Cloning, Characterisation and Site-selected P-element Mutagenesis of Genes Encoding V-ATPase in *Drosophila*

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

By

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September 1996

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Abbreviations

AA	amino acid(s)
ATP	adenosine triphosphate
ATPase	ATP hydrolysing enzyme
BCIP, X-phosphate	5-bromo-4-chloro-3-indoyl-phosphate
bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary DNA
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DNA	2' deoxyribonucleic acid
DNase I	deoxyribonuclease I
dATP	2' deoxyadenosine triphosphate
dCTP	2' deoxycytidine triphosphate
dGTP	2' deoxyguanosine triphosphate
dNTP	2' deoxy (nucleotide) triphosphate
dTTP	2' deoxythymidine triphosphate
dUTP	2' deoxyuridine triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid (disodium salt)
EtBr	ethidium bromide
g	gram
g	centrifugal force equal to gravitational acceleration
h	hour
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
IPTG	isopropyl-b-D-thio-galactopyranoside
kb	kilobases
kDa	kiloDaltons

Klenow	Klenow fragment of *E. coli* polymerase I
l	litres
M	molar
mg	milligram
mM	milliMolar
min	minutes
ml	millilitres
MOPS	3-morpholinopropanesulfonic acid
mRNA	messager RNA
ng	nanograms
nM	nanmolar
nm	nanometres
NTB	4-nitro blue etrazolium chloride
OD	optical density
ORF	open reading frame
PCR	Polymerase chain reaction
PEG	polyethylene glycol
pH	acidity [-log10(Molar concentration of H^+ ions)]
polyA$^+$	poly adenosine tailed RNA molecule
ppi	pyrophosphate
RNA	ribonucleic acid
RNase A	ribonuclease A
RP49	ribosomal protein 49 (*Drosophila*)
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
Tris	Tris (hydroxymethyl) aminomethane
tRNA	transfer RNA
UTR	untranslated region
U	units

UV	ultraviolet
V-ATPase	vacuolar H^+-transporting adenosine triphosphatase
vha14	gene encoding V-ATPase F-subunit in *Drosophila.*
vha26	gene encoding V-ATPase E-subunit in *Drosophila.*
vha68-1	gene encoding V-ATPase A-subunit in *Drosophila.*
vha68-2	gene encoding V-ATPase A-subunit in *Drosophila.*
Vol	volume
Xgal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
µCi	microCuries
µl	microlitres
µg	micrograms
3'	three prime
5'	five prime

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Acknowledgements

I wish to take this opportunity to thank Dr. Kim Kaiser and Dr. Julian A. T. Dow for the excellent supervision, the critical reading and correcting this thesis. Their patience and generosity throughout all the years is greatly appreciated.

I would like to thank the past and present members of KK group, JATD group and media ladies. Special thanks would go to Ann Gillan and Xueqiong (Sally) Zhang for help with plasmid rescue; Zhongsheng (Susan) Wang for *in situ* hybridisation of polytene chromosome; Peggy Ennis for mutant screening; and James Clark for reading the manuscript. Great thanks are also due to Dr. Kevin O'Dell, Dr. Minyao Yang, Dr. Yong Yu, Dr. Douglas Amstrong, Andy Mousey, Xin (Cindy) An, Audrey Davidson, Dr. Philippe Rosay, Dr. Shireen Divies, David Kelly, Ali Sozen and Shirley Graham for the many valuable discussions and technical tips.

I thank Professor Helmut Wieczorek (in Germany) for kindly providing the *Manduca* cDNAs; Dr. Istvan Kiss and Dr. Peter Deck (in Huangary) for providing the thousands of P-element fly lines.

Thanks also go to Professor R. Wayne Davies and Dr. Richard Wilson for being my Auditors, their suggestion "you should stop bench work as soon as possible " is highly appreciated.

Thanks all those people in the Division of Molecular Genetics, IBLS, University of Glasgow, who provide help and support during this work.

Summary

Over the last few years, thousands of lines carrying lethal P-element insertions have been produced by the *Drosophila* community, which must presumably have inactivated a large number of essential genes. This thesis describes a fast and efficient approach to correlating cloned genes with mutant fly lines carrying P[*lacW*] insertions in the second chromosome (Török *et al.*, 1993). We have made use of the fact that P[*lacW*] contains a plasmid replicon to establish a collection of rescued plasmids containing genomic DNA flanking sites of transposon insertion. Plasmids representing a total of 1836 lines were individually rescued, and pooled in batches of 10 and 100. Pools of 100 plasmids were screened by hybridisation with cDNAs corresponding to cloned second chromosome loci. Hybridising pools were then narrowed down to single plasmids by a process of subdivision and rehybridisation, and corresponding mutant lines were obtained. Initial screening with 40 cDNAs has detected positive hybridisation for more than 10 genes. Mutations for 7 genes have been confirmed, of which insertions in genes encoding the A and c subunits of *Drosophila* V-ATPase are included.

V-ATPase is a proton pump made of multiple subunits. The genes and cDNAs for A, E, and F subunits of V-ATPase have been cloned from *Drosophila melanogaster via* homology with the corresponding *Manduca sexta* genes. *vha68-1* and *vha68-2,* genes encoding two isoforms of V-ATPase A subunit, have also been isolated and sequenced. Both isoforms are composed of a polypeptide of 614 amino acids with a predicted molecular mass of 68.4 kDa and 68.3 kDa respectively. The *vha68-2* gene is punctuated by four introns. The chromosomal location of both genes is at 34A on the second chromosome. Northern analysis of total RNA reveals that both isoforms are expressed in a similar pattern. They are ubiquitously expressed in head, thorax and abdomen of the adult fly. Developmental Northern blots of embryo, larvae, pupae and adult total RNA show general expression, but at a much reduced level during metamorphosis. A fly line (25/8) carrying a single P[*lacW*] insertion in *vha68-2* was

isolated by screening pools of rescued plasmids. The transposon is inserted into the first intron, in front of the translation start codon of *vha68-2*. The enhancer detector reporter gene carried by the P-element (β-galactosidase) was generally activated, but particularly strongly in the gut and Malpighian tubes of both larvae and adults. The insertion largely reduces the transcript of the *vha68-2* isoform which leads to a homozygous lethal phenotype at first instar larvae. The homozygous lethal phenotype can be reverted by 'jumping out' the insertion. Imprecise excision or internal deletion of the P-element created a set of novel hypomorphic or null alleles, with phenotypes which range from the first instar larvae lethal, as in the original P-element insertion line, to sub-lethals of different phenotype.

A gene and a cDNA encoding the E subunit of V-ATPase have been characterised. The gene contains three small introns. Its deduced translation product has 226 amino acids and a molecular weight of 26.1 kDa. *vha26* is present as a single copy at cytological position 83B1-4 on the third chromosome and gives rise to an mRNA species of 2.3 kb, with an expression pattern similar to that of *vha68*. A fly line carrying a single lethal P[*lacW*] insertion within *vha26* gene has been identified.

The deduced translation product of the cDNA (*vha14*) for the F subunit is a 124 amino acid polypeptide with a molecular mass of 14 kDa. *vha14* is present as a single copy at cytological position 52B on the second chromosome, and gives rise to an mRNA species of 0.65 kb. Unlike *vha68* and *vha26*, the *vha14* transcript shows relatively little variation during development and between adult head, thorax and abdomen, suggesting that the F subunit is a relatively ubiquitous component of the V-ATPase.

Chapter 1

Introduction

1.1 *Drosophila melanogaster*

The fruit fly *Drosophila melanogaster* has a lot to offer as an experimental organism. It has a distinguished history as a subject of classical genetic analysis. Many of the major principles of genetics, principles that we tend to take for granted, were established by work with *D. melanogaster* (Ashburner, 1989b). A large number of easily recognisable genetic markers, a generation time of only 10 days, simple culture methods and a large body of literature and technical information are readily available to the investigator. Additionally, establishing the chromosomal location of a newly-cloned gene is particularly straightford, as the salivary gland polytene chromosomes are large and easy to map. This means that a newly discovered gene can be reconciled rapidly with the sum of existing knowledge of the *Drosophila genome* (Dow, 1994; Dow *et al.*, 1996). Transposable elements, and in particular the enhancer trap P-element, have played a pivotal role as mutagens, as molecular tags, and as germ-line transformation vectors (Rubin, 1988; Kaiser, 1995; Sentry and Kaiser, 1995). *D. menalogaster* is now widely used not only in classical and molecular genetics but also in research on more complex phenomena, such as those of developmental biology and neurobiology.

My PhD project will use *Drosophila* to address the issues of (i) systematic site-selected P-element mutagenesis of second chromosome genes and (ii) the molecular genetic analysis of genes encoding V-ATPase subunits.

1.2 The P-element of *Drosophila*

A large number of transposable elements are known to exist in *Drosophila melanogaster*, of which the P-element family is the most heavily exploited. P-element technology has revolutionised *Drosophila* molecular genetics, not only in terms of providing important insights into the mechanism of eukaryotic transposition, but also use as important tools for gene transfer, insertional mutagenesis, enhancer trapping and gene cloning (See Kaiser, 1990; Kaiser, 1993 and Kaiser *et al.*, 1995 for recent reviews).

1.2.1 P-element Biology

P-elements are a family of transposable elements found in *Drosophila melanogaster*. They have been shown to be the causal agents of P-M hybrid dysgenesis, a syndrome whose traits include high rates of sterility, mutation, and chromosomal rearrangements (Engels, 1987; Engels, 1989; Rio, 1990). P-element transposition is genetically regulated, occurring at very high frequency only in the progeny from a cross between males of a 'P strain' and females of an 'M strain'. The distinguishing characteristics of P strains are that their eggs have "P cytotype", a condition that results in repression of P-element transposition, and that they carry autonomous 2.9 kb full-length P-elements which encode transposase. Transposition in a P- strain is repressed by a product of the full-length P-element itself, thus the P-element is normally quiescent but becomes highly mobile in the progeny of females that lacks repressor (Black *et al.*, 1987; Engles *et al.*, 1990). M strains, by contrast, lack autonomous P-elements, and lay eggs that are permissive for P-element transposition (M cytotype). No dysgenic traits are observed in the progeny of the reciprocal M male by P female cross or in the progeny from P x P or M x M crosses. Moreover, as transposition is restricted to cells of the germline, phenotypic results are not observed until further generations.

The first P-element to be cloned was a defective element, identified by virtue of having disrupted the *white* locus. The defective element was then used as a molecular probe to clone a complete element which was further confirmed for its transpositional activity when injected into embryos of a M strain - it transposed from a plasmid into the *Drosophila* genome (Spradling *et al.*, 1982). Molecular analysis indicated that the P-elements present in P- strains could be divided into two classes; a class of full-length 2.9kb elements and a heterogeneous class of internally deleted P-elements (Figure 1.1). P-element sequences required in *cis* for transposition are contained within 138 bp at the 5' end and 150 bp at the 3' end. These include 31 bp terminal inverted repeats. Full-length P-elements include four long open reading frames encoding an 87 kDa transposase, the activity of which is restricted to the germline due to differential splicing because the third intron is not removed in somatic cells. (Rio, 1991; Handler *et al.*, 1993). An element with the third intron removed (Δ2,3) is able to transpose in somatic cells but lacks the capacity to establish a P- cytotype (Laski, *et al.*, 1986). Internally deleted elements of various lengths can occur in both P- strain and M strains as well. Though unable to produce active transposase, such elements can nonetheless be mobilised in the presence of full-length elements.

When P-elements transpose they excise from the donor site and leave behind a double-stranded break, repair of which appears to require a template (Figure 1.2; Engels *et al.*, 1990; reviewed by Sentry and Kaiser, 1992; Weaver, 1995). Excision of the P-element can either be 'precise' or 'imprecise'. The phenomenon of precise and imprecise excision could be explained by a double-stranded break repair model (Engels *et al*, 1990; Gloor *et al.*, 1991; Daniels and Chovnick, 1993). Sister chromatids or homologous chromosomes of the broken molecule are used as templates for repair. If the template contains the P-element, double stranded repair will mostly produce a chromosome identical in appearance to the donor chromosome prior to transposition. In such a case, P-element sequences seem to have been retained at the donor site. In a few cases, however, repair can be interrupted, resulting in the generation of nonautonomous P-element deletion

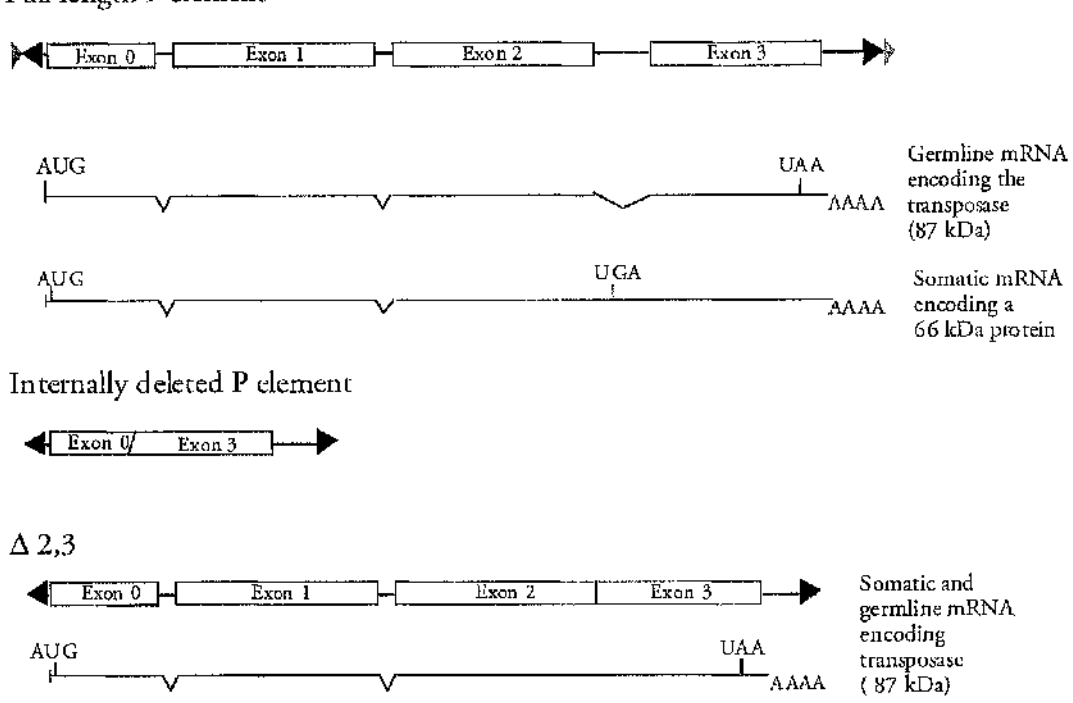


Figure 1.1 Structure of P-elements. The full length 2.9 kb P-element has four long ORF's separated by introns. The P-element is bounded by 31 bp inverted repeats (large arrowheads). Insertion of a P-element causes an 8 bp target site duplication (Small arrowheads). Germline transcripts, spliced as shown, provide functional transposase. Somatic transcripts, which retain the intron between exon 2 and 3, encode a prematurely truncated and thus non-functional transposase. Internally deleted P-elements do not produce functional transposase and thus non-autonomous, but they retain *cis*-acting determinants that allow their mobilisation in the presence of a transposase source. Δ2,3 elements, from which the third intron has been removed by *in vitro* manipulation and produce transposase in both germline and somatic tissues (Diagram kindly provided by Dr. Kim Kaiser).

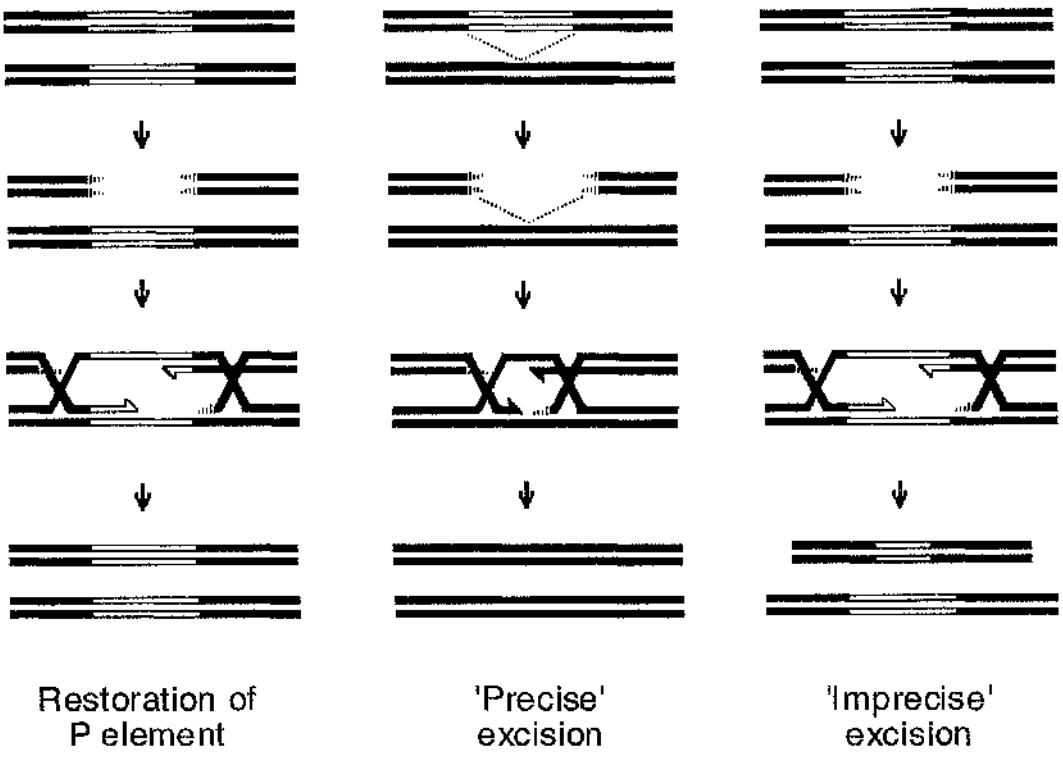


Figure 1.2 Model for template-dependent gap repair following P-element excision. Excision of a P-element (open bars) induces a double-strand break that can be subject to widening by exonucleases. Free 3' ends invade the template duplex, which serves as a substrate for DNA synthesis. In the left panel, the template is a second copy of the P-induced allele, most commonly provided by a sister chromatid. The result is restoration of a P-element at the locus. Less frequently, the template can be a wild-type allele present on a homologous chromosome (centre panel). This will give the impression of precise excision. Interruption of the repair process, in this case where the sister chromatid is the template, followed by pairing of partially extended 3' ends, may give the impression of an 'imprecise excision' (right panel). This can take the form of internal deletion of the P-element, or more extremely a deletion that extends into flanking DNA, usually when the template is a wild-type allele present on a homologous chromosome. (Diagram kindly provided by Dr. Kim Kaiser).

derivatives. A different result is obtained if the template does not contain the P-element (i.e. is a wild-type allele) at the site corresponding to the P-element donor site. In this case, double stranded break repair restores the donor site to its wild-type pre-insertion sequence; thus appearing as if the P-element had excised precisely from the donor site. Loss of sequences flanking a P-element, together with some or all of the element itself, would result from incomplete repair of a gap that had been widened by exonuclease activity. The involvement of double-strand gap repair was also suggested by the fact that reversion frequencies for heterozygous P-element insertion mutants are 100 times higher than those for homozygous mutants (Engels *et al.*, 1990).

1.2.2 Germ-line transformation

Introduction of cloned and manipulated genes into the germline DNA is a valuable tool for analysing many problems in *Drosophila* molecular genetics. The P-element transposon was first engineered as a transformation vector and used for the generation of transgenic flies by Rubin and Spradling in 1982. A plasmid construct bearing a nonautonomous P-element, into which the gene of interest had been inserted, was injected into embryos undergoing the transition between syncitial and cellular blastoderm (Figure 1.3). P-element DNA injected into the pole region can become internalised during cellularisation, and can transpose to the genome. Transposition is not frequent on a per molecular basis, but nonetheless provides acceptable transformation efficiencies. Newly integrated elements in the germ cells will be inherited by the progeny of individuals that survive the injection.

An autonomous P-element provides its own transposase. P-elements engineered as vectors dispense with this ability, but retain sequences required in *cis* for transposition. In this respect they resemble the defective elements (Kaiser *et al.*, 1995). It is therefore necessary to provide transposase from another source. Transpose can be supplied in a number of ways: co-injection of an element that produces transposase but that cannot itself

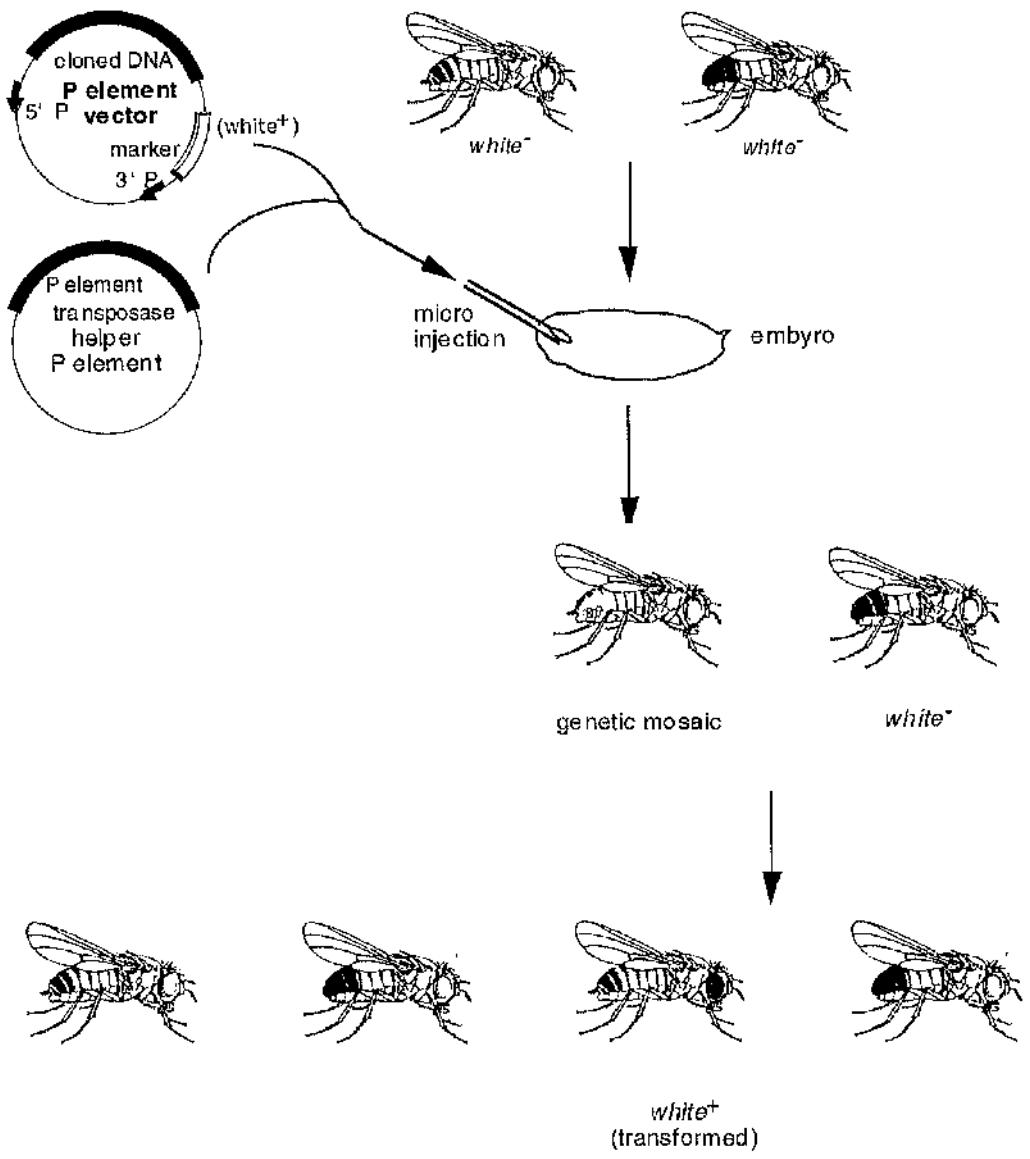


Figure 1.3 Germ-line transformation. A plasmid construct bearing a nonautonomous P-element, into which the gene of interest has been inserted, is injected into young M cytotype embryos prior to the cellularisation of the germline. P-element DNA injected into the embryo can become internalised during cellularisation, and can transpose to the genome. Transformed individuals can then be recovered in the surviving progeny; usually the transposon of interest carries a phenotypic marker to allow identification of transformations. (Diagram kindly provided by Dr. Kim Kaiser).

transpose - e.g. a wings-clipped element (Karess *et al.*, 1984); co-injection of purified transposase (Kaufman *et al.*, 1991); injection of the a construct into embryos that express transposase endogenously, such as the carrying the P[*ry*$^{+}$ Δ2,3] (99B) element which generates high levels of transposase activity without establishing a P cytotype. Generation of a line with a stable insertion of the construct requires selection against Δ2,3 in a subsequent generation. A dominant marker on the P[ry+Δ2,3] (99B) chromosome makes it possible to select stable transformed progeny that have lost the transpose source by segregation. Transformed individuals can then be recovered in the surviving progeny, and usually the transposon of interest carries a phenotypic marker to allow identification of transformants. Markers that rescue a visible phenotypic defect, such as loss of eye colour (rosy, white, vermilion), loss of body pigmentation (yellow), or abnormal eye morphology (rough) are easily scored (Bingham *et al.*, 1989; Ashburner, 1989b; Fridell *et al.*, 1991; Patton *et al.*, 1992; Lockett *et al.*, 1992). Alternatively, *adh* and neomycin-resistance genes confer the ability to survive on selective media (Goldberg *et al.*, 1983; Steller *et al.*, 1985). The frequency with which transformants are recovered appears inversely related to transposon length (Spradling, 1986). Nonetheless, transformation with cosmid sized pieces greater than 40 kb can achieved (Haenlin *et al.*, 1985).

There can be pronounced position effects on the expression of genes contained within a P-element transformation construct. It is advisable to obtain lines containing a number of independent insertions. These can be generated either as primary transformants, or *via* remobilisation of a construct by a cross that provides Δ2,3. P-element transposition is non-random with respect to insertion site. Moreover, sequences contained within a P-element construct can have a pronounced effect on insertion specificity (Kassis *et al.*, 1992). Markers in the P-element can themselves be sensitive to position effects. Levels of marker expression may be a useful guide to whether a transgene will be expressed at a reasonable level (Kaiser *et al.*, 1995).

Other transposable elements, such as *hobo, minos*, have been successfully transferred into germ-line of *Drosophila* (Blackman *et al.*, 1989; Loukeris *et al.*, 1995a). And a transposable element in *Drosophila hydi* has been transferred into medfly (Loukeris *et al.*, 1995b).

Germ-line transformation experiments have had two major impact on *Drosophila* molecular genetics: firstly, P-element vectors can be used to transform cloned genes to rescue a mutant phenotype to prove that a DNA fragment carries the corresponding gene; secondly, genes manipulated *in vitro* can be reintroduced into the animal and its biological consequences assayed *in vivo*.

1.2.3 Remobilisation of P-elements

Three events (local jumping, precise and imprecise excision) would happen when the P-element was supplied with a transposase:

Local jumping

Recent evidence indicates that mobilisation of P-elements in the female germline leads to a high frequency of insertion within a hundred kb or so of the donor site (Tower *et al.*, 1993; Zhang *et al.*, 1993). P-element transposition is not always accompanied by loss of the donor element (Golic, 1994; Johnsonschlitz *et al.*, 1993). It may thus not be easy to score a local jump based on the marker that the transposon contains. Site-selected mutagenesis by PCR may be the most efficient approach (Kaiser *et al.*, 1990; Littleton *et al.*, 1993). In case of more than one P-element, segregation might separate the insertion of interest from others (Kaiser *et al.*, 1995).

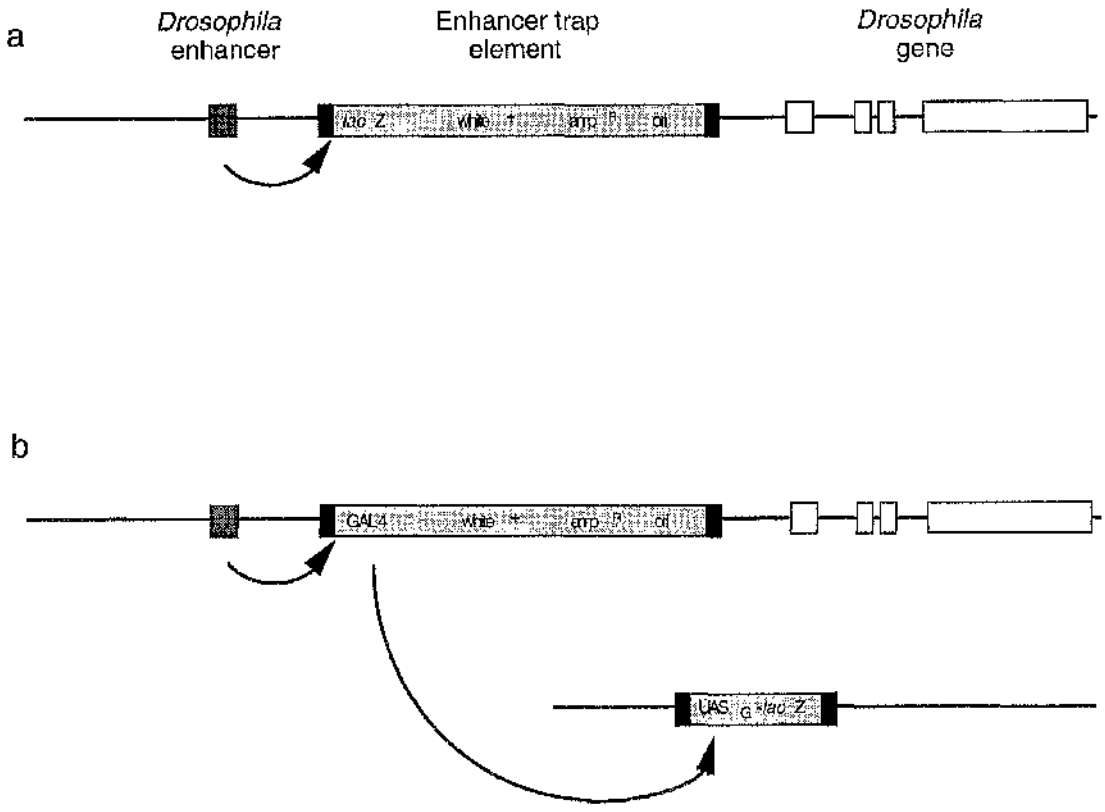


Figure 1.4 Enhancer-trapping. (A) A first generation enhancer-trap element inserted within a *Drosophila* gene. The pattern and timing of expression of the reporter, *lacZ*, is dependent upon the specific genomic context in which it is integrated. *white*$^{+}$ is a marker that confers red eye colour in a *white*$^{-}$ genetic background, and thus allows flies containing new insertions to be recognised. The ampicillin resistance determinant (*amp*^{R}) and *E. coli* origin of replication (*Ori*) facilitate plasmid rescue of flanking sequences. (B) A GAL4 enhancer trap element. The pattern and timing of GAL4 expression is similarly context dependent, and can be used to drive expression of a secondary reporter gene linked to the GAL4-responsive promoter, UAS_G (Diagram kindly provided by Dr. Kim Kaiser).

Precise and imprecise excision

Reversion of a P-induced mutation by precise loss of the transposon may be the only unambiguous means of demonstrating that a phenotypic change is indeed the consequence of a lesion in a tagged or targeted gene (Kaiser *et al.*, 1995). Such losses can be selected following remobilisation of the P-element, preferably from a background in which it is the only P-element remaining. Remobilisation can also result in imprecise excision, leading to the generation of a range of new alleles of varying severity (Voelker *et al.*, 1984; Tsubota *et al.*, 1986; O'Hare *et al.*, 1987; Salz *et al.*, 1987). Once a P-element lies close to rather than within genes of the interest, imprecise excision may be a necessary step in further analysis (Kaiser, 1990).

1.2.4 Enhancer-trap element

An enhancer-trap element is a modified P-element, close to one end of which lies a 'reporter' gene (Figure 1.4). Due to the lack of a transcriptional enhancer, the reporter has a negligible level of intrinsic expression. In order for it to be expressed at a significant level, the transposon must insert close to an endogenous *Drosophila* enhancer. Reporter activity in a line with only one insertion thus reflects the temporal and spatial expression characteristics of a flanking gene (O'Kane and Gehring, 1987; Dorn *et al.*, 1993).

First generation enhancer-trap elements contain the reporter gene *lacZ*, encoding the enzyme β-galactosidase. The presence of β-galactosidase activity in a tissue can be detected simply by its conversion of the chromogenic substrate X-gal. In addition to the reporter gene, enhancer trap elements carrying a marker gene such as *white* enables flies with insertions to be recognised, and most include sequences that allow plasmid rescue of the flanking DNA.

P[*lacW*] is a widely used enhancer-trap element of the first generation. It is 10.6 kb long which carries the *lacZ*, *beta-lactamase* and *mini white* genes (Bier *et al.*, 1989). The *LacZ* gene permits detection of gene expression pattern by staining with X-gal. The *mini-white* gene permits rapid scoring of flies heterozygous or homozygous for a P[*lacW*] insertion. P[*lacW*] contains a bacterial origin of replication and the *beta-lactamase* gene coding for ampicillin resistance at the 3' end - this feature permits easy cloning of DNA flanking the insertion site (Cooley *et al,* 1988; Hamilton *et al,* 1991; Guo *et al.*, 1996c).

One potential disadvantage of the first generation enhancer trap elements is that they express β-galactosidase fused to the N-terminal nuclear localisation signal of the P-element transposase (Bier *et al.*, 1989). Nuclear staining has its uses but precludes visualisation of cell architecture, a particular problem in the study of cells with long processes, such as neurons (Kaiser *et al.*, 1995; Yang *et al.*, 1995).

A second generation enhancer-trap element P[GAL4] has now been developed (Fisher *et al.*, 1988). Instead of β-galactosidase the reporter of P[GAL4] is a yeast transcription factor that is functional in *Drosophila*, and that can be used to direct expression of other transgenes placed under the control of a GAL4-dependent promoter (UAS_G). A cross between a fly with a P[GAL4] insertion and a fly containing UAS_G-lacZ, for example, causes β-galactosidase to be expressed in a pattern that reflects GAL4 activity. Unlike the nuclear localisation signal in the first generation enhancer trap, GAL4 can nicely detect the signals in whole cells, including the long processes in neurons (Yang *et al.*, 1996). A another particularly attractive feature of this system is that any UASG-transgene construct can be used in conjunction with any P[GAL4] line. (Sentry *et al.*, 1993; Sentry *et al.*, 1994a; Sentry *et al.*, 1994b; Sweeney *et al.*, 1995).

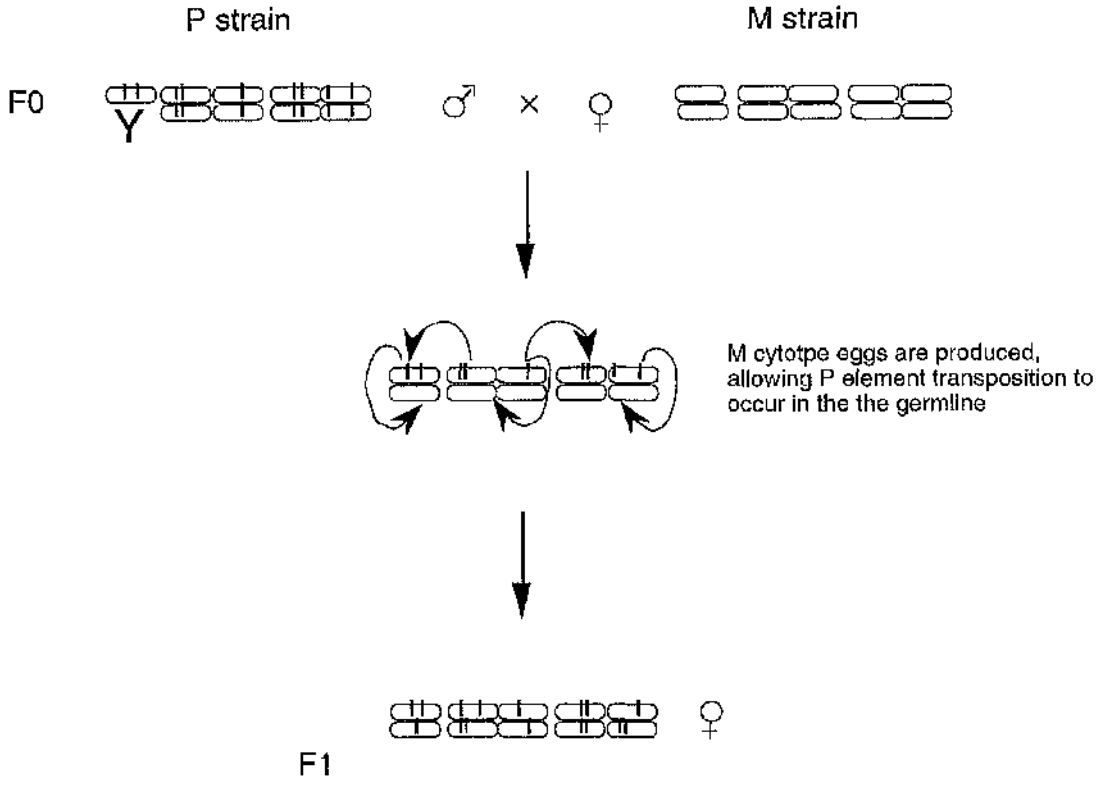


Figure 1.5 P-element mutagenesis. P strain males, carrying autonomous and non-autonomous P-elements, are mated with M strain females. The fertilised eggs are of M cytotype, allowing P-element transposition to occur in the developing germline. As a result, each germline cell contains a new configuration of P-elements. Phenotypic consequences are observed in subsequent generations. (Diagram kindly provided by Dr. Kim Kaiser).

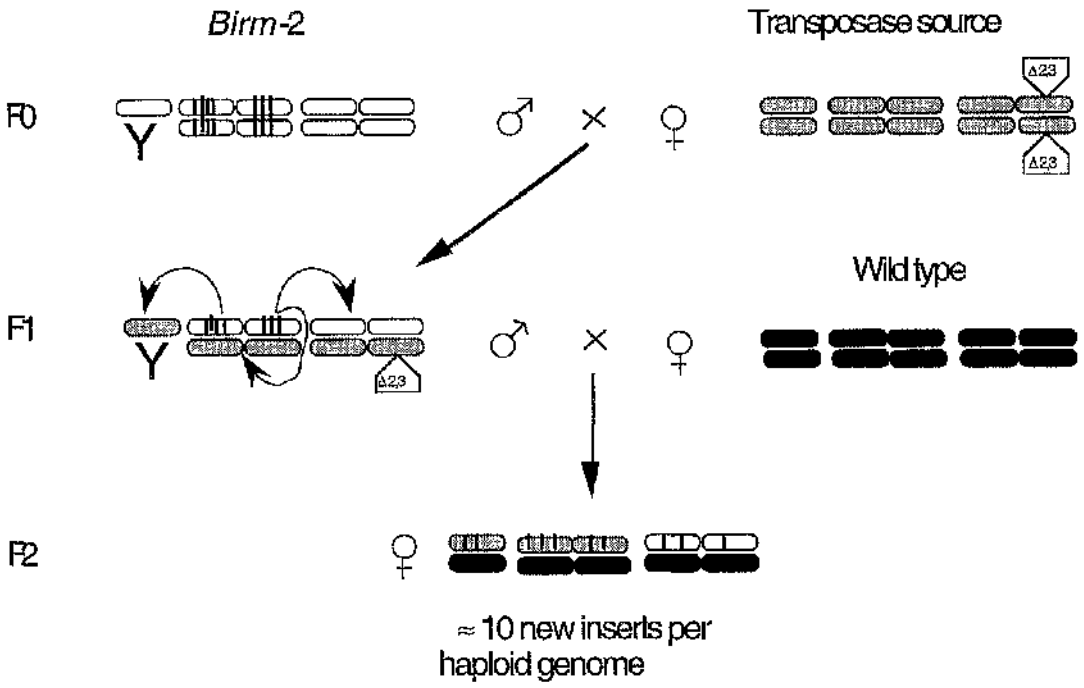


Figure 1.6 A controlled P-element mutagenesis strategy. *Birm-2*, a strain with 17 internally deleted P-elements on each of its second chromosomes, mated with a strain containing the Δ2,3 element. The P-elements are mobilised by the Δ2,3 transposase in germline cells of F_1 males. Each of their sperm has a different spectrum of new insertions. Selection against the transposase source in the F_2 generation ensures that new insertions remain stable (Diagram kindly provided by Dr. Kim Kaiser).

1.2.5 P-element mutagenesis

P-elements are particularly useful as mutagens because of their high transposition frequency and the availability of strains without P-elements. The latter property allows backcrossing to eliminate all P-elements from a line other than the one in the gene of interest. A typical protocol would be as follows: P strain males and M strain females are mated, leading to the induction of P-element transpositions in the germline of their progeny. These progeny are bred and their offspring are screened or selected for mutations in the gene of interest (Kidwell, 1987; Figure 1.5).

The most efficient general mutagenesis strategy (Figure 1.6) involves crossing *Birmingham* 2, a strain with 17 internally deleted P-elements on each of its second chromosome (Engels *et al.*, 1987), with a strain in which Δ2,3 has become irreversibly inserted near to the dominant eye phenotype locus *Dr* (Robertson *et al.*, 1988); an immobile source of transposase linked to a dominant marker simplifies selection for loss of transposase in subsequent generations. Unlike crosses involving wild-type strains, the direction of the above cross is irrelevant. Eggs laid by Δ2,3 females have M cytotype. One disadvantage of using Δ2,3 is transposase activity in the soma. This reduces the viability of dysgenic individuals. The problem can be minimised by performing the cross at 16°C.

The generation of strains containing only a single marked P-element has many advantages as a method of mutagenesis (Zhang and Spradling, 1994). Phenotypic and molecular analyses of new mutations are greatly simplified. The mutant gene can be mapped, cloned and reverted. New alleles could be generated by imprecise excision of the P-element. A drawback with marked elements is their size; they are invariably much larger than unmarked elements, and so transpose at lower frequencies. In addition, the one or few copies of the marked P-element per genome make the target-mutagenesis less efficient. Nonetheless, large collections of single P-element insertions, many plasmid-rescuable, are being assembled through the collective efforts of the international

Drosophila community (e.g. Cooley *et al.*, 1988; Török *et al.*, 1993; P. Deak, personal communication). It is thus increasingly likely that a colleague or stock centre will hold a line with a marked P-element in the region of one's target gene. Site-selected mutagenesis, either by PCR or by plasmid rescue, provides a means of screening such collections *en masse. In situ* hybridisation to polytene chromosome can be used to confirm that a P-element indeed lies in the region to which a mutant maps. Sequencing the rescued plasmids would reveal the exact position of the P-element insertion.

1.2.6 Site-selected mutagenesis

Although traditional genetics relies on the cloning and characterisation of a pertinent gene after a recognition of a mutant phenotype, a large number of novel genes have been cloned by virtue of their DNA sequence homology to a already known genes or on the basis of an interesting expression pattern. Only rarely, however, has such a gene been found to correspond to a pre-existing *Drosophila* mutation. It is therefore desirable for a reverse genetics approach to find a corresponding mutant from the cloned gene. One such approach is site-selected mutagenesis, a means of identifying *Drosophila* lines with P-element transposons inserted within or near to target genes by either PCR (Ballinger *et al* 1989; Kaiser *et al*, 1990) or *via* plasmid rescue (Hamilton *et al.*, 1991; Hamilton, 1994; Guo *et al.*, 1996c)

PCR-based screen for P-element insertion events

The PCR method amplifies a specific region of the target gene lying between a gene specific primer and a newly inserted transposon (defined by a transposon-specific primer) (Figure 1.7). Insertions are detected initially within a population of flies, and are then followed as specific amplification products while the population is subdivided. Detection at the molecular rather than the phenotypic level facilitates fast and efficient screening and can be performed on heterozygous individuals (Ballinger *et al* 1989; Kaiser *et al*, 1990; Banga *et al.*, 1992). A similar approach has been adapted for screening natural

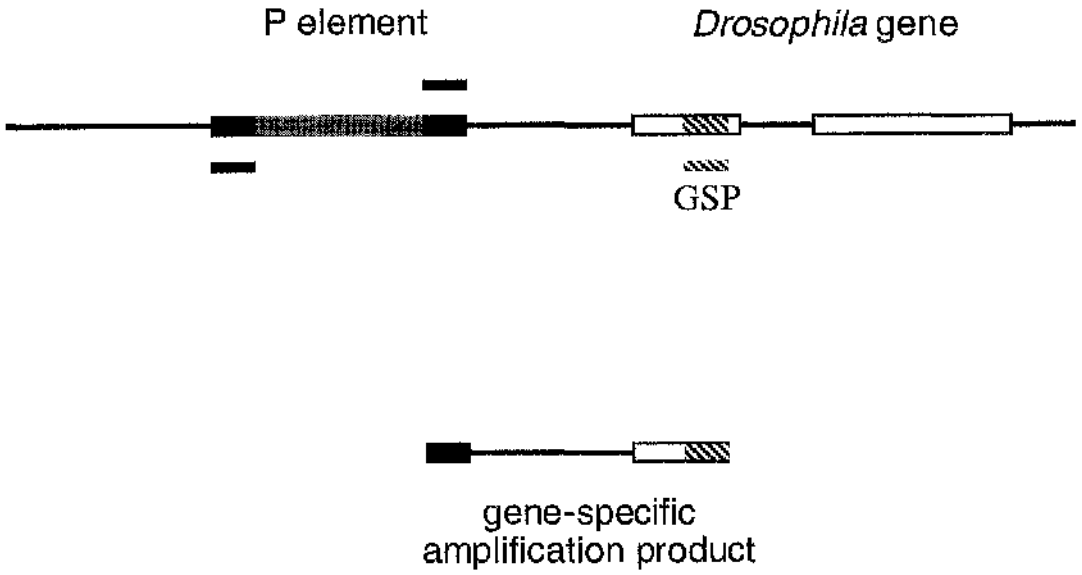


Figure 1.7 Site-selected mutagenesis. Juxtaposition of a P-element and a target gene uniquely provides a template for amplification between a gene-specific primer (GSP) and a transposon-specific primer based on the P-element 31bp inverted repeat. Open boxes represent exons of a hypothetical *Drosophila* gene (Diagram kindly provided by Dr. Kim Kaiser).

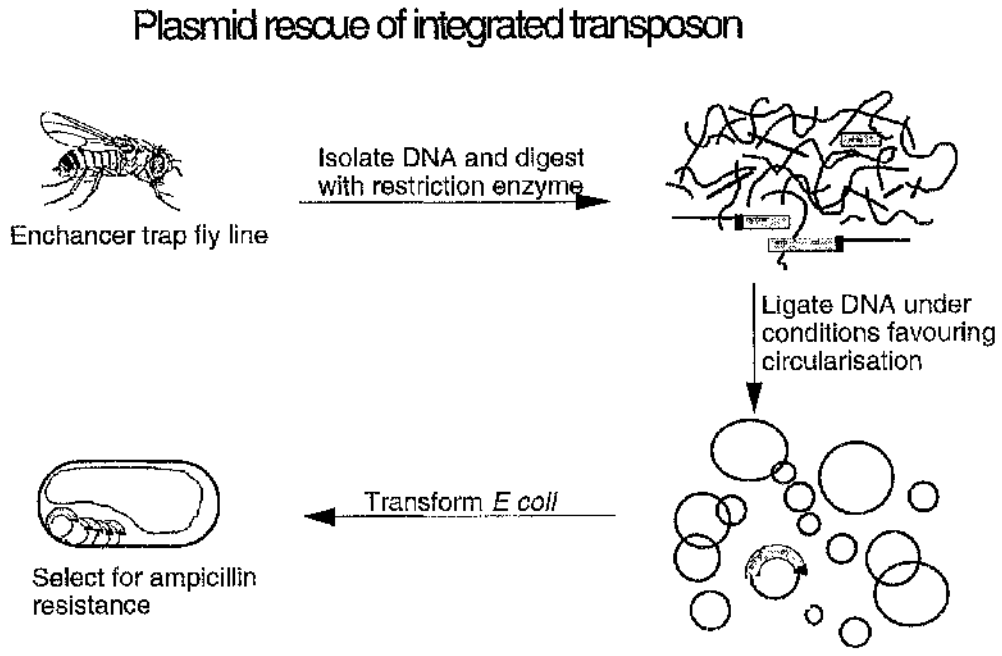


Figure 1.8 Plasmid rescue. DNA is isolated from a line with a single engineered P-element (here an enhancer-trap element) containing an *E. coli* origin of replication (*ori*) and a drug-resistance determinant (amp^R). The DNA is cleaved with an appropriate restriction enzyme, ligated under conditions that favour intra-molecular ligation, and used to transform *E. coli*. Plasmids recovered from ampicillin-resistant colonies contain *Drosophila* DNA from adjacent to the site of P-element insertion (Diagram kindly provided by Dr. Kim Kaiser).

populations of *D. melanogaster* to obtain P-element insertions in or near the target gene (Clark *et al.*, 1994).

Site- selected mutagenesis *via* Plasmid rescue

P-elements engineered to contain a plasmid origin of replication and a drug-resistance determinant allow one-step recovery of *Drosophila* genomic DNA flanking the site of insertion (Figure 1.8). This procedure is known as plasmid rescue (Pirrotta *et al.*, 1986; Steller *et al.*, 1986). Genomic DNA from the flies with the engineered P-element such as

P[*lacW*] and P[GAL4], is digested with an appropriate enzyme that cuts the polylinker in the P-element and somewhere in the flanking DNA. This enzyme is subsequently inactivated and the fragments are cloned as plasmids allowing them to be transformed into *E. coli*. Only those *E. coli* containing the plasmids can survive in the medium with antibiotics. Such rescued plasmids can also be used for a form of site-selected mutagenesis (Hamilton *et al.*, 1991; Guo *et al.*, 1996c). A pool of plasmids rescued from a population of flies with different insertion sites contains sequences representative of every flanking region. Hybridisation between the pool and a specific cDNA/genomic DNA clone is diagnostic of an insertion in or near the gene of interest.

1.3 V-ATPase

1.3.1 Proton pumps

Proton pumps (H^+-ATPases) function in biological energy conversion in every known living cells and they fall into three types. One belongs to the family of P-ATPases which is integral membrane proteins and operates with a phospho-enzyme intermediate (Nelson 1992a). Na^+/K^+-ATPases and gastric H^+-ATPases are notable members of the P-ATPase family. The function of this proton pump is primarily in the plasma membrane of plant and fungal cells and in specialised mammalian cells such as partietal cells in the stomach.

The other families of F-and V-ATPases operate without an apparent phospho-enzyme intermediate (Pedersen *et al.*, 1987; Nelson, H. *et al.*, 1989; Nelson *et al.*, 1992a; Bowman *et al.*, 1993). F- and V-ATPases are more universal proton pumps and at least one of them is present in every living cell (Nelson, 1992a).

F-ATPase and V-ATPase share a common structure and mechanism of action and have a common evolutionary ancestry. F-ATPases function in eubacteria, chloroplasts and mitochondria, and V-ATPase is present in archaebacteria and the vacuolar system of eukaryotic cells. Eukaryotic F-ATPases are confined to the semiautonomous organelles, chloroplasts and mitochondria that contain their own genes encoding some of the F-ATPase subunits. F-ATPase is also vital for every known eubacterium acting in photosynthetic or respiratory ATP formation and/or in generating proton-motive-force (pmf) by the reaction of ATP dependent proton pumping. In contrast, V-ATPases are composed only of nuclear gene products and are present in organelles of the vacuolar system and in the plasma membrane of specialised cells (Nelson, 1992a).

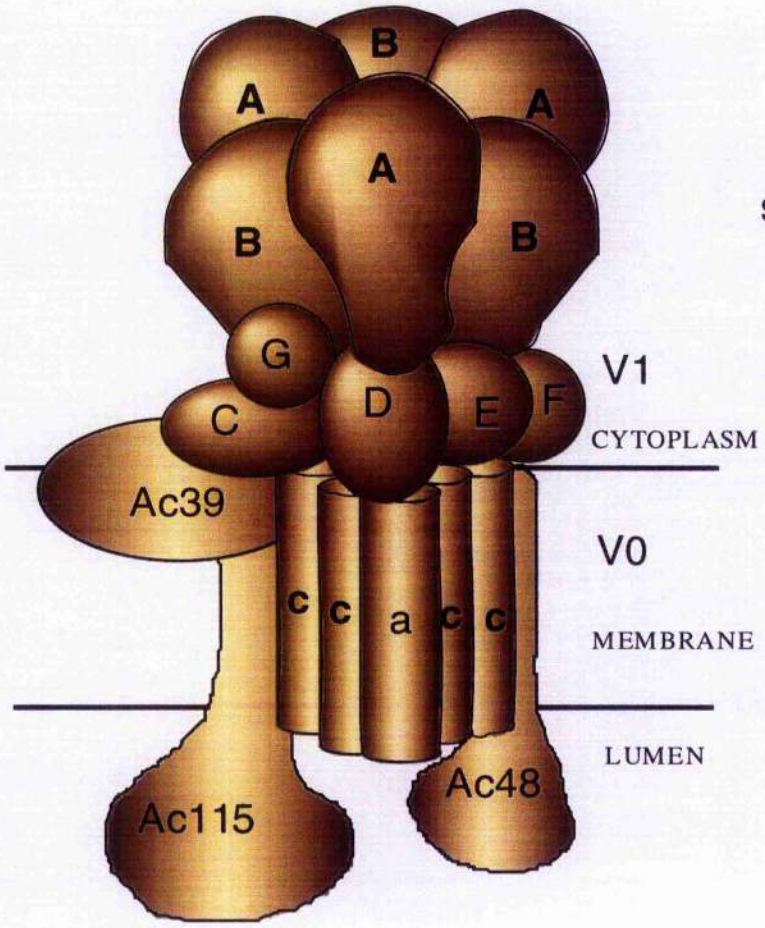
One of the most notable distinctions between F- and V-ATPases is in their function in ATP formation. While the primary function of F-ATPases in eukaryotic cells is to form ATP at the expense of pmf generated by electron transport chains, the main function of V-ATPases is to generate a pmf at the expense of ATP and to cause limited acidification of the internal space of several organelles of the vacuolar system. The pmf generated by V-ATPases in organelles is utilised as a driving force for numerous secondary uptake processes. Several metabolic processes that take place in the internal membrane network of eukaryotic cells may be dependent or influenced by the function of V-ATPase (Nelson 1994).

1.3.2 Structure of V-ATPase

V-ATPases are multi-subunit protein complexes built from distinct catalytic and membrane sectors (Figure 1.9). The catalytic sector (V1) contains six different polypeptide donated as A, B, C, D, E and F (Nelson, 1992a; Nelson, 1994; Nelson *et al.*, 1994; Gräf *et al.*, 1994a; Graham *et al.*, 1994b; Nelson *et al.*, 1995; Guo *et al.*, 1996b). The stoichiometry of these subunits excluding F was determined to be 3:3:1:1:1, respectively (Arai *et al.*, 1988; Supek *et al.*, 1994). The function of the catalytic sector is to provide the ATP binding site and to catalyse the ATP formation and/or ATPase activities of the enzymes. The main function of the membrane sectors is to conduct protons across the membrane. A proteolipid (subunit c) is confirmed to present in the membrane sector of all the V-ATPase. A stoichiometry of six proteolipids per enzyme has been reported for V-ATPases from clathrin-coated vesicles and plant vacuoles (Arai *et al.*, 1988; Jones *et al.*, 1995).

It was only since 1988 that cDNAs and genes encoding subunits of V-ATPases were cloned and sequenced (Bowman *et al*, 1988; Zimniak *et al.*, 1988; Hirsch *et al.*, 1988; Mandel *et al.*, 1988). The sequences revealed valuable information on the structure, function and evolution of the various subunits as well as the evolution of F- and V-ATPases (Nelson, N. *et al.*, 1989; Nelson 1994). It became apparent that subunits A and B of V-ATPases and subunit β and α of F-ATPases evolved from a common ancestral gene.

The proteolipids of F- and V-ATPases also evolved from a common ancestral gene. The proteolipid has been found to be the principal protein component of gap junctions, at least in invertebrates. (Finbow *et al.*, 1992; Finbow and Pitts, 1993; Finbow *et al.*, 1994a), thus subunit c of V-ATPase was also called ductin. Gap junctions are aggregates of paired connexon channels that allow the intercellular movement of cytoplasmic solutes up to Mr. 1000 within tissues of metazoan animals (Finbow *et al.*, 1994b).



Subunit	M_r(Da)
A	68
B	57
C	44
D	28
E	26
F	14
G	13
a	20
c	16
Ac115	96
Ac48	51
Ac39	36

Figure 1.9 Schematic subunit structure of V-ATPase. The catalytic vector (V0) is composed of A, B, C, D, E, F, G subunits, the membrane sector (V1) is composed of subunit a, c, Ac 115, Ac 48 and Ac 39. Genes encoding subunit A, B, C, D, E, Ac115, Ac 48, Ac39 and the proteolipid (subunit c) has been cloned from chromaffin granules. Genes encoding subunit A, B, C, D, E, F, G and c has been cloned from *M. sexta*. More V-ATPase subunits are likelyto exist. (This diagram is modified from Nelson's (1994) and Dow's).

An analogy to the membrane sector of F-ATPases suggests that additional subunits should function in the membrane sector of V-ATPases. While the membrane sector of the archaebacterial V-ATPase may be composed only of the proteolipid (Denda *et al.*, 1990) the membrane sector of mammalian V-ATPase may composed of at least five different subunits (Zhang *et al.*, 1992; Nelson, 1992a). The genes or cDNAs encoding four of the subunits (M115, M45, M39 and proteolipid) have been cloned and sequenced from bovine, yeast and several other sources (Wang *et al.*, 1990; Perin *et al.*, 1991; Bauerle *et al.*, 1993). More subunits may function in proton conduction through the membrane and/or in the assembly of the V-ATPase membrane sector.

A novel 13 kDa subunit of V-ATPase has been cloned from yeast (*Vma10p*) *Manduca* (subunit G), and bovine (M16) (Lepier *et al.*, 1996; Supekova *et al.*, 1996). The deduced protein is significantly homologous to the b subunit of bacterial F-ATPases, but contains no apparent transmembrane segment in its N terminus. While *Vma10p* in yeast behaved like a V-0 subunit, the *Manduca sexta* 13 kDa subunit behaved like a V1 subunit, since it could be stripped from the membrane by treatment with the chaotropic salt KI and by cold inactivation, thus this subunit was considered to be a new member of the catalytic sector (V_1) and was designated as subunit G (Lepier *et al.*, 1996)

Gene disruption experiments in yeast that led to a complete loss of V-ATPase activity gave no indications for multiple isoforms in *Saccharomyces cerevisiae* (e.g. Nuomi *et al.*, 1991; Foury, 1990). Also, in other fungi only one gene per subunit has been identified (Gogarten *et al.*, 1992b). However, In the case of human, animal and higher plants, different genes encoding the same subunit type have been found. Two isoforms have been reported for A subunit from hunan, chicken and plants (van Hille *et al.*, 1993b; Hernando *et al.*, 1995; Gogarten *et al.*, 1992b); B subunit in human and bovine (Bernasconi *et al.*, 1990; Puopolo *et al.*, 1992; Nelson *et al.* 1992; Berkelman *et al.*, 1994); E subunit in Mammal (Hemken *et al.*, 1991), c subunit in yeast and maize (Umemoto *et al.*, 1991; Vieveck *et al.*, 1996) and 100-kDa subunit in bovine (Peng *et al.*,

1994). The presence of different isoforms might allow differential targeting and regulation of cell-, organelle- or plasma membrane- specific V-ATPases.

1..3.3 Plasma membrane V-ATPase

V-ATPases usually reside in the membranes of acidic organelles. However, they are also present in the plasma membrane of several cell type. Although having a similar structure and subunit constitutes as that of endomembrane V-ATPase, the plasmid membrane V-ATPases in arthropod and vertebrate cells share several features that are not generally observed in the V-ATPases in intracellular membranes (Bowman *et al.*, 1993; Gluck, 1992). Plasmid membrane V-ATPases are present at high densities, far greater than the densities on intracellular membranes. However, the amplification of plasma membrane V-ATPase is limited to specific cell types. In insects, high densities of V-ATPase on the plasma membrane are observed in the midgut goblet cell and the enveloping cells of sensilla (Klein *et al.*, 1991a, 1991b). Similarly, high densities of plasma membrane V-ATPase are found in the mitochodria-rich cell of toad bladder (Brown *et al.*, 1987) and frog skin (Harvey, 1992), in the intercalated cells of the mammalian kidney collecting tubule (Brown *et al.*, 1988; Brown, 1992; Gluck *et al.*, 1992a; Gluck *et al.*, 1992b; Gluck *et al.*, 1994), in insect Malpighian tubules (Dow, 1994; Garoyoa *et al.*, 1995) and in insect midgut (Wieczorek *et al.*, 1989). In bone only the osteoclast cells have the immunocytochemically detectable plasma membrane V-ATPase (Baron, 1994).

1.3.4 Functions of V-ATPase

V-ATPase is a proton pump required for acidification of many types of eukaryotic vacuole. These include lysosomes, plant and fungal vacuoles, synaptic vesicles, coated vesicles and Golgi (Nelson, 1992a). The participation of V-ATPases in numerous aspects of endocytosis, secretion and sorting has been amply recognised (Forgac, 1989; Mellman *et al.*, 1986; Lukacs et al., 1996). In fungi, plants and most animal cells, V-ATPases

energise selected intracellular membrane compartments of the vacuolar system, acidifying the interior of these compartments and providing an electrochemical driving force for the transport of solutes (reviewed by Nelson, 1992a; Nelson, 1994).

V-ATPase functions not only in the vacuolar system but also in the plasma membrane of specialised cells. The roles of V-ATPase in kidney function and bone reabsorption is well understood. The kidney plays a vital role not only in cleaning the body of waste products but also in the acid-base balance of mammals. Hydrogen ion excretion involves several processes including bicarbonate reabsorption, carbonic anhydrase activity and regulated pumping of protons across the plasma membrane by V-ATPase. In epithelial cells of the proximal urinary tubule, V-ATPase is present in the apical membrane and functions in proton secretion. In the collecting duct V-ATPase may be found either in apical or basolateral membranes of specialised intercalated cells. These cells shuttle V-ATPase between intracellular vesicles and the plasma membrane in response to changes in the acid-base balance of the animal. It was shown that the distribution of V-ATPase, in apical or basolateral membranes of intercalated cells, changes during adaptation to acidosis or alkalosis. The cells increase the number of V-ATPase enzymes in their apical membrane during acidosis and decrease their number during alkalosis. Therefore, V-ATPase plays a major role in maintaining pH homeostasis in mammals and other animals (Gluck, 1992).

The involvement of V-ATPase in bone reabsorption has been well reviewed by Baron *et al.* (1994). Bone reabsorption is necessary for bone growth, remodelling and repair. Osteoclasts are multinucleated and highly motile cells that migrate between the bone and bone marrow and function in bone reabsorption. They attach to the mineralised bone matrix forming a close space to which hydrolytic enzymes are secreted. The optimal activity of these enzymes require low pH which is provided by V-ATPase located in the part of the plasma membrane in contact with the bone. And protons are required for the release of each calcium ion from the mineral. The osteoclast V-ATPase provides all protons necessary for calcium reabsorption. The pharmacological value of studying the

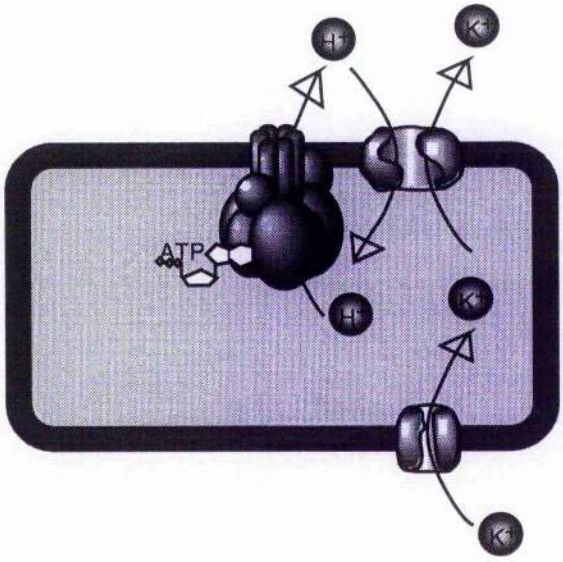


Figure 1.10 Generalised model for insect epithelia. An apical plasma-membrane V-ATPase pumps proton out of the cell. These are exchanged for alkali metal cations (Na^+ or K^+) to produce a net ATP-dependent flux. Entry through the basal plasma membrane is not defined in the basic model, but is thought to be *via* channels, cotransports or ATPases in various insect tissues (Diagram kindly provided by Dr. Julian A. T. Dow).

osteoclast V-ATPase is apparent because a specific slow down in its activity may prevent the onset of osteoporosis.

The plasma membrane V-ATPase in vertebrate cells functions primarily for proton transport. In contrast, The plasma membrane V-ATPases of insects generate a membrane potential, which is used to drives an electrogenic K^+/H^+ antiporter operating in parallel in the same membrane (Wieczorek, 1991; Wieczorek, 1992; Klein, 1992; Wieczorek and Harvey, 1995), This "Wieczorek model" for the K^+ pump in insect midgut is now generally accepted for all insect epithelia which appear to have an apical, electrogenic pump for sodium or potassium. Essentially, it is that an apical plasma membrane V-ATPase energises an exchanger more or less similar to the vertebrate Na^+/H^+ exchanger, and that this coupling is normally so tight that on a macroscopic scale, the ion pumped appears to be the metal ion, rather than the proton (Figure 1.10). Unlike the vertebrate use of the pump in kidney epithelium and plasma membrane, the V-ATPase does not appear to be used directly to acidify the extracellular space; rather, it is used as a driving force, employed to move a different ion (Dow, 1994; Azuma *et al.*, 1995). In *M. sexta* midgut this results in extreme alkalisation of the lumen of the midgut to pH>11 (Dow, 1984; Dow, 1986; Dow, 1989; Dow, 1992). Similarly, V-ATPases are the primary driving force generating a membrane potential which drive salt and water fluxes in the Malpighian tubules and the rectum (Moffett, 1992).The V-ATPase-generated membrane potential in the enveloping cells of the sensillum drives the signalling currents initiated by activation of the sensory cells (Klein, 1992).

However, the 'Wieczorek' model has recently been challenged by an alternative explanation, based on the insensitivity of electrical measurements of the insect trichogen sensilla to amiloride or harmaline (Küppers and Bunse, 1996). On this basis, they argue that no exchanger exists and that the apical V-ATPase is primarily a proton ATPase, but with the additional ability to transport alkali metal cations. Given that the intracellular pH is 7, and that intracellular K^+ is around 100mM, even if the pump were 10^5:1

selective in favour of H^+ over K^+, under normal conditions the two ions would be transported at nearly equal rates (Dow *et al.*, 1996). However, given that an exchanger has been demonstrated functionally in *Manduca* midgut (Azuma *et al.*, 1995), this alternative model requires further supporting evidence.

In addition to the straightward endosomal acidification, an increasing number of cellular processes are being shown to be dependent on V-ATPase function (reviewed by Dow *et al.*, 1996). *Polycomb* may be modulated by hemizygosity for *vha55*, a gene encoding a proton pump B subunit (Davies *et al.*, 1996); V-ATPases have been implicated in the regulation of cytoplasmic pH (Dow *et al.*, 1996); the proteolipid subunit of V-ATPase was implicated as the main structural protein in gap junctions (Finbow, 1992) and in neurosecretion of acetycholine (Birman *et al.*, 1990); V-ATPases have also been found to colocalise with calcineurin, an important Ca^{2+}-sensitive phosphatase, suggesting an important role for V-ATPases in regulating intracellular calcium (Garrettengele *et al.*, 1995; Tanida *et al.*, 1995). Three transmembrane subunits of the V-ATPase (proteolipid, Ac39 and Ac116) were found to coexist with synaptobrevin and synaptophysin in rat synaptosome (Galli *et al.*, 1996), and the 39 kDa subunit of the V-ATPase has been identified as a synaptic-vesicle binding protein (Siebert *et al.*, 1994). These observations further suggest a role of V-ATPase in the neurotransmission. It is also possible that some human genetic disease may be associated with haploabnormality for a V-ATPase gene (Goldstein *et al.*, 1991; Baud *et al.*, 1994; Mears *et al.*, 1995; Gottlieb *et al.*, 1995; Koralnik, 1995; DeFranco *et al.*, 1995).

1.3.5 Mutational analysis of V-ATPases

The yeast *S. cerevisiae* V-ATPase closely resembles the V-ATPases from other fungi, plants and animals, both in its overall structure and in the sequences of the subunit genes that have been cloned (Anraku *et al.*, 1992; Kane, 1992). Yeast has been used as a model system for mutational analysis of V-ATPase. Mutation for the 100, 69, 60, 42, 27, and

17 kDa subunits have been constructed (Kane, 1992; Liu et al., 1996). Deletions in any of these subunit genes yield a well-defined set of phenotypes, which includes a complete loss of vacuolar acidification, absence of all ATPase activity in isolated vacuoles and failure to grow in media buffered to neutral pH (Nelson and Nelson, 1990). Mutations in the ATPase subunits also result in precursor accumulation and missorting of both soluble and membrane vacuolar proteins (Yaver *et al.*, 1993; Ho *et al.*, 1993).

Gene replacement in yeast has been a powerful method to generate V-ATPase null mutants, but such approaches are not yet feasible in higher eukaryotes (Gogarten *et al.*, 1992a), and yeast V-ATPases mainly play endomembrane role (Dow, 1994). As an alternative approach, Gogarten *et al* (1992a) used antisense mRNA to inhibit gene expression of V-ATPase A subunit in higher plants. Carrot root cells were transformed with the coding or 5' noncoding regions of the carrot V-ATPase A subunit cDNA cloned in the antisense orientation. Regenerated plants containing the antisense constructs exhibited altered leaf morphologies and reduced cell expansion. It was inferred that the antisense constructs specifically blocked expression of a tonoplast-specific isoform of the V-ATPase A subunit in carrot. The degree of antisense mRNA inhibition is variable in different tissues and rarely completely block the gene. Moreover, in some animals, antisense mRNA has not been so successful. As a solution to this problem, *Drosophila* may provide an ideal model organism for mutational analysis of genes encoding different subunits of V-ATPases (Dow, 1994; Davies *et al.*, 1996, Dow *et al.*, 1996). A pilot study for gene inactivation shows that transposable P-elements can be easily inserted into the *Drosophila* ductin *vha16* gene. Although without phenotypic consequences, these can serve as a starting point for generation of null alleles (Finbow *et al.*, 1994a). *vha55,* the gene encoding the B-subunit of *Drosophila* V-ATPase has been cloned recently. Inactivation of the gene reveals a larval lethal phenotype (Davies *et al.*, 1996).

1.4 The aim of this project

The aim of this project is to clone and characterise genes encoding A, E, F subunits in *Drosophila* V-ATPase and subsequently inactivate these genes. The mutagenesis work began with a large scale plasmid rescue of P[*lacW*] lethal insertion lines (generated by the laboratories of Istvan Kiss and Peter Deck in Hungary) and was followed by screening for the specific mutations. The target genes, apart from components of V-ATPase, will also include a range of neurotransmitter receptors, neuronal kinases, *et al*. Once a mutation is isolated, a detailed molecular, physiological and behavioural study will subsequently follow to address the functions of the genes.

