Genetic Analysis of G protein-coupled Signalling Pathways in *Drosophila melanogaster*

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

Summary

The aim of this project is to investigate the function of selected cloned genes primarily expressed in the adult nervous system of *Drosophila melanogaster*. To this end a reverse genetic approach of site-selected mutagenesis was undertaken. A number of genes were chosen as targets for mutagenesis and P element insertions were obtained in three genes encoding two classes of G protein alpha subunit ($DG\alpha_i$ and $DG\alpha_o$; Provost *et al.*, 1988; Yoon *et al.*, 1989) and the gene encoding a catalytic subunit of cAMP-dependent protein kinase (DC0; Kalderon and Rubin, 1988). Strains containing P element insertions were homozygous viable and presented no discernable phenotype. The P element insertion in the gene encoding $DG\alpha_o$ (*dgo*) was mobilised during a secondary mutagenesis in an attempt to create deletions via imprecise P element excision. A variety of alterations were detected in the vicinity of the original P element insertion in the *dgo* locus by Southern analysis, but the majority of these had no phenotypic consequence. Eight lines had a recessive lethal phenotype, and four exhibit embryonic lethality. This lethal phase is consistent with a subsequent report that mutations in the *dgo* gene result in embryonic lethality with associated defects in the developing embryonic central nervous system (Guillén *et al.*, 1995). Complementation analysis between the recessive lethal lines show that the mutations do not fall into the same complementation group. Two lines, 273 and 459, with embryonic lethality have had a deletion of DNA corresponding to the *dgo* coding region and are therefore null mutations in the *dgo* gene. In addition to having a mutation in the *dgo* gene, 273 and 459 also fail to complement two other recessive lethal mutations that map to the same chromosomal location. Similar to *dgo*, *longitudinals lacking* (*lola*; Seeger *et al.*, 1993) and *bumper-to-bumper* (*btb*; Kania *et al.*,

1995), are both required in the developing embryonic nervous system. Thus, 273 and 459 have large deletions of flanking DNA that encompass at least two additional essential loci.

The functional requirement of *dgo* during the embryonic stage of development makes it difficult to assess the role *dgo* may play in the adult nervous system. Taking advantage of the fact that DGα_o is the sole substrate for pertussis toxin (PT) in *Drosophila*, flies were transformed with a pertussis toxin transgene within a GAL4-responsive vector, pUAST. PT transformants were crossed to strains that express GAL4 to activate expression of the PT transgene. The PT transgene is expressed and active, since it is able to perturb development in some GAL4 lines. Although experiments with the PT transgene described in this study are preliminary, PT transformants will provide a means to dissect the role of the *dgo* gene in specific sub-domains of the adult central nervous system, and at different developmental stages.

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ABBREVIATIONS

Chemicals

ATP	adenosine triphosphate
DNA	2' deoxyribonucleic acid
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
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
MOPS	3-morpholinopropanesulfonic acid
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
Tris	Tris (Hydroxymethyl) aminomethane
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Measurements

bp	base pair
Ci	Curies
cpm	counts per minute
°C	degrees Centigrade
g	grammes
g	centrifugal force equal to gravitational acceleration
hr	hours
kb	kilobase paris (10^3 bp)
kDa	kilodalton (10^3 dalton)
l	litres
mg	milligrammes
min	minutes
ml	millilitres
M	Molar
mM	millimolar

µM	micromolar
ng	nanogrammes
nm	nanometres
nmol	nanomoles
OD	optical density
pfu	plaque forming units
pH	acidity [$-\log_{10}$(Molar concentration of H^+ ions)]
pmol	picomoles
rpm	revolutions per minute
sec	seconds
U	units
µCi	microCuries
µg	microgrammes
µl	microlitres
V	volts
vol	volume
W	watts

Miscellaneous

cDNA	complementary DNA
ORF	open reading frame
DNaseI	deoxyribonuclease I
mRNA	messenger RNA
RNase A	ribonuclease A
UV	ultra violet light

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Chapter 1

Introduction

1.1 Analysis of nervous system function in *Drosophila*

Experimental analysis of the nervous system in any higher eukaryotic organism constitutes a major challenge because of its complex function. This task is made less daunting in *Drosophila melanogaster* since its brain, although comprising around 200,000 neurons, is a great deal less complex than the mammalian brain. The nervous system in the fly does however have several characteristics in common with vertebrate nervous systems. For example, *Drosophila* has a well developed sensory system including relatively advanced visual, olfactory and gustatory systems. In addition, flies exhibit intricate courtship behaviour and have the capacity to learn and memorise. A smaller number of neurons in the *Drosophila* head is reflected by a smaller number of RNA species which has been estimated at around 11,000 (Levy and Manning, 1981), compared to the number of transcription units in the human brain at somewhere around 20,000 (Sutcliffe, 1988). In mammals and *Drosophila* the number of genes expressed in the nervous system constitutes a significant proportion of the number of transcripts expressed in the organism as a whole, but few are exclusive to the nervous system. So it would appear neuronal processes have evolved from common strategies and many 'neuronal' genes are the result of alternative RNA splicing or are members of large multigene families (for e.g. see Jan and Jan, 1990; Schaeffer *et al.*, 1989).

Biochemical and molecular studies have shown that neuronal functions from one species to the next are frequently accomplished by molecules that are highly conserved. At the protein level, it has been demonstrated that nearly 50% of the monoclonal antibodies made to the adult *Drosophila* brain cross-react specifically to the human central nervous system, including the spinal cord, hippocampus, cerebellum, and optic nerve (Miller and Benzer, 1983; Hinton *et al.*, 1988). Molecular

characterisation of many of the genes expressed in the nervous system of *Drosophila* have shown them to be highly conserved in structure and function (Buchner, 1991).

1.1.1 *Classical genetics*

The traditional genetic approach to analysing the function of a given gene or particular process in *Drosophila* relies on the availability of mutants. If a particular process is under study, such as oogenesis, sex determination or learning, a screen for mutations affecting these processes can be carried out (Rubin, 1988). Methods used to induce mutations include radiation (X-rays and gamma rays) chemicals (EMS) and transposons (reviewed in Ashburner, 1989). Classical genetic dissection however, is dependent on there being an observable consequence to an alteration in any particular gene. If phenotypic mutations are subtle, they could go unnoticed when screening a large number of individuals that result in a typical mutagenesis experiment. This is likely to be a particular problem when studying such complex structures as the nervous system. Never the less, specific screens have been devised to successfully detect mutations in more subtle features such as circadian rythmns, courtship and learning (for reviews see Dudai, 1988; Hall, 1985). Genetic analysis has also allowed identification of genes involved in the development of the central nervous system (Campos-Ortega, 1988; Doe and Scott, 1988), peripheral nervous system (Ghysen and Dambly-Chaudiere, 1989), eye development, (Tomlinson, 1988; Ready, 1989) and membrane excitability (Ganetzky and Wu, 1986). A number of structural brain mutants have also been isolated where specific structures in the adult nervous system are deleted (Fischbach and Heisenberg, 1984).

When the phenotype of a mutation in a particular gene is known, the molecular cloning of the gene can be achieved using two general approaches. The first involves classical genetic mapping using the breakpoints of deletions or other rearrangements (Lindsley and Zimm, 1992). This can be followed by chromosome walking (Bender *et al.*, 1983), or microdissection of the chromosomal region and the DNA used for construction of a library in bacteriophage lambda (Pirrotta, 1986). In the second approach, the gene is tagged using a transposable element, usually the P-element, which can then be used to aid in the cloning of the gene (Bingham *et al.*, 1981).

1.1.2 *Molecular genetics*

There are some obstacles to using classical genetic screens for mutations affecting the nervous system. One is the difficulty of predicting possible mutant phenotypes as they may cause only subtle morphological or behavioural defects. Moreover, mutations in some genes may be lethal at an early developmental stage, obscuring additional roles in later neural processes. This has prompted many researchers to explore 'reverse genetic' strategies to study genes starting from DNA or protein and going back to the mutant phenotype (Sentry *et al.*, 1994). Thus, genes are frequently isolated based on their pattern of expression, spatially or developmentally, or by virtue of sequence homology to genes expressed in other organisms.

Cloning a gene from its protein

A speculative screen for genes of unknown function that were expressed in the adult fly brain was initiated by Seymour Benzer (Fujita *et al.*, 1982). Using monoclonal antibodies generated to proteins isolated from the

adult brain, the expression pattern of cell- or region-specific proteins was visualised by immunolocalisation. The protein can be purified using the same monoclonal antibody, and from a small region of amino acid sequence, a number of small nucleotide sequences are deduced and used as probes to clone and characterise the corresponding gene. Alternatively, labelled monoclonal antibodies can be used to screen a cDNA expression library where each clone produces the protein it is capable of encoding.

Cloning a gene from its expression pattern

There are a variety of strategies available to isolate genes based on their expression in a specific tissue or at a particular developmental stage. These include differential hybridisation screens, or construction of tissue-specific or subtracted libraries. A differential screen by Levy *et al.*, (1982), was used to specifically select clones that were expressed in head tissue, but not body. Subtraction-cloning methods have been used to prepare a head-minus-embryo library (Palazzolo *et al.*, 1989; Hyde *et al.*, 1990; Lee *et al.*, 1990), and an eye-not-head library (Shieh *et al.*, 1989; Shaeffer *et al.*, 1989). A PCR-based approach can be used to isolate cDNAs present in low abundance or from very small amounts of starting material (Pikielny *et al.*, 1994).

Enhancer-trapping is a more recent and extremely powerful approach to identify genes with tissue specifc expression patterns (O'Kane and Gehring, 1987; see below). An engineered P element vector containing the bacterial β-galactosidase gene (*lacZ*) under the control of a weak promoter is mobilised to new random chromosomal sites. If the P element integrates close to a tissue specific enhancer, the enhancer will regulate *lacZ* transcription in a defined subset of cells which can be visualised histochemically using antibodies against *lacZ* or with the

chromogenic substrate X-gal (Figure 1.6a). Enhancer-trapping has been used to analyse the expression patterns of genes expressed during embryonic development in *Drosophila* (Wilson *et al.*, 1989). Extensive enhancer detector screens have been performed for genes expressed in the developing central and/or peripheral nervous system (Bellen *et al.*, 1989; Bier *et al.*, 1989). Staining patterns are visualised by staining whole-mount embryos, larvae or sections of adult flies. If the P element contains a plasmid origin of replication, sequences flanking the insertion can be rescued and used to isolate cDNA clones (Pirrotta, 1988).

Cloning genes using heterologous probes

Genes that have analagous function in different organisms may share nucleotide or amino acid sequences due to evolutionary conservation. Thus, a DNA sequence specific for a gene of interest can be used as a probe for isolating a homologous gene from another organism. A heterologous probe can also be used to isolate additional members of a gene family within the same organism or to identify genes containing a specific structural motif such as a zinc-finger or homeodomain. A large number of genes that are expressed in the nervous system of *Drosophila* have been isolated using heterologous probes (see section 1.3.2; section 3.1.2). These include genes encoding neurotransmitters, neuromodulators and proteins involved in second messenger systems such as protein kinases and G proteins (see section 3.1.2; for reviews see Restifo and White, 1990; Buchner, 1991)

1.1.3 *Determining the function of a cloned gene*

Once a gene has been cloned, information regarding function can be obtained first with sequence comparisons, and then by analysing

expression patterns using *in situ* hybridisation to sections, or by generating antibodies to fusion proteins derived from the cloned cDNA. From this, one might be able to obtain a great deal of circumstancial information, but a very important step in the functional analysis is the isolation of mutants in the gene. By generating a mutation in an otherwise normal individual, a single gene product is altered and the effect of this particular genetic modification on the phenotype can be examined *in vivo*, in the context of the whole organism. If one is lucky, there will be a strain available containing a transposable element, or a chromosomal deletion spanning the particular chromosomal region. Homozygotes for such mutations, if obtained, can be examined by Southern, Northern or Western blot analyses to look for disruption in the gene, size or abundance of transcript, or loss of protein product. Alternatively, rescue of the mutation by transformation of the cloned gene can prove the mutation disrupts the gene of interest. If no pre-existing mutations or deletions are available, a mutagenesis experiment must be carried out to disrupt the function of the gene of interest.

Disruption can be achieved using 'dominant negative' mutations in which the normal function of the wild-type gene product is altered by the expression of a modified gene copy introduced using germline transformation (Drain *et al.*, 1991; Griffith *et al.*, 1993). If an antibody to the gene is available, mutant flies can be screened for the loss of an epitope on Western blots or in embryos or tissue sections (Van Vactor, *et al.*, 1988; Dolph *et al.*, 1993). At the level of RNA, antisense RNA strategies can be used to produce 'phenotypic mutants' where the target gene mRNA is either degraded when complexed to antisense RNA, or is blocked at translation. In *Drosophila*, antisense RNA can be provided either by direct injection into the embryo or by expression of the antisense

gene integrated in the genome of the fly (Rosenberg, 1985; Qian *et al.*, 1988). By regulating the expression of the antisense RNA by an inducible promoter it is possible to assess the phenotypic effects at any stage in the *Drosophila* life cycle. However, antisense RNA strategies are generally ineffective. Another approach to gene inactivation is the use of catalytic antisense RNA, or 'ribozymes', that act specifically to cleave the target gene transcripts (Heinrich *et al.*, 1993; Zhao and Pick, 1993).

The following sections describe methods available to create mutations in a gene of interest that result from manipulation of the P element, including insertion or deletion mutations, or by targeted gene replacement (Kaiser *et al.*, 1995).

1.2 P elements in *Drosophila*

1.2.1 *P element biology*

The P element family of transposable elements in *Drosophila melanogaster* is responsible for the P-M system of hybrid dysgenesis, a syndrome of abnormal germline traits which includes chromosomal rearrangements, male recombination, high mutability and temperature sensitive gonadal sterility (Engels, 1989). Hybrid dysgenesis is germline specific and occurs only in the progeny of a cross between females devoid of P elements (M strains) and males which carry P elements scattered throughout the genome (P strains). In the progeny of the reciprocal cross, as well as in the progeny of P and M strains themselves, no dysgenesis occurs. P strains contain two types of P elements: autonomous (full-length) elements of 2.9 kb that encode their own transposase, and non-autonomous (defective) elements that result from internal deletions of various sizes (O'Hare and Rubin, 1983). Transposition in P strains, which

contain both full-length and defective P elements, is tightly regulated and is repressed by a product of the full-length P element. This state is known as 'P cytotype'. Since most, if not all of the P elements within M strains are defective, they lay eggs that are permissive for P element transposition ('M cytotype'). Thus, transposition and hybrid dysgenesis can only occur when active P elements are introduced into the permissive environment provided by the female parent.

Molecular analysis of P elements shows that they are diverse with respect to size, but display almost no sequence variation (O'Hare and Rubin, 1983). Full-length elements have four open reading frames encoding an 87 kDa transposase (Figure 1.1; Karess and Rubin, 1984). The germline specificity of transposition is achieved by an alternative splicing of the third intron which occurs exclusively in cells of the germline (Laski *et al.*, 1986). An engineered P element with the third intron removed (Δ2,3) is able to transpose in somatic cells, and cannot establish P cytotype (Laski *et al.*, 1986). The P element sequences required in *cis* for transposition comprise 138 bp at the 5' end and 150 bp at the 3' end and includes the 31 bp inverted repeat (Mullins *et al.*, 1989). Defective P elements derived from the larger element by internal deletion are unable to produce a functional transposase, but can transpose if they retain parts of the sequence required and transposase is provided in *trans* (see Figure 1.1).

1.2.2 *P element transposition*

Until only recently, it was unclear if P element transposition in *Drosophila* occurred by a replicative mechanism or nonreplicative mechanism. In favour of the nonreplicative mechanism, precise loss of the transposon had been observed, albeit rarely. However, interpretation was complicated by the failure to correlate the frequency of P element loss

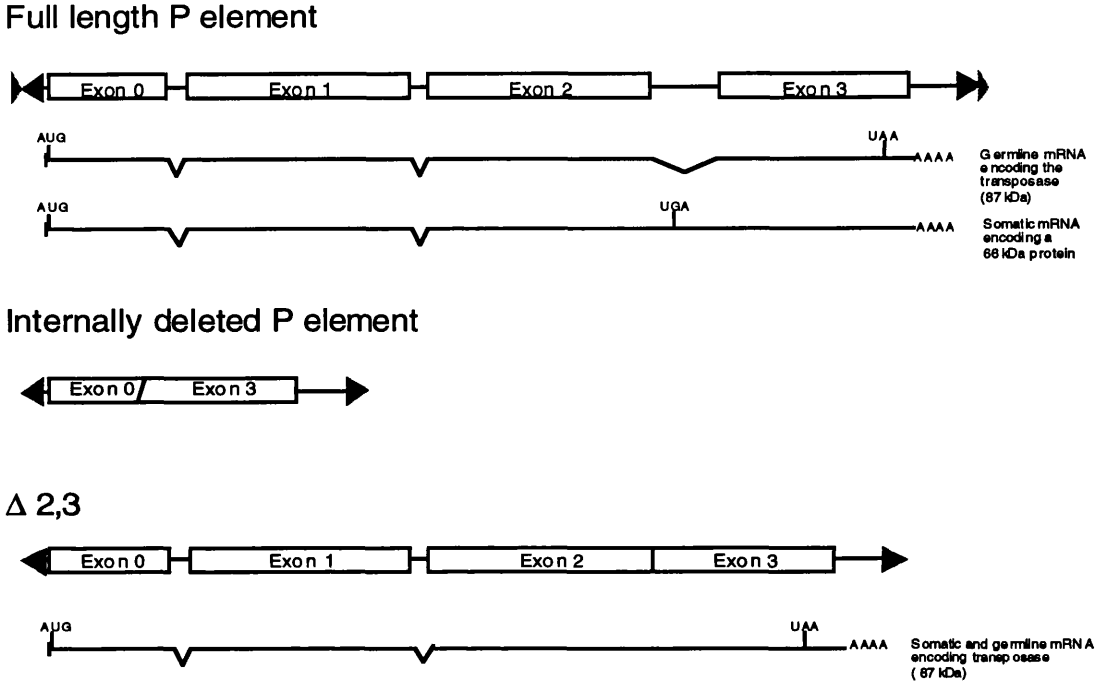


Figure 1.1 P element structure.

Full-length P elements of 2.9 kb contain four ORFs separated by introns. The terminal 31 bp inverted repeats are indicated by large arrowheads, and the 8 bp target site duplication are indicated by shaded arrowheads. The germline transcript is spliced to provide functional transposase. Somatic transcripts retain the 2-3 intron and encode a prematurely truncated, non-functional transpoasase. Internally deleted P-elements do not produce transposase, but can be mobilised. The Δ2-3 element has been modified in vitro to remove the third intron, and so produces transposase in both germline and somatic tissues (Diagram taken from Sentry and Kaiser, 1993).

with the rate of transposition to new sites. In an elegant series of experiments, W.R. Engels and colleagues addressed this problem using various alleles of the X-linked *white* gene combined with a P element induced *white* allele (w^{hd}). They measured the rate of precise P excision using the eye colour phenotype as a marker. These experiments indicated that a high frequency of precise excision occurs when a homologous *white* gene is present (Engels *et al.*, 1990). No precise P element loss could be observed when a homologous *white* sequence was absent (males), or if a *white* allele containing a deletion covering the P element site was present on the homologous chromosome. The high frequency of precise P elements excision observed with an allele containing wild-type DNA sequence opposite the insertion point suggested a double-strand break repair model of P element transposition (Figure 1.2). In this model, P element transposition produces a double-strand gap that may be enlarged by exonucleases. The gap is repaired by homologous recombination, using either a template provided by a sister chromatid or less often a homologous chromosome. If a sister chromatid is used for repair, P element sequences will be restored at the locus giving the impression of replicative transformation (0% reversion rate). When the template is a homologue lacking a P element, precise excision will appear to have taken place as a result of gene conversion by gap repair (13% reversion). The model proposed can also be used to explain the origin of internally deleted P elements and other examples of imprecise excision (Figure 1.2). As the gap repair model requires a template for repair, it would be expected that any reduction in the ability of the w^{hd} allele and its homologue to pair would affect the reversion rate. Indeed, a lower rate of precise P element loss was observed when females were heterozygous for w^{hd} allele and an X chromosome containing multiple rearrangements (3% reversion). Finally, the observation that an ectopic copy of the *white*

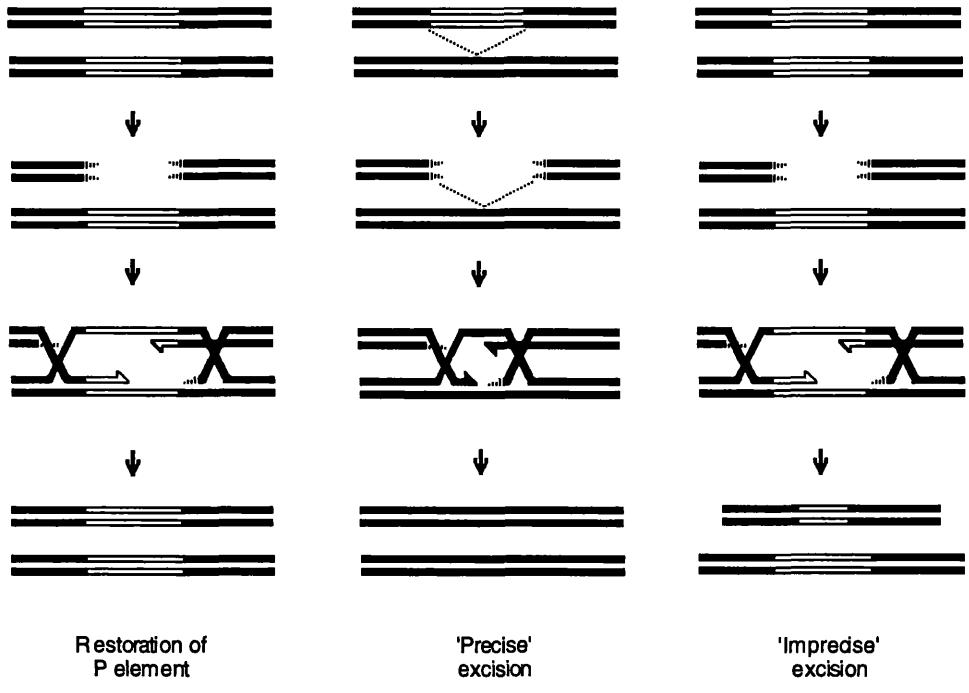


Figure 1.2 Model for template-dependent gap repair following P element excision.

Excision of a P element (open bars) induces a double-strand break that can be subject to widening by exonucleases. Free 3' ends invade the template duplex, which serves as a substrate for DNA synthesis. In the left panel, the template is a second copy of the P-induced allele, most commonly provided by a sister chromatid. The result is restoration of a P element at the locus. Less frequently, the template can be a wild-type allele present on a homologous chromosome (centre panel). This will give the impression of precise excision. Interruption of the repair process, in this case where the sister chromatid is the template, followed by pairing of partially extended 3' ends, may give the impression of an 'imprecise excision' (right panel). This can take the form of internal deletion of the P-element, or more extremely a deletion that extends into flanking DNA, usually when the template is a wild-type allele present on a homologous chromosome (Diagram taken from Sentry and Kaiser, 1993).

gene elsewhere on the X chromosome could act as a template for repair highlighted the potential of this mechanism to direct targeted changes to the genome (see section 1.2.9).

1.2.3 *Germline transformation*

The use of P elements as vectors to insert specific DNA segments into the *Drosophila* germline has revolutionised the study of gene regulation and function in *Drosophila* (Rubin and Spradling, 1982). In germline transformation, the gene under study is placed between P element ends contained within a plasmid, and injected into pre-blastoderm embryos. If injected P element DNA is internalised within germline precursor cells during cellularisation, the integrated DNA can be inherited by the progeny of surviving transformed flies (Figure 1.3). The P element vector usually contains a marker that allows identification of transformants, such as the eye colour genes *rosy* or *white* (Pirrotta, 1988). As the P element vector is essentially a defective P element, a transposase source must be provided. Purified transposase protein can be co-injected with plasmid DNA (Kaufman *et al.*, 1991), or a 'helper' plasmid can be co-injected, such as a *wings-clipped* element that produces transposase but which cannot itself integrate into the genome (Figure 1.3; Karess and Rubin, 1984). Alternatively, plasmid DNA can be injected into embryos that have an endogenous transposase source, such as P[$ry^{+}\Delta 2,3$](99B) (Robertson *et al.*, 1988). A dominant marker on the $\Delta 2,3$ chromosome allows stable transformants lacking the transposase gene to be selected among the progeny. The frequency of transformation is related to the size of the inserted sequence: the larger the insert the lower the frequency. However, large inserts of around 40 kb have been successfully transformed (Haenlin *et al.*, 1985). In addition, the specificity of insertion

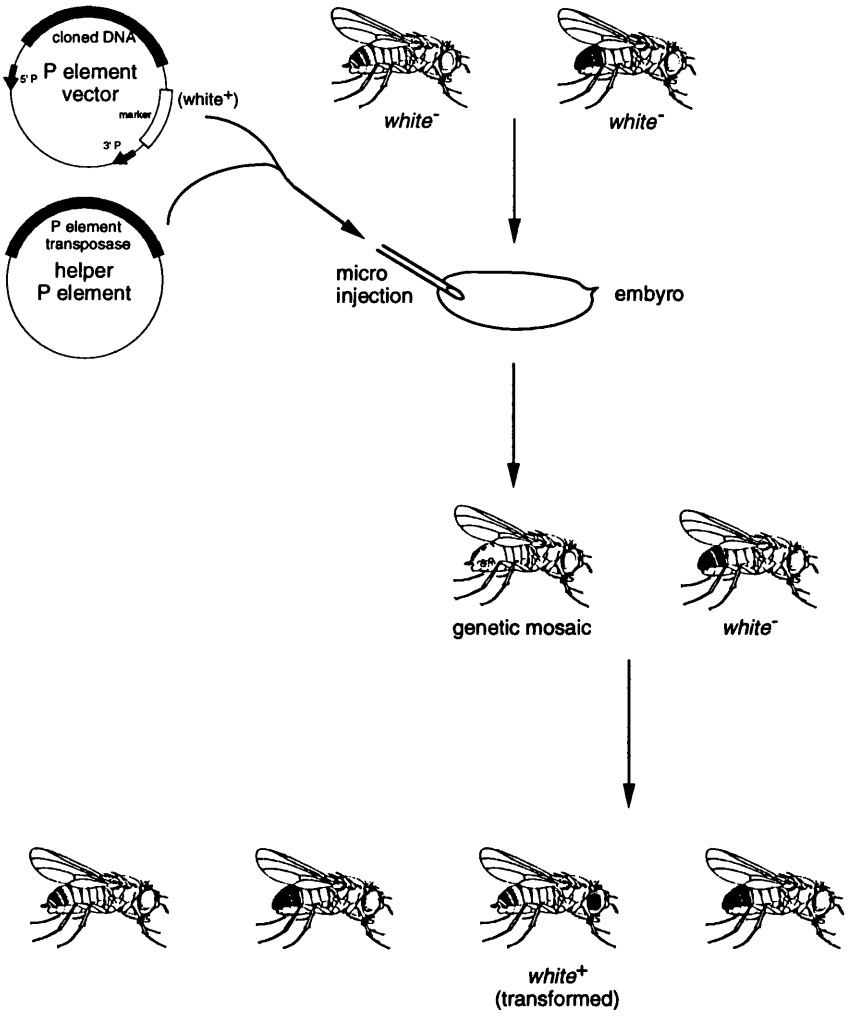


Figure 1.3 Germline transformation.

A plasmid construct containing a DNA sequence within P element ends, is injected into embryos prior to the cellularisation of the germline. The transgene contains a marker gene that enables transformants to be recognised. Transposase can be supplied by co-injection of a helper element that produces transposase (shown), by co-injection of purified protein, or by injecting Δ2-3 embryos that express transposase endogenously. If injected DNA is incorporated into pole cells prior to cellularisation, transposition of the transgene to the *Drosophila* genome may occur. Adults that develop from injected embryos will contain the transgene in the germline, but a further generation is required before transformed individuals can be recognised. (Diagram taken from Sentry and Kaiser, 1993).

can be influenced by sequences contained within the P element (Kassis *et al.*, 1992).

A disadvantage associated with P-mediated transformation is the position effect of the DNA surrounding the insertion site on the expression of the transformed gene. It is advisable to isolate several lines containing independent insertions that can be obtained as primary transformants, or generated by remobilisation of a construct with Δ2,3. The expression of the marker contained within the P element is often sensitive to position effects and can be used as an indicator of the level of expression of the transgene.

Germline transformation methods have had a major impact on *Drosophila* molecular genetic experiments. DNA can be inserted into a P element vector and used to rescue a mutant phenotype, providing evidence that the cloned DNA corresponds to the mutation. The temporal and spatial activity of regulatory sequences can be examined by fusing promoter or enhancer sequences to a reporter gene (Serano *et al.*, 1994). A gene of interest such as a toxin gene or peptide inhibitor can be expressed under the control of an inducible promoter (Drain *et al.*, 1991; Fitch *et al.*, 1993).

1.2.4 *P element mutagenesis*

There are two instances when a P element mutagenesis may be required: when a gene is known only by the phenotype of its mutations, or when mutations are required in a gene known only by its DNA sequence. In both cases, mutations in the gene can be provided by P element mutagenesis.

When the phenotype of a mutation is known, new mutant alleles can be created by a P element insertion which allows cloning by transposon 'tagging'. In a typical P element mutagenesis experiment, P-strain males are mated to M-strain females, resulting in P element transpositions in the germline of their progeny. The progeny are bred and their offspring are screened for a new mutation in the gene of interest (Figure 1.4). A library can then be constructed from the new mutant strain and screened with a P element probe. Hybridising clones should contain genomic DNA flanking the gene of interest.

A more efficient and controlled mutagenesis can be performed using engineered strains containing stable transposase producing elements and defective, but mobile P elements that serve as 'ammunition' (Figure 1.5; Cooley *et al.*, 1988a). The P elements are provided by a Birmingham 2 strain (*Birm*-2) that contains 17 defective P elements on each of its second chromosomes (Engels *et al.*, 1987). This strain is crossed to the P[*ry*$^{+}$Δ2,3](99B) strain containing a P element that provides a high level of transposase activity, but is itself extemely stable (Roberston *et al.*, 1988). When ammunition and transposase come together in the germline cells of the F1 progeny, transposition of the P elements occurs. The P[*ry*$^{+}$Δ2,3](99B) element is integrated close to the dominant eye marker, *Dr*, allowing selection against the transposase in subsequent generations. One problem encountered in this mutagenesis is the reduced viability of dysgenic individuals due to the somatic transposase activity of the Δ2,3 combined with the defective P elements (Robertson *et al.*, 1988). The problem can be overcome by performing the cross at a reduced temperature of 16^{o}C.

Using this mutagenesis scheme, Robertson *et al.*, (1988), estimated that about 10 new insertions could be obtained per mutant genome. Since the

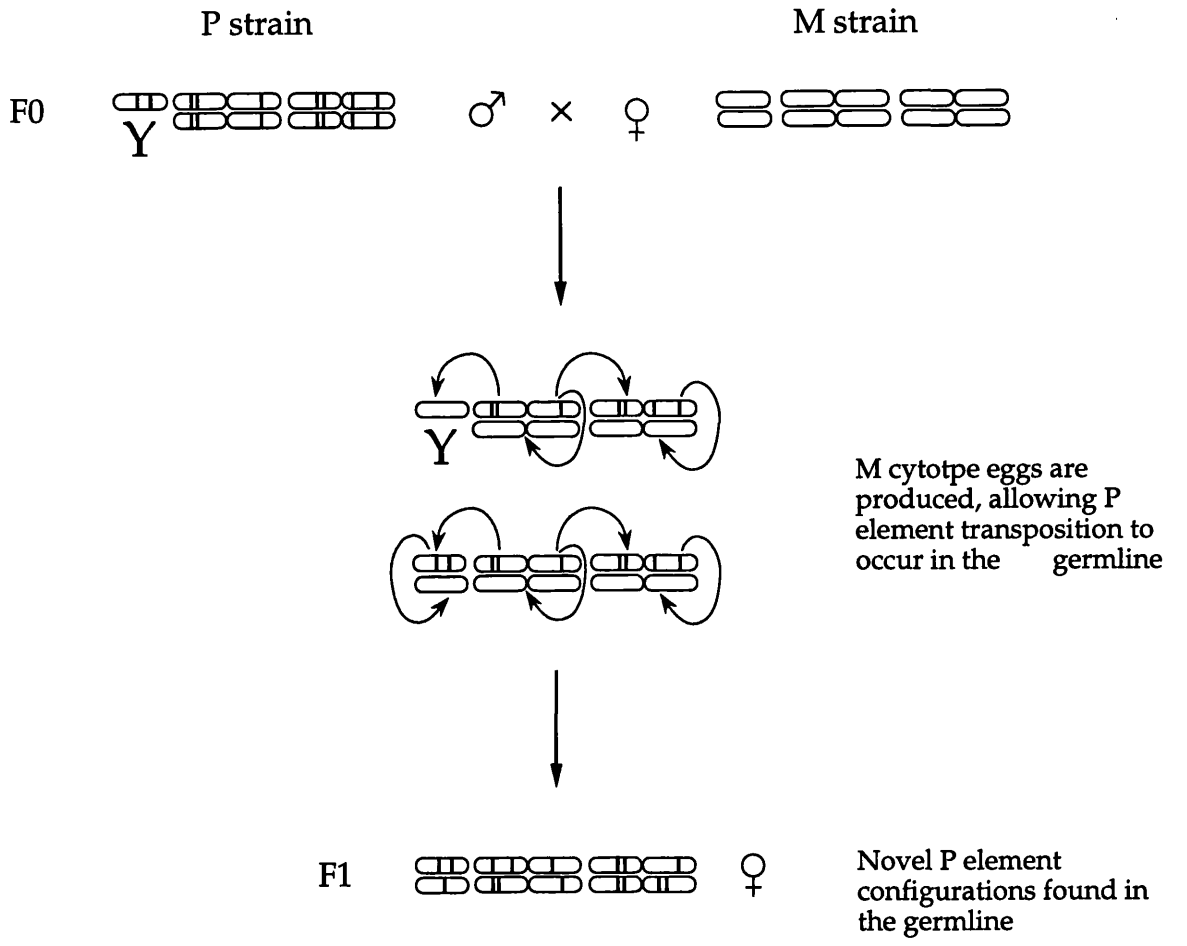


Figure 1.4 Standard P element mutagenesis.

P-strain males and M-strain females are mated. The fertilised eggs are of M cytotype, providing a background in which P element transposition can occur in the developing germline. Each germ cell contains a new configuration of P elements and the phenotypic consequences of these can be assessed in subsequent generations. (Diagram kindly provided by J. Sentry).

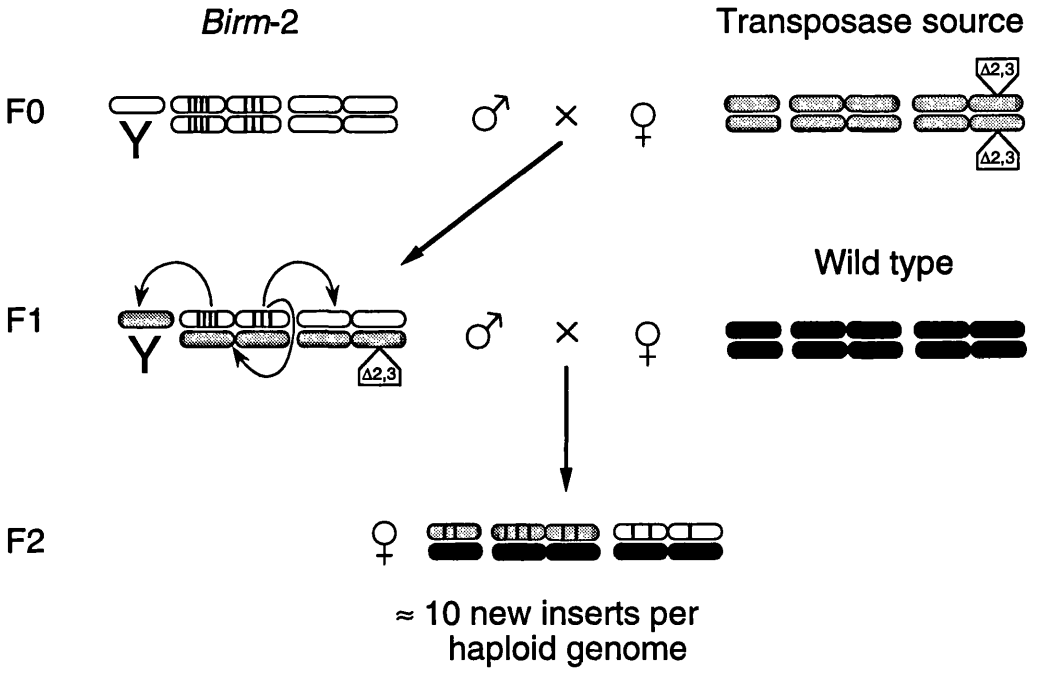


Figure 1.5 A controlled mutagenesis strategy.

A *Birm-2* strain with 17 defective P elements on each of its second chromosomes, is mated with a strain containing the Δ2,3 element. When ammunition and transposase come together in the germline cells of the F_1 progeny, transposition occurs. The germline of both male and female F_1 have a different spectrum of new insertions. Selection against the transposase source in the F_2 generation ensures that new insertions are stable.

Drosophila genome is approximately 1.65 x 10^5 kb, the production of 10,000 mutagenised females would give an insertion approximately every 1.5 kilobases along the genome and a reasonable chance of a hit in a particular gene. It cannot be guaranteed that a P element will be recovered in any particular target gene since P element insertion is non-random. The mutation rate varies greatly from one locus to another, ranging from 10^{-2} to 10^{-7} of all progeny (Kidwell, 1987; Engels, 1989).

As an alternative to defective P elements as ammunition, P elements containing a visible marker such as the *rosy* eye colour gene can be used (Cooley *et al.*, 1988b). The advantage of using a single marked P element is that subsequent analysis, such as screening for excision mutants, is greatly simplified. However, obtaining such insertions requires more effort: The transposition frequency of larger marked elements is reduced relative the the smaller elements in *Birm2*, and they are present at only one to a few copies per genome.

1.2.5 *Detection of P element insertions*

Many genes cloned from *Drosophila* are isolated on the basis of DNA sequence homology to related genes in other organisms. Since it is difficult to predict for many genes what the mutant phenotype will be, other strategies are required to detect mutations. A number of approaches based on molecular rather than phenotypic criteria are available to detect P element insertions in or near target genes.

In site-selected mutagenesis, the detection of a P element insertion is achieved using the polymerase chain reaction (PCR) (see section 3.1; Ballinger and Benzer, 1989; Kaiser and Goodwin, 1989). A primer based on target gene DNA sequence is used in conjunction with a primer

specific to P element sequences to amplify DNA between a gene and a newly inserted transposon. Gene specific primers are usually designed to detect insertions at the 5' region of the gene where P elements preferentially insert. In addition, a primer based on the 31 bp terminal inverted repeat of the P element will detect P element insertions in either orientation. The sensitivity of PCR makes it possible to detect a novel P element insertion in DNA isolated from a population of flies, even if it is present in only one fly per thousand. Taking advantage of this, DNA is extracted from a pool of potential mutants and amplified with the gene specific primer and P element primer. Since DNA is prepared from the eggs laid by F2 individuals (see Figure 1.5), a population containing a fly with the desired P element insertion can be subdivided sequentially until the individual female is identified.

An alternative detection strategy to detect P element insertion is inverse PCR (Ochman *et al.*, 1989; Dalby *et al.*, 1994; Sentry and Kaiser, 1994). In this approach, pools of genomic DNA from mutagenised individuals is digested and circularised so that sequences flanking the insertion are amplified. Another procedure known as plasmid rescue, uses engineered P elements containing a bacterial origin of replication and antibiotic resistance (Hamilton *et al.*, 1991). Plasmid DNA prepared from a mutagenised population of flies will contain DNA sequence flanking the P element insertion site. In both inverse PCR and plasmid rescue, captured flanking DNA sequences can be hybridised with specific cDNA or genomic clones to identify an insertion in or near the gene of interest.

1.2.6 *Local jumping*

Several recent observations suggest that mobilised P elements often jump to nearby regions of the genome, within a range of a hundred kilobases or

so from the donor site (Tower *et al.*, 1993). Moreover, the rate of local transposition appears to be much higher in the female germline (Zhang and Spradling, 1993). This phenomenon can be exploited using the ever increasing number of strains available from laboratories and stock centres with single P element insertions at a defined chromosomal location. Local jumping, in combination with site-selected mutagenesis, has been used to successfully target a gene encoding the synaptic vesicle protein, synaptotagmin (Littleton *et al.*, 1993a).

1.2.7 *Precise and imprecise excision*

Remobilisation of a P element from an existing site can lead to precise or imprecise excision of the element, deletions or chromosomal rearrangements. Reversion of a P-induced mutation by precise excision can unambiguously demonstrate that an associated phenotype is the consequence of a transposon insertion. Imprecise excisions can involve loss of sequences internal to the P element, or loss of the entire P element and DNA sequence flanking the transposon. When P elements transpose, they leave behind a double-strand break that can be enlarged by exonucleases (Engels *et al.*, 1990; Sentry and Kaiser, 1993). Imprecise excision is presumed to be the consequence of an aberrant repair event of the enlarged gap resulting in loss of sequences flanking the insertion (see Figure 1.2). Excisions resulting in loss of flanking DNA can be used to generate new alleles of a gene (Tsubota and Schedl, 1986; Salz *et al.*, 1987; Ségalat *et al.*, 1992). Since P elements prefer to insert in the 5' region of a gene, often with no phenotypic consequence, the generation of imprecise excisions may be a necessary step in the analysis of the gene.

1.2.8 *Enhancer-trap elements: the GAL4 system*

As described above, enhancer-trap elements provide a powerful method to search for genes expressed in specific cell types in the nervous system and to subsequently examine their function using genetic analysis (see section 1.1.2; Figure 1.6a). These 'first generation' enhancer-trap elements have been used to extensively examine the development of the peripheral and central nervous systems in *Drosophila* (Bellen *et al.*, 1989; Bier *et al.*, 1989).

A second generation enhancer-trap element has been developed that utilises enhancer detection to express a yeast transcriptional activator GAL4 in specific cells and tissues (Brand and Perrimon, 1993). The GAL4 transcription factor, which was shown to function in *Drosophila* (Fischer *et al.*, 1988), is used to activate the expression of a transgene under the control of a GAL4-dependent promoter (UAS_G) (Figure 1.6b). The GAL4 protein and its target gene are initially present in two distinct transgenic flies. When the two lines are crossed, the target gene will be expressed in a pattern that reflects GAL4 activity. Expression patterns can be visualised by crossing a strain containing a new GAL4 insertion with a fly containing a UAS_G-*lacZ* construct. The GAL4 system is being used to examine many aspects of nervous system function. Since GAL4 activated *lacZ* expression results in β-galactosidase expression in the cytosol, specific neurons and their projections can be visualised. This feature is proving extremely useful in generating three-dimensional reconstructions of the adult brain (Yang *et al.*, 1995), and analysing the structure of neurons in the peripheral nervous system (Smith and Shepherd, 1996). The GAL4 system as a means to misexpress genes in specific cell types and disrupt processes such as cell fate determination and courtship behaviour (Halder *et al.*, 1995; O'Dell *et al.*, 1995). GAL4

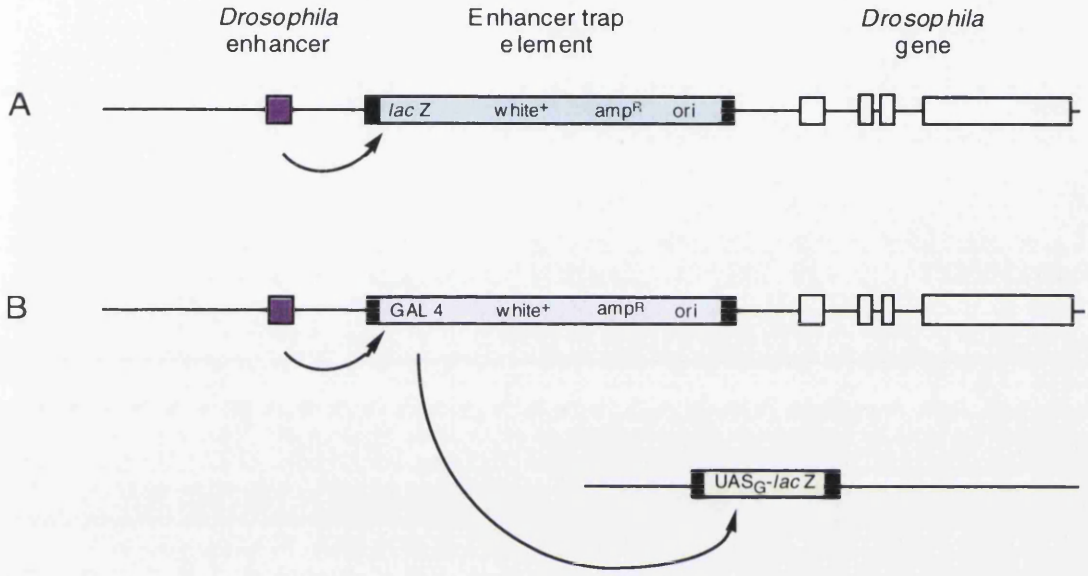


Figure 1.6 Enhancer trapping.

