STUDIES OF THE *DROSOPHILA* BRAIN USING P[GAL4] ENHANCER TRAP LINES

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by

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The research reported in this thesis is my own original work, except where otherwise stated, and has not been submitted for any other degree.

Ming Yao Yang

This thesis is dedicated to my wife and my daughter.

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Abbreviations

amp	ampicillin
BCIP,X-phosphate	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
bp	base pairs
bis	N, N'-methylenebisacrylamide
cDNA	complementary DNA
Ci	Curies
cpm	counts per minute
DAB	diaminobenzidine
DEPC	Diethylpyrocarbonate
DMSO	dimethylsulphoxide
DNAse I	deoxyribonuclease I
$d(N_6)$	Random Hexanucleotides
dNTP	deoxyribonuleotide triphosphate
DTT	dithiotheritol
EDTA	ethylendiamine tetra-acetic acid
EtBr	ethidium bromide
IPTG	isopropyl-ß-D-thiogalactopyranoside
kb	Kilobases/kilobasepairs
LMP	Low melting point
MOPS	morpholino propane sulphonic acid
mRNA	messenger ribonucleic acid
NaPPi	sodium pyrophosphate
NTB	4-nitrobluetetrazoliumchloride
OD	optical density
PEG	polyethylene glycol
pfu	plaque forming units
RF	RNase Free
RNAse A	ribonuclease A
rpm	revolutions per minute
SDS	sodium dodecylsulphate
TEMED	N, N, N' N', -tetramethylenediamine
tRNA	transfer RNA
µci	microcuries
UV	ultraviolet light
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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SUMMARY

This thesis is concerned with the application of enhancer trap technology to illustrate the structures of *Drosophila* brain and identify the genes relevant to the central complex function in the brain.

Over 1400 novel enhancer trap lines bearing P[GAL4] insertions were generated by genetic crosses and they were then screened for GAL4-directed ß-gal expression in cryostat sections of the *Drosophila* head. More than 300 lines display interesting patterns in the brain from an anatomical perspective. Of these, as many as 100 are more or less restricted to specific regions or neuronal sub-populations of brain. Particularly exciting are lines that express GAL4 in the mushroom bodies and the central complex, structures that have been implicated in associative learning and memory. For most of lines, the chromosomal locations of P[GAL4] insertions were identified by *in situ* hybridisation to polytene chromosomes.

P[GAL4] expression patterns have suggested multiple roles for certain *Drosophila* brain structures in integration of signals. In the mushroom bodies, the blue staining patterns have revealed axonal processes corresponding to Kenyon cells and showed that the *Drosophila* mushroom bodies are compound neuropils in which parallel sub-components exhibit discrete patterns of gene expression. A strong prediction that different sub-sets of Kenyon cells perform different functional roles is made. In the ellipsoid body of the central complex, four different ring structures are revealed by P[GAL4] expression patterns. Developmental analysis indicates that the *lac*Z expression in the central complex lines begins at early pupal stages to the adults.

The central complex of the *Drosophila* brain has been shown to act as a higher centre for locomotor activity and other behaviours. To identify the genes relevant to central complex function, seven P[GAL4] enhancer trap lines with staining patterns specific to the central complex were selected. Genomic DNAs flanking each insertion site

were cloned by plasmid rescue. Rescued genomic DNAs from some of lines were used as probes for screening a cDNA head library. Corresponding cDNA clones were isolated.

In the case of line c507, P[GAL4] staining is restricted to the ellipsoid body of the central complex of brain and to the Malpighian tubules in the *Drosophila*. Three genes, two located downstream and one upstream of the P[GAL4] element, are identified. Sequencing of a 1.8 kb cDNA clone from pMY51 which is located downstream of the P[GAL4] reveals a protein with significant homology to the alkaline phosphatase gene family in other organisms. The other cDNA clone, closely linked with pMY51, represented by pMY8 is sequenced and a full length predicted amino acid sequence identified. It has head-elevated expression as judged by Northern blot analysis. The gene which is located upstream of the P[GAL4] element is identified as calcineurin A1, the Ca^{2+}/calmodulin-stimulated protein phosphatase.

in situ hybridisation to tissue sections using cDNA probe generated from a whole insert of pMY51 reveals that the gene has expression patterns in the cell bodies of the ellipsoid body and the Malpighian tubules consistent with the X-Gal staining. Results indicate that the *Drosophila* "alkaline phosphatase" enhancer is trapped and the corresponding gene has been cloned by enhancer trap approach.

Chapter 1

Introduction

1.1 The P element as a tool for the study of *Drosophila* genetics

1.2 The enhancer trap approach

1.3 The structure of brain

1.4 Aims of the project

This chapter provides a background to some of the topics discussed in this thesis. A general introduction to P elements and their applications is presented first. This is followed by a review of the "enhancer trap" system. Then, the structure of the *Drosophila* brain is described. The final section of this chapter describes the initial aims of this project and the strategies used to achieve them.

1.1 The P element As A Tool for the Study of *Drosophila* Genetics

1.1.1 The P Element

Transposable genetic elements are segments of DNA with the special ability to jump from place to place on the chromosomes. They are typically found in many copies scattered about the genome, and their position is widely variable between individuals of *Drosophila melanogaster.* Of these, the P element family is the most extensively studied and widely used in the molecular biology of *Drosophila.* The P elements are of particular interest because their transpositional activity is potentially high but under strict genetic control (Engels, 1989).

P elements have been shown to be the causal agents of P-M hybrid dysgenesis, a syndrome whose traits include high rates of sterility, mutation, and chromosomal rearrangements (Engels, 1989;). P element transposition is genetically regulated, occurring at very high frequency only in the progeny from a cross in which P element containing males (P strains) are mated to females lacking P elements (M strain). No dysgenic traits are observed in the progeny of the reciprocal M male by P female cross or in the progeny from PxP or MxM crosses. Moreover, transposition is restricted to cells of the germ-line. Thus, P element transposition is regulated in two ways: genetically and tissue specifically. The distinguishing characteristic of P strains is that they contain full-length autonomous P elements, encoding their own transposase. P strains also contain defective, generally internally deleted, P elements. Transposition in a P strain is tightly regulated. It is repressed by a product of the full-length P element itself. This condition is known as a "P cytotype". M

strains, in comparison, lack autonomous P elements and are permissive for P element transposition ("M cytotype"). Thus, transposition and hybrid dysgenesis are induced when potentially active P elements are introduced into the permissive cellular environment of M cytotype which is transmitted by the female parent.

Molecular analysis indicated that the complete P element is 2.9 kb in length (Fig. 1.1). Four long open reading frames encode an 87 kD transposase, activity of which is restricted to the germ-line as the result of differential splicing (Rio, 1991). Inverted 31 bp terminal repeats, together with as much as 138 bp and 216 bp at the both ends respectively, are the *cis* acting determinants of transposition (Mullins *et al.*, 1989). In addition to full-length P elements, most P strains contain a range of internally deleted elements varying in length from 500 to 2500 bp. These P elements are non-autonomous because they are unable to produce functional transposase. But many such elements retain *cis*-acting determinants that allow their mobilisation in the presence of full-length elements (Engels, 1989). It is noted that an engineered P element with the third intron removed (Δ2-3), as shown in Fig. 1.1, can produce transposase in both somatic and germ line cells (Laski *et al.*, 1986). It is often used to mobilise internally deleted P elements in *Drosophila* genetics.

When P elements transpose they leave behind a double-stranded break that can be the subject of widening by exonucleases and the repair of which appears to require a template. Usually the template is provided by a sister chromatid, in which case P element sequences are restored at the site. Occasionally it is provided by an homologous chromosome. Where this carries a wild-type allele the impression will be given of precise excision from locus. Remobilisation can also result in imprecise excision, presumably reflecting failure of the repair process (Engels, 1992).

The applications of P elements will be briefly discussed below.

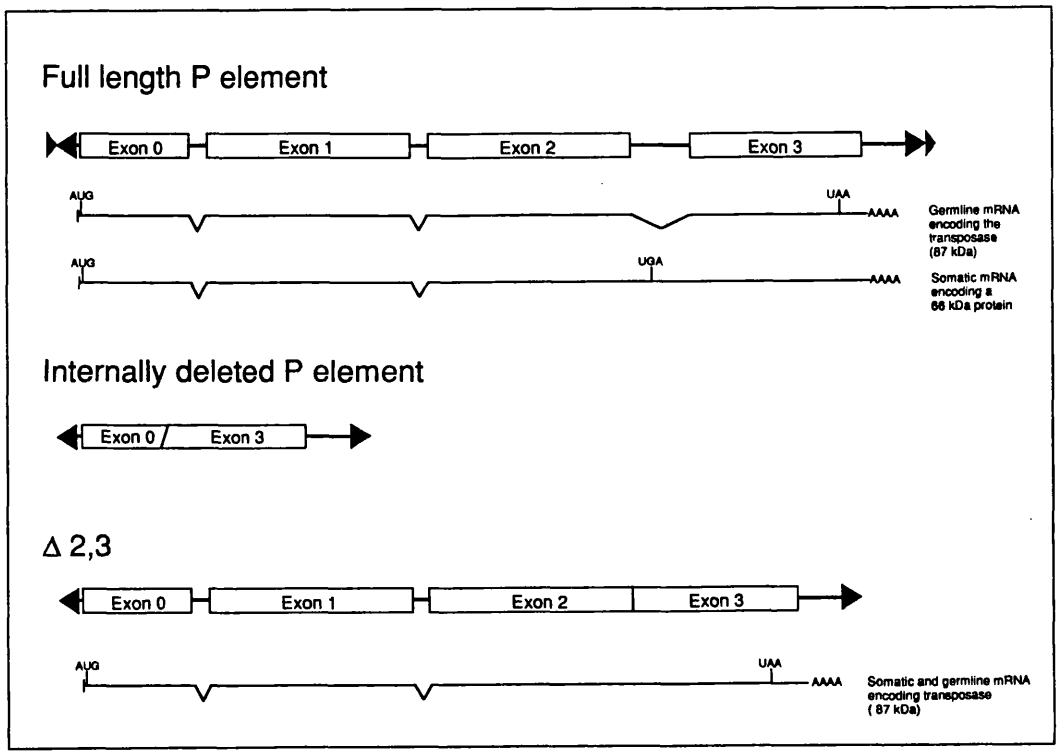


Figure 1.1. Structure of the full length 2.9 kb P-element, internally deleted P-element and Δ 2-3 element. The terminal 31 bp inverted repeat and the direct 8 bp target site duplication are denoted by arrow heads (black and shaded, respectively). Translation initiation and termination codons are shown for the full length P-element and the Δ 2-3 element. (Diagram taken from Sentry and Kaiser, 1993)

1.1.2. Germ-line Transformation

DNA-mediated germline transformation is an indispensable tool for analysing many problems in *Drosophila* molecular genetics. Germ-line transformation of *Drosophila* is achieved by the injection of cloned and suitably manipulated P element DNA (as a component of a bacterial plasmid) into embryos undergoing the transition between nuclear syncitium and cellular blastoderm (Rubin and Spradling,1982; Spradling and Rubin, 1982).

P element vectors, carrying a marker, are the defective P elements which lack a functional transposase gene but have all of the *cis*-acting determinants necessary for transposition. Transposase can be supplied by co-injection of a plasmid that encodes it, by co-injection of purified protein, or by injection of Δ2-3 embryos. DNA injected at the posterior pole prior to cellularisation will become incorporated into germ-line precursors, and occasional transposition will occur from the injected plasmid to the *Drosophila* genome. Adults that develop from injected embryos are genetic mosaics with respect to the presence of the transposon in their germ-line. True transformed individuals can be recovered in the next generation, usually the transposon of interest carries a phenotypic marker to allow identification of transformants (Sentry and Kaiser, 1993).

Germ-line transformation experiments have had a major impact on *Drosophila* molecular genetics for obvious reasons. P element vectors can be used to transform flies with cloned genes to rescue a mutant phenotype, to prove that a DNA fragment carries the corresponding gene, and can be used to identify *cis*-acting regulatory sequences involved in its correct spatial and temporal expression (Bargiello *et al.*, 1984; Bourois and Richards, 1985; Haenlin *et al.*, 1985; Fischer and Maniatis, 1986). In addition, genes manipulated *in vitro* can be reintroduced into the animal and their biological consequences assayed *in vivo*.

1.1.3. Controlled Mutagenesis

In order to control P-element insertional mutagenesis, flies containing two types of defective element are used; "mutator" element, which is defective in both transposase and P-repressor function while retaining intact 31bp P element end and other *cis*-acting sequences required for transposition, so it can be mobilised when provided with a source of transposase *in trans*. Appropriate phenotypic markers such as *Adh* (Goldberg *et al.*, 1983), *ry* (Rubin and Spradling, 1982) and *w* (Bier *et al.*, 1989) are included on the mutator element in order to trace their integration. Transposase is supplied by a "jumpstarter" element whose terminal repeat structures have been mutated to eliminate self-mobilisation. In other words, jumpstarter elements produce transposase and can therefore catalyse transposition of mutator elements but not themselves. Both elements can be maintained in stock, in isolation, and brought together in a dysgenic cross. Mobilisation then occurs within a proportion of the germ cells from F_1 individuals. Novel insertions are stabilised in the next generation by segregation of the jumpstarter chromosome from the target chromosomes, facilitated by selection against genetic markers closely linked to the jumpstarter element (Fig. 1.2).

The jumpstarter element, Js-1 (Cooley *et al* .,1988) can produce low levels of germ-line restricted transposase. This element, however, has the disadvantage that at low frequencies (1-2%) it will self-mobilise, requiring outcrossing at each generation and occasional confirmation of genomic position. Currently, the most efficient jumpstarter element available appears to be P[ry^+ Δ2-3] (99B), reported by Robertson *et al* ., (1988) lying at position 99B7-10 on the third chromosome. The Δ2-3 element can cause mobilisation of other elements at unusually high frequencies, yet is itself remarkably stable. It produces very high levels of active transposase.

Depending on the application, multiple or single insert lines can be produced by a combination of different mutator and jumpstarter chromosomes to control the rate and frequency of transposition (Cooley *et al.*, 1988; Robertson *et al.*, 1988).

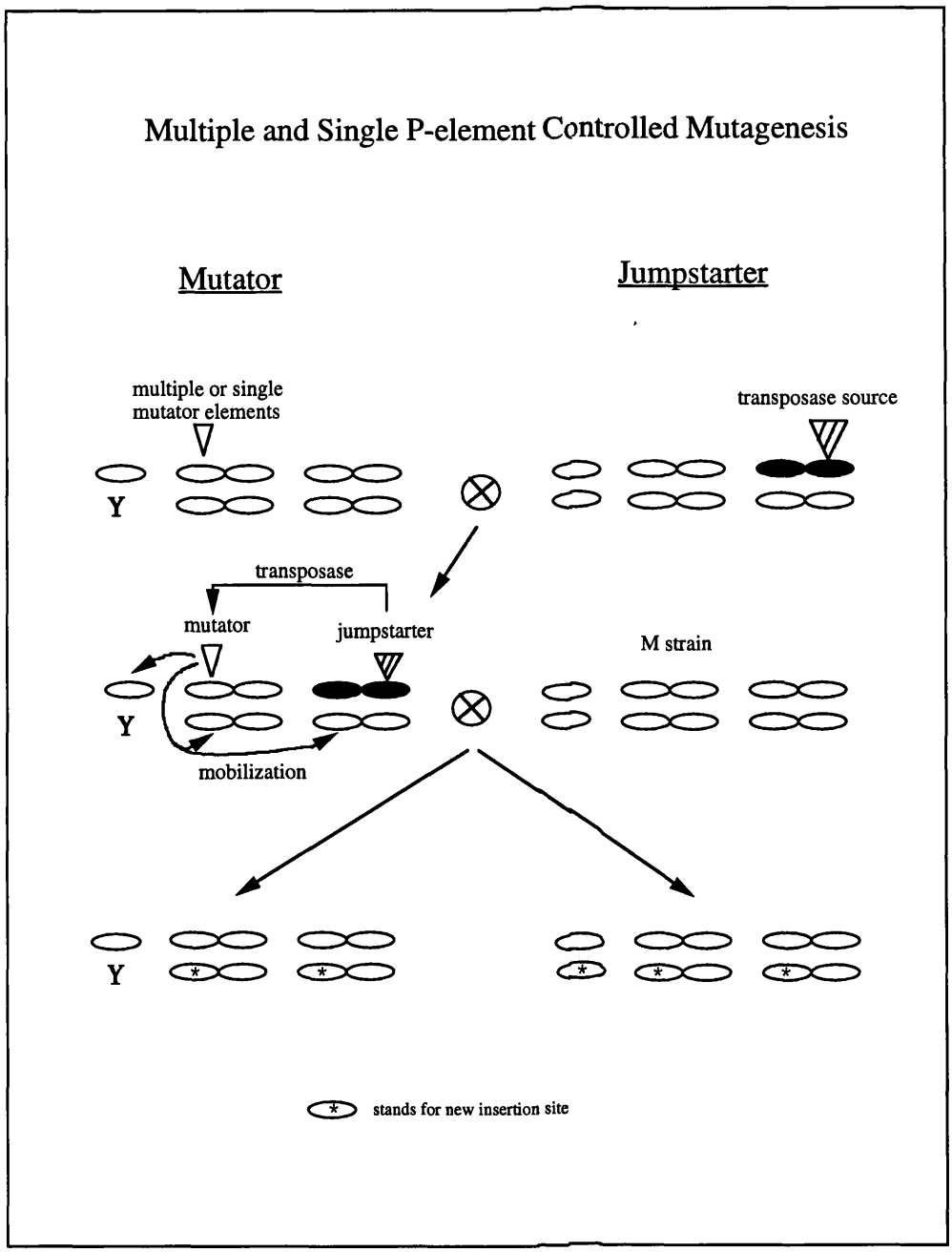


Figure 1.2. A controlled P-element mutagenesis strategy. An enhancer trap P element (mutator) is mobilised by the Δ 2-3 transposase (jumpstarter) in the germ line cells of F_1 males. Each sperm carries a different spectrum of new insertions. Selection against the transposase source in the F_2 generation ensures that new insertions remain stable.

The generation of lines containing only a single marked mutator element has many advantages as a method of mutagenesis. Phenotypic and molecular analyses of new mutations are greatly simplified. The mutant gene can be mapped by identifying the transposon insertion site using *in situ* hybridisation to polytene chromosomes. DNA flanking the insertion site can be cloned simply by screening a library for P element homology. Revertants, including new alleles generated by imperfect excision, can be recovered by reintroducing a jumpstarter element and scoring for loss of the phenotype specified by the mutator element's marker gene. In addition, single-insert lines have intrinsic long-term value for manipulating the *Drosophila* genome (Cooley, *et al* 1988; Sentry and Kaiser, 1995).

1.1.4. Other Applications

The P element has also been applied to other areas. For example, it can be used to clone genes by transposon tagging (Bingham *et al* ., 1981; Searles *et al* ., 1982), for precise and imprecise excision (Tsubota and Schedl, 1986; Salz *et al.*, 1987) and local jumping (Tower *et al.*, 1993; Zhang and Spradling, 1993). It can be also used to create double-strand DNA breaks, in experiments where the flanking DNA is replaced with modified sequences (Gloor *et al.*, 1991).

"Site-selected" P element mutagenesis is a PCR-based screen for P-element insertion events. It allows the detection and isolation of a P element transposon into or near a cloned gene of interest (Ballinger and Benzer, 1989; Kaiser and Goodwin, 1990; Sentry and Kaiser, 1994). The corresponding mutants can be used to analyse the function of the gene (S. Goodwin, per com.).

P elements can be also engineered with reporter genes and used to identify the timing and tissue distribution of the expression of genes that happen to lie near the insertion site (O'Kane and Gehring 1987). For this point, I will review in more detail in the next section.

1.2 The Enhancer Trap Approach

1.2.1. What Is An Enhancer ?

Enhancers are regulatory sequences which stimulate transcription from eukaryotic promoters with which they are associated. They are probably the major mechanism for regulating gene expression in eukaryotes. They are activated by the binding of a specific protein and then act as sites for the assembly of transcriptional initiation complexes. They differ from most regulatory sequences due to the following reasons: (1) They may be located either upstream or downstream of the gene and several kilobases away from the gene whose transcription they control. (2) They act in either orientation and so can simultaneously influence the expression of two genes, one on each side of the enhancer sequence. (3) They must be located on the same molecule of DNA as the regulated gene, but the sequence can be on either DNA strand. (4) Enhancers are not gene-specific but they are tissue-specific. Preferentially enhancers stimulate transcription from the nearest promoter (Smith-Keary, 1991).

1.2.2. An Enhancer Trap Element

In *Drosophila,* the "enhancer trap" method was initially described by O'Kane and Gehring (1987) and has since been modified in various ways (Bellen *et al.*, 1989; Bire *et al.*, 1989; Brand and Perrimon 1993). The major advantage of the enhancer trap is that instead of identifying genes by means of the phenotype caused by a mutation, they are identified by their pattern of expression. The underlying assumption is that a developmentally important gene will show a specific temporal and spatial expression pattern related to its function. This notion is supported by many examples (see review, Freeman, 1991).

Enhancer trap constructs are defective P-elements within boundaries of intact 31 bp terminal repeats and other essential *cis*-acting sequences. Such constructs can be introduced

into the *Drosophila* genome by coinjection with a helper element into embryos, as in the germline transformation technique mentioned as section 1.1.2. The reporter gene in the enhancer trap is a translational fusion that bring the *E. coli* ß-galactosidase gene under the control of a weak promoter (usually the P-transposase promoter). The coding sequence of the ß-galactosidase gene is fused in frame to a sequence in the second exon of the P transposase gene. Following chromosomal integration of the P element, the transposase promoter may be influenced by nearby genomic enhancers, leading to developmental regulation of ß-galactosidase expression (Fig. 1.3a). Of the first generation enhancer trap element, report gene is *lacZ*. In addition, the element carries a dominant eye colour gene such as w^+ or ry^+ to identify flies that contain it, and plasmid sequences that facilitate the cloning of flanking genomic DNA.

The presence of ß-galactosidase expression pattern can be easily visualised *in situ* by staining whole-mount embryos, larvae or adult flies with a chromogenic substrate for the enzyme. Generally, staining is localised to cell nuclei, by virtue of the nuclear localisation signal present on the N-terminal fraction of the P-transposase gene to which the reporter is fused (Grossniklaus et al 1989). Although nuclear staining allows visualisation of the location of the neuronal cell-body, cytoplasmic localisation is favourable for the analyse of cells with extensive processes, such as axons and dendrites of neurons. Some efforts have been made to address this problem (Smith and O'Kane, 1991; Giniger *et al.*, 1993; Callahan and Thomas, 1994).

Since P element transposition is, to a first approximation, a random process, lines in which enhancer trap elements have become integrated at new locations in the genome can be generated easily by following a P-element mutagenesis strategy descried as section 1.1.3. For example, the *lacZ* reporter gene is used as "mutator" element and P[ry^+ Δ2-3] as the "jumpstarter" element, the number of new enhancer trap lines can be produced by genetic crosses instead of by embryo microinjection.

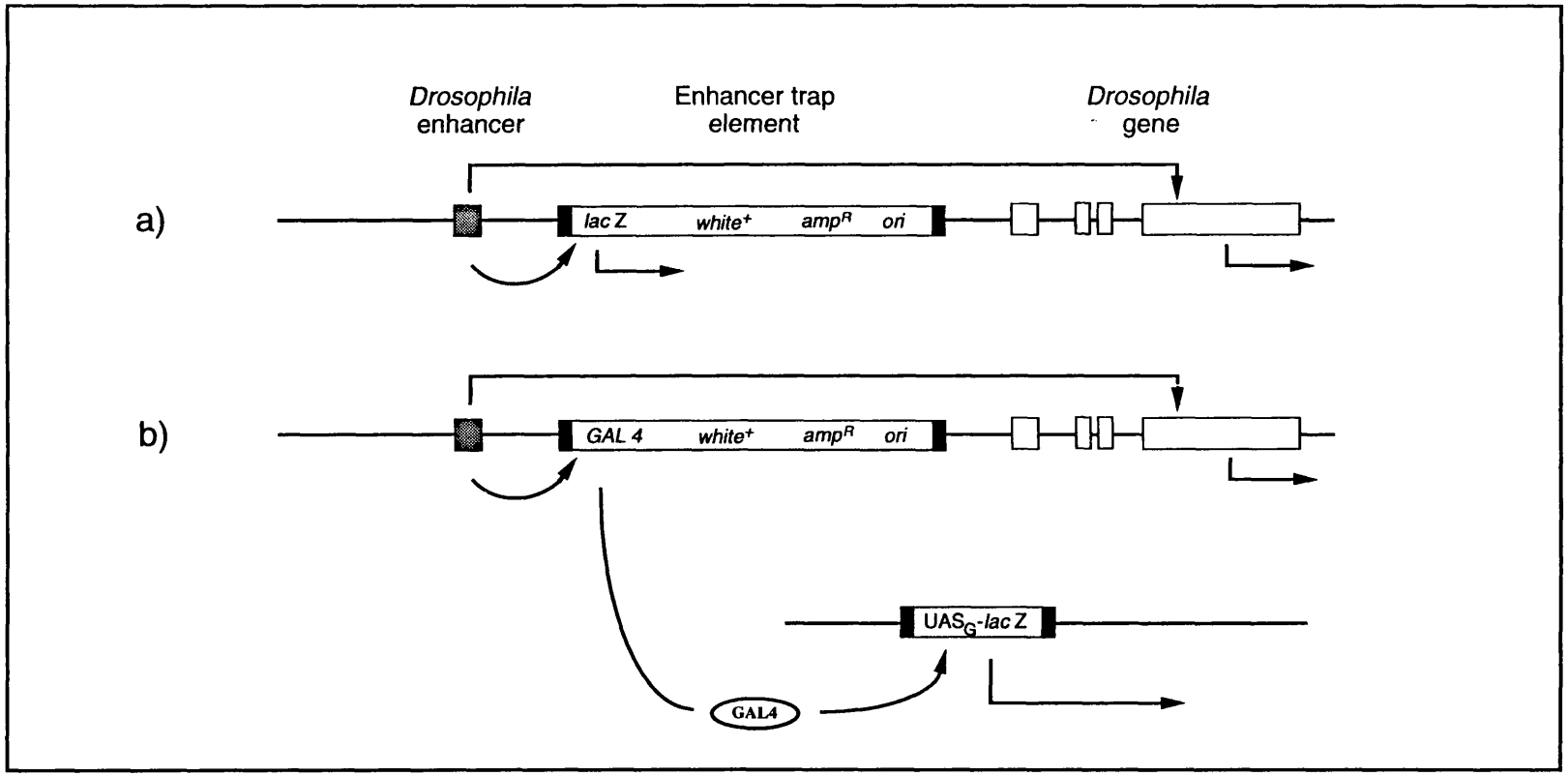


Figure 1.3 Enhancer trap strategies

a). First generation enhancer trap element. After integration into the genome, the reporter may be influenced by nearby genomic enhancers, leading to developmental regulation of *lacZ* expression. Enhancer trap elements have a dominant eye colour gene (w^+) which allows flies with insertions to be recognised. The ampicillin resistance (amp^R) determinant and *E coli* origin of replication (*ori*) facilitate plasmid rescue of flanking sequences.

b). A GAL4 enhancer trap element. As above, except that GAL4 expression is influenced by a nearby enhancer. GAL4 activates transcription from a second reporter gene linked to the GAL4-responsive promoter, UAS_G. (Diagram slightly modified from Sentry *et al.*, 1994)

The insertion of an element may or may not be mutagenic itself (approximately 10% of enhancer trap lines contain insertions that are mutagenic themselves), depending upon its precise location with respect to the gene. Even if the insertion of the element does not itself disrupt gene function, then the element can be remobilised to generate imprecise excisions. This approach is facilitated by a dominant eye-colour marker carried by the enhancer trap element, whose loss can be easily scored in an appropriate excision screen.

1.2.3. ß-gal Expression in the Nervous System

The enhancer trap method has been used to observe ß-gal expression specifically in the cells of the central nervous system (CNS) and peripheral nervous system (PNS). (Bellen *et al.*, 1989; Bire *et al.*, 1989; Wilson *et al.*, 1989; Ghysen and O'Kane, 1989; Klambt *et al.*, 1991; Smith and O'Kane, 1991; Han *et al.*, 1992; Hartenstein and Jan, 1992; Skoulakis *et al.*, 1993; Giniger *et al.*, 1993; Callahan and Thomas, 1994).

Bier *et al.*(1989) found that about 31% of their lines were expressed in the embryonic nervous system. Bellen *et al* (1989) described approximately 50% of all lines stained specifically in the embryonic nervous system with around 20% of these showing either CNS or PNS specific staining. Nose *et al.* (1992) reported that they screened about 11000 enhancer trap lines and found a number of lines stained in the nervous system.

Besides the embryonic nervous system, the enhancer trap method has been recently applied to the adult nervous system by several groups. Schneuli and Heisenberg (Wurzberg, personal com.) found that as many as 70% of the lines showed some degree of staining in the brain, with a smaller though significant subset of these showing more restricted patterns of expression, specific to certain anatomical domains or groups of cells. Han *et al.* (1992) screened approximately 5300 enhancer trap lines and found about 90 of these lines showed preferred or exclusive expression of *lacZ* in mushroom body cells. Skoulakis *et al.* (1993) have isolated a *DCO* (the catalytic subunit of protein kinase A) mutant by an enhancer trap

screen for genes preferentially expressed in the mushroom bodies. In our lab, Paterson and Kaiser (1993) generated and screened about 350 new enhancer trap lines. They found that approximately 65% of the lines showed *lacZ* expression within the adult brain.

Enhancer trapping has become a widely used approach for the generation of cell and tissue makers in *Drosophila*. For example, Klambt *et al.*, (1991) isolated enhancer trap lines as markers for specific midline lineages of CNS. Hartenstein and Jan (1992) analysed enhancer trap marker lines for the embryonic development and specific subdivision of the embryo. In the CNS, for example, perineurial cells can be clearly divided into two layer, an inner layer and an outer layer. Nelson and Laughon (1993) used enhancer trap lines as a marker to demonstrate the glial architecture and development. Using BrdU incorporation and enhancer trap lines, Prokop and Technau (1994) identified a reproducible spatial and temporal pattern of DNA replicating cells in the abdominal larval CNS (A3-7 neuromeres) of *Drosophila*. Some of marker lines are not only useful for studying normal development, but they can also be employed in the analysis of defects in mutant flies (Han *et al.*, 1992; Skoulakis *et al.*, 1993).

1.2.4. Staining Patterns Represent the Expression Patterns of Neighbouring Genes

The enhancer trap system can be used to analyse many different genes in *Drosophila*, including those that cannot be identified or characterised initially by classical genetics. More evidence has accumulated to show that known P element location lie close to genes with known expression patterns which match, closely or exactly, the observed ß-galactosidase pattern of the construct (Bellen *et al.*, 1989; Bier *et al.*, 1989; Wilson *et al.*, 1989; Ghysen and O'Kane 1989; Fasano *et al.*, 1991; Han *et al.*, 1992; Bier *et al.*, 1992; Mlodzik and Hiromi, 1992; Nose *et al.*, 1992; Skoulakis *et al.*, 1993; Callahan *et al.*, 1995).

By an enhancer trap screen, seven lines were isolated with P element insertions in the cytogenetic vicinity of the learning and memory gene, *rutabaga*. (Han *et al.*, 1992). For another learning gene *DC0*, mutations have also been isolated in a such screen. (Lane and Kalderon, 1993; Skoulakis *et al.*, 1993). Their expression patterns of *lacZ* are the same as those of genes which are predominantly elevated in the mushroom bodies. Mlodzik and Hiromi (1992) showed examples that two insertion lines displayed an identified subset of the expression patterns observed for the endogenous genes (*Toll* and *fasIII*) in the embryonic CNS. Bellen *et al.* (1989) reported that six lines were identified in which the staining pattern seemed to reflect the expression pattern of a gene adjacent to the insertion by mapping 68 insertion lines cytologically. Wilson *et al.* (1989) confirmed this at the molecular level in two cases. In addition, they tested whether the cloned genomic fragments encode transcripts expressed in the pattern predicted by the embryonic ß-galactosidase staining of the corresponding insertion line. Therefore, they estimated that at least 25% of enhancer trap lines will reflect the expression of a neighbouring gene. Since the expression pattern of the vast majority of *Drosophila* genes is not yet known, it is difficult to assess the efficiency of gene detection from this data. However, genes may be identified and cloned solely on the basis of their expression pattern by this method, with no requirement for information regarding associated function.

As the enhancer trap element carries "plasmid sequences", it is possible to facilitate the plasmid rescue of flanking genomic DNA. For example, "designer element", P[lArB] (Wilson *et al.*, 1989) contains a bacterial origin of replication (*ori*) and antibiotic resistance gene (amp^R). They allow production of a clonable plasmid which contains *Drosophila* DNA from adjacent to the site of P element insertion. Figure 1.4 demonstrates the procedure of plasmid rescue. Firstly, genomic DNA from flies carrying an enhancer trap insertion is digested with a restriction enzyme that cuts in one of the polylinker sequences in the construct. This produces many fragments including one that contains the *ori* and amp^R sequences and adjacent genomic sequences extending to the next restriction site. Secondly, dilution of the digested DNA and subsequent ligation leads to intramolecular ligation of the

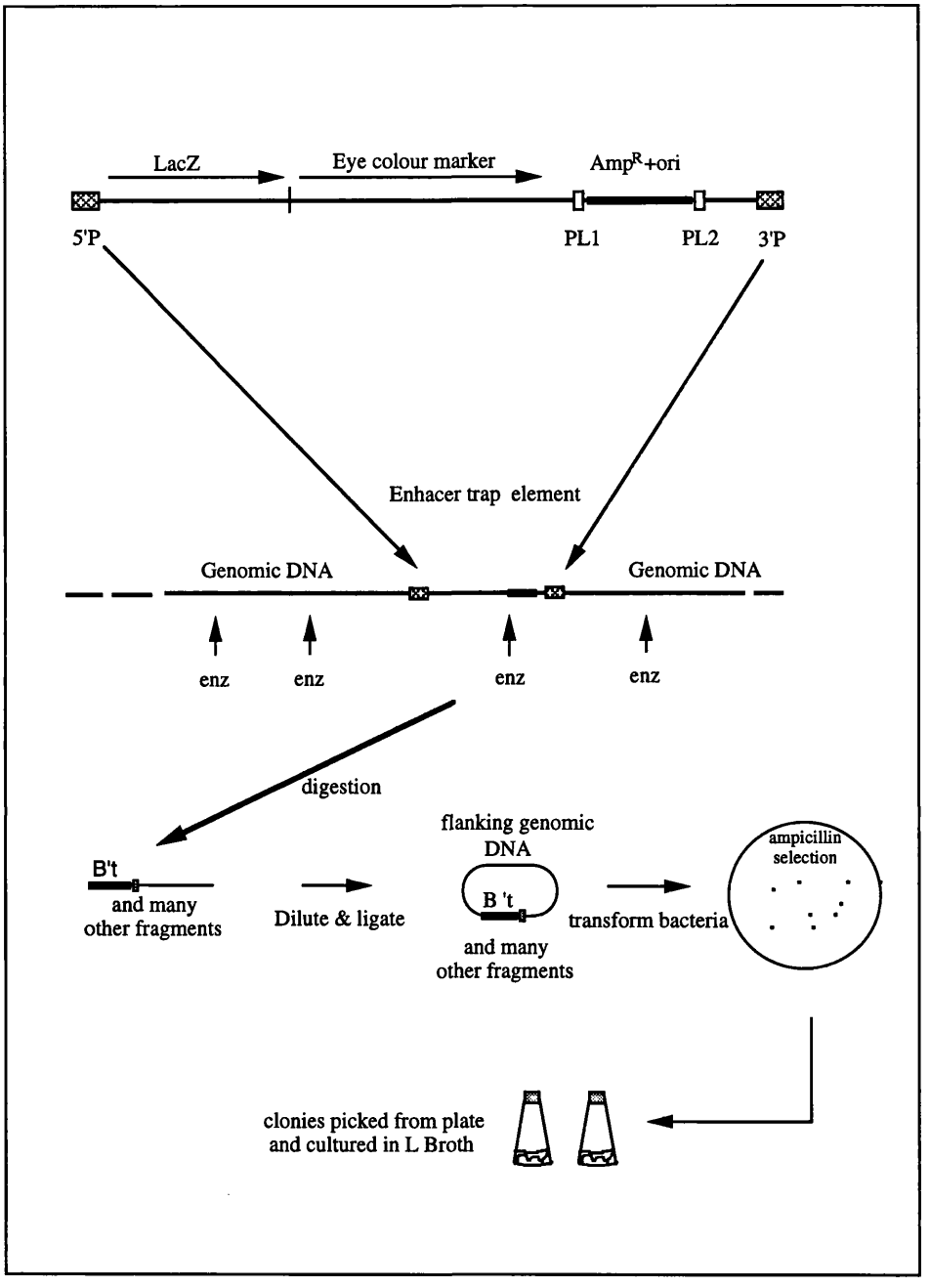


Figure 1.4. Diagram of plasmid rescue technique. PL=polylinker, enz=enzyme. See text for full description. (Diagram was redrawn and slightly modified from Bellen *et al*., 1990). See text for further details.

different fragments. If these circular molecules are introduced into *E coli*, only the cells containing the bacterial origin of replication and the resistance gene will grow on plates containing ampicillin. The surviving colonies carry a plasmid which contains genomic sequences directly adjacent to the enhancer trap element. Digestion with a suitable restriction enzyme can then produce a fragment containing 3' (or 5') P-element sequences and all of the adjacent cloned genomic DNA. The genomic fragment generated from such a digest of plasmid DNA can be isolated on a gel and labelled as a probe for Southern and Northern analysis or *in situ* hybridisation to tissue. On the other hand, the rescued plasmid can be used to probe a wild type library to identify clones of interest. Following this method, Wilson *et al* (1989) have cloned genomic regions flanking the insertion sites in 23 lines. Within our lab, Y. Guo, and A. Gillan (personal comm.) have obtained the rescued plasmids from about 2000 lines.

1.2.5 "Second Generation" Enhancer Trap System

More recently, to exploit a method for turning on genes in a tissue-specific manner during any stage of development, "second generation" enhancer trap systems have been developed (Brand and Perrimon 1993, Kaiser 1993). This method separates the activator from its target gene in distinct lines, to ensure that the individual parent lines are viable: in one line the activator protein is present but has no target gene to activate, in the second line the target gene is silent. When the two lines are crossed, the target gene is turned on only in the progeny of the cross, allowing dominant phenotypes (including lethality) to be conveniently studied. This two part system consists of the yeast transcriptional activator, GAL4, and a gene of interest, which is transcriptionally controlled by the GAL4 upstream activator sequence (UAS_G).

To assay transactivation by GAL4, for example, flies that express GAL4 are crossed to those bearing a *lacZ* gene whose transcription is driven by GAL4 binding sites. Their progeny will contain both the GAL4 enhancer trap element and the UAS_G-*lacZ* gene and will,

therefore, express ß-galactosidase only in those cells in which GAL4 is first expressed (Fig. 1.3b). The ß-galactosidase encoded by the UAS_G-*lacZ* construct is localised in the cytoplasm rather than in the nuclei (Brand and Perrimon 1993). It will be very useful for one to visualise the neuropil structures in the brain and axons for neuronal pathfinding and synaptic connectivity.

The advantage of this system is that different markers can be tested in a single GAL4 enhancer trap line if the suitable marker becomes available (Brand and Perrimon 1993; Greig and Akam 1993; Ferveur *et al.*, 1995; Sweeney *et al.*, 1995; Yeh *et al.*, 1995). For instance, it might be useful to cross a cell-surface marker into an enhancer trap line that expresses GAL4 in neurones, thereby allowing the axonal processes to be traced. More specifically, fusion between *lacZ* and genes encoding the microtubule-associated proteins kinesin (Giniger *et al.*, 1993) and tau (Callaghan and Thomas, 1994) may allow complete mapping of long, microtubule-rich neuronal projections. Furthermore, the gene attached to the UAS_G need not simple encode a marker. By linking a UAS_G to a gene involved in development, the consequences of ectopically expressing that gene in the cells in which the enhancer trap is active can be assayed.

One of the most powerful uses of the second generation enhancer trap system will be the ability for cell ablation. In *Drosophila*, cell ablation experiments can be done by transactivation of ricin or diptheria toxin expressing constructs (Kunes and Steller, 1991; Moffat *et al.*, 1992; Bellen *et al.*, 1992). Recently Sweeney *et al.*, (1995) reported that targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioural defects; in one case, the olfactory escape response is reduced. Hidalgo *et al.*, (1995) showed that targeted ablation of glia disrupts axon tract formation in the *Drosophila* CNS. Such a strategy could be used to address a range of questions concerning the development and function of specific groups of cells (O'Kane and Moffat, 1992; Sentry *et al.*, 1993)

1.3 The Structure of the *Drosophila* Brain

P[GAL4] enhancer trap lines are not only suitable to facilitate the cloning of flanking genes as mentioned before, but also useful as neuronal markers for anatomical analysis (Yang *et al.*, 1995; Smith and Shepherd, 1995; S. Renn, personal com.; R. Stocker, personal com.) and for functional studies in *Drosophila* brain (Ferveur *et al.*, 1995; Sweeney *et al.*, 1995; O'Dell *et al.*, 1995). In this section I will briefly summarise the structures of the *Drosophila* adult brain.

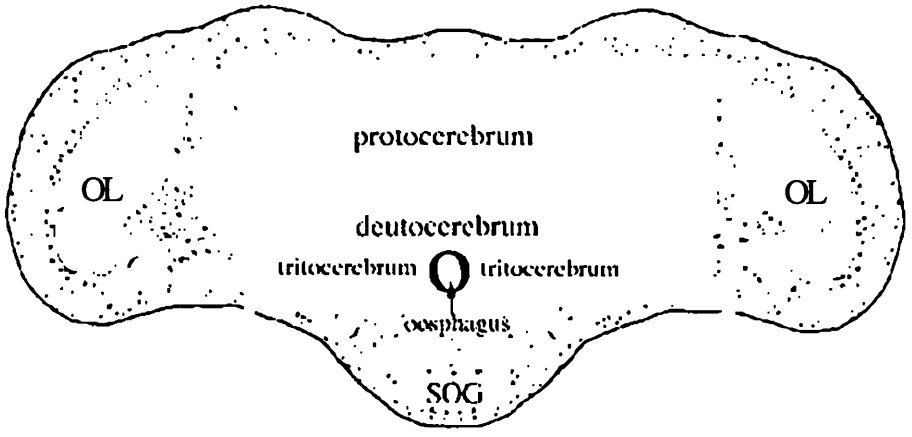


Fig 1.5 Schematic diagram of the *Drosophila* brain.