Chapter 2

Materials and Methods

2.1 *Drosophila*

The main *Drosophila* stocks used in this work are described below:

Strain/Genotype	Reference
Oregon R	Lindsley and Zimm, 1992
Canton S	Lindsley and Zimm, 1992
w; Sb P[ry$^{+}\Delta$2,3)/TM6	Robertson et al., 1988

Mutations used are listed in Appendix 3.

Flies were routinely raised on Glasgow medium. Culture temperature was 25°C, unless otherwise stated. A grape juice agarose medium was used to obtain eggs. Third instar larvae, used for *in situ* hybridisation to polytene chromosomes, were reared on a rich medium.

Glasgow medium: 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 per litre of water.

Grape juice agarose medium: 19.8 g agarose, 52.2 g glucose, 26 g sucrose, 7 g dried yeast, 9% (v/v) red grape juice (Safeway) per litre of water.

Rich medium: 100 g glucose, 100 g dried yeast, 20 g agar per litre of water.

2.2 *E. coli*, plasmids and bacteriophages

The *E. coli* strains used in this work are all derivatives of *E. coli* K12. They are listed below with their genotypes:

strain	Genotype	Reference
XL1-Blue	*recA1, endA1, gryA96, thi-1, hsdR17, supE44.*	Bullock (1987)
NM621	*hsdR,mcrA,mcrB,supE44,recD1009.*	Whittaker *et al,* 1988
DH5α	F⁻, *deo*R, *pho*A, *sup* E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *relA1*	Gibco BRL

Plasmids and bacteriophages used in this study, other than those whose construction is described elsewhere, are listed below.

Plasmids/	Description	Source/ Reference
Bacteriophage		
pBR*rp49*	*Eco*RI-*Hind*III fragment of the *Drosophila* ribosomal protein 49 gene in pBR322	O'Connell & Rosbash, 1984
pBluescript®IISK+/-		Mead *et al.*, 1985
P[*lacW*]	Whole P[*lacW*] sequence	Bier *et al.*, 1989
EMBL3	λ Vector for genomic DNA	Frischauf *et al.*,1983

2.3 *E. coli* Growth medium

L-Broth:	10 g Bacto-tryptone (Difco), 5 g yeast extract (Difco), 10 g NaCl, per litre of water and adjust to pH 7.0 with NaOH.
L-Agar	As L-broth with the addition of Bacto-agar (Difco) to 1.5%.

BBL Broth	10 g trypticase peptone (BBL), 5 g sodium citrate, made up to 1 litre with distilled H_2O.
BBL agar:	As BBL broth with the addition of Bacto-agar to 1.5%.
BBL top agarose	As BBL broth with the addition of gel quality agarose to 0.7%.
2xYT Broth:	10 g Bacto-tryptone (Difco), 10 g yeast extract (Difco), 5 g NaCl made up to 1 litre with distilled H_2O
φ–Broth	20 g Bacto-tryptone (Difco), 5 g yeast extract (Difco), 4.93 g MgSO4, 0.58 g, NaCl, 0.37 g KCl, made up to 1 litre with distilled H_2O

All culture media was sterilised by autoclaving at 120°C for 15 min at 15 psi. Where required, L-broth and BBL top agar were supplemented with 10 mM MgSO4 for growth of bacteriophage lambda and its derivatives.

2.4 Antibiotics and indicators

Ampicillin, at a final concentration of 100 µg/ml (100 mg/ml stock solution in sterile distilled water) was added to broth or agar to select transformed *E. coli*. When necessary, tetracycline, at a final concentration of 7.5 µg/ml (15 mg/ml stock solution in absolute ethanol), was added to broth or agar. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were added to molten agar (50°C) in order to detect recombinant clones. X-gal was dissolved in dimethylformamide and IPTG in sterile distilled water. Both were stored at -20°C as 20 mg/ml solutions, and used at a final concentration of 20 µg/ml.

2.5 Competent cells and transformation

2.5.1 Preparation of competent cell

$CaCl_2$ method

This method is modified from that of Hanahan (1985). 20 ml of L-broth was inoculated with 0.4 ml of an overnight culture of XL1-Blue, and grown with aeration at 37°C until cells had entered the logarithmic growth phase (OD_{600}=0.4-0.6). The cells were then pelleted at 4000 g for 5 min at 4°C in a bench-top centrifuge, the supernatant removed, and the resulting pellet resuspended in 10 ml ice-cold 100 mM $CaCl_2$ solution. After a 20 min incubation on ice, the cells were repelleted as above, and then suspended in 2 ml ice-cold 100 mM $CaCl_2$. Competent cells were either used fresh, or frozen for later use after adding 25% of glycerol .

RbCl method

A single colony was picked off a freshly streaked LB agar plate and dispersed in 20 ml of φ-broth. The culture was incubated with agitation overnight. 4 ml of the overnight culture was added to 200 ml of φ–Broth and incubated at 37°C with agitation in a 2 litre flask until OD_{600}=0.5. The cells were then pelleted at 1300 g for 10 min at 4°C. The pellet was resuspended by gently shaking in 50 ml pre-chilled RF1 buffer and incubated on ice for 30 min. Cells were pelleted again as above and then resuspended in 15 ml of chilled RF2 buffer. The competent cells, after being flash frozen in liquid nitrogen, were stored at -70°C for later use.

RF1

Compound	Concentration	Amount/litre
RbCl	100 mM	12 g
$MnCl_2.4H2O$	50 mM	9.9 g
Potassium acetate	30 mM	30 ml (1 M stock pH 7.5)
$CaCl_2.2H_2O$	10 mM	1.5 g
Glycerol	15% (W/V)	150 g

Adjust the pH to 5.8 with 0.2 M acetic acid. Sterilise by filtration through a pre-rinsed 0.22 μ membrane.

RF2

Compound	Concentration	Amount/litre
MOPs	10 mM	20 ml (0.5M stock pH7.5)
RbCl	10 mM	1.2 g
$CaCl_2.2H_2O$	75 mM	11 g
Glycerol	15% (W/V)	150 g

Adjust pH to final pH 6.8 with NaOH (if necessary) and sterilise by filtration through a pre-rinsed 0.22 μ membrane.

Competent cells for eletroporation

4 ml of fresh overnight culture was added to 400 ml of L Broth at 37°C with vigorous shaking to an OD=0.5-0.7. The cells were pelleted at 4°C in cold centrifuge bottles in a cold rotor at 2000 g for 10 min. The pellets were gently resuspended in 400 ml of ice-cold 10% glycerol and repelleted as above. The step was repeated twice with the pellet being resuspended in 200 ml of ice-cold 10% glycerol for the first repeat, and in 100 ml of ice-cold 10% glycerol for the second repeat. Finally the cells were resuspended in 1.5-2 ml of ice-cold 10% glycerol. This suspension of competent cells can be used fresh or can be frozen in aliquots in liquid nitrogen and stored at -70°C.

2.5.2 Transformation of *E. coli*

50-100 ng of DNA in a volume up to 10 μl was added to 200 μl of competent cells and left on ice for 15 min. The mixture was subjected to a heat-shock at 42°C for 90 seconds and quickly chilled on ice for a few mininutes. The cells were either plated immediately, or after incubation in 800 μl 2XYT with agitation at 37°C for 0.5 -1 hr., onto L-agar plates containing the appropriate antibiotics and indicators. The plates were incubated overnight at 37°C to select for transformants.

Electroplation was performed according to the manual provided with that *E. coli* Pulser apparatus (BIO-RAD). 40 µl of the cell suspension was mixed with 1 to 2 µl of DNA in a cold, 1.5 ml polypropylene tube and left on ice for 0.5-1 min. Immediately after electroplation the mixture was plated on an ampicillin selective plate.

2.6 Nucleic Acid Isolation

2.6.1 Plasmid DNA

Large scale plasmid isolation was carried out by the alkaline-lysis method of Birnboim and Doly (1979) as described in Sambrook et al. (1989). Small scale plasmid preparations were made by the alkaline-lysis or boiling method (Sambrook *et al.*, 1989), or with the Magic^{TM} DNA purification system (Promega) using the protocol recommended by the manufacturer.

2.6.2 Bacteriophage λ DNA

Isolation of λ DNA was performed by a modification of the protocol of D. Chisholm (1989).

Host Cell Preparation

1 ml of an overnight culture of NM621 was added into 100 ml of L-broth to grow until OD_{600} was ≈0.3 (about 3hrs). The cells were pelleted and resuspended in 10 mM $MgSO_4$ to a final OD_{600}=1.

Growing Lamda Lysates

$2X10^6$ phage was added to 500 µl ($4X10^8$) of plating cells. The culture was incubated at 37°C for 30 min to allow the phage to be absorbed to the bacteria. The mixture was then

added to 37 ml of NZCYM in a 250 ml flask and grown with vigorous shaking until lysis was apparent (12-15 hrs).

Isolation of Phage

The above mixture was transferred to Falcon tubes containing 100 µl chloroform with thorough shaking. 370 µl of nuclease solution (50 mg DNAse 1, 50 mg RNAse A, in 10 ml of 50% glycerol, 30 mM NaOAc, pH 6.8; stored at -20°C) was added and the mixture was incubated at 37°C for 30 min. 2.1 g of NaCl was added and the mixture shaken gently until the salt was dissolved. Debris was pelleted (4000 rpm, 20 min, 4°C) and 3.7 g PEG8000 was added to the supernatant. The sample was placed on ice for 1 hr after the PEG had dissolved at room temperature. The phage were pelleted (10,000 rpm for 20 min at 4°C) and resuspended in 500 µl of phage buffer. This phage suspension was mixed with an equal volume of chloroform and the phases separated by centrifugation.

Isolation of Phage DNA

The aqueous layer was transferred into a new Eppendorf and 20 µl 0.5M EDTA, 5 ul of 20% SDS, and 2.5 µl proteinase K (10 mg/ml) were added. After incubation at 65°C for 30 min, the supernant was extracted with phenol and then with chloroform. DNA was precipitated and dissolved in 300 µl of TE. Yields for EMBL3 derivatives were generally 50-100 µg.

2.6.3 *Drosophila* DNA

Rapid single fly DNA isolation for PCR

Single-fly DNA was prepared by the method modified from Gloor, G and Engels, W (1991). A single fly was homogenised in an 1.5 ml Eppendorf microcentrifuge tube with an micropestle in 50 µl of homogenisation buffer (10 mM Tris-HCl, pH 8.3, 1 mM EDTA, 25 mM NaCl, 200 µg/ml Proteinase K, from a 20 mg/ml stock solution in sterile

distilled water). And after incubation for 30 mins at 37°C, the homogenate was then heated to 95°C for 2 min, 2 µl of the homogenate was used directly in a 20 µl volume of PCR reaction.

Genomic DNA isolation from adult flies

Adult genomic DNA was prepared by a modification of the method of Hamilton *et al.* (1991). 15-20 flies were homogenised in a 1.5 ml Eppendorf microcentrifuge tube with a motorised pestle in 400 µl of lysis buffer (80 mM NaCl, 5% sucrose, 0.5% SDS, 50 mM EDTA, 100 mM Tris-HCl pH8.5). Following 30 min at 70°C, KOAc was added to a final concentration of 0.6 M, and the tube was placed on ice for 30 min. Debris was pelleted by centrifugation at 4°C for 15 min, and genomic DNA present in the supernatant was carefully removed to a fresh tube. The following stage (A, B, or C) is slightly variable according to the quality requirements for the DNA:

(A) The supernant was extracted once with an equal volume of phenol, once with an equal volume of phenol/$CHCl_3$ (1:1) and finally with an equal volume of $CHCl_3$. The DNA was then precipitated with 0.6 volume of isopropanol. The pellet was washed with 70% ethanol, dried and resuspended in 50 µl of TE with RNase A at 20 µg/ml.

(B) 0.5 volume of PEG solution (13% PEG8000, 1.6 M NaCl) was added to the supernant, mixed well and centrifuged at 4°C for 5 min. The pellet was washed with 70% ethanol, dried and resuspended in 100 µl of TE.

(C) The supernant was pelleted with 0.6 volume of isopropanol and washed with 70% ethanol, dried and resuspended in 100 µl of TE.

Genomic DNA purified by either method (A) or method (B) can be cleaved by restriction enzymes for genomic Southern blot analysis. Genomic DNA prepared using (C) suffices for plasmid rescue.

2.6.4 *Drosophila* RNA

Total RNA was isolated using TRIzol™ (Gibco BRL). 40 adult flies (or the same volume of larvae, pupae or embryos) were homogenised in a 1.5 ml Eppendorf with 1ml of TRIzol™ reagent and left at room temperature for 5 min. 0.2 ml of chloroform was added, mixed well and incubated at room temperature for 2-3 min. The mixture was centrifuged at 12000 g at room temperature for 15 min. The aqueous phase (about 600 µl) was carefully removed to a fresh 1.5 ml Eppendorf and 500 µl of iso-propanol was added. After incubation at room temperature for 10 min, the sample was centrifuged at 4°C for 10 min and washed with 70% EtOH. The pellet of total RNA was dissolved in 40 µl of RFW (RNase free water). 40 adult flies can result in 200 -300 µg of total RNA.

2.7 Quantification of nucleic acids

For quantitating the amount of DNA or RNA in a sample, readings were taken at wavelengths of 260 nm or 280 nm. An OD_{260} corresponds to 50 µg/ml for double stranded DNA, 40 µg/ml for RNA and 33 ug/ml for oligonucleotides. When samples had limiting concentrations of DNA (<250 ng/ml), the quantity of DNA was estimated by spotting the sample and known standards onto the surface of a 1% (W/V) agarose gel containing EtBr (0.5 µg/ml). The gel was photographed using short-wavelength UV illumination (254 nm) and the concentration of the DNA sample was estimated by comparing the intensity of fluorescence in the sample with those of known DNA concentration standards.

2.8 Labelling nucleic acids

2.8.1 ^{32}P labelling of DNA

Labelled gel-purified fragments or linearised plasmids were prepared by random priming, a method slightly modified from Feinberg and Vogelstein (1984). Briefly, to 5-100 ng of denatured DNA (in 27 µl of distilled water), 10µl of 4X random priming buffer, 3 µl of [α-^{32}P] dCTP (30µCi; 3000 Ci/mmole) and 1 µl of Klenow DNA polymerase (5 U/µl) were added. The mixture was then incubated for 1 to 4 hr. Probes were purified by Sephadex G50 (Pharmacia) chromatography, in columns prepared from disposable 1 ml syringes (Sambrook *et al.*, 1989).

The 4x Random priming buffer is "home -made" based on the original recipe. The random priming mix is made from three individual components (solutions 1 to 3). These are mixed together to make a batch of random priming buffer that is then aliquoted and stored at -20°C.

Solution 1:	MixΦ*	1 ml
	β–mercaptoethanol	5 µl
	100mM each of dA, dG, dT	5 µl
	Φ*: 1.25 M Tris HCl pH 8.0 and 0.125 M $MgCl_2$.	
Solution 2:	2 M Hepes pH 6.6	
Solution 3:	Hexanucleotides at 90 OD units per ml. The Pharmacia 50 OD unit aliquots of hexanucleotides were dissolved in 0.55 ml water.	

4x buffer	solution 1	solution 2	solution 3
ratio	2:	5:	3
for 0.5 ml	100 µl	250 µl	150 µl

2.8.2 DIG-labelling of DNA

Fragments used to generate probes were excised from the appropriate vector and separated by agarose electrophoresis. 200 ng of this gel purified fragment (See Section 2.9.1) was then used to produce each DIG labelled probe. Briefly, the DNA was denatured at 100°C for 5 min and quickly chilled on ice before addition to the labelling mixture. Distilled water was added to make a volume of 20 µl and the sample incubated at 37°C overnight. The reaction was stopped by the addition of 2 µl of 0.2 M EDTA (pH8.0) solution. The probe was precipitated by adding 2.5 µl of 4 M LiCl and 75 µl prechilled (-20°C) ethanol followed by incubation at -70°C for 30 min. The probe was then pelleted and resuspended in TE (pH8.0).

2.8.3 Nick translation

Labelled plasmid DNA was prepared by nick translation (Sambrook *et al.*, 1989). Briefly, 2.5 µl of 10X Nick Translation Buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M $MgSO_4$, 1 M DTT, 500 µg bovine serum albumin; fraction V; Sigma), 20 nmole each of dATP, dGTP and dTTP (Pharmacia) and 50 µCi; 3000 Ci/mmole of [α–^{32}P] dCTP were added to approx 0.5 µg of plasmid DNA and the volume was made up to 21.5 µl with distilled water. After chilling (0°C) the mixture, 2.5 µl of DNase I (10 ng/ml in ice-cold 1X Nick Translation Buffer containing 50% glycerol) and 2.5 U of *E. coli* DNA polymerase I were added. The reaction was then incubated for 60 min at 16°C and stopped by the addition of 0.04 volume of 0.5 M EDTA, pH 8.0. For probes for chromosomal *in situ* hybridisation the reaction was performed in the presence of 1 mM biotin 16 dUTP (Boeringer Mannheim). A trace [α–^{32}P]dCTP (10 µCi) was also added

progression of the synthesis reaction. The precipitated probe from 500 ng of cDNA plasmid was resuspended in 75 µl of chromosomal *in situ* hybridisation solution (0.6 M NaCl, 50 mM NaPO4, pH 6.8, 5 mM $MgCl_2$, 0.02% ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone).

2.9 Electrophoresis

2.9.1 Agarose gel electrophoresis for DNA

This method was performed as described in Sambrook *et al.*, 1989. DNA was electrophoresed in agarose in 1X TBE (90 mM Tris, 90 mM boric acid, pH8.3, 2 mM EDTA). The marker was a 1 kb ladder (Gibco BRL). DNA fragments were purified from 1% (w/v) LMP (Low Melting Point agarose, Gibco BRL) agarose gel in 1X TAE (40 mM Tris-acetate, pH 7.6, 1 mM EDTA), using the Magic™ DNA purification system from Promega, or by using the silica suspension method (Boyle and Lew, 1995).

2.9.2 Denaturing agarose gel electrophoresis for RNA

Prior to electrophoresis, RNA samples (up to 5 µl) were denatured by the addition of 10 µl of formamide, 2 µl of 5X MOPS buffer (200 mM MOPS, pH 7.0, 50 mM sodium acetate, 5 mM EDTA, 11 M formaldehyde), 3.5 µl of formaldehyde (12.3 M), 1 µl of EtBr (1mg/ml stock), and heated to 70°C for 5 min. Prior to loading, 2.5 µl of loading dye (30% (w/v) Ficoll 400, 1 mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) was added. The RNA was electrophoresed in 1% (w/v) agarose formaldehyde gel (Sambrook *et al.*, 1989), using 1X MOPS, with constant circulation from anode to cathode chambers in order to maintain a constant pH.

2.9.3 Polyacrylamide gel for DNA sequencing

Products of DNA sequencing reactions were separated on denaturing polyacrylamide gels: 6% (w/v) acrylamide (Acrylamide: N, N'-methylenebisacrylamide, 19:1), 7 M urea, in 1X TBE. Polymerisation was initiated by the addition of 1 ml of 10% (w/v) ammonium persulfate and 50 µl of TEMED (N, N, N', N', -tetramethylenediamine) to 150 ml of 6% acrylamide/urea mixture. The gel was allowed to polymerise overnight before use. Samples were denatured for 5 min at 80°C and then loaded onto the gel. Gels were run for various lengths of time, depending on the size of DNA to be resolved, and then dried for 1-2 hr at 80°C on Whatman 3MM paper under vacuum. Autoradiography was carried out without intensifying screens at room temperature.

2.10 Nucleic acid hybridisation

2.10.1 Southern blotting and hybridisation

Agarose gels containing DNA were transferred to nylon membranes (Hybond-N), by capillary action and fixed to the membrane by UV treatment as instructed by the manufacturer (Amersham UK). DNA/DNA hybridisation was carried out at 65°C in hybridisation solution (5X SSPE, 10X Denhart's solution, 1% SDS, 0.005% sodium pyrophosphate and 100µg/ml of denatured sonicated salmon sperm DNA) or in Church buffer (7% SDS, 1% BSA, 1 mM EDTA, 0.25 M Na_2HPO_4 pH 7.2). Filters were pre-hybridised at 65°C for at least 1hr before addition of the denatured radioactive probe (10^5-10^6 cpm/ml of hybridisation solution) and hybridised for between 4 hr and overnight according to the type and amount of DNA on the filters. After hybridisation, the blot was then washed at 65°C in 2x SSPE, 0.1% SDS for 30 min; 0.5x SSPE, 0.1% SDS for 30 min; and finally in 0.1x SSPE, 0.1 % SDS for 30 min. The washed filters

were covered in Saran Wrap™ and then subjected to autoradiography between intensifying screens at -70°C.

2.10.2 Northern blotting and hybridisation

Agarose formaldehyde gels containing RNA were transferred to reinforced nitrocellulose (Hybond C+) by capillary action. RNA was fixed to the membrane by UV treatment as instructed by the manufacturer (Amersham UK). Pre-hybridisation and hybridisation was carried out at 42°C in RNA hybridisation buffer (50% formamide, 5X SSPE, 2X Denhardt's solution and 0.1% SDS) or at 55°C in Church buffer (7% SDS, 1% BSA, 1 mM EDTA, 0.25 M Na_2HPO_4, pH 7.2). Filters were pre-hybridised for at least 3 hr before addition of the denatured radioactive probe (10^5-10^6cpm/ml hybridisation solution) and then hybridised for a minimum of 16hr. The blots were washed at 42°C (or 55°C if the hybridisation was in Church buffer) in 2x SSC, 0.1% SDS for 30 min; 0.5x SSC, 0.1% SDS for 30 min; and finally in 0.1x SSC, 0.1% SDS for 30 min. The washed filters were then covered in Saran Wrap™ and exposed to Fuji X-ray film for 1-3 days. Size was determined with respect to an RNA ladder (Gibco BRL).

2.11 Oligonucleotide synthesis

Oligonucleotides were synthesised by the solid state method on an Applied Biosystems Inc. PCR-MATE 391 DNA Synthesiser, employing phosphoramidite chemistry. After ammonium hydroxide cleavage and deprotection, oligonucleotides were evaporated to dryness under vacuum and resuspended in water or TE. Typically primers were 18-31 nt in length having about 50% G+C composition (Appendix 2)

2.12 DNA sequencing

Sequencing of double-stranded DNA was carried out by the dideoxy chain-termination method recommended in the Sequenase Version 2.0 manual supplied by the manufacturers (United States Biochemical Corporation).

2.13 PCR

Generally PCR reactions were carried out on 100-200 ng of template DNA in 20 µl of 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at room temperature), 1.5 mM $MgCl_2$, 0.01% (w/v) Triton X-100®, 200 µm dATP, 200 µm dCTP, 200 µm dGTP, 200 µm dTTP, primers (each at between 0.33-1 µM) and 1 unit of *Taq* polymerase (Promega). Samples were overlaid with an equal volume of mineral oil (Sigma) and PCRs were performed in a Hybaid Thermal Reactor (Hybaid) with an initial denaturation step of 3 min at 94°C, followed by a three step routine that consisted of 1 min annealing at 55-60°C, extension at 72°C for 3 min and denaturation at 94°C for 1 min. A total of 30 cycles were carried out, followed by a return to 55-60°C for 5 min, a further 20 min extension step at 72°C, and a return to room temperature.

2.14 *In situ* hybridization to polytene chromosomes

Salivary gland chromosome squashes were prepared as described by Ashburner (1989). Chromosomes were probed with a biotinylated, random-primed DNA probe, and hybridisation was detected using streptavidin-conjugated alkaline phosphatase.

2.15 Isolation of cDNA and genomic clone

A λZapII (Stratagene) and a NM1149 (Dorssers and Postmes, 1987) oligo-dT primed cDNA library representing the heads of *eyes absent Drosophila* (S.R.H. Russell, unpublished) was used to screen for cDNAs encoding *Drosophila* V-ATPase A, E, and F subunit. Probes were either Dig-labelled or [α–^{32}P] labelled, random-primed probes of the cDNAs encoding the A, E, and F subunit of *Maduca* V-ATPas. To isolate genomic DNA clones a *D. melanogaster* genomic DNA library in the vector EMBL3 was screened by plaque hybridisation with an [α–^{32}P] labelled random-primed cDNA probe. Positives were purified by second or third round of screening. Genomic DNA fragments were subcloned in pBluescript SK-.

2.16 Generation of unidirectional deletions for rapid DNA sequencing

Generation of unidirectional deletions was with the Erase-a-Base system (Promega), using the method described by the manufacturer. The Erase-a-Base system is designed for the rapid construction of plasmid subclones containing progressive unidirectional deletions of inserted DNA, thus allowing efficient sequencing of large DNA fragments. The system makes use of the ability of exonucleaseIII (*Exo*III) to digest DNA from a 5' protruding or blunt end, while leaving a 4 base 3' protruding end or an α–phosphorothioate filled end intact. The uniform rate of digestion of the enzyme allows a series of deletions of increasing size to be made by removing timed aliquots from the reaction. See Section 6.3.2 and Promega's protocols for detailed procedures.

2.17 Plasmid rescue and mutation screening

The laboratory of Istvan Kiss in Szeged (Hungary) has generated approximately 2300 fly lines with homozygous lethal mutant of a P[*lacW*] element on the second chromosome, which were balanced over *CyO* (Török *et al.*, 1993)

Genomic DNA was prepared by a modification of the method of Hamilton *et al.*, (1991) (see Section 2.6.3) and resuspended in 50 µl of 1X React 2 buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM $NaCl_2$) by heating at 70°C for 15 min. After cooling to room temperature, another 50 µl of React 2 buffer was added, together with 10 units of *Eco*RI, and the tube was placed at 37°C for 3-4 hours. Digestion was halted by heat-inactivation at 70°C for 15 min, and, after cooling to room temperature, ligation was initiated by adding an equal volume of 2x modified ligase buffer (10 mM $MgCl_2$, 4 mM ATP, 20 mM DTT, 30 mM Tris-HCl pH 7.4) and 0.5 µl T4 DNA ligase (Promega, 3 u/µl).

Competent *E. coli* (DH5α or XL1-blue) were prepared using the RbCl method (Section 2.5.1). 200 µl of competent cells were mixed with 40µl of ligated DNA, placed on ice for 15min, heat-shocked at 42°C for 90 sec, again placed on ice for 5 min, and then mixed with 0.5 ml of 2xYT broth. The culture was shaken at 37°C for 1 hr, diluted into 25 ml of LB containing ampicillin at 150 µg/ml, and then shaken overnight at 37°C. Approximately 80% of overnight cultures showed evidence of growth. 1ml from each 25ml culture was stored at -70°C in the presence of 20% glycerol. As a check on contamination, plasmid DNA isolated from 50µl of sampled overnight cultures was characterised by gel electrophoresis.

The remainder of the overnight culture (24 ml) was mixed with cultures representing nine other P[*lacW*] lines, and plasmid DNA was prepared by the alkaline lysis method and the resulted DNA was resuspended in 1 ml of TE. Portions of each pool were then

mixed to make pool of plasmids representing 100 lines for screening (See Chapter 3 for detail).

2.18 Histochemical Staining and Immunocytochemistry

β-Galactosidase expression in larval and adult tissues was detected by X-Gal staining (method modified from Bellen *et al.*, 1989). Adults or larvae were dissected in 1X PBS and tissues were fixed in 1% glutaraldyde for 10-15 min. After washing with 1X PBS twice, tissues were stained in X-gal solution overnight.

Embryo staining required more steps. Embryos were collected from yeasted apple/grape juice agar plates and dechorionated by dipping into 50% bleach (sodium hypochlorite solution, Safeway's bleach, freshly diluted 1:1) for 90 seconds. After washing with water, the embryos were fixed in a mixture of 0.35 ml 4% paraformaldehyde in 1X PBS and 0.7 ml n-heptane for 15-20 minutes at room temperature. The embryos were then washed at least twice with 800 µl 1XPBS + 0.1%Triton X-100 and stained in X-gal solution until the colour appeared.

For staining with anti β-Galactosidase primary antibodies the tissue was fixed in 4% paraformaldehyde (in 1 X PBS) for 15 mins and washed twice in 1 X PBS, 3% triton X-100 and then preincubated in PAT (1 X PBS, 1% BSA, 1% Triton X-100) for 1 hour. The primary antibody, at a dilution of 1:2000 in PAT and 3% normal goat serum, was added and incubated overnight. The tissue was rinsed several times in PBS then reacted with an FITC-cojugated secondary antibody (1:250 for 1 hour). After washing in PAT, the tissue was then mounted in VectaShield for detection.

2.19 Isolation of viable revertants and new alleles with P-element excision

Once a specific mutation line is isolated, it is necessary to isolate a viable revertant to prove the lethality is due to the insertion. If the insertion is on the 2nd chromosome,

female mutants are crossed to males carrying Sb, Δ2,3 on their third chromosome over the TM6b balancer. This cross yields F0 "jumpstart" male carrying both P[*lacW*] and the Δ2,3 element, and thus the P[*lacW*] will be mobilised. The crossing scheme is shown in Figure 2.1. Where the insertion is not within the gene, but at a site near the gene, local jumping combined with the strategy of PCR screening can identify other insertions within the target gene. The P-element loss may be precise or imprecise (Klambt *et al*, 1992; Tower *et al*, 1993). The identification of viable revertants proves that lethality was due to the P-element insertion.

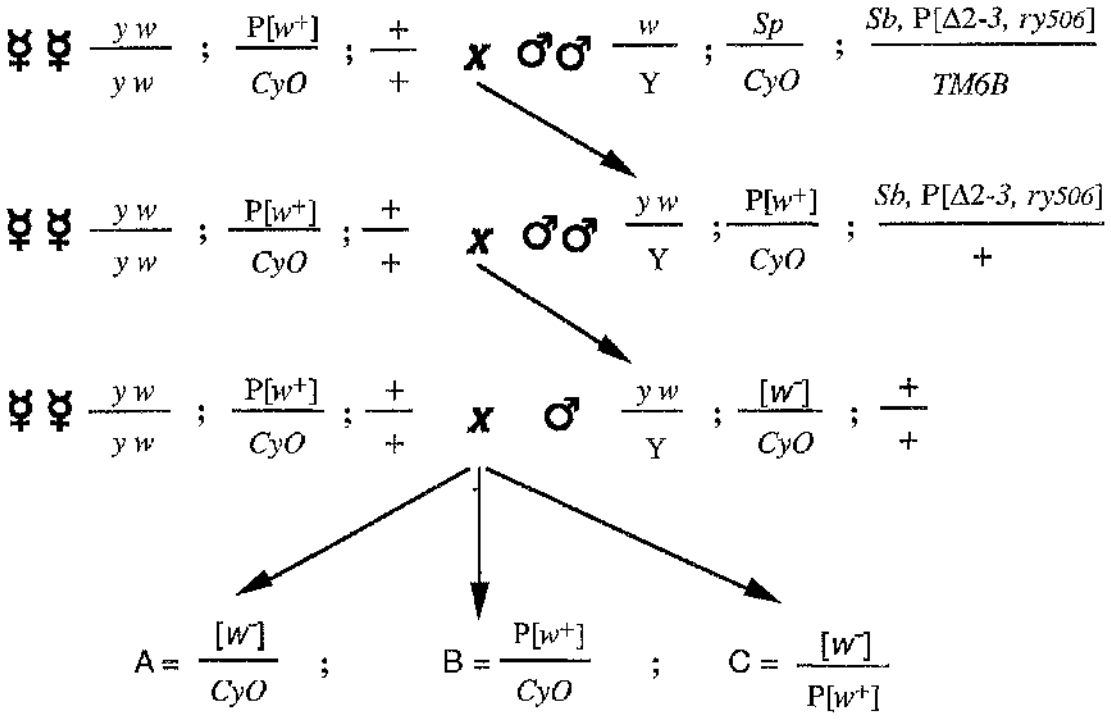


Figure 2.1 Scheme for isolation of viable revertants and deficiency strains. P[*W*+] stands for the P[*lacW*], [*w*-] stands for loss of the *w*+ marker.

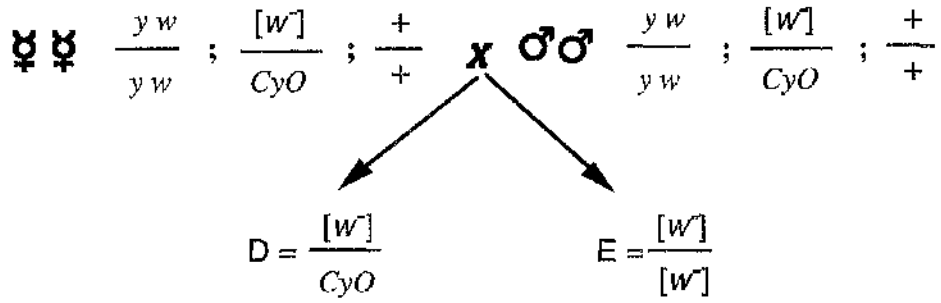
The numbers of adults with phenotypes A, B and C were recorded.

If the numbers of A, B and C are equal, there has been a clean reversion of the homozygous lethal phenotype.

If the number of type C is less than A and B, it suggests that type C are suffering deleterious effects following remobilisation, i.e. a new allele with internal deletion within the original P-element or imprecise deletion of the gene.

If C=0, it is likely to be a new lethal allele due to deletion caused by imprecise excision or by internal deletion within P[*lacW*].

The survival efficiency of homozygous [*w*-]/[*w*-]can be further evaluated by the following cross.



The number of adults with phenotypes D and E was recorded.

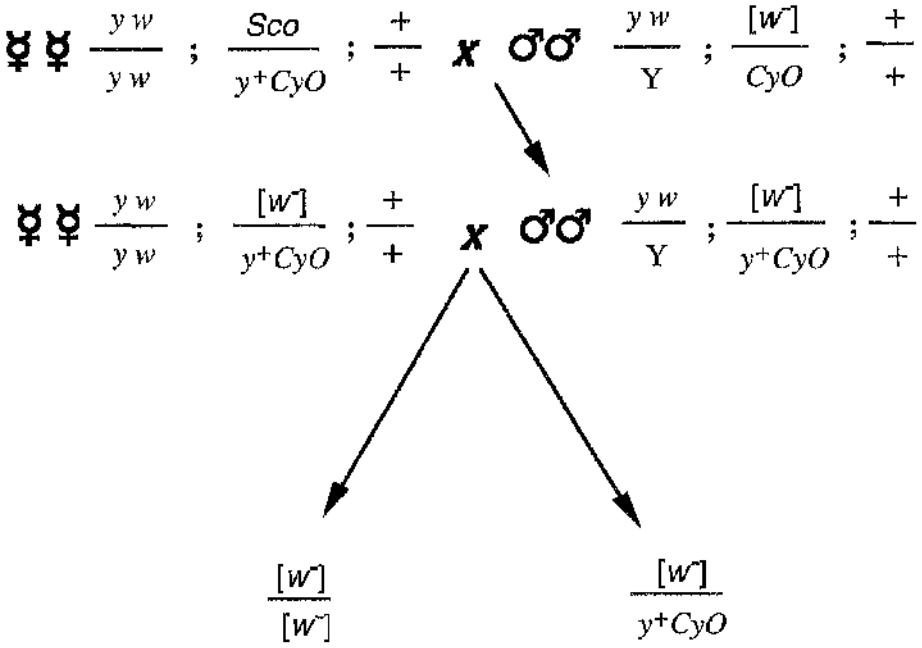
If E=D/2, there has been clean reversion.

If 0<E<D/2; then the excision event has had some deleterious effects.

If E=0; then it is a new lethal allele with imprecise deletion or internal deletion of the P-element.

2.20 Determination of lethal phase of the mutations

In order to determin the developmental phase for lethalities the original *CyO* balancer was replaced with a modified *CyO* balancer marked with a copy of y^+. Embryos were collected overnight from *y w*; P[*lacW*]/y^+*CyO* females crossed with yw; P[*lacW*]/y^+*CyO* males (See the following cross scheme). Eggs were laid out on an apple juice agar plate and incubated at 25°C. At regular intervals over a 48 hour period, the plate was examined to determine how many larvae had hatched. The phenotype of the larvae was determined by examination of their mouth hooks, homozygous y larvae possessing gold brown mouth hooks while heterozygous y^+ larvae have brown/black mouth hooks.



Hence, offsprings with phenotype D and E can be distinguished as early as first instar larvae, allowing the lethal stage of the homozygous flies to be determined.

Chapter 3

Site-Selected Mutagenesis of the *Drosophila* Second Chromosome *via* Plasmid Rescue of Lethal P-Element Insertions

3.1 Summary

This chapter describes a fast and efficient approach to correlating cloned genes with mutant phenotypes in *Drosophila*. We make use of a large collection *D. melanogaster* lines with recessive lethal insertions of a P[*lacW*] transposon on their second chromosome. Within this collection there must clearly be many insertions corresponding to *Drosophila* genes that have been cloned and characterised, but for which mutant phenotypes have yet to be identified. We have made use of the fact that P[*lacW*] contains a plasmid replicon to establish a collection of rescued plasmids containing genomic DNA flanking the sites of transposon insertion. Plasmids representing a total of 1836 lines were *independently* rescued, and pooled in batches of 10 and 100. Pools of 100 plasmids were screened by hybridisation with cDNAs corresponding to cloned second chromosome loci. Hybridising pools were then narrowed down to single plasmids by a process of subdivision and rehybridisation, and corresponding mutant lines were obtained.

3.2 Introduction

Many cloned *Drosophila* genes have yet to be correlated with a mutant phenotype. Site-selected transposon mutagenesis (SSM) is a reverse genetics solution to this problem. As originally described it involves the use of PCR between gene- and transposon-specific primers to identify individuals in which a P element transposon had inserted in or close to a target gene (Ballinger and Benzer, 1989; Kaiser and Goodwin, 1990; Banga *et al.*, 1992). The sensitivity of PCR allows a new insertion to be detected initially within a

population of mutagenised flies, after which it can be followed, as a specific amplification product, while the population is sub-divided. A similar strategy has been applied to mutagenesis of *Caenorhabditis elegans* (Rushforth *et al.,* 1993; Zwaal *et al.,* 1993) and maize (Das and Martienssen, 1995).

P elements engineered to contain a plasmid origin of replication and a drug-resistance determinant allow a different form of SSM, involving plasmid rescue of DNA flanking the site of insertion (Figure 3.1; Hamilton *et al.,* 1991; Hamilton and Zinn 1994; Guo *et al.,* 1996c). Pools of plasmids are created, each representing a population of flies with different insertion sites. Hybridisation between a pool and a specific cDNA/genomic DNA fragment is diagnostic of an insertion in or near to the gene of interest. The relevant pool is then narrowed down to a single hybridising plasmid, and thus to the corresponding *Drosophila* line, by a process of subdivision and re-hybridisation (Hamilton *et al.,* 1991; Guo *et al.,* 1996c).

Generation of large numbers of P element insertion lines is labour-intensive, as is their maintenance. In any case, only a small fraction of all new P element insertions is associated with phenotypic consequences. Thus, SSM tends to involve relatively transient collections of lines that are discarded or dispersed soon after screening. Even allowing for simultaneous screening with a number of target genes, this tends to reduce the generality of SSM. Further, plasmid rescue SSM tends to be performed on pools of lines (Hamilton *et al.,* 1991; K. Basler and E. Hafen, personal communication), rather limiting the amount of plasmid DNA that can be generated per individual line, and inevitably leading to misrepresentation of the individual plasmids. If time and resources allowed, it would clearly be preferable to rescue each line independently.

A recent large scale screen for P[*lacW*] transposon insertions on the *D. melanogaster* second chromosome forms the background to a means by which some of the above

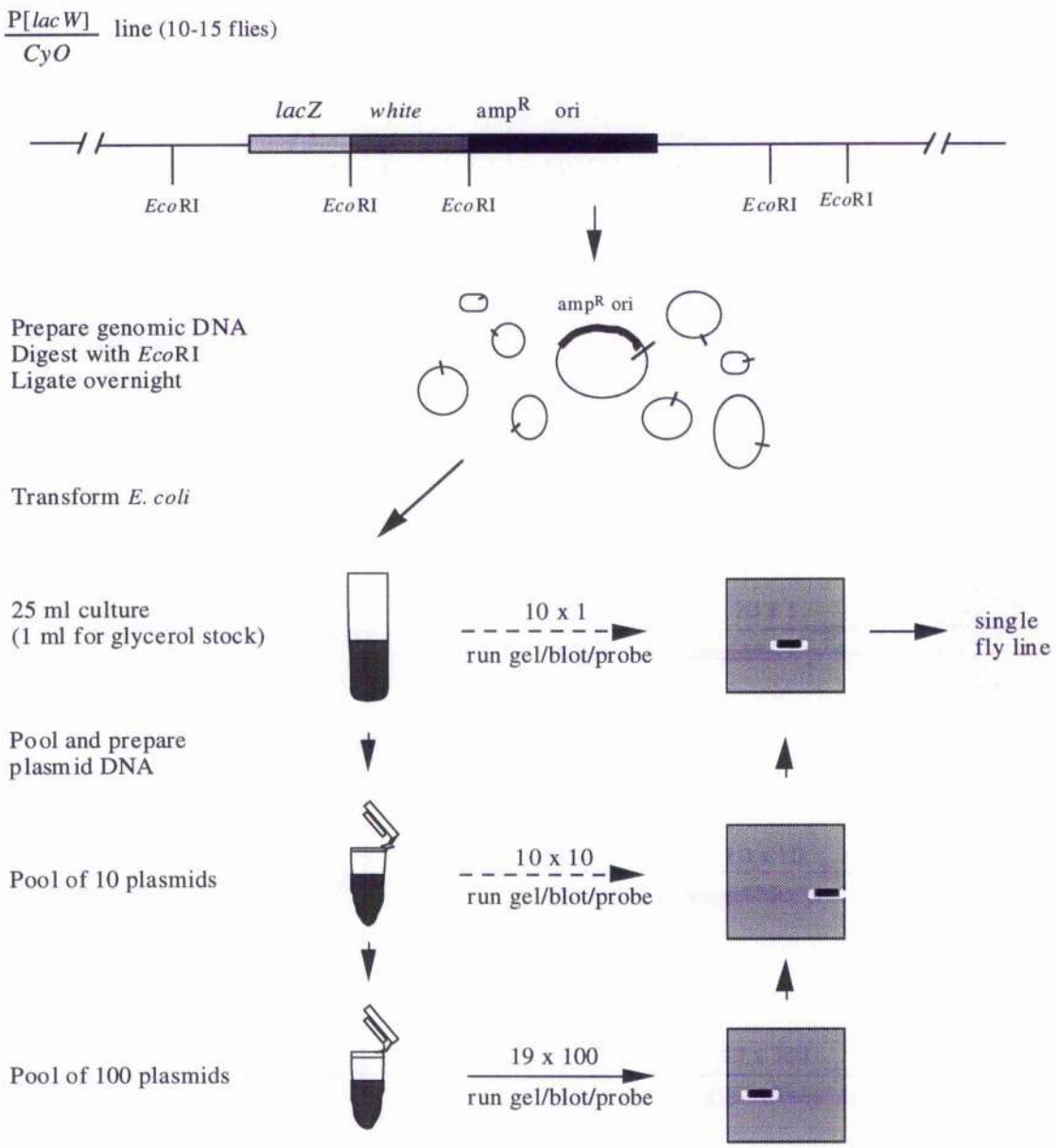

P[lacW] / CyO line (10-15 flies)
lacZ
white
ampR ori
EcoRI
EcoRI
EcoRI
EcoRI
EcoRI
ampR ori
Prepare genomic DNA
Digest with EcoRI
Ligate overnight
Transform E. coli
25 ml culture
(1 ml for glycerol stock)
10 x 1
run gel/blot/probe
single
fly line
Pool and prepare
plasmid DNA
Pool of 10 plasmids
10 x 10
run gel/blot/probe
Pool of 100 plasmids
19 x 100
run gel/blot/probe

Figure 3.1 Overview of the plasmid rescue strategy. The essential structure of the P[*lacW*] transposon is shown at the top of the figure. Each line is maintained as a 'balanced lethal' in which only one of the pair of second chromosomes carries a recessive lethal P[*lacW*] insertion. The other second chromosome, the balancer *CyO*, confers a dominant visible phenotype (curly wings), is homozygous lethal, and suppresses recombination. Balanced lethal lines are thus easily maintained, since viable progeny have the same chromosomal constitution as their parents (see Ashburner 1989). P[*lacW*] contains an ampicillin resistance determinant (amp^R) and a plasmid origin of replication (*ori*). This plasmid replicon is separated from the rest of the transposon by a unique site for *Eco*RI. Rescued plasmids therefore contain DNA extending to the right of the transposon up to the nearest flanking *Eco*RI site (complete digestion), or to a more distant site (partial digestion). Full arrows in anticlockwise direction show the order in which particular steps were carried out. Dashed arrows show source of plasmid DNA for second and third rounds.

problems can be overcome. 2308 independent recessive lethal mutations and 403 'semi-lethal' mutations were generated, each of which was saved in the form of a balanced lethal stock, and the lethal phase determined (Török *et al.,* 1993). P-induced lethals, though infrequent, must almost by definition correspond to insertions within genes. Inevitably the collection is likely to include many examples of genes that have been 'hit' more than once. There is also an unexpectedly high frequency (≈ 50%) of lethals that do not coincide with an inserted P element (Kiss, I person. Com., 1996). Nevertheless, the collection represents a substantial proportion of the 2000 or so lethal complementation groups estimated to be present on the second chromosome (13/48 of the lethal complementation groups within the 1.8 Mb 34D-36A region, for example; Spradling *et al.,* 1995). Moreover, even non-lethal insertions are useful starting points for the secondary mutagenesis of flanking loci. The lines will be maintained in Szeged (Hungary), and possibly in other stock centres, for the conceivable future.

3.3 Plasmid Rescue

P[*lacW*], a modified P element transposon 10.6 kb in length, was designed as an enhancer-trap element (Bier *et al.,* 1989). It carries a *lacZ* reporter gene, the eye-colour marker *white*$^{+}$, and a plasmid replicon with poly-linker (Figure 3.1). Insertion *within* a *Drosophila* gene of such a large element might be expected often to have significant consequences for gene expression (Spradling *et al.,*1995). Plasmid rescue using the enzyme *Eco*RI was attempted independently for 2210 of the lines of Török *et al.,* (1993), as described in Materials and Methods.

Independent rescue and transformation allowed each transformant to be propagated without the risk of competitive growth. Rescue was successful in the case of 1836 of the 2210 lines (83%). Recalculated in the context of available *in situ* hybridisation data (Refer Encyclopaedia of *Drosophila*), this corresponds to 77% rescue of lines containing a single P[*lacW*] element, and 89% rescue of lines containing more than one P[*lacW*]

element. Because we were concerned that such a large series of transformations could present a contamination problem, small scale plasmid preparations of at least 500 transformants were analysed by agarose gel electrophoresis. Plasmid sizes varied considerably, with no evidence of contamination at any stage (not shown). Since most lines contain just one P[*lacW*] transposon (data not shown; Török *et al.*, 1993), rescue usually involved a single flanking region. Partial cleavage of genomic DNA by *Eco*RI can give rise to a series of related plasmids, however, and it is also possible for unrelated *Eco*RI fragments to be 'co-cloned'.

A 25 ml culture was generated for each P[*lacW*] line, and a small quantity was put into long-term storage in the form of a glycerol stock. The remainder was pooled together with cultures representing nine other lines, and plasmid DNA was isolated. Equal volume samples of ten such plasmid preparations were then mixed to create effective pool sizes of 100 plasmids. The amount of plasmid DNA generated will be sufficient for many screenings.

3.4 Screening

Plasmid DNAs in each of the 19 pools of 100 plasmids are separated in twenty slot agarose gels (Figure 3.2). The final slot is used for hybridisation controls and size markers. To screen for an insertion in the vicinity of a cloned gene, a blot of the gel is hybridised with a relevant cDNA or genomic DNA fragment. If the fragment has been cloned using a vector that contains plasmid sequences, it is essential that the fragment be gel-isolated before use. Here we show the results of screening several interesting *Drosophila* genes, of which *vha68-2* and *ductin* are the genes encoding *Drosophila* V-ATPase subunit A and c respectively.

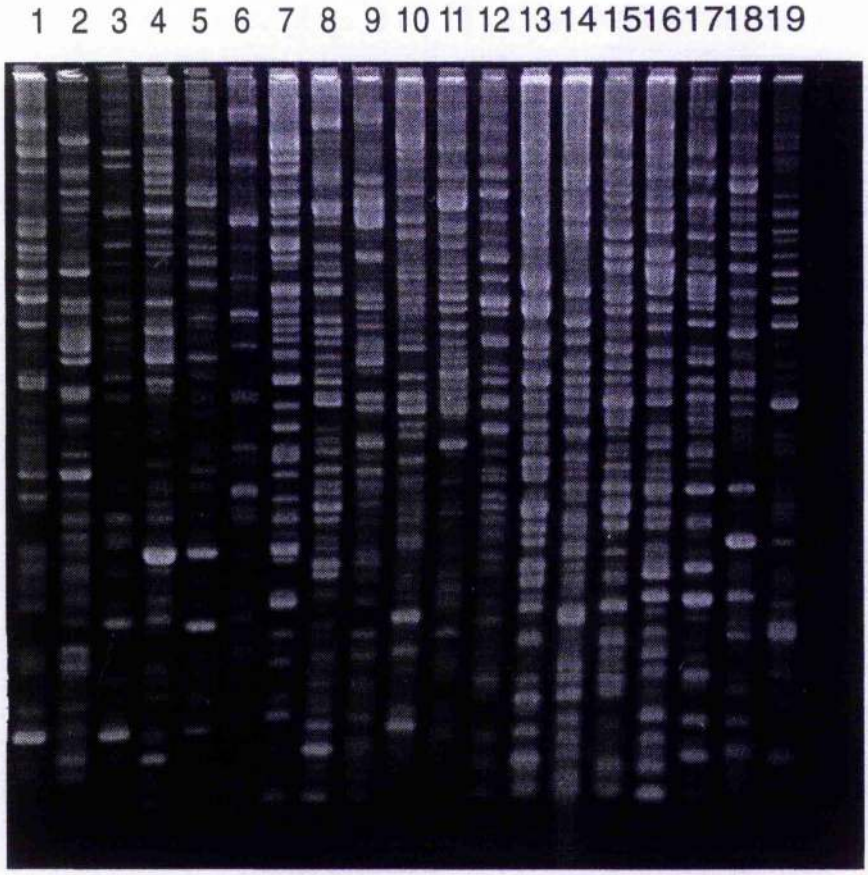


Figure 3.2 19 pools of 100 plasmids separated by electrophoresis in a 0.8% agarose gel.

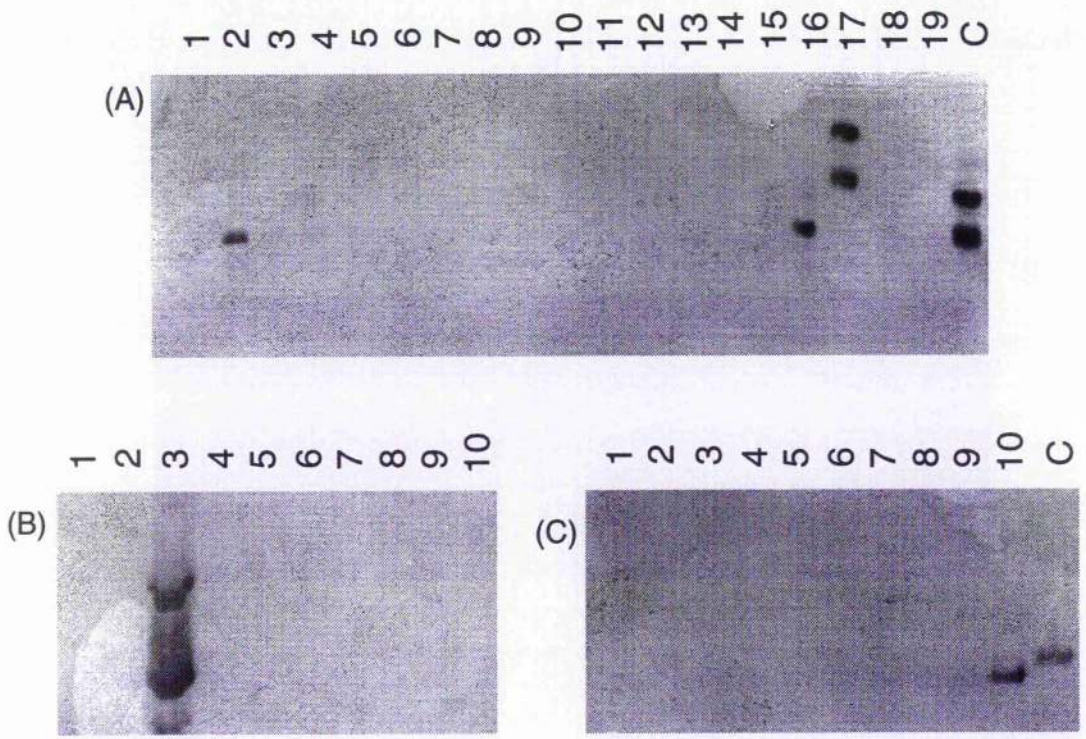


Figure 3.3 Screening for insertions in *vha68-2* the gene encoding subunit A of the *Drosophila* V-ATPase. (A) Three pools of 100 plasmids showed cross-hybridisation with *vha68-1* cDNA probe (lanes 2, 16, and 17). (B) Screening the ten pools of ten plasmids corresponding to lane 2 further narrowed down this particular insertion (lane 3). (C) Hybridisation was eventually assigned to a plasmid isolated from a single glycerol stock (lane 10). C indicates a positive hybridisation control (*vha68-1* cDNA).

3.4.1 *vha68-2*, the gene encoding V-ATPase A-subunit

Figure 3.3 are results of screening with a *vha68-2* cDNA fragment representing the gene encoding subunit A of the *Drosophila* vacuolar ATPase (See Chapter 5). Bands of hybridisation are seen in three lanes of 100 plasmids (Figure 3.3A). One such band was followed through subdivision to the relevant ten batches of ten plasmids (Figure 3.3B), and was eventually narrowed down to a single glycerol stock (Figure 3.3C). Detailed analysis of this P[*lacW*] insertion line is reported in Chapter 5.

3.4.2 *Ductin*, the gene encoding the V-ATPase c-subunit

Ductin, the 16 kDa proteolipid c-subunit of V-ATPase is the major component of the vacuolar H^+-ATPase membrane sector, responsible for proton translocation (Meagher *et al.*, 1990; Finbow *et al.*, 1994). Screening the pool of rescued plasmids found lines 16/1 and 76/16 hybridised to the genomic DNA probe (Figure 3.4). Line 16/1 has an insertion in the second intron (Figure 3.7A). Although the rescued plasmids from line 76/16 can hybridise to the *ductin* probe, the sequence near the P element do not align to *ductin* genomic DNA sequence. It is likely that the insertion in line 76/16 is near the gene, but outside of the reported genomic DNA sequence (GenBank accession no. X77936). Further analysis of these two lines is being carried out by Miss Shirley Graham in this department.

3.4.3 *CalpA*, the gene encoding calpain

CalpA is a highly tissue-specific calpain gene from *Drosophila*, specifically expressed in a small set of nerve, midgut and blood cells (Theopold *et al.*, 1995). This calpain is involved in the dynamic changes in the embryonic cytoskeleton, especially actin-related structures, during early embryogenesis prior to cellularization (Emori and Saigo, 1994). The gene is located at 56C-D on the second chromosome. Using *CalpA* cDNA as a

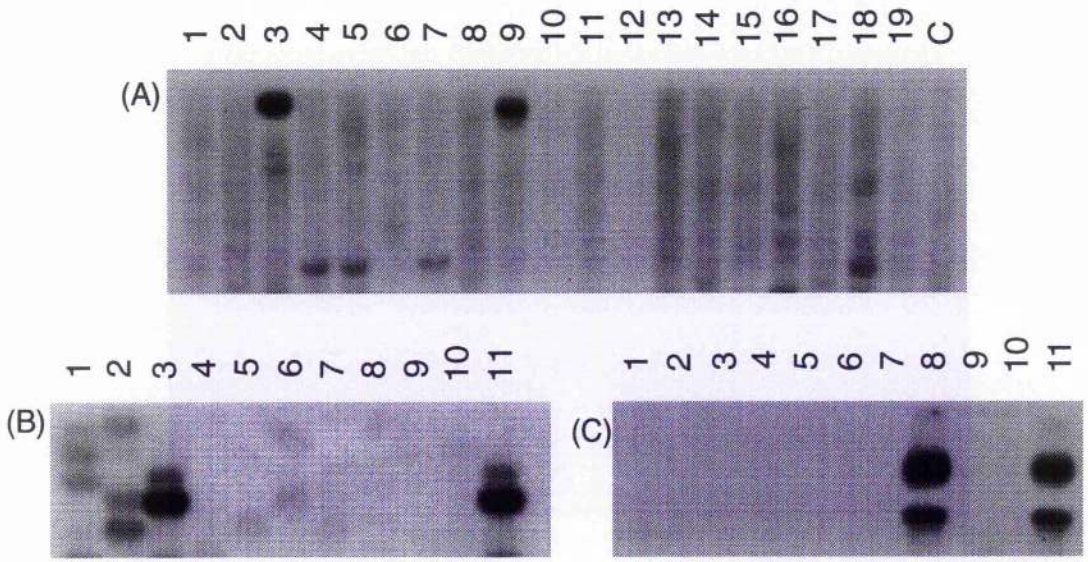


Figure 3.4. Screening for insertions in *ductin*, encoding subunit c of *Drosophila* V-ATPase. (A) Two pools of 100 plasmids showed cross-hybridisation with a *ductin* cDNA probe (lanes 3 and 9). Lane C did not contain a control for the *ductin* probe (blots are reused). (B) Screening the ten pools of ten plasmids corresponding to lane 3 further narrowed down this particular insertion (lane 3). Lane 11 represents the previous pool of 100. (C) Hybridisation was eventually assigned to a plasmid isolated from a single glycerol stock (lane 8). Lane 11 represents the previous pool of 10.

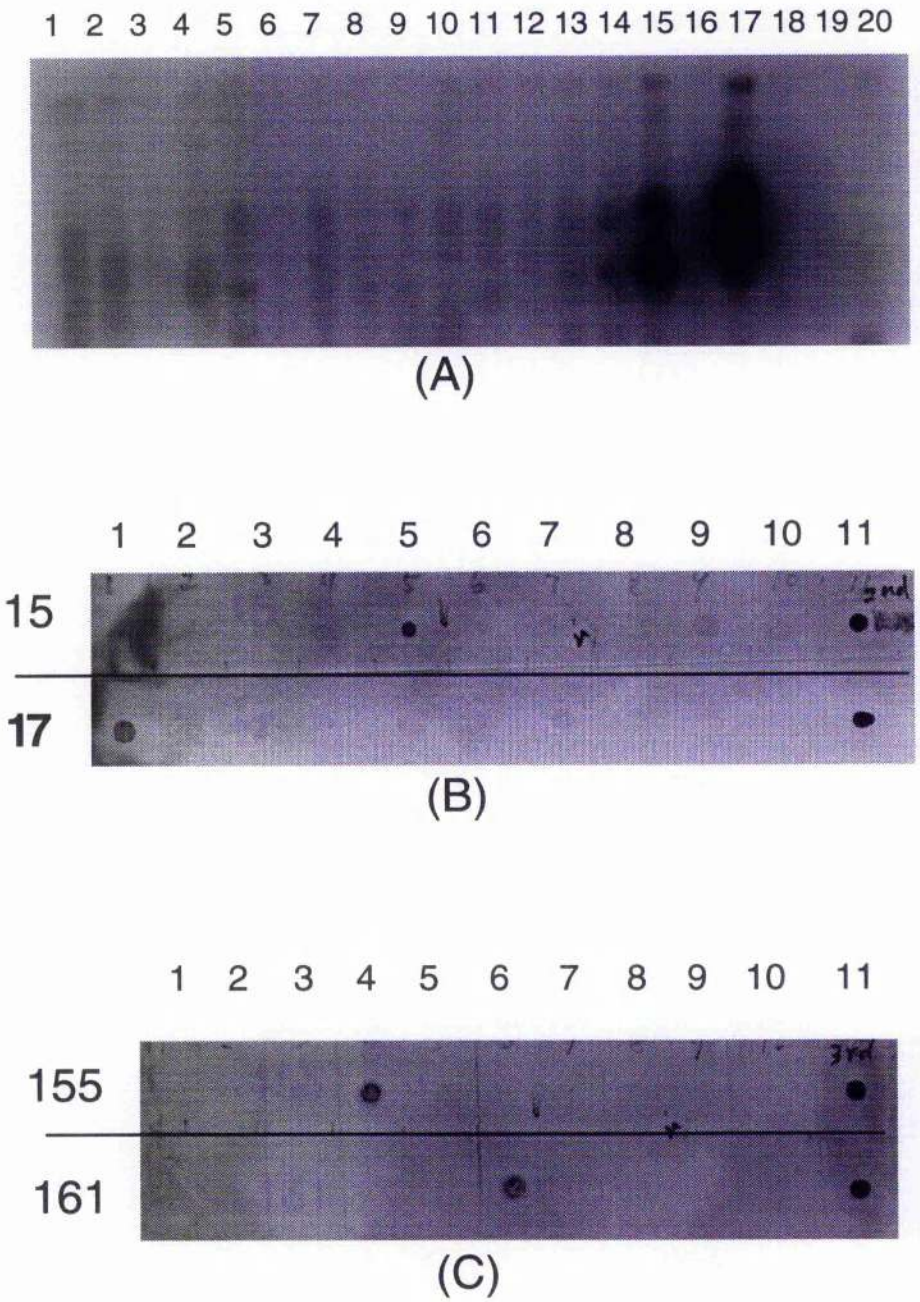


Figure 3.5 Screening for insertions in *CalpA*, a *Drosophila* calpain homolog. (A) Two pools of 100 plasmids showed cross-hybridisation with *CalpA* cDNA probe (lanes 15, 17). (B) Screening the ten pools of ten plasmids corresponding to lane 15 and 17 by dot hybridisation, further narrowed down these particular insertions to dots 5 and 1 respectively. Dot 11 is the former pooled 100 as control. (C) A further round of dot hybridisation eventually identified two single glycerol stocks (Dot 4 and dot 6). Dot 11 is the former pooled 10 as a control.

probe to screen the pool of rescued plasmids found the 15th and 17th lanes showed positive hybridisation (Figure 3.5 A). Subdivision by DNA dot hybridisation assigned the two positive bands to two individual lines: 145/23 and 169/13 (Figure 3.5 B, C). Line 162/14 has an insertion between *CalpA* and *hu-li-tai-shao* (Ding *et al.,* 1993) It is likely the insertion is at the regulatory region of *CalpA*. However, insertion in line 145/23 is in the nearby gene, *hu-li-tai-shao* (Figure 3.7 B). Further analysis is carried out by Dr. Philippe Rosay in this laboratory. He is trying to remobilise the P-elements into the *CalpA* gene.

3.4.4 *DC0* the catalytic subunit of cAMP-dependent protein kinase

DC0 is the gene encoding the catalytic subunit of cAMP-dependent protein kinase (Kalderon and Rubin 1987; Figure 3.6). The *DC0* cDNA was used as probe to screen the pool of rescued plasmids and bands of hybridisation are seen in three lanes of 100 plasmids. One such band was followed through subdivision to the relevant ten batches of ten plasmids, and was eventually narrowed down to a single glycerol stock from line 8/4. The insertion is within the first intron. (Figure 3.7C).

3.4.5 *Syb*, a gene encoding synaptobrevin

Synaptobrevin is a major constituent of the membranes of synaptic vesicles. *Syb* is a *Drosophila* gene encoding an isoform of synaptobrevin that abounds in non-neuronal cells. The *Syb* transcripts show no enrichment in the nervous system and are present in very early embryos, well before neurogenesis. The greatest concentration of *Syb* transcripts has been found in cells of the gut and Malpighian tubules. It has been suggested that *Syb* may be involved in membrane trafficking and in the secretion of digestive enzymes (Chin *et al.,* 1993). However, the precise function of *Syb* is unknown.

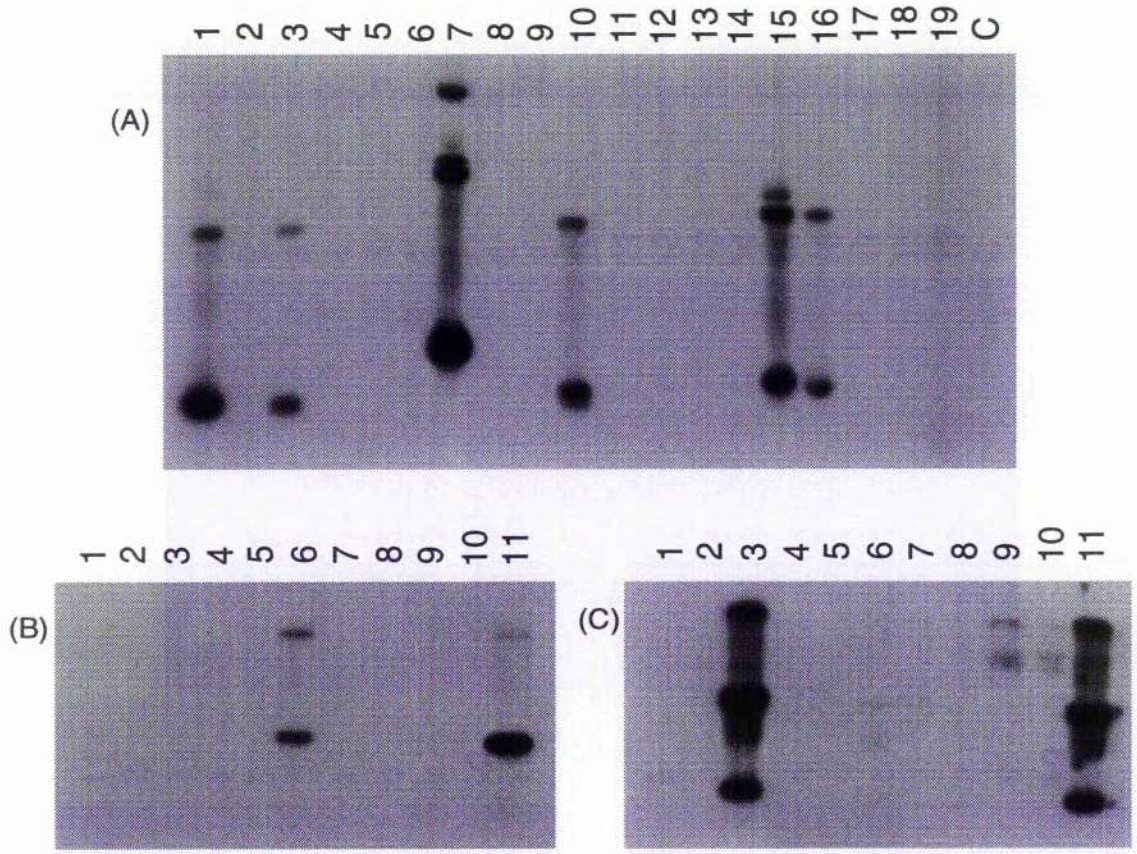


Figure 3.6 Screening for insertions in *DC0*, encoding a catalytic subunit of cAMP-dependent protein kinase. (A) Several pools of 100 plasmids showed cross-hybridisation with a *DC0* cDNA probe. (B) Screening the ten pools of ten plasmids corresponding to lane 1 further narrowed down this particular insertion (lane 6). Lane 11 represents the previous pool of 100. (C) Hybridisation was eventually assigned to a plasmid isolated from a single glycerol stock (lane 3). Lane 11 represents the previous pool of 10.

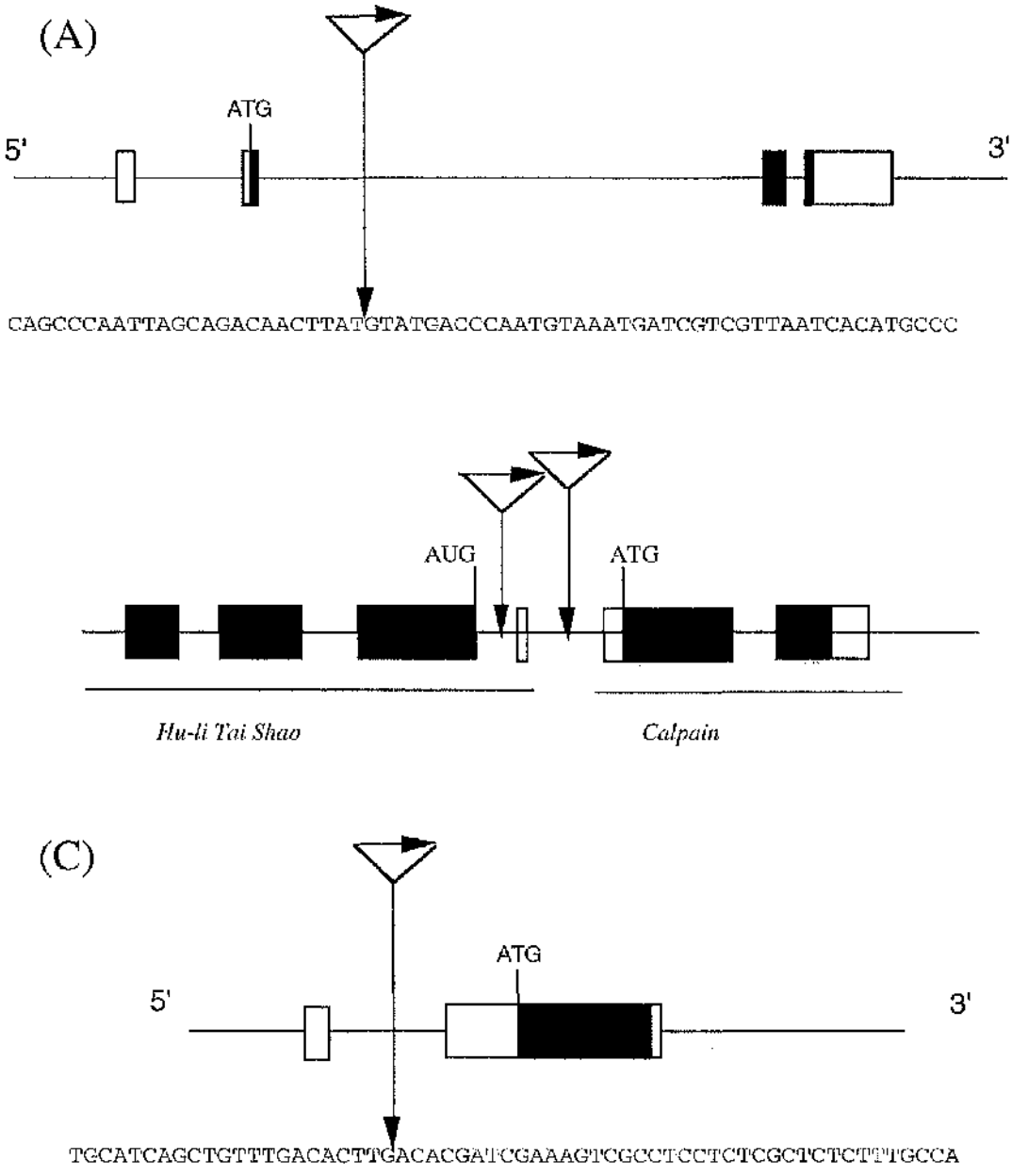


Figure 3.7 Insertion in *ductin*, *CalpA* and *DC0*. (A) Insertion in gene of *ductin*, the subunit c of V-ATPase (GenBank accession no. X77936); (B) Insertions in or near gene encoding calpain. (GenBank accession no. X78555, Z46891, Z46892) (C) insertion in *DC0*, the catalytic subunit of cAMP-dependent protein kinase (GenBank accession no. X16969). Arrow on P-element denotes the sense of P-*lacZ* reporter gene.