(A) First generation enhancer trap element. After integration into the genome, the reporter may be influenced by nearby genomic enhancers, leading to enhancer-specific regulation of β-galactosidase expression. The enhancer trap element has a dominant eye colour gene (w^+) which allows flies with insertions to be recognised. A bacterial origin of replication (*ori*) and antibiotic resistance (amp^R) facilitate plasmid rescue of flanking sequences. (B) A GAL4 enhancer trap element. As above, except that GAL4 expression is influenced by a nearby enhancer. GAL4 expression then activates transcription from a second reporter gene linked to the GAL4 responsive promoter, UAS_G.

targeted expression of toxin genes has been used to ablate specific cell types required for axon outgrowth (Hildalgo *et al.*, 1995), and to disrupt the function of proteins required for synaptic transmission (Sweeney *et al.*, 1995).

1.2.9 *Targeted gene replacement*

The experiments by Engels *et al.*, (1990) described above highlighted the potential of the double-strand gap repair model as a basis for targeted alterations to the genome (Sentry and Kaiser, 1992). The suggestion that gene conversion was possible was confirmed by Gloor *et al.*, (1991), who illustrated that homologous, but slightly altered sequences from an ectopic chromosomal position are able to convert the sequence at the P element insertion. As in the experiments of Engels *et al.*, (1990) the template for conversion was the P-induced w^{hd} allele of the *white* gene. The 'donor' sequence was an ectopic copy of the transgene engineered to contain novel restriction sites. This feature permitted Gloor *et al.*, to follow the conversion tracts up to 2000 bp on each side of the original P element insertion point. In these experiments, the average conversion tract length observed was 1400 bp. More recent experiments have shown that insertions (up to 8 kb in size) and deletions can also be copied into the gap as efficiently as single base pair changes (Johnson-Schlitz and Engels, 1993; Nassif *et al.*, 1994). Although most of the conversion events analysed have been at the *white* locus, gene conversion has also been demonstrated at two sites in the *bithorax* complex (McCall and Bender, 1993).

1.3 G proteins

1.3.1 *G proteins in vertebrates*

In order that cells can communicate with their surroundings, they have developed signal transduction mechanisms in which external information is conveyed through specific membrane receptors and transduced into an intracellular signal. In all eukaryotic systems, a family of GTP-binding proteins (G proteins) play an important role in linking many cell-surface receptors to intracellular effectors (Stryer and Bourne, 1986; Gilman, 1987; Simon *et al.*, 1991). The external signals can be in the form of hormones, neurotransmitters and growth factors, or sensory signals such as light and odourants.

G proteins are heterotrimers made up of α subunits (39-46 kDa), β subunits (35-37 kDa) and γ subunits (8 kDa). The α subunits are the most diverse and have been traditionally used to define the heterotrimeric G protein. The β and γ subunits exist as a tightly bound complex that can be shared among different G protein classes. The receptors that interact with G proteins generally have a characteristic seven transmembrane spanning domain structure and also have considerable amino acid sequence identity (Ross, 1989; Bockaert, 1991). The G protein cycle begins when an external signal, either chemical or physical, stimulates a receptor and produces a conformation change (Figure 1.7). The activated receptor associates with the α subunit and accelerates the exchange of GDP, bound to the α subunit, for GTP. This exchange of GDP for GTP leads to the dissociation of the α-GTP complex from the βγ subunits. The activated state lasts until the GTP is hydrolysed to GDP by the intrinsic GTPase activity of the α subunit, after which α and βγ subunits reassociate and return to the inactive state. A single receptor can activate many G

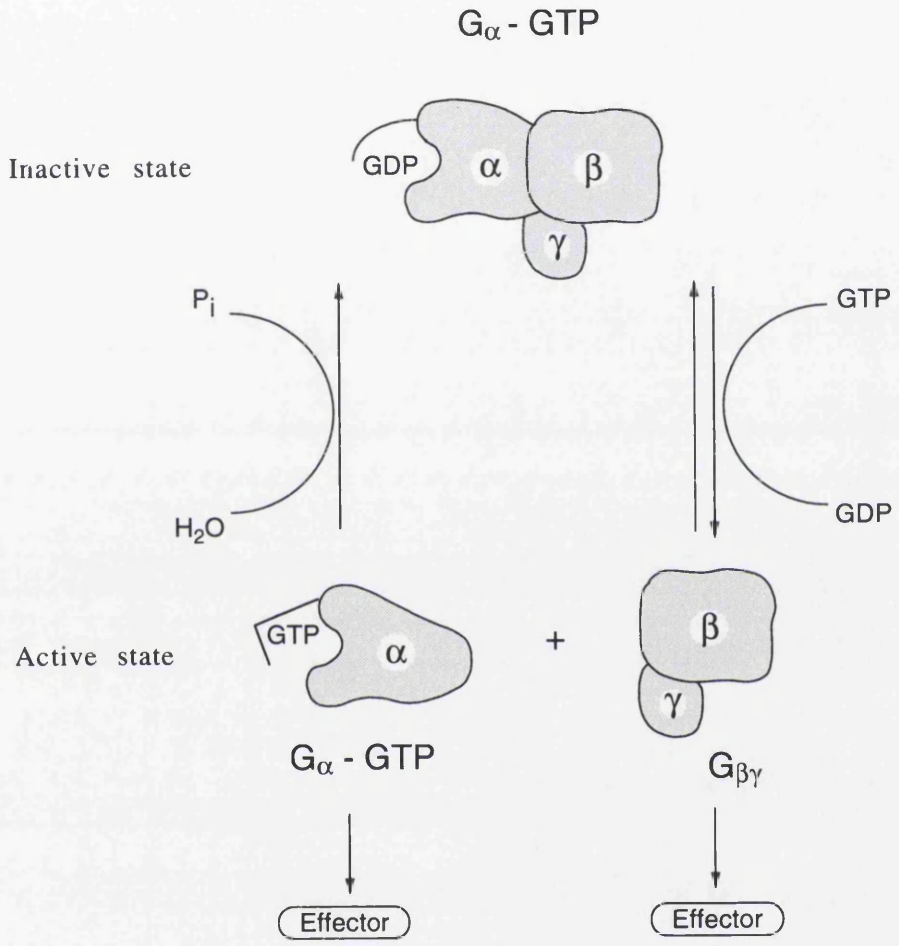


Figure 1.7 The G protein cycle.

The functional state of a G protein is determined by its bound nucleotide. With GDP bound, the G protein is inactive and subunit association is favoured. With bound GTP, the G protein is activated and the affinity between its α and βγ subunits is markedly reduced. Receptors stimulate G proteins by catalysing exchange of GTP for GDP. Activated α subunits (αGTP) and βγ subunits can interact with different effectors.

proteins thus amplifying the initial cell-surface signal. Most of the understanding of this cycle has come from experiments with the β-adrenergic stimulated adenylyl cyclase system and the specialised light-activated cGMP phosphodiesterase pathway in retinal rods. The GTP-bound α subunit of G_s (*s*timulatory) associates with adenylyl cyclase which increases the concentration of the second messenger cAMP (Stryer and Bourne, 1986). The cAMP produced activates cAMP-dependent protein kinase which phosphorylates target proteins within the cell. In the visual system rhodopsin is coupled to the G protein transducin (G_t) which activates a phosphodiesterase that cleaves cGMP and closes sodium channels in the plasma membrane (Stryer, 1991).

α subunits

Cloning and sequencing techniques have led to the discovery and classification of new α, β and γ subunits. So far, at least twenty-one α subunits (seventeen gene products), four β subunits and six γ subunits have been described from a variety of mammalian tissue and cell types (Simon *et al.*, 1991). Using amino acid sequence similarity, α subunits can be divided into four classes; G_s, G_i, G_q and G_{12} (Hepler and Gilman, 1992; Neer, 1995). A summary of α subunits and some of their properties is given in Table 4.1. The amino acid sequence identity between α subunits ranges from 56% to 95%, with those in the same group generally having greater than 70% identity. The α subunits contain regions necessary for interactions with the βγ subunit, receptors and effector. The domains required for GTP-binding and GTPase activity are particularly highly conserved.

The G_s group consists of $G\alpha_s$ and $G\alpha_{olf}$, which share 88% amino acid identity, activate adenylyl cyclase to produce cAMP, and are sensitive to

Table 1.1 Properties of mammalian G protein α subunits

Class/members	*% amino acid identity* [a]	*Modifying toxin*	*Some functions*
G_s			
$\alpha_{s(s)}$[b]	100	Cholera	Stimulate adenylyl cyclase,
$\alpha_{s(L)}$[b]	-	Cholera	regulate Ca^{2+} channels.
α_{olf}	88	Cholera	Stimulate adenylyl cyclase.
G_i			
α_{i1}	100	Pertussis	
α_{i2}	88	Pertussis	Inhibit adenylyl cyclase,
α_{i3}	94	Pertussis	regulate K^+ and Ca^{2+} channels,
$\alpha_{o.a}$[b]	73	Pertussis	activate phospholipase C. (?)
$\alpha_{o.b}$[b]	73	Pertussis	
α_{t1}	68	Cholera, pertussis	Activate cGMP
α_{t2}	68	Cholera, pertussis	phosphodiesterase.
α_g	67	Pertussis	?
α_z	60		?
G_q			
α_q	100		
α_{11}	88		Activate phospholipase C.
α_{14}	79		
α_{15}	57		?
α_{16}	58		Activate phospholipase C (?)
G_{12}			
α_{12}	44		?
α_{13}	44		?

[a]percentage amino acid identity compared with the first member of each family.
[b]splice variants of α_s and α_o. $\alpha_{s(s)}$, short form; $\alpha_{s(L)}$, long form.

cholera toxin (CT), but not pertussis toxin (PT) (Simon *et al.*, 1991). The $G\alpha_s$ protein is distributed ubiquitously, but $G\alpha_{olf}$ is found exclusively in olfactory neuroepithelium. Apart from activating adenylyl cyclase, purified $G\alpha_s$ has also been shown to regulate voltage-gated calcium channels in heart and skeletal muscle (for reviews see Schultz *et al.*, 1990; Dolphin, 1990).

Members of the G_i group have greater than 60% amino acid identity, and include all of the G proteins known to be substrates for pertussis toxin. The G_i group consists of α subunits $G\alpha_i$, $G\alpha_o$, $G\alpha_t$, $G\alpha_g$ and $G\alpha_z$ (Simon *et al.*, 1991). In addition to the well studied $G\alpha_{t1}$ mediated cGMP phosphodiesterase pathway in retinal rods, another transducin, $G\alpha_{t2}$, is expressed in cone cells where it is presumed to link cone opsins to a phosphodiesterase in a similar way to $G\alpha_{t1}$ (Stryer, 1991). A G protein with 80% amino acid identity with transducin α subunits, termed gusducin or $G\alpha_{gust}$, is found exclusively in taste buds where it is presumed to link odourant receptors to adenylyl cyclase (McLaughlin *et al.*, 1992). The α subunit $G\alpha_z$ was initially purified from bovine brain where it is associated with neurons that have long axonal processes, but its function is so far unknown (Simon *et al.*, 1991). The α subunits of the G_i class are ubiquitously expressed, and in addition to their well studied role as inhibitors of adenylyl cyclase, $G\alpha_i$ has also been linked to the regulation of ion channels (Brown and Birnbaumer, 1990; Brown, 1990). The three isoforms of $G\alpha_i$ ($G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$) have been shown to activate the opening of a potassium channel in cardiac myocytes (Yatani *et al.*, 1988). A potassium channel in cloned pituitary cells was also shown to be coupled to $G\alpha_{i1}$ (Yatani *et al.*, 1987).

The G_o alpha subunit was initially purified from bovine brain as an abundant protein sensitive to pertussis toxin (Sternweis and Robishaw,

1984: Neer *et al.*, 1984), which was subsequently cloned from rat brain (Itoh *et al.*, 1986), and human brain (Lavu *et al.*, 1988). The cDNAs isolated encode a protein 354 amino acids long that is highly conserved among bovine, rat and humans which share 98% amino acid identity. Two forms of vertebrate $G\alpha_o$ generated by alternative splicing differ in about 25 amino acids contained within the C-terminal region (Hsu *et al.*, 1990; Strathmann *et al.*, 1990). Studies using antibodies to $G\alpha_o$ show the protein predominantly localised to neuronal tissues (Sternweis and Robishaw, 1984; Gierschick *et al.*, 1986; Homburger *et al.*, 1987) and that it constitutes about 1% of the total membrane protein in brain (Sternweis and Robishaw, 1984, Gierschick *et al.*, 1986). A role for $G\alpha_o$ in axonal growth and guidance is suggested by the fact that the protein is found at high levels in the growth cone membrane, where it is regulated by the intracellular growth cone protein GAP-43 (Strittmatter *et al.*, 1990). In the nervous system, modulation of calcium and potassium channels has been attributed to $G\alpha_o$ (Brown and Birnbaumer, 1990; Brown, 1990). Purified $G\alpha_o$ protein has been shown to regulate the closing of voltage-sensitive calcium channels when coupled to opiate receptors in neuroblastoma-glioma cells (Hescheler, 1987), and to neuropeptide Y receptors in rat dorsal root ganglia (Ewald *et al.*, 1988). Antibodies to $G\alpha_o$ have been used to demonstrate that inhibition of voltage-sensitive calcium channels in snail neurons (Harris-Warrick *et al.*, 1988), and neuroblastoma-glioma cells (McFadzean *et al.*, 1989), are both specifically mediated through $G\alpha_o$. Purified $G\alpha_o$ has also been shown to activate several potassium channels in cultured hippocampal neurons (VanDongen *et al.*, 1988).

The activity of pertussis toxin has provided an important tool in obtaining information regarding G protein function (Ui, 1990; see section

6.1). The toxin acts to ADP-ribosylate some α subunits and uncouples receptor/G protein interactions. PT can be used to completely or partially inhibit the activity of hormones or neurotransmitters that act through PLC, an effector enzyme that catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce the second messengers inositol trisphosphate (IP_3) and diacylglycerol. The sensitivity of some hormone pathways to PT has been attributed to G_i and G_o proteins. However, some hormone systems are PT insensitive and by default, those G proteins not modified by the toxin were implicated in these pathways. Members of the $G\alpha_q$ class of G proteins are now understood to mediate PT-insensitive PLC activation: $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{16}$ subunits have been found to activate the β1 isoform of PLC (for review see Sternweis and Smrcka, 1992). The G_q, G_{11} and G_{14} alpha subunits are found in many tissues, with the exception of G_{15} and G_{16} which are found specifically in haematopoietic cells (Simon *et al.*, 1991).

The two members of the G_{12} group of G protein α subunits, $G\alpha_{12}$ and $G\alpha_{13}$ were isolated using the polymerase chain reaction (Strathmann *et al.*, 1989). Both α subunits are ubiquitously expressed and little is known regarding the function of these proteins .

βγ subunits

There are five known mammalian β subunit isoforms four of which, β_1, β_2, β_3 and β_4, share greater than 80% amino acid identity (Neer, 1995). All of the β subunits contain seven repetitive segments, each 43 amino acids long called a WD-repeat. This repeat structure is also found in a large number of other unrelated proteins and is thought to help in the assembly of large protein complexes (Neer *et al.*, 1994). The six known γ subunits are a more heterogeneous group than α or β subunits. For

example, the γ_1 subunit expressed in photoreceptors is only 38% identical to γ_2 that is found in a variety of tissues (Simon *et al.*, 1991). The association of β and γ can be very selective: β_1 can associate with γ_1 and γ_2, but the very similar β_2 can only form a dimer with γ_2 and not with γ_1 (Schmidt *et al.*, 1992). The choice of γ subunit appears to define the function of the $\beta\gamma$ dimer since $\beta_1\gamma_1$ found only in the retina has a different activity to that of $\beta_1\gamma_2$ found in brain (Clapham and Neer, 1993). G protein α subunits bind and hydrolyse GTP and interact with effectors, whereas the $\beta\gamma$ subunit was only thought necessary to inhibit α subunit activation and to anchor the more soluble α to the plasma membrane. However $\beta\gamma$ subunits, once released, are now known to regulate downstream effectors (Ross, 1992; Clapham and Neer, 1993). This was first suggested when purified $\beta\gamma$ was shown to activate a potassium channel in cardiac tissue (Logothetis *et al.*, 1987). Subsequently, $\beta\gamma$ has been shown to directly activate effectors, including phospholipase A_2 in rod photoreceptor outer segments (Jelsema and Axelrod, 1987), and certain isoforms of PLC-β (Camps *et al.*, 1992).

The diversity of G proteins together with the number of G protein receptors discovered so far, combine to produce a complex picture of cellular signalling. A single cell can express several types of α subunit, a couple of β subunits and several γ subunits. The specificity of the association of α subunits with $\beta\gamma$ dimers is unknown, but can be very selective: In GH_3 cells, activated muscarinic and somatostatin receptors both act to inhibit calcium channels. When GH_3 cells are depleted of individual G protein subunits by injection of antisense oligonucleotides, each receptor interacts with a different alternatively spliced form of $G\alpha_o$ combined with a different $\beta\gamma$ complex to inhibit calcium channels (Kleuss *et al.*, 1991, 1992, 1993). Around 50 neurotransmitter receptors and several

hundred odourant receptors have been identified by cloning (Dohlman *et al.*, 1991; Buck and Axel, 1991). Often a single transmitter has several closely related receptor subtypes; at least five different muscarinic receptors, six α-adrenergic, three β-adrenergic, and five different serotonin receptors have been identified (Hille, 1992). Characterisation of effectors in cloning studies has revealed that they too often belong to extensive gene families. For example, six types of adenylyl cyclase (Kuprinski, 1989), and five PLC isotypes (Rhee *et al.*, 1989) have been described. A given transduction pathway could involve any combination of these molecules so that a single neurotransmitter, hormone or growth factor can produce a variety of signalling patterns in different cell types.

1.3.2 *G proteins in invertebrates*

Homologues of G proteins have been found in yeast (*Saccharomyces cerevisiae*); two genes encoding α subunits, one gene for a β subunit and one gene for a γ subunit have been isolated (for review see Blumer and Thorner, 1991). One of the heterotrimeric G proteins found in yeast is involved in the yeast mating pheromone pathway. However, the activation of effector in this pathway is not achieved directly by the α subunit, which is a negative regulator of the mating response, but via the βγ complex (Whiteway *et al.*, 1989). In the slime mould, *Dictyostelium discoideum*, eight α subunits and a β subunit have been isolated (for reviews see Firtel *et al.*, 1989; Devreotes, 1994). So far, no γ subunit has been isolated. During the various stages of the life cycle of the slime mould: in the transitions from amoebae to aggregate, from slug to stalk and spore, new G proteins are expressed at each stage (Firtel *et al.*, 1989; Devreotes, 1994). In the nematode, *Caenorhabditis elegans*, genes encoding $G\alpha_s$ and $G\alpha_o$ proteins (Lochrie *et al.*, 1991) and a Gβ protein

(van der Voorn *et al.*, 1990) have been isolated. Other genes encoding G protein α subunits have been isolated from *C. elegans* that do not clearly belong to previously defined classes of α subunit (Fino Silva and Plasterk, 1990; Lochrie *et al.*, 1991). G proteins are involved in signalling pathways that control several behavioural responses in *C. elegans*. Mutations in the *goa-1* gene, which encodes a $G\alpha_o$ homologue, lead to defects in locomotion, egg-laying and male mating (Mendel *et al.*, 1995; Segalat *et al.*, 1995).

Representatives of each of the major vertebrate classes of G protein α subunits have been isolated from *Drosophila*. A gene encoding a *Drosophila* homologue of $G\alpha_s$ ($DG\alpha_s$) has been described that is expressed in neuronal cell bodies of the adult brain and eye (Quan *et al.*, 1989). In common with mammalian $G\alpha_s$, the $DG\alpha_s$ gene is alternatively spliced (Quan and Forte, 1990), and $DG\alpha_s$ protein is a substrate for modification by cholera toxin (Quan *et al.*, 1991). Two forms of $DG\alpha_s$ protein are produced, a long form ($DG\alpha_sL$) and a short form ($DG\alpha_sS$), which are 70% identical to mammalian $G\alpha_s$ (Quan and Forte, 1990). Using an antibody raised against mammalian $G\alpha_s$ that also recognises $DG\alpha_s$, the protein has been localised to the neuropils of brain and thoracic ganglia (neuropil consists of axons, fibre tracts and synapses, but no cell bodies), with lower levels in the lamina of the eye (Wolfgang *et al.*, 1990). When $DG\alpha_sL$ and $DG\alpha_sS$ are expressed in a S49 lymphoma cell line deficient for endogenous $G\alpha_s$ mRNA and protein, both are capable of activating mammalian adenylyl cyclase and so have a functional activity expected of a stimulatory α subunit (Quan *et al.*, 1991).

Genes encoding G protein α subunits $G\alpha_i$ (Provost *et al.*, 1988) and $G\alpha_o$ (de Sousa *et al.*, 1989; Thambi *et al.*, 1989; Yoon *et al.*, 1989) have also been isolated from *Drosophila*, DGα1 and $DG\alpha_o$, that have 78% and 82%

amino acid sequence identity to vertebrate $G\alpha_{i1}$ and $G\alpha_o$, respectively. DGα1 lacks a cysteine four residues from the carboxyl terminus that is the substrate for pertussis toxin, found in all members of the G_i class of α subunits in vertebrates. The *DGα1* gene is expressed in embryos and pupae, with no expression detected in the nervous system. Hybridisation to *DGα1* mRNA in tissue sections and embryos show staining in early embryos, in nurse cells and late stage oocytes within the ovary (Provost *et al.*, 1988). Immunolocalisation of DGα1 protein to tissue sections shows low levels of DGα1 in the nervous system but with higher levels in eye structures, specifically at the terminations of photoreceptor cells in the lamina and medulla (Hurley *et al.*, 1990; Wolfgang *et al.*, 1990). DGα1 is also detected in ovaries, particularly within the nuclei of nurse cells and follicle cells (Hurley *et al.*, 1990). The gene encoding *Drosophila* $G\alpha_o$ (*dgo*) is alternatively spliced to produce two proteins that differ in only seven amino acids in the amino terminus (de Sousa *et al.*, 1989; Thambi *et al.*, 1989; Yoon *et al.*, 1989), which are subject to modification by pertussis toxin (Thambi *et al.*, 1989). The *dgo* gene is expressed throughout development and is found, by *in-situ* hybridisation, at high levels in the central nervous system (de Sousa *et al.*, 1989; Thambi *et al.*, 1989; Yoon *et al.*, 1989; see section 4.1). Lower levels of *dgo* mRNA were also detected in nurse cells and oocytes (de Sousa *et al.*, 1989). The distribution of DGα_o protein, visualised by immunocytochemistry, is very similar to that observed for DGα_s with staining found at high levels in central nervous system (Wolfgang *et al.*, 1989; see section 4.1). The genes encoding both DGα1 and DGα_o are expressed in early embryos and ovaries, that suggests RNAs are maternally transcribed and deposited into the egg (Provost *et al.*, 1988; de Sousa *et al.*, 1989).

A homologue of the $G\alpha_q$ class has been described in *Drosophila*, $DG\alpha_q$, that has 76% amino acid sequence identity with the vertebrate protein (Lee *et al.*, 1990; Strathmann and Simon, 1990). The *dgq* gene is spliced to produce two putative proteins, DGq1 and DGq2, which based on amino acid sequence are insensitive to pertussis toxin (Lee *et al.*, 1990). Since *in-situ* hybridisation to head sections revealed *dgq* expression in the retina and ocellus of the eye, with no hybridisation to brain tissue, it was proposed that $DG\alpha_q$ acted in a similar way to vertebrate transducin in the fly (Lee *et al.*, 1990). In the visual system of *Drosophila*, light activation of rhodopsin is coupled to a PLC, encoded by the *norpA* locus (Bloomquist *et al.*, 1988), which catalyses the breakdown of PIP_2 to IP_3 and diacylglycerol. The increase in the intracellular concentration of IP_3 eventually leads to the opening of cation channels in the photoreceptor cell membrane, producing a receptor potential and neurotransmitter release (Ranganathan *et al.*, 1995). In experiments using an antibody that specifically recognises DGq1 (Lee *et al.*, 1994), in conjunction with DGq1 and DGq2 mutants, Scott *et al.*, (1995), showed that DGq1 was essential for the activation of the phototransduction cascade. In addition to the $DG\alpha_q$ found in the visual system, another G_q α subunit has been described in *Drosophila*, DGq3, that is 89% identical to DGq1 (Talluri *et al.*, 1995). The gene encoding DGq3 is expressed in chemosensory cells of olfaction and taste structures, as well as in the cells of the brain.

In *Drosophila*, G protein α subunits are involved in developmental processes. The *concertina* (*cta*) gene is a maternal effect gene required during gastrulation in *Drosophila* (Schüpbach and Wieschaus, 1989). The *cta* gene is most similar to the vertebrate $G\alpha_{12/13}$ class of α subunits, particularly in the domains of the protein required for GTP-binding (Parks and Weischaus, 1991). The G protein encoded by *cta* is presumed

to be involved in transducing signals that, through cytoskeletal components, lead to cell shape changes and movements required during gastrulation (Parks and Weischaus, 1991). Another G protein α subunit that is likely involved during development in *Drosophila* has been isolated, designated $G\alpha_f$, that shares only 30%-38% identity with vertebrate α subunits (Quan *et al.*, 1993). Northern analysis shows $G\alpha_f$ mRNA in the earliest stages of embryonic development up to late pupal stages, and by *in-situ* hybridisation localised to the developing midgut.

In addition to α subunits, genes encoding two β subunits (Yarfitz *et al.*, 1988, 1991) and a γ subunit (Ray and Ganguly, 1992) have been isolated from *Drosophila*. The β subunit gene *Gbb* encodes the protein Gβb that is 84% identical in amino acid sequence to bovine transducin β1 (Yarfitz *et al.*, 1988). The other β subunit protein in *Drosophila*, Gβe, has only 43% amino acid sequence identity with Gβb, and is found exclusively in the eye (Yarfitz *et al.*, 1991). In a GTP-γS binding assay (GTP-γS is a non-hydrolysable analogue of GTP), a *gbe* mutant (gbe^1) was unable to mediate the activation of rhodopsin (Dolph *et al.*, 1994; Yarfitz *et al.*, 1994). A gene encoding a γ subunit in *Drosophila*, DGγ1, is 52%-60% identical in amino acid sequence to bovine γ subunits (Ray and Ganguly, 1992). The *DGγ1* gene is expressed in the central nervous system of adults, specifically in the cortex of the brain and optic lobe, in a pattern very similar to that previously described for $DG\alpha_o$, $DG\alpha_s$ and DGβ (Provost *et al.*, 1988; Yoon *et al.*, 1989; Yarfitz *et al.*, 1991).

1.3.3 Project aims

The principle aim of this Thesis is to obtain P element induced mutations in previously isolated genes using site-selected mutagenesis. This

method provides a fast and efficient means to mutate a gene of interest. Advantages of this method are that no predications need be made regarding the phenotypic consequence of the mutation. In addition, apart from limited sequence information, no other materials are required such as antibodies or specifically constructed libraries. Of particular interest are genes expressed in the central nervous system of the adult fly. While considerable progress has been made using classical genetic and molecular analyses described above to investigate neural processes, there remains a large number of cloned genes expressed in the nervous system for which no functional information is available.

Chapter 2

Materials and Methods

2.1 *DROSOPHILA*

2.1.1 *Stocks*

A description of *Drosophila* stocks used in this work are listed below. A full description of all mutations used can be found in Lindsley and Zimm, 1992.

Table 2.1 ***Drosophila*** **stocks**

Strain/Genotype	*Reference*
Canton S	Lindsley and Zimm, 1992
Birm-2 ; ry ^{506}	Engels *et al.*, 1987
w ; Dr P[*ry* $^{+}$ Δ2-3](99B)/TM6B, *Tb, Hu, e*	Robertson *et al.*, 1988
w ; Dr P[*ry* $^{+}$ Δ2-3](99B)/TM6B, *Tb, Hu, e; CyO/Sco*	Robertson *et al.*, 1988
CyO/Sp	Lindsley and Zimm, 1992
CyO/Sco	Lindsley and Zimm, 1992
w ; TM3, *Sb, Ser, e* /TM6B, *Tb, Hu, e*	Lindsley and Zimm, 1992
w^{1118}	Hazelrigg *et al.*, 1984
lola ^{5D2}	Giniger *et al.*, 1994
99/1 (allele of *bumper-to-bumper*)	Kania *et al.*, 1995
1/1 (P element-induced second chromosome lethal - plasmid rescued)	Guo *et al.*, 1996
2/33 (P element-induced second chromosome lethal - plasmid rescued)	Guo *et al.*, 1996

2.1.2 *Culture*

All stocks were maintained on Glasgow medium at 25^{o}C unless stated otherwise. A grape juice agar medium was used to rear flies in sib-selection procedures, and for embryo collection.

Glasgow medium

10g agar, 15g sucrose, 30g glucose, 35g dried yeast, 15g maize meal, 10g wheat germ, 30g treacle, 10g Soya flour, per litre of water and 0.1% (v/v) Nipagen M (4-hydroxybenzoic acid methylester, 10% w/v) to inhibit fungal growth.

Grape juice agarose medium

19.8g agarose, 52.2g glucose, 26g sucrose, 7g dried yeast, 9% (v/v) red grape juice per litre of water and 0.1% (v/v) Nipagen M (4-hydroxybenzoic acid methylester, 10% w/v).

2.1.3 *Germline transformation*

pUAST-PT5 was prepared for microinjection using a Qiagen mid-prep column following the instructions of the manufacturer. DNA was coinjected at a concentration of 600 µg/ml, with the helper plasmid phs-π-Δ2,3 (Misra and Rio, 1990) at a concentration of 100µg/ml in injection buffer (0.1mM Na phosphate buffer, pH 6.8, 5mM KCl) into w^{1118} embryos using standard procedures (Spradling, 1986). Briefly, needles for injection were prepared using borosilicate glass capillaries (1.0mm O.D. x 0.78 I.D., Clark Electromedical Instruments) and pulled on a Campden Instruments electrode needle puller, following manufacturers instructions. DNA was centrifuged to pellet any suspended particles and back-filled into needles using a finely drawn out pasteur pipette. Embryos were collected on grape juice agar plates at room temperature at 40 min intervals and lined-up, without dechorionation, on coverslips lightly smeared with a glue prepared from 3MM tape soaked in heptane. Embryos were then covered with Voltalef 10S oil and viewed using a Nikon inverted stage microscope and injected at 18°C using a Leitz micromanipulator. Approximately 500 embryos were injected and

allowed to hatch on grape juice agar plates at 18°C. Newly hatched larvae were collected and transferred to individual food vials and grown at room temperature. Individual G0 adults were mated with w^{1118} flies and G1 progeny screened for red/orange eyes. The chromosomal location of insertions in two independent transformant lines were mapped by crossing flies homozygous for the insertion to the balancer stock *w; Sco/CyO; +/+*. Homozygotes can be distinguished from heterozygotes as having darker eye pigmentation. The progeny from this crossed were again mated to *w;Sco/CyO;+/+* and the location of insertions determined relative to the segregation of w^+ and Sco/CyO chromosomes. Transformant line PT5.8(II) had a single P element insertion on the third chromosome, and PT5.9(I) a single P element insertion on the second chromosome.

2.1.4 *Lethal phase analysis*

Embryos were collected from adults heterozygotes for a lethal mutation. Flies were placed in plastic beakers inverted over grape juice agar plates and allowed to lay eggs for ~45 mins at 25°C. Approximately 100 eggs were transferred to a fresh grape juice agar plate which was incubated at room temperature until hatching. The number of embryos that hatched were counted.

2.2 BACTERIA AND BACTERIOPHAGE

2.2.1 *Strains and plasmids*

Bacterial strains

XL1-Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44*
(Bullock, 1987)	*relA1, lac,* F'[*proAB, lacI* qZΔ*M15,* Tn*10* (tet^r)]

ER1647 *serB28*, Δ(*mrr-hsdRMS-mcrB*), *mcr*A122, *recD*

Bacteriophage strains

EMBL4 (Frischauf *et al.*, 1993) λ*sbh*Iλ1^{o}, *b*189 <polycloning site *int* 29, *nin*L44, *trpE*, polycloning site> KH54, *chi*C, *sr*Iλ4^{o}, *nin*5, *sr*Iλ5^{o}

Plasmids used in this work, not described elsewhere in this thesis, are listed below.

Table 2.3 Plasmids

Plasmid	*Description*	*Source/Reference*
pBluescript II KS+/-		Mead *et al.*, 1985 Stratagene USA
pBluescript II SK+/-		Mead *et al.*, 1985 Stratagene USA
pBR*rp49*	*Eco*RI-*Hin*dIII fragment of the *Drosophila* ribosomal protein gene 49 in pBR322.	O'Connell & Rosbash, 1984
pπBS25.7wc	P-element sequences from position 39 to 2882 inserted between the *Hin*dIII and *Sal*I sites of pBluescript.	Rubin and Spradling, 1983
phs-π-Δ2,3	P element transposase gene with 2 - 3 intron removed, expressed under the control of the heat shock promoter.	Misra and Rio, 1990

DGo12	$G\alpha_o$ class I cDNA, similar to DGo59 (Thambi *et al.*, 1989), minus 3' untranslated sequence. Subcloned into *Eco*RI site of pBluescript.	Thambi *et al.*, 1989
DC01.8	1.8 kb *Eco*RI-*Eco*RI fragment containing the 5' transcription and coding sequence of DC0.	

2.2.2 *Culture media and phage buffer*

L-broth: 10g Bacto-tryptone (Difco), 5g yeast extract (Difco), 10g NaCl, per litre of water and adjusted to pH 7.0 with NaOH.

L-agar: As above with the addition of Bacto-agar (Difco) to 1.5% (w/v).

BBL-top: 10g BBL trypticase peptone, 5g NaCl, 6g bacto-agar (Difco).

Phage buffer: 7g Na_2HPO_4, 3g KH_2PO_4, 5g NaCl, 10ml 1M $MgSO_4$, 10ml 0.01M $CaCl_2$, 1ml 1% gelatin.

2.2.3 *Bacteria and bacteriophage growth*

Bacterial growth

Bacterial colonies were grown on inverted plates, or in liquid culture with vigorous shaking, at 37°C, with appropriate selection when necessary.

Bacteriophage growth

ER1647 host cells were prepared by innoculating 100ml of L-broth containing 0.2% maltose with 100µl of an overnight culture of ER1647,

which were grown at 37°C with vigorous aeration until cells had entered logarithmic growth phase (OD_{600} of 0.3-0.4). Cells were pelleted by brief centrifugation and resuspended in 0.2 culture volumes of 10mM $MgSO_4$. Phage particles were mixed with 100µl of host cells and incubated at 37°C for 20-30 mins to allow adhesion of phage to cells. This was mixed with 3ml of warm BBL-top overlay and poured onto dried, pre-warmed L-broth plates. Plates were inverted once set and incubated for 8-10 hours at 37°C.

Storage

Bacteria were stored as glycerol stocks (1.5ml glycerol added to 0.85ml of culture) at -70°C. Bacteriophage were picked as single plaques and stored as agarose plugs in 1ml phage buffer with a drop of chloroform at 4°C.

2.2.4 *Antibiotics and indicators*

When selection of bacteria in culture was necessary either ampicillin, at a final concentration of 50µg/ml (50mg/ml stock solution in sterile distilled water), or tetracycline, at a final concentration of 12.5µg/ml (12.5mg/ml stock solution in absolute ethanol) were added to broth or agar. In order to detect recombinant clones, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) were added to molten agar (50°C) to a final concentration of 20µg/ml.

2.2.5 *Transformation of* E. coli

Preparation of competent cells

20ml of L-broth was innoculated with 0.4ml of an overnight culture of XL1-Blue, and grown with aeration at 37°C until cells had entered the logarithmic growth phase (OD_{600} = 0.3-0.4). The cells were then pelleted at 4000g for 5 min at 4°C in a microcentrifuge, the supernatant removed, and the resulting pellet gently resuspended in 10ml ice-cold 50mM $CaCl_2$ solution. After a 20 min incubation on ice, the cells were again pelleted as above and gently resuspended in 2ml ice-cold 50mM $CaCl_2$ solution. Competent cells were either used immediately, or after overnight storage at 4°C.

Transformation

Routinely, half the volume of ligation reactions (10µl) was added to 200µl of competent cells, the mixture placed on ice for 45 min, heat-shocked at 42°C for 2 min and then cooled on ice for 1 min. 0.4ml of L-broth was added to each transformation and incubated at 37°C for 20 min to allow expression of antibiotic resistance. Half of the final volume (300µl) was spread onto pre-dryed L-agar plates containing the appropriate antibiotics and indicators, and incubated overnight at 37°C.

2.2.6 *Plasmid DNA isolation*

Large scale plasmid isolation was carried out by the alkaline-lysis method of Birnboim and Doly, (1979), as described in Sambrook *et al.*, (1989). Small scale plasmid isolation was carried out with the Magic™ or Wizard™ DNA purification system (Promega), using the conditions recommended by the manufacturers.

2.2.7 *Bacteriophage DNA isolation*

Small amounts of bacteriophage DNA was prepared by the plate lysate method, essentially as described in Sambrook *et al.*, (1989). Single isolated plaques were stored as agar plugs in 1ml phage buffer containing a drop of chloroform. Approximately 10^5 pfu of eluted phage were added to 100µl of ER1647 host bacteria and incubated at 37°C for 30 mins, then mixed with 3ml of warm (50°C) BBL-top agarose and poured onto pre-warmed L-agar plates. Plates were incubated until confluent, usually 8-10 hrs. Phage particles were collected by overlaying plates with 5ml phage buffer and agitating at 4°C for 4 hr. The recovered supernatant was incubated with RNase and DNase each at a final concentration of 1µg/ml at 37°C for 30 min. Solid NaCl was added to a final concentration of 1M, dissolved by swirling and incubated on ice for 1 hr. To remove debris, the solution was centrifuged at 11,000g for 10 mins at 4°C. Solid PEG8000 was added to the supernatant to a final concentration of 10% (w/v) and the mixture placed on a 'rock and roller' at room temperature until the PEG8000 had dissolved. After incubation on ice for 1 hr, phage particles were collected by centrifugation at 11,000g for 10 min. The pellet was resuspended in 500µl of phage buffer and the solution transferred to a microcentrifuge tube. To release DNA from phage heads, 5µl of 0.5M EDTA, 10µl of 10% (w/v) SDS and 2.3µl of proteinase K at 10mg/ml was added and incubated at 70°C for 15 min. The solution was extracted once with an equal volume of phenol/chloroform (1:1) and then once with chloroform. DNA was precipitated with an equal vol of isopropanol for 15 min at room temperature, and collected by centrifugation . The DNA pellet was washed in 70% ethanol, dried and resuspended in 50µl of TE (1mM EDTA, 10mM Tris-HCl, pH 8.0). Typical yield was 5-10µg DNA.

2.2.8 *Screening of bacteriophage libraries*

Screening of a *Drosophila* genomic library was as described in Sambrook *et al.*, (1989). Approximately 2 x 10^4 recombinant phage were screened. Duplicate filters were pre-hybridised for 5 hr at 65°C in hybridisation solution (5X SSPE, 10X Denhardt's solution, 1% (v/v) SDS, 0.005% (w/v) sodium pyrophosphate and 100µg/ml of denatured sonicated salmon sperm DNA). Filters were hybridised with with labelled probe in hybridisation solution at 65°C for 16 hr, and washed with 2X SSC at room temperature for 15 min, then 1X SSC, 0.1% SDS at 65°C for 15 min with a final wash of 0.1X SSC, 0.1% SDS at 65°C for 5 min. 17 positively hybridising phage were isolated and six showing the strongest hybridisation signals were purified.

2.3 GENERAL MOLECULAR BIOLOGY

2.3.1 *Nucleic acid isolation*

Large scale DNA isolation

High molecular weight chromosomal DNA from *Drosophila* was prepared according to Levis *et al.*, (1982), with modifications. 1g of flies was ground in liquid N_2 with a pre-cooled mortar and pestle and homogenised with 10ml of ice cold homogenisation buffer (30mM Tris-HCl, pH 8.0, 10mM EDTA, 100mM NaCl, 10mM β-mercaptoethanol, 0.5% (v/v) Triton X-100) in a Wheaton 15ml homogeniser. The solution was decanted through nylon gauze into a sterile 30ml corex tube on ice, then the nuclei pelleted at 4,000g for 10 min at 4°C. The supernatant was carefully removed and the nuclei resuspended in 1ml of ice cold homogenisation buffer (minus Triton X-100) by gentle pipetting, then

5ml of nuclear lysis buffer (100mM Tris-HCl, pH 8.0, 100mM EDTA, 100mM NaCl, 0.5mg/ml Proteinase K) was added. The nuclei were lysed by addition of 0.2ml of 30% (w/v) sarkosyl followed by gentle swirling of the tube, and the lysate incubated at 37°C for 12 hr. 1.25g CsCl was added per gram of lysate and the solution loaded into a Beckman polyallomer Quick-Seal™ centrifuge tube, topped up with 1.25g CsCl/ml water and sealed. After centrifugation for 48 hr at 45,000 rpm at 25°C, the tube was punctured with a 18G needle at the top, and the gradient collected in 0.5 ml fractions through an 18G needle near the bottom. The concentration of DNA in each fraction was determined by spotting onto EtBr plates (1% agarose, 0.5µg/ml EtBr). The fractions containing DNA were pooled and dialysed extensively against TE (1mM EDTA, 10mM Tris-HCl, pH 8.0). Yields were generally 100-200µg/g of starting material.

Rapid DNA isolation

High molecular weight chromosomal DNA was prepared from 20 flies by the method described by Hamilton *et al.*, (1991) with modifications. Flies were homogenised in a 1.5 ml Eppendorf microcentrifuge tube with an Eppendorf micropestle in 500µl of 5% sucrose, 80mM NaCl, 0.1M Tris-HCl, pH 8.5, 0.5% SDS (w/v), 50mM EDTA. The homogenate was stored at -20°C until frozen then incubated at 70°C for 30 min. Each sample was then made 160mM in KOAc and placed on ice for 30 min. After centrifugation to remove the precipitate, the aqueous phase was collected and extracted once with an equal vol of phenol/chloroform (1:1), and once with chloroform. DNA was precipitated with 0.7 vol of isopropanol and collected by centrifugation. The DNA pellet was washed with 70% EtOH, dried and resuspended in 50µl of TE (1mM EDTA, 10mM Tris-HCl, pH 8.0) containing 10µg/ml of RNase A. The yield was typically 15µg of DNA/20 flies.

