BEHAVIOURAL AND MOLECULAR STUDIES OF THE *DROSOPHILA* BRAIN USING THE P[GAL4] ENHANCER-TRAP

A thesis submitted for the Degree of Doctor of Philosophy
at the University of Glasgow.

by

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Abbreviations

amp	ampicillin
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indoyl-phosphate
bp	base pair
BSA	bovine serum albumin
Ci	Curies
cDNA	complementary DNA
°C	degrees Centigrade
DEPC	diethyl pyrocarbonate
DNA	2' deoxyribonucleic acid
DNaseI	deoxyribonuclease I
dATP	2' deoxyadenosine triphosphate
dCTP	2' deoxycytidine triphosphate
dGTP	2' deoxyguanosine triphosphate
dNTP	2' deoxy(nucleotide) triphosphate
dTTP	2' deoxythymidine triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid (disodium salt)
EtBr	ethidium bromide
g	grams
g	centrifugal force equal to gravitational acceleration
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs (10^3 bp)
kDa	kilodalton (10^3 dalton)
l	litres
M	Molar

µCi	microCuries
mg	milligrams
µg	micrograms
ml	millilitres
µl	microlitres
mM	millimolar
µM	micromolar
MOPS	3-morpholinopropanesulfonic acid
mRNA	messenger ribonucleic acid
NaPPi	sodium pyrophosphate
NBT	4-nitrobluetetrazoliumchloride
ng	nanograms
nmol	nanomoles
OD	optical density
PEG	polyethylene glycol
pfu	plaque forming units
pH	acidity [$-\log_{10}$(Molar concentration of H^+ ions)]
pmol	picomoles
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
Tris	Tris (Hydroxymethyl) aminomethane
U	units
UV	ultra violet light
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Summary

The P[GAL4]/UAS$_G$ enahancer-trap system of *Drosophila* has been used to determine the cell-type specific expression patterns of genes flanking the transposon, and also to target expression of any specific desired gene products to the marked cells. In this thesis, P[GAL4] enhancer-trap lines with specific patterns of expression in the adult brains have been used to address the relationship between neural structure and function of sexual orientation and to identify enhancer-trapped genes according to their patterns of expression.

Gynandromorphs of specific sub-domains within the male brains of 24 P[GAL4] lines were generated by GAL4-mediated expression of female-specific *transformer (tra)* transcripts, and the subsequent courtship behaviour towards male and female targets were tested. Feminisation of the mushroom bodies, which are thought to be involved in the olfactory pathway, appears to lead to non-discriminatory behaviour.

The mini-*white*$^+$ gene (in heat-shock construct pHSBJCaSpeR) has been reported to lead to male-male courtship (Zhang and Odenwald, 1995). As the mini-*white*$^+$ gene is used as a marker in both P[GAL4] and UAS$_G$-*tra* constructs, male-male courtship was tested by mutagenesis of the mini-*white*$^+$ gene in both the UAS$_G$-*tra* line and the P[GAL4] line 201Y which has specific expression in the mushroom body and shows a transformed bisexual behaviour when *tra* is expressed through the GAL4/UAS$_G$ system (O'Dell, et al., 1995). Significant male-male courtship was only observed in 201Y mini-*white*$^+$ homozygotes, but not 201Y mini-white $^{+/-}$ heterozygotes. Mini-*white*$^+$ has no such effect in the UAS$_G$-*tra* line. All the 201Y/UAS$_G$-*tra* flies, no matter whether they were homozygous or heterozygous for mini-*white*$^+$, or lacked it entirely, displayed bisexual behaviour, which shows that the transformed behaviour of line 201Y is absolutely determined by *tra* expression, and not a consequence of mini-*white*$^+$. Further, RT-PCR examination of the transformed dorsal brain tissue in male 201Y showed the female

transcripts of *doublesex (dsx)* and *fruitless (fru)* beside the male transcripts of these genes, providing evidence of the expression and functioning of female *tra* in the targeted cells.

An analysis of the genomic DNA flanking 10 P[GAL4] insertions were carried out by plasmid rescue. These lines have brain specific expression patterns of the mushroom bodies, the central complex and the optic lobes. Detailed genomic restriction maps around the insertion sites were generated of mushroom body expression lines c739 and c772, and line c819 which shows expression in the ellipsoid body of the central complex.

The cDNA of the AMP-activated protein kinase (AMPK) γ subunit gene was isolated by cDNA library screening using the downstream region of the c819 insertion as a probe. The central complex of the *Drosophila* brain has been shown to act as a higher centre for locomotor activity and other behaviours. Anti-β-gal antibody staining shows the expression in the central complex of line c819 begins at later pupal stages and continues to the adults. Developmental Northern and tissue *in situ* hybridization in the brain show that the AMPK γ gene is expressed from the pupal stage and seems to have a specific expression pattern in the cell bodies of the ellipsoid body and the optic lobe. These results imply that the *Drosophila* AMPK γ enhancer is likely to have been trapped by the c819 insertion and the corresponding gene has been cloned through the P[GAL4] enhancer-trap.

Chapter 1

Introduction

1.1 The Genetics of courtship behaviour in *Drosophila*

Over the last century, the fruit fly *Drosophila melanogaster* has been used as a major research animal in the field of genetics, from the discovery of some basic principles of classic genetics to the recent achievements of molecular biology. It remains a favourite experimental animal because of its small (but not too small) size, ease of culture, short generation time, large number of progeny, low chromosome number, and giant salivary chromosomes. Through the intensive research of these decades, a large number of easily recognisable genetical markers have been discovered and provide adequate tools. The collection of data in Flybase (http://flybase.edu) contains information on 38,000 alleles of more than 11,000 genes, 7,000 genomic clones and so on. Among metazoan organisms, *Drosophila* is perhaps unique in its sophisticated genetics and readily applied genetic techniques, due to which it has proven invaluable as an aid to study the mechanism of the nervous system.

Compared to the size of the 100 billion neuron human brain, the structure and function of the *Drosophila* brain is relatively simple. Complex traits such as circadian rhythm and behaviour can also be studied and *Drosophila* has been proven to be a useful model system for the genetic analysis of behaviour. It is possible to systematically screen for genes that can mutate to produce a given phenotype. Locomotor behaviour (Strauss and Heisenberg, 1993), and, even more complicated, learning and memory (Tully, 1991) have been studied. Among the behaviours, courtship receives lots of attention as the process consists of a series of actions, each of which is accompanied by the exchange of visual, auditory, olfactory, tactile and chemosensory signals between males and females (Hall, 1994; Spieth and Ringo, 1983).

Flies isolated as eggs and kept singly until maturity are capable of recognising an appropriate target, and of directing the entire routine of sex-specific behaviours. It is therefore clear that the ability to perform sex-specific behaviour is genetically

programmed into the individual. It might be hoped that a classical genetic study of courtship would reveal mutants and help us understand how genes act to build and operate a nervous system.

1.1.1 An overview of *Drosophila* courtship behaviour

The courtship in *Drosophila melanogaster* involves a series of behaviours (Fig. 1.1; Hall, 1994; Spieth and Ringo, 1983; Jallon, 1984; Cline and Meyer, 1996). The first step is orientation (Fig. 1.1A). Once the male senses a potential mate within some reasonable proximity (perhaps on a food source or when experimentally put together in a mating chamber), he slightly elevates his body, turns to face the potential mate, and approaches it. The response of the potential mate to the male's behaviours results in information being transmitted which enables the two individuals to distinguish conspecifics from non-conspecifics, males from females.

Having made physical contact with the potential mate, usually by tapping, the male moves to the rear of the approached individual and positions himself close to and facing the tip of the target's abdomen. If the target is a virgin female, he follows her during most of the time that she is moving (no courtship occurs in flight). As the male orients to a stationary female, including circling her, or follows a mobile one, he frequently displays one wing or the other and flutters. The vibration of the wing produces a species-specific rhythm, the so-called "love song" (Fig. 1.1B).

Several seconds to a few minutes after the two flies have begun to interact, the male extends his proboscis and licks the female's genitalia (Fig. 1.1C). This action is almost immediately followed by the males first copulation attempt, which involves an abdominal bending by the male (Fig. 1.1D; E). If an attempt fails, the male may cease courting for some moments. Thus, overt courtship interactions occur about 60 to 80% of the time when the male and female are together (Tompkins, 1984).

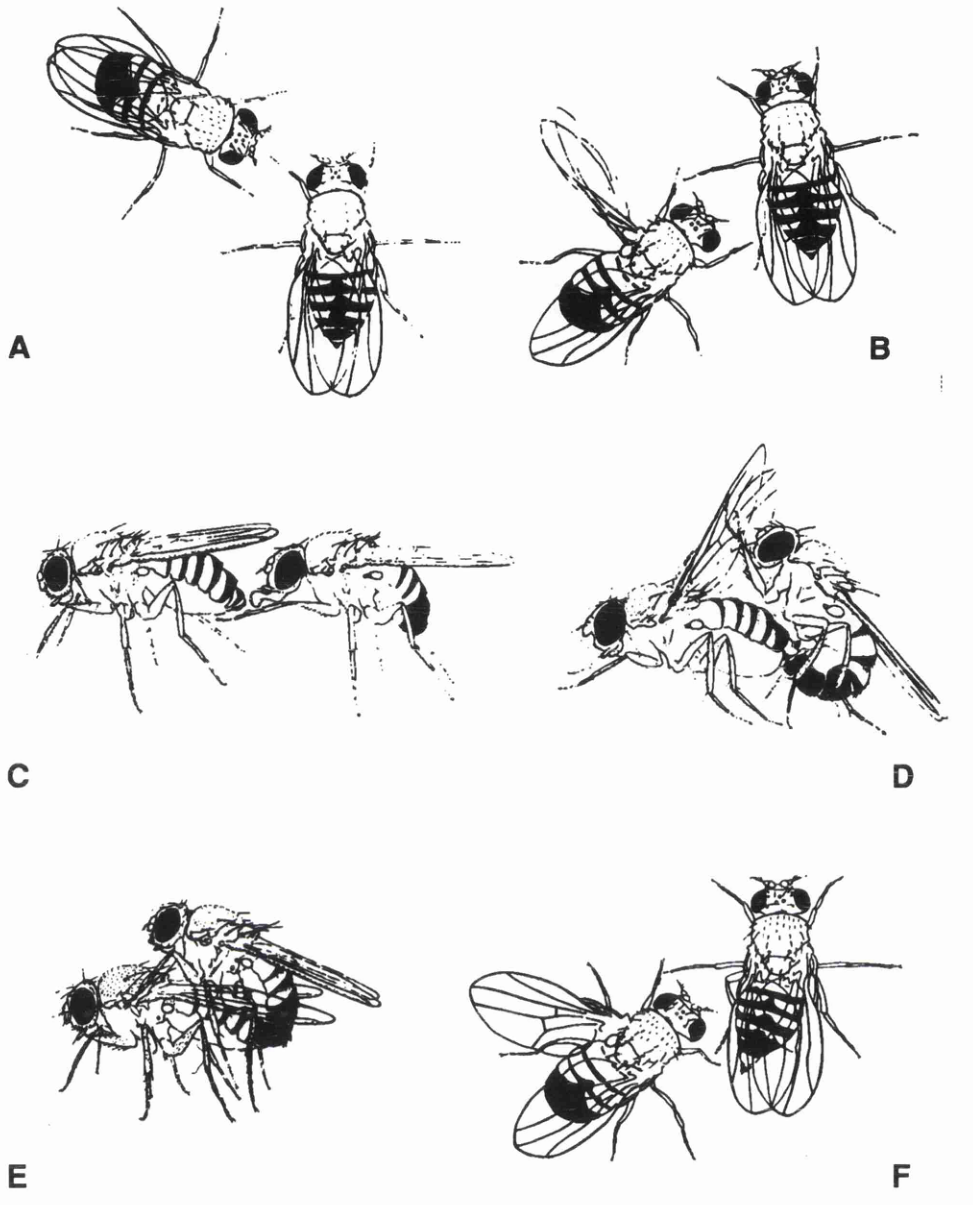


Figure 1.1 Courtship behaviour of male *Drosophila melanogaster*. A: orientation of the male to the female. B: wing vibration by the male. C: the male licks the female's genitalia with his proboscis. D: the male curls his abdomen. E: flies in copulation. F: A rejection response by the female to courtship. (After Burnet and Connolly, 1974)

When the male resumes courting, he almost always drops back to the orientation and following or singing stages (not to tapping or licking) and continues through the rest of the sequence. Non-receptive females, like fertilized females, usually give rejection signals to the courting male, such as kicking with the hind legs and curling the abdomen to the side (Fig. 1.1F). *Drosophila* are normally heterosexual, with almost no courtship between wild-type males. A male target usually spins about and faces the courting male and engages in wing flicking or wing fluttering and foreleg striking movements.

Female courtship actions in *Drosophila melanogaster* are not immediately obvious to the human observer, but the female is not thoroughly passive. Sexual receptivity in virgin females is switched on at a species-specific time, which in *Drosophila melanogaster* occurs about 48 hours posteclosion (Manning, 1967). When the female is generally receptive to the males advances, she responds by performing acceptance signals eventually, such as slowing down and stopping moving (Spieth and Ringo, 1983). When females refuse to mate, they perform rejection responses like fleeing. One-day old virgins reject mostly by flicking, older virgins reject mainly by kicking, and fertilized females reject almost exclusively by extruding their ovipositor (Connolly and Cook, 1973).

Some chemical messages exchanged by *Drosophila* also participate in sex and species recognition. In *Drosophila melanogaster*, females produce several molecules (e.g. 7, 11-heptacosadiene) which act as aphrodisiacs to male *Drosophila melanogaster*. This chemical sex appeal might be a sufficient signal for male Canton-S to distinguish females from males (Jallon, 1984). Male chemicals have a small effect on attraction to females (Venard, 1980), as females may stop moving in response to some olfacory signals produced by males. Males also produce another kind of compounds, like vaccenyl acetate, to inhibits the courtship from other males (Jallon, 1984). The production of these chemicals, including contact pheromones, is under polygenic control. It might be

triggered by sex-determination genes which control the structural genes for pheromone biosynthesis enzymes (Ferveur, et al., 1997).

1.1.2 Genes and courtship behaviour

Although, mutations of many genes can affect courtship, most of these genes are also serving other functions. Identification of their biochemical roles may help us to understand this complicated behaviour. For example, lower activity mutations may lead to defective courtship, such as, *yellow* (Burnet and Wilson, 1980) and *ebony* (Kyriacou, 1981) body colour mutations cause neurotransmitter defects, and males carrying either of these mutations court abnormally. Some more specific defects, like *white* (Heisenberg and Wolf, 1984) and *smellblind* (Markow, 1987), affect the sensory system directly, and they court in a mediocre manner. This indicates that visual and olfactory functions are important within courtship. In addition, altered sexual orientation has been correlated with misexpression of the *white* gene in the brain (Zhang and Odenwald, 1995; Hing and Carlson, 1996). Rhythm mutants (such as *period*, Kyriacou, et al., 1990), and learning and memory mutants (*dunce*, Kyriacou and Hall, 1984) all show some modification of courtship behaviour. These add credence to the notion that courtship (and other complex behaviours) are regulated by multiple genes acting together. However, this contributes little to our understanding of the true genetic control of courtship.

Two genes with relatively specific effects on courtship are *fruitless*, mutations of which cause abnormal sexual preference (Ryner, et al., 1996; Ito, et al., 1996), and *dissatisfaction*, mutations of which cause low sexual activity (Finley, et al., 1997). Interestingly, both of them are under the control of *transformer (tra)*, a sex-determing gene. These studies provide strong support for a special relationship between the genetics of courtship and that which revolves around somatic sex-determination.

1.1.3 Courtship of sex-determining mutants

The somatic sexual phenotype of *Drosophila* is cell-autonomous (Baker and Ridge, 1980), which means the sex of each cell is independent from its neighbours. In the sex-determination hierarchy (Fig.1.2), the *Sxl* gene is initially activated in response to an X-chromosome to autosome ratio of 1:1 in female cells. *Sxl* is positively autoregulated (Cline, 1984). Once this initial female-specific, embryo-specific activation occurs, the Sxl protein is capable of directing female-specific splicing of the regulated region of *Sxl* (Bell, et al., 1991). Male and female *Sxl* RNAs are similar in structure except for the presence of the male-specific exon 3 that contains the stop codon UGA in-frame with the AUG start codon of exon 2 (Bell, et al., 1988 and Fig1.3.A). Sxl protein is not produced in male cells (X:A=1:2). The Sxl product regulates not only *Sxl* splicing but also the splicing of RNA derived from the *transformer (tra)* gene (Bell, et al., 1991), thus leading to the production of an active Tra product.

Just under *Sxl* in the genetic cascade of somatic sex determination is *tra*, which also encodes an RNA binding protein (Belote, et al, 1989). The *tra* gene has alternative RNA-splicing regulation between females and males (Fig. 1.3.B), and is functional in females but not in males. The RNA derived from *tra* is the prime target of the Sxl protein (Nagoshi, et al., 1988). In females, in the presence of Sxl protein, more than half of the *tra* RNA uses an alternative 3' splice site for the first intron. This effectivly removes the stop codon and produces the RNA that codes for the active Tra protein (McKeown, et al., 1988). In males, the RNA produced by default splicing contains stop codons that renders it non-functional (Boggs, et al., 1987). The region preceding the regulated splice-site contains a striking sequence identity to the male-specific 3' splice site region of *Sxl* and this region is essential for *tra* regulation (Inoue, et al., 1990). These suggest that the regulation of both *tra* and *Sxl* results from a direct competition between Sxl protein and the basal splicing machinery for the use of particular splice sites.

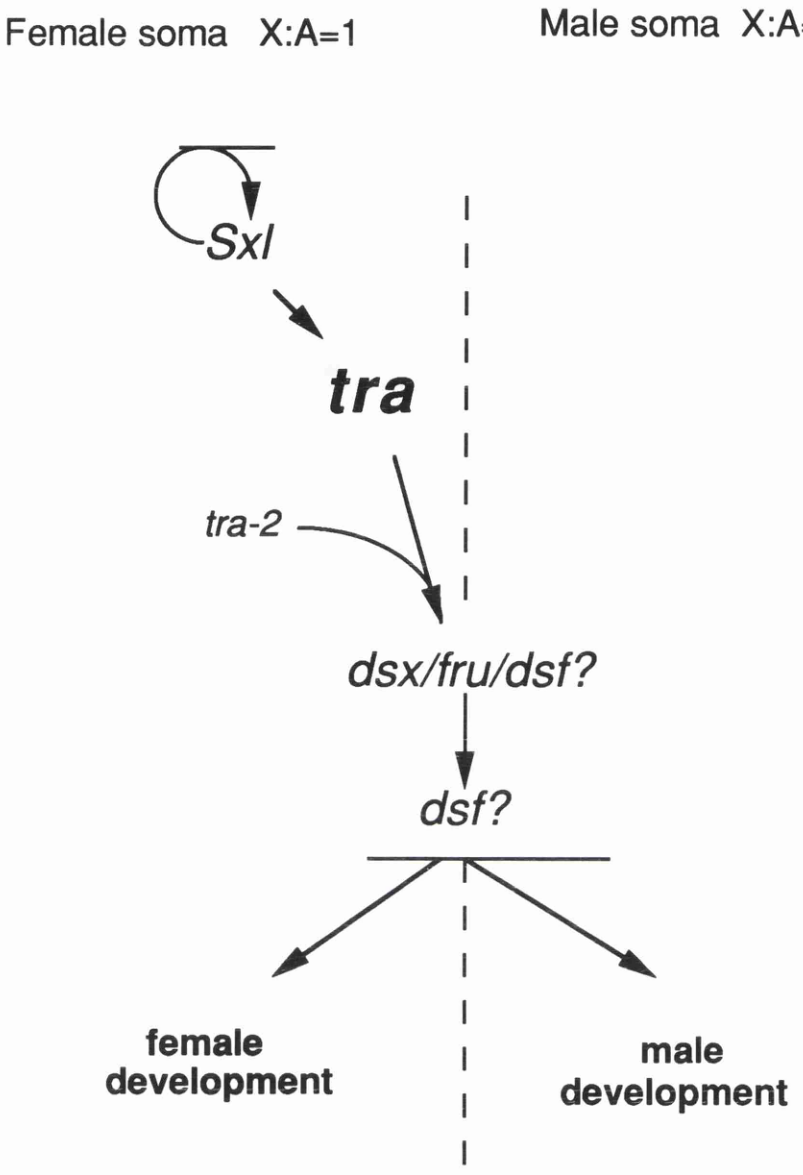


Figure 1.2 The sex differentiation hierarchy. X:A, the ratio of X chromosomes relative to sets of autosomes. Arrows indicate activation of gene function. It is still uncertain the position of *dsf* in the sex-determination pathway.

The *tra-2* product appears to be present in both males and females (Mattox and Baker, 1991). It is necessary in females for *doublesex (dsx)* female splicing (Tian and Maniatis, 1993). It can have multiple activities, like autoregulation in the germline, depending on the RNA and protein it interacts with (Ryner and Baker, 1991; Hedley and Maniatis, 1991).

Mutations in *tra, tra-2* or *Sxl* transform not only the fly's external morphology but also its sexual behaviour. Viable mutants of *Sxl* (Tompkins and McRobert, 1989) and *tra* or *tra-2* null mutations (McRobert and Tompkins, 1985) cause chromosomally female flies to behave as males; they court females but elicit very little courtship (unlike mature wild-type males); they can even copulate, but they are sterile because their internal abdominal anatomy is not fully transformed. The XX flies homozygous for *tra* mutations show an essentially normal male manner of wing vibration (Kulkarni and Hall, 1987).

A gain-of-function *Sxl* mutation in chromosomal males leads to ectopic expression of the female version of *Sxl*; they perform less courtship than wild-type males do and elicit a very high level of interest from other normal flies (Tompkins and McRobert, 1989).

The next gene in the sex-determination cascade is *doublesex (dsx)*. It has different transcript versions in female and male, as well as in different developmental stages (Baker and Wolfner, 1988). The *dsx* gene is transcribed to produce a common primary transcript that is alternatively spliced and polyadenylated to yield male- and female-specific mRNAs (Fig 1.3.C). They share common 5' ends, but possess alternative sex-specific 3' exons (Nagoshi and Baker, 1990). This give rise to alternative *dsx*-encoded proteins with common amino-terminal regions and sex-specific carboxy-termini. Genetic and molecular data suggest that sequences including those at, and adjacent to, the female-specific splice acceptor site play an important role in the regulation of *dsx* expression by the *tra* and *tra-2* proteins (Burtis and Baker, 1989). Both male (DSX^m) and female (DSX^f) proteins bind to three sites within a 127-bp enhancer that directs sex-and tissue-

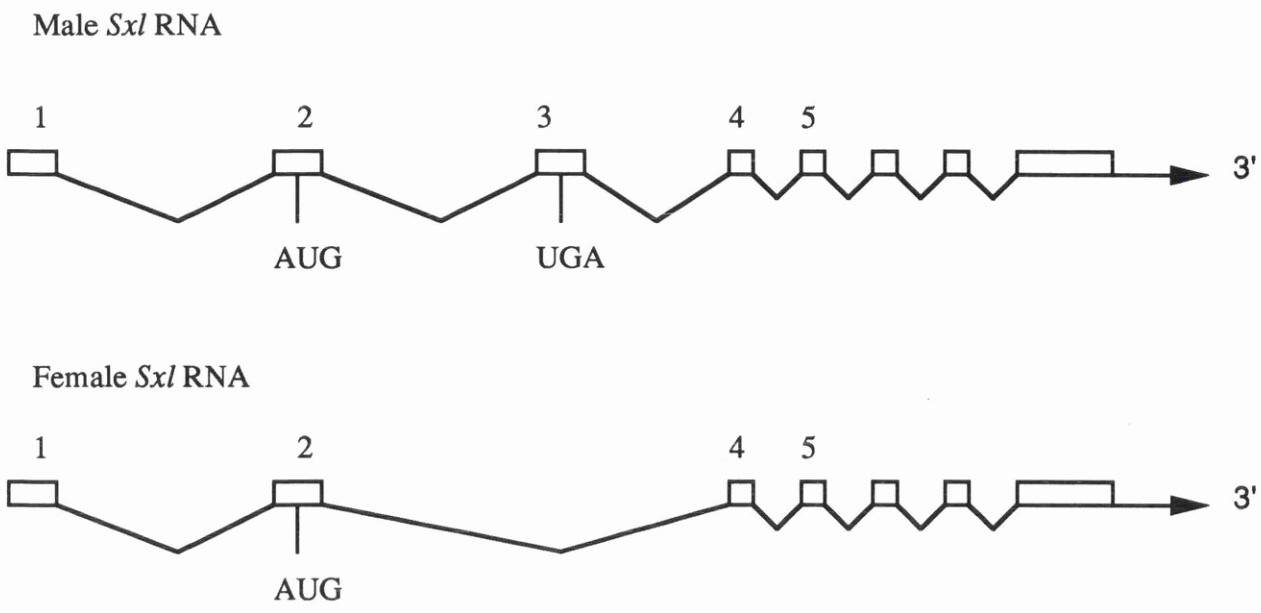


Figure 1.3 A Processing of *Sex-lethal (Sxl)* RNAs. Male and female *Sxl* RNAs are similar in structure except for the presence of the male-specific exon 3 that contains the stop codon UGA in frame with the AUG start codon of exon 2 (Samuels and Cline, 1991). The exons (1-5) are represented as the rectangles. Additional 3' alternative processing events are not shown.

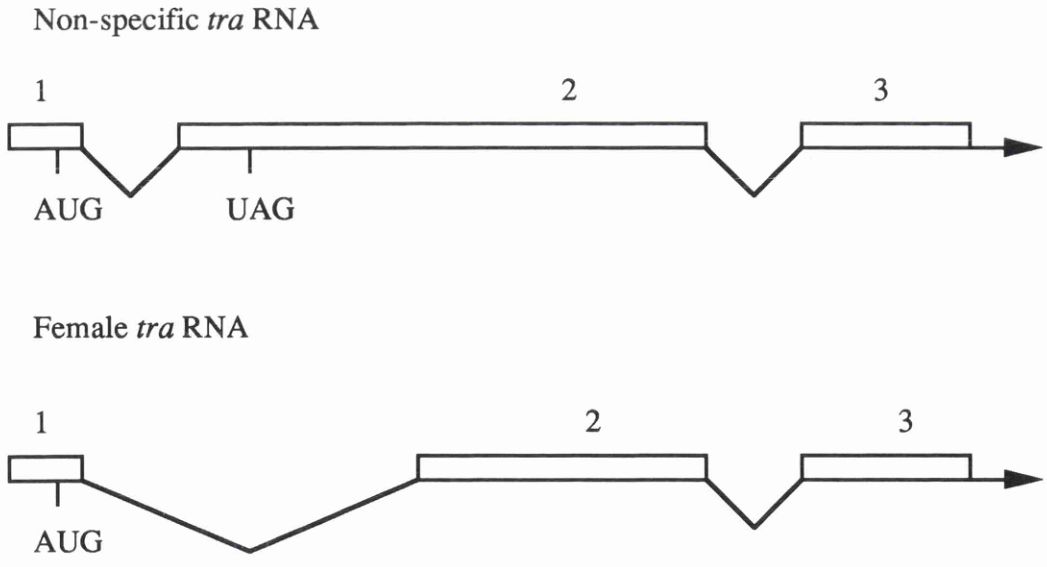
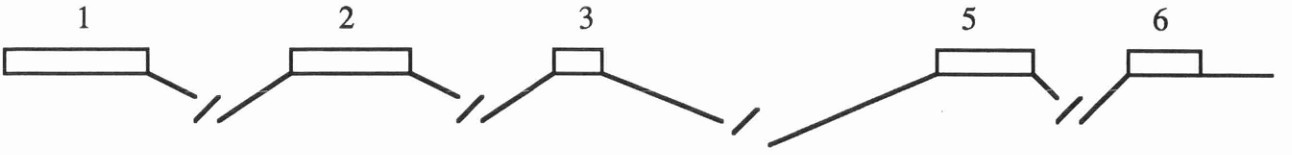


Figure 1.3 B Processing of *transformer (tra)* RNAs. The exons are represented as the rectangles (1-3). The non-specific and female-specific *tra* RNAs differ only in the splice site they use at the start of exon 2. The non-specific RNA contains stop codons (represented by the UAG) in the regions unique to it, thus blocking translation from the start codon (AUG) in exons 1 (After McKeown, 1992).

Male *dsx* RNAs



Female *dsx* RNAs

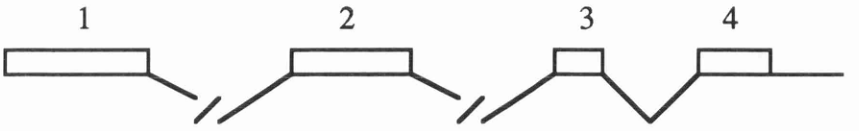


Figure 1.3 C Processing of *doublesex (dsx)* RNAs. The exons are represented as rectangles. The *dsx* RNAs contain three common exons (1-3) followed by male-specific (5,6) and female-specific (4) terminal exons. Gaps in the sequence are necessitated by the large size of introns (After McKeown, 1992).

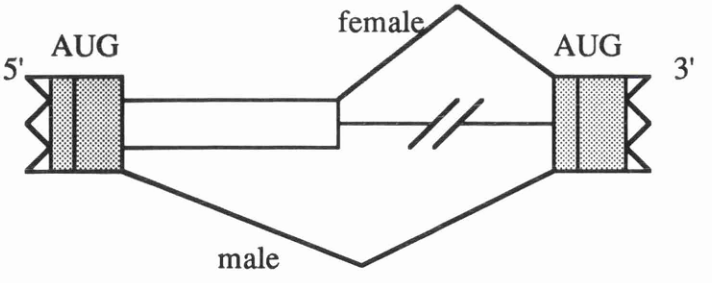


Figure 1.3 D Schematic drawing of the alternative 5' splice sites of *fru* that are joined to a common 3' exon. Exons are indicated by rectangles; jagged sides indicate that only a portion of the exon is shown; thin lines represent the introns, Shaded regions have protein coding potential (Ryner, et al., 1996).

specific transcription of Yolk protein genes. The female product activates transcription (Coschigano and Wensink, 1993), while the male product represses transcription (Bownes and Nothiger, 1981).

The effect of *dsx* mutations on courtship is as complicated as the gene itself. When a null mutation of *dsx* was tested, it gave an intersexual courtship behaviour (McRobert and Tompkins, 1985). But, the formation of the Muscle of Lawrence (MOL) — a male-specific muscle in the abdomen, was found to be immune to allelic variation at the *dsx* locus (Taylor, 1992). The development of MOL requires innervation by genetically male motor neurons emanating from the abdominal ganglion (Lawrence and Johnston, 1986), and also requires *tra* and *tra-2* expression (Taylor, 1992). However, recently the affects of *dsx* mutations on sex-specific behaviours began to be called into question. Taylor, et al. (1994) re-examined the affects of one particular *dsx* mutation (dsx^1) on courtship. They showed that mutant (chromosomal) males' courtship towards females seemed qualitatively normal in most aspects of male courtship except for copulation, because these mutants are physically intersexual. Moreover, although externally they look like normal males, XX flies expressing a *dsx* allele that causes constitutive production of DSX^m, exhibited no courtship whatsoever toward normal females. So, it has been suggested that elements of neural/behavioural sex-specificity are *dsx*-independent (Taylor, et al., 1994).

Another recent report (Villella and Hall, 1996) uncovered a new behavioural anomaly: dsx^{15} and dsx^{16} mutations caused chromosomal males to court other males at abnormally high levels. However, unlike *fru*-like courtship, no "chaining" behaviour has been observed. Considering *in situ* expression of *dsx* in adults has been monitored only indirectly with respect to genic targets of DSX action in certain tissues (An and Wensink, 1995), whether *dsx* products are present and functioning in the imaginal nervous system is an open question.

Among the numbers of genes affect courtship performance of *Drosophila* males, *fruitless (fru)* is the first gene in a branch of the sex-determination hierarchy functioning specifically in the central nervous system (CNS). It is also one of the very few that appears to be specific to male courtship (Taylor, et al., 1994). In *fru* mutant males, the later steps of courtship, from singing through copulation, are abnormal or absent. Because *fru* mutant males fail to copulate, they are sterile. In addition, *fru* males court both males and females indiscriminately. When *fru* mutant males are grouped together, they form male-male courtship chains in which each male is both courting and being courted. An additional *fru* phenotype is that the male-specific MOL is incompletely formed or absent (Gailey et al., 1991). So far, there are no reported phenotypic effects of *fru* in females (Hall, 1994).

Recently, the *fru* gene has been cloned by two groups independently (Ryner, et al., 1996; Ito, et al., 1996). The gene spans approximately 140kb along the genome. It is identified as a sequence-specific transcriptional regulator, encoding a zinc finger protein with a BTB-domain (Zollman et al., 1994). BTB is for BR-C (Broad-complex), *ttk (tramtrack)* and *bab (bric à brac)*. These three genes all contain a common motif of approximately 115 amino acids. This BTB-domain defines a gene family in *Drosophila.* The motif is found primarily at the N terminus of Zinc finger proteins. These genes are expressed in overlapping, but different, cell-specific patterns (Read, et al., 1992; Emery et al., 1994; von Kalm et al., 1994). Thus, the proteins may regulate different target genes in a cell-specific manner. *fru* produces multiple transcripts, including sex-specific transcripts that are alternatively spliced (Fig 1.3D) in a *tra-* and *tra-2* - dependent manner. Both female and male versions of *fru* have common 3' exons, which are joined to alternative 5' splice sites.

fru controls a range of male-specific nervous system functions (Ryner, et al., 1996). The mutants and mutant combinations disrupt the early steps of courtship (orientation, following, and wing vibration) as well as the later steps (courtship song and attempted

copulation). None of the published *fru* mutant alleles interrupt the coding sequence. Some mutations are lethal in both sexes. These facts indicate that *fru* encodes a vital function and is essential in both sexes (Ryner, et al., 1996).

fru locates downstream of *tra* in the sex-determination hierarchy, and likely at the top of a new branch, for its expression is independent from *dsx*. Possibly, like *dsx*, *fru* is the final regulatory gene in its branch of the hierarchy. If so, *fru* would directly control the expression of downstream genes responsible for governing sex-specific MOL development, sexual orientation and the behaviours that comprise male courtship. Although courtship behaviour in *Drosophila* can be modified to a limited degree by experience (Greenspan, 1995), the genetically programmed part seems controlled by the same hierarchy that rules all other aspects of sex-development.

In *Drosophila* brain, only 500 of the roughly 10^5 neurons of the CNS had detectable *fru* expression by tissue hybridization (Ryner, et al., 1996); there was no signal in other tissues of the body. The small number and the locations of the *fru*-expressing neurons suggest that *fru* is directly involved in only some of the sensory and motor systems necessary for courtship behaviour. *fru*-positive neurons were found most commonly as small groups and distributed in similar areas of the brain and ventral nerve cord in males and females. A set of nine neuron groups, ranging from 10-30 cells, was detected in the CNS of males in positions likely to be involved in particular male courtship behaviours. For example, the sex-specific transcripts of *fru* are abundantly expressed in a group of primary sensory interneurons in the antennal lobe involved in the processing of chemosensory information (Stocker, 1994). In females, six of these comparable locations were found. Surprisingly, some *fru*-expressing cells were detected only in females. This may suggest that *fru* has specific functions in the female, with the female phenotypes being too subtle to have been detected.

More recently, another *dsx*-independent but *tra*-dependent gene, *dissatisfaction* (*dsf*) has been identified (Finley, et al., 1997). It affects sex-specific courtship and neural differentiation. *dsf* males exhibit bisexual behaviour with abnormal copulation (different from *fru* males which fail to curl abdomen). Unlike the lack of abnormalities for *fru* females, *dsf* females show phenotypes in both courtship and fertility. They are reluctant to mate and unable to lay mature eggs. The multiple differences between *fru* and *dsf* indicate these genes act in separate regulatory pathways, each of them is required for appropriate function.

1.2 *Drosophila* brain structures and function

The brain is the major part of the central nervous system (CNS) of the fly and contains two regions, the central brain and optic lobes (Fig 1.4 B). The oesophagus runs through the centre of the brain. Neural cell bodies lie in a thin cortex surrounding the bulk of neural tissue (Power, 1943). Each cell body sends a single process or neurite inwards, which then gives rise to separate axonal tracts and large regions of synapsis known as neuropil. Unlike most *Drosophila* cells, only a few CNS neurons can be characterised by cell body position alone (Armstrong and Kaiser, 1997).

About 4 hours after the onset of the embryonic development, some cells segregate from the ectoderm. They are the neuroblasts that make the primordial nervous system, and subsequently begin to divide asymmetrically (Hartenstein, et al., 1987). Neurons make synaptic connections with each other during the late embryonic development (Ito, 1990). Neurons are small round cells just after the division. As they mature, they send fibers into the neuropil. The shape of the brain changes drastically during metamorphosis (Kanket et al., 1980). Degeneration or re-modelling of the larval neurons occurs during the early pupal stage (Technau and Heisenberg, 1982). The basic structure of the adult

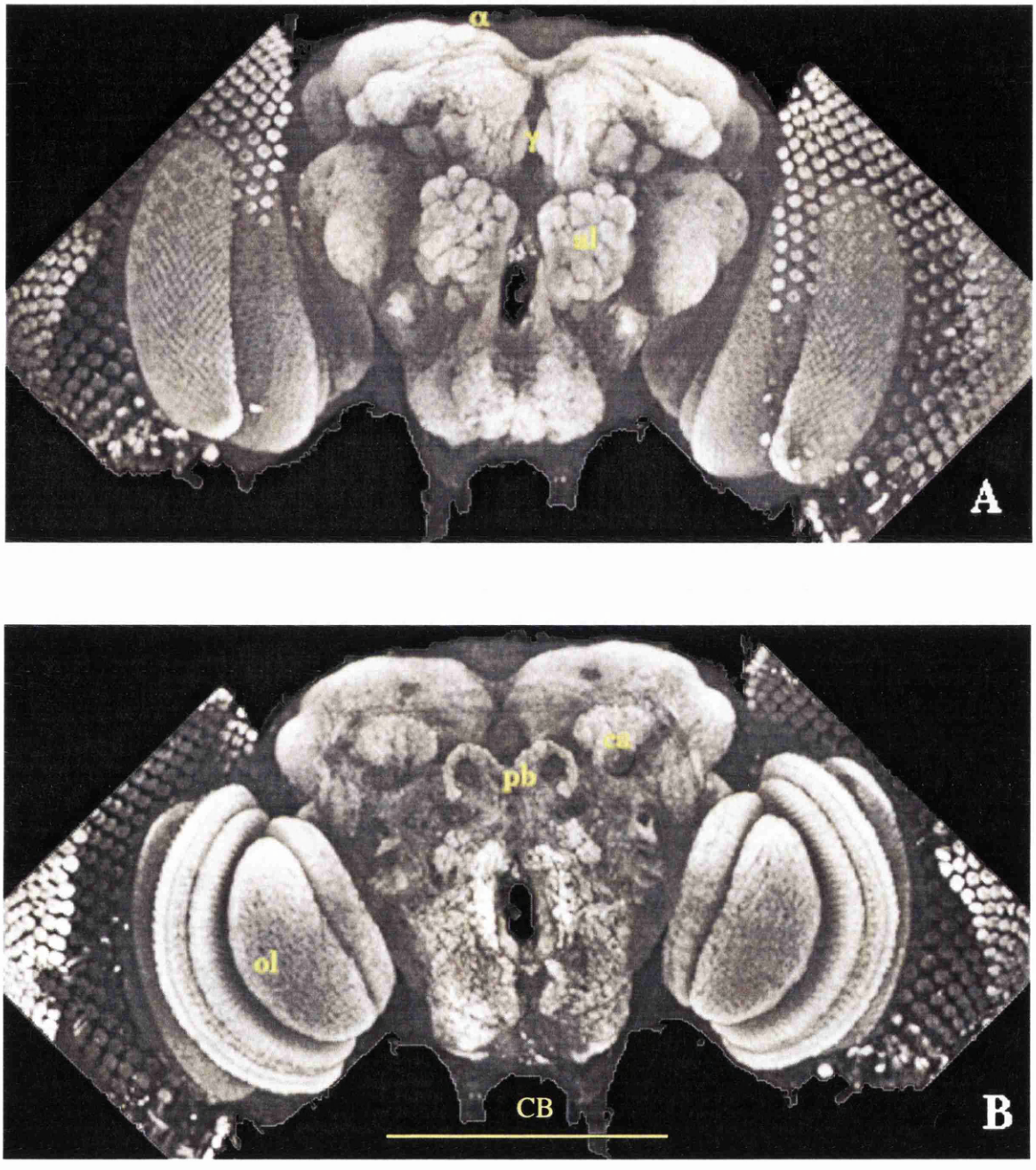


Figure 1.4 Three-dimensional image of *Drosophila* brain structures. The pictures were kindly provided by Dr. X.J. Sun. **A**: From the front. α and γ indicate the α and γ lobe of the mushroom body; al means the antennal lobe. **B**: From behind. ca represents the calyx of mushroom body; pb indicates the protocerebral bridge of the central complex; ol means the optic lobe; and CB means the central brain.

nervous system is built up simultaneously. No major change except for maturation occurs in the nervous system from pupa to adulthood.

Certain portions of the CNS of *Drosophila melanogaster* are essential for different features of male reproductive behaviour. This conclusion comes from investigations of genetic mosaics that are part male, part female (Hotta and Benzer 1976; Hall, 1977). A detailed study (Hall, 1979) revealed the association between different elements of the courting routine and various parts of the central nervous system (Fig. 1.5). Hall (1979) concluded that initiation of courtship required male cells in one side or the other of the mushroom bodies. Later steps in courtship, especially those demanding precise motor coordination, require male tissue in additional parts of the nervous system such as the thoracic ganglion.