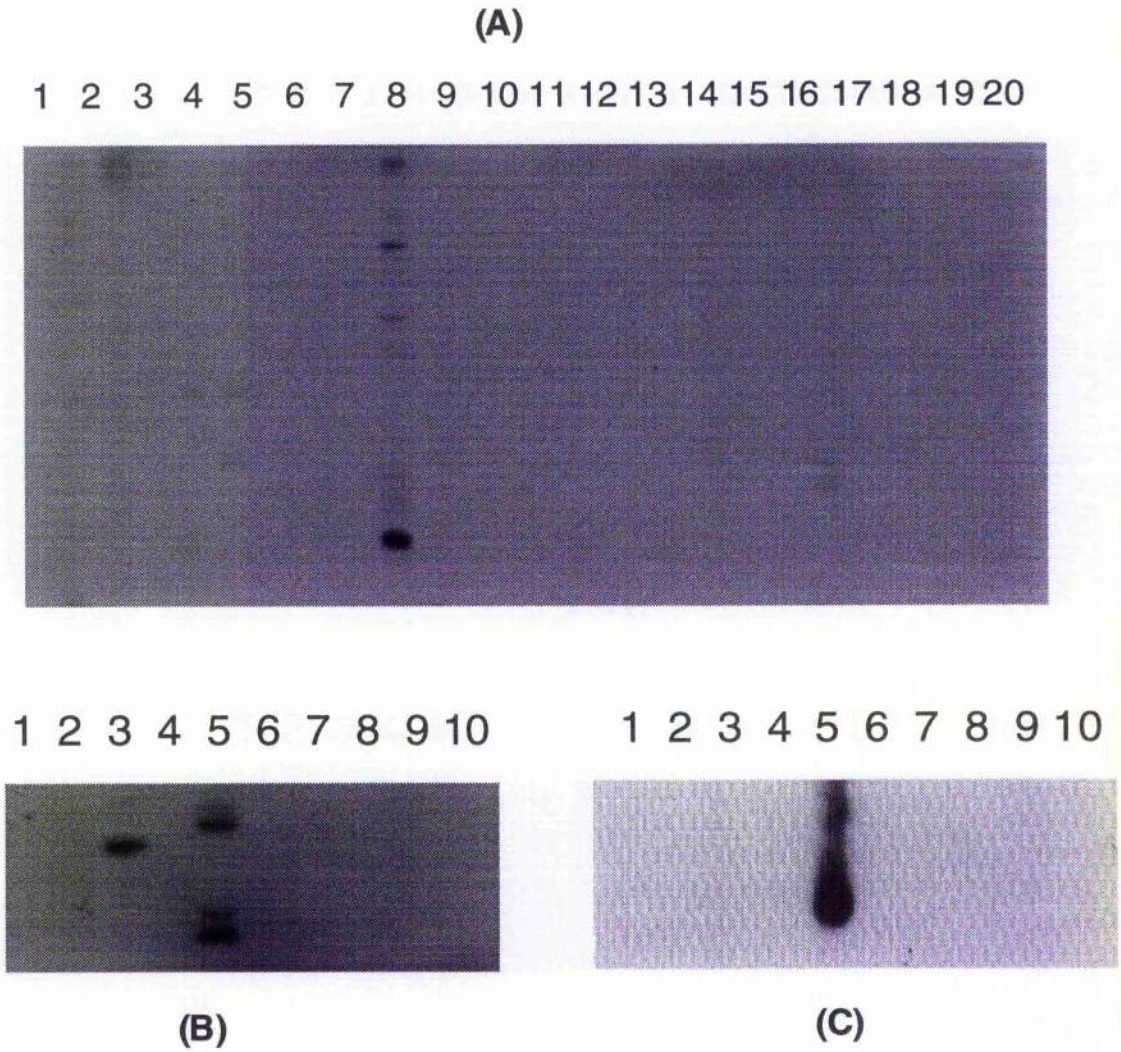


Figure 3.8 Screening for insertion in *syb*, the gene encoding synaptobrevin (A) One pool of 100 plasmids showed cross-hybridisation with a *syb* cDNA probe (Lane 8). (B) Screening the ten pools of ten plasmids corresponding to lane 8 further narrowed down this particular insertion to two pools of 10 (Lane 3 and 5). (C) Subdivision of the pool of 10 in lane 5 eventually assigned the positive band to a plasmid isolated from a single glycerol stock (lane 5).

(A)

```
                                        10        20        30
p958.s                                  TTGTTATTTCATCATGGCTCAGCGCACAAAAGCAAGGA
                                                  ||||||||||||||||||||||||||||
l14270  ACAACGAGTAAGTGGTGGAAGTCCATCGAATCAACAGGCTCAGCGCACAAAAGCAAGGA
              910       920       930       940       950       960

        40        50        60        70        80        90
p958.s AAATCCCATACAGTGACGTCACCTGCGTCATATGGGCCACAGCGAACCGGAAGTAAAGTC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
l14270 AAATCCCATACAGTGACGTCACCTGCGTCATATGGGCCACAGCGAACCGGAAGTAAAGTC
              970       980       990      1000      1010      1020

      100       110       120       130       140       150
p958.s TTCGGACTTCACTTTCGGACATTGGAATACCGTAAACGTATGCTGCTGCCCAAGCGGTAG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
l14270 TTCGGACTTCACTTTCGGACATTGGAATACCGTAAACGTATGCTGCTGCCCAAGCGGTAG
              1030      1040      1050      1060      1070      1080

      160       170       180       190       200       210
p958.s CTTCAGTTGTAGTTTTGAAAATCAATTAGGGTCATTTAAAAGCATTCAGTTAAGTT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||
l14270 CTTCAGTTGTAGTTTTGAAAATCAATTAGGGTCATTTAAAAGCATTCAGTTAAGTTGAGA
              1090      1100      1110      1120      1130      1140

l14270 TAGTGTAGAGTCAGCTGCTATCTTAGATGGAATATTAATGTGAAATGGCAAATTAACTCG
              1150      1160      1170      1180      1190      1200
```

(B)

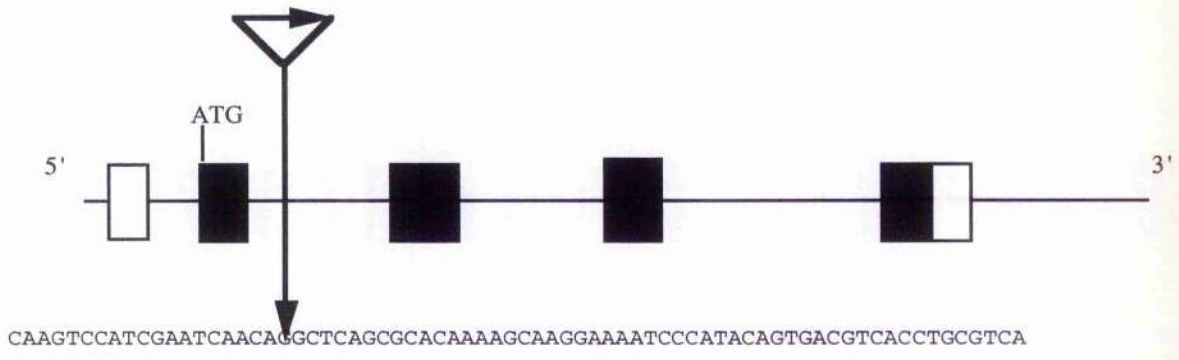


Figure 3.9 Insertion in *Syb*. (A). Alignment of sequence of rescued plasmid p958 from mutant line 77/5 to *syb* genomic DNA sequence. (B) Position of insertion in *syb*, the gene encoding synaptobrevin (Chin *et al.*, 1993; GenBank accession no. L14270)

The filter with rescued plasmids was screened with a *Syb* cDNA probe (provided by Dr. Cahir O'Kane in Cambridge) and lane 8 showed positive hybridisation (Figure 3.8A). After subdivision of this pool of plasmids of 100 plasmids, lanes 3 and 5 show positive hybridisation (Figure 3.8B). Subdivision of the two lanes identified that the two plasmids from line 75/2 and 77/5 showed cross-hybridisation to the *Syb* probe. The sequence flanking the site of insertion in line 77/5 is identical to part of *Syb* gene. The exact position of p[*lacW*] is in the second intron (Figure 3.9A, B; Chin *et al* ., 1993). However, the insertion in line 75/2 is not relevant to *Syb*. The hybridisation of the plasmid from line 75/2 is due to a *Syb* fragment co-cloned during plasmid rescue. Repeated rescued plasmids from this line do not hybridise to the *Syb* probe.

Southern blotting of 77/5 and Canton S genomic DNA probed with *Syb* cDNA detected a 3.4 kb *Eco*RI band in addition to the wild type 5.1 kb band (Figure 3.10A). The band shift is due to the P-element insertion. Northern blotting showed a reduction of *Syb* RNA in the P[*lacW*]/+ heterozygotes (Figure 3.10 B). Homozygous flies usually died shortly during the stage of the first instar larvae. Remobilising of the P-element produced many revertants and new alleles. Reversion indicated that the lethal phenotype was indeed caused by the P-element insertion. Further examination of the defect of the *Syb* mutant is being carrying out collaboratively with Dr. Cahir O'Kane's group in Cambridge.

3.4.6 *KLP38B*, a mitotic kinesin-related protein

KLP38B (Kinesin-Like-Protein-at-38B) is a new member of the kinesin superfamily in *Drosophila*. *KLP38B* was isolated through its binding to the catalytic subunit of type 1 serine/threonine phosphatase (PP1) in the two-hybrid interaction trap. Seven lines with P[*lacW*] insertions in the intron of *KLP38B* were isolated (Figure 3. 11). See Alphey *et al* . (1996) for detailed analysis of these mutants.

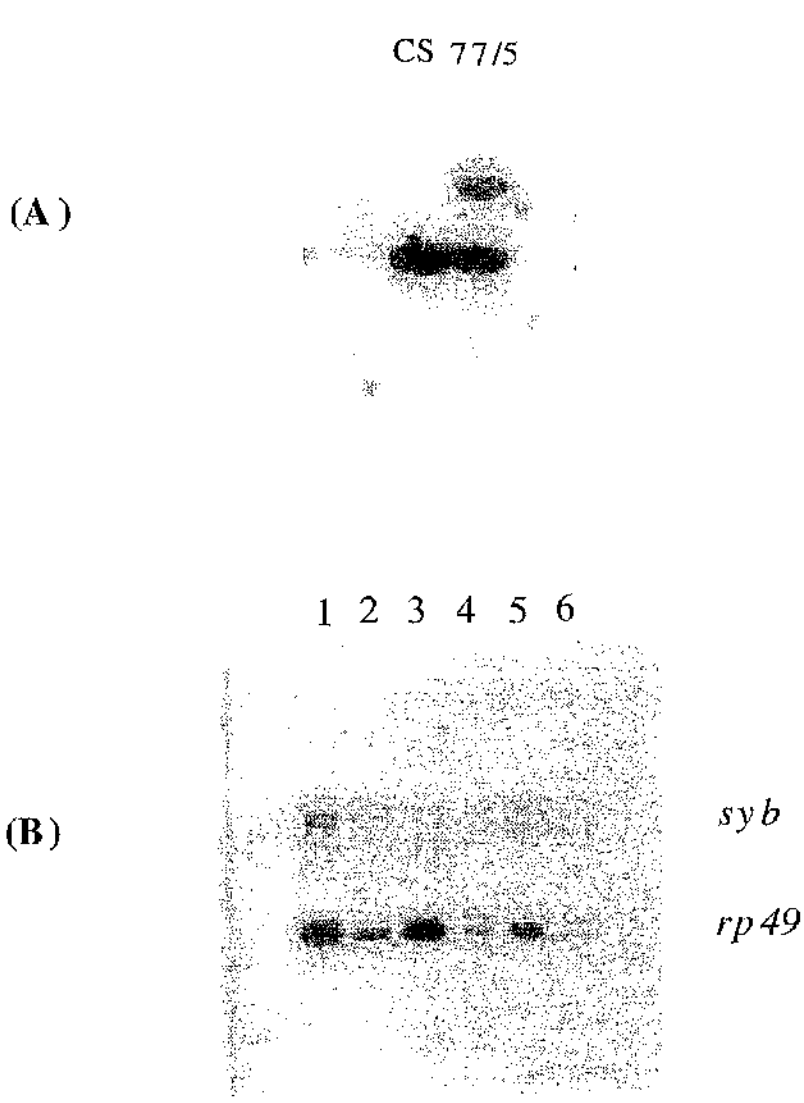


Figure 3.10 Southern blot and Northern blot analysis of *Syb* mutant (A) Southern blot of *Syb* mutant line 77/5 showing a band shift due to P[*lacW*] insertion. The first lane is Canton S genomic DNA, the second lane is line 77/5 genomic DNA, cut by *Eco*RI, probed with *Syb* cDNA. (B) Northern analysis of *Syb* mutant line to show the reduction of RNA transcript. Total RNA, isolated from adult Canton S and 77/5, was hybridised with *Syb* cDNA and *rp49* as a control for loading. Lane 1, Canton S 15 µg; Lane 2, Canton S 30 µg; Lane 3, 77/5 15 µg; Lane 4, 77/5 30 µg; Lane 5, 25/8 15 µg; Lane 6, 25/8 30 µg.

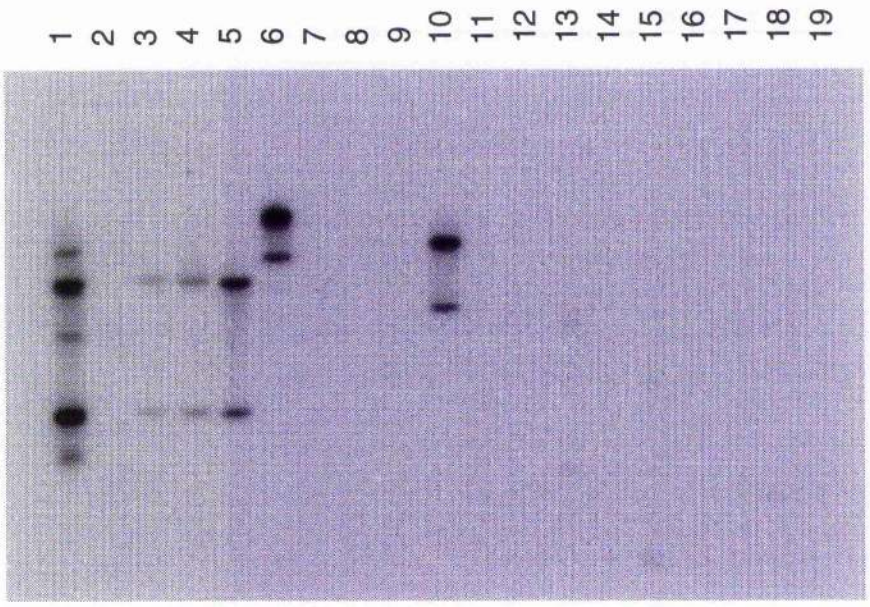


Figure 3.11 Screening for insertions in the gene of *KLP38B*. Six pools of 100 plasmids showed cross-hybridisation with *KLP8B* probe (lane 1, 3, 4, 5, 6, 10). Subdivision of the pools of plasmids with positive hybridisation signals further narrowed down these positive signals to 7 particular insertion lines: 8/2 (lane 1), 49/13 (lane 1), 39/3 (lane 3), 48/5 (lane 4), 57/2 (lane 5), 86/23 (lane 10).

3.4.7 *PP2A-28D*, the gene encoding protein phosphatase 2A

PP2A-28D is a gene encoding protein phosphatase 2A in *Drosophila*. The line 98/22 which carried a P[*lacW*] insertion in 251 bp upstream of the initiating ATG. By excision of the P-element, it has been proved that this insertion had caused the lethality . A mutational analysis has been performed in Dr. Partritia Cohen's group in Dundee (Snaith *et al*., 1996).

3.4.8 Mutations in other genes

Apart from the mutations reported above, we have presently correlated each of the following cloned genes to P[*lacW*] mutant lines. *D-Gγ1*, a gene encoding a G protein γ subunit (Ray *et al*., 1994); *shaw*, a *Shaker* cognate gene (Butler *et al*., 1989; Butler *et al* ., 1990); *Drongo* and 5 other genes.

3.5 One-step screening

As an alternative to screening pools of plasmids, we have used a one-step screening procedure involving grids of colonies created by a robotic device. The entire grid is visualised by hybridisation with a ^{35}S probe for the plasmid replicon, while individual colonies corresponding to particular insertion sites are visualised with a ^{32}P probe specific to the gene of interest (not shown). This one step screening work was done by Mrs. Ann Gillan in collaboration with Zeneca.

3.6 Verification

Once an individual glycerol stock has been identified as containing the hybridising plasmid, the corresponding balanced lethal line is obtained from the stock collection in Szeged. At this stage it is crucial to verify that the plasmid and *Drosophila* line do indeed correspond. This can be easily done by repetition of plasmid rescue. In the case of the

insertion reported in this chapter, plasmids of identical size and hybridisation characteristics were rescued again from the identified fly lines (data not shown). Were some unrelated *Eco*RI fragment to have been 'co-cloned' during the initial rescue, it is highly unlikely that the same event would occur a second time.

To confirm that identified lines each contain only a single insertion, we hybridised the blot of mutant genomic DNA with a P[*lacW*] specific probe. All the 4 lines tested appeared to contain only one insertion (Figure 3.12).

Other important concerns are whether the P element has indeed inserted within the target gene (a 'gene-specific' probe may unexpectedly hybridise to other sites in the genome), and whether insertion is truly the cause of lethality. In the case of the gene for subunit A of the *Drosophila* vacuolar ATPase, the rescued plasmid hybridised *in situ* to a single polytene chromosome band corresponding to the known location of the gene and sequencing of the rescued plasmid showed insertion within the first intron of *vha68-2* gene, loss of which is associated with reversion of lethality (see Chapter 7). Similar work was or is being carried out for other mutant lines.

In total, approximately 40 cDNA fragments corresponding to second chromosome genes have been used as probes. Positive hybridisation signals were seen in 13 cases and in seven cases shown to represent genuine insertions within or near to target genes (Table 3.1). In five of the seven cases, P[*lacW*] insertion had occurred 5' to the reported coding sequence. In the other two cases, insertion occured within the intron. That P elements prefer to insert near to the 5' ends of genes has been observed in other studies (Spradling *et al*., 1995).

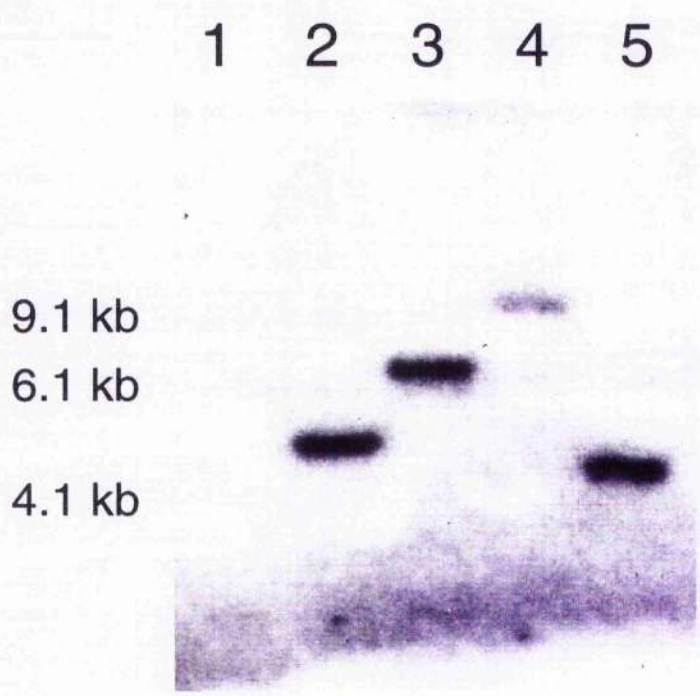


Figure 3.12 Southern blot of genomic DNA of the P[*lacW*] insertional lines to show the single insertion. Each lane is genomic DNA isolated from 10 flies, digested by *Eco*RI, hybridised with the 1.9 kb fragment of P[*lacW*] that correspond to pBluescript. lane 1: Canton S wild type; lane 2: 25/8, with insertion in *vha68-2*; the gene encoding subunit A of V-ATPase; lane 3: 16/1, with insertion in *ductin*, the gene encoding subunit c of V-ATPase; lane 4, 77/5, with insertion in *Syb*, the gene encoding synaptobrevin; lane 5, 8/4, with insertion in *DC0*, the gene encoding the catalytic subunit of cAMP-dependent protein kinase.

Table 3.1 Summary of screening results

Target gene	Accession no.	First round	Verified	Reference
vha68-2	U59147	3	3	Chapter 3, 4
ductin[d]	X77936	2	2	Chapter 3
DCO[d]	X16969	6	1[a]	Chapter 3
PP2A-28D	X55199	1	1	Snaith *et al*., 1996.
KLP38B		7	7	Alphey *et al*., in preparation
Syb	L14270	2	1[b]	McCabe *et al*., 1996.
CalpA	Z46891	2	2	Rosay *et al*., unpublished
vha14	Z26918	1	0[b]	Guo *et al*., 1996.
D-Gγ-1		4		Ray *et al*., 1994
Shaw		3		Butler *et al*., 1989
α–adaption		1	1	Nick Gay in Cambradge
Cliper		1	1	Chunyang Bai in New York
La		1		P. Tolias in New York
? gene		1	1	P. Wes in Crag Montell lab
3 gene		5		Myles Axton in Oxfod
A21		2		B. Srinivasan in Purdue
A22		1		B. Srinivasan in Purdue
6356 DNA		0		B. Retinker
LRL1-5 5 genes		0		M. Cann in Cornell
2a9		0		C. Coelho in Koln
32c2		0		C. Coelho in Koln
47c1		0		C. Coelho in Koln
G808		0		Y. Grau in France
CAM-kinase-like gene		0		
Simon's 51		0		
Simon's 123		0		
Serotonin recepter 2A		0		
Serotonin recepter 2A		0		
Gf alfa		0		
Gs alfa		0		
Igloo		0		
pbprp-5		0		
PKC		0		
PKG-2cDNA		0		
PLC		0		
NPY recepter		0		
muscarinic acetylcholine recepter		0		

[a] Only one of the six putative insertions was chosen for further subdivision. [b] One first round hybridisation signal was a 'co-cloning' artefact. [c] No first round signal. [d] Genes for which P element insertions has been previously described (Finbow *et al*., 1994; Skoulakis *et al*., 1993).

3.7 Discussion

The strategy described here permits rapid identification of mutant lines corresponding to specific cloned genes. This is illustrated by Figure 3.3, detailing the identification of a line with a P element insertion in the gene for subunit A of the *Drosophila* vacuolar ATPase. Three novel and important features of this strategy are as follows. First, we carried out plasmid rescue independently for each of many lines. Plasmid rescue from pools of lines (e.g. Hamilton *et al*., 1991) leads to misrepresentation both because transformation efficiency varies with the size of rescued plasmid, and because it is difficult to avoid competitive growth. By allowing each transformant to grow independently we avoided misrepresentation, and were able to generate sufficient plasmid DNA for screening with any number of target genes. Second, unlike previous examples of SSM by plasmid rescue, the lines described here were generated with the intention of creating and maintaining only lethal insertions of P[*lacW*] (lethals represent only a small proportion of all P element insertions). Though homozygous chromosomal lethality turned out to be associated with P element insertion in only approximately half of the lines, even non-lethal insertions can be useful for secondary mutagenesis. Third, there is a commitment to maintain the entire collection of balanced lethal lines in Szeged for the conceivable future. This is unlike most previous site-selected mutagenesis experiments, in which lines were discarded soon after screening, and were thus unavailable to the wider research community.

Approximately one in four cases of screening with cDNA probes has proved successful. cDNA probes will often fail to detect an insertion in a target gene, merely because the rescued plasmid contains no transcribed sequences. Such occasions will arise when an *Eco*RI site lies between the transposon and the nearest exon. It would of course have been preferable to rescue each line using a range of different enzymes, and to rescue DNA on both sides of the transposon. This would have been prohibitively laborious, however. A simpler way to increase the probability of a 'hit' is via screening with genomic DNA

fragments representing non-transcribed in addition to transcribed sequences (though not a fragment that contains repetitive DNA sequences).

Even so, one should not expect all second chromosome genes to be represented by P[*lacW*] insertions within the Szeged collection since: a) P[*lacW*] mutagenesis was not carried out to saturation; b) not all *Drosophila* genes are good targets for P element insertion; c) not all *Drosophila* genes correspond to lethal complementation groups. Where a pre-existing mutation cannot be found, it may prove fruitful to probe with genomic DNA more distant to the gene of interest, and thereby detect an insertion in a nearby gene. Such an insertion could be used for 'local jumping', an elevated rate of transposition within 100 kb or so on either side of a 'donor' P element (Tower *et al*., 1993; Zhang and Spradling, 1993).

Once one has obtained a line with a single P[*lacW*] transposon within the gene of interest, it is necessary to verify that the insertion is indeed the cause of the mutant phenotype. Spontaneous recessive lethal mutations are common within *Drosophila* populations and can become fixed on the same balanced chromosome as a P element. It is thus essential to demonstrate, as for the *vha68-2* insertion, that remobilisation of the inserted transposon can lead to reversion of the phenotype. Even then it may not be a simple matter to deduce, just from a single allele, the precise role of the gene and its product in *Drosophila* development or physiology. Remobilisation can also result in imprecise 'excision', however, and thus generation of a range of new alleles of varying severity (e.g. Klambt *et al*., 1992). The presence of an eye colour marker (*white*) on P[*lacW*] makes loss of the transposon easy to score. Further, P[*lacW*] was designed as an enhancer-trap element, the *lacZ* component serving as a reporter for gene expression in the vicinity of the insertion site (Bier *et al*., 1989). The pattern and timing of β-galactosidase expression may provide useful information concerning the tissue-specificity and developmental regulation of gene expression.

The collection of P element lethal mutants generated by Török *et al*. (1993) is finding many uses in *Drosophila* genetics and genome mapping. As described here, it provides a simple means of correlating a cloned *Drosophila* gene with a mutant phenotype. Sufficient plasmid DNA has been prepared to allow screening for many targets. An added dimension would be provided by performing large scale correlation of cDNA library clones with the Szeged lines. This would provide access to many as yet unknown, but nonetheless essential, *Drosophila* genetic loci.

One simple way this could be carried out is as follows. Probes of rescued plasmids could be labelled and used to screen a cDNA library to correlate individual clones within the *Drosophila* cDNA library to the corresponding fly lines bearing P[*lacW*] insertions. The whole rescued plasmids could be labelled for screening cDNA library in vector, such as *lambda* NM1149, which shares no sequence homology with the P-element sequence in the rescued plasmids. Each pair is highly likely to represent a mutation of a gene, and, alternatively, imprecise excision will generate mutations where the initial insertion does not. The cDNA library can be screened as arrays of plaques laid out in a rectangular grid by a robotic device.

Chapter 4

Characterisation of *vha68-1* and *vha68-2*, the Genes Encoding Two Isoforms of V-ATPase A Subunit in *Drosophila*

4.1 Summary

vha68-1 and *vha68-2*, genes encoding two isoforms of the V-ATPase A subunit in *Drosophila melanogaster*, have been cloned and sequenced. Both isoforms are composed of a polypeptide of 614 amino acids with a predicted molecular mass of 68417 Da and 68338 Da respectively. The coding sequences of the cDNAs for the two isoforms share 85.5% identity while the translated proteins are 90.7% identical. The gene *vha68-2* is punctuated by four introns. *In situ* hybridisation of the cDNA of *vha68-1* to salivary gland chromosome squashes reveals only one band at 34A on the second chromosome, suggesting that the two genes are at the same location. Northern analysis of total RNA reveals that both isoforms are expressed in a similar pattern. They are expressed in head, thorax and abdomen of the adult fly. Developmental Northern blots of embryo, larvae, pupae and adult total RNA show general expression, but at a much reduced level during metamorphosis.

4.2 Introduction

V-ATPases, found in all eukaryotic cells, are required for the acidification of intracellular organelles such as lysosomes, endosomes, the Golgi apparatus, secretary vesicles, and clathrin-coated vesicles, as well as plant and fungal vacuoles (Nelson, 1992a). They are also located in the apical membrane of cells specialised in H^+ secretion, such as osteoclasts (OCs), kidney intercalated cells, and insect midgut (Baron *et al.*, 1994;

Brown, *et al.,* 1987; Blair *et al.,* 1989; Dow, 1994). Although the organelle and plasma V-ATPases appear similar in composition, it is clear that cells can differentially target these enzymes and thereby regulate the pH of the various intracellular compartments and luminal spaces (Hernando *et al.,* 1995). The mechanisms for this targeting is accomplished remains unclear, but several hypotheses have been proposed. The simplest hypothesis is the putative existence of organelle- or cell-specific isoforms of particular V-ATPase subunit. Only one gene per subunit and per genome has been identified in *S. cerevisiae* and other fungi (Gogarten *et al.,* 1992). Gene disruption experiments in yeast that led to a complete loss of V-ATPase activity gave no indications for multiple isoforms in *S. cerevisiae* (Umemoto et al., 1990; Neumi et al., 1991; Foury, 1990). And only a single gene encoding subunit A from *M. sexta* (Gräf *et al.,* 1992) and bovine (Pan *et al.,* 1991). However, two isoforms of subunit A have been reported from plant, human and chicken (Gogarten *et al.,* 1992b; van Hill *et al.,* 1993; Hernando *et al.,* 1995). In higher plants, two genes encoding the A subunit differ by the size of an intervening sequence. The two genes exhibit a coding region of the same length but differ in the length of the intron (Gogarten *et al.,* 1992b; Stark *et al.,* 1995). In human the VA68 isoform of V-ATPase subunit A is expressed in all tissues whereas the expression of a second isoform, HO68, has been found only in osteoclastomas, tumours enriched in osteoclasts (van Hill *et al.,* 1993). In chicken, alternative splicing of a single gene generates two polypeptide isoforms of the A subunit. However, both isoforms seems to be ubiquitously expressed (Hernando *et al.,* 1995). The putative existence of different isoforms of particular V-ATPase subunits and thus the specific assembly of different isoforms of some of the subunits may allow differential targeting and the regulation of cell-, organelle- or membrane-specific V-ATPases.

All of the V-ATPases purified to date share similar functions and structural features (Forgac, 1989). They are multimeric proteins with at least three common subunits: a catalytic subunit A, a regulatory subunit B, and a proton channel subunit c with relative molecular masses of approximately 70,000, 60,000 and 17,000 respectively (Gräf *et al.,*

1992). cDNAs and genes encoding subunit A were first cloned from plant (Zimniak *et al.*, 1988), fungi (Bowman *et al.*, 1988) and the archaebacterium *Sulfolobus acidocaldarius* (Denda *et al.*, 1988). It immediately became apparent that the enzyme that functions in ATP-synthesis in archaebacteria is also a V-ATPase, and that subunit A is homologous to the β subunit of F-ATPases. It was also revealed that a *S. cerevisiae* gene involved in trifluoperazine resistance, cloned the same year, encodes a larger protein that undergoes protein splicing to give the mature subunit A (Shih *et al.*, 1988; Hirata *et al.*, 1990; Kane *et al.*, 1990). Aligning the amino acid sequences of A and β subunits from various sources produced a wealth of information. The conserved glycine-rich loop in the A-subunit was implicated as a primordial common structure for nucleotide binding. It is thought that the A subunit, as the β subunit of F-ATPase, is the catalytic subunits of the V-ATPase.

A cDNA encoding an *M. sexta* V-ATPase A-subunit has been previously cloned by screening a larval midgut cDNA expression library with monoclonal antibodies to the midgut plasma membrane subunit A (Gräf *et al.*, 1992). It shared considerable homology to cDNAs encoding subunit A from other sources. Using *Manduca* cDNA as a probe, we have successfully isolated two corresponding *Drosophila* genes, *vha68-1* and *vha68-2*, which encode different isoforms of the V-ATPase A subunit. This chapter will report the isolation and characterisation of cDNAs and genomic DNA of the two genes.

4.3 Isolation of two different cDNAs encoding the catalytic A subunit

4.3.1 Isolation of *vha68-1* cDNA

A *Drosophila* head λZap II cDNA library was screened by plaque hybridisation with a digoxygenin-random-primed probe of cDNA encoding the *Manduca* V-ATPase A-subunit. Positives were obtained at approximately 1:10,000 and were purified by a further round of plating. Nineteen clones were obtained and inserts of four recombinant

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1                                                   31
AAT TTT CAT AAG AGC TGG TGA AAC AAA TCC AAC GAA CGA TTT GAC CGT TAC CGA AGC AGA
61                                                  91/3
AGA AGA AGA GCA GCA ACC GCG ACC ATG CCC AAC TTG AGG AAA TTC AAA GAC GAG GAG CGC
                                    M   P   N   L   R   K   F   K   D   E   E   R
121/13                                              151/23
GAG TCG GAA TAT GGC CGT GTC TAC GCG GTA TCC GGA CCA GTG GTC ACC GCT GAG GCC ATG
 E   S   E   Y   G   R   V   Y   A   V   S   G   P   V   V   T   A   E   A   M
181/33                                              211/43
TCT GGA TCA GCT ATG TAC GAG TTG GTC CGC GTC GGC TAC TAC GAG CTG GTG GGC GAG ATC
 S   G   S   A   M   Y   E   L   V   R   V   G   Y   Y   E   L   V   G   E   I
241/53                                              271/63
ATC CGT CTG GAG GGC GAC ATG GCC ACC ATC CAG GTG TAC GAG GAG ACC TCT GGC TTG ACT
I   R   L   E   G   D   M   A   T   I   Q   V   Y   E   E   T   S   G   L   T
301/73                                              331/83
GTC GGC GAT CCG GTG CTG CGT ACC GGC AAA CCT CTT TCC GTG GAA CTT GGA CCC GGC ATT
 V   G   D   P   V   L   R   T   G   K   P   L   S   V   E   L   G   P   G   I
361/93                                              391/103
ATG GGC AGC ATC TTC GAC GGC ATC CAA CGT CCT TTG CGG GAC ATT GGT GTC ATG ACC AAC
 M   G   S   I   F   D   G   I   Q   R   P   L   R   D   I   G   V   M   T   N
421/113                                             451/123
TCC ATC TAT ATA CCC AAA GGT GTC AAC ACA ACT GCT TTG TCG CGC TCG GAG ATG TGG GAA
S   I   Y   I   P   K   G   V   N   T   T   A   L   S   R   S   E   M   W   E
481/133                                             511/143
TTT AAT CCG CTG AAT GTG CGG GTG GGA TCC CAC ATC ACC GGA GGA GAT CTG TAT GGA GTG
 F   N   P   L   N   V   R   V   G   S   H   I   T   G   G   D   L   Y   G   V
541/153                                             571/163
GTA CAC GAG AAC ACG CTG GTG AAG CAG CGC ATG ATT GTG GCA CCG AGG GCT AAG GGA ACC
 V   H   E   N   T   L   V   K   Q   R   M   I   V   A   P   R   A   K   G   T
601/173                                             631/183
GTT CGA TAC ATT GCC CCC GCG GGC AAC TAC AAC CTG GAG GAC ATT GTC CTG GAG ACG GAG
 V   R   Y   I   A   P   A   G   N   Y   N   L   E   D   I   V   L   E   T   E
661/193                                             691/203
TTC GAC GGC GAG ATC ACC AAG CAC ACC ATG TTG CAG GTC TGG CCA GTG CGG CAG GCA CGT
 F   D   G   E   I   T   K   H   T   M   L   Q   V   W   P   V   R   Q   A   R
721/213                                             751/223
CCC GTC ACA GAG AAG CTG CCA GCC AAC CAT CCG CTC TTC ACG GGC CAA CGC GTC CTT GAC
 P   V   T   E   K   L   P   A   N   H   P   L   F   T   G   Q   R   V   L   D
781/233                                             811/243
TCG CTC TTC CCC TGC GTA CAG GGC GGC ACC ACT GCC ATC CCC GGT GCC TTT GGC TGC GGC
 S   L   F   P   C   V   Q   G   G   T   T   A   I   P   G   A   F   G   C   G
841/253                                             871/263
AAG ACC GTC ATT TCG CAG GCC CTG TCC AAG TAC TCC AAC TCT GAT GTG ATC ATC TAC GTC
 K   T   V   I   S   Q   A   L   S   K   Y   S   N   S   D   V   I   I   Y   V
901/273                                             931/283
GGT TGC GGC GAG CGC GGT AAC GAG ATG TCT GAG GTA CTG CGT GAC TTT CCC GAA CTG ACC
 G   C   G   E   R   G   N   E   M   S   E   V   L   R   D   F   P   E   L   T
961/293                                             991/303
TGC GAC ATA GAT GGC GTC ACC GAG TCC ATT ATG AAG CGA ACT GCT CTG GTG GCC AAC ACC
 C   D   I   D   G   V   T   E   S   I   M   K   R   T   A   L   V   A   N   T
1021/313                                            1051/323
TCC AAC ATG CCG GTG GCA GCT CGT GAG GCC TCC ATT TAC ACT GGT ATC ACT CTG TCT GAA
 S   N   M   P   V   A   A   R   E   A   S   I   Y   T   G   I   T   L   S   E
1081/333                                            1111/343
TAC TTC CGT GAT ATG GGC TAC AAC GTA GCC ATG ATG GCT GAT TCC ACC TCC CGT TGG GCT
 Y   F   R   D   M   G   Y   N   V   A   M   M   A   D   S   T   S   R   W   A
1141/353                                            1171/363
GAG GCA CTT CGT GAG ATT TCG GGT CGT TTG GCT GAG ATG CCT GCC GAT TCT GGC TAC CCG
 E   A   L   R   E   I   S   G   R   L   A   E   M   P   A   D   S   G   Y   P
1201/373                                            1231/383
GCT TAT CTA GGA GCT CGT CTG GCC ACA TTC TAC GAG CGT GCT GGG CGC GTC AAG TGC TTG
 A   Y   L   G   A   R   L   A   T   F   Y   E   R   A   G   R   V   K   C   L
1261/393                                            1291/403
GGT AAC CCG GAG CGC GAG GGA TCC GTG TCC ATT GTC GGA GCT GTG TCT CCT CCT GGT GGT
 G   N   P   E   R   E   G   S   V   S   I   V   G   A   V   S   P   P   G   G
1321/413                                            1351/423
GAC TTC TCC GAT CCC GTG ACC TCC GCC ACT TTG GGT ATC GTG CAG GTG TTC TGG GGT CTC
 D   F   S   D   P   V   T   S   A   T   L   G   I   V   Q   V   F   W   G   L
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1381/433                                                1411/443
GAC AAG AAA TTG GCC CAG CGC AAG CAC TTC CCC TCG ATC AAC TGG CTC ATC TCC TAC TCG
 D   K   K   L   A   Q   R   K   H   F   P   S   I   N   W   L   I   S   Y   S
1441/453                                                1471/463
AAG TAC ATG CGT GCT CTG GAT GAA TAC TAT GAC AAG AAC TAC CCC GAG TTC GTG CCA CTA
 K   Y   M   R   A   L   D   E   Y   Y   D   K   N   Y   P   E   F   V   P   L
1501/473                                                1531/483
CGC ACC AAG GTC AAG GAG ATC CTG CAG GAG GAG GAG GAT CTG TCT GAG ATC GTT CAG CTG
 R   T   K   V   K   E   I   L   Q   E   E   E   D   L   S   E   I   V   Q   L
1561/493                                                1591/503
GTG GGC AAA GCA TCA CTG GCC GAG ACC GAC AAG GTG ACC CTG GAA GTG GCA AAG CTG CTG
 V   G   K   A   S   L   A   E   T   D   K   V   T   L   E   V   A   K   L   L
1621/513                                                1651/523
AAG GAC GAC TTT CTG CAA CAG AAC TCC TAC TCA CCA TAC GAT CGC GTT TGT CCC TTC TAC
 K   D   D   F   L   Q   Q   N   S   Y   S   P   Y   D   R   V   C   P   F   Y
1681/533                                                1711/543
AAG ACC GTG GGC ATG CTG AGA AAC ATC ATG GCC TTC TAT GAG ACC GCC CGG CAT GCC GTT
 K   T   V   G   M   L   R   N   I   M   A   F   Y   E   T   A   R   H   A   V
1741/553                                                1771/563
GAG TCC ACA GCC CAG TCG GAC AAC AAG ATC ACA TGG AAC ACC ATC AGG GAA TCG ATG GGC
 E   S   T   A   Q   S   D   N   K   I   T   W   N   T   I   R   E   S   M   G
1801/573                                                1831/583
GGA ATT ATG TAC CAG CTG TCG TCG ATG AAG TTC AAGGAC CCT GTG AAA GAT GGC GAG CAA
 G   I   M   Y   Q   L   S   S   M   K   F   K D   P   V   K   D   G   E   Q
1861/593                                                1891/603
AAG ATC AAG GCG GAC TAC GAC CAG CTG TAC GAG GAT CTG CAG CAG GCC TTC CGA AAT CTG
 K   I   K   A   D   Y   D   Q   L   Y   E   D   L   Q   Q   A   F   R   N   L
1921/613                                                1951
GAG GAC TAA GCG GAA ACG GCC AGA AAC CAT CTG CGG GCT TTC CTA GCG GGA GGA ATG GAA
 E   D   *
1981                                                    2011
AAT GAA GCA AAC CAA ACG AAA TAA GTA ACC AAA ACT AGG TTA TTA TTC GAA TTC CCC ATT
2041                                                    2071
CAA TCT AGT CAT ATT TAC ATA ATG CAT AAT AAG ATA TTT GAA TCC AAG TTT ACT TAT AAG
2101                                                    2131
TTT AAC AAA CAG TTT GGC CCG CTT CAG GTC TAG TCA GGT CAG AAT CGA ATC ACC AGA AGA
2161                                                    2191
TAC GCA AAA CGA AAG GAA AGA CGA ACA ATA ATT AGT CGG TAG CGC AAA TGG AAC GCA GTT
2221                                                    2251
AAA CCA GCC ATA TAC ATA AAT ACC ATA CAT ATA TGA CAC ATA TGT ATA ATT ATC TAT GTT
2281                                                    2311
GAT ATA TAA ATA TAA TTC ACA GCT ATG TAT TGG TAG TAA ATT TTC ATA TAG TTA TCG ATT
2341                                                    2371
GTG TTC GTT ACC CTA TTG TGT GAA ACT AAA CCA ACT AAA CGA CGA GTC TAA AGG GCG TTT
2401                                                    2431
GAA TCT TTA CGA AAT TAC AAA ATA TTA TAT TCC TAC ATA TTA TAG ACC TTT AAA GAA CGC
2461                                                    2491
AAT AAC AAC GTA GCC CCA AAA GCA TGT ACC TCT ACT ACC AAA GGA TAG CTA TTT CAG TAA
2521                                                    2551
CTT GTG TGT GTT GCA AAT GGA GCT ATG GAA ATA AAA TGT ATT ATG AAT GTT ACA AA
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Figure 4.1 cDNA and putative aa sequence of *vha68-1*. The presumed polyadenylation signal is underlined. The start of the poly A tail is marked in bold. This cDNA sequence has been published in the GenBank database under the accession number U19745.

phages were excised as pBluescript plasmids. Double-stranded sequencing was performed according to the Sequenase™ II protocol (US Biochemical, Cleveland, OH), with the aid of synthetic oligo primers. All of the four clones have the same 3' end, except for differing lengths of the poly A tails. The 5' end sequence of three cDNA clones, p68A1, p68B1 and p68E1, were found to be identical, except for small differences in the length of the 5' end. However, p68C1 is the shortest of the four clones beginning at nucleotide 663. The longest cDNA p68A1 was sequenced from both DNA strands, using synthetic oligonucleotides to extend the reading. The resulting sequence consists of 2576 bp. A long open reading frame encodes a putative polypeptide of 614 amino acids (Figure 4.1) with a M_r of 68417 Da which is clearly a V-ATPase A subunit. The gene has been named *vha68-1*. The open reading frame is preceded by a 5' untranslated region (UTR) of 84 bp. The 3' UTR of 644 bp long contains a poly A addition signal between nucleotides 2550-2556, 19 bases upstream of the poly A tail.

4.3.2 Isolation of *vha68-2* cDNA

A NM1149 cDNA library representing adult heads of the *D. melanogaster eyes absent* (*eya*) mutant was screened by plaque hybridisation with the genomic DNA fragment of the plasmid rescued from the fly line l(2)k02508 (See Figure 3.3 in Chapter 3). Plaques giving both strong and weak hybridising signals were picked. More than 20 positive plaques were obtained, of which five recombinant phages were purified. cDNA inserts in the recombinant phages were excised by *Eco*RI and *Hind*III. There were three types of cDNAs according to digestion map and the intensity of the hybridisation to the genomic DNA probe (Figure 4.2). The inserts were subcloned into pBluescript SK⁻ and sequenced by the universal primers T3 and T7 from the both ends. While the sequence of p68c-5 was identical to that of *vha68-1* cDNA, the digestion maps and sequences of p68c-1, p68c-2 and p68c-3 are different from *vha68-1* cDNA. Sequences of the three inserts are identical except for small length differences at the 5' end. The longest cDNA, p68c-1, was sequenced from both strands, using synthetic oligonucleotides to extend

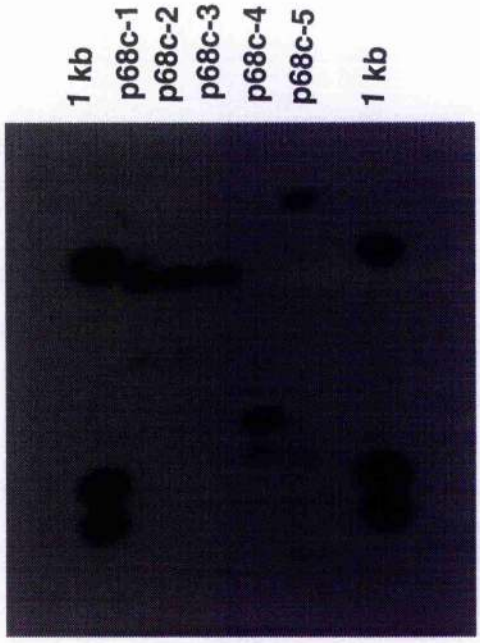

Figure 4.2 Three types of cDNA inserts hybridised to *vha68* probes. cDNA inserts in the recombinant phages were excised by *Eco*RI and *Hind*III. The Southern blot was probed with the genomic DNA fragment of the plasmid rescued from the fly line l(2)k02508.

```
1                                                   31
GTT CGT TCT GTT GGA GAA AAG CAG CAA TCA CAC GTT CGC AAG GTG AAC GCG AAG ACA CAG
61                                                  91/2
CAA ATC GAA AAA ACA GAA TAA AGC AAA ATG TCC AAC CTT AAG CGT TTC GAT GAT GAG GAG
                                        M   S   N   L   K   R   F   D   D   E   E
121/12                                              151/22
CGT GAG TCC AAA TAT GGA CGT GTC TTC GCT GTC TCC GGT CCT GTC GTC ACC GCC GAG GCC
R   E   S   K   Y   G   R   V   F   A   V   S   G   P   V   V   T   A   E   A
181/32                                              211/42
ATG TCT GGA TCA GCT ATG TAC GAG TTG GTC CGC GTC GGC TAC TAC GAG CTG GTG GGC GAG
M   S   G   S   A   M   Y   E   L   V   R   V   G   Y   Y   E   L   V   G   E
241/52                                              271/62
ATC ATC CGT CTG GAG GGT GAC ATG GCC ACC ATC CAG GTG TAC GAG GAG ACC TCT GGC GTA
I   I   R   L   E   G   D   M   A   T   I   Q   V   Y   E   E   T   S   G   V
301/72                                              331/82
ACT GTC GGA GAT CCG GTG CTG CGT ACC GGC AAG CCT CTT TCC GTG GAG CTG GGA CCC GGT
T   V   G   D   P   V   L   R   T   G   K   P   L   S   V   E   L   G   P   G
361/92                                              391/102
ATC ATG GGC AGC ATC TTT GAC GGT ATC CAG CGT CCC CTG AAG GAC ATT AAC GAG CTG ACC
I   M   G   S   I   F   D   G   I   Q   R   P   L   K   D   I   N   E   L   T
421/112                                             451/122
GAA TCC ATC TAC ATT CCC AAG GGT GTG AAC GTG CCC AGT TTG TCC CGC GTG GCC AGC TGG
E   S   I   Y   I   P   K   G   V   N   V   P   S   L   S   R   V   A   S   W
481/132                                             511/142
GAG TTC AAC CCC CTG AAC GTC AAG GTC GGC TCC CAC ATC ACC GGA GGT GAC CTG TAC GGT
E   F   N   P   L   N   V   K   V   G   S   H   I   T   G   G   D   L   Y   G
541/152                                             571/162
CTG GTG CAT GAG AAC ACT CTG GTC AAG CAC AAG ATG ATT GTG AAC CCC CGC GCC AAG GGA
L   V   H   E   N   T   L   V   K   H   K   M   I   V   N   P   R   A   K   G
601/172                                             631/182
ACA GTG CGC TAC ATC GCC CCC TCC GGC AAC TAC AAG GTC GAC GAT GTC GTC CTG GAG ACC
T   V   R   Y   I   A   P   S   G   N   Y   K   V   D   D   V   V   L   E   T
661/192                                             691/202
GAG TTC GAT GGA GAG ATC ACC AAG CAC ACC ATG TTG CAG GTG TGG CCA GTC CGT CAC CAC
E   F   D   G   E   I   T   K   H   T   M   L   Q   V   W   P   V   R   H   H
721/212                                             751/222
GCT CCC GTG ACC GAG AAG CTG CCC GCC AAC CAC CCC CTG CTC ACC GGA CAG CGT GTG CTC
A   P   V   T   E   K   L   P   A   N   H   P   L   L   T   G   Q   R   V   L
781/232                                             811/242
GAC TCG CTC TTC CCC TGT GTC CAG GGC GGT ACC ACC GCC ATT CCC GGA GCT TTC GGT TGC
D   S   L   F   P   C   V   Q   G   G   T   T   A   I   P   G   A   F   G   C
841/252                                             871/262
GGC AAG ACT GTG ATC TCG CAG GCT CTG TCC AAG TAC TCC AAC TCC GAT GTC ATC ATC TAC
G   K   T   V   I   S   Q   A   L   S   K   Y   S   N   S   D   V   I   I   Y
901/272                                             931/282
GTC CGT TCC GGT GAG CGT GGT AAC GAG ATG TCT GAG GTA CTG CGT GAC TTC CCC GAG CTG
V   G   C   G   E   R   G   N   E   M   S   E   V   L   R   D   F   P   E   L
961/292                                             991/302
TCC GTG GAG ATC GAT GGT GTG ACC GAG TCC ATC ATG AAG CGT ACC GCC CTT GTG GCC AAC
S   V   E   I   D   G   V   T   E   S   I   M   K   R   T   A   L   V   A   N
1021/312                                            1051/322
ACC TCC AAC ATG CCT GTG GCT GCT CGA GAG GCC TCC ATC TAC ACT GGT ATC ACC TTG TCC
T   S   N   M   P   V   A   A   R   E   A   S   I   Y   T   G   I   T   L   S
1081/332                                            1111/342
GAA TAC TTC CGT GAT ATG GGT TAC AAC GTG TCC ATG ATG GCT GAT TCC ACC TCC CGT TGG
E   Y   F   R   D   M   G   Y   N   V   S   M   M   A   D   S   T   S   R   W
1141/352                                            1171/362
GCT GAG GCT CTT CGT GAA ATT TCT GGT CGT CTC GCT GAG ATG CCT CGC GAT TCC GGC TAC
A   E   A   L   R   E   I   S   G   R   L   A   E   M   P   R   D   S   G   Y
1201/372                                            1231/382
CCA GCC TAC TTG GGA GCT CGT CTG GCC TCC TTC TAC GAG CGT GCC GGT CGC GTT AAG TGC
P   A   Y   L   G   A   R   L   A   S   F   Y   E   R   A   G   R   V   K   C
1261/392                                            1291/402
TTG GGT AAC CCC GAG CGC GAG GGA TCC GTG TCC ATT GTC GGA GCT GTG TCT CCT CCT GGT
L   G   N   P   E   R   E   G   S   V   S   I   V   G   A   V   S   P   P   G
1321/412                                            1351/422
GGT GAC TTC TCC GAT CCC GTA ACC TCC GCC ACT CTG GGT ATC GTG CAG GTG TTC TGG GGT
G   D   F   S   D   P   V   T   S   A   T   L   G   I   V   Q   V   F   W   G
```