As shown in Figure 1.5, the brain of the *Drosophila* , in common with that of other insects, can be divided into two regions: the supra-oesophageal and sub-oesophageal ganglion (SOG). The supra-oesophageal ganglion is made up of two hemispheres. They can be divided into three regions, namely the protocerebrum, the deutocerebrum and the

tritocerebrum. The protocerebrum occupies most of the ganglion mass. The deuterocerebrum lies under it, just above the oesophageal foramen (A hole through which the oesophagus passes is called the oesophageal foramen). The tritocerebrum is a region at the side of the oesophageal foraman. The sub-oesophageal ganglion lies under the oesophageal foramen. Its neuropils are linked to the upper brain by a pair of connectives situated either side of the oesophageal foramen (Power, 1943; Strausfeld, 1976; Mobbs, 1987).

The *Drosophila* brain has two layers. The inner region of the brain is called the neuropil. The peripheral layer that surrounds the neuropil is called the cortex. The neural cell bodies situated on the cortical rind and the processes extend into the neuropil, which is the site of synaptic interactions. In the underlying neuropil, at least four conspicuous main structures can be observed. They are (1) the mushroom body, (2) the central complex, (3) the antennal lobe and (4) the optic lobe. Figure 1.6 shows the schematic picture of the whole brain. In the following sections, more details are described.

The *Drosophila* brain is highly variable in size. Most neuropil regions such as calyx, lobule are continuously reorganised throughout life in response to specific living conditions (Technau, 1984; Heisenberg *et al.*, 1995).

1.3.1. The Mushroom Body

The structural organisation and connectivity of the mushroom bodies (MBs) in the *Drosophila* brain has been investigated by Power (1943), Heisenberg *et al.* (1985), Davis (1993) and Yang *et al.* (1995).

The mushroom bodies comprise two symmetrically arranged parts of characteristic shape in the protocerebral brain hemispheres. They can be divided into three major parts: (1) an upper cap-shaped part called the calyx; (2) a stalk-like structure, the pedunculus; and (3) three lobes named the alpha, beta and gamma lobes respectively (Fig. 1.6).

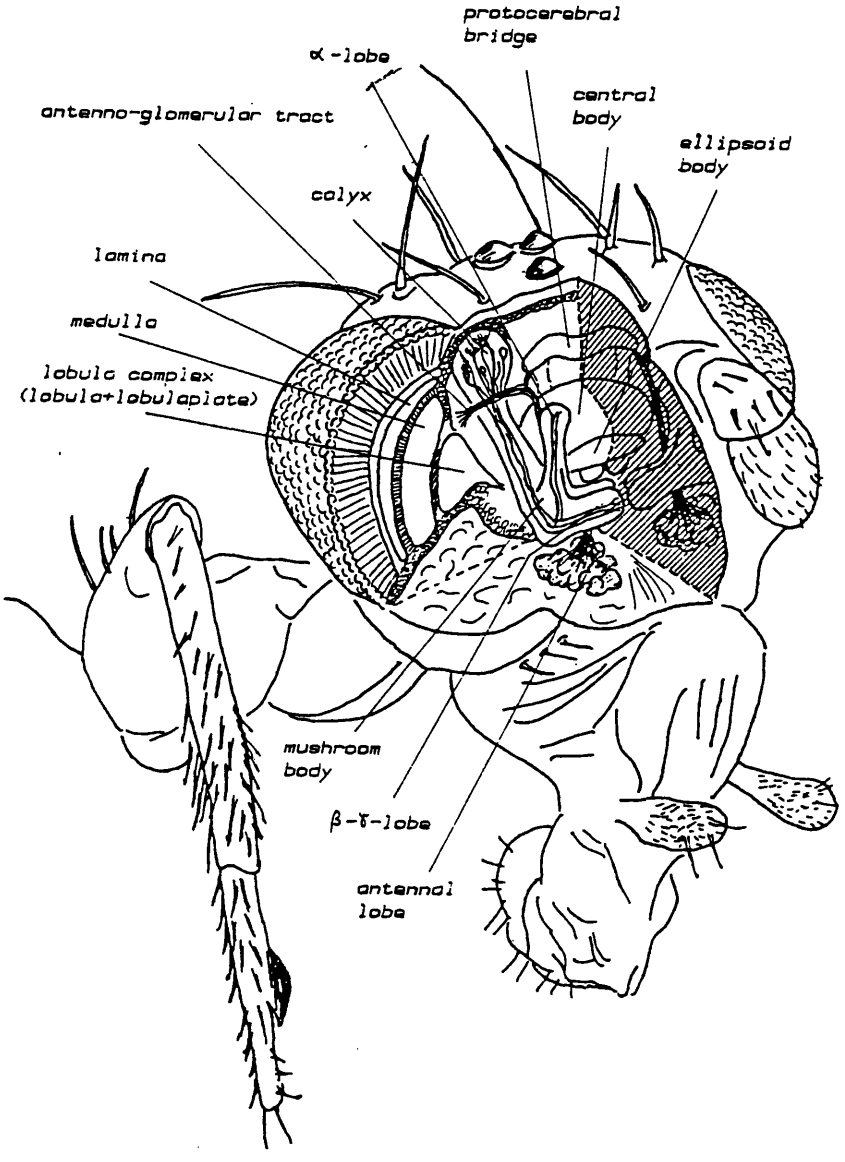


Figure 1.6. Schematic drawing of an opened head capsule of *Drosophila* showing the most prominent parts (enlarged) of its central brain (Taken from Heisenberg *et al.*, 1985).

The MBs are composed of two main types of neurons: extrinsic and intrinsic. The extrinsic neurons can be divided into three classes: input neurons projecting from sensory neuropils to the calyxes, output neurons projecting from the lobes of the MBs to other parts of the brain, and feedback neurons connecting the lobes of the MBs with the calyxes. The intrinsic neurons, or Kenyon cells, are restricted to the MBs. They arise from dense clusters of cells bodies dorsal and posterior to each brain lobe. Their dendrites form the mushroom bodies calyces, large regions of input from the antennal lobes and other central, while beneath each calyx the Kenyon cell axons converge to form the pedunculus. The pedunculus extends almost to the front of the brain, at which point it gives rise to three lobes: a dorsally-projecting α lobe, and β/γ lobe complex projecting towards the mid-line.

The MBs have been implicated in associative learning and memory, and in controlling a variety of complex behavioural repertoires (Davis, 1993; deBelle, 1995). In the case of *Drosophila*, single gene mutations that cause defective mushroom body anatomies have been shown to interfere significantly with olfactory associative learning (Heisenberg *et al.*, 1985; Heisenberg, 1989). Ablation of mushroom body neuroblasts at an early stage of development, selectively causing mushroom body absence from the adult brain, has an even more profound effect (de Belle and Heisenberg, 1994). Three *Drosophila* learning/memory genes *dunce*, *rutabaga and DC0* , all of which encode components of the cAMP signalling pathway, have a significant component of their expression in the MBs (Nighorn *et al.*, 1991; Han *et al.*, 1992; Skoulakis *et al.*, 1993). Gynandromorph analysis implicates MBs, or adjacent neuropils, in control of the male courtship repertoire (Hall, 1979), a behaviour that relies heavily on olfaction. Taken together, the picture that emerges is of a specialised neuropil involved in associating and storing multimodal sensory information, thereby providing the organism with memory, and predictive behaviour.

More recently, enhancer trap expression patterns have revealed subdivision of the *Drosophila* MBs (Yang *et al.*, 1995). Rather than being homogenous, MBs are compound neuropils in which parallel sub-components exhibit discrete patterns of gene expression.

Different patterns correspond to hitherto unobserved differences in Kenyon cell trajectory, and placement. It is possible that different sub-sets of Kenyon cells perform different functional roles. This notion is supported by selective feminisation of genetically defined subdivision of MB in terms of sex-specific behaviour (O'Dell *et al.*, 1995).

1.3.2. The Central Complex

Along with the mushroom bodies, the central complex (CC) is one of the most fascinating structures in the insect brain. It is a compact neuropil and highly symmetrically organised. A similar structure and location were found in all the insects (Williams, 1975; Strausfeld, 1976; Hanesch *et al.*, 1989). The CC resides in the middle between the two protocrebral hemispheres just above the oesophagus, flanked on either side by the pedunculi of the mushroom bodies. In *Drosophila*, the CC consists of four interconnected main neuronal region or substructures: (1) the protocerebral bridge; (2) the fan-shaped body; (3) the ellipsoid body; and (4) the paired noduli (Fig. 1.6). In addition, two accessory structures are closely associated with the CC, the ventral bodies and the lateral triangles. The structural organisation of the CC may be characterised as columnar small-field elements linking different substructures, or regions in the same substructure and tangential large-field neurons forming strata perpendicular to the columns (Hanesch *et al.*, 1989).

In insects, the CC has connections to many other parts of the brain. There are three main systems of tracts entering or leaving the CC. The first tracts are ones entering at the end or along the protocerebral bridge. The second tracts connect the central complex to the lateral accessory lobes (the ventral bodies in *Drosophila*) and the posterior lateral protocerebrum. The third tracts are called the anterior bundles, connecting the central complex to the anterolateral protocerebrum (Homberg, 1987). In *Drosophila* the main input to the CC is through large-field neurons such as ring neurons and fan-shaped neurons and the main candidate for outputs from the central complex is the class of small-field neurons projecting with clublike terminal to the ventral bodies (Hanesch *et al.*,1989).

For the possible function of the CC, early experimental data from surgery and electrical stimulation have suggested a role in the regulation of behavioural activity (Homberg, 1987). Recently more systematic studies on genetic lesions in *Drosophila* have led to similar conclusions. Mutants with defects in the CC show a variety of behavioural impairments in locomotion, vision and learning (Heisenberg *et al.*, 1985, Strauß *et al.*, 1992, Strauß and Heisenberg, 1993, Bouhouche *et al.*, 1993 , Ilius *et al.*, 1994 and Bausenwein *et al.*, 1994).

1.3.3. The Antennal Lobes

Another prominent structure is called the antennal lobe (AL). The AL is situated in the anterior part of the *Drosophila* brain, at the level of the oesophagus, as a pair of protrusions (Fig. 1.6). The ALs are the first order neuropils of the olfactory chemosensory pathway. They are divided into a series of spherical neuropil compartments called glomeruli. There are 35 glomeruli in each antennal lobe of the *Drosophila* brain, of which 30 are located in the periphery of the lobe and 5 in its centre. No obvious sexual dimorphism regarding the number, size or location of glomeruli has been observed although there is sexual dimorphism in other insects, e.g. moths (Rospars, 1983). The glomerular organisation is a reflection of the architecture of the olfactory and gustatory receptor terminals, the intrinsic interneurons, and the extrinsic output and feedback cells within the deutocerebrum. The ALs are thought to be a centre of topographic, multimodal and numerical convergence (Stocker *et al.*, 1990; Stocker, 1994).

The major input and output tracts of the ALs are the antennal nerve (about 1800 axons) as a principal input tract, the antennal commissure (about 2500 fibres) as the connection of the two lobes and three tracts extending into higher brain centres as output.

The antennal lobe receives afferents from antennal, maxillary, and pharyngeal sensilla and connects a considerable number of afferents with a smaller number of relay interneurons.

The three output tracts of the ALs were described by Stocker *et al.* (1990): (1) The inner antenno-cerebral tract (iACT; synonyms: antenno glomerular tract ATG). It runs from the postero-dorsal end of the lobe straight up to the median dorso-posterior protocerebrum, then turns laterally towards the calyx of the mushroom bodies and extends further into the lateral protocerebrum. (2) The middle antenno-cerebral tract (mACT). This tract branches off the iACT laterally some distance behind the antennal lobe and runs straight into the lateral protocerebrum. Occasionally, fibres leave the mACT halfway and extend along the pedunculus of the mushroom bodies to the calyx. (3) The outer antenno-cerebral tract (oACT), emerges from the antennal lobe lateral to the iACT and extends into the lateral protocerebrum.

Three major types of interneurons have been reported in the ALs of flies (Stocker *et al.*, 1990). They are local interneurons, relay interneurons and the giant bilateral neurons. The local interneurons branch in many of the glomeruli of one antennal lobe and appear to connect glomeruli. The relay interneurons densely arborize in a single glomerulus or more than one glomerulus and extend into the ipsilateral calyx of the mushroom bodies and the lateral protocerebrum or exclusively into the lateral protocerebrum. The giant bilateral neurons are characterised by extensive mirror-symmetric arborizations in both antennal lobes, a pair of giant processes leading towards a second arborization region in the posterior brain, and a cell body located in the ventral midline of the SOG.

Based on data from *Drosophila* and other insects, Stocker (1994) further described the connectivity of the antennal lobe in *Drosophila*. The antennal lobe appears to be constructed of four types of glomeruli: monosensillar type-1 glomeruli, which are targets of specialised sensilla; monosensillar type-2 glomeruli, receiving a wider spectrum of information from a single type of olfactory sensillum; polysensillar type-1 glomeruli, receiving olfactory input from different types of antennal sensilla; and polysensillar type-2 glomeruli, which are targets of antennal and nonantennal sensilla. Each glomerulus occupies

a specific and constant position and is associated with particular groups of receptor fibres. In other word, individual glomeruli are functionally specialised and represents the convergence of inputs with some similarity of response.

1.3.4. The Optic Lobes

It is well known that the optic lobes , flanking lateral to the fly midbrain, comprise four pairs of neuropils, namely the lamina, medulla, lobula and lobula plate (Fig. 1.6). Distally (farthest from the centre of the brain) lies the lamina beneath the compound eye, and proximal to this are the medulla and lobula and lobula plate. Lamina and medulla are connected by the first (outer) optic chiasm and medulla, lobula, and lobula plate by the second (inner) optic chiasm (Fischbach and Dittrich, 1989).

The lamina receives input from the retinal terminal of short visual fibres and *en-passant* input from the long visual fibres that run on into the medulla. In the lamina the neurological map is retinotopic. This map is reversed in the horizontal axis by the first optic chiasm, the medulla receiving a mirror image of the retinal map. This image is subsequently re-reversed by the second optic chiasm by fibres running into the lobula. As the output neuropil, there are various tracts connecting the optic lobe with the central brain, such as the anterior optic tract and the optic foci. Of the visual neuropils, the lobula is most intimately connected to the central brain (Strausfeld, 1976; Fischbach and Dittrich, 1989).

According to the different neuronal cell types in optic lobes, they can be classified as either columnar or tangential elements. Fischbach and Dittrich (1989) have demonstrated that the columnar neurons establish multiple and stacked retinotopic maps in the optic lobe. They connect the distinct cellular regions, such as retina, lamina, medulla, lobula, lobula plate, and optic foci with the central brain. The number of columns in each neuropil corresponds to the number of ommatidia in the compound eye. In the fly, sets of eight photoreceptors lie beneath the lenslet of each ommatidium. Six of these cells project to the first order neuropil,

the lamina, and the remaining pair (R7 and R8) projects directly to the medulla via the first optic chiasm. There are many neurons connecting medulla with lobula and lobula plate, such as transmedullary neurons (Tm), transmedullary Y cells (TmY) and T2-4 cells.

It is clear that different lamina monopolar cells communicate with different set of Tm and TmY. The larger number of columnar neurons reflects the segregation of many paralleled functional pathways, which are all retinotopically organised.

The tangential elements are oriented perpendicularly to the columns. The arborizations of different tangential neurons are restricted to different layers of the optic neuropils, within such layers their dentritic fields may span the entire retinotopic field or only part of it. The medulla neuropile can be subdivided into ten layers (M1-M10), the lobula plate into four layers (Lop1-Lop4), and the lobula into six layers (Lo1-Lo6). Strikingly, the serpentine layer is formed by tangential neurons entering the medulla anteriorly via Cuccatti's bundle. The cell bodies of these tangential neurons lie clustered in front of the medulla neuropile. Some send their axons via the posterior optic tract into the contralateral medulla (Fischbach and Dittrich, 1989).

Other neuropil structures, such as suboesophageal ganglion, thoracic ganglia and giant commissure were not described in this chapter.

1.4 Aims of the Project

The structure and function of the brain is one of the major outstanding problems in biology. A study of brain structure-function relationships at the molecular and genetic level is a big challenge. The complexity of the nervous system of most organisms make them refractory to detailed analysis. For this reason, much work has concentrated on invertebrate model systems. The fruitfly *Drosophila melanogaster* has proved to be an invaluable model system in the field of neurobiology, since it is particular suited to genetic and molecular

analyses of neural development and function (Rubin, 1988). Though vastly simpler than the human brain, the fly brain is still immeasurably more sophisticated than the best man-made computational device. The research on the fly brain will illuminate many of the basic mechanisms which can be applied to other organisms.

Currently several new approaches have been developed in the field of *Drosophila* neurobiology. A powerful new tool, "enhancer trapping", has been used successfully to identify and clone genes expressed more or less specifically in the nervous system of the fly (Bier *et al.*, 1989; Bellen *et al.*, 1989; Wilson *et al.*, 1989; Ghysen and O'Kane, 1989; Klambt *et al.*, 1991; Bier *et al.*, 1992; Nose *et al.*, 1992). In the main this has involved looking for staining of the embryonic nervous system, at least in part because of the ease with which whole-mount of embryos can be prepared. Searching for staining of the adults brain is more labour intensive because it requires the generation of cryostat sections from each independent line. However, investigation of region-specific staining in the adult brain has great significance for an elucidation of relationships between structure and function because sections of adult flies can show clearly brain structures in greater detail. In addition, some adult specific genes required in the CNS can be identified by enhancer trap expression patterns.

In order to study the structure-function relationships in the *Drosophila* brain and identify some genes required in the adults central nervous system, I have embarked upon such studies by using an "enhancer trapping" approach mentioned as section 1.2.

Chapter 2

Material and Methods

2.1 Basic materials

2.2 Histochemistry and immunohistochemistry

2.3 *in situ* hybridisation

2.4 General methods for molecular biology

This chapter contains the general procedures used in the experiments which make up the basis of this thesis. This chapter is divided into 4 main sections for convenience. Section 2.1 is about basic materials used, section 2.2 is involved in the techniques of histochemistry and immunohistochemistry, section 2.3 contains the methods for *in situ* hybridisation and the final section 2.4 describes the methods for molecular biology.

2.1 BASIC MATERIAL

2.1.1 *Drosophila* Strains

The *Drosophila* stocks used were listed as follows. Some of them were described by Lindsley and Grell (1968) or Lindsley and Zimm (1992). Others were given for references. The stocks were maintained at either 25°C or 18°C.

Wild types strain: (1) Canton S, (2) Oregon R.

White eye strains: (1) w^{1118} (2) *w*(SC10) (Dura *et al.*, 1993)

Transposase strains: (1) Dr, Δ 2.3/TM6B, (2) CyO/Sp; Dr, Δ2-3/TM6, (Robertson *et al.*, 1988).

P[GAL4] strains: (1) GAL4/FM7, (2) GAL4,CyO, (Brand and Perrimon 1993; Tully, personal comm.).

***lacZ* strain:** UAS_G-lacZ on the second chromosome (Brand and Perrimon 1993).

2.1.2 Bacterial Strains, Plasmids and Phage Vectors

XL1-Blue: *rec* A$^-$. a derivative of *Escherichia coli* K-12. Bullock (1987); Stratagene.

NM621: *hsd*R *mcr*A *mcr*B *supp*E44 *rec*D 1009 *thy*+. Whittaker *et al.*, (1983).

MC1061: *hsdR mcrB ara D139 (ara ABC-leu) 7679* Meissner *et al* ., (1987).

pBluescript II (KS+/- and SK+/-): *amp*^{r} Short *et al.*, (1988); Stratagene, USA.

pBR*rp49:* The *Drosophila* ribosomal protein 49 gene. O'Connell & Rosbash (1984).

pST41: A cDNA clone of *nina E* gene (Zucker *et al.*, 1985), major opsin of *D. melanogaster.* Gift from S.Tomlinson (Glasgow Genetics Department).

α-tublin: Kalfayan and Wensink, (1982).

The Genomic DNA libraries: One was constructed using the bacteriophage lambda GEM-11 replacement vector. The other was constructed using the bacteriophage lambda vector EMBL3 (Russell and Kaiser, unpublished)

The male head cDNA library: It was constructed using the lambda NM1149 replacement vector supplied. (S. Russell, unpublished)

2.1.3 Culture Media for *E. coli* and *Drosophila*

L-Broth: 10g Bacto-tryptone (Difco), 5g yeast extract (Difco), 5g NaCl, 1g glucose made up to 1 litre in distilled water and adjusted to pH 7.0 with NaOH.

0.7% (w/v) Top Agarose: 0.7g agarose added to 100ml of L-broth, containing 10mM $MgSO_4$ heated to dissolve the agarose and left to cool to 50°C before use.

Glasgow fly food: 10g Bacto-agar (Difco), 15g sucrose, 30g glucose, 35g yeast, 15g maize meal, 10g wheatgerm, 30g treacle, 10g Soya flour, 0.1% (v/v) Nipagen and 0.5% (v/v) proprionic acid per litre of water.

Fly food for egg laying plate: 20g Agar, 52.5g Glucose, 26g Sucrose, 7g Yeast, 9ml Red grape juice and 6ml Nipagin per litre of water.

Rich Larval media: 100g glucose, 100g yeast, 20g agar, 0.1% (v/v) Nipagen per litre of water.

2.1.4 Enzymes

Restriction enzymes , T4 DNA ligase and 10X buffers supplied by BRL and Promega.

2.1.5 Chemicals and Reagents

The chemicals and reagents used in this thesis were supplied by one of following companies: BDH, Sigma, Boehringer Mannheim, BRL, Pharmacia, Amersham, United States Biochemical, Vector, Capell, Whatman.

2.1.6 Buffers and Solutions

X-Gal Staining buffer: (FeNaP buffer) 10mM $NaH_2PO_4.H_20$, 10mM $Na_2HPO_4.2H_2O$, 150mM NaCl, 1mM $MgCl_2.6H_2O$, 3.1mM $K_4(Fe^{2+}CN)_6$, 3.1mM $K_3(Fe^{3+}CN)_6$, 0.3% Triton X-100, pH 7.8

Biotin Prehybridisation solution: 0.6M NaCl, 50mM Sodium Phosphate pH 6.8, 1xDenhardts, 0.5mM $MgCl_2$.

10mg/ml RNase/DNase solution: DNase I and RNase A were mixed to give a 10mg/ml stock solution. Stored at -20°C.

RNA Denaturing solution: 4M guanidinium thiocyanate, 0.1M Tris.HCl pH 8.0, 10µl antifoam A, made up to 100ml with DEPC treated water. Added 0.1M ß-mercaptoethanol immediately before use.

DNA Homogenisation buffer: 0.03M Tris.HCl pH 8.0, 0.01M EDTA, 0.1M NaCl, 10mM ß-mercaptoethanol.

Nuclear lysis buffer: 0.1M Tris.HCl pH 8.0, 0.1M EDTA, 0.1M NaCl, 0.5 mg/ml Proteinase K.

Oligo (dT) cellulose binding buffer: 0.5M NaCl, 10mM Tris.HCl pH 8.0, 1.0% (v/v) Sarcosine, 1mM EDTA. Made up to 500ml for 2x binding buffer, 1 litre for 1x binding buffer.

5x 1st strand cDNA buffer: 250mM Tris.HCl pH 8.3, 700mM KCl, 50mM $MgCl_2$, 50mM DDT.

4x Random Priming buffer: 250mM Tris.HCl, pH 8.0, 25mM $MgCl_2$, 100µM dNTPs, 1M Hepes pH 6.6, 27 A_{260} units/ml random hexanucleotides.

10x Nick Translation Buffer: 0.5M Tris.HCl pH 7.5, 0.1M $MgSO_4$, 1mM DDT, 500µg/ml BSA (Fraction V).

Spermidine: 1M stock solution, dissolve 2.54g in 10ml water. stored at -20°C.

Ampicillin: 50mg/ml stock solution in sterile distilled water. Stored at -20°C.

Tetracycline: 12.5mg/ml stock solution in absolute ethanol. Stored at -20°C.

X-Gal: X-Gal was dissolved in dimethylformamide to give a 20mg/ml stock solution, and stored at -20°C.

IPTG: It was dissolved in sterile distilled water as 20mg/ml stock solution. Stored at -20°C.

4% (w/v) Paraformaldehyde: 40g Paraformaldehyde added to 800ml warm PBS, made up to 1L with PBS.

5x Agarose gel loading buffer: 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 25% (w/v) ficoll, 0.5% (w/v) SDS, 50mM EDTA

PBS: 8g NaCl, 0.2g KCl, 1.44g NaP_2HPO_4 and 0.24g KH_2PO_4, made up to 1 litre (pH adjusted to 7.4 using HCl).

PBT: PBS containing 0.1% (v/v) Tween 20.

PAT: 1% (v/v) Triton X-100, 1% (w/v) BSA (fraction V) in PBS.

Other solutions used in this thesis were made as described by Sambrook *et al.*, (1989).

2.1.7. Micoscopes and Films

Nikon stereoscopic zoom microscope; Zeiss inverted microsocope, Leica Orthoplan microsocope; Molecular Dynamics Multiprobe laser scanning confocal microscope. Kodak film (ASA 100 or 25); Fuji film (ASA 100); Polaroid 545 land film; Fuji NIF RX X-ray film.

2.2 HISTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

2.2.1. X-Gal staining of frozen cryostat sections

2.2.1.1. Embedding of Adult Fly Heads

For frontal section:

Flies are anaesthetised with CO_2 and threaded up side by side into a fly collar by their necks, and a well-known staining pattern fly is set beside them as a control (the fly collar was modified from Heisenberg and Böhl 1979). A drop of freezing medium OCT 4583 (TISSUE-TEK, USA) is added onto the fly heads and left to soak for 5-10 min. The fly heads are embedded on a Cryocassetter of Cryotme 620 (Anglia Scientific). Firstly, a drop of OCT is put onto the Cryocassetter and the Cryocassetter is placed on the Peltier Element.

When OCT is almost frozen, more drops of OCT are added and the fly collar is placed onto the Cryocassetter immediately (fly heads are embedded into OCT). The fly collar is then pressed down with the copper 'heat sink'. When the OCT is frozen the fly collar is taken away. Only fly heads are left in the frozen block. Another drop of OCT is added on the top of block and the copper 'heat sink' is immediately applied again. The Cryocassetter with the block is placed on the Cryocassetter Holder after a few minutes and block is cut into rectangle shape and trimmed.

For horizontal section:

The procedure of embedding is the same as above Frontal Section except re-embed the frozen block for the fly proboscis faces down.

For saggittal section:

The procedure of embedding is the same as the Frontal Section except flies should be mounted in a fly collar with different orientation.

2.2.1.2. Embedding of Whole Fly

After the fly is anaesthetised with CO_2, a drop of OCT is added onto the Cryocassetter on the Peltier element. When the OCT is almost frozen, more drops of OCT are added and a whole fly is placed into OCT with the needed orientation. Then, a drop of OCT is added on the top of block and the copper 'heat sink' is applied immediately. The Cryocassetter with the block is placed on the Cryocassetter Holder and block is cut into rectangle shape and trimmed.

2.2.1.3. Sectioning and Staining

The frozen block with flies is cut in thickness of 12μm using Cryotome 620 (Anglia scientific) at -18°C. The ribbon of sections are collected on gelatinised slides and placed directly on a slide warmer at about 35°C for 1 min. Serial sections may be collected onto one slide provided the slide is not heated for more than 5 minutes in total. Then, they are fixed in

a coplin jar containing 1% glutaraldehyde in PBS and left for 15 min. at 4°C. After washing three times in PBS for 10 minutes, the slides are rinsed in the FeNaP staining buffer (see 2.1.6). Slides are then placed in a humid box and added 200 µl of pre-warmed staining solution with 0.2% *X-gal* (diluted from an 8% stock solution in DMSO). A coverslip is carefully placed on top to spread out the solution over the specimen. The box is left at 37°C for 1-2 hrs. After rinsing in PBS for 5-10 min., the slides are dehydrated by immersing them in solutions of increasing concentrations of ethanol at room temperature, i.e. 30%, 70%, 95%, 2X100%, and mounted in glycerol gelatin (Sigma).

2.2.2. X-gal Staining of Embryo, Larval, Pupal and Adult Brains

2.2.2.1. X-gal Staining of Embryo

Embryos are collected from yeasted apple/grape juice agar plates into a container with a nylon mesh screen at the bottom, dechorionated by dipping into 50% bleach for 4 minutes and washed thoroughly with water. Embryos are placed into an Eppendorf tube containing a mixture of 0.35ml fix solution (1% glutaraldehyde in PBS) and 0.7ml n-heptane and fixed for 15 minutes at room temperature on a rotating mixer. After removing heptane and fix solution from tube, embryos are washed three times for 10 min. in PBS and 0.1% Triton X-100, and resuspended in staining buffer with 0.2% X-gal for 1-2 hours at 37°C. After staining, staining solution are removed and about 400µl of mixture solution (Glycerol : staining buffer = 2:1) are replaced. Embryos can then be mounted on a slide in a coverslip chamber.

2.2.2.2. X-gal Staining of Larval, Pupal and Adult Brains

Brains are dissected in PBS, and fixed in 4% paraformaldehyde for 30-60 minutes. They are then washed three times for 20 minutes in PBS, and stained with staining buffer and 2% X-gal for 1-2 hours at 37°C. They are then washed for 20 minutes in PBS, cleared overnight at 4°C with PBS/12.5% hydrogen peroxide, washed for 10 min. with PBS, dehydrated through graded ethanol, and mounted in glycerol gelatin.

2.2.3. Immunohistochemistry and Confocal Microscopy

2.2.3.1. Anti-ß-gal Antibody Staining of Adult Brains

Intact adult brains are dissected under PBS, fixed in 4% paraformaldehyde for 30 minutes and washed twice for 1 hour in PAT (1 X PBS; 1% bovine serum albumin; 1% Triton X-100). They are then incubated overnight with rabbit polyclonal anti-ß-gal antibody (Cappel) diluted 1:2000 in PAT, 3% normal goat serum (SAPU); washed three times in PAT for 1 hour; incubated overnight with secondary antibody (fluorescein-labelled goat anti-rabbit IgG; Vector Labs) diluted 1:250 in PAT; washed twice for 1 hour in PAT, and once for 5 minutes with PBS. All of the above procedures are carried out at room temperature. Stained brains are mounted in VectaShield (Vector).

2.2.3.2. Confocal Microscopy

Intact brains are examined with a Molecular Dynamics Multiprobe laser scanning confocal microscope. Excitation (480nm) and emission (530±15nm) filters are appropriate to the fluorescein-based labels of the goat antibody used. Three dimensional reconstructions are performed using the programme 'ImageSpace' (Molecular Dynamics). Pseudo colour is added using the programme 'NIH-Image' (National Institutes of Health, Washington).

2.3 *in situ* HYBRIDISATION

2.3.1. *in situ* Hybridisation to Tissue Sections

The method is essentially described by Nighorn *et al.*, (1991), but some modifications are made. Wild type flies (Canton S) are cut as 12 µm sections in a cryostat (Anglia Scientific, Cryotome 620) described as section 2.2.1., placed on gelatinised slides, and postfixed in freshly made PLP fixative (4% (w/v) paraformaldehyde, 0.01M sodium metaperiodate, 0.075 M lysine, 0.044M NaCl, 0.037M phosphate buffer, pH 7.2) for 10 min. After two washes in PBS, they are treated with Proteinase K (10µg/ml) at 37°C for 15 min. After wash in PBS, slides are re-fixed in 4% paraformaldehyde in PBS for 20 min, and

acetylated with 0.25% (v/v) acetic anhydride and 0.1M triethanolamine in PBS for 10 min. After the sections are dehydrated though graded methanol, they are incubated with 200 µl of prehybridisation solution at 55°C for 1-2 hrs. They are then incubated with DIG RNA labelling probes at 55°C. After overnight incubation, the sections are washed in 50% formamide, 2XSSC, then in the following mixes of 50% formamide/ 2XSSC: 3:1; 2:2; 1:3 and finally in 2xSSC at 55°C. After washes once in 1 X SSC and once in 0.5 X SSC at room temperature, a final wash is performed in low salt buffer (2mM NaPPi, 1mM $NaPO_4$, 1mM EDTA, pH 7.2) at 45°C.

Colorimetric Detection with NBT and X-phosphate

For immunological detection of the hybridised probes, the sections are washed in PBT and incubated with 200 µl of 5% (v/v) sheep serum in PAT for 2-3hr. The sections are then incubated with 150-200 µl of anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim) diluted at 1:500 PAT for 2-3 hr at room temperature or overnight at 4°C. The sections are washed twice in PBT. After extensive washes in 100mM Tris-HCl, pH 9.5, 100mM NaCl, 50mM $MgCl_2$, 2mM levamisole, the sections are placed with 200 µl of diluted chromogenic substrate solutions (NTB and BCIP, X-phosphate) following the manufacturer's instructions (Boehringer Mannheim), incubated in the dark at room temperature for 2-4 hrs. The reaction is stopped by washing in PBT for 20 min and mounted with glycerol gelatin (Sigma).

Detection with DAB and H_2O_2

The sections are washed in PBT and incubated with 200 µl of 5% (v/v) sheep serum in PAT for 1-2 hr. They are then incubated with 150-200 µl of anti-digoxigenin-horse radish peroxidaes (anti-DIG-HRP) Fab fragments (Boehringer Mannheim) diluted at 1:500 PAT for 2-3 hr at room temperature or overnight at 4°C. After extensive washes, the sections are detected with diaminobenzidine and hydrogen peroxide according to condition recommended by manufacturer (Boehringer Mannheim). The reaction is stopped by washing in PBT for 20 min and mounted with glycerol gelatin (Sigma).

2.3.2 *in situ* Hybridisation to Embryos and Whole Brain

The method is adapted from Jowett and Lettice (1994) and employs a Boeringer Mannheim Digoxygenin (DIG) labelling and detection kit.

2.3.3. *in situ* Hybridisation to Polytene Chromosome using Biotin Labelled Probes.

The procedure for *in situ* hybridisation to polytene chromosomes is essentially as described by Pardue (1986).

Preparation of chromosome squashes

Larvae are grown at 18°C using food with extra yeast up to 3rd instar, washed in 0.7% NaCl solution to remove food and dissected in 45% glacial acetic acid on a clean slide. 4-8 glands are transferred onto a drop (10µl) of 1:2:3 (Lactic Acid:dH_2O:GAA) on a siliconised coverslip (18 mm^2) and fixed for 5 minutes. It is covered by a clean subbed slide. To spread out chromosomes, the coverslip is tapped with a pencil for at least one minute. The slides are left for a week at 4°C. After that, slides are placed on dry ice for 10 minutes for flipping off the coverslips with a razor blade, immersed immediately into freshly made ethanol:acetic acid (3:1) for 10 minutes and dehydrated in 100% ethanol for 10 minutes. After drying, the slides are examined using a phase contrast microscope. The slides with well spread chromosomes are selected for hybridisation.

Pretreatment of chromosomes for hybridisation

The slides are incubated in preheated 2xSSC for 30 minutes at 65°C, washed in 2xSSC for 2 min. at room temperature and acetylated in 0.1M triethanolamine-HCl (pH 8.0) and 0.125% Acetic Anhydride for 10 minutes. After twice washes in 2xSSC for 2 min., slides are dehydrated twice in 70% ethanol and in 95% ethanol for 5 minutes respectively. Then, they are denatured in 0.07N NaOH for 3 min. (exactly), washed in 2xSSC for 5 min.

and dehydrated again in 70% and 95% ethanol for 5 min. respectively. After drying, slides are ready for hybridisation.

Hybridisation and washes

The biotinylated DNA probe prepared as described in section 2.4.5.3. is denatured. Then, 20µl probe is applied to per slide with a 18x18mm coverslip. The edges of coverslips are sealed with Cow Gum thinned with diEthyl Ether. The slides are incubated in a moist chamber at 58°C for 12-18 hours. Cow Gum and coverslips are peeled off in 2xSSC with fine forceps at 53°C. The slides are washed three times in 2xSSC for 20 minutes each at 53°C.

Signal detection

After twice washes in 1xPBS for 5 minutes, once in PBT (1xPBS and 0.1% Triton X-100) for 2 minutes and rinse in 1xPBS at room temperature, slides are placed in a humid box, added 300 µl Vectastain mix (Vector Lab) and incubated at 37°C for 45 min. Again after twice washes in 1xPBS, once in PBT and rinse in 1xPBS at the room temperature, slides are placed in a tray and covered with 250µl-500µl DAB solution (0.5mg/ml DAB and 1/100 volume of 1% H_2O_2). They are then incubated in dark at room temperature for 1 hour. The slides are washed in 1x PBS and stained with Giemsa (2.5ml Giemsa in 50ml 10mM Na buffer) for 12 min. After staining, they are washed in water and mounted with a drop of DPX mountant.

2.4. GENERAL METHODS FOR MOLECULAR BIOLOGY

Generally, molecular biology techniques are performed as described by Sambrook *et al.*, (1989) unless otherwise described.

2.4.1 Isolation of Genomic DNA

Approximately 1g of flies are added to a mortar and pestle which have been precooled in liquid Nitrogen. Before the liquid Nitrogen evaporated completely, the flies are ground to a fine powder. Using a small paint brush, (cooled in N_2) the powder is transferred from the mortar into a 15ml Wheaton homogeniser (on ice) containing 9 ml of ice cold homogenisation buffer (HB) and 0.5 ml of 10% (v/v) Triton X-100. This is homogenised thoroughly and the resulting homogenate decanted through gauze into a sterile 15ml tube on ice. They are spun immediately at 4000 K for 10 min at 4°C in a cooled rotor. The supernatant is decanted and the nuclear pellet is resuspended in 1ml of ice cold HB. 5ml of nuclear lysis buffer and 200µl of 30% (v/v) Sarkosyl are added to this solution. This is mixed by swirling until lysis had occurred. The lysate is incubated overnight at 37°C. After centrifugation to remove the debris, the supernatant is decanted into a preweighed Falcon Tube and 1.25g CsCl per ml of homogenate is added. The solution is loaded into Polyallomer tubes which are filled up with 1.25g/ml CsCl/ Water. The tubes are ultracentrifuged at 45K for 24 hours in Ti70 rotor at 25°C. Samples were collected by dripping the gradient through an 18 gauge needle at the bottom of the centrifuge tube. 1.5 ml fractions were collected initially, and then 0.5 ml fractions once it appeared more viscose. The concentration of the DNA samples is roughly estimated using EthBr plates. The best fractions are pooled and dialysed against TE. The yield of genomic DNA obtained are generally 100-200µg/g of starting material.

2.4.2 Isolation of Bacteriophage DNA

2.4.2.1 Host Cell Preparation

1.0 ml of overnight culture of NM621 is added into 100 ml L Broth with 1ml 20% Maltose, 100µl 1M $MgSO_4$ and grown at 37°C with shaking to OD_{600} of 0.3. After centrifugation, cells are resuspended in 10mM $MgSO_4$ to a final A_{600} of 1.0.

2.4.2.2. Isolation of Phage DNA

This protocol is adapted from Chisholm (1989). About $2x10^6$ eluted phage are incubated with 500µl ($4x10^8$) of NM621 plating cells for 30 min. at 37°C. Then they are transferred into a flask with 37ml of L-broth and are grown with shaking at 37°C for 15 hours. After lysising, the mixture is transferred into 50 ml Falcon tube containing 100 µl chloroform. After adding 370 µl of nuclease solution (50mg DNase I, 50mg RNase A, in 10 ml of 50% glycerol, 30mM NaoAc), the mixture is incubated at 37°C for 30 min. Then the mixture 2.1g NaCl is added and then centrifuged at 4K for 20 min. at 4°C. The supernatant is transferred to a new tube containing 3.7g PEG 8000. It is left on a 'rock and roller' until all the PEG had dissolved. The solution is left for one hour at 4°C to precipitate the phage. In order to pellet the phage, the solution is spun at 10K for 20 minutes. when the pellet is resuspended in 500 µl phage buffer, 500µl chloroform is mixed and centrifuged for 10 min.. The phage are decanted into a centrifuge tube, then EDTA is added to give a final concentration of 20mM, Proteinase K to a final concentration of 50µg/ml and SDS to a final concentration of 0.2% (v/v). This is incubated at 65°C for 1 hour. The solution is then extracted by phenol and chloroform and precipitated by isopropranol. The pellet is washed with 70% ethanol and resuspend in 200 µl of TE. This usually gives a yield of 50-100µg bacteriophage DNA.

2.4.3 Isolation of Plasmid DNA

2.4.3.1 Large Scale Plasmid Preparation

Large scale preparations of plasmid DNA are prepared by alkaline lysis method. The plasmid containing bacteria are inoculated into L-broth containing the appropriate antibiotic. This is grown with shaking at 37°C. The culture is spun down and the bacterial pellet resuspended in 20ml solution I (50mM glucose, 25mM tris.HCl pH 8.0, 10mM EDTA), to this suspension is added 20ml of freshly prepared solution II (0.2N NaOH, 1% SDS). The contents are mixed by inversion and incubated at room temperature for 5-10 min.. 20ml of ice cold solution III (5M KOAc pH4.8) is added and the solution shaken vigorously before

incubating on ice for 10 min. The white precipitate is removed by centrifugation at 4000 rpm for 15 minutes at 4°C. The supernatant is mixed with 0.6 vol. of isopropanol and left at 4°C for 10 minutes. After centrifugation, the pellet is washed with 70% ethanol, left to dry and resuspended in 10ml of TE pH 8.0. The plasmid DNA is purified using CsCl gradients. 1g CsCl is added per ml of DNA solution. In addition, 0.5ml of EthBr (10mg/ml) is added. The solution is transferred to a polyallomer tube and ultracentrifuged at 49K for 18 hour in a Ti70 rotor at 20°C. The resulting plasmid band is removed from the tube, extracted with water saturated butanol to remove the EthBr and dialysed against 1xTE. Yields of 1-2mg are obtained.

2.4.3.2 Small Scale Plasmid Preparation

The above protocol is also used to produce small scale plasmid preparation. In this instance, 10ml of the overnight culture is used, 300µl of solution I, 300µl of solution II, 300µl of solution III and 600µl isopropanol. The resulting plasmid DNA is resuspended in 100µl of TE pH 8.0 containing RNase A. Yields of 20µg are obtained. In some cases, the Promega wizard™ preparations are used to isolate small amounts of DNA for checking rescued plasmids or for sequencing reactions. Procedure is followed as described by the manufacturer.

2.4.4. Transformation of *E.coli.*

2.4.4.1. Electrotransformation

Electrocompetent cell preparation.

1.0 ml of overnight culture of MC1061 is added into 400 ml L Broth and grown at 37°C with shaking to $O.D_{600}$ of 0.4-0.5 (approx. 3 hrs.). After chilling on ice for 30 min., they are centrifuged in Jouan at 4000rpm and 4°C for 10 min. Then cells are washed twice in 200 ml ice-cold distilled water and resuspend in 100ml ice-cold 10% glycerol. Then, cells are centrifuged again and resuspend in the final volume 1.0ml 10% glycerol. They are stored at -70°C.

