Identification and the characterisation of a novel gene required for the development of the larval head of *Drosophila melanogaster*.

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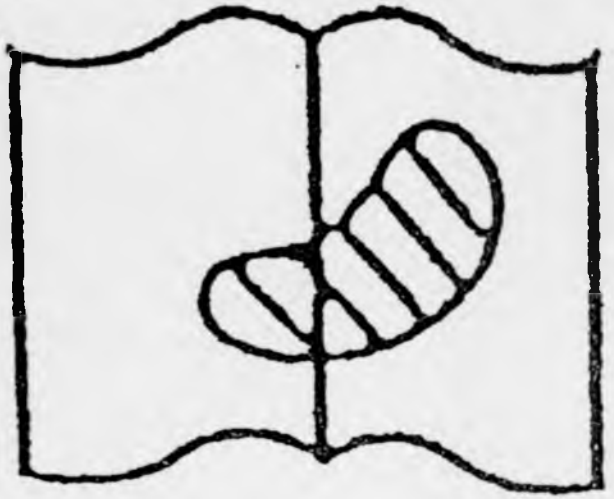

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Abbreviations used in the thesis

BSA	bovine serum albumin
DNA	deoxyribonucleic acid
cDNA	complementary DNA
Df	deficiency
DIG	digoxigenin
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylene diamine tetracetic acid
EtBr	ethidium bromide
OD	optical density
PBS	phosphate buffered saline
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TE	Tris/ EDTA (10mM Tris/ 1mM EDTA)
UAS	upstream activating site
UV	ultra-violet
V	volts
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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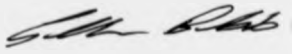
Declaration

The work presented in this thesis is, to the best of my knowledge, original except as acknowledged in the text. I hereby declare that I have not submitted this material, in whole or part, for a degree at this or any other institution.



Gillian Bubb

Abstract

The aim of this study was to identify novel genes required during early embryonic development, of *Drosophila melanogaster*.

This thesis describes the identification of a gene required for development of the larval head. This gene has not been detected previously in conventional mutagenesis screens and was detected using the technique of 'enhancer trapping'. This gene was selected for further investigation on the basis of the ß-galactosidase expression pattern from the P[lArB]enhancer detector transposon insertion, A350.1M2. The expression in this line was considered to be interesting since it occurs very early in a spatially restricted manner. The insertion was mapped to 59F1-3 on chromosome 2R. The P-element insertion was shown to be hemizygous lethal over Df(bwS46) and this lethality can be reverted by excision of the P-element. This showed that the insertion must have disrupted a gene that is essential for development.

The P[ClrB] insertion D26, is homozygous viable, and viable when hemizygous over $Df(2R)bw^{S46}$. It had been shown to have a similar expression pattern and map position to the A350.1M2 insertion, and it was therefore likely that it had inserted near to the same essential gene disrupted by A350.1M2. This was later confirmed by non complementation of ΔD26 excision chromosomes with A350.1M2.

The ß-galactosidase expression pattern of A350.1M2 in early gastrulating embryos, corresponds to cells that are the precursors of the midgut, Malpighian tubules and the proctodeum in the posterior, and in the anterior, the anterior midgut and head ectoderm. In germband extended embryos expression corresponds to cells of the anterior and posterior gut, gnathal and hypopharyngeal segments of the head and a large number of cells of the nervous system.

Genomic clones from the region flanking the insertion were obtained from a previously isolated plasmid rescue clone and a cosmid clone from the *brown* gene walk. Two fragments from this region known to contain only unique sequences were used to screen a cDNA library. Three cDNA clones were isolated. cDNA clone 19 and cDNA clone 3a3 were shown to be overlapping, cDNA 19 being a 5' trunkated form of 3a3. cDNA clone 6 seemed to be a different gene which may be from a different genomic location.

Fragments from each of these three clones were used to perform in situ hybridisation to whole mount *Drosophila* embryos. cDNA clones 19 and 3a3 gave expression patterns strongly reminiscent of the ß-galactosidase pattern of A350.1M2. The expression of these cDNA clones occurs slightly earlier than in A350.1M2, in two broad stripes in the anterior and posterior of the embryo. These cells correspond to the

same cells that express ß-galactosidase slightly later in line A350.1M2. By germband extension the expression is very similar to that of A350.1M2. cDNA clone 6 is not expressed during embryogenesis.

All three cDNA clones map back to the genomic walk. cDNA 3a3 is 6 kb to the right of the A350.1M2 insertion, and approximately 50 kb to the right of the *brown* gene. cDNA 3a3 was sequenced in both strands and the protein sequence was predicted in all three frames. The frames program on GCG showed there was no long open reading frame in any of the frames. The two longest frames occur in the same frame ORF1 and ORF2, which encode 92 and 88 amino acids respectively. The best homologies to known protein sequences are a sodium channel protein from rat cardiac muscle which shares 28.1% homology over 32 amino acids for ORF1, and a minor core protein V which has 35.1% identity over 37 amino acids, for ORF2. The sequence has provided little information about the function of the gene.

The viable D26 insertion was excised using transposase mediated excision and imprecise excision events were selected by lethality over $Df(2R)bw^{S46}$. Ten imprecise excision events were selected and were all shown to be embryonic lethal when hemizygous. These excision events were placed into two complementation groups.

The cuticular structure of dead first instar larvae showed that some of the excision events had defects in the head skeleton. These defects include reduced lateralgrate and disintegrated dorsal bridge. There were no obvious homeotic transformations accompanying these defects. There were no obvious defects in the corresponding earlier embryos. The morphological defects observed in mutant larvae correspond to a small subset of the total expression domain of the 3a3 transcript.

Chapter 1

Introduction.

The aim of my research has been to identify novel genes involved in the early development of *Drosophila melanogaster*, in particular development of the nervous system.

Apart from its powerful genetic technology *Drosophila* provides an excellent system for such studies for the following reasons: embryonic development occurs externally, and there are a large number of easily identifiable morphological landmarks at all stages of development facilitating the study of pattern formation and identification of mutant phenotypes; the genome is well characterised genetically and is amenable to genetic manipulation. *Drosophila* is also useful for the study of neurogenesis since it has a relatively small nervous system, there are approximately two hundred and fifty neurons per hemisegment in the ventral nerve cord, although the brain is larger and more complex.

Following fertilisation the zygotic nuclei divide in a common cytoplasm, the syncitial blastoderm (stage 1-2; 0 - 1:05 hr; fig 1.1). After eight synchronous divisions most of these nuclei begin to migrate to the periphery of the embryo (stage 3; 1: 05 - 1:20 hr; fig 1.1). Pole bud formation and nuclear division nine take place during stage three. Three protuberances bud off at the posterior pole. These are the polar buds which will divide twice and immediately after the second division the buds will pinch off forming 12-14 pole cells (Foe and Alberts, 1983). As the pole cells form, the remaining nuclei continue to divide in near but not perfect synchrony until they have divided thirteen times. During stage five the plasma membrane extends between the nuclei resulting in cellularisation (Mahowald, 1963)(stage 5; 2:10 - 2:50 hr; fig 1.1). Gastrulation then involves three main sets of cell movements. First a strip of the most ventral cells of the blastoderm embryo invaginates to create a two layered embryo (stage 6-7; 2:50 - 3:10 hr; fig 1.1). The inner layer, the mesoderm, forms many of the internal organs, such as muscles, and the outer layer, the ectoderm will form the epidermis, central and peripheral nervous systems. Secondly, presumptive endoderm

invaginates as two pockets at the anterior and posterior extremes of the ventral furrow, these are the forerunners of the anterior and posterior halves of the midgut (stage 8; 3:10 - 3:40 hr; fig 1.1). Thirdly, at the posterior pole a roughly ovoid cell plate corresponding approximately to the posterior midgut anlagen begins to move rapidly in an anterodorsal direction and simultaneously invaginates, carrying with it and internalising the pole cells (stage 9; 3:40 - 4:20 hr; fig 1.1). This anterodorsal movement of the posterior pole marks the onset of germ band extension. The germ band consists of the main trunk of the future embryo, the part that will become segmented. A transverse cephalic furrow forms laterally at the anterior extreme of the ventral furrow. Before germ band extension is complete, during stage nine, cells that will become the central nervous system begin to delaminate from the ventral neuroectoderm along the segmented region of the embryo, and also from the procephalic neuroectoderm in the head region. These neuroblasts form an intervening layer between the mesoderm and ectoderm, and subsequently divide a number of times to form the central nervous system (CNS). By stage 10, (4:20 - 5:20 hr; fig 1.1), the epidermis displays evenly spaced grooves and inside the mesoderm is arranged in a series of bulges. The epidermal grooves demarcate fourteen parasegments which correspond to the anterior edge of the posterior compartment of the three gnathal, three thoracic and eight abdominal segments. During stage eleven (5:20 - 7:20 hr; fig 1.1) the germ band retracts and in stage twelve (7:20 - 9:20 hr; fig 1.1) the anterior and posterior midgut fuse. By this stage the body plan and major tissue types of the larva are obvious and the remainder of embryonic development consists largely of differentiation of specialised cells within these tissues. Stage 13 (9:20 - 10:20 hr) is initiated at the completion of germ band shortening. This stage begins when the prospective anal plate is at the posterior egg pole and ends with the beginning of head involution. During stage 14 (10:20 - 11:20 hr) and stage 15 (11:20 - 13 hr) the epidermal layer of both sides of the embryo moves dorsally covering the amnioserosa towards the dorsal midline, ultimately leading to dorsal closure. The gut forms a closed tube that completely contains the yolk sac. On the lateral prospect the gut

shows a constriction which gives it a characteristic heart shape. Head involution occurs simultaneously with the movement of the epidermis. Head involution occurs by the caudalwards retraction of the clypeolabrum and the inward movement of the labial segment which results in the displacement of the opening of the salivary gland into the mouth. Ventrally the hypopharyngeal lobes have been displaced into the stomodeum. The gnathal appendages have moved antero medially and become located behind the lateral border of the stomodeum and the lateral walls of the atrium. Stage 16 (13 - 16 hr) ends when the dorsal ridge has completely overgrown the tip of the clypeolabrum (the morphogenic movements of head involution are summarised in Fig 1.2). Several further constrictions appear in the gut and the ventral nerve cord shortens until its posterior tip corresponds to 40% egg length. Stage 17 lasts until hatching of the embryo during which time the tracheal tree becomes apparent and retraction of the ventral nerve cord continues.

Maternal contribution to pattern formation.

Positional cues are established in the egg before or shortly after it is fertilised or laid; these are due to the activities of gene products expressed in the germ cells or follicle cells of the mother (Fig. 1.3). During ovarian development the female germ cells divide mitotically to produce sixteen cells that are in electrical contact via cytoplasmic channels. One of the most posterior cells develops into the oocyte and stays diploid while the others become the polyploid nurse cells. The group of sixteen cells is surrounded by about 1000 follicle cells which are derived from the mesoderm of the mother (fig 1.2). Early experiments by Sander (1978) (cited by Ingham, 1988) in *Euscelis*, and later in *Drosophila* (Nusslein-Volhard *et al*., 1987), which involved separating the embryo into two halves and then allowing them to develop separately, showed that the anterior posterior and the dorsal ventral axis are established independently. These experiments also showed that the anterior-posterior axis is generated progressively during early embryogenesis, under the influence of organising centres at the two poles of the embryo.

The anterior posterior pattern is set up by three systems which determine anterior, posterior, and terminal identity in the embryo.

To enable investigators to be able to describe the location of structures or gene expression domains within the *Drosophila* embryo a reference system of percentage egg length has been developed. EL 0% refers to the posterior pole and 100% to the anterior pole.

Anterior pattern.

There are three levels of gene activity that potentially regulate cephalic patterning: a set of maternally expressed factors and two levels of region specific zygotic gene expression. Maternal patterning systems control the organisation of the cephalic region: the *bicoid* anterior-posterior gradient, and the *torso* terminal signalling system described in the next section.

The gene *bicoid* was one of the genes identified in the screen for maternal genes that affect embryonic pattern (Nusslein-Volhard *et al.*, 1987). Embryos mutant for this gene develop normal posterior segments but have disrupted anterior abdominal segments, and no proper head or thoracic segments. The *bicoid* gene has been cloned, and from in situ hybridisation experiments *bicoid* mRNA has been shown to be transcribed in the nurse cells and apparently transported to the anterior pole of the oocyte (Berleth *et al.*, 1988; St. Johnson *et al.*, 1989). At least three gene products are necessary for the correct positioning of *bicoid* RNA; these are *exuperantia*, *swallow* and *staufen* (Frohnhofer and Nusslein-Volhard, 1987; St. Johnston *et al.*, 1991). After fertilisation, a concentration gradient of bicoid protein is established by translation of the *bicoid* mRNA at the anterior pole followed by diffusion posteriorly from the anterior source (Driever and Nusslein-Volhard, 1988a). This concentration gradient is thought to be maintained by diffusion from the anterior source counteracted by a uniform rate of degradation of the short-lived bicoid protein. By the time pole cell formation has occurred the detectable protein gradient extends from 100% to 30% egg length (Berleth *et al.*, 1988).

The sequence of the *bicoid* gene reveals that its product has a homeo domain; it has also been isolated by homology to a sequence (PRD-repeat) in the segmentation gene *paired* (Berleth *et al.*, 1988; Frigerio *et al.*, 1986). This suggests that *bicoid* encodes a DNA-binding protein which acts by directly regulating zygotic target genes.

In the syncitial blastoderm embryo seven gap genes are induced in overlapping domains along the anterior posterior axis. Two of these gap genes *huckebein* and *tailless* are expressed in the terminal regions. The expression of these two genes are partially activated independantly both by the *torso* signalling system and by the high levels of the bicoid gradient (Bronner and Jackle, 1991; Pignoni *et al.*, 1992). The role of the terminal gap genes differs at the posterior and anterior ends of the embryo; the pattern of *tailless* expression at the anterior end of the embryo is controlled by the *bicoid* gene.

Another zygotic target of bicoid is the gap gene *hunchback* which is required for correct development of the thorax and part of the head. Zygotic *hunchback* RNA occurs in a broad anterior domain which extends to about 50% egg length (Bender *et al.*,1987; Lehmann and Nusslein-Volhard, 1987b; Tautz *et al.*, 1987). This expression is dependent on moderate levels of *bicoid* expression. In *bicoid* mutant embryos zygotic *hunchback* is absent, whereas when *bicoid* gene dosage is increased the domain of *hunchback* expression extends posteriorly (Schroder *et al.*, 1988; Tautz, 1988; Struhl *et al.*, 1989). A number of bicoid-binding sites are present in the *hunchback* upstream region, including three strong and three weak binding sites, in the three hundred base pairs immediately 5' to the major start site of zygotic *hunchback* transcription (Driever and Nusslein-Volhard, 1989). Bicoid protein has been shown to be a morphogen, since its presence allows head patterning in a dose-dependent fashion. If the number of *bicoid*+ doses in the mother is increased the boundary of *hunchback* transcription shifts more anteriorly (Driever and Nusslein-Volhard, 1988b). A model for how this occurs has been suggested by analysis of the ability of different bicoid-binding sites to direct the embryonic activation of a basic promoter (Driever and Nusslein-Volhard, 1989b). The model suggests that promoters with low affinity

bicoid-binding sites require high concentrations of protein to be activated and are therefore expressed only in the most anterior regions. Conversely promoters with high affinity sites can bind bicoid at lower concentrations and direct expression in larger domains which extend more posteriorly (Struhl *et al.*,1989). In this way the smooth protein gradient can be translated into a number of discrete domains of zygotic gene expression, which define several anterior positional values.

Since ***hunchback*** alone is not sufficient to specify head development and loss of ***hunchback*** causes less severe defects than loss of ***bicoid*** the system requires at least one additional zygotic gap gene, which is directly regulated by ***bicoid***, to be expressed in a smaller more anterior domain than ***hunchback*** (Driever and Nusslein-Volhard, 1989).The remaining four gap genes expressed in the cephalic region are ***orthodenticle***, ***empty spiracles***, ***buttonhead*** and ***giant***. Mutations in these genes delete partially overlapping, adjacent regions of the head anterior to that affected by ***hunchback*** mutations and these genes are therefore likely also to be head gap genes that are regulated by bicoid (Dalton *et al.*, 1989, Cohen and Jurgens, 1990, Fig. 1.3).

In the trunk regions of the embryo segmental boundaries are determined by the expression of gap genes that direct localised expression of pair-rule genes, which in turn direct the localised expression of the segment polarity genes. No pair-rule genes are expressed in the cephalic region so the overlapping domains of gap gene expression must define segmental boundaries (for reveiw see Cohen and Jurgens, 1991). The phenotype of mutants of the gap class of genes is the deletion of one or more segments.

Embryos mutant for the ***buttonhead*** gap gene show incomplete involution of the embryonic head, lack mandibular, hypopharyngeal and antennal segments (Cohen and Jurgens, 1991). The ***buttonhead*** transcript is expressed in an anterior stripe at 80-65% EL which becomes visable during nuclear cycle 12/13 and an anterior dorsal spot which appears at cellular blastroderm (Wimmer, 1992).

orthodenticle is expressed during syncytial blastoderm and its expression is limited to the circumferential anterior region extending to the pole. By cellular

blastoderm *orthodenticle* expression has retracted from the pole and is confined to a broad stripe extending from 70-90% egg length. Formation of the cephalic furrow occurs just posterior to the domain of *orthodenticle* expression. The orthodenticle protein has a homeodomain suggesting it may be a transcriptional regulator. Mutations in *orthodenticle* cause embryonic lethality. The associated morphological phenotype is failure of head involution and deletion of the antennal and preantennal segments. The clypeolabrum, hypopharyngeal and gnathal (mandibular, maxillary and labial) segments develop but are often disrupted. There is no evidence for homeotic transformation or duplication of head structures (Fickelstein and Perrimon, 1990).

The expression of *empty spiracles* (*ems*) is also controlled by *bicoid*. The *ems* gene is expressed from cellular blastoderm in the developing head in a single anterior band extending from 70-76%EL dorsally and 74-89%EL ventrally. Later in embryogenesis *empty spiracles* is expressed in the lateral region of each segment, where the tracheal pits form and lateral neuroblasts originate, as well as in the posterior spiracles. The empty spiracles protein has a homeodomain suggesting a role in transcriptional regulation of other genes. The N-terminal portion of the predicted protein sequence is very proline rich whereas the C-terminus has an acidic profile. This is also consistent with the role of a transcription factor. In mutants of *empty spiracles* head involution fails to occur, the anterior border of the mandibular lobe and the entire optic lobe are absent. The maxillary lobe has a slightly perturbed shape compared to wildtype (Walldorf and Gehring, 1992).

Another possible target for *bicoid* regulation is the anterior of the *giant* expression domain, which is also dependent on *bicoid* concentration (Eldon and Pirrotta, 1991; Kraut and Levine, 1991). Mutations in the *giant* gene result in some head defects affecting the clypeolabrum and the labial segment resulting in incomplete head involution and loss of parts of the cephalopharyngeal skeleton (Petschek *et al.*, 1987; Mohler *et al.*, 1989).

During cellularisation of the blastoderm the second phase of region specific anterior gene expression occurs. Genes in this tier include segmental homeotic genes

of the Antennapedia complex: *labial*, *Deformed*, *Sex combs reduced and proboscipedia;* and the region specific homeotic genes *spalt* and *forkhead*. In the trunk homeotic selector genes specify segmental identity. Mutations in these genes result in transformation of segmental identities, without affecting the number of segments. In the head, no mutations have been found that alter the identity of the cephalic segments in the embryo.

At the same time the two zygotic gap genes *tll* and *giant* shift from initial broad domains of expression to distinct late expression patterns which are maintained throughout the germ band extended stage. The late expression of the *giant* gene is three stripes corresponding to 94%, 77% and 65% EL (Mohler *et al*, 1989). The expression domain of *tailless* extends from 75- 88% EL (Pignoni *et al*.,1992).

The *Deformed* gene contributes to the specification of maxillary segment identity and is expressed at 67% EL (Regulski *et al.*, 1987). *Sex combs reduced* contributes to the specification of the labial and first thoracic segments. The expression domain is from 56-64% EL (Kaufman *et al.*, 1990; Riley *et al.*, 1987). Mutations in the gene *labial* result in defects in all derivatives of the gnathocephalic segments, head involution fails but there is no homeotic transformation, the gene is expressed at 72% EL (Merrill *et al.*, 1989; Diederich *et al.*, 1989). *proboscipedia* is necessary for the correct specification of the labial and maxillary segments. Null mutations of *proboscopedia* result in simultaneous transformation of the labial segment to prothoracic legs and of the maxillary palps towards antennae (Pultz *et al.*, 1988).

The homeotic gene *spalt* is also required for the correct specification of the labial segment in the *Drosophila* head. The anterior expression of this gene has two domains, one 60-70% EL and a dorsal spot at 85% EL (Frei *et al.*, 1988).

Other anterior structures included in the *Drosophila* head are the foregut, anterior midgut and salivary glands. *forkhead* was originally detected as a homeotic gene promoting terminal as opposed to segmental development in the ectodermal parts of the gut (Jurgens and Weigel, 1988). *forkhead* is expressed in the most anterior part of the cellular blastoderm from 95-100% EL, *forkhead* is also expressed in the salivary

gland placodes. The salivary gland placodes are established by a collaboration of *Sex combs reduced*, which acts positively, *decapentaplegic*, and genes of the *spitz* group, which act negatively to limit the dorsal and ventral extent of the placode. *huckebein* is also required for invagination of the salivary placode (Panzer and Fong, 1992).

tramtrack has a complicated expression pattern during embryogenesis of two alternatively spliced transcripts. The pattern of one of these transcripts (408) during germ band elongation is strikingly similar to the expression of *forkhead* in the foregut, anterior midgut, posterior midgut and hindgut. *tramtrack* encodes a zinc-finger protein and plays a role in the regulation of the two pair rule genes *fushi tarazu* and *evenskipped* (Read and Manley, 1992). The expression domains of genes required for head development are shown in Figure 1.4.

Posterior pattern.

The pole plasm at the posterior pole contains two localised determinants. The posterior determinant which controls the development of the abdomen and a signal that directs the formation of the pole cells. There are ten posterior group genes that give a disruption of abdominal patterning. Eight of these genes *cappuccino*, *spire*, *staufen*, *oskar*, *vasa*, *valois*, *tudor* and *mago nashi* are also required for pole cell formation. These mutations do not remove the posterior activity but interfere with its localisation to the posterior of the egg (for review see, St. Johnston and Nusslein-Volhard, 1992). The two remaining genes, *nanos* and *pumilio* are only involved in abdominal development. No posterior determining activity is found at any stage in ovaries or eggs mutant for *nanos* (Lehman and Nusslein-Volhard, 1991). Injection of wildtype *nanos* RNA can rescue the abdominal deletions produced by all other posterior gene mutations. *nanos* is therefore the gene most downstream in the pathway, that is it encodes the posterior factor. *nanos* has been cloned and its RNA as expected is localised at the posterior pole of the egg (Wang and Lehmann, 1991). Since *nanos* activity is required in the presumptive abdominal region, while its RNA is localised at the posterior pole, *nanos* protein must move to this more anterior region.

In *pumilio* mutants insufficient *nanos* activity appears to reach the abdomen although it is present in their pole plasm. The role of *pumilio* is to enhance the activity of nanos protein. The expression and distribution of nanos protein is indistinguishable from wild-type in embryos derived from *pumilio* mutant females (Barker *et al.*, 1992). Unlike *bicoid* the embryo does not respond point by point to *nanos* protein concentration. The role of *nanos* is to remove maternal *hunchback* from the posterior half of the embryo, thus allowing posterior development to occur (Irish *et al.*, 1989). In fact, if maternal *hunchback* expression is removed from the embryo by making germ line clones there is no need for the *nanos* gene. The mechanism of control of *hunchback* by nanos protein is unknown as there are no strong sequence similarities with other known genes. However nanos protein affects the distribution of both maternal *hunchback* RNA and *hunchback* protein (Tautz, 1988; Tautz and Pfeifle, 1989). This has been confirmed by the discovery of a short sequence that occurs twice in the 3' untranslated region of the *hunchback* transcript that is required for *nanos* regulation. The presence of these two sites in a heterologous transcript is sufficient to allow nanos-dependent repression in the posterior of embryos (Wharton and Struhl, 1991). Ectopic anterior expression of *nanos* which occurs in *Bicaudal*-D mutations, results in loss of the *bicoid* gradient at the anterior pole (Wharton and Struhl, 1989; Wang and Lehmann, 1991). In these embryos bicoid protein is degraded prematurely even though the RNA is present when the protein is normally translated. The 3' untranslated region of the *bicoid* transcript has been found to contain sequences similar to those found in the *hunchback* transcript and its translation may therefore be repressed by the same mechanism. Since *hunchback* encodes a protein with six zinc finger domains (Schroder *et al.*, 1988) it is likely that it prevents abdomen formation by directly repressing the expression of *knirps* and *giant*, which are both gap genes required for abdominal pattern formation (Tautz *et al.*, 1987; Stanojevic *et al.*, 1989; Treisman and Desplan, 1989). *giant* is expressed in a posterior domain from 0-30% EL at syncitial blastoderm, localising to 25-35% EL at cellular blastoderm (Kraut and Levine, 1991). *giant* activity is required for the correct specification of abdominal

segments A5-A8. In embryos mutant for the gap gene *empty spiracles* the posterior spiracles and filzkorper are disrupted (Walldorf and Gehring, 1992).

Homeotic genes expressed in the posterior include *Abdominal* B of the *Bithorax complex* specifies development of the abdominal segments A5-A10 and at least part of the genitalia. The mutant adult phenotype is transformation of all the abdominal segments posterior to A5 towards A4. Posterior to A8 only the spiracles were affected and are either reduced or absent (Sanchez-Herrero *et al.*, 1985).

The mutant phenotype in the posterior of embryos lacking *spalt* expression is the transformation of the fate of abdominal parasegments 14 and 15 to parasegment 13 (Frei *et al.*, 1989). The posterior domain of *spalt* expression corresponds to 10-20% EL (Jurgens, 1988).

Mutations in the homeotic gene *Polycomb*, develop homeotic cuticular features of abdominal segment 8 in more anterior body segments (Denell and Frederick, 1983).

The posterior region of the embryo also includes the hindgut, posterior midgut and malpighian tubules. *forkhead* a homeotic gene, and *tramtrack* are both expressed in, and required for, the correct development of the posterior midgut and hindgut. The position of the posterior domain of *forkhead* expression is determined by *tll* and *hkb*. There are two complementary subdomains of the posterior *forkhead* domain, one includes hindgut and anal pads, the other one the malpighian tubules and hindgut. Mutations in *forkhead* affect PS16 and PS17, the anal pads and malpighian tubules are abolished (Jurgens and Weigel, 1993). The terminal gap genes *tll* and *hkb* are expressed at 0-15% and 0-12% EL respectively (Bronner and Jackle, 1991). Mutations in the central gap gene *Kruppel* produce embryos in which the cells of the malpighian tubule anlagen fail to enter their normal pathway but contribute to hindgut, suggesting that it acts as a homeotic gene in the malpighian tubules (Harbecke and Janning, 1989). Figure 1.5 shows the expression domains of genes involved in posterior determination.

Terminal system.

Most of the genes involved in this system are also maternal effect, homozygous

mutant mothers lay eggs that lack both anterior and posterior extremes of the embryo, the acron and the telson respectively. The seven maternally active genes of the terminal class which have been identified by classical mutation screens are: *torso* (*tor*), *trunk* (*trk*), fs(1) *Nasrat* [fs(1)*N*], fs(1)*pole hole* [fs(1)*ph*], *torso-like* (*tsl*), *pole hole* [*ph*1] and *corkscrew* (*csw*) (Perrimon *et al.*,1985; Klinger *et al.*, 1988; Nishida *et al.*, 1988; Schupbach and Wieschaus, 1989; Degelmann *et al.*, 1990; Stevens *et al.*, 1990 and Perkins *et al.* 1992).

The three terminal class genes which have been cloned show significant homology to other signaling proteins. The gene *torso* has been extensively studied and encodes a tyrosine kinase receptor (Casanova and Struhl 1989; Sprenger *et al.*, 1989). *torso* RNA is synthesised during oogenesis but is not translated until after fertilization. The *torso* receptor is distributed evenly on the egg plasma membrane of the oocyte (Casanova and Struhl, 1989).

The pole hole (*ph*1) gene encodes the *Drosophila* homologue of Raf 1 a serine-threonine kinase which is involved in a number of signal transduction pathways (Mark *et al.*, 1987; Nishida *et al.*, 1988). *c-raf* which encodes Raf 1 has been implicated in the signal transduction pathways of several vertebrate receptor tyrosine kinases (Morrison *et al.*, 1988).

corkscrew encodes a non-receptor tyrosine phosphatase that is homologous to the mammalian PTP1C protein. PTP1C has been shown to bind physically to the activated EGF receptor. (Perkins *et al.*, 1992). Both the maternally transcribed *ph* 1 and *corkscrew* transcripts are distributed throughout the mature oocyte and early embryo (Nishida *et al.*, 1988; Ambrosio *et al.*, 1989; Perkins *et al.*, 1992).

Mutations in the gene *torso* include alleles which give either loss or gain of function. *torso* loss-of-function proteins have no tyrosine kinase activity, whereas the gain-of-function proteins are constitutively activated. Loss-of-function alleles give the terminal pattern defects. The gain-of-function alleles give the opposite phenotype, embryos develop normal terminal structures but have defects in the segmented region (Klingler *et al.*, 1988). These gain-of-function phenotypes are suppressed in embryos

that are also mutant for the gap gene *tailless* (Klingler *et al.*, 1988; Strecker *et al.*, 1988; Weigel *et al.*, 1990). The phenotype is therefore probably due to the ectopic expression of *huckebein* and *tailless*, whose functions are to repress central gap gene activity in the termini, resulting in the loss of expression of the central gap genes.

Mutations in *ph*1 and *csw* cause a terminal pattern phenotype when the maternal contribution is removed and suppress the *torso* gain-of-function phenotype, suggesting that they are downstream of *torso* in the signal transduction pathway (Ambrosio *et al.*, 1989; Klingler, 1989; Perkins *et al.*, 1992). The mutant phenotype of *ph*1 in larval imaginal discs indicates that it has a major role in the regulation of cell proliferation like the mammalian c-raf and it is also required for the development of the adult compound eye (Perrimon *et al.*, 1985; Nishida *et al.*, 1988).

The genes upstream of *torso* are involved in the localised production of the ligand at the two termini of the egg. The ligand is thought to be produced and localised in subpopulations of the somatic follicle cells. The three genes *trunk*, *fs(1)Nasrat*, and *fs(1) pole hole* are all required in the germline nurse cell- oocyte complex (Schupbach and Weishaus, 1986). By contrast *torsolike* acts in the somatic follicle cells and not in the germline. Follicle cell mosaics mutant for *torso-like* show that *torso-like* is specifically required in the terminal follicle cells; this suggests that these cells produce the terminal signal (Stevens *et al.*,1990). Which of these genes upstream of *torso* encodes the ligand for the *torso* receptor is not known. The ligand that activates torso has been shown to be freely diffusible and limited in quantity (Sprenger and Nusslein-Volhard, 1992; Struhl and Casanova, 1993). *torso* is transiently activated during the syncytial blastoderm and this activation is produced by the binding of its ligand. The activation of *torso* initiates a signal transduction pathway which results in the activation of the gap genes *tailless* and *huckebein* in the termini of the embryo.

In an attempt to identify other genes in the terminal pathway a large scale mutagenesis screen for dominant suppressors of the *tor* gain-of-function allele was carried out by Doyle and Bishop (1993). Forty five mutants that suppress the *tor* gain-of-function phenotype were identified and these fell into seven complementation

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groups two of which correspond to mutations in *ras*-1 and *Sos*.

The signal transduction cascade resulting in the R7 photoreceptor cell fate also involves a receptor tyrosine kinase receptor. This receptor is encoded by *sevenless* and the ligand of this receptor is the product of the *Boss* locus presented by the neighbouring R8 cell (for review, see Rubin, 1991). The intermediates of this transduction pathway are very similar to those in the *torso* pathway, except the end results are different, and in this case probably lead to the activation of the *sina* transcription factor.

Complementation tests between the dominant suppressor of *torso* gain-of-function alleles and mutations identified as enhancers of a hypomorphic allele of *sev* [*E(sev) loci*] , showed that four of these mutations disrupt the *Son of sevenless* (*Sos*) or the *ras-1* loci (Simon *et al.*, 1991). In addition to *Sos* and *ras-1* they showed that three other enhancers of sevenless mutations suppress the *tor* gain-of-function phenotype E(*sev*)1A, E(*sev*)2B and E(*sev*)3A (Simon *et al* , 1991). The E(*sev*)2B encodes a protein containing SH2 and SH3 domains (Simon *et al.*, 1993) The gene *downstream of receptor kinases (drk)*, was independently cloned by identification as a mutation in a screen for second site modifiers of both gain and loss of function *sev* alleles, and has been shown to be the same as E(*sev*)2B (Olivier *et al* , 1993). The biological activity of this protein correlates with binding of its SH2 domain to activated receptor tyrosine kinases and concomitant localisation of drk protein to the plasma membrane. In vitro, drk protein has been shown to bind directly to the C-terminal tail of *Sos*, a guanine nucleotide releasing protein, which leads to the activation of the D*ras*-1 protein. These results suggest that *drk* is the linking molecule between receptor tyrosine kinases and *Sos* through its SH3 domains thereby coupling receptor tyrosine kinases to *Ras* (Olivier *et al* ,1993).

A screen for dominant suppressors of the eye phenotype (reduced number of ommatidia and rough eye surface [Perrimon *et al.*, 1985]) and eclosion defects of the weak D-*raf*^{C110} (*ph*1^{C110}) allele, identified Dominant *suppressor of Raf* (D*sor*1) (Tsuda *et al.*, 1993). Loss of function mutants of D*sor* die during larval/pupal stages

with severe defects in tissues containing proliferating cells inside normal looking larvae. Embryos derived from germline clones homozygous for D*sor* which lack any maternal contribution of the gene, have both anterior and terminal defects, showing that D*sor* is essential for the development of the termini. D*sor* has been cloned and encodes a serine/threonine kinase similar to MAP kinase activator, or extra cellular signal regulated protein kinase (ERK's)(Tsuda *et al.*, 1993). *Raf* 1 has been shown to activate MAP kinase activator (Kynakis *et al.*,1992; Howe *et al.*, 1992). Since the raf kinase and MAP kinase activator are highly conserved between *Drosophila* and vertebrates a MAP kinase homologue would be expected to function downstream of D*sor*. Dm-ERK-A a *Drosophila* homologue of ERK may be the candidate (Biggs and Zipursky, 1992). The multifunctional nature of *D-raf* indicates that *D-raf* is involved in a variety of transmembrane signalling processes associated with both cellular proliferation and differentiation. These experiments provide conclusive evidence of the extensive overlap between the *torso* and *sevenless* receptor tyrosine kinase pathways (Fig 1.6). The *sevenless* signal transduction pathway results in activation of the *sina* transcription factor resulting in R7 photoreceptor cell fate. The end result of the *torso* signal transduction pathway is the localised zygotic transcription of the terminal gap genes *huckebein* and *tailless*. For this to happen there is presumably one more widely distributed transcription factor which is activated as a result of this pathway, thereby leading to the localised transcription of *huckebein* and *tailless*. This localisation of terminal gap gene expression requires the activity of the *bicoid* gene in the anterior pole.

The zygotic gap genes *tailless* and *huckebein* are transcribed in domains restricted to the poles of the embryo, and have mutant phenotypes similar to the maternal terminal class genes (Pignoni *et al.*, 1990; Bronner and Jackle 1991). The domains of expression of the terminal gap genes *tll* and *hkb* are dependent on torso activity. The spatial control of *hkb* and *tll* expression does not involve interactions with each other, or any regulatory input from the central gap genes.

The terminal gap genes prevent metamerisation by repression of the central gap genes, thereby distinguishing the segmented trunk from the unsegmented terminal regions of the embryo (Klingler *et al.*, 1988; Strecker *et al.*, 1988; Weigel *et al.*, 1990).

Neurogenesis in *Drosophila*

Much of what is known about the cellular basis of neural development in the nervous system of *Drosophila*, comes from comparisons with work first carried out in grasshopper embryos. The model of how the array of neurons arises, was originally obtained by microscopic observation and laser ablation studies. Grasshopper embryos and their individual cells are five to tenfold larger than in *Drosophila*, and their embryonic development is about twenty fold slower. Most of the neurons in the grassshopper CNS arise from neuroblasts, about 30 of these cells per hemisegment differentiate in a reproducible order from the ventral ectoderm (Doe and Goodman, 1985). Ablation of a particular neuroblast leads to its replacement by differentiation of another ectodermal cell (Doe and Goodman, 1985b), suggesting that stochastic processes determine which cell will become a neuroblast and that when one forms, it inhibits its neighbours from also becoming neuroblasts. These experiments also suggest that the identity of a particular neuroblast is dependent on where it forms in the array. Each neuroblast is a stem cell with a characteristic lineage; it buds off a series of ganglion mother cells (GMC's) which each divide once to give two neurons. The identity of this neuron pair is dependent on the identity of its parent GMC, which in turn is dependent on the identity of the parental neuroblast (Doe and Goodman, 1985b). Ablation experiments also suggest that the two neurons derived from each GMC are initially identical, their final identity being determined by stochastic interactions between them (Kuwada and Goodman, 1985). During grasshopper development segments are generated sequentially as the cells of the embryo proliferate. By contrast, in insects such as *Drosophila* the entire body plan is established simultaneously at the blastoderm stage. Despite the different early

embryonic events in grasshoppers and *Drosophila*, the neuronal anatomy of these two organisms is remarkably similar (Thomas *et al.*, 1984).

At cellular blastoderm the two halves of the presumptive neurogenic region are separated by the most ventral cells of the embryo, which invaginate to form the mesoderm. During gastrulation the invagination of the mesoderm along the ventral furrow causes joining of the two halves of the neuroectoderm at the ventral midline (Fig. 1.7). During the four hours following gastrulation the neurogenic region exhibits three waves of neuroblast formation. Neuroblasts are formed from single ectodermal cells which enlarge and delaminate into the embryo.

The first two waves of neuroblast formation (SI and SII) produce three columns of neuroblasts which extend along the anterior-posterior axis on each side of the embryo giving medial, intermediate and lateral neuroblasts in each half of the neurogenic region. The first ten neuroblasts of the SI class form in the following columns, four lateral, two intermediate and four medial neuroblasts (Jimenez and Campos-Ortega, 1990). The next set of neuroblasts, the SII class, results in the addition of two neuroblasts to the medial column and between five and six to the intermediate column (Hartenstein and Campo-Ortega, 1984). The last wave of neuroblast formation, SIII adds neuroblasts throughout the neurogenic region resulting in approximately twenty five neuroblasts arranged roughly in four columns and six rows (Doe *et al.*, 1988, Jimenez and Campos-Ortega, 1990).

A neuroblast is a stem cell and soon after formation it begins to produce an apparently stereotyped cell lineage. Each neuroblast undergoes approximately five asymmetrical divisions to produce five smaller ganglion mother cells. Each ganglion mother cell divides to produce two neurons. Studies in a variety of insects show that an identified neuroblast generally produces a characteristic chain of progeny (Taghert and Goodman, 1984) (Fig. 1.7b).

Neuroblast formation.

There are two main classes of gene known to be involved in this process, the proneural and the neurogenic genes. These genes have broadly opposite roles in neuroblast determination. Loss of function of the proneural genes results in a decrease in the number of neuroblasts formed (Jimenez and Campos-Ortega, 1990), whereas absence of function of the neurogenic genes results in a larger number of neuroblasts being formed (Lehmann *et al.*, 1983).

The proneural genes include *daughterless*, and the *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) and *asense* (*ase*) genes of the *achaete-scute* complex (*AS*-C) . The loss of function of the proneural genes results in the formation of fewer neuroblasts (Jimenez and Campos-Ortega, 1990; Cabrera *et al.*, 1987). Adding additional copies of the wild-type gene results in excessive neuroblast formation (Brand and Campos-Ortega, 1988). The loss and gain of function phenotypes of the *AS*-C mutations suggest that the role of the proneural genes is to repress epidermal development and promote the neural fate.

The *ac* protein is present in four clusters of four to six ectodermal cells, each in stereotyped positions within each hemisegment of the neurogenic region. One neuroblast will form from each cluster, at which point the ectodermal cells will lose *ac* expression; *achaete* expression, however, persists in the neuroblast until just before it divides. Later *ac* expression reappears in a more complex pattern which preceeds SII neuroblast formation. Presumably one neuroblast is formed from each of these clusters (Cabrera *et al.*, 1987; Cabrera, 1990).

l'sc protein is also detected in clusters of four to six ectodermal cells, with each cluster producing one *l'sc* positive neuroblast. *l'sc* seems to be expressed in every neuroblast (Cabrera, 1990). Expression of *AS-C* genes is directly related to neural fate; only those cells that maintain expression of either *ac* or *l'sc* become neuroblasts. Genes of the *achaete-scute* complex encode proteins with basic helix-loop-helix DNA-binding motifs and appear to form heterodimers with the daughterless protein which act as neural-specific transcription factors (Cabrera and Alonso, 1991). Further

experiments looking at under and over expression of either *ac* or *sc* in sensory precusors concluded that *AS-C* genes are involved in triggering neural fate, but are not involved in determining the identity of the neuron or sensory precursor.