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1381/432                                                1411/442
CTC GAC AAG AAG TTG GCC CAG CGC AAG CAT TTC CCC TCG ATC AAC TGG CTC ATC TCC TAC
L   D   K   K   L   A   Q   R   K   H   F   P   S   I   N   W   L   I   S   Y
1441/452                                                1471/462
TCG AAG TAC ATG CGT GCT CTG GAT GAC TTC TAT GAC AAG AAC TTC CCG GAA TTC GTG CCG
S   K   Y   M   R   A   L   D   D   F   Y   D   K   N   F   P   E   F   V   P
1501/472                                                1531/482
CTG CGT ACC AAG GTC AAG GAG ATC CTG CAG GAG GAG GAG GAT CTG TCT GAG ATC GTG CAA
L   R   T   K   V   K   E   I   L   Q   E   E   E   D   L   S   E   I   V   Q
1561/492                                                1591/502
CTG GTC GGC AAG GCC TCT CTC GCC GAA ACC GAC AAG ATC ACG CTG GAG GTG GCC AAG CTG
L   V   G   K   A   S   L   A   E   T   D   K   I   T   L   E   V   A   K   L
1621/512                                                1651/522
CTG AAG GAC GAT TTC CTG CAG CAG AAC TCC TAC TCC TCG TAC GAT CGC TTC TGC CCC TTC
L   K   D   D   F   L   Q   Q   N   S   Y   S   S   Y   D   R   F   C   P   F
1681/532                                                1711/542
TAC AAG ACC GTG GGC ATG TTG AGG AAC ATC ATC GAC TTC TAC GAC ATG GCC CGT CAC TCC
Y   K   T   V   G   M   L   R   N   I   I   D   F   Y   D   M   A   R   H   S
1741/552                                                1771/562
GTG GAG TCT ACG GCT CAG TCT GAG AAC AAG ATC ACC TGG AAC GTG ATT CGT GAG GCA ATG
V   E   S   T   A   Q   S   E   N   K   I   T   W   N   V   I   R   E   A   M
1801/572                                                1831/582
GGC AAC ATT ATG TAC CAG CTG TCA TCC ATG AAG TTC AAG GAC CCC GTT AAG GAT GGT GAG
G   N   I   M   Y   Q   L   S   S   M   K   F   K   D   P   V   K   D   G   E
1861/592                                                1891/602
GCC AAG ATC AAG GCT GAC TTC GAG CAG CTG CAC GAG GAC CTG CAG CAG GCC TTC AGA AAT
A   K   I   K   A   D   F   E   Q   L   H   E   D   L   Q   Q   A   F   R   N
1921/612                                                1951
CTG GAG GAC TAG AGA CCG ACG ACT GGC CCT ACT TTT ACA CTC TAA TCT TAT ATT TGT TAT
L   E   D   *
1981                                                    2011
ATA GTT AAC GTT TAA AAA TGA AAG CAG TCA AAA ACC ATC CGA AAA AGC CTA ATC AAA CAC
2041                                                    2071
CAA CAA TTC CAG CTG CAT TCG ATG AAA AAC AAA AGT CCA ACA AAT ACC ATA ACT TCT TGG
2101                                                    2131
TGC CTG CGA GAG ATG TAA ACA TTC CGG CCT GCG GTT AAT ACT TTC CCC TAA CCA CGC CCC
2161                                                    2191
CTC CGC CCC TTG AAG GGC AAC TCT AGG CAA CAG CAA CTA CAA CGT CCT GCT ATG TAC TTC
2221                                                    2251
CAT TTA CAA CAA CAA CAC CAA CAT ACA CTT GAA TAA AAG TAC ACG GAC ACT GGC GCA CAC
2281                                                    2311
ACA ACA CAT ACA TAA AAG ACA CAA ATA CAA ATG CAT GCA TAA ATA GTA TTA TTG TTT AAT
2341                                                    2371
GAA TGG AAA TTC TTG TTT ATT TGT GAA AAA AGT CAT GTC TTC TCC CTG TTT GTT TGT TAA
2401                                                    2431
ATT TAT GTA AAT ATT TAA AGT ATG AAA TAT TAA ATG TAC GAA TAA AGT GCA ACA ACA AAT
2461
ACA TTT AAT GTA AA
```

Figure 4.3 cDNA and predicted amino acid sequence for *vha68-2*. The presumed polyadenylation signal is underlined. The beginning of the poly A tail is marked in bold. The cDNA sequence has been published in the GenBank database under the accession number U59146.

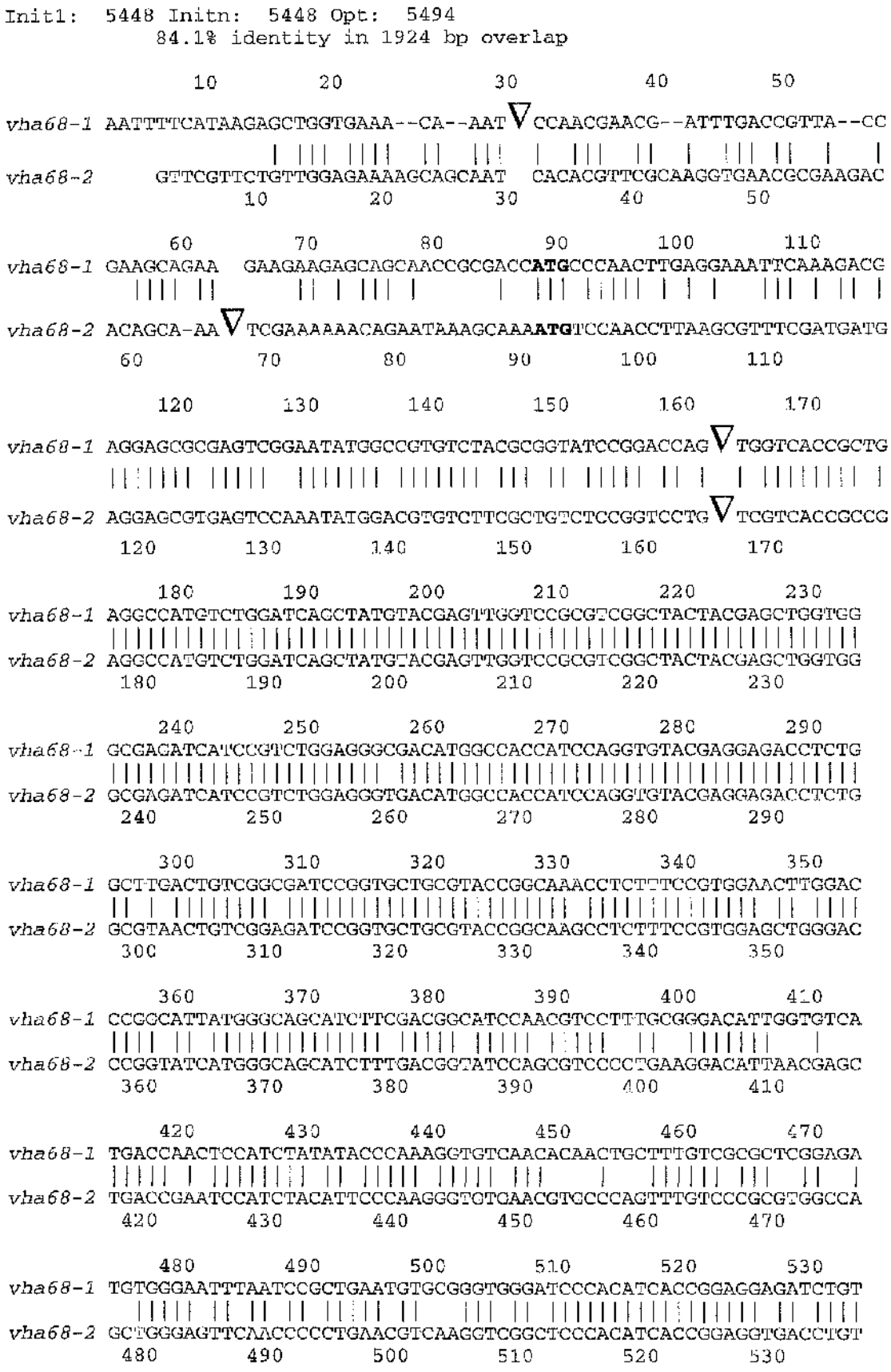
readings. It is 2474 bp long. The long open reading frame encoded a putative polypeptide of 614 amino acids (Figure 4.3) with a molecular mass of 68338 Da. The high homology of this cDNA sequence with that of *vha68-1* cDNA (Figure 4.4) and with sequences for A subunits from other sources in the GenBank database (Figure 4.5) suggests that this new cDNA encodes a second isoform of the catalytic A subunit of the *Drosophila* V-ATPase. Accordingly, the gene was named as *vha68-2*. The 5' UTR of *vha68-2* cDNA is 88 bp long, the 3' UTR 542 bp. There is a poly A addition signal between nucleotides 2446-2451, 24 bp upstream of the poly A tail.

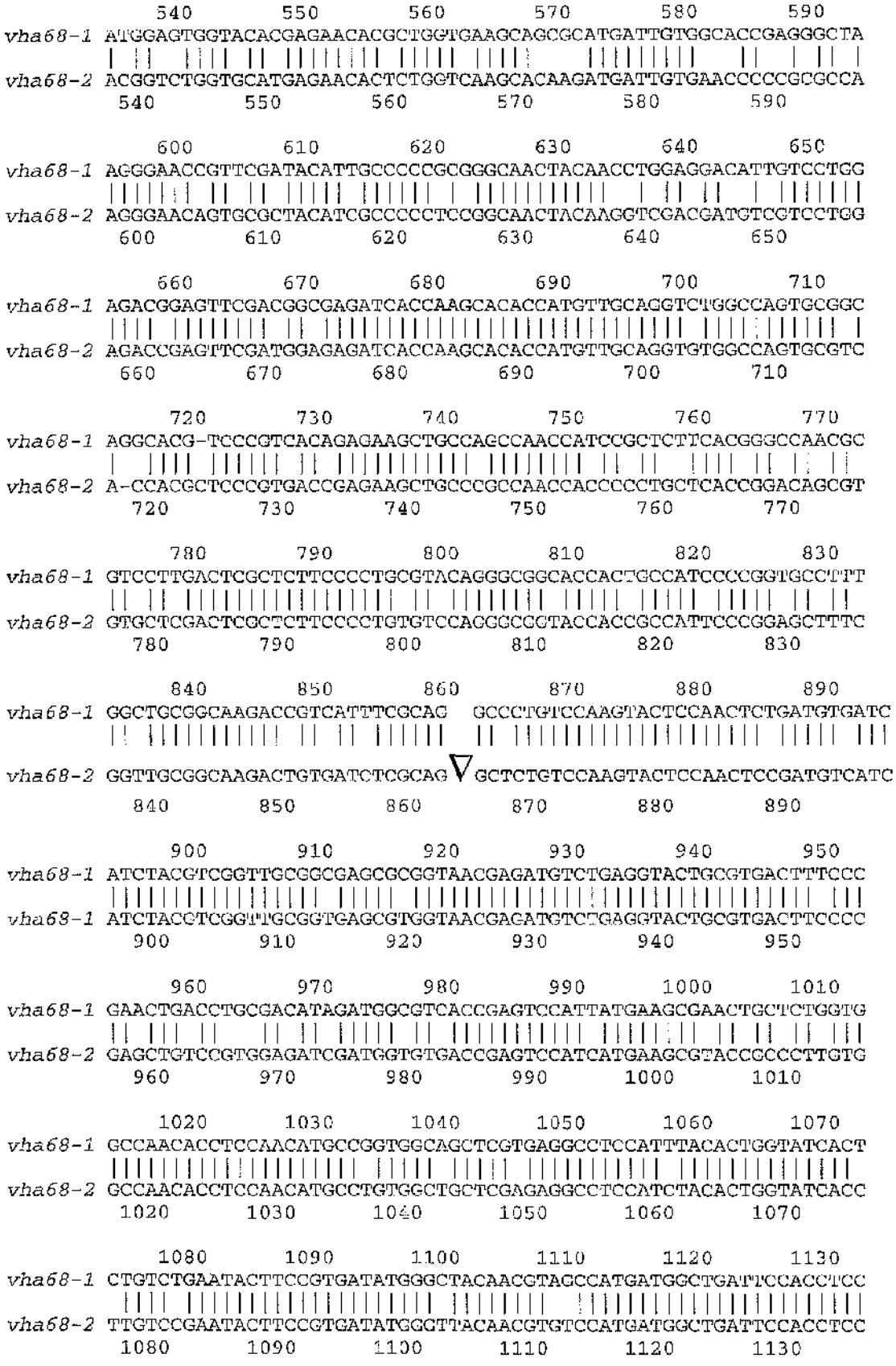
The digestion map of p68c-4 is different from both *vha68-1* and *vha68-2* cDNA. Whether this insert represents a third *vha68* cDNA awaits confirmation by sequencing the insert.

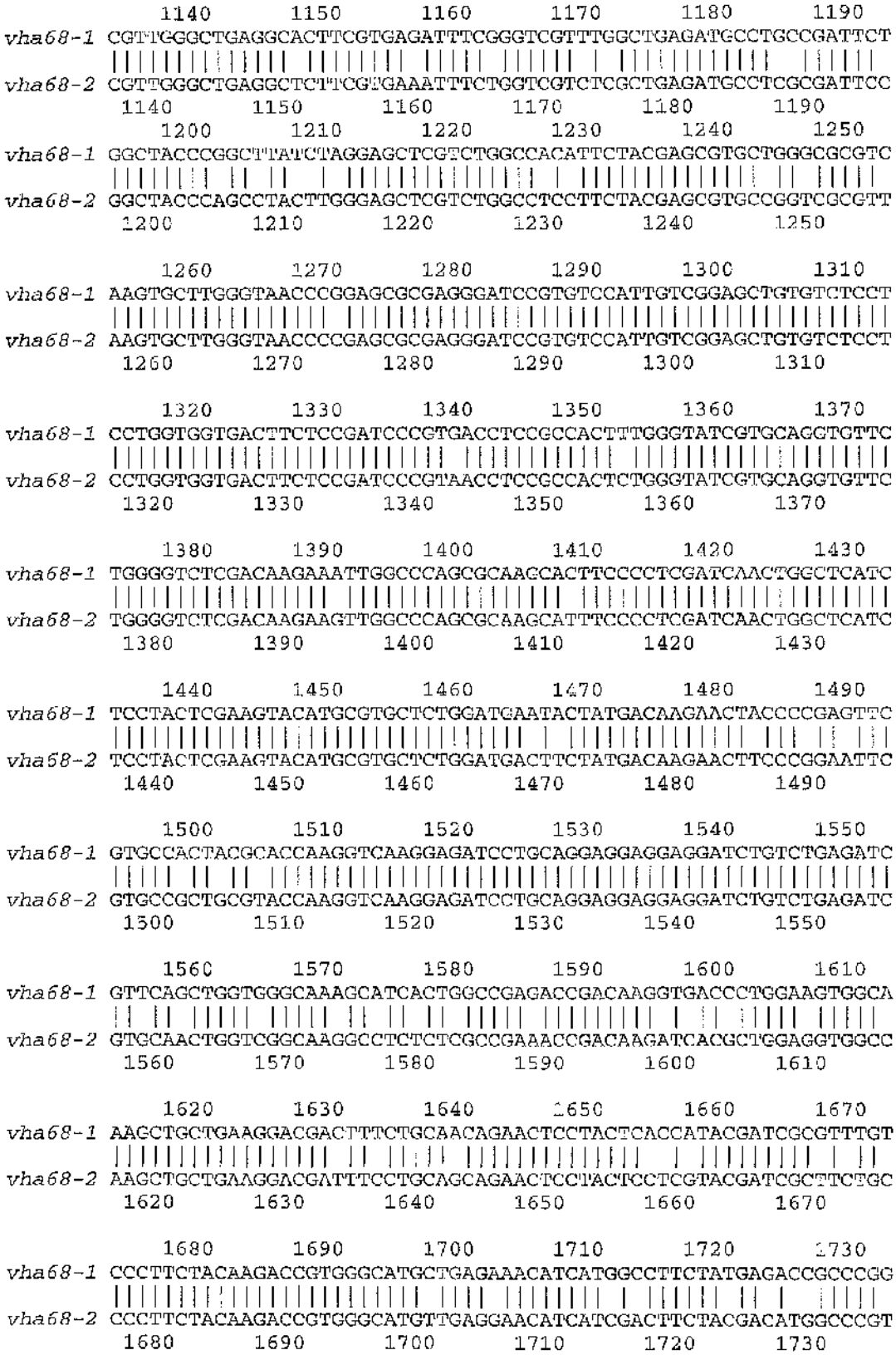
4.3.3 Comparison of the two isoforms

The length of the two cDNAs are similar. *vha68-1* is 2576 bp while *vha68-2* is 2474 bp, about 100 bp shorter. Both cDNAs have a long open reading frame of 1842 bp which encodes a polypeptide of 614 amino acids ≈68 kDa. The two polypeptides share 91% aa identity. The coding DNA sequences share 85.5% identity. However, the homology between the 5' and 3' noncoding sequence is very low or without homology (Figure 4.4). The 5' UTRs in the two longest cDNA of *vha68-1* and *vha68-2* are almost of the same size, but the 3' UTR of *vha68-1* is 102 bp longer than that of *vha68-1*. The poly A tail signal AATAAA was found near the poly A tails of both cDNAs.

The predicted translation start site of *vha68-2* CAAAA**T**G is the same as that of *vha26* (See chapter 6) which is in perfect match with this consensus start site (C/A)AA(A/C)**ATG** (Cavener, 1987). However, *vha68-1* has a different start site GACCA**TG**. *vha68-1* uses TAA for the translation stop codon but *vha68-2* uses TAG as the stop codon.


Init1: 5448 Initn: 5448 Opt: 5494
84.1% identity in 1924 bp overlap
vha68-1 AATTTTCATAAGAGCTGGTGAAA--CA--AAT∇CCAACGAACG--ATTTGACCGTTA--CC
vha68-2 GTTCGTTCTGTTGGAGAAAAGCAGCAAT CACACGTTCGCAAGGTGAACGCGAAGAC
vha68-1 GAAGCAGAA GAAGAAGAGCAGCAACCGCGACCATGCCCAACTTGAGGAAATTCAAAGACG
vha68-2 ACAGCA-AA∇TCGAAAAAACAGAATAAAGCAAAATGTCCAACCTTAAGCGTTTCGATGATG
vha68-1 AGGAGCGCGAGTCGGAATATGGCCGTGTCTACGCGGTATCCGGACCAG∇TGGTCACCGCTG
vha68-2 AGGAGCGTGAGTCCAAATATGGACGTGTCTTCGCTGTCTCCGGTCCTG∇TCGTCACCGCCG
vha68-1 AGGCCATGTCTGGATCAGCTATGTACGAGTTGGTCCGCGTCGGCTACTACGAGCTGGTGG
vha68-2 AGGCCATGTCTGGATCAGCTATGTACGAGTTGGTCCGCGTCGGCTACTACGAGCTGGTGG
vha68-1 GCGAGATCATCCGTCTGGAGGGCGACATGGCCACCATCCAGGTGTACGAGGAGACCTCTG
vha68-2 GCGAGATCATCCGTCTGGAGGGTGACATGGCCACCATCCAGGTGTACGAGGAGACCTCTG
vha68-1 GCTTGACTGTCGGCGATCCGGTGCTGCGTACCGGCAAACCTCTTTCCGTGGAACTTGGAC
vha68-2 GCGTAACTGTCGGAGATCCGGTGCTGCGTACCGGCAAGCCTCTTTCCGTGGAGCTGGGAC
vha68-1 CCGGCATTATGGGCAGCATCTTCGACGGCATCCAACGTCCTTTGCGGGACATTGGTGTCA
vha68-2 CCGGTATCATGGGCAGCATCTTTGACGGTATCCAGCGTCCCCTGAAGGACATTAACGAGC
vha68-1 TGACCAACTCCATCTATATACCCAAAGGTGTCAACACAACTGCTTTGTCGCGCTCGGAGA
vha68-2 TGACCGAATCCATCTACATTCCCAAGGGTGTCAACGTGCCCAGTTTGTCCCGCGTGGCCA
vha68-1 TGTGGGAATTTAATCCGCTGAATGTGCGGGTGGGATCCCACATCACCGGAGGAGATCTGT
vha68-2 GCTGGGAGTTCAACCCCCTGAACGTCAAGGTCGGCTCCCACATCACCGGAGGTGACCTGT


540 550 560 570 580 590
vha68-1 ATGGAGTGGTACACGAGAACACGCTGGTGAAGCAGCGCATGATTGTGGCACCGAGGGCTA
vha68-2 ACGGTCTGGTGCATGAGAACACTCTGGTCAAGCACAAGATGATTGTGAACCCCCGCGCCA
540 550 560 570 580 590
600 610 620 630 640 650
vha68-1 AGGGAACCGTTCGATACATTGCCCCCGCGGGCAACTACAACCTGGAGGACATTGTCCTGG
vha68-2 AGGGAACAGTGCGCTACATCGCCCCCTCCGGCAACTACAAGGTCGACGATGTCGTCCTGG
600 610 620 630 640 650
660 670 680 690 700 710
vha68-1 AGACGGAGTTCGACGGCGAGATCACCAAGCACACCATGTTGCAGGTCTGGCCAGTGCGGC
vha68-2 AGACCGAGTTCGATGGAGAGATCACCAAGCACACCATGTTGCAGGTGTGGCCAGTGCGTC
660 670 680 690 700 710
720 730 740 750 760 770
vha68-1 AGGCACG-TCCCGTCACAGAGAAGCTGCCAGCCAACCATCCGCTCTTCACGGGCCAACGC
vha68-2 A-CCACGCTCCCGTGACCGAGAAGCTGCCCGCCAACCACCCCCTGCTCACCGGACAGCGT
720 730 740 750 760 770
780 790 800 810 820 830
vha68-1 GTCCTTGACTCGCTCTTCCCCTGCGTACAGGGCGGCACCACTGCCATCCCCGGTGCCTTT
vha68-2 GTGCTCGACTCGCTCTTCCCCTGTGTCCAGGGCCGTACCACCGCCATTCCCGGAGCTTTC
780 790 800 810 820 830
840 850 860 870 880 890
vha68-1 GGCTGCGGCAAGACCGTCATTTCGCAC GCCCTGTCCAAGTACTCCAACTCTGATGTGATC
vha68-2 GGTTGCGGCAAGACTGTGATCTCGCAG∇GCTCTGTCCAAGTACTCCAACTCCGATGTCATC
840 850 860 870 880 890
900 910 920 930 940 950
vha68-1 ATCTACGTCGGTTGCGGCGAGCGCGGTAACGAGATGTCTGAGGTACTGCGTGACTTTCCC
vha68-1 ATCTACCTCGGTTGCGGTGAGCGTGGTAACGAGATGTCTGAGGTACTGCGTGACTTCCCC
900 910 920 930 940 950
960 970 980 990 1000 1010
vha68-1 GAACTGACCTGCGACATAGATGGCGTCACCGAGTCCATTATGAAGCGAACTGCTCTGGTG
vha68-2 GAGCTGTCCGTGGAGATCGATGGTGTGACCGAGTCCATCATGAAGCGTACCGCCCTTGTG
960 970 980 990 1000 1010
1020 1030 1040 1050 1060 1070
vha68-1 GCCAACACCTCCAACATGCCGGTGGCAGCTCGTGAGGCCTCCATTTACACTGGTATCACT
vha68-2 GCCAACACCTCCAACATGCCTGTGGCTGCTCGAGAGGCCTCCATCTACACTGGTATCACC
1020 1030 1040 1050 1060 1070
1080 1090 1100 1110 1120 1130
vha68-1 CTGTCTGAATACTTCCGTGATATGGGCTACAACGTAGCCATGATGGCTGATTCCACCTCC
vha68-2 TTGTCCGAATACTTCCGTGATATGGGTTACAACGTGTCCATGATGGCTGATTCCACCTCC
1080 1090 1100 1110 1120 1130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vha68-2 CGTTGGGCTGAGGCTCTTCGTGAAATTTCTGGTCGTCTCGCTGAGATGCCTCGCGATTCC
1140 1150 1160 1170 1180 1190
1200 1210 1220 1230 1240 1250
vha68-1 GGCTACCCGGCTTATCTAGGAGCTCGTCTGGCCACATTCTACGAGCGTGCTGGGCGCGTC
vha68-2 GGCTACCCAGCCTACTTGGGAGCTCGTCTGGCCTCCTTCTACGAGCGTGCCGGTCGCGTT
1200 1210 1220 1230 1240 1250
1260 1270 1280 1290 1300 1310
vha68-1 AAGTGCTTGGGTAACCCGGAGCGCGAGGGATCCGTGTCCATTGTCGGAGCTGTGTCTCCT
vha68-2 AAGTGCTTGGGTAACCCCGAGCGCGAGGGATCCGTGTCCATTGTCGGAGCTGTGTCTCCT
1260 1270 1280 1290 1300 1310
1320 1330 1340 1350 1360 1370
vha68-1 CCTGGTGGTGACTTCTCCGATCCCGTGACCTCCGCCACTTTGGGTATCGTGCAGGTGTTC
vha68-2 CCTGGTGGTGACTTCTCCGATCCCGTAACCTCCGCCACTCTGGGTATCGTGCAGGTGTTC
1320 1330 1340 1350 1360 1370
1380 1390 1400 1410 1420 1430
vha68-1 TGGGGTCTCGACAAGAAATTGGCCCAGCGCAAGCACTTCCCCTCGATCAACTGGCTCATC
vha68-2 TGGGGTCTCGACAAGAAGTTGGCCCAGCGCAAGCATTTCCCCTCGATCAACTGGCTCATC
1380 1390 1400 1410 1420 1430
1440 1450 1460 1470 1480 1490
vha68-1 TCCTACTCGAAGTACATGCGTGCTCTGGATGAATACTATGACAAGAACTACCCCGAGTTC
vha58-2 TCCTACTCGAAGTACATGCGTGCTCTGGATGACTTCTATGACAAGAACTTCCCGGAATTC
1440 1450 1460 1470 1480 1490
1500 1510 1520 1530 1540 1550
vha68-1 GTGCCACTACGCACCAAGGTCAAGGAGATCCTGCAGGAGGAGGAGGATCTGTCTGAGATC
vha68-2 GTGCCGCTGCGTACCAAGGTCAAGGAGATCCTGCAGGAGGAGGAGGATCTGTCTGAGATC
1500 1510 1520 1530 1540 1550
1560 1570 1580 1590 1600 1610
vha68-1 GTTCAGCTGGTGGGCAAAGCATCACTGGCCGAGACCGACAAGGTGACCCTGGAAGTGGCA
vha68-2 GTGCAACTGGTCGGCAAGGCCTCTCTCGCCGAAACCGACAAGATCACGCTGGAGGTGGCC
1560 1570 1580 1590 1600 1610
1620 1630 1640 1650 1660 1670
vha68-1 AAGCTGCTGAAGGACGACTTTCTGCAACAGAACTCCTACTCACCATACGATCGCGTTTGT
vha68-2 AAGCTGCTGAAGGACGATTTCCTGCAGCAGAACTCCTACTCCTCGTACGATCGCTTCTGC
1620 1630 1640 1650 1660 1670
1680 1690 1700 1710 1720 1730
vha68-1 CCCTTCTACAAGACCGTGGGCATGCTGAGAAACATCATGGCCTTCTATGAGACCGCCCGG
vha68-2 CCCTTCTACAAGACCGTGGGCATGTTGAGGAACATCATCGACTTCTACGACATGGCCCGT
1680 1690 1700 1710 1720 1730

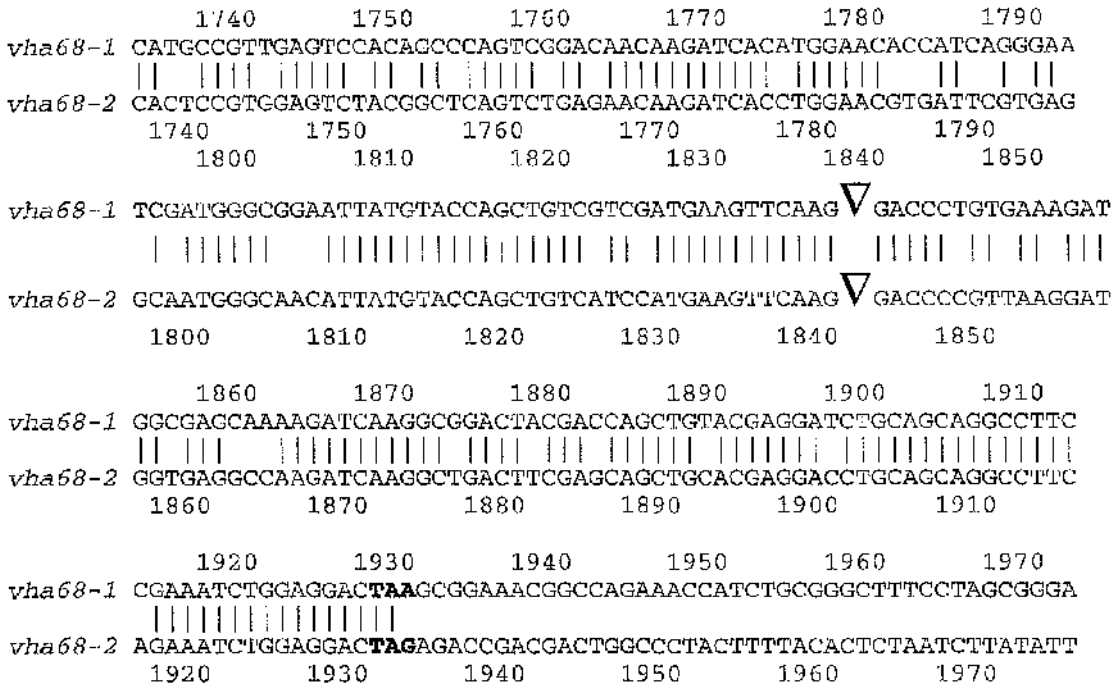
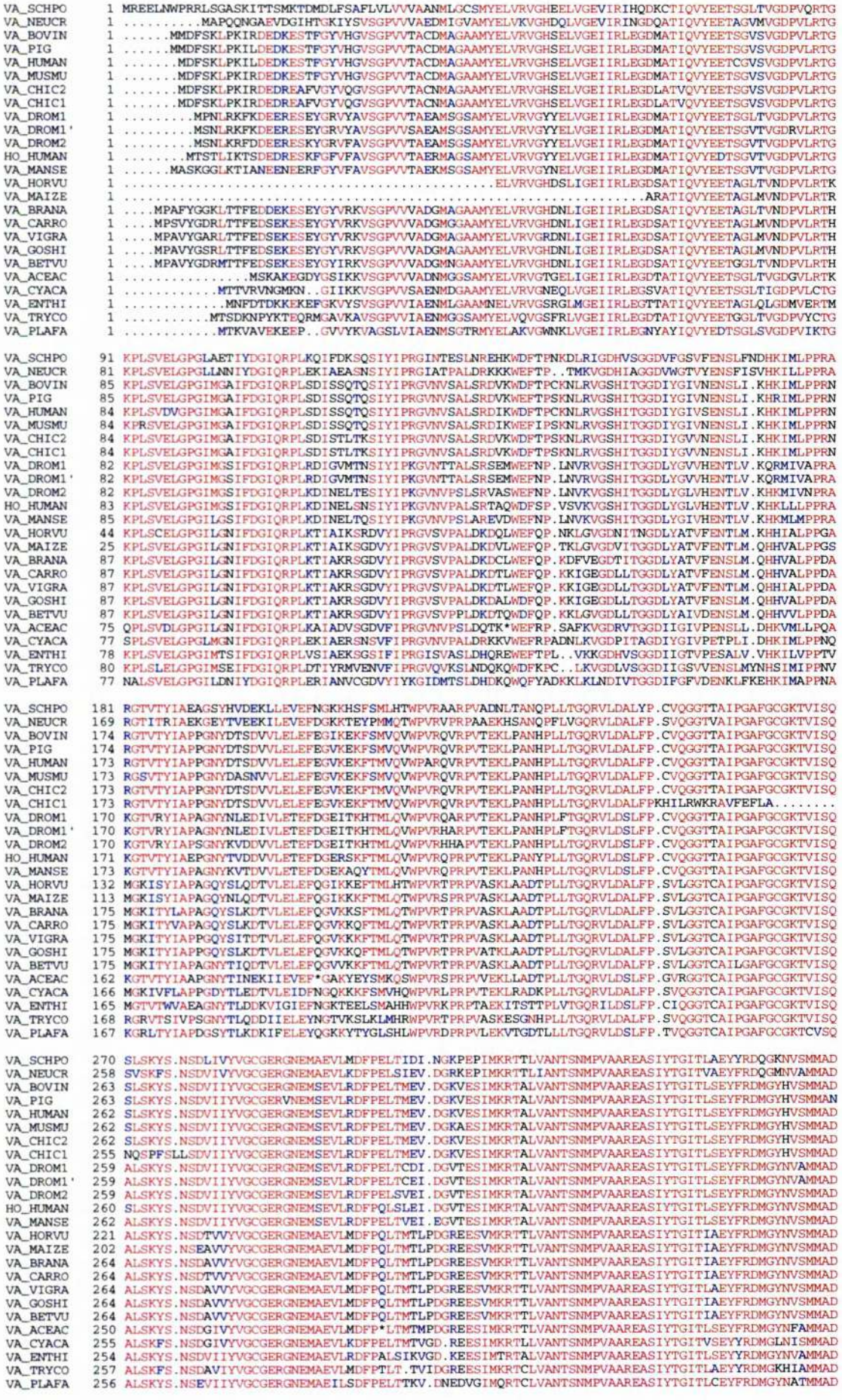


Figure 4.4 Alignment of the two cDNA by FASTA in GCG. The positions of the introns were marked by "∇" according the information of the genomic sequence. See Section 4.4 for genomic sequence of *vha68-2*. Refer Accession number: U19742 in GenBank database for genomic sequence of *vha68-1*.


VA_SCHPO 1 MREELNWPRRLSGASKITTSMKTDMDLFSAFLVLVVVAANMLGCSMYELVRVGHEELVGEVIRIHQDKCTIQVYEETSGLTVGDPVQRTG
VA_NEUCR 1 .....MAPQQNGAEVDGIHTGKIYSVSGPVVVAEDMIGVAMYELVKVGHDQLVGEVIRINGDQATIQVYEETAGVMVGDPVLRTG
VA_BOVIN 1 .....MMDFSKLPKIRDEDKESTFGYVHGVSGPVVTACDMAGAAMYELVRVGHSELVGEIIRLEGDMATIQVYEETSGVSVGDPVLRTG
VA_PIG 1 .....MMDFSKLPKILDEDKESTFGYVHGVSGPVVTACDMAGAAMYELVRVGHSELVGEIIRLEGDMATIQVYEETSGVSVGDPVLRTG
VA_HUMAN 1 .....MDFSKLPKILDEDKESTFGYVHGVSGPVVTACDMAGAAMYELVRVGHSELVGEIIRLEGDMATIQVYEETCGVSVGDPVLRTG
VA_MUSMU 1 .....MDFSKLPKIRDEDKESTFGYVHGVSGPVVTACDMAGAAMYELVRVGHSELVGEIIRLEGDMATIQVYEETSGVSVGDPVLRTG
VA_CHIC2 1 .....MDFSKLPKIRDEDREAFVGYVQGVSGPVVTACNMAGAAMYELVRVGHSELVGEIIRLEGDLATVQVYEETSGVSVGDPVLRTG
VA_CHIC1 1 .....MDFSKLPKIRDEDREAFVGYVQGVSGPVVTACNMAGAAMYELVRVGHSELVGEIIRLEGDLATVQVYEETSGVSVGDPVLRTG
VA_DROM1 1 .....MPNLRKFKDEERESEYGRVYAVSGPVVTAEAMSGSAMYELVRVGYYELVGEIIRLEGDMATIQVYEETSGLTVGDPVLRTG
VA_DROM1' 1 .....MSNLRKFKDEERESEYGRVYAVSGPVVSAEAMSGSAMYELVRVGYYELVGEIIRLEGDMATIQVYEETSGVTVGDRVLRTG
VA_DROM2 1 .....MSNLKRFDDEERESKYGRVFAVSGPVVTAEAMSGSAMYELVRVGYYELVGEIIRLEGDMATIQVYEETSGVTVGDPVLRTG
HO_HUMAN 1 .....MTSTLIKTSDEDRESKFGFVFAVSGPVVTAERMAGSAMYELVRVGYYELVGEIIRLEGDMATIQVYEDTSGVTVGDPVLRTG
VA_MANSE 1 .....MASKGGLKTIANEENEERFGYVFAVSGPVVTAEKMSGSAMYELVRVGYNELVGEIIRLEGDMATIQVYEETSGVTVGDPVLRTG
VA_HORVU 1 .....ELVRVGHDSLIGEIIRLEGDSATIQVYEETAGLTVNDPVLRTK
VA_MAIZE 1 .....ARATIQVYEETAGLMVNDPVLRTR
VA_BRANA 1 ....MPAFYGGKLTTFEDDEKESEYGYVRKVSGPVVVADGMAGAAMYELVRVGHDNLIGEIIRLEGDSATIQVYEETAGLTVNDPVLRTH
VA_CARRO 1 ....MPSVYGDRLTTFEDSEKESEYGYVRKVSGPVVVADGMGGAAMYELVRVGHDNLIGEIIRLEGDSATIQVYEETAGLMVNDPVLRTH
VA_VIGRA 1 ....MPAVYGARLTTFEDSEKESEYGYVRKVSGPVVVADGMAGAAMYELVRVGRDNLIGEIIRLEGDSATIQVYEETAGLMVNDPVLRTH
VA_GOSHI 1 ....MPAVYGSRLTTFEDSEKESEYGYVRKVSGPVVVADGMAGAAMYELVRVGHDNLIGEIIRLEGDSATIQVYEETAGLMVNDPVLRTH
VA_BETVU 1 ....MPAVYGDRMTTFEDSEKESEYGYIRKVSGPVVVADGMNGAAMYELVRVGHDNLIGEIIRLEGDSATIQVYEETGGLTVNDPVLRTH
VA_ACEAC 1 .....MSKAKEGDYGSIKKVSGPVVVADNMGGSAMYELVRVGTGELIGEIIRLEGDTATIQVYEETSGLTVGDGVLRTK
VA_CYACA 1 .....MTTVRVNGMKN..GIIKKVSGPVVSAENMDGAAMYELVRVGNEQLVGEIIRLEGSVATIQVYEETSGLTIGDPVLCTG
VA_ENTHI 1 .....MNFDTDKKEKEFGKVYSVSGPVVIAENMLGAAMNELVRVGSRGLMGEIIRLEGTTATIQVYEETAGLQLGDMVERTM
VA_TRYCO 1 .....MTSDKNPYKTEQRMGAVKAVSGPVVIAENMGGSAMYELVQVGSFRLVGEIIRLEGDTATIQVYEETGGLTVGDPVYCTG
VA_PLAFA 1 .....MTKVAVEKEEP..GVVYKVAGSLVIAENMSGTRMYELAKVGWNKLVGEIIRLEGNYAYIQVYEDTSGLSVGDPVIKTG
VA_SCHPO 91 KPLSVELGPGLAETIYDGIQRPLKQIFDKSQSIYIPRGINTESLNREHKWDFTPNKDLRIGDHVSGGDVFGSVFENSLFNDHKIMLPPRA
VA_NEUCR 81 KPLSVELGPGLLNNIYDGIQRPLEKIAEASNSIYIPRGIATPALDRKKKWEFTP..TMKVGDHIAGGDVWGTVYENSFISVHKILLPPRA
VA_BOVIN 85 KPLSVELGPGIMGAIFDGIQRPLSDISSQTQSIYIPRGVNVSALSRDVKWDFTPCKNLRVGSHITGGDIYGIVNENSLI.KHKIMLPPRN
VA_PIG 85 KPLSVELGPGIMGAIFDGIQRPLSDISSQTQSIYIPRGVNVSALSRDVKWEFTPSKNLRVGSHITGGDIYGIVNENSLI.KHRIMLPPRN
VA_HUMAN 84 KPLSVDVGPGIMGAIFDGIQRPLSDISSQTQSIYIPRGVNVSALSRDIKWDFTPCKNLRVGSHITGGDIYGIVSENSLI.KHKIMLPPRN
VA_MUSMU 84 KPRSVELGPGIMGAIFDGIQRPLSDISSQTQSIYIPRGVNVSALSRDIKWEFIPSKNLRVGSHITGGDIYGIVNENSLI.KHKIMLPPRN
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VA_CHIC1 84 KPLSVELGPGIMGAIFDGIQRPLSDISTLTKSIYIPRGVNVSALSRDVKWDFTPSKNLRVGSHITGGDIYGVVNENSLI.KHKIMLPPRN
VA_DROM1 82 KPLSVELGPGIMGSIFDGIQRPLRDIGVMTNSIYIPKGVNTTALSRSEMWEFNP.LNVRVGSHITGGDLYGVVHENTLV.KQRMIVAPRA
VA_DROM1' 82 KPLSVELGPGIMGSIFDGIQRPLRDIGVMTNSIYIPKGVNTTALSRSEMWEFNP.LNVRVGSHITGGDLYGVVHENTLV.KQRMIVAPRA
VA_DROM2 82 KPLSVELGPGIMGSIFDGIQRPLKDINELTESIYIPKGVNVPSLSRVASWEFNP.LNVKVGSHITGGDLYGLVHENTLV.KHKMIVNPRA
HO_HUMAN 83 KPLSVELGPGIMGSIFDGIQRPLKDINELSNSIYIPKGVNVPALSRTAQWDFSP.VSVKVGSHITGGDLYGLVHENTLV.KHKLLLPPRA
VA_MANSE 85 KPLSVELGPGILGSIFDGIQRPLKDINELTQSIYIPKGVNVPSLAREVDWEFNP.LNVKVGSHITGGDLYGIVHENTLV.KHKMLMPPRA
VA_HORVU 44 KPLSCELGPGILGNIFDGIQRPLKTIAIKSRDVYIPRGVSVPALDKDQLWEFQP.NKLGVGDNITNGDLYATVFENTLM.KHHIALPPGA
VA_MAIZE 25 KPLSVELGPGILGNIFDGIQRPLKTIAIKSGDVYIPRGVSVPALDKDVLWEFQP.TKLGVGDVITGGDLYATVFENTLM.QHHVALPPGS
VA_BRANA 87 KPLSVELGPGILGNIFDGIQRPLKTIAKRSGDVYIPRGVSVPALDKDCLWEFQP.KDFVEGDTITGGDLYATVFENSLM.QHHVALPPDA
VA_CARRO 87 KPLSVELGPGILGNIFDGIQRPLKTIAKRSGDVYIPRGVSVPALDKDTLWEFQP.KKIGEGDLLTGGDLYATVFENSLM.QHHVALPPDA
VA_VIGRA 87 KPLSVELGPGILGNIFDGIQRPLKTIAKRSGDVYIPRGVSVPALDKDTLWEFQP.KKIGEGDLLTGGDLYATVFENTLM.QHHIALPPDA
VA_GOSHI 87 KPLSVELGPGILGNIFDGIQRPLKTIAKRSGDVYIPRGVSVPALDKDALWDFQP.KKIGEGDLLTGGDLYATVFENSLM.QHHVALPPDA
VA_BETVU 87 KPLSVELGPGILGNIFDGIQRPLKTIAKRSGDVYIPRGVSVPPLDKDTQWDFQP.KKLGVGDLLTGGDLYAIVDENSLM.QHHVVLPPDA
VA_ACEAC 75 QPLSVDLGPGILGNIFDGIQRPLKAIADVSGDVFIPRGVNVPSLDQTK*WEFRP.SAFKVGDRVTGGDIIGIVPENSLL.DHKVMLLPQA
VA_CYACA 77 SPLSVELGPGLMGNIFDGIQRPLEKIAERSNSVFIPRGVNVPALDRKKVWEFRPADNLKVGDPITAGDIYGIVPETPLI.DHKIMLPPNQ
VA_ENTHI 78 KPLSVELGPGIMTSIFDGIQRPLVSIAEKSGSIFIPRGISVASLDHQREWEFTPL..VKKGDHVSGGDIIGTVPESALV.VHKILVPPTV
VA_TRYCO 80 KPLSLELGPGIMSEIFDGIQRPLDTIYRMVENVFIPRGVQVKSLNDQKQWDFKPC..LKVGDLVSGGDIIGSVVENSLMYNHSIMIPPNV
VA_PLAFA 77 NALSVELGPGILDNIYDGIQRPLERIANVCGDVYIYKGIDMTSLDHDKQWQFYADKKLKLNDIVTGGDIFGFVDENKLFKEHKIMAPPNA
VA_SCHPO 181 RGTVTYIAEAGSYHVDEKLLEVEFNGKKHSFSMLHTWPVRAARPVADNLTANQPLLTGQRVLDALYP.CVQGGTTAIPGAFGCGKTVISQ
VA_NEUCR 169 RGTITRIAEKGEYTVEEKILEVEFDGKKTEYPMMQTWPVRVPRPAAEKHSANQPFLVGQRVLDALFP.SVQGGTVAIPGAFGCGKTVISQ
VA_BOVIN 174 RGTVTYIAPPGNYDTSDVVLELEFEGIKEKFSMVQVWPVRQVRPVTEKLPANHPLLTGQRVLDALFP.CVQGGTTAIPGAFGCGKTVISQ
VA_PIG 174 RGTVTYIAPPGNYDTSDVVLELEFEGVKEKFSMVQVWPVRQVRPVTEKLPANHPLLTGQRVLDALFP.CVQGGTTAIPGAFGCGKTVISQ
VA_HUMAN 173 RGTVTYIAPPGNYDTSDVVLELEFEGVKEKFTMVQVWPARQVRPVTEKLPANHPLLTGQRVLDALFP.CVQGGTTAIPGAFGCGKTVISQ
VA_MUSMU 173 RGSVTYIAPPGNYDASNVVLELEFEGVKEKFSMVQVWPVRQVRPVTEKLPANHPLLTGQRVLDALFP.CVQGGTTAIPGAFGCGKTVISQ
VA_CHIC2 173 RGTVTYIAPPGNYDTSDVVLELEFEGVKEKFTMVQVWPVRQVRPVTEKLPANHPLLTGQRVLDALFP.CVQGGTTAIPGAFGCGKTVISQ
VA_CHIC1 173 RGTVTYIAPPGNYDTSDVVLELEFEGVKEKFTMVQVWPVRQVRPVTEKLPANHPLLTGQRVLDALFPKHILRWKRAVFEFLA.....
VA_DROM1 170 KGTVRYIAPAGNYNLEDIVLETEFDGEITKHTMLQVWPVRQARPVTEKLPANHPLFTGQRVLDSLFP.CVQGGTTAIPGAFGCGKTVISQ
VA_DROM1' 170 KGTVRYIAPAGNYNLEDIVLETEFDGEITKHTMLQVWPVRHARPVTEKLPANHPLFTGQRVLDSLFP.CVQGGTTAIPGAFGCGKTVISQ
VA_DROM2 170 KGTVRYIAPSGNYKVDDVVLETEFDGEITKHTMLQVWPVRHHAPVTEKLPANHPLLTGQRVLDSLFP.CVQGGTTAIPGAFGCGKTVISQ
HO_HUMAN 171 KGTVTYIAEPGNYTVDDVVLETEFDGERSKFTMLQVWPVRQPRPVTEKLPANYPLLTGQRVLDSLFP.CVQGGTTAIPGAFGCGKTVISQ
VA_MANSE 173 KGTVTYIAPAGNYKVTDVVLETEFDGEKAQYTMLQVWPVRQPRPVTEKLPANHPLLTGQRVLDSLFP.CVQGGTTAIPGAFGCGKTVISQ
VA_HORVU 132 MGKISYIAPAGQYSLQDTVLELEFQGIKKEFTMLHTWPVRTPRPVASKLAADTPLLTGQRVLDALFP.SVLGGTCAIPGAFGCGKTVISQ
VA_MAIZE 113 MGKISYIAPAGQYNLQDTVLELEFQGIKKKFTMLQTWPVRSPRPVASKLAADTPLLTGQRVLDALFP.SVLGGTCAIPGAFGCGKTVISQ
VA_BRANA 175 MGKITYLAPAGQYSLKDTVLELEFQGVKKSFTMLQTWPVRTPRPVASKLAADTPLLTGQRVLDALFP.SVLGGTCAIPGAFGCGKTVISQ
VA_CARRO 175 MGKITYVAPAGQYSLKDTVLELEFQGVKKQFTMLQTWPVRTPRPVASKLAADTPLLTGQRVLDALFP.SVLGGTCAIPGAFGCGKTVISQ
VA_VIGRA 175 MGKITYIAPPGQYSITDTVLELEFQGVKKKFTMLQTWPVRTPRPVASKLAADTPLLTGQRVLDALFP.SVLGGTCAIPGAFGCGKTVISQ
VA_GOSHI 175 MGKITYIAPPGQYSLKDTVLELEFQGVKKQFTMLQTWPVRTPRPVATKLAADTPLLTGQRVLDALFP.SVLGGTCAIPGAFGCGKTVISQ
VA_BETVU 175 MGKITYIAPAGNYTIQDTVLELEFQGVVKKFTMLQTWPVRTPRPVASKLAADTPLLTGQRVLDALFP.SVLGGTCAILGAFGCGKTVISQ
VA_ACEAC 162 KGTVTYIAAPGNYTINEKIIEVEF*GAKYEYSMKQSWPVRSPRPVVEKLLADTPLLTGQRVLDSLFP.GVRGGTCAIPGAFGCGKTVISQ
VA_CYACA 166 MGKIVFLAPPGDYTLEDTVLEIDFNGQKKFSMVHQWPVRLPRPVTEKLRADKPLLTGQRVLDSLFP.SVQGGTCAIPGAFGCGKTVISQ
VA_ENTHI 165 MGTVTWVAEAGNYTLDDKVIGIEFNGKTEELSMAHHWPVRKPRPTAEKITSTTPLVTGQRILDSLFP.CIQGGTCAIPGAFGCGKTVISQ
VA_TRYCO 168 RGRVTSIVPSGNYTLQDDIIELEYNGTVKSLKLMHRWPVRTPRPVASKESGNHPLLTGQRVLDALFP.SVQGGTCAIPGAFGCGKTVISQ
VA_PLAFA 167 KGRLTYIAPDGSYTLKDKIFELEYQGKKYTYGLSHLWPVRDPRPVLEKVTGDTLLLTGQRVLDSLFP.TVQGGTCAIPGAFGCGKTCVSQ
VA_SCHPO 270 SLSKYS.NSDLIVYVGCGERGNEMAEVLMDFPELTIDI.NGKPEPIMKRTTLVANTSNMPVAAREASIYTGITLAEYYRDQGKNVSMMAD
VA_NEUCR 258 SVSKFS.NSDVIVYVGCGERGNEMAEVLKDFPELSIEV.DGRKEPIMKRTTLIANTSNMPVAAREASIYTGITVAEYFRDQGMNVAMMAD
VA_BOVIN 263 SLSKYS.NSDVIIYVGCGERGNEMSEVLRDFPELTMEV.DGKVESIMKRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYHVSMMAD
VA_PIG 263 SLSKYS.NSDVIIYVGCGERVNEMSEVLRDFPELTMEV.DGKVESIMKRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYHVSMMAN
VA_HUMAN 262 SLSKYS.NSDVIIYVGCGERGNEMSEVLRDFPELTMEV.DGKVESIMKRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYHVSMMAD
VA_MUSMU 262 SLSKYS.NSDVIIYVGCGERGNEMSEVLRDFPELTMEV.DGKAESIMKRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYHVSMMAD
VA_CHIC2 262 SLSKYS.NSDVIIYVGCGERGNEMSEVLRDFPELTMEV.DGKVESIMKRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYHVSMMAD
VA_CHIC1 255 NQSPFSLLSDVIIYVGCGERGNEMSEVLRDFPELTMEV.DGKVESIMKRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYHVSMMAD
VA_DROM1 259 ALSKYS.NSDVIIYVGCGERGNEMSEVLRDFPELTCDI.DGVTESIMKRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYNVAMMAD
VA_DROM1' 259 ALSKYS.NSDVIIYVGCGERGNEMSEVLRDFPELTCEI.DGVTESIMKRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYNVAMMAD
VA_DROM2 259 ALSKYS.NSDVIIYVGCGERGNEMSEVLRDFPELSVEI.DGVTESIMKRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYNVSMMAD
HO_HUMAN 260 SLSKYS.NSDVIIYVGCGERGNEMSEVLRDFPQLSLEI.DGVTESIMKRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYNVSMMAD
VA_MANSE 262 ALSKYS.NSDVIIYVGCGERGNEMSEVLRDFPELTVEI.EGVTESIMKRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYNVSMMAD
VA_HORVU 221 ALSKYS.NSDTVVYVGCGERGNEMAEVLMDFPQLTMTLPDGREESVMKRTTLVANTSNMPVAAREASIYTGITIAEYFRDMGYNVSMMAD
VA_MAIZE 202 ALSKYS.NSEAVVYVGCGERGNEMAEVLMDFPQLTMTLPDGREESVMKRTTLVANTSNMPVAAREASIYTGITIAEYFRDMGYNVSMMAD
VA_BRANA 264 ALSKYS.NSDAVVYVGCGERGNEMAEVLMDFPQLTMTLPDGREESVMKRTTLVANTSNMPVAAREASIYTGITIAEYFRDMGYNVSMMAD
VA_CARRO 264 ALSKYS.NSDTVVYVGCGERGNEMAEVLMDFPQLTMTLPDGREESVMKRTTLVANTSNMPVAAREASIYTGITIAEYFRDMGYNVSMMAD
VA_VIGRA 264 ALSKYS.NSDAVVYVGCGERGNEMAEVLMDFPQLTMTLPDGREESVMKRTTLVANTSNMPVAAREASIYTGITLAEYFRDMGYNVSMMAD
VA_GOSHI 264 ALSKYS.NSDAVVYVGCGERGNEMAEVLMDFPQLTMTLPDGREESVMKRTTLVANTSNMPVAAREASIYTGITIAEYFRDMGYNVSMMAD
VA_BETVU 264 ALSKYS.NSDAVVYVGCGERGNEMAEVLMDFPQLTMTLPDGREESVMKRTTLVANTSNMPVAAREASIYTGITIAEYFRDMGYNVSMMAD
VA_ACEAC 250 ALSKYS.NSDGIVYVGCGERGNEMAEVLMDFP*LTMTMPDGREESIMKRTTLVANTSNMPVAAREASIYTGITLSEYFRDMGYNFAMMAD
VA_CYACA 255 ALSKFS.NSDGIVYVGCGERGNEMAEVLKDFPELTMTVGD.REESIMKRTTLLVANTSNMPVAAREASIYTGITVSEYYRDMGLNISMMAD
VA_ENTHI 254 ALSKYS.NSDVIIYVGCGERGNEMAEVLRDFPALSIKVGD.KEESIMTRTALVANTSNMPVAAREASIYTGITLSEYYRDMGYNVAMMAD
VA_TRYCO 257 ALSKFS.NSDAVIYVGCGERGNEMAEVLMDFPTLT.TVIDGREESIMKRTCLVANTSNMPVAAREASIYTGITLAEYYRDMGKHIAMMAD
VA_PLAFA 256 ALSKYS.NSEVIIYVGCGERGNEMAEILSDFPELTTKV.DNEDVGIMQRTCLVANTSNMPVAAREASIYTGITLCEYFRDMGYNATMMAD

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VA_SCHPO   358 STSRWAEALREISGRLAEMPADSGYPAYLGAKLASFYERAGRARCLGSPDREGTVSIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
VA_NEUCR   346 SSSRWAEALREISGRLGEMPADQGFPAYLGAKLASFYERAGKVQALGSPPREGSVSIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
VA_BOVIN   351 STSRWAEALREISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPEREGSVSIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
VA_PIG     351 STSRWAEALREISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPEREGSVTIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
VA_HUMAN   350 STSRWAEALREISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPEREGSVSIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
VA_MUSMU   350 STSRWAEALREISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPEREGSVSIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
VA_CHIC2   350 STSRWAEALREISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPEREGSVTIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
VA_CHIC1   344 STSRWAEALREISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPEREGSVTIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
VA_DROM1   347 STSRWAEALREISGRLAEMPADSGYPAYLGARLATFYERAGRVKCLGNPEREGSVSIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
VA_DROM1'  347 STSRWAEALREISGRLAEMPADSGYPAYLGARLATFYERAGRVKCLGNPEREGSVSIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
VA_DROM2   347 STSRWAEALREISGRLAEMPRDSGYPAYLGARLASFYERAGRVKCLGNPEREGSVSIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
HO_HUMAN   348 STSRWAEALREISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPDREGSVSIVGAVSPPGGDFSDPVTTATLGIVQVFWGLDKKL
VA_MANSE   350 STSRWAEALREISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPDREGSVSIVGAVSPPGGDFSDPVTAATLGIVQVFWGLDKKL
VA_HORVU   310 STSRWAEALREISGRLAEMPADSGYPAYLASRLASFYERAGKVQCLGSPDRTGSVTIVGAVSPPGGDFSDPVTSATLSIVQVFWGLDKKL
VA_MAIZE   291 STSRWAEALREISGRLAEMPADSGYPAYLAARLASFYERAGKVKCLGSPDRNGSVTIVGAVSPPGGDFSDPVTSATLSIVQVFWGLDKKL
VA_BRANA   353 STSRWAEALREISGRLAEMPADSGYPAYLAARLASFYERAGKVKCLGGPERNGSVTIVGAVSPPGGDFSDPVTSATLSIVQVFWGLDKKL
VA_CARRO   353 STSRWAEALREISGRLAEMPADSGYPAYLAARLASFYERAGKVKCLGGPERNGSVTIVGAVSPPGGDFSDPVTSATLSIVQVFWGLDKKL
VA_VIGRA   353 STSRWAEALREISGRLAEMPADSGYPAYLAARLASFYERPGKVKCLGGPERTGSVTIVGAVSPPGGDFSDPVTSATLSIVQVFWGLDKKL
VA_GOSHI   353 STSRWAEALREISGRLAEMPADSGYPAYLAARLASFYERAGKVKCLGGPERTGSVTIVGAVSPPGGDFSDPVTSATLSIVQVFWGLDKKL
VA_BETVU   353 SGSRWAEALREISGRLAEMPADSGYPAYLAARLASFYEAAGKVKCLGGPERNGSVTIVGAVSPPGGDFSDPVTSATLSIVQVFWGLDKKL
VA_ACEAC   338 STSRWAEALREISGRLAEMPADSGYPAYLGARLASFYERSGRVACIGSPEREGSVTIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
VA_CYACA   343 STSRWAEALREISGRLAEMPADSGYPAYLAARLASFYERAGKVSCLGSPNRQGSITIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
VA_ENTHI   342 STSRWAEALREISGRLAEMPADSGYPAYLAARLASFYERAGMVECLGSPKRIGSVSIVGAVSPPGGDFSDPVTTSTLNIVQVFWGLDKKL
VA_TRYCO   345 STSRWAEALREISGRLAEMPADGGYPAYLSARLASFYERAGRVTCIGGPKREGSVTIVGAVSPPGGDFSDPVTSATLGIVQVFWGLEKRL
VA_PLAFA   344 STSRWAEALREISGRLAEMPADSGYPAYLGARLASFYERAGKVKCIGSPSRIGSITIVGAVSPPGGDFSDPVTTATMSIVQAFWGLDKKL

VA_SCHPO   448 AQRKHFPSINTSLSYSKYINALQPWYEERVPGFNTLRDQIKQIIQQEDSMLEIIQLVGKSALSETDKVTLDIAGIIKNDFLQQNGYSDYD
VA_NEUCR   436 AQRKHFPSINTSVSYSKYLTILDKWYEREYPDFPRLRDRIRQLLSDSEELDQVVQLVGKSALSDPDKITLDMATLIKEDFLQQNGYSDYD
VA_BOVIN   441 AQRKHFPSVNWLISYSKYMRALDEYYDKHFTEFVPLRTKAKEILQEEEDLAEIVQLVGKASLAETDKITLEVAKLIKDDFLQQNGYTPYD
VA_PIG     441 AQRKHFPSVNWLISYSKYMRALDEYYDKHFTEFVPLRTKAKEILQEEEDLAEIVQLVGKASLAETDKITLEVAKLIKDDFLQQNGYTPYD
VA_HUMAN   440 AQRKHFPSVNWLISYSKYMRALDEYYDKHFTEFVPLRTKAKEILQEEEDLAEIVQLVGKASLAETDKITLEVAKLIKDDFLQQNGYTPYD
VA_MUSMU   440 AQRKHFPSVNWLISYSKYMRALDEYYDKHFTEFVPLRTKAKEILQEEGDLAEIVQLVGKASLAETDKITLEVAKLIKDDFLQQNGYTPYD
VA_CHIC2   440 AQRKHFPSVNWLISYSKYTRALDEYYDKHFTEFVPLRTKAKEILQEEEDLAEIVQLVGKASLAETDKITLEVAKLIKDDFLQQNGYTPYD
VA_CHIC1   434 AQRKHFPSVNWLISYSKYTRALDEYYDKHFTEFVPLRTKAKEILQEEEDLAEIVQLVGKASLAETDKITLEVAKLIKDDFLQQNGYTPYD
VA_DROM1   437 AQRKHFPSINWLISYSKYMRALDEYYDKNYPEFVPLRTKVKEILQEEEDLSEIVQLVGKASLAETDKVTLEVAKLLKDDFLQQNSYSPYD
VA_DROM1'  437 AQRKHFPSINWLISYSKYMRALDEYYDKNYPEFVPLRTKVKEILQEEEDLSEIVQLVGKASLAETDKVTLEVAKLLKDDFLQQNSYSPYD
VA_DROM2   437 AQRKHFPSINWLISYSKYMRALDDFYDKNFPEFVPLRTKVKEILQEEEDLSEIVQLVGKASLAETDKITLEVAKLLKDDFLQQNSYSSYD
HO_HUMAN   438 AQRKHFPSINWLISYSKYMRALDDFYDKNFPEFVPLRTKVKEILQEEEDLSEIVQLVGKASLAETDKITLEVAKLLKDDFLQQNSYSPYD
VA_MANSE   440 AQRKHFPSINWLISYSKYMRALDDFYEKNYPEFVPLRTKVKEILQEEEDLSEIVQLVGKASLAETDKITLEVAKLLKDDFLQQNSYSSYD
VA_HORVU   400 AQRKHFPSVNWLISYSKYSTALEGYYEKFDPGFIDMRTKAREVLQREDDLNEIVQLVGKDALGESDKITLETAKLLREDYLAQNAFTPYD
VA_MAIZE   381 AQRKHFPSVNWLISYSKYSKALESFYEKFDPDFIDIRTKAREVLQREDDLNEIVQLVGKDALAESDKITLETAKLLREDYLAQNAFTPYD
VA_BRANA   443 AQRKHFPSVNWLISYSKYSTALESFYEKFDSDFIDIRTKAREVLQREDDLNEIVQLVGKDALAEGDKITLETAKLLREDYLAQNAFTPYD
VA_CARRO   443 AQRKHFPSVNWLISYSKYSTALESFYEKFDSDFIDIRTKAREVLQREDDLNEIVQLVGKDALAETDKITLETAKLLREDYLAQNAFTPYD
VA_VIGRA   443 AQRKHFPSVNWLISYSKYSTALESFYEQFDPDFINIRTKAREVLQREDDLNEIVQLVGKDALAEGDKITLETAKLLREDYLAQNAFTPYD
VA_GOSHI   443 AQRKHFPSVNWLISYSKYSGALESFYEKFDPDFISIRTKAREVLQREDDLNEIVQLVGKDALAETDKITLETAKLLREDYLAQNAFTPYD
VA_BETVU   443 AQRKHFPSVNWLISYSKYSGALESFYEKFDSEFIDIRTKAREVLQREDDLNEIVQLVGKDALAETDKITLDTAKLLREDYLAQNAFTAYD
VA_ACEAC   428 AQRKHFPSVNWLISYSKYLNALEPFYEKFDSDFVTLRQVAREVLQKEDELNEIVQLVGKDALAESDKIILETARFLKEDYLQQNSFTKYD
VA_CYACA   433 AQRKHFPSVNWLISYSKYMKALEPYYEERFPEFLNYQQKAREILQTEDDLMEIVQLVGKDSLAENDKITLEVAKMIREDFLAQNSFTEYD
VA_ENTHI   432 AQRKHFPAVNWNISFSKYIKSLDSYYNSKDEEFVPLRDKIKEILQMEEGLLQIVQLVGQDSLAETDKLTLEIARVIKDDFLQQNSYTPYD
VA_TRYCO   435 AQRKHFPSVNWLISYSKYLNALEPFFNTLDPDYMRLRSVAAEILQREEELQEIVQLVGKDSLSESDKIILETAKVIREEFLQQNAFTPYD
VA_PLAFA   434 AQRKHFPSVNWSTSFSKYVRQLEQYFDNFDQDFLSLRQKISDILQQESDLNDIVQLVGKDSLSEDQKVVMEVAKIIREDFLQQNAFSDYD

VA_SCHPO   538 RCCPLYKTYHMMRNMIAYYTKAKSAVETG........SVPWSKIKESTSDIFYELTSMKFENP.NEGEKEIVEHYETLHKKIEDKFHTLT
VA_NEUCR   526 QFCPIWKTEWMMKLMMGFHDEAQKAIAQG........Q.NWNKVREATQDLQAQLKSLKFEVP.SEGQEKICKKYEAIQQQMLDKFASVI
VA_BOVIN   531 RFCPFYKTVGMLSNMIAFYDMARRAVETTAQ...SDNKITWSIIREHMGEILYKLSSMKFKDPVKDGEAKIKADYAQLLEDMQNAFRSLE
VA_PIG     531 RFCPFYKTVGMLSNMIAFYDLARRAVETTAQ...SDNKITWSIIREHMGEILYKLSSMKFKDPVKDGEAKIKADYAQLLEDMQNAFRSLE
VA_HUMAN   530 RFCPFYKTVGMLSNMIAFYDMARRAVETTAQ...SDNKITWSIIREHMGDILYKLSSMKFKDPLKDGEAKIKSDYAQLLEDMQNAFRSLE
VA_MUSMU   530 RFCPFYKTVGMLSNMISFYDMARRAVETTAQ...SDNKITWSIIREHMGEILYKLSSMKFKDPVKDGEAKIKADYAQLLEDMQNAFRSLE
VA_CHIC2   530 RFCPFYKTVGMLSNMIAFYDMRRRAVENTAQ...SDNKITWSIIRENMSEILYRLTSMKFKDPVKDGETKIKADYAQLFEDMQNAFRSLE
VA_CHIC1   524 RFCPFYKTVGMLSNMIAFYDMRRRAVENTAQ...SDNKITWSIIRENMSEILYRLTSMKFKDPVKDGETKIKADYAQLFEDMQNAFRSLE
VA_DROM1   527 RVCPFYKTVGMLRNIMAFYETARHAVESTAQ...SDNKITWNTIRESMGGIMYQLSSMKFKDPVKDGEQKIKADYDQLYEDLQQAFRNLE
VA_DROM1'  527 RVCPFYKTVGMLRNIMAFYETARHCLESTAQ...SDNKITWNTIKESMGGIMYQLSSMKFKDPVKDGEQKIKADYDQLYEDLQQAFRNLE
VA_DROM2   527 RFCPFYKTVGMLRNIIDFYDMARHSVESTAQ...SENKITWNVIREAMGNIMYQLSSMKFKDPVKDGEAKIKADFEQLHEDLQQAFRNLE
HO_HUMAN   528 RFCPFYKTVGMLKNMIAFYDMSRHAVESTAQ...SENKITWNVIRDSMGNILYQLSSMKFKDPVKDGEAKIKADFEQLHEDIQQAFRNLE
VA_MANSE   530 RFCPFYKTVGMLKNIISFYDMSRHAVESTAQ...SDNKVTWNVIRDAMGNVLYQLSSMKFKDPVKDGEAKIKADFDQLLEDMSAAFRNLE
VA_HORVU   490 KYCPFYKSVWMMRNIIHFNQLANQAVERAAN..ADGHKITYAVVKSRMGDLFYRLVSQKFEDPA.EGEDVLVAKFQKLYDDLTAGFRNLE
VA_MAIZE   471 KFCPFYKSVWMMRNIIHFNTLANQAVERAAG..TDGHKITYSVIKHRLGDLFYRLVSQKFEDPA.EGEEALVGKFKKLYDDLTTGFRNLE
VA_BRANA   533 KFCPFYKSVWMMRNIIHFYNLANQAVERGAG..MDGQKISYSLIKHRLGDLFYRLVSQKFEDPA.EGEDVLVGKFKKLHDDLTSGFRNLE
VA_CARRO   533 KFCPFYKSVWMMRNIIHFYNLANQAVERGAG..MDGQKISYTLIKHRLGDLFYRLVSQKFEDPA.EGEDVLVGKFKKLHDDLTSGFRNLE
VA_VIGRA   533 KFCPFYKSVWMMRNIIHFYNLANQAVERGAG..SDGQKITYSLIKHRVGDLFYRLVSQKFEDPA.EGEAALVGQFQKLHEDLSTGFRNLE
VA_GOSHI   533 KFCPFYKSVWMMRNIVHFNALANQAVEKAAG..MDGQKITYSLIKHRLGDLFYRLVSQKFEDPA.EGEEALVAKFKKLNEDLTAGFRALE
VA_BETVU   533 KFCPFYKSVWMMRNIIHFYNLANQAVERGAG..SDGQKITYSLIKLRLGDLFYRLVSQKFEDPA.EGEDALVAKFKKLNEDLTAAFRNLE
VA_ACEAC   518 KYCPFYKSVGMMRNIVTFHRLATQAIERTAAGNVDGQKITFNIIKAKLGDLLYKVSS*KFEDPS.DGEGVVTAHLNELNEELKEKFRALE
VA_CYACA   523 RFCPFYKSVLMLRNMIHFYELANKAVE.....GSGEQHLTLAQIKEQMGETIYKISGMKFLDPA.QGWSLF...................
VA_ENTHI   522 FSCPFYKTCGIIRNIIHFYNEAFQALSVD....YEDHKITWATIKSAMSDLLVRISRMKYEEPS.QGEQVINEKYGELYRDITTRFATLL
VA_TRYCO   525 KYCPPYKTCWMLRNIVAFYEESQRVVAESA....GELKITWNYIREMIPHIYTGLTEMKFRDP.QEGEEANVEFYRKQNEEIVSAFASLL
VA_PLAFA   524 YMCPLQKTVGMMRIICHFYAQCLRTLQ...EYDSRERKIGWGSIYNTLRPTINKITHMKFENP.KNSDEYFKKYFKALEEEITVGLRNLM

VA_SCHPO   619 E....
VA_NEUCR   606 DE...
VA_BOVIN   618 D....
VA_PIG     618 D....
VA_HUMAN   617 D*...
VA_MUSMU   617 D....
VA_CHIC2   617 D....
VA_CHIC1   611 D....
VA_DROM1   614 D....
VA_DROM1'  614 D....
VA_DROM2   614 D....
HO_HUMAN   615 D....
VA_MANSE   617 D....
VA_HORVU   577 DEAR.
VA_MAIZE   558 DEAR.
VA_BRANA   620 DETR.
VA_CARRO   620 DETR*
VA_VIGRA   620 DETR.
VA_GOSHI   620 DETR*
VA_BETVU   620 DETR.
VA_ACEAC   606 DEYR*
VA_CYACA       .....
VA_ENTHI   607 E....
VA_TRYCO   610 Q*...
VA_PLAFA   610 EK...
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Figure 4.5A. Alignment of known V-ATPase A subunits (VA) aa sequences. All sequences are deduced from cDNAs. The source tissues, accession number and references for each sequences are list below:

VA_SCHPO: fission yeast, *Schizosaccharomyces pombe*, X68580 (Ghislain *et al.*, 1992);

VA_NEUCR: *Neurospora crassa*, J03955 (Bowman *et al.*, 1988);

VA_BOVIN: *Bos primigenius taurus*, X58386 (Pan *et al.*, 1991);

VA_PIG: *Sus scrofa*, X62338 (Sander et al., 1992);

VA_HUMAN: *Homo sapiens*, isoform VA68, L09235 (van Hille *et al.*, 1993b);

VA_MUSMU: *Mus musculus*, U13837 (Laitala *et al.*, 1986);

VA_CHIC1: Chicken, *Gallus gallus*, A1 isoform, U22077 (Hernando, 1995);

VA_CHIC2: Chicken, *Gallus gallus*, A2 isoform, U22076 (Hernando, 1995);

VA_DROM1: *Drosophila melanogaster*, isoform *vha68-1*, U19745 (Guo *et al.*, 1996d);

VA_DROM2: *Drosophila melanogaster*, isoform *vha68-2*, U59146 (Guo *et al.*, 1996d);

VA_DROM1': *Drosophila melanogaster*, isoform *vha68-2*, U19742 (Chio *et al.*, 1995);

HO_HUMAN: *homo sapiens*, isoform HO68, L09234 (van Hille *et al.*, 1993b);

VA_MANSE: *Manduca sexta*, X64233 (Crä f *et al.*, 1992);

VA_BRANA: *Brassica napus*, U15604 (Orr *et al.*, 1995);

VA_CARRO: carrot, *Daucus carota*, J03769 (Zimniak *et al.*, 1988);

VA_VIGRA: *Vigna radiata*, U26709 (Chiu *et al.*, 1995);

VA_GOSHI: *Gossypium hirsutum*, L03186 (Vilkins, 1993);

VA_HORVU: Barley, Hordeum vulgare, U36939;

VA_MAIZE: *Zea mays*, U36436; VA_ACEAC: *Acetabularia acetabulum* D50528;

VA_ACEAC: *Acetabularia acetabulum* D50528;

VA_CYACA: *Cyanidium caldarium*, U17100 (Ziegler *et al.*, 1995);

VA_ENTHI: *Entamoeba histolytica*, U04849 (Yi *et al.*, 1994).

VA_PLAFA: Plasmodium falciparum, A48582 (Karcz et al., 1993);

VA_BETVU: *Beta vulgaris*, X98767;

VA_TRYCO: *Trypanosoma congolense*, Z25814.

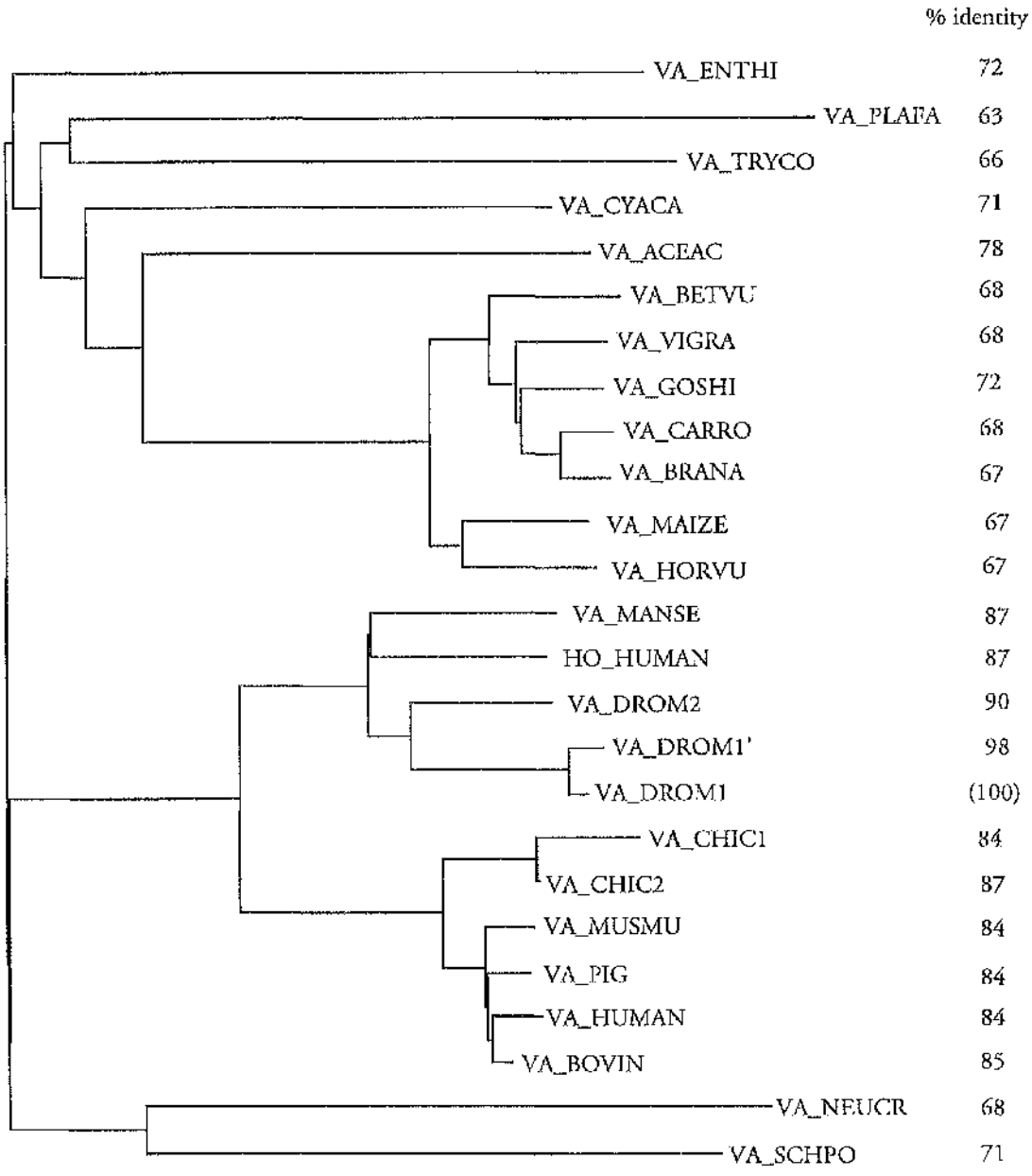

Figure 4.5B Phylogenetic tree of V-ATPase A subunits. This figure was generated by ClustalW and N-J plot from the multiple alignment in Figure 4.5A.. See the legend of Figure 4.5A for the sources of aa sequence.

4.3.4 Homology of *vha68* to subunit A of V-ATPases from other sources

The alignment in figure 4.5A showed both isoforms share high homology with V-ATPase A subunit of other organisms. There is greater than 60% identity at the aa level for all the compared sequence of the V-ATPase A-subunits. Figure 4.4B is the phylogenetic tree of the V-ATPase A-subunits generated by GCG, ClustalW and N-J plot.

4.3.5 Comparison of vha68 to β chain of F-ATPase

Alignment of the two isoforms of *Drosophila* V-ATPase A subunit with several β-chains of F-ATPases, including that of *Drosophila,* is shown in Figure 4.7 In general, the V-ATPase subunit shows significant homology to that of F-ATPases.

The homology is remarkably evident in the region that has already been identified in F0F1-ATPases as areas of probable importance for function or assembly (Zimniak, *et al.*, 1988; Taiz *et al.*, 1994). The most important of these is the proposed nucleotide binding site; GXXXXGKT and RXXXGXXXX***D. (* represents hydrophobic residents) are well conserved in both isoform (marked in bold in Figure 4.6). The homology between V-ATPase and F-ATPase of *Drosophila* proved again that the catalytic subunits from the two classes of ATPase share similar structure for the catalytic domain.

4.4 Genomic structure analysis of *vha68-2*

4.4.1 Restriction mapping of genomic DNA and subcloning

Four recombinant phage were isolated from an EMBL3 genomic DNA library by hybridisation with a *vha68-1* cDNA probe. DNAs prepared from each recombinant phage were cleaved first with *Sal*I and it was found that the four clones contain an

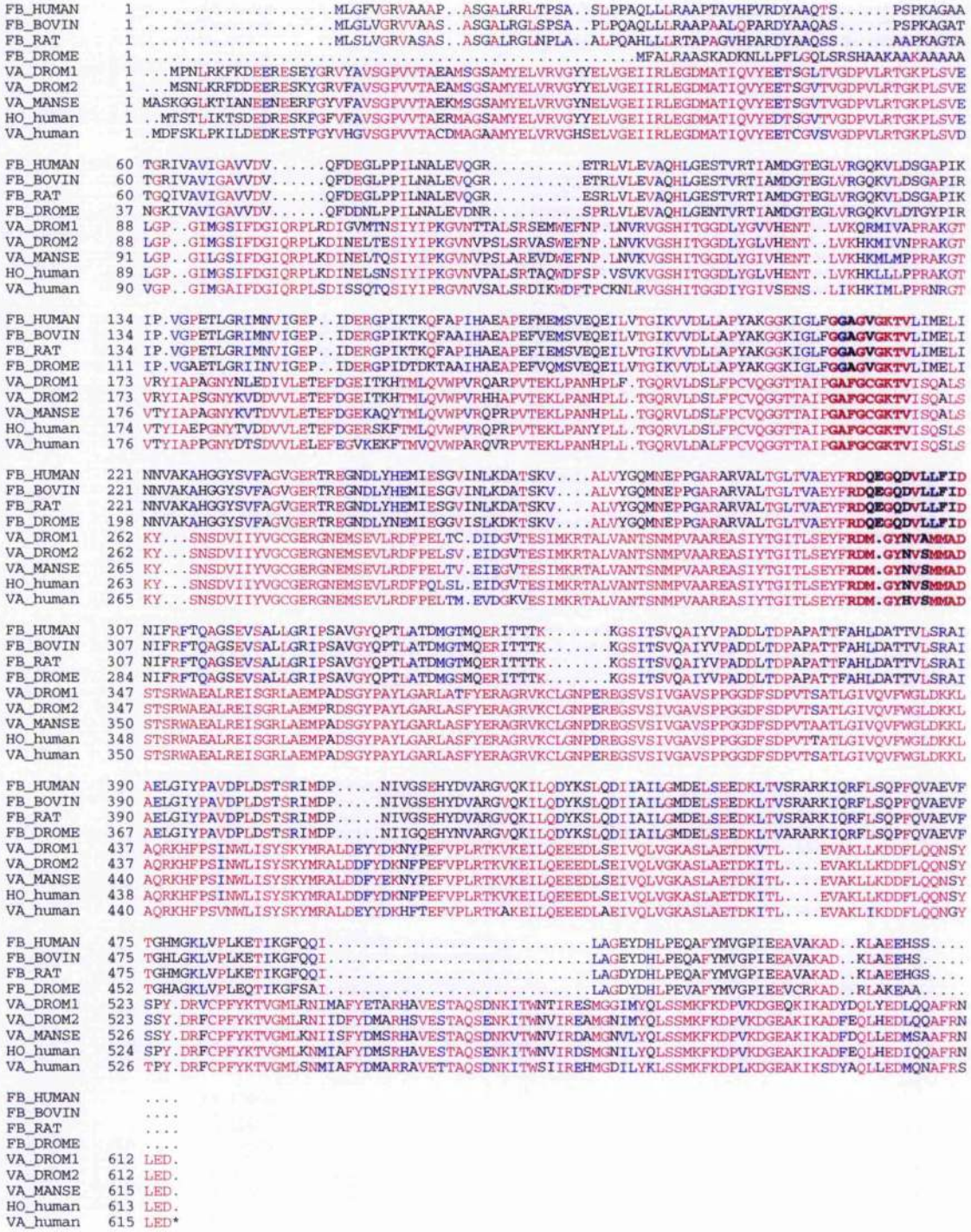

Figure 4.6 Alignment of the V-ATPase A subunit (VA) and F-ATPase β subunit (FB). All sequences are deduced from cDNA: The source tissues, accession number of FB and references are listed below: FB_HUMAN: *homo sapiens*, P06576; FB_BOVIN: *Bos primigenius taurus*, P00829; FB_RAT: P10719, FB_DROME: *Drosophila melanogaster*, Q05825. See the legend of Figure 4.5 for those of V-ATPase. The proposed nucleotide binding sites are marked in bold.

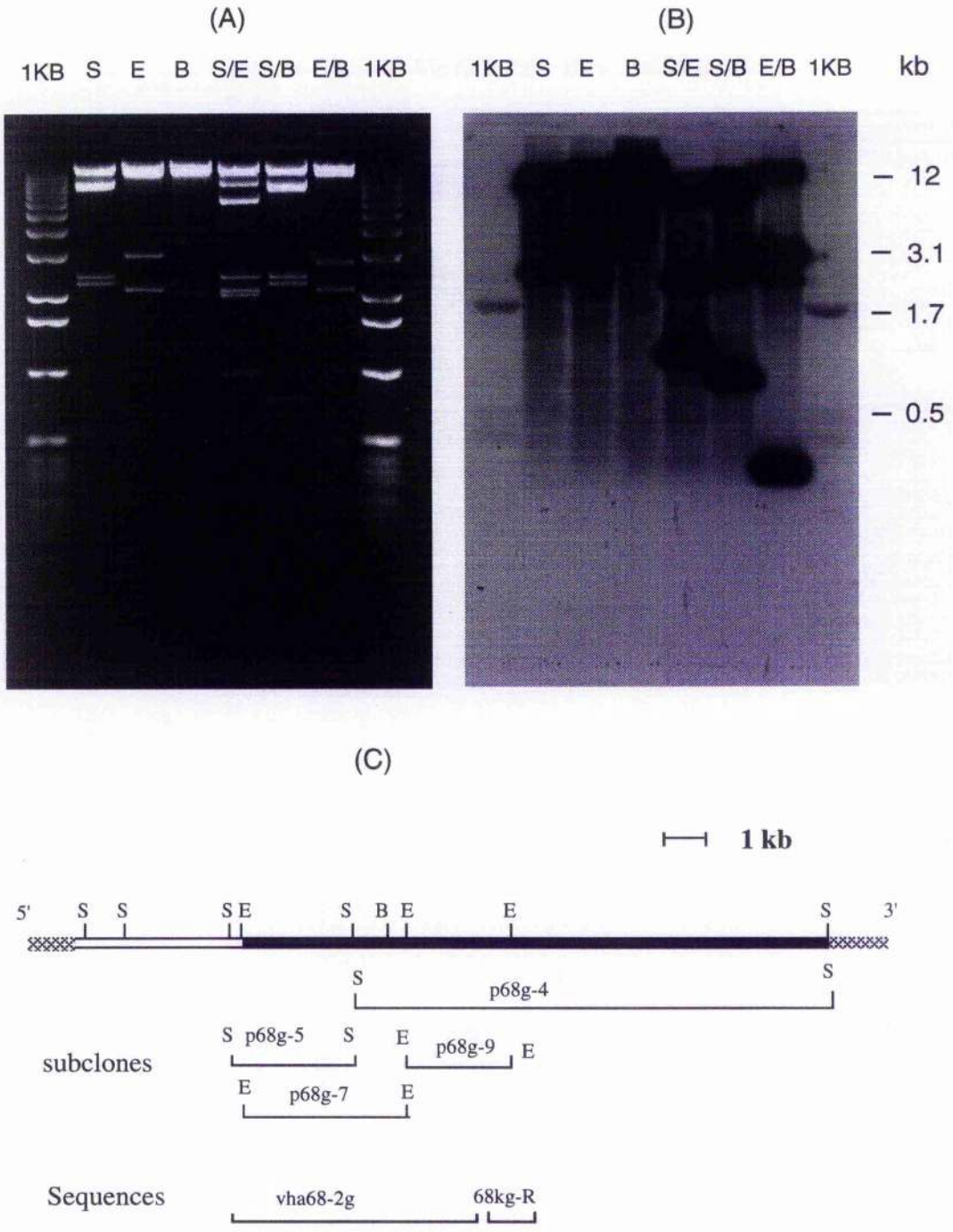

Figure 4.7 Restriction map of genomic ph68A . (A) photo of agarose gel in which the phage ph68A was cleaved by various enzymes. S, *Sal*1; E, *Eco*RI; B, *Bam*HI; S/E, *Sal* I/*Eco*RI; S/B, *Sal*1/*Bam*H1; E/B, *Eco*RI/*Bam*HI. (B) The blot of the gel A hybridised with *vha68-1* cDNA. (C) Digestion map of ph68A deduced from the (A) and (B). Fragments which hybridised to the probe were subcloned into pBluescript SK$^-$. Black indicated hybridising fragments

identical genomic fragment of 12 kb long. ph68A was then chosen for constructing the restriction map. The DNA was cleaved with *Sal*I, *Eco*RI, *Bam*HI and every possible double digestion of the three enzymes (Figure 4.6). Fragments that hybridised to *vha68-1* cDNA were subcloned into pBluescript SK$^-$. (See Figure 4.7).

4.4.2 Genomic DNA analysis

The four subclones of genomic DNA shown in Figure 4.7 were sequenced, first by T3 and T7, then with synthesised oligo nucleotide primers. 4405 bp of genomic DNA has been sequenced, comprising 68kg-5, 68kg-7 and part of 68kg-9 sequences (Figure 4.8). Although ph68A was identified by a *vha68-1* cDNA probe, the genomic sequence actually appears to be correspond to *vha68-2* (Figure 4.8). Moreover, the digestion map and the sequence of ph68A is corresponded to the genomic DNA in the rescued plasmid from fly line l(2)k02508, suggesting that the P[*lacW*] insertion in this line is in *vha68-2* , rather than in *vha68-1* (See Chapter 5).

4.4.3 A comparison of the *vha68-1* and *vha68-2* genes.

It is clear from this work and from Choi *et al* (1995) that there are two *vha68* genes encoding the *D. melanogaster* V-ATPase A subunit. *vha68-2* cDNA was punctured by 4 introns of 1165, 405, 108 and 66 bp at nucleotides 66-67, 166-167, 864-865 and 1843-1844 of the cDNA (Figure 4.4, 4.8and 4.9). The first intron is at 23 bp upstream of the ATG translation start site. The other three introns are within the coding sequence. Unfortunately, among the 4 genomic fragments identified by a *vha68-1* probe, none of them corresponded to *vha68-1*. However, a partial genomic sequence corresponding to *vha68-1* has been reported (Choi *et al.*, 1995; GenBank accession number: U19742), which makes it possible to compare the genomic structure of the two different genes. Instead of having 4 introns *vha68-1* has 3 introns at nucleotides 31-32, 163-164 and 1840-1841 of the cDNA (Figure 4.4, 4.9). The first intron is at 59 bp upstream of the