Electrotransformation

When electrocompetent cells are thaw, *E. coli* Pulser cuvettes are placed on ice. 5µl of DNA (DNA should be salt free) and 40µl of cell suspension are mixed in a cold eppendorf tube and allowed to sit on ice for one min. The mixture is subsequently transferred to 0.1 cm cuvettes. When the cuvette is placed in pulsing chamber, pulse are applied (set E.coli Pulser to 1.8kV). 1.0ml of LB is added immediately. They are transferred to glass tube and incubated at 37°C for 30 min. in shaking water bath. After centrifugation, 200µl of cells are plated onto LB plates containing the appropriate antibiotics (i.e. 100µm/ml ampicillin for plasmid-rescue purposes) and incubated overnight at 37°C.

2.4.4.2. Chemical Transformation

Competent cell preparation

1.0 ml of overnight culture of XL1 Blue cells is added into 100 ml L Broth and grown at 37°C with shaking to $O.D_{600}$ of 0.5. After centrifugation, cells are resuspended in 10ml of ice-cold 50mM $CaCl_2$ solution. The cells are pelleted again, resuspended in 1ml of ice-cold 50mM $CaCl_2$.solution. Competent cells are either used fresh, or stored at -70°C.

Chemical Transformation

Transformations are carried out in sterile 1.5 ml microfuge tube. An aliquot of ligation mixture (or plasmid) containing up to 100ng plasmid DNA is added to 200µl aliquots of competent cells and they is incubated on ice for at least 30 min.. The DNA/cell mixture is then heat shock at 42°C for 90 seconds chilled on the ice for 2 min. 800µl of L-broth is added to the tube and incubated at 37°C for 30 min.. The cells are then plated onto LB plates containing the appropriate antibiotic/chromogenic substances and incubated overnight at 37°C to select for transformants.

2.4.5. Growth of *E. coli*

Liquid cultures of *E. coli* strains from which plasmids are to be isolated are grown in L broth with the appropriate antibiotic selection (usually ampicillin at 50μg/ml). The volume of L broth inoculated depended on the quantity of plasmid required. Routinely, 1.5 ml and 400 ml cultures are used for mini and large scale plasmid preparations respectively (section 2.4.3). The cultures are incubated at 37°C in an orbital shaker at ca. 250 rpm.

2.4.6. Plasmid Rescue Techniques

The basic methods for plasmid rescue is described by Pirrotta (1986). However, some modifications are made.

2.4.6.1. Isolation of Genomic DNA from Flies

In addition to the method described as section 2.4.1., the following method is applied for obtaining a small amount of genomic DNA. About 100 flies are ground in 1ml cold Homogenisation buffer (5% sucrose, 80mM NaCl, 0.1M Tris pH8.0, 0.5%SDS, 50mM EDTA) in 15ml Wheaton homogeniser. The samples are transferred to a tube and frozen for 10 minutes. They then are incubated at 70°C for 30 minutes. When KAc (pH4.8) is added to final concentration of 160mM, the samples are placed on ice for 30 minutes. After centrifugation, the supernatant is removed to a fresh tube and extracted twice with an equal volume of phenol/choroform and chloroform. DNA solution is precipitated by the addition of 0.75 volumes of isopropanol. After mixing, the DNA is pelleted by centrifugation. The pellet is washed in 70% (v/v) ethanol and dry. Genomic DNA is then resuspended in 0.5ml of TE containing RNAse A(20μg/ml).

2.4.6.2. Digestion of Genomic DNA

1μg Genomic DNA from the insertion line is digested using appropriate enzyme and buffer at 37°C for 3 hours. Digestion is checked on a mini-gel.

2.4.6.3 Ligation of DNA Fragments

Ligations are performed usually in 20µl of 1x ligation buffer provided by BRL, containing 1U of T4 ligase per µg of DNA. The reactions are incubated for 4 hours at room temperature or overnight at 16°C.

2.4.6.4. Transformation of *E. coli.*

Transformation of *E.coli* is described in section 2.4.4. Afterward a single colony is grown in 10ml LB of the glass tube with ampicillin at 50µg/ml described as section 2.4.6.

2.4.6.5. Isolation of Plasmid DNA

The mini prep of plasmid DNA isolation is described in section 2.4.3.2. Then the rescued plasmid containing the flanking DNA is analysed by restriction enzyme mapping.

2.4.7 Isolation of RNA

2.4.7.1 Isolation Total RNA

Separation of fly heads from bodies

About 15 ml of stunned flies is placed into a Falcon tube. Liquid nitrogen is slowly poured in, a perforated cap screwed on and vortexed for 30-60 seconds until all the N_2 boils off. More N_2 are added and the vortex is repeated. Then, the contents of the tube are poured into a 710 µm sieve already immersed in N_2 with porcelain basin. The preparation is checked that all heads have detached from the bodies. When flies from about 10 Falcon tubes are poured into the same sieve, the cool nylon bristled brush is used to force heads through the sieve using a circular "grinding" motion. The body preparation on this sieve are knocked into a Falcon tube and stored in liquid Nitrogen until needed. The mixture containing heads, appendages and some bodies in the basin are transferred into a 600µm sieve which will retain all the remaining bodies. Most of the heads and appendages will go through the sieve into the basin below. To separate heads from appendages, this mixture are put through the 425µm

sieve, all heads are retained and stored into a pre-cooled Falcon tube as a pure head preparation.

Isolation of fly head or body RNA

This procedure is adapted from the protocols described in Chomczynski and Sacchi (1987). 4g of fly heads or bodies are homogenised in 25ml of RNA Denaturing solution (4M Guanidine thiocyanate, 0.1M Tris.HCl. pH8.0, 2% ß-mercaptoethanol and 0.1µl/ml antifoam A.) using a Kinematica Polytron. After spinning, the homogenate is transferred to RF 50ml Falcon tube. Then, 1/10 vol. 2M Na acetate pH 4.0, an equal volume phenol and 1/5 vol. chloroform are added. This is shaken vigorously before being incubated on ice for 15 min.. The solution is centrifuged for 15 minutes at 15,000 rpm. The clear upper phase is removed into a fresh tube. An equal volume of isopropanol is added and the RNA precipitated at -20°C for an hour. After this time, the solution is centrifuged for 10 minutes at 10,000 rpm. The supernatant is discarded and the RNA pellet resuspended in 5ml denaturing solution. The RNA is again precipitated using an equal volume of isopropanol followed by incubation for an hour at -20°C. After centrifugation the RNA is resuspended in 5 ml of DEPC treated water. The RNA is stored as an ethanol precipitate at -20°C. Yield is 1mg/gram flies.

2.4.7.2 Isolation of Poly (A)$^+$ mRNA

5g of oligo (dT) are equilibrated in 10ml of 1x binding buffer (BB). This is left to swell for 1 hour at 4°C. A 5ml syringe is plugged with RF glass wool and filled up with oligo dT cellulose to give a bed volume of 1ml. The column is washed with 10ml of 0.1M NaOH and rinsed with several volumes of RF water until the pH of the effluent is less than pH 8.0. The column is washed with 10-20 volumes of 2xBB. The RNA is dissolved in 2xBB, heated to 65°C, cooled on ice and added to the column. The effluent is collected, reheated and re-applied to the column, this procedure is repeated so that the effluent is applied to the column three times. The column is now washed with 10-20 volumes of 1xBB. The bound RNA is eluted from the column using RF water that had been heated to

65°C (usually 3ml of water were used). An equal volume of 2xBB is added to the eluted RNA. The column is treated with NaOH as before and the whole procedure repeated. Once the RNA has been eluted, it is precipitated using 1/10 vol. of NaOAc pH 5.2 and 2.5 vol. of ethanol and left at -20°C overnight. Generally, yields of 20-50μg of poly(A)$^+$ mRNA/g of tissue are obtained.

2.4.8. Quantification of Nucleic Acids

In order to determine the concentration of DNA or RNA in a sample, readings are taken at wavelengths of 260nm. An OD_{260} =1 corresponds to 50μg/ml for double-stranded DNA, 40μg/ml for RNA and 33μg/ml for oligonucleotides. In other instances, the concentration of DNA is estimated by spotting the sample and known standards onto the surface of a 1% (w/v) agarose gel containing EtBr (0.5μg/ml). The gel is photographed using short-wavelength UV illumination (254nm) and the concentration of the DNA sample is estimated by comparing the intensity of fluorescence in the sample with those of known DNA concentration standards.

2.4.9. Labelling of Nucleic Acids

2.4.9.1. First Stand cDNA Probe for Reverse Northern

In order to produce high specific activity 1st strand cDNA probes, 150μCi 600 Ci/mmole of [a-^{32}P]dCTP is dried down in a siliconised microfuge tube, to this is added 4μl 5x 1st strand buffer, 1μl 80mM NaPPi, 30U RNAGUARD, 2μl Oligo $(dT)_{12-18}$, 1μg Poly A$^+$ RNA, 20U AMV reverse transcriptase and made up to a final volume of 20μl using RF water. The reaction is incubated at 42°C for 30 min, at which time 1μl of 10mM dCTP is added and the incubation continued for a further 30 min. Hydrolysis of the RNA is achieved by the addition of 1 volume of 0.6M NaOH, 20mM EDTA followed by incubation at 65°C for 30 min. Unincorporated nucleotides are separated using Sephedex G-50 columns (Sambrook *et al.*, 1989). Incorporation is assessed in a scintillation counter using

Cherenkov counting on a small aliquot of the probe. Specific activities of $1x10^8$ cpm/μg RNA are normally obtained.

2.4.9.2. Random Primed DNA Labelling with ^{32}P

DNA fragments are purified from 1% (w/v) LMP (Low Melting Point agarose, BRL) agarose gels in 1x TAE or using the Magic™ DNA purification system from Promega, using the condition recommended by the manufacturers.

The DNA labelling procedure is essentially as described in Feinberg and Vogelstein (1983). Between 25-50ng of denatured DNA sample in 12μl of water is mixed with 6μl 4x Random priming Buffer, 30μCi 600 Ci/mmole of [a-^{32}P]dCTP and 5U of Klenow enzyme(Promega) The mixture is usually incubated for at least 12hr at room temperature. Probes are purified using Sephadex G-50 columns prepared in 1ml syringes. Incorporation of radioactive precursor is calculated using the scintillation counter and Cherenkov counting. For random primed probes specific activities of 10^8-10^9 cpm/μg are normally obtained.

In some cases, "ready-to-go™ DNA labelling kit " is used as labelling DNA , using the condition recommended by the manufacturers (Pharmacia Biotch).

2.4.9.3. Nick Translated Biotin Probe

In order to produce biotin labelled probes for *in situ* hybridisation, 0.5μg of plasmid DNA, 2.5μl of 10x Nick translation buffer, 2.5μl of dNTP mix (0.3mM of each base), 2μl of Bio-16-dUTP (Boehringer), 1μl of ^{32}P (trace), , 1.5μl of DNase I diluted (1:1000 in 10mM Tris pH7.5, 10mM $MgCl_2$), made up to 25μl with distilled water and 10U of DNA Polymerase I are mixed in an eppendorf. This is incubated at room temperature for 60 min. The probe is separated by the addition of 1μl of 0.2M spermidine followed by incubation for 30 min on ice and spun down at 4°C for 30 min. When the supernatant is removed and stored, the pellet is resuspended in 75μl Hybridisation buffer. Incorporation is roughly

estimated by comparing the resuspended pellet with the supernatant. An incorporation of between 25-30% is found to produce optimal probes.

2.4.9.4. DIG RNA Labelling Probe

RNA probe synthesis is according to the method recommended by Boehringer Mannheim.

2.4.10 Electrophorses and Blotting

2.4.10.1 DNA Electrophoresis and Southern Blots

DNA is electrophoresed on agarose gels which are made and run in 1xTBE. A range of agarose concentrations (0.8-1.2%, w/v) is used depending on the sizes of fragments to be resolved (Sambrook *et al.*, 1989). Applied voltages varies between 2 and 10V/cm, depending on the time of running. Markers are usually the 1kb ladder supplied by the manufacturers (BRL). Gels are photographed on the UV transilluminator after staining agarose gels with EtBr or adding EtBr to the sample (0.5µg/ml) using a Polaroid camera loaded with 667- land film and fitted with a Kodak Wratten filter No. 23A. The DNA is transferred onto Hybond N membrane following the procedure of Southern (1975). After electrophoresis, the DNA is immersed in Denaturing solution for 20 min, followed by soaking in two changes of Neutralising solution for 20 min each on a 'rock-and-roller'. The gel is gently agitated during this time. It is left to transfer onto the membrane overnight. DNA is fixed to the membrane by baking at 80°C for two hours or by UV irradiation using a Stratalinker™ (Stratagene).

Hybridisation Conditions.

All hybridisations are carried out in hybridisation tubes in a Hybaid oven at 65°C. The membrane is prehybridised in SSC prehybridisation solution for at least two hours before the addition of the probe. The probe (described as section 2.4.9) is boiled for 5 min before being added to the filter and left to hybridise for at least 10 hrs. Filters are washed in

2xSSC at room temperature followed by wash for 15 min in low stringency wash at 65°C, and two washes for 15 min in the high stringency wash also at 65°C. The filters are blotted dry and covered in Saran Wrap™. Autoradiography of probed filters is carried out at -70°C, using intensifying screens and Fuji NIF RX X-ray film. Films are developed using a Kodak X-Omat film processor.

2.4.10.2. Reverse Northern

1µg of plasmid DNA restricted with the appropriate enzymes are run in duplicate on a 0.8% (w/v) TBE agarose gel. The gel is blotted as described above. The filter is cut in half, one half probed with the head cDNA probe, the other with the body cDNA probe (section 2.4.9.1). Filters hybridisation and washing conditions are as described above.

2.4.10.3. RNA Electrophorses and Northern Blots

5µg mRNA and RNA ladder (BRL) in a volume of 5µl are loaded onto a 1.0% (w/v) MOPS/Formaldehyde denaturing gel. Before being loaded onto the gel the samples are denatured by the addition of 10µl of formamide, 2µl of 5xMOPS buffer, 3.5µl of formaldehyde, 1µl of EtBr (1mg/ml stock), and heated to 70°C for 15 min, cooled immediately on ice. Prior to loading 2µl of formaldehyde loading buffer are added to each sample (Sambrook *et al.*, 1989). The gels are run in 1xMOPS, with constant circulation from anode to cathode chambers in order to maintain a constant pH. The gels are photographed as before. After photography, the gel is soaked in 0.2M NaOH for 20 min followed by soaking for 30 min in 20xSSC. The gels are transferred to Hybond C^+. RNA is fixed to the membrane by baking for 2hr at 80°C.

Hybridisation conditions

DNA/RNA hybridisations are carried out at 42°C in Formaldehyde prehybridisation solution. Filters are pre-hybridised at 42°C for at least 3hr before addition of the denatured radioactive probe and hybridised for a minimum of 16hr. After hybridisation, the filters are washed in 2xSSC at room temperature followed by two 20 min washes in low stringency

wash solution at 65°C, followed by one 15 min. wash in high stringency wash solution at 65°C. The filters are blotted dry and covered with Saran Wrap™ before autoradiography.

2.4.11. Screening Lambda Genomic DNA and cDNA Libraries

2.4.11.1. First Round Screening

Screening of lambda Genomic DNA and cDNA libraries is essentially described by Sambrook *et al.*, (1989). Briefly, cells from a prepared bacterial suspension (as described in section 2.4.2.1) are infected with phage from the bacteriophage lambda libraries. Then, about 2 x 10^3 pfu are plated onto 10x10 cm prtri dishes using 8ml of 7% (w/v) top agarose in LB. The plates are incubated at 37°C for about 8 hours or until the phage are just visible. Replica filters are lifted from each plate, denatured for 3 min, neutralised twice for 5 min and washed in 2xSSC for 15 min. They are air-dried before using crosslinking. The prehybridised and hybridisations described in section 2.4.10.1. The positive plaques are stabbed out of the plates using the big end of a sterile glass Pasteur pipette. The plug is left in 1ml of phage buffer with 100µl chloroform for two hours at room temperature (or overnight at 4°C) to allow bacteriophage particles to diffuse out of the agar.

2.4.11.2. Secondary Screening

Similarly, the host cells are infected with phage from the first round screening bacteriophage. Then, they are plated onto a 9mm circular petri dishes. The following steps are as same as the first round screening.

An individual plaque of interest is picked from the plate using the narrow end of a sterile glass Pasteur pipette. The plug is left in 1ml of phage buffer with 100µl chloroform for two hours at room temperature (or overnight at 4°C). Then the protocol of isolation of bacteriophage DNA is followed in section 2.4.2.

2.4.12. DNA Sequencing Techniques

2.4.12.1 Preparation of Polyacrylamide Gel

6% (w/v) denaturing polyacrylamide gel are used for sequencing. The gel are prepared from the following stock solution:

For 100 ml:

40% (w/v) acrylamide stock	15 ml
urea	55 g
10x TBE	10 ml
dH_2O	35 ml

The urea is dissolved by heating the mix to 37°C and then cooled to room temperature. Before pouring the gel, 1ml of freshly prepared 10% (w/v) Ammonium persulphate and 40µl of TEMED are added to the gel solution.

2.4.12.2. Preparation of Glass Plates and Pouring the Gel

The sequencing plates are cleaned thoroughly with SDS, alcohol and water and assembled using two spacers along the vertical sides and 3MM Whatman paper (cut as a spacer) along the bottom of the gel. The entire assembly is held in place by clamps. The gel solution is applied using a 50ml syringe on one edge of the plates while tilting the plates at an angle of about 30°. The plates are then laid at an angle of 5° and the sharks tooth combs inserted on the flat side to provide an even surface of the top of the gel. The gel is usually left to polymerise overnight before use.

2.4.12.3. Preparation of DNA Sequencing Samples

Double stranded sequencing reactions using the dideoxy chain termination method (Sanger *et al.*, 1977) are carried out as described in the Sequenase Version 2.0 manual (United States Biochemical Corporation).

2.4.12.4. Electrophoresis of Sequencing Gel

The gel is pre-electrophoresed for 1hr in 1xTBE at a constant power of 100W, after which time, the gel temperature is normally 50°C. Before loading, the samples are denatured for 2 min at 75°C. Gels are run for 2 hours for a short run (150 bp) or for 5 hours for a long run (300 bp) on constant power, and then dried for 1-2hr at 80°C onto Whatman 3MM paper under vacuum. Autoradiography is carried out without intensifying screens at room temperature.

2.4.12.5. Computer-assisted Sequence Analysis

The DNA sequences are input into MacVector™ 4.1.4. programme using IBI Gel Reader. The DNA sequences are then analysed using the following programmes.

MacVector™ 4.1.4. and AssemblyLIGN™ (Sequence Analysis Software); Fasta, Pileup. Distances and Growtree (GCG, Program Manual see the Wisconsin Package); Blast searches, Prosite search (National Centre of Biotechnology Information, National Institutes of Health).

Chapter 3

Screening of P[GAL4] Enhancer Trap Lines

This chapter describes the results of generating and screening novel P[GAL4] lines for patterns of expression in the adult *Drosophila* brain. The chapter then details some specific staining patterns in the brain. Finally, the chapter lists the chromosomal locations of P[GAL4] insertions.

3.1 Introduction

As mentioned in section 1.2., one disadvantage of the first generation enhancer trap elements is that they express ß-gal fused to the N-terminal nuclear localisation signal of the P element transposase. Nuclear staining has its uses but precludes visualisation of cellular geometry, an important requirement in the study of cells with long processes such as neurons. In *Drosophila*, the cell bodies of the central brain neurons lie in a thin rind just beneath the cuticle, whereas the structural elements (neuropil) described by conventional anatomy consist of axonal and dendritic projections and the synapses between them. In order to visualise neuropil it is therefore necessary to use a reporter gene that directs expression to the cytoplasm and whose product will be actively transported.

A second generation enhancer trap element has now been developed (Brand and Perrimon, 1993; Kaiser, 1993) that provides for expression of a cytoplasmically-localised reporter and that in addition can be used to target expression of any desired gene product to the marked cells. The reporter of P[GAL4] is a yeast transcription factor that is functional in *Drosophila* (Fisher *et al.*, 1988), and that can be used to direct expression of other transgenes placed under the control of a GAL4 -dependent promoter (UAS_G). A cross between a fly with a new GAL4 insertion and a fly containing UAS_G-*lacZ*, for example, causes ß-gal to be expressed in a pattern that reflects GAL4 activity (Fig. 1.3.). Any number of other transgenes can then substitute for *lacZ* (Greig and Akam, 1993; Ferveur *et al.*, 1995; Sweeney *et al.*, 1995; O'Dell *et al.*, 1995; Yeh *et al.*, 1995; Brand, 1995).

P[GAL4] was created by modification of plwB, in which p, l, w, and B stand for plasmid, *lacZ*, w^+ eye marker and Bluescript respectively (Wilson *et al.*, 1989). Briefly, the vector plwB was first digested with *Hin*dIII to remove the *lacZ* gene and the N-terminus of the P-transposase gene. These were replaced with the entire GAL4 coding region behind the TATA box of the P-transposase gene (Brand and Perrimon 1993). Although the frequency of transposition of the GAL4 element is less than that of first-generation elements, it is not prohibitively so. Therefore, a large number of new P[GAL4] insertion lines can be created by controlled P-element mutagenesis as described in section 1.1.3.

The reporter construct, the UAS_G-*lacZ*, was constructed as follows (Brand and Perrimon 1993): First, pUAST was designed to direct GAL4-dependent transcription of a gene of choice. It contains five tandemly arrayed, optimised GAL4 binding sites, followed by the *hsp70* TATA box and transcriptional start site, a polylinker with unique restriction sites, and the SV40 small t intron (SV40 small t plays a supplementary role in both the establishment and maintenance of transformation) and polyadenylation site. Then, an *Adh-lacZ* fusion gene was removed from pCaSpeR-AUG-ß-gal (Thummel *et al.*, 1988) and subcloned in pUAST between the TATA box and SV40 transcriptional terminator.

Investigation of region-specific staining in the adult brain has great significance for an elucidation of relationship between structure and function because adult flies can show clearly brain structures in greater detail compared with larva and pupae. As discussion before, the GAL4 enhancer trap system provides us novel means to do this. When specific expression pattern or individual cell staining pattern in particular structure of the brain are found, they can be used to further analyse for brain anatomy and flanking genes cloning. Furthermore, these specific expression patterns can be used for ablation for function and behavioural studies. In order to pursue such a study, I began a search for specific expression patterns in adult brain using the GAL4 enhancer trap system. In the following sections, the results of screening 1400 newly generated P[GAL4] lines are reported.

3.2 Generation of New P[GAL4] Insertion Lines

We used two different strains as P[GAL4] donors. One had a recessive lethal insertion on the X chromosome (Brand and Perrimon 1993). Another P[GAL4] insertion strain has a single insertion on the second chromosome balancer *Cy*O (kindly provided by Tim Tully, Cold Spring Harbour). To mobilise the insertions from the above strains to new chromosomal locations, the "jumpstart technique" (Cooley *et al.*, 1988) for P element mutagenesis was employed. Two controlled P element mutagenesis schemes are shown in Figure 3.1 and 3.2.

In the first crossing scheme (Fig. 3.1), females carrying the P[GAL4] insertion on the X chromosome over the balancer FM7 were mated *en masse* with males carrying a stable genomic transposase, the P[ry^+ Δ2-3] element on the third chromosome with a dominant marker *Drop* (*Dr*), over the TM6, *Ubx* balancer (Robertson *et al* 1988). This cross yields F1 "jumpstart" females carrying both the P[GAL4] insertion and the transposase gene. Therefore, virgin females with flies red eye colour and *Dr* bar eye shape were selected for the next cross. The Δ2-3 transposase in the germ-line cells of these females can excise the P[GAL4] element and, in some cases, insert it at new locations. When a single jumpstart female was mated with *white* males (w^{1118}), the male progeny bearing new P[GAL4] insertion were selected by their coloured eyes (the original P[GAL4] insertion is male lethal). Then, males carrying new insertions were individually crossed to w^{1118} females to establish a set of 280 independent lines which were labelled from line 1Y to 280Y.

For the above crossing scheme, It is found that the frequencies with which new P[GAL4] insertion were recovered was very low. This might be attributed to the alterations in the pGawB that allow GAL4 to be expressed from its own AUG start codon, rather than as a P-transposase-GAL4 fusion protein (Brand and Perrimon 1993). In addition, when recovering F_2 progeny, only males can be selected because it is impossible

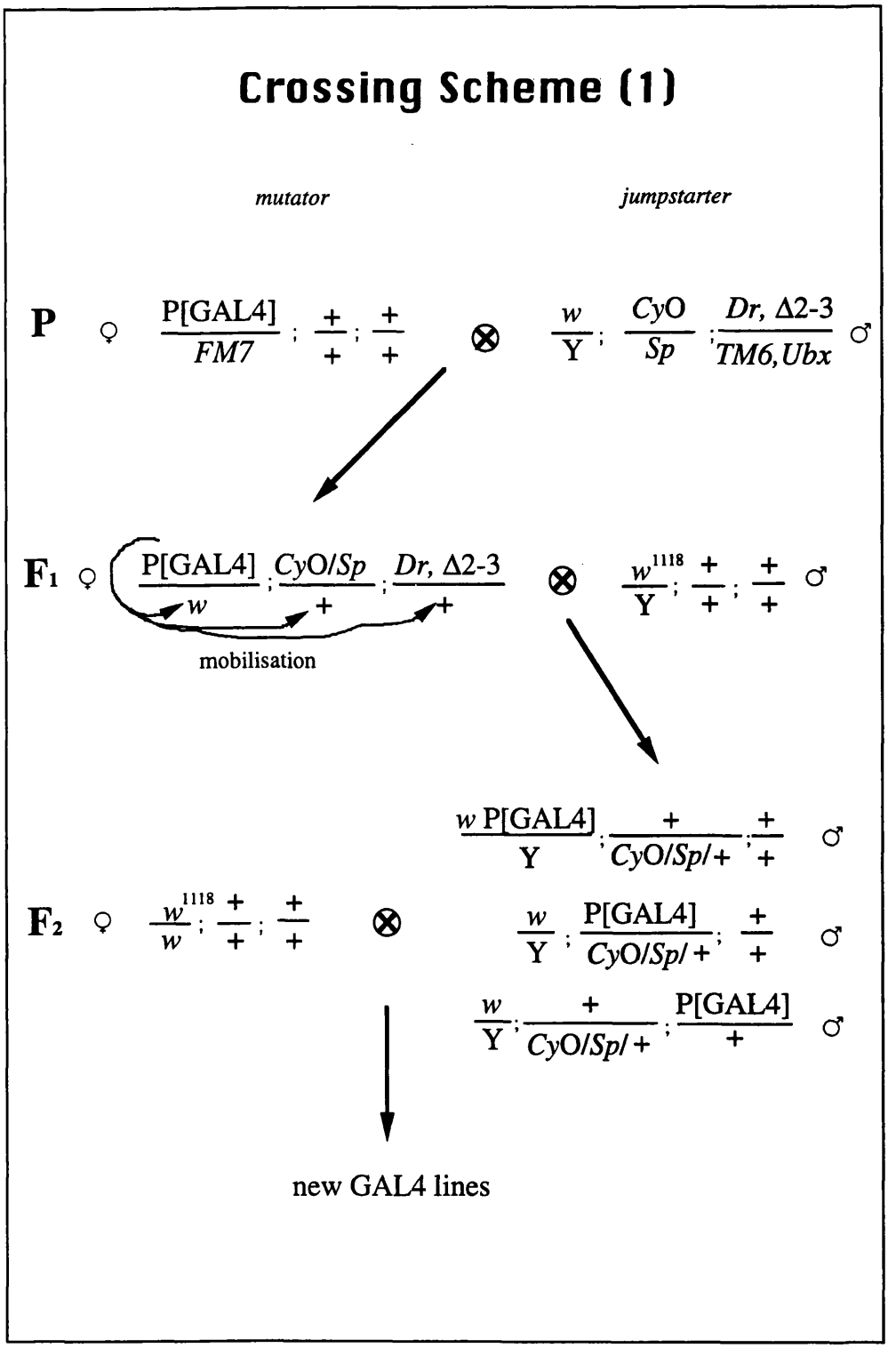


Figure 3.1. Genetic scheme for mobilization of a recessive lethal P[GAL4] on an X-chromosome. For details see text in section 3.2.

to distinguish between the original P[GAL4] insertion and new P[GAL4] insertion in females at this stage.

Due to above reasons, I set up a second crossing scheme (Fig. 3.2) when a P[GAL4],*Cy*O strain became available (Tully, *et al.*, personal comm.). In the second crossing scheme, similarly, flies carrying a stable genomic P-transposase source P[ry^+; Δ2-3] were crossed to the P[GAL4],*Cy*O insertion strain. When males that carried both GAL4 and Δ2-3 P elements were mated with *w* (CS10) females (Dura *et al.*, 1993), F_2 males and females with red but normally shaped eyes and wild-type wings could be selected against *Cy*O or *Dr,* Δ2-3 carrying progeny. Then, these transformed flies were backcrossed to *w* (CS10) again and 1125 new P[GAL4] lines were established (line c1 to line c856). Sometimes, more than one transformed fly came from one individual vial in F_1 cross, they were also used to establish lines. These sublines were sub-marked as a, b, c, etc. For example c123a, c123b and c123c.

Finally, in order to make the novel P[GAL4] lines into the Cantonised background for further analysis, they were crossed with *w* (CS10) at least two generations. The *w* (CS10) strain was derived by backcrossing w^{1118} flies to wild-type (Canton-S) flies for ten generations (Dura *et al.*, 1993).

During the establishment of new P[GAL4] lines, it was estimated that about 8% insertion lines were sterile in F_2 stage. This was probably due to P-element insertion.

Insertions segregating with the X chromosome were detected by examining the progeny of crosses between the P[GAL4] line and flies for homozygous UAS_G-*lacZ.* About one-fifth of the GAL4 lines have insertions on the X chromosome. This event is in accord with assumption that P-element insertion occur randomly on a chromosomal scale (Spradling 1986).

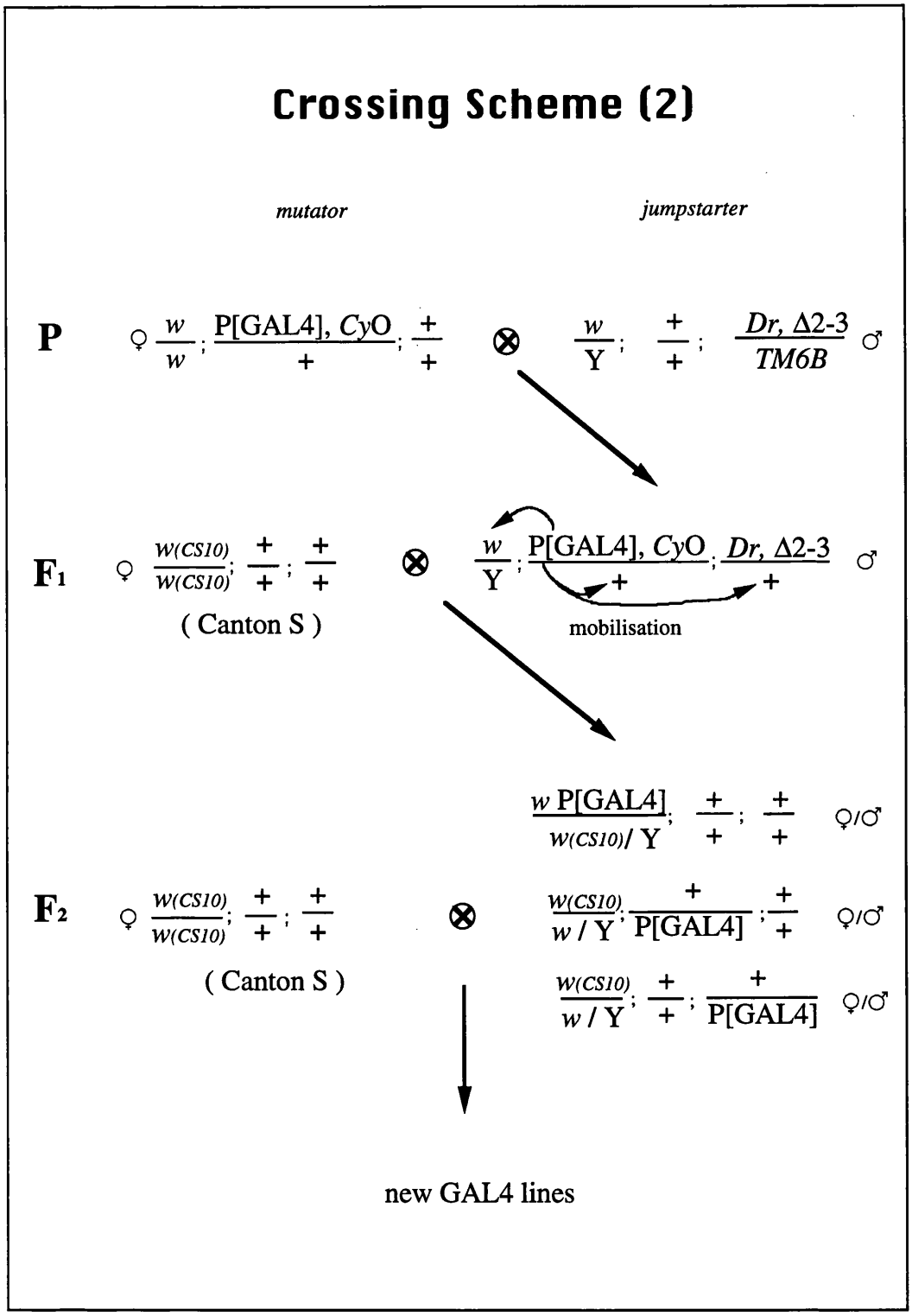


Figure 3.2. Genetic scheme for mobilization of P[GAL4] from the balancer, *Cy*O on the second chromosome. For details see text in section 3.2.

3.3 Screening of New P[GAL4] Insertion Lines

To screen a large number of lines for ß-gal expression in fly heads, it is preferable to perform serial cryostat sections and use a histochemical detection method. It is quick and convenient compared with whole-mount staining and antibody *in situs*.

In screening, rather than to visualise GAL4 expression directly, it is used to drive a secondary reporter gene linked to a GAL4-dependent promoter, UAS_G-*lacZ*, and thus ß-galactosidase is expressed in a pattern that reflects GAL4 activity. ß-gal activity is readily detected as a blue stain produced by conversion of the chromogenic substrate X-Gal. Each of the 1405 P[GAL4] lines was crossed to flies homozygous for a UAS_G-*lacZ* transgene on the second chromosome (Brand and Perrimon 1993) and their progeny were examined for GAL4-directed ß-gal expression in the fly head.

The recombinant flies (males and females) carrying both P[GAL4] and P[UAS_G-*lacZ*] were mounted side by side in "fly collars" and soaked in OCT for 10 minutes. They were then frozen and embedded heads separated from bodies. 12µm serial frontal head sections were cut in a cryostat (Anglia Scientific) at -18°C. The sections were then placed on slides and stained according to the methods described in section 2.2.1.3. When an interesting staining pattern was found, it was always checked again from a new cross.

A very large proportion of the lines showed some ß-galactosidase expression in the brain. A summary of the ß-gal expression patterns is given in Table 3.1.

As can be seen from the results, about 10% of GAL4 lines had no staining pattern in the brain within four hours of staining. However, the other 90% of insertion lines exhibited a wide range of different expression patterns. In the "general staining" group, as many as 80% of the lines showed some degree staining in the brain. The staining patterns vary from a little blue staining to "all over" staining, and from weak to strong staining. Some of lines are stained in complex pattern that mark many different tissues and cells.

Table 3.1 GAL4-directed Expression Patterns in the Brain

STAINING REGION	NUMBER OF LINES	PERCENTAGE
no brain staining	139	9.9%
general staining	1132	80.6%
mushroom body (MB)	24	1.7%
central complex (CC)	20	1.4%
antennal complex (AC)	18	1.3%
optic lobe (OL) & eye	17	1.2%
MB+CC	7	0.5%
MB+AC	7	0.5%
MB+OL	10	0.7%
CC+AC	8	0.6%
CC+OL	7	0.5%
specific tracts+others	16	1.1%
TOTAL	1405	100%

Such patterns might reflect the presence of multiple regulatory elements near the P[GAL4] insertion, each one of which controls a different gene (Wilson *et al.*, 1989). Often, we observed quite complicated patterns which were difficult to interpret.

A small proportion of staining patterns showed a specific staining in one or more substructure of the brain. Approximately 1.7% had ß-gal expression principally in the mushroom bodies, around 1.4% had specific staining patterns in the central complex, about 1.3% had staining patterns more or less restricted to the antennal complex (antennal lobes and antennal nerves). Another 1.2% had staining patterns restricted to the optic lobe and eye, and nearly 3% showed staining patterns in more than one substructure of the brain. About 1.1% of GAL4 lines showed the blue staining in specific tracts or other

regions of the brain. In these groups, the percentage includes staining patterns that stain weakly all over but that still have a strong superimposed tissue-specific pattern. These interesting expression patterns will be useful for further analysis.

As mentioned above, when more transformed flies came from an individual vial in F_1 cross of the second crossing scheme, they were sub-marked. During the screening, it was found that about 60% of the sub-marked progeny showed the same ß-gal expression patterns as a sibling P[GAL4] line. In such cases it was inferred that each of these lines carried an insertion at the same location and probably was the result of the same premeiotic transposition event in the germ line of the male parent as its sibling. The rest of sub-marked lines showed different staining patterns and were considered as independent new insertions. Such lines were obtained due to the following results. (1) the P[GAL4] was inserted at the different chromosomal location as the sibling line and (2) P[GAL4] insertion came from different "jumpstart male" in the same vial, in which there were three "jumpstart males" together.

In some cases, the same or very similar patterns of GAL4-directed expression are observed in different independent lines. This is probably due to the same chromosomal location of the inserted P[GAL4] element and to staining resolution which is not high enough to distinguish these patterns. On the other hand, it is also possible that the same or a similar pattern could arise from insertion at different sites (see section 3.5).

An attention was given to the choice of staining conditions that minimise the fly's endogenous ß-galactosidase activity. The endogenous ß-galactosidase activity could be eliminated at higher pH values and in a shorter staining time allowing visualisation of enhancer-trap specific *lacZ* expression. Staining solutions at pH 7.8 and staining times of 1-4 hours were used routinely. An alternative way to remove the endogenous ß-gal staining would be to delete the endogenous ß-gal gene in flies (Fargnoli *et al.*, 1985). On the other hand, It was also found that flies bearing both P[GAL4] and UASG-*lacZ* were

more sensitive to ß-gal staining than those bearing P[*LacW*]. This is probably due to the high level expression of GAL4 in those flies.

After further observation of the staining patterns, more than 300 lines were kept which display interesting patterns in the brain from an anatomical perspective. Of these, as many as 100 lines are restricted to specific regions or neuronal sub-populations of brain. The examples of these patterns will be given in the next section.

3.4 Examples of Interesting Patterns in the Brain

As mentioned in section 1.3, at least four conspicuous main structures can be observed in the *Drosophila* brain. They are (1) the mushroom body, (2) the central complex, (3) the antennal lobe and (4) the optic lobe. In some of P[GAL4] lines, the ß-gal expression was more or less restricted to these regions.

3.4.1 The Mushroom Bodies

As has been mentioned in Chapter One, the mushroom bodies are the most fascinating structure in the *Drosophila* brain, a region thought to play a central role in learning and memory. As can be seen from the screening result, a number of P[GAL4] lines showed the staining patterns in the mushroom bodies. Figure 3.3 showed a series of sections through the brain of line 30Y, in which ß-gal expression was a good match for the classical view of *Drosophila* mushroom body architecture (Heisenberg, 1980). Note that the staining extended from the cell body layer to the tips of the lobes, i.e. the Kenyon cell body layer, the calyx, the pedunculus and α, ß, and γ lobes of the mushroom body. Only Kenyon cells have such a trajectory project out of the calyx to form the pedunculus and lobes (Pearson, 1971) thus, the ß-gal staining patterns must represent Kenyon cells expression in the mushroom bodies.

More blue staining patterns in the mushroom bodies are depicted in Figure 3.4 (a-f).

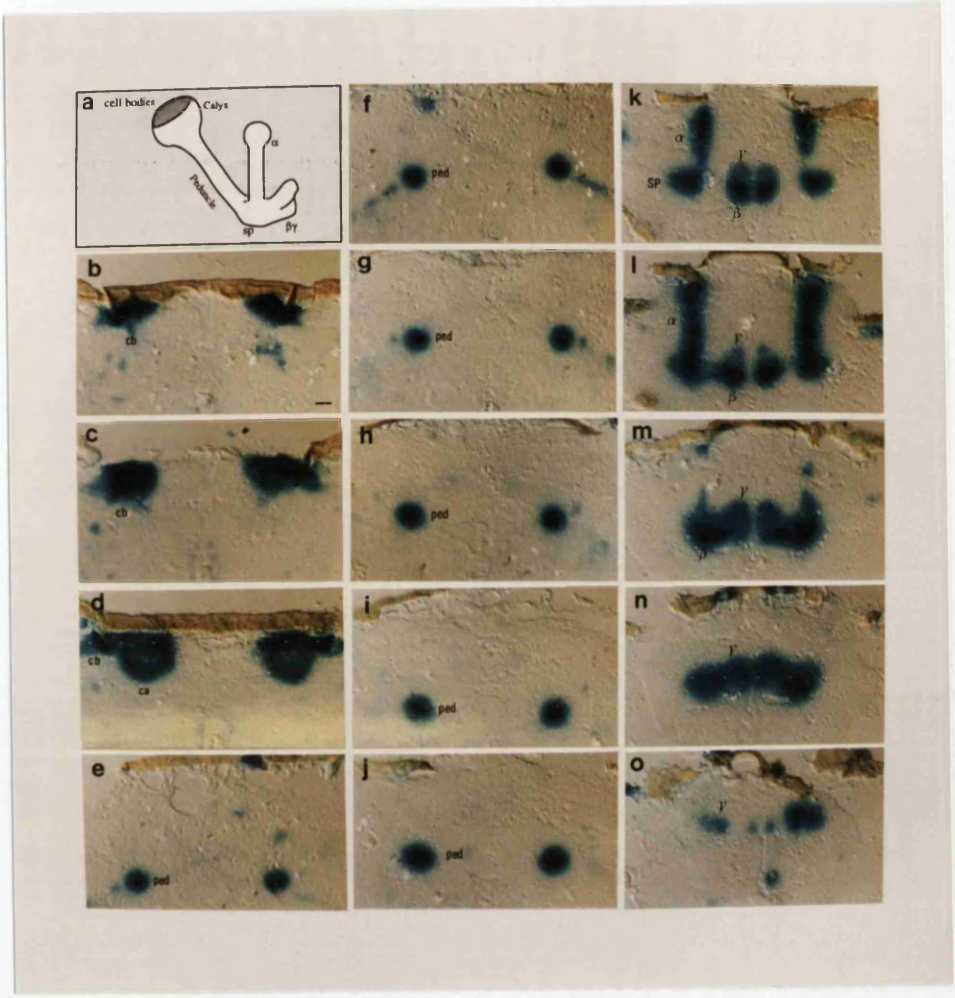


Figure 3.3. Mushroom body structures

(**a**). Schematic representation of the *D. melanogaster* mushroom bodies. The sagittal view of dense clusters of Kenyon cell bodies (cb), above and behind the calyx (ca). The calyx is formed by Kenyon cell dendrites and afferents from the olfactory lobes. Beneath each calyx, Kenyon cell axons converge to form a stalk-like structure, the pedunculus (ped). This extends almost to the front of the brain, where it divides into a dorsally-projecting α lobe, and a β/γ complex projecting towards the mid-line. (**b**) to (**o**) showing ß-gal expression revealed by X-Gal staining in representative 12μm cryostat frontal sections through the head of P[GAL4] line 30Y. (**b-d**) show blue staining in the cell bodies and the calyx; (**e-j**) show blue staining in the pedunculus; and (**k-o**) show expression in the three lobes of the mushroom bodies. Scale bar: 10μm.