The neurogenic class of genes include *Notch*, *Delta*, *mastermind*, *bigbrain*, *neuralised*, and several genes of the *Enhancer of split* complex and *shaggy* (Lehmann *et al.*, 1983; Bourouis *et al.*, 1989). Loss of function of these genes results in all the cells of the neuroectoderm developing into neuroblasts rather than into epidermis. An increase in neurogenic gene function results in fewer neuroblasts being formed. Embryos lacking neurogenic gene function initially show normal expression of *ac* and *l'sc*, but expression is never restricted to a single neuroblast. Since expression persists in all the cells of the cluster they all develop into neuroblasts. Neurogenic genes limit *AS-C* expression to a single cell per cluster by cell interactions described as lateral inhibition (Wigglesworth, 1940). The lateral inhibition signal and its receptor are thought to be mediated by *Delta* and *Notch* respectively. They both encode membrane proteins with extracellular EGF like repeats (Wharton *et al.*, 1985; Kopozynski *et al.*, 1989). The protein encoded by *shaggy* is a putative serine/threonine kinase and is therefore a candidate in a process leading to the phosphorylation/inactivation of *AS-C* proteins. This signal ultimately leads to loss of *AS-C* expression in cells adjacent to the neuroblasts and these cells differentiate into epidermis. The genes of the Enhancer of split complex have been shown to act downstream of all the other neurogenic loci (Vassin, Vielmetter and Campos-Ortega 1985; De la Concha *et al* 1988). The function of E(spl) is to receive or to process signals that regulate the epidermal pathway of development within the neuroectoderm (Technau and Campos-Ortega, 1987). As several of the E(Spl) genes encode helix-loop-helix proteins, they may act to specify epidermal development analogously to the role of the AS-C genes in specifying neural development.

The pattern of neuroblasts within the embryonic CNS show segment specific differences, particularly between the thoracic and abdominal segments (Bate, 1976). Homeotic genes are thought to regulate the spatial expression of the proneural genes,

thereby producing different neuroblast patterns in different segments.

Cell fate within the CNS must be controlled, at least conceptually, by three classes of genes.

1) Neuroblast identity is thought to be determined by genes regionally expressed within the neuroectoderm, which provide two dimensional positional cues along the anterior-posterior and dorsal-ventral axes. The neuroblast identity genes would translate these positional cues into specific neuroblast lineages.

2) GMC identity genes are expressed in subsets of GMC's in response to neuroblast identity genes. These genes control the identity of individual GMC's and their progeny.

3) Neuronal identity genes are expressed in the neurons as they are generated by asymmetric division of a ganglion mother cell.

Few NB identity genes have been identified, one example is the gene *prospero* (Doe *et al.*, 1991). Homeo box genes such as *fushi tarazu* (*ftz*) and *evenskipped* (*eve*) are expressed in GMC's and control the fate of them and their neuronal progeny. Table one shows a list of genes known to control neural fates.

Using enhancer detector transposons to detect novel *Drosophila* genes.

In the past screening for developmentally active genes has relied on identification of obvious morphological disruptions caused by the loss of function of the gene. This can be problematic if the phenotype is subtle or affects an internal organ such as the CNS. Genes can also function at more than one time during development. Mutations in these genes may produce phenotypes associated with only the earliest function. If the gene product can be replaced in part by another gene, that is the gene's function is at least partially redundant, then a mutation in such a gene may not give a detectable phenotype.

The technique of 'enhancer trapping' can detect genes that may have been missed in classical genetic screens for the reasons discussed above, since it allows genes to be detected by their patterns of expression rather than by their mutant phenotypes. The technique relies on the assumption that developmentally important genes are expressed

in temporal and spatial patterns related to their function, an assumption which over the years has been shown to be often, but not always the case. An enhancer detector transposon consists of the weak, basal P-element promoter fused to the *Escherichia coli lacZ* gene which encodes ß-galactosidase. When this transposon is introduced into the *Drosophila* genome, the ß-galactosidase gene is only active if the transposon integrates close to a transcriptional enhancer. The enhancer directs expression from the weak promoter, resulting in ß-galactosidase expression, in a pattern which often reflects the expression pattern of the neighbouring gene normally regulated by the enhancer (O'Kane and Gehring, 1987). The presence of ß-galactosidase can be detected in situ using either the chromogenic substrate X-gal or antibodies raised to ß-galactosidase. Although large numbers of discrete lines can be produced by micro-injection, it is now easier to produce different insertions by transposase-mediated mobilisation of the transposon. Fortunately a stable genomic source of P-element transposase is available in *Drosophila* (Robertson *et al.*, 1988). New enhancer-trap insertion lines can be made by crossing flies carrying the enhancer detector transposon to flies which contain a disabled P-element which encodes transposase but cannot jump.

Another advantage of screening for novel genes using enhancer traps is that it is relatively staightforward to clone the gene flanking the transposon. In many cases to date a gene with the expected pattern of expression has been found near to the insertion site eg. *fasciclin* III and *collagen* IV (Bellen *et al.*, 1989). The number of enhancer trap insertions which show specific expression patterns is fairly high, Bellen *et al.* (1989), found that 65% of lines are expressed in a tissue specific pattern during embryogenesis. Also 35% of lines are expressed in the embryonic CNS (Bier *et al.*, 1989). Only 10% of P-insertions cause homozygous lethality or an obvious phenotype (Freeman, 1992), but given a source of transposase the P-element can excise producing small deficiencies at low frequency. Many of the genes detected by enhancer detector transposons have transcription patterns similar to the *lacZ* expression shown by the detector transposon. Below are a number of examples of

genes that have either been detected and cloned by the use of enhancer detector transposons, or were previously identified mutations that have been able to be cloned by this method.

The gene *prospero* was identified in an 'enhancer trap' screen by its expression in a subset of neuroblasts, sensory neuron precursors, and identified glial precursors. It is not expressed in neurons. Neuroblasts lacking *prospero* gene function generate abnormal cell lineages, producing incorrectly specified progeny that differentiate into neurons exhibiting pathfinding defects. *prospero* is therefore required in neuroblasts to specify neuronal fate. The expression pattern of *prospero* transcripts as seen by in situ hybridisation with *prospero* cDNA is very similar to the original *lacZ* expression pattern. *prospero* was cloned using a P-lacW insertion 150 base pairs away from the site of the start of transcription (Doe *et al.*, 1991).

The gene *deadpan* was also isolated from an enhancer trap screen looking for a gene expressed in all the neurons and their precursors. The function of *deadpan* is essential for viability. Complete lack of *deadpan* gene function affects the function but not the gross morphology of the CNS. Again the expression of *deadpan* transcripts is pan-neural closely resembling the original *lacZ* expression . The lack of a morphological phenotype, explains why this gene was not detected in previous genetic screens for genes involved in CNS function. The protein encoded by the *deadpan* transcript has a helix-loop-helix domain . A P-lacW element less than 1000 base pairs 5' of the cDNA was used as the molecular entry point for cloning the gene (Bier *et al.*,1992).

couch potato was also identified by P-element mediated enhancer detection on the criteria that it expressed *lacZ* in the sensory mother cells (SMC's) of the embryonic PNS and more differentiated cells of the PNS. This gene is essential for embryonic development and is re quired for normal adult behaviour. Again no obvious morphological defects have been observed in the PNS. Bellen *et al.*, 1992, also showed that the P-element enhancer detectors are inserted into key regulatory elements of *couch potato*. All the P-element insertions into *couch potato* are clustered

within a genomic fragment 200 base pairs upstream of the 5' end of the cDNA. A genomic fragment carrying these sequences was fused to a heterologous promoter driving expression of *lacZ*. Embryos of two independently transformed lines stained for ß-galactosidase activity show the expression pattern of *lacZ* essentially corresponds to the expression pattern in the enhancer detector strains (Bellen *et al.*, 1992). The two proteins encoded by the *couch potato* gene contain similar, but nonidentical, RNA-binding domains that are most homologous to the RNA-binding domains of the *Drosophila embryonic lethal abnormal vision* gene and a human brain protein.

The *sloppy paired* (*slp*) locus was cloned by P-element mediated detection. Two cDNA clones were isolated that map to the *sloppy paired* loci, *slp*1 and *slp*2. *slp*1 is less than 500 base pairs from one P-lacZ insertion 509; *slp*2 is ~1.5 kb from another insertion 208, about 8 kb away from the first insertion. Each of these insertions are to the 5' of the transcriptional units. The distribution of the *sloppy paired* mRNA's closely resembles the expression of *lacZ* in the original enhancer detector strain. This expression is segmentally repeated and reminiscent of the pair-rule and segment polarity genes. Loss of function of these transcripts produces a pair-rule phenotype and head defects which correspond to the pattern in which it is expressed. The *slp* transcripts encode proteins with a putative DNA binding domain, the *forkhead* domain (Grossniklaus *et al.*, 1992).

The reporter gene insertion in the gene *teashirt* was expressed in a gap gene like pattern covering the trunk region of the embryo. In-situ hybridisation of the *teashirt* cDNA probe showed that this gene is expressed in a similar pattern to the reporter gene. The insertion is less than 1 kb to the 5' of the *teashirt* cDNA. Mutations in this gene show that it is required for the normal development of the trunk region of embryos which correlates with the spatial expression of the gene in the anterior posterior axis but not in the dorsal ventral axis. *teashirt* encodes a protein with three distantly spaced zinc finger motifs (Fasano *et al.*, 1991).

An insertion into the gene *apterous* was detected in flies homozygous for a P-element insertion which exhibited a wingless and haltereless phenotype; characteristic of the *apterous* mutation. The nature of the defects are consistent with the pattern of *apterous* expression in the larval imaginal wing and haltere discs. *apterous* is also expressed in a subset of cells in the embryonic CNS and larval PNS. The location of the rk568 insertion was found to be 42 bp upstream of the 5' end of the longest cDNA clone. The cDNA clone encodes for a protein with a homeo domain and a cysteine/ histidine rich domain known as the LIM domain (Cohen *et al.*, 1992).

The gene *ming* was detected by its expression in a subset of neuroblasts at reproducible points in their cell lineage. The mRNA pattern of the *ming* gene is indistinguishable from the *lac*Z expression of the enhancer detector strain. Defects in *ming* result in precise alterations in the CNS gene expression, defects in axonogenesis and embryonic lethality. The insertion is less than 1 kb from the cDNA, which encodes for a protein with a zinc finger motif (Cui and Doe, 1992).

Other genes have been cloned using this technique that I shall not discuss in detail, genes such as *germcell less* (Jongens *et al.*,1992), *scabrous* (Baker and Rubin, 1990), *escargot* (Whitely *et al.*, 1992), *argos/ giant lens* (Freeman *et al.*, 1992; Kretzschmar *et al.*,1992) and *rutabaga* (Levin *et al.*,1992).

Second generation enhancer traps.

A second generation enhancer trap system has been developed (Brand and Perrimon, 1993). In these enhancer detector transposons the reporter gene is the yeast transcriptional activator GAL4. This transcription factor has been shown to function in *Drosophila*, and activates genes which have a GAL4 binding site linked to their promoters. This binding region is known as the upstream activating site (UAS). This system enables any cloned gene to be expressed in the cells in which a particular GAL4 enhancer trap is active.The gene attached to the UAS can encode markers for cell surfaces, the nucleus or cytoplasm, this will allow precise characterisation of the position and morphology of many cell types. If UAS is linked to a gene involved in

development, the effect of ectopic expression in the cells expressing GAL4 can be determined. In our laboratory this system is being used to express a cold sensitive ricin a subunit under the control of UAS, in the same cells as GAL4 enhancer trap lines. This system should provide a mechanism of ablating specific cells and then looking for a consequence of this ablation.

Enhancer detectors with ß-galactosidase directed cytoplasmically rather than nuclearly have been made in the hope that axon fibres of neural cells expressing *lac*Z would become visible. P[ClrB] made by removing the nuclear targeting sequence from the P-*lac*Z proved to be not wholly satisfactory; while the fusion was cytoplasmically localised it did not fill axons in sufficient quantity (Smith and O'Kane, 1991).

A recent approach to construct an enhancer detector with cytoplasmic *lac*Z expression, has been to fuse the *Drosophila kinesin* gene, that encodes a microtubule-associated motor protein, to *lac*Z (Giniger *et al.*, 1993).

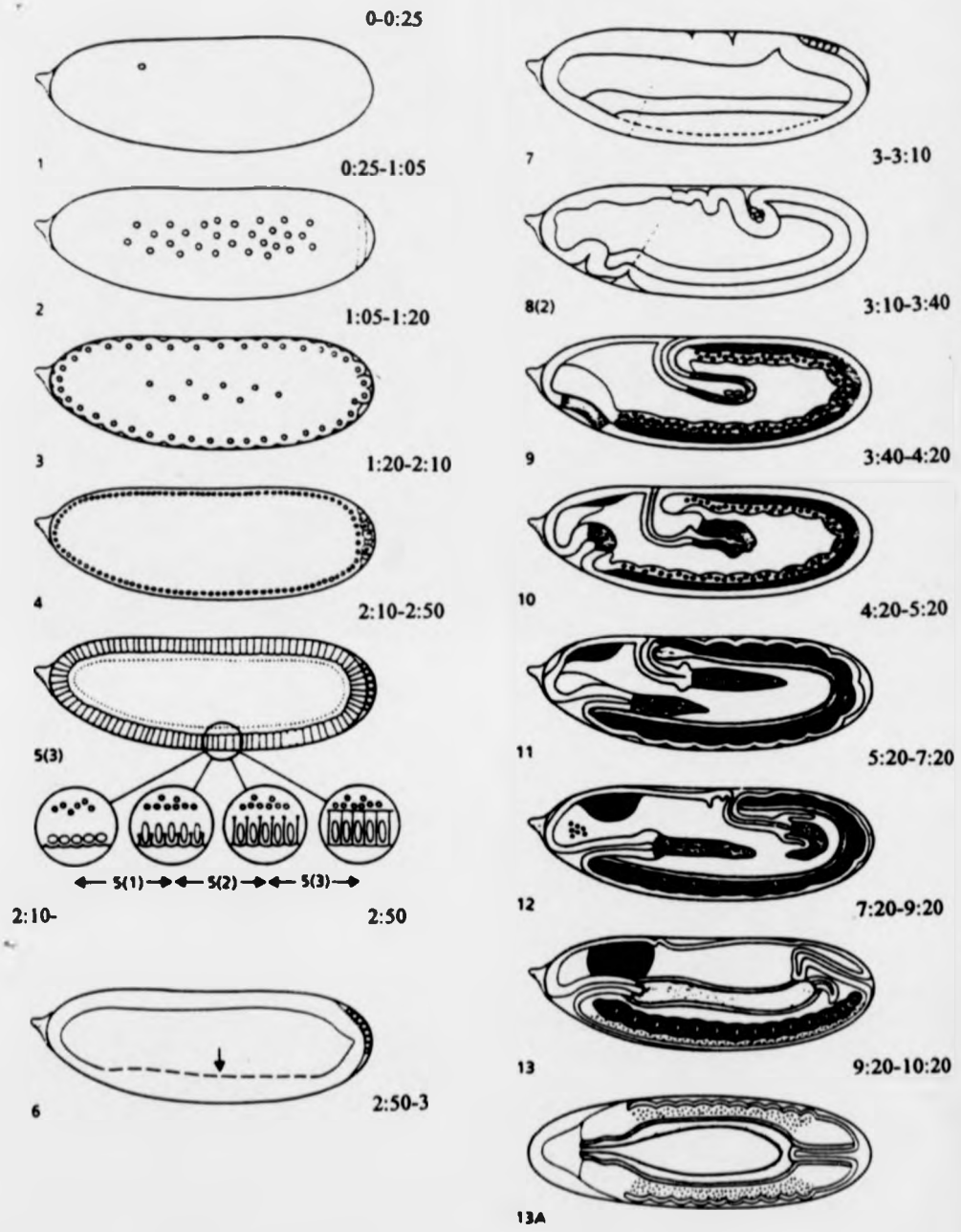


Figure 1.1 Embryogenesis of *Drosophila melanogaster*. Numbers refer to developmental stage. Time refers to number of hours after fertilisation at 25° C. Embryos are orientated anterior left, posterior right, dorsal top and ventral bottom. (adapted from Lawrence, 1992).

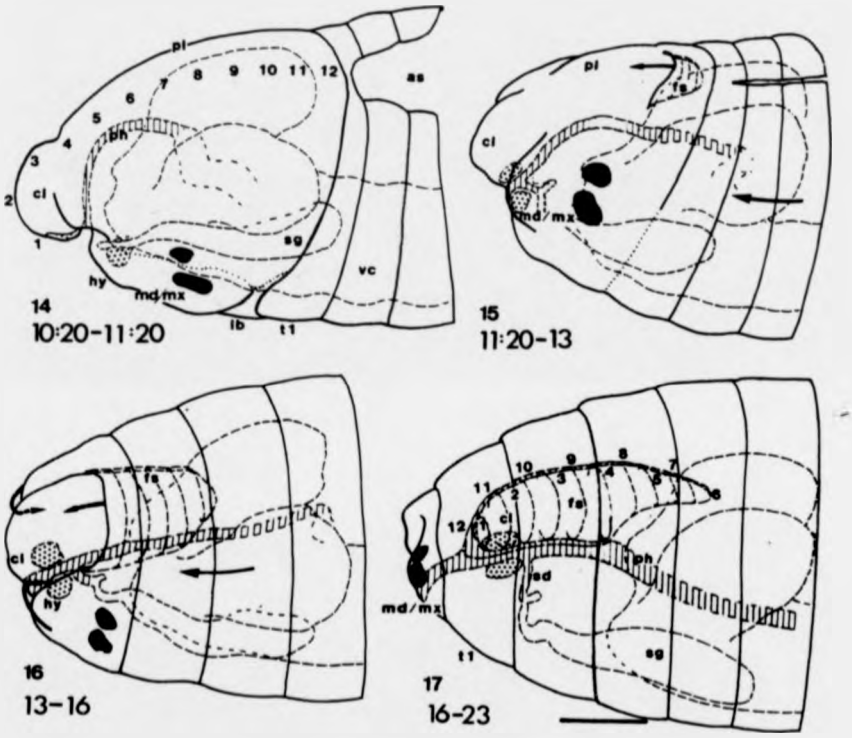


Figure 1.2 Embryonic head involution. Head involution is initiated by dorsal closure at the end of stage 13. By stage 14 the gnathal buds (md/ mx/ lb) have moved orally and the md/ mx have reached the lateral margins of the atrium. The dorsal ridge has moved anteriorly initiating the formation of the frontal sac (fs). Progression of the dorsal fold and further deepening of the frontal sac continues during stage 15 and 16 until the dorsal subdivisions of the antenno maxillary complex attain their position at the anterior.
Bold numbers refer to developmental stage. Numbers 1-12 arbitarily label various regions of the procephalic lobe (pl) and the clypeolabrum (cl) before and after head involution. (taken from Campos-Ortega, 1985).

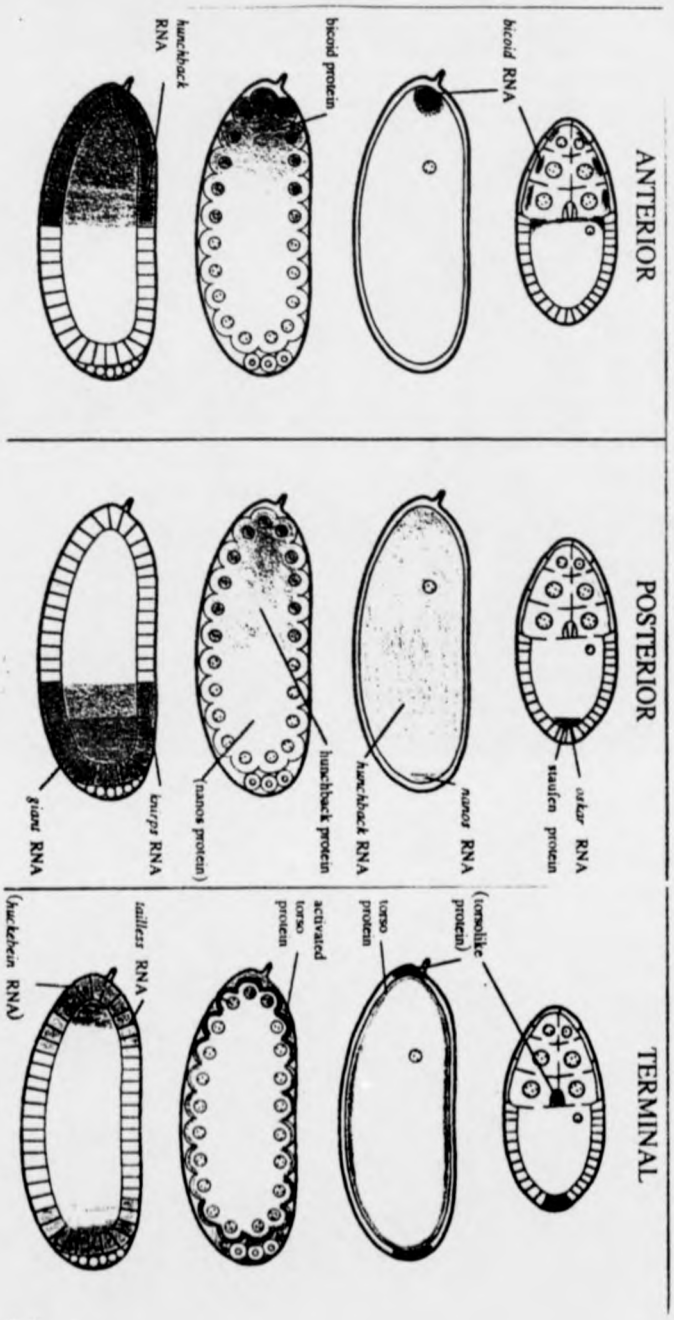


Figure 1.3 The major maternal gene products required for determining polarity in the early Drosophila embryo. (taken from St. Johnston and Nusslein- Volhard, 1992).

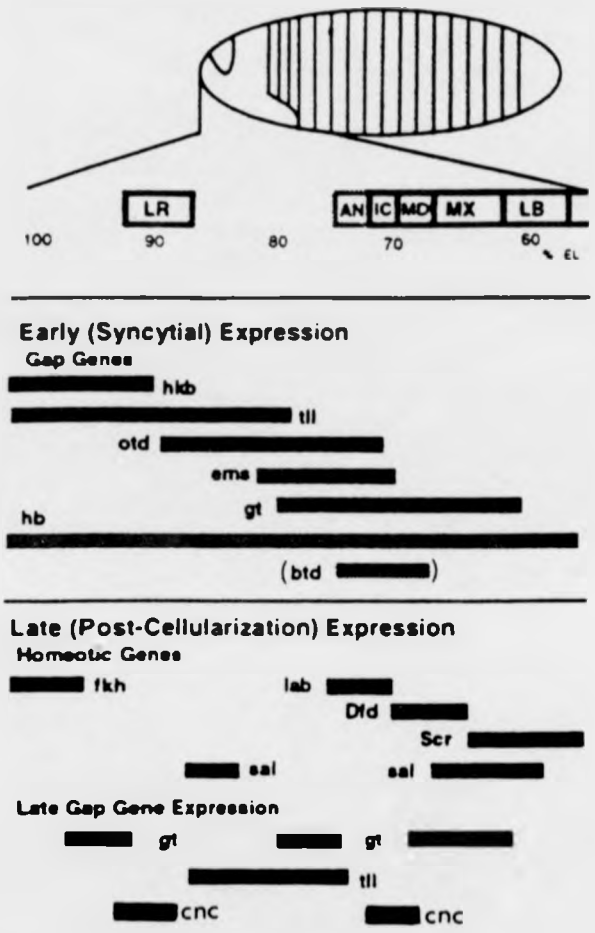


Figure 1.4 The spatial domains of gene expression in the cephalic region of the Drosophila embryo (adapted from Mohler, 1993)

Abbreviations: **hkb**, huckebein, **tll**, tailless, **otd**, orthodenticle, **ems**, empty spiracles, **gt**, giant, **hb**, hunchback, **btd**, buttonhead, **fkh**, forkhead, **lab**, labial, **Dfd**, deformed, **Scr**, sex combs reduced, **sal**, spalt, **cnc**, cap and collar

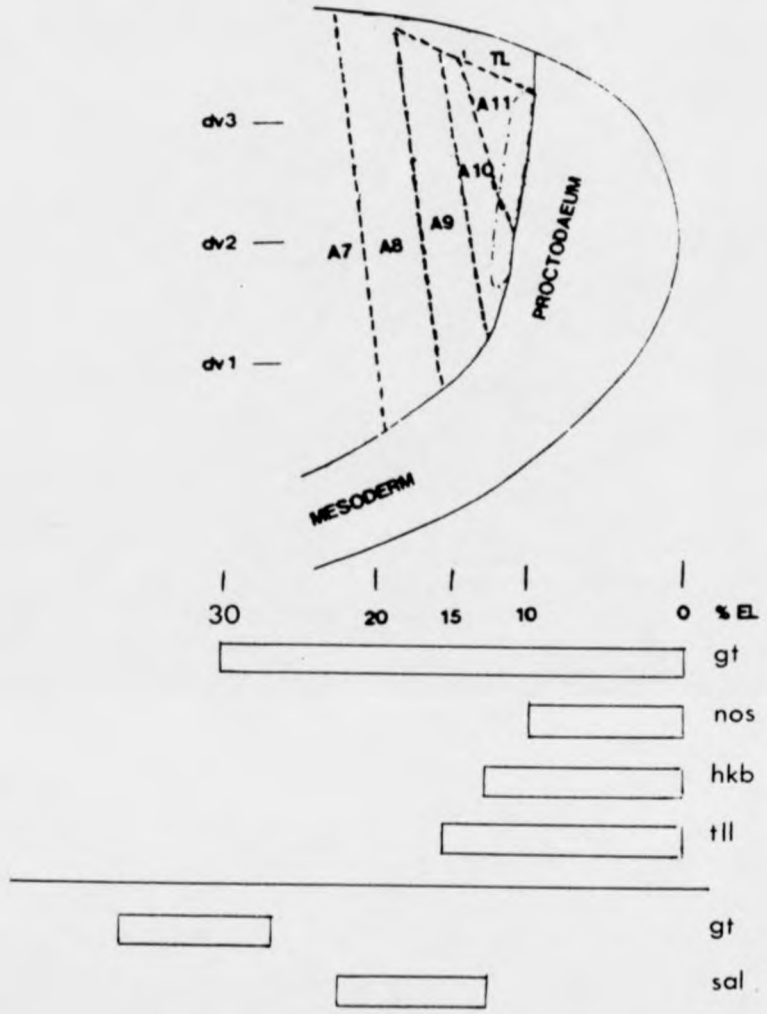


Figure 1.5 Gene expression domains in the posterior of the *Drosophila* embryo.

Abbreviations: **gt**, giant, **nos**, nanos, **hkb**, huckebein, **tll**, tailless, **sal**, spalt

Figure 1.6: A comparison of the *torso* and *sevenless* tyrosine kinase signal transduction pathways (adapted from Tsuda et al., 1993 and Dickson et al, 1992).

A: *torso* teminal signal transduction pathway- the *torso* receptor tyrosine kinase is activated by the binding of its ligand. The genes *tsl, trk, fs*(1)ph, *fs*(1)N are upstream of *torso*. D-*raf* acts downstream of *torso*. The protein drk is thought to link the signal transduction from the tyrosine kinase receptor to *Sos* through its SH3 domains. *Sos* activates *ras*1 which is upstream of D-*raf* and *csw*. A non receptor phosphatase encoded by the *corkscrew* gene *csw* also acts downstream of *torso* and in concert with D-*raf*, the interaction between *csw* and D-*raf* is not known. D-*sor*1 acts downstream of D-*raf* and *csw* and is a protein kinase similar to MAP kinase activator. This predicts a MAP kinase like molecule downstream of D-*sor*, Dm-ERK-A may be the candidate. The transcription factor which activates *hkb* and *tll* is still not known. Embryos double mutant for the zygotic genes *huckebein* and *tailless* show terminal defects and are the target genes of the terminal system. The expression pattern of *tailless* suggests that the terminal signal transduction pathway regulates *tll* positively at the posterior but negatively at the anterior end. *bcd* is also involved in the control of *tailless* expression at the anterior end.

B: The *sevenless* signal transduction pathway-Activation of *sev* results after interaction with *Boss* ligand which leads to an increase in the amount of active GTP-bound Ras1 protein either by stimulation of *Sos* (a putative guanine nucleotide exchange factor homologue) or by inhibition of Gap1 (a putitive Ras GTPase activating protein) or both. D-*raf* acts downstream of Ras1 playing a crucial role in the transduction of the signal towards the nucleus where a possible target the *sina* protein is localised.

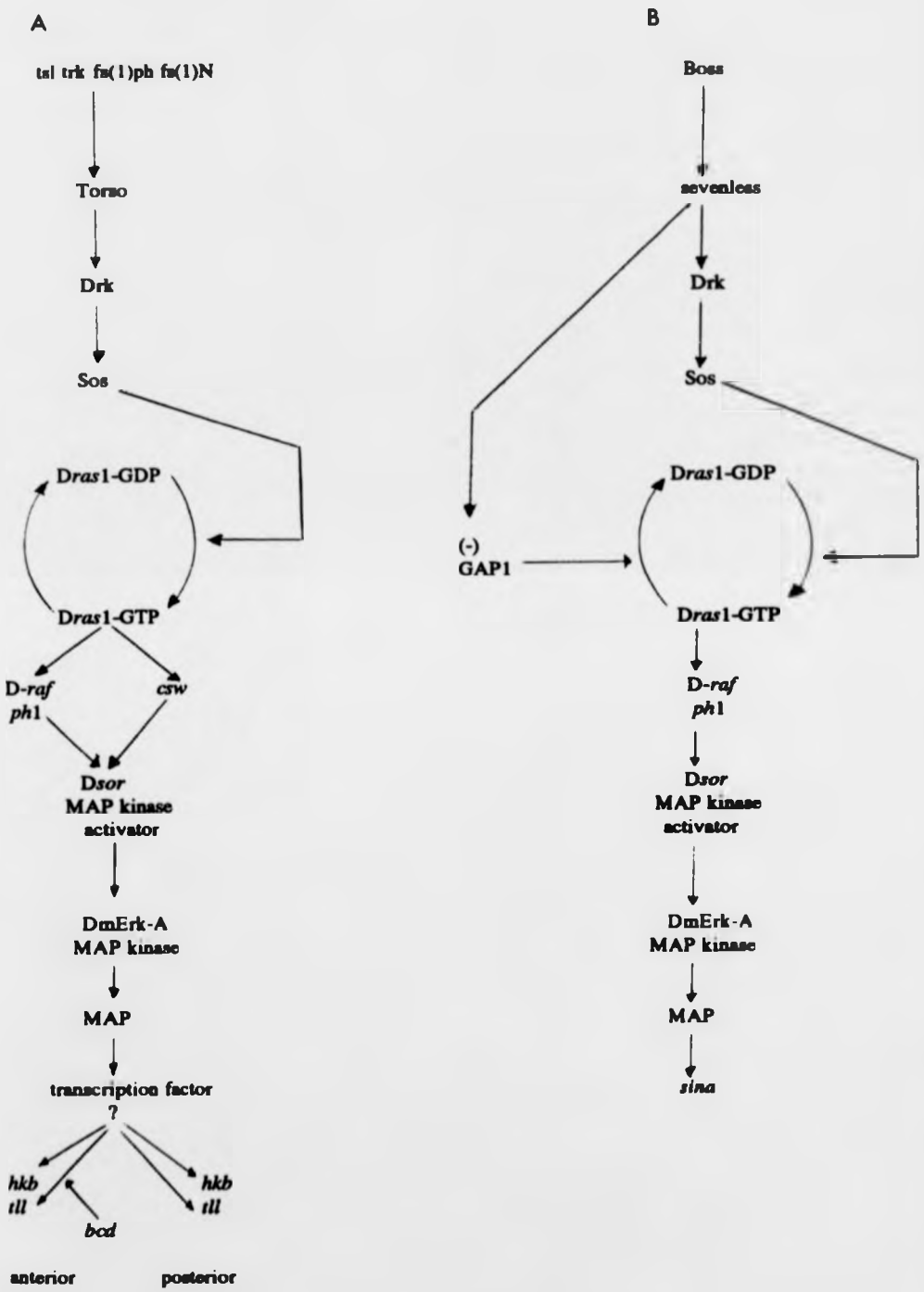
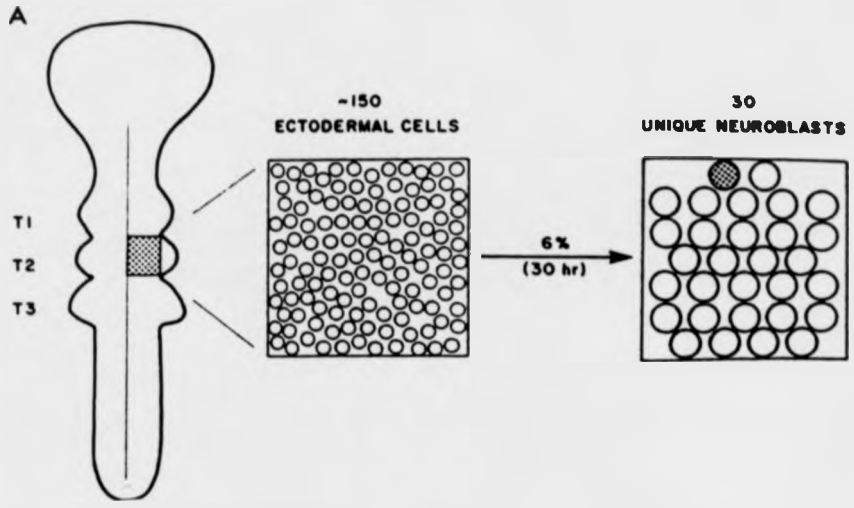
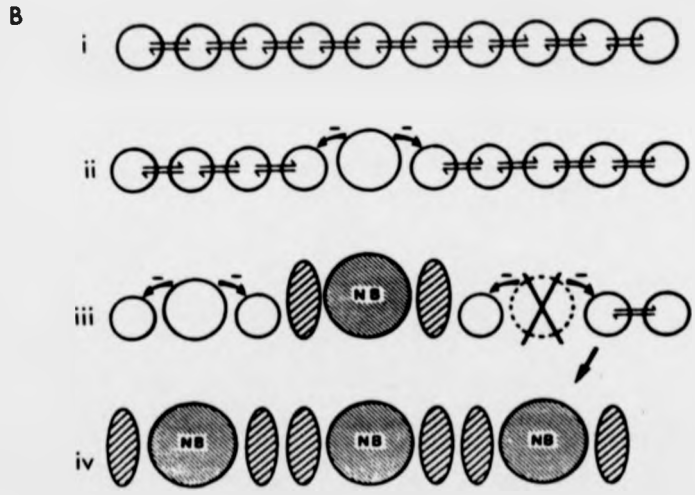
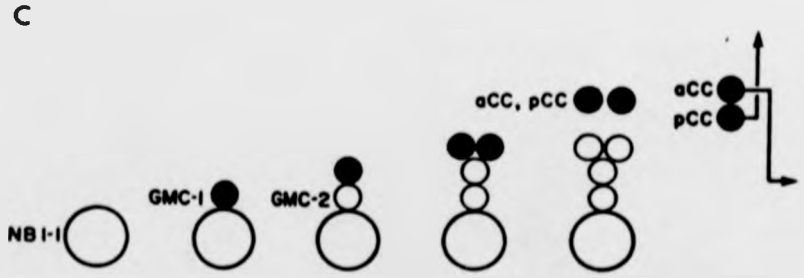

A
tsl trk fs(1)ph fs(1)N
Torso
Drk
Sos
Dras1-GDP
Dras1-GTP
D-raf
ph1
csw
Dsor
MAP kinase
activator
DmErk-A
MAP kinase
MAP
transcription factor
?
hkb
tll
hkb
tll
bcd
anterior
posterior
B
Boss
sevenless
Drk
Sos
Dras1-GDP
(-)
GAP1
Dras1-GTP
D-raf
ph1
Dsor
MAP kinase
activator
DmErk-A
MAP kinase
MAP
sina

Figure 1.7 Neurogenesis in the Grasshopper.
A: The transformation of a uniform epithelial sheet of ectodermal cells into the stereotyped pattern of unique neuronal precursor cells.
B: Lateral inhibition
i: All the cells of the neuroectoderm are initially equivalent and interact with each other.
ii: One cell begins to enlarge into a neuroblast and inhibits the adjacent cells from doing so.
iii: If an enlarging neuroblast is ablated the adjacent neuroectodermal cells are released from inhibition and one will enlarge to replace it.
iv: Eventually all the cells differentiate into either neuroblasts or nonneuronal support cells.
C:The second step of neurogenesis is the production of a characteristic family of neurons from each neuroblast.
(taken from Doe and Goodman, 1985)


A
~150
ECTODERMAL CELLS
30
UNIQUE NEUROBLASTS
T1
T2
T3
6%
(30 hr)


B
i
ii
iii
NB
iv
NB
NB
NB


C
NB 1-1
GMC-1
GMC-2
aCC, pCC
aCC
pCC

Gene	Phenotype	Neuroectoderm	neuroblasts	ganglion mother cells	neurons	Protein motifs
Pre-neuroblast gene activity	Loss of nervous system					
AS-group						
lethal of scute		subset clusters	most	-	-	helix-loop-helix
scute		subset clusters	subset	-	-	helix-loop-helix
achaete		subset clusters	subset	-	-	helix-loop-helix
daughterless		constitutive	constitutive	?	?	helix-loop-helix
Post-neuroblast gene activity						
Neuroblast group						
asense	loss of nervous system	-	most	?	-	helix-loop-helix
snail	?	-	subset	subset	subset	Zn finger
seven-up	?	subset clusters	subset	?	?	Zn finger
hunchback	?	no	all	most	most	Zn finger
runt	?	-	subset	subset	subset	?
prospero	Abnormal fasciculation	-	most	most	-	homeodomain
pox-neuro	?	-	subset	subset	subset	homeodomain
cut		-	subset	subset	subset	homeodomain
gooseberry BHS4	?	-	subset	subset	subset	homeodomain
Kruppel	?	-	?	?	subset	Zn finger
dPOU-19 & 28	?	-	subset	-	-	POU domain
GMC group						
fushi-tarazu	Sibling transformation	-	-	subset	subset	homeodomain
even-skipped	sibling transformation	-	-	subset	subset	homeodomain

Table 1.8: Neural fate genes (taken from Cabrera, 1992).

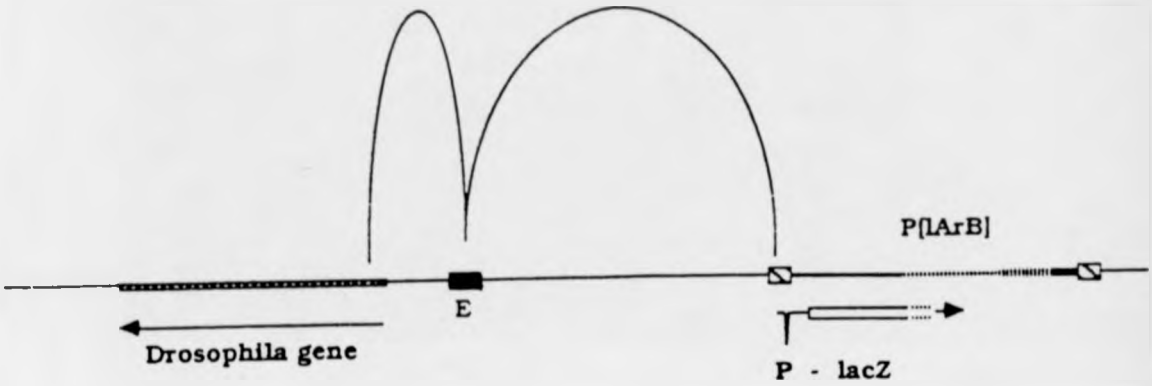


Figure 1.9 Detection of genomic regulatory elements with enhancer detector transposons.

E-represents a genomic regulatory element (enhancer). Genomic enhancer elements can act at a distance and in either direction to stimulate expression from a promoter. The enhancer detector element consists of the *E.coli* ß-galactosidase gene fused to a weak promoter. If the transposon integrates such that it is near to a genomic enhancer then ß-galactosidase will be expressed under the control of that genomic element.

Chapter 2
Materials and Methods

2.1 Standard Materials for growth of *E.coli.*

LB: 1% Bacto-tryptone (w/v); 0.5% Bacto-yeast extract (w/v); 0.5% NaCl (w/v); adjusted to pH 7.

LB agar plates: 1.5% agar dissolved in L.B.

SOB: 2% Bacto-tryptone (w/v); 0.5% Bacto-yeast extract (w/v); 0.05% NaCl (w/v); 2.5mM KCl (add as a solution); pH to 7.0 with 5M NaOH.

SOC: SOB +20mM glucose

2.2 Making competent *E.coli* cells.

FSB: KCl 100mM, $MnCl_2 4H_2O$ 45mM, $CaCl_2$ H_20 10mM, Hexammine cobalt chloride 3mM, K acetate 10mM, redistilled glycerol 10% (w/v).

Bacteria were made competent to take up exogenous DNA by the procedure of Hanahan (1986). Stocks of bacteria were maintained as 1ml liquid cultures made 25% for glycerol and stored at -70°C. A small quantity of this glycerol stock was removed using a sterile loop and then streaked across an L-agar plate and grown overnight at 37°C to obtain single colonies. A single colony was used to inoculate 500 mls of L-broth and shaken at 37°C in an orbital shaker until the $O.D._{550}$ had reached approximately 0.5. The culture was then transferred to a sterile 500 ml tube and the cells were pelleted by centrifugation at 7000 r.p.m. for 15 minutes. After this the supernatant was removed and the pellet was gently resuspended in 1/3 culture volume of FSB and incubated on ice for 10-15 minutes. The cells were pelleted by gentle centrifugation and resuspended in 1/12.5 of the original volume. DMSO was added to a final concentration of 3.5% (v/v) into the middle of the culture and swirled for 5-10 seconds, it was then left on ice for 5 minutes. A second aliquot of DMSO was added so that the final concentration was 7% (v/v) the cells were left for 10-15 minutes on ice. Cells were aliquoted into screw cap cryogen tubes, flash frozen in liquid nitrogen and stored at -20°C.