```
1                                                   31
gtc gac gtt tta ttt ctg cgg ctc agt cgg ttt taG TTC GTT CTG TTG GAG AAA AGC AGC
61                                                  91
AAT CAC ACG TTC GCA AGG TGA ACG CGA AGA CAC AGC AAA gta agc cct tcc ccc cac caa
121                                                 151
cac aca cac cca ccc aaa gca aat aag taa aaa taa ata atg gaa tgg ctg gaa gac ggt
181                                                 211
tct ggg cga ttt aaa caa tta gcg aaa gaa agc ggc att gaa atc cgt ctt gaa ttc gcc
241                                                 271
ccg aaa aag tga cga agc agc gat caa agc gca gag caa aac acg cac aca gac tgc aag
301                                                 331
tgt gtt aca taa taa gtg cag cac aag tcc aca ctt gag taa aat aat ccc taa aaa agc
361                                                 391
cga ata tca att agt ttt cca agg agc ttg aaa aag tgc cgg tat gaa aac gtg aaa att
421                                                 451
tcc gcg tgg aaa att atc ttc cct tgt cag ctg acc ccc ttc ccc gtg ttc gct cca tcc
481                                                 511
ctg tcg cac cgc ggg tct tgt gat cgc cgc cgc tct tgc gct cgc ttg ctc tcc cat ttc
541                                                 571
gaa act cga aac aga agt ggg agt tat tcg tat tcc gat aat gaa aaa cca ata tgg aga
601                                                 631
acg agc gac gta aaa aag gcg gcc caa aga ctt tta cca ttt ccc tta cac act ttt ttt
661                                                 691
tca ttt gtc agc tga cgg caa tga cag tag tct tgt gat caa cgt caa aag caa ttg tca
721                                                 751
aat att cga act cga atg gag agc gag aga gcc aga gcg aga gtt gct ctc cca ctc cac
781                                                 811
cct ctc ttg ttt ttc ttt gct gat aat tat gaa aac ccg cat att ttg aaa aac atg cat
841                                                 871
ttc agt tac att cct ccg ttg aat ttg tca acc tgt ggt tgt ttt ttc aca gct ctt att
901                                                 931
tta ttt att tag cga tta gtt tga caa att gct ttc ttc gaa ctt tca aag ctc tgt cac
961                                                 991
gtg aaa cga aag ctc tgc ttt taa agt ttt acg cag cat aat caa aga agg gga gtt aaa
1021                                                1051
aaa aat aat taa atc aat cga aat tat tag ctg cta acc tac aac ttt ata acc tat aat
1081                                                1111
cga aaa ttt ggg agc tgt ggg ctg tac aaa aac tta acc tgt aaa tgt agc aga tac acc
1141                                                1171
tgc ccc ttg cca gct gac aga ggg ctg agc aag aaa tta gtg ata aga aaa tgt tca cct
1201                                                1231
tta tct tcg ccc ttt tgc agc cag cat tta aca att ttc ctc ttc tat ttt ccc tcc att
1261                                                1291/2
gca gTC GAA AAA ACA GAA TAA AGC AAA ATG TCC AAC CTT AAG CGT TTC GAT GAT GAG GAG
                                        M   S   N   L   K   R   F   D   D   E   E
1321/12                                             1351/22
CGT GAG TCC AAA TAT GGA CGT GTC TTC GCT GTC TCC GGT CCT Ggt aag cac cta act ata
 R   E   S   K   Y   G   R   V   F   A   V   S   G   P
1381                                                1411
ctg agt aac cat aac tca tgc tat cta aaa gtt aat aaa aat aaa tta ata ata cct gtg
1441                                                1471
aac tca aac cta gtc tag aac tta cac ttc tgt gtg aaa taa tgg caa ctt tag aaa tgt
1501/501                                            1531/511
gtc cac cta ttt gtg att aat att caa aca act caa aca ttg gtt tca tta ttc aaa att
1561/521                                            1591/531
aaa tgt gaa taa ttt taa taa tta att aat tgt ttc ttt aaa ctt ttt tct ata att cta
1621/541                                            1651/551
aca aaa aca tca tca agt atc ata aat aat aaa aaa ttt taa aag aaa atg ttc aag gcc
1681/561                                            1711/571
gaa atg gaa cct atc ttg gtt ggc aaa gtt ata aaa act tct tga atg aaa tgt atc ccc
1741/581                                            1771/27
cct aac cca acc aac cgt ttc att cca gTC GTC ACC GCC GAG GCC ATG TCT GGA TCA GCT
                                        V   V   T   A   E   A   M   S   G   S   A
1801/37                                             1831/47
ATG TAC GAG TTG GTC CGC GTC GGC TAC TAC GAG CTG GTG GGC GAG ATC ATC CGT CTG GAG
 M   Y   E   L   V   R   V   G   Y   Y   E   L   V   G   E   I   I   R   L   E
1861/57                                             1891/67
GGT GAC ATG GCC ACC ATC CAG GTG TAC GAG GAG ACC TCT GGC GTA ACT GTC GGA GAT CCG
 G   D   M   A   T   I   Q   V   Y   E   E   T   S   G   V   T   V   G   D   P
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1921/77                                                    1951/87
GTG CTG CGT ACC GGC AAG CCT CTT TCC GTG GAG CTG GGA CCC GGT ATC ATG GGC AGC ATC
 V   L   R   T   G   K   P   L   S   V   E   L   G   P   G   I   M   G   S   I
1981/97                                                    2011/107
TTT GAC GGT ATC CAG CGT CCC CTG AAG GAC ATT AAC GAG CTG ACC GAA TCC ATC TAC ATT
 F   D   G   I   Q   R   P   L   K   D   I   N   E   L   T   E   S   I   Y   I
2041/117                                                   2071/127
CCC AAG GGT GTG AAC GTG CCC AGT TTG TCC CGC GTG GCC AGC TGG GAG TTC AAC CCC CTG
 P   K   G   V   N   V   P   S   L   S   R   V   A   S   W   E   F   N   P   L
2101/137                                                   2131/147
AAC GTC AAG GTC GGC TCC CAC ATC ACC GGA GGT GAC CTG TAC GGT CTG GTG CAT GAG AAC
 N   V   K   V   G   S   H   I   T   G   G   D   L   Y   G   L   V   H   E   N
2161/157                                                   2191/167
ACT CTG GTC AAG CAC AAG ATG ATT GTG AAC CCC CGC GCC AAG GGA ACA GTG CGC TAC ATC
 T   L   V   K   H   K   M   I   V   N   P   R   A   K   G   T   V   R   Y   I
2221/177                                                   2251/187
GCC CCC TCC GGC AAC TAC AAG GTC GAC GAT GTC GTC CTG GAG ACC GAG TTC GAT GGA GAG
 A   P   S   G   N   Y   K   V   D   D   V   V   L   E   T   E   F   D   G   E
2281/197                                                   2311/207
ATC ACC AAG CAC ACC ATG TTG CAG GTG TGG CCA GTG CGT CAC CAC GCT CCC GTG ACC GAG
 I   T   K   H   T   M   L   Q   V   W   P   V   R   H   H   A   P   V   T   E
2341/217                                                   2371/227
AAG CTG CCC GCC AAC CAC CCC CTG CTC ACC GGA CAG CGT GTG CTC GAC TCG CTC TTC CCC
 K   L   P   A   N   H   P   L   L   T   G   Q   R   V   L   D   S   L   F   P
2401/237                                                   2431/247
TGT GTC CAG CGC GGT ACC ACC GCC ATT CCC GGA GCT TTC GGT TGC GGC AAG ACT GTG ATC
 C   V   Q   G   G   T   T   A   I   P   G   A   F   G   C   G   K   T   V   I
2461/257                                                   2491
TCG CAG gtg aga gtc cca caa att gag aat tta agg agc gat gcc tcg tgt agc ctc cat
 S   Q
2521                                                       2551
aca ctc aag ttt cat aaa aac aca atc cct aat aaa tca ttt act tgc ttg cag GCT CTG
                                                                             A   L
2581/261                                                   2611/271
TCC AAG TAC TCC AAC TCC GAT GTC ATC ATC TAC GTC GGT TGC GGT GAG CGT GGT AAC GAG
 S   K   Y   S   N   S   D   V   I   I   Y   V   G   C   G   E   R   G   N   E
2641/281                                                   2671/291
ATG TCT GAG GTA CTG CGT GAC TTC CCC GAG CTG TCC GTG GAG ATC GAT GGT GTG ACC GAG
 M   S   E   V   L   R   D   F   P   E   L   S   V   E   I   D   G   V   T   E
2701/301                                                   2731/311
TCC ATC ATG AAG CGT ACC GCC CTT GTG GCC AAC ACC TCC AAC ATG CCT GTG GCT GCT CGA
 S   I   M   K   R   T   A   L   V   A   N   T   S   N   M   P   V   A   A   R
2761/321                                                   2791/331
GAG GCC TCC ATC TAC ACT GGT ATC ACC TTG TCC GAA TAC TTC CGT GAT ATG GGT TAC AAC
 E   A   S   I   Y   T   G   I   T   L   S   E   Y   F   R   D   M   G   Y   N
2821/341                                                   2851/351
GTG TCC ATG ATG GCT GAT TCC ACC TCC CGT TGG GCT GAG GCT CTT CGT GAA ATT TCT GGT
 V   S   M   M   A   D   S   T   S   R   W   A   E   A   L   R   E   I   S   G
2881/361                                                   2911/371
CGT CTC GCT GAG ATG CCT CGC GAT TCC GGC TAC CCA GCC TAC TTG GGA GCT CGT CTG GCC
 R   L   A   E   M   P   R   D   S   G   Y   P   A   Y   L   G   A   R   L   A
2941/381                                                   2971/391
TCC TTC TAC GAG CGT GCC GGT CGC GTT AAG TGC TTG GGT AAC CCC GAG CGC GAG GGA TCC
 S   F   Y   E   R   A   G   R   V   K   C   L   G   N   P   E   R   E   G   S
3001/401                                                   3031/411
GTG TCC ATT GTC GGA GCT GTG TCT CCT CCT GGT GGT GAC TTC TCC GAT CCC GTA ACC TCC
 V   S   I   V   G   A   V   S   P   P   G   G   D   F   S   D   P   V   T   S
3061/421                                                   3091/431
GCC ACT CTG GGT ATC GTG CAG GTG TTC TGG GGT CTC GAC AAG AAG TTG GCC CAG CGC AAG
 A   T   L   G   I   V   Q   V   F   W   G   L   D   K   K   L   A   Q   R   K
3121/441                                                   3151/451
CAT TTC CCC TCG ATC AAC TGG CTC ATC TCC TAC TCG AAG TAC ATG CGT GCT CTG GAT GAC
 H   F   P   S   I   N   W   L   I   S   Y   S   K   Y   M   R   A   L   D   D
3181/461                                                   3211/471
TTC TAT GAC AAG AAC TTC CCG GAA TTC GTG CCG CTG CGT ACC AAG GTC AAG GAG ATC CTG
 F   Y   D   K   N   F   P   E   F   V   P   L   R   T   K   V   K   E   I   L
3241/481                                                   3271/491
CAG GAG GAG GAG GAT CTG TCT GAG ATC GTG CAA CTG GTC GGC AAG GCC TCT CTC GCC GAA
 Q   E   E   E   D   L   S   E   I   V   Q   L   V   G   K   A   S   L   A   E
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3301/501                                                3331/511
ACC GAC AAG ATC ACG CTG GAG GTG GCC AAG CTG CTG AAG GAC GAT TTC CTG CAG CAG AAC
 T   D   K   I   T   L   E   V   A   K   L   L   K   D   D   F   L   Q   Q   N
3361/521                                                3391/531
TCC TAC TCC TCG TAC GAT CGC TTC TGC CCC TTC TAC AAG ACC GTG GGC ATG TTG AGG AAC
 S   Y   S   S   Y   D   R   F   C   P   F   Y   K   T   V   G   M   L   R   N
3421/541                                                3451/551
ATC ATC GAC TTC TAC GAC ATG GCC CGT CAC TCC GTG GAG TCT ACG GCT CAG TCT GAG AAC
 I   I   D   F   Y   D   M   A   R   H   S   V   E   S   T   A   Q   S   E   N
3481/561                                                3511/571
AAG ATC ACC TGG AAC GTG ATT CGT GAG GCA ATG GGC AAC ATT ATG TAC CAG CTG TCA TCC
 K   I   T   W   N   V   I   R   E   A   M   G   N   I   M   Y   Q   L   S   S
3541/581                                                3571/591
ATG AAG TTC AAG gtg ggt taa cac gca aac tta gcc att gcc tag aca cgg gtg acc aca
 M   K   F   K
3601                                                    3631/589
ttt ttc aat cca ttt cag GAC CCC GTT AAG GAT GGT GAG GCC AAG ATC AAG GCT GAC TTC
                        D   P   V   K   D   G   E   A   K   I   K   A   D   F
3661/599                                                3691/609
GAG CAG CTG CAC GAG GAC CTG CAG CAG GCC TTC AGA AAT CTG GAG GAC TAG aga ccg acg
 E   Q   L   H   E   D   L   Q   Q   A   F   R   N   L   E   D   *
3721                                                    3751
ACT GGC CCT ACT TTT ACA CTC TAA TCT TAT ATT TGT TAT ATA GTT AAC GTT TAA AAA TGA
3781                                                    3811
AAG CAG TCA AAA ACC ATC CGA AAA AGC CTA ATC AAA CAC CAA CAA TTC CAG CTG CAT TCG
3841                                                    3871
ATG AAA AAC AAA AGT CCA ACA AAT ACC ATA ACT TCT TGG TGC CTG CGA GAG ATG TAA ACA
3901                                                    3931
TTC CGG CCT GCG GTT AAT ACT TTC CCC TAA CCA CGC CCC CTC CGC CCC TTG AAG GGC AAC
3961                                                    3991
TCT AGG CAA CAG CAA CTA CAA CGT CCT GCT ATG TAC TTC CAT TTA CAA CAA CAA CAC CAA
4021                                                    4051
CAT ACA CTT CAA TAA AAG TAC ACG GAC ACT GGC GCA CAC ACA ACA CAT ACA TAA AAG ACA
4081                                                    4111
CAA ATA CAA ATG CAT GCA TAA ATA GTA TTA TTG TTT AAT GAA TGG AAA TTC TTG TTT ATT
4141                                                    4171
TGT GAA AAA AGT CAT GTT TTC TCC CTG TTT GTT TGT TAA ATT TAT GTA AAT ATT TAA AGT
4201                                                    4231
ATG AAA TAT TAA ATG TAC GAA TAA AGT GCA ACA ACA AAT ACA TTT AAT GTa att gaa agt
4261                                                    4291
gaa ttt cac tgg cag cag aat gga tat taa aaa tgt gtc aac tcg ata aaa aga taa taa
4321                                                    4351
gtt aaa ata ttt ttt tga att ttg aaa cct tca tta tat aaa cat act tga cta tat gaa
4381
agc taa gaa aat ggg aat ata ttg t
```

Figure 4.8 Genomic DNA and putative aa sequence of *vha68-2*. (GenBank accession no.: U59147). cDNA sequence is shown in upper case.

ATG translation start site. The other two introns are within the coding sequence at exactly the same sites as the two introns of the *vha68-2* gene.

4.4.4 Evidence for additional complexity at the *vha68-2* locus

A genomic DNA fragment just 3'to *vha68-2* gene also shows hybridisation to the *vha68* probe (Figure 4.7). 68kg-R, a partial sequence around the *Eco*RI site in subclone p68g-4 has been obtained (Figure 4.10). The DNA sequence is 61% identical with the genomic sequence of *vha68-2* (Figure 4.11), which contains a long open reading frame with a translated polypeptide 73% identical to *vha68-2* (Figure 4.12). Thus this may be a gene encoding another isoform of V-ATPase A subunit. However, it is also possible that this fragment offers an alternative splicing as it is very close to *vha68-2*; or it is a pseudogene without transcription? Hopefully, information of longer sequence of 68kg-R and the sequence of p68c-4 cDNA clone (See section 4.2) would help to answer this question.

4.5 Southern blot analysis of genomic DNA with *vha68-1* and *vha68-2* cDNA probes

D. melanogaster (CS) genomic DNA was cleaved with a range of restriction endonucleases. Southern blots were probed with the coding region of *vha68-1* cDNA . After hybridisation and washing at high stringency, more than one band was revealed at each of the lanes (Figure 4.13A). The band sizes were same as that predicted from the digestion map of *vha68-1* and *vha68-2* genomic DNA clones. However, probing with the 3' non-coding sequence of either *vha68-1* or *vha68-2*, which is gene-specific, reveals only one band in most of the lanes (Figure 4.13B, C), suggesting that the two cDNAs are the products of two different genes and each gene has only one copy.

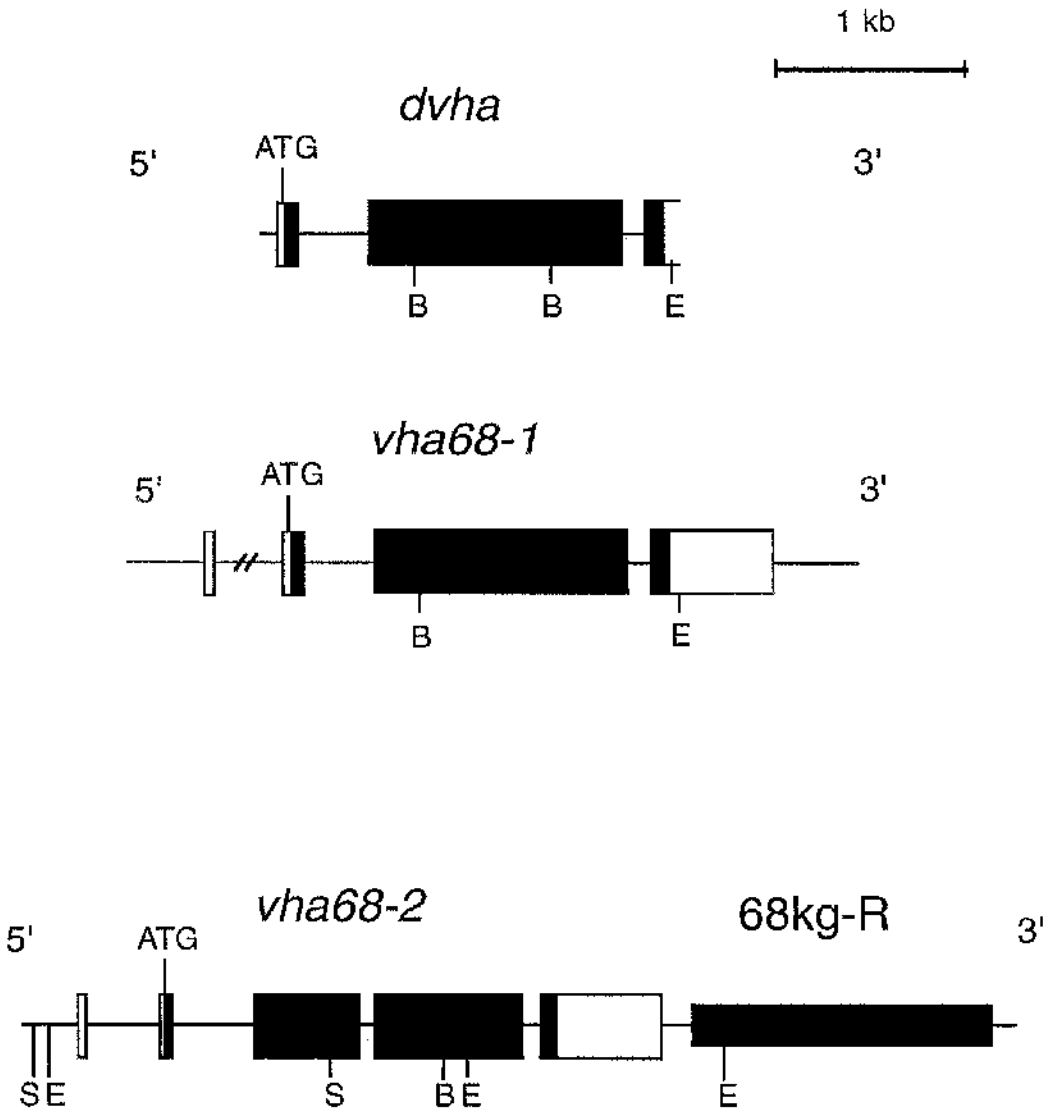

Figure 4.9 Structure of the genes encoding the two isoforms of the *D. melanogaster* V-ATPase A subunit. The exons are represented as rectangles of which coding regions are in dark. *dvha* is the partial genomic sequence for *vha68-1* from Chio et al (1995). As the genomic sequence is incomplete, the length of the first intron in *vha68-1* is uncertain, and whether the 3' UTR has an intron or not awaits confirmation. Here we assume there is no intron in the 3' UTR of *vha68-1*. As 68kg-R has not been completely sequenced, here it is presented as a small filled rectangle. E: *EcoR* I; B, *BamH*I; S, *Sal*I.

```
1                                                   31
CGG TAC CCC CTC CCC CTC AAT CAC GCA AGC CTG TAG CCG ACC CGG AAA GCC CCC ATG ATA
61                                                  91
CAG TCA ATG ACG AGG ACA GTT TGA AGG ACT TGA GAC GTT CGA CGG ACC AAT CCC ACA AGA
121                                                 151
GCG CTC ACA TCG CCT TGG AGA AGA ATG AGG ACT CGG GTT TTG TGA TCG AGC AGG TGG TTG
181                                                 211
ATA CGC ACA AAT ATT CGT CGG ATG AAG AAG AGG AGG AGG CGA CGA TGG GTC GCA TTT TCG
241                                                 271
GAT GTC CCC GGC CCG GTG GTC AAT GCC GAG GAG ATG GCC GGC GCA GCC ATG TAC GAG CTG
301                                                 331
GTT CGC GTT GGA CAC TCC CAG CTT GTT GGT GAG ATC ATT CGA CTG GAG GGT GAT ATG GCC
361                                                 391
ACC ATT CAG GTT TAC GAG GAT ACT TCG GGT GTG AGC GTG GGT GAT CCC GTC TAC CAG ACG
421                                                 451
GGA AAG CCA CTC TCC GTT GAA TTG GGA CCC GGC ATC ATG GGC AGC ATC TTT GAT GGT ATC
481                                                 511
CAG CGA CCA TTG AGG TCC ATC AGT GAA CTA ACC AAC TCC ATA TAC GTG CCC AAG CGC ATC
541                                                 571
GAT ACG CCC TCC CTG CCC AGG AAC ATT GCG TAC GAA TTC ACA CCC GGA AAA TTG AAG ATC
601                                                 631
GAT GCT CTG ATC ACC GGC GGA GAC ATC TAC GGA TCT GTT TTC GAA AAC AGC ATG ATG CAC
661                                                 691
GAT CAC CGC CTG ATA CTA CCG CCC CGC ACC AAG GGG CGC ATC CGG TGG TTG GCA CCG CCC
721                                                 751
GGG AAC TAC TGC GTG GAC GAG GTG ATC GTG GAG ACG GAG TTC AAC GAC GAG ATC ACC AAG
781                                                 811
CAC ACC ATG CTC CAG GTG TGG CCC GTA CGG AGG TGT CGT CGG TGG AGG ATA AGC TCC CCC
841                                                 871
AGC AAT TCA CCA CTC TTG ACT GGC CAG CGC GTC CTG GAC CGA TTC TTT CCA TGT GTC CAG
901                                                 931
GGC GGA ACC ACT GCC ATT CCA GGA GCG TTT GGA TGT GGA AAG ACC GTC ATC TCG CAG GTG
961                                                 991
AGA CGG TTT CTA AGA GTT TAG TTG ACA AAT GAT TAC ATT CCA ATC AAC TTA TAC CCC TAG
1021                                                1051
GCC CTG TCC AAA TAC TCC AAC TCA GAT GTC ATC ATC TAC GTG GGC TGC GGT GAG CTC GGG
1081
AAC GAA ATG TCC GAG GTT CTT ATG GAC TTT CC
```

Figure 4.10 Partial sequence of 68kg-R. The *EcoR*I site is marked in bold.

```
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     Length Weight:  0.300   Average Mismatch:  0.000

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             Ratio:  0.564               Gaps:      6
Percent Similarity: 60.956   Percent Identity: 60.956

vha68-2.g.seq x 68kg-R. seq

vha68-2   1451 tagtctagaacttacacttctgtgtgaaataatggcaactttagaaatgt 1500
                                                              ||
68kg-R       1 ..........................................cggt 4

vha68-2   1501 gtccacctatttgtgattaatattcaaacaactcaaacattggtttcatt 1550
                 || |         || |           | | | |        |
68kg-R       5 acccccteccccteaatcacgcaagcctgtagccgagccggaaagcccce 54

vha68-2   1551 attcaaaattaaatgtgaataatttttaataattaattaattgtttctttta 1600
                 ||   | | | ||||   | |   |  ||  | || |    |||
68kg-R      55 atgatacagtcaatgacgaggacagtttgaaggacttgagacgttcgacg 104

vha68-2   1601 aacttttttctataattctaacaaaaacatcatcaagtatcataaataat 1650
                 ||    | | | ||       | | :  | |  || |  || |  | |
68kg-R     105 gac.caatcccacaagagcgctcacatcgccttggagaagaatgaggact 153

vha68-2   1651 aaaaaatttttaaaagaaaatgttcaaggccgaaatggaacctatcttggt 1700
                    || | |  ||  | ||    ||  || | | |       | |
68kg-R     154 cgggttttgtgatcgagcaggt....ggttgatacgcacaaatattcgtc 199

vha68-2   1701 tggcaaagttataaaaacttcttgaatgaaatgtatccccctaacccaa 1750
                 |  |||      |     |    |||    | ||   |      ||
68kg-R     200 ggatgaagaagaggaggaggcgacgatgggtcgcattttcggatgtcc.. 247

vha68-2   1751 ccaaccgtttcattccagTCGTCACCGCCGAGGCCATGTCTGGATCAGCT 1800
                   |  || || ||||  |||||||  ||| | ||  ||||
68kg-R     248 .........CCGGCCCGGTGGTCAATGCCGAGGAGATGGCCGGCGCAGCC 288

vha68-2   1801 ATGTACGACTTGGTCCGCGTCGGCTACTACGAGCTGGTGGGCGAGATCAT 1850
                 |||||||| |||| ||||| ||  ||| | |||| || || |||||||
68kg-R     289 ATGTACGAGCTGGTTCGCGTTGGACACTCCCAGCTTGTTGGTGAGATCAT 338

vha68-2   1851 CCGTCTGGAGGGTGACATGGCCACCATCCAGGTGTACGAGGAGACCTCTG 1900
                 || |||||||||||| |||||||||| |||||  |||||||  || || |
68kg-R     339 TCGACTGGAGGGTGATATGGCCACCATTCAGGTTTACGAGGATACTTCGG 388

vha68-1   1901 GCGTAACTGTCGGAGATCCGGTGCTGCGTACCGGCAAGCCTCTTTCCGTG 1950
                 | || |  || ||  |||| ||    |  || || |||||  || |||||
68kg-R     389 GTGTGAGCGTGGGTGATCCCGTCTACCAGACGGGAAAGCCACTCTCCGTT 438

vha68-2   1951 GAGCTGGGACCCGGTATCATGGGCAGCATCTTTGACGGTATCCAGCGTCC 2000
                 ||  ||||||||||| |||||||||||||||||||| ||||||||||| ||
68kg-R     439 GAATTGGGACCCGGCATCATGGGCAGCATCTTTGATGGTATCCAGCGACC 488

vha68-2   2001 CCTGAAGGACATTAACGAGCTGACCGAATCCATCTACATTCCCAAGGGTG 2050
                 ||| |  ||| |  || || ||| |  ||| ||| |  |||||||||
68kg-R     489 ATTGAGGTCCATCAGTGAACTAACCAACTCCATATACGTGCCCAAGGGCA 538

vha68-2   2051 TGAACGTGCCCAGTTTGTCCCGCGTGGCCAGCTGGGAGTTCAACCCCCTG 2100
                 |  |  ||||   || || |          |  || ||||  |||
68kg-R     539 TCGATACGCCCTCCCTGCCCAGGAACATTGCGTACGAATTCACACCCGGA 588

vha68-2   2101 AACGTCAAGGTCGGCTCCCACATCACCGGAGGTGACCTGTACGGTCTGGT 2150
                 ||  | ||| |||   | |  |||||||| || ||| | |||||   ||
vha68-2    589 AAATTGAAGATCGATGCTCTGATCACCGGCGGAGACATCTACGGATCTGT 638
```

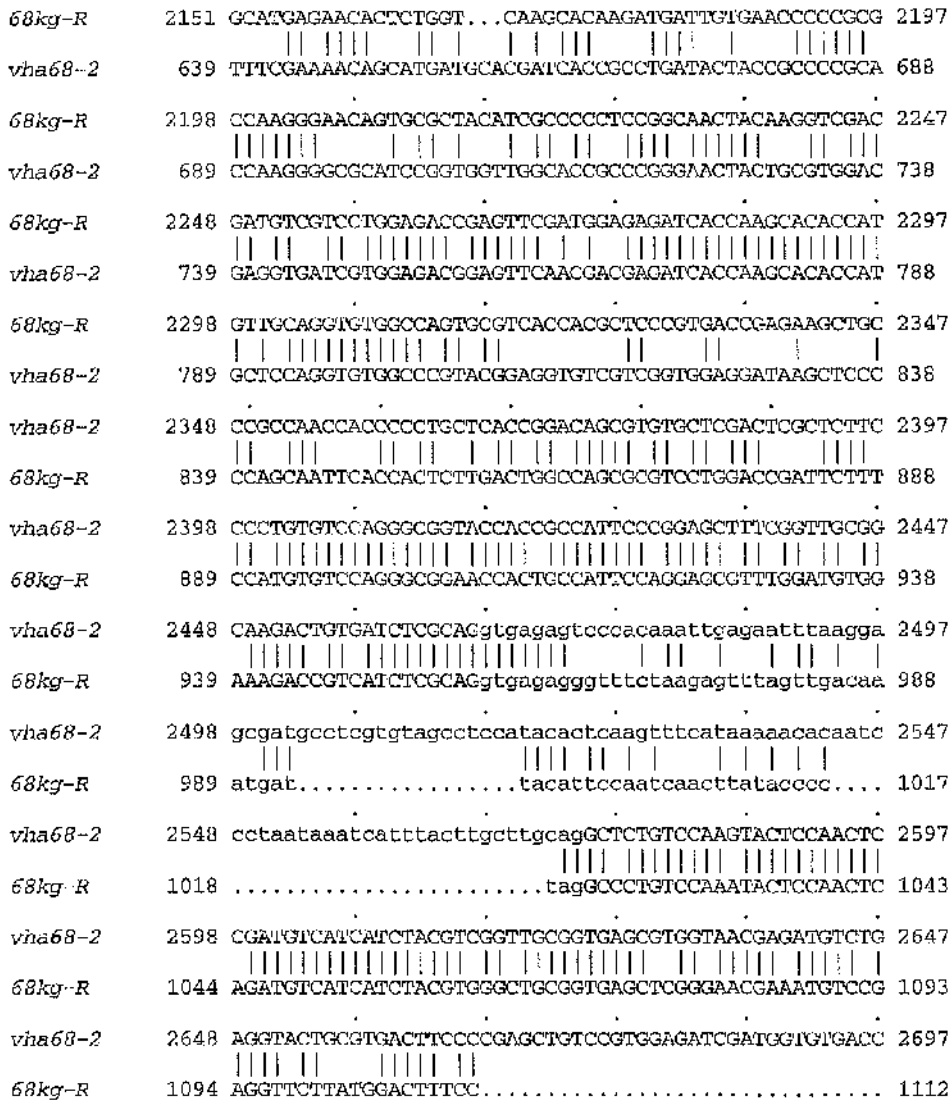

Figure 4.11 Homology between *vha68-2* genomic DNA and partial 68kg-R sequence.

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     Length Weight:  0.100   Average Mismatch: -0.396

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             Ratio:  1.186               Gaps:       1
Percent Similarity: 83.650   Percent Identity: 73.384

vha68-2 x 68kg-R  ..
```

```
vha68-2       1 MSNLKRFDDEEERESKYGRVFAVSGPVVTAEAMSGSAMYELVRVGYYELVG 50
                                          ||.||.|.|.|||||||||. :|||
68kg-R        1 .......................VVNAEEMAGAAMYELVRVGHSQLVG 25

vha68-2      51 EIIRLEGDMATIQVYEETSGVTVGDPVLRTGKPLSVELGPGIMGSIFDGI 100
                |||||||||||||||||:||||.|||||..|||||||||||||||||||||
68kg-R       26 EIIRLEGDMATIQVYEDTSGVSVGDPVYQTGKPLSVELGPGIMGSIFDGI 75

vha68-2     101 QRPLKDINELTESIYIPKGVNVPSLSRVASWEFNPLNVKVGSHITGGDLY 150
                |||||:.|.|||:|||:|||::.|||.|  .:||.| .:|::. |||||:|
68kg-R       76 QRPLRSISELTNSIYVPKGIDTPSLPRNIAYEFTPGKLKIDALITGGDIY 125

vha68-2     151 GLVHENTLV.KHKKIVNPRAKGTVRYIAPSGNYKVDDVVLETEFDGEITK 199
                | | ||.:: .|::|: ||.|| :|::||.||| ||:|::||||::||||
68kg-R      126 GSVFENSMMHDHRLILPPRTKGRIRWLAPPGNYCVDEVIVETEFNDEITK 175

vha68-2     200 HTMLQVWPVRHHAPVTEKLPANHPLLTGQRVLDSLFPCVQGGTTAIPGAF 249
                |||||||||:  .   . |.| ||||||||||.:|||||||||||||||
68kg-R      176 ITMLQVWPVRRCRRWRISSPSNSPLLTGQRVLDRFFPCVQGGTTAIPGAF 225

vha68-2     250 GCGKTVISQALSKYSNSDVIIYVGCGERGNEMSEVLRDFPELSVEIDGVT 299
                |||||||||||||||||||||||||| ||||||||.||
68kg-R      226 GCGKTVISQALSKYSNSDVIIYVGCGELGNEMSEVLMDF.......... 264
```

Figure 4.12 Homology between the translated proteins of *vha68-2* and 68kg-R partial sequence.

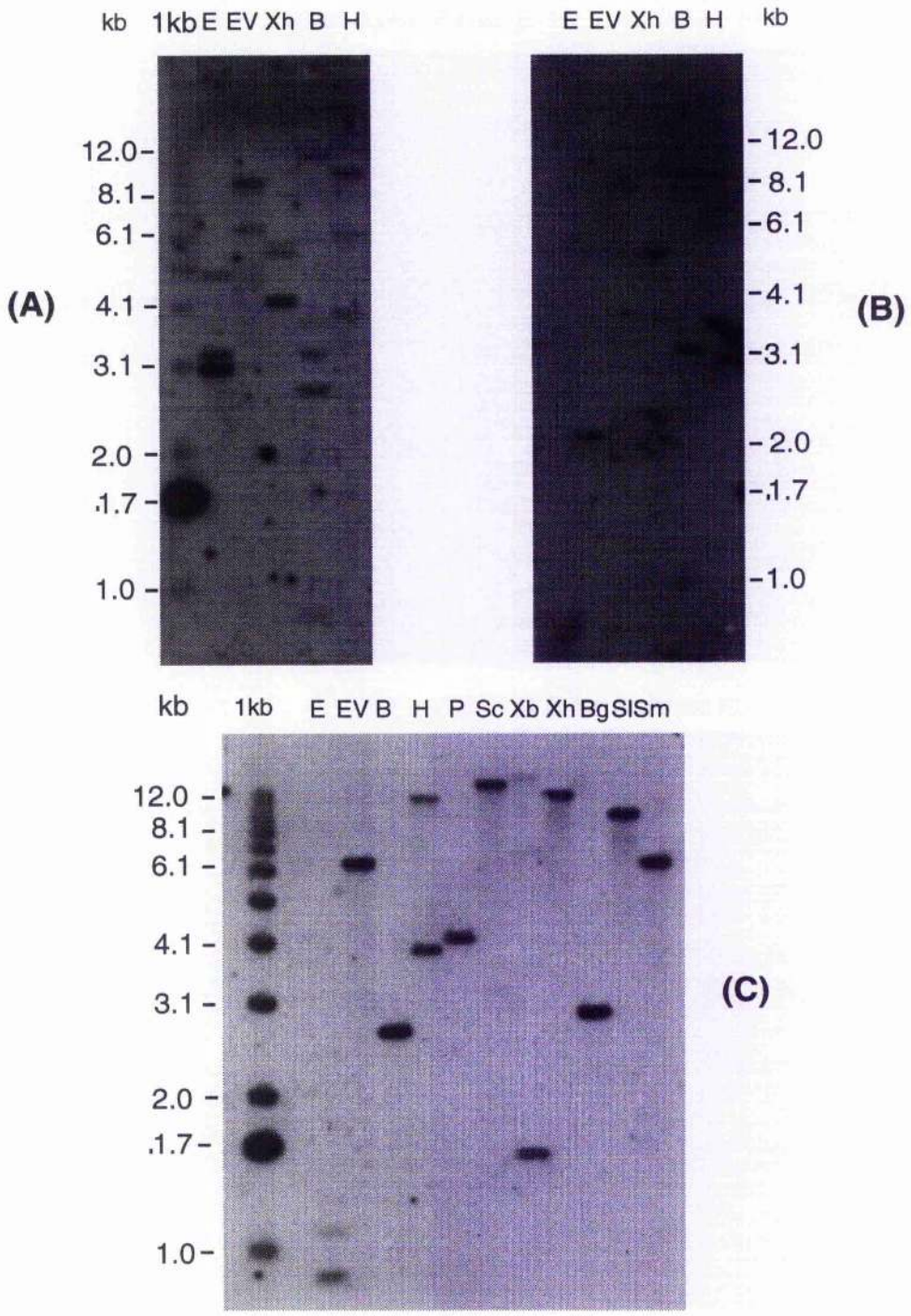


Figure 4.13 Southern blots of genomic *D. melanogaster* DNA. (**A**) Probed with *vha68-1* coding sequence; (**B**) probed with *vha68-2* 3' isoform-specific sequence; (**C**) Probed with vha68-1 3' isoform-specific sequences. E, *Eco*RI; EV, *Eco*RV; Xh, *Xho*I; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; Sc, *Sac*I; Xb, *Xba*I; Bg, Bg*l*II; Sl, *Sal*I; Sm, *Sma*I.

4.6 Chromosomal location

Salivary gland chromosome squashes probed with *vha68-1* cDNA revealed only one site of hybridisation band at polytene chromosome 34A (Figure 4.14). As both *vha68-1* and *vha68-2* share significant homology and cross hybridise in Southern blots, the *vha68-1* cDNA probe should also hybridise to *vha68-2*. Thus, *vha68-2* may also be at 34A. This has been further supported by the localisation at 34A of the P-element in fly line l(2)k02508 (Refer to Encyclopaedia of *Drosophila*). In next chapter we will show that this P-element is in the first intron of *vha68-2*.

4.7 Northern blot analysis of *vha68-1* and *vha68-2*

Northern blots of total RNA, using the whole *vha68 -1* cDNA as a probe, detected only a single band equivalent to mRNA(s) of ≈2.6 kb. The single band probably corresponds to both *vha68-1* and *vha68-2* transcripts. A developmental Northern of embryo, larval, pupal and adult total RNAs showed that the genes are almost equally expressed at embryo, larval and adult stages, but at much reduced level at the pupal stage (Figure 4.15). Tissue-based Northern analysis of adult head, thorax and abdomen total RNAs showed the genes to be almost equally expressed (Figure 4.16) as would be expected for a putative housekeeping gene. The same blots, probed with *vha68-1* or *vha68-2* specific 3' prime non-coding fragments, found that both genes to be similarly expressed (Figure 4.15 & 4.16).

4.8 Discussion

The V-ATPase A subunit has been previously reported to be encoded by a single gene in all the animals and microorganisms studied. Although multiple genes have been found in plants only a single type mRNA has been reported. Therefore, it has been originally concluded that there is just a single isoform of the A subunit (Bowman *et al.*, 1988; Hirata *et al.*, 1990; Puopolo *et al.*, 1991; Zimniak et al, 1988; Gräf *et al.*, 1992).

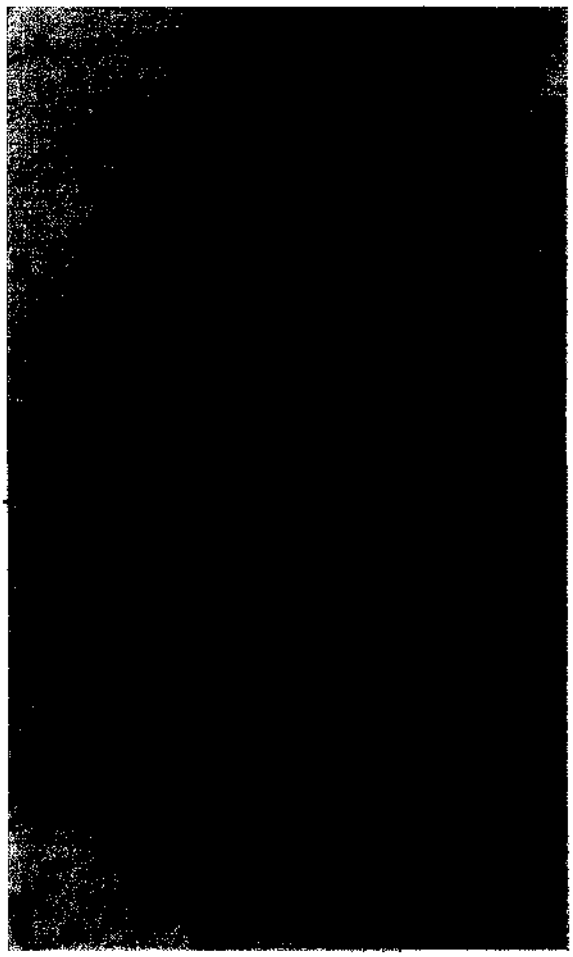


Figure 4.14 Chromosomal localisation of *vha68*. Salivary gland chromosome squashes were prepared by standard techniques (Ashburner, 1989). Chromosomes were probed with biotinylated, random-primed *vha68-1* cDNA and hybridisation was detected using streptavidin-conjugated peroxidase and diaminobenzidine.

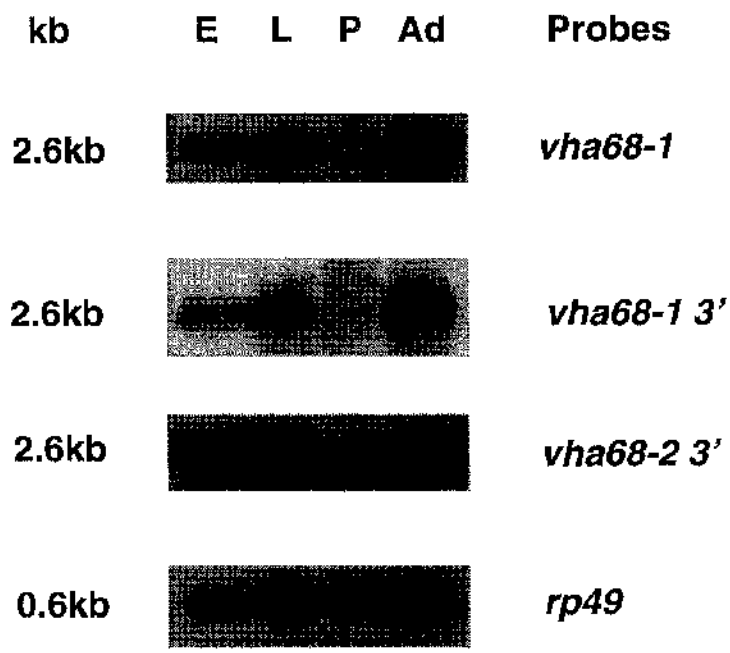


Figure 4.15 Developmental Northern blot analysis of the *vha68* genes. Total RNA was isolated from Canton S embryos, larvae, pupae and adults. The RNA was separated by electrophoresis in a 1% formaldehyde-agarose/MOPS gel, blotted to nitrocellulose and hybridised with ^{32}P-labelled random-primed probes. The filters was then exposed to Fuji X-ray film for 1-3 days. Sizes were determined with respect to an RNA ladder (Gibco BRL). E, Embryo; L, third instar larva; P, pupa; Ad, adult. The filter was first hybridised with whole *vha68-1* cDNA, then stripped and reprobed with isoform-specific cDNA fragments and *rp49* as a control for differences in RNA loading.

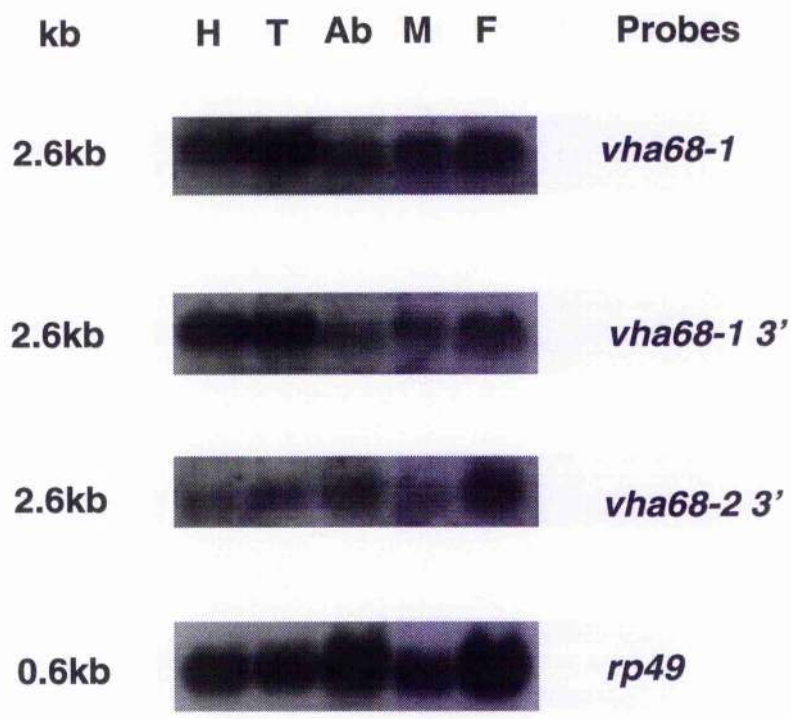


Figure 4.16 Tissue specific Northern blot analysis of the *vha68* genes. Total RNA of adult head, thoraces and abdomens, as well as male and female adults was isolated. The RNA was separated by electrophoresis in a 1% formaldehyde-agarose/MOPS gels, blotted to nitrocellulose, and hybridised with ^{32}P-labelled random-primed probes. The filters was then exposed to Fuji X-ray film for 1-3 days. Sizes were determined with respected to an RNA ladder (Gibco BRL). H, head; T, thorax, Ab, abdomen; M, males; F, females. The filter was first hybridised with whole *vha68-1* cDNA, then stripped and reprobed with isoform-specific cDNA fragments and *rp49* as a control for differences in RNA loading.

The existence of two isoforms of the A subunit was first reported in human (van Hille, 1993). The VA68 isoform is expressed in all tissues whereas the HO68 isoform was detected only in osteoclastoma, a tumour enriched in osteoclasts (Chambers *et al.*, 1985). In chicken, two isoforms of the A subunit are generated by differently splicing of two mutually exclusive exons from the same gene. Unlike the classical A1 isoform, the chicken A2 isoform does not contain either the ATP-binding consensus sequences (the p-loop) or the pharmacologically relevant Cys^{254} in its polypeptide. Both isoforms appear to be ubiquitously expressed (Hernando *et al.,* 1995). In this chapter two *D. melanogaster* A subunit genes, *vha68-1* and *vha68-2,* have been described. The two isoforms share 91% identity at the polypeptide level. A genomic DNA fragment correspond to *vha68-2* was identified and sequenced. A partial genomic DNA fragment for *vha68-1* was already available (Chio *et al,* 1995). Both genes are found to have a similar structure, the two introns are at the exact same sites but *vha68-2* has a small extra intron. Sequences of introns and of 3' and 5' prime non-coding fragments are different. However, since the coding sequence and corresponding polypeptides share high homology, the two genes presumably arise from a duplication of a single gene present in an ancestor. If the two isoforms have the same function the purpose of the two copies of the gene might be to compensate for an increased need for the protein product. The presence of two isoforms could also impart different properties or provide alternative sorting to cell compartments (such as vacuolar or plasma membrane). Although Northern blot of *D. melanogaster* total RNA suggests both genes are ubiquitously expressed, this does not necessary mean that both isoforms are present in the same cellular population or subcellular compartment. It is still possible one of the isoforms might be involved in plasma membrane V-ATPase while another may be implicated in endomembrane V-ATPase function. The reporter detector of P[*lacW*] insertion in *vha68-2* reveals this gene is highly expressed in Malpighan tubules, midgut etc. where the plasma membrane V-ATPase should have a role (See Chapter 5). However, the functional implications of the presence of two isoforms of the V-ATPase A subunit are still not clear.

Chapter 5

Mutational Analysis of *vha68-2*, a Gene Encoding One of the Two Isoforms of the *Drosophila* V-ATPase A-subunit

5.1 Summary

A *Drosophila* line (l(2)k02508) carrying a single P[*lacW*] insertion in *vha68-2*, a gene encoding one of the two isoforms of the *Drosophila* V-ATPase A subunit, was isolated by screening pools of rescued plasmids. Molecular characterisation demonstrates that the transposon is inserted within the first intron, and thus lies 5' to *vha68-2* translation start codon. Expression of the enhancer detector reporter gene carried by the *lacZ* (β-galactosidase) was widespread, but was particularly strong in the gut and Malpighian tubules of both larvae and adults. The insertion significantly reduces the accumulation of *vha68-2* mRNAs and causes homozygous lethality during the first larval instar. The lethal phenotype can be reverted by excision of the inserted P-element. Imprecise excision or internal deletion of the P-element created a set of novel hypomorphic or null alleles, with phenotypes ranging from first instar lethality to sub-lethals of various classes.

5.2 Introduction

Chapter 4 described the identification and characterisation of two genes, *vha68-1* and *vha68-2*, both of which encode V-ATPase A subunits. Both *vha68-1* and *vha68-2* are widely expressed. In order to address the *in vivo* functions of the two genes, it would be useful to partially or entirely inactivate them. For this purpose, *Drosophila* had the considerable advantages that it is genetically well characterised and amenable in several ways to mutational analysis. Once the chromosomal location of a gene has been specified, there is often a large amount of available information related to that chromosome location that can help with the analysis. For example, the P-element insertions in *vha26* (Chapter 6) and *vha55* (Davies *et al.*, 1996) were identified by screening available P-

element lines corresponding to the approximate locations of the genes. In the case of *vha68* gene, no such lines had been described. Fortunately, however, a collection of more than 2000 lines with recessive lethal P[*lacW*] insertions on the *Drosophila* second chromosome was available (Török, 1993) and plasmids representing the insertion sites of 1864 of these had been rescued (See Chapter 3). Southern blotting of the rescued plasmids and hybridisation with *vha68-1* cDNA identified 3 lanes containing related plasmids. One of these plasmids was traced to a single rescued plasmid (P184) corresponding to fly line l(2)k02508 (See Figure 3.3). A "mini-*white*" gene (Pirrotta, 1988) has been inserted in the middle of P[*lacW*]. As a genetic marker, mini-*white* provides advantages. First, flies heterozygous for mini-*white* in a genetic background null for the *white* locus generally have orange eyes, whereas flies homozygous for the same element have red eye pigmentation. Eye colour also tend to be darker in flies with multiple insertions (Kiss, 1996, Personal com.). Second, once P-element has been detected in a region of interest, it can be remobilised in the presence of transposase, and by screening for loss of eye pigmentation one can isolate revertants (precise excision) or new alleles (imprecise excision). At the 5' end of P[*lacW*] is the *lacZ* reporter gene which may give clues to the expression pattern of the target gene.

5.3 l(2)k02508 contains a single insertion in *vha68-2*

Southern blotting of genomic DNA from fly line l(2)k02508, cleaved by *EcoR*I and probed with *vha68-1* cDNA, shows band shifts due to P[*lacW*] insertion (Figure 5.1A). Probing with a 1.9 kb P[*lacW*] fragment corresponding to the plasmid replicon detected only a single band (Figure 5.1B), suggesting that line l(2)k02508 contains a single P[*lacW*] insertion in or near one of the two *vha68* genes. This is supported by *in situ* hybridisation to polytene chromosomes with a P-element probe, which shows line l(2)k02508 to contain a single insertion at 34A3-4 (refer to Encyclopaedia of *Drosophila* for information on l(2)k02508). As reported in Chapter 4, *in situ* hybridisation to

polytene chromosomes with *vha68-1* cDNA also detects a single band at 34A, the probable location of both A subunit genes.

5.4 The insertion in l(2)k02508 lies within *vha68-2*

Comparison of the restriction maps of the plasmid P184 and *vha68-2* showed the insertion to be in the first intron, less than 1 kb 5' to the translation start site (Figure 5.2). Sequencing of the rescued plasmid produced unequivocal evidence for the insertion within the *vha68-2* gene. The insertion has occured between 703 and 704 in the *vha68-2* genomic DNA sequence (Figure 5.3). The sequence generated by primer PR is exactly the same as a region of the first intron of *vha68-2*. PR is a P-element primer reading out of the P-element into flanking DNA, i.e. into the rescued DNA (Figure 5.3 A). Sequence generated by primer 68T7-6 shared more than 97% homology among the 218 base pairs (Figure 5.3 B), with no changes found in the coding sequences.

5.5 Lethality in l(2)k02508 is caused by insertion of the P[*lacW*] element

That the P[*lacW*] insertion is indeed responsible for the homozygous lethality of the l(2)k02508 was shown by the generation of viable revertants following precise P-element excision. P[*lacW*] was remoblised by the cross shown in Figure 2.1. *white*$^-$ progeny of various classes was generated (Table 5.1). One class was homozygous viable for the original second chromosome. Lethality in the l(2)k02508 was then due to P-element insertion rather than to some other accidently fixed events elsewhere on the same chromosome.

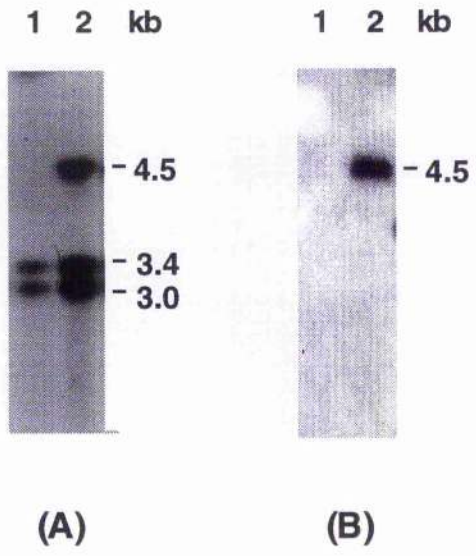


Figure 5.1 Southern blotting of genomic DNAs confirms that line l(2)k02508 contains a single P[*lacW*] insertion in *vha68*. (**A**) Canton S (lane 1) and l(2)k02508 (lane 2) DNAs cleaved by *Eco*RI and hybridised with *vha68-1*. (**B**) Probed with the 1.9 kb P[*lacW*] fragment corresponding to the plasmid replican.

Figure 5.2 Correspondance of the rescued plasmid and *vha68-2* genomic DNA fragment. S, *Sal*I; B, *Bam*HI; E, *Eco*RI.

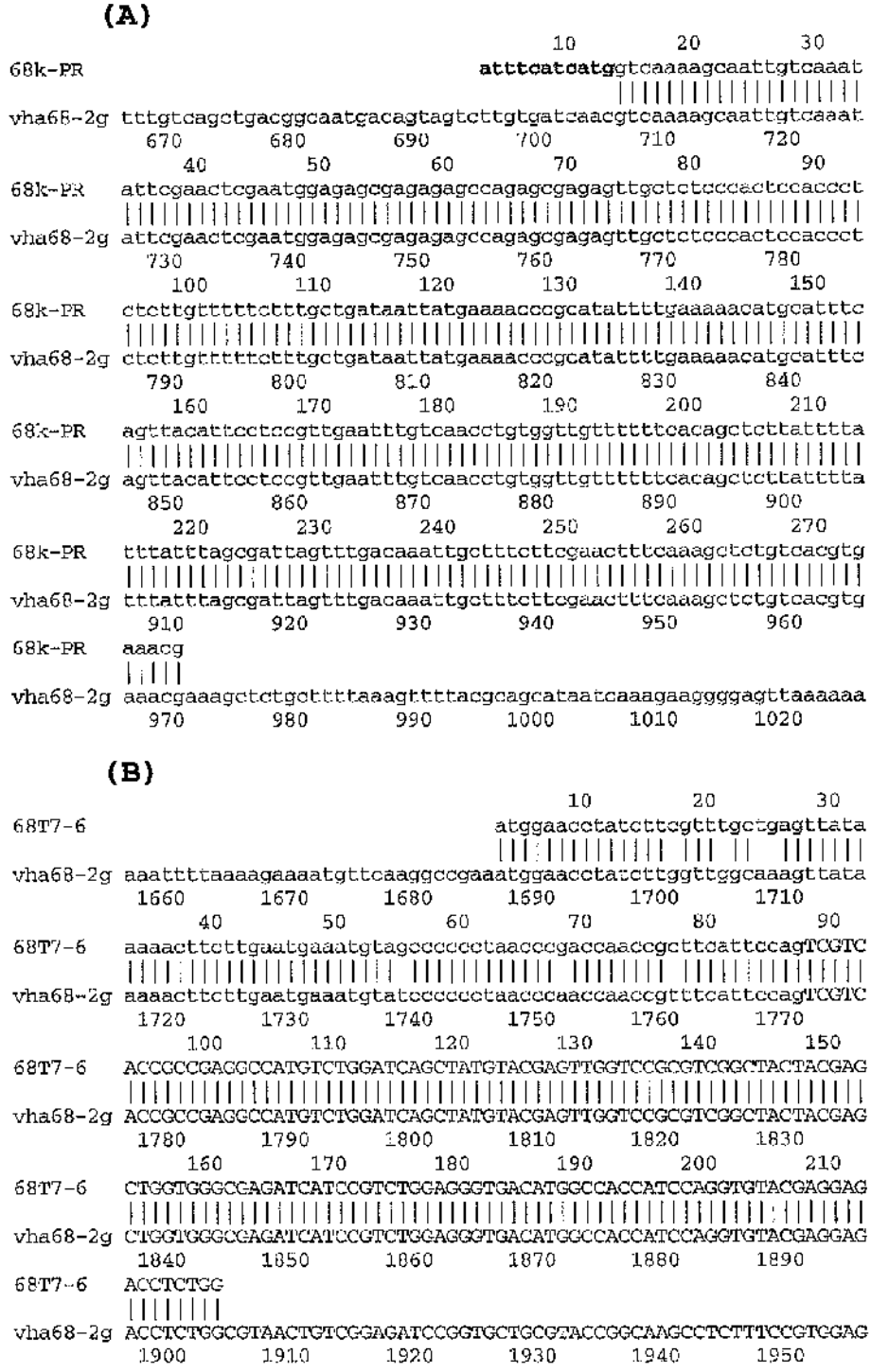


Figure 5.3 Sequence homology of rescued plasmid and *vha68-2*. (A) 68k-PR is the sequence reading out of rescued plasmid from primer PR. Bold indicates the end of the P[*lacW*] insertion. (B) 68T7-6 is the sequence of rescued plasmid generated by primer 68T7-6 which is in *vha68-2* gene.

5.6 Imprecise excision generates a range of new alleles

Remobilisation of a P-element, apart from the precise excision, often generates flanking sequence deletions by imprecise excision (Daniels *et al.,* 1994; Salz *et al.,* 1987; Voelker *et al.,* 1984). Remobilisation may also generate local reinsertions that can often be selected by scoring the dominant marker on the transposon (Tower, *et al.,* 1993).

About 200 lines which lost eye colours were selected and backcrossed to the original line l(2)k02508 to test survival to the adult stage. The survival rate showed a range of differences (Table 5.1 and Figure 5.4). Interestingly, several lines showed a temperature-sensitive phenotype. The homozygous flies of these lines can survive at high temperature (25-30°C) but they die before reaching adult stage if they are reared at 16°C (Table 5.1 and Figure 5.5). A genomic Southern blot of the new alleles found that alleles 68S-6 and 68S-10 are likely to have deletions in gene *vha68-2* (Figure 5.6). Of the five temperature-depedent alleles, 68S-27 has an internal deletion with the plasmid replicon still there. However, the hybridisation patterns of other three alleles, 68S-22, 68S-25 and 68S-38, looks the same as that of Canton S. It is possible that these alleles still contain deletions but the deletions are too small to be detected by genomic Southern blot.

5.7 Reporter gene expression

Line l(2)k02508 contains a single P[*lacW*] insertion, located in the first intron of *vha68-2.* Since *lacZ* enhancer detector element is in the same orientation as *vha68-2* transcript, it might be expected that the *lacZ* expression pattern would mirror at least in part the expression pattern of *vha68-2.*

The first evidence for *lacZ* expression was in gastrulating embryos (Figure 5.7) The heaviest staining was initially in a loop of embryonic midgut, with staining soon becoming general. In larvae, pupae and adults, most or all tissues eventually stain, as

would be expected for a ubiquitously expressed gene; however, staining in shorter time showed certain tissues, the labial palps, a region of the midgut, the main segments of the Malpighian tubules and rectal pads to be conspicuously labelled. This is significant, because it neatly delineates those tissues in which V-ATPases play a plasma-membrane, rather than an endomembrane role (Davies et al., 1996). Although P-element enhancer detectors do not necessarily report faithfully the entire expression pattern of their neighbouringtranscription units, as they may be unduly influenced by short-range

Table 5.1 New alleles and revertants after excision of the P[*lacW*] in line l(2)k02508

fliy lines	25°C					30°C		16°C	
	A	B	C	D	E	D	E	D	E
S1	24	46	5	156	9	65	3	69	1
S2	25	26	14	97	52	42	31	36	21
S3	20	16	10	137	30	32	22	52	4
S4	14	18	19	78	56	18	24	39	23
S6	30	32	0	131	0	108	0	74	0
S8	22	29	19	96	19	39	16	67	1
S9	19	21	11	97	33	62	37	79	41
S10	40	45	0	163	1	71	0	25	0
S11	17	28	2	166	14	33	4	60	3
S13	15	36	21	45	16	50	21	65	37
S22	48	48	54	67	11	17	8	83	0
S25	23	23	6	112	21	40	5	74	0
S27	27	81	18	85	10	81	18	181	2
S29	13	19	7	92	28	59	15	110	27
S33	15	23	1	191	7	35	12	86	3
S35	13	13	12	58	24	64	24	50	21
S36	20	32	0	138	0	77	0	89	0
S37	21	48	24	89	26	22	5	75	24
S38	28	68	28	122	21	16	3	108	3

A, B, C, D, E, F stand for different phenotypes, See Method section 2.18 for the meaning.

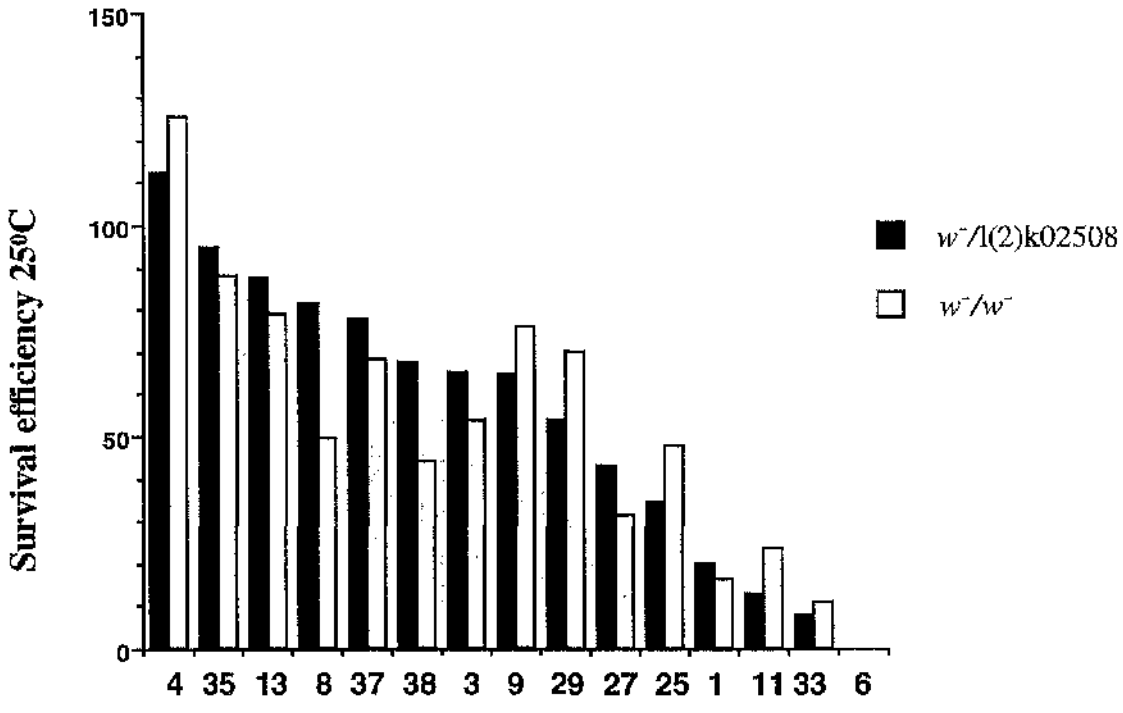


Figure 5.4 New alleles with different survival efficiency after remoblisation of the P-element in strain l(2)k02508. Filled boxes show the % survival when heterozygous with the l(2)k02508 chromosome; Empty boxes show % survival when homozygous for a new allele.

$$\text{Survival efficiency (\%)} = \frac{\text{Actual ratio of certain progeny}}{\text{Expected ratio of certain progeny if without detrimental effects}}$$

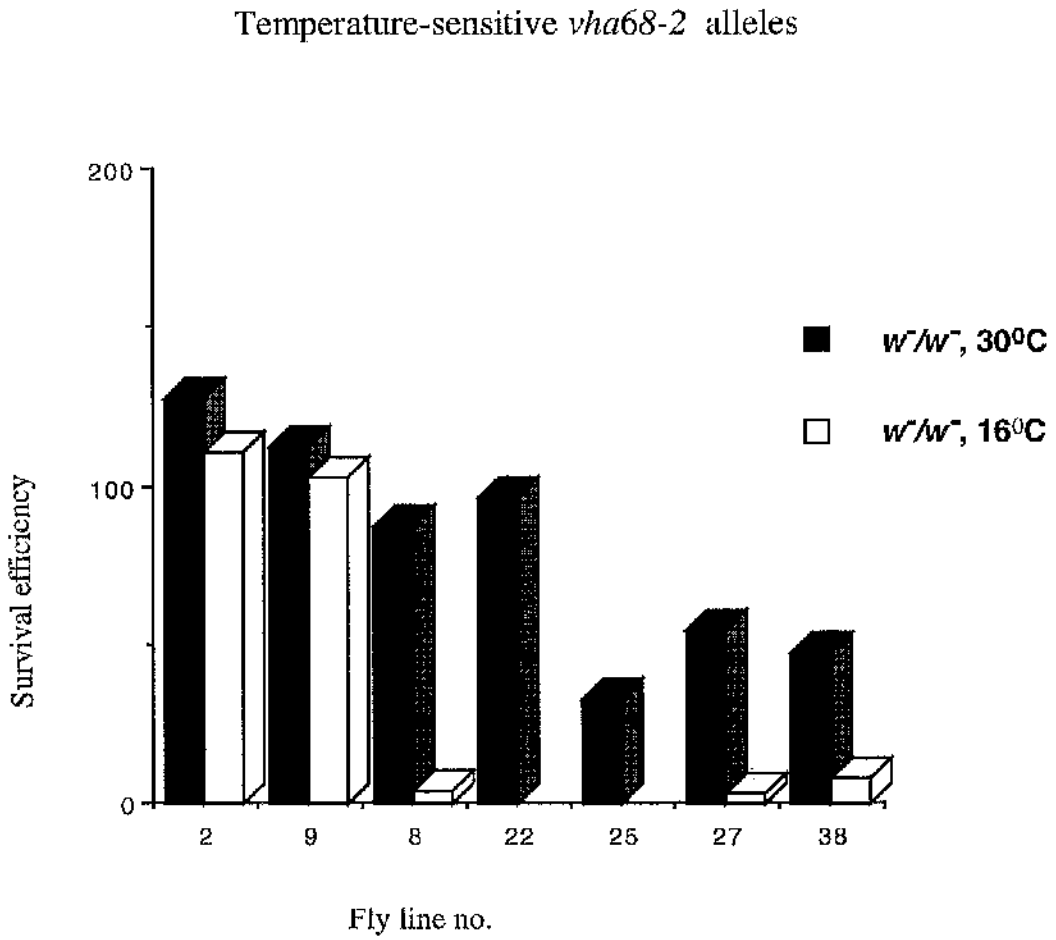


Figure 5.5 Alleles with temperature-dependent survival. Filled boxes show survival at 30°C, empty boxes show the survival at 16°C.

$$\text{Survival efficiency (\%)} = \frac{\text{Actual ratio of certain progeny}}{\text{Expected ratio of certain progeny if without detrimental effects}}$$

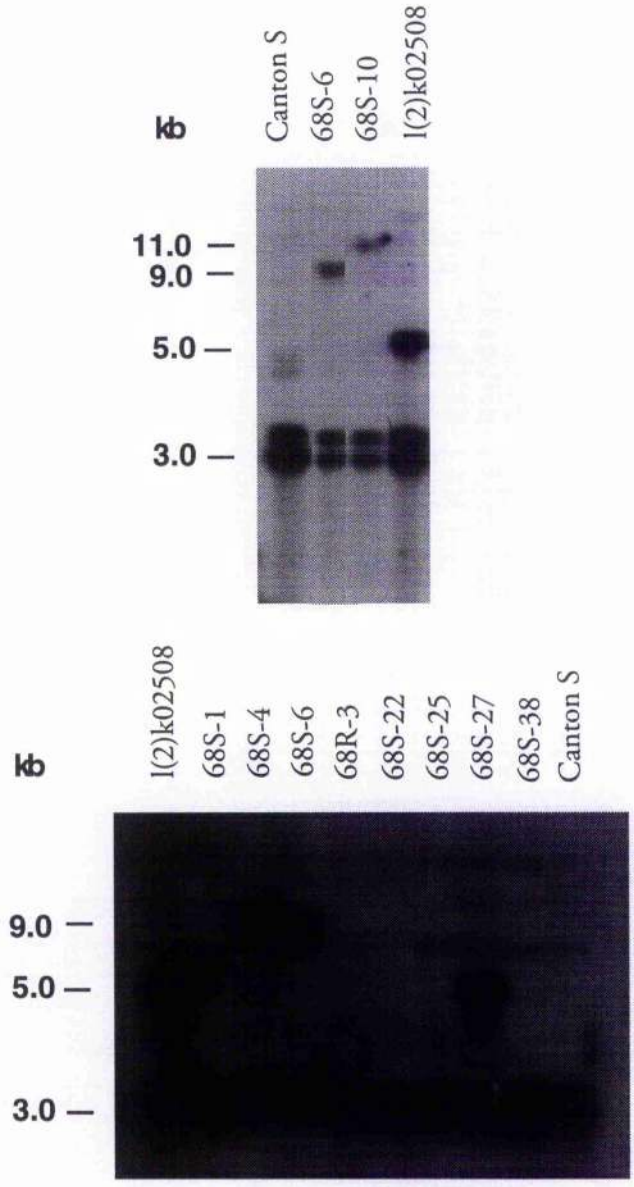


Figure 5.6 Genomic Southern blot of *vha68-2* mutant flies. Genomic DNA was digested with *Eco*RI, run out on a 1% agarose gel and blotted to Hybond N. The both filters were hybridised with probe of *vha68-1* cDNA.

