Reference
Bovine chromaffin granules	(Mandel, 1988)
Mouse cerebellum	(Hanada <i>et al.</i> , 1991)
Rat liver	(Nezu, 1992)
Human	(Gillespie, 1991)
Oat	(Lai <i>et al.</i> , 1991)
Cotton	(Hasenfratz <i>et al.</i> , 1995)
<i>Arabidopsis</i>	(Perera <i>et al.</i> , 1995)
<i>Mesembryanthemum crystallinum</i>	(Tsiantis <i>et al.</i> , 1996)
Maize	(Vierek, 1996)
<i>Kalanchoe diargemontiana</i>	(Bartholomew, 1996)
<i>Drosophila melanogaster</i>	(Meagher <i>et al.</i> , 1990)
<i>Manduca sexta</i>	(Dow <i>et al.</i> , 1992)
<i>Heliothis virescens</i>	(Pietrantonio and Gill, 1993)
<i>Aedes aegypti</i>	(Gill, 1998)
<i>Nephrops norvegicus</i>	(Finbow <i>et al.</i> , 1992)
<i>Dictyostelium</i>	(Xie <i>et al.</i> , 1996)
<i>Neurospora crassa</i>	(Bowman, 1992)
<i>Acetabularia acetabulum</i>	(Ikeda, 1997)
<i>Giardia intestinalis</i>	(Hilario, 1998)
<i>Saccharomyces cerevisiae</i>	(Nelson and Nelson, 1989)
<i>Schizosaccharomyces pombe</i>	(Toyama, 1991)
<i>Entamoeba histolytica</i>	(Descoteaux, 1994)

Table 1.3 Cloning of subunit c in various organisms and organelles.

How this molecule transports protons is still relatively unknown, but the use of specific inhibitors and comparisons with F_1F_0 -ATPases can give us clues to how it functions. Present in all observed V-ATPase proteolipid subunits and also their 8 kDa proteolipid counterpart in the F_1F_0 ATP synthase, is a conserved glutamate residue in helix IV that is thought to be the site for binding and translocation of protons. Evidence for this is provided by the fact that this residue has been shown to react covalently with the lipophilic inhibitor *N,N'*-dicyclohexylcarbodi-imide (DCCD) (Sebald, 1980; Manolson, 1985). This interaction irreversibly abolishes proton translocation. V-ATPases also show sensitivity to alkyl tin compounds that interact with the proteolipid near to the DCCD binding site. These compounds also block proton translocation, as do the bafilomycin antibiotics, that reversibly inhibit proton translocation, however the site of binding of these compounds is thought to be in the 100 kDa subunit (Zhang *et al.*, 1994).

The V-ATPase proteolipid is also interesting in the fact that it has been implicated in other cellular roles, those of cell-cell communication and neurotransmitter release. These roles shall be discussed in the following section.

1.1.6 Alternative proteolipid roles

Gap junctions

Gap junctions have been widely studied in vertebrate tissues. Viewed under EM they are seen as hexagonal arrays of closely packed channels formed by proteins called connexins. Each cell contains a hemichannel that connects to a similar channel in a neighbouring cell, forming a pore that is 1.5-2 nm in diameter. These channels are involved in cell-cell communication and allow movement of low molecular weight solutes between cells (Warner, 1988). They also play important developmental roles as interference with these channels can cause developmental disruption (Guthrie, 1989). Over a dozen connexin proteins exist in vertebrates but surprisingly no such proteins have been discovered in non-vertebrates such as *Caenorhabditis elegans*, *Nephrops norvegicus* and *Drosophila melanogaster*. This is despite the fact that gap junction-like structures in these organisms have been observed, that physically resemble connexons under electron microscopy and have similar properties (Leitch, 1990; Ryerse, 1989a; Ryerse, 1989b).

Now that the entire *Drosophila melanogaster* genome has been sequenced it has been observed that no homologues to connexin genes exist in this organism. As no connexin genes are present in *Drosophila*, which proteins take over the role of forming these important structures? Two main types of candidate proteins have been studied.

The first was isolated from gap junctional preparations of *Nephrops norvegicus*. These preparations contained arrays of protein hexamers with a central pore of around 2 nm. This *Nephrops* protein was named ductin. Sequencing of the protein isolated from these junctions showed that it had identity to the V-ATPase 16 kDa proteolipid of other organisms (Finbow *et al.*, 1992). Gap junctional preparations from *Manduca sexta* also isolate the same protein (Finbow *et al.*, 1994). The observation that this 16 kDa protein functions as a gap junctional pore and a V-ATPase subunit is a subject of much contention, but some evidence exists to support this hypothesis. Immunological studies have shown that antibodies raised to the *Nephrops* protein inhibit dye-coupling between cells in *Drosophila* and have effects on development (Bohrmann, 1993; Bohrmann, 1998).

One difficulty with this protein playing separate cellular roles is that it is required to sit in two orientations within the membrane to function. In the gap junction the C and N termini face the cytoplasmic side of the membrane, while with the V-ATPase the C and N must termini face the vacuolar side. However evidence to support this dual orientation theory exists in the fact that antibodies to the N-terminal region of the protein block gap junctional communication but do not block V-ATPase activity, implying the N-termini are accessible to the antibody in the cytoplasm (Finbow *et al.*, 1993), and are not accessible to the intracellular compartments. The N-terminal

antibodies do interact with V-ATPases if the chromaffin granule membranes are disrupted (Finbow *et al.*, 1993).

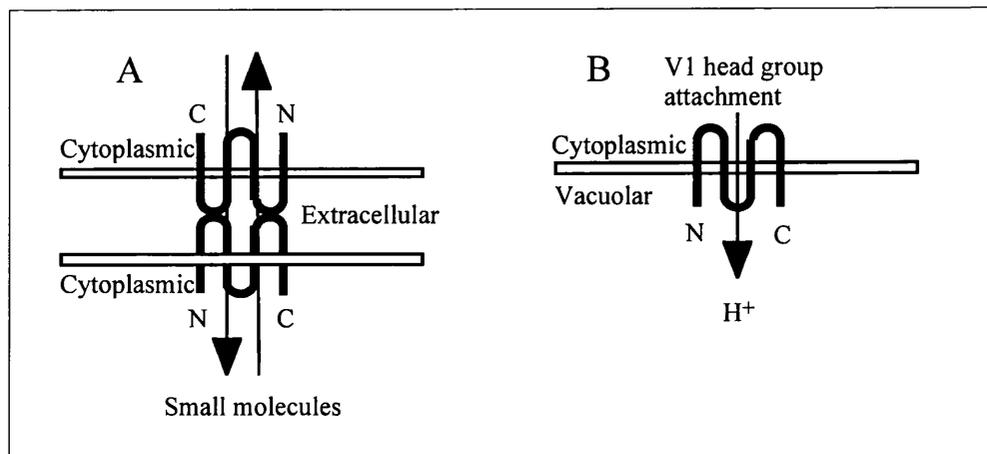


Figure 1.5 **Schematic diagram showing orientation of the 16 kDa protein in gap junctions and V-ATPases.**

The diagram shows, A) the C and N terminals of the 16 kDa protein in the gap junction facing the cytoplasm, allowing the conserved loops to interact and B) the opposite orientation in the V-ATPase, with the C and N terminals facing the vacuole and the conserved loops interacting with the V₁ head group subunits.

Alternative proteins have also been implicated in a gap-junctional role in invertebrates. These proteins, from the OPUS family (also termed invertebrate connexins) are topologically similar, but not homologous to connexins or ductin. Mutations in one of these proteins, *shaking -B* (or *Passover*), causes disruption of synapses in the giant fibre system of *Drosophila* and lack of this protein inhibits dye-coupling in the cells (Boekema, 1999; Phelan, 1996).

Even though two different types of protein have been observed to be present in the gap-junctions of arthropods and much contention has arisen as to whether the 16 kDa proteolipid is indeed a gap junctional component or not, the evidence is building to accept both types of protein in this important role.

Mediatophores

A similar 16 kDa protein isolated from V-ATPases and gap junctions has also been isolated from a third location in an invertebrate, called the mediatophore. Mediatophores are acetyl choline transporters present in presynaptic plasma membranes of the *Torpedo marmorata* electric organ, that respond to an influx of calcium. They exist as multi-subunit protein but are simpler in structure than V-ATPases and consist of at least five 15 kDa proteins (Israel, 1998) which, when purified and sequenced were observed to be highly homologous to the initially cloned chromaffin granule proteolipid except at the N-terminus (Birman *et al.*, 1990).

This project focused on the multiple roles of the c subunit of the vacuolar ATPase. Although much of the characterisation of V-ATPases has been performed in the yeast system it is by no mean a perfect organism for the study of these proteins. Being in a single-celled organism, the system has been of great value particularly due to the *vma⁻* phenotype, but it has its limits and cannot tell us much about V-ATPase subunits in specialised cell types and tissues. Therefore a higher eukaryote is required in which to study these complex proteins in a whole organism context. An ideal one exists in the form of the fruit fly, *Drosophila melanogaster*, an organism where many V-ATPase subunits have now been, or are being cloned. The usefulness of this organism is discussed in the next section.

1.2 *Drosophila melanogaster*

1.2.1 *Drosophila* as a model organism

Drosophila melanogaster is an extremely useful and intensely studied organism and researchers have a wide array of tools that can be used to mutate and manipulate the genome of *Drosophila*. *Drosophila* is an excellent higher eukaryotic model in that they have a short generation time of only several days, they are small and easy to culture and large volumes can be kept in relatively small spaces. They have few chromosomes, making genetic analysis simpler and in certain tissues such as the salivary glands these chromosomes are polytene, allowing accurate mapping and localisation of genes by *in situ* hybridisation with gene specific probes. *Drosophila* studies are aided greatly by the existence of a comprehensive database of literature, biological techniques and genetic information that is instantly and freely available on the Internet. This database also contains a vast amount of sequence data thanks to the *Drosophila* genome project that is succeeding in sequencing the entire euchromatic genome. Another useful tool in *Drosophila* technology is the existence of balancer chromosomes that allow the maintenance of recessive lethal mutations without any requirement of selective breeding at every generation. However, it is the existence and engineering of the natural mobile genetic element in *Drosophila melanogaster*, the P-element, that has proved to be the most useful aspect of *Drosophila* biology. These will be discussed in section 1.3.

1.2.2 V-ATPases in *Drosophila*

Several V-ATPase subunits were cloned in *Drosophila* in the last ten years (table 1.4). Many of these subunits correlate directly with those so far identified in yeast (table 1.2), although there are some differences, e.g. homologues of the yeast accessory assembly proteins have not been observed in *Drosophila*, neither has the c' protein. The small proteolipid subunit M9.7, however has only been observed in higher eukaryotes.

Subunit	Gene(s)	Ref.
V₁		
A	<i>vha67-1</i> <i>vha67-2</i> <i>vha67-3</i>	(Guo, 1996a)
B	<i>vha55</i>	(Davies, 1996)
C	<i>vha44</i>	(Dow, 1999)
D	<i>vha36</i>	(Dow, 1999)
E	<i>vha26</i>	(Guo <i>et al.</i> , 1996b)
F	<i>vha14</i>	(Guo <i>et al.</i> , 1996a)
G	<i>vha13</i>	(Dow, 1999)
(H) SFD	<i>vhaSFD</i>	(Dow, 1999)
V_o		
100 (a)	<i>vha100-1</i> <i>vha100-2</i> <i>vha100-3</i>	(Dow, 1999)
AC39 (d)	<i>vhaAC39</i>	(Dow, 1999)
c	<i>vha16-1</i> <i>vha16-2</i> <i>vha16-3</i> <i>vha16-4</i>	(Meagher <i>et al.</i> , 1990) (Finbow <i>et al.</i> , 1994) This study
PPA1 (c'')	<i>vhaPPA1-1</i> <i>vhaPPA1-2</i>	(Dow, 1999)
M9.7 (e)	<i>vhaM9.7-1</i> <i>vhaM9.7-2</i>	(Dow, 1999)

Table 1.4 V-ATPase subunits identified in *Drosophila melanogaster*. Listed are subunits which have been identified in *D. melanogaster* either by cloning or inferred by homology to subunits cloned in other organisms.

Despite V-ATPases only having been described in the late 1980's, the first (unrecognised) V-ATPase knockout was performed in the 1970's by (Gausz *et al.*, 1979) when attempting to delete the chromosomal location 87C. One of the mutations generated was subsequently demonstrated to have inactivated the *vha55* gene (subunit B) (Davies, 1996). The lethality of this mutation showed that deleting a subunit of the vacuolar ATPase resulted in death of the organism. An interesting phenomenon was observed with these mutations in that completely null alleles died at an early larval stage, death presumably occurring when the maternal supply of V-ATPase was exhausted. However, some EMS (ethylmethansulphonate) alleles, probably containing point mutations, died earlier at the embryonic stage. An explanation for this lies in the structure of the V-ATPase. Each holoenzyme requires three B subunits to function, but if mutant subunits are present they can be incorporated into the maternal holoenzyme supply, rendering it useless. These data also imply that mutations in other V-ATPase subunits, that are present in multi-copies in the enzyme, may display a dominant negative phenotype (Davies, 1996). Dying larvae from the *vha55* null alleles have an interesting Malpighian tubule phenotype, that of transparent tubules. Hypothetically this is because the V-ATPase in these mutants is unable to acidify the tubules, a process which normally leads to accumulation of white uric acid crystal within the tubule lumen. This mutant phenotype correlates with more recent data using P-elements to knock out V-ATPase subunits. These show that lethality occurs when subunits A or B are interrupted with P-elements (Davies, 1996; Guo, 1996b). These studies also show, via *lacZ* reporter genes contained in the elements, that V-ATPases have elevated expression in those cells where V-ATPases have been hypothesised to play a plasma membrane role within *Drosophila* such as antennae, maxillary palps and Malpighian tubules (Dow, 1995). These results however require confirmation via *in situ* hybridisation as expression data from enhancer traps may be influenced by enhancers other than those of the gene interrupted.

P-element mutagenesis is an extremely useful tool for *Drosophila* biologists. They can be used to interrupt genes to determine whether they are lethal, and enhancer trap elements can observe the activity of genes both spatially and temporally. They can also be used to introduce DNA sequences into the *Drosophila* genome. These elements are discussed in the next section.

1.3 P-elements

1.3.1 P-element history and engineering

A number of different types of mobile genetic elements exist in the *Drosophila melanogaster* genome (Maside, 2000), with the P-element being the most heavily studied and utilised. The massive amount of information available about the *Drosophila* genome relies heavily on the information received using engineered variants of these elements. P-elements can be used to knock-out genes to observe mutant phenotypes, to re-introduce genes back into the germline, to provide expression data and can also be used to rescue sequences flanking their points of insert.

P-elements were discovered in the '70s when they were observed to be the causative agent of a genetic phenomenon called P-M hybrid dysgenesis (Engels, 1979). This occurs when males from a 'P' strain are mated to females of an 'M' strain with the resulting offspring showing high degrees of chromosomal rearrangements, mutations and sterility. This is due to the presence of a mobile P-element, that in the parental P-strain stays repressed, but becomes de-repressed in the M strain due to lack of this repressor. This allows the element to transpose uncontrollably in the germ cells of the progeny, causing insertion mutations and chromosome rearrangements due to element excision and misrepair.

When the P-element was eventually isolated it was observed to be around 2.9 kb in length, but smaller, internally deleted elements were also observed. The element contains 4 exons which in the germline encode an 87 kDa polypeptide, the transposase (figure 1.6). In somatic cells, the intron between exons 2 and 3 is maintained, this contains a stop codon and as a result a non-functional truncated 66 kDa protein is produced. The element also has 31 bp inverted terminal repeats that are absolutely necessary for transposition (O'Hare and Rubin, 1983).

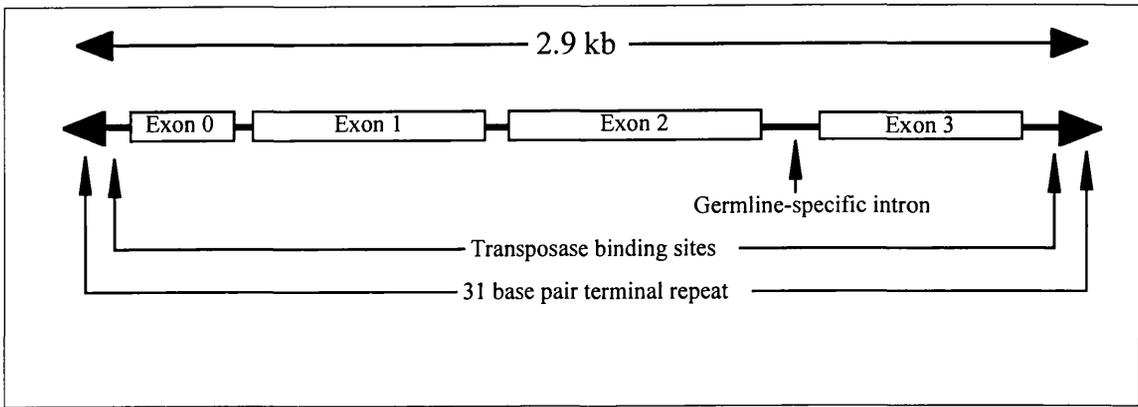


Figure 1.6 **Schematic diagram showing the structure of a wild-type P-element.**

The diagram shows the exon structure, the sites where transposase binds and the 31 base pair terminal repeats which are required for transposition. Also shown is the germline-specific intron, which is spliced out in germline cells and produces active transposase (O'Hare and Rubin, 1983)..

1.3.2 P-element technology and germline transformation

Rubin *et al* (Rubin and Spradling, 1982) hypothesised that P-element sequences could be used to introduce cloned DNA sequences back into the *Drosophila* genome, using an *in trans* transposase source.

In order to microinject DNA into a *Drosophila* embryo the stages of development of the embryo have to be taken into consideration. Until 3 h post fertilisation the *Drosophila* embryo is a syncytium of nuclei that are able to take up exogenous DNA. However, in order for the injected DNA to be inheritable it must be inserted into the germ cells. These are set aside early in the embryo and cellularise earlier than the rest of the nuclei, (at 90 min post fertilisation at 25 °C) therefore any DNA to be inserted must be injected before this stage.

The requirements for the initial Rubin and Spradling experiment were to introduce a suitable gene, xanthine dehydrogenase, into a P vector lacking the transposase gene, and to microinject it into embryos alongside a plasmid containing a source of transposase in this case an intact P-element plasmid (Rubin and Spradling, 1982).

The M-type strain into which the DNA was injected was *rosy*⁻ and the presence of the introduced xanthine dehydrogenase gene led to a reversion of the eye colour phenotype to wild type. The injected embryos themselves would not have the phenotype as only the germ cells would be transformed. Therefore the resulting progeny was crossed back to the original injected line and revertants scored for in the next generation. Transformants were observed, indicating that *Drosophila* are indeed

amenable to genetic transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982).

The source of transposase in this experiment was the complete P-element sequence. This was not entirely suitable as a this element would also insert into the genome and keep supplying supply transposase, leading to continuous transposition. This led to the engineering of a generation of marked elements, supplying transposase but without the ability to transpose themselves, termed helper plasmids, that could be selected against in transformed flies (Robertson *et al.*, 1988), therefore leading to stable integration of the transgene.

A schematic diagram of how germline transformation is performed is shown in figure 1.7. The DNA to be introduced into the germline is cloned into a transformation vector which also contains a gene encoding the *white* marker. This vector is co-transformed with a transposase-producing vector into the pole cells of syncitial embryos of a white-eyed fly line. Survivors, which putatively contain the transgene stably integrated into their germ cells, are then crossed back to the white-eyed line. Progeny from this cross, if transformed, display the red-eyed phenotype encoded by the *white* gene.

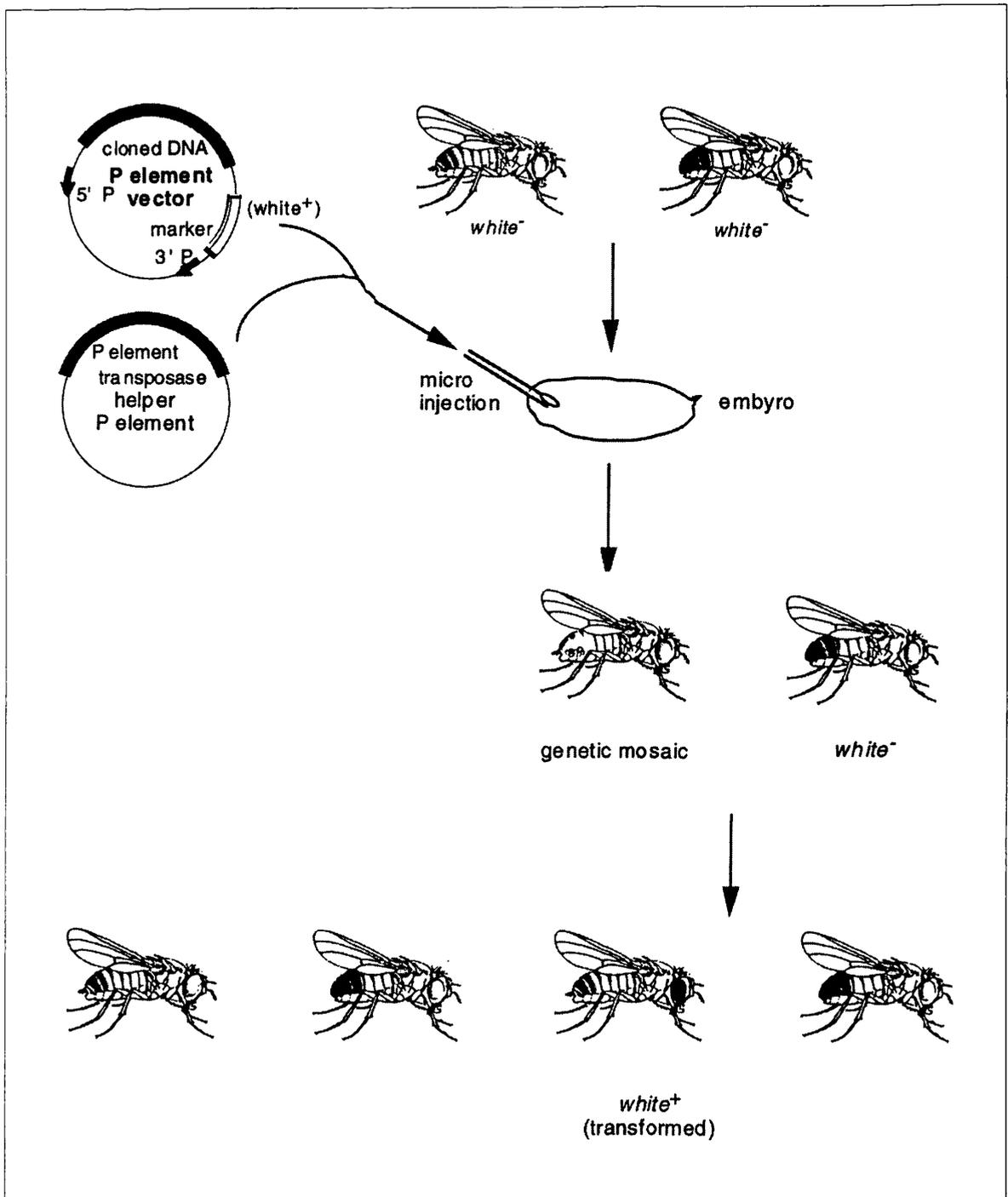


Figure 1.7 Schematic diagram showing the processes involved in germline transformation of *Drosophila melanogaster* embryos. Reproduced from (Guo, 1996a). The cloned DNA for re-introduction into the germline is inserted into a P-element vector carrying a *white*⁺ marker. This is then co-injected into *Drosophila* embryos (*white*⁻) with a transposase producing helper plasmid. Flies which reach adulthood putatively contain the transgene in their germline and are crossed back to the original white-eyed fly strain. Progeny from the next generation, if transformed, carry the *w*⁺ marker, giving the flies red eyes.

1.3.3 Enhancer trapping

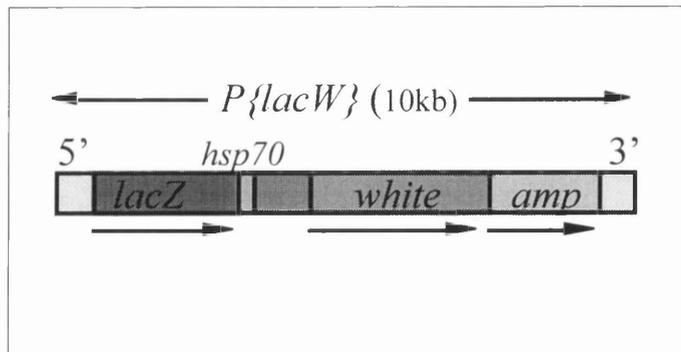


Figure 1.8 **Schematic diagram of the enhancer trap element $P\{lacW\}$.** The element contains the gene encoding β -galactosidase, the *white* gene and the *E. coli ampicillin* gene and origin of replication. The bacterial sequences allow plasmid rescue of sequences surrounding the point of P-element insertion (Bier, 1989).

Enhancer traps are engineered P-elements that contain reporter genes. If they insert close to an enhancer in the *Drosophila* genome the expression of the reporter gene represents the status of that particular enhancer, both spatially and temporally. A common reporter utilised is *lac Z* (such as in the construct $P\{lacW\}$, figure 1.8) that can easily be detected by a simple staining assay (see section 2.12) using the chromogenic substrate X-gal. This results in a blue colour appearing in cells in which the *lac Z* gene is expressed.

However, due to the nature of enhancers, which can act over large distances, expression patterns obtained from enhancer trap data have to be confirmed by other means, such as *in situ* hybridisation, to rule out other genomic effects.

Also present in the $P\{lacW\}$ element is the bacterial ampicillin resistance gene and origin of replication, these allow sequences flanking the P-element to be elucidated by a procedure called plasmid rescue (section 3.3.1).

1.4 Malpighian tubules and fluid secretion

In order to study V-ATPases in a plasma membrane context we require an epithelial phenotype that is amenable to physiological studies. Although *Drosophila* are small, a factor that is often detrimental to physiological studies, a tissue present in *Drosophila* is amenable to such experiments. This is the Malpighian tubule, which is the insect equivalent of the kidney.

1.4.1 Malpighian tubules

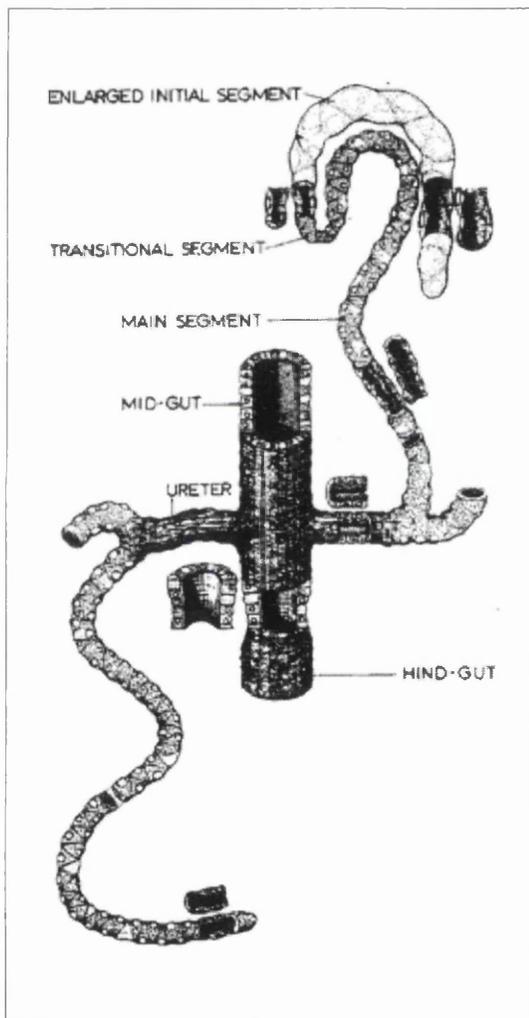


Figure 1.9 Morphology of the *D. melanogaster* Malpighian tubule. Reproduced from (Wessing and Eichelberg, 1978).

Malpighian tubules are one cell thick, tubular epithelia consisting of around 149 cells (Sözen *et al.*, 1997), that have a urinary function analogous to that of the kidney in vertebrates. *Drosophila melanogaster* have 4 tubules, arranged in pairs, one posterior and one anterior. Each pair shares a common ureter that attaches to the gut at the midgut/hindgut boundary (figure 1.9). Their main function is to clean the haemolymph of waste products by transporting them across the membrane into the

tubule lumen (Dow, 1995), from where the waste is transported into the gut and excreted with the remainder of the digestive waste. Tubule fluid secretion and secondary transport is energised by the V-ATPase, evidence for this being provided by the abolition of fluid secretion by the addition of specific V-ATPase inhibitors such as bafilomycin.

1.4.2 The fluid secretion assay

The tubule secretion assay allows quantitation of fluid secretion rates from the Malpighian tubules. It has previously been shown that secretion rates can be modulated by the activation of pathways such as the nitric oxide signalling pathway (Dow *et al.*, 1998). Activation of this pathway by compounds such as the cardioacceleratory peptide, CAP_{2b} (Davies, 1995, Terhzaz, 1999), result in an upregulation of the apical V-ATPase in the principal cells of the tubules (figure 1.10). This increase in fluid secretion can be measured via the fluid secretion assay.

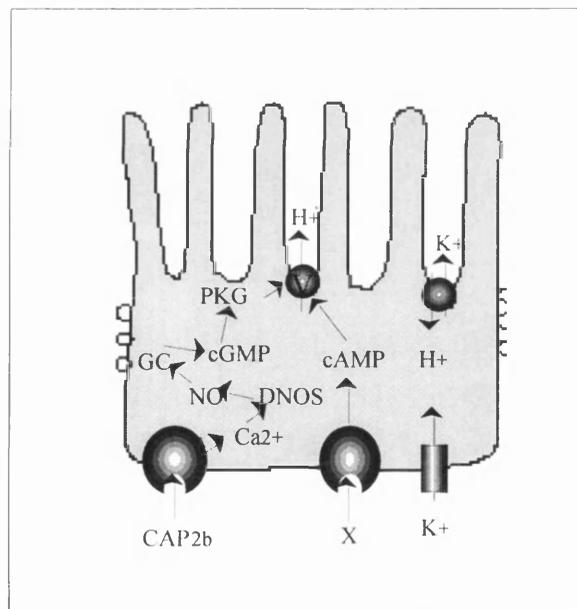


Figure 1.10 **Signal transduction pathway involved in stimulation of fluid secretion with CAP_{2b}** in the principal cell of the Malpighian tubule. CAP_{2b} binds to a basolateral membrane receptor (which is as yet unknown) which results in an upregulation of intracellular calcium (Ca²⁺). This in turn activates *Drosophila* nitric oxide synthase (DNOS), which produces nitric oxide (NO) which activates a soluble guanylate cyclase (GC) which produces cGMP (Dow, 1994). cGMP stimulate a protein kinase G (PKG) which in turn upregulates the vacuolar H⁺-ATPase (V) in principal cells of the Malpighian tubule (Davies, 1995, Terhzaz, 1999).

Fluid secretion assays involve the dissection of pairs of tubules that are placed into a drop of a suitable osmotic and nutrient medium which is overlaid by paraffin oil. One tubule of the pair is drawn out into the paraffin and wrapped around a small pin with the ureter between the pin and the drop. The fluid secreted by the remaining tubule left in the drop collects at the ureter and by the means of a fine glass rod can be removed at timed intervals (figure 1.11).

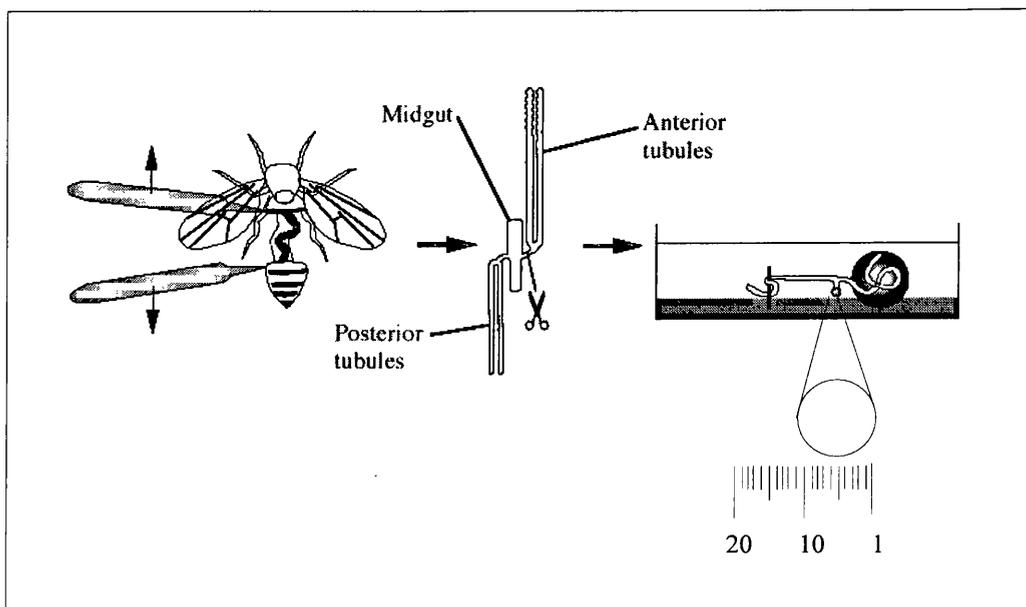


Figure 1.11 **Schematic diagram showing the steps in the fluid secretion assay.** Reproduced and modified from (Dow *et al.*, 1994). Flies are anaesthetised on ice and their tubules dissected in *Drosophila* Schneiders Saline. Each pair of tubules is placed in a bubble of saline under paraffin oil and one of the tubules is drawn out of the bubble and wrapped around a pin. Bubbles of secreted liquid collect at the ureter and are removed at timed intervals and their diameter measured using an eyepiece graticule.

Once removed, the secreted droplets form spheres due to the presence of the hydrophobic oil, allowing their diameter to be measured by an eyepiece graticule. The actual diameter of the droplet is calculated taking into account the magnification factor of the microscope and the volume of the droplet measured by use of the equation, $\frac{4}{3}(\pi r^3)$. This enables the measurement of the rate of secretion by measuring the droplets every ten minutes. This assay allows us to test the effect of agonists/ antagonists on the secretion rate from tubules with wild-type and mutant backgrounds (Dow *et al.*, 1998; Dow *et al.*, 1994) and potentially allows an assay for epithelial function of V-ATPase.

1.5 Aims and Objectives

The aims of this project were to study the multiple roles of the V-ATPase subunit c in an animal model, using a combination of physiological, molecular and genetic techniques.

The specific aims were;

- to isolate and characterise a lethal P-element mutation in the *vhal6-1* gene by screening a series of rescued plasmids.
- to characterise and clone the emerging subunit c gene family.
- to try and dissect the roles of subunit c in an animal.

This work also led to;

- attempts at rescuing the P-element mutant phenotype with *Drosophila melanogaster* DNAs and sequences from other organisms.
- cloning and partial characterisation of previously unknown V_o sector proteins.

Chapter 2

Materials and Methods

2.1 *Drosophila melanogaster*

2.1.1 *Drosophila* stocks

Listed below are the various *Drosophila* lines utilised in this study, their phenotypes and application.

Strain	Genotype	Purpose
Oregon R	Wild type	Southern and Northern analyses.
<i>w¹¹¹⁸</i> (Hazelrigg <i>et al.</i> , 1984)	<i>w¹¹¹⁸</i>	Microinjection.
1799	<i>w[*]</i> ; P{ <i>w⁺mC=GAL4-Hsp70.PB</i> } <i>89-2-1</i> / P{ <i>w⁺mC=GAL4-</i> <i>Hsp70.PB</i> } <i>89-2-1</i>	Heat shock inducible GAL4 on third chromosome. Flybase ID FBrf0105495, from Bloomington stock centre
$\Delta 2,3$ (Robertson <i>et al.</i> , 1988).	<i>w[*]</i> ; <i>wg^{Sp-1}/CyO</i> ; <i>ry⁵⁰⁶ Sb¹</i> P{ <i>ry⁺t7.2=Delta2-3</i> } 99B/TM6B, <i>Tb¹</i>	Transposase source for P-element excision
16/1 (Török, 1993)	<i>y/w</i> ; P- <i>lacW</i> /CyO; +/+	Lethal P-element insert line containing the <i>lacZ</i> gene, mini- <i>white</i> marker and bacterial <i>ori</i> of replication.
76/16 (Török, 1993)	<i>y/w</i> ; P- <i>lacW</i> /CyO; +/+	Lethal P-element insert line containing the <i>lacZ</i> gene, mini- <i>white</i> marker and bacterial <i>ori</i> of replication.
Trix/CyO (from K. O'Dell)	<i>w/B^sY</i> ; Trix/CyO; +/+	To determine chromosomal location of germline transformants on chromosome 2.
TM3Sb/TM6Tb (from K. O'Dell)	<i>y/w</i> ; +/+; TM3Sb/TM6Tb	To determine chromosomal location of germline transformants on chromosome 3.

Table 2.1 *Drosophila melanogaster* strains used in this study.

All lines used were lab stocks, except the P{*lacW*} lines which were provided by the Kiss lab in Szeged, Hungary.

2.1.2 *Drosophila* rearing

Flies were reared in vials on standard *Drosophila* medium at 25°C (appendix 1) in a 12:12 light:dark cycle. If large quantities of flies were required, rearing was in large bottles on standard medium. For egg collection flies were reared in cages and egg collection was from standard grape juice agar plates (appendix 1). Egg collection for microinjection was from acetic acid/ethanol plates (appendix 1)

2.2 *Escherichia coli*

2.2.1 *E. coli* strains and plasmids

Strain	Genotype
TOP10 competent cells (Invitrogen).	(F ⁻ <i>mcrA</i> , Δ (<i>mrr-hsdRMS-mcrBC</i>), ϕ 80 <i>lacZ</i> Δ M15, Δ <i>lacX74</i> , <i>recA1</i> , <i>deoR</i> , <i>araD139</i> , Δ (<i>ara-leu</i>)7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> , (Str ^R), <i>endA1</i> , <i>nupG</i>).
DH5 α TM subcloning efficiency competent cells (Gibco BRL).	(F ⁻ ϕ 80 <i>dlacZ</i> D15, Δ (<i>lacZYA-argF</i>), U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (<i>rK</i> ⁻ , <i>mK</i> ⁺), <i>phoA</i> , <i>supE44</i> , λ ⁻ , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>).