The *Drosophila* brain varies in size throughout the lifetime of the fly and may also vary in response to specific living conditions (Technau, 1984; Heisenberg et al., 1995). The volume changes are observed in most neuropil regions such as the calyx, central complex and optic lobes. The differences in size of the calyx reflect differences in the numbers of Kenyon cell fibers. The size of the calyx is influenced by the larval density of the cultures, food condition, and the sex of the partner, for example, females have larger calyces if they are grown with females than if grown with males (Heisenberg et al., 1995).

In the underlying neuropil, we are interested in four conspicuous structures: the mushroom body, the central complex, the antennal lobe and the optic lobe, which are now described in turn.

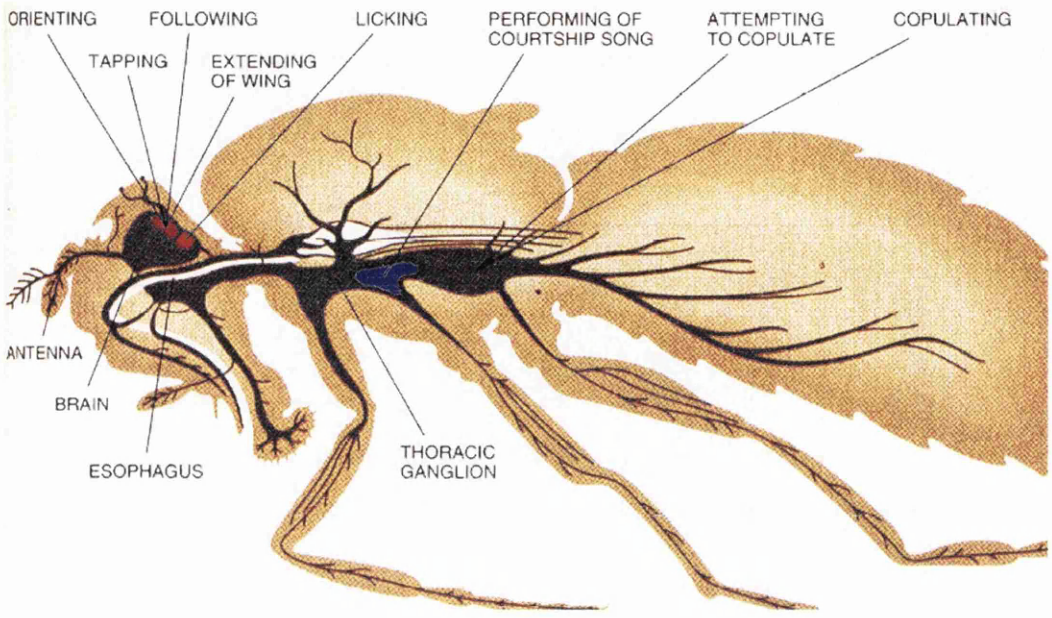


Figure 1.5 Sites in the central nervous system (brown) that control the steps of courtship in *Drosophila* male. The positions have been mapped by gynandromorphs studies (Hall, 1979). The domineering focuses for the early steps of courtship (orientation, following, tapping, extending of wing and licking) are located on the dorsal brain (red). The thoracic ganglion (blue) is required for performing courtship song. Other different sections of thoracic ganglion are required for copulation. (After Greenspan, 1995).

1.2.1 The mushroom body

Mushroom bodies are large phylogentically conserved insect brain elements that have been implicated in associative learning and memory (Balling, et al., 1987), and in a variety of complex functions including courtship (deBelle, 1995), motor control, etc. (Mizunami et al., 1993). The mushroom bodies of *Drosophila melanogaster* are bilateral clusters of about 2500 cells. The fundamental computational properties of mushroom bodies are provided by the intrinsic neurons (Mauelshagen, 1993), known as Kenyon cells. They are postsynaptic to the fibers from the antennal lobe. Their fibers form the calyx, the pedunculus, and the α, β, γ lobes of the mushroom body in *Drosophila* (Technau, 1984) (Fig. 1.4 A and B). Chemosensory signals from the antennae and other chemoreceptors enter the mushroom body via the antennal lobe.

In *Drosophila*, single gene mutations (e.g. *mushroom body miniature*) that cause defective mushroom body anatomies have been shown to interfere significantly with olfactory associative learning (Heisenberg, et al., 1985; Heisenberg, 1989). Olfactory learning is even more profoundly affected by ablating neuroblasts at an early stage of development, depleting the adult brain of mushroom body intrinsic neurons (deBelle and Heisenberg, 1994). Additional support for a role of *Drosophila* mushroom bodies in olfactory learning derives from studies of "biochemical" learning mutants as expression of the "learning" genes *dunce*, *rutabaga* and *DCO* is elevated in the mushroom bodies (Nighorn, et al., 1991; Han, et al., 1992; Davies, et al., 1993). Finally, gynandromorph analysis implicates *Drosophila* mushroom bodies, or adjacent neuropils, in the control of the early stage of 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.

By using the enhancer-trap technique, subdivisions of *Drosophila* mushroom bodies have been revealed (Yang, et al., 1995). Rather than being homogenous, mushroom bodies 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 the mushroom body in terms of sex-specific behaviour (O'Dell et al, 1995). This work suggested that certain region(s) of the mushroom body are involved in determining sexual preference.

Unlike many other elements of the CNS, the mushroom bodies are present throughout development, in the embryo, larva and pupa, and as well as the adult brain (Ito and Hotta, 1992). During metamorphosis, reorganisation of the mushroom bodies takes place; some elements of the larval lobes may remain through pupation, but most would appear to undergo partial or complete degeneration (Armstrong, 1995).

1.2.2 The central complex

The central complex is a group of neurons lying at the center of the brain, located just above the oesophagus. It is the only unpaired neuropil, so in general the central complex may coordinate information processing in the two hemispheres and regulate behavioural activity (Heisenberg, et al., 1985). In *Drosophila*, the central complex consists of four interconnected main neuronal regions or substructures: the protocerebral bridge (Fig. 1.4B); the fan-shaped body; the ellipsoid body and the paired noduli. Since different putative neurotransmitters are involved in the different substructures of the central complex. These substructures may exert different functional roles (Hanesch, et al., 1989).

The possible function of the central complex has in part been revealed by early surgery and electrical stimulation experiments. Both inhibitory and excitatory effects were observed in walking, escape responses and feeding behaviour (Homberg, 1987). Genetic lesion studies in *Drosophila* with single gene mutations which affect the central complex structure (Heisenberg, 1980), have led to similar behavioural phenotypes. These exhibited slow initiation of activity and slow walking. Brain activity mapping, a technique using radio-labelled glucose to trace neuronal activity, also suggests that the central complex plays a role in the processing of visual information (Bausenwein et al., 1994). Although research has focused on the possible roles of the central complex in locomotor behaviour (Strauβ and Heisenberg, 1993), there is some evidence that the central complex may play a role in olfactory associative learning and memory (Heisenberg, 1989).

The ellipsoid body seems to be a speciality of dipterans (Williams, 1972 and Strausfeld, 1976). In *Drosophila* mutants the ellipsoid body is opened up ventrally to varying degrees and may appear as a flat glomerulus (Strauβ and Heisenberg, 1993). The staining patterns of P[GAL4] enhancer trap lines reveal different R-type neurones (Armstrong, et al., 1997), which are the most extensively studied ring neurons in the ellipsoid body. It is presumed that different R-type neurons could give rise to the different integrative functions of the ellipsoid body, although the main role of the ellipsoid body is thought to be inhibitory control of behaviour due to most of the R-type neurons showing dense GABA immunocytochemical staining which is known as an inhibitory neurotransmitter (Hanesch, et al., 1989 and Bausensein, et al., 1994).

The structures of the central complex appear late in development. The region can not be identified in the larval stage. During metamorphosis, it grows constantly. The shape is almost identical with that of the adult by the second day after puparium formation. Like the other neuropil regions, it grows further with the maturation of the whole fly body.

1.2.3 The antennal lobe

The antennal lobes are the first order neuropils of the olfactory chemosensory pathway. They are prominent structures situated in the anterior part of the *Drosophila* brain (Fig 1.4 A), at the level of the oesophagus, as a pair of protrusions. No obvious sexual dimorphism has been observed regarding the size or shape or location. The antennal lobes are thought to be the primary olfactory association centre (Stocker, et al., 1990; Stocker, 1994). Their subunits, the glomeruli, are organised into odor-discriminating areas (Rodrigues, 1988). The antennal lobe receives afferents from the antennae, and the output tracts run straight into the calyx of the mushroom bodies and lateral protocerebrum.

The antennal system is the receptor for the anti-aphrodisiacs emanating from mated females (Stocker and Gendre, 1989). "Feminized" antennal lobes cause *Drosophila* males to display courtship towards both female and male targets (Ferveur, et al., 1995). These phenomena maybe due to incorrect processing of the inhibitory odor cues that come from mature males.

1.2.4 The optic lobes

The optic lobes, whose size is as large as the central brain, have three regions: lamina, medulla and lobula complex (Fig. 1.4 B). The lamina is the lateral most region of the CNS, just beneath the compound eyes. 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 (Fischback and Dittrich, 1989).

The study of a large number of neuronal cell types and their connections (Fischback and Dittrich, 1989) suggests that the parallel networks of the optic lobe filter different kinds

of visual information and thus represent structurally separated functional subunits of the optic lobe.

1.3 The P-element in the biology of *Drosophila*

The P-elements are a transposon family of *Drosophila*. They can transpose directly from DNA to DNA within a cell. In nature, transposition of P-elements occurs in the offspring of males that have P-elements and females that lack them (Engels, 1989). Transpositions are restricted to the germ-line cells and thus become manifest only in later generations.

The first P-element to be cloned was a defective element, identified by virtue of disrupting the *white* locus (Sprading, et al., 1982). Autonomous P-elements are 2.9kb in length and have 31bp inverted terminal repeats that are essential for transposition. Full-length elements have four exons encoding an 87kDa transposase (Karess and Rubin, 1984). Restriction of P-element activity to the germ-line results from differential splicing of the mRNA (Rio, 1991). In addition to full-length P-elements, most P strains contain a range of internally deleted elements varying in length from 500bp to 2.5kb. These P-elements are non-autonomous because they are unable to produce functional transposase. But many such elements are still mobilised in the presence of a full-length element (Engels, 1989). It is noted that an engineered P-element with the third intron removed can produce transposase in both somatic and germ line cells (Laski, et al., 1986), and it is often used to mobilise internally deleted P-elements in *Drosophila* genetics. These observations are the basis of the experimental manipulation of P-element transposition, as well as the development of P-elements as transformation vectors and as enhancer traps.

The P-elements serving as transformation vectors are used for modification and manipulation of the *Drosophila* genome in several ways, such as germ-line

transformation, mutagenesis via the imprecise excision strategy, enhancer-trap, gene cloning by transposon tagging, etc. We are particularly interested in the application of P-elements as enhancer-traps.

1.3.1 Enhancer-trapping

Targeting gene expression to specific cell types in a multicellular organism is a powerful tool for studying the development and normal function of group of cells. Enhancer-traps provide a successful technique to achieve this goal, and a new way to identify genes by their pattern of expression instead of means of phenotype caused by a mutation. Developmentally important genes usually show specific temporal and spatial expression pattern related to their function. In the context of *Drosophila*, enhancer-traps are usually modified P-elements, which can insert pseudo-randomly within the *Drosophila* genome (Kaiser, 1993). The modification include incorporation a very poorly expressed reporter gene, initially *lacZ*. They are not autonomous, but like the internally deleted P-elements, they can be mobilised. Due to the lack of a transcriptional enhancer, the reporter has a negligible level of intrinsic expression. In order for it to be expressed at significant levels, the transposon must insert close to an endogenous *Drosophila* enhancer. Enhancers are *cis*-acting sequences that can greatly increase transcription rates from promoters on the same DNA molecule. They are not gene-specific but tissue-specific. Such tissue-specific enhancer elements appear to be rather common in the genome. Bellen et al. (1989) found that tissue-specific patterns in the embryo were observed in about 65% of their enhancer-trap lines; Bier et al. (1989) found that about 35% of their lines were showing expression in the embryonic nervous system. The high frequency of tissue-specific expression patterns may reflect the fact that enhancers can act at long distances, so the insertion can be quite far from the enhancer are still be influenced by it (Wilson, et al., 1989).

Enhancers can function in either orientation, operating either upstream or downstream from the promoter they are enhancing (Griffiths, et al., 1993). Theoretically, the expression pattern of the reporter gene in the enhancer-trap element reflects the temporal and spatial expression pattern of a flanking gene (Sentry, et al., 1994). There is evidence (Nose, et al., 1992) to show that the β-gal blue patterns of the construct match the known expression patterns of the genes which are close to the P-element insertion site. In the case of the learning gene *DCO*, recessive lethal mutation has been isolated (Lane and Kalderon, 1993; Skoulakis, et al., 1993) by an enhancer-trap screen for genes preferentially expressed in the mushroom bodies. Some genes have been cloned and analysed solely on the basis of the expression patterns through this method (Yang, et al., 1997; Pignoni, et al., 1995).

First generation enhancer trap elements (O'Kane and Gehring, 1987 and Fig 1.6 A) contained the reporter gene *lacZ*, encoding the enzyme β-galactosidase (β-gal). The presence of β-gal activity in tissue can be detected simply by its conversion of the chromogenic substrate Xgal. In addition to the reporter gene, enhancer trap elements carry a marker gene (e.g. *white* or *rosy*) that enables flies with insertions to be recognised. In order to facilitate cloning of the genomic sequences flanking the P-element insertion point, a plasmid origin of replication (*ori*) and an antibiotic resistance gene (Amp^r) gene are included. This design allows the performance of plasmid rescue method (Pirrotta, 1986). The procedure is demonstrated in Figure 1.7. Genomic DNA from the flies carrying an enhancer trap element is digested with an appropriate restriction enzyme that cuts the polylinker in the engineered P-element and somewhere (could be upstream or downstream of the insertion) in the flanking DNA. By the subsequent self-ligation, the fragments are cloned as plasmids allowing them to be transformed into *E. coli* and surviving the antibiotic medium. The rescued genomic fragments could be used for further chromosomal walking or other analysis.

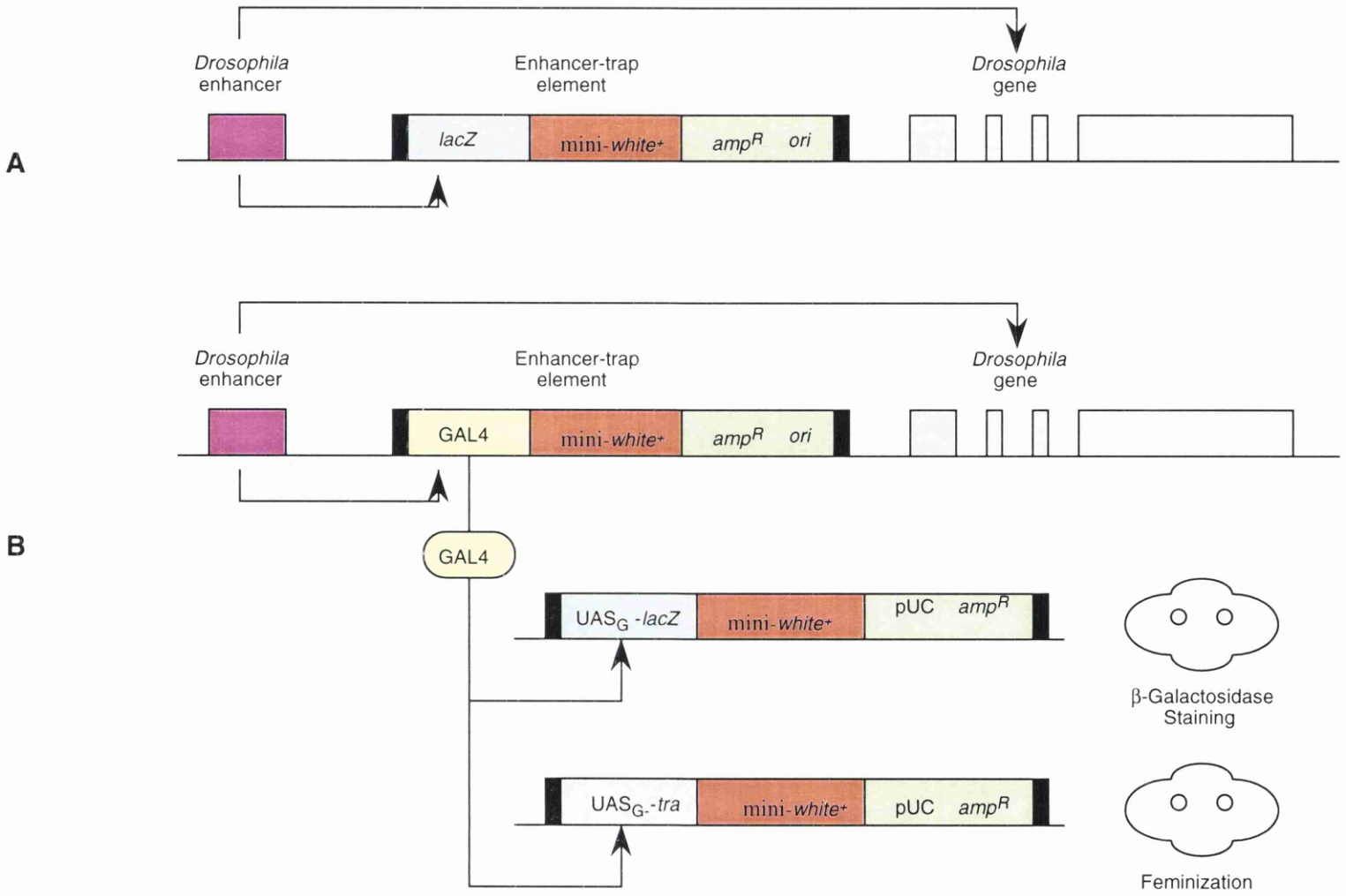


Figure 1.6 Enhancer trap strategies. **A**: P[lacZ] construct. **B**: P[GAL4]/UAS_G system constructs.

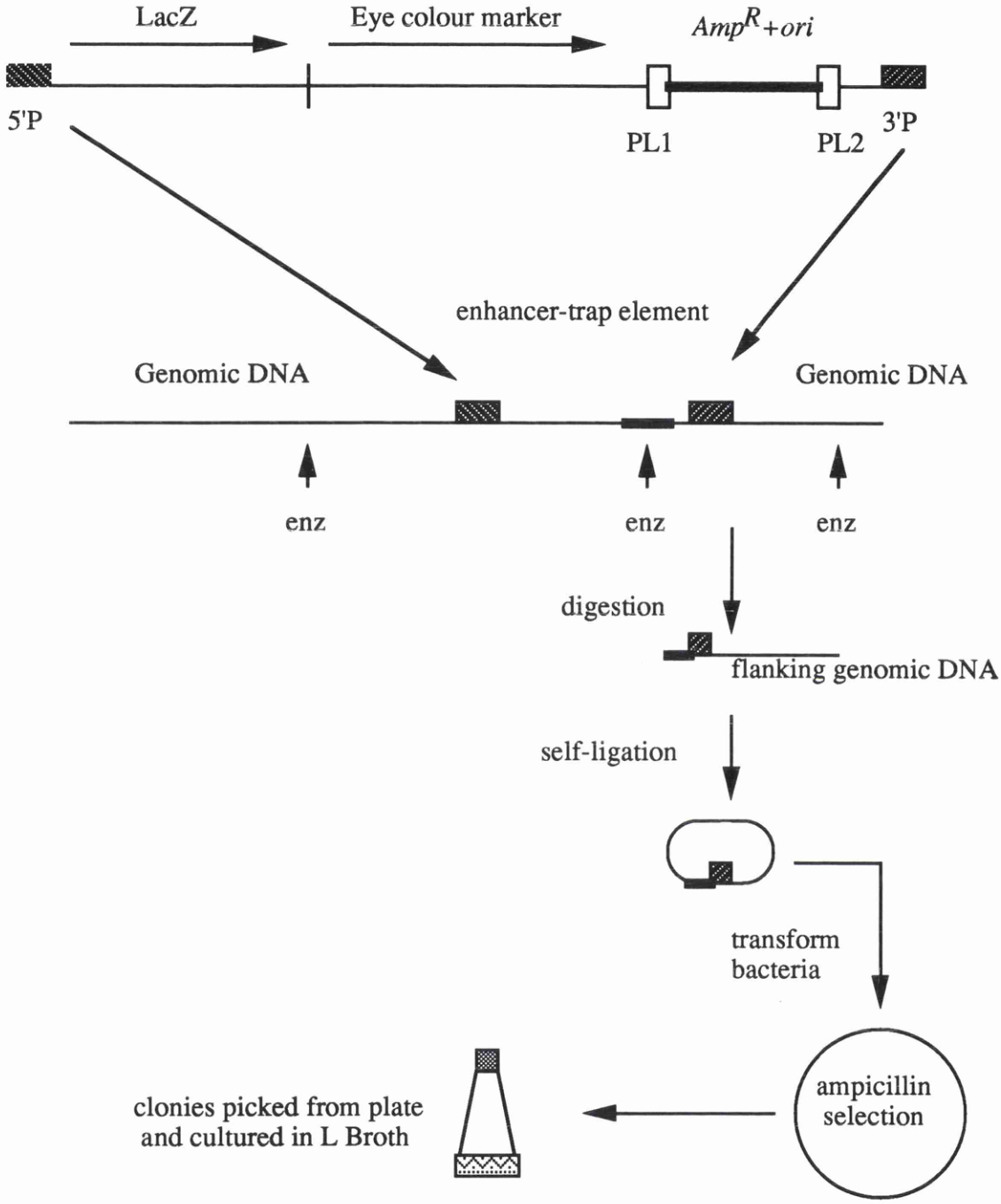


Figure 1.7 Diagram of plasmid rescue technique. PL: polylinker; enz: restriction enzyme.

See text for full description (Diagram was redrawn and modified from Bellen et al., 1990).

One feature of most "first generation" enhancer-trap elements is that they express β-gal fused to the N-terminal nuclear localisation signal of the P-element transposase (Grossniklaus, et al., 1989). It reveals only cell nuclei. Although nuclear localisation of the lacZ product makes it easier to visualise and count single cells, cytoplasmic localisation is favourable for the analysis of cells with extensive processes such as central neurons (axons and dendrites) in the brain. Smith and O'Kane (1991) addressed this problem by constructing an enhancer-trap element lacking the N-terminal signal, thus allowing cytoplasmic localisation.

Considering the insect nervous system as a collection of interacting cells, then the identity of these cells is defined by the genes expressed within them. Enhancer-traps are effective markers of gene expression. They have been used to trace cell lineages (O'Kane and Gehring, 1987) and for anatomical markers (Hartenstein and Jan, 1992) of neurogenesis.

The classical and standard approach to studying genetics in *Drosophila* is to identify mutations that disrupt the process of interest, and then to characterise the gene both by its biological function and by cloning and studying it at the molecular level. Even though this approach has been extraordinarily successful (Hafen, et al., 1987), relying on the phenotypes of mutations in genes does have its shortcomings (Freeman, 1991). First, genes may have functions at more than one time in development, and mutations in such genes may only produce phenotypes associated with one of those functions. Sometimes lethal mutations conceal other information and leave these genes undetectable. Second, in a system with great diversity, like the CNS, it is technically difficult to detect some subtle phenotypes. A third general problem with the classic mutational approach is that, often, in the absence of a functional gene product, another one can at least partially replace the lost function; in this case, a mutation in such a gene may not give a detectable phenotype. On the other hand, extensive genetic and molecular analysis of *Drosophila* embryonic pattern formation have revealed that the majority of genes controlling cell

identities in the early embryo do so by being expressed in a spatially restricted pattern that correlates with their regional requirements (Ingham, 1988).

1.3.2 GAL4/UAS$_G$ system

To exploit the basic enhancer-trap principle even further, "second generation" enhancer-trap systems have been developed (Brand and Perrimon, 1993; Kaiser, 1993). As shown in Figure 1.6 B, the β-gal reporter gene in the previous enhancer-trap element has been replaced by the gene encoding the yeast transcription factor, GAL4. This transcription factor can function in *Drosophila* (Fischer, et al., 1988), where it only activates the expression of genes that have the GAL4 binding site, known as the upstream activating site (UAS). Crossing a fly having a new GAL4 insertion with a fly containing a UAS$_G$-*lacZ* construct causes β-gal to be expressed in a pattern that reflects GAL4 activity in the progeny. In the case of β-gal free of a nuclear localisation signal, the expression leads to the transport of β-gal away from the cell body. Such a construct allows precise characterisation of the position and morphology of cells of many kinds, Particularly for visualising the neuropil structures in the brain and axons for neuronal path finding and synaptic connectivity. This second generation enhancer-trap provides for expression of a cytoplasmically-localised reporter, and makes it possible to express any cloned marker gene in the cells in which a particular GAL4-enhancer trap is active.

The advantage of the GAL4/UAS$_G$ system is that any gene placed downstream of a UAS$_G$ element can then be crossed into the same GAL4 enhancer-trap line and be expressed in the same tissue specific pattern. This has been shown to be the case with Figure 1.6 B. Therefore, in most cases we can map the expression of a GAL4 enhancer-trap line by observing the pattern of blue X-gal staining in a P[GAL4]/UAS$_G$-*lacZ* line, and be confident that another UAS$_G$ construct, such as UAS$_G$-*tra*, will result in expression of *tra* in the same pattern previously visualised when crossed into the same P[GAL4] background (O'Dell, et al., 1995). By using this system of P[GAL4]/UAS$_G$-

tra to some enhancer-trap lines with specific expression patterns, the functions of particular neural tissues could be addressed according to the developmental, morphological and behavioural consequences.

One of the most powerful uses of this feature is the ability to ablate specific cells by expressing cell autonomous toxin genes, such as ricin or diptheria toxin (Kunes and Steller, 1991; Moffat et al., 1992). Sweeney, et al. (1995) successfully expressed tetanus toxin light chain in embryonic neurons, and eliminated synaptic transmission. The targeted expression of toxin also produced the specific behavioural defect of a reduced olfactory escape response. This approach could allow us to interpret some of the connections between structure and function during the development of neurons (Sentry, et al., 1993).

1.4 The aim of the project

Most behaviour are generated by complex neuronal circuits, which are themselves difficult to unravel. Courtship is one of them. By combining the P[GAL4] lines with specific expression pattern in the *Drosophila* brain and the subsequent *transformer* gene expression under UAS_G construct, chromosomally male flies with feminised subregions of the brain can be created. The study of courtship behaviour toward male and female targets will reveal some connection between brain structure and fly sexual orientation. The effect of both mini-*white* and *transformer* gene on the male-male courtship has also been tested.

In order to identify some genes required in the adults central nervous system, several P[GAL4] enhancer-trap lines, with expression pattern in specific neurons, have been employed for plasmid rescue and gene cloning strategy. This approach will contribute to the understanding of the molecular mechanisms in the *Drosophila* brain.

Chapter 2

Materials and Methods

This chapter describes the general methodology used for the experiments which were carried out for this thesis. Any modifications and necessary additional descriptions of methods are found in the relevant later chapters. This chapter contains 4 main sections; (2.1) Materials, (2.2) *Drosophila*, (2.3) General methods of molecular biology, (2.4)*in situ* hybridization of *Drosophila* tissue and immunohistochemistry.

2.1 Materials

2.1.1 Chemicals and biochemicals

Antibiotics, Xgal, IPTG, ethidium bromide and SDS were obtained from Sigma chemical Co.

Radiochemicals were from ICN.

DMSO and polyethylene glycol 8000 were obtained from BDH Chemicals.

Agarose and phenol were obtained from Gibco-BRL Ltd.

Deoxynucleoside triphosphates were obtained from Promega.

Oligonucleotides were synthesised on an Applied biosystems(Model 280A) DNA Synthesiser at the Department of Genetics, University of Glasgow, using reagents from Cruachem.

General chemicals and solvents were from BDH and Sigma.

2.1.2 Enzymes and kits

Restriction endonucleases, other DNA modification enzymes, e.g. T4 DNA ligase, Klenow fragment, and reverse transcriptase were obtained from Gibco-BRL, Stratagene, Promega and Boehringer Mannheim. Proteinase K, RNase A, DNase I and lysozyme were obtained from Sigma. Automatic Sequencing Kits were from Perkin-Elmer Appli Gene. DNA labelling kits for both α-P^{32} and digoxygenin (DIG) were obtained from Boehringer Mannheim. A kit for RNA labelling was obtained from Promega. The RT-PCR kit used was from Gibco-BRL.

2.1.3 *Escherichia coli* strains and maintenance

Three strains were employed in this work:

The strain XL1 - Blue (Bullock et al, 1987) was used for most of the plasmid transformation and cloning procedures including white-blue selection. The genotype is as follows: *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, *supE*44, *relA*1, *lac*, [F'*proAB*, *lacI*^{q}*ZΔM*15, Tn10(tet^{r})].

The propagation of bacteriophage was carried out in the host strain NM621 (Whittaker et al, 1988), its genotype is: *hsdR, mcrA, mcrB,lac,SupE*44, *recD*1009.

For the preparation of better quality templates for double stranded sequencing (Perkin Elmer, 1995), the strain DH5α (Bethesda Research Laboratories, 1986; Hanahan,1983) was used. The genotype of this host is: *supE*44, *Δlac* U169 (ϕ80 *lacZ*ΔM15), *hsdR*17, *recA*1, *gyrA*96, *thi*-1, *relA*1.

Bacterial strains were stored in 80% LB(0.1% NaCl, 0.1% bacto-typtone, 0.05% bacto-yeast extract, 0.01% glucose), 20% glycerol at -70^{o}C.

2.1.4 Plasmid vectors

Plasmid pBluScript II SK^+ *amp*^{r} (Mead et al, 1985) was used as the vector for the subcloning of DNA fragments.

2.1.5 λ phage libraries

A *Drosophila* genomic DNA library was constructed using the EMBL3 (Frischauf et al., 1983) replacement vector. The library was kindly supplied by R. Blackman.

A *Drosophila* male adult head cDNA library was made in the vector λ NM1149 (Murray, 1983) by Steven Russell (1989).

2.2 *Drosophila*

2.2.1. *Drosophila melanogaster* strains

Canton-S; From Steve de Bell, Biologische Kybernetik, Max-Planck-Institut. A wild type strain used as a source of DNA, RNA and in situ hybridization experiments and as a control for behavioural tests.

w(CS10); white-eye Canton-S, derived by backcrossing w^{1118} flies to wild-type (Canton-S) flies for ten generations (Dura et al., 1993). This strain was used to make Cantonised background P[GAL4] lines for behavioural analysis.

P[GAL4] lines (Brainbox: http://brainbox.gla.ac.uk; Yang, et al., 1995; Yang, 1996; Armstrong, 1995; Armstrong, et al., 1997; J.D.Armstrong, personal comm.; M.Yang, personal comm.):

(1) mushroom body expression lines: 201Y, c772, c747, c739, 72Y, 43Y, c97, c184, 30Y, c532, 117Y, 121Y, c302, c309, c253, 238Y. (2) central complex expression lines: c819, c522, c5, 7. (3) optic lobe expression lines: c827, c829. (4) antennal lobe expression line: c287. (5) great commissure expression line: 82Y. (6) general staining lines: 21Y.

All these lines have been Cantonised in Dr. K. Kaiser's laboratory by various researchers.

UAS_G- lines: (1) UAS_G-*lacZ* on the second chromosome (Brand and Perrimon, 1993). (2) UAS_G-*tra* on the second chromosome (O'Dell, et al., 1995).

Balancer lines: From Kevin O'Dell, Department of Genetics, University of Glasgow *CyO/Sp*, and *CyO/Ddc* (Lindsley and Zimm, 1992).

2.2.2. Rearing conditions

Basic technique for the laboratory culture of *Drosophila* were as described by Ashburner (1989) and Roberts (1986). During large scale amplification in bottles, fly density was controlled by not allowing over-crowding to the extent of reducing adult body size. The number of parent flies were around 10 males and 20-30 females, and they were removed prior to hatching of the next generation. The fly stocks were maintained in yeast-glucose food plastic vials and bottles, at 18°C and 25°C.

The food recipe is: 1%(w/v) bacto-agar, 1.5%(w/v) sucrose, 3%(w/v) glucose, 3.5%(w/v)active dried yeast, 1.5%(w/v) maize meal, 1% (w/v) wheat germ, 3%(w/v) treacle, 1%(w/v) soya flour, Simmered for 20 minutes, then supplemented with 0.5%(v/v) propionic acid and 0.1%(w/v) nipagin M, once cooled to below 70°C. For embryo collection, flies were kept in population cages at 25°C and fed on grape juice agar (52g glucose, 26g sucrose, 7g yeast, 20g agar, 58.8ml grape juice and 6ml 10%

Nipagen per litre) plates. Live liquid yeast was smeared onto the plates to encourage egg laying, The plates were changed daily to wash off the eggs.

2.2.3 Ethyl methanesulphonate (EMS) mutagenesis

This method was adapted from Roberts (1986). Newly eclosed males were placed into bottles with food for 3 days at room temperature. Prior to the treatment, they were transfered to an empty bottle at room temperature for 2 hours. This pre-conditioning supposedly enticed the flies to ingest the "unpalatable" EMS-sucrose solution.

EMS is an oily liquid that will initially form droplets when added to aqueous solution. These droplets were dispersed by a disposable syringe after removing 0.26ml EMS to 100ml 1% sucrose solution. One piece of Whatman 2V (125mmØ) filter paper was placed on the bottom of each clean empty half-pint bottle and wetted with 2ml EMS-sucrose solution. Approximately, 100-200 male flies were put into the bottle and left in the fume hood for 24 hours. The male flies were removed from treatment bottles to empty bottles for 2 hours and then to fresh medium and allowed to feed and recover for about 24 hours prior to mating.

Denaturing solution was made up by dissolving 20g of NaOH in 500ml of water and 2.5ml of thioglycolic acid before begining any handling of EMS. Every container or syringe contaminated with EMS was treated with denaturing solution immediately after use and allowed to stand for approximately 24 hours.

2.2.4 Tests of courtship behaviour

For behavioural tests, the crosses typically were of two males and three virgin females per vial. Thus larval density was always substantially greater than five larvae per ml of food, therefore the larval population density effect on mushroom body size was not

relevant (Heisenberg, et al., 1995). Live liquid yeast was added to standard fly media to enrich the food. *Drosophila* were maintained on a 12 hour dark: 12 hour light cycle at 25°C and 35%-45% relative humidity.

When experiments were performed with virgin males, they were collected under CO2 anaesthesia within 4 hours of eclosion, and kept separately in yeasted vials for 4-6 days before testing. The flies were transferred to fresh media on the day prior to observation. Target flies were housed in group of ten of the same sex, aged for five days, and decapitated approximately 15 minutes before use. Courtship were observed at 25°C for 5 minutes in mating wheel with 4 chambers (8mm diameter). After being transferred to the chamber, the courting flies were allowed to recover for 5-10 minutes before being introduced to the target. The observation for each class was repeated ten times to provide n=10.

The courtship index (CI, Siegel and Hall, 1979) was measured for a five-minute observation period. The performance of wing vibration, known as the sex-appeal parameter (SAP, Jallon and Hotta, 1979) was also scored as an additional control index for the courtship. Canton-S males and virgin females were used as targets. Statistical manipulation and analyses (two-tailed t-test and correlation coeffecient) were performed using JMP2 software (SAS Institute, Inc.) and Minitab 10.5 (Minitab Inc.).

2.3 General methods of molecular biology

Molecular biology techniques were performed as described by Sambrook et al. (1989) unless otherwise described.

2.3.1 Manipulation of nucleic acids

(a) Agarose gel electrophoresis

For the restriction analysis of DNA, 0.7-1.2% agarose gels were produced in 1xTBE (90mM Tris-HCl pH8, 90mM boric acid, 1mM EDTA) by boiling. The solution was allowed to cool to about 60°C, and ethidium bromide was added to a concentration of 0.05 μg/ml before pouring. 0.2 volume of loading dye (40% w/v Ficoll, 1mM EDTA pH8.0, 0.1% w/v bromophenol blue) was mixed with the DNA samples. The horizontal gels were run submerged in 1xTBE at 1.5-10 volts/cm. Nucleic acids were visualised under UV irradiation. 1kb DNA markers (BRL) were used on all gels as a size standard and for quantification of the amount of DNA by comparing the intensity of bands to those of the samples.

(b) Recovery of DNA from agarose gel

A silica suspension was made by mixing 10g of silica (Sigma) in 100ml of PBS and allowing the silica to settle for 2 hours. The supernatant was removed and the procedure was repeated. Then the silica was resuspended in 3M NaI at 100mg/ml (Boyle and Lew, 1995).

The DNA band of interest was excised from the agarose gel, 2 volumes of 6M NaI solution were added to the agarose block and followed by incubation at 55°C for 5 min. The silica suspension was used as a DNA-binding matrix by mixing 10-50μl with the melted gel. After washing the pellet twice with 0.5 ml of the wash buffer (50mM NaCl, 10mM TrisHCl pH7.5, 2.5mM EDTA, 50% v/v ethanol), the DNA was eluted in one pellet volume of distilled water.

(c) Restriction endonuclease digestion of DNA

For general purpose digestion of DNA, normal concentration restriction endonuclease preparations were used in the suppliers' recommended buffer, with between 2-fold and 10-fold excess enzyme. Incubation at the recommended temperature was for 1-4 hours,

and the reaction was terminated by addition of electrophoresis loading buffer, or by heating to 70 °C for 5 minutes, or by phenol extraction.

(d) Double stranded DNA ligation and subcloning

Ligations were usually performed in 20-40µl of 1xligation buffer (BRL), containing 1u of T4 DNA ligase. The reactions were incubated overnight at 16°C.

DNA fragments to be subcloned and plasmid vectors were both digested with appropriate restriction enzymes. For the ligation, the molar ratio of insert DNA to vector was 10:1. Transformation was carried out afterwards. Recombinant clones were analysed by restriction digestion and/or hybridization.

(e) Measurement of nucleic acid

the concentration and purity of nucleic acid were determined by spectrophotometry. An absorbance value of 1.0 at 260nm corresponds to 50µg/ml double stranded DNA, 40µg/ml RNA and 20µg/ml oligonucleotides. Pure preparations of RNA and DNA have an A_{260}/A_{280} of 1.8 and 2.0 respectively. Contaminating protein or phenol significantly lowers these values.

(f) Nucleic acid labelling

Radioactive labelling of DNA fragments was performed using a random primer DNA labelling kit (Boehringer Mannheim). [α-^{32}P] dCTP was incorporated into the synthetic oligonucleotides. Labelled probe was separated from unincorporated nucleotide by chromatography through Sephadex G50 columns (Sambrook et al., 1989) prepared in disposable 1ml syringes.

The DNA probes labelled with digoxygenin (DIG)-dUTP were obtained by using random-primed labelling Kit (High Primer, Boehringer Mannheim). The labelled DNA fragment was precipitated with ethanol and can be kept at -20°C for 1 year.

RNA probes were synthesised by utilising the Riboprobe® *in vitro* Transcription System (Promega).

For a "reverse Northern", the first strand cDNA probe was made from 1 μg of *Drosophila* total RNA. The RNA was annealed at 70°C for 5 minutes with 100ng oligo $dT_{(14-18)}$ (Boehringer Mannhleim) and chilled on ice. This template was incubated with 70μCi α^{32}PdCTP (800μCi /mMol); 500mM each dATP, dGTP and dTTP; 10mM DTT; 50mM Tris-HCl pH8.3; 75mM KCl; 3mM $MgCl_2$ and 200 units of AMV Reverse Trancriptase (Boehringer Mannheim) for 90 minutes at 45°C. Then, the reaction was "chased" with cold dCTP (to produce longer probes) at a final concentration of 500μM and a further 80 units of enzyme for 45 minutes at 45°C. After this step, EDTA was added to a final concentration of 20mM and NaOH was added to a final concentration of 600mM and the reaction incubated at 68°C, for 45 minutes, to hydrolyse the RNA template. Finally, labelled single stranded DNA was separated from unincorporated nucleotides by Sephadex G-50.

2.3.2 Isolation of plasmid DNA

Colonies picked from plates were incubated in 10ml LB, with 100 μg/ml Ampicillin, shaking overnight at 37°C. The culture was harvested by centrifugation (5,000rpm for 5-10 minutes). The cell pellet was resuspended in 300μl of cold solution 1 (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0). Next, 300μl of solution 2 (0.2 M NaOH, 1% SDS) was added and mixed, then placed on ice for 5 minutes. 300μl of 3.0M Potassium acetate (pH4.8) solution was added and mixed gently on ice for 5 minutes. Cell debris was removed by centrifugation at 4, 000rpm for 15 minutes. The

supernatant was incubated with 20μg/ml RNase A (DNase-free) at 37°C for 20 minutes. then, extracted with an equal volume of 1:1 phenol/chloroform and the phases separated by centrifugation (10,000rpm, 2 minutes). The aqueous phase was transferred to 0.6 volume of isopropanol and left at room temperature for 10 minutes to precipitate the nucleic acid. After spinning for 15 minutes at 14,000rpm, the pellet was washed with 1ml of 70% ethanol, air dried briefly, and then resuspended in 50-100μl of TE (10mM TrisHCl, 1mM EDTA pH8.0).

2.3.3 Transformation of *E. coli* with plasmid DNA

(a) Preparation of competent cells

An overnight culture of XL1-Blue or DH5α strain was diluted 100-fold into 50ml LB. The culture was shaken vigorously for 2 hours at 37°C to reach the mid log phase (OD_{650}=0.4-0.5). The cells were harvested at 5,000rpm, 4°C in centrifuge for 10 minutes, then resuspended in 25ml of ice-cold 50mM $CaCl_2$ and incubated on ice for 30 minutes. The cells were pelleted again, and resuspended in 2.5ml of $CaCl_2$. The efficiency of transformation increases four-six fold during the first 12-24 hours of storage at 0-4°C and then decreases to the original level (Sambrook et al., 1989).