2.3 Transformation of competent *E.coli*

To transform competent bacteria 5-50 ng of DNA in a maximum volume of 15µl of TE, was added to a 100 µl aliquot of competent cells. This was incubated on ice for 30 minutes and then a 30 second heat shock at 42°C was performed, then the cells were returned to the ice for 5 minutes. After this time 1 ml of SOC was added and the culture was incubated at 37°C for 30 minutes. The cells were gently pelleted by spinning at 6000 r.p.m. for two minutes in a MSE microcentaur and resuspended in 100 µl of L. broth. The bacterial cells were then spread on a L-agar plate containing 50µg Ampicillin per ml. The plates were incubated overnight at 37°C and then examined for transformants. Transformation frequencies of $1x10^6$ - $1x10^7$ colonies per µg of DNA were routinely obtained for *E.coli* XLI-blue when using this protocol.

2.4 Isolation of plasmid DNA (Alkaline lysis method).

Solutions for routine DNA isolation

TE: 10mM Tris Cl (pH 8); 1mM EDTA (pH 8).
Isopropanol: 100%.
Ammonium Acetate: 7.5M.
E.D.T.A.: 0.5M (pH 8).
LiCl: 4M.
ethanol: 100%.
ethanol: 70%.

2.4.1 Small scale 'minipreps'.

Materials.
Solution I: Lysis solution: 25mM Tris Cl (pH 8); 10mM EDTA (pH 8); 10% glucose; 0.1mg / ml RNAse (DNAse free); 1mg / ml lysozyme (added just before use).
Solution II: Alkaline SDS: 0.2M NaOH; 1% SDS.
Solution III: Plasmid Hi-salt solution: 3M potassium acetate; 11.5 % acetic acid (v/v)

A single bacterial colony was inoculated into 10 ml L-broth with Ampicillin 50µg/ ml and grown overnight at 37 °C in an orbital shaker. 1.5 ml of culture was pelleted by centrifugation at 6000 r.p.m. for 5 minutes in a microcentaur and the pellet was then resuspended in 100µl of solution 1. 200µl of solution 2 was added and mixed by inverting the Eppendorf tube, followed by 150µl of solution 3. The now lysed bacteria were incubated on ice for 10 minutes and then centrifuged at 12000 r.p.m. for 10 minutes to

pellet the bacterial debris. The supernatant was transferred to a new Eppendorf tube and an equal volume of isopropanol was added (500µl). After inversion the tube was centrifuged for 20 minutes at 13000 r.p.m. to precipitate the nucleic acids. The DNA was phenol extracted, chloroform extracted and ethanol precipitated as described in section 2.4.3 and 2.4.4. Typically 1-2 µg of plasmid DNA was obtained using this method.

2.4.2 Large scale 'maxipreps'.

Materials (as for small scale)
Ethidium bromide: 10 mg/ml.
Caesium chloride
Isopropanol saturated with S.TE buffer: 10mM Tris (pH 8); 100mM NaCl; 1mM **EDTA** 0.5M (pH 8).

Method.

A 10 ml bacterial culture was grown overnight as above. This was then used to inoculate 500 ml of L-broth where the concentration of Ampicillin was 50 µg/ ml. The culture was shaken vigourously in an orbital shaker for 18 hours at 37 °C. The cells were harvested in two 250 ml Beckman plastic bottles and pelleted at 7000 r.p.m. in a MSE centrifuge for 20 minutes at 4 °C. The pelleted cells were resuspended in 4 mls of solution 1 and placed on ice. 8 mls of solution 2 was added and after a further 15 minutes on ice 12mls of solution 3 was added. The tube was mixed by gentle inversion. The mix was spun at 13000 r.p.m. for 30 minutes at 4 °C in a Beckman centrifuge. The supernatant was then decanted into fresh oakridge tubes. 0.6 volumes of isopropanol was mixed with the supernatant and incubated at room temp for 15 minutes. The oakridge tubes were then spun at 10000 r.p.m. for 20 minutes. The supernatant was discarded and the pellet air dryed following a wash with 70% ethanol. The pellet was resuspended in 5.5 ml of TE, and 6.05 g of CsCl and 0.7 ml of 10 mg/ ml EtBr was added. The solution was then transferred to a Beckman vti 65 heat seal tube. The tube was ultracentrifuged at 45000 r.p.m. for 18 hours in a Beckman (vti 65) rotor. The CsCl gradient produced by ultracentrifugation causes the DNA to become localised in two discrete bands. The lower band is composed of supercoiled plasmid DNA and was extracted using a syringe and the Et Br was extracted with STE saturated isopropanol. Four volumes of TE were added and

the DNA was precipitated with 3 volumes of 100% ethanol. This was spun at 13000 r.p.m. for 30 minutes, washed with 70% ethanol and vacuum dried. The pellet was resuspended in 1 ml of TE.

2.4.3 Phenol/ chloroform extraction of protein from DNA

Phenol /(chloroform/isoamyl alcohol, 24:1) 1:1

An equal volume of phenol chloroform mixture was added to the aqueous DNA solution. The two phases were mixed and then separated by centrifugation for five minutes at 6500 r.p.m. The upper aqueous layer was removed leaving the protein residue at the interface. This procedure was repeated until the interface was clean.

2.4.4 Ethanol precipitation of DNA.

Ethanol 100%
3M Sodium Acetate pH 5.2

Sodium acetate was added so that it was one tenth of the volume of the DNA solution. Ice cold Ethanol was added until the final volume was three times that of the original DNA solution. The solution was placed at -70°C for thirty minutes or in dry ice for ten minutes and then centrifuged for thirty minutes at 13000 r.p.m. The pellet was washed in 70% ethanol vacuum dried and resuspended in T.E. to a concentration of 1µg/µl.

2.5.1 Restriction digestion of DNA.

DNA was routinely cut with restriction enzymes as described in Maniatis *et al.*, 1982 Generally DNA was cut in a volume of between 10 µl and 100 µl containing 1X reaction buffer (appropriate to the enzyme) and <1/10th volume restriction enzyme. Digests were incubated at 37°C for 3 hours. Restriction enzymes and buffers were supplied by Gibco BRL.

2.5.2 Size separation of DNA fragments.

Fragments of DNA were separated on the basis of their size by electrophoresis in agarose gels by the standard method in Maniatis *et al.* 1982. In outline, for the purposes of visualisation of the sizes of various DNA fragments, DNA was mixed with loading buffer containing glycerol and coloured dyes (bromophenol blue and xylene cyanol) and loaded into wells in a 0.7% to 2% agarose gel in tris/ borate/ EDTA buffer. This gel was then run at between 100 and 150mA until the DNA fragments had migrated far enough to become separated. Ethidium bromide (0.5µg/ml) was added to the gel to allow visualisation of the DNA fragments by U.V. illumination (254 nM).

If a DNA fragment was to be purified from the gel by geneclean, the gel was made and run in a tris/acetate /E.D.T.A. buffer.

2.5.3 Transfer of DNA to nylon membrane.

Fragments of DNA which had been size fractionated in agarose gels were often transferred to nylon membranes for the purposes of performing Southern hybridisations.

Materials.

Alkali buffer: 0.5M NaOH. 1.5M NaCl.
Ammonium acetate: 1M.
Nylon membrane: Hybond N (Amersham).
SSC

Method

After electrophoresis the DNA on the gel was denatured by incubating in alkali buffer for 30 minutes. The DNA was neutralised by washing twice in ammonium acetate (15 minutes per wash). The DNA was transferred to the nylon membrane by capillary action. A piece of 3mm Whatman paper (wetted with 20X SSC) was placed on a glass plate. The gel was then placed on top of the Whatman and then the nylon membrane (cut to size and pre-wetted in SSC) was placed on top of the gel. This was followed by three more layers of Whatman (again pre-wetted and cut to size) and finally on top 10 cm of tissues. Another glass plate was placed on top of the tissues and a moderately heavy weight (500

g) was placed on the glass plate. Transfer was allowed to proceed for between 1 hour and overnight.

After transfer the nylon membrane was removed from the stack, allowed to air dry and then the DNA was crosslinked to the membrane by U.V. illumination (254 nM) for 2 to 3 minutes. Membranes were stored between sheets of Whatman paper before use.

2.6 Preparation of DNA probes

2.6.1 Preparation of Digoxygenin labelled probe.

This protocol is taken from the non-radioactive DNA labelling kit supplied by Boehringer Mannheim.

Materials
10x Hexanucleotide Mix: Boehringer Mannheim.
10X Dig DNA labelling mix: Boehringer Mannheim.
Klenow fragment of DNA polymerase 1: B.R.L. (6 units per ml).
LiCl 4M

Method

Linearised template DNA was denatured by boiling for 10 minutes in a water bath and then chilling quickly in a dry ice/ethanol bath. The following components were then added to an eppendorf on ice: 100 ng denatured linearised DNA; 2 µl 10x hexanucleotide mix; 2 µl 10x DIG labelling mix; 1 µl Klenow enzyme; dH_2O to make the volume to 20µl. The reaction was then incubated at 37°C for 1 hour and then the probe was precipitated by adding 12 µl LiCl and 330 µl prechilled ethanol and by incubating at -70°C for 30 minutes or more. The precipitate was spun down in a MSE microfuge at 13,000 R.P.M. for 30 minutes and then washed with 1 ml of prechilled 70% ethanol. The pellet was dried in a vacuum dessicator and then resuspended in 50 µl TE.

2.6.2 Preparation of Biotin labelled probe.

Materials.
10x Hexanucleotide mix: Boehringer Mannheim.
10x Nucleotide mix: 1mM dATP; 1mM dCTP; 1mM dGTP; 0.5mM dTTP.
Biotinylated dUTP: 0.5mM (Boehringer Mannheim).
Klenow fragment of DNA polymerase 1: B.R.L. (6 units/ml).
Hybridisation Buffer: 600mM NaCl; 1X Denhardts; 50mM $NaPO_4$ (pH7.2); 5mM $MgCl_2$.

Method

Linearised template DNA was denatured by boiling for 10 minutes in a water bath and then chilling quickly in a dry ice/ethanol bath. The following components were then added to an eppendorf on ice: 100 ng linearised denatured DNA; 2 µl hexanucleotide mix; 2 µl nucleotide mix; 1µl biotinylated dUTP; 1µl Klenow enzyme: dH_2O to a volume of 20µl.

The reaction was incubated at 37°C for 20 hours and then stopped by adding 2 µl E.D.T.A. The probe was then precipitated by adding 10 µl Herring sperm DNA, 11 µl Na Acetate and 330 µl ethanol and then incubating at -70°C for 30 minutes. The probe was centrifuged in a MSE microfuge at 13,000 R.P.M. for 30 minutes and then washed with prechilled 70% ethanol. Finally the pellet was dried in a vacuum dessicator, resuspended in 20 µl TE and 280 µl of hybridisation buffer was added.

2.6.3 Preparation of a radioactively labelled probe.

Radioactively labelled DNA probes were prepared by random priming.

Materials.
Solution A: 1.25M Tris (pH 8); 0.25M $MgCl_2$; 0.018% 2-mercaptoethanol; 1mM dATP; 1mM dCTP; 1mM dTTP.
Solution B: 2M Hepes (pH 6.6).
Solution C: Random hexamers (Boehringer Mannheim) at 90 OD units/ml in TE
OLB: Solution A: Solution B: Solution C at 100:250:150 ratio
B.S.A.: Ultra pure (10mg/ml).
α^{32}P labelled dGTP: 10 µCi/µl
Klenow fragment of DNA polymerase 1: B.R.L. (6 units/ml).

Method

Approximately 50 ng template was dissolved in 32.5 µl dH_20 and then denatured at 100°C for 3 minutes. The template was then incubated at 37°C for 5 minutes and then the following components were added in order; 10 µl OLB, 2µl B.S.A., 5 µl α^{32}P dGTP, 1µl Klenow. The solution was mixed by pipetting and then incubated at room temperature overnight. The unincorporated nucleotides were separated in one of two ways. Either the volume was made up to 100 µl with TE and the unincorporated nucleotides were removed using a G-50 sephadex column as described in 2.6.4 or the unincorporated nucleotides were removed by precipitation. In this case 10 µg tRNA, 12.5 µl sodium acetate and 50 µl

isopropanol were added. The solution was incubated on ice for 30 minutes and then spun for 30 minutes in a MSE microfuge to pellet the probe. The pellet was washed once briefly in ice-cold 70% ethanol and then allowed to air dry before resuspension in 50 µl TE.

2.6.4 Separation of unincorporated nucleotides.

Unincorporated nucleotides were separated from DNA probes as described in Maniatis *et al.* 1982

G-50 Column preparation.

Materials.G-50 Sephadex: Resuspended in TE.
TE: 10mM Tris (pH 8); 1mM E.D.T.A. (pH 8).

Method.

To prepare a column the plunger was removed from a 1 ml plastic syringe (Becton Dickenson) and a small amount of siliconised glass wool was added and pushed down to the bottom of the syringe using the plunger. The syringe was then filled with G-50 sephadex. The column was then inserted through a small hole in the lid of a Falcon 2097 tube. An eppendorf was placed at the bottom of a 2097 tube to collect fractions from the column. The 2097 tube was then spun at 1600 g for 4 minutes in a Mistral 2000 centrifuge. The column was washed twice with 100 µl TE. The TE was removed by spinning 1600g for 4 minutes. The eppendorf was replaced with a fresh one before the column was used.

Use of the G-50 Column

The volume of the probe to be separated from unincorporated nucleotides was made up to 100 µl with TE and this was added to the top of the column. The column was then spun at 1600g in a Mistral 2000 centrifuge for 4 minutes and the elutant was collected.

2.7 Detection of labelled DNA hybridised to Southern blots.

Labelled DNA was hybridised to southern blots and detected in one of 3 ways. The hybridisation of radioactively labelled probe was detected by autoradiography using X-ray film (Fuji). Digoxygenin labelled DNA probes were detected by either a colour reaction using N.B.T. and X-Phosphate (dig DNA detection kit), or by chemiluminescense (A.M.P.P.D.).

2.7.1 Hybridisation and detection of radioactively labelled probe on Southern blots.

Materials.
Components of hybridisation solutions

Boiled, sonicated, Herring sperm DNA: 10mg/ml.
50x Denhardts: 5% Ficol (w/v); 1% Polyvinylpyrrolidone (w/v), 1% B.S.A. (w/v).
tRNA: 10 mg/ml.
20x S.S.C.: 17.53% NaCl (w/v), 8.82 % Na citrate (w/v); Adjusted to pH 7.0 with NaOH.

Prehybridisation solution: 50% formamide, 5x Denhardts, 5x S.S.C., 0.1% S.D.S., 100µg/ml denatured, sonicated herring sperm DNA.

Washing solution 1: 2X S.S.C., 0.1% S.D.S.
Washing solution 2: 0.1x S.S.C., 0.1% S.D.S.

Method.

The membrane, on to which DNA had been transferred was placed in a glass tube (Hybaid) and was prehybridised for at least an hour in 50 mls hybridisation solution at 42°C. The radioactively labelled probe, was boiled in a water bath for 5 minutes (to make it single stranded) and then cooled in an ice/water bath. Most of the prehybridisation solution was then poured away from the blot to leave just enough to keep the filter evenly wet (5 to 10 mls). The single stranded probe was then added to the prehybridisation solution and the tube was incubated rotating for 12 hours at 42°C in a Hybaid oven.

The probe was then poured off and the blot was then washed twice in washing solution 1 for 10 minutes at room temperature. The blot was washed twice in 100 mls washing solution 2 at 68°C for 45 minutes each wash. The filter was then allowed to drain (but not dry out), wrapped in cling film and exposed to X-ray film (Fuji) in an a

autoradiograph cassette for between 1 and 16 hours. The X-ray film was developed under standard conditions described in Maniatis *et al.* (1982).

2.7.2 Hybridisation and Detection of Digoxygenin labelled probe on southern blots using the colour reaction generated by N.B.T. and X-phosphate.

DNA blots were probed essentially as described in the protocol supplied with the Dig DNA detection Kit. (Boehringer Mannheim).

Materials.
Antibody conjugate: Anti digoxygenin fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim).
Malate buffer: 100mM Maleic acid; 150mM NaCl; Adjusted to pH 7.0 with NaOH.
10x Blocking buffer: Blocking reagent (Boehringer Mannheim) 10% in Malate buffer.
Prehybridisation Solution: 5x SSC; 1% Blocking reagent (1 in 10 dilution of 10x blocking buffer); 0.1% n-laurylsarcosine; 0.02% S.D.S.
Blocking Buffer: 10x Blocking buffer diluted 1 in 10 with Malate buffer.
Colour reaction solution: NaCl (100mM); Tris pH 9.5 (100mM); $MgCl_2$ (50mM)
N.B.T.: nitroblue tetrazolium salt 75 mg/ml in DMF (Boehringer Mannheim).
X-phosphate: 5-bromo-4-chloro-3 indolyl phosphate toluidinium salt 50 mg/ml in DMF (Boehringer Mannheim).
Wasning solutions 1 and 2 as in section 2.7.1

Method

DNA blots, were prehybridised for 1 hour in prehybridisation solution (30 mls) at 68^{o}C. Digoxygenin labelled DNA probes were made single stranded by boiling for 10 minutes and then cooling in an ethanol / dry ice bath for 5 minutes. Prehybridisation solution was poured away to leave a volume of 10 mls. Single stranded probe (30 µl) was added and the blot was incubated at 68^{o}C for 12 hours.

The probe solution was poured off and the blot was washed twice in 50 mls of washing solution 1 at room temperature. The blot was then washed twice in 50 mls of washing solution 2 (45 minutes per wash) at 68^{o}C. The blot was next incubated for 2 minutes in Malate buffer 1 and then incubated for 30 minutes in blocking solution. Anti-digoxygenin antibody was diluted 1 in 5000 in blocking solution. The blot was sealed in a plastic bag containing 10 mls of diluted conjugate and incubated at room temperature for 30 minutes. The blot was then washed twice in 100 mls of Malate buffer (containing 0.1% Tween 20) for 15 minutes each wash. Next, blots were washed twice for 5 minutes in

100 mls colour reaction buffer. During these washes, colour developing solution was made by adding 45 µl N.B.T. and 22.5 µl X-phosphate to 10 mls colour reaction buffer. The blot was then incubated with the colour developing solution for the desired length of time (typically between 10 minutes and several hours.) The colour reaction was then stopped by washing the blot twice for 10 minutes in 10x TE

2.7.3 Hybridisation and Detection of Digoxygenin labelled probe on southern blots using chemiluminescense.

DNA blots were probed essentially as described in the protocol supplied with the Dig DNA detection Kit with the exception that the colour reaction was replaced with a chemiluminescent substrate of alkaline phosphatase (A.M.P.P.D. Tropix-NBS Biologicals).

Materials.

Antibody conjugate: Anti digoxygenin fab fragments conjugated to Alkaline Phosphatase (Boehringer Mannheim).

Assay buffer: 0.1M Diethanolamine, 1mM $MgCl_2$.

2.4 mls Diethanolamine were added to 200 mls dH_2O and the pH was adjusted to 10.0 with concentrated HCl. $MgCl_2$ was then added to a concentration of 1mM and the volume was made up to 250 mls.

A.M.P.P.D.: disodium 3-(4-methoxyspiro [1,2 dioxetane-3,2'-tricyclo[3.3.1.1.^{3,7}] decan] -4-yl) phenyl phosphate ,24mM (N.B.S).

Method.

The blot was hybridised and detected as described in up to the point where the blot would have been washed in colour reaction solution. Instead the blot was incubated twice in 100 mls of assay buffer (5 minutes each) and then sealed in a plastic bag with 5 mls of A.M.P.P.D. (diluted 1 in 100 in assay buffer) and incubated for 5 minutes. The A.M.P.P.D. was then removed and the blot was placed in an autoradiograph cassette and exposed to x-ray film (Fuji) for 30 minutes. The blot was then exposed to x-ray film for a further period determined by the result of the first exposure.

2.8 Separation of DNA away from impurities, salts and agarose.

DNA to be used in ligations or as template in probe making reactions was restriction digested and then run on a 0.7% TAE agarose gel to separate the fragments. The required fragment was then cut from the gel using a razor blade and separated from the agarose using either the Geneclean II kit (Bio 101 inc.) Gene clean relys on DNA preferentially binding to glass beads at high salt concentrations (6M NaI).

2.8.1 Separation using the Geneclean II kit.

Materials.
NaI: 6M (Bio 101).
Glassmilk: Suspension of silica matrix (Bio 101).
New wash: NaCl, Tris, E.D.T.A. made up in ethanol and stored at -20°C. (Bio 101).

Method.

The DNA band was cut from the gel (visualised using a long wave U.V. lamp (302 nm) and transferred to an eppendorf. The gel slice was weighed and dissolved in 3 volumes of NaI at 55°C. (0.3 mls NaI per 0.1 g gel slice). 5 µl of Glassmilk was added and mixed by inverting the capped Eppendorf several times. The solution was then placed on ice for 5 minutes to allow binding of the DNA to the silica matrix. The glassmilk was then pelleted by spinning for 15 seconds in a MSE microfuge. The NaI was then removed and the pellet was washed three times with 700 µl 'New Wash' (-20°C). After the third wash the 'New Wash' was removed the pellet was spun for a further 15 seconds to bring down any residual 'New Wash' adhering to the sides of the Eppendorf. This last wash was removed and the pellet was then resuspended in 10 µl TE and incubated at 50°C for 3 minutes to elute the DNA. The glassmilk was then pelleted by spinning for 1 minute and the supernatant containing the eluted DNA was transferred to as fresh tube. The DNA was eluted twice.

2.9 Isolation of genomic DNA from adult *Drosophila.*

Materials.
Homogenisation Buffer: 10mM Tris (pH 8.0); 60mM NaCl; 10mM EDTA; 0.15mM **Spermidine**; 0.5% Triton X-100 (v/v).
Sarkosyl: 20%.
Sodium Acetate: 3M (pH 6.3).

Method.

A Dounce homogeniser was cleaned and then pre-chilled on ice. Approximately 100 to 200 flies were homogenised in 3 mls of homogenisation buffer. The homogenate was filtered through a fine gauze into a 15 ml plastic tube (Falcon 2059). The filtrate was then spun at 7,000 r.p.m. for 7 minutes in a Beckman centrifuge at 4°C. The supernatant was removed and the pellet was resuspended in a further 3 mls of homogenisation buffer and respun at 7,000 R.P.M. for 7 minutes at 4°C. The supernatant was decanted and the pellet was resuspended in 1.8 mls of homogenisation buffer and 200µl of 20% sarkosyl was added. The solution was mixed by gentle inversion and 20µl proteinase K (µg / µl) was added. The solution was then incubated at 50°C for 2 to 3 hours. Following proteinase K treatment, 200 µl sodium acetate (3M) was added, and protein was extracted with 250 µl phenol. A equal volume of chloroform/isoamyl alcohol was added to removed any traces of phenol. Nucleic acid was precipitated with 3X the volume of 100% ethanol at -20°C for 30 minutes, and then spun at 10,000 R.P.M. for 15 minutes at 4°C to pellet the nucleic acid. The pellet was washed to remove salt with 10 mls of ice-cold 70% ethanol and then air dried. The pellet was resuspended in approximately 50µl - 100µl of TE.

2.10 RNA Manipulations

2.10.1 Isolation of total RNA from *Drosophila* tissue.

Total RNA was isolated from *Drosophila* embryos, using the following protocol (Ashburner, 1989).

Materials.
Homogenisation buffer: 100g Guanidinium isothiocyanate, 100 ml deionised H_20, 10.6 ml 1M Tris HCl (pH 7.6), 10.6 ml 0.2M EDTA.
P.C.I.: Phenol/Chloroform/ Isoamyl Alcohol (25:24:1)
Rnase-free dH_2O:

Falcon 2059 tubes: Freshly opened bag.
15 ml Dounce homogeniser: RNAse-free.

Method.

Precautions to prevent contamination with RNAses.

Glassware was made RNAse free by baking at 250°C overnight. Sterile plastic ware is essentially RNAse free and so, fresh bags of eppendorfs, yellow and blue disposable tips and Falcon 2059 tubes were used each time the experiment was performed. Disposable gloves were worn at all times and changed frequently throughout the experiment.

RNA extraction.

A 15 ml RNAse free Dounce homogeniser and a 15 ml 2059 Falcon tube were placed on ice for 5 minutes. Approximately 300mg of tissue (fresh or frozen at -70°C) was homogenised in 1ml homogenisation buffer. The homogenate was transferred to a Falcon 2059 tube where it was deproteinised with 1ml of phenol/ chloroform. The tube was then spun in a beckman centrifuge at 10,000 R.P.M. for 20 minutes at 4°C. The upper aqueous phase was then removed and the RNA was precipitated by adding 100µl of sodium acetate and 3X the volume of 100% ethanol, this was mixed by inversion and incubated at -20°C for 30 minutes. The RNA was then pelleted by spinning at 10,000 R.P.M. for 15 minutes at 4°C. The pellet was washed and resuspended in 50µl of RNAse free dH_2O.

The concentration and purity of the RNA was estimated spectrophotometrically. Absorbance readings were taken at 280 nm, 260 nm and 230 nm respectively. The 260 nm value was used to assess the concentration of the RNA (using the approximation that a 40 µg/ml solution of RNA will have an absorbance at 260 nm of 1 O.D. unit). The 260/280 ratio was used to assess levels of contamination with protein (RNA with a value of 1.7 or above was used).

2.10.2 Electrophoresis of RNA.

Materials
Formamide gel: 50% deionised Formamide, 1.2% Agarose, 1XTPE, RNAse free H_20
10X TPE pH8.0: Tris base 4.36g/l, NaH_2PO_4 4.68g/l, EDTA 0.37g/l

Method

Dissolve agarose in TPE and H_20 in a microwave measure formamide in fume hood and add to hot agarose and mix. Set at 4°C.RNAse free loading buffer was added to the RNA samples which were heat denatured at 65°C before loading. Run gel at 20mA in 1X TPE for 6 hours.After running the gel was soaked in 20X SSC for 30 mins, the gel was then stained in Ethidium bromide at 1µg/ ml for 30 mins. The gel was destained in RNAse free water for 15 mins. The gel was veiwed on a transilluminator and photographed.

2.10.3 Transfer of RNA to nylon membrane.

RNA species were size fractionated on a formamide gel . The gel was then soaked in 0.5M NaOH for 20 minutes to partially hydrolyse the RNA which aids transfer. The gel was the soaked in RNAse free 20X SSC for 45 mins and then transfered to a nylon membrane in exactly the same way as for DNA.

2.10.4 Hybridisation of DNA probes to RNA bound to nylon membranes

Random prime labelled $\alpha^{32}P$ probes were synthesised as described in section 2.6.3. the filter was prehybridised and the probe was denatured by boiling before adding to the filter. The filter was hybridised overnight and detected as for southerns with radioactive probes 2.7.1.

2.11 Screening a plasmid cDNA library. (Adapted from the protocol of Brown and Kafatos, (1988) of high density library screening)

Epicuran blue (Statagene) supercompetent cells were transformed with the 4-8hr embryonic cDNA library of N.Brown. The library was titered and then plated onto 9 plates at a density of 50,000 colonies per plate.

2.11.1 Preparation of filters.

Each plate was grown at 37°C for ~ 10 hours. At this stage the colonies are just visible less than 0.2mm diameter. A square of nylon membrane was placed onto each plate and keyed using a hypodermic needle. The filter was carefully removed and a replica filter

was prepared. The printing press method was used. A dry nylon filter is placed on top of the colony side of the lifted filter, this sandwich is squashed in the press, keyed with a needle and then wrapped in tin foil and autoclaved for five minutes with slow exhaust. This lyses the colonies and denatures the DNA. DNA was fixed to the filters by UV irradiation for five minutes. The master plates were stored at 4°C.

2.11.2 Screening the Library

To screen the library, P^{32} labelled probe was made to selected fragments of genomic DNA. The filters and their replicas were washed in PW for 2hours at 65°C for two hours, prehybridised, hybridised and detected as described in 2.7.1.

Isolation of positives.

Positives which appeared on both the original and replica filters were identified and a 5mm circle was picked. Permanent stocks were made from these cultures by adding 0.5ml sterile 50% glycerol to 1 ml of culture and freezing at -70°C.

Secondary screen

For the secondary screen the plates were plated at a density of 1000 colonies per plate. The secondary replicas were then prehybridised, hybridised and detected as described for the primary replicas. Positives identified on both replicas and which could be identified as single colonies were picked into 5 mls of LB(amp) and grown overnight at 37°C. Approximately 3 positives per plate in the secondary screen were picked. From each of these cultures DNA was prepared and restriction enzyme digested. Permanent stocks were made from these cultures by adding 0.5 ml sterile 50% glycerol to 1 ml of culture and freezing at -70°C.

2.12 Double stranded sequencing of DNA.

2.12.1 Preparation of the sequencing gel.

Materials.
Cream cleaner, detergent, ethanol: 100%.**Siliconising solution:** Repelcote (B.D.H.)
Vaseline:
5x T.B.E.: 5.4% Tris base (w/v); 2.75% Boric acid (w/v); 10mM E.D.T.A. (pH 8).
Urea: 46.7% Urea (w/v); 5x T.B.E..
Acrylamide solution: 19.3% Acrylamide (w/v); 0.67% Methylene-bis-acrylamide (w/v); 46.7% Urea (w/v); 1x T.B.E..
Ammonium persulphate: 10% in dH_2O.
TEMED: N,N,N',N'-tetramethylethylenediamine

Method.

Two glass sequencing plates (one notched) were cleaned thoroughly using cream cleaner and detergent. The plates were rinsed thoroughly with water and then dried. The plates were then cleaned with 100% ethanol. 0.4mm thick teflon spacers were scrubbed and then wiped with 100% ethanol. The spacers were positioned along the sides and bottom of the plate. Two small blobs of Vaseline were added at the intersections between the bottom and the two side spacers. The notched plate was coated with siliconising solution and was placed siliconised side down on top of the non-notched plate. The two plates were then clamped together using several bulldog clips. The gel was prepared using, 80 mls urea solution mixed with 20 mls acrylamide solution in a 200 ml beaker (final acrylamide concentration of gel = 4%). To this 140 µl TEMED and 400 µl (100mg/ml) ammonium persulphate were added.Using a 50 ml syringe (Becton Dickenson). The solution was poured between the two plates which were angled to allow the solution to run down one side and fill the space up from the bottom of the gel. The wells were formed by inserting a sharks tooth comb (flat side inwards) into the polymerising gel. Once the gel was set, the bulldog clips, comb and the bottom spacer were removed. The well and edges of the gel were washed with water to remove any unpolymerised acrylamide. Wells were produced by inserting a sharkstooth comb, teeth down into the top of the gel. Gels were used after 30 mins to 1 hour after setting.

2.12.2 Preparation of single stranded DNA.

Single stranded DNA was prepared essentially as described in the protocol supplied with the sequencing kit as follows;

Approximately 5 µg template DNA was used for each set of sequencing reactions. The DNA was denatured by adding 1/10th volume of 5M NaOH and incubating at 20°C for 10 minutes. NaOH was removed from the template by centrifuging through a Sepharose column.

Sepharose column preparation.

The column was prepared in a 0.5ml Eppendorf. A 19 guage needle was used to pierce the base of the tube. Three mm of glass beads were placed into the column followed by the Sepharose. The column was placed inside a 1.5 ml Eppendorf and spun 3X for 30sec. 20µl of H_20 was added to the top of the column and spun for 30sec, this was repeated until the volume coming out of the bottom was also 20µl.

2.12.3 Sequencing reactions.

The sequencing reactions were performed as described in the protocol supplied with the Sequenase version 2.0 kit (U.S.B.). This technique of sequencing using chain terminators is adapted from that of Sanger *et al.* (1977).

Materials.
Single stranded DNA: See above.
Primer: 300ng
Reaction Buffer: 200mM Tris Cl (pH 7.5).; 100mM $MgCl_2$; 250mM NaCl; (U.S.B.)
DTT: 0.1M (U.S.B.)
Labeling mix: 7.5 µM dGTP; 7.5 µM dTTP; 7.5µM dCTP; (U.S.B.).
α-^{35}S dATP: 10 µCi/µl.
Sequenase: Version 2.0 enzyme (U.S.B.).
Enzyme dilution buffer: 10mM Tris (pH 7.5).; 5mM DTT; 0.5 mg/ml B.S.A; (U.S.B.)
ddGTP termination mix: 80 µM dGTP; 80 µM dATP; 80 µM dCTP; 80 µM dTTP; 8 µM ddGTP; 50mM NaCl; (U.S.B.).
ddATP termination mix: 80 µM dGTP; 80 µM dATP; 80 µM dCTP; 80 µM dTTP; 8 µM ddATP; 50mM NaCl; (U.S.B.).

ddTTP termination mix: 80 µM dGTP; 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 8 µM ddTTP; 50mM NaCl; (U.S.B.).
ddCTP termination mix: 80 µM dGTP; 80 µM dATP; 80 µM dCTP; 80 µM dTTP, 8 µM ddCTP; 50mM NaCl; (U.S.B.).
Stop solution: 95% Formamide (v/v); 20mM E.D.T.A.; 0.05% Bromophenol blue (w/v); 0.05% Xylene cyanol (w/v); (U.S.B.).

Method.

Annealing template and primer.

For each set of sequencing reactions, 1µl primer (300ng) was added to 2 µl reaction buffer and 7µl DNA 5µg (denatured as described above). The DNA and primer were allowed to anneal at 37°C for 30 minutes. During this incubation, labelling mix was diluted 5 fold in dH_2O and stored on ice.

Labelling reaction.

After annealing the primer and the template, the solution was placed on ice for 5 minutes. The following were added to the annealed mix; 1 µl DTT; 2 µl diluted labelling mix; 0.5 µl α-^{35}S dATP; 2 µl freshly diluted Sequenase (Sequenase enzyme diluted 1 in 8 in ice-cold enzyme dilution buffer). The solution was mixed thoroughly and incubated for 2 to 5 minutes at room temperature.

Termination reaction.

During the annealing reaction, 2.5 µl ddGTP termination mix was added to tube G, 2.5 µl ddATP was added to tube A , 2.5 µl ddCTP was added to tube C and 2.5 µl ddTTP was added to tube T.

The tubes were capped and prewarmed to 37°C, 3.5 µl of the labelling mix was removed and transferred to each of the tubes A, C, G and T. The solutions were mixed and incubated at 37 °C for 5 minutes. After 5 minutes, 4 µl stop solution was added to each tube. The sequencing reactions were stored at -20°C until they were required.

2.12.4 Running the sequencing gel.

Sequencing gels were run using standard electrophoresis equipment for sequencing gels (Maniatis et al). The running buffer used was 1X T.B.E (6.1.3)

Method.

Sequencing gels prepared as described above were pre-run at 1500 V, 200 amps and 40Watts for 15 minutes. The wells were then flushed out with 200 µl 1x T.B.E. Prior to loading, the sequencing reactions were heated to 85°C. Approximately 3.5 µl of each sequencing reaction was loaded per track and the gel was run for 3 hours per 1 run of the gel.

Fixation and transfer of sequencing gels.

Materials.
MeOH/Glacial acetic acid: 10% MeOH (v/v; 10% Galcial acetic acid (v/v).

Method

After electrophoresis, the combs, spacers and non-siliconised plate were removed. The gel was fixed in 10%MeOH / 10%glacial acetic acid for 30 minutes. After fixation the gel was removed from the solution and allowed to air dry slightly. A piece of dry Whatman paper was then placed on the gel. The gel becomes stuck to the paper allowing it to be moved. The gel was covered in cling film and then placed on a heated vacuum drier for 40 minutes at 80°C. The cling film was then removed and the dried gel was exposed to X-ray film (Fuji) overnight.

2.13 In situ hybridisation of DNA probes to *Drosophila* polytene chromosomes.

Biotin labelled probes were synthesised and these were detected using Streptavidin conjugated to Horse Radish Peroxidase.

2.13.1 Preparation of polytene chromosome squashes.

Materials.
1x PBS: 0.1M NaH_2PO_4/Na_2HPO_4 (pH 6.8), 0.15M NaCl
3:1 fixative: 75% ethanol (v/v), 25% Glacial acetic acid (v/v).
Acetic Acid: 45%.
Glass slides: Subbed
Coverslips: Siliconised in repelcote (BDH).
Pencil: With rubber on the end.

Method.

Fly stocks were transferred daily on rich food and then the larvae were cultured at 18°C until they reached the late third instar. The larva was dissected in 1x PBS and the salivary glands were removed. These were then transferred to a drop of 45% acetic acid on a coverslip. The coverslip was then lifted with a subbed slide and positioned coverslip up. The coverslip was then tapped firmly with the rubber end of the pencil until the chromosome arms had spread. Strong pressure was then applied to the coverslip with the thumb to flatten the chromosomes. The slide was then placed on dry ice for 5 minutes and the coverslip was flicked off with a razor blade. The chromosomes were fixed immediately in 3:1 ethanol acetic acid. The chromosomes were fixed for 10 minutes followed by dehydration in 100% ethanol for 10 minutes. Finally the slide was allowed to air dry before storage or pretreatment.

2.13.2 Pretreatment and hybridisation of slides.

Materials.
Triethanolamine:7.5M (pH 8).
Acetic anhydride
Rubber cement

Method.

The chromosomes were heat treated by incubating them at 68°C for 30 minutes in 2x S.S.C.. The slides were then transferred to 2x S.S.C. at room temperature for 2 minutes. 500 mls of 0.1M triethanolamine was rapidly agitated using a magnetic stirrer and to this 0.625 mls of acetic anhydride was added. After 5 seconds the stirrer was turned off and the slides were incubated immediately in this solution for 10 minutes. The slides were then transferred to fresh 2x S.S.C. for 4 minutes at room temperature. The slides were then washed 4 more times in 2x S.S.C. for 4 minutes each at room temperature. The slides were then dehydrated by washing then twice in 70% ethanol for 5 minutes and then once in 100% ethanol for 5 minutes. The chromosomes were then denatured in freshly prepared 0.07M NaOH (pH 12.5) for 3 minutes at room temperature. The slides were washed in 2x S.S.C. for 5 minutes at room temperature three times. The chromosomes were dehydrated

by washing twice in 70% ethanol for 5 minutes and then once in 100% ethanol for 5 minutes. Finally they were allowed to air dry. Pretreated slides were kept for up to a month before they were hybridised.

Slides were hybridised with biotin labelled probes. The probe was boiled for 10 minutes and then cooled on dry ice/ethanol for 5 minutes. To each slide 30 µl probe was added and then a large coverslip (22mm x 22mm) was laid on top. The edges of the coverslip were sealed with rubber cement and the slides were incubated in a moist chamber at 58°C for 12 hours.

2.13.3 Detection of biotin labelled probes.

Materials.
Triton X-100:
ABC reagent A: Vectastain ABC Elite kit (Vector laboratories).
ABC reagent B: Vectastain ABC Elite kit (Vector laboratories).
B.S.A.: Boehringer Mannheim.
DAB substrate mix: 0.02% H_2O_2 (v/v); 1 mg/ml DAB; 0.1M Tris (pH 7.2).
Giemsa staining solution: 5% Giemsa (v/v); 10mM PBS.
DePeX: BDH.

Method.

After hybridisation the coverslips were removed and the slides were washed 3 times for 20 minutes in 2x S.S.C. at 53°C. During the last wash the following components were added to an Eppendorf and mixed on a blood mixer for 30 minutes; 972 µl PBS, 9 µl ABC reagent A; 9 µl ABC reagent B; 10 µl B.S.A.. During this incubation the slides were washed once for 2 minutes in 1x PBS, once for 2 minutes in 1x P.B.S\0.1% Triton X-100 and once for 5 minutes in 1x PBS. The ABC/PBS/B.S.A. mixture was added (100 µl per slide) and allowed to incubate for 30 minutes. The slides were washed once in 2x S.S.C. for 5 minutes, once in 1X PBS/0.1% triton X-100 for 5 minutes and once in 1x PBS for 5 minutes. DAB substrate mix was added to each slide and allowed to incubate for 8 minutes. The slides were washed twice in dH_2O for 3 minutes and then dehydrated in 70% ethanol (2 x 2 minutes) and 100% ethanol (1 x 2 minutes). Slides were then counter-stained with Giemsa for 90 seconds, washed extensively in dH_2O and then mounted in DePeX.

2.14 In situ hybridisation to whole mount *Drosophila* embryos.

2.14.1 Preparation of probe for in situ hybridisation.

Probes for use in in situ hybridisations were prepared by a modification of the standard method for the synthesis of digoxygenin labelled probes. This method utilises a high concentration of the random hexamers to reduce the average length of the probe molecules. This substantially reduces background.

Materials.
Random primers: 20 mg/ml (Boehringer mannheim).
Vogel buffer: 0.95M Pipes (pH 6.6); 50mM $MgCl_2$; 0.36% β-mercaptoethanol
dNTP mix: 1mM dATP; 1mM dCTP; 1mM dGTP.
dTTP: 1mM
Dig-dUTP: 1mM (Boehringer mannheim).
DNA polymerase 1 (Klenow fragment): 6 units/µl (BRL)
Hybridisation buffer: 50% Deionised formamide; 5X SSC; Sonicated ,boiled ,Herring sperm DNA (0.1mg/ml); tRNA (0.1mg/ml); Heparin (0.05mg/ml); Tween 20 (0.1%).

Method.