Rapid single fly DNA isolation for PCR

Genomic DNA was prepared from a single fly by the method of Gloor and Engels (S. Goodwin, pers. comm.). Single flies were homogenised in a 1.5ml Eppendorf microcentrifuge tube with an Eppendorf micropestle in 50µl of homogenisation buffer (10mM Tris-HCl, pH 8.3, 1mM EDTA, 25mM NaCl, 200µg/ml Proteinase K). The homogenate was incubated for 30 min at 37°C and then heated to 95°C for 2 min. This solution was extracted with an equal vol of phenol/chloroform (1:1) and the aqueous phase removed. DNA was precipitated by the addition of 0.1 vol of 3M NaOAc, pH 5.2 and 2.5 vol of EtOH, and incubated at room temperature for 15 min. The DNA was pelleted in a microcentrifuge, washed with 70% EtOH, dried and resuspended in 50µl of TE (1mM EDTA, 10mM Tris-HCl, pH 8.0) containing 10µg/ml of RNase A.

Egg DNA isolation

Eggs collected on grape juice agar plates were washed off in tap water and homogenised in 500µl of homogenisation buffer (10mM Tris-HCl, pH 7.4, 10mM EDTA, 60mM NaCl, 0.15mM spermine, 0.15mM spermidine, 0.5% Triton X-100) in an Eppendorf microcentrifuge tube with an Eppendorf micropestle. The homogenate was extracted once with an equal volume of phenol/chloroform (1:1) and the DNA in the aqueous phase precipitated by the addition of 0.1 vol of 3M NaOAc, pH 5.2 and 2.5 vol of EtOH for 1-2 hr at -20°C. The DNA was pelleted in a microcentrifuge at 4°C, then washed with 70% EtOH, dried and resuspended in 100-1000µl of TE (1mM EDTA, 10mM Tris-HCl, pH 8.0) containing 10µg/ml of RNase A.

Total RNA isolation

Adult heads and bodies were collected as previously described (Levy and Manning, 1981). Total RNA was isolated by a modification of Chomzynski and Sacchi (1987), that is described in the laboratory manual of K. Stanley (EMBL, Heidelberg). 1g of tissue was homogenised at full speed with a Kinematica Polytron® homogeniser for 2-3 min in 10ml of denaturing solution (4M guanidinium isothiocyanate, 0.1M Tris-HCl, pH 8.0, 0.1M ß-mercaptoethanol, 0.1% (v/v) antifoam-A). The following were added sequentially to the homogenate, 0.1 vol 2M NaOAc pH 4.0, an equal volume of phenol and 0.2 vol chloroform. The mixture was vortexed, left to stand on ice for 15-30 min and centrifuged at 12,000g for 10 min at room temperature. The aqueous phase was removed to a fresh tube, 1 vol of isopropanol added and the mixture left at -20°C for 1 hr to precipitate the RNA. The RNA was collected by centrifugation at 12,000g for 10 min at room temperature and resuspended in 5ml of denaturing solution. The RNA was precipitated again as described above and stored in EtOH at -70°C. Yield was typically 1mg of RNA/gram of starting tissue.

Poly (A)$^+$ RNA isolation

Isolation of poly(A)$^+$ mRNA was prepared by batch purification using oligo(dT)-cellulose. Approximately 0.5mg of total RNA prepared as above was resuspended in 1ml of oligo(dT)-cellulose binding buffer (0.5M NaCl, 10mM Tris-HCl, pH 7.5, 1mM EDTA, 0.1% (w/v) sarkosyl). 0.2g of oligo(dT)-cellulose was hydrated overnight at 4°C in sterile distilled water, treated for 15 min at room temperature with 0.1M NaOH, washed 5 times with 10ml sterile distilled water and then 5 times with 10ml binding buffer. The RNA was heated to 68°C for 5 min, chilled on ice, thoroughly mixed with the oligo(dT)-cellulose in a 15ml Falcon tube and incubated at room temperature for 30 min with gentle agitation. The

oligo(dT)-cellulose was collected by centrifugation at 12,000g for 10 min at room temperature and washed 5 times with 10ml of binding buffer. The poly(A)$^+$ mRNA was eluted from the oligo(dT)-cellulose by the addition of 0.5ml of sterile distilled water to the resin and incubating at 58^{o}C for 15-30 min with occasional shaking. The oligo(dT)-cellulose was pelleted at 3,000g, 10 min at room temperature and the eluate transferred to a 1.5ml microcentrifuge tube. RNA was precipitated by the addition of 50µl 3M NaOAc, pH 5.0, and 1ml EtOH, stored at -20^{o}C for 1 hr and collected by centrifugation in a microcentrifuge for 30 min at 4^{o}C. The RNA was resuspended in 10µl of sterile distilled and stored at -70^{o}C. Yields were generally 10µg of poly(A)$^+$ mRNA/0.5mg of total RNA.

2.3.2 *Oligonucleotide synthesis*

Oligonucleotides were synthesised on an Applied Biosystems Inc. PCR-MATE 391 DNA Synthesiser by the solid state method, employing phosphoramidite chemistry. After ammonium hydroxide cleavage and deprotection, oligonucleotides were dried under vacuum and resuspended in sterile distilled water. PCR primers were generally 21-31 nucleotides in length with a G+C composition of 50 to 60%, so that the T_ms for primer pairs where as close as possible. T_ms were estimated by adding 2^{o}C for A or T and 4^{o}C for G or C according to the calculation of Thein and Wallace, (1986). A description of PCR primers used, in addition to those described in chapter 3, is given in Table 2.2. Oligonucleotides used in sequencing were generally 17 bp long and are described in section 2.3.10.

Table 2.3 PCR primers

Primer	*Sequence (5' - 3')*	T_mS	*Reference*
P31	CGACGGGACCACCTTATGTTATTTCAT CATG	90°C	O'Hare and Rubin, 1983
PR	AGCATACGTTAAGTGGATGTCTC	66°C	O'Hare and Rubin, 1983
PL	GTGTATACTTCGGTAAGCTTCGG	68°C	O'Hare and Rubin, 1983
PTE	GAAAGGAATTCACACAATGGACGATC CTCCCGCCACCGTATAC	130°C	Nicosia *et al.*, 1986; Fitch *et al.*, 1993
Go1	TATTTGCTATTCATTGCAACG	56°C	Yoon *et al.*, 1989

2.3.3 *Quantification of nucleic acids*

For quantitating the amount of DNA or RNA in a sample, readings were taken in a spectrophotometer at a wavelength of 260nm. An $OD_{260} = 1$ corresponds to ~50µg/ml for double-stranded DNA, and ~40µg/ml for RNA. When only small samples of DNA were available, the quantity of DNA was estimated by spotting the sample, and DNA standards of known concentration, onto the surface of a 1% (w/v) agarose gel containing EtBr (0.5µg/ml). The DNA samples were visualised using UV illumination and the concentration estimated by comparing the fluorescence of the sample with that of the DNA standards. The concentration of oligonucleotides was determined by calculating the extinction coefficient (E) using the values: A = 15.4, T = 8.8, C = 7.3, G = 11.7 for each nucleotide, and measuring the absorbance of the oligonucleotide at a wavelength of 260nm. The E value, combined with the absorbance reading, can be used to determine the concentration of the oligonucleotide using the equation:

[oligo] µM/ml = $A_{260nm} \div E$

2.3.4 *Restriction enzyme digests*

DNA was digested with restriction enzymes supplied by Promega or BRL with the appropriate buffer recommended and provided by the manufacturer. At least 2 units of enzyme/μg of DNA was used and incubations were carried out at 37°C. Plasmid DNA was digested for ~1 hr and genomic DNA for ~4 hr.

2.3.5 *PCR*

PCR reactions were carried out in a volume of 20μl containing 100-200ng of template DNA in 1X buffer (50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5mM $MgCl_2$, 0.01% (w/v) Triton X-100), 200μM dATP, 200μM dCTP, 200μM dGTP, 200μM dTTP, with primers each at a concentration of between 0.5μM-0.33μM and 1 unit of *Taq* polymerase (Promega). Samples were overlaid with mineral oil (Sigma) and reactions performed in a Hybaid Thermal Reactor (Hybaid). A standard programme used was: initial denaturation of 3 min at 94°C, followed by 30 cycles of 1 min annealing at 55-65°C, 2 min extension at 72°C and 1 min denaturation at 94°C, with a final step of 55-65°C for 5 min and then 72°C for 10 min. Amplified products were analysed by electrophoresis on 1.5% agarose gels.

2.3.6 *Purification of PCR products*

When purified PCR products were required, PCR reactions were separated on 1% (w/v) LMP (low melting point) agarose gel in 1X TAE (40mM Tris-acetate, pH 7.6, 1mM EDTA). DNA fragments were visualised by staining with EtBr (0.5μg/ml) and excised in a small slice of agarose gel. The agarose slice was incubated at 70°C until liquid and then

purified with the Magic™ (now Wizard™) DNA purification system (Promega), using the conditions recommended by the manufacturers.

2.3.7 *Cloning PCR products*

PCR products were cloned into pBluescript II by taking advantage of the terminal transferase activity of *Taq* polymerase (Clark, 1988). 1µg of pBluescript II was digested with *Eco*RV and incubated with *Taq* polymerase (1 unit/20µl vol) using standard PCR buffer conditions in the presence of 2mM dTTP for 2 hrs at 72°C. The reaction was then purified using the Magic™ (now Wizard™) DNA purification system (Promega), using the conditions recommended by the manufacturers. DNA ligations were carried out in a final volume of 20µl, using 100ng of T-tailed pBluescript II, with a 4 to 6-fold excess of insert DNA to vector.

2.3.8 *DNA ligation*

Ligations were carried out in a final volume of 20µl using 4µl of 5X ligation buffer (250mM Tris-HCl: pH 7.6, 50mM $MgCl_2$, 25% (w/v) PEG 8000, 5mM DTT, 5mM ATP), 1 unit of T4 DNA ligase (Promega) with the appropriate volumes of insert and vector DNA. Ligations were incubated overnight at 14°C.

2.3.9 *Labelling of nucleic acids*

Gel-purified DNA fragments, linearised plasmid DNA or bacteriophage λ DNA was labelled by random priming (Feinberg and Vogelstein, 1983). Approximately 50ng of DNA was mixed with distilled water in a final vol of 12µl and denatured at 95°C for 2 min (DNA fragments) or 8 min for plasmid or phage DNA, then quenched on ice for 1 min. Added to this

was 6µl of 4X random priming buffer (250mM Tris-HCl, pH 8.0, 25mM $MgCl_2$, 5mM β-mercaptoethanol, 100µM dNTPs, 1M Hepes, pH 6.6, 27 A_{260} units/ml random hexanucleotides), 50µCi of [α-^{32}P]dCTP (3000 Ci/mmole) and 5 units of Klenow enzyme (Promega). The mixture was then incubated for at least 12 hr at room temperature and the reaction stopped by heating at 100°C for 2 min. Probes were also prepared by random priming using the "Ready-to-Go" kit (Pharmacia) following the instructions of the manufacturer. Probes were separated from unincorporated radionucleotides by Sephadex G50 (Pharmacia) chromatography, in columns prepared from disposable 1ml syringes (Sambrook *et al.*, 1989). Probes generally had a specific activity of 10^8-10^9 cpm/µg of DNA.

2.3.10 *Dideoxy sequencing*

Sequencing of double-stranded DNA was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the Sequenase Version 2.0 kit (U.S. Biochemical Corporation) following instructions provided by the manufacturers. PCR products subcloned into pBluescript II were sequenced using T3 and T7 sequencing primers (BRL). Other primers used were:
(5'-3') pU_L: CAATCTGCAGTAAAGTG; pU_R: GTCCAATTATGTCACAC; PT181: ACCGAGGTGTATCTCGA.

2.3.11 *Gel electrophoresis of nucleic acids*

Loading buffer

Before DNA samples were loaded onto gels, 0.1 vol of 10X loading buffer (25% (w/v) Ficoll 400, 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol) was added to each. 10X loading buffer for RNA samples is the same as for DNA with the addition of 1mM EDTA.

Agarose gel electrophoresis

DNA was electrophoresed on agarose gels which were prepared and run in 1 X TBE (89mM Tris-borate, pH 8.3, 2mM EDTA). A range of agarose concentrations [0.8-1.5% (w/v)] was used depending on the sizes of fragments to be resolved (Sambrook *et al.*, 1989). Applied voltages varied between 2 and 10 V/cm, depending on the time of running. DNA size markers used were the 123bp or 1kb ladder supplied by the manufacturers (BRL).

Low melting point agarose gels

DNA fragments were purified (Sambrook *et al.*, 1989), from 1% (w/v) LMP (low melting point agarose, BRL) agarose gels in 1 X TAE (40mM Tris-acetate, pH 7.6, 1mM EDTA). DNA fragments were excised in gel slices, heated at 70°C until the agarose melted and DNA purified with the Magic™ (now Wizard™) DNA purification system (Promega), using the conditions recommended by the manufacturers.

Denaturing agarose gel electrophoresis

Prior to electrophoresis, RNA samples (up to 4.5µl vol) were denatured by the addition of 10µl of formamide, 2µl of 5X MOPS buffer (200mM MOPS, pH 7.0, 50mM sodium acetate, 5mM EDTA, 11M formaldehyde),

3.5µl of formaldehyde (12.3M), 1µl of EtBr (1mg/ml stock), and heated to 65°C for 15 min. The RNA was electrophoresed on 1% (w/v) agarose gels containing 2.2M formaldehyde (Sambrook *et al.*, 1989), using 1 X MOPS, in a Hybaid 'Buffer Puffer™' gel tank which provides a constant circulation of buffer from anode to cathode chambers necessary to maintain a constant pH.

Denaturing polyacrylamide gel electrophoresis

Products of DNA sequencing reactions were separated on denaturing polyacrylamide gels prepared with 6% (w/v) acrylamide (N,N'-methylenebisacrylamide, 19:1), 7M urea, in 1 X TBE. Polymerisation was catalysed by the addition of 1ml of 10% (w/v) ammonium persulfate and 50µl of TEMED (N,N,N'N'-tetramethylenediamine) to 150ml of acrylamide/urea mix. Polymerised gels were pre-run at 110W until they reached 50°C. Before loading, samples were denatured for 2 min at 75°C and gels were run for 2-5 hr. Gels were dried for 2 hr at 80°C under vacuum onto Whatman 3MM paper and then exposed to X-ray film overnight at room temperature without intensifying screens.

2.3.12 *Visualisation and photography of gels*

DNA was visualised by UV induced fluorescence after staining agarose gels in EtBr (0.5µg/ml), and RNA by adding EtBr directly to the sample before loading, on a short wave (254nm) transilluminator. Gels were photographed using a Polaroid camera loaded with 545- or 667- land film fitted with a Kodak Wratten filter No. 23A.

2.3.13 *Blotting and nucleic acid hybridisation*

Southern blotting

The DNA separated in an agarose gel was transferred to nylon membranes (Hybond-N), either by capillary transfer (Sambrook *et al.*, 1989) or vacuum blotting (Stratagene), and fixed to the membrane by UV treatment as instructed by the manufacturer (Amersham UK). Filters were pre-hybridised for a minimum of 4 hr at 65°C in hybridisation solution (5X SSPE, 10X Denhardt's solution, 1% (v/v) SDS, 0.005% (w/v) sodium pyrophosphate and 100μg/ml of denatured sonicated salmon sperm DNA). Hybridisation with a denatured radioactive DNA probe was carried out at 65°C for a minimum of 12 hr in fresh hybridisation solution. After hybridisation, the filters were washed in 2X SSC at room temperature for 15 min, 1X SSC, 0.1% SDS at 65°C for 15 min and then a final wash of 0.1X SSC, 0.1% SDS at 65°C for 15 min.

Northern blotting

RNA separated in an agarose formaldehyde gel was transferred to reinforced nitrocellulose (Hybond C^+, Amersham), by capillary action. RNA was fixed to the membrane by UV treatment. Filters were pre-hybridised at 42°C for at least 3 hr in 50% (v/v) formamide, 5X SSPE, 2X Denhardt's solution, 0.1% SDS. The denatured radioactive DNA probe was added and hybridised at 42°C for a minimum of 16 hr. After hybridisation, the filters were washed in 1X SSPE, 0.1% SDS at room temperature for 20 min, then 3 times in 0.2X SSPE, 0.1% SDS at 42°C for 20 min.

Prior to autoradiography, filters were blotted dry and covered in clingfilm.

2.3.14 *Autoradiography*

Autoradiography of radioactive filters was carried out at -70°C with intensifying screens with exposure to Fuji NIF RX X-ray film. Films were developed using a Kodak X-Omat film processor.

2.3.14 *Phosphoimaging*

The intensity of ^{32}P emissions from labelled filters was quantified using a Fuji BAS-1500 phosphoimager. Filters were exposed to a Fuji Imaging Plate for 4-6 hr and then processed using MacBas Ver. 2x software.

Chapter 3

Mutagenesis of Selected Cloned Genes

3.1 Introduction

3.1.1 *Site-selected mutagenesis*

Site-selected mutagenesis is a controlled and efficient method of generating mutations in cloned genes (Ballinger and Benzer, 1989; Kaiser and Goodwin, 1990; Kaiser, 1990). This procedure utilises a transposable element, the P element, that can be mobilised during hybrid dysgenesis to transpose to new sites in the *Drosophila* genome (see section 1.2.4). The detection of a P element insertion in or near a specific gene within a large population of mutagenised flies relies on the sensitivity of the polymerase chain reaction, PCR (Saiki *et al.*, 1986). Using primers derived from P element sequences in conjunction with primer(s) from specific gene sequences, a P element can be found in the vicinity of the target gene. P elements contain terminal 31 bp inverted repeats, so a primer based on these sequences gives the ability to detect a P element irrespective of orientation upon insertion. Once a P element insertion is detected in a target gene, the starting population is subdivided and re-screened by PCR. By a process of elimination, the individual containing the desired P element insertion can be identified. As mutants are isolated using molecular criteria only, no predictions need be made regarding phenotype(s) resulting from disruption of a particular gene.

3.1.2 *Genes selected for mutagenesis*

The chosen targets for site-selected mutagenesis were genes isolated on the basis of sequence homology in other organisms that are predominantly expressed in the nervous system of *Drosophila* or that had a role in signal transduction processes (Sentry *et al.*, 1994). Moreover, there were no known

mutants corresponding to these genes in *Drosophila*. The genes selected as targets encode proteins for three classes of G protein alpha subunit (DGα1: Provost *et al.*, 1988; DGα_s: Quan *et al.*, 1989; DGα_o: de Sousa *et al.*, 1989; Thambi *et al.*, 1989; Yoon *et al.*, 1989), the catalytic subunit of cAMP-dependent protein kinase (Foster *et al.*, 1984; Kalderon and Rubin, 1988), Protein kinase C (Schaeffer *et al.*, 1989), calcium/calmodulin-dependent protein kinase II (Cho *et al.*, 1991), the Amyloid Precursor Protein (Rosen *et al.*, 1989), and the synaptic vesicle proteins synaptobrevin and synaptotagmin (Südhof *et al.*, 1989; Perin *et al.*, 1990).

G proteins

G proteins belong to a family of GTP-binding proteins that mediate cellular responses to extracellular signals such as neurotransmitters, peptide hormones, light and odourants (Stryer and Bourne, 1986; Gilman, 1987; see section 1.3). They function as a heterotrimeric complex of α, β and γ subunits to transmit signals from cell-surface receptors to intracellular effectors that include ion channels, adenylyl cyclase and phospholipase C. G proteins were originally classified based on the interaction of α subunits with effectors in the cell. For example, transducins (G_t) are responsible for the transduction of light in the retina to an activation of cGMP phosphodiesterase; G_s and G_i stimulate and inhibit adenylyl cyclase respectively; and G_o has no effect on adenylyl cyclase so is termed 'other'. Molecular cloning has shown that homologues of each of the major vertebrate classes of Gα subunit are expressed in *Drosophila* (see section 1.3). Mammalian G protein gene probes have been used to isolate genes encoding a $G\alpha_i$ homologue DGα1 (Provost *et al.*, 1988), a $G\alpha_s$ homologue $DG\alpha_s$, (Quan *et al.*, 1989), and a $G\alpha_o$ homologue $DG\alpha_o$ (de Sousa *et al.*, 1989; Thambi *et al.*, 1989; Yoon *et al.*, 1989). The

Drosophila Gα proteins share a high degree of amino acid sequence identity with their vertebrate counterparts; 78% for DGα1, 71% for $DG\alpha_s$ and 82% for $DG\alpha_o$. A gene encoding a homologue of the vertebrate $G\alpha_q$ class of α subunit, $DG\alpha_q$, has been isolated by subtraction cloning (Lee *et al.*, 1990) and by PCR using degenerate primers (Strathmann and Simon, 1990). $DG\alpha_q$ is likely to be an analogue of vertebrate transducin as it is expressed solely in the visual system in adult *Drosophila* (Lee *et al.*, 1990). In addition to Gα subunits, genes encoding Gβ and Gγ subunits have been isolated from *Drosophila* (Yarfitz *et al.*, 1988; Ray and Ganguly, 1992).

Selected for mutagenesis were the genes encoding $DG\alpha_s$ (*DGα$_s$*), $DG\alpha_o$ (*dgo*) and DGα1 (*DGα1*). Analysis of the expression of $DG\alpha_s$ and $DG\alpha_o$ by *in-situ* hybridisation to RNA and protein have shown them to be predominantly expressed in the nervous system of the fly (Quan *et al.*, 1989; Yoon *et al.*, 1989; Hurley *et al.*, 1990; Wolfgang *et al.*, 1990). DGα1 is predominantly expressed in embryos and pupae (Provost *et al.*, 1988).

Catalytic Subunit of PKA

cAMP is a ubiquitous second-messenger within the nervous system and elsewhere (Gilman, 1984). When cell-surface receptors are activated by ligand binding, G proteins in turn act to stimulate or inhibit adenylyl cyclase, which catalyses the formation of cAMP within the cell. Although cAMP may act directly to activate ion-channels, the major effector for cAMP within the cell is cAMP-dependent protein kinase (PKA; Edelman *et al.*, 1987). At low cAMP levels PKA exists as a tetramer of two catalytic and two regulatory subunits. When intracellular cAMP levels increase, cAMP binds to regulatory subunits resulting in the release of catalytic

subunits, which are then free to phosphorylate target proteins (Taylor *et al.*, 1990). Genes encoding a regulatory and a catalytic subunit of PKA have been cloned and characterised from *Drosophila*. A type I regulatory subunit (RI) and catalytic subunit (DC0) have 71% and 82% amino acid identity, respectively, to mouse subunits (Foster *et al.*, 1988; Kalderon and Rubin, 1988). The gene encoding DC0 is associated with at least four transcripts which vary in expression during different stages of development. The two largest transcripts are enriched in the head of the adult fly (Kalderon and Rubin, 1988).

In *Drosophila*, the role of cAMP in learning and memory processes has been clearly demonstrated (Davis, 1993). Two well characterised behavioural mutations, *dunce* and *rutabaga*, both directly affect the cAMP cascade; *dunce* mutants are defective in cAMP phosphodiesterase and *rutabaga* mutants in a calcium/calmodulin-responsive adenylyl cyclase (Byers *et al.*, 1981; Chen *et al.*, 1986; Levin *et al.*, 1992).

Protein Kinase C

Protein kinase C (PKC) describes a family of serine-threonine kinases that require phospholipid for activity and which are activated by the second messenger diacylglycerol (Schulman, 1991). They are involved in a diverse range of biological processes including mitosis, production of lymphokines, hormone and neurotransmitter release, modulation of conductance channels and long term potentiation. At least nine distinct isoforms of PKC have been identified in vertebrates which differ in their substrate specificity, sensitivity to calcium or diacylglycerol and cellular and intracellular localisation (Schulman, 1991). Three PKC genes have been cloned in

Drosophila to date. A PKC gene at chromosomal position 98F, *dPKC98F*, is the subject of this study. It is transcribed throughout development, with specific expression in the neuronal cell bodies in the brain of the adult fly (Schaeffer *et al.*, 1989). Two other PKC genes are located at cytological position 53E: *dPKC53Ebr* is expressed in the adult brain (Rosenthal *et al.*, 1987; Schaeffer *et al.*, 1989), and *dPKC53Eey* has restricted expression in adult heads, specifically the visual system (Schaeffer *et al.*, 1989). All three *Drosophila* PKCs have structural features typical of the mammalian PKC family: a regulatory domain containing a pseudosubstrate sequence, a catalytic domain and an ATP-binding site. The specific function of PKC isoforms and their substrates are unknown, but the action of PKC has been implicated in hippocampal long-term potentiation, a form of synaptic regulation that may be involved in early stages of associative learning and memory (Malinow *et al.*, 1988; Malenka *et al.*, 1989).

Calcium/Calmodulin-dependent Protein Kinase II

When a stimulus is received at a nerve terminal, the major component that responds to the influx of calcium is calcium/calmodulin-dependent protein kinase II (CaM kinase). CaM kinase is a serine/threonine kinase which is found in abundance in the mammalian brain, particularly the hippocampus, where it is found as a large multimeric protein that consists of approximately twelve distinct, but homologous, α and β subunits (Schulman, 1991). A distinctive property of CaM kinase is its ability to autophosphorylate in the presence of calcium/calmodulin, which converts it to a calcium-independent state of activity. This autoregulation leads to a prolonged effect of the initial calcium trigger and has led to the proposal that CaM kinase is involved in plasticity and long term potentiation in the synapse (Malinow *et*

al., 1988; Kennedy, 1989; Malenka *et al.*, 1989). Using an antibody to rat brain type II CaM kinase, *Drosophila* has been shown to have three polypeptides of similar size to those in rat (Cho *et al.*, 1991; Ohsako *et al.*, 1993). Four alternatively spliced transcripts have been shown to originate from a single *Drosophila* CaM kinase gene, *cam*, which is 77% identical in nucleotide sequence to the α subunit from rat brain and contains expected sites for autophosphorylation, including one which controls calcium-independent activity (Ohsako *et al.*, 1993; Cho *et al.*, 1991). The *Drosophila* CaM kinase gene is expressed at high levels in the CNS of late embryos and abundant in adult heads (Ohsako *et al.*, 1993).

Synaptic Vesicle Proteins

Small synaptic vesicles are specialised organelles that function in the uptake, storage and release of neurotransmitters. Release of neurotransmitter occurs when synaptic vesicles dock with the presynaptic membrane and, following the influx of extracellular calcium, fuse and release their contents into the synaptic cleft (Südhof and Jahn, 1991; Trimble *et al.*, 1991). Genes encoding synaptic vesicle proteins have been cloned and characterised in *Drosophila*, including synaptobrevin, rab3 and synaptotagmin (Südhof *et al.*, 1989; Johnston *et al.*, 1991; Perin *et al.*, 1991).

Synaptobrevin, a small protein of 115-120 amino acids that is intrinsic to the membrane of synaptic vesicles, was originally isolated from Torpedo (Trimble *et al.*, 1988), and rat (Baumert *et al.*, 1989). The specific function of synaptobrevin is unknown, but the proteolysis of synaptobrevin by tetanus toxin (an inhibitor of neurotransmitter release) suggests that it is essential for secretion of neurotransmitter (Schiavo *et al.*, 1992). Using an oligonucleotide probe based on bovine synaptobrevin cDNA sequence, a

gene encoding *Drosophila* synaptobrevin, *syb*, has been isolated (Südhof *et al.*, 1989). The protein encoded by *syb* shares an overall 49% amino acid sequence identity with rat synaptobrevin, but this increases to 78% in a highly conserved cytoplasmic domain (Südhof *et al.*, 1989). Further studies revealed that *syb* was expressed at low levels in the nervous system, but at higher levels in the gut (Südhof *et al.*, 1989; Chin *et al.*, 1993), and a more likely candidate for the neuronal specific synaptobrevin was isolated, designated *n-syb* (DiAntonio *et al.*, 1993a). This gene is associated with two transcripts that are expressed throughout development until adulthood, when one transcript highly enriched in head tissue predominates.

Synaptotagmin is an abundant integral membrane protein of synaptic vesicles (Perin *et al.*, 1990; Südhof and Jahn, 1991). A synaptotagmin gene isolated from *Drosophila*, *syt*, has 57% amino acid sequence identity to vertebrate synaptotagmin (Perin *et al.*, 1991). Antibodies show the protein localised at synaptic contact sites in the embryo, neuromuscular junctions in the larvae and in the neuropil of the adult brain (DiAntonio *et al.*, 1993a; Littleton *et al.*, 1993a). It has been proposed that synaptotagmin functions as the calcium sensor for the release of neurotransmitter: it has been shown to bind calcium (in the presense of phospholipids) and is associated with N-type calcium channels at the synapse (Kelly, 1995).

Amyloid Precursor Protein

Alzheimers disease in humans is characterised by the extracellular deposition of amyloid protein aggregates, or plaques, in the brain. The major component of the plaques is the β-amyloid protein, a small peptide of 42 amino acids that is proteolytically cleaved from the larger integral

membrane Amyloid Precursor Protein (APP), (Selkoe, 1989). A *Drosophila* APP-like gene (*APPL*) has been identified and the protein it encodes found to have features in common with human APP (Rosen *et al.*, 1989). It has a single membrane-spanning domain near the carboxyl terminal, two conserved extracellular domains and a conserved cytoplasmic domain. In common with human APP, *Drosophila* APP is synthesised as a membrane-associated protein precursor that is cleaved to give a secreted, 130 kDa form (Luo *et al.*, 1990). However, the APP-like protein in *Drosophila* lacks the β-amyloid sequence. A single transcript from *APPL* is expressed in the nervous system of embryo, pupae and adult flies (Rosen *et al.*, 1989; Martin-Morris and White, 1990).

Presented in this chapter are the results of a screen for P element insertions in the genes described above, using a site-selected mutagenesis strategy. Also presented is preliminary molecular characterisation of P element insertions obtained in or near the genes *DGα1*, *dgo* and *DC0*, with further characterisation of an insertion in *dgo*.

3.2 P element mutagenesis

The generation of random P element transpositions was carried out using a controlled mutagenesis strategy in which the activity of P elements and the transposase required to mobilise them are under experimental control (Robertson *et al.*, 1988; Cooley *et al.*, 1988b). Mutator P elements were provided by a homozygous *Birm-2; ry*^{506} strain that contains 17 defective P elements on the second chromosome. A high level of transposase was supplied by *w;Dr* P[*ry*$^{+}$Δ2,3](99B)/TM6 which contains a non-mobilisable P

element insertion that has the third intron of the transposase gene removed *in-vitro* (Laski *et al.*, 1986). *Birm-2;ry*^{506} males were mated to *w;Dr* P[*ry*$^{+}$Δ2,3](99B)/TM6 females at 16^{o}C (Figure 3.1). This cross was carried out at 16^{o}C to avoid the larval and pupal lethality due to the somatic activity of Δ2,3 transposase acting on the *Birm-2* chromosome (Robertson *et al.*, 1988). F1 *Dr* male progeny were selected and crossed to wild-type females at 18^{o}C. *Dr*$^{+}$ virgin F2 females were then mated with wild-type males in ten batches of 200. The *Dr* marker is closely linked to P[*ry*$^{+}$Δ2,3](99B) and so selection against the *Dr* phenotype ensured that new insertion events were stable. Due to the high levels of transposase produced by the Δ2,3 element, and the presence of 17 defective P elements in *Birm-2*, the scheme described generates a high level of insertion rate, producing approximately 10 new insertions per mutagenised genome (Robertson *et al.*, 1988). This is comparable to the rate observed in a strong P/M hybrid dysgenesis, giving a similar mutation rate in the *singed* locus of 2 x 10^{-3} (Robertson *et al.*, 1988). Flies were screened for new P element insertions in four batches containing approximately two thousand F2 virgin females each.

3.3 PCR detection of insertions and sib-selection

The eggs produced by F2 females represent a spectrum of new insertion events. Batches of 200 virgin females were introduced to approximately 50 wild-type males and placed in a cage. Females were given a day to mature, then eggs produced in each cage during a 24 hour period were collected and DNA prepared from them. PCR analysis was carried out on DNA from each batch with gene specific primers and a P element primer (Kaiser and Goodwin, 1990). Any batch(es) that contained a transposon insertion in or

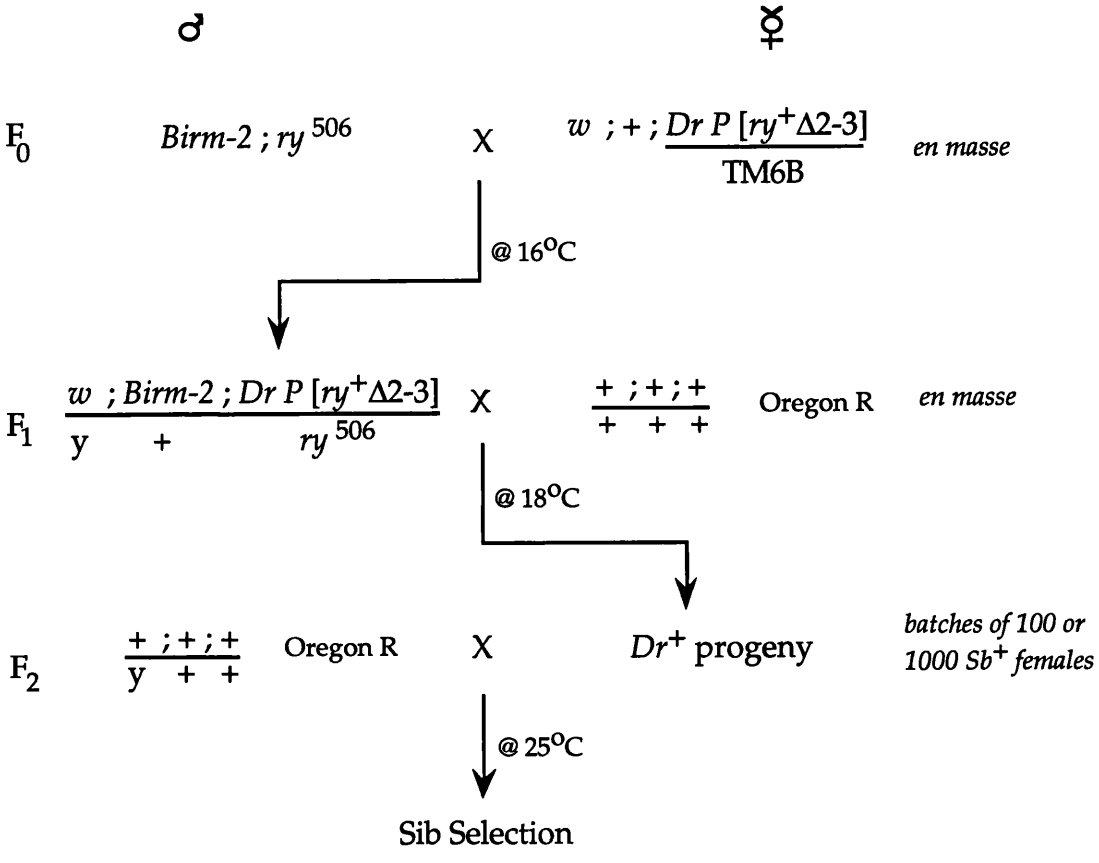


Figure 3.1 P-element mutagenesis.

The *Birm-2* strain, containing 17 defective P elements was crossed to Δ2,3 which provides a stable source of transposase, that can be selected against in the F_2 generation (Robertson *et al.*, 1988; see Figure 1.5).

near the gene of interest was identified by the visualisation of a PCR product on an ethidium bromide stained agarose gel. PCR products dependent upon amplification between gene and P element primers were distinguished from non-specific amplification products by hybridisation with gene specific DNA sequences. Verification that insertions were genuine was further demonstrated by performing PCR with two additional P element primers, Pleft and Pright (PL and PR), (Kaiser and Goodwin, 1989; see section 3.4). Positive batches were subdivided into groups of twenty and PCR performed on egg DNA as before. By a process of elimination, outlined in Figure 3.2, a single female of interest was isolated. This female was placed in a vial, allowed to produce progeny, and then killed for DNA extraction and subsequent PCR analysis. The progeny of a female positive for the desired insertion were crossed to appropriate balancer stocks.

3.4 PCR primer design

It appears that P elements show a preference for inserting near the transcription start site of genes (Tsubota, 1985; Kelley *et al*, 1987; Engels, 1989). With this in mind, two gene specific primers were designed to screen both the 5'-flanking and coding regions of target genes (Figure 3.3). The nucleotide sequences of target gene primers were based on published DNA sequences available at the time, in most cases only cDNA sequences. However, in those instances when more information was available such as genomic sequence and organisation, primers were designed to avoid introns located in the upstream control area of target genes. The primers chosen for each target gene and their positions, relative to published DNA sequences, are summarised in Table 3.1. Primer pairs were based on sequences unique

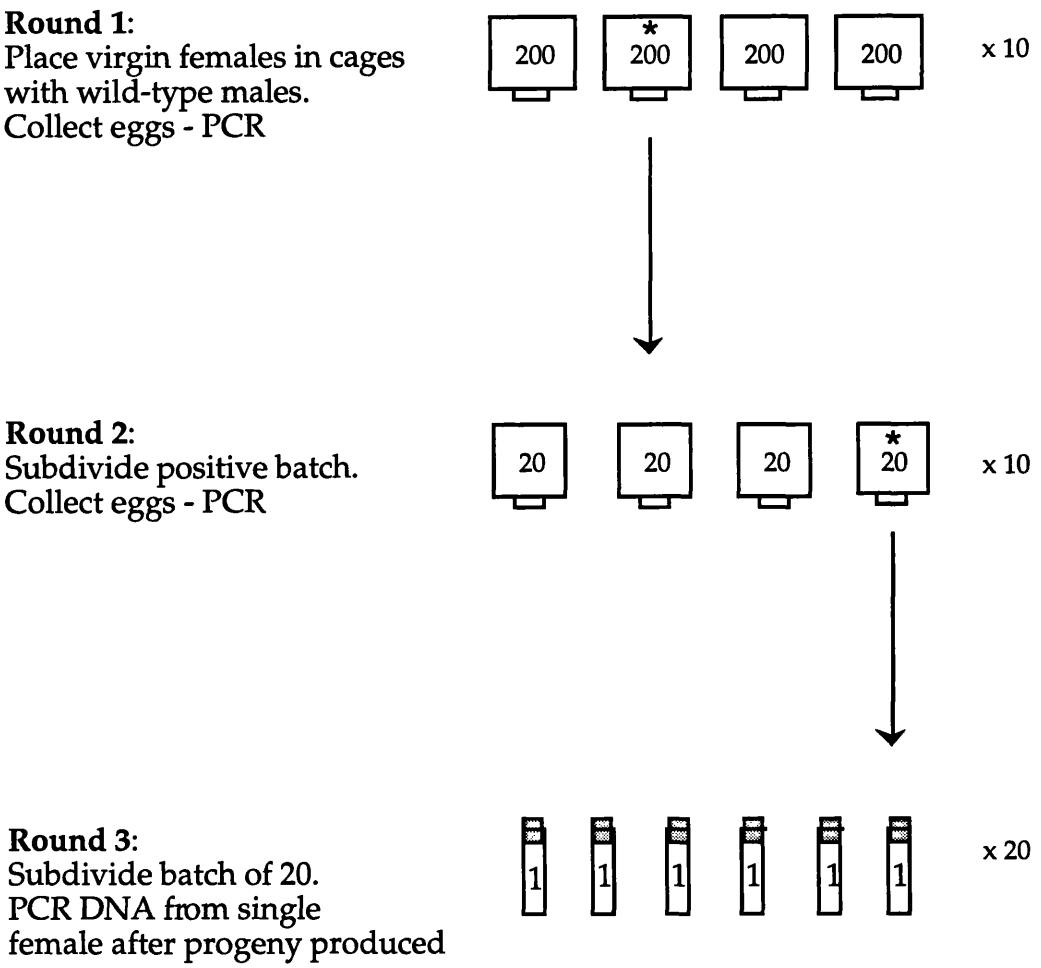


Figure 3.2 Sib-selection procedure.

A large population of flies are placed in cages and allowed to lay eggs. DNA is prepared from eggs and analysed by PCR using gene specific primers. The appropriate positive batch is then subdivided for the next round. In this way, a single female with the desired P element insertion can be isolated.

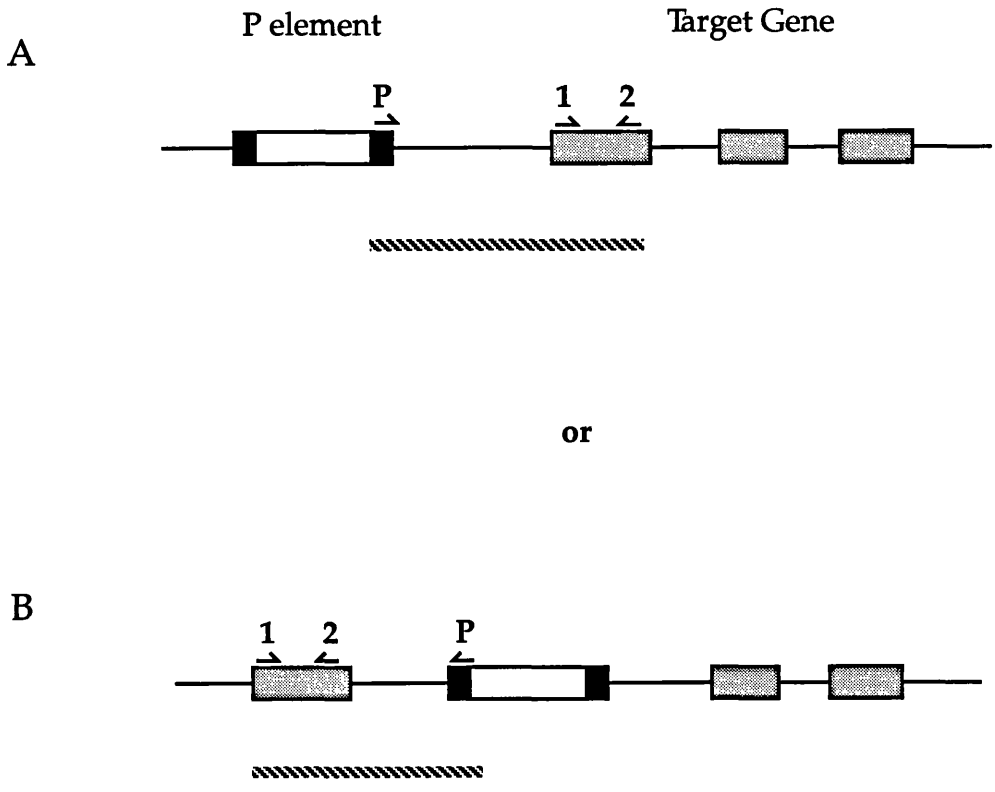


Figure 3.3 Detection of a P element in a target gene.

When a P element (open box with closed ends) inserts in a target gene (shaded boxes), a unique substrate for amplification is created (hatched line). Amplification can occur between gene specific primers (1 & 2) and a P element primer (P). In (A), a P element has inserted upstream of the gene and will be detected with primers P and 2. In (B), a P element has inserted within the gene and will be detected with primers P and 1.

Table 3.1. Genes selected for mutagenesis and PCR primers.

Target Gene	*Gene Symbol*	*Chromosome Location*	*Left*	*Right*	*PCR Primers Sequence 5' - 3'*	*Position **	*Reference*
G_i alpha subunit	*DGα1*	65C	Gi4	Gu	GCTTGCGTGCGCTGCGCATG ACCCAGCAGCAG(CG)AGTTT(CG)A	-195 to -176 121 to 104	Provost *et al.*, 1988
G_o alpha subunit	*dgo* (I) *dgo* (II)	47A	Go2 Go3	Gu	CTCGTTGCAATGAATAGCAAAT GTACAGTACAACATCCATAGAGCG ACCCAGCAGCAG(CG)AGTTT(CG)A	-714 to -693 -394 to -417 118 to 101	Yoon *et al.*, 1989
G_s alpha subunit	*DGα$_s$*	60A	Gs6	Gs5	CTCGCAACCGAGTCGTGCACCTA GCCCAGGAGGAGCAGCCTGTG	-298 to -275 150 to 130	Quan *et al.*, 1989
Catalytic subunit of PKA	*DC0*	30C	DC12	DC11	CAGACGGAATAGAACCCGCGC CACACTCAGCGCTCACCTAAC	264 to 284 693 to 673	Kalderon and Rubin, 1988
Protein Kinase C	*dPKC98F*	98F	P1	P2	GATCACGACCAGCAATAGGATCT TGGCCATGAACTTGTGCCCATTC	-372 to -350 229 to 207	Schaeffer *et al.*, 1989
Calcium/calmodulin dep. protein kinase II	*cam*	102E-F	CK1	CK2	TCGGTATCTAGCTGTGTGTTTCG AGTTCACCACCGGTTACAAGGTC	-248 to -226 293 to 271	Cho *et al.*, 1991
Synaptobrevin	*syb*	46E-F	S1	S2	ATACACAGCAGTATTCTACAGGC ACCAGAACGATGATGAGCAGCAC	32 to 54 458 to 436	Südhof *et al.*, 1989
Synaptotagmin	*syt*	23B	ST1	ST2	CCTCACTAGCCAAAGCCAGTCAC TCCACCTGTGCGATTCTTTGACT	20 to 42 565 to 543	Perin *et al.*, 1991
Amyloid Precursor Protein-like	*APPL*	1B	A1	A3	CAGACGGCGGCCATAGTGCC CAGAGGCTCCTCAGCAACAAATT	26 to 46 752 to 730	Martin-Morris and White, 1990

*Position relates to published sequence and numbering in references listed.
Nucleotides underlined in primer Gu are positioned in the first intron of *DGα1* and *dgo*.

to each target gene, with the exception of the Gu (universal) primer that was used to detect P element insertions in DGα1 and in both cDNA classes of DGα_o. Gu is positioned in a region of sequence homology shared by DGα_o (cDNA classes I and II) and DGα1, that differs at only two nucleotide positions. To allow for this small sequence variation, Gu was synthesised to contain either a C or G nucleotide at the two variable positions (see Table 3.1).