Table 2.2 *E. coli* strains used in this study.

Plasmid	Purpose
pCR [®] 2.1	For cloning of PCR products according to the TA cloning kit protocol (Invitrogen).
pCR [®] 2.1TOPO	For cloning of PCR products according to the TOPO TA cloning kit protocol (Invitrogen).
<i>pP</i> { <i>UAST</i> }	For germline transformation of cloned sequences under control of the <i>UAS</i> enhancer sequence. (Brand and Perrimon, 1993)
<i>pP</i> { Δ 2-3}	Transposase source for germline transformation. (Spradling, 1983)
pBluescript	Used as a probe for Southern detection of ampicillin resistance gene in <i>p</i> { <i>lacW</i> } (Stratagene).
pYes2/pDm16k	Containing original <i>vha16-1</i> cDNA, for PCR and probes (Finbow <i>et al.</i> , 1994).
pEGFP-N1	Contains enhanced GFP sequence for creating fusion protein with <i>vha16-1</i> (Clontech).
pBR <i>rp49</i>	pBR322 containing the <i>EcoRI-HindIII</i> fragment of the <i>Drosophila</i> gene <i>rp49</i> used as a Northern blot loading control.

Table 2.3 Plasmids utilised in this study.

2.2.2 Transformation of *E. coli*

Plasmids were transformed into DH5 α TM subcloning efficiency competent cells by adding 50-100 ng of plasmid to 50 μ l of cells on ice and leaving for 30 min. The cells were heat shocked for 20 s at 37°C then left on ice for 2 min before adding 200 μ l L-broth before allowing 30 min to allow expression of the *amp^R* gene. 100 μ l of the transformations were then spread onto L-agar (appendix 2) plates containing 100 μ g/ml ampicillin.

Plates containing transformations were grown overnight at 37°C. Transformants were removed as single colonies and were grown overnight (with shaking) at 37°C in 10 ml L-broth (appendix 2) containing 100 μ g/ml ampicillin.

Transformation of pCR[®]2.1 plasmids (containing cloned PCR products) into TOP10 cells is described in section 2.8.4.

2.2.3 Plasmid selection

All plasmids used contained the ampicillin resistance gene encoding β -lactamase and so were selected for by the presence of 100 μ g/ml ampicillin when being grown on L-agar or in L-broth. This antibiotic was made as a 50 mg/ml stock solution (w/v in H₂O), filter sterilised and stored at -20°C.

pCR[®]2.1 plasmids containing inserts require further selection using X-gal. 40 μ l of a 40 mg/ml (w/v) stock solution in DMF (dimethylformamide) (stored at -20°C) was spread onto an ampicillin plate an hour before use.

2.2.4 Storage of bacterial cultures

0.5 ml of bacterial culture was added to 1 ml of a 2% peptone, 40% glycerol solution (in H₂O) before being frozen under liquid nitrogen. Frozen stocks were stored at -70°C.

2.3 Nucleic Acid Isolation and Quantification

2.3.1 Plasmid DNA isolation

Small scale plasmid DNA preparation was with the Qiaprep Spin Miniprep Kit and large scale preparation for germline transformation was with the Qiagen EndoFree Maxi- Kit, both according to manufacturers instructions (Qiagen).

2.3.2 Isolation of *Drosophila* genomic DNA

200 adult flies were anaesthetised on ice and homogenised in a mortar and pestle in 5 ml of homogenisation buffer (10 mM Tris-HCl (pH 7.5), 60 mM NaCl, 10 mM EDTA (pH 8)).

The homogenate was placed into four Eppendorf tubes and fly debris removed by centrifugation at 1 000 rpm for 1 min.

The supernatant was removed and centrifuged for 5 min at 8 000 rpm to pellet the nuclei. The nuclear pellet was resuspended in 0.5 ml of homogenisation buffer and proteinase K added to a final concentration of 100 µg/ml (from a stock solution of 25 mg/ml w/v in H₂O).

After mixing, SDS (from a stock solution of 10% w/v in H₂O) was added to a final concentration of 1% and the solution incubated for 1 h at 37°C.

The DNA was extracted twice with phenol to extract protein and salts and once with chloroform to remove any residual phenol, then precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.5) and two volumes of 100% ethanol at room temperature with gentle rocking.

The DNA was removed with a glass hook, washed with 70% ethanol (v/v in H₂O), air dried and resuspended overnight in 500 µl TE buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8)). The DNA was re-precipitated as before then resuspended in 200 µl TE buffer for several hours and stored at -20°C.

2.3.3 Single fly DNA preparation

Quick single fly DNA preparations for utilisation in PCR reactions were performed according to (Gloor and Engels, 1991). Briefly, an anaesthetised single fly was placed in a 0.5 ml Eppendorf tube. 50 µl of 'squishing buffer' (10 mM Tris-HCl (pH 8.3), 1 mM EDTA, 25 mM NaCl, 200 µg/ml proteinase K) was drawn up into the pipette tip and the fly squashed by the tip, without expelling the liquid.

Once the fly was suitably squashed, the liquid was expelled and the reaction incubated for 30 min at 37°C. The proteinase K was inactivated by heating the

reaction for 1.5 min at 95°C. 1 µl of the reaction was then utilised for PCR. DNA from squashed flies could be maintained for several months at -20°C.

2.3.4 Preparation of total RNA

Prior to RNA isolation all solutions were made RNase free with a 0.1% (v/v) DEPC solution where required. Fresh plasticware and pipette tips were used and gloves worn at all times. The homogeniser was washed thoroughly with ethanol and rinsed in RNazol B (Biogenesis) before use.

For large scale total RNA extraction 100 flies were frozen in liquid nitrogen in a Sterilin tube and homogenised with a Polytron homogeniser in 1 ml RNazol B (guanidinium isothiocyanate phenol solution), then transferred to fresh Eppendorf tubes.

0.1 volumes of chloroform was added and the samples were shaken for 15 s and left on ice for 5 min.

The samples were centrifuged for 15 min at 12 000 g (4°C). The aqueous phase was removed to a fresh tube to which isopropanol of an equal volume was added before leaving for 15 min on ice.

The samples were centrifuged as before and the supernatant carefully removed from the RNA pellet. The pellet was washed with 75% ethanol, vortexed, then centrifuged at 8 000 g for 8 min (4°C).

The pellet was left to air dry then dissolved in an appropriate volume of 0.5% SDS (initially 200 µl then more added if necessary). When separate head and body RNA was required the frozen flies were passed through graded sieves (initially a 355 µM sieve which allows passage of legs and wings, then a 710 µM sieve to allow separation of heads), to separate heads and bodies.

For larval collection, flies were allowed to lay on egg plates and the eggs allowed to hatch. A small amount of boiled yeast paste was added to each plate and the larvae allowed to mature.

Larvae were collected in a Falcon tube and 1 ml of mixed stage larvae was used in each extraction. A similar 'volume' of mixed stage pupae was used.

For RNA extraction from embryos, three egg plates were set up for 24 hr with ca. 200 flies in each cage. RNA was extracted from the mixed age embryos produced. Embryos were dechorionated with 50% bleach (v/v in H₂O) and washed before being added to RNazol B.

2.3.5 Quantification of nucleic acids

Nucleic acid concentrations were estimated by spectrophotometry at $A_{260/280}$, where an OD of 1 at 260 nm corresponds to 50 $\mu\text{g/ml}$ of double-stranded DNA and 40 $\mu\text{g/ml}$ of single-stranded DNA and RNA. Readings were zeroed with the solution in which the samples had been diluted. The ratio of $A_{260/280}$ provided an estimation of nucleic acid purity. Values between 1.8 and 2.0 indicated pure preparations

Where concentrations of DNA were extremely small the quantity of DNA was estimated by dropping 1 μl onto a 1% agarose (w/v in H_2O) plate containing 0.5 $\mu\text{g/ml}$ ethidium bromide (EtBr), allowing it to dry and comparing its intensity to that of a series of known DNA standards (6, 12, 25, 50, 100 and 200 ng) under UV illumination at 254 nm on a transilluminator.

2.4 Restriction Digests and Electrophoresis

2.4.1 Restriction digests

DNAs were restricted for 1 hour at 37°C in single strength REact® buffer appropriate to the restriction enzyme being used (all Gibco BRL). When double digestion was required the reaction was initiated with the enzyme with the lower strength buffer and after heat inactivation (at 60°C for 10 min) an appropriate amount of the necessary salt was added before addition of the second enzyme.

Typical amounts of DNA in a restriction digest were 1 µg of plasmid DNA and 5 µg of genomic DNA.

2.4.2 Agarose gel electrophoresis of DNA

DNAs were separated in 1% agarose (unless indicated otherwise in main text) in 1x TBE (90 mM Tris, 90 mM boric acid (pH 8.3), 2 mM EDTA) containing 0.1 µg/ml EtBr, as described in (Sambrook *et al.*, 1989), using 1x TBE as the electrophoresis buffer. Sizes were compared to a 1 kb ladder (Gibco BRL). Prior to loading 3 µl of loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol in water) was added to the samples.

2.4.3 Purification of DNA from gels: the "freeze squeeze" method

DNA bands were excised with a scalpel blade, placed in a 0.5 ml centrifuge tube and frozen at -70°C for 15 min or -20°C overnight. Before thawing, a tiny hole was pierced in the bottom of the tube and the frozen gel mashed with a needle. The 0.5 ml tube was placed inside a 1 ml tube which was spun at 7 000 rpm for 10 min. The liquid collected was cleaned with a phenol/chloroform step to remove any residual gel and precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.5) and two volumes of 100% ethanol for at least half an hour at -20°C. The precipitated DNA was recovered by centrifugation at 13 000 rpm for 15 min and resuspended in 20 µl distilled water.

2.4.4 Electrophoresis of RNA

40 µg RNA (per lane) was denatured by the addition of;

3.5 µl formaldehyde

10 µl formamide

2 µl 5x MOPS (0.1 M MOPS (pH 7), 40 mM sodium acetate, 5 mM EDTA (pH 8)) and heating to 70°C for 10 min, then snap-chilling on ice prior to the addition of 2.5 µl loading dye (as above).

The RNA was electrophoresed overnight in a denaturing gel (20% (v/v) 5x MOPS, 18% (v/v) formaldehyde, 1% agarose in DEPC-treated water) in 1x MOPS, containing 0.01 µg/ml EtBr, as the electrophoresis buffer. Sizes were compared to the Gibco BRL 0.24-9.5 kb RNA ladder and gels were photographed with a ruler.

2.4.5 Polyacrylamide gel electrophoresis for DNA sequencing

Denaturing polyacrylamide gel electrophoresis was used to separate the products of sequencing reactions. Sequencing gels were composed of 6% (w/v) acrylamide/bis (acrylamide:N, N'-methylenebisacrylamide, 19:1), 7 M urea in 1x TBE (provided as a complete solution by Anachem). Gels were polymerised via the addition of 1 ml 10% (w/v in H₂O) APS (ammonium persulphate) and 50 µl TEMED in a Bio-Rad sequencing apparatus. Gels were allowed to set for at least half an hour before use and pre-run to reach the desired temperature (50°C). The samples to be electrophoresed were denatured for 5 min at 90°C and snap-chilled on ice before loading. Gels were run for two to four hours (in 1x TBE) at 50°C depending on the size of DNA to be resolved before drying down onto Whatman 3MM paper under vacuum. Dried gels were exposed to Fuji X-ray film for a sufficient number of days to produce a clear signal.

2.5 Labelling of Nucleic Acids

2.5.1 Labelling of DNA with ³²P

Radioactive probes were generated by the random priming method modified from Feinberg and Vogelstein (Feinberg and Vogelstein, 1983).

30-50 ng of gel-purified DNA or linearised plasmid in 30 µl of water was denatured at 90°C for 5 min and snap-chilled on ice before the addition of;

2 µl of BSA (1 mg/ml in H₂O)

10 µl 5x OLB (oligo labelling buffer (appendix 3))

3 µl of [α -³²P] dCTP (30 µCi: 3 000 Ci/mMolar (ICN))

5 µl of Klenow DNA polymerase (Boehringer Mannheim)

The mix was incubated at 37°C for 1-3 hr. Before adding to filters, the probes were boiled for 5 min and snap-chilled on ice.

2.5.2 Labelling of DNA with Digoxigenin

Digoxigenin labelled probes were generated by PCR with the digoxigenin-dUTP being supplied by the 10x DIG DNA Labelling Mix (Boehringer Mannheim), which contains digoxigenin-conjugated dUTP as well as dTTP (10x mixture contains, 1 mM dATP, 1 mM dGTP, 0.65 mM dTTP and 0.35 mM DIG-UTP). Incorporation of DIG was detected by agarose gel electrophoresis of 5 µl of the PCR product, where the observed PCR products run at a heavier size than expected due to DIG-dUTP being heavier than dTTP (manufacturers instructions state that DIG-UTP is incorporated every 20-25 nucleotides in the newly synthesised DNA). Before addition to filters, DIG probes were boiled for 10 min at 95°C and snap-chilled on ice.

2.6 Nucleic Acid Hybridisation

2.6.1 Southern blotting

Agarose gels were treated in order to denature the DNA for 20 min (in 1.5 M NaCl, 0.5 M NaOH), washed in distilled water and neutralised for 20 min (in 1 M Tris-HCl (pH 8), 1.5 M NaCl) prior to capillary blotting to a nylon membrane (Hybond-N, Amersham) with 20x SSC (3 M NaCl, 300 mM sodium citrate) buffer. After overnight transfer, the DNA was fixed to the membrane by UV crosslinking at $12 \times 10^4 \mu\text{J}/\text{cm}^2$ (Spectrolinker, Spectronics Corporation)

2.6.2 Southern Hybridisation

Filters were prehybridised in Church buffer (7% SDS, 1% (w/v) BSA, 1 mM EDTA, 0.25 M Na_2HPO_4 (pH 7.2)) for radioactive probing, or in DIG pre-hyb solution (5x SSC, 0.1% (w/v) sodium lauryl sarcosine, 0.2% SDS, 1% (w/v) Marvel milk powder) for DIG probing, for several hours in a Techne hybridisation oven at 65°C, with regular shaking of the filter. Denatured probe was added and allowed to hybridise overnight. Filters were then washed as follows;

low stringency wash

2x SSC, 0.1% SDS for 30 min at 25°C

high stringency wash

2x SSC, 0.1% SDS for 30 min at 25°C

0.1x SSC, 0.1% SDS for 30 min at 65°C

2.6.3 Northern blotting

Gels were treated for 20 min in 0.05 N NaOH then soaked in 20x SSC for 40 min, prior to capillary blotting to a nylon membrane (Hybond-N, Amersham) with 20x SSC buffer. After overnight transfer the RNA was fixed to the membrane by UV crosslinking as above.

2.6.4 Northern hybridisation

Filters were prehybridised for several hours in Church buffer containing formamide (2:1 buffer:formamide) at 42°C. The denatured probe was added and hybridised overnight. Filters were washed as follows;

2x SSC, 0.1% SDS for 20 min at 25°C

0.2x SSC, 0.1% SDS for 30 min at 65°C

Size was determined with respect to the Gibco BRL 0.24-9.5 kb RNA ladder.

2.6.5 Autoradiography

Filters hybridised to radioactive probes were wrapped in Saran wrap and exposed to Fuji X-ray film for a sufficient number of days at -70°C (between intensifying screens) to produce a clear signal. Film were developed in an X-OMAT Ltd, X-2 X-ray film processor.

2.6.6 Detection of digoxigenin-dUTP

Digoxigenin was detected using the DIG colour detection system (fully described by the manufacturer in the "DIG Users Guide" by Boehringer Mannheim) in which the digoxigenin is detected by a specific antibody attached to a alkaline phosphatase which is in turn detected by the addition of a chromogenic substrate (NBT (nitro-blue-tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate)). Buffers used in this procedure were;

DIG 1, 150 mM NaCl, 100 mM maleic acid (pH 7.5).

DIG 2, DIG 1 plus 1% Marvel milk powder.

DIG 3, 100 mM Tris H-Cl (pH 9.5), 100 mM NaCl, 50 mM MgCl₂.

Antibodies were diluted 1:10 000 in DIG 2. NBT was stored as a stock solution of 75 mg/ml in 70% (v/v in H₂O) DMF at -20°C and BCIP stored as a 50 mg/ml solution in 100% DMF at -20°C.

2.6.7 Re-screening filters

When filters were required to be re-screened with a different probe they were first soaked in boiling 0.1% SDS for 10 min to remove the previous probe. They were pre-hybridised again for several hours before the addition of the new probe and repetition of the hybridisation procedure.

2.7 Oligonucleotides

Oligonucleotide synthesis

Some oligonucleotides were synthesised on an Applied Biosystems Inc. PCR-MATE 391 DNA synthesiser on 0.2 μ M columns (Cruachem). These required overnight cleavage and deprotection by ammonium hydroxide, before being vacuum dried and dissolved in distilled water. Remaining oligonucleotides were synthesised by the Gibco BRL custom primer service and were provided deprotected at 50 nmol. These were resuspended in TE buffer to a concentration of 100 μ M. All primers were stored at -20°C. A list of primers used in this study and not included in the text are provided in appendix 4.

2.8 Polymerase Chain Reaction

2.8.1 Standard PCR

Standard PCR protocols were used in the everyday amplification of DNAs. Amounts of template DNA varied, with 0.5 µg genomic template DNA used per reaction and 0.1 µg or less of plasmid template. For reactions using Boehringer Mannheim *Taq* polymerase, dNTPs (Boehringer Mannheim) were added at 200 µM each to single strength PCR buffer, left and right primers at a concentration of 0.5 µM with 1 u of *Taq* polymerase. When Applied Biosystems Reddy Load Mix *Taq* was used, only template and primers at the same concentrations as above were added to the pre-aliquoted mix.

Cycling was performed in thin walled PCR tubes in a Hybaid Omne or Hybaid PCR Sprint machine.

Cycling procedures were typically:

94°C for 1-2 min to ensure denaturation of template.

Then 20-30 cycles of;

denature at 94°C, 30 sec

anneal at 50-60°C, 30 sec

extend at 72°C, 1 min

then a final 2 min extension step of 72°C.

Annealing temperatures depended on the primers used.

2.8.2 High fidelity PCR

The Expand™ High Fidelity PCR System (Boehringer Mannheim) was used to generate PCR products with fewer PCR induced errors (error rate of 8.5×10^{-5} compared to *Taq* which has error rate of 2.6×10^{-5}). The PCR was set up as two separate master mixes.

Mix 1 contained;

dNTPs each at 200 µM

primers at 300 nM

0.5 µg template in a 50 µl volume.

Mix 2 contained

single strength buffer

2.6 u of enzyme mix in a total volume of 50 µl.

The mixes were kept separate until cycling began to prevent cleavage of the template or primers due to the 3'-5' exonuclease activity of *Pwo* DNA polymerase. Cycling was according to specific manufacturers instructions. Extension was at 68°C because the products expected were less than 3 kb.

The reaction was cycled as follows

94°C, 2 min

15 cycles

94°C 15 sec

50-60°C 30 sec

68°C 45 sec)

15 cycles

94°C 15 sec

50-60°C 30 sec

68°C 45 sec (plus cycle elongation of 45 sec per cycle)).

2.8.3 Reverse transcriptase PCR

PolyA⁺ RNA was obtained using the magnetic Dynabeads mRNA DIRECT kit (Dyna^l®) according to manufacturers instructions. Various tissues of up to six flies were used in the extraction. Tissues were ground in Treff tubes with matching homogeniser.

Once the mRNA was extracted a reverse transcriptase reaction was set up.

This reaction contained;

0.2 mM of each dNTP

40 u RNAsin (Promega)

10 mM dithiothreitol (DTT)

1x first strand buffer ,

and H₂O in a volume of 18 µl.

2 µl SuperscriptTM II RNase H⁻ Reverse Transcriptase (Gibco BRL) was then added to start the reaction.

Reactions were incubated at 42°C for >30 min, with occasional tapping to resuspend the beads. The beads were then collected using the Dynal MPC magnet, washed and suspended in 20 µl TE buffer, the suspension being stored at -20°C. 1 µl of the Dynabead solution was sufficient template for a standard PCR reaction.

2.8.4 Cloning of PCR products

PCR products were cloned using the Invitrogen TA cloning or TOPO TA cloning kits into pCR[®]2.1 or pCR[®]2.1 TOPO according to manufacturers instructions and transformed into pCR[®]2.1.

pCR[®]2.1 transformations were accomplished by first adding 2 µl of β-mercaptoethanol to 50 µl of competent TOP10 cells then gently stirring in 1 µl of ligated product on ice. (Ligations were performed according to manufacturers instructions (Invitrogen)).

The cells were left on ice for 30 min then heat shocked at 42°C for 30 s. The cells were put back on ice for 2 min before adding 250 µl SOC medium (appendix 2) then transformations were shaken on their side for 30 min at 37°C.

100 µl of the transformation was spread onto L-agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. These plates also contained X-gal (see below). The white transformants were removed as single colonies and were grown overnight (with shaking) at 37°C in 10 ml L-broth (appendix 2) containing 100 µg/ml ampicillin.

2.9 DNA Sequencing

2.9.1 Manual

Double-stranded DNA sequencing was carried out using the T7 Sequenase version 2.0 DNA sequencing kit (Amersham) or the T7 Sequenase quick denature plasmid sequencing kit (Amersham) using the methods described by the manufacturer. Both employ the dideoxy chain termination method of DNA sequencing using ^{35}S - α -dATP (ICN).

2.9.2 Automated

A single stranded PCR was performed with template and primers supplied at 1 μg and 3.2 pmol respectively, with a PCR mix containing fluorescently labelled dideoxynucleotides. Samples were run on an agarose gel with the nucleotides being detected on an ABI automated DNA sequencer. Automated sequencing procedures and materials were carried out by the Glasgow University Molecular Biology Support Unit. Analysis was with the Applied Biosystems automated sequence analysis programme.

2.10 Plasmid Construction for Microinjection

2.10.1 Preparation of vector

20 µg of plasmid DNA was digested with 50 u *EcoRI* for 3 hours then the DNA extracted with phenol/chloroform.

The plasmid was precipitated with 2 volumes of ethanol for 15 min at 0°C, spun for 10 min at 4°C and dissolved in 90 µl 10 mM Tris-HCl (pH 8.3).

To prevent self-ligation of the *pP{UAST}* vector, it was treated with Calf intestinal alkaline phosphatase (CIP) (Sambrook *et al.*, 1989).

10 µl of 10x CIP buffer and 1 µl of CIP enzyme (Promega) was added and incubated at 37°C for >30 min.

SDS and EDTA (pH 8) were added to 0.5% and 5 mM respectively and the reaction incubated at 56°C for 30 min after the addition of proteinase K to 100 µg/ml.

The DNA was extracted once with phenol and one with phenol/chloroform and precipitated with 0.1 volumes of 3 M sodium acetate (pH 7) and 2 volumes of 100% ethanol at 0°C for 15 min and centrifuged as before.

The DNA was washed with 70% ethanol, dried and dissolved in 200 µl TE buffer (pH 7.6).

2.10.2 Preparation of insert

5 µl of pCR®2.1TOPO containing the insert of choice was digested with *EcoRI* and run on a 1% agarose gel. The insert was purified (see 2.4.3) and dissolved in 5 µl distilled water.

2.10.3 Ligation

A vector:insert ratio of 3:1 was used.

Ligation reactions generally contained;

0.5 µl vector

5 µl insert

2 µl 10x T4 ligase buffer

1 µl T4 DNA ligase (Gibco BRL)

1.5 µl distilled water.

The reactions were incubated at 14°C overnight and 2 µl of each ligation was used in a transformation.

2.11 Plasmid Rescue

This technique allows the determination of the insertion site of a P{*lacW*} element as a result of pBluescript sequences present in this type of element.

2.11.1 DNA isolation

(Guo, 1996b) 15 flies were homogenised in an Eppendorf with a pestle in 400 µl of lysis buffer;

(80 mM NaCl, 5% (w/v) sucrose, 0.5% SDS, 50 mM EDTA, 100 mM Tris-HCl (pH 8.5)) and incubated for 30 min at 70°C.

Potassium acetate was added to a final concentration of 0.6 M and the tube placed on ice for 30 min.

Centrifugation for 15 min at 4°C removed debris and the supernatant was removed to a fresh tube.

The DNA was precipitated with 0.6 volumes of isopropanol, spun down and washed in 70% ethanol, dried and resuspended in 50 µl 1x REact 2 buffer (Gibco BRL).

The solution was heated at 70°C for 15 min, cooled, and another 50 µl added before the addition of 10 u *EcoR*I.

The reaction was incubated at 37°C for 3-4 hours. The enzyme was heat inactivated at 70°C for 15 min and cooled to room temperature before the ligation step.

2.11.2 Ligation

An equal volume of 2x modified ligase buffer was added (10 mM MgCl₂, 4 mM ATP, 20 mM DTT, 30 mM Tris-HCl (pH 7.4)) to the isolated DNA before addition of 0.5 u T4 DNA ligase (Promega). The reaction was incubated at 14°C overnight.

2.11.3 Transformation

40 µl of the ligated mixture was transformed into 200 µl competent cells. The cells were not plated out but were grown overnight in 25 ml L-broth containing 100 µg/ml ampicillin.

2.11.4 Plasmid detection

50 μ l of cracking buffer (200 mM NaOH, 2% SDS, 0.2% sucrose, 20 mM EDTA) was added to 50 μ l of the overnight growth and incubated at 70°C for 5 min. The reaction was cooled and 12 μ l of 50:50 4 M KCl:loading buffer was added, incubated on ice for 5 min and spun for 5 min, prior to running on a 0.8% agarose gel (Guo, 1996b). Gels were blotted as normal and probed with DIG probes.

2.12 Detection of β -galactosidase

Flies containing P{*lacW*} insertions were anaesthetised on ice, decapitated and dissected in PBS (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄) then pinned out to display their internal organs in a Sylgard coated petri-dish. They were fixed with 1% (v/v) glutaraldehyde in PBS for 20 min, washed thoroughly in PBS twice then stained overnight in Fe-NaP staining buffer (10 mM NaH₂PO₄, 10 mM Na₂HPO₄, 150 mM NaCl, 1 mM MgCl₂, 3.1 mM K₄(Fe²⁺CN)₆, 3.1 mM K₃(Fe³⁺CN)₆, 0.3% Triton-X-100, (pH 7)) containing 0.2% X-gal (from an 8% (w/v) stock in DMSO), at 37°C overnight. Flies were then washed three times in PBS then viewed under a light microscope and photographed.

2.13 P-element Excision

When a P-element insertion has been isolated, proof that the lethality is caused by that insertion can be given by showing that excision of the element reverts the lethality. Excision can be accomplished by crossing the P-element to a “jump-starter” line containing a source of transposase. Line 16/1 was crossed to a line containing the $\Delta 2-3$ transposase element on its third chromosome (Figure 2.1). This cross produced males carrying both the P-element and the “jump-starter” chromosomes, therefore the P-element could be mobilised. The crossing scheme below indicates how jump-outs could be obtained. As the progeny of each cross were always mated back to the original lethal 16/1 mutant line after cross 3, any viable straight-winged progeny (i.e. those that can survive without the balancer chromosome) could only have been as a result of the excision of the lethal P-element. Progeny of type 1 from the figure below were collected and mated to sibs to produce a balanced excision line.

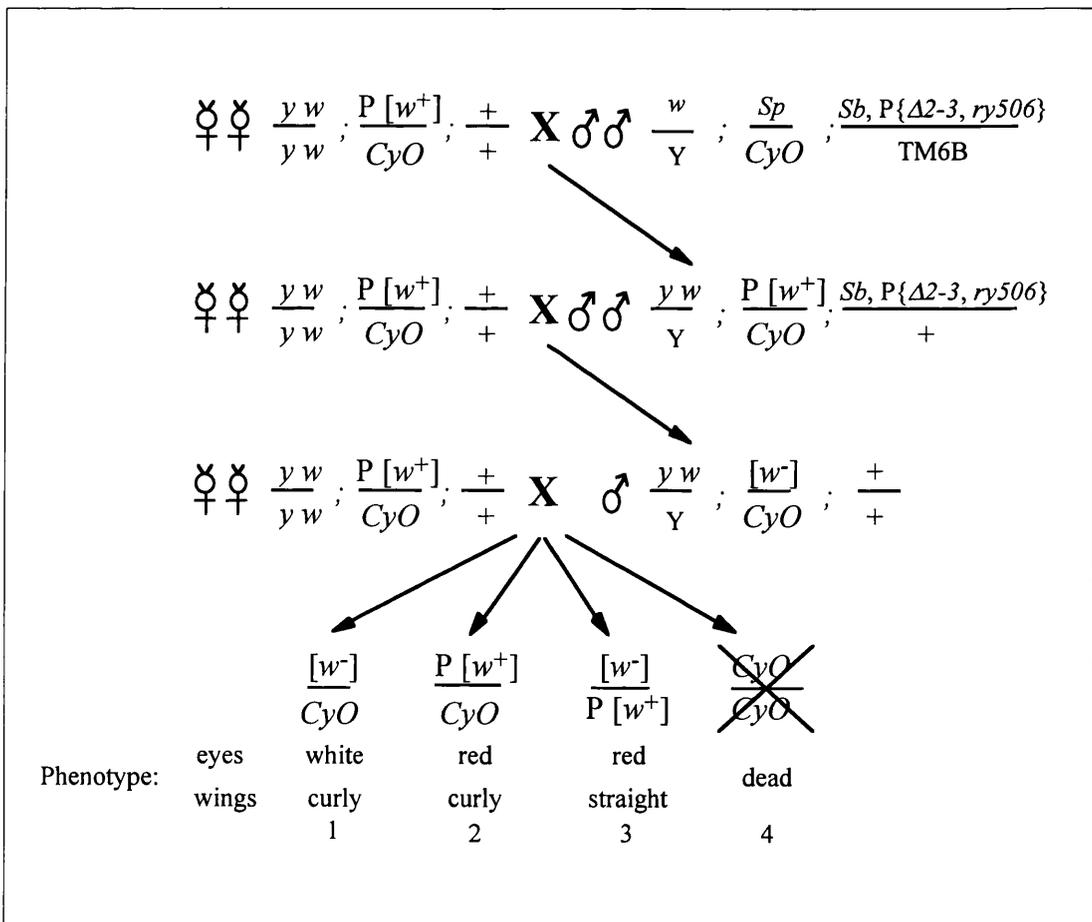


Figure 2.1 P-element excision. Crossing scheme for the generation of P-element excisions.

2.14 Lethal Phase Analysis

200 flies (Oregon R, 16/1 or outcrossed 16/1) were set up in separate fly cages at 25°C. 50 embryos were transferred to fresh plates and allowed to hatch. The number of hatched larvae were counted and transferred to a fresh food vial. This process was repeated three times. Numbers of surviving larvae and adults were counted and analysed in StatView Student v1.0 (Abacus Concepts Inc.). Data was displayed in CricketGraphIII (Computer Associates International Inc.).

2.15 Germline Transformation

2.15.1 Embryo collection and preparation

500 flies (*w¹¹¹⁸*) between 3 and 7 days old were set up in an egg collection cage the night before embryos were required. Next day, plates covered in a thin layer of yeast paste were changed hourly until sufficient eggs were produced for injection (> 100) in a 20-25 min period. The eggs were removed to a slide and dechorionated with fine forceps under a light microscope and lined up on the edge of the slide which had a thin layer of glue (Scotch tape dissolved in heptane) along the edge. The embryos were placed with the posterior pole facing outwards, dehydrated for 5 min and covered with 10s Volatef oil.

2.15.2 Needle preparation

Needles were pulled on a Campden Instruments moving-coil microelectrode puller, model 753, from borosilicate glass capillary tubes (Clark Genetic Instruments) of dimensions 1.0 mm (O.D.) x 0.78 mm (I.D.), with the puller settings on,

pre-pull heating time	40
pull force	30
heater control	350

Needles were backfilled with DNA solution and the remaining space filled with 10s Volatef oil. When ready to inject the sealed tip was broken back by a slight tap on the edge of the slide.

2.15.3 Microinjection

The embryos were injected with a mixture of *pP{UAST}* containing the sequence of interest (200 ng) and the helper plasmid *pP{Δ2-3}* (50 ng), which had been coprecipitated with ethanol/sodium acetate and washed twice in 70% ethanol before dissolution in injection buffer (0.1 mM Na₂HPO₄, 0.1 mM NaH₂PO₄, 5 mM KCl, pH 7.8). Embryos were injected with this solution using a hydraulic oil pressure system at 1-2 drops per second into the posterior end. Embryos were viewed under a Nikon inverted stage microscope.

2.15.4 Post-injection care

Slides containing injected embryos were removed to a fresh grape-juice agar plate. The embryos were left to recover for 1-2 d and any hatched larvae transferred to standard food. Surviving adults were mated individually back to the host strain and the progeny scored for eye colour transformation. Transformants were crossed again to the host strain and transformed progeny of this cross were mated to sibs.

2.16 Fluid Secretion Assays

Adult *Drosophila* tubules were dissected out into Schneiders revised *Drosophila* medium (Gibco BRL) and pairs of tubules were transferred to a 9 µl drop of a 1:1 (Schneiders:*Drosophila* saline) mix (*Drosophila* saline, 117.5 mM NaCl, 20 mM KCl, 2 mM CaCl₂, 8.5 mM MgCl₂, 10.2 mM NaCO₃, 4.3 mM Na₂HPO₄, 8.6 mM HEPES with 20 mM glucose added before use).

One tubule was drawn from the drop and looped around a pin, ensuring that the ureter (from where secretion issues), was out of the drop.

Every ten minutes the droplets excreted from the ureter were removed with a fine rod and their diameter measured using an eyepiece graticule. For a schematic diagram see figure 1.10

When agonists were to be added they were first diluted in Schneiders:*Drosophila* saline if required before addition of 1 µl of the compound to the bubble.

Results were analysed using a spreadsheet in Excel 5.0 (Microsoft).

2.17 DNA and Protein Sequence Analysis

DNA sequences were viewed and manipulated in MacVector 6.5.1 (Oxford Molecular Group PLC.). This program was used to deduce restriction enzyme sites and was also used to translate sequences into protein using the translation feature.

DNA sequences were aligned with a MacVector sister program, Assemblylign, set on default settings.

Protein alignments were performed using ClustalWPPC (Thompson, 1994) on the default settings and bootstrap tree plots were also performed in ClustalWPPC on default settings. Protein alignments were viewed in SeqVu 1.0.1 (Garvan Institute of Medical Research) and Tree alignments in TreeView PPC version 1.5.3 (Roderic D M. Page).

Chapter 3

Identification and Characterisation of a Lethal P-element Insertion in *vha 16-1*

3.1 Summary

This chapter describes the isolation and characterisation of a lethal P-element insertion in the gene *vha16-1* which forms the transmembrane pore of the V-ATPase. It describes the isolation of the insert line and discusses where in the gene the P-element is inserted and what effect this has on expression and fluid secretion, whether it is present as a single copy and at what stage it causes lethality. It also shows β -galactosidase staining to observe where the gene is expressed in the adult fly and describes the excision of the P-element and whether this reverts this lethality. The chapter also discusses the *vha16-1* gene itself, showing the number of transcripts, its developmental regulation and whether it is single copy within the genome.

3.2 Introduction

As has been discussed, *Drosophila melanogaster* is an extremely useful model organism, particularly due to the powerful molecular genetic tools that have been designed to engineer and interfere with the *Drosophila* genome. The most powerful of these tools is the P-element, and when studying a particular gene it is most convenient to have a P-element insert within the gene as deductions can be made about the gene function from the mutant phenotype.

The genomic sequence of the vacuolar ATPase 16 kDa subunit gene of *Drosophila melanogaster* has already been characterised, but as this gene is part of an extremely complex enzyme and may have more than one role within the organism an attempt was made to isolate a marked P-element insert within the gene that would be a starting point in a genetical analysis.

3.3 Isolation of a P-element insert in *vha16*

3.3.1 Screening rescued plasmids

The 16 kDa vacuolar-ATPase subunit gene, cloned by Meagher *et al* (Meagher *et al.*, 1990) (GenBank accession no. X77936), is present on the second *Drosophila* chromosome at the location 42B. It had been previously observed in (Finbow *et al.*, 1994) and (Goodwin, 1994) that when performing "site selected" P-element mutagenesis with unmarked P-elements, the *vha16-1* gene was a P-element insertion hotspot.

Available to us in the lab were a series of nearly two thousand rescued plasmids that had been rescued from lethal P-element-inserted ($P\{lacW\}$) lines which had been generated by the Kiss lab in Hungary. (Török, 1993). These P-elements were all known to be inserted randomly into the second chromosome. As this genomic region is a hot-spot for transposon insertion it is probable that one of these lethal elements is inserted in this gene.

As mentioned in section 1.3.3 $P\{lacW\}$ is a large P-element which carries the *ampR* gene which allows the plasmid rescues of sequences flanking the element. Plasmid rescue of these lines was undertaken and the resulting plasmids were collected into a series of pools as described in Guo *et al* (Guo, 1996b). A schematic outline of this procedure is shown in figure 3.1.