(b) The procedure of transformation and the selection of recombinant clones

5-40μl ligation mix (or plasmid) was added to 50-200μl aliquots of competent cells, the mixture was incubated on ice for 30 minutes, and then heat shocked at 42°C for 90 seconds. 800μl 2YT (0.16% bacto-typtone, 0.1% yeast extract, 0.05% NaCl) was added to the cells. The culture was shaken at 37°C for 1 hour to allow genes conferring antibiotic resistance to be expressed. 50-200μl of the culture was plated on to LB agar (LB with the addition of 15g/l bacto-agar) plates containing the appropriate antibiotic (e.g. 50μg/ml of Ampicillin)/chromogenic substances (X-gal and IPTG) and incubated overnight at 37°C.

X-gal was used in conjunction with IPTG to identify *E. coli* containing pBlueScript vectors with inserts in their multiple cloning sites. Recombinants containing inserts are generally white while those lacking inserts are blue.

X-gal was stored at a concentration of 20mg/ml in dimethylformamide (DMF) at -20°C while IPTG was stored at a concentration of 200mg/ml in dH_2O at -20°C. X-gal and IPTG were added to LB agar plates to a final concentration of 40μg/ml and 100μg/ml respectively.

2.3.4 Techniques for handling *E. coli* bacteriophage λ

(a) Preparation of plating cells

100ml of LB supplemented with 0.1% (w/v) maltose, 10mM $MgSO_4$, was incubated with 1ml of overnight culture of *E.coli* strain NM621, and grown at 37°C on a shaker for 2-3 hours to a density of approximatly 10^8 cells /ml (OD_{600} = 0.45-0.55). The cells were pelleted by centrifugation (4,000rpm, 10 minutes, 4°C) and resuspended in 40ml of sterile, ice cold, 10mM $MgSO_4$. The cell suspension was stored at 4°C and remained viable for 3-4 weeks.

(b) Plating and titring of λ phage

Serial 10-fold dilutions of λ phage libraries were prepared in phage buffer (20mM Tris-HCl pH7.4, 100mM NaCl, 10mM $MgSO_4$). The infection of cells with bacteriophage was achieved by adding 1-2ml aliquots of each dilution to 100ml of the plating cells. The samples were incubated at 37°C for 20 minutes, 3-5ml of melted top agarose (0.1% trytpone, 0.05% yeast extract, 0.05% NaCl, 0.065% agarose) at a temperature of 45°C was added and the mixture was poured onto LB agar plates. Once set, the plates were

incubated at 37°C for 6-10 hours. The plaques were counted and the titre determined for each dilution assayed.

(c) Isolation of λ phage DNA

The plaque of interest was pulled from plate by the narrow end of a sterile glass pasteur pipette. The phage plug was left in 500µl of phage buffer and 50µl of chloroform (to kill bacterial cells) for 2 hours at room temperature to allow phage particles to be soaked out into the buffer. 20µl of the diffused phage was used to infect 200µl NM621 plating cells. Then the cells were grown on 37ml NZCYM (0.1% NZamine, 0.05% NaCl, 0.05% baco-yeast extract, 0.01% casamino acids, 0.02% $MgSO_4$) at 37°C with vigorous shaking overnight until lysis was apparent. The culture was treated with 1.8mg of DNase I and 1.8mg of RNase A at 37°C for 30 minutes. Then 100µl of chloroform along with 1.1g NaCl was added and dissolved by a further 15 minutes shaking. The debris was pelleted by centrifugation (4,000rpm, 15minutes) and 3.7g of PEG 8000 was dissolved in the recovered supernatant at room temperature and the samples were left at 0°C for 90 minutes to precipitate the phage. The phage was spun down at 10,000rpm for 20 minutes and resuspended in 500µl phage buffer. Then the suspension was extracted with 500µl of choloform. The aqueous phase was incubated with 25µg proteinase K at 65°C for 30 minutes. After purification by phenol and chloroform extractions, DNA was precipitated and resuspended in TE.

(d) Screening of genomic and cDNA libraries

The libraries were plated on 10x10cm square plates. The plaque density was around $1x10^6/cm^2$. The plates containing phage were incubated under normal conditions until plaques were visible, but still isolated. Duplicated nylon filters were marked using a syringe needle and lifted. The denaturing, neutralization and hybridization were

performed according to the manufacturer's (Sartorius) recommendations and standard procedures.

The secondary screening were carried out by 1/100 dilution of the primary phage plug; single plaques could be picked at this stage.

2.3.5 Isolation of *Drosophila* DNA

30 flies were collected in a 1.5ml eppendorf tube and chilled on ice. 500µl of lysis buffer (100mM Tris-HCl pH8.5, 80mM NaCl, 5% sucrose, 0.5% SDS, 50mM EDTA pH8.0) was added and the mixture ground for a few moments The homogenate was left at room temperature for 10 minutes, then transferred to a 70°C heating block for another 30 minutes. Approximately 50µl of 6M potassium acetate (pH4.8) was mixed with the homogenate to make a final concentration of about 1M. After 30 minutes incubation on ice, the debris were spun down (12,000rpm) at 4°C for 15 minutes. The supernatant was removed and precipitated by 0.6 volume isopropanol. The genomic DNA was pelleted by centrifugation (14,000rpm, 10 minutes) at room temperature and resuspended in TE.

2.3.6 Plasmid rescue

The procedure started with DNA preparation and restriction digestion of interesting P-element insertion lines. After performing self-ligation, the DNA mixture was transformed into *E.coli* competent cells. The recombinant plasmids contained both plasmid sequence and *Drosophila* genomic DNA fragments.

2.3.7 Southern blot and hybridization

(a) Southern blot

After electrophoresis and photography, DNA was denatured by leaving the gel in denaturing solution (0.5M NaOH, 1.5M NaCl) for 45 minutes. Then the gel was removed to neutralising solution (1.5M NaCl, 0.5M TrisHCl pH7.2, 1mM EDTA) for another 45 minutes. DNA was transferred to a Nylon filter by capillary action (disposable nappies proved a particularly useful absorbent material for driving the transfer process) with 20xSSC solution (3M NaCl, 0.3M tri-sodium citrate). Nucleic acids were fixed to Nylon membranes by automatic UV crosslinking in a Stratalinker^{TM}.

(b) Hybridization and autoradiography with radioactive probes

The prehybridization was carried out with 20-40ml of Church buffer (7% SDS, 1% fatty acid free BSA, 1mM EDTA, 0.25M Na_2HPO_4) at 65°C for 1-4 hours. Probes in 0.5ml TE were boiled for 5 minutes and quenched on ice for denaturing, then added to prehybridized filters. Hybridization lasted for 14-18 hours. Afterwards, DNA blots were washed briefly in 50-100ml 2xSSC, 0.01% SDS (w/v) at 65°C; secondly, in 0.5xSSC; finally, in 0.1xSSC. The filter was sealed in Saran Wrap and exposed to Kodak X-OMAT film in a metal cassette with intensifying screens. The exposure of the autoradiographic images was performed at -70°C. The X-ray films were developed using a compact X-OMAT automatic processor, model X-2.

(c) Hybridization and detection with DIG-probe

Prehybridization of the filter was carried out in a solution of 5xSSC, 1% blocking reagent (w/v), 0.1% N-laurylsarcosine Na-salt (w/v), and 0.02% SDS for 4 hours at 65°C, followed by the addition of 10µl freshly denatured DIG-DNA probe and overnight incubation. After removing the probe by washing with a different stringency,

the filter was briefly rinsed in buffer 1 (0.1M Maleic acid, 0.15M NaCl, pH7.5), and incubated in buffer 2 (1% blocking reagent in buffer 1) for 30 minutes. Anti-DIG-AP, Fab-fragments (Boehringer Mannheim) were diluted to 0.15u/ml in 30ml buffer 2 and incubated with the filter for a further 30 minutes. The unbound conjugates were removed by further washing in buffer 1. Then the filter was equilibrated for 2-5 minutes in 20ml buffer 3 (0.1M Tris HCl pH9.5, 0.1M NaCl and 50mM $MgCl_2$). The colour development was performed by NBT and BCIP essentially as described in the manual from Boehringer Mannheim.

2.3.8 Isolation of *Drosophila* RNA and Northern hybridization

RNase is a very persistent enzyme and precautions were taken against contamination. All solutions were made RNase free with the addition of 0.1% diethyl pyrocarbonate (DEPC).

(a) RNA preparation

Fly tissue or whole flies weighing roughly 50-100mg was homogenised in 1ml of TRIZOL Reagent (Gibco-BRL) for 60 seconds and left at room temperature for 5 minutes. 200µl chloroform was added and shaken to mix for 30 seconds. After 2-3 minutes of incubation at room temperature, the homogenate was spun at 12,000 rpm for 15 minutes. The aqueous phase was removed and precipitated with an equal volume of isopropanol. The RNA was pelleted by centrifugation at 4°C, and 14,000 rpm for 10 minutes.

Poly A^+ RNA was isolated from total RNA using the Quik® mRNA Isolation Kit (Stratagene) according to the manufacture's protocol.

(b) Northern hybridization

For RNA electrophoresis, the denaturing gels were made from 1% agrose, 7.2% Formaldehyde and 1xMOPS (20mM 3-(N-morpholino) propane sulfonic acid, pH7.0; 8mM sodium acetate; 1mM EDTA, pH8.0). The RNA samples were denatured by heating at 70°C for 5 minutes and mixed with EtBr (at a final concentration of 50ng/ml) prior to loading. The RNA size marker (Gibco BRL, 0.24-7.5kb) was also employed in determining sizes of the signal bands. RNA was transfered and fixed to Nitrocellulose (Hi-Bond) as described in Sambrook et al, (1989).

The prehybridization was carried out at 42°C, with a solution containing 50% Formamide (v/v), 5xSSPE (750mM NaCl, 45mM NaH_2PO_4, 5mM EDTA) pH7.4, 2xDenhardt's (0.04% Ficoll, 0.04% BSA and 0.04% PVP), 0.1% (w/v) SDS. Then the hybridization lasted for 16-24 hours with the addition of 300µl denatured probe. When an RNA probe was used, the temperature of hybridization was 55°C.

For washing, lower salt concentration and high temperature are more stringent, so the procedures were: one brief wash in 2xSSC, 0.1% SDS at 42°C, one 15-minute wash in 1xSSC, 0.1% SDS at 42°C, and another 15-minute wash in 0.5xSSC, 0.1% SDS at 55°C. The autoradiography was similar to that used for Southern hybridization.

2.3.9 Sequencing

(a) Automated Cycle sequencing

Sequencing of subcloned DNA fragments was carried out by the protocol recommended in the Ready Reaction Dye Deoxy™ terminator Cycle Sequencing Kit (ABI). The reaction was driven by thermally stable DNA polymerase (Ampli Taq®). Four dye-labelled dideoxynucleotides are incorporated into the DNA as the terminating bases. The samples were detected and analysed on an ABI automated DNA sequencer (Model 373).

(b) Sequence analysis

The DNA sequences from the ABI 373 DNA sequencing system were first assembled by SEQUENCE NAVIGATOR™1.0 (Applied Biosystems), then input into the MacVector™ programme. Prediction of open reading frames and translation into protein sequences were run on MacVector™4.1.4. For homology and structure comparison, database searches by Blast and Beauty were performed through Netscape 2.1 (http://dot.imgen.bcm.tmc.edu:9331/seq-search/Options/).

2.3.10 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Messenger RNA was isolated by the DYNAL mRNA Direct Kit, the oligo dT moiety of the magnetic beads being used both to capture the mRNA and as a primer for the reverse transcriptase (Gibco-BRL) to synthesise the first strand cDNA. The reaction was carried out with 1mM each dNTP, 5mMDTT, 1xbuffer (supplied with enzyme) and 20 units RNAsin (Promega) at 42°C for 30 minutes. The first strand cDNA covalently attached to the magnetic particles was collected and washed and resuspended. 1/20-1/30 of the beads were used as a template for PCR.

The PCR reaction was carried out in a final volume of 30µl, in the presence of 0.33µM oligonucleotide primers, 200µM of each dNTP, in 1xPCR buffer (supplied with the enzyme) and with 1unit of *Taq* DNA polymerase. Samples were incubated in Hybaid thermal cyclers. A typical cycling profile would be: 40 cycles of incubation at 93°C for 30 seconds; χ°C for 30 seconds; 72°C for 2 minutes. χ was the empirically derived annealing temperature for each pair of oligonucleotides which was judged to generate the least background. If optimisation was not possible, a temperature of 5°C below the T_m of the primer was used.

2.4. *Drosophila* tissue *in situ* hybridization and immunohistochemistry

2.4.1 *in situ* hybridization to *Drosophila* brain sections

The method was essentially as described by Nighorn et al., (1991), but some modifications were made according to Yang (1996). The Digoxygenin (DIG) labelling and detection kit from Boehringer Mannheim was employed.

(a) Sectioning and fixation of tissues

After being anaesthetised, the flies were placed in a Heisenberg collar (Heisenberg and Boehl, 1979) with the needed orientation, and soaked in OCT mountant (BDH) for 10 minutes before being frozen in a cryostat (Anglia Scientific, Cryotome 620). 12μm sections were cut at -18°C. The ribbons of sections were placed onto gelatinised slides and allowed to dry at room temperature for 1-2 minutes. Then they were fixed in freshly made PLP (2% paraformaldehyde, 0.01M sodium metaperiodate, 74mM lysine and 1xPBS) for 10 minutes.

(b) Hybridization and detection of the DIG labelled probe

After two washes in PBS, the sections were treated with 10μg/ml Proteinase K at 37°C for 10 minutes. Then another PBS wash followed by 20 minutes of re-fixation in 4% paraformaldehyde in PBS. The slides were washed twice in 2xSSPE. Each slide was bathed in 200μl prehybridization solution and covered with a cover slip. After 4 hours of prehybridization at 42°C, the slides were then incubated with a denatured (boiled) DIG DNA probe in prehybridization solution (50ng/ml) overnight at 42°C in a humid box. After hybridization, the slides were washed twice in 2xSSPE, once in 1xSSPE and once

in 0.5xSSPE. These washes were all carried out at room teperature for 15 minutes. The final wash was in 2mM NaPPi, 1mM $NaPO_4$ and 1mM EDTA at 42°C.

For detection, the sections were first rinsed in buffer A (0.1M TrisHCl pH7.5, 150mM NaCl) then incubated with 200µl of blocking solution (2% sheep serum, 0.3% Triton X-100 in buffer A) for 1 hour. 150µl of a 1:500 dilution of preabsorbed Anti-DIG-AP was added to each slide and incubated 3 hours at room temperature. After 4 washes in buffer A, the sections were placed in buffer 3 for 2-3 minutes, then 200µl levamisole solution with NBT and X-phosphate was applied to each slide. The sections were incubated in the dark at room temperature for 2-4 hours. The reaction was stopped by washing in PBT for 20 minutes and mounted with glycerol gelatin (Sigma).

2.4.2 Developmental staining of Anti-β-gal antibody on larval, pupal and adult brains

The intact brains were dissected from male and female 3rd instar larvae; four-hour-interval pupae and adults. The sex of the larvae and pre-pupae was judged by the size of the gonads (Ashburner, 1989). For timed pupal series, the newly immobile pre-pupae (Bainbridge, and Bownes, 1981) were collected every 30 minutes throught the day. The slide containing the pupae was then placed in an empty food bottle to maintain a similar temperature and humidity to that of the original bottle. Pupae's dissection times were accurate to within +/- 10 minutes, the collection times were accurate to within +/- 30 minutes giving an overall accuracy of +/- 40 minutes well within the +/- 1 hour used in previous studies (Ito, 1990)

The brain tissues were fixed in 4% paraformaldehyde for 30 minutes and washed twice for 1 hour in PAT (1% bovine serum albumin, 1% Triton X-100, 1xPBS). They were incubated overnight in 3% normal goat serum (SAPU) containing rabbit polyclonal anti-β-gal antibody (Cappel) diluted 1:2000 in PAT, then washed three times in PAT for 20

minutes each. 1/250 dilution of secondary antibody (fluorescein-labelled goat anti-rabbit IgG; Vector Labs) was incubated overnight with the brains. Then, two washes for 1 hour in PAT were followed by a 5-minute wash in PBS. All of the above was carried out at room temperature. Stained brains were mounted in Vecta Shield (Vector).

Whole-mount stained brains were examined with a molecular Dynamics Multiprobe laser scanning confocal microscope. The excitation (480nm) and emission (530+/-15nm) barrier filters used were appropriate to the fluorescein-based label of the secondary antibody. Three dimensional reconstructions were performed using the programme "ImageSpace 3.1" (Molecular Dynamics). Pseudo colour was added to the reconstructed view using the programme "NIH-Image" (National Institutes of Health, Washington). Montages were assembled in "Adobe Photoshop". The image processing was kindly carried out by Dr. Douglas Armstrong.

2.4.3 X-gal staining of adult brains

Brain dissection and fixation were as described before. After PBS washes, the brains were stained with staining buffer (10mM NaH_2PO_4, 10mM Na_2HPO_4, 150mM NaCl, 1mM $MgCl_2$, 3.1mM $K_4(Fe^{2+}CN)_6$, 3.1mM $K_3(Fe^{3+}CN)_6$, 0.3% TritonX-100, pH7.8) and 2% X-gal for 1-2 hours at 37°C. They were then washed for 20 minutes in PBS. The tissues were mounted on a slide in a cover slip chamber.

Chapter 3

The effects of ectopic *white* and *transformer* expression on male-male courtship

3.1 Introduction

The *white* gene was first reported by Morgan (1910). Over the decades, it has served as a prototype for numerous studies concerning gene regulation, insertional mutagenesis, and behavioural analysis of mutants (Levis et al., 1984; Hazelrigg, 1987; Geer and Green, 1962). It is located at the distal end of the X chromosome, 3C2 (Lefevre and Wilkins, 1966). The major transcript has been identified as a 2.6kb poly(A^+)RNA (Fig. 3.1A) found in embryos, larvae, pupae and adults (O'Hare et al., 1983; Fjose et al., 1984; Pirrotta and Brockl, 1984). It is predicted to encode a 687 amino acid member of the ATP-binding, transmembrane, transporter superfamily (O'Hare, et al., 1984; Pepling and Mount, 1990). Specifically, the products of the *white* and *scarlet* (Tearle et al., 1989) genes are believed to form a heterodimer that functions in the transport of tryptophan; the products of the *white* and *brown* (Dreesen et al., 1988) genes are believed to form a heterodimer that acts in guanine transport. Tryptophan and guanine are precursors to the eye pigments essential to normal eye colour. In *Drosophila, white* is required for pigment production in the light-screening cells of the compound eye, ocelli pigment cells, sheath cells of the testes, and the larval Malpighian tubules.

From the early stages of P-element transformation experiments, along with *rosy*, *white* genomic DNA was used as a marker to follow integration, but the transformation frequency seemed to be highly size-dependent (Rubin and Spradling, 1983) . In order to obtain a more suitable transformation vector, the size of the *white* gene was reduced by removing a large part from its first intron and 3' non-coding sequence (Klemenz et al., 1987). This genetically engineered *white* construct is known as the "mini-*white*" and still encodes a full-length protein. During the last decade, it has been extensively used as an eye marker to identify P-element mediated germ-line transformants in *white*$^-$ recipient lines.

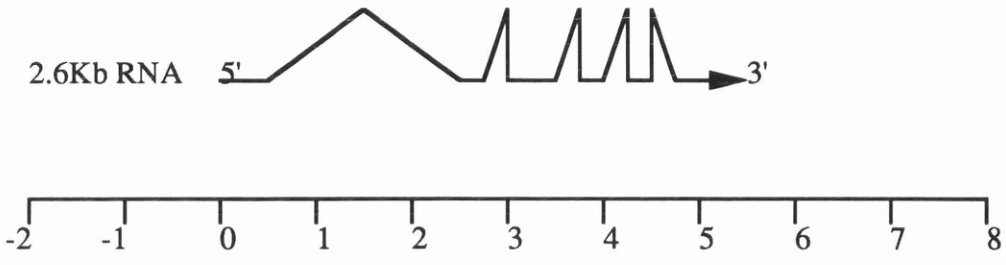


Figure 3.1 A Structure of the major 2.6 kb poly(A)$^+$ RNA encoded by *white* (Hazelrigg, 1987). The transcription initiation site marks the 0 point of the coordinate system (scale in kb).

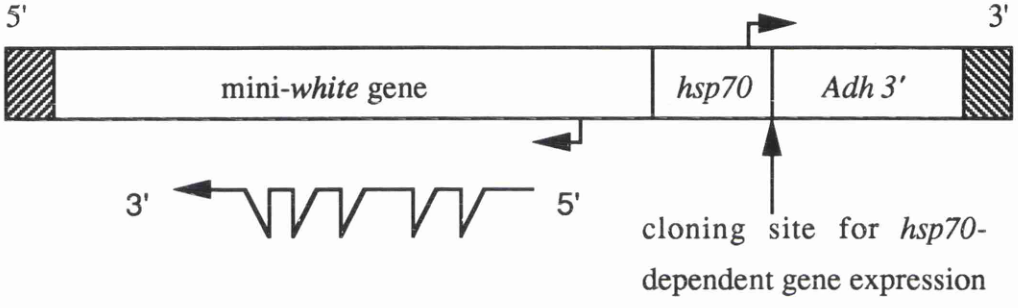


Figure 3.1 B The pHSBJCaSpeR vector (Malicki, et al., 1993). 290bp of the *hsp70* promoter/ transcription start site are flanked by mini-*white* and by the 3' untranslated trailer of the Alcohol dehydrogenase (*Adh*) gene (Kreitman, 1983). The mini-*white* gene includes 300bp of the endogenous promoter, a transcribed domain lacking most of its 5' intron, and 630bp of DNA flanking its poly(A) addition site (Pirrotta, 1988). Hatching represents the P-element ends and the bent arrows indicate transcription initiation sites and directions for the mini-*white* and *hsp70* promoters.

Heat shock promoters are employed in numerous gain-of-function experiments studying ectopically expressed proteins *in vivo*; they are turned on by an increase of the environmental temperature. *hsp*-70 is one of these promoters. P-element vector pHSBJCaSpeR (Fig. 3.1B) was designed containing an *hsp*-70 promoter and a mini-*white* gene marker (Malicki et al., 1993). It has been reported (Zhang and Odenwald, 1995) that the heat shock vector's mini-*white* gene was also activated after heat induction, and this misexpression of *white* led to a remarkable homosexual behaviour in mature adult males. The heat-shocked males vigorously court other males, forming courtship chains: males follow each other in lines, occasionally forming lariats and rings. This effect was observed in a variety of the strains containing insertions of pHSBJCaSpeR constructs in which different transgenes had been placed under the control of the heat-shock promoter. Zhang and Odenwald used EMS treatment to create *white*$^-$ mutants of pHSBJCaSpeR. Ablation of the mini-*white* transgene function also abolished the inducible behaviour.

Loss of *white* gene function does not appreciably affect viability and fertility, but the absence of light-screening pigments in the compound eyes causes poor courtship. However, males of *Drosophila* will search for females when in the dark. Crossley (1970) observed that wild type *Drosophila melanogaster* males and the *white* mutants court under darkness in a similar manner. A *white* male's optomoter responses to moving females is impaired, however, and it "tracks" the female in a particularly anomalous way (Heisenberg and Wolf, 1984).

As already mentioned in Chapter 1, the somatic sexual phenotype of *Drosophila* is cell-autonomous (Baker and Ridge, 1980), and the performance of male courtship requires genotypically male cells in the central nervous system (CNS). Further, the *transformer (tra)* gene is necessary for all aspects of female somatic sexual differentiation (McKeown, et al., 1988), and sexual behaviour (McRobert and Tompkins, 1985). To investigate the neural basis of sexual orientation, chromosomally male flies feminized in

certain regions of the central brain have been generated by the P[GAL4]/UAS$_G$*tra* system (Ferveur et al., 1995; O'Dell, et al., 1995). The *tra* gene under UAS$_G$ control produces the female specific transcript. A P[GAL4] transposon that has "trapped" a tissue-specific enhancer can be subsequently used to drive ectopic female *tra* expression (See Fig. 1.6B). Though *tra* is normally transcribed in both sexes, only the female-specific splice isoform leads to the production of an active *tra* product, TRA^F, which works together with TRA-2 as a splicing factor. They bind to primary transcripts of the downstream genes, *dsx* and *fru* (Tian and Maniatis, 1993; Ryner, et al., 1996), and commit the latter pre-mRNAs to a female-specific splicing pattern. The transcripts of both *dsx* and *fru* are thus sex-specifically controlled by *tra* and *tra-2* (See Chapter 1).

A study concerning brain structures relevant to male courtship behaviour (through GAL4 dependent transformation of male neurons by expressing *tra*) has been previously carried out in this laboratory (O'Dell et al., 1995). In total 6 lines have been analysed, three of them (c35, c739, 201Y) show mushroom body expression patterns, one (c232) has neuronal expression restricted to the ellipsoid body, one (c123a) reveals antennal lobe and antennal nerve expression, and one (c127) has no visible staining in the adult brain. The behavioural results came out as follows: two mushroom body lines (201Y and c739) and the antennal lobe line performed non-discriminatory courtship towards female and male targets, while the others behaved more or less as the wild type control. The study of Ferveur et al. (1995) also suggested that brain feminization in a portion of the antennal lobes or in the mushroom bodies changed the sexual orientation of male flies, so they courted both male and female targets. The conclusion drawn was that the feminization of some subdomains of mushroom body and antennal lobes, elements of the olfactory pathway, may be responsible for the behavioural transformation.

However, both the P[GAL4] and the UAS$_G$-*tra* P-element constructs have the mini-*white* gene as an eye-marker. Therefore, the courtship result faced a challenge: Is the observed male-male courtship observed due to *tra* or mini-*white*?

To address this question further, we decided to knock out the mini-*white* gene in both P[GAL4] and UAS$_G$-*tra* constructs. 201Y was chosen as the P[GAL4] line for mutagenesis, because of its strong behavioural transformation under UAS$_G$-*tra*. No abdominal transformation has been observed for this line, and the transformed males are not attactive to wild-type males (O'Dell, et al,. 1995). Besides, 201Y has a very restricted neuronal expression pattern in the mushroom bodies (Fig. 3.2). Staining is almost exclusively in Kenyon cells belonging to core elements of the α and β lobes, and to a subset of fibres within the γ lobe (Yang et al., 1995). The staining pattern starts during the larval stage and lasts into adulthood (Armstrong, 1995).

Mutagenesis was carried out by EMS treatment. Mutant lines were then subjected to functional tests, courtship observation and analysis. My results confirm that ectopic expression of *tra* rather than of *white* is responsible for the behavioural transformation seen in line 201Y/UAS$_G$-*tra* .

I have also used RT-PCR to study the molecular consequences of GAL4-induced *tra* expression in the brain. The *dsx* and *fru* genes are both dependent on *tra*, but are independent of each other. The work presented in this chapter shows that the female versions of *dsx* and *fru* transcripts are generated in the 201Y brain under *tra*- control.

3.2 Results

3.2.1 Ethyl methanesulphonate (EMS) mutagenesis

This was carried out mainly according to the protocol described by D. B. Roberts (1986). Approximately 100-200 201Y and UAS$_G$-*tra* males were collected within a day of eclosion and fed with EMS-sucrose (See Chapter 2). After 24 hours of recovery, crosses were set up with the ratio of 1 male to 2-3 newly eclosed *w;CyO/Sp* virgin

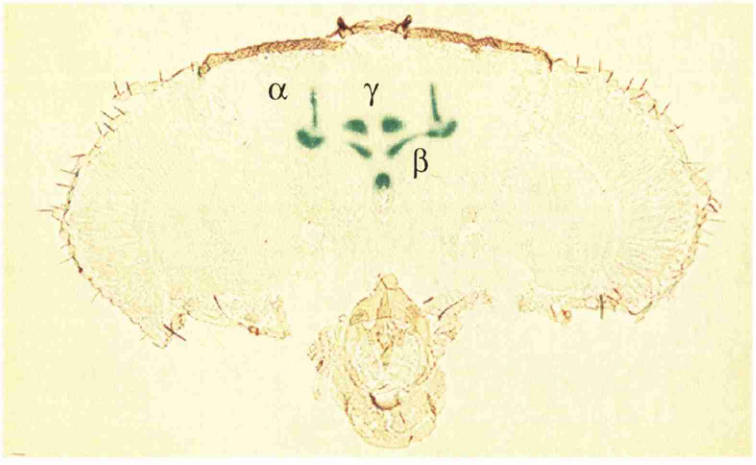


Figure 3.2 The expression pattern of P[GAL4] line 201Y. The panel is a 12 μm frontal cryostat section through the adult head, stained using X-gal, a chromogenic substrate for β-gal. A thin central component of the α lobe, a thin component of the β lobe, and a dorsal component of the γ lobe can be seen at this plane of section (Yang , et al., 1995).

females. Parent flies were removed to another fresh medium bottle after 3 days to allow them to lay more eggs. Prior to hatching of the next generation, all the parent flies were removed.

3.2.2. Selection of the mutated flies

The crossing scheme for selection of mutated flies is shown in Figure 3.3. The P element insertions of both 201Y and UAS$_G$-*tra* transgenic lines are located on the second chromosome. *CyO* is a second chromosome balancer (Lindsley and Zimm, 1992) marked with *Curly (Cy)*, with the dominant visible phenotype of curly wings. The dominant visible phenotype of *Sternopleural (Sp)*, also on the second chromosome, is an increase in the number of sternopleural bristles. Both mutations are homozygous lethal; heterozygotes have good viability and fertility. The cross of *w; CyO/Sp* virgin females and mutagen-treated males allowed identification of knocked out mini-*white* genes on the P-element constructs. After the first generation of selection, *white*$^-$ progeny were backcrossed to the balancer. Then, F_2 inbreeding of 201Y*/*CyO* or UAS$_G$-*tra**/*CyO* was used to establish homozygous mutant lines (* stands for *white*$^-$).

One 201Y* line was established from a screen of 4,000 F1 progeny, and two white-eye UAS$_G$-*tra* lines from 5,000 F1 progeny.

3.2.3 Tests of GAL4 and UAS$_G$-*tra* function in the mutant lines

The purpose of creating the mini-*white* mutants was to drive expression of *tra* in the mushroom bodies without expression of mini-*white*. It was thus essential to confirm that expression of GAL4 and of *tra* were unaffected by the mutagenesis

For line 201Y*, X-gal staining has been done to whole mounts of brains of 201Y*/UAS$_G$-*lacZ*. Staining is strongly concentrated within the mushroom bodies

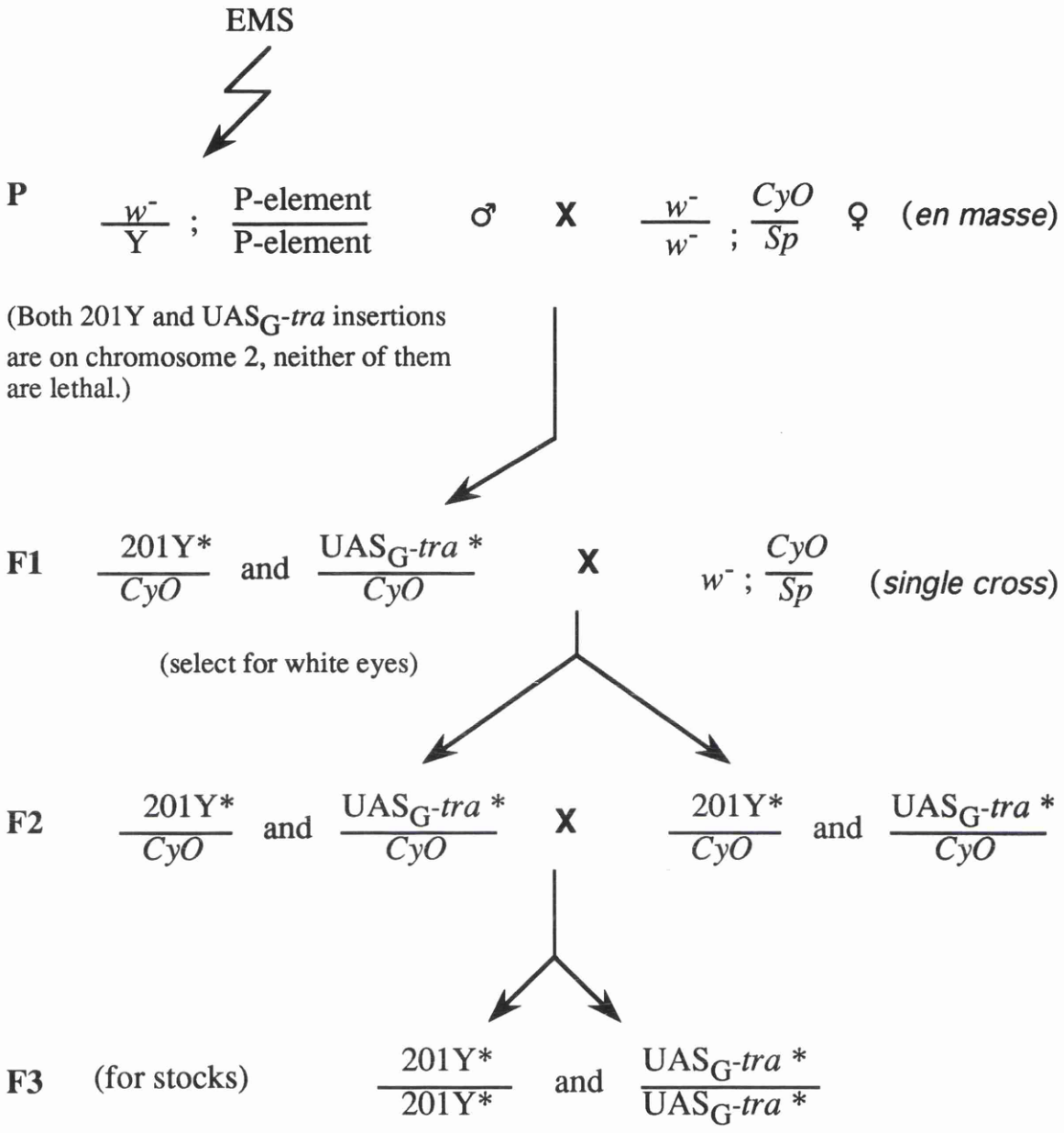


Figure 3.3 Scheme for isolation of *white*$^-$ derivatives (*) of 201Y and UAS$_G$- *tra*.

(Fig.3.4), as in 201Y/UAS$_G$-*lacZ*. The trapped enhancer of line 201Y* is thus driving expression of a functional GAL4 gene from the mutated P-element construct in the same area of the brain as does 201Y.

Because *tra* is required for all aspects of somatic feminisation, ectopic expression of *tra* can cause morphological feminisation in some P[GAL4] backgrounds (e.g. line c739). Of the two putative UAS$_G$-*tra** lines, one, when crossed to c739, showed the expected incomplete feminisation of the male abdomen, though not of the sex combs on the front legs (Fig. 3.5). This demonstrates that the *tra* gene of the mutated construct remain functional. The other UAS$_G$-*tra** line, for unknown reason(s), did not give rise to any morphological change.

3.2.4. *white* gene transcripts in the mutant lines

As the phenotype of the EMS-induced mutations is observable as white eyes, it was interesting to test if there was any decrease in the level of *white* transcription. Northern blotting was used to detect steady state mRNA levels. Total RNA from equal numbers of 201Y, 201Y*, UAS$_G$-*tra*, UAS$_G$-*tra** and *w; Cyo/Sp* adults (both sexes) was isolated, followed by gel electrophoresis and transfer (Chapter 2).

A complementary RNA probe specific to the 3' end of the *white* gene was generated using T7 RNA polymerase and the mini-*white* gene (from P[lacW] (Bier, et al., 1989)) pBluescript subclone as a template. The blot was rehybridized with a ribosomal protein rp49 (O'Connell and Rosbash, 1984) DNA probe as a loading control.

Northern analysis revealed (Fig. 3.6) that both *white*$^-$ mutation lines (201Y* and UAS$_G$-*tra**) had lower *white* mRNA levels than did controls (201Y, UAS$_G$-*tra* and *w; CyO/Sp*). The function of the *white* gene in the mutated lines (201Y* and UAS$_G$-*tra**) has therefore almost certainly been knocked out by reducing the level of mRNA

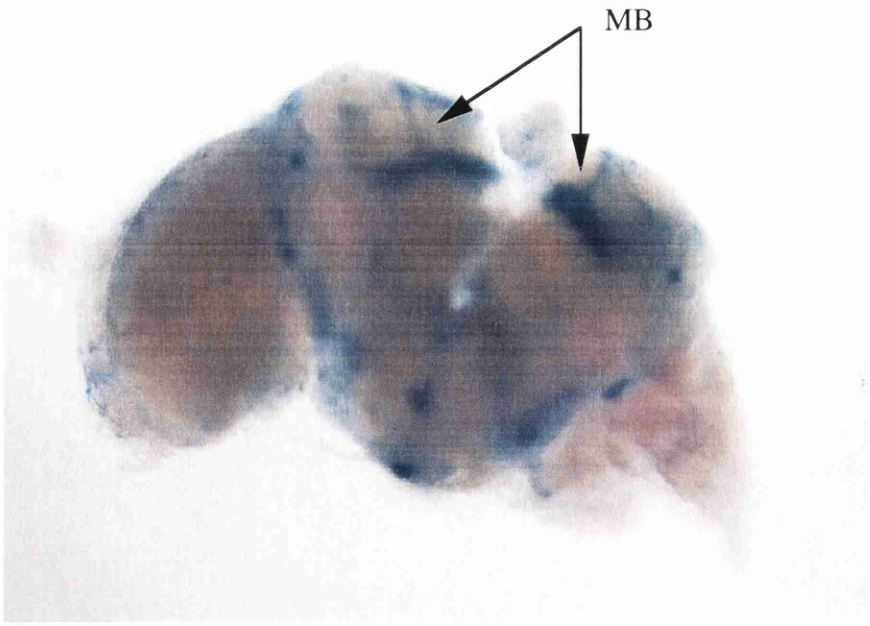


Figure 3.4 Whole mount of the adult 201Y* brain (from the front) stained by X-gal. The pattern is identical to that of line 201Y. Blue dots around the central brain are staining of the surrounding membrane. The GAL4 gene in 201Y* is thus still functional and triggered by the same enhancer. MB stands for mushroom body.

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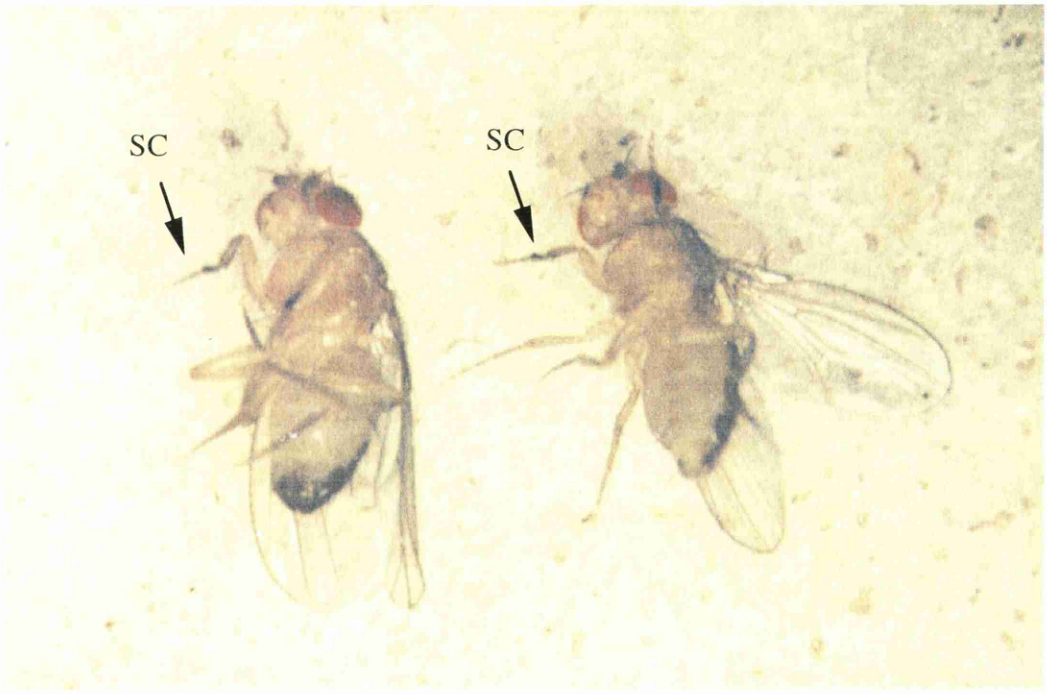


Figure 3.5 Functional test of UAS$_G$-*tra**. Left: wild-type (Canton-S) male; right: c739/UAS$_G$-*tra** male. Although sex combs (SC) are retained on the front legs, c739/UAS$_G$-*tra** shows morphological feminization of abdominal pigmentation and genital structure. The *tra* gene of c739/UAS $_G$-*tra** is thus unaffected by the mini- *white* mutation.