Fragments to be used as templates were generally isolated from agarose gels by geneclean (Bio 101). Approximately 100 ng template was added to 5 µl random primers and the volume was then made up to 12.5 µl. This mixture was then boiled for 4 minutes to denature the template and then quick chilled in ice/water. The following components were then added; 2 µl Vogel buffer (10x), 2 µl dNTP mix, 1.3 µl dTTP, 0.7 µl dig-dUTP and 1.5 µl klenow. The reaction was incubated overnight at 14°C. The following morning another 1 µl klenow was added and the reaction was incubated for a further 4 hours at room temperature. The reaction was stopped by adding 2 µl E.D.T.A. and heating to 65°C for 10 minutes. The probe was precipitated by adding 1 µl tRNA, 80 µl dH_2O, 10 µl LiCl and 300 µl ethanol and incubating at -20°C overnight. The probe was pelleted by spinning in a MSE microfuge for 30 minutes. The pellet was then washed once in 100% ethanol, air dried and resuspended in 60 µl hybridisation buffer. Probes were stored at -20°C before use.

2.14.2 In situ hybridisation of DNA probes to wholemount *Drosophila* embryos.

This protocol is based on that described by Tautz and Pfiefle (1987).

Materials.
Sodium Hypochlorite: 8%.
n-Heptane:
Formaldehyde: 37% (B.D.H.).
Methanol: 100%..
PBS: 0.1M NaH_2PO_4 / Na_2HPO_4 (pH 6.8); 0.15M NaCl
PBT: P.B.S; 0.1% Tween 20.
Glycine: 2mg/ml.
Proteinase K: 10mg/ml
Hybridisation solution: 50% Deionised formamide; 5X SSC; Sonicated ,boiled ,Herring sperm DNA (0.1mg/ml); tRNA (0.1mg/ml); Heparin (0.05mg/ml); Tween 20 (0.1%).
Antibody conjugate: Digoxygenin fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim).
Detection buffer: 100mM NaCl; 50mM $MgCl_2$, 100mM Tris HCl (pH 9.5); 0.1% Tween 20. Filtered through 0.23 µm filter and then Levamisol added to 1mM.
N.B.T.: 50 mg/ml in DMF (Boehringer Mannheim).
X-phosphate: 50 mg/ml in DMF (Boehringer Mannheim).
Histoclear
G.M.M.: 2g/ml Canada balsam in methylsalicylate.

Method.

Embryo collection and fixation.

Approximately 50 *Drosophila* were placed in a small cage and allowed to lay onto a grape juice agar plate. After timed layings, embryos were collected from the plate using a fine paintbrush into a collecting basket immersed in dH_2O. The embryos were placed in sodium hypochlorite to remove the chorion (3 minutes). The embryos were then transferred to a 20 ml glass vial containing a two phase mixture of equal volumes of n-Heptane and 10% formaldehyde (diluted in PBS), and fixed by shaking vigorously for 20 minutes. The lower aqueous phase and some of the heptane was removed and 10 mls of methanol was added. The embryos were devitelinised by shaking vigorously for 10 seconds. The devitelinised embryos sink, and were transferred to an Eppendorf. These embryos were washed several times with methanol. Finally the embryos were washed three times in ethanol and stored at -20°C.

2.14.3 Pretreatment.

All washes were performed in 1ml, on the rotostat, unless stated otherwise.

Embryos were washed twice for 2 minutes in methanol and then twice for 2 minutes in a mixture of equal volumes of methanol and 5% formaldehyde in PBS. The embryos were post fixed for 20 minutes in 5% formaldehyde in 0.5 x PBT. Next they were washed 3 times in PBT (5 minutes per wash). During the last wash 1 ml PBT was preheated to 37°C and 2.5µl freshly thawed proteinase K 20mg/ml was added (final concentration 50µg/ml). After washing the embryos were incubated in the proteinase K buffer for 3 minutes at 37°C, digestion was stopped by rinsing twice for 2 minutes with glycine solution (2mg/ml in PBT). The embryos were rinsed twice in PBT (5 minutes per wash) and then post fixed for 20 minutes in 5% formaldehyde in PBT. Finally the embryos were washed 5 times in PBT (5 minutes per wash).

2.14.4 Hybridisation.

An aliquot of embryos was set aside for antibody preabsorbtion and the remainder were rinsed twice in a mixture of equal volumes of PBT and hybridisation solution for 10 minutes each wash. The embryos were then washed in hybridisation solution for 20 minutes. The embryos were prehybridised by incubating at 48°C for 2 hours in 100 µl hybridisation solution. Antibody conjugate was diluted 1 in 100 in PBT and preabsorbed to wildtype embryos overnight at 4°C. Following prehybridisation as much hybridisation solution as possible was removed and replaced with 90 µl hybridisation solution and 10 µl denatured probe. The embryos were hybridised overnight at 48°C.

2.14.5 Detection

After hybridisation the embryos were rinsed in hybridisation solution at room temperature for 2 minutes. Embryos were washed for 20 minutes in hybridisation solution (at 48°C), and then for 20 minutes in a mixture of equal volumes of hybridisation solution and PBT(at 48°C). They were then washed 4 times (20 minutes each) in PBT also at 48°C. The preabsorbed antibody conjugate was removed from the embryos. The antibody

was diluted 1 in 20 in PBT. The embryos were incubated in 100 µl of diluted antibody conjugate for 2 hours, at room temperature, on a blood mixer. Following this the antibody conjugate was removed and the embryos were washed for 2 minutes, 10 minutes and 3 times 20 minutes in PBT. The embryos were then rinsed 3 times in detection buffer (2 minutes per rinse). The colour reaction was produced by adding 4.5 µl N.B.T. and 2.5 µl X-phosphate to the last wash (PBT + embryos). Finally the embryos were transferred in the detection solution to a 24 well, multiwell plate and incubated until the colour reaction occurred. The reaction was stopped by rinsing 5 times in TE (2 mls per rinse).The embryos were dehydrated through an ethanol series: 50% ethanol, 70% ethanol, 90% ethanol. They were then rinsed 3 times for 1 minute in 100% ethanol, and for 10 minutes (or longer) in 100 µl of histoclear. Finally the embryos were mounted in G.M.M.

2.15 Antibody labelling of *Drosophila* embryos.

Antibody labelling was standardly performed by incubating with a mouse or rabbit primary antibody followed by a biotin labelled anti-mouse or anti-rabbit secondary antibody. The ABC component is a complex consisting of streptavidin bound to horse radish peroxidase. Streptavadin binds to the biotin labelled secondary antibody. The peroxidase activity was detected using the substrate diamino benzoate (DAB) which gives a black precipitate when cleaved by the peroxidase. DAB is carcinogenic and so all operations after the addition of the DAB were performed on a plastic tray and everything which might have come into contact with DAB was washed in sodium hypochlorite before disposal. The secondary antibody, and streptavidin/peroxidase complex were obtained from the ABC Elite kit from Vector laboratories.

Materials.
Pretreated embryos: 6.6.2
Primary Antibody:
PBS: 0.1M NaPO4 pH 6.8, 0.15M NaCl.
PBT: P.B.S, 0.1% Triton-X, 0.2% BSA.
PBTN: PBT, 5% Swine serum
B.S.A.: 10% (w/v) in PBT
Secondary Antibody: ABC Elite Kit (Vector laboratories.)
Solution A: ABC Elite Kit (Vector laboratories.)
Solution B: ABC Elite Kit (Vector laboratories.)

DAB solution: 0.1M Tris (pH 7.5); 0.5 mg/ml DAB; 0.00012% H_2O_2 (v/v)

Method.

All of the following incubations were performed at room temperature whilst mixing on a blood mixer unless stated otherwise.

2.15.1 Antibody Preabsorbtion.

Primary antibodies were preabsorbed (where possible) in the following manner. Embryos for preabsorbtion were isolated and pretreated as for 2.14.2 . Embryos were then rehydrated by washing them in 30%, 60% and 100% ethanol diluted with PBT (2 minutes per wash). Non-specific binding was blocked by incubating the embryos in B.S.A. for 30 minutes. The antibody was preabsorbed at 1 in 50 dilution (in PBT), overnight at 4°C.

2.15.2 Antibody binding.

Embryos were isolated, pretreated and rehydrated as described for preabsorbtion. The primary antibody (preabsorbed or not as required) was incubated with the embryos at a dilution of between 1 in 2 and 1 in 500 in PBT (depending upon the antibody) at 4°C overnight. During this time the secondary antibody (anti-rabbit or anti-mouse depending upon the primary antibody) was also preabsorbed as described for primary antibody preabsorbtion. The embryos were washed 3 times for 1 minute in PBT (1 ml per wash) and then 3 times for 20 minutes in PBT (1ml per wash). The embryos were incubated in PBTN for 30 minutes.The embryos were then incubated with the preabsorbed secondary antibody at a dilution of between 1 in 200 and 1 in 500 in PBTN (depending upon the primary antibody) at 4°C overnight, or for 4 hours at room temperature.

The embryos were washed 3 times for 1 minute in P.B.T. (1ml per wash).They were then given three 20 minutes washs in PBT (1 ml per wash). During the three 20 minute washes, the two components of the ABC kit were allowed to complex by adding 16 µl solution A and 16 µl solution B to 1 ml PBT and incubating for 1 hour. The embryos were then incubated in the ABC complex (500 µl) for 30 minutes. The embryos were washed for 1 minute in PBT (1 ml per wash) and then 3 times for 20 minutes in PBT (1 ml per wash). The colour reaction was performed in 500 µl DAB solution until the colour

developed. The reaction was stopped by washing several times in PBT. Embryos were dehydrated and mounted in GMM.

2.16 X-Gal staining

2.16.1 Staining for β-galactosidase activity in *Drosophila* embryos.

Method: Cahir O'Kane, pers. comm.

Materials.
Grape juice agar plates
Fix: 4% formaldehyde; 50mM EGTA; 1X PBS.
X-Gal: 8% in DMSO.
Staining solution: 10mM $NaH_2PO_4.H_2O/Na_2HPO_4.2H_20$ (pH 7.2); 150mM NaCl; 1mM $MgCl_2$; 10mM $K4[Fe^{II}(CN)_6]$;10mM $K3[Fe^{III}(CN)_6]$; 0.3% Triton X-100.
PBS: 0.1M NaPO4 pH 6.8; 0.15M NaCl.
P.B.X.: 0.3% Triton X-100 in PBS.

Method.

Embryos were washed and then dechorionated in sodium hypochlorite (about 3 minutes). The dechorionated embryos were then washed several times in PBS. The embryos were fixed in 0.5 ml of fix solution and 0.5 ml of n-heptane and shaken vigorously for 15 minutes. During the fixation period 12.5 µl X-gal was added to 500 µl staining solution and prewarmed to 37°C. After fixation the heptane and fix were removed and the embryos were washed several times in P.B.X. until they no longer stuck to the sides of the eppendorf. The P.B.X. was then replaced with the preincubated X-gal staining solution and incubated at 37°C until the blue colour developed. Embryos were mounted in 70% glycerol/30% staining solution beneath supported coverslips.

2.16.2 Detection of β-galactosidase activity in *Drosophila* imaginal discs

Method: Cahir O'Kane, pers. comm.
Materials.
PBS: 0.1M NaPO4 pH 6.8; 0.15M NaCl.
PBT: P.B.S; 0.3% Triton X-100.
Fixative: 0.00375% gluteraldehyde in PBT.
X-Gal: 8% in DMSO.
Staining solution: 10mM $NaH_2PO_4.H_2O/Na_2HPO_4.2H_20$ (pH 7.2); 150mM NaCl; 1mM $MgCl_2$; 10mM $K4[Fe^{II}(CN)_6]$; 10mM $K3[Fe^{III}(CN)_6]$; 0.3% Triton X-100.
Ethanol series: 30%, 50%, 70% and 100%.
G.M.M.: 2g/ml Canada balsam in methyl salicylate.

Method.

Imaginal discs were dissected out of well fed 3rd instar larvae in PBT and then transferred to fixative for 15 to 20 minutes. During the fixation 25 µl X-gal was added to 1 ml staining solution (preheated) and then incubated at 37°C. The fix was then removed and the discs were washed 3 times for 2 minutes in PBT. The discs were then transferred to the staining solution and incubated until the colour developed. The colour reaction was stopped by washing several times in PBT.

Mounting.

The discs were taken through an ethanol series (3 minutes in each of 50%, 70%, 90%, and 3X in 100% ethanol). They were then transferred to a glass slide and mounted in G.M.M.

2.17 Cuticle preparations of first instar larvae.

Clearing solution: Lactic acid : 70% Ethanol (9:1)
Hoyers mountant.

The unhatched larvae were collected at least twenty four hours after being laid. They were hand dechorionated by placing onto double sided sticky tape and removing with a sharp pair of forceps. The procedure was repeated but this time under a drop of PBS to devitellinise the larvae. The larvae were transferred to clearing solution and left at 55°C to clear overnight. The larvae were then transfered to a drop of Hoyers mount on a slide and a coverslip was placed on top.

2.18 Miscellaneous

2.18.1 Maintenance of fly stocks.

Fly stocks were maintained at either 25°C or 18°C in vials or bottles of food prepared as described below.

2.18.2 Fly food

Flies were generally maintained on standard food. On occasions where large numbers of flies were required the parents were allowed to lay on rich food. This significantly increased the number of hatching offspring.

Materials.

Standard food;
To make 1 litre of standard food;
Maize meal: 104g.
Sugar (granulated): 94g.
Agar: 5.5g.
Yeast: 18.5g.
Nipagin: 15 mls (10% in ethanol).
H_2O: To make volume to 1 litre.

Rich food;
To make 1 litre of rich food;
Yeast: 100g.
D-Glucose: 100g.
Agar: 20g.
Nipagin: 15 mls (10% in ethanol).
H_2O: To make volume to 1 litre.

Method.

Both standard and rich food were made in the same way. The yeast was dissolved in the water and then transferred to a large pan. The maize, sugar and agar were then added and the mixture was brought to the boil whilst stirring continuously. The food was then simmered for 10 minutes. The nipagin was added and the pan was kept on a low heat whilst it was poured into either plastic vials or bottles. The food was allowed to cool (protected by muslin) and then live yeast was dropped onto the surface of the food and the bottles and vials were stoppered with cotton wool bungs.

2.18.3 Grape juice agar plates.

Grape juice agar plates were used to collect embryos for either in situ hybridisations, antibody labellings or detection of β-galactosidase activity.

Materials.
To make 1 litre of Grape juice agar;
Grape Juice: 50 mls.
Sugar: 80g.
Agar: 25g.
Water: to make volume up to 1 litre.

Method

All the materials were combined in a large saucepan and then heated until the solution boiled. The solution was cooled a little then poured into tissue culture dishes (5.5cm diameter). Once set plates were stored at 4°C until required. Before use the plates were allowed to reach room temperature, and a small amount of moist yeast was added to the plate.

2.18.4 Subbed slides.

Materials.
Gelatin
Chrome alum
Glass slides

Method

Glass slides were washed thoroughly in detergent and then three times in water. Gelatin was then dissolved to 0.1% in hot water. When this solution had cooled chrome alum was added to 0.1% and the slides were dipped into the subbing solution and then allowed to air-dry.

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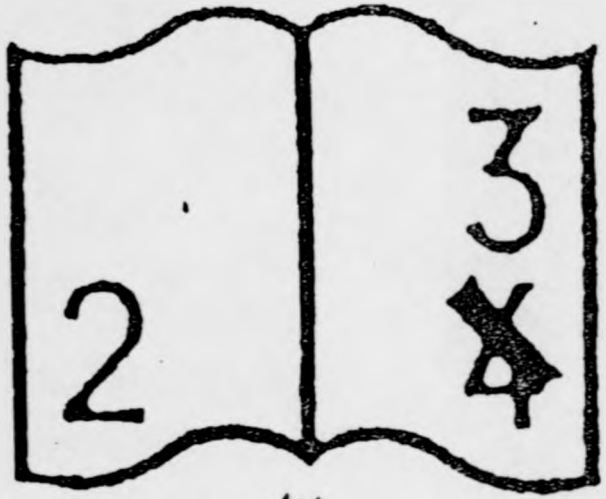

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Chapter 3

Initial characterisation of the P[lArB] insertion A350.1M2.

3.1 Introduction

The temporal and spatial pattern in which a gene is expressed is often indicative of its function. One example is the gene *twist* which is expressed at the beginning of gastrulation in a ventral band of cells. These are the cells that invaginate during formation of the ventral furrow that will go on to form the mesoderm. In embryos lacking the *twist* gene function gastrulation fails. The *twist* gene product is a helix-loop-helix transcription factor and remains active in the mesoderm cells until they begin to differentiate and may be required to specify mesoderm (Thisse *et al.*, 1988). As mentioned in Chapter 1 *bicoid* is required for anterior patterning and its mRNA is localised in the anterior pole of the embryo (Frigerio *et al.*, 1986). Likewise the *nanos* gene product is required for abdominal development and is localised in the posterior pole of the embryo (Wang and Lehmann, 1991). The products of all these genes are localised in a temporally and spatially restricted manner that is related to their function. There are exceptions however. The gene *patched* is expressed in a spatially restricted pattern that never includes the posterior compartment of each segment in the Drosophila embryo. The phenotype of *patched* mutations is the loss of the middle region of each segment (Nakano *et al.*, 1988). Ubiquitous expression of *patched* under the control of a heat shock promoter in the embryo has no obvious phenotype, suggesting that its localised expression is not essential for its function (Sampedro and Guerrero, 1991). Another exception is the gene *torso*, its product is localised ubiquitously in the blastoderm embryo, but is required only in the terminal regions where it is activated (Klingler *et al.*, 1988).

It should be noted that genes required for setting up the body plan are expressed very early during embryogenesis and that genes which elaborate this plan are expressed slightly later. The gap genes are expressed in broad overlapping domains along the anterior -posterior axis and embryos mutant for these genes

develop with large chunks of the body pattern missing. The gap genes regulate the expression domains of each other and then later define the positions of the pair rule genes.

Some genes have multiple functions; for example, *ftz* has an early role in segmentation, and is expressed later in specific subsets of neurons in the central nervous system. In *ftz* mutants these particular neurons are defective (Doe *et al.*, 1988).

On the basis of its temporal and spatial pattern of expression, I therefore decided that P[lArB] insertion A350.1M2 (originally reported by Bellen *et al.*, 1989) was worthy of further investigation. The most interesting features of the expression pattern in this line are 1) the expression is very early, during gastrulation in the amnioproctodeal invagination and in the cells of the anterior midgut and the cephalic epidermis and 2) the later expression is in most of the cells of the ventral nerve cord, and in the epidermis of the hypopharyngeal and gnathal segments of the head.

Before undertaking more extensive work, however, it was necessary to determine whether the insertion was likely to be in a gene already known to be involved in head, gut or nervous system development. This can be performed by mapping the P-element insertion on the cytological map of the *Drosophila* genome. The known mutations around this region can then be tested for complementation with any mutations caused by insertion or excision of the P-element.

Genomic insertion of P-elements has previously been used as a form of mutagenesis (reviewed by Cooley *et al.*, 1988), so it is reasonable to test whether the P-element insertion has disrupted a gene adjacent to the enhancer that it has detected. Individual flies homozygous or hemizygous (i.e. heterozygous over a deficiency in the region) for the P-element insertion can be checked for a phenotype. Homozygous or hemizygous individuals may not be present if the insertion is in a gene that is essential for viability. If such individuals are present then one possibility is that the gene near which the P-element has inserted is essential, but the P-element has not inactivated it.

If the P-element insertion line has a phenotype, it is then necessary to determine whether any phenotype observed is associated with the P-element insertion, as opposed to an unrelated mutation on the same chromosome. Excisions of the P-element can be made using transposase-mediated excision (Robertson *et al.*, 1988). If the insert excised precisely (or nearly precisely) then the mutant phenotype should be reverted to wild-type if it has been caused by the insertion.

Here I shall explain precise excision of transposons from the genome of *Drosophila*. Later in Chapter Five imprecise excisions of P-elements to generate small genomic deletions will be discussed.

The method employed to excise P-elements from genomic DNA is by transposase mediated excision. Fortunately a stable source of transposase is available in *Drosophila*, the defective P-element P[ry$^+$ Δ 2.3](99B) (Robertson *et al.*, 1988). The P[ry$^+$ Δ 2.3](99B) element produces transposase but can not excise. Presence of the P[ry$^+$ Δ 2.3](99B) element in a fly line also containing a P-element results in transposition and loss of the P-element from some of its cells. In the germ-line of males this event will result in the production of sperm lacking the element. The P-element is marked in the case of P[lArB] with the *rosy*$^+$ gene and reversion of this to a *rosy*$^-$ phenotype can be used to screen for loss of the element.

A model of the molecular mechanism of these excision events has been proposed; the gap repair model of P transposition. When the P-element is excised it produces a double-strand break, which is widened to varying degrees by exonuclease activity. The gap is shown with 3' overhangs (Fig. 3.1b). The broken ends and the complementary template find each other, and strand invasion is initiated at the overhanging 3' ends (Fig. 3.1c). Polymerisation occurs from both broken ends filling in the gap and leaving an intermediate structure which has two Holliday junctions (Fig. 3.1d). This intermediate structure is resolved and the result is a noncrossover product in which the template duplex is unchanged (Fig. 3.1e) (for review, see Gloor *et al.*, 1991).

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If the insertion causes no obvious phenotype, then it may not have inactivated the adjacent gene; 20% of insertions on the third chromosome cause lethality, 5% of insertions on the X cause lethality (Bellen *et al.*, 1989). However, mutations in genes flanking the insertion can be generated by imprecise excision of flanking DNA. Imprecise excision events result in the removal of flanking genomic DNA along with the P-element. The P-element can be excised given a transposase source, these excision events can be tested for imprecise events by checking for loss of viability over a deficiency in the region.

3.2 P[lArB] insertion A350.1M2.

The ß-galactosidase expression pattern of A350.1M2 detected with anti-ß-galactosidase antibody and horse radish peroxidase.

3.2.1 Embryonic expression pattern.

Embryos were staged according to the description of development of wildtype *Drosophila* embryos (Campos-Ortega and Hartenstein, 1985).

The first expression can be seen during early gastrulation, stage 7, (3 - 3:10 hrs). After formation of the ventral furrow, cells of the amnioproctodeal invagination can be seen to express ß-galactosidase (Fig. 3.2.1b). As the cell plate deepens during stage 8 (3:10 - 3:40 hrs), the expression becomes more extensive in the cells of the primordia of the posterior midgut. In the anterior region of the embryo expression can be seen in the cephalic ectoderm and anterior midgut (Fig. 3.2.1c and d). By stage 11 (5:20 - 7:20 hrs) the expression can be seen in the cells of the posterior midgut, Malpighian tubules and proctodeum. Expression can also be seen in the region of the anterior midgut (Fig.3.2.1e). Many cells of the ventral nerve cord can be seen to be expressing ß-galactosidase in a segmentally repeated pattern. Expression is also present in the cephalic neurogenic region. The expression is strong in the epidermis of the three gnathal segments of the head (mandibular, maxillary, and labial) and in the hypopharyngeal lobe (Fig. 3.2.1f). After germ band shortening during stage 12 (7:20 - 9:20 hrs), expression is seen in much of the head epidermis (except the clypeolabrum), and in a large number of cells of the central nervous system. Expression can still be

seen in the posterior midgut, Malpighian tubules and the proctodeum and is also visible in the larval salivary glands (Fig. 3.2.1g).The dorsal view of the stage 13 (9:20 - 10:20 hrs) embryo shows staining in the Malpighian tubules (Fig. 3.2.1h).

3.2.2 Larval expression pattern.

ß-galactosidase expression is present in the larval brain in the brain lobes and in the ventral nerve cord (Fig. 3.2.2a). Only one of the larval discs shows any ß-galactosidase expression and that is the dorsal prothoracic discs which are located at the anterior-most end of the trachea (Fig. 3.2.2b). This larval disc gives rise to the humerous a small plate on the anterior corner of the notum of the adult fly.

3.2.3 Adult expression

In the central nervous system a small amount of expression is found in a few cells of the adult brain lobes and in a striped pattern in the ventral nerve cord (Fig. 3.2.3a). The nuclear localisation of the *lac*Z gene product makes it impossible to visualise the shapes of these cells, and hence it is unclear what types of cell these are.

Other adult structures include the pericardial cells of the heart (Fig. 3.2.3b) and a structure at the posterior end of the gut (Fig. 3.2.3c) which both contain endogenous ß-galactosidase activity.

3.3 A350.1M2 maps to cytological position 59F

A clone of genomic DNA flanking the A350.1M2 insertion was obtained from Dr. Clive Wilson. This clone had previously been used to localise the insertion to 59E-F (Wilson *et al.*, 1989). A biotin-labelled probe was made from this plasmid rescue clone which was then hybridised to wild-type *Drosophila* polytene chromosomes. Detection of the probe revealed a unique signal at cytological band 59F1-3 as shown in Figure 3.3.1.

3.4 The A350.1M2 insertion is associated with hemizygous lethality.

It was not possible to tell directly whether the A350.1M2 insertion was homozygous lethal because it is on the CyO balancer chromosome, which itself is homozygous lethal. However given the knowledge of the cytological map position of the insertion, one can test for hemizygous lethality by crossing the insertion chromosome to a chromosome carrying a deficiency which is thought to cover the insertion site. The A350.1M2 chromosome was tested over Df(2R)bw^{S46} (Simpson, 1983), which has the breakpoints -59D8; 60A7 (Fig. 3.4.1). From the results of the cross to Df(2R)bw^{S46}, the A350.1M2 chromosome has a lethal in the region of the deficiency, but is this lethality due to the insertion of P[lArB]? This can be tested by precise excision of the P-element to ask whether excision reverts the chromosome to viability.

3.5 The A350.1M2 insertion causes hemizygous lethality.

The crossing scheme used to excise the A350.1M2 insertion and results are shown in (Fig. 3.5.1). In the F zero generation flies containing the insertion to be excised (A350.1M2) were crossed to flies containing the P[ry^+ Δ 2.3](99B) element. From the F1 generation flies containing both the A350.1M2 insertion and the P[ry^+ Δ 2.3](99B) element (jumpstart males) were selected. These jumpstart males were crossed singly to ry^- virgins, and flies from the F2 generation were selected that had lost the transposase-producing chromosome and also were lacking at least the ry^+ marker of A350.1M2. These flies (a single progeny fly of each jump-start male) were then crossed to the deficiency Df(2R)bw^{S46} to test for viability of the excised chromosome over the deficiency.

After excision of P[lArB] the CyO A350.1M2 chromosome is reverted to viability over deficiency Df(2R)bw^{S46}. The P-element insertion A350.1M2 had therefore produced the lethality, suggesting that the gene it has disrupted is essential for viability.

3.6 Determination of the lethal phase of the genotype A350.1M2/ Df(2R)bwS46 .

The hatch frequency of embryos from the cross shown in Figure 3.6.1 was determined by lining up fertilized eggs on a grape juice plate, leaving for twenty four hours at 25°C, and then counting the number of unhatched eggs. A quarter of the progeny of this cross will be CyO A350.1M2/ CyO and are embryonic lethal. If 75% of the embryos hatch then CyO A350.1M2/ Df(2R)bwS46 individuals are not embryonic lethal. If 50% of the embryos hatch then A350.1M2/ Df(2R)bwS46 embryos are embryonic lethal. The results in Figure 3.6.1 show that 29% of the embryos did not hatch, i.e. 69% of the embryos hatched. This suggests that A350.1M2/ Df(2R)bwS46 do not die during embryogenesis. In control experiments with wildtype embryos >90% of fertilised eggs hatch.

3.7 P[ClrB] insertion D26.

The P[ClrB] construct is essentially similar to the P[lArB] detector transposon except that it carries a truncated P-*lacZ* fusion gene. The predicted protein from this fusion has the first two amino acids of P-transposase fused to the *lacZ* product. The nuclear localisation of the fusion product in P[lArB] was thought to be due to the presence of the N-terminal 126 amino acids of the P-transposase, the removal of this region results in cytoplasmic localisation of the ß-galactosidase in P[ClrB]. Eighty eight independent lines were generated and the ß-galactosidase expression pattern of these lines were examined (Smith and O'Kane, 1991).

3.8 ß-galactosidase expression pattern of P[ClrB] line D26.

The ß-galactosidase expression pattern in this line is similar to that of the A350.1M2 insertion during embryogenesis. In early gastrulating embryos expression can be seen in the cells of the amnioproctodeal invagination and in the head ectoderm. Once the germ band has elongated expression occurs in a segmentally repeated pattern in the ventral nerve cord; this expression is particularly strong in the epidermis

of the gnathal and hypopharyngeal segments of the head, a characteristic of the A350.1M2 expression pattern (Fig. 3.8).

3.9 D26 maps at cytological position 59F and is homozygous and hemizygous viable.

D26 has been shown to map to the same cytological position on wildtype polytene chromosomes as the A350.1M2 insertion, at cytological band 59F (H. Smith, pers. comm.). The D26 chromosome is homozygous viable showing that the insertion has not disrupted the flanking gene. The D26 insertion was tested for viability over the Df(2R)bw^{S46} by looking at the progeny of the cross shown in Fig. 3.9.1. The results show that D26 insertion is viable over Df(2R)bw^{S46}.

3.10 Discussion

3.10.1 A350.1M2

This line is interesting because the expression of ß-galactosidase in this line occurs in a spatially restricted manner early during embryogenesis. The expression pattern of this line is shown diagrammatically in Figure 3.10. Genes expressed this early during development are good candidates to be involved in determining cell fate decisions. The expression pattern of the P-*lacZ* insertion suggests that any adjacent gene with a similar expression pattern might be involved in determination of the posterior gut, the head or the CNS. The fact that the A350.1M2 insertion causes hemizygous lethality shows that it has disrupted (at least partially) an adjacent gene, which is essential for development. For a P-element insertion to disrupt the function of a gene it must insert within the coding region of the gene (structural gene), in the regulatory elements or in such a place that it separates the coding region from its control elements.

3.10.2 D26

The expression pattern and map position of this insertion suggests that it is probably near the same essential gene which is disrupted by the A350.1M2 insertion. The D26 chromosome is homozygous viable and is also hemizygous viable over $Df(2R)bw^{S46}$.

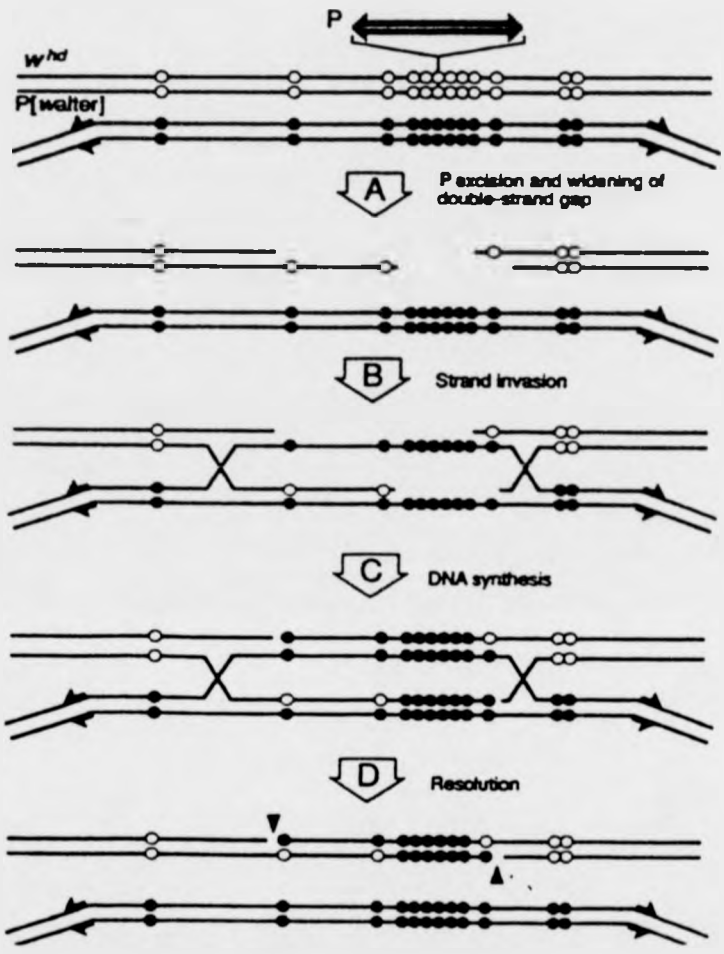

Figure 3.1 The Gap repair model.
A: P-element excision leaves a double-strand gap, which is widened to various extents by exonuclease activity. The gap is shown with 3' overhangs.
B: The broken ends and the template find one another, then strand invasion occurs at the overhanging 3' ends.
C: Polymerisation occurs from both broken ends filling in the gap and leaving an intermediate structure with two Holliday junctions.
D: The double-Holliday junction intermediate is resolved, resulting in a noncrossover product in which the template duplex is unchanged. The repaired duplex has a central region of converted sites flanked by heteroduplex regions and single strand nicks. The nicks are ligated.

Figure 3.2.1
The embryonic ß-galactosidase expression pattern of P[lArB] insertion A350.1M2.

A-stage 5 (2:10 - 2:50hrs)
At cellularisation there is no expression.

B-stage 7 (3 - 3:10hrs)
At the onset of germ band extension, expression can first be seen in the cells of the amnioproctodeal invagination.

C-stage 8 (3:10 - 3:40hrs)
This is the rapid phase of germ band extension, expression can be clearly seen in the primordia of the posterior midgut and the proctodeum. Expression is now apparent in the region of the anterior ectoderm and the anterior midgut primordium.

D-stage 8 (Dorsal view)
The anterior midgut invagination can be seen to be faintly expressing ß-galactosidase (ring of cells).

E-stage 11 (5:20 - 7:20hrs)
Expression can be seen in a segmentally repeated pattern in the cells of the ventral nerve cord. Cells in the cephalic neurogenic region also strongly express ß-galactosidase. Staining is still intense in the posterior midgut and proctodeum. Expression is strong in the stomodeum and faint in the anterior midgut.

F-stage11 (5:20 - 7:20hrs)
The expression is most intense in the epidermis of the three gnathal segments (mandibular, maxillary and labial) and also in the hypopharyngeal lobe. In the maxillary and labial segments the expression is mainly in the ventral half of each segment. The salivary glands invaginate from the labial segment.

G-late stage 12 (9hrs)
Expression can be seen in most of the cells of the ventral nerve cord, cephalic neurogenic region and most of the head epidermis except the clypeolabrum. At the anterior the salivary glands are expressing ß-galactosidase. The posterior midgut, Malpighian tubules and proctodeum are still expressing ß-galactosidase.

H-stage 13 (9:20 - 10:20hrs; Dorsal view)
This view shows the expression of ß-galactosidase in the embryonic Malpighian tubules.

Embryos are orientated anterior left, posterior right. Lateral views are dorsal top, ventral bottom, except where stated otherwise.
Abbreviations: am; anterior midgut primordium, **api**; amnioproctodeal invagination, **ce**; cephalic ectoderm,**CNR**; cephalic neurogenic region, **hp**; hypopharyngeal lobe, **lb**; labial bud, **md**; mandibular bud, **mt**; malpighian tubules, **mx**; maxillary bud, **pm**; posterior midgut primordium, **pr**; proctodeum, **sg**; salivary glands, **VNC**; ventral nerve cord, **B**;brain.

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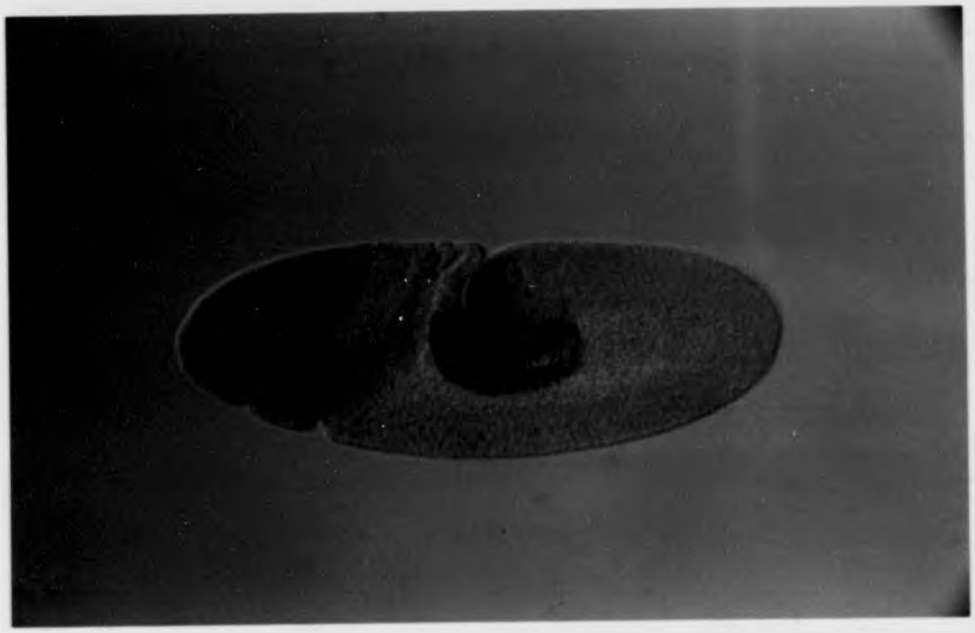


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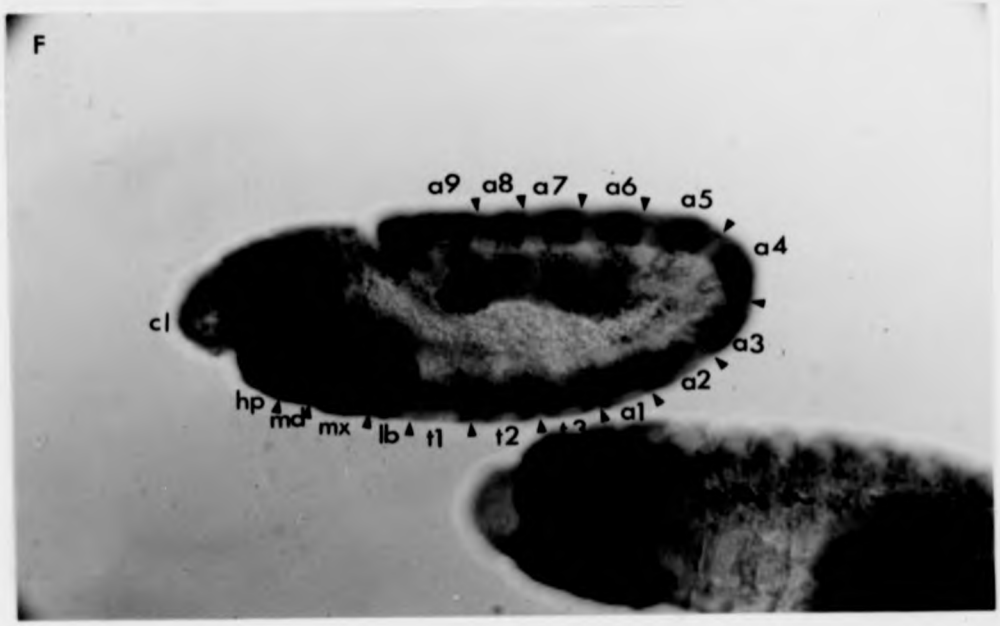
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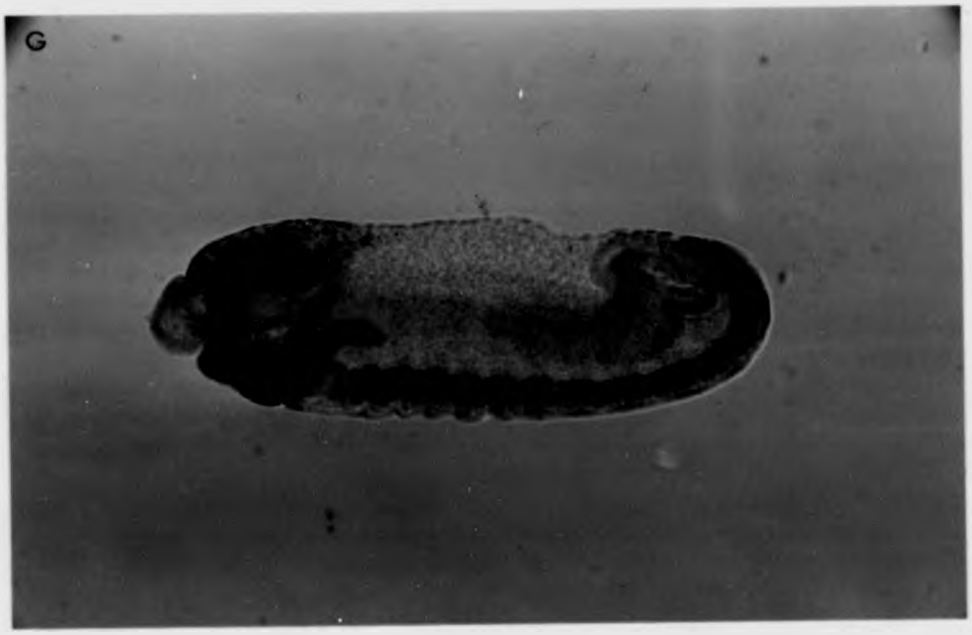
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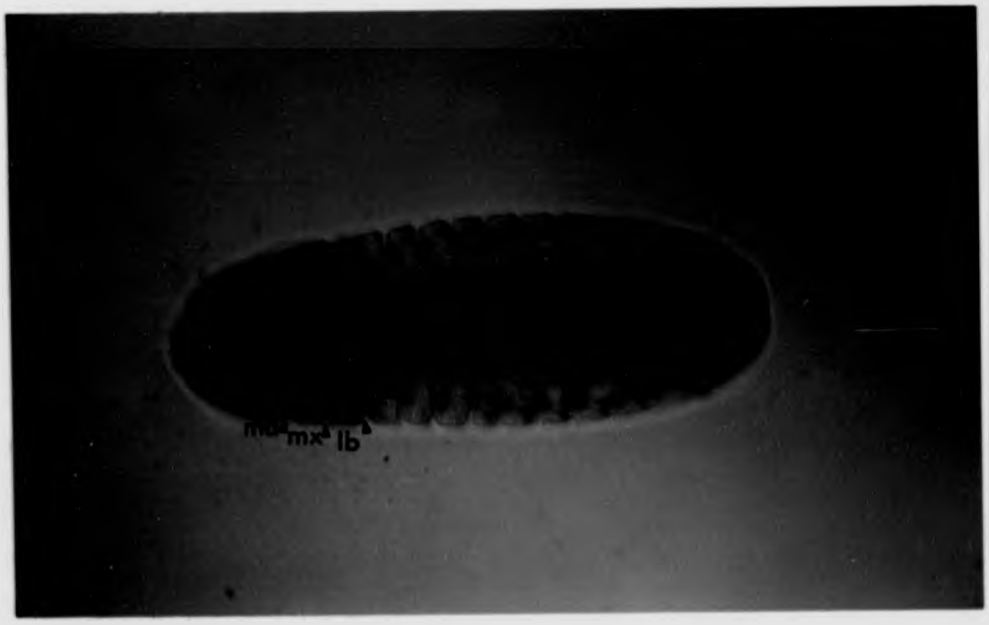
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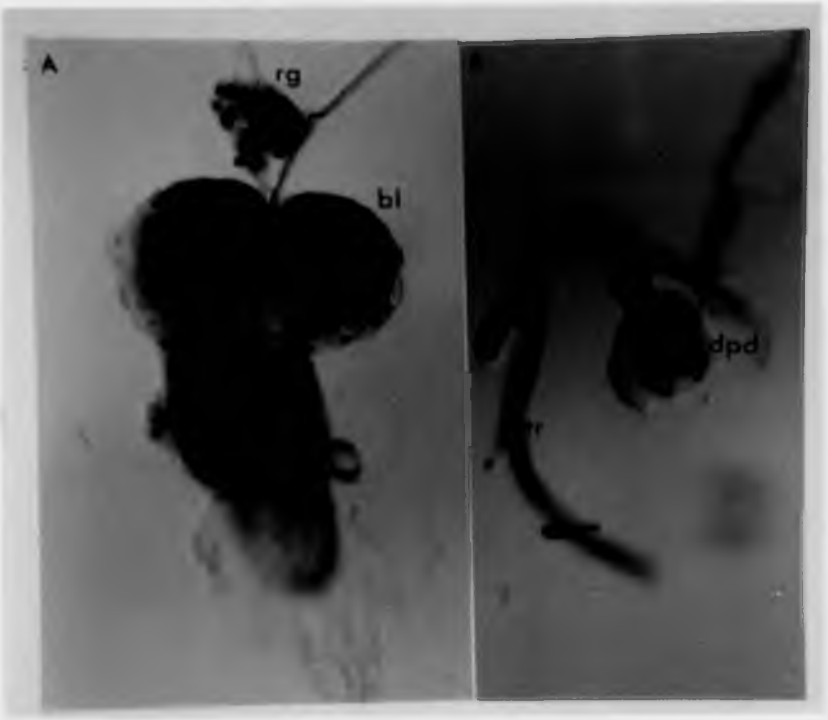


Figure 3.2.2 The ß-galactosidase expression pattern in the third instar larvae of line A350.1M2.