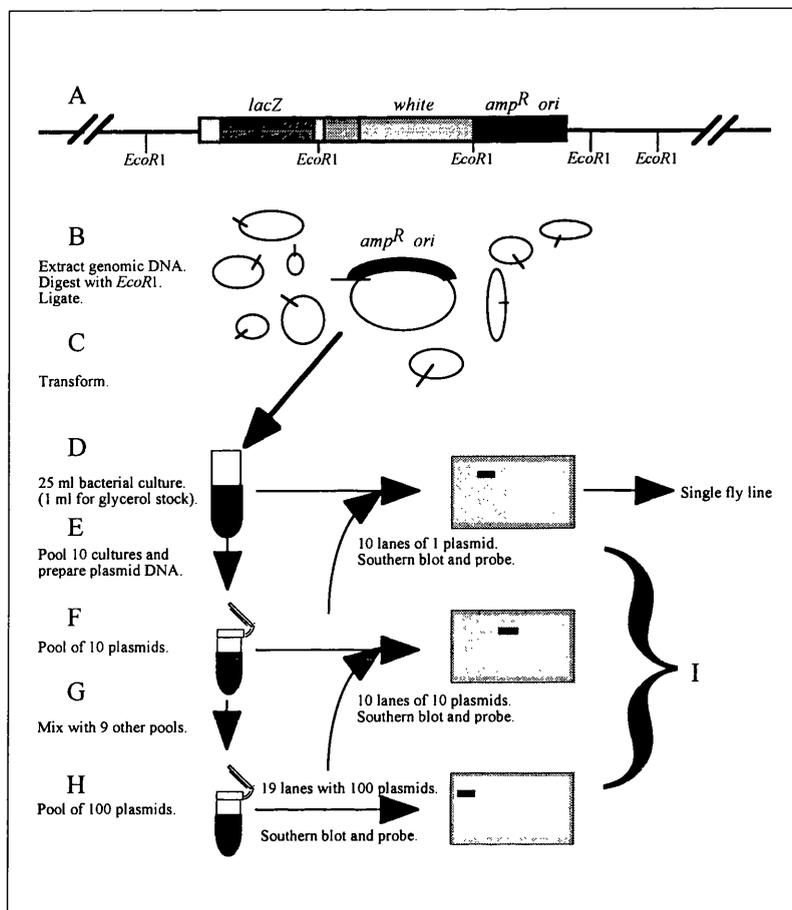


Figure 3.1 **Plasmid rescue and plasmid pooling protocol** undertaken in order to isolate, in three steps, P-elements of interest inserted in the second chromosome of *Drosophila melanogaster*. Plasmid rescue of genomic sequences flanking the P-element insertion is undertaken as follows, A) genomic DNA is extracted from the two thousand individual fly lines with second chromosome inserts and digested with *EcoRI*. B) this genomic digest is re-ligated and only the plasmids containing the *amp^R* gene and bacterial origin of replication present in the P-element can propagate when transformed (C) into *E. coli*. D) some of each individual culture is pooled with nine others (with the remaining culture stored frozen) and E) the plasmid DNA isolated. This gives a pool of ten plasmids (F) which are pooled with nine others (G), giving a pool of 100 plasmids (H), nineteen of these were produced. As well as being pooled, a proportion of the individual stages, F), and H) were stored. I) the 19 pools of 100 plasmids can be run on an acrylamide gel and screened via Southern blotting with specific probes to identify any hybridising plasmids. If a pool is seen to hybridise then it can be subdivided into the ten subpools which had been collected together. These subpools can then be probed in the same manner, leading to identification of the first pool which contains ten plasmids. The ten frozen cultures containing these plasmids can then be mini-prepped and these plasmids probed again to lead to an individual P-element containing fly line.

These plasmid pools were screened in an attempt to find a lethal P-element insert in the *vhal6* gene. This procedure involved the initial screening of nineteen plasmid pools (figure 3.2a), each of which contained ten subpools of ten individual plasmids of known fly line origin. This enabled the screening of the entire plasmid population on one agarose gel. After Southern blotting the filter was probed with a genomic *vhal6* digoxigenin probe and two positive lanes were observed, corresponding to plasmid subpools (21-30) and (81-90). The screening process was repeated again (figure 3.2b), with the smaller subpools being run on an agarose gel, Southern blotted and probed, with a positively hybridising lane now corresponding to one of ten rescued plasmids. In this case, three pools of ten gave a positive signal; pools 22 (plasmids 50-134), 23 (plasmids 138-153) and 84 (plasmids 940-958). The individual plasmids from which the pools were made had been stored as frozen bacterial cultures. The cultures were then defrosted and prepped and the plasmids electrophoresed on agarose gels, blotted and probed a final time (figure 3.2c-d). This procedure resulted in the isolation of two individual plasmids, numbers 151 and 945, which strongly hybridised to the genomic probe. The fly lines from which these rescued plasmids had been obtained (lines 16/1 and 76/16 respectively) were ordered from Szeged in Hungary where they had been generated.

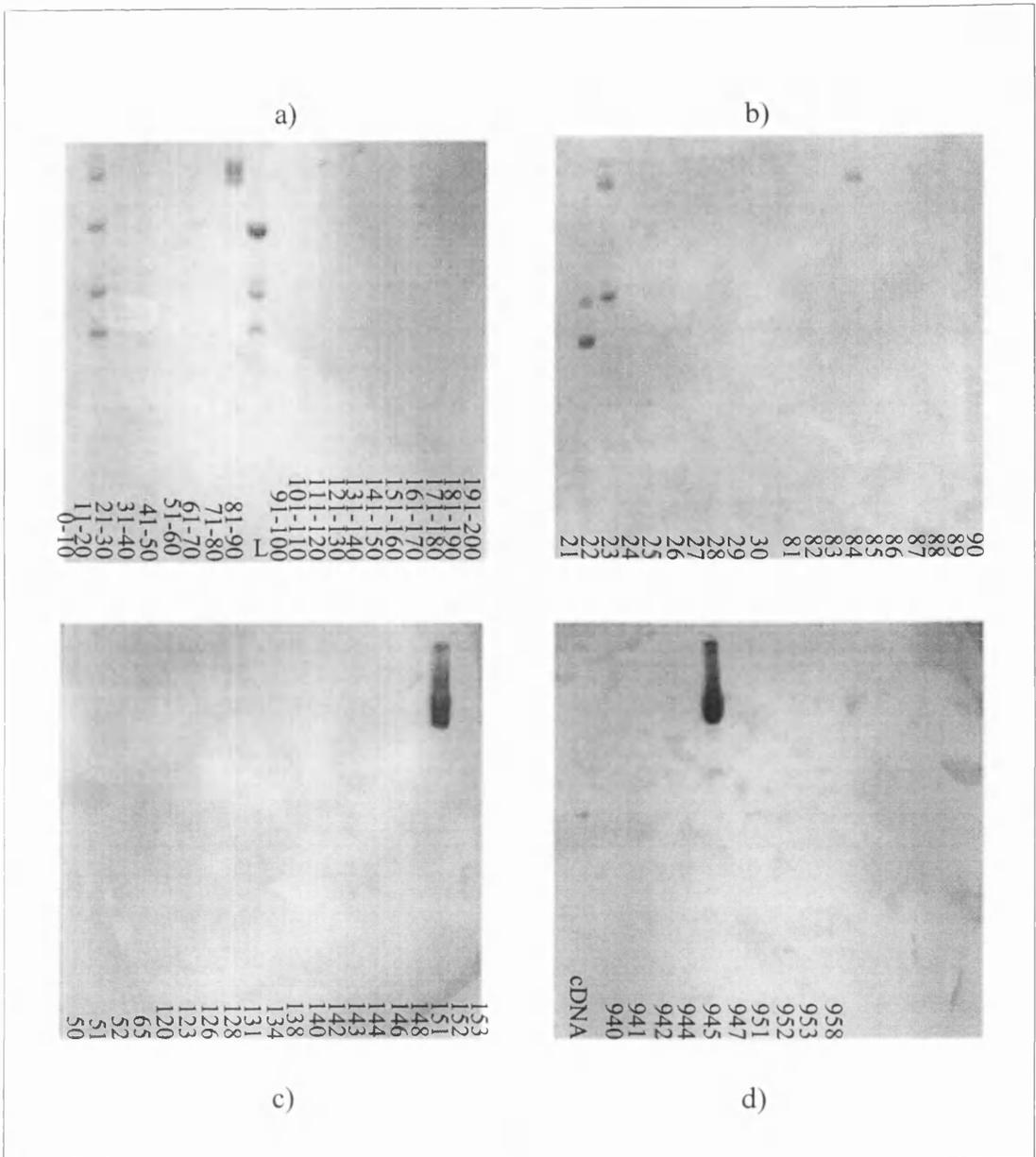


Figure 3.2 **Isolation of P-element inserts in *vha16-1***, a) Southern blot shows nineteen lanes, each containing one hundred rescued plasmids. The two lanes which hybridised to the genomic DIG probe were traced back to the ten pools of ten from which they were made (b).

b) Southern blot of the these pools of ten showing three that hybridise. The thirty lines that made up these pools were run again (c and d), and upon probing, two individual lines were isolated.

These were traced to the original fly lines (16/1 and 76/16), that had been plasmid rescued. These flies were then obtained from Szeged.

3.3.2 Sequencing of rescued plasmids

Plasmid rescue was performed upon receipt of the fly lines and the resulting plasmids sequenced (from a P-element specific primer, P_R), to determine where the elements were inserted and whether they were indeed within *vhal6-1*. Plasmid 16/1, upon sequencing, was observed to be inserted at base 3005 of the genomic DNA sequence of *vhal6-1*, in the large intron within the gene. A schematic diagram of the P-element and where it is inserted in the *vhal6-1* gene is depicted in figure 3.3 a-b.

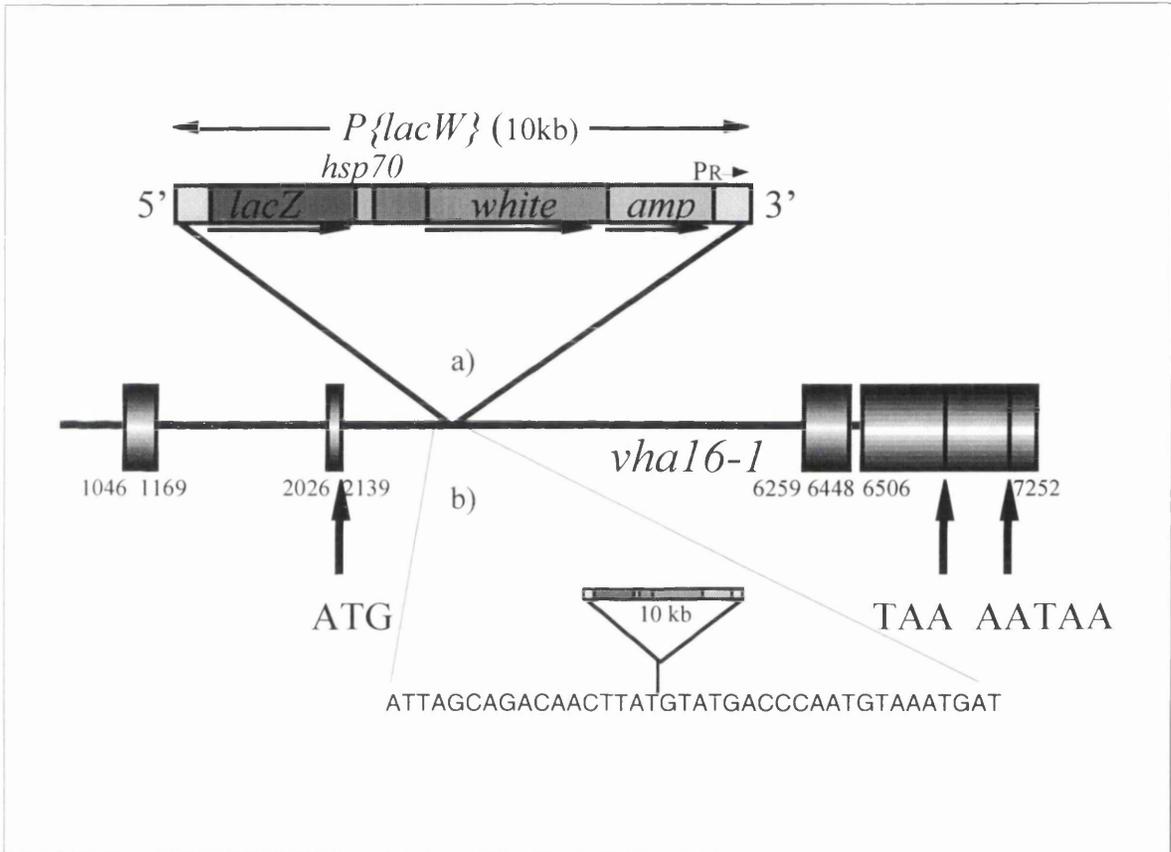


Figure 3.3 a) **Schematic diagram showing the gene structure of *vhal6-1*** and the relative position of the P-element from line 16/1 within it, and b) the sequence around the point of insertion. Shown in the diagram (top) is the P-element $P\{lacW\}$ and the genes contained within it. The middle section shows the genomic structure of *vhal6-1* Genbank accession X77936 (Finbow *et al.*, 1994) with the shaded boxes representing introns and the line representing the exons. The lower section shows a blown up section of the *vhal6-1* gene and where the P-element is inserted within it.

Although line 76/16 produced a rescued plasmid that hybridised to the *vha16-1* genomic DIG probe, no sequence recognisable as that of *vha16-1*, or any other known *Drosophila* sequence at that time, was encountered when sequencing out of the P-element. This was believed to be due to the insert being present in an unsequenced region up or downstream of the *vha16-1* sequence and due to the rescued plasmid being large (>20 kb) it would contain enough, if not all, of the *vha16-1* sequence for the probe to hybridise, therefore appearing as a positively hybridising plasmid. Complementation tests showed the two P-element fly lines complemented each other, indicating further that 76/16 did not interrupt *vha16-1*. As line 76/16 was not inserted within the gene, only line 16/1 was used in further studies.

Very recently, the *Drosophila* Genome Project published sequence confirming the theory relating to the 76/16 plasmid. Genomic sequence was made available on the database for the upstream region of *vha16-1*. This region contains a gene called *trap1*, a Hsp90-related protein and 76/16 was inserted upstream of this, around 4 kb upstream of *vha16-1*. A map of the region is shown in figure 3.4.

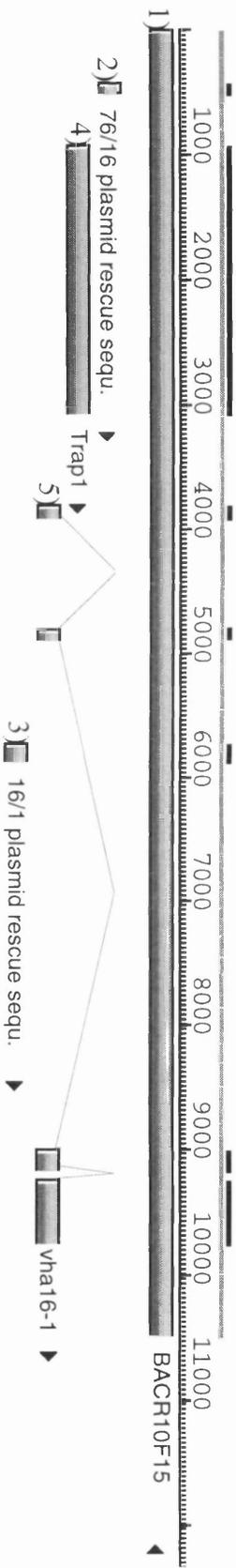


Figure 3.4 **Map created by the Assemblylign program** of Mac Vector (modified in ClarisDraw), showing a genomic BAC clone ((1) BACR10F15, Genbank accession AC007624) which contains both sequences obtained upon sequencing out of the plasmids (2) 76/16 and (3) 16/1. The picture shows the relationship between (5) *vha16-1*, (4) *trap1* (Genbank accession AF115775) and the plasmid rescue sequences. Default parameters were used to perform the assembly.

3.3.3 Southern verification of insert within *vha 16-1*

When probed with *vha16-1* cDNA, a Southern blot of genomic DNA from line 16/1 and wild type Oregon R restricted with *EcoRI*, showed a change in restriction pattern (figure 3.5) between the two lines. The Oregon R lane only had one hybridising band while the 16/1 lane had two, the upper band being due to the larger DNA fragment created by the P-element insert and the lower band due to the wild-type allele present on the balancer chromosome.

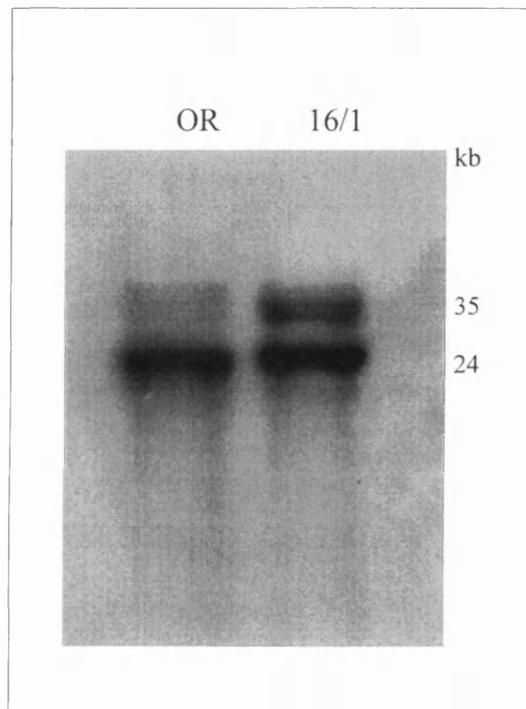


Figure 3.5 **Southern blot with a *vha16-1* cDNA probe** showing a band shift in the genomic DNA due to insertion of the P-element. 5 μ g of genomic DNA (lane 1, wild-type Oregon R, lane 2, *vha16-1* mutant line 16/1) was digested with *EcoRI*, separated on a 1% agarose gel and blotted onto nylon membrane (Boehringer Mannheim). Washing was at high stringency, 0.1% SSC, 0.1% SDS at 65°C. The filter was exposed to film for six nights.

3.3.4 P-element copy number

Although sequence data shows that a P-element is inserted into *vha16-1*, this does not prove that the lethal phenotype is a direct result of disruption of this gene as there may be some other genetic factor involved, such as another P-element insertion being present. To show the copy number of the P-element within the 16/1 genome, a genomic Southern blot of DNA isolated from line 16/1 had to be performed. If the DNA is digested with a number of enzymes (which do not cut within the element), then the number of bands that appear, after hybridisation with a P-element specific probe, reflects the number of elements inserted in the genome. The probe used in this case was the pBluescript plasmid. This contains many of the same sequences (such as the origin of replication and ampicillin resistance gene), as the plasmid sequences present in the P-element, therefore serving as a useful probe against P-element insertions. Results of this genomic Southern showed a single band in every lane (figure 3.6), therefore the P-element is single copy. The copy number of the P-element could have also been determined by *in situ* hybridisation to polytene chromosomes

The fact that there is one copy of the element in the genome makes it likely that it is indeed the this element present within *vha16-1* that causes the lethal phenotype and not another.

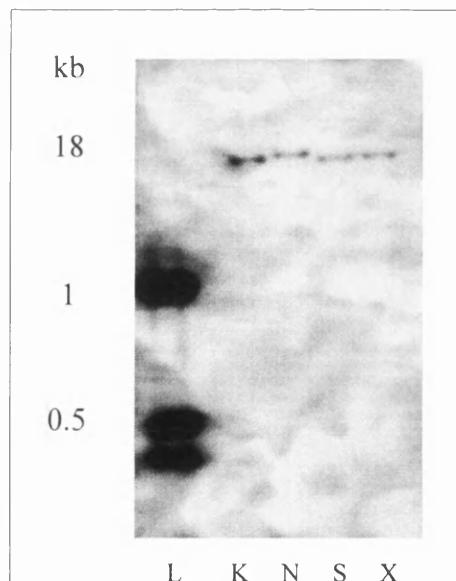


Figure 3.6 **Southern blot probed with pBluescript** showing that line 16/1 contains a single P-element insertion. 5 μ g of genomic DNA isolated from *vha16-1* mutant (16/1) flies was restricted with *KpnI* (K), *NotI* (N) *SphI* (S), and *XhoI* (X) and run on a 1% agarose gel. DNA was blotted onto a nylon membrane (Boehringer Mannheim). Filter was washed at high stringency, (0.1% SSC, 0.1% SDS) and exposed to film for six nights. Ladder (L) is Gibco BRL 1 kb.

3.4 Consequences of P-element Insertion

3.4.1 Expression patterns reported by the *lacZ* gene

The P-element mutator P{*lacW*}, used by Török *et al* (Török, 1993) in the second chromosome mutation screen were first generation elements that contain a *lacZ* reporter gene which expresses β -galactosidase in cell nuclei. As this gene has a minimal promoter, when it comes under the influence of an endogenous enhancer the β -galactosidase is then expressed in a spatial and temporal manner akin to that of the gene which it normally controls. This *lacZ* production can be detected by the addition of the chromogenic substrate X-gal, which, when cleaved by β -galactosidase, produces a blue colour in the nuclei of the cells within which it is expressed.

Line 16/1 was dissected, fixed and treated for the detection of β -galactosidase activity. Also, two lines containing P-element inserts in *vha55* and *vha67* were stained to observe their patterns.

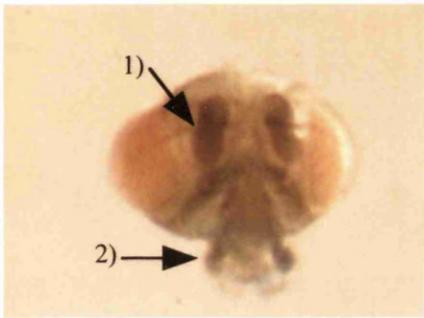
For 16/1 staining was observed throughout the fly, being strongest in antennae, maxillary palps, rectal pads, Malpighian tubules, and in female male and reproductive organs (figure 3.7 a-f). These results correlate with those obtained from enhancer trap data from two other V-ATPase subunits (Davies, 1996; Dow *et al.*, 1997), with expression in areas with V-ATPase energised membranes. In the Malpighian tubules the staining occurs only in the principal cell nuclei, with none in the secondary cells. The major differences between the stained lines occur in the reproductive organs. The *vha55* line has more extensive staining in the ovarian follicle cells than the *vha16-1* and *vha67* lines. The *vha67* insert line shows no staining in the testis but as three genes encode this subunit in *Drosophila* one of the others may function in this tissue. However, results obtained from enhancer trap data have to be confirmed using *in situ* hybridisation as the expression patterns generated could be due to an alternative enhancer acting upon the reporter gene.

vha16-1

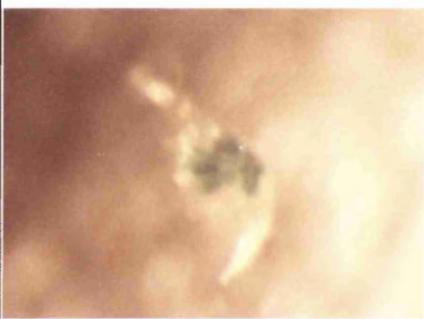
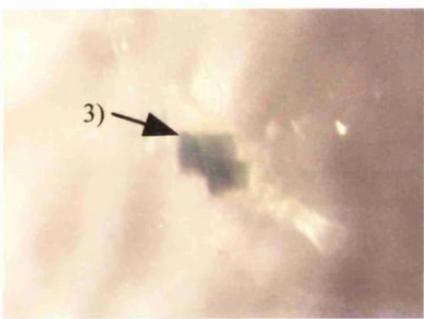
vha55

vha67

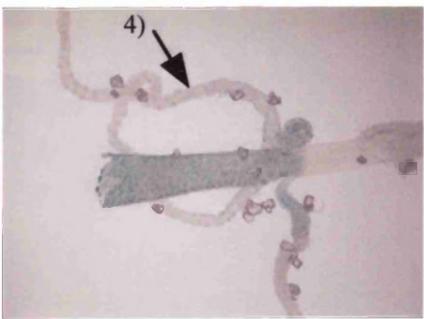
a)



b)



c)



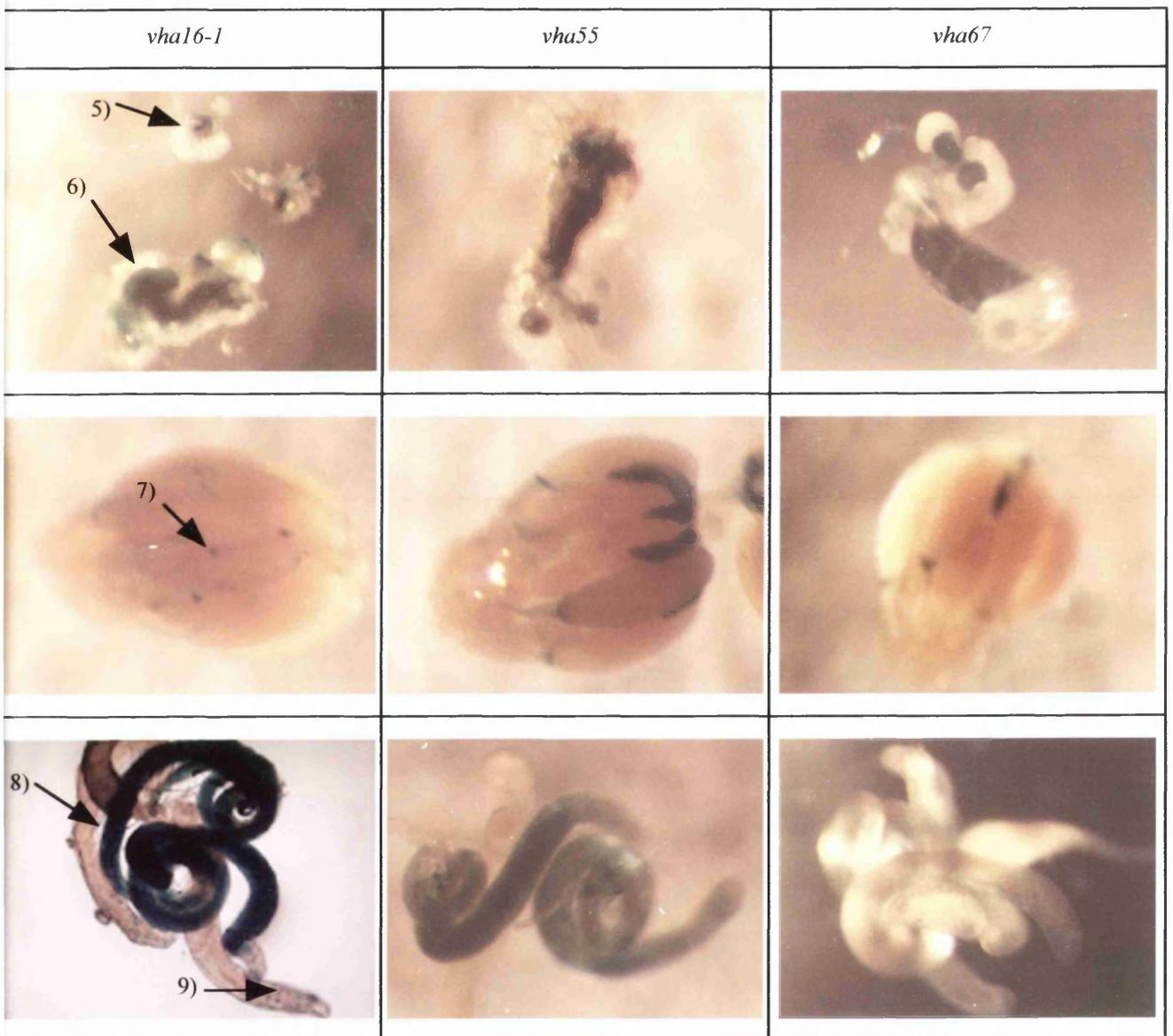


Figure 3.7 **X-Gal staining patterns**. Flies were anaesthetised, dissected to isolate internal organs, fixed and stained with X-gal at 37°C overnight.

Panels show staining in: a) antennae (1) and maxillary palps (2), b) rectal pads (3) c) Malpighian tubules (4), d) spermathacae (5) and uterus (6), e) ovarian follicles (7), f) testis (8) and accessory gland (9).

3.4.2 Lethal phase of the P-element insertion

The P-element insert is known to be lethal, but at which stage in the *Drosophila* life-cycle does lethality occur? Eggs from Oregon R wild-type flies and the 16/1 mutant were collected then gridded out on grape juice agar plates. After incubation for two days at 25°C larvae were collected, counted and put onto standard fly food. Numbers of adult flies were counted as shown in the table 3.1. These results were collated and shown graphically in figure 3.8.

Fly Line	No. of eggs	Larvae	Eclose
Oregon R	50	39	39
	50	42	40
	50	44	37
16/1	50	31	12
	50	40	21
	50	20	10

Table 3.1 Survival studies determining the lethal phase of 16/1.

Showing numbers of larvae hatching and pupae eclosing from 150 Oregon R and 16/1 embryos.

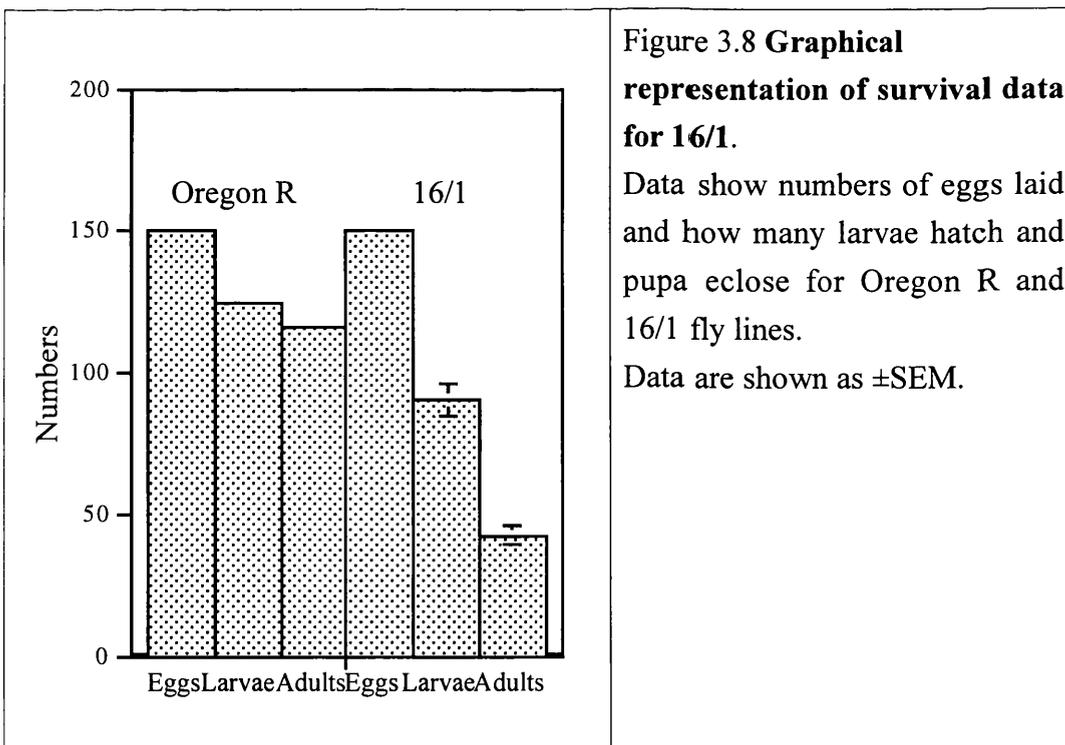


Figure 3.8 Graphical representation of survival data for 16/1.

Data show numbers of eggs laid and how many larvae hatch and pupa eclose for Oregon R and 16/1 fly lines.

Data are shown as \pm SEM.

The graph (figure 9.8) shows with wild-type Oregon R flies 80% of the eggs hatch and 90% of these reach eclosion. A small proportion of the eggs and larvae that die may be due to mishandling upon transfer.

Genetically 25% of the 16/1 embryos should die as a result of obtaining a double dose of the balancer chromosome. If the P-element insertion is lethal in the embryo then 50% of the eggs should die. For the mutant flies 60% of the eggs gridded out hatched and only around 50% of these eclosed. This implies that a small proportion of eggs that hatch die as larvae. Therefore, late embryo/early larval was initially thought to be the lethal phase but upon PCR no larvae ever had two P-element insertions, i.e. wild-type chromosome was always present. Further investigation led to the discovery that a double dose of the Curly of Oster balancer, while generally embryonic lethal, can occasionally escape into the larval phase.

In order to avoid the balancer problem, 16/1 was out-crossed to Oregon R and straight winged progeny from this cross mated *inter se*. The experiment was repeated and now 75% of the eggs laid should advance to eclosion if the insert is embryonic lethal and 100% if larval lethal. Results (table 3.1 and figure 3.9) show that 70% of the embryos hatch and 90% of the larvae reach adulthood, indicating the P-element causes lethality in the embryo.

Another method of determining the lethal phase of the P-element is to use balancer chromosomes which express the GFP (green fluorescent protein) marker. This theoretically allows us to determine which embryos do not contain the balancer chromosome as they should not fluoresce. Unfortunately the embryos contain a significant amount of autofluorescence due to yolk proteins and it is difficult to differentiate between those containing the balancer and those which do not.

Fly Line	No. of eggs	Larvae	Eclose
Oregon R	50	40	37
	50	40	36
	50	39	35
Outcrossed 16/1	50	33	28
	50	35	29
	50	36	30

Table 3.2 **Survival studies determining the lethal phase of outcrossed 16/1.** Showing numbers of larvae hatching and pupae eclosing from 150 Oregon R and outcrossed 16/1/+ embryos.

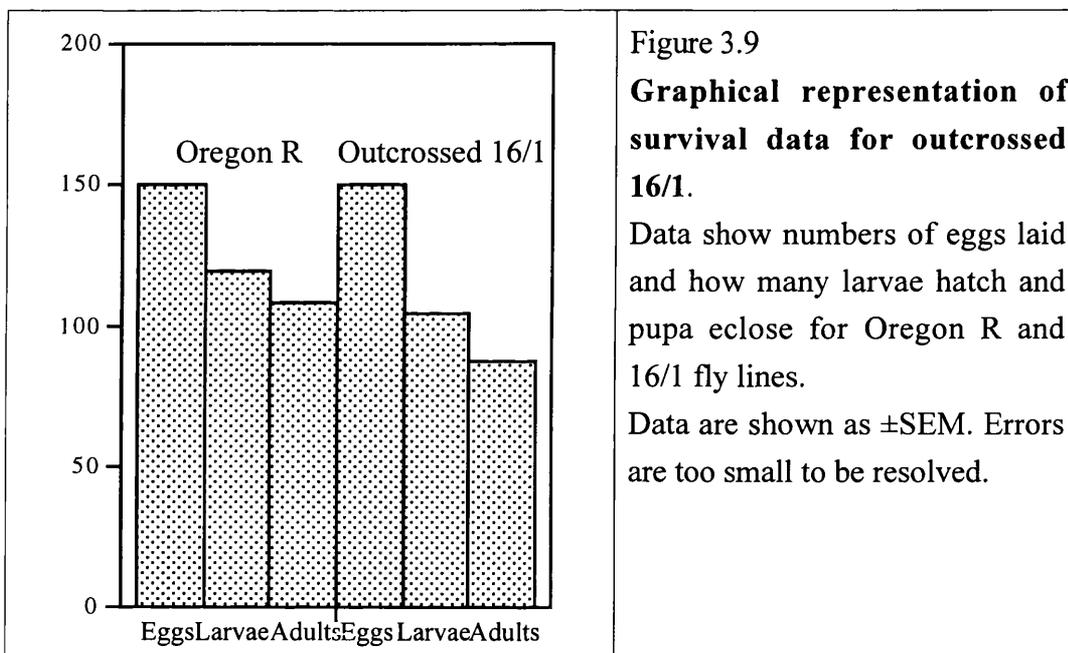


Figure 3.9
Graphical representation of survival data for outcrossed 16/1.

Data show numbers of eggs laid and how many larvae hatch and pupa eclose for Oregon R and 16/1 fly lines.

Data are shown as \pm SEM. Errors are too small to be resolved.

A two-tailed χ^2 test performed on these data show a significant difference ($P \leq 0.05$) in egg hatching and larval survival between the balanced and outcrossed 16/1 lines.

3.4.3 Northern analysis of *vha16-1*

As the *P{lacW}* P-element encodes the *Drosophila white* gene, it is plausible that splicing of the *vha16-1* gene may be impaired by the presence of the *white* splice sites. This could result in production of aberrant transcripts which do not encode functional proteins. Northern analysis was performed to see if the presence of the P-element in line 16/1 affected the 1.8 kb *vha16-1* transcript (figure 3.10).

There was no observable difference in the transcript size, but a significant reduction in the amount of mRNA produced relative to wild-type Oregon R flies was observed. This implies that there is no up regulation of the balancer chromosome allele at the transcript level to compensate for the mutant allele. However, if this experiment was to be repeated it would be advantageous to develop the blot using a phosphorimager as this would allow quantification of the bands relative to the control to determine if this result is real.

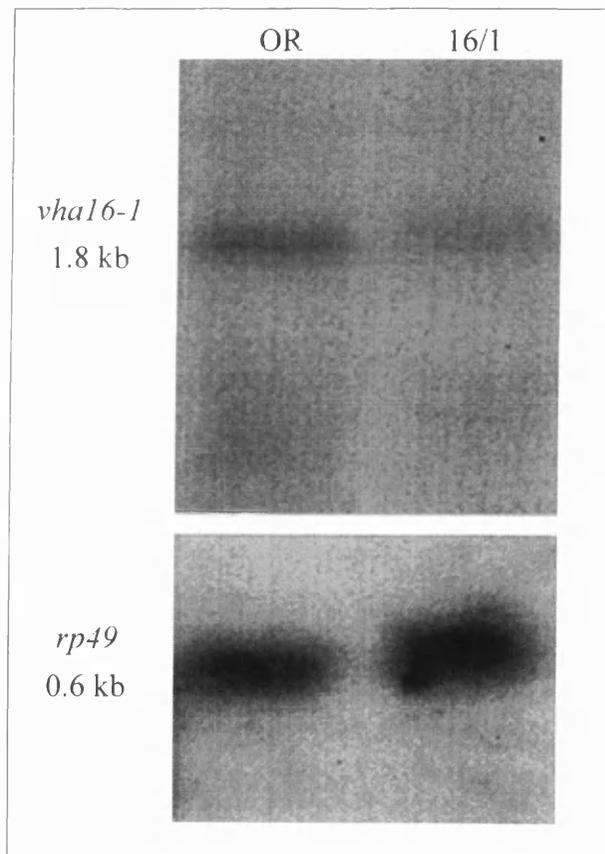


Figure 3.10 **Northern analysis of Oregon R and 16/1.** Northern blot of wild type (Oregon R) and 16/1 P-element insert RNA. 40 μ g of total adult RNA was run on a 1% agarose gel, blotted and probed with *vha16-1* cDNA (top panel). The blot was stripped and reprobed with *rp49*, a housekeeping gene, used as an RNA loading control (bottom panel). Blots were washed to high stringency, (0.1x SSC, 0.1% SDS, 65°C) and exposed to film for four and one nights respectively.