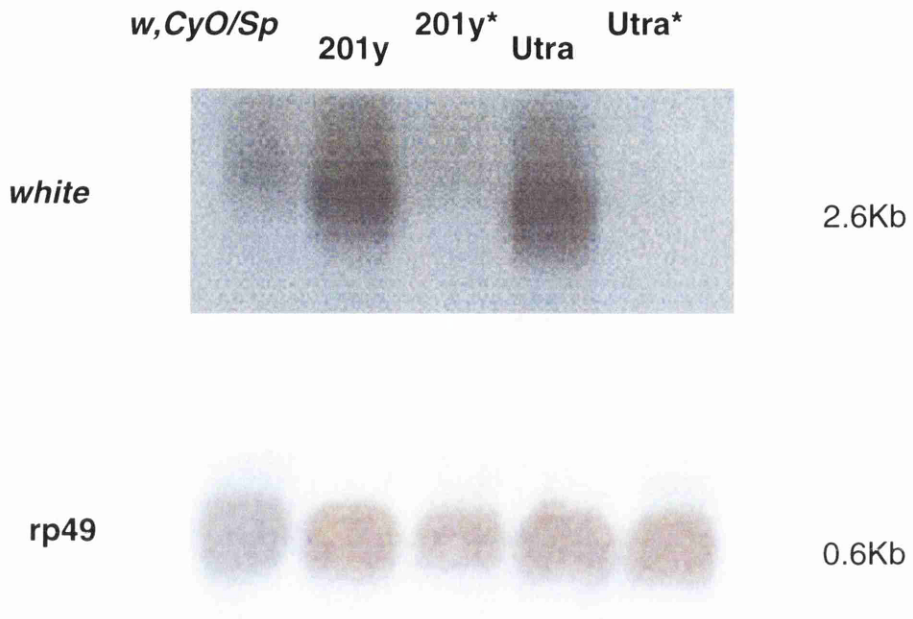


Figure 3.6 Detection of *white* gene expression by Northern hybridization. RNA electrophoresis and transfer were performed as described in Chapter 2. The blot was probed with a ^{32}P-labelled *white* RNA probe. After hybridization, the filter was stripped and reprobed with *rp49* (O'Connell and Rosbach, 1984) as a control for differences in RNA loading. Sizes of 2.6Kb and 0.6 Kb were determined with respect to an RNA size marker (Gibco BRL).

transcription. It is similar to the result obtained by Zhang and Odenwald (1995) for their mini-*white* mutations.

3.2.5. Introduction of an X-linked wild-type *white* gene

Mutation of *white* results in poor visual acuity and impairs a male's ability to visually track a potential mate. It does not effect the mating efficiency in complete darkness, but reduces the "lights on" mating success compared to *white*$^+$ males (Heisenberg and Wolf, 1984). In order to ensure that courting males have normal eye colour, a wild-type *white* gene has been introduced into the 201Y, UAS$_G$-*tra*; 201Y* and UAS$_G$-*tra** backgrounds.

The balancer used for the crossing scheme was *Ddc/CyO* isogenised into a Canton-S genetic background. The *Ddc* (*Dopa decarboxylase*; Wright, et al., 1976) mutation is homozygous lethal and located on the second chromosome. The crossing scheme is shown in Figure 3.7. Males resulting from the first cross all carried an X chromosome derived from their maternal parents and a P-element from their paternal parents. Half of them contained the *CyO* balancer. When F_1 P-element/*CyO* males were backcrossed to Cantonised *Ddc/CyO* virgin females, all the F_2 progenies had an X-linked wild-type *white* gene. The F_2 inbreeding established the final generation.

3.2.6. Courtship behaviour

Courtship behaviour towards wild-type male targets was tested in 3 groups.

a) 201Y/201Y, 201Y/201Y* and 201Y*/201Y*.

b) UAS$_G$-*tra*/UAS$_G$-*tra*, UAS$_G$-*tra*/UAS$_G$-*tra** and UAS$_G$-*tra**/UAS$_G$-*tra**.

c) 201Y/UAS$_G$-*tra*, 201Y*/UAS$_G$-*tra*, 201Y/UAS$_G$-*tra** and 201Y*/UAS$_G$-*tra**.

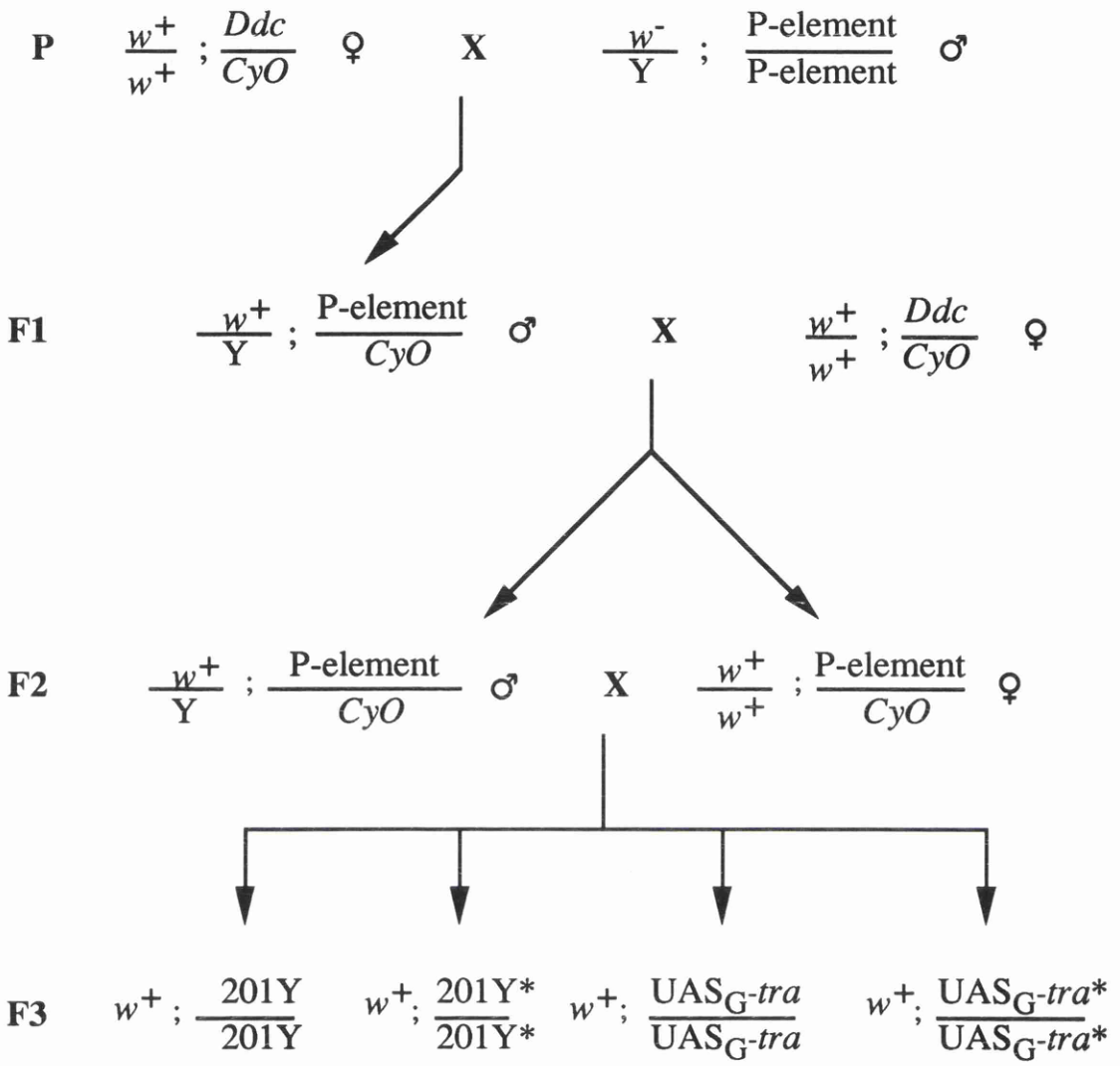


Figure 3.7 Scheme for introducing a wild-type *white* gene into the 201Y, 201Y*, UAS$_G$-*tra* and UAS$_G$-*tra** backgrounds.

Canton-S flies were used as controls. The first and second groups also served as controls of the third group. They tested the effect of different dosages of mini-*white* without *tra* expression. The third group expressed *tra* in the same mushroom body neurons, but with different dosages and sources of mini-*white*.

Figure 3.8 shows the results for the first group. When there was expression of two copies of the mini-*white* gene in 201Y, the flies performed significantly more courtship than did the wild-type control. If only one copy (201Y*/201Y) or no copy (201Y*/201Y*) of mini-*white* was present, no significant male-male courtship was observed. These results show that mini-*white* alone in the P[GAL4] construct can cause non-discriminatory courtship. The behaviour can be triggered when mini-*white* is homozygous, but not when heterozygous.

Figure 3.9 shows the results for the second group. None of the UAS_G-*tra* genotype displayed significant courtship. This shows that the UAS_G-*tra* construct, including the mini-*white* gene, does not affect behaviour.

Figure 3.10 shows the result for the third group. All of the tested genotypes showed significantly more courtship towards male targets than did control flies. Variation in mini-*white* dosage caused no significant difference. These results indicate that, in the presence of *tra* expression, and in heterozygotes, there is no effect of the mini-*white* gene.

Taken together, the results of the three groups, in the absence of *transformer* expression, point to bisexual behaviour in homozygous 201Y males being a consequence of ectopic mini-*white* expression. On the other hand, feminization of the mushroom body in line 201Y by *tra* expression leads to non-discriminatory courtship, no matter what the level of *white* gene expression.

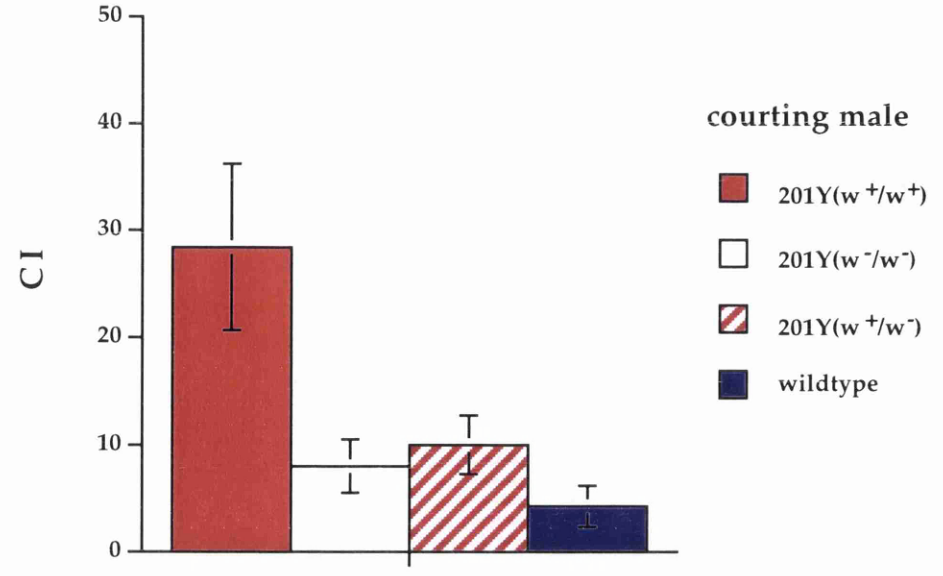


Figure 3.8 Courtship of 201Y males with different dosages of the mini- *white* gene towards wild-type males. CI (courtship index) indicates the percentage of time spent courting. The height of each bar represents the mean score (with SEM) for 10 individuals of each line. 201Y (w $^{+}$/w^{+}) has significantly higher CI than the other genetic constitutions tested.

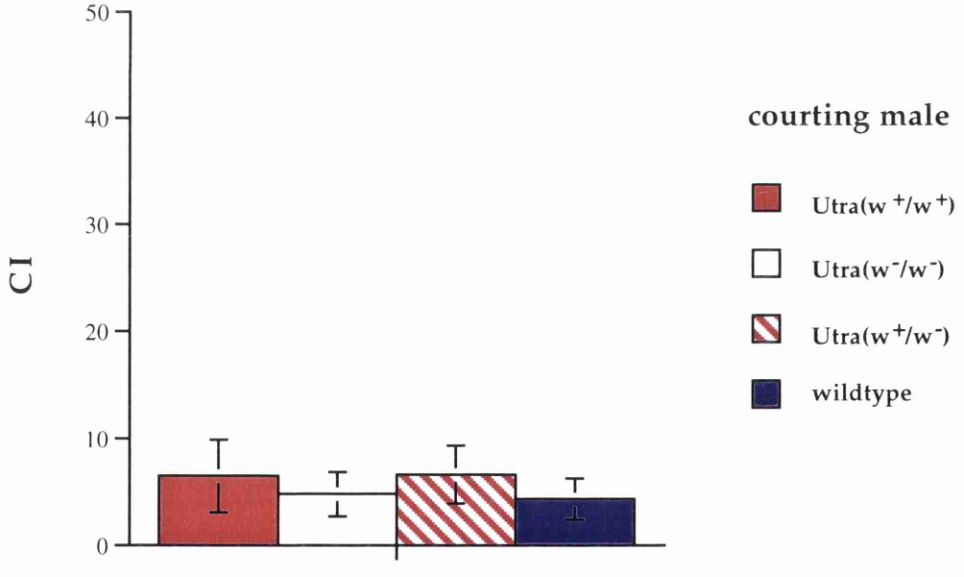


Figure 3.9. Courtship of UAS G-*tra* males with different dosages of the mini*white* gene towards wild-type males. The height of each bar represents the mean score (with SEM) for 10 individuals of each line. There is no difference between these four genotypes.

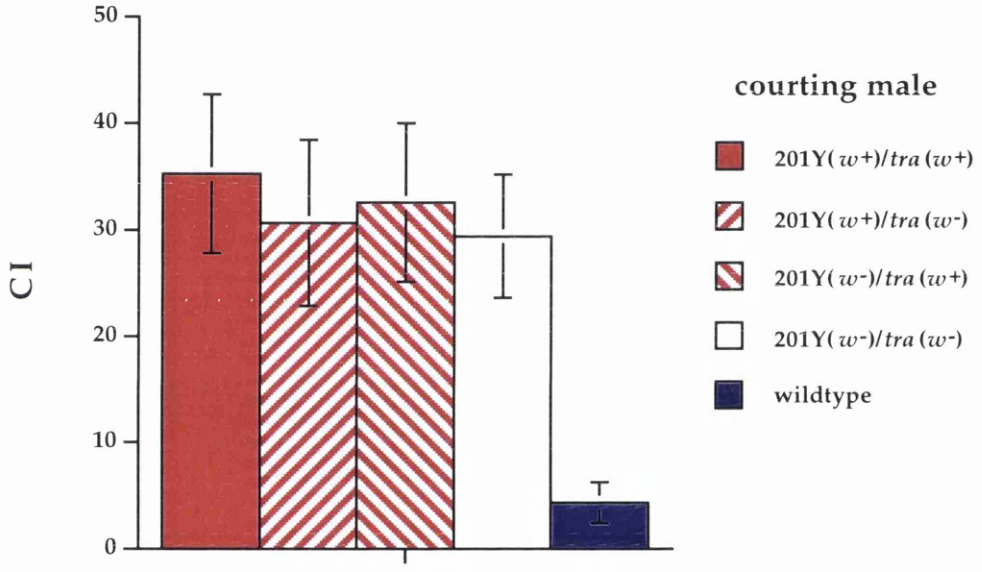


Figure 3.10 Courtship of 201Y/UAS G-*tra* males with different dosages of the mini-*white* gene towards wild-type males. The height of each bar represents the mean score (with SEM) for 10 individuals of each line. The four tested genotypes all have a significantly high CI than the wild-type control.

3.2.7 *tra*-induced *dsx* and *fru* expression

The brains of Canton-S, 201Y/UAS$_G$-*tra* males, 201Y/UAS$_G$-*lacZ* males and UAS$_G$-*tra* males were dissected in PBS. Tissues of the dorsal brain, in which the neurons of the mushroom bodies are mainly gathered, were dissected, and RT-PCR reactions were carried out as described in Chapter 2.

The splicing pattern of *fru* is shown in Figure 3.11A. The design of gene specific primers fm, ff and fc is from Ryner, et al. (1996). Figure 3.11B and C show that a female-specific *fru* RT-PCR product (496bp) is generated from wild-type females, while a male-specific product (322bp) is generated from wild-type males. In the dorsal brains of 201Y/UAS$_G$-*tra* males, both male and female versions of the *fru* transcripts are found. Both controls, 201Y/UAS$_G$-*lacZ* and UAS$_G$-*tra*, show more or less normal splicing of *fru*. Thus, in the presence of GAL4 induction in UAS$_G$-*tra* males, TRA^F leads to generation of a female *fru* transcript.

The splicing pattern of *dsx* is shown in Figure 3.12A, together with the positions of primers dm, df and dc. RT-PCR on RNA from Canton-S males and females indicates that the design of the the primers enables us to amplify sex-specific *dsx* transcripts (Fig. 3.12B and C). Again, in the 201Y/UAS$_G$-*lacZ* and UAS$_G$-*tra* males, RT-PCR could only amplify the male-specific region, while in 201Y/UAS$_G$-*tra* males, both male and female versions of *dsx* were amplified. Female splicing of *dsx* thus occurs as a consequence of GAL4-induced *tra* expression.

3.3 Discussion

We do observe an effect of mini-*white* on male-male courtship. The phenomenon only occurred in flies homozygous for the mini-*white* gene in P[GAL4] line 201Y. The same

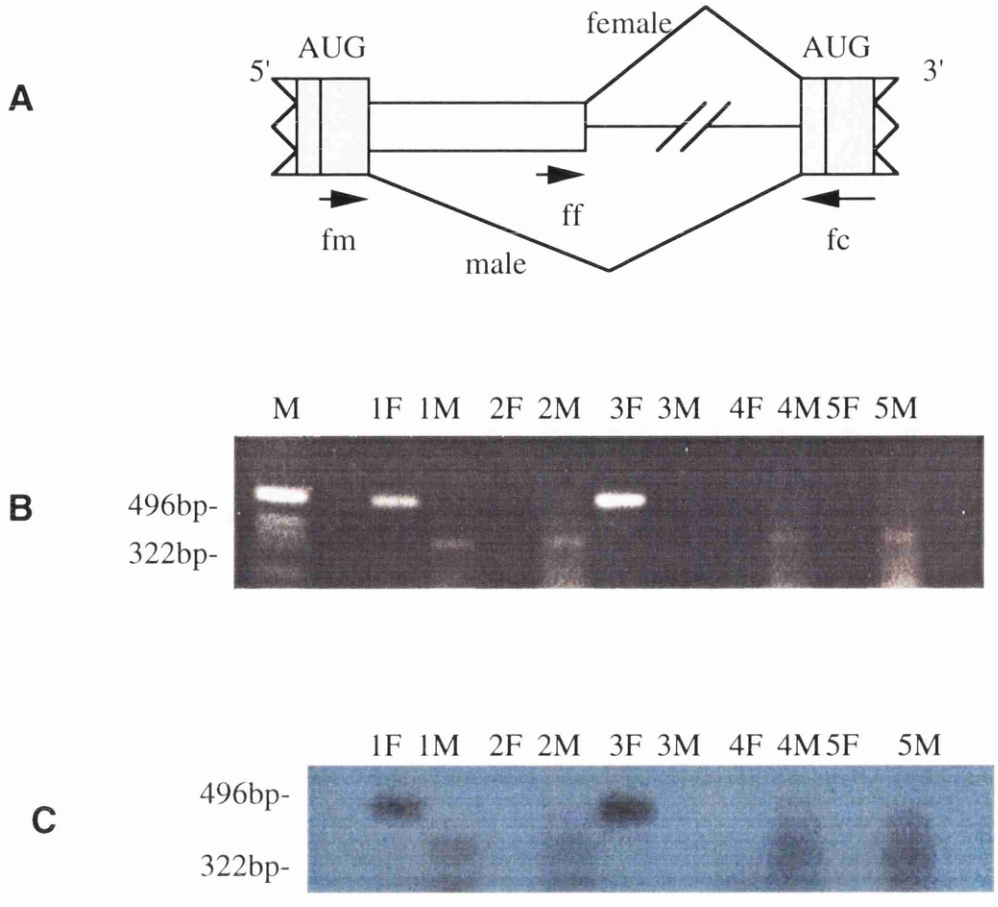


Figure 3.11 Sex-specific processing of *fruitless (fru)* .

A Schematic drawing of the alternative 5' splice sites of *fru* that are joined to a common 3' exon. Exons are indicated by rectangles; jagged sides indicate that only a portion of the exon is shown; thin lines represent the introns. Positions of primers fm, ff and fc used for RT-PCR are indicated by short lines with arrowheads. Shaded regions have protein coding potential.

B RT-PCR products generated using primer fc and sex-specific primers (F = fc + ff; M = fc + fm). The numbers above each lane indicate that the RNAs are from the dorsal brains of: 1, 201Y/UAS_G-*tra* males; 2, Canton-S males; 3, Canton-S females; 4, 201Y/UAS_G-*lacZ* males; and 5, UAS_G-*tra* males. Lane M contains DNA size markers.

C Southern blot analysis of B using primer fc as a probe. Both male (322bp) and female-specific (496bp) transcripts of *fru* are detected in 201Y/UAS_G-*tra* males.

Figure 3.12 Sex-specific processing of *doublesex (dsx).*

A Processing of *doublesex (dsx)* RNAs. Exons are represented as rectangles. The *dsx* RNAs contain three common exons (1-3) followed by male-specific (5,6) or female-specific (4) terminal exons. Gaps in the figure are necessitated by the large size of introns. Positions of primers dm,df and dc used for RT-PCR are indicated by short lines with arrowheads.

B RT-PCR products generated using primer dc and sex-specific primers (F = dc + df; M = dc + dm). The numbers above each lane indicate that the RNAs are from the upper parts of the brains of: 1, 201Y/UAS_G-*tra* males; 2, Canton-S males; 3, Canton-S females; 4, 201Y/UAS_G-*lacZ* males; and 5, UAS_G-*tra* males. Lane M contains DNA size markers.

C Southern blot analysis of B using primer dc as a probe. Both male (800bp) and female-specific (1154bp) transcripts of *dsx* are detected in 201Y/UAS_G-*tra* males.

effect is seen for a large number of homozygous P[GAL4] insertions, as will be discussed in the next Chapter.

Considering that mini-*white* did not affect the behaviour of UAS_G-*tra* flies, the result with 201Y homozygotes might be merely a dosage effect. Different lines can have different degrees of eye pigmentation, presumably due to position effects associated with different chromosomal sites of insertion (Klemenz, et al., 1987). Because of a position effect, the eye colour of UAS_G-*tra* flies is orange rather than red (but gets darker with age). Maybe insufficient *white* function in this line is the reason for no bisexual behaviour being observed.

On the other hand, there are no P[GAL4]/CS heterozygote males which have shown male-male courtship. It seems to be a homologous pair-dependent effect. Transvection is a possible explanation for the mini-*white* effect. Transvection is defined as synapsis-dependent interaction between loci. The *white* locus is known to be subject to synapsis-dependent suppression of expression by mutant forms of the *zeste* gene (Hazelrigg, 1987); *zeste* suppresses *white* expression only when two copies of *white* are present on homologous chromosomes. This effect persists even when the two copies of the *white* locus are present as transgenes at a new chromosomal location; the effect operates in *trans*. The *zeste* gene product is known to have a sequence-specific DNA-binding activity and to recognise binding sites at numerous places in the genome (Pirrotta, 1991). Thus, it acts as a transcription factor and mediates transvection phenomena at several loci. Hazelrigg and Petersen (1992) observed a wide range of *white* expression and responses to $zeste^1$ depending on the insertion positions of the P-element-borne copies of the *white* gene. Recently, Judd (1995) proposed the concept that it is the insertion of the P-borne construct itself, perturbing the invaded chromatin domain, that confers pairing sensitivity by changing the way that proteins recognise and combine with the target sequences to activate or silence the domain. Our observation of 201Y, in terms of their effects on male courtship, appears to fit the above theory. The ability to

create compound heterozygotes which bear two copies of P[GAL4] associated mini-*white* genes, but at different loci, should allow us to detect pairing (or synapsis)-dependence.

The biochemical mechanisms for the effect of *white* on courtship behaviour are currently unknown. Ectopic expression of the tryptophan/guanine transporter, which is encoded by *white*, may modulate levels of these raw materials in cells that utilize them for purposes other than pigment production and thereby affect additional physiological processes. Relevant examples from other animals (cats, rats and rabbits) show that reduction of serotonin levels induces male homosexual activity (Fratta, et al., 1977; Ferguson, et al., 1970). The misexpression of a tryptophan/guanine transporter may lower the synthesis of serotonin by altering the level of guanine and tryptophan (the serotonin precursor) in serotonin-producing neurons. In *Drosophila*, the connection between such a biochemical pathway and courtship behaviour is still uncertain.

What is the physiological basis of male-male courtship behaviour? Visual, olfactory and gustatory functions have been tested in males with heat-shock induced *white* misexpression (Hing and Carlson, 1996). All appear normal. It seems that male-male courtship does not in this case depend on the reception of olfactory information, nor on the reception or generation of auditory cues, although sensory cues are very important in male-female courtship behaviour. Only under dim red light was courting activity reduced, to one-fourth of control values; this may suggest some visually dependent mechanisms of courtship.

Although 201Y homozygotes and pHSBJCaSpeR subjected to heat-shock perform some male-male courtship, there are several differences between them. First, the behaviour of line 201Y homozygotes is bisexual rather than homosexual. The data of Figure 3.13 show that the courting flies also find female targets attractive. However, heat-shocked pHSBJCaSpeR males predominantly court males. Secondly, according to Zhang and

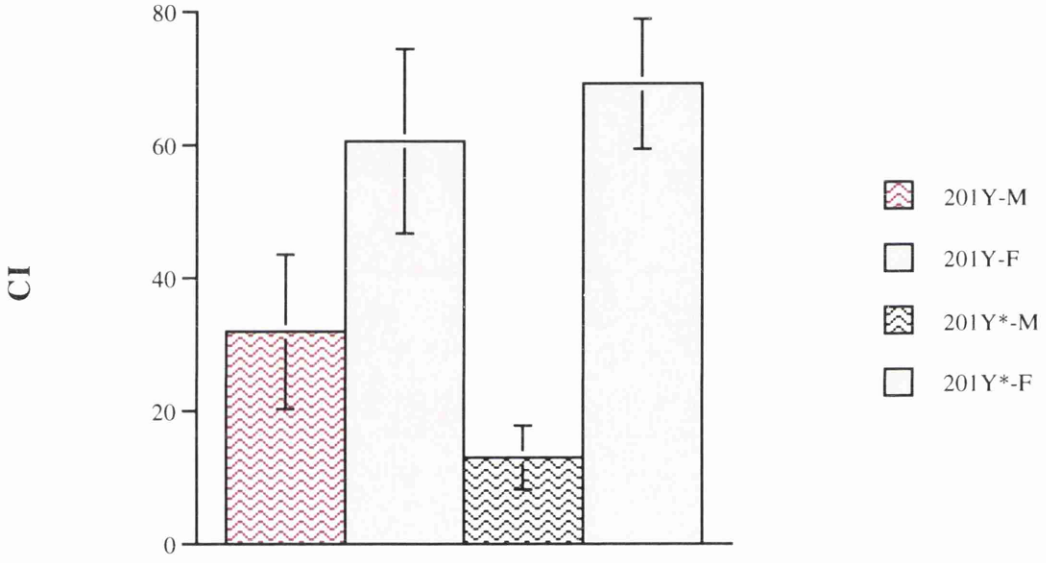


Figure 3.13 Courtship of homozygous 201Y and 201Y* males towards wild-type males and virgin females. -M: male targets; -F: virgin female targets. The height of each bar represents the mean score (with SEM) for 10 individuals of each test.

Odenwald (1995), the homosexual courtship of pHSBJCaSpeR were observed in the bottles containing hundreds (<600) of flies. While, our experiments were carried out by isolated pair tests, and the target flies were decapitated, thus precluding behaviour of the targets from influencing that of the courter. Another study (Hing and Carlson, 1996) confirmed that the male-male courtship in mini-*white* heat shock flies did not depend on sensory cues produced only by large populations; the phenomenon could be induced within a single pair of male flies.

As to the molecular basis of male-male courtship, the potential contributions of *dsx* and *fru* are still unclear. So far, there has been no report about the relation of the detailed splicing of the *fru* gene to different aspects of behaviour. Expression of the female *fru* transcript in chromosomal males may or may not be the reason for male-male courtship, although the male-specific behavioural change does resemble the non-discriminatory courtship of *fru* mutations (Ryner, et al., 1996). The discovery of new genes involved in sex-specific behaviour, such as *dsf* (Finley, et al., 1997), introduce added complexity. *dsf* is a *tra*-dependent and *dsx*-independent gene. *dsf* males show a bisexual phenotype. There are even more uncertainties about *dsx* than *fru* on its involvement in courtship (Villella and Hall, 1996).

The RT-PCR results demonstrate that the female versions of both *dsx* and *fru* transcripts are generated as the result of GAL4-mediated *tra* expression. Whatever the function of these "down-stream" effects, the ectopic expression of the "up-stream" gene *tra* has been confirmed to be responsible for male-male courtship.

Chapter 4

The identification of *Drosophila* brain structures associated with courtship behaviour

4.1 Introduction

As described in Chapter 1, most aspects of sexual behaviour of *Drosophila* appear to be under genetic control (Hall, 1994 and Greenspan, 1995). The somatic sex is determained in a cell-autonomous manner in *Drosophila* (Baker and Ridge, 1980), which indicates the sex of each cell is independent from its neighbour cells. The study of gynandromorphs showed that the performance of male courtship requires genotypically male cells in higher centres of the nervous system (Hall, 1977 and 1979).

In contrast to the standard gynandromorph technique, which produces unique patterns of sex mosaicism in each individual (Hotta and Benzer, 1972), GAL4 enhancer-trap strains produce stable mosaic lines. This allows the detection of probabilistic aspects of behaviour and the examination of the anatomical staining patterns in greater detail. A series of P[GAL4] enhancer trap lines with specific expression patterns have been used (O'Dell, et al., 1995; Ferveur, et al, 1995). The GAL4/UAS_G system drives the expression of the sex-determining gene *transformer*, upon which the female somatic sexual phenotype, including neuronally-based aspects of sexual behaviour (McRobert and Tompkins, 1985), appears absolutely dependent. At least, *doublesex, fruitless* and *dissatisfaction* are all regulated by *transformer* (Tian and Maniatis, 1993; Ryner, et al., 1996 and Finley, et al., 1997), even through *dsx* and *fru* are independent of each other. Therefore, using the P[GAL4]/UAS_G system to express the female-specific splice isoform of *transformer* (Boggs et al, 1987) should lead to feminisation of chromosomally male cells to give flies that are predominately male, but with GAL4 expressing cells feminised (O'Dell and Kaiser, 1997). These sex mosaic flies also present the advantage of having cells that are neither abnormal (mutant) nor missing (ablated) but that are either male or female.

Gynandromorph analysis implicates the mushroom bodies, or adjacent neuropil, in control of some early aspects of the male courtship repertoire (Hall, 1979). Previous

studies revealed that the mushroom body neurons, located in the posterior dorsal brain, are involved in olfactory processing and learning (de Belle and Heisenberg, 1994). In other insects, the mushroom bodies have been invoked as centres for courtship behaviour (Wahdepuhl, 1983). In *Drosophila*, the number of Kenyon cells, which are the intrinsic components of mushroom bodies, is sexually dimorphic (Technau, 1984).

Recently, a marked bisexual (*fru*-like) behaviour is observed as the consequences of GAL4/ UAS_G-*tra* mediated feminisation, particularly associated with specific subdomains of the antennal lobes (Ferveur, et al, 1995) and the mushroom bodies (O'Dell et al, 1995). These results suggest that smell perception and processing in the olfactory system may have an important role in sexual orientation.

The work described in this chapter is the screening for changes in sex discrimination courtship behaviour in a set of P[GAL4] lines expressing *transformer* in different neuronal subsets in the brain. The brain major structures that have been studied in this chapter are shown in Figure 4.1. The colours and patterns of different parts in the diagram are the same for the later results figures. The aim is to find the correlation between brain structure and function with respect to sexual orientation, and identify brain structure that mediate sexual behaviour.

4.2 Results

4.2.1 The expression patterns of P[GAL4] lines

A total of 24 P[GAL4] lines were tested in this experiment. GAL4 expression in the fly brain can be revealed using a UAS_G-*lacZ* reporter. Staining with X-gal and anti-β-gal antibodies show the locations of GAL4-directed β-gal activity (Fig. 4.2). Table 4.1 summarises the major patterns of these lines. In 14 lines, staining could be seen in the mushroom bodies (Yang, 1996; Yang, et al., 1995; Armstrong, 1995 and J.D.Armstrong,

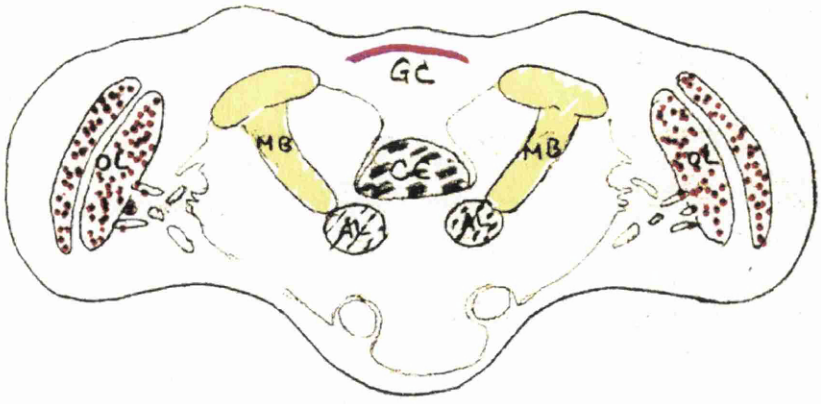


Figure 4.1 Schematic diagram of the *Drosophila* brain (from the front). AL: antennal lobe; CC: central complex; GC: great commissure; MB: mushroom body; OL: optic lobe. The colours and patterns of these structures are the same as shown in the later results figures.

Table 4.1 Summary of the expression patterns of the P[GAL4] lines used in this study

Line	Pattern	Line	Pattern
c253③	Mushroom body	59Y①	Antennal lobe and mushroom body
43Y②	Mushroom body	72Y①	Widespread in neuropil, raised in mushroom bodies
c747①	Widespread in neuropil, raised in mushroom bodies	c827⑦	Optic lobe
c97⑥	Mushroom body	c829③	Optic lobe
c184⑥	Mushroom body	c469③	Optic lobe
c532③	Mushroom body	c5③	Central complex
30Y①	Widespread in neuropil, raised in mushroom bodies	c522①	Central complex
117Y①	Mushroom body	7①	Central complex
121Y①	Mushroom body and Central complex	c819④	Central complex
c309②	Mushroom body	c287⑥	Antenna glomerular tract
c302⑤	Mainly mushroom body, some other neuropil	82Y③	Great commissure
238Y⑤	Mainly mushroom body, some other neuropil	21Y⑦	General staining

① Brainbox: http://brainbox.gla.ac.uk; ② Armstrong, 1995; ③ Yang, 1996; ④ Armstrong, et al., 1997; ⑤ Yang, et al., 1995;

⑥ J.D.Armstrong, personal comm.; ⑦ M.Yang, personal comm.

In the later figures of the Results section, different structures are represented by different colours and patterns. Mushroom body: yellow; central complex: grey; optic lobe: red; antennal lobe: brown stripe; great commissure: pink; general staining: blue; and wild-type: white.

Figure 4.2 Expression patterns of the P[GAL4] lines used in this study. The lines are (in order from upper left to lower right): c253; 43Y; c747; c97; c184; c532; 30Y; 117Y; 121Y; c309; c302; 238Y; 59Y; 72Y; c827; c829; c469; c5; c522; 7; c819; c287; 82Y and 21Y. The picture of line c287 is the confocal image of anti-β-gal activity. The others are all X-gal staining of 12mm frontal cryostat section through adult heads (See Table 4.1).

personal comm.). Three lines have a pattern of expression in the optic lobe (Yang, 1996; M.Yang, personal comm.). Four lines (c5, c522, 7 and c819) show staining of central complex structures (Armstrong, et al., 1997 and Yang, 1996). Staining of line c287 occurs in the antenna glomerula tract (J.D. Armstrong, personal comm.). Line 82y has strong staining in the great commissure (Yang, 1996.). There is also a general staining line (M.Yang, personal comm.). More expression patterns of P[GAL4] lines have been described in "Brainbox", an online atlas and database of the *Drosophila* nervous system. Brainbox can be accessed via the World Wide Web from servers in Glasgow (http://brainbox.gla.ac.uk).

Although the expression patterns of these lines are described as "mushroom body", "central complex", etc., these descriptions reflect the strongest areas of staining. However, in some lines, staining is also seen elsewhere in the brain at a lower level. For example, from a series of cryostat sections through the adult heads of line c469, faint staining can also be seen in the antennal lobe besides the major expression of the optic lobe.

4.2.2 Courtship behaviour

Drosophila melanogaster males are normally heterosexual. They direct intense courtship towards virgin females, but very little courtship-related activity towards mature males (Jallon, 1984). The screening of courtship behaviour was performed as described in Chapter 2. Courting activity was measured by the CI (courtship index: the percentage of time spent courting (Siegel and Hall, 1979)). Wing vibration was scored as another index, the SAP (sex-appeal parameter: the percentage of time spent wing-vibrating (Jallon and Hotta, 1979)). All the tests have been performed with Canton-S wild type flies as control. For each line, individual courting males were introduced to mature male and virgin female targets respectively. Comparison between the performance of P[GAL4] lines and Canton-S control towards male and female targets can show if there

is any discrimination in mating. Every courtship combination was repeated ten times for the calculation of mean and standard error.

As shown in in Chapter 3, no matter what the dosage of mini-*white*, UAS_G-*tra*/UAS_G-*tra*, UAS_G-*tra**/UAS_G-*tra* and UAS_G-*tra**/UAS_G-*tra** all perform very little courtship towards males, just like wild-type flies. The results indicate that the UAS_G-*tra* construct does not affect courtship behaviour in the absence of GAL4-driven expression.

The GAL4 mediated expression of TRA^F in 12 lines (c253, 121Y, 30Y, 117Y, 238Y, 21Y, c469, c829, c5, 82Y, c827 and 7) can cause feminisation of the abdomen (Fig. 4.3), thus proving *tra* is functional. The observations of the courtship behaviour performed by 24 transformed P[GAL4] lines are summarized in Table 4.2. The courtship towards females is in all cases more or less normal (Fig. 4.4), even that of males with transformed morphology, which can not bend their abdomen to attempt copulation. Comparing the courtship behaviour towards female targets of transformed P[GAL4] males to Canton-S controls, the data show significant high CI score of c747 and 59y ($p<0.05$), and significant high SAP score of c184, 59y and c5 ($p<0.05$). Because inbreeding problems always go hand in hand with the control flies, that may cause Canton-S control flies to be less active than the out-bred flies (P[GAL4]/UAS_G-*tra*). The transformed 21y had a physical lesion, the abdomen kept sticking on the medium and this prevented them from walking properly, and they displayed very little courtship, and hardly any wing vibration. For this tested group toward female targets, the SAP and CI were highly correlated for the entire set of 24 lines (the correlation of SAP and CI = 0.82).

For 12 lines, the paired two-tailed t-test revealed no significant differences between wild type Canton-S controls for the courtship toward male targets under GAL4-mediated *tra* expression (Fig. 4.5 and Table 4.2), which means the transformed neurons in these lines do not affect this aspect of behaviour. By contrast, the rest of the tested P[GAL4] lines

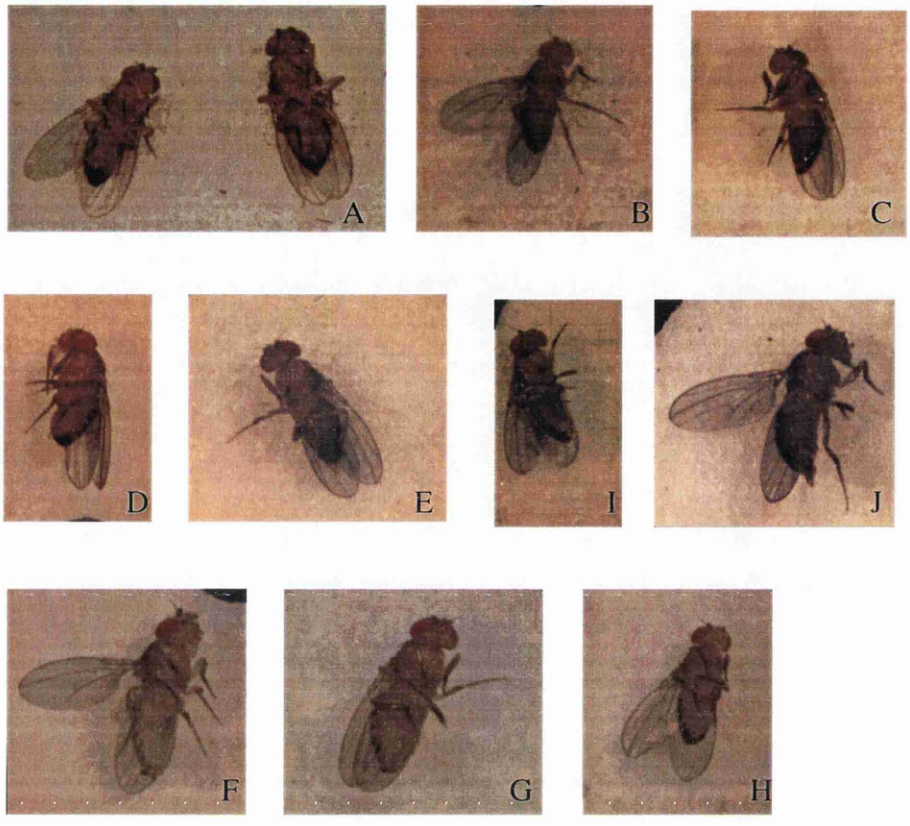


Figure 4.3 Some of the morphologically transformed P[GAL4] male flies. A: Canton-S wild type fly, left: male; right: female. B; 121Y ; C: c5 ; D: c253 ; E: c287 ; F: c309 ; G: 30Y ; H: 238Y ; I: 82Y ; J: 117Y .