One line, 201Y, has a very restricted neuronal expression pattern. Staining is almost exclusively in Kenyon cells belonging to core elements of the α and ß lobes, and to most of fibres within the γ lobe. Similarly, line 103Y showed the dark blue staining in the α and ß lobes corresponding to the same neurones as 201Y and relative weak staining in other parts of the mushroom bodies . On the other hand, line c772 showed staining in the α and ß lobes with an unstained core, and in all the γ lobe. Line 11Y showed strong staining patterns only in α and ß lobes but no staining of the γ and the spur. In line c532, all the mushroom bodies pattern can be seen, but the intensity differences between γ lobe with a spur and other lobes of the mushroom body are observed. But line c253 has a strong staining in part of α lobe, all the ß and γ lobes of mushroom bodies.

Based on observations of staining patterns, it is found that groups of Kenyon cell axons disposed at "characteristic", often concentric, positions within the pedunculus, beyond which they segregate to specific regions of the lobes. A spur-like structure is observed at the branch point of the pedunculus and the lobes. The α and ß lobes are organised concentrically and λ lobe that is correlated with a spur seems to be different in its organisation.

For most of MB lines, P[GAL4] insertions are localised by *in situs* (see the next section). Different lines have a P[GAL4] insertion at a different chromosomal location and display quite different patterns even though these lines have wild-type mushroom bodies as judged by interference phase-contrast microscopy or autofluorescence. Therefore, different staining patterns represent GAL4 expression in different, genetically specified, subsets of Kenyon cells in the mushroom bodies.

Based on further examination of whole-mount brain preparations stained by X-Gal (data not shown) and confocal sections stained by the anti-ß-gal antibody (JD. Armstrong, personal comm.), the covert anatomical subdivision of the mushroom bodies is confirmed. More details about subdivision of the mushroom bodies by expression patterns can be seen in Appendix 2 (Yang *et al.*, 1995).

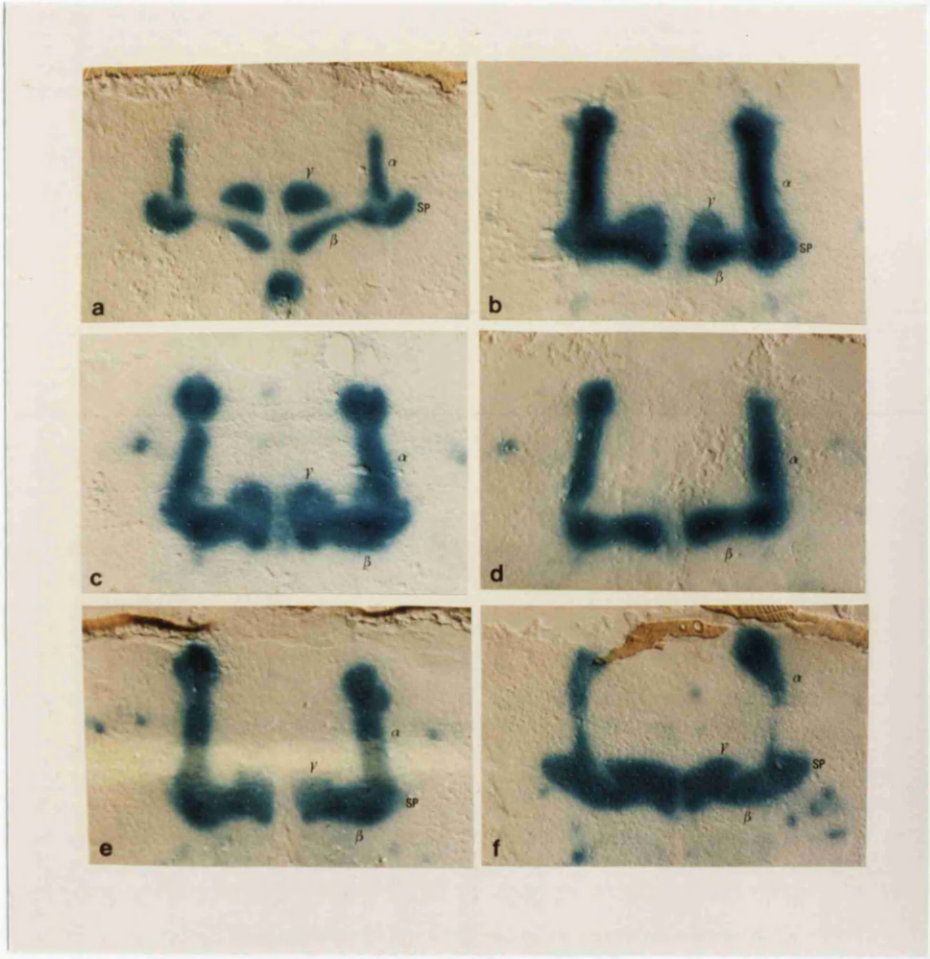


Figure 3.4. Cryostat frontal sections (12 μm) showing X-Gal staining in the mushroom body of six P[GAL4] lines. (a) line 201Y, (b) line 103Y, (c) line c772, (d) line 11Y, (e) line c532, and (f) line c253. Abbreviations are as in Fig. 3.4. See text for full description.

3.4.2 The Central Complex

The structure and the connection of the central complex have been described in Chapter One. In a number of P[GAL4] lines the different substructures of the central complex are clearly revealed by expression patterns.

Figure 3.5 shows some sections through the brain of one such line, c161. The blue staining can be seen in the protocerebral bridge, the fan-shaped body, the ellipsoid body and the noduli. More than one kind of neuron may be responsible for this staining pattern (Hanesch *et al.*, 1989). In this line, connection fibres between the substructures of the central complex, such as the "vertical fibre system" (VFS) (Hanesch *et al.*, 1989), are stained. The cell bodies of the VFS lies in the dorso-caudal cortex. Its main fibre passes the protocerebral bridge, sending spiny arborization into one glomerulus, enters the fan-shaped body ipsilaterally in layer 4 and takes a downward turn to the contralateral noduli. The ß-gal expression of this line is a good match for the classical view of *Drosophila* central complex structure (Fig. 3.5a, Hanesch *et al.*, 1989). The further characterisation of expression patterns in the central complex will present in Chapter Four.

3.4.3 The Antennal Lobes

The antennal lobe is the major brain neuropil that receives olfactory input in *Drosophila*. (Fig. 1.6) The olfactory information from the antennae is conveyed to the antennal lobe via the antennal nerve. Relay neurons from the antennal lobe project via a large tract, (antenno-glomerular tract AGT), to synapse on the dendrites of mushroom body cells in the calyx (Stocker *et al.*, 1990). More output tracts from the antennal lobes to the other parts of the central brain were described in Chapter One.

As can be seen in Table 3.1, about 1.2% of GAL4 lines has expression patterns in the antennal complex, representing a substantial fraction of its cellular components.

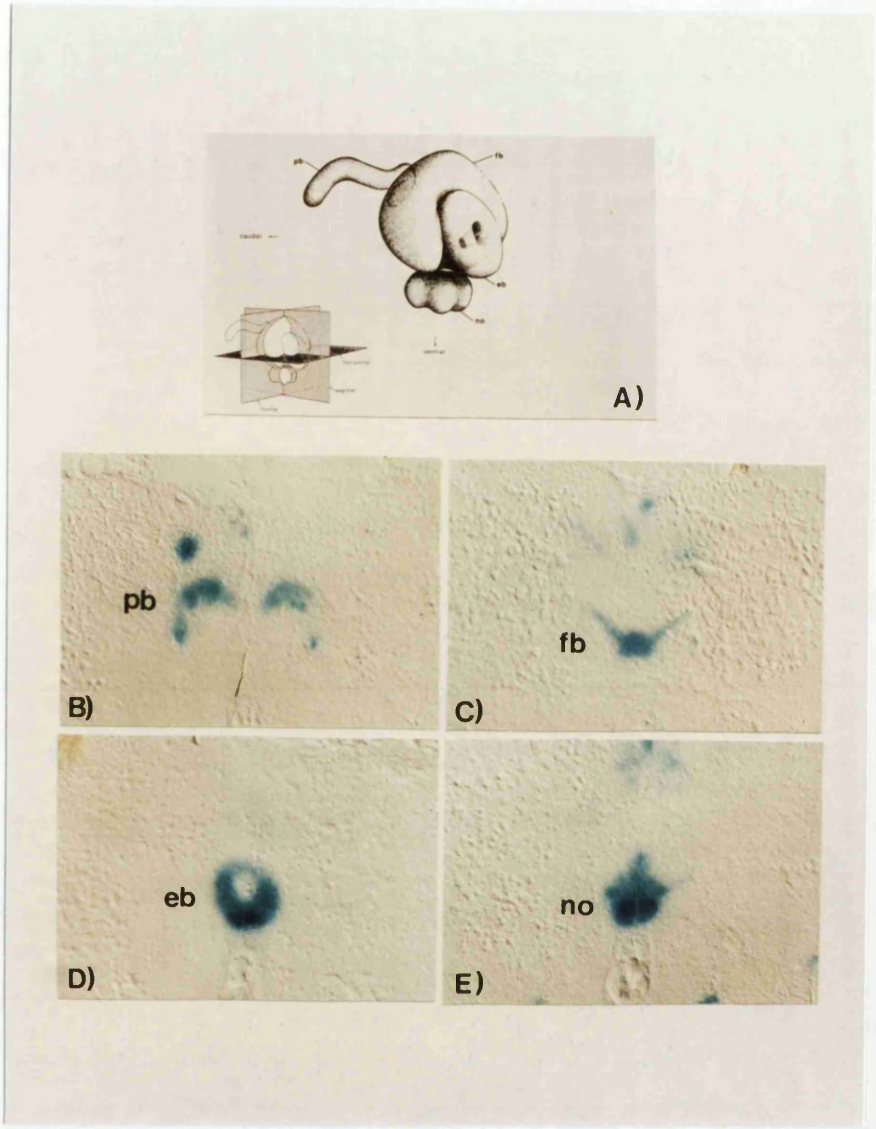


Fig. 3.5. Central complex structures.

A: Schematic diagram of the *Drosophila* central complex (taken from Hanesch *et al.*, 1989). The four substructures: the protocerebral bridge (pb), the fan-shaped body (fb), the ellipsoid body (eb) and the paired noduli (no). **B**(a-d): ß-gal expression revealed by X-Gal staining in the main neuronal region of the central complex in representative 12µm cryostat frontal sections through the central complex of line c161. (a) Blue staining in the protocerebral bridge; (b) in the fan-shaped body; (c) in the paired noduli and (d) in the ellipsoid body.

Examples of lines which have a staining pattern in the antennal lobes and antennal nerve are given (Figure 3.6).

Line 253Y shows stained subsets of fibres in the antennal nerve, in which about two thirds of the volume of axons is stained, whereas, in lines c588 the whole antennal nerve appears labelled. A strong staining in the glomeruli of the antennal lobe is observed in line 59Y. Line c503a shows a staining pattern in the antennal lobes, the antennal nerve and in the antennal commissure. However, line c133 has an expression pattern only in the periphery of the antennal lobes. Interestingly, line c492b showed intense staining in the antennal lobes and the mushroom bodies. Staining in the AGT connection between these substructures is also observed in this line.

It is well known that the antennal complex (lobes and nerve) plays an important role in passing and organising olfactory and gustatory information (Stocker, 1994). Recently, Ferveur *et al.*, (1995) and O'Dell *et al.*, (1995) used P[GAL4] antennal complex lines to study courtship behaviour. They were able to feminize specific regions of the male antennal complex by targeted expression of the female form of the *transformer* sex determination gene. They found that these males can court both males and females, suggesting a role for these regions of antennal complex in sexual orientation. For analysis of structure-function in antennal lobes and antennal nerve, these P[GAL4] lines are indeed good markers .

3.4.4 The Optic Lobe and Eye

Among the lines investigated, a number of lines have shown specific GAL-directed expression patterns in the optic lobes and eye. More specifically, Figure 3.7 showed examples of the *lacZ* expression in the compound eyes (line 54Y), glia cells between the retina and the lamina (c340), lamina (line c246), the first optic chiasm (line c829), medulla (line c577), the second optic chiasm (line c637a) and lobula complex (line c469) These classes of lines can be used as marker for further analysis.

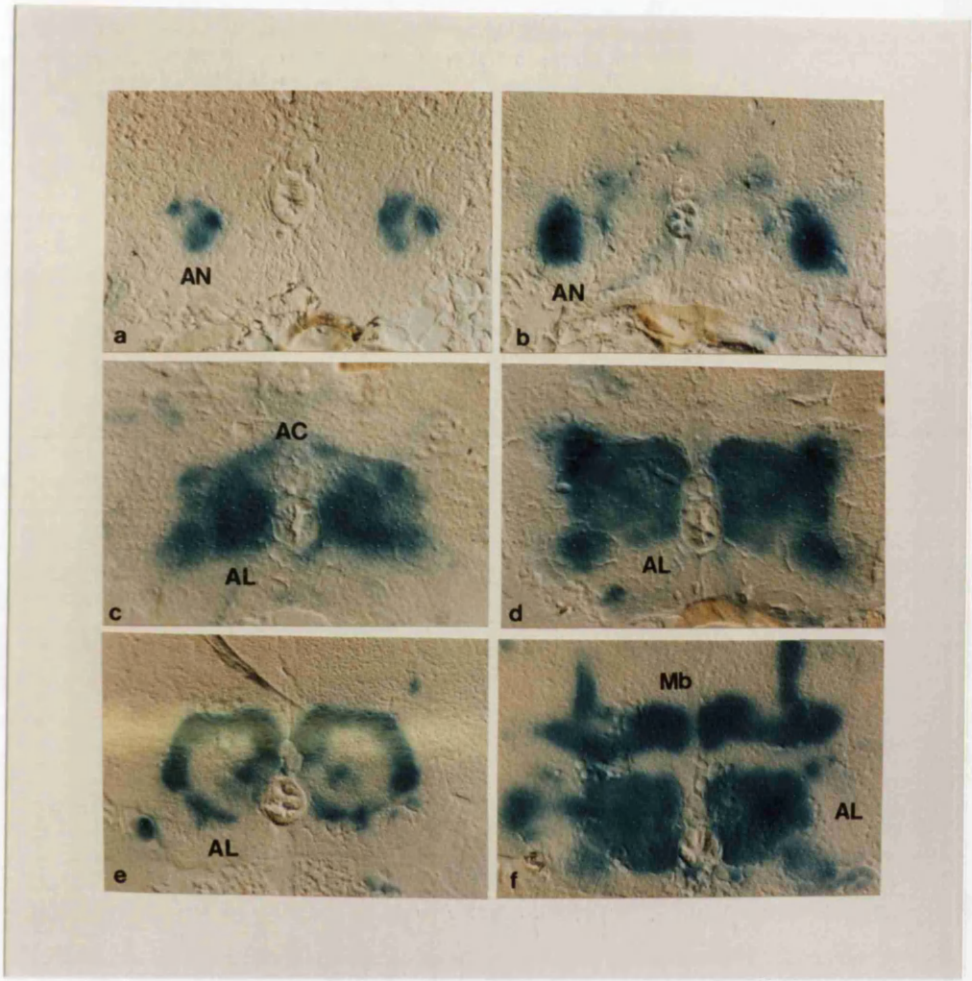


Fig. 3.6 Antennal complex staining

The ß-gal expression patterns in the antennal lobes (AL), the antennal nerves (AN) and the antennal commissure (AC), and the mushroom bodies (Mb). (a) line 253Y, (b) line c588, (c) line 59Y, (d) line c503a, (e) line c133, and (f) line c492b. For a description of these lines, see the text.

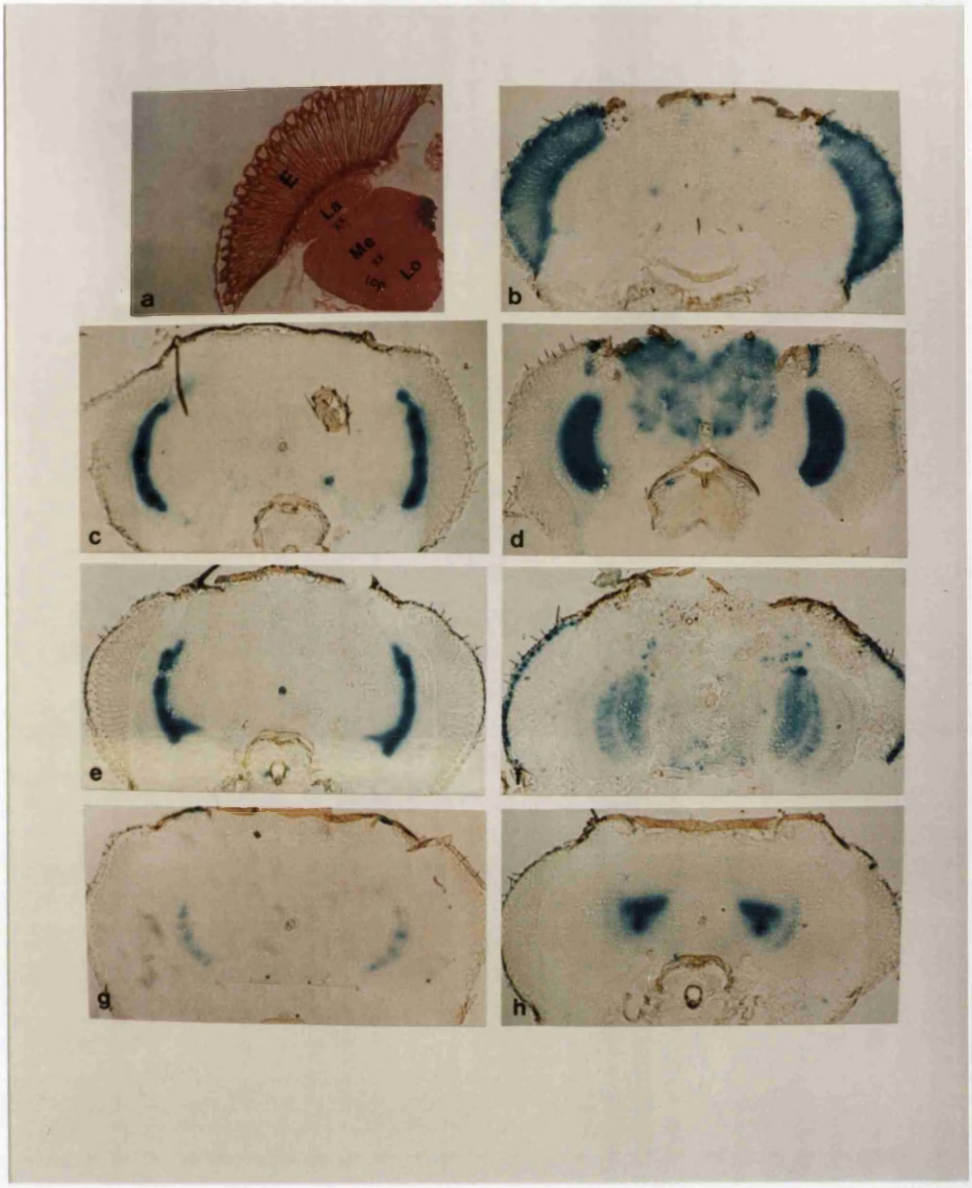


Fig. 3.7. The optic lobe and eye staining

(**a**) A semithin horizontal section (1.5µm) for the optic lobe and eye of a wild type fly (method see Tix and Fischbach, 1992). Eye (E), Lamina (La), the first optic chiasm (X1), medulla (Me), the second optic chiasm (X2), lobula complex (Lo) are marked. (**b-h**) showing the ß-gal expression patterns in the optic lobe and eye. (**b**) line 54Y showing the staining in the eyes, (**c**) line c340 showing lacZ expression in the glia cells between the retina and the lamina. Blue staining patterns expressing in (**d**) lamina (line c246), (**e**) the first optic chiasm (line c829), (**f**) medulla (line c577), (**g**) the second optic chiasm (line c637a) and (**h**) lobula complex (line c469).

3.4.5 Other Interesting Patterns

Apart from the staining patterns described as above, there are also other interesting patterns in the brain. Although some tracts and dots stained have not been identified, some examples are showed in Figure 3.8. Line 18Y has a staining pattern in the Great Commissure that connects both hemisphere of the protocerebrum and in line c215 a tract is stained within deuterocerebrum which is possibly related to the antennal lobe. Lines c518 and c600 show dots staining patterns in the brain that have not been identified yet. In the frontal sections, some dots are stained in the brain. These dots seem to be a part of tracts or neurons.

In a summary, GAL4-directed expression patterns are indeed a unique window for anatomy. They can be used to analyse the mushroom bodies (Yang *et al.*, 1995), the central complex (Armstrong *et al.*, submitted), antennal lobes (R. Stocker, personal comm.) and also to study brain developmental (S. Renn, personal comm.; JD. Armstrong, personal comm.). In addition, they have been used to study the structure-function roles in the *Drosophila* brain by GAL4-directed expression patterns (Ferveur *et al.*, 1995; Sweeney *et al.*, 1995; O'Dell *et al.*, 1995; deBelle, 1995)

More staining patterns of P[GAL4] lines have been described in "Flybrain", an on-line atlas and database of the *Drosophila* nervous system (Armstrong *et al.*, 1995). Flybrain can be accessed via the World Wide Web from servers in Glasgow (http://flybrain.gla.ac.uk).

3.5 Chromosomal Locations of P[GAL4] Insertion Lines

Drosophila are able to over-replicate their chromosomes in some tissues such as the salivary glands. This over replication enables the visualisation of the chromosomes, allowing the determination of the chromosomal location of the particular clone. As P[GAL4] element contains the pBluescript (pBS), its location can be determined by

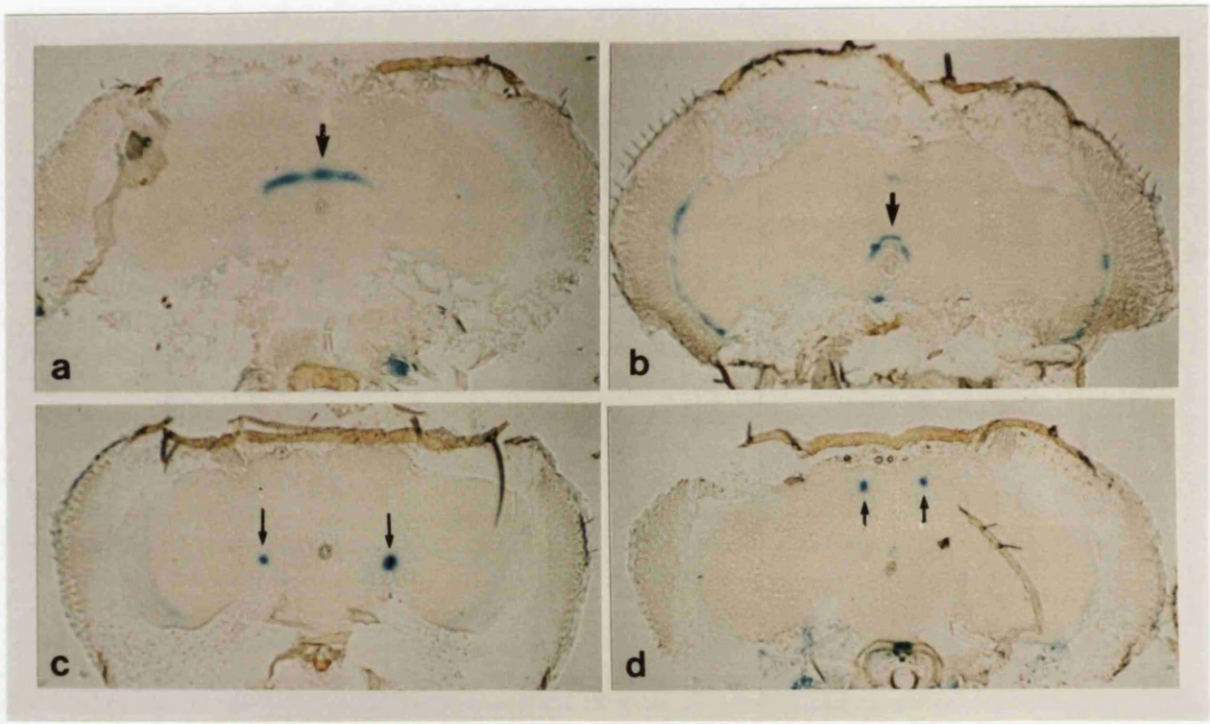


Figure 3.8 Other staining patterns

(**a**) Line 18Y showing a staining pattern in the Great Commissure. (**b**) Line c215 has a staining in a tract. (**c**) Line c518 and (**d**) line c600 showing the blue staining dots in the brain. More detail see text.

probing pBS to the polytene chromosomes of the salivary glands. The pBS probe is hybridised to the chromosome spreads and the signal detected as described in Section 2.3.3.

P[GAL4] insertions were localised by *in situ* hybridisation to polytene chromosomes for 70 lines showed interesting GAL4 expression patterns in the brain. Two examples are given in Figure 3.9. These insertions mapped to 63 distinct chromosomal locations. Cytological mapping of insertions is shown in Table 3.2.

Only one line (c492b) is showed to carry two independent insertions and these are on the same chromosome. The rest of lines contained a single P element insertion. Therefore, their simple or complex staining patterns result from the effects of genomic regulatory elements on a single P element. Certain lines which give very similar staining patterns have the same P element locations (e.g. line c105 and line c561). But only a few lines which give the different staining patterns have similar location (e.g. line c61 and line 277Y). This is because P[GAL4] was not inserted into the exact same position in the genome. The polytene chromosomal location does not provide precise information on the site. This problem can be solved by mapping the rescued flanking genomic DNA (see chapter 5). On the other hand, the different locations give different staining patterns in the brain although some similar patterns were observed. All the staining pattern is reproducible among individuals of the same insertion line. No apparent sexual dimorphisms are found.

Enhancer-trap elements are not mere anatomical markers. Besides revealing intriguing cellular arrangements within brain structures, each staining pattern implicates a flanking gene in its function. In Table 3.2, the possible genes related to P element insertions are listed and named as candidate genes. These genes are selected by virtue of their functions in the nervous system. From P element locations, we may predict that some P[GAL4] elements were inserted into or near to the interesting genes related to their functions. For example, in the case of line 43Y, P[GAL4] at the location 2C was

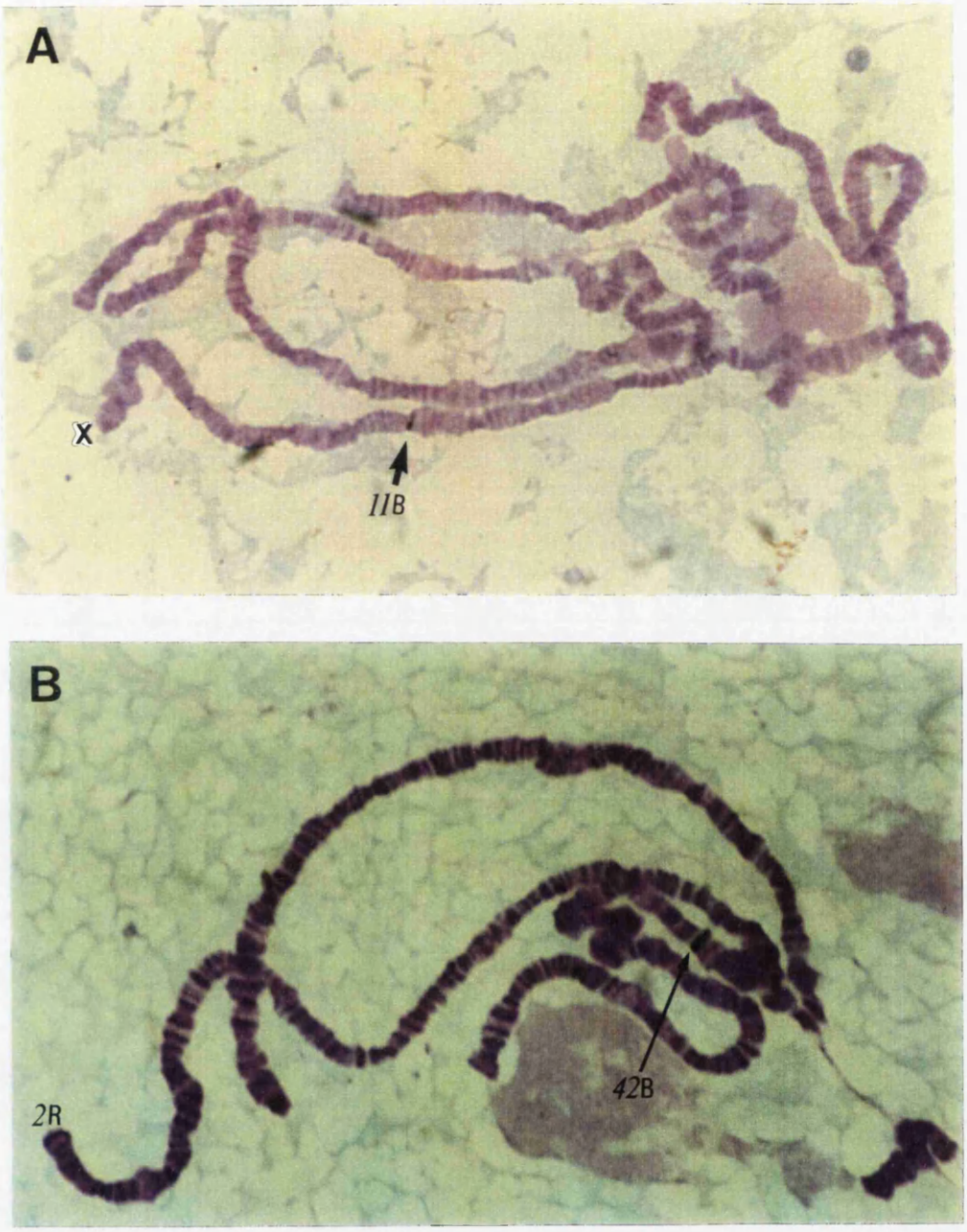


Figure 3.9 Polytene chromosomal *in situ* using a pBluescript probe.
(A) shows a set of polytene chromosomes displaying the chromosomal arms radiating from the chromocentre. Arrow indicates the position of the hybridisation signal for P[GAL4] insertion (line 277Y). (B) shows the P[GAL4] insertion in the polytene chromosome of line c831. Arrow indicates the position of the hybridisation signal.

supposed to insert into or near to the known genes named *usp* (ultraspiracle) or *Actn* (α-actinin). When sequencing the flanking DNA of this line, it proved that the P[GAL4] was indeed inserted between genes of "*usp*" and "*Actn*". When the corresponding antibodies were applied to determine the expression pattern of the protein in fly heads, "*Actn*" expression was largely restricted to the mushroom bodies in a manner very similar to that of enhancer trap expression in line 43Y. In other words, GAL4 expression pattern reflects the true expression pattern of nearby gene, in this case, "*Actn*" which could play a key role in regulating neuronal plasticity in the mushroom bodies (A. Mounsey, Glasgow, personal comm.)

Another line 189Y, P[GAL4] at the location of 24A, has a insertion in the gene named "foraging" which is related to locomotor activity of the central complex function (Varnam and Sokolowski, 1994; M. Sokolowski, personal comm.). Another example is provided by line c481 whose insertion is located at 18A. This region has a candidate gene called Neural Conserved at 18A (Nc18A), whose RNA *in situ* distribution mainly in the brain and thoracic ganglia (Perelygina *et al.*, 1992). The X-Gal staining in the brain has a similar distribution (data not shown).

A mutation can be caused by P[GAL4] itself, if it inserted into the gene and disrupt gene function. For example, line c161 is a recessive lethal mutation. P[GAL4] is inserted within an essential gene at 66A, leading homozygous lethal at 2nd instar larva stage. (D. Shepherd, Southampton, personal comm.). Line c549 exhibits the "*sn*" phenotype which gives severe defect in "bristle morphology" when homozygous. For this line, P[GAL4] was situated at chromosomal band of 7D1, the position of the *singed* bristle locus thought to be hot spot of P element insertion (Roiha *et al.*, 1988).

Over 1400 novel P[GAL4] enhancer trap lines were generated and screened for GAL4-directed ß-gal expression in cryostat sections of the *Drosophila* head. More than 100 display interesting patterns which are more or less restricted to specific regions or neuronal sub-populations of brain. They are unique markers for anatomy analysis and

developmental study. For most of these lines, the chromosomal locations of P[GAL4] insertions were identified by *in situ* hybridisation to polytene chromosomes. These insertion lines carrying a single P element are readily amenable to a genetic and molecular analysis (Wilson *et al.*, 1989, Bier *et al.*, 1989). The studies at the molecular level for some of these lines will be presented in Chapter 5 and 6.

Table 3.2 Cytological Locations of P[GAL4] Insertions

line	staining pattern	location (arm)	candidate genes
11Y	MB	71C (3L)	
30Y	MB	70E (3L)	*gnu*
43Y	MB	2C (X)	*usp, Actn*
46Y	MB	29E (2L)	
52Y	CC	69E (3L)	
59Y	AL	11E (X)	
64Y	CC	70B (3L)	
72Y	MB	21B (2L)	*mbm, plc21C*
78Y	CC	84D (3R)	*ids*
93Y	CC	11E (X)	
103Y	MB	2D (X)	*aperB*
104Y	CC	26D (2L)	
117Y	MB	34C (2L)	
121Y	MB	71B (3L)	
154Y	MB	61C (3L)	
181Y	MB	57B (2R)	
187Y	CC	97D (3R)	
188Y	CC	54D (2R)	*klp 54D*
189Y	CC	24A (2L)	*for, Pkg24A*
201Y	MB	56D (2R)	
203Y	MB	1C (X)	
210Y	CC	70B (3L)	
227Y	MB	1A (X)	*ewg*
238Y	MB	48C (2R)	
252Y	CC	42B (2R)	
253Y	AL	4D (X)	
259Y	CC	57F (2L)	
277Y	MB	11B (X)	
C5	CC	88B (3R)	
C35	MB	44A (2R)	*Myc*
C61	CC	1A (X)	*ewg*
C62	CC	48A (2R)	*inv*
C105	CC	12F (X)	*rut, ste*
C107	CC	19F (X)	*slgA, sol*
C115	MB	77A (3L)	*polo*
C123a	AL	68A (3L)	
C133	AL	11D (X)	*rad*
C161	CC	66A (3L)	*Tph*
C232	CC	100B(3R)	*chp*
C245	MB	82D (3R)	
C253	CC	49D (2R)	
C263	AL	34F (2L)	
C271	CC	70C (3L)	*Gl*
C302	MB	18C (X)	
C401	CC	39E (2L)	
C481	CC	18A (X)	*Nc18A*
C486	CC	1A (X)	*ewg*
C492b	AL	49C/30B (2R)	
C502	MB	3A (X)	*hypo A*

C502b	MB	12C (X)	*rdgB*
C503a	AL	95F (3R)	*Esm*
C507	CC	100B(3R)	*chp*
C522	CC	64D (3L)	*Klp64D*
C532	MB	47E (2R)	
C547	CC	61B (3L)	
C561	CC	12F (X)	*rut, ste*
C681	AL	45A (2R)	*pk45C*
C737	MB	49D (2R)	
C739	MB	40A (2L)	
C742	AL	8C (X)	
C747	MB	42A (2R)	*EcR*
C753	CC	78A (3R)	
C758	MB	42A (2R)	*EcR*
C761	AL	93D (3R)	
C767	CC	86D (3R)	
C772	MB	42A (2R)	*neuro*
C819	CC	93C (3R)	*gustM*
C831	MB	42B (2R)	
C855a	CC	79E (3L)	*Csp*
C549	CC	7D (X)	*sn*

MB = Mushroom bodies, CC = Central complex, AL = Antennal lobes.

X = X-chromosome. 2L = the left arm of the second chromosome, 2R = the right arm of the second chromosome. 3L = the left arm of the third chromosome, 3R = The right arm of the third chromosome.

Chapter 4

Characterisation of GAL4-directed Expression Patterns in the Central Complex

This chapter will further detail GAL4-directed ß-gal expression patterns in the different substructures of the central complex of adult brain. Then, the chapter will describe the central complex expression patterns at different developmental stages.

4.1 Introduction

As mentioned above, the central complex (CC) refers to a series of intimately related neuropils in the midbrain. Even though different terminology is used to describe them, the CC has a similar structure and location in all insects (Williams, 1975; Strausfeld, 1976; Mobbs, 1982; Homberg, 1985; Hanesch *et al.*, 1989).

In *Drosophila* the CC is located in the centre of the brain (Fig. 1.6) and receives projections from both hemispheres (Power, 1924; Hanesch *et al.*, 1989). Using silver staining, Hanesch *et al.* (1989) revealed the general neuropil architecture of the CC. A schematic diagram of the *Drosophila* central complex is given in Figure 4.1. It includes four substructures: the protocerebral bridge (*pb*), the fan-shaped body (*fb*), the ellipsoid body (*eb*) and the paired noduli (*no*). In addition, there are two accessory structures: the ventral bodies (*vbo*) and the lateral triangles (*ltr*).

By Golgi staining, about 30 single neurons of the CC were observed (Hanesch *et al.*, 1989). They were classified as either small-field or large-field elements. The small-field neurons connect different substructures or regions within a single substructure. The majority of these neurons are intrinsic to the CC. In contrast to the small-field elements, the large-field neurons (e.g. Ring neurons and Fan-shaped neurons) reside only in a single substructure and link it to one or two central brain regions outside the CC. These neurons are thought to be the main input to the central complex.

The CC is the only unpaired neuropil in the central area of the insect brain. Therefore, its function is probably related to an integration of information from the right and left halves of the brain (Homberg, 1987). This notion was supported by early

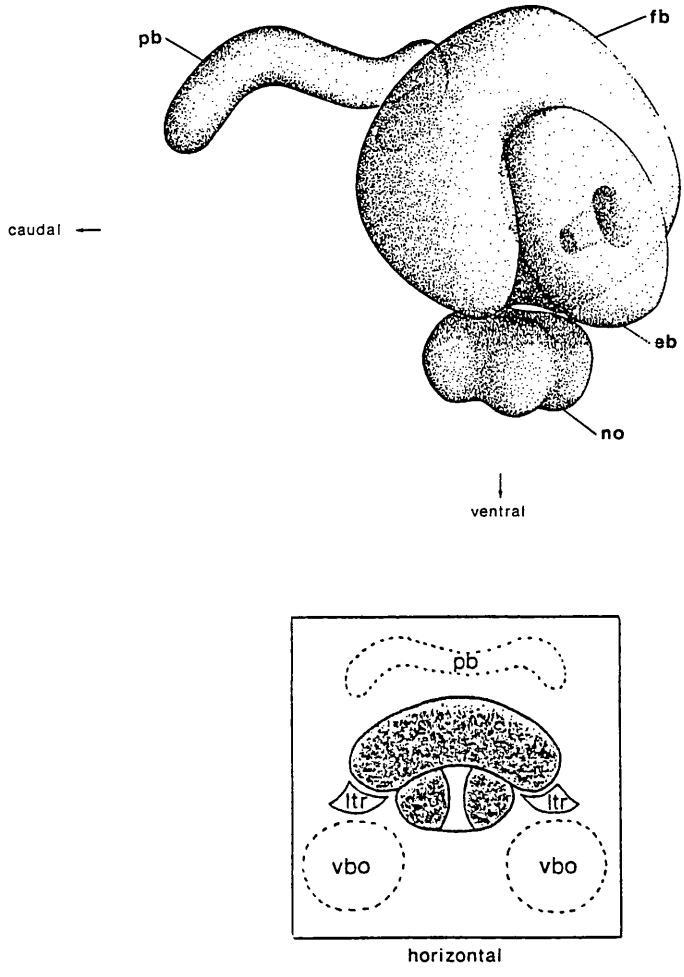


Figure 4.1 Schematic diagram of the *Drosophila* central complex (taken from Hanesch *et al.*, 1989). The four substructures: the protocerebral bridge (pb), the fan-shaped body (fb), the ellipsoid body (eb) and the paired noduli (no). Two accessory structures: the ventral bodies(vbo) and the lateral triangles (ltr).

experimental evidence from surgery and electrical stimulation. In these experiments, stridulation, walking, escape, respiration, and feeding behaviour were affected. Both inhibitory and excitatory influences, depending on the position of the electrode, were observed (Homberg, 1987, for a review). Recently more experiments in *Drosophila* have demonstrated the similar conclusion that the central complex is a control centre for behavioural activity. Mutants with defects in the central complex anatomy show a variety of behavioural impairments such as locomotor (Strauss and Heisenberg 1993) and learning (Bouhouche *et al.*, 1993).

In the Heisenberg laboratory, they have identified and characterised structural mutants of eight independent genes with behavioural phenotypes. The apparent specificity of the structural phenotypes and experimental results derived from the use of mosaics, point to the central complex as the major brain centre for motor control. All mutants show slow initiating activity, slow walking, and disturbed leg coordination during turns and start-stop manoeuvres (Strauss and Heisenberg 1993). One of the mutants, *no-bridge* (*nob*) showed frequent spasmodic attacks and withdrawal from sensory stimulation, the protocerebral bridge apparently being the only affected component of the central complex. In a walking test, consisting of tracking the fly's path back and forth between two stripes (Buridan's test), the mutant flies tested perform quite abnormally in terms of trajectory and speed (Strauss *et al.*, 1992).