A developmental Northern was also performed to detect whether transcript levels of *vhal6-1* in wild-type flies differ during *Drosophila* development. Embryonic, larval, pupal and adult head and body total RNA was probed with *vhal6-1* cDNA. Results (figure 3.11) appear to show that the RNA levels are decreased in the pupae but when normalised to the *rp49* control (table 3.3), there appears to be no downregulation of this gene in the pupa.

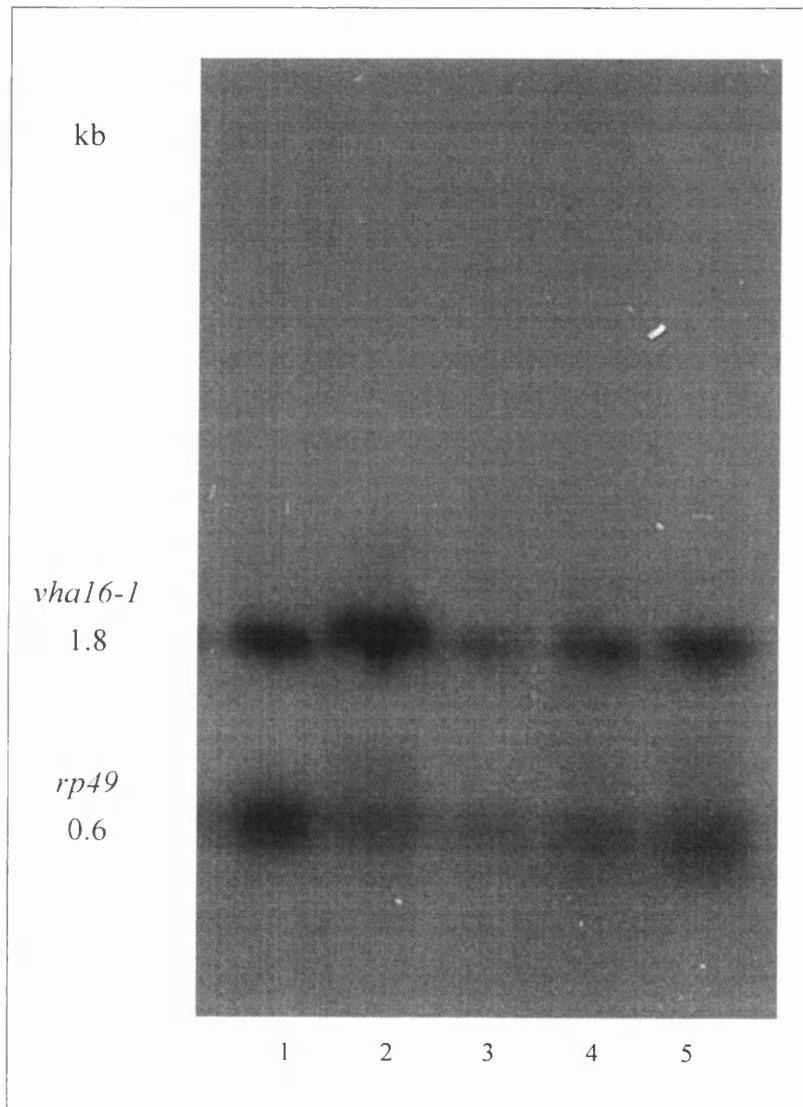


Figure 3.11 **Developmental northern blot.**

25 µg of total wild-type Oregon R RNA from:

Lane 1) mixed embryo, 2) mixed larvae, 3) mixed pupae, 4) head, 5) body, were run on a 1% agarose gel, blotted and probed with *vhal6-1* cDNA (top panel). Blot was stripped and reprobed with *rp49* (bottom panel) used as a loading control. Blots were washed to high stringency (0.1x SSC, 0.1% SDS, 65°C) Filters were exposed to film for six hours.

Band rp49	PSL rp49	PSL vha16-1	PSL-BG rp49	PSL-BG vha16-1	Ratio vha16-1/rp49
Embryo	3839	6724	3119	6622	2.12
Larva	2471	9714	1751	9605	5.5
Pupa	1762	2813	1042	2781	2.6
Head	2055	4721	1358	4611	3.4
Body	2616	6132	1919	6027	3.1
Background (BG)	702	111	-	-	-

Table 3.3 Analysis of *vha16-1* phosphorimage data.

Intensities of bands (PSL-phosphostimulated luminescence) for each developmental stage probed with *rp49* (column 2) and *vha16-1* (column 3) were measured in the quantification mode of the MacBAS programme (Fuji). The background reading (BG) was subtracted from each (columns 4 and 5) and the ratios between the two values calculated (column 6).

3.4.4 Effects on fluid secretion

As was observed from the Northern blot data, the level of *vha16-1* transcript is reduced in the P-element inserted line. Fluid secretion assays were performed to observe whether the function of the V-ATPase was affected by this.

Flies were outcrossed to a wild-type strain before performing secretion assays as it had been observed previously in the lab (personal communication) that the presence of the *CyO* balancer chromosome lowered secretion rates. Results showed (figure 3.12) that basal secretion rates between the two lines were similar and upon addition of 10^{-7} M CAP_{2b} they were stimulated to the same degree (see figure 1.9 for mode of action of CAP_{2b}). This implies that, despite the differing levels of transcription, the enzyme activity is unaffected.

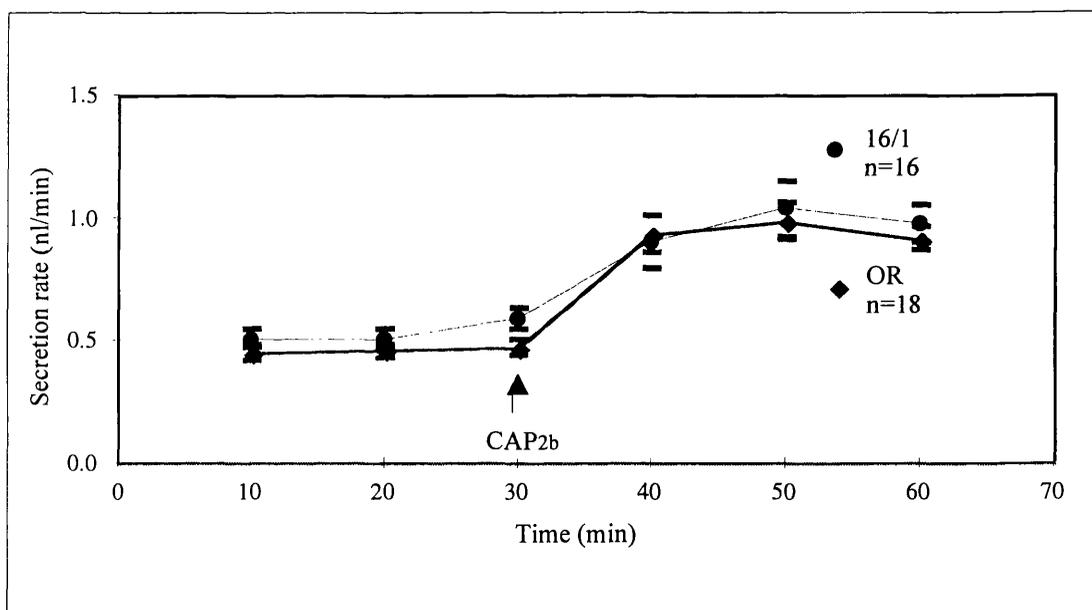


Figure 3.12 **Comparison of fluid secretion by outcrossed 16/1/+ and wild-type tubules.**

Heterozygous $16/1/+$ (16/1 n=16) or Oregon R (OR n=18) adult flies were dissected and both resting and CAP_{2b} stimulated (10^{-7} M, 30 min) rates of fluid secretion were measured. Data are shown as mean \pm SEM. There are no significant differences between data points (Student's t test, two tailed, $P < 0.05$).

3.4.5 P-element excision

Although we know in line 16/1 the P-element is inserted in the *vha16* gene and that the element is single copy, we cannot prove this insertion causes the lethality as it remains possible that some other genetic accident during the mutagenesis may have created a second site lethal. This can be tested by two strategies; precise excision of the element and observing whether the lethality is reverted, and rescue with a wild-type cDNA. This section describes the results using the first strategy.

To excise a P-element a jump-starter fly line expressing transposase has to first be crossed to the P-element line to initiate transposition. The line used in this case was w^* ; wg^{Sp-1}/CyO ; $ry^{506} Sb^1 P\{ry^{+7.2}=\Delta\alpha 2-3\} 99B/TM6B, Tb^1$, with the crossing scheme as shown in figure 2.1.

Figure 3.13 shows a schematic diagram of how P-element excision can be determined by PCR. The top part A) shows the 10 kb P-element inserted within the *vha16-1* gene and the relative positions (not to scale) of the primers L (16/1 P-el_L) and R (16/1 P-el_R) with the distance between the primers being too large to amplify a product. B) shows that after an excision event the wild-type distance between primers is restored.

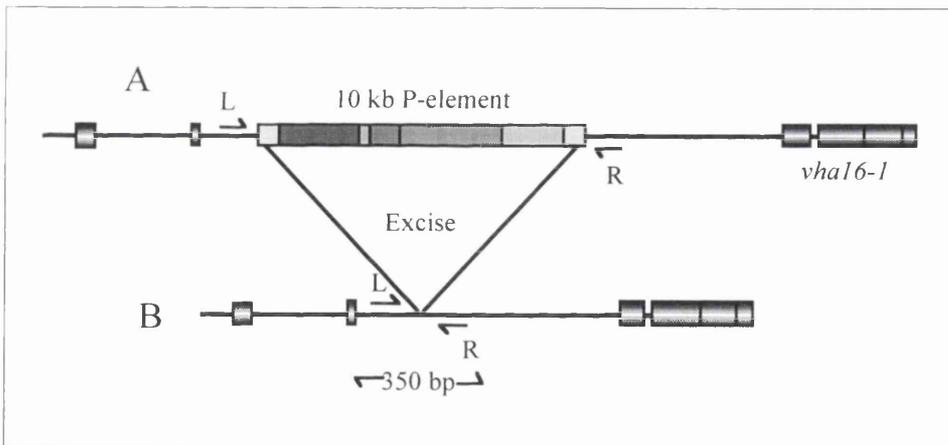


Figure 3.13 **Schematic diagram of determination of P-element excision by PCR.** The diagram shows A) the *vha16-1* gene interrupted by the 10 kb P-element and the relative positions (not to scale) of primers L, (16/1 P-el_L) and R (16/1 P-el_R). B) shows that after excision the distance between the primers is reduced (350 bp in wild-type) and the size of this product indicates whether the element has excised precisely.

Figure 3.14 shows the PCR results from five excision lines (using gene specific primers 16/1 P-el_L and P-el_R, appendix 4), called A-E, which can exist as homozygotes (i.e. straight-winged flies that do not require the balancer chromosome for survival). Line C appears to be a precise excision as the band is of the same size (~350 bp) as the controls, lines 16/1 and wild type. Lines A, B, D and E give PCR products of larger sizes indicating the element has not excised cleanly. A and B in particular produce PCR products of more than 1 kb larger than expected, but these sequences are not lethal. Perhaps this is due to the inserts being present in the large intron of the *vha16-1* gene, and the extra DNA is not enough to interfere critically with normal splicing mechanisms. These imprecise excision lines, thought not lethal, are potentially useful, as they could represent hypomorphic alleles.

This result shows that precise excision of the P-element reverts the lethal phenotype, therefore lethality is a result of the P-element insertion in *vha16-1*. Further proof of this would be to rescue the mutant phenotype by reintroducing the wild-type cDNA via germline transformation. This will be discussed in Chapter 5.

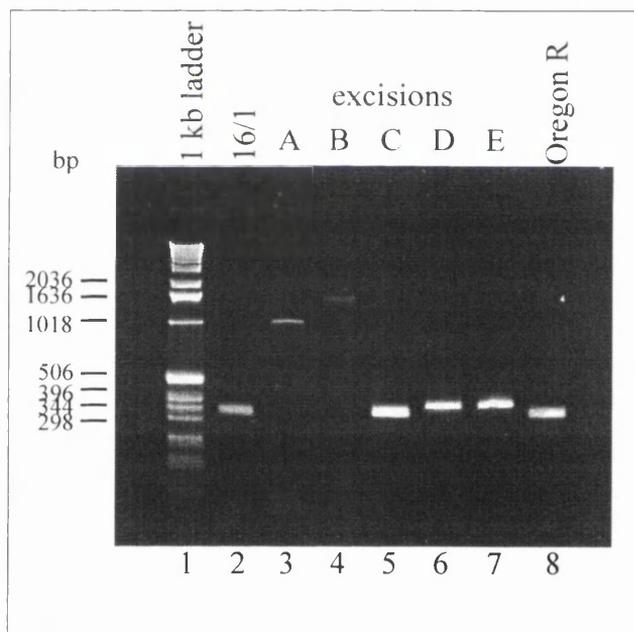


Figure 3.14 **PCR from five P-element excision lines.**

PCR was performed on DNA from 'squished' 16/1, five excision lines and wild-type Oregon R. DNA's were separated on a 1% acrylamide gel. Lane 1 is Gibco 1 kb ladder, lane 2 P-element inserted line 16/1, lanes 3-7, P-element excisions, lane 8, Oregon R.

Fluid secretion assays were performed on lines A and B (from figure 3.14) to observe whether these lines had a hypomorphic phenotype due to the remaining P-element DNA within the intron, which could interfere with normal splicing. Figure 3.15 shows that Line B has a significantly different higher rate of basal secretion, but no difference is observed upon stimulation.

Line A, apart from the first time point, has no significant difference compared to control. However this line, when homozygous, does not propagate well and therefore has some deficiency.

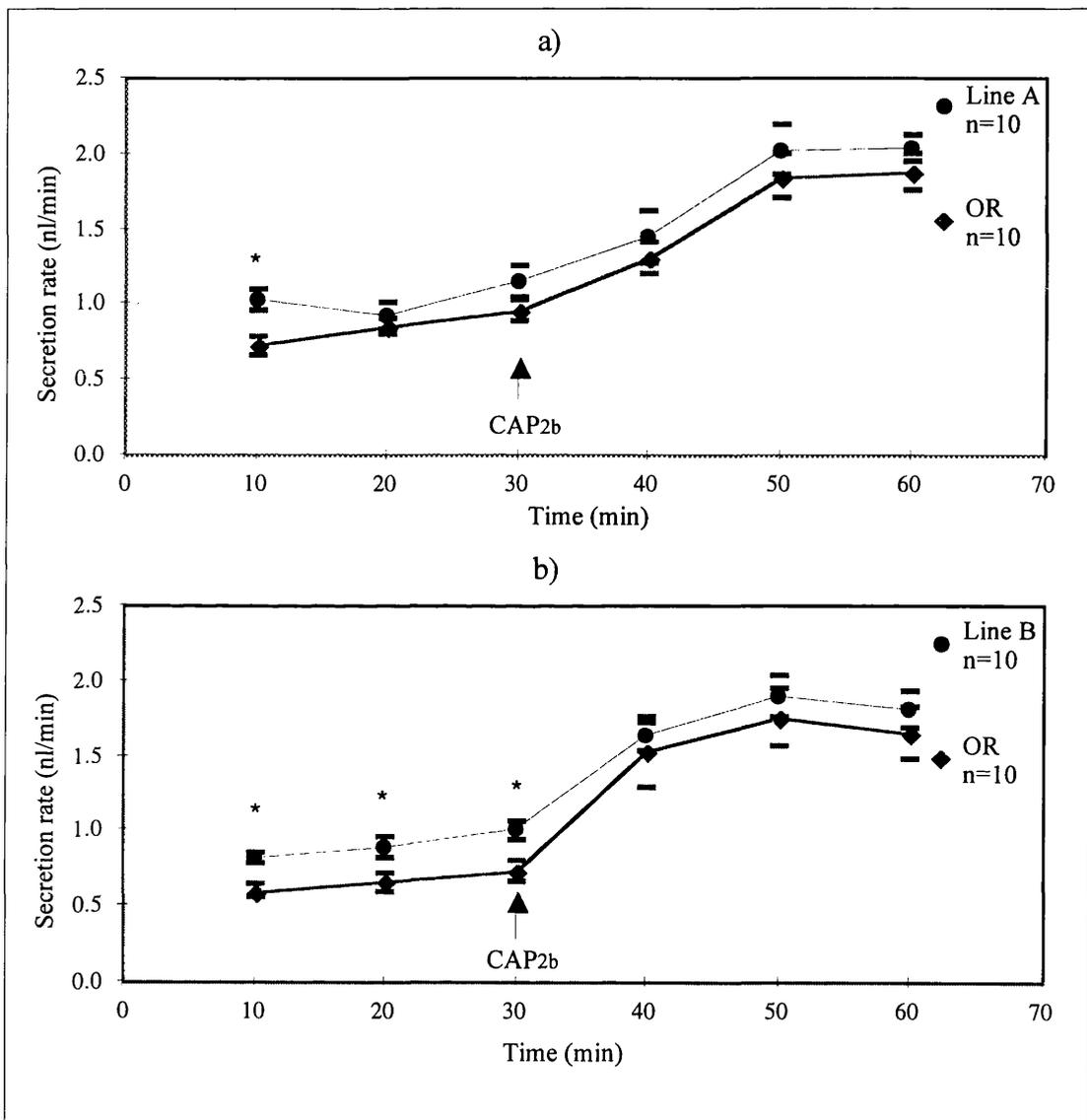


Figure 3.15 Comparison of fluid secretion by excision lines A and B and wild-type Oregon R tubules.

Homozygous excision lines A (n=10), B (n=10) or Oregon R (OR n=10) adult flies were dissected, and both resting and CAP_{2b} stimulated (10⁻⁷ M, 30 min) rates of fluid secretion were measured. Data are shown as mean ±SEM. * indicates significant difference between timed data points (Student's t test, two tailed, P<0.05).

3.5 Is there a *vha16* gene family?

When genomic restriction digests were probed at a low stringency with the cDNA used to isolate the lethal P-element insert line, several strongly hybridising bands were observed (figure 3.16 a). This indicated that there could be related member of a gene family within the *Drosophila melanogaster* genome. However, when probed at a high stringency, only one band in each lane remained, indicating that there is only one identical copy (figure 3.16 b). These Southern blot results are consistent with those obtained in (Goodwin, 1994), Finbow *et al.*, 1994). This observation is the subject of Chapter 4.

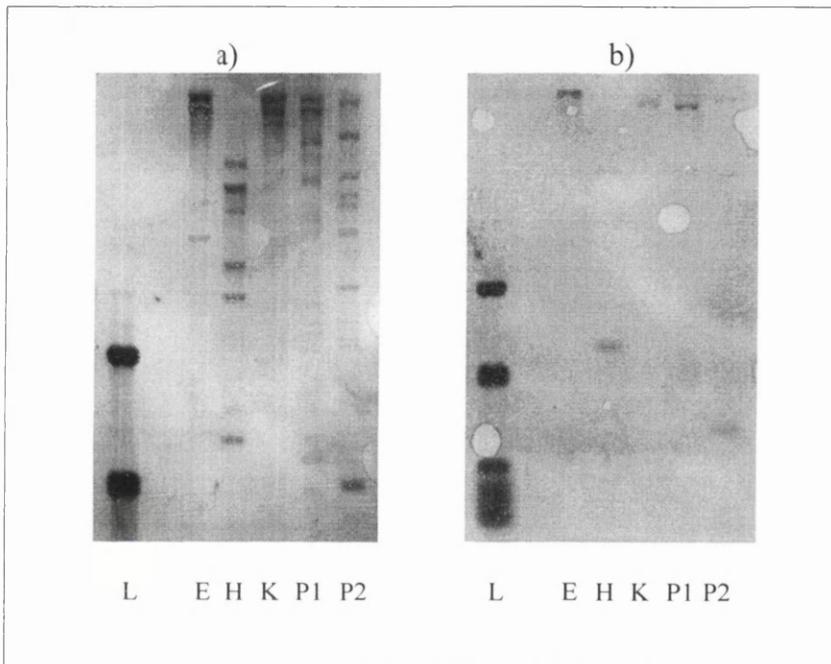


Figure 3.16 **Southern blots of *Drosophila melanogaster* DNA** probed with *vha16-1* cDNA (DIG) and washed at a) low stringency (2x SSC, 0.1% SDS, 25°C) and b) high stringency (0.1x SSC, 0.1% SDS, 65°C). Lane 1 corresponds to the Gibco BRL 1 kb ladder and the others correspond to 5 µg of genomic DNA cleaved for 5 hours with *EcoRI* (E), *HincII* (H), *KpnI* (K), *PstI* (P1) and *PvuII* (P2). Digests were run on a 1% agarose gel and blotted onto Hybond-N.

3.6 EST analysis

During the course of this project much sequence information about the *Drosophila melanogaster* genome has become known due to the work of the genome sequencing project. One of the most useful aspects of the BDGP has been the generation of ESTs (expressed sequence tags) which relate to transcribed sequences from the genome. Greater than 80 000 ESTs have been generated from a variety of cDNA libraries covering all *Drosophila* tissues and developmental stages.

An interesting observation is that the more highly expressed the gene, the more ESTs exist for that gene (although this is a generalisation). An extremely large number of ESTs (>120) exist for *vhal6-1* (appendix 5). An interesting feature, revealed when compiling pileup data for these ESTs, was that three main consensus sequences appeared. These were termed 1, 2 and 3 in an arbitrary order. These sequences were identical over their coding regions, and differed only in their 5' untranslated regions. When these regions were compared to the genomic sequence it was observed that they match different areas of the upstream coding region. One of these sequences, Consensus 2, matches the original *vhal6* exon 1 as described by Finbow (Finbow *et al.*, 1994; Meagher *et al.*, 1990). This was by far the most common upstream EST sequence (appendix 5). Figure 3.17 shows how the three consensus sequences are related to each other and to the genomic DNA sequence.

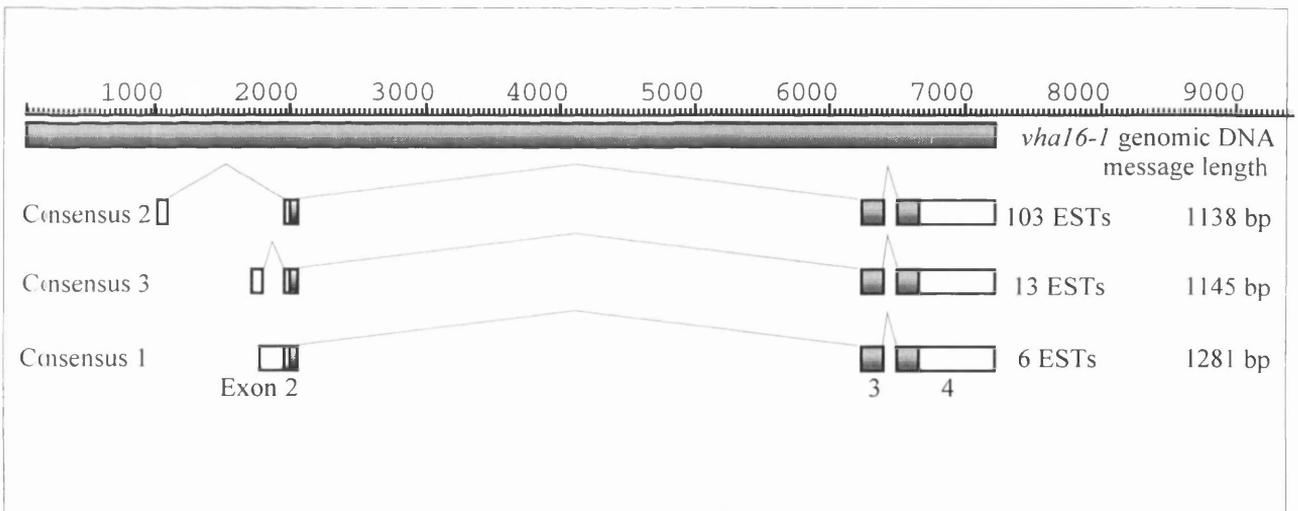


Figure 3.17 **Pileup of consensus sequences obtained from BDGP EST clone data.** Also included are exons two, three and four of the originally described *vhal6-1* gene and the genomic DNA sequence (DMCSDUC X77936). Sequences were aligned using Assemblylign. For the consensus sequences the white boxes correspond to untranslated regions and shaded boxes to coding regions. Message length does not include Poly A⁺ tails as the lengths of these are unknown.

The Northern blot data in figure 3.10 indicated a single transcript size of 1.8 kb. The transcript sizes in figure 3.17 are the minimal sizes expected before addition of poly A⁺ tail. Although the size observed in the Northern blot was 1.8 kb and the direct sequence size of the most common transcript is 1.16 kb, long poly A⁺ tracts are not uncommon. The Northern data given here shows a *vha16-1* transcript of 1.8 kb, however, the Northern data from (Finbow *et al.*, 1994) and (Goodwin, 1994) indicated a transcript size of 3.1 kb, but this is unlikely to be correct.

The two 'new' transcripts from the EST data do not appear on the total RNA blot but as all three are similar in size and the abundance of the other transcripts is much lower than the original, they are perhaps masked by the signal from the most common sequence. It is possible however that these transcripts are merely a result of mis-splicing of the gene.

The 5' sequences from these new transcripts could be used as probes in Northern blots, and/or *in situ* hybridisation to see where and when they are expressed.

3.7 Discussion

These experiments addressed the aims of isolating and analysing a lethal P-element insertion in the *vha16-1* gene, the proteolipid subunit of the vacuolar ATPase.

This involved screening, by Southern blotting with a genomic *vha16-1* probe, a series of rescued plasmids obtained from a random P-element ($P\{lacW\}$) mutagenesis of the second chromosome of *Drosophila melanogaster* by (Török, 1993). This mutagenesis had produced greater than two thousand lethal P-element inserted lines, 1900 of which had been successfully rescued by (Guo, 1996b). The plasmids had been collected into a series of pools which could lead to the isolation of a P-element insert within three steps.

In this study this screen isolated two separate plasmids, and therefore fly lines, which hybridised to the probe, therefore containing sequences corresponding to *vha16-1*. This however did not prove the P-elements were inserted within the gene of interest and so the plasmids were manually sequenced to determine their points of insertion. The initial mutagenesis screen called these fly lines 16/1 and 76/16, and these names were carried over in this study.

The sequence data showed that the P-element 16/1 was inserted within the *vha16-1* gene, in the large intron between exons two and three. Southern analysis with a range of restriction enzymes showed there was only one P-element present in the genome and Northern analysis showed that the amount of 1.8 kb transcript in these flies was greatly reduced. This implied that there was no upregulation of the balancer chromosome allele to compensate for the mutant allele.

Line 76/16, upon sequencing out of the P-element, gave no sequence homologous to that of *vha16-1*. The plasmid which had been rescued was extremely large and was presumed therefore to be inserted some distance up or downstream of the gene of interest and that the plasmid rescue had coincidentally carried the *vha16-1* gene sequences. After determining line 76/16 did not contain an insert in *vha16-1* it was not used in subsequent experiments. The exact point of insertion was determined much later when more data was made available at this locus. The element was seen to be inserted at the other side of the gene neighbouring *vha16-1*, called *trap1*.

The P-element used as the mutator in the mutagenesis by Török, was $P\{lacW\}$, which contains the *lacZ* reporter gene, under control of a minimal promoter. When inserted near an endogenous enhancer, the reporter gene is expressed in the same manner as the gene normally under control of that enhancer. This allows us to observe, via detection of the *lacZ* gene product (β -galactosidase), where and where the gene containing the P-element is normally expressed, both spatially and temporally. Line 16/1 was dissected and fixed, then the presence of β -galactosidase detected using the chromogenic substrate X-Gal. Results showed staining in several fly tissues such as Malpighian tubules, rectal pads, antennae, maxillary palps and

male and female sex organs. These are tissues known to contain highly V-ATPase energised membranes. This staining was compared to that of P-element insertions in two other V-ATPase subunits, *vha55* and *vha67*. The *vha55* line had staining in the same tissues as the 16/1 line. *vha55* has been shown to be single copy by (Davies, 1996), therefore has to be a part of every V-ATPase holoenzyme and therefore is likely to be expressed in every V-ATPase containing cell. The *vha67* line, however had staining in many of these tissues, except for the male sex organs, where no staining was observed. However, *vha67* is a multicopy gene and the role is presumably encoded by an alternative allele in this tissue.

However, the reporter gene expression results need confirming by *in situ* hybridisation as there may be other enhancers acting upon the reporter and influencing the β -galactosidase expression

The P-element insertion in *vha16-1* is embryonic lethal, unlike the insertions in *vha55* and *vha67*, where the P-element insertions are larval lethal and a clear Malpighian tubule phenotype is observed (Davies, 1996). Although Malpighian tubules are present in the late embryo, this phenotype cannot be observed with the 16/1 line as the embryos blacken and die too early. This difference in lethality could be explained by the fact that *vha16-1* undertakes multiple roles in the cell, therefore the maternal copies of the protein present in the embryo will run out earlier in embryogenesis than the other subunits.

Although the P-element was shown to be single copy in a Southern blot, this does not prove that it is indeed the P-element that causes this lethality, as another genetic accident could have occurred during the mutagenesis. Lethality due to the presence of a P-element can be proven in two ways, either by excision of the P-element to revert the sequence back to wild-type or re-insertion of the wild-type gene back into the genome by germline transformation.

Excision of the P-element by crossing line 16/1 to another fly line producing an exogenous source of transposase showed that it was indeed the interruption of the *vha16-1* gene by the P-element that caused the lethality and not some other genetic factor, as lethality was reverted upon excision. This reversion could be seen by the existence of straight-winged flies at the outcome of the excision cross i.e. the balancer chromosome (which carries the curly wing phenotype) was no longer required to keep the flies alive. PCR was performed using primers flanking the P-element insert site to show that the element had indeed excised, and that the wild-type sized PCR fragment was restored. Although it is still conceivable that the insertion may be in the control element of a distant gene it is extremely likely that the lethality was caused by interruption of *vha16-1*.

The PCR results also led to the observation that in two of the excision lines, the P-element had not excised cleanly, leaving behind fragments of DNA behind. As this P-element DNA could contain sequences which interfered with splicing of *vha16-1* it was determined whether the flies had an observable phenotype via fluid secretion assays. Homozygotes of line B had a higher basal rate of secretion than wild-type Oregon R, but did not have a significantly different CAP_{2b} stimulated rate. Line A while not having an observable tubule phenotype do not thrive as homozygotes, therefore may have a reproduction phenotype.

A developmental Northern was performed to observe the expression of the *vha16-1* gene through development. This showed that the gene is not developmentally regulated, with no downregulation of the transcript in the pupa, a result observed for V-ATPase subunits A and E (Guo, 1996b; Guo *et al.*, 1996b).

At the start of the project it was known that there was only one copy of the gene encoding the *Drosophila* V-ATPase proteolipid. Low stringency probing of a genomic Southern blot with the *vha16-1* coding region, however, gives rise to several bands, indicating the presence of a gene family. Therefore the gene in which the P-element is inserted, although essential, may not be the only gene encoding a 16 kDa protein of the V-ATPase. However, none of these copies of the gene (if they do indeed encode protein) can be able to compensate for the P-element disruption of *vha16-1*. This does not mean however that these genes are not themselves essential as they may have their own vital roles to play in the fly, perhaps in a particular cell type or developmental event.

The Berkeley *Drosophila* Genome Project has provided us with an enormous amount of *Drosophila* sequence data. Part of this project involved the sequencing of a great numbers of ESTs (expressed sequence tags) extracted from different developmental stages of the *Drosophila* life cycle. There exists a rough correlation between how highly a gene is expressed and the number of ESTs produced for that gene. A large number of EST clones (>120) exist on the database which are identical to the *vha16-1* mRNA. There are none, however, that correspond to sequences from any alternative genes.

An interesting feature arising from the EST data was that the majority of ESTs corresponded to the previously cloned *vha16-1* mRNA. This was originally thought to be the only transcript, as no other appear in a Northern blot, but the EST library shows us that other transcripts do exist, albeit at a much lower level, and it would be interesting to know where they are expressed.

These mRNAs only differ from the original in their 5' untranslated regions. There may be a physiological reason for this or they may merely be wrongly spliced products.

Chapter 4

Characterisation of New Members of the *vha16* Gene Family

4.1 Summary

This chapter describes the isolation and cloning of homologues of *vha16-1* in *Drosophila melanogaster*. It describes how these sequences were found on the Berkeley *Drosophila* Genome Project database and how they were cloned via PCR. It deduced the putative proteins encoded by these sequences and shows how similar they are to the original *Drosophila* protein, and protein sequences from a variety of organisms. RT-PCR indicates that these genes are expressed, despite the lack of corresponding EST clones.

4.2 Introduction

The Berkeley *Drosophila* Genome Project is a project that aims to sequence the *Drosophila melanogaster* genome by the sequencing of BAC clones and ESTs, (expressed sequence tags). These sequences are freely available on the database alongside any information about the sequence, e.g. homology to known genes. As the ESTs are derived from PolyA⁺ RNAs they are therefore almost always the products of transcribed genes, and if these are highly expressed genes then this is reflected in the number of EST clones that are available for that sequence.

Previously *vha16-1* was thought to be single copy within the *Drosophila* genome but in this study, low stringency Southern analysis (figure 3.8 and (Finbow *et al.*, 1994).) showed the likelihood of there being other members of a 16 kDa subunit gene family in *Drosophila*. When the BDGP started producing vast amount of sequence data from P1 and BAC clones, BLAST searching this database with *vha16-1* sequence isolated clones which are not identical but similar to *vha16-1*.

Some plant species contain more than one gene encoding this subunit (Hasenfratz *et al.*, 1995; Perera *et al.*, 1995) which are expressed in temporal and tissue specific manners. Therefore the presence of a gene family could help explain the multiple roles the subunit plays within eukaryotes, with the BDGP being an ideal way of beginning to search for homologues in *Drosophila*.

4.3 Isolation of homologues of *vha16*

4.3.1 Database searching

A search of BDGP sequences was performed using the mRNA sequence of the cloned *vha16-1* as the input sequence and initially one P1 clone (DS 03786, AC004438) was isolated which, in two places, was highly similar to this cDNA. This clone had a different chromosomal localisation (68C as opposed to 42B) from the previously cloned subunit. Subsequent searching isolated yet another P1 clone (DS 06306, AC004641), which had a different chromosomal location (53E). No ESTs were isolated in the search which would imply that any transcripts would not be abundant (unlike *vha16-1* which, in a search of the BDGP database, isolated a relatively large number of clones, see appendix 5). The sequences were named *vha16-2*, *vha16-3* (from DS 03786) and *vha16-4* (from DS 06306).

Specific primers were designed towards these P1 sequences (table 4.1). Sequences were amplified using a high fidelity *Taq* polymerase from genomic DNA and were cloned into the pCR[®]2.1 TA cloning vector. As these primers amplify products directly from a genomic DNA template they presumably exist as intronless genes within the genome. These sequences also appear to maintain the intron-exon boundaries of *vha16-1* as they can be directly translated into proteins which are homologous to the *vha16* protein (figure 4.3). The clones were sequenced to verify their identity using sequencing primers specific to the cloning vector (pCR[®]2.1 forward and reverse, appendix 4).

Upon publication of the entire *Drosophila* genomic sequence (Adams *et al* 2000) another putative proteolipid subunit was reported. It was termed *vha16-5* and was derived from sequence at chromosomal location 32A (AC007174, BACR19N18). Many of the results presented in this chapter do not include *vha16-5* as it was isolated too late in this project.

Gene	Location	P1/BAC clone	No. ESTs	% Identity to <i>vha16-1</i> protein
<i>vha16-1</i>	42B	BACR10F15	122	-
<i>vha16-2</i>	68C	DS03786	0	61
<i>vha16-3</i>	68C	DS03786	0	74
<i>vha16-4</i>	53E	DS06306	0	49
<i>vha16-5</i>	32A	BACR19N18	0	56

Table 4.1 Summary of data obtained by homology searching the BDGP with the *Drosophila vha16-1* protein. Sequences were isolated using a tBLASTn search using the *Drosophila vha16-1* protein (Genbank accession P23380) as input sequence.

4.3.2 Southern analysis of *vha16* DNAs

High stringency Southern analysis was performed to determine the copy number of the putative *vha16* sequences (figure 4.1). One band was observed in each lane indicating single identical copies of each sequence.