Table 4.2 Courtship behaviour of transformed P[GAL4] lines and control.

	P[GAL4] /UAS$_G$-*tra* toward male targets				P[GAL4] /UAS$_G$-*tra* toward female targets			
Line	CI (%) Mean (Std)	P-value of CI	SAP (%) Mean (Std)	P-value of SAP	CI (%) Mean (Std)	P-value of CI	SAP (%) Mean (Std)	P-value of SAP
c253	33.17 (28.65)	0.05*	7.46 (7.64)	0.04*	75.04 (33.34)	0.20	17.25 (17.49)	0.41
43y	34.15 (38.67)	0.11	7.30 (8.66)	0.051	34.44 (40.69)	0.32	3.56 (4.46)	0.10
c747	38.11 (41.61)	0.17	9.67 (11.02)	0.10	85.22 (20.45)	0.042*	21.28 (12.18)	0.13
c97	44.78 (34.30)	0.017*	16.56 (15.07)	0.049*	53.50 (33.80)	0.99	13.89 (13.13)	0.64
c184	47.33 (32.78)	0.024*	18.57 (14.80)	0.019*	77.00 (17.59)	0.084	35.91 (18.35)	0.009*
c532	38.19 (16.57)	0.001***	8.19 (5.19)	0.002**	49.41 (39.79)	0.83	11.67 (10.95)	0.85
30y	32.39 (32.25)	0.16	7.39 (9.18)	0.13	58.78 (33.42)	0.75	18.95 (18.25)	0.33
117y	43.59 (39.56)	0.039*	12.70 (15.18)	0.045*	47.96 (29.86)	0.73	11.96 (13.02)	0.83
121y	50.22 (17.96)	<0.001***	10.39 (5.50)	0.007**	28.05 (10.06)	0.064	0.72 (1.16)	0.056
c309	59.00 (35.66)	0.019*	14.50 (11.14)	0.029*	51.28 (34.26)	0.91	10.72 (10.52)	0.98
c302	52.33 (37.42)	0.024*	14.86 (11.25)	0.016*	73.05 (18.54)	0.15	17.43 (14.11)	0.34
238y	23.78 (31.51)	0.36	5.22 (10.67)	0.34	26.84 (34.18)	0.16	4.45 (8.10)	0.28
59y	29.33 (41.35)	0.28	9.19 (12.81)	0.13	90.67 (15.71)	0.01**	31.76 (11.79)	0.005**
72y	19.53 (27.27)	0.37	5.00 (8.26)	0.13	55.87 (32.72)	0.87	10.13 (10.82)	0.93
c827	37.67 (30.85)	0.028*	5.52 (8.60)	0.13	68.91 (21.43)	0.27	16.72 (9.04)	0.28
c829	5.73 (6.34)	0.40	1.27 (2.25)	0.47	37.70 (40.00)	0.39	14.43 (24 00)	0.70
c819	2.44 (4.13)	0.15	0.52 (1.14)	0.77	50.37 (35.26)	0.86	17.74 (16.44)	0.34
c5	29.27 (24.20)	0.056	13.53 (13.70)	0.016*	73.50 (31.12)	0.19	30.66 (20.35)	0.032*
c522	28.80 (31.42)	0.12	6.03 (9.35)	0.10	51.50 (46.75)	0.93	13.70 (20.55)	0.72
7	11.93 (21.02)	0.84	2.70 (3.62)	0.84	40.89 (45.22)	0.52	11.44 (16.33)	0.91
21y	5.33 (13.06)	0.51	0.67 (1.63)	0.99	5.19 (15.56)	0.001***	1.19 (3.56)	0.067
c287	13.80 (22.52)	0.69	5.77 (9.89)	0.14	34.69 (31.15)	0.23	8.97 (11.31)	0.77
82y	10.10 (16.04)	0.98	1.53 (2.00)	0.26	40.70 (28.68)	0.39	11.27 (15.03)	0.92
c469	66.41 (34.53)	<0.001***	20.59 (11.87)	0.001***	78.11 (20.03)	0.058	13.26 (5.81)	0.55
CS	10.26 (14.22)	—	0.67 (1.00)	—	53.24 (28.32)	—	10.62 (11.09)	—

P value is obtained by two-tailed t-test comparing the score of CI or SAP of each line to that of Canton-S wild type control correspondingly.
* significant ($p<0.05$); ** very significant ($p<0.01$); *** highly significant ($p<0.001$); $p\geq0.05$, not significant.

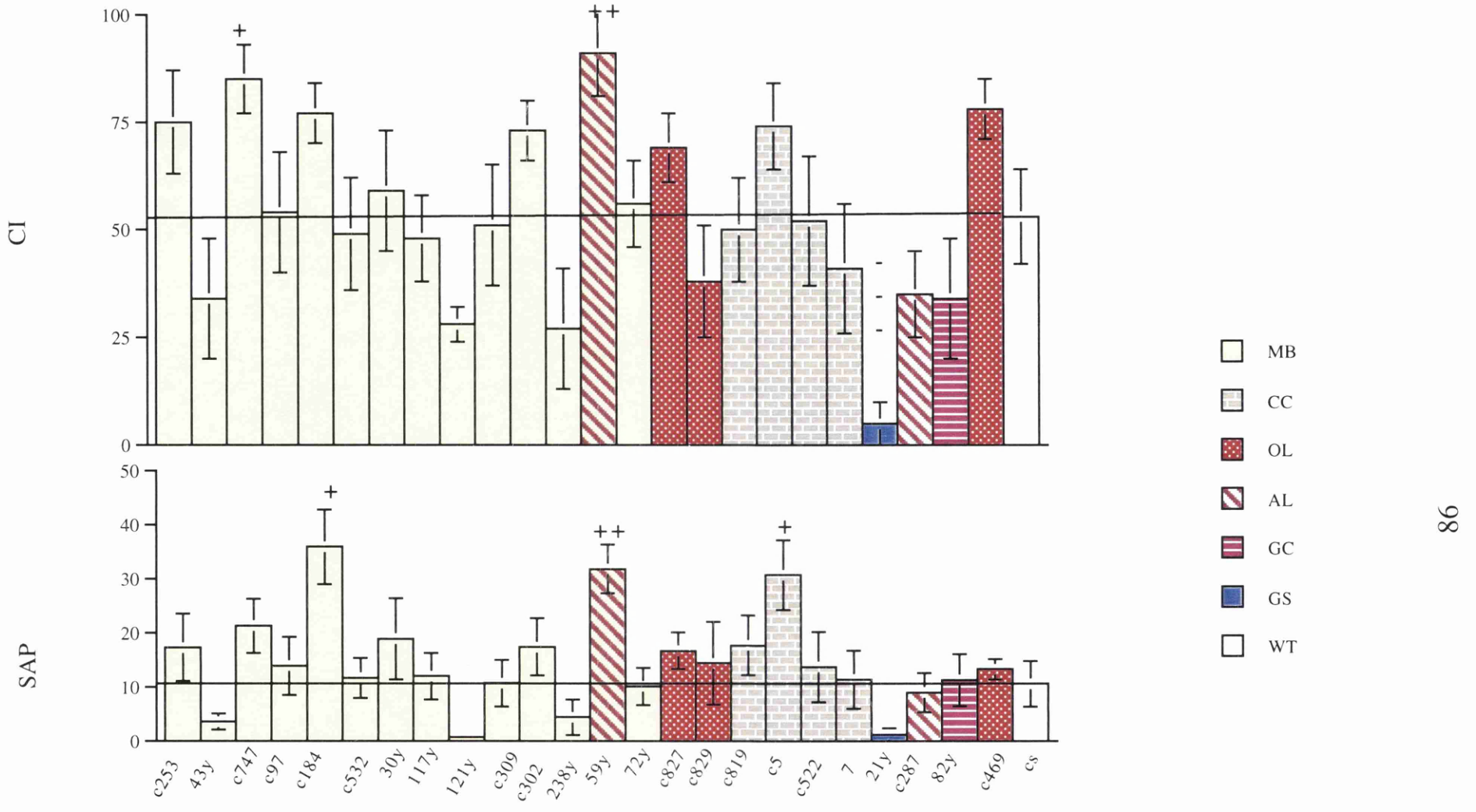


Figure 4.4 Courtship behaviour of transformed P[GAL4] males toward female targets. CI (courtship index) means the percentage of time spent courting. SAP (sex appeal parameter) means the percentage time spent wing-vibrating. Flies were observed for 5 minutes, the height of each bar represents the mean score (with SEM) for 10 individuals of each P[GAL4] line. MB: mushroom body; CC: central complex; OL: optic lobe; AL: antennal lobe; GC: great commissure; GS: general staining; WT: Canton-S wild-type.

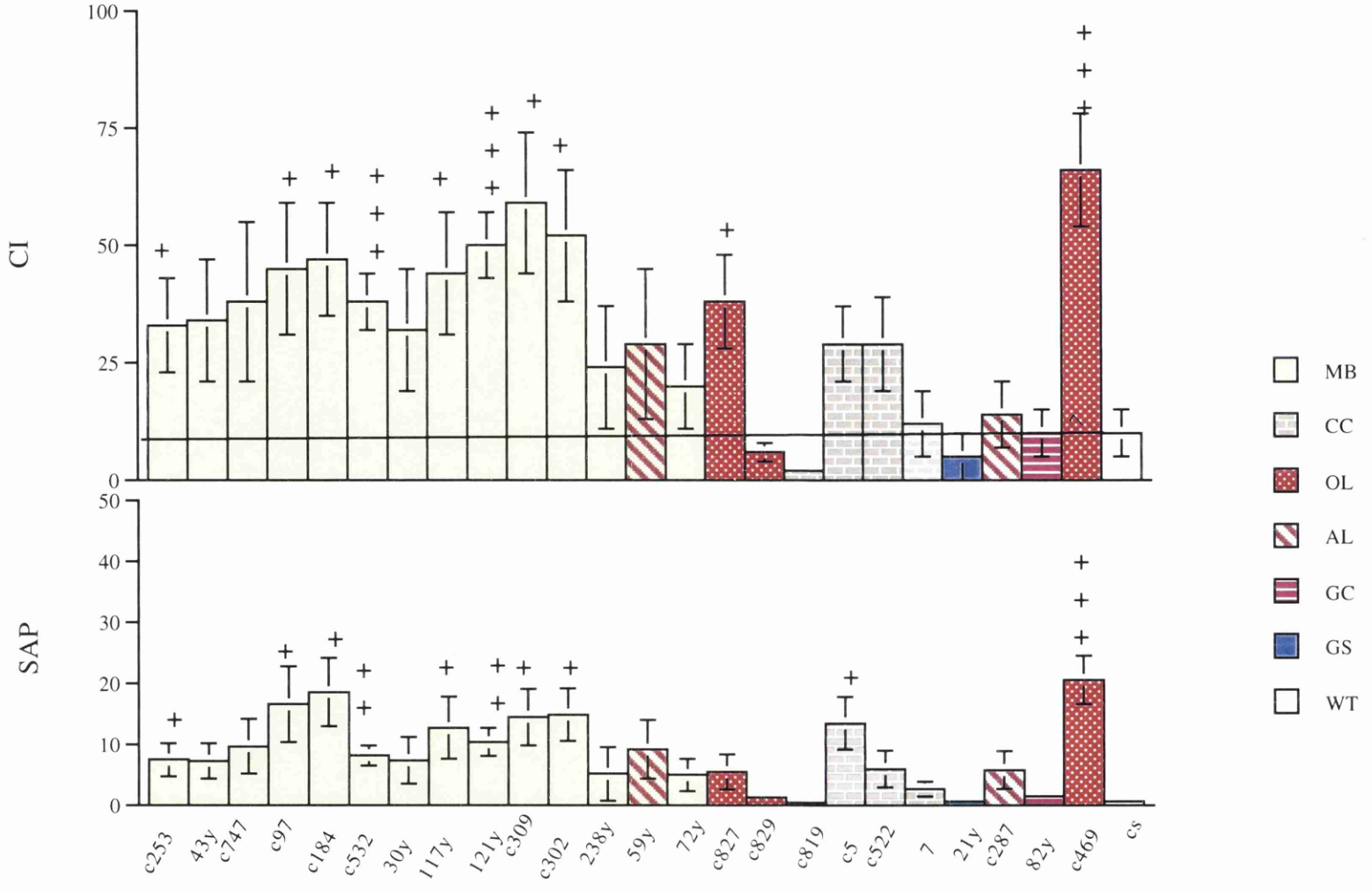


Figure 4.5 Courtship behaviour of transformed P[GAL4] males toward male targets. CI (courtship index) means the percentage of time spent courting. SAP (sex appeal parameter) means the percentage time spent wing-vibrating. Flies were observed for 5 minutes, the height of each bar represents the mean score (with SEM) for 10 individuals of each P[GAL4] line. MB: mushroom body; CC: central complex; OL: optic lobe; AL: antennal lobe; GC: great commissure; GS: general staining; WT: Canton-S wild-type.

displayed male-male courtship induced by TRA^F (for lines c253, c97, c184, 117Y, c309, c302 and c827, $0.01<p<0.05$; for lines c532, 121Y and c469, $p<0.001$). Line 121Y was extremely abnormal, it spent 50% of the testing time courting mature males, but only 28% of the time courting virgin females. It showed a significant preference for male targets ($p=0.03$), and it is the only line exhibiting such a preference.

As a control for the P[GAL4] insertion effect itself, I screened 17 P[GAL4] homozygous flies. Because these flies lack of TRA^F, they should serve as the control for this experiment. The CI and SAP toward female targets (Fig.4.6 and Table 4.3) were all within the range of Canton-S control flies, except the CI scores of lines c97 and c184 which were as high as 90%, and the SAP scores of lines c97 and 121Y were higher than that of Canton-S. On the other hand, the data from male targets were a little bit confusing (Fig 4.7 and Table 4.3). Lines c253, 43Y, c309, c302, c819, c827, 21Y and c469 all displayed more courtship than Canton-S males ($p<0.05$). The overall picture of CI and SAP scores (Fig 4.6 and 4.7) were similar. They were correlated with each other (the correlation of male targets = 0.83; female targets = 0.67), which means these two index for measuring courtship are in good agreement.

The high proportion of male-male courtship shown by some homozygous P[GAL4] males is unlikely to be ascribable to the insertion site of the P-element, it seems more likely to be some effect of the P[GAL4] construct itself. The study in Chapter 3 shows that the mini-*white* gene expression in the P[GAL4] construct alone can cause non-discriminating courtship in line 201Y when it is homozygous.

The mini-*white* effect on male-male courtship is not seen when there is one copy of mini-*white* in the genome. So, P[GAL4]/Canton-S heterozygotes males were used as another group of control. The CI towards female targets were all on the same scale (Fig. 4.8 and Table 4.4). The males of 82Y/CS showed higher courtship intensity (p value for SAP = 0.04). As to the male targets, their CI and SAP scores were all within the range of

Table 4.3 Courtship behaviour of P[GAL4] homozygous and control males.

	P[GAL4] homozygous males toward male targets				P[GAL4] homozygous males toward female targets			
Line	CI (%) Mean (Std)	P-value of CI	SAP (%) Mean (Std)	P-value of SAP	CI (%) Mean (Std)	P-value of CI	SAP (%) Mean (Std)	P-value of SAP
c253	40.07 (38.97)	0.041*	12.92 (16.40)	0.081	78.30 (20.76)	0.27	21.26 (9.11)	0.085
43Y	31.48 (26.42)	0.034*	8.30 (8.01)	0.056	65.18 (34.53)	0.99	10.41 (12.89)	0.89
c747	8.89 (14.38)	0.86	2.96 (5.45)	0.64	68.82 (31.24)	0.80	13.59 (9.54)	0.69
c97	19.50 (18.00)	0.18	3.72 (4.32)	0.44	90.28 (9.55)	0.047*	35.66 (13.62)	0.006**
c184	14.00 (11.30)	0.37	3.17 (3.11)	0.54	95.11 (3.77)	0.022*	22.11 (10.74)	0.13
c532	8.56 (7.95)	0.90	1.78 (2.19)	0.99	45.28 (40.79)	0.31	9.50 (12.72)	0.80
30Y	12.91 (17.23)	0.52	4.14 (7.21)	0.44	66.19 (44.85)	0.95	29.90 (23.16)	0.094
117Y	11.63 (8.95)	0.49	0.96 (1.07)	0.60	62.44 (21.33)	0.83	4.78 (6.38)	0.19
121Y	7.95 (9.27)	0.97	1.86 (2.64)	0.98	82.19 (18.12)	0.18	45.57 (18.55)	0.002**
c309	41.62 (21.30)	0.002**	5.29 (4.52)	0.15	75.48 (23.61)	0.45	23.14 (13.90)	0.12
c302	22.17 (8.80)	0.043*	3.06 (2.23)	0.55	70.33 (22.92)	0.71	23.00 (9.09)	0.083
238Y	10.71 (19.97)	0.72	0.58 (1.05)	0.47	50.17 (42.94)	0.42	13.25 (14.15)	0.79
59Y	34.33 (28.42)	0.052	9.24 (9.25)	0.086	76.67 (16.84)	0.34	25.67 (16.32)	0.089
c819	47.59 (27.31)	0.002**	11.74 (8.40)	0.007**	70.52 (20.23)	0.64	10.41 (8.59)	0.86
c827	43.85 (28.04)	0.005**	6.33 (4.80)	0.058	55.76 (25.69)	0.52	5.43 (4.88)	0.28
21Y	35.76 (25.39)	0.013*	12.48 (9.00)	0.020*	54.15 (29.86)	0.46	12.59 (14.79)	0.86
c469	27.71 (19.86)	0.033*	9.52 (8.58)	0.061	55.00 (36.32)	0.57	14.19 (12.84)	0.68
CS	7.70 (13.93)	—	1.81 (4.60)	—	65.00 (26.05)	—	11.33 (12.39)	—

P value is obtained by two-tailed t-test comparing the score of CI or SAP of each line to that of Canton-S wild type control correspondingly.

* significant ($p<0.05$); ** very significant ($p<0.01$); $p\geq0.05$, not significant.

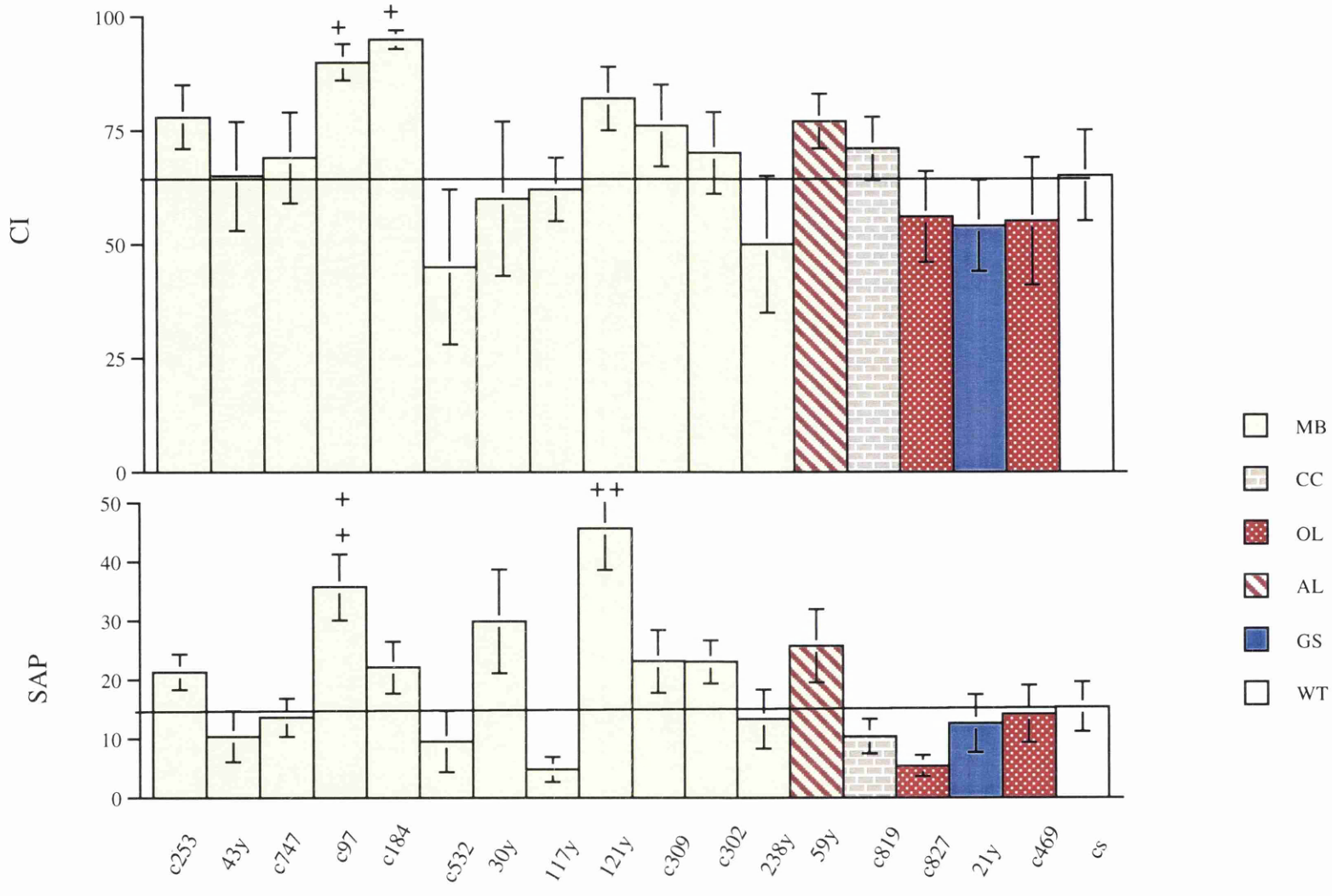


Figure 4.6 Courtship P[GAL4] homozygous males toward female targets. CI (courtship index) means the percentage of time spent courting. SAP (sex appeal parameter) means the percentage time spent wing-vibrating. Flies were observed for 5 minutes, the height of each bar represents the mean score (with SEM) for 10 individuals of each P[GAL4] line. MB: mushroom body; CC: central complex; OL: optic lobe; AL: antennal lobe; GC: great commissure; GS: general staining; WT: Canton-S wild-type.

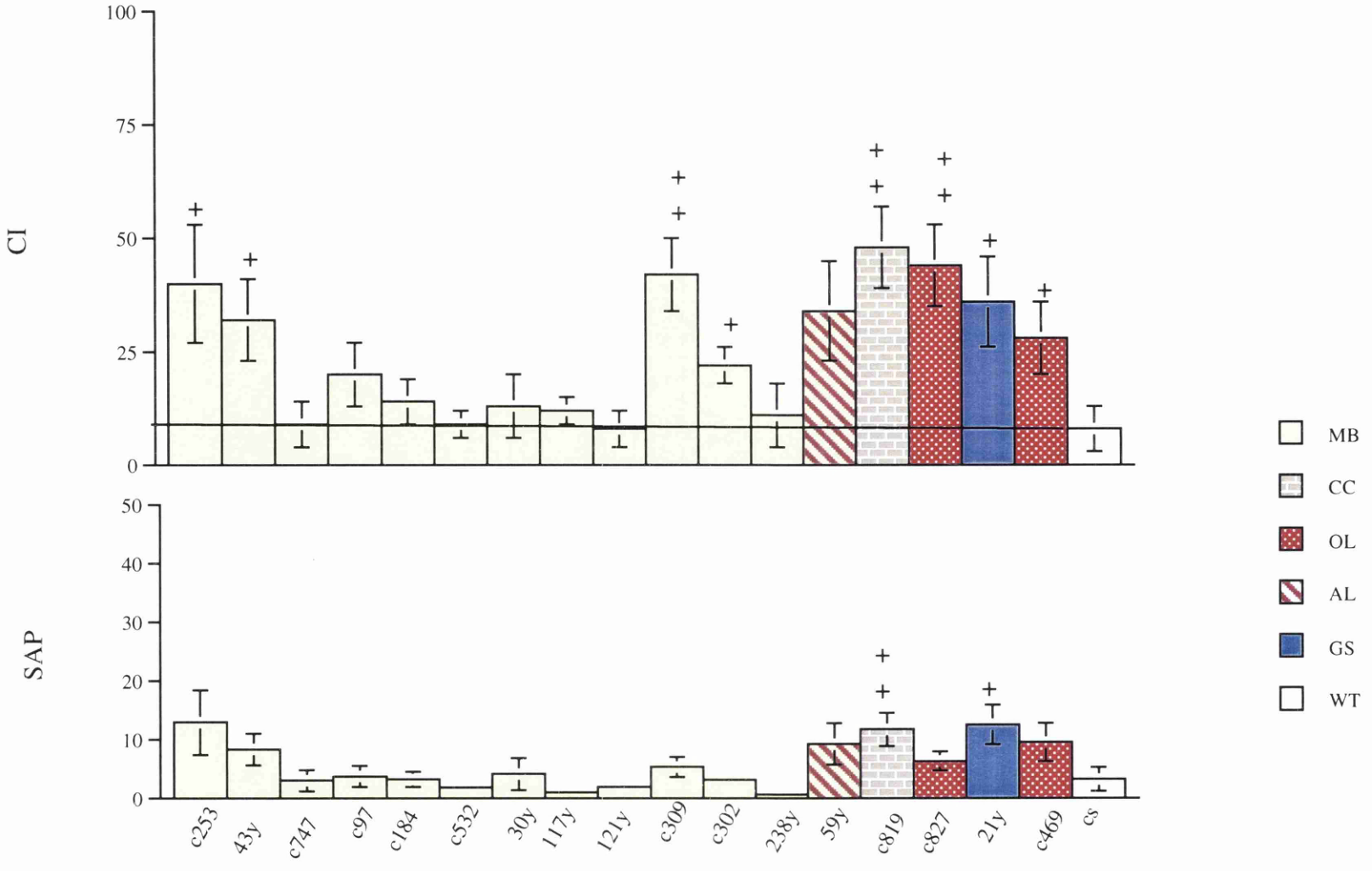


Figure 4.7 Courtship behaviour of P[GAL4] homozygous males toward male targets. CI (courtship index) means the percentage of time spent courting. SAP (sex appeal parameter) means the percentage time spent wing-vibrating. Flies were observed for 5 minutes, the height of each bar represents the mean score (with SEM) for 10 individuals of each P[GAL4] line. MB: mushroom body; CC: central complex; OL: optic lobe; AL: antennal lobe; GS: general staining; WT: Canton-S wild-type.

Canton-S controls (Fig. 4.9 and Table 4.4), which means that they are as heterosexual as wild type flies. These results show that in P[GAL4] heterozygous lines no bisexual behaviour has been observed without expression of *tra* in specific brain neurons.

4.3 Discussion

The courtship data from the GAL4-mediated *transformer* expression lines are quite complicated. This is not entirely unexpected as the connection between the patterns and behaviour may reveal the complexities of the relationship between brain structure and function. The *in situ* hybridization with an antisense probe presented in the results of O'Dell et al. (1995) confirmed that *tra* expression in P[GAL4] lines reflected that of *lacZ*. So, we are confident that the feminisation patterns are the same as has been observed by X-gal staining. We should remember that in some P[GAL4] lines, there is also other staining in the brain apart from the major patterns, and these stained neurons may be involved in sexual orientation.

The technique of P[GAL4] enhancer trapping enables the identification and feminisation of different subdomains and neurons of the mushroom body. Previous studies suggest the mushroom body (O'Dell, et al, 1995) and the antennal lobe (Ferveur, et al., 1995) have a role in mate discrimination by involvement with olfactory processing. Among the 14 mushroom body expression lines tested in this experiment, 8 lines (c253, c97, c184, c532, 117Y, 121Y c309 and c302) showed more male-male courtship than Canton-S controls ($p<0.05$). Two optic lobe lines (c827 and c469) also displayed significant courtship toward males. However, some faint staining is also seen in the antennal lobe of line c469. Lines with predominant expression patterns of the central complex (4 lines), the great commissure (1 line), general staining (1 line) and antenna glomerular tract (1 line), showed relatively lower male-male courtship, they are not significantly different from Canton-S control. Of these lines, only transformed line c5 performed increased

Table 4.4 Courtship behaviour of P[GAL4]/Canton-S and control.

	P[GAL4]/Canton-S males toward male targets				P[GAL4]/Canton-S males toward female targets			
Line	CI (%) Mean (Std)	P-value of CI	SAP (%) Mean (Std)	P-value of SAP	CI (%) Mean (Std)	P-value of CI	SAP (%) Mean (Std)	P-value of SAP
82Y	17.33 (19.77)	0.242	7.57 (9.56)	0.12	70.10 (30.77)	0.726	31.10 (21.30)	0.04*
c829	16.13 (17.63)	0.27	7.23 (10.60)	0.18	61.27 (28.11)	0.785	17.40 (13.73)	0.37
c5	9.67 (10.98)	0.74	1.93 (2.80)	0.95	57.37 (40.52)	0.64	25.83 (24.88)	0.18
c522	7.90 (13.93)	0.98	2.97 (6.59)	0.67	66.77 (30.50)	0.90	23.23 (16.85)	0.13
7	17.78 (17.67)	0.20	5.04 (5,45)	0.19	66.73 (23.15)	0.89	19.67 (14.20)	0.23
c287	15.93 (24.74)	0.39	6.30 (12.87)	0.34	63.70 (27.85)	0.92	22.63 (19.84)	0.20
72Y	18.17 (26.69)	0.31	6.77 (9.56)	0.17	64.23 (33.87)	0.96	24.67 (22.22)	0.17
238Y	14.60 (15.47)	0.32	6.00 (7.39)	0.16	60.89 (38.11)	0.81	27.00 (23.33)	0.13
CS	7.70 (13.94)	—	1.81 (4.60)	—	65.00 (26.05)	—	11.33 (12.39)	—

P value is obtained by two-tailed t-test comparing the score of CI or SAP of each line to that of Canton-S wild type control correspondingly.

* significant ($p<0.05$); $p\geq0.05$, not significant.

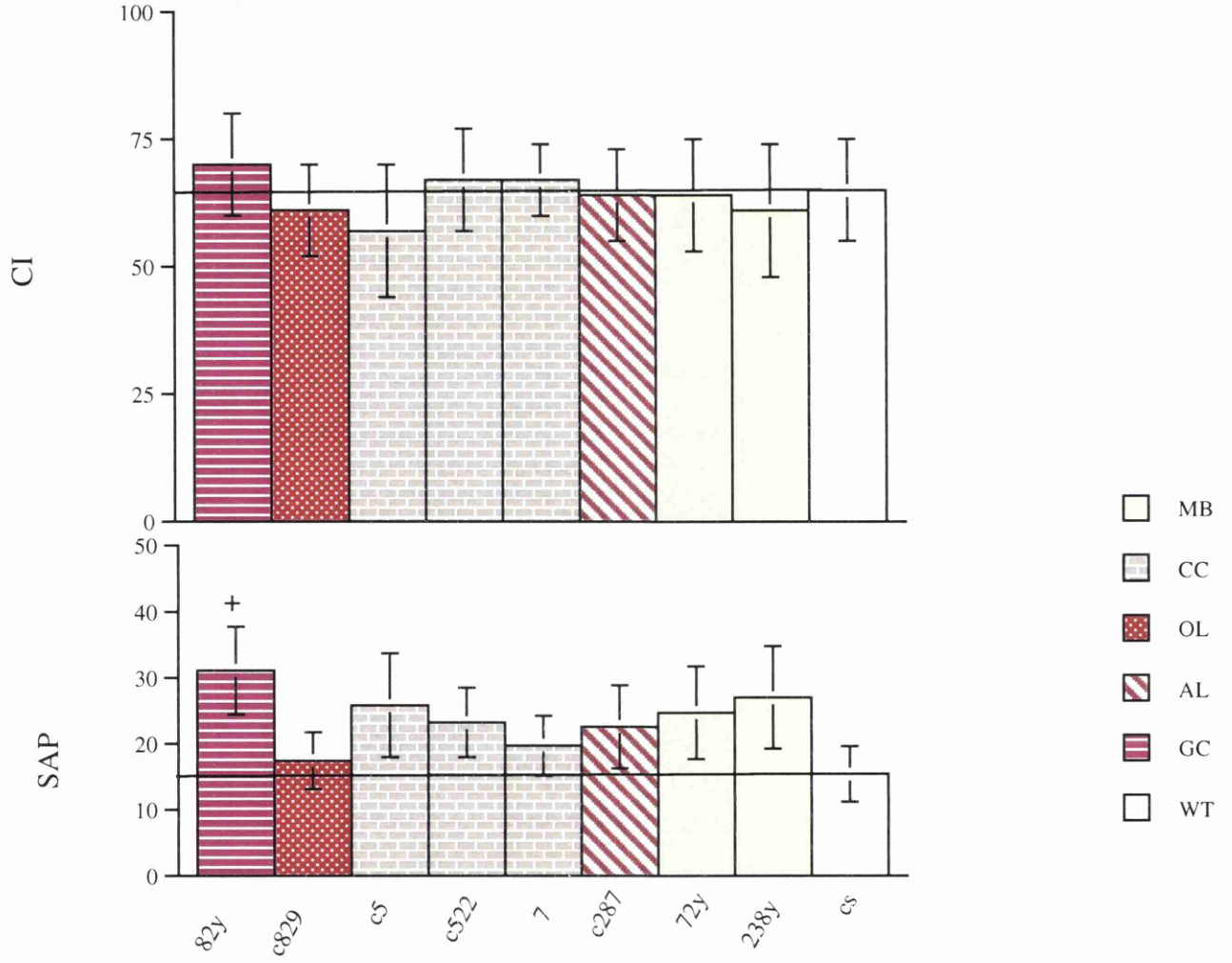


Figure 4.8 Courtship behaviour of P[GAL4]/Canton-S males toward female targets. CI (courtship index) means the percentage of time spent courting. SAP (sex appeal parameter) means the percentage time spent wing-vibrating. Flies were observed for 5 minutes, the height of each bar represents the mean score (with SEM) for 10 individuals of each P[GAL4]/Canton-S line. MB: mushroom body; CC: central complex; OL: optic lobe; AL: antennal lobe; GC: great commisure; WT: Canton-S wild-type.

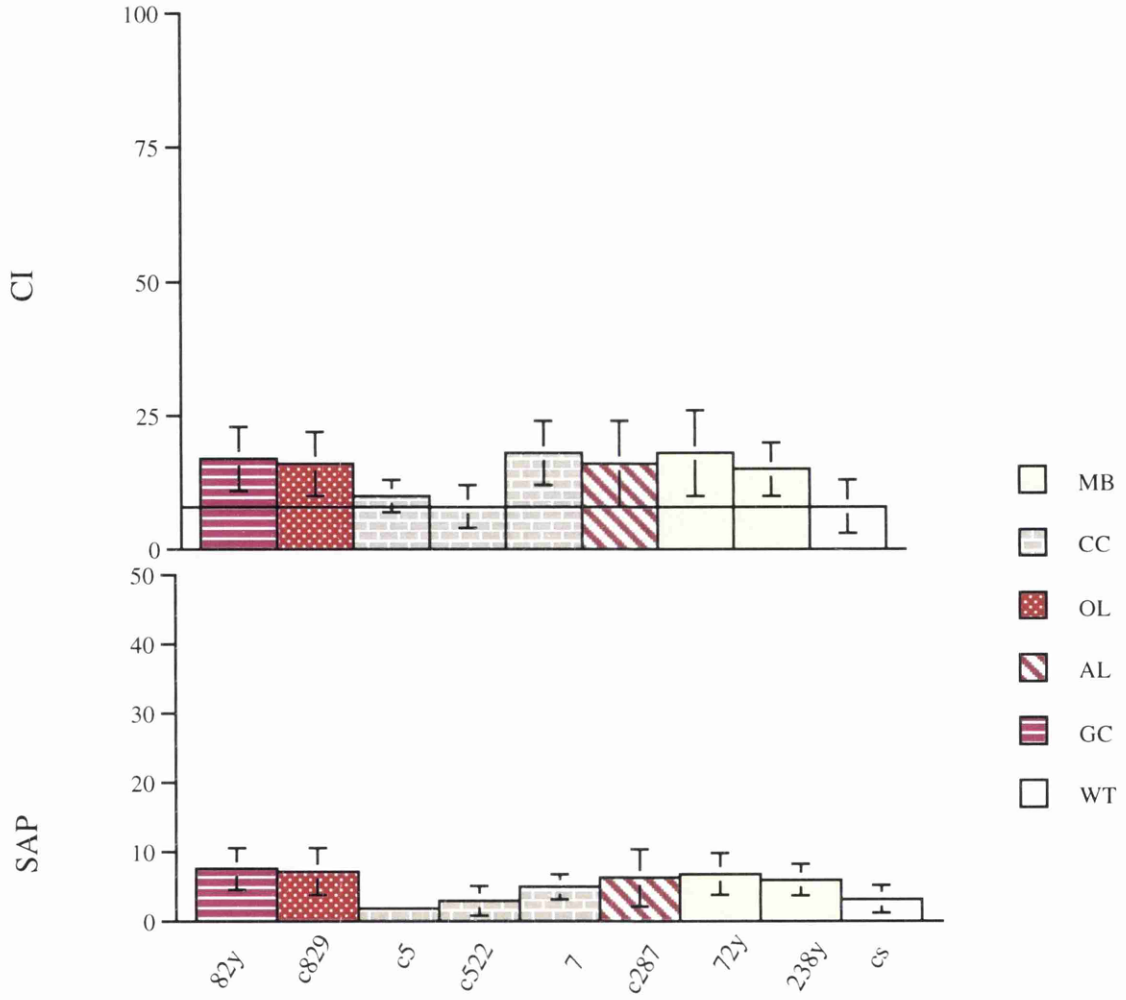


Figure 4.9 Courtship behaviour of P[GAL4]/Canton-S males toward males. CI (courtship index) means the percentage of time spent courting. SAP (sex appeal parameter) means the percentage time spent wing-vibrating. Flies were observed for 5 minutes, the height of each bar represents the mean score (with SEM) for 10 individuals of each P[GAL4]/Canton-S line. MB: mushroom body; CC: central complex; OL: optic lobe; AL: antennal lobe; GC: great commissure; WT: Canton-S wild-type.

intensity of wing vibration to both males and females. In conclusion, the feminisation of specific subdomains of mushroom bodies leads to non-discrimination behaviour. These results confirm the previous studies (Ferveur, et al., 1995; O'Dell, et al., 1995) and are consistent with the importance of olfactory processing in male sexual orientation.

If it is true that feminisation of specific subdomains of the mushroom body leads to non-discriminatory courtship, then, it is likely due to the sex change of the olfactory pathway. Mating choice in flies probably depends on detecting volatile chemical pheromones (Ferveur, et al., 1989). The mushroom body may have a function in responding to pheromones. Male chemicals have a small effect on attractiveness to females (Venard, 1980), whereas the predominant male-specific molecules tends to inhibit male excitation. (Jallon, 1984). The feminisation of the olfactory system by ectopic *transformer* expression may cause a female type recognition of the attraction of the male aphrodisiac chemical(s), or destroy male flies' ability to detect the inhibitory compounds. Either of the above possibilities could be connected with non-discriminatory courtship.

The behavioural differences between these mushroom body expression lines also implicate that the different subdomains may perform different functional roles in the context of mate discrimination. Because the differences between the staining patterns in differently behaving fly lines seem to concern all the lobes of mushroom body, it is premature to draw conclusions as to specific neuronal elements. This part of the analysis is still carrying on in the lab.

Along with the mushroom body, the antennal lobe is another part of the olfactory processing region, which is connected with the mushroom body by the antenna glomerular tract (Stocker, 1994). It is the major brain neuropil that receives olfactory input in *Drosophila*. The feminisation of the antennal lobe has also been reported to lead to non-discriminatory mating behaviour (Ferveur, et al., 1995). One of the lines (c469)

showing transformed bisexual behaviour has faint staining in the antennal lobe. Nevertheless, two lines (59Y and c287) with antennal lobe expression tested in this experiment showed no significant male-male courtship. As in line c469, the major staining pattern is in the optic lobe, so the possibility that the optic lobe neurons may also associate with sexual orientation could not be ruled out.

Vision research in insects has emphasised the existence of parallel visual pathways (Fischbach and Heisenberg, 1984). The compound eyes of insects may contain functionally specialised regions and that the structural separation of functional subsystems begins at the receptor level (Fischbach and Dittrich, 1989). In *Calliphora* and *Musca* examples for a nearly complete regional separation of visual functions include the sexual dimorphism in chasing behaviour (Land and Collett, 1974). A dorso-frontal region of the eye of male *Musca domestica* has specialised receptors (Franceschini, et al, 1981). At the isotopic position of the lobula, male-specific interneurons have been found (Hausen and Strausfeld, 1980). These give reasons to presume the functional role of the optic lobe in the sexual orientation of courtship behaviour. On the other hand, the gynandromorph study (Hall, 1979) did not find the *Drosophila* optic lobe was a governing part of courtship, while the genetically male mushroom body was essential for male behaviour.

The transformed mushroom body expression line 121Y is the only line which appeared to display more courtship toward male targets than female targets. The reason is unclear. This line also shows some staining in the central complex. There may be other mechanisms interacting with the affected neurons in this specific line.

The homozygous UAS_G-*tra* fly does not show abnormal courtship (Chapter 3), external morphology change or sterility in the absence of a P[GAL4] element, which means the expression of *tra* needs to be driven by GAL4. In the presence of UAS_G-*tra*, some P[GAL4] lines (Fig. 4.3) showed incomplete feminisation of their morphology, which is

clear evidence that the *transformer* construct is functional. It is known that TRA^F is responsible for female splicing of *dsx* and *fru* (Tian and Maniatis, 1993; Ryner, et al., 1996). The study of *doublesex* and *fruitless* expression in GAL4/UAS_G lines carried out in Chapter 3 shows that the female isoform of *transformer* existed in 201Y/ UAS_G-*tra* males supporting the notion that UAS_G-*tra* is functional at a molecular level.