Three primers were designed to detect P element sequences. The DNA sequence of P31, PL and PR with their locations within the full length P element are shown in Figure 3.4. The P31 primer is designed to take advantage of the fact that all mobilisable P elements have a 31 bp inverted repeat at their termini. This primer will therefore detect a P element insertion irrespective of its orientation. PL and PR are positioned inside the inverted repeat and are therefore specific to the left and right ends of the P element. After initial rounds of PCR with P31, PL and PR can be used to determine the orientation of the P element and to confirm that amplification products are specific to P-sequences.

Before gene specific primers were used in a screen for P element insertions, a preliminary test PCR reaction with each gene primer pair was carried out to determine conditions for PCR, such as optimal annealing temperature, and to confirm that amplification gave a PCR product of the anticipated size. The substrates for the PCR reaction was DNA prepared from whole flies or from a cDNA library. For most target genes, amplification with genomic DNA and cDNA yielded products of equal and anticipated size. However, amplification with primers to *dPKC98F* and *syb* produced larger PCR products with genomic DNA than with cDNA. The introns between primer

Left end:

```
         10          20          30          40          50          60
CATGATGAAA TAACATAAGG TGGTCCCGTC GAAAGCCGAA GCTTACCGAA GTATACACTT
GTACTACTTT ATTGTATTCC ACCAGGGCAG CTTTCGGCTT CGAATGGCTT CATATGTGAA
```

Right end:

```
          2860        2870        2880        2890        2900
AA GCATACGTTA ACTGGATGTC TCTTGCCGAC GGGACCACCT TATGTTATTT CATCATG
TT CGTATGCAAT TCACCTACAG AGAACGGCTG CCCTGGTGGA ATACAATAAA GTAGTAC
```

5' CGACGGGACCACCTTATGTTATTTCATCATG **3' P31**

5' GTGTATACTTCGGTAAGCTTCGG **3' Pleft; PL**

5' AGCATACGTTAAGTGGATGTCTC **3' Pright; PR**

Figure 3.4 P-element specific primers.

DNA sequence of the left and right ends of the 2.9 kb P element. Primers used in PCR reactions are indicated in colour. (Sequence taken from O'Hare and Rubin, 1983; accession no. V01520).

pairs for *dPKC98F* and *syb* are approximately 400 bp and 1010 bp, respectively (Figure 3.5a). For *syb*, this increase in size corresponds to the first two introns described in a later study by Chin *et al.*, (1993). Gene primers to *syt* and *cam* gave PCR products of anticipated size with cDNA, but no product with genomic DNA (Figure 3.5b and 3.5c). Further analyses published on the exon-intron structure of both the *syt* and *cam* genes confirmed there are introns of sufficient size to prevent amplification with the primers chosen (Littleton *et al.*, 1993b; Ohsako *et al.*, 1993).

3.5 Isolation of insertions in *dgo*, *DGα1* and *DC0*

Eight thousand mutagenised females were generated in batches of two thousand each. Eggs produced by females were screened using PCR and sib-selection as described above (see section 3.3). Eight thousand females were screened with some gene primer pairs, but not others. The number of females screened with respect to each target gene, and the results, are presented in Table 3.2. Of the target genes selected, putative P element insertions were obtained in or near the genes encoding $DG\alpha_o$, DGα1 and DC0. The original isolation of these insertions was carried out by T. Davis (Lineruth *et al.*, 1992). Subsequent verification that insertions were within these genes was carried out as described below. No P elements were detected in the genes encoding PKC, APPL, CAM kinase, synaptobrevin or synaptotagmin.

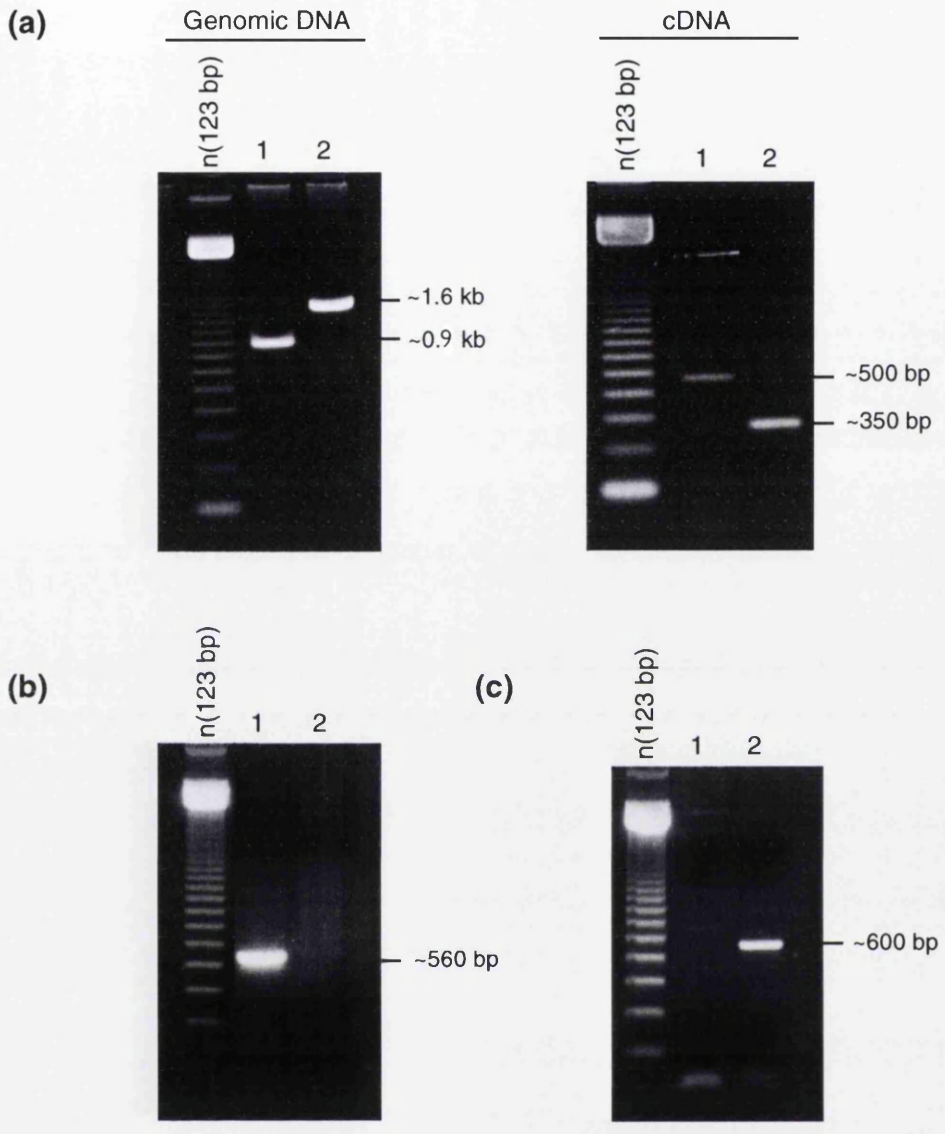


Figure 3.5 Amplification of genes encoding PKC, synaptobrevin and CAM kinase II using genomic DNA*vs* cDNA templates.

(a) Genomic DNA and DNA prepared from a cDNA library was amplified with (1) *PKC98F* gene primers (P1 and P2); and (2) *syb* gene primers (S1 and S2).

(b) DNA prepared from a cDNA library (1), and genomic DNA (2), was amplified with *cam* gene specific primers (CK1 and CK2).

(c) DNA prepared from a cDNA library (1), and genomic DNA (2), was amplified with *syt* gene specific primers (ST1 and ST2).

Amplification products were visualised on 1% agarose gels, stained with ethidium bromide. n(123 bp) corresponds to 123 bp DNA ladder (BRL).

Table 3.2 Results of Mutagenesis Screen.

Gene	*No. of mutagenised females screened*	*No. of insertions obtained*
G proteins		
DGα1	6,000	3*
dgo	6,000	3
$DG\alpha_s$	6,000	0
Second messenger-related genes		
DC0	4,000	3
dPKC98F	8,000	0
cam	2,000	0
Synaptic vesicle proteins		
syn	8,000	0
syt	6,000	0
APPL	6,000	0

*Only one insertion in DGα1 has been confirmed by DNA sequence analysis.

3.5.1 *Insertions in* dgo

Four independent P element insertions were detected in *dgo* gene using the gene primer Gu and P element primer P31. Three insertions gave a PCR product of ~1.4 kb, and were designated dgo^1, dgo^2 and dgo^3. A fourth insertion, designated dgo^4, gave a PCR product of ~1.6 kb. The organisation of the *dgo* locus is illustrated in Figure 3.6, and shows the putative *dgo* transcripts that correspond to the two classes of cDNA described by Yoon *et al.*, (1988). Also shown in Figure 3.6 is the position of gene specific primers

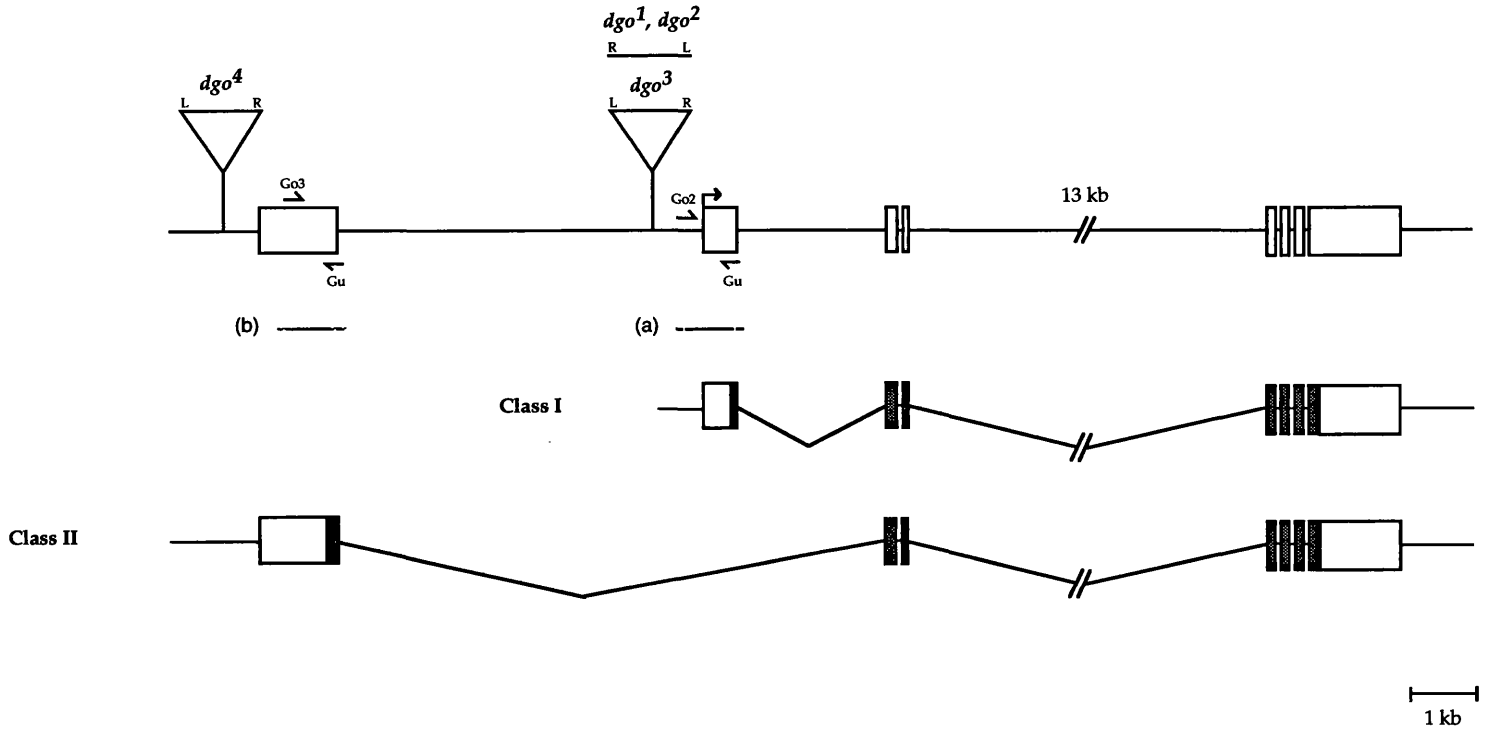


Figure 3.6 P element insertions in *dgo*.

The structure of the *dgo* locus is as described by Yoon *et al.*, (1989). Two classes of cDNAs isolated, Class I and Class II are shown below. The positions of cDNA exons are indicated as open boxes. P element insertions are indicated by inverted triangles, and the *dgo* specific PCR primers used to detect insertions indicated by small arrows. Non-coding regions in Class I and Class II cDNA are indicated by open boxes, and the coding regions are indicated by shading. Class I and Class II specific PCR products used as probes are labelled (a) and (b).

and P element insertions. To determine the orientation of the P element, PCR was carried out using P element primers PL and PR in conjunction with Gu. Amplification of dgo^1, dgo^2 and dgo^3 insertions was dependent upon PCR with primers PL, PL and PR respectively. Amplification of dgo^4 was dependent upon PR and Gu primers. PCR products, visualised on an ethidium bromide stained agarose gel, are shown in Figure 3.7a. Insertions upstream of the first exon of Class I or Class II transcripts were distinguished by hybridisation to Class specific probes generated by PCR: a Class I specific PCR product was amplified with primers Gu and Go2, and a Class II with primers Gu and Go3 (see Figure 3.6). A Southern blot of PCR products hybridised with specific ^{32}P-labelled DNA probes is shown in Figure 3.7b and 3.7c. Based on the size of PCR products dgo^1, dgo^2 and dgo^3 have P elements at the same position ~680 bp upstream of the putative start of transcription for the Class I transcript. The dgo^4 insertion had a P element positioned ~1.4 kb upstream of the translational start site found in the first exon of the Class II transcript. The Class II transcription initiation site has not been described, but does not reside in the sequences between P element and the start of translation (Yoon *et al.*, 1989).

3.5.2 *Insertions in* DGα1

A P element amplified by primers Gu and P31 gave a PCR product of ~520 bp. The result of PCR analysis with Gu and PR, separated on an agarose gel and stained with ethidium bromide, is shown in Figure 3.8a. The PCR product was hybridised with a ^{32}P-labelled *DGα1* specific DNA probe (amplified from *DGα1* specific primers) is shown in Figure 3.8b. The structure of the *DGα1* locus, based on information given in Provost *et al.*, (1988), is illustrated in

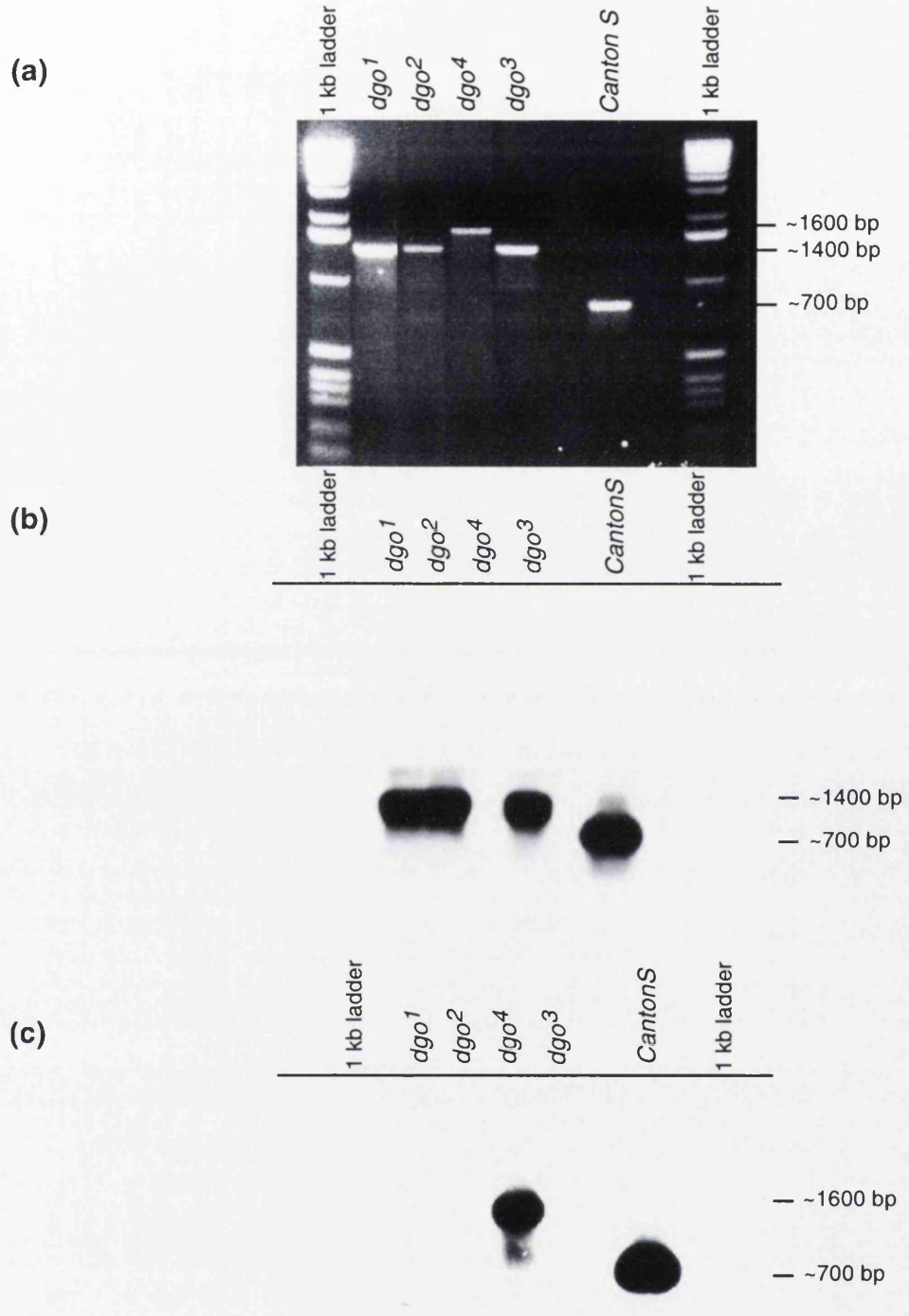


Figure 3.7 PCR detection of insertions in *dgo*.

(a) The P element insertions near Class I and Class II *dgo* cDNAs were amplified with gene specific primers: Class I, Gu and Go2; Class II, Gu and Go3. *Canton S* DNA was amplified with Go2 and Gu. PCR products were visualised in a 1% agarose gel, stained with ethidium bromide. n(123 bp) corresponds to 123 bp DNA ladder (BRL).

(b) and (c) Autoradiographs. *dgo* specific PCR products were probed with Class I specific PCR probe, (b); and Class II specific PCR probe, (c) [see Figure 3.6].

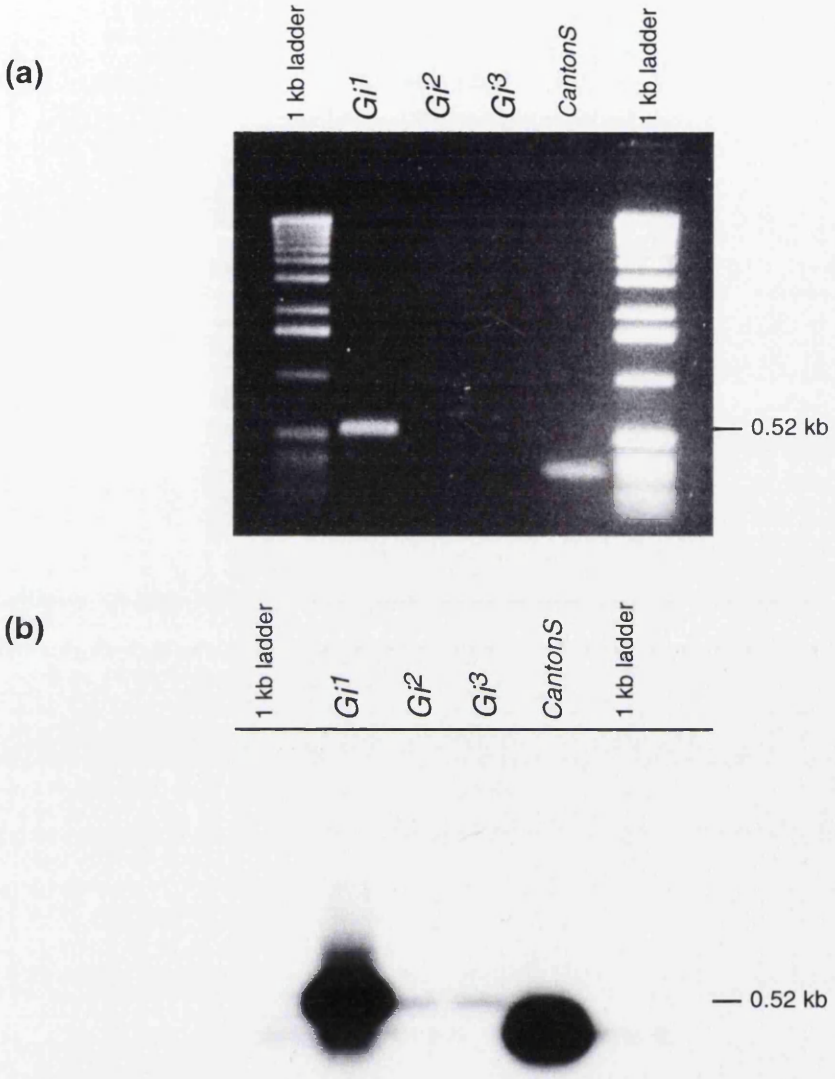


Figure 3.8 PCR detection of P element insertions in *DGα1*.

(a) Amplification of the P element in *DGα1* with gene specific primers Gu and Gi4. *Canton S* DNA was amplified with Gi4 and Gu. PCR products were visualised in a 1% agarose gel, stained with ethidium bromide. I kb ladder corresponds to 1 kb DNA ladder (BRL).
(b) Autoradiograph. PCR products were hybridised with a *DGα1* specific PCR probe (see Figure 3.9).

Figure 3.9. Also shown in Figure 3.9 is the position of gene specific primers and P element insertion. Based on the size of PCR fragments, the P element in Gi^1 is positioned upstream of the $DG\alpha 1$ gene, approximately 400 bp from the start of translation (Provost *et al.*, 1988).

In addition to Gi^1, two other hybridisation signals were detected in Southern blots using the $DG\alpha 1$ specific PCR probe (see Figure 3.8b). The hybridising fragments migrate at a similar position to Gi^1, but no amplification products corresponding to the signals were seen on an ethidium bromide stained agarose gel (see Figure 3.8a). The origin of these amplified fragments is unclear, but the absence of a visible PCR product hampered purification and subsequent sequence analysis. These insertions are therefore tentatively designated Gi^2 and Gi^3.

3.5.3 *Insertions in* DC0

Three independent P element insertions were isolated in or near the *DC0* gene and gave amplification products of approximately 1.8 kb, 300 bp and 900 bp (Figure 3.10a). The structure of the *DC0* gene, showing the position of primers and P element insertions, is illustrated in Figure 3.11 (based on information presented by Kalderon and Rubin, 1988). The insertion associated with a PCR product of ~1.8 kb, designated $DC0^1$, was amplified with DC11 and PL. A PCR product of ~900 bp, designated $DC0^3$, was amplified with primers DC12 and PL. The smallest PCR fragment of ~300 bp, designated $DC0^2$, was amplified with DC11 and PR. The insertion in DC0-2 could also be amplified with DC12 and PR to give a DNA fragment of ~250 bp

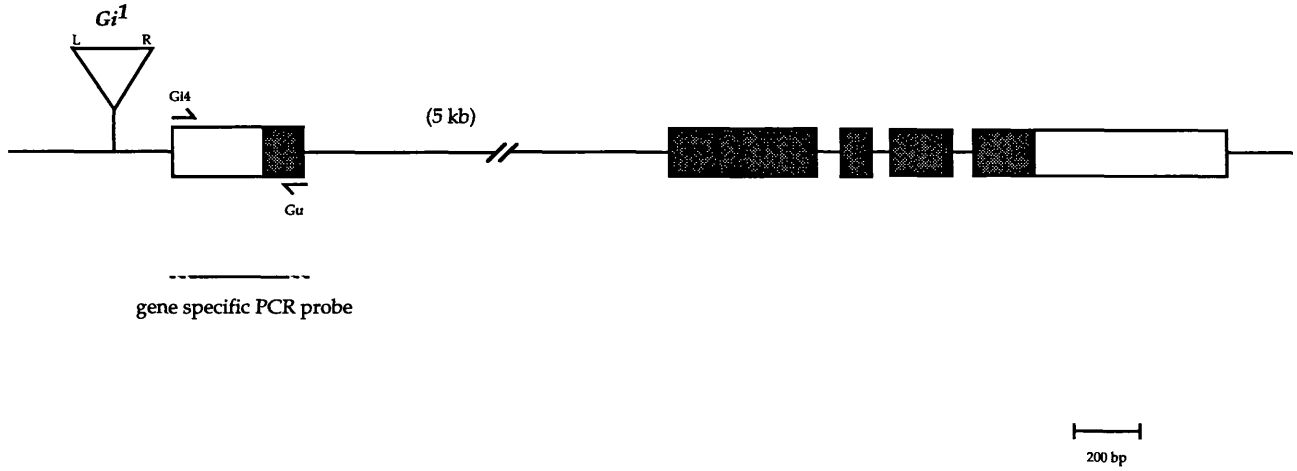
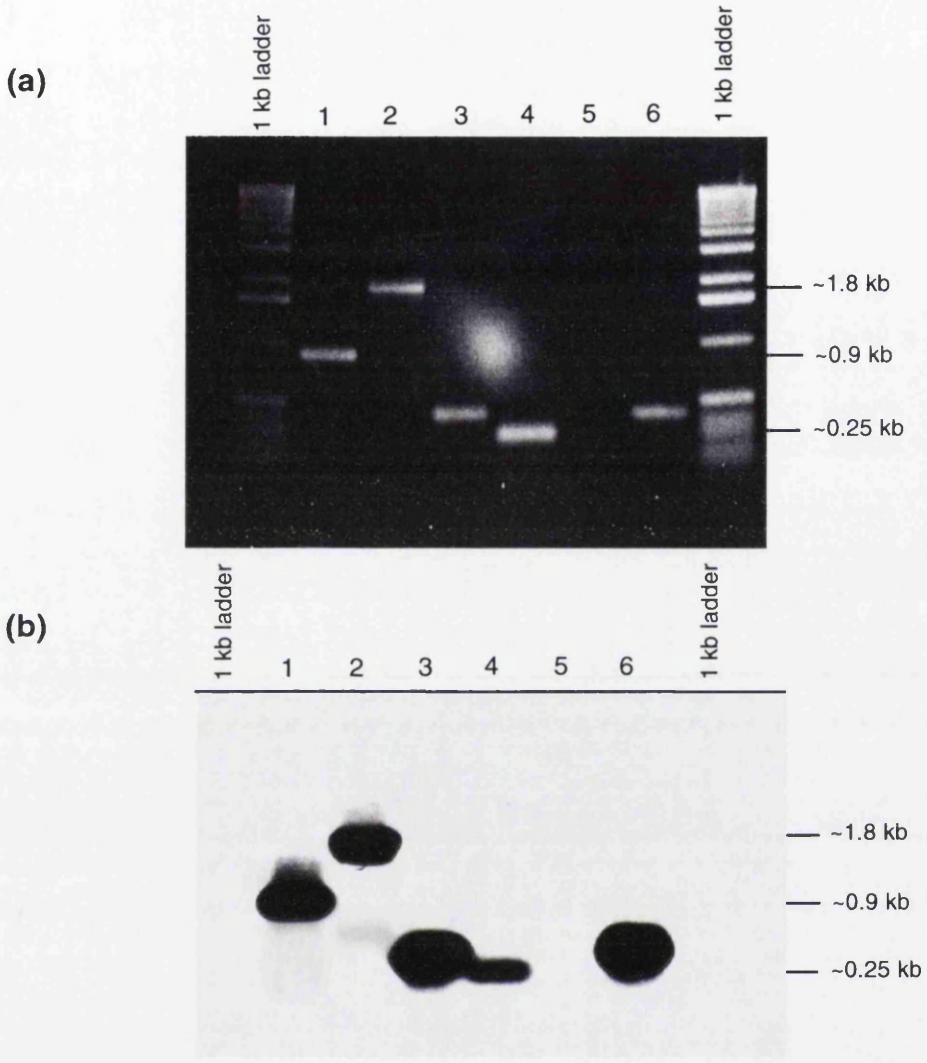


Figure 3.9 P element insertions in *DGα1*.

The organisation of the *DGα1* locus is as described by Provost *et al.*, (1988). Non-coding regions are indicated by open boxes, coding regions are indicated by shaded boxes. The P element insertions are shown as inverted triangles, and the PCR primers used are indicated by small arrows. The *DGα1* specific PCR probe is shown below.



Lane 1: *DC0*^{3} amplified with DC12 and PL.

Lane 2: *DC0*^{1} amplified with DC11 and PL.

Lane 3: *DC0*^{2} amplified with DC11 and PR.

Lane 4: *DC0*^{2} amplified with DC12 and PR.

Lane 5: *DC0*^{2} amplified with PR only.

Lane 6: *Canton S* amplified with DC11 and DC12.

Figure 3.10 PCR detection of P element insertions in *DC0*.

(a) Amplification of P element insertions in the *DC0* locus with primer combinations shown above. PCR products were visualised in a 1% agarose gel, stained with ethidium bromide. 1 kb ladder corresponds to 1 kb DNA ladder (BRL).

(b) Autoradiograph. PCR products were hybridised with a *DC0* specific PCR probe (see Figure 3.11).

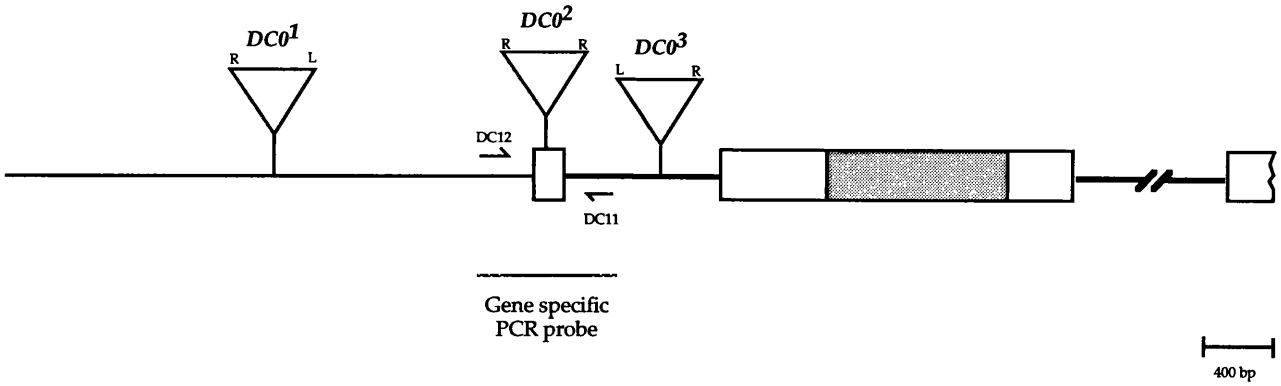
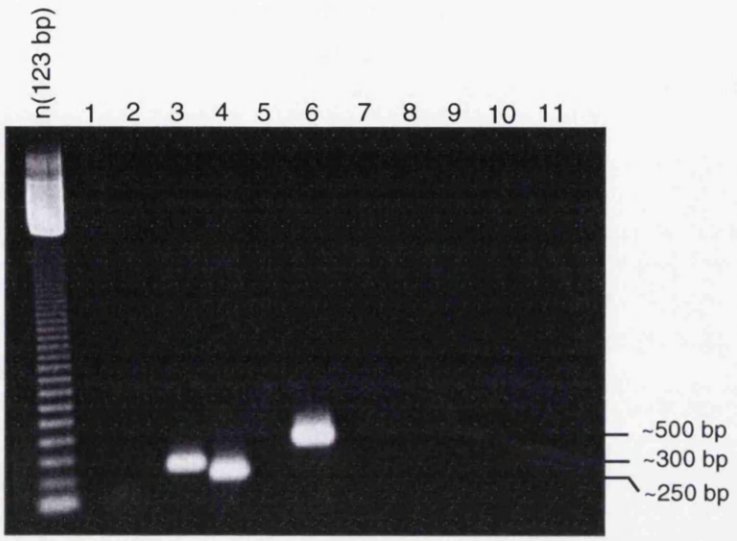


Figure 3.11 P element insertions in the *DC0* locus.

The organisation of the *DC0* locus is as described by Kalderon and Rubin, (1988). Non-coding exons of the *DC0* gene are indicated by open boxes, coding region by shaded boxes. The P element insertions are indicated by inverted triangles, and gene specific primers by small arrows. Shown below is the *DC0* specific PCR product used as a probe.

(see Figure 3.10a). PCR products were hybridised with a *DC0* specific ^{32}P-labelled probe, corresponding to the sequence between gene primers DC11 and DC12 (Figure 3.10b).

Based on the size of PCR products, the P element in $DC0^1$ is located upstream of the *DC0* gene ~1.6 kb from the start of transcription, and $DC0^3$ is located in the first intron (see Figure 3.11). $DC0^2$ contains a P element inserted between primers DC11 and DC12 in the first non-coding exon of the gene, close to the transcription initiation site (Kalderon and Rubin, 1988). The P element in $DC0^2$ was unusual since gene primers that flank the insertion site give PCR products in conjunction with PR only, and not with PL and PR as expected. To obtain further information regarding the overall configuration of the P element and surrounding DNA, amplification with various primer combinations was carried out with DNA prepared from flies homozygous for the chromosome containing the $DC0^2$ insertion. As shown in the ethidium stained agarose gel in Figure 3.12, a PCR product was observed only when PR was used in conjunction with DC11 and DC12. No amplification occurred with primers DC11 and DC12 on $DC0^2$ homozygotes. This lack of amplification across the P element insertion site could be explained by the size of the insertion, for example if two P elements had inserted end-to-end. To determine the size of the inserted P element, genomic DNA was prepared from $DC0^2$ homozygotes, and from $DC0^3$ heterozygotes. DNA was digested with *Bam*HI and *Sma*I, separated on an agarose gel and probed with ^{32}P-labelled pDC01.8 which contains sequence corresponding to the *DC0* transcription unit (clone kindly provided by D. Kalderon; see Figure 3.11). As shown in Southern analysis, the hybridising *Bam*HI-*Sma*I fragment observed in wild-type DNA had increased in size from ~7 kb to ~8 kb in both



Lane 1: *DC0*^{2} amplified with DC11 and PL.
Lane 2: *DC0*^{2} amplified with DC12 and PL.
Lane 3: *DC0*^{2} amplified with DC11 and PR.
Lane 4: *DC0*^{2} amplified with DC12 and PR.
Lane 5: *DC0*^{2} amplified with PL and PR.
Lane 6: *Canton S* amplified with DC11 and DC12.
Lane 7: *DC0*^{2} amplified with DC11 and DC12.
Lane 8: *DC0*^{2} amplified with DC11 only.
Lane 9: *DC0*^{2} amplified with DC12 only.
Lane 10: *DC0*^{2} amplified with PL only.
Lane 11: *DC0*^{2} amplified with PR only.

Figure 3.12 PCR analysis of P element insertion in *DC0*^{2}.

Amplification of the insertion line *DC0*^{2} with various primer combinations. PCR products were separated on a 1% agarose gel stained with ethidium bromide. Amplification of *DC0*^{2} occurs when PR is in combination with DC11 or DC12. No amplification is observed with PL with either gene specific primer. n(123 bp) corresponds to 123 bp ladder (BRL).

DC0^2 and *DC0*^{3} (Figure 3.13). The P element is inserted in both lines is approximately 1 kb and therefore the size of inserted DNA is unlikely to prevent amplification across the insertion site. The basis of this PCR result is so far unknown.

3.6 Sequence analysis

To determine the precise location of P element insertions, PCR products were purified, sub-cloned into pBluescript and sequenced (see sections 2.3.6, 2.3.7 and 2.3.10).

dgo^1, *dgo*^{2}, *dgo*^{3} *and* *dgo*^{4}

The nucleotide sequence from the P element side of the insertions in *dgo*^{1}, *dgo*^{2} and *dgo*^{3} is shown in Figure 3.14. Sequence analysis verified that the point of insertion is identical for *dgo*^{1}, *dgo*^{2} and *dgo*^{3}. Sequence obtained from the gene specific side of the PCR fragment for the three insertion lines, aligned to Class I cDNA sequence confirmed that insertions were in the *dgo* gene. Shown in Figure 3.15 is the sequence obtained from the gene specific side of the PCR fragment from *dgo*^{1}, aligned to Class I cDNA sequence, as an example of the insertions in *dgo*^{1}, *dgo*^{2} and *dgo*^{3} (Figure 3.15; Yoon *et al.*, 1989).

The PCR product from *dgo*^{4} was sequenced from both ends and aligned to *dgo* Class II cDNA sequences. Sequence analysis from the gene specific side of the PCR product confirmed that *dgo*^{4}had a P element insertion upstream of the Class II cDNA (Figure 3.16a; Yoon *et al.*, 1989). Also shown is the sequence obtained flanking the P element insertion site, aligned to sequences upstream of the Class II first exon (Figure 3.16b).

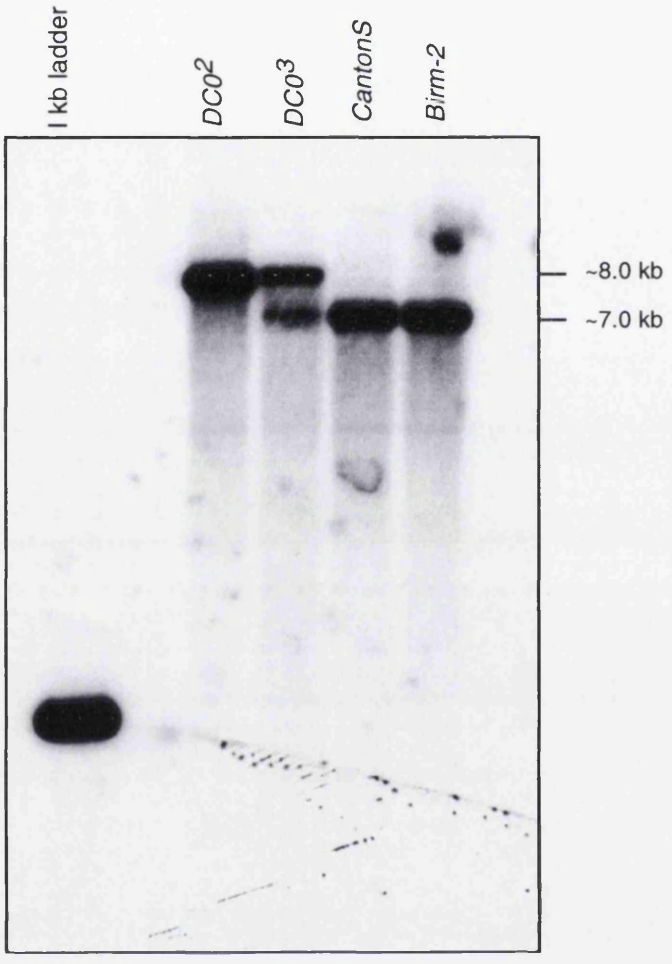


Figure 3.13 Genomic Southern analysis of *DC0²*.

Genomic Southern blot analysis to determine the size of the P element inserted in *DC0²* and *DC0³*. Genomic DNA from *DC0²* and *DC0³* homozygotes, *Canton S* and *Birm-2* was digested with *Bam*HI and *Sma*I. DNA was separated on a 0.8% agarose gel, blotted and hybridised with a *DC0* probe, pDC01.8. 1 kb ladder corresponds to 1 kb DNA ladder (BRL).

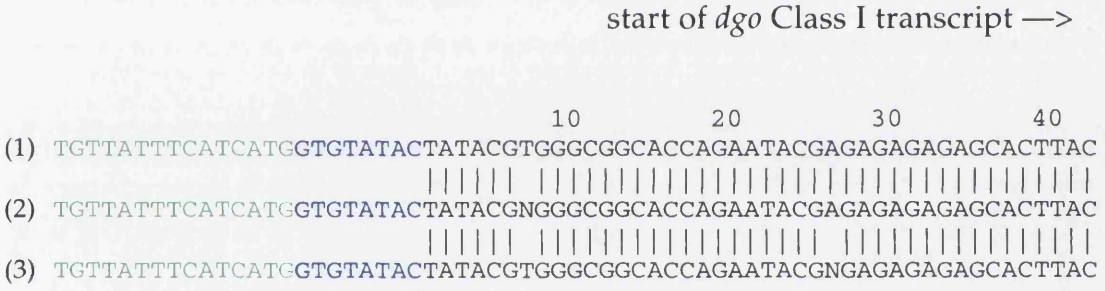


Figure 3.14 DNA sequence flanking P element insertions in *dgo*^{1}, *dgo*^{2} and *dgo*^{3}.

DNA sequence flanking the P element in *dgo*^{1}, (1); *dgo*^{2}, (2); and *dgo*^{3}, (3), is shown in black, with the putative 8 bp duplication shown in blue (only sequenced from one side). Sequence from the P element 31 bp terminal inverted repeat of the P element is shown in green.

```
SCORES      Initl:  391  Initn:  427 Opt:  455
              98.1% identity in 107 bp overlap

                  730         740         750         760         770
dgo1    TGCGCACAGTCTGCCGAGGAGCGAGCCGCAGCCACCAGGAGTCGCCTCATC
        ||||||||||||||||||||||||||||||| ||||||||||||||||||
Goa-dm  TGCGCACAGTCTGCCGAGGAGCGAGCCGCAGCCGCCAGGAGTCGCCTCATC

                780         790         800         810         820
dgo1    GAGCGCAATCTCAAGGAGGACGGCATACAGGCGGCAAAGGCACTCAA-CT
        |||||||||||||||||||||||||||||||||||||||||||||| ||
Goa-dm  GAGCGCAATCTCAAGGAGGACGGCATACAGGCGGCAAAGGCACTCAAACT

                830
dgo1    CCTGCTGCTGG
        ||||||||||||
Goa-dm  CCTGCTGCTGG
```

Figure 3.15 DNA sequence from *dgo*^{1}.

DNA sequence of the *dgo*^{1} PCR fragment from the gene specific primer side, aligned to *dgo* Class I cDNA (Yoon *et al.*, 1989). Gene specific primer, Gu, is shown in blue (see Table 3.1). Numbering refers to sequence of the *dgo* Class I cDNA under Accession number J05083.