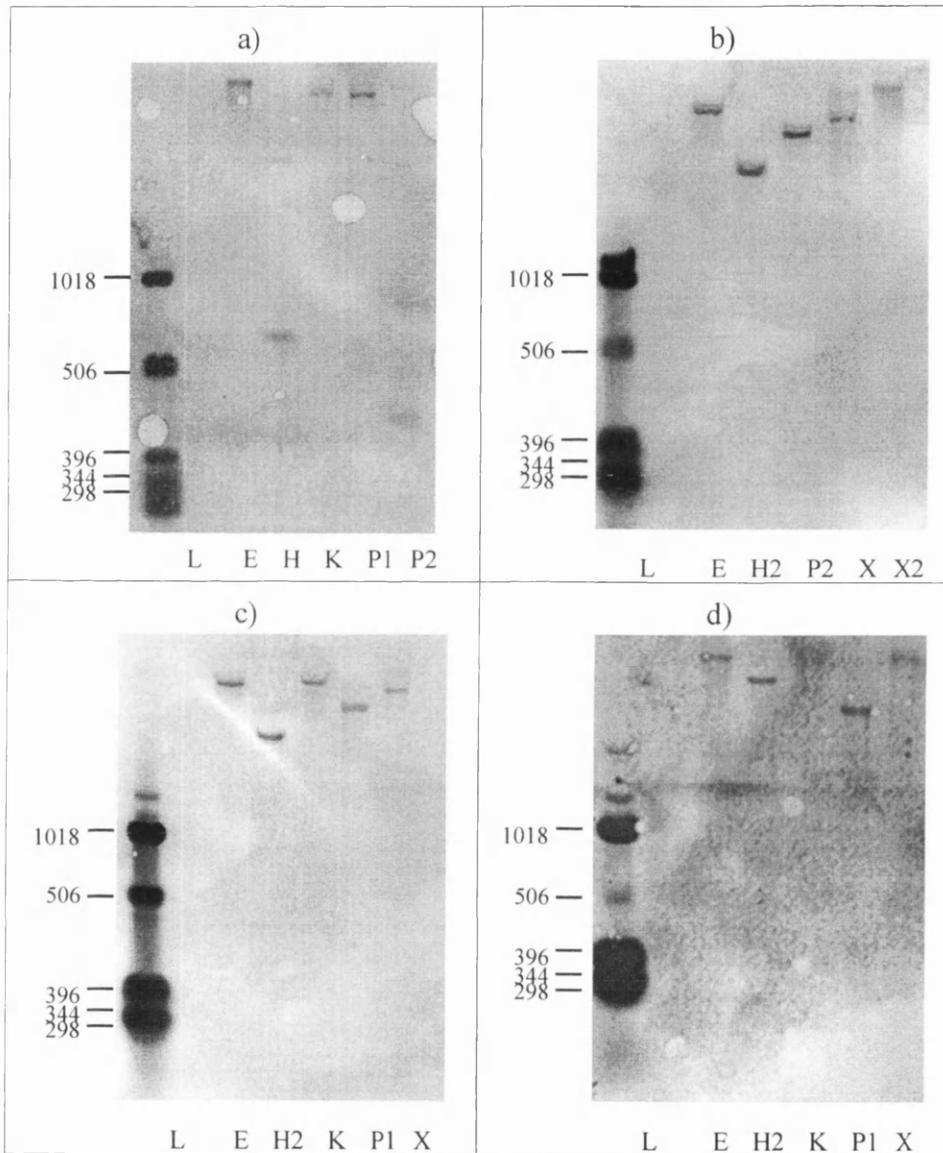


Figure 4.1 **High stringency Southern blots** showing single copies of a) *vha16-1*, b) *vha16-2*, c) *vha16-3*, d) *vha16-4*. 5 µg of genomic DNAs were digested with a selection of the following enzymes, *Eco*RI (E), *Hind*III (H), *Hinc*II (H2), *Kpn*I (K), *Pst*I (P1), *Pvu*II (P2), *Xba*I (X) or *Xho*I (X2). Digests were electrophoresed on a 1% agarose gel and blotted onto Hybond-N. Filters were then probed with the corresponding cDNA (DIG labelled), then washed to high stringency (0.1x SSC, 0.1% SDS, 65°C) before visualisation.

4.3.3 Cloning of three *vha16* homologues

PCR primers were designed to amplify the open reading frame encoding the putative 16 kDa proteins (figure 4.2). The primers were designed to amplify *vha16-2* and *-3* had added *EcoR1* sites to enable subsequent subcloning. PCR products were cloned into the PCR product cloning vector pCR2.1 and sequenced to confirm their identity.

vha16-2 F	GGG AAT TCA TGG TTA CTG CCG CTT TAA AT
vha16-2 R	GGG AAT TCT AAA GCT TAG TGT ACA AAT AGA
vha16-3 F	GGG AAT TCA TGG CAA CAG ATG CAG CTG ACA AG
vha16-3 R	GGG AAT TCT TAC TTG GTG TAC AGG TAA ATG GCG
vha16-4 F	TCG TTG GAT GAA CCG CAA TG
vha16-4 R	TAT GAG ACC ATA CAG ACC CA

Figure 4.2 **Primers used to amplify sequences similar to the published *vha16-1* cDNA.** Primers were designed against the sequences obtained from the BDGP. *vha16-2* and *-3* primers were designed with *EcoR1* restriction sites, in bold, to enable subsequent subcloning (see Chapter 5).

The deduced open reading frame sequences were translated into protein using the translation function of the MacVector programme (figure 4.3). This allowed us to compare the predicted protein structures against proteolipid subunits from a variety of species (figure 4.4) to observe whether they prospectively contain transmembrane regions and other features of the *vha16* protein such as the DCCD binding site.

a) *vhal6-2*

```

      10      20      30      40      50
ATGGTTACTGCCGCTTTAAATGAAGAGCCATCATACGCCTTCTTCTTGGG
TACCAATGACGGCGAAATTTACTTCTCGGTAGTATGCGGAAGAAGAACCC
M V T A A L N E E P S Y A F F L G
      60      70      80      90     100
ATGCACGGGGCTGCAGTTGCGATTATATTCACGACCTTGGGAGCGTCTT
TACGTGCCCCGACGTCAACGCTAATATAAAGTGCTGGAACCCCTCGCAGAA
C T G A A V A I I F T T L G A S Y
      110     120     130     140     150
ACGGAACAGCAGTTTCCGGTGTCCGAATCGCCAAGATGGCGGTCAATCGA
TGCCTTGTCTGTCAAAGGCCACAGCCTTAGCGGTCTTACCGCCAGTTAGCT
G T A V S G V G I A K M A V N R
      160     170     180     190     200
CCGGATATGATCATGAAGGCCATCATTCCGGTTCGTAATGGCGGGAATTAT
GGCCTATACTAGTACTTCCGGTAGTAAGGCCAGCATTACCGCCCTTAATA
P D M I M K A I I P V V M A G I I
      210     220     230     240     250
TGCATCTACGGATTGGTGGTATCCGTCTGATAGCCGGATCGATTGGCG
ACGCTAGATGCCTAACCACCATAGGCAGGACTATCGGCCTAGCTAACCGC
A I Y G L V V S V L I A G S I G D
      260     270     280     290     300
ATGATTATACGATGGAAGACAGTTATGTGCATCTGGGCGCCGGATTGTCC
TACTAATATGCTACCTTCTGTCAATACACGTAGACCCGCGGCCTAACAGS
D Y T M E D S Y V H L G A G L S
      310     320     330     340     350
GTCGGACTTCCAGGACTCACCGCAGGAGTAGCCATTGGAATCGCAGGCGA
CAGCCTGAAGGTCTGAGTGGCGTCCTCATCGGTAACCTTAGCGTCCGCT
V G L P G L T A G V A I G I A G D
      360     370     380     390     400
TGCTGGAGTCCGGGGTACCGCCGAACAGCCACGCCTTTTCGTGGGCATGG
ACGACCTCAGGCCCCATGGCGGCTTGTCCGGTGCGGAAAAGCACCCGTACC
A G V R G T A E Q P R L F V G M V
      410     420     430     440     450
TCCTGATCCTGATCTTCCGCCGAGGTCTGGCTCTATACGGCCTCATTGTG
AGGACTAGGACTAGAAGCGGCTCCAGGACCGAGATATGCCGGAGTAACAC
L I L I F A E V L A L Y G L I V
      460     470
GCCATCTATTTGTACACTAAGCTTTAG
CGGTAGATAAACATGTGATTCGAAATC
A I Y L Y T K L *

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b) *vhal6-3*

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      10          20          30          40          50
ATGGCAACAGATGCAGCTGACAAGGATAAGCCGGCGTACTCGTTCTTCTT
TACCGTTGTCTACGTGCGACTGTTCCCTATTTCGGCCGCATGAGCAAGAAGAA
M A T D A A D K D K P A Y S F F F
      60          70          80          90         100
TGGTTCGATGGGAGCGGCATCGGCCATCATCTTTTCAGCCTTGGGAGCGG
ACCAAGCTACCCTCGCCGTAGCCGGTAGTAGAAAAGTCGGAACCCCTCGCC
G S M G A A S A I I F S A L G A A
     110         120         130         140         150
CATATGGAACGGCCAAGTCGGGCACTGGGATTGCAGCATGGCCGTAATG
GTATACCTTGCCGGTTCAGCCCGTGACCCTAACGTCGGTACCGGCATTAC
Y G T A K S G T G I A A M A V M
     160         170         180         190         200
CGACCCGAATTGATCATGAAATCGATTATTCCCGTTGTGATGGCTGGTAT
GCTGGGCTTAAGTAGTACTTTAGCTAATAAGGGCAACACTACCGACCATA
R P E L I M K S I I P V V M A G I
     210         220         230         240         250
CATTGCGATCTATGGCCTGGTGGTCTCCGTTCTGATTGCCGGATCTCTGT
GTAACGCTAGATAACCGGACCACCAGAGGCAAGACTAACGGCCCTAGAGACA
I A I Y G L V S V L I A G S L S
     260         270         280         290         300
CGGATTTCGTATAACCATTTCGAAGGGATACATCCACCTGGCAGCCGGATTA
GCCTAAGCATATGGTAAGCGTTCCTATGTAGGTGGACCGTCGGCCTAAT
D S Y T I R K G Y I H L A A G L
     310         320         330         340         350
TCGGTGGGTTTCGCCGGATTGGCGGCTGGATTGCCATTGGGATTGTGGG
AGCCACCCAAAGCGGCCTAACCGCCGACCTAAACGGTAACCCTAACACCC
S V G F A G L A A G F A I G I V G
     360         370         380         390         400
TGATGCCGGAGTGCGGGGCACTGCCCAGCAGCCACGCCTTTTGTGGGCA
ACTACGGCCTCACGCCCCGTGACGGGTGTCGGTTCGGGAAAAACACCCGT
D A G V R G T A Q Q P R L F V G M
     410         420         430         440         450
TGATCCTGATCCTGATCTTCGCCGAGGTAAGGTTCTGTATGGGCTGATC
ACTAGGACTAGGACTAGAAGCGGCTCCATGACCCAGACATACCCGACTAG
I L I L I F A E V L G L Y G L I
     460         470
GTCGCCATTTACCTGTACACCAAGTAA
CAGCGGTAAATGGACATGTGGTTCATT
V A I Y L Y T K *

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c) *vha16-4*

```

      10          20          30          40          50
ATGGAGCTCTCGTTGGATGAACCGCAATGCGCATCCTTCTTCTGCATCCT
TACCTCGAGAGCAACCTACTTGGCGTTACGCGTAGGAAGAAGACGTAGGA
M E L S L D E P Q C A S F F C I L
      60          70          80          90         100
GGGTGCCGTGTGCGCCATTGTCTTTTCGACATTGGGCGCCGCCTATGGAA
CCCACGGCACACGCGGTAACAGAAAAGCTGTAACCCGCGGGGATAACCTT
G A V C A I V F S T L G A A Y G T
     110         120         130         140         150
CAGCAAAGGCTTCTGTGGGAATTTCTTCGATGTCAATCAAGCATCCGCAG
GTCGTTTCCGAAGACACCCTTAAAGAAGCTACAGTTAGTTCGTAGCGCTC
A K A S V G I S S M S I K H P Q
     160         170         180         190         200
CTGATCATGAAGGCGATTGTTCCAGTGGTTATGGCTGGCATTATAGCCAT
GACTAGTACTTCCGCTAACAAGGTCACCAATACCGACCGTAATATCGGTA
L I M K A I V P V V M A G I I A I
     210         220         230         240         250
TTATGGACTGGTGATCGCGGTCCTGCTTGCTGGATCACTTAGCAGCCCCT
AATACCTGACCACTAGCGCCAGGACGAACGACCTAGTGAATCGTCGGGGA
Y G L V I A V L L A G S L S P Y
     260         270         280         290         300
ATAGCGCCTACAAGGGTTTCCTAAACCTCAGTGCTGGACTGGCGGTGGGA
TATCGCGGATGTTCCCAAAGGATTTGGAGTCACGACCTGACCGCCACCCT
S A Y K G F L N L S A G L A V G
     310         320         330         340         350
GTCTCTGGGATGGGGGCTGGAATTGCTATTGGCGTGGTGGGAGAAGCTGG
CAGAGACCCTACCCCGACCTTAACGATAACCGCACCACCCTCTTCGACC
V S G M G A G I A I G V V G E A G
     360         370         380         390         400
AGTCCGTGCATCTGCCAGCAGCCAAAACCTCTTTGTGGCCATCATTTTAA
TCAGGCACGTAGACGGGTCGTCGGTTTTGAGAAACACCGGTAGTAAAATT
V R A S A Q Q P K L F V A I I L I
     410         420         430         440         450
TATTGATATTTGCCGAGGTCTTGGGTCTGTATGGTCTCATAGTGGCCATT
ATAACTATAAACGGCTCCAGAACCCAGACATACCAGAGTATCACCGGTAA
L I F A E V L G L Y G L I V A I
     460
TATTTGTTTTTCCAAG
ATAAACAAAAGGTTT
Y L F S K
```

d) *vha16-5*

