A P Element Excision-Derived Mutation In *Drosophila melanogaster*

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Summary

The purpose of the work described in this Thesis was to isolate and mutate novel genes essential in the development of the *Drosophila melanogaster* nervous system. Two marked P element enhancer trap strains were found to express the reporter gene *lacZ* in spatially and temporally regulated patterns in the nervous system. Excisions of the P element construct in one of these strains, A22, were detected in approximately 68% of the A22-derived progeny which were recognised by the loss of the *rosy* gene. 19 strains were subsequently found to have a recessive mutant phenotype believed to be a result of imprecise excision of the P element. This represents a mutation rate of approximately 24%. Each of these 19 strains had a recessive mutation that resulted in abnormal wing development. Other mutant phenotypes were not detected in any of the excision-derived strains. No similar mutations are known to be located at the site of P element insertion of the ancestral A22 strain and therefore these excision-derived mutants are considered novel. Four excision-derived mutants were selected for further characterisation and these were found to be cold-sensitive. The severity and frequency of the mutant phenotype was increased when these four strains were raised at 20°C rather than 25°C. When raised at 16°C, these four mutants were found to have a recessive pupal lethal phenotype. Complementation analysis using all possible combinations of these four mutants confirm each strain is mutant at the same locus. The mutant phenotype indicates no disruption to embryonic neurogenesis at the temperatures investigated. In addition, the mesothoracic disc of the A22 ancestral strain gives no evidence of reporter gene activity. Thus the *lacZ* expression pattern of the A22 P element insertion strain can not be correlated with the abnormal wing development. Reporter gene activity was detected in the larval and adult central brain. This may indicate the cold-sensitive pupal lethality results from abnormal CNS development during metamorphosis. The available data at this time suggests the gene disrupted by P element imprecise excision may not be the gene regulated by the enhancer element directing *lacZ*

expression during embryonic development. Further analysis of these four mutant strains when raised at both 20°C and 25°C, show they are able to jump, excluding the possibility of abnormal leg development. Flight ability tests show these homozygous mutants are unable to fly when raised at the higher temperature of 25°C while those raised at 20°C have normal flight behaviour. Those homozygous progeny raised at the lower temperature of 20°C with phenotypically normal wings are able to initiate and maintain flight, while those raised at 25°C with apparently normal wings are unable to fly. Characterisation of these four mutants show imprecise excision of the A22 P element has resulted in the generation of a paradoxical phenotype where normal development during the pupal stages is cold-sensitive and flight behaviour is heat-sensitive.

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I feel like a cross between Humpty Dumpty and Rip Van Winkle - I have fallen off the wall and suddenly awake I find all the pieces of me, before me.

There are more parts than I began with.

All the King's horses and all the King's men, cannot put Humpty together again.

Brian Keenan

Dutch Embassy,

Damascus,

24th Aug 1990

I dedicate this Thesis to my Mum, Helen, who has so much courage and love, and deserves so much more, and to Andy

Abbreviations

Chemicals

cDNA	complementary DNA
DAB	diaminobenzidine
EDTA	ethylene diamine tetra-acetic acid (disodium salt)
EtBr	ethidium bromide
IPTG	isopropylthio-β-D-galactoside
PEG	polethylene glycol
SDS	sodium dodecyl sulphate
Tris	Tris (hydroxymethyl) aminomethane
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Measurements

bp	base pair
Ci	Curies
cm	centimetres
cpm	counts per minute
g	grammes
hr	hours
kb	kilobase pairs (10^3 bp)
l	litres
M	Molar
mg	milligrammes
min	minutes
ml	millilitres
mM	milliMolar

ng	nanogrammes
OD	optical denstiy
pH	acidity [-log10(Molar concentration of H+ ions)]
rpm	revolutions per minute
µCi	microCuries
µg	microgrammes
µl	microlitres
^{o}C	degrees centigrade

Miscellaneous

Ab	antibody
β-gal	β-galactosidase
BSA	bovine serum albumin
dH_2O	distilled water
DNase I	deoxyribonuclease
HRP	horseradish peroxidase
mAb	monoclonal antibody
Nb	neuroblast
O/N	overnight
Rnase A	ribonuclease A
U.V	ultra violet light

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Chapter 1

Introduction

1.1 The Development of *Drosophila melanogaster*

Complex molecular and cellular interactions are required for a multicellular organism to develop from a single fertilised egg. Many different cell types must be generated and organised into the various tissues and structures, which ultimately give rise to the complete adult form. *Drosophila melanogaster* must pass from the embryo through three larval instar stages and a pupal stage to generate the adult form, and is thus a holometabolous insect. The embryo develops a larval form, and then hatches. The structure changes little through the three instars, but shows a marked increase in size. The third larval instar is then followed by pupation when metamorphosis changes the larval body form to the adult fly structure. This involves drastic changes, mediated by histolysis of most of the larval tissues, growth and differentiation of the imaginal disc tissue generating a radically altered form.

Embryogenesis - Overview

The fertilised egg develops as a synctial blastoderm with the nuclei lying just under its surface of the egg. These nuclei, become enveloped by a plasma membrane, in a process named cellularisation. Gastrulation then occurs. *Drosophila* are classified as long germ-band insects since metameric germ-band arises, without growth, by partition of the early embryo into segmental units.

The primary pattern of the *Drosophila* embryo is initiated by four different maternal patterning systems that have been extensively reviewed (St Johnston and Nüsslein-Volhard, 1992; Stein and Stevens, 1991; Small and Levine, 1991; Dessain, and McGinnis, 1991; Irish *et al.*, 1989). The anterior system establishes the anterior polarity, chiefly by the graded expression of the Bicoid protein, while the posterior polarity of the embryo is established chiefly by *nanos* gene expression. The terminal system requires the correct spatial expression of *tailless* to establish terminal structure patterns. The dorsoventral system requires the activity of a number of genes such as

Toll and *dorsal* for the accurate formation of the embryonic dorsoventral polarity. The maternal patterning systems are activated prior to fertilisation, being supplied by the nurse cells of the ovary. The maternal contribution to embryonic pattern formation has been extensively reviewed (St Johnston and Nüsslein-Volhard, 1992; Stein and Stevens, 1991). The metameric pattern of the embryo is established prior to gastrulation by a complex cascade of gene activity. The gap genes are expressed in single bell-shaped domains prior to cellularisation of the embryo. Specific combinations and concentration gradients of the gap gene proteins define the position of various pair-rule gene expression domains. These pair-rule genes are expressed in alternating stripes along the anterior/posterior axis of the embryo, thus establishing the basic metameric pattern of the embryo. The pair-rule genes are responsible for the activation of the segment polarity genes, that function primarily to establish the anterior/posterior polarity of each embryonic segment. The pair rule genes have been reviewed by Small and Levine (1991), while the segment polarity genes have been reviewed by Ingham (1991). The final group of genes involved in pattern determination are the homeotic genes (reviewed in Dessain, and McGinnis, 1991). These genes control the development and identity of segmental structures, and are expressed in response to the gap and pair-rule gene activities (e.g., Irish *et al.*, 1989), and later their expression patterns are refined as development proceeds.

1.2 The *Drosophila* Nervous System

The nervous system is the most complex and intricate organ possessed by multicellular organisms. The nervous system allows the animal to detect, interpret, and respond to both the internal and external environments. The mechanisms used to provide a fully integrated and responsive nervous system are not well understood. If we are to begin to unravel the complex nature of how nervous systems develop and function, then we must first ask basic, simple questions. Which neurons synapse with what cells? How do the neurons develop and form the synapse? What function

does that synapse have? What happens when that synapse is disrupted? What genes are required for the generation of the large number of cell types within the nervous system? How do they exert their effects?

These questions require, at least initially, the study of a relatively simple system, such as *C. elegans* or *D. melanogaster* that have a variety of advantages experimentally. The ontogeny of the small number of *C. elegans* cells has been fully mapped, although the genetics of this organism has not been as extensively characterised. *D. melanogaster* has provided a wealth of genetic information over the last century, facilitating the dissection of many developmental processes. This organism has a small nervous system allowing easy access, and the analysis of mutations that may perturb the development and/or functions of specific subsets of neural cells. However, the small size of *Drosophila* makes many experimental techniques such as cell transplantation (Technau and Campos-Ortega, 1986; Stuttem and Campos-Ortega, 1991; 1986) and the ablation of specific cells by physical means difficult. Such experimental approaches have made use of the larger grasshopper embryos that are believed to have many cell types and developmental mechanisms in common with *Drosophila* (Doe and Goodman, 1985). Additionally *D. melanogaster* has several well-defined behaviours that may permit analysis of many functions of the nervous system.

The central and peripheral nervous systems, especially in both the embryonic and the adult stages, have received much attention (e.g., Bates and Martinez Arias, 1993). These neural tissues have so far proved productive in the search for the molecular and genetic mechanisms involved in specifying cell fate, and cell-cell interactions throughout important morphological changes as development builds an ever increasingly intricate tissue.

The Larval and Pupal CNS

The larval central nervous system (CNS) consists of the two rudimentary brain lobes and the ventral nerve cord. It is the development of the ventral nerve cord that has received most attention, and will be discussed in detail later. The larval CNS, generated during embryogenesis, provides the neural functions necessary for survival in a semi-liquid environment. The larval CNS is remodelled rather than replaced during metamorphosis, (although there is also a significant level of cell death) unlike most of the larval structures that degenerate during this period (reviewed in Truman *et al.*, 1993). Neurogenesis, first detected in the embryo, also occurs during larval life (White and Kankel, 1978; Booker and Truman, 1987; Truman and Bate, 1988). For example, the four mushroom body neuroblasts are mitotically active at the time of larval hatching, and continue dividing almost to the end of metamorphosis (Truman and Bate, 1988; Ito and Hotta, 1992, de Belle and Heisenberg, 1994.). During the first 24 hours of metamorphosis, there are detectable levels of cell death in the CNS, occurring predominantly, although not exclusively, in the abdominal ventral ganglia (reviewed in Truman *et al.*, 1993) and a second phase of cell death occurs after eclosion, primarily in the abdominal neuromeres.

The Adult CNS

The adult CNS consists of the subeosophageal ganglia and the supraosephageal ganglia, which together comprise the brain, and the ganglia located in the thorax. The neurons of the CNS are motoneurons and interneurons and these are connected to the periphery by the axons of the peripheral nerves (reviewed by Truman *et al.*, 1993).

The Peripheral Nervous System

The larva and adult posses a diverse set of sensory organs, both external and internal, many of which develop at constant, reproducible positions, and so have been assigned individual names, providing easily scoreable characteristics, that have facilitated the genetic analysis of *Drosophila* development (reviewed by Ghysen and Dambly-Chaudière, 1992.)

The external organs contain an external process, the trichogen, and a socket cell, the tormogen. These organs have an inner support cell, the thecogen, that surrounds the dendrite (or dendrites) of the sensory neuron. The mechanosensory organs respond to touch and have an external, pointed bristle. The chemosensory organs have an external, open-tipped bristle and are responsive to chemical stimulation of the dendrite. The external organ of the campaniform sensilla is a flat dome, responsive to stresses acting on the cuticle. The campaniform sensilla and the mechanosensory organs are mono-innervated, while the chemosensory organs are innervated by at least two neurons. Each sensory neuron sends out an axon that makes contact with the appropriate target cells within the CNS.

The chordotonal organs are the best understood internal sensory organ of the larva, consisting of a neuron, a thecogen cell and two other support cells. The neuron is bipolar in that it has a single dendrite similar to the neurons of the external sense organs. There are a second group of internal sense organs that consist of a single neuron with multiple dendrites. Although not well understood, many of these organs are thought to act as touch receptors in the larvae. The internal sense organs of the adult fly have not been characterised.

1.3 Embryonic Neurogenesis

The developing embryonic peripheral nervous system (PNS) facilitates the study of the nervous system as the pattern of cells is relatively simple to distinguish

and occurs in a highly reproducible manner. The precursors of the larval sensilla can first be detected after 5 hours of embryonic development and by 9 hours of development all the cells of the sensory organs are born (Bodmer *et al.*, 1989).

The initial events in nervous system development require the emergence of progenitor cells, known as neuroblasts, in the CNS and sensory mother cells in the PNS. The ventral neurogenic region (NR), or neuroectoderm of the embryo gives rise to progenitors of the epidermis, CNS and PNS (Hartenstein and Campos-Ortega, 1984; Technau and Campos-Ortega, 1986). Foe (1989) has divided the developing embryo into a number of regions on the basis of mitotic synchrony. Mitotic domain 11 corresponds to the dorsal ectoderm that gives rise to epidermis and a subset of PNS cells. Mitotic domain M encompasses the ventral ectoderm that gives rise to the SI, SII, SIII neuroblasts of the CNS and epidermoblasts, and mitotic domain N gives ride to SI and SII neuroblasts in addition to both PNS precursors and epidermoblasts. In contrast, all the cells of the dorsal epidermal anlage, another ectodermal region, follow the epidermal developmental fate. The neural progenitors of the ventral ectoderm then divide, producing a variety of neuronal cell types and sensory organ precursors that have specific identities as illustrated in Figures 1.1 and 1.2 (reviewed in Goodman and Doe, 1993; Campos-Ortega, 1993). The final stage of neurogenesis involves the differentiation of the neurons, and the sensory organs. The neurons send out axons, and make specific connections with target cells, allowing communication between the PNS, muscles, and other tissues, with the CNS.

Cellular Events (Early Neurogenesis)

The cells of the CNS become segregated from the ectoderm 4-6 hours after fertilisation, near the end of, or soon after, gastrulation (stage 8). This process is complete when the CNS separates wholly from the ectoderm, after germ band shortening. The segregation of the neuroblasts occurs in three distinct waves producing the SI, SII, and SIII neuroblasts (Hartenstein and Campos-Ortega, 1984;

Campos-Ortega and Hartenstein, 1985). Each neural precursor cell enlarges, and delaminates from the ectoderm layer, moving into the embryo. The first wave of neuroblast segregation gives rise to 10 neuroblasts in each hemisegment. Along the presumptive ventral nerve cord of the embryo there are three columns of these neuroblasts with four rows across each segment such that they partially form a 3 x 4 array (Jiménez and Campos-Ortega, 1990). As the second and third wave neuroblasts segregate the pattern of NBs becomes more complex, making identification of individual cells difficult.

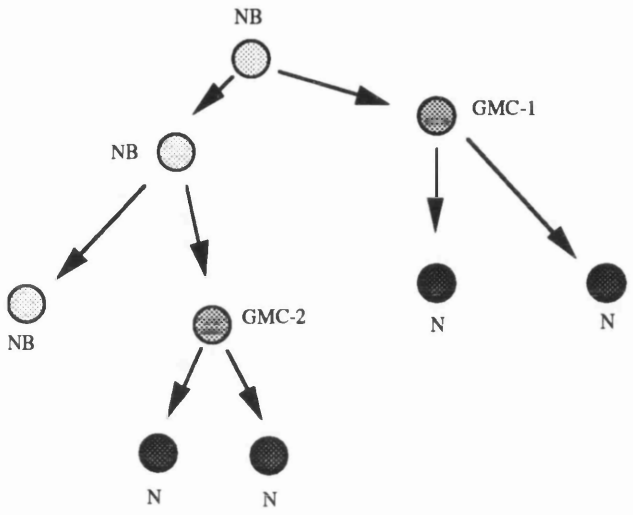
The Ventral Midline

The midline cells separate the two neurogenic regions of the embryo and can first be detected molecularly at the cellular blastoderm stage when they initiate expression of the *single-minded* gene (Thomas *et al.*, 1988; Crews *et al.*, 1988). 8 midline cells in each segment have been characterised during the early stages of development. These 8 cells have an invariant lineage generating approximately 25 glia and neurons per segment (Klämbt and Goodman, 1991) and have proved useful in the study of axogenesis in the CNS since these cells provide the initial scaffold for neural processes as they search for their appropriate targets (Klämbt *et al.*, 1991).

Patterns of Cell Division

An important stage in the development of the nervous system is the creation of a three-dimensional tissue from a two-dimensional arrangement of neuroblasts. Each neuroblast divides asymmetrically in a stem cell mode, giving rise to between 5 (reviewed in Goodman and Doe, 1993) and 9 (discussed in Campos-Ortega, 1993) progeny cells named ganglion mother cells (GMCs) and further neuroblasts. Each GMC divides once to give rise to two neurons as illustrated in Figure 1.1. The larger parental cell remains just below the epidermal sheet, while the smaller progeny cells

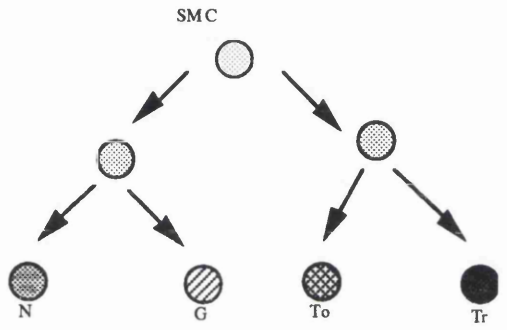
Figure 1.1 The Asymmetric Cell Division Pattern of Neuroblast Cells



NB	Neuroblast.
GMC	Ganglion Mother Cell.
N	Neuron.

The neuroblast divides producing a daughter neuroblast and a ganglion mother cell. The ganglion mother cell divides producing two daughter neurons. The daughter neuroblast continues in this cell division pattern, producing ganglion mother cells and neuroblasts for a number of generations.

Figure 1.2 The Symmetric Cell Division Pattern of the PNS Precursor Cells



The sensory mother cell or sensory organ precursor cell divides to produce two progeny. These two progeny divide to produce the four cells of the sensory organ. Abbreviations: N: neuron, G: glial cell, To: Tormogen cell, Tr: trichogen cell.

are found below the parental cells. Many neurons have been shown to have an invariant cell lineage, such that a given neuroblast always divides to produce the same first GMC, and in some cases the same second and third GMCs (Goodman *et al.*, 1984; Raper *et al.*, 1984). This GMC always divides to produce the same pair of neurons. An example of this is the progeny of the NB 1-1 neuroblast in both the grasshopper and *Drosophila* embryos. This neuroblast always gives rise to the aCC and pCC neurons, after the GMC division (Raper *et al.*, 1984; Doe *et al.*, 1988; Doe *et al.*, 1988). This must be due, at least in part, to the fact that identified neuroblasts and their progeny are born in highly reproducible sites.

The cell division patterns of the sensory mother cells gives rise to four progeny cells (see Figure 1.2). The midline cells are notable in that they do not follow this asymmetric division pattern, but rather have a symmetric cell division pattern similar to that of the SMCs of the PNS , (reviewed in Goodman and Doe, 1993).

1.4 The Genetics and Molecular Biology of Neurogenesis

1.4.1 Neural Development Mutants

A number of genes in *Drosophila* have been identified, that when mutant, perturb the normal development of the nervous system in both embryos and adults. In the embryo, loss of function (LOF) mutations have shown *achaete* (*ac*) and *scute* (*sc*) are involved in the development of the same set of external sensory organs (Ghysen and Dambly-Chaudière, 1988), while *asense* (*ase*) is required for the formation of a complementary subset of external organs (Dambly-Chaudière and Ghysen, 1987).

In the CNS, the genes of the AS-C are believed to play an important role in the development of neuroblasts (Cabrera *et al.*, 1987; Jiménez and Campos-Ortega, 1990). LOF mutations of the *lethal of scute* (*l'sc*) gene cause a reduction in the number of neuroblasts in the embryonic CNS (Martin-Bermudo *et al.*, 1991) and this

reduction is enhanced when either *ac* or *sc* LOF mutations are present (Jiménez and Campos-Ortega, 1987) illustrating in part, the functional overlap that exists between the *sc* and *ac* gene products. This LOF mutation does not affect sensory organ development of adults (García-Bellido, 1979). In adult neurogenesis, using weak alleles that allow homozygous animals to reach adulthood, and the study of mitotic clones, many of these genes have been found to play an important role in adult neurogenesis. LOF mutations of *ac* remove mostly microchaete, while *sc* LOF mutations display mainly a loss of macrochaete (García-Bellido and Santamaria, 1978). Flies doubly mutant for both *ac* and *sc* develop without the bristles and sensory organs of the adult epidermis (Ghysen and Dambly-Chaudière, 1988). LOF mutations have implicated *asense* to be required for the development of some sensory organs of the adult thorax (García-Bellido, 1979) and of the wing (Dominguez and Campuzano, 1993). Gain of function (GOF) mutations of the *ac* and *sc* genes result in ectopic formation of sensory organs (Campuzano *et al.*, 1986; Balcells *et al.*, 1988), supporting the notion that these genes are intimately involved in the development of sensory organs.

Mutations of the *daughterless* (*da*) gene lead to defects in the larval CNS and failure of the larval sense organs to develop (Brand and Campos-Ortega, 1988; Caudy *et al.*, 1988; 1988; Dambly-Chaudière *et al.*, 1988), suggesting the *da* gene product is required for PNS and CNS development. The Daughterless protein has been shown to form DNA-binding heterodimers with the products of the *achaete-scute* complex (Murre *et al.*, 1989; Cabrera and Alonso, 1991) indicating the *daughterless* gene product interacts with the genes of the AS-C during neurogenesis.

1.4.2 Proneural Clusters

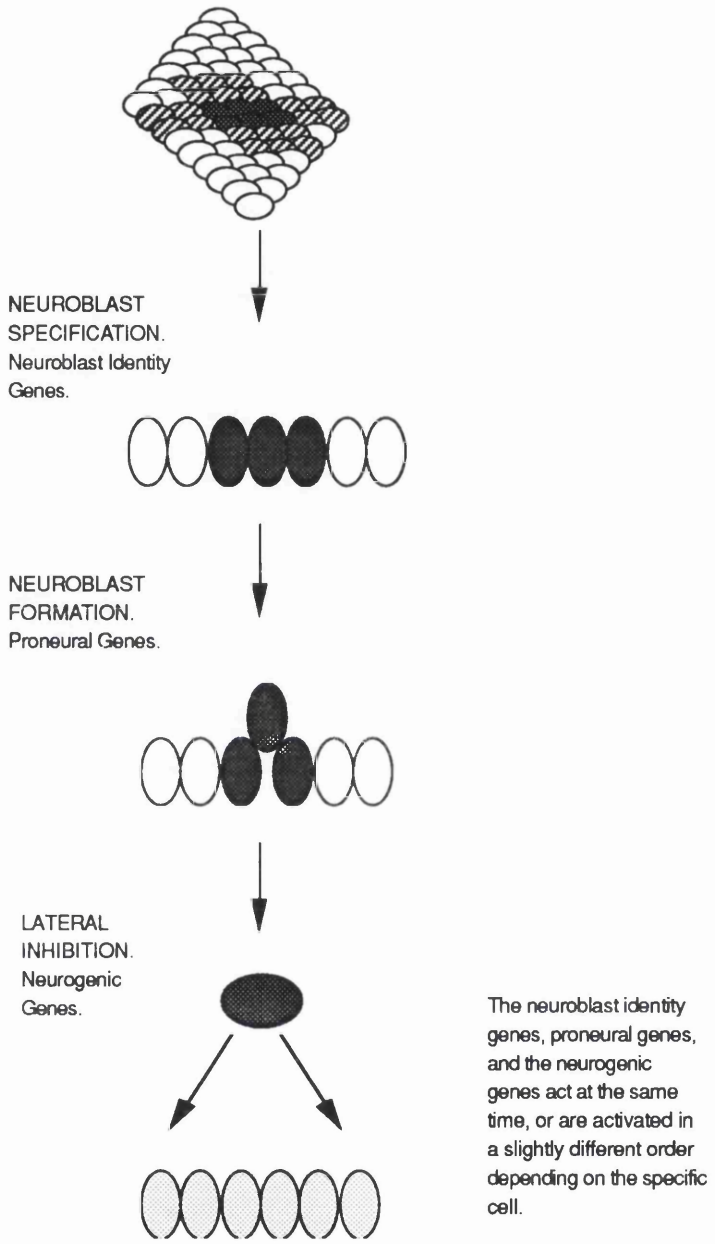
Genetic mosaic analysis demonstrated that when the appropriate cells were mutant for *ac*, the dorsocentral bristle failed to arise (Stern, 1954). However, a dorsocentral bristle arises ectopically, near the site where its development should

have occurred (Stern, 1954). This suggests that a group of cells, rather than a single cell, are capable of following the dorsocentral bristle cell fate. These cell groups are called proneural clusters. Once one of the cells in this group has begun to follow this fate, the remaining cells can not do likewise, and so follow an alternative, epidermal fate. The mechanism that is thought to prevent the remaining cells in the group from following the same developmental fate is termed lateral inhibition and is illustrated in Figure 1.3.

1.4.3 Lateral Inhibition

In neurogenesis, lateral inhibition occurs in groups of cells, or proneural clusters, and is thought to be essential to the process of neurogenesis (reviewed by Ghysen and Dambly-Chaudière, 1992; Jiménez and Modolell, 1993; Jan and Jan, 1993.). Each cell in the cluster has the potential to become a neuroblast or a sensory mother cell. When each cell of the cluster has the potential to form a sensory organ, the cells are said to have reached the competent stage. Lateral inhibition is the mechanism by which a single cell from the cluster is singled out to become the neural progenitor, while the others loose their neural potential and revert to the epidermal fate. In the embryo, each neural precursor cell forms in a specific region and at a specific time in the neuroectoderm. The early development of the grasshopper and *Drosophila* embryos are similar in that there are homologous cells that show similar developmental and cell division fates. Laser ablation experiments in the grasshopper embryo illustrated the importance of cell-cell communication in neuroblast specification (Doe and Goodman, 1985). Here laser ablation of the pre-neuroblast as it begins to enlarge (determined stage) induces one of the neighbouring cells in the cluster, to take on a neural fate rather than the epidermal fate. There are at least two groups of genes known to be involved in the specification of the PNS neural progenitor, the SMC, and the CNS of neural progenitor, the NB. The first group, the proneural genes, confer the neural fate potential, while the second group, the

Figure 1.3 Lateral Inhibition



This figure illustrates the process of lateral inhibition. Redrawn from F. Jiménez and J. Modolell. (1993). A group of cells form the proneural cluster and a single cell is singled out by the action of the proneural genes. The neurogenic genes act to prevent the other cells of the cluster following the neural fate.

neurogenic genes, are responsible for the lateral inhibition mechanism itself (reviewed by Ghysen and Dambly-Chaudière, 1992; Jiménez and Modolell, 1993; Jan and Jan, 1993).

1.4.4 The Proneural Genes

The proneural genes comprise *achaete* (*ac*), *scute* (*sc*), and *lethal of scute* (*l'sc*), from the AS-C, *atonal* (*ato)* and *daughterless* (*da*). Some of these genes are found on the X chromosome in a gene complex, although *ato* is located on the third chromosome. The genes of this class are related at the molecular level (Alonso and Cabrera, 1988; Caudy *et al.*, 1988; Villares and Cabrera, 1987), suggesting they have arisen by gene duplication, but have evolved different roles in the development of the embryonic and adult nervous systems. Both *da* and *sc* have been shown to be required in the sex determination pathway, as is *sc* (Torres and Sanchez, 1989; Cline, 1984).

achaete

Ghysen and Richelle (1979) suggested the *achaete* gene might be the gene responsible for invoking neural competence on cells within the proneural clusters. This was later supported by Romani *et al.*, (1989) who demonstrated that *ac* is expressed at the sites of sensory organ development, and by Skeath and Carroll, (1992) who have demonstrated all the initial neuroblasts to segregate in the embryonic CNS (SI neuroblasts) are derived from clusters of cells expressing one or more of the AS-C proneural genes.

The *ac* protein is synthesised in a repeating pattern in four ectodermal cell clusters, in each hemisegment of the embryo, at around stage 8 of embryogenesis. This expression is seen in five to seven cells in each cluster, but only one of these cells maintains this *ac* expression (Skeath and Carroll, 1992). In neurogenic mutants

such as *Notch*, all cells in these clusters are found to maintain *ac* expression, and all subsequently enlarge and delaminate from the ectoderm, developing as neuroblasts. It seems therefore, an important function of the neurogenic genes is to act by repressing the expression of the proneural genes. Using an anti-*ac* antibody to study the expression and translation of the *ac* protein (Skeath and Carroll, 1992), one cell in each of the clusters has increased levels of *ac* protein relative to the other cells of the cluster. It is this cell that delaminates, becoming the neuroblast, while the remaining cells lose expression of *ac*. The newly formed neuroblast also loses *ac* expression, but later at around stage 10 of embryogenesis, the expression of *ac* is reactivated (Skeath and Carroll, 1992). Therefore *ac* is expressed transiently in the neuroblast, inducing the neural fate, but is not essential for the maintenance of this trait.

The precursors of the embryonic nervous system are therefore derived from a group of cells that are positioned periodically along the anterior/posterior axis and dorsal/ventral axis of the ventral neuroectoderm. Using a variety of embryonic pattern mutants such as *fushi tarazu* (*ftz*), *paired* (*prd*), *engrailed* (*en*), and *decapentaplegic* (*dpp*), and a panel of antibodies raised against the AS-C gene products, Skeath *et al.*, (1992) have found the expression of *ac*, and therefore the position of the proneural clusters is regulated by the products of the pair rule genes in the anterior/posterior axis, and dorsal/ventral polarity genes along the dorsal/ventral axis.

scute in the Wing Disc

In the developing wing disc of third instar larvae, the expression of *sc* and *ac* have been found to be located in clusters of cells at the sights where sensory organs are thought to differentiate (Romani *et al.*, 1989). The transcription and translation of *sc* is then increased in a single cell and lost in the rest of the cells of the cluster (Cubas *et al.* 1991; Skeath and Carroll, 1991). This is similar to the elevated

expression of *ac*, which is detected in the cell that will delaminate and progress through the neuroblast developmental fate as previously discussed. Analysis of *sc* expression in *sc* LOF mutants confirms a requirement for *sc* function for the appearance of specific proneural clusters and the subsequent sensory organ (Skeath and Carroll, 1991). In *sc* GOF alleles, all cells in the proneural cluster show increased levels of transcription of the *scute* gene (Campuzano *et al.*, 1986; Ruiz-Gómez and Modolell, 1987; Balcells *et al.*, 1988) and supernumerary bristles are produced. The expression of *ac* in the imaginal wing disc has been found to be identical to that of *sc* and has been shown to be due to these genes *trans*-activating each other (Skeath and Carroll, 1991). However *sc* and *ac* are known to be involved in the development of complementary and overlapping sensory organs in the adult fly. This specificity is achieved in part, by the negative regulation of *sc* by *extramacrochaete* (*emc*), while *ac* is negatively regulated by *hairy* (*h*). These regulators are differentially active in the wing disc, both temporally and spatially (reviewed by Ghysen and Dambly-Chaudière, 1992)

This data has led to the proposal that the *sc* pattern of expression is due to the array of regulatory sequences spread over approximately 40 kb 3' to the *scute* gene (Ruiz-Gómez and Modolell, 1987; Leyns *et al.*, 1989), where each regulatory site is responsible for the expression of *scute* within a specific cluster in the imaginal wing disc. At the molecular level, the proneural clusters can be defined as groups of cells in the developing epidermis, or neuroectoderm that express the proneural genes (Simpson, 1990).

atonal

So far only the role of the proneural genes in the development of the external sensory organs has been discussed. However, proneural genes are also required for internal sensory organ development. It is suggested that the genesis of these chordotonal organs follow a similar mechanism to that employed for the development

of the external sensory organs, since mutations of the *daughterless* gene prevent formation of the entire PNS, including the internal sensory organs (Caudy *et al.*, 1988). The isolation of the gene *atonal* has identified a novel proneural gene specifically involved in the development of these organs. *atonal* is expressed in embryos and the imaginal discs, coincident with the sites of chordotonal organ proneural clusters. Analyses of deletions that remove the *atonal* gene, show a failure in the development of the internal sensory organs, in an essentially complementary pattern to the PNS phenotype found in flies that are mutant for the *achaete-scute* complex (Jarman *et al.*, 1993).

Structure of the Proneural Gene Products

All four genes of the *achaete-scute* complex, *hairy* (*h*), and the *daughterless* gene encode proteins with a basic helix-loop-helix motif, homologous to the mammalian *myc* gene (Villares and Cabrera, 1987; Caudy *et al.*, 1988; Alonso and Cabrera, 1988; Rushlow *et al.*, 1989). The *atonal* gene product also has a basic helix-loop-helix domain. This suggests that these proteins are transcriptional activators: *l'sc* has been shown to act as a transcription factor (Cabrera and Alonso, 1991). The *sc*, *ac*, and *l'sc* proteins have been found to dimerise with the product of the *daughterless* gene, and these complexes bind to specific DNA sequences (Cabrera and Alonso, 1991; Murre *et al.*, 1989), supporting the genetic evidence that suggests these gene products functionally interact.

1.4.5 Other Genes With Functional Activity in Proneural Clusters

Other genes have been identified which, when mutated, have the opposite effect on neurogenesis from the AS-C genes. *Hairy-wing* (*Hw*) mutations induce the appearance of additional microchaetes on the wing, *extramacrochaete* (*emc*) increases the number of macrochaetes on the notum, and *hairy* (*h*) mutations lead to

an increase in the microchaetes on both the notum and wing, with no effect on the macrochaetes. The *extramacrochaete* (*emc*) gene encodes a protein that has the helix-loop-helix motif, but lacks the basic region required for the DNA binding activity found in the other genes (Ellis *et al.*, 1990). Since *emc* can form dimers with the other proneural genes, but does not bind to DNA sequences, it has been proposed that this protein acts to limit the activity of these genes (*ac*, etc.) by sequestering them, to render them unable to bind DNA (Ellis *et al.*, 1990; Garrell and Modolell, 1990; Doren *et al.*, 1991).

1.4.6 The Neurogenic Genes

Neurogenic Gene Activity in Embryos

The neurogenic genes include *Notch*, *Delta*, *big brain*, *mastermind*, *neuralised*, *shaggy* and the Enhancer of split-Complex (Lehmann *et al.*, 1983; Bourouis *et al.*, 1989). LOF mutations of these genes lead to increased numbers of neuroblasts and SMCs during development of both the larval and adult nervous systems, at the expense of epidermal cells, while GOF mutations in these genes cause a reduction in the number of cells that follow the neural developmental fate, with a subsequent gain in epidermis (Lehmann *et al.*, 1983; Brand and Campos-Ortega, 1988; Heitzler and Simpson, 1991).

Detailed analysis of the embryonic CNS in LOF neurogenic mutants reveals initially normal proneural gene expression in the proneural clusters. However, all cells of the cluster maintain the expression of the proneural genes with subsequent neural differentiation of every cell in the cluster (Cabrera, 1990; Skeath and Carroll, 1992). This illustrates wild type expression patterns of the neurogenic genes are necessary to restrict proneural gene expression to only one cell of each cluster. LOF neurogenic mutations have been found to have a similar effect on the proneural clusters of the developing PNS (Lehmann *et al.*, 1983; Brand and Campos-Ortega, 1988; Heitzler and Simpson, 1991).

The Activity of *Notch* and *Delta* in the Adult PNS

The genesis of bristles on the adult notum has been extensively studied as they are easily identified sensory structures. Genetic mosaic analysis has revealed each bristle is formed from a small equivalence group, or proneural cluster. Each cell of the cluster has the potential to form either a bristle mother cell or an epidermal precursor cell (Simpson and Cateret, 1990; Heitzler and Simpson, 1991) as is the case with the proneural clusters of the embryo. Heitzler and Simpson (1991) constructed mosaic flies, with patches of cells mutant for *Notch*, adjacent to patches of cells that were wild type for *Notch*. In the centre of the mutant patches, increased numbers of bristles developed, demonstrating the requirement for *Notch* in epidermal development. In the border region, between the wild type and *Notch* mutant patches, the proneural clusters were a mixture of mutant and wild type cells. All the *Notch* mutant cells gave rise to bristles, while the wild type cells became epidermal cells, illustrating the cell autonomous nature of *Notch*.

Delta activity was also studied using mosaic analysis. When a proneural cluster developed in a region entirely mutant for a strong allele of *Delta*, increased numbers of bristles appeared. In the border regions between patches of cells mutant for *Delta* and patches of cells that were wild type, most mutant cells gave rise to epidermal cells, while wild type cells generated bristles. This border region is several cells wide. However during pupal development there are two rounds of mitosis after the development of the epidermal cells. Therefore, the border region may be only one cell wide at the time of its appearance. These experiments indicate that *Delta* is also required for epidermal development, but that *Delta* is not cell autonomous. Heitzler and Simpson, 1991 and de Celis *et al.*, 1991 suggest that *Notch* functions as a receptor for the intercellular signal of *Delta* . Alternative studies suggest *Notch* acts as a cell adhesion molecule (Kidd *et al.*, 1989; Greenspan *et al.*, 1990). Evidence for a direct association between *Notch* and *Delta* comes from *in*

vitro studies that demonstrate aggregation between cells expressing *Notch* and cells expressing *Delta* (Fehon *et al.*, 1990).

Structure of the Neurogenic Gene Products

Molecular analysis of the genes *Notch* and *Delta* indicate that they encode transmembrane proteins suggesting that they may be involved in cell-cell interactions. The extracellular domain of the Notch protein contains 36 EGF-like elements, while the cytoplasmic domain has 6 tandem cdc10 repeats, believed to be involved in protein-protein interactions (Wharton *et al.*, 1985; Kidd *et al.*, 1986; Kopczynski, 1988; Vassin *et al.*, 1987). The extracellular and intercellular domains of the Notch protein have been shown to be homologous to the *lin-12* gene products, a protein thought to function as a receptor for an inhibitory signal in the nematode (Greenwald, 1989).

1.5 The Specification of Cell Fate in the PNS

Once the proneural genes and the neurogenic genes have selected the neuronal precursor, the cell delaminates from the epidermal layer and follows a developmental programme different from that of its epidermal neighbours. By implication, the neuroblast expresses a group of genes necessary for neuronal differentiation, while the epidermal cells, express other genes necessary for the equivalent epidermal development. The pan-neural genes are believed to be the early acting genes of the neural differentiation programme. Some of these genes, such as *deadpan* (Bier *et al.*, 1992), *prospero* (Doe *et al.*, 1991; Vaessin *et al.*, 1991), and *asense* (Alonso and Cabrera, 1988; Gonzalez *et al.*, 1989), are expressed in most or all of the neuroblasts, while others, such as *couch potato* (Bellen *et al.*, 1992) are expressed in only a subset. Importantly, these genes are not expressed by the neighbouring epidermal cells. These pan-neural genes may promote the neuronal developmental programme

by a variety of mechanisms, but have not been characterised sufficiently to allow a clear hypothesis of their function.

More progress has been achieved in the isolation and characterisation of the genes required for later events required in the differentiation programme of the PNS precursor cells. Expression of the *cut* locus is required if the external sensory organs are to achieve their correct identity. In LOF mutations, the external sensory organs develop as the internal chordotonal organs, that do not express *cut.* When this protein is expressed inappropriately by the induction of the *cut* gene under the control of heatshock regulatory sequences in the precursor cells of chordotonal organs, they acquire an external sensory organ fate (Bodmer *et al.*, 1987; Blochlinger *et al.*, 1988; 1990; 1991).

LOF mutations of the *pox-neuro* gene result in cells of the poly-innervated external sensory organs following the differentiation pathway of mono-innervated external sensory organs. This gene is normally expressed by the poly-innervated external sensory organs, but when mis-expressed in the mono-innervated external sensory organs, they are transformed to poly-innervated external sensory organs (Bopp *et al.*, 1989; Dambly-Chaudière *et al.*, 1992).

Chordotonal organs have been found to be a heterogeneous class of sensory organ at the genetic level, since different subsets are affected by a variety of mutant loci. When *pointed, rhomboid spitz* or *Star* are mutant, two of the five chordotonal organs in an abdominal hemisegment fail to develop. This is an important observation since these genes are involved in the initial organisation of the dorsal/ventral patterning of the embryo (Mayer and Nüsslein-Volhard, 1988). The *rhomboid* gene is normally expressed in the precursor cell of the two lost chordotonal organs suggesting this gene is essential for the development of subsets of chordotonal organs (Bier *et al.*, 1990).

The cells of a sensory organ are clonally related, being derived from a single primary precursor cell (although there is an exception to this found in the adult wing margin). A number of genes have been isolated and shown to be involved in

specifying the identity of each of these cells of the sensory organ. Using a temperature sensitive allele of the *Notch* gene, Cagan and Ready (1989), and Hartenstein and Posakony (1990), have found this gene to be involved in the determination of cell type in the sensory organs. Mutant *Notch* clones induced late in development lack sensory bristles, since the bristle cells develop as neurons. The Notch protein is a membrane protein, and may be involved in cell-cell communication during the differentiation of the immature cells of the sensory organ.

In *numb* mutants, the neuron and glia of the sensory organ follow the differentiation programme of the socket and hair cells (Uemera *et al.*, 1989), whereas overexpression of the Numb protein leads to the opposite transformation. Interestingly, Numb has been shown to be spatially restricted to one half of the sensory organ precursor, raising the possibility that the asymmetric distribution of the Numb protein is involved in the determination of the neuron-glial and shaft-socket precursors (Rhyu *et al.*, 1994).

A recently identified gene, *musashi*, encodes a neural-specific RNA-binding protein, has been isolated and is believed to be required for the formation of the SOP progeny in the adult PNS. Nakamura *et al.*, (1994) have the proposed that *musashi* has the role of regulating postranscriptional processing of gene transcripts. *musashi* mutants are characterised by the development of addition extra shaft and socket cells of mechanosensory bristles at the expense of the neuron and glia of the sensory organ, possibly as a result of symmetric rather than asymmetric, division of the SOP.

BarH1 and *BarH2* are two genes believed to be involved in the specification of the type of external structure formed by the trichogen cell of external sensory organs. When both genes are deleted hairs rather than papillae are formed. These genes are expressed in the neural cells of the sensory organ located below the trichogen cell, suggesting cell-cell communication is involved in specifying the fate of the tormogen cell (Higashijima *et al.*, 1992).

1.6 The Specification of Cell Fate in the CNS

Many of the early genes involved in the establishment of the embryonic metameric pattern in *Drosophila* are reactivated later in embryonic development, in new temporal and spatial patterns (reviewed by Goodman and Doe, 1993). In the developing CNS *fushi tarazu* and *even-skipped* are reactivated in specific subsets of ganglion mother cells and their progeny. The first progeny cell generated by the neuroblast 4-2 expresses both *ftz* and *eve*. This *eve*$^+$, *ftz*$^+$ cell divides to produce a cell of unknown identity and the RP2 neuron, while an adjacent GMC expresses *ftz* but not *eve*. In experiments where the neural-specific regulatory sequences were deleted from the *fushi tarazu* gene, the GMC of NB 4-2 loses *eve* expression and the subsequent progeny morphologically resemble the RP1 and RP3 neurons, that are the normal progeny of the *ftz*$^+$ *eve*$^-$ neighbouring GMC. By using a temperature sensitive allele of the *eve* gene, Doe *et al.*, (1988; 1988) demonstrated that it is the specific loss of *eve* expression rather than *ftz* expression that results in the alteration of cell fate in the NB 4-2 progeny . The analysis of these genes has led to the identification of a number of genetic interactions required to confer cellular and lineage identity. Indeed, many have begun to provide clues as to the processes and mechanisms, beyond the expression and repression of a variety of transcription factors, that are used to produce one cell type as opposed to another.

A major problem that remains to be resolved is the terminal differentiation of the component cells of the sensory organs, and the cells of the CNS. Mature neurons express a wide variety of neural-specific genes such as neurotransmitters and neurotransmitter receptors. Many cells of the nervous system also have a distinct morphology. Neurons have long axons and structures named dendrites, while the external sensory organ has a socket cell derived from the tormogen cell and a sensory structure, such as a bristle. The mechanisms that induce the formation of these specific structures are not well understood. What induces the growth and position of the extending axon? What molecular mechanisms are activated within the cell that

induce the development of the external sensory structure, and what determines its cellular position?

Axonal Guidance

The final step in the construction of a fully integrated nervous system is the projection of the neural axons to their target cells (muscles, other neurons, etc.), allowing cell-cell communication to be established. Because the *Drosophila* embryo is compact and the cells are small, much of the early analysis of axogenesis was focused on the much larger grasshopper embryo. During differentiation, the neurons send out cytoplasmic processes, or growth cones that reach out to establish contact with the appropriate target cell. A variety of experimental approaches have shown these growth cones respond to positional cues provided by cell surface and extracellular molecules (reviewed by Goodman and Doe, 1993). Cellular recognition of the large variety of molecules that could potentially provide guidance cues, and the subsequent processing of this information, allowing the appropriate decisions to be made by the growth cone, is a complex phenomenon. Simple systems, using morphological criteria and molecular markers for a small number of molecules thought to be involved, have proved useful in the initial analyses of these processes. For example laminin, an extracellular matrix molecule, has been found to act as a substrate adhesion molecule, promoting the extension of neural growth cones (Lander *et al.*, 1985). In the grasshopper embryo, a pair of pioneer neurons have been shown to recognise guidance cues provided by the limb bud epithelium and another pair of neurons (Bastiani *et al.*, 1986). Using the *Drosophila* imaginal wing disc, Blair *et al.*, (1987), have shown the epithelia and the extracellular matrix provide positional cues for growing axons as they move towards the CNS.

Many interneurons have axons that traverse the midline of the CNS. These interneurons only follow the longitudinal axon tract after they have crossed the midline, and so finally reach the appropriate target cells. This introduces the

intriguing aspect of asymmetry to the CNS, and is likely to be the result of differential, or asymmetric gene expression. Four cell surface glycoproteins have been found to be dynamically expressed on different, although overlapping subsets of growth cones, glial cells and axons of the CNS during embryogenesis (Bastiani *et al.*, 1987; Patel *et al.*, 1987; Bieber *et al.*, 1989). These molecules are believed to play an important role in axon guidance. A number of interneurons express the glycoprotein FasI on their commissural processes, but express FasII, another cell-surface glycoprotein, on their longitudinal axon after crossing over the midline, suggesting these proteins are intimately involved in longitudinal and commissural pathway recognition (Bastiani *et al.*, 1987; Patel *et al.*, 1987).