The observed developmental abnormalities (Fig. 4.2) in some transformed flies made the males infertile. Some classes of these males have been shown to be attractive to wild type males (O'Dell, et al, 1995). This is most likely because the female abdomen is a focus for female cuticular pheromone synthesis (Jallon, 1984), which is known to be under the control of *transformer* (McRobert and Tompkins, 1985). According to the observation, there is no evident connection between the transformed morphology and non-discriminatory behaviour, although some abdominally feminised males tended to have lower SAP score. This point of view is also confirmed by Ferveur, et al., (1997). In their experiment, *tra* is expressed in adult oenocytes via GAL4 direction. The female pheromones can be found in these resulting males which elicited homosexual courtship from other males, but these abdominally feminised males still exhibited male heterosexual orientation. The male-male courtship induced by *tra* expression in some subregions of the brain, such as mushroom bodies, could be explained as the interference of either the processing or the response of chemical cues. The neurons in these subdomains are involved in this function.

Reanalysis of the data using arcsin transformations and a two-way ANOVAs and tests of variance on the groups of data may show more significance differences among the strains, targets and controls.

Chapter 5

The molecular analysis of P[GAL4] line c819

5.1 Introduction

As estimated on the basis of RNA-DNA hybridization experiments (Levy and Manning, 1981), several thousand genes appear to be more or less specifically expressed in the *Drosophila* brain. The cloning and functional characterisation of such genes is a major challenge. Enhancer-trap approaches provide a promising means of identifying genes with neural-specific expression patterns (Mlodzik and Hiromi, 1992).

The central complex is a phylogenetically conserved structure in the insect brain. It has been implicated in a variety of behaviours, in particular, visual associated memory (Heisenberg, 1989) and general motor activity (Bouhouche, et al., 1993). As described in Chapter 1, the *Drosophila* central complex comprises several interconnected subunits, the ellipsoid body being one of them. The characteristic structure of the ellipsoid body is shown in Figure 5.1. It is built up from several isomophic neuronal subtypes (R1-R4). R-type neurons have their cell-bodies lateral to the central complex, from which arise neurites that project to the mid-line via the lateral triangle. Of the 4 subclasses of R-type neurons, the axonal processes of R1-3 arborise from the centre of the ring outward whilst the axons of R4 arborise from the periphery of the ring inward. The *Drosophila* ellipsoid body is thought to exert inhibitory control of motor behaviour (Hanesch, et al., 1989 and Bausenwein, et al., 1994).

Yang et al. (1997) recently identified the first *Drosophila* alkaline phosphatase gene (*dALP1*) via two P[GAL4] lines (c507 and c232) which have identical expression patterns in the ellipsoid body of the brain, and in the Malpighian (renal) tubules in the abdomen. Reassuringly, both tissue *in situ* hybridization with *dALP1* probes and direct histochemical determination of alkaline phosphatase activity precisely matched the enhancer-trap patterns.

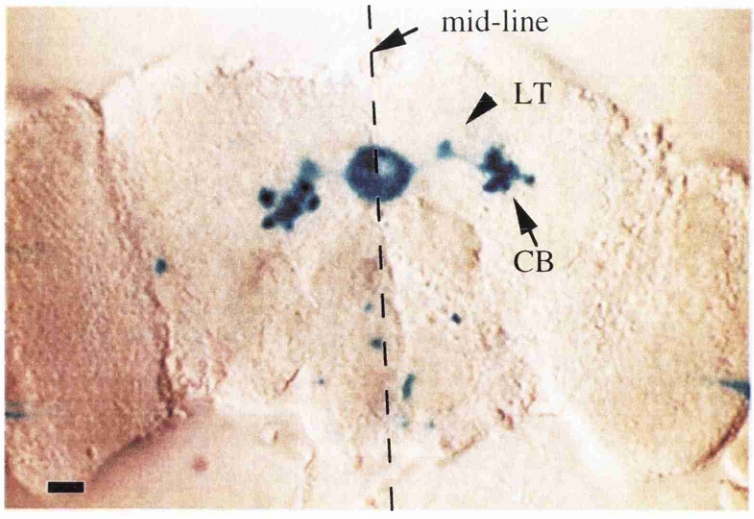


Figure 5.1 Whole mount X-gal staining of the adult c819 brain (from the front), showing GAL4 dependent expression ofβ-gal in the ellipsoid body- a component of the central complex (Picture taken from Yang, 1996). LT: lateral triangle; CB: cell body cluster. Bar: 10μm.

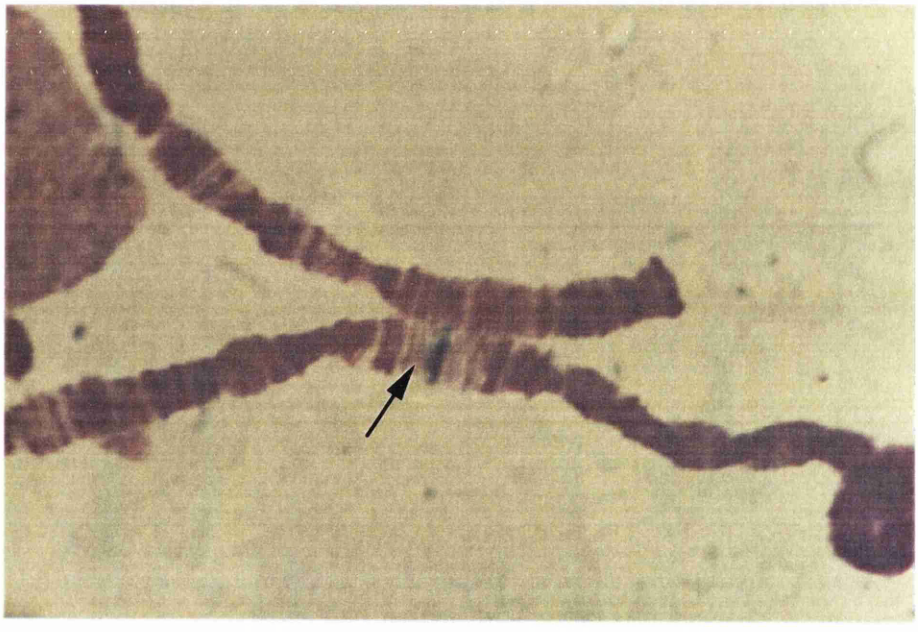


Figure 5.2 Chromosomal localisation of the P-element insertion of line c819. The *in situ* hybridization of a biotin-labelled pBluescript probe to spread polytene chromosomes shows signals at 93C-93D, as indicated with the arrow. (Courtesy of Susan Wang).

Among a number of P[GAL4] lines with central complex expression patterns (Yang, 1996), c819 shows intense and quite specific staining of the outer ring layer of the ellipsoid body (Fig. 5.1). The pattern matches the positions of R2 and/or R4 neurons (Hanesch et al., 1989). The P-element insertion of line c819 was mapped to polytene chromosome location 93C (Fig. 5.2) by *in situ* hybridization to salivary chromosomes of third instar larvae.

5.2 Developmental study of line c819 by anti-β-gal immunohistochemistry

Line c819 was crossed to UASG-*lacZ* according to standard procedures (Chapter 2). Third instar larvae, four-hour-interval pupae and adults were collected. The intact brains were then dissected and stained by anti-β-gal antibody as described in Chapter 2 (2.4.3). The time series of staining patterns is shown in Figure 5.3. In the larval brain, several unidentified structures, presumably unrelated to the central complex, were stained. The staining pattern does not change much during the early stages of pupal development. After this period, staining of the ellipsoid cell bodies became visible around 30 hours after puparium formation (APF). Then a few faint fibres became visible, followed by the gradual appearance of a ring pattern around 40 hours APF. The lateral triangles became visible at 44 hours APF. The number of cell bodies increased to about 24-28 in each hemisphere at 48 hours APF. After that, the pattern of the ellipsoid body was almost complete, although the subsequent increase of staining intensity may suggest growth and enlargement of the structure, or a merely the accumulation of β-gal.

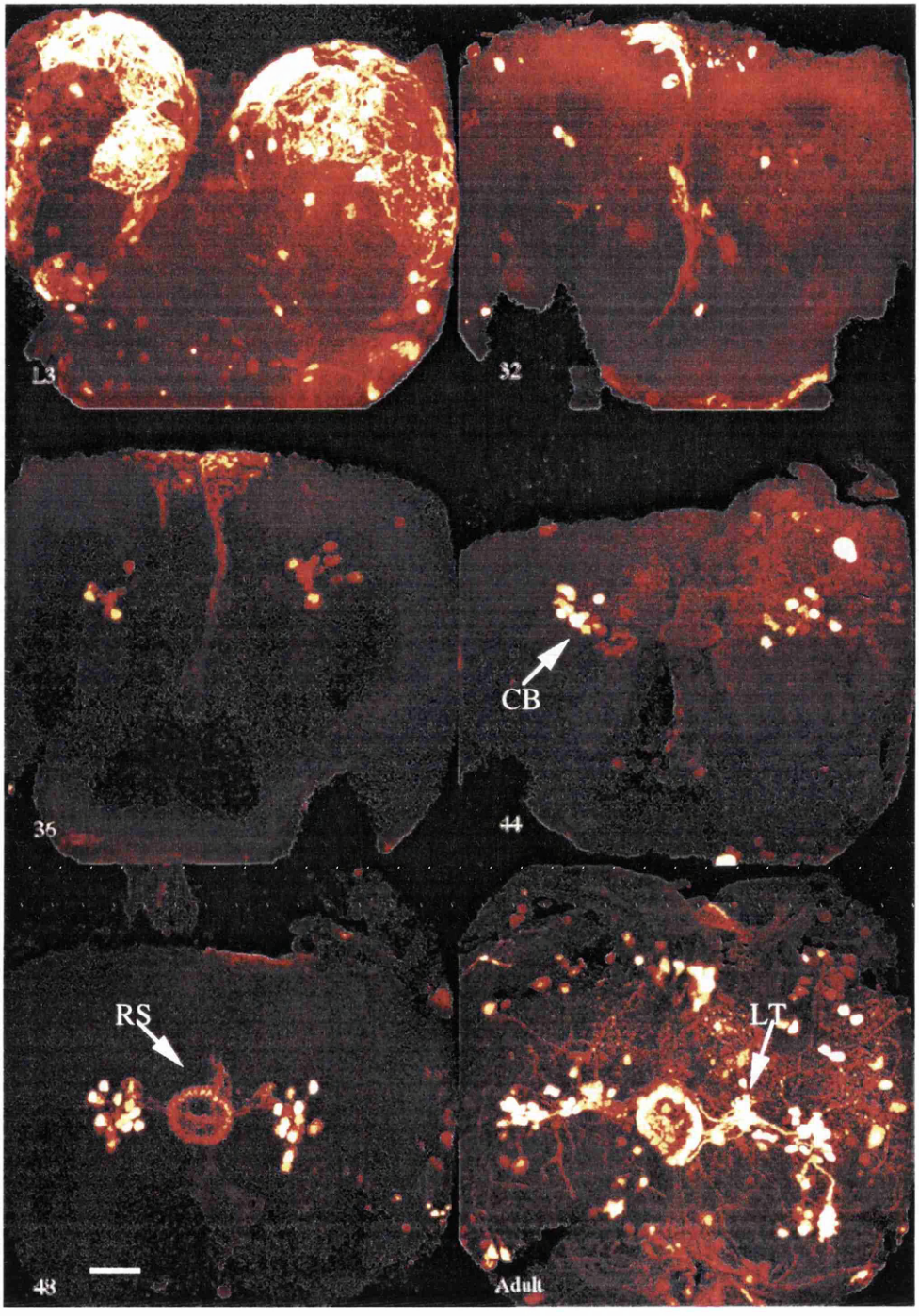


Figure 5.3 Three dimensional confocal reconstructions of GAL4-directed β-gal expression in the developing c819 brain (from the front) revealed by anti-β-gal antibody and florescein-labelled secondary-antibody (Chapter 2). L3: third instar larva brain; 32, 36, 44 and 48 indicate number of hours after puparium formation (APF). CB: cell bodies; RS: ring structure; LT: lateral triangle; Bar: 10 μm.

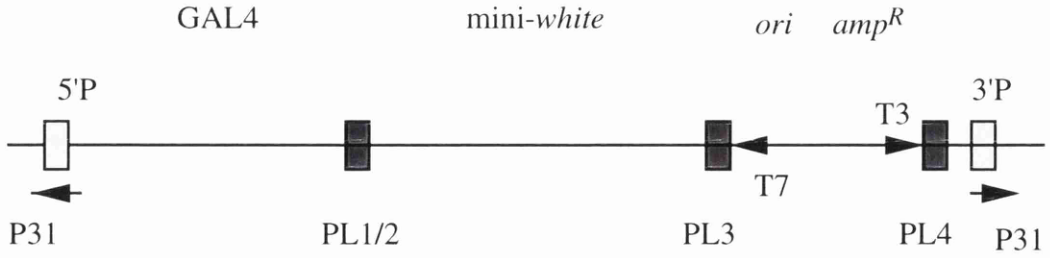
5.3 Mapping of the c819 insertion region

5.3.1 Plasmid rescue of flanking genomic DNA

Plasmid rescue was carried out according to the principles and procedures described in Chapters 1 and 2. Figure 5.4 shows the structure of the P[GAL4] element. The plasmid sequences (*ori* and amp^R) contained in the construct enable the rescue of genomic DNA flanking the insertion as part of a plasmid replicon. The unique restriction enzyme *Sst*I, present in polylinker 3 but not in polylinker 4, was chosen to clone DNA downstream of the c819 insertion. pBluescript sequences and adjacent genomic DNA extending to the next *Sst*I site were separated from the rest of the genome by digestion, and formed a plasmid by self-ligation. The resulting plasmid p819 contained a 9kb fragment of genomic DNA.

In polylinker 4, there are two unique restriction enzyme sites, *Kpn*I and *Sfi*I. Due to its long recognition sequence, *Sfi*I sites are very rare in the genome, so *Kpn*I was chosen for rescue of upstream genomic DNA. Unfortunately, the attempt at cloning DNA upstream of line c819 by this means was never successful. P[GAL4] is itself 12 kb in length (Fig. 5.4), and it is relatively difficult to capture large plasmids due to their low transformation efficiency.

Both p819 and genomic DNA of line c819 were digested with *Sst*I, and Southern blotting was performed by hybridization to a pBluescript probe. As shown in Figure 5.5, the sizes of the bands are identical, which means that the rescued plasmid indeed contained the region downstream of the c819 insertion. Moreover, the genomic Southern detected only one band, indicating that there is just one P-element insertion in line c819.



Restriction enzyme sites of the polylinkers: PL1/2: *Hind*III, *EcoR*V, *EcoR*I, *Pst*I, *Sma*I, *BamH*I, *Spe*I and *Xba*I. PL3: ***Pst*I**, ***Sal*I**, ***Xho*I**, ***BstX*I**, *Sst*II, ***Sst*I**. PL4: ***Kpn*I**, *Sma*I, *Sst*II, ***Sfi*I**, *Spe*I, *BamH*I. Restriction sites in bold are unique and could thus be used for plasmid rescue. *ori*: plasmid origin of replication. *amp*^{R}: ampicillin resistance determinant.

Figure 5.4 Detailed map of the P[GAL4] construct, based on the PlwB (Wilson, et al., 1989) and pGawB (Brand and Perrimon, 1993) vectors. 3'P and 5'P are P-element sequences at the ends of the construct. P31 represents the 31bp inverted repeat sequence of the P-element. Multiple cloning sites PL3 and PL4 are flanked by T7 and T3 RNA promoters.

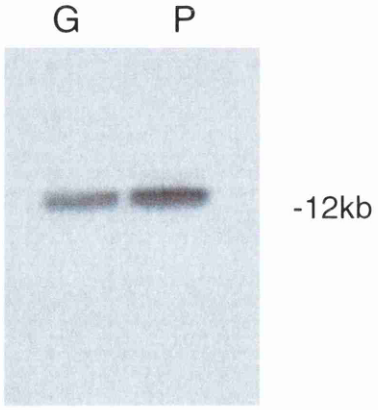


Figure 5.5 Genomic Southern analysis of the rescued plasmid from line c819. Genomic DNA from line c819 (G) and p819 (P) were digested with Sst I and run on a gel side by side. The blot was probed with pBluescript. The identical size of the two bands indicates that the correct fragment has been cloned.

5.3.2 Cloning of DNA upstream of the insertion site

In order to obtain DNA fragments representing the region upstream of the c819 insertion, the insert of p819 was used as a probe to screen a *Drosophila* genomic DNA library. The library had been constructed by inserting size-selected *Sau*3A fragments (14-22kb) between the *BamH*I sites of the bacteriophage λ vector EMBL3. Digestion with *Sal* I clearly separates the phage arms from inserts.

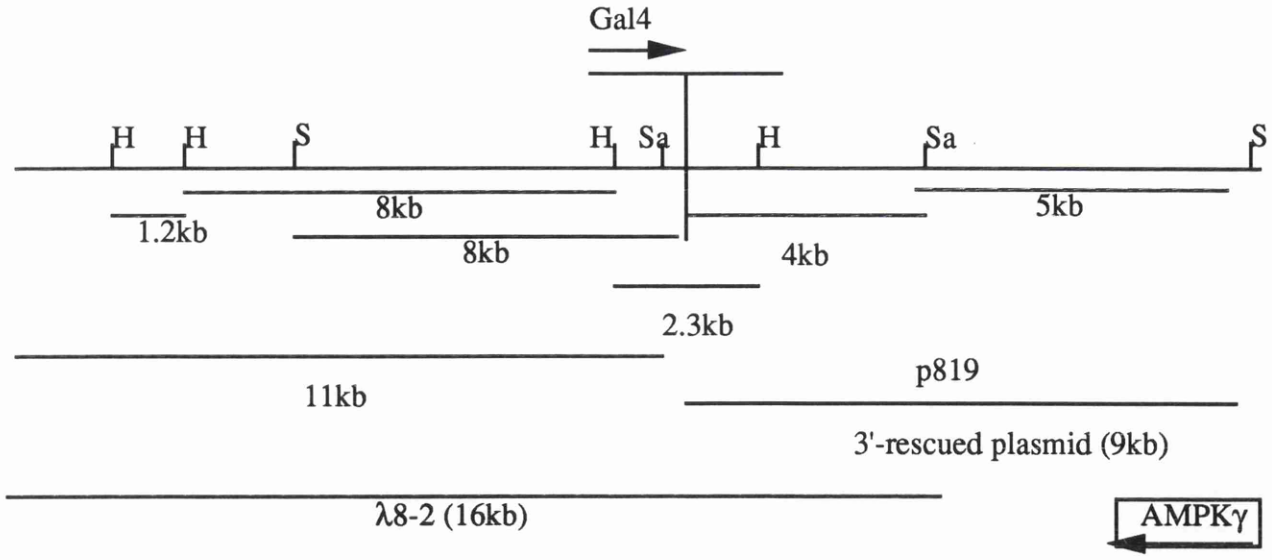
Three positive clones were isolated. DNA prepared from the phage clones was first cleaved with *Sal* I, *Hind* III and with both enzymes. Insert sizes were as follows: λ8–1 = 14kb, λ8–2 = 16kb, λ8–3 = 14.5kb. Southern blotting confirmed that these clones and the rescued DNA region overlapped each other. λ8–2 was found to cover the most upstream DNA (Fig. 5.6A).

For the convenience of later operations, subcloning into pBluescript was performed from the original λ phage DNAs (Fig. 5.6A). *Sal* I and *Hind* III restriction enzyme sites were chosen and 4 fragments were subcloned (1.2kb, 2.3kb, 8kb *Hind* III fragments and 11kb *Sal* I fragment).

5.3.3 "Reverse Northern" analysis

The term "Reverse Northern" (Fryxell and Meyerowitz, 1987) describes a technique by which many genomic DNA fragments can be simultaneously screened for hybridization to a probe corresponding to a complex mixture of RNA species, in this case, a first strand cDNA probe copied from total adult head (Canton-S) mRNA was used.

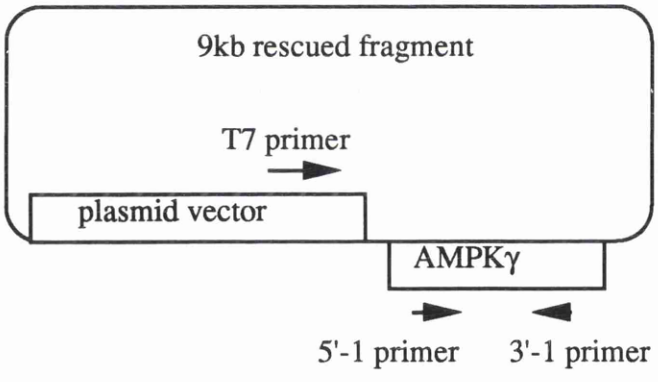
It had been previously observed that pBluescript hybridizes to head cDNA probes under Reverse Northern conditions (Milligan, 1995; Yang, 1996), although the reason is unclear. To avoid interference from pBluescipt, subcloned and rescued DNA fragments



A. Restriction map of genomic DNA surrounding of the c819 insertion.

AMPKγ indicates the position and orientation of the AMPKγ-subunit gene in the genome.

H: *Hind*III; Sa: *Sal*I; S: *Sst*I.



B. Structure of p819 and orientation of the AMPKγ gene. The direction of the AMPKγ transcription was identified by PCR with the rescued plasmid as template. T7 indicates the promoter for T7 RNA polymerase flanking polylinker 3 of the P[GAL4] construct (See Fig. 5.4). 3'-1 and 5'-1 are sequencing primers designed for cDNA clone pc8-1 (See Fig. 5.8). Amplification occurs with T7 and 3'-1 primers, but not with T7 and 5'-1 primers.

Figure 5.6 Genomic organisation of DNA surrounding the c819 insertion site, and orientation of the AMPKγ gene.

were separated from the vector by enzyme digestion and agarose electrophoresis (Fig. 5.7). For rescued plasmid p819, digestion with *Sst*I, *Sst*II and *Sal*I released fragments of 2.9 kb (pBluescript), 4kb and 5kb. For the fragments subcloned from λ8-2, *Sal*I and *Hind*III were employed to excise the inserts (1.2kb, 8kb, 2.3kb and 11kb). These DNA bands were transferred to a Nylon filter and prehybridized before adding probes.

Heads were collected *en masse*, and mRNA isolated, as described in Chapter 2. A cDNA probe was made by oligo dT primed reverse transcription in the presence of α-^{32}P dCTP. Hybridization conditions were as described in Section 2.3.7. After washing and autoradiography, the only fragment detected was the 5kb fragment derived from p819. The absence of a hybridization signal for other fragments implied either that the transcript level was too low to be detected, or that they contained no transcribed sequences.

5.4 cDNA cloning and analysis

5.4.1 cDNA library screening

A *Drosophila* male head cDNA library was screened with p819. The library was constructed starting with Canton-S male head mRNA and cloned into the λ vector NM1149 (Russell, 1989). Inserts are flanked by an *EcoR*I site at their 5' end and a *Hind*III site at their 3' end.

By using conventional screening techniques with nitrocellulose as the support membrane, 300,000 phage clones have been screened. The screen was performed in duplicate and single plaques were isolated after secondary screening. Just one cDNA was isolated. Double digestion (*EcoR*I + *Hind*III) of the DNA extracted from the positive clone excised a 2.2kb insert, so there were either no internal *EcoR*I or *Hind*III site, or they must be very close to the ends.

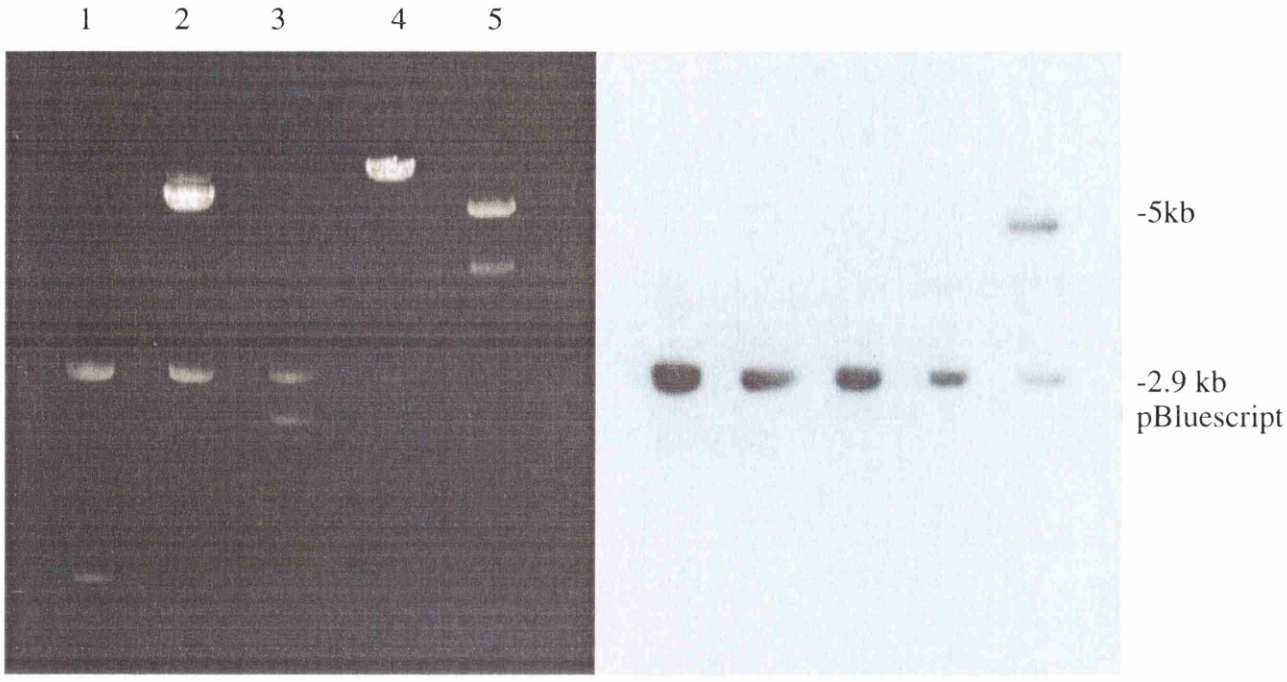


Figure 5.7 "Reverse Northern" analysis of DNA flanking the P-element insertion of line c819. Subcloned DNA fragments were separated from the plasmid vector by restriction digestion and gel electrophoresis. The blot was hybridized with a cDNA probe copied from total adult head mRNA. Lane 1: 1.2kb *Hind*III fragment; lane 2: 8kb *Hind*III fragment; lane 3: 2.3kb *Hind*III fragment; lane 4: 11kb *Sal*I fragment; lane 5: rescued plamid digested with *Sst*I, *Sst*II and *Sal*I.

In order to determine the corresponding position of the transcribed region within the genome, the genomic DNA clones were restricted and probed with the cDNA insert. The transcribed region is located towards the 3' end of the rescued fragment, as illustrated in Figure 5.6 A.

For sequencing and other analysis, the cDNA fragment was subcloned into pBluescript using *EcoR*I and *Hind*III. This recombinant plasmid is called pc8-1.

5.4.2 Sequence analysis

(a) Sequencing strategy

The multiple cloning site of pBluescript SK contains a series of unique restriction enzyme sites. If there is a site for such an enzyme in the cDNA, then digestion with the enzyme and re-ligation can be used to create a deletion, and sequencing from T3 or T7 primers can be used to obtain internal sequences. Alternatively, sequences internal to the cDNA can be captured by subcloning (for example, *BamH*I and *Xba*I, Fig. 5.8) into pBluescript.

A series of partially deleted recombinant plasmids and subclones was generated. Sequencing was carried out from both ends of each clone with T3 and T7 primers. Then, oligonucleotide primers were designed to cover the remaining regions for both strands. This sequencing strategy is summarised in Figure 5.8.

(b) General sequence features

The full sequence of the pc8-1 cDNA, together with the predicted translation product, is shown in Figure 5.9. The cDNA is 2,226 bp long. At the 3' end, there is the polyadenylation signal AATAAA (Proudfoot and Whitelaw, 1988), which aligns well with the polyA tail.

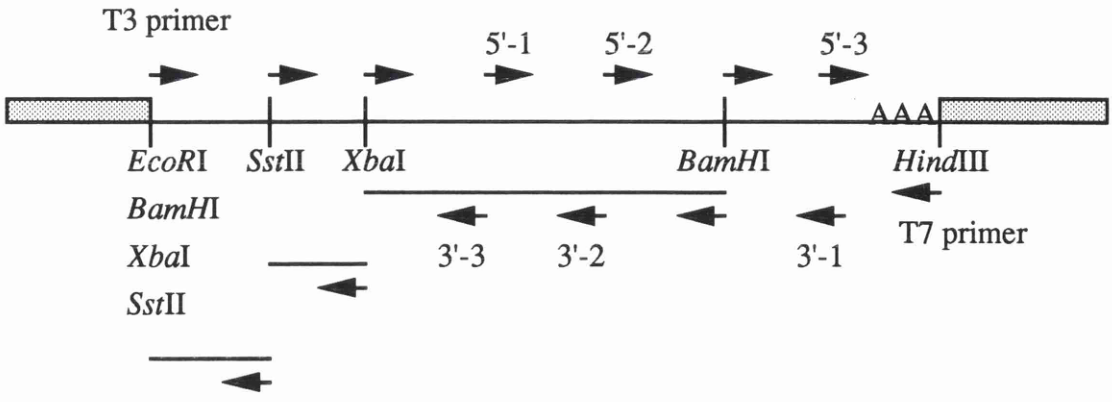


Figure 5.8 Sequencing strategy for pc8-1. Partial deletion subclones were generated by cleavage with *BamH*I, *Sst*II and *Xba*I. Subclones were also generated by double digestion (*BamH*I/*Xba*I, *Xba*I/*Sst*II and *EcoR*I/*Sst*II). Oligonucleotide primers 3'-1, 3'-2, 3'-3 and 5'-1, 5'-2, 5'-3 were used to complete the sequence on both strands. AAA: poly A tail.

The identification of open reading frames was performed by the MacVector™ programme. One long open reading frame of 350 amino acids was identified spanning the region from 553bp to 1602bp. The area around the putative translation start site was found to fit the consensus sequence proposed by Cavener and Ray (1991).

By using the programme BLASTN, which compares a nucleotide query sequence against a nucleotide sequence database, the cDNA sequence was found to have a significant homology (68%) with the open reading frame of the AMP-activated protein kinase (AMPK) γ subunit gene of mammals (Gao, et al., 1996). No *Drosophila* homologue has so far been described. The predicted protein sequence was analysed with the FASTA program (Pearson and Lipman, 1988). At the amino acid level, it shows 53.8% identity to the mammalian AMPK γ subunit. A multiple alignment of the *Drosophila* polypeptide and various mammalian AMPK γ subunits is shown in Figure 5.10.

Different mammalian AMPK γ subunit polypeptides show 75-80% identity with each other. A more distantly related polypeptide is the yeast *S. cerevisiae* Snf4p (Celenza, et al. 1989), which is required for expression of many glucose-repressible genes. Evolutionary relationships between these AMPK γ subunits were determined using the programme PAUP 3.1.1 (Swofford, 1993). The phylogenetic tree is shown in Figure 5.11.

The programme TMpred (Hofmann and Stoffed, 1993 and http://ulrec3.unil.ch/software/TMPRED-form.html), available through Netscape, was used for analysing various sequence features related to protein sorting signals. No signal sequence has been recognised.

```
   ATCTGCTGGA TCCCTCCTCC GCCGACAGCA GCGCCACCAG CAGCACCTAC TCCGTTTCCG GCGGCACAGG CCCGGCCACG 80
   AGCTCGGCAG CAGGTGGAGC AGGAGGAGCG GCGGCAGCAG CAGGACAGGG CGCAGCGGGA GGAGCCGGTG GCCTTATGAA 160
   CTCCATGAAG GTGGCATGCA GAACTTTAGC ATCGCCAGCA TCCCGCCGTG ACGATAACGA GCGCCGACGG CACGCAATCA 240
   ACGGAAAGAG CAAGTACAAA GACGGGAGTG CCATCCGCAT CAAGGAGGGA CGCGCAGTAT TACCACACGG TGACGGCGGT 320
   GCGTCCAAAC TCTTCCCAAC GGTCGCGATG ACCAGGTCAT GGTCTGTGCG GCATCGATCC AGCTCGGTTG TCAGCGAAGC 400
   CGACAAACGC AAAGCGCGCG CGGGGGCATC AGCAACAGTT GGTGTGCAAA GTGCTCACAT GCGTCGTGCC TCCGCGGATT 480
    TGGAGAAACG TCGTGCATCA GTTGGTGCCG CAGGTCGAGG ACTGCGAGGG GATGGTACTT TGGATCCACA CCATG CAG 558
 1                                                                              M   Q
   CCA TCC TCT TCA GAG ACT CAC GAG GGT TGC CTG TCG CTG ATC CGT TCT AGA GAA AGT AAT CTA TCA 624
 3 P   S   S   S   E   T   H   E   G   C   L   S   L   I   R   S   R   E   S   N   L   S
   GAT CTG GAA GAG GAC GAC TCA CAG GAT CTT CGT GAA GTT CTT CGT TTT CAC AAG TGC TAT GAT CTG 690
25 D   L   E   E   D   D   S   Q   D   L   R   E   V   L   S   F   H   K   C   Y   D   L
   ATA CCC ACC TCC GCC AAG TTG GTT GTC TTC GAC ACC AGC TCT GTA AAG AAG GGC TTC TAC GCC CTC 756
47 I   P   T   S   A   K   L   V   V   F   D   T   S   S   V   K   K   G   F   Y   A   L
   GTC TAC AAC GGT GTG CGA GCG GCA CCG CTC TGG GAT TCG GAG AAG CAA CAG TTC GTG GGC ATG CTA 822
69 V   Y   N   G   V   R   A   A   P   L   W   D   S   E   K   Q   Q   F   V   G   M   L
   ACC ATC ACG GAC TTT ATC AAG ATC CTG CAA ATG TAT TAC AAA TCG CCA AAT GCG TCC ATG GAG CAG 888
91 T   I   T   D   F   I   K   I   L   Q   M   Y   Y   K   S   P   N   A   S   M   E   Q
   CTG GAA GAG CAC AAA CTG GAC ACG TGG CGG AGC GTG CTG CAC AAC CAG GTG ATG CCG TTG GTC AGC 954
113 L   E   E   H   K   L   D   T   W   R   S   V   L   H   N   Q   V   M   P   L   V   S
   ATC GGA CCG GAT GCG TCC CTC TAC GAT GCC ATC AAA ATT CTC ATC CAC AGG CGC ATA CAT CGC CTG 1020
135 I   G   P   D   A   S   L   Y   D   A   I   K   I   L   I   H   R   R   I   H   R   L
   CCC GTC ATC GAT CCG GCG ACC GGC AAT GTC CTC TAC ATC CTG ACA CAT AAA CGC ATA CTT AGG TTC 1086
157 P   V   I   D   P   A   T   G   N   V   L   Y   I   L   T   H   K   R   I   L   R   F
   CTT TTC CTA TAC ATT AAT GAA TTA CCA AAG CCG CGT ACA TGC AAA AAG TTT GCG GAA CTG AAG ATT 1152
179 L   F   L   Y   I   N   E   L   P   K   P   R   T   C   K   K   F   A   E   L   K   I
   GGC ACC TAT AAC AAC ATC GAG ACC GCC GAC GAG ACG ACG AGC ATC ATC ACG GCG CTC AAG AAA TTT 1218
201 G   T   Y   N   N   I   E   T   A   D   E   T   T   S   I   I   T   A   L   K   K   F
   GTG GAG CGA CGA GTC TCA GCC CTG CCA CTA GTG GAT TCC GAT GGT CGC CTC GTT GAC ATT TAC GCA 1284
223 V   E   R   R   V   S   A   L   P   L   V   D   S   D   G   R   L   V   D   I   Y   A
   AAG TTT GAT GTG ATT AAT CTC GCC CCC GAG AAA ACC TAC AAC GAT CTC GAT GTT TCG CTG CGC AAA 1350
245 K   F   D   V   I   N   L   A   P   E   K   T   Y   N   D   L   D   V   S   L   R   K
   GCC AAC GAG CAC CGG AAC GAG TGG TTC GAG GGC GTG CAG AAG TGC AAT CTG GAC GAA TCG CTC TAC 1416
267 A   N   E   H   R   N   E   W   F   E   G   V   Q   K   C   N   L   D   E   S   L   Y
   ACG ATC ATG GAA CGA ATC GTC CGC GCC GAA GTA CAT CGA CTG GTG GTG GTC GAC GAG ATT CCA AGT 1482
289 T   I   M   E   R   I   V   R   A   E   V   H   R   L   V   V   V   D   E   I   P   S
   GAT CGG CAT AAT CTC CTG TCC GAT ATA CTG CTC TAC CTC GTC CTG CGA CCA AGC GGT GAA GGC GTC 1548
311 D   R   H   N   L   L   S   D   I   L   L   Y   L   V   L   R   P   S   G   E   G   V
   GGT GGC TCG GAG AGC TCA TTG CGT GCG TCC GAT CCC GTT CTC TGC CCA AAG TGC TGA GGTTGAAATAC 1616
333 G   G   S   E   S   S   L   R   A   S   D   P   V   L   C   P   K   C   *
   CAGCGACACC GCAGCGGCGA CGACAACAAC CCCGCCTCGC AGTCCATCGG CCGGATCCGC AATCGCAGCC TGATCGAGGA 1696
   CATACCCGAA GAGGAGACGG CGCCGGCGAG GAGCGACGAT GCCGACAGTG ATAACAATAT GTCCGCCAGT GAGGATAACG 1776
   ACAACAATAA CCAGCACGAC CAGACGACGA CGGTGCGACA GCTAATGGTG ATAGCAACAA CAGCCCGTTG AAGTGTCCTT 1856
   TGCGATGAGG GCAGGAAGAA GAAGCTCCCG ACCAGGTCGA GCGCAGCAAT TGTGATGATG ATGACCAGCC AGCGTTAGCG 1936
   GAGATGAGCC AGAATGCATC GATGGACGAC GACGAGGACG ATGGGATGAG CAGCGCCGTG TCCCTGCCNC CGCGTTGGCC 2016
   AGTCGCTGAC GCCCGCGCGC GAGAAATGCT TGTTAGTGAA TAAACCTAAA CCTACACCTT AACACTAAGT TAAACTTATG 2096
   CTAATGAGAT ACAGCTGTTA CAGACCAAAA GAAACAAAAA AAACAATGCT AAACAATAAC TAAGAAACCC AAACACAGCA 2176
   TTAATGATAA AGCAGATGAA CATTATATTT GAATATGAAT AAAAAAAAAA
```

Figure 5.9 The sequence of the pc8-1 cDNA. The sequence is numbered from the 5' end. The predicted amino acid sequence of the long open reading frame is shown underneath the DNA sequence with the single letter abbreviation aligned to the second base of each codon. The putative polyadenylation signal is underlined. Bold indicates the consensus *Drosophila* translation start site (Cavener and Ray, 1991). * indicates the stop codon.

```
        1                                                                  80
Human-L ...METVI.. SSDSSPAVEN EHPQETPESN NSVYTSFMKS HRCYDLIPTS SKLVVFDTSL QVKKAFFALV TNGVRAAPLW
  Rat-L ...MESV... AAESAPAPEN EHSQETPESN SSVYTTFMKS HRCYDLIPTS SKLVVFDTSL QVKKAFFALV TNGVRAAPLW
Human-B ...METVF.. SSDSSPAVEN EHPQETPESN NSVYTSFMKS HRCYDLIPTS SKLVVFDTSL QVKKAFFALV TNGVRAAPLW
Dros.-B MQPSSSETHE GCLSLIRSRE SNLSDLEEDD SQDLREVLSF HKCYDLIPTS AKLVVFDTS. SVKKGFYALV YNGVRAAPLW
 SNF4-Y .......... ..MKPTQDSQ EKVSIEQQLA VESIRKFLNS KTSYDVLPVS YRLIVLDTSL LVKKSLNVLL QNSIVSAPLW
        81                                                                 160
Human-L DSKKQSFVGM LTITDFINIL HRYYKSALVQ IYELEEHKIE TWREVYLQDS FKPL..VCIS PNASLFDAVS SLIRNKIHRL
  Rat-L DSKKQSFVGM LTITDFINIL HRYYKSALVQ IYELEEHKIE TWREVYLQDS FKPL..VCIS PNASLFDAVS SLIRNKIHRL
Human-B DSKKQSFVGM LTITDFINIL HRYYKSALVQ IYELEEHKIE TWRELYLQET FKPL..VNIS PDASLFDAVY SLIKNKIHRL
Dros.-B DSEKQQFVGM LTITDFIKIL QMYYKSPNAS MEQLEEHKLD TWRSV.LHNQ VMPL..VSIG PDASLYDAIK ILIHRRIHRL
 SNF4-Y DSKTSRFAGL LTTTDFINVI Q.YYFSNPDK FELVDKLQLD GLKDIERALG VDQLDTASIH PSRPLFEACL KMLESRSGRI
       161                                                                 240
Human-L PVIDPESGN. ...TLYILTH KRILKFLKLF ITEFPKPEFM SKSLEELQIG TYANIAMVRT TTPVYVALGI FVQHRVSALP
  Rat-L PVIDPESGN. ...TLYILTH KRILKFLKLF ITEFPKPEFM SKSLEELQIG TYANIAMVRT TTPVYVALGI FVQHRVSALP
Human-B PVIDPISGN. ...ALYILTH KRILKFLQLF MSDMPKPAFM KQNLDELGIG TYANIAFI.P DTPIIKALNI FVEXRISALP
Dros.-B PVIDPATGN. ...VLYILTH KRILRFLFLY INELPKPRTC KK.FAELKIG TYNNIETADE TTSIITALKK FVERRVSALP
 SNF4-Y PLIDQDEETH REIVVSVLTQ YRILKFVALN CRE...THFL KIPIGDLNII TQDNMKSCQM TTPVIDVIQM LTQGRVSSVP
       241                                                                 320
Human-L VVDEKG.RVV DIYSKFDVIN LAAEKTYNNL DVSVTKALQH RSHYFEGVLK CYLHETLETI INRLVEAEVH RLVVVDENDV
  Rat-L VVDEKG.RVV DIYSKFDVIN LAAEKTYNNL DVSVTKALQH RSHYFEGVLK CYLHETLEAI INRLVEAEVH RLVVVDENDV
Human-B VVDESGKRVV DIYSKFDVIN LAAEKTYNNL DITVTQALQH RSQYFEGVVK CNKLEILETI VDRIVRAEVH RLVVVNEADS
Dros.-B LVDSDG.RLV DIYAKFDVIN LAPEKTYNDL DVSLRKANEH RNEWFEGVQK CNLDESLYTI MERIVRAEVH RLVVVDEIPS
 SNF4-Y IIDENGY.LI NVYEAYDVLG LIKGGIYNDL SLSVGEALMR RSDDFEGVYT CTKNDKLSTI MDNIRKARVH RFFVVDDVGR
       321                                     362
Human-L VKGIVSLSDI LQALVLTG.G EKKP
  Rat-L VKGIVSLSDI LQALVLTG.G EKKP
Human-B IVGIISLSDI LQALILTPAG AKQKETETE
Dros.-B DRHNL.LSDI LLYLVLRPSG EGVGGSESSL RASDPVLCPK C*
 SNF4-Y LVGVLTLSDI LKYILLGSN
```

Figure 5.10 Multiple alignment (Pileup: Feng and Doolittle, 1987) of AMP-activated protein kinase (AMPK) γ-subunit polypeptide sequences. Amino acid residues conserved among the species are represented in red. Human-L and Human-B are from liver and brain respectively; SNF4-Y is from the yeast *S. cerevisiae*.