A- Larval CNS, A number of cells in the brain lobes and the ventral nerve cord can be seen to be expressing ß-galactosidase.

B- The prothoracic disc is the only disc to express ß-galactosidase in the third instar larva.

abbreviations: **rg**; ring gland, **bl**; brain lobes, **dpd**; dorsal prothoracic disc, **tr**; trachea.

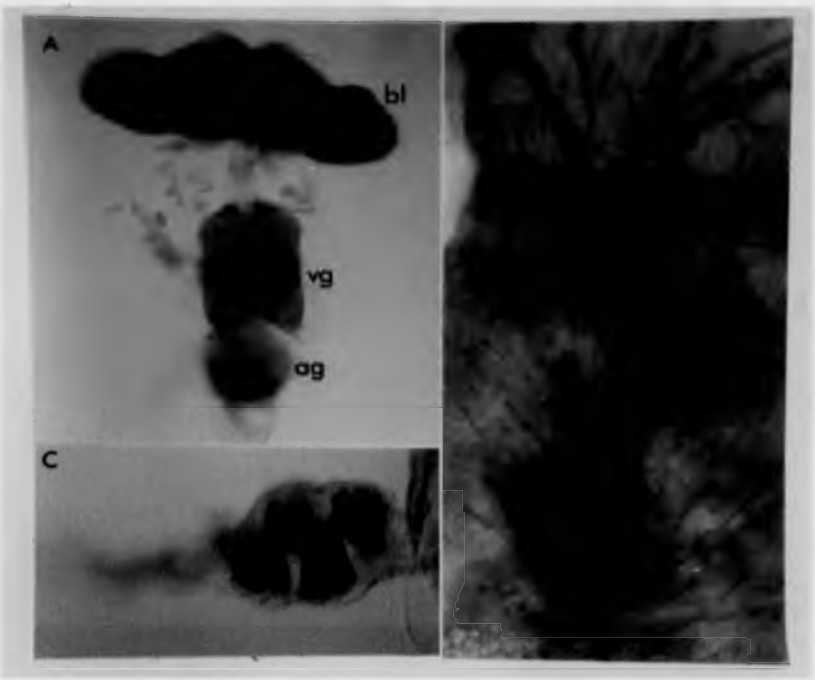


Figure 3.2.3 ß-galactosidase expression pattern in the adult fly of A350.1M2 heterozygotes.

A- The adult CNS, staining can be seen in a small number of cells in the brain lobes and in a striped pattern in the thoracic ganglion (anterior top, posterior bottom).

B- Some of the pericardial cells of the heart express ß-galactosidase (anterior top, posterior bottom). This is due to endogenous ß-galactosidase activity in these cells.

C- Part of the male testis (anterior left, posterior right). This is also due to endogenous ß-galactosidase activity in this structure.

abbreviations **bl**, brain lobes, **vg**, ventral ganglion, **ag**, abdominal ganglion.

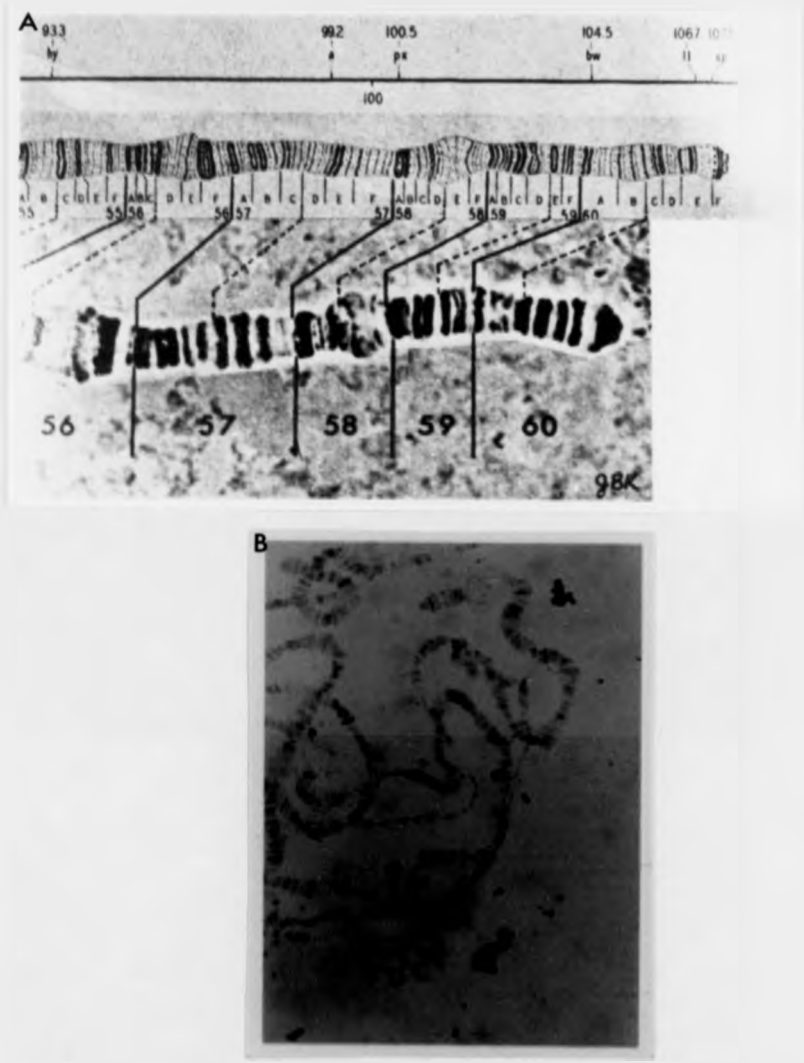
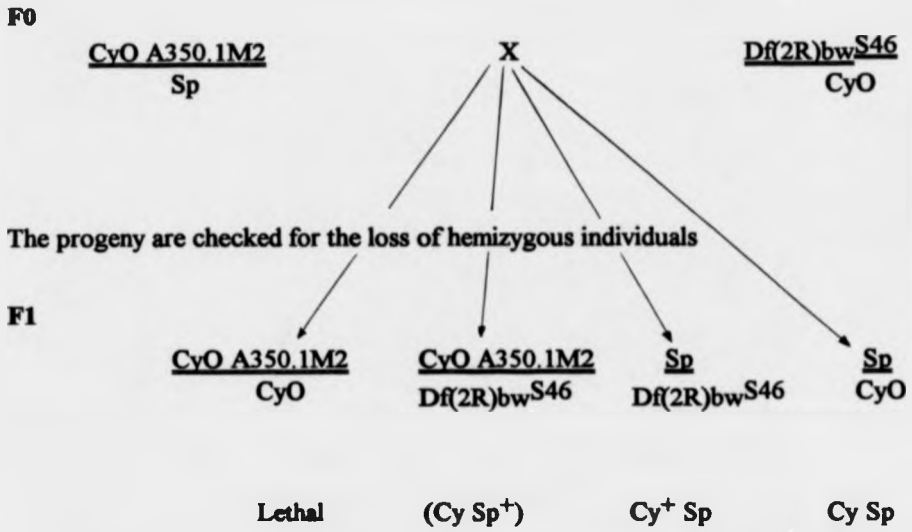


Figure 3.3.1 Cytological map position of A350.1M2 P-element insertion.

A; The cytological map of Lefevre (1976). B: The A350.1M2 plasmid rescue clone was hybridised to wild-type *Drosophila* polytene chromosomes. The signal can be seen at cytological bands 59F1-3.

Flies carrying the insertion of interest are crossed to a Deficiency in the region.

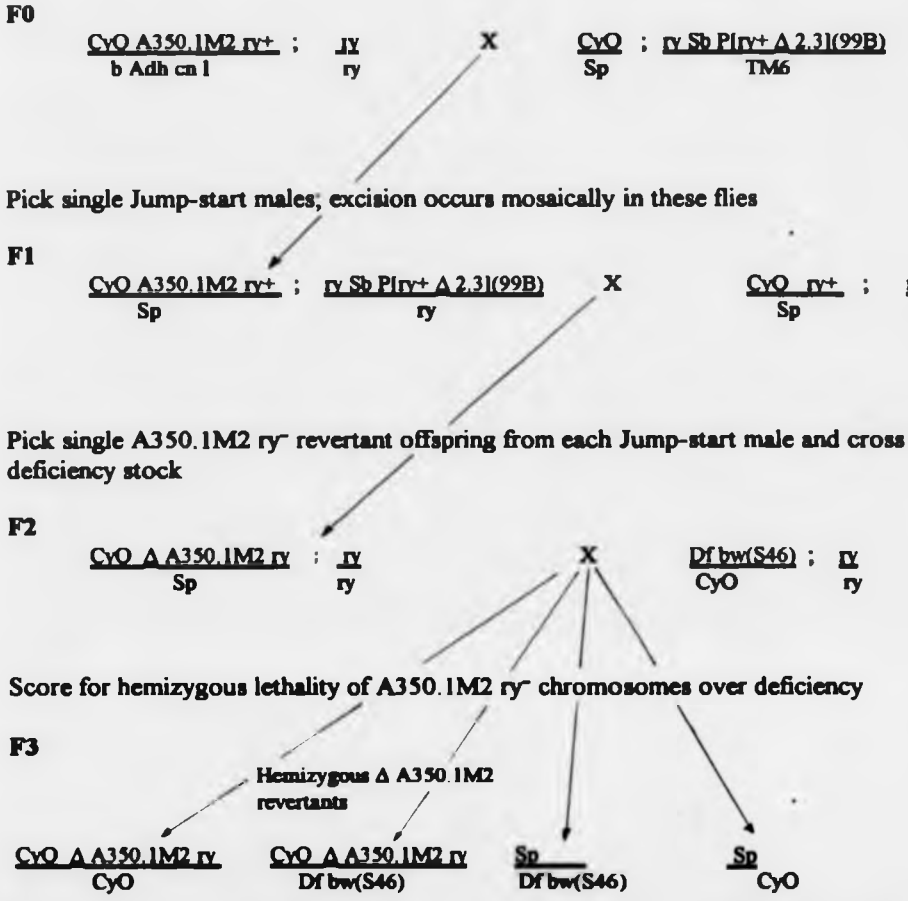


	Number of flies counted			
#1	none	none	136	143
#2	none	none	192	178
#3	none	none	206	198

Figure 3.4.1 Test for hemizygous lethality of the A350.1M2 insertion over Df(2R)bwS46.

There were no Cy Sp^+ progeny, indicating that the A350.1M2 insertion chromosome is lethal over Df(2R)bwS46.

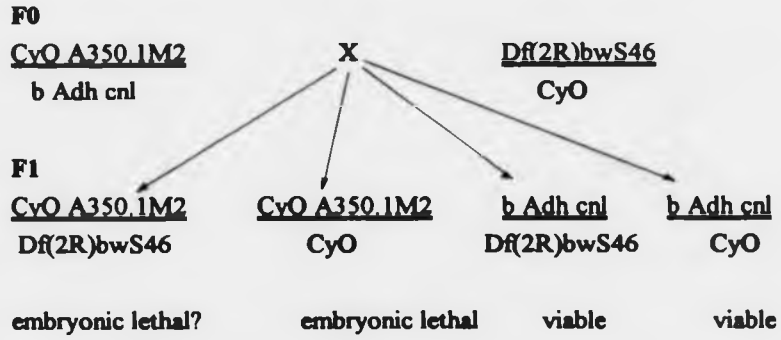
Cross A350.1M2 females to transposase-producing stock.



Lethal	Cy, Sp^+	Cy^+, Sp	Cy, Sp	Phenotype
none	20	18	20	#1
none	20	18	30	#2
none	16	17	14	#3
none	12	17	16	#4
none	6	9	13	#5
none	19	18	19	#6

Figure 3.5.1. Precise excision of A350.1M2

Cy Sp^+ flies were present in the offspring showing that CyO Δ A350.1M2 is viable over Df(2R)bw S46. This shows that the P-insertion must have caused the lethality since the chromosome is viable when the element is excised.



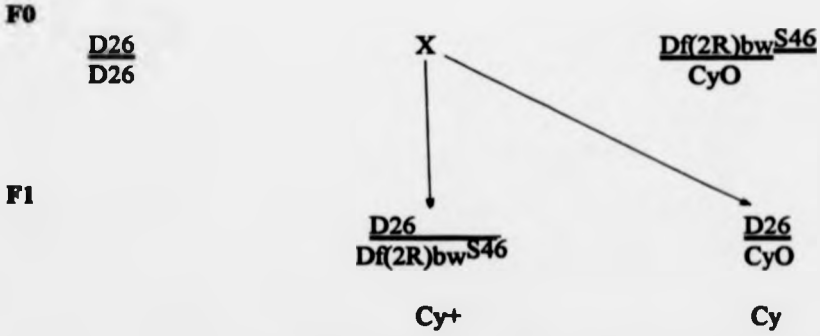
total number of embryos	number of unhatched embryos	% of unhatched embryos
400	115	29

3.6.1. Test for embryonic lethality of the genotype A350.1M2/ Df(2R)bw^{S46}.

25% of the offspring are CyO A350.1M2/ CyO and die as embryos. 71% of the embryos hatched indicating that A350.1M2/ Df(2R)bwS46 individuals are not embryonic lethal.

Figure 3.8 The embryonic ß-galactosidase expression pattern of P[ClrB] insertion D26.

Germ-band extended embryo stained with antibodies to β-galactosidase, expression can be seen to be in a segmentally repeated pattern in the ventral nerve cord. The expression is strong in the epidermis of the gnathal segments of the embryonic head. This is a characteristic of the ß-galactosidase expression pattern of the A350.1M2 P[lArB] insertion line.



	Number of flies counted	
#1	137	158
#2	89	102
#3	97	85

Figure 3.9.1 Test to determine whether D26 insertion is viable over Df(2R)bw^{S46}.

The presence of non Cy flies in the F1 progeny indicates that the D26 insertion chromosome is viable over Df(2R)bwS46.

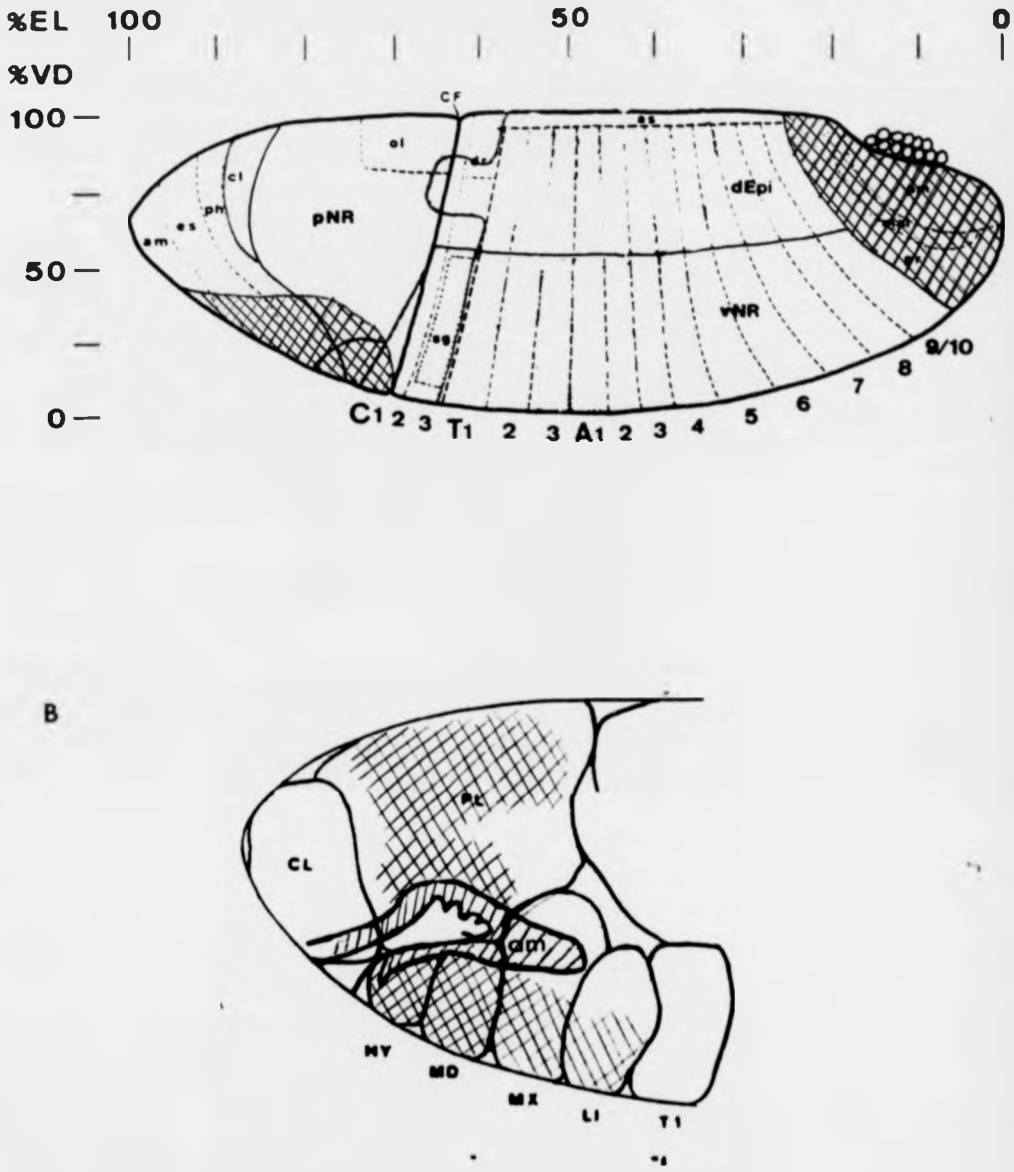


Figure 3.10 Diagrammatic representation of the ß-galactosidase expression in early A350.1M2 embryos. (Fate maps adapted from (Jurgens *et al.*, 1987).

A: Expression domains have been drawn onto the fate map at the gastrula stage.

B: Expression in the head region at germ band extension.

Chapter 4

Molecular characterisation of the genomic region flanking the A350.1M2 insertion.

4.1 Introduction.

In this chapter I describe the strategy taken to molecularly clone a gene that flanks the A350.1M2 enhancer detector transposon and has a similar expression pattern to it. Molecular cloning of a gene requires the identification of transcriptional units in the genomic region, to enable the characterisation of these transcripts to be performed. Characterisation of the transcript can provide information about any protein encoded by this gene and information about possible functions of the gene.

The P[lArB] transposon contains Bluescript plasmid sequences allowing plasmid rescue of flanking genomic sequences. Cloning of genomic DNA is facilitated by the presence of a polylinker, (PL3) in the vector, which contains a number of restriction sites which are not found downstream in the construct (Fig. 4.1.1.) Consequently the next site downstream will be in the genomic sequences. After restriction enzyme digestion to produce linear genomic fragments and ligation to recircularise them, these fragments can be transformed into competent *E.coli* cells. Checking that the transformants are in fact plasmid rescue clones can be done both by restriction mapping and by labelling the insert DNA with biotin and hybridising to wildtype polytene chromosomes.

Restriction mapping involves digesting the DNA with a large range of enzymes and then sizing the resulting fragments by electrophoresis. The fragments can be ordered by comparing fragment sizes produced by different combinations of digests using two enzymes. Restriction mapping of putative plasmid rescue clones indicates which clones are likely to be real plasmid rescue clones on the basis of the sizes of the fragments produced. A digest which liberates the vector from insert is useful since the size of the vector band is known, and from this digest the size of the insert of genomic DNA can also be calculated. Producing a restriction map is useful as it allows

subsequent selection of smaller genomic fragments which can be used for further experiments.

Hybridisation of the clone to polytene chromosomes shows that the clone contains only unique DNA sequences and also shows the cytological map position of the clone.

If more genomic DNA than that contained in the plasmid rescue clone is required, it can be obtained by screening a genomic DNA library using the plasmid rescue clone as a probe. If the region is already well characterised, a genomic walk from a nearby gene with clones covering the region may already exist. Once genomic clones covering the region are obtained they can also be restriction mapped.

When clones of DNA flanking the insertion are available and have been restriction mapped, it is then necessary to identify transcribed sequences in this region. These transcripts can then be tested for an expression pattern resembling that of the P-*lacZ* insert, and to identify and characterise cDNA clones in order to determine the nature of any gene product encoded by appropriate transcripts.

cDNA clones are DNA clones made by reverse transcribing mRNA molecules and they therefore represent potential protein coding sequences. Screening a cDNA library with genomic DNA fragments will therefore yield cDNA clones derived from mRNAs transcribed from those fragments. The difficulty of this depends on the abundance of the relevant cDNA clones in the library. Any cDNA clones recovered can then be mapped and orientated with respect to the genomic walk. Whole mount in situ hybridisation to *Drosophila* embryos using the cDNA as a probe detects the developmental expression pattern of the transcript. This pattern can then be compared to the original P-*lacZ* expression pattern the line was selected for. The sequence of the cDNA clone provides information regarding any protein encoded by the transcript, and may predict a possible function for such a protein.

4.2 Isolation of genomic DNA flanking the A350.1M2 insertion, and the production of a restriction map.

Genomic DNA flanking the 3' end of the insertion had previously been obtained by plasmid rescue (Wilson et al., 1989). The plasmid rescue clone was generated from a *Hin*dIII digest of A350.1M2 genomic DNA, and contained 11kb of DNA 3' to the insertion. This clone was digested with a range of enzymes and the resulting fragments were sized by electrophoresis to generate a restriction map. The restriction sites in PL3 and PL4 of the plasmid rescue clone are shown in Figure 4.1.1. The gels used to size these fragments are shown in Fig. 4.2.1 and this information is summarised in Table 4.2.2. The restriction map and the rationale behind the mapping is presented in Fig. 4.2.3. Genomic DNA 5' to the insertion was obtained from a genomic cosmid clone from the genomic walk covering the region of the *brown* gene (Dreesen et al., 1988). This walk extends approximately from 59D/E-59F1, so there was a possibility that it contained genomic DNA from the region of the A350.1M2 insertion at 59F. A Southern blot of *Eco*RI and *Xho*I digests of cosmid clone cPn31 was probed with the radioactively labelled *Xho*I-*Not*I fragment from the plasmid rescue clone. Cross hybridisation to the large 12kb XhoI fragment was seen (data not shown). The cosmid fragments were ordered by probing *Eco*RI and *Xho*I digests of cPn31, cPn124 and the plasmid rescue clone with this 12kb *Xho*I fragment of cPn31. The gel showing digests of cPn31 and cPn124, and the sizes of these fragments are shown in Fig. 4.2.4. and Table 4.2.5. A restriction map of the cosmid vector cosPneo is shown in Figure 4.1.1.

From the data of Dreesen and co-workers I have positioned the plasmid rescue clone with respect to the *brown* walk as approximately 50kb to the right of the *brown* gene on chromosome 2R. Dreesen (personal communication) has also placed cPn124 as being to the left of cPn31, and overlapping with cPn 722 and cPn121. As I have found that cPn124 overlaps only with the region of the A350.1M2 clone nearest the P-insertion, whereas cPn31 probably covers the entire clone. This has allowed me to orientate the restriction map with respect to the polytene map (Fig.4.2.3.).

4.3 Isolating cDNA clones from the region adjacent to the A350.1M2 insert.

Before fragments from the genomic region described were used for further experiments it was first necessary to check that they did not contain repetitive sequences. The *Not*I-*Hin*dIII fragment from the plasmid rescue clone, fragment a) and the 12kb *Xho*I fragment from cPn31, fragment b) (these fragments are shown in Fig. 4.2.3) were labelled with biotin by the method of random priming, and hybridised to wildtype polytene chromosomes. The result for the *Not*I-*Hind*III fragment of the A350.1M2 plasmid rescue clone is shown in the previous chapter. Both fragments gave a single signal at cytological position 59F indicating that these fragments contained only unique sequences (Fig. 4.3.1 shows *Xho*I fragment).

Before screening the cDNA library I made numerous attempts to size any transcript that might be encoded by the *Not*I-*Hin*dIII plasmid rescue clone by Northern analysis. Although my Northern blots were not of the highest quality, they did suggest that the plasmid rescue clone was transcriptionally active (Fig. 4.3.2).

Given the evidence for a transcript within the *Not*I-*Hin*dIII plasmid rescue clone, I decided to screen for cDNA clones derived from this genomic fragment. The Nicholas Brown embryonic cDNA libraries (Brown and Kafatos,1988), were available in our laboratory and because of the early ß-galactosidase expression (3 hrs onwards) in the A350.1M2 P-*lac*Z insertion line, I chose to screen the 4-8hr embryonic library. The complexity of this library (ie. the number of initial transformants) was 3.5×10^6. The 4-8hr embryonic cDNA library was transformed into Epicurian Blue competent cells. I screened 450,000 colonies over 9 plates (~50,000 per plate). In the primary screen 23 independent putative positive colonies were picked and by the tertiary screen three of these original positives were represented by three types of cDNA clone.

The cDNA clones were characterised into one of the three types on the basis of their restriction patterns when digested with the two enzymes *Sau*IIIa and *Eco*RI. The cDNA clones were restriction mapped using *Eco*RI and *Hin*dIII, this also enabled an estimation of the size of each clone to be made. The gel showing *Hin*dIII and

*Eco*RI digests of each of the clones are shown in Fig. 4.3.3. This information is summarised in Table 4.3.4.

The 5' and 3' end of each of the cDNA clones was sequenced using the dideoxy T termination reaction only (ie. T-tracked) in order to determine which of the cDNA clones were the same. From the T-tracking experiment the 3' ends of cDNA clones type 19 and 3a3 were the same but they differed at the 5' end. The sequence of cDNA clone 6 differed from the other two clones at both its 5' and 3' end. These results suggest that clones 19 and 3a3 are overlapping (ie. the same gene) whereas the other clone is non overlapping (ie. a different gene). (Data not presented).

4.4 Mapping cDNA clones back to the genomic walk.

Fragments containing non vector sequences from each of the cDNA clones were labelled with Digoxigenin by random priming and used to probe filters of *Eco*RI digests of cPn31 and the plasmid rescue clone. The positions of these fragments on the genomic walk are shown in Figure 4.2.3. Each filter had an *Eco*RI digest of the cDNA clone it was probed with as a positive control. The gel blotted to make these filters is shown in Fig. 4.4.1. The hybridisation was detected colourmetrically on the filter Fig 4.4.1. Clones 19 and 3a3 map between co-ordinates +6 and +8.3 kb on the restriction map. All the cDNA clones hybridised to the 8kb fragment from the plasmid rescue clone fragment and to the 10kb fragment from cPn31. The 8kb fragment contains bluescript so hybridisation to this band is probably due to contaminating vector sequences in the probe. The 10kb cPn31 fragment may also be vector, if this is real hybridisation the fragment is further away either to the left or to the right of the insertion site and is therefore of less interest. cDNA clone 6 hybridised weakly between co-ordinates +6 and +7.4 kb.

4.5 In-situ hybridisation of the cDNA clones to whole mount *Drosophila* embryos.

Fragments from *Eco*RI -*Hin*dIII digests of each of the cDNA clones corresponding to non vector sequences were used to make random-primed Digoxigenin probes and were hybridised to whole mount *Drosophila* embryos. These fragments are shown on the restiction maps of the cDNA clones shown in Table 4.3.3. Two expression patterns were detected in wildtype embryos.

1) cDNA clones of type 19 and 3a3 gave a very similar expression pattern to that originally detected by the Enhancer detector transposon (Fig. 3.2.1). The expression of cDNA clone 3a3 is shown in detail in Figure 4.5.1. The homologous transcript corresponding to cDNA clone19 and 3a3 is expressed very early in an anterior and a posterior stripe during the blastoderm stage (between 2:20 - 2:50 hrs); the cells at the anterior are the anlagen of the anterior midgut, hypopharyngeal lobe and brain, the cells at the posterior are the precursors of the posterior midgut and proctodeum. These are the same cells that showed *lac*Z expression in A350.1M2 but at a slightly later stage. The A350.1M2 shows the posterior expression in the cells of the amnio proctodeal invagination (anlagen of the posterior midgut and proctodeum) in early gastrulating embryos (stage 7; 3 hrs). The anterior expression of *lac*Z in A350.1M2 in the cephalic region occurs slightly later stage 8 (3:10-3:40 hrs). During germ band elongation the expression is in a segmentally repeated pattern in the ventral nerve cord as in A350.1M2. A striking similarity with A350.1M2 enhancer detector expression is the more intense staining in the gnathal segments of the head. A more detailed description is given in the legend of Figure 4.5.1.

2) cDNA clone 6 showed no expression throughout embryogenesis.

4.6 Precise mapping of cDNA 3a3 to the genomic region flanking the A350.1M2 insertion.

cDNA clone 3a3 was shown to detect a transcript which was expressed in a similar pattern to the *lacZ* expression pattern of the original A350.1M2 enhancer

detector line (Fig. 4.5.1). The cDNA clone 3a3 was mapped more precisely to the digests of the DNA flanking the original insertion site to determine how far it was from the site of insertion of the P-element. Digests of the cosmid clone cPn 31 and the plasmid rescue clone were probed with the cDNA clone (Fig 4.6.1 and summarised in Table 4.6.2. The cDNA maps somewhere between map co-ordinates +6 and +9.3 kb (Fig.4.2.3). cDNA clone 3a3 was orientated with respect to the genomic region by making a probe from a fragment from the 5' end of the clone (ie. 1.2kb *Hin*dIII, *Eco*RI fragment of cDNA clone 3a3; restriction map Table 4.3.3). The 5' fragment from cDNA 3a3 hybridised only to the 1.45kb *Eco*RI site (+6 - +7.4 kb) (Fig 4.6.3; Table 4.6.4), orientating the clone as shown with respect to the genomic region (Fig 4.2.3.). The restriction map of clone 3a3 also corresponds to the genomic map.

cDNA clone 6 also hybridised map between co-ordinates +6 - +7.4 kb of the genomic walk. No expression was detected by in situ hybridisation to embryos, and sequencing of the ends of the clone show that it has no homology to cDNA 3a3. This is suprising as one would expect substantial overlap with clone 3a3. I have not resolved this but one possibility is that cDNA 6 maps at a different location and shares some homology with this genomic region. The restriction map of cDNA 6 does not correspond to the genomic.

4.7 DNA sequence of cDNA clone 3a3.

cDNA clone 3a3 was larger than the cDNA clone 19 and was less likely to be truncated and was therefore the one that I decided to sequence. Both strands of the clone were sequenced using sequential primers. The DNA sequence was 2887 base pairs long (Fig.4.7.1) and was translated in all three frames to its peptide sequence, in an attempt to find translational start sites and an open reading frame. In all three frames there appears to be no long open reading frame. I used frames on GCG to locate all the possible start and stop sites in each of the three frames and also of the clone in reverse (Fig. 4.7.2). The stop sites occur very frequently in all three frames. In frame two there are two short potential open reading frames, these have been

labelled ORF1 and ORF2. ORF1 encodes 92 amino acids starting at the methionine before it stops, ORF2 encodes 88 amino acids from the methionine. I performed a homolgy search of the Swissprot database witheach of these peptide sequences. The best ten homologies to ORF1 and ORF2 are shown in table 4.7.3. None of these provide any information about what the 3a3 transcript is doing in normal development. I also searched each of the ORF's for protein structural motifs but neither of them had any. There are a small number of regions where there could be a mistake in the sequence (these were areas of high secondary structure in the template causing compressions in the sequence, these compressions were present in the sequence of both strands) these have been clearly marked on the sequence (Fig. 4.7.1). Even if all of these areas resulted in a frame shift it still would not produce a long reading frame.

Since I had a shorter version of the same clone ie. cDNA clone 19 it occured to me that there was a possibility that cDNA 3a3 might be an unspliced version of clone 19, which would account for the lack of a long open reading frame. From the restriction map of clone 19 the 5' most *Hin*dIII *Eco*RI fragment was shown to be 650 basepairs shorter (550 bp) than the most 5' *Hin*dIII *Eco*RI fragment (1.2 kb) in clone 3a3. We know that the sequence from the 3' ends of 19 and 3a3 are the same, if clone 19 is a spliced form of 3a3 then the 5' end of clone 19 will be closer than 650 base pairs from the start of the 5' end of clone 3a3. The 5' end of clone 19 was sequenced to determine where the sequence was in clone 3a3. The 5' end of clone 19 starts 671 base pairs into clone 3a3 (Fig. 4.7.1). The start of the 5' sequence of clone 19 is indicated on the sequence of clone 3a3 (Fig.4.7.1). This data suggests that clone 19 is a 5' truncated form of clone 3a3.

100 base pairs of sequence from the 3' end of cDNA 6 was compared to 3a3 and no homology was seen. This DNA sequence from cDNA 6 is given in Fig 4.7.4.

4.8 Discussion.

4.8.1 The restriction map of the genomic DNA flanking the A350.1M2 insertion.

Genomic DNA flanking the original A350.1M2 insertion was obtained from plasmid rescue of sequences 3' to P[lArB] and from a cosmid clone cPn 31 (from the *brown* gene genomic walk) which extends both to the 5' and the 3' of the insertion. This region has been restriction mapped and orientated with respect to the cytological map 50kb to the right of *brown* on chromosome 2R. It was determined that the plasmid rescue clone and the 12kb *Xho*I genomic fragment contained only unique sequences and could therefore be used as a probe for cDNA clones in the region. I also produced tentative evidence that the plasmid rescue clone was transcriptionally active.

4.8.2 cDNA clones from the genomic region adjacent to the A350.1M2 insertion.

A number of cDNA clones were isolated from a cDNA library screen using the plasmid rescue clone as the probe. These clones were categorised into three types on the basis of their restriction patterns. The cDNA clones 19 and 3a3 both mapped back to the genomic region flanking the A350.1M2 insertion. cDNA 3a3 and cDNA 19 appear to be the same from their sequence at their 3' ends but cDNA 19 is truncated by 671 bp at the 5' end. cDNA 6 also mapped to 1.45 kb *Eco*RI (between +6 and +7.4 kb on the restriction map) fragment which is suprising since the sequence of cDNA clone 6 is not contained in cDNA 3a3.

4.8.3 In-situ hybridisation of the cDNA clones to whole mount *Drosophila* embryos.

The immense similarity between the original *lacZ* expression of A350.1M2 and the hybridisation pattern of the cDNA clone suggests that cDNA 3a3 represents the transcript of the gene originally detected by the enhancer trap line A350.1M2

4.8.4. The sequence of cDNA clone 3a3.

The DNA sequence of cDNA clone 3a3 has not yielded conclusive information as to the nature of the protein if any, encoded by this transcript. Perhaps this gene has its effect as an RNA molecule? Or maybe one of the short reading frames does encode a protein product? The other possibility, is that there may be another transcript in the region that is disrupted by the A350.1M2 insertion. I have not conclusively proved that the 3a3 transcript is the only transcript in the region or that this transcript is disrupted by the insertion.

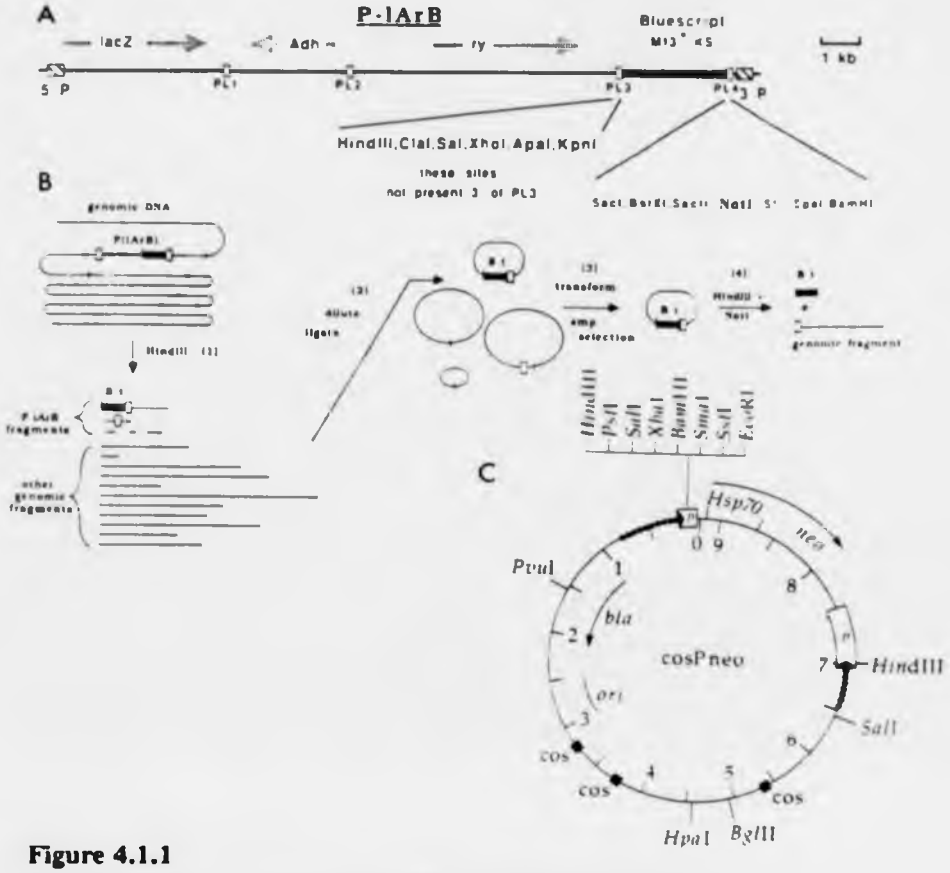


Figure 4.1.1
Plasmid rescue of sequences flanking the P[lArB] transposon.

A: structure and function of the P-element enhancer detector P[lArB], the P-lArB plasmid contains a P-*lacZ* fusion gene, the *Adh* gene, the ry^+ gene as an eye colour marker and Bluescript plasmid sequences. The transposon is flanked by the 5' and 3' P-element sequences necessary for transposition (hatched boxes). The polylinker 3 (PL3) contains six restriction enzyme sites that are not present farther 3' in the construct and therefore may be used for plasmid rescue.

B: plasmid rescue with P[lArB]. Genomic DNA from the P[lArB] transposant of interest is digested with each of the six enzymes that cut in PL3, in this case *Hind*III. The genomic fragments are ligated in large dilution. The ligation mixture is used to transform competent *E.coli*, only those molecules containing bluescript sequences can replicate and confer Ampicillin resistance and therefore are rescued. Digestion of the rescued clone with the enzyme used for the rescue and *Not*I produces a fragment containing 3' P-element sequences and all of the adjacent cloned genomic DNA.