```

          10          20          30          40          50
ATGGAATGGATTACTATCTGCCAGTGATAACGGATCAAATTCGGTGGA
TACCTTTACCTAATGATAGACGGTCACATATTGCC TAGTTTAA GCCACCT
M E M D Y Y L P V Y N G S N S V D
          60          70          80          90         100
CGTGCCGGGGCTGATTGAAAGTCTCAAGCTGAAGGAAACCAGTATATCGC
GCACGGCCCCGACTAACTTTCAGAGTTCGACTTCCTTTGGTCATATAGCG
V P G L I E S L K L K E T S I S P
          110         120         130         140         150
CAATCGTGCTGGATCGCTATCCGCCGTACTCCCCGTCTATGGCGTGATG
GTTAGCACGACCTAGCGATAGGCGGCATGAGGGGCAAGATACCGCACTAC
I V L D R Y P P Y S P F Y G V M
          160         170         180         190         200
GGCGTCGTATTCTCCAGTGTGCTGACCTCCGCTGGAGCGGCCTACGGAAC
CCGCAGCATAAGAGGTCACACGACTGGAGGCGACCTCGCCGGATGCCTTG
G V V F S S V L T S A G A A Y G T
          210         220         230         240         250
TGCTGTTTCCGGAAC TGAATCGCCGCCACAGCGGTGATGCGTCCCGAGC
ACGACAAAGGCCTTGACCTTAGCGGCGGTGTCGCCACTACGCAGGGCTCG
A V S G T G I A A T A V M R P E L
          260         270         280         290         300
TGGTGATGAAGTCCATCATTCCGGTGGTCATGGCGGGCATCATTGCCATA
ACCACTACTTCAGGTAGTAAGGCCACCAGTACCGCCCGTAGTAACGGTAT
V M K S I I P V V M A G I I A I
          310         320         330         340         350
TACGGTCTGGTGGTATCCGTGCTCCTATCTGGGGAACTAGCGCCTGCTCC
ATGCCAGACCACCATAGGCACGAGGATAGACCCCTTGATCGCGGACGAGG
Y G L V V S V L L S G E L A P A P
          360         370         380         390         400
CAAATACTCGCTGCCACC GGCTATGTACACCTGGCCGCTGGACTTTTCGG
GTTTATGAGCGACGGGTGGCCGATACATGTGGACCGGCGACCTGAAAGCC
K Y S L P T G Y V H L A A G L S V
          410         420         430         440         450
TGGGATTTGCTGGACTGGCTGCCGGATATGCAGTGGGTGAAGTCGGCGAA
ACCCTAAACGACCTGACCGACGGCCTATACGTACCCACTTCAGCCGCTT
G F A G L A A G Y A V G E V G E
          460         470         480         490         500
GTGGGCGTGCGGCACATTGCCCTGCAGCCACGCCTCTTTATCGGCATGAT
CACCCGCACGCCGTGTAACGGGACGTCCGTTGCGGAGAAAATAGCCGTA
V G V R H I A L Q P R L F I G M I
          510         520         530         540         550
CCTGATCCCTTATTTTCGCCCGAGGTGCTCGGTCTCTACGGCCTGATCATTG
GGACTAGGAATAAAAAGCGGCTCCACGAGCCAGAGATGCCGGACTAGTAAC
L I L I F A E V L G L Y G L I I G
          560         570         580
GGATTTATTTGTACACGGTCAACATTAAGTAG
CCTAAATAAACATGTGCCAGTTGTAATTCATC
I Y L Y T V N I K *

```

Figure 4.3. Nucleotide sequences and deduced amino acid sequences obtained from the new *vha16* DNAs. Shown are the sequences of the predicted cDNAs a)-d) of the new members of the *vha16* gene family. Numbers represent the number of bases in the cDNAs with the DNA sequences being shown in plain type. Plus strands of these sequences were translated with the translation function of MacVector and the deduced protein sequences are shown in bold. Primers used to amplify these sequences by PCR are shown in italics.

The deduced proteins, alongside a selection of other *vha16* proteins from a variety of organisms were aligned by ClustalWPPC (set on default parameters) and viewed in SeqVu (figure 4.4), with shaded and boxed residues corresponding to amino acid identity. Alignments show that these proteins show a high degree of identity to the sequences already known, with *vha16-3* showing the highest degree of identity to the original *Drosophila* protein (74%). *Vha16-2*, -3, -4 and -5 also all contain the conserved DCCD reactive site.

However it is unknown whether functional protein would be produced from these DNAs, particularly in the case of *vha16-2* that has a proline at position 104 which, at the beginning of transmembrane helix three is thought to be at a position where the amino acid present has to be small (Malcolm Finbow, personal communication). Proline, which has a bulky five-membered ring is thought to be too large. This idea comes from protein modelling experiments and is at present hypothetical.

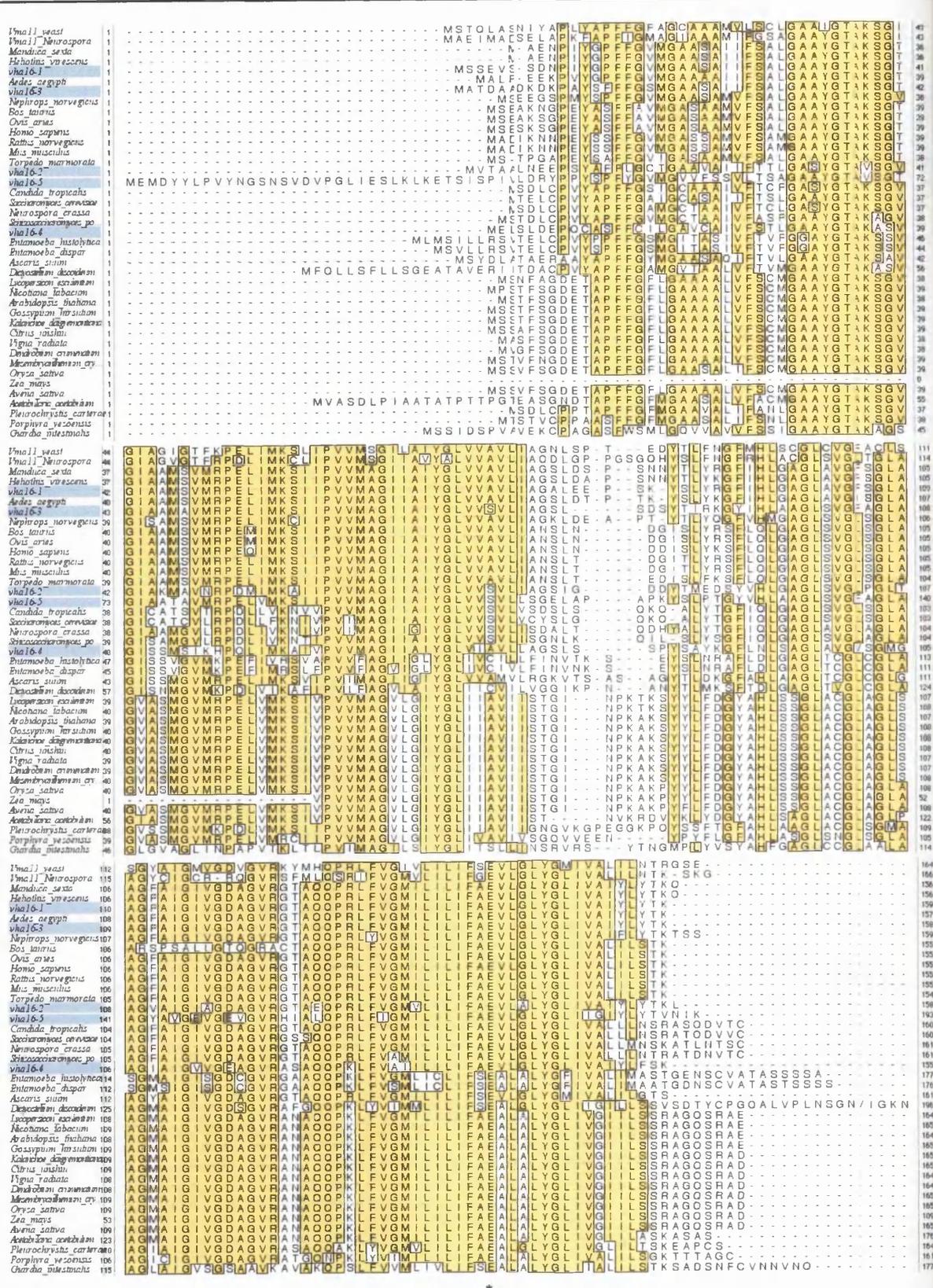


Figure 4.4 Pile-up of several 16 kDa V-ATPase subunits (and vma11) from a variety of species. Sequences were aligned using ClustalwPPC and viewed with SeqVu. Shaded and boxed residues correspond to identity. *Drosophila* sequences are highlighted. The asterisk corresponds to the inferred conserved DCCD reactive site.

The ClustalW alignment shows that predicted protein sequences are conserved at the amino acid level. They were then subjected to hydropathy analysis to observe whether the putative membrane spanning domains are disrupted (Kyte and Doolittle, 1982).

Kyte-Doolittle hydrophobicity plots (figure 4.5), when compared to the original vha16-1 protein show that the membrane spanning domains (which, in order to span the membrane have to contain around twenty hydrophobic amino acids), remain intact, therefore the proteins can potentially form membrane spanning domains.

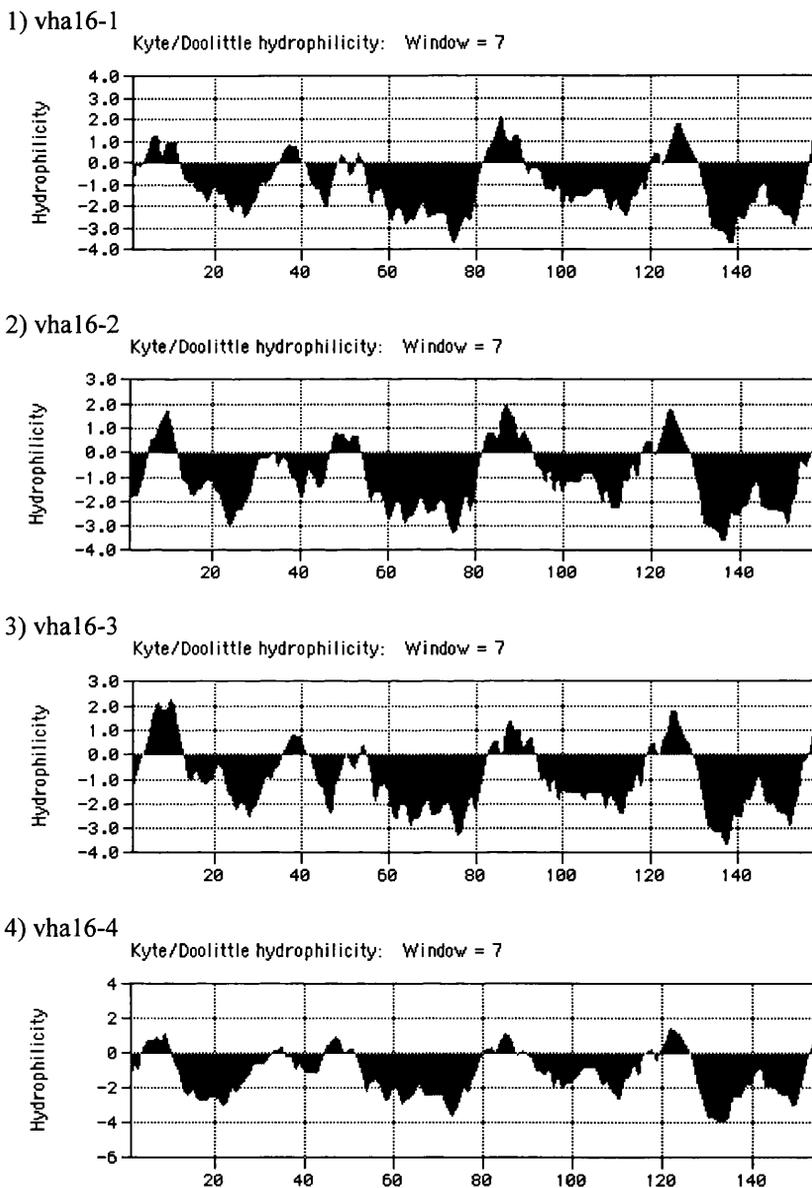


Figure 4.5 **Hydrophobicity analysis of vha16-1, -2, -3 and -4.** Shown are Hydropathy plots predicted for each of the proteins (1-4) by MacVector using the Kyte-Doolittle hydrophilicity algorithm. Values above the axis denote hydrophilic regions, while values below the axis indicate hydrophobic regions, which are likely to be membrane spanning if the region contains around twenty amino acids.

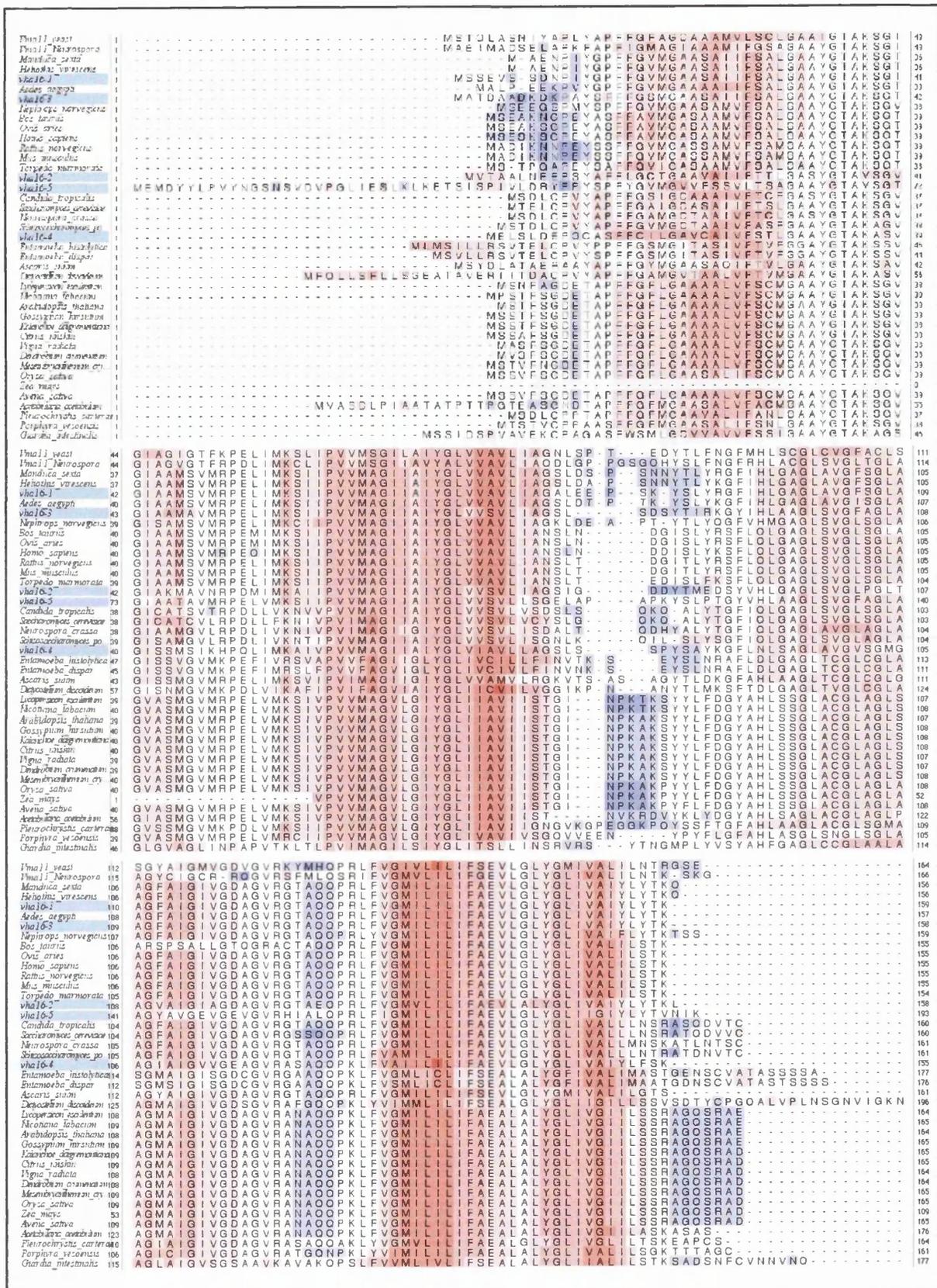


Figure 4.6 Pile-up of several 16 kDa V-ATPase subunits (and vma11) from a variety of species. Sequences were aligned by ClustalwPPC and viewed with SeqVu. Red residues are most hydrophobic, pink less so. Purple residues are most hydrophilic, with lilac less hydrophilic. *Drosophila* sequences are highlighted.

Figure 4.6 shows a hydropathy view of the ClustalW alignment viewed in SeqVu. This shows a different outlook on the conservation of the hydrophobic domains. The dark red residues are the most hydrophobic, with the different shades going down to pink indicating a lesser degree of hydrophobicity. The purple residues are most hydrophilic, with the lighter shades being less so. This view allows us to observe the membrane spanning domains when all the sequences are aligned together. The new proteins still maintain the hydrophobic sequences which can form the four membrane spanning domains.

In an unrooted bootstrap tree plot of these sequences (figure 4.7), the sequences fall into distinct groups depending on the type of organism from which they were cloned. With the exception of *M. crystallinum*, the plant sequences (31-40) fall into two main subgroups, distinct from the other sequences. *vha16-3* clusters alongside *vha16-1* and other insect subunits (11-14) as well as the crustacean *Nephrops norvegicus*. Interestingly, in this plot, *vha16-5* is in a cluster with the *vma11* proteolipid proteins (6-7), *vma11* is also termed c'. The function of c' is uncertain, but critical in *S. cerevisiae*, and is thought to contribute to one subunit of the proteolipid hexamer. *vha16-5* has an N-terminus extension which is not seen in the other c subunits (figure 4.4). *vha16-2* does not fall into any obvious group but is of equal distance from the insect subgroup and the mammalian subgroup. From the plot, *vha16-4* branches earlier than the other *Drosophila* sequences so is less similar.

As these sequences are so similar it is impossible to define a suitable outgroup with which to root the tree. This means the plot can only infer the similarity between the individual proteins and cannot give any evolutionary divergence data.

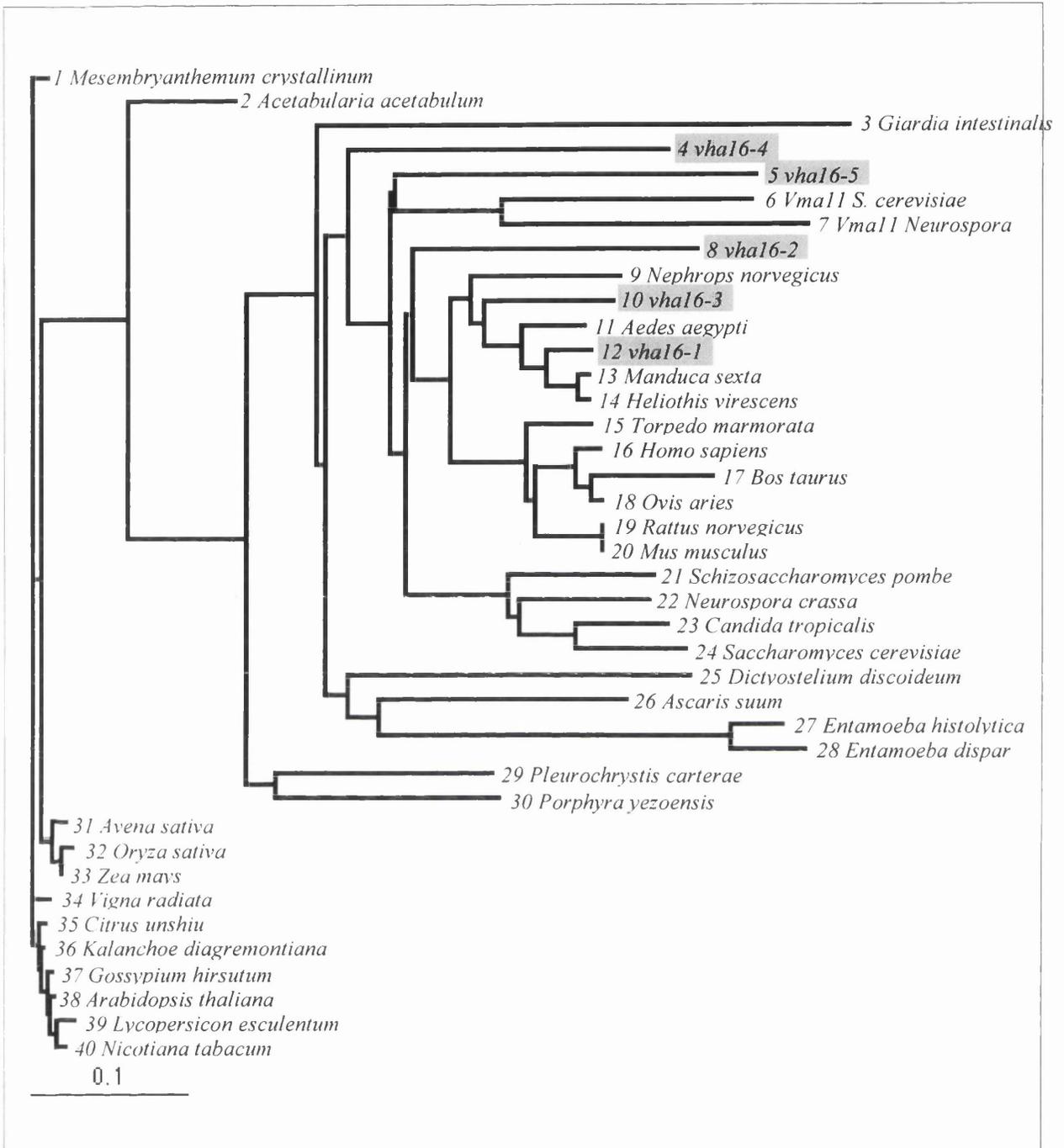


Figure 4.7 **Bootstrap analysis of V-ATPase proteolipid sequences.** Sequences were aligned using ClustalwPPC using default parameters and viewed with TreeView, *Drosophila* proteins highlighted. This tree is unrooted as defining an outgroup is not possible with the proteins being so similar. Genbank accession numbers and references for each protein sequence are as follows:

- 6 VMA11 *Neurospora crassa* AAD45120
- 7 VMA11 *Saccharomyces cerevisiae* BAA01367 (Umemoto *et al*, 1991)
- 1 *Mesembryanthemum crystallinum* Q39437 VATL_MESCR (Tsiantis *et al*, 1996)
- 2 *Acetabularia acetabulum* BAA21683 *Acetabularia acetabulum* (Ikeda, 1997)
- 3 *Giardia intestinalis* AAC06133 (Hilario, 1998)
- 9 *Nephrops norvegicus* Q26250 VATL_NEPNO (Finbow *et al*, 1992)
- 11 *Aedes aegypti* O16110 VATL_AEDAE (Gill, 1998)
- 12 *Drosophila melanogaster (vha16-1)* P23380 VATL_DROME (Meagher *et al*, 1990)
- 13 *Manduca sexta* P31403 VATL_MANSE (Dow *et al*, 1992)
- 14 *Heliothis virescens* P55277 VATL_HELVI (Pietrantonio and Gill, 1993)
- 15 *Torpedo marmorata* Q03105 VATL_TORMA (Birman *et al*, 1990)
- 16 *Homo sapiens* P27449 VATL_HUMAN (Gillespie, 1991)
- 17 *Bos taurus* P23956 VATL_BOVIN (Mandel, 1988)
- 18 *Ovis aries* O18882 VATL_SHEEP
- 19 *Rattus norvegicus* JX0226 VATL_RAT (Nezu, 1992)
- 20 *Mus musculus* P23967 VATL_MOUSE (Hanada *et al*, 1991)
- 21 *Schizosaccharomyces pombe* P50515 VATL_SCHPO (Toyama, 1991)
- 22 *Neurospora crassa* P31413 VATL_NEUCR (Bowman, 1992)
- 23 *Candida tropicalis* Q00607 VATL_CANTR
- 14 *Saccharomyces cerevisiae* P25515 VATL_YEAST (Nelson and Nelson, 1989)
- 25 *Dictyostelium discoideum* P54642 VATL_DICDI (Xie *et al*, 1996)
- 26 *Ascaris suum* Q17046 VATL_ASCSU
- 27 *Entamoeba histolytica* Q24810 VATL_ENTHI (Descoteaux, 1994)
- 28 *Entamoeba dispar* Q24808 VATL_ENTDI (Descoteaux, 1994)
- 29 *Pleurochrystis carterae* Q43362 VATL_PLECA
- 30 *Porphyra yezoensis* BAA87945 (Kitade, 1999)
- 31 *Avena sativa* (oat) P23957 VATL_AVESA (Lai *et al*, 1991)
- 32 *Oryza sativa* (rice) Q40635 VATL_ORYSA
- 33 *Zea mays* (maize) Q41773 VATL_MAIZE (Vierek, 1996)
- 34 *Vigna radiata* (mung bean) O22552 VATL_PHAAU
- 35 *Citrus unshiu* BAA89594 (in press)
- 36 *Kalanchoe diargremontiana* Q96473 VATL_KALDA (Bartholomew, 1996)
- 37 *Gossypium hirsutum* (wheat) Q43434 VATL_GOSHI (Hasenfratz *et al*, 1995)
- 38 *Arabidopsis thaliana* Q39039 VATL_ARATH (Perera *et al*, 1995)
- 39 *Lycopersicon esculentum* O24011 VATL_LYCES
- 40 *Nicotiana tabacum* (tobacco) Q40585 VATL_TOBAC

4.4 Transcription of the Homologues

4.4.1 RT-PCR

The next question to be answered was whether these 'genes' were indeed transcribed or were merely silent sequences. This was initially determined by RT-PCR, which amplifies mRNA sequences which have been reverse transcribed into DNA. If a PCR product is observed then mRNA is indeed transcribed, this mRNA however is not necessarily translated into protein. Flies were dissected into five tissues to determine whether differential expression is observed within these tissues.

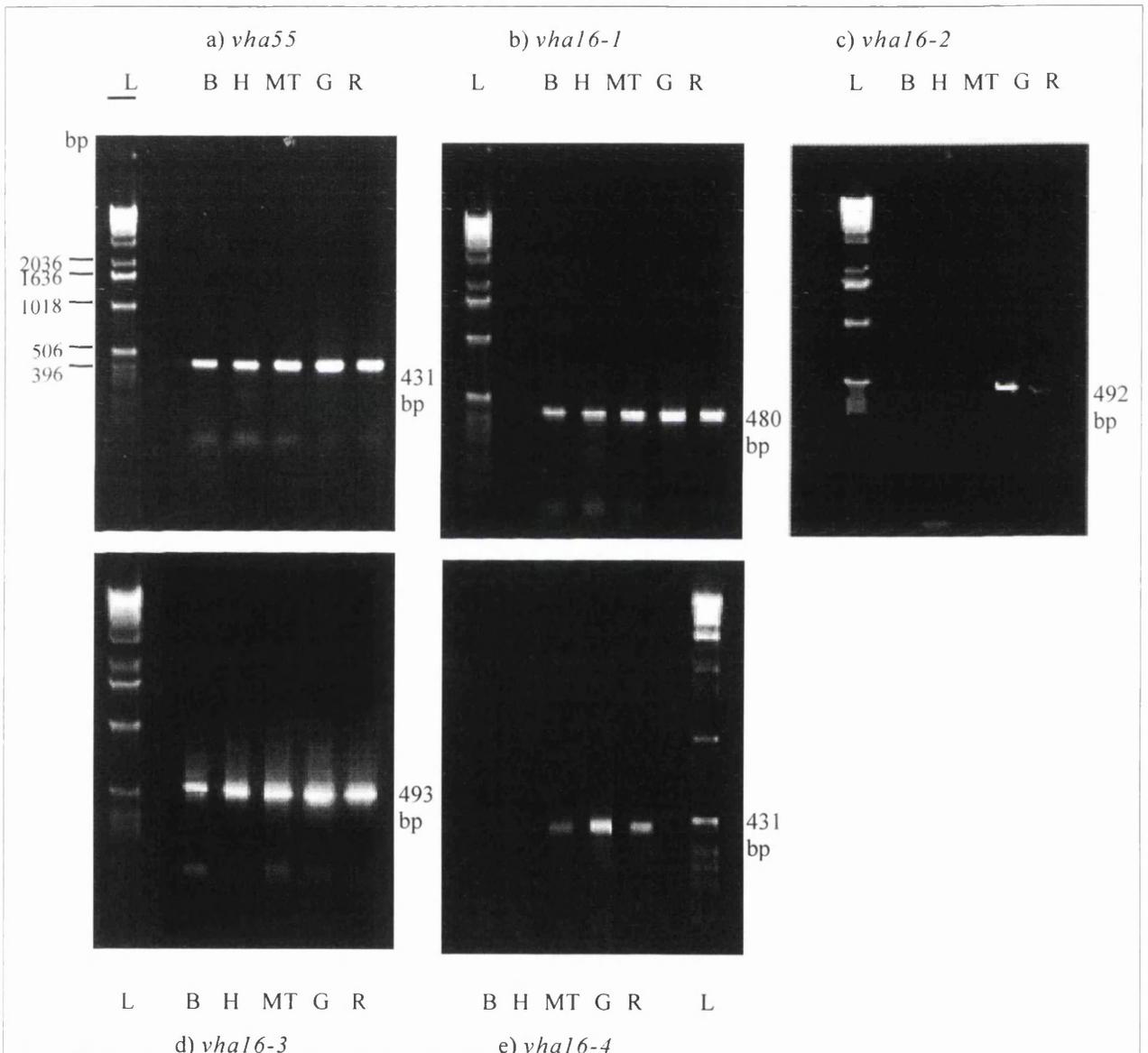


Figure 4.8 **Reverse transcriptase polymerase chain reaction (RT-PCR)** of the same *Drosophila* tissue template cDNA from 1) brain 2) head 3) Malpighian tubule 4) gut 5) remainder. Primers specific for *vha55*, *16-1*, *16-2*, *16-3* and *16-4* (see figure 4.1 for primer sequences for *16-2*, *16-3* and *16-4*, figure 5.3 for *vha16* and appendix 4 for *vha55*) correspond to pictures a, b, c, d and e), and show differential expression

in these tissues (L= Gibco BRL 1 kb ladder). A *vha55* control PCR was performed with primers bracketing an intron, as *vha16-2* to *-4* are intronless. No genomic band was observed (1.2 kb) indicating no genomic contamination.

The RT-PCRs (figure 4.8) show *vha16-1* and *-3* are expressed in all these tissues. *vha16-2* has expression in the gut lane. *vha16-4* is also expressed in gut, Malpighian tubules and the remainder tissue, which contains the carcass and the male and female gonads. These experiments show that mRNA is transcribed from these genes, but it is not yet known whether these mRNAs are translated into protein.

However, despite there being no band in the *vha55* control, the gel was not Southern blotted so the possibility exists that there may still be some genomic contamination which the *vha16-2*, *-3*, and *-4* primers may be priming from. As these primers do not bracket an intron they cannot be internally controlled for genomic contamination. These experiments would have to be repeated with much more stringent controls to rule out genomic contamination. Such controls could be either: no superscript, which means no cDNA is synthesised, therefore only genomic contaminants can be amplified, or DNase treatment of the RNA samples prior to reverse transcription to degrade any DNA present.

4.4.2 Northern analysis

Northern analysis of PolyA⁺ RNA extracted from *Drosophila* head and body resulted in no bands being observed for *vha16-2* and *-3*, even when 8 µg of PolyA⁺ RNA was probed. It may be that these genes are transcribed at very low levels in particular tissues and/or developmental stages.

4.5 Discussion

This chapter describes the discovery of four previously undescribed homologues of the 16 kDa V-ATPase proteolipid pore gene. The new sequences are seen to exist as cDNA copies within the genomic DNA and contain no intronic sequences which is unusual, especially when compared to *vha16-1*.

Reverse transcriptase PCR studies indicated that mRNA is produced by these sequences in different tissues throughout the fly, although Northern blots did not isolate any transcripts for these homologues. It may be that they produce levels of mRNA only detectable by RT-PCR, a more sensitive technique, which can detect. It has also been mentioned in the previous chapter that no ESTs exist for any of these sequences, but if they are only expressed in a few cells, or at critical developmental time points then this is unsurprising.

The sequences lying 5' and 3' to these DNA will also have to be examined to see if they contain the sequences necessarily for transcription such as promoter, enhancer and polyadenylation sequences.

We also need to ask if these transcripts encode protein and if they do will they form a functional pore, either on their own or in combination with the originally described subunit. It is possible that they may form a specific type of V-ATPase pore in a certain site in a particular cell type. Unfortunately there are no genomic aberrations at these sites so we do not know if deletion of these sequences are lethal.

Multiple copies and transcripts of subunit c exist in plants (Lai *et al.*, 1991; Lehr, 1999; Perera *et al.*, 1995; Tsiantis *et al.*, 1996). These isoforms are regulated in response to environmental, such as salt stress (Tsianitis *et al.*, 1996), and developmental stimuli (Vierek, 1996). There are also reports of expression of alternative isoforms in different plant tissues (Hasenfratz *et al.*, 1995; Lehr, 1999). The presence of multiple functional isoforms of the proteolipid in non-plant species has not been described. There is a report in human (Hasebe, 1992), but only one isoform is thought to be functional and the others merely pseudogenes. In *C. elegans* (Oka, 1998) there are three genes, expressed in cell specific manners, and one of these, *vha3*, is intronless (Oka, 1998).

Pseudogenes in *Drosophila* are uncommon but reports do exist. The pseudogenes in most of these cases, however, tend to be nonsense proteins, containing stop codons and/or deletions, e.g. a cecropin pseudogene, (Ramos-Onsins, 1998) and a gene at the lcp (larval cuticle protein) cluster (Pritchard, 1997). Only one report mentions a 'cryptic' pseudogene which putatively encodes an esterase (Balakirev, 1996).

Determining whether these genes encode protein would involve their introduction into a protein expression system and if protein was indeed synthesised, antibodies

could be generated against them to observe their cellular location. To observe whether they could substitute for *vha16-1* they could be transgenically expressed in a *vha16-1* null background This second strategy is discussed in Chapter 5.

Chapter 5

Rescue of P-element Mutant Phenotype

5.1 Summary

This chapter describes the attempted rescue of a mutant phenotype caused by a P-element insertion in *vhal6-1*, by introducing the wild-type *vhal6-1* cDNA back into the mutant background. Rescue of this phenotype was also attempted with cDNAs of *vhal6-2*, *vhal6-3*, *Nephrops norvegicus* and *Manduca sexta*. A *vhal6-1/GFP* construct was also made to observe where the protein targets in the cell. The chapter describes the cloning of sequences, synthesis of the constructs and how rescue can be carried out. Also included are results of expression of *vhal6-1*, *-2*, *-3* and *-4* in the *S. cerevisiae vma3⁻* mutant.

5.2 Introduction

We assume from Chapter 3 that lethality in the fly line 16/1 is a result of a P-element insertion in the *vhal6-1* gene, because P-element excision reverts this lethality. However, this falls short of demonstrating that lethality is indeed caused by disruption of *vhal6-1* as it remains possible that the insertion's lethality is due to disruption of another gene nearby. One way to test this is to attempt rescue by germline transformation with the *vhal6-1* cDNA.

In a similar manner it would also be of value to introduce the newly discovered homologues of *vhal6* into the mutant background as this would tell us whether they could substitute for the major gene *in vivo*. This would provide an interesting *in vivo* test of concepts of gene redundancy and compensatory gene networks.

The 16 kDa proteolipid is an incredibly well conserved membrane protein in all species in which it has been studied (figure 4.4). It has been observed that a proteolipid deficient mutant in *Saccharomyces cerevisiae* (*vma3⁻*) can be rescued by the introduction of a cDNA from entirely different species, the crustacean *Nephrops norvegicus* (Harrison *et al.*, 1994), and also by the *Drosophila* cDNA (Finbow *et al.*, 1994). In this system, however, the *vhal6-1* cDNA only partially rescues the yeast mutation.

With these data in mind, it was decided to attempt rescue with cDNAs from alternate species. The crustacean *Nephrops norvegicus* was selected as it, like *Drosophila*, is an arthropod and also chosen was the sequence from another insect, the moth *Manduca sexta*.

GFP (green fluorescent protein) has been engineered with the *Drosophila melanogaster* codon preference in mind and an attempt was also made to tag this protein on the C-terminus of the *vhal6-1* coding region to observe where the protein targets to in the cell.

The *GAL4/UAS* transposon mediated method was chosen to re-introduce sequences back into the P-element mutant background. In this system, the sequences are transformed into flies under control of the yeast *UAS* promoter, which does not allow transcription of the transgene until the flies are crossed to lines containing a *GAL4* expressing element under control of an endogenous enhancer. Knowledge of in which cells these enhancers act can allow us to select where the transgene is transcribed.

5.3 Plasmid construction

5.3.1 Transformation vector

The transformation vector $pP\{UAST\}$ (figure 5.1 (Brand and Perrimon, 1993)) was used to introduce the cDNA sequences into the *Drosophila* germline. This vector contains a multiple cloning site, of which the *EcoRI* site was chosen to clone the sequences. The construct has five tandemly arranged GAL4 binding sites (*UAS* -upstream activation sequence), followed by a *Hsp70Bb* promoter, the multiple cloning site and the SV40 polyadenylation signal.

The construct is itself incapable of promoting transposition but can easily transpose into the host genome with the aid of a helper plasmid $pP\{\Delta 2-3\}$.

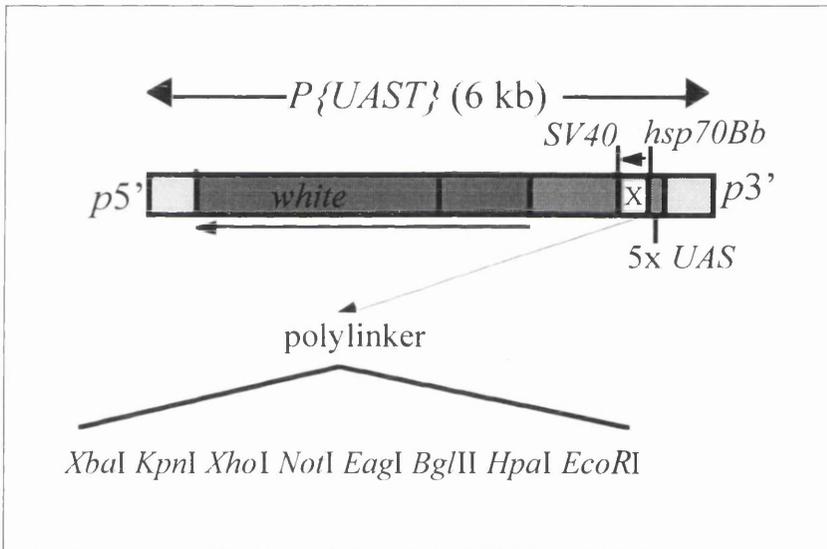


Figure 5.1 **Diagram showing the transposon $p\{UAST\}$.**

Schematic diagram of the germline transformation transposon, $p\{UAST\}$. X corresponds to the inserted gene. 5x *UAS* refers to the five tandemly arranged, optimised GAL4 binding sites. This is followed by the *Hsp70Bb* promoter, the polylinker, the cloned sequence then the *SV40* polyadenylation signal (Brand and Perrimon, 1993).

This transformation vector was chosen as it enables selective expression of the gene of choice in different tissues by crossing to fly lines that are known to express the GAL4 protein in particular tissues (figure 5.2). This allows us to observe what happens when the genes are overexpressed in these tissues, or indeed in the whole fly.

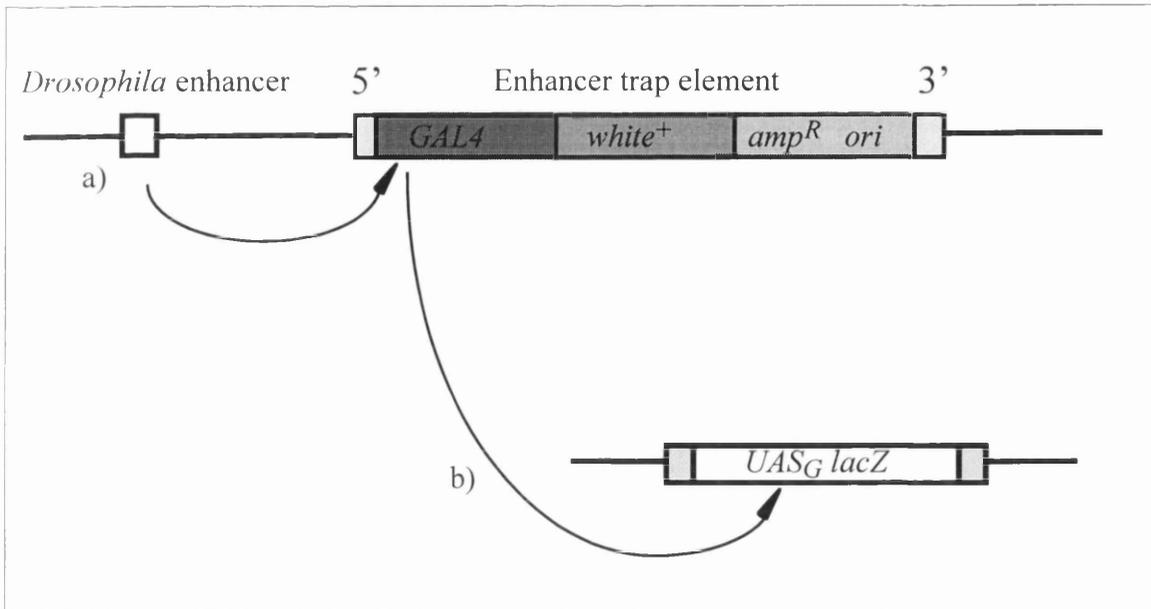


Figure 5.2 **Schematic diagram of *GAL4* /*UAS* system.**

This system utilises the yeast transcription factor GAL4, which, in one fly is expressed under control of an endogenous enhancer. Contained in a different fly is an element containing a gene under control of the *UAS_G* promoter (in this case the *lacZ* gene). This promoter is only active in the presence of GAL4. This gene is silent until the two flies are crossed, when a) the GAL4 under control of the endogenous enhancer can activate transcription b) of the *lacZ* gene. The gene under *UAS* control can be any gene of choice (Kaiser, 1993).

5.3.2 Obtaining insert DNAs

vha16-1 L-F	GGG AAT TCA TGT CTT CTG AAG TGA GCA G
vha16-1 R-R	GGG AAT TTT ATT TCG TGT ACA GGT AAA
Manduca L-F	GGG AAT TCC CGT GGA GTG AGT GTG TGC A
Manduca R-R	GGG AAT TCT TAC AGT AGT TGT TTT CAA AA
Nephrops L-F	GGA ATT CAT GTC TGA AGA GGG TAG TCC T
Nephrops R-R	GGG ATT CTT ACG TGG AGG TCT TGG TGT A
vha16-2 F	GGG AAT TCA TGG TTA CTG CCG CTT TAA AT
vha16-2 R	GGG AAT TCT AAA GCT TAG TGT ACA AAT AGA
vha16-3 F	GGG AAT TCA TGG CAA CAG ATG CAG CTG ACA AG
vha16-3 R	GGG AAT TCT TAC TTG GTG TAC AGG TAA ATG GCG
Dros-EGFP-F	CCA TTT ACC TGT ACA CGA AAA TGG TGA GCA AGG GCG AGG A
Dros-EGFP-R	TCC TCG CCC TTG CTC ACC ATT TTC GTG TAC AGG TAA ATG G
EGFP-R	GGG AAT TCT TAC TTG TAC AGC TCG TCC A

Figure 5.3 **Primers used in cloning the rescue DNA sequences.** *EcoRI* restriction enzyme sites, shown in bold, were added to aid cloning into the *pP{UAST}* vector. F indicated forward facing and R indicated backward facing.

Sequences were amplified by PCR using a high fidelity *Taq* polymerase with the primers used in amplification (figure 5.3) containing terminal *EcoRI* sites to enable the subsequent subcloning into the *EcoRI* site of *pP{UAST}*. Products were initially ligated into the pCR[®]2.1TOPO cloning vector. *vha16-1* was amplified from a pYES2 vector containing the originally cloned cDNA, and *vha16-2* and *-3* were amplified from genomic DNA isolated from Oregon R. GFP sequence was amplified from pEGFP-N1. *Nephrops* cDNA was gifted by Prof. Malcolm Finbow, and the *Manduca* cDNA gifted by Prof. Julian Dow. Primers were designed against Genbank Accession numbers DMVHATP (*vha16-1*), S40059 (*Nephrops*), and MSVATP16S (*Manduca*).

To make the GFP C-terminal in-frame fusion construct, a PCR mutagenesis was first performed to splice the sequences together (figure 5.4). Three separate PCRs, PCRs 1, 2 and 3 on the diagram, were required. Firstly, reaction 1 using primers vha16-1 L-F and Dros-EGFP R and reaction 2 using primers Dros-EGFP L and EGFP R were carried out. For PCR 1 this results in the *vha16-1* coding region (minus stop codon) to be tagged with the first twenty bases of the *GFP* sequence. For PCR 2 the resulting product is the *GFP* preceded by the last twenty bases of *vha16-1*. When the products of these two reactions are mixed and heated to denature the double strands, the region of twenty base pairs overlap can anneal and a third PCR performed using vha16-1 L-F and EGFP R as primers. The *Taq* polymerase can then fill in the recessed ends of these overlapping molecules, providing a double-stranded template for the PCR reaction. This method produced an insert of the correct size (1.2 kb). The third PCR would only work using normal *Taq* polymerase and consistently failed with the Hi-fidelity *Taq*.

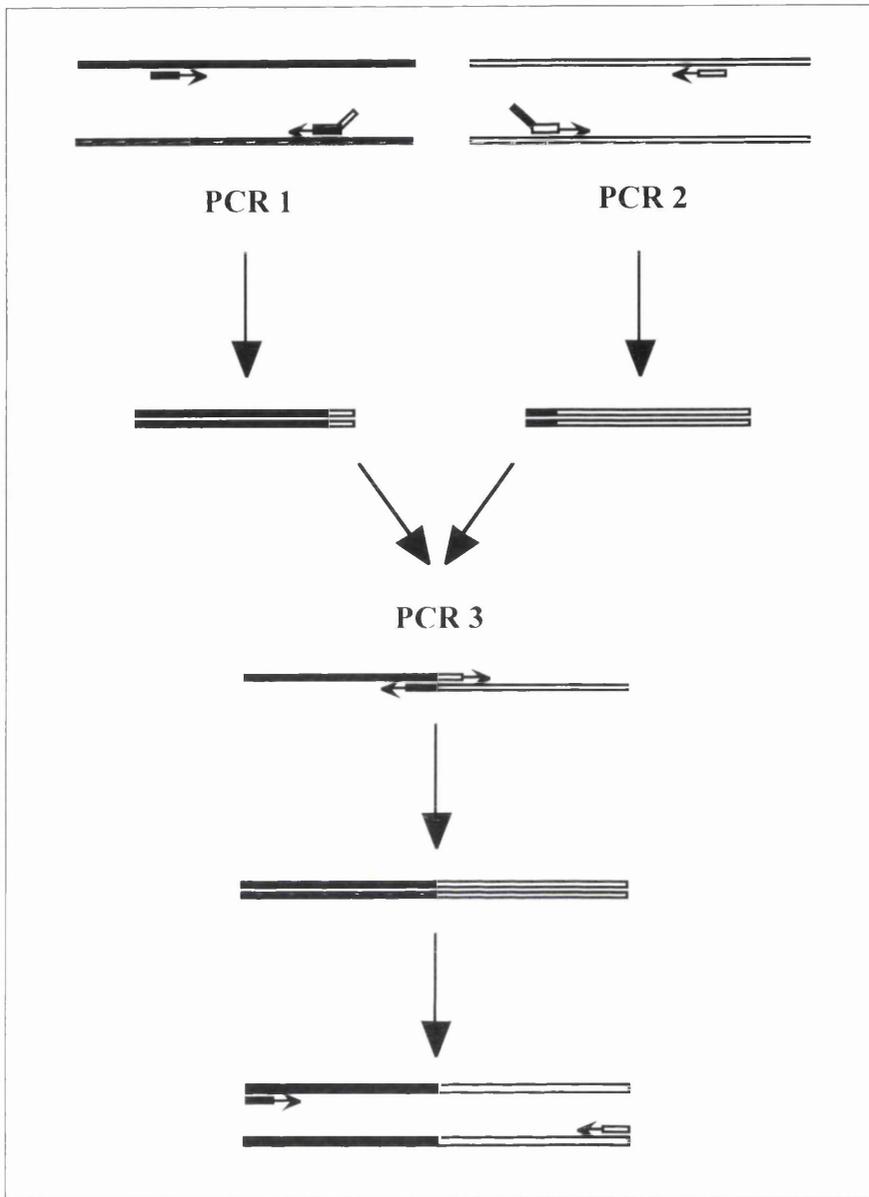


Figure 5.4 **PCR mutagenesis strategy** used to generate a *vha16-1* cDNA (black box) with an in-frame GFP tag (white box). Primers Dros-EGFP R (minus sense) and Dros-EGFP L (plus sense) contained the last 20 bases before the stop codon (which was removed) of *vha16-1* and the first 20 bases of the GFP sequence. This provides an area of overlap, allowing the ends of the different strands to anneal. In PCR 3 the recessed ends act as primers and are filled in by the *Taq* polymerase allowing outer primers (*vha16-1* L-F and EGFP R) to anneal in subsequent cycles.

5.3.3 Verification of plasmids

Plasmids were first sequenced with pCR[®]2.1TOPO specific sequencing primers to ensure no errors were introduced by the *Taq* during the amplification process. DNAs were then excised with *Eco*RI, run on a 1% agarose gel, band purified and ligated into *p{UAST}* and digested with *Eco*RI to observe the size of insert (figure 5.5) before being sequenced again with a *p{UAST}* specific primer (appendix 4) to choose constructs with the insertion in the correct orientation. To observe whether the join between the two separate sequences in the *Dros*/GFP construct was as expected the splice point was sequenced using a primer near the end of the *Drosophila* cDNA sequence (for primer sequences see appendix 4). Figure 5.6 shows the sequences around the splice site and it can be observed that the coding sequence is maintained between the two sequences.

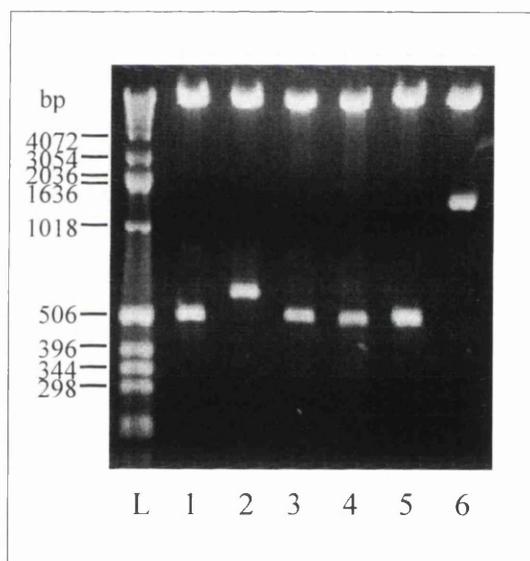


Figure 5.5 **Restriction digest of cloned coding sequences in the *pP{UAST}* vector.**

Plasmids were digested with *Eco*RI for two hours prior to running on a 1% agarose gel. Lane 1) Gibco BRL 1 kb ladder, 2) *vha16-1*, 3) *Manduca*, 4) *Nephrops*, 5) *vha16-2*, 6) *vha16-3*, 7) *vha16-1/GFP*.

	440	460	480	500	520
Consensus	TCTCTACGGCTTGATTGTGGCCATTTACCTGTACACGAAA . . . ATGGTGAGCAAGGGCGAGGAGCTGTTACACGGGGTGGTGC				
<i>vha16-1</i> coding ▶	TCTCTACGGCTTGATTGTGGCCATTTACCTGTACACGAAATAA				
Dros-EGFP-F ▶		CCATTTACCTGTACACGAAA . . .	ATGGTGAGCAAGGGCGAGGA		
Dros-EGFP-R ◀		CCATTTACCTGTACACGAAA . . .	ATGGTGAGCAAGGGCGAGGA		
<i>vha16-1</i> /GFP join		ACGAAA . . .	ATGGTGAGCAAGGGCGAGGAGCTGTTACACGGGGTGGTGC		
EGFP ▶			ATGGTGAGCAAGGGCGAGGAGCTGTTACACGGGGTGGTGC		
		A I Y L Y T K M V S K G E E L F T G V V			

Figure 5.6 **Sequence around the splice point of the *vha16-1*/GFP sequence.**