(a)

```
Goaii.rev    120 ACCCAGCAGCAGGAGCTTGATGTCCTTGGCCGCCTGGATTCCATCCT  73
                 ||||||||||||||||||||||||||||||||||||||||||||||
dgo4         120 ACCCAGCAGCAGGAGCTTGATGTCCTTGGCCGCCTGGATTCCATCCT  73

Goaii.rev    72  CCTTTAGATTCTTCTCGATCTGTTTGGATCGCTGGATGGCGGCGCGT  26
                 ||||||||||||||||||||||||||||||||||||||||||||||
dgo4         72  CCTTTAGATTCTTCTCGATCTGTTTGGATCGCTGGATGGCGGCGCGT  26
```

(b)

```
                  1474
Goaii        AGTACCTCGCTTGCTTCGTCTCTCTCGTCGATTTAAAAGCGTGTGGTCTT  1518
                  ||||||||||||||||||||||||||||||||||||||||||||||
dgo4              CTCGCTTGCTTCGTCTCTCTCGTCGATTTAAAAGCGTGTGGTCTT  1518

Goaii rev    TTCACGTTAAATGGT  1533
             ||||||||||||||||
dgo4         TTCACGTTAAATGGT  1533
```

Figure 3.16 DNA sequence from *dgo*^{4}.

(a) DNA sequence from the gene specific end of the *dgo*^{4} PCR product. Gene specific primer, Gu, is shown in red. Numbering refers to *dgo* Class II cDNA sequence (Yoon *et al.*, 1989; accession no. J05983).
(b) DNA sequence from the P element end of the *dgo*^{4} PCR product. The last 5 nucleotides of the P element terminal inverted repeat are shown in green. The putative 8 bp duplication is shown in blue.
Numbering as above.

Gi^1

The PCR product from *Gi^1* was sequenced from both ends. Sequence obtained from the gene specific side of the PCR product is shown aligned to *DGα1* gene sequence (Figure 3.17a; Provost *et al.*, 1988). Sequence analysis confirmed that *Gi^1* had a P element inserted in the *DGα1* gene. Also shown is the sequence obtained flanking the P element insertion site, upstream of the *DGα1* gene (Figure 3.17b).

$DC0^2$ and $DC0^3$

The nucleotide sequence obtained from the ends of the PCR product purified from *$DC0^3$* are shown aligned to *DC0* gene sequences in Figure 3.18. Sequence analysis confirmed the insertion in *$DC0^3$* lies within the first intron of the *DC0* gene (Kalderon and Rubin, 1988). The insertion in line *$DC0^2$* lies between gene primers DC11 and DC12, and so DNA sequence flanking the insertion point was obtained. Sequence analysis confirmed the position of the P element within the first non-coding exon of *DC0* (Figure 3.19a). DNA sequence from the junction of the transposon and the *DC0* gene contains an 8 bp sequence duplication, a feature commonly found at the site of insertion of P elements (Figure 3.19b; O'Hare and Rubin, 1983; see section 3.9). The insertion in *$DC0^2$* is located precisely 71 bp downstream of the transcription start site of the *DC0* gene.

3.7 Isogenisation of insertion lines

A disadvantage of a multiple P element mutagenesis is the resulting extraneous P elements in the genome. To gauge the number of P elements in each strain, DNA was prepared from *dgo^1*, *dgo^2*, *dgo^3*, *dgo^4*, *$DC0^1$*, *$DC0^2$*,

(a)

```
SCORES      Initl:  140  Initn:  239  Opt:  292
              98.8% identity in 110 bp overlap

Gi1 rev  133 GGTCGTCGTCGTCAAAGTGGAGGCTCCGCCGGGCGAGCGGGAGGC 88
             ||||||||||||||||||||||||||||||||||||||||||||
Gia.dm   133 GGTCGTCGTCGTCAAAGTGGAGGCTCCGCCGGGCGAGCGGGAGGC 88

Gi1 rev   89 GGGCGT--CGCGCTAGCTACAAGAAGCTGGCGAGCTACCGGAGGA 44
             ||||||  ||||||||||||||||||||||||||||||||||||
Gia.dm    89 GGGCGTCCCGCGCTAGCTACAAGAAGCTGGCGAGCTACCGGAGGA 44

Gi1 rev   43 ATAGCGCACGACATGAGTGTC 23
             |||||||||||||||||||||
Gia.dm    43 ATAGCGCACGACATGAGTGTC 23
```

(b)

```
                          10          20          30          40
TATTTCATCATGGCGCACAGCAAAATCAGCGGATAAGCAAATTAAAGCGACAAAAGAA

   50          60          70        78
TATTAAATCGGCTCTGCTGCAGCAAGAGCGTG
```

Figure 3.17 DNA sequence from *Gi*[1].

(a) DNA sequence of the *Gi*[1] PCR product from the gene specific primer, Gu, shown in red. Sequence is aligned to the *DGα1* gene (Provost *et al.*, 1988). Numbering is according to the *DGα1* gene DNA sequence under Accession number M23094.

(b) DNA sequence from the P element side of the *Gi*[1] PCR product. The P element 31 bp terminal inverted repeat is shown in green, and the putative 8 bp duplication is shown in blue.

Figure 3.18 DNA sequence from *DC0*^{3}.

DNA sequence from the *DC0*^{3} PCR product. Upper panel is the sequence from the gene specific primer, DC12, shown in red. Numbering refers to the published *DC0* gene sequence (Kalderon and Rubin, 1988) under Accession number X16969. Lower panel is DNA sequence from the P element end of the PCR product. Nucleotides from the terminal inverted repeat are shown in green, and the putative 8 bp duplication is shown in blue. Numbering is as upper panel.

(a)

```
SCORES      Initl:  232  Initn:  404  Opt:  412
              99.0% identity in 100 bp overlap

dco.dm   264 CAGACGGAATAGAACCCGCGCAGTAGGGATTACTCATGATTGCGCCGCAG 313
             ||||||||||||||||||||||||||||||||||||||||||||||||
dco2     264 CAGACGGAATAGAACCCGCGCAGTAGGGATTACTCATGATTGCGCCGCA- 313

dco.dm   314 CGGTTCACGCTGAGCGAGCTGCCTATCGCTGGCCTACTCACACCTGCGTG 363
             |||||||||||||||||||||||||||||||||||||||||||||||||
dco2     314 CGGTTCACGCTGAGCGAGCTGCCTATCGCTGGCCTACTCACACCTGCGTG 363
```

(b)

```
DC0+                    (444) GGTATTTTTAATCTAAGCCATCAATCCA

                                    <--PR          PR-->
DC02                TTTAATCTAAGCCATGATG------CATCATGATCTAAGCCATC

Gene primers  DC12 -->                                  <-- DC11
```

Figure 3.19 DNA sequence from *DC0*^{2}.

(a) DNA sequence from the gene specific side of the PCR product from *DC0*^{2} aligned to *DC0* gene sequence (Kalderon and Rubin, 1988). Gene primer DC12 is shown in red. Numbering refers to *DC0* gene sequence under Accession number X16969.
(b) DNA sequence changes at the P element insertion point in the *DC0* gene. P element sequence is indicated in green, and the 8 bp duplication found at the ends of the inserted P element is shown in blue. The sequence of the wild-type *DC0* gene (*DC0*$^+$) is shown above with numbering as in (a). The orientation of the P element primer PR and gene specific primers, DC12 and DC11 are shown with arrows.

DC0^3 and *Gi*^{1}, digested with the restriction enzyme *Bam*HI, separated on an agarose gel and blotted. The filter was probed with ^{32}P-labelled pBS25.7wc, an almost complete P element, and the result is shown in Figure 3.20 (pBS25.7wc is missing 29 bp of one inverted repeat; Karess and Rubin, 1984). Southern blot analysis indicates that *Bam*HI digested *Birm-2* DNA does not give the expected 17 bands, therefore only the minimum number of P elements could be determined. The number of P elements in each strain varied, from a minimum of two in *DC0*^{1} to a minimum of ten in *DC0*^{2}. The large number of P elements seen in some lines may represent P elements from the *Birm-2* chromosome which did not transpose or that jumped to sites in the same chromosome (see section 7.1).

To eliminate extraneous P elements, females positive for P element insertions in *dgo*^{1}, *DC0*^{2}, *DC0*^{3} and *Gi*^{1} were isogenised with a wild-type strain (*Canton S*) by outcrossing and were selected by PCR to allow free recombination to occur. Outcrossing of *Gi*^{1} required six generations, but *dgo*^{1}, *DC0*^{2} and *DC0*^{3} required twelve generations. After isogenisation, DNA was prepared from *DC0*^{2}, *DC0*^{3}, *dgo*^{1} and *Gi*^{1} was digested with *Bam*HI, separated on an agarose gel, blotted and probed with pBS25.7wc. Southern analysis indicated that only one P element remained in each line (Figure 3.21).

The insertion lines described were crossed to balancer strains; *Gi*^{1} to *TM3/TM6B*; *dgo*^{1}, *DC0*^{2}, *DC0*^{3} to *Sp/CyO*. In all insertion lines, flies homozygous for the P element containing chromosome were viable, fertile and presented no phenotype.

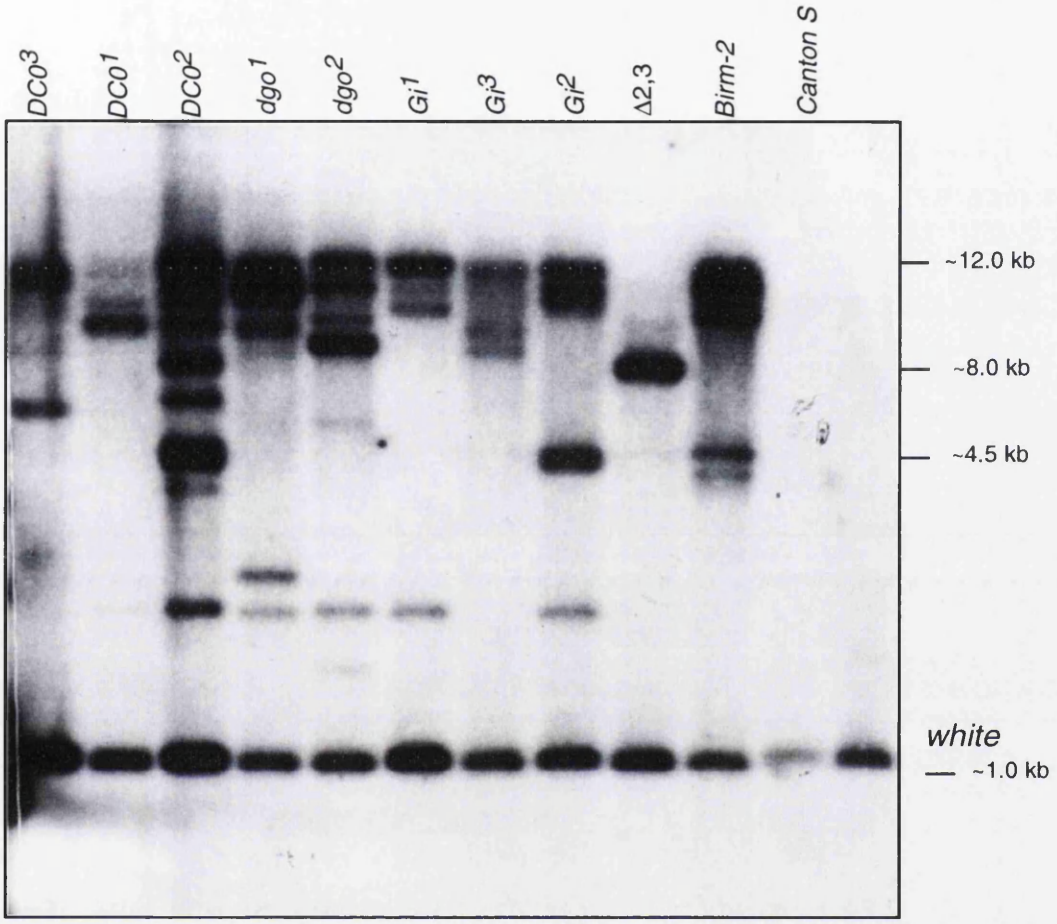


Figure 3.20 Genomic Southern analysis: number of P elements in insertion lines.

Genomic DNA from the indicated insertion lines, Δ2,3, *Birm-2* and *Canton S* was digested with *Bam*HI, separated on a 0.8% agarose gel, blotted and hybridised with a P element specific probe, pπ25.7wc. The common ~2.0 kb hybridising band labelled *white*, is a consequence of a small portion of the *white* locus captured within pπ25.7wc (Karess and Rubin, 1984).

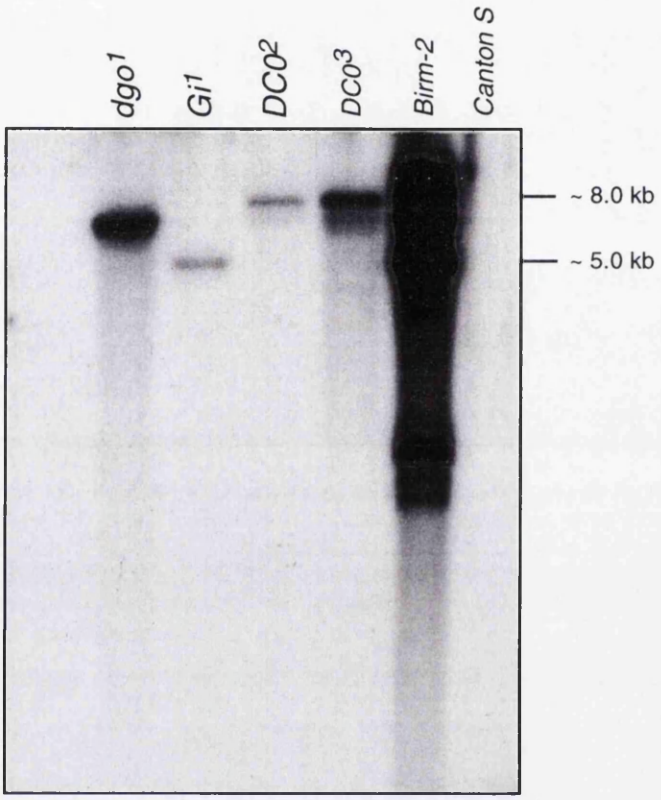


Figure 3.21 Genomic Southern analysis: number of P elements remaining in insertion lines.

Genomic Southern blot analysis to determine the number of P elements remaining in insertion lines after isogenisation. Genomic DNA from homozygous *dgo*^{1}, *Gi*^{1}, *DC0*^{2} and *DC0*^{2}; *Canton S* and *Birm-2* was digested with *Bam*HI. DNA was separated on a 0.8% agarose gel, blotted and hybridised with a P element probe, pBS25.7wc.

At this stage, further analysis continued with the *dgo*^{1} insertion line only. This decision was based on information that investigation of a DC0 mutant, isolated in the lab of R.L. Davis at Cold Spring Harbour Laboratories, was in advance of this work. Since DGα_o is expressed predominantly in the central nervous system of *Drosophila*, I considered *dgo* a more interesting gene to pursue in the context of possible mutant phenotypes that could arise from excision-induced disruption of the *dgo* locus (see chapter 4).

3.8 Disruption of the *dgo* locus does not affect transcription

DNA prepared from homozygous *dgo*^{1} flies was digested with *Bam*HI and *Sma*I, separated in an agarose gel and probed with a PCR product amplified from *dgo*^{1} DNA with primers Gu and PL. Southern blot analysis indicates that disruption of the *dgo* locus had occurred within a *Bam*HI-*Sma*I fragment spanning the insertion site. The wild-type 2.8-kb *Bam*HI-*Sma*I fragment has increased in size to 5.6 kb (Figure 3.22).

To investigate whether the P element insertion in *dgo*^{1} has any effect on *dgo* expression, Northern analysis was carried out. The *dgo* gene is the source of at least three distinct transcripts with different tissue distributions and developmental profiles (Thambi *et al.*, 1989; de Sousa *et al.*, 1989; Yoon *et al.*, 1989). The largest transcript of 5.4 kb is present primarily in RNA prepared from adult head, with very low levels in larvae and pupae. A 3.9 kb transcript is present in both head and body, and throughout development. The smallest transcript of 3.5 kb can only be detected in adult bodies. Although the P element in *dgo*^{1} is approximately 680 bp upstream of the

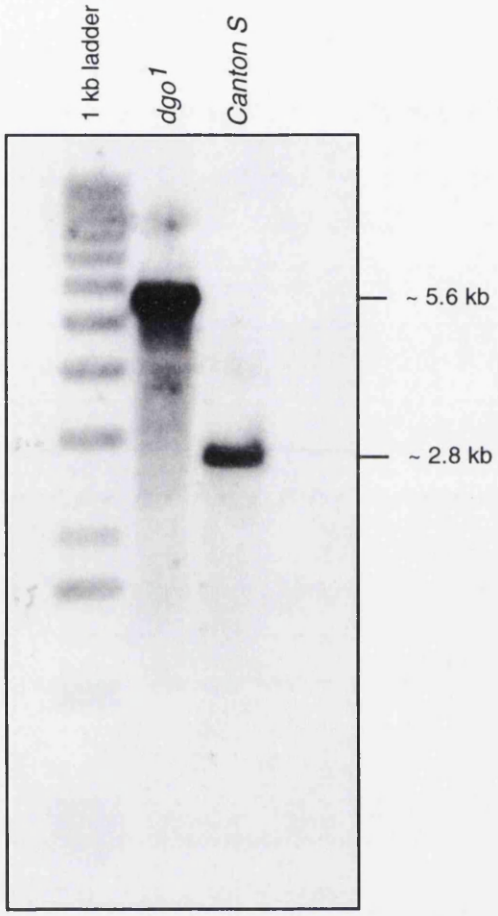


Figure 3.22 Genomic Southern analysis: disruption of the *dgo*^{1} locus.

Genomic DNA prepared from *dgo*^{1} homozygotes and *Canton S* was digested with *Bam*HI and *Sma*I. Digested DNA was separated on a 0.8% agarose gel, blotted and probed with a *dgo*^{1} specific PCR product, amplified using PCR primers and Gu and PL (see section 3.5.1). 1 kb ladder corresponds to 1 kb DNA ladder (BRL).

proposed transcription start site, P elements inserted near control regions have been shown to affect transcription of genes nearby (Tsubota *et al.*, 1985).

To determine if transcription from *dgo* is affected in dgo^1 mutants, poly(A)$^+$ RNA was prepared from heads and bodies of wild-type and dgo^1 homozygotes. Isolated RNA was hybridised with a DGα_o cDNA probe, DGo12, which corresponds to Class I cDNA (see Figure 3.7; DGo12 is as DGo59 described in Thambi *et al.*, (1989) less a region of 3' untranslated sequence, kindly provided by M. Forte). Northern analysis indicates that a transcript approximately 5.4 kb is present at high levels in head RNA and at very low levels in the body (Figure 3.23). A transcript of approximately 3.9 kb is present in head and body, and a 3.4-kb transcript is observed in bodies only. No change in expression in *dgo* can be detected in dgo^1 compared to wild-type.

3.9 Discussion

This chapter describes the use of site-selected mutagenesis to create P element insertion mutations in previously cloned genes in *Drosophila*. This is a general method that can be applied to any gene for which sequence data is available. Since isolation of P-induced mutations is accomplished by molecular methods, no information on possible mutant phenotypes is required. Thus silent mutations that could be overlooked in conventional screening strategies will be detected. In addition to the insertions described here, this method has been used to produce P-induced mutations in the gene encoding a microtubule associated protein (Pereira *et al.*, 1992), the *pourquois-pas* gene (Ségalat *et al.*, 1993), and a regulatory subunit (*RI*) of PKA (Goodwin, 1994). Site-selected mutagenesis is not limited to *Drosophila*.

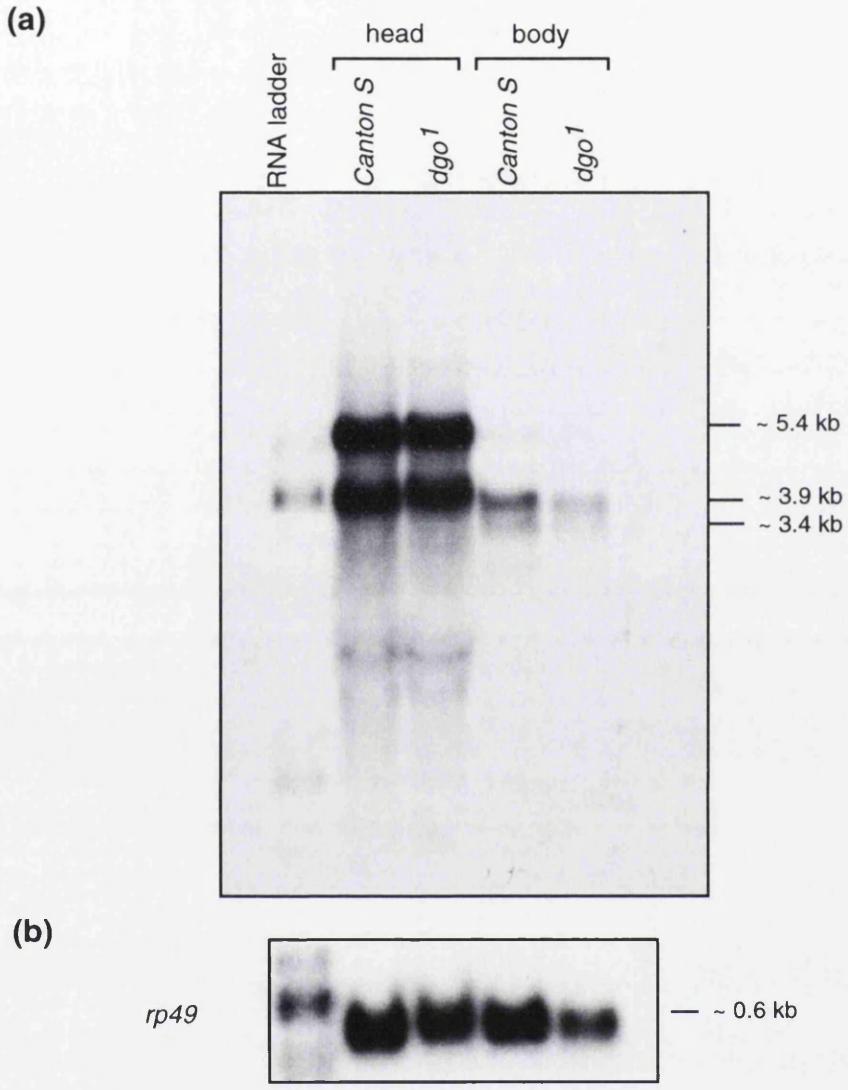


Figure 3.23 Northern analysis of *dgo*^{1}.

(a) Poly(A)$^{+}$ RNA (~5µg per lane) was prepared from adult head and body of *dgo*^{1} homozygotes and *Canton S*. RNA was separated on a 1% agarose/formaldehyde gel, blotted and hybridised with a *dgo* Class I cDNA probe, DGo12.

(b) The same blot was probed with a ribosomal probe, *rp49*, to check RNA integrity and the relative quantity of RNA in each lane.

Similar procedures have been used to isolate Tc1-induced mutations in a myosin light chain gene in *C. elegans*, (Rushforth *et al.*, 1993), and mutations at the *hcf106* locus in maize utilising the Mu1 element (Das and Martienssen, 1995).

Site-selected mutagenesis was used to successfully detect transposons in or near the genes encoding DGα_o, DGα1 and DC0 (Lineruth *et al.*, 1992). Flies containing these insertions are viable and fertile, showing no obvious phenotype. No insertions were obtained in the genes encoding PKC, APPL, CAM-kinase, synaptobrevin or synaptotagmin. As outlined in section 3.4, amplification with some primer pairs was complicated by the presence of introns between primer sites. Based on empirical observation, I estimate the maximum distance that a P element can be detected from a gene specific primer to be 3 kb or so. Thus the target area covered by only two primers in each gene described here is around 6 kb. Detection of a transposon in upstream regulatory regions of the genes encoding synaptobrevin, and in particular CAM kinase and synaptotagmin, would be hampered by the presence of introns which would effectively further reduce a relatively small target window for insertion. Using the mutagenesis scheme described, Robertson *et al.*, (1988), estimated that about 10 new insertions could be obtained per mutant genome. Since the *Drosophila* genome is approximately 1.65×10^5, the production of 10,000 mutagenised females would give a P element insertion approximately every 1.5 kilobases along the genome and a reasonable chance of a hit in a particular gene. So it could be argued that the relatively small number of flies screened for insertions for some target genes may have limited the probability of obtaining an insertion (Table 3.2). Another factor to consider, in addition to the number of flies

screened, is the non-random nature of P element insertion. The frequency of mutation of certain loci varies widely, from more than 10^{-3} at *singed* to less than 10^{-6} for the *ADH* gene (Kidwell, 1986; 1987; Engels, 1989). However, even if the maximum rate of 10 new insertions per genome is achieved and the number of flies screened exceeds 10,000, there is still no guarantee that a particular gene will be mutated.

The specificity of P element insertion is not clear, but may involve the 8 bp target sequence duplicated at the site of P element insertion, chromatin structure, or both (Engels, 1989). Based on the analysis of eighteen insertions at the *white* locus, O'Hare and Rubin, (1983), described an 8 bp target site consensus sequence: GGCCAGAC. From the sequences that flank the P element insertions, $DC0^2$, dgo^1, dgo^4 and Gi^1 match the consensus in 4 out of 8 bp. $DC0^2$ has a match of only 2 bp out of 8 bp. Although insertion site sequence cannot fully explain insertional specificity, P elements do transpose to preferred sites known as 'hot spots'. These have been observed in the *singed*, *white* and *notch* genes (Engels, 1989). The isolation of an enhancer trap element at the same nucleotide position as $DC0^2$ (DCO^{581}; Skoulakis *et al.*, 1993) and the isolation of three identical, independent P element insertions in *dgo* reinforces the theory of target site preference.

It has also been suggested that P elements insert near the transcription start sites of genes. In a general, but not comprehensive, survey of P element insertion sites published, it appears that insertions in 5' flanking sequences are more common (Tsubota *et al.*, 1985; Chia *et al.*, 1986; Searles *et al.*, 1986; Kelley *et al.*, 1987; Salz *et al.*, 1987; Roiha *et al.*, 1988; Levin *et al.*, 1992; Pereira *et al.*, 1992; Ségalat *et al.*, 1992). With the exception of $DC0^3$, all of the insertions described in this study are localised to 5' flanking

sequences. Insertions in the gene encoding $G\alpha_o$,*dgo*^{1}, *dgo*^{2} and *dgo*^{3}, are 680 bp from the transcription start site of the Class I transcript and the insertion in *DC0*^{2} is only 71 bp upstream of the transcription start site of *DC0*. Although *DC0*^{2} had a P element in the same position as the enhancer element insertion line DC0^{581}, it does not result in a recessive lethal phenotype. This could be due the size of the DC0^{581} element which is much larger than the defective P element in *DC0*^{2}, at approximately 18.8 kb. In addition to its larger size, the enhancer element also contains coding sequence for *lacZ* and *rosy* which may interfere with normal expression of the *DC0* gene (Horowitz and Berg, 1995).

Although site-selected mutagenesis was used with success to isolate P element insertions in *DC0*, *dgo* and *DGα1*, there are some features that limit its usefulness. Screening for insertions in a large number of target genes requires individual PCR reactions and hybridisations. Also, to ensure detection of all possible insertions it would be necessary to use several gene specific primers per target gene. While it is possible to use multiple primers in each PCR reaction, spurious products often result that can reduce the yield of specific ones (Clark *et al.*, 1994). One approach that overcomes these problems is inverse PCR in which pools of genomic DNA from mutagenised individuals is digested and circularised so that sequences flanking the insertion are amplified (Ochman *et al.*, 1989; Dalby *et al.*, 1994). Another approach utilises engineered P elements, P[*lacW*], containing a bacterial plasmid origin of replication and antibiotic resistance that allow plasmid rescue of flanking genomic DNA (Hamilton *et al.*, 1991). In both methods, captured DNA sequences can be labelled and used as probes to filters containing previously cloned cDNAs. Strategies involving capture of

flanking DNA allow assays for insertions to be carried out on a large number of cloned loci simultaneously so that insertions within and adjacent to genes will be readily detected.

The P elements in or near *dgo*, *DGα1*, and *DC0* are viable and fertile, showing no obvious phenotype. Although silent insertions are of limited use, they can be used to create new mutations in the gene by mobilising the P element from its original location. Such excision events are often imprecise and frequently result in removal of sequences flanking the P element insertion site (Ségalat *et al.*, 1992; Littleton *et al.*, 1993). The following chapter describes the mobilisation of the P element in dgo^1 and the subsequent analysis of excision events.

Chapter 4

P element Excision Mutagenesis

4.1 Introduction

The G protein alpha subunit, $G\alpha_o$, was originally purified from bovine brain as an extremely abundant 39 kDa protein and substrate for pertussis toxin (Sternweis and Robishaw, 1984; Neer *et al.*, 1984; see section 1.3.1). The gene encoding $G\alpha_o$ was subsequently cloned from rat brain (Itoh *et al.*, 1986) and shown to encode two proteins generated by alternative splicing (Hsu, *et al.*, 1990; Strathmann *et al.*, 1990). An antibody raised against vertebrate $G\alpha_o$ detects a 39-40 kDa protein in brain membranes from vertebrates, and invertebrates such as molluscs and insects (Homburger *et al.*, 1988). In addition, a pertussis toxin sensitive protein of 39 kDa was detected in membranes prepared from *Drosophila* heads, but not bodies (Hopkins *et al.*, 1988). These results suggested that a $G\alpha_o$-like protein was present in *Drosophila*, that could be utilised in neuronal signal transduction pathways similar to those in mammals.

The gene encoding the $G\alpha_o$ subunit in *Drosophila* (*dgo*) was cloned using bovine transducin and rat $G\alpha_o$ probes (de Sousa *et al.*, 1989: Thambi *et al.*, 1989; Yoon *et al.*, 1989). Similar to vertebrate $G\alpha_o$, *dgo* is alternatively spliced: two transcripts are encoded by seven exons, with the first exon being unique to each and spliced to six exons common to both (see Figure 4.1). This splicing results in two proteins 354 amino acids long that differ in only seven amino acids at the amino-terminus. Both forms of $DG\alpha_o$ have been shown to be pertussis toxin substrates and share 82% amino acid identity with vertebrate $G\alpha_o$ (Thambi *et al.*, 1989). Three *dgo* transcripts are detected in Northern blots of approximately 5.5 kb, 3.9 kb and 3.4 kb. The 5.5 kb transcript is expressed in the adult head, but not body, and is found at low levels during development (Yoon *et al.*, 1989). The 3.9 kb transcript is expressed in both heads and bodies in adult

Drosophila and is present throughout development. The smallest transcript of 3.4 kb is expressed in adult bodies only. The distribution of *dgo* RNA by *in-situ* hybridisation to tissue sections shows a strong signal in the central nervous system, with labelling in the cortex of the brain and lamina of the optic lobes. Expression was also detected in the cortex of the thoracic ganglion. Low levels of *dgo* RNA were detected in nurse cells and oocytes, suggesting the gene is maternally transcribed (de Sousa *et al.*, 1989).

The distribution of $DG\alpha_o$ protein in the central nervous system of *Drosophila* has been examined using antibodies against both $DG\alpha_o$ (Schmidt *et al.*, 1989), and vertebrate $G\alpha_o$ (Wolfgang *et al.*, 1990). Both classes of antibodies recognise a 39 kDa protein in membranes prepared from *Drosophila* heads. As observed with *in-situ* hybridisation, $DG\alpha_o$ is found in abundance in the central nervous system, but is found at higher levels in the neuropils of the brain and thoracic ganglion with lower levels in the cortical regions. Lower levels of $DG\alpha_o$ are also found in the neuropil of the optic lobe. This is consistent with $DG\alpha_o$ transcripts being detected in the cell bodies (cortices) and the protein being transported to the axon tracts (neuropil). During development, $DG\alpha_o$ protein is expressed during the earliest stages of embryogenesis which may represent maternal gene expression (Guillén *et al.*, 1991). At later stages there is a marked increase in $DG\alpha_o$ levels at the beginning of axonogenesis with expression localised to the axonal scaffold of the CNS.

The specific function of $DG\alpha_o$ in *Drosophila* is unknown, but its preferential localisation in the developing and adult nervous systems suggest a role in a neuronal transmembrane-signalling pathway. An increase in the levels of $DG\alpha_o$ has been observed in the *Drosophila*

behavioural mutants *rutabaga* and to a lesser extent, *turnip* (Guillén *et al.*, 1990).

The most direct way to determine the *in vivo* function of DGα_o in *Drosophila* is to create a mutation in the gene. P elements in or near a gene can be mobilised to create new alleles of the gene by imprecise excision, chromosomal rearrangements, or other changes (Tsubota *et al.*, 1986; Salz *et al.*, 1987; Ségalat *et al.*, 1992; Littleton *et al.*, 1993a; Engels, 1989; see section 1.2.7). By combining a source of transposase (Δ2,3) with a P element strain in a genetic cross, the types of event that could occur include precise loss or internal deletions of the P element, deletion of flanking sequences (generally unidirectional and can involve a few base pairs up to several kb of DNA), or P elements inserted at new sites in the genome. P element transposition leaves behind a double-strand break which is repaired using homologous sequences as template, usually a sister chromatid, but can be an homologous chromosome (Engels *et al.*, 1990; Gloor *et al.*, 1991). The widening of the DNA gap and an aberrant repair process is thought to lead to imprecise excision events. The rate of imprecise excision can be increased if, during the template-dependent repair process, pairing is prevented using a homologous chromosome containing a deletion or multiply inverted balancer chromosome (Engels *et al.*, 1990).

Described in chapter 3 was the isolation of P element insertions near the *dgo* gene (see section 3.6). Flies homozygous for the P element chromosomes are viable and present no phenotype. The following chapter describes the results of a second mutagenesis carried out to recover deletions generated by imprecise P element excision.

4.2 Mutagenesis and selection strategy

Four P element insertions were recovered near the *dgo* gene (see section 3.5.1; see Figure 3.6). Three insertions, dgo^1, dgo^2 and dgo^3, were at the same position ~680 bp upstream of the putative transcription start of Class I cDNA, and another ~1.4 kb upstream of the first exon of the Class II transcript (Yoon *et al.*, 1989). The dgo^1 insertion was mobilised since loss of flanking DNA by imprecise excision to the left or right could remove either Class I or Class II first exons, or both if the deletion was large enough. Also, the proximity of the P element in dgo^1 (~3.5 kb upstream) to the six coding exons shared by both classes of transcript increased the probability of removing coding sequences, and thereby creating a null phenotype. In an attempt to maximise the number of imprecise versus precise excision events, the P element bearing chromosome was heterozygous with a balancer chromosome during the mutagenesis.

The P element insertion in dgo^1 did not have a visible phenotype and no deletions were available at the *dgo* locus. This necessitated a molecular rather than genetical screen for transposase induced mutation. A PCR-based strategy was employed to detect the loss of a dgo^1 specific amplification product in DNA samples prepared from individual mutagenised lines. The PCR-fragment can be amplified with a primer positioned upstream of *dgo* Class I cDNA sequence and a P element primer (Figure 4.1). No amplification would occur if a deletion by imprecise excision removed one or both primer sites. It was hoped that this approach would identify events that include deletions that result in precise excision of the P element, precise excision of the P element and flanking DNA, and/or excisions originating from within the P element that extend to the left of the P element insertion site. Any internal

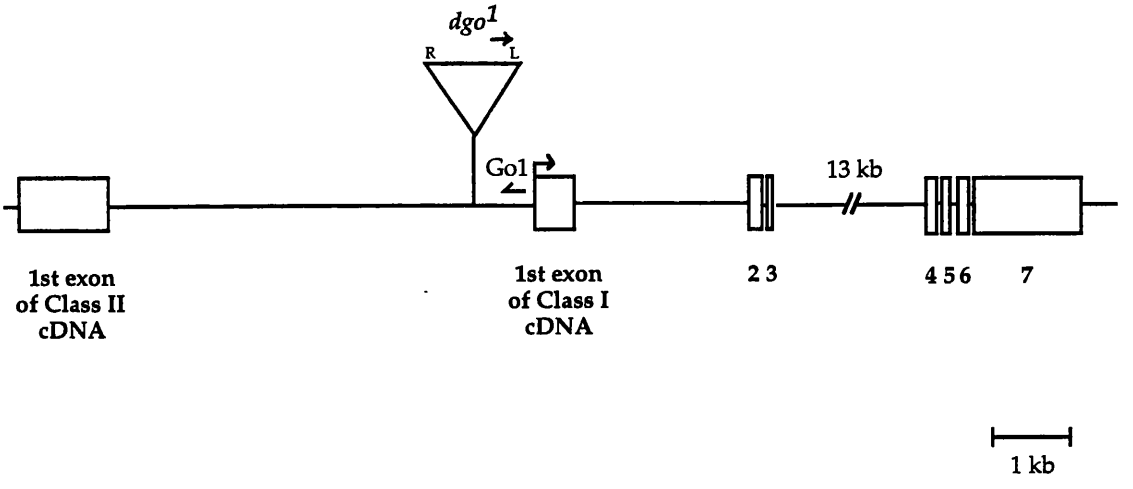


Figure 4.1 PCR strategy to detect deletions within the *dgo* locus.

The organisation of the *dgo* locus showing the position of both Class I and Class II first exons. Numbers below open boxes indicate exons. The position of the P element within dgo^1 is ~680 bp upstream of the Class I first exon and ~10.5 kb downstream of the Class II first exon. The position the gene specific primer (Go1) and P element primer (PL) used to screen for excision events are shown.

deletions within the P element that leave the left end of the P element intact, or deletions that originate within the P element and extend to the left of the insertion site would not be detected with this approach. Since there was no prior information available regarding possible mutant phenotypes occurring from disruption of the *dgo* gene, all fly lines that displayed any alteration during PCR analysis were retained for further genomic Southern analysis.

4.3 P element mobilisation

The dgo^1 insertion line contains a single P element as ascertained in Southern blot analysis (see Figure 3.22). In the genetic scheme outlined in Figure 4.2, flies homozygous for the dgo^1 insertion were crossed *en masse* to *w ; Sp/CyO ; Dr* P[ry^+ Δ2,3](99B)/TM6. F_1 *Dr;CyO* were then crossed *en masse* to the *Sco;CyO* balancer strain. Individual F_2 Dr^+;*CyO* progeny were mated with *Sco;CyO* to establish single lines. Approximately 1020 single lines were generated using this scheme and 1000 were screened for the loss of a PCR product as described below. Finally, brother/sister matings were made of lines retained after the PCR analysis to reveal any recessive phenotypes resulting from the mutagenesis.

4.4 PCR detection of possible deletion events

The P element insertion in dgo^1 can be amplified with primers Go1 and PL to give a PCR product of ~600 bp (see Figure 4.1). The Go1 primer is located in sequences upstream of the transcription start of the *dgo* Class I transcript (Figure 4.3), and PL is located just inside the P element terminal 31 bp inverted repeat (see Figure 3.4). As described above, Dr^+; *CyO* males and females were mated with *Sco/CyO* to establish single lines. After a

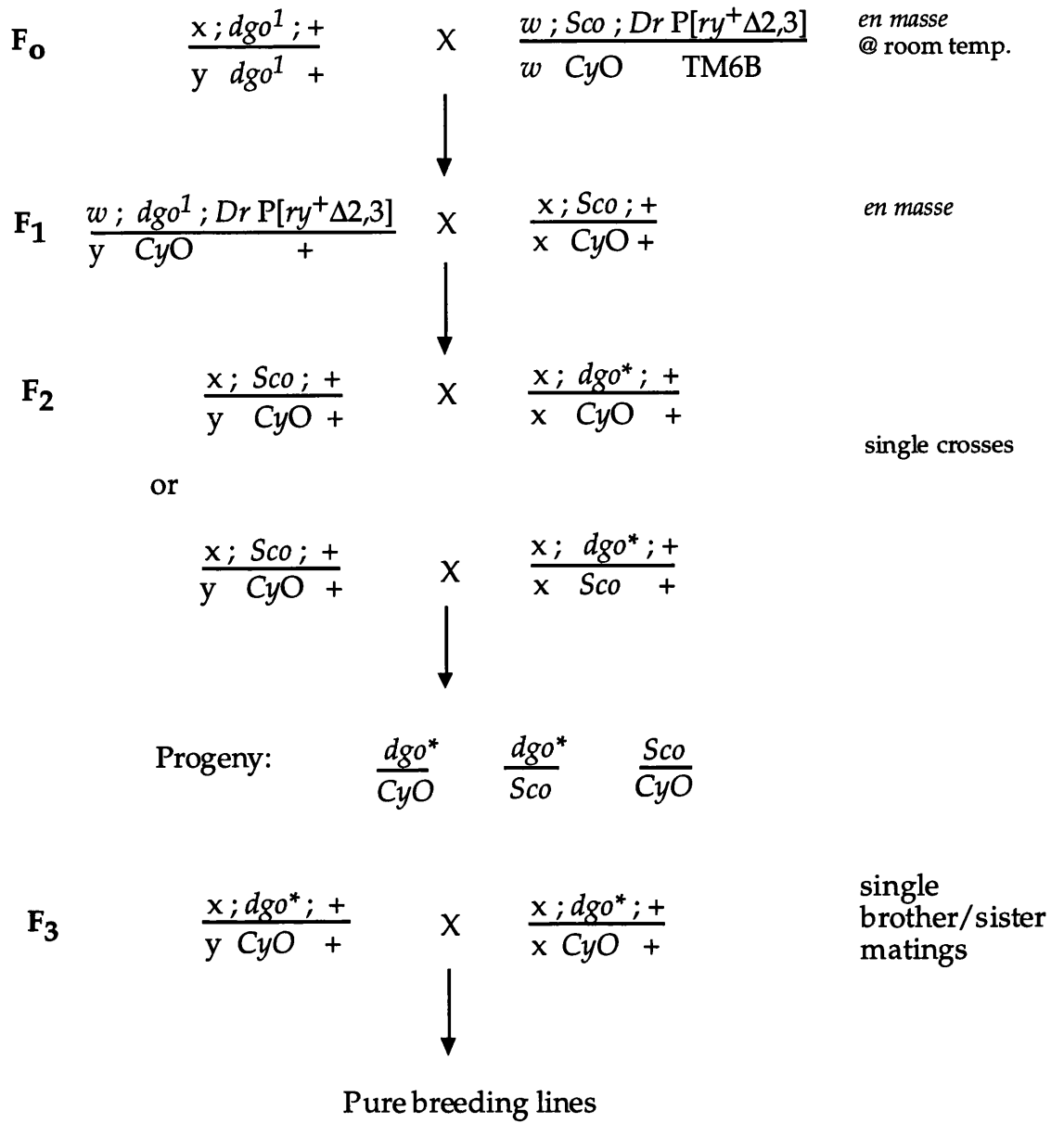


Figure 4.2 Generation of DGα_o excision lines.

The P element in *dgo*^{1} homozygotes was mobilised from the second chromosome by introducing a source of transposase, Δ2,3(99B) (Table 2.1). The chromosomes that could contain a excision are indicated with an asterisk (*). Reciprocal crosses were performed for all generations.

<-- P element in *dgo*^{1}

```
-714
ctcgttgcaatgaatagcaaatattacaaaaaaataacattttcacataaaaatataaa
-655
ataaaagaaaatgcagcagtcgtatATTATTGATTTTTGCCATATTTGTACGCATTTTG
-596
AGTTTTGGTTAATGAAAACCACGCTCCTTAATTACACATTTTTCCATGGCTCGAAAAAC
-537
CCTGGCCTAGAGCTTTTCTATTCGCTGCTTTCACTCTCTCTCTCCTTCGTTTCTAGTTT
-478
TTCCTAGCCAAAGACAACAAAATTTCCCGAGTTTCGTAGCGAGTAACGGGTTTCCTCGC
-419
TCTTGGACATGCGGGTTTTTCAGTTTACAATTAGACTCTACCGCTAGCGGTCAAATCGG
-360
GTATCCTTGAGAATCCCTTTTGCGCGGATCACAACAAAATGCGCAGATAAAAACAAAAC
-301
TGAATTGTTTGGTGACATAATTATATGTTGCAGTGTTGAATTGAAGCAAACCAAAGTTC
-242
AAAAACCTGAAAAAACCGAAAAGAAGTGATTGAAAAATCGAATATCGAGTTGAAGTGCC
-183
GTACTGAAAATTAAAGTCCAGTGACACGATCGAATCCCTCGGATAGCGGAGTTAGTTTA
-124
GCCCCCCGAATTCGAGTCCCCGCACGTTGTACACCTGGTTTTTCTCGCTGGCAACGTAG
-65
TCGGCCATTGAGTTGGCCGATACCAAACGACCTTCAAAACGTTTTGCGTCGAGGCAATA
-6
CGCACC.ATG.GGC.TGC.GCA.CAG.TCT.GCC.GAG.GAG.CGA.GCC.GCA.GCC.
        M   G   C   A   Q   S   A   E   E   R   A   A   A
39
GCC.AGG.AGT.CGC.CTC.ATC.GAG.CGC.AAT.CTC.AAG.GAG.GAC.GGC.ATA
 A   R   S   R   L   I   E   R   N   L   K   E   D   G   I
84
CAG.GCG.GCA.AAG.GAC.ATC.AAA.CTC.CTG.CTG.CTG
 Q   A   A   K   D   I   K   L   L   L   L
```

Figure 4.3 Position of primer Go1 in *dgo* DNA sequence.