In addition, most of mutant flies learn poorly in olfactory and visual discrimination tasks, and they show complex abnormalities in visual flight control (Heisenberg *et al.*, 1985; Bouhouche *et al.*, 1993; Ilius *et al.*, 1994; Bausenwein *et al.*, 1994). These data further support earlier notions that the central complex is involved in the initiation and organisation of behaviour and that it integrates visual data of the two brain hemispheres.

The behavioural complexity implies a more complex neuronal organisation and function. To study the relationship between structure and function, it is important to find the specific expression patterns in the substructure, or subdivision, of the central complex.

Then, further manipulation can be carried out, such as specific cell marking, targeted gene expression and gene cloning.

Among a large of collection of P[GAL4] lines, certain lines have shown the staining patterns more or less restricted to the substructure of the central complex (Table 3.1). Here more details will be described.

4.2 Specific Staining Patterns in the Ellipsoid Bodies

The large-field Ring neurones innervate the ellipsoid body (*eb*). The ringlike arborizations fill in the *eb* structure. Figure 4.2 shows the staining patterns in the ring neurones (R-neurones) of the *eb* and in the lateral triangles, presumably input region to ring neurones. The cell body clusters of ring neurones are located at the rostral cellular cortex and the axons run in a prominent fibre tract, the lateral ellipsoid body tract, to the ipsi-lateral triangles. Then it extends further towards the midline and ends in the *eb*.

Three types of ring neurones (R-neurones, ExR1-neurones and ExR2-neurones) can be distinguished based on their cell body locations and their arborizations according to Hanesch *et al.* (1989). Among them, R-neurones were the most abundant and can be subdivided into four types of ring structures. R1-R3 arborize from the *eb* canal outward and R4 arborizes from the periphery of the ring inward. The branches of R1 terminate within the inner region of the *eb*. R2 and R4 -neurones innervate the outer ring of the *eb* and R3-neuron branch in the inner and outer ring. ExR1 has a large cell body in the pars intercerebralis, and spiny arborizations in the *eb*, the dorsal *fb,* the *vbo*, and other parts of the medial protocerebrum ipsilateral to the cell body. ExR2 has its branches in the median protocerebrum and most likely in the *vbo*.

On the basis of our observation, the ellipsoid body ring structure can be divided into four layers. They are inner-ring (**A**), inner mid-ring (**B**), outer mid-ring (**C**), and outer-ring (**D**) (Fig. 4.3a). The different ring neurones arborize into different parts of ring structures.

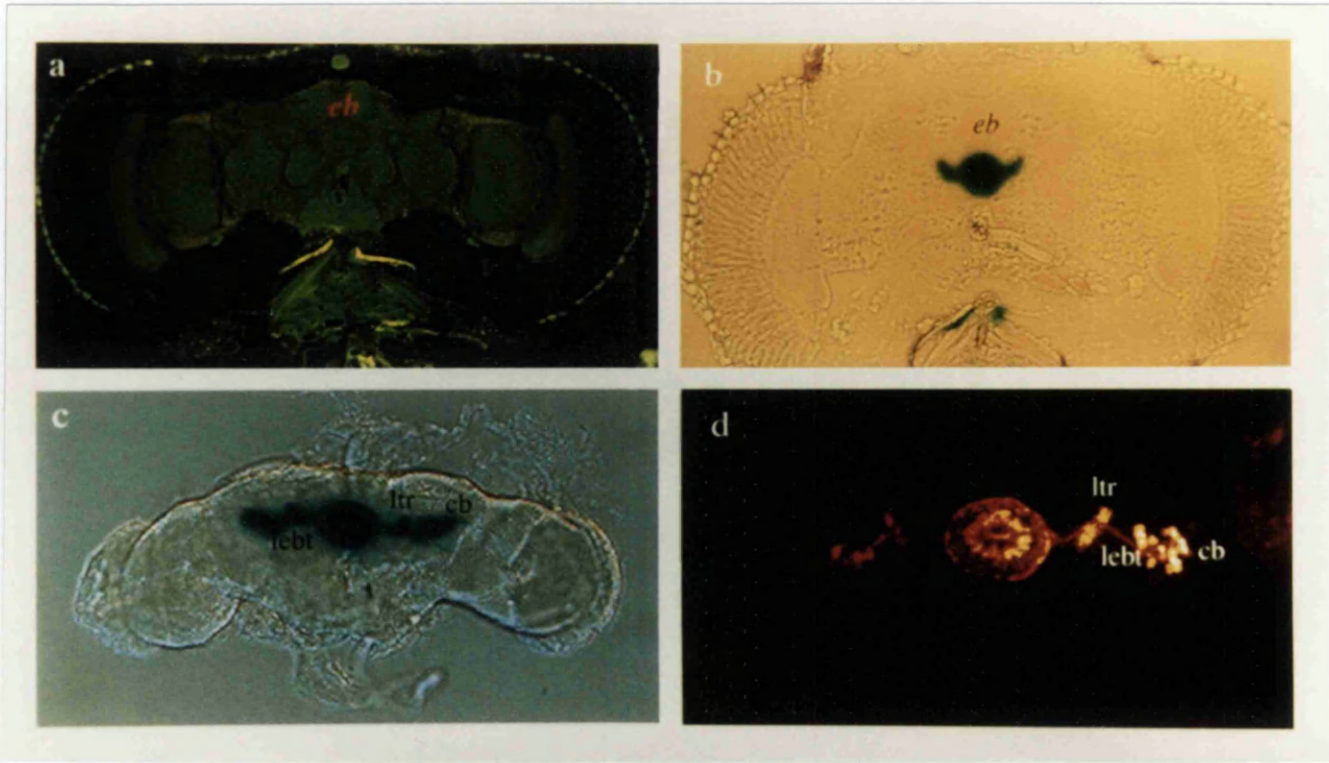


Figure 4.2 The staining patterns in the ellipsoid body (*eb*).

(a) shows a paraffin section from a wild type fly. (b) shows a cryostat section of line c507 stained by X-Gal. (c) shows the whole mount staining by X-Gal for the same line. (d) shows a confocal image stained by the anti-ß-gal antibody for the same line (provided by JD. Armstrong). The ellipsoid body (***eb***), cell bodies (**cb**) of the *eb*, the lateral ellipsoid body tract (**lebt**) and the lateral triangles (**ltr**) are marked.

In the preparations, the staining patterns can be seen in different layers of the *eb* ring structure. As seen in Fig. 4.3b-l, the blue staining in line 198Y occurs in layer A and line 189Y has a dark blue pattern in A region and light staining in B layer. But in line c105, GAL4-directed expressed only in the B ring layer of the *eb*. These expression patterns correspond to R1 neurones described by Hanesch *et al.* (1989). In this type of line, besides cell bodies having their branches in the *eb*, they also send a small branch with bleblike terminals into the *vbo* area in median protocerebrum.

In addition, line 64Y showed staining in all A, B and C layers of the *eb*. And line 78Y showed a strong staining in A and B regions. Line c547 has a strong staining in C layer and a light blue staining in B and D layers. These patterns correspond to R2 and R3 neurons.

Lines c93, c819 and c42 showed the specific staining in outer ring layer of the *eb*. However line c42 has a slight difference in staining density because less cells were stained. These patterns match for R2 or R4 neurones described by Hanesch *et al.*, (1989).

Moreover, extensive staining in the whole ring structure was found in different lines. For example, line c107 showed the strong blue pattern in the layer D and light staining in the rest of layers. But line c232, the staining pattern was seen in all ring structure including the centre of ring, in which A, B and D layers had a strong staining and C layer had a relative weaker staining. Comparing these types of lines with other eb lines, these lines have more cell bodies stained. In case of c232, about 30 cells can be seen in each hemisphere.

Using an enhancer trap approach, we can subdivide the *eb* ring structure into four different layers rather than two rings (Hanesch *et al.*, 1989).By comparing the classification of ring neurones from Hanesch *et al* (1989) and ring structures from us, it is obvious that there are some overlapping each other (Table 4.1). Our results, combined with theirs, can provide more detailed substructures of the *eb*. Furthermore, staining patterns by ß-gal

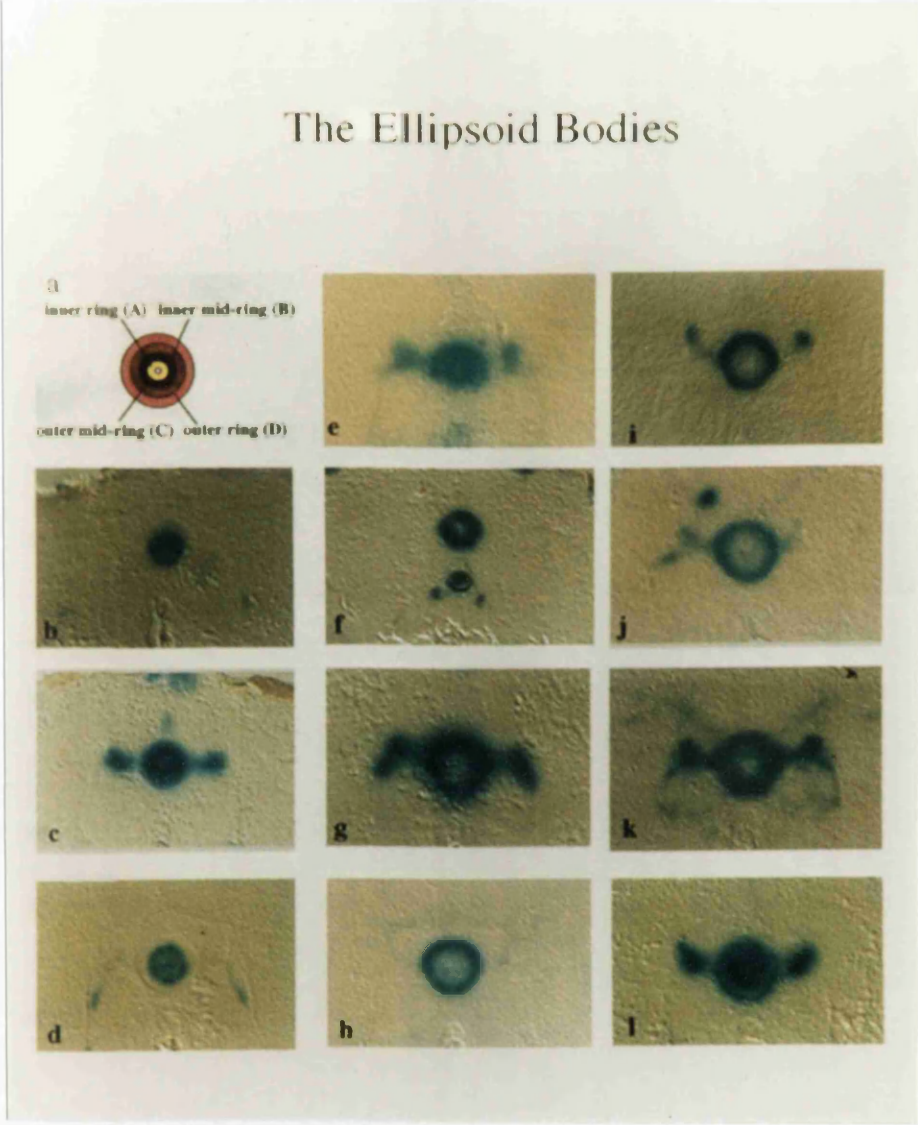


Figure 4.3 GAL4-directed staining patterns in the subdivision of the ellipsoid body (*eb*). (a) Schematic diagram for 4 layers *eb* ring structure: the inner-ring (**A**), inner mid-ring (**B**), outer mid-ring (**C**), and outer-ring (**D**). (b) Line 198Y showing the staining in the layer A. (c) 189Y showing the staining in the layers A and B. (d) c105 showing the staining in B layer. (e) 64Y showing the staining in the layers A, B and C. (f) 78Y showing the staining in the layers A and B. (g) Line c547 showing the staining in the layers B to D. (h-j) Lines c93, c819 and c42 showing the staining in the layer D. (k) Line c107 showing the strong staining in the layer D and faint staining in other layers. (l) Line c232 showing the staining in all the layers of the *eb*. For full description, see text .

expression reveal genetically specified subdivision of the ring structures. They also suggest that there is a functional difference between the R-neurons as they express different genes.

Table 4.1 Comparison of the ring structures and ring neurones of the *eb*

line	The layer of the ring structure	R-neurones Hanesch *et al.*, (1989)
198Y	A	R1+R3
189Y	A+B	R1+R3
c105	B	R1
64Y	A+B+C	R1+R2+R3
78Y	A+B	R1+R3
c547	B+C+D	R2+R3
c93	D	R2+R4
c819	D	R2+R4
c42	D	R2 or R4
c107	All layers	All R-neurones
c232	All layers	All R-neurones

The ellipsoid body seems to be specific in dipterans. It is a perfectly round "doughnut with hole" shape. In other insects such as *Schistocerca gregaria* its homologue is a half circular structure (Williams, 1975). In the ellipsoid body mutants of *Drosophila* , the *eb* is opened up ventrally to varying degrees and may appear as a flat glomerulus (Strauss and Heisenberg, 1993). GAL4-directed staining patterns selectively expressed in the different ring structures provide further insight into the neuroarchitecture of the *eb*. Different ring structure of the *eb* may have different functional roles. The main role of the *eb* is thought to be inhibitory control of behaviour due to most of the ring neurones showing dense GABA immunocytochemical staining which is known as an inhibitory neurotransmitter (Hanesch *et al.* 1989; Bausenwein *et al.* 1994). However, octopamine immunoreactivity in the *eb* was observed recently (Monastirioti *et al.* 1995). This neurotransmitter has excitatory modulatory actions in some muscles e.g. locust flight muscle (Malamud *et al.* 1988). It is likely that this biogenic amine has an excitatory role in the *eb*. Therefore, we presume that different ring structure or ring neurones could give rise

to the different integrative function of the *eb*. Targeted toxin ablation of different ring neurones may eventually help to elucidate their role in behaviour.

4.3 Expression Patterns in the Fan-shaped Bodies

The fan-shaped body (*fb*) is the largest substructure in the central complex. The fb is saucer-shaped with the convex side pointing dorso-posteriorly (Fig. 4.1). It is a regular structure of horizontal layers and vertical segments. Its long axis is perpendicular to the plane of symmetry of the brain. The *fb* can be divided into six roughly horizontal layers. They run parallel to each other and are the consequence of stratification of large-field neurones. Along the transverse axis the *fb* is divided into eight segments according to the regular arrangement of bundles of medium-sized fibre from the protocerebral bridge (Hanesch *et al.*, 1989).

In the collection of P[GAL4] lines, a number of expression patterns representing a substantial fraction of its cellular components in the *fb* were found. Some type of neurones can be attributed to certain horizontal layers or vertical segments of the *fb* (Fig 4.4a-h). Line c5 only showed staining pattern in the first layer of the *fb*. Line 121Y showed a strong staining in the first two layers corresponding to "superior arch" described by Strausfeld (1976). In line 210Y, a strong staining pattern was found in the 1st, 2nd and the 6th layers of the *fb*. In addition, line c522 showed the staining in the 4th layer of the *fb* and 13Y has the staining pattern in 5th layer region. Line c61 showed the staining in the lowest division of the fan-shaped body. On the other hand, a number of lines have staining patterns in different segments longitudinally. Such a line 188Y showed the dense staining in both outer segments and pale staining in the rest segments longitudinally. And line 34Y showed the blue staining only in 3 and 4 segments.

Above *lacZ* expression patterns in the *fb* are consistent with the *fb* neuronal architecture described by Hanesch *et al.* (1989).

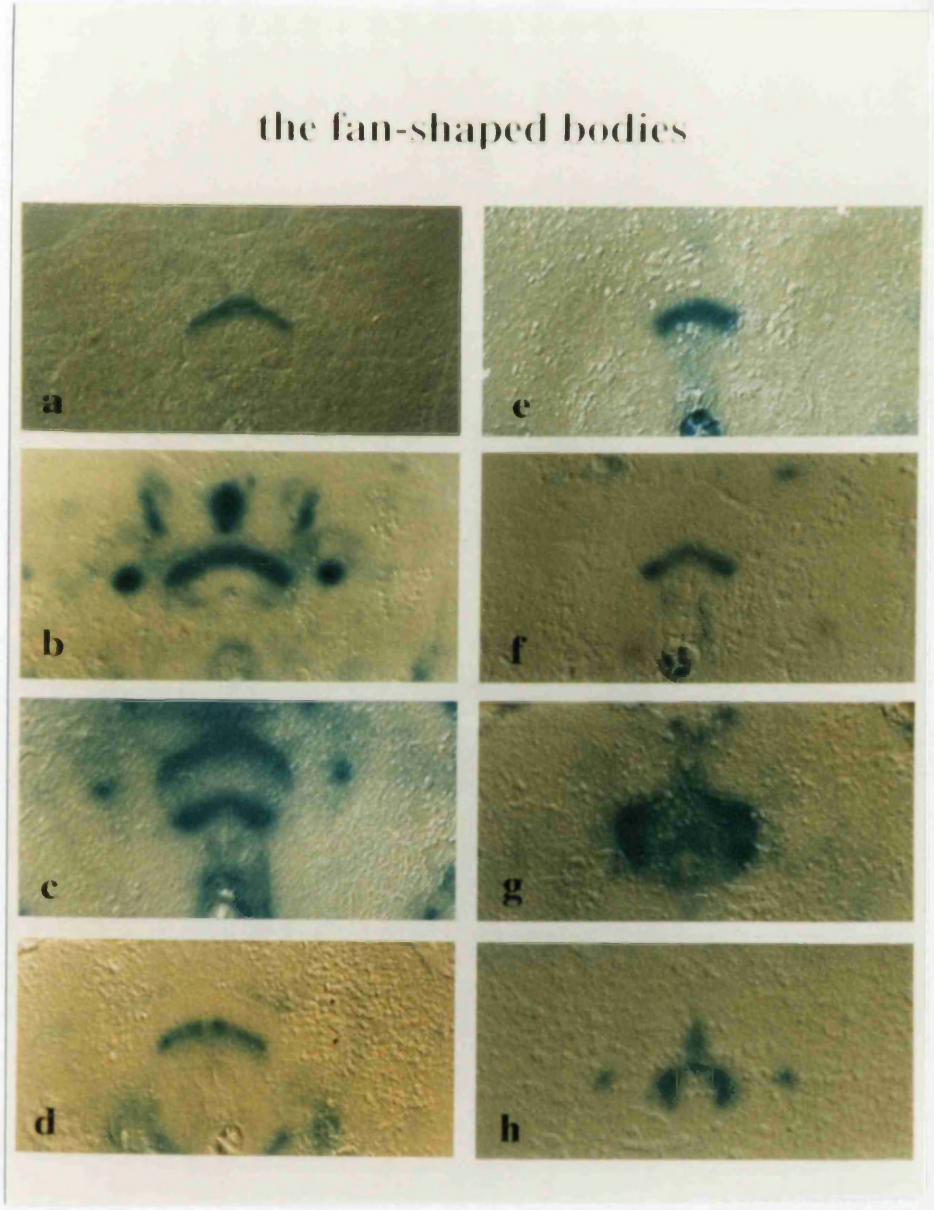


Figure 4.4 *lacZ* expression in the fan-shaped bodies (*fb*).
Staining patterns were observed in different layers of the *fb*. in (a) line c5, (b) line 121Y, (c) line 210Y, (d) line c522, (e) line 13Y, and (f) line c61. Staining patterns were observed in different segments of the *eb* in (g) line 188Y and (h) line 34Y. See text for full description.

4.4. Expression Patterns in Other Substructures of the Central Complex

A pair of noduli lie ventrally to the *fb*. These are roughly spherical glomeruli each segmented into two subunits along the anteroposterior axis (Fig. 4.1). As seen in Figure 4.5 (a-c), line c767 showed a strong staining in the paired noduli and the lower division of *fb*. The linking fibres (Hanesch *et al.* 1989) from the *fb* and the contralateral noduli were also observed. In line c252, upper parts of the noduli have a faint staining compared to lower parts which have a strong blue staining. Line 78Y also stains in the noduli.

The protocerebral bridge is composed of 16 glomeruli in a row, 8 on each side of the midline. It looks like the handlebar of a bicycle. Figure 4.5d shows the protocerebral bridge of wild type fly using autofluorescent (Heisenberg and Boehl, 1979). In P[GAL4] lines, 78Y expressed the ß-gal staining in all rows of the protocerebral bridge (Fig. 4.5e) and c161 showed staining in the protocerebral bridge which was restricted to some of the glomeruli (Fig. 4.5f).

The two ventral bodies were thought to be the main output areas from the CC (Hanesch *et al.*, 1989). But we have not found any specific staining pattern in these substructure of the CC so far.

GAL4-directed expression was observed in the fan-shaped body, noduli and the protocerebral bridge and their connecting fibres. These regions were thought to be the excitatory control centre in the fly because acetylcholine, an excitatory neurotransmitter, was found in these substructures (Buchner *et al.*, 1986).

The confocal examination of expression patterns in the central complex revealed that some types of neurones indeed show morphological specialisation which had not been noted previously (JD. Armstrong, Glasgow, personal comm.) It will be helpful for us to interpret the behavioural complexity.

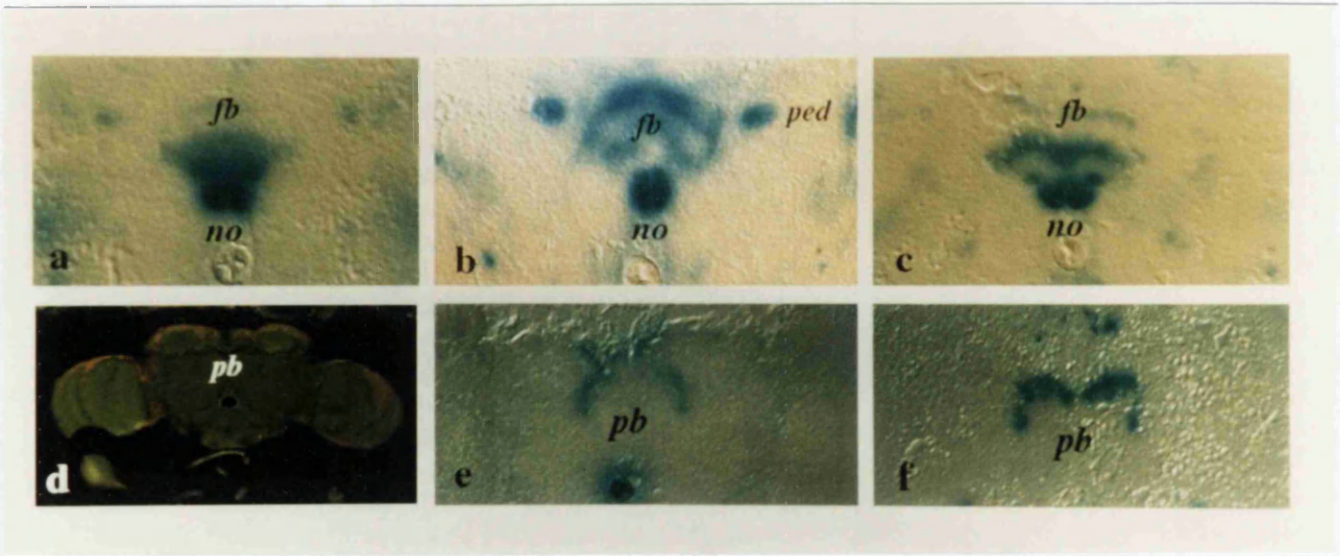


Figure 4.5 Expression patterns in other substructures of the central complex

(a) Line c767 showing strong staining in the paired noduli (***no***) and the lower division of the ***fb***. (b) Line c252 showing the staining in the lower parts of the ***no*** and a faint staining in upper parts. The pedunculus (***ped***) of the mushroom body are also marked. (c) Line 78Y showing the staining in the paired ***no*** and the lower division of the ***fb***. (d) showing the protocerebral bridge (***pb***) in the wild type fly. (e) and (f) showing the staining in the protocerebral bridge in lines 78Y and c161.

4.5 Developmental Study of the Central Complex

The central complex of the *Drosophila* brain appears late in development (Hanesch *et al.*, 1989). At the third instar larvae stage, only few cell bodies were observed in some of lines. For most of lines, the *lacZ* expression began at early pupal stages and their levels varied during metamorphosis. Based on the whole-mount *lacZ* expression at different developmental stages, the blue staining pattern in the ellipsoid body and the fan-shaped body will be described in detail.

***eb* line, c232:** (Fig 4.6)

Embryo stage: No staining except in salivary glands was found. In P[GAL4] lines, it seems to be quite a common feature that the salivary glands were stained. Brand and Perrimon (1993) examined 220 P[GAL4] lines and found about 80% of lines had staining in the salivary glands. This suggests that in constructing the GAL4 vector, a position-dependent salivary gland enhancer was generated.

Larva stage: No staining is seen in the brain at the 1st instar. At the 2nd and 3rd instar, one pair of large cell bodies are stained in the pars intercerebralis of the brain. Their axonal branches extended into the ventral ganglion can be seen faintly. There are lots of cell bodies stained in the ventral ganglion.

During metamorphosis: Cell bodies of the *eb* are stained at about 30 hr after puparium formation (APF) and inner ring pattern (pale staining) can be seen at 41 hr APF. Whole ring patterns and the lateral triangles can be seen at 45 hr. The number of cell bodies increases onwards to about 25 in each hemispheres at 48 hr. After that, the pattern of the *eb* is almost same as that in adult although it is still growing and enlarging.

Adult: The staining only in the *eb* and in the lateral triangles can be seen. It has a very strong staining in inner ring and strong staining in outer ring. About 30 cells can be counted in each hemisphere.

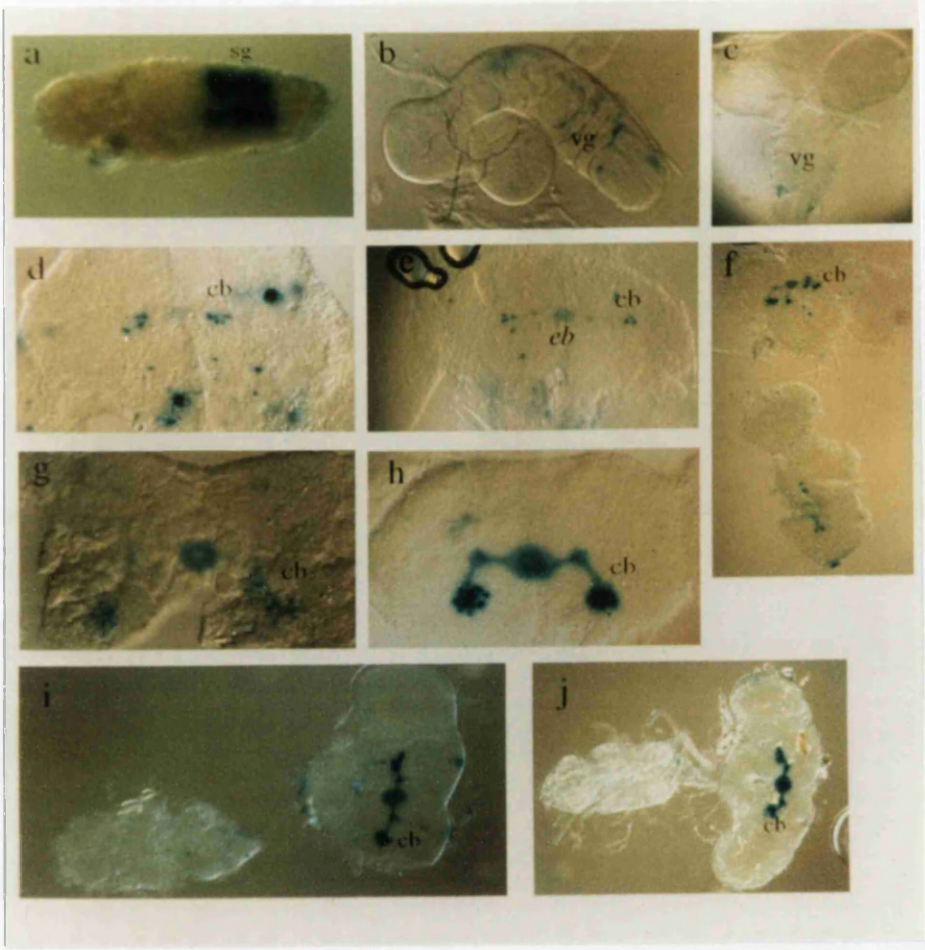


Figure 4.6 *lacZ* expression in the *eb* line c232 at the different developmental stages (a) showing staining in salivary glands of the embryo (**sg**). (b) showing *lacZ* expression in the central brain and the ventral ganglion (**vg**) of the second instar larva, (c) showing *lacZ* expression in the central brain and the **vg** of the third instar larva, (d) showing the cell bodies (**cb**) of the *eb* ring neurons at about 30 hr after puparium formation (APF), (e) showing the ***eb*** ring neurons structure appeared at 41 hr APF, (f) at 45 hr APF. (g) at 48 hr APF, (h) 66 hr APF, (i) 70 hr APF and (j) adult. See text for full description.

On the other hand, the staining of the cell bodies in ventral ganglion begins at 2nd instar larvae. At 3rd instar, most staining disappeared, staining is restricted to ventral ganglion (T3 region) and abdominal neuromeres region. This staining gradually disappeared during metamorphosis. It possibly reflects an activity of relevant gene.

***eb* line, c561a:** (Fig 4.7)

The staining in embryo is the same as line c232. From the 1st instar larvae onwards, more cells are stained than those seen in line c232, which do not belong to the *eb* cell. The *eb* pattern can be seen at about 43 hr APF. Up to adult, about 6-8 cell bodies can be counted in each hemispheres. In the ventral ganglion, discrete clusters staining can be seen. Interestingly, the staining pattern of a motor neuron in T1 region of thoracic ganglion lasts from 40 hr APF up to adult. This is probably related to locomotor activity.

***fb* line, c5:** (Fig 4.8)

At the third instar larvae, staining is found in the brain but it is not sure that these cell bodies are a component of the *fb*. On the other hand, an extensive staining in ventral ganglion can be seen. At about 16 hr APF few cell bodies can be seen which is likely to be cell bodies of the fan-shaped body. More other cell bodies are stained during after 24 hr. At about 80 hr APF, the staining in the fan-shaped body can be clearly seen. This staining continues into adulthood. With respect to the ventral CNS, the staining is found from the larvae onwards through adulthood although some of the staining disappears later.

In *Drosophila*, metamorphosis is accompanied by the degeneration of most larval tissues and the construction of adult structures from imaginal discs and clusters of imaginal precursor cells. However, in the central nervous system (CNS), most neurones of the larvae do not die, but rather they persist through metamorphosis and join with groups of new adult-specific neurones to form the CNS of the adult. (Truman *et al.*, 1993). In other words, the neurones of the adult consist of "old neurones" from larvae and "new adult-specific" neurones formed during metamorphosis. Based on the observation of the *lacZ* expression

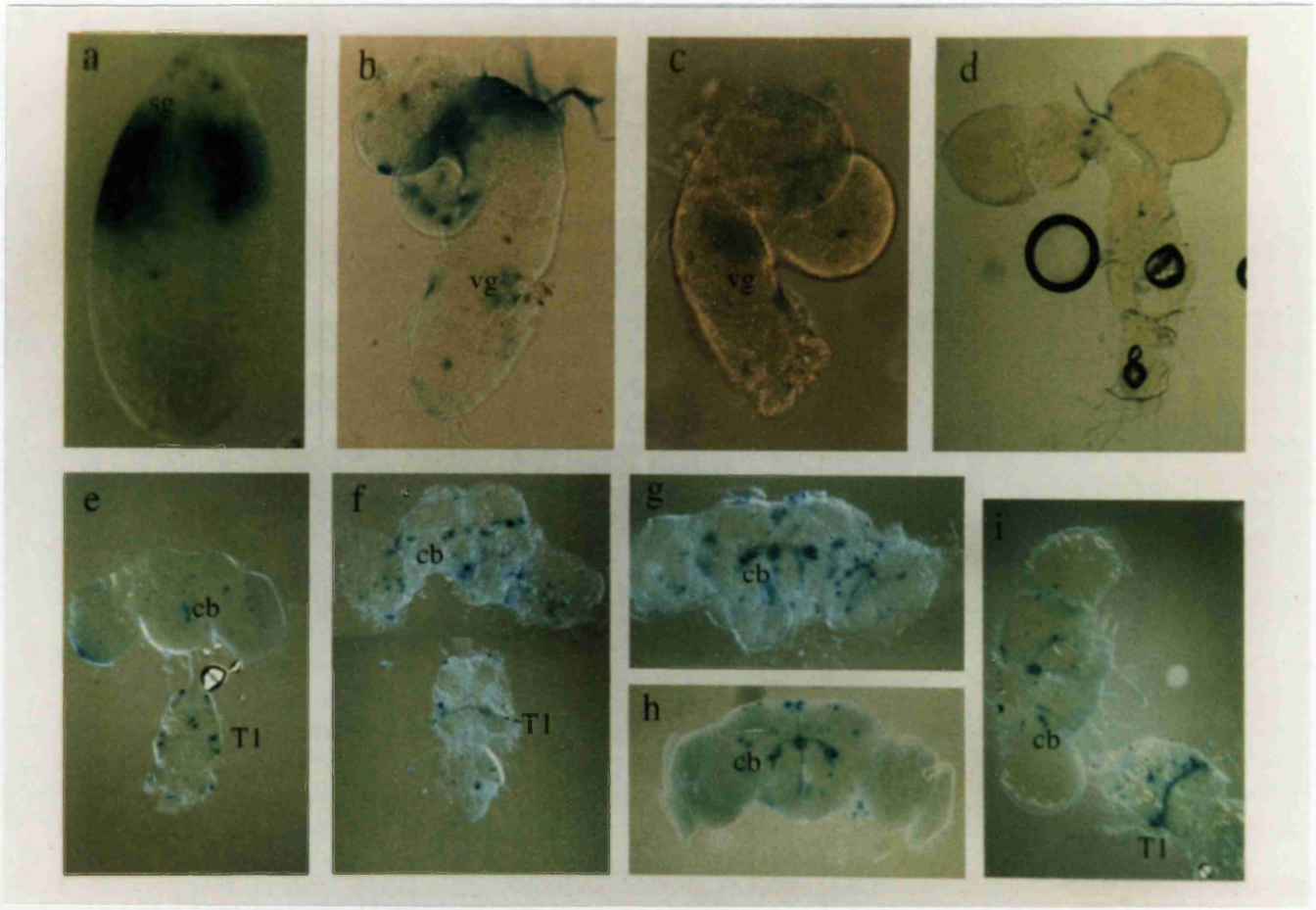


Figure 4.7 *LacZ* expression in the *eb* line c561a at the different developmental stages.
(a) showing staining in salivary glands of the embryo. (b) showing the staining in the central brain and the ventral ganglion (**vg**) of the first instar larva, (c) showing the staining in the central brain and the **vg** of the second instar larva, (d) showing the staining in the central brain and the **vg** at 8 hr APF. (e) showing the staining in the cell bodies (**cb**) of the *eb* ring neurons at about 40 hr APF. A motor neuron in the T1 region of the thoracic ganglion is marked. (f) showing the *eb* staining at 43 hr APF, (g) at 60 hr APF, (h) at 74 hr APF and (i) in the adult. See text for full description.

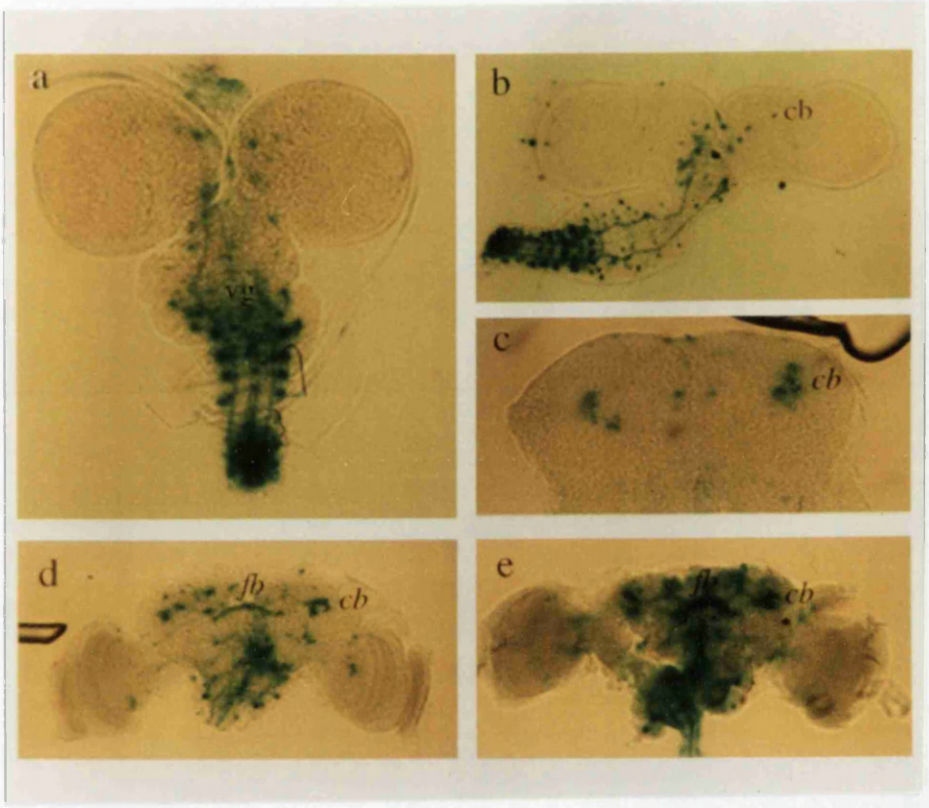


Figure 4.8 *lacZ* expression of the *fb* line c5 at the different developmental stages (a) showing the staining in the central brain and the ventral ganglion of the third instar larva. (b) showing stained cell body (***cb***) of the fan-shaped body (*fb*) at 24 hr APF. (c) showing the staining in the part of the ***fb*** at 66 hr APF. (d) and (e) showing the staining in the part of the ***fb*** and other regions in the brain at 80 hr APF and in the adult.

patterns at different developmental stages, it is suggested that the neurones of the central complex belong to new adult-specific neurones which appear during metamorphosis.

We observed that the *lacZ* expression started at early pupal stages in the *eb* and the *fb* lines. However, there is a possibility that some cells in the central complex may pre-exist but *lacZ* has not be activated until a later stage due to the delay of GAL4-directed ß-gal expression by the two-step promoter interaction (Ito *et al.* 1995). The staining of antibody raised against GAL4 protein can solve this problem when the antibodies become available in *Drosophila.*

It is interesting that all the central complex lines have expression patterns not only in the substructures of the central complex but also in motor neurones of ventral or thoracic ganglion. It seems to be that these two classes of neurones have some degree of connection functionally. These staining patterns may provide more evidence for the notion that the central complex is related to locomotor control.

Using the P[GAL4] system, we observed that specific expression patterns in the CC of the *Drosophila* brain. Furthermore, the *eb* can be divided into four different ring structures genetically by P[GAL4] expression patterns. Developmental analysis indicates that the *lacZ* expression in the central complex lines begins at early pupal stages although there is a possibility that some cellular components of the CC already exist at earlier stage. As discussed before, P[GAL4] lines are not only used for anatomical analyse, but are also used for the cloning of flanking genes. In the next two chapters, I will present the results of analysis of the central complex lines at the molecular level.

Chapter 5

Plasmid Rescue of the Flanking Genomic DNA

5.1 Introduction

5.2 GAL4-directed expression patterns of the central complex lines for plasmid rescue

5.3 Plasmid rescue of flanking genomic DNA of the central complex lines

5.4 "Reverse Northern" analysis

5.5 Isolation of genomic DNA clones corresponding to lines c507 and c161

This chapter describes the plasmid rescue experiments for the central complex lines. Then, "reverse Northern" data and genomic clones of some lines are presented.

5.1 Introduction

Understanding the molecular and genetic control of brain function remains one of the most challenging problems of modern biology. Although a number of brain structural and functional mutants have been isolated, their number and diversity is small compared to the complexity of the *Drosophila* nervous system and is also small compared with the large number of transcription units expressed in the brain (Flybase, 1994).

In *Drosophila*, several strategies may be used to identify genes involved in brain structure and function. The classical approach has been to utilise mutagenic screens to identify genes whose mutant phenotype results in perturbation of the developmental pathway being studied. This approach has been used successfully to identify genes involved in brain structure and function (Delaney *et al.*, 1991; Miyamoto *et al.*, 1995). The pioneering work of Heisenberg and Böhl (1979) used the mass histological technique, following chemical mutagenesis, to isolate structural brain mutants. There are more than 40 mutant strains with structural defects in the mushroom bodies, the central complex and the optic lobes (Fichbach and Heisenberg, 1981; Heisenberg *et al.*, 1985). Subsequently, Delaney *et al.*, (1991) cloned one of structural brain genes, the small optic lobes (*sol*). They found that two major transcripts were produced from this locus and present throughout the entire life cycle. Miyamoto *et al.*, (1995) recently reported they have characterised the mushroom body deranged (*mbd*) locus. A putative *mbd* transcriptional unit, which is transcribed at various developmental stages, has been identified and isolated as a cDNA clone. However, with the classical mutagenesis screen, it is easy to miss some mutations with subtle phenotypes. So screens based on mutant phenotype alone may lead to an underestimation of the number of genes required for brain development.

On the other hand, due to the availability of molecular techniques a large number of novel genes relevant to brain function have been cloned in *Drosophila* by virtue of their sequence conservation to already known genes or on the basis of an interesting expression pattern.

One method for cloning genes is by screening with a mammalian probe. This technique requires the use of a variety of methods of homology screening. It makes the assumption that structural features of the functionally defined locus e.g. DNA or amino acid sequence are sufficiently conserved between *Drosophila* and mammals. It also makes the assumption that should a motif be conserved at the polypeptide level, it will perform the same function in the target organism. By homology screening a wide variety of genes have been successfully cloned. These include transmitter related genes such as acetylcholine receptor, *ard*, (Hermanns-bergmeyer *et al.*, 1989); second messenger genes such cAMP dependent protein kinase (PK-A) (Foster *et al.*, 1988), and genes involved in physiological functions including Na^+/K^+ATPase α-subunit (Lebovitz *et al.*, 1989). Another similar method involves a screen for gene expression patterns using panels of monoclonal antibodies raised against specific parts or whole of the organism (Fujita *et al.*, 1982). These screens based on homology, however, have their own limitations. These include the nature of the DNA or the protein probes used and the fact that these depend, *a priori*, on functional conservation of the genes in question. In addition, most genes that are cloned are generally not amenable to immediate genetic analysis.

An alternative way is to use 'Reverse Genetic' approaches such as differential screening or enhancer-trapping to isolate genes expressed in a particular tissues.