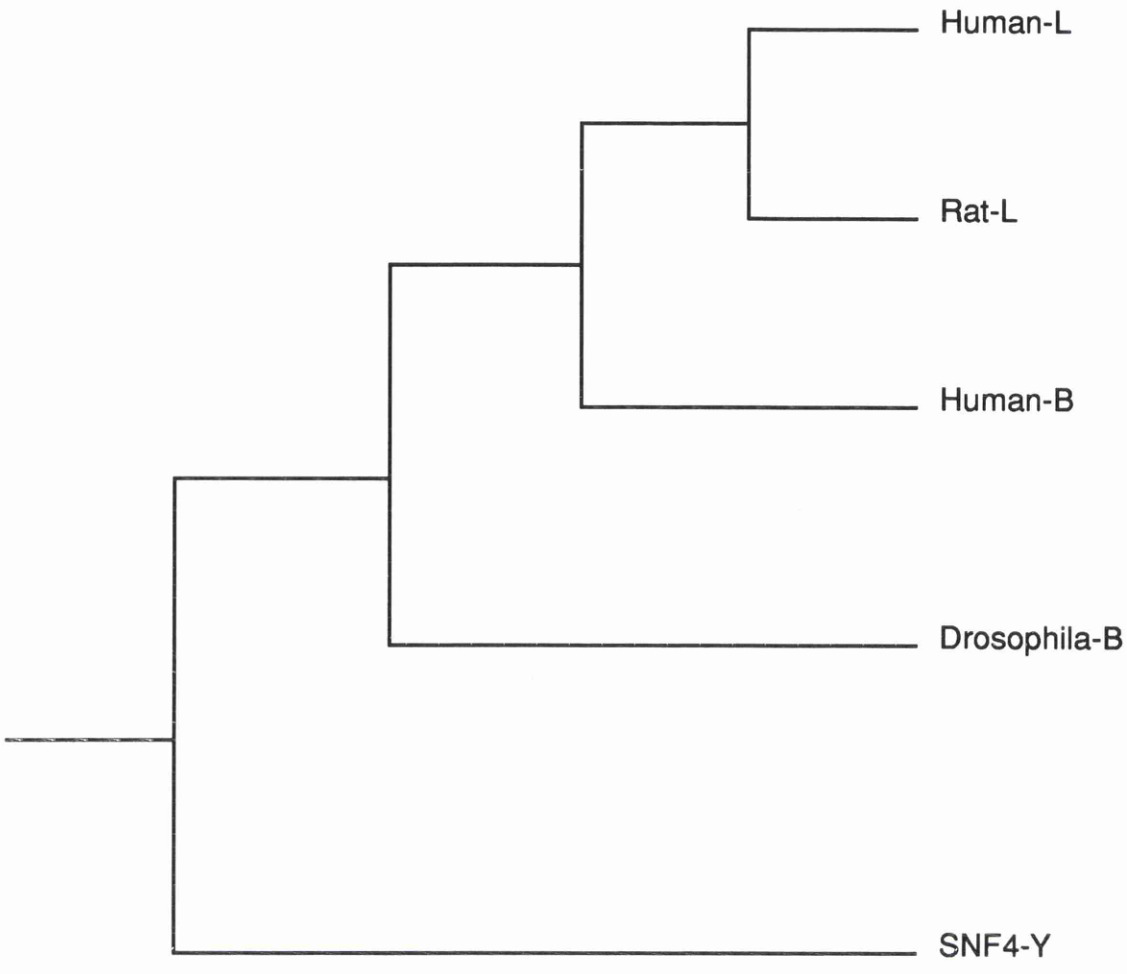


Figure 5.11 Phylogenetic relationship of AMPK γ-subunits. The tree was generated using the PAUP 3.1.1 programme. GenBank accession numbers are as follows.

Human-L (from human liver) accession no. :HSU42412;

Human-B (from human brain) accession no.: F06918;

Rat-L (from rat liver) accession no.: X95578;

SNF4-Y (from *S. cerevisiae*) accession no.: Z72637

As expected, the three mammalian genes are clustered together yet show more homology to the *Drosophila* sequence than to the yeast SNF4 sequence. The relationship reflects closely the evolutionary distance of the organisms from which these polypeptide sequences were derived.

5.4.3 Genomic organisation

To examine the genomic organisation of the AMPK γ-subunit gene, a Southern blot of wild-type *Drosophila* genomic DNA digested with different restriction enzymes (*SalI* and *SstI*) was probed with the *EcoRI/Hind*III fragment of pc8-1. As can be seen in Figure 5.12, there is no obvious evidence for more than one copy of the gene. A strong hybridization band of approximately 17 kb is seen in *SstI* digested genomic DNA, as expected. It is possible that the 3' rescued fragment may not cover the whole genomic region of the AMPK γ-subunit gene, so other bands from the *SstI* digestion would have been acceptable. However, the hybridization does not show any obvious bands besides the 17kb one. For the *SalI* digestion, one hybridizing band of roughly 6kb in size is seen.

In order better to identify the direction and the position of the transcriptional unit, PCR reactions were set up with the rescued plasmid as template and primed with different sequencing primers (3'-1 and 5'-1, See Figure 5.8) and the T7 primer, which neighbours the *SstI* site of polylinker 3 on the P[GAL4] construct (Fig. 5.4 and Fig 5.6 B). T7 and 3'-1 primers gave a 2.1kb amplified fragment. There was no amplification with T7 and 5'-1 primers. This result suggests that the AMPKγ-subunit gene is transcribed in the opposite orientation to the GAL4 gene in the P-element construct, and that its 3' end is located approximately 7kb away from the c819 insertion site (Fig. 5.6 A).

Whether the gene contained any intron(s) could be analysed by using the sequencing primers on genomic DNA, but due to a lack of time I concentrated on a transcriptional study, and left the identification of intron(s) unfinished.

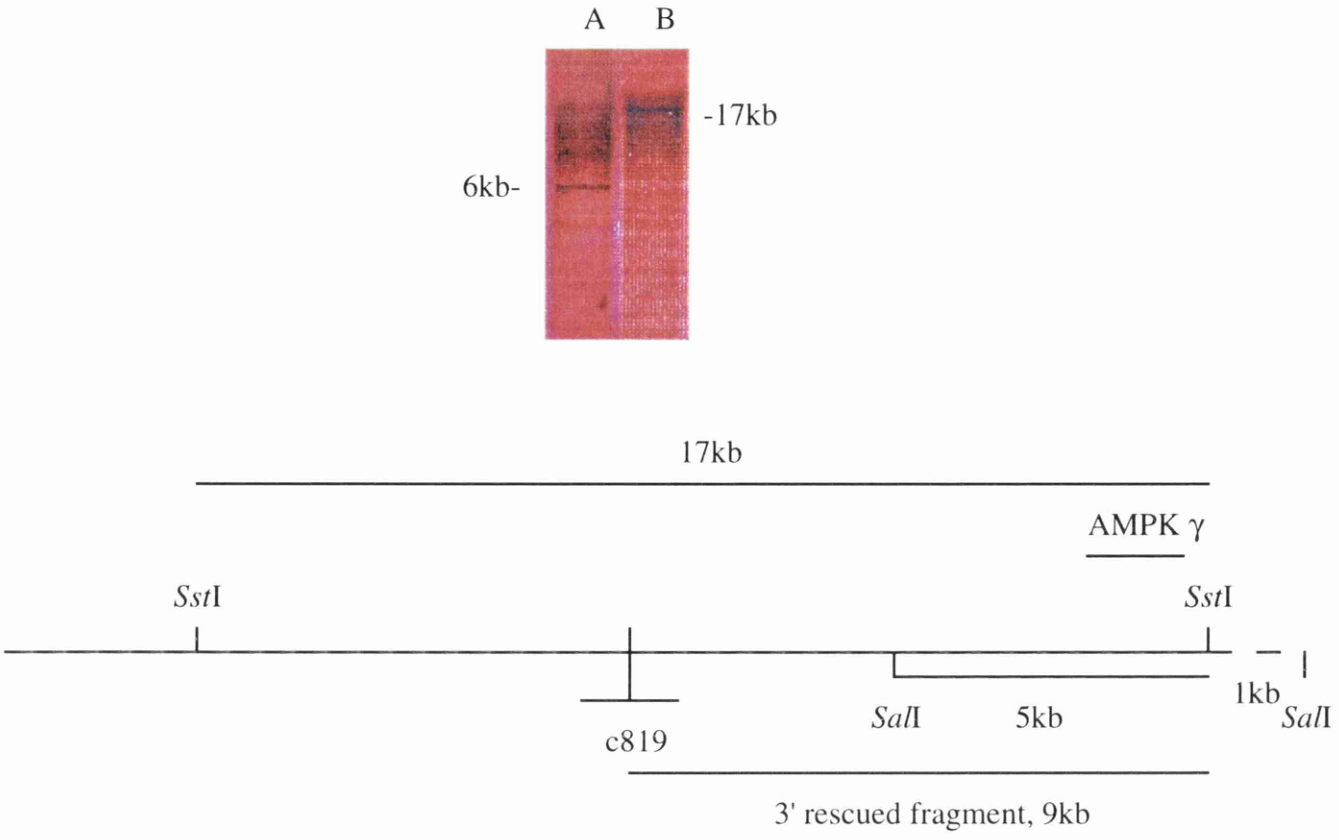


Figure 5.12. Southern blot of *Drosophila* (Canton-S) genomic DNA cleaved with the restriction enzymes:*Sal*I (lane A) and*Sst*I (lane B). The blot was probed with the Dig-labelled*Drosophila* AMPK γ-subunit gene (See Chapter 2). The diagram shows the genomic map and the corresponding position of the AMPK γ-subunit cDNA. A 6kb *Sal*I fragment implied a*Sal*I site 1kb beyond the end of the rescued fragment. There is no obvious evidence for more than one copy of the gene.

5.4.4 Transcriptional analysis

(a) Northern blotting

For this study, Canton-S wild type *Drosophila* were used as an RNA source. Total RNA was extracted from different developmental stages (mixed embryo, mixed third instar larvae and mid-pupae), from adults of both sexes, and from adult heads and bodies. Between 30-40μg of these total RNA samples were electrophoresed through denaturing formaldehyde gels and blotted (See Chapter 2). The filter was probed with antisense RNA transcribed from the pc8-1 subclone (Fig. 5.8). The pBluescript SK vector contains a T7 promoter adjacent to the *Hind*III site, which defines the end of the poly A tail of the cDNA fragment. Antisense RNA was thus synthesised by T7 RNA polymerase.

The resulting autoradiograph (Fig 5.13A) showed that there are two sizes of transcript, approximately 2.3kb and 2.0kb. As the pc8-1 cDNA insert is 2,226b, it is very likely that this clone contains almost a full length copy of the messenger RNA. As indicated by the rp49 (O'Connell and Rosbash, 1984) loading control, the RNA samples were evenly loaded in general, however, the lanes of female adults and adult bodies gave stronger hybridization signals than the others. It can be seen that the 2.0kb transcript is expressed at similar levels in both head and body, female and male, and through all the developmental stages. The 2.3kb transcript only appears from the pupal stage, and is expressed equally in female and male, head and body.

A Northern blot of RNA isolated from Canton-S and line c819 homozygotes was hybridized with the same probe to check if the P-element insertion had disturbed gene expression. The hybridization bands showed no difference in size and expression level between wild type and c819 (Fig. 5.13B), suggesting that the insertion had no effect on gene expression and function.

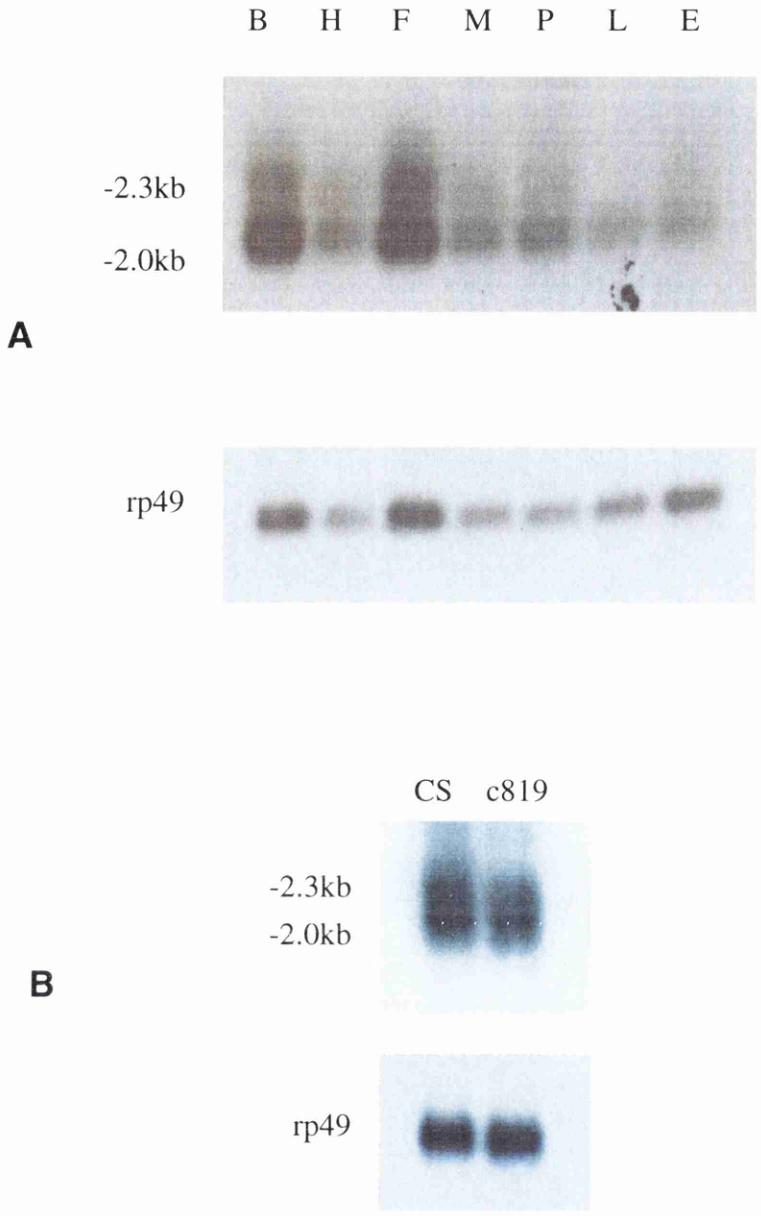


Figure 5.13 Northern blot probed with a ^{32}P-labelled antisense RNA probe corresponding to the *Drosophila* AMPK γ-subunit cDNA. The RNA electrophoresis and transfering were performed as described in Chapter 2. Both A and B were re-probed with *rp49* (O'Connell and Rosbash, 1984) as a loading control. Sizes were determined with respect to an RNA size marker (Gibco BRL). **A**. Developmental analysis of RNA samples from: adult body (B); adult head (H); female (F); male(M); pupa (P); larva (L) and embryo (E). **B.** There is no change of size or expression level between RNA samples from adult Canton-S (CS) and c819 homozygotes.

(b) *in situ* hybridization

An *in situ* hybridization to adult head tissue sections was carried out as described in Chapter 2. The whole cDNA fragment was used as a probe by labeling with digoxygenin. Positive (*opsin*, O'Tousa, et al., 1985) and negative (pBluescript) control probes were used alongside. The hybridization signals match the position of ellipsoid body cell bodies, and cell bodies in the medulla of the optic lobes (Fig. 5.14). Because *in situ* hybridization detects RNA, which is found only in the cell bodies, it would not be expected to see axonal projections. Therefore, no "ring shape" was seen. There is also some other heavy staining on the cryostat section. It is difficult to tell if it belongs to brain tissue or whether it could be heavy background staining.

5.5 Discussion

From previous studies of mammalian 5'-AMP-Activated Protein Kinase (AMPK), it is known to be a heterotrimeric protein consisting of α–, β– and γ–subunits. The α–subunit is the catalytic subunit, others being non-catalytic (Stapleton, et al., 1994). So far as AMPK biochemical function is concerned, it belongs to the SNF1 (sucrose non-fermentor) kinase family (Woods, et al., 1994), and it was first recognised as a regulator of fatty acid and sterol synthesis through its phosphorylation of acetyl-CoA carboxylase, hydroxymethylglutaryl-CoA reductase and hormone-sensitive lipase (Hardie and Mackintosh, 1992). In particular, AMPK mediates responses of these pathways to several metabolic or other cellular stresses, including glucose depletion, heat shock, and ATP depletion (Kudo, et al., 1995); it plays a primary role in protecting cells from stress by switching off ATP-consuming biosynthetic pathways.

Mammalian AMPK γ–subunit is a homologue of *S. cerevisiae* Snf4p. Snf4p was shown to interact with the Snf1p (Celenza and Carlson,1989) protein in participating in glucose

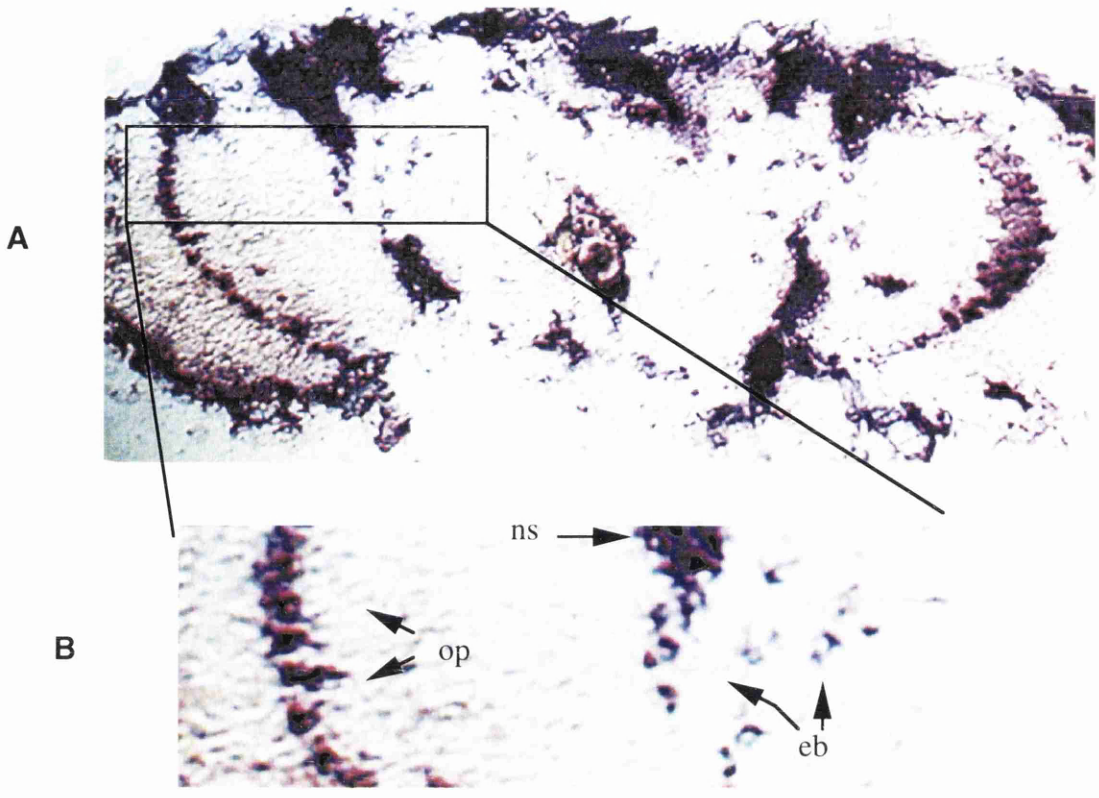


Figure 5.14 *Drosophila* AMP-activated protein kinase (AMPK) γ-subunit expression in the adult brain. Dig-labelled AMPK-γ DNA probe was used for this *in situ* hybridization to a 14 μm frontal cryostat section through the adult head (**A**). Expression is seen in cell bodies which match the position of ellipsoid body cell bodies. There is also strong expression in optic lobe cell bodies (**B**). The hybridization signals are indicated by arrows. op: optic lobes; eb: ellipsoid body; ns: non-specific staining. The latter staining do not show round-shape cell bodies, so it is difficult to tell if it belongs to brain tissue.

regulation of gene expression (Stapleton, et al. 1994), although the mechanism by which Snf4p activates Snf1p is not known.

In addition to the homology of the AMPK γ–subunit to Snf4p, examination of the database reveals several different mammalian proteins highly similar to the human and rat liver AMPK γ–subunit sequences (Gao, et al., 1996). This indicates that there is a mammalian isoform family of potential AMPK γ–subunits, each perhaps with different tissue expression (brain, heart, breast and placenta) and regulatory roles. From tissue-specific Northern analysis, the AMPK γ–subunit mRNA appears to be most highly expressed in heart and brain (Gao, et al., 1996).

The similarity of sequences between the mammalian AMPK γ–subunit and Snf4p from yeast emphasises the likelihood that the role of these genes has been highly conserved throughout evolution, and suggests that the cloned *Drosophila* cDNA gene may have the same function.

The signal observed in brain tissue by *in situ* hybridization matches the position of the ellipsoid body cell bodies. But, the strong expression observed in the optic lobe cell bodies does not correspond with the enhancer-trap pattern. There were just a few, if any, X-gal stained cells in the optic lobe, and even in the anti-β-gal antibody immunohistochemistry study, the signal in the optic lobe was weaker than it was in the ellipsoid body. The reason for this difference could be that the enhancer trapped by the P-element is different from the one enhancing AMPK γ-subunit gene transcription, or the AMPK γ–subunit is controlled by both ellipsoid body and optic lobe enhancers, but the GAL4 expression is driven only by the ellipsoid body enhancer. Alternatively, even if it is the same enhancer for both GAL4 and AMPK γ–subunit, the efficiency may be different between different types of cells and different genes. If there is a molecular genetic link between the optic lobe and ellipsoid body in terms of enhancer activity within the brain, perhaps they share physiological characteristics.

Northern hybridization of pc8-1 detected two bands of 2.0kb and 2.3kb. These may reflect isoforms of AMPK γ–subunits in *Drosophila*. The mRNA of 2.3kb became evident from the pupal stage (Fig. 5. 13). This result tallies well with the observations of the developmental immunohistochemistry study in section 5.2. It provides another piece of evidence that the starting time for expression from the trapped enhancer is the same as the expression of the AMPK γ–subunit. Therefore, from the developmental point of view, the enhancer responsible for GAL4 expression seems to be the one that enhances the transcription of the AMPK γ–subunit.

Taken together, the AMPK γ–subunit gene may activate AMPK as a "metabolic sensor" to transmit certain signals and control the regulation of behavioural activities. The functional role of the central complex can be understood better by knowing more about the genes that are expressed in these neurons.

As far as the length and structure of the 5' untranslated region (UTR) of the pc8-1 cDNA insert are concerned, it is longer than 150bp, and contains 6 potential translation starts (ATG codons). These facts may suggest that this is a "double-cloning" which means that two cDNA sequences have been joined together accidentally. To prove whether this is the case or not, genomic Southerns could be done with the 5' UTR and the rest of the cDNA insert as probes. By comparing the resulting signals, we could confirm whether the 5' UTR and coding sequences are neighbouring each other in the genome. Alternatively, when the genomic clones covering the whole chromosomal region are available, PCR, restriction site analysis and sequencing could be applied. Data base search of the 5' UTR did not find any homology, so the sequence does not belong to any plasmid or phage vector. If it is not a part of *dAMPKγ*, the Northern, *in situ* and Southern results will require reinterpretation. The PCR result from the T7 and 3'-1 primers (Fig. 5.6B) shows that the 3' of the pc8-1 cDNA insertion is within the rescued genomic region of line c819.

Chapter 6

Flanking genomic DNA analysis of some P[GAL4] insertions

6.1 Introduction

Apart from P[GAL4] line c819, the plasmid rescue procedure has also been applied to another 9 lines which have brain specific expression patterns. Most of them have been used for the selective feminisation study in Chapter 4 (Table 4.1). Here, we are interested in finding genes with specific patterns of expression. For further transcript searching, it would be useful to obtain genomic DNA clones that span the site of the insertion and extend further 3' and 5'. Detailed genomic analysis of lines c739 and c772 will also be described in this Chapter.

6.2 The brain expression patterns of the P[GAL4] lines for plasmid rescue.

Genomic fragments flanking the P-element insertions have been rescued from 9 P[GAL4] lines. Their major expression patterns in their brains are summarised in Figure 6.1 and Table 6.1. There are 6 mushroom body lines (45Y, 121Y, c184, c253, c772 and 72Y), one central complex line c481, and 2 optic lobe lines (c469 and c827). X-gal staining elsewhere in the brain is also observed for several lines. Besides the strong expression in the optic lobe of line c469, faint staining can be seen in the antennel lobe as well. The patterns of these lines have also been described in other studies (Armstrong, et al., 1997; Yang, et al., 1995 and Yang, 1996) and "Brainbox" (http://brainbox.gla.ac.uk).

6.3 Plasmid rescue of the flanking genomic DNA fragments

According to the P[GAL4] construct shown in Figure 5.4, plasmid rescue (Chapter 1 and 2) was carried out by using the restriction enzyme *Sst*I for the downstream (3')

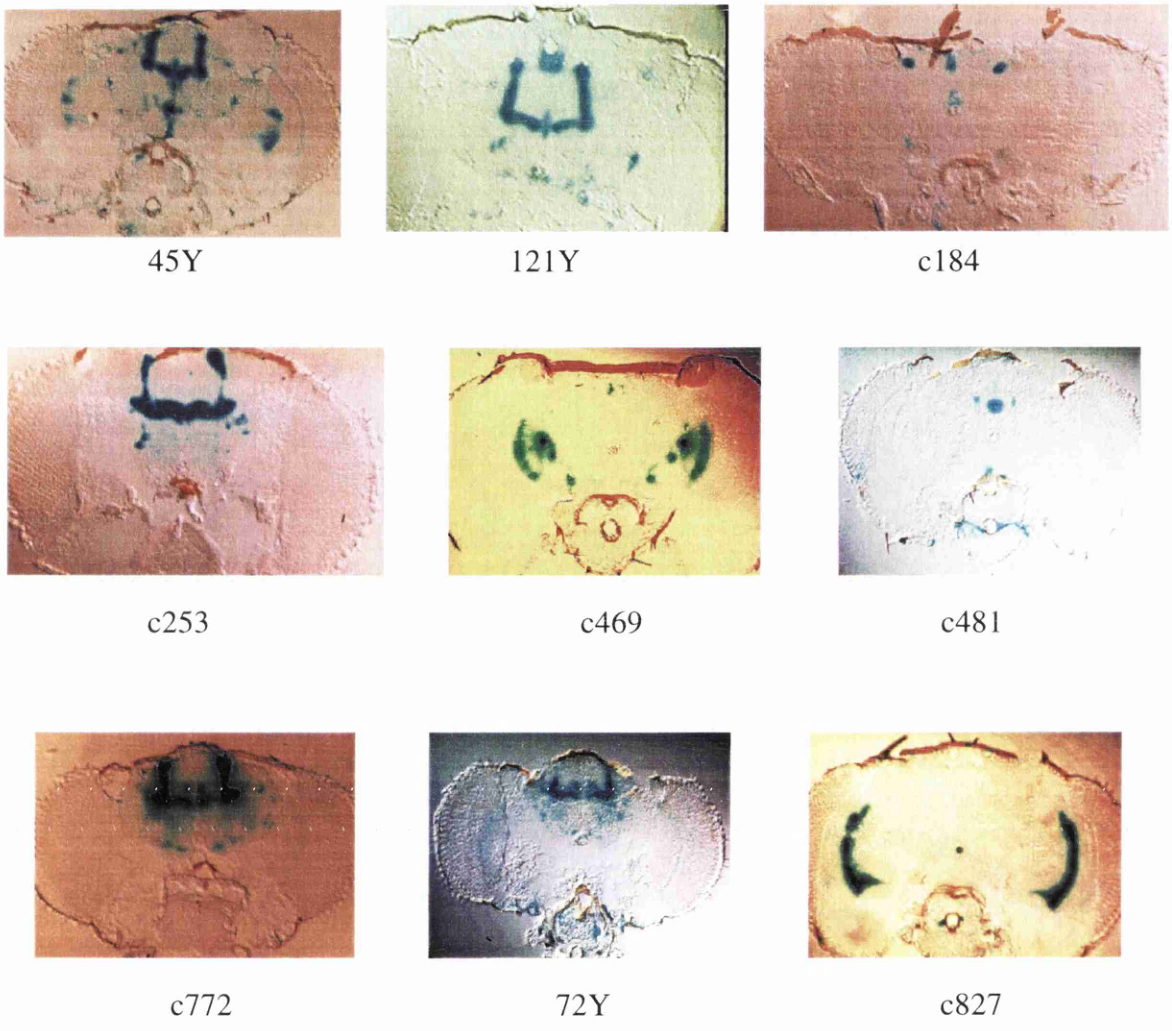


Figure 6.1 GAL4-directed β-gal expression patterns of the lines used for plasmid rescue. Each panel is a 12 μm frontal cryostat section. Lines 45Y, 121Y, c184, c253 c772 and 72Y have patterns of mushroom body expression. Line c481 shows staining on the ellipsoid body of central complex. The major expression patterns of lines c469 and c827 are optic lobes. Line c469 also has faint staining on the antennal lobe.

fragments and *Kpn*I for the upstream (5') fragments. The results are presented in Table 6.1.

Table 6.1 The results of plasmid rescue from nine P[GAL4] enhancer-trap lines.

Line	expression pattern	3' rescued plasmids	5' rescued plasmids	Chromosomal location
45Y⑤	mushroom body	6.5kb	-	-
121Y⑥	mushroom body *	12kb	-	71B
c184④	mushroom body	4.5kb	-	-
c253①	mushroom body	10.5kb	-	49D
c469①④	optic lobe**	9kb	-	-
c481②④	central complex	11kb	-	18A
c772③	mushroom body	5.4kb	12.7kb	42A
72Y⑥	mushroom body	11.5kb	-	21B
c827⑤	optic lobe	5kb	20kb	-

The sizes of 3' rescued plasmids include 2.9kb pBluescript and the adjacent genomic fragments. The sizes of 5' rescued plasmids include the adjacent genomic fragments and the 12kb P[GAL4] construct (pBluescript, mini-*white* and GAL4). *: the staining can also be seen in the central complex. **: there is also faint staining in the antennal lobe of line c469. The polytene chromosome locations are kindly provided by Susan. Wang. ① Yang, 1996; ② Armstrong, et al., 1997; ③ Yang, et al., 1995; ④ J.D.Armstrong, personal comm.; ⑤ M.Yang, personal comm.; ⑥ Brainbox: http://brainbox.gla.ac.uk

Genomic fragments from the 3' direction are obtained by digestion with *Sst*I and *Sst*II, while genomic fragments from the 5' direction are obtained by digestion with *Kpn*I and *Hind*III. Among the rescued fragments, the longest one extends 9kb downstream of the 121Y insertion. The shortest fragment begins only 0.7kb from 5' of the c772 insertion.

In order to confirm that the sizes of the rescued plasmids are as expected, genomic DNAs from the 9 P[GAL4] lines were digested by *Sst*I for checking the size of 3' fragments, and *Kpn*I for checking the size of 5' fragments. The Southern blot was performed as standard procedure (Chapter 2) with pBluescript probe. As seen in Figure 6.2, the hybridization bands are the same sizes as the rescued plasmids (Table 6.1). Since there is only one band in each line, there is only one insertion in each line.

6.4 Mapping the genomic region of the c739 insertion

P[GAL4] line c739 shows an intense staining within components of the α and β lobes of the mushroom body, but no staining of the spur and γ lobe (Fig. 6.3). Through confocal microscopy, four groups of Kenyon cell bodies can be seen and they supply four tracts in the calyx that enter the pedunculus. In the pedunculus these four tracts fuse into two, then subsequently fuse again into a single tract near the anterior end of the pedunculus. Close examination reveals a very narrow unstained core region in both α and β lobes. The intact brain also contains some weak staining in the antennal nerve (Armstrong, 1995).

Line c739 has been chosen as a mushroom body specific expression line for the study of neuron subregions relating to courtship behaviour by selective feminization (O'Dell, et al., 1995). The ectopic expression of *transformer* driven by GAL4 in c739 can result in imcomplete abdominal feminization and sterility of male progeny, and these males displayed male-male courtship in addition to male-female courtship.

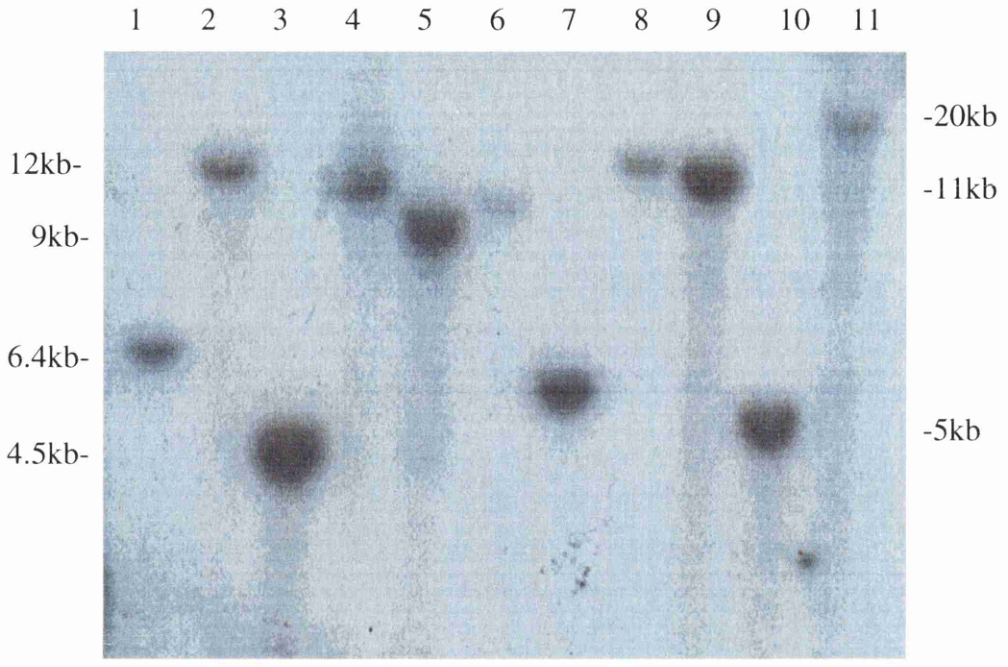


Figure 6.2 Genomic Southern analysis of rescued plasmids. Genomic DNAs from 9 P[GAL4] lines were digested with *Sst* I (for checking 3' rescued plasmids) and *Kpn* I (for checking 5' rescued plasmids). They were hybridized with pBluescript probe. The sizes of the bands should be the sizes of the rescued plasmids (See Table 6.1).

Lane 1: 45Y/ *Sst* I , 6.5kb
Lane 2: 121Y/ *Sst* I, 13kb
Lane 3: c184/ *Sst* I, 4.5kb
Lane 4: c253/ *Sst* I, 11kb
Lane 5: c469/ *Sst* I, 9kb
Lane 6: c481/ *Sst* I, 10kb
Lane 7: c772/ *Sst* I, 5.4kb
Lane 8: c772/ *Kpn* I, 14kb
Lane 9: 72Y/ *Sst* I, 11kb
Lane 10: c827/ *Sst* I, 5kb
Lane 11: c827/ *Kpn* I, 20kb

Both sides of the genomic flanking region of c739 were previously rescued by Andrew Mounsey. The upstream fragment is 2.8kb, and the downstream fragment is 0.7 kb. Approximately, $3x10^5$ phage from a *Drosophila* EMBL3 genomic library were probed with the 2.8kb rescued DNA fragment. Three positive clones were recovered. They are λ9.1, λ9.2 and λ9.3. *SalI* digestion separated the inserts: λ9.1 = 14.5kb, λ9.2 = 19kb, λ9.3 = 15.5kb. In order to map genomic lambda clones, the phage DNAs were restricted with a combination of different enzymes. The constructed restriction map is shown in Figure 6.4.

Plasmids were independently rescued from a collection of recessive lethal insertions of a P[lacW] transposon on *Drosophila* chromosome 2 (Torok, et al., 1993). These plasmids containing genomic DNA flanking the sites of transposon insertion represented 1836 *Drosophila* lines, and were pooled in batches of 10 and 100. Pools of 100 plasmids were screened with genomic clones representing the sequences adjacent to the c739 insertion point, which is known to be at second chromosome 40A (S. Wang, personal comm.) Hybridizing pools were then narrowed down to single plasmids by a process of subdivision and rehybridization (Guo, et al., 1996). Line l(2)K09017 was thereby identified. The rescued plasmid from line l(2)K09017 matches the 5' region of the c739 insertion by Southern hybridization. According to the information provided by the Berkeley *Drosophila* Genome Project (http://shoofly. bdgp.berkeley.edu/), this line contains two P-element insertions located at 35D1-D2 and 39E1-E4. Chromosomal location 39E1-E4 is very close to the cytological position of the P[GAL] insertion of line c739, 40A. However, it is still unknown if the insertion at 39E is responsible for the lethal phenotype of P[lacW] line l(2)K09017.

Figure 6.3 Expression pattern in the brain (from the front) of P[GAL4] enhancer-trap line c739. X–gal staining indicates the *lacZ* expression which is driven cytoplasmically by GAL4, and reveals the α and β lobes of the mushroom bodies in the brains (Yang, 1996).

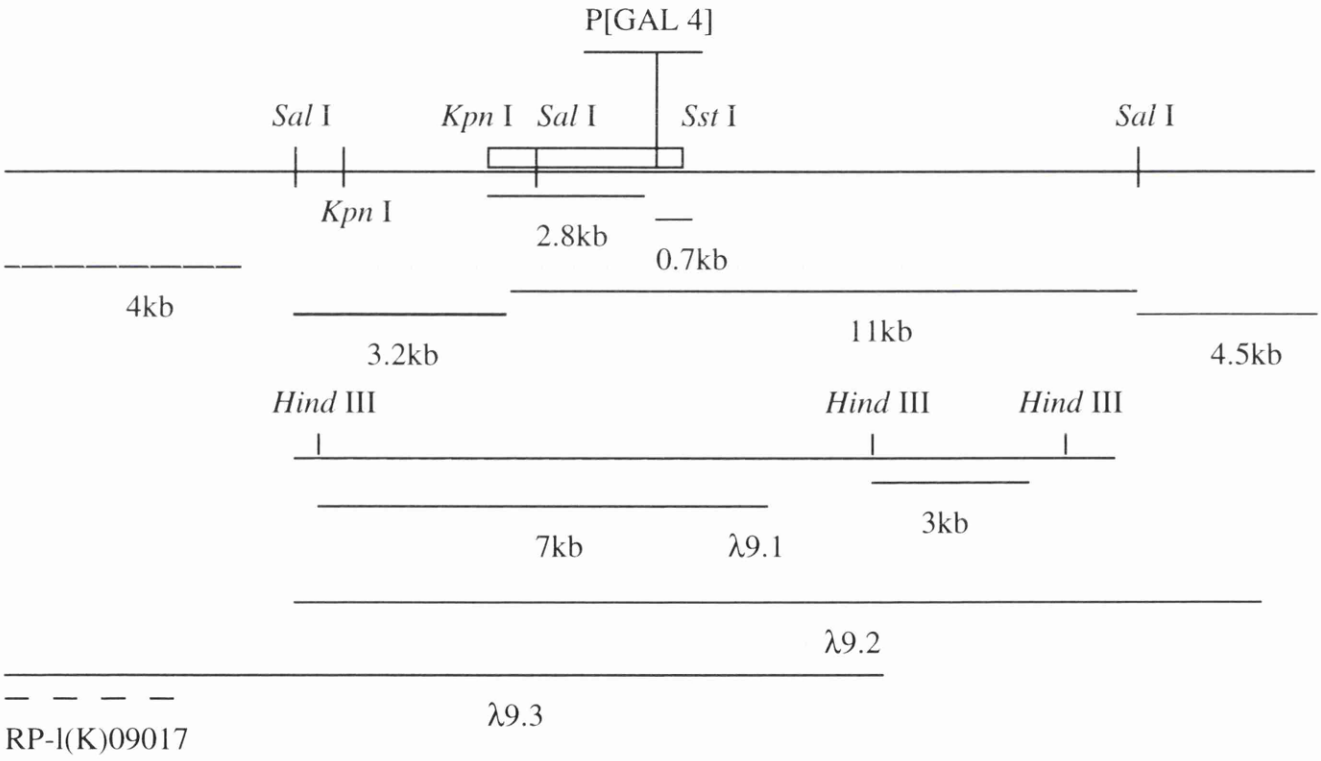


Figure 6.4 Genomic organisation of the region surrounding c739 insertion. The restriction map was generated from the analysis of λ genomic clones (λ 9.1, λ 9.2, and λ 9.3). The plasmid rescued fragments flanking the P[GAL4] insertion are referred as rectangles. The rescued plasmid identified from the pooled second chromosome P[lacW] lethal lines (RP-l(K)09017) match the position of the 4kb *Sal* I fragment in the 5' side of the c739 P[GAL4] insertion. This fragment is shown as dashed line.