Genomic sequence 5' to the start of *dgo* Class I cDNA and sequence of the first exon. Genomic sequence is indicated in lower case and transcribed sequence in upper case. The putative TATA box and transcription initiation site for Class I cDNA are indicated in red. The translation product of Class I cDNA first exon is given in single-letter code below cDNA sequence. The gene specific primer, Go1, used to screen for excision events is shown in blue.

few days, the parents were removed and their DNA extracted using a rapid single-fly DNA prep (see section 2.3.1). DNA from 1000 lines was amplified with Go1 and PL and analysed on agarose gels. Shown in Figure 4.4 are the results of PCR on 92 samples presented as typical of those obtained for the entire experiment. In most cases, a ~600 bp amplified fragment was observed, indicating no loss or disruption of primer sites in those lines. An absence of an amplified fragment in some lines (e.g. see lanes 323 and 955) could be due to an imprecise excision or rearrangement, but could also be attributed to failure of the PCR reaction. To distinguish these possibilities, samples were amplified with another set of gene primers (DC11, DC12) that give a PCR product of ~430 bp, and again with Go1 and PL (Figure 4.5). In some samples, a ~600 bp fragment was observed in the second amplification with Go1 and PL, indicating a failure of the first PCR reaction (e.g. see lanes 3 and 597). Failure to amplify either the ~600 bp or ~430 bp fragments was most likely due to a poor quality DNA preparation and the corresponding fly lines were discarded (e.g. see lanes 22 and 156). In 96 samples a ~430 bp fragment was amplified, but a ~600 bp fragment was not (e.g. see lanes 13, 178 and 606). Fly lines corresponding to these samples were retained for genomic Southern analysis to determine if any disruption or deletion within the *dgo* locus had occurred.

4.5 Genomic Southern analysis of excision lines

Of 96 lines retained following PCR analysis, 89 were examined in genomic Southern blots. While examining individual fly lines for recessive phenotypes, eight lines were found to have a recessive lethal phenotype. Three further lines were scored as *Sp/CyO*, a consequence of some *w; Sp/CyO; Dr* P[ry^+ Δ2,3](99B)/TM6 non-virgin females in the F_0

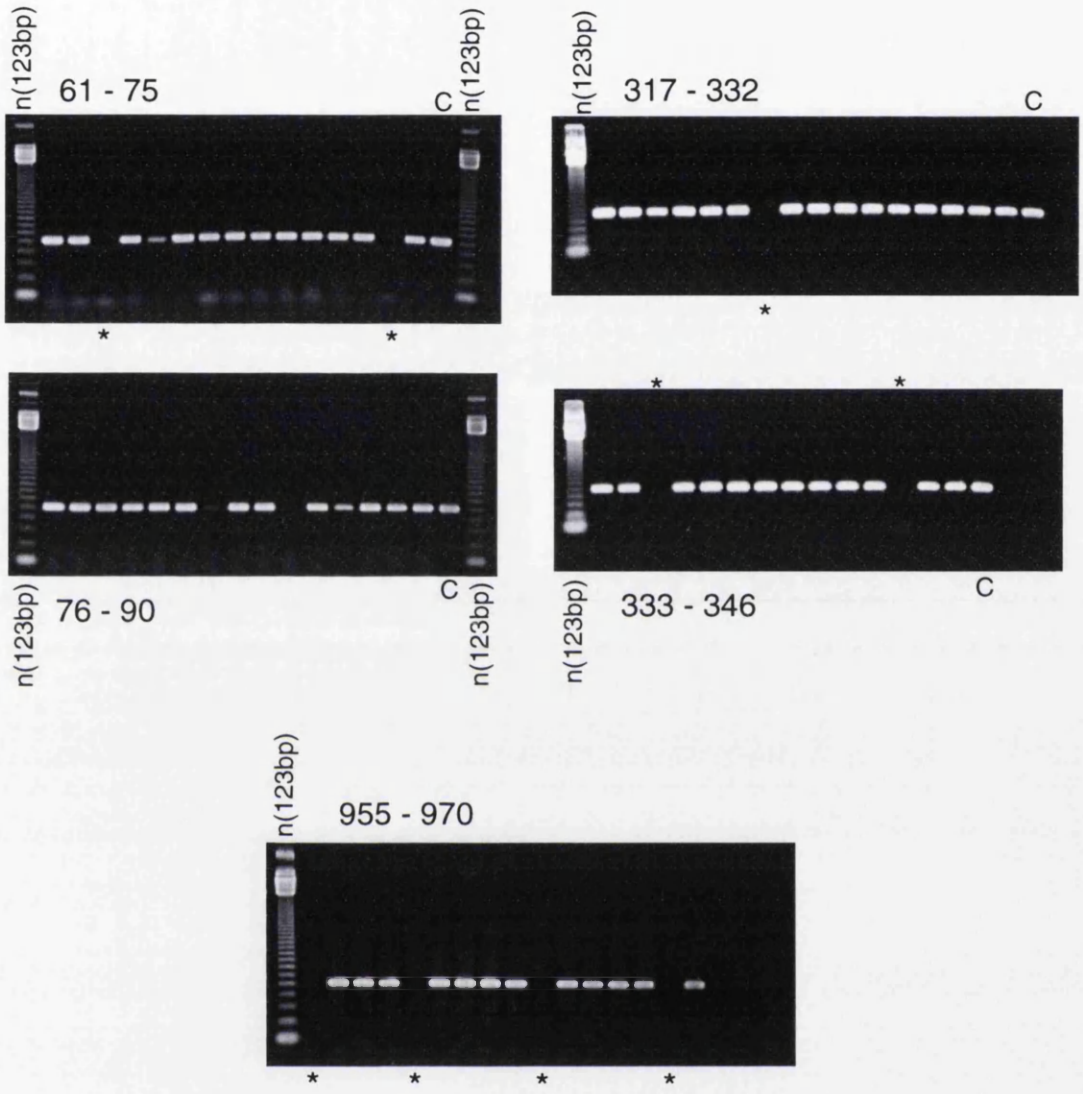


Figure 4.4 PCR detection of excision events.

The P element in *dgo*^{1} gives a ~600 bp PCR product when *dgo*^{1} DNA is amplified with primers Go1 and PL (lane C). Sample numbers are shown above, and those lines that may have an absence of the ~600 bp fragment are indicated with an asterisk (*). n(123bp) corresponds to 123 bp DNA size marker (BRL). PCR products were separated on a 1.0% agarose gel stained with eithidium bromide.

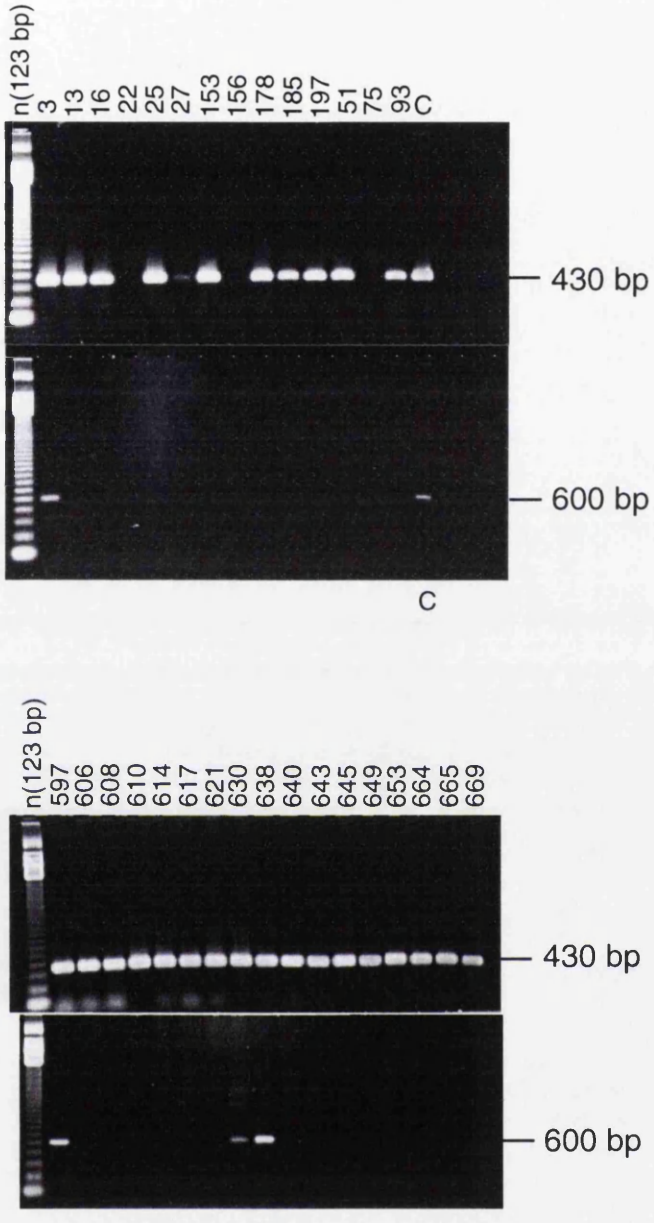


Figure 4.5 PCR as a test of DNA quality.

DNA samples that did not yield a PCR product upon amplification with Go1 and PL (Figure 4.4) were again amplified with Go1 and PL (bottom), and another pair of gene specific primers, DC11 and DC12 (top). Genomic DNA prepared from *Canton S* flies amplified with DC11 and DC12 yields a PCR product of ~430 bp (top, lane C). n(123)bp corresponds to 123 bp DNA size marker (BRL). PCR products were separated on a 1% agarose gel stained with Ethidium bromide.

generation, and were discarded (see Figure 4.2). Four lines failed to grow or produce sufficient progeny for genomic Southern analysis. Thus, genomic DNA was prepared from 89 lines: 81 homozygous for the mutagenised chromosome, and from 8 heterozygote recessive lethal lines. DNA was also prepared from dgo^1 homozygotes, *Sco/CyO* and *Canton S*. DNA was digested with *Bam*HI and *Sma*I, separated in an agarose gel and probed with DGo12, a Class I cDNA probe (Figure 4.6). *Bam*HI and *Sma*I do not cut within P element sequence. Southern analysis shows that the ~2.8 kb *Bam*HI-*Sma*I fragment encompassing the insertion site observed in *Canton S* and *Sco/CyO* had increased in size to ~5.6 kb in dgo^1 homozygotes. The restriction pattern of *Sco/CyO* was identical to *Canton S* and so genomic DNA from the balancer strain was used as the control in subsequent Southern blots. The probe also hybridises to a *Sma*I-*Bam*HI fragment of ~12 kb, and a ~2.0 kb *Bam*HI-*Bam*HI fragment in genomic DNA from all lines. No alteration to the 12 kb or 2.0 kb fragment is observed in any of the lines tested, suggesting that deletions to the right of the P element insertion are restricted to the 2.8 kb *Bam*HI-*Sma*I fragment. The events observed are somewhat repetitive and can be classified into a number of groups, shown diagrammatically in Figure 4.7.

A hybridisation pattern the same as that in dgo^1 homozygotes, i.e. a 5.6 kb fragment, was observed in six lines tested suggesting that mobilisation of the P element has not occurred (e.g. see lines 27, 178 and 460). Precise or near precise excision of the P element from the insertion site, resulting in a wild-type restriction pattern, was seen in 39 lines (e.g. see lines 74, 306 and 484). Deletions of DNA to the right of the P element insertion site would result in a loss of DNA complementary to the probe and should be observed as an absence of the 5.6 kb fragment. This type of excision event,

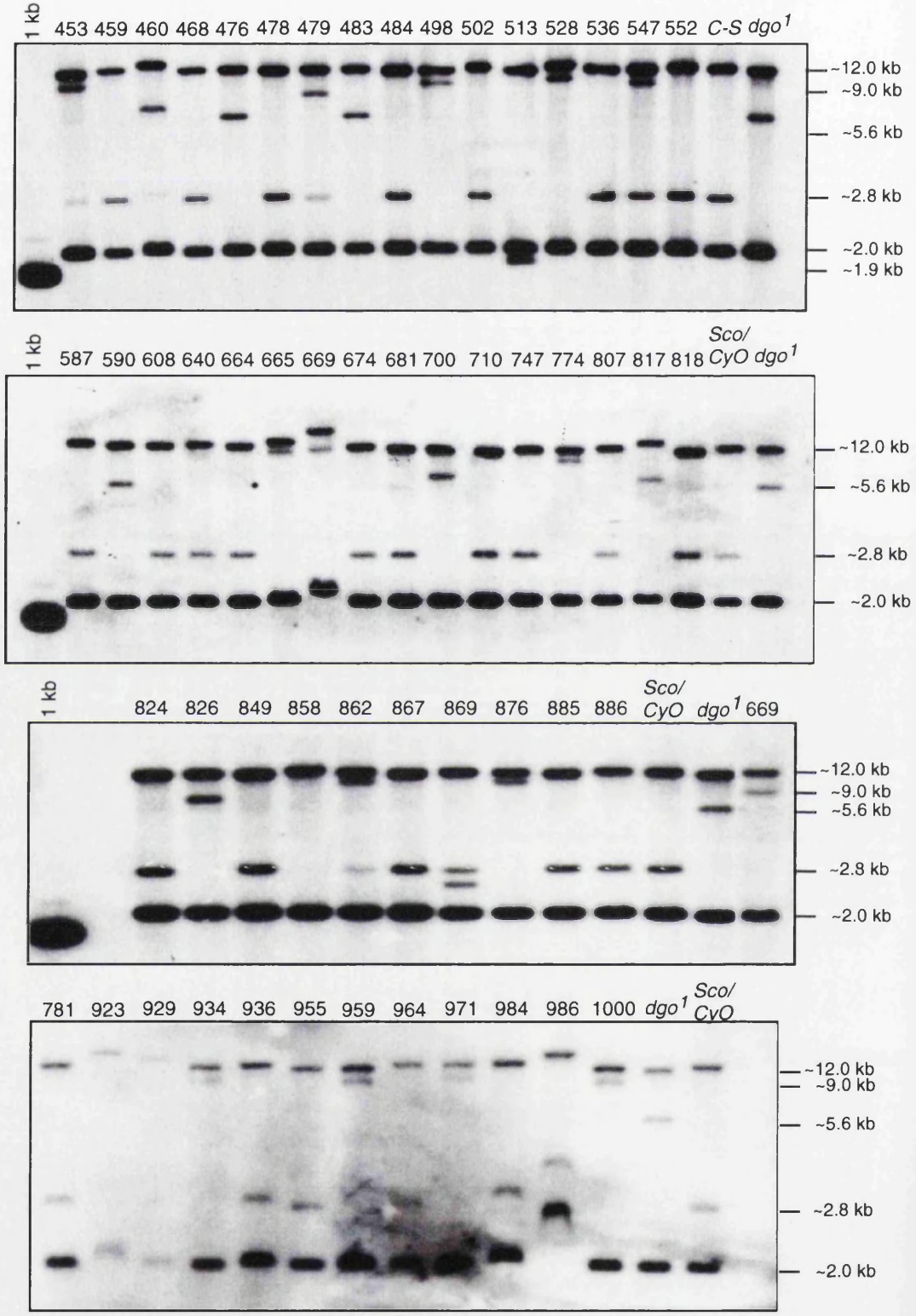


Figure 4.6 See facing page for legend.

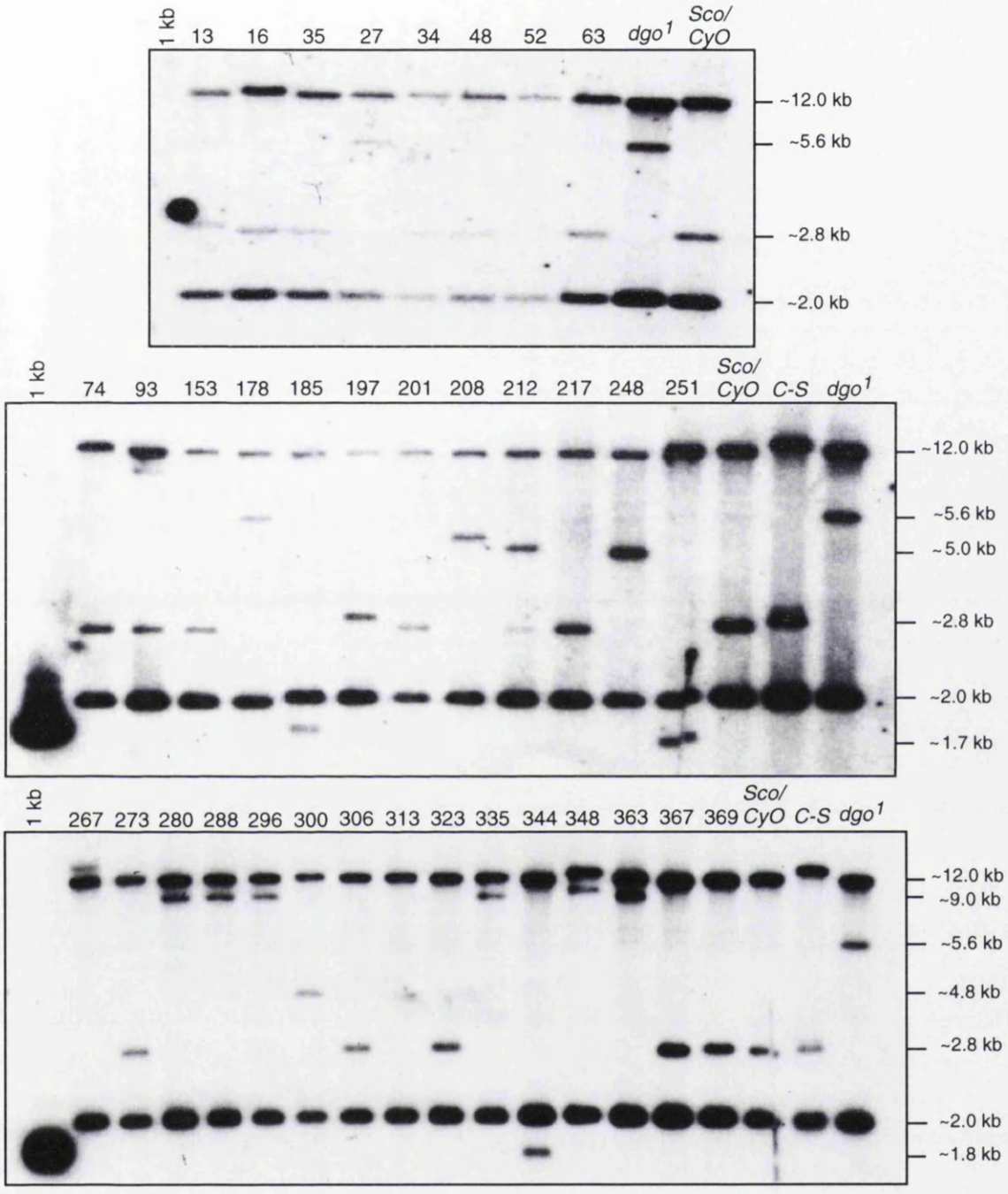


Figure 4.6 Genomic Southern blot analysis of excision lines.
Genomic Southern blot analysis of homozygous *dgo*^{1} mutants, *Sco/CyO* balancer strain, homozygous excision lines and heterozygous recessive lethal lines. DNA was digested with *Bam*HI and *Sma*I, separated in a 0.8% agarose gel, blotted and probed with a ^{32}P-labelled *dgo* cDNA probe, dGo12. The size of appropriate restriction fragments in *dgo*^{1} and wild-type are indicated.

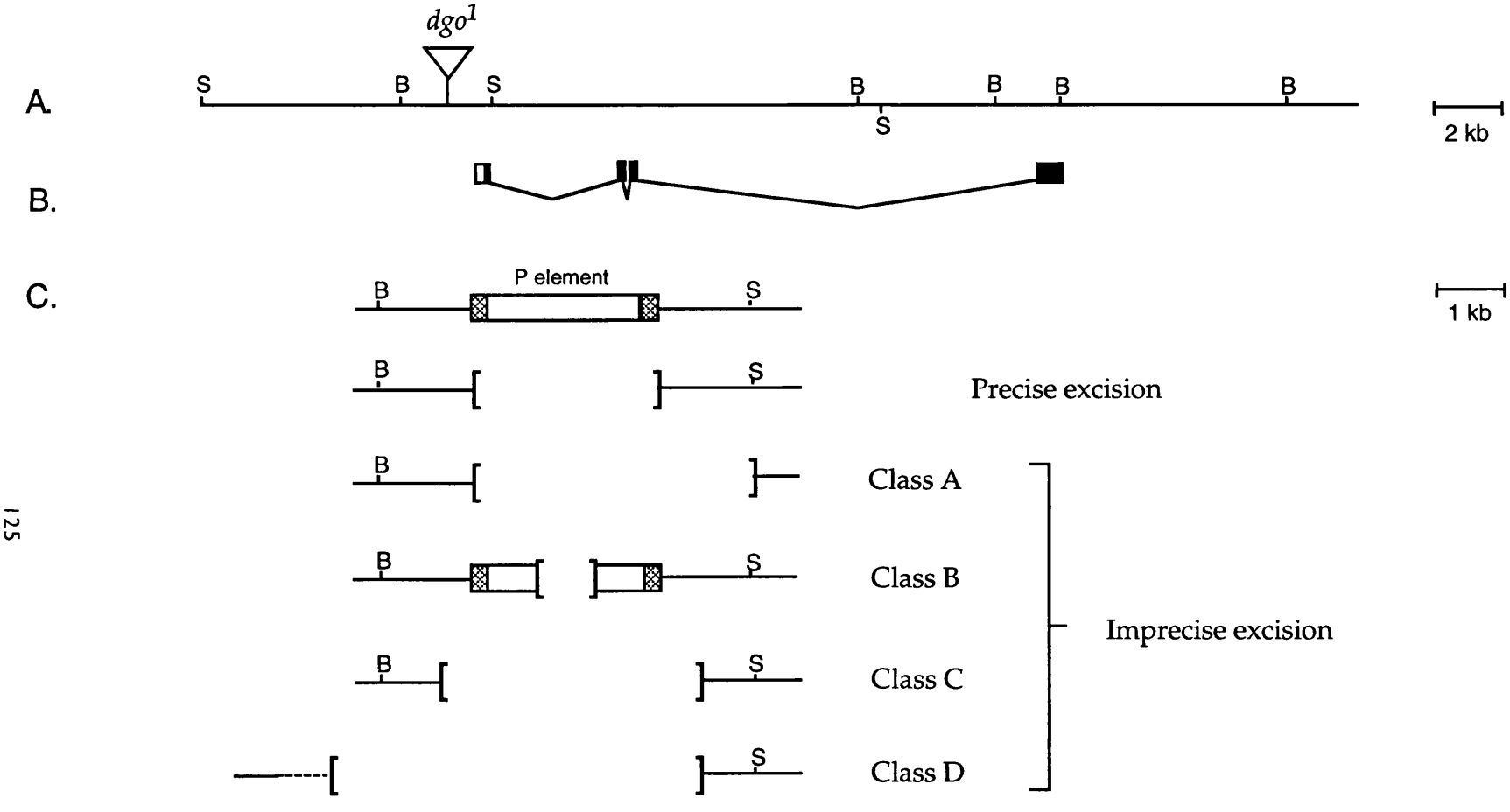


Figure 4.7 **Structure of the *dgo* locus and excision events.**

(A) A restriction map of the *dgo* locus showing B, *Bam*HI and S, *Sma*I sites. The position of the dgo^1 insertion that was mobilised is shown (triangle).

(B) The Class I cDNA probe is shown below the restriction map. Noncoding regions are indicated by open boxes; shaded boxes indicate coding sequences.

(C) The extent of imprecise excisions produced by mobilisation of dgo^1. The P element is shown as a open box with shaded 31 bp terminal repeats. Deletion breakpoints are indicated by brackets. The dashed line in class D imprecise excision indicates a variable extent of deletion beyond the *Bam*HI site.

designated class A, was seen in 3 lines tested (e.g. see lines 52, 858 and 923). Those designated as class B imprecise excisions had a restriction fragment less than 5.6 kb, but greater than the wild-type 2.8 kb fragment, and are suggestive of a deletion of part of the P element and/or flanking DNA with retention of some P element sequences (e.g. see lines 212, 248 and 300). Imprecise excisions of class C had a reduction in size of the 5.6 kb fragment to a size less than 2.8 kb, indicative of a deletion of the P element and DNA within the wild-type 2.8 kb fragment (e.g. see lines 185, 251, 344 and 513). In 23 lines, an increase in the size of the 5.6 kb fragment was observed. Seventeen of these 23 lines had the same ~9 kb hybridising fragment (e.g. see lines 288, 348 and 528). This could be attributed to either a gain of DNA such as P element sequences, or a deletion to the left of the P element that extends far enough to remove the *Bam*HI site and so give rise to a larger hybridising fragment (e.g. see lines 267, 479 and 700). The explanation based on such an excision event is illustrated as class D in Figure 4.7.

The recessive lethal lines were analysed as heterozygotes and so had a background wild-type restriction pattern from the *CyO* chromosome. Four lethal lines, 63, 217, 273 and 459, exhibited a wild-type restriction pattern. Line 929 in Figure 4.6 was difficult to interpret due to a poor quality hybridisation, but Southern analysis described elsewhere showed that it had a wild-type restriction pattern (see section 5.3). However, lines 273 and 459 appear to have a reduced intensity of hybridisation relative to neighbouring lanes that contained at least an equal quantity of DNA (as estimated from ethidium stained agarose gels, not shown). Two lines, 93 and 547, had a ~9 kb hybridising fragment in addition to the 2.8 kb wild-type fragment. In line 869, an additional fragment of ~2.5 kb was observed.

4.6 Southern analysis with a P element probe

In an attempt to confirm some of the predictions made regarding transposase-induced events at the *dgo* locus, the Southern blots in Figure 4.6 were re-probed with a ^{32}P-labelled P element probe (pBS 25.7wc, see section 2.2.1). The results of Southern blot analysis with the P element probe for all lines are shown in Figure 4.8.

Six lines were classified from the pattern of hybridisation with the DGo12 probe as having retained a P element at the original site as indicated by a ~5.6 kb fragment. In all six lines the same ~5.6 kb fragment hybridises with the P element probe (e.g. see lines 27, 178 and 460). Thirty nine lines were scored as precise or near precise excision of the P element: 26 of these appear to have lost all P element sequences (e.g. see lines 34, 74 and 807), and 13 have hybridising fragments of various sizes that may represent new P element insertions (e.g. see lines 153, 478 and 824).

Class A imprecise excisions predicted to have lost sequences complementary to the DGo12 probe to the right of the P element insertion had no hybridisation to P element sequences (e.g. see lines 52, 858 and 923). This suggests that an imprecise excision has occurred that involves the loss of the P element and *dgo* sequences corresponding to the DGo12 probe. Six excision lines of class B were predicted to have undergone a deletion of part of the P element and/or flanking DNA since the fragments were greater than the 2.8 kb wild-type fragment. In five lines, the same DNA fragment that hybridised with the DGo12 probe also hybridised with P element sequences supporting the prediction that some P element sequences remain (e.g. see lines 197 and 300). One line in class B, line 208, has a P element hybridising fragment at an entirely different

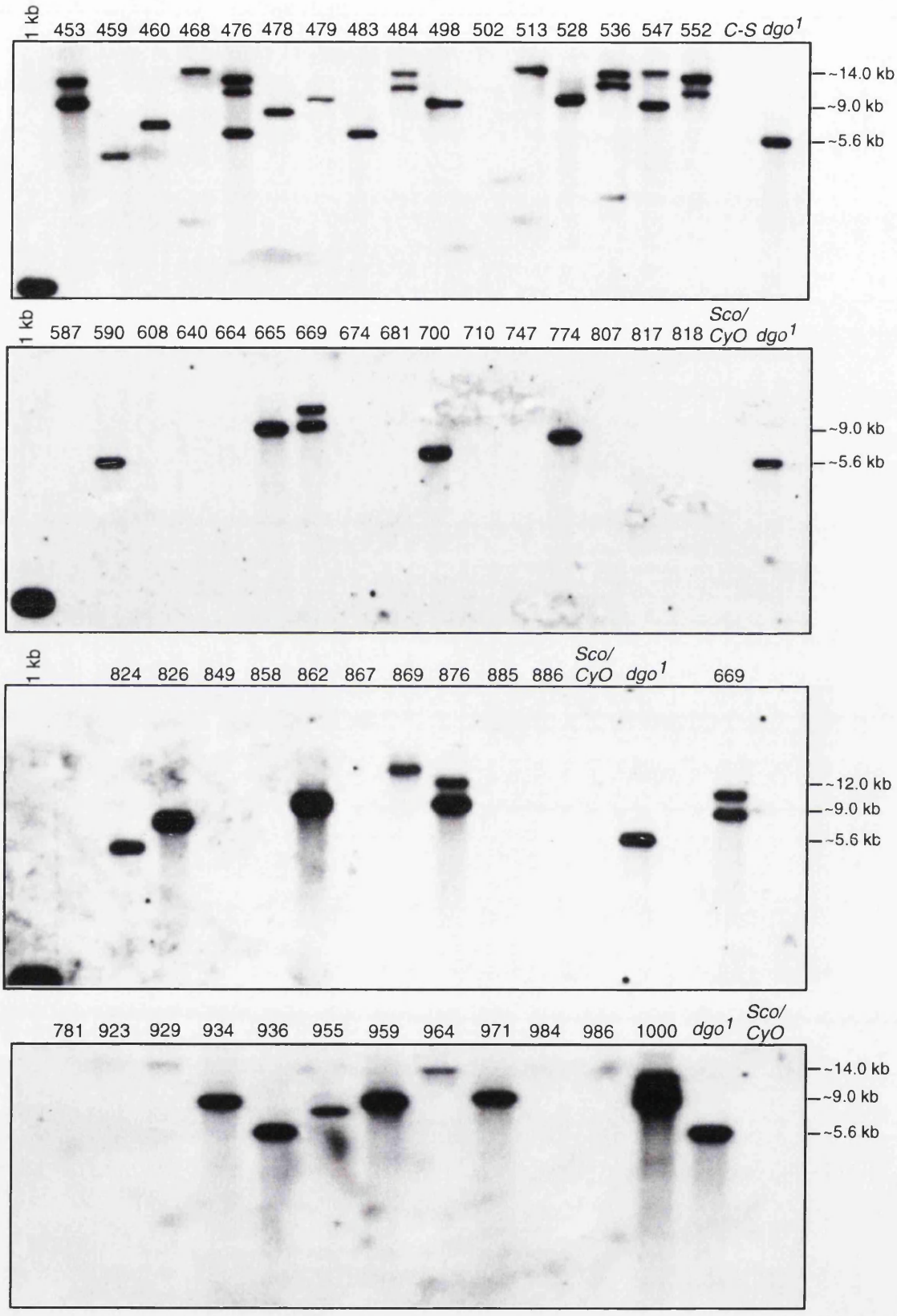


Figure 4.8 See facing page for legend.

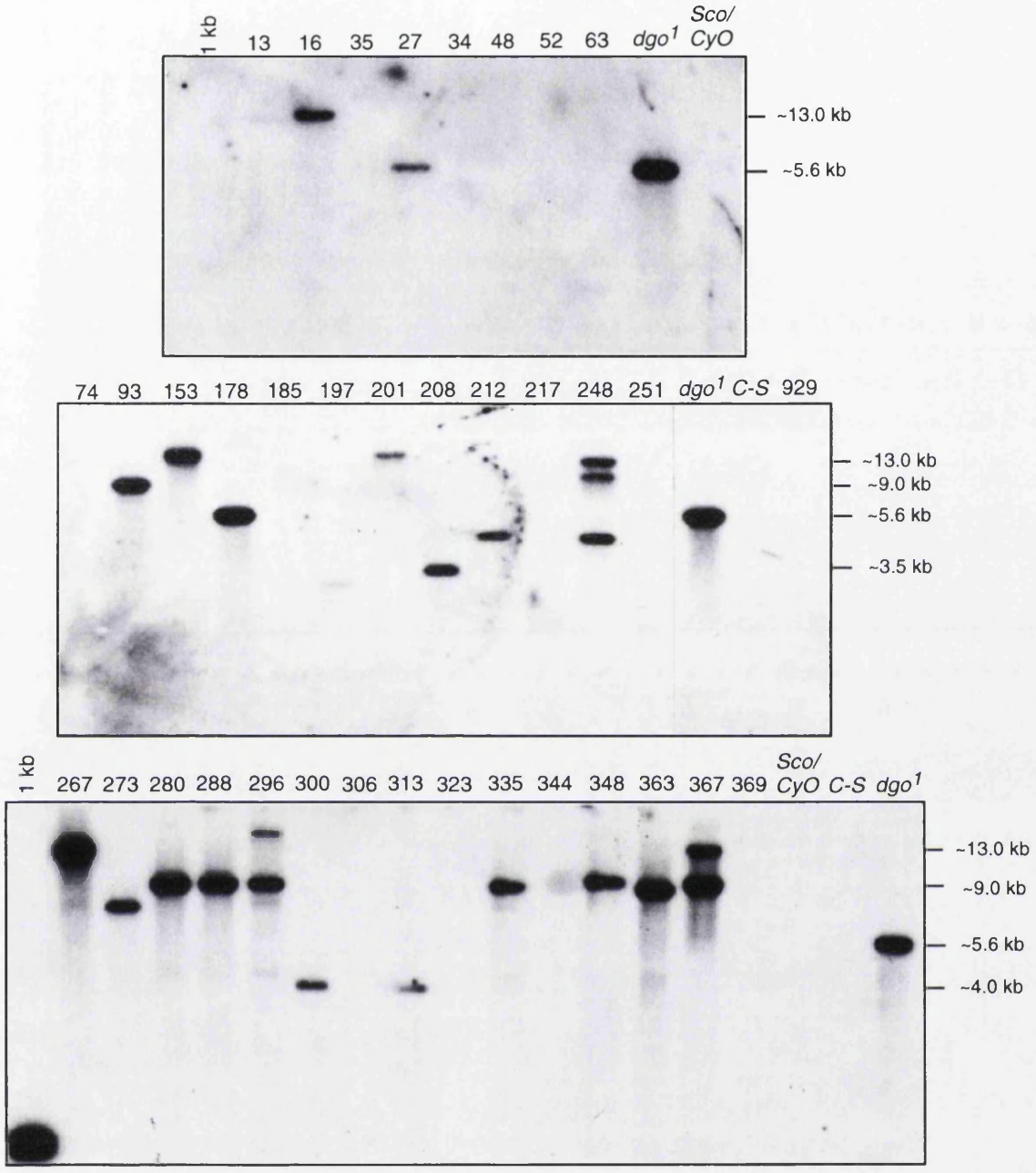


Figure 4.8 Genomic Southern blot analysis of excision lines using a P element probe.

Genomic Southern blots shown in Figure 4.7 were re-probed with ^{32}P-labelled P element sequence, isolated from pπBS25.7wc (section 2.). The size of appropriate fragments are indicated.

position than that observed with DGo12 and therefore likely represents another class of event (e.g. class D, see below). Four class C excision lines were predicted to have a deletion of all P element sequences and some flanking DNA to give a *Bam*HI-*Sma*I fragment less than 2.8 kb (e.g. see lines 185, 251, 344 and 513). This explanation is still plausible since in all four lines, these altered fragments did not hybridise with P element sequences. However one line, 513 did have another P element hybridising fragment at ~14 kb that could represent a secondary P element insertion.

Twenty three lines had a fragment greater than 5.6 kb seen in Southern analysis of dgo^1 homozygotes probed with DGo12: in 21 lines, the same larger fragment also hybridised with the P element probe (e.g. see lines 267, 669, 700 and 826). This includes the 17 lines with a similar ~9 kb fragment (e.g. see lines 288, 363 and 862). In these lines, DNA flanking the P element insertion and P element sequences have been retained. These lines have been interpreted as a complex class of event in which additional DNA sequences have been accrued within the *Bam*HI-*Sma*I fragment. In two lines, 479 and 817, the ~9 kb fragment observed with the DGo12 probe did not hybridise with P element DNA. The excision events in lines 479 and 817 have therefore been classed as imprecise excision of class D, interpreted as deletions that include all P element sequences, DNA to the left of the insertion site and the *Bam*HI restriction site, creating a larger restriction fragment.

Of the eight recessive lethal lines, five retain P element sequences (lines 93, 273, 459, 547 and 869) and three do not (lines 63, 217 and 929). The size of the P element hybridising fragments in the five lines vary in size and could represent secondary P element insertions.

4.7 Discussion

The P element within strain *dgo*^{1} was mobilised in an attempt to recover new mutant alleles of the *dgo* gene. Using a PCR screen, 96 lines were isolated as having lost one or both PCR primer sites. The types of excision events that have occurred, interpreted from Southern analyses, are summarised in Table 4.1. A direct comparison on the overall frequency of transposase induced events in this study with published excision rates is difficult as they vary widely depending of the type of selection used. Those based on the reversion of a mutant phenotype to wild-type show typical excision frequencies in the range of 1% (Chia *et al.*, 1986; Tsubota *et al.*, 1986; Salz *et al.*, 1987; Roiha *et al.*, 1988). When a loss of a visible marker gene carried by a P element such as wild-type alleles of *white* or *rosy* are used to measure excision rates, figures in the order of 5% are typical (Daniels *et al.*, 1986; Levis *et al.*, 1985; Robertson *et al.*, 1988). This increased excision rate is associated with a higher level of internal excisions within the P element, possible due to the larger size of the marked P element. In this study, approximately 10% of lines analysed by PCR displayed a transposase induced event that precluded PCR amplification with P element and gene specific primers. The detection of events was entirely molecular, with no bias toward reversion of mutant phenotype or loss of visible marker. Thus, in this study events often not selected in other screens were retained and may have contributed to the higher rate of excision. The excision lines retained for further analysis are unlikely to represent entirely independent events. The F_1 generation was carried out *en masse* with a number of excision events arising from the same cross, some of which are likely to represent premeiotic clusters.

In an attempt to increase the rate of imprecise versus precise excision, the *dgo*^{1} insertion was mobilised with Δ2,3 in the presence of a balancer

Table 4.1 Summary of excision events.

Description of Excision Event	*Line number*[a]	*Total*
P element retained at original site:	27, 178, 460, **476**, 483, 590	6
Precise excisions:	13, **16**, 25, 34, 48, 74, **153**, **201**, 306, 323, **367**, 369, **468**, **478**, **484**, 502, **536**, **552**, 587, 608, 640, 664, 674, 681, 710, 747, 781, 807, 818, **824**, 849, 867, 885, 886, **936**, **955**, **964**, 984, 986	39
Gain of P element or other sequence at insertion point[b]:		
With similar larger ~9 kb fragment	280, 288, 296, 335, 348, 363, 453, 498, 528, 665, 774, 862, 876, 934, 959, 971, 1000	17
Others	267 (~13 kb), **669** (~7 kb), 700 (~6.5 kb), 826 (~7 kb)	4
Imprecise excisions:		
Class A, deletion of P element and DNA to right of insertion site	858, 923	2
Class B, deletions that leave portion of P element gving fragments > 2.8 kb, but < 5.6 kb	52, 197, 212, 248, 300, 313	6
Class C, deletion of P element and *dgo* sequence giving fragments < 2.8 kb	185, 251, 344, **513**	4
Class D, deletion of P element and sequence to left of insertion point, giving fragments > 5.6 kb	**208**, **479**, 817	3
Recessive lethal phenotype:		
Wild-type restriction pattern with no P element remaining	63, 217, **273**, **459**, 929	5
Larger ~9 kb fragment associated with P element sequence	93, **547**	2
Imprecise excision of ~1.3 kb of *dgo* sequence plus P element	**869**	1

[a]numbers shown in bold represent fly lines with additional P elements elsewhere in the genome. Numbers in brackets are the size of dGo12 and P element hybridising fragments within each fly line.

[b]an alternative interpretation of the excision event in these lines is an imprecise excision similar to class D, but with some portion of P element sequence remaining.

chromosome. A high frequency (13%) of precise excision of a P element in the *white* locus was observed when heterozygous with a wild-type version of the gene (Engels *et al.*, 1990). This rate of reversion was reduced to 3% when a multiply inverted chromosome was used. In this experiment, 89 lines were examined by genomic Southern analysis and of these, 39 were judged as having had precise loss of the P element. Precise excisions were scored as a restoration of the wild-type pattern of restriction fragments probed with a *dgo* cDNA that also fail to hybridise with a P element probe, although small pieces of P element sequences would not be detected within the limits of Southern analysis. With this in mind, the observed rate of precise excision at 3.9% is very similar to the rate of 3% described by Engels *et al.*, (1990) using a balancer chromosome.

Of the 39 excision lines scored as precise, 13 appear to have new P element insertions elsewhere in the genome. The model proposed for P element transposition is a nonreplicative mechanism with the P element leaving behind a double-strand gap that is repaired using the sister chromatid or less often, a homologous chromosome (Engels *et al.*, 1990). Since the sister chromatid is estimated to be utilised in approximately 85% of the P element induced gap repair events, P element transposition resembles a replicative mechanism, with restoration of a P element at the donor site. In the selection protocol used here, the use of the sister chromatid as repair template and retention of a P element at the original site would result in a dgo^1 specific PCR product, so that events that result in a loss of P element at the *dgo* locus would be positively selected for, irrespective of the fate of the transposed P element. Exceptions to this were observed however as six lines that did not amplify the diagnostic PCR fragment had a restriction pattern identical to that for dgo^1 homozygotes in

Southern analysis. It is possible that in these rare cases an imprecise excision too small for detection within the limits of Southern analysis disrupted the P element primer site.

A relatively high number of the lines tested (23 of 89) had an increase in the size of the restriction fragment spanning the insertion site that hybridised with both *dgo* cDNA and P element probes. These have been interpreted as a gain of P element or other sequences. An alternative hypothesis of the data is possible based on a deletion extending beyond restriction sites to the left of the insertion point, retaining a portion of P element and resulting in a larger hybridising fragment. However, such a excision event would seem unlikely to have occcurred in those 17 lines with a similar altered fragment since it necessarily requires a deletion breakpoint in the same position in every case. The larger fragments in the 17 lines could be associated with extra P element or *dgo* sequences. P elements have been observed to integrate directly adjacent to or very close to the donor element (Roiha *et al.*, 1988; O'Hare *et al.*, 1992; Daniels and Chovnick, 1993; Tower *et al.*, 1993; Zhang and Spradling 1993; Delattre *et al.*, 1995). Complex rearrangements associated with P element excision that involve the duplication of sequences surrounding the P element insertion site have also been described (Salz *et al.*, 1987; Gloor *et al.*, 1991; Nassif *et al.*, 1994).

A variety of events were observed that were classified as imprecise excision of P element and/or *dgo* sequences. None of the lines so classified displayed any phenotype. This was not unexpected of imprecise excisions of class B and C, which involve deletion of P element sequences (B) and sequences upstream of the *dgo* Class I cDNA first exon (C), since they would not disrupt transcribed or coding sequences. Imprecise excisions of class D could disrupt the first exon of Class II cDNA.

Although the precise location of deletion breakpoints were not determined for class D imprecise excisions that extend to the left of the insertion point, the size of the altered fragment visualised with the *dgo* cDNA probe preclude them extending much beyond the *Bam*HI site to the left of the P element insertion (confirmed in Southern analysis using *Sma*I, not shown). Of particular interest are class A excisions interpreted as having had deletion of sequences that include the upstream regulatory regions and start of the Class I transcript. These excision mutations have no discernable phenotype which suggests the function(s) of the *dgo* gene are fully met by expression of the Class II transcript.