The CNS of the *Drosophila* embryo has been particularly useful in the study of axonal guidance. Two mutants, *commissureless* (*comm*) and *roundabout* (*robo*), have been isolated that are potential candidates for either signals or receptors involved in providing growth cone guidance cues (Seeger *et al.*, 1993). The *comm* mutants, the CNS neuron growth cones do not grow over the midline, and the commissures never form. In *robo* mutant embryos, the growth cones that normally never cross the midline now do so. It has been suggested that *comm* encodes a component of an attractant signalling system, while *robo* may be a component of a repulsive signalling system (Seeger *et al.*, 1993). The embryos of *longitudinals lacking* (*lola*) mutants have many longitudinal axon tracts in the CNS are not detected. In the embryonic segments of *lola* mutants, the MP1 and vMP2 growth cones extend and reach the longitudinal glia, but then stall, indicating the required interaction between the growth cone and the longitudinal glial is disrupted (Seeger *et al.*, 1993).

The characterisation of these genes has permitted initial dissection of the complex processes that allow the nervous system to achieve precise connections.

1.7 Positional Information

Within an embryonic segment, there are a variety of sensory neurons, each uniquely and reproducibly positioned although there are clear differences in the embryonic sensory organ complement between thoracic and abdominal segments. For example, thoracic segments have four chordotonal organs while the abdominal segments have eight.

As previously discussed in Section 1.1, the embryo is sequentially divided into distinct domains, determined by the expression or repression of genes that establish the anterior/posterior and dorsal/ventral polarities. The gap, pair rule and segmentation genes divide the embryo into segments, or parasegments, while the dorsal/ventral patterning genes create molecularly distinct, longitudinal along the length of the embryo. These two patterning systems combine to create a grid-like pattern of cells that differ both in position and the particular genes they express. Superimposed on this gene expression pattern are the distinct expression domains of the homeotic genes. It is likely, the information required for the determination of neural cell type number and position is derived from this grid of differential gene expression.

Ingham *et al.*, (1988) have proposed the pair rule gene products subdivide each embryonic segment into four molecularly and developmentally distinct regions, each generating a single row of SI neuroblasts of the CNS. In support of this hypothesis, expression of the proneural gene, *ac*, in SI neuroblasts is altered in a pair rule gene mutant background, while in a segmentation gene mutant background, *ac* gene expression in SI neuroblasts is not disrupted (Ingham *et al.*, 1988).

1.8 Aims

The aim of this project is to characterise previously unidentified genes expressed in the developing central and peripheral nervous system of the *Drosophila melanogaster* embryo. As described above, much knowledge has been gained from classical genetic and molecular approaches, namely the isolation and mutation number of genes and the identification of the roles they play in neurogenesis. Much of the molecular processes, however, have so far remained elusive. For example, what proteins are required for the internalisation of the SI, SII and SIII neuroblasts, and how is this movement physically achieved? Many of the previously characterised genes are DNA-binding proteins, presumably involved in regulating the expression of other genes that function downstream in the developmental programme. These downstream genes are likely to have diverse functions, perhaps regulating cell shape changes for example. The protein content of each cell from a particular cell lineage will vary not only between lineages but also at each developmental stage. For example, the pointed bristle of a mechanosensory external sense organ will have a different protein content from that of the flat dome of a campaniform sensillum. The mechanisms that give such differential expression and subsequently different functions and morphologies must be thoroughly characterised if a more extensive understanding of neuronal development is to be achieved. It is hoped the isolation of novel genes expressed in these cell types and their mutations, will allow further characterisation of the many developmental processes required to give a fully formed and integrated nervous system in *Drosophila*.

1.9 Potential Approaches

There are several approaches that may be taken to permit the isolation of novel genes specifically expressed in the developing nervous system of *Drosophila*. Genetic screens may be undertaken, using a variety of mutagenic protocols (reviewed

by Buchner, 1991), and screening the resultant progeny for external defects in nervous system morphology and/or function. This approach has been a popular one, but requires large scale organisation and personnel not to mention the ability to detect specific malfunctions at some point throughout development. This is clearly an impossible approach if microscopic defects are likely to be induced in tissues and cell types that are yet not well characterised. Similarly, the specific defect may be difficult to identify if the induced lesion is lethal, since many developmental processes may be disturbed as a consequence of a single developmental disruption. For example, the *daughterless* gene product is required not only in the development of the nervous system, but also in sex determination (Caudy *et al.*, 1988)), . Such pleiotropic functions may be difficult to identify when disruption of one developmental process i.e., neurogenesis, leads to lethality before the second developmental event i.e., sex determination becomes detectable. In addition, the molecular characterisation of such mutants is generally formidable, since the chromosomal position of such loci must be assessed accurately by recombination analysis, before such techniques as chromosomal walking can be used to isolate the correct DNA sequence.

An alternative approach may be to isolate behavioural mutants which are likely to have defects in the structure or function of the nervous system These loci may be identified genetically by designing a variety of behavioural tests, and isolating mutagenised strains that exhibit abnormal behaviours when tested in the paradigms. The parameters of these screens however, limit the isolation of mutants with defects specific to the behaviour pattern being examined, i.e., screens designed to isolate flightless flies will identify flies that can not fly properly (Homyk and Shepperd, 1977). The anatomical structures that, when defective, give rise to a behavioural defect are varied. If a fly is found to be unable to fly, the defect may lie in the signalling mechanisms between the wing and the muscles required for flight. Alternatively, the defect may be due to abnormalities of the flight muscles. Obviously if a fly is blind, then it will appear to have a variety of defects in its

behaviour including reduced flight ability as was by Homyk and Shepperd (1977). Similarly, if there is a defect in the functioning of an enzyme in the brain, such as in *dunce* mutants (Chen *et al.*, 1986), there may be a behavioural phenotype. Therefore, there is a considerable range of genetic defects that may give rise to a behavioural phenotype, and a variety of behavioural tests must be applied, if the true behavioural defect is to be characterised.

Another approach is to initiate differential screens, or subtractive hybridisations (Levy *et al.*, 1982). Subtractive hybridisation techniques, although undertaken in a number of organisms, are not applicable to this study as a relatively large amount of the tissue of interest, in this case the embryonic nervous system, must be cleanly isolated. Although this is a potentially productive method in the isolation of tissue-specific clones (Pikielny *et al.*, 1994), it does not provide the opportunity for genetic analysis of the clones. Differential screens (Tomlinson, 1995.), again are technically difficult, and are likely to produce many clones that need extensive characterisation to ensure the tissue specificity of such clones, and are therefore not of the required sensitivity. Again, this approach is entirely dependant on the representation of the libraries used, with limited genetic possibilities.

An further approach is to exploit the enhancer-trap technique, recently developed in *Drosophila* (O'Kane and Gehring, 1987). This can lead to the isolation of genes expressed in a specific, or novel patterns during the developmental stage chosen for study, facilitating both genetic and molecular characterisations of the insertion locus. The reporter gene technique was first developed by Casadaban and Cohen (1979) while working with *E. coli,* who used *lacZ* to identify genes in prokaryotes, and has now been refined and applied to higher eukaryotes, such as *Drosophila*, *C. elegans* (Hope, 1991), plants (Topping *et al.*, 1991), zebrafish (Bayer and Campos-Ortega, 1992) and the mouse (Skarnes *et al.*, 1992). In *Drosophila* this has involved using molecular genetic techniques to modify transposable elements, specifically the P element, which when integrated into the genome will "report" the activity of neighbouring enhancers.

Enhancer-trapping allows mutagenic screens to be especially productive since the identification and isolation of transposon insertion lines with interesting patterns of enhancer activity, can be rapid. This approach has the potential to provide information about gene expression patterns before the gene has been cloned and the transposon facilitates cloning of the appropriate DNA sequences, since the inserted construct provides a necessary "tag". If the insertion induces a mutant phenotype, the function of the trapped gene may be indicated, otherwise a mutant phenotype may be obtained by the imprecise excision of the P element construct from the *Drosophila* genome. *In situ* hybridisation experiments with insert-specific probes to the salivary gland chromosomes of each insertion line, may allow the identification of any possible alleles previously characterised, that may have a similar expression pattern. This technique will be discussed in more detail later in this Introduction.

The enhancer trap approach has been chosen as the most versatile method available for the purposes of this Thesis. The insertion lines used in this study have a first-generation P element and have been identified as showing *lacZ* expression patterns in the developing embryonic nervous system. Our primary interest in these lines is the identification, characterisation and mutation of genes that may be involved in the development of both the peripheral and central nervous systems. It is hoped that the study of many of these insertion lines will lead to an increase in our knowledge of molecular events involved in embryogenesis, in addition to aiding the understanding the major problem of how neurons make specific connections with their targets. For the purposes of this Thesis two enhancer trap strains, A11 and A22 have been selected in the hope that they will facilitate the isolation of novel, neural-specific genes.

1.10 Enhancers

The Activity and Function of Enhancers

Gene expression is regulated at a variety of levels. Many *cis*-acting DNA sequence motifs are essential for accurate spatial and temporal transcription. Enhancers function to permit expression or increased expression of a particular gene, usually in a tissue-specific manner, in response to a variety of physiological stimuli. A broad definition of an enhancer would be DNA sequence elements that increase the level of transcription of a gene, in a distance and orientation independent manner (Atchison, 1988). Enhancer sequences show a large variation in size, and are usually found at the 5' end of the gene, often several kilobases upstream, or even downstream of the gene. Clusters of sequence motifs, such as CCAAT and the GC box may act as enhancers, within certain contexts, thus confusing the issue as to what sequences constitute an enhancer as opposed to a promotor.

The mechanism of enhancer activity is much debated (Cook, 1994) and likely to be diverse. One model suggests enhancers induce changes in the chromatin state of the chromosome, and thus the DNA becomes more exposed to the activity of the transcriptional machinery. This model is supported by the mapping of DNase I hypersensitive sites to many enhancer sequences (Cremisi, 1981; Mills *et al.*, 1983). A second model proposes that enhancers function, when exposed, to bind the DNA to the nuclear matrix via DNA-binding proteins. The DNA is then in the correct "compartment" of the nucleus for transcription. This model is supported by the finding of scaffold attachment regions, or SARs close to enhancer/promotor regions of *Drosophila* genes (Gasser and Laemmli, 1986) and the immunoglobulin κ chain enhancer (Cockerill and Garrard, 1986). However, this model fails to suggest the mechanism employed to initially expose the DNA to the transcriptional machinery.

The most actively studied area of enhancer activity is the binding of a variety of transcription factors to enhancer sequences. Many of these studies include the *in*

vitro site-directed mutagenesis of these sequences in an attempt to either increase or reduce enhancer activity by altering its ability to bind these protein factors. Without doubt, enhancers do function by the binding of a variety of specific protein complexes, which then increase levels of the appropriate transcription. Similarly, DNA-binding factors may repress the transcription of a gene by binding the DNA at specific repressor sequences, (Ptashne, 1986; Hatzopoulos *et al.*, 1988) and act to block the activity of the transcriptional machinery.

Tissue Specificity of Enhancers

Different enhancers have a number of possible modes of action. They can be constitutively active, tissue-, developmentally-, or sex-specific. They may be activated by growth factors, or by shock to the cells, such as heat, etc., (Atchison, 1988). These elements tend to consist of a variety of modules, or short sequence motifs. For example, the yolk protein enhancer of *Drosophila* confers sex- and developmental-expression patterns on the gene (Garabedian *et al.*, 1986; Shermoen *et al.*, 1987). Another example is a temporal and tissue-specific enhancer has been discovered in the chicken β–globin gene, located 400 bp from the polyadenylation signal at the 3' end of the gene (Choi and Engels, 1986; Hesse *et al.*, 1986).

Multiple Enhancers

Multiple enhancers are a common feature of gene expression in eukaryotes. Many genes are expressed in complex and highly dynamic patterns, requiring elaborate controls to ensure the timing and pattern of expression of the gene is adequate. The control is thus provided by a variety of individual "elements" each with influence over a particular aspect of the gene's activity. For example, the pair rule gene *hairy* has multiple enhancer elements, each defining a single stripe of *hairy* expression in the embryo and

the activity of each of these enhancer elements has been functionally dissected (Howard and Struhl, 1990).

There are many examples where an enhancer, or regulatory element need not act on the gene that lies directly downstream (see Figure 4.4). It may act on a gene several coding units downstream, e.g., AS-C, without affecting the expression of the more adjacent genes that are in between the two units.

Promotor Activity, Structure and Function

In eukaryotic genes, there is frequently a bipartite promotor, responsible for accurate and efficient transcription by RNA polymerase II and associated proteins. A TATA box motif, common to housekeeping genes, is located approximately 30 bp upstream of the CAP site, that functions by binding general transcription factors. The second element of the promotor is positioned approximately 70 bp upstream and is required for efficient transcription. This second element varies depending on the gene e.g., CCAAT, the GC box, and the octamer motif. The transcription of ribosomal genes by RNA polymerase I also requires the presence of upstream bipartite promotor sequences, while RNA polymerase III that transcribes a number of small RNAs such as tRNAs, recognise promotor sequences that are located both upstream and within the coding regions of such genes (see Transcription and Splicing Eds. B. D. Hames. D. M. Glover. IRL Press, 1988.)

A good example of the complex nature of gene expression control is the *pointed* gene. This gene has two alternative transcripts, each expressed differentially in specific embryonic and adult cell types. There are two promotors that allow initiation of expression of the two different transcripts encoded by *pointed.* The P1 promotor allows expression of a 4.2 kb transcript in the longitudinal glial cells and the glial support cells of the VUM cluster amongst other embryonic cell types, while the P2 promotor drives expression of a second 4.0 kb transcript in the midline glial cells. The P1 coding region spans over 8 kb of genomic DNA, while the second P2

message is encoded by exons spread over some 45 kb. An enhancer that directs the stage and tissue-specific expression of the second, P2 transcript lies 3' to the coding sequences (Scholz *et al.*, 1993).

The control of gene expression within AS-C and at the *pointed* locus illustrates some of the potential control points in gene expression. The promotors, which act in a distance-dependant manner, can then be used selectively by different cell types, allowing the synthesis of different proteins from a single locus. This selection will be influenced by the availability of different transcription factors in the different cell types, required for RNA polymerase II activity in the different cell types.

1.11 The P Element

P-M hybrid dysgenesis is a phenomenon detected by either increased frequency of sterility in F1 progeny or a variety of mutational events in the F2 progeny of female M strain and male P strain crosses, but not in the reciprocal male M strain x female P cross (Engels, 1983).

During the early 1980's the underlying mechanism of hybrid dysgenesis was rapidly elucidated, when a transposable element, called a P element, was found in an allele of *white*, which had been induced in a hybrid dysgenic fly (Rubin *et al.*, 1982). The *white* gene had previously been cloned using the P element as a "gene tag" (Bingham *et al.*, 1981). Confirmation that the P transposable elements were responsible for the hybrid dysgenesis phenomenon was achieved when these elements were shown to be present at the genomic sites susceptible to mutations induced by hybrid dysgenesis (Bingham *et al.*, 1982).

P Element Structure

The complete P element is 2 907 bp (O'Hare and Rubin, 1983) in length with a 31 bp inverted terminal repeat with a further 11 bp subterminal repeat starting 125 bp from each of the termini. There is an 8 bp direct repeat of genomic sequence on either side of the P element termini, that probably forms as a result of the insertion mechanism. A subgroup of cryptic P elements exists, composed of elements that have internal deletions, or defective termini, either of which leave the P element immobile, although internally deleted elements may transpose in the presence of exogenous transposase (O'Hare and Rubin, 1983).

An allele of *singed* (*sn*), sn^w, was shown to be stable when there were no other P elements in the genome (Engels, 1984) indicating the P elements encoded a gene product, transposase, necessary for transposition and excision. Spradling and Rubin (1982) found full length P elements were capable of transposition as assayed by sn^w hypermutability, supporting the notion of a transposon-encoded enzyme necessary for P element transposition. Frame shift mutations introduced into each of the four P element open reading frames, when re-introduced to the *Drosophila* genome, failed to produce transposase activity as measured by the lack of transposition at the sn^w site. Since complementation among the four different mutations did not occur, it was concluded the element carried a single gene consisting of four open reading frames (Karess and Rubin, 1984). The P element encoded transposase has been shown to recognise subterminal sequences of the P element (Kaufman *et al.*, 1989; Mullins *et al.*, 1989).

1.12 Mechanism of Transposition and Excision of the P Element

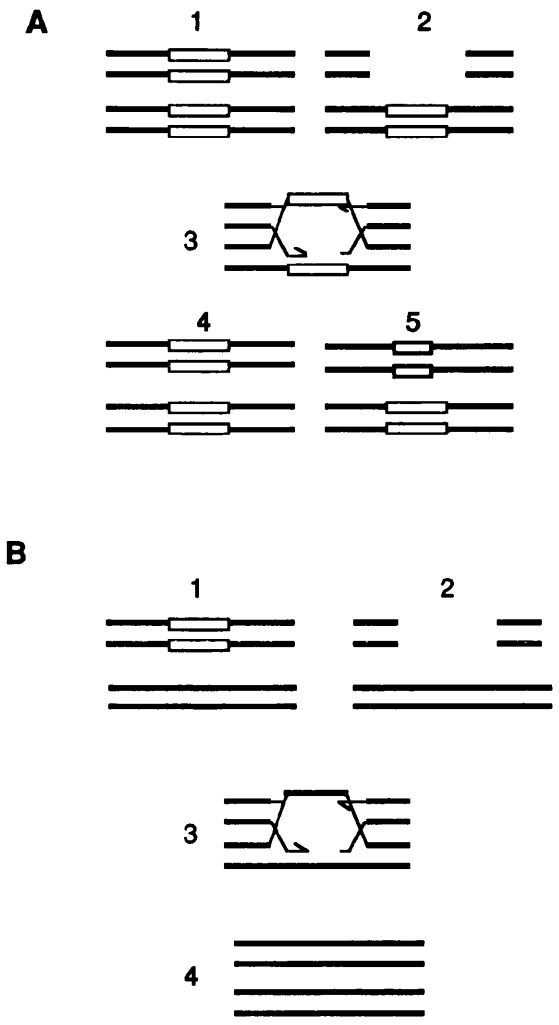
P elements can excise from the genome, either precisely or imprecisely. P elements may excise imprecisely such that a proportion of the termini are left remaining in the genome creating an internal deletion of the element. Alternatively,

genomic DNA sequences may be removed with either all or part of the P element (see Figure 4.1). It is difficult to assess the frequencies of these events, since the various methods used do not generally detect all potential excision events (Engels, 1983). The requirement for transposase in P element excision has been clearly shown both *in vitro* and *in vivo* (Engels, 1984; Spradling and Rubin, 1982; Kaufman *et al.*, 1992; Engels, *et al.*, 1990), although the mechanism has not been fully characterised. Transposition of P elements has been shown to require transposase and the 31 bp and 11 bp inverted repeats (Karess and Rubin, 1984). Since the majority of precise excision events are believed to be internal excisions, where very small sequences of the P element, usually from the direct repeats are left in the genome (Rio *et al.*, 1986; Searles *et al.*, 1986; Tsubota and Schedl, 1986), it is likely the major mechanism of excision by the transposase enzyme relies on breakpoint formation between short direct repeats (Engels, 1983).

The molecular mechanism of P element excision and transposition has been much debated since simple models of replicative and non-replicative transposition do not account for all the available data (Engels, 1983). A simple replicative mechanism does not explain the precise excision events, while simple non-replicative models fail to explain the increase of P elements within the genome. Engels *et al.*, (1990) in their analysis of precise P element excision have proposed a "cut-and-paste' mechanism with the involvement of double-strand gap repair.

As the P element excises, a double-stranded gap with 3' overhanging sequences is left behind that may be extended by exonuclease activity (see Figure 1.4). The repair process is initiated, using the sister DNA strand as a template (Gloor *et al.*, 1991). This model not only accounts for the presence of internally deleted P elements, but since the repair mechanism can potentially replace or repair the excised P element on the sister strand, the result is an increase in the number of P elements in the genome. Additionally, homologous sequences, other than those present on the sister strand may act as template for the repair process, providing the opportunity of directly mutating specific DNA sequences *in vivo*. This model has been supported by

Figure 1.4 The Double-Stranded Gap Repair Model of P Element Transposition and Excision



Redrawn and modified from Engels *et al.*, 1990. White boxes represent the P element sequence, while the thick lines represent the single-stranded DNA. In A, the homozygous P element (1) is initially excised (2), followed by double strand gap repair (3) after the free 3' ends have invaded the double-stranded template, using the homologous chromosome as a template. Two products are envisaged, namely, the entire P element is copied from the homolog (4), or the gap repair process is interrupted, giving an incomplete copy of the P element (5) that is detected as an internally deleted element. In B, the P element is in the heterozygous state (1), and, after excision (2), wild type genomic sequences are used to repair any exonuclease activity (3) at the site of P element excision. The product is wild type sequences left in the site of P element excision (4).

the *in vitro* analysis of Kaufman and Rio (1992) and by Gloor *et al.*, (1991) who have replaced the wild type *white* sequence, where P element excision occurred with a mutant *white* sequence carried by a modified P element.

Further analysis of P element transposition has demonstrated the tendency of these elements to transpose to local chromosomal sites (Tower *et al.*, 1993; Zhang and Spradling, 1993). Those elements inserting within or 5' to the donor element lie in a head-to-head orientation, while those that inserted near to the donor were normally inserted into the genome in a tandem orientation relative to the donor element (Zhang and Spradling, 1993). The molecular mechanisms of this local transposition remain unidentified, although the P element constructs carrying DNA derived from the *engrailed* locus, have a higher frequency of inserting in the vicinity of the *engrailed* gene, indicating homologous genomic sequences are important in the P element insertion mechanism (Kassis *et al.*, 1992). The ability of a P element to transpose to a more distant locus in the genome may be inhibited by the state of chromatin packaging, indicated by the work of Bownes (1990).

Repression of Transposition

P element transposition occurs at high levels in germline cells, but does not occur in the somatic cells (Engels, 1983). The germline specificity of transposition was demonstrated by Laski *et al.*, (1986) to be controlled by mRNA splicing mechanisms. In somatic cells, removal of the intron between exons 2 and 3 does not occur, and functional transposase is not produced. They concluded that when this intron is spliced out of the transcript in the germline, functional transposase is synthesised, allowing transposition events to occur. In support of this, the Δ2-3 P element that has the third intron deleted, produces high levels of transposase activity in the germline and somatic cells (Laski *et al.*, 1986).

As mentioned above, the first level of repression in the somatic cells is achieved by alternative splicing of the P element transcript (Laski *et al.*, 1986), thus

preventing transposition and excision, when the flies can be genetically classed as M cytotype. The P cytotype is the genetic state in which P element excision and transposition are suppressed in both somatic and germline cells. Progeny from M cytotype females and P cytotype males are dysgenic. When P cytotype females are crossed to M cytotype males, dysgenesis is observed at a much reduced frequency. Dysgenesis is not detected at any appreciable levels in crosses where both parents are of the P cytotype, or of the M cytotype.

The simplest model to explain this phenomenon is to predict the expression of a P element repressor protein. An alternative splice product from the P element ORFs produces a smaller, 66 kDa protein, that has been postulated to act as the repressor of P element transposition in the somatic tissues (Laski *et al.*, 1986). Since the complete P element is required to encode the transposase enzyme, the repressor must be encoded by overlapping sequence. In support of this, modified P elements that have frameshift mutations in exons 0, 1, or 2 failed to repress transposase activity, indicating these three exons are essential for repressor function (Robertson *et al.*, 1989). It was thought that titration of a repressor allowed dysgenesis to occur in the M ♀ X P ♂ crosses, and that the P cytotype is due to a repressor, encoded by the P element itself.

1.13 The P Element as a Molecular and Genetic Tool

In two simultaneous papers, Rubin and Spradling were able to demonstrate genetically engineered P elements could be used to transform *Drosophila melanogaster*, and that such elements were functional, if a source of transposase was supplied via a helper plasmid (Rubin and Spradling, 1982; Spradling and Rubin, 1982), allowing the modified P elements to integrate into the genome.

The *rosy* (*ry*) gene was cloned into the P element, in order to allowing selection of flies that had P element constructs in integrated in their *ry*$^-$ genome. The *rosy* gene product, xanthine dehydrogenase is not cell autonomous, and levels of the

product, xanthine dehydrogenase, as low as 50% will restore wild type eye colour in the *rosy*$^-$ backgrounds. The P element constructs successfully transposed from plasmids in *Drosophila* embryos with "helper plasmids". This P element was then able to induce transposition of the element at the *sn*^{w} allele, indicating that the modified P element was functional.

When these experiments were repeated with four mutated constructs (frameshift mutations in each of the four reading frames), the P element at the *sn*^{w} site was not mobilised (Karess and Rubin, 1984). They concluded therefore, that the P element encoded the enzyme required for transposition. The introduction of an amber mutation, and mutations at the intron/exon boundaries failed to produce the transposase (Laski *et al.*, 1986) demonstrating a potential control point in the expression of the P element gene.

P Element Insertions

The phenomenon of hybrid dysgenesis has proved to be useful in the generation of novel mutations in the *Drosophila* genome (Eisenberg and Elgin, 1987; Howes *et al.*, 1988). There are many reported instances of a P element inserting in the 5'-untranslated sequence, or within the 5'-control sequences, upstream from the gene (Salz *et al.*, 1987; Tsubota *et al.*, 1985; Tsubota and Schedl, 1986 Voelker *et al.*, 1984). Such insertions may disrupt the appropriate expression of the neighbouring gene; however, the newly generated mutant flies must have the large number of additional P elements also present, removed by extensive "back-crossing" if a specific insertion event is to be attributed with inducing the novel mutation. In 1981, the *Drosophila* P element was used as a "tag" in the cloning of the *Drosophila white* gene (Bingham *et al.*, 1981), and to clone sequences from the *RNA polymerase II* locus (Searles *et al.*, 1982). These are the first reports of the P element being used as a molecular tool, and mark the beginning of a reverse genetics approach in *Drosophila*.

P Element Excisions

The P element may bring about a mutation as it imprecisely excises from the genome. P elements have been shown, in a variety of experiments to remove as much as 7 kb of genomic sequences that flank the element, at a wide range of frequencies (Engels, 1983).

Speculation surrounds the frequency of imprecise excision. The current view, based upon the believed mechanism of excision, suggests that imprecise excisions are detected at elevated frequencies if the P element is heterozygous, for example, over a deletion, or balancer chromosome (Daniels and Chovnick, 1993; Engels *et al.*, 1990 Gloor *et al.*, 1991). Heterozygosity removes sequence information that could be used to repair any losses brought about by the excision, via a gap repair mechanism (Daniels and Chovnick, 1993; Engels *et al.*, 1990 Gloor *et al.*, 1991). It is almost certain that the type of P element is important in the rates of excision. The modified enhancer trap elements will almost certainly have different frequencies of excision because they are much larger than wild type. The rate of precise and imprecise excisions for a given P element type would be invaluable data for experimental design. As many insertions have no detectable phenotype, relatively simple imprecise excision events may provide the desired mutations.

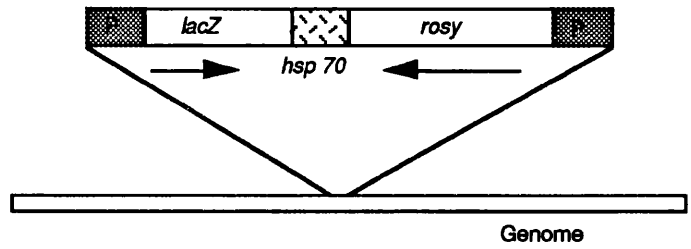
The P Element As an Enhancer Trap

The power of the P element as a molecular tool was realised by O'Kane and Gehring (O'Kane and Gehring, 1987), when they cloned the reporter gene, *lacZ* into the P element creating a *Drosophila*-specific reporter construct. They had suggested that such constructs could be used as gene-, or enhancer-traps, analogous to the techniques previously applied to *E. coli.* (Casadaban and Cohen, 1979).

An element was constructed with the *E. coli* gene *lacZ* at the 5'-end of the element. The *hsp70* polyadenylation signal was included for accurate processing of

the prokaryote gene. The *Drosophila rosy* gene was cloned into the 3'-end of the element, and is transcribed towards the 5'-end of the P element construct (see Figure 1.5). This acts as a selectable marker for the appropriate flies. Only the P element ends were left intact to allow for transposition, and the weak, constitutively active promotor of the P element, to drive expression of the *lacZ* gene (see Figure 1.5). Thus, the P element construct has no transposase source, and will remain immobile in the genome, unless a transposase source is supplied by a second P element (O'Kane and Gehring, 1987).

Figure 1.5 The P Element Construct



Arrows denote direction of transcription. P denotes P element sequences. Not to scale. Illustration of the P element insert in the lines A11 and A22. The *lacZ* gene is transcribed in the 5' to 3' direction of the P element, under control of the P element minimal promotor. If the P element promotor is influenced by an enhancer, then *lacZ* is expressed. The *rosy* gene is transcribed in the opposing direction under the control of its own regulatory elements, allowing selection for insertion strains in a mutant *rosy* genetic background.

In their initial experiments 76% of the insertion lines showed a variety of spatially regulated expression patterns of *lacZ,* indicating the technique would be useful in the identification of previously unknown genes expressed in specific tissues or subsets of cells in a variety of developmental stages. This expression is presumably dependant upon the spatial and temporal activity of upstream regulatory

elements in the genome. Additionally, 20% of these insertion lines exhibit a mutant phenotype as a result of the P element insertion. It is claimed such mutant phenotypes are indicative of the function of the gene under the control of this enhancer (O'Kane and Gehring, 1987).

1.14 Does the Expression of *lacZ* Accurately Represent that of the Gene?

A P[*lac* ry^+]A construct was detected just upstream of the *Drosophila hairy* gene. Since the temporal and spatial expression patterns of this gene have been characterised, comparisons between *hairy* transcription patterns and *lacZ* expression patterns would indicate the faithfulness of the reporter gene's activity. Fasano *et al.*, were able to find only minor differences between the reporter gene and the *hairy* transcription pattern during embryogenesis (Fasno *et al.*, 1988).

Mellerick *et al.*, (1992) isolated a P element enhancer construct within the 3' untranslated region of *pollux* gene. Expression of the *pollux* gene was not disrupted by this insertion. However, the *lacZ* expression pattern detected by the reporter gene was that of *castor*. This situation is notable, since the *lacZ* gene is expressed in the same direction as the *pollux* transcript. The authors postulate that the minimal promotor within the construct and the *pollux* enhancers are not compatible. This would suggest that a variety of genes within the genome may not be detected using the enhancer-trap technique, even if the P element inserts within the relevant region of the genome. This is an important study since it demonstrates *in vivo*, the direction-independence of enhancers. In support of this suggestion of enhancer-promotor compatibility, Li and Noll (1994) have studied the mechanisms that control the expression patterns of *gooseberry* and *gooseberry neuro*. They have found that the closely apposed enhancers of these two genes have no influence on the other gene. They propose that each enhancer-promotor combination requires specific, non-complementing transcription factors. Other possibilities, are i) the binding proteins

are not available in some cell types, ii) the binding proteins are available, but the binding specificities of the DNA sequences are different.

Figure 1.6 P Element Insertion in the *pollux* Locus

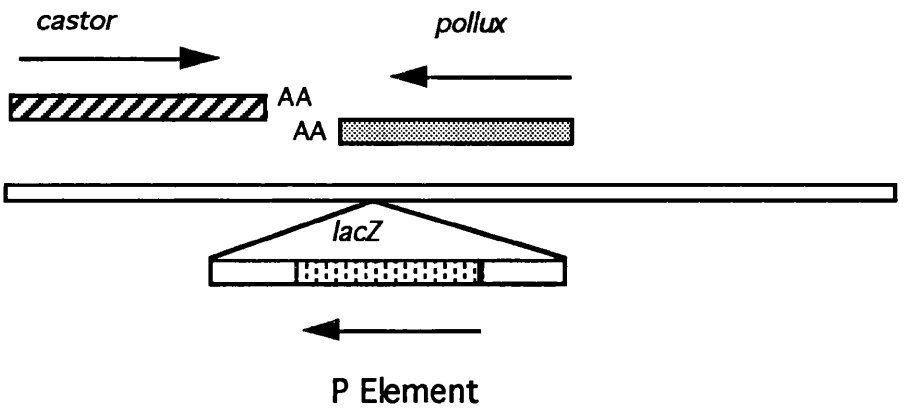


Illustration of the P element insertion in the *pollux* locus. Illustration taken from Mellerick *et al.*, 1992. The P element construct inserted in the *pollux* gene drives *lacZ* expression in response to the regulatory regions that regulate *castor* gene expression. Arrows denote direction of transcription. Not to scale.

Many studies of *Drosophila* gene expression have now utilised the enhancer-trap technique with satisfactory results. Bellen *et al.*, (1989) identified four P element insertion lines, with *lacZ* expression patterns, which were similar to, although not always identical to the embryonic expression pattern of the neighbouring gene. Wilson *et al.*, (1989) were able to demonstrate that twelve lines had elements inserted close to known genes, and describe the pattern of seven of these genes. Using *in situ* hybridisations they found that four of the seven insertions expressed *lacZ* in response to regulatory elements in patterns similar to that of the known genes. The expression patterns of other genes have been shown to be accurately reflected by the activity of *lacZ* in these types of P element constructs (Rothberg *et al.*, 1990; Kim and Crews, 1993 and references therein.).

Thus these insertion vectors appear to be reasonably good indicators of the spatio-temporal expression pattern of a large variety of genes.

Activity of Different Promotors Within the P element Constructs

Perrimon *et al.*, (1991) compared the activity of four different P element constructs used in screens as markers for specific cell types, for use in studying *Drosophila* development and cell lineage analysis. Weak promotors of either the P element itself, found in P[lArB], *engrailed, ftz*, or the *eve* genes were used in these constructs. They found that amongst those transformed lines that exhibited tissue-specific patterns, there was a correlation with the promotor used and the tissue that gave detectable *lacZ* expression patterns. Constructs with the P element promotor tend to show specific staining patterns in the nervous system. The *ftz* promotor shows a higher frequency of activity in the nervous system, while the *eve* promotor has an increased level of tissue specificity in the cells derived from the mesoderm. The *engrailed* promotor is preferentially active in the nervous system and the epidermis. The authors speculate that the insertional event near a tissue-specific gene, giving appropriate reporter gene expression, is dependent upon the specific promotor within

the construct. Alternatively, the different promotors used may preferentially function in response to "tissue-type" specific enhancers, inferring "compatibility" between promotors and enhancers is an important factor in controlling gene expression. This hypothesis is compatible with the data of Mellerick *et al.*, (1992) and Li and Noll (1994). This work provides useful information, that could aid the design of P element screening techniques. Specific constructs could be designed to use in screens for genes active in specific tissues, increasing the efficiency of the screen (Perrimon *et al.*, 1991).

The enhancer trap technique provides a great deal of versatility permitting not only the swift isolation of flanking DNA, but also aids the generation and subsequent genetic and molecular analysis of induced mutations. In addition, this technique should allow the identification of those cell types that may be disrupted by any induced mutations and so should prove useful in functional analysis of the insertion site. Therefore, for the purpose of this thesis, two *Drosophila* enhancer trap strains, A11 and A22 were selected for study, in the hope that they would be useful in the isolation of novel genes required for embryonic neurogenesis. Such novel genes and their subsequent mutational analysis may ultimately lead to a greater understanding of the molecular and cellular mechanisms required in the generation of a fully functional nervous system.

Chapter 2

Materials and Methods

2.1 *Drosophila* Husbandry

Unless otherwise stated, all flies were maintained at room temperature, and passed on to fresh food every two or three weeks. All crosses were of three virgin females and a single male in a small vial unless otherwise stated. Single male x virgin female crosses were used in the temperature sensitivity and complementation tests.

Table 2.1 *Drosophila melanogaster* Strains

Strain/Genotype	Source
ry^{506}	C. O'Kane (Cambridge)
TM3 ry^{506} *Sb e/ rf 10*	C. O'Kane (Cambridge)
Oregon-R	Glasgow Stocks
A11	M. Bownes
A22	M. Bownes
A37	A. Giangrande
Df(3L)th^{ss102}, *h ri* e^{s}/TM6C	Bloomington Stock Centre
Df(3L)sf^{13}, *Ki roe* p^{p}/TM6B	Umeå Stock Centre
Df(3L)*brm11*/TM6C	Bloomington Stock Centre
Sb P(ry^{+} Δ2-3)(99B)/TM6	C. O'Kane (Cambridge)
dumpy	R. McNab (Glasgow)
hypoC	K. O'Dell (Glasgow)

All fly stocks are described in Lindsley and Zimm except A37 (Ghysen, A., C. O'Kane. 1989.), *Sb* P(ry^{+} Δ2-3)(99B)/TM6 (Robertson *et al.*, 1988), and A11 and A22 P element insertion strains which were generated in the Laboratory of Prof. J. Merriam.

Drosophila Media

Glasgow Fly Food

10g agar, 15g sucrose, 30g glucose, 35g yeast, 15g maize meal, 10g wheatgerm, 30g treacle, 10g soya flour. 10ml of Nipagin and 5ml of propionic acid were added (once the boiled media had cooled to 60°C) and made up to 1 litre with sterilised dH_2O. Nipagin is *p*-hydroxybenzoic acid methyl ester (25g dissolved in 250ml ethanol).

***Drosophila* Egg Collection Plates**

19.8g agar, 90ml grape juice, 52.2g glucose and 26g sucrose, 7g yeast. 10ml of Nipagin was added once the media had cooled to 60°C and made up to 1l with sterilised dH_2O.

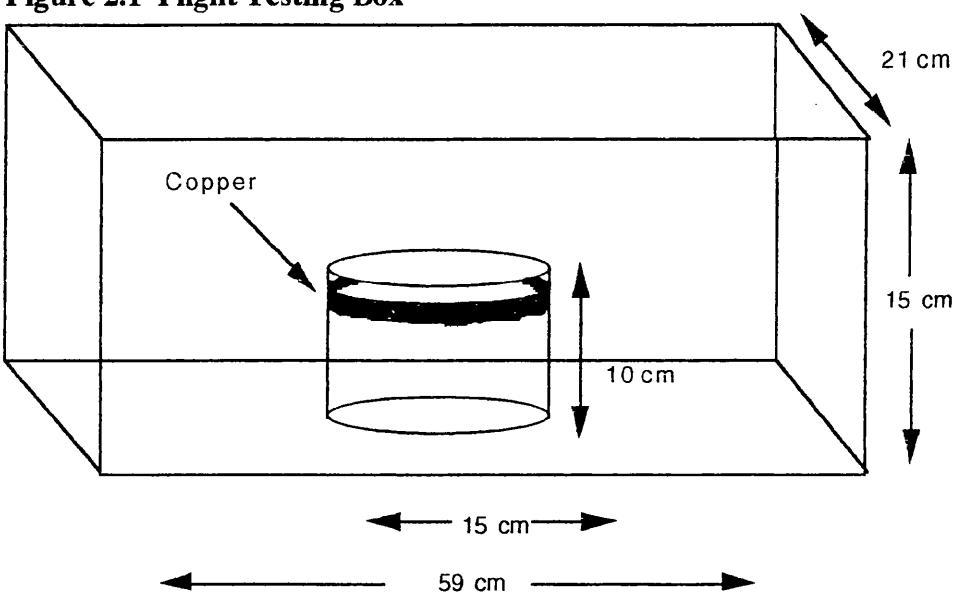
2.2 Behavioural Assays

The jumping assay used here is a modification of that of Homyk and Sheppard (1977). Male flies, between 2 and 4 days old had their wings gently removed under light CO_2 anaesthesia, and were left to recover at the temperature they were reared O/N. Each fly was then placed on a piece of card marked with 1cm grid lines, and subjected to repeated banging on the bench in order to startle the fly, and so induce a jump response. For each fly, the longest estimated jump distance was recorded. Each fly was labelled with a code number and scored blind to avoid any bias in the scoring of the jump assay.

Flight was assayed in a perspex flight chamber (Sheppard, 1974; Homyk and Sheppard, 1977) constructed by Glasgow University workshop (see Figure 2.1). A cylindrical perspex, open-topped cylinder measuring 15cm in diameter and 10cm high, was fixed to the bottom of a large perspex box measuring 59cm by 15cm by

21cm, with a removable lid. The upper inside surface of the cylinder was covered with copper wire/plate, that was connected to 120-150 Volts. Flies aged 3-5 days old were gently dropped into the central chamber, and the power quickly connected. The chamber was then vigorously shaken for 1-3 min. Thus only flies capable of initiating and maintaining flight can escape from the central chamber. Those flies that attempt to escape by walking will receive an electric shock from the copper. The number of flies that had/had not escaped the central chamber were then counted.

Figure 2.1 Flight Testing Box



The flight testing box, based on that of Sheppard, 1974.

Statistical Analysis

The data obtained from both the flight assay and the temperature sensitivity tests were compared using the $\chi 2$ test (Bailey, 1981).

2.3 Cytology Buffers and Methods

X-gal Staining of *Drosophila* embryos

Embryos were collected from the egg laying plates described earlier after the desired length of development. Embryos were immersed in 50% bleach (Safeways), for 90-120 seconds and then rinsed thoroughly, before being fixed in 5ml of heptane and 5ml 4% paraformaldehyde in PBS (150mM NaCl, 450mM KCl, 20mM $MgCl_2.6H_2O$, 100mM KH_2PO4/K_2HPO_4, pH 6.8) for 20-30 minutes with occasional shaking. If desired, the lower phase was removed and 5ml methanol was added to remove the vitelline membrane. Methanol treatment is essential for antibody staining of the embryos. The embryos were then collected and washed extensively in PBT (1% Triton X-100®, in PBS). The embryos were then left in a 300µl volume of the X-gal Staining Solution (prewarmed to 37°C) with 0.2% X-gal, in the dark at 37°C. The embryos were periodically checked for signs of staining. When the embryos were judged to have stained, they were washed in PBS and mounted either in a 1:2 (v/v) mixture of PBT and glycerol or JB-4 (Polysciences). The X-gal staining solution was 10mM $NaH_2PO_4.H_20$ /$Na_2HPO_4.2H_20$ (pH. 7.2), 150mM NaCl, 1.0mM $MgCl_2.6H_20$, 6.2mM K_2 $[Fe^{II}(CN)_6]$, 6.2mM, $K_3[Fe^{III}(CN)_6]$, 0.3% Triton -100®, modified from Simon *et al.*, (1985). The β-galactosidase enzyme encoded by the P element construct, enzymatically converts 5-bromo-4-chloro-indole-β-D-galactoside (X-gal) to the colourless bromochloro-indoxyl, that then dimerises to the chlorobromoindigo.

X-gal staining of Other *Drosophila* Tissues

Larval and pupal tissues were dissected in PBS and fixed in 4% paraformaldehyde for 30-60 minutes. Adult flies were mounted side by side in a fly collar and soaked in O.C.T. (Lab-Tek Division) for 30 min and frozen at -18°C.

7-12μm sections were then taken using a cryostat (Anglia Scientific, Cryotome 620), and placed on subbed slides. The tissues and sections were extensively washed in PBT and then stained as for the embryos. Staining would typically take from 4 hours to O/N with 10 animals of a particular developmental stage for each strain. A11, A22, A37 and OR-R were all stained in parallel. Only those staining patterns that were consistently seen in the stained tissues have been reported.

Antibody Staining

Embryos and tissues were collected as for X-gal staining. All embryos were methanol treated. All embryos were washed extensively in PBT after fixing with 4% paraformaldehyde as previously described. All antibody incubations were done in PAT (1% Triton X-100®, 1% BSA fraction V in PBS) with the presence of appropriate serum at 4-10%, reducing the levels of background. Primary antibody incubations were at 4°C O/N. The secondary antibodies were preabsorbed to similarly prepared wild type embryos O/N to ensure appropriate blocking. The secondary antibody incubation was for 2-4 hours at room temperature in PAT, after extensive washing in PAT with serum to remove the primary antibody. The avidin and biotinylated horseradish peroxidase complex was then produced using the Vectastain Elite ABC kit. The colour reaction was performed as above with H_2O_2 and DAB.

Double Antibody Labelling Experiments

In the double labelling experiments, embryo preparation was as before, and differential colour of the two different antibodies was achieved using $NiCl_2$ and $CoCl_2$ (Raz and Shilo, 1993) in the HRP reaction (3μl of 8% solution in a 300μl reaction mix) to produce the blue colour.

Table 2.2 Antibodies

Antibody	Final concentration
anti-HRP (Cappel)	1 : 5 000
anti-β-gal (Cappel)	1 : 4 000
anti-β-gal (Promega)	1: 300
22C10 (N Patel)	1 : 10
anti-rabbit (Cappel)	1 : 500
anti-rabbit (Texas red labelled)	1 : 100 - 1 : 500
anti-mouse (FITC labelled)	1 : 500
anti-mouse (Promega)	1 : 600

The Texas red (Molecular Probes) and FITC (fluorescin, Vector Laboratories) labelled antibodies were a kind gift from D. Armstrong (Glasgow).

Fluorescently labelled embryos were examined using a Multiprobe Confocal Laser Scanning Microscope using Image Space 3.1 software (Molecular Dynamics) Embryos and other tissues were photographed using a Leitz Vario-orthomat camera system and Normaski optics attached to a Leitz microscope with Ektar 25 film (Kodak). Adult flies were photographed using a Canon 200mm Camera attached to a Leitz dissecting binocular microscope.