Differential (+/-) hybridisation screening is a method that allows the isolation of genes based solely on their pattern of expression. The technique is able to detect messages that comprise of as low as 0.1% of the mRNA population (Sambrook *et al.*, 1989). It has

been used successfully in various systems to identify tissue or stage-specific transcription units including those involved in the nervous system. For example, head-specific genes such as *nina*E (Levy *et al.*, 1982) and Na^+/K^+ATPase β-subunit (Tomlinson *et al.*, submit). This technique relies on generating two different mRNA populations, one of which is enriched in the sequences of interest, the other, in which these transcripts are reduced or lacking. A differential screening approach can be used to identify genes whose transcription is tissue-specific or is highly regulated in a spatial or temporal fashion. It does not however, facilitate the detection of transcripts which show differential splicing.

A powerful approach to identify new genes has been the use of the technique of enhancer trapping, pioneered in *Drosophila* by O'Kane and Gehring in 1987. A major advantage of this technique is its ability to detect genes which do not give an obvious morphological defect when mutated but are required for normal development. Enhancer-trapping has been used to identify transcription units which are expressed in a particular tissue of interest, in this case, identifying genes that are expressed in the brain. The details of enhancer trapping has been discussed in Chapter One.

So, in the case of a head/body differential screen, when one gets genes whose transcription is tissue-specific, it would still be necessary to perform *in situ* hybridisation to head sections to see which, if any, had restricted expression patterns. The enhancer-trapping technology, by comparison, gives us immediate access to tissue-specific patterns which represent gene expression patterns. Even these may not be 'gene' patterns, rather they may just represent a fraction of the gene total expression pattern (i.e. just the enhancer). It is still useful for expressing something under GAL4 control to manipulate specific cells. So, the enhancer-trapping technique is indeed a novel tool for identification and developmental characterisation of *Drosophila* genes.

As discussed before, in the enhancer trap system, the P[GAL4] element contains a bacterial origin of replication (*ori*) and the amp^R gene conferring resistance to the ampicillin (see Fig. 1.3). Therefore, it provides the simplest and quickest way for cloning of adjacent genomic DNA and then of adjacent genes.

One of the aims of this project is to identify and clone the genes relevant to the structure-functional in *Drosophila* brain by enhancer trap approach. When the interesting expression patterns become available, cloning of genes will be pursued.

5.2 GAL4-directed Expression Patterns of the Central Complex Lines for Plasmid Rescue

Seven P[GAL4] enhancer trap lines, which specifically stained in the substructures of central complex, were selected for plasmid rescue. Examples of the patterns revealed by X-Gal staining in frontal sections of the adult brain are shown in Figure 5.1. Line c561a and c105 appear to have the same staining pattern in the inner ring region of the ellipsoid body. In addition, these two lines have the same chromosomal locations of the P[GAL4] insertion. Line c819 has a blue staining almostly exclusively in the outer ring neurons of the ellipsoid body. Line c232 and c507 have the same chromosomal locations of the P[GAL4] and the same staining patterns in almost all neurons of the ellipsoid body. In addition, all lines also showed the blue staining in the lateral triangles, a presumed input region to the ring neurons (Hanesch *et al.*, 1989).

Staining in line c61 is restricted to the lower part of the fan-shaped body. However, line c161 showed staining in all the substructures of the central complex, e.g. in the protocerebral bridge, the fan-shaped body, the ellipsoid body and the noduli. This insert is homozygous lethal at the second instar larvae (D. Shepherd, personal comm.) and thus P[GAL4] is probably inserted in an essential gene. More detail concerning the

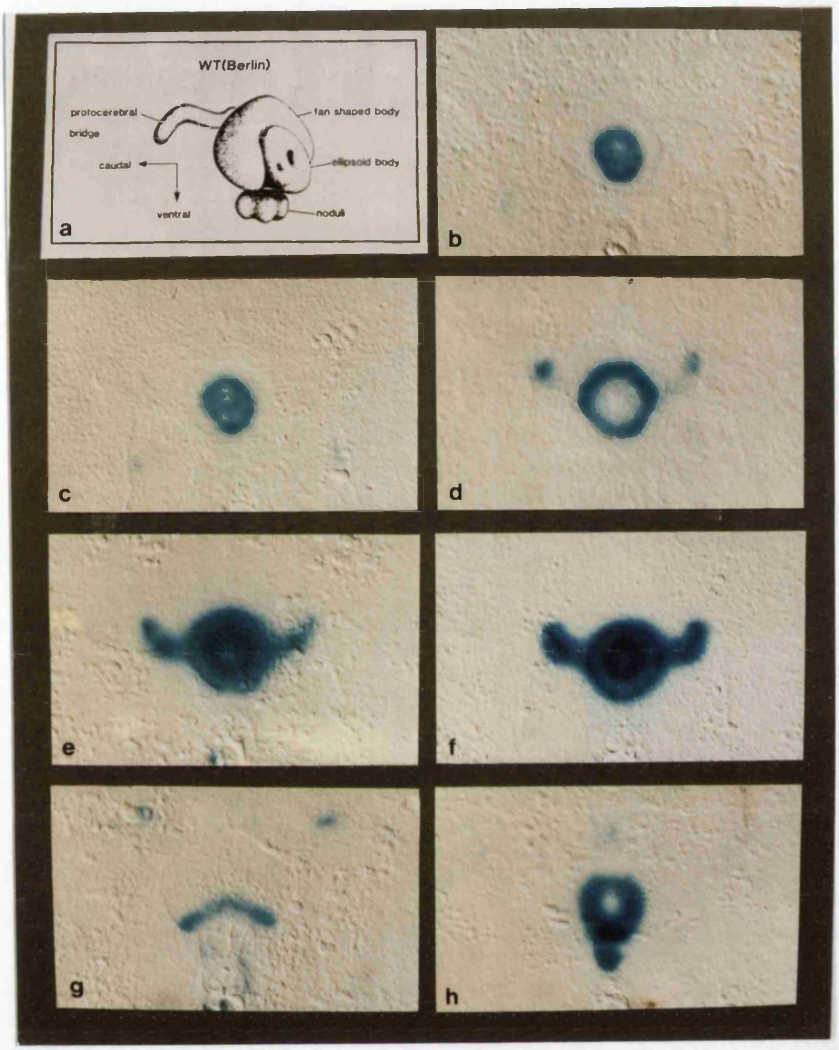


Figure 5.1. GAL4-directed ß-gal expression patterns of the central complex lines used for plasmid rescue. Each panel is a 12µm frontal cryostat section. (**a**) A schematic diagram of the *Drosophila* central complex (taken from Strauß and Heisenberg, 1993). The four substructures are the protocerebral bridge, the fan-shaped body, the ellipsoid body and the paired noduli. (**b**) line c561a and (**c**) line c105 show the same staining patterns in the inner ring region of the ellipsoid body. (**d**) line c819 showing blue staining in the outer ring neurons of the ellipsoid body. (**e**) c232 and (**f**) line c507 showing staining patterns in almost all neuronal sub-type of the ellipsoid body. (**g**) line c61 exhibits staining in the lower part of the fan-shaped body. (**h**) line c161 showing staining in all the substructures of the central complex (in this section only the ellipsoid body and parts of noduli can be seen. Other substructures of this line were described in Figure 3.5).

the characterisation of P[GAL4] expression patterns in the central complex can be seen in Chapter 4.

5.3 Plasmid Rescue of Flanking Genomic DNA of the Central Complex Lines

First of all, the P[GAL4] element construct is drawn in Figure 5.2. on the basis of the plwB (Wilson *et al.*, 1989) and the pGawB (Brand and Perrimon, 1993) vectors. As can be seen in this diagram, whole P[GAL4] construct contains a number of sites, e.g. *Pst* I, in polylinker 3 which are not found 3' of this polylinker. Therefore, these sites can be used for plasmid rescue experiments (described as section 2.4.6.) to clone genomic sequence downstream of the P element. Similarly, polylinker 4 also contains unique sites, e.g. *Kpn* I, which can be used to subclone sequences upstream of the P[GAL4] element.

To clone genomic sequence downstream of the P[GAL4] element, genomic DNAs from seven P[GAL4] lines were digested with *Pst* I that cut in PL3 and other sites of genomic DNA. This produced many fragments including one that contained the *ori* and amp^R sequences and adjacent genomic sequences extending to the next *Pst* I site. After dilution and ligation, transformation of competent *E.coli* cells was performed. In this experiment, the electro-transformation technique (described as section 2.4.4.1) was used because of its high efficiency. By selection on Amp-plates, only the cells containing the Bluescript can replicate and confer ampicillin resistance. Therefore, the surviving colonies carry a plasmid which contained genomic sequences directly adjacent to P[GAL4] element. These rescued plasmids were named pPC507, pPC819, etc.

In order to cut out the vector and isolate the genomic sequences flanking the insertion site, double digestion with *Pst* I and *Bam* HI was performed to produce a fragment containing just the 3' P-element sequence (vector) and all of the adjacent cloned genomic

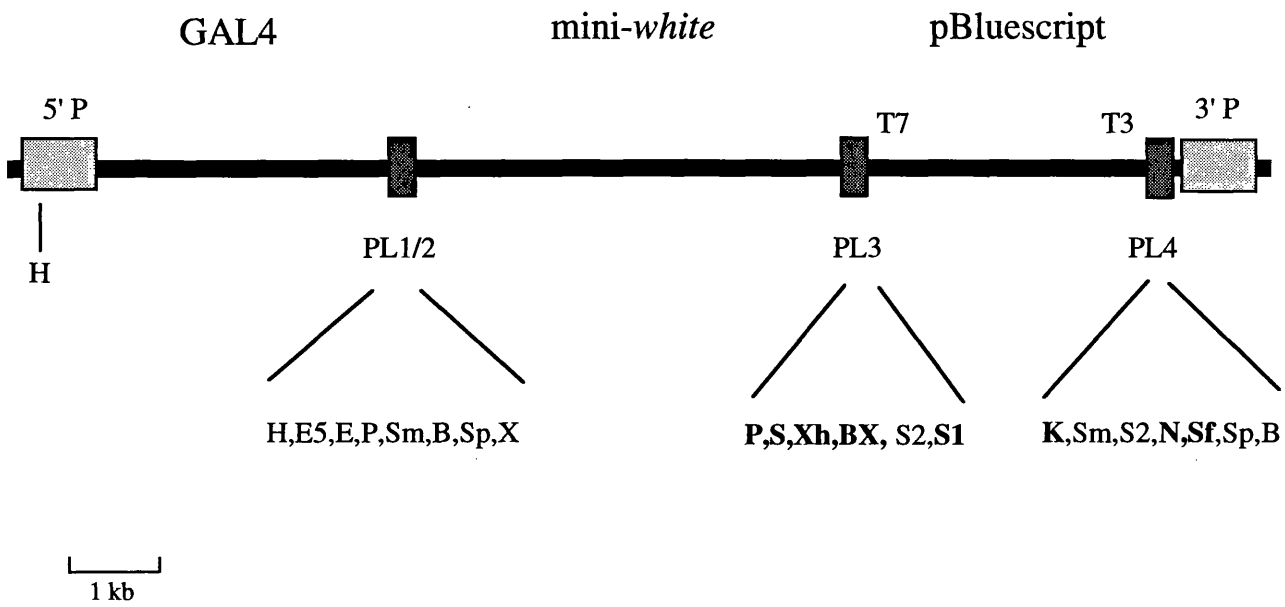


Figure 5.2 Map of P[GAL4] construct, based on the plwB (Wilson *et al.*, 1989) and pGawB (Brand and Perrimon, 1993) vectors.

5' (3') P= the 5' (3') end of the construct. T7 (T3)= T7 (T3) direction of pBluescript. PL= polylinker. B= *Bam*HI, BX=*Bst*XI, E=*Eco*RI, E5=*Eco*R V,H=*Hin*dIII, K=*Kpn*I, N=*Not*I, P=*Pst*I, S=*Sal*I, S2=*Sac*II, Sf=*Sfl*I, Sm=*Sma*I, Sp=*Spe*I, X=*Xba*I, Xh=*Xho*I. Restriction sites in bold are unique.

DNA. Of seven P[GAL4] lines, the size of rescued plasmids and genomic DNA fragments were summarised in Table 5.1. The longest genomic DNA fragment was 4.6 kb from pPC507. The shortest one was 0.3 kb from pPC819. From line c105 and c561a, rescued fragments of the same size were obtained.

Genomic DNA fragments generated from such digests of plasmid DNA were isolated on a gel and labelled as a probe for further analysis, such as Southern and Northern blots and the screening of genomic and cDNA libraries.

In order to check that each of the rescued plasmids was identical in size to the expected fragment, all the isolated plasmids downstream of the P[GAL4] element were analysed by the genomic Southern blots. Genomic DNAs from seven P[GAL4] lines and corresponding plasmid DNAs from rescue experiment were run on the agarose gel side by side. Before loading the gel, DNAs were adjusted to approximately equivalent amounts. After they were transferred onto Hybond N membrane, they were hybridised with the pBluescript probe. As seen in Figure 5.3, the hybridisation bands of the genomic DNA were same sizes as those in resulting plasmids. This indicated that these rescued plasmids were as expected. Moreover, the genomic Southern showed only one band in each P[GAL4] line, indicting only one insertion in each line and also confirming the chromosomal *in situ* hybridisation data.

On the other hand, it is possible that genes related to enhancer trap element were located at upstream of the P[GAL4] element. Therefore, *Kpn* I was used to digestion for cloning genomic sequence upstream of the P[GAL4] element. The results can be seen in Table 5.1. Plasmid rescue from the 5' end in five lines were successful. These rescued plasmids were named as pKC507, pKC161, etc. The genomic sequences adjacent to the 5' end of the P[GAL4] insertion can be obtained by digestion of above plasmids with *Kpn* I and *Hin*dIII. For the other two lines genomic clones flanking insertion were not obtained with

line	c61	c105	c161	c232	c507	c561a	c819
staining pattern	lower fan-shaped body	inner ring ellipsoid body	all central complex	whole the ring ellipsoid body	whole the ring ellipsoid body	inner ring ellipsoid body	outer ring ellipsoid body
chromosomal location	1A	12F	66A	100B	100B	12F	93B
name of 3' plasmid	pPC61	pPC105	pPC161	pPC232	pPC507	pPC561a	pPC819
size of 3' plasmid	4.8 kb	4.3 kb	4.3 kb	3.8 kb	7.6 kb	4.3 kb	3.3 kb
size of genomic fragments (3')	1.8 kb	1.3 kb	1.3 kb	0.8 kb	4.6 kb	1.3 kb	0.3 kb
name of 5' plasmid	pKC61	pKC105	pKC161		pKC507	pKC561a	
size of of 5' plasmid	14.3 kb	25.9 kb	14.5 kb		18.2 kb	25.9 kb	
size of genomic fragments (5')	3.3 kb	14.9 kb	3.5 kb		7.2kb	14.9 kb	

Table 5.1. The result of plasmid rescue from seven P[GAL4] enhancer trap lines

The size of 3' plasmids includes pBluescript and the adjacent genomic fragment. The size of 5' plasmids includes pBluescript, mini-*white*, GAL4 and the adjacent genomic fragments. The genomic fragments from (3') direction were obtained by digestion with *Pst* I and *Bam* HI. The genomic fragments from (5') direction were obtained by digestion with *Kpn* I and *Hind* III.

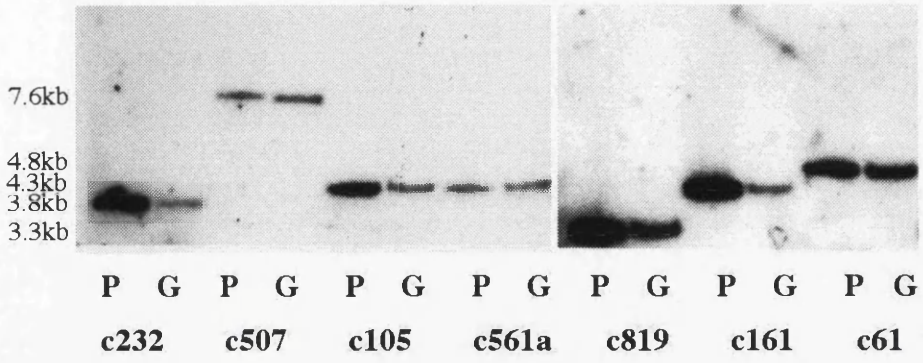


Figure 5.3 Genomic Southern analysis of rescued plasmids.

Of seven different GAL4 lines, genomic DNAs (**G**) and rescued plasmids (**P**) from 3' ends were digested with *Pst*I and run on gel side by side. They were hybridised by pBluscript probe.

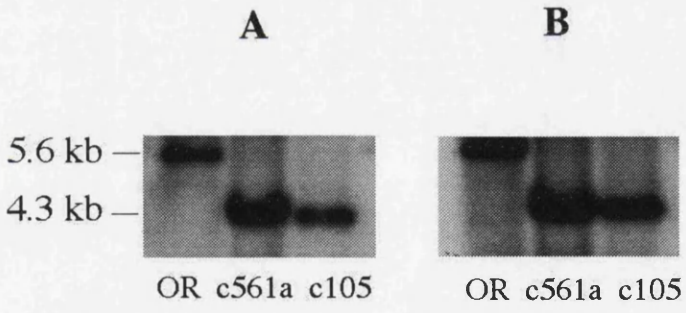


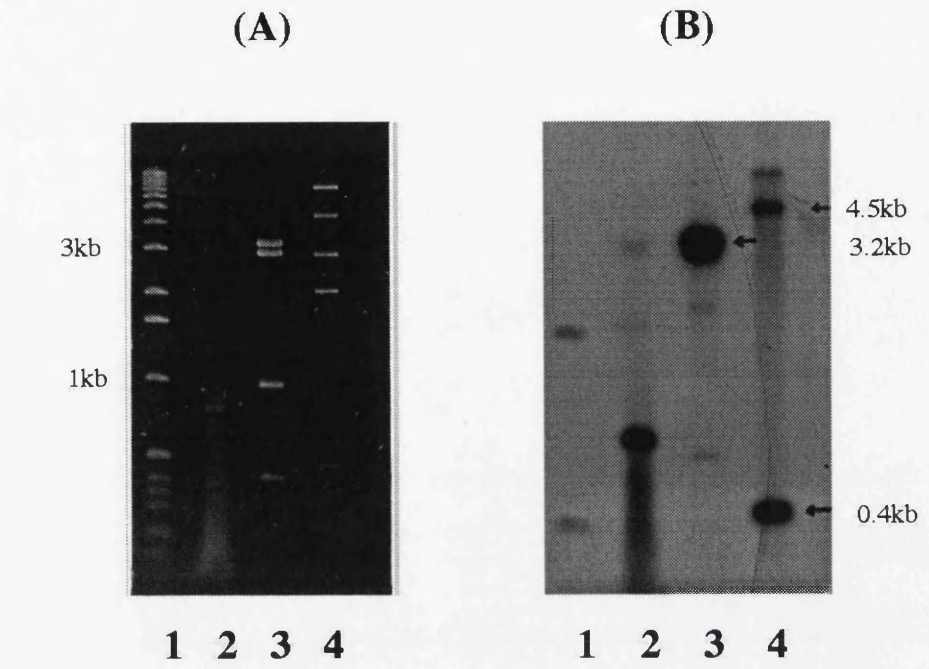
Figure 5.4 Genomic Southern analysis showing the relationship between lines c105 and line c561a.

The genomic DNA from wild type (OR) flies and from P[GAL4] line c561a and c105 were digested with *Pst*I and run on duplicate gels. Two filters from duplicate gels were probed with the insert from pPC561a (**A**) and pPC105(**B**) respectively.

*Kpn*I enzyme. It seems likely that this is due to an unusual distribution of enzyme sites near the insert. Perhaps the *Kpn* I site is too far away from the insertion site. Even though corresponding larger plasmids were obtained, it was difficult to achieve transformation due to low transformation efficiency.

As can be seen in Table 5.1, line c105 and c561a give rescued plasmids of the same size, in addition to having at the same chromosomal locations and with the same blue staining patterns. In order to check that these rescued plasmids were identical, Southern blots were employed. Duplicate genomic DNA blots were prepared from wild type flies and P[GAL4] line c105 and line c561a and probed with both rescued fragments from the 3' ends. The result showed they had an identical hybridisation signals and did cross-hybridise with each other (Fig. 5.4). Thus it proved that these two lines not only had same position of P[GAL4] insertion, but also had same captured fragments.

Interestingly, line c232 and c507 give a same chromosomal location and similar blue staining patterns, but they had different sized rescued fragments from the 3' end. In order to examine further the relationship between those two lines, Southern analysis was performed. In the case of line c507, three genomic fragments (3.3, 0.9 and 0.45 kb) from the 3' end (pPC507) were released by a *Pst* I/*Bam*HI double digest. And three genomic fragments (0.4, 4.5 and 2.2 kb) from the 5' end (pKC507) were obtained by a double digest with *Kpn* I and *Hin*d III (Fig. 5.5a). When they were probed with a rescued genomic fragment from line c232, hybridisation signals can be seen in bands of 3.3 kb from the 3' direction, and 0.4 kb and 4.5 kb from the 5' direction (Fig. 5.5b). From this information and the sequencing data surrounding two P elements (data not shown), the map of relationship between line c232 and c507 was drawn in Figure 5.5c. Two P[GAL4] elements were inserted into the *Drosophila* genome only 46 bp away from each other but in different orientation. Their expression patterns were probably activated by the same enhancer. This also implied that the enhancer could act throughout the entire length of the P[GAL4] element. On the other hand, it is noted



(C)

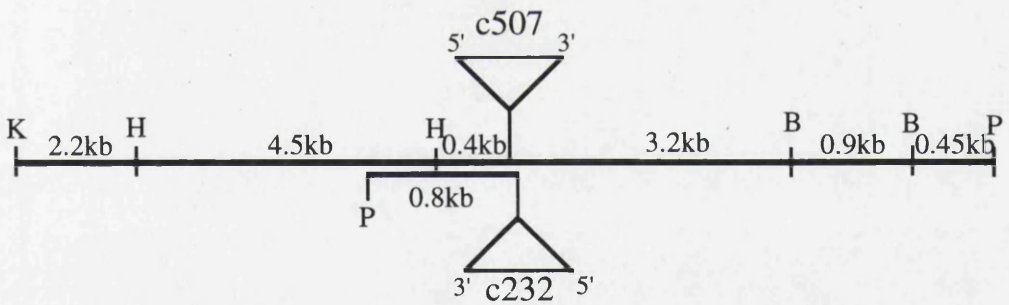


Figure 5.5. The relationship between the line c232 and c507.
(**A**) shows an ethidium bromides stained gel prior to blotting. In track 1, the 1kb ladder is a size marker from Gibco-BRL. In track 2 a genomic fragment released by a *Pst*I/*Bam*HI double digest from pPC232. In track 3, genomic fragments released by a *Pst*I/*Bam*HI double digest from pPC507; The track 4 shows the genomic fragments digested by *Kpn*I/*Hin*dIII from pKC507. (**B**) shows the blot hybridised with a probe from a whole insert of pPC232. (**C**) shows the restriction map of the genomic region encompassing the P[GAL4] insertion of line c507 & c232. Restriction sites shown are **B**-*Bam*HI, **P**-*Pst*I, **K**-*Kpn*I, **H**-*Hin*dIII. See text for more details.

that although the polytene chromosomal location looks like same, the precise genomic site of P element insertion is slight different.

5.4 "Reverse Northern" Analyses

As a first step towards determining whether rescued plasmids contained transcribed sequences, "Reverse Northern" analysis was performed. The mRNA was used as a probe to simultaneously screen DNA clones encoding transcribed sequences, a process called reverse Northern blot analysis (Fryxell and Meyerowitz, 1987). In this case, head mRNA and body mRNA were transcribed into cDNA and then made into the cDNA probes described in Section 2.4.9.1.

A Reverse Northern does not assess the range or stage specificity of different transcripts, what is assessed here are all transcripts that hybridise to a particular DNA fragments. Each plasmid from the 3' end were double digested with *Pst* I and *Bam*HI, while plasmids from the 5' end were double digested with *Kpn* I and *Hin*dIII (Fig. 5.6a, d). The digested plasmid DNAs were run in duplicate on a 0.8% agarose gel and transferred to nylon filters. The filters were hybridised in parallel with cDNA probes produced by reverse transcription of head and body mRNA. The autoradiographs were compared to determine which fragments exhibited a head-elevated pattern of hybridisation. In order to check the efficiency of probes, two controls were included on each filter. One was λST41, a clone of *Nina*E gene (Zucker *et al.*, 1985) that only expressed in the head and other gene was α1-tublin (Kalfayan and Wensink, 1982) which was expressed in head and body (Kindly provided by S. R. Tomlinson, Glasgow).

All of the plasmids from the 3' end when digested with *Pst* I and *Bam*HI released a fragment of 2.968 kb that was the pBluescript and genomic flanking sequences. The plasmids from the 5' end when digested with *Kpn* I and *Hin*dIII released two fragments of 8

and 3 kb corresponding to the pBluescript plus the mini-*white* gene and the GAL4 element in addition to genomic flanking sequences. As seen in Figure 5.6, pPC507 from line c507 releases three fragments of about 3.3, 0.9 and 0.45 kb. Two fragments of 0.9 and 0.45 kb hybridise strongly to head cDNA and body cDNA probes (Fig. 5.7b-c). And pPC61 from line c61 had a fragment of 1.8 kb. This fragment was identified (Fig. 5.7b-c) which showed hybridisation to the head and body probes. Other lines did not show any hybridisation signal in the plasmid rescued fragments. This suggested either that the transcript was at too low a level to be detected, or that transcribed sequences are further away. If the reason is the former, these fragments should be checked by Northern blots because Northern blotting is a more sensitive technique than reverse Northern (Sambrook, *et al.*, 1989). If rescued fragments are too short to contain the transcribed sequences, this problem can be solved by following ways. First, digestion with a different unique enzyme (e.g. *Xho*I) for genomic DNA of P[GAL4] lines will possibly produce longer genomic fragments which contain the transcribed sequences. The second way is to screen genomic library using rescued fragments as probes to get longer genomic DNA clones and then find the transcribed sequences.

As can be seen in Figure 5.6, about 3 kb common bands of pBluescript had a hybridisation signal as well. This phenomena was observed by other researchers in our laboratory. It is probably due to a few basepairs DNA homology between probes and pBluescript, or other unknown reasons.

Unfortunately, when the 5' end rescued fragments were run on the gel for "Reverse Northern", they were not digested well enough to identify the size of fragments (Fig. 5.6.d). Therefore, it is difficult to identify whether these fragments include transcribed sequences or not when they were hybridised by head and body cDNA probes (data not shown).

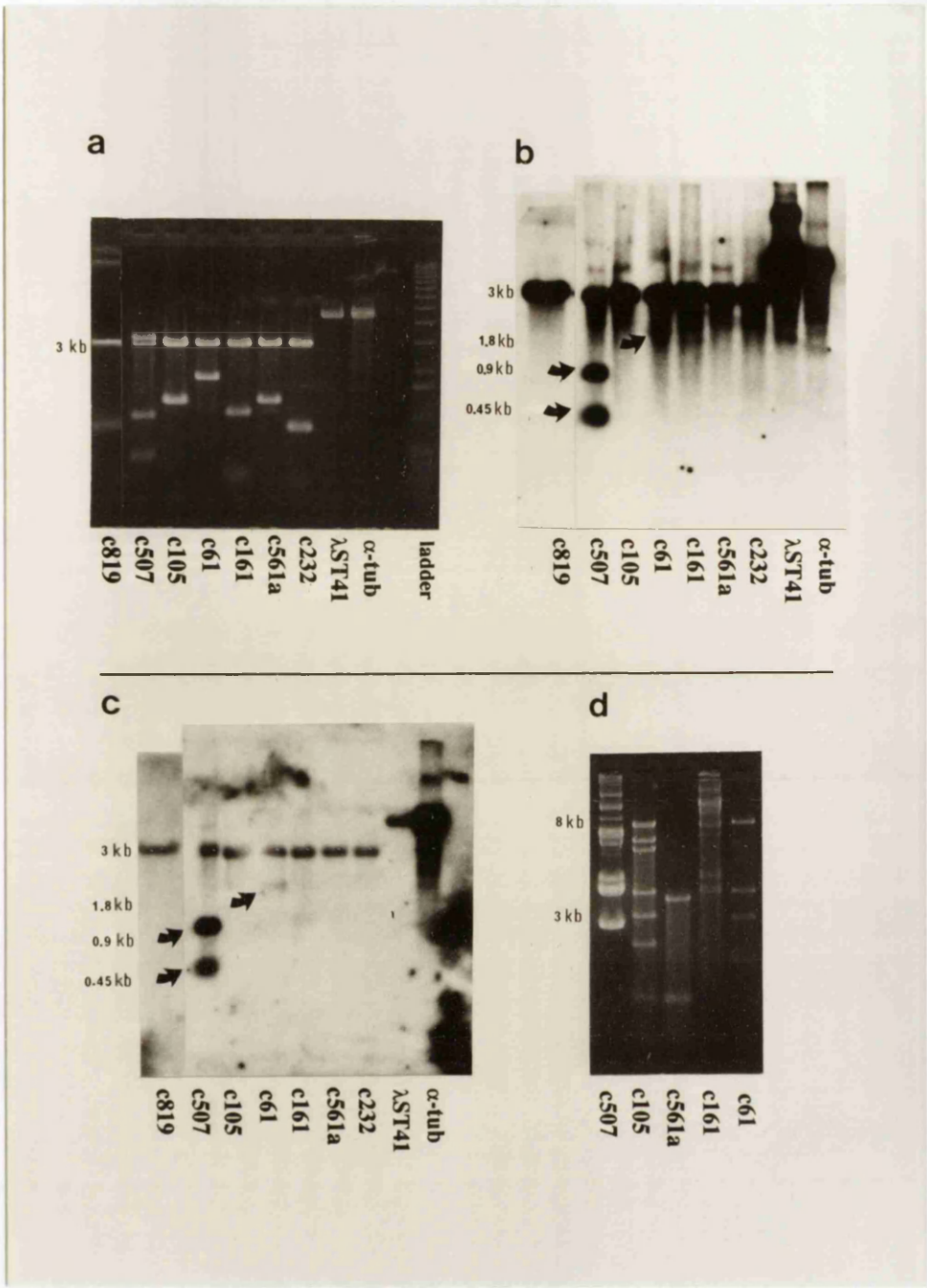


Figure 5.6. "Reverse Northern" analysis

(**a**) shows an ethidium bromide stained gel prior to blotting. Each plasmid from the 3' end is restricted with *Pst*I and *Bam*HI to release the insert, except λST41 and α tubulin which are taken as positive controls. Blot filters are probed with head (**b**) and body (**c**) cDNA probes of almost equivalent activity. The arrows indicate the hybridised fragments that are transcribed regions. (**d**) shows an ethidium bromides stained gel prior to blotting. Each plasmid from 5' end restricted with *Kpn*I and *Hin*dIII to release the insert. Again blot filters are probed with same head and body cDNA probes (data not shown).

5.5 Isolation of Genomic DNA Clones Corresponding to Lines c507 and c161

As "reverse Northern blot" analysis, only two rescued plasmids pPC507 and pPC61 contain transcribed sequences. In order to find more transcribed regions for these lines and other lines, it would certainly be useful to obtain genomic lambda DNA clones that span the site of insertion and extends further 3' and 5' ends.

Two λ libraries of *Drosophila* genomic DNA have been constructed. One library was constructed in the lambda vector EMBL3 and contains 9-22 kb inserts derived from Oregon-R DNA partially digestion with *Sau*3A (K. Kaiser, unpublished). Another library was constructed in the lambda GEM-11 (Russell and Kaiser, unpublished). The screening was performed as described in section 2.4.11. The rescued fragments from the 3' end were used as probes to clone the longer genomic DNA fragments.

For line c507, four identical positive clones of 14 kb (λ1-4) were recovered from the genomic library in the lambda GEM-11 after screening twice, but these clones only extend the 3' end. Therefore I decided to screen the different library. When the EMBL3 genomic library was used, two positive clones (λ5=13kb; λ6=14.5kb) were purified. In order to map genomic lambda clones, the phage DNAs were restricted with a combination of the different enzymes. Comparing restriction map of rescued fragments (see section 5.3) and their cDNA clones (see section 6.2), they provided sufficient information to construct a restriction map for line c507 which spans the site of insertion and extends further 3' and 5' ends. The resulting restriction map is shown in Figure 5.7a.

A similar screen has been performed for lines c161, c105, c61 and c819. In the case of c161, when the EMBL3 genomic library was used, four genomic lambda DNA clones were obtained. Two of them were mapped and showed in Figure 5.7b. For other lines some

"positives" plaques were identified which, due to lack of time, have not yet been resolved to single plaques or isolated to lambda phage DNAs.

When the longer genomic clones were obtained from these lines, they should also be analysed by "reverse Northern" blot analyses to find transcribed sequences in both sites of the P element and search for the head specific or head elevated genes. But due to lack of time I have not done that yet. As two lines (c507 and c61) have already shown the transcribed sequences, I decided to screen the cDNA library to find corresponding cDNAs for further analysis. The further characterisation of these lines at the molecular level will be discussed in the subsequent chapters.

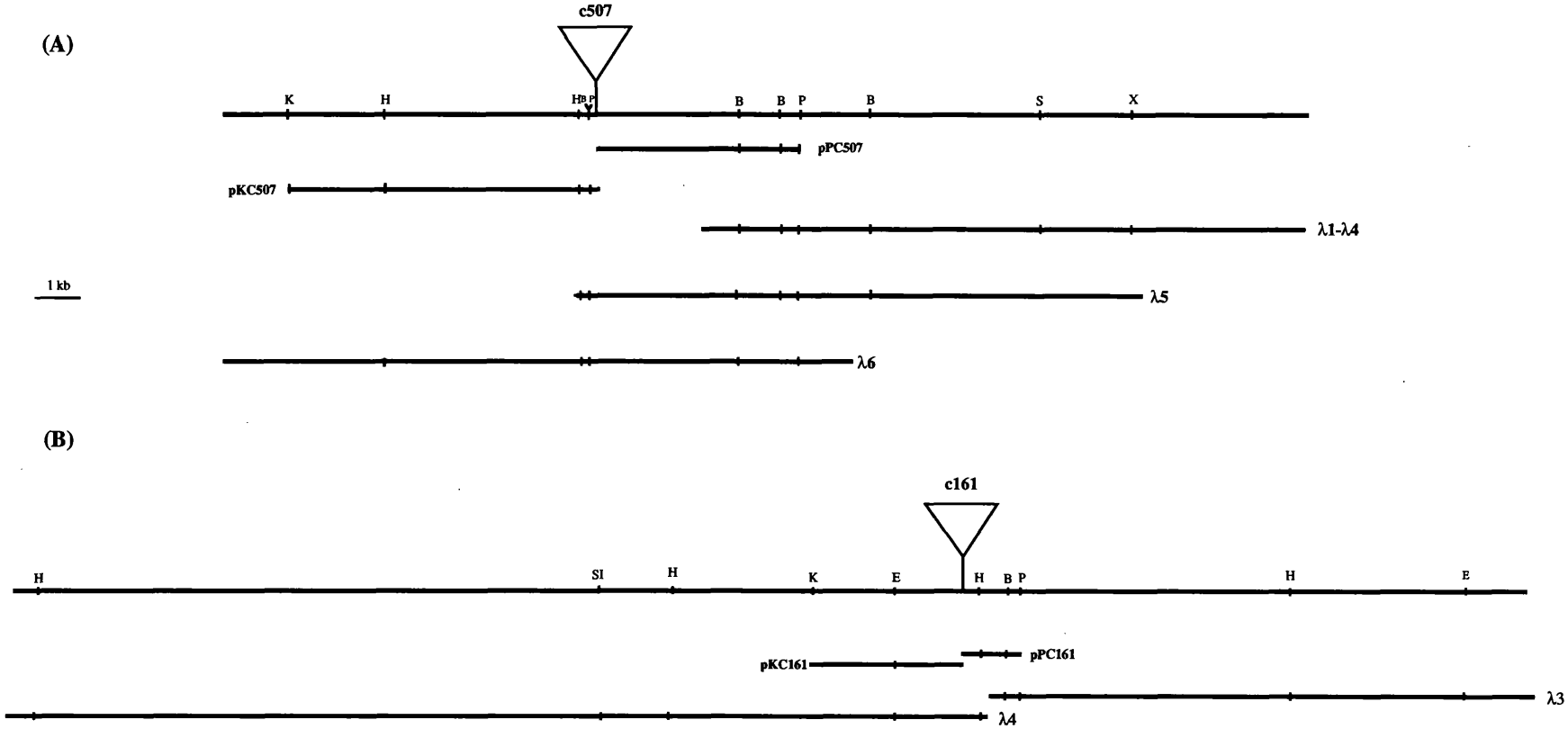


Figure 5.7. Maps of the λ genomic DNA clones

(**A**) The restriction map of the λ genomic DNA clones of line c507. (**B**)The restriction map of the λ genomic DNA clones of line c161. Restriction sites shown are **B**-*Bam*HI, **P**-*Pst*I, **S**-*Sst*I, **X**-*Xho*I, **E**-*Eco*RI, **K**-*Kpn*I, **H**-*Hin*dIII, **SI**-*Sal*I.

Chapter 6

Cloning of Genes Neighbouring the P[GAL4] insertion in lines c507 and c232

6.1 Introduction

As has been described in Chapter Five, genomic DNAs flanking P[GAL4] insertions have been obtained from seven central complex lines. They have been further analysed at the molecular level. So far, I have obtained and sequenced a number of cDNA clones for these lines, however, I do not intend to present all the data in this thesis. I will focus on the analysis of line c507 and c232, in which their P elements were identified to insert in the genome about 500 bp away from each other but in different orientation (see Fig. 6.1a). This chapter will first describe isolation of cDNA clones for line c507 and c232. The chapter will then report the sequencing analysis. Finally, the Northern analysis and coexpression patterns will be presented and the conclusion will be made.

6.2 Isolation of cDNA Clones Related to c507 Flanking DNA

To isolate cDNA clones of both sides of the P[GAL4], a conventional cDNA library screening approach was chosen. A male head cDNA library in λ-phage NM1149 (which was made by S. Russell 1989) was available for use in our laboratory.

6.2.1 cDNA clones from the "downstream" side of the P[GAL4] element in line c507

As discussed in section 5.4, the rescued plasmid from pPC507 produced a 0.9 kb *Bam*HI fragment which contains a transcribed sequence judged by "reverse Northern" analysis. This fragment was used as a probe for isolation of cDNA clones related to the flanking genes downstream of the P[GAL4] element. Four independent cDNA clones were obtained and designated pMY3, pMY5, pMY8, and pMY10 (Fig. 6.1).

In order to determine the relationships between cDNA clones, they were digested with the enzymes *Kpn*I, *Pst*I, *Bam*HI, *Xho*I, *Sal*I and *Sst*II. The result has provided sufficient information to construct a restriction map for these cDNA clones. It appears to

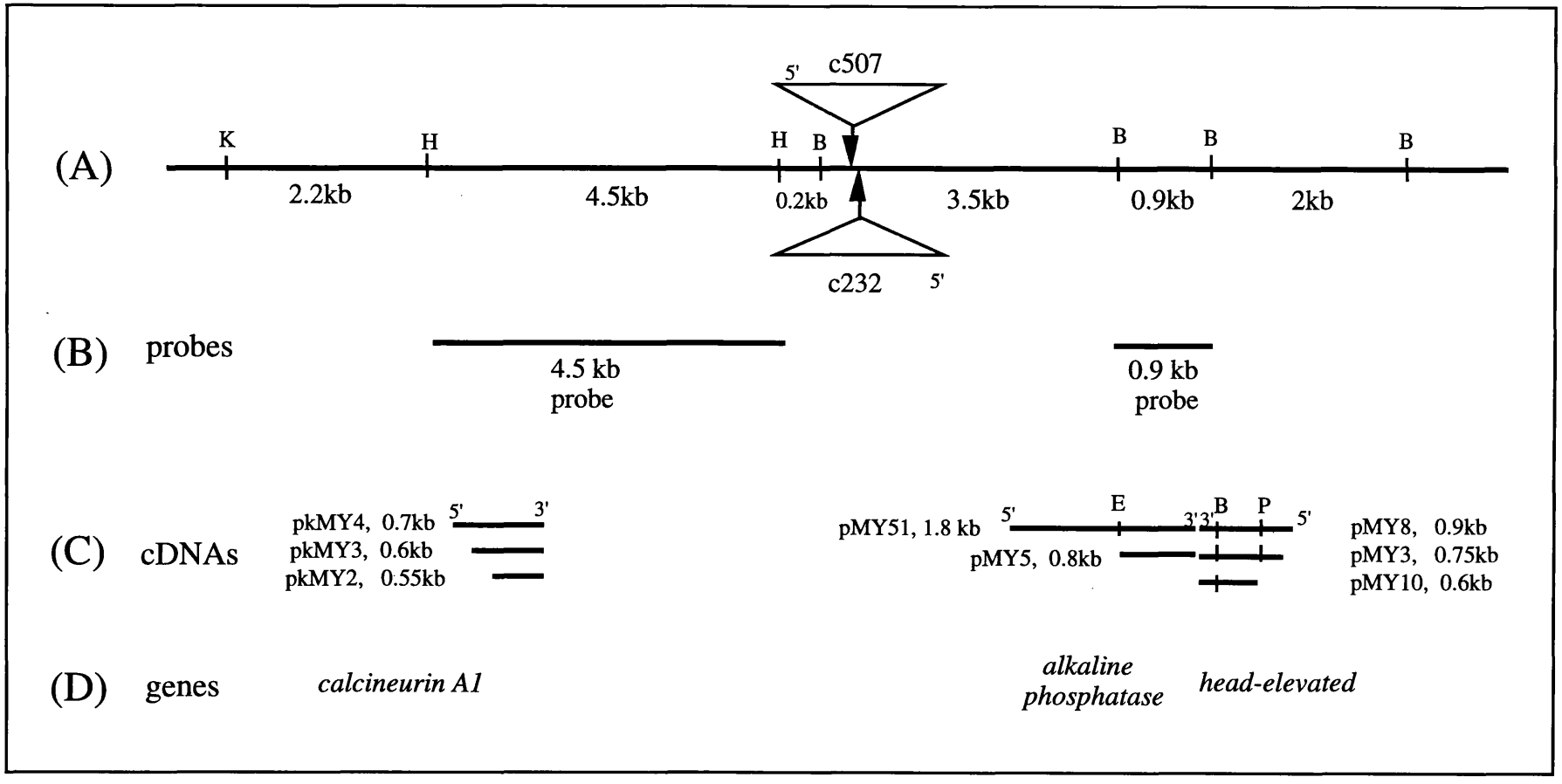


Figure 6.1 The organisation of the region surrounding the P[GAL4] elements in lines c507 and c232.
(**A**) shows the restriction map of the genomic region encompassing the P[GAL4] insertion of line c507 and c232. Two P[GAL4] elements were inserted into the *Drosophila* genome only 46 bp away from each other but in different orientation. (**B**) shows two probes from the genomic DNA fragments used for screening cDNA library. (**C**) shows one group cDNAs matched upstream of the P[GAL4] and two groups of transcript units in the downstream of the P[GAL4] insertion in line c507. (**D**) shows the names of genes. The restriction sites shown are **K**-*Kpn*I, **B**-*Bam*HI, **P**-*Pst*I, **H**-*Hin*dIII, **E**-*Eco*RI. NB: It should not be assumed that the cDNAs are necessarily co-linear with the genomic DNAs.

divide these cDNA into two groups. Group one consists of the pMY3 and pMY8, pMY10. The longest clone pMY8 hybridised with the other two clones of this group. So, they were thought to be same transcribed sequences but different length. This result was also supported by sequence data (data not shown). But the pMY5 belongs to a different group of cDNA. It had no restriction cleavage site for the above enzymes and did not hybridise with other cDNA clones.