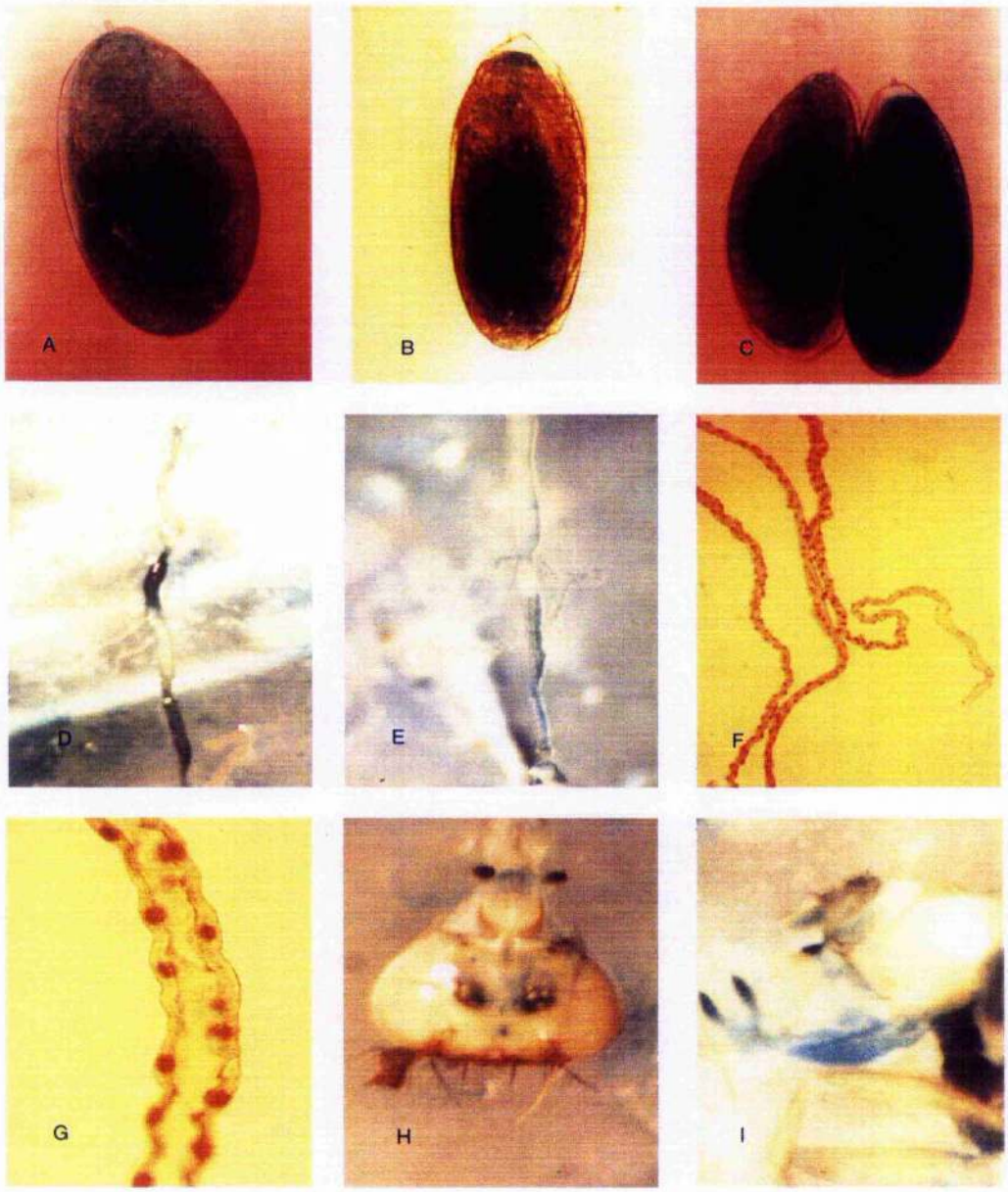


Figure 5.7 *lacZ* expression patterns of l(2)k02508. **(A)** embryonic, showing a loop of the midgut staining; **(B)** embryonic, showing Malpighian tubule and midgut staining; **(C)** embryonic with longer staining; **(D)** Larval gut showing the mid gut and Malpighian tubule staining; **(E)** Adult gut showing the Malpighian tubules and midgut staining; **(F)** Adult Malpighian tubules, showing staining confined to nuclei of main segment; **(G)** Enlarged view of the adult Malpighian tube staining; **(H)** Front view of adult head, showing staining of antennal bases and labial palps; **(I)** Side view of adult head, showing the staining of antennae and labial palps.

enhancers, the pattern of expression reported here is precisely what would be expected for a V-ATPase gene (Figure 5.7). Antibody staining for β−galactosidase shows a similar expression pattern. Figure 5.8 shows the antibody staining of Malpighian tubules in larvae.

5.8 Phenotypic analysis of l(2)k02508 and new alleles

The original P-element strain l(2)k02508 and the two new alleles 67S-6 and 67S-10 are homozygous lethal and are maintained over balancer *CyO*. Flies homozygous for balancer *CyO* are lethal at late embryo or early larvae stage, but flies heterozygous for *CyO* are viable with curly wings (Lindsley and Zimm, 1992). If flies homozygous for the *vha68*$^{-}$ could survive to adult stage they should have distinctive straight wings. However, it is difficult to distinguish the difference earlier than the adult stage. To facilitate the analysis of lethal phase the *CyO* balancer chromosome was first replaced with wild type to observe whether embryos homozygous for the P-element can hatch. 468 larvae hatched from 483 eggs laid by parents P[*lacW*]/+. The hatch rate is 97%, approximately the same hatch rate for the wild type flies. Of the 15 unhatched eggs, 7 eggs are unfertilised. This high hatch rate means that the homozygous P[*lacW*] can survive to larval stage. To distinguish the homozygous [*vha68-2*$^{-}$/*vha68-2*$^{-}$] larvae from the heterozygous larvae the original balancer *CyO* was replaced by the y^{+}*CyO* chromosome which then could distinguish the homozygotes [*vha68-2*$^{-}$/*vha68-2*$^{-}$] from heterozygotes[*vha68-2*$^{-}$/y^{+}*CyO*] as early as the first instar larvae. The heterozygous fly has a black hook while the homozygous flies have yellow hooks (figure 5.9A).

For the three mutant lines, l(2)k02508, 68S-6, and 68S-10, the homozygotes can survive the embryo stage. The new hatched larvae wiggled around slowly and were not as active as the healthy one. The homozygous [*vha68-2*$^{-}$/*vha68-2*$^{-}$] larvae were observed dying in first instar larvae.

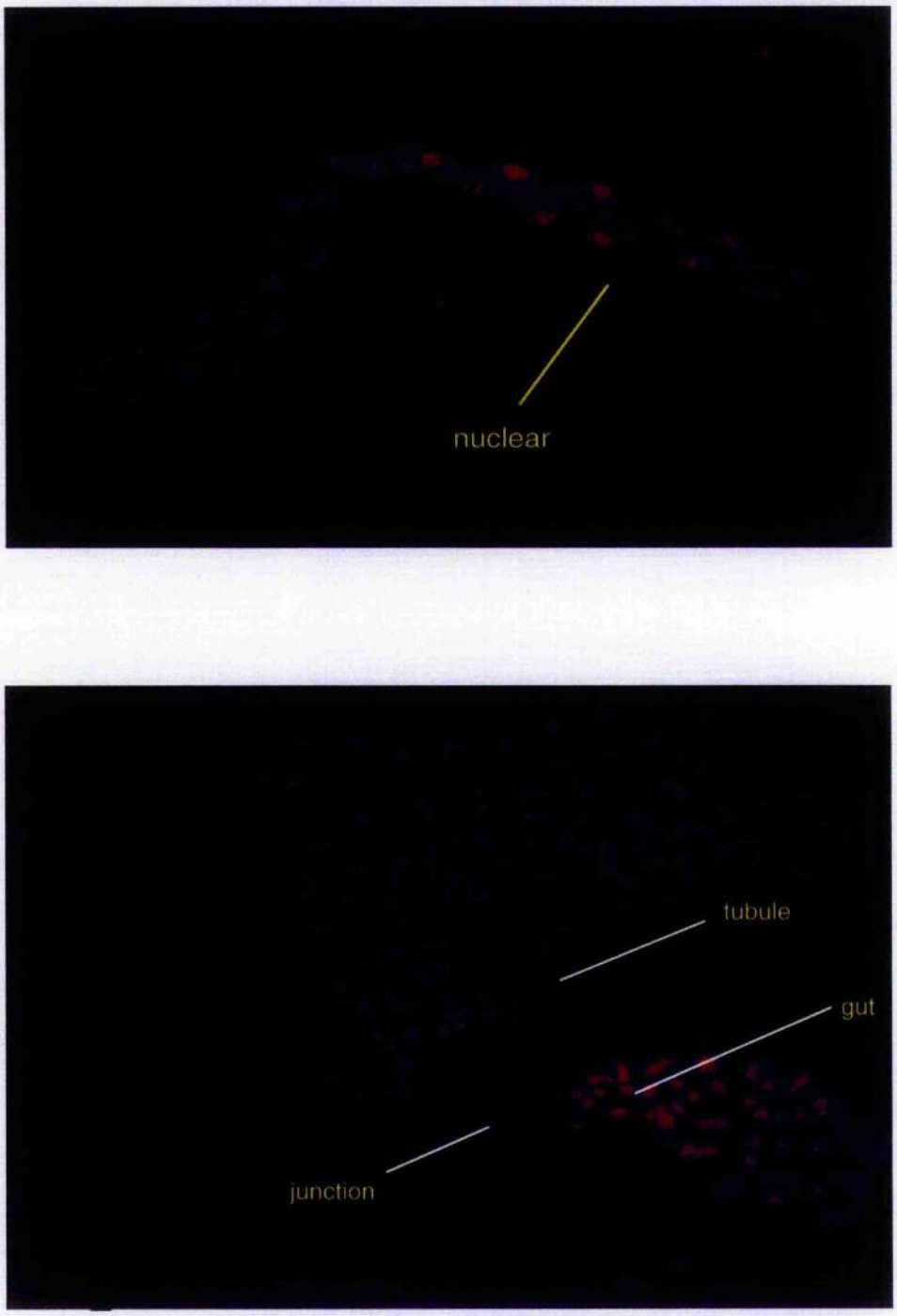


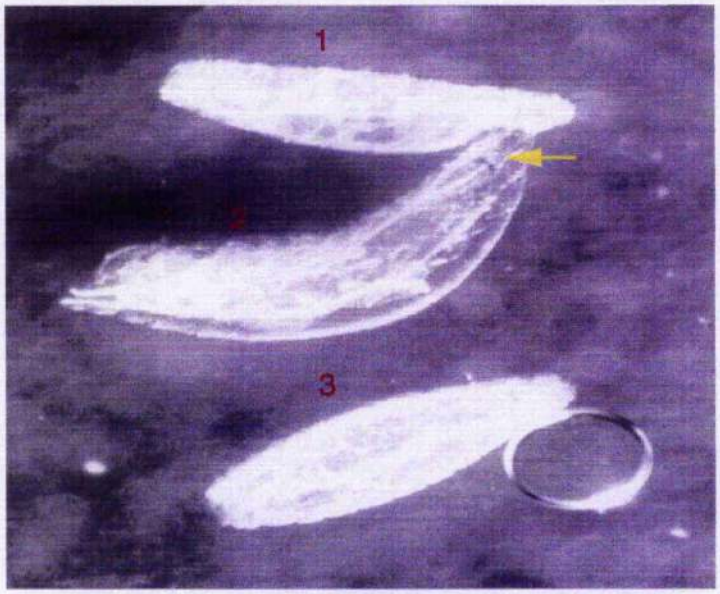
Figure 5.8 Antibody staining of β-galactocidase in the Malpighian tubules. (**A**) Third instar larval Malpighian tubules showing nuclear staining in the principal cells. (**B**) Malpighian tubules and gut of third instar larvae showing the nuclear staining of gut and Malpighian tubules, and the unstaining junction.

Examination of the Malpighian tubules in the homozygous larvae indicates the mutation affects the morphology of this organ, especially the anterior segment. Tubules are responsible for the clearance of the waste products. The anterior segment of the Malpighian tubules normally stores the primary urine in the form of crystalline concrements of uric acid, calcium phosphate, etc (Maddrell and O'Donnell, 1992). The concrement play an important role in the process of osmoregulation and they are either absent or severely reduced in the original P-element mutant and the two deletion alleles (Figure 5.9B).

5.9 Northern blot analysis of mutant flies

The above results indicated that the l(2)k02508 strain and the two alleles 68S-6 and 68S-10 were hypomorphic for V-ATPase function. I therefore was interested to test whether a decrease also occurred at the level of transcription of the *vha68* gene in line l(2)k02508. Total RNA was isolated from adult of wild-type Canton S, the heterozygous P-element insertional line l(2)k02508, two homozygous revertants, 67R-2 and 67R-4. The RNA was separated by electrophoresis in 1% formaldehyde-agarose/MOPS gels and blotted to nitrocellulose. The blot was probed with *vha68-1* cDNA (Figure 5.10). For comparison of RNA loading, the blots were stripped and probed with *Rp49* cDNA. All the 4 lines has the same 2.6 transcript of *vha68*, but fly strain l(2)k02508, even being heterozygous and that the probe used here can be expected to hybridise to transcripts of both *vha68-1* and *vha68-2,* shows an appreciable reduction in overall *vha68* levels in the mutant lines. The revertant line 67R-4 has the same RNA level as that of wild type, but The revertant line 67R-2 has less RNA ltranscript which is the same level as that of the heterozygous l(2)k02508. Thus, it can be strongly suggested that the l(2)k02508 are also a hypomorphic mutation at the level of transcription.

(A)



(B)

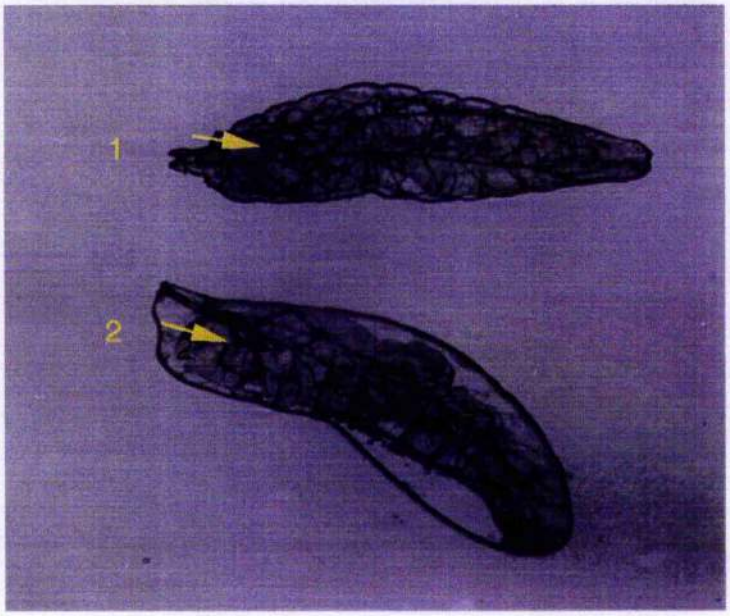


Figure 5.9 Phenotype of 68S-6. **(A)** Difference of hook colour between homozygous and heterozygous larvae of 68S-6. (1) and (3) are homozygous dying larvae with yellow hook, (2) is heterozygous larvae with black hook.**(B)** defects in Malpighian tubules in dying homozygous larvae of 68S-6, (1) is the dying homozygous the larvae in which the white precipitates are reduced or absent. (2) is the heterozygous larvae with normal Malpighian tubules which contain a white precipitate of uric acid and calcium salts. (here seen as black by transmitted light).

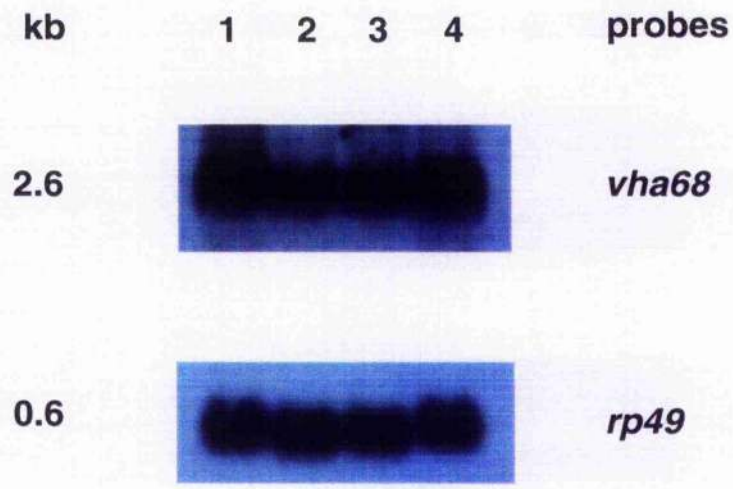


Figure 5.10 Northern blot analysis of the mutant flies of *vha68-2.* Total RNA was isolated from the adult flies using TRIzol^{TM} (Gibco BRL). The RNA was separated by electrophoresis in 1% formaldehyde-agarose/MOPS gels, blotted to nitrocellulose, and hybridised with ^{32}P-labelled random-primed probes. The filters was then exposed to Fuji X-ray film for 1-3 days. Sizes were determined with respected to an RNA ladder (Gibco BRL). The filters were first hybridised with whole *vha68-1* cDNA, then the same blots were stripped and reprobed with *rp49* to control for differences in RNA loading. Lane 1. Canton S; Lane 2, P-element insertional mutant l(2)k02508; lane 3, homozygous revertant 68R-2; Lane 4, homozygous revertant 68R-4.

5.10 Discussion

The identification of a P[*lacW*] insertion in *vha68-2* is of great help in addressing the function of the gene. Inactivation of just *vha68-2* leads to the homozygous lethality at first instar larvae, which suggests *vha68-2* to be an essential gene. Although the sequence of the two isoforms is highly homologous at DNA and protein levels, the presence of only *vha68-1* is insufficient for proper function. The Northern blots of total RNA of both isoforms detected a very similar pattern of ubiquitous expression. However, this does not necessarily mean that both isoforms are present in the same cellular population or subcellular compartment. The X-gal staining of the strain l(2)k02508 with a P-element in *vha68-2* reveals a general expression pattern, but highly enriched in the midgut and Malpighian tubules, suggesting a plasma membrane role for the *vha68-2* isoform. This staining pattern is similar to the x-gal staining pattern of fly lines with a P-element in genes encoding other subunits, such as the E, B and c subunits of Drosophila V-ATPases. Such a expression pattern may be applied to other subunits of V-ATPase and thus may provide a general means of screening P-element for mutations for V-ATPases and related genes.

The new alleles generated by excision of P-element in l(2)k02508 show phenotypes with different severity; and in particular, five temperature-sensitive alleles. However, the molecular mechanism underlying these potentially important alleles needs further investigation.

As *vha68-1* and *vha68-2* are both at 34A and remobilisation of P-element tends to reinsert into the local sites around the original P-element, it should not be too difficult to identify a fly carrying a P[*lacW*] in *vha68-1* by the PCR strategy (Kaiser and Goodwin, 1990) following the local jumping of the P-element in line l(2)k02508. Analysis of the mutants of both *vha68-1* and v*ha68-2* should help in elucidation of the function differentiation of the two isoforms of the V-ATPase A subunit in *Drosophila*.

Chapter 6

Characterisation and Inactivation of *vha26*, the Gene Encoding an E-Subunit of the V-ATPase

6.1 Summary

A *D. melanogaster* gene and a cDNA for the 26 kDa E subunit have been cloned utilising homology with the corresponding *M. sexta* gene. The *Drosophila* gene contains three small introns. Its deduced translation product has 226 amino acids and a molecular weight of 26.1 kD. The polypeptide shares 76.5% identity with the M. sexta polypeptide, 62.8% with that of human and 34.3% with that of yeast. The *Drosophila* gene (*vha26*) is present as a single copy at cytological position 83B1-4 on the third chromosome and gives rise to an mRNA species of 2.3 kb. Abundance of the latter, relative to an *rp49* control, shows relatively little variation within adult head, thorax and abdomen, suggesting that the E subunit is a relatively ubiquitous component of the V-ATPase. *vha26* is, however, relatively less expressed during metamorphosis, as is also the case for the *D. melanogaster* V-ATPase A subunit (Chapter 4). A fly line carrying a single lethal P[*lacW*] insertion within *vha26* gene has been identified. This will greatly facilitate study of the *in vivo* function of the E subunit.

6.2 Introduction

Subunit E is a constituent of the catalytic sector of the V-ATPase. It was one of the first subunits to be identified in kidney V-ATPase by immunological studies, and the cDNA encoding the kidney subunit has been cloned and sequenced (Hirsh *et al.*, 1988). Studies with monoclonal antibodies, supported by partial DNA sequencing, reveal the existence of at least two isoforms of subunit E in the kidney. While V-ATPase isolated from kidney

microsomes contains one form of subunit E, the enzyme from the kidney brush-border contains at least one additional form of subunit E. Presently a cDNA for subunit E has been cloned and sequenced from *M. sexta*. The deduced polypeptides show high homology with the E subunit from other sources. Although at least two isoforms for the E subunit may exist in human, only one gene encoding the *M. sexta* E subunit has been detected in Southern and Northern blots (Gräf *et al.*, 1994a). The precise function of the E subunit is unknown but it has been suggested that E subunit may play an analogous role in the V-ATPase to the γ–subunit in F-ATPases (Bowman *et al.*, 1995) and as such should be considered to form part of the catalytic headgroup. The corresponding yeast gene *vma4*, has been cloned, sequenced and mutagenised (Foury, 1990). The mutant exhibits a similar phenotype to all other yeast V-ATPase nulls. While the protcolipid assembles into the membrane, all subunits of the catalytic sector did not assemble. Consequently, the mutant is unable to grow in medium buffered at pH 7.5 (Ho *et al.*, 1993). This suggests that subunit E may be necessary for the functional assembly of the enzyme. In vertebrates, it has been suggested that E subunit co-localises immunocytochemically with plasma membranes, rather than microsomes in kidney (Hemken, *et al.*, 1992), implying that E subunit may be important in assembly of the holoenzyme on the plasma membrane of certain epithelia. Here, as first step to clarify this issue, I report the cloning, characterisation and mutagenesis of the gene encoding subunit E of V-ATPase in *D. melanogaster*, a species which is particularly suited to genetic analysis.

6.3 Identification of a cDNA encoding a 26 kD E-subunit

6.3.1 cDNA cloning

A *D. melanogaster* head λ–ZapII cDNA library was screened by plaque hybridisation with a *M. sexta* E-subunit cDNA probe and one positive plaque was purified by successive rounds of screening. The purified clone was excised as pBluescript and the cDNA insert

cDNA clones were obtained and subcloned into pBluescript SK-. Sequences from both ends of all five clones were identical except for differences in length at the 5' end. The longest insert (p26CD) was 2.1 kb long.

6.3.2 Generation of unidirectional deletions of p26CD for sequencing

p26CD was isolated and purified on a Promega column. *Exo*III was used to generate a set of deletions of p26CD DNA for sequencing. Two pairs of enzyme (*Sac*I /*Eco*RI and *Hin*dIII/*Kpn*I) were selected for digesting DNA which can then be further digested by *Exo*III to make deletions from both ends (Figure 6.2A). The cDNA insertion has no digestion site for any of the 4 enzymes. *Sac*I and *Kpn*I can generate the 3' *Exo*III-protected end, while *Eco*RI and *Hin*dIII generate the 5' overhang which is digested by *Exo*III. In the case of making deletions which can be sequenced by primer T3, 20 μg of p26CD plasmid was first digested with 50 units of *Sac*I for 3 hours. A sample of this digest was electrophoretically separated on a 1% agarose TBE gel to assess the extent of digestion. After completion of the digestion, buffer condition was adjusted with NaCl for *Eco*RI digestion for another 3 hours. Double digested DNA was digested by *Exo*III at 37°C and samples were taken every 30 seconds. The first 15 samples were treated with S1 nuclease and were electrophoretically separated through an agarose gel (Figure 6.2B). From the figure we can see the digestion rate was about 200 bp/min. This rate of digestion is less than described by the manufacturer of Erase-a-Base system (Promega). However the *Exo*III digestion indeed produced progressive deletions.

Each timepoint sample was treated with the Klenow fragment of *E. coli* DNA polymerise to generate flush DNA termini and was then recircularised with DNA ligase. Ligation products were used to transform DH5α competent cells (see methods section). 50 to 1000 colonies were obtained for each timepoint transformation. Three colonies from each of the first 12 transformations were selected at random and miniprep DNAs

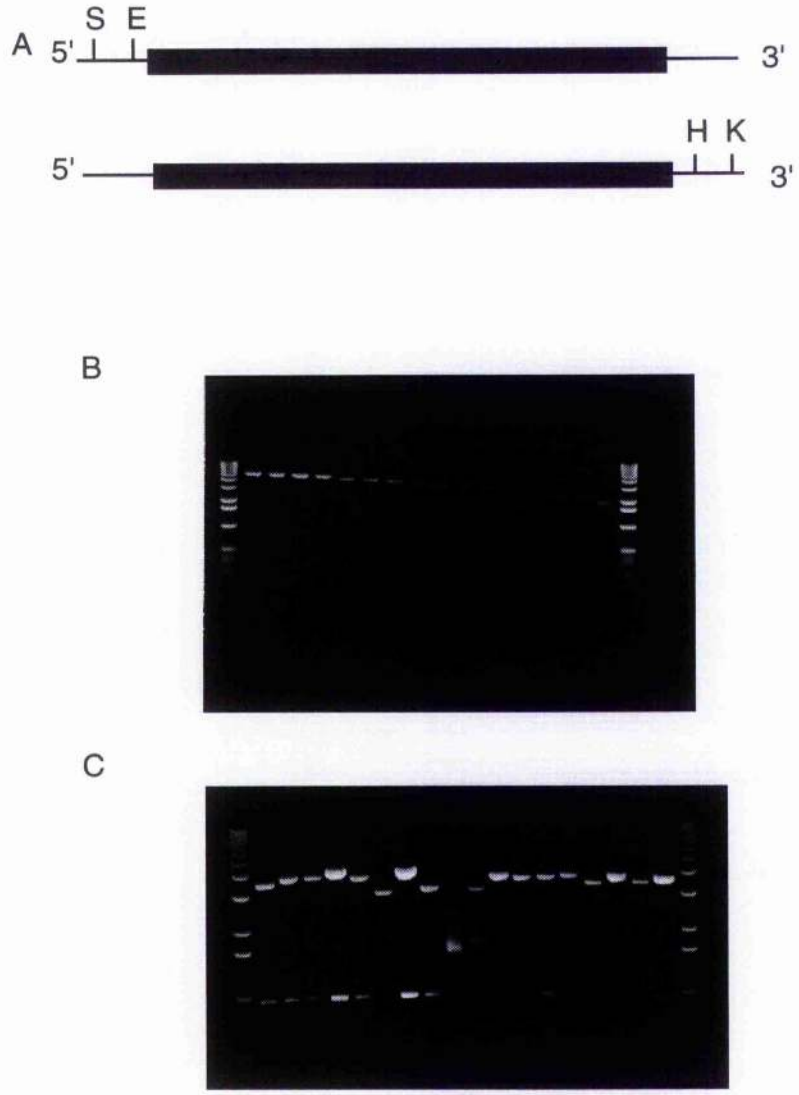


Figure 6.1 *Exo*III deletion of the p26CD insert. (**A**) p26CD structure showing the restriction enzymes selected to make *Exo*III protected and unprotected termini. *EcoR1* and *Hin*dIII generate 5' overhanging termini, *Sac*I and *Kpn*I generate protected termini. (**B**) The products of *Exo*III and S1 nuclease digestion of *Sac*I/*Eco*RI digested p26CD. Samples of the *Exo*III reaction were removed at intervals of 30 seconds. (C) Plasmid minipreps from the deletion experiment after digestion with *Xho*I and *Xba*I.

(Method Section) were digested with *Xho*I and *Xba*I (Figure 6.1C). Subclones with different size of deletions were selected for sequencing by primer T3.

Similarly, DNA from the double digestion of p26CD by *Hin*dIII and *Kpn*I was digested by *Exo*III to generate deletions which can be sequenced from the opposite end using primer T7. The 2.1 kb cDNA insert of p26CD was completely sequenced from both directions.

6.3.3 DNA sequence analysis of *vha26* cDNA

The 2.1 kb contig of p26CD has an open reading frame corresponding to a 226 amino acid polypeptide of M_r 26.1 kDa (Figure 6.3). This is clearly a V-ATPase E-subunit, sharing 76.5% amino acid identity with the E-subunit of *M. sexta*, 62.8% with that of human, but only 34.3% identity with that of *S. cerevisiae* (Figure 6.3). In accordance with the nomenclature for other *D. melanogaster* V-ATPase loci, the gene has been named *vha26*. Although we cannot at present exclude the possibility that longer transcripts exist, the longest 5' UTR of the 5 cDNA clones is 77 bp. This is in good agreement with the length of 5' UTRs reported for other V-ATPase subunits in *Drosophila*, 84 and 88 bp for the two genes encoding 67 kDa A-subunit (see Chapter 4); 86 bp for the 55 kDa B-subunit (Davis, *et al.*, 1996); 116 bp for the 17 kDa c-subunit (Meagher, *et al.*, 1990); and 42 bp for the 14 kDa F-subunit (see Chapter 7). The sequence of the start site CAAAATG matches the consensus start site (C/A)AA(A/C)**ATG** perfectly (Calvener, 1987). The 3' UTR is 1307 bp long, with a canonical **AATAAA** signal centred 26 bases upstream of the polyA tail.

```
1                                                   31
GCA CGG TTG TTG TAC GTG GGC TTC TTT AAA ACA CTT GAA TTT CCT TTC GGT TTG TGC AGT
61                                                  90/5
GAA AAA AAT CAG TCA AA  ATG GCA CTG AGC GAT GCT GAT GTA CAA AAG CAG ATC AAG CAC
                        M   A   L   S   D   A   D   V   Q   K   Q   I   K   H
120/15                                              150/25
ATG ATG GCG TTC ATT GAG CAG GAG GCC AAT GAG AAA GCC GAG GAG ATC GAT GCC AAG GCC
 M   M   A   F   I   E   Q   E   A   N   E   K   A   E   E   I   D   A   K   A
170/35                                              210/45
GAG GAG GAG TTC AAC ATT GAG AAG GGA CGC CTG GTC CAG CAG CAG CGT CTC AAG ATC ATG
 E   E   E   F   N   I   E   K   G   R   L   V   Q   Q   Q   R   L   K   I   M
240/55                                              270/65
GAA TAC TAC GAG AAG AAG GAG AAG CAA GTT GAG CTG CAG AAG AAG ATT CAG TCC TCC AAC
 E   Y   Y   E   K   K   E   K   Q   V   E   L   Q   K   K   I   Q   S   S   N
300/75                                              330/85
ATG CTC AAC CAG GCT CGT CTG AAG GTG CTG AAA GTG CGC GAG GAC CAT GTG AGC AGC GTG
 M   L   N   Q   A   R   L   K   V   L   K   V   R   E   D   H   V   S   S   V
360/95                                              390/105
CTG GAT GAT GCC CGC AAG CGT CTC GGC GAG GTC ACC AAG AAT CAG TCC GAG TAC GAG ACT
 L   D   D   A   R   K   R   L   G   E   V   T   K   N   Q   S   E   Y   E   T
420/115                                             450/125
GTG CTG ACC AAG CTC ATC GTC CAG GGC CTG TTC CAG ATC ATG GAG CCC AAG GTG ATC CTG
 V   L   T   K   L   I   V   Q   G   L   F   Q   I   M   E   P   K   V   I   L
480/135                                             510/145
CGC TGC CGC GAG GTG GAC GTC CCC CTG GTA CGT AAC GTC CTG CCT GCC GCT GTG GAG CAA
 R   C   R   E   V   D   V   P   L   V   R   N   V   L   P   A   A   V   E   Q
540/155                                             570/165
TAC AAG GCC CAG ATC AAT CAG AAC GTC GAG CTG TTC ATC GAC GAG AAA GAC TTC CTC TCT
 Y   K   A   Q   I   N   Q   N   V   E   L   F   I   D   E   K   D   F   L   S
600/175                                             630/185
GCT GAT ACC TGC GGT GGT GTT GAG CTG CTG GCC CTC AAC GGA CGC ATC AAG GTG CCC AAT
 A   D   T   C   G   G   V   E   L   L   A   L   N   G   R   I   K   V   P   N
660/195                                             690/205
ACG CTG GAG TCC AGA TTA GAC CTC ATT TCG CAG CAG CTG GTG CCC GAG ATT CGT AAC GCA
 T   L   E   S   R   L   D   L   I   S   Q   Q   L   V   P   E   I   R   N   A
720/215                                             7510/225
CTT TTC GGC CGC AAC GTC AAT CCC AAA TTC ACC GAC TAA ATT CTA TAA GTG CAA AAC AAA
 L   F   G   R   N   V   N   R   K   F   T   D
780                                                 810
ACA TAA CTA ACC AGA AAG AGA ACC AGC ATC AAC ACC TAT TCA GCA GGA ACA GTT CAA GTT
840                                                 870
ATT ACA CAG AGC TCC ACC CAC TAA ATA TTG AAC CCA AGT AAA CTT ATC CTT TGG CAG TCA
900                                                 930
GGA GGC AAC AGC TAG GAT ATA TTG ATT GTC AAA ATA CTT TTG CCG TTG TCT TGT AAA GTG
960                                                 990
AAA TTG AAA CAC TCA AGA ACA TTT CGG TCC TTG TGT ACG CAA CAG TTT TAA TAG TAA CCA
1020                                                1050
CAC TAA ACG CGC ATA TAT ACT CTC CGA TAT ATA TGT CTG TAT GCC AAT ACT TAT TAT ATA
1080                                                1110
GTT TAG AGG ACA CGA TCC TAG GAG CAT ACG AAA GCA TAA TAC GAA GTT TGT TAA AGT TTG
1140                                                1170
TTC GTT TTT TTT TTA CAT ATG CAC ATG TTT CTG AGC AGT AGG TCT AGA TAT GTG CTT ATA
1200                                                1230
TTG TAT ACA TAC ACT TTA AAA TTT TGC ATA CAT TCC TGT CCA AGA ATT TTT ATT TCA GTT
1260                                                1290
TTC CCC TTG TTT ATT GTA CAT TAT TTT CTG TAG TCT TTG TTA ACT TTT TAT ATG TCT ATG
1320                                                1350
TCG TTT ATG TTC GTA ATT ATC AAG TGC ACG TTC AGG AGG AAC AAC GGC AGT GGA TCG CCC
1380                                                1410
CTT TTA CAG ACC GCT GGC AGG TTG CGA TGC GAC CAC ACA GCA TTG TTG CTC AGC GAA GCA
1440                                                1470
CCG AAA TGG ACC TAA ACC CCC GAT TTC GCT TCT TCG AGG GCA ACG GAC GCT TGT GCA ACT
1500                                                1530
GCC ACT GGC TCA ACG AAA GCC CCG AAA ATC ATC AAT GTC TGT TGT TGT TGA GAT ACC GAG
1560                                                1590
AGT AGA GAA TAC ACA CTG CTT AGC ACG CGA CAC TTA ATA CCC ATT CAT TAC ACA TGC ACC
1620                                                1650
ACG ACG ATG AAG TTT GCC AAG TAG CTA AGT TGT TGA CCT GAC CAT CAA GTG CAG CTT TCA
1680                                                1710
```

```
CAC CCT CAT ATA ACT ACT TAA AGA AAA TAT AGA AAA ATG GAA ATT AGT TTT GCA ATT TAG
1740                                    1770
GCC ACT GCC GAA CTG CCA CCG TTT CCA CCT GAC GTG CGC CAT CAT ATC AGG CTC TAA AAA
1800                                    1830
TCA ACA CAC CAT GTT CAA ACA CAC GAC TAG CAT ACA GGA GCA GGA GCT ACA GTA AAT TTG
1860                                    1890
AAC CTT GTA TTC GCA TGT TCG CCA ATG TTC ATA GTC TAT TCT TCA AGC TCA TTT TCT AAC
1920                                    1950
CAA GTT ACC AAG TTC AAT ATG ATG AAT AAC TAC AAG ATT AGC AAA CAA ATA CAA GTA GCA
1980                                    2010
TAT GCG TTA TTA TAT AAC ATC GAG TAC TAT ATA CAT CAC ATG AAA TAC AAA ATG CAA GAA
2040                                    2070
AAA TTA CTT TTA AAC AAA ATT TAT GTT GAA TAA AAA ACA GTA TTT CCA AAA ACT AAA
```

Figure 6.2 Sequence of a *vha26* cDNA (p26CD) and deduced amino acid sequence of the *Drosophila* E-subunit (GenBank accession no. is U38198). Double-stranded sequencing of the cloned genomic DNA fragment was performed according to the Sequenase™ II protocol (USB) by generation of unidirectional deletions with the Erase-a-Base system (Promega), and with the aid of synthetic oligo primers when required. The putative polyadenylation signal is underlined. The start of poly A is marked as bold.

6.4 Genomic structure of *vha26*

6.4.1 Genomic DNA clones corresponding to *vha26*

An Oregon R genomic DNA library in vector EMBL3 was used to isolate the gene represented by the *vha26* cDNA. Approximately 40,000 phage from the library were plated on four Petri dishes (150mmX150mm). Plaque-lifts probed with random-primed p26CD cDNA, revealed three "positive" signals. Plaques from the corresponding spots were re-plated at 50-200 pfu per 90 mm Petri dish and re-screened: two individual and overlapping positive clones were obtained (ph26A and ph26B). Restriction digests of ph26A are shown in Figure 6.3A. The deduced map is shown in Figure 6.4. Probing of ph26A with *vha26* cDNA reveals the sequence homology between the genomic fragment and *vha26* (Figure 6.3B). A 5 kb *Bam*HI fragment that hybridises with the cDNA was subcloned into pBluescript SK$^-$, and named p26kg.

6.4.2 *vha26* is a single copy gene

D. melanogaster genomic DNA, cleaved with various restriction enzymes, was blotted and probed at high stringency with the part of *vha26* cDNA (1183-2096 bp in Figure 6.2). The single band of hybridisation seen in each lane suggests a single genetic locus. This is consistent with the structure and sequence of cloned genomic DNA and *in situ* hybridisation to polytene chromosome squashes which identifies a single locus at 83B1-4 on the right arm of chromosome 3 (Figure 6.10). The 188 kb 83B interval contains three identified genes: *gorp*, a gene implicated in meiosis (Castrillon *et al.*, 1993), *nmdaR* , a glutamate receptor (Ultsch *et al.*, 1993), and a tRNA gene (Dunn, *et al.*, 1979). However, there are also several lethal P-element insertions, suggesting that inactivation of the *vha26* locus by "local jumping" of the P-element may be feasible, or even that an existing P-element insertion might already represent a lethal allele of this gene.

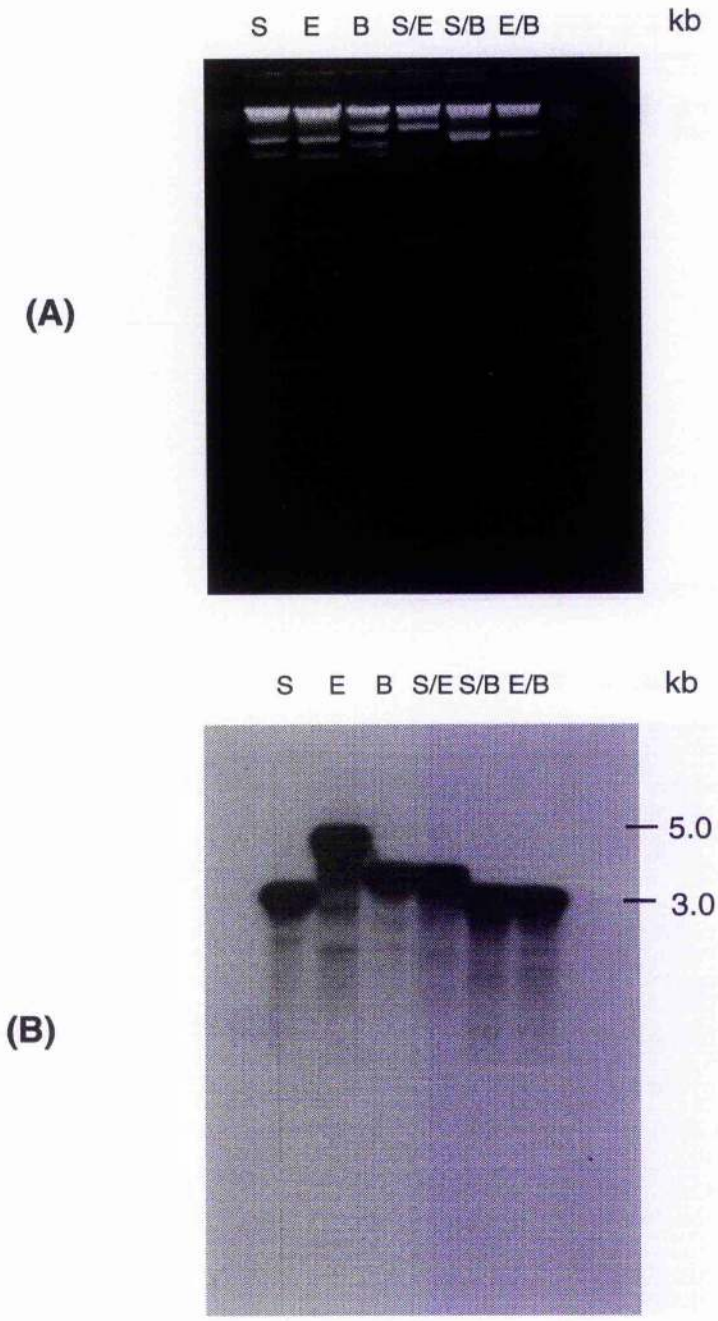


Figure 6.3 A: Agarose gel of ph26A phage DNA cleaved with *Bam*HI (B), *Eco*RI (E) , *Sal*I(S),SalI/*Eco*RI (S/E), *BamHI*/*EcoR*I (B/E) and *Sal*I/*BamH*I (S/B). **B**: A blot of the above gel probed with *vha26* cDNA.

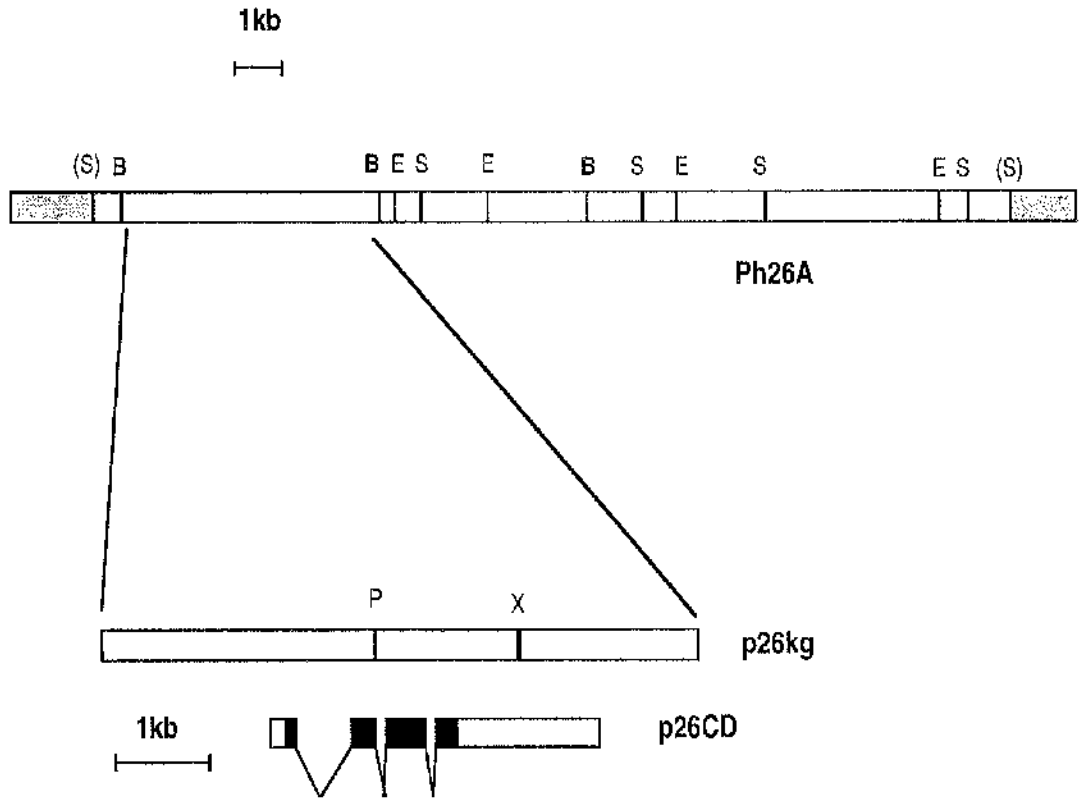


Figure 6.4 Genomic organisation of the *vha26* locus. Above: Restriction map of ph26A DNA. The estimated length of the insert is 10 kb? Below: map of p26kg and p26CD subclone of p26kg. S; *Sal*I; B; *Bam*HI; E: *Eco*RI; P:*Pst*I; X: *Xba*I.

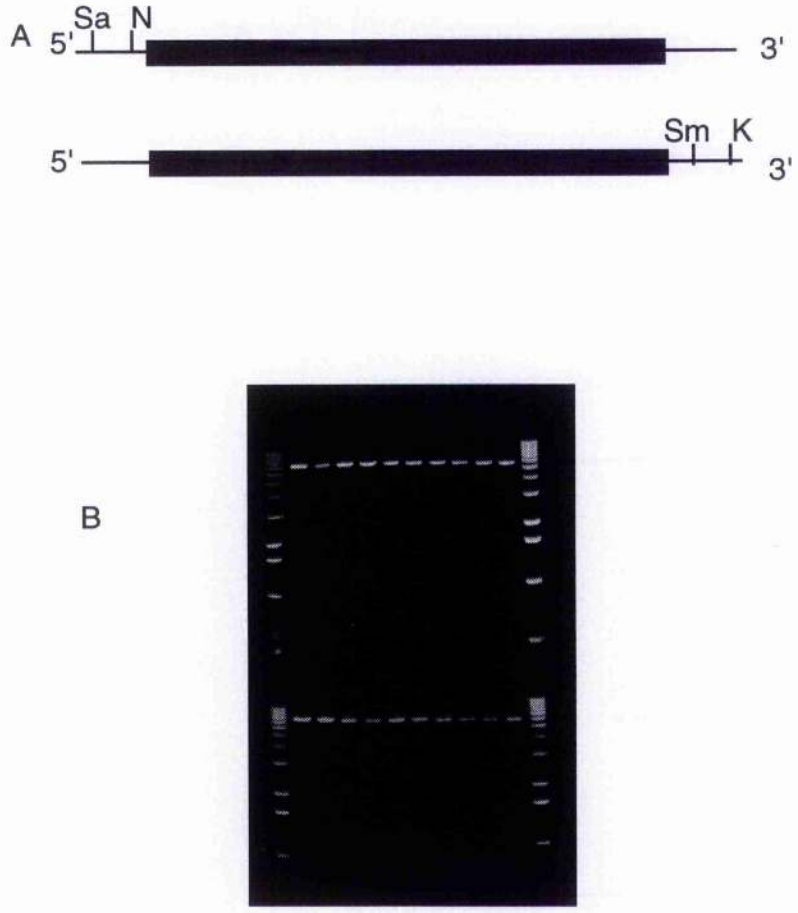
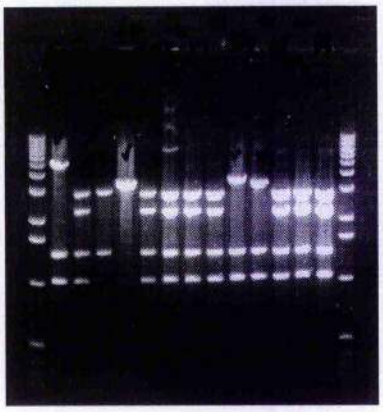


Figure 6.5 *Exo*III deletion of the p26kg insert. (**A**) p26kg structure showing the restriction enzymes selected to make *Exo*III protected and unprotected termini. *Not*I and *Sma*I generate 5' overhanging and thus unprotected termini; *Sac*I and *Kpn*I generated protected termini. (**B**) The first 10 samples of *Exo*III and S1 nuclease digestion of *Sac*I/*Not*I digested p26kg. (**C**) The first 10 samples of *Exo*III and S1 nuclease digestion of *Sma*I /*Kpn*I digested p26kg. Samples of the *Exo*III reaction were in both cases, removed at interval of 30 second.

A



B

Figure 6.6 (A). Plasmid minipreps from the*Sac*I/*Not*I deletion experiment digested with *Xba*I and *Pst*I. **(B)** Plasmid minipreps from the*Sma*I/*Kpn*I deletion experiment digested with *Xba*I and *Kpn*I.

In Section 6.8 we will see that a fly line with a P[*lacW*] insertion in the first intron of *vha26* can indeed be identified.

6.4.3 Generation of unidirectional deletions of p26kg DNA for sequencing

Two pairs of enzyme (*Sac*I/*Not*I and *Sma*I/*Kpn*I) were selected for digesting p26kg, and the resulting DNA fragments are treated with *Exo*III to make deletions from each end (Figure 6.5A). p26kg has no digestion site for any of the four enzymes. 20 timepoints were taken for each *Exo*III digestion. Figure 6.5B and 6.5C shows the first 10 digestions by *Exo*III from either ends. Two colonies from each of the first 9 transformations were selected at random, and plasmid DNAs were digested with *Xba*I and *Pst*I (Figure 6.6 A, B). From the size of the bands we know how far the DNA has been deleted. A set of subclones with different sizes of deletions (Figure 6.6A) were selected for sequencing using primer T3. Another set of subclones was sequenced using primer T7. A genomic DNA fragment covering all of the *vha26* cDNA was sequenced on both strands.

6.4.4 Correlation of genomic and cDNA sequences

The cDNA sequence of p26CD is contained within the 5 kb *Bam*HI fragment of p26kg. It is punctuated by three small introns with in-frame boundaries (Figure 6.7). This is the first description of a genomic DNA sequence, and thus of intron placement in the gene for an in animal E subunit. Intron placement frequently marks functional boundaries within proteins; however, the only other genomic DNA sequence available, for *Neurospora crassa vma4* (Bowman, *et al.*, 1995), shows that intron placement is not precisely conserved between animals and fungi; however, as further genomic sequences are obtained, they may be informative. As with the *N. crassa* gene *vma4*, no TATA or CAAT boxes could be seen upstream of the putative transcriptional start site in the available sequence for *vha26*. This is commonly the case for ubiquitously expressed genes.

```
1                                                   31
caa caa ata cac att ttt acc ctc gca atc gca ggg tca cac ttt cgt gaa atc ata tga
61                                                  91
tcg att tgc agt gaa aat ttt cag acg ttg ggc aga agg caa aag taa ctt atc gtt ttc
121                                                 151
cac ttt cct cgt gtt ggg ccg ccg ttt cca act cag ttc ggc tgt gaa tgt att agc tta
181                                                 211
att aaa ttt caa tta ttt cca gGC ACG GTT GTT GTA CGT GGG CTT CTT TAA AAC ACT TGA
241                                                 271
ATT TCC TTT CGG TTT GTG CAG TGA AAA AAA TCA GTC AAA ATG GCA CTG AGC GAT GCT GAT
                                                                M   A   L   S   D   A   D
301/8                                               331
GTA CAA AAG CAG gta att gaa aac ttg gat tgg gaa cgg gca ggc gat caa ggt cgt agg
 V   Q   K   Q
361                                                 391
gaa aca agc aaa acg aga ggc ttc gtt tgc ctt ttt gcc ttt gca att tgc ctt tgc aat
421                                                 451
aaa gat ggc gaa gtc atg gga tct ccc agg tca tgt gaa ctt ttc acc gcc agt agt cca
481                                                 511
att aga ctg aca tcc ttc caa atc ggc ccg gtc att tgg gag ttg ccg gag ttt tga cat
541                                                 571
att tgt tgg cta atg aag aca cat caa ttt att tgt cca gat agt ttg cgt aaa aag tga
601                                                 631
gta aaa att cgt gct ggt cat gtg aca cgg ccc ccg cat tgg agc aat gtg ttg gag cga
661                                                 691
gac gac tag ccc tgc acc cca cac tcg tac tct ctg tca cac gac cag cga ccc cct tac
721                                                 751
gtt atc aaa act tta acg aaa ata aat aga ggc tag ggt ctt gga cgt ctc cct ttt cca
781                                                 811
ttt atc atg tcc agt tat cat gtg aca cac agg caa cta cta aac agg acg act gtt tca
841/12                                              871/21
gATC AAG CAC ATG ATG GCG TTC ATT GAG CAG GAG GCC AAT GAG AAA GCC GAG GAG ATC GAT
  I   K   H   M   M   A   F   I   E   Q   E   A   N   E   K   A   E   E   I   D
902/32                                              932/42
GCC AAG GCC GAG GAG GAG TTC AAC ATT GAG AAG GGA CGC CTG GTC CAG CAG CAG CGT CTC
 A   K   A   E   E   E   F   N   I   E   K   G   R   L   V   Q   Q   Q   R   L
962/52                                              992/62
AAG ATC ATG GAA TAC TAC GAG AAG AAG GAG AAG CAA GTT GAG CTG CAG AAG AAG ATT CAG
 K   I   M   E   Y   Y   E   K   K   E   K   Q   V   E   L   Q   K   K   I   Q
1022/72                                             1052/82
TCC TCC AAC ATG CTC AAC CAG GCT CGT CTG AAG gtg cgt gtc gtc cag ttg gtg gcc cta
 S   S   N   M   L   N   Q   A   R   L   K
1082                                                1112
aca tat acc gga aaa cac ctt att ctt aat cat tcg taa tgt acc ctg tag GTG CTG AAA
                                                                            V   L   K
1142/86                                             1172/93
GTG CGC GAG GAC CAT GTG AGC AGC GTG CTG GAT GAT GCC CGC AAG CGT CTC GGC GAG GTC
 V   R   E   D   H   V   S   S   V   L   D   D   A   R   K   R   L   G   E   V
1202/106                                            1232/116
ACC AAG AAT CAG TCC GAG TAC GAG ACT GTG CTG ACC AAG CTC ATC GTC CAG GGC CTG TTC
 T   K   N   Q   S   E   Y   E   T   V   L   T   K   L   I   V   Q   G   L   F
1262/126                                            1293/136
CAG ATC ATG GAG CCC AAG GTG ATC CTG CGC TGC CGC GAG GTG GAC GTC CCC CTG GTA CGT
 Q   I   M   E   P   K   V   I   L   R   C   R   E   V   D   V   P   L   V   R
1322/146                                            1352/156
AAC GTC CTG CCT GCC GCT GTG GAG CAA TAC AAG GCC CAG ATC AAT CAG AAC GTC GAG CTG
 N   V   L   P   A   A   V   E   Q   Y   K   A   Q   I   N   Q   N   V   E   L
1382/166                                            1412/176
TTC ATC GAC GAG AAA GAC TTC CTC TCT GCT GAT ACC TGC GGT GGT GTT GAG CTG CTG GCC
 F   I   D   E   K   D   F   L   S   A   D   T   C   G   G   V   E   L   L   A
1442/186                                            1472
CTC AAC GGA CGC ATC AAG gtg agt act gtc ctt tcg gtg gag aga gag caa tcc caa ctg
 L   N   G   R   I   K
1502                                                1533/196
atc taa caa acc act tca g GTG CCC AAT ACG CTG GAG TCC AGA TTA GAC CTC ATT TCG CAG
                          V   P   N   T   L   E   S   R   L   D   L   I   S   Q
```

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1563/206                                                    1551/213
CAG CTG GTG CCC GAG ATT CGT AAC GCA CTT TTC GGC CGC AAC GTC AAT CGC AAA TTC ACC
 Q   L   V   P   E   I   R   N   A   L   F   G   R   N   V   N   R   K   F   T
1623/226                                                    1653
GAC TAA AT TCT ATA AGT GCA AAA CAA AAC ATA ACT AAC CAG AAA GAG AAC CAG CAT CAA
 D   *
1682                                                        1712
CAC CTA TTC AGC AGG AAC AGT TCA AGT TAT TAC ACA GAG CTC CAC CCA CTA AAT ATT GAA
1742                                                        1772
CCC AAG TAA ACT TAT CCT TTG GCA GTC AGG AGG CAA CAG CTA GGA TAT ATT GAT TGT CAA
1802                                                        1832
AAT ACT TTT GCC GTT GTC TTG TAA AGT GAA ATT GAA ACA CTC AAG AAC ATT TCG GTC CTT
1862                                                        1892
GTG TAC GCA ACA GTT TTA ATA GTA ACC ACA CTA AAC GCG CAT ATA TAT TCT CCG ATA TAT
1922                                                        1952
ATG TCT GTA TGC CAA TAC TTA TTA TAT AGT TTA GAG GAC ACG ATC CTA GGA GCA TAC GAA
1982                                                        2012
AGC ATA ATA CGA AGT TTG TTA AAG TTT GTT CGT TTT TTT TTT ACA TAT GCA CAT GTT TCT
2042                                                        2072
GAG CAG TAG GTC TAG ATA TGT GCT TAT ATT GTA TAC ATA CAC TTT AAA ATT TTG CAT ACA
2102                                                        2132
TTC CTG TCC AAG AAT TTT TAT TTC AGT TTT CCC CTT GTT TAT TGT ACA TTA TTT TCT GTA
2162                                                        2192
GTC TTT GTT AAC TTT TTA TAT GTC TAT GTC GTT TAT GTT CGT AAT TAT CAA GTG CAC GTT
2222                                                        2252
CAG GAG GAA CAA CGG CAG TGG ATC GCC CCT TTT ACA GAC CGC TGG CAG GTT GCG ATG CGA
2282                                                        2312
CCA CAC AGC ATT GTT GCT CAG CGA AGC ACC GAA ATG GAC CTA AAC CCC CGA TTT CGC TTC
2342                                                        2372
TTC GAG GGC AAC GGA CGC TTG TGC AAC TGC CAC TGG CTC AAC GAA AGC CCC GAA AAT CAT
2402                                                        2432
CAA TGT CTG TTG TTG TTG AGA TAC CGA GAG TAG AGA ATA CAC ACT GCT TAG CAC GCG ACA
2462                                                        2492
CTT AAT ACC CAT TCA TTA CAC ATG CAC CAC GAC CAT GAA GTT TGC CAA GTA GCT AAG TTG
2522                                                        2552
TTG ACC TGA CCA TCA AGT GCA GCT TTC ACA CCC TCA TAT AAC TAC TTA AAG AAA ATA TAG
2582                                                        2612
AAA AAT GGA AAT TAG TTT TGC AAT TTA GGC CAC TGC CGA ACT GCC ACC GTT TCC ACC TGA
2642                                                        2672
CGT GCG CCA TCA TAT CAG GCT CTA AAA ATC AAC ACA CCA TGT TCA AAC ACA CGA CTA GCA
2702                                                        2732
TAC AGG AGC AGG AGC TAC AGT AAA TTT GAA CCT TGT ATT CGC ATG TTC GCC AAT GTT CAT
2762                                                        2792
AGT GTA TTC TTC AAG CTC ATT TTC TAA CCA AGT TAC CAA GTT CAA TAT GAT GAA TAA CTA
2822                                                        2852
CAA GAT TAG CAA ACA AAT ACA AGT AGC ATA TGC GTT ATT ATA TAA CAT CGA GTA CTA TAT
2882                                                        2912
ACA TTA CAT GAA ATA CAA AAT GCA AGA AAA ATT ACT TTT AAA CAA AAT TTA TGT TGA ATA
2942                                                        2972
AAA AAC AGT ATT TCC AAA AAC TAA Act taa ctg tat aac aac ttc ctt ttg caa tgt tct
3002                                                        3032
aat gat cct aaa aac aag aca tgg ggt aaa cta ttt taa gaa att caa tct agg act caa
3062
tag tct ata gta cca
```

Figure 6.7 Sequence of *vha26* genomic DNA and deduced amino acid sequence of the *Drosophila* E-subunit (GenBank accession No. is U389510. Double-stranded sequencing of the cloned genomic DNA fragment was performed according to the Sequenase™ II protocol (USB) by generation of unidirectional deletions with the Erase-a-Base system (Promega) and also with the aid of synthetic oligo primers when required. The putative polyadenylation signal is underlined.

Although the cDNA (Canton S) and genomic DNA (Oregon R) came from different *D. melanogaster* strains, apart from the genomic DNA having three small introns, the sequences are identical.

6.5 Phylogenetic analysis of the E subunit

The recent availability of deduced sequence from a broad range of phyla allows new insights into the structure of the E subunit. Although the primary sequence is poorly conserved across phyla, the substitutions are generally conservative, even in the distantly related halophilic archaebacterial *Haloferax volcanii* gene. Similarly, the predicted secondary structure is conserved; all members of the family appear to encode predominantly hydrophilic α-helical proteins with conserved N- and C-termini, as noted previously (Bowman, *et al.*, 1995). However, there is a clearer dichotomy between animal and plant/fungal sequences than we have observed for other *D. melanogaster* V-ATPase subunits, suggesting that the E-subunit may have a distinctive role in animals (perhaps plasma membrane or epithelial targeting), which requires the conservation of regions of primary sequence. As the gene appears to be single-copy both in *Manduca* (Gräf, *et al.*, 1994) and *Drosophila*, it is likely that the same gene product serves both endomembrane and plasma membrane roles, so we speculate that in epithelia there may be as yet unidentified conserved accessory proteins which bind conserved domains. For example, an extended 22-aa N-terminal motif DVQKQIKHMMAFIEQEANEKAEE is absolutely conserved in all known animal sequences across a 400 million year evolutionary span, but only 15 residues are conserved in plants, 11 in fungi and 6 in *H. volcanii* (Figure 6.8). Further in the sequence, the motifs QRLKIMEYYEKKEKQ and QKKIQ(S/M)SN(L/M)(L/M)NQARLKVL are absolutely conserved in animals, while being poorly conserved in plants; they also have a particularly high surface probability (as calculated by Mac Vector, IBI). Similarly, at the C-terminus, the motif NTLESRL(D/E)LI(A/S)QQ is conserved only in animals.

(A)

```
VE_arath    1 .......MNDGDVSRQIQQMVRFIRQEAEEKANEISVPAEEEFNIEKLQLVEAEKKKIRQ
VE_mescr    1 .......MNDTDVQNQIQQMVRFMRQEAEEKANEISVSAEEEFNIEKLQLVEAEKKKIRQ
VE_huma1    1 .....MALSDADVQKQIKHMMAFIEQEANEKAEEIDAKAEEEFNIEKGRLVQTQRLKIME
VE_huma2    1 .....MALSDADVQKQIKHMMAFIEQEANEKAEEIDAKAEEEFNIEKGRLVQTQRLKIME
VE_huma3    1 .....MALSDADVQKQIKHMMAFIEQEANEKAEEIDRKAEEEFNIEKGRLVQTQRLKIME
VE_bovin    1 .....MALSDADVQKQIKHMMAFIEQEANEKAEEIDAKAEEEFNIEKGRLVQTQRLKIME
VE_mans1    1 .....MALSDADVQKQIKHMMAFIEQEANEKAEEIDAKAEEEFNIEKGRLVQQQRLKIME
VE_mans2    1 .........DADVQKQIKHMMAFIEQEANEKAEEIDAKAEEEFNIEKGRLVQQQRLKIME
VE_drome    1 .....MALSDADVQKQIKHMMAFIEQEANEKAEEIDAKAEEEFNIEKGRLVQQQRLKIME
VE_yeas1    1 MSSAITALTPNQVNDELNKMQAFIRKEAEEKAKEIQLKADQEYEIEKTNIVRNETNNIDG
VE_yeas2    1 MSSAITALTPNQVNDELNKMQAFIRKEAEEKAKEIQLKADQEYEIEKTNIVRNETNNIDG
VE_neucr    1 .MSQVHALSDDQVGQELRKMTAFIKQEAEEKAREIQIKADEEFAIEKSKLVRQETDAIDS

VE_arath   54 DYEKKEKQADVRKKIDYSMQLNASRIKVLQAQDDIVNAMKDQAAKDLLNVSRDEYAYKQL
VE_mescr   54 EYERKAKQVDVRRKIEYSMQLNASRIKVLQAQDDLVNAMKEAASKELLLVSGDHHQYRNL
VE_huma1   56 YYEKKEKQIEQQKKIQMSNLMNQARLKVLRARDDLITDLLNEAKQRLSKVVKDTTRYQVL
VE_huma2   56 YYEKKEKQIEQQKKIQMSNLMNQARLKVLRGRDDLITDLLNEAKQRLSKVVKDTTRYQVL
VE_huma3   56 YYEKKEKQIEQQKKIQMSNLMNQARLKVLRARDDLITDLLNEAKQRLSKVVKDTTRYQVL
VE_bovin   56 YYEKKEKQIEQQKKIQMSNLMNQARLKVLRARDDLITDLLNEAKQRLSKVVKDTTRYQVL
VE_mans1   56 YYEKKEKQVELQKKIQSSNMLNQARLKVLKVREDHVRNVLDEARKRLAEVPKDIKLYSDL
VE_mans2   52 YYEKKEKQVELQKKIQSSNMLNQARLKVLKVREDHVRNVLDEARKRLAEVPKDIKLYSDL
VE_drome   56 YYEKKEKQVELQKKIQSSNMLNQARLKVLKVREDHVSSVLDDARKRLGEVTKNQSEYETV
VE_yeas1   61 NFKSKLKKAMLSQQITKSTIANKMRLKVLSAREQSLDGIFEETKEKLSGIANNRDEYKPI
VE_yeas2   61 NFKSKLKKAMLSQQITKSTIANKMRLKVLSAREQSLERIFEETKEKLSGIANNRDEYKPI
VE_neucr   60 AYAKKFKQAQMSQQITRSTMANKTRLRVLGARQELLDEIFEAASAQLGQATHDLGRYKDI

VE_arath  114 LKDLIVQCLLRLKEPSVLLRCREEDLGLVEAVLDDAKEEYAGKAKVHA.PEVAVDTKIFL
VE_mescr  114 LKELIVQSLLRLKEPAVLLRCREEDKHHVHRVLHSAREEYGEKACVSH.PEVIVD.DIHL
VE_huma1  116 LDGLVLQGLYQLLEPRMIVRCRKQDFPLVKAAVQKAIPMYKIATKNDV..DVQIDQESYL
VE_huma2  116 LDGLVLQGLYQLLEPRMIVRCRKQDFPLVKAAVQKAIPMYKIATKNDV..DVQIDQESYL
VE_huma3  116 LDGLVLQGLYQLLEPRMIVRCRKQDFPLVKAAVQKAIPMYKIATKNDV..DVQIDQESYL
VE_bovin  116 LDGLVLQGLYQLLEPRMIVRCRKQDFPLVKAAVQKAIPVYKVATKRDV..DVQIDQEAYL
VE_mans1  116 LVTLIVQALFQLVEPTVTLRVRQADKALVESLLGRAQQDYKAKIKKDV..VLKIDNENFL
VE_mans2  112 LVTLIVQALFQLVEPTVTLRVRQADKALVESLLGRAQQDYKAKIKKDV..VLKIDNENFL
VE_drome  116 LTKLIVQGLFQIMEPKVILRCREVDVPLVRNVLPAAVEQYKAQINQNV..ELFIDEKDFL
VE_yeas1  121 LQSLIVEALLKLLEPKAIVKALERDVDLIESMKDDIMREYGEKAQRAPLEEIVISNDYLN
VE_yeas2  121 LQSLIVEALLKLLEPKAIVKALERDVDLIESMKDDIMREYGEKAQRAPLEEIVISNDYLN
VE_neucr  120 LRDLILEGFYAMNEPELVIRARQADYDAVREAAGWASAQYKHKTDKDVKATIDAENPV..