C: Restriction map of cosmid P neo

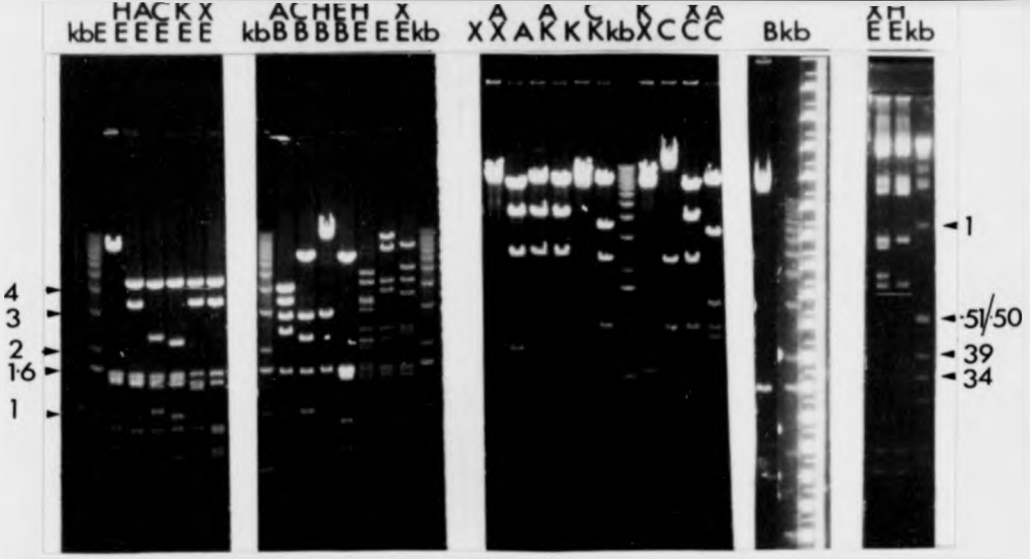


Figure 4.2.1 Cosmid clone Cpn 31 and the plasmid rescue clone A350 digested with a range of restriction endonucleases.

These gels were used to calculate the sizes of the fragments used to produce the restriction map (Fig 4.2.3).

A-*Apa*I, B-*Bam*HI, C-*Cla*I, E-*Eco*RI, H-*Hind*III, K-*Kpn*I, X-*Xho*I

A350 E	A350 E&H	A350 E&A	A350 E&C	A350 E&K	A350 E&X	A350 B&A	A350 B&C	A350 B&H	A350 B&E	C.31 E&H	C.31 E	C.31 E&X	1 kb ladder
kb	kb	kb	kb	kb	kb	kb	kb	kb	kb	kb	kb	kb	kb
9	4.75	4.75	4.75	4.75	4.75	3.8	6.1	10	5.7	4.75	10	8	12.21
1.6	3.6	2.4	2.3	3.6	3.6	3.3	2.65	2.75	1.6	4.55	7.1	5.1	11.19
1.5	1.6	1.6	1.6	1.6	1.6	2.65	2.15	1.6	1.5	3.5	4.55	4.55	10.18
1.45	1.5	1.5	1.5	1.45	1.45	2.25	1.6		1.45	3.35	3.8	3.65	9.16
0.9	1.45	1.45	1.45	1.4	0.9	1.6	1.0		0.9	3	2.5	2.5	8.14
0.61	0.9	1.15	1.0	0.9	0.86				0.61	2.5	2.15	2.15	7.12
0.2	0.61	0.9	0.9	0.61	0.64				0.2	2.15	1.6	1.6	6.10
0.18	0.2	0.56	0.61	0.2	0.61				0.18	1.95	1.5	1.5	5.09
	0.18	0.2	0.2	0.18	0.2					1.65	1.45	1.45	4.07
		0.18	0.18		0.18					1.6	0.94	0.94	3.05
										1.5	0.9	0.9	2.03
										1.45	0.69	0.84	1.63
										1.4	0.61	0.69	1.01
										1.25	0.2	0.66	.517
										0.94	0.18	0.61	.506
										0.9		0.57	.396
										0.84		0.46	.344
										0.69			.298
										0.61			.220
										0.46			.201

A350 X	A350 A&X	A350 A	A350 K&A	A350 K	A350 C&K	A350 K&X	A350 C	A350 C&X	A350 C&A
kb	kb	kb	kb	kb	kb	kb	kb	kb	kb
7.8	7.1	8.1	7.8	6.7	8.2	6.7	10.5	7.0	7.8
	4.3	4.3	4.3		3.7	0.56	2.3	3.9	3.2
	2.4	2.4	2.4		2.3		1.0	2.3	1.3
	0.7		0.24		1.0			1.0	1.0
									0.86

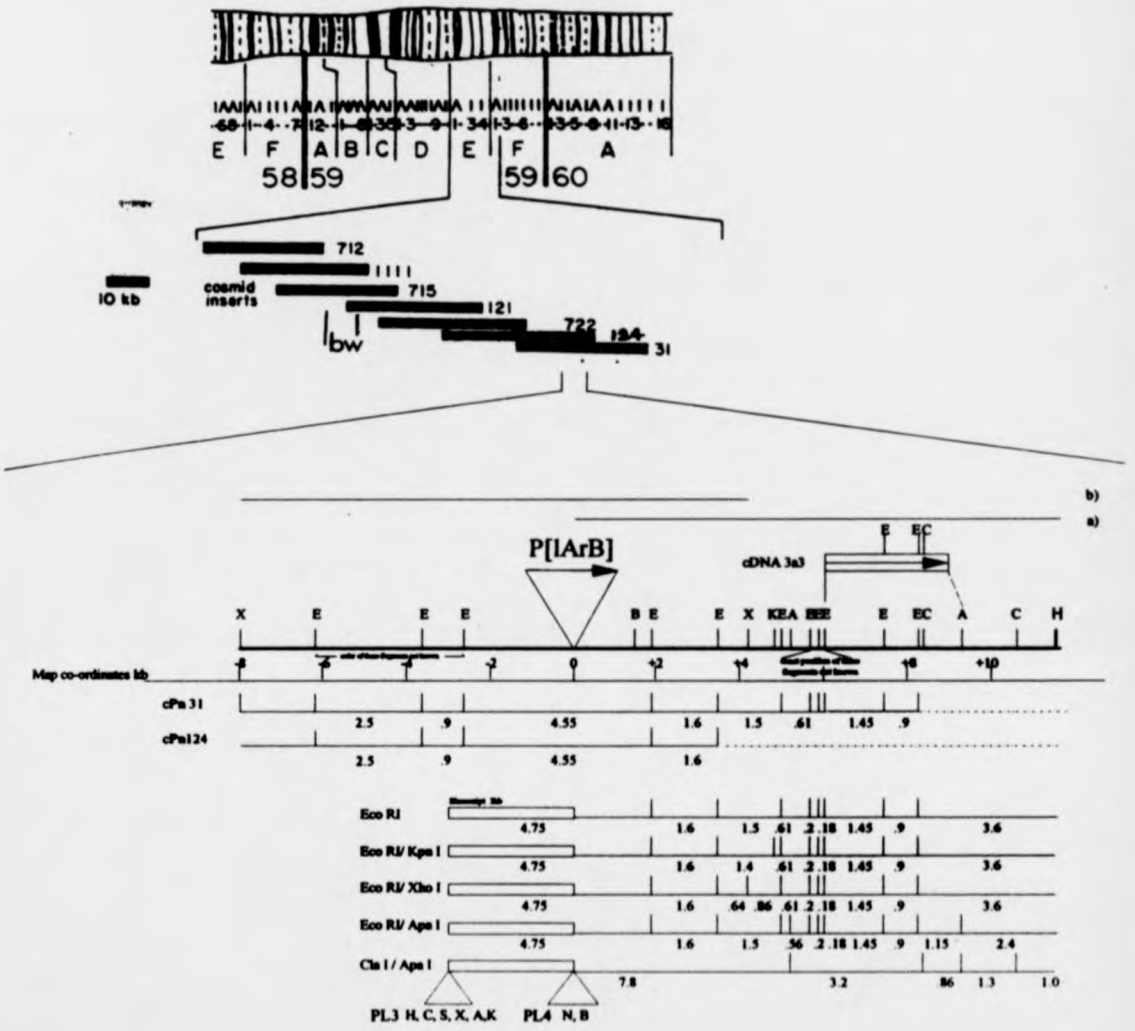
Table 4.2.2 The sizes of restriction fragments produced from clones Cpn31 and the A350 plasmid rescue clone when digested with the enzymes indicated.

The fragment sizes were calculated from several gels to give the most accurate value in kb, these gels are shown in Fig 4.2.1. This information was used to produce the restriction map of the genomic region flanking the A350.1M2 P-element insertion Fig. 4.2.3

Figure 4.2.3 Restriction map of the genomic region flanking the A350.1M2 insertion.

None of the restriction enzyme sites in PL3 are in Bluescript.
Map co-ordinates refer to the distance in kb to the left (-) or right (+) of the A350.1M2 P-element insertion.

1) A single digest of *Bam*HI produces two fragments of 14kb and 1.6 kb. There are no *Bam*HI sites in Bluescript and a *Bam*HI site in PL4, therefore the *Bam*HI site in the clone must be 1.6 kb to the right of PL4.
2) A single digest of *Apa*I produces a 8.1kb, a 4.3kb and a 2.4kb fragment. There is an *Apa*I site in PL3, the 2.4kb fragment is to small to contain Bluescript, so it must be in either the 4.3kb or 8.1kb fragments.
3)A single digest of *Cla*I produces the following fragments 10.5kb, 2.3kb, and 1.0kb. *Cla*I cuts in PL3,only the 10.5kb fragment is large enough to contain Bluescript, and extends to the right of PL3 and places a *Cla*I site 3.3kb away from the right hand end of the plasmid rescue clone. The other two fragments lie to the right of this site.
4) The double digest of *Cla*I and *Apa*I results in the 4.3kb and the 2.4kb *Apa*I fragments being cut. Therefore each of the two *Cla*I sites must lie in one of each of the two *Apa*I fragments. Since we know that one *Cla*I site is 3.3kb from the right hand end of the clone and the other *Cla*I site is within this, the order of the *Apa*I fragments is 8.1kb, 4.3kb and 2.4kb from left to right. The 2.3kb *Cla*I fragment is cut by *Apa*I so the order of the *Cla*I fragments is 10.5kb, 2.3kb and 1.0kb from left to right.
5) *Xho*I cuts to produce a doublet of ~7.8kb, it has one site in PL3 therefore cuts in the middle of the clone.
6) *Xho*I and *Cla*I double digests place the *Xho*I site 3.9kb to the left of the most left *Cla*I site.
7) *Xho*I and *Apa*I double digest place the *Xho*I site 0.7kb to the left of the most left *Apa*I site.
8) *Kpn*I single digest also produces a doublet of about 6.7kb , it also has a site in PL3 and the second site is therefore in the middle of the clone close to the *Xho*I site.
9) A *Cla*I and *Kpn*I double digest puts the *Kpn*I site 3.7kb to the left of the most left *Cla*I site suggesting that the *Xho*I site is to the left of the *Kpn*I site. The distance is confirmed by a *Kpn*I and *Xho*I double digest which suggests they are separated by 0.56kb. *Kpn*I and *Apa*I double digest places the *Kpn*I site 0.24kb to the right of the most left *Apa*I site.
10) Digestion with *Eco*RI produces the following Fragments 9kb, 1.6kb, 1.5kb, 1.45kb, 0.9kb, 0.61kb, 0.2kb and 0.18kb. These fragments have been positioned as follows.The 9kb fragment must contain Bluescript and from the *Eco*RI and *Hind*III double digest it is shown to extend 3.6 kb in one direction and 4.75 kb in the other direction from the *Hind*III site in PL3. *Eco*RI, *Cla*I and *Eco*RI , *Apa*I double digests both result in the 3.6kb fragment being cut. This places an *Eco*RI site 3.6kb from the right hand end of the clone, and one 4.75kb to the right of PL3.
11) The 1.5kb *Eco*RI fragment is cut by *Xho*I into two small fragments of 0.64kb and 0.86kb. This positions two *Eco*RI sites 0.64kb and 0.86kb away from this *Xho*I site, the orientation of these sites are not known. The *Kpn*I and *Eco*RI double digest cuts the 1.5kb fragment producing a new 1.4 kb fragment, reconfirming that the *Kpn*I site lies to the right of the *Xho*I site.
12) The 0.61kb *Eco*RI fragment can be positioned adjacent to and to the right of the 1.5kb *Eco*RI fragment on the basis that it is cut by *Apa*I to a fragment of 0.56kb.
13) The southern (Fig 4.2.4 & 4.2.5) where the probe was the large 12kb *Xho*I fragment from Cpn 31 showed hybridisation to the 1.6kb *Eco*RI positioning it to the left of the 1.5kb *Eco*RI fragment. This means that the 0.9kb and the 1.45kb fragment must lie to the right of the 0.61kb fragment. The small fragments (0.2 and 0.18) were not on this gel so their positions are not accounted for.
14)The 1.45kb *Eco*RI fragment is to the left of the 0.9kb *Eco*RI fragment since the 5' end of the cDNA hybridised only to the 1.45kb fragment.
15) Since cPn 124 overlaps the region of the A350.1M2 clone nearest the P-element insertion whereas cPn31 probably covers the entire clone. This allows orientation of this restriction map with respect to the polytene map; centromeric left, telomeric right.


E F A B C D E F A
58 59 59 60
10 kb
cosmid inserts
712
715
121
722
124
31
bw
b)
a)
P[lArB]
cDNA 3a3
X E E E B E E X KEA E EC A C H
Map co-ordinates kb
-3 -2 0 +2 +4 +8 +10
cPn 31
cPn124
2.5 .9 4.55 1.6 1.5 .61 1.45 .9
2.5 .9 4.55 1.6
Eco RI
Eco RI/ Kpn I
Eco RI/ Xho I
Eco RI/ Apa I
Cla I / Apa I
4.75 1.6 1.5 .61 .2 .18 1.45 .9 3.6
4.75 1.6 1.4 .61 .2 .18 1.45 .9 3.6
4.75 1.6 .64 .86 .61 .2 .18 1.45 .9 3.6
4.75 1.6 1.5 .56 .2 .18 1.45 .9 1.15 2.4
7.8 3.2 .86 1.3 1.0
PL3 H, C, S, X, A,K
PL4 N, B

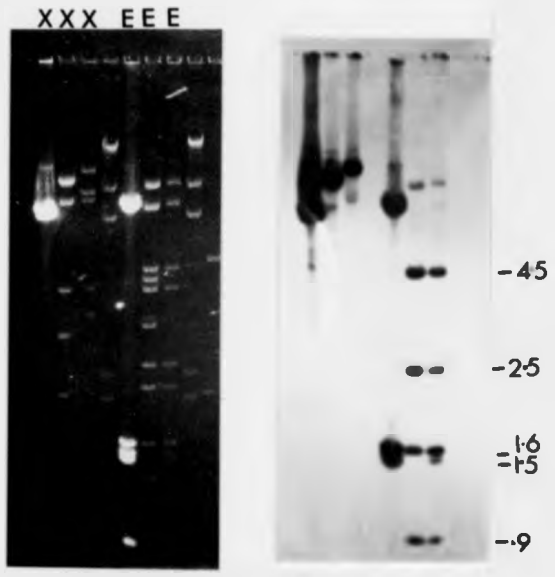


Figure 4.2.4
XhoI and EcoRI digests of cPn31, cPn124 and the plasmid rescue clone A350 were run on an agarose gel. The gel was blotted and hybridised with a radioactively labelled 12kb *Xho*I fragment from cPn31. The autoradiograph shows which bands the probe hybridised to. This data is summarised in Table 4.2.5.

A350 X	C.124 X	C.31 X	A350 E	C.124 E	C.31 E
7.8	10.5	12	9	9.5	10
	9.4	9.8	1.6	6.8	7.1
	4.0	9.4	1.5	4.55	4.55
	2.95	4	1.45	4.2	3.8
	2.1	3.4	0.9	3.8	2.5
	1.5	1.5	0.61	3.4	2.15
	1.45	1.45	0.2	2.2	1.6
			0.18	2.5	1.5
				2.15	1.45
				1.6	0.93
				1.25	0.9
				0.93	0.69

Table 4.2.5
Summarises the information from the gel shown in figure 4.2.4 probed with the 12kb *Xho*I fragment.

double lined hatched box indicates the bands which the probe hybridised to.

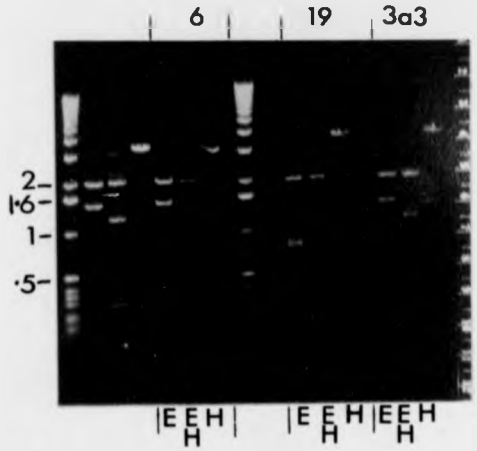
Figure 4.3.1 In situ hybridisation of the 12kb *Xho*I fragment of Cpn 31 to wildtype polytene chromosomes.

An unique signal can be seen at cytological band 59F showing that the 12 kb fragment only contains unique sequences.

Figure 4.3.2 Northern blot of Wildtype 0-24hr total RNA probed with the A350.1M2 *Not*I *Hin*dIII fragment.

A northern blot of total RNA from 0-24 hour embryos two lanes were loaded with 20µg and 10µg of RNA. The two arrows indicate the position of the ribosomal bands.

Hybridisation can be seen suggesting that this fragment is transcriptionally active. The size of the hybridising bands varied between gels so an estimation of transcript size could not be made.



cDNA clone 6			cDNA clone 19			cDNA clone 3a3		
E	EH	H	E	EH	H	E	EH	H
2.15	2.15	3.5	2.15	2.15	4.0	2.15	2.15	4.0
1.5	0.96	0.22	0.88	0.8	0.74	1.5	1.2	1.45
	0.22		0.8	0.71		0.88	0.8	
	0.21			0.55		0.8	0.71	
				0.29			0.29	
				0.22			0.22	

Figure 4.3.3 Gel showing *Hin*dIII *Eco*RI digests of the cDNA clones 6, 19 and 3a3, the fragment sizes in kb are shown in the table above.

Primary Screen	Probe which detected colony	cDNA number	Size of insert	Expression pattern	Restriction map
1a	A350NH	19	2.23kb	A350-like	
3a	A350NH	3a3	2.88kb	A350-like	
5b	A350NH	6	1.07kb	no expression during embryogenesis	

Table 4.3.4 cDNA clones isolated from screening the Nick Brown cDNA library.

The origin of each clone and the probe which it was identified by. The cDNA clones have been classified into three types on the basis of their restriction patterns. The non vector fragment used for in situ hybridisation to embryos is indicated by the asterisk.

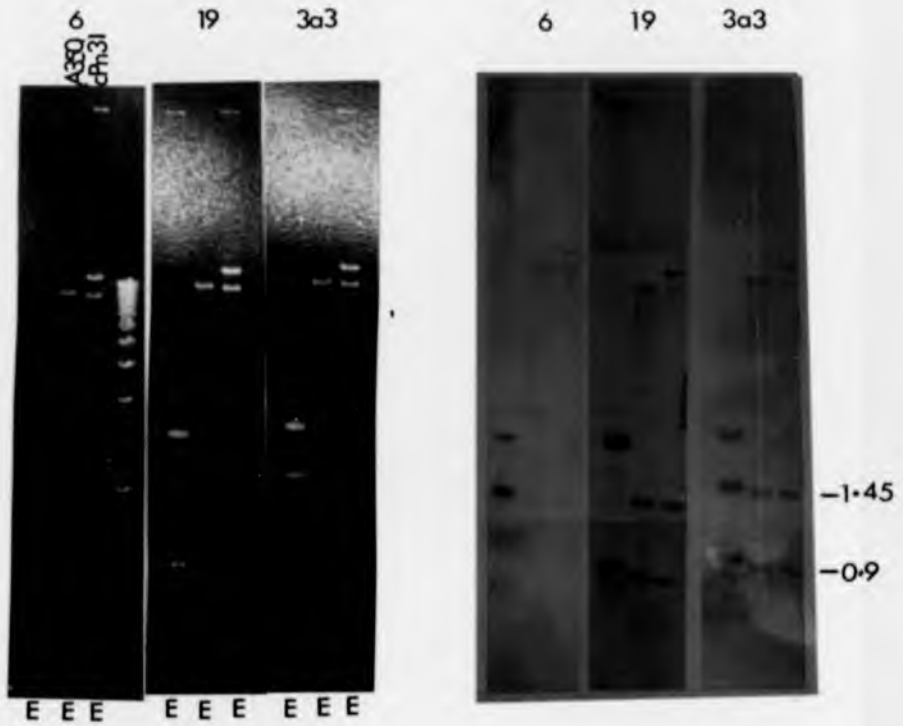


Figure 4.4.1 Mapping cDNA clones to the genomic map.

Gel showing *Eco*RI digests of A350.1M2 plasmid rescue clone and cPn 31 DNA clones 6, 19 and 3a3 were also run as positive controls. Each was probed with non vector sequences from one of the cDNA clones. The probe was labelled with digoxigenin and detected colourmetrically with NBT and X-phosphate.

Fig. 4.5.1 The pattern of hybridisation of cDNA clone 3a3 in whole mount *Drosophila* embryos.

A. Cellularisation occurs during stage 5 (2:10 - 2:50) this stage has been divided equally into three depending on the depth of invaginating membranes. The time between these stages has been taken to be approximately equal (according to Campos Ortega and Hartenstein,1985; Lawrence and Johnston,1989). The embryo is approximately old stage 5(1) 2:23 hrs,the 3a3 transcript can first be seen to be expressed at this stage. The expression is in two bands one at the anterior and one at the posterior end of the embryo. The anterior band at the dorsal surface extends from 92-76% egg length and from 92-78% egg length on the ventral suface. The posterior band is from 12-0% egg length. There may be low levels of expression around some of the other peripheral nuclei. There is no expression in the yolk nuclei or the pole cells.

B. stage 5(2) 2:23 -2:36 hrs,the expression pattern of 3a3 transcript is unchanged from stage 5(1). This blastoderm expression is shown diagramatically on the blastoderm fate map (Fig 5.7). This map indicates that the cDNA expression is in the cells which are the anlagen of the proctodeum and the posterior midgut in the posterior and precursors of part of the anterior midgut, hypopharyngeal lobe and the procephalic neurogenic region in the anterior of the embryo. These are approximately the same cells that minutes later (ie. 3hours in the posterior, and 3:10 hrs in the anterior) show the early lac-Z expression in the A350.1M2 enhancer trap line.

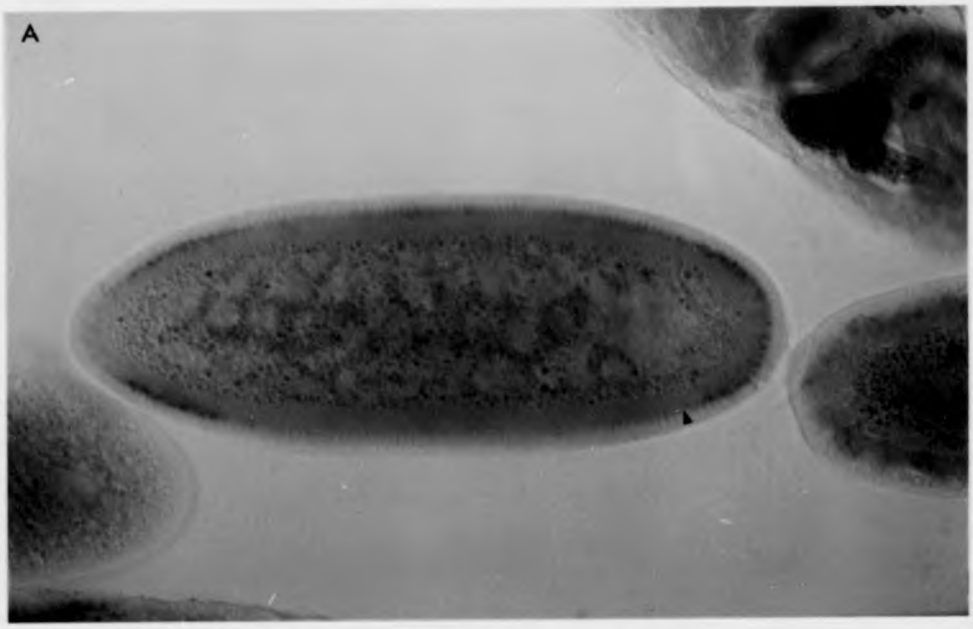
C. Late stage 5(3) 2:50 hrs, just prior to the formation of the ventral furrow 3a3 can still be seen to be expressed in two terminal stripes.

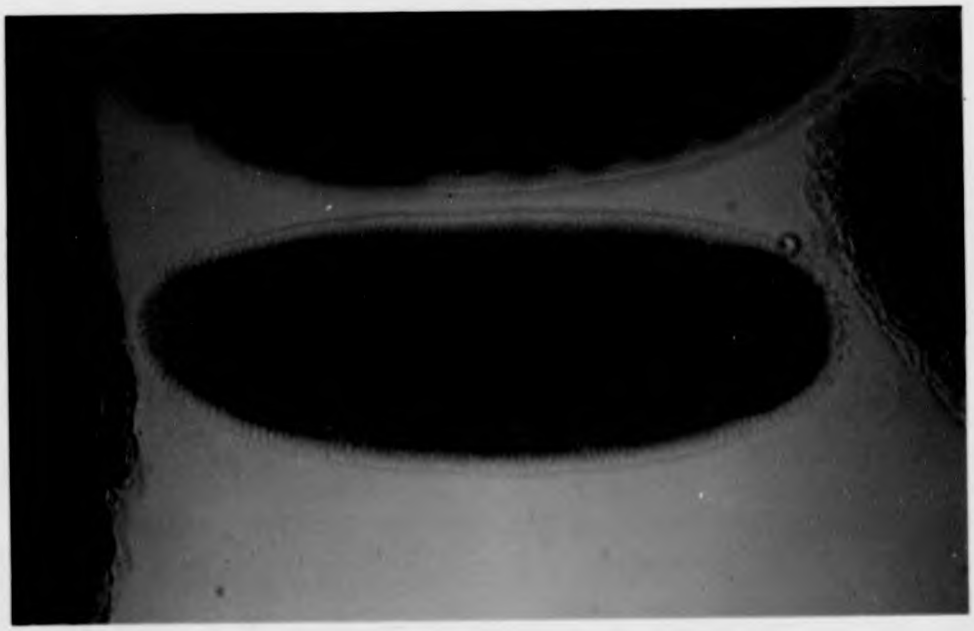
D. The cephalic furrow has started to form stage 6 (2:50 - 3 hrs). cDNA 3a3 can be seen to be expressed in cells anterior to the cephalic furrow.

E. This embryo is approximately stage 8 . The amnioproctodeal invagination has formed,this invagination of endoderm becomes the primordia of the posterior midgut and the germ band is extending. 3a3 expression is strong in these cells. The embryo is viewed dorsally and shows expression in the posterior midgut and cephalic ectoderm. This expression is similar to the original A350.1M2 *lac*Z line.

F. Stage 11 (5:20 - 7:20 hr),segmentation has occured and expression can be seen in a segmentally repeated pattern in the ventral nerve cord. Expression is strong in the posterior midgut, Malpighian tubules and proctodeum. The expression is strongest in the gnathal segments and the hypopharyngeal lobe of the head a characteristic of A350.1M2.

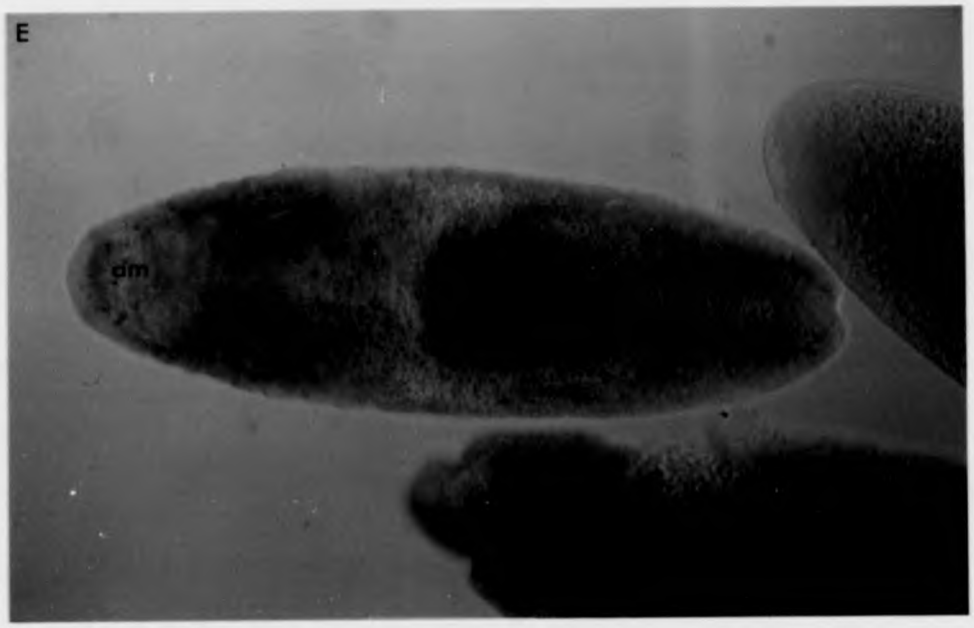
G. By stage 12 (7:20 - 9:20 hrs) the expression pattern is strongly reminiscent of the A350.1M2 lac-Z line. There are a large number of cells of the ventral nerve cord showing expression, and this expression is strongest in the gnathal segments of the head. There is also expression in the cephalic region and in the salivary glands.

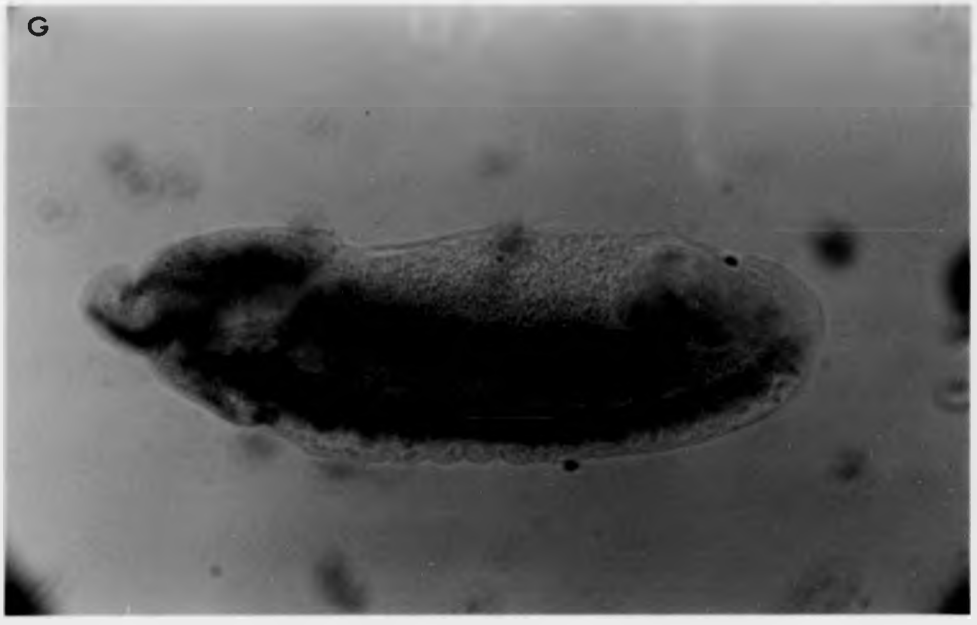

A




C


D


E
gm



Arrowheads indicate the depth of invaginating membranes.

abbreviations: **VNC**; ventral nerve cord, **pr**; proctodeum, **pmg**; posterior midgut, **cf**; cephalic furrow, **am**; anterior midgut.

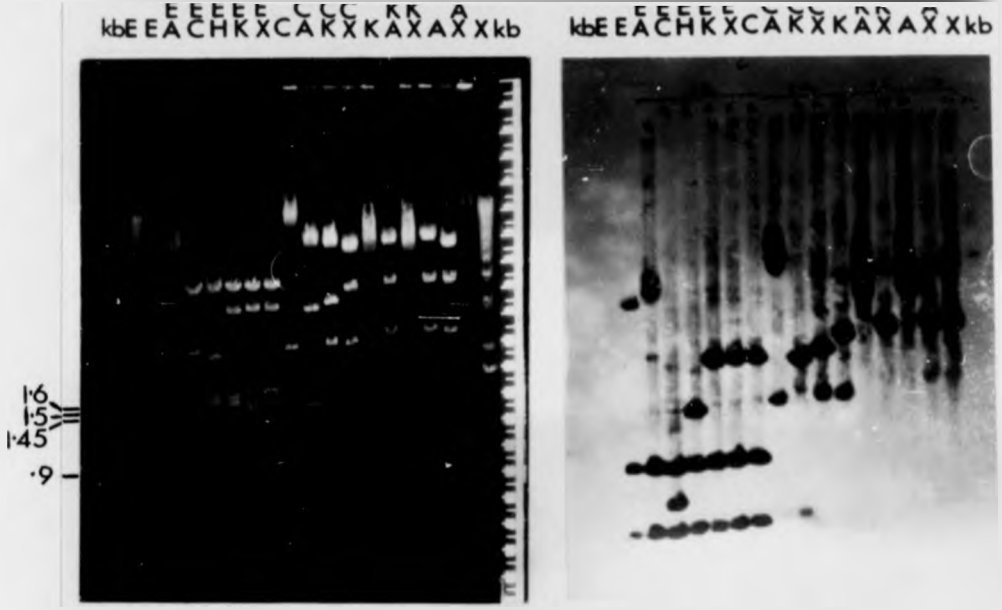


Figure 4.6.1 Accurate mapping of cDNA clone 3a3 to the genomic region.

Gel showing digests of A350.1M2 plasmid rescue clone and Cpn 31. The gel was blotted and probed with the whole cDNA 3a3 clone

C.31 E	A350 E	A350 E&A	A350 E&C	A350 E&H	A350 E&K	A350 E&X	A350 C	A350 C&A	A350 C&K	A350 C&X	A350 K	A350 K&A	A350 K&X	A350 A	A350 A&X
Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb
10	9.0	4.75	4.75	4.75	4.75	4.75	10.3	7.8	8.2	7	6.7	7.8	6.7	8.1	7.1
7.1	1.6	2.4	2.3	3.6	3.6	3.6	2.3	3.2	3.7	3.9		4.3	0.56	4.3	4.3
4.55	1.5	1.6	1.6	1.6	1.6	1.6	1.0	1.3	2.3	2.3		2.4		2.4	2.4
3.8	1.45	1.5	1.5	1.5	1.45	1.45		1.0	1.0	1.0		0.24			0.7
2.5	0.9	1.45	1.45	1.45	1.4	0.9		0.86							
2.15	0.61	1.15	1.0	0.9	0.9	0.86									
1.6		0.9	0.9	0.61	0.61	0.64									
1.5		0.56	0.61			0.61									
1.45															
0.94															
0.9															
0.69															
0.61															
0.46															

Table 4.6.2

DNA fragments generated by restriction enzyme digestion of the two clones A350 and Cpn 31 were calculated from figure 4.6 are summarised in this figure. The filter was probed using the whole of cDNA clone 3A3 as a probe.

Double outlined hatched box indicates which bands the probe hybridised to.
A - *Apa*I, B - *Bam*HI, C - *Cla*I, E - *Eco*RI, H - *Hin*dIII, K - *Kpn*I, X - *Xho*I.

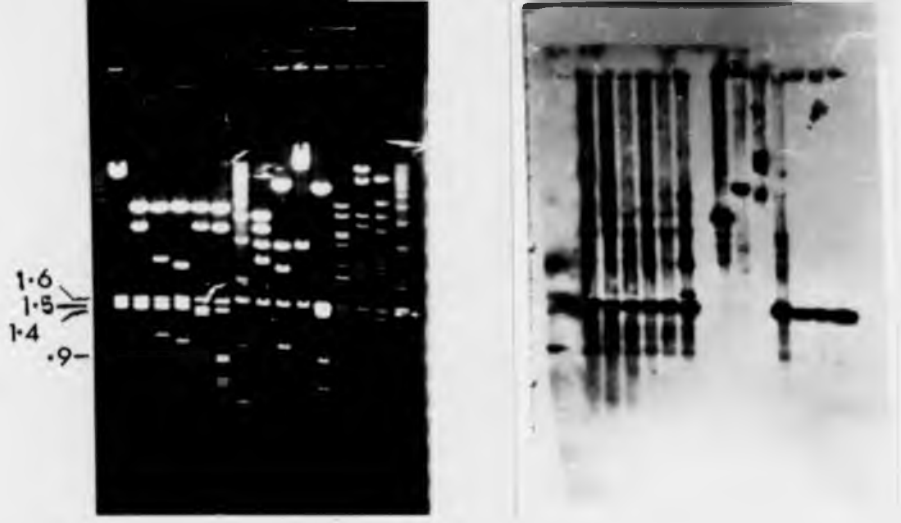


Figure 4.6.3. Orientating the cDNA clone 3a3 with respect to the genomic walk.

Gel showing digests of A350.1M2 plasmid rescue clone and Cpn 31. This gel was probed with the 5' end of cDNA clone 3a3.

A350 E	A350 E&H	A350 E&A	A350 E&C	A350E &K	A350 E&X	A350 B&A	A350 B&C	A350B &H	A350 B&E	C.31 E&H	C.31 E	C.31 E&X
Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb
9	4.75	4.75	4.75	4.75	4.75	3.3	3.1	10	5.7	4.75	10	8
1.6	3.6	2.4	2.3	3.6	3.6	3.3	2.65	2.75	1.6	4.55	7.1	5.1
1.5	1.6	1.6	1.6	1.6	1.6	2.65	2.15	1.6	1.5	3.5	4.55	4.55
1.43	1.5	1.5	1.5	1.43	1.43	2.25	1.6		1.43	3.35	3.8	3.65
0.9	1.43	1.43	1.43	1.4	0.9	1.6	1.0		0.9	3	2.5	2.5
0.61	0.9	1.15	1.0	0.9	0.86				0.61	2.5	2.15	2.3
	0.61	0.9	0.9	0.61	0.64					2.15	1.6	1.6
		0.56	0.61		0.61					1.95	1.5	1.5
										1.65	1.43	1.43
										1.6	0.94	0.94
										1.43	0.9	0.9
										1·4	0.69	0.84
										1.25	0.61	0.69
										0.94	0.46	0.66
										0.9		0.61
										0.84		0.57
										0.69		0.46
										0.61		
										0.46		

Table 4.6.4 Summarises the information shown by the gel and autoradiograph shown in figure 4.6.3.

Gel was blotted and probed with the 5' end of cDNA 3a3 to orientate it with respect to the genomic DNA. Bands which the probe hybridised to are shown by double lined boxes.

Hatched box indicates fragments which the probe hybridised to.
A - *Apa*I, B - *Bam*HI, C - *Cla*I, E - *Eco*RI, H - *Hin*dIII, K - *Kpn*I, X - *Xho*I.

Figure 4.7.1. The nucleotide sequence of cDNA clone 3a3.