Meanwhile, genomic DNA was probed with the cDNAs from pMY8 and pMY5. The two groups of cDNA hybridised to the closely linked positions in the genome. The region of hybridising unit from pMY5 is nearer to the P[GAL4] element while that of hybridising unit from pMY8 is located further away. A schematic diagram of the organisation of the transcription units relative to the genomic DNA is shown in Figure 6.1. In this diagram, however, it should not be assumed that the cDNAs are necessarily co-linear with the genomic DNAs because the mapping of potential intron/exon boundaries has not been carried out completely.

6.2.2 Isolation of A "Full Length" cDNA Clone Related to pMY5

Using a ^{32}P labelled DNA probe generated from the insert sequence of pMY5, a preliminary Northern blot from head poly (A) mRNA demonstrated the presence of two transcripts of about 2.0 kb and 3.7 kb. The 2.0 kb band had a strong signal which indicates the size of main transcript of this gene (Fig. 6.2). This result also suggested that pMY5 (0.8 kb) was not the full length of the cDNA. Therefore it is necessary to find longer cDNA clones related to pMY5.

When the screening of the cDNA library again, the longer cDNA clone (1.8 kb) was obtained and named as pMY51. It is presumed that the 1.8 kb clone is a full length of cDNA because this gene has a abundant transcript in about 2.0 kb (Fig. 6.2). In fact, the longer clone is an extended version of the clone pMY5 which has a internal *Eco*RI site (Fig. 6.1). This suggests that when the cDNA library was constructed, the methylation used to protect against restriction of internal *Eco*RI site was not fully functional.

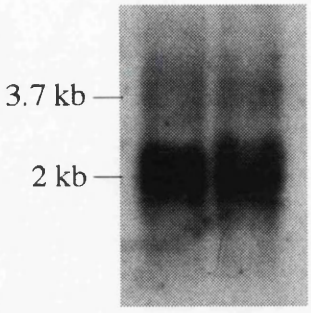


Figure 6.2 A Northern blot using a insert probe from pMY5.
A Northern filter with the heads poly(A)$^+$ RNA prepared from the wild type adults (Canton S) was hybridised to a cDNA probe from pMY5.

6.2.3 cDNA clones from the "upstream" side of the P[GAL4] element in line c507

It is possible that a gene relevant to enhancer trap expression patterns is located upstream side of the P[GAL4] insertion. Therefore, it was necessary to get cDNA clones from this side. A 4.5 kb *Hin*dIII fragment from the rescued plasmid, pKC507, was used a probe to screen a head cDNA library. Five "positive" plaques were identified. Three independent cDNA clones were purified and designated pkMY2, pkMY3 and pkMY4. The longest clone pkMY4 showed hybridisation with others, suggesting that these three cDNA clones come from the same transcribed sequence but are different versions (Fig. 6.1c).

In order to determine the corresponding position of the transcribed region within the genome, the genomic DNA clones were restricted with different enzymes and hybridised with the cDNA probe generated from the whole insert of pkMY4. The approximate corresponding position of the cDNA is illustrated in Figure 6.1 as well.

6.2.4 Genomic Southern for three different groups of cDNA clones

In order to determine if a gene has a single copy in *Drosophila*, genomic DNA derived from Canton-S was probed with representative cDNAs. Figure 6.3B showed that genomic DNA was restricted with *Pst*I and hybridised with the cDNA probe of pMY51. A strong hybridisation signal in band of about 5 kb was found which was expected, and two faint bands were seen in 7 kb and 9.5 kb. This result could be explained in two way: Either this gene possibly has two more copies in the genome or has a homology with other genes of their family.

When whole insert from pMY8 was probed for genomic DNA restricted with *Bam*HI, one strongly hybridising band of 2 kb and one weaker band of 0.9 kb were seen from Figure 6.3C. This is because the main insert of the pMY8 cDNA clone corresponds to the 2 kb of *Bam*HI fragment and a few base pairs DNA are homologous to the 0.9 kb band in the genome. Similarly, when the genomic DNA was digested with *Hind*III and probed with the insert of pkMY4, one band of 4.5 kb was seen (Fig. 6.3A). Above results suggest that these two clones represent single copy genes in *Drosophila*. In addition, the number and size of bands observed in these cases agree with the restriction map derived earlier.

6.3 Analysis of Sequence

Three representative cDNAs (pMY8, pMY51 and pkMY4) were identified to locate at both sides of the P[GAL4] insertion (Fig. 6.1). They all have been sequenced. Then, their open reading frames, putative terminators and protein sequence obtained from translation of the DNA sequence were also analysed.

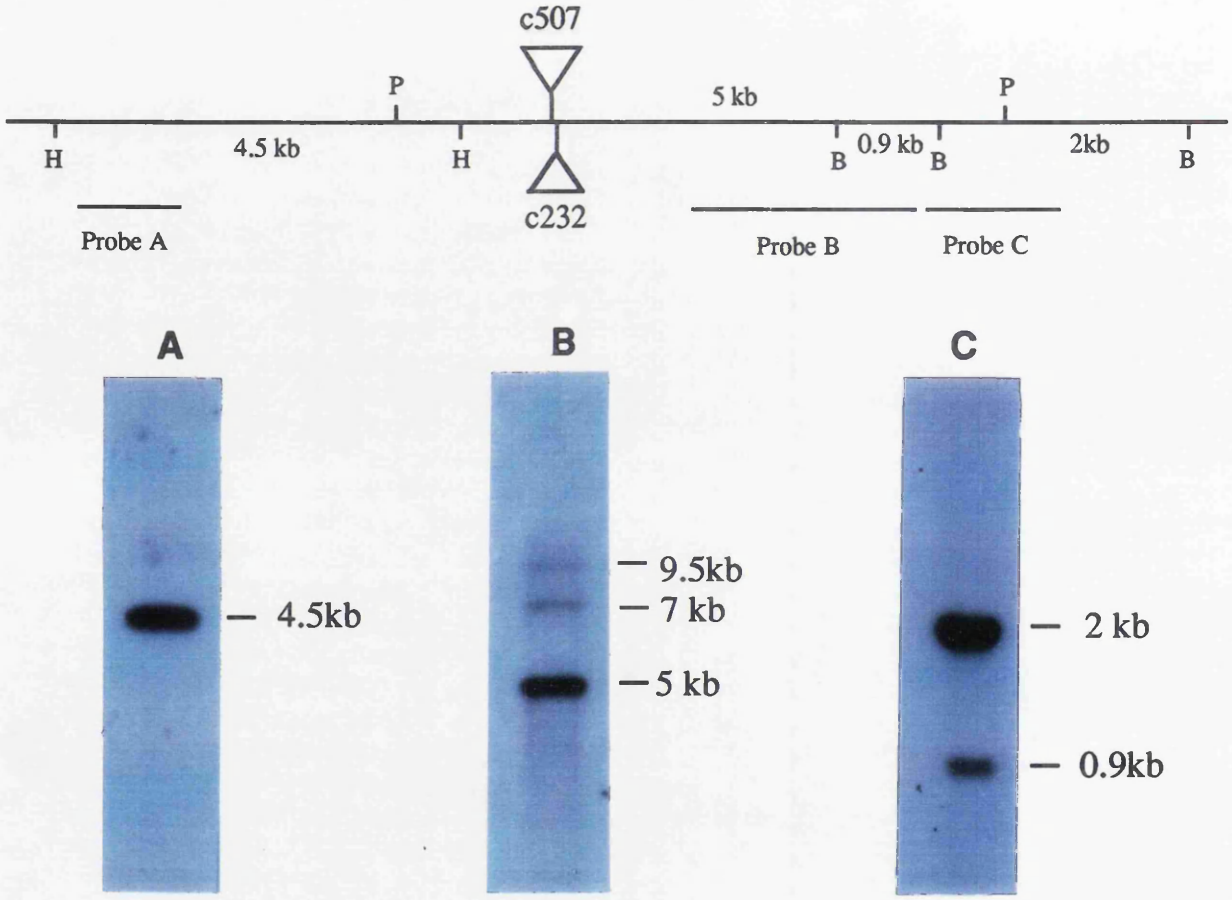


Figure 6.3 Southern blot of *Drosophila* genomic DNA (wild type CS).

Upper diagram shows the genomic map and corresponding cDNA clones surrounding the P[GAL4] element of line c507 & c232. **H** (*Hin*dIII); **P** (*Pst*I); **B** (*Bam*HI).
A Genomic DNA was digested with *Hin*dIII and probed with whole insert of pkMY4 (probe A).
B Genomic DNA was restricted with *Pst*I and probed with whole insert of pMY51 (probe B).
C Genomic DNA was restricted with *Bam*HI and probed with whole insert of pMY51 (probe C).

6.3.1 General Sequence Features Of the pkMY4 cDNA clone

When sequencing clone pkMY4, the partial sequences were put through the GenEMBL database as a DNA search using Blastn. The result (Fig. 6.4) shows the alignment of the published sequence of the *Drosophila* Calcineurin A1 and the 5' sequence from pkMY4. Clearly pkMY4 is a cDNA clone of a Calcineurin A1 transcript (Guerini *et al.*, 1992). When comparing the amino acids between the calcineurin A1 and the pkMY4, they are also identical. The amino acids derived from the pkMY4 are parts of the coding region of the calcineurin A1. (Fig. 6.4).

Calcineurin, Ca^{2+}/calmodulin-stimulated protein phosphatase, has been implicated in synaptic transmission, cytoskeletal dynamics and other calcium-regulated events (Tallant and Cheung, 1986; Klee *et al.*, 1987). It is composed of two subunit: a catalytic subunit, calcineurin A and a Ca^{2+}-binding regulatory subunit, calcineurin B. The Ca^{2+}stimulation of calcineurin is mediated by two different Ca^{2+}-binding proteins, calcineurin B and calmodulin; Ca^{2+} binding to calcineurin B promotes a small basal activity, whereas Ca^{2+} binding to calmodulin facilitates calmodulin's interaction with calcineurin A and results in a ten-fold further activation of the enzyme (Klee *et al.*, 1987). Calcineurin A can be divided into two classes of clones, calcineurin A1 and calcineurin A2, based on the restriction mapping, sequence analysis and the Northern blot (Guerini and Klee, 1989).

In *Drosophila*, calcineurin A was originally cloned by homology to the human sequence. The cDNA sequence of calcineurin A1 contains a 1704 bp open reading frame (ORF) coding for a 568 amino acid protein. The putative ATG initiator codon is preceded by a 60-bp open reading frame without initiator codon. The coding region consists of 12 exons. The 263-bp 3'-noncoding sequence lacks a poly(A) tail and the polyadenylation signal (Guerini *et al.*, 1992). However, from my sequencing data of the clone pkMY4, a poly(A) tail and the polyadenylation signal AATAAA were found, in addition to the sequence overlapped with the calcineurin A1 (data not shown). This success resulted from the different cDNA library used.

(A) . The sequence alignment

DROCALCA Drosophila melanogaster calcinuerin A1 (CNA1) mRNA, complete cds.

Length = 2039

```
CNA1: 1518  CAACGAACGGATGCCGCCCCGTCGTCCTCTTTTGATGAGTGCCAGCAGCAGCAGCATCAC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pkMY4: 11   CAACGAACGGATGCCGCCCCGTCGTCCTCTTTTGATGAGTGCCAGCAGCAGCAGCATCAC

CNA1:       CACGGTCACAAGGAGCAGCAGCAGCAGCAGCAACAACAACAACAATAACAGCAACACCAGCA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pkMY4:      CACGGTCACAAGGAGCAGCAGCAGCAGCAGCAACAACAACAACAATAACAGCAACACCAGCA

CNA1:       GCACCACGACGACAAAGGACATCAGCAACACCAGCAGTAAT  1680
            ||||||||||||||||||||||||||||||||||||||||
pkMY4:      GCACCACGACGACAAAGGACATCAGCAACACCAGCAGTAAT  173
```

(B) . The amino acids alignment

```
                451                                                 500
CNA1            PTGALPVGAL SGGRDSLKEA LQGLTASSHI HSFAEAKGLD AVNERMPPRR
pkMY4           .......... .......... .......... .......... ..NERMPPRR
                1                                                     8

                501                                                 550
CNA1            PLLMSASSSS ITTVTRSSSS SSNNNNNNSN TSSTTTTKDI SNTSSNDTAT
pkMY4           PLLMSASSSS ITTVTRSSSS SSNNNNNNSN TSSTTTTKDI SNTSSN....
                9                                                    54

                551                         578
CNA1            VTKTSRTTVK SATTSNVRAG FTAKKFS*
pMY4            .......... .......... .......
```

Figure 6.4 pkMY4 is a cDNA clone of a calcineurin A1 transcript.

(A) shows that the Blastn search of pkMY4 sequence derived from extension from the T3 primer (**pkMY4**) aligned to the published sequence of Guerini *et al.*, 1992 (**CNA1**). Numbering is in base pairs from the 5' end of the cDNA. (**B**) shows the alignment of amino acids between the calcineurin A1 and pkMY4.

6.3.2 General Sequence Features Of the pMY51 cDNA Clone

Figure 6.5 shows the full length sequence of the pMY51 cDNA clone which has 1888 bp long including 22 poly A^+ tail remnant. This cDNA contains a 547 amino acids ORF identified by the MacVector™. The long ORF of pMY51 is translated in the first frame, beginning from nucleotide 43 and ending at nucleotide 1758. It is followed by a TGA stop codon encoded by DNA residues 1759-1861. By searching the database, this predicted polypeptide has a significant homology to the alkaline phosphatase (ALP) gene family in other organisms at protein level (Fig. 6.6). No *Drosophila* homologue has so far been described. Like most ALP, the predicted polypeptide from pMY51 has five potential N-linked glycosylation signals. These sites vary with different species (Manes *et al.*, 1990; Itoh *et al.*, 1991). By the Prosite search (Fuchs, 1994), pMY51 has a matching pattern with the APL, which is "VPDSAGTAT". Unexpectedly, the normal polyadenylation site is not found in the 3' untranslated region of cDNA.

Alkaline phosphatase (EC 3.1.3.1) (ALP) is a zinc and magnesium-containing metalloenzyme which hydrolyses phosphate esters, optimally at high pH. It is found in nearly all living organisms, with the exception of some plants. In *E. coli*, ALP (gene *pho*A) is found in the periplasmic space. In yeast it (gene *PHO*8) is found in lysosome-like vacuoles and in mammals, it is a glycoprotein attached to the membrane by a GPI-anchor (McComb *et al.*, 1979; Trowsdale *et al.*, 1990).

In mammals, four different isozymes are currently known. Three of them are tissue-specific: the placental, placental-like (germ cell) and intestinal isozymes. The fourth form is tissue non-specific and was previously known as the liver/bone/kidney isozyme. Each isozyme is encoded by a separate gene (Harris, 1989; Manes *et al.*, 1990). In *Drosophila*, at least four allelic forms of the ALP were reported by histochemical studies (Yao, 1950; Beckman and Johnson, 1964; Schneiderman *et al.*, 1966; Harper and Armstrong, 1972; 1973), but there is no idea about relationship between mammals and *Drosophila.*

```
GTGCCAACTGGATCTCACTGAGATGGGCAATTAATACGTGACATGGCACTTGACTTACCCAGCGATTTTCGATCC  75
                                                  M  A  L  D  L  P  S  D  F  R  S  11
GATTTAGTTTCAATCTGTGAATCCATTTCAGACGCGGAATTCTGGCACAACGTGGGCCTGAGGCAGCTGGAGAAG  150
 D  L  V  S  I  C  E  S  I  S  D  A  E  F  W  H  N  V  G  L  R  Q  L  E  K  36
ACCATTAAGCAGGCGCAGCGCGTGAAGGAGGACTCCTACCAGAAAAAGGCCCGGAATATCATCATCTTCATCGGA  225
 T  I  K  Q  A  Q  R  V  K  E  D  S  Y  Q  K  K  A  R  N  I  I  I  F  I  G  61
GACGGCATGGGAATATCCACGATCAGTGCTGGTCGCATCTACAAGGGGCAGTACCTGAAGCACGGCTACGGCGAG  300
 D  G  M  G  I  S  T  I  S  A  G  R  I  Y  K  G  Q  Y  L  K  H  G  Y  G  E  86
GAGGAAACCCTCGTCTTCGACGATTTCCCAAACACTGGAATGGCCAAAACCTACAACGTGGACAAACAAGTGCCG  375
 E  E  T  L  V  F  D  D  F  P  N  T  G  M  A  K  T  Y  N  V  D  K  Q  V  P  111
GATTCGGCGGGCACTGCCACTGCGATCTTCTCGGGTTCGAAAACCCATTACGGAGCCATTGGAATGGACGCCACC  450
 D  S  A  G  T  A  T  A  I  F  S  G  S  K  T  H  Y  G  A  I  G  M  D  A  T  136
CGCTCCAAGAAGAATGGGCAGCAAGGCAGGGTCCAGAGCGTCATGGAGTGGGCCCAGAAGGAGGGCAAGCGCACC  525
 R  S  K  K  N  G  Q  Q  G  R  V  Q  S  V  M  E  W  A  Q  K  E  G  K  R  T  161
GGCGTGGTCACCACAACCAGGATCACGCACGCCACGCCCGCCGCCACCTACGCCCACATCTACGACCGGGACTGG  600
 G  V  V  T  T  T  R  I  T  H  A  T  P  A  A  T  Y  A  H  I  Y  D  R  D  W  186
GAGTGCGACACGGAAGTGCCCGCGGAATCGGTGGGCTTTCATGTCGATATTGCCCGTCAGTTGGTGGAGAATGCT  675
 E  C  D  T  E  V  P  A  E  S  V  G  F  H  V  D  I  A  R  Q  L  V  E  N  A  211
CCGGGAAATCGGTTCAATGTGATCCTGGGCGGAGGAATGTCGCCCATGGGCATCCTGAATGCCTCCGAGGTGAAG  750
 P  G  N  R  F  N  V  I  L  G  G  G  M  S  P  M  G  I  L  N  A  S  E  V  K  236
ACTACTATTTTCGAAGGACCCACGGAAACAATTTGCACCCGCGGTGATAACAGAAACCTTCCTGCCGAGTGGCTG  825
 T  T  I  F  E  G  P  T  E  T  I  C  T  R  G  D  N  R  N  L  P  A  E  W  L  261
GCCCATCACGCCAACGACACAGTTCCTCCAGCATTGGTACATAACCGTAAGGATCTGCTTAATGTGAATGTCAAG  900
 A  H  H  A  N  D  T  V  P  P  A  L  V  H  N  R  K  D  L  L  N  V  N  V  K  286
AAGGTGGACCATTTGATGGGCCTGTTCCGAAACAATCACATTACGTACTCCATAGCCAGGGAGGCGGGAGAGCCT  975
 K  V  D  H  L  M  G  L  F  R  N  N  H  I  T  Y  S  I  A  R  E  A  G  E  P  311
TCCCTGCAAGAGATGACGGAGACGGCCTTGGGAATCTTGGAAAGGGACGATGAGTCCAACGGCTTTGTGCTCCTG  1050
 S  L  Q  E  M  T  E  T  A  L  G  I  L  E  R  D  D  E  S  N  G  F  V  L  L  336
GTGGAAGGAGGTCGCATTGACCACGGTCACCACATGAACTACGCCCGTGCTGCTCTGCACGAGCTCTACGAATTC  1125
 V  E  G  G  R  I  D  H  G  H  H  M  N  Y  A  R  A  A  L  H  E  L  Y  E  F  361
GATTTGGCAATCCAAGCGGCCGTGAACAATACGGATCCCGACGAAACGTTGATCCTGGTGACGGCAGACCATTCC  1200
 D  L  A  I  Q  A  A  V  N  N  T  D  P  D  E  T  L  I  L  V  T  A  D  H  S  386
CACGCGGTCACCTTTAATGGTTACGCGCTCCGTGGAGCTGATATCCTGGGGACAGCCAATTCACACGAGAAAAAC  1275
 H  A  V  T  F  N  G  Y  A  L  R  G  A  D  I  L  G  T  A  N  S  H  E  K  N  411
GATCCCATGTTCTACGAGACCATCTCGTATGCCAATGGTCCTGGCTATTGGGATCACTTGGCGAATGACTCCAGA  1350
 D  P  M  F  Y  E  T  I  S  Y  A  N  G  P  G  Y  W  D  H  L  A  N  D  S  R  436
CCTCAGAACAGCTCCAACATGTGGATGCCCCTGAAGCATTTTACCGCTGAGGAGCGGGCTGCTCCCACTTATCGC  1425
 P  Q  N  S  S  N  M  W  M  P  L  K  H  F  T  A  E  E  R  A  A  P  T  Y  R  461
CACTTGGAGCCGGTTCCCAGAAAGGACGAAACGCACGGCGGCGAGGATGTGGCTGTCTTTGCATATGGACCTGGT  1500
 H  L  E  P  V  P  R  K  D  E  T  H  G  G  E  D  V  A  V  F  A  Y  G  P  G  486
TCCAGTTTGATTCGCGGGGTCTTCGAGCAGAACTACTTGGCCTATGTGATGAGCTACGCGGCTGTTTGGGTCCCG  1575
 S  S  L  I  R  G  V  F  E  Q  N  Y  L  A  Y  V  M  S  Y  A  A  V  W  V  P  511
CCAAGGACTTCGATGACTCCTGTGAGGATCACAAGGATGGGCAAAAGGATAGGCCGCTGGACAAACCCAATCCAA  1650
 P  R  T  S  M  T  P  V  R  I  T  R  M  G  K  R  I  G  R  W  T  N  P  I  Q  536
AGAGAAGTGGCGCCTCTGTTGTGGGAGCCTCCTTGATCCCCATTTTGACTGCTGCCACTGCGGCTATTTTGCGCT  1725
 R  E  V  A  P  L  L  W  E  P  P  ***                                        547
GTCACGGGCTGTAATTAATGTTGTTTTAATTATATTAATTGTTATTAAAGTTTATAGACGTACGGATGGCTTCGT  1800

TGAATGAGAGTGAGCGAGATGGAGAAGTGATCGTTGCAACTTAACAAATAGATACACTAAAAAATAAAAAAAAAA  1875

AAAAAAAAAAAAA                                                                1888
```

Figure 6.5 The nucleotide and deduced amino acid sequence of the pMY51 cDNA clone. The nucleotide sequence of the full-length alkaline phosphatase cDNA is shown along with the amino acid sequence of the longest deduced open reading frame starting with a methionine, numbering is in bold. Numbering of the DNA starts at the most 5' end base of the cDNA. Sites of putative N-glycosylation are underlined. The stop codon TGA is marked by asterisks.

In order to compare the pMY51 clone, presumably encoding a ALP in *Drosophila*, to the ALP from other species, the putative amino acids sequence is aligned with those deduced for the ALP from different species, using the PILEUP programme in GCG package (see section 2.4.12.5). The result from the pileup's can be seen in Figure 6.6. Amino acid positions that are identical in all eight proteins, or in at least six proteins (one is Drosophila) are marked by stars and quotation marks respectively. Among the *Drosophila* and other species, there is about 40% homology (24% amino acid identity and 15% partial homology) in 533 overlap amino acids. There is over 70% similarity between *Drosophila* ALP and any one individual ALP in question by FastA search (data not shown).

It is accepted that knowledge of the intron-exon organisation of a gene helps in identifying possible functional domains in the protein molecule. However, in the case of ALPs, the intron-exon junctions do not correspond to anything that could loosely be defined as a catalytic domain. Instead, the residues that constitute the "active site pocket" are spread throughout the molecular (Millan *et al.*, 1988). The metal and substrate phosphate binding sites determined in the *E. coli* ALP by X-ray crystallography (Sowadski *et al.*, 1985) are shown and marked with "@" in Figure 6.6. These conserved sites in *Drosophila* are identical to other species, reflecting essential functional domains. A significant conservation in residues coordinating the active pocket suggested that *Drosophila* ALP has a common ancestral gene with other species APL genes.

In order to analysis the evolutionary relationships among the ALPs (Human, Mouse, Bovine, Rat, *Drosophila*, *Bombyx* and *E coli*) at protein level, "growtree and distances" programmes from GCG package were used (see section 2.4.12.5). Based on the above result, a phylogenetic tree of parts of ALPs is constructed and shown in Figure 6.7. The diagram shows that mammalian ALPs are more closely related than insects or *E coli*; two insects (*Drosophila* and *Bombyx*) are more related than others; *E coli* APL seems to be separate from others, although they all have a common ancestral root. These data are consistent with previous homologue analysis. There is more homology among the mammalian ALPs (Millan, 1988; Manes *et al.*, 1990). There is higher homology between

The Result of Protein Pileup for Alkaline Phosphatase

```
                1                                                                                                   @                 100
Dro_pMY51_      MALDLPSDFR .......... .......... SDLVSICESI SDAEFWHNVG LRQLEKTIKQ AQRVKEDSYQ KKARNIIIFI GDGMGISTIS AGRIYKGQYL
    A40172      MISPFSVLAI GTCL...... .........T NSF..VPEKE RDPSYWRQQA QETLKNALKL .QKLNT.... NVAKNVIMFL GDGMGVSTVT AARILKGQ.L
PPBT_MOUSE      MISPFLVLAI GTCL...... .........T NSF..VPEKE RDPSYWRQQA QETLKNALKL .QKLNT.... NVAKNVIMFL GDGMGVSTVT AARILKGQ.L
  PPBT_RAT      MILPFLVLAI GTCL...... .........T NSF..VPEKE KDPSYWRQQA QETLKNALKL .QKLNT.... NVAKNIIMFL GDGMGVSTVT AARILKGQ.L
PPBT_BOVIN      MISPFLLLAI GTCF...... .........A SSL..VPEKE KDPKYWRDQA QQTLKNALRL .QTLNT.... NVAKNVIMFL GDGMGVSTVT AARILKGQ.L
PPBT_HUMAN      MISPFLVLAI GTCL...... .........T NSL..VPEKE KDPKYWRDQA QETLKYALEL .QKLNT.... NVAKNVIMFL GDGMGVSTVT AARILKGQ.L
PPBI_MOUSE      MQGPWVLLLL GLRL...... .........Q LSLSVIPVEE ENPAFWNKKA AEALDAAKKL .QPIQT.... S.AKNLIIFL GDGMGVPTVT ATRILKGQ.L
PPB_BOMMO_      MVVSVVAAAA AAGLVRAEDR YHPERLAAGE ASAATRSAAE SEASFWVREA QEAIERRERE GAGAKQAA.. GHAKNVVMFL GDGMSVPTLA AARTLLGQ.R
                *                                          *   *   *       *         *           * * * *  ***** **   * *" "** *

                101                                  @@                                                                    @ @
Dro_pMY51_      KHGYGEEETL VFDDFPNTGM AKTYNVDKQV PDSAGTATAI FSGSKTHYGA IGMDATRSKK ..N..GQQG. RVQSVMEWAQ KEGKRTGVVT TTRITHATPA
    A40172      HHNTGEETRL EMDKFPFVAL SKTYNTNAQV PDSAGTATAY LCGVKANEGT VGVSAATERT RCN..TTQGN EVTSILRWAK DAGKSVGIVT TTRVNHATPS
PPBT_MOUSE      HHNTGEETRL EMDKFPFVAL SKTYNTNAQV PDSAGTATAY LCGVKANEGT VGVSAATERT RCN..TTQGN EVTSILRWAK DAGKSVGIVT TTRVNHATPS
  PPBT_RAT      HHNTGEETRL EMDKFPFVAL SKTYNTNAQV PDSAGTATAY LCGVKANEGT VGVSAATERT RCN..TTQGN EVTSILRWAK DAGKSVGIVT TTRVNHATPS
PPBT_BOVIN      HHSPGEETKL EMDKFPYVAL SKTYNTNAQV PDSAGTATAY LCGVKANEGT VGVSAATQRS QCN..TTQGN EVTSILRWAK DAGKSVGIVT TTRVNHATPS
PPBT_HUMAN      HHNPGEETRL EMDKFPFVAL SKTYNTKAQV PDSAGTATAY LCGVKANEGT VGVSAATERS RCN..TTQGN EVTSILRWAK DAGKSVGIVT TTRVNHATPS
PPBI_MOUSE      EGHLGPETPL AMDRFPYMAL SKTYSVDRQV PDSASTATAY LCGVKTNYKT IGLSAAARFD QCN..TTFGN EVFSVMYRAK KAGKSVGVVT TTRVQHASPS
PPB_BOMMO_      RGQTGEEASL HFEQFPTLGL AKTYCVNAQV PDSSCTATAY LCGVKANQGT PGVTAAVPRH DCEASTDVTK RVQSIAEWAL ADGRDVGIVT TTRITHASPA
                 *  ***  *   * **      ****  ** **********   * *  *  * *       *    **  * *  **    **  * ** ***  ****

                201     @                                                                                              300
Dro_pMY51_      ATYAHIYDRD WECDTEVPAE ..SVGFHVDI ARQLVENAPG NRFNVILGGG MSPMGILNAS EVKTTIFEGP TETICTRGDN RNLPAEWLAH HANDTVPPAL
    A40172      AAYAHSADRD WYSDNEMPPE ALSQG.CKDI AYQL..MHNI KDIDVIMGGG RKYMYPKNRT DVE...YELD EKARGTRLDG LDLISIWKSF KPRHKHS.HY
PPBT_MOUSE      AAYAHSADRD WYSDNEMPPE ALSQG.CKDI AYQL..MHNI KDIDVIMGGG RKYMYPKNRT DVE...YELD EKARGTRLDG LDLISIWKSF KPRHKHS.HY
  PPBT_RAT      AAYAHSADRD WYSDNEMPPE ALSQG.CKDI AYQL..MHNI KDIDVIMGGG RKYMYPKNRT DVE...YELD EKARGTRLDG LDLISIWKSF KPRHKHS.HY
PPBT_BOVIN      ASYAHSADRD WYSDNEMPPE ALSQG.CKDI AYQL..MYNI KDIEVIMGGG RKYMFPKNRT DVE...YELD EKARGTRLDG LNLIDIWKSF KPKHKHS.HY
PPBT_HUMAN      AAYAHSADRD WYSDNEMPPE ALSQG.CKDI AYQL..MHNI RDIDVIMGGG RKYMYPKNKT DVE...YESD EKARGTRLDG LDLVDTWKSF KPRHKHS.HF
PPBI_MOUSE      GTYVHTVNRN WYGDADMPAS ALREG.CKDI ATQL..ISNM .DINVILGGG RKYMFPAGTP DPE...YPND ANETGTRLDG RNLVQEWLS. ..KHQGS.QY
PPB_BOMMO_      GTFAKVANRN WENDNDVKQE GHDVNRCPDI AHQLIKMAPG NKFKVIFGGG RREFLPTTQV D........E EGTRGLRTDG RNLIEEWQND KESQKVSYKY
                " """  "*" *  *   " "   " "   ** * **          ** ***    "   "    "      "        "* *    *   *

                301                                                                                 @     @   @                  400
Dro_pMY51_      VHNRKDLL.. NVNVKKVDHL MGLFRNNHIT YSIAREA.GE PSLQEMTETA LGILERDDES NGFVLLVEGG RIDHGHHMNY ARAALHELYE FDLAIQAAVN
    A40172      VWNRTELL.. ALDPSRVDYL LGLFEPGDMQ YELNRNNLTD PSLSEMVEVA LRILTKN..L KGFFLLVEGG RIDHGHHEGK AKQALHEAVE MDQAIGKAGA
PPBT_MOUSE      VWNRTELL.. ALDPSRVDYL LGLFEPGDMQ YELNRNNLTD PSLSEMVEVA LRILTKN..L KGFFLLVEGG RIDHGHHEGK AKQALHEAVE MDQAIGKAGA
  PPBT_RAT      VWNRTELL.. ALDPSRVDYL LGLFEPGDMQ YELNRNNLTD PSLSEMVEVA LRILTKN..P KGFFLLVEGG RIDHGHHEGK AKQALHEAVE MDEAIGKAGT
PPBT_BOVIN      VWNRTDLL.. ALDPHSVDYL LGLFEPGDMQ YELNRNNATD PSLSEMVEMA IRILNKN..P KGFFLLVEGG RIDHGHHEGK AKQALHEAVE MDQAIGQAGA
PPBT_HUMAN      IWNRTELL.. TLDPHNVDYL LGLFEPGDMQ YELNRNNVTD PSLSEMVVVA IQILRKN..P KGFFLLVEGG RIDHGHHEGK AKQALHEAVE MDRAIGQAGS
PPBI_MOUSE      VWNREQLIQK AQDPS.VTYL MGLFEPVDTK FDIQRDPLMD PSLKDMTETA VKVLSRN..P KGFYLFVEGG RIDRGHHLGT AYLALTEAVM FDLAIERASQ
PPB_BOMMO_      LWNRQELLK. .LGSSPPDYL LGLFEGSHLQ YHLEGDESTE PTLAELTDVA IRVLSRN..E RGFFLFVEGG RIDHAHHDNY AHLALDETIE MDRAVKVATD
                " **  **         "" *  ***       "   "      *** "" " * *   **        ** ****** *******    *  **** "  * *"  *
```

```
           401          @@ @                                                                  500
Dro_pMY51_ NTDPDETLIL VTADHSHAVT FNGYALRGAD ILGTANSHEK NDPMFYETIS YANGPGYWDH LANDSRPQNS SNMWMPLKHF TAEERAAPTY RHLEPVPRKD
    A40172 MTSQKDTLTV VTADHSHVFT FGGYTPRGNS IFGLAPMVSD TDKKPFTAIL YGNGPGYKV. VDGE.....R ENVSM.V... ...DYAHNNY QAQSAVPLRH
PPBT_MOUSE MTSQKDTLTV VTADHSHVFT FGGYTPRGNS IFGLAPMVSD TDKKPFTAIL YGNGPGYKV. VDGE.....R ENVSM.V... ...DYAHNNY QAQSAVPLRH
  PPBT_RAT MTSQKDTLTV VTADHSHVFT FGGYTPRGNS IFGLAPMVSD TDKKPFTAIL YGNGPGYKV. VDGE.....R ENVSM.V... ...DYAHNNY QAQSAVPLRH
PPBT_BOVIN MTSVEDTLTV VTADHSHVFT FGGYTPRGNS IFGLAPMVSD TDKKPFTAIL YGNGPGYKV. VGGE.....R ENVSM.V... ...DYAHNNY QAQSAVPLRH
PPBT_HUMAN LTSSEDTLTV VTADHSHVFT FGGYTPRGNS IFGLAPMLSD TDKKPFTAIL YGNGPGYKV. VGGE.....R ENVSM.V... ...DYAHNNY QAQSAVPLRH
PPBI_MOUSE LTSERDTLTI VTADHSHVFS FGGYTLRGTS IFGLAPL.NA LDGKPYTSIL YGNGPGY.V. GTGE.....R PNVTA.A... ...ESSGSSY RRQAAVPVKS
PPB_BOMMO_ ALKEDESLVV VTADHTHVMS FNGYSPRGTD VLGTVRSL.D SNRMPFMVLS YTNGPGARIQ QNGV.....R PDVTTDA... ...NFGALRW RTHTDVPLDS
            "    **   *******  " * **  **   " * "       "       "  * *****"                "  "           "   "      **

           @                                                                                  600
Dro_pMY51_ ETHGGEDVAV FAYGPGSSLI RGVFEQNYLA YVMSYAAVWV PPR..... ..... .....TS MTPVRITRMG KRIGRWTNPI QREVAPLLWE
    A40172 ETHGGEDVAV FAKGPMAHLL HGVHEQNYIP HVMAYASCIG ANLDHCAWAG SG..... ..... ..... ..SAPSPGAL LLPLAVLSLR
PPBT_MOUSE ETHGGEDVAV FAKGPMAHLL HGVHEQNYIP HVMAYASCIG ANLDHCAWAG SG..... ..... ..... ..SAPSPGAL LLPLAVLSLP
  PPBT_RAT ETHGGEDVAV FAKGPMAHLL HGVHEQNYIP HVMAYASCIG ANLDHCAWAS SA..... ..... ..... ..SSPSPGAL LLPLALFPLR
PPBT_BOVIN ETHGGEDVAV FAKGPMAHLL HGVQEQNYIP HVMAYAACIG ANRDHCASAS SS..... ..... ..... ..GSPSPGPL LLLLALLPLG
PPBT_HUMAN ETHGGEDVAV FSKGPMAHLL HGVHEQNYVP HVMAYAACIG ANLGHCAPAS SA..... ..... ..... ..GSLAAGPL LLALALYPLS
PPBI_MOUSE ETHGGEDVAI FARGPQAHLV HGVQEQNYIA HVMASAGCLE PYTD.CGLAP PADESQTTTT TRQTTITTTT TTTTTTTTPV HNSARSLGPA TAPLALALLA
PPB_BOMMO_ ETHGGDDVTV FAWGVHHWMF SGLYEQTHVP HRMAWAACMG PGRHVCVSA. ..... ..... ..... ...ATVPTAA LLSLLLAAFI
           ********** ** **   *   ** ****    ** **                                            *

           601        613
Dro_pMY51_ PP..... ...
    A40172 TLF..... ...
PPBT_MOUSE TLF..... ...
  PPBT_RAT TLF..... ...
PPBT_BOVIN SLF..... ...
PPBT_HUMAN VLF..... ...
PPBI_MOUSE GMLMLLLGAP AES
PPB_BOMMO_ TLRHQCFL.. ...
```

Figure 6.6. Multiple sequences alignment for alkaline phosphatase

Stars (*) indicate identity to the corresponding residue of the pMY51 (**DRO_pMY51**) predicted polypeptide. Quotation marks (") indicate that most of the residues at that position are identical. @ indicate those residues that participate in the active site in *E.coli* alkaline phosphatase (Sowadski *et al.,* 1985). The sequences used are Mouse alkaline phosphatase precursor (**A40172**, from Hahnel and Schults, 1990); Mouse alkaline phosphatase, tissue-nonspecific isozyme precursor (**PPBT_MOUSE**, from Terao and Mintz, 1987); Rat alkaline phosphatase, tissue-nonspecific isozyme precursor (liver/bone/kidney isozyme) (**PPBT_RAT**, from Thiede *et al.*, 1988); Bovine alkaline phosphatase, tissue-nonspecific isozyme precursor (liver/bone/kidney isozyme) (**PPBT_BOVIN**, from Garattini *et al.,* 1987); Human alkaline phosphatase, tissue-nonspecific isozyme precursor (liver/bone/kidney isozyme) (**PPBT_HUMAN**, from Weiss *et al.*, 1988); Mouse alkaline phosphatase, intestinal precursor (**PPBI_MOUSE**, from Manes *et al.,* 1990); Silk moth membrane-bound alkaline phosphatase precursor (**PPB_BOMMO**, from Itoh *et al.,* 1991).

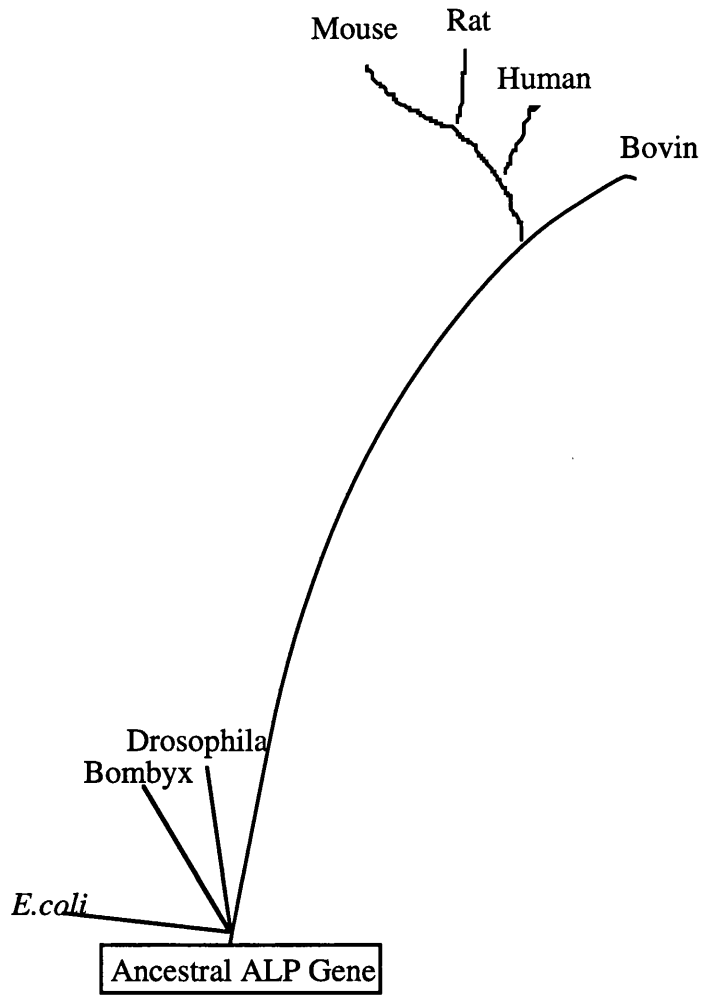


Figure 6.7 Schematic tree of alkaline phosphatase genes.

Diagram showing the postulated evolutionary relationships among Mouse ALP, tissue-nonspecific isozyme precursor (Terao and Mintz, 1987); Rat ALP, tissue-nonspecific isozyme precursor (Thiede *et al.*, 1988); Bovine ALP, tissue-nonspecific isozyme precursor (Garattini *et al.*, 1987); Human ALP, tissue-nonspecific isozyme precursor (Weiss *et al.*, 1988); Silkmoth (*Bombyx*) membrane-bound ALP precursor (Itoh *et al.*, 1991); *Drosophila* ALP (pMY51); *E. coli* ALP (Bradshaw *et al.*, 1981). (diagram is not to scale)

insects than mammalian ALPs (this section) and less homology between *E. coli* ALP and mammalian or insects ALPs (Millan, 1988; Itoh *et al.,* 1991). This may reflect the evolutionary history of the ALP gene family. They appear to have evolved from a common ancestral gene by a series of successive gene duplications.