6.5 Isolation of genomic DNA clones corresponding to line c772

The 2.4kb rescued genomic fragment from the 3' direction was used as a probe for the screening of a *Drosophila* EMBL 3 genomic library. Two positive clones were isolated. The size of λ7.1 is 14kb. The other one, λ7.2 contains a 15kb insertion. The phage DNA was extracted, and then cleaved by *Sal*I, *Hind*III and a double digestion. The restriction map (Figure 6.5) was constructed by hybridization to the 2.4kb probe.

Interestingly, lines c772 and c747 have the same expression patterns, which show X-gal staining in the α and β lobes with an unstained core, and in all the γ lobe (Armstrong, 1995). Both insertions are located at chromosomal position 42A (S. Wang, personal comm.). The plasmid rescue work on line c747 was done previously by Andrew Mounsey. The 5' rescued plasmids of lines c772 and c747 both contain 1.9kb genomic fragments. The 3' plasmid rescue of line c747 was obtained through *Pst*I digestion. The size of the genomic insertion is 1.2kb. The 3' rescued fragment of line c772 contains one *Pst*I site, whose position is roughly 1.2kb from the insertion. Also, the captured fragments from the two lines cross-hybridize with each other. Thus it is shown that these two lines have the same, or very close to the same position of P-element insertion. Their expression patterns are probably activated by the same enhancer.

A "Reverse Northern" strategy was applied using head and body cDNAs to probe the rescued fragments from lines c739 and c747. They showed no hybridization signal (A. Mounsey, personal comm.). Unfortunately, in the genomic region of the c739 and c772 insertions, no transcript sequence has been found through cDNA library screening.

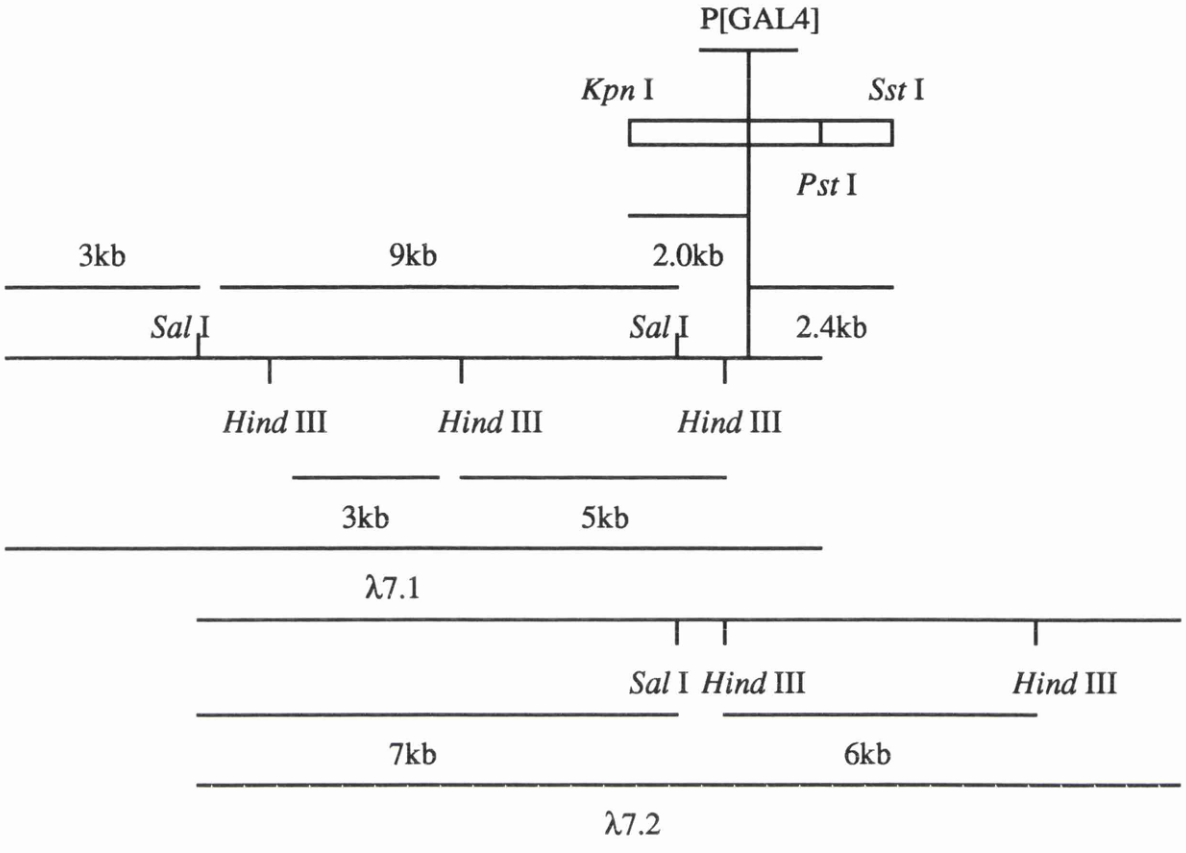


Figure 6.5 The genomic organisation of the region surrounding the P[GAL4] insertion in line c772. The restriction map was generated from the analysis of the 3' and 5' rescued plasmids (referred as rectangles), and the λ genomic clones (λ 7.1 and λ 7.2).

Chapter 7

Cloning and characterisation of a transcript sequence *dKAL*

7.1 Introduction

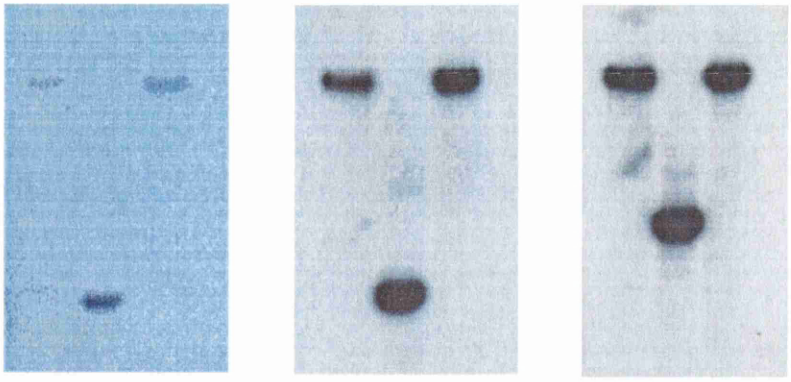
During the analysis of the genomic region adjacent to the P[GAL4] insertion of line c739, one lambda clone was picked up by similarity to repetitive sequences from the screening of the EMBL3 genomic library. Two transcript sequences have been found in this genomic region by probing the NM1149 cDNA library with the cloned genomic fragments. One cDNA clone (pc-MAPK) was identified as a member of the family of mitogen-activated protein (MAP) kinases. Comparisons of the nucleotide sequence to data bases shows that it shares a high homology with p38, which has been cloned from mammalian cells. Dr. E. Martin-Blanco et al. (1995) has also cloned the *Drosophila* p38 MAP kinase. The gene is located in polytene band 95E. The P1 clones corresponding to the 95E genomic region were probed with both of the transcript sequences that we have cloned. They all showed some hybridization signal (Fig. 7.1). These indicate that this lambda genomic clone and the transcripts are located on 95E. The analysis of the other cDNA clone (pc-KAL) is presented in this chapter.

7.2 The cloning and analysis of *dKAL*

This cDNA clone was isolated from an NM1149 cDNA library representing adult heads of the *Drosophila melanogaster eyes absent (eya)* mutant. cDNA inserts in the recombinant phage were excised by *EcoR*I and *Hind*III, then subcloned into pBluescript SK^-.

7.2.1 Sequencing and analysis

Within the sequence, there is one *Xho*I site and one *BamH*I site. The sequence was determined by using synthetic oligonucleotides. The full length sequence of the cDNA clone is 1734bp long including a 12bp polyA tail. However, the normal polyadenylation



A B C

Figure 7.1 Southern blot of the P1 clones from genomic region 95E.
A: probed with the p38 MAP kinase gene cloned by Dr. Martin-Blanco; B: probed with pc-MAPK; C: probed with pc-KAL. The blot of P1 clones was kindly provided by Dr. Martin-Blanco. These results show that pc-MAPK and pc-KAL are located at the same chromosomal region of 95E.

signal site is not found in the 3' untranslated region. When the restriction map of the cDNA was predicted by MacVector™, it showed very close correspondence to that obtained using restriction enzymes.

The sequence obtained was used to search the databases with the programme BLAST (Kailin and Altschul, 1993). At the nucleotide level, no significant homology was detected. The sequence has an Open Reading Frame (ORF) corresponding to a 522 amino acid polypeptide (Fig. 7.2), starting from nucleotide 37 and ending at nucleotide 1602. It is followed by a TAG stop codon. The deduced protein sequence displayed certain homology to a large number of neural cell adhesion molecules in a wide range of species. Upon further investigation, the homology was shown to be due to the presence of a four-disulphide-core domain (Drenth et al, 1980) and fibronectin type III repeats (Odermatt, et al., 1985). Among them, Kallmann syndrome protein precursor (KAL-human, Legouis, et al. 1991; Franco, et al. 1991) has the highest score. They share 28% and 45% amino acid identity in the two major domains. Figure 7.3 shows the alignment of the deduced KAL protein sequences from human, chicken and *Drosophila*. The cDNA we got from *Drosophila* is therefore named as *dKAL*.

By the PSORT (prediction of protein localisation sites, Nakai and Kanefisa, 1992; http://psort.nibb.ac.jp/ form.html) search, the predicted protein from dKAL seems to have a cleavable N-terminal signal peptide sequence from amino acid 6 to 24, and 6 N-glycosylation sites. No transmembrane segments have been recognised.

7.2.2 Northern blot analysis

Northern blot of different RNA samples using a *dKAL* cDNA antisense riboprobe, identified a single band with the same size of approximately 1.9kb (Fig. 7.4). So, it seems that the cDNA clone contains the complete transcribed sequence; there is no evidence for alternative splicing.

```
CGTGCTGAAT TCAAGCCGAG GATCGGAGCG TTTCAC ATG GGC AGC ATG CAA GTG GCG CTG CTG GCG CTG   69
1                                        M   G   S   M   Q   V   A   L   L   A   L
  CTT GTT CTC GGC CAG CTA TTC CCA AGC GCC GTG GCT ATG GAT CCT CCT CCT ATA GTT CCA CCT 132
12 L   V   L   G   Q   L   F   P   S   A   V   A   M   D   P   P   P   I   V   P   P
  CCA CAT CCG CAT CGA ATC AGC TGC AGC GAC AGA AGC TGG CAC ACT GGT TCC GGG ATA GCA ATG 195
33 P   H   P   H   R   I   S   C   S   D   R   S   W   H   T   G   S   G   I   A   M
  ATG TTA AGG ATA AGA TCC TGG AGC TGC AAT GCC TGG CGA AGT GTG GCA GAA TCC CAC AAC CAA 258
54 M   L   R   I   R   S   W   S   C   N   A   W   R   S   V   A   E   S   H   ■   Q
  AGC TGG ACG GGA ACA GTG CCT GAA CAA GTG CAT CCA GGA GCT TTT GCT GGG ACC AGA GCC GGC 321
75 S   W   T   G   T   V   P   E   Q   V   H   P   G   A   F   A   G   T   R   A   G
  AGT TGC CCC AAA ATT GGA AGG CAA TCG CGT GCC AGA CTC TCC TGC CTG GAC AAC TGT CAG TAC 384
96 S   C   P   K   I   G   R   Q   S   R   A   R   L   S   C   L   D   N   C   Q   Y
  GAT CAT GAA TGC CCA GAG GTG CAG AAG TGT TGT CCC TCC AGT TGC GGA CCC ATG TGC GTG GAA 447
117D   H   E   C   P   E   V   Q   K   C   C   P   S   S   C   G   P   M   C   V   E
  CCT CTC GGC GTT AGG AAC AAC ACA CAG CTT CCG CCC ATA CCG AAG ATT TTG TAT TTC CGG AGA 510
138P   L   G   V   R   N   N   T   Q   L   P   P   I   P   K   I   L   Y   F   R   R
  TCG CGA GGT CAT GCT GTC GAT CTG AAG ATC GAG TCC TCG CTA CTG GTC TAC TAC TTC CAT GTG 573
159S   R   G   H   A   V   D   L   K   I   E   S   S   L   L   V   Y   Y   F   H   V
  GAG GTA AGA TCC CAC ATA GGA CGG CAT TTT GCA GCC AGA AAA CTG GGT CCT TGG CAA TGG CAG 636
180E   V   R   S   H   I   G   R   H   F   A   A   R   K   L   G   P   W   Q   W   Q
  AAG GTG GAG AAG TAC CAT GGA GAG AAC ATC GGA CAC AGC AAG CAT ACT TAC ATC TTC CTT CAC 699
201K   V   E   K   Y   H   G   E   ■   I   G   H   S   K   H   T   Y   I   F   L   H
  ATG CGA CCT GGT CGG TGG TAT GAG GTT CGA GTG GCA GCC GTA AAC GCC TAC GGG TTC CGT GGA 762
222M   R   P   G   R   W   Y   E   V   R   V   A   A   V   N   A   Y   G   F   R   G
  TAT TCC GAG CCA AGC GAT CCA TTT CCC TCG ACG GGC AAC CCA AAG CCC CCA AAG TGT CCG AAC 825
243Y   S   E   P   S   D   P   F   P   S   T   G   N   P   K   P   P   K   C   P   ■
  GAT TCG AAG ATC ATC GGC AAG CAG TTG ATG GAC GCT ACA GTA CCC TTA AGC TGG TGT GGT GCC 888
264D   S   K   I   I   G   K   Q   L   M   D   A   T   V   P   L   S   W   C   G   A
  CGT CCA AGT CCG ACG TGC CTG TCG AGG GAG TAC AAG ATC AAC TGG TCA TTG CAA GTA ACA GTG 951
285R   P   S   P   T   C   L   S   R   E   Y   K   I   N   W   S   L   Q   V   T   V
  CCA AGG CTT GAT GAT TAC GGA CAG TCT AAG TTA AGG ATA CCC ACC AGT TTG AAA TTA AGG ATG 1014
306P   R   L   D   D   Y   G   Q   S   K   L   R   I   P   T   S   L   K   L   R   M
  TAC CCA ATA TCT ATG CAA TCC AAG TGC AGG CCA TAT TCT AAC TGG TTT TGG TAT GCC TTA AGT 1077
327Y   P   I   S   M   Q   S   K   C   R   P   Y   S   N   W   F   W   Y   A   L   S
  ATC GAG CAG TGG TTG ATG TGT ATT AAA GAC GGT GCA ACT ATT GGA GCC AAT TAC ACC GGA TCA 1140
348I   E   Q   W   L   M   C   I   K   D   G   A   T   I   G   A   ■   Y   T   G   S
  GTG GAT CCG GGA ATG GCA ATA GGG GAC GGC ATA TCA ATC ACA ATA GCA GGA CTA GTA GGT CGG 1203
369V   D   P   G   M   A   I   G   D   G   I   S   I   T   I   A   G   L   V   G   R
  GAC GGG CCA CAA CCT AGG AGC CAG TTG CCA TCA ATG GAG ATC CGC CCA CGA TTA CAA ACC GGA 1266
390D   G   P   Q   P   R   S   Q   L   P   S   M   E   I   R   P   R   L   Q   T   G
  CAT CGG CCG CCG CCA CGT ATG AAG TGG TTT CCG GTT AAA CCG GAA GTT CGG CAT GAT TGT GCA 1329
411H   R   P   P   P   R   M   K   W   F   P   V   K   P   E   V   R   H   D   C   A
  GAT TCT GGC TTC CAA GCC ACA CAA GGA GAA GTC CTA TTG AAA CTG TGT CCC CAG GAG ACG AAC 1393
432D   S   G   F   Q   A   T   Q   G   E   V   L   L   K   L   C   P   Q   E   T   ■
  TGC GAG CAG CGA GAG TTC CGC GCG ATT CGC GCA AAA AAG ACC CGC TGG AGT TCA GCA AAC GTA 1455
453C   E   Q   R   E   F   R   A   I   R   A   K   K   T   R   W   S   S   A   N   V
  AGT ACA ACA CCA CGT ATG TGC GTA GGA TTC CCC GCT TCC AGT CCC AAT TCC GTG TTG GAC GAC 1518
474S   T   T   P   R   M   C   V   G   F   P   A   S   S   P   N   S   V   L   D   D
  TCC AGA AAT GTC TTT ACC TTC ACC ACG CCT AAA TGT GAA AAT TTC CGC AAG AGA TTT CCC AAG 1581
495S   R   N   V   F   T   F   T   T   P   K   C   E   ■   F   R   K   R   F   P   K
  CTG CAG ATC AAG TGC AGC GAC TAG CCTTCTCTGT ATGGCTCAAC GCACTGCTGA CCATGAGATT ACATTAGATT 1655
516L   Q   I   K   C   S   D   *
 GTAAATAATT GTATGTAAAA CTTAACTTTT ACTTATCGTT AGGCCTAAGA ACAAACAACA AGAAACTAAA AAAAAAAAA 1734
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Figure 7.2 The sequence of the *dKAL* cDNA. The sequence is numbered from the 5' end. The predicted amino acid sequence of the long open reading frame is shown underneath the DNA sequence with the single letter abbreviation aligned to the second base of each codon. Bold indicates Cavener's consensus *Drosophila* start site. The four-disulfide-core-domain is underlined. The fibronectin type III domain is also underlined (dashed). The potential N-linked glysosylation sites are outlined.

```
        1                                                                                                  80
 Human- MVPG VPGAV LTLCLWLAAS SGCLAAGPGA AAARRLDESL SAGSVQRAPC ASRCLSLQIT RISAFFQHFQ NNGSLVWCQN
Chick.- MVSERAPGAS LALLLWVTAV SGS PAGPGA ATARRQDEAF S  TA R  C TSRCLSLQIT RISAFFKHFQ NNGSLAWCQN
 Dros.- MGSMQV  AL LAL L VLGQ L FPSAV  A MDPP PIVPP P HPH RISC SDR  SWH T  GSG IAMML RIRS WSC N
        81                                                                                                160
 Human- HKQCSKCLEP CKESGDLRKH QCQSFCEPLF PKKSYECLTS CEFLKYILLV KQGDCPAPEK ASGFAAACVE SCEVDNECSG
Chick.- HKQCSKCLEP CKESWDLKKN HCQSFCEPLF PKKNYECLTS CEFLKYILSV KQGDCPAPEK ASGFAAACVE SCEADSECSG
 Dros.- AW RS VAES HNQSW      TGTV PEQVH PGA F AGT            RAGSCPKIGR QSRARLSCLD NCQYDHECPE
        161                                                                                               240
 Human- VKKCCSNGCG HTCQVPKTLY KGVPLKP RK ELRF.TEL.Q SGQLEVKWS. SKFNISIEPV IYVVQRRWNY  GIHPSE DD
Chick.- VKKCCSNGCG HTCQVPKNLY KGVPLKP RK ELKF.IEL.Q SGDLEVKWS. SKFNISIEPV IYVVQRRWNQ  GIHPSE DD
 Dros.- VQKCCPSSCG PMCVEPLGVR NNTQLPPIPK ILYFRRSRGH AVDLKIESSL LVYYFHVE V RSHIGRHFAA RKLGPWQWQK
      241                                                                                                 320
 Human- ATHWQTVAQT TDERV QLTD IRPSRWYQFR VAAVNVHGTR GFTAPSKHFR SSKDPSAPPA PANLRLANST VNS DGSVTV
Chick.- ATNWQTVAQT TDERV QLSD IRASRWYQFR VAAVNVHGTR GFTAPSKHFR SSKDPSAPPA PSNIRIANIS ANN DGTVNV
 Dros.- VEKYHGENIG HSKHTYIFLH MRPGRWYEVR VAAVNAYGFR GYSEPSDPFP STGNPKPPKC P NDSKI IG KQLMDATV P
        321                                                                                               400
 Human- TIVWDLPEEP DIPVHHYKVF WSW MVSSKS LVPTKKKRRK TTD GFQNSV I LEKL QPD CD YVVELQA ITYWGQTRLK
Chick.- MITWDLPEEP DIPVHHYKVF WSW  TYSKY VIPAKKKRRK ITD GPQNYV V LEGL QPN SN YNVELQA VTRWGQIRLK
 Dros.- LSWCGARPSP TCLSREYKIN WSLQVTVPRL DDYGQSKLRI PTSLKLRMYP ISMQSKCRPY SNWFWYAL S IEQWLMCIKD
        401                                                                                               480
 Human- SAKVSLHFTS THA TNNKE QLVKTRKGGI QTQLPFQRRR PTRPLEVGAP FYQDGQLQVK  VYW K KTE  DPT VNR YH
Chick.- SAKVSLHFST AQD NRNNNE QTSAGKPP K GLVDP YPTF Q RRKPTR F LKIG TPFYQ DNQLQVKV Y WKK TDINMN
 Dros.- GATIGANYTG SVDPGMAIGD GISITIAGLV GRDGP QPRS QLPSMEIRPR LQTGHRPPPR MKWFPVKPEV RHDCADSGFQ
        481                                                                                               560
 Human-  VR  WFPEA CAHNRTTGSE ASSGMTHENY I ILQDLSFS CKYKVTVQ P IRPK S HSK AEAVF FTTP PCSALKGKSH
Chick.- QFQVHSLLES CVHNDTKGLE KVTELTYENY M ILKDLSFS CKYKVTVL P AKSK S RFK AESIFFV TP SCSAFKEKTH
 Dros.- ATQGEVLLKL CPQE TNC E QREFRAIRAK KTRWSSANVS TTPRMCVGFP ASSPNSVLDD SRNVFTFTTP KCENFRKRFP
        561                                                                                               640
 Human- KP IGCLGEA GHVLSKVLAK PENLSASFIV QDVNITGHFS WKMAKANLYQ PMTGFQVTWA EVTTESRQNS LPNSIISQSQ
Chick.- KY INCAAEE VPVLPKVLAK PENLSASFIV QEGNITGHFS WKISKAVLHQ PMTGFQVTWA EVTTESRQNS LPNSIISQSQ
 Dros.- KLQIKCSD*
        641
 Human- ILPSDHYVLT VPNLRPSTLY RLEVQVLTPG GEGPATIKTF RTPELPPSSA HRSHLKHRHP HHYKPSPERY
Chick.- ILPADHYVLT VPNLRPSMLY RLEVQVLTTG GEGPATIKLF RTPDLPPFLP HRPHLKQHHP HHYKPPPEKY
```

Figure 7.3 Multiple alignment of KAL polypeptide sequences from human, chicken (Chick.) and *Drosophila* (Dros.). Amino acid residues conserved among the species are represented in red. The alignment was visualised using the MacVector programme. GenBank accession no. of the Human KAL gene is M97252, and the accession no. of the Chicken KAL gene is L12144.

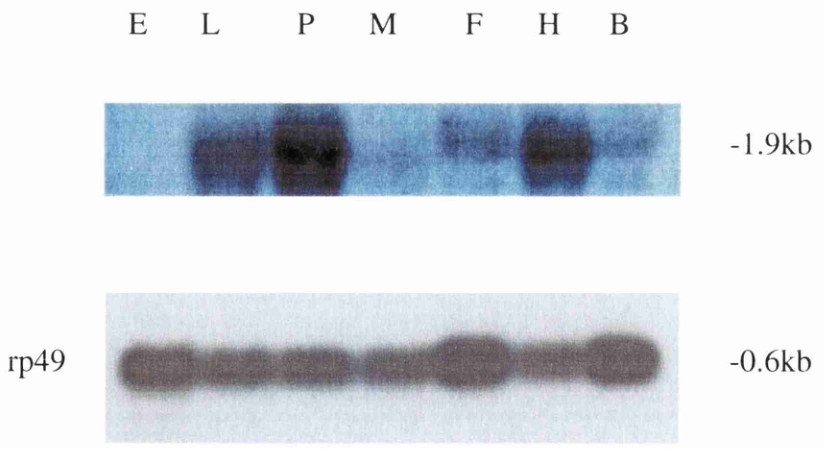

Figure 7.4 Developmental Northern hybridization with a ^{32}P-labelled RNA probe generated from *dKAL*. The RNA electrophoresis and transferring were performed as described in Chapter 2. The RNA samples were from: embryo (E); larva (L); pupa (P); adult male (M); adult female (F); head (H) and body (B). After the hybridization, the filter was stripped and reprobed with *rp49* as a control for differences in RNA loading. Sizes of 1.9kb and 0.6 kb were determined with respect to an RNA size marker (Gibco BRL).

A developmental Northern (Fig. 7.4) of embryo, larval, pupal and adult total RNAs showed that the gene begins its expression from the larval stage. There is hardly any hybridization signal in the embryo. A tissue-based Northern showed that *dKAL* gene expression is elevated in the adult head. No expression difference has been observed between male and female.

7.2.3 Immunohistochemistry

A series of monoclonal antibodies and rabbit immune serum have been raised against the KAL protein purified from transfectant CHO (Chinese hamster ovary) cell clones in Dr. Petit's laboratory (Soussiyanicostas, et al., 1996). They kindly provided us MAbs 9-4, 73-14, 1-4 and the immune serum. An antibody staining was performed on whole mount fly brains. The secondary antibodies (fluorescein-labelled goat anti-mouse IgG and goat anti-rabbit IgG) were applied respectively, followed by confocal immunofluorescence analysis.

The MAbs 1-4 and the immune serum revealed the same labelling patterns (Fig. 7.5). The large cell bodies (4-8mm) lying among the Kenyon cells of the mushroom bodies have been stained strongly. These cells send processes anteriorly, passing the mushroom body calyx. There are arborisations in a region anterior to the calyx within the dorsal-protocerebrum. These neurons may also send projections to the contralateral hemisphere. The region of arborisation is close to a region that receives many projections from central complex and optic lobe neurons. Projections extend frontally through the lateral protocerebrum dorsal to the mushroom body pedunculus. There is no clear evidence of staining in the mushroom body pedunculus. At the frontal margin of the brain, the processes extend to the extreme lateral and dorsal margin of the protocerebrum.

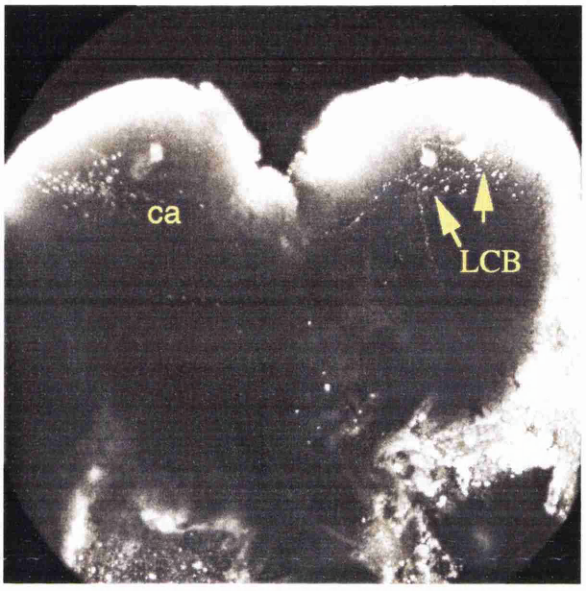

Figure 7.5 Anti-KAL antibody staining of *Drosophila* whole mount brain (from the front) viewed by confocal microscopy (Chapter 2). The staining shows large cell bodies (LCB, 4-8μm) lying amongst the Kenyon cells of the mushroom bodies (MB). These cells send processes anteriorly (frontally), passing the MB calyx (ca). There are arborisations (synapses) in a region just anterior to the calyx within the dorsal protocerebrum. These neurons may also send projections to the contralateral (opposite) hemisphere. The region of arborisation is close to a region that receives many projections from central complex and optic lobe neurons.

7.3 Discussion

The KAL gene in humans is responsible for Kallmann syndrome which is clinically characterised by hypogonadism and an inability to smell (Legouis, et al. 1991). Significant homology was detected in primate (chimpanzees, gorillas, etc.), bovine, rabbit and chicken DNA but not in hamster, mouse or *Drosophila melanogaster* DNA (Franco, et al. 1991).

The analysis of the predicted protein of the KAL gene has revealed the presence of three conserved domains: first, a four-disulphide-core domain (Drenth et al, 1980), described in many protease or ATPase inhibitor activities (Dear and Kefford, 1991). The predicted dKAL protein also contains this domain. The similarity of this domain between these proteins may suggest a related function. This putative inhibiting function might be involved in cell adhesion (Edelman and Crossin, 1991). The adhesion molecules termed "repulsin" modify the shape of the cell (Cox et al., 1990) by way of evoking the action of proteases. Extracellular ATPase is also involved in several adhesion processes (Legouis, et al. 1991), and the cell adhesion molecule CAM105 has been reported to be identical to an ecto-ATPase (Aurivillius et al, 1990).

Secondly, two domains of about 30 amino acids each, both related to part of the fibronectin-like type III (FNIII) domain (Odermatt, et al., 1985), are present in the KAL gene. Significant homology with these two domains was found in some protein phosphatases and kinases known to be involved in cell growth, and in many of the neural cell adhesion molecules which play an important part in axonal path finding by both cell-cell adhesion (Dodd and Jessell, 1991) and neurite outgrowth-promoting mechanisms (Furley, et al, 1990). The predicted dKAL protein only contains one of the FNIII domains.

There are also some other similarities between KAL protein and the deduced dKAL protein. They both contain an N-terminal leader peptide, but no transmembrane helical segment. The potential N-glycosylation sites suggest they are glycoproteins which may be important to the cell-surface membrane . The immune fluorescence result with anti-KAL antibodies showed a strong labelling at the synapses (Fig. 7.5). This agrees with the analysis of the predicted protein, which seems to be a neural cell adhesion molecule. This gene is expressed in cells with larger cell bodies than the Kenyon cells of the mushroom body. The axons of these cells extend symmetrically to both sides of the hemisphere of the brain. The KAL protein is considered to have a specific role in neuronal migration. The phenomenon of expressing of dKAL in the synapses might also be associated with some kind of function in the *Drosophila* brain.

Chapter 8

Discussion and future work

8.1 The brain, behaviour and genes of *Drosophila*

One part of my PhD project was to analyse the sexual orientation of *Drosophila* with the aim of finding out the relevant functional roles of various neurons and/or gene(s). This purpose has been achieved by using the P[GAL4]/UAS_G system to feminise different region of the *Drosophila* brain with ectopic *transformer* expression.

In total, 24 P[GAL4] lines have been tested for courtship behaviour towards both male and female targets. The expression patterns cover several major structures of the *Drosophila* brain, including the mushroom bodies, central complex, optic lobes, antennal lobes and great commisure.

The transformed males of 9 lines out of 14 lines with mushroom body expression patterns showed a certain level of bisexual behaviour, which indicates that the Kenyon cells of the mushroom body play a role in sexual orientation. Mushroom bodies have been invoked as centres for courtship behaviour in other insects (Wahdepul, 1983), and have also been implicated in olfactory processing and learning (Davis, 1993, deBelle and Heisenberg, 1994). Gynandromorph analysis concluded that *Drosophila* mushroom bodies, or adjacent neuropils, were involved in control of the male courtship repertoire (Hall, 1979), a behaviour that relies heavily on olfaction. The non-discrimination behaviour (O'Dell, et al., 1995) of mosaic flies, which were generated by GAL4-mediated *tra* expression in selected areas of mushroom bodies, indicated the flies had lost the ability to distinguish male from female and became equally interested in both. Compared to previous reports (O'Dell, et al., 1995; Ferveur, et al., 1995), the study presented in Chapter 4 shows larger sample sets. The results confirm the role of the mushroom body in mate discrimination and emphasize the importance of olfaction in male sexual orientation. The study of *mushroom body miniature (mbm)* mutants combined with mushroom body ablation revealed that the function of mushroom bodies in mating behaviour is likely to be a "female courtship focus" (O'Dell, et al., 1996). The

feminisation caused by *tra* expression may lead to gain (or change) of function in the male brain, so, the male courters found male targets as attractive as female targets.

Surprisingly, the feminisation of some flies with dominant expression patterns in the optic lobes also displayed a certain level of male-male courtship. This phenomenon in *Drosophila* has not been reported before. Although, one line has faint staining in the antennal lobes besides the optic lobes, it is still possible that neurons in the optic lobes play a role in the mating choice. Recently, the rhythm modulatory neurons which express the *period* gene have been found in the structure of the optic lobe (Meinertzhagen and Pyza, 1996). These results may open up new opportunities to examine the regulation of behaviour in the optic lobe neurons.

By contrast, the feminisation of the other parts of the brain did not show significant bisexual behaviour.

The effect of the mini-*white* gene on male-male courtship has been analysed by mutagenesis of that gene in both P[GAL4] and UAS_G-*tra* constructs. The behaviour tests reached following conclusions: First, mini-*white* does appear to have an effect on male-male courtship, this effect has been observed in one homozygous P[GAL4] line, 201Y. Secondly, the transformation of behaviour from heterosexual to bisexual in P[GAL4] enhancer-trap line 201Y is absolutely determined by *tra* expression, and not some consequence of mini-*white*. The feminization of the mushroom body is responsible for the non-discrimination courtship.

Furthermore, the regulation of genes down-stream of *transformer* in the sexual differentiation pathway has been investigated. In the dorsal brain tissue containing Kenyon cells, of 201Y/UAS_G-*tra* males, the RT-PCR detected both male and female versions of *doublesex* and *fruitless* specific transcripts. These results are further evidence for the phenotype of bisexual behaviour of transformed 201Y males at the

molecular level. For the next step, we would like to see whether this is a consequence of *fru* rather than *dsx* misexpression. Another experiment has been designed by creating a line that expresses GAL4-mediated *tra* in a *dsx* male line. The observation of male courtship behaviour towards both male and female targets will answer the question.

Recently, a second *transformer*-dependent *doublesex*-independent gene involved in sexual behaviour has been described as *dissatisfaction* (Finley, et al, 1997). This gene affects sex-specific courtship behaviours and neural differentiation in both sexes. *dsf* males actively court both mature males and females. So, the gene regulatory consequences of *transformer* expression are more complicated than we imagined at the beginning. Appropriate sexual behaviour may require all the elements to be fully functional.

In *Drosophila*, the genetic control of the sexual behaviour is clearly shown. Added to knowledge of anatomy, physiology and biochemistry, the "black box" between genes and behaviour will be finally opened. There is already evidence for the preservation of gene structures and functions during evolution. Human counterparts have been discovered for a number of genes originally identified in the fly, such as *ether-a-go-go* (*eag*, Griffith, et al., 1994), which participates in the potassium channels and also has an effect on courtship (Griffith, et al., 1993). Within limits, the genetic study of *Drosophila* behaviour should bear some relevance to other creatures (like human beings). From the pure genetic point of view, research on *Drosophila* tells us that most genes underlying the construction of behaviour serve more than one function in the body. Identical genes may also be used for somewhat different purposes. This understanding has improved on the early hypotheses of "single gene control" or "multiple hereditary donations" of human behaviour.

The work presented in this thesis makes a certain contribution to the comprehension both of the genetic control of *Drosophila* sexual orientation, and of the neural structures that

subserve it. There is a need to refine the anatomical story to the level not just of identical cells, but of the functional circuits to which they belong. The vital roles of *fru, dsf* and other downstream genes are also expected to be discovered.

8.2 Molecular analysis

For reverse genetic studies, enhancer-trapping provides a powerful tool to clone genes expressed by specific cell types, rather than searching for genes responsible for certain phenotypes. Proceeding from this point of view, we started DNA analysis of some P[GAL4] lines with specific expression patterns, such mushroom body and central complex. These structures are known to perform some major neurological function related to a fly's behaviour, like learning and memory, courtship and motor behaviour. Ideally, gene(s) involved in these functions could be found by this reverse genetic approach.

Some of the P[GAL4] lines that I used for plasmid rescue are also employed in the behaviour tests. The main reason for choosing these lines is their staining patterns. Secondly, homozygous males of lines c253, c819, c469 and c827 showed higher level of male-male courtship than wild-type flies. Initially, we tried to explain this phenomenon by gene disruption, and so started with DNA analysis. However, in general it appears to be the effect of the mini-*white* gene in the P[GAL4] construct (Chapter 3).

During the study of genomic clones, we noticed that the P-element is likely to occur at some "hot spots" in the genome, which may be surrounded by repetitive sequence (Karpen and Spradling, 1992). When short DNA fragments next to the insertions were used as probes for genomic library screening, usually, some clones would be picked up by the similarity of these repetitive sequences, but not the real flanking genomic regions.

I happened to find identical clones in the analysis of different lines with different chromosomal sites of insertion.

As the AMPK γ-subunit cDNA has been cloned and sequenced, many further experiments need to be continued. To map the precise position of the gene in the genome and find the potential intron/exon boundaries, the corresponding genomic DNA should be sequenced completely.

In line c819, there is no abnormal phenotype observed in the homozygotes as the direct result of P-element insertion. The reason is because the P[GAL4] element did not insert into the gene, there is 7kb distance between them. In order to address the function of the AMPK γ-subunit gene in *Drosophila*, it is useful to make mutants. First, 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 gene. Alternatively, flanking deletions can be generated so as to produce true nulls (Tsubota and Schedl, 1986; Selze et al., 1987). Both methods for gene disruption take the advantage of the controlled mobility of the P-element.

On the other hand, the rescued plasmid pools for the P[lacW] recessive lethal insertion lines on both the second and the third chromosomes is now available in the lab (Guo, et al., 1997; Y. Guo and M. Yang, personal comm.). The three cDNAs, AMPK γ-subunit, *dKAL* and MAPK p38 *Drosophila* homologue were used to the screen these plasmid pools. One line for the AMPK γ-subunit gene (l(3)K111611), and one line for the MAPK p38 *Drosophila* homologue (l(3)K70215) have been isolated. Detailed works are ongoing. Hopefully, the mutant of the AMPK γ-subunit will provide more information for the study of this gene. The P[lacW] insertion for chromosomal site 95E will give some reference to the function of the MAPK p38 *Drosophila* homologue. Because *dKAL* and MAPK p38 *Drosophila* homologue neighbour each other, l(3)K70215 could be a good start for local jumping or excision experiments for the *dKAL* gene. As human KAL

gene mutations have a phenotype of anosmia and nystagmus, it would be interesting to find out what mutation in this gene can do in *Drosophila.*

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Appendix

Table A-1 Courtship toward males performed by 201Y, UAS_G-*tra*, and 201Y/UAS_G-*tra* with different dosage of mini-*white*.

Courting males	CI (%)	SE (CI)	SAP (%)	SE (SAP)
201Y/201Y	28.43	7.75	7.87	3.35
201Y*/201Y	10.00	2.73	0.90	0.30
201Y*/201Y*	8.03	2.50	0.90	0.31
UAS_G-*tra*/UAS_G-*tra*	6.47	3.40	1.17	0.89
UAS_G-*tra**/UAS_G-*tra*	6.60	2.69	1.00	0.49
UAS_G-*tra**/UAS_G-*tra**	4.80	2.07	0.40	0.20
201Y/UAS_G-*tra*	35.27	8.17	7.97	1.77
201Y*/UAS_G-*tra*	32.57	7.44	7.50	2.52
201Y/UAS_G-*tra**	30.67	7.79	8.87	3.66
201Y*/UAS_G-*tra**	29.40	5.79	7.97	3.10
Canton-S	4.33	1.91	0.93	0.65

Table A-2 Courtship behaviour of 201Y and 201Y* toward males and virgin females.

Courting males	Targets	CI (%)	SE (CI)	SAP (%)	SE (SAP)
201Y	males	31.92	11.67	13.08	6.27
201Y	virgin females	60.58	13.85	31.92	11.54
201Y*	males	12.93	4.82	2.30	0.86
201Y*	virgin females	69.17	9.77	20.60	5.14

Table A-1 and Table A-2 are the raw data described in Chapter 3. CI Stands for the courtship index (percentage of time spent courting); SE stands for standard error; SAP stands for sex-appeal parameter (percentage of time spent wing-vibrating).

Table A-3 List of primers used for RT-PCR reactions described in Chapter 3.

Primers	Sequences (5'-3')	Sex-specific transcripts*
fc	GGAAATCGTCTCGAAGTAGGAC	in both male and female *fru*
fm	TGCATTACGCGGCCTTGGACTT	in male *fru* only
ff	GGGAATTCGAGGACGTGTGACGAT	in female *fru* only
dc	AGGTGGTAGGTCATCGGGAACATC	in both male and female *dsx*
dm	ACGTTGCGATACTGCTACGTGG	in male *dsx* only
df	CCTAGTTTTCTTCTCGATCCCCCTTTG	in female *dsx* only

* These primers correspond to different sex-specific transcript regions of *fru* or *dsx*.