Three recessive lethal lines have restriction fragment length polymorphisms. Lines 93 and 547 have a new fragment of ~9 kb that, similar to the 17 non-lethal lines, hybridised to both DGo12 and P element probes. Whether the recessive lethal phenotype in these lines is directly associated with the observed restriction length fragment polymorphisms, or are associated with the additional P element insertions, cannot be determined from this analysis. Line 869 has a reduction in size of the fragment encompassing the insertion site corresponding to a deletion of approximately 300 bp or so of flanking DNA. The size of the deletion in 869 makes it unlikely that transcription from the *dgo* locus, particularly expression of the Class II transcript is affected. In two lethal lines, 273 and 459, an apparent reduction in the intensity of hybridisation signal with the cDNA probe was observed. If these lines have a reduced DNA content, and therefore a deletion, across the extent of the cDNA probe, they could represent large deletion events that remove *dgo* coding sequences (see section 5.3).

The proposed configuration of excision events presented in Table 4.1 are based on results obtained in Southern analysis with a P element probe and a cDNA probe that covers a relatively small region of the *dgo* locus. Southern analysis using more comprehensive probes, such as genomic clones, would be necessary to locate breakpoints of deletion events. Confirmation using PCR and sequence analysis would also be required to define the precise conformation of sequences at the sites of insertion or deletions (Nassif *et al.*, 1994; Johnson-Schlitz and Engels, 1994).

The following chapter describes further molecular and genetic analysis of eight excision lines displaying a recessive lethal phenotype.

Chapter 5

Analysis of Excision-derived Recessive Lethals

5.1 Introduction

In addition to the mutations in *dgo* described in this study, another *dgo* allele, Goα-B97 has been isolated by P element insertion mutagenesis (Guillén *et al.*, 1995). Homozygous Goα-B97 mutants with no $DG\alpha_o$ immunoreactivity exhibit embryonic lethality. When combined with a deficiency covering the *dgo* locus, Goα-B97 mutants have defects in the nervous system and dorsal vessel that can be completely rescued with *dgo* cDNA.

The *dgo* gene is located at chromosomal position 47A where at least two other embryonic lethal complementation groups have been mapped. While screening for mutations that affect the pattern of axonal pathways in the developing CNS, Seeger *et al.*, (1993), isolated a mutation, *longitudinal lacking* (*lola*) also located at 47A. The *lola* mutation is observed as a loss or reduction of axons along the longitudinal tracts connecting the segmental ganglia in the CNS (Seeger *et al.*, 1993; Giniger *et al.*, 1994). In an enhancer detector screen both for genes expressed in the embryonic PNS and CNS, and/or lethal mutations arising from P element insertion, Bier *et al.*, (1989), isolated eleven P[*lac*W] insertions at 47A. Six of these were embryonic lethal and allelic, with defects in the developing CNS. The P[*lac*W] insertions were later shown to be alleles of *lola* (Giniger *et al.*, 1994). Subsequent molecular cloning of the *lola* gene by plasmid rescue of DNA flanking the P[*lac*W] transposon in five insertion lines revealed *lola* to be a zinc-finger protein, related to a family of transcription factors that include the products of the *Drosophila* genes *tramtrack* and *broad-complex* (Giniger *et al.*, 1994; Zollman *et al.*, 1995).

The second complementation group that maps to chromosomal position 47A is defined by the *bumper-to-bumper* (*btb*) mutation which was also

identified in a screen for P element mutations affecting the developing embryonic PNS (Kania *et al.*, 1995). The phenotype observed in *btb* mutant embryos shows neuronal pathfinding and connectivity defects in the developing PNS.

This chapter describes additional Southern analyses of recessive lethal excision lines with genomic clones encompassing the *dgo* locus. The results of complementation tests, of recessive lethal lines crossed *inter se* and with other mutations that map to chromosomal position 47A, are also presented.

5.2 Isolation of genomic clones

To further investigate possible deletion or disruption events within the *dgo* locus, more comprehensive probes were required. Particularly spanning the insertion site in dgo^1 mutants. A Class I cDNA probe, dGo12, was used to screen a genomic library in EMBL4 (kindly provided by S. Russell). Twenty thousand phage, approximately two genome equivalents, were screened. Seventeen positively hybridising clones were isolated and seven with the strongest hybridisation signal were purified for further analysis. To establish whether the genomic clones relate to the previously published restriction map of the *dgo* locus (Yoon *et al.*, 1989), purified phage DNA was digested with *Eco*RI and *Hin*dII, separated in an agarose gel, blotted and probed with labelled dGo12 (Appendix I). Two clones were subsequently selected as probes, λDG2 and λDG14, that together contain the extent of the locus encompassed by the seven isolated phage inserts (Figure 5.1). None of the seven clones analysed had inserts that extended beyond λDG14 at the 3' end of the locus, and so

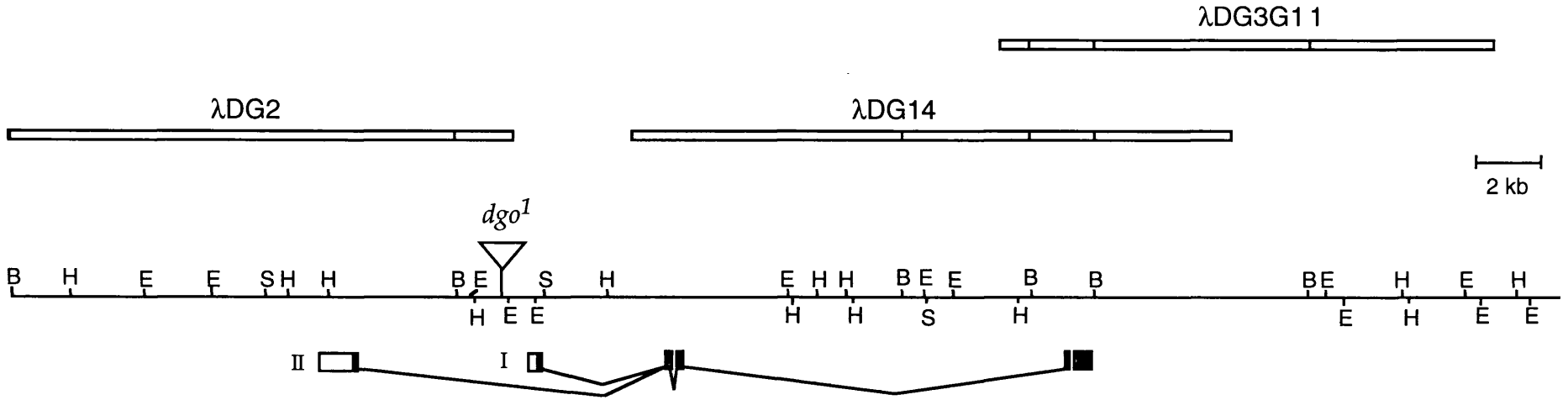


Figure 5.1 Structure of the *dgo* locus with genomic clones.

Restriction map of the *dgo* genomic region: *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*dIII; *S*, *Sma*I. The restriction map is as described by Yoon *et al.*, (1989). The position of the P element insertion mobilised in dgo^1 is shown as an inverted triangle. Shown above the restriction map are the genomic clones covering the *dgo* locus used as probes. λDG3G11 is as described by Yoon *et al.*, (1989) [kindly provided by M. Forte]. The closed bars in phage inserts correspond to *Bam*HI sites. Shown below are the exons of Class I and II cDNA: noncoding regions are indicated by open boxes; shaded boxes indicate coding sequences.

genomic clone λDG3G11 (Yoon *et al.*, 1989) that contains this region was obtained (Figure 5.1; λDG3G11 was kindly provided by M. Forte).

5.3 Southern analysis of excision lethals using genomic clones

Genomic DNA was prepared from heterozygous recessive lethal lines, dgo^1 homozygotes and *Canton S* flies, digested with *Bam*HI and *Sma*I, separated in an agarose gel and blotted. Southern blots were probed sequentially with labelled phage DNA from λDG3G11, λDG14 and λDG2.

λDG3G11

In Southern analysis, λDG3G11 hybridised to fragments of approximately 8.6 kb, 6.8 kb, 3.5 kb and 2.0 kb in genomic DNA from *Canton S* (Figure 5.2). The origin of the weaker signals at ~6.0 kb and ~11 kb are unknown. No change in the pattern of restriction fragments can be seen in lethal lines when compared with dgo^1 homozygotes or *Canton S*. A decreased intensity of signal is visible in lines 273 and 459, previously noted with a *dgo* cDNA probe (see section 4.5; see below). None of the lethal lines have deletion breakpoints within the DNA encompassed by the λDG3G11 probe.

λDG14

Southern blots probed with λDG14 give hybridising fragments of approximately 12 kb, 6.8 kb, 3.5 kb and 2.0 kb in genomic DNA from *Canton S* (Figure 5.3a). Southern analysis shows that no disruption has occurred in any of the lethal lines and that the pattern of hybridisation is the same as *Canton S*. To investigate further the apparent decreased intensity of hybridisation observed in lines 273 and 459, the ^{32}P-signal from a Southern blot probed with λDG14 was quantified using a phosphoimager. To correct for differences in the amount of DNA loaded

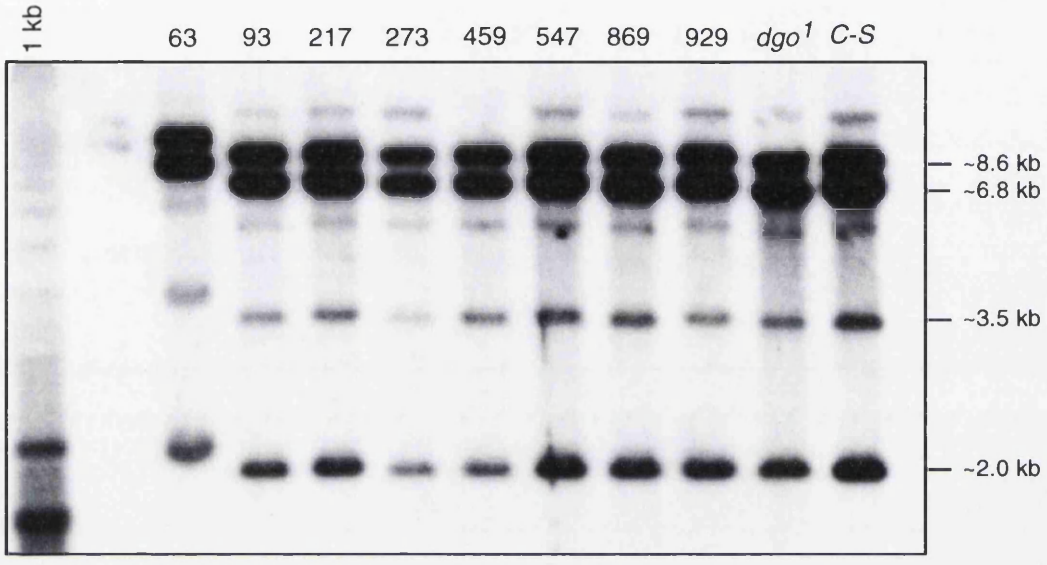


Figure 5.2 Genomic Southern analysis of excision lethals probed with λDG3G11.

Genomic DNA from heterozygote excision lethal lines, dgo^1 homozygotes and *Canton S*, digested with *Bam*HI and *Sma*I. Digested DNA was separated in an 0.8% agarose gel, blotted and probed with ^{32}P-labelled λDG3G11. The size of appropriate restriction fragments is indicated.

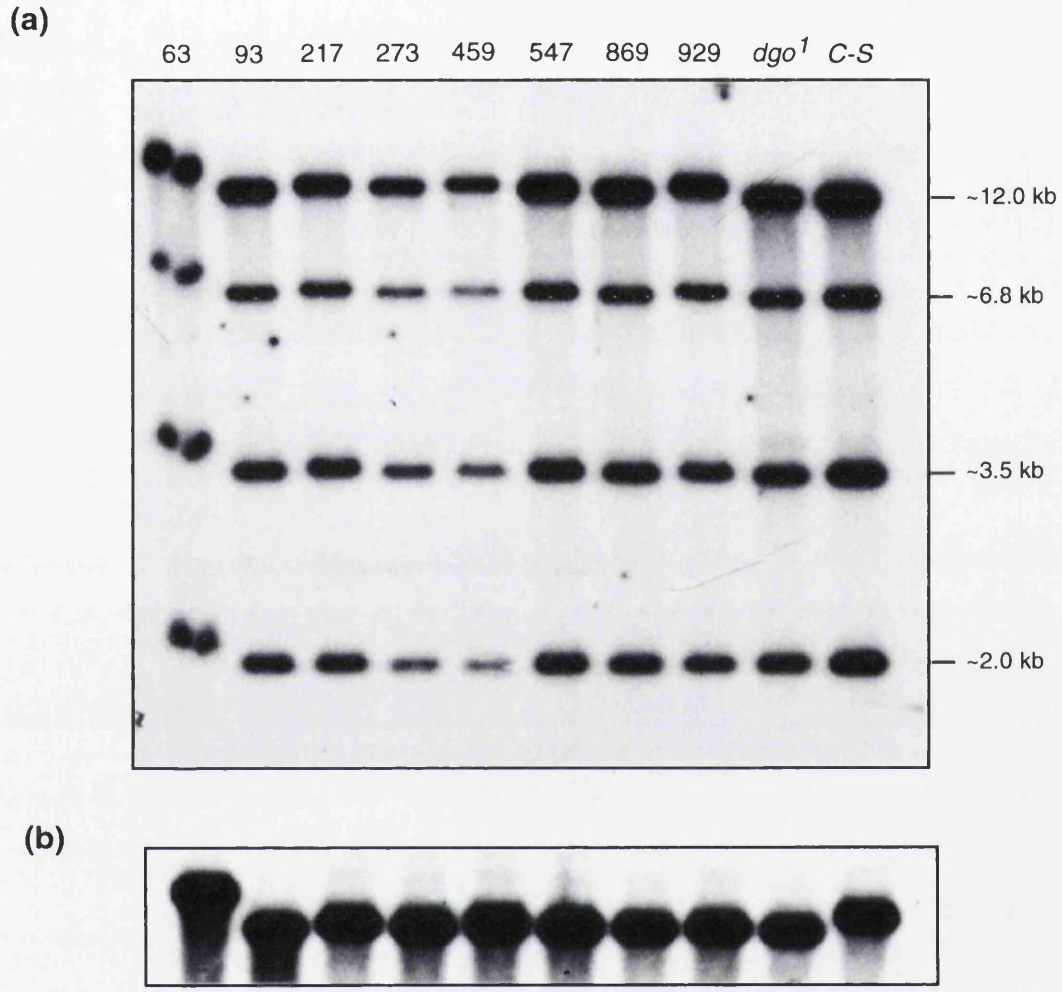


Figure 5.3 Genomic Southern analysis of excision lethals probed with λDG14.

(A) Genomic DNA from heterozygote excision lethal lines, dgo^1 homozygotes and *Canton S*, digested with *Bam*HI and *Sma*I. Digested DNA was separated in an 0.8% agarose gel, blotted and probed with ^{32}P-labelled λDG14. The size of appropriate restriction fragments is indicated. (B) The same filter probed with a control gene probe to determine the relative amount of DNA within each lane prior to quantification of the intensity of hybridisation signals using a phosphoimager.

onto the gel, the filter was then labelled with a control gene probe (Figure 5.3b). The amount of DNA was standardised to the amount of the control gene signal in each lane (Appendix II). No reduction in the signal from the four hybridising fragments was detected in lines 63, 93, 217, 547, 869 or 929. In both 273 and 459, the intensity of the 12.0 kb, 6.8 kb, 3.5 kb and 2.0 kb fragments each have half or less the value of the corresponding fragment of *Canton S* (Table 5.1). This suggests that 273 and 459 have a deletion that minimally spans the DNA complementary to the λDG14 probe, which includes the coding region shared by both classes of *dgo* transcript (see Figure 5.1).

Table 5.1 Quantification of signal intensity in lines 273 and 459.

Fragment size (kb)		*Relative value standardised to control*[a]
12.0 :	*Canton S*	1.00
	273	0.50
	459	0.45
6.8 :	*Canton S*	1.00
	273	0.37
	459	0.33
3.5 :	*Canton S*	1.00
	273	0.38
	459	0.33
2.0 :	*Canton S*	1.00
	273	0.40
	459	0.37

[a]see Appendix II for calculations of relative signals.

λDG2

A Southern blot probed with λDG2 gives hybridising fragments of approximately 12.0 kb, 5.6 kb and 2.8 kb in genomic DNA from *Canton S*

(Figure 5.4). The λDG2 phage insert spans the P element insertion site in *dgo*^{1}, which is observed as a ~5.6 kb fragment in DNA from *dgo*^{1} homozygotes. Southern analysis with λDG2 shows that lines 93 and 547 have an additional fragment of approximately 9 kb which was observed previously with the *dgo* cDNA probed (DGo12). From this and previous analysis with the dGo12 probe (see Figure 4.6), this altered fragment does not affect the large ~12 kb *Sma*I-*Bam*HI fragment downstream of the insertion site. Whether this fragment arises from a deletion that extends just beyond the *Bam*HI site upstream of the insertion site to create a larger fragment, or additional sequences within the ~5.6 kb fragment in *dgo*^{1} homozygotes was not determined. The only other alteration occurs in line 869, which has an additional fragment of ~2.5 kb. This can be explained by a deletion of the P element and ~300 bp of DNA from within the ~2.8 kb wild-type fragment. From this analysis it is clear that no deletion breakpoints occur within the ~5.6 kb *Bam*HI-*Sma*I fragment upstream of the insertion site in any of the lines tested.

5.4 Embryonic lethality

In a previous study it was reported that *dgo* mutants with an absence of DGα_o immunoreactivity, have an embryonic lethal phenotype (Guillén *et al.*, 1995). To test for embryonic lethality, eggs laid by excision lethal lines heterozygous for a *CyO* balancer chromosome were assayed for hatching. Eggs were collected from all lines (except 547) onto egg laying plates and allowed to develop for approximately 30 hours. Normal embryos hatch around 22-24 hours after egg laying. An embryonic lethal phenotype would be observed as a 50% failure to hatch, 25% being attributed to the *CyO* balancer chromosome. A minimum of one hundred eggs were counted for each line and the results are presented

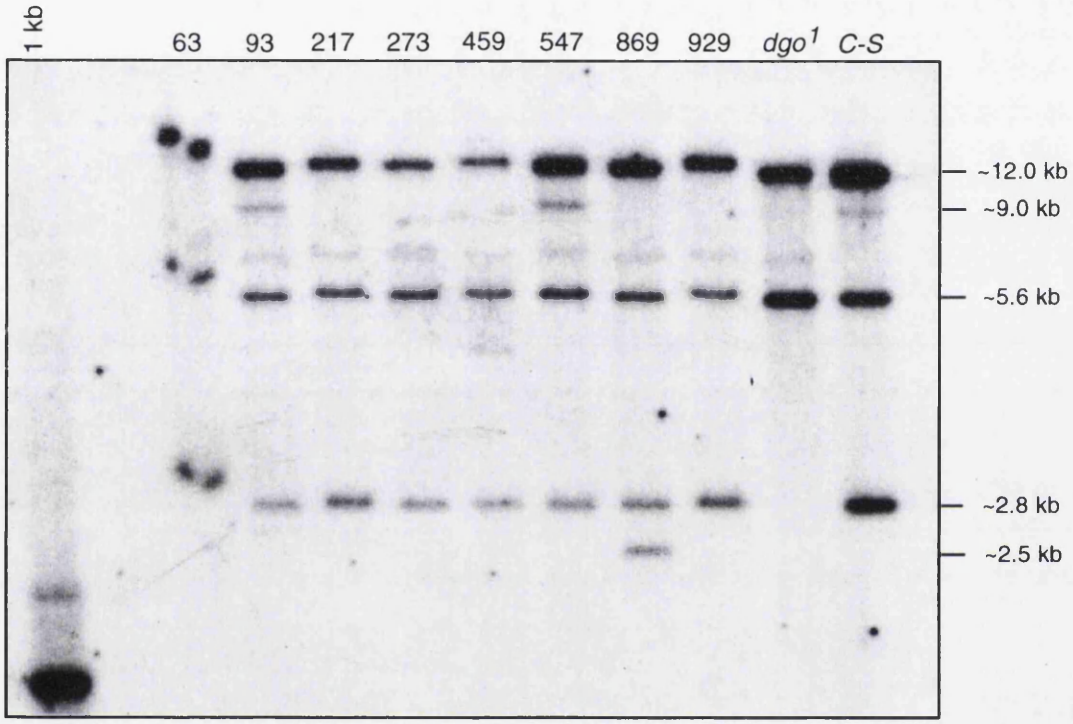


Figure 5.4 Genomic Southern analysis of excision lethals probed with λDG2.

Genomic DNA from heterozygote excision lethal lines, dgo^1 homozygotes and *Canton S*, digested with *Bam*HI and *Sma*I. Digested DNA was separated in an 0.8% agarose gel, blotted and probed with ^{32}P-labelled λDG2. The size of appropriate restriction fragments is indicated.

graphically in Figure 5.5. Excision lines 93, 273, 459 and 929 are embryonic lethals: Each have embryonic lethality greater than 50%. A percentage greater than 50% could be attributed to the increased embryonic lethality of +/*CyO* flies (28% compared to 25% expected) or to unfertilised eggs. Excision line 217 has 42% embryonic lethality, which is significantly greater than +/*CyO* flies. Lines 63, 547 and 869 do not have an embryonic lethal phenotype. Since no pupal or adult lethality was detected in any of the lethal strains, 63, 547 and 869 must, by default, have a larval lethal phenotype. However, this has not been directly observed.

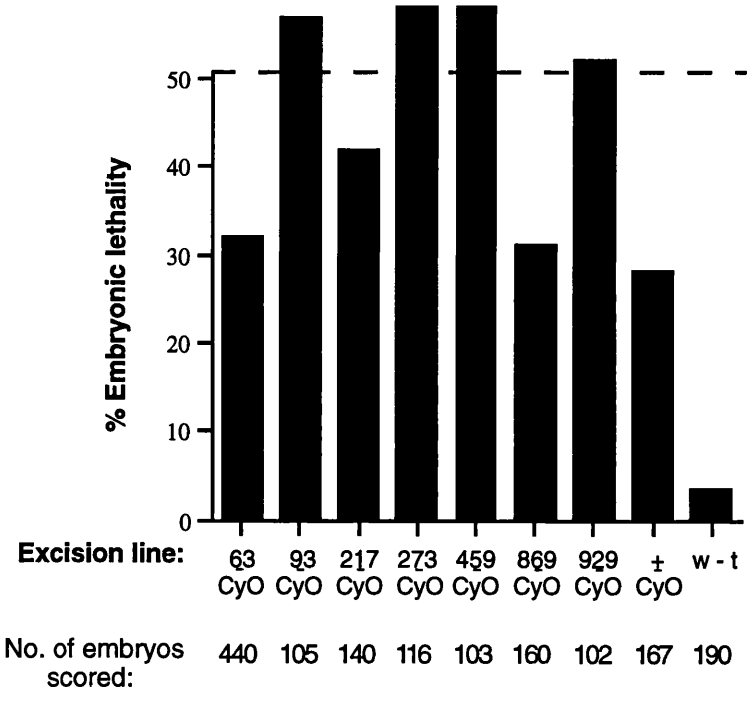


Figure 5.5 Embryonic lethality of excision lines.

Recessive lethal excision lines were scored for embryonic lethality approximately 30 hr. after egg laying (normal embryos hatch after 22-24 hr). Embryonic lethality, in combination with the *CyO* balancer chromosome, should result in 50% failure to hatch. Lines, 93, 273, 459 and 929, show greater than 50% embryonic lethality.

5.5 Complementation analysis

To establish if the lethal mutations in the excision lines map to the same location, a complementation test between each line was carried out. The crosses were performed reciprocally to test for any maternal influences. The number of non-*CyO* transheterozygote progeny produced for each heteroallelic combination was counted (Table 5.2). Because no maternal influences were observed, data are presented for only one of each reciprocal cross.

Table 5.2 Complementation analysis of lethals, crossed *inter se*.

	Female parent							
male parent	**63**	**93**	**217**	**273**	**459**	**547**	**869**	**929**
63	-							
93	+	-						
217	+	+	-					
273	+	+	+/-	-				
459	+	+	-	-	-			
547	+	+	+	+	+	-		
869	-	+	+	+	+	+	-	
929	+	+	-	+	+	+	-	-

(-) = no transheterozygous progeny recovered; (+) = greater than 50% of the expected transheterozygous progeny recovered; (+/-) = less than 50% of the expected transheterozygous progeny recovered. See Appendix III for numerical data.

Complementation analysis shows that the lethals fall into two overlapping complementation groups. One group associated with line 459 fails to complement both 217 and 273. The heteroallelic combination of 217 and 273 produces less than 50% of expected transheterozygote progeny. The second complementation group is associated with line 929

which fails to complement 217 and 869. Lines 217 and 869 fully complement each other. Line 217 fails to complement one member from each group and may therefore have more than one lethal mutation. Line 63 fails to complement line 869, and lines 547 and 93 complement all other lines.

Two other genes, *lola* and *btb*, map to chromosomal position 47A. To establish if the recessive lethal lines isolated in this study disrupt *lola* or *btb*, complementation tests were performed with an allele of *lola*, $lola^{5D2}$, and an allele of *btb* (99/1). The mutations in *btb* and *lola* result in embryonic lethality with defects in the developing embryonic nervous system. Complementation tests were also performed with two lines that could contain lethal P element insertions in *dgo* that were isolated from a collection of 2,000 second chromosome lethal insertions generated by Török *et al.*, (1993). DNA flanking the transposon insertion sites for approximately 1,800 lines was plasmid rescued in this laboratory (Guo *et al.*, 1996, in press). A filter containing rescued plasmids from the 1,800 lines was probed with a *dgo* cDNA probe. Rescued plasmids isolated from two lines, 1/1 and 2/33, had sequences complimentary to the *dgo* cDNA probe (Yiquan Guo; pers. comm.). These embryonic lethal lines may therefore represent P[*lac*W] insertions in or near the *dgo* gene. The results of complementation analysis with $lola^{5D2}$, 99/1, 1/1 and 2/33 are presented in Table 5.3.

Table 5.3 Complementation analysis with mutations that map to chromosomal position 47A, and potential alleles of *dgo*.

	female parent[a]			
	lola	*btb*	?	?
	5D2	99/1	1/1	2/33
male parent				
63	+	+	+	+
93	+	+	+	+
217	+	+	+	+
273	-	-	+	+
459	-	-	+	+
547	+	+	+	+
869	+	+	+	+
929	+	+	+	+

[a]the reciprocal cross was also made with similar results (see Appendix IV). (-) = no transheterozygote progeny recovered; (+) = transheterozygote progeny recovered.

Complementation analysis shows that lines 273 and 459 both fail to complement mutations in $lola^{5D2}$ and 99/1. All other excision lethals complement these alleles. All excision lethals also complement 1/1 and 2/33. Thus, lines 1/1 and 2/33 contain mutations that do not map to the same chromosomal region as *dgo*, *lola* or *btb* at cytological location 47A.

5.6 Discussion

This chapter describes additional molecular and genetic characterisation of eight recessive lethal lines isolated as a result of a mobilisation of a P element approximately 680 bp upstream of the *dgo* Class I transcript. To more fully characterise the extent of possible deletions in some recessive

lethal lines, genomic clones containing a large portion of the *dgo* locus were isolated. Southern analysis with genomic clones as probes revealed little additional information than was initially obtained with a cDNA probe (see chapter 4). Three lines show alterations in the size of restriction fragments, 93, 547 and 869, and two have a reduction in the intensity of hybridisation signal in almost all restriction fragments visualised (273 and 459). The intensity of the signal in hybridising fragments was measured for lines 273 and 459 and the values obtained for all fragments measured were at least half of the values of the equivalent fragment in genomic DNA from *Canton S* flies. The fragments correspond to the coding region shared by both classes of *dgo* transcript and thus 273 and 459 likely represent null mutations in the *dgo* gene (see below). Two of the lines, 93 and 547, have the same altered restriction pattern in the *dgo* locus with an additional larger hybridising fragment. However, the change in restriction pattern in 93 and 547 are unlikely to be directly associated with the lethal phenotype since these lines complement each other for lethality. One line, 869, has a deletion of approximately 300 bp of DNA flanking the site of the original P element insertion, which is unlikely to affect *dgo* transcription.

$DG\alpha_o$ protein is found localised in the developing embryonic nervous system where it is necessary for subsequent embryonic development (Guillén *et al.*, 1991; Guillén *et al.*, 1995): Mutants with no $DG\alpha_o$ immunoreactivity have defects in the developing CNS and dorsal vessel (Guillén *et al.*, 1995). Four of the excision lethals lines tested for embryonic lethality display an embryonic lethal phenotype. This includes lines 273 and 459 which are null mutations in the *dgo* gene. One line, 217, has a partial embryonic lethal phenotype. Two of the lines tested, 63 and 869, do not die as embryos. As all of the excision-derived

lethal lines were analysed for pupal and adult lethality, strains that did not die as embryos must die as larvae, although this has not been tested directly.

Complementation analysis with pairwise combinations of lethal lines reveals the mutations in these strains do not belong to a single complementation group. A complex pattern of complementation is observed which places the mutations into two overlapping complementation groups. This is consistent with the data obtained for embryonic lethality of the excision derived lethals which suggest that mutations act during at least two distinct lethal phases; embryo and larvae. However, there are examples of mutations that affect a single product having different stage-specific phenotypes (Kiss *et al.*, 1988). This type of interaction could explain the absence of complementation between 869 and 929, which have different lethal phases, but still fail to complement.

The two complementation groups are associated with lines 459 and 929. One group consists of 459, 273 and 217 and the other group 929, 869, 63 and 217. Line 217 has a mutation in common with both groups as it fails to complement at least one member of each. Both 273 and 459 fail to complement lethal alleles of *lola* and *btb* which map to chromosomal position 47A (Seeger *et al.*, 1993; Giniger *et al.*, 1994; Kania *et al.*, 1995). Consequently, the deletions in lines 273 and 459 remove, in addition to *dgo*, at least two other essential loci. Since the breakpoints of the deletions associated with the two strains occur beyond the bounds of genomic probes used in Southern analysis, it cannot be determined if the extent of the deletions are identical in 273 and 459. The heteroallelic combination of 217 and 273 produces transheterozygotes with a partial

lethal phenotype. Line 217 could be a hypomorphic allele of *dgo*; it complements both $lola^{5D2}$ and *btb* (99/1), but partially fails to complement the mutation in 273. Line 217 must also have another lethal mutation elsewhere in the genome as it fails to complement a member of the 'second' complementation group. However, the interpretation that 217 is a hypomorphic allele of *dgo* is complicated by the fact that it does not have any discernable alteration in the *dgo* locus in Southern analysis.

The members of the 'second' complementation group, 929, 869 and 63, fully complement lines 273 and 459 for lethality. In addition, lines 869 and 63 do not have an embryonic lethal phenotype expected of mutations in the *dgo* gene. The lethal phenotype in these strains could be the result of 'background' mutations that would only become obvious when lines were made homozygous. A high frequency of such background mutations was observed by Cooley *et al.*, (1988a) during a large scale P element mutagenesis experiment. These mutations could have arisen in fly stocks prior to the mobilisation experiment, or alternatively, could be the result of a transposase induced event such as a chromosomal rearrangement.

Chapter 6

GAL4-driven Pertussis Toxin Expression

6.1 Introduction

The covalent modifications of G proteins by cholera toxin and pertussis toxin are well characterised (Gilman, 1987; see section 1.3.1). Cholera toxin and pertussis toxin both catalyse the transfer of the ADP-ribose from NAD to a specific residue of certain G protein α-subunits. Modification of an α-subunit at a specific arginine residue by cholera toxin reduces the rate of hydrolysis of bound GTP to GDP, which traps the protein in an activated state. The action of pertussis toxin (PT), however, has the opposite effect. The inactive state of the α-subunit is the substrate for PT, which prevents receptor-mediated G protein activation. Naturally occurring PT is synthesised by *Bordetella pertussis* and is composed of five subunits, S1, S2, S3, S4 and S5. In common with cholera and diptheria toxins PT can be grouped into two functional units, an A chain and a B chain. The A chain of PT consists of the toxic subunit S1, and the B chain comprises S2, S3, S4 and S5 which bind to the surface of eukaryotic cells and translocate S1 across the cell membrane. The S1 subunit then catalyses the transfer of ADP-ribose from NAD to a specific cysteine residue at the carboxyl-terminus of the α-subunit. Substrates for PT action in mammals include α-subunits G_i, G_o and transducin (Gilman, 1987). As experimental tools, these toxins have been important in distinguishing different classes of G protein α-subunits and in understanding their physiological importance (Stryer and Bourne, 1986, Gilman 1987; see section 1.3.1).

In *Drosophila*, a single PT substrate has been detected and identified as the α-subunit of G_o, designated $DG\alpha_o$ (Hopkins *et al.*, 1988; Thambi *et al*, 1989). Other G protein α-subunits found in *Drosophila* lack the required cysteine residue and are not modified by the toxin. This specificity of PT

action was exploited by Fitch *et al.*, (1993), to investigate, *in vivo*, the role of $DG\alpha_o$. Flies were transformed with the S1 subunit under the control of the hsp70 heat-shock promoter which should lead to expression of the PT S1 subunit in all *Drosophila* tissues. Unexpectedly, in adults expression of the S1 RNA and protein was restricted to the anterior and posterior midgut, photoreceptors and the lamina of the optic lobe. Low level expression was also observed in the brain cortex and ovarioles. In embryos, PT expression in virtually homogeneous. Although $DG\alpha_o$ is primarily expressed in the embryonic CNS (Guillen *et al.*, 1991), PT expression at this stage is only partially lethal with 18-35% of embryos developing to adults. When PT was induced by heat-shock in adults, flies were unable to absorb nutrients and had altered visual responses. The effects of PT in the visual system are associated with the expression of PT in the lamina of the optic lobe, since photoreceptor function remains unaffected. This is consistent with the pattern of expression $DG\alpha_o$ which is found in the lamina of the optic lobe, but not photoreceptors (de Sousa *et al.*, 1989).

A method to directly express a toxin gene, or indeed any gene, is provided by the GAL4 enhancer-trap detection system (Brand and Perrimon, 1992; Kaiser, 1993; Brand and Dormand, 1995; see section 1.2.8). In this system a transposon containing the yeast transcriptional activator GAL4 (P[GAL4]) is inserted randomly in the *Drosophila* genome where, depending upon the site of integration, expression is directed by one of many genomic enhancers. GAL4 is then used to activate a target gene fused to a GAL4-responsive promoter (or UAS_G). By crossing flies containing P[GAL4] to those with a UAS_G-linked transgene, such as UAS_G-*lacZ*, the target gene can be expressed in a pattern which reflects enhancer-driven GAL4 activity. The target gene can therefore be activated in different tissues at

varying developmental stages by crossing the UAS_G-responsive gene to a collection of GAL4-expressing lines.

The ability to direct when and where GAL4-responsive genes are expressed is particularly useful in cell ablation studies, since toxin expression can be lethal to the organism. This problem has been encountered in previous experiments with diptheria toxin for example, where it has been necessary to control its action by inserting a suppressible amber codon in the coding region (Kunes and Stellar, 1991), or by generating temperature-sensitive mutations in the gene (Bellen *et al.*, 1992). Directed cell ablation in conjunction with the GAL4 system has been used to study the process of axon pathfinding in the developing embryonic CNS, where the small size of the *Drosophila* embryos make laser ablation of specific neuronal cells difficult. By expressing UAS_G-ricin to ablate glial cells at various embryonic stages, Hildago *et al.*, (1993), were able to test the role of glial cells in pathfinding of pioneer growth cones, and the subsequent maintenance of axon tracts once formed. Using an antibody to a glial-specific protein, it was shown that cells targeted were killed rapidly and efficiently. In addition, ablation was found to be cell-autonomous since neighbouring cells were unaffected.

The specificity of pertussis toxin action to $DG\alpha_o$ in *Drosophila*, together with the availability of a large collection of P[GAL4] lines generated in this laboratory (e.g. Yang *et al.*, 1995), provides an ideal opportunity to express pertussis toxin and disrupt $DG\alpha_o$ function in different tissues and at various development times. The S1 subunit gene of pertussis toxin was cloned into pUAST to give a GAL4-responsive PT gene (Brand and Perrimon, 1993), and transformants carrying the transgene were obtained. These transformants were crossed to a number of P[GAL4] lines to assess

toxin activity. PT expression with some P[GAL4] lines resulted in death primarily in larvae, while a small number of pupae that formed were unable to hatch.

6.2 Generation of a GAL4-responsive PT transgene

The S1 subunit gene cloned into the vector pUAST was isolated from the transgenic line PT37 described by Fitch *et al.*, (1993) [PT37 strain kindly provided by J. Hurley]. The S1 subunit of PT is normally secreted into the extracellular medium by *B. pertussis* as a polypeptide precursor containing an amino terminal secretory signal (Nicosia *et al.*, 1986). To remove this signal sequence and prevent secretion of the toxin in *Drosophila* tissues, the S1 gene in line PT37 had been previously modified by amplifying the gene with a primer (PT1) containing sequences complimentary to the first 21 bp of the mature S1 coding region, thereby eliminating the secretory signal sequences (for a detailed description see Fitch *et al.*, 1993). Amplification with primer PT1 also introduced a *Bam*HI restriction site and sequences encoding the translation initiation of the *Drosophila* hsp70 gene upstream of S1 coding sequences.

The S1 gene within line PT37 was used as a substrate for PCR and the amplification product cloned into pUAST using a similar strategy as Fitch *et al.*, (1993) (Figure 6.1). pUAST is a vector into which a gene can be subcloned behind five tandemly arranged GAL4 binding sites, resulting in a GAL4-responsive transgene (Brand and Perrimon, 1993). Genomic DNA prepared from line PT37 was amplified with a new primer, PTE, and a P element primer P31 (complementary to P element termini, see section 3.4). Primer PTE is identical to PT1 except that it contains an *Eco*RI restriction site instead of *Bam*HI. Amplification with PTE and P31

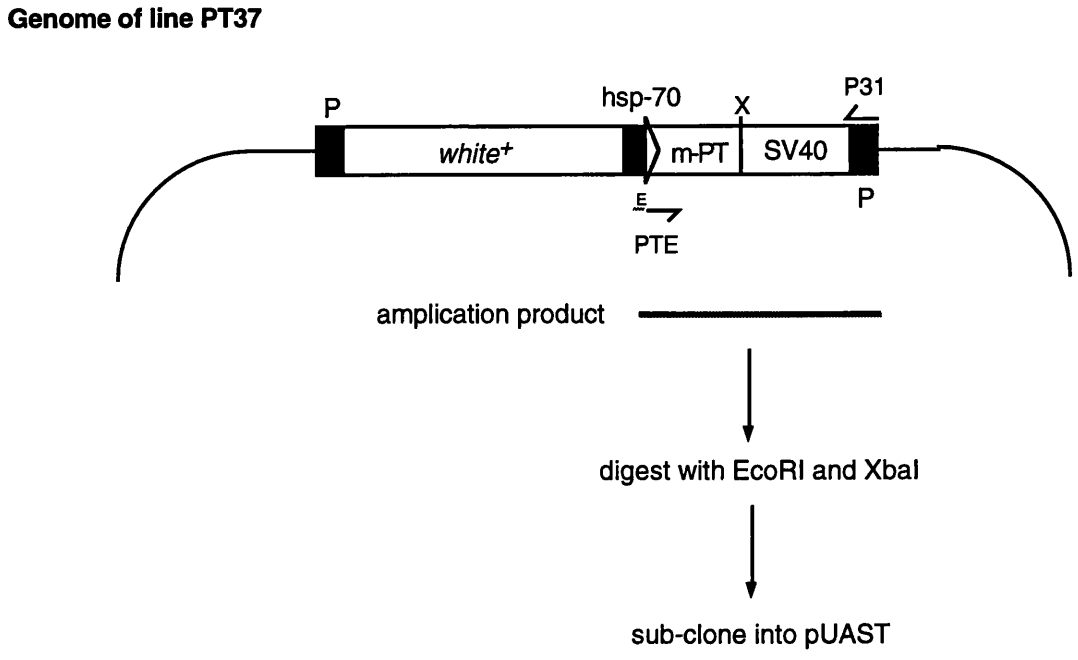


Figure 6.1 Strategy used to isolate the S1 subunit gene.

The PT-transgene within the genome of line PT37 is shown as a box. P element ends are indicated by black boxes labelled P. The primers used for amplification of the mature S1 gene (m-PT) and SV40 polyadenylation sequence, PTE and P31, are indicated by small arrows. For cloning purposes, primer PTE was synthesised with an *Eco*RI restriction site at its 5' end (see below). The resulting PCR product was digested with *Eco*RI and *Xba*I to liberate m-PT and this fragment was then subcloned into pUAST.

(PTE: 5'-3'

GAAAG**GAATTC**ACACAATGGACGATCCTCCCGCCACCGT)

gives a fragment of ~2 kb which in addition to S1 coding sequences, includes SV40 polyadenylation sequences, followed by part of the left end of the P element (Figure 6.2A; see Figure 6.1). The purified amplification product was digested with *Eco*RI and *Xba*I and a 700 bp fragment containing the S1 gene isolated (Figure 6.2B). This fragment was purified and inserted into plasmid pUAST which had previously been digested with the same restriction enzymes. The resulting plasmid, pUAST-PT (Figure 6.3) has an eye colour selection marker (*white*$^+$), five GAL4 binding sites and the *Drosophila* hsp70 promoter in front of the mature S1 gene, followed by the SV40 polyadenylation site and small t intron.

6.3 Sequence analysis of the S1 gene in pUAST-PT

Since the PT gene cloned into pUAST was isolated using PCR and could contain nucleotide substitutions frequently observed with amplification with *Taq* polymerase, the complete nucleotide sequence of pUAST-PT was determined by dideoxy chain termination. Using primers that flank the polylinker in pUAST (pU_L, pU_R), sequence was obtained from both ends of the S1 gene. Five pUAST-PT clones were analysed and only one, pUAST-PT5, had no base substitutions or other changes. The complete sequence of the S1 gene within pUAST-PT5 was established using a sequencing primer that sits within the S1 gene (PT181). An alignment of pUAST-PT5 with the nucleotide sequence of the S1 subunit gene is shown in Figure 6.4. Plasmid pUAST-PT5 was used for subsequent microinjection of *Drosophila* embryos.

6.4 Germline transformation with pUAST-PT

To obtain a PT transformant, pUAST-PT was injected into w^{1118} embryos with a helper P element plasmid phs-π-Δ2,3 (Misra and Rio, 1990) as a source of transposase. The 48 surviving G0 adults were crossed

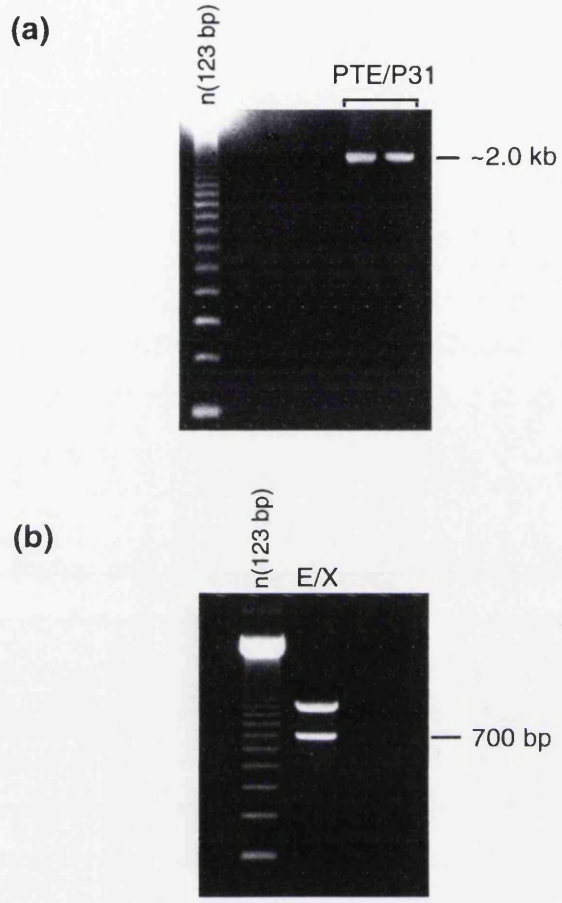


Figure 6.2 PCR amplification and isolation of the S1 gene.