The strong sequence homology between this locus in *Drosophila* and in other organisms is overwhelming evidence that this cDNA (pMY51) encode a alkaline phosphatase in *Drosophila*, which can be classified as tissue non-specific isozyme.

6.3.3 General Sequence Features Of the pMY8 cDNA clone

The pMY8 cDNA was sequenced on both strands. The full length sequence is 887 bp long excluding the 3' 20 bp polyA tail remnant as shown in figure 6.8. Eighty two nucleotides upstream of 3' poly (A) tracts in the cDNA clone is the sequence TATAAA. This is not the most common of polyadenylation signals but is used occasionally (Manley, 1988). When the MacVector™ map program was used to predict the restriction map of the cDNA, it showed very close correspondence to that obtained using restriction enzymes.

The MacVector™ was also used to identify ORF and the predicted polypeptide. Figure 6.8 also shows that this cDNA contained a long ORF with a hypothetical translation product of 223 amino acids. This ORF is bounded at the 5' end by a potential ATG start site at DNA residues 64-66 and terminated at the 3' end by a TAG stop codon encoded by DNA residues 733-735. The remainder of the cDNA is presumed to be the 3' untranslated region. But the deduced amino acid sequence data available does not identify pMY8 as being related to other proteins in the BLAST, Swiss-Protein, or Pro-Site database. The gene, therefore, appears to encode a novel protein possibly involved in neural function because the Northern blot shows that this clone has a head-elevated expression (see section 6.4.1).

```
GCCCTGTAGTTTTTCACCGAAAAGGGTTTTGCTCCACGAACAGCAGCTAGAGGA   54

ACTTCAAAGATGGGAGCCCGACAGTCTCAATCCCGCGAGCCCCGAACCGTTTCA  108
          M  G  A  R  Q  S  Q  S  R  E  P  R  T  V  S     15
ATGGAAAACCCAACTCCTGCCGGCGTTATTGATATATCCGACGATGTGGTCAAG  162
 M  E  N  P  T  P  A  G  V  I  D  I  S  D  D  V  V  K     33
CGACTGAAGGCGGGAATATCCCAGCAGGCTCATGAGCACGCAGCCGCTGCGGAG  216
 R  L  K  A  G  I  S  Q  Q  A  H  E  H  A  A  A  A  E     51
GAATCGAAACCAGTGCCCAAGCCAACGACGAAGGCTGCCGCCAAGCCAGCCGCA  270
 E  S  K  P  V  P  K  P  T  T  K  A  A  A  K  P  A  A     69
TCCTCGCCAGCTGCTTCTCCTGCTCCGAAAGTATCCTCCTATCCCGCTGCAGTG  324
 S  S  P  A  A  S  P  A  P  K  V  S  S  Y  P  A  A  V     87
CCCATTTACGTCCAGGGAGGAGGACACACCATTAGCGCGGCCGATGTGCAGCGC  378
 P  I  Y  V  Q  G  G  G  H  T  I  S  A  A  D  V  Q  R    105
CAGATGAACCAGGAGCTAATCAAGAATGACGAGCTGTGGAAGGAGCGCATGGCG  432
 Q  M  N  Q  E  L  I  K  N  D  E  L  W  K  E  R  M  A    123
AAGCTGGAGGAGAACCTCAAGAAGACCAACACCATCCTGGAGAAGGAGTACGCC  486
 K  L  E  E  N  L  K  K  T  N  T  I  L  E  K  E  Y  A    141
AATGCTGTAGAAAATGTGCACAAGCGATTCGTCAGCACCGCGTCGTCGCACAAA  540
 N  A  V  E  N  V  H  K  R  F  V  S  T  A  S  S  H  K    159
GTGCCTCCCTGCCAGGACCTGAAATCCCAGCTGCTTGCCTGCTACCGCGCGCAT  594
 V  P  P  C  Q  D  L  K  S  Q  L  L  A  C  Y  R  A  H    177
CCCGGGGAGACCTTGAAGTGCATGGAGGAGGTGGCCCAATTCCGACAGTGCATC  648
 P  G  E  T  L  K  C  M  E  E  V  A  Q  F  R  Q  C  I    195
GATCTACATCGCGTCCAGAAGCTGGATGCGGAACCAGAGTCGTCGAAAGCGACC  702
 D  L  H  R  V  Q  K  L  D  A  E  P  E  S  S  K  A  T    213
TCCAAGGCCACCGTTCCTGCCAAGGCGGCCTAGGATCCTGGCAGATCCTTTTTG  756
 S  K  A  T  V  P  A  K  A  A  <                         223
TCCTGATTTGTTGACTTTTCTTTGAGGCCCCTTATGGGTGTTGTATAAACGATT  810

GAGCGATCGATGGGGATGCACTCAAAACTTAAGCAAAAGTGTCATTGCGAGCGA  864

TAGTAAACTAACTTAAATGAATCAAAAAAAAAAAAAAAAAAA              907
```

Figure 6.8 The nucleotide and derived amino acids sequence of the cDNA clone pMY8

Shown is the complete sequence of cDNA clone pMY8 with its hypothetical 223 amino acid translation product. Numbering of the DNA starts at the most 5' end base of the cDNA and numbering (in bold) of the protein starts at the first methionine of the open reading frame. The putative polyadenylation signal sequences is underlined.

6.4 Northern Analyses

In order to investigate the above genes expression in the *Drosophila*, Northern blots are performed to analysis these cDNA clones.

6.4.1 Northern Blotting Using Head and Body mRNA

The whole insert from pMY8 was excised using *Eco*RI and *Hind*III and used to probe a Northern blot made with both head and body poly(A)$^+$ mRNA. The resulting auto radiograph (Fig. 6.9a) shows hybridising transcripts to the pMY8 probe were found abundantly in the head but relatively weakly in the body. By comparison to the *rp*49 (O'Connell and Rosbach, 1984) load control, the head RNA was approximately evenly loaded relative to that of the body, suggesting pMY8 has a head-elevated expression. From the Northern blot estimates of the band size is around 0.9 kb. As the cDNA pMY8 insert is about 0.9 kb, it is very likely that this clone contains a full length copy of the message. This is also supported by sequence data (Fig. 6.8).

Figure 6.9b shows a Northern blot hybridised with a cDNA probe generated from the whole insert sequence of pMY51 (ALP). The probe clearly identifies at least two transcripts in both head and body. They are approximately 2.0 kb and 3.7 kb. It can be seen from blot that there are similar transcripts expression in the head and body. This blot was reprobed with *rp*49 as a loading control. By comparison to the *rp*49 load control the head RNA was approximately evenly loaded relative to that of the body, suggesting that "alkaline phosphates" gene in *Drosophila* has expression in both head and body. This agrees with the result of *in situ* hybridisation to tissue sections (see section 6.5.2).

The head and body Northern blots probed with the insert from pkMY4 of calcineurin A1 failed to detect a clearly discernible band (data not shown). Guerini *et al.*, (1992) reported the same result. Presumably this gene is expressed at very low levels. It may be a specialised form required in only a few cell type at specific time during

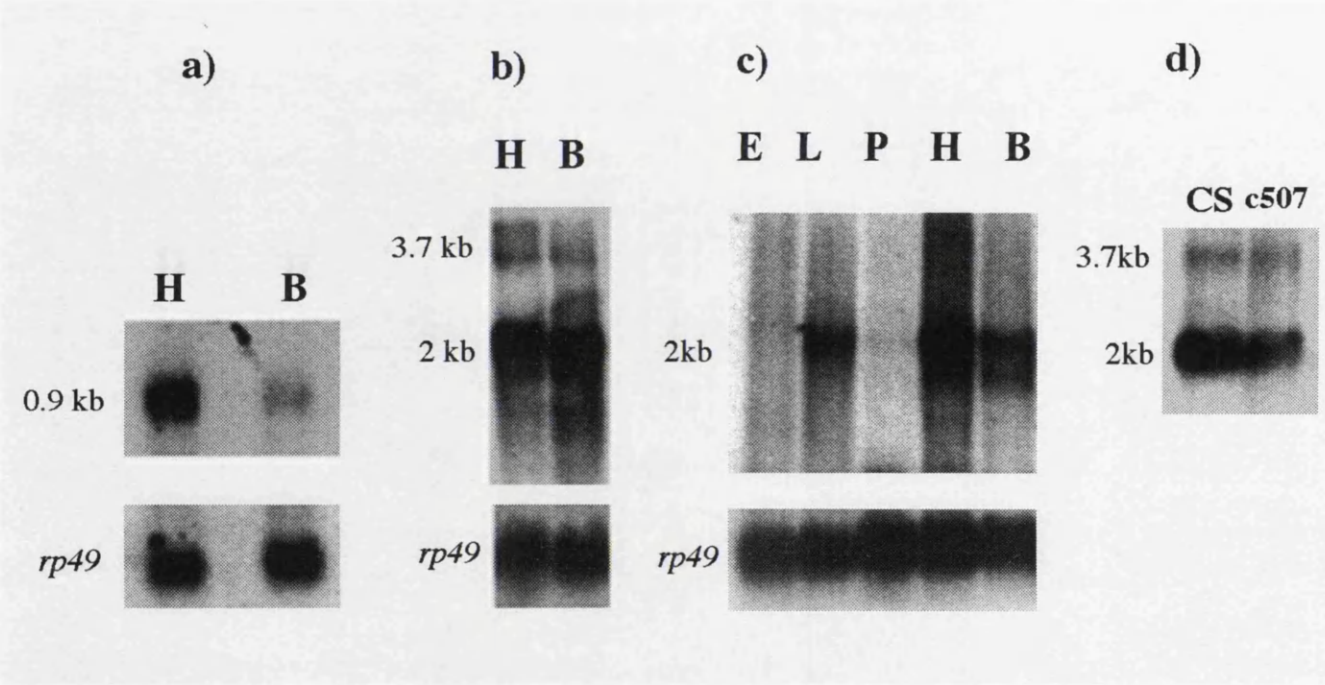


Figure 6.9 Northern blots analysis

(**a**). RNA blot of adult head and body poly(A)$^+$ RNA (10 µg per lane) were hybridised to a cDNA probe from pMY8. (**b**) RNA blot of adult head and body poly(A)$^+$ RNA (10 µg per lane) were hybridised to a whole insert probe generated from the pMY51 clone. (**c**) A Northern filter containing 10 µl of mRNA from each of the stages indicated was hybridised to a whole insert probe from pMY51. The mRNA was prepared from *Drosophila* Canton-S mixed embryo (E), mixed third instar larvae (L), mid-pupal (P), adult head (H) and adult body (B). Subsequent these filters were reprobed with a ribosomal protein probe, *rp49 Eco*RI-*Hin*dIII fragment, as a loading control. (**d**) The poly(A)$^+$ RNAs (10 µg per lane) prepared from the P[GAL4] line c507 and wild type strain (CS) were hybridised to a whole insert probe from pMY51.

development.

6.4.2 A Developmental Northern Blot

The cDNA clone pMY51 was used to probe Northern blot of the mRNA prepared from various developmental stages, e.g. the mixed embryo, mixed third instar larvae, mid-pupal, adult head and adult body of the Canton-S wild type. The blot result is shown as figure 6.9c, together with an autoradiograph produced after the blot was re-probed using *rp*49 as a loading control. The developmental RNA blotting experiment indicated that the abundance of alkaline phosphatase RNA was expressed during the larvae and adult stages. But no detectable transcription was observed in embryo and pupal stages. This may imply that the gene has not switched on during the embryonic stage and thus it will start to express at the larvae stage. The dramatic increase of alkaline phosphatase activity in third instar larvae before the secretion of pupal cuticle suggested a role for this enzyme in cuticle formation and possibility of its regulation by the ring gland (Schneiderman *et al.*, 1966). Afterwards, the gene expression reduces to undetectable level by Northern blot during pupal stages. Then the gene has abundant expression in the adults. It is important for adults to have transport function in the brain and the Malpighian tubules (see section 6.6). The developmental Northern observation is in good agreement with the observation of the development study using X-Gal staining in section 4.5. It also supports the earlier data that alkaline phosphatase activities in insect, are highest during the active larval and early adult stages and lowest during pupation (McComb *et al.*, 1979).

6.4.3 Northern Analysis for Line c507 and Wild Type Flies

To determine whether insertion of P element has disturbed the gene expression, Northern blot was performed. In this experiment, the poly(A)$^+$ RNA prepared from wild type (Canton-S) and P[GAL4] line c507 flies was hybridised with cDNA probe generated from a whole insert of pYM51. The result (Fig. 6.9d) shows that these lanes have hybridisation signals at about 2 kb. No difference between wild type and line c507 was

observed. This is because that the P[GAL4] was not inserted into the gene of this line, so insertion didn't disturb the gene expression and its function. But the imprecise excision can be used to generate total loss-of-function alleles (Engels, 1989). The imprecise excision experiment is being carried out.

6.5 *in situ* Hybridisation

in situ hybridisation techniques are used to localise specific nucleic acids sequences in morphologically preserved chromosomes, cells or tissue sections. In combination with immunocytochemistry, *in situ* hybridisation is capable of relating microscopic topological information to gene activity at the DNA, mRNA and protein level.

6.5.1 Localisation of the pMY51, pMY8 and pkMY4 cDNA clones to Polytene Chromosomes

In order to ensure the cDNA fragments corresponding to the flanking regions of P[GAL4] insertion, polytene *in situ* s were performed as described in section 2.3.3 using the whole inserts of pMY51, pMY8 and pkMY4 as probes, respectively. When these probes were hybridised to polytene chromosome of wild type flies, the hybridisation signals were seen in the band 100B on the third chromosome (Fig. 6.10). This band is the identical region as the P[GAL4] element insertion (section 3.5).

Guerini *et al.*, (1992) localised the calcineurin A1 gene into 21EF on the second chromosome. However we found this gene located at the third chromosome. A more likely explanation is that they may have made wrong chromosomal localisation. By comparison to the sequence from both of us, we cloned the same gene. It should be at the same position on the polytene salivary gland chromosome because the genomic southern has shown only one copy of this cDNA in the *Drosophila* (see section 6.2.4.) Another evidence that the P[GAL4] was inserted into the third chromosome came also from genetic crosses for line 507. Furthermore, both cDNA pMY51 and pkMY4 hybridised genomic DNA clone λ6

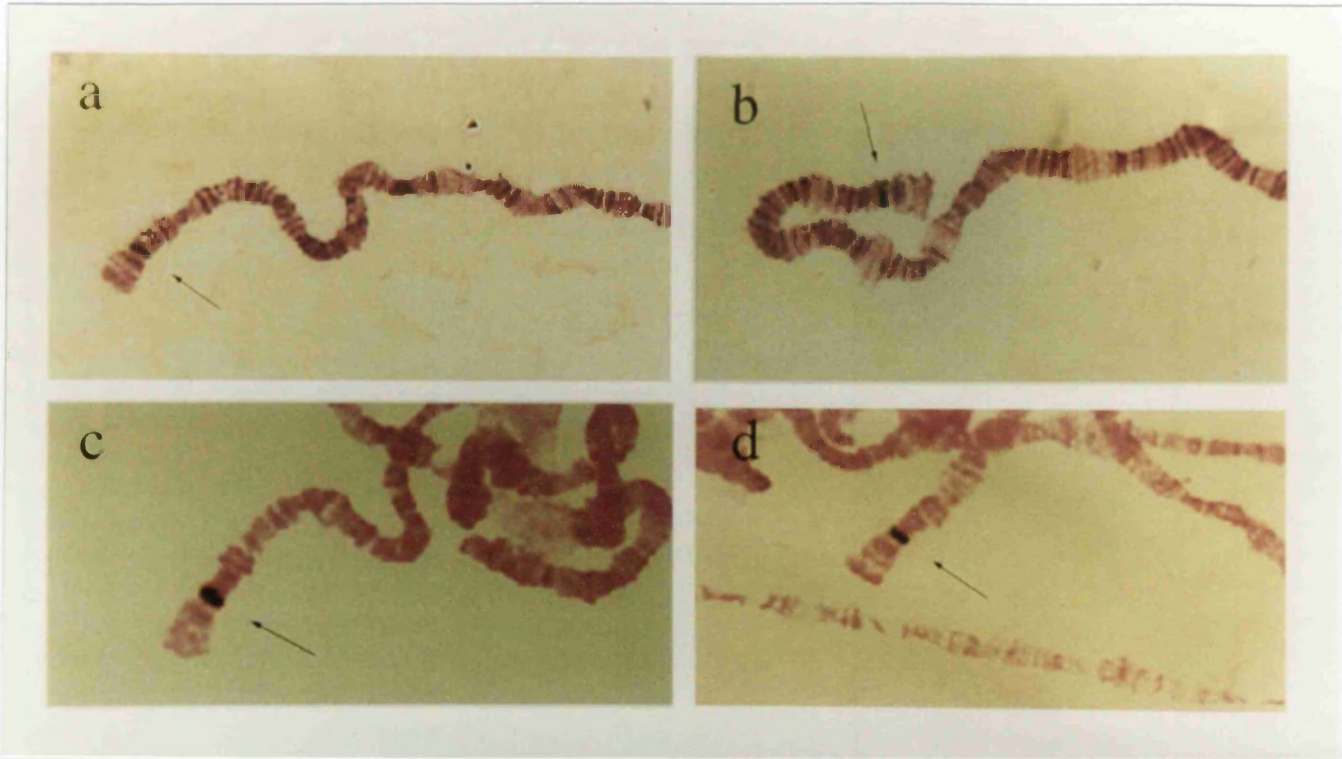


Figure 6.10 Polytene chromosomal *in situs* using different probes from pBluescript, pMY51, pMY8 and pkMY4 cDNA clones.

Arrows indicate the position of the hybridisation signals at 100B on the third chromosomes. **A** shows a set of chromosomes from line c507 displaying the hybridisation signal using pBS probe, which hybridises the P[GAL4] element. **B, C and D** show a different set of polytene chromosomes from wild type strain displaying the hybridisation signals as same position as **A** when probes from the pMY51, pMY8 and pkMY4 cDNA clones are used respectively.

(section 5.5). Therefore, we can say positively that calcineurin A1 gene is located at 100B on the third chromosome rather than at 21EF.

6.5.2 Co-localisation of *LacZ* and Alkaline Phosphatase Gene Expression

With respect to the enhancer trap system, it makes the assumption that expression pattern of the reporter gene will reflect that of a nearby *Drosophila* gene. In other words, a relevant endogenous gene should have same expression pattern as that of the reporter. In the case of line c507, GAL4-directed ß-gal expression patterns were found in the ellipsoid body of the brain and Malpighian tubules in body. In order to determine whether the alkaline phosphates gene cloned by plasmid rescue has same expression patterns in wild type fly, *in situ* hybridisation to the head and body using digoxygenin labelled RNA probes was performed. The DIG RNA probes were generated from pMY51 according to Boeringer Mannheim Digoxygenin (DIG) labelling kit and hybridised to 14µm cryostat head and body sections (described as in section 2.3.1) The hybridisation patterns were detected using the NBT/X-Phosphate colour reaction. Positive and negative controls are performed along side the pMY51 *in situ* and for this other clones (as negative) and pST41 insert (a Opsin clone as a positive control) were used.

Figure 6.11 shows that co-expression of alkaline phosphatase gene in the cell bodies of the ellipsoid body and the cell bodies of the Malpighian tubules with X-gal staining by *in situ* hybridisation to tissue sections. Therefore, it is reasonable to say that the gene relevant to enhancer trap element has been found.

As above *in situ* hybridisation using RNA or DNA probe only allows the reaction to occur in the nuclear of the cell bodies, it would not be expected to see the ring shape (axonal projections) of the ellipsoid body. Hybridisation signals can only be seen in the cell bodies of the ellipsoid body and the Malpighian tubules. If antibodies against relevant genes are available, the antibody staining can be used to improve result.

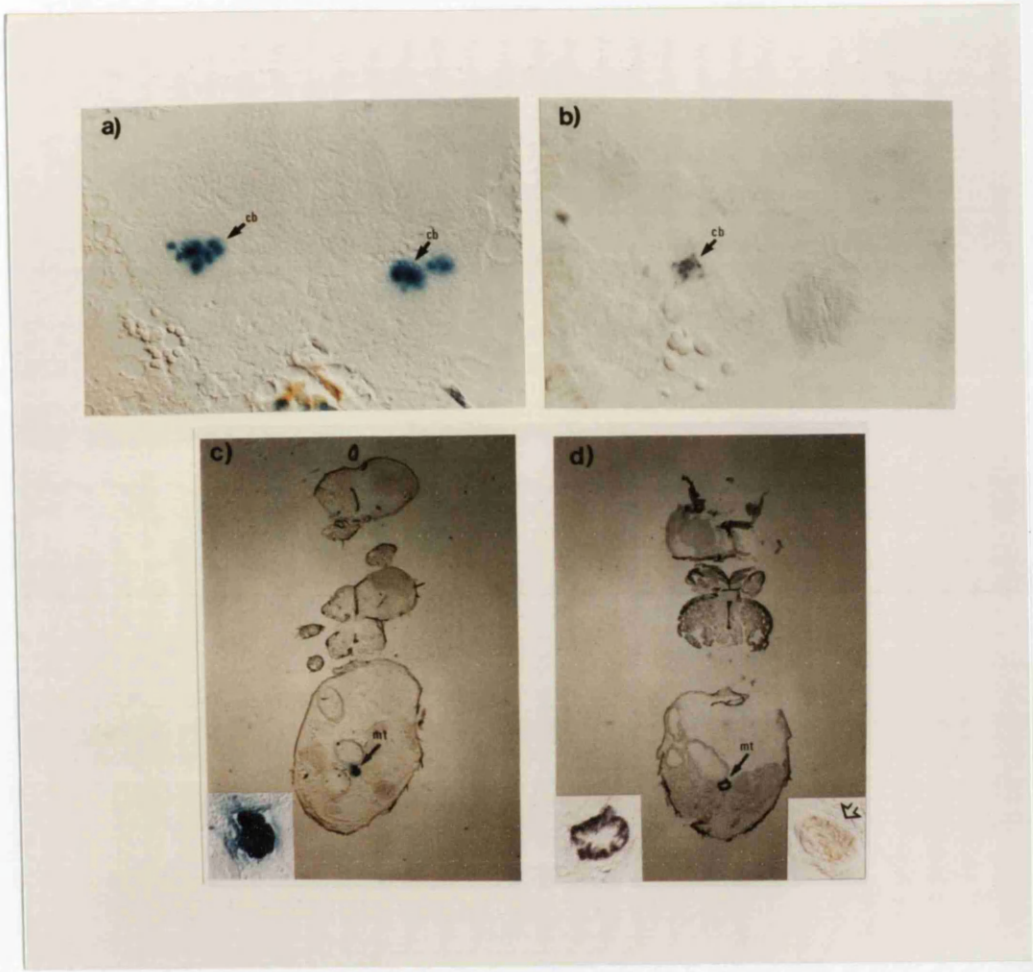


Figure 6.11 Co-localisation of expression patterns in the tissue sections by *in situ* hybridisation .

(**A**) In P[GAL4] line c507, the cell bodies of the ellipsoid body (*eb*) are stained by X-Gal. (**B**) For wild type fly, the coexpression of pMY51 RNA is detected in the same cell bodies of the *eb* by DIG-RNA labelling probe. The hybridisation signals are arrowed. (**C**) In P[GAL4] line c507, the Malpighian is stained by X-Gal. (**D**) For wild type fly, the coexpression of pMY51 RNA is detected in the same tissue by DIG-RNA labelling probe. The hybridisation signals are arrowed. The closer view of hybridisation regions are stuck in the corners of photos. The open arrow indicates the hybridisation signals with different colour detection system.

During the colour detection of this experiment, the fresh lavamisole was used to block endogenous phosphatase expression (McComb *et al.*, 1979), even though residual alkaline phosphatase activity was usually lost during hybridisation. On the other hand, in order to avoid directly staining by the NBT and X-phosphate (J. Dow, per comm.), different colour detection system with diaminobenzidine (DAB)/H_2O_2 (as described method in section 2.3.1) was used to confirm the result (Fig. 6.11).

Whole mount embryonic *in situ* was performed using DIG labelled RNA probe from pMY51. No expression patterns were detected. This result was consistent with that of X-Gal staining and developmental Northern blot.

On the other hand, when whole insert from pkMY4 (calcineurin A1) was used as a DIG probe for *in situ* hybridisation to the adult head and body sections, no staining patterns were matched with the patterns stained by X-Gal. In the head sections, staining was found in all the cortex layer of the brain but pale staining pattern was observed in the body (data not shown).

Taken together these data suggest that a "correct" enhancer was trapped and "correct" gene, *Drosophila* alkaline phosphatase, was identified by an enhancer trap approach.

6.6 Conclusion

We have successfully identified and cloned the genes flanking P[GAL4] element of line c507 by enhancer trap system. Three different genes located at 100B were cloned and characterised. One of them is called calcineurin A1, the Ca^{2+}/calmodulin-stimulated protein phosphatase (Guerini *et al.*, 1992) which is located at the upstream of the P[GAL4] element. Other two closely linked genes are localised at the downstream of the P[GAL4] element. The cDNA clone pMY51 near to the P[GAL4] has a significant homology to alkaline phosphatase from a variety of organisms. And other cDNA clone pMY8 which sits

further away from P[GAL4] has a head-elevated expression judged by Northern blot, but it is yet to be identified as being a member of a known gene family. *in situ* hybridisation to tissues sections of flies reveals that the cDNA clone pMY51 is from a nearby gene which has same expression patterns with those stained by X-Gal. This gene, *Drosophila* alkaline phosphatase, has expression patterns in the ellipsoid body of the brain and in the Malpighian tubules of *Drosophila* . What is it possible function in the *Drosophila* ellipsoid body and Malpighian tubules ?

Although the function of alkaline phosphatase is not well understood, a large number of experiments demonstrate its possible functions, such as hydrolysis of phosphate esters at high pH, transfer of phosphates from a high-energy to a lower-energy state, transport of a number of substances (e.g. Na^+, K^+, inorganic phosphate,) across membranes (McComb *et al.*, 1979).

Alkaline phosphatase activities in the brain are generally lower than those in liver and kidney, however, its distribution in the brain has been reported in different species (McComb *et al.*, 1979). More specific to the nervous tissue, intense alkaline phosphatase activity has been located in nerve cell bodies and processes in some parts of central and peripheral nervous systems of various animals such as mouse (Sood and Mulchandani, 1977), rat (Nandy and Bourne, 1963), guinea-pig (Song *et al.*, 1994), monkey (Friede, 1966) and fish (Sood and Sinha, 1983). At the ultrastructural level, the enzyme has been localised on the outer surface of plasma membranes of nerve cell bodies and dendrites (Mayahara et al., 1967; Mori and Nagano, 1985), postsynaptic membrane and synaptic vesicles (Sugimura and Mizutani, 1979). Biochemical studies also suggested that alkaline phosphatase is associated with synaptic vesicles isolated from bovine cerebral cortex (Zisapel and Haklai, 1980). In view of these facts that the ALP reactivity has been localised to discrete subsets of neurones in different species, it is suggested that the ALP has a function related to particular features of the reactive neurones. From all available evidence, it appears that the ALP may play some role in transmembrane transport and cell differentiation or proliferation in the nervous tissue. Moreover, the synaptic alkaline phosphatase may play

an important role in the storage and release of transmitter, postsynaptic reception of chemical stimuli, and Ca^{2+} transport following stimulatory excitation of the membrane.

In our preparations, X-Gal and "alkaline phosphatase" cDNA clone have co-expression patterns in the ellipsoid body of the brain and the Malpighian tubules of the abdomen in *Drosophila* (see section 6.5.2). Therefore, it is reasonable to say that the ellipsoid body neurones are alkaline phosphatase-reactive neurones which have the same function as discussion above. More specifically, it is possible that the ALP is functional in the storage and release of transmitter in neurones of the ellipsoid body. On the other hand, In vertebrates, evidences show that alkaline phosphatase is related to absorption, transport and secretion in intestine and kidney (McComb *et al.*, 1979). It is therefore not surprising that the alkaline phosphatase distributes in the Malpighian tubules of *Drosophila* abdomen because this tissue has similar functions as vertebrates, e.g. transport, secretion and absorption (Maddrell and O'Donnell, 1992; Dow *et al,* 1994).

Chapter 7

Discussion

7.1 Introduction

7.2 General discussion

7 3 Future work

7.1 Introduction

Although most of the results were discussed within their own chapters, this general discussion aims to link relevant finding from different chapters and to assess their significance. It also attempts sum up the questions that have arisen in light of the data presented and suggestion of future work which would address these question.

7.2 General Discussion

The enhancer trap technique has allowed us to study structures of the *Drosophila* brain and identify some genes relevant to the central complex function in the brain.

Over 1400 of P[GAL4] enhancer trap lines have been analysed. They show a great variety of ß-galatosidase expression patterns, reflecting the influence of many different genomic regulatory elements on the P[GAL4] reporter gene. More than 300 display interesting patterns in the brain from an anatomical perspective. Of these, as many as 100 are more or less restricted to specific regions or neuronal sub-populations of brain. It is obvious that these P[GAL4] lines are a resource that can be exploited in a number of different contexts including: domain specific expression of toxins or inhibitors, domain specific rescue of behavioural mutations, neuro-anatomical studies etc. (Sweeney *et al.*, 1995; Hidalgo *et al.*, 1995; Yang *et al.*, 1995; Ferveur *et al.*, 1995; O'Dell *et al.*, 1995; Smith and Shepherd, 1995)

Many P[GAL4] lines we isolated can be used for various purposes as marker lines for anatomical analysis. The expression patterns of P[GAL4] lines do reveal novel substructures of the neuropil most of which are not apparent in silver stained material. We have been able to observe the subdivision of the *Drosophila* mushroom bodies (Yang *et al.*, 1995) and subdivide the ellipsoid bodies of the central complex into the four genetically distinct regions (see chapter four). Stocker (pers. comm.) also found novel interneurons in

the antennal lobe of the *Drosophila* brain. Smith and Shepherd (1995) reported that they used one of our P[GAL4] line to visualise the central projections of proprioceptive sensory neurons of the thorax and abdomen. In the abdomen, they found GAL4 was expressed in Wheeler's organ and a segmentally repeated array of internal sensory neurons that have not been previously described. In addition to adults, Ito *et al.* (1995) used GAL4 as a marker to reclassify the glial cells during late embryogenesis and generate a three-dimensional map of the glia in the embryonic nervous system. More recently, the green fluorescent protein (GFP) from the jelly fish has been used as a cell marker, combined with P[GAL4] system, to label neurons in living embryos, larvae, pupae and adults for developmental study *in vivo* without invasive manipulation (Yet *et al.*, 1995; Brand, 1995; Y. Yu, Glasow). Taken together, P[GAL4] enhancer trap lines are indeed excellent neuronal markers for anatomical and developmental study.

The GAL4 expression patterns were used to analysis the substructures and development in the *Drosophila* central complex (see chapter four). The expression patterns in neuronal organisation may implicate the multiple integrative role for the central complex. Developmental study revealed that the central complex in *Drosophila* appears from pupal stage to adults. It seems that the central complex has not formed in early stages such as embryo and larvae. This result is consistent with that of other researchers (Hanesch, 1989; S. Renn, personal comm.).

In *Drosophila*, the "central complex" is a term used to describe a series of intimately related neuropils in the midbrain (Hanesch *et al.*, 1989). However, in most insects the "central body" is termed to refer to main neuropils in the midbrain (Williams, 1975; Strausfeld, 1976; Mobbs, 1982; Homberg, 1987). In order to be consistent with other insects, it is suggested that the term of "central body complex" for *Drosophila* should be used in future. It includes four main substructures: the protocerebral bridge (*pb*), the fan-shaped body (*fb*), the ellipsoid body (*eb*) and the paired noduli (*no*) and two accessory structures: the ventral bodies (*vbo*) and the lateral triangles (*ltr*).

Enhancer-trap elements are not mere anatomical markers. Besides revealing intriguing cellular arrangements within the brain, each staining pattern implicates a flanking gene in their functions. A large number of staining patterns provide an abundance of target site for brain function-specific gene actions. To identify the genes relevant to central complex function, seven P[GAL4] enhancer trap lines with staining patterns specific to the central complex were selected. Genomic DNAs flanking each insertion site were cloned by plasmid rescue (Pirrotta, 1986) "Reverse Northern" analysis were performed to identify the transcribed units. Rescued genomic DNAs were used as probes for screening a cDNA head library. Corresponding cDNA clones were isolated

In case of line c507, three different genes around P[GAL4] were cloned and characterised. Insertion site of P[GAL4] was mapped to between genes of the calcineurin A1 and the alkaline phosphatase (see Fig. 6.1).

One of gene located at the upstream side of the P[GAL4] is called calcineurin A1, the Ca^{2+}/calmodulin-stimulated protein phosphatase (Guerini *et al.*, 1992). Calcineurin is a brain enriched phosphatase that plays an important role in the regulation of brain physiology, including synaptic transmission, cytoskeletal dynamics and other calcium-regulated events (Tallant and Cheung, 1986; Klee *et al;*. 1987). This gene is very interesting for our group for further analysis even if it is not relevant to the enhancer trap expression pattern.

Other two closely linked genes are localised at the downstream side of the P[GAL4] element. The 1.8 kb pMY51 cDNA near to the P[GAL4] contains a 356 amino acids ORF producing a predicted polypeptide that shows homology to the alkaline phosphatase from other organisms at protein level (see section 6.3.3). *in situ* hybridisation to tissues sections of flies reveals that this cDNA clone represents a nearby gene which has same expression patterns in the ellipsoid body and the Malpighian tubules with those stained by X-Gal. Northern blot also supports this result. The other cDNA clone pMY8, which sits further away from P[GAL4], has a head-elevated expression judged by Northern blot, but it is yet

to be identified as being a member of a known gene family.

The ellipsoid body of the central complex is mainly though to have an inhibitory role in its function because of distribution of inhibitory neurotransmitter in these neurons (Hanesch *et al.*, 1989; Bausenwein *et al.*, 1994). Localisation of alkaline phosphatase activity in the ellipsoid body also supported this notion as alkaline phosphatase activity was confined to inhibitory motor neurons of the guinea-pig as well (Song *et al.*, 1994).

Alkaline phosphatase (ALP) are glycoproteins and there are at least four isozyme forms of APL in human. They are tissue nonspecific ALP, also known as "liver/bone/kidney' ALP, intestinal ALP, placental ALP and placental-like APL. Each isozyme is encoded by a separate genes. Expression of the intestinal, placental, and placental-like ALPs is limited to a few specific tissues as their names indicate, whereas tissue nonspecific ALP is present in many cell types (McComb *et al.*, 1979; Harris, 1989; Manes *et al.*, 1990). Therefore, the *Drosophila* alkaline phosphatase encoded by pMY51 cDNA can be grouped into "tissue nonspecific ALP". It is not surprising that this ALP express in the ellipsoid body of the brain and the Malpighian tubules of the gut in *Drosophila.* It is controlled by the third chromosome locus, 100B, corresponding to one of *Drosophila* alkaline phosphatase described by Beckman and Johnson (1964). This type ALP is different from other ALP, from adult hindgut, which is controlled by the second chromosome locus Aph-2 (Schneiderman *et al.*, 1966).

It is interesting to find that Alkaline Phosphatase (EC. 3.1.3.1) and Protein Phosphatase (also called calcineurin) (EC. 3.1.3.16) are located in the same region of polytene chromosome at 100B. They are only a few kb away each other. Another kind of phosphatase, Acid Phosphatase(EC. 3.1.3.2) is located at 99C of the same chromosome based on deletion mapping (Frisardi and MacIntyre, 1984). All the above three phosphatases are classified as "phosphoric monoester hydrolases" by IUB for Enzyme Nomenclature in 1978. It is likely that these three similar functional genes are clustered in the same region of the chromosome. A similar phenomenon was observed in human

alkaline phosphatases (Harris, 1989). If this is assumed to be the case, it is possible for us to predict that more "phosphoric monoester hydrolases" genes will be found near that region.

As mentioned before, the P[GAL4] system has allowed to ablate a selected cells by expressing cell autonomous toxin genes under the control of a UAS. In *Drosophila* nervous system, for example, targeted expression of tetanus toxin light chain specifically eliminated synaptic transmission and caused behavioural defects; in one case, the olfactory escape response was reduced (Sweeney *et al.*, 1995). Again, ablation of the interface glia early in development led to a complete loss of the longitudinal axon tracts, and ablation of the glia later in embryonic development resulted in defects comprising of weakening and loss of axon fascicles within the connectives (Hidalgo *et al.*, 1995). For us, such a strategy could be used to ablate the specific cells in the mushroom bodies or central complex of the brain to test their functions and other roles. This system is a simple and efficient method of targeted cell ablation because cell killing is rapid due to the expression of the wild-type toxin, no invasive manipulation of the animal is required and cell ablation is autonomous (O'Kane and Moffat, 1992; Sentry *et al.*, 1993; Hidalgo *et al.*, 1995).

Enhancer trapping, however, is not without problems. It is generally accepted that P elements do not insert randomly in the genome (Engels, 1989; Smith *et al.*, 1993). Some genes are "hot spots" for P element insertion, whereas others are "cold spots". It is, therefore, easy to miss some important genes which are "cold spots" for P element insertion during our screening. On the other hand, the insertion site may also be close to more than one enhancer element and give a false pattern of expression. Consequently, the gene that is isolated may have a pattern of expression that bears little relation to that seen in the enhancer-trap line (Bolwig, *et al.*, 1995).

It is also noted that an interesting expression pattern may not always be indicative of the true domains of genetic function of the endogenous gene. For instance, in the many other eye photoreceptor cells in addition to R7 photoreceptor express *sevenless* even after

their determination (Banerjee *et al.*, 1987). In addition, in an enhancer trap screen one may easily miss or overlook the genes such as *Notch* that are expressed ubiquitously but play important developmental roles in specific regions or cell types.

Nevertheless, the advantages and utilities of the enhancer trap technique outweigh some of its disadvantages. It is, therefore, widely used in *Drosophila* biology.

7.3 Future Work

Using P[GAL4] enhancer trap system, we have successfully analysed some of the lines for their expression patterns in mushroom bodies and central body complex of *Drosophila* brain (Yang *et al.*, 1995; Armstrong *et al.*, submit; also see chapter four). However, a large collection of P[GAL4] lines still need to be characterised in details for expression patterns in other substructures of the brain when our anatomical knowledge accumulates in further.

As mentioned above, P[GAL4] enhancer trap system allows us to cross different UASG-marker for function analysis. For example, the next thing I would like to do is to cross these P[GAL4] lines with UASG-toxin for cell ablation to address range of questions concerning the function and development of specific groups of cells (O'Kane and Moffat, 1992; Sentry *et al.*, 1993; Sweeney *et al.*, 1995; Hidalgo *et al.*, 1995). More evidence will be added to support the hypothesise that different genetic identities reflect different functional roles in the brain.

Now that genes have been successful cloned and sequenced (see chapter six), there are many further experiments which need to be carried out.

In order to map the precise position of these genes to genome and find the potential intron/exon boundaries, corresponding genomic DNA will be sequenced completely.

A cDNA clone pMY51 encoded alkaline phosphatase in *Drosophila* and other cDNA clone pkMY4 encoded calcineurin A1 gene were identified by P element insertion. However, we have not observed any abnormalities phenotype in the homozygotes as the direct result of transposon insertion . The reason is that the P[GAL4] was not inserted into the genes themselves but just flanking these genes. In order to study the function of these genes in *Drosophila*, it is useful to generate flanking deletions so as to produce true nulls (Tsubota and Schedl, 1986; Salz *et al.*, 1987). Candidate lines will be analysed genetically, by Southern blot and Northern blot or PCR approach analysis, and where the deletions are non-lethal for anatomical and behavioural abnormalities. This imprecise excision experiment is being carried out.

On the other hand, to make new mutants, P[GAL4] insertion can be used for the local jumping (Tower *et al.*, 1993; Zhang and Spradling, 1993) to re-mobilise P element into the alkaline phosphatase gene or calcineurin A1 gene. These mutants will be analysed further. Now the mutational studies are facilitated by the tag that the gene carries.

The clone pMY8 is yet to be identified as having homologies to known *Drosophila* genes or to genes from other organism. But its expression pattern in the Northern blot is of continuing interest in the laboratory. It would be very informative to do a more complete study of the expression of this locus.

In short, P[GAL4] enhancer trap lines are a rich resource and can be used for *Drosophila* research in many aspects in the future.

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Appendix 1. Summary of DNA Clones

Listed are the DNA clones including the rescued plasmid clones, λ Genomic clones and cDNA clones used during this thesis.

A 1.1. Rescued plasmid clones

line	clone number	approx. size	description & comments
c61	pPC61	4.8 kb	pBS + DNA insert
c61	pKC61	14.3 kb	pBS+*w*+GAL4 + DNA insert
c105	pPC105	4.3 kb	pBS + DNA insert
c105	pKC105	25.9 kb	pBS+*w*+GAL4 + DNA insert
c161	pPC161	4.3 kb	pBS + DNA insert
c161	pKC161	14.5 kb	pBS+*w*+GAL4 + DNA insert
c232	pPC232	3.8 kb	pBS + DNA insert
c507	pPC507	7.6 kb	pBS + DNA insert
c507	pKC507	17.9 kb	pBS+*w*+GAL4 + DNA insert
c561a	pPC561a	4.3 kb	pBS + DNA insert
c561a	pKC561a	25.9 kb	pBS+*w*+GAL4 + DNA insert
c819	pPC819	3.3 kb	pBS + DNA insert

A 1.2. Genomic lambda DNA clones

line	clone number	approx. size of insert	description & comments
c507	λ1(c507)	14 kb	isolated from GEM-11
c507	λ2(c507)	14 kb	isolated from GEM-11
c507	λ3(c507)	14 kb	isolated from GEM-11
c507	λ4(c507)	14 kb	isolated from GEM-11
c507	λ5(c507)	13.5 kb	isolated from EMBL3
c507	λ6(c507)	14.5 kb	isolated from EMBL3
c161	λ3(c161)	12.4 kb	isolated from EMBL3
c161	λ4(c161)	16 kb	isolated from EMBL3

A 1.3. cDNA clones

line	clone number	approx. size of insert	description & comments
c507	pMY3	0.75 kb	E/H frag. in pBluescript
c507	pMY5	0.8 kb	E/H frag. in pBluescript
c507	pMY8	0.9 kb	E/H frag. in pBluescript
c507	pMY10	0.6 kb	E/H frag. in pBluescript
c507	pMY51	1.8 kb	E/H frag. in pBluescript
c507	pkMY2	0.55 kb	E/H frag. in pBluescript
c507	pkMY3	0.6 kb	E/H frag. in pBluescript
c507	pkMY4	0.7 kb	E/H frag. in pBluescript
	rp49	0.6 kb	E/H frag. in pBluescript
	λ41	1.5 kb	in NM1149
	α1 tubulin	1.5 kb	α1 3' in pBR322