2.4 General Microbiology

Bacterial Strains

Table 2.3 *E. coli* Strains

Strain	Reference/Source
DS 941	Kind gift from M. Burke (Glasgow)
XL1-Blue.	Bullock *et al* (1988), Stratagene
XL1-Blue MRF'	Stratagene
P2392	Kind gift from V. Graham (Glasgow)

Bacteriophage Strains

Both the EMBL4 lambda cloning vector, a kind gift from V. Graham (Glasgow) and lambda ZAP Express™ (Strategene) were used.

Bacterial Media

L-broth and L-agar

10g Bacto-tryptone (Difco), 5g yeast extract (Difco), 10g NaCl, made up to one litre with dH_2O. This was adjusted to pH 7.0 with NaOH. L-agar was made as L-broth, but with the addition of Bacto-agar to 1.5%.

BBL Media

10g trypticase peptone (BBL), 5g sodium citrate, made up to 1 litre with dH_2O. BBL agar was made as BBL broth, but with the addition of Bacto-agar to 1.5%. BBL top agarose was as BBL broth, but with the addition of gel quality agarose to 0.7%.

2X YT Broth

10g Bacto-tryptone (Difco), 10g yeast extract (Difco), and 5g NaCl, made up to 1 litre with dH_20.

NZCYM Medium

10g NZ amine, 5g NaCl, 5g Bacto yeast extract, 1g casamino acids, 2g $MgSO_4.7H_2O$, made up to 1 litre and adjusted to pH 7.0 with NaOH. NZCYM agar was as NZCYM Medium, but with the addition of Bacto-agar to 1.5%.

All bacterial media was sterilised by autoclaving at 120°C for 15 min at 15 psi.

Bacterial Growth

Bacterial colonies were grown on inverted plates, with appropriate selection when necessary, overnight at 37°C. Stationary liquid bacterial cultures were obtained by inoculating 20ml of liquid media, with appropriate antibiotic selection where necessary, overnight with vigorous shaking at 37°C.

Antibiotics and Indicators

Where appropriate, antibiotic selection was used to maintain the presence of plasmids in bacterial cultures. Ampicillin at a final concentration of 50µg/ml (50 mg/ml stock solution in sterile dH_2O), or tetracycline at a final concentration of 12.5µg/ml (12.5mg/ml stock solution in ethanol) was added to broth or warm agar. X-gal and IPTG were used to indicate the synthesis of β-galactosidase, and added to warm agar at a final concentration of 20µg/ml. X-gal was dissolved in DMF at 20mg/ml and stored at -20°C, while IPTG was dissolved in sterile dH_2O at 20mg/ml at -20°C.

Bacterial Density Measurements

Bacterial cell density was measured using a Beckman DU-50 spectrophotometer, with a wavelength of 600nm ($O.D_{600nm}$ = 0.5).

Bacterial Storage

All bacterial strains were stored as both stab cultures at room temperature, and glycerol stocks at -70°C.

Bacteriophage Buffers

All lambda bacteriophage isolates were stored in phage buffer at 4°C.

Phage Buffer

For 1 litre of phage buffer, 18g of Disodium Hydrogen Orthophosphate dodecahydrate ($Na_2.HPO_4.12.H_2O$), 3g of Potassium dihydrogen Orthophosphate anhydrous (KH_2PO_4), 5g Sodium chloride (NaCl), 10ml of 0.1 M Magnesium sulphate ($MgSO_4$), 2ml of 50mM Calcium chloride ($CaCl_2$) and 1ml of a 1% gelatin solution were mixed and brought up to 1 litre.

Bacterial Growth and Manipulation

Unless stated otherwise, all bacterial cultures were grown at 37°C.

Preparation of Competent Cells

20ml of L-broth was inoculated with 0.4 ml of an overnight culture of bacteria, and grown with vigorous shaking at 37°C for 90 min or until the cells had entered the logarithmic growth phase ($O.D._{600}$=0.4), and then pelleted at 4000g for 5 min at 4°C. The pellet was then resuspended in 10ml of ice cold 50mM $CaCl_2$ solution and incubated on ice for 20 min. The cells were then pelleted and resuspended in 2ml of ice cold $CaCl_2$ solution.

Bacterial Transformation

50-100ng of DNA in a volume no greater than 10µl was added to 200µl of competent cells and stored on ice for 1 hr, followed by a heat shock at 42°C for 2 min and then cooled on ice for 1 min. the bacteria were then plated on L-agar plates with the appropriate selection, and incubated O/N at 37°C.

Bacteriophage Growth and Manipulation

Plate Culture

The appropriate *E. coli* strain, taken from an overnight culture, were cultured with fresh media and vigorous aeration at 37°C until they had entered logarithmic growth ($O.D_{600nm}$ 0.4-0.5). These cells were then pelleted and resuspended in 0.4 volumes of 10mM $MgSO_4$. Phage particles were mixed with fresh plating cells and allowed to adhere to the cells at 37°C for 15-20 minutes. This was then mixed with 5ml of cooled NZCYM agar overlay and poured onto dry NZCYM plates. The plates were inverted after the overlay had set, and incubated overnight at 37°C, or until plaques were apparent.

Bacteriophage Titres

Bacteriophage stocks were titred by making serial dilutions of phage stocks with phage buffer. Each dilution was plated on BBL plates with BBL top agar as described in plate culture.

2.5 General Molecular Biology

General Solutions and Buffers

T.E.

10mM Tris-HCl, 1mM Na_2EDTA (pH. 8.0)

20X SSC

3M NaCl, 0.3M sodium citrate (pH. 7.0)

Denaturing solution

1.5M NaCl, 0.5M NaOH

Neutralising solution

1.5M NaCl, 0.5M Tris-HCl pH. 7.2

Phenol

All phenol used in the purification of nucleic acids was distilled. For DNA isolation it was equilibrated first with 1M Tris-HCl (pH. 8.0) then with T.E.

Phenol/Chloroform

A 1:1 mixture of phenol and chloroform.

10X TBE Buffer

109g Tris-HCl, 55g boric acid, 9.3g $Na_2EDTA.2H_2O$ made up to 1 litre with distilled H_2O (pH. 8.3)

10X TAE

48.4g Tris-HCl, 16.4g sodium acetate, 3.6g $Na_2EDTA.H_2O$ made up to 1 litre with distilled H_2O (pH. 8.2)

10 X gel loading buffer

Ficoll, 0.5% SDS, 0.06% bromophenol blue, 0.06% xylene cyanol FF

Hybridisation Solution

6X SSC, 5X Denhardt's solution [0.1% polyvinylpyrolidine (MW. 360 000)] 0.1% Ficol (MW. 400 000), 0.1% BSA (fraction V; Sigma), 50mM sodium phosphate

(pH. 6.8), 1% SDS, 100μg/ml sheared herring sperm DNA, 1mM EDTA, 0.05% sodium pyrophosphate.

Drosophila Genomic DNA Isolation

Homogenisation Buffer

0.1M NaCl, 0.003M Tris pH 8.0, 0.01M EDTA, 10mM β-mercaptoethanol

Nuclear Lysis Buffer

0.1M Tris pH. 8.0, 0.1M EDTA, 0.5mg/ml proteinase K, 0.1M NaCl

Drosophila Genomic DNA Preparation

Large Scale Preparation

A modified protocol of Levis *et al.*, (1982) was used to prepare high molecular weight DNA. 1g of flies or dechorionated embryos were added to a pre-cooled mortar and pestle with liquid N_2, and ground to a fine powder. This powder was transferred to a 15ml Wheaton homogeniser, on ice with 9ml of DNA Homogenisation Buffer. 500μl of 10% Triton X-100® was added, and then homogenised. The homogenate was then decanted through a piece of nylon gauze, into a sterile 30ml corex tube on ice and the nuclei pelleted (4000g, 10 min) at 4°C.

The nuclear pellet was gently resuspended in 1ml of ice cold HB (with no Triton X-100®), followed by the addition of 5ml Nuclear Lysis Buffer. Finally, 200μl of 30% Sarkosyl was added and mixed by gentle swirling, lysing the nuclei. Incubation O/N at 37°C allowed the proteinase K to degrade excess protein. 1.25g CsCl/g of homogenate is added and allowed to slowly dissolve, and then loaded into Quick-Seal™ tubes (Beckman), and filled with 1.25g CsCl/ml water, such that the final density is 1.7g/ml and sealed. The DNA was then ultracentrifuged at 45000 rpm for up to 60 hours at 25°C. The DNA band was collected in fractions from the tube using two 18 gauge needles. The fractions were then run out in a 0.7% agarose gel to check for the appropriate fraction and DNA integrity. The best fractions were pooled

and concentrated by dialysis over PEG 8 000. Yields were usually 100-200µg/g of flies.

On occasion genomic DNA was obtained from *Drosophila*, using a protocol essentially the same as above except the DNA was phenol/chloroform twice, and then chloroform treated, rather than purified on a CsCl gradient. This DNA was then ethanol precipitated and resuspended in an appropriate volume of T.E.

Plasmid Purification

Large and small scale plasmid isolation was carried out using the alkali-lysis method of Birnboim and Doly (1979). The large scale plasmid purifications were purified further by CsCl/EtBr equilibrium centrifugation, as described by Sambrook *et al.*, (1989). On occasion, small scale plasmid preparation was by Magic™ Miniprep (Promega).

DNA Fragment Isolation

DNA fragments were isolated by a variety of techniques including electroelution from TAE gels, centrifugation of smaller fragments (< 5 kb) from the gel slice, through glass wool, followed by passage through Promega Magic™ DNA purification system as directed by the manufacturer.

Quantification of DNA

The quantity of DNA was measured using a Beckman DU-50 spectrophotometer. At a wavelength of 260nm, a reading of 1 corresponds to ~ 50µg/ml. When samples were limited, the quantity of DNA was estimated by spotting the sample onto the surface of a 1% agarose gel containing EtBr at 0.5µglml. The gel was photographed, and the concentration of DNA estimated by comparison to the intensity of fluorescence with that of known samples, using short-wavelength U. V. illumination (254nm).

Restriction Enzymes

DNA was digested using a minimum of 2 units of enzyme/μg of DNA at the appropriate temperature for 1 hr (plasmid) or 3 hr (genomic). The enzymes were obtained from either BRL or Promega, and the appropriate buffer, supplied by the manufacturer was used.

Size Fractionation in Agarose Gels

Most agarose gels were run with a TBE buffer. The concentration of agarose used was 0.8-1.5% (w/v) depending on the size of fragments to be resolved (Sambrook *et al.*, 1989). TAE was used in preference to TBE when DNA fragments were to be purified from the gel. LMP agarose was occasionally used with TAE, when DNA molecules were to be purified for ligations. DNA size markers were lambda DNA digested with *Eco* RI and *Hind* III (BRL), or lambda 1kb ladder. The DNA was visualised by U.V. fluorescence after staining the gel with Ethidium bromide.

DNA Visualisation and Photography of Gels

DNA was visualised by U. V. induced fluorescence after staining the gel in 0.5mg/ml EtBr, on a short (254nm) or long (302nm) wave transilluminator. Gels were photographed using with a Polaroid camera loaded with 545- or 667- land film and fitted with a Kodak Wratten filter # 23A.

Radio-labelling of Nucleic Acids, and Hybridisation to Filters

DNA probes in LMP were labelled to high specific activity by the random priming method (Sambrook *et al.*, 1989), using a Boehringer Mannheim kit according to the manufacturer's instructions. Labelled probe was then separated from the unincorporated nucleotides, through a Sephadex G-50 (Pharmacia) column chromatography, prepared in a 1ml syringe. Probes generally had a specific activity of 10^7-10^9 cpm/μg.

Southern Blots

Gels were soaked in denaturing solution for 20 min and then rinsed in dH_2O twice. This process was repeated using neutralising solution. Genomic and plasmid DNAs were then transferred to nylon membranes (Hybond-N) or nitrocellulose membranes (Hybond-C) by capillary action using 2XSSC, as instructed by the manufacturer, and the DNA was then fixed to the membrane by baking at 80°C for 2 hours as described by Sambrook *et al.*, (1989). For genomic Southern blots, the gels were treated with 0.25M HCl for 15-20 min prior to blotting. Alkali transfer that does not require the initial denaturing and neutralising steps, was also used on occasion.

Library Construction and Screening

Subgenomic libraries were constructed according to the manufacturer's instructions (see Chapter 5).

Screening of Bacteriophage Libraries

After plaque formation, the plates were left to cool at 4°C for 1 hour. Duplicate filters were then taken from each plate. Alignment was achieved by piercing the first filter with a needle while on top of the plaques. The second filter was marked at the same points. The first filter was placed on the plate and gently removed when completely wet. The second filter was left on the plate for 30-60 seconds. Each filter, phage side facing upwards, was then placed on 3MM (Whatman) paper soaked in denaturing solution for 1-2 minutes, then on a sheet of 3MM paper soaked with neutralising solution for 5 minutes and to a second sheet of Whatman paper soaked in neutralising solution for a further 5 minutes. The filters were then rinsed in 2 X SSC for a minimum of 10 minutes, left to dry, and then baked for 2 hours at 80°C.

Hybridisation of Filter-bound DNA

Hybridisation of filter bound DNA was carried out overnight at 65°C with fresh hybridisation solution and appropriate probe, after an overnight prehybridisation step at 65°C. All hybridisations were carried out in aTechne Hybridisation oven.

DNA Amplification

All PCR reactions were performed using Promega *Taq* polymerase and Pharmacia dNTPs under mineral oil using a Hybaid PCR machine. Oligonucleotide primers used are presented in Table 2.4. The PCR reaction profiles are described in Chapter 5.

Autoradiography

Filters were stored at -70°C in an autoradiography cassette with intensity screens for the desired length of time. Autoradiographs were then developed using a benchtop X-Omat developer.

Table 2.4 PCR Primers

Primer	Sequence (5' - 3')	Reference
L1	GCGGTGACCCGGGAGATCTGAATTC	J. Sentry
L2	GAATTCAGATC	J. Sentry
TOP 1	GATCCAAGGAGAGGACCAATGGCCTCGAGGCCTATTGAGCTCTATTCGTTATGAGAAGGGAGAGT	J. Sentry
BOT 1	ACTCTCCCTTCTCAGTCATAACCTGCAGGATGGAGTTCAGGACGAGCTCCTACGTCCTCTCCTTG	J. Sentry
EXT 1	AGTCATAACCTGCAGGATGGAGTTC	J. Sentry
P31	CGACGGGACCACCTTATGTTATTTCATCATG	O'Hare and Rubin, (1983)
cWS1	TCGTCCGCACACAACCTTTCCTCTC	J. Sentry
cWS2	CTTTCACTCGCACTTATTGCAAGC	J. Sentry
snA	GTCTGTCGTCACACCCTTCACTTCGCC	S. F. Goodwin (1994)

L1, L2, Bot 1 and Top 1 are linkers. L2 will anneal to L1 and the oligonucleotide Ext 1 will anneal to Bot 1. Top 1 and Bot 1 will aneal at the termini but not in the central region. The primer P31 will anneal to both 5' and 3' terminal sequences of the P element while cWS1 will anneal to the 5' and cWS2 will anneal to the 3' terminal sequences.

Chapter 3

lacZ Expression Profiles of the *Drosophila* P Element Insertion Strains A11 and A22

3.1 Introduction

In this Chapter, the spatial and temporal expression pattern of the reporter gene of both A11 and A22 insertion strains will be presented. These enhancer trap strains are believed to express *lacZ* in the developing central and peripheral nervous systems, and therefore may be useful tools in the isolation and characterisation of novel genes required in the development of the *Drosophila* embryo. This may indicate which cell types express the gene regulated by the trapped enhancer. As many genes are pleiotropic, being required during various developmental stages and tissues, expression of the *lacZ* transgene in both insertion strains at a variety of developmental stages will be described, providing a developmental profile of the activity of the enhancer. The information gained from these analyses should facilitate the characterisation and dissection of any mutants derived by the imprecise excision of the P element constructs. If imprecise excision of the P element generates mutants that have no obvious external phenotypes, or are lethal, the expression patterns should indicate where any subtle or internal changes may be located.

The P element construct of A22 has previously been identified as inserted in chromosome bands 72BC of the third chromosome, and that of A11 to 25E on the second chromosome (E. Zador unpublished data). If these *lacZ* expression patterns are similar to any genes previously located to these bands then they may be used as convenient tools in the analysis of such genes. If there are no previously identified genes in the vicinity of the P element insertion site, then they may lead to the identification of novel *Drosophila* genes.

3.2 Developmental Stages of *Drosophila melanogaster*

The developmental processes of *Drosophila* are complex. The fruit fly undergoes various morphological changes as it develops. The embryo hatches after 24 hours (at 25°C), to give the first instar larvae, which subsequently hatches into the second instar larvae. This stage gives rise to a third instar larva after a further moult. This larva then undergoes metamorphosis, giving rise, finally, to the fully formed adult fly. Embryogenesis encompasses the developmental processes involved in the formation of a larva from a single fertilised cell. The subsequent larval instars show little change in morphology, except growth. During metamorphosis, many of the larval structures are histolysed, and the adult structures form from sheets of epidermal cells, or imaginal discs, first formed in the developing embryo.

There are a number of volumes that have described the morphological events that occur during embryogenesis (Campos-Ortega and Hartenstein, 1985; Bownes, 1982), allowing embryonic development to be subdivided into different phases or stages, as judged by specific structural changes at a variety of time points. Here, I have chosen the most recent text and staging protocol (Bate and Martínez-Arias, 1993) for embryogenesis.

Embryogenesis

Embryogenesis begins as the diploid nucleus undergoes 13 cell divisions. These nuclei divide without cellular division and migrate to the periphery of the synctial egg (stages 1-4). When cell membranes encompass these nuclei, and the germ cells are located at the posterior of the egg, the embryo has reached the cellular blastoderm stage (stage 5). Gastrulation occurs as the blastoderm invaginates during stages 6-8, and results in an embryo with three germ layers, rather than one layer of cells. This stage also sees the formation of the ventral and cephalic furrows, while

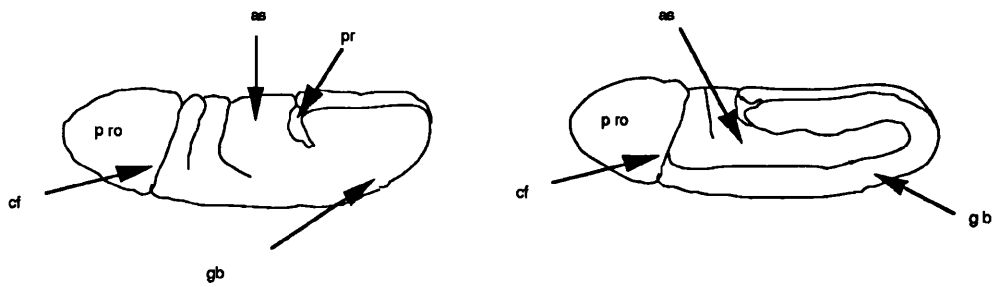
the anterior and posterior midgut invaginations form and the initiation of germ band elongation. During this stage the proneural clusters of the CNS and PNS form.

During stages 9-11 the germ band continues to extend until the posterior of the embryo folds back, until it reaches the head regions. In addition the ectoderm begins to form organ primordia which includes the epidermis, CNS, mid- and fore-gut. During stage 11, the tracheal pits and parasegmental furrows appear, showing the metameric organisation of the embryo.

Stage 12 is characterised by the start and completion of germ band shortening. During stage 13, dorsal closure and head involution initiates, and differentiation of the neuronal precursors begins and will continue through development until the end of stage 15.

Figure 3.1 Embryo Stages 8 and 9

A Stage 8 Embryo **B Stage 9 Embryo**



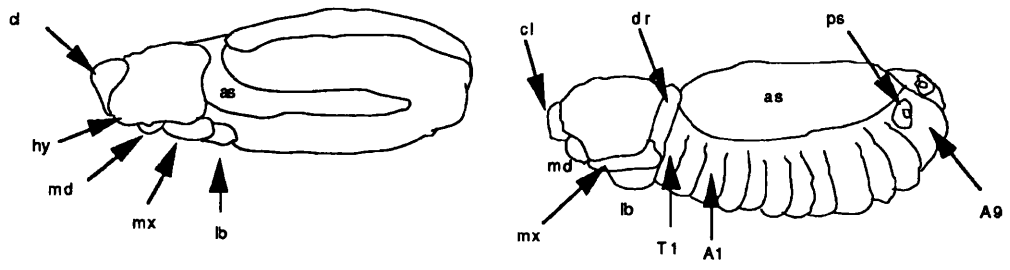
as: amnioserosa, cf: cephalic furrow, gb: germ band, pr: proctodeum, pro: procephalon. Not to scale.

Stage 14 is marked by the beginning of head involution, where most of the head structures are internalised, and dorsal closure continues. The ventral nerve cord condenses during stage 14, and this process is complete in the stage 17 embryo. Stage 15 ends when dorsal closure is complete. Stage 17 sees the final stage of embryogenesis, where the embryo becomes more larval-like on its surface, and head involution has terminated and the cuticle layer is completed.

Embryogenesis is complete when the first instar larva hatches. The three larval instars have growth of the larval tissues as their primary function, while their gross morphology changes little. At the end of the third larval instar stage the animal enters metamorphosis. This is an important stage in the development of *Drosophila*, where most of the larval tissues are histolysed, and the adult structures differentiate from single-layered sheets of epidermal cells, called the imaginal discs, and histoblasts. At the end of the pupal period, the young fully formed fly frees itself from the pupal case.

Figure 3.2 Embryo Stages 11 and 13

A Stage 11 Embryo **B Stage 13 Embryo**



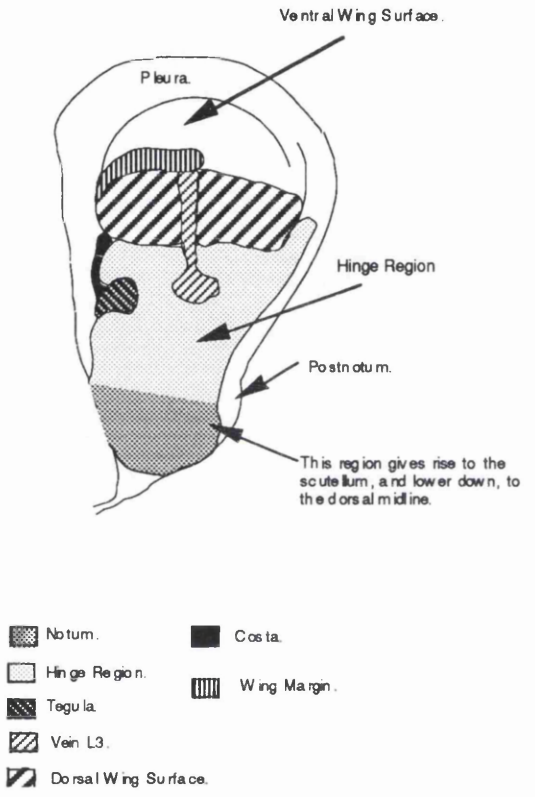
as: amnioserosa, cl: clypeolabrum, dr: dorsal ridge, hy: hypopharyngeal, lb: labium, md: mandible, mx: maxilla, ps: posterior spiracle, T1: first thoracic segment, A1: first abdominal segment, A9: ninth abdominal segment. Not to scale.

The *Drosophila* Imaginal Discs

During metamorphosis the larval tissues are destroyed while the adult tissues develop from the imaginal discs and histoblasts. The imaginal discs consist of a monolayer of epidermal cells and a peripodial membrane. As the discs age, the cells of the disc proper become folded in a characteristic manner, providing morphological landmarks that allow the identification of each disc. These features have aided the determination of imaginal fate maps constructed by Bryant (1975). The imaginal discs also have a number of adepithelial cells that are believed to differentiate as muscle cells (Mandaron, 1973; Ursprung *et al.*, 1972). At the time of pupation the imaginal discs undergo eversion, giving a thin or tubular appendage, while the larval cells are histolysed. The appendages of the fly form from the "pouch" regions of these discs, while the body regions of the different discs fuse.

Due to its large size, and the easy accessibility of the adult wing the wing disc has received much attention. Using the *Minute* technique, García-Bellido *et al.*, (1973) were able to show that several clonal restrictions become apparent in the wing disc during larval development, that progressively subdivide the disc into regions or domains. These clonal restrictions or compartments are believed to derive from groups rather than single cells, and so are termed polyclonal (Crick and Lawrence, 1975). The first compartment boundary to form is the anterior-posterior compartment (García-Bellido *et al.*, 1976; Morata and García-Bellido, 1976) early in embryonic development. During the first larval instar, the dorsoventral and the wing-notum compartment boundaries are established. During the second instar larvae, further boundaries that define the thoracic structures are laid down (García-Bellido *et al.*, 1976), and in the third instar larvae, the boundary that distinguishes the proximodistal axis of the wing is induced. At the end of larval development the imaginal discs have reached their final shape and size, have been compartmentalised, and the disc cells are competent to differentiate cuticular structures during metamorphosis.

Figure 3.3 Fate Map of the *Drosophila* Imaginal Wing Disc



Representation of the dorsal mesothoracic disc fate map. Redrawn from Campuzano and Modolell, (1992).

3.3 Results. X-gal Staining

3.3.1 The Insertion Strain A11

A11 Embryos

Synctial and Cellular Blastoderm Stages (stages 1-5)

There is no evidence of X-gal staining in the synctial and cellular blastoderm stages, or during gastrulation in the A11 P element insertion strain embryos. The pole cells at this stage have no X-gal staining (not shown).

Germ Band Elongation

During early stage 9 of embryogenesis, X-gal staining becomes discernible in a dorsal cluster and a smaller posterior cluster of cells of the procephalon (Plate 3.1A). During this stage the amnioserosa develops and shows staining that is maintained throughout development (Plate 3.1A). Later in stage 9 the staining of the procephalon increases in intensity, while the amnioserosa increases in cell number. As development proceeds, staining in the procephalon encompasses more cells, but does not include the clypeolabrum (Plate 3.1B). As the embryo enters stage 11, staining in a subset of ectodermal cells can be seen developing in each segment (Plate 3.1C). The ectodermal pattern of *lacZ* expression consists of pairs of cells, seen more clearly in Plate 3.1D, and is believed to be the early progenitors of the PNS, and is maintained throughout embryonic development (Plate 3.1E).

The segmentally repeated ectodermal pattern becomes more elaborate as stage 11 proceeds. These cells, believed to be precursors of the dorsal cluster of the PNS, show *lacZ* activity first. Other cells in the embryonic ectoderm thought to be the precursors of the ventral cluster of the PNS then show staining at around the same time as a number of cells along the length of the midline. These cells along the midline show a segmentally repeated pattern that extends into the head (Plate 3.1E).

The amnioserosa can be easily identified during stages 12 and 13, due to the large nuclei expressing *lacZ*. Each abdominal segment has a small dorsal and a small ventral cluster. These clusters are believed to progenitors of the PNS. Here (Plate 3.1F) small clusters of three to four cells can clearly be detected in the epidermis of abdominal segments four, five and six. In the first thoracic segment, the more dorsal clusters consists of two to three cells (Plate 3.9B).

During embryonic stage 14, X-gal staining in the head region can still be detected. There are two clusters of five to six cells just dorsal to the clypeolabrum and there is a third, smaller cluster, just ventral to these two clusters. In addition to a variety of smaller patches, extensive staining in a large, central region of the embryonic head can be seen situated both dorsally and ventrally to the larger central patch. There appear to be two smaller clusters of *lacZ* expressing cells in the maxilla segment. The first thoracic segment has intense staining in two regions located in the posterior segment. The most dorsal patch appears to be of six to seven intensely stained cells, while the second appears as three or four intensely staining cells, arranged in a linear arrangement directly ventral from the first cluster (Plate 3.2A, B).

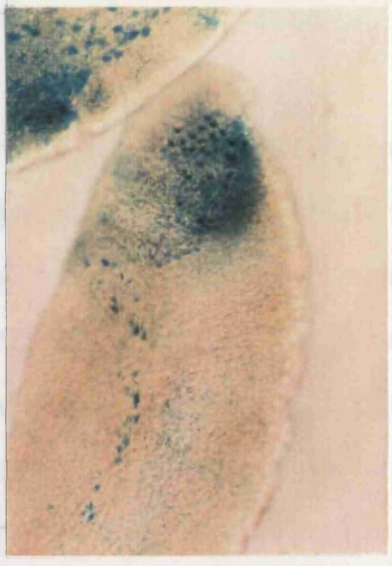
The ventral midline of A11 embryos of stages 12-14 have intensely stained cells forming a four by three array at the segmental furrows in the head (Plate 3.2B), thoracic and abdominal segments. It is not clear, especially in the head segments, but it appears that two rows of this array are located in the anterior of the segment and the third row is in the posterior of neighbouring segment.

Plate 3.1 Legend

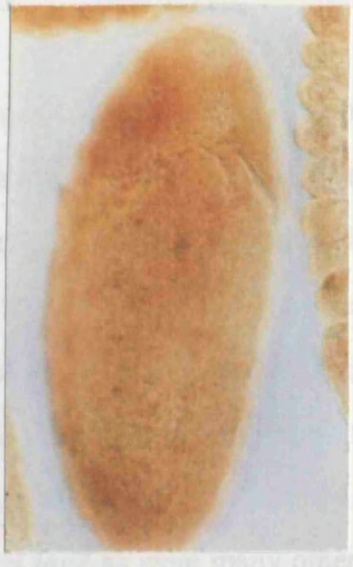
Lateral view of A11 embryos stained for transgenic β-galactosidase activity with X-gal. **A, B** (stage 9), **C** (stage 11), anterior is up, and dorsal is to the right. **A** shows staining in two regions of the head and staining of the amnioserosa is apparent. In **B** staining on the surface of the head is visible. The progenitors of the PNS is beginning in embryo **C. D:** a stage 11 A11 embryo labelled with an anti-β-galactosidase antibody, anterior is up and dorsal is to the right. The peripheral clusters in **D** can be seen to consist of two closely positioned nuclei and a third more ventral nucleus in the abdominal segments. **E:** ventral view of a late stage 11/early stage 12 A11 embryo. Staining of the head and midline is evident. Cells of the developing PNS can be seen in both dorsal and ventral positions. **F:** Dorso-lateral view of the abdominal segments of an A11 stage 13 embryo labelled with an anti-β-galactosidase antibody. The dorsal clusters not consist of four clustered nuclei in the abdominal segments.

Plate 3.1


A


B


C


D


E


F

A11 Larval Tissues

A11 Larval Wing Disc

The A11 imaginal wing disc shows *lacZ* activity in three small regions that correspond to the presumptive hinge region, shown in Plate 3.2C (Bryant, 1975; Campuzano and Modolell, 1992). This staining is seen to be more intense in a slightly older imaginal wing disc (Plate 3.2D). This older disc shows weak staining in that part of the disc that will give rise to the third vein of the wing, in addition to weak staining at the "top" of the disc in a region that will give rise to the pleura of the adult fly.

A11 Eye/Antennal Disc

The eye/antennal disc has a distinct spatial *lacZ* expression pattern, that elaborates as metamorphosis is initiated. Staining is initially detected as two broad bands (Plate 3.2E), one of which corresponds to the developing ommatidia, while the second band of *lacZ* staining appears to be in the presumptive head region. As development continues, the number of cells that express *lacZ* in this imaginal disc increases and becomes more intense (Plate 3.2E, F). The initial expression domains expand and begin to abut each other (Plate 3.2F).

The male gonad disc was found to express *lacZ* as were many other imaginal discs (Plate 3.3.B). For example, the leg imaginal discs all appear to express the reporter gene prior to pupation (not shown).

A11 Larval Brain Lobes

Initially, a dense cluster of staining cells is detected in each brain lobe, possibly the developing medulla, in addition to a few scattered towards the "midline" (Plate 3.2E). The staining of these cells becomes clustered in dense regions as development proceeds (Plates 3.2F, 3.3A), extending from the anterior to the posterior of each lobe. Increased numbers of cells express *lacZ* in these dense

clusters. During this period, another dense cluster of cells is detected on the surface at the anterior of each lobe (Plate 3.2F) and on a different focal plane from the initially detected cluster. This may correspond to the antennal nerve. The ventral ganglia of A11 in third instar larvae, initially in a central region that may be analogous to the embryonic midline show *lacZ* expression, and later in scattered cells throughout the ventral ganglia (Plates 3.2E, F, 3.3A). This staining shows major difference between thoracic and abdominal regions.

A11 Pupae

Reporter gene activity is clearly detected in the developing ommatidia, before pigmentation is evident (Plate 3.3C, D), and in the developing ocelli (Plate 3.3D). In the extended legs *lacZ* expression can be detected in the pretarsus and at each of the leg joints (Plate 3.3C). On the head between the eyes there is strong staining in two circular structures that remain unidentified (Plate 3.3C). There is also strong staining in the region of the thorax where the haltere has developed and at the region of the wing/thorax boundary forming a braid-like pattern (Plate 3.3D).

Later in pupal development, as the eye gains pigment, the staining in the thorax becomes more refined. The posterior and anterior scutellar bristles, the supraalar bristles and the dorsocentral bristles all show specific *lacZ* staining, as does the scutellum ridge (posterior line) and possibly the notopleural setae (Plate 3.3F). The wing base and the wing margin exhibit *lacZ* activity as do small clusters of cells on the pupal legs (Plate 3.3E).

A11 Adults

Whole Mounts

There is a cluster of *lacZ* expressing cells on the femur of the prothoracic leg, and on the trochanter or the coxa of the metathoracic and mesothoracic legs, while a patch on the notal wing process and two cells above the wing on the dorsal thorax itself express *lacZ*. Two cells located just above the wing, possibly the supraalar

Plate 3.2 Legend

A, B are stage 14 A11 embryos stained for *lacZ* expression with X-gal. **A:** lateral view of an embryo, where X-gal staining is evident in the head segments, and in the first thoracic segment. The staining posterior to the clypeolabrum may be the antenno-maxillary organ. **B:** in this ventro-lateral view, staining of cells covering the ventral nerve cord is detected. **C, D, E,** and **F** are tissues dissected from wandering third instar imaginal tissues and stained for *lacZ* expression with X-gal. In **C**, *lacZ* expression is detected in three clusters of the imaginal wing disc, that begin to merge in the wing disc shown in **D**. In **E**, expression of *lacZ* in the larval brain lobes and progenitors of the ommatidia of the adult eye and ventral nerve cord is seen, while in **F**, the expression is seen to include many more cells in the brain lobes and ventral nerve cord of a slightly older larva.

Plate 3.2

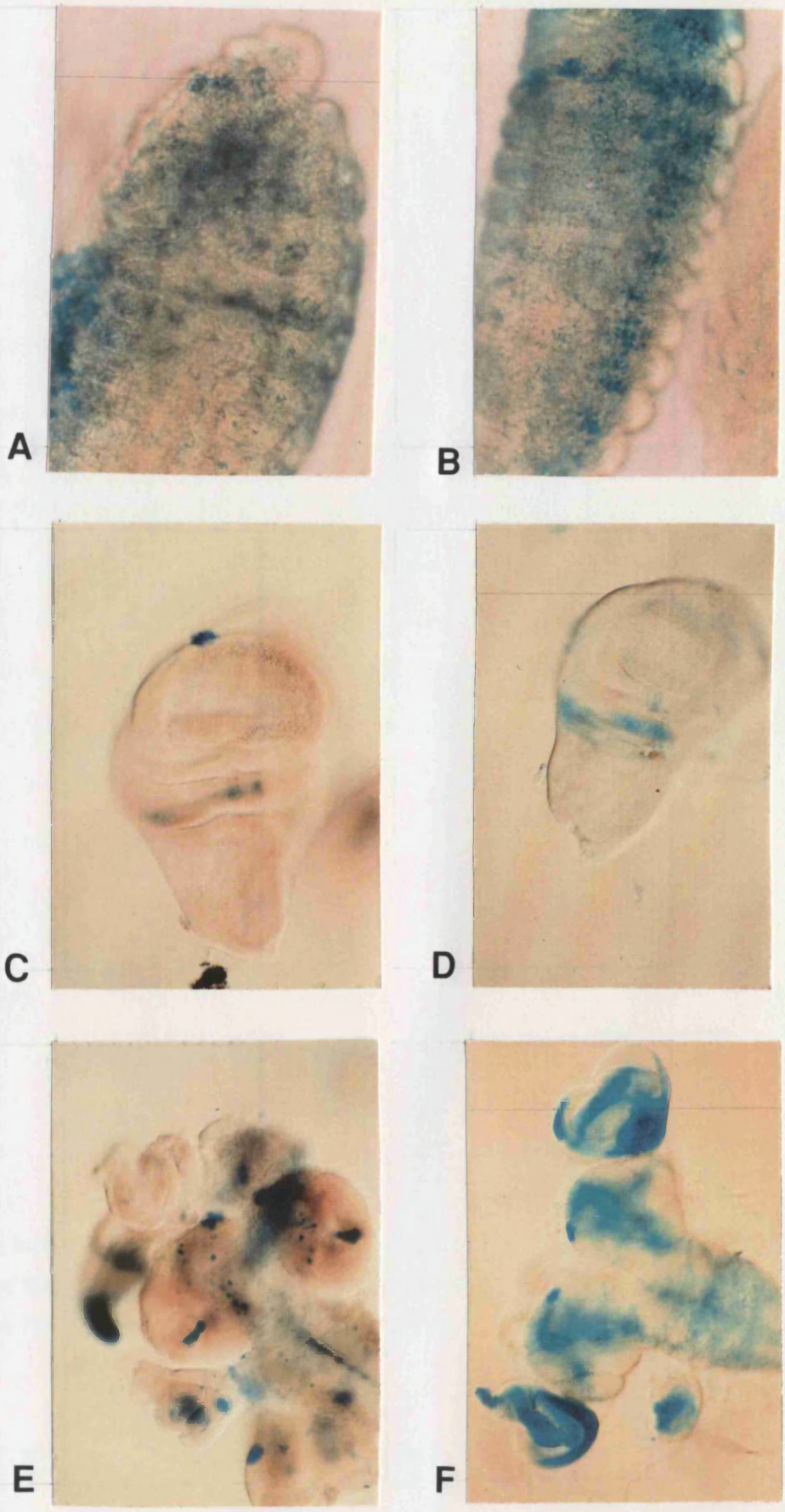
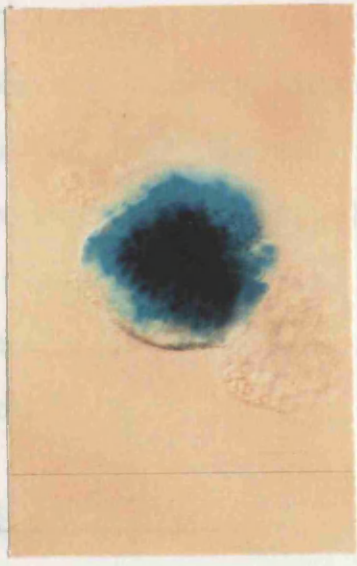


Plate 3.3 Legend

A11 X-gal staining of A11 larval tissues and pupae. **A:** brain lobes and ventral ganglia of wandering instar A11 larva. **B:** A11 male gonad disc dissected from a wandering third instar larva. **C:** ventral view and **D:** dorsal view of an A11 pupa of stage P5ii-P6. **E:** ventral, and **F:** dorsal view of an A11 pupa of stage P10-14.

Plate 3.3

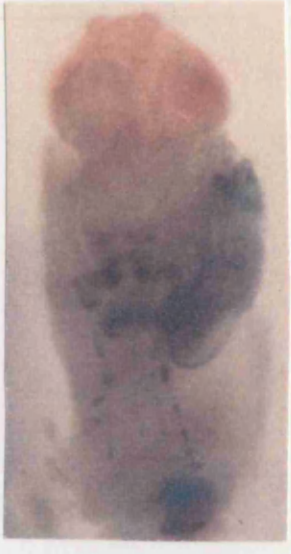
A



B

C

D



E

F

bristles, and a cluster of two to three cells just dorsal to the haltere express *lacZ*. The humeral region has a cluster of three to four cells, and the notopleurals exhibit *lacZ* expression. On the scutellum, there is a single intensely staining cell located on the posterior rim, midway between the posterior scutellars. On the abdominal segments no X-gal staining, other than two clusters on the first and second abdominal segments, situated ventrolaterally were detected (not shown). On the head, there are two to three cells on the cuticle that rims the ommatidia (the ptilinal ridge), and there is occasional, weak staining in the region of the ocelli.

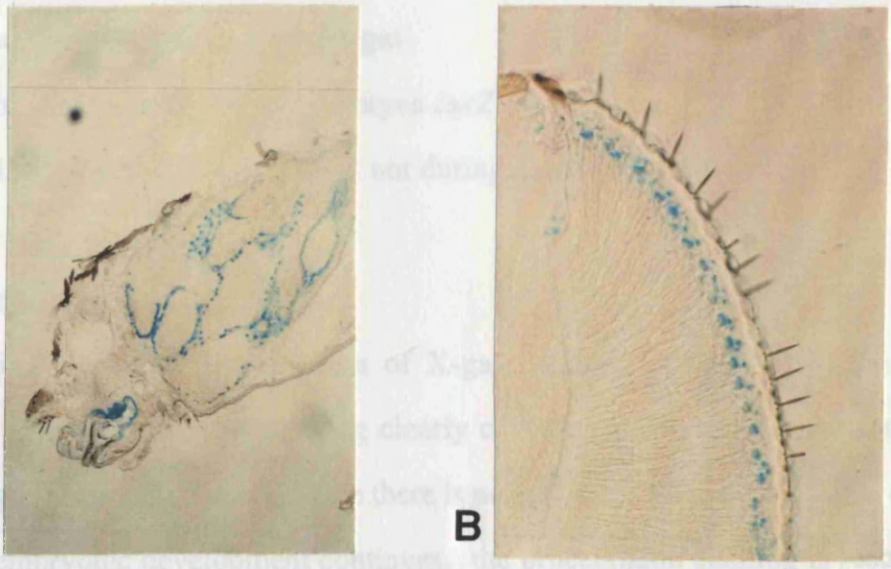
A11 Adult Cryostat Sections

These sections show A11 females have staining in the follicle cells (Plate 3.4A), thought to be of stage 9 (M. Todman, Sussex, pers. comm.). *lacZ* activity is apparent in a distinct, repeating pattern in the ommatidia of the adult eye in cells that have been tentatively identified as a subset of cone cells (Plate 3.4B). In addition to the staining in the eye, there is also staining in the adult brain cortex (not shown).

Plate 3.4 Legend

A: Adult A11 female cryostat section of abdomen, showing follicle cells, and, **B:** A11 adult head cryostat section showing eye detail.

Plate 3.4



3.3.2 The Insertion Strain A22

A22 Embryos

Synctial and Cellular Blastoderm Stages

In the A22 insertion strain embryos *lacZ* activity is not detected during the synctial and cellular blastoderm stages, nor during gastrulation (Plate 3.5A).

Germ Band Elongation

During stage 9 a large region of X-gal staining is detected in the A22 embryonic procephalon. This staining clearly consists of two expression domains that converge (Plate 3.5B). At this time there is no staining in the amnioserosa.

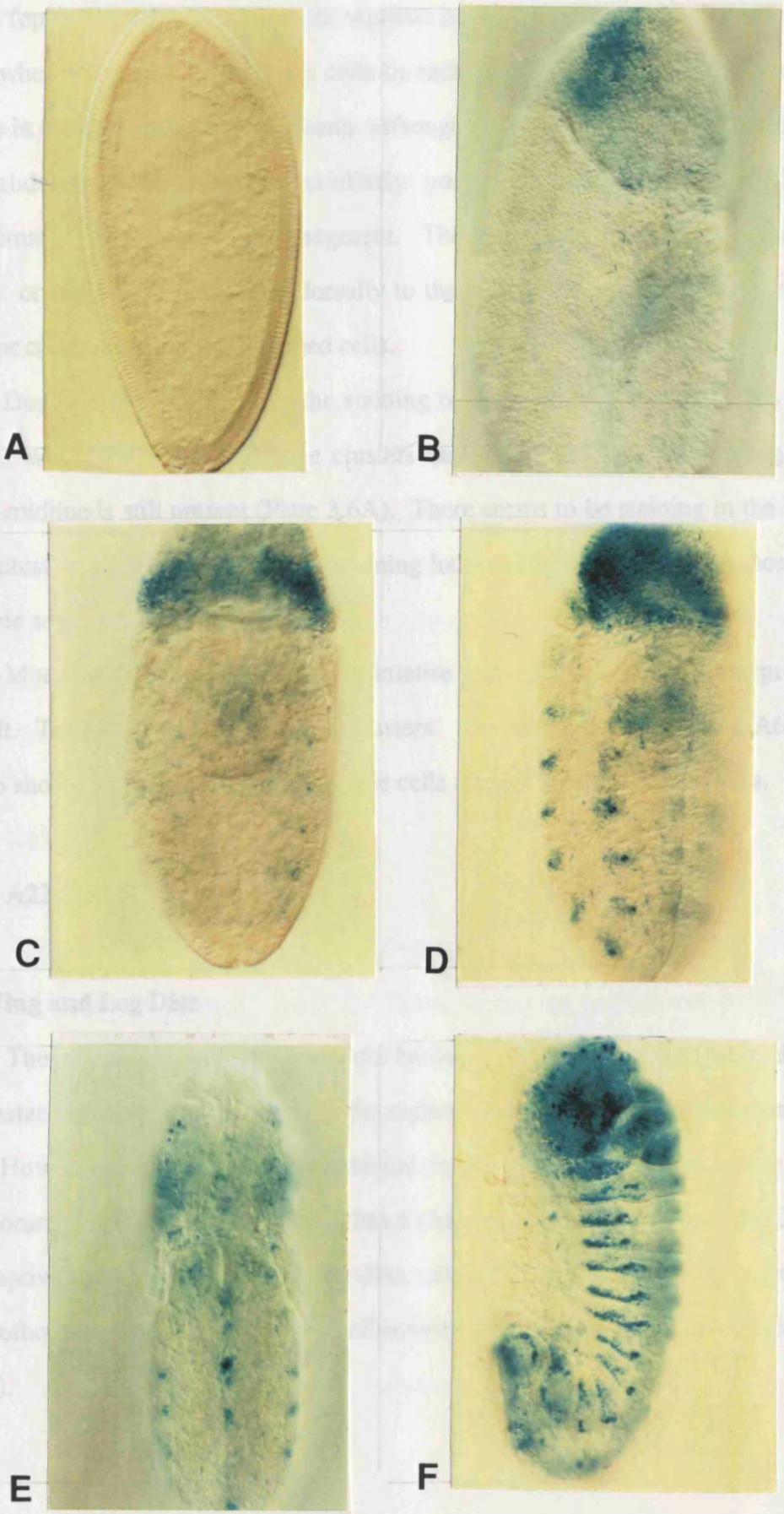
As embryonic development continues, the procephalon staining is restricted posteriorly. Individual cells can be seen at the cephalic furrow (not shown) and in the ectoderm of stage 11 embryos that express *lacZ* (Plate 3.5C). Staining in the developing PNS, possibly the dorsal cluster, can be detected as two cells per cluster (Plate 3.5D). As stage 11 proceeds, but before stage 12 begins, staining begins to become apparent in the mesectoderm and in discrete cells in the dorso-ventral procephalon (Plate 3.5E). As the development of A22 embryos proceeds from stage 11 to stage 12 staining in the ventral midline, particularly in the head regions becomes intense, while staining in the PNS is maintained.