(A) Amplification of the S1 gene from PT37 flies with gene primer PTE and P element primer P31. The amplification product of ~2 kb includes a 700 bp fragment containing the S1 gene, SV40 polyadenylation sequences and ~300 bp of the right end of the P element (see Figure 6.1).
(B) The amplification product digested with restriction enzymes *Eco*RI and *Xba*I. The 700 bp DNA fragment containing the S1 gene is marked.

In (A) and (B) DNA was separated on a 1% agarose gel and visualised by ethidium bromide staining. n(123bp) is 123bp DNA size marker (BRL).

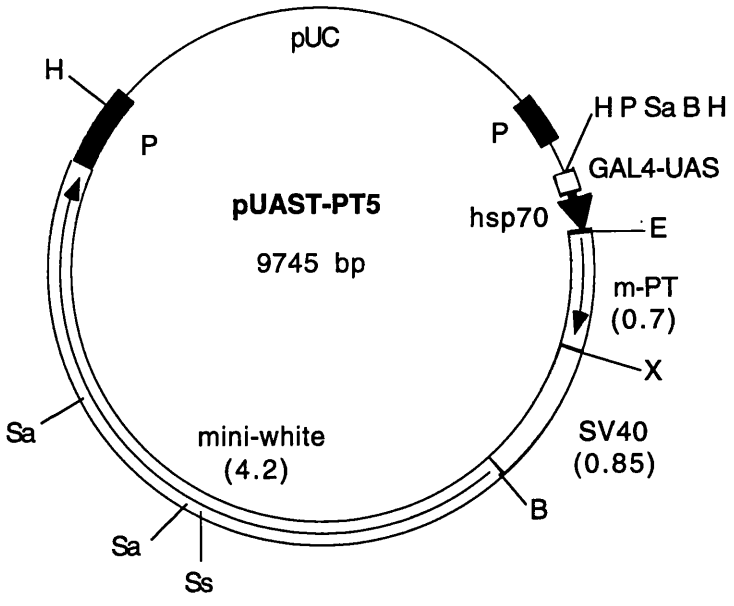


Figure 6.3 Plasmid pUAST-PT used for P element-mediated transformation.

A ~2 kb PCR fragment containing the mature S1 gene of pertussis toxin (m-PT), SV40 polyadenylation signal, small t intron (SV40), and P element right end (P), was amplifed from line PT37 (Fitch *et al.*, 1993, see Figure 6.2A). A 700 bp, *Eco*RI-*Xba*I restriction fragment containing the m-PT gene was inserted into pUAST (Brand and Perrimon, 1993). Other abbreviations: hsp70, *Drosophila* hsp70 promoter; GAL4-UAS, yeast transcriptional activator GAL4 upstream activation sequence; mini-white, *Drosophila white*$^+$ gene; *E*, *Eco*RI; *B*, *Bam*HI; *H*, *Hin*dIII; *P*, *Pst*I; *Sa*, *Sal*I; *Ss*, *Sst*I; *X*, *Xba*I.

```
|M14378|BPETOXS Bordetella pertussis (strain BP165) toxin gene encoding subunits
              S1, S2, S3, S4, S5.
              Length = 4936

Score = 3460  (956.1 bits),  Expect = 2.3e-280,  P = 2.3e-280

Identities = 692/692  (100%), Positives = 692/692 (100%)

PT5:        16 GACGATCCTCCCGCCACCGTATACCGCTATGACTCCCGCCCGCCGGAGGACGTTTTCCAG 75
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTOX:     609 GACGATCCTCCCGCCACCGTATACCGCTATGACTCCCGCCCGCCGGAGGACGTTTTCCAG 668

PT5:        76 AACGGATTCACGGCGTGGGGAAACAACGACAATGTGCTCGACCATCTGACCGGACGTTCC 135
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTOX:     669 AACGGATTCACGGCGTGGGGAAACAACGACAATGTGCTCGACCATCTGACCGGACGTTCC 728

PT5:       136 TGCCAGGTCGGCAGCAGCAACAGCGCTTTCGTCTCCACCAGCAGCAGCCGGCGCTATACC 195
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTOX:     729 TGCCAGGTCGGCAGCAGCAACAGCGCTTTCGTCTCCACCAGCAGCAGCCGGCGCTATACC 788

PT5:       196 GAGGTCTATCTCGAACATCGCATGCAGGAAGCGGTCGAGGCCGAACGCGCCGGCAGGGGC 255
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTOX:     789 GAGGTCTATCTCGAACATCGCATGCAGGAAGCGGTCGAGGCCGAACGCGCCGGCAGGGGC 848

PT5:       256 ACCGGCCACTTCATCGGCTACATCTACGAAGTCCGCGCCGACAACAATTTCTACGGCGCC 315
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTOX:     849 ACCGGCCACTTCATCGGCTACATCTACGAAGTCCGCGCCGACAACAATTTCTACGGCGCC 908

PT5:       316 GCCAGCTCGTACTTCGAATACGTCGACACTTATGGCGACAATGCCGGCCGTATCCTCGCC 375
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTOX:     909 GCCAGCTCGTACTTCGAATACGTCGACACTTATGGCGACAATGCCGGCCGTATCCTCGCC 968

PT5:       376 GGCGCGCTGGCCACCTACCAGAGCGAATATCTGGCACACCGGCGCATTCCGCCCGAAAAC 435
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTOX:     969 GGCGCGCTGGCCACCTACCAGAGCGAATATCTGGCACACCGGCGCATTCCGCCCGAAAAC 1028

PT5:       436 ATCCGCAGGGTAACGCGGGTCTATCACAACGGCATCACCGGCGAGACCACGACCACGGAG 495
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTOX:    1029 ATCCGCAGGGTAACGCGGGTCTATCACAACGGCATCACCGGCGAGACCACGACCACGGAG 1088

PT5:       496 TATTCCAACGCTCGCTACGTCAGCCAGCAGACTCGCGCCAATCCCAACCCCTACACATCG 555
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTOX:    1089 TATTCCAACGCTCGCTACGTCAGCCAGCAGACTCGCGCCAATCCCAACCCCTACACATCG 1148

PT5:       556 CGAAGGTCCGTAGCGTCGATCGTCGGCACATTGGTGCGCATGGCGCCGGTGATAGGCGCT 615
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTOX:    1149 CGAAGGTCCGTAGCGTCGATCGTCGGCACATTGGTGCGCATGGCGCCGGTGATAGGCGCT 1208

PT5:       616 TGCATGGCGCGGCAGGCCGAAAGCTCCGAGGCCATGGCAGCCTGGTCCGAACGCGCCGGC 675
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTOX:    1209 TGCATGGCGCGGCAGGCCGAAAGCTCCGAGGCCATGGCAGCCTGGTCCGAACGCGCCGGC 1268

PT5:       676 GAGGCGATGGTTCTCGTGTACTACGAAAGCAT 707
               ||||||||||||||||||||||||||||||||
BPTOX:    1269 GAGGCGATGGTTCTCGTGTACTACGAAAGCAT 1300
```

Figure 6.4 **Alignment of nucleotide sequences from pUAST-PT5 with PT gene nucleotide sequence.**

Sequence alignment begins at the start of the mature S1 gene, nucleotide 609 of pertussis gene sequences (Nicosia *et al.*, 1986; accession no. M14378). A vertical line indicates identity. Nucleotides corresponding to those in primer PTE are in blue, and the position of internal sequencing primer, PT181, is in red.

individually to w^{1118} flies and G1 progeny screened for the *white*$^+$ eye colour marker. Two lines produced transformants and were numbered I and II. Since the *white*$^+$ offspring from lines I and II could represent unique insertional events, as many as possible were collected and mated individually to w^{1118}. Thirteen individuals were isolated from vial I and four from vial II, and were again mated with w^{1118}. These lines were retained for further analysis.

6.5 Molecular analysis of transformants

To determine whether transformants had single or multiple insertions, genomic DNA was prepared from flies from each of the 17 lines and digested with *Eco*RI which cuts once upstream of the PT gene in pUAST. Thus the size of the genomic fragment containing the PT gene will depend on where the gene has integrated. Digested genomic DNA was separated on an agarose gel, blotted and probed with ^{32}P-labelled PT-DNA. Southern analysis indicates that G0 transformant lines I and II produced progeny with multiple insertions, for example lines PT3.1(I) and PT2.4(II) (Figure 6.5). The DNA samples prepared from PT3.2(I), PT2.3(II), PT5.1(I) and PT5.12(II) were of low quality and the result therefore difficult to interpret. There were nine transformants with a single insertion and these fall into two classes. Genomic DNA was isolated from two examples of each class, digested with *Eco*RI, separated on an agarose gel, blotted and probed with labelled PT DNA. Southern analysis indicates that in PT1.6(I) and PT5.9(I) the probe hybridises to a fragment of ~9 kb, and in PT2.3(II) and PT5.8(II) to a fragment of ~11 kb (Figure 6.6a). The PT-hybridising fragments observed in classes I and II are of a different size and therefore represent a P element insertion at a single unique site in each.

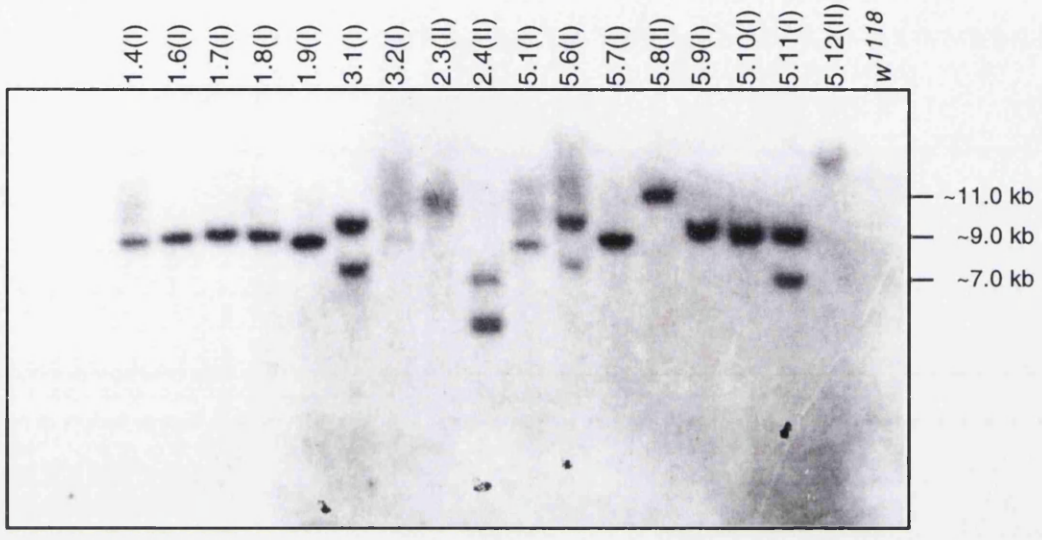


Figure 6.5 Southern analysis of transformant lines.

Genomic DNA from 17 transformant lines and w^{1118} was digested with *Eco*RI, separated in a 0.8% agarose gel, blotted and hybridised with labelled PT DNA. (1 kb ladder) is a 1 kb DNA size marker (BRL).

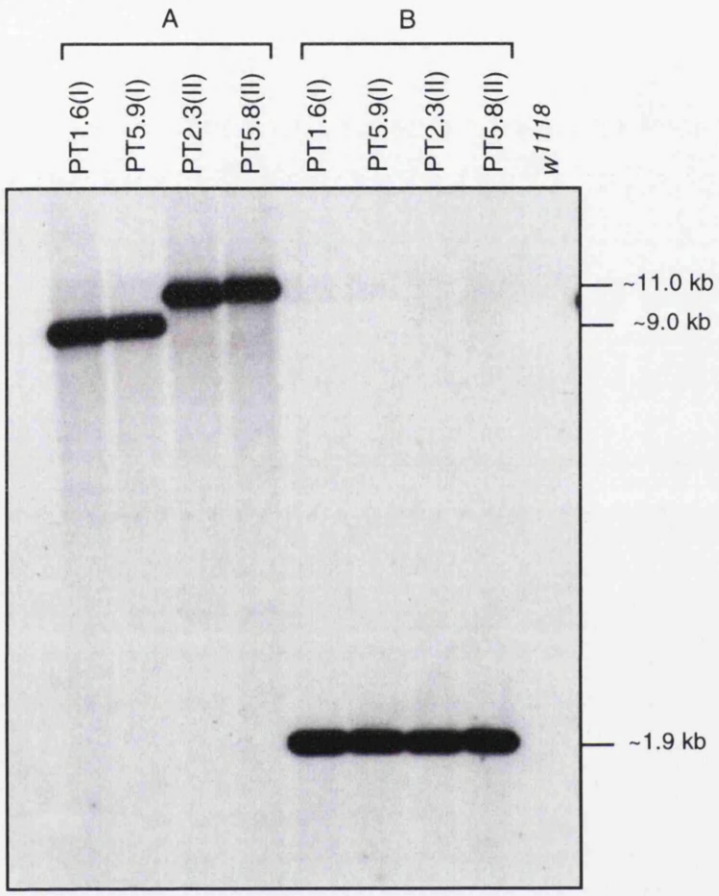


Figure 6.6 Southern analysis of class I and II transformants.

(A) Genomic DNA from PT1.6(I), PT5.9(I), PT2.3(II) and PT5.8(II) digested with *Eco*RI, and (B) PT1.6(I), PT5.9(I), PT2.3(II), PT5.8(II) and w^{1118} digested with *Bam*HI, separated on a 0.8% agarose gel, blotted and hybridised with ^{32}P-labelled PT DNA.

To determine if the PT transgene had integrated into the genome intact, the same DNA samples were digested with *Bam*HI, separated on an agarose gel, blotted and probed with ^{32}P-labelled PT DNA. *Bam*HI cuts pUAST-PT twice to give a 1.9-kb fragment (see Figure 6.3). In Southern analysis only a single hybridising fragment of ~1.9 kb is observed which suggests that the PT transgene is intact (Figure 6.6b).

6.6 PT transgene expression and activity

6.6.1 *Northern analysis of PT transgene expression*

The PT transgene is expressed only when a transformant line is combined in a cross with a GAL4 enhancer trap line. To induce PT expression, PT5.9(I) and PT5.8(II) flies were mated with 201Y, which has GAL4 expression in the brain and abdomen of the adult fly (Yang *et al.*, 1995, O'Dell *et al.*, 1995). Total RNA was prepared from the progeny of this cross and from PT5.8(II) and PT5.9(I) homozygous for the transgene. Total RNA was also prepared from P[GAL4] line 201Y and wild-type flies. RNA was separated on a denaturing agarose gel, blotted and probed with labelled PT-DNA. Northern analysis indicated that the toxin is expressed only when PT5.8(II) and PT5.9(I) are combined in a cross with 201Y, and that the transcript of ~1.7 kb was more abundant in PT5.9(I) (Figure 6.7). The source of the observed larger hybridising RNA species is unknown.

6.6.2 *PT transformants combined with GAL4 lines*

To determine if the toxin was active, both transformant lines were crossed to ten P[GAL4] lines. These were from a large collection of lines generated in this laboratory which were screened for GAL4 expression in the adult brain (Yang *et al.*, 1995). PT5.8(II) and PT5.9(I) females were

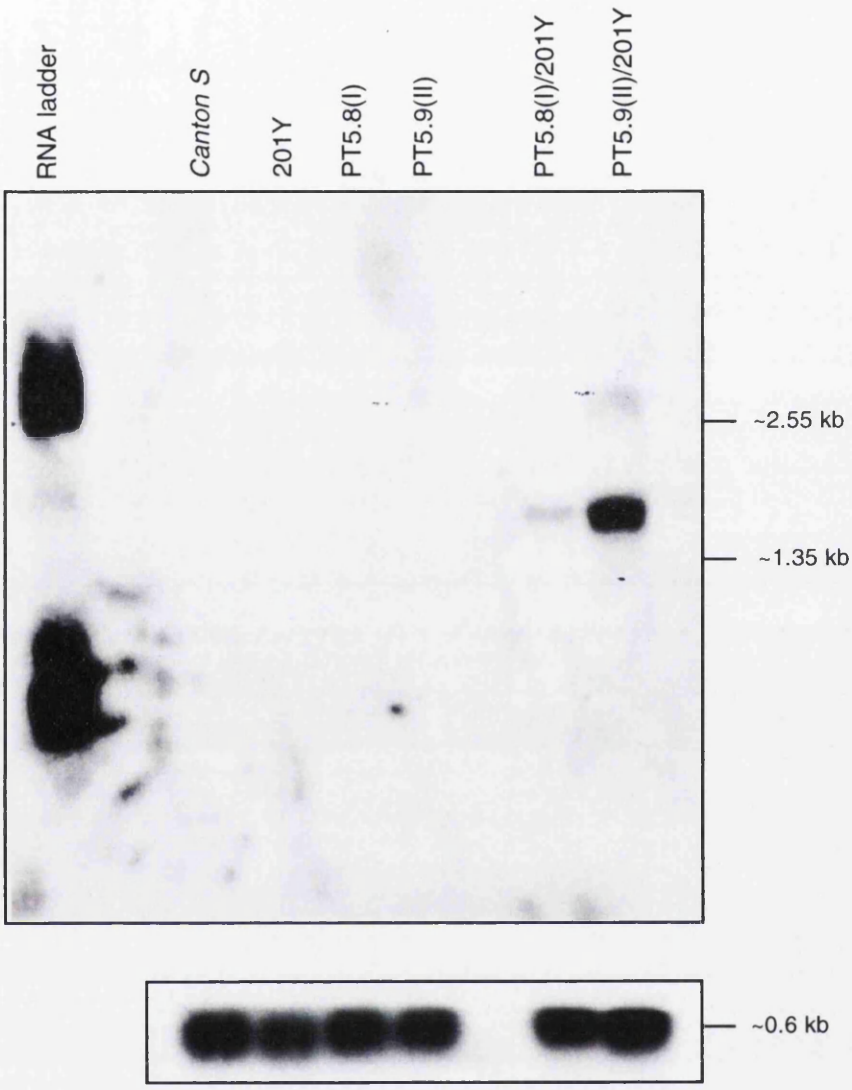


Figure 6.7 Northern blot analysis of PT expression.

Total RNA (15 µg each) was separated on a 1% agarose/formaldehyde gel, blotted and probed with a 700 bp *Eco*RI-*Xba*I fragment representing the S1 gene (see Figure 6.2b). A transcript of ~1.7 kb is observed only when PT5.8(II) and PT5.9(I) are combined in a cross with GAL4 line 201Y. The same blot (lower panel) was probed with a ribosomal probe, *rp49*, to check RNA integrity and the relative quantity of RNA in each lane.

mated to males from each P[GAL4] line and the cultures observed for any effect of PT expression. In eight of the ten crosses, viable adults were produced that appeared normal. However, when PT was expressed in two lines, C35 and 188Y, no adults were produced. C35 has GAL4 expression in mushroom bodies other neurons in the brain from the third instar to adult stages, and in the adult abdomen (O'Dell *et al.*, 1995). Line 188Y has GAL4 expression throughout the brain of the adult, but little is known regarding GAL4 expression during development. To establish the lethal phase of PT action, embryos were collected from a cross of PT5.9(I) with C35 and 188Y, and placed on grape juice agar plates at 18°C. Toxin expression in these GAL4 lines results in lethality in all larvae produced. Larvae develop normally to the second instar, then they begin to move sluggishly and eventually die. Embryos collected from w^{1118}, PT5.9(I) and PT5.8(II) homozygotes grown under the same conditions develop normally to adulthood.

To assess the consequence of PT expression in embryos, PT5.9(I) was crossed to P[GAL4] line C133 which has a high level of GAL4 activity in the embryo. It is not known specifically, however, what structures in C133 embryos have GAL4 activity (J. Clarke, pers. comm). PT expression combined with C133 resulted in 21% embryonic lethality (52 embryos hatch from a total of 240) as compared to 8.4% for w^{1118} (174 hatch from a total of 190) and 9.3% for PT5.9(I) (136 hatch from a total of 150).

In a separate experiment, PT5.9(I) was crossed to fifty lines from the same collection of P[GAL4] enhancer trap lines (Z. Wang, pers. comm). PT5.9(I) virgin females were mated to P[GAL4] males and scored for viable adult progeny. In eight of the fifty crosses, PT expression caused lethality prior to adulthood. As observed previously, PT5.9(I) in conjunction with C35

produced lethality primarily in second instar larvae, with no pupae developing. Lethality in second instar larvae was also observed in crosses with lines 30Y and 181Y. PT5.9(I) in conjunction with 121Y, 238Y, C107 and C532 resulted in only one or two pupae that did not eclose. 30Y has GAL4 expression in the mushroom bodies of the brain from third instar larvae up to adult stages. Line 238Y has GAL4 activity in mushroom body neuroblasts in the embryo, and in the mushroom bodies in the brains of larvae, pupae and adults (D. Armstrong, pers. comm). Information on the developmental expression pattern of GAL4 in lines 121Y, C107 and C532 is limited, but they are known to have GAL4 activity in adult brain structures.

6.7 Discussion

The GAL4 enhancer trap technique has been used to express pertussis toxin in an attempt to modify the DGα_o subunit *in vivo*, which has been shown previously to be the sole substrate for this toxin in *Drosophila* (Thambi *et al.*, 1988). A previous study using a heat-inducible PT gene has indicated the utility of this approach in disrupting G_o function in flies (Fitch *et al.*, 1993). The GAL4 system can be used to express any gene of interest in specific cells or tissues, or at any particular stage in development (Brand and Perrimon, 1993). GAL4-driven expression of other toxin genes has been used with success to analyse nervous system function. Specific pioneer neurons in the developing CNS have been selectively ablated with expression UAS_G-diptheria toxin (Lin *et al.*, 1995), and UAS_G-tetanus toxin has been used to specifically cleave synaptobrevin in a defined neuromuscular junction in the embryo (Sweeney *et al.*, 1995).

The PT-transgene in PT5.8(II) and PT5.9(I) is expressed as mRNA and produces an active S1 subunit. When crossed to ten GAL4 lines out of sixty tested, toxin expression results in lethality, primarily in second instar larvae. GAL4 activated transcription has been observed in salivary glands of larvae in 51% of GAL4 insertion lines (Brand and Perrimon, 1993). However, PT expression in salivary glands alone cannot account for the observed toxic effect since larval lethality was observed in only ten out of sixty GAL4 lines tested. In addition, salivary glands are non-essential for viability. The $DG\alpha_o$ protein is observed, using immunolocalisation, in the developing CNS of the embryo (Guillén *et al.*, 1991) and $DG\alpha_o$ mRNA is expressed in all developmental stages of the *Drosophila* life-cycle (Thambi *et al.*, 1989). If $DG\alpha_o$ is required for the proper development of the CNS, it might be expected that PT modification of $DG\alpha_o$ in the embryo would result in lethality at this stage. Expression of PT in GAL4 line C133, which has a high level of GAL4 activity in the embryo, resulted in a slight reduction in viability of embryos but not complete lethality. This is similar to the results of Fitch *et al.*, (1993), who similarly did not observe complete embryonic lethality even though PT expression was almost homogeneous in the embryo. Since it is known that $DG\alpha_o$ is essential for embryonic development in *Drosophila* with mutants showing defects in the developing CNS (Guillén *et al.*, 1995), these results suggest that *in vivo* modification of $DG\alpha_o$ by PT does not completely disrupt $DG\alpha_o$ function. However, two points are worth noting in this regard. First, in the experiments of Fitch *et al.*, 20% of $DG\alpha_o$ protein in the adult remained unmodified by pertussis toxin. Although it was not determined how much $DG\alpha_o$ is modified by PT in the embryo, it is possible that a sufficient fraction of $DG\alpha_o$ protein is left unmodified to fulfil $DG\alpha_o$ function at this stage. Second, the partial embryonic lethality observed with PT expression in GAL4 line C133

cannot be clearly attributed to PT acting on $DG\alpha_o$ in the CNS as it is not known for certain if the GAL4 activity in C133 is in the embryonic central nervous system. In the experiments described here, the developmental phase which is most sensitive to PT action is the larval stage. Whether this is a non-specific effect of PT expression or reflects GAL4 activity in some lines at this time is not clear. However, in those GAL4 lines that show an effect with PT, when the expression pattern during earlier developmental stages is known, all have GAL4 activity in structures within the CNS in third instar larvae at least.

The experiments described in this chapter suggest that the PT transformants isolated produce active toxin which can perturb normal cellular processes, particularly in second instar larvae. However, before any firm conclusions can be drawn regarding $DG\alpha_o$ function in *Drosophila* from experiments with the PT transformants described here, it would be necessary to show that the toxin is acting to modify endogenous $DG\alpha_o$ when expressed in GAL4 lines. The ADP-ribosyltransferase activity of PT in transformants could be assayed in membrane fractions using [^{32}P]-NAD. It would also be necessary to show, using an antibody specific to PT, that the toxin is expressed in the same cell types as the GAL4 transcription factor, originally visualised in a cross to UAS_G-*lacZ*. Once these questions are addressed, a selection of GAL4 lines could be used to express PT in those regions in the adult CNS where $DG\alpha_o$ protein is known to be localised, and its role within individual structures assessed. Also, expression of PT within the developing nervous system may shed some light on where and when $DG\alpha_o$ protein is required for subsequent development.

Final Conclusions

7.1 Final conclusions

Screen for P element insertions

Site-selected mutagenesis was used to successfully isolate flies containing P element insertions in genes encoding G protein alpha subunits, DGα1 and DGα_o, and DC0, the catalytic subunit of cAMP-dependent protein kinase. All P element insertions, apart from one, were detected within upstream 5' gene sequences. P element mutations were silent, with no phenotypic consequence. However, the position of transposons near upstream regulatory regions provides a valuable resource for disruption of the nearby gene using imprecise P element excision.

Two of the genes successfully targeted, *dgo* and *DC0*, are located on the second chromosome at cytological position 47A and 30C, respectively. The source of the transposons in this mutagenesis was *Birm*-2 which contains a second chromosome with seventeen defective P elements. Interestingly, when the cytological positions of the P elements on the *Birm*-2 second chromosome are examined by *in-situ* hybridisation to polytene chromosomes, P elements are found at positions 47A and 30C (J. Lim and C. Preston, pers. comm.). This raises the possibility that the insertions were 'local jumps'; transpositions of the P element from its existing site to another site nearby on the chromosome (Tower *et al.*, 1993).

Since this screen for P element insertions was initiated, other reverse genetic approaches have been used to examine the function, in *Drosophila*, of some of the genes selected in this study as targets for mutagenesis. The role of PKA in learning and memory processes was tested using a 'dominant negative' approach. Flies harbouring transgenes containing a specific peptide inhibitor, or fragments of regulatory or catalytic subunits of PKA, displayed a reduction in learning

and memory capability (Drain *et al.*, 1991). Mutations in the *DC0* gene have been isolated from an enhancer element screen (Skoulakis *et al.*, 1993), and by chemical mutagenesis using EMS (Lane and Kalderon, 1993). The involvement of PKA in learning processes has been more clearly demonstrated with flies carrying a combination these mutations: DC0 hypomorphs with less than 20% of normal PKA activity have impaired ability in an associative learning/memory test (Skoulakis *et al.*, 1993). DC0 has also been shown to be necessary for developmental processes. Adults heterozygous for a strong and a weak *DC0* allele fail to lay eggs and have defects in oogenesis (Lane and Kalderon, 1993). Using a local jump strategy and PCR detection of insertion, Littleton *et al.*, (1993a), were able to generate a lethal insertion within the gene encoding synaptotagmin. Subsequent imprecise excision of this P element to create new mutations, in conjunction with EMS-induced mutations, have led to an extensive analysis of the *in-vivo* function of synaptotagmin in *Drosophila* (Littleton *et al.*, 1993b; DiAntonio *et al.*, 1993b; Broadie *et al.*, 1994; Littleton *et al.*, 1994).

Secondary mutagenesis of dgo

A PCR-based strategy was used to identify fly lines with disruption in the vicinity of the P element insertion in the insertion line, dgo^1. Approximately 10% of lines exposed to transposase had undergone a disruption that prevented amplification between the P element and gene primer. Within these lines there are likely to be clustering of identical events, as the dysgenic individuals were generated in mixed cultures and premeiotic events were not excluded. This may account for the repetitious nature of the observed events. Only eight lines from a total of 89 had an associated phenotype. Analysis of one type of imprecise excision that disrupts the transcription of the class I *dgo* transcript (Yoon

et al., 1989), shows that *dgo* function is intact as far as can be observed in these lines.

Complementation analysis between eight lines with a recessive lethal phenotype show that they affect more than one essential loci. The mutations in two lines, 273 and 459, fail to complement and have a deletion that spans *dgo* coding sequences. Thus, they correspond to null mutations in the *dgo* gene. Recessive lethal lines that complement 273 and 459 have mutations that most likely arose in the starting P element insertion stock, or the second chromosome balancer stock, prior to the initiation of the mobilisation experiment. Such background mutations have been observed previously in large scale transposon mutagenesis experiments (Cooley *et al.*, 1988; Bellen *et al.*, 1989). One recessive lethal line, 217, has a partial lethal phenotype in combination with 273. No disruption is observed in line 217 in Southern analysis with probes that encompass the *dgo* locus. However, this line could have a P element insertion within the promoter region of the Class II transcript which must be located 5' to the locus as described by Yoon *et al.*, (1989). Line 217 has however, in addition to a mutation in common with 273, another lethal mutation that map elsewhere.

The $G\alpha_o$ subunit in *Drosophila* is found localised in the central nervous system of the embryo (Guillén *et al.*, 1991). $DG\alpha_o$ protein accumulates in the embryonic nervous system during the period of intense axonogenesis (stage 13) and coincides with the development of the axonal scaffold (Guillén *et al.*, 1995). It is interesting to note that in mammalian neuroblastoma cells in culture, global $G\alpha_o$ levels increase during differentiation when cells extend long neurites (Brabet *et al.*, 1988). It has also been shown in mammals that $G\alpha_o$ is a major component of the growth cone membrane, where it can be regulated by GAP-43, a protein involved in axonal growth (Stritmatter, *et al.*, 1990). These data together

suggest a role for $G\alpha_o$ in axonal growth and guidance. In *Drosophila*, $DG\alpha_o$ mutants result in embryonic lethality and show defects in the developing nervous system (Guillén *et al.*, 1995). Four of the recessive lethal lines isolated in this study have embryonic lethal phenotypes. This includes lines 273 and 459 which are null mutations in *dgo*. Unfortunately, attempts to analyse the structure of the developing nervous system in mutant lines with an embryonic lethal phenotype using a $DG\alpha_o$ specific antibody have so far been unsuccessful.

The mutations in excision lethal lines 273 and 459 fail to complement two other mutations that map to chromosomal position 47A: *longitudinals lacking* (*lola*) and *bumper-to-bumper* (*btb*). The *lola* mutation was isolated in a screen for mutations that affect the development of the CNS axon pathways in the embryo (Seeger *et al.*, 1993). Embryos that are *lola*$^-$ have defects in longitudinal axon pathways which are absent or greatly reduced. Analysis of *lola* mutants for PNS defects in the developing embryo show that lateral chordotonal organs were displaced dorsally, or occasionally posteriorly, from their wild-type position (Giniger *et al.*, 1994). The *btb* mutation was isolated in an enhancer-detector screen for genes expressed in the developing embryonic PNS (Kania *et al.*, 1995). The insertion in *btb* was one of the rare P element insertions isolated that complemented mutations in *lola*, an insertional hot-spot. Similar to *lola* mutants, *btb* mutants also have misplaced lateral chordotonal organs, which are positioned posteriorly relative to wild-type (Kania *et al.*, 1995). The phenotype of *btb* in the developing CNS has not been described. It is interesting that three genes mapping to chromosomal location 47A have very similar staining patterns in the CNS of the embryo with specific antibodies, and result in mutants with defects in the developing nervous system. Molecular cloning of the *lola* gene shows it to be a transcription factor, related to

products from the *tramtrack* and *BR-C* genes (Giniger *et al.*, 1995). The gene encoding the *btb* product has not been cloned so far. Thus, *lola* may regulate the transcription of both *dgo* and *btb*. In addition, no data presented here or elsewhere excludes the possibility that the *btb* mutation is actually a mutation in *dgo*. Further information on the phenotype of a specific *dgo* mutation (Guillén *et al.*, 1995), may help clarify this matter, as will molecular characterisation of DNA flanking the P[*lacW*] element in *btb*. Plasmid DNA from the same collection of insertion lines that contained the *btb* mutation has been screened with a *dgo* specific DNA (chapter 5), but the insertion in *btb* could have been missed as DNA was rescued from only one side of the transposon insertions (Guo *et al.*, 1996).

Pertussis toxin expression

In the adult nervous system, DGα_o protein is found localised primarily in the neuropil of the brain, with staining in the optic lobes, and in the neuropil of the thoracic and abdominal ganglia (Schmidt *et al.*, 1989). However, the specific role of *dgo* in these tissues is unknown. The requirement of *dgo* in the genesis of the nervous system poses a problem in the analysis of *dgo* function in the adult. A very useful tool to dissect *dgo* function is provided by the specificity of pertussis toxin action to DGα_o in *Drosophila*, previously exploited by Fitch *et al.*, (1993). To more specifically direct expression of the toxin, the S1 gene of PT was cloned into a pUAST vector. When expressed in lines with GAL4 activity, PT is active and disrupts developmental processes, particularly in larvae. There are available a library of GAL4 lines in the laboratory of K. Kaiser with defined expression in a variety of structures in the adult nervous system. Thus it will be possible to assess the role of DGα_o in defined subdomains of the adult central nervous system.

7.2 Future work

It would be essential to define the nature of the mutations in the recessive lethal strains isolated here. Complementation analysis with a specific *dgo* mutant would be first choice. However, mutations could be mapped by other means. For example, visible genetic markers flanking the chromosomal location could be introduced via recombination to allow replacement of the rest of the genome. Alternatively, there are now available three deficiency stocks that remove chromosomal location 47A, and neighbouring regions, that could be used to map mutations. Further analysis on the embryonic phenotypes of recessive lethal lines using antibodies specific to *dgo* would be of interest. Two other mutations at 47A, *lola* and *btb*, also have defects in the developing central nervous system. It would be of interest to ascertain if the embryonic lethal phenotype in lines 273 and 459 resemble *lola* or *btb*. Such analysis could help determine the hierarchy of function of the three genes. Finally, if none of the recessive lethal lines have mutations in *dgo*, then the deficiencies in lines 273 and 459 could be used for a further round of mutagenesis, with the chemical mutagen EMS for example. Using the background of the deficiency, and testing any resulting mutant lines for allelism with *btb* or *lola*, a specific *dgo* mutant could be isolated.

The availability of a GAL4-responsive pertussis toxin transgene could pave the way for a full analysis of the role of *dgo* in the adult nervous system. Once the specificity of action of the toxin to $DG\alpha_o$ protein is determined, in a pertussis toxin assay using ^{32}P-NAD, expressing the transgene will provide a means to dissect the role of $DG\alpha_o$ in those tissues where it is normally expressed. Flies with modified $DG\alpha_o$ could be tested for aberrant behaviours, such as locomotory defects, visual defects or failure to learn in an associative learning paradigm.

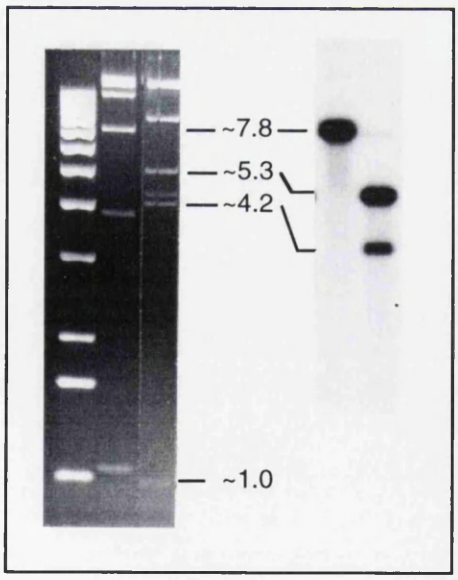
Appendices

Appendix I

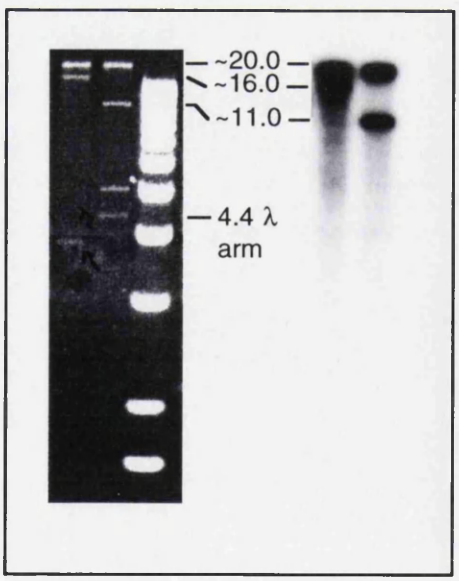
Restriction data for λDG2 and λDG14.

DNA was isolated from λDG2 and λDG14, digested with *Eco*RI and *Hin*dII and separated on 1% agarose gels, blotted and probed with dGo12, a Class I *dgo* cDNA. Restriction digests were compared to a map of the *dgo* locus previously described by Yoon *et al.*, (1989). EtBr stained agarose gels and autoradiographs are shown below with the size of appropriate fragments indicated. The size of restriction fragments in *Eco*RI and *Hin*dIII digests for λDG2 and λDG14 are given in Tables A1.1 and A1.2 respectively, with brief descriptions of their locations within the map shown in Figure 5.1.

λDG2



λDG14



Appendix I cont'd.

Table A1.1 λDG2 restriction fragments.

Restriction enzyme	*Size* (kb)[a]	*Description*[b]
*Eco*RI:	~20.0	Large λ arm at right of phage insert.
	~13.0	λ small arm + ~4.0 kb of DNA at left side of phage insert.
	~7.8	*Eco*RI fragment hybridising to exons 2 and 3 of cDNA.
	~4.0	Right *Eco*RI fragment of phage insert.
	~1.1	Small fragments within 2.8 kb *Bam*HI-*Sma*I fragment.
*Hin*dIII:	>20	Large λ arm at right of phage insert.
	~8.5	4.4 kb fragment of λ small arm + ~4.0 of DNA at left of phage insert.
	~5.3	Central fragment, hybridising to exons 2 and 3 of cDNA.
	~4.4	4.4 kb fragment of λ small arm.
	~4.2	Fragment hybridising to exon 1 of Class I cDNA.
	~1.0	Fragments at left of phage insert.

[a]restriction fragments in bold hybridise with cDNA probe.

[b]descriptions refer to locations within the *dgo* locus shown in Figure 5.1.

Small λ arm = 8.8 kb; large λ arm = 19.9 kb. The small arm of λ has a *Hin*dIII site that results in two 4.4 kb fragments. Left and right in descriptions refer to orientation of phage inserts as illustrated in Figure 5.1.

Table A1.2 λDG14 restriction fragments.

Restriction enzyme	*Size* (kb)[a]	*Description*[b]
*Eco*RI:	**>20.0**	Large λ arm + DNA at left of phage insert.
	~16.5	Small λ arm + ~8 kb of DNA at right of phage insert.
	~4.0	Central fragment of insert.
	~1.0	Fragment not observed.
*Hin*dIII:	**>20.0**	Large λ arm + ~5.0 kb of DNA at left of phage insert.
	~11.0	4.4 kb fragment of small λ arm + ~7.0 kb of DNA at right of phage insert.
	~5.2	Central fragment.
	~4.4	4.4 kb fragment of λ small arm.
	~1.0	Fragments not observed.

[a] and [b] - see legend to Table A1.1.

Appendix II

Quantification of the ^{32}P signals obtained from Southern blots of excision lines 273 and 459 probed with λDG14.

Table A2.1 Southern blot probed with control gene probe.

Strain	PSL[a]	Area (mm^2)	PSL/mm^2
Canton S	1152.00	51.20	22.50
273	1194.00	55.20	21.63
459	1111.00	52.20	20.13

[a]PSL = photostimulated luminescence.

Table A2.2 Southern blot probed with λDG14.

Fragment size (kb)	Strain	PSL	Area (mm^2)	PSL/mm^2	Relative value standardised to control[a]
12.0	*Canton S*	3397.00	64.00	53.07	1.00
	273	1568.00	61.44	25.53	0.50
	459	1318.00	61.44	21.46	0.45
6.8	*Canton S*	1705.00	64.00	26.65	1.00
	273	590.20	61.44	9.61	0.37
	459	483.00	61.44	7.86	0.33
3.5	*Canton S*	2129.00	64.00	33.27	1.00
	273	744.00	61.44	12.11	0.38
	459	608.60	61.44	9.90	0.33
2.0	*Canton S*	1613.00	64.00	25.20	1.00
	273	599.90	61.44	9.76	0.40
	459	518.90	61.44	8.45	0.37

[a]The PSL value for each DNA fragment in lines 273 and 459 was divided by the corresponding value obtained for the control gene probe. The quotients were then standardised to *Canton S* with control probe, which was given the arbitrary value of 1.00.

Appendix III

Table A3.1 Complementation analysis of excision mutants, crossed *inter se*.

Number of homozygote (no. of balancer heterozygotes) with female parent

male parent	**63**	**93**	**217**	**273**	**459**	**547**	**869**	**929**
63	0 (82)	21 (48)	38 (68)	24 (54)	26 (48)	21 (48)	0 (92)	20 (35)
93	18 (39)	0 (78)	18 (55)	21 (49)	34 (73)	22 (52)	27 (66)	36 (77)
217	32 (69)	21 (47)	0 (91)	24(135)	0 (70)	24 (46)	24 (78)	0 (71)
273	20 (62)	30 (63)	29(120)	0 (73)	0 (82)	30 (68)	17 (31)	28 (76)
459	30 (48)	37 (79)	0 (95)	0 (68)	0 (89)	25 (45)	25 (86)	27 (68)
547	35 (76)	18 (45)	32 (50)	18 (66)	24 (51)	0 (87)	20 (37)	29 (73)
869	0 (63)	23 (52)	36 (49)	13 (35)	30 (63)	28 (56)	0 (67)	0 (66)
929	20 (55)	31 (76)	0 (68)	12 (41)	21 (78)	28 (42)	0 (16)	0 (72)

Appendix IV

Data from complementation analysis with mutations that map to chromosomal position 47A, and potential alleles of *dgo*.

Table A4.1a Complementation with *lola, btb* and potential alleles of *dgo*.

Number of transheterozygotes (no. of balancer heterozygotes) with female parent

male parent	*lola* 5D2/*CyO*	*btb* 99/1	? 1/1	? 2/33
63	24 (52)	26 (70)	35 (63)	20 (64)
93	24 (48)	28 (84)	26 (42)	32 (60)
217	18 (36)	20 (72)	24 (58)	22 (44)
273	0 (77)	0 (75)	35 (88)	27 (41)
459	0 (83)	0 (85)	26 (64)	22 (68)
547	28 (74)	23 (30)	15 (40)	24 (54)
869	44 (86)	30 (70)	28 (72)	20 (52)
929	23 (69)	27 (64)	15 (49)	22 (74)

Table A4.1b Complementation with *lola, btb* and potential alleles of *dgo*.

Number of transheterozygotes (no. of balancer heterozygotes) with female parent

male parent	**63**	**93**	**217**	**273**	**459**	**547**	**869**	**929**
lola 5D2/*CyO*	16 (32)	25 (43)	45 (82)	0 (168)	0 (84)	24 (69)	74(152)	23 (60)
btb 99/1	23 (54)	20 (38)	41(114)	58(110)	78(103)	29 (66)	44 (75)	18 (58)
? 1/1	20 (44)	17 (59)	27 (56)	31 (70)	49(100)	16 (28)	38 (88)	14 (30)
? 2/33	20 (56)	21 (68)	23 (63)	44 (82)	11 (37)	19 (55)	21 (59)	33 (73)

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