Sequence obtained from sequencing out of the *vha16-1* region of the *vha16-1*/GFP plasmid was aligned with the primer and template sequences used in its construction. Underneath lies the translated sequence showing the reading frame of GFP is maintained and no frame-shift occurs. The TAA stop codon from the *vha16-1* sequence had been removed when designing the primers.

5.4 Germline Transformation

Plasmids containing correctly oriented cDNAs were microinjected along with the helper plasmid into embryos of the fly line *w¹¹¹⁸*. Numbers of transformed flies from each injected plasmid and chromosomes into which the elements had inserted are listed in table 5.1.

Construct	Eggs injected	No. Transformants	Chromosome(s)
Puast/ <i>vha16-1</i>	254	8	3
Puast/ <i>vha16-2</i>	398	0	-
Puast/ <i>vha16-3</i>	339	12	2
Puast/ <i>Manduca</i>	204	15	2 and 3
Puast/ <i>Nephrops</i>	421	10	2 and 3
Puast/ <i>vha16-1</i> /GFP	237	0	-

Table 5.1 **Numbers of transformants obtained from each rescued plasmid and chromosomes on which they were inserted.**

Transformants were crossed individually to balancer chromosomes to determine on which chromosome the sequences had inserted.

In order to rescue the 16/1 P-element mutation the inserted sequences are required to be on the third or sex chromosomes, this is because rescue can only be performed in a *vha16-1* null background, i.e. there have to be two P-element inactivated copies of *vha16-1* in the fly when the transgene is expressed. So if a transformed sequence inserts on chromosome two, the wild-type gene will also be present and can only be crossed out by genetic recombination, a long and difficult process. If inserted on a different chromosome, however, the necessary genetic elements can be crossed in and out as required.

Unfortunately all the 16-3 inserts were on the second chromosome so rescue in this case was impossible. This element can potentially be re-mobilised to insert into chromosome three.

PCR was performed on the surviving lines using a *P{UAST}* specific primer and the reverse transgene primer to ensure that the inserted DNAs were indeed present in the genome and that they were in the correct orientation within the P-element (figure 5.7).

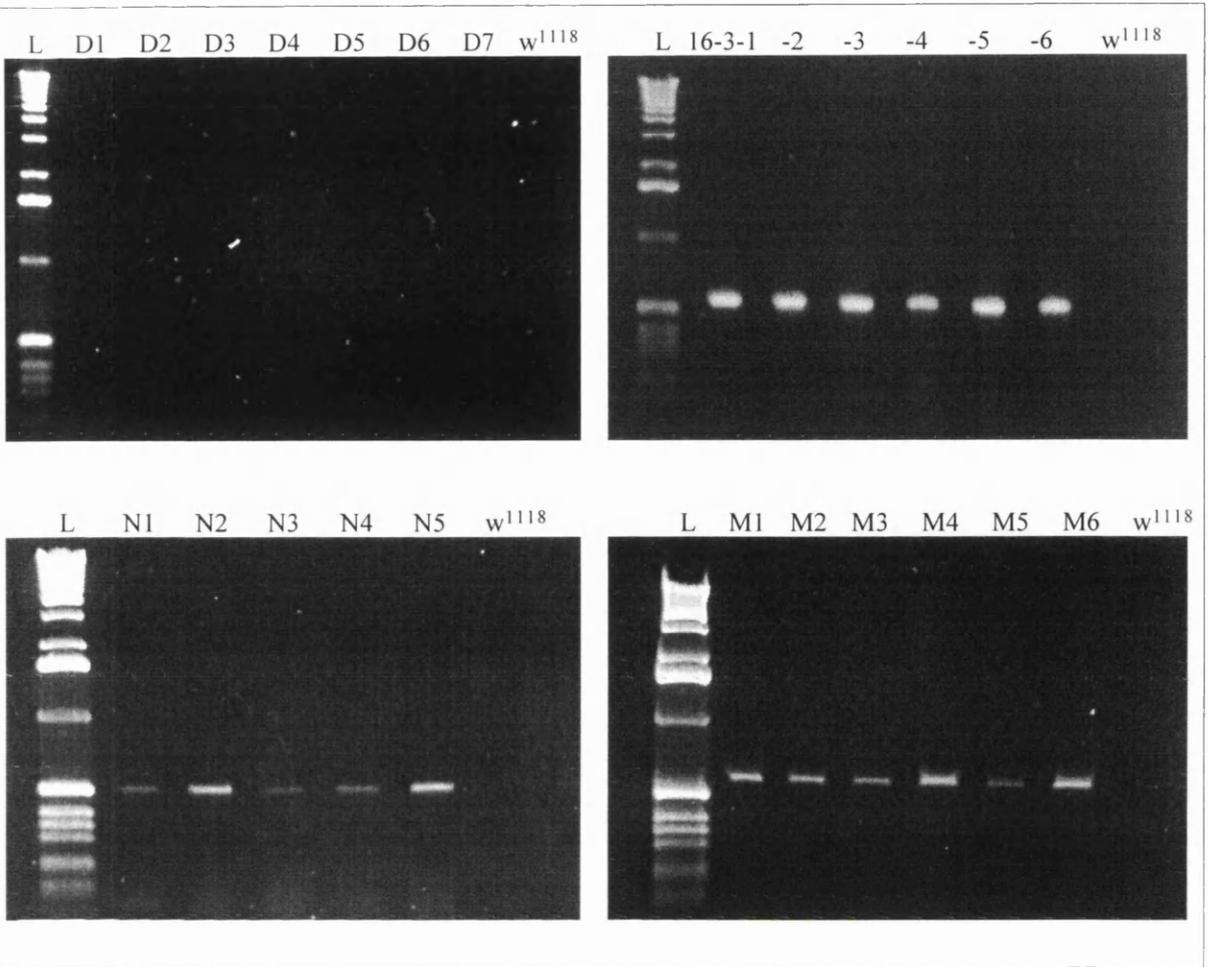


Figure 5.7 PCR with pUAST seq primer and reverse insert primers on the transformed lines.

DNA obtained from the surviving fly lines (*vha16-1*, *vha16-3*, *Nephrops* and *Manduca*) was extracted, and PCR performed to observe the presence and correct orientation of the insert. Primers used were pUAST seq and the appropriate reverse primer from figure 5.3. Ladder is Gibco BRL 1 kb and sizes were compared to those obtained from restriction digests of the plasmids used for transformation.

The flies containing the *vha16-1* insert, although red eyed, gave no bands after PCR, implying there was no element inserted, even though the balancer cross indicated that the third chromosome only was carrying the red eyed mutation.

A DIG Southern blot was carried out, probing with the *vha16-1* sequence cloned into *P{UAST}* to observe whether this sequence was present in the flies genomes (figure 5.8).

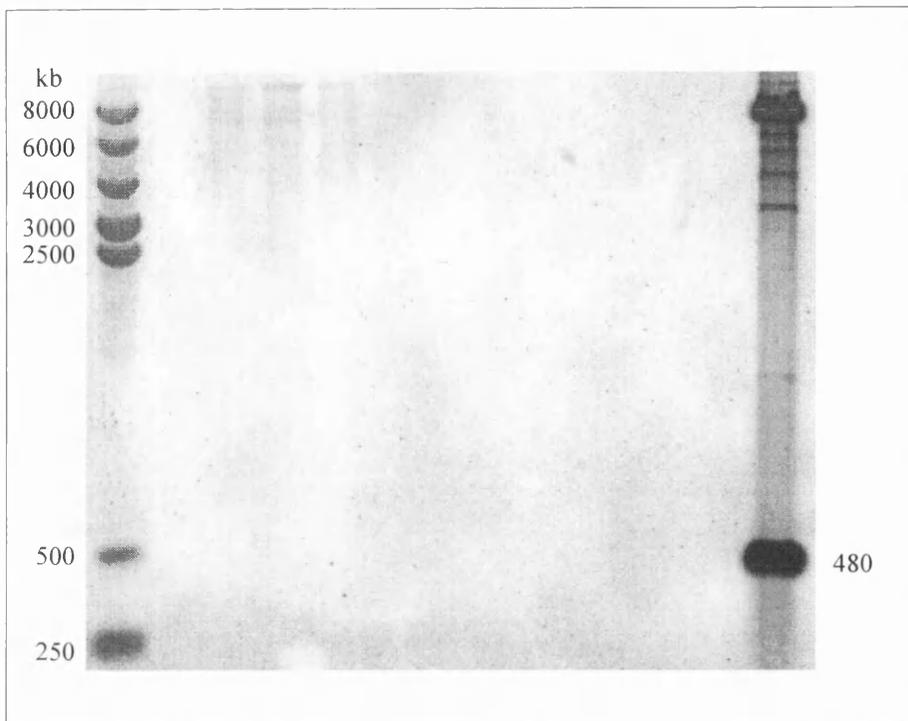


Figure 5.8 Southern blot of *vha16-1* transformed flies probed with the DIG-labelled *vha16-1* coding region.

2.5 μ g of genomic DNA from flies D1, D2, D3, D4, D5, D6, D7 and *w¹¹¹⁸* (as a negative control) and 0.5 μ g of *Puast/vha16-1* plasmid (as a positive control) were digested with *Eco*R1 and electrophoresed on a 1% agarose gel, prior to blotting onto nylon membrane (Boehringer Mannheim). Ladder is Promega 1 kb. Washing was to high stringency, 0.1% SSC, 0.1% SDS at 65°C.

This Southern blot shows the probe hybridising to the plasmid control, but no hybridising band were observed in any of the "transformed" flies indicating they had not been transformed at all. The blot could also be probed with *P{UAST}* sequences to determine if these are present and a deletion event has occurred.

The *Manduca* and *Nephrops* lines containing the third chromosome inserts were then put individually into the rescue cross as follows (figure 5.9).

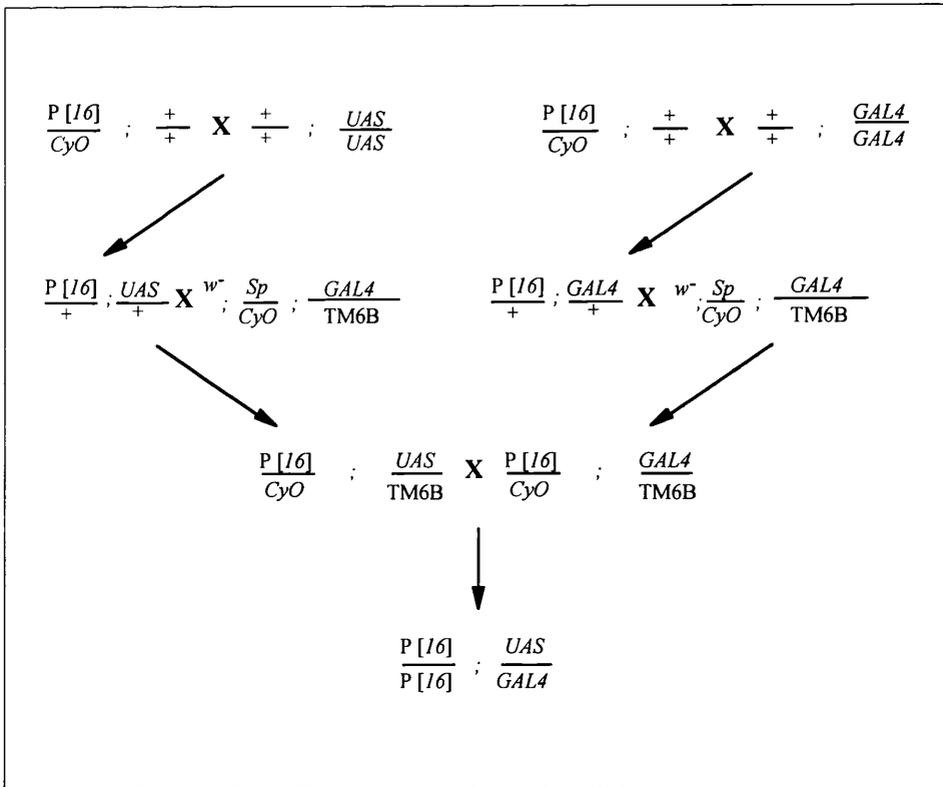


Figure 5.9 **Crossing scheme involved in the rescue of the mutant P-element phenotype.** P[16] refers to the original P-element mutation on chromosome two. *UAS* refers to the cloned insert under *UAS* control and *GAL4* is the line which produces *GAL4* upon heat-shock.

For the final cross, flies were transferred to egg plates, also containing fly food, and were heat shocked at 37°C for half an hour every morning, as were resulting eggs, larvae and pupae. Upon pupal eclosion straight-winged flies were sought, however, after several attempts none were found for any of the lines. The singly P-element inserted curly-winged flies which also resulted from the final cross did survive to eclosion, so therefore the daily heat-shocking regime was not deleterious to any of the developmental stages of the flies.

5.5 Rescue of *vma*⁻ phenotype

As was mentioned previously, the *Nephrops norvegicus* 16 kDa proteolipid (Harrison *et al.*, 1994) and *Drosophila* cDNA (Finbow *et al.*, 1994) were shown to rescue the *vma3*⁻ mutation in yeast. The pCR2-TOPO cloned DNAs, of *vha16-2*, *-3* and *-4*, were sent to Malcolm Finbow's lab, where these experiments had been carried out, to observe if they rescued the yeast Vat c strain which is deficient in the *vma3* gene. The mutant yeast strain is able to grow at the permissive pH of 5.5, but is unable to grow at pH 7.5. In this experiment yeast are transformed and replica plated on medium of these pHs. If the expressed sequence rescues the mutation, the strain will grow at pH 7.5.

Unfortunately in this case, none of the sequences rescued the mutation, i.e. the transformed cells were unable to grow at the restrictive pH of 7.5 (figure 5.10).

However, the experiment also shows the *vha16-1* protein only partially rescues this phenotype, and that the *Nephrops* gene only rescues it intermediately and not fully (Malcolm Finbow, personal communication). This implies that the proteolipid sequences from different species, even though highly homologous, are not simply interchangeable.

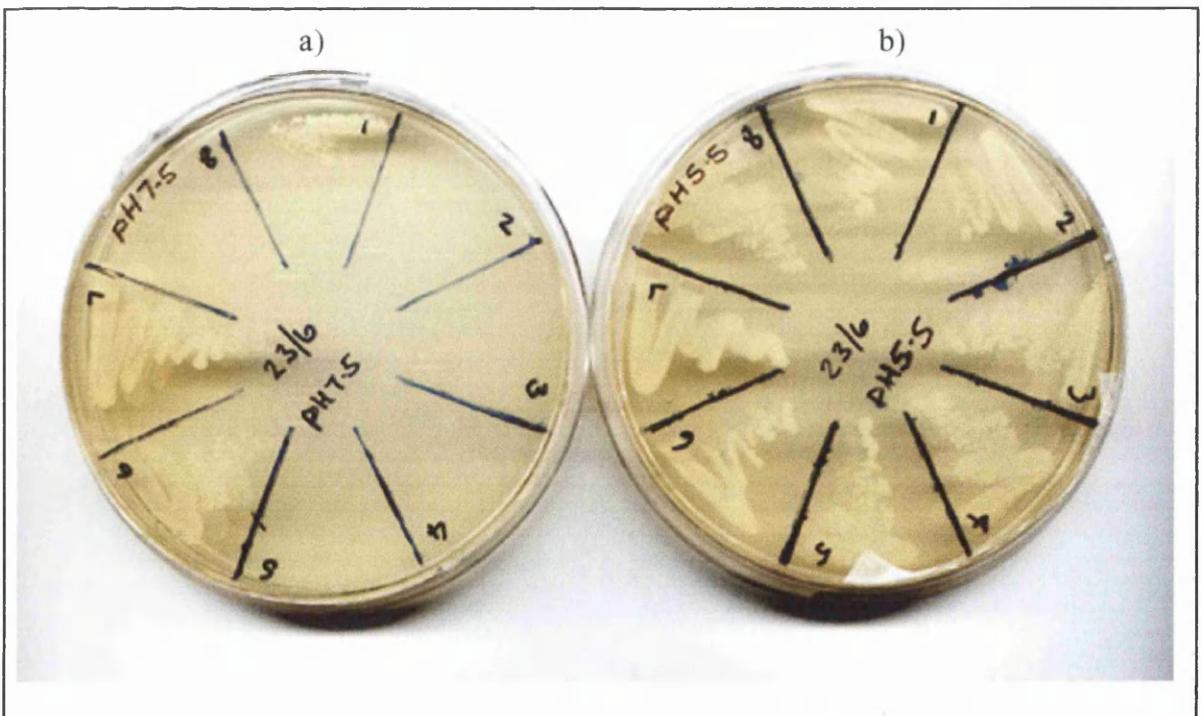


Figure 5.10 **Expression of *vha16-2*, *-3* and *-4* in the *S. cerevisiae vma*⁻ mutant.**

Sequences were cloned into the pYES2 high expression vector expression vector and replica plated at a) pH 7.5 and b) pH 5.5. Each plate contains transformation with: 1) *vha16-1*, 2) *vha16-2*, 3) *vha16-3*, 4) *vha16-4*, 5) *Nephrops norvegicus* (low expression vector), 6) *Nephrops norvegicus* (high expression vector), 7) wild type *S. cerevisiae*, 8) no DNA.

5.6 Discussion

These experiments attempted to rescue the lethal P-element mutation in *vha16-1*, via germline transformation with a series of sequences encoding the 16 kDa proteolipid. These sequences included two of the newly discovered proteins, *vha16-2* and *vha16-3*, a *vha16-1*/GFP fusion protein and the proteins from *Manduca sexta* and *Nephrops norvegicus*. This experiment was also an attempt to see if the new genes encoded a functional protein and if sequences from different species would rescue a mutation in *Drosophila*.

This involved cloning of the sequences, using a high fidelity *Taq* polymerase, first into a PCR cloning vector and then into the germline transformation vector *pP{UAST}*. The plasmids were co-injected into *Drosophila* embryos alongside a plasmid providing a transposase source.

The transformation of *vha16-2* and the GFP C-terminal fusion unfortunately failed, but the others appeared to successfully transform and their presence within the genomic DNA was determined by PCR and the flies crossed to balancer chromosomes to determine where they had inserted. The exact position of the successful transformants could be elucidated either by inverse PCR or by *in situ* hybridisation to polytene chromosomes, providing us with more information about their points of insertion. Although the *vha16-1* transformed flies had the red eye colour phenotype indicating a successful transformation, and the fact that this eye colour only segregated on chromosome three, indicating only one chromosome carried the gene, the *vha16-1* sequences could not be amplified by PCR from these flies. A Southern blot also showed the sequence was not present in these flies.

Despite several attempts, no rescue with the *Manduca* and *Nephrops* sequences was obtained, whether this was because the proteins were not functional in *Drosophila*, or if the sequences were not transcribed at all or at a critical correct time point, temporally and/or spatially as the heat shock only occurred for a half hour every day. It may also be merely that overexpression of these proteins is lethal to the cells.

These results, alongside the yeast data, indicate that the V-ATPase proteolipid genes, while incredibly similar, cannot simply be substituted for one another in different organisms. These proteins exist in many cell type and organelles, where the V-ATPase enzyme has different functions. This would demand a much more sophisticated level of control than either of these transformation systems could provide. As *Drosophila* is phylogenetically a much higher organism than yeast, which only shows partial rescue with alternative proteolipids, it is perhaps unsurprising that the *Nephrops* and *Manduca* proteins cannot rescue the *Drosophila* phenotype.

If these germline transformation experiments were to be repeated it would be more prudent to clone either the entire genomic sequences of these DNAs, or the upstream promoter sequences along with the coding regions to obtain correct spatial and temporal transcription of these genes as these experiments controlled the transcription of the transgenes in a very crude manner, via heatshock.

Chapter 6

Isolation of Other V-ATPase Proteolipid Genes

6.1 Summary

This chapter describes the discovery and cloning of two new proteolipid subunits of the V-ATPase in *Drosophila melanogaster*. It deduces their amino acid sequences and compares them to homologues in other organisms. It also shows basic expression data in the form of tissue specific reverse-transcriptase PCR and developmental Northern blots.

6.2 Introduction

Although *vha16-1*, as a hexamer, forms the transmembrane pore of the vacuolar ATPase holoenzyme it is not the only membrane associated protein as discussed in Chapter 1. A search of the BDGP database was undertaken to find evidence, in *Drosophila*, of these other proteolipid genes in other organisms. The theory behind cloning these subunits is that they are closely associated with the other V_o subunits in the membrane and therefore if they exist in *Drosophila* we can try to look for differences in expression patterns between these subunits and *vha16-1* which is thought to have multiple roles.

One of these V_o sector proteins is encoded by *PPA1* (or *VMA16*), a gene initially discovered in yeast (Hirata, 1997). This protein has 35% identity to the yeast Vma3p protein, which encodes the 16 kDa proteolipid pore in this organism. It, like Vma3p contains a critical glutamic acid residue in a transmembrane domain which binds DCCD, and experiments show that this protein, along with another similar protein, Vma11p, contributes to proton translocation (Powell, 2000).

The second protein is M9.7, a small highly glycosylated protein present in *Manduca sexta* (Merzendorfer, 1999) V-ATPase holoenzyme. This protein is present as a constitutive component of the holoenzyme in *Manduca* although it has been postulated that it has structural similarity to the yeast V-ATPase assembly factor Vma12p, which is only present in the endoplasmic reticulum of yeast.

M9.7 also has a human homologue, M9.2 (Ludwig, 1998), this protein however is not thought to be glycosylated, although the authors also postulate structural similarity to Vma12p.

6.3 Isolation of *Drosophila* homologues of *PPA1*

6.3.1 Database searching

The yeast *ppa1p* (*Vma16*) protein sequence was used as the input sequence for a tBLASTn search of the BDGP database. This search resulted in the isolation of two genomic DNA sequences, several ESTs and a P-element insertion. An alignment of these sequences is shown in figure 6.1.

For *PPA1* two areas of identity were found on the same genomic BAC clone, BACR48E23 (*AL079199*), 3 kb distant from each other. This clone, according to the database, had a cytological location of 88D.

All the ESTs predicted by the BDGP corresponded to only one of the genomic sequences. This was given the name *vhaPPA1-1*. The other sequence had no corresponding ESTs and was named *vhaPPA1-2*. A pileup of these sequences is shown in figure 6.1 and data summarised in table 6.1

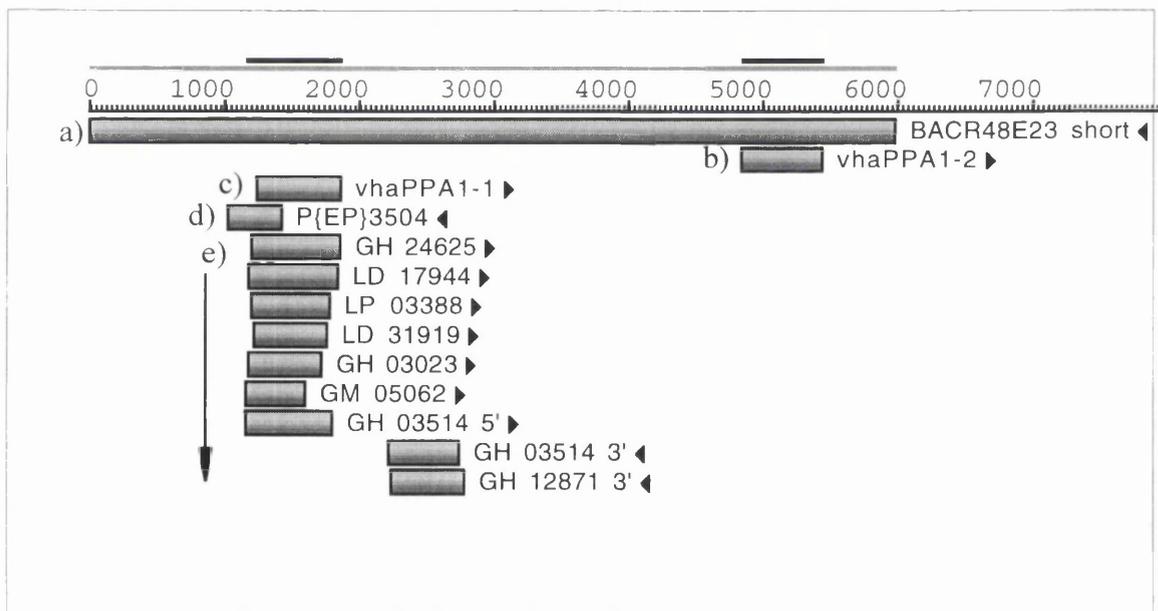


Figure 6.1 **Assemblylign pileup of sequences isolated with homology to yeast *PPA1***. Figure shows an Assemblylign alignment (default settings) of a) the relevant length of genomic BAC clone (BAC48E23), b) and c) the putative *Drosophila* open reading frames (*vhaPPA1-1*, *vhaPPA1-2*), c) a P-element insertion (P{EP}3504) and e) EST clones obtained from the BDGP. The letters next to the EST clones refer to the cDNA library from which they were obtained, LD-embryo, GH-adult head, LP-larvae, pupae, GM-ovary.

Gene	Location	BAC clone	No. of ESTs	% homology to yeast protein
<i>vhaPPA1-1</i>	88D	BACR48E23	8	48
<i>vhaPPA1-2</i>	88D	BACR48E23	0	39

Table 6.1 Summary of data obtained by homology searching the BDGP with the yeast *ppa1* protein. Sequences were isolated using a tBLASTn search using the yeast *ppa1p* as input sequence.

6.3.2 Cloning of *vhaPPA1* sequences

Primers were designed from the BDGP genomic sequences to clone the putative new genes. Primer sequences are shown in figure 6.2. PCR products were cloned into the pCR®2.1 TA cloning vector and sequenced to verify their identity.

PPA1-1 L	CAA GAT GGC GGC CCA AAT ACG
PPA1-1 R	CTC CTT GTC GCC CAT CTT GG
PPA1-2 L	AAA GAT GAT CTC TAA GAT GAG
PPA1-2 R	ATT AAT GGT TTC CGC CTT GG

Figure 6.2 PCR primers used to clone *PPA1* sequences. Primers were designed against the sequences obtained from the BDGP in order to clone the entire coding region. These primers are highlighted in italics in figure 6.2.

The sequences were translated by the MacVector translation function and aligned with the DNA from which they were encoded (figure 6.3), with a putative polyadenylation site for *vhaPPA1-1* highlighted in bold. No such site was observed for *vhaPPA1-2*, this may be due to an error in sequencing or it may be further downstream in the sequence.

vhaPPA1-2

20 40 60
ATGTTCTATAAGAAATGATTATCTCTGTGAGTTGCGAAAAGATTTTCATTCCACCGTGCCAACTTAAAATCG
TACAAGATATTCTTTACTAATAGGACAGCTCAAGCTTTTCTAAAAGTAAGGTGGCACGGTTGAATTTTAGC

80 100 120
ATTCTTTATATCTGTTAATTCTGAAGCTTTTCGCTTAAAAGATGATCTCTAAGATGAGTTTTGGATTAT
TAAGAAATATAAGACAATTAAGCTTCGAAAAGCGAATTTTCTACTAGAGATTCTACTCAAAAACCTAATA
M I S K M S F G L>

140 160 180 200
TCTTTTATGCTGTTCGTACTGGGCGCCGCTCGTTGGGCGCGGTTGGCATAAATCTTCGCCAACGTGATGG
AGAAATACGACAAGCATGACCCGCGCGGAGCAACCCGCGCCAACCGTATTAGAAGCGGTTGCACTACC
F F M L F V L G A A S L G A V G I I F A N V M>

220 240 260
TCAATATGGGTGAGCGCATGGGGCTCGGCTGGTTCCTGTACACCTCGAATCCCTTCTGTGGTCCGGCA
AGTTATACCCACTCGCGTACCCGAGCCGACCAAGGACATGTGGAGCTTAGGGAAGGACACCAGGCCGT
V N M G E R M G L G W F L Y T S N P F L W S G>

280 300 320 340
TGGGCATCTTCTCGCCTGCGCCCTGTCCGTTCTGGGCGCCAGTGGAACTACATGATTGGATGCA
ACCCGTAGAAGGAGCGGACGCGGGACAGGCAAGACCCGCGCGGTCACCTTAGATGACTAACCTACGT
M G I F L A C A L S V L G A A S G I Y M I G C>

360 380 400
GTGTGGCGGGCGGAGTGGCATCGCCTCGCATCAAGACCAAGAACCCTGATCTCAGTCATCTTTTGGC
CACACGCCCCGCTCAGCTAGCGGAGCTAGTTCTGGTCTTGGACTAGAGTCAGTAGAAAACGC
S V A G G G V R S P R I K T K N L I S V I F C>

420 440 460 480
AGGCGTGGCCATCTATGGCCTGATCACGGCCATCCTGCTGTCCGGCAACGTCAACAAGTTGAGCAGCG
TCCGCCACCGGTAGATACCGGACTAGTGCCCGGTAGGACGACAGGCCGTTGCAGTTGTTCAAGTCGTCGC
E A V A I Y G L I T A I L L S G N V N K F S S>

500 520 540
TGCGTCTGATCACCGACAGCAGGTCATGGCGACCAATATGTTTACGGGATTTCGCTACTTTTGGGGCAG
ACGCAGACTAGTGGCTGTCGTGCCAGTACCCTGGTTATACAAATGCCCTAAGCGATGAAAACCCCGTC
V R L I T D S T V M A T N M F T G F A T F G A>

560 580 600 620
GTCTCTGCGTGGAAATGGTGAACGTGGCCCTGCGGCATTGCTGTGGGCATCGTGGGATCCGGCGCTGCC
CAGAGACGCAACCTTACCCTTGCACCCGAGCCGTAACGACACCCGTAGCACCCCTAGGCCGCGACGGG
G L C V G M V N V A C G I A V G I V G S G A A>

640 660 680
TGGCGGATGCGGCCAACTCGGGCTCTTCGTCAAGATCCTCATTTGTGGAGATTTTGGCTCGGCTATCG
ACCCCTACCGGTTGAGCCGCGAGAAGCAGTTCTAGGAGTAACACCTCTAAAACCGGACCGGATAGC
L A D A A N S A L F V K I L I V E I F G S A I>

700 720 740
GACTGTTTGGCCTGATTTGTGGCCATCTACATGACCTCCAAGGCGGAAACCATTAATTAGACCATTATAT
CTGACAAACCGGACTAACACCGTAGATGACTGGAGGTTCCGCCTTTGGTAATTAATCTGTAATATA
G L F G L I V A I Y M T S K A E T I N *>

760 780 800 820
AAACGCGACTTAATTGAAAAGCTAACATTTATTTGGTAATTAAGCTATTTCTCCCCATCATCGTAATC
TTTGGCCTGAATTAACCTTTTCGATTGTAATAAACCATTAATTTGATAAGAAGGGGTAGTAGCATTAG

840 860 880
GCTGTCATCAGCTTCATCATTGTGACCCCTCCTCGTCTGCTCTTCTCGTCCCAGTGTGATCTTCTT
CGACAGTAGTCGAAGTAGTAACACTGGGAGGAGCAGCAGCAGAAGGAGCAGGGTCACGAAC TAGAAGAA

900 920 940 960
GTTGGTCATGAAGTCCTCGCGCAGCACACTCCATTCCGACTTCTTTTTGCCGCTGGAGCCCTTGTCTTC
CAACCAGTACTTCAGGAGCGCGTCTGTGAGGTAAGCCTGAAGAAAAACGGCGACCTCGGGAACAGAAG

980 1000 1020
GTCGTGTCATCCGTTTTCTGTTCTTCTTTACAGCGTTATCTACGGGTGTGGATTTGGCGCGCTT
CAGCAGCAGTAGGCAAGAACAAGGAGAAAGAAATGTCGCAATAGATGCCACACCTAAACCGCGCGAA

1040 1060 1080 1100
CCCCTCATCAGGACGTCAAAAACTTTTCGCTTGTGATGTTGTTTAGCACCGCATCCTGTGCGCTGTC
GGGCGAGTAGTCTGCAGTTTTTTGAAAGCGAACAAC TACAACAAATCGTGGCGTAGGACAGCCGACAG

1120 1140 1160
CAGCGGACCCGCTTCCTCCAGCTGCTGCTGCAGATCCTTCTGCTGAATGCGCACGGCATTAAAGAAGCTG
GTCGCTGGGCGAAGGAGGTCGACGACGAGCTTAGGAAGACGACTTACGCGTGCCGTAATTTCTTGAC

1180 1200 1220 1240
CACCCTCCACGAGTGGCAACTTTGCGGAGGTTCTGTTACGCTCCATGTCCCGGAAACTGGGCTTCAC
GTGGTGAGGTGCTCACCGTTGAAACGCCTCGCAAGCAAGTGCAGGTTACAGGGCCTTTGACCCGAAGTG

1260
ACGCAGCTGGAGGGGCACATTCTTAC
TGCGTCGACCTCCCGGTGAAGAATG

Figure 6.3 DNA and predicted protein sequences of the putative *Drosophila* PPA1 homologues. Protein sequences were generated by MacVector and aligned with the cDNA from which they were generated. Primers used to amplify the sequences are indicated in italics. The putative polyadenylation signal is highlighted in bold.

Homologues from human, *S. cerevisiae*, *S. pombe*, *C. elegans* and *Arabidopsis* were aligned by ClustalW (on default parameters) and viewed with SeqVu (figure 6.4). This is viewed in the same way as the vha16 alignment in figure 4.6. It shows a hydropathy view with red amino acids being most hydrophobic and pink ones less so. The purple/lilac residues are hydrophilic. The alignment shows that five hydrophobic tracts (red/pink) are present in all sequences, indicating five membrane spanning regions. This protein is also DCCD reactive and the predicted residue for DCCD reactivity is marked with an asterisk.

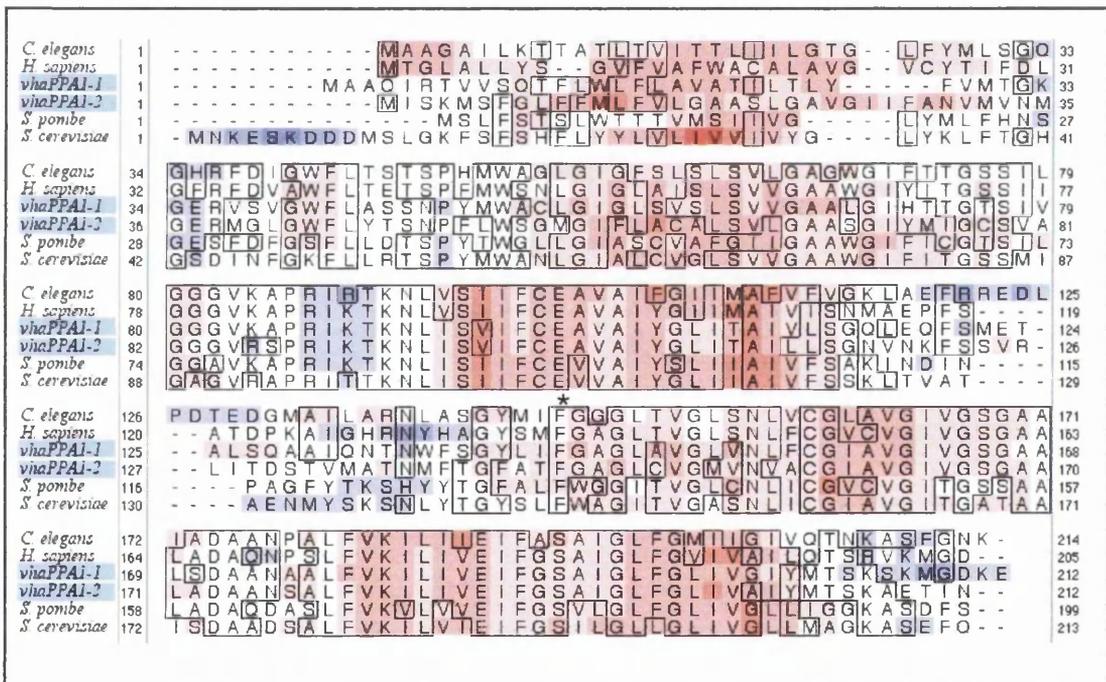


Figure 6.4 Pile-up of PPA1 V-ATPase subunits from a variety of species. Sequences were aligned using ClustalwPPC (on default parameters) and viewed with SeqVu. Boxed residues correspond to identity, red/pink areas are hydrophobic and purple/lilac hydrophilic. The asterisk corresponds to a DCCD binding site and *Drosophila* sequences are highlighted.

Protein sequence accession numbers were as follows:

C. elegans BAA22597 (Oka, 1997)

S. cerevisiae P23968 (Apperson *et al.*, 1990)

S. pombe CAB16373

H. sapiens BAA13753

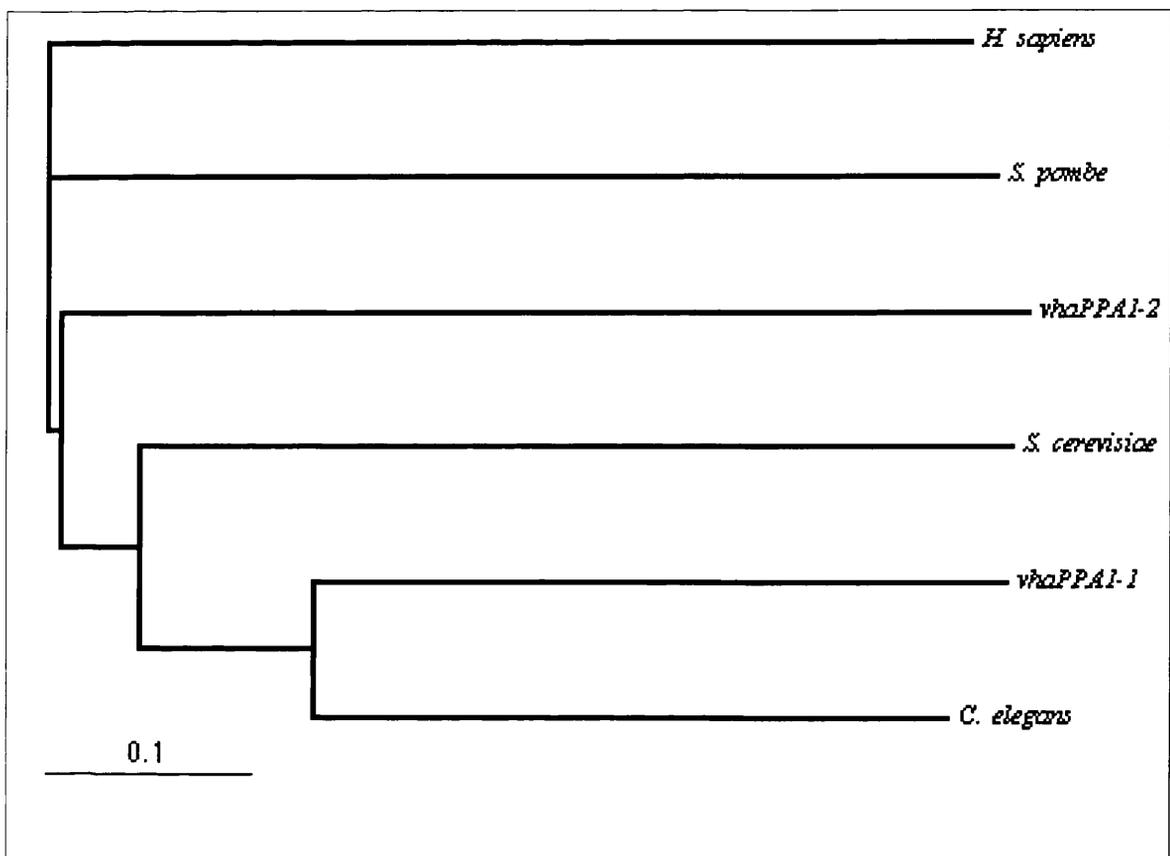


Figure 6.5 **Bootstrap analysis of homologues of ppa1p.** Sequences were aligned using ClustalW (on default parameters) and viewed with TreeView. This tree is unrooted as an outgroup cannot be defined.

The bootstrap analysis (figure 6.5) indicates that the protein encoded by *vhaPPA1-1* is most closely related to the protein present in *C. elegans* and that the protein encoded by *vhaPPA1-2* is not closely related to any of the other proteins. These are the only organisms from which this protein has been isolated. This leads to a difficulty in defining an outgroup to root the tree, so it remains unrooted.

6.3.3 RT-PCR

The presence of several ESTs for *vhaPPA1-1* indicate that it is expressed. RT-PCR was performed to see whether both of the new 'genes' generated transcripts in various tissue types. The tissues observed were: brain, head, Malpighian tubule, gut, male and female gonad and the remains of the carcass. Figure 6.6 contains the RT-PCR results and shows *vhaPPA1-1* is expressed in all these tissues and that *vhaPPA1-2* shows strong expression is gonad. The primers used to amplify these sequences did not bracket an intron so do not control for genomic contamination of the cDNA. The cDNA used was the same as that used in figure 6.13, where results show a very small amount of genomic contamination in the samples. This indicates that the bands could be as a result of the primers priming off genomic DNA. DNase treatment of the mRNA samples prior to cDNA synthesis would rule out the genomic contamination problem.

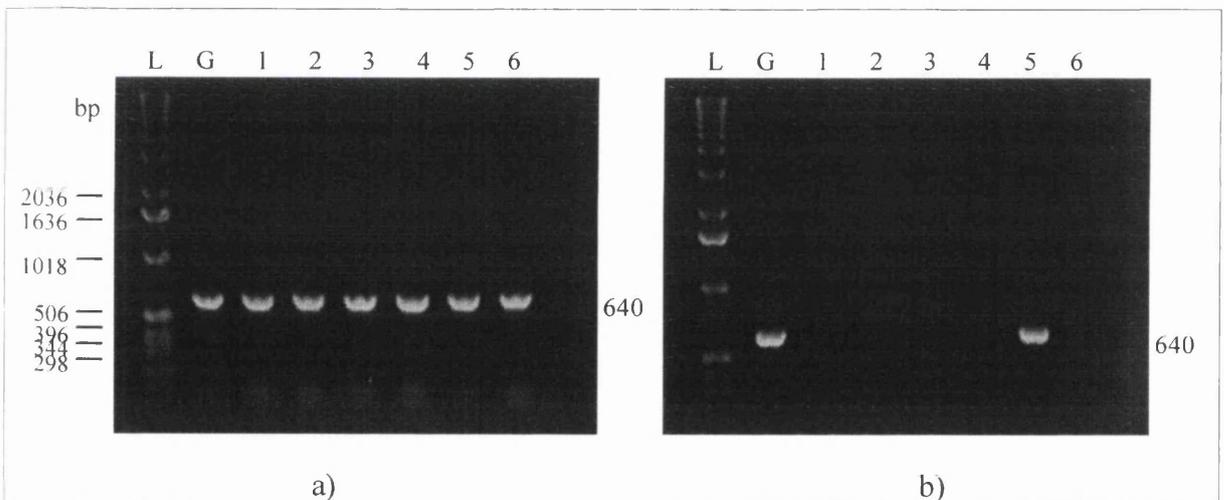


Figure 6.6 **RT-PCR of *Drosophila* tissues.** *Drosophila* tissue template cDNA from 1) brain, 2) head, 3) Malpighian tubules, 4) gut, 5) gonad, 6) carcass. G) is the genomic DNA control. Panel a) shows *vhaPPA1-1* and panel b) shows *vhaPPA1-2*. L= Gibco BRL 1 kb ladder., DNAs were separated on a 1% acrylamide gel.

6.3.4 Northern Analysis

As several ESTs exist for *vhaPPA1-1* and RT-PCR indicates expression, a developmental Northern analysis was performed to observe if there is any developmental regulation of the gene.

Results (figure 6.7) show hybridisation in each lane indicating gene expression throughout development. When band intensities were normalised to the *rp49* control (table 6.2), it was observed that expression was at similar levels at each stage and there was no downregulation in pupae, unlike the genes encoding subunits A and E (Guo, 1996b; Guo *et al.*, 1996b).

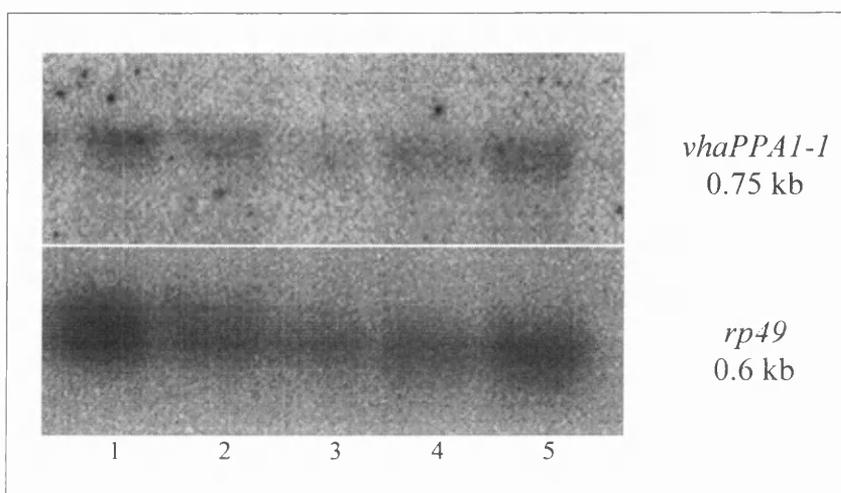


Figure 6.7 **Developmental northern blot.** 25 μ g of total RNA from:

Lane 1) mixed embryo, 2) mixed larvae, 3) mixed pupae, 4) head, 5) body, were run on a 1% agarose gel, blotted and probed with *vhaPPA1-1* cDNA (top panel). The blot has previously been probed with *rp49* (bottom panel) used as a loading control. Blots were washed to high stringency (0.1x SSC, 0.1% SDS, 65°C) Filters were exposed to a phosphorimage plate for twelve hours and four hours respectively.

Band rp49	PSL rp49	PSL PPA1-1	PSL-BG rp49	PSL-BG PPA1-1	Ratio PPA1-1/rp49
Embryo	3839	870	3119	641	0.21
Larva	2471	708	1751	461	0.26
Pupa	1762	461	1042	208	0.20
Head	2055	679	1358	432	0.32
Body	2616	809	1919	557	0.29
Background (BG)	702	247	-	-	-

Table 6.2 Analysis of *vhaPPA1-1* phosphorimage data.

Intensities of bands (PSL-phosphostimulated luminescence) for each developmental stage probed with *rp49* (column 2) and *vhaPPA1-1* (column 3) were measured in the quantification mode of the MacBAS programme (Fuji). The background reading (BG) was subtracted from each (columns 4 and 5) and the ratios between the two values calculated (column 6).

6.4 Isolation of *Drosophila* homologues of *M9.7*

6.4.1 Database searching

The *Manduca* M9.7 protein sequence (CAA06822) (Merzendorfer, 1999) was used as the input sequence for a tBLASTn search of the BDGP database. Two areas of homology were observed, one on the genomic clone AC017581 that is located at 78E and the other on genomic clone BACR48M07 (64B). The first clone was named *vhaM9.7-1* and has four corresponding ESTs, the second, *vhaM9.7-2* has one EST associated with it. A similar protein had been isolated from various other species but has been termed M9.2 due to a difference in size from the *Manduca* protein.

Figure 6.8 shows an Assemblylign pileup (on default settings) of the *Drosophila* EST and predicted coding sequences. Table 6.3 shows a summary of this data.

Very recently, another two homologues were suggested by the *Drosophila* sequencing project (Adams, 2000). These have been named *vhaM9.7-3* (AAF47874 at 64B) and *vhaM9.7-4* (AAF55365 at 89E). There are no ESTs on the database for these sequences.

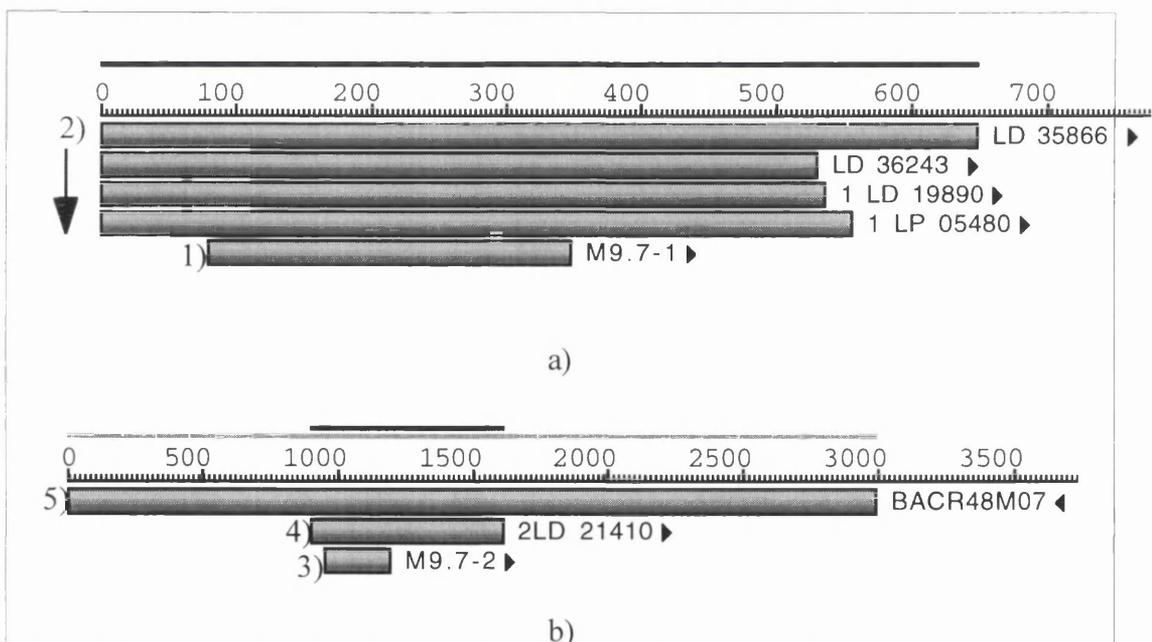


Figure 6.8 **Assemblylign pileup of sequences isolated with homology to *Manduca* M9.7.** Shown is an alignment (default settings) of the putative *M9.7* genes. a) *vhaM9.7-1* (1) and the four ESTs described by the BDGP. (2), b) *vhaM9.7-1* (3) with the genomic BAC clone (5) and the one EST clone (4). The letters next to the EST clones refer to the cDNA library from which they were obtained, LD-embryo, LP-larvae, pupae.

Gene	Location	Genomic clone	No. of ESTs	% homology to <i>Manduca</i> protein
<i>vhaM9.7-1</i>	78E	AC017581	3	63
<i>vhaM9.7-2</i>	64B	BACR48M07	1	50
<i>vhaM9.7-3</i>	64B	AAF47874	0	60
<i>vhaM9.7-4</i>	89E	AAF55365	0	47

Table 6.3 **Summary of data obtained by homology searching of the BDGP with the *Manduca* M9.7 protein.** Sequences were isolated using a tBLASTn search using the yeast *Manduca* M9.7 as input sequence.

6.4.2 Cloning of DNA sequences

Primers were designed against the putative genes from the EST sequences to clone the coding region. Primer sequences are shown in figure 6.9, and are highlighted in italics on figure 6.10. PCR products cloned into the pCR®2.1 TA cloning vector.

M9.7 1 L	CAA AAT GGT ATC CGA GTG GG
M9.7 1 R	GAC GGT GAC AGC CAT GGT GTC C
M9.7 2 L	AAT CAT GGA AGT TTT CCT AAC
M9.7 2 R	TCC CTC CTT GAT GGG ATT TCC

Figure 6.9 **Primers sequences used to clone the *vhaM9.7-1* and-2 genes.**

Primers were designed against the sequences obtained from the BDGP in order to clone the entire coding region. These primers are highlighted in italics in figure 6.10.

The sequences were translated in MacVector with the translation function, and aligned with the sequence from which they were derived, (figure 6.10). Putative polyadenylation sites for *vhaM9.7-1* are indicated in bold. No such site was observed for *vhaM9.7-2*.

vhaM9.7-1