Intense, diffuse staining is found in the first three segments (mandible, maxilla, labium) in addition to individual cells of the head surface (epithelia) and diffuse staining of the dorsal ridge in stage 12-13 embryos (Plate 3.5F).

Plate 3.5 Legend

A22 embryos stained for transgenic *lacZ* activity with X-gal. Anterior is up. **A:** a cellular blastoderm embryo with no evidence of X-gal staining, **B:** lateral view of an embryo of stage 9 with X-gal staining in the ventral regions of the head, **C:** dorsal view of a stage 11 A22 embryo exhibiting X-gal staining in the head and progenitor cells of the PNS, **D:** dorso-later view of an embryo (stage 11) expressing *lacZ* in the head and pairs of progenitor PNS cells in each segment, **E:** dorsal view of an A22 embryo (stage 11), **F:** dorso-lateral view of a stage 12-13 embryo expressing *lacZ* in the head and clusters of cells in each segment.

Plate 3.5



Each thoracic and abdominal segment has strong staining in the last posterior column of cells (epithelia). The first thoracic segment has two adjoining clusters of staining cells, where there are at least two cells in each cluster. Similarly, there are two clusters in the second thoracic segment, although these are positioned more dorsally. Each abdominal segment has similarly positioned clusters, consisting of approximately four cells in each segment. The thoracic segments have a smaller cluster, or single cell positioned dorsally to the double cluster, and anterior to the posterior column of positively stained cells.

During stages 12 and 13, the staining in the head region of the CNS is still intense, while the number of these clusters has increased, and the staining in the ventral midline is still present (Plate 3.6A). There seems to be staining in the ventral (or v') clusters of the PNS and dense staining located laterally, on the surface of the mandible segment.

Much of the midline staining is intense and diffuse, making interpretation difficult. They seem to be of 2-4 cell clusters. However, in segment A5/A6 of the embryo shown in Plate 3.6A and 3.6B, the cells are in a single row of 6 cells.

A22 Larval Tissues

A22 Wing and Leg Disc

The only X-gal activity that could be detected in the imaginal wing discs of third instar larvae was a single cell in the region of the presumptive third vein (Plate 3.7). However, *lacZ* staining is detected in the presumptive limb tissue of the metathoracic leg disc and this staining has a clear "mirror" pattern (Plate 3.6C). The presumptive thoracic tissue in this leg disc, shows no evidence of *lacZ* expression. The prothoracic leg disc also shows *lacZ* activity in the presumptive limb region (not shown).

A22 Larval CNS

The larval brain hemispheres show intense staining almost completely restricted to the proximal half, since in the region of optic ganglia there is only one or two positive cells on the surface. The ventral ganglia have many positive cells arranged in horizontal clusters throughout the abdominal and thoracic neuromeres located on many focal planes (Plate 3.6D).

A22 Pupae (not shown)

The pupal head shows evidence of *lacZ* activity within the developing brain (stage P5(ii) to P6), while head structures that are either the antennae or the ptilin sac also show *lacZ* expression. The developing legs of these pupae exhibit *lacZ* expression in many cells that are located internally along the lengths of the legs, and diffuse staining is evident on the thorax at the source of the developing wing. The wing staining however, was not consistently detected, but was never present in wild type Oregon-R (OR-R) negative controls.

A22 Adults

Whole Mount (not shown)

Three cells oriented along a dorsal/ventral axis on the mesopleura exhibit *lacZ* expression, as does a small cluster of cells on the notal region just dorsal to the haltere, and a single cell of the ventral mesothoracic leg femur. There are a few scattered cells that express *lacZ* on the head between the eyes and proboscis.

A22 Adult Sections

The A22 adult head shows clear staining in the supraoesophageal and suboesphageal ganglia (Plate 3.6E). There is no evidence of *lacZ* expression in any other tissues, other than the endogenous enzyme expressed in the gut.

Plate 3.6 Legend

A: dorso-lateral view of a stage 13-14 A22 embryo exhibiting segmentally repeated *lacZ* expression in the PNS and midline cells of the CNS, and **B:** on higher magnification, **C:** an imaginal leg disc dissected from a wandering third instar A22 larva, and **D:** imaginal third instar brain lobes and ventral ganglia, **E:** adult cryostat section of an adult A22 head.

Plate 3.6

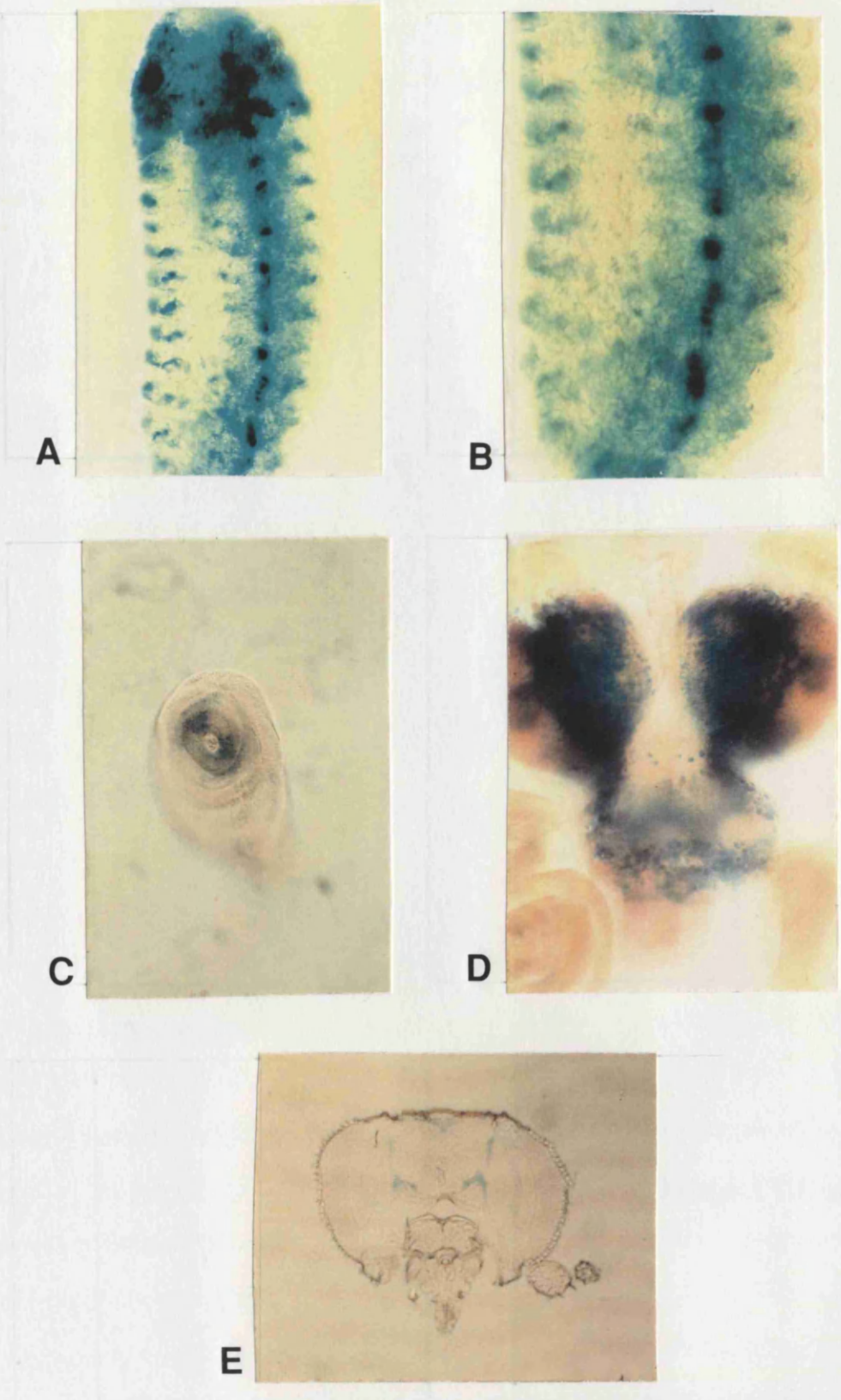
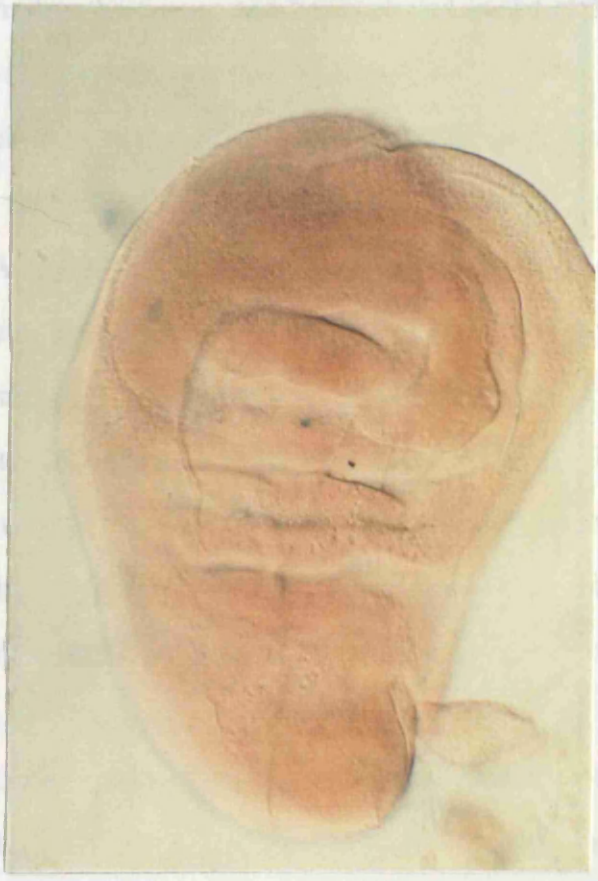


Plate 3.7 Legend

A mesothoracic imaginal wing disc dissected from an A22 wandering instar larva.

Plate 3.7



3.4 Antibody Labelling of A11 and A22 Embryos

The X-gal staining technique has its limitations since the products produced by β-galactosidase activity tend to diffuse. As a consequence the analysis of the staining patterns will be especially difficult if the β-gal expression level is high, or many neighbouring cells express the enzyme. This can be overcome in a variety of ways. For example, the tissues can be incubated in the staining mix at a reduced temperature, or for less time or both.

The precise identification of those embryonic cells that express *lacZ*, or any other marker on the basis of cellular morphology is difficult since the embryo is small and compact. A second difficulty is the nuclear localisation of the transgenic *lacZ*. The characteristic cytoplasmic extensions formed by neurons are therefore not labelled, preventing identification of cells on the basis of morphology and specific cell-cell contacts. A battery of antibodies that specifically bind cell type specific epitopes can be used to assist identification. Such antibodies may prove useful if they label those cells, or subsets of cells that express *lacZ* also, or they may label neighbouring cells, and so act as sign posts, allowing identification of cells as judged by their relative positions.

Initially, the embryos were double labelled with anti-β-gal and anti-horse radish peroxidase (HRP) antibodies. The anti-HRP antibody recognises a carbohydrate epitope expressed on all neurons and their axons after germ band retraction (Jan and Jan, 1982). MAb 22C10, an antibody that recognises an epitope present in the cytoplasm of a subset of neurons (Zipursky *et al.*, 1984), was a kind gift from N. Patel. The use of this antibody was intended to aid the identification of those embryonic cells that express the reporter gene, and perhaps identify any subtle phenotypes in the embryonic nervous system as a result of the P element insertion. However, the fluorescently labelled anti-β-galactosidease antibody was of poor quality, and subsequently these double-labbelled preparations gave little information,

other than the insertion strains A11 and A22 had no obvious defects in the axon pathways labelled by MAb 22C10 (data not shown).

3.4.1 Double Antibody Labelling of A11 Embryos

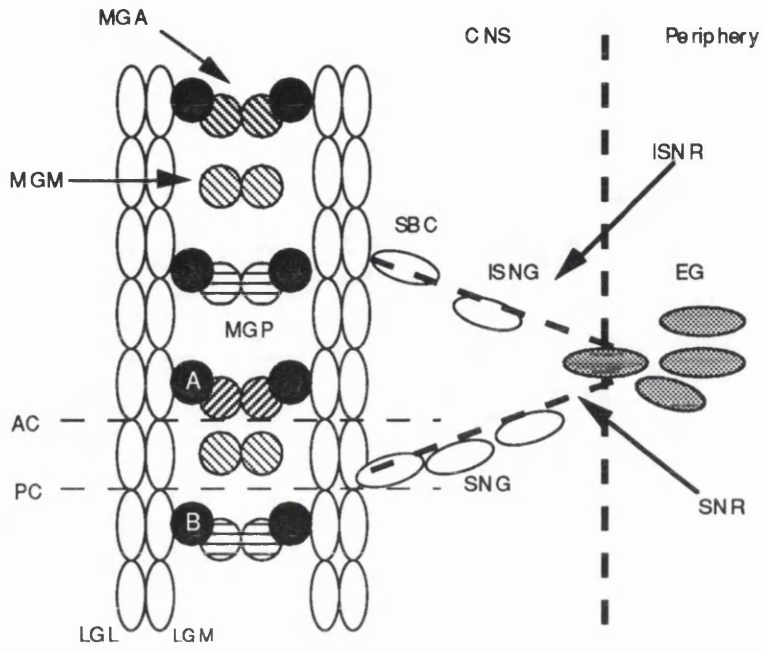
In A11 embryos where the ventral nerve cord has developed (stage 14-15), anti-HRP antibodies label the neural axons that are densely packed in a distinctive ladder-like pattern. Cells that are labelled by the anti-β-gal antibody can be seen lying directly on top of both the anterior and posterior commissures in each segment (Plate 3.8A).

In each embryonic segment, there is a cluster of four cells where the segmental and intersegmental nerves emerge from the CNS and enter the periphery. In each of these clusters, two or three of these cells express *lacZ*. The intersegmental and segmental nerves merge in this area, and travel dorsally, just anterior to this "delta" cluster of *lacZ* positive cells. Notably, there is an anterior cell in this cluster that does not express *lacZ*. (Plate 3.8B).

The embryonic brain of A11 is labelled by both the anti-β-gal and anti-HRP antibodies (not shown), and pairs of cells located underneath the epithelia express *lacZ*. These pairs of cells are likely to be sensory organ cells. High concentrations of the anti-HRP antibody gives considerable background and consequently the lower concentrations of this antibody used does not strongly label the PNS cells.

The column of *lacZ* expressing cells in the first thoracic segments detected by X-gal staining is confirmed by the anti-β-gal antibody preparations. Furthermore, there are clear segmental differences. Each abdominal segment during stage 13 has *lacZ* expressing cells, probably of the dorsal PNS cluster (Plate 3.9B). However the second abdominal segment has a third *lacZ* expressing cell, just dorsal to the pair of cells found in all the other abdominal segments. Later, as dorsal closure completes *lacZ* expression is detected in the dorsal and ventral clusters of the PNS (Plate 3.9A).

Figure 3.4 The Embryonic CNS and Exit Junction Glia



The glial cells of the embryonic ventral nerve cord are presented. **A**, **B**: large glia cells, AC: anterior commissure, PC: posterior commissure, LGL: longitudinal glia (lateral), LGM: longitudinal glia (medial), MGA: midline glia (anterior), MGM: midline glia (medial), MGP: midline glia (posterior), SBC; segmental boundary cell, SNG: segmental nerve root glia, ISNG: intersegmental nerve root glia, SNR: segmental nerve root, ISNR: intersegmental nerve root, EG: exit glia. Redrawn from Goodman and Doe, (1993).

Plate 3.8 Legend

Ventral view of A11 embryos (anterior to the top), labelled with both an anti-HRP antibody (brown) and an anti-β-galactosidase antibody (grey). **A**: anti-β-galactosidase labelled cells located above the anterior and posterior commissures of the CNS, **B**: anti-β-galactosidase labelled cells located out with the CNS, at the exit junction. Note the anti-HRP labelled neurons (brown). See text for details.

Plate 3.8

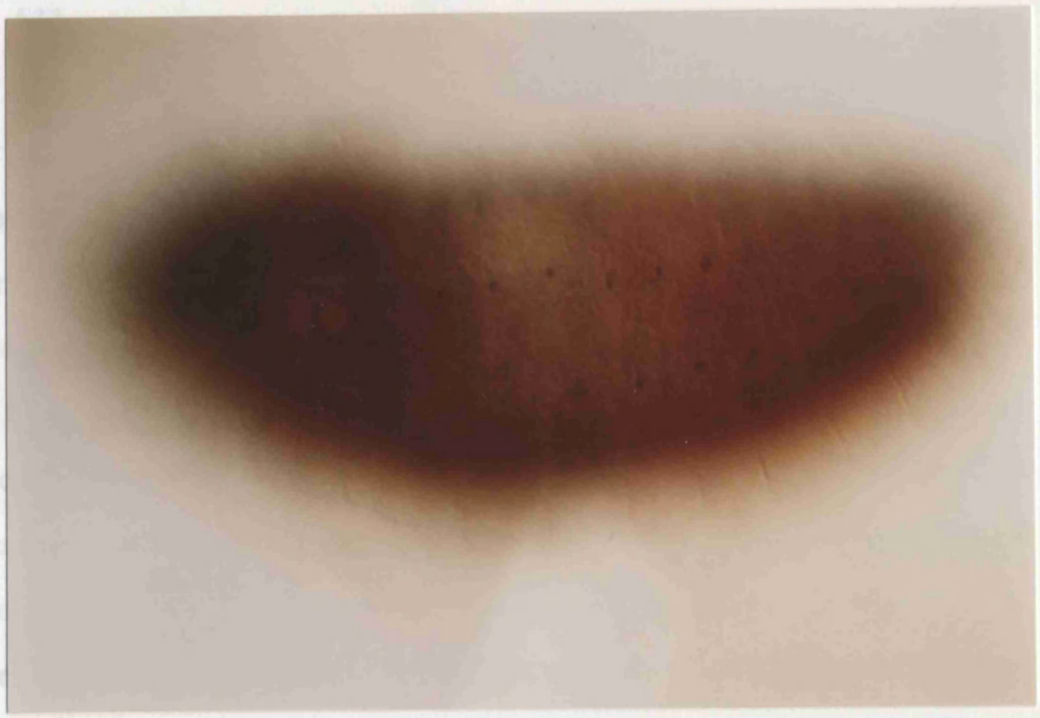
A

B

Plate 3.9 Legend

A: anti-β-galactosidase labelled cells in the PNS in an embryo where dorsal closure is almost complete, **B:** lateral view of thoracic and abdominal segments from an A11 stage 13 embryo. *lacZ* expressing cells are labelled (see text for details). Note the amnioserosa cells just out of focus.

Plate 3.9



A

B

3.5 Discussion

A11

lacZ expression in A11 embryos is initially detected in the head region, and in a segmentally repeated pattern of ectodermal cells at the time germ band elongation has completed. The expression in the head region appears to occur in mitotic domain δ_{145} as described by Foe (1989), believed to give rise to the pharynx roof and the dorsal surface of the head that carries both the dorso-medial papilla of the maxillary sense organ and the antennal sense organ (Foe, 1989; Jurgens *et al.*, 1986). The ectodermal cells that express the reporter gene as the embryos enter stage 11 are believed to be the dorsal cluster progenitors of the PNS. Later, ectodermal cells, believed to be progenitors of the ventral cluster of the PNS express *lacZ*. Each thoracic and abdominal segment has the same pattern of *lacZ* positive cells with one exception. The third thoracic segment has three cells rather than the two cells seen in the first and second thoracic and all nine abdominal segments (Plate 3.9B).

The progenitor cells of the amnioserosa span the length of the germ band before elongation has begun. This sheet of columnar cells becomes buckled by germ band elongation, and appears as a narrow strip on either side of the embryo. At this stage, the amnioserosa cells begin to change in morphology from columnar to squamous (Foe, 1989). The amnioserosa cells of A11 embryos initiate *lacZ* expression at this time, and this expression is maintained thereafter.

The double labelling antibody experiments using anti-β-gal and anti-HRP have allowed the identification of a subset of exit glia cells as expressing *lacZ*, and confirmed the expression of *lacZ* in cells located above the anterior and posterior commissures of the CNS (compare Plate 3.2B and Plate 3.8A, Figure 3.4).

It is equally important to note the embryonic cell types and structures that do not express the reporter gene. The pole cells, muscle (derived from the mesoderm), embryonic gut (derived from both the endoderm and the ectoderm) and the tracheal

system (derived from the dorsal ectoderm) do not express *lacZ*. Thus there is a fundamental restriction, not only of cell type, but of germ layer origin of those cells that do and do not express *lacZ*.

Wandering and immobile third instar larvae were dissected, allowing characterisation of *lacZ* activity in the imaginal tissues. *lacZ* expression in the larval brain was found to be dynamic in the A11 insertion strain. In the central brain lobes and the ventral nerve cord, increasing numbers of cells are found to express *lacZ* in specific regions as the larvae age. There are two large clusters of *lacZ* positive cells, the lateral of which may be the developing medulla, that later fuse and increase in size as development proceeds giving a cluster with an almost "pronged" structure, on each hemisphere that remain unidentified. There is a smaller cluster of *lacZ* expressing cells on each brain hemisphere that may be the developing antennal nerve, while the optic lamina regions of the larval brain lobes exhibit no *lacZ* expression. The larval CNS of A11 clearly exhibits clear changes in expression of *lacZ* in the brain lobes as metamorphosis approaches. These changes are difficult to compare with expression in the adult brain, due to the complex remodelling during pupation, compactness, complexity, and the limited anatomical information available. The LacZ reporter protein has a nuclear localisation signal. This prevents the identification of the axons and their target cells, and so any changes in fasciculation that may occur during pupation are not obvious.

The larval ventral nerve cord has *lacZ* expression in two longitudinal, bilateral stripes and a third central stripe that occupies a midline position. There are many other *lacZ* expressing cells scattered throughout the thoracic and abdominal neuromeres, and as the larvae near metamorphosis increasing numbers of cells express *lacZ*. This observed increase in the number of the *lacZ* expressing cells in the ventral nerve cord, especially in the abdominal neuromeres is notable since neurogenesis in the abdominal neuromeres occurs between 50 and 90 hours after hatching, and ceases before the onset of metamorphosis. This is the period when the larvae leave the semi-liquid food. The thoracic neuromeres continue neurogenesis up

to 30 hours after puparium formation (Truman *et al.*, 1993; Truman, 1990). This suggests that *lacZ* expression is not activated immediately following mitosis, but is activated in these cells over a relatively long time period. Alternatively, these cells may not be neural.

lacZ expression in the developing eye is first detected in the eye/antennal disc, located mainly, although not exclusively, in the developing ommatidia. As can be seen in the pupae and the adult cryostat sections, the expression in the ommatidia is maintained throughout pupal development. The ocelli, derived from the posterior half of the eye/antennal disc, as are the ommatidia, show *lacZ* expression in pupae (P10-P11) and in the adult. This activity may be located in the light-sensing ocelli structures, the two large ocellar bristles, or the six smaller interocellar bristles. The staining detected in the larval brain and eye/antennal imaginal disc is consistent with the staining in the outer adult eye, the *lacZ* expression detected in the adult brain, and with the expression of the reporter gene in the embryonic CNS.

The dorsal mesothoracic discs of A11 third instar larvae exhibit spatially and temporally regulated expression of *lacZ*. The expression of *lacZ* in the presumptive hinge region (Bryant, 1975; Campuzano and Modolell, 1992) is maintained and elaborated during pupal development (Plate 3.2A, Plate 3.2B, see Figure 3.3). The pupal wing margin expresses *lacZ* although no staining is apparent in the presumptive wing margin of the imaginal disc. This indicates that although *lacZ* in the A11 strain is expressed in these sensory organs, *lacZ* is not expressed in the proneural cluster or the sensory mother cell at the time of its selection, as defined by the time of expression of the proneural genes *ac* and *sc* (Romani *et al.*, 1989) and by the expression of *lacZ* in the enhancer trap strain A37 (Ghysen and O'Kane, 1989). The progenitor cells of the major notal macrochaete do not express *lacZ* in either the imaginal wing disc of L3 or during the pupal stage P5(ii) to P6, or in the adult macrochaete. However, *lacZ* is expressed at the sites where these sensory organs have developed during the pupal stages P10 to P11. This *lacZ* expression in A11 is detected after the terminal differentiation of these organs, a process known to occur

for up to forty hours after puparium formation (Hartenstein and Posakony, 1989). Expression in these sensory organs was detected in only two or three adults implying the trapped enhancer may be temporally regulated or indicative of some other variation in the activity of the enhancer. Alternatively, X-gal staining of whole animals may less reliable than desired.

The expression in the leg imaginal discs (not shown) is elaborated and maintained throughout the pupal period. The legs of *Drosophila* are rich in sensory organs, while the pupae exhibit *lacZ* expression in a subset of sensory organs. Very few cells were found to express the reporter gene in the whole mount preparations were detected. However, this may not accurately reflect enhancer trap expression as the fly is relatively large and the cuticle impermeable, so the X-gal and other components may not have reached all the sensory organs in the leg.

Adult females of the insertion strain A11 have been found to express *lacZ* in specific abdominal cells. These cells have been identified as stage 9 follicle cells of the ovary (M. Todman, Sussex, pers. comm.). The follicle cells are derived from the mesoderm while the nurse cells and the oocyte are derived from the germ line (Bownes, 1982). These somatic cells have been found to play an important role in embryogenesis by providing the informational cues for the establishment of the dorsoventral polarity (Schüpbach, 1987). A requirement for *Notch* in the somatic follicle cells has been demonstrated (Ruohola *et al.*, 1991), since LOF (loss of function) mutations in this gene lead to an excess of posterior follicle cells similar to the embryonic neural hyperplasia exhibited by *Notch* mutants and to mislocalisation of the *bicoid* transcript, disrupting the initial embryonic pattern. Other genes have been found to play a major role in the development of the nervous system, for example *cut* (Blochlinger *et al.*, 1993), are also expressed in the follicle cells, indicating such genes direct the development of at least more than one cell type. *lacZ* expression has not been detected in early embryos during the time when the maternal gene products are active. However the transgenic *lacZ* transcript will not be actively transported into the developing oocyte (since it does not have the appropriate signal

sequences), while the transgenic protein has a nuclear localisation signal, but not a signal permitting transport from the follicle cells to the oocyte. This precludes any judgement as to the location and function of the downstream gene product in the follicle and the oocyte.

Male larvae of this insertion strain exhibit *lacZ* expression in gonad, indicating the trapped enhancer may be active in the development of the male reproductive system, and is interesting in the light of *lacZ* activity in the follicle cells of the adult ovary. The smaller female larval gonad has not been examined for expression of the reporter gene.

The expression of *lacZ* in the embryonic and adult central and peripheral nervous systems, and the ovary raises the possibility that the trapped enhancer of the A11 strain may be required for the activity of a novel gene that is expressed mainly in the developing PNS and CNS.

A22

In the A22 embryos *lacZ* expression is detected in the head region that apparently corresponds to mitotic domains $\delta_{14}1$ and $\delta_{14}3$ described by Foe (1989) that merge and give rise to the labral sense organs, or to domain $\delta_{14}9$ that gives rise to cells that remain on the surface of the procephalon and central brain neuroblasts. *lacZ* expression is also apparent in a double row of cells just anterior to the cephalic furrow, corresponding to mitotic domain $\delta_{14}20$, the dorsal ridge. This double row of cells may be expressing a background pattern of *lacZ* in response to regulatory sequences within the P element construct (O'Kane and Gehring, 1987; Bellen *et al.*, 1989). As embryogenesis proceeds, the staining in the head segments becomes intense and diffuse while columns of epithelial *lacZ* expressing cells in the posterior of each thoracic and abdominal segment becomes discernible. Like the staining located just anterior to the cephalic furrow, this staining may be due 'background' activation of the reporter gene. The germ band extended embryo has *lacZ* expression

in the midline cells that is maintained throughout embryogenesis, developing as a single row of cells that runs the length of the ventral nerve cord into the head region. Reporter gene expression in these cells may indicate the gene regulated by the trapped enhancer plays an important role in the architecture of the CNS.

In the thoracic segments there is clear staining in clusters of cells that are located near to the stripes of epithelial cells believed to express background levels of *lacZ*. These clusters of cells are tentatively identified, as judged by their position, to be cells of the chordotonal organ dch3, while in the abdominal segments the *lacZ* positive cells are tentatively identified as subsets of cells from the dorsal group neurons. The sensory organ dch3 consists of both internal chordotonal organs and external sensory organs while the dorsal cluster of the abdominal segments have no associated chordotonal organs (Jan and Jan, 1993).

In third instar wandering larvae, there is a spatially restricted *lacZ* expression pattern in the brain hemispheres. This staining encompasses almost all of the medial half of each lobe, but does not include the presumptive lamina. This staining is seen to occur in more cells as the larvae approach puparium formation. Expression of *lacZ* in the larval brain hemispheres is consistent with that of the adult brain. In the larval ventral nerve cord there are many nuclei that express the reporter protein, seen as scattered nuclei in wandering third instar larvae. In slightly older larvae, this pattern of expression seems to encompass approximately eight rows of cells on one focal plane, while anteriorly in a deeper focal plane another eight rows of cells approximately express *lacZ*, creating a "three-dimensional segmental pattern".

The imaginal ventral thoracic discs of A22 larvae express *lacZ* in the central pouches of the discs that will give rise to the legs, consistent with the expression seen in the pupal legs. In the dorsal mesothoracic disc a single cell in the region of the presumptive third wing vein expresses *lacZ*, casting further doubt on the inconsistent *lacZ* expression in the region of the wing hinge in pupae.

In summary, during A22 development, the expression of *lacZ* is restricted to specific cell types in the CNS and PNS, originating from the ectodermal germ layer,

while there is no expression in muscle cells or their progenitors, the gut or the reproductive system. Importantly, there are no known genes that are located within the vicinity of the P element insertion site, that have a similar expression pattern to that of the reporter gene. This enhancer trap line, therefore appears to be a useful candidate strain for the isolation of a novel gene that is required in the development of the CNS and PNS.

3.6 Future Work

Identification of the embryonic cells that express *lacZ* has been based mainly on their position and the age of the embryo and correlated with the known embryonic developmental events (Bate and Martínez-Arias, 1993). There are a variety of approaches that can be used to aid the identification of specific cells in *Drosophila.* Genetic approaches would be a valuable approach in the context of this work. For example, by constructing an embryo that carries the P element construct of interest and a mutation such as *atonal*, and assaying the expression of *lacZ,* it may be possible to discern which of the PNS organs express *lacZ*, since if the *lacZ* expressing cells are deleted, then they belong to the group of cells that require the activity of *atonal* for their development (Jarman *et al.*, 1993). Similarly, the construction of embryos that carry both the P element insertion of interest and the A37 chromosome should aid in the identity of the cells that express the reporter gene in response to the P element insertion being studied. If the embryos carrying both constructs express *lacZ* in identical cells, then it is certain that the cells are all PNS precursors. However if the *lacZ* expression patterns do not overlap, then the cells that express *lacZ* under the direction of the new construct are not neural. Alternatively, X-gal stained tissues may be non-isotopically probed using a previously cloned and characterised gene such as *achaete* or *scute* in *in situ* hybridisation experiments. Those cells labelled by both markers may then be conclusively identified.

The use of cell type specific antibodies, when available, with *lacZ* expression assays could also aid the identification and cell lineage analysis of the enhancer trap strains enormously. For example, the A11 embryo has been found to have *lacZ* expressing cells that lie directly above the ventral nerve cord and may be glial cells. A glial-specific antibody would be valuable in confirming, or discounting this tentative identification.

Chapter 4

Excision Mutagenesis of the A22 P Element Insert

4.1 Introduction

In order to demonstrate gene function, and indicate the biological processes in which it is involved, a mutation in the gene is required. As the lines A11 and A22 show no detectable mutant phenotype as a consequence of the P element insertion, then it is desirable to induce one. Therefore, as part of this project, a mutation was sought in the A22 line. Such a mutation can be obtained by imprecisely excising the P element in such a way as to delete part or all of the "trapped" gene. The A22 line has been chosen for mutagenesis, since it shows a simpler *lacZ* expression pattern, not only in the embryo, but also in the adult, suggesting the derived mutants should have a restricted number of defects, simplifying their analyses.

4.2 A22 Mutagenesis

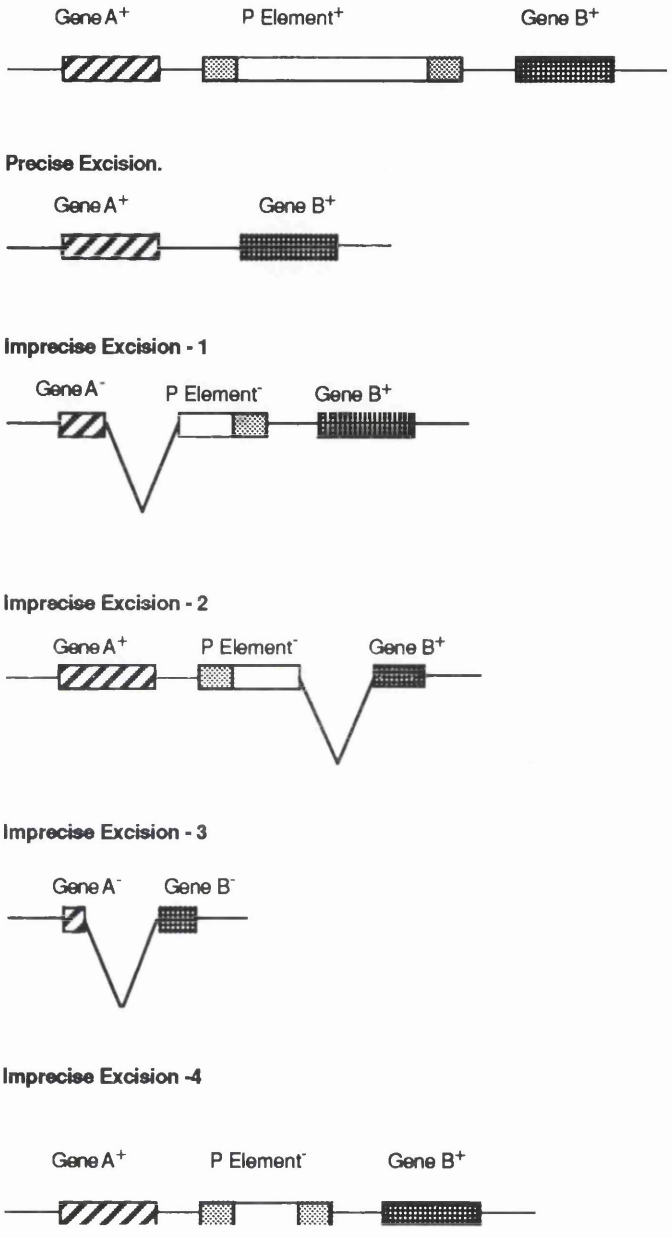
The line A22 has a single P element construct inserted on the third chromosome. It has been cytologically localised to position 72B-C on the salivary gland polytene chromosomes (E. Zador., unpublished data). This line has no obvious phenotype brought about by the P element insertion. Additionally, there are no identified genes at this position of the chromosome, which have been shown to be active in the nervous system of the embryo or in the brain of the adult fly (Lindsley and Zimm, 1992). This raises the possibility that the *lacZ* expression pattern seen in this line is symbolic of a previously unidentified gene.

A simple, standard "Jump Out" strategy was used in an attempt to create a mutation in the trapped gene of A22 by inducing the P element construct to excise. Any excision events generated in the experiment may be internal deletions, precise excisions, or imprecise excisions that cause the deletion of a stretch of flanking sequence either at the 5' or the 3' end, or at both ends, of the element as illustrated in Figure. 4.1 (Engels, 1983). Since the P element is marked with the wild type allele of the xanthine dehydrogenase gene, *rosy*, and the flies are homozygous for the

endogenous *rosy*^{506} allele, the excision events can be selected for by the loss of wild type eye colour. The progeny could also be scored for the loss of *lacZ* expression but this is more expensive and time consuming.

Since the P element of A22 has had the transposase gene deleted, it is necessary to supply a transposase source, if excisions or transpositions are to be generated. The Δ2–3 element from Laski *et al.* (1986) is known to produce high levels of transposase in both somatic and germ line cells. Robertson *et al.*, (1988) found this element to be exceptionally stable, transposing at a rate of only 1 in 36000 chromosomes and is thought to be incapable of regulating its transposase levels. Thus, the high level of transposase expression from this defective element has frequently been used as an effective means of inducing P element excisions and so was used in this experiment.

Figure 4.1 Potential P Element Excision Events



This illustrates the potential excision products derived from the mutagenesis strategy. +/- denotes the wild type or mutant nature of the endogenous genes. The P element may excise precisely. Alternatively, the P element may excise imprecisely deleting flanking genomic sequence from either side of the insertion site (Imprecise excision 1 and 2), or both sides (Imprecise excision 3). The P element may excise imprecisely leaving the P element termini intact, but deleting P element internal sequences (Imprecise excision 4).

4.3 Mutagenesis Strategy

As discussed in the Introduction Chapter, the temperature at which the excision events take place is directly correlated with excision frequency. A low temperature, (16°C) permits a low frequency of excision events, while at higher temperatures, (25°C-29°C), high levels of male infertility are associated with a higher frequency of excision. In this experiment, I have chosen to allow the flies to mate and develop at room temperature, which was at 18°C to 20°C. This temperature allows for reasonable levels of transposase activity and excision events, as well as ensuring a sufficient number of males were fertile for mating in the next generation.

It has been suggested the rate of imprecise excision of a P element increases if the P element is heterozygous over a deletion, since the mechanism of excision is thought to be mediated via a gap repair process (Gloor *et al.*, 1991; Daniels and Chovnick, 1993), depicted in Figure 1.4. If the appropriate homologous stretches of DNA are not available for the repair process, then this will in effect, increase the number of detectable excision events. I have attempted to increase the rate of excision in this experiment by having the A22 P element heterozygous over a balancer chromosome. Thus, there are no homologous P element sequences suitable for the "cut and paste" mechanism of double-stranded gap-repair process (Engels *et al.*, 1990). This should decrease the rate of precise excisions that may be recovered, and increase the frequency of detected imprecise excision events. In addition, any genomic sequences that may be deleted by excision of the P element imprecisely should not be repaired if the P element chromosome and the balancer chromosome can not pair in the normal manner.

4.4 The Mutagenesis Experiment

The A22 P element insertion chromosome was kept heterozygous over the TM3 *Sb*$^{-}$ *ry*^{506} *e*^{s} chromosome. Each stock is listed in Chapter 2 or in Lindsley and Zimm, (1992) and each of the crosses in the excision mutagenesis experiment have been illustrated in Figure 4.2.

The P1 cross, (labelled cross 1 in Figure 4.2) three virgin females carrying the Δ2–3 element (*CyO /Sp*; TM6 *bx*^{34e} *e*^{s}; *ry*^{506}/*Sb*$^{-}$ *ry*^{506} P[*ry*$^{+}$ Δ2–3]99B) were mated to a single male heterozygous for the A22 insertion and the TM3 *Sb*$^{-}$ *ry*^{506} *e*^{s} balancer chromosome. Twenty cultures were left at room temperature (18°C-20°C), and the progeny were subsequently collected and scored (data not shown). The progeny were of the following genotypes:

A22/TM6 *bx*^{34e} *e*^{s};
A22/*Sb*$^{-}$ *ry*^{506} P[*ry*$^{+}$ Δ2–3]99B,
TM3 *Sb*$^{-}$ *ry*^{506} *e*^{s} /*Sb*$^{-}$ *ry*^{506} P[*ry*$^{+}$ Δ2–3]99B,
TM6 *bx*^{34e} *e*^{s} /TM3 *Sb*$^{-}$ *ry*^{506} *e*^{s}.

Homozygosity for the *Stubble* allele causes lethality in the larval stages (may be embryonic lethal), and so such flies are not present in the progeny. Heterozygosity for the TM3 chromosome with the TM6 chromosome is partially lethal. Any flies of this genotype that emerge are homozygous for *e*^{s} and can easily be distinguished from the A22/*Sb*$^{-}$ *ry*^{506} P[*ry*$^{+}$ Δ2–3]99B flies. This is important since both classes of progeny are heterozygous for the *Stubble* allele.

The next cross in the scheme (labelled cross 2 in Figure 4.2) involved the mating of TM3 *Sb*$^{-}$ *ry*^{506} *e*^{s} */rf 10 e*^{s} males to A22/*Sb*$^{-}$ *ry*^{506} P[*ry*$^{+}$ Δ2–3]99B/ virgin females, and allowed isolation of excision event progeny. Since the rate of excision can be highly variable, I chose to set up 100 of these crosses, again with three virgins and one male in each culture.

The progeny from these crosses were scored for the *Sb*+, *e*^{s} and *ry*^{506} markers. The progeny from this cross are as follows:

TM3 *Sb*$^{-}$ *ry*^{506} *e*^{s} / *Sb*$^{-}$ *ry*^{506} P[*ry*+ Δ2–3]99B,

Sb^{-} *ry*^{506} P[*ry*+ Δ2–3]99B / *rf 10 e*^{s},

TM3 *Sb*$^{-}$ *ry*^{506} *e*^{s} / A22 P element present,

TM3 *Sb*$^{-}$ *ry*^{506} *e*^{s} / A22 P element excised,

rf 10 e^{s}/A22 P element present,

rf 10 e^{s} /A22 P element excised.

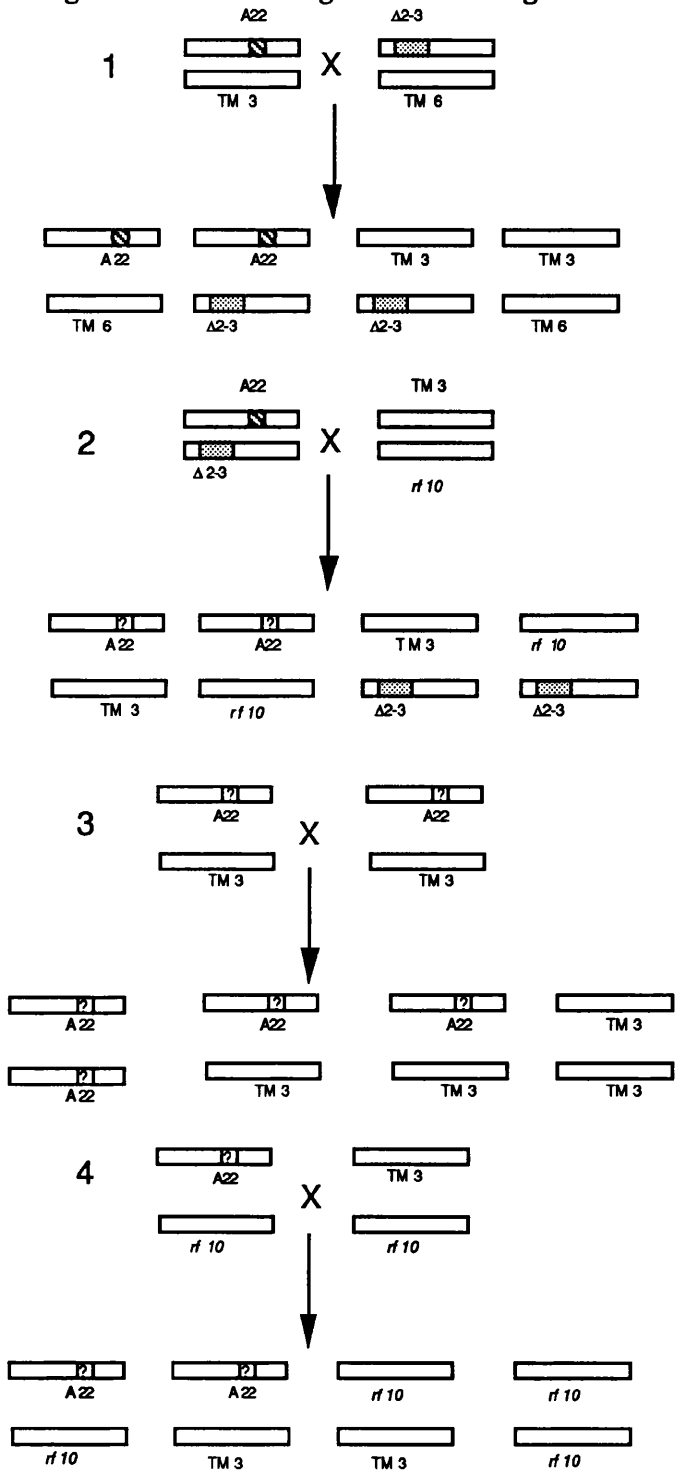
Again, 25% of the progeny will be homozygous for the *Stubble* marker that is lethal. The collected data is presented in Appendix 1, and summarised in Table 4.1. Theoretically 25% of the progeny should be *Sb*$^{-}$ *ry*^{506} P[*ry*+ Δ2–3]99B/*rf 10 e*^{s}, but in real terms, due to lethality of flies carrying two copies of the *Sb*$^{-}$ allele, they represent 33% of the progeny. There are four other classes of progeny produced as a result of the excision of the A22 P element, scored by the expression of the *rosy* marker. Flies that have lost the wild type eye colour, and have *rosy* eyes instead have lost, at least partially, the P element. Those flies that express the wild type eye colour have retained the *rosy* marker carried on the A22 insertion. This information is displayed in Figure 4.3 and presented in Appendix 1.