VE_arath  173 PPPPKSNDPHGLHCSGGVVLASRDGKIVCENTLDARLDVAFRMKLPVIRKSLFGQVTA..
VE_mescr  172 PPAPTSYDSHELSCSGGVVMASRDGKIVFENTLDARLEVAFRKKLPQIRKQLFAV.....
VE_huma1  174 PE.........DIAGGVEIYNGDRKIKVSNTLESRLDLIAQQMMPEVRGALFGANANRK
VE_huma2  174 PE.........DIAGGVEIYNGDRKIKVSNTLESRLDLIAQQMMPEVRGALFGANANRK
VE_huma3  174 PE.........DIAGGVEIYNGDRKIKVSNTLESRLDLIAQQMMPEVRGALFGANANRK
VE_bovin  174 PE.........EIAGGVEIYNGDRKIKVSNTLESRLDLIAQQMMPEVRGALFGANANRK
VE_mans1  174 PP.........DTCGGIELIAAKGRIKISNTLESRLELIAQQLLPEIRNALFGRNPNRK
VE_mans2  170 PP.........DTCGGIELIAAKGRIKISNTLESRLELIAQQLLPEIRNALFGRNPNRK
VE_drome  174 SA.........DTCGGVELLALNGRIKVPNTLESRLDLISQQLVPEIRNALFGRNVNRK
VE_yeas1  181 KD.........LVSGGVVVSNASDKIEINNTLEERLKLLSEEALPAIRLELYGPSKTRK
VE_yeas2  181 KD.........LVSGGVVVSNASDKIEINNTLEERLKLLSEEALPAIRLELYGPSKTRK
VE_neucr  178 PE.........GSAGGIIIVGGNGKIDIDNTFEARLTLLKDSALPAMRKALFGENPNRK

VE_arath      ...
VE_mescr      ...
VE_huma1  224 FLD
VE_huma2  224 FLD
VE_huma3  224 FLD
VE_bovin  224 FLD
VE_mans1  224 FTD
VE_mans2  220 FTD
VE_drome  224 FTD
VE_yeas1  231 FFD
VE_yeas2  231 FFD
VE_neucr  228 FFD
```

(B)

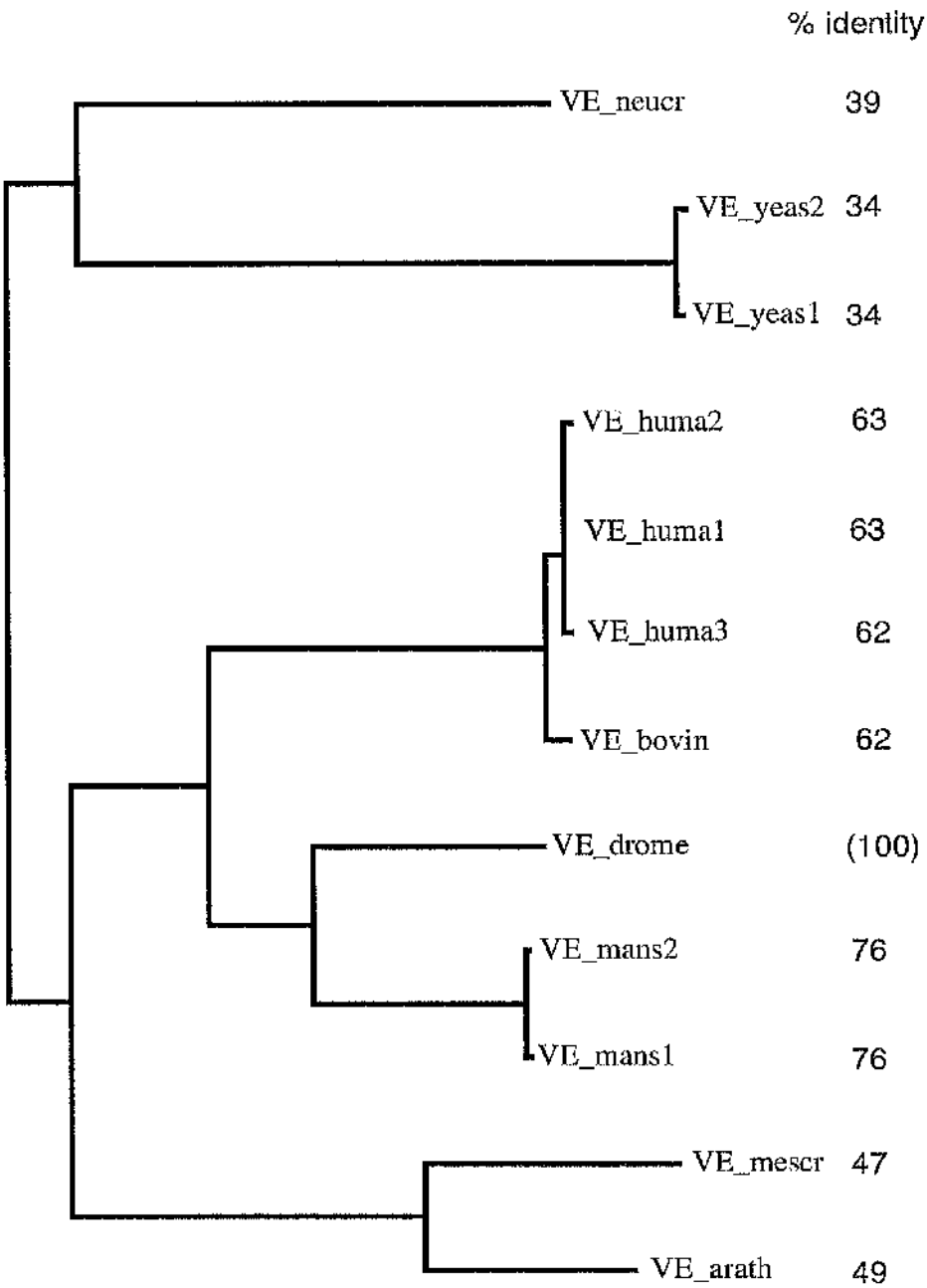

% identity
VE_neucr 39
VE_yeas2 34
VE_yeas1 34
VE_huma2 63
VE_huma1 63
VE_huma3 62
VE_bovin 62
VE_drome (100)
VE_mans2 76
VE_mans1 76
VE_mescr 47
VE_arath 49

Figure 6.8 A: PILEUP (GCG) of polypeptides related to the *Drosophila* E subunit. All sequences are deduced from cDNA. **B:** Phylogenetic tree of V-ATPase E-subunits generated by ClustalW and N-J plot using the PILEUP data.. GenBank accession numbers are as follows.

VE_drome *Drosophila melanogaster* ACCESSION NO.:U38198 and U38951

VE_mans1 *Manduca sexta* accession no.: P31402

VE_mans2 *Manduca sexta* accession no.: S25014

VE-huma1 *Homo sapiens* accession no.: P36543

VE_huma2 *Homo sapiens* accession no.: A42666

VE_huma3 *Homo sapiens* accession no.: JN0909

VE-bovin *Bos taurus* accession no.: P11019

VE_arath *Arabidopsis thaliana* accession no.: X92117

VE_neucr *Neurospora crassa* accession no.: U17641

VE_mescr *Mesembryanthemum crysta* accession no.: X92118

VE_ycas1 *Saccharomyces cerevisiae* accession no.: Z49821

VE_yeas2 *Saccharomyces cerevisiae* accession no..: P22203

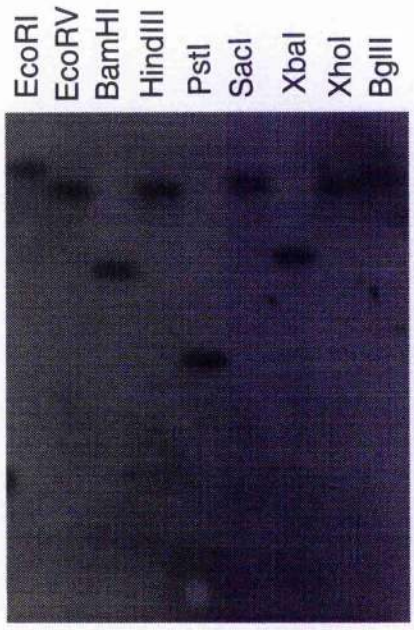


Figure 6.9 Genomic Southern blot of the *vha26* locus. Southern blot of genomic *D. melanogaster* DNA. Genomic DNA purified from wild-type *D. melanogaster* (Canton S) was cleaved with a range of restriction endonucleases, separated by electrophoresis in a 0.8% agarose gel, blotted to Hybond N (Amersham), and hybridised with a ^{32}P-labelled random-primed probe of *vha26* cDNA. Prehybridisation was in Church buffer (7% SDS, 1% BSA, 1 mM EDTA, 0.25 M Na_2HPO4, pH 7.2) at 65 °C for 3 hours, and hybridisation was in Church buffer overnight. The filter was then washed at 65 °C in 2XSSPE, 0.1% SDS for 30 min; 0.5X SSPE, 0.1% SDS for 30 min; and finally in 0.1XSSPE, 0.1% SDS for 30 min and exposed to X-ray film for 1-2 days.

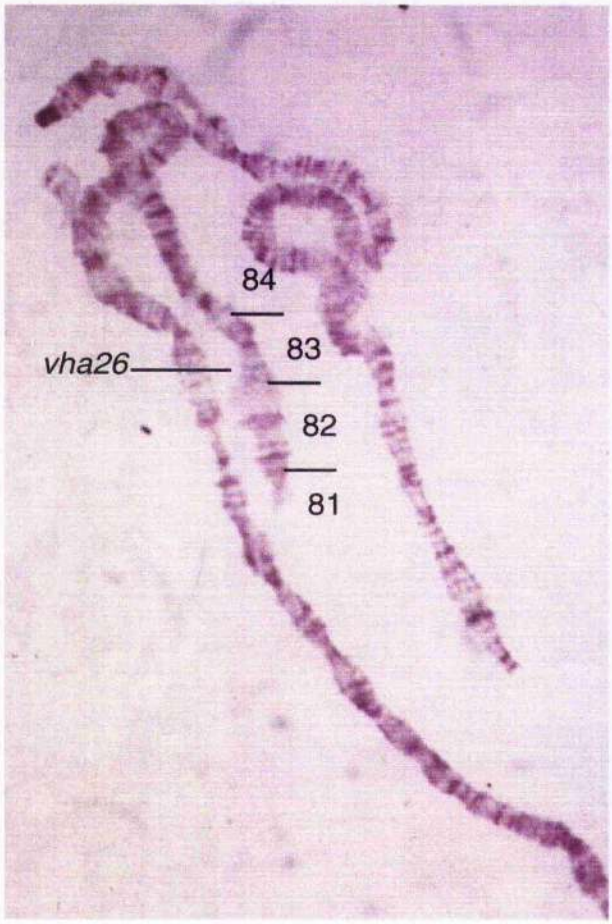


Figure 6.10 Chromosomal localisation of *vha26*. Salivary gland chromosome squashes were prepared by standard techniques (Ashburner, 1989). Chromosomes were probed with biotinylated, random-primed *vha26* cDNA and hybridisation was detected using streptavidin-conjugated peroxidase and diaminobenzidine (Courtesy of Ms. Zhongsheng Wang).

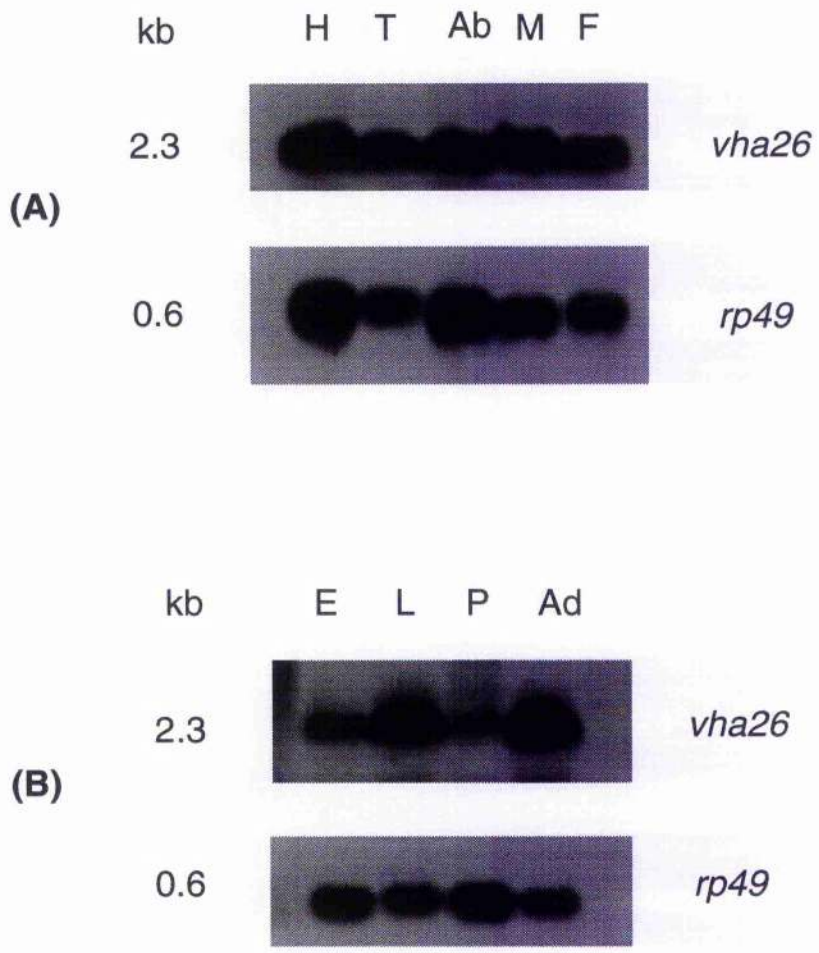


Figure 6.11 Northern blot analysis of vha26 gene expression. Total RNA was isolated using RNA zol^{TM} from Canton S embryos, larvae, pupae and adults; from adult head, thoraces and abdomens; and from male and female adults. The RNA was separated by electrophoresis in 1% formaldehyde-agarose/MOPS gels, blotted to nitrocellulose and hybridised with ^{32}P-labelled random-primed probes. (**A**) Adult tissues. H, head; T, thorax, Ab, abdomen; M, males; F, females. (**B**) Developmental Northern. E, embryo; L, third instar larva; P, pupa; Ad, adult. The filter was first hybridised with a *vha26* cDNA probe, then the same blot was stripped and reprobed with *rp49* as a control for differences in RNA loading.

Recently, it has been shown in *M. sexta* that V-ATPase activity can be controlled hormonally via reversible association and dissociation of the V1 headgroups from the V0 transmembrane sector (Sumner, *et al.*, 1995), and that V-ATPases in *D. melanogaster* tubules are controlled by cAMP and cGMP (Dow, *et al.*, 1994). In this context, it is interesting to note that the insect genes share a C-terminal PKA/PKG phosphorylation site consensus (RKFT) at residues 222-5, although the target threonine is not preserved in other phyla.

6.6 Gene expression

Northern blots of total RNA probed with *vha26* cDNA identify a single band equivalent to a transcript (s) of approximately 2.3kb (Figure 6.11). Different cloned cDNAs differed only in the length of their 5' UTRs, and the genomic sequence identified so far does not contain alternative exons that could be spliced to yield a product of the same size. The simplest interpretation is therefore that a single mRNA species is transcribed from the gene. Equivalent levels of expression are found in adult head, thorax and abdomen (Figure 6.11A) as might be expected for a "housekeeping" gene. The RNA is, however, much reduced during pupation (Figure 6.11B), as is the case with RNA for the *D. melanogaster* 68 kD A subunit (See Chapter 4). In contrast, the 14 kD V-ATPase F subunit RNA is expressed at similar levels during all development (Chapter 7; Guo *et al.*, 1995). In *M. sexta*, it has been suggested that some of the V-ATPase subunits disappear as the midgut pump shuts down during larval moults (Sumner, *et al.*, 1995); it is possible that downregulation of certain critical mRNA species may be involved.

6.7 Identification of a fly line carrying a P[*lacW*] insertion in *vha26*

In situ hybridisation for polytene chromosome places *vha26* at 83B1-4 in chromosome 3. From the Bloomington *Drosophila* Stock Center and the *Drosophila* Genome Center

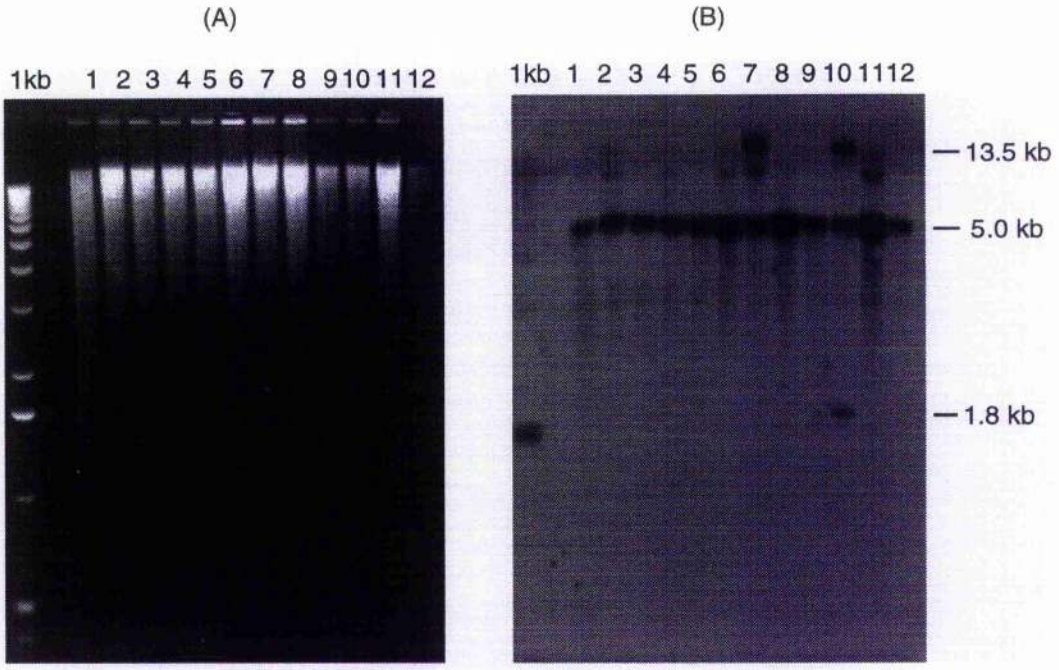


Figure 6.12 Southern blotting of gemonic DNA identified a line carrying a P[*lacW*] insertion in or near the *vha26* gene. (**A**) Photo of Agarose gel of genomic DNA cleaved by *Bam*HI, each lane containing genomic DNA from 10 adult flies. Each lane represents a line with a P-element insertion at 83B. 1, p1560; 2, p1581; 3, p1520; 4, p1609; 5, p1636; 6, p1540; 7, p1644; 8, p1529; 9, l(3)s1938; 10, l(3)j3E7; 11, l(3)j9B6;12, l(3)j5E7. Lines 1-8 were provided by the Bloomington stock centre; Lines 9-12 were from the Drosophila Genome Centre at the Carnegie Institute of Washington. (**B**) Southern blot of the genomic DNA gel (A) probed with p26kg, the 4 kb genomic fragment that includes *vha26*.

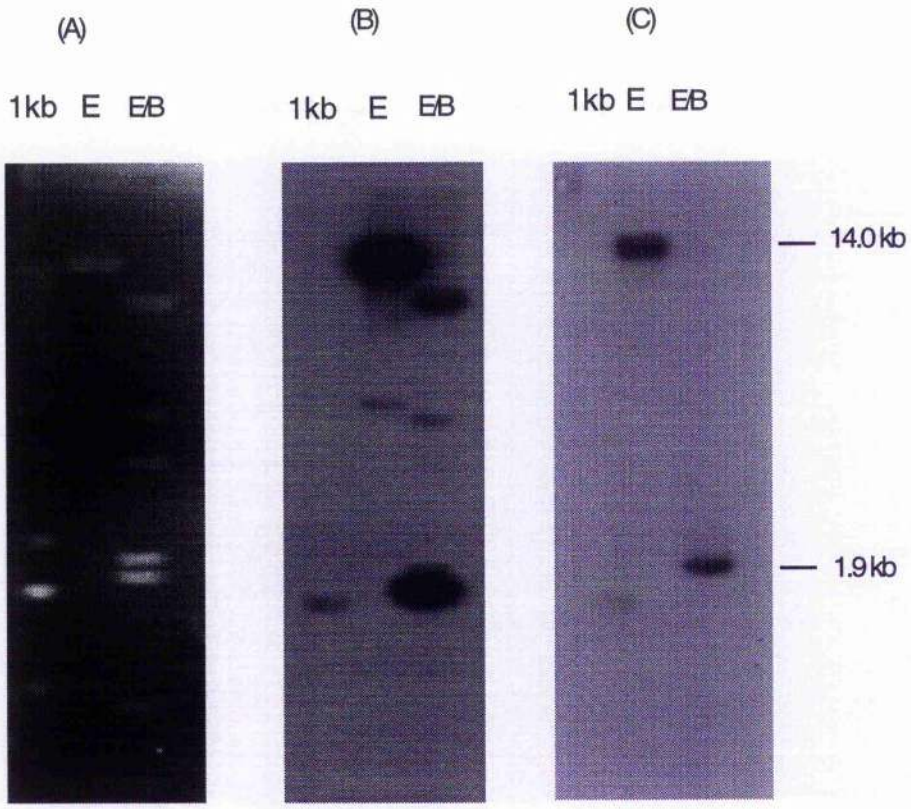


Figure 6.13 Plasmid rescue of DNA flanking the P[*lacW*] element in l(3)j3E7. The restriction enzyme for plasmid rescue was *Eco*RI. (**A**) Restriction digests of rescued plasmid. (**B**) Southern blot of gel (A) probed with p26kg. (**C**) Same filter as (B) stripped and reprobed with the 1.9 kb P[*lacW*] fragment corresponding to the plasmid sequences. E, *Eco*RI ; B, *Bam*HI.

(A)

```
1                                                  31
TTA AGT GGA TGT CTC TTG CCG ACG GGA CCA CCT TAT GTT ATT TCA TCA TGG ATC ATA TGA
61                                                 91
TTT CAC GAA AGT GTG ACC CTG CGA TTG CGA GCG TAA AAA TGT GTA TTT GTT GTC GCT GTC
121                                                151
AGA CCA CCG ATA GAC GAT GTA ATT GTT ATC GCA TTT GTA ACA GAG GCT TCA CTT TAA TCG
181                                                211
ACT AGG TAG AAA AAT CAT GCG ATA TAA TCT ATA TAT GAT AAT GAA AAA TCA ATT TGG CTC
241                                                271
TTT AAA TAT CAT TAT TAT ATT ACT CGA ATA ATC GAG CGT TAA TTT ATA CAT CTG CAT TCC
301                                                331
CGA AAT CCA CAT TAA TTG CCA GTG TGA TCG GAG TAT AAT AAC CTG ACA ATA ATA TGA TGT
361                                                391
GAC AAT ATA AGC CAT CCC TGC TTT ATT GTA AGT GTA TTT TTT AAT GTA CAC ACG CTG ACA
421
AAA GTT GTG TTT CCT TCG GGA TTT CGC TAA GT
```

(B)

```
SCORES            Init1:   248 Initn:   248 Opt:   253
          98.5% identity in 65 bp overlap
         139       129       119       109        99        89
p26k.pr TTACATCGTCTATCGGTGGTCTGACAGCGACAACAAATACACATTTTTACCCTCGCAATC
                                                  ||||||||||||||||||||||||||||
vha26.g                                           CAACAAATACACATTTTTACCCTCGCAATC
                                                            10        20        30
          79        69        59        49        39        29
p26k.pr GCAGGGTCACACTTTCGTGAAATCATATGATCCATGATGAAATAACATAAGGTGGTCCCG
        ||||||||||||||||||||||||||||||
vha26.g GCAGGGTCACACTTTCGTGAAATCATATGATCGATTTGCAGTGAAAATTTTCAGACGTTG
                 40        50        60        70        80        90
          19        9
p26k.pr TCGGCAAGAGACATCCACTTAA

vha26.g GGCAGAAGGCAAAAGTAACTTATCGTTTTCCACTTTCCTCGTGTTGGGCCGCCGTTTCCA
                100       110       120       130       140       150
```

(C)

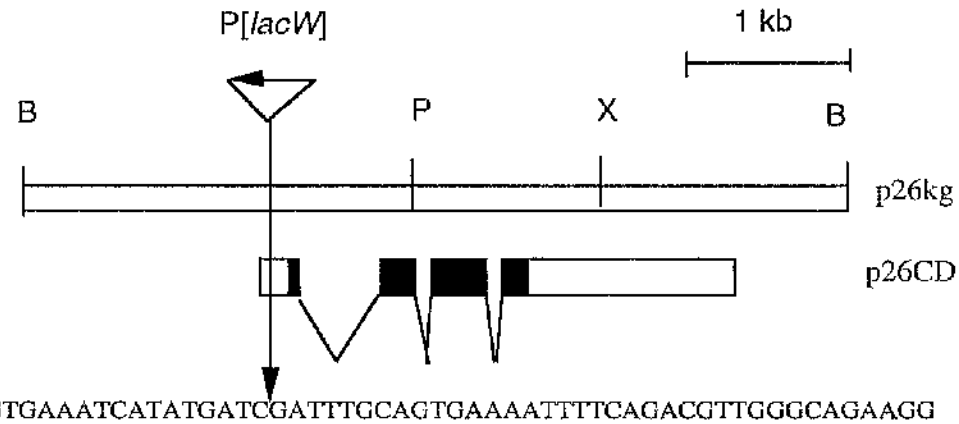


Figure 6.14 (A) Sequence reading out of the rescued plasmid from primer PR-1. (B) Sequence homology of rescued plasmid from line l(3)j3E7 and *vha26*. Underlined indicates the end of the P[*lacW*] insertion. (C) Position of the P[*lacW*] insertion in line l(3)j3E7.

at the Carnegie Institute of Washington, 12 fly lines carrying P-element insertions in this region were obtained. Adult genomic DNA isolated from each line was cleaved by *Bam*HI and separated in 0.8% agrose gel (Figure 6.12 A). A Southern blot of this gel was hybridised with a dro26kg fragment probe (Figure 6.12B) All lanes exhibited a ≈5kb band which hybridised with the 5 kb *vha26* genomic fragment (See Figure 6.4). However, Lane 10 corresponding to fly line l(3)j3E7, exhibited two extra bands of ≈1.8 kb and ≈13.5 kb. This fly line carries a single P[*lac*W] insertion at 83B1-2 (Refer to Encyclopaedia of *Drosophila*). The 5 kb size band in this lane was from the balancer chromosome. The other two extra bands were likely come from the chromosome with the P-element which inserted in gene *vha26*.

P[*lacW*] is an enhancer-trap element that which includes a *lacZ* reporter and bacterial plasmid sequences for rapid plasmid rescue (Bier *et al.*, 1989). *Eco*RI was chosen for digestion of the genomic DNA used for plasmid rescue of line l(3)j3E7 (See Chapter 2 and 3 for methods). Figure 6.13 A shows the rescued plasmid digested with *Eco*R1 (lane 1) and doubly digested with *EcoR*I and *Bam*HI (lane 2). The plasmid digested with *Eco*RI produced two bands of ≈14 kb and ≈4.1 kb. Hybridisation with a dro26kg probe (Figure 6.13B) and with plasmid sequence (Figure 6.13C) shows that the 14 kb band contains both the 1.9 kb plasmid sequence and flanking genomic DNA which hybridises to *vha26* genomic DNA. The 4.1 kb fragment comes either from incomplete digestion or from "co-cloning" in the process of plasmid rescue. The plasmid after double digestion with *Eco*RI and *Bam*HI released a 1.8 kb *vha26* genomic fragment which is of a same size as the band found in the genomic Southern blot (Figure 6.12).

Figures 6.12 and 6.13 strongly suggested that the P[*lacW*] insertion in line l(3)j3E7 is in the *vha26* gene. As the rescued plasmid by *Eco*RI was 14+4.1 kb, the orientation of the insertion should be opposite to *vha26*gene, otherwise the rescued plasmids should be much smaller because there are several *Eco*RI sites immediately 3' prime to the

dro26kg fragment (See Figure 6.4). Sequencing the rescued plasmid specified the P[*lacW*] insertion to the 5' of *vha26* (Figure 6.14).

6.8 Discussion

This chapter reports the first genomic sequence and chromosomal localisation for a V-ATPase E-subunit in an animal. Alignment with a few E subunit sequences clearly shows that *Drosophila* gene to be conserved across eukaryote and prokaryote phyla. It has been possible to identify extended motifs diagnostic of either all members or merely animal members of the family. Expression studies suggest that *vha26* mRNA may fall into a subclass of V-ATPase subunits which is not expressed continually during the life of the insect. This characterisation of *vha26* is the first step to elucidate further the function of the subunit in an organismal context by *Drosophila* genetics.

The isolation of a P[*lacW*] insertion in gene vha26 might be of great use for analysis the function of V-ATPase E-subunit in *Drosophila*. The *lacZ* gene in P[*lacW*] may allow detection of the domain of expression of the gene. Precise and imprecise excision of the P-element will generate new alleles. More detailed mutational analysis based on the P[*lacW*] insertion line will be carried out in the near future. See chapter 5 for examples of this kind of analysis.

Chapter 7

vha14, the Gene Encoding a 14 kDa F Subunit of the V-ATPase

7.1 Summary

A *Drosophila melanogaster* cDNA for the 14 kDa F-subunit has been cloned via homology with the corresponding *M. sexta* gene. Its deduced translation product is a 124 amino acid polypeptide sharing 90% identity with the *M. sexta* polypeptide and 50% identity with an analogous polypeptide of *Saccharomyces cerevisiae*. Homology was also found with expressed sequence tags from a variety of other species, indicating that the subunit is phylogenetically conserved. The *Drosophila* gene (*vha14*) is present as a single copy at cytological position 52B on the second chromosome, and gives rise to an mRNA species of 0.65 kb. Abundance of the *vha14* transcript, relative to an *rp49* control, shows relatively little variation during development and between adult head, thorax and abdomen, suggesting that the F-subunit is a relatively ubiquitous component of the V-ATPase.

7.2 Introduction

The gene encoding F-subunit of V-ATPases was first identified from Tobacco hornworm midgut (*Manduca sexta*) and subsequently from yeast and mammalian. Cloning of a cDNA for the F-subunit and demonstration that the polypeptide is indeed a component of the *M. sexta* V-ATPase, was carried out as follows (Gräf et al., 1994b). A polyclonal antiserum against *M. sexta* plasma membrane V-ATPase was used to screen a cDNA expression library, leading to characterisation of a gene that encodes a 14 kDa polypeptide (Gräf *et al.*, 1994). A fusion protein was then used to purify monospecific

antibodies against the gene product. Such antibodies both cross-reacted with the F-subunit on a Western blot and were able to abolish *M. sexta* V-ATPase activity *in vitro* (Gräf *et al.*, 1994). Though Western blotting failed to detect membrane components from other species (Gräf *et al.*, 1994), a related *S. cerevisiae* gene (*VMA7*) was subsequently described, null mutations of which show properties characteristic of other classes of V-ATPase null (Graham *et al.*, 1994; Nelson *et al.*, 1994). Another related gene (*NtpG*) appears to encode a component of the Na^+-pump from the microbe *Enterococcus hirae* (Takase *et al.*, 1994). While these results confirm the F subunit as an essential component of some V-ATPases, it is not clear whether it is a general component, or instead serves a specialised role in holoenzymes from particular tissues. In principle, the powerful genetic tools unique to *Drosophila* (Rubin, 1988) may allow a more detailed resolution of this question. As a first step to such an analysis, this chapter reports the cloning and characterisation of *vha14*, the *D. melanogaster* gene encoding the F-subunit.

7.3 cDNA cloning and DNA sequence analysis

A *D. melanogaster* head λZapII cDNA library was screened by plaque hybridisation with a cloned cDNA for the *M. sexta* F-subunit. Hybridisation signals were obtained at approx. 1:10,000 and three plaques were purified by successive rounds of screening. One of these cDNAs was excised as pBluescript and sequenced on both strands, using synthetic oligonucleotides to extend the reading. The 595 bp contig contains an open reading frame corresponding to a 124 amino acid polypeptide of $M_r \approx 13.9$ kDa (Figure 7.1), which is clearly a V-ATPase F-subunit, sharing 90.3% identity with the F-subunit of *M. sexta* (insect), and 49.6% identity with that of *S. cerevisiae* (Figure 7.2). In accordance with the nomenclature for other *D. melanogaster* V-ATPase loci, the gene has been named *vha14*.

```
TCCACATCGCTCGTAAGAAAAAATTAGAAAAAACCAATCGAAATGGCTCTGCACTCGGCA    60
                                                M  A  L  H  S  A     6
ATCAAGGGAAAACTGATCAGCGTTATCGGCGACGAGGACACCTGTGTGGGCTTTCTGCTC   120
 I  K  G  K  L  I  S  V  I  G  D  E  D  T  C  V  G  F  L  L     26
GGCGGAGTGGGCGAGATCAACAAGAATCGCCATCCCAACTTTATGGTGGTCGACAAAAAT   180
 G  G  V  G  E  I  N  K  N  R  H  P  N  F  M  V  V  D  K  N     46
ACGGCCGTCAGCGAACTGGAGGACTGTTTCAAGCGTTTCCTTAAGCGGGACGATATCCAC   240
 T  A  V  S  E  L  E  D  C  F  K  R  F  L  K  R  D  D  I  D     66
ATCATTCTAATCAACCAGAACTGCGCCGAGCTTATTCGTCATGTGATCGATGCCCATACG   300
 I  I  L  I  N  Q  N  C  A  E  L  I  R  H  V  I  D  A  H  T     86
TCGCCCGTGCCCGCTGTTTTGGAGATTCCCTCCAAGGACCATCCGTACGACGCCAGCAAG   360
 S  P  V  P  A  V  L  E  I  P  S  K  D  H  P  Y  D  A  S  K    106
GACTCCATTCTGCGTCGCGCCCGCGGCATGTTCAATCCGGAGGATCTGGTGCGCTAATTC   420
 D  S  I  L  R  R  A  R  G  M  F  N  P  E  D  L  V  R  *       124
CTCGAATTCTGCTCGAGGACACTGTTTCGTATTGCTGCAACCGCCAGAGTATTGCTTTAC   480
ACCCTGTAAACAACTATCCATACATTCAGTGCTTCGCCTTTGTTCTTATCGTGTATTTAA   540
AGACATTTATTAAATGGTTTTCGTTGTATAAATAGATTAAA                      581
```

Figure 7.1 Sequence of a *vha14* cDNA, and deduced amino acid sequence of the *Drosophila* F-subunit (GenBank accession no. Z26918).

The putative start codon between nucleotides 43-45 is embedded within a region of perfect agreement with the canonical eukaryotic translation initiation sequence, RNNMTGG. A 3' UTR of 164 bp separates the stop codon at nucleotide position 415-417 from a 16 residue poly(A) tract. As in the case of the cloned cDNA for the *D. melanogaster* 16 kDa subunit (Meagher *et al.*, 1990), there is no canonical polyadenylation signal. There is, however, the motif ATTAAA between nucleotides 548-552, centred 26 bp before the start of the poly-A tract. In *M. sexta*, there are two F-subunit transcripts, distinguished by the length of 3' UTR (Gräf *et al.*, 1994). The shorter of the two has a AATAAA motif, though unusually close to its poly(A) tract, whereas the longer has in addition an ATTAAA motif centred 17 bp before the poly(A) tract . Thus this may be a polyadenylation signal for these RNAs.

7.4 Amino acid sequence comparisons

In addition to matches to *M. sexta* and *S. cerevisiae* F-subunit sequences, a search of the GenBank database using the programmes TFASTA (GCG) and BLAST (NCBI) revealed matches to expressed sequence tags (ESTs) from human fetal lung, spleen, and brain; from the plants *Arabidopsis thaliana* and *Oryza sativa*; from the nematode worms *Caenorhabditis elegans* and *briggsiae*; and from the malarial parasite *Plasmodium falciparium* (Figure 7.2A). Probably due to EST sequencing errors, it was occasionally necessary to switch reading frames in order to maximise alignment (see legend to Figure 7.2A). We can thus extend greatly the known phylogenetic base for the occurrence of the F-subunit, which is clearly distributed widely and conserved in plants, animals and fungi (Figure 7.2B). We can also add greatly to the authority of the suggestion of similarity between the Na^+ ATPase of the bacterium *Enterococcus hirae* and the V-ATPases, as most of the residues identified as matching the *M. sexta* sequence can now be seen to be conserved among all the V-ATPase subunits (Figures 7.2A and 7.2B)

(A)

```
VF_ATTS       1 MAGSSYIPARNSALIAMIADEDTVVGLIMAGVGNVDIRRKTNYLIV..DSKTTVXQIEDA
VF_RICC       1 MAGRPSIPTNSSALIAIIADEDTVTGFLLAGVGNVDLRKKTNYLIV..DNKTIVKQIEDA
VF_CELEG      1 .....MASAAKGKILAVIGDEDTVVGFLLGGVGELNKARKPNYLIV..DKQTTVQEIEFA
VF_R02891     1 ............................GGVGELNKARKPTTLIV..DKQTTIQEIEDA
VF_F06548     1 ........AGRGKLIAVIGDEDTVTGFLLGGIGELNKXRHPNFLVV..EKDTTXNEIEDT
VF_F07836     1 ........AGRGKLIAVIGDEDTVTGFLLGGIGELNKXRHPNFLVV..EKDTTXNEIEDT
VF_F08542     1 ........AGRGKLIAVIGDEDTVTGFLLGGIGELNKXRHPNFLVV..EKDTTIXEIEDT
VF_D31181     1 ....AAGMAGRGKLIAVIGDEDTVTGFLLGGIGELNKNRHPNFLVV..EKDTTINXIEDT
VF_DROME      1 ...MALHSAIKGKLISVIGDEDTCVGFLLGGVGEINKNRHPNFMVV..DKNTAVSELEDC
VF_MANSE      1 ...MALHAAVKGKLISVIGDEDTCVGFLLGGIGEINKNRHPNFMVV..DKNTPVSEIEEC
VF_T57982     1 ..........................................................IEDT
VF_YEAST      1 .......MAEKRTLIAVIADEDTTTGLLLAGIGQITPETQEKNFFVYQEGKTTKEEITDK
VF_T02519     1 ..........................................................AREEV
VF_NTPG       1 ........................MTYKIGVVGDKDSVSPFRLFGFDVQHGTTKTEIRKT

VF_ATTS      59 FKEFS.GXDDIAIILSSHFIANMIRFLVDSYNKPV.PXILEIPSKDHPYDPDFESVLSRV
VF_RICC      59 FKEFT.TREDIAIVLISQYVANMIRYLVDSYNRPV.PAILEIHSKDHPYDQDRFCSFWVK
VF_CELEG     54 FNGFC.ARDDIAIILINQHIAEMIRYAVDNHTQSI.PAVLEIPSKEAPYDPSKDSILNRA
VF_R02891    30 FKGFC.ARDD..XILINQHIAEMIRYAVDQHTQSI.PAVLEIPSKEAPYDPSKDSILNRA
VF_F06548    51 FRQFL.NRDDIGIILINQYIAEMVRHALDAHQQSI.PAVLEIPSKEHPYDX.........
VF_F07836    51 FRQFL.NRDDIGIILINQYIAEMVRHALDAH*QSI.PAVLEIPSKEHPYDAA........
VF_F08542    51 FRQFL.NRDDIGIILINQYIAEMVRHALDAHXQSI.PAVLEIPSKEHP............
VF_D31181    55 FRQFL.NRDDIGIILINQYIAEMVRHALDGHQQSI.PAVLGIPFKE..............
VF_DROME     56 FKRFL.KRDDIDIILINQNCAELIRHVIDAHTSPV.PAVLEIPSKDHPYDASKDSILRRA
VF_MANSE     56 FKRFV.KRDDIDIILINQNVAELVRHVIDAHTAPV.PSVLEIPSKDHPYDASKDSILRRA
VF_T57982     5 FRQFL.NRDDIGIILINQYIAEMVRHALDAHQQSI.PAVLEIPSKEHPYDAAKDSILRRA
VF_YEAST     54 FNHFTEERDDIAILLINQHIAENIRARVDSFTNAI.PAILEIPSKDHPYDPEKDSVLKRV
VF_T02519     6 FKEYS.SKHDCGVILINQQIADEIRYLVDLHDKIL.PIVLEIPSKDKPFDPNKDSIIQRV
VF_NTPG      37 IDEM..AKNEYGVIYITEQCANLVPETIERYKGQLTPAILIPSHQGTLGIGLEEIQNSV