```

      20          40          60
AAAAAGTTCGGTGATCGCTTTGTCCACCTTTTTATTTTTATTTGCCCAAAGCACTTTGCGTCGATCCT
TTTTTCAAGCCACTAGCGAAACAGGTGGAAAAATAAAAAATAAACGGGTTTCGTGAAACGCAGCTAGGA

      80          100         120
AGAGCCTGC AAAATGGTATCCGAGTGGGTGGCACCAATCGTTATCACCAGCATTGGGCCTTCATTGGC
TCTCGGACGTTTTACCATAGGCTCACCCACCGTGGTTAGCAATAGTGGTCGTAAACCCGGAAGTAACCG
      M V S E W V A P I V I T S I W A F I G>

140          160          180          200
ATCATCTGCCCTTCTTCGCCCAGGACCCAAACAGGGGGGTGACTCAATGCTGCCTGATGCTCACC GCA
TAGTAGACGGGGAAGAAGCGGGTCCTGGGTTGTCCCCCACTGAGTTACGACGGACTACGAGTGGCGT
      I I C P F F A R G P N R G V T Q C C L M L T A>

      220          240          260
GCAACTTGCTGGCTGTCTGGCTGTGCTGTACATGACGCAGCTGAACCCCTCATCGGACCCAAACTA
CGTTGAACGACCGACAAGACCGACACGACGATGTACTGCGTCGACTGGGGGAGTAGCCTGGGTTTGAT
      A T C W L F W L C C Y M T Q L N P L I G P K L>

      280          300          320          340
AGCATGAACGAAATCATGATCATGGCCCGGAGTGGGGCAATGAGATCAAGGACACCATGGCTGTCACC
TCGTA CTGCTTTAGTACTAGTACCGGGCGCTCACCCGTTACTCTAGTT CCTGTGGTACCGACAGTGG
      S M N E I M I M A R E W G N E I K D T M A V T>

      360          380          400
GTCTAATGTCTGTACCCTAATGTATTGTTTCAATTGTTTTGTTATTGTTTCTTTATCCGGATTTTC
CAGATTACAGACAGTGGGATTACATAAGCAAAGTTAACAAAACAATAACAAAGAAATAGGGCCTAAAAG
      V *>

      420          440          460          480
CGACATTCTGTATTATTAATATCCACTTATTGTTCGAAGCATAAAATAAATTGTAACCGCGGTTCGCTCT
CGTGTAAGACATAAATAATTATAGGTGAATAACAAGCTTCGTATTTTATTTAACATTGGCGCCAGCGAGA

      500          520          540
TGGCAAACACGTTGTAGAAAAATCATAGATAAACAACATATTTTTATGCTTTGTACGCGCAAAGATGTAA
ACCGTTTGTGCAACATCTTTTAGTATCTATTTGTTGTATAAAAAATACGAAACATGCGCGTTTCTACATT

ACTTAACGTTGCCTTTTTGGCCAAGCATCCCAATAATCGAGTGTCTTAAAATGAAGATTGAAACCTAA
TGAATTGCAACGAAAAACCGGTTTCGTAAGGGTTATTAGCTCACAGAAATTTACTTCTAACCTTGGATT

      640
AATAAAGTTATATTTACACAATTCTGTGTTC
TTATTTCATATAAATGTGTTAAGACACAAG
```

vhaM9.7-2

```

                20                40                60
CCTGTGGTAATTTGACGTAAATAAAGGAAAGTGGAGAAAATAAGCAACAATCATGGAAGTTTTCTAAC
GGACAGCATTAAACTGCATTTATTTCCCTTCACCTCTTTTATTCGTTGTTAGTACCTTCAAAGGATTG
                M E V F L T>

                80                100               120
AATCGTCTTCTTCACCATCTTCTGGGCGGCGGTGGCGAAGTACGGACCCATTCGTTCACCAAGCAACC
TTAGCAGAAGAAGTGGTAGAAGACCCGCCGCCACCGCTTCATGCCTGGGTAAGACAAGTGGTTCGTTGG
  I V F F T I F W A A V A K Y G P I L F T K Q P>

140                160                180                200
GCACGATGACCTGGTGGCGCTGCATTTTCCCTTCTGACCGCCGTCGTCTGCTGGCTTTTCTGGCTGTGCTG
CGTGCTACTGGACCACGGACGTAAGAAAGGAAAGACTGGCGGCAGCAGACGCCAAAAGACCGACACGGAC
  H D D L V R C I F L L T A V V C W L F W L C C>

                220                240                260
CTATTTGGCGCAACTGAATCCACTGCTGGGGCCGAAACTCAATGGAAACACCATTCGGATCATTCGCTC
GATAAACCGCGTTGACTTAGGTGACGACCCCGGCTTTGAGTTACCTTTGTGGTAAGCCTAGTAACGGAG
  Y L A Q L N P L L G P K L N G N T I R I I A S>

280                300                320                340
GTCCTGGGAAATCCCATCAAGGAGGGATAAGGAAAGCCACATAGGAATCTCCACCTAGCTAATCTT
CAGGACCCCTTAGGGTAGTTCCTCCCTATTCCTTTCGGGTGTATCCTTAGAGGGTGGATCGATTAGAA
  S W G N P I K E G *>

                360                380                400
TGGCGGTTGCTTCTTCCCGTATTTCCGGATCTTAAGCCACATCACACACAGTCGGTGAAACCACGGAA
ACCGCCAACGAAGAAGGCGCATAAAGGCTAGAAATTCGGTGTAGTGTGTGCAGCCACTTTGGTGCCCTT

                420                440                460                480
GCAGTGGCTTCGCGNAACCCTTTTGTACTTCAATACTGGCAGCAAGTGGACAAGGTTGGGCTACGGCT
CGTCACCGAAGCGCNTTGGGAAAAACATGAAGTTATGACCGTCGTTACCTGTTCCAACCCGATGCCGA

                500                520                540
ATCCAAGCCCCAGATACGCCCCACTATTTCCAAACTAACCACATTAACCTCTGGACGTAAACTTTCACT
TAGGTTCCGGGCTATGCGGGGTGATAAAGGTTTGATTGGTGAATTGGAGACCTGCATTTGAAAGTGA

                560                580                600                620
GTACATTTTGTGTTGCTTATAGCTCAATATCTCTGATCAGCATATTTCTTTATGTTTACTGCAAAACAT
CATGTAAAACAACAACGAATATCGAGTTATAGAGACTAGTCGTATAAGAAATACAAATGACGTTTGTGA

                640                660                680
AAGCCATGTATTCACCCAAAAATCCAATTATTGCAATTAATTCTCAACAACTGGCCACATGAGCGA
TTCGGTACATAAGGTGGGTTTTTAGGTTAATAACGTTAAATTAAGAGTTGTTTGAACGGTGTACTCGCT

                700                720
TTTTTGGTAGTCAGATTATTATTTACCTATT
AAAAACCATCAGTCTAATAATAATGGATAA

```

Figure 6.10 DNA and predicted protein sequences of the putative *Drosophila* M9.7 homologues. Protein sequences were generated by MacVector and aligned with the cDNA from which they were generated. Primers used to amplify the sequences are highlighted in italics. Putative polyadenylation sites are highlighted in bold.

Proteins from *Manduca sexta*, human, mouse, *C. elegans* and *Arabidopsis thaliana* were aligned with ClustalW (on default settings) and viewed in SeqVu, showing hydropathy as before (figure 6.11). Two hydrophobic tracts are observed (red/pink) indicating two membrane spanning domains in all of these proteins.

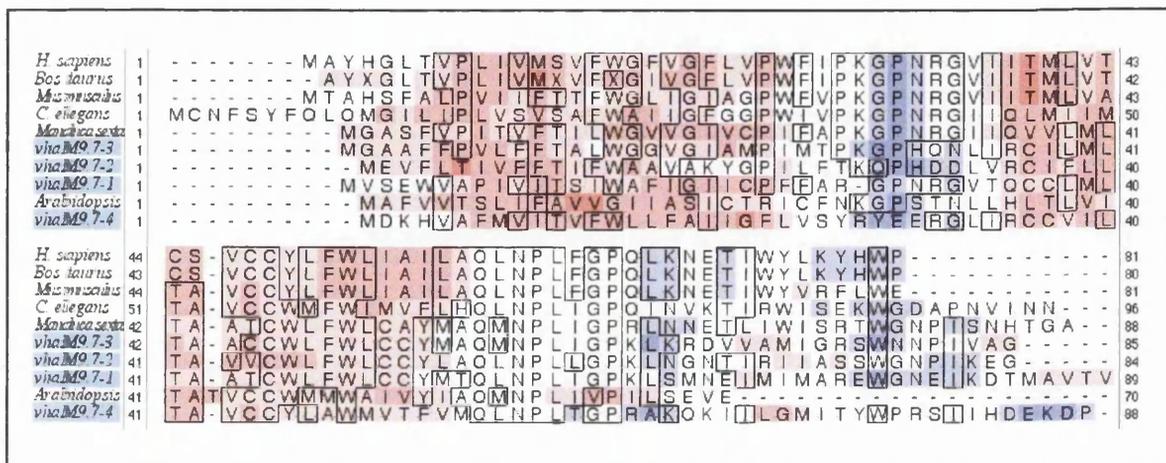


Figure 6.11 **Pile-up of M9.7 and M9.2 V-ATPase subunits from a variety of species.** Sequences were aligned using ClustalwPPC (on default parameters) and viewed with SeqVu. Boxed residues correspond to identity, red areas are hydrophobic and purple are hydrophilic, indicating the protein contains two transmembrane sectors.

Sequence accession numbers were as follows:

M. sexta CAA06822 (Merzendorfer, 1999)

C. elegans Q20591

H. sapiens CAA75571 (Ludwig, 1998)

A. thaliana CAB36517

Mus musculus translated from AW107146

Bos taurus P81103 (Ludwig, 1998)

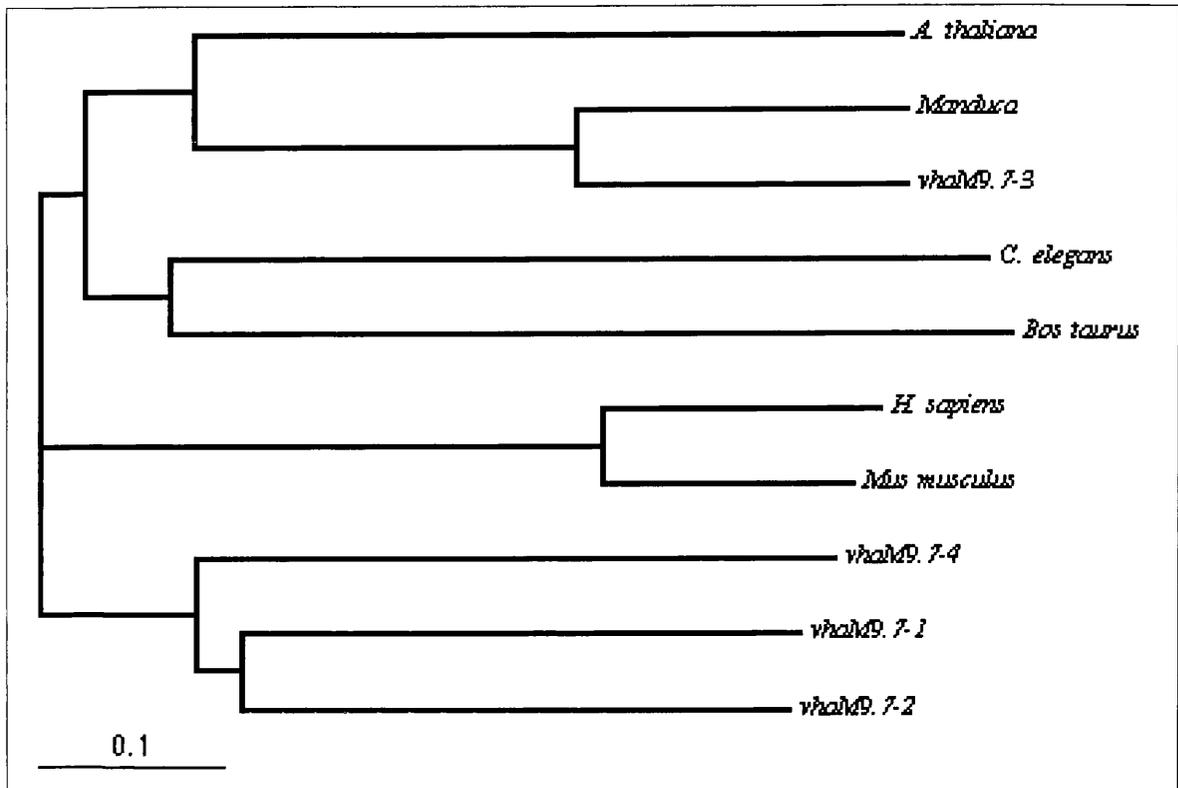


Figure 6.12 **Bootstrap analysis of homologues of M9.7.** Sequences were aligned using ClustalW and viewed with TreeView. As no outgroup could be defined the tree is unrooted.

Bootstrap protein analysis (figure 6.12) shows three of the *Drosophila* proteins, encoded by *vhaM9.7-1*, *-2* and *-4* clustered together with the protein encoded by *vhaM9.7-3*, being most closely related to the *Manduca sexta* protein.

An interesting feature of the protein observed in *Manduca sexta* is that it is extensively glycosylated (Merzendorfer, 1999), however no glycosylation sites are observed in any of the *Drosophila* proteins. The human protein is also thought not to be glycosylated, despite containing a putative glycosylation site (Ludwig, 1998).

6.4 3 RT-PCR

Tissue expression patterns of the *vhaM9.7-1* and *-2* genes were determined by RT-PCR. Tissues were dissected into brain, head, Malpighian tubules, gut, gonad and the remains of the carcass. Results show *vhaM9.7-1* and *vhaM9.7-2* had expression in all the tissues (figure 6.13). The M9.7 primers bracket an intron and the genomic control shows a small amount of genomic DNA contamination in the samples. Again, this genomic contamination problem could be solved by treatment of the mRNA with DNase before DNA synthesis.

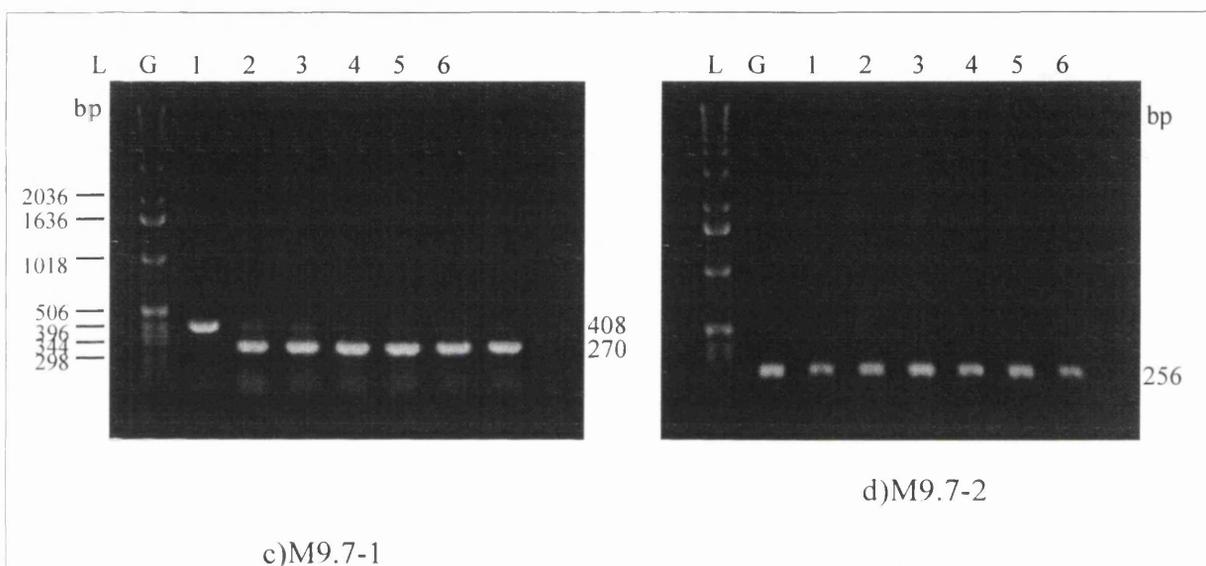


Figure 6.13 RT-PCR of *Drosophila* tissues. *Drosophila* tissue template cDNA from 1) brain, 2) head, 3) Malpighian tubules, 4) gut, 5) gonad, 6) carcass. G) is a genomic DNA control. Panel a) shows *vhaM9.7-1* and panel b) shows *vhaM9.7-2*. L= Gibco BRL 1 kb ladder.

6.4.4 Northern Analysis

A developmental Northern analysis was performed with *vhaM9.7-1* to observe if there is any developmental regulation of this gene (figure 6.14).

When normalised to the *rp49* control there appeared to be no developmental regulation of *vhaM9.7-1* (table 6.4).

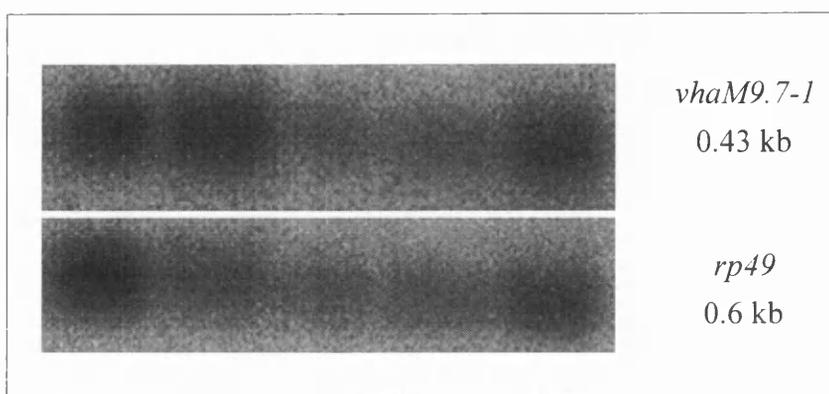


Figure 6.14 **Developmental northern blot.** 25 µg of total RNA from: Lane 1) mixed embryo, 2) mixed larvae, 3) mixed pupae, 4) head, 5) body, were run on a 1% agarose gel, blotted and probed with *vhaM9.7-1* cDNA (top panel). The blot has previously been probed with *rp49* (bottom panel) used as a loading control. Blots were washed to high stringency (0.1x SSC, 0.1% SDS, 65°C) Filters were exposed to a phosphorimage plate for twelve hours and four hours respectively.

Band rp49	PSL rp49	PSL M9.7-1	PSL-BG rp49	PSL-BG M9.7-1	Ratio M9.7-1/rp49
Embryo	3839	2978	3119	2341	0.75
Larva	2471	4230	1751	3424	2.0
Pupa	1762	1773	1042	1061	1.0
Head	2055	2203	1358	1435	1.0
Body	2616	3199	1919	2394	1.24
Background (BG)	702	728	-	-	-

Table 6.4 **Analysis of *vhaM9.7-1* phosphorimage data.**

Intensities of bands (PSL-phosphostimulated luminescence) for each developmental stage probed with *rp49* (column 2) and *vhaM9.7-1* (column 3) were measured in the quantification mode of the MacBAS programme (Fuji). The background reading (BG) was subtracted from each (columns 4 and 5) and the ratios between the two values calculated (column 6).

6.5 Discussion

The V_o transmembrane sector of the V-ATPase has long been known to consist of subunits other than the 16 kDa pore forming subunit, but none of these had been observed in *Drosophila melanogaster*. This chapter describes the isolation of two of these previously undescribed V-ATPase subunit genes, the first of which is homologous to the yeast *PPA1* gene and the second to the *Manduca sexta* M9.7 gene. These subunits were initially observed using the powerful tool of the Berkeley *Drosophila* Genome Project, by tBLASTn searching with the protein sequences. Homologous *Drosophila* sequences were observed for both subunits.

With *PPA1*, two separate sequences were found in tandem on a single BAC clone. These genes were named *vhaPPA1-1* and *vhaPPA1-2*. PCR primers were designed to perform RT-PCR to determine whether these genes were indeed transcribed. RT-PCR data indicated the genes were expressed in many tissues throughout the fly. For *vhaPPA1-1* a developmental Northern showed that this gene is expressed throughout the life cycle of the fly

The BDGP EST project then corroborated this data in the case of *vhaPPA1-1* by sequencing a series of ESTs, proving that this genes at least is expressed. No ESTs however have appeared for *vhaPPA1-2*.

The deduced protein sequences were then aligned with homologues from other organisms to observe their similarity. As with many other V-ATPase subunits, a high degree of homology is maintained between the species. The *Drosophila* sequences maintain the five transmembrane regions and also contain the predicted DCCD reactive site observed in *ppa1p* in yeast.

The existence of *PPA1* in *Drosophila* is unsurprising as this protein has now been implicated in forming the proton pore with 16 kDa protein in *S. cerevisiae*. (Powell, 2000).

In situ hybridisation needs to be carried out with these sequences to observe in what cell types they are expressed and it would also be of benefit to study the 5' and 3' sequences of these sequences to observe whether they share similar promoter/enhancer sequences with each other and with other V-ATPase encoding genes.

The M9.7 protein has not been well described and it's function is so far unknown, however data shows that it is indeed a true subunit of the V-ATPase holoenzyme in *Manduca sexta* (Merzendorfer, 1999). and humans. (Ludwig, 1998) having cloned the human M9.2 protein (a slightly smaller homologous protein) suggested a structural

similarity to vma21p, a protein required in yeast for V-ATPase assembly, but which is not part of the holoenzyme itself.

The *Manduca* protein sequence was used in a tBLASTn search of the BDGP sequence database and initially two regions of homology were observed, both of which had associated ESTs. RT-PCR showed they were transcribed in all of the tissues studied, except for *vhaM9.7-2* which gave no product in the brain. Much later in this project two other homologous, but not identical ESTs were sequenced by the BDGP. All of these *Drosophila* proteins appear to contain two predicted membrane-spanning domains

An interesting observation is that none of the *Drosophila* homologues of M9.7 have glycosylation sites, unlike the *Manduca* M9.7 protein which is extensively glycosylated. However (Ludwig, 1998) suggest that the M9.2 protein is also not glycosylated.

Again, *in situ* hybridisation with these sequences would have to be carried out along with promoter/enhancer studies.

Antibodies exist against the *Manduca* M9.7 protein and it would be interesting to observe their localisation in immunocytochemistry.

Summary

The 16 kDa subunit of the vacuolar H⁺-ATPase is a multifunctional protein, functioning, as a hexamer, in the V-ATPase, mediators and gap junctions. This project aimed to dissect these various roles by mutagenesis, therefore invoking a reverse genetic approach.

When this project was initiated this protein was thought to be encoded by a single gene, but this work, and that of the *Drosophila* Genome Project, showed us that this important assumption was incorrect.

Although this work was complicated by the size of this gene family, the aims of this project were broadly fulfilled by a mutagenesis and phenotype analysis.

Mutagenesis of *vha16-1*.

A novel screening technique was invoked to isolate lethal P-element mutations in the *vha16-1* gene. This involved screening of a series of plasmid rescued lines, rescued from random P-element insertions on the second chromosome. Three screens of these different pools, by probing Southern blots with a genomic probe, could lead to the isolation of a single P-element insertion line. This study isolated two hybridising lines, one of which, 16/1, upon sequencing, proved to be inserted within *vha16-1*.

Although the P-element line isolated in *vha16-1* was lethal, the fact still remained that this lethality could have been caused by a second lethal site within the genome, as is often the case with P-element mutagenesis. The lethal nature of the element can be identified by its excision, and observing whether the lethality is reverted. This excision was undertaken with line 16/1, and lethality was reverted. This could be observed directly by the existence of straight-winged flies which did not require a balancer chromosome for survival. Excision was confirmed by PCR showing the allele reverting back to its original size.

As one would expect when inactivating a multifunctional gene, lethality occurs in the embryo. This is comparable to the lethal phase observed for mutations in other V-ATPase genes, *vha55* and *vha67*, which are larval lethal, with the dying larva exhibiting the phenotype of clear Malpighian tubules. Although tubules are formed in late embryos no similar phenotype was observed for line 16/1 as the embryos died too early. Embryos contain maternal copies of the V-ATPase genes, which are diluted as cell division takes place. Vha16 functions as a hexamer, and has multiple roles, therefore the maternal proteins could be exhausted earlier in development than the other subunits.

The P-element excision resulted in two imprecise excision lines, with P-element sequences being left in the genome. One of these lines exhibited a tubule

secretion phenotype, with basal secretion rates being higher than those of control flies, however, upon stimulation with CAP_{2b}, the rates rose to similar values. The other line, while having no tubule secretion phenotype, exhibited a failure to thrive upon propagation of the homozygotes.

At this point in the project, the genome project sequence data indicated the presence of alternative V-ATPase genes in the genome which could potentially encode the proteolipid.

Rescue of the P-element Mutant Phenotype.

The theory behind this work was to re-introduce the wild-type *vha16-1* cDNA, two proteolipid subunits from other organisms, and two of the newly discovered *Drosophila* proteolipids, back into the mutant background to rescue the P-element's lethal phenotype. This at first seemed straightforward, as subunits from two alternate species, *Nephrops norvegicus* and *Drosophila melanogaster* had rescued the yeast phenotype, however it was later observed that this rescue was only partial. The data showed the *Nephrops* and *Manduca* proteins failed to rescue. This could have been for several reasons, either the proteins from a different species do not function in *Drosophila*, the overexpression of this protein was toxic, or more likely, the control of expression was far too crude.

However, the lack of rescue may well indicate that these genes are not as interchangeable as initially thought, as the extreme sequence conservation would imply. This is borne out by the rescue attempts in yeast of the *Drosophila* proteins. Also, the rescue sequences were under control of a heat-shock promoter which was only activated once a day. Finer gene control is probably required in a higher organism such as *Drosophila* where the V-ATPase functions in many cellular systems.

The EST data showed the existence of three 5' splice variants for *vha16-1*, these 5' sequences were lost in the rescue as only the minimal coding areas had been cloned. Therefore vital targeting information may have been lost.

The *vha16* Gene Family

The work of the Genome Project led to the isolation of several DNA sequences which would putatively encode 16 kDa proteolipids. These sequences, while lacking introns, appear to contain complete open reading frames. Do any of these sequences compensate for the original? It is possible that they do in some cell types, but they do not compensate globally as the P-element insertion is lethal. Equally, these sequences themselves may be vital and expressed in a spatio/temporal pattern that cannot be replaced by an alternative subunit. However, there are no known genetic aberrations in these areas, such as P-elements, which could be utilised to knock out these sequences and resolve these questions.

Recently published data (Kennerdell, 2000), showed how genes can be silenced in *Drosophila* by germline transformation of constructs containing hairpin looped double-stranded RNA sequences. These are stably inheritable and under *UAS* control, therefore can be expressed in selected cells by crossing to GAL4 expressing fly lines. This allows selective deletion of genes in particular cell types and therefore could be a useful way to knock-out the new *vha16* genes.

Discovery of New V-ATPase V_o Sector Proteins in *Drosophila melanogaster*

It had been observed in other organisms that the V_o sector of the V-ATPase contained subunits other than the 16 kDa pore-forming subunit.

One of these proteins, ppa1p was initially observed in *S. cerevisiae* and is thought to contribute to pore formation. This work, with the aid of the BDGP, led to the isolation and partial characterisation of two genes which prospectively encode this protein.

Another V_o sector protein identified in *Drosophila melanogaster* by this work, was the small transmembranous M9.7 subunit. Four prospective genes encoding this protein were identified and two were partially characterised.

Future Work

Future work, leading from these experiments would include:

In situ hybridisation to observe whether the *lac Z* staining results for *vha16-1* and the other V-ATPase subunits coincide with the real expression patterns.

Determining whether the 5' splice variants observed from the EST data are real and not as results of mis-splicing. This could be undertaken by RT-PCR, Northern blotting and *in situ* hybridisation to observe if particular cell types express particular isoforms.

The new members of the *vha16* gene family need to be characterised in much more detail. *In situ* hybridisation would determine whether they are expressed in a particular cell type or time point. Expression of the proteins in *Drosophila* cell culture would allow us to observe whether these subunits, either on their own or in a complex with the original protein, could form functional proton pores. The surrounding genomic regions of these DNA sequences have to be studied to see if they contain the promoter and enhancer sequences required for transcription of *Drosophila* genes. As no genomic aberrations are present in the genomic regions of these genes it would be useful to inactivate them e.g. by RNA interference, to observe if this would cause lethality.

The rescue of the mutant phenotype could be attempted again, using the entire genomic sequences of the various DNAs, allowing correct temporal and spatial expression of these genes. It would be useful to try to transform the C-terminal GFP construct again (and also synthesise an N-terminal fusion construct) to observe where this protein localises to within the *Drosophila* cells to hopefully finally answer the V-ATPase/Gap junction conundrum.

The V-ATPase subunits PPA1 and M9.7 also need further characterisation, again by *in situ* hybridisation to observe their expression patterns. Again, analysing the promoter regions of these genes would give us an insight into their transcription patterns and if they share similarities with the promoter regions of other V-ATPases genes. It would also be of interest to knock-out these sequences to see if this causes lethality in the organism. Examining the gene region of the *vhaPPA1* genes (88D) shows the existence of several P-element in the area. It would be advantageous to obtain these and attempt to mutagenise the genes by local jumping and observe the effects. P-element insertions also exist near the *vhaM9.7* genes and this inactivation by P-element insertion would also apply.

Appendices

Appendix 1: *Drosophila Media*

Standard growth media	per litre of water 10 g agar 15 g sucrose 30 g glucose 35 g dried yeast 15 g maize meal 10 g wheat germ 30 g treacle 10 g soya flour
Grape juice agar.	per litre of water 20 g agar 52 g glucose 26 g sucrose 10 g dried yeast 10% (v/v) red grape juice 1% (v/v) Nipagin
Microinjection media	per 100 ml of water 1.5 g agar 0.3 g NaCl boil and allow to cool before adding 3 ml glacial acetic acid 5 ml 100% ethanol

Appendix 2: *Escherichia coli* growth media

L-broth	per litre of water
	10 g Bacto-tryptone
	5 g yeast extract
	10 g NaCl
L-agar	per litre of water
	10 g Bacto-tryptone
	5 g yeast extract
	10 g NaCl
	15 g Bacto-agar
SOC broth	2% tryptone
	0.5% yeast extract
	10 mM NaCl
	2.5 mM KCl
	10 mM MgCl ₂
	10 mM MgSO ₄
	20 mM glucose

Appendix 3: Oligo Labelling Buffer

5x OLB

To a tube of Pharmacia (50 OD units) hexamers add:

550 ml TE

917 ml 2 M HEPES (pH 6.6)

350 ml Solution Φ *

6.6 ml b-mercaptoethanol

1.85 ml of 100 mM stock of dATP, dGTP, dTTP

Store in 100 ml aliquots at -20°C.

*Solution Φ

1.25 mM Tris (pH 8)

0.125 mM MgCl₂

Appendix 4: Primers Sequences Not Included in Main Text

pUAST seq	GAA ATC TGC CAA GAA GT
pCRII Forward	AGC TCG GAT CCA CTA GTA
pCRII Reverse	CTC GAG CGG GGC CCA GTG
P₃₁	CGA CGG GAC CAC CTT ATG TTA TTT CAT CAT G
P_R	AGC ATA CGT TAA GTG GAT GTC TC
16/1 P-el_L	CCC TCG CCT TTT GCT CTT TG
16/1 P-el_R	AAT GAG TGG TCT TGA TAA GCC C
vha55L	AAT TCT CGA AAT TTG CAG TGC AC
vha55R	GAA TTG GGG GTC CCT TGT CGA TG
DROS/GFP Seq	TGG GCA TGA TCC TGA TTC

Appendix 5: List of ESTs corresponding to *vha16-1*

ESTs obtained from the BDGP. ESTs correspond to consensus sequence two except for those prefixed by -1 or -3. The letters indicate from which library the ESTs were obtained:

- LD embryo
- GM ovary
- HL adult head
- GH adult head
- LP larvae, pupae
- SD Schneider cells

1 GH 07362	LD 30434	LD 28434
1 GH 09407	LD 32987	LD 28587
1 GH 14086	LD 33934	LD 29125
1 GH 28772	LD 34563	LD 29221
1 LD 31888	GH 07362	LD 30434
1 LD 34887	GH 09407	LD 32987
GH 01476	GH 14086	LD 33934
GH 03503	GH 28772	LD 34563
GH 04092	LD 31888	LD 38224
GH 04751	LD 34887	LD 38607
GH 08505	GH 01476	LD 38814
GH 12266	GH 03503	LD 40981
GH 15892	GH 04092	LD 43604
GH 24719	GH 04751	LD 43958
GH 25406	GH 08505	LD 46975
GM 06270	GH 12266	LD 47662
GM 07432	GH 15892	LD 15895
GM 10427	GH 24719	LP 02050
LD 01123 3'	GH 25406	LP 02281
LD 02872	GM 06270	LP 03043
LD 03026	GM 07432	LP 03265
LD 04667	GM 10427	LP 05074
LD 07239	LD 01123 3'	LP 05877
LD 07287	LD 02872	LP 10107
LD 07692	LD 03026	LP 11794
LD 09023	LD 04667	SD 02719
LD 09526	LD 07239	SD 10440
LD 09925	LD 07287	3 GH 01415
LD 10243	LD 07692	3 GH 28014
LD 11173	LD 09023	3 GM 07745
LD 11440	LD 09526	3 HL 01915
LD 13991	LD 09925	3 LD 20064
LD 15372	LD 10243	3 LD 29725
LD 17008	LD 11173	3 LD 33235
LD 21204	LD 11440	3 LD 35744
LD 23401	LD 13991	3 LP 02093
LD 24727	LD 15372	3 LP 05450
LD 28434	LD 17008	3 SD 03710
LD 28587	LD 21204	3 SD 05981
LD 29125	LD 23401	3 SD 07890
LD 29221	LD 24727	

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