The heterozygous flies showed no obvious dominant phenotype. At this point, 100 lines were selected for single brother-sister matings in order to identify any recessive phenotypes that may have been induced by the excision protocol. The new stocks were re-labelled 1 to 100. These brother/sister crosses (labelled as cross 3 in Figure 4.2) were set up for those flies carrying the TM3 balancer chromosome in a screen for any recessive mutations that may have been induced by loss of the A22 P element. Such crosses were of single male/single female matings, allowing a balanced stock of each line to be established. Those flies that appeared to have lost

Figure 4.2 Legend

The A22/Δ2-3 female progeny from cross 1 are selected, having wild type eyes and the *Sb*$^-$ marker, and crossed to the TM3/*rf 10* balancer stock in cross 2. *rosy* flies with either the TM3 or *rf 10* chromosome are excision events. TM3/Δ2-3 progeny are lethal due to the double dose of *Sb*$^-$. Flies that have wild type eyes and are *Sb*$^+$, having the *rf 10* chromosome, are failed excision events. Progeny that have red eyes and the *Sb*$^-$ marker are of two classes - either Δ2-3 with the *rf 10* chromosome, or are failed A22 excision events with the TM3 chromosome. In crosses 3 and 4, *rosy*$^-$ marked progeny are mated to the TM3 balancer stock. The *rosy*$^-$/TM3 progeny from these crosses were used to establish balanced stocks, that permit any deletions to be maintained and recessive homozygous phenotypes to be identified. The TM3 homozygotes are lethal due to the *Sb*$^-$ homozygosity, while the *rf 10* homozygotes are also lethal.

Figure 4.2 The Mutagenesis Crossing Scheme



The crossing scheme used to generate excision of the A22 P element. In cross I the P element transposase is provided, allowing excision events, and is removed in cross 2. Crosses 3 and 4 illustrate the isolation of balanced stocks from those progeny that appear to have lost the *rosy* marker.

the A22 P element while the other third chromosome was *rf 10* were again crossed to the TM3/*rf 10* stock (similar to the cross labelled 3 in Figure 4.2) to obtain balanced lines. The TM3/A22 derived chromosomes were then used in brother/sister matings as represented in cross 4 of Figure 4.2.

Eighty balanced stocks were finally established. These were scored for any external defects that may have resulted from the excision of the P element.

4.5 Results

The progeny scored in each genetic class from the excision cross, labelled cross 2 in Figure 4.2 have been presented in Appendix 1, and summarised in Table 4.1.

Table 4.1. Summary of The Excision Mutagenesis Cross

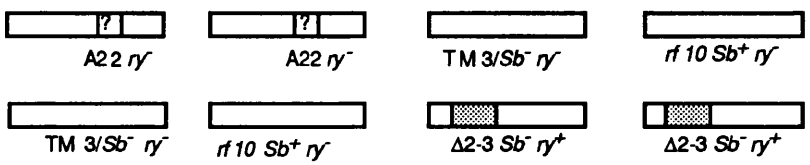
Progeny Class	Genotype	Total Scored
Δ2–3, and failed TM3/P	ry^+, Sb^-	1 421
failed *rf 10* /P	ry^+, Sb^+	414
Excision/ TM3	ry^-, Sb^+	712
Excision/ *rf 10*	ry^-, Sb^-	828
Total.		3 375

This Table summarises the data derived from the excision cross 2. The non-virgin female crosses have been omitted. Those progeny carrying the *rf10* chromosome with the A22 chromosome are scored as losing at least part of the P element if they have a ry^- phenotype , while those that have wild type eyes are scored as having maintained the P element. Progeny found to have the *Sb* and ry^- eyes were scored as having lost the P element while those with the Sb^- marker with wild type eyes were scored as mixed genotypic class, being either A22 chromosomes that had maintained the P element heterozygous with the TM3 chromosome, or progeny that carried the Δ2-3 P element heterozygous with the *rf10* chromosome.

As discussed previously, theoretically, 33% of the progeny are *Sb*$^{-}$ *ry*^{506} *e*^{s} P[*ry*$^{+}$ Δ2–3]99B. Failed excision events, when heterozygous with the TM3 chromosome can not be differentiated from the Δ2-3 progeny since such progeny have a wild type copy of the *ry* allele and the mutant *Sb* allele, and therefore these failed excision events must be estimated. Using the corrected total that removes the non-virgin female crosses, this is estimated as 3 375/3 = 1 125 *Sb*$^{-}$ *ry*^{506} *e*^{s} P[*ry*$^{+}$ Δ2–3]99B progeny, therefore, 296 of the *Sb*$^{-}$, *ry*$^{+}$ are estimated to be failed excision events

For the purposes of clarity, the numbers of flies that belong to the various genotypic classes have been presented in pictorial form below in Figure 4.3.

Figure 4.3 Excision Cross Progeny



296 *ry*$^{+}$	414 *ry*$^{+}$	Estimated as	Lethal
828 *ry*$^{-}$	712 *ry*$^{-}$	1 125	

Here, the various classes and numbers of progeny scored, generated from the excision cross (cross 2 of Figure 4.2) are presented. The numbers scored are taken from Total A.

This allows the rate of excision to be calculated by adding all progeny that have lost the P element as judged by the loss of the *ry*$^{+}$ marker and dividing by the sum of all progeny that had the potential to excise the A22 P element. This has been converted to a percentage value.

$$\frac{828 + 712}{296 + 828 + 712 + 414} \times 100 = 68.4\ \%.$$

4.6 The Induced Phenotype

Of the eighty established excision lines, nineteen showed a distinctive wing phenotype, or were lethal (see Plate 4.1, 4.2, and Table 4.2). Two pairs of these mutant strains, A22-30 and A22-31, A22-32 and A22-33, may be related, since they were derived from the same excision culture, and so may represent the same, premeiotic excision event. This represents a mutation rate of approximately 24%, (or 21% if premeiotic events occurred).

Primary scoring of these stocks indicated 19 strains with a semi lethal phenotype in eight, viable in nine and lethal in two strains, had been generated. Observed escapers in the 2 lethal strains were seen to have an extreme wing phenotype like that of the flies of the other two classes. This phenotype initially presented itself as one wing that appeared to be wild type, while the second wing was swollen, melanised, and filled with a dark fluid, making the wing look like a large, black, bulbous sac. The wing veins are clearly visible, even though the two epithelia are not attached, indicating development was perturbed after vein formation. In these wings the wing margin can be seen to be normal (see Plate 4.2), indicating that both the sensory organs of the margin are not perturbed and that there is no gross disruption to the mechanisms required for wing axis formation. Frequently, the flies become trapped in the pupal case as they try to emerge. This could possibly be due to the deformed wings becoming caught inside the case and so the imago can not free itself from the case. Alternatively, there could be a weakness in the legs, and so the young fly is unable to pull itself completely free from the pupal case. As the flies age they frequently appear darker than normal. Many of those flies with severely deformed wings become trapped in the semi-liquid food and die.

After four or five generations, the lethality and semi lethality were lost. The wing phenotype was still found in all stocks although when present, usually in a less extreme form. Occasionally, both wings were short and puffed, sometimes, although at very reduced frequency, the original phenotype could be seen. The flies

Plate 4.1 Legend

Lateral view of an A22-34 mutant female derived by imprecise excision of the P element construct in the A22 insertion strain. One wing exhibits the mutant phenotype "coal", while the second shows no phenotype other than a slight increase in melanisation of the wing veins near the hinge.

Plate 4.1

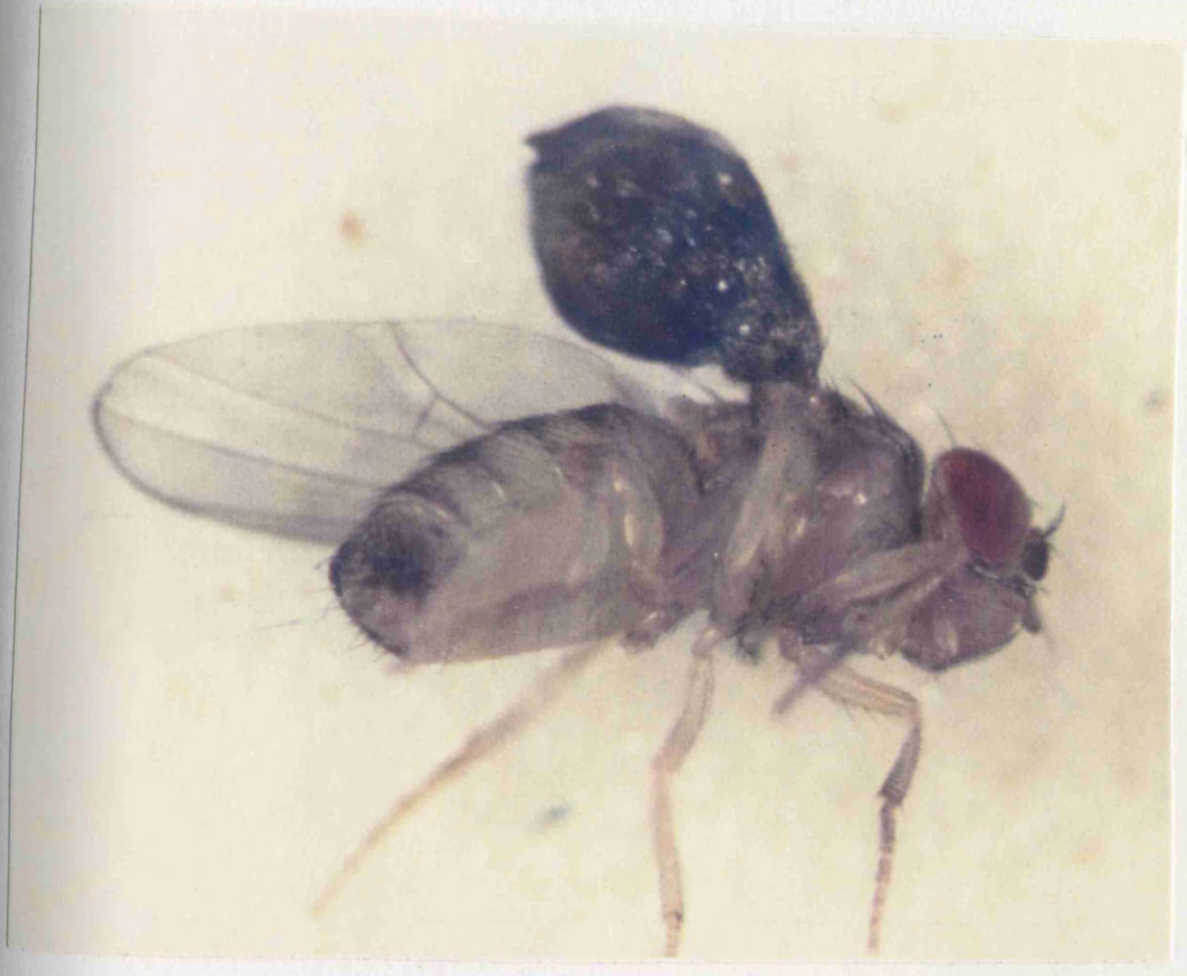


Plate 4.2 Legend

A22 excision mutagenesis-derived mutant male (strain A22-32). Here both wings exhibit "spooning". Note the presence of the wing veins and the anterior margin.

Plate 4.2

were sometimes noted to have wings that appeared wild type, except either one or both of the wings were occasionally shorter than normal, usually only as long as the fly's body. These flies were not, therefore, of wild type genotype. Other flies appeared to have completely wild type wings that were perfectly normal in shape and size.

Some of the flies that appear to have normal wings seem to be unable to fly. I have noticed that many of these apparently wild type flies can hop around the bench, but do not have the ability to fly. In addition to this, when they hop, they often land on their backs and subsequently struggle to get themselves upright. Interestingly, many of these flies seem to have a skew in their strength, since when they jump, they tend to land at approximately a 90^{o} angle from their starting direction. There does seem to be a defect in the mobility of the fly, suggesting tissues other than the wing are abnormally formed. It may be there is a fault in the connectivity of the adult nervous system, leading to loss of co-ordination. Alternatively, there may be a defect in the jump muscles, being expressed in either one side, or both sides of the body axis; this may be a similar asymmetric defect to that exhibited by many of the flies that have only one deformed wing. During routine maintenance the flies appear to hold usually one wing at a time, at an angle of 45^{o} for a few seconds even when walking, indicating abnormal control of wing movement.

Table 4.2 Identified Mutant Strains

Strain	Initial Phenotype
A22-04	Lethal
A22-07	Semi-lethal
A22-30	Semi-lethal
A22-31	Semi-lethal
A22-32	Viable
A22-33	Viable
A22-34	Semi-lethal
A22-36	Viable
A22-44	Semi-lethal
A22-47	Viable

Strain	Initial Phenotype
A22-64	Viable
A22-65	Lethal
A22-70	Semi-lethal
A22-76	Viable
A22-83	Viable
A22-88	Semi-lethal
A22-90	Semi-lethal
A22-94	Semi-lethal
Cross X	Semi-lethal

The original phenotypic classification of the flies homozygous for the mutant chromosome are presented. The mutants A22-30 and A22-31 may be related, as may A22-32 and A22-33 since they are derived from the same excision culture. Pre-meiotic events may result in identical excision events.

4.7 Discussion

4.7.1 The Excision Events

I have described a strategy to induce a mutation in the gene thought to be under the control of the enhancer element that directs the expression of the *lacZ* gene within the P element construct. Since 19 independent fly strains showing a recessive mutant phenotype were recovered from the mutagenesis, then the experiment, at this stage can be assumed to be successful.

P elements are known to be capable of reintegration into the genome, after excision. If any of the P elements were to do so, keeping the *rosy* gene intact, then it would have been scored as a "non-jumper" in the protocol. Similarly, partial deletion of the P element in its upstream domain may have left the *rosy* sequence in the genome in a functional state. Such events would also be scored as non-jumpers since loss of the *lacZ* sequence was not used as a selectable marker for excision events.

It seems very unlikely that the P element has reinserted at a new location in the genome 19 times, producing the same mutation in 19 strains. Normally, wild type P elements will reintegrate in the genome at a frequency of approximately 0.1% (Salz *et al.*, 1987). Since the P element here is considerably larger, it may reintegrate less readily.

However, it may be that the gene that has been mutated is different from the gene controlled by the "trapped enhancer". It is possible that the mutated gene lies in close proximity to the P element, while the trapped enhancer regulates a different, more distant gene as illustrated in Figure 4.4. Further experiments are required to demonstrate which gene has been mutated, and where it lies with respect to the enhancer-trap.

Figure 4.4 Possible Spatial Organisation of Regulatory and Coding Regions

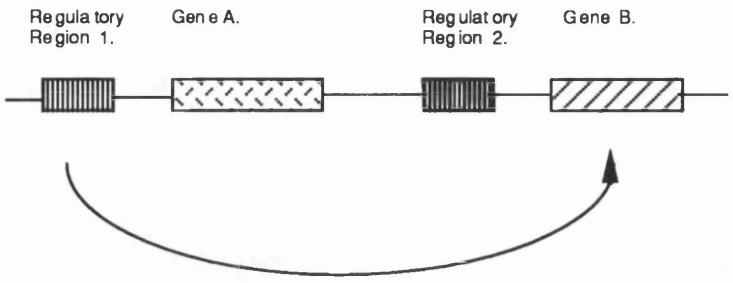


Diagram illustrating the potential complexity of genomic position of genes and their regulatory elements, with respect to position.

It is important to note that in this excision experiment, there is a bias in the data. Only excision events that either delete completely or partially the *rosy* gene, have been selected. It is possible that induced mutations that delete completely or partially the *lacZ* gene while leaving the *rosy* gene in a functional state, were products of the excision cross. "Jump-outs" with this polarity will not be present in the isolated flies of the excision class, but will have been discarded, being scored as non-jumpers.

Published Excision/Mutation Rates

It is important that estimated rates of excision are available for the large variety of P element constructs that are now available. This type of data will allow a realistic estimate of the magnitude of experiment about to be undertaken. Obviously such data can serve only as a guide, but will be desirable nevertheless. The rates of such excisions will facilitate our understanding not only of the biology of P elements, but also allow us to understand the way in which P element function is disrupted when genetically engineered in a similar fashion to those used in this study. Unfortunately excision rates are not normally published.

The mutation rate, as opposed to the excision rate, is also an important parameter, and is influenced by both the P element insertion site, and the source of transposase (Robertson *et al.*, 1988). In a small survey of the literature there is a considerable range of mutation rates generated by P element excision, as can be seen in the examples presented in Table 4.3 below. Unfortunately, neither the excision rate, nor the experimental conditions are usually reported in the literature preventing any realistic comparisons.

Table 4.3 Published Excision Rates

Reference.	Construct.	Mutation Rate.
Boedigheimer and Laughon. 1993	P[lacW]	89% internal, 5% precise revertants and 6% imprecise.
Nakamura *et al.*, 1994	P[*lacZ*, ry^+]	7.3% imprecise mutation
C. Henchcliffe *et al.* 1993.	pUChsneo P element	7.6% imprecise mutation.
Klämbt. 1993	P[*lacZ*, ry^+]	44% precise revertants
D. Montell *et al.* 1992	PZ	80% precise revertants
H. E. Vaessin *et al.* 1991.	P[lacW]	53% precise revertants

In the experiment here, nineteen of the eighty surviving homozygous strains (24%) show a distinct phenotype. This is a reasonably high percentage. A low number of mutants isolated from such an experiment might indicate the generation of a low number of deletions large enough to remove regulatory or coding sequences, or a large number of internal P element deletions. The rate of mutation in this experiment may indicate that the A22 P element is relatively close to the gene now mutated and that precise excision of the P element did not occur preferentially with this construct.

4.7.2 Wing Development and the Nature of the Mutated Gene

The phenotype of these mutant lines indicates the mutated gene may be involved in specific processes in the development of the wing or associated structures.

Sensory Organ Development

Since the embryo exhibits a distinctive *lacZ* expression pattern in the nervous system, then any *lacZ* expression in the developing wing might be found in the sensory organs. The gross morphology of the adult wing does not depend on the proper formation of the sensory organs and the outgrowth of their axons to the correct target in the thorax (Blair *et al.*, 1985). Therefore, inappropriate development of the sensory organs in these mutants is unlikely to be a reasonable focus for the mutations in the strains generated in this experiment. In addition the wing margin, rich in sensory organs does not appear to be disrupted in even the more severe form of the phenotype.

Pupal Wing Development

The anatomy of the developing wing throughout pupal development has previously been studied by a number of authors including Waddington (1940) and Fristrom *et al.* (1993). The third instar imaginal disc changes shape in the prepupal period, forming an epithelial bilayer. The apposed epithelial layers begin to separate at around 11 hours, although the layers at this point remain connected by cytoplasmic processes. At the onset of pupation proper, the head is everted by the forces of abdominal muscle contraction. At the same time, the haemolymph that moves into the developing wing, forces the dorsal and ventral surfaces of the immature wing blade further apart, breaking the cytoplasmic, or transalar processes, which had previously held the two surfaces in contact. Between 20 and 35 hours of pupal

development, the two epithelial layers come back together, forming a thinner wing blade, which has a spongy appearance. As this process of reapposition continues, the haematocytes in the wing become restricted to the vein regions. This process is complete around 45 hours of development. At around 60 hours of pupal development, separation of both dorsal and ventral surfaces occurs again, although not to the same degree as previously. The defect in the wings of these flies could occur at either of these separation processes.

Alternatively, the phenotype may be a consequence of the failure of the wing hinge region to develop properly. Pupation occurs approximately 12 hours after the prepupa has formed, when contraction of the abdominal muscles evert the head. At the same time an influx of haemolymph through the presumptive hinge structure forces the dorsal and ventral wing surfaces further apart until the wing is a bloated sac. This separation causes disruption of most of the transalar processes, except for those at the very periphery of the wing. If the haemolymph can not escape from the cavity, then the wing will not form into a thin epithelial bilayer. The haemolymph may not escape if, during the inflated stage, a constriction at the hinge region develops.

Adhesion of the Wing Epithelia

A number of genes have been shown to be directly involved in the adhesion processes that result in the two epithelial layers of the wing being held together. An example of this is the lethal *myospheroid* gene, shown to encode the position-specific integrin β subunit (Leptin *et al.*, 1989; MacKrell *et al.*, 1988). These mutants show a blister phenotype on the wing blade as a result of the failure of the adhesion mechanisms. In addition, the wings frequently have a "held-out" phenotype (Wilcox *et al.*, 1989). Another example of this is the *inflated* locus, known to encode the αPS2 subunit of the PS integrins (Wilcox *et al.*, 1989). When flies mutant at both the *inflated* locus and the *myospheroid* locus are generated, they show more severe

blisters than when seen if only one of these genes is mutant (Wilcox *et al.*, 1989). In the imaginal wing disc the αPS1 antigen is located in the dorsal half of the wing blade domain, while the αPS2 antigen is found in the ventral half (Wilcox *et al.*, 1981; Brower *et al.*, 1984; 1985). It has been shown that the PS integrins are localised at the sites of adhesion between the dorsal and ventral epithelia of the wing blade in the pupal and adult fly (Fristrom *et al.*, 1993). Interestingly, the *myospheroid*^{nj42} allele has a non-jumper phenotype (De Le Pompa, 1989).

The black appearance of the most extremely deformed wings may be interpreted as excessive melanisation and chitin formation, described by Waddington (1940), when the immature wing fails to come back together and adhere, forming the thin blade, in mutants such as *bloated*, and *balloon*.

Variation Within the Strains

There is a variation in the wing phenotype of the mutant flies generated in this experiment. It may be possible that the severely melanised, balloon wings result as a failure of the adhesion process that normally occurs at the earlier time of 11 hours. This would result in the excessive melanisation. Those wings that are blistered, but do not have an excessive amount of melanin, may be due to failure of the second adhesive process, which occurs at around 60 hours. This failure may be caused by a defect in the expansion of the wing blade cells, since the wings of this type are frequently shorter than normal. This shorter wing may be due to inappropriate cell death, and so inadequate cell expansion, or just failure of the cells to spread appropriately. The homozygous flies that have only one mutant wing are not unusual since the *inflated* mutants also exhibit this asymmetry (N. Brown, Cambridge, pers. comm).

These lines were selected on the basis of the embryonic expression pattern of *lacZ*. Therefore, if the intended gene has been mutated, then it is required for an

unknown function in the embryonic nervous system, and in the adult wing, where disruption of its expression is essential for proper development of the wing.

4.7.3 Other Known Alleles

There are many known mutations throughout the *Drosophila* genome that have similarly described phenotypes to the mutants generated by the excision of the A22 P element (Lindsley and Zimm, 1992). However, there are no phenotypically similar mutations previously located to bands 72BC, the site of the ancestral insertion (E. Zador, unpublished data).

Crimp (*Cm*), thread (*th*), dark hairy margins (*dhm*), *Washed eye* (*We*), and *minus bar* (*mb*) have all been placed in chromosome band 72B. *Cm* is a dominant, homozygous lethal mutation that exhibits a temperature sensitive wing phenotype when heterozygous. At 25°C the wings are crimped, and ruffled on the rear edge, while at 19°C the phenotype is difficult to score. *dhm* is a recessive mutation that results in dark hairy margins, and the wing veins are thicker. *th* is a recessive mutation that results in the loss of the side-branch hairs on the antennal aristae. *mb* is a modifier of *Bar*, and homozygous females are infertile. *We* is a dominant modifier of *white*, producing partial reversion of the *white* mutant phenotype.

Cm and *dhm* have different descriptions from the mutants generated here, but either could possibly be an allele. *Cm*, first discovered by Bridges in 1928 (Lindsley and Zimm, 1992) no longer exists thus preventing further analysis for allelism.

Known Genes Functional in Both the Embryo and the Wing

It has been noted that many genes involved in the development of the embryo are also functionally important in the later adult structures. For example, there are a variety of *veinlet* (*ve*) mutant alleles, some of which are lethal and the viable alleles show vein defects. Homozygous *ve* embryos have dorsoventral defects, and defects in the midline cells such that the glia and neurons either do not develop, or are abnormal. A narrower CNS can be seen, with abnormalities in the pathfinding abilities of the neurite outgrowths. At least two of the stretch receptors have been shown to fail to develop in the embryonic PNS. Sequence analysis of the cloned gene indicates that the *ve* protein is located at the cell surface (Waddington, 1940; 1984; Jurgens *et al.*, 1984; Mayer *et al.*, 1988; Bier *et al.*, 1990; Diaz-Benjumea and García-Bellido, 1990).

4.8 Summary

This experiment does not provide us with enough information to confirm the molecular nature of the gene. Indeed it is impossible at this stage to determine what proportion of the gene has been mutated as a result of the excision events. It may be that enhancer elements have been deleted. This would explain why all mutants generated here have the same phenotype, and the apparent lack of various alleles, such as consistently homozygous viable, or lethal. Alternatively the gene product may not be essential for the viability of the fly. This will require detailed molecular characterisation of the mutants to answer these issues. What could be said however, is the gene seems to be "functionally overlapping" with other, unidentified genes, since modifiers have apparently collected in these stocks, implying that other genes can, in part, supply the missing functions.

There is always the possibility that the gene mutated in this experiment is a gene neighbouring the gene reflected in the expression of *lacZ* (see Figure 4.4). This can only be resolved by cloning the flanking regions, and thus the gene. *In situ*

hybridisation experiments with this clone to the original A22 embryos will show that it either has the same expression pattern, and is therefore the gene sought, or it has a different expression pattern from the *lacZ* line, and is therefore not the "trapped" gene.

Chapter 5

Molecular Biology

5.1 Introduction

If the molecular function of a gene is to be elucidated, then the gene must be cloned and sequenced as a preliminary to molecular analyses. A simple, though potentially time consuming approach would be to initiate a chromosome walk along the chromosome, using a previously cloned gene, located near the chromosomal band of interest to begin the walk. Isolated genomic clones could then be used to molecularly map the P element insertion site, and thus the gene, or genes, in the vicinity of the enhancer trap may be slowly isolated. In the case of P element insertion strains, however, the P element rather than a distantly located gene can act as a useful genomic "tag" (Bingham *et al.*, 1981), thus avoiding burdensome chromosome walking techniques. By constructing a genomic library for each unique insertion strain, it is possible to rapidly isolate the appropriate genomic sequences, by using a P element construct specific probe to screen the library. This approach allows flanking genomic fragments to be directly isolated from each P element insertion locus.

Similar principles can be applied to the construction of subgenomic libraries. By restriction mapping the P element insertion site, it may be possible to identify a DNA fragment of suitable length that could be cloned directly into a plasmid vector. This approach is advantageous if such target fragments can be purified from the rest of the genomic DNA, reducing the number of clones that must be screened and the number of subsequent subcloning steps are minimised. The use of plasmid vectors in library construction, however, are relatively inefficient but can be replaced by using bacteriophage λ based cloning vectors, that are specifically designed for cloning small DNA molecules normally generated in the construction of cDNA libraries (Ausuber *et al.*, 1990).

A variety of polymerase chain reaction (PCR) approaches have been developed for the cloning of specific genomic DNA sequences (e.g., Ochman *et al.*, 1990, Riley *et al.*, 1990). The PCR reaction requires two primers that anneal to

single-stranded target DNA, and then directed DNA synthesis. Denaturation of double-stranded DNA products and continued DNA synthesis by a thermostable DNA polymerase achieves DNA amplification of specific DNA fragments *in vitro* as discussed in Innis *et al.*, (1990).

A basic PCR strategy requires the design of two primer oligonucleotides based on previously sequenced DNA. However, such information is not normally available when attempting to clone novel genomic DNA. This obvious obstacle to PCR approaches may be overcome by the *in vitro* modification of the target DNA molecule, if a proportion of the sequence has previously been characterised, for example, the P element sequences. Ligation-mediated PCR strategies have been developed and may be applied to projects such as enhancer trap cloning (e.g. Riley *et al.*, 1990, Arnold and Hodgson, 1991, Sentry and Kaiser, 1994).

Both library construction and linker-mediated PCR techniques were chosen as appropriate methods to clone the genes believed to have the same expression patterns as the reporter gene of A11 and A22 insertion strains.

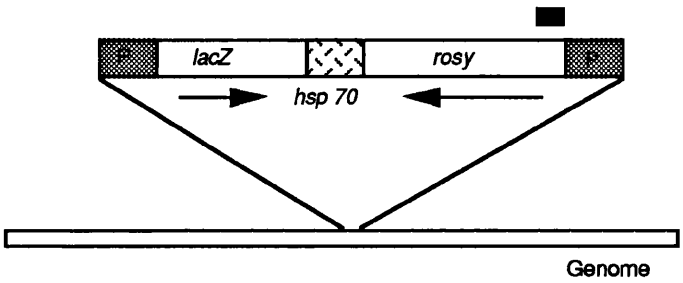
5.2 Results

As a prelude to using the P element insertion as a genomic "tag", it is necessary to ensure only a single P element is present in each genome. If multiple P elements are present then subsequent cloning procedures will be complicated, as will the interpretation of any mutant phenotypes obtained by P element excision mutagenesis protocols and their association with any given locus. The genetic background of both insertion strains carry the *rosy*^{506} (*ry*^{506}) mutation that has the coding sequence deleted while the upstream regulatory regions remain intact (Cote *et al.*, 1986). This genetic background allows any P element insertion event, where *rosy* has been cloned into the modified P element, to be detected by the restoration of the wild type eye colour, or molecularly by the detection of *ry* coding sequences

either by Southern blotting or by *in situ* hybridisation to polytene chromosomes. Here, the presence of *ry* coding sequence has been employed to generate a restriction map of the genomic region downstream of each insertion strain. By using a probe specific to the regulatory sequences of *ry* (see Figure 5.1) and the appropriately selected restriction enzyme, each strain should give two hybridisation bands. One band should be specific to the endogenous sequence and should be of the same length in each unique insertion strain. The second band should be specific to each strain, and therefore of different lengths, depending not only on the restriction enzyme used, but also on the P element insertion site. If multiple P element constructs are present in the genome, then multiple bands should be detected, only one of which should be common to each strain. Such Southern blot analysis has been performed initially to ensure each strain has a single P element construct, but also to provide a restriction map of each locus.

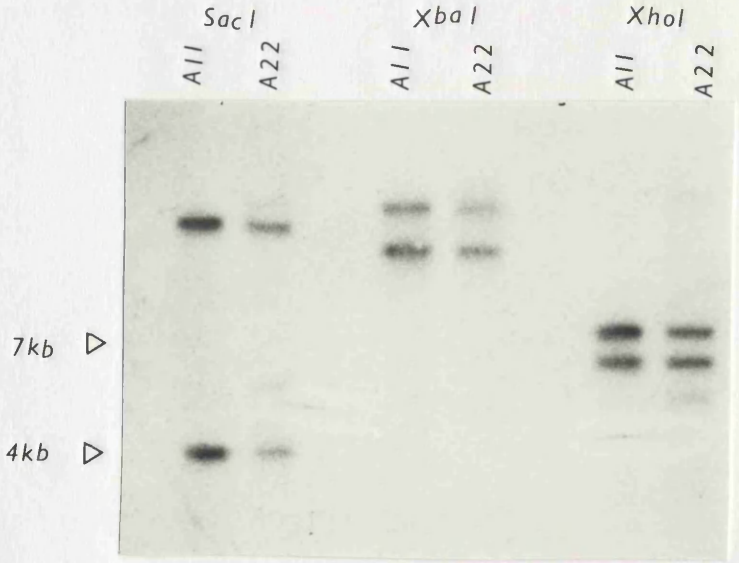
Initially, previously frozen A11 and A22 flies were used to prepare DNA and genomic Southern blots, and were probed with the *ry* 5' *Hind* III-*Acc* I DNA fragment of approximately 1.2 kb as shown in Figure 5.1. The autoradiograph obtained is presented in Figure 5.2, and as can been seen, there are two hybridisation signals in each track indicating severe contamination of the frozen fly stocks that had been previously prepared and stored at -70°C. The DNA prepared from these stocks was therefore discarded immediately, and the live strains were assayed for the previously determined *lacZ* expression patterns and for contamination. The *lacZ* expression patterns were found to be as expected and therefore new genomic DNA was prepared and the genomic Southern blots repeated.

Figure 5.1 The P Element



By restriction digesting the genomic DNA with an enzyme that cuts once between the *hsp 70* fragment and once in the genome but not in the P element terminal sequences, the probe indicated (approximately 1.2 kb) can be used to construct a restriction map of each P element insertion. The probe illustrated is a *Hind* III-*Acc* I 5' fragment of the *rosy* gene obtained from the plasmid p41 (a kind gift from C. O'Kane, Cambridge).

Figure 5.2 Autoradiograph of A11 and A22 Genomic DNA Probed With a *rosy* -specific Probe



Approximately 0.7 µg of restriction enzyme digested genomic DNA was run out in a 1% agarose gel and transferred to a Hybond N membrane. The prehybridised filter was then probed using the 5' probe (see Figure 5.1) labelled with P^{32} to high specific activity.

Figure 5.3 Autoradiograph of A11 and A22 Genomic DNA Probed With a *rosy*-specific Probe

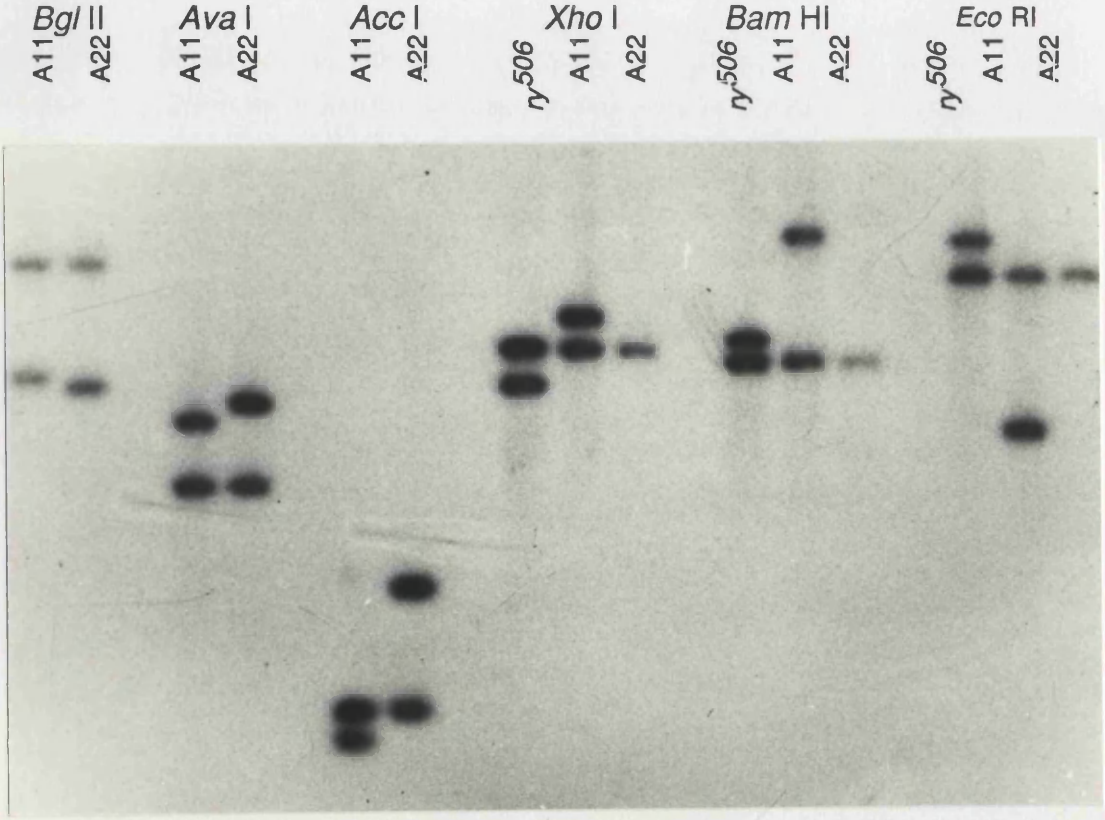


Figure 5.3 Legend

Autoradiograph of A11 and A22 genomic DNA restriction enzyme digested and Southern blotted. Approximately 0.7 μg of restriction enzyme digested genomic DNA was ran out in a 1% agarose gel and transferred to a Hybond N membrane. The prehybridised filter was then probed using the 5' probe depicted in Figure 5.1, labelled with P^{32} to high specific activity. The endogenous ry^{506} sequences gave hybridisation signals as follows: *Acc* I, 1.2 kb; *Ava* I, 3.0 kb; *Bam*HI, 4.8 kb; *Bgl* II, 12.5 kb; *Cla* I, 2.1 kb; *Eco* RI, 9.6 kb; *Mlu* I, 4.6 kb; *Sac* I, 3.4 kb; *Xba* I, 15 kb; *Xho* I, 5.1 kb. The P element sequences of A11 gave hybridisation signals of *Acc* I, 2.0 kb; *Ava* I, 4.3 kb; *Bam*HI, 14 kb; *Bgl* II, 4.6 kb; *Cla* I, 20 kb; *Eco* RI, 3.5 kb; *Mlu* I, 3.3; *Sac* I, 18 kb; *Xba* I, 19 kb; *Xho* I, 7.0 kb. The genomic DNA of A22 gave hybridisation signals of *Acc* I, 1.0 kb; *Ava* I, 3.9 kb; *Bam*HI, 5.8 kb; *Bgl* II, 4.9 kb; *Cla* I, 2.1 kb; *Eco* RI, 13.5 kb; *Mlu* I, 21 kb; *Sac* I, 1.4 kb; *Xba* I, 20 kb; *Xho* I, 4.5 kb.

Figure 5.3B Autoradiograph of A11 and A22 Genomic DNA Probed With a *rosy*- -specific Probe

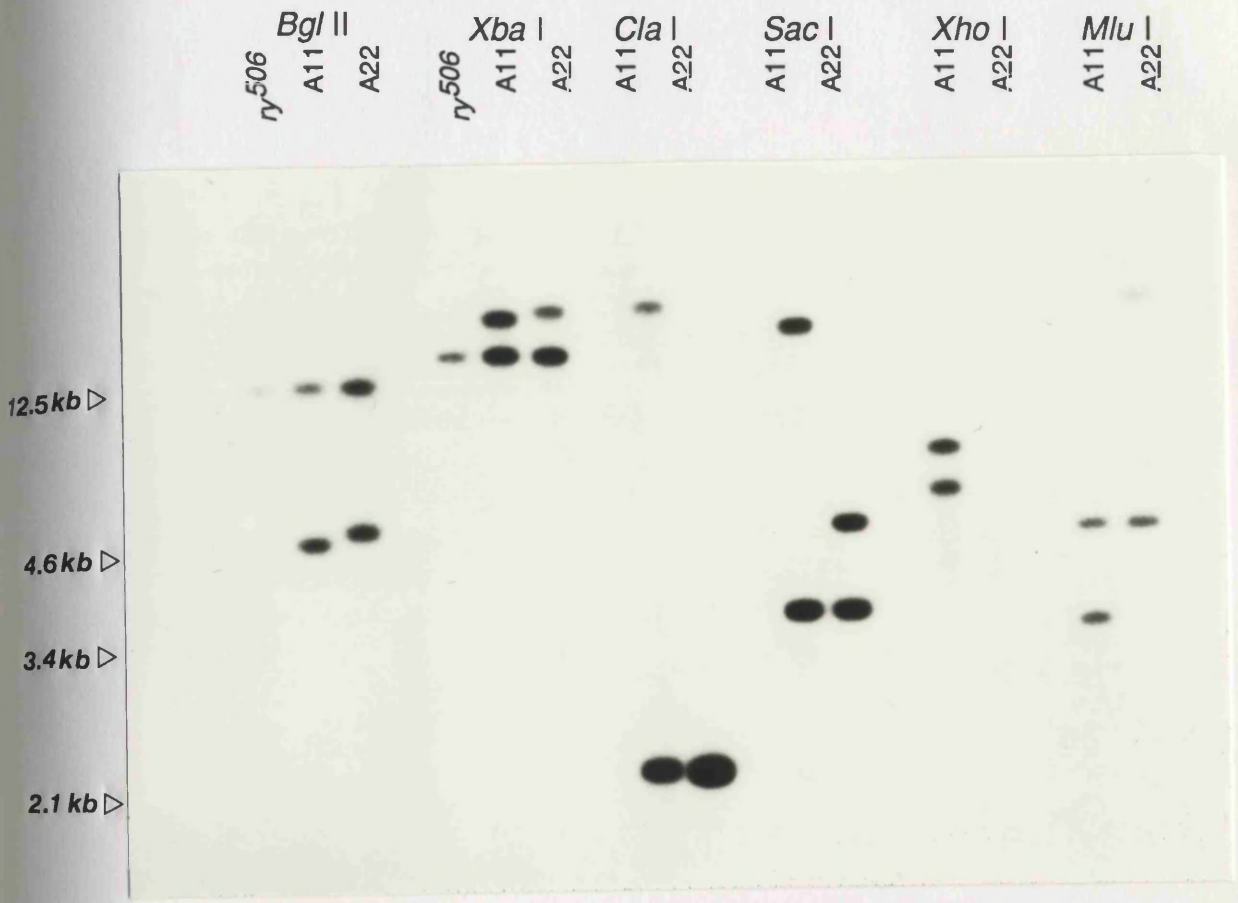


Figure 5.4 Legend

The restriction map of the genomic DNA down stream of both the A11 and A22 P element constructs are depicted. The length of genomic DNA within each hybridising fragment detected in Figure 5.3 can be calculated simply by subtracting the known length of P element sequence from the size of the hybridising fragment. For A11, *Eco* RI and *Mlu* I cuts very close to the insertion locus, *Bam*HI, 10.7 kb downstream of the insertion locus; *Xho* I, 3.7 kb; *Ava* I, 1 kb; *Bgl* II, 4.1 kb; *Sac* I, 15.7 kb; *Cla* I, 17.7 kb; and *Xba* I, 16.2 kb. For A22, *Eco* RI cuts 9.7 kb downstream of the insertion locus, *Bam*HI, 2.5 kb; *Xho* I, 1.2 kb; *Ava* I, 0.6 kb; *Bgl* II, 4.4 kb; *Mlu* II, 17.7 kb and *Xba* I, 16.7 kb, while *Cla* I cuts very close to the 3' insertion site. The vertical lines on each depicted chromosome indicate 1 kb of DNA.