The clone was sequenced in both strands using the primers marked. The sequence was translated and the two longest potential reading frames are shown ORF1 and ORF2. Areas of uncertainty are marked between two x's. The position of the 5' end of cDNA clone 19 is marked by a large arrowhead.

```
                                  Z
                    ──────────────►
   1  ccccccaaaa gtttggtcaa aataacagaa gggggtcagc gcatttatac

  51  ttagacataa aaatgatatt ctttttagct tcaagataga agttcaattt

 101  ctgtaagata caattgagaa catccaatta tatatctctg cttaatatct

 151  gagtagctga agagtttctt taggatagtt actgcgtgcc ttggccaatc

 201  cttgagctag ccaaagatga gataagataa gtactccttg cctaagtgcc

                                  3
                    ──────────────►
 251  tatatgcgct cgaaaagaga ttgaagattg ccaagttcga taccagatgt
                                                ◄──────────

                          x     x
 301  gcacaacaat gattaacccc ggcaattgcc gtcgcttgca gtttgttctt
      ──────────►
           4

 351  ggtcgctatg tatctgtaag atactttcat tccgttcgct ggcataactt
                                                        ▲

 401  atgcttcaac gcgtaccatt taggcataaa tcgcgctgtc accccgatca
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                                  5
                    ──────────────►
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        P  F  V   I  I  M   R  A  A  L   A  S  F   F  F        ORF1

 501  atttgattgt tttcgttgtc gttaaaatat tttttgggtg tgttctgtat
        D  L  I   V  F  V   V  V  K  I   F  F  G   C  V  L  Y

 551  tttaatagcg cactgcgaag agaagccagc acttccttgc gtctcacaca
        F  N  S   A  L  R   R  E  A  S   T  S  L   R  L  T  H
      ◄──────────

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        P  F  C   P  A  T   S  A  C  L   I  S  F   E  L  V

 651  ggcaggggga ccaaaccaaa ccgaaagtaa catgatttca gtttttactc
        G  Q  G   D  Q  T   K  P  K  V   T
                              ▲
                           cDNA19

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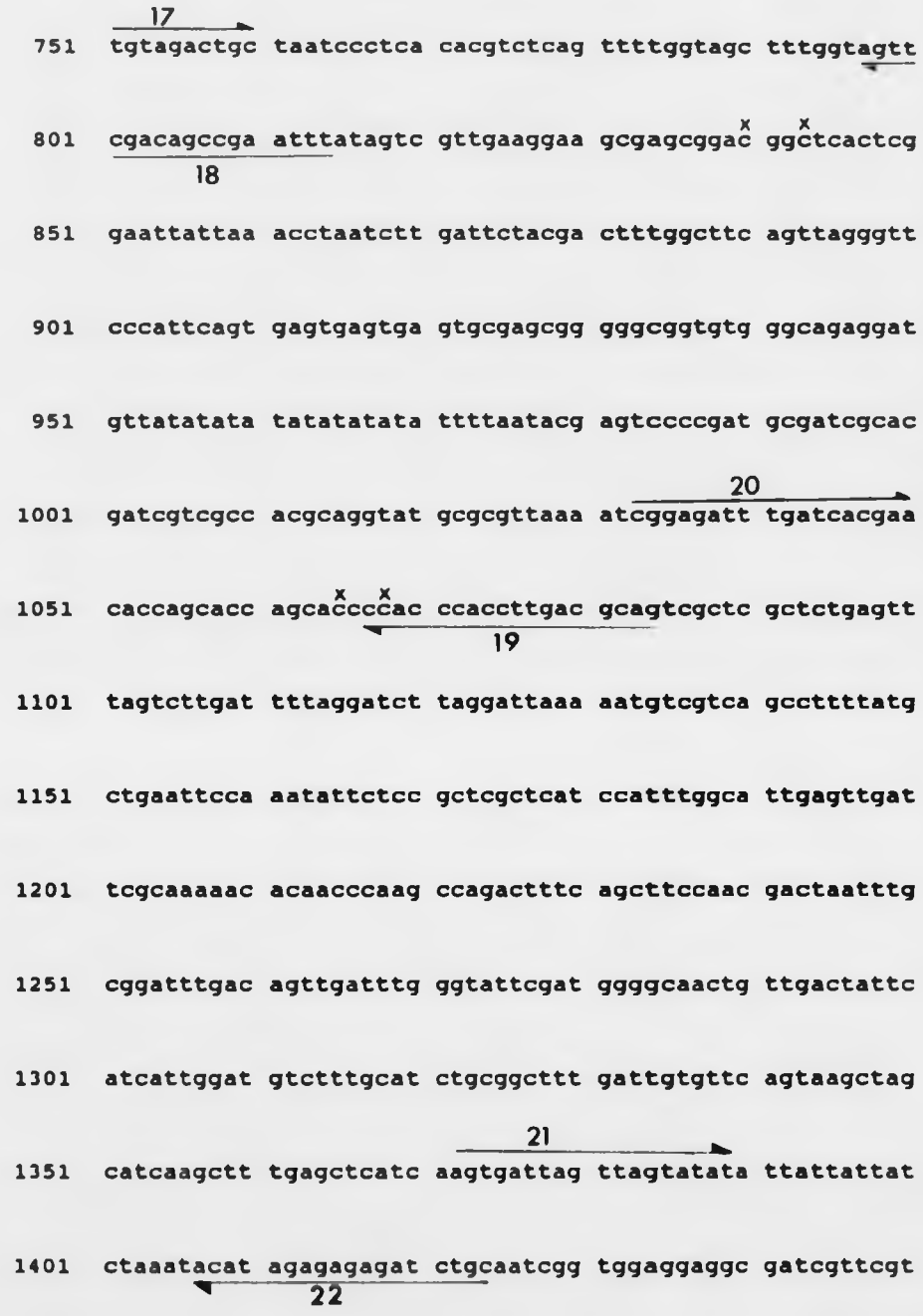
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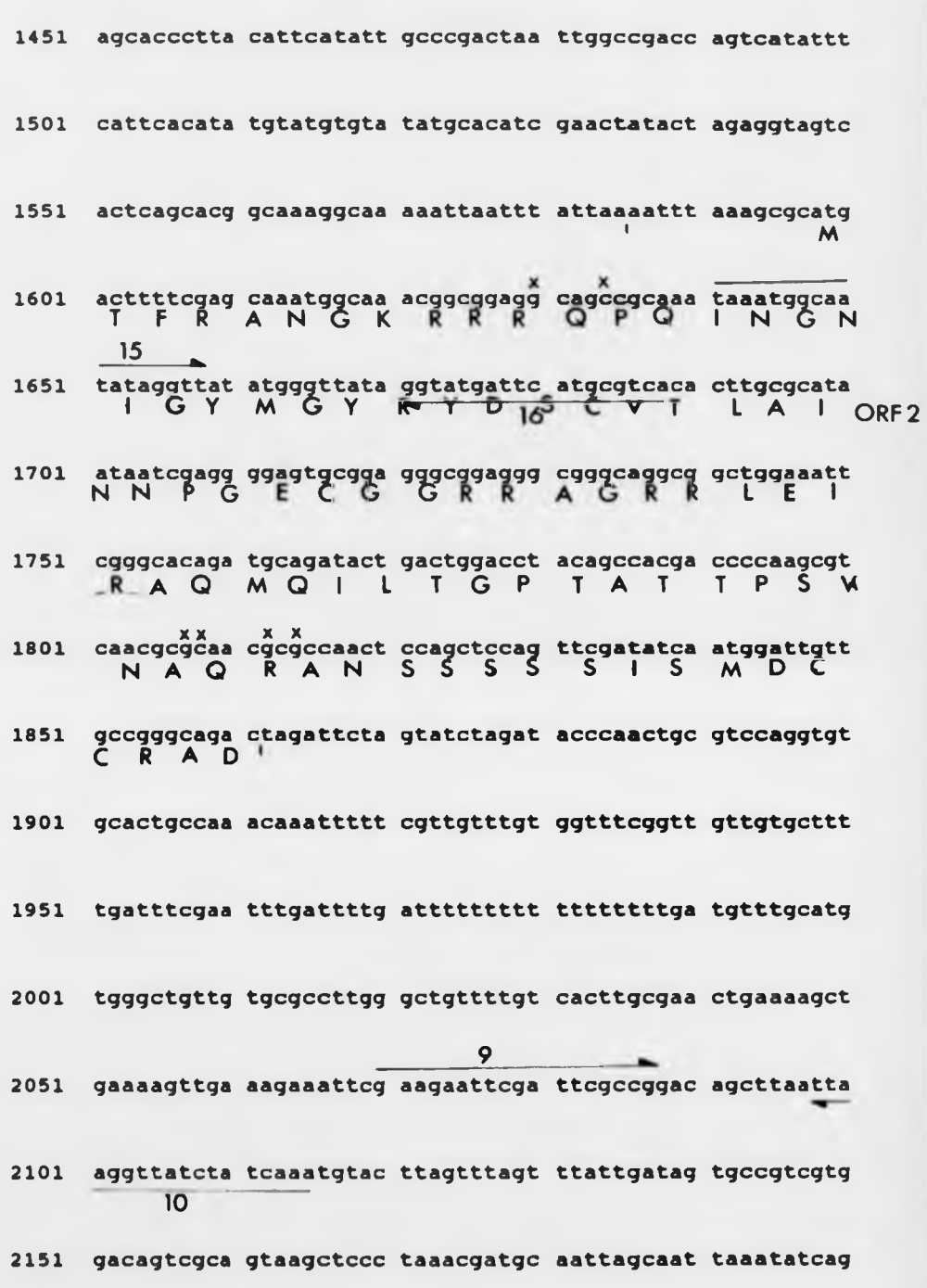
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15
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I G Y M G Y R Y D S C V T L A I ORF2
16
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C R A D
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2201 ttgacttaat gctaggcaaa gtctcgacga cttatttatc aatttcagat

2251 acaaatacaa cgatgtgtgc gacatcgata aacaaaagag ctctaaatta

8

2301 tgtgtagtct tagcatttgt tattcctgtt gaccaacaca cacacgcaca

7

2351 ctcgaagcca atcgttccca tgactttaat tacaatttaa tatttattta

2401 gttcgataag gccggggtga ttatttttgg atactcatga ctgcacccag

2451 cactcgcata cttacgtgca cagatacaga tacagataca gatacagata

2501 cagatacaca tgcgtacata tctttacgag tgcgtatttg tttttgtgtt

2551 actatcaatt gggcctaatc aaatgcctaa gtaaattgct cagcaaatca

2

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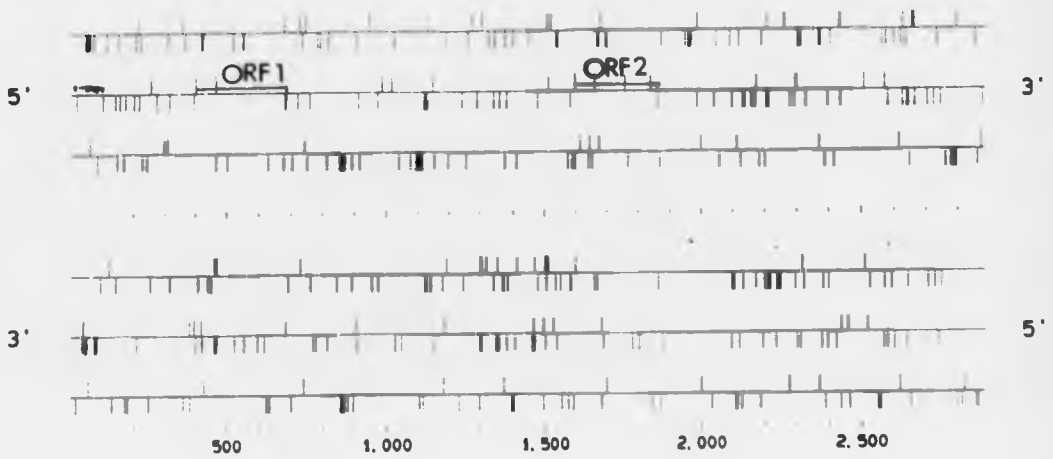


Figure 4.7.2 Potential start and stop sequences in the cDNA clone 3a3 translated in three frames.

The longest potential open reading frames are present in frame two . These are labelled ORF1 and ORF2.
Upwards lines represent starts and downwards lines represent stops.

ORF 1		ORF 2	
gene	homology	gene	homology
Na^+ channel protein, rat cardiac muscle	28.1% over 32 aa	minor core protein V	35.1% over 37 aa
Quinate permease	43.8% over 16 aa	Major core protein precursor VII	30.0% over 40 aa
Cl^- channel protein	21% over 78 aa	Von Willebrand factor precursor Human	29.0% over 31 aa
regulatory protein BLAR1	22.9% over 35 aa	S-Laminin precursor rat	33.3% over 24 aa
chitin synthase 2 yeast	27.3% over 33 aa	human bone morphogenetic protein 4	34.8% over 23 aa
complement factor H precursor mouse	23.8% over 21 aa	human bone morphogenetic protein 4 precursor	34.8% over 23 aa
cytochrome B chick	28.1% over 32 aa	isopentenyl transferase	40% over 15 aa
cytochrome B (fragment)	34.4% over 32 aa	insect toxin 4	36.4% over 22 aa
cytochrome BXenopus	28.1% over 32 aa	ryanodine receptor rabbit	87.5% over 8 aa
L-(α-aminoadipyl)-L-cystein-D-valine synthetase	34% over 26 aa	ryanodine receptor human	87.5% over 8 aa

Table 4.7.3

This table shows the ten best homologies to the peptide sequence of ORF1 and ORF2.