VF_ATTS     117 KYLFSAESVSQR
VF_RICC     117 NCFL*.......
VF_CELEG    112 RGLFNPEDFR..
VF_R02891    86 RGLFNPEGFR..
VF_F06548       ............
VF_F07836       ............
VF_F08542       ............
VF_D31181       ............
VF_DROME    114 RGMFNPEDLVR.
VF_MANSE    114 KGMFNPEDLVR.
VF_T57982    63 RXLFTAEDLR..
VF_YEAST    113 RKLFGE......
VF_T02519    64 KLFFGGDISHL.
VF_NTPG      95 EKAVGQNIL...
```

(B)

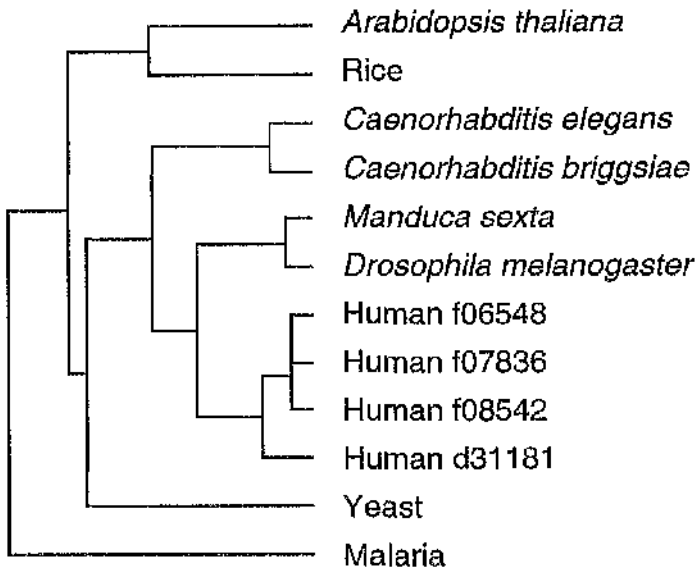


Figure 7.2 A: Alignment of known 14-kDa F-subunit aa sequences. All sequences are deduced from cDNA. GenBank accession numbers are as follows:

A. thaliana, ATTS2695 and ATTS 3474;

Oryza sativa (rice) callus, RICC1365A;

C. elegans, Z49073;

C. briggsiae, R02891 and R02892;

H. sapiens infant human brain, F06548, F07836, F08542;

H. sapiens fetal lung, D31181;

D. melanogaster head, Z26918;

M. sexta midgut, X67130;

S. cerevisiae; U10073; *P. falciparum*, T02519; *ntpG*, D17462.

B: Phylogenetic tree of V-ATPase F-subunits generated by PILEUP using default parameters.

The many human ESTs show some differences in amino-acid sequence (Figure 7.2A); but it should be noted that they are all at least 98% identical at the DNA level, with many of the differences being ambiguous nucleotides in their sequences. It seems likeliest at present that the human ESTs are all cDNAs from the same human gene.

In common with other F-subunits (Figure 7.2), the N-terminus of the *Drosophila* polypeptide lacks a known membrane targeting sequence. Since the polypeptide is also hydrophilic and is accessible to antibodies (Gräf *et al.*, 1994), this would be compatible with it being synthesised cytoplasmically. A search of the Prosite polypeptide motif database also revealed extended similarity to a casein kinase II phosphorylation site, beginning at amino acid 50 (SELED), and the motif is conserved in the F-subunit of *M. sexta* (though not in other F-subunits). Although there are few clues as to how V-ATPases might be regulated (Sumner *et al.*, 1995), and there is not yet evidence for the action of any particular kinase, V-ATPases demand a large fraction of the cellular energy budget (Dow and Harvey, 1988), and are known to be hormonally regulated in both *Manduca* midgut (Sumner*et al.*, 1995) and *Drosophila* Malpighian tubules (O'Donnell *et al.*, 1995).

7.5 *vha14* is a single copy gene

D. melanogaster genomic DNA, cleaved with various restriction enzymes, was blotted and probed at high stringency with *vha14* cDNA (Figure 7.3). The single band of hybridisation seen in each lane suggests a single genetic locus. This is consistent with *in situ* hybridisation to polytene chromosome squashes, which identifies a single locus at 52B on the right arm of chromosome 2 (not shown). Several uncharacterised lethal alleles have been mapped to 52A-D as part of more detailed studies of two neighbouring loci, *hexokinase-C* and *pox-N*. For example, eight lethal complementation groups (*l(2)52ACa-h*) uncovered by Df(2R)XTE-18 have been documented (Davis and MacIntyre, 1988).

Drosophila genes encoding several other V-ATPase subunits have recently been cloned and characterised. Chapter 4-6 has reported the characterisation and mutagenesis of the A and E subunit genes. Inactivation of *vha26* or *vha68-2* lead to a homozygous lethal phenotype. This Glasgow group has also been working on the B and c subunits of *Drosophila* V-ATPase. *vha55*, the gene for the B-subunit, corresponds to a known lethal complementation group, *SzA* (Davies *et al.*, 1995; Gausz *et al.*, 1979), extreme alleles of which are recessive embryonic or early first instar larval lethals. Malpighian tubules of dying individuals are transparent, a defect that is cell-autonomous in transplants (Gausz *et al.*, 1979). Such a phenotype can be reconciled with the critical role of V-ATPases in transporting epithelia (Dow, 1994; Wieczorek, 1992). Since one might predict a similar phenotype associated with null alleles of other essential V-ATPase subunits, this may provide a way of screening candidate lethals at the*vha14* locus.

7.6 Gene expression

Northern blots of total RNA probed with *vha14* cDNA identify a single band equivalent to a transcript(s) of approximately 0.65 kb (Figure 7.4). Normalisation with respect to an*rp49* control indicates little modulation during development (Fig. 7.4A)
Moreover, equivalent levels of expression are found in adult head, thorax and abdomen (Figure 7.4B), as might be expected for a gene involved in the basic aspects of function.

M. sexta cDNAs corresponding to the F-subunit differ by 97 bp in the length of their 3' UTRs (Gräf*et al.*, 1994). While all three cDNAs isolated here have the same 3' end, it cannot be ruled out that the single band seen in chromosomal *in situ* hybridisation comprises more than one transcript class.

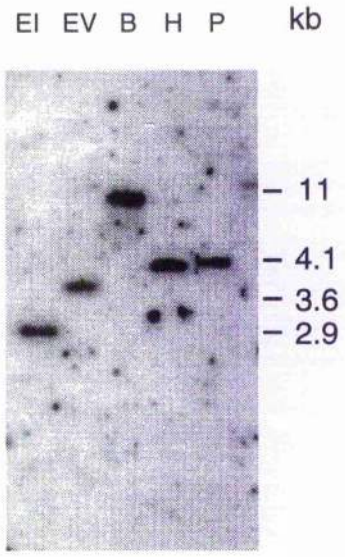
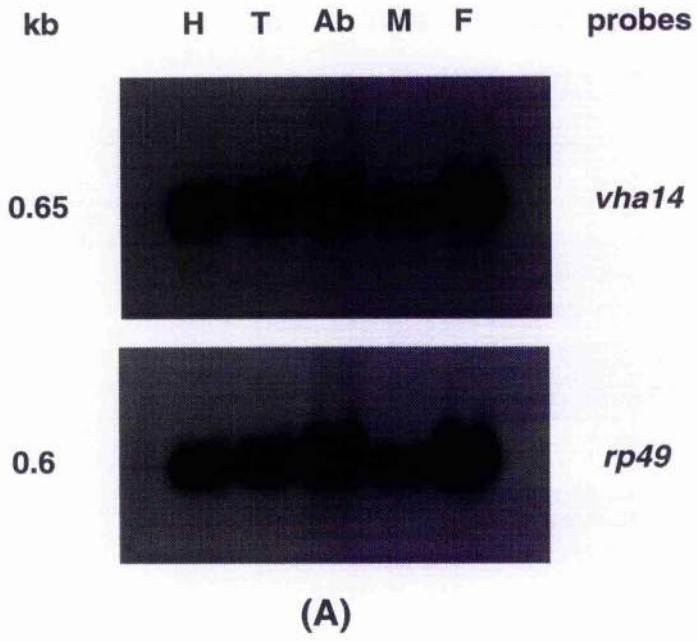


Figure 7.3 Southern blot of *D. melanogaster* genomic DNA cleaved with the following enzymes: lane 1, *Eco*RI; lane 2, *Eco*RV; lane 3, *Bam*H1; lane 4, *Hin*dIII; lane 5, *Pst*I. The blot was probed with a 400 bp *Xho*I/*Xba*I fragment of *vha14* cDNA, which contains no sites for the above enzymes.



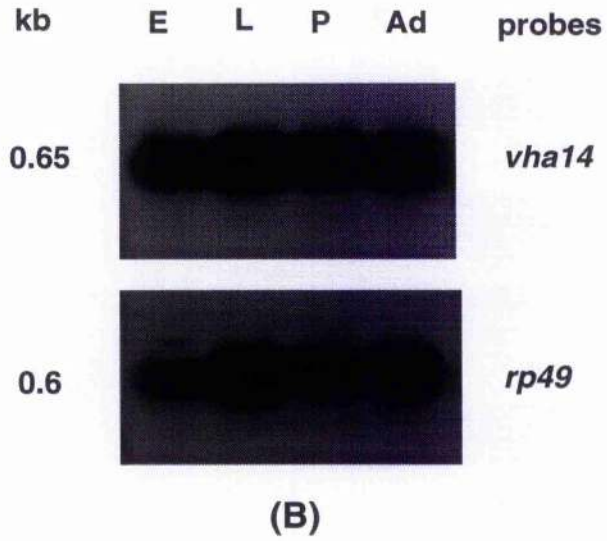


Figure 7.4 Northern blot analysis of *vha14* gene expression. (**A**) Adult tissues. H, head; T, thorax; Ab, abdomen; M, adult males; F, adult females. (**B**) Developmental stages. E, embryo; L, third instar larva; P, pupa; Ad, adult. The lower panels in both (A) and (B) show the same blots, stripped and reprobed with cDNA for the ribosomal protein gene, *rp49*, This controls for differences in RNA loading.

7.7 Discussion

The *Drosophila vha14* has been cloned by homology with a gene thought to encode a subunit of *M. sexta* V-ATPase, and that is expressed in *M. sexta* midgut. An analogous subunit has been identified by homology in another V-ATPase model, the yeast *S. cerevisiae* , and has been shown to be essential for proper assembly of the yeast V-ATPase holoenzyme (Graham *et al.*, 1994). Is the F-subunit a genuine V-ATPase subunit, or an accessory; and is it a specialisation for either a plasma membrane or endomembrane role of the V-ATPase? The widespread tissue distribution implied by the human ESTs and the broad phylogenetic distribution implied by ESTs from other species would suggest that this cannot be uniquely a subunit of a plasma-membrane form of the V-ATPase. The ubiquitous spatial and temporal expression of *vha14* in *D. melanogaster* reported here further supports the suggestion that this is a general subunit which exists in all V-ATPases. A definitive demonstration of an essential role of *vha14* in animal V-ATPase function will depend on the future identification of a null allele, for which *Drosophila* is likely to be a uniquely suitable model. Possibly a pre-existing mutant corresponding to the locus can be identified can be identified (as described earlier). Alternatively, a novel allele could be generated by P-element mutagenesis. Such studies should help in elucidating the function of F subunit in V-ATPase.

Chapter 8

Discussion and Future Work

This thesis consists of two main parts: (i) a set up of a fast and efficient method to correlate cloned genes to P-element mutants and (ii) cloning, characterisation and mutagenesis of genes encoding *Drosophila* V-ATPase. Chapter 3 described the approach of site-selected mutagenesis of *Drosophila* genes *via* plasmid rescue. 1836 fly lines have been plasmid rescued individually and a simple procedure to screen mutants for a target genes has been set up. Initially screening has isolated mutations for more than 10 genes. Sufficient plasmid DNA has been prepared to allow screening for many targets.

8.1 One-step screening to correlate cloned gene to P-element lines

As an alternative to screening pools of plasmids, an one-step screening procedure involving grids of colonies created by a robotic device has been tried. The entire grid is visualised by hybridisation with a ^{35}S probe for the plasmid replicon, whist individual colonies corresponding to particular insertion sites are visualised with a ^{32}P probe specific to the gene of interest. Unfortunately the robotic equipment is unavailable in Glasgow and the hybridisation to the grids was not as sensitive as that described in Chapter 3. Here, I propose an improved screening procedure which reduces the former three rounds of screening to one single hybridisation while still retaining the sensitivity (Figure 8.1). A large cube made of 1000 small cubes each representing the plasmid(s) from a *Drosophila* line. The 1000 plasmids are pooled into 10 pools from each dimension of the cube with each pool containing 100 plasmids. By pooling from the three dimensions a total of 30 pools of plasmids are obtained which can be loaded into a gel of 30 lanes. A single hybridisation of the Southern blot could easily assign any positive signal to the corresponding fly line. Screening for the 1836 plasmids from the second chromosome

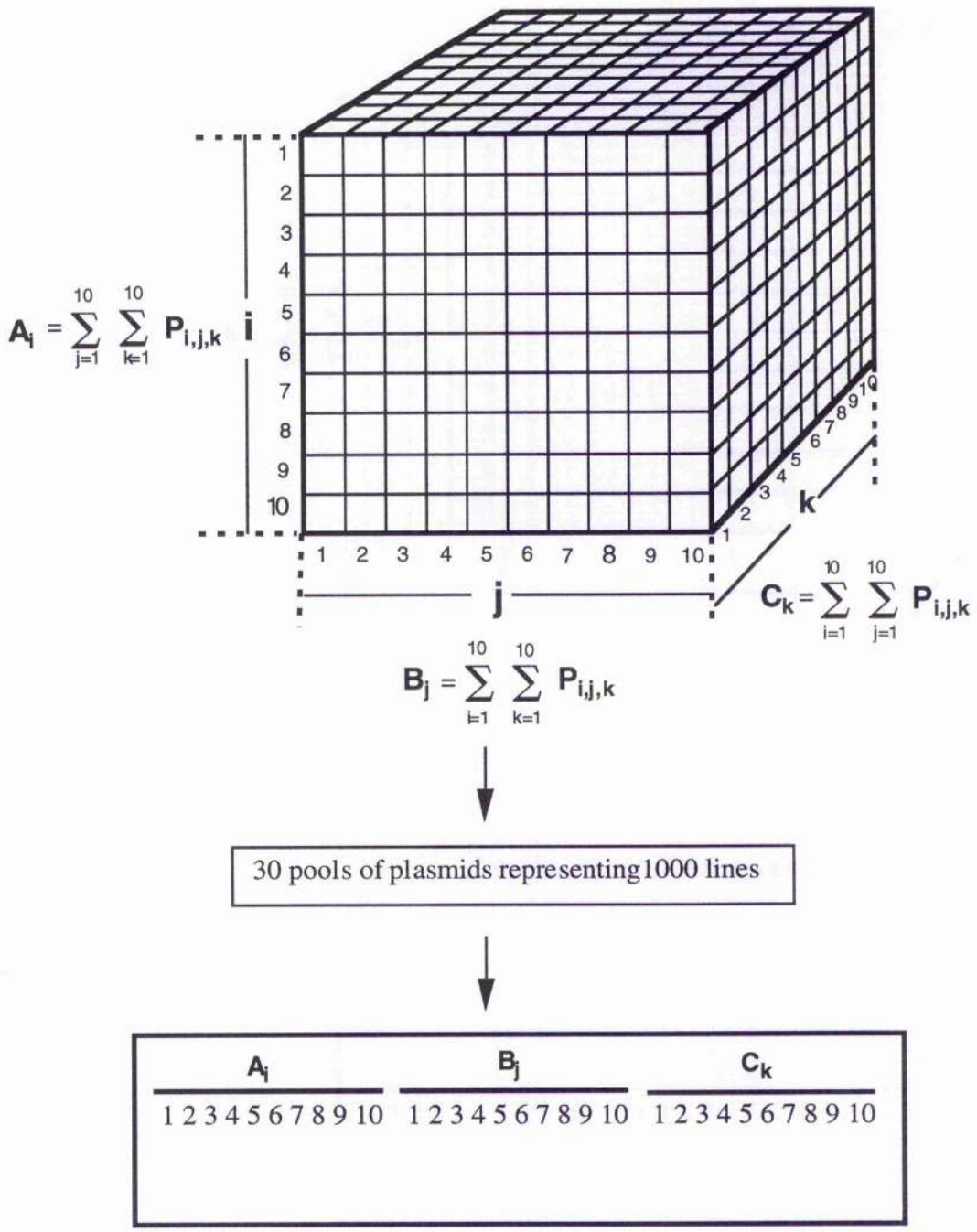


Figure 8.1 A strategy of pooling plasmids for One-step screening. The cube represents plasmids from 1000 individual *Drosophila* lines. $P_{i,j,k}$ (i,j,k=1, 2, 3, ..., 10) stand for the individual plasmid. P_i, P_j and P_k (i, j, k=1, 2, 3, ..., 10) stand for the pool of 100 plasmids pooling from each of the three dimensions. All the 30 pools of DNA could be loaded in a single gel. A single hybridisation of the Southern blot could easily assign any positive signal to the corresponding fly line.

insertion line (see Chapter 3) could be simplified if the individual plasmids are re-pooled according to Figure 8.1. This pooling strategy will be applied to the work of the third chromosome lines. Approximately 2500 fly lines with P-element in third chromosome are being plasmid rescued individually (collaborated with Dr. Peter Deak). The resulting transformed *E. coli* will be pooled from three directions for maxi DNA preparation.

8.2 The correlation of cDNA library clones with the P-element lines

Except for the use in site-selected mutagenesis, the large amount of rescued plasmids can also be utilised in the correlation of individual clones within *Drosophila* cDNA library with the individual flies bearing a P-element. This would provide access to many unknown but essential *Drosophila* genetic loci. A procedure likely to be suitable for large scale screening for cDNA clones with our rescued plasmids is proposed (Figure 8.2). The whole rescued plasmids (including the vector) can be directly labelled if the cDNA library is in a vector such as *lambda* NM1149, which shares no sequence homology with the vector sequence of the rescued plasmids. The cDNA library are laid out as plaques in a rectangular grid by a robotic device constructed by this group (Mackenzie *et al.*, 1989). The device can easily generate 6 or more arrays of 1000 clones and produce as many filter replicas of each as desired. The filter can be screened by probes of pooled plasmids representing 10 or 100 lines depending on the sensitivity of the probe. As the plaque is laid out individually in the grid, positive plaques will represent a single cDNA clone without need for a further round of screening. However, as the probe is labelled from a pool of plasmids, the cDNA clone needs to be further labelled to screen the filter of plasmids (obtained as in figure 8.1) to be correlated to the mutant flies, thus a pair of cDNA and mutant is obtained. This pair, very possibly, represents a mutation of a gene. In cases wherever insertion is near the gene, local jumping or deletion could possibly mutate the gene. For flies being homozygous lethal there is high possibility for each of the rescued plasmid to detect one cDNA and hence one informative insertion. The resulting cDNA/P-element line pair would be subjected to preliminary studies: Lines

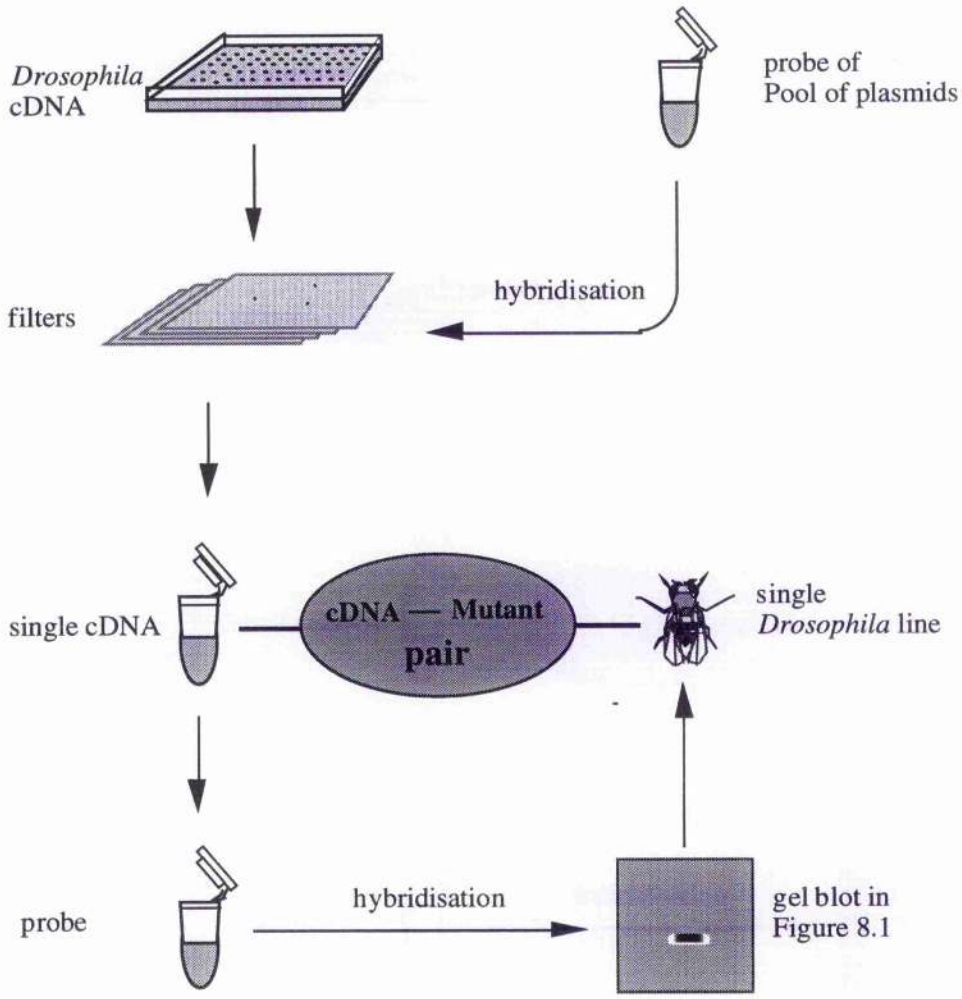


Figure 8.2 Large scale correlation of *Drosophila* cDNA clones to P-element insertional mutants. The pools of plasmids are labelled to screen filters of cDNA clones. Any positive cDNA clone is further labelled to screen the gel blot of the pooled plasmids (as in Figure 8.1) to identify the corresponding *Drosophila* line.

could be examined initially for obvious phenotypes in the homozygote and for *lacZ* expression. Sequence of the cDNA and deduced peptide, in association with the phenotype exhibited by the mutant , provide valuable information in the study of gene function as well as other purposes such as in the searching for novel insecticides.

8.3 PCR amplification of cDNA corresponding to the rescued plasmids

Cloning cDNAs corresponding to the locus of P-element insertion in large scale can be an arduous task. Here I suggest a simple strategy which is modified from Straus and Ausubel (1990). The method is diagrammed in Figure 8.3. An excess of biotinylated rescued plasmids is mixed with a small amount of purified cDNA library (in a vector sharing no homology with that of P-element vector). The mixture is denatured and then allowed to reassociate. The corresponding cDNA will hybridise to biotinylated strands of rescued plasmid. The biotinylated DNA, together with the cDNA reassociated with it, is bounded to avidin-coated polystyrene beads. The bound cDNA is thus separated from other cDNAs and is then released from the beads for PCR amplification.

8.4. The *Drosophila* V-ATPase

In this thesis I have reported the cloning and characterisation of genes and cDNA for subunit A, E and F of V-ATPases in *Drosophila*. Subunit c and B have also been cloned by the Glasgow research group (Meagher *et al.*, 1990; Davies *et al.*, 1996). Two further subunits have been cloned unintentionally, one from an enhancer-trap study (Harvie and Bryant, 1996), and one from a yeast two-hybrid study of cytoskeletal proteins (He and Kramer, 1996). Adding all this together, genes encoding seven subunits have been cloned (Table 8.1).

In spite of the overwhelming advantage (Rubin, 1988);), *Drosophila* as a model system had a major drawback (Dow, 1994; Dow *et al.*, 1996). The extremely small size of the

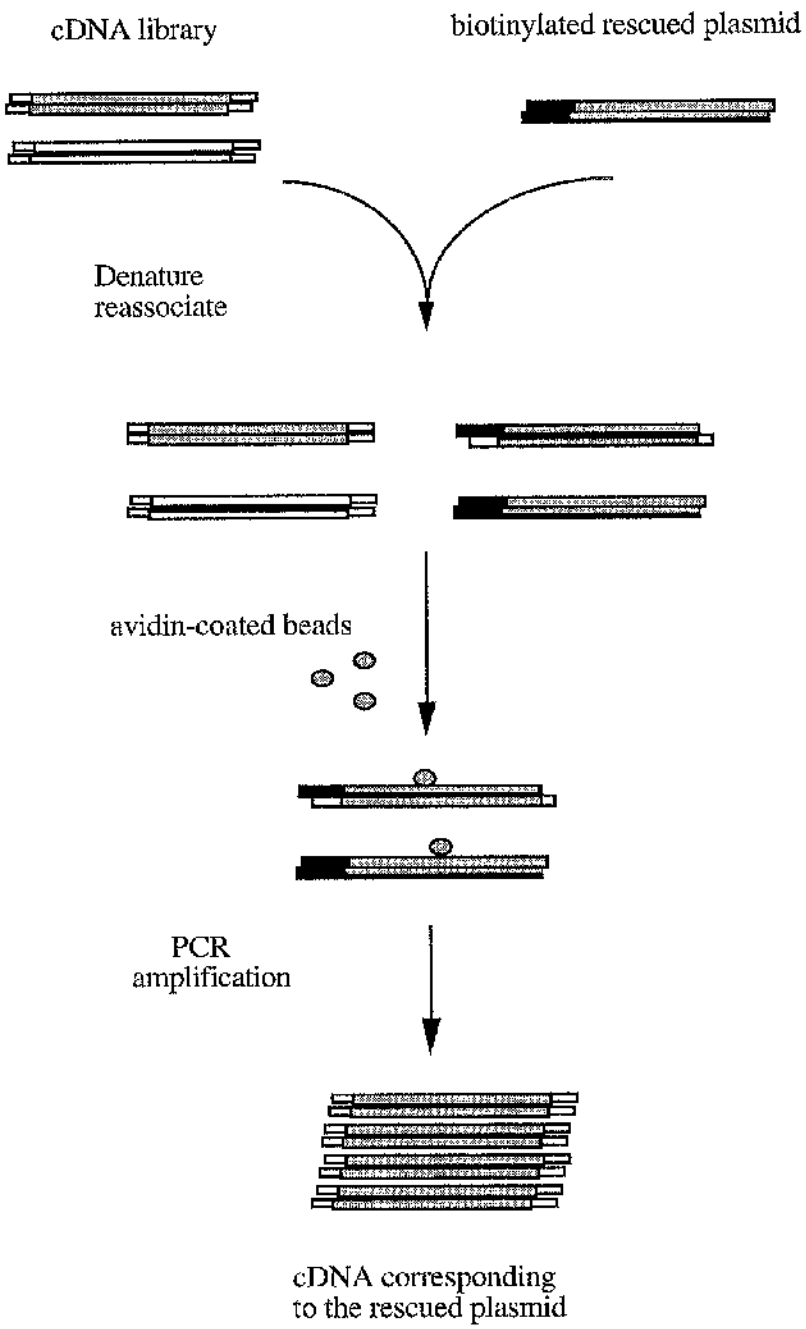


Figure 8.3 Schematic representation of PCR amplification of cDNA corresponding to the rescued plasmids. The biotinylated rescued plasmids are reassociated with the corresponding DNA in the cDNA library.The cDNAs hybridised to the biotinyted DNA are bound to avidin-coated heads and separated from the rest cDNAs. The bound cDNA is then released and is subject to PCR amplification.

organism compared with vertebrate make it difficult to perform physiological analysis of the V-ATPase function. Nonetheless, a delicate assay of the Malpighian tubule has been developed (Dow, 1994; Dow *et al.*, 1996). The insect Malpighian tubule performs a unction analogous to that of the vertebrate kidney tubule. Despite its small size, the *D. melanogaster* tubule is remarkably robust and provides a valuable physiological phenotype (Dow *et al.*, 1994). Potentially, then the *D. melanogaster* Malpighian tubule may prove a useful tool for the study of plasma membrane V-ATPase function.

Table 8.1 Characterisation of *D. melanogaster* genes encoding V-ATPase subunits

subunit	gene		transcript	deduced peptide			Citation
	name	location	(kb)	size (kb)	identity (*human*)	identity (*Manduca*)	
A	*vha68-1*	34A	2.6	68	87.1 (VATO) 81.9 (VATA)	87.4	Chapter 4
A	*vha68-2*	34A	2.6	68	91.7 (VATO) 82.4 (VATA)	 91.2	Chapter 4 Chapter 4
B	*vha55*	*87C*	2.8, 2.3	55	93 (brain) 89(kidney)	97	Davies *et al* 1996
C			1.8		66		Harvie *et al.* 1996
D							He *et al.* 1996
E	*vha26*	83B	2.3	26	63	77	Chapter 6
F	*vha14*	52B	0.65	14	71	90	Chapter 7
c	*vha17*	42B	1, 1.2	16	87	93	Meagher *et al.* 1990

8.5 The V-ATPase mutants in *Drosophila*

The cloning of a gene in *D. melanogaster* and identification of the chromosomal location unlocks a wealth of information. It is possible that the existing mutations in the region include alleles of the gene under study. Over the last few years, the probability of such findings has been increased greatly by the systematic physical mapping of the genome, the production of comprehensive panels of thousands of lines carrying lethal P-element insertions, which must presumably have inactivated a large number of essential genes (Török *et al.*, 1993). The development of site-selected mutagenesis of target genes by PCR (Kaiser and Goodwin, 1990) and via plasmid rescue (Chapter 3) allow the easy identification of candidate lines for a particular genes. This thesis reported the identification of P[*lacW*] mutant lines for genes encoding subunit A, E and c of *Drosophila* V-ATPase. Together with mutations for genes encoding subunit B (Davies *et al.*, 1996) and subunit C (Harvie *et al.*, 1996), P-element mutations for five V-ATPase genes have been identified (Table 8.2).

Table 8.2 P-element mutations of genes encoding *Drosophila* V-ATPase

subunit and gene name	fly No.	position of the insertion	homozygous phenotype	citation
A, *vha68-2*	25/8	before ATG, in intron.	first instar larvae lethal	Chapter 5
B, *vha55*	l(3)j2E9	after ATG, in intron	embryonic lethal to viable	Davies *et al* 1996
C		before ATG	second instar to pupal lethal	Harvie *et al.* 1996
E, *vha26*	l(3)j3E7	after ATG, in intron	lethal	Chapter 6
c, *vha17*	16/1	after ATG, in intron	third instar lethal	Dow *et al.* ,1996 Chapter 3

There is no detectable heterozygous phenotype of any of the available V-ATPase mutations, but total RNA reduction for *vha68* has been observed even in the heterozygous mutant flies. The homozygous lethal phenotype has been observed in all the five P-element lines. Although the lethal phase is varied for mutations of different subunits (Dow *et al.*, 1996) all the null alleles seem to be able to live past the embryo stage. The V-ATPase needed is likely to be provided by their mother. It has been found that the mutation of *vha68-2*, as well as mutation in *vha55*, shows a homozygous detectable tubule phenotype. The mutant homozygotes which survived to late embryonic or early larval stages showed transparent Malpighian tubules, without the luminal white material observed in healthy larvae. This phenotype is considered to be a characteristic of mutations of genes of V-ATPase subunits and mutations in any genes essential for plasmid membrane V-ATPase function are likely to show this characteristic phenotype as well (Dow *et al.*, 1996).

The LacZ expression in the P-element lines for *vha68-2*, *vha55*, *vha26* and *vha17* seems to have a similar staining pattern (Chapter 5; Davies *et al.*, 1996; Dow *et al.*, 1996). The expression is strongly detected in epithelia known to be energised by V-ATPases, the Malpighian tubules, the antennal palps and rectum. If this expression is a general pattern for P-element insertion in genes encoding any of the V-ATPase subunits, it could be as a general marker to screen for P-element insertions in other V-ATPase genes. However, the *lacZ* expression of lines with a insertion in gene of sununit C gives a different pattern from the gene (Harvie *et al.*, 1996), This *lacZ* expression may be affected by other nearby promoters.

Appendix 1.
List of publications from or partially from this study

1. Yiquan Guo, Ann Gillan, Tibor Török, Istvan Kiss, Julian A. T. Dow and Kim Kaiser. 1996. Site-selected mutagenesis of the *Drosophila* second chromosome *via* plasmid rescue of lethal P-element insertions.*Genome Research* 6:972-979.

2. Yiquan Guo, Zhongsheng Wang, Andrew Carter, Kim Kaiser and Julian Dow. 1996 . Characterisation of *vha26,* the *Drosophila* gene for a 26kDa E-subunit of the vacuolar ATPase. *Biochemica et Biophisica Acta* 1283, 4-9.

3 Yiquan Guo, Kim Kaiser, Helmut Wieczorek, and Julian A. T. Dow. 1996. The *Drosophila melanogaster* gene *vha14* encoding a 14-kDa F-subunit of the vacuolar ATPase. Gene 172: 239-243.

4. Luke Alphey, Louise Parker, Gillian Hawcroft, Yiquan Guo, Stephen Elledge, Kim Kaiser and Gareth Morgan. 1996. KLP38B - a mitotic kinesin-related protein from *Drosophila* which associates with PP1. Submitted to *Cell.*

5. Hilary A. Snaith, Christopher G. Armstrong, Yiquan Guo, Kim Kaiser and Patricia T. W. Cohen. 1996. Deficiency of protein phosphatase 2A uncouples the nuclear and centrosome cycles in *Drosophila* embryos. *Journal of Cell Science* (in press).

6. Y. Guo, J. A. T. Dow, A Gillan, I. Kiss and K. Kaiser. 1996. Molecular characterisation and inactivation of the 68 kDa A-subunit of V-ATPase in *Drosophila*. 37th American *Drosophila* Conference, San Diego. 91B.

7. B. McCabe, Y. Guo, S. Sweeney, E. Goldstein, K. Kaiser, C. O'Kane Investigation of the function of synaptobrevin proteins in Drosophila melanogaster. 37th American *Drosophila* Conference, San Diego. 102 B.

8. Dow, J. A. T., Davis, S. A., Guo, Y., Graham, S., Finbow, M. and Kaiser, K. (1996). Molecular genetic analysis of V-ATPase function in *Drosophila melanogaster*. *J. Exp. Biol.* 202 (in press).

Appendix 2 List of primers used in this study

primers	sequences (5'-3')	genes	orientation	position
P31	CGACGGGACCACCTTATGTTATTTCATCATG	P-element	+/-	
PR	AGCATACGTTAAGTGGATGTCTC	P-element	+	
PL	GTGTATACTTCGGTAAGCTTCGG	P-efement	-	
gt10rev	GGCTTATGAGTATTTCTTCCAGGGTA	nm1149 vector		
nm1149him	AACCTTCAGCCAGAATCCATTGCC	nm1149 vector		
14KT3-1	AACTGGAGGACTGTTTCAAG	vha14c	+	194-213
14KT7-1	TGGCGTCGTACGGATGGTCC	vha14c	-	336-354
G14T3-2	GGTGCGCTAATTCCTCGAAT	vha14c	+	426-427
G14T7-2	TCGACCACCATAAAGTTGGG	vha14c	-	154-172
28T3-1	GAAGAAGATTCAGTCCTCCA	vha26g	+	1009-1028
28T3-2	GAACGTCGAGCTGTTCATCG	vha26g	+	1369-1388
28T3-3	CAGTCAGGACGCACAGCTAGGA	vha26g	+	1769-1786
28T3-5	AGTAGCTAAGTTTGTTGACCTG	vha26g	+	2509-2529
28T7-1	GTTATATAATAACGCATATGTAC	vha26g	-	2848-2866
28T7-2	CGATGAACAGCTCGACGTTC	vha26g	-	1369-1387
28T7-3	CACGCTGCTCACATGGTCCTC	vha26g	-	1148-1167
28T7-4	CGCATATGCTACTTGTATTTG	vha26g	-	2835-2854
28T7-6	TCCTAGCTGTGCGTCCTGACTG	vha26g	-	1764-1786
28T7-5	CAGGTCAACAAACTTAGCTACT	vha26g	-	2509-2528
28g-1	CACTGCACAAACCGAAAGGAAA	vha26g	-	242-262
28g-2	CATCGAGTACTATATACATTA	vha26g	+	2867-2887
28g-3	GCAGGCGATCAGGTCGTA	vha26g	+	340-358
28g-4	CGTCCAAGACCCTAGCCTCTA	vha26g	-	747-766
28g-10	GATCCACTGCCGTTGTTCCTCC	vha26g	-	2224-2244
28g-4	CGTCCAAGACCCTAGCCTCTA	vha26g	-	747-766
G67T3-1	CGACATGGCCACCATCCAGG	vha68-1c	+	255-274
G67T3-2	AGATGGCGAGCAAAAGATCA	vha68-1c	+	1840-1867
G67T3-4	GAAAGTCACGCAGTACCTCA	vha68-1c	-	930-948
G67T3-3	CTACAACCTGGAGGACATTG	vha68-1c	+	627-646
G67T3-8	CGGTAGCTGAAATGGAACG	vha68-1c	+	2197-2215
G67T3-9	CTGTCCAAGTACTCCAACTC	vha68-1c	+	862-881
67T3-20	TCTGTCTGAATACTTCCGTG	vha68-1c	+	1071-1090
G67LT3-1	TTCAGCTGGTTGGCAAAGCA	vha68-1c	+	1553-1572
G67T7-1	GTCCTTTAGTCCCGCTTACC	vha68-1c	-	
G67T7-2	TGATCTTTTGCTCGCCATCT	vha68-1c	-	1847-1866
G67T7-3	CAATGTCCTCCAGGTTGTAG	vha68-1c	-	627-645
G67T7-4	TGAGGTACTGCGTGACTTTC	vha68-1c	-	930-949
G67T7-5	AGGGTAACGAACACAATCGA	vha68-1c	-	2335-2353
G67T7-8	CGTTCCATTTCAGCTACCG	vha68-1c	-	2197-2234

Appendix 2 List of primers used in this study, cont.

primers	sequences (5'-3')	genes	orientation	position
67T7-10	CCCGTGAAGAGCGGATGGTT	vha68-1c		745-763
67T7-20	TGCGTAGTGGCACGAACTCGG	vha68-1c	-	1484-1503
G67LT7-1	TCGGAGAAGTCACCACCAGG	vha68-1c	-	1332-1330
67LT7-2	GAACACCTGCACGATACCCAAA	vha68-1c	-	1349-1370
PS67-1	GAGCTGGTGAAACAAATCCAACG	vha68-1c	+	12-34
PS67-2	GCGATTAGTTTGACAAATTGC	vha68-2g	+	912-932
PS67-3	TAACTCAGCAAACGAAGATAGG	vha68-2g	+	1690-1700
67T3-5	TCCATTTACACTGGTATCACT	vha68-1c	+	1051-1071
G67T7-6	TCCAAGTTCCACGGAAAGAG	vha68-1c	-	332-350
67CP-1	AGAAGAAGAAGAGCAGCAACCGCGACC	6vha68-1g		
67GP-1	ATTGCAGTCGAAAAAACAGAATAAAGCAAA	vha68-2g	+	1258-1287
67CP-2	GTAACATTCATAATACATTTTATTTCC	vha68-1c	-	2547-2572
EHT7-1	GCATGCATTTGTATTTCTGTCT	vha68-2g	-	4076-4097
EHT7'	AAGTCATGTTTTCTCCCTGTTTG	vha68-2c	+	2370-2392
EHT7	GTTGCACTTTATTCGTACATT	vha68-2c	-	2432-2452
67KG-10	CACCAACAATTCCAGCTGCAT	vha68-2g	+	3817-3838
67KG-PS-2'	CCTTCTTTGTTATGCTGCG	vha68-2g	-	991-1009
67KG-9-3-2	TTCAATCCATTTCAGGACC	vha68-2g	+	3604-3622
67KG-9-7-3	ATCCTCGGCATTGACCACCGG	vha68-2g	-	
67KG-9-7-3'	AACGCATAGTGCAGCAGCGAC	vha68-2g	-	
PS-9'	AACATCATCAAGTATCAT	vha68-2g	+	1626-1643
5T3-1'	GGTATCATGGGCAGCATCTT	vha68-2g	+	1963-1982
67KR-1	ACCTGGCTCATCTCCTACTCG	vha68-2g	+	3136-3156
67KG-9-7-1	CGTCTGGTAGACGGATCACCA	vha68-2g	-	
67KG7T3-1'	ACTTGCAGTCTGTGTGCGTGTT	vha68-2g	-	280-301
67KG9T7-2	ATGGACCTCAATGGTCGCTGGA	vha68-2g		
67KG9T7-1'	TCCAGCGACCATTGAGGTCCAT	vha68-2g		
67KG9T3-1	CCTGCAGCAGAACTCCTACT	vha68-2g	+	3348-3367
67KG5T7-1	AGTGACGAAGCAGCGATCAA	vha68-2g	+	248-267
67KGT3-1	TGTAGATGGATTCGGTCAGC	vha68-2g	+	2018-1037
67KG-PS14	TCGATGATGAGGAGCGTGAGT	vha68-2g	+	1307-1327
67KG9T7-2'	AGGTGTCGTCCGGTGGAGGATAA	67kg-mid	+	813-834
PS-7	GACCGTTACCGAAGCAGAAGA	vha68c-1	+	43-63
PS-8	CGCGTAGACACGGCCATATT	vha68-2g		
PS-9	CCAACCAAGATAGGTTCCAT	vha68-2g	-	1683-1702
PS-10	TTGCCGTCAGCTGACAAATG	vha68-2g	-	661-682
PS-12	ATGTAGCAGATACACCTGCC	vha68-2g	+	1125-1144
PS-13	GTGCGGTATGAAAACGTGAA	vha68-2g	+	397-416

Appendix 3. List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*]

Notes for some items in the table:

1. The Glycerol stock in the table is the rescued plasmid transformed in *E. coli* which was stored at -70°C. Plasmid DNAs were isolated by pool of 10 line.

2. Lethal phase and chromosomal sites of the P-elements were kindly provided by Dr. Istvan Kiss. P: Pupae; L: larvae; 8A: Pharate adult; A±: Adult (semi-lethal); E; Embryo; L<n : Larvae maller than normal; L<<n: Larvae much smaller than normal. L>n: Larvae larger than normal.

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 1

Plasmid pool 1			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
380	46/8	8A	
384	48/2	E	
391	48/11	8A	
392	49/1	E	
395	49/9	E	26B5-6 42E3-4
396	49/10	L<<n	
397	49/11	E	
398	49/12	E	
399	49/13	E	44F1-2
401	49/16	E	

Plasmid pool 2			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
402	49/17	E	
403	50/1	P	
404	50/2	E	
405	50/7	E	
406	51/3	E	47F8-9
407	51/23	$A^{\pm}$	
408	51/5	E	56D8-11
409	51/6	$A^{\pm}$	
410	51/8	E	
411	51/13	E	30B5-6 83F1-2

Plasmid pool 3			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
412	51/14	$A^{\pm}$	
413	51/15	E	
414	51/19	E	
416	51/24	E	
417	51/4	E-L	
418	51/25	E	
419	52/1	8A	47A3-5
420	52/2	E	
421	52/4		57F5-6
422	52/5	E	

Plasmid pool 4			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2	1/3	E	51C1-2
3	1/7	8A-$A^{\pm}$	43E1-3
4	1/8	8A	
5	1/9	E	
6	1/10	8A	
7	1/12	$A^{\pm}$	
8	1/14	L<<n	
9	1/15	P-8A	
10	1/16		57A4-8
27	2/28	E	

Plasmid pool 5			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
30	2/31	P	46A2-3
31	2/32		46A2-3
32	2/33	L<<n	42A15-19
33	2/35	E	
35	2/37	E	
37	3/2	L-P	34B8-9
38	3/3	8A	34B8-9
39	3/4	8A-$A^{\pm}$	43F5-6
40	3/5		53F3-5 47A11-14
89	8/4	E	

Plasmid pool 6			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
42	3/8	E	
45	3/13	P	45D1-2
46	3/14	E	
47	3/15	E	
49	4/3	P	
53	4/12	E	
54	4/13	P	45A4-8
56	4/18	E	
58	4/20	8A	
90	8/5	P-8A	

Plasmid pool 7			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
59	4/23	L<<n	38A5-6
60	4/24	E	30D1-2 44F1-2
62	5/3	P	42B1-3
63	5/4	E	
67	6/4	P-8A	
68	6/5	L<<n	27A1-2
69	6/6	E	
70	6/7	P-8A	
71	6/9		23F5-6
203	26/10	E	

Plasmid pool 8			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1	1/1	E	43E1-3
11	1/19	P	
13	2/1	8A-$A^{\pm}$	
14	2/3	E	46A2-3
16	2/7	E	
19	2/11	E	
20	2/12	$A^{\pm}$	59A1-3
23	2/22	E	
24	2/23		23D3-4
25	2/24	L≤n	

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 2

Plasmid pool 9			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
28	2/29	$A^{\pm}$	
34	2/36	E-L	25C1-2
17	2/8	L<<n	50C17-19
72	6/10	E	23A4-6
74	6/12		
76	6/15	E	
78	6/17		48E1-2
79	6/18	L<<n	
83	7/3	$E\text{-}A^{\pm}$	51B4-5 83B6-7
84	7/5	P-8A	56D7-9

Plasmid pool 10			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
85	7/6	E	
87	8/2		
88	8/3	P-8A	51B1-5
91	8/6	E	56D5-6
93	8/8	$8A\text{-}A^{\pm}$	60D6-8
95	8/11	8A	
98	8/15	L<<n	
99	9/1		
100	9/9	L<<n	47C1-2
101	9/12	E	

Plasmid pool 11			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
102	10/2	P	
103	10/4		
104	10/5	E	47A11-14 70D4-5
106	10/8		
107	10/9	8A	
111	10/15	L<<n	60A10-14
109	10/12	E	
108	10/10		
113	10/18		

Plasmid pool 12			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
6/13	6/13	P	
8/9	8/9		49E1-2 28D1-2
12	1/20	E	43F1-2
12/7	12/7		
48	4/1	E	
387	48/7	$8A^{\pm}$	
423	52/6	E	
424	52/7	$8A\text{-}A^{\pm}$	53D11-14
425	52/9	E	

Plasmid pool 13			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
25/23	25/23		42D1-2
25/21	25/21	E	
25/20	25/20	E	42D1-2
25/17	25/17	P	
25/16	25/16	E	
25/13	25/13		
25/12	25/12		
25/11	25/11		52E5-7
25/8	25/8	E	34A3-4

Plasmid pool 14			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
25/26	25/26	E	
25/6	25/6		30C7-8
26/5	26/5	E	31F3-4
26/6	26/6		
26/5	26/5	E	
26/4	26/4	E	
27/8	27/8	E	
27/7	27/7	P	
27/6	27/6	E	

Plasmid pool 15			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
26/8	26/8	E	57B1-5 59E-F
27/4	27/4		57D11-12 22E1-2
47/4	47/4		
28/1	28/1		
28/2	28/2	E	50C20-23
28/6	28/6		
28/7	28/7		32E1-2
28/8	28/8	$A^{\pm}$	
44/5	44/5	P-8A	

Plasmid pool 16			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
28/3	28/3		
28/9	28/9	E	
28/11	28/11	E	
28/12	28/12	$A^{\pm}$	
28/14	28/14		27C4-5
28/17	28/17		
29/1	29/1	E	
29/3	29/3	$A^{\pm}$	
36/3	36/3	E	

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 3

Plasmid pool 17			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
42/9	42/9	E	60B1-2
42/17	42/17	L<<n	49D1-3 33C4-5
42/21	42/21	E	
44	3/11	$A^{\pm}$	36A11-12
45/1	45/1	E	
45/4	45/4	L-P	42A10-16
45/10	45/10		48F5-6
45/12	45/12	E	45B1-2
46/5	46/5	E	

Plasmid pool 18			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
42/10	42/10	P-8A	
47/3	47/3	8A	24A1-2
48/1	48/1	L<<n	
53/11	53/11	8A	
54/20	54/20	E-L	46B4-5
54/22	54/22	L-P	46B3-13

Plasmid pool 19			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
54/24	54/24	E	
54/25	54/25	E-L	
54/26	54/26		
54/27	54/27		
54/31	54/31		
54/32	54/32		
54/34	54/34	E	50B1-2 50C11-15
54/35	54/35		26D7-8
56/36	56/36		

Plasmid pool 20-			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
54/38	54/38	E	
54/39	54/39		50C14-16
54/41	54/41		46B4-5 59F1-2
54/42	54/42		53E1-2
54/45	54/45		
54/47	54/47		21D3-4
54/48	54/48		33F1-2
55/2	55/2	E	56A1-2
54/29	54/29	E	39F1-2 76B3-4

Plasmid pool 21			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
46/1	46/1	L-P	
54/40	54/40		53B1-2
25/7	25/7	E	
27/5	27/5		
46/7	46/7	$A^{\pm}$	
55/4	55/4	E	
25/5	25/5	8A-$A^{\pm}$	

Plasmid pool 22			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
50	4/5	E	25C1-2
51	4/6	E	
52	4/7	8A	47B15-16
65	5/8	E	60B4-5
120	11/7	L-P	
123	11/10	E	31F4-5 42D4-5
126	12/2		
128	12/5		60D15-16
131	12/8		60B4-5
134	12/11	E-$A^{\pm}$	

Plasmid pool 23			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
138	13/1	$A^{\pm}$	45F1-2
140	13/3	E	
142	13/7	E	
143	13/8	E	
144	13/10	$A^{\pm}$	
146	14/3	P	
148	15/1	E	26B8-9
151	16/1	E	
152	16/3	E	
153	17/1		25C1-2

Plasmid pool 24			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
155	18/2	E	
156	19/1	L-P	21B4-6
160	20/4	E	
161	21/2		
162	21/4		
163	21/7	E	
164	22/1	E	
167	22/6	E-L	57A8-9
168	22/8	$A^{\pm}$	

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 4

Plasmid pool 25			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
170	22/14		
171	23/1	8A	47A11-14
172	23/2	E	
174	24/1	P	
175	24/3	P	
176	24/5	L<<n	35D1-4
177	24/6	$A^{\pm}$	38B3-5 27F3-6
178	25/1	E	
179	25/2	E	
180	25/3	L<<n	

Plasmid pool 26			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
230	29/5	L<<n	49F4-5
232	30/2	L<<n	
234	30/4	$A^{\pm}$	49B1-2 94F1-2
236	30/7	L-P	55E1-2 23A5-6
237	30/8	E-$A^{\pm}$	49E1-2 94F1-2
89	8/4	E	
246	31/7		32C1-2
248	31/10	$A^{\pm}$	44C1-2
250	31/12	$A^{\pm}$	
242	31/1	E	60B3-5

Plasmid pool 27			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
89s	8/4	E	
251	31/13	P	42A1-2
252	31/14	pP	42A1-2
255	31/17	P-8A	55C9-12 54B15-16 90D
256	32/1	E	26D6-9
304	36/14	$A^{\pm}$	42B1-3
257	32/2		
258	32/3	L<<n	
259	32/4	8A-$A^{\pm}$	
303	36/11	E	

Plasmid pool 28			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
308	37/1	$A^{\pm}$	51B1-3
309	37/3	E	46F1-2
315	39/3	$A^{\pm}$	38B3-4
327	42/6	8A	36A1-2
333	42/16	8A	
336	42/20	E-$A^{\pm}$	28B1-4
341	43/1	P	27F4-5 50D5-6
344	43/4	E	
348	43/8	$A^{\pm}$	46F1-2
354	44/3	E	

Plasmid pool 29			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
363	44/15	P-8A	
368	45/8	E-L	
369	45/9	E	35D1-2
372	45/13	E-$A^{\pm}$	
374	46/2	L<<n	
386	48/6	P	
390	48/10	8A-$A^{\pm}$	53E1-2
414	51/19	E	
428	52/12	8A	
429	52/13	E	

Plasmid pool 30			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
430	52/14	E	
439	53/2	E	
441	53/4	8A-$A^{\pm}$	
444	53/9	P	
445	53/10	E	
461	54/6		
462	54/7		
464	54/10		
456	53/34	E	50B1-2 50C11-15
459	52/4	8A-$A^{\pm}$	57F5-6

Plasmid pool 31			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
357	44/8	P	
454	53/29	L-P	
465	54/11	E	
467	54/13		
468	54/14		
472	54/19		
485	54/33	E	53E1-2
496	54/45	P-8A	
495	54/44	E	
499	55/1	L-P	

Plasmid pool 32			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
443	53/7	E	
394	49/7	E	50E4-7
346	43/6	E	24D1-2
365	45/2	E	
312	38/1	E-$A^{\pm}$	
338	42/22	$A^{\pm}$	54B1-2
310	37/4	E	60E8-9
376	46/4	E-L	
322	41/1	L<<n	
453	53/28	L-P	56F10-13

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[lacW] Box 5

Plasmid pool 33			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
57	4/19	8A-A$^{\pm}$	
82	7/2	8A	
81	6/20	L<<n	42C1-2
116	11/2	P-8A	
112	10/17	E	46F5-6
119	11/6	8A	
130	12/7	E	43F1-2
132	12/9	L<<n	54B4-8
141	13/4	A$^{\pm}$	
124	11/15	E-L	

Plasmid pool 34			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
3-24	26/12	E	
336	42/20	L-8A	28B1-4
362	44/13	E	
311	37/6	A$^{\pm}$	29D1-2
348	43/8	A$^{\pm}$	46F1-2
302	36/10	E	46F1-2
326	42/5	E	
385	48/5	E	
363	44/15	P-8A	
233	30/3	P-8A	34B8-9

Plasmid pool 35			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
485	54/33	E	53E1-2
442	53/5	P	
437	52/24	8A-A$^{\pm}$	
438	52/25	A$^{\pm}$	
435	52/31		
457	54/1		21B4-6
466	54/12		
488	54/36		
467	54/13		
471	54/18		

Plasmid pool 36			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
139	13/2	E	25D1-2
238	13/1	A$^{\pm}$	45F1-2
231	30/1	E	
243	31/2	E	25C1-2
246	31/7		32C1-2
245	31/6	L-P	
253	31/15	8A-A$^{\pm}$	46B1-2
240	30/11	E-A$^{\pm}$	
264	33/1	E	
263	32/10	E	

Plasmid pool 37			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
265	33/2		37A1-2
272	33/11		
271	33/10		
275	33/16	L<<n	51B7-8
266	33/3	A$^{\pm}$	54E1-2
277	34/2	E	53C1-4
283	35/1	P-8A	
281	34/8	E	
270	33/9	L<<n	
269	33/8	P	

Plasmid pool 38			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
296	36/1	E	
291	35/11	E	
290	35/10	E	
293	35/13	E	
287	35/5	E	36F11-12
288	35/6		35D1-2 89B9-10
292	35/12	E	
295	35/14	E	47F1-2
298	36/4	E	
300	36/8	E	

Plasmid pool 39			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
36	3/1	A$^{\pm}$	36A11-12
41	3/7	E	
43	3/10	E	
55	4/14	E-L	
64	5/7	pP	
66	6/2	P	
73	6/11	P-A$^{\pm}$	58F4-5
77	6/16	L<<n	21B7-8
26	2/17		
86	8/1		

Plasmid pool 40			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
90s	8/5	P-8A	
97	8/13	L<<n	
110	10/13	8A-A$^{\pm}$	
117	11/3	E	48F3-6
129	12/6	E	21A1-4
139	13/2	E	25D1-2
165	22/3	8A-A$^{\pm}$	44C1-2
185	25/10		30C6-7
189	25/14	E	35D3-4

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal location(s) of the P[*lacW*] Box 6

Plasmid pool 41			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
196	26/1	E	
197	26/2	E	35D3-4
198	26/3	E	
204	26/11	E	
206	26/15	L-P	52E3-4
208	27/3	P-8A	
215	27/13	E	
239	30/10	E	
244	31/5	E	
261	32/7	E	48F3-4

Plasmid pool 42			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
21	2/13	P-8A	27C2-3
22	2/15	E	
122	11/9	8A	41F8-9
127	12/3		30E1-2
125	11/17	E-A$^{\pm}$	
159	20/3	8A-A$^{\pm}$	
158	20/2	P-8A	
247	31/9	E-L	
219	28/5	E	
267	33/4	P-A$^{\pm}$	
268	33/7	E	

Plasmid pool 43			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
274	33/15	E	
275	33/16	L<<n	51B7-8
280	34/6	E	
284	35/2	E	
289	35/9	A$^{\pm}$	29E1-2
440	53/3	8A-A$^{\pm}$	52E5-8
203	26/10	E	
351	43/14	L-P	42A1-2
460	54/5	E	
260	32/5	E	

Plasmid pool 44			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
294	35/15	E	
299	36/7	E-L	
301	36/9	E	53C1-2
313	38/2	E	
314	39/1	L-P	
317	39/4	A$^{\pm}$	43F5-9
318	39/5	E	48C5-6
321	40/4	A$^{\pm}$	
324	42/3	P	
340	42/24	E	

Plasmid pool 45			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
328	42/7	P-8A	35D1-2
332	42/13	E	48F5-6
335	42/18	E	
350	43/11	A$^{\pm}$	
352	44/1	E	55D1-2
353	44/2	8A	28C7-8
357	44/8	P	
358	44/9	E	59A1-3
360	44/11	P	26B8-9
361	44/12	E	

Plasmid pool 46			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
393	49/6		
400	49/14	E	
415	51/22	P-A$^{\pm}$	
426	52/10	E	
427	52/11		35F1-2 60B10-11
431	52/15	E	
432	52/18		
433	52/19	L<<n	
436	52/23		
449	53/17	P	56F10-12

Plasmid pool 47			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
517	55/16	P-8A	
518	55/17	P	35D1-4 37C6-7 82E6-7
519	55/18	E	
520	55/19	L<<n	
521	55/23	8A-A$^{\pm}$	
524	55/32	P	
511	55/7	E	
512	55/8	P-8A	
514	55/12	P	
516	55/15	E	

Plasmid pool 48			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
455	53/32		
458	54/2		
447	53/13	L-P	
451	53/19	P	
501	55/3	P-8A	
480	54/28	E	
482	54/30		
549	56/33	E	53E1-2

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal location(s) of the P[*lacW*] Box 7

Plasmid pool 49			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
530	56/6	L<<n	
535	56/10	E	
536	56/11	E	
537	56/13	$A^{\pm}$	28B1-2
539	56/15	E	
532	56/8	E	
534	56/12	E-L	
527	56/2	E	
543	56/24	E	
542	56/23	P	

Plasmid pool 50			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
546	56/29	E	
547	56/30	E	
548	56/32	E	
545	56/27	E	36A6-7
549	56/33	E	60B4-5
544	56/25	E-L	
541	56/17	$A^{\pm}$	
554	56/40	E	
553	56/39	8A	51B4-5 98C1-2
550	56/35	E	

Plasmid pool 51			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
555	56/41		
557	56/47	P-8A	
556	56/42	E	
574	57/17	E	
561	56/51	E	51B5-6 51B9-10
570	57/12	E	30C3-4
567	57/8	E-L	
565	57/2	$A^{\pm}$	
538	56/14	E	57F5-6 35D1-2
533	56/9	E	50D1-2 57F9-11

Plasmid pool 52			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
704	64/1	E	
721	64/19	8A-$A^{\pm}$	
728	65/6	P-8A	21C4-5
718	64/16	E-L	30E1-2
716	64/13	8A-$A^{\pm}$	
725	65/3	$A^{\pm}$	53C14-15
726	65/4	E	
731	65/11		
732	65/14	P	
733	65/15	E	58D1-2

Plasmid pool 53			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
734	65/20	8A	
739	66/3	E	
740	66/5	E	
743	66/8	P-8A	31A1-2 60B1-2
752	66/20	E	
749	66/17	L<<n	58D6-7
748	66/14	P-8A	
750	66/18	E	50E6-7
746	66/12	E	48D1-2
578	57/25	E-L	

Plasmid pool 54			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
650	61/20	E	
652	61/22	E	
653	61/25	8A-$A^{\pm}$	
654	61/26	8A-$A^{\pm}$	42A8-9
656	61/31	E	
657	61/32	E	
658	61/33		
660	62/2	E	54C1-4
663	62/5	L-P	46B1-2
665	62/7	L<<N	

Plasmid pool 55			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
666	62/10		42A10-12
667	62/11	E	35D1-4
685	63/21	E	34D5-6
687	63/24	L<<n	31A1-2
688	63/26	E-L	
689	63/27	P-8A	28E3-4
764	67/14	E-L	
765	68/1	P-8A	
766	68/2	E	
169	22/10	$A^{\pm}$	

Plasmid pool 56			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
529	56/5	E	
566	57/5	E	
577	57/22	E	
583	58/6	$A^{\pm}$	
584	58/7	L-P	23D3-4 47F1-2
585	58/8	E	
588	58/11	E	37F1-2 92D7-8
592	58/16	$A^{\pm}$	23D1-2
596	58/21	E	50D1-2
768	68/4	L<<n	

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 8

Plasmid pool 57			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
805	70/14	P-A$^{\pm}$	44E1-2
808	70/18	E-L	50C14-15
809	70/20	P	36B1-2
810	70/24	E	43D1-2
214	27/10	E	
249	31/11	E	46B1-2
279	34/5		
282	34/9	8A	
133	12/10	E	
154	18/1	L-P	

Plasmid pool 58			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
759	67/9	E	
762	67/12	E	
763	67/13	E	
771	68/8	A$^{\pm}$	
772	68/10	E	
773	68/11	E	
799	70/5	P	21C4-5
802	70/11	A$^{\pm}$	
803	70/12	E-L	37A2-3
804	70/13	P-8A	

Plasmid pool 59			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
794	69/20	P-8A	
795	69/22	P-8A	
797	70/2	E	
798	70/3	8A-A$^{\pm}$	
701	63/42	P-A$^{\pm}$	
702	63/43	E	
754	67/1	E	
755	67/3	E	
756	67/4	P-8A	
757	67/6	E	

Plasmid pool 60			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
785	69/4	L<<n	
786	69/6	E	
784	69/3	P-8A	
787	69/8	P-A$^{\pm}$	
788	69/9	E-A$^{\pm}$	
789	69/10	E-L	
790	69/15	E	34C3-5
791	69/16	E	
792	69/18	E	
793	69/19	P	

Plasmid pool 61			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
774	68/13	E	
775	68/14	8A	
776	68/15	8A-A$^{\pm}$	
777	68/17	E-A$^{\pm}$	47A7-8
778	68/18	L-8A	
779	68/19	E	
780	68/20	P-8A	
781	68/21	P-8A	
782	69/1	E	
783	69/2	E	

Plasmid pool 62			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
601	59/1	E	
602	59/2	A$^{\pm}$	
604	59/7	E-L	
605	59/8	E	
607	59/10	E	
610	59/13	E	
612	59/16	P	54B10-14
616	59/20	8A-A$^{\pm}$	
619	60/4	L<<n	
621	60/6	E	44F3-4

Plasmid pool 63			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
623	60/8	E-L	
625	60/10	E	
626	60/11	8A	
627	60/15	P	
628	60/18	E	
629	60/19	P-8A	21B4-6
630	60/21	pP	45B7-8
631	60/22	E	
632	60/24	E	47A11-14 47C4-7
635	61/1	E	21C6-7

Plasmid pool 64			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
636	61/3	E	
637	61/4	E-L	
638	61/5	E	
640	61/7		
641	61/8	E	
644	61/12	E	47A11-14
646	61/14	8A-A$^{\pm}$	
647	61/15		
648	61/17	E	
649	61/19		

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 9

Plasmid pool 65			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
661	62/3	L<<n	51A1-5
668	62/13	8A-A$^{\pm}$	
672	63/3	L<<n	29C3-4
674	63/5	L<<n	
677	63/9	E-A$^{\pm}$	
683	63/18	E	
767	68/3	P-8A	
897	73/36	P-A$^{\pm}$	46E1-2
869	72/39	E	
822	71/17	E	

Plasmid pool 66			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
307	36/19	A$^{\pm}$	
460	54/5	E	
458	54/2	E	37B7-10 32A1-2
526	55/31	8A-A$^{\pm}$	32A1-2
813	71/5	L-A$^{\pm}$	
814	71/6	P-A$^{\pm}$	
816	71/9	E	25F1-2 36E3-4
815	71/8	E-A$^{\pm}$	56D7-9
821	71/16	L-8A	
824	71/20	L-P	

Plasmid pool 67			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
858	72/26	E	53E1-2
860	72/28		53E1-2
866	72/36	E-L	52E5-6
862	72/31	L<<n	
829	71/27	E-L	53C1-2
846	72/11	E	49E6-7
842	72/4	E	57F5-6 47A11-14
854	72/20	8A-A$^{\pm}$	53E1-2
867	72/37	L<<n	46C1-2
864	72/33	E	

Plasmid pool 68			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
870	72/40	L-P	
879	73/6	E	
872	72/44	P-8A	
875	73/1	E	53B1-2
882	73/10	E	21C4-5
884	73/12	P-8A	41C 39B1-2
888	73/21	E	
885	73/13	E	
889	73/22	E-A$^{\pm}$	
890	73/24	P	59F1-2

Plasmid pool 69			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
894	73/31	8A	
893	73/27	E	
896	73/33	E	
892	73/26	E-A$^{\pm}$	
825	71/23	8A	
634	60/28	L<<n	
820	71/15	E	56D8-9
823	71/18	8A-A$^{\pm}$	29C1-3
826	71/24	L-A$^{\pm}$	
830	71/29	A$^{\pm}$	36B1-2

Plasmid pool 70			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
828	71/26	E	
836	71/38	E	
839	72/1	E-L	49E6-7
850	72/16	E	49E6-7
852	72/18	E	25C1-2 26A5-6
863	72/32	P-8A	
891	73/25	E	
933	76/2	8A-A$^{\pm}$	
934	76/3	E	
936	76/5	E	

Plasmid pool 71			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
949	76/20	pP	
960	77/8	pP	
961	77/11	E	
965	77/17	L<<n	32D1-2
957	77/4	E	29D1-2
963	77/13	E	
970	77/27	pP	
973	77/36	E	23F5-6
974	77/37	E	45A1-2
975	77/38	P	

Plasmid pool 72			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
979	78/5	E	
982	78/9	A$^{\pm}$	
983	78/10	E-L	47A7-8
1013	79/7	P	
1014	79/8	E	
1016	79/12	L<<n	
1019	79/16	E-A$^{\pm}$	55B5-10
1021	79/19	pP	
1023	79/21	L<<n	
1025	79/23	P-A$^{\pm}$	

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 10

Plasmid pool 73			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1028	79/27	L<<n	
1029	79/28	L<<n	
1031	79/31	E-L	55B5-6
1032	79/32	L<<n	
1033	80/1	E	
1035	80/3		
1036	80/4	L<<n	21D3-4
1037	80/5	E-L	
1042	80/11	8A	42D4-5
1047	80/16	P	

Plasmid pool 74			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1045	80/14	8A-A$^{\pm}$	
1046	80/15	E	51D3-5
1048	80/17	E	
1049	80/18	L<<n	44A1-2
1050	80/19	P	
1051	80/21	E	
1053	80/23	E	51D3-5
1055	80/25	L-A$^{\pm}$	
1056	80/27	E	22D4-5
1058	80/29	L<<n	

Plasmid pool 75			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1059	80/31	P-8A	
1060	80/32	E	
1062	80/34	E	39E3-4
1063	80/35	E	
1069	80/45	E	
1072	81/1	E	
1073	81/2	A$^{\pm}$	21B4-6
1077	81/6	L-A$^{\pm}$	35F11-12 35F4-5 21B4-5 34C4-5 42C1-2
1083	81/12	A$^{\pm}$	
1084	81/13	E	

Plasmid pool 76			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1086	81/16	p-A$^{\pm}$	
1087	81/18	E	
1088	81/19		
1090	81/21		
1093	81/24		
1094	81/25	E	23C1-2
1095	81/27		
1097	81/34	E-P	59B1-2
1101	81/39	pP	
1102	81/41	pP	

Plasmid pool 77			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
511	55/7	E	
569	57/11	L-A	
575	57/18	8A-A$^{\pm}$	
579	58/1	8A-A$^{\pm}$	
581	58/4	E	
589	58/12	E	32C4-5
591	58/15	8A-A$^{\pm}$	39E3-4
593	58/17	8A-A$^{\pm}$	
594	58/18	E	
597	58/23	8A	

Plasmid pool 78			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
606	59/9	E	
613	59/17	P	26B8-9
615	59/19	E	
643	61/11	8A-A$^{\pm}$	
655	61/30	P	
670	62/21	L<<n	
683	63/18	E	
692	63/30	E	47A11-14
696	63/35	E	31A1-2
699	63/40	L<<n	

Plasmid pool 79			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
678	63/10	E	
693	63/32	L<<n	
700	63/41	E	
703	63/44	P-8A	
711	64/8	A$^{\pm}$	46C7-8
715	64/12	A$^{\pm}$	
731	65/11	E-P	
758	67/8	E-L	
923	75/15	L<<n	
924	75/16	E	

Plasmid pool 80			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
678	63/10	E	
693	63/32	L<<n	
700	63/41	e	
703	63/44	P-8A	
711	64/8	A$^{\pm}$	46C7-8
715	64/12	A$^{\pm}$	
731	65/11	E-A$^{\pm}$	
758	67/8	E-L	
923	75/15	L<<n	
924	75/16	E	

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 11

Plasmid pool 81			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
851	72/17	E	
855	72/22	E	37F1-2 73D1-2
859	72/27	L<<n	46F1-2
865	72/34	E	
868	72/38		
878	73/5	E	53B1-4 50A12-14
881	73/9	E-L	46B1-2
886	73/15	E	
887	73/20	E	
895	73/32	E	34A1-2

Plasmid pool 82			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
901	74/4	E	34A1-2
902	74/5	E	
905	74/9	P	
908	74/22	E	
909	74/23	E	49B5-6 47A11-14
910	74/31	L≤n	42E,51B 58D,60F
911	74/33	E	
913	75/2	P	26D1-2
915	75/5	E	57B1-3
916	75/7	8A-A±	48C7-8

Plasmid pool 83			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
917	75/9	8A-A±	54E1-2
919	75/11	P-8A	
920	75/12	E	
921	75/13	E	43E7-10
925	75/17	E-L	46A3-4
927	75/19	E	
929	75/21	E	44C1-2
930	75/25		
935	76/4	8A	
937	76/6	8A	

Plasmid pool 84			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
940	76/10	8A-A±	52E5-6
941	76/11	E	
942	76/12	8A-A±	
944	76/15	E	
945	76/16	P-8A	42C1-2
947	76/18	L<<n	56E3-6
948	76/19	8A-A±	43E15-16
951	76/23	E	60E1-2
952	76/24	P-8A	43F1-2
958	77/5	E	46F5-6

Plasmid pool 85			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
966	77/19		
967	77/21	P-8A	
968	77/22	P-8A	
972	77/35	E	
976	77/39	E	23F5-6
984	78/11	P-8A	44C4-5
986	78/13	L-P	
987	78/14	E	55E1-2
989	78/16		
990	78/17	E	

Plasmid pool 86			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
991	78/18	A±	
993	78/21	E	
994	78/22	P	
995	78/23	E	
996	78/24	E	53C1-4
998	78/27	E	
1002	78/31	A±	
1103	78/32	P-8A	34B6-7
1006	78/39	E	57A5-6
1015	79/9		

Plasmid pool 87			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1008	79/2	P	
1011	79/5	P	
1034	80/2		56F6-9
1040	80/9	L<<n	
1054	80/24	E	
1062	80/34	E	39E3-4
1067	80/40	8A-A±	44F11-12
1068	80/41	E	
1074	81/3	E	
1075	81/4	L<<n	35D1-4 68C1-2

Plasmid pool 88			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1076	81/5	E	
1078	81/7	E	
1080	81/9	E	
1082	81/11	8A-A±	
1106	82/3	E	56D8-11
1108	82/8	E	
1110	82/11		
1113	82/17	P-8A	
1114	82/18		21C4-5
1115	82/19	E	

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 12

Plasmid pool 89			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1116	82/20	E-A$^{\pm}$	
1117	82/21	E	
1119	82/24	E	
1121	82/27	E	50D1-2 53F4-5
1124	82/31	E	
1126	82/33	P	
1127	82/39		
1129	82/45	A$^{\pm}$	
1130	82/47	E	
1134	83/1	8A	52E5-6

Plasmid pool 90			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1135	83/2	L<<n	
1137	83/4	E-A$^{\pm}$	
1137/1	83/5	A$^{\pm}$	57A5-6
1138	83/6	A$^{\pm}$	
1139	83/7	P-8A	53C1-2 60A8-11
1140	83/8		
1141	83/10	E	
1144	83/16	E	58E1-2
1146	83/19	P	
1147	83/20	P	

Plasmid pool 91			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
571	57/13	E-L	
580	58/3	E	50C11-15
598	58/26	E	
572	57/14	P-8A	
528	56/3	A$^{\pm}$	58D8-10
680	63/12	E	
698	63/39	E	
563	56/54	P-8A	
540	56/16	E	
551	56/36	L<<n	

Plasmid pool 92			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
697	63/38	P-A$^{\pm}$	
712	64/9	8A-A$^{\pm}$	57B1-3
747	66/13	E	
807	70/16	E	
932	76/1	8A	
962	77/12	8A-A$^{\pm}$	43F3-4
1148	83/21		
1149	83/22		

Plasmid pool 93			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1150	84/1	8A	
1151	84/2	8A-A$^{\pm}$	42F1-2 57E9-10
1153	84/4	L-P	
1156	84/7		52D1-2
1157	84/8	E	30D1-2
1158	84/9	E	
1162	84/15	E	
1166	85/4	A$^{\pm}$	44A1-2
1167	85/5	E	
1168	85/6	L-8A	

Plasmid pool 94			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1169	85/7		48A5-6 60A5-6
1171	86/2	E	
1172	86/4	E	
1173	86/5	E	
1174	86/9		
1176	86/11	E	
1177	86/12	E	
1178	86/13	E	
1181	86/17	E	
1184	86/21	E	

Plasmid pool 95			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1185	86/23	E	
1187	86/25	P	43D1-2
1188	86/29	P	
1189	86/30	E	
1192	87/5	E	
1194	87/7	P-A$^{\pm}$	
1195	87/8	E	
1196	87/12	A$^{\pm}$	35A3-4
1197	87/13	E	56C1-2
1198	87/4	E	

Plasmid pool 96			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1201	88/4	8A-A$^{\pm}$	
1202	88/5		53F4-5
1203	88/6	E	
1204	88/8	E	
1205	88/9	E	25C1-2
1206	88/10	L<<n	56A1-2
1207	88/12	L<<n	
1208	88/13	E	
1210	88/7	E-L	44F
1211	88/18		

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 13

Plasmid pool 97			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1212	88/19		
1213	89/1		54B15-16
1214	89/2		55F5-6
1215	89/3		
1217	89/6	E	
1218	89/7	E	
1220	89/9	E	
1225	89/18	E	
1226	89/19	E	
1228	89/21	E	37F1-2 55E6-7

Plasmid pool 98			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1261	91/5	A±	
1269	92/1	8A-A±	60A8-11
1271	92/3	L-8A	
1273	92/5	L-A	
1274	92/8	L-P	
1275	92/9	8A	
1276	92/10	A±	
1277	92/14	E	
1279	92/17	P-A	52D11-12
1280	92/18	8A-A±	

Plasmid pool 99			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1235	90/3	E	25C1-2
1237	90/8	E	
1240	90/15	A±	
1247	90/24	P	47A11-14
1248	90/25	E	60A8-11 86F1-2
1251	90/30	E	
1253	90/32	E	
1258	90/41	L<<n	25C1-2
1259	91/2	E	
1260	91/4	E	32C4-5

Plasmid pool 100			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1283	92/21	L<<n	46B1-2
1286	92/24	E	
1287	92/25	L<<n	
1288	92/29	E	
1293	92/38	A±	28A1-2
1294	92/39	A±	26D1-2
1295	92/40	E-A±	26D1-2
1297	92/44	E-L	
1299	92/47		
1302	93/6	E	25C1-2

Plasmid pool 101			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1303	93/10		
1304	93/12	E	
1310	93/19	E	
1312	93/22	A±	
1315	94/2	E	
1316	94/3	P-A±	
1317	94/4	8A	
1321	94/11	L<<n	28D1-2
1323	94/13	E	
1324	94/14	L<<n	39B1-2

Plasmid pool 102			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1233	89/31	L-8A	55B5-10
1236	90/5	E	
1292	92/36	A±	
1296	92/42	E	46F5-7
1250	90/28	E	
1290	92/32	L<<n	
1327	95/2	L<<n	
1330	95/6	E	
1333	95/13	E	44B7-8
1353	96/6		

Plasmid pool 103			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1342	95/33	A±	48F3-4
1344	95/36	E-L	
1346	95/38	E	46F5-6
1347	95/39	E	
1348	95/41	E	
1350	96/2	8A-A±	
1354	96/8	E	
1358	96/17	E	57F10-11
1360	96/19	pP	
1365	96/29	E	

Plasmid pool 104			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1370	97/10	E	55F1-3
1373	97/16	A±	43D1-4
1374	97/17	A±	
1378	98/3		56D3-6
1379	98/5	A±	
1380	98/8	E-A±	58D4-5
1381	98/9	8A-A±	
1382	98/10	8A-A±	56D5-6
1383	98/11		58D4-5
1384	98/12	E	

Plasmid pool 105			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1387	98/15	E	
1389	98/17	P-A	
1391	98/20	E	
1395	98/24	E	25C1-2
1401	98/34	E	35F1-5
1409	98/47	E	26D1-2
1411	98/50	P-A	26D1-2
1417	99/2	8A	
1418	99/3	A±	22D1-2
1422	99/8	8A-A±	

Plasmid pool 106			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1434	99/32	8A-A±	22C1-2
1451	101/11	E	
1456	101/18	E	
1457	101/19	E	34A5-6
1461	101/26	8A	
1513	104/16	E	
1516	104/23	E	36B1-2
1519	105/2	8A	60D1-2
1522	105/5	E	
1523	105/6	L<<n	

Plasmid pool 107			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1526	106/4	L<<n	
1360	96/19		
1375	97/18	E	
1396	98/28	E	
1418	99/3	22C1-2	22D1-2
1515	104/20	L<<n	
1531	106/10	P	
1532	106/12		
1533	106/13	L-P	
1534	106/15	L<<n	

Plasmid pool 108			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1537	107/1	8A	51B1-5 78D4-5
1538	107/2	A±	
1547	108/3	E	
1548	108/4	E-L	
1552	108/11	L<<n	
1553	108/12	L<<n	
1555	108/17	E	35D1-2
1562	109/5	A±	
1566	109/9	L-8A	
1568	109/16	P-8A	

Plasmid pool 109			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1574	110/11	A±	43E4-5 58C1-2
1575	110/12	E	54B15-16
1577	110/16	E	
1581	110/24	P	35F4-5 93D3-5
1584	110/31	A±	
1588	111/1	A±	28D10-11
1589	111/2		
1591	111/6	E	
1593	111/8	8A-A±	
1594	111/9	8A	

Plasmid pool 110			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1596	111/15	A±	
1597	111/16	E	
1600	111/20	8A-A±	45D4-5
1601	112/1	E	45B1-2
1602	112/2	E	56D7-10
1603	112/3	L<<n	
1605	112/5	8A-A±	45B1-2
1610	112/10	E	
1612	112/12	L-P	
1613	112/13	P	50F4-7

Plasmid pool 111			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1615	112/15	E-P	
1616	112/17	L-8A	
1618	112/22	E	
1620	112/25	E	50D1-2 58D6-7
1623	112/34	L-P	
1624	112/31	E	
1642	113/29	L-A±	
1660	115/8	8A-A±	
1518	105/1	E	48D1-2
1536	106/22	pP	

Plasmid pool 112			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1611	112/11	P-8A	26A5-6 46A1-2 47A11-14
1619	112/23	A±	
1628	113/1		53F4-5
1697	119/12		
1699	120/2	E	
1709	123/1	pP	
1714	124/1	L<<n	
1715	124/2	8A-A±	45D4-5
1717	124/5	E	
1721	126/2	P	

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 15

Plasmid pool 113			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1336	95/18	A±	48F3-5
1631	113/10	A±	
1633	113/12	L<<n	
1635	113/15	E	
1636	113/18	E	25D1-2 49B3-4
1640	113/25		21D1-2 98F1-2
1641	113/28	P	
1644	113/31	P-8A	
1657	115/5	E	54F1-2
1660	115/8	8A-A±	37C5-7

Plasmid pool 114			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1661	115/9	A±	
1662	115/11	E	25E5-6
1663	115/13	PO	
1665	115/15	E	
1672	115/26	pP	
1674	115/30	pP	
1677	115/33	A±	57E3-4 86E9-10
1681	115/42	L<<n	
1691	118/5	E	25D4-5
1692	119/1	L-8A	43E1-5

Plasmid pool 115			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1696	119/5		
1698	120/1	E	49D1-3
1703	121/1	L≤n	
1704	121/2	E-L	46A1-2
1707	121/4		
1710	123/2	P	
1712	123/4	E	
1716	124/3	P-A	30C1-2
1718	124/8	E	30C7-8
1720	125/3	E	

Plasmid pool 116			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1152	84/3	E-A±	
1154	84/5	8A-A±	32B1-3
1193	87/6	L<<n	
1227	89/20	E	
1239	90/11		
1243	90/18	E	
1244	90/20	L-P	47A11-14
1245	90/22	E	27C1-2
1252	90/31	E	
1265	91/16	P-A±	31F4-5

Plasmid pool 117			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1270	92/2	E	54A1-2
1272	92/4	E	44C4-5 57E3-4
1285	92/23	E	52E5-6 78C1-2
1298	92/46	A±	
1301	93/4	8A	48F1-6
1306	93/15	E-L	
1331	95/7	A±	45D4-5
1352	96/5	E	
1359	96/18	E	
1361	96/23	L<<n	

Plasmid pool 118			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1376	97/20	E	48E6-9
1390	98/19		42C1-2
1392	98/21	E	45D1-2
1398	98/31	E	
1508	104/9	E	
1528	106/7	E-L	49F7-8 21F1-2
1540	107/4	A±	
1541	107/5	A±	
1542	107/6	8A-A±	53D10-13 54C7-8
1543	107/12	P	

Plasmid pool 119			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1554	108/15	L-P	53F6-9
1564	109/7	E	
1582	110/26	E	50C3-4 53C1-4
1590	111/4	8A-A±	39F1-2
1567	109/14	A±	54B1-2
1595	111/10	E	43E4-6
1598	111/17	A±	
1606	112/6	A±	25C5-6
1611	112/11	P-8A	26A5-6 46A1-2 47A11-14
1621	112/26	P-8A	39C1-2

Plasmid pool 120			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1629	113/2	L<<n	
1631	113/10	A±	
1639	113/24	E-A±	21C1-2
1641	113/28	P	34A5-6
1642	113/29	L-A±	
1646	114/2	E	
1647	114/8	E	45F5-6 54C1-4
1651	114/7	E	47A3-4 61F6-8
1652	114/11	E	
1654	114/13	8A	33A3-4 79E1-2

Plasmid pool 121			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1650	114/6	P-A	53F1-2
1655	115/2	E	
1656	115/4	P-8A	
1658	115/6	P	
1668	115/21	A±	
1669	115/23	8A	
1671	115/25	E-L	
1679	115/37	L<<n	
1693	119/2	8A	43E7-10 86A4-5
1694	119/3		

Plasmid pool 122			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1242	90/17	8A-A±	35D1-2 39E1-4
1256	90/37	P	
1337	95/19	E	
1397	98/29	E	
1399	98/32		
1400	98/33	A±	49B7-8
1404	98/41	E	
1405	98/42	E	
1406	98/44	8A-A±	
1407	98/45	E	

Plasmid pool 123			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1413	98/52	E	
1420	99/6	P-A±	
1421	99/7	L≤ n	53D12-14
1423	99/10	E	52E7-8
1425	99/18	E	56F8-15
1426	99/19	L<<n	
1428	99/21	L≤ n	
1430	99/23		26D1-2
1458	101/21	E	51B4-5 30A7-8
1459	101/22	P-8A	

Plasmid pool 124			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1462	101/27	A±	25C1-2
1502	104/1	8A	
1503	104/2	E	
1508	104/9	E	
1509	104/10	A±	
1514	104/19	L-P	
1529	106/8	A±	
1535	106/17	E-L	27C6-8
1539	107/3	8A	
1544	107/14	E	

Plasmid pool 125			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1545	108/1	E-8A	
1549	108/5	8A	
1550	108/7	8A	
1556	108/18	P	
1558	108/21	E	50C14-15 49F1-2
1559	108/24	E	
1585	110/35	L<<n	
1587	110/38	8A-A±	22D1-2
1599	111/18	E	
1614	112/14	L<<n	

Plasmid pool 126			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1625	112/37	L<<n	
1632	113/11	L<<n	54E1-2
1637	113/19	E	
1645	114/1	E-L	
1653	114/12	L-8A	
1659	115/7	A±	54C1-2
1664	115/14	E	
1666	115/16	A±	27C1-2
1667	115/19	E	
1680	115/38	8A-A±	37C5-6

Plasmid pool 127			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1683	116/3	E	
1695	119/4		
1705	121/3	E	60B4-5
1706	122/1	A±	
1708	122/2	8A-A±	
1713	123/5	E	47A11-14 46F5-6
1722	126/4	pP	
1725	126/9		
1740	130/3	E	
1742	130/9	E	

Plasmid pool 128			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1745	131/3	8A-A±	
1784	133/22	E	
1785	134/1	E	
1795	134/21	A±	
1830	136/24	P-A	21B4-6
1843	137/6	E-L	57B1-3
1851	137/16	E	26A5-6 61D1-2 66F1-2
1853	137/19	L<<n	
1856	138/1	L-A	
1858	138/3	L<<n	57C1-2

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 17

Plasmid pool 129			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1859	138/4	$A^{\pm}$	
1860	138/5	8A-$A^{\pm}$	
1862	138/9		50A12-14
1863	138/10	E	
1869	138/22	$A^{\pm}$	
1870	138/25	P-8A	
2242	115/20		
2244	105/14		
1852	137/17	E	
1883	140/2	E	

Plasmid pool 130			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1844	137/7		
1889	140/10	E	35D1-2
1891	140/14	E	
1893	140/18	L-P	
1898	140/25	E	
1902	140/29	P-8A	
1903	140/36	E	48B6-7
1905	140/38	$A^{\pm}$	
1908	140/41	E-$A^{\pm}$	
1910	141/4	E	

Plasmid pool 131			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1913	141/9	L-P	
1918	142/4	L<<n	30D1-2
1928	143/1	8A	
1929	143/4	E	
1935	143/12	E	49F7-8
1938	144/1	L≤n	31A
1942	144/7	E	29B1-2 42A15-19
1944	144/9	E	
1950	144/16	E	
1952	144/21	E	

Plasmid pool 132			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1953	144/22	8A-$A^{\pm}$	35D5-7
1954	144/23	8A-$A^{\pm}$	
1956	145/2	E	30F5-6
1957	145/3	L<<n	
1960	145/5	8A-$A^{\pm}$	39C1-2
1961	145/6	L<<n	
1969	145/18	E	
1970	145/20	E	
1979	146/10	$A^{\pm}$	
1980	146/12	P-8A	

Plasmid pool 133			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1981	146/14	E	
1985	147/2	8A	39B1-2 60A5-6
2007	149/1	$A^{\pm}$	
2008	149/2	8A-$A^{\pm}$	29B1-2
2036	154/3	E	
2039	154/9	E	
2041	154/11	E	
2042	154/14	L<<n	
2043	154/15	E-P	
2071	158/3		

Plasmid pool 134			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2074	158/11	E-L	
2100	160/6	E	26A5-8 45B7-8
2115	161/17	E	
2117	161/20	E	44B5-6
2247	167/12		
2248	161/15	$A^{\pm}$	29E1-2
2204	168/21	E	
2243	113/9	E	35D1-2 54B12-16
2245	114/3	8A	
2246	104/15	L-P	

Plasmid pool 135			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1388	98/16	E	45C7-8
1393	98/22	E	28D1-2
1403	98/39	P	
1416	99/1	E	
1419	99/5	8A	52E5-8
1424	99/17	E	28B1-2
1546	108/2	E	
1557	108/20	8A	
1634	113/13	$A^{\pm}$	
1643	113/30		

Plasmid pool 136			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1744	131/2		
1787	134/3	$A^{\pm}$	48A3-4
1788	134/9	L-P	60B3-5
1793	134/18	8A	
1878	139/11	E	
2082	158/22	E	
2101	160/8	L<<n	
2102	160/9	E-L	39B1-2 40B1-2
2103	160/10	E-L	
2106	161/2	L<<n	

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 18

Plasmid pool 137			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2108	152/2	L<<n	
2112	161/11	E	
2118	161/22	P-8A	
2119	161/24	E	23C1-2 61F3-4 68C1-2
2120	161/25	P-8A	
2124	161/30	8A-A±	
2125	161/31	E	
2128	162/5	A±	
2131	162/8		47A11-14 30E1-2
2132	162/10	P	56F1-2

Plasmid pool 138			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2135	162/15	P	36A4-5
2139	162/21	E-L	43F3-6 55C7-8
2140	162/24	pP	
2141	162/25	E	
2160	166/1	E	
2164	166/5		
2169	166/15	L<<n	25C1-2
2173	166/19	A±	
2177	167/4	E	
2179	167/6	E	

Plasmid pool 139			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2180	167/8	E	42E5-6 39A1-2
2187	168/1	E	31E1-2 60F1-2
2191	168/5	L<<n	
2193	168/7	E	23B5-6
2197	168/12	E	25C1-2
2198	168/13	E	
2216	170/2	E-L	58F4-5
1238	90/10	E	30D3-4
1241	90/16	E	60A8-11
1269	92/1	8A-A±	

Plasmid pool 140			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1092	81/23	P-A	
1096	81/31	E	50A9-10 77B4-5
1098	81/35	E	
1128	82/43	E-A±	37F1-2
1131	82/53		
1133	82/58	E	
1150	84/1	8A	32B2-3 38B5-6
1161	84/14	P	
1163	84/16	E	21B2-3 96C1-2
1186	86/24	A±	

Plasmid pool 141			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1219	89/8	E-L	
1221	89/10	P-8A	
1222	89/15	A±	21C5-6
1300	93/3	P	53F4-5
1307	93/16	P	
1946	144/12	8A-A±	42C1-2
1955	145/1	E	54B15-16
1966	145/14	P	25F3-4 27D3-6
2076	158/16	E	56C20-21 78A1-2
1339	95/24	A±	

Plasmid pool 142			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1159	84/10	E-A±	
1160	84/12	E	
1311	93/21	E	
1318	94/6		26A5-6 38F1-2 57F5-6
1319	94/7		
1328	95/3	E	
1338	95/21	E	
1340	95/26	8A-A±	
1341	95/31	E	
1322	94/12	E	28E3-4

Plasmid pool 143			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1345	95/37	E	23C1-2 48E4-5
1363	96/25	8A	
1364	96/28	E	21B1-2 42A10-12
	96/40	L<<n	39B, 42F 50D
1372	97/15	P	
1700	120/3	E	
1701	120/4	L<<n	34C4-5
1702	120/5	P-8A	
1733	129/9	P	
1734	129/10	P	

Plasmid pool 144			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1738	129/13	L<<n	21C3-4
1745	131/3	8A-A±	
1746	131/4	E-L	56F10-11 55E1-2
1749	131/7	pP	49A10-11 48E4-7
1750	131/8	pP	60A3-4 38C1-23
1772	133/4	A±	30A1-2
1775	133/7	E-L	
1779	133/14	8A-A±	53A3-5
1782	133/19	P-8A	
1888	140/7	8A	

Plasmid pool 145			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1900	140/27	8A	42C1-2 22B4-5
1947	144/13	E	
1958	145/4	8A	21B7-8
1971	145/23	8A-A±	56F5-6
2018	152/2	L<<n	
2022	152/10	E	
2039	154/9	E	
2087	159/3	L<<n	
2220	170/6	A±	
1892	140/17	8A-A±	52E5-6

Plasmid pool 146			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1071	80/47	E	
1199	88/1	E	
1230	89/23	E	
1332	95/8	L<<n	
2238	170/36		22F1-2 38F1-3
1907	140/39	L-P	
1906	140/40	8A	
1909	141/3	E	
1915	141/14	8A	
1917	142/2	E	

Plasmid pool 147			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1726	127/2	pP	
1727	128/1	A±	
1728	129/1	A±	23C1-2
1730	129/5	L<<n	57F5-7
1732	129/7	A±	
1729	129/2	A±	
1736	129/12	A±	
1737	129/14	E	29C1-2
1739	130/1	E	
1748	131/6	A±	

Plasmid pool 148			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1752	131/10	E	
1753	131/13	E	
1754	131/16	E	28F1-2 93B9-11
1755	132/1		
1758	132/7		
1759	132/8	L<<n	45F1-2
1761	132/11	E	58D6-7
1762	132/14	E	
1763	132/15	E-L	
1764	132/17	P	

Plasmid pool 149			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1765	132/18	E	35B3-5
1767	132/21	E	
1771	133/12	A±	
1773	133/5	8A-A±	30F5-6
1774	133/6	E	48E1-2 102D5-6
1776	133/10	L<<n	
1777	133/12	A±	48D5-6
1778	133/13	E	
1786	134/6	E	
1789	134/10	P-8A	

Plasmid pool 150			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1790	134/11	E	
1791	134/15	8A	
1792	134/16	E-P	
1794	134/20	E	25C1-2
1796	134/27	8A	
1797	134/22	L<<n	41C 48D5-6
1798	134/30	E	
1799	135/1	E	
1800	135/2	E-L	21B4-6 82C1-2
1801	135/4	E	

Plasmid pool 151			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1802	135/5	E	
1803	135/7	8A-A±	35A1-2
1807	135/10	P	28E3-4
1808	135/16		
1809	135/17	L<<n	
1811	135/19	E	
1812	135/20	E	
1813	135/21	E	
1817	136/2	E	
1818	136/3	A±	45D4-5

Plasmid pool 152			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1820	136/5	A±	
1823	136/9	L<<n	
1836	136/38	E	
1846	137/9	L<<n	
1824	136/12	E	
1826	136/15	L<<n	
1832	136/27	P	
1833	136/30	L<<n	
1835	136/32	L<<n	
1840	137/3	A±	

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 20

Plasmid pool 1			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1845	137/8	L<<n	
1847	137/12	8A	
1848	137/13	L<<n	28B1-2
1849	137/12	$A^{\pm}$	21C7-8
1850	137/15	$A^{\pm}$	38F1-2 87C6-7
1852	137/17	E	
1854	137/20	P-$A^{\pm}$	26C2-3
1855	137/21	E	
1861	138/6	L<<n	
1864	138/11	8A	32C3-5

Plasmid pool 1			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1865	138/12	P-8A	
1867	138/14	E	
1868	138/17	8A	
1871	138/27	E	
1872	139/2	E	
1873	139/3	E	
1874	139/5	E	36A10-11
1875	139/6	E	46D1-2
1876	139/9	P-8A	
1879	139/12	E	

Plasmid pool 1			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1800	135/2	E-L	21B4-6 82C1-2
1881	139/14	P	
1882	140/1	E	23C4-5
1883	140/2	E	
1885	140/4	E	
1886	140/6	8A	22B3-5 42B2-3
1887	140/5	$A^{\pm}$	
1901	140/28		38A
1904	140/37	E	
1894	140/19	pP	

Plasmid pool 1			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1234	90/2	E	
1343	95/34		
1349	96/1	L<<n	
1371	97/13	E-$A^{\pm}$	25D4-5
1735	129/11		
1747	131/5	$A^{\pm}$	
1769	133/1	E	
1770	133/2	E	37B6-9
1780	133/15	E	27B1-2
1828	136/19	L-A	

Plasmid pool 1			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1041	80/10	E	23A5-6 75C3-4
1066	80/39	E	
1415	98/55	E	
1437	100/3	pP	21B4-6
1438	100/4	8A-$A^{\pm}$	25B1-2
1489	103/6	$A^{\pm}$	
2002	148/17	$A^{\pm}$	34C4-5
2003	148/20	E-L	
1013	79/7	P	
2016	151/3	$A^{\pm}$	

Plasmid pool 1			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2020	152/5	pP	47A11-14 49D1-3 57B1-3
2021	152/6	E-L	28B1-2
2023	152/11	L<<n	
2026	153/3	E-$A^{\pm}$	
2030	153/11	$A^{\pm}$	
2035	154/2	E	
2037	154/4	8A-$A^{\pm}$	
2040	154/10	E	
2044	154/18	E	
2048	155/12	pP	54C5-8

Plasmid pool 1			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2050	155/16	E	
2053	156/5	E	
2054	156/6	E	50C17-19
2055	156/8	E	
2057	156/12	E-L	21B4-5
2058	156/14	L<<n	
2060	156/17	$A^{\pm}$	52E1-2
2061	156/19	E-L	
2062	156/20	L<<n	
2068	157/15	L<<n	

Plasmid pool 1			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2091	159/9	E	
2092	159/11	E-$A^{\pm}$	
2093	159/13	E	
2095	159/15	P-8A	53f1-5
2105	161/1	P	43b1-2
2109	161/6	L<<N	
2110	161/8	E	
2111	161/9	E	44f3-4
2113	161/13	L<<n	27e1-2 53a1-2 61d1-2
2114	161/14	L<<n	

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 21

Plasmid pool 161			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2116	161/18	$A^{\pm}$	34B1-2 44F1-2
2123	161/29	L<<n	
2126	162/3	$A^{\pm}$	
2129	162/6	E	43C3-4
2130	162/7	L<<n	56D1-2
2134	162/14	E	
2137	162/18	E	44F2-6
2138	162/19	E	
2142	163/4	L<<n	
2143	163/15	P	

Plasmid pool 162			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2144	164/1	E	30C1-2
2145	164/3	$A^{\pm}$	39E1-4
2149	165/3	E	44A4-5
2148	165/2	E-L	
2151	165/7	P	
2152	165/10	$A^{\pm}$	21B4-6
2162	166/3	E	
2163	166/4	E	
2166	166/8	E	
2170	166/16	8A	

Plasmid pool 163			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2174	167/1	E	
2181	167/14	L<<N	60F2-3 60D1-2
2183	167/19	E	
2185	167/22	$A^{\pm}$	42E3-4
2186	167/24	E-L	
2189	168/3	E	
2190	168/4	E	
2199	168/14	L-P	23B5-6
2201	168/16	E-L	
2202	168/17	$A^{\pm}$	

Plasmid pool 164			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2208	169/1	E	53C1-4
2209	169/2	E	
2210	169/4	L≤n	
2213	169/13	E	
2215	169/19	E	28E3-4
2216	170/2	E-L	58F4-5
2221	170/10	E	51A 53C-E
2222	170/11	E	
2223	170/2	E	53C1-4
2227	170/20	E	

Plasmid pool 165			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
319	39/6	E	31F1-2 51B4-5
512	55/8	P-8A	
514	55/12	P	
516	55/15	E	
522	55/21		28C4-5 47C1-4
523	55/27	E	
524	55/32	P	
564	56/55	E	
552	56/38	E-L	
801	70/9		

Plasmid pool 166			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
955	77/1	E	
1012	79/6	P-8A	
1018	79/15		
1026	79/25	L<<n	
1052	80/22	E	50F1-2 87D1-2
1070	80/46	8A-$A^{\pm}$	
1143	83/15		48F4-5 49C1-3
1175	86/10		
1200	88/2		29E5-6
1231	89/24	E	45F1-2

Plasmid pool 167			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1264	91/11	E	
1308	93/17	E	60B4-5
1313	93/24	P-$A^{\pm}$	
1386	98/14		
1408	98/46	P-8A	
1414	98/54	$A^{\pm}$	53F4-5
1468	102/13	$A^{\pm}$	45F1-2
1447	101/6	$A^{\pm}$	45A4-8
1465	102/9	P	53E1-2
1440	100/9	8A-$A^{\pm}$	

Plasmid pool 168			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1478	102/27	E	
1479	102/28	E	48B6-7
1499	103/21	pP	47A13-14
1756	132/2	8A	
1757	132/6	$A^{\pm}$	32A4-5
1586	110/37	L<<N	
1569	110/1	$A^{\pm}$	
1576	110/14	E	
1571	110/4	E	
1578	110/18	E	47C3-4

Plasmid pool 169			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1579	110/21	E	22B1-2
1580	110/23	A±	29C1-2
1670	115/24	E-L	35B6-7 93D3-5
1678	115/34	8A	58F1-2
1719	124/9	P	26B8-9 26C1-2 26D4-5
1743	131/1	pP	48E4-7
1766	132/19	8A	53B1-2
1804	135/8	L≤n	
1829	136/23	P-A	42D1-2
1932	143/9	E	50F4-7 57B4-6

Plasmid pool 170			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1940	144/4		55C9-12
1995	148/7	E	
1989	147/7	E	51C1-2
2000	148/14	E	
2001	148/16	P	29A3-5 23D1-2
2009	149/4	8A	
2045	154/19		22F3-4
2080	158/20	E	26A5-6 29A1-2 67B1-2
2059	156/16	L<<n	42A15-16
2067	157/13	E	

Plasmid pool 171			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2083	158/26	8A-A±	47C3-4
2104	160/11	E	39B1-2 40B1-2
2121	161/26	P	
2133	162/13	E	27E1-2 53A1-2 61D1-2
2136	162/16	P	
2146	164/6	8A-A±	
2147	165/1	L<<n	25A6-7 28D1-2
2153	165/14	A	
2184	167/21	E	25C1-2
2155	165/16	P	36A10-11 39C1-2 40B1-2

Plasmid pool 172			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2157	165/21	E-L	21B4-6
2161	166/2	E-L	43E7-10
2165	166/6	A±	
2167	166/10		21D3-4
2168	166/11	E	
2171	166/17	E-L	47A11-14
2188	168/2	P	26B8-9
2192	168/6	A±	45D4-5
2194	168/8	8A-A±	34B1-2 60F1-3
2200	168/15	L<<n	

Plasmid pool 173			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2203	168/18	P-8A	27D1-2
2204	168/21	E	
2206	168/28	L<<n	
2211	169/7	E	
2212	169/10		
2214	169/18	E-L	
2217	170/3		
2218	170/4	L<<n	34A3-4
2219	170/5	P-A	47F4-9
2224	170/13	E	

Plasmid pool 174			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
2226	170/19	L<<n	49B3-4 49E1-2
2230	170/24	E	
2231	170/25	8A-A±	33E7-8
2232	170/26	E	58D6-7
2233	170/27	E	35F10-11
2235	170/31	L-P	45F1-2 50F1-2
2237	170/35	E-L	45F1-2
2239	170/37	E	
2229	170/23	L<<n	
2243	113/9	E	

Plasmid pool 175			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
510	55/6	8A	
517	55/16	P-8A	
518	55/17	P	35D1-4 37C6-7 82E6-7
525	55/29	E	
586	58/9	E	
587	58/9	8A	
595	58/19	E	24F1-2
614	59/18	E	
620	60/5	E	48E8-11
645	61/13	E-L	39E5-6

Plasmid pool 176			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site
603	59/5	P	26B8-9
662	62/4	E	46C1-2
684	63/20	L<<n	60C7-8 67C5-8
679	63/11	E-L	
694	63/33	E	31A1-2
729	65/7	A±	
741	66/6	E	30B1-2 21B4-6
800	70/7	E	
856	72/23	E	34A5-6
926	75/18	P-8A	

List of rescued plasmids with corresponding fly lines, lethal phases and chromosomal locations of the P[*lacW*] Box 24

Plasmid pool 185				Plasmid pool 186				Plasmid pool 1				Plasmid pool 1			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site	Glycerol stock	Fly line	lethal phase	P[*lacW*] site	Glycerol stock	Fly line	lethal phase	P[*lacW*] site	Glycerol stock	Fly line	lethal phase	P[*lacW*] site
1926	142/13	$A^{\pm}$		1976	146/3	L<<n									
1930	143/6	E		1977	146/7	P-8A									
1931	143/8	E		1978	146/8	L<<N									
1933	143/10	E		1900	140/27	8A	48B6-7 56D1-2								
1934	143/11	E-L		1991	147/10	E	42A15-19								
1939	144/3	pP		1992	147/15	E									
1941	144/5	L<<n		1993	147/16	E	70A1-2								
1943	144/8	L<<n		1997	148/11	E-$A^{\pm}$									
1945	144/10	L<<n		1027	79/26	E	47A11-14 62E6-7								
1974	146/1	$A^{\pm}$	54E1-2	1975	146/2	L≤n	48B1-2 67B1-2								
Plasmid pool 1				**Plasmid pool 1**				**Plasmid pool 1**				**Plasmid pool 1**			
Glycerol stock	Fly line	lethal phase	P[*lacW*] site	Glycerol stock	Fly line	lethal phase	P[*lacW*] site	Glycerol stock	Fly line	lethal phase	P[*lacW*] site	Glycerol stock	Fly line	lethal phase	P[*lacW*] site

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