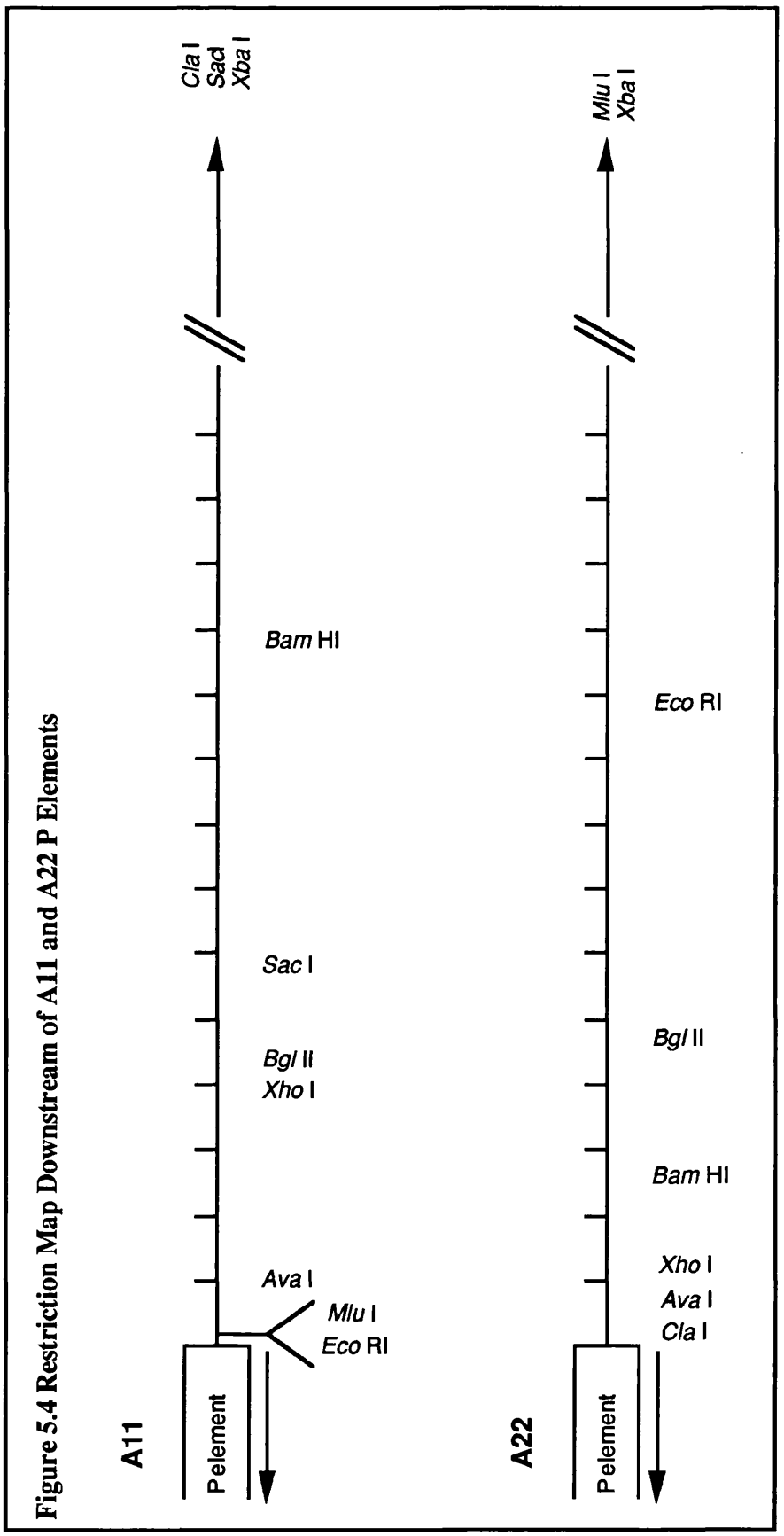


Figure 5.4 Restriction Map Downstream of A11 and A22 P Elements

5.3 Library Construction.

From the autoradiograph, The *Bgl* II fragments were identified as the most suitable for subgenomic library construction. For both strains the *Bgl* II fragments fall within the size range required for ligation into a vector normally used for cDNA library construction. Both *Bam*HI and *Bgl* II restriction enzymes generate compatible cohesive ends, and so a *Bgl* II fragment can be ligated into a vector prepared for *Bam*HI fragments. Importantly, only one type of vector is required for the construction of both A11 and A22 subgenomic libraries, thus considerably reducing cost. The vector used was λ ZAP since this vector allows rapid subcloning of the isolated genomic DNA and *Bam*HI pre-digested vector is commercially available (Stratagene).

Subgenomic library construction requires a large amount of digested DNA if detectable levels of the subsequently isolated DNA fraction is to be used (Ausuber *et al.*, 1990.). Approximately 250 μg of A22 genomic DNA was therefore restriction digested and size fractionated through a 0.8% TAE agarose gel cast with a long slot comb. The appropriate size ranges of DNA were cut away from the gel slab, electroeluted from each agarose slice and purified using the Promega Magic™ DNA purification system. The integrity of each fraction was established by gel electrophoresis (see Figure 5.5A) and the appropriate fraction identified by hybridisation (see Figure 5.5B) with a radiolabelled *Acc* I/*Hind* III 5' probe, indicated in Figure 5.1. 0.12 μg of insert DNA was ligated to 1 μg of λ ZAP vector in a final concentration of 5 μl. The ligation mix was incubated at room temperature for one hour and then at 4°C for two days. 2.5 μl of this ligation mix was then packaged with Gigapack® II packaging extract (Stratagene) according to the manufacturers instructions, except the packaged library was stored in 5 ml of phage buffer rather than 550 μl. The library was immediately titred using X-L1 Blue MRF' cells and calculated as 9.4×10^4. The percentage of phage that had no insert was calculated as 4% by the addition of X-gal and IPTG to the phage plates prior to plating the phage.

In parallel, Gigapack® II test DNA was packaged and titred and gave a total phage count of 2.8 x 10^7/μg DNA, while a plate of cells with no added phage gave a confluent lawn of cells with no plaques. Approximately 80 000 phage were then screened as described in Chapter 2 in a primary screen that involved duplicate filters of each plate seeded with approximately 10 000 phage particles each. As a control, restriction digested p41 plasmid was included in a dot blot (serially diluted) and probed with the same batch of 5' radiolabelled probe. No positive plaques were isolated, while the p41 dot blot gave a hybridisation signal (not shown).

In a further attempt to clone the genomic DNA flanking the A22 P element insertion, genomic DNA was restriction digested with *Eco* RI, as previously described for the *Bgl* II subgenomic library. In this instance the endogenous ry^{506} fragment will be 9.6 kb while the A22-specific *ry* fragment will be 13.5 kb (see Figures 5.3 and 5.3). As before, the gel fractions were electrophoresed through a 1.0 % TAE agarose gel (Figure 5.6A) allowing DNA integrity to be monitored, and the appropriate fraction selected for by Southern blot analysis using the 5' p41 probe. As can be seen in Figure 5.6B, two bands were detected, one the endogenous sequence, the other specific to the P element construct sequence. The smaller size fraction was selected and used to construct a λ EMBL4 library (kind gift from V. Graham, Glasgow), a vector that can accept inserts of 9-23 kb in length. Approximately 25 ng of fractionated A22 DNA was ligated to the EMBL4 arms and incubated as previously described. On titring, this library was found to consist of 18 000 phage particles/μg. Although this titre is low, the library was propagated using the *E. coli* strain P2392 that permits recombinant but not wild type λ growth. This library was therefore screened in duplicate as before, but no positive plaques were isolated.

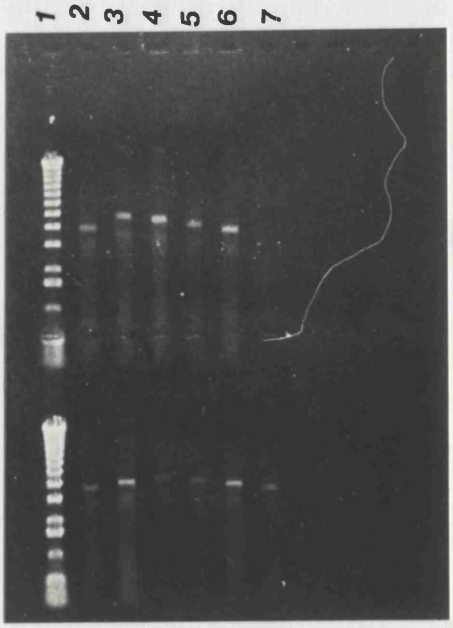
Figure 5.5 Legend

In **A**. A22 *Bgl* II genomic fractions were excised from an agarose gel and re-fractionated in agarose after electroelution. Apart from some smearing of the genomic fractions, the DNA seems to be of reasonably good quality, and the library was therefore constructed. In both the top and bottom tracks the 1 kb ladder size marker has been loaded, while in the lanes 2-6 of the top gel and lanes 2-7 of the bottom gel have *Bgl* II genomic fractions.

In **B**, the gel was probed using the 5' *AccI/Hind* III fragment and the resultant autoradiograph is depicted. Tracks 2- 6 of the top gel and lanes 2-7 of the bottom gel gives hybridisation signals, indicating poor fractionation. Therefore, those bands that gave the strongest signal (lanes 1, 2 and 3) were chosen for ligation into the λ ZAP vector.

Figure 5.5 Genomic Fractions

A



B

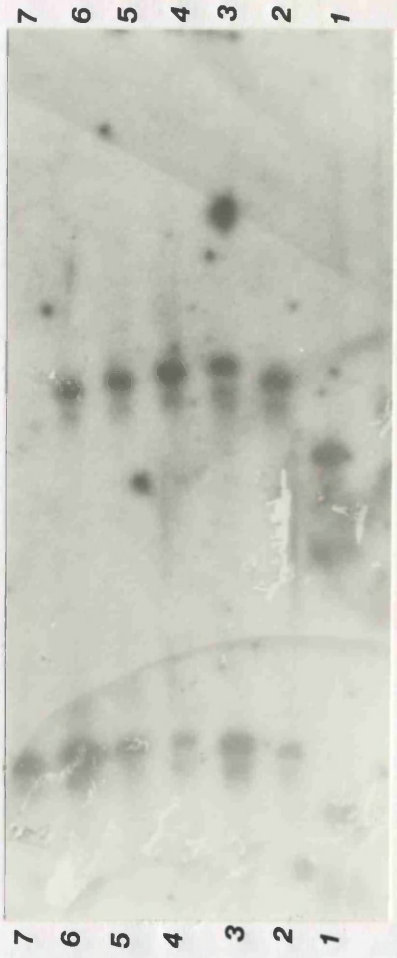
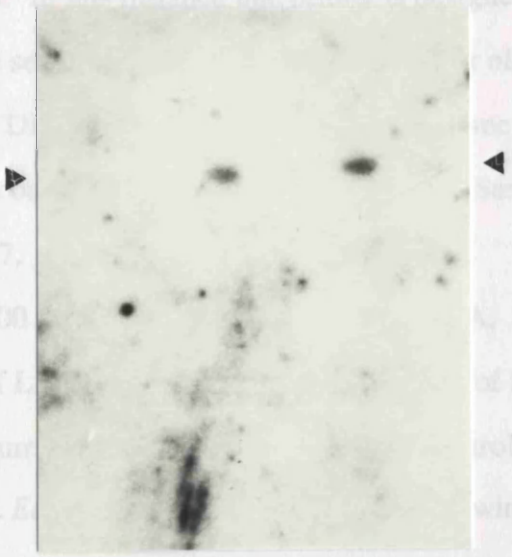


Figure 5.6 Autoradiograph of Genomic Fragments



Autoradiograph of *Eco* RI digested and size selected A22 DNA. The *rosy*-specific probe used here is depicted in Figure 5.1. From the samples loaded in gel lanes 2-8 (lane 1 was the 1 kb ladder size marker), it can be seen that two of the A22 *Eco* RI genomic fractions give a hybridisation signal, one specific to the endogenous *rosy* sequence in lane 4 and the other specific to the P element construct in lane 6.

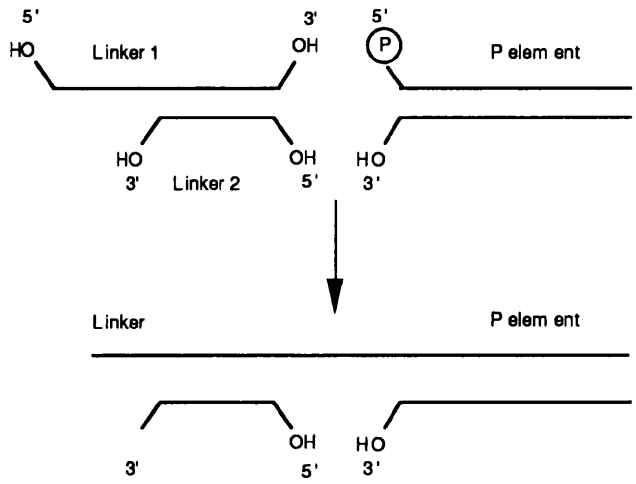
5.4 PCR Cloning Approaches

A second approach, using PCR technology, was undertaken in an attempt to amplify the genomic sequences flanking the A11 and A22 P element inserts, in parallel with the experiments of J. Sentry. As previously stated, one of the two required oligonucleotides can not be synthesised, as the required sequence is unknown. In this instance one primer is designed to anneal to the P element termini, while the second primer will anneal to a linker oligonucleotide ligated to fragments of genomic DNA generated by restriction enzyme digestion and this Linker-mediated PCR approach (Arnold and Hodgson, 1991, Sentry and Kaiser, 1994) is depicted in Figure 5.7.

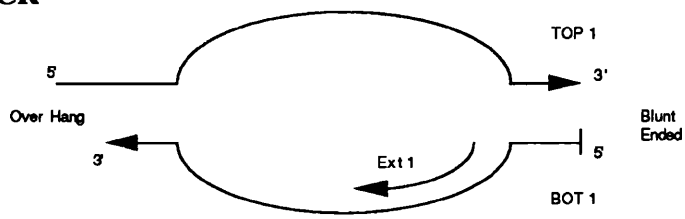
200 ng of A11 and A22 genomic DNA, digested with *Hae* III was ligated to 300 ng of L1+L2, providing a 20-fold excess of linker : genomic DNA ends, at room temperature for 2.5 hours. In a parallel control, the T4 DNA ligase was found to ligate a λ *Eco* RI/*Hind* III DNA ladder. Following heat inactivation of the ligase, the DNA was then used as a template for PCR amplification using L1 and P31 as primers. 1.25 mM dNTPs, 2.2 μg P31, 2.2 μg L1 and PCR buffer (Promega) were added to the ligation mix, to a final volume of 45 μl. After incubation at 94°C for 3 minutes, the reaction volume was increased to 50 μl by the addition of 1 unit *Taq* polymerase (Promega). The PCR reaction was then continued with 30 cycles of 60°C, 1 minute; 72°C, 3 minutes; 94°C, 1 minute, and completed with a single cycle of 60°C, 5 minutes and 72°C for 30 minutes. A11 and A22 genomic DNAs digested with the restriction enzyme *Rsa* I were used in parallel ligation and PCR reactions. Under the same conditions, the PCR was controlled using genomic DNA (CFL3) prepared from a *Drosophila* strain carrying a P element insertion at the *singed* locus, and the primers snA and P31 for amplification.

Figure 5.7 Ligation-Mediated PCR Approaches

A Linker-Mediated PCR



B Bubble PCR

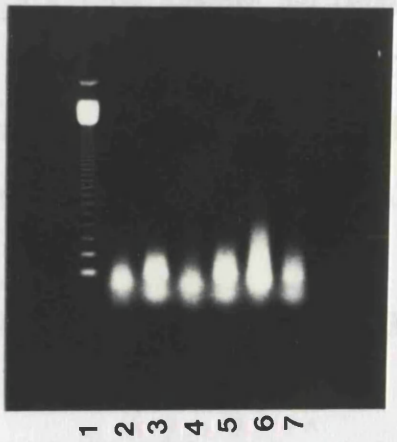


A (Linker-Mediated PCR, Arnold and Hodgson, 1991, Sentry and Kaiser, 1994), the linker will ligated by the formation of a phosphodiester bond between the 5' P and the 3' OH, if a second linker has annealed and makes it, in effect double-stranded and blunt ended. Since there is no phosphoryl group available for phosphodiester bond between the two short linkers, these will remain separate and can be denatured away from the ligated molecule. In this approach the genomic DNA is digested with restriction enzymes that produce blunt ended fragments, and have a 4 base pair recognition sequence, and should therefore produce a fragment of amplifiable length.

B (Bubble PCR, Riley *et al.*, 1990., Arnold and Hodgson, 1991), the same principles apply. However, a number of primers may be used in a nested PCR approach, thus sequentially reducing smearing that may result. TOP 1 and BOT 1 refer to the linkers that provide known sequence permitting a the use of the PCR primer Ext1 in a PCR approach.

The amplified DNA was electrophoresed through a 1.5% agarose gel. Each PCR reaction produced a considerable smear of DNA, with no identifiable amplified DNA fragments (not shown). This non-specific amplification was believed to be due to either inappropriate PCR cycling conditions and/or poor quality P31 primer.

Figure 5.8 Bubble PCR



In these "bubble" PCRs (Riley *et al.*, 1990), genomic DNA was digested with *Sau* 3AI and the control reactions have no *Taq* polymerase. Lane 1, 123 bp ladder; lane 2, A11 control; lane 3, A11 PCR; lane 4, A22 control; lane 5, A22 PCR; lane 6, CFL3 PCR; lane 7, CFL3 control. Only the primers and some smearing are detected by Ethidium bromide staining of the 1.5% agarose gel.

This PCR approach was subsequently modified by using another set of linkers and fresh P31 primer. In these "bubble" PCR reactions (Riley *et al.*, 1990., Arnold and Hodgson, 1991) template genomic DNA was ligated to the linkers TOP 1 and BOT 1 as before (as illustrated in Figure 7.7B), and similar controls were employed. The ligation mix was then used as a template for PCR using 297 ng/µl cWS1, 264 ng/ml cWS2, 937 ng/ml Ext1 primers, 1.25 mM dNTPs and PCR buffer in a final volume of 45 µl. As before, a "hot start" was used before adding *Taq* polymerase to a final volume of 50 µl, and the same cycles were used as before. In this experiment, the CFL3 genomic DNA was again used as a PCR control as were A11 and A22 genomic DNAs with no *Taq* polymerase. The completed PCR reactions were then electrophoresed through a 1.5% agarose gel (Figure 5.8). Unfortunately, no fragments representing the genomic DNA flanking of either strain were amplified in these experiments. Since the DNA ligase was found to ligate a λ *Eco* RI/*Hind* III DNA ladder, then it can be assumed the PCR reaction conditions were inappropriate.

5.5 Molecular Analysis of The Mutant A22-30

Molecular characterisation of the mutants derived by imprecise excision of the A22 P element construct is required for further study of the induced developmental defect. The isolation of genomic sequences from the A22 insertion locus would permit eventually, the analysis of any neighbouring transcription units, and the chromosomal location of the induced mutation could be confirmed. Lack of genomic sequence at this time does not exclude initial molecular characterisation of the A22-derived mutants. Using P element sequences to probe Southern blots of mutant genomic DNA, it is possible to estimate the extent of P element sequences loss.

Figure 5.9 Autoradiograph of A22-30 Genomic DNA Using a *rosy*-specific Probe

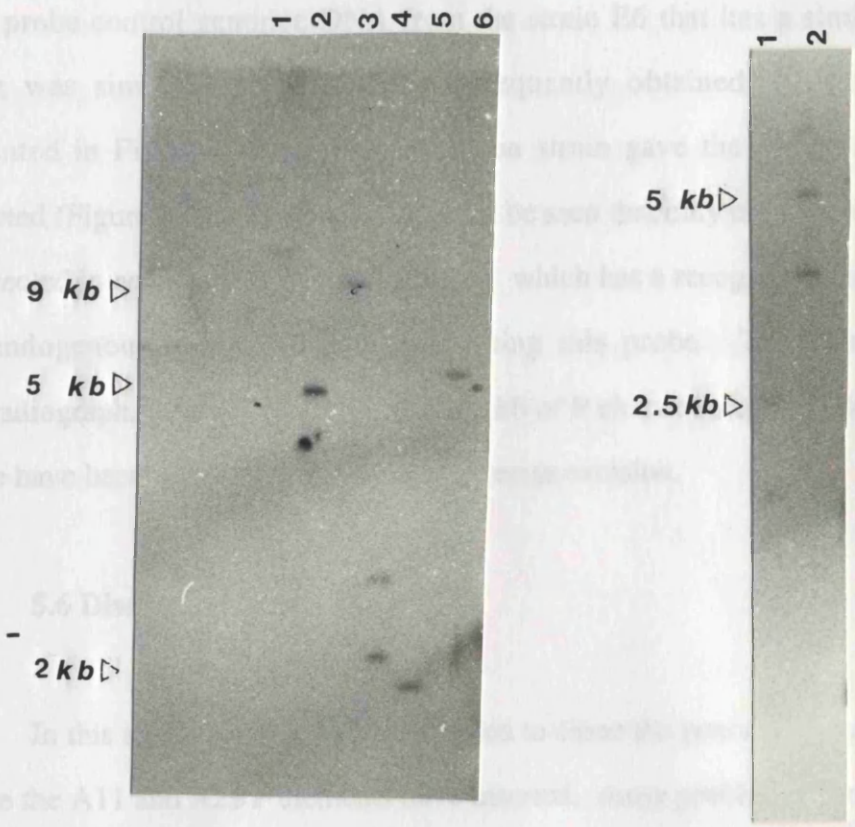


Figure 5.9 Legend

In **A**, A22-30 digested genomic DNA gave hybridisation signals as follows: 1) *Xba* I > 12 kb, 2) *Xho* I 4.9 kb, 3) *Eco* RI 9.0 kb, 4) *Acc* I 2.2 and 3.5 kb 5) *Cla* I 1.8 kb, 6) *Bam*HI 5.1 kb. In **B** the control insertion strain E6 gave hybridisation signals as follows: 1) *Cla* I 2.5 kb, 1.8 kb, and *Bam*HI 5.3 kb, 3.4 kb.

To begin this characterisation genomic DNA from the mutant strain A22-30 was digested with a variety of restriction enzymes, size fractionated in an agarose gel and probed with a 3.7 kb *Hind* III/*Xba* I fragment of the *ry* gene from the 5' region. As a probe control genomic DNA from the strain E6 that has a similar P element insert was similarly probed. The subsequently obtained autoradiographs are presented in Figure 5.9. The E6 insertion strain gave the hybridisation signals expected (Figure 5.9B). In Figure 5.9 it can be seen that only one hybridisation signal is detected in each sample except for *Acc* I, which has a recognition sequence within the endogenous *ry* sequence detected using this probe. Therefore, from this autoradiograph, it can be deduced that 3.7 kb of P element sequence detected by this probe have been lost during P element imprecise excision.

5.6 Discussion

In this series of experiments intended to clone the genomic DNA in the region where the A11 and A22 P elements have inserted, many problems were encountered. With hindsight, the attempted construction of subgenomic libraries was probably the wrong approach to be adopted. As with all libraries, the quality of insert DNA largely determines the level of success achieved. The CsCl method used to purify high molecular weight DNA (as described in Chapter 2) generally yields DNA of high quality, as was the case here. However, the selected size range of DNA, obtained by size fractionation on agarose gels may be of a much lower quality after restriction enzyme digestion and agarose fractionation, thus reducing the number of suitable ends available for ligation into any vector. Perhaps the use of high quality low melting point agarose may have been beneficial. The construction of standard genomic libraries using partially digested genomic DNA size fractionated by either sucrose or NaCl gradients may have been a more productive approach. A major problem with this approach was the size range of the fragments to be used as insert DNA. The restriction enzyme *Bgl* II has a hexanucleotide base pair recognition

sequence. If such a sequence is considered to occur randomly throughout the genome, then it will produce fragments on average of 4.1 kb in length, while the target sequence for A11 is 4.6 kb and 4.9 kb for A22 as determined by genomic Southern blot analysis (see Figure 5.3). Therefore, a large number of clones would have to be generated and screened if each desired fragment is to be isolated.

Despite several approaches used here in attempts to clone genomic sequences neighbouring the P elements of both A11 and A22, these sequences remain to be isolated. As the mutants isolated by imprecise excision of the A22 P element insertion and described in Chapter 4 have an interesting phenotype, further genetic characterisation of these mutants may provide interesting and potentially useful data on the nature of the ancestral A22 insertion locus.

Chapter 6

Genetic Analysis of the Excision-Derived Mutants

6.1 Section I Temperature Sensitivity Tests

6.1.1 Introduction

Each of the 19 recessive mutant strains generated by excision of the A22 P element were initially classified as either viable, semi-lethal or lethal as described in Chapter 4. Once balanced stocks were established, the semi-lethal and lethal classifications were found to be no longer valid, since the homozygotes were present in the correct Mendelian proportion among the balanced stocks. In addition, many of the recessive flies had either no obvious mutant wing phenotype, or the phenotype was less severe allowing homozygous stocks to be maintained in the absence of the TM3 balancer chromosome. There are a number of credible explanations that could account for this reduced severity of these mutations.

It has long been understood that the effects produced by a gene/mutation vary in dependence on both the environment and the genetic background. Timofeff-Ressovsky (1931) has proposed classifying such variations in terms of expressivity, penetrance and specificity. Expressivity is a measure of the amount of effect shown by the gene/mutation and is dependant on both the gene and the environment. The *Drosophila* mutant *Bar* demonstrates clearly the concept of expressivity, since the number of eye facets in *Bar* mutants is greatly influenced by temperature (Lindsley and Zimm, 1992). Penetrance is a measure of the frequency of which the gene shows any effect. The *Drosophila* mutant *giant* is a larval-specific mutation and under conditions of severe larval competition, very few *giants* will emerge, even though all *giant* larvae that show the phenotype, exhibit little or no variation in size (Lindsley and Zimm, 1992). Penetrance is eloquently described by Waddington (1950) "The rest of the genotype may be taken to define a 'landscape' along the main valley of which the character-producing reaction moves: but there are side valleys, and a gene acting at the right time may push the reaction out of its normal course into one of these. A gene with low penetrance provides a push that occasionally carries the

reaction out of the main valley: One with high penetrance is usually always successful in this". Specificity is used to describe the qualitative nature of the gene/mutation, i.e., "the variation in the course of the side valleys, gives a variation in the type, but not the effect of the gene mutation" (Waddington, 1950).

Such variations in phenotype may be influenced by a variety of environmental factors, such as temperature, nutrition and humidity. A variety of genetic components may also be involved, such as modifier genes that may act positively or negatively.

In the A22 excision-derived balancer stock flies exhibiting the mutant wing phenotype could still be seen many months after their initial isolation. Recessive modifier genes that act to reduce the severity of the phenotype and so improving the fitness of the mutant fly would normally be under positive selection in the mutant stocks. Those flies that do not carry such modifiers in their genome would have reduced fitness, unable to compete for food and mates. This may suggest the presence of modifying genes in the variable genetic background of the original mutant stocks. As observed in Chapter 4, the severe wing phenotype apparently reduces the life span of the fly by inhibiting movement on the damp food. Similarly, enhancing effect modifiers are not likely to play any important role in the phenotypic variation. Firstly, the mutants were generated by the mutation of a single locus, implying modifiers with an enhancing effect must have been present in the genome prior to the excision event. Such modifier mutations could possible arise in the mutant stocks after P element excision, but this occurring in 19 independently is highly unlikely. Secondly, such enhancers would act to decrease the fitness of the fly and would therefore, be selected against in the stocks, resulting in loss of the modifier. Clearly selection for recessive suppressors and against recessive enhancer loci has not occurred among the mutant strains since the variety of phenotypes can still be seen in the stocks.

Dominant modifier genes may also have as selective advantage and thereby become fixed in the population. Dominant modifier genes however, may not be

present in all mutant individuals, resulting therefore, in variation in the phenotype within the stocks. The reduced frequency of the wing phenotype might therefore be due to dominant modifier loci.

An important mechanism that gives rise to variations in phenotype is the phenomenon of position effect variagation (Judd, 1988; Tartoff and Henikoff, 1991). When a chromosomal region of heterochromatin becomes juxtaposed to a region of euchromatin. As a consequence of DNA rearrangements, the dense packing of the heterochromatic DNA may spread into the euchromatic domain, thus reducing the euchromatic domain. The amount of heterochromatic spread is thought to vary from cell to cell. The effect of this is to inhibit the expression of any genes that lie in those regions of chromatin that have become more tightly packaged. The A22 P element has previously been localised to chromosome bands 72BC by *in situ* hybridisation to the salivary gland polytene chromosomes (E. Zador, unpublished data). Salivary chromosome sections 1-19, 21-39, 42-60, 61-79 and 82-100 are considered euchromatic. Most known loci within these chromosomal regions have been found to variagate in appropriate chromosomal rearrangements (Spofford, 1976). Therefore the variation in phenotype of these mutants could possibly be due to this phenomenon. However, the high frequency with which these mutants were generated would suggest simple deletions rather than complex chromosomal rearrangements involving chromosome section 61-79 and heterochromatic domains, are responsible for the mutation.

The existence and identification of modifier genes that influence the severity and frequency of the mutant wing phenotype requires the initiation of a number of long-term genetic experiments. Initially, the entire genome of the A22-derived excision stocks, except the mutation itself, must be made wild type (isogenised) by out crossing to a wild type strain and by recombination to replace as much of the third chromosome as possible. If modifier genes have a suppressive effect on the mutant phenotype then the isogenised strains should show a severe phenotype. The modifier genes may then be localised by the construction of progeny that have the mutant

homozygous gene, and defined regions from the mutant genome. Those regions of the genome, and eventually any modifiers that have a major effect on the phenotype may then be identified.

Before embarking on such painstaking analysis it is wise to exclude the possibility that other, more simple phenomena may play a role in this variability. It has long been established that the effect of environment on phenotype is important. For example, children who carry the two copies of a defective phenylalanine hydroxylase gene will develop severe mental retardation when phenylalanine is present in the diet. If this amino acid is not consumed, then no mental retardation will occur. In *Drosophila* many mutant phenotypes have been found to be affected by temperature, such as *Cy* O (Lindsley and Zimm, 1992) and *Moonrat*, an allele of *hedgehog* (Felsenfeld and Kennison, 1995). Therefore, a wide variety of environmental effects may result in the phenotypic variation exhibited by these excision-derived mutants. In this respect it is worth noting that while the newly generated stocks were being balanced, and examined for recessive phenotypes, they were raised at a range of temperatures ranging from 16°C to 20°C, and have since been maintained at room temperature. The room temperature, as would be expected, varies significantly during the day. These changes in developmental temperature may be of significance. Two mutant strains that were initially scored as semi-lethal, A22-30 and A22-34, and two strains that were initially classified as viable, A22-32 and A22-33 were therefore selected for further characterisation in an attempt to determine the importance of temperature during development on the variation of the wing phenotype, and the semi-lethal and viable classifications.

Method.

Single male x female crosses for each of the selected mutant strains were incubated at 16°C, 20°C and 25°C. Oregon-R, a wild type strain was also raised and scored for wing defects as a positive control. For each strain and temperature, the same batch of fresh fly media was used to ensure any effects due to differences in food were minimised. After 4-5 days the parental flies were removed from the vials except those crosses incubated at 16°C, where the parents were allowed to mate and lay for 10-12 days. This longer laying period allows similar numbers of progenies to be scored, and the vials will be of relatively similar densities. Where possible, at least 1000 flies were scored for wing defects. Variation in the resulting mutant wing phenotype requires the mutants to be classified as "wild type", "dark", "blister", "coal", and "spoon". Dark wings are those that appear slightly darker and usually have one wing slightly shorter than the other and are considered as weak expressors. "Blister" refers to any form of blistering and includes a slight increase in melanisation of the wing. "Spoon" wings are short, dark, and concave, while "coal" wings are the severest form of blistering, where the wing forms a large black sac-like structure, often filled with a black fluid, and frequently has bubble-like structures, or vesicles within the swollen wing. Included in this group are few *vestigial*-like flies, where the wings are very dark and crusty, and shrivelled, which seem to be due to bursting or crushing the "coal" wings and the fluid escaping. A22-32 flies raised at 20°C are shown in Plate 6.1.

6.1.2 Results

The flies scored for each of the strains at each of the temperatures have been presented below as percentages in Table 6.1.1. The raw data for each strain has been presented in Appendix 2. The Oregon-R wild type strain did not give any progeny that had similar wing phenotypes scored as mutant in the three A22-derived mutant strains at either of the three temperatures.

Table. 6.1.1 The Percentage of Mutant Progeny and their Phenotypes Scored at Three Different Temperatures

Strain	Temp	Percentage Mutant Progeny With Each Phenotype Class								
		wt	dark	blister	spoon	coal	A_{lethal}	P_{lethal}	E_{lethal}	T_{mut}
A22-30	16°C	N.D	N.D	N.D	N.D	N.D	65.1	27.0	7.8	100
A22-30	20°C	98.9	0.7	0.4	0	0	N.D	N.D	N.D	1.1
A22-30	25°C	97.9	2.1	0	0	0	N.D	N.D	N.D	2.1
A22-32	16°C	N.D	N.D	N.D	N.D	N.D	37.9	48.1	14.0	100
A22-32	20°C	51.4	14.6	15.2	15.6	3.2	N.D	N.D	N.D	48.6
A22-32	25°C	91.3	6.9	0.4	1.2	0.1	N.D	N.D	N.D	8.6
A22-33	16°C	N.D	N.D	N.D	N.D	N. D	34.8	56.5	8.7	100
A22-33	20°C	23.3	20.9	34.2	17.7	3.9	N.D	N.D	N.D	76.7
A22-33	25°C	84.8	10.2	1.4	3.6	0	N.D	N.D	N.D	15.2
A22-34	16°C	N.D	N.D	N.D	N.D	N. D	74.7	15.0	10.3	100
A22-34	20°C	59.1	19.8	6.9	12.6	1.6	N.D	N.D	N.D	40.9
A22-34	25°C	93.6	6.0	0	0.3	0	N.D	N.D	N.D	6.3

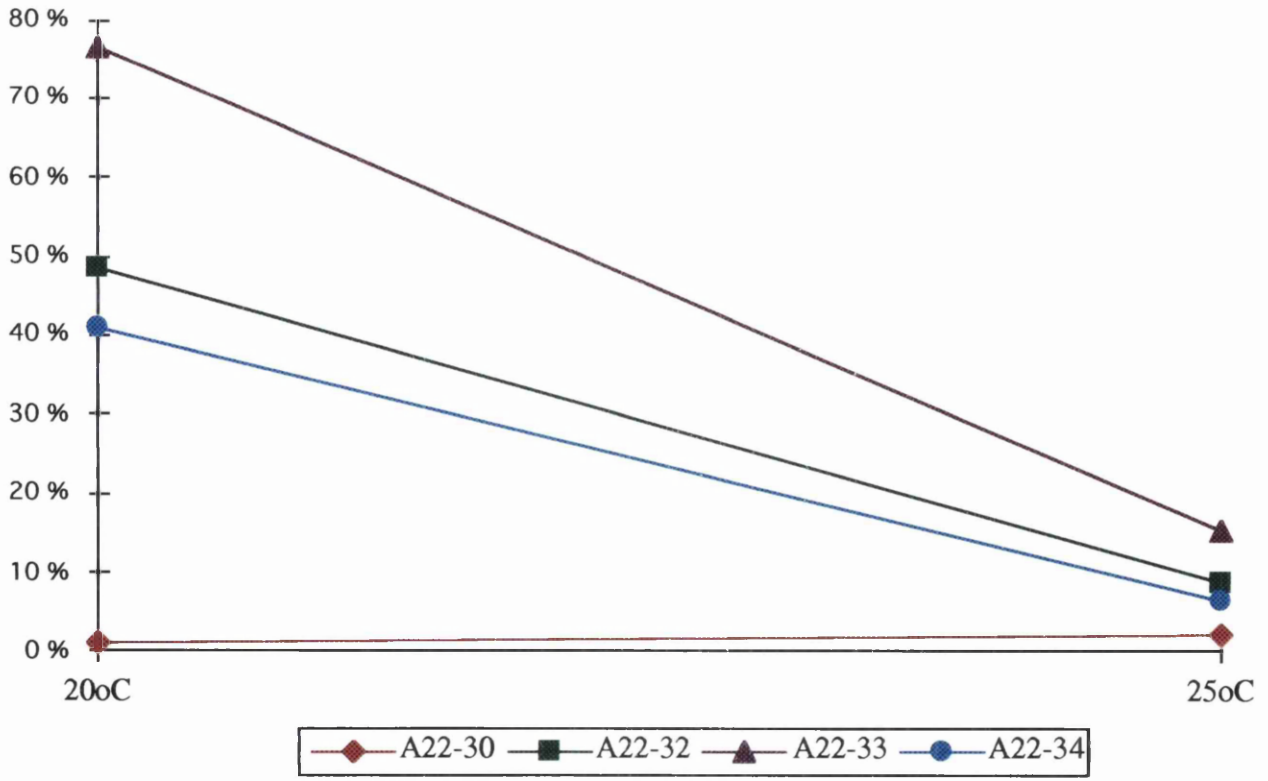
The percentage of flies of each phenotypic class are shown. A_{lethal}; adult lethal, P_{lethal}; pupal lethal, E_{lethal}; eclosion lethal. T_{mut} refers to the percentage flies that have the mutant wing phenotype, or are lethal at 16°C. N.D.-Not Determined. For each strain, $n > 250$. At 20°C and 25°C the three lethal classes were not scored, since extensive lethality was not expected at these temperatures and no noticeable levels of pupal lethality were noticed. The variation in wing phenotype was difficult to determine in dead pupae and adults found dead in the semi-liquid food. The numbers scored for each strain in each phenotypic class for each of the temperatures are presented in Appendix 2. The OR-R wild type strain with similar phenotypes at either of the three developmental temperatures.

There are clear differences within each of the mutant strains when grown at different temperatures. This is most easily demonstrated by examining the number of flies scored in each phenotypic class as a percentage of the total flies scored. At 25°C there are very low percentages of phenotypically mutant flies in each of the four strains, A22-33 having the highest level of 15.2%. The levels of phenotypically mutant flies increase greatly in each strain when the mutants are raised at 20°C except for A22-30, which has only 1.1% of expressors at this temperature. 48.6% of A22-32, 76.7% of A22-33 and 40.9% of A22-34 flies were classed as phenotypically mutant at 20°C. This similarity is graphically presented in Figure 6.1.1. Clearly, the gradients are closest for A22-32 and A22-34, while A22-30 and A22-33 clearly have very different gradients, and therefore different sensitivities to developmental temperature.

From the data collected, it is clear that for at least three of the mutant wing phenotype is temperature dependent. The "dark" phenotype is the least severe manifestation of the mutations, while the "spoon" classification, at least visibly, does not seem to be as severe as the "blister" and "coal" phenotypes. The percentages for all four mutants have presented in Figure 6.1.2. Here, the "blister" and "coal" phenotypes have been grouped since both involve the separation of the wing epithelia.

From Figure 6.1.2 and the data, a number of generalisations can be drawn. As the temperature of development is decreased all strains except A22-30 exhibit an increase in the frequency of "dark" wings, "spoon" wings, and "blister" wings. At 20°C, A22-32 had a higher level of "coal" than "spoon" wings, while the "dark" wings were the least frequent. The frequency of "blistered" wings, including the "coal" wings was found to be much higher than "dark" wings in the strain A22-33. The least frequent phenotype in this strain is "spoon". These raw data have been tested using the χ^2 test where possible (A22-30 raised at 20°C, and the data collected for development at 25°C can not be used in these tests since some of the scores are less than 5). If the severity of the mutation is reflected by the severity of the defect during wing development, then these generalisations should allow the strains to be

Figure 6.1.1 Relationship of Wing Phenotype Variation with Temperature



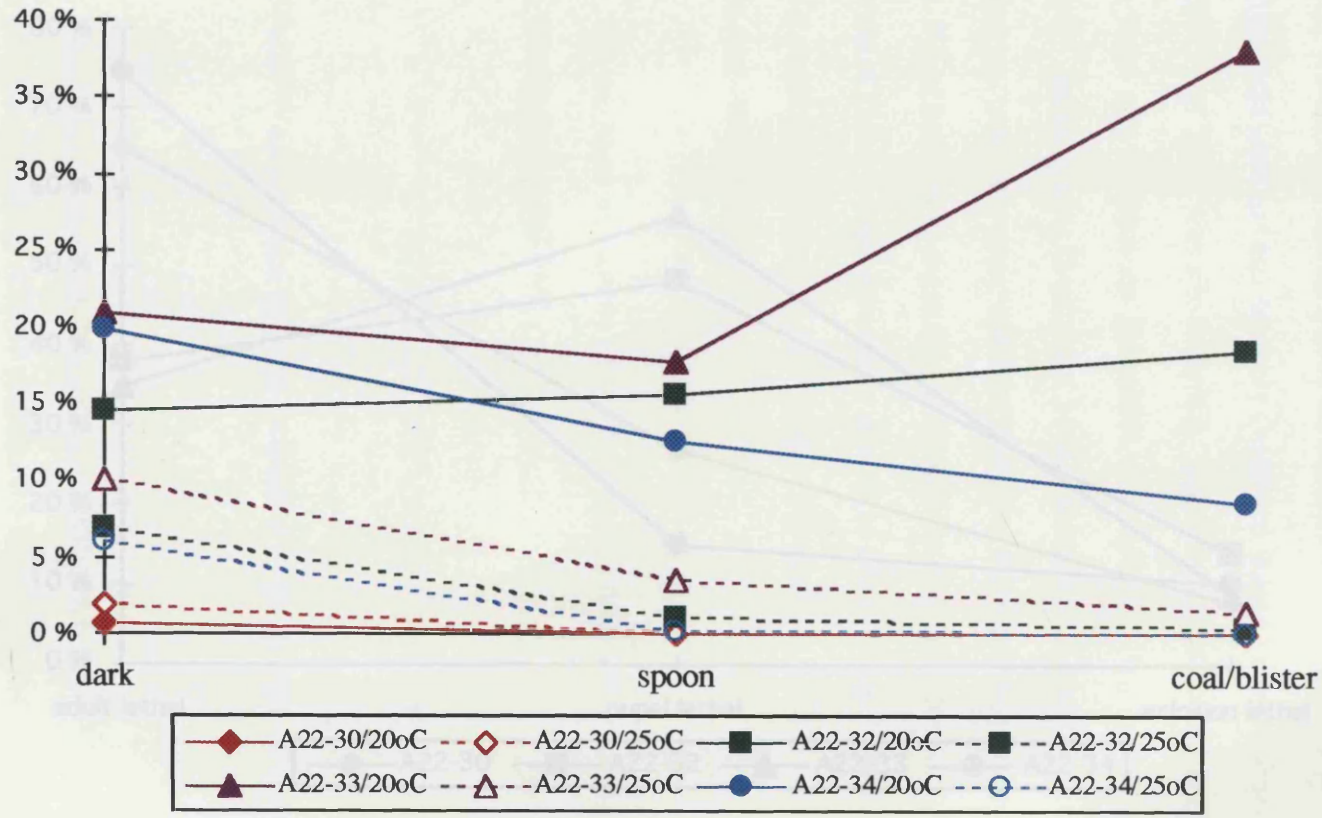
The percentage of all wing phenotypes versus developmental temperature are illustrated for four of the A22-excision-derived mutant strains (see Table 6.1.1). All wing phenotypes is described in the methods section of the text and the raw data is presented in Appendix 2. The frequency of the wing phenotype increases as the developmental temperature is lowered, except for the strain A22-30.

Plate 6.1 Legend

Variation in the wing phenotype exhibited by A22-32 mutants when raised at 20°C; top left male with crushed "coal" wing, top right male with slightly blistered wing, centre left male with "coal" wing, centre female with both wings short and blistered, centre right female with one wing short and blistered, bottom left female with one "spoon" wing, bottom right male with single weak "spoon" wing. Definitions of wing phenotypes are described in the text.

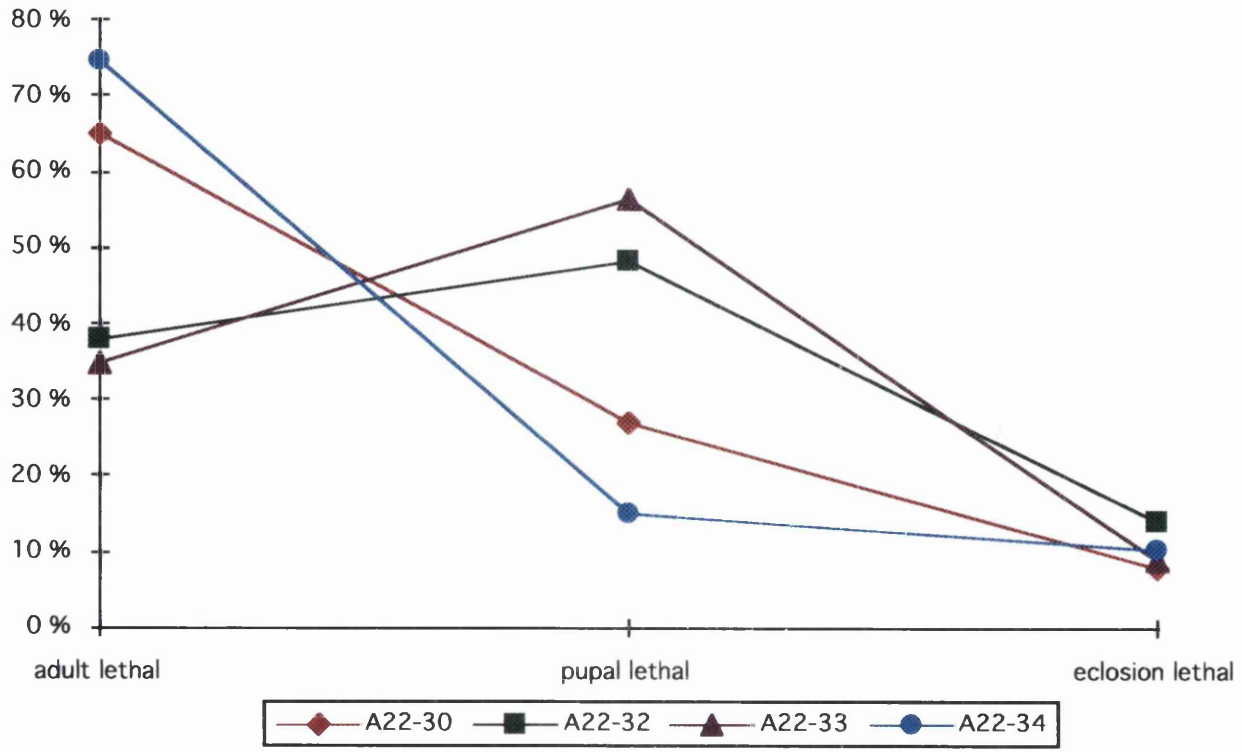
Plate 6.1

Figure 6.1.2 Phenotypic Severity and Relationship with Temperature



The percentages of the different mutant wing phenotypic classes are presented (see Table 6.1.1). Each mutant wing class is described in the Method section of the text. The classes "coal" and "blister" have been combined. Clearly, the frequency of phenotypically mutant wings is greatly increased for each of the strains A22-32, A22-33 and A22-34 are raised at 20°C rather than 25°C.

Figure 6.1.3 Percentage Progeny in the Three Lethal Classes Raised at 16°C



The percentages of the three lethal classes detected when four of the excision-derived mutant strains raised at 16°C are illustrated (see Table 6.1.1). Each lethal class is described in the Method section of the text and the raw data is presented in Appendix 2. A22-30 and A22-34 appear to have similar frequencies as do A22-32 and A22-33.

classified in an allelic series. The A22-33 mutation seems to be more sensitive to the effect of temperature than A22-32, while A22-32 seems to be more sensitive than A22-34. A22-30 is the least sensitive to the effect of the lower temperature of 20°C.