```
  1  ggcaccatnt ntgggttcgc ggtttacgtt ttacatagga acttttcgct
 51  cagtataatc gaaacagtaa gcccagcacc gtcgccacca ccaaccccca
101  aaacaaaatt tt
```

Figure 4.7.4 Nucleotide sequence of the 3' end of cDNA 6.

This sequence was found to share no homology with cDNA 3a3.

Chapter 5

Genetic analysis of A350.1M2

5.1 Introduction.

The classical genetic approach of determining the wild-type function of a given gene is to remove its activity and then to look for developmental consequences. There are a number of ways of removing the wild-type activity of a gene. Mutagenesis can be performed using either X-rays or chemicals. To generate a mutation in any single gene requires screening two to ten thousand mutagenised sperm. After mutations are generated, a means of identifying a mutation in the gene of interest is also required. One way of doing this is to cross mutagenised males to a stock carrying an existing allele of the gene of interest, and screen the progeny for new mutations that fail to complement the original allele.

As discussed in chapter one P-element insertions can be used as a form of mutagenesis. The P-element insertion sometimes disrupts gene function. If the P-insertion has not disrupted a nearby gene it can be used to generate imprecise excision events; which can be then be screened for mutation in the flanking gene.

Targeted mutagenesis screens allow identification of P-element insertions near to a gene of interest. The strategy requires two oligonucleotides; one complementary to part of the gene of interest, and one complementary to the terminal sequence of the P-element. The segment of DNA between the two primers will be the target for amplification by the polymerase chain reaction; only in a fly where the insertion is within two kilobases of the gene primer (Ballinger and Benzer, 1989; Sentry and Kaiser, 1992).

If the P-element insertion has not caused a mutation it can be excised by transposase mediated excision, to generate imprecise excision events. This allows easy genetic characterisation of genes identified by enhancer detection (eg. Bellen *et al.*, 1989; Fasano *et al.*, 1991). P-element jumpouts displaying a homozygous mutant

phenotype are recovered at high frequency. Mobilisation of the viable sloppy paired insertion A208 created ~30% lethal jumpouts (Grossniklaus *et al.*, 1992).

It is quite possible that the gene detected by enhancer trapping may have a redundant function; this is because identification relies on expression pattern rather than mutant phenotype. This would also account for the gene not being detected in previous screens.

One might expect the phenotype of a mutation affecting the 3a3 transcript to affect the regions where it is expressed. The screen for cuticular patterning genes did not detect a gene in the region 59F (Nusslein-Volhard *et al.*, 1984).The mutation affecting the 3a3 transcript is either one of the few remaining mutations not detected, or it could be partially redundant and more than one gene has to be disrupted to see a phenotype eg. *gooseberry* (Baumgartner, *et al.*, 1987).

The types of defect one might predict in the absense of the 3a3 transcript are, those affecting the development of the cephalic regions (especially the gnathal and hypopharyngeal lobes), the posterior terminus, parts of the gut or the CNS.

Complementation tests with A350.1M2 and all the available point mutations in the region had previously been performed (Bhatia pers. comm.). Table 5.1 shows the mutations from the region which were tested. The results showed that the A350.1M2 mutation was not in any of these genes.

5.2 Deficiency mapping

A number of deficiencies in the region generated by Bruce Reed (Ph.D. thesis, Cambridge University) whilst working on the gene *morula* were used to perform complementation tests to map them with respect to the A350.1M2 insertion. The cytology of these deficiencies are tentative but the results of complementation tests with the A350.1M2 insertion suggest the approximate Deficiency map in Fig 5.2.

5.3 Generation of additional alleles of A350.1M2

A350.1M2 insertion has been shown to be a lethal insertion (Chapter 3) but the lethal phase was shown to be after embryogenesis. The tendency of P-elements is to integrate in regulatory regions rather than structural genes, and hence they frequently cause hypomorphic rather than amorphic mutations. This would account for the A350.1M2 insertion not causing embryonic lethality. I therefore wanted to generate additional alleles of A350.1M2, particularly with the hope of isolating some with a more severe phenotype. I was particulary interested in generating embryonic lethal mutations which might have defects corresponding to the expression pattern of the gene flanking this insertion, the 3a3 transcript (or a neighbouring, so far undetected, transcript with a similar expression pattern). Given the localised expression pattern of the A350.1M2 enhancer trap and the nearby 3a3 transcript, it seemed likely that an amorphic mutation (eg. a deletion of 3a3 transcript) would give rise to an embryonic lethal mutation, possibly with a cuticular phenotype.

The D26 PClrB line described in chapter 3 maps close to the A350.1M2 insertion on the cytological map, and its expression pattern is also very similar to the A350.1M2 insertion suggesting that they are being regulated by the same enhancer elements. The D26 insertion is viable when homozygous and when hemizygous over Df(2R)bw^{S46}. Hence, mutations that give rise to loss of the insertion and any neighbouring essential gene, can easily be screened for simultaneous loss of the insertion and mutation towards lethality. Imprecise excisions of the P-element from the D26 chromosome were generated by remobilisation mediated by transposase and selected by lethality over Df(2R)bw^{S46} (Fig. 5.3.1.).

36 individual D26 chromosomes were used to generate the jumpstart males. Jumpstart males contain both the source of transposase and the element to be excised, and are referred to as the F0 generation. Each of these chromosomes was followed through subsequent generations to check that I did not select an already existing hemizygous lethal mutation on any of the chromosomes.

The P[ry^+ Δ 2-3 (99B)] chromosome was used as an exogenous source of transposase (Robertson *et al.*, 1988). Jumping which occurs in the germline of the jumpstart males produces offspring with precise and imprecise excision events. Many of the progeny from a single jump-start male may have the same excision event. Hence only one of the offspring of each jump-start male which carried a ry^- excision event (*Fig.* 5.3.2 F1) was tested for the nature of its excision event as described below. In all, two hundred and seven individual F0 jump-start males were sucessfully used to generate (F1) 'jump-outs'. F1 offspring were selected which had lost the ry^+ marker, indicating loss of the P[ClrB] transposon from the D26 chromosome; the source of transposase was removed by selecting only flies that did not carry the Sb marker on the P[ry^+ Δ 2-3(99B)] chromosome.

Imprecise excision events that extended into a neighbouring essential gene were lethal over the Df(2R)bw^{S46} deficiency, allowing selection of imprecise events by the lack of Δ D26/ Df(2R)bw^{S46} hemizygous progeny. Of the two hundred and seven F1 progeny carrying excision events that were tested for viability over Df(2R)bw^{S46}, ten were lethal over the deficiency; these were therefore imprecise excision events which affected a nearby essential gene.

All the one hundred and ninety seven hemizygous viable excision events were checked for obvious adult morphological defects and were tested for female sterility since the excision may have just removed a possible maternal contribution of a neighbouring gene. Hemizygous viable excisions of D26 over the Df(2R)bw^{S46} were crossed to Df(2R)bw^{S46}/CyO Roi to check a) whether the excisions cause a maternal effect lethal and b) whether the excisions caused a maternal effect lethal which could be paternally rescued. If the excision has caused embryonic lethality by loss of maternal expression of a gene there would be no offspring, but if paternal rescue occurs there would be only Cy progeny (Fig. 5.3.2). None of the hemizygous viable excisions tested had an obvious phenotype or were female sterile, or showed a maternal effect lethality that could be paternally rescued.

5.4 Classification of the Δ D26 chromosomes into complementation groups.

In order to identify the minimum number of adjacent genes affected by the excision of the D26 insertion, the ten imprecise excision events were used to perform complementation tests in an attempt to classify them into complementation groups. The raw data are presented in Table 5.4.1. This information is summarised in Table 5.4.2. The results of these complementation tests suggest that A350 and 33E lie in one complementation group and that 27B and 31A lie in another. 1A, 6C cover both of these groups. 27A and 31H partially cover both groups. 24K and 32E also cover most of the deficiencies in the two groups but they complement each other. 11K is not complemented by 1A or 6C but is partially complemented by all the other deficiencies (Fig. 5.4.3).

5.5 *lac*Z staining of the Δ D26 excision stocks.

Embryos from each of the stocks were collected and stained with the chromogenic substrate of ß-galactosidase, X-gal. 1A, 6C, 27A and 32E showed ß-galactosidase activity (this information is shown in Table 5.4.2).

5.5 Determination of the lethal phase of the Δ D26 chromosomes.

The imprecise excision chromosomes were selected using the criterion that they were hemizygous lethal over Df(2R)bw^{S46}. The lethal phase of each of these chromosomes was determined by looking at hatch frequencies of embryos from the following cross, Δ D26/ CyO X Df(2R)bw^{S46}/ CyO. One quarter of the progeny should be homozygous for the CyO balancer chromosome, these embryos die during embryogenesis. If the observed hatch frequency is 75%, then the dead embryos are the CyO homozygotes and the Δ D26 chromosome over Df(2R)bw^{S46} is not embryonic lethal. If however the hatch frequency is 50%, then the Δ D26 chromosome over Df(2R)bw^{S46} is embryonic lethal. All ten of the crosses showed hatch frequencies of approximately 50%, indicating that the hemizygous lethality occured during embryogenesis (Fig. 5.5.1).

5.6 Cuticular phenotype of dead 1st instar Δ D26/ Df(2R)bwS46 and ΔD26/ ΔD26 larvae.

Hemizygous phenotypes of the excision chromosomes were scored by examining embryos of the following cross D26/ CyO X Df(2R)bw^{S46}/ CyO. Unhatched embryos were collected after 48 hrs and mounted for microscopic inspection. Half of the dead embryos were homozygous for the CyO balancer chromosome and die without any obvious morphological defects. The other half of the dead embryos were the following genotype Δ D26/ Df(2R)bwS46, and were examined for a phenotype.

Embryos were scored for homozygous defects by collecting embryos from each of the deficiency stocks ΔD26/CyO X ΔD26/ CyO. The dead embryos were examined after 24 hours and are of the genotype ΔD26/ ΔD26.

The embryonic head in a large number of the embryos examined showed a phenotype, no obvious defects could be detected in the posterior of these embryos. The number of embryos of each stock tested were counted for wild-type and mutant phenotypes (Table 5.6.1).

The head structures which I scored for in terminally differentiated embryos were as follows: mouth hooks, cirri, maxillary sense organs, median tooth, antennal sense organs, ectostomal sclerite, hypostomal sclerite, epistomal sclerite, cross piece of H-piece, lateralgrate, dorsal bridge, ventral arms, vertical plates, dorsal arms and the presence of the dorsal pouch. These structures in the wild-type first instar head are shown diagramatically in Figure 5.6.2.

The structures in the tail region which I scored for are: anal pads, Filzkorper and the spiracular opening.

In a number of cases, the D26 excisions appeared to cause head defects. No defects were detected in tail cuticular structures. In mutant embryos none of the structures scored for in the embryonic head were missing, but a number of them were disrupted. The Lateralgrate in these embryos is grossly shortened. The ectostomal,

hypostomal and epistomal sclerites are present but disorganised. The dorsal bridge is present but fragmented in appearance (Fig 5.6.3.).

5.7 Discussion.

The generation of excisions from the D26 insertion produced ten imprecise excision events from two hundred and seven independent excision events (4.8%). The criteria used for selection of imprecise excision events was hemizygous lethality over $Df(2R)bw^{S46}$. From the surviving hemizygous viable excision events I recovered none with any obvious adult morphological defects, none of them were maternal effect lethal or maternal effect lethal that could be paternally rescued. As most of these Δ D26 excisions are lethal over A350.1M2, this confirms that the D26 insertion is close to the A350.1M2 insertion, as had been suggested by their similar map positions and *lacZ* expression patterns.

The ten imprecise excision events fell into two complementation groups. A350 and 33E are in one of them and 31A and 27B are in the other, 1A, 6C, cover both of these complementation groups. 24K, 27A, 31H, and 32E partially cover both complementation groups. 11K partially complements all the excisions except 1A and 6C and cannot be accurately placed with respect to the complement groups. The exact genomic deletions contained in these deficiencies can only be determined by genomic southern analysis.

Two complementation groups suggest two genes, to the right of the D26 insertion. The other explanation is that there is only one gene mutated, but at least two sets of regulatory elements. One of the complementation groups seems to only contain hypomorphic alleles 33E and A350.1M2, ie. 33E is homozygous viable, but hemizygous lethal and A350 is post-embryonic lethal. This suggests that this complementation group may not correspond to a structural gene.

All the ten imprecise excision events were shown to be embryonic lethal. The dead embryos of Δ D26 lines 1A, 6C, 27B and 31A showed head skeleton defects. These are likely to be amorphic (null) alleles of the gene responsible for the phenotype

since defects were seen in both homozygous and hemizygous embryos. This tentatively supports the idea that mutations in only one structural gene are being detected.

Many of the excisions are still *lacZ*$^{+}$. These suprisingly are excisions which affect both complementation groups. These therefore are not simple deletions of the D26 insertion. This is further circumstantial evidence that only one gene is being detected by the excision events.

The head defect shows shortening of the lateralgrate and disruption of the dorsal bridge. These excision events are likely to be null alleles of the gene.

These results suggest that there is only one gene being affected by the D26 excisions, whose null phenotype is a head skeleton defect.

Correlation of 3a3 expression with mutant defects.

3a3 expression domains in the embryo are correlated with the head fate maps of Jurgens *et al.*, (1986) in Figure 5.7. The cells that express the 3a3 transcript at the cellular blastoderm are fated to give rise to the hypopharyngeal segment, part of the mandibular segment the anterior midgut and the brain (Fig 5.7a). The dorsal bridge of the head skeleton is included in the 3a3 expression domain. The dorsal bridge is present in the mutant embryos, but is fragmented. The lateralgrate is reduced in size in mutant embryos, but at the blastoderm is not included in the 3a3 expression domain.

In the fate map of the extended germ-band (Fig 5.7b), head structures included in the 3a3 expression domain includes all the structures of the head skeleton. The disruption of the lateralgrate and dorsal bridge is consistent with the expression pattern at this stage. None of these structures have been deleted in the mutant embryos.

The ectostomal, epistomal and hypostomal sclerites appear to be present but disorganised. The epistomal sclerite is included in the 3a3 blastoderm expression domain, but the hypostomal sclerite is outside this domain, and the ectostomal sclerite is not marked on the fate map of Jurgens *et al.*, 1986.

Mutation	Map position genetic cytological	Phenotype scored for	Mutation/A350.1M2 Complements (+)
heavy vein - hv	104.0	thickened wing veins	hv+
abbreviated - abb	105.5 59E2;60B10	smaller bristles, especially posterior scutellars	abb+
minus - mi	104.7 59D6;E4	bristles almost as small as hairs	mi+
slight - slt	106.3	small fly, short thin bristles	slt+
Plexate - Px	107.2 60C6;D	viability	+
purploid - pd	106.4 59E2;60B10	dark pinkish, maroon eyes	pd+
lanceolate - ll^2	106.7 59E2;60B10	wings narrow at tips, small bulging eyes, narrow head	ll+
seizure - sei	106.0 60A7;B10	paralysed at temperatures above 38°C	sei+
morula - mr	106.7 59E2;60B10	rough eye, irregularly reduced bristle size and number.	+

Table 5.1 Mutations in the region surrounding the A350.1M2 insertion and results of complementation tests.

+ fully complements
- does not complement.

The results of the complementation tests were obtained from D. Bhatia (pers. comm.).
Map positions were obtained from 'the red book', Lindsley and Zimm, (1992)

A

	map position cytological	Phenotype scored for	Mutation / A350 complements +
Df(2R)bwDRj	59C5;59F6-8*	viability	+
Df(2R)bwDRa	59E1;60A4-5*	viability	(+) 6%
Df(2R)bwDrp	59D3-4;59F5-6*	viability	(+) 6%
In(2LR)610#7.5	Df(2R)59F3;60B1, In(2LR)	viability	+
Df(2R)bw^{S46}	59D8-11;60A7	viability	-
D26 P[ClrB] insertion	59F	viability	+

B

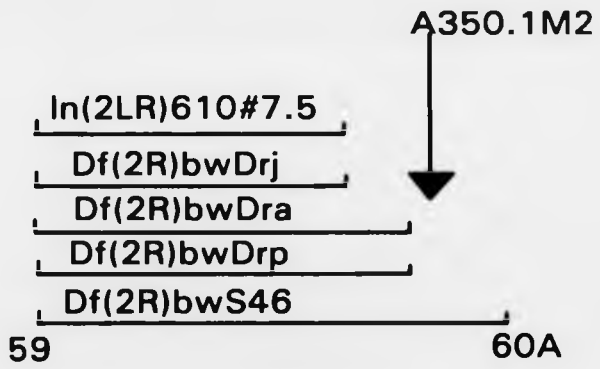


Figure 5.2
A Complementation tests between A350.1M2 and several deficiencies in the region.
B Approximate deficiency map suggested by the results of the complementation tests in A.

\+ shows that the A350.1M2 insertion is fully complemented by the deficiency
\- shows that the A350.1M2 insertion is not complemented by the deficiency.
(+) shows partial complementation, the % figure refers to the number of these individuals observed if the expected number is 100%.
* the cytology of these deficiencies is tentative (Bruce Reed, pers. comm.) but my results suggest the approximate map given above.

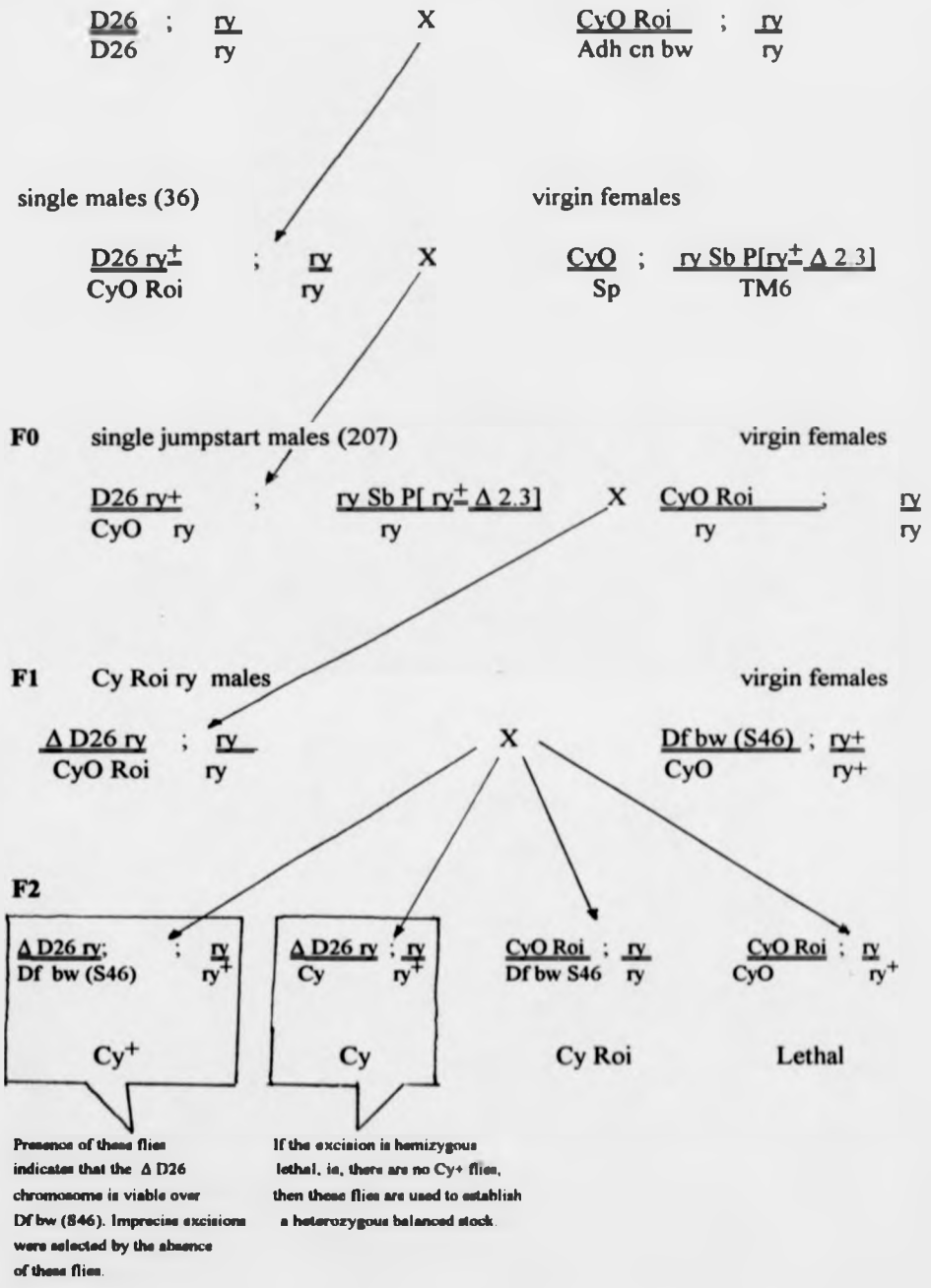


Figure 5.3.1 Strategy for the generation of imprecise excisions of the D26 insertion.

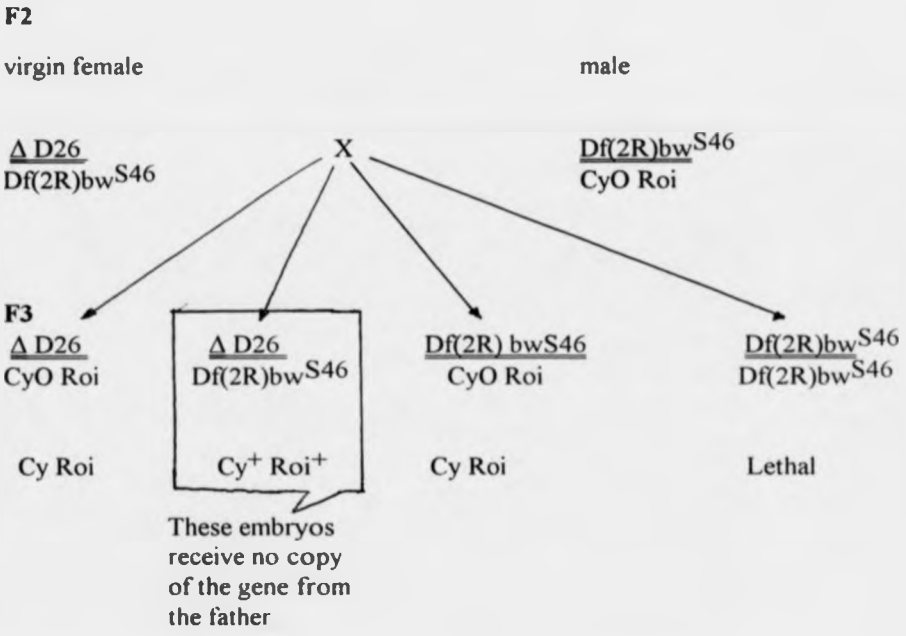
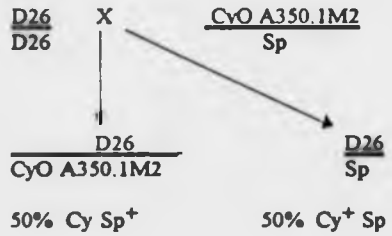


Figure 5.3.2. Surviving hemizygous excision events over the Df(2R)bwS46 were tested for maternal effect lethality using the above strategy.

If the excision event has knocked out a possible maternal expression of a gene then there will be no offspring, but if paternal rescue occurs there will be offsping but there will be no straight winged flies.

1)



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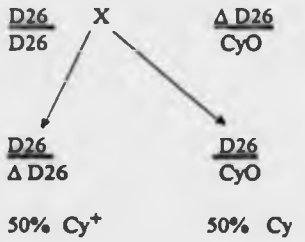
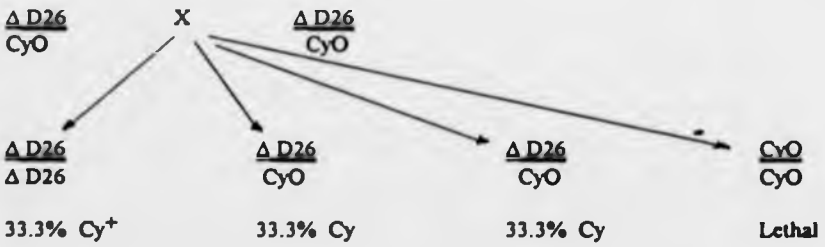


3)



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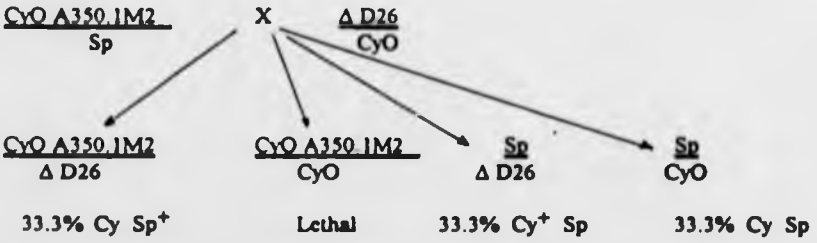


The expected number of offspring of a genotype of interest was calculated as the number of flies one would expect to hatch if that genotype was totally viable. In cases where the flies were either non viable or partially viable the expected figure is calculated from the number of flies of the other genotypes obtained.

	D26	A350	Df546	1A	6C	11K	24K	27A	27B	31A	31H	32E	33E
D26 E		46	95	90	134	67	74	57	71	61	70	70	66
O		39	82	67	97	59	70	43	56	45	59	49	68
%	100%	84%	86%	74%	72%	88%	95%	75%	79%	74%	84%	70%	103%
A350 E			49	65	57	48	53	35	29	22	36	42	55
O			0	0	0	37	9	6	24	26	0	0	0
%		0%	0%	0%	0%	79%	17%	17%	83%	118%	0%	0%	0%
Df546 E			82	98	76	88	79	67	70	91	47	59	57
O			0	0	0	0	0	0	0	0	0	0	0
%			0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
1A E				116	110	84	81	90	74	84	75	70	85
O				0	2	0	0	0	0	0	0	0	0
%				0%	2%	0%	0%	0%	0%	0%	0%	0%	0%
6C E					130	49	69	70	60	66	73	83	88
O					0	0	0	0	0	0	0	0	0
%					0%	0%	0%	0%	0%	0%	0%	0%	0%
11K E						78	82	99	77	96	41	82	92
O						0	4	36	11	3	5	1	22
%						0%	5%	36%	14%	3%	12%	1.2%	24%
24K E							47	86	48	100	78	72	89
O							0	0	0	0	0	17	0
%							0%	0%	0%	0%	0%	24%	0%
27A E								83	74	47	79	75	72
O								0	0	0	6	0	22
%								0%	0%	0%	8%	0%	31%
27B E									75	68	104	81	94
O									0	0	17	0	59
%									0%	0%	16%	0%	63%
31A E										47	72	79	119
O										0	0	0	34
%										0%	0%	0%	29%
31H E											66	59	40
O											0	0	6
%											0%	0%	15%
32E E												74	74
O												0	0
%												0%	0%
33E E													88
O													41
%													47%

Table 5.4.1 Complementation tests between A350.1M2, D26, Df(2R)bwS46 and the ten Δ D26 imprecise excision chromosomes.

E- expected number of offspring of the genotype represented by that square if they are viable. The crossing schemes opposite (page 33) show the various expected percentage of the genotype of interest.
O- is the observed number of offspring of that genotype
%- is the percentage of offspring observed if the expected number is 100%

	D26	A350	Df346	1A	6C	11K	24K	27A	27B	31A	31H	32E	33E
D26	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A350	+++	-	-	-	-	+++	+	+	+++	+++	-	-	-
Df346	+++	-	-	-	-	-	-	-	-	-	-	-	-
1A	+++	-	-	-	(-)	-	-	-	-	-	-	-	-
6C	+++	-	-	(-)	-	-	-	-	-	-	-	-	-
11K	+++	+++	-	-	-	-	(-)	++	+	(-)	+	(-)	++
24K	+++	+	-	-	-	(-)	-	-	-	-	-	++	-
27A	+++	+	-	-	-	++	-	-	-	-	+	-	++
27B	+++	+++	-	-	-	+	-	-	-	-	+	-	+++
31A	+++	+++	-	-	-	(-)	-	-	-	-	-	-	++
31H	+++	-	-	-	-	+	-	+	+	-	-	-	(-)
32E	+++	-	-	-	-	(-)	++	-	-	-	-	-	-
33E	+++	-	-	-	-	++	-	++	+++	++	(-)	-	+++
lacZ activity	+	+	-	+	+	-	-	+	-	-	-	+	-
[illegible]	viable	viable	n.t.	+	+	-	-	-	+	+	-	(+?)	viable
[illegible]	viable	viable	n.t.	+	+	n.t.	-	n.t.	+	n.t.	-	n.t.	(+?)

Table 5.4.2 Summary of Complementation test results with D26, A350, Df bw^{S46} and the ten imprecise excision chromosomes.

The crosses used in the complementation tests and the numbers of each genotype expected in the progeny are shown on the facing page.
the following only refer to the complementation tests:

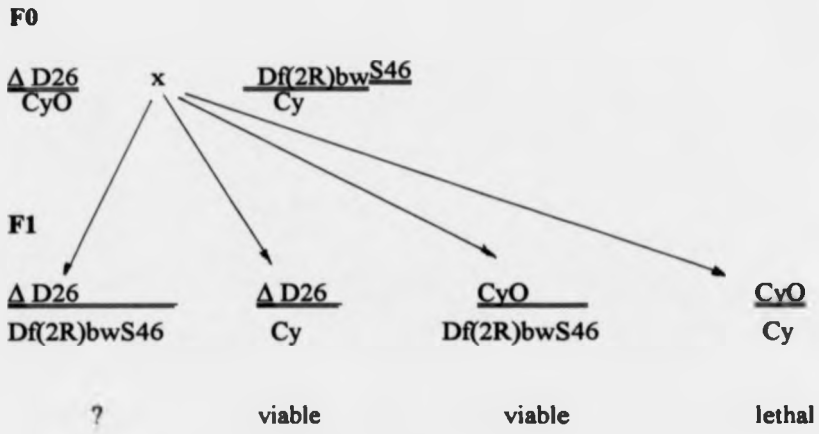
+++ >40% of the expected number of flies of this genotype
++ 20-40% of the expected number of flies of this genotype
+ 5-20% of the expected number of flies of this genotype
(-) 0-5% of the expected number of flies of this genotype
- No flies of this genotype (does not complement)

lacZ expression in the deficiency lines.

+ positive for ß-galactosidase activity
- negative for ß-galactosidase activity

Head phenotypes in unhatched larvae.

homozygous individual are of the genotype D26/ D26
hemizygous individuals are of the genotype D26/ CyO
+ means disrupted head structures were present.
n.t. means that this genotype was not tested.
0 no mutant larvae.



line being tested Δ D26	Total number of embryos	number of embryos unhatched after 48hrs	% of embryos unhatched	Embryonic lethal
Df(2R)bw^{S46}	900	519	58%	yes
1A	275	123	45%	yes
6C	780	403	52%	yes
11K	167	82	49%	yes
24K	464	197	42%	yes
27A	620	408	66%	yes
27B	158	84	53%	yes
31A	504	320	63%	yes
31H	237	133	56%	yes
32E	432	270	63%	yes
33E	405	261	64%	yes

Figure 5.5.1 Testing for embryonic lethality by determination of hatch frequencies of the deficiency and the ten imprecise excisions when hemizygous over Df(2R)bw^{S46}.

These imprecise excision events were selected by lethality over the Df(2R)bw^{S46} chromosome, but the stage at which they die had not been determined. One quarter of the progeny will die because of the recessive lethals on the CyO chromosome. If 25% do not hatch then the lethality does not occur during embryogenesis. If 50% do not hatch then then the lethality does occur during embryogenesis.

All of the excision events over the deficiency had hatch frequencies of ~50% and were therefore embryonic lethal.

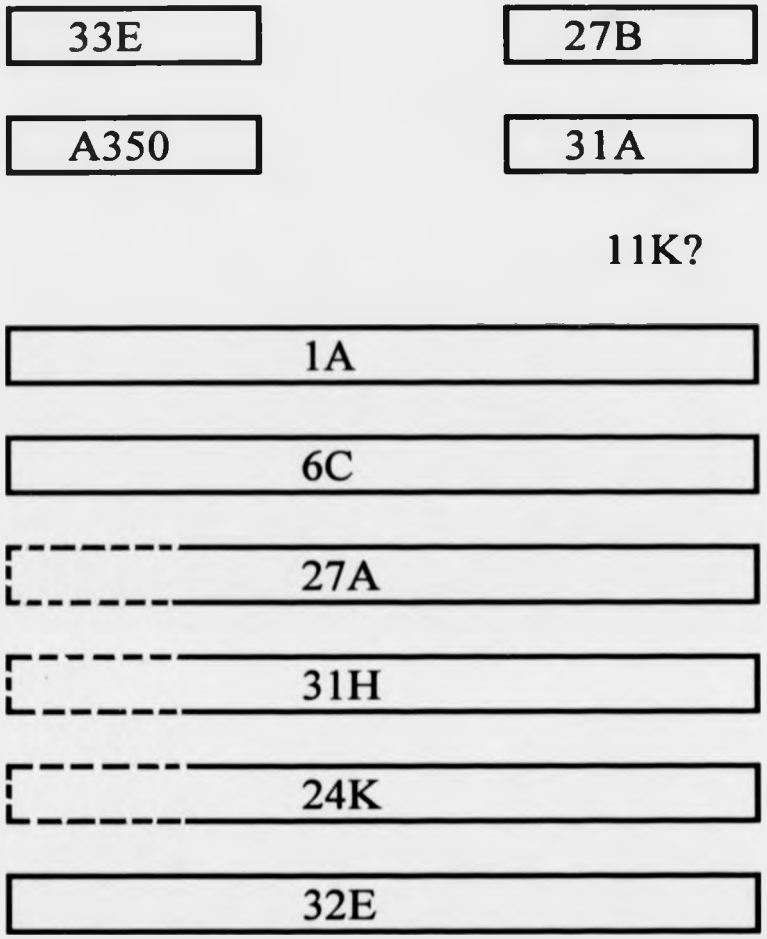


Figure 5.4.3. Proposed complementation groups for A350.1M2 and the ten Δ D26 chromosomes.

Homozygous Δ D26 chromosomes	Wild-type	head defect	% of mutant larvae	Hemizygous Δ D26 chromosomes	Wild-type	head defect	% of mutant larvae	null alleles
1A/1A	25	28	53%	1A/Df	10	3	23%	+
6C/6C	13	9	41%	6C/Df	6	2	25%	+
11K/11K	27	0	0%					
24K/24K	21	0	0%	24K/Df	25	0	0%	
27A/27A	37	0	0%					
27B/27B	30	27	47%	27B/Df	15	4	21%	+
31A/31A	23	10	30%					(+)
31H/31H	17	0	0%	31H/Df	19	0	0%	
32E/32E	6	5	45%					(+)
33E/33E	21	0	0%	33E/Df	17	10	37%	

Table 5.6.1. The cuticular phenotype of unhatched larvae derived from the Δ D26 imprecise excision stocks.

Unhatched larvae homozygous for the ΔD26 chromosome were collected from each of the stocks ΔD26/ CyO X ΔD26/ CyO. Half of the unhatched larvae are of the genotype CyO/ CyO which die with no obvious morphological defects. If Δ D26/ΔD26 homozygotes have a phenotype then 50% of the unhatched larvae are expected to show defects.

Unhatched larvae hemizygous for the ΔD26 excision chromosome were collected from the following cross ΔD26/ CyO X Df(2R)bw^{S46}/ CyO. Again half of the unhatched larvae will be CyO/ CyO, the other half will be ΔD26/ Df(2R)bw^{S46}. If the hemizygous individuals have a mutant phenotype it should be present in 50% of the unhatched larvae.

\+ null alleles show a head phenotype both as homozygotes and hemizygotes.
(+) possible null alleles, homozygotes show head defects. Hemizygotes have not been examined.

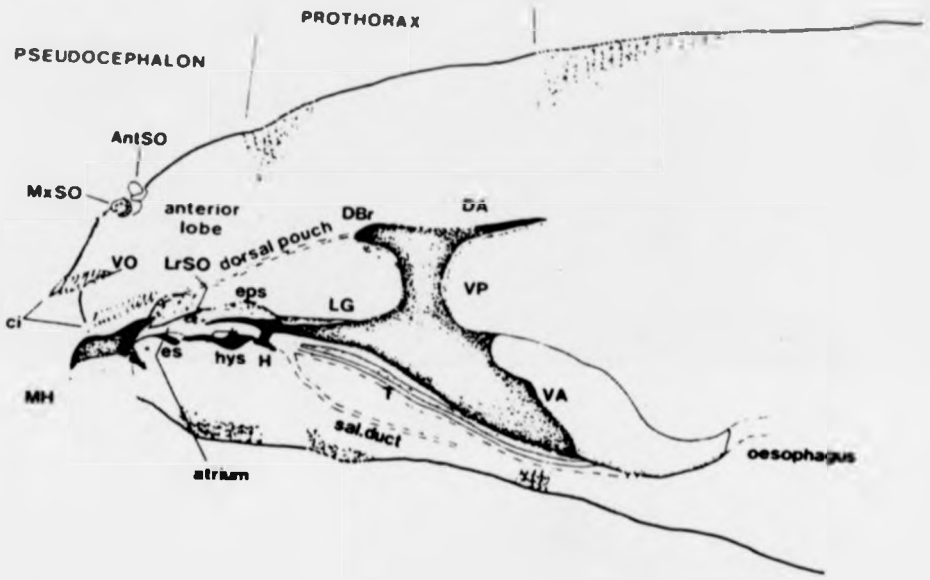


Figure 5.6.2. Camera lucida drawing of the head of wild-type first-instar larvae.

Abbreviations: *AntSO*- antennal sense organ; *ci*- cirri; *DA*- dorsal arm; *D Br*- dorsal bridge; *eps*- epistomal sclerite; *es*- ectostomal sclerite; *hys*- hypostomal sclerite; *LG*- lateralgrate; *MH*- mouth hook; *VA*- ventral arm; *VP*- vertical plate (Jurgens, 1987).

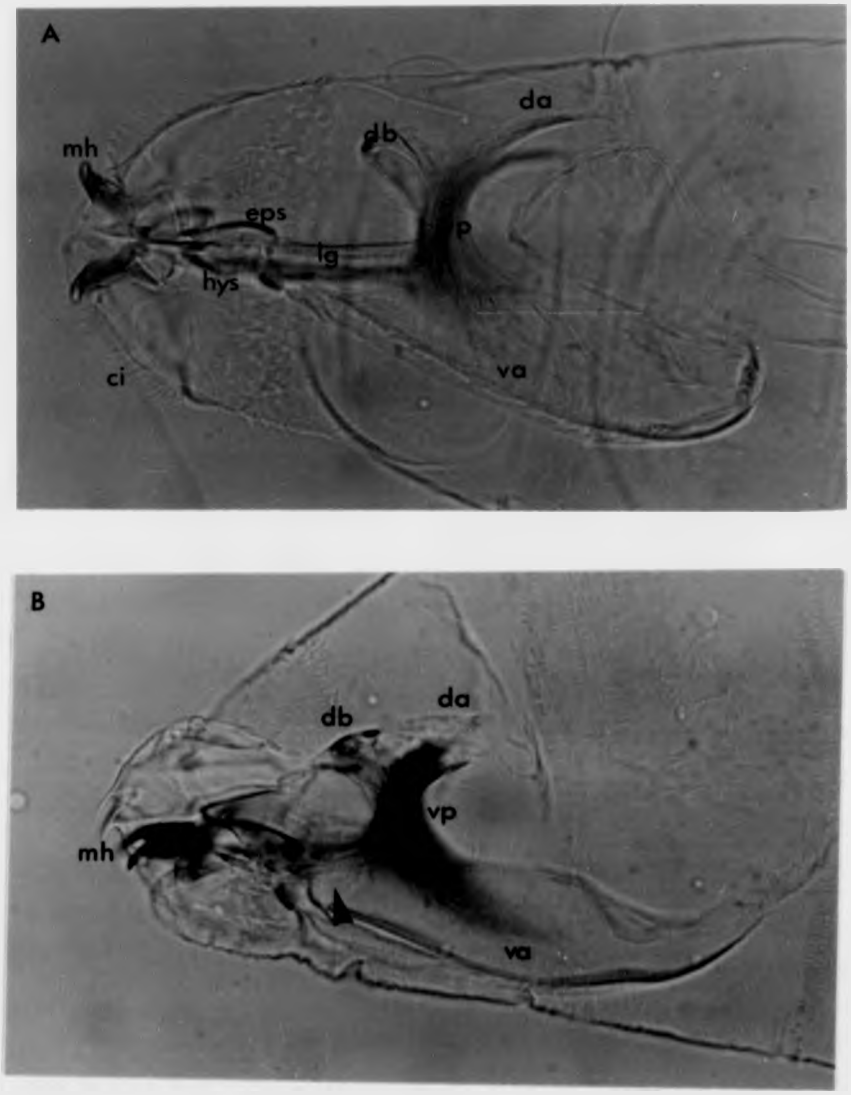


Figure 5.6.3. The phenotype of ΔD26 null alleles.

A- shows the cuticular structures present in the head of a wild-type 1st instar larva.

B- the head skeleton of ΔD26 null alleles.

Abbreviations: *ci*- cirri; *DA*- dorsal arm; *D Br*- dorsal bridge; *eps*- epistomal sclerite; *es*- ectostomal sclerite; *hys*- hypostomal sclerite; *LG*- lateralgrate; *MH*- mouth hook; *VA*- ventral arm; *VP*- vertical plate.

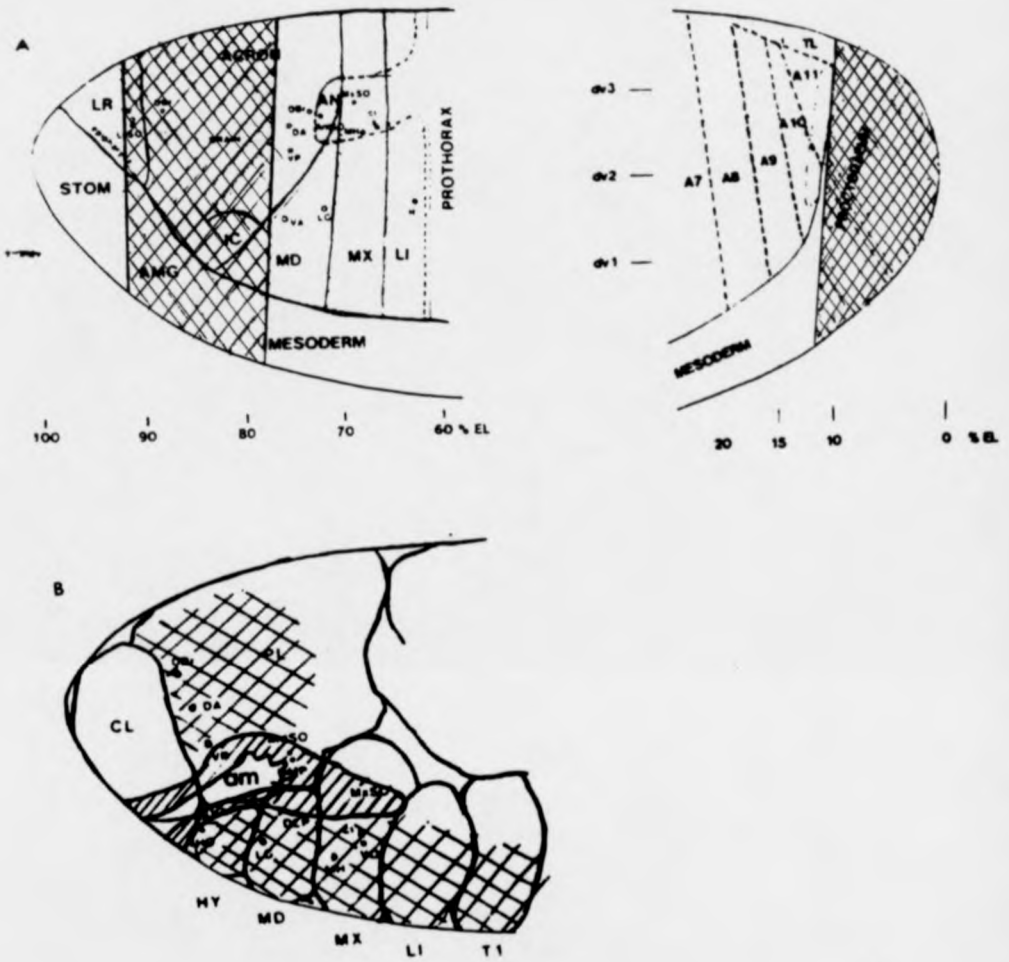


Figure 5.7 Fate maps of head structures at the blastoderm **A**, and extended germband stage **B** of Drosophila embryos (Jurgens *et al.*, 1987). The pattern of hybridisation of the cDNA 3a3 has been draw onto these maps and is indicated by cross hatching.

Chapter 6

Other Enhancer detector lines studied

6.1 Introduction.

A number of P-transposon enhancer trap strains which had been generated in a previous screen, and had been shown to be homozygous lethal were available in the laboratory (Bellen *et al.*, 1989). These lines were potentially interesting since the P-insertion must have disrupted a gene which was essential for viability.

I screened these twenty lines for embryonic morphological and CNS defects. Since the nervous system is an internal structure, a method of marking these cells was required. The cell markers I used were the antibody to horse radish peroxidase (HRP), which fortuitously recognises *Drosophila* neuronal cell surfaces. This antibody labels the entire neuropile (Jan and Jan, 1982), and therefore enabled me to detect any gross morphological defects. I also used antibodies to the engrailed and even-skipped proteins. These antibodies each label small subsets of segmentally repeated neurons (Frasch *et al.*, 1987; DiNardo *et al.*,1985), and enabled me to detect alterations in any of these cells.

If one of these lines should have an interesting phenotype, the presence of the P-element insertion would allow it to be immediately amenable to genetic and molecular analysis.

6.2 Screening the lethal P-element stocks for a embryonic phenotype.

The lethal P-element insertions were maintained as heterozygotes over a balancer chromosome. For this reason only one quarter of their offspring will be homozygous for the insertion, and should therefore exhibit any phenotype.

From the twenty lethal P-elements screened only two lines showed mutant morphological phenotypes.

6.3 Enhancer trap line A326.2F3

6.3.1 Phenotype

Antibody staining of line A326.2F3 with antibodies to HRP protein showed that A326.2F3 mutant embryos had no ventral epidermis, since neural specific staining extended right to the ventral surface of the embryo. Antibodies to engrailed and even-skipped also showed that these embryos had more neural tissue than wild-type embryos (Fig 6.3.1).

This phenotype is consistent with a mutation in one of the neurogenic genes Complete lack of function of any one of the neurogenic genes, results in all the cells of the neural ectoderm developing into nervous system at the expense of epidermis (de la Concha *et al.*, 1988).

The ß-galactosidase expression pattern of A326.2F3 is strong in the proctodeum, midgut and oesophagus of germ band extended embryos. In late embryos (stage 11, 5:20 hrs) ß-galactosidase expression can be seen faintly in the CNS and in a few cells of the PNS (Data not shown).

6.3.2 Precise excision of the P-element from line A326.2F3 to check that the P-insertion is responsible for the lethal phenotype.

The P-element was excised from line A326.2F3 by transposase mediated excision. The cross performed is shown in (Fig. 6.3.2).

The result of this experiment showed that the homozygous lethality of line A326.2F3 could be reverted to viability by excision of the P-element. Therefore the lethality must be due to the presence of the insertion and not due to another mutation elsewhere on the chromosome.

6.3.3 Determination of the cytological map position of the A326.2F3 insertion.

A biotin labelled *lacZ* probe was hybridised to A326.2F3 polytene chromosomes, and was shown to be 92A (Fig. 6.3.3). This map position corresponds to the neurogenic gene *Delta*.

6.3 Enhancer trap line A326.2F3

6.3.1 Phenotype

Antibody staining of line A326.2F3 with antibodies to HRP protein showed that A326.2F3 mutant embryos had no ventral epidermis, since neural specific staining extended right to the ventral surface of the embryo. Antibodies to engrailed and even-skipped also showed that these embryos had more neural tissue than wild-type embryos (Fig 6.3.1).

This phenotype is consistent with a mutation in one of the neurogenic genes. Complete lack of function of any one of the neurogenic genes, results in all the cells of the neural ectoderm developing into nervous system at the expense of epidermis (de la Concha *et al.*, 1988).

The ß-galactosidase expression pattern of A326.2F3 is strong in the proctodeum, midgut and oesophagus of germ band extended embryos. In late embryos (stage 11, 5:20 hrs) ß-galactosidase expression can be seen faintly in the CNS and in a few cells of the PNS (Data not shown).

6.3.2 Precise excision of the P-element from line A326.2F3 to check that the P-insertion is responsible for the lethal phenotype.

The P-element was excised from line A326.2F3 by transposase mediated excision. The cross performed is shown in (Fig. 6.3.2).

The result of this experiment showed that the homozygous lethality of line A326.2F3 could be reverted to viability by excision of the P-element. Therefore the lethality must be due to the presence of the insertion and not due to another mutation elsewhere on the chromosome.

6.3.3 Determination of the cytological map position of the A326.2F3 insertion.

A biotin labelled *lacZ* probe was hybridised to A326.2F3 polytene chromosomes, and was shown to be 92A (Fig. 6.3.3). This map position corresponds to the neurogenic gene *Delta*.

6.3.4 Complementation with a point mutation in Delta.

The neurogenic phenotype and cytological map position both suggest that the insertion is in the gene *Delta*. The expression pattern are similar to but weaker than *Delta*. To test whether the A326.2F3 insertion is in *Delta* it was crossed to a *Delta* point mutation. A326.2F3 failed to complement a point mutation in *Delta* and therefore must have disrupted this gene (Fig. 6.3.4).

6.4 Enhancer trap line A131.1F3

6.4.1 Phenotype

The embryonic phenotype seen in A131.1F3 embryos is variable, but it does consistently show some kind of segmental defect. The embryos often show irregularities in segment width or depth of the segmental furrows in a few thoracic or abdominal segments and adjacent segments are sometimes partially fused. Staining with even-skipped antibodies showed that in some but not all mutant embryos, there is some disruption of the embryonic CNS (Fig. 6.4.1).

The ß-galactosidase staining pattern in line A131.1F3 occurs in the dorso-posterior part of each segment and is stronger in alternate segments (data not shown). This expression pattern has been reported to occur in a large number of P-insertion lines, and is thought to be due to some regulatory component in P[lArB] rather than due to a nearby genomic enhancer.

6.4.2 Precise excision of the P-element from the A131.1F3 chromosome to determine that the insertion was responsible for the lethality.

The same crossing scheme as for A326.2F3 was used to excise the P-element. The results of this experiment showed that the lethality of A131.1F3 was due to the P[lArB] insertion (Fig. 6.3.2).

6.4.3 Determination of the cytological map position of the P-insertion A131.1F3.

A biotin labelled probe to *lac*Z was hybridised to polytene chromosomes from line A131.1F3. The map position of this insertion was shown to be 65C-D (Fig. 6.4.3.1). I tested all the available point mutations in the region for complementation with A131.1F3 and showed that it was in none of these genes (Table 6.4.3.2). There are also four cloned genes known in this region also shown in Table 6.4.3.2).

6.4.4 Is the morphological phenotype associated with embryos homozygous for the P-insertion?

Due to the variability of the phenotype it was necessary to confirm that embryos with this phenotype were in fact homozygous for the P-element insertion. A way of unambiguously identifying embryos that are homozygous for the insert is to make the insertion heterozygous with a balancer chromosome with a strong ß-galactosidase expression pattern. Embryos homozygous for the insertion will be the only ones that do not exhibit the strong expression of the balancer chromosome. The embryos homozygous for the insertion also have ß-galactosidase expression but this expression pattern is different from the balancer.

The cross and the genotypes of the resulting progeny are shown in Figure 6.4.4. The embryos were collected and stained with antibodies to *lac*Z, and then examined by microscopy. 75% of the embryos showed the strong expression associated with the blue balancer. The other 25% were homozygous for the A131.1F3 insertion. The phenotype could not be seen in many of the embryos which were homozygous for the insertion and also the phenotype could be seen in some embryos that carried the *lac*Z marked balancer. This result showed that the phenotype was not associated with the P-insertion.

6.5 Discussion

6.5.1 A326.2F3

This insertion has been shown to be in the neurogenic gene *Delta*. The neurogenic phenotype in this line is consistent with this as is the ß-galactosidase expression patttern. The cytological map position is the same as the gene *Delta* (Vassin *et al.*, 1987) and most conclusively, insertion A326.2F3 failed to complement a point mutation in *Delta*.

Delta has already been extensively studied both genetically and molecularly. I therefore discontinued working on this line.

6.5.2 A131.1F3

The variable phenotype in line A131.1F3 can be explained in a number of ways. First it might have produced a hypomorphic mutation in the gene it has inserted into. In a hypomorphic mutation the gene function is reduced rather than removed completely. Secondly the insertion may have caused a null mutation of the gene, but the function of the gene is partially redundant; ie., there are one or more other genes whose function can partially compensate for the loss of the first gene.

The ß-galactosidase expression pattern of this line is not particularly enlightening, since it seems to be specific to P[lArB] rather than to a nearby genomic regulatory element. This means that one cannot correlate the expression pattern to the morphological defects observed.

The P-insertion in line A131.1F3 mapped to cytological region 65C-D, there are a number of point mutations and four molecularly cloned genes in this region. A131.1F3 insertion is in none of the point mutations tested. None of the cloned genes in this region are likely to be associated with the kind of phenotype observed in this line.

The result that the phenotype was not associated with the P-element insertion meant that I discontinued working on this line.

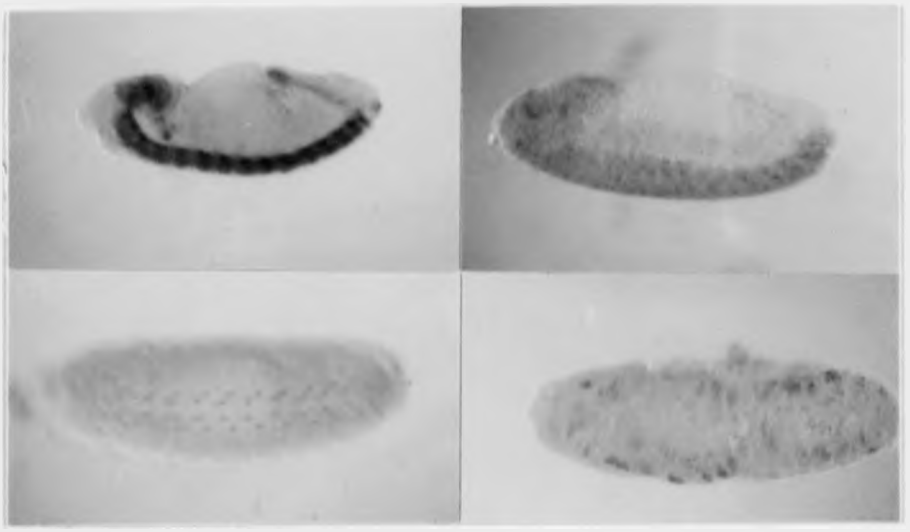


Figure 6.3.1 The phenotype of mutant enbryos from the A326.2F3 insertion line.

The two upper embryos are labelled with antibodies to HRP which light up all neural tissue. The lower two embryos are labelled with antibodies to even-skipped which light up a subset of the cells of the CNS.
The embryos on the left are wildtype, those on the right are homozygous for A326.2F3. The evenskipped and HRP staining show the presence of an abnormally large number of neural cells. The HRP staining shows that this embryo has no epidermis.

abbreviations: **nc**; neural cells, **e**; epidermis

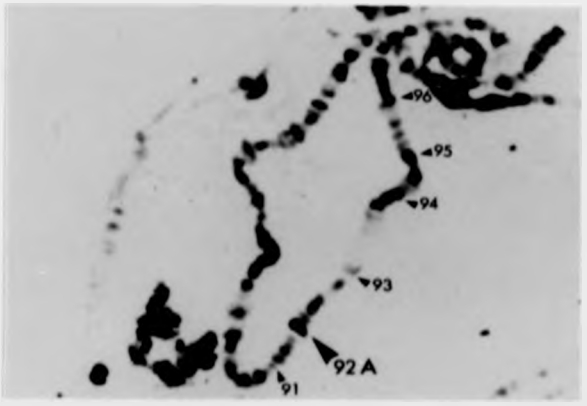
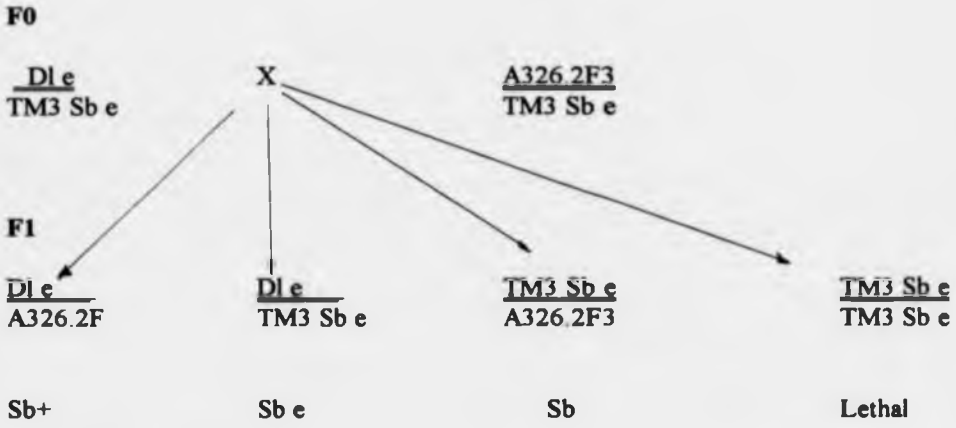


Figure 6.3.3 The cytological map position of P-insertion A326.2F3

Polytene chromosomes from line A326.2F3 were probed with a lacZ probe to identify the insertion site of the P-element. Hybridisation can be seen at cytological band 92A.



Number of flies of each genotype counted		
None	148	123

6.3.4 Complementation test between A326.2F3 and a point mutation in Delta.

There were no Sb+ progeny indicating that A326.2F3 does not complement the point mutation in Delta.

F0

Cross the heterozygous P-insertion stock to the transposase producing strain.

P[ry+] A326.2F3 / TM3 Sb X P[ry+ Δ 2-3](99B) / TM6 Ubx

F1

Select Sb Ubx+ males; these carry both the transposase producing chromosome and the P-insertion chromosome.

P[ry+] A326.2F3 / P[ry+ Δ 2-3](99B) Sb X TM3 Sb ry / rf10

F2

Select ry brothers and sisters for individual sibling matings; loss of ry+ markers means that some kind of excision event has occured.

ΔA326 ry / TM3 Sb X ΔA326 ry / TM3 Sb

F3

Check for viability of excised A326.2F3 ry homozygotes

ΔA326.2F3 ry / TM3 Sb	ΔA326.2F3 ry / TM3 Sb	ΔA326.2F3 ry / ΔA326.2F3 ry	TM3 Sb / TM3 Sb
			lethal

F3 expected phenotypes

Sb	Sb	Sb+

If there are no Sb+ progeny then A326.2F3 homozygotes are lethal.

Observed phenotypes

	matings where only Sb flies are observed in F3	matings where Sb+ and Sb+ flies are observed
A326.2F3	13	7
A131.1F3	15	3

Figure 6.3.2 Reversion cross to test viability of the A326.2F3 and the A131.1F3 chromosomes after excision of the P-element.

The presence of Sb^+ flies in the F3 generation shows that the homozygous excision chromosome is viable. The viability after excision suggests that the P-insertion was responsible for the lethality.

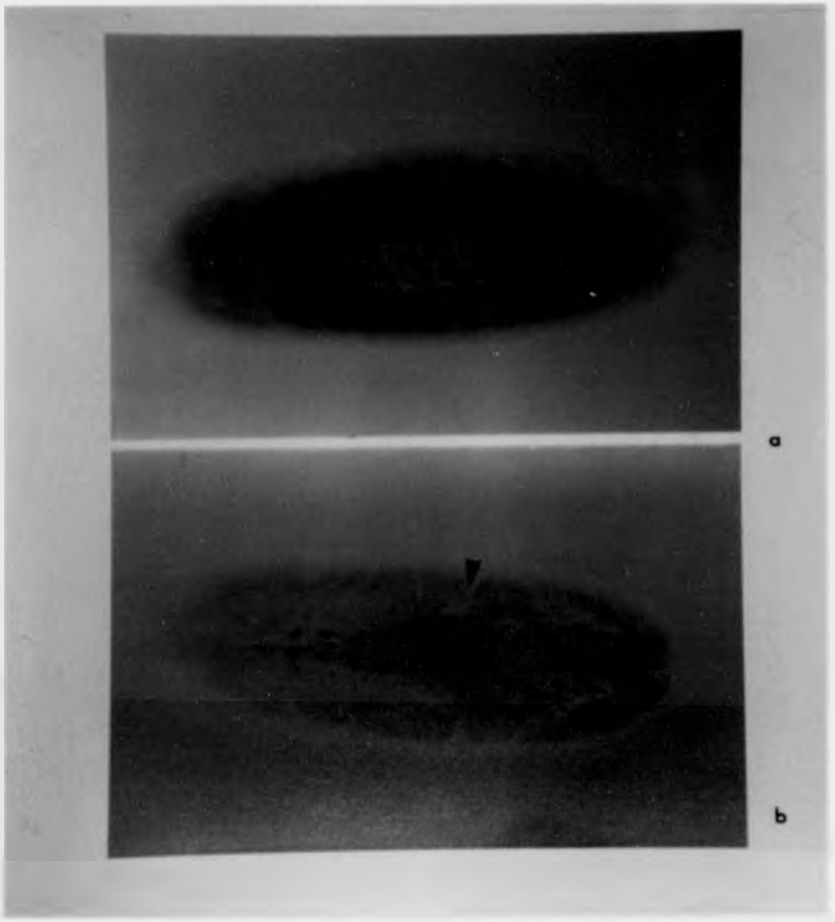


Figure 6.4.1 The embryonic phenotype in line A131.1F3.

A- ventral view of a wildtype embryo after germ band retraction (stage13). The segments are regularly spaced and the segmental furrows are of a constant depth. The embryos have been stained with antibodies to evenskipped protein.

B- is a ventral view of a mutant embryo at an equivalent stage. Irregularities in segmental width and depth of the segmental furrows (arrowheads) can be seen. The staining of evenskipped can be seen to be disrupted in the abdominal regions.

Figure 6.4.3.1 The cytological map position of the P-insertion in line A131.1F3.

Map position was determined by hybridisation of a *lac*Z probe to polytene chromosomes from A131.1F3. The P-insertion maps at 65C/D.

name	map position		Complemented by A131.1F3 (+)
Moire	64C12-65E	3.19.2	+
javelin	64C12-65E	3.19.2	+
shrew		3.15	+
nudel		3.17	+
spook		3.19	+
l(3)tr		3.20	+
ple		3.18.8	+
SG8		3.19.2	+
SG9		3.22.2	+
SG10		3.23.3	+
tyrosine hydrolase pale	65C		
G protein α subunit (DGal)	65C		
POU III domain (Cf1a) DNA binding transfactor	65D		
PRD gene 3 paired homology	65D		

Table 6.4.3.2. The point mutations and cloned genes in the region around 65C-D.

These are the point mutations in the region that I tested for complementation with A131.1F3. A131 was shown not to be in any of these genes.

F0
insertion line A131.1F3 is crossed to the stock carrying the Balancer marked with *lac*Z P[C41]

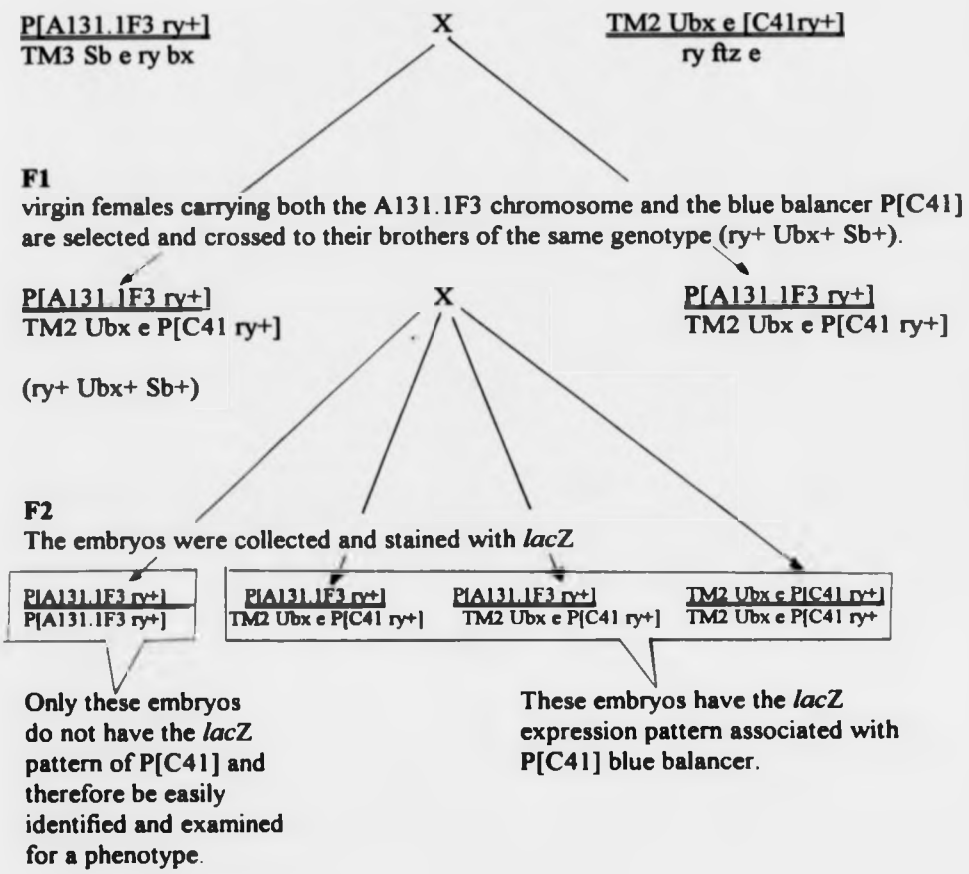


Figure 6.4.4 Cross to allow unambiguous identification of embryos homozygous for A131.1F3.

Chapter 7

General Discussion.

The work presented in this thesis describes the identification of a novel gene required for pattern formation of the larval head. This gene had not been previously identified in conventional mutagenesis screens. The gene was detected by an enhancer trap screen on the basis of the ß-galactosidase expression pattern of an enhancer detector transposon insertion, A350.1M2.

Initial characterisation of the P[lArB] insertion A350.1M2.

The early ß-galactosidase expression pattern of this line was considered to be of interest, because expression occurs in a spatially restricted manner very early during embryogenesis. The early nature of the expression pattern of this line, suggests that a flanking gene, with a similar pattern of expression, may be involved in early cell fate decisions. The insertion was mapped to 59F1-3 on chromosome 2R. The A350.1M2 insertion was shown to cause hemizygous lethality. This shows that it has disrupted at least to some extent an adjacent gene that is essential for development. For the insertion to have disrupted the function of this essential gene the insertion must have disrupted either the structural gene or the regulatory elements. Another explanation is that the insertion separates the structural gene from the regulatory elements.

A P[ClrB] insertion, D26, also seemed to be inserted near this same essential gene. This was suggested by its expression pattern and cytological map position. This was subsequently confirmed by non complementation of deletions made by imprecise excision of the D26 insertion, with A350.1M2. The D26 insertion is homozygous and hemizygous viable. This suggests that the insertion has not disrupted the gene.

The ß-galactosidase expression from the enhancer detector transposon A350.1M2 has been drawn onto the *Drosophila* blastoderm fate map and the fate map of the head at germ band extension, to determine the final fate of the labelled cells.

During early gastrulation the posterior expression corresponds to cells which become the posterior midgut, Malpighian tubules and proctodeum. Expression in the anterior corresponds to cells which become the anterior midgut and cephalic neural and epidermal cells. By germ band extension the expression corresponds to the gnathal segments and the hypopharyngeal lobe of the head, and a large number of cells of the CNS.

Molecular characterisation of the genomic region flanking the A350.1M2 insertion.

Genomic DNA flanking the insertion was obtained from a previously isolated plasmid rescue clone and a cosmid clone from a walk close to the *brown* gene. The genomic region was restriction mapped and orientated onto the cytological map. Two fragments from this region which had been shown to contain only unique sequences were used to screen a cDNA library. cDNA clones were isolated with only one of these fragments the A350.1M2 plasmid rescue clone. The clones were characterised by their restriction patterns and the sizes of these clones were determined. Sequencing showed that clone 3a3 and 19 were the same at the 3' end but differed at the 5' end, the other clone 6 was different from these clones at both 5' and 3' ends. Clone 19 seems to be the same gene as 3a3 but is truncated at the 5' end. The other cDNA clone 6 seemed to be a different gene which may actually map at a different chromosomal location.

Fragments from each of these cDNA clones were used as probes to detect the RNA expression in situ in whole mount *Drosophila* embryos. cDNA clones 19 and 3a3 gave expression patterns which were strongly reminiscent of the ß-galactosidase expression pattern in A350.1M2. The expression of cDNA 3a3 can be seen slightly earlier than the first expression in A350.1M2. During cellular blastoderm this expression occurs in two broad stripes in the anterior and posterior of the embryo. These cells correspond to the same cells that slightly later express *lacZ* in A350.1M2. By germ band extension the expression is very similar to that of A350.1M2. The other cDNA clone 6 shows no expression during embryogenesis.

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The cDNA clones were mapped onto the genomic walk clone 19 and 3a3 map between +6 and +8.3kb on the genomic map. The cDNA clone 3a3 is 6kb to the right of the A350.1M2 insertion, and approximately 50 kb to the right of the *brown* gene.

The cDNA clone 3a3 was sequenced in both strands and this sequence was used to predict the peptide sequence in all three frames. The frames program on GCG which shows all possible translational starts and stops showed that there was no long open reading frame in any of the three frames. The two longest open reading frames ORF1 and ORF2 are 92 and 88 amino acids long and both occur in the same frame. The best homologies to known protein sequences on the Swissprot data base are, a Sodium channel protein from rat cardiac muscle which shares 28.1% homology over 32 amino acids, and a minor core protein V which has 35.1% identity over 37 amino acids, for ORF1 and ORF2 respectively. The sequence has provided little information about the function of the gene.

There is a possibility that there is another transcript in the region that I did not detect. *sloppy paired* has two transcripts that are expressed in very similar patterns. In the case of *sloppy paired* both transcripts have long open reading frames.

Genetic characterisation of A350.1M2.

The A350.1M2 insertion had partially disrupted the gene which it had inserted into since it was hemizygous lethal over a deficiency in the region. The A350.1M2 insertion did not cause embryonic lethality. I wanted to isolate some more severe alleles in particular ones which were embryonic lethal. The viable insertion D26 was excised by transposase mediated excision and imprecise excision events were selected by hemizygous lethality over Df(2R)bw ^{S46}. The imprecise excisions fell into two complementation groups. All ten of the imprecise excisions were embryonic lethal. The cuticular structure of dead first instar larvae was determined by microscopy. Some of the imprecise excisions showed a defect in the head skeleton in the dead first instar larvae. These defects include reduced lateralgrate and disintegrated dorsal bridge. I could not detect any morphological defects in corresponding earlier embryos. This may

be because at earlier stages the defects are very subtle or they only occur during the formation of the head skeleton, which occurs after embryonic head involution. No obvious homeotic transformations accompany these defects. The morphological defects observed correspond to a small subset of the total extent of the expression domain of the transcript in the respect that the head is affected. The head segments are not deleted however; although some of the cuticular head structures arising from these structures are perturbed.

Approaches to further illucidate the whereabouts of the gene responsible for this head phenotype are discussed below.

Determining the genomic limits of the deletions could be acheived by genomic southern analysis. *Eco*RI digests of genomic DNA from each of the deletions would be probed with known *Eco*RI fragments from the walk to determine which ones are missing. This information would make it easier to interpret the complementation groups, and would also determine whether the 3a3 transcript is included in these deficiencies.

Whether or not the transcript homologous to cDNA 3a3 is present in embryos homozygous for the Δ D26 deficiencies could be checked by in situ hybridisation. This would give information regarding whether the transcript was present. It would not however determine whether this transcript was altered in some way.

Northern analysis could be used to check the whole genomic region for transcriptional activity and determine whether 3a3 is indeed the only gene in this region. There is a possibility that there may be another transcript in the region, with a similar expression pattern to A350.1M2, which might have a more enlightening sequence.

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