At the lower temperature of 16°C, there is a high level of pupal and adult lethality. Those flies that do eclose do not survive long enough to reproduce. Many dead flies were seen to have severely deformed wings. The level of pupal lethality at both 25°C and 20°C was not recorded, since significant numbers of dead pupae or adults were not obvious on the sides of the vials, while many adults eclosed. However, these mutant strains can been maintained as homozygous stocks at room temperature, implying pupal and adult lethality at these temperatures does not readily occur. Pupal lethality is most frequently seen in the mutant strains A22-32 and A22-33, while A22-34 is least frequently scored as pupal lethal. The mutant strain A22-30 has a pupal-lethal frequency somewhere between the A22-32, A22-33 frequencies and the A22-34 frequency.

On the basis of mutant wing phenotype frequency, a tentative allelic series can be constructed as follows:

$$\text{A22-33} > \text{A22-32} = \text{A22-34} > \text{A22-30}$$

where A22-33 exhibits the greatest frequency of deformed wings at both 20°C and 25°C.

On the basis of percentage pupal lethality seen at 16°C a similar allelic series can be constructed where A22-33 exhibits the highest degree of pupal lethality.

$$\text{A22-33} = \text{A22-32} > \text{A22-30} \geq \text{A22-34}$$

This tentative allelic series is supported when the data is presented as a graph (Figure 6.1.3). From this graph, A22-30 and A22-34 appear similar, as do the mutant strains A22-32 and A22-33. The apparent similarities and differences seen when the percentages are examined, have been tested using the $\chi 2$ statistical test in an attempt to confirm the four tentative allelic series. These calculations have been presented in Table 6.1.3..

Table 6.1.2 χ^2Values Comparisons of the Four Mutant Strains Exhibiting Lethality at 16°C

Strain	Strain	Temp (°C)	d.f	χ^2	P
A22-30	A22-32	16	2	185.5	0.01
A22-30	A22-33	16	2	198.8	0.01
A22-30	A22-34	16	2	16.8	0.01
A22-32	A22-33	16	2	18.4	0.01
A22-32	A22-34	16	2	118.8	0.01
A22-33	A22-34	16	2	139.5	0.01

Each strain is statistically pair-wise compared to each of the others at each developmental temperature using the χ^2 test. For each test the numbers of progeny of each strain at 16°C scored as adult lethal, pupal lethal and eclosion lethal (presented in Appendix 2) were used. d.f is degrees of freedom. P is the probability. With 2 degrees of freedom, the probability of observing a χ^2 value greater than 13.82 is 1%. A χ^2 value greater than this would indicate the two populations are not significantly different.

Table 6.1.3 χ^2Values Comparisons of the Four Mutant Strains Exhibiting The Wing Phenotype when raised at 20°C

Strain	Strain	Temp (°C)	d.f	χ^2	P
A22-30	A22-32	20	1	625.9	0.01
A22-30	A22-33	20	1	1043.1	0.01
A22-30	A22-34	20	1	489.4	0.01
A22-32	A22-33	20	1	125.4	0.01
A22-32	A22-34	20	1	13.0	0.01
A22-33	A22-34	20	1	195.5	0.01

Each strain is statistically pair-wise compared to each of the others at each developmental temperature using the χ^2 test. For each test the numbers of progeny of each strain at 20°C scored as adult lethal, pupal lethal and eclosion lethal (presented in Appendix 2) were used. d.f is degrees of freedom. P is the probability. With 1 degree of freedom, the probability of observing a χ^2 value greater than 10.83 is 1%. A χ^2 value greater than this would indicate the two populations are not significantly similar.

Table 6.1.4 χ^2Values Comparisons of the Four Mutant Strains Exhibiting The Wing Phenotype when raised at 25°C

Strain	Strain	Temp (°C)	d.f	χ^2	P
A22-30	A22-32	25	1	49.7	0.01
A22-30	A22-33	25	1	129.0	0.01
A22-30	A22-34	25	1	21.52	0.01
A22-32	A22-33	25	1	21.31	0.01
A22-32	A22-34	25	1	2.9	0.01
A22-33	A22-34	25	1	28.8	0.01

Each strain is statistically pair-wise compared to each of the others at each developmental temperature using the χ^2 test. For each test the numbers of progeny of each strain at 25°C scored as adult lethal, pupal lethal and eclosion lethal (presented in Appendix 2) were used. d.f is degrees of freedom. P is the probability. With 1 degree of freedom, the probability of observing a χ^2 value greater than 10.83 is 1%. A χ^2 value greater than this would indicate the two populations are not ssignificantly similar.

6.1.3 Discussion

On the basis of the χ^2 calculations, presented in Tables 6.1.2, 6.1.3 and 6.1.4, there are appears to be a small difference in the developmental time of lethality exhibited by the strains A22-32 and A22-33 when raised at 16°C. There appears to be no difference in the frequency of the mutant wing phenotype in strains A22-32 and A22-34 when raised at 25°C, although there is a difference between these two mutant strains when they are raised at 20°C.

The four mutant strains derived from the A22-mutagenesis and characterised here, are clearly cold-sensitive. At high developmental temperatures a low frequency of homozygous flies have defects in wing development. At an intermediary developmental temperature, increased frequencies of flies in 3 of the 4 strains tested have phenotypically mutant wings. At the lower developmental temperature all four of the mutant strains are late pupal/adult lethal. The cold-sensitivity is recessive since balanced stocks can be maintained at the lower temperature of 16°C.

Many mutants in a wide variety of developmental systems such as T4 (Scotti, 1968), *E. coli* (O'Donovan, 1965), yeast and *Drosophila* (Falke and Wright, 1975) have been shown to be cold-sensitive. Many of these mutations result in the disruption of the regulatory properties of allosteric proteins (O'Donovan, 1965) or in the defective assembly of the ribosomes (Guthrie *et al.*, 1969). In *Drosophila*, a number of loci clustered on the X chromosome were found to be cold-sensitive and required for ribosome assembly (Falke and Wright, 1975).

Since the mutants used in this study were derived by P element excision mutagenesis using a marked P element, it is unlikely each of the mutants produce a protein that is mutant due to a single amino acid substitution that renders it unstable at low temperatures, but rather are mutant due to the deletion of a stretch of DNA that encodes sevseral amino acids or of regulatory sequences. Mutants such as *bobbed* that are defective in ribosome assembly, frequently exhibit thin, short bristles and abnormal abdomens (Wright, 1973) but these external phenotypes have not been

detected in the mutants studied here. Additionally, the mutations that disrupt ribosome assembly in *Drosophila* appear to be clustered on the X chromosome (Steffenson, 1973). These observations argue that the A22-derived mutants are not defective in either ribosome assembly, or in the allosteric regulation of the mutant product.

There may be a variety of developmental processes, that when disrupted could lead to the cold-sensitive phenotype exhibited by these mutants. The adhesion of the wing epithelia to the extracellular matrix, as previously discussed in the Chapter 4 may be defective. If this is so, the affected process is worsened by lower temperatures of development, at least in three of the mutants studied here. Defects in such adhesion mechanisms could explain the pupal/adult lethality seen at 16°C. The mutants *inflated* and *myospheroid* have defects in muscle attachment in the pupae, and this is thought to inhibit the flies escaping from the pupal case, since their legs are weakened considerably (N. Brown, Cambridge, pers. comm.). Such loose attachments may degenerate in those flies that succeed in eclosion, leading to premature lethality.

In the yeast *S. cerevisiae*, two genes believed to be required in the mechanism of protein folding, namely the chaperonins, have been found to be cold-sensitive. These two mutations result in defective tubulin and actin assembly *in vivo* (Chen *et al.*, 1994), and a cold-sensitive mutation in an actin gene in yeast has also been isolated (Kolling, 1994). Proteins such as actin and tubulin are involved in many aspects of cell structure and motility. This is suggestive of inappropriate cell shape during development. For example, during pupal development, the cytoskeleton is essential in the formation of the transalar arrays during the inflated stages of wing development (Fristrom *et al.*, 1993). If these structures are not properly formed, inflated phenotypes may result (Waddington, 1941).

6.2 Section II Complementation Analysis

6.2 1 Introduction

A number of predictions can be made about the 19 mutant strains. The mutants were generated by the excision of a single marked P element, thereby limiting the loci that can be mutated be imprecise excision, to either one or both sides of the P element insertion sites illustrated in Figure 4.1. Of the mutants, two were scored as lethal, 8 as semi-lethal, and 9 as viable, when initially isolated. This may indicate the generation of three allelic classes. The 19 strains have the same type of wing defects (including after several generations the two lethal lines) making it likely the same gene has been mutated in each of the strains. The lethality may indicate, the mutation (at least partially) of an essential gene in addition to the gene being required for proper wing development. Given that the flies are temperature sensitive in both terms of lethality and the wing syndrome, the most plausible explanation is that the mutation of a single locus has occurred, at least until this has been shown not to be the case. This has been tested by complementation, at both 16°C and 20°C.

Method

The same four A22-derived mutant strains used in the temperature sensitivity tests were again selected to test for complementation. Each combinations was tested by single male x female matings, again, by setting up a number of vials of each cross. Each complementation test was carried out at both 20°C and 16°C, allowing the complementation properties of both the mutant wing syndrome and the pupal/adult lethality to be tested.

6.2.2 Results

Table 6.2.1 Complementation Analysis. Percentage Progeny Scored at 16°C

	Percentage Heterozygous Progeny With Each Phenotype Class								
	20°C					16°C			
Genotype	wt	dark	blister	spoon	coal	A_{lethal}	P_{lethal}	E_{lethal}	T_{mut}
A22-30/A22-33	N. D	N. D	N. D	N. D	N. D	43.1	49.0	7.9	100
A22-30/A22-33	60.4	12.8	15.0	7.5	4.3	N. D	N. D	N. D	39.6
A22-30/A22-34	N. D	N. D	N. D	N. D	N. D	68.9	21.2	9.9	100
A22-30/A22-34	82.8	5.7	1.8	4.9	4.7	N. D	N. D	N. D	17.1
A22-32/A22-30	N. D	N. D	N. D	N. D	N. D	54.8	36.1	9.1	100
A22-32/A22-30	87.2	2.4	4.8	4.8	1.0	N. D	N. D	N. D	13.0
A22-32/A22-33	N. D	N. D	N. D	N. D	N. D	34.2	59.0	6.8	100
A22-32/A22-33	41.3	18.8	22.5	15.9	1.4	N. D	N. D	N. D	58.6
A22-32/A22-34	N. D	N. D	N. D	N. D	N. D	52.6	37.6	9.8	100
A22-32/A22-34	55.4	12.7	10.8	17.2	3.9	N. D	N. D	N. D	44.6
A22-34/A22-33	N. D	N. D	N. D	N. D	N. D	73.5	17.9	8.6	100
A22-34/A22-33	47.4	22.0	10.5	17.8	2.4	N. D	N. D	N. D	52.7

The percentage of flies of each phenotypic class are shown. A_{lethal}; adult lethal, P_{lethal}; pupal lethal, E_{lethal}; eclosion lethal. T_{mut} refers to the percentage flies that have the mutant wing phenotype, or are lethal at 16°C. N.D.-Not Determined. For each test n > 100. At 20°C and 25°C the three lethal classes were not scored, since no noticeable levels of pupal lethality were noticed. The variation in wing phenotype was difficult to determine in dead pupae and adults found dead in the semi-liquid food. The numbers scored for each strain in each phenotypic class for each of the temperatures are presented in Appendix 3.

This complementation analysis has confirmed the mutant loci in each of the strains are the same since the progeny of the complementation crosses all lie within the three lethal classes seen for the homozygous progeny at 16°C.

The severity of the mutation varies among each strain as described in the Temperature sensitivity tests in Section I of this Chapter. Using the percentages obtained for each lethal class in the heterozygotes and the homozygotes as presented in Tables 6.1.1 and 6.2.1, it may be possible to generally classify the severity of the four mutant chromosomes with respect to each other. However, such strict comparisons between the two experiments are inappropriate as these complementation crosses were not performed in parallel with the temperature sensitivity tests.

Complementation at 16°C

1. A22-32 homozygotes have approximately twice the rate of A22-30 pupal lethality, and the A22-32/A22-30 heterozygote has a level of pupal lethality midway between the two parental homozygotes, indicating the A22-30 chromosome may give a less severe phenotype than A22-32.
2. The A22-32 homozygotes have approximately three times the rate of pupal lethality seen for A22-34, while the A22-32/ A22-34 heterozygote level of pupal lethality has approximately twice the level of that seen for A22-34. Therefore, the heterozygote has one third of the A22-32 pupal lethality and two thirds of the A22-34 level. This may indicate A22-32 flies have a more severe mutation than A22-34 flies.
3. The A22-30 homozygotes have a pupal lethality level approximately twice the rate of A22-34 homozygotes, while the A22-30/A22-34 heterozygous progeny have a level of pupal lethality midway between the parental homozygous strains. Here, A22-30 can be generally judged to have a more severe mutation than that of the A22-34 strain.

4. A22-33 homozygotes show almost four times the ratio of pupal lethality exhibited by A22-34 homozygotes. The A22-34/A22-33 heterozygotes seem to exhibit a level of pupal lethality almost equal to the frequency of A22-34 homozygotes.

Complementation at 20°C

1. The A22-30/A22-33 heterozygous progeny have a frequency of mutant wing phenotypes approximately midway between that of the parental homozygous mutants.
2. The heterozygous A22-32/A22-30 progeny have a higher percentage of phenotypically mutant flies than the A22-30 homozygotes, but lower than the A22-32 homozygous progeny.
3. The A22-34/A22-30 flies have a higher frequency of phenotypically mutant flies than is seen for the A22-30/A22-30 strain.
4. The heterozygous A22-33/A22-32 flies have a mutant wing frequency that is midway between the frequencies of phenotypically mutant wings scored for the parental A22-33 and A22-32 homozygous flies.
5. The A22-32/A22-34 flies have a similar percentage of flies scored as having mutant wings to the A22-32 and A22-34 homozygous strains.
6. The heterozygous flies, A22-33/A22-34, have a level of phenotypically mutant wings approximately midway between A22-33 and A22-34 homozygous strains.

Comparing the proportion of each phenotypic class of both the homozygous and heterozygous flies raised at 20°C, supports the notion that in general, the heterozygous progeny express the mutant phenotypes at a frequency midway between, or very similar to the parental homozygous frequencies. Some differences are notable however. The heterozygous A22-30/A22-34 shows a considerable difference in the frequency of 'dark" but similar level of "coal/blister" wings when compared to A22-34/A22-34. The frequency of each phenotypic class in the

heterozygous progeny are almost equal, although low, while the frequency of each class in A22-34 decreases as the severity of the phenotype increases. The A22-30/A22-33 heterozygous progeny have very similar levels of "spoon" wings but very different frequencies of "coal/blister" wings when compared to the homozygous A22-33. The homozygous A22-33 and A22-34 mutant strains, as with the A22-33/A22-34 heterozygous progeny have very similar levels of "dark" and "spoon" frequencies. The heterozygous progeny, however has a similar level of frequency of "blister/coal" progeny, to A22-34. The high frequency of this phenotype seen in the A22-33 strain is not reflected in the A22-33/A22-34 heterozygous flies.

6.2.3 Discussion

The wing phenotype of the A22-derived mutants clearly is due to a single mutation since none of the four strains are capable of complementing the others. Similarly, the lethality exhibited by these mutants when raised at 16^{o}C is due to the same mutation since the four strains fail to complement at the reduced temperature of development. This single complementation class is indicative of a single gene being mutated in each of these strains. As discussed previously, it is possible that two different genes may be mutated by imprecise excision of the P element (see Figure 4.1, -1 and -2) and two complementation groups may be generated.

If the gene mutated in these mutant strains is to be considered to be more severely disrupted in some strains than in others, then the frequency of the different phenotypes in the heterozygous progeny would be expected to be somewhere between the two homozygous strains frequencies particularly with respect to the wing phenotype. Wing development in each individual will not occur at precisely the same time point, and the variation may reflect small fluctuations in temperature during the larval and pupal stages. Care should be taken not to overstate the comparisons between the temperature sensitivity and the complementation tests can be informal only since the homozygous and heterozygous progeny were not raised in parallel.

6.3 Section III Deficiency Mapping

6.3.1 Introduction

Deficiency mapping of the four selected A22-derived mutants have been undertaken, in an attempt to genetically confirm the localisation of the P element on the salivary gland chromosomes. If this localisation is accurate, then the A22-derived mutants should be uncovered when made heterozygous with deletions known to span. The *in situ* hybridisation experiments could not distinguish between bands 72B and 72C, and so by using deficiencies known to have a deletion breakpoint in either band, B or C, it may be possible, genetically, to distinguish in which of these bands, the A22 P element was inserted.

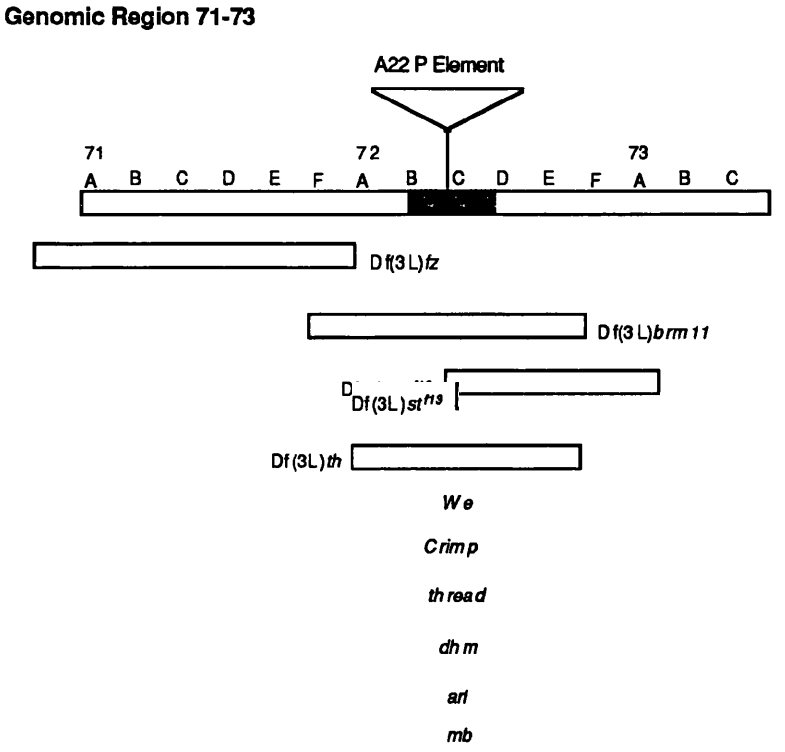
Four Deficiency strains were obtained from the Stock Centres (see Materials and Methods) and the genomic region deleted in each chromosome are depicted in Figure 6.3.1.

Df(3L)*fzM21* should complement the mutant phenotype while Df(3L)*brm 11* and Df(3L)th^{ss102} should not. The deficiency chromosome Df(3L)st^{f13} may or may not uncover the A22-derived mutation. If the new mutation is within 72B, then no mutant phenotype will be seen when in combination with the Df(3L)st^{f13}. However if the new deletion is located within 72C, then Df(3L)st^{f13}/A22-derived mutation heterozygous flies should exhibit the wing phenotype.

The temperature sensitivity tests have shown the phenotype to be cold-sensitive, the A22-32 mutation being homozygous pupal lethal at 16°C. There are a number of homozygous lethal loci located in bands 72A and 72D. If the A22 P element localisation was inaccurately interpreted, the mutants may be allelic to any one of these lethal loci, although none of these recorded lethals have been reported to be temperature sensitive, or associated with abnormally developed wings.

The presence of a large number of lethal loci in chromosome band 73A is conspicuous; l(3)*Ab* and l(3)*Ag*, both located in this band are pupal or pupal/adult lethals, while l(3)73*Af*, also located in band 73A has been classed as L3/pupal lethal (McKeowan *et al.* 1987).

Figure 6.3.1 Deletions and Known Genes in the Vicinity of the A22 Insertion Region



The genomic region where the A22 P element construct is detected by *in situ* hybridisation to polytene salivary gland chromosomes. Previously identified mutations and their location are indicated (as described in Lindsley and Zimm, 1992) Each deletion is represented as an open rectangle.

6.3.2 Results

The Df(3L)*brm11* deficiency in combination with the Df(3L)th^{ss102} or the Df(3L)st^{f13} deficiency is lethal as is the Df(3L)th^{ss102}/Df(3L)st^{f13} combination. This provides a suitable control in establishing the authenticity of these deletions, and the appropriate crossing schemes confirmed the identity of these three chromosomes, and the deletion analysis was then initiated. Since each of the mutants are derived from the excision of a single marked P element, and each should therefore have a mutation in the same locus, only one of the strains was used in these analyses. The crosses for each of the deletion tests were raised at 25°C, 20°C and 16°C, except for the Df(3L)*fz* crosses which were raised at 25°C and 20°C and scored for the wing phenotype or the lethal phenotypes as appropriate and as previously established in Section I of this Chapter.

From the data collected and presented in Appendix4, the mutation generated by excision of the A22 P element is complemented by the Df(3L)*fz* chromosome that covers chromosome bands 71A-F, as expected if the *in situ* data is accurate.

The Df(3L)*brm 11* deficiency gave a very low frequency of flies that were defective in the development of wing disc derivatives when in combination with the A22-32 mutant chromosome at the developmental temperature of 25°C. A single female had a small blister on one wing blade, while a second female had a severely misshapen thorax on one side. The wing on this side had failed to expand and a small lump of crusty material replaced the haltere, while the other side of the fly had developed normally. A third female had "floppy" wings, that were not convincingly deformed, but seem to be due to damage incurred in the vial. Both wings in a male fly had failed to expand. Remarkably a male fly was found to have three apparently healthy wings (see Plate 6.2).

Plate 6.2 Legend

The generation of this fly was as described in the text. A: frontal view of the Df(3L)*brm 11*/A22-32 fly with three wings. B: dorsal view of the same fly, note the lack of dorsal cuticle on that side of the fly that has developed the additional wing.

Plate 6.2

A

B

6.3.3 Discussion

The wing disc on one side of the fly had apparently developed and differentiated two wing blades rather than a wing blade and the dorsal thorax. This fly, not surprisingly, failed to mate with a number of virgin females. Since this male fly was particularly impressive, but only detected at very low frequency, this cross was repeated as before, in a number of male x female pair-wise crosses. No defective progeny were found in the repeat experiment, or when the crosses were raised at 20°C. In both tests, no significant levels of pupal lethality were detected, as would be expected at both 20°C and 25°C if the Df(3L)*brm 11* deficiency did not complement the A22-derived mutation. These phenotypes, including the fly with three wings, are likely, therefore, to be just "background deformities" since they are at such a low frequency.

The A22-32 deletion was also complemented by both the Df(3L)th^{ss102} and Df(3L)st^{f13} deletion chromosomes indicating in the*in situ* hybridisation data was inaccurate. These deletion analyses is discussed further in Chapter 8.

Chapter 7

Behaviour Analysis of the Excision-Derived Mutants

7.1 Introduction

The variation in wing phenotype of the A22-derived mutants, as previously shown in Chapter 6, is dependant on developmental temperature. During routine maintenance of the mutant stocks, the flies appeared to have poor flight ability and mobility as previously discussed in Chapter 4. Those mutants that exhibit severe disruption of wing development will obviously be unable to fly. However those that exhibit little or no abnormal wing phenotype may have poor flight ability as a result of defects that may be present but are not directly visible. Such defects may be neural, muscular or cuticular.

Flight is a complex behaviour requiring both sensory and mechanical precision in addition to strong cuticular structures. Many defects in the anatomy of the fly may therefore hinder flight. The fact that the *myospheroid*^{nj42} allele has defects in its jumping ability in addition to the wing defects (de le Pompa *et al.*, 1989) is not surprising since strong alleles show defects in muscle development during embryogenesis (Leptin *et al.*, 1989).

Two easily assayed flight behaviour components are the jump reflex, that enables the fly to become air borne, and the wing motions that maintain flight. There are a number of assays that may be employed to test flying ability, and great care must be taken in choosing the appropriate test or paradigm. The wing motion required to maintain and propel the fly in the air can be assayed in a number of paradigms, the simplest of which is to induce flight by shaking the flies inside a container and observing any flies that may fly as described by Homyk (1977). Wild type *Drosophila* may be induced to jump when startled either by banging a vial containing the fly or banging an object in close proximity to the fly. However, a number of mutants have been found to be bang-sensitive, and although able to jump and fly, they do not do so in response to the bang stimulus. Alternatively, they may be startled and induced to jump by an on/off light stimulus. Such a response depends

on the perception and accurate processing of the visual stimulation. As an initial investigation of the four selected mutants derived by the imprecise excision of the A22 P element and previously characterised in Chapter 6, I have tested their ability to jump in response to a banging stimulus following the work of Homyk (1977). Firstly, this is a simple test and avoids light perception that may not be normal in these *ry*⁻ mutants. Secondly, Palka (1994) has reported abnormal walking phototaxis and no phototaxis in the Benzer countercurrent apparatus in flies that have damaged wings. Therefore, no response to a light on/off stimulus may be a result of many diverse anatomical defects, and more specifically in these mutants, due to the defective, however visibly minor, wing rather than the jump behaviour itself. However, if the mutants fail to jump in response to the test chosen, other paradigms should then be considered.

7.2 Results

7.2.1 Flight Ability

The ability of the mutant flies to fly was tested using the apparatus described in Chapter 2. Here, each of the four selected mutants were tested as were the control strains*dumpy*, that does not fly but can jump, the original A22 insertion strain and the wild type strain OR-R. The original A22 strain was included since it has not previously been tested and any flying defect may possibly be present in the original strain, and not as a direct result of the excision events, while OR-R was used as a positive control. Each strain was raised in parallel at 25°C and 20°C, and tested for flight ability, at 5-10 days after eclosion. Only those flies that had no severe disruption to wing development were tested for flight. The flies raised at 25°C, were not exposed to CO_2 anaesthesia, being scored for wing defects following the flight assay, while those raised at 20°C were allowed to recover from anaesthesia O/N at 20°C. Flies raised at this temperature exhibit the mutant wing phenotype more

frequently than those raised at 25°C as demonstrated in Chapter 6, and so those with abnormal wings were removed from the population prior to the flight test.

For each flight assay, both male and female flies were dropped into the central chamber of the flight box described in Chapter 2, the lid replaced and the electricity was turned immediately on. The box was then rigorously shaken and bumped for approximately one minute. When the mutant strains were being tested, the box was left still for a period of 10-20 minutes, before the flies were sprayed with 70% ethanol after the electricity was turned off. The number of flies that flew out of the central chamber and those that did not are presented in Table 7.1.

Table 7.1 Flight Ability

Strain	Temp (°C)	fliers	non-fliers	Total	% non fliers
OR-R	20	64	29	93	31.2
dumpy	20	73	56	129	43.4
A22	20	59	64	123	52.0
A22-30	20	67	59	126	46.8
A22-32	20	78	89	167	53.3
A22-33	20	82	91	173	52.6
A22-34	20	48	66	114	57.9
OR-R	25	74	23	97	23.7
dumpy	25	4	283	287	98.6
A22	25	67	77	144	31.5
A22-30	25	6	213	219	97.3
A22-32	25	0	287	287	100
A22-33	25	4	369	373	98.6
A22-34	25	4	308	312	98.7

The developmental temperature for each strain tested for flight ability is in °C. Those flies that were able to escape the central flight chamber by flying are labelled as fliers while those that could not escape are labelled as non-fliers.

The flight assay clearly demonstrates these mutants are unable to fly. As presented in Table 7.1, a minimum of 97.3% of the excision mutants raised at 25°C fail to escape the central flight box chamber, and the *dumpy* mutants exhibit a similar percentage of flies that also fail to fly in this experiment. These A22-derived mutants within the central chamber of the flight testing box frequently rolled in their backs and struggled to right themselves. This behaviour was not exhibited by the *dumpy*, A22 and OR-R strains. These mutants also failed to disperse across the large outer chamber over a period of 10-15 minutes, while there was no shaking and banging of the chamber, as was also the case for the *dumpy* mutants. The wild type strains, in contrast to the mutants appeared to be much more active in the flight chamber and there was no need to allow them time to disperse, since so many immediately flew from the central chamber. The A22-derived mutant flies appeared to have a normal phototactic/geotactic response since they climbed up the sides of the chamber but could not escape because on making contact with the copper plates, they were electrically shocked and dropped back to the bottom of the chamber.

7.2.2 Jumping Ability

The jumping ability of 10 males of each strain raised at 20°C and 25°C was assessed in parallel. Each mutant male raised at 20°C had "blister"/"spoon" wings while those raised at 25°C had "dark" wings. The exception here was A22-30 males, which had "dark" wings since in this line blistered wings are very rare. *hypoC* mutants were used as a negative control, since it has previously been shown to have reduced jumping frequency (O'Dell and Burnet, 1988), while the OR-R wild type strain was used as a positive control. The original insertion strain A22 was also tested here as a control since this fly strain has not previously been tested in such a paradigm. The inclusion of A22 controls for the possibility that any defect detected

may be inherent in the ancestral strain. The control strains were raised and prepared under the same conditions.

Each male, aged from 5-10 days, had each wing gently removed under CO_2 anaesthesia, and left to recover overnight at the original temperature of development. The flies were then tested for jumping ability on a sheet of cardboard printed with a 1 cm^2 grid, by banging the bench firmly, in close proximity to the fly. The distance the fly jumped on the grid was carefully estimated by eye to the nearest 0.5 cm in Table 7.2. In Table 7.3 the average distance jumped is presented with its standard deviation.

Table 7.2 Distance Jumped

Strain	Temp	Distance Jumped (cm)									
OR-R	20	2.5	2.5	2.5	2.5	3	3.5	3.5	3.5	3.5	3.5
hypoC	20	1	1.5	1.5	1.5	1.5	2.5	2.5	2.5	2.5	2.5
A22	20	1.5	2.5	2.5	2.5	2.5	3.5	3.5	3.5	3.5	3.5
A22-30	20	1	1.5	1.5	1.5	1.5	1.5	2	2.5	2.5	2.5
A22-32	20	0	0	1	1	1	1	1	1.5	1.5	1.5
A22-33	20	0	0	1	1.5	2	2.5	2.5	2.5	2.5	3
A22-34	20	1	1	1	1.5	1.5	1.5	1.5	1.5	2.5	3
OR-R	25	2.5	2.5	2.5	2.5	2.5	3.5	3.5	3.5	3.5	3.5
hypoC	25	0	1	1	1.5	1.5	2.5	2.5	2.5	2.5	2.5
A22	25	1.5	1.5	2.5	2.5	2.5	2.5	3.5	3.5	3.5	3.5
A22-30	25	1.5	1.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
A22-32	25	0	1	1	1	1.5	1.5	2.5	2.5	2.5	3
A22-33	25	0	1	1.5	2	2	2.5	2.5	2.5	2.5	3
A22-34	25	1	1.5	1.5	1.5	2.5	2.5	2.5	2.5	2.5	2.5

In both Table 7.1.1 and 7.1.2, the distance jumped by each fly has been rounded up to the nearest 0.5 cm and the developmental temperature (Temp) is in ^oC.

Each of the mutant strains are able to jump when startled when raised at both 20°C and 25°C. Therefore, if any defects in flight ability are present in these mutants, it is probably not in the jump that is required to initiate flight. This jump test also indicates the mutants are not bang-sensitive since they do not fall over and struggle to right themselves when startled by the bang. This was anticipated since during routine maintenance they are not noticeably sensitive.

Table 7.4 Jump Analyses

Strain	Temp	n	mean	σ_n	S. E
OR-R	20	10	3.05	0.47	0.15
hypoC	20	10	1.95	0.57	0.18
A22	20	10	2.90	0.66	0.21
A22-30	20	10	1.80	0.51	0.16
A22-32	20	10	0.95	0.52	0.16
A22-33	20	10	1.75	1.03	0.33
A22-34	20	10	1.60	0.62	0.20
OR-R	25	10	3.0	0.50	0.16
hypoC	25	10	1.75	0.84	0.27
A22	25	10	2.70	0.75	0.24
A22-30	25	10	2.30	0.40	0.13
A22-32	25	10	1.65	0.90	0.28
A22-33	25	10	1.95	0.85	0.27
A22-34	25	10	2.05	0.57	0.18

The standard deviation (σ_n), standard error, and average distance jumped for each strain tested is presented. n is the number of individuals tested.

7.2.3 Discussion

These experiments were intended to identify any behavioural defects present in the four mutant strains generated by the excision of the A22 P element. As these mutant flies exhibit a cold-sensitive wing phenotype, it seemed any behavioural defects might be due to subtle phenotypes in derivatives of the wing imaginal disc. From the data presented in Table 7.1, the A22-derived mutants have a clear defect in flight behaviour when raised at 25°C and there is no severe disruption to the development of the wing blade. Similarly the data presented in Table 7.2 indicates the four mutant strains are able to jump when raised at both 20°C and 25°C.

The indirect flight muscles move the wing when they are stimulated by contraction of the jump muscles (Costello and Wyman, 1986 and references therein) This would suggest that the flightless phenotype does not result from abnormal jump muscles but may be due to a disturbance in the interaction between the jump and flight muscles. However, cuticular and neural defects, in addition to defects in the flight muscles can not be excluded.

In Section I of Chapter 6, the severity and frequency of the wing phenotype and pupal lethality was shown to be cold-sensitive. Therefore the percentage of non-fliers were much lower than expected, when raised at 20°C, being in the range of 46.8-57.9%. This would indicate the excision induced mutation affects flight behaviour in a heat-sensitive manner. The work of Tasaka and Suzuki (1973) suggests that about half of the third chromosome cold-sensitive mutations are also heat-sensitive.

As previously stated above (see Chapter 4), when the mutant flies were shaken in the flight box many flies were observed struggling to get upright. This behaviour was not observed in those flies that had their wings removed in the jump test. This behaviour suggests the flies are unable to control the movement of their wings, as previously suggested in Chapter 4. When jumping on a flat surface, those flies that had at least one abnormal wing, the mutants were observed to land at an

angle of at least 90° from their starting position. This behaviour would perhaps implicate abnormal jump muscle function, but can be excluded as a mutant phenotype since it was frequently observed in "wingless" mutant and wild type flies raised at both 20°C and 25°C. These behaviour data obtained from the jump and flight experiments are further discussed in Chapter 8.

Chapter 8

Discussion

As a first step in the identification of novel genes required for the normal development of the *Drosophila* nervous system two enhancer trap strains A11 and A22 were selected from a panel gifted to J. Davies by M. Bownes. Both strains have no deleterious or obvious mutant phenotype as a result of the P element insertion and they are easily maintained as homozygous lines. The *lacZ* expression patterns of these two strains have been characterised during embryogenesis, the third larval instar stage, pupation and in the adult (see Chapter 3). It can be concluded from these investigations that both lines express *lacZ* in response to at least one enhancer that is active in the nervous system during development. Both insertion strains were found to have normal CNS and PNS structures as expected from viable and fertile lines.

The A11 insertion strain was found to have a complex spatial and temporal *lacZ* expression pattern. As discussed in Chapter 3, *lacZ* is expressed in the developing nervous system throughout each of the developmental stages examined. In addition, *lacZ* activity was also detected in the follicle cells of the ovaries and the male gonad disc in the third instar larvae. Such expression patterns, although not conclusive, indicate the enhancer trapped in this strain may be essential in the development and function of diverse cell types. Therefore, any phenotypes that result from imprecise excision of the A11 P element construct may be difficult to interpret. For example, deletion of DNA flanking the insertion site may result in infertility or lethality in the early embryo. The role played by the enhancer and the gene under its regulation during neurogenesis would therefore be difficult to establish. The A22 insertion strain also exhibits *lacZ* expression during neurogenesis. This strain, however, has a more restricted pattern than the A11 strain indicating any phenotypes induced by excision mutagenesis may be neural and so of direct relevance to the aims of this project. The limited expression pattern of the reporter gene relative

to that of A11 suggests any excision derived mutations should be less complicated to interpret.

As a consequence, the A22 P element insertion strain was chosen as suitable for P element excision mutagenesis. The aim of this was to induce the imprecise excision of the marked P element and so generate mutant strains that exhibit a neural phenotype during embryogenesis. Excision mutagenesis of this P element was achieved by genetically providing the P element transposase on the Δ2-3 chromosome (Robertson *et al.*, 1988). Progeny that had lost the *rosy* marker carried by the A22 P element were then isolated from the Δ2-3 chromosome and balanced strains were subsequently established as described in Chapter 4.

No dominant phenotypes were detected among these balanced stocks. Nineteen excision strains representing a mutation rate of 24% were found to have a recessive mutant wing phenotype. All nineteen mutants were found to have the same phenotype and no other mutant phenotype was detected among the *rosy*$^-$ strains, suggesting the same gene had been disrupted in each of the isolated mutants. As described in Chapter 4, the homozygotes were observed to have deformed wings of varying severity, and flies that had one defective wing and one morphologically normal wing were frequently observed among the phenotypically mutant homozygotes (see Plates 4.1 and 4.2).

The use of enhancer traps facilitates the cloning of nearby genes by providing a molecular "tag" (Bingham *et al.*, 1981). As discussed in Chapter 5, a number of attempts were made to clone genomic DNA located at the insertion loci of both the A11 and A22 insertion strains. Such clones when isolated, should lead to rapid identification of the genes regulated by the trapped enhancers and therefore allow their functions to be characterised. Such data is of particular interest in the light of the expression pattern of the A22 insertion strain, and the unexpected mutant phenotype.

It was hoped that the excision mutagenesis would generate strains that exhibit abnormal development of either the CNS, PNS or possibly both. Such abnormal

development, if severe enough would lead to embryonic lethality. The mutant phenotype can not be correlated with the known *lacZ* expression patterns of the ancestral A22 insertion strain at this time. *lacZ* expression in the imaginal wing disc and the pupal wings of the A22 insertion strain as previously, mentioned were inconsistent. Indeed, only a single cell was found to express *lacZ* in a single imaginal wing disc of the third instar wandering larvae. This discrepancy between the mutant phenotype and the expression pattern of the reporter gene indicates the trapped enhancer has little or no influence on the disrupted gene in the excision-derived strains. However, it is possible the enhancer is active and drives the expression of a gene in the embryonic cells that express *lacZ*. For example, the gene *vestigial* (*vg*) is known to be expressed in the developing embryonic and adult CNS (Williams *et al.*, 1991). In vg^- null adult flies there are minor defects in a number of sensory organs in addition to the elimination of the wings and halteres. The function of Vg in the embryonic neural progenitor cells remains unknown, and any defects that may occur in vg^- null embryos is assumed to be very minor with no lethal effect (Williams *et al.*, 1991). It is therefore possible the gene responsive to the trapped enhancer and the gene mutant in the A22 excision-derived strains are the same. This would imply a very minor role for the gene product during neurogenesis, or perhaps some degree of functional overlap with another unknown gene product.

As discussed in Chapter 1, previously characterised enhancer-trap strains provide data not necessarily predicted by the expression pattern of *lacZ*. The P element construct inserted in the *pollux* gene (Figure 1.6) was surprisingly found to express *lacZ* in response to the regulatory elements of *castor*, but not *pollux* (Mellerick, *et al*,. 1992). In Chapter 4, an alternative chromosomal organisation that may explain the conflicting *lacZ* expression pattern and the mutant phenotype derived by A22 P element imprecise excision is depicted in Figure 4.4. Another possibility is the *lacZ* expression pattern of the A22 line results from activation by genomic sequences that have no regulatory effect on any *Drosophila* genes. This problem,

common to the mouse enhancer-trap technique has been overcome by the use of promotor traps (Skarnes, *et al.*, 1992).

There are a vast number of *Drosophila* mutants that exhibit abnormal wing development (summarised in Lindsley and Zimm, 1992). However, no similar phenotype has been previously detected in mutants known to be located on the third chromosome at bands 72BC or in the surrounding bands of the salivary gland chromosome (Lindsley and Zimm, 1992). These mutant strains are therefore considered novel.

The observed variation and penetrance of the induced phenotype was initially considered problematic. When a very low percentage of homozygous progeny exhibit a mutant phenotype, then any data obtained from subsequent genetic tests may be difficult to interpret and unreliable. In an attempt to resolve such difficulties, four of the mutant strains were selected for further phenotypic characterisation. Each of the four selected strains were raised at three different temperatures in parallel experiments. As described in Section I of Chapter 6, the mutant wing phenotype was detected more frequently among those flies raised at 20°C than 25°C, with the exception of the strain A22-30. The mutations were found to be lethal when the homozygotes were raised at 16°C. This lethality has been classified as either pupal lethal, eclosion lethal, or adult lethal where those flies that emerge die soon after.

Behavioural analysis in the excision-derived mutants in an attempt to further characterise the phenotype. Fewer homozygous strains exhibit the abnormal wing phenotype at 25°C, than 20°C. Does this mutation have other, more subtle effects on wing development ? This was addressed by testing the flight ability of homozygous mutants that had no visibly obvious wing defects. The homozygous progeny raised at 25°C, although able to jump were unable to fly indicating the flight muscles or some process required for flight may be abnormal. Those progeny that appeared phenotypically normal when raised at 20°C were found to be able to initiate and maintain flight. Thus, these four recessive mutations appear to have a heat-cold-

sensitive mutation. Further anatomical analysis may reveal other, as yet unidentified, defects in these mutants.

EMS mutagenesis of the *Drosophila* third chromosome indicates cold-sensitive mutations occur with similar frequencies to heat-sensitive mutations (Tasaka and Suzuki, 1973). Of the isolated mutations none exhibited a wing phenotype and about one third of these heat-sensitive mutations are also cold-sensitive, and many were found to cluster between the *Sb* and *st* loci (Tasaka and Suzuki, 1973).

At this time many questions concerning the nature of these mutations remain unanswered. What gene has been mutated, and how is this gene product involved in wing development? How many other *Drosophila* structures and organs require its wild type expression for accurate development? Why are these mutations cold-sensitive?

The temperature sensitivity tests have provided clues useful in the identification of the developmental function perturbed in these mutants. At 20°C-25°C normal wing development is disrupted, while at 16°C an essential biological process is lost. This suggests those flies raised at 20°C and possibly 25°C may have other more subtle phenotypes, that remain so far undetected. For example, any number of internal structures may also develop abnormally at these temperatures. Such structures may be more severely disrupted at 16°C leading to lethality.

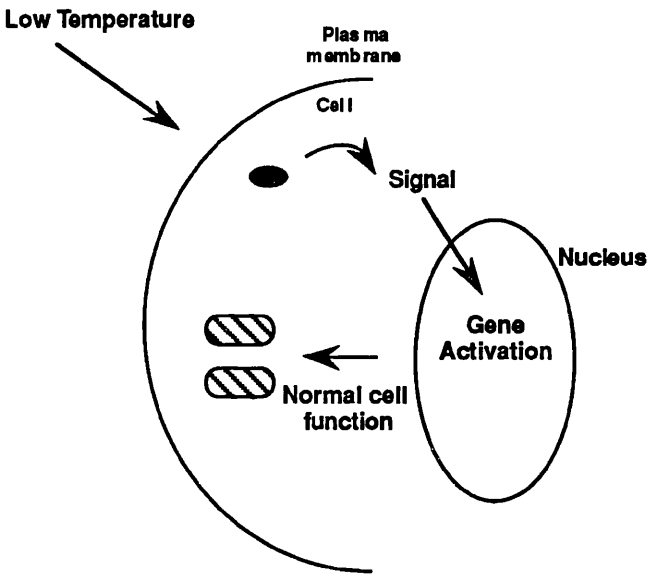
A number of interpretations may be made at this stage, one of which is the P element induced mutation results in abnormal gene regulation. The more severe phenotype observed at 16°C may imply increased levels of gene product are required for normal development. Disruption or complete deletion of regulatory elements may therefore prevent or reduce the necessary increase in expression. This model requires a number of components. For example the initial component is detection of the lower developmental temperature. The cell must then normally respond by expressing the specific transcription factor(s) required for the activation of a number of enhancer elements. This leads ultimately to increased expression of genes essential for normal development at reduced temperatures (see Figure 8.1). Such a biological process may

be considered analogous to the regulation and function of the heat-shock proteins (reviewed by Hendrick and Hartl, 1993). Any one of these hypothetical components may be disrupted in the A22-derived mutants, the resolution of which requires cloning and molecular analysis of the mutant loci.

Here, the original enhancer trap expression studies may prove useful in attempts to establish the relevance of this hypothesis. As previously discussed in Chapter 3, *lacZ* expression analysis of the imaginal wing discs and the A22 pupae were inconsistent. As with all *Drosophila* developmental stages characterised for *lacZ* activity and described in this Thesis (Chapter 3), the pupae were raised and prepared at room temperature. Would pupal *lacZ* activity be detected if the pupae were raised at 16°C? Expression of the A22 reporter gene in pupae raised at 16°C would provide good evidence that the gene is indeed regulated by an enhancer normally active only at low temperatures. The mutations would therefore have lost either the cold-responsive enhancer or the gene it activates.

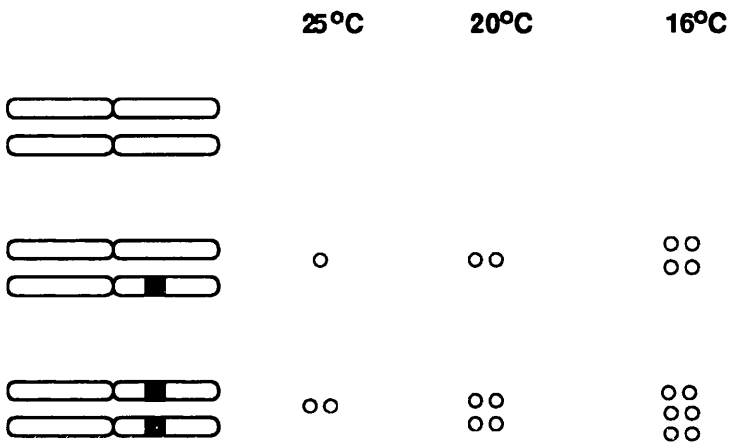
The above hypothesis assumes a LOF (loss of function) mutation has been induced. Alternatively, the imprecise excision events may have deleted, for example, a repressor element. These mutants would therefore have GOF (gain of function) mutations as depicted in Figure 8.2. The mutant phenotype may then result from inappropriate spatial and temporal expression of some unknown gene product. In these mutants, this abnormal expression or its poisonous effects must be more severe at the lower developmental temperature. In this model, progeny that have one wild type and one mutant chromosome, when raised at 16°C should have a similar mutant phenotype to that observed for mutant homozygotes raised at 20°C or 25°C. From the data presented in Section III of Chapter 6, the mutants are complemented by the deficiency chromosomes and so this model appears to be inappropriate. The mutants were often stored as balanced stocks at 16°C and these heterozygous stocks similarly should no abnormal wing development, as would be predicted by this model. The *Moonrat* mutation is one example of this type of mutation that is affected by temperature (Felsenfeld and Kennison, 1995).

Figure 8.1 Model of Cellular Response to Reduced Temperature



In this model, the reduced temperature is detected by a "thermometer" (black circle) and a signal is subsequently received in the nucleus that ultimately results in the synthesis of a gene product (represented by the hatched boxes) that permits cellular processes to continue at the lower temperature. Gene activation may result by the activation of an enhancer or by the derepression of a repressor element. Any component of this pathway may be disrupted, resulting in defective cell physiology at reduced temperatures.

Figure 8.2 Repressor Element Deletion Model



The shaded area represents repressor element mutation on the chromosome and the circles represent an abnormally expressed gene product. At 25°C the heterozygote expresses a low level of the product and no phenotype is detected while the homozygote expresses a slightly higher level similar to that of the homozygote raised at 25°C and infrequently a mutant phenotype is observed. The heterozygote when raised at 16°C expresses moderate levels of gene product also associated with development of the homozygous progeny raised at 20°C and the phenotype is observed frequently. At 16°C the homozygotes express high levels of gene product that results in the most severe form of the phenotype.

Future Work.

The cytological localisation of the A22 ancestral strain and the mutation must be re-established since the *in situ* hybridisation data has been found genetically to be inaccurate. Although the molecular characterisation of these mutants is at early stages, further Southern blot analysis should allow the extent of P element excision to be established and may also indicate the size of the deletion induced by imprecise excision of the P marked element. The nature of the disrupted gene product and the function it plays during development may be established by DNA isolation and sequence analysis, in addition to *in situ* hybridisation experiments that may indicate its spatial and temporal expression pattern. This expression pattern is unlikely to have major similarities to that of the reporter gene of the A22 insertion strain. However, once flanking genes have been cloned, the molecular organisation of the locus, with particular respect to the *lacZ* expression pattern may be more fully understood.

In light of the pupal lethal phenotype observed in these mutants it is expected that the developing embryonic CNS and PNS will proceed normally. However, there may be subtle differences that have no detrimental effect on embryonic development proceeding on to the larval stage. Analysis of the development of these cell types in the mutants may be of some interest.

The complete analysis of any mutation requires the use of a great diversity of techniques, the selection of which are dependant on the data derived at each progressive step. The mutants that have been generated as part of this Thesis may be of use in the study of a variety of biological phenomena. The cold-sensitivity properties are of special interest merely by the method used to generate them. Many mutations previously characterised in *Drosophila* have been obtained by EMS mutagenesis (Tasaka and Suzuki, 1973), a mutagen that induces point mutations in DNA. Such mutants are likely to result from instability in protein products as a result of a single amino acid change. However the A22-derived mutants have been derived

by P element mutagenesis, and therefore it is likely the phenotype is due to the deletion of a region of genomic DNA. This may indicate the gene product that may be involved in stabilising other protein products or "buffering" biological processes when the developing pupa is exposed to environmental stress. Thus, these mutants may provide a valuable resource in understanding physiological processes that occur at low temperatures.

An alternative hypothesis involving DNA rearrangements may be proposed. The previously noted high frequency of P element excision and mutation could be suggestive of sequences within the vicinity of the P element insertion point being unstable. The presence of the P element construct and high levels of transposase may promote DNA rearrangements between the P element and a putative susceptible region or "hot-spot" for recombination. If such events were found to have occurred in theses excision-derived mutants, then the mutation may be located at the breakpoints some distance away from the insertion site and thus would not have been uncovered by the deficiency stocks employed in the genetic analyses of these mutants (Chapter 6).

The determination of the lethal phase at 16^{o}C is essential in the further analysis of these mutants. Although lethality is observed at very late stages of pupal development, the perturbation that results in this lethality may occur much earlier in the pupal stage. Indeed this perturbation in development need not be restricted to the pupal stage but could occur in the larval stages. For example the imaginal discs and histoblasts may not grow normally during the larval instar stages, or their normal morphological movements and subsequent differentiation may become abnormal in the third instar larvae or early pupal stages. The effective lethal phase of these mutations can be established by temperature shift experiments, where the homozygous progeny are allowed to develop at the permissive temperature until a pre-determined developmental stage and then shifted to the non-permissive temperature. Similar experiments where initial development occurs at the non-

permissive temperature prior to shifts to the permissive temperature should establish the period of development that is specifically abnormal in these mutants.

The observed heat-cold-sensitive nature of this mutation must be further studied. The possibility that two separate genes have been mutated in these four selected strains must be considered. The method used to generate these mutants has the potential to disrupt two neighbouring genes. Either a gene on each side of the P element insert has been disrupted simultaneously, or two genes both located downstream, or upstream of the P element may be disrupted by the deletion of a stretch of DNA required for the activity and normal expression of both genes. Each of the nineteen mutants should be tested for flight behaviour. The identification of one or more mutant strains with normal flight ability would indicate the four selected mutants had mutations that disrupt two genes. Complementation analysis between the non-fliers and any fliers and scoring for flight ability would be an appropriate genetic test. Possibly, those flies that had lost the *rosy* marker and did not have deformed wings, were actually excision progeny that had disrupted the gene involved in flight behaviour, but not tested. These strains would have been invaluable in the determination of the number of closely linked genes mutated in the excision mutagenesis.

The current lack of DNA sequence information, although important, does not prevent further characterisation of these mutants. Genetic recombination may be employed to establish the location of these mutations on the third chromosome more accurately than the *in situ* data allowed. The interaction of these mutants with carefully selected mutants that have previously been characterised both functionally and molecularly should permit the affected developmental processes to be identified more clearly. If the lethal phase is established accurately and the mutant focus is indicated, this may aid the choice of mutations useful in establishing genetic interactions with other loci.

Appendices.

Appendix. 1. Excision Mutagenesis.

The number of flies scored in the excision cross, determined by the loss of the *rosy* marker, allowing the calculation of excision rate of the A22 P element, are presented in the following table.

Here the number of flies scored in each phenotype class are presented. The final column shows the level of contamination due to females that were not virgins at the time of collection. Homozygous *ebony* progeny indicates the parental females were not virgins at the time of collection. Total A is the number of progeny scored that were derived from vials that did not have any homozygous *ebony* progeny, indicating the parental females were all virgins. Total B is the total number of flies scored for each genotype, including those vials that had a number of homozygous *ebony* progeny.

Table A1. Excision Progeny.

CROSS	$Sb^- ry^+$		$Sb^- ry^-$		$Sb^+ ry^-$		$Sb^+ ry^+$		TM3/rf 10
VIAL #	♂	♀	♂	♀	♂	♀	♂	♀	
1	6	12	3	4	2	6	2	3	N
2	All	ALL	0	0	0	0	0	0	Y
3	3	9	5	10	2	8	1	1	N
4	0	7	2	11	3	6	4	0	N
5	10	10	4	4	0	1	0	3	N
6	11	22	5	12	1	4	1	5	N
7	3	3	6	7	1	4	2	2	N
8	7	15	2	4	3	5	1	5	N
9	8	20	12	30	6	20	0	1	N
10	ALL	ALL	0	0	0	0	0	0	Y
11	15	32	3	10	2	6	6	11	N
12	7	15	3	5	5	7	1	2	N
13	ALL	ALL	0	0	0	0	0	0	Y
14	ALL	ALL	0	3	0	4	0	0	Y
15	3	3	0	1	1	4	0	0	N
16	2	2	1	1	2	2	2	1	N
17	19	34	6	19	9	18	5	13	N
18	8	17	2	0	0	0	0	0	N
19	6	10	0	0	0	0	0	0	Y
20	5	7	3	6	0	0	0	0	N
21	16	48	11	17	3	10	13	21	N
22	19	41	0	0	0	0	0	0	Y
23	17	58	11	18	7	19	7	14	N
24	ALL	ALL	0	0	0	0	0	0	Y
25	2	4	19	40	12	42	24	24	N
26	11	18	7	14	5	12	0	0	N
27	14	36	2	8	6	12	3	6	N
28	14	30	2	4	9	12	5	11	N
29	ALL	ALL	0	0	0	0	0	0	Y
30	0	28	0	5	0	5	0	6	N
31	0	11	0	7	0	6	0	0	N
32	ALL	ALL	0	0	0	0	0	0	Y
33	2	4	0	1	1	3	0	1	N
34	ALL	ALL	0	0	0	0	0	0	Y
35	0	4	2	2	1	1	5	8	Y
36	ALL	ALL	0	0	0	0	0	0	Y
37	11	28	3	9	4	7	8	11	N
38	8	30	4	10	6	11	0	0	N
39	4	7	3	7	3	7	0	2	N
40	9	10	0	0	1	1	0	2	N
41	ALL	ALL	0	0	0	0	0	0	Y
42	ALL	ALL	0	0	0	0	0	0	Y
43	ALL	ALL	0	0	0	0	0	0	Y
44	9	19	7	12	7	15	0	0	N
45	6	10	2	4	0	4	2	5	N
46	14	36	4	13	1	7	4	9	N
47	16	34	11	26	11	22	0	0	N
48	3	16	4	8	2	6	1	1	N
49	8	25	13	26	9	17	9	9	N

CROSS	Sb^{-} ry^{+}		Sb^{-} ry^{-}		Sb^{+} ry^{-}		Sb^{+} ry^{+}		TM3/rf 10
VIAL #	♂	♀	♂	♀	♂	♀	♂	♀	
50	8	19	8	9	10	11	0	16	N
51	17	36	5	13	4	12	10	13	N
52	ALL	ALL	0	0	0	0	0	0	Y
53	5	10	6	17	2	10	1	2	N
54	ALL	ALL	0	0	0	0	0	0	Y
55	18	41	14	33	13	40	2	2	N
56	11	48	25	46	14	52	1	3	N
57	3	18	4	8	6	14	0	0	N
58	5	12	4	14	3	6	0	0	N
101	8	12	3	4	4	0	2	5	N
102	14	16	4	5	0	0	0	0	N
103	15	29	8	4	6	10	12	5	N
104	1	3	1	0	1	3	4	1	N
105	11	11	7	1	3	1	4	7	N
106	ALL	ALL	0	0	0	0	0	0	Y
107	12	9	14	8	7	6	0	0	N
108	3	3	2	2	1	2	3	5	N
109	ALL	ALL	0	0	0	0	0	0	Y
110	15	18	5	8	5	4	5	4	N
111	ALL	ALL	0	0	0	0	0	0	Y
112	ALL	ALL	0	0	0	0	0	0	Y
113	ALL	ALL	0	0	0	0	0	0	Y
114	ALL	ALL	0	0	0	0	0	0	Y
115	ALL	ALL	0	0	0	0	0	0	Y
116	7	16	9	8	3	6	0	5	N
117	ALL	ALL	0	0	0	0	0	0	Y
118	5	2	2	7	1	6	15	6	N
119	1	1	0	0	0	2	0	1	N
120	5	11	1	2	5	3	4	2	N
121	ALL	ALL	0	0	0	0	0	0	Y
122	7	4	1	3	2	1	1	3	N
123	13	7	2	10	6	5	7	5	Y
Total A.	442	979	283	545	214	498	165	249	3375
Total B.	480	1041	287	560	221	508	177	262	

Appendix 2. Temperature Sensitivity Tests.

The data presented in the following tables is of the Temperature Sensitivity Tests presented in summary form in the Genetics Chapter.

Table A2.1. A22-30 homozygous progeny raised at 25°C.

	Progeny										
	Homozygotes										
	wild type		dark		blister		coal		spoon		
Vial	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	Total
1	33	29	0	2	0	0	0	0	0	0	64
2	35	37	0	3	0	0	0	0	0	0	75
3	18	27	2	4	0	0	0	0	0	0	51
4	13	14	0	0	0	0	0	0	0	0	27
5	36	25	0	1	0	0	0	0	0	0	62
6	28	28	0	0	0	0	0	0	0	0	56
7	50	44	0	0	0	0	0	0	0	0	94
8	13	16	0	0	0	0	0	0	0	0	29
9	22	18	2	1	0	0	0	0	0	0	43
10	41	44	0	0	0	0	0	0	0	0	85
11	36	31	0	1	0	0	0	0	0	0	68
12	29	38	0	0	0	0	0	0	0	0	67
13	19	16	3	0	0	0	0	0	0	0	38
14	28	24	0	0	0	0	0	0	0	0	52
15	46	44	0	4	0	0	0	0	0	0	94
16	17	12	1	0	0	0	0	0	0	0	30
17	22	29	0	0	0	0	0	0	0	0	51
18	48	56	0	2	0	0	0	0	0	0	106
19	23	28	0	0	0	0	0	0	0	0	51
20	34	50	0	0	0	0	0	0	0	0	84
Total	591	610	8	18	0	0	0	0	0	0	1227

Table A2.2. A22-30 homozygous progeny raised at 20°C.

Vial	Progeny Homozygotes										Total
	wild type		dark		blister		coal		spoon		
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
1	46	50	1	0	0	0	0	0	0	0	97
2	57	59	0	0	0	0	0	0	0	0	116
3	25	25	0	3	0	0	0	0	0	0	53
4	30	27	0	0	0	0	0	0	0	0	57
5	43	44	0	0	0	0	0	0	0	0	87
6	51	35	1	0	0	0	0	0	0	0	87
7	65	47	0	0	1	0	0	0	0	0	113
8	29	25	0	0	0	0	0	0	0	0	54
9	39	43	0	0	0	2	0	0	0	0	84
10	18	15	0	0	0	0	0	0	0	0	33
11	53	59	0	2	0	0	0	0	0	0	114
12	27	42	0	0	1	0	0	0	0	0	70
13	31	22	0	0	0	0	0	0	0	0	53
Total	514	493	2	5	2	2	0	0	0	0	1018

Table A2.3. A22-30 homozygous progeny raised at 16°C.

Vial	eclosed pupae	dead pupae	semi-eclosed	Total
1	23	27	5	55
2	29	8	4	41
3	8	3	2	13
4	30	7	5	42
5	58	22	8	88
6	18	13	2	33
7	14	11	8	33
8	55	15	2	72
9	40	21	4	65
10	8	1	0	9
11	11	4	0	15
12	36	11	3	50
13	17	11	2	30
14	54	20	7	81
15	49	26	12	87
16	35	14	4	53
17	26	8	2	36
18	66	25	8	99
19	67	24	8	99
20	32	5	3	40
21	19	7	0	26
22	13	10	2	25
23	28	11	2	41
24	8	1	1	10
25	10	11	0	21
26	29	14	2	45
27	77	35	10	122
28	38	8	2	48
Total	898	373	108	1 379

A2.4. A22-32 homozygous progeny raised at 25°C.

Vial	Progeny: Homozygotes: wild type ♂	wild type ♀	dark ♂	dark ♀	blister ♂	blister ♀	coal ♂	coal ♀	spoon ♂	spoon ♀	Total
1	12	19	2	1	0	0	0	0	0	0	34
2	10	20	2	1	0	0	0	0	0	0	33
3	11	7	1	0	0	0	0	0	0	0	19
4	8	5	0	0	0	0	0	0	0	1	14
5	15	6	0	1	0	0	0	0	0	0	22
6	5	7	0	0	0	0	0	0	0	0	12
7	12	15	1	0	0	0	0	0	0	0	28
8	18	18	1	1	0	0	0	0	0	0	38
9	6	8	0	0	0	0	0	0	0	0	14
10	10	11	0	0	0	0	0	0	0	0	21
11	3	2	0	0	0	0	0	0	0	0	5
12	3	13	0	1	1	0	0	0	0	0	18
13	10	23	1	1	0	0	0	0	0	0	35
14	12	8	1	1	0	0	0	0	0	0	22
15	4	4	0	1	0	0	0	0	0	0	9
16	9	11	0	0	0	0	0	0	0	0	20
17	6	11	0	0	0	0	0	0	0	0	17
18	6	7	0	0	0	0	0	0	0	0	13
19	8	6	2	0	0	0	0	0	0	0	16
20	5	8	1	1	0	0	0	0	0	1	16
21	13	3	0	0	0	0	0	0	0	2	18
22	9	7	0	0	0	0	0	0	0	0	16
23	18	15	0	2	0	0	0	0	0	0	35
24	15	26	1	0	0	0	0	0	0	0	42
25	10	21	2	2	0	0	0	0	0	0	35
26	15	10	2	2	0	0	0	0	0	0	29
27	6	11	2	2	0	0	0	0	0	0	21
28	13	24	2	0	0	0	1	0	0	1	41
29	9	25	0	4	0	0	0	0	0	0	38
30	10	11	0	0	0	0	0	0	0	0	21
31	6	6	1	0	0	0	0	0	0	0	13
32	11	12	2	1	0	0	0	0	2	3	31
33	13	12	2	2	0	0	0	0	0	0	29
34	8	10	1	1	1	0	0	0	0	1	22
35	11	21	1	1	2	0	0	0	0	1	37
36	8	14	3	3	0	0	0	0	0	0	28
37	5	3	2	0	0	0	0	0	0	0	10
38	24	29	1	1	0	0	0	0	0	0	55
39	12	13	1	0	0	0	0	0	0	0	26
40	8	18	2	0	0	0	0	0	0	1	29
41	20	33	2	2	0	0	0	0	0	0	57
42	4	6	2	0	0	0	0	0	0	0	12
Total	421	539	41	32	4	0	1	0	2	11	1051

Table A2.5. A22-32 homozygous progeny raised at 20°C.

Vial	Progeny Homozygotes										Total
	wild type		dark		blister		coal		spoon		
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
1	1	3	0	0	1	0	0	0	0	0	5
2	19	14	0	0	0	0	0	0	0	0	33
3	6	9	6	2	3	0	0	0	3	4	33
4	10	12	0	4	6	2	1	0	5	2	42
5	1	2	0	0	0	0	0	1	0	0	4
6	4	6	0	2	0	1	0	0	0	0	13
7	5	7	0	4	2	0	2	0	3	5	28
8	2	8	0	3	2	6	0	0	5	1	27
9	16	28	5	4	5	3	1	0	3	1	66
10	14	17	4	8	5	11	1	1	6	2	69
11	4	16	1	7	3	4	2	0	3	5	45
12	16	16	2	4	0	0	2	1	1	0	42
13	33	33	6	5	1	1	0	0	4	4	87
14	6	11	2	2	6	0	0	0	1	1	29
15	5	7	3	4	11	11	3	0	2	5	51
16	2	2	0	0	1	2	1	0	3	2	13
17	8	17	2	2	1	2	0	1	2	3	38
18	7	14	5	6	3	5	2	1	3	6	52
19	1	11	1	6	4	3	0	2	4	4	36
20	15	16	2	3	6	0	0	0	9	2	53
21	18	33	2	3	11	5	1	1	5	4	83
22	6	4	3	3	1	1	1	0	7	0	26
23	5	7	3	5	3	1	1	1	4	5	35
24	4	12	3	4	6	3	1	0	9	7	49
25	5	6	0	4	1	0	1	0	4	5	26
26	5	4	2	4	3	0	2	0	4	4	28
27	2	8	1	4	4	0	1	0	0	0	20
28	10	5	4	4	1	6	0	1	0	4	35
29	3	8	3	5	4	7	2	1	3	4	40
Total	233	336	60	102	94	74	25	11	93	80	1108

Table A2.6. A22-32 homozygous progeny raised at 16°C.

Vial	eclosed pupae	dead pupae	semi-eclosed	Total
1	29	46	11	86
2	21	31	8	60
3	17	13	5	35
4	30	34	21	85
5	34	35	7	76
6	31	36	11	78
7	7	18	4	29
8	20	34	11	65
9	3	13	3	19
10	27	26	8	61
11	15	39	3	57
12	7	15	4	26
13	10	18	5	33
14	6	6	3	15
15	18	17	6	41
16	26	23	4	53
17	40	40	18	98
18	37	34	7	78
19	22	26	10	58
20	14	17	2	33
21	15	24	7	46
Total	429	545	158	1132

Table A2.7. A22-33 homozygous progeny raised at 25°C.

Vial	Progeny: Homozygotes: wild type ♂	wild type ♀	dark ♂	dark ♀	blister ♂	blister ♀	coal ♂	coal ♀	spoon ♂	spoon ♀	Total
1	4	4	1	0	0	0	0	0	0	0	9
2	5	5	2	1	0	0	0	0	0	0	13
3	2	7	0	0	0	0	0	0	0	0	9
4	7	8	1	0	0	0	0	0	0	0	16
5	5	1	0	0	0	0	0	0	0	0	6
6	7	8	0	0	0	0	0	0	0	0	15
7	6	11	0	2	0	0	0	0	0	0	19
8	1	6	0	0	0	0	0	0	0	0	7
9	6	6	0	0	0	0	0	0	0	0	12
10	5	2	0	0	1	0	0	0	0	0	8
11	5	7	1	0	0	0	0	0	0	0	13
12	3	2	0	0	0	0	0	0	0	0	5
13	20	25	4	2	0	1	0	0	0	0	52
14	10	27	4	1	1	0	0	0	0	0	43
15	27	26	2	5	0	0	0	0	0	0	60
16	17	15	3	3	0	0	0	0	0	0	38
17	16	28	5	6	3	0	0	0	0	0	58
18	23	43	5	2	0	0	0	0	0	0	73
19	17	33	6	3	1	1	0	0	0	0	61
20	17	18	2	1	0	0	0	0	0	0	38
21	5	3	1	0	0	0	0	0	0	0	9
22	6	14	2	2	0	0	0	0	0	0	24
23	3	8	0	0	1	0	0	0	0	0	12
24	14	26	7	3	0	0	0	0	0	0	50
25	24	39	1	6	0	1	0	0	9	0	80
26	24	26	3	2	2	2	0	0	8	1	68
27	21	25	2	2	0	0	0	0	7	4	61
28	26	22	0	0	0	0	0	0	01	0	49
29	26	17	1	2	0	0	0	0	3	0	49
30	23	32	3	6	0	0	0	0	3	1	68
Total	375	494	56	49	9	5	0	0	31	6	1025

Table A2.8. A22-33 homozygous progeny raised at 20°C.

Vial	Progeny: Homozygotes: wild type ♂	wild type ♀	dark ♂	dark ♀	blister ♂	blister ♀	coal ♂	coal ♀	spoon ♂	spoon ♀	Total
1	0	2	1	3	2	0	0	0	0	0	8
2	1	0	0	1	2	0	3	0	0	2	9
3	1	1	0	0	5	5	0	0	0	2	14
4	0	1	1	1	3	5	1	0	0	2	14
5	1	5	2	4	5	6	1	0	2	3	29
6	0	2	0	2	6	4	0	0	1	1	16
7	0	2	1	3	2	0	0	0	0	0	8
8	1	0	0	1	2	0	3	0	1	2	10
9	1	1	0	0	5	5	0	0	0	2	14
10	0	1	1	1	3	5	1	0	0	2	14
11	1	5	2	4	5	6	1	0	2	3	29
12	0	2	0	2	6	4	0	0	1	1	16
13	1	3	0	7	6	8	0	0	3	4	32
14	3	3	3	5	0	2	0	0	0	0	16
15	6	2	5	2	1	1	0	0	5	3	25
16	2	3	5	5	3	2	1	0	5	2	28
17	0	0	0	1	2	1	0	0	2	0	6
18	3	6	5	8	6	2	0	0	3	2	35
19	2	6	3	5	5	3	0	0	3	1	28
20	3	9	2	5	6	4	1	2	3	5	40
21	1	2	0	2	0	0	0	0	0	0	5
22	2	9	2	6	10	7	0	0	3	9	48
23	2	8	0	7	11	11	0	0	3	3	45
24	1	2	0	3	3	1	4	1	1	0	16
25	1	3	0	0	1	0	2	0	1	3	11
26	2	4	0	3	5	7	1	0	2	0	24
27	6	8	2	2	1	1	0	0	3	2	25
28	3	4	2	4	4	3	1	0	5	2	28
Total	44	94	37	87	110	93	20	3	49	56	593

Table A2.9. A22-33 homozygous progeny raised at 16°C.

Vial	eclosed pupae	dead pupae	semi-eclosed	Total
1	0	13	3	16
2	16	16	3	35
3	32	62	9	103
4	11	22	2	35
5	10	28	3	41
6	25	32	5	62
7	39	25	0	64
8	12	23	2	37
9	8	9	0	17
10	20	58	9	87
11	30	34	10	74
12	5	20	4	29
13	11	13	6	30
14	9	4	1	14
15	13	32	4	49
16	14	17	2	33
17	14	29	4	47
Total	269	437	67	773

Table A2.10. A22-34 homozygous progeny raised at 25°C.

Vial	Progeny: Homozygotes: wild type ♂	wild type ♀	dark ♂	dark ♀	blister ♂	blister ♀	coal ♂	coal ♀	spoon ♂	spoon ♀	Total
1	11	10	3	0	0	0	0	0	0	0	24
2	15	17	0	1	0	0	0	0	1	0	34
3	9	8	3	0	0	0	0	0	0	0	20
4	6	7	2	0	0	0	0	0	0	0	15
5	1	4	0	0	0	0	0	0	0	0	5
6	4	3	0	0	0	0	0	0	0	0	7
7	7	2	0	0	0	0	0	0	0	0	9
8	2	0	0	0	0	0	0	0	0	0	2
9	8	5	0	0	0	0	0	0	0	0	13
10	2	4	0	0	0	0	0	0	0	0	6
11	3	2	0	0	0	0	0	0	0	0	5
12	18	20	2	1	0	0	0	0	0	0	41
13	13	8	2	0	0	0	0	0	0	0	23
14	14	11	0	1	0	0	0	0	0	0	26
15	3	11	0	0	0	0	0	0	1	0	15
16	2	6	0	0	0	0	0	0	0	0	8
17	12	8	0	0	0	0	0	0	0	0	20
18	13	7	0	0	0	0	0	0	0	0	20
19	14	22	3	1	0	0	0	0	0	0	40
20	13	5	1	0	0	0	0	0	0	0	19
21	17	7	0	1	0	0	0	0	0	0	25
22	11	6	0	1	0	0	0	0	0	0	18
23	7	6	0	0	0	0	0	0	0	0	13
24	16	20	2	1	0	0	0	0	0	0	39
25	12	18	1	1	0	0	0	0	0	0	32
26	8	5	2	3	0	0	0	0	0	0	18
27	17	27	0	2	0	0	0	0	0	0	46
28	32	36	1	2	0	0	0	0	0	0	71
Total	290	285	22	15	0	0	0	0	2	0	614

Table A2.11. A22-34 homozygous progeny raised at 20°C.

Vial	Progeny Homozygotes										Total
	wild type		dark		blister		coal		spoon		
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
1	18	20	4	8	0	0	1	0	3	4	58
2	15	16	8	11	4	1	0	0	3	4	62
3	12	6	2	1	1	0	0	0	0	6	28
4	42	33	9	14	2	6	3	1	16	10	136
5	21	15	4	7	2	1	0	0	3	3	56
6	31	37	11	12	6	2	0	0	7	8	114
7	21	29	4	8	2	0	0	0	3	2	69
8	34	27	13	10	4	2	3	1	7	7	108
9	43	29	16	9	6	6	1	0	7	4	121
10	52	65	14	27	7	12	2	4	15	9	207
11	24	29	6	10	5	3	1	0	6	5	89
Total	313	306	91	117	39	33	11	6	70	62	1048

Table A2.12. A22-34 homozygous progeny raised at 16°C.

Vial	eclosed pupae	dead pupae	semi-eclosed	Total
1	31	3	1	35
2	29	3	1	33
3	16	5	2	23
4	10	1	3	14
5	14	4	2	20
6	8	1	0	9
7	11	2	3	16
8	24	5	7	36
9	12	1	3	16
10	34	13	4	51
Total	189	38	26	253

Table A2.13. OR-R Negative Control For The Temperature Sensitivity Tests.

Flies raised at 16°C			Flies raised at 20°C			Flies raised at 25°C		
Vial	Progeny wt wings	mutant wings	Vial	Progeny wt wings	mutant wings	Vial	Progeny wt wings	mutant wings
1	41	0	1	66	0	1	127	0
2	16	0	2	78	0	2	107	1
3	82	0	3	118	0	3	80	0
4	72	0	4	61	0	4	121	0
5	42	0	5	116	0	5	17	0
6	37	0	6	54	0	6	74	0
7	26	0	7	86	0	7	17	0
8	64	0	8	86	0	8	119	0
9	96	1	9	100	0	9	20	0
10	121	0	10	141	0	10	18	0
11	43	0	11	161	0	11	51	3
12	48	0	12	141	0	12	57	0
13	23	0	13	59	2	13	37	0
14	47	0				14	21	0
15	25	1				15	150	0
16	36	0				16	120	0
17	60	0				17	149	2
18	38	0				18	35	0
19	19	0				19	108	0
20	23	2				20	100	0
21	35	0				21	107	0
22	93	0				22	137	0
						23	53	0
						24	157	0
						25	35	0
						26	52	0
						27	117	0
						28	96	0
						29	124	0
Total	1087	4		1267	2		2406	6

Those flies scored as mutant, were found to have either weak blistering or one or both wings had failed to expand. Those wings that were obviously damaged, by being in contact with the semi-liquid food, or were torn, were scored as wild type.

Appendix 3. Complementation Tests.

The data presented in the following tables is of the Complementation Tests presented in summary form in the Genetics Chapter.

Table A3.1 A22-30/A22-33 heterozygous progeny raised at 16°C

Vial	Progeny			
	A_{lethal}	P_{lethal}	E_{lethal}	Total
1	35	32	6	73
2	18	10	2	30
3	19	26	3	48
4	16	22	0	38
5	34	30	8	72
6	6	13	0	19
7	32	37	6	75
8	37	43	9	89
9	22	36	6	64
Total	219	249	40	508

Table A3.2 A22-32/A22-30 heterozygous progeny raised at 16°C

Vial	Progeny			
	A_{lethal}	P_{lethal}	E_{lethal}	Total
1	56	27	6	89
2	28	18	5	51
3	24	9	6	39
4	21	13	3	37
5	8	20	1	29
6	14	21	4	39
7	15	4	2	21
8	16	10	2	28
9	47	6	5	58
10	32	34	11	77
11	8	17	3	28
12	50	31	5	86
Total	319	210	53	582

Table A3.3 A22-30/A22-34 heterozygous progeny raised at 16°C

Vial	Progeny			
	A_{lethal}	P_{lethal}	E_{lethal}	Total
1	42	8	6	56
2	44	8	4	56
3	37	12	3	52
4	37	15	9	61
5	33	10	1	44
6	44	20	11	75
Total	237	73	34	344

Table A3.4 A22-32/A22-33 heterozygous progeny raised at 16°C

Vial	Progeny			
	A_{lethal}	P_{lethal}	E_{lethal}	Total
1	13	22	1	36
2	21	32	6	59
3	7	13	1	21
4	5	14	3	22
5	7	18	4	29
6	14	19	3	36
7	17	27	2	46
8	21	36	1	58
Total	105	181	21	307

Table A3.5 A22-32/A22-34 heterozygous progeny raised at 16°C

Vial	Progeny			
	A_{lethal}	P_{lethal}	E_{lethal}	Total
1	32	6	2	40
2	22	40	6	68
3	29	27	12	68
4	32	25	6	63
5	17	7	2	26
6	40	21	5	66
7	39	15	6	60
8	57	47	6	110
9	21	13	3	37
10	24	11	5	40
11	11	15	4	30
12	22	14	2	38
13	38	30	9	77
14	37	30	11	78
Total	421	301	79	801

Table A3.6 A22-34/A22-33 heterozygous progeny raised at 16°C.

Vial	A_{lethal}	P_{lethal}	E_{lethal}	Total
1	16	6	2	24
2	10	3	0	13
3	25	4	1	30
4	35	8	7	50
Total	86	21	10	117

Table A3.7 A22-32/A22-30 heterozygous progeny raised at 20°C

Vial	Progeny					
	wt	dark	blister	spoon	coal	$Total_{mut}$
1	32	2	1	1	2	38
2	17	0	3	1	0	21
3	26	2	3	2	0	33
4	15	1	0	3	1	20
5	13	0	0	0	0	13
6	55	0	2	0	0	57
7	38	0	2	0	0	40
8	41	3	0	5	0	49
9	34	0	3	4	0	41
10	22	0	2	0	0	24
Total	293	8	16	16	3	336

Table A3.8 A22-30/A22-33 heterozygous progeny raised at 20°C

Vial	Progeny					
	wt	dark	blister	spoon	coal	$Total_{mut}$
1	12	3	2	4	0	21
2	36	6	8	3	2	55
3	31	9	6	2	4	52
4	16	4	2	2	2	26
5	18	2	10	3	0	33
Total	113	24	28	14	8	187

Table A3.9 A22-30/A22-34 heterozygous progeny raised at 20°C

Vial	Progeny					
	wt	dark	blister	spoon	coal	$Total_{mut}$
1	26	0	2	1	0	29
2	42	4	0	1	0	47
3	67	11	1	6	21	106
4	52	3	2	0	1	58
5	36	0	0	2	0	38
6	39	2	0	4	0	45
7	57	3	1	2	0	63
8	46	4	1	5	0	56
9	19	0	2	0	0	21
10	35	2	0	4	2	43
Total	419	29	9	25	24	506

Table A3.10 A22-32/A22-33 heterozygous progeny raised at 20°C

Vial	Progeny					
	wt	dark	blister	spoon	coal	$Total_{mut}$
1	9	4	6	2	0	21
2	10	6	4	3	1	24
3	7	3	4	6	1	21
4	7	3	3	0	0	13
5	5	1	1	3	0	10
6	12	7	5	5	0	29
7	7	2	8	3	0	20
Total	57	26	31	22	2	138

Table A3.11 A22-32/A22-34 heterozygous progeny raised at 20°C

Vial	Progeny					
	wt	dark	blister	spoon	coal	$Total_{mut}$
1	11	3	5	5	1	33
2	15	4	7	2	0	28
3	9	1	1	3	0	17
4	25	8	4	9	0	47
5	22	3	2	6	2	37
6	31	7	3	10	5	56
Total	127	26	22	35	8	218

Table A3.12 A22-34/A22-33 heterozygous progeny raised at 20°C

Vial	Progeny					
	wt	dark	blister	spoon	coal	$Total_{mut}$
1	10	6	0	4	0	20
2	16	7	3	8	0	34
3	9	4	2	5	0	20
4	14	8	5	5	1	33
5	12	6	3	7	0	28
6	21	10	4	6	4	45
7	12	5	1	3	0	21
8	17	9	5	3	0	34
9	25	8	7	10	2	52
Total	136	63	30	51	7	287

Appendix 4. Deletion Analysis.

Table A4.1 A22-32/Df(3L)*brm 11* raised at 25°C

Vial	Progeny						Total
	TM6C/A22-32		Df(3L)*brm 11* /A22-32				
			"wild type"		"mutant"		
	♂	♀	♂	♀	♂	♀	
1	1	2	1	2	0	0	6
2	3	5	5	11	0	0	24
3	20	17	11	10	0	1	59
4	3	7	6	6	0	0	22
5	4	2	1	1	0	0	8
6	2	1	7	2	0	0	12
7	5	12	10	7	0	0	34
8	9	13	7	10	0	0	39
9	10	20	10	9	0	0	49
10	11	10	10	5	0	0	36
11	2	1	3	2	0	0	8
12	6	7	7	3	0	0	23
13	0	3	2	0	0	0	5
14	10	3	3	0	0	0	16
15	5	6	3	7	0	0	21
16	16	14	11	13	0	0	54
17	13	11	9	12	0	0	45
18	16	19	20	15	0	0	70
19	13	13	9	15	0	0	50
20	3	1	2	1	0	0	7
21	2	1	1	0	0	0	4
22	5	9	9	10	0	0	33
23	7	8	13	7	0	1	36
24	11	20	9	19	0	0	59
25	10	10	8	5	0	0	33
26	11	5	2	3	1	0	22
27	5	3	1	5	0	0	14
28	2	1	1	1	0	0	5
28	17	14	16	15	1	0	63
30	5	4	2	7	0	0	18
31	3	1	2	2	0	0	8
32	15	6	11	7	0	0	39
33	9	11	6	8	0	1	35
34	2	3	0	2	0	0	7
35	12	12	8	9	0	0	41
36	3	6	6	11	0	0	26
37	2	5	5	4	0	0	16
38	5	4	2	4	0	0	15
39	1	1	1	4	0	0	7
40	2	1	3	3	0	0	9
41	3	4	2	3	0	0	12
42	7	13	6	4	0	0	30
43	0	2	0	4	0	0	6
Total	291	311	251	268	2	3	1126

Table A4.2 A22-32/Df(3L)*brm 11* raised at 25°C

Vial	Progeny						Total
	TM6C/A22-32		Df(3L)*brm 11* /A22-32				
			"wild type"		"mutant"		
	♂	♀	♂	♀	♂	♀	
1	6	9	7	4	0	0	26
2	5	3	6	7	0	0	21
3	14	16	18	19	0	0	67
4	3	9	6	10	0	0	28
5	8	11	7	6	0	0	32
6	6	2	4	4	0	0	16
7	0	3	2	1	0	0	6
8	7	4	3	0	0	0	14
9	12	11	16	14	0	0	53
10	5	4	8	3	0	0	20
11	2	6	3	3	0	0	14
12	14	15	12	11	0	0	52
13	9	13	7	8	0	0	37
14	9	11	4	8	0	0	32
15	4	6	5	2	0	0	17
16	11	5	6	3	0	0	25
17	10	9	11	9	0	0	39
18	7	6	9	10	0	0	32
19	15	14	8	10	0	0	47
20	6	11	6	8	0	0	31
21	13	15	16	11	0	0	55
22	3	5	4	1	0	0	13
23	9	6	7	12	0	0	34
24	5	8	10	6	0	0	29
25	12	7	4	5	0	0	28
26	16	9	8	14	0	0	47
27	7	9	9	6	0	0	31
Total	218	227	206	195	0	0	846

Table A4.3 A22-32/Df(3L)*brm 11* raised at 20°C

	Progeny						
	TM6C/A22-32		Df(3L)*brm 11* /A22-32				
			"wild type"		"mutant"		
Vial	♂	♀	♂	♀	♂	♀	Total
1	12	13	17	17	0	0	59
2	21	18	16	16	0	0	71
3	5	3	9	4	0	0	21
4	7	7	6	3	0	0	23
5	9	15	6	12	0	0	42
6	4	3	3	7	0	0	17
7	7	10	2	5	0	0	24
8	23	11	18	10	0	0	62
9	7	11	0	4	0	0	22
10	14	12	12	9	0	0	47
11	6	1	6	2	0	0	15
12	4	11	6	9	0	0	30
13	11	6	4	2	0	0	23
14	8	9	11	14	0	0	42
15	7	15	6	11	0	0	39
16	3	7	3	8	0	0	21
17	5	10	2	3	0	0	20
18	6	4	2	2	0	0	14
19	12	9	14	9	0	0	44
20	13	8	5	5	0	0	31
21	5	2	8	5	0	0	20
22	15	10	10	10	0	0	45
Total	202	195	166	167	0	0	732

Table A4.4 A22-32/Df(3L)*brm 11* raised at 16°C

	Progeny						
	TM6C/A22-32		Df(3L)*brm 11* /A22-32				
			"wild type"		"mutant"		
Vial	♂	♀	♂	♀	♂	♀	Total
1	12	9	4	10	0	0	35
2	7	8	9	6	0	0	30
3	12	14	7	12	0	0	45
4	9	11	16	8	0	0	44
5	8	13	9	12	0	0	42
6	11	16	10	17	0	0	54
7	8	5	9	5	0	0	27
8	13	10	10	7	0	0	40
9	6	8	5	9	0	0	28
10	2	7	3	4	0	0	16
11	8	7	6	6	0	0	27
12	8	11	5	9	0	0	33
Total	104	119	93	105	0	0	421

Table A4.5 Df(3L)st^{f13}A22-32 raised at 25°C

	Progeny						
	TM6C/A22-32		Df(3L)st^{f13} /A22-32				
			"wild type"		"mutant"		
Vial	♂	♀	♂	♀	♂	♀	Total
1	10	16	12	11	0	0	49
2	2	2	2	0	0	0	6
3	5	3	3	3	0	0	14
4	1	0	0	3	0	0	4
5	6	6	7	5	0	0	24
6	8	10	8	7	0	0	33
7	3	4	2	3	0	0	12
8	3	4	2	1	0	0	10
Total	38	45	36	33	0	0	152

Table A4.6 Df(3L)st^{f13}A22-32 raised at 20°C

	TM6C/A22-32		Df(3L)st^{f13} /A22-32				
			"wild type"		"mutant"		
Vial	♂	♀	♂	♀	♂	♀	Total
1	5	7	9	5	0	0	26
2	4	2	2	3	0	0	11
3	11	6	7	4	0	0	28
4	4	5	3	5	0	0	17
5	2	5	4	4	0	0	15
6	9	7	3	7	0	0	26
7	4	3	6	5	0	0	18
8	4	5	4	2	0	0	15
9	10	6	8	5	0	0	29
10	9	5	7	2	0	0	23
Total	62	51	53	42	0	0	208

Table A4.7 Df(3L)st^{f13}A22-32 raised at 16°C

	TM6C/A22-32		Df(3L)st^{f13} /A22-32				
			"wild type"		"mutant"		
Vial	♂	♀	♂	♀	♂	♀	Total
1	5	3	2	4	0	0	14
2	1	3	3	0	0	0	7
3	3	6	1	4	0	0	14
4	5	7	2	6	0	0	20
5	2	5	4	4	0	0	15
6	5	8	9	3	0	0	25
7	6	3	0	5	0	0	14
Total	27	35	21	26	0	0	109

Table A4.8 Df(3L)*fz*M21/A22-32 raised at 25°C

	Progeny						
	TM6C/A22-32		Df(3L)*fz* M21/A22-32				
			"wild type"		"mutant"		
Vial	♂	♀	♂	♀	♂	♀	Total
1	12	9	8	7	0	0	36
2	3	1	6	1	0	0	11
3	9	14	6	7	0	0	36
4	7	5	4	2	0	0	18
5	5	8	3	6	0	0	22
6	9	11	14	8	0	0	42
7	13	8	13	11	0	0	45
8	10	11	15	12	0	0	48
Total	68	67	69	54	0	0	258

Table A4.9 Df(3L)*fz*M21/A22-32 raised at 20°C

	Progeny						
	TM6C/A22-32		Df(3L)*fz* M21/A22-32				
			"wild type"		"mutant"		
Vial	♂	♀	♂	♀	♂	♀	Total
1	14	13	14	20	0	0	61
2	4	6	9	8	0	0	27
3	4	8	2	7	0	0	21
4	11	7	13	14	0	0	45
5	4	9	7	5	0	0	25
6	6	8	4	11	0	0	29
7	5	9	7	7	0	0	28
Total	48	60	56	72	0	0	236

Table A4.10 Df(3L) th^{ss102}/A22-32 raised at 25°C

	Progeny						
	TM6C/A22-32		Df(3L)th^{ss102}/A22-32				
			"wild type"		"mutant"		
Vial	♂	♀	♂	♀	♂	♀	Total
1	3	6	2	3	0	0	14
2	8	4	4	9	0	0	25
3	7	3	6	4	0	0	20
4	4	4	3	6	0	0	17
5	4	7	5	9	0	0	25
6	8	2	3	0	0	0	13
Total	34	26	23	31	0	0	114

Table A4.11. Df(3L) th^{ss102}/A22-32 raised at 20°C

	Progeny						
	TM6C/A22-32		Df(3L)th^{ss102}/A22-32				
			"wild type"		"mutant"		
Vial	♂	♀	♂	♀	♂	♀	Total
1	3	0	0	1	0	0	4
2	4	1	7	3	0	0	15
3	2	7	1	3	0	0	13
4	5	7	3	2	0	0	17
5	8	3	3	7	0	0	21
6	4	7	4	1	0	0	16
7	0	6	5	2	0	0	13
8	2	6	0	3	0	0	11
Total	28	37	23	22	0	0	110

Table A4.12 Df(3L) th^{ss102}/A22-32 raised at 16°C

	Progeny						
	TM6C/A22-32		Df(3L)th^{ss102}/A22-32				
			"wild type"		"mutant"		
Vial	♂	♀	♂	♀	♂	♀	Total
1	1	4	2	1	0	0	8
2	5	3	4	0	0	0	12
3	4	2	2	4	0	0	12
4	3	1	5	2	0	0	11
5	1	3	0	2	0	0	6
6	3	1	1	4	0	0	9
7	5	3	8	4	0	0	20
8	4	8	3	0	0	0	15
9	0	4	1	2	0	0	7
10	1	3	0	1	0	0	5
11	3	2	2	5	0	0	12
Total	30	34	28	25	0	0	117